Sp5 and Sp8 recruit β-catenin and Tcf1-Lef1 to select enhancers to activate Wnt target gene transcription

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The ancient, highly conserved, Wnt signaling pathway regulates cell fate in all metazoans. We have previously shown that combined null mutations of the specificity protein (Sp) 1/Klf-like zinc-finger transcription factors *Sp5* and *Sp8* (i.e., *Sp5/8*) result in an embryonic phenotype identical to that observed when core components of the Wnt/β-catenin pathway are mutated; however, their role in Wnt signal transduction is unknown. Here, we show in mouse embryos and differentiating embryonic stem cells that Sp5/8 are gene-specific transcriptional coactivators in the Wnt/β-catenin pathway. Sp5/8 bind directly to GC boxes in Wnt target gene enhancers and to adjacent, or distally positioned, chromatin-bound T-cell factor (Tcf) 1/lymphoid enhancer factor (Lef) 1 to facilitate recruitment of β-catenin to target gene enhancers. Because *Sp5* is itself directly activated by Wnt signals, we propose that Sp5 is a Wnt/β-catenin pathway-specific transcripton factor that functions in a feed-forward loop to robustly activate select Wnt target genes.

Sp5/Sp8 | transcription | stem cells | Wnt | Tcf/Lef

Signaling pathways in multicellular organisms have evolved over millions of years to accommodate complex programs of tissue-specific gene expression. One such pathway, the Wnt/β-catenin pathway, regulates gene expression by elevating the cytosolic levels of the transcription coactivator β-catenin (1). Stabilized β-catenin translocates to the nucleus, where it interacts with the DNA-bending, DNA-binding Tcf1 and Lef1 transcription factors (TFs), which subsequently replace Groucho/Tcf3 repressor complexes on Wnt target gene enhancers (2). β-Catenin interacts with cell context-dependent cofactors (web.stanford.edu/group/nusselab/cgi-bin/wnt/) to associate with RNA polymerase II and the general transcription apparatus to activate transcription. However, the nature of the β-cateninTcf/Lef enhancer-binding protein complex and the mechanisms that facilitate its association with regulatory elements at Wnt target genes remain poorly understood.

The formation of a Wnt signaling center during gastrulation is essential for animal development (3). Secreted Wnts emanating from the primitive streak regulate the fate of posterior progenitors, including the neuromesodermal progenitor (NMP), an embryonic cell that depends upon *Wnt3a* for self-renewal and mesodermal differentiation and that gives rise to the spinal cord, dermis, and musculoskeletal system of the trunk and tail (4–6). Embryos lacking *Wnt3a*, *Ctnnb1* (β-catenin), T-cell factor (*Tcf*) 1 and lymphoid enhancer factor (*Lef*) 1, or specificity protein (*Sp*) 5 and *Sp8* display similar severe posterior truncations caused by the loss of NMPs (7–10). These genes define a syn-phenotype group that, together with the genetic interactions observed between *Sp5* and *Wnt3a*, suggests that Sp5/8 could be effectors of Wnt signaling. Sp5/8 are closely related to Sp1, which is one of the first identified eukaryotic TFs (11) and is frequently associated with the regulation of housekeeping genes. In contrast to the ubiquitously expressed *Sp1*, *Sp5* expression is restricted to sites of Wnt activity (12). Here we show that Sp5/8 bind DNA, directly interact with Tcf1/Lef1, and promote the association of β-catenin with chromatin to activate Wnt target genes, suggesting that Sp5/8 are new components of a Wnt-directed transcription complex.

Results

Sp5/8 Activate Wnt/β-Catenin Target Gene Expression. To elucidate the potential role of *Sp5/8* as transcriptional effectors of the Wnt/β-catenin pathway, we overexpressed *Sp8* (13) in NMPs, in vivo, using the *T-Cre* driver, and a Cre-activated B6.Cg-*Gt(ROSA)26Sortm1(rtTA,EGFP)Nagy*/J (rtTA)-expressing mouse line (4, 14) (i.e., *T-Cre;Sp8*^{GOF}) and assayed for the expression of several well-established Wnt/β-catenin target genes, including *T* (15), *Sp5* (16), and *Axin2* (17). *T-Cre;Sp8*^{GOF} embryos showed a dramatic expansion of *T*, which is indicative of an expanded embryonic posterior progenitor population, as well as highly elevated expression of the universal Wnt/β-catenin target genes, *Axin2* and *Sp5* (Fig. 1*A*), indicating that *Sp8*, and likely *Sp5*, can activate at least some Wnt/β-catenin target genes. Conversely, the expression of *T* reached only 25% of control levels when *Sp5/8* double knockout (DKO) embryonic stem cells (ESCs) were treated with the Wnt agonist CHIR99021, indicating that Sp5/8 are required for ESC to properly respond to a Wnt stimulus (Fig. 1*B*). *T* expression was similarly impaired upon in vitro differentiation of *Sp5/8* DKO ESCs (Fig. 1*C*), indicating an impaired response to endogenous Wnts. Taken together, these results suggest that Sp5/8 transduce Wnt signals.

Significance

Deciphering the mechanisms that underlie stem cell growth and differentiation is key to understanding how embryos develop and will lead to important applications in regenerative medicine. Wnt proteins are powerful regulators of stem cells. We have determined that the Sp1-like transcription factors, Sp5 and Sp8, are components of the Wnt/β-catenin signaling pathway. Sp5/8 promote the differentiation of pluripotent progenitors into the multipotent mesoderm progenitors that largely generate the trunk musculoskeletal system. Unexpectedly, Sp5/8 functions to recruit the transcriptional coactivator β-catenin to select enhancers to stimulate expression of a subset of Wnt target genes. This study reveals a more refined level of Wnt/β-catenin target gene regulation and suggests previously unidentified ways to manipulate the expression of specific Wnt targets.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE73084).

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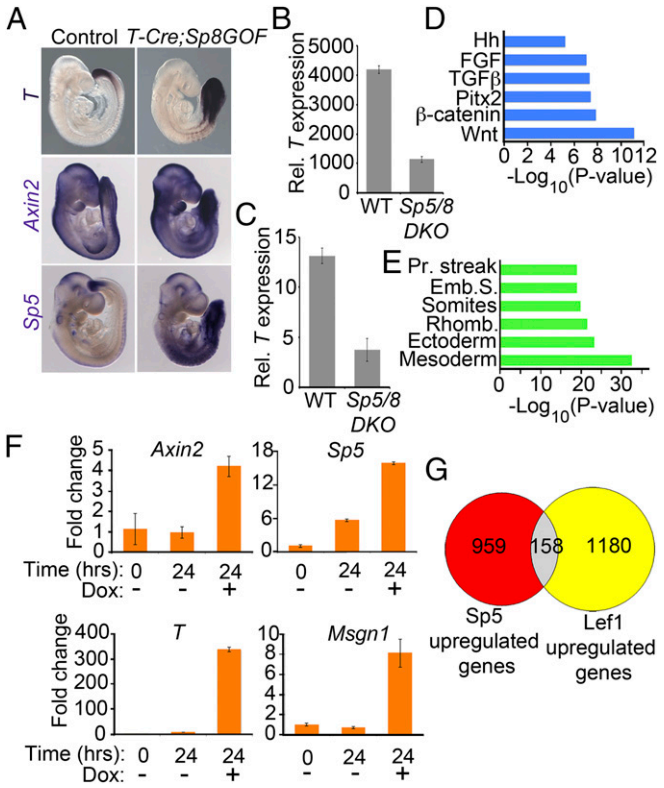

Fig. 1. Sp5/8 regulate Wnt/β-catenin target gene expression. (*A*) Whole-mount in situ hybridizations analysis of *T-Cre;Sp8*^{GOF} mutants shows that *Sp8* overexpression promotes expansion of Wnt-dependent progenitors and target gene expression. (Total magnification: 63×.) (*B*) Induction of *T* expression by CHIR99021 is impaired in *Sp5/8* DKO ESCs. (*C*) Induction of *T* expression by endogenous Wnt is impaired in *Sp5/8* DKO ESCs. (*D*) GO statistical analysis of DEGs in F-Sp5–expressing ESCs for enrichment of signaling pathway genes. (*E*) GO analysis of DEGs in F-Sp5–expressing ESCs assessed by tissue type. (*F*) RT-qPCR analysis of candidate F-Sp5 target gene expression relative to *Gapdh*$^{-/+}$ Dox treatment (24 h), normalized to day 2 ($t = 0$ h) expression levels. For *Sp5* expression, only endogenous transcripts were measured. Error bars = 1 SD for this representative experiment. (*G*) Venn diagram representing the overlap (gray) in genes induced by iF-Sp5 (red) or iF-Lef1 (yellow). The overlap is highly statistically significant ($P < 6.916e^{-62}$, hypergeometric test). All RT-qPCR is normalized to *Gapdh* levels. Emb. S., embryonic structures; Fgf, fibroblast growth factor; Hh, hedgehog; Pr., primitive; rel., relative; Rhomb., rhombencephalon; Tgfβ, transforming growth factor β.

To address whether Sp5/8 broadly regulate Wnt/β-catenin–dependent developmental gene programs, we generated doxycycline (Dox)-inducible, Flag (F)-epitope–tagged Sp5 and Sp8-expressing ESC lines (i.e., *iF-Sp5* and *iF-Sp8*) to identify the Sp5/8 transcriptome (Fig. S1 *A* and *B*). Comparisons of the global gene-expression patterns in untreated and Dox-treated *iF-Sp5* ESC 24 h after Dox administration identified 2,057 differentially expressed genes (DEGs) ($q < 0.05$, fold-change ≥ 1.5) (Fig. S1*C*). Gene Ontology (GO) analysis of these DEGs showed that genes associated with the Wnt/β-catenin pathway, and mesoderm development, were significantly up-regulated by Sp5 (Fig. 1 *D* and *E*). This finding was confirmed by quantitative RT-PCR (RT-qPCR) expression analysis of the universal Wnt target genes *Axin2*, *Sp5* (only endogenous *Sp5* transcripts were assessed), and the embryo Wnt target genes *T* and *Msgn1* (Fig. 1*F*) (15–19). Sp8 similarly activated the same four Wnt/β-catenin target genes, suggesting that Sp5 and Sp8 have equivalent activity (Fig. S1*D*). Notably, GO analysis of down-regulated genes revealed that Sp5 generally inhibits genes associated with neural development, but not the Wnt/β-catenin pathway (Fig. S1 *E* and *F*), consistent with a role for Sp5 in both neural and mesodermal fate selection. By comparing the genes induced by F-Sp5 with those induced by overexpression of the Wnt/β-catenin effector F-Lef1 (Fig. S1 *A*–*C* and *G*), we identified 158 genes that were up-regulated by both factors but only 47 that were down-regulated by both (Fig. 1*G* and Fig. S1*H*). In addition to *Axin2*, *T*, and *Sp5*, common genes also included the well-characterized Wnt targets *Apcdd1* and *Tnfrsf19* (15–17, 20), strongly suggesting that Sp5/8 regulate Wnt/β-catenin gene programs.

We next asked if Sp5 was binding to regulatory DNA to directly activate Wnt target genes. ChIP-seq analysis of Dox-induced *iF-Sp5* cells identified 9,428 Sp5 DNA binding events, associated with 6,236 unique genes, that were enriched in proximal regulatory regions 5′ of the transcriptional start site (TSS) (i.e., –5 kb to TSS) (Fig. 2 *A* and *B* and Fig. S2 *A* and *B*). Motif analysis determined that Sp5 primarily associated with GC boxes, similar to other Sp/Klf Zn^{2+}-finger TFs (Fig. 2*C* and Fig. S2*C*).

Integration of the F-Sp5 RNA-seq and ChIP-seq datasets identified 892 DEGs bound by Sp5 (i.e., direct Sp5 target genes) (Fig. 2*D* and Dataset S1). Separate GO pathway analysis of up-regulated (517 of 892) and down-regulated (375) genes showed

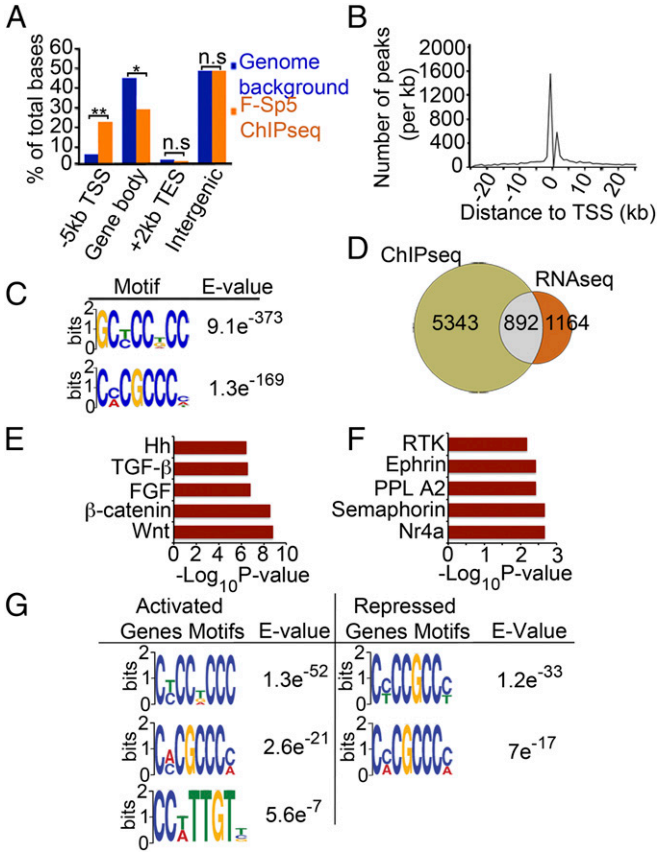

Fig. 2. ChIP-seq characterization of the genome-wide, Sp5 DNA-binding profile. (*A*) Genome distribution of Sp5 binding events relative to TSS and transcription end sites (TES). $**P = 2.2 \times 10^{-9}$; $*P = 3.4 \times 10^{-5}$; n.s., not significant. (*B*) Graphical representation of the Sp5 binding frequency relative to TSS. (*C*) The two most significant sequence motifs enriched at Sp5 ChIP-seq peaks are Sp5-binding GC boxes. (*D*) Integration of Sp5-regulated genes (RNA-seq) with the Sp5 ChIP-seq dataset identified 892 candidate direct Sp5 target genes. (*E*) GO pathway analysis of the up-regulated genes in the Sp5 target gene set. (*F*) GO pathway analysis of the down-regulated genes in the Sp5 target gene set. (*G*) DREME analysis identifies sequence motifs associated with activated and repressed genes in the Sp5 target gene set (see Fig. S2C for complete list). Fgf, Fibroblast growth factor; Hh, hedgehog; Nr4a, nuclear receptor subfamily a; PPL, phospholipase; RTK, receptor tyrosine kinase; Tgfβ, transforming growth factor β.

that genes associated with the Wnt/β-catenin pathway were significantly enriched in the up-regulated gene set only (Fig. 2 *E* and *F*). Sp5-binding GC-box motifs (see, for example, Fig. S4*J*) were associated with both up and down-regulated genes; however, Tcf/Lef binding sites were only associated with the activated gene set (Fig. 2*G* and Fig. S2*C*), consistent with Sp5 activation of Wnt/β-catenin target genes. Together, these results suggest that the activator or suppressor activity of Sp5 depends upon interactions with other TFs.

To investigate whether Sp5 could be interacting with the Tcf1/Lef1 TFs to activate Wnt target genes, we first cross-referenced the direct Sp5 targets and Lef1 RNA-seq datasets and found that Sp5 bound to 123 genes up-regulated by F-Lef1 (Fig. 3*A* and Dataset S2). Examination of the *T* and *Axin2* loci identified F-Sp5 binding peaks in *cis*-regulatory regions that were subsequently validated by ChIP-qPCR for both Sp5 and Sp8 (Fig. 3 *B* and *C* and Fig. S3 *A* and *B*). Importantly, overexpression of F-Sp5 strongly activated the proximal *T* enhancer/promoter (15) (which includes ChIP-seq peak 3,668) in luciferase reporter assays (Fig. 3*D*, *Left*), but did not activate the *Axin2* peak 1,619 reporter, which contained the most Sp/Klf Zn^{2+}-finger motifs among the three *Axin2* peaks (Fig. S3*E*). Interestingly, the *T* regulatory region possesses both Sp5 and Tcf/Lef binding sites, whereas *Axin2*-1619 has only Sp5 binding sites, suggesting a corequirement for Tcf/Lef and Sp5 for gene activation (Fig. S3 *C* and *D*). Consistent with this finding, mutation of either the two Tcf/Lef or two Sp5 binding sites blunted the *T* promoter reporter activation by endogenous factors in differentiating ESCs (Fig. 3*D*, *Right*). Taken together, these results strongly suggest that Sp5 directly activates the expression of Wnt/β-catenin target genes.

To determine if F-Sp5 or F-Sp8–mediated transactivation of Wnt/β-catenin target genes requires active Wnt signaling, Dox-induced *iF-Sp5* and *iF-Sp8* ESCs were treated with recombinant (r) DKK1 protein, a potent extracellular inhibitor of Wnt/β-catenin signaling (21). rDKK1 blocked the endogenous activation of *T* in differentiating noninduced ESCs, demonstrating that 400 ng/mL of rDKK1 is sufficient to antagonize endogenous Wnt ligands (Fig. 3*E*). Treatment of Dox-induced *iF-Sp5* and *iF-Sp8* ESCs with rDKK1 significantly reduced *T* expression (Fig. 3*E* and Fig. S3*F*). Moreover, simultaneous treatment of *iF-Sp5* and *iF-Sp8* ESCs with Dox and small molecule inhibitors that deregulate β-catenin [i.e., iCRT-14 and *endo*-IWR1 (22, 23)] dramatically inhibited *T* expression activated by rWnt3a or F-Sp5/8 (Fig. 3*F* and Fig. S3 *G* and *H*). These data suggest that the amplification of Wnt target gene expression by F-Sp5/8 depends upon β-catenin.

Our demonstration that Sp5/8 activity requires β-catenin predicts that Sp5/8 might synergize with rWnt3a to activate gene expression. Using serum-free, feeder-free culture conditions to minimize the influence of other signaling pathways on *T* activation, we found that Dox-induced F-Sp5 was insufficient to activate *T* expression in the absence of rWnt3a, but synergized with rWnt3a to activate *T* expression above the levels induced by Wnt3a alone (Fig. 3*G*). These data are consistent with Sp5/8 functioning as signal amplifiers in the Wnt/β-catenin pathway.

Sp5/8 Are Novel Components of the β-Catenin-Tcf/Lef Complex. To define the molecular mechanism underlying β-catenin–dependent Sp5/8 activity, we asked whether Sp5/8 proteins could interact with the β-catenin–Tcf/Lef transcription complex. Coimmunoprecipitation (Co-IP) experiments from Dox-induced *iF-Sp5* and *iF-Lef1* ESCs demonstrated that F-Sp5 and F-Sp8 interacted with endogenous β-catenin and Tcf1, and that F-Lef1 interacted with endogenous Sp5 and β-catenin, respectively (Fig. 4*A* and Fig. S4*A*). Similar protein interactions were also observed in heterologous 293T cells (Fig. S4*B*). In vitro binding assays were performed to determine if Sp5/8 bound directly to Tcf1, Lef1, or β-catenin proteins. Pairwise analysis of GST-Sp5/8 fusion proteins with in vitro translated TCF1, LEF1, or β-catenin in individual pulldown assays demonstrated that the Sp5/8 Zn^{2+}-finger domain interacted directly with the HMG domain of Tcf1 and LEF1 but not with β-catenin (Fig. 4*B* and Fig. S4 *C*–*H*). Close physical associations (<30–40 nm) between endogenously expressed Sp5 and Tcf1/Lef1 were confirmed in situ using a proximity ligation assay (PLA) (Fig. 4*C*). Importantly, no signal was detected in control *Sp5/8* DKO ESCs using the same antibodies. Remarkably, comparisons of Sp5 and β-catenin (24) genome-wide binding profiles revealed that 3,517 genes (56% of Sp5 target genes, 43% of β-catenin target genes) were bound by both factors (Fig. 4*D*). Of these commonly bound genes, 273 are known to be associated with the Wnt/β-catenin pathway (Fig. 4*E*) and include many well-characterized direct target genes such as *Axin2*, *Sp5*, *Myc*, *T*, and *Lgr5* (Dataset S3). Indeed, 1,341 of the commonly bound genes showed peaks at the

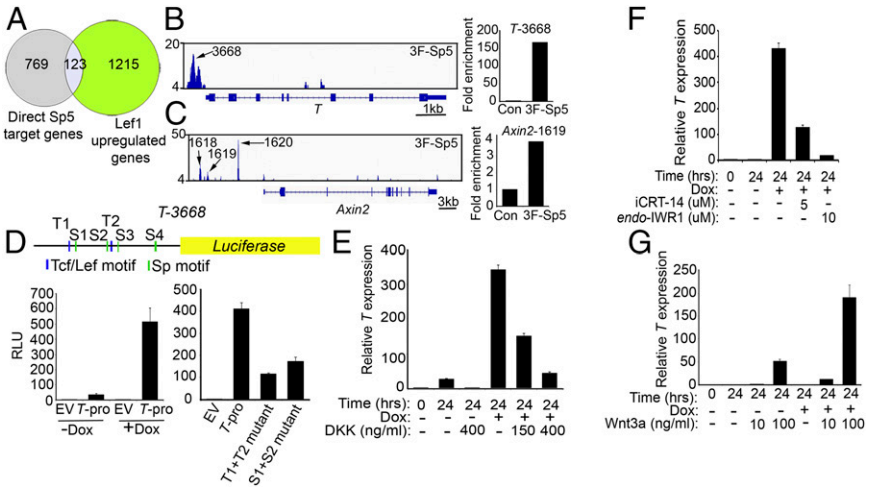

Fig. 3. Sp5 requires β-catenin to active Wnt target genes. (*A*) Comparisons of the lists of direct Sp5 target genes and Lef1 up-regulated genes identified 123 common genes. (*B* and *C*) Visualization of Sp5 ChIP-seq peaks (*Left*) and ChIP-qPCR validation (*Right*) of Sp5 binding to *T* and *Axin2* cis-regulatory regions. (*D*) Schematic of *T*-promoter luciferase reporter (*Upper*). T, Tcf/Lef binding site (BS); RLU, relative luciferase activity; S, Sp5 BS. (*Left*) Dox induction of F-Sp5 activates *T*-promoter in ESCs. EV, empty vector. (*Right*) Mutations in Tcf/Lef or Sp BS abrogates *T*-reporter activation. (*E*) *T* activation by F-Sp5 expression is diminished by rDKK administration. (*F*) Inhibiting β-catenin activity blocks F-Sp5 activation of *T*. (*G*) F-Sp5 and rWnt3a synergistically activate *T* expression. RT-qPCR in *E*–*G* is normalized to *Gapdh* levels.

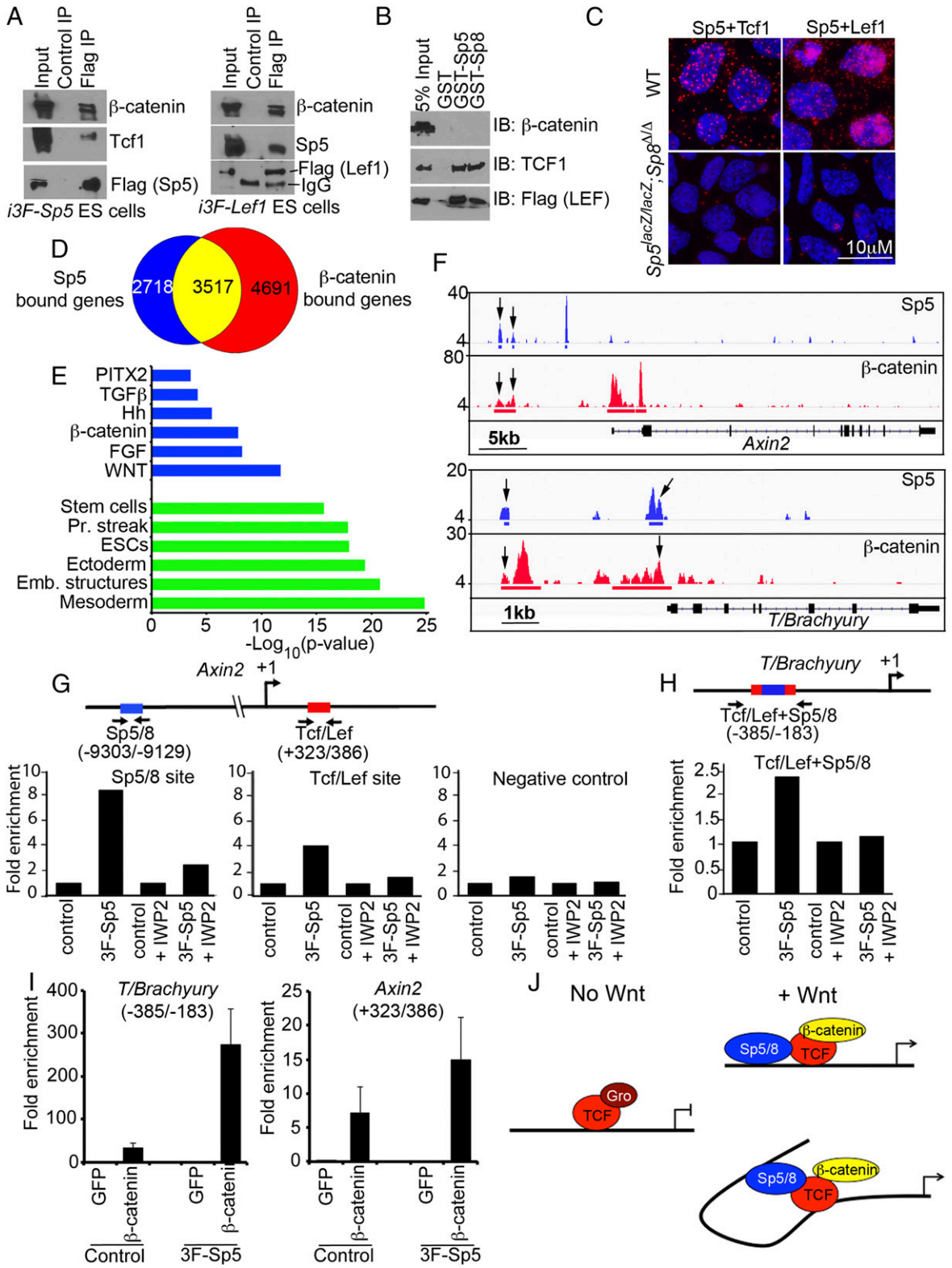

Fig. 4. Sp5/8 directly interacts with Tcf1/Lef1 proteins and enhances β-catenin recruitment to enhancers. (*A*) Co-IP analysis of overexpressed F-Sp5 and F-Lef1 and endogenous Wnt transcription complex core components. (*B*) GST-pulldown assay shows GST-Sp5/8 directly interacts with in vitro-translated TCF1/LEF1 proteins. (*C*) PLA analysis in *Sp5/8* DKO and wild-type ESCs show in situ interactions between endogenously expressed Sp5 and Tcf1/Lef1 proteins. (*D*) Venn diagram depicts common Sp5 and β-catenin bound genes. (*E*) GO analysis of the genes bound by Sp5 and β-catenin at the same genomic location (also see Fig. S4*I*). (*F*) β-Catenin and Sp5 bind to similar *cis*-regulatory regions at *Axin2* and *T* (arrows). (*G* and *H*) ChIP-qPCR analysis of a representative experiment shows F-Sp5 simultaneously bound to Sp5 sites and Tcf/Lef enhancer elements in *Axin2* and *T*, and is dependent on active Wnt signaling. (*I*) ChIP-qPCR indicates Sp5 overexpression promotes the localization of β-catenin to target gene enhancers. (*J*) Schematic depicting proposed Sp5/8 function in the β-catenin-Tcf/Lef complex. Emb., embryonic; Fgf, fibroblast growth factor; Hh, hedgehog; PITX, paired-like homeodomain transcription factor; Pr., primitive; Tgfβ, transforming growth factor β.

same genomic location (Fig. 4*F* and Fig. S4*I*). Thus, multiple lines of evidence demonstrate that Sp5/8 directly interact with the β-catenin–Tcf1/Lef1 complex on many, but not all, Wnt/β-catenin target genes.

Interaction Between Tcf/Lef and Sp Regulatory Elements. Interestingly, Tcf/Lef binding motifs were among the most significant, nonzinc-finger motifs identified by Sp5 ChIP-seq (Fig. S2*C*) but were not directly bound by Sp5 (Fig. S4 *J* and *K*). Considering the Sp5 DNA-binding profile and the physical interaction with Tcf1/Lef1, we asked whether Sp5/8 bound to distal enhancers could recruit known, distantly located Tcf/Lef-bound DNA elements. To study the dynamics of Sp5-mediated chromatin interactions, we identified 9 h of Dox treatment as a time point sufficient for F-Sp5 to induce some (ex. *Sp5* and *T*) but not all (for example, *Axin2*) Wnt target genes (Fig. S4 *L–N*). ChIP-qPCR analysis of F-Sp5, F-Sp8, or F-Lef1 simultaneously precipitated chromatin ∼9.3 kb upstream of *Axin2* (i.e., *Axin2* 1,619 peak) and a distant Tcf/Lef regulatory element located in the first intron (+323/+386) (25), indicating an association between these distant DNA elements (Fig. 4*G* and Figs. S3*B*, *Right*, and S4 *O* and *P*). Antagonizing the Wnt/β-catenin pathway with IWP2, a Porcupine (PORCN) inhibitor (23), disrupted the interaction between Tcf/Lef and Sp5. Intriguingly, IWP2 also reduced exogenous F-Sp5 binding to DNA (Fig. 4*G*), suggesting Sp5 may require active β-catenin–Tcf/Lef complexes for DNA binding. It should be noted that Sp5/8 and Tcf/Lef regulatory elements do not need to be separated by large genomic distances to be functional, as exemplified by the close proximity of their binding sites in the *T* promoter (Fig. 4*H* and Figs. S3*D* and S4*Q*).

In light of these findings, we asked if Sp5 affected the binding of β-catenin to DNA. ChIP-qPCR analysis of β-catenin occupancy on *T* and *Axin2* enhancers after induced F-Sp5 expression suggested significantly higher amounts of β-catenin were associated with chromatin compared with control ESCs (Fig. 4*I*). This result suggests that Sp5 promotes the recruitment of β-catenin to enhancers.

Discussion

Our data demonstrate that Sp5/8 are enhancer/promoter-selective TFs that function in the β-catenin–Tcf/Lef transcription complex to amplify Wnt target gene expression (Fig. 4*J*). We propose that Sp5/8 promotes the localization of β-catenin to Wnt target gene enhancers by binding to both *cis*-regulatory DNA and Tcf1/Lef1. Our genome-wide analyses of Sp5 activity indicate that Sp5/8 directly regulates many but not all established Wnt target genes, thereby suggesting a "fine-tuning" mechanism for selective amplification of a subset of Wnt target genes.

The observed bimodal occupancy pattern of Sp5 near the TSS is notably different from that observed for TCF4 (26), but is reminiscent of the H3K4me3 marks at CpG islands associated with active and poised transcription (27). We speculate that Sp5/8 could interact with activated histone complexes to recruit β-catenin complexes to transcriptionally competent sites.

Despite our observations that Sp5 is broadly expressed at sites of Wnt activity, we cannot presently conclude that it plays a global role in Wnt gene regulation because it is not bound to all known Wnt target genes. Consistent with this statement, *Sp5/8* double-mutants display a *Wnt3a*-like phenotype in the posterior embryo (7) but not, for example, a *Wnt1*-like phenotype in the mid-hindbrain (28). However, *Sp8* is not coexpressed at all sites of *Sp5* expression, suggesting that other Sp family members could play a redundant role with Sp5 in other cell contexts. In addition to our demonstration that Sp5/8 are critical effectors of Wnt/β-catenin signaling during mouse gastrulation (7), Sp TFs similarly mediate the responses to Wnt signals from developmental signaling centers in the limb bud, and in the zebrafish gastrula and hindbrain (29–32). We conclude that the interaction between Sp proteins and the β-catenin–Tcf/Lef complex is a conserved feature of the vertebrate pathway. Nevertheless, our study clearly demonstrates that Sp5/8 play a major role in the propagation of Wnt signals.

Materials and Methods

Detailed material and methods are included in *SI Materials and Methods*.

Mice. The transgenic *Sp8* gain-of-function, *tetO-Sp8-ires-EGFP,* (hereafter called $Sp8^{GOF}$), *T-Cre*, and $Sp5^{lacZ/lacZ}$;$Sp8^{+/\Delta}$ mice are as described previously (7, 13, 14). rtTA were obtained from The Jackson Laboratory and bred to the $Sp8^{GOF}$ line to generate a Cre- and Dox-inducible mouse line. After crossing $Sp8^{GOF}$;*rtTA* with the *T-Cre* line to activate *rtTA* expression in *T*-expressing cells, including NMPs, the $Sp8^{GOF}$ allele was induced by feeding pregnant females with Dox-supplemented chow (Bio-Serv, S3888) and water [1.6 mg/mL (wt/vol) Dox in 5% (wt/vol) sucrose] for 24 h [i.e., embryonic days (E) 8.5–9.5]. Mouse experiments were carried out in strict accordance with the *Guide for the Care and Use of Laboratory Animals* (33) of the National Institutes of Health and using Frederick National Laboratory Animal Care and Use Committee-approved protocols (Animal Study Proposal #12–408). All mice were euthanized by CO_2 inhalation in accordance with the most recent American Veterinary Medical Association guidelines on euthanasia (34).

Whole-Mount in Situ Hybridization. *T/Brachyury*, *Sp5*, and *Axin2* probe synthesis and whole-mount in situ hybridizations were performed as described previously (7).

Plasmids, Recombinant Proteins, and Small Molecules. The *T*-promoter and *Axin2-1619* luciferase reporters, the pGex4T-1-Sp5/8 series, and the pcDNA-3xFlag-Lef1 construct, were generated by PCR cloning. pcDNA3-p45(TCF1) and pcDNA-Myc-β-catenin plasmids were gifts from H. Clevers, Hubrecht Institute, Utrecht, The Netherlands, and F. McCormick, University of California, San Francisco, respectively. The LEF1 deletion series (35) was modified by N-terminal insertion of annealed oligos encoding a 2xTy1 epitope tag.

Recombinant DKK1 and Wnt3a proteins (R&D Systems, 5439-DK and 1324-WN, respectively), and iCRT-14, *endo*-IWR2, and IWP2 (Tocris Bioscience, 4299, 3532, and 3533, respectively) were reconstituted according to the manufacturer's instructions.

ESC Line Generation and Culture. Dox-inducible ESC lines were generated using the A2.Lox.Cre inducible cassette exchange system (36). Briefly, 1x and 3xFlag epitope, N-terminally tagged Sp5, Sp8, and Lef1 cDNA's were PCR cloned into the MluI/AflII restriction sites of the P2.Lox vector. Targeting vectors were electroporated into A2.Lox.Cre cells 24 h after Dox-induced Cre expression and positive clones were selected for G418 resistance.

$Sp5^{lacZ/lacZ}$;$Sp8^{\Delta/\Delta}$ (i.e., DKO) ESCs were established from E3.5 blastocysts harvested from pregnant females obtained from $Sp^{lacZ/lacZ}$;$Sp8^{+/\Delta}$ intercrosses.

RNA-seq. Total RNA was extracted from three replicates of *iFlag*, *iF-Sp5*, and *iF-Lef1* ESC using TriReagent (Ambion), 24 h after addition of Dox. RNA quality was assessed by Agilent Bioanalyser to have RNA integrity number values of 9–10. Libraries were generated using Illumina TruSeq (FC-122-1001) kit and sequenced on an Illumina HiSeq2000 sequencer using PhiX as sequencing control.

ChIP. ChIP experiments were performed as described previously (37) using M2 antibodies or M2-beads for immunoprecipitation. See Dataset S4 for antibody information and qPCR primers.

Duplicate ChIP-seq libraries were prepared using TruSeq v3 library-construction protocol (Illumina). After passing Agilent Bioanalyzer (high-sensitivity chip)-based quality control, libraries were sequenced on an Illumina GAIIx sequencer on a single-end read 36-cycle flowcell. Base calling was performed with the RTA 1.9.35.0 software. All samples had over 24 million pass-filtered reads with 92% of the bases having qualities ≥ Q30. Samples were aligned to the mouse mm9 build using Bowtie with default parameters.

qPCR Analysis. qPCR analysis were performed as described previously (7) and in *SI Materials and Methods*.

EMSA. EMSAs were performed using annealed, $^{\gamma\text{-}32}$P-ATP end-labeled DNA probes with purified GST-tagged Sp5 or in vitro-translated 3xFlag-LEF1 proteins. Protein complexes were resolved on 6% (vol/vol) native gels.

Bioinformatic Analysis. GO analyses of gene lists generated from the RNA-seq and ChIP-seq datasets were performed using GePS literature mining software (Genomatix.de). BED file comparison of the Sp5 and β-catenin ChIP-seq datasets to determine the overlap in position of genome binding was also performed in Genomatix. Motif analysis of ChIP-seq peak DNA sequences

was performed using the Discriminative Regular Motif Elicitation (DREME) program (38). ChIP-seq peaks were visualized using the Integrative Genomics Viewer (IGV) program. Tiled data files (TDF) and BED files were used to generate the peak tracks and underlying lines to indicate statistically significant peaks, respectively.

Accession Numbers. Sp5 RNA-seq and ChIP-seq datasets and the Lef1 RNA-seq dataset have been deposited into the Gene Expression Omnibus with accession no. GSE73084. β-Catenin ChIP-seq data were obtained from GEO43597.

Western Blot, Co-IP, and Immunofluorescence. Western blots and Co-IP were performed using nuclear protein extracts. Immunofluorescent staining was performed on ESCs grown on gelatin coated IBIDI slides or coverslips. See Dataset S4 for antibodies.

Luciferase and *Renilla* Assays. *iFlag* and *iF-Sp5* ESCs were plated on gelatin-coated plates and differentiated for 2 d. On day 2, luciferase reporter constructs and TK-*Renilla* plasmids were cotransfected into ESCs using FugeneHD (Promega, E2311). After 24 h, ESCs were harvested in 1× passive lysis buffer (Promega, E194A), luciferase activity was measured (Promega, E1501), and transfections normalized to TK-*Renilla* activity according to the manufacturer's protocol (Promega, E2820). All luciferase and *Renilla* assays were performed in triplicate in three independent experiments. Errors are SDs of triplicate samples for the representative experiment that is shown.

Proximal Ligation Assay. The PLA kit (Sigma DUO92105) was used following the manufacturer's protocol. Imaging was performed using the Zeiss 710 confocal LSM and imaging software. Images were exported to ImageJ (FIJI) before brightness and contrast enhancement using Photoshop (CS5).

GST Pulldowns. GST and GST-Sp5/8 fusion proteins were expressed in BL21 competent cells and purified from sonicated protein lysates with GST Sepharose beads. GST protein-bead complexes were incubated with in vitro translated proteins. Protein interactions were assayed by Western blotting.

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