Supplementary Materials

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

Atish Mohanty[1], Natalie Sandoval[1], An Phan[1], Thang V. Nguyen[2], Robert W. Chen[3], Elizabeth Budde[3], Matthew Mei[3], Leslie Popplewell[3], Lan V. Pham[4], Larry W. Kwak[5], Dennis D. Weisenburger[6], Steven T. Rosen[7], Wing C. Chan[6], Markus Müschen[1] and Vu N. Ngo[1,6*]

Supplementary Methods

Antibodies and chemicals

The following antibodies were used: SOX11 (Abcam, Cambridge, MA), HA (C29F4), HDAC1, HDAC2, Histone H3, STAT3 and pY705 STAT3 (Cell Signaling Technology, Danvers, MA), CCND1 (M20), GAPDH, and LAMIN A/C (Santa Cruz, Dallas, TX), and H3K9/14Ac (Diagenode Inc., Denville, NJ). The following chemicals were used: cyclohexamide (Sigma, St. Louis, MO), SAHA, C646, AZD1480 (Selleck Chemicals, Houston, TX), and JAK inhibitor I (MilliporeSigma, Burlington, MA). IL21 was from R&D Systems (R&D Systems, Inc., Minneapolis, MN).

DNA transfection and viral transduction

DNA transfection was performed by mixing DNA with JetPrime (PolyplusTransfection, New York, NY) and following the manufacturer's instructions. For retroviral transduction, a retroviral vector and a mixture of helper plasmids for viral envelope and *gag*/*pol* were transfected into HEK293T cells using JetPrime. Retroviral supernatants were harvested 48 hours after transfection and were used to transduce ecotropic receptor-expressing

target cells by centrifugation at 1200 xg for one hour in 8 μg/ml polybrene. For lentiviral transduction, a lentiviral vector and a mixture of helper plasmids for viral envelope VSV-G (pMD2.G) and *Gag*/*Pol*/*Rev*/*Tat* (psPAX2) were transfected into HEK293T cells using JetPrime. Lentiviral supernatants were combined 48 and 72 h after transfection and concentrated by centrifugation (100,000 xg for 2 h). Lentiviral transduction of target cells was performed by centrifugation at 1200 xg for one hour in 8 μg/ml polybrene.

Cell viability measurement

Cell viability was assessed by flow cytometric analysis for propidium iodide (PI) negative population. In some experiments, shRNA knockdown vectors also co-express eGFP and the fractions of PI-, eGFP+ were analyzed by flow cytometry over time and compared with the early time fractions. A reduction in the GFP+ fractions over time indicates reduced cell viability.

Immunoprecipitation analyses

Cells were lysed at $4X10^7$ cells/ml in IP buffer from Pierce Biotechnology (25 mM Tris•HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol), in presence of 1mM PMSF, 10 mM glycerophosphate, 1x concentration of Protease and Halt Phosphatase cocktail inhibitors for 30 min on ice. Lysates were cleared by centrifuging for 15 minutes at 14,000 xg at 4°C. Five micrograms of IgG1 isotype control was mixed with 50 ml of 1:1 slurry of PBS and protein A agarose beads and the mixture was added to 1 ml of lysate followed by one-hour incubation on a rotating mixer at 4°C. Lysates were cleared again by centrifugation for one minute at 2400 xg at 4°C. Supernatants were quantified for protein concentrations using the BCA protein assay. Approximately,

1.5 mg of lysate from each sample was incubated with 40 ml of 1:1 slurry of PBS and HA antibody (IgG1)-conjugated agarose beads (Sigma, St. Louis, MO) overnight on a rotating mixer at 4°C. Agarose beads were washed 4 times in 1ml of PBS containing 0.5% NP40 for 10 min each and pelleted by centrifugation at 2400 xg for 5 min. After discarding the supernatant, washed agarose beads were suspended in 100 μl of 1x sample buffer containing 5 μl of beta-mercaptoethanol and heat denatured for 5 min at 95°C. Samples were separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes for western blot analysis.

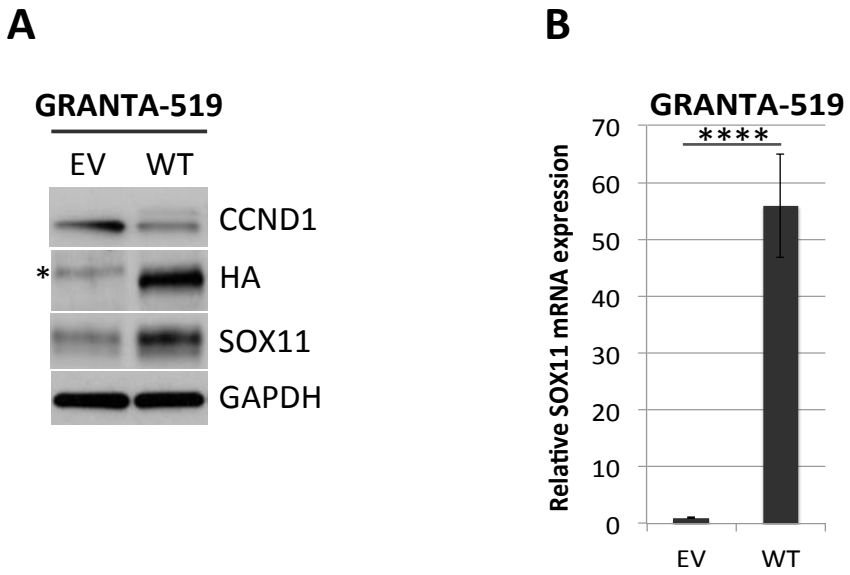
Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed using the ChIP Assay Kit (Millipore, Temecula, CA) according to manufacturer's instructions. Briefly, two million formaldehyde-fixed cells were lysed in 200 μl of SDS lysis buffer and diluted to 2 ml in ChIP dilution buffer in the presence of protease inhibitors. Lysates were sonicated for 9 cycles of 30-second pulse at 30% power with 1 min gap on ice. Lysates were pre-cleared in salmon sperm DNA and protein A agarose by centrifugation. Prior to addition of antibody, 10% of the lysate was used for input and the remaining lysate was divided into two equal parts, one for IgG control and other for H3K9 AC9/14 or HDAC1 antibody. DNA was extracted by phenol-chloroform method and assayed by SYBR® Green-based RT-PCR. Phosphorylated STAT3 ChIP was performed with one million cells following the manufacturer's protocol for EZ-magna ChIP A/G (Millipore, Temecula, CA). Primer sequences for ChIP-qPCR are shown in **Supplementary Table S5**.

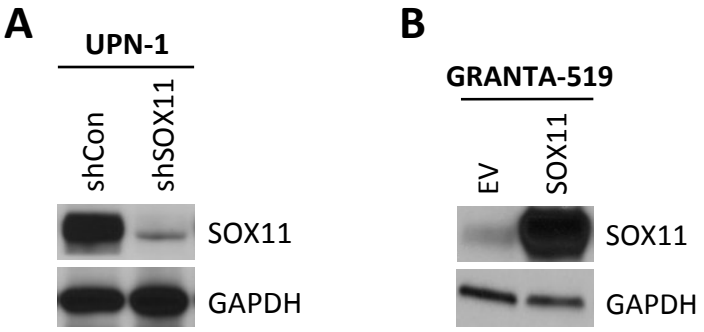
Cell fractionation

Five million cells were harvested and washed twice in PBS. The cell pellet was suspended in 300 μl of nuclear isolation buffer (NIB, 15 mM Tris-HCl pH 7.6, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 5.5% sucrose, 1mM DTT, 1x protease inhibitor and 1x phosphatase inhibitor cocktails). To these suspended cells, 300 μl of NIB buffer containing 0.6% of NP40 was added and incubated on ice for 5 min followed by centrifugation at 2000 r.p.m. for 5 min at 4°C and transferred to a new tube, followed by denaturation in the presence of 2-mercaptoethanol and 200 μl of 4x sample buffer at 95°C. The pellet was washed twice in NIB buffer and re-suspended in 300 μl of nuclear extraction buffer (NEB, 25 mM Tris-HCl pH 8.0, 250 mM NaCl, 1mM EDTA, 10% glycerol, 0.5% NP-40, 1x protease inhibitor and 1x phosphatase inhibitor cocktails). DNA amount in the lysate was quantified and DNAse I was added to a ratio of 1U of enzyme to 4 μg of DNA for 1 h on ice. The reaction was stopped by adding EDTA and centrifuged at 13,000 r.p.m. for 30 min at 4°C. Supernatant was collected, denatured in the presence of 100 μl of 4x sample buffer and used as nuclear soluble fraction. The pellet was washed again in NEB buffer, sonicated and denatured in 200 μl of 1x sample buffer and used as nuclear insoluble fraction.

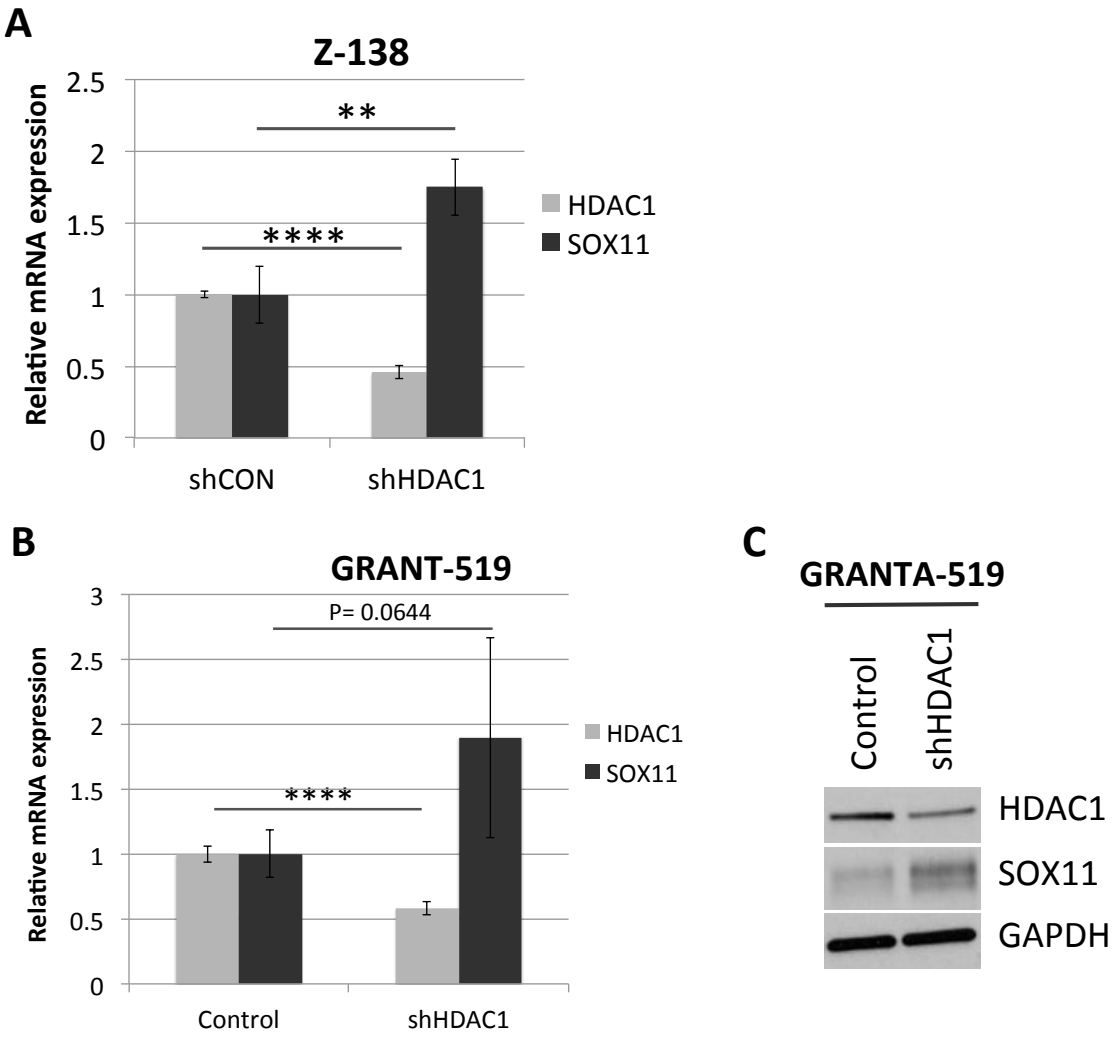
Supplementary Figures



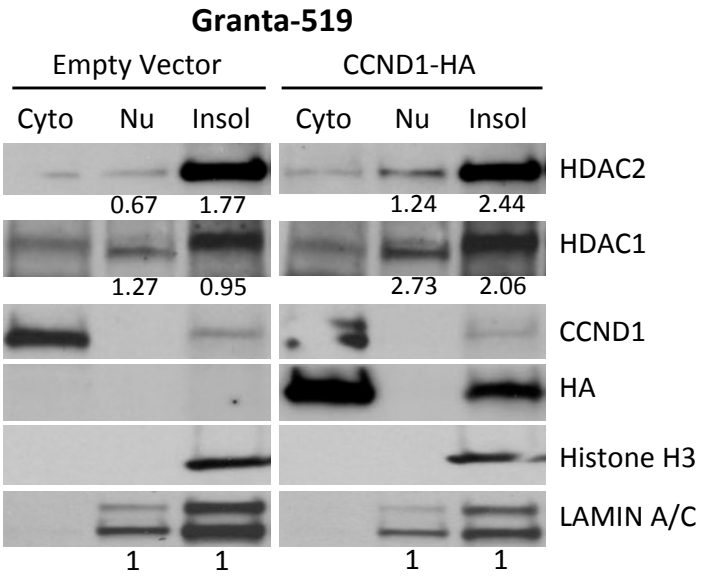
Supplementary Figure S1. CCND1 upregulates SOX11 expression. A. Immunoblot analysis of GRANTA-519 cells stably transduced with empty vector (EV) or WT CCND1-HA constructs. Cell lysates (30 μg per lane) were separated by SDS-PAGE gel and immunoblotted with indicated antibodies. * non-specific band. **B.** 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<0.0001 by a 2-sided Student t-test.



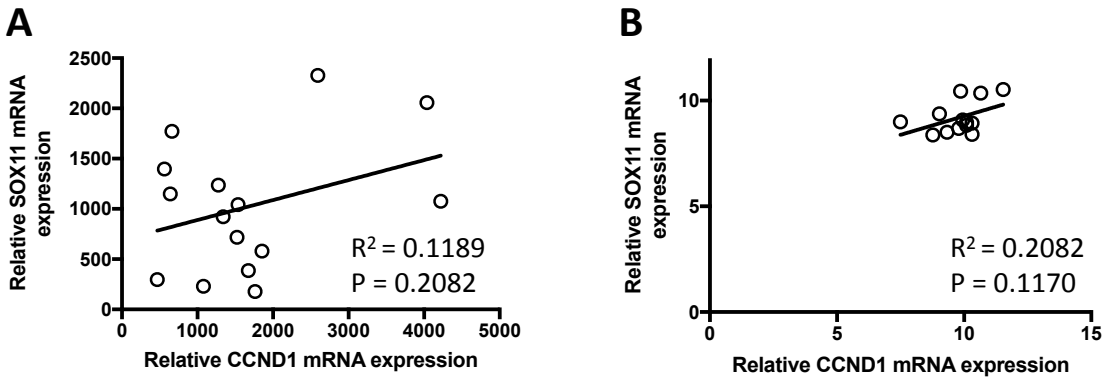
Supplementary Figure S2. Validation of SOX11 antibody specificity. A. UPN-1 cells were stably transduced with control or SOX11 shRNA and cell lysates were immunoblotted with indicated antibodies 2 days after transduction. **B.** GRANTA-519 cells were stably transduced with empty vector (EV) or SOX11-expressing vector and cell lysates were immunoblotted with indicated antibodies. The reduction or increase in the immunoblot signals after *SOX11* gene-specific knockdown (A) or overexpression (B), respectively, indicates the specificity of the SOX11 antibody.



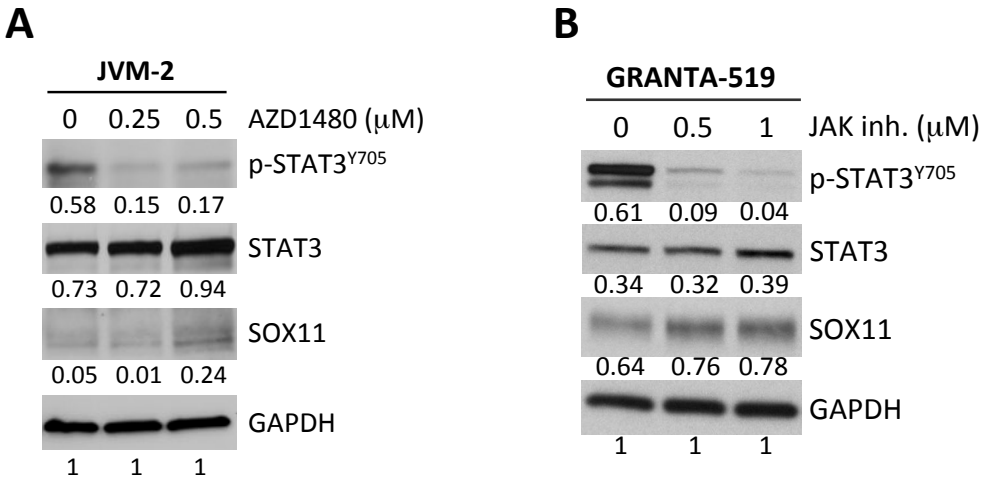
Supplementary Figure S3. HDAC1 depletion upregulates SOX11 expression. A. qPCR analysis of HDAC1 and SOX11 mRNA expression in Z-138 cells transduced with control or HDAC1 shRNA. Shown are the means of mRNA expression levels after normalization to GAPDH signals from four independent amplification experiments. **B.** qPCR analysis of HDAC1 and SOX11 mRNA expression in GRANTA-519 cells stably transduced with control or HDAC1 shRNA. Shown are the means of mRNA expression levels after normalization to GAPDH signals from four independent amplification experiments. Error bars, SD. ** P<0.01, **** P<0.0001 by a 2-sided Student t-test. **C.** GRANTA-519 cells were generated as described in (**B**) and protein expression was analyzed by immunoblot analysis with indicated antibodies 3 days after transduction.



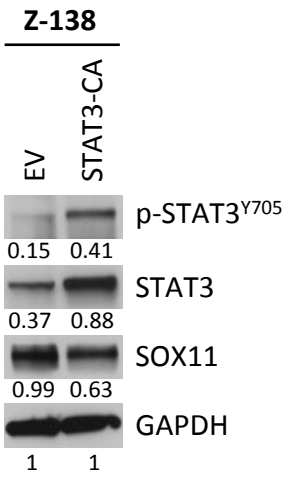
Supplementary Figure S4. Sequestration of HDAC1 from chromatin in CCND1 overexpressing cells. Cytosolic, soluble and insoluble nuclear extracts were prepared as described in Materials and Methods from GRANTA-519 cells that stably expressed empty vector or WT CCND1-HA. The extracts were immunoblotted with indicated antibodies. LAMIN A and histone H3 were used to confirm nuclear fractions. Cyto, cytoplasmic; Nu, soluble nuclear fraction; Insol, insoluble nuclear fraction. Numbers below immunoblots are relative densitometric values of corresponding bands after normalization to LAMIN A signals.



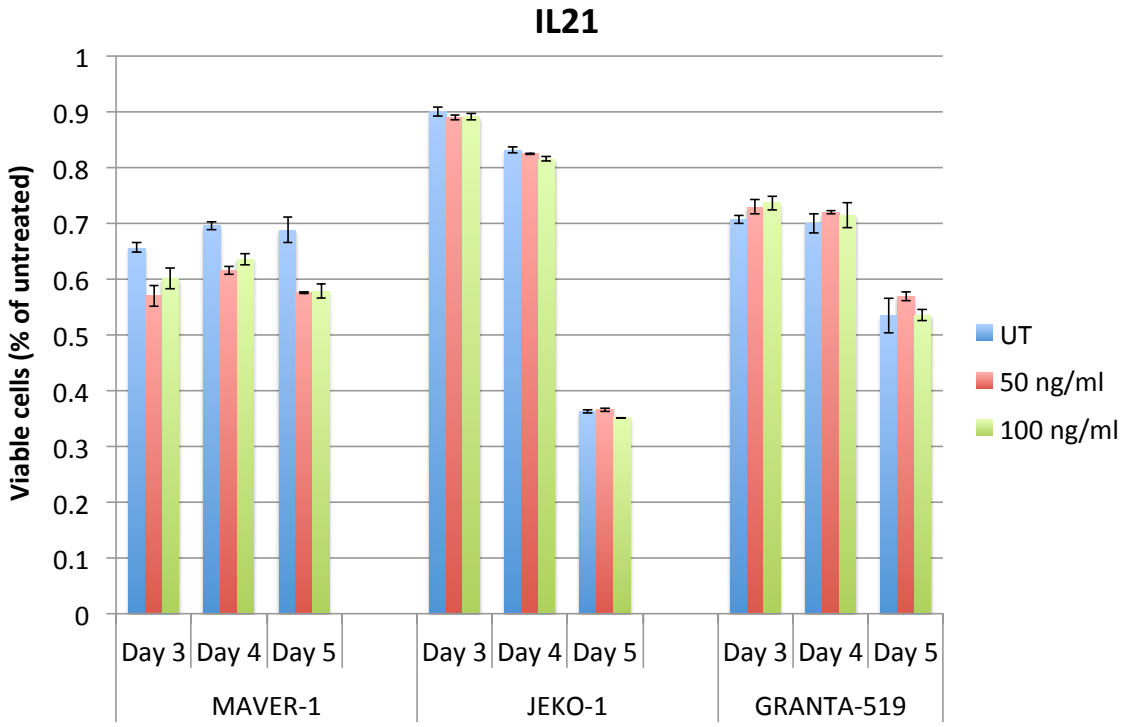
Supplementary Figure S5. Correlation of SOX11 and CCND1 mRNA levels. Linear regression analysis of SOX11 and CCND1 mRNA levels from SOX11 positive cases (see **Supplementary Table S6** for expression data in **A** and **Supplementary Table S7** for expression data in **B**) using GraphPad Software.



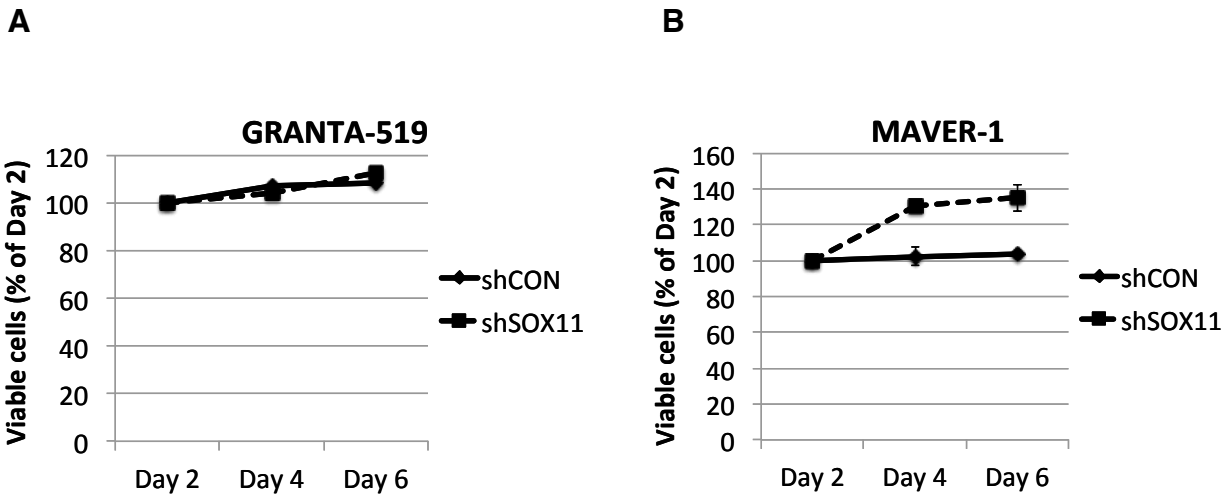
Supplementary Figure S6. Effect of STAT3 inhibition on SOX11 expression. A. JVM-2 cells were treated with indicated doses of the JAK1/2 inhibitor AZD1480 for 16 h and immunoblotted with indicated antibodies. **B.** GRANTA-519 cells were treated with indicated doses of the JAK inhibitor I (MilliporeSigma, Burlington, MA, USA), an inhibitor of STAT3, for 16 h and immunoblotted with indicated antibodies.



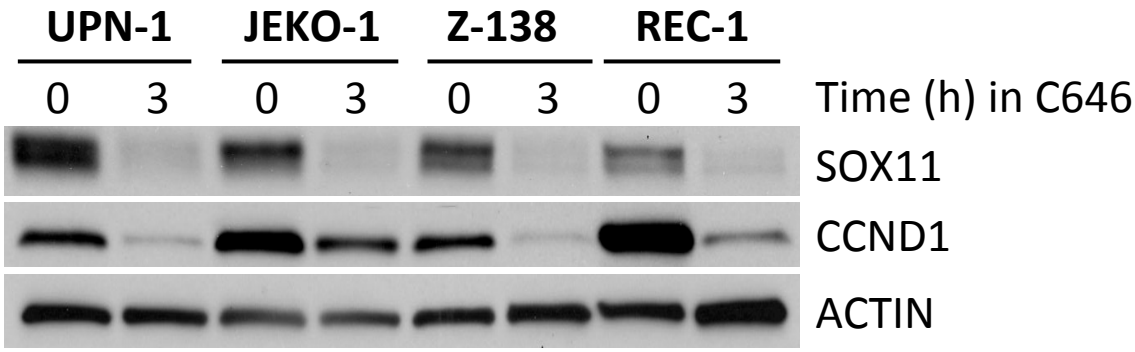
Supplementary Figure S7. Effect of increased STAT3 activity on SOX11 expression. Z-138 cells were transduced with EV or constitutive active STAT3 (STAT3-CA) and cell lysates were immunoblotted with indicated antibodies.



Supplementary Figure S8. Little effect of IL21 on cell viability in some MCL cell lines. Indicated MCL cell lines were treated with 50 or 100 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 (UT) samples from at least two independent experiments.



Supplementary Figure S9. Effect of SOX11 knockdown on MCL cell viability. GRANTA (**A**) and MAVER-1 (**B**) cells 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.



Supplementary Figure S10. EP300 inhibition reduces SOX11 expression. Immunoblot analysis of indicated cells treated with 10 μM of C646 in serum-free medium for indicated times before cell lysates were prepared and separated by SDS-PAGE gel, followed by immunoblotting with indicated antibodies.

Supplementary Tables

Supplementary Table S1. Results of cell line authentication by short tandem repeat DNA profiling.

	Short tandem repeat profile			
Locus	**JEKO-1 (a)**	**Z-138 (a)**	**GRANT-519 (b)**	**UPN-1 (c)**
TH01	7	6	7, 8	8, 9.3
D5S818	10, 13	11, 13	9, 13	12, 13
D13S317	8, 9	9, 12	9, 11	13
D7S820	10, 11	8	10, 14	11, 13
D16S539	12	11	12, 12	13
CSF1PO	9, 12	10, 11	10, 10	12
Amelogenin	X	X, Y	X, X	X
vWA	14	15, 18	17, 17	16, 18
TPOX	8	8	8, 8	8, 9

(a) Profile matches ATCC Reference Database profile

(b) Profile matches DSMZ Reference Database profile

(c) Profile not present in ATCC and DSMZ Databases

Supplementary Table S2. MCL patient-derived xenografts.

Case	Name	Age	Gender	Disease Stage	Cytogenetics/Karyotype	Exome-seq
98848	MCL PDX 4	81	M	Relapse	*IGH-CCND1* by FISH	*NOTCH1*^{P2514fs*} *KRAS*^{S136R}
44685	MCL PDX 5	63	M	Relapse	t(11;14)(q13;q32) *IGH-CCND1*	*ATM*^{V1671fs*} *WHSC1*^{E1009K} *CREBBP*^{Q2257H}
91438	MCL PDX 7	75	M	Progressive	*IGH-CCND1* by FISH	*TET2*^{N1103fs*}
96069	MCL PDX 9	72	F	Refractory	t(11;14)(q13;q32) *IGH-CCND1*	*BIRC3*^{L548fs*}

Supplementary Table S3. List of shRNA sequences.

Gene	Name in manuscript	RNAi sequences	Used in figures
CCND1	shCCND1	GCCGAGAAGCTGTGCATCTAC	1C, D
SOX11	shSOX11	CGCCAGCCAGAGCCCAGAGAA	1E, S2A
HDAC1	shHDAC1	GAGGAAAGTCTGTTACTACTA	3C; S3A-C
HDAC2	shHDAC2	CAGTCTCACCAATTTCAGAAA	3D
STAT3	shSTAT3	GCTGACCAACAATCCCAAGAA	5H, I

Supplementary Table S4. Validation of SOX11 mRNA quantitative PCR assays.

	Ct values	
	RT+ cDNA	RT- mock
SOX11 primer 1	23.8 ± 0.05	39.0 ± 0.59
SOX11 primer 2	25.2 ± 0.24	36.4 ± 0.81
GAPDH primer	23.2 ± 0.13	38.6 ± 0.38

RT, reverse transcriptase; Ct, cycle threshold. Ct values are means of four independent amplication experiments ± S.D.

Supplementary Table S5. List of PCR primers.

Name	Sequence (5'-3')	Assay	References
GAPDH primer F	AAGGGCTCATGACCACAGTC	RT-PCR	
GAPDH primer R	GGATGACCTTGCCCACAG	RT-PCR	
SOX11 primer 1-F	ACTCCAATGTCTCTTTTGC	RT-PCR	Hamborg 2012
SOX11 primer 1-R	GAACGGACTTTTTTTTTTTTTTTTTGAAGAT	RT-PCR	Hamborg 2012
SOX11 primer 2-F	CATGTAGACTAATGCAGCCATTGG	RT-PCR, ChIP-qPCR (amplicon 2)	Vegliante 2011
SOX11 primer 2-R	CACGGAGCACGTGTCAATTG	RT-PCR, ChIP-qPCR (amplicon 2)	Vegliante 2011
SOX11 primer 3-F	GAGAGCTTGGAAGCGGAGA	ChIP-qPCR (amplicon 1)	Vegliante 2011
SOX11 primer 3-R	AGTCTGGGTCGCTCTCGTC	ChIP-qPCR (amplicon 1)	Vegliante 2011
SOX11 primer 4-F	GAGAGCTTGGAAGCGGAGA	ChIP-qPCR (amplicon 1)	Vegliante 2011
SOX11 primer 4-R	CGTTCGATCTTGGACCATACC	ChIP-qPCR (amplicon 1)	
SOX11 primer 5-F	GCAGGTGTTTGATAATGCCTC	ChIP-qPCR (amplicon 3)	
SOX11 primer 5-R	GCCAGAGCAATACAGTGAGTAATC	ChIP-qPCR (amplicon 3)	
SOX11 primer 6-F	TTGGCTTTAGAATGAGGG	ChIP-qPCR (amplicon 4)	
SOX11 primer 6-R	TCACACTGAAGTTTTGATGGG	ChIP-qPCR (amplicon 4)	

Supplementary Table S6. mRNA expression data from GSE16455 (ref. 34).

Cases (SOX11 positive)	SOX11			CCND1		SOX11	CCND1
	204915_s_at	204914_s_at	204913_s_at	208712_at	208711_s_at	average	average
Conventional.MCL.1	993.6	2779.6	2397	4288.1	3788	2056.733	4038.05
Conventional.MCL.10	546.7	2167.4	1475.6	504.8	617.9	1396.567	561.35
Conventional.MCL.11	683	2394.5	2237.8	633	694.7	1771.767	663.85
Conventional.MCL.13	460.8	1297.6	1468.2	4027.8	4413.9	1075.533	4220.85
Conventional.MCL.14	184.2	344.8	163	1071.3	1088	230.6667	1079.65
Conventional.MCL.15	384	1062.5	1320	1496.1	1184	922.1667	1340.05
Conventional.MCL.2	1087.1	2530.4	3369.9	3833.6	1351.1	2329.133	2592.35
Conventional.MCL.3	483.6	739.6	932	1824.7	1224.7	718.4	1524.7
Conventional.MCL.4	465.5	1719.3	1263.6	749.2	532.2	1149.467	640.7
Conventional.MCL.5	502.9	1307.6	1317.2	1671.9	1403.9	1042.567	1537.9
Conventional.MCL.6	296.1	708.6	735	2416	1293	579.9	1854.5
Conventional.MCL.7	148.9	328.2	415.6	434.7	500	297.5667	467.35
Conventional.MCL.9	555	1742.2	1415	561.6	1988.6	1237.4	1275.1
Indolent.MCL.4	239.2	553.3	368.2	2118.8	1231.1	386.9	1674.95
Indolent.MCL.2	137.7	222.5	174.7	2200.5	1324	178.3	1762.25
Cases (SOX11 negative)	**SOX11**			**CCND1**		**SOX11**	**CCND1**
	204915_s_at	204914_s_at	204913_s_at	208712_at	208711_s_at	average	average
Conventional.MCL.8	24.7	11.9	21.5	2.1	4951.4	19.36667	2476.75
Conventional.MCL.12	4.8	1.4	32.5	1349.8	906.3	12.9	1128.05
Indolent.MCL.1	12.5	2.3	0.6	857.4	508.1	5.133333	682.75
Indolent.MCL.3	2.9	25.4	24.7	2203	1398.5	17.66667	1800.75
Indolent.MCL.5	7.5	74	29.9	2055.6	922.4	37.13333	1489
Indolent.MCL.6	4.9	10.3	3.5	1485.1	746.7	6.233333	1115.9
Indolent.MCL.7	3.1	19.7	4.3	96.5	4792.7	9.033333	2444.6

Supplementary Table S7. mRNA expression data from GSE36000 (ref. 16).

Cases (SOX11 positive)	SOX11			CCND1		SOX11	CCND1
	204915_s_at	204913_s_at	204914_s_at	208712_at	208711_at	average	average
MCL_IGHV_UNMUT (R1394)	10.3621	10.0579	11.1429	11.8866	11.1948	10.5209667	11.5407
MCL_IGHV_UNMUT (R1306)	8.90176	9.34356	9.88933	8.99182	9.06118	9.37821667	9.0265
MCL_IGHV_UNMUT (R1332)	8.72074	8.6211	9.92335	10.1699	9.72807	9.08839667	9.948985
MCL_IGHV_UNMUT (R1587)	9.5396	7.53551	9.92012	2.67941	12.3112	8.99841	7.495305
MCL_IGHV_UNMUT (R1680)	7.40145	9.46408	9.93824	9.83827	10.7875	8.93459	10.312885
MCL_IGHV_UNMUT (268-01-5TR)	8.29182	7.66145	9.18539	9.05927	8.48165	8.37955333	8.77046
MCL_IGHV_UNMUT (043-01-4TR)	11.3831	8.93468	11.0159	10.9781	8.74049	10.44456	9.859295
MCL_IGHV_UNMUT (R1338)	7.88213	8.53114	8.80149	10.4192	10.1986	8.40492	10.3089
MCL_IGHV_UNMUT (R1388)	9.02642	8.16175	9.71772	10.1339	10.0619	8.96863	10.0979
MCL_IGHV_MUT (R1400)	8.05998	9.09335	9.34785	9.95061	10.2426	8.83372667	10.096605
MCL_IGHV_MUT (R1628)	9.68681	10.8834	10.5004	10.9729	10.3333	10.35687	10.6531
MCL_IGHV_MUT (R1629)	8.32904	9.00908	8.72256	9.70786	9.86063	8.68689333	9.784245
MCL_IGHV_MUT (R1938)	8.65126	7.52828	9.32923	9.47836	9.17358	8.50292333	9.32597

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

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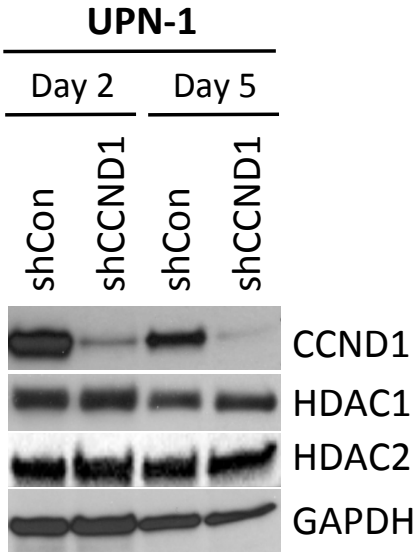

Supplementary Figure S11. Immunoblot analysis shows effect of CCND1 knockdown on HDAC1 and HDAC2 expression. UPN-1 cells were transduced with control or CCND1 shRNA and cell lysates at the indicated times were immunoblotted with indicated antibodies.

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

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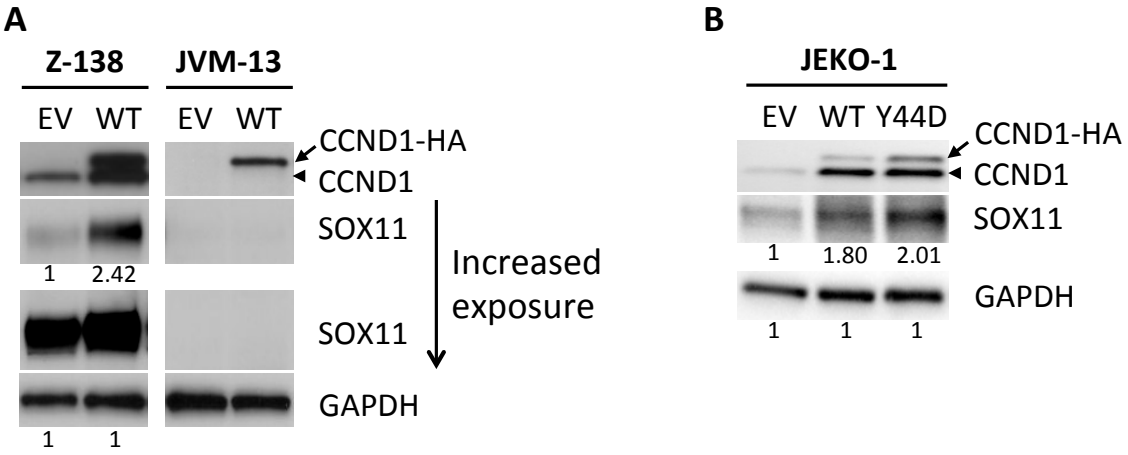

Supplementary Figure S12. CCND1 upregulates SOX11 expression in Z-138 and JEKO-1 cells. A. Immunoblot analysis of indicated MCL cell lines stably transduced with empty vector (EV), WT or Y44D mutant CCND1-HA constructs. Cell lysates were separated by SDS-PAGE gel and immunoblotted with indicated antibodies. Arrow indicates a mobility shift of the CCND1-HA protein. Arrowhead indicates endogenous CCND1. Numbers below immunoblots are relative densitometric values of corresponding bands after normalization to GAPDH and respective control signals.

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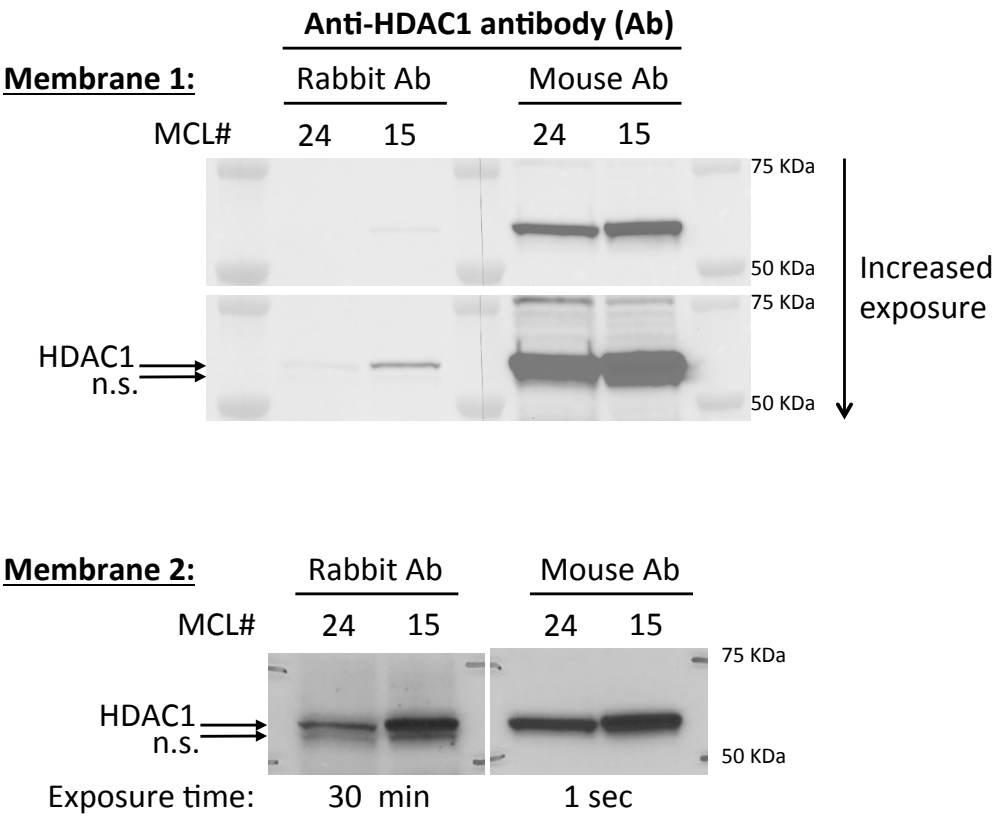

Supplementary Figure S13. Comparison of rabbit and mouse anti-HDAC antibodies. Lysates from MCL# 15 and 24 were immunoblotted with rabbit and mouse anti-HDAC1 antibodies. In membrane 1, the blot was imaged using a BioRad Imaging system and two different exposures were shown. In membrane 2, the blot was exposed to X-ray films at two different times to normalize the signal intensities from the two different antibodies. In both membranes, the single band specific for HDAC1 was detected by the mouse antibody and used as a positive control for the HDAC1-specific band. This HDAC1 specific band lines up with the upper band detected by the rabbit antibody. This result suggests that the bottom band detected by the rabbit antibody is non specific (n.s.).