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FOXP1 Potentiates Wnt/ β -catenin Signaling in Diffuse Large B-cell Lymphoma

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Abstract

The transcription factor FOXP1 is a master regulator of stem and progenitor cell biology. In diffuse large B-cell lymphoma (DLBCL), copy number amplifications and chromosomal translocations result in overexpression of *FOXP1*. Increased FOXP1 protein abundance in DLBCL predicts poor prognosis and resistance to therapy. To connect gene overexpression with phenotype, we developed a genome-wide mass spectrometry-coupled gain-of-function genetic screen, revealing that FOXP1 potentiates β -catenin-dependent Wnt signal transduction. Gain-of-

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function and loss-of-function studies in cell models and zebrafish confirmed that FOXP1 was a general and conserved co-activator of Wnt signaling. In a Wnt-dependent fashion, FOXP1 co-complexed with β -catenin, TCF7L2, and the acetyltransferase CBP, and bound the promoters of Wnt target genes. FOXP1 promoted the acetylation of β -catenin by CBP, and acetylation was required for FOXP1-potential of β -catenin-dependent transcription. In DLBCL, we found that FOXP1 promoted sensitivity to Wnt pathway inhibitors and knockdown of FOXP1 or Wnt signaling slowed xenograft tumor growth. These data connect FOXP1 overexpression with β -catenin-dependent signal transduction, and provide a new molecular rationale for Wnt-directed therapy in DLBCL.

Introduction

The Wnt/ β -catenin signal transduction pathway controls cell fate and proliferation during development, adult tissue homeostasis and in disease. In the absence of Wnt ligand, a complex of cytosolic proteins, which is referred to as the destruction complex and includes adenomatous polyposis coli (APC), AXIN, glycogen synthase kinase β (GSK3B), casein kinase 1 α (CSNK1A) and β TrCP1 and β TrCP2 (BTRC and FBXW11), sequentially phosphorylates and ubiquitinates β -catenin, resulting in its proteasomal degradation (1, 2). Wnt ligand brings together a Frizzled receptor and a LRP co-receptor, resulting in a series of phosphorylation events that transiently represses β -catenin phosphorylation by the destruction complex (3). Consequently, β -catenin protein stability is increased, allowing it to translocate to the nucleus where it functions as a transcriptional co-activator for members of the TCF/LEF family of transcription factors. Aberrant Wnt/ β -catenin pathway activity is a hallmark of several human cancers, including colon and liver cancer in which mutations in core signaling components result in constitutive pathway activity and oncogenesis (4, 5). Many tumors, however, display increased Wnt/ β -catenin signaling in the absence of mutations in components of this pathway, suggesting that heretofore unrecognized genome alterations contribute to Wnt/ β -catenin activation in cancer (6).

Functional genomic screening technologies provide a powerful approach to identify genetic determinants of Wnt/ β -catenin signaling, and more broadly to connect genotype with phenotype. Loss-of-function approaches, including random mutagenesis and RNA interference, have revealed hundreds of Wnt/ β -catenin regulatory genes, many of which are altered in cancer (7-11). Alternatively, gain-of-function screens can more accurately model activating mutations, copy number amplifications and gene overexpression. In contrast to loss-of-function approaches, a single gain-of-function genetic screen of the Wnt pathway has been reported (12). Beyond the Wnt pathway, limited technical resources and high costs have hampered the use of gain-of-function screens in many areas of cancer research.

Here, we used a new gain-of-function screening approach to identify the FOXP1 transcription factor as an enhancer of Wnt/ β -catenin signal transduction. Like Wnt/ β -catenin signaling, the Forkhead box protein P1 (FOXP1) transcription factor makes fundamental yet widely varied contributions to development, adult tissue homeostasis, regeneration and disease (13, 14). Tissue-specific differences in FOXP1 alternative splicing and FOXP1 protein-protein interactions diversify target gene selection and influence whether FOXP1

acts to promote or inhibit transcription. In embryonic stem (ES) cells, a specific FOXP1 splice variant maintains pluripotency by promoting *OCT4*, *NANOG*, *NRFA2*, and *GDF3* expression while simultaneously suppressing the expression of genes involved in differentiation (15). During lung development and regeneration, FOXP1 directs progenitor cell differentiation to balance epithelial and goblet cell numbers (16). Within the developing heart, FOXP1 promotes cardiomyocyte proliferation within the myocardium, but represses proliferation within the endocardium (17). FOXP1 is also required for proper B-cell development; FOXP1 deficient lymphoid stem cells fail to differentiate beyond the pro-B cell stage and overexpression of FOXP1 inhibits differentiation at a later stage of B-cell maturation (18, 19).

As in development, FOXP1 plays context-dependent roles in human disease; FOXP1 demonstrates oncogenic characteristics in B-cell lymphoma but tumor suppressive functions in several epithelial cancers. *FOXP1* is located within a cluster of tumor suppressor genes on 3p13, and is lost or silenced in kidney and colon cancer (20, 21). Conversely, high *FOXP1* expression carries a favorable prognosis in breast and lung cancer (22, 23). Conversely, recurrent copy number amplifications and chromosomal translocations contribute to its overexpression and poor prognosis in several types of B-cell lymphoma (24, 25). Functionally, FOXP1 directly represses pro-apoptotic genes, thus providing direct evidence for the role of FOXP1 as an oncogene in B-cell lymphomas (26). Therefore, FOXP1 may act as both a tumor suppressor and an oncogene, although the underlying molecular mechanism for this disparity is not clear. Alterations in FOXP1 contribute to other human diseases as well. Genomic deletions, nonsynonymous mutations and gene overexpression have been reported in congenital heart disease and autism spectrum disorders (27, 28).

Here, we showed that FOXP1 overexpression potentiated Wnt/ β -catenin signaling in diverse cancer cell types, including B-cell lymphoma, colorectal, melanoma, and in zebrafish embryos. We found that CBP-mediated acetylation of β -catenin was required for FOXP1-induced β -catenin transcriptional activity. Further, FOXP1 co-complexed with a β -catenin transcriptional complex on chromatin, resulting in enhanced β -catenin-dependent transcription. FOXP1 overexpression in B-cell lymphoma cell lines moderately promoted sensitivity to small molecule inhibitors of the Wnt/ β -catenin pathway. Consistent with these results, mouse xenograft experiments demonstrated that FOXP1 and the Wnt/ β -catenin pathway promoted the growth of B-cell lymphoma. Together, these data identify FOXP1 as a transcriptional enhancer of the Wnt/ β -catenin signaling pathway in human cancer.

Results

CDt/MS identifies FOXP1 as a Wnt signaling enhancer

We used a mass spectrometry-coupled lentiviral ‘CD-tagging’ mutagenesis approach to identify genes that activate Wnt/ β -catenin signaling (Fig. 1A) (29, 30). Human A375 melanoma cells containing a β -catenin-driven GFP (green fluorescent protein) transcriptional reporter were transduced with CDBF lentivirus (Fig. 1A). When integrated near an expressed and spliced gene, the cytomegalovirus (CMV) promoter of the CDBF vector drives constitutive BFP (blue fluorescent protein) expression and by virtue of the splice donor (SD) sequence, an overexpressed FLAG-tagged fusion of the targeted gene.

Depending on where within the gene locus the CDBP vector integrates, the resulting overexpressed gene product may be full length or truncated at the N-terminus. Fluorescence activated cell sorting (FACS) was used to isolate BFP+/GFP+ (Wnt active) or BFP+/GFP- (Wnt inactive) A375 cells. We reasoned that if successful, FLAG epitope tag immunopurification and mass spectrometry-based identification of the overexpressed fusion proteins would be cheaper and faster and would provide more information than traditional PCR-based detection. FLAG immunopurification followed by a series of high salt washes, on-bead tryptic digestion and shotgun mass spectrometry (MS) identified 20 high-confidence proteins specific to Wnt-active cells (table S1). The high-salt washes removed associated proteins from the FLAG-tagged bait proteins. The FOXP1 transcription factor ranked as the top screen hit, as determined by spectral count abundance and the CompPASS WD-score across four biological replicate screens (31).

FOXP1 potentiates Wnt signaling

To validate FOXP1 as a promoter of Wnt/ β -catenin signaling, we performed gain- and loss-of-function experiments across an array of human cell lines. Overexpression of FOXP1 in A375 cells enhanced the expression of a β -catenin-driven fluorescent reporter gene, both in the absence and presence of exogenous Wnt3a ligand (Fig. 1B, 1C). FOXP1 overexpression induced the expression of a β -catenin-activated luciferase reporter (BAR) in A375 melanoma cells, HEK293T embryonic kidney cells and HT1080 fibrosarcoma cells (Fig. 1D, fig. S1A-1B). FOXP1 overexpression also promoted BAR in HCT116 colorectal carcinoma cells and DLD1 colorectal adenocarcinoma cells, both of which harbor mutations within the Wnt pathway resulting in constitutive activity (Fig. 1E-F). In two DLBCL cell lines, DB and KARPAS422, FOXP1 expression similarly resulted in increased BAR activity (Fig. 1G, fig. S1C). In loss-of-function experiments, two non-overlapping short interfering RNAs (siRNAs) specific to *FOXP1* suppressed β -catenin-dependent reporter expression in HEK293T, HCT116, HT1080, DB and KARPAS422 cells, suggesting that endogenous FOXP1 promotes Wnt/ β -catenin pathway activity in diverse cell types, including B-cell lymphoma (Fig. 1H-J and fig. S1D-F). To rule out reporter-based artifact, the expression of β -catenin target genes *AXIN2* and *NKDI* was quantified after *FOXP1* overexpression or silencing. FOXP1 gain-of-function induced and loss-of-function decreased *NKDI* and *AXIN2* in multiple human cell lines (Fig. 1K-1N and fig. S1G-1J).

Because FOXP1 is a transcription factor, we hypothesized that FOXP1 induces Wnt signaling downstream of the destruction complex within the nuclear compartment. If true, an active Wnt signaling pathway would be required for FOXP1 to promote β -catenin-dependent transcription. Indeed, a series of epistasis experiments revealed that FOXP1 is not sufficient to activate Wnt signaling on its own, but strongly enhances an active pathway. First, FOXP1 potentiated β -catenin-dependent transcription in colon cancer cells harboring pathway-activating mutations (Fig. 1E-F). HCT116 cells contain a mutant form of β -catenin that cannot be phosphorylated by GSK3 β and DLD1 cells contain truncated and inactive APC. Second, FOXP1 enhanced β -catenin dependent transcription in the presence of the selective GSK3 β inhibitor CT990201 (Fig. 2A). Third, FOXP1 overexpression did not affect the steady-state abundance of β -catenin (Fig. 2B). Fourth, FOXP1-induced β -catenin-dependent transcription was blocked by XAV939, a tankyrase inhibitor that stabilizes AXIN protein

and increases β -catenin degradation by the destruction complex (Fig. 2C) (32). Fifth, in cell lines containing an intact Wnt signaling pathway, FOXP1 required Wnt ligand to promote β -catenin-dependent transcription. DKK1, a receptor-level Wnt antagonist, blocked FOXP1-induced BAR activity in the absence of Wnt3a treatment, indicating the presence of an autocrine signaling loop (Fig. 2C and fig. S1K). Confirming this result, the porcupine inhibitor C59, which blocks Wnt ligand secretion, inhibited FOXP1-driven Wnt signaling (Fig. 2C and fig. S1K) (33). Lastly, FOXP1 failed to promote BAR activity when β -catenin was knocked down (Fig. 2D). Together, these data argue that FOXP1 promotes β -catenin-dependent transcription downstream of the β -catenin destruction complex, which is consistent with its role as a transcription factor.

We next tested whether FOXP1 promoted Wnt signaling *in vivo*. During zebrafish development, Wnt/ β -catenin signaling controls cell fate decisions to instruct anterior-posterior patterning. Ectopic Wnt activity posteriorizes the developing embryo, resulting in exaggerated posterior structures with concomitant loss of the anterior (34). Varying degrees of Wnt activation yield well-characterized morphological phenotypes, which can be binned into 4 classes of phenotype severity (Fig. 2E). Using this approach, embryos were microinjected with zebrafish *foxp1b* mRNA, the closest homolog of human *FOXP1*, and scored based on overall morphology (Fig. 2E and 2F). *Foxp1b* injection induced phenotypes closely resembling those induced by ectopic Wnt activation (Fig. 2F). *Foxp1b* synergized with a hypomorphic dose of *wnt8* to increase the severity of the Wnt phenotype (Fig. 2G), suggesting that the phenotypes seen were due to increases in Wnt activity. To confirm that *Foxp1b* promotes Wnt/ β -catenin signaling, we asked whether *Foxp1b* overexpression was sufficient to alter the expression a transcriptional reporter of Wnt/ β -catenin signaling in *6 $\times$ Tcf/Lef-miniP:d2EGFP^{isi04}* transgenic zebrafish. *In situ* hybridization at 8 hours post fertilization (hpf) indeed revealed an expansion of *6 $\times$ Tcf/Lef-miniP* driven *EGFP* expression compared to controls (Fig. 2H). In agreement with the reporter data, *Foxp1b* overexpression increased expression of *Axin2* and *Bozozok*, 2 canonical Wnt target genes (Fig. 2I). These data support our hypothesis that FOXP1 functions in part to promote Wnt signaling. Lastly, we tested whether FOXP1 expression regulates the activity of additional signaling pathways. Overexpression of FOXP1 did not impact the transforming growth factor β (TGF β) or nuclear factor κ B (NF κ B) signaling pathways, suggesting selectivity for the Wnt pathway over those tested (Fig. 2J). In total, these data show that FOXP1 can promote β -catenin-dependent transcription.

FOXP1 isoforms have different effects on Wnt signaling

Differential expression of FOXP1 splice isoforms is associated with B-cell differentiation, stem-cell maintenance and hematological malignancies. Seven FOXP1 isoforms were tested for their ability to modulate Wnt signaling in a GOF setting (fig. S2A). Overexpression of FOXP1 isoforms 1, 6 and 9 stimulated BAR activity (fig. S2B,C). By contrast, the severely truncated isoform 5 and isoform 12, which lacks a DNA binding domain, significantly attenuated Wnt signaling, possibly by acting as dominant negatives. Isoform 3 lacks the coiled coil domain and NR box and did not impact reporter activity. In chronic lymphocytic leukemia (CLL), a mutation in *SF3B1* causes aberrant splicing of *FOXP1* leading to the overexpression of a novel isoform termed FOXP1w (35). This CLL-derived mutant protein

promoted Wnt signaling. In mice, an ES-specific *Foxp1* isoform (*Foxp1-ES*) maintains stem cell pluripotency (15). Both *Foxp1-ES* and *mFoxp1* significantly stimulated BAR activity (fig. S2D). Lastly, we found that the total protein abundance and isoforms of FOXP1 present varied in a panel of human cancer cell lines (fig. S2E).

FOXP1 does not potentiate Wnt signaling through SOX17

A previous study hypothesized that FOXP1 indirectly promotes Wnt/ β -catenin signaling through transcriptional suppression of SOX17 (17). Two experiments suggest direct involvement of FOXP1 in β -catenin potentiation. First, *SOX17* is epigenetically silenced and not expressed in HCT116 cells (36), and we observed strong enhancement of β -catenin-dependent reporter activity in HCT116 cells (Fig. 1E). Second, we used siRNAs to silence *SOX17* before FOXP1 overexpression and BAR activity quantitation (fig. S3A). FOXP1 potentiated Wnt signaling in the absence of SOX17, which supports a direct mechanism of action (fig. S3B).

FOXP1 increases β -catenin acetylation through CBP

To determine the mechanism by which FOXP1 promotes β -catenin-dependent transcription, we used small molecules to inhibit nuclear components of Wnt signaling. Two pan-histone acetyltransferase (HAT) inhibitors, anacardic acid and CPTH2, suppressed FOXP1 potentiation of Wnt signaling without affecting basal reporter activity (Fig. 3A). Moreover, the small molecule ICG001, which blocks the association of the CBP/CREBBP HAT with β -catenin, strongly inhibited FOXP1-driven β -catenin activation (Fig. 3A) (37). Consistent with this result, siRNA-mediated silencing of *CBP* blocked FOXP1-induced Wnt signaling (Fig. 3B). Therefore, we tested whether FOXP1 promoted CBP-dependent acetylation of β -catenin (38, 39). Indeed, FOXP1 expression induced acetylation of Lys⁴⁹ in β -catenin in the nucleus without affecting the total abundance of β -catenin (Fig. 3C-D). The acetylation of β -catenin at Lys⁴⁹ was suppressed by siRNA-mediated silencing of *FOXP1* in HEK293T cells (Fig. 3E) and in DB cells (Fig. 3F). As expected, CBP-specific siRNAs blocked FOXP1-induced acetylation of β -catenin without affecting FOXP1 abundance (Fig. 3G). Mutation of Lys⁴⁹ (K49R) blocked the ability of FOXP1 to promote Wnt signaling (Fig. 3H), suggesting that FOXP1 induced potentiation of Wnt signaling depends upon CBP-mediated acetylation of Lys⁴⁹.

FOXP1 co-complexes with β -catenin, TCF7L2 and CBP

Next, we used protein-protein interaction experiments to evaluate associations between FOXP1, CBP, β -catenin and TCF7L2, one of the transcription factors through which β -catenin functions. Overexpressed FOXP1 co-complexed with endogenous CBP, TCF7L2, and β -catenin (Fig. 4A). Reciprocally, immunoprecipitation of exogenous β -catenin revealed an interaction with FOXP1 (fig. S4). Endogenous FOXP1 immunoprecipitated TCF7L2, CBP, β -catenin and acetylated β -catenin in HCT116 and HEK293T cells (Fig. 4B, 4C). Consistent with our epistasis data, these interactions required Wnt3a stimulation in HEK293T cells (Fig. 4C). Therefore, in the presence of an active Wnt pathway, FOXP1 associates with a CBP/ β -catenin/TCF7L2 protein complex.

We used siRNA-mediated silencing and chromatin immunoprecipitation (ChIP) experiments to test if FOXP1 regulates protein-chromatin interactions at β -catenin target genes. β -catenin and FOXP1 associated with the *AXIN2* promoter in HEK293T, but only after Wnt3a treatment (Fig. 4D). FOXP1 also bound the Wnt-responsive promoters of *AXIN2* and *PDE4B1* in DB and HCT116 cells (Fig. 4E, and fig. S5A-C). The FOXP1 ChIP signal was suppressed by *FOXP1* siRNA, thus establishing antibody specificity (Fig. 4E and fig. S5A-C). We next tested the effect of FOXP1 on TCF7L2, CBP, and β -catenin protein-protein and protein-chromatin associations. siRNA-mediated silencing of FOXP1 decreased binding between TCF7L2 and β -catenin (fig. S4) as well as decreased β -catenin and CBP occupancy on the *AXIN2* promoter and other Wnt-responsive promoters in HCT116 and DB cells (Fig. 4E-G and fig. S5A, S6A-D). FOXP1 silencing also suppressed histone acetylation at the *AXIN2* promoter, which is consistent with a role for FOXP1 in recruiting the HAT CBP (fig. S6E).

The ChIP data and the FOXP1 isoform data suggest that FOXP1 DNA binding is required for Wnt potentiation. To test this notion and to evaluate functional relationships between FOXP1 and the TCF7L2 and LEF1 transcription factors, we performed four gain-of-function reporter experiments. First, we found that FOXP1 and TCF7L2 synergistically promoted β -catenin-dependent transcription, but this synergy was markedly less pronounced with LEF1 (Fig. 4H, S7A). Second, FOXP1 did not affect a GAL4 reporter in cells expressing β -catenin fused to the GAL4-DNA binding domain (Fig. 4I). Third, a FOXP1 DNA binding mutant was unable to potentiate Wnt signaling (Fig. 4J). Lastly, TCF7L2 knockdown blocked the ability of WT FOXP1 to stimulate BAR activity (fig. S7B). These data suggest that the ability of FOXP1 to potentiate β -catenin-dependent transcription requires the FOXP1/TCF7L2 interaction.

To determine if TCF7L2 and FOXP1 sites tend to co-occur, we identified the location of both motifs genome-wide using HOMER (40) and measured their relationship by testing relative distance (Fig. 4K). If the locations of the two motifs were independent of each other, the relative distance will exhibit a uniform distribution between 0 and 0.5 (41). However, we observed that the relative distance between the two motifs was much closer than expected with a median distance of 60 bp (Fig. 4K). We then investigated the enrichment of FOXP1 binding sites near experimentally determined TCF7L2 binding sites by ChIP-seq across multiple cell lines (Fig. 4L) (42). FOXP1 and TCF7L2 sites were significantly co-localized in each cell line evaluated (Fig. 4M). Reciprocal analysis using FOXP1 ChIP-Seq data could not be done because these experiments were done in the absence of Wnt activation, which was required for FOXP1 occupancy at Wnt target genes (Fig. 4D) (15, 17, 26). FOXP1 motifs were identified in 7.8% of the combined set of TCF7L2 binding sites, slightly less than the 12.2% of TCF7L2 motifs identified in the same set (Fig. 4L). The FOXP1 motif ranked among the top 10% of all motifs tested (Fig. 4L). Therefore, our analyses support a physical co-localization of FOXP1 and TCF7L2 based on both *in silico* and *in vitro* experiments.

FOXP1 isoform 1 expression correlates with Wnt activity in DLBCL

Patients with DLBCL characterized by high FOXP1 protein abundance show decreased progression-free survival and increased resistance to R-CHOP, the frontline DLBCL chemotherapy that consists of the therapeutic antibody rituximab (R) and the chemotherapy drugs cyclophosphamide, doxorubicin, vincristine, and prednisone, which are collectively known as CHOP (24, 43). β -catenin protein abundance positively correlates with DLBCL disease progression, which is consistent with its oncogenic role in many epithelial tumors (44). Therefore, we speculated that FOXP1 might promote Wnt/ β -catenin signaling in human DLBCL. To begin to test this, we used 9 human B-cell lymphoma cell lines to determine if FOXP1 correlated with: (i) β -catenin abundance, (ii) β -catenin-target gene expression and (iii) cellular responsiveness to Wnt/ β -catenin small molecule inhibitors. First, as was previously reported, FOXP1 abundance and alternative splicing varied widely across a panel of 9 DLBCL cell lines (Fig. 5A). DB and KARPAS422 cells had FOXP1 isoform 1 as well as high β -catenin abundance (Fig. 5A). In contrast, RCK8 and MEDB1 cells had no detectable FOXP1 or β -catenin. Second, we quantitated *AXIN2* and *NKD1* gene expression before and after treatment with the Wnt inhibitor C59, reasoning that C59 treatment would decrease the expression of Wnt target genes in cells containing an active pathway. Both Wnt target genes were reduced in DB and KARPAS422 cells, but not in FOXP1-low or FOXP1-negative cell lines (fig. S8). Third, we found that FOXP1 abundance correlated with cellular sensitivity to two distinct Wnt pathway inhibitors: DKK1 and XAV939 (Fig. 5B,C). DB and KARPAS422 cells showed the greatest sensitivity to the inhibitors. MEDB1 and RCK8 cells were comparatively resistant to Wnt inhibition. shRNA-mediated silencing of FOXP1 partially rescued XAV939 and DKK-induced death in DB cells (Fig. 5D). Together, these data suggest that within the panel of tested cell lines, Wnt/ β -catenin signaling is active in DB and KARPAS422, which are positive for FOXP1 isoform 1.

FOXP1 promotes Wnt signaling in DLBCL cells

Our data suggest that Wnt inhibitors may show efficacy in DLBCL tumors with high abundance of FOXP1 isoform 1. As a first step in testing this hypothesis, we asked if Wnt pathway antagonists, XAV939 and C59, synergized with doxorubicin and 4-Hydroxycyclophosphamide, which are components in the R-CHOP regimen. Both XAV939 and C59 sensitized FOXP1 expressing cells—but not FOXP1-negative cells—to doxorubicin and 4-Hydroxycyclo-phosphamide (Fig. 5E-G, S9). We also evaluated the importance of FOXP1 and Wnt signaling for DLBCL tumor growth using DB cells in a mouse xenograft model. FOXP1 silencing by shRNA reduced the growth of DB cells, although this reduction was not statistically significant (Fig. 5H,I). To test the effect of Wnt signaling, we overexpressed dominant negative TCF7L2, which binds DNA but lacks the ability to bind β -catenin (45). Overexpression of dnTCF7L2 blocked Wnt signaling in DB cells, as determined by decreased expression of Wnt target genes (Fig. 5J), and significantly inhibited tumor growth ($p < 0.0001$) in a xenograft mouse model (Fig. 5K).

Discussion

We suggest a model in which FOXP1 promotes formation of a protein complex comprising FOXP1, TCF7L2, CBP, and β -catenin on the promoters of Wnt/ β -catenin target genes. The

presence of FOXP1 promotes the acetylation of β -catenin by CBP, and consequently increases the transcription of β -catenin/TCF7L2 target genes. FOXP1 is therefore a potentiation factor for an active Wnt/ β -catenin signaling pathway. Mechanistically, there are many unanswered questions, likely involving molecular effectors that have not yet been identified. FOXP1 was originally characterized as a transcriptional repressor that functions through recruitment of specific transcriptional repressors (46). However, gene expression and ChIP-chip analysis in B-cells suggests that FOXP1 acts as both a transcriptional repressor and activator (18). Because DNA binding motifs do not differ between activated or repressed genes, the polarity of FOXP1 gene regulation is likely dependent upon other cofactors (18). A more complete mechanistic understanding of the coregulators that affect FOXP1 induced potentiation could provide insight into how FOXP1 controls gene transcription both in the context of Wnt signaling and in the regulation of other signaling pathways. For example, the exact mechanism by which FOXP1 recruits CBP is not known. Although other FOX family proteins interact directly with CBP, reciprocal functional analysis on FOXP1 and CBP has not yet been done.

In contrast to the power, throughput and low cost of loss-of-function genetic screening technologies, comparable gain-of-function approaches have been lacking. Given this and the underlying importance of assigning phenotype to gain-of-function genotypes, we began modifying the CD-tagging viral insertional gene trap system for high throughput screening (29) by optimizing the use of mass spectrometry as a means to identify CD-tagged genes within a phenotypically enriched pool of cells. Because mass spectrometry enables quick and relatively inexpensive gain-of-function screening with a robust phenotype, CDt/MS screens can be completed in less than 3 weeks and for a fraction of the cost of sequencing-based target identification (~\$500/screen). Another benefit is that CDt/MS analysis of monoclonal cell lines can reveal the overexpressed tagged protein, the protein-protein interaction network and the associated posttranslational modifications, which provides more information than DNA/RNA-based detection. Moreover, an appreciable percentage of top candidates identified by sequencing-based approaches are likely to be loss of function events caused by viral integration either out of frame or within a promoter. In CDt/MS experiments, only proteins that are overexpressed with a FLAG-tag will be detected, thus decreasing the likelihood of a false positive and streamlining the downstream analysis. That said, insertions that generate truncation protein products are likely, which can be identified by mapping identified peptides to the protein sequence. Lastly, technical improvements to the CDt/MS approach will likely strengthen its value. As opposed to using the FLAG epitope, the biotin ligase system (AviTag) has much stronger binding affinity allowing for more stringent wash conditions and decreasing false positives. Further, re-engineering the fusion tag with an intervening proteotypic linker peptide will permit detection of the tryptic peptide spanning the epitope tag and the N-terminus of the targeted exon.

Diffuse large B-cell lymphoma is the most common subtype of non-Hodgkin lymphoma (NHL), affecting approximately one-third of the 70,000 NHL patients in the U.S. every year. The standard treatment for DLBCL is R-CHOP, which combines a therapeutic antibody with four chemotherapy drugs. Even with the addition of rituximab to the CHOP regimen, more than one-third of patients are refractory to or relapse after first line R-CHOP therapy; their cure rate is only ~10% (47). This has spurred multiple attempts to improve outcome.

However, after more than 30 years of research and numerous clinical trials, advancements to the CHOP chemotherapy backbone remain elusive. Here, we provide data that collectively supports the development, testing and possible future clinical use of Wnt/ β -catenin inhibitors in specific populations of lymphoma. We show that FOXP1 potentiates Wnt/ β -catenin signal transduction through CBP-dependent acetylation of β -catenin on the promoters of Wnt target genes in DLBCL. DLBCL tumors with high FOXP1 protein abundance show poor prognosis and decreased response to R-CHOP therapy (24, 43), thus supporting our hypothesis that specific FOXP1 protein isoforms may possibly control or correlate with sensitivity to Wnt pathway inhibitors, several of which are in clinical trials. In agreement with this notion, FOXP1 knockdown and Wnt pathway inhibition significantly decreased xenograft growth of a FOXP1 high DLBCL cell line. This xenograft work is first step towards the potential use of Wnt inhibitors in DLBCL patients.

Aberrant Wnt activation is a hallmark of many epithelial tumors. Comparatively, the impact of Wnt signaling in lymphoma is less understood, although various studies suggest increased Wnt signaling in at least a subset of DLBCL patients (44, 48-51). First, increased nuclear β -catenin protein, a marker of Wnt pathway activation, is detected in some DLBCL tumors, which correlates with poor outcome (44). Second, Wnt3a-dependent signaling within and between clonal cell populations in DLBCL maintains population equilibrium; preventing Wnt signaling in this context results in decreased cell growth and colony formation (51). Consistent with this finding, the Wnt antagonist-encoding *DKK1* is the most induced transcript in Rituximab-treated DLBCL cell lines, and high *DKK1* mRNA abundance predicts improved patient outcome (52). Third, constitutive Wnt pathway activity occurs in a large fraction of mantle cell lymphomas (53, 54). Fourth, the Wnt pathway is constitutively active in multiple myeloma, owing in part to increased abundance of the B cell lymphoma 9 (BCL9) protein, which activates β -catenin (55). A small molecule inhibitor of the BCL9- β -catenin interaction blocks multiple myeloma cell growth (56). Fifth, three Burkitt's lymphoma cell lines have been reported to be unresponsive to Wnt3a ligand, supporting the complexity of Wnt pathway activity in lymphomagenesis (50).

Beyond lymphoma, functionally connecting FOXP1 to the Wnt signaling pathway may have ancillary benefits. *FOXP1* RNA expression positively correlate with *ER α* expression in breast and ovarian tumors, raising the possibility that Wnt pathway antagonists might show therapeutic potential in endocrine resistant ER α tumors (22, 57, 58). Increased FOXP1 abundance promotes glioblastoma growth; although Wnt activation in glioblastoma is common, the mechanisms underlying this increased activation are unclear (59-61). Outside of cancer, *de novo* mutations in *FOXP1* are associated with intellectual disability, autism, and language impairment, and interestingly, neural specific deletion of *Foxp1* causes autism like behavior in mice, but as of yet have not been connected to altered Wnt signaling (62-65).

Although definitive functional data are lacking across multiple tumor types, comparative analysis of RNA and protein abundance of various cancers and matched normal tissues suggest that FOXP1 may behave as both a tumor suppressor and an oncogene, depending on tissue type and disease stage (20, 66-68). The abundance of specific splice variants of FOXP1 is thought to figure prominently in determining its impact on cancer initiation and

progression, although the correlative studies of FOXP1 expression in cancer must be interpreted with caution, as most did not quantify isoform-specific expression. Our mouse xenograft study suggests that FOXP1 isoform 1 promotes tumor growth, a result consistent with the oncogenic functions of Wnt signaling in epithelia cancers and lymphoma (44, 51). In agreement with our result, diagnostic chromosomal translocations place the *FOXP1* gene downstream of strong immunoglobulin heavy chain (*IG*) promoters in some DLBCL tumors, resulting in increased abundance of FOXP1 isoform 1 (68). Non-*IG/FOXP1* translocations often result in increased abundance of N-terminal truncations of FOXP1. These truncation products have been postulated to promote lymphoma tumor progression, rather than initiation, possibly by acting in a dominant negative fashion (66, 67). On this point, we found that like isoform 1, several of the smaller FOXP1 splice isoforms promote Wnt signaling and two of the isoforms block Wnt pathway activation. How the abundance of FOXP1 isoforms—either alone or in combination—impacts specific cellular targets such as Wnt signaling *in vivo* remains a pressing question for the field.

Taken together, our results suggest that FOXP1 promotes β -catenin-dependent transcription through CBP-mediated protein acetylation, resulting in increased Wnt signaling. As several human diseases show altered FOXP1 expression and splicing, we suggest that it may be beneficial to explore possible therapeutic efficacy of Wnt inhibitor therapies in FOXP1-altered disease.

Materials and Methods

Expression Constructs

pGL3-BAR, pSL9-BAR-LS, pSL9-BAR-Venus, CMV-Renilla, pGLUE-CTNNB1, pGLUE-point mutant- β -catenin, pGLUE-TCF4, pGLUE-LEF1, and FLAG- β -catenin were described previously (7, 69, 70). The SD-1 mutagenesis vector was a kind gift from George Stark and was modified to replace GFP with BFP to make the CDBF vector (29). All three reading frame versions of the SD-1 vector were converted to the CDBF vector and named CDBF, CDBF +1, and CDBF+2. pDONR223-FOXP1 was obtained from the Plasmid Database (Harvard University) and cloned into pHAGE-GW vector (70). pSL9-BAR-NLS cherry was made from mutating pSL9-BAR-Luciferase using site directed mutagenesis to allow for subcloning of NLS-Cherry. The NLS-Cherry construct was a gift of Jon Lane (71). FOXP1 isoform 6 and 8 were cloned from pooled cDNA from DLBCL cell line panel. Isoforms 3 and 12 were made by Q5-Site-Directed mutagenesis (New England Biolabs, Ipswich, Massachusetts). Isoform 5 was obtained Human ORFeome Collection. mFoxp1, mFoxp1-ES, and pLKO-FOXP1 15 were obtained from Addgene (15).

Chemicals and Antibodies

FLAG-M2 (F3165) and TUBB (T8453) were purchased from Sigma Aldrich (St. Louis, MO). TCF7L2 (2569), acH3K9 (9649), CBP (7389), FOXP1 (4402), β -catenin (8480), Lys⁴⁹ acetylated- β -catenin (9534) and rabbit IGG (3900) were all purchased from Cell Signaling (Danvers, MA). LEF1 (A303-486A) is from Bethyl Laboratories (Montgomery, TN). Mouse β -catenin (610153) antibody was from BD transduction. CT99021 was purchased from Axon Med Chem (Groningen, Netherlands). Trichostatin A, Doxorubicin

HCL, Etoposide, XAV938, Anacardic Acid, and CPTH2 were from Sigma Aldrich (St. Louis, MO). DKK1, Wnt3a, TNF α , and TGF β 1 were purchased from Peprotech (Rocky Hill, NJ). C-59 was purchased from Cellagen Technology (San Diego, CA). 4-Hydroxycyclophosphamide was a gift of Howard McLeod. Wnt3a conditioned media was made according to the ATCC protocol.

Cell Culture

All cells were cultured at 37°C and 5% CO₂. HEK293T, HCT116, DLD1, HT1080, L-Wnt3a, and L-control cells were obtained from the ATCC. A375 cells were from Channing Der's lab (University of North Carolina). A375 BAR-Venus was a gift from Dr. Randy Moon. A375 BAR-Cherry, HCT116 BAR/REN, HT0180 BAR/REN, DLD1 BAR/REN, and HEK293T BAR/REN cells were stably infected and grown in 1 μ g/mL puromycin. Adherent cell lines were grown in DMEM with 10% FBS. DB and KARPAS422 cells were from the ATCC. BJAB, FARAGE, MEDB1, OCI-LY3, RCK8, and SUDHL2 were gifts from Dr. Sandeep Dave (Duke University). DB, KARPAS422, RCK8, and MEDB1 were grown in RPMI with 15% FBS. OCI-LY3 cells were grown in RPMI with 20% human serum. BJAB, FARAGE, HBL1, and SUDHL2 were grown in RPMI with 10% FBS. The cell lines are subtyped as GCB (BJAB, DB, FARAGE, KARPAS422, and RCK8), ABC (Hwath BL1, OCI-LY3, and SUDHL2) or PMBL (MEDB1) (72, 73).

CDt/MS protocol

CDt/MS vector virus was made using equal amounts of CDBF, CDBF +1, and CDBF+2 and titrated using Lenti-X p24 rapid titer kit. 1 $\times$ 10⁷ A375 BAR-Venus cells were infected at MOI of 0.5 in a 15cm plate overnight. Three-five days post infection, GFP/BFP positive cells were obtained using Fluorescence Automated Cell Sorting. A BFP only population was also sorted as a negative control. The GFP/BFP population was sorted 2 additional times to ensure a highly pure population. The initial sort yielded few cells (~1000), while each additional sort increased the BFP/GFP positive population. Purity was determined on the LSRII Flow Cytometer. All FACS and Flow Cytometry experiments were done at UNC-Chapel Hill – Flow Cytometry core facility.

After three sorts, five - 15cm plates were grown up for each condition. Cells were washed twice on ice with cold PBS. Cells were lysed in cold RIPA buffer with protease and phosphatase inhibitors and immunoprecipitated for 1 hour at 4°C with FLAG M2 beads from Sigma Aldrich (St. Louis, MO). Cells we washed 3 $\times$ with cold RIPA buffer and then washed 3 $\times$ with cold RIPA buffer with 1M NaCl. All spins were done at 4°C and 2,000RPM for 5 minutes. The resulting beads were then prepared for MS using the FASP purification system from Expedon (San Diego, CA) per manufacturer's instruction. Mass spectrometry and peptide identification were done as described previously (74). Proteins were scored by two methods. The CompPASS WD-score was used to determine which proteins were bona fide hits in the CDt/MS experiments as compared to other FLAG APMS experiments from the Major Lab (31). Fold change was calculated for each hit by taking the ratio of total spectral counts between CDt/MS and control experiments, using a pseudo-count of 1 (75). High confidence hits had a top 5% CompPASS score and a fold change above 3.

Transfections and siRNA

Transit 2020 or Transit293 was used in all gain of function experiments according to the manufacturer's instructions (Mirus Bio, Madison, WI). Lipofectamine 2000 was used in co-transfection experiments with siRNA and plasmid DNA (Invitrogen, Carlsbad, CA). RNAiMAX was used for siRNA only transfections. All siRNAs used were Stealth siRNA from Invitrogen and sequences are listed in Supplemental Table 3 (Carlsbad, CA).

Luciferase Assays

BAR assays were done in 96 well plates and transfected for 24 hours with 10ng CMV-*Renilla*, 20ng BAR-pGL3, and 70ng of indicated constructs. Unless stated otherwise, all treatments were for 16 hours. For B-cell derived luciferase assays, the 7TFC luciferase reporter (Addgene) was used and normalized using the internal Cherry control or co-transfected CMV-*Renilla*. Luciferase was detected using Promega Dual-Luciferase Reporter Assay system (Madison, WI) and read on Enspire plate reader from Perkin Elmer (Waltham, MA). Compounds were used at the following concentrations: CT990201 - 10 μ M, C59 - 1 μ M, XAV938- 10 μ M, and DKK - 200ng.

Concanavalin A Pulldown-

293T cells were transfected in 6 well plates. After 24 hours, cells were split into two 10cm plates and allowed to adhere overnight to achieve a density of 50% confluence. Cells were treated for 2 hours before lysis in 500 μ l of RIPA buffer with protease inhibitors. Lysates were cleared on pre-washed Concanavalin A beads for 2 hours.

Lys⁴⁹ Acetylation Detection

Cell lines were transfected with indicated constructs for 48 hours. Prior to lysis, cells were treated with Wnt3a conditioned media and 1 μ M TSA for 2 hours. Cells were lysed in RIPA buffer with protease inhibitors and 1 μ M TSA.

Zebrafish

Whole zebrafish RNA was extracted from 24-hour post fertilization (hpf) old embryos and cDNA was synthesized using oligodT primers. The zebrafish *foxp1b* open reading frame was amplified with forward primer 5'-ATT ATA GAA TTC CCA CCA TGA TGC AAG AGT CGG GGA CAG-3' and reverse primer 5'-TAT AAT CCG CGG TTAC AGC ATG TCC TCG GTG CC-3' and cloned into the pCS2⁺ plasmid vector. Capped mRNA was synthesized from this template using the Ambion mMessage mMachine kit (Thermo Fisher Scientific, Waltham, USA) according to manufacturer's instructions. Zebrafish *foxp1b* or control (membrane bound *Renilla* luciferase) mRNA was injected into the cytoplasm of one-cell-stage zebrafish embryos, which were analyzed at 24 hpf. For over-expression, 100 pg of *foxp1b* or control mRNA was injected per embryo. To test for synergy with Wnt8, 20 pg of *foxp1b* or control mRNA was injected into embryos heterozygous for the *Tg(hsp70l:wnt8a-GFP)*^{w34} transgene followed by a heat shock at 70% epiboly (1 hr at 37°C) (76). Phenotypes were scored as described previously (77) and significance was determined using the Chi-square test.

Microinjection and Whole-Mount In Situ Hybridization

The $6\times Tcf/Lef-miniP:d2EGFP^{isi04}$ transgenic fish line was used (78). Capped sense RNA was synthesized *in vitro* using mMessage mMachine kits (Ambion). Heterozygous $6\times Tcf/Lef-miniP:d2EGFP$ zebrafish were crossed out followed by RNA injection into the cytoplasm of one-cell stage zebrafish embryos using standard procedures. 100 pg of *foxp1b* RNA was injected per embryo. Equimolar amounts of *Renilla* luciferase RNA was used as control. At 8 hours post fertilization embryos were fixed overnight in 4% PFA. Whole-mount *in situ* hybridization was performed as described previously (79). A digoxigenin-labeled antisense RNA probe was prepared from a template encoding *d2EGFP* (corresponding to residues 422–461 of mouse ornithine decarboxylase) (80).

Fluorescence Reporter Assays

A375 BAR-NLS-Cherry reporter cells were transfected for 24 hours before treatment with Wnt3a or L-cell control media. Fluorescence was quantified and analyzed every 2 hours on Incucyte Cell Player by Essen Biosciences (Ann Arbor, MI).

Stable Knockdown

shRNA against FOXP1 was obtained from Addgene (15) and pLKO-control was a gift of the Der Lab (UNC-Chapel Hill). Virus was made using psPAX2 and PMD2.G according to Addgene protocol. Cell lines were spin-infected for 2 hours at 2,000RPM with 0.8 μ g/mL polybrene and then incubated in virus over night with 0.15 μ g/mL polybrene. Cells were selected in 1 μ g/mL puromycin.

Gene expression Analysis

RNA was isolated from cells using Trizol from Invitrogen (Carlsbad, CA). 1 μ g of RNA was reverse-transcribed using cDNA synthesis kit from Thermo-Fisher using both Oligo-dT and random hexamers primers. qPCR was done using Fast Syber Green Master Mix from Applied Biosystems (Carlsbad, CA) per manufacturer's instructions on Applied Biosystem 7400HT. Primers listed in Supplemental Table 4 and were designed using Primer3plus or were published previously (7, 81).

ChIP Protocol

All ChIP experiments were done as described previously with the primers listed in Supplemental Table 5 (82).

Cell Viability

30,000 cells were plated in 150 μ L of media in a 96 well plate and incubated overnight. For single compound treatments, cells were treated with indicated compounds for 48 hours before reading with PrestoBlue from Invitrogen according to manufactures instructions (Carlsbad, CA). Fluorescence was quantified on Perkin Elmer Enspire plate reader (Waltham, MA). For synthetic lethality, cells were treated with indicated Wnt antagonists 2 hours prior to treatment with chemotherapeutics and then read as previously indicated.

Xenograft Mouse study

1×10^7 DB cells with scrambled shRNA or shRNA against *FOXP1* were injected subcutaneously into NOD.CB17-*Prkdc*^{scid}/J (NOD scid) mice in Matrigel as previously described(83). Tumors reached measurable size within 2-3 weeks post injection. Tumor length and width were then measured daily with digital carbon fiber calipers to the nearest millimeter. Volume of tumors was calculated by the following formula:

where width is the shorter of the 2 measurements. The study was ended when the largest tumor was measured at 2000mm³. Linear mixed models, allowing both a random intercept and slope, were used to compare changes in tumor volume over time, and p-values for the difference in slopes are reported.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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One Sentence Summary: A mass-spectrometry coupled gain-of-function screen connects FOXP1 expression to Wnt/ β -catenin signal transduction in B-cell lymphoma.

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Figure 1. Identification of FOXP1 as a promoter of Wnt signaling (A)

Schematic of the CDt/MS approach. IRES: internal ribosomal entry site, LTR: long terminal repeat. **(B,C)** A375 cells harboring a β -catenin activated reporter (BAR), which reports mCherry protein expression, were transfected with *FOXP1* (splice isoform 1) or *GFP* control; mCherry fluorescence intensity quantitation is shown in (B). At 24 hours post-transfection, cells were treated with control or Wnt3a conditioned media (C). Fluorescence intensity was quantified every hour using the Incucyte Cell Player. Error bars represent standard error between 3 biological replicate wells. **(D)** Quantification of BAR-driven luciferase reporter assay in HEK293T cells transfected with *FOXP1* or control and treated with Wnt3a or control conditioned media. **(E-G)** Quantification of β -catenin-dependent reporter activity in HCT116, DLD1, and DB cells after *FOXP1* overexpression. **(H-J)** Quantification of BAR activity in the indicated stable BAR/Renilla infected cell line after transfection with control, FOXP1-A, or FOXP1-B siRNA. Cells were treated with control or Wnt3a conditioned media, as indicated. **(K,L)** Quantification of *AXIN2* and *NKDI* mRNA following FOXP1 overexpression. **(M,N)** *AXIN2* and *NKDI* mRNA quantification following transfection with the indicated siRNAs, as determined by qRT-PCR. **(B-N)** Significance was determined by Wilcoxon Signed Rank across a minimum of three independent experiments. * < 0.05; **<0.005; ***< 0.0005. Error bars represent SEM.

Figure 2. FOXP1 potentiates Wnt signaling downstream of the destruction complex
(A) Quantification of BAR activity after FOXP1 overexpression in the presence or absence of CT99021 treatment. **(B)** Western blot of β -catenin in Concanavalin A (CONA) stripped lysates. HEK293T cells were transfected with control or *FOXP1* before treatment with control or Wnt3a conditioned media. **(C)** Quantification of BAR luciferase reporter assay in HEK293T cells transfected with *FOXP1* or control and then treated with Vehicle (VEH), C59, rhDKK1, or XAV939. **(D)** Quantification of BAR activity in HEK293T cells transfected with control or *FOXP1* and control or β -catenin siRNA. **(E)** Representative images of different classifications of Wnt or *foxp1b* overexpressing embryos at 24 hours post fertilization (hpf). The classes represent increasing severity of anterior truncations (reduction and loss of eyes and midbrain-hindbrain-boundary, arrow in classes 1-3) and reduction in dorsal mesoderm derivatives (notochord in class 4, arrow). **(F)** Quantification of *foxp1b* injected embryos binned into classes representing severity of Wnt phenotype. Embryos were injected with *foxp1b* (n=63) or control RNA (n=55). **(G)** Quantification of *foxp1b* or control injected embryos overexpressing *wnt8*. 25pg of *foxp1b* or control RNA was injected in *hs:wnt8* transgenic embryos and *wnt8* expression induced by heat-shock during gastrulation. Control (n=70), *foxp1b* (n=64), control+*wnt8* (n=49), and *foxp1b* + *wnt8* (n=79) **(H)** At 8 hpf, expression of GFP RNA in $6\times Tcf/Lef1-miniP:d2EGFP^{isi04}$ transgenic embryos is expanded in embryos injected with 100 pg *foxP1b* RNA (n=52 embryos, 100% of the embryos show this phenotype) compared to embryos injected with equimolar amounts of control *RLuc* RNA (n=69 embryos). **(I)** Quantification of Wnt target genes in *foxp1b* injected embryos 24 hours post fertilization. Target genes were normalized to GAPDH gene and presented as fold-induction over GFP control. (N=4 embryos per condition) **(J)**

HEK293T cells were transfected with the indicated transcriptional reporter, CMV-*Renilla* and either *GFP* control or *FOXPI*. Cells were then treated with the indicated recombinant protein before luciferase quantitation. **(A-D,F-G, I-J)** Significance was determined by Wilcoxon Signed Rank across a minimum of three independent experiments. * < 0.05; **<0.005; ***< 0.0005. Error bars represent SEM.

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Figure 3. FOXP1 promotes acetylation of β -catenin by the acetyltransferase CBP

(A) β -catenin reporter quantitation in HCT 116 cells following FOXP1 overexpression in the presence or absence of Anacardic Acid, CPTH2, or ICG-001. (B) Quantification of BAR activity in HEK293T cells transfected with control or *FOXP1*, and control, β -catenin, CBP-A, or CBP-B siRNA. Inset: Western blot showing CBP protein abundance following siRNA transfection. (C) Western blot analysis of β -catenin acetylated at Lys⁴⁹ and total β -catenin after transfection with increasing amounts of FOXP1 plasmid. LI-COR-based quantification is shown above. (D) Western blot analysis of β -catenin acetylated at Lys⁴⁹ and total β -catenin in nuclear and cytosolic extracts from HEK293T cells expressing control or FLAG-FOXP1. (E) HEK293T cells transfected with control or *FOXP1*-specific siRNAs, treated with Wnt3a and TSA, and Western blotted for the indicated proteins. (F) DB cells stably expressing control or FOXP1 shRNA were analyzed by Western blot for FOXP1, β -catenin, and acetylated- β -catenin. (G) Western blot of the acetylation of β -catenin at Lys⁴⁹ in HEK293T cells after transfection with control or FOXP1 plasmid and the indicated siRNAs. (N=3 biological replicates) (H) Quantification of BAR luciferase reporter after transfection with β -catenin T41A, β -catenin T41A, K49R, or GFP control and FOXP1 or control. (C-F) LI-COR-based quantification of the Western blots are shown as a ratio of acetyl- β -catenin/total β -catenin. Error bars represent S.E.M. of tubulin-normalized biological triplicates. (A-H) Significance was determined by Wilcoxon Signed Rank across a minimum of three independent experiments. * < 0.05; ** < 0.005; *** < 0.0005. Error bars represent SEM.

Figure 4. FOXP1 co-complexes with TCF7L2, β-catenin, and CBP

(A) HEK293T cells were transiently transfected with *FLAG-FOXP1* or *FLAG-HC-RED* as control before protein extraction and FLAG immunoprecipitation. Co-complexed endogenous proteins were detected by Western blot analysis. (N=3 biological replicates) (B) Immunoprecipitation and Western blot analysis of endogenous FOXP1 in HCT116 cells revealed association with endogenous β-catenin, acetylated β-catenin, CBP, and TCF7L2. (N=3 biological replicates) (C) Immunoprecipitation and Western blot analysis of endogenous FOXP1 in HEK293T cells revealed association with endogenous β-catenin and acetylated β-catenin, but only after 2 hours of Wnt3a treatment. (N=3 biological replicates) (D) ChIP analysis of the *AXIN2* promoter Wnt response element (WRE) or *AXIN2* open reading frame (ORF) in HEK293T cells after treatment with control or Wnt3a conditioned media. Control IgG, FOXP1 or β-catenin ChIP antibodies were used. (E) ChIP analysis of stable knockdown DB cells using FOXP1 or β-catenin antibodies. (F,G) ChIP analysis on *AXIN2* WRE and *AXIN2* ORF of either β-catenin (F) or CBP (G) following FOXP1 knockdown in HCT116 cells. (H) Quantification of BAR activity in HEK293T cells transfected with equal amounts of control or FOXP1 and/or TCF7L2 or LEF1. Significance was determined by paired T-tests across a minimum of three independent experiments. * < 0.05; Error bars represent SEM. (I) Quantification of GAL4 reporter in cells transfected with GAL4-DBD-β-catenin and Control, FOXP1, or EP300. Data is presented as fold induction over GAL4-DBD alone. (J) Quantification of BAR-luciferase transfected with control, FOXP1-WT, or FOXP1 DNA binding mutant. (D-J) All experiments were performed in biological triplicate and p-values were determined by Wilcoxon Signed Rank test. * < 0.05; ** < 0.005. Error bars represent SEM. (K) Relative distances between TCF7L2 (blue) and

FOXP1 (red) motifs were calculated. The observed distribution of the relative distance between the two motifs was plotted (red dot) together with relative distribution after randomization (grey dot). $p < 2.2 \times 10^{-16}$ Kolmogorov–Smirnov test. **(L,M)** All HOMER derived motifs were searched in the set of TCF7L2 ChIP-seq peaks. The significance of the association for each motif was plotted by rank order. FOXP1 is highlighted (red). Inset shows the fraction of TCF7L2 motifs (blue) and FOXP1 motifs (green) in the set of regions enriched by TCF7L2 ChIP. A randomized background is shown for control.

Figure 5. FOXP1 potentiates Wnt signaling in DLBCL

(A) Cell lysates were Western blotted for FOXP1, β-catenin, and acetylated-β-catenin. (N=3 biological replicates) (B,C) Viability of cell lines treated with XAV939 or rhDKK. (D) Cell viability was determined in stable knockdown DB cells treated with rhDKK or XAV939. Viability was normalized to DMSO control. p-value = * <0.05 was measured between the 2 stable cells lines for indicated treatment. (E-G) Cell viability of the indicated cell lines treated with XAV939 and increasing doses of doxorubicin. p-value < 0.05 between treatments at given doxorubicin dose. (H) Quantification of tumor size in DB cell mouse xenograft in 2 independent experiments with 6 mice per group per replicate (N=12). P=0.07 as determined by linear mixed models. (I) RNA was isolated from resected tumors and *FOXP1* gene expression was determined by qRT-PCR analysis. p-values were determined by Wilcoxon test on all mouse tumors. * $p<0.05$ (N=6 mice per group). (J) RNA was isolated from dnTCF4 stable cell lines treated with DMSO or CT99021. (N=3 biological replicates) (K) Quantification of tumor size in DB cell mouse xenograft in 2 independent experiments with 7 or 9 mice per group per replicate (N=16). Significance was determined by linear mixed models. (A-G) were performed in biological triplicate and p-values were determined by Wilcoxon Signed Rank test. * <0.05 ; ** <0.005 ; *** < 0.0005 . Error bars represent SEM.