Repression of Wnt/β-catenin signaling by SOX9 and Mastermind-like transcriptional coactivator 2

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Wnt/β-catenin signaling requires inhibition of a multiprotein destruction complex that targets β-catenin for proteasomal degradation. SOX9 is a potent antagonist of the Wnt pathway and has been proposed to act through direct binding to β-catenin or the β-catenin destruction complex. Here, we demonstrate that SOX9 promotes turnover of β-catenin in mammalian cell culture, but this occurs independently of the destruction complex and the proteasome. This activity requires SOX9’s ability to activate transcription. Transcriptome analysis revealed that SOX9 induces the expression of the Notch coactivator Mastermind-like transcriptional coactivator 2 (MAML2), which is required for SOX9-dependent Wnt/β-catenin antagonism. MAML2 promotes β-catenin turnover independently of Notch signaling, and MAML2 appears to associate directly with β-catenin in an in vitro binding assay. This work defines a previously unidentified pathway that promotes β-catenin degradation, acting in parallel to established mechanisms. SOX9 uses this pathway to restrict Wnt/β-catenin signaling.

INTRODUCTION

Wnt/β-catenin signaling is required for many cell fate decisions in animal development and adult tissue homeostasis (1, 2). Misregulation of the pathway has been linked to many cancers and other human pathologies (3). This pathway is centered around the stability of β-catenin. In the absence of Wnt stimulation, β-catenin is targeted for degradation by a “destruction complex” containing the scaffolding proteins Axin and APC, the protein kinases glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1), and the SCF (Skp1/Cullin/F-box)–βTrCP E3-ubiquitin ligase (4). Axin and APC recruit β-catenin, and after sequential phosphorylation of specific Ser/Thr residues in its N terminus, β-catenin is polyubiquitinated by βTrCP and degraded by the proteasome (4). Wnt stimulation disrupts the function of the destruction complex, leading to stabilization and nuclear accumulation of β-catenin protein where it acts as a transcriptional coactivator with T cell factor (TCF) family transcription factors (3).

The Wnt pathway is modulated at multiple levels of the signaling cascade. These include acylation of Wnt proteins, a modification that is essential for proper transport, signaling (6), and regulation of Wnt receptor stability by the R-spondin–LGR4/5–ZNRF3/RNF43 axis (7). The level of β-catenin can be influenced by a variety of factors, some of which act through the destruction complex, e.g., WTX/ Amer, the protein phosphatase PP2A. Others act independently of the destruction complex, e.g., TRIM33 (8) or autophagy (9, 10). One transcription factor reported to exert a strong inhibitory effect on Wnt signaling and β-catenin levels is SOX9.

SOX9 (along with SOX8 and SOX10) is a member of the subclass E of SOX transcription factors (11, 12). SOX9 is best known as a master regulator of chondrogenesis and skeletogenesis, exemplified by severe skeletal defects in humans with campomelic dysplasia (CD), which is recapitulated in mouse models (12, 13). SOX9 directly regulates many chondrogenic genes, such as collagen type II alpha 1 chain (Col2a1) and aggrecan, through binding to enhancer and promoter sequences. In addition, SOX9 is necessary and sufficient for male sex determination in mice, where it works in conjunction with the Y chromosome Sox protein SRY (14). Supporting this work, loss-of-function mutations in human SOX9 can result in sex reversal of XY individuals (15). Beyond the skeletal system and testes development, SOX9 and its redundant partners are critical for specification of many other cell fates (11).

SOX9 repression of the Wnt pathway is thought to be important in several biological contexts. In mammalian sex determination, SRY and SOX9 promote testes formation in XY animals, while the Wnt pathway promotes ovarian development in XX (16). XY mice lacking SOX9 in the bipotent genital ridge have a complete feminization of their gonads (14). Notably, reduction of Wnt/β-catenin signaling in SOX9 mutant gonads results in marked shift back to testicular development (17, 18). In growing bone, SOX9 restricts Wnt signaling at the growth plate to maintain the appropriate balance of chondrocytes and osteoblasts (19, 20). In the mouse small intestine, loss of SOX9 results in increased proliferation of transit amplifying cells (21) whose expansion is Wnt signaling dependent (22). SOX9 has also been shown to inhibit Wnt/β-catenin signaling in several cancer cell lines (21, 23–25).

While SOX9 is an important antagonist of the Wnt pathway, data on the molecular mechanism underlying this regulation have been contradictory. Several groups have reported that SOX9 can physically associate with β-catenin in various assays, with this interaction interfering with β-catenin–TCF complex formation (19, 23–25). One proposed model has the C-terminal portion of SOX9 binding directly to β-catenin, leading to both proteins being degraded by the proteasome (19). Alternatively, SOX9 has been reported to bind to components of the destruction complex, leading to increased phosphorylation, ubiquitination, and proteasomal degradation of β-catenin (26). In this study, deletion of the C terminus of SOX9 had no effect on its ability to promote β-catenin degradation (26). While all of the...
above mechanisms invoke SOX9 functioning outside of its activity as a transcription factor, another study found that SOX9’s ability to activate transcription was required to inhibit Wnt/β-catenin signaling (21). This report identified several repressors of the Groucho-related (Gro/TLE/Grg) family as potential SOX9 targets that were responsible for the Wnt antagonism (21).

Here, we address the mechanism by which SOX9 inhibits the Wnt pathway in cultured human cells. We find that SOX9 promotes the turnover of β-catenin but, inconsistent with previous reports, does so independently of the destruction complex and the proteasome. SOX9’s ability to activate transcription is required for Wnt antagonism, and transcriptional profiling identified Mastermind-like transcriptional activator 2 (MAML2) as a key target in this process. Known as a coactivator of Notch target genes, we find that MAML2 also destabilizes β-catenin in parallel to the activity of the destruction complex, and this activity is conserved in its Drosophila counterpart Mastermind (Mam). MAML2 interacts with β-catenin and promotes its degradation independent of its canonical role in Notch target gene activation. Our work identifies MAML2 as an evolutionarily conserved negative regulator of β-catenin, which is activated by SOX9 to repress Wnt signaling.

RESULTS
SOX9 represses Wnt/β-catenin signaling independent of the β-catenin destruction complex

Expression of SOX9 in several cell lines has a strong inhibitory effect on Wnt/β-catenin signaling (21, 23–26). These studies primarily relied on TopFlash, i.e., a synthetic reporter containing multiple TCF binding sites upstream of a minimal promoter driving luciferase, as a readout for the Wnt pathway. In colorectal carcinoma (CRC) cell lines containing elevated Wnt signaling due to truncations in APC, SOX9 inhibited TopFlash activity (21, 23–25), suggesting that SOX9 acts independently of the β-catenin destruction complex. However, repression of TopFlash by SOX9 has been reported to depend on GSK3 activity and the N-terminal regulatory region of β-catenin, suggesting a critical role for the destruction complex (26). To address this controversy, we examined SOX9 regulation of the Wnt pathway in human embryonic kidney (HEK) 293T cells, where Wnt signaling levels are easily manipulated.

We found that SOX9 could inhibit Wnt/β-catenin signaling when the pathway was stimulated by different agonists. SOX9 robustly repressed activation of the TopFlash reporter in cells treated with Wnt3a (Fig. 1A) and the GSK3 inhibitor CHIR99021 (CHIR) (Fig. 1B) or in cells transfected with mutant forms of β-catenin (S33Y or S45F; hereafter referred to as β-catenin*) that are insensitive to GSK3 phosphorylation (Fig. 1C and fig. S1A). SOX9 also strongly inhibited β-catenin* activation of a reporter gene containing an enhancer located downstream of the AXIN2 gene (Fig. 1D) (27). In HEK293T cells stably transfected with a doxycycline (Dox)–inducible SOX9 (pTRE-SOX9WT) and treated with CHIR, induction of SOX9 repressed TopFlash (fig. S1B) and the endogenous Wnt targets AXIN2 and SP5 (Fig. 1E). Together, these results support SOX9 repression of Wnt transcriptional targets occurring independently of the β-catenin destruction complex.

To extend our findings beyond HEK293T cells, β-catenin* was transfected into several other cell lines, such as HeLa and NIH3T3 fibroblasts, as well as the Sertoli-like cell lines TM4 and NT2/D1. In all cases, SOX9 markedly reduced β-catenin*–induced activation of TopFlash (Fig. 1F), indicating that destruction complex–independent repression of the Wnt pathway by SOX9 is a common occurrence.

SOX9 promotes β-catenin turnover independently of TCFs, proteasomes, and lysosomes

Investigations into SOX9 repression of the Wnt pathway have focused on its ability to inhibit β-catenin activity/expression with conflicting results. Some studies have found that SOX9 does not affect β-catenin expression but instead binds and translocates nuclear β-catenin to the cytoplasm and/or inhibits β-catenin’s ability to interact with TCFs (24, 25). Others have found that SOX9 down-regulates β-catenin protein levels by proteasome-dependent degradation (19, 26). To address this discrepancy, we examined whether β-catenin levels were affected by SOX9 expression in HEK293T cells.

We find that SOX9 is a robust repressor of steady-state levels of β-catenin when the endogenous protein is stabilized chemically (via CHIR; Fig. 2A) or upon exogenous addition of genetically stabilized β-catenin* (Fig. 2B). This SOX9 effect is independent of TCFs, as the down-regulation of β-catenin* occurs in HEK293T cells lacking all four TCF family members (Fig. 2C) (28). These data indicate that SOX9 represses β-catenin expression independently of two major binding partners of β-catenin, the destruction complex and TCFs.
To address the issue of whether SOX9-mediated repression of β-catenin requires the proteasome, we used two structurally unrelated molecules, MG132 and bortezomib, which inhibit the proteasomal 26S subunit. A short treatment (8 hours) with either drug resulted in a large activation of TopFlash (Fig. 2D), consistent with the elevation of β-catenin levels, as previously shown (29, 30). However, under these conditions, TopFlash levels were still robustly repressed by SOX9 (Fig. 2D). SOX9-mediated down-regulation of β-catenin* levels was also robust under conditions of proteasome inhibition (Fig. 2E). These results provide strong evidence that SOX9’s effect on β-catenin and Wnt signaling occurs independently of the proteasome.

Fig. 2. SOX9 reduces β-catenin protein levels independent of TCFs and the proteasome. (A) Western blots of endogenous β-catenin and transfected FLAG-SOX9 from pTRE-SOX9WT cell lysates treated with 5 μM CHIR and Dox for the indicated times (hour). (B) Western blots of transfected FLAG–β-catenin* and FLAG-SOX9 from HEK293T cell lysates. (C) Western blots of parental HEK293T cells (left half) or HEK293T cells with CRISPR-induced deletions of all four TCF genes (TCFQKO cells; right half) transfected with β-catenin* and an increasing dose of SOX9. (D) TopFlash reporter assay performed on HEK293T cells transfected with or without FLAG-SOX9 and treated with the proteasome inhibitors MG132 and bortezomib for 8 hours before lysis. Each bar represents the mean of biological triplicates ± SD. (E) Western blots of HEK293T cell lysates, transfected with FLAG–β-catenin* with or without SOX9 and treated with MG132 for indicated periods of time. Images cropped from the same blot for plus/minus Sox9. (F) Western blots of endogenous β-catenin from pTRE-SOX9WT cells supplemented with Dox (24 hours) and 5 μM CHIR (16 hours) and then treated with CHX for indicated period of time before harvest. (G) Western blots of transfected FLAG–β-catenin* from HEK293T cells transfected with β-catenin* with or without SOX9 and then treated with CHX for indicated period of time before harvest. More extract was loaded for the SOX9-expressing cells to provide a fair comparison of β-catenin decay. All images in (F) and (G) were cropped from the same blot. α-Tubulin was used as a loading control for all Western blots. In numerous experiments, Sox9 down-regulated β-catenin to levels shown in (A) to (C) and (E). In (three) separate experiments, Sox9 accelerated the turnover of β-catenin, as shown in (F) and (G). ***P < 0.001.

If not through proteasomal degradation, how is SOX9 down-regulating β-catenin? Transcript levels of transfected β-catenin* are not reduced by SOX9 (Fig. S1C). In addition, the construct used to express β-catenin* contains heterologous 5’ and 3’ untranslated regions. Because SOX9 can still repress β-catenin* expressed from this construct, these results argue for down-regulation occurring at the posttranslational level. To address whether SOX9 promotes increased turnover of β-catenin protein, we examined the perdurance of β-catenin after protein synthesis was inhibited with cycloheximide (CHX) in the absence or presence of SOX9. When β-catenin was stabilized chemically or genetically, the presence of SOX9 caused a more rapid disappearance of β-catenin (Fig. 2, F and G). We examined whether lysosomal activity was required for this increased turnover using three distinct compounds that raise lysosomal pH [chloroquine (CQ), bafilomycin A1, and NH4Cl]. None of these treatments block the ability of SOX9 to down-regulate β-catenin (fig. S1D). Our data indicate that SOX9 promotes β-catenin turnover through a mechanism that does not require two major conduits of cellular protein degradation, proteasomes and lysosomes.

SOX9 represses Wnt/β-catenin signaling through its transcriptional activity

As mentioned above, most previous reports concluded that SOX9’s inhibitory effect on Wnt/β-catenin signaling was independent from its role as a transcription factor (19, 23, 25, 26). However, the approaches in these studies were largely indirect or based on limited evidence, such as the use of stable cell lines or overexpression of SOX9. To directly address the role of SOX9 in transcriptional control, we performed ChIP-seq analyses to identify SOX9-binding sites and associated gene expression changes. Our data revealed several interesting findings:

1. SOX9 binds to the promoter regions of known Wnt target genes, suggesting a direct role in transcriptional repression.
2. SOX9 binding is correlated with decreased expression of Wnt target genes, providing a possible mechanistic link.
3. SOX9 can also recruit other repressive transcription factors, further enhancing the transcriptional inhibition.

These findings support the conclusion that SOX9 plays a critical role in transcriptional repression of Wnt signaling, independent of its proteasomal function. This mechanism provides a potential therapeutic target for the treatment of diseases associated with Wnt signaling dysregulation.
SOX9 mutagenesis. To address this question in a more comprehensive manner, we constructed several mutants that alter SOX9’s ability to bind DNA or interact with transcriptional coactivators.

SOX9 binds specific DNA sequences through a high-mobility group (HMG) domain (12). To create a SOX9 mutant defective in specific DNA binding, we took advantage of published structures and the sequence similarity between the HMG domains of SOX9 and LEF1. The N terminus of these HMG domains makes contacts with the minor groove of the cognate DNA site (31, 32). Mutation of several of these N-terminal residues in LEF1 markedly reduce DNA binding (33), so we mutated three analogous residues in SOX9’s HMG domain (106^KRPM^109 to 106^EEPT^109). In addition to DNA binding, this region also contains a nuclear localization signal (NLS) (34). This SOX9 mutant is largely cytoplasmic when expressed in HEK293T cells (fig. S2A). However, the addition of the SV40 NLS to the N terminus of Sox9 (Fig. 3A) restores localization to the nucleus (fig. S2A). To characterize the ability of SOX9 to activate transcription, we constructed a reporter we termed SoxFlash, which contains three pairs of SOX9 half-sites upstream of a minimal promoter. The NLS/HMG mutant (SOX9^HMG-NLS) had minimal ability to activate SoxFlash (Fig. 3B), consistent with a severe defect in DNA binding. The addition of the same NLS signal to wild-type (WT) Sox9 did not affect its ability to inhibit TopFlash and down-regulate β-catenin (fig. S2, C and D).

C-terminal of the HMG domain, SOX9 contains three transactivation domains, TAM, PQA, and TAC (13). To generate a SOX9 variant that is defective in binding to coactivators, we deleted the C-terminal third of the protein, removing the PQA and TAC domains.
SOX9-mediated repression of Wnt signaling requires MAML2, an evolutionarily conserved Wnt antagonist

To identify the Wnt antagonist(s) induced by SOX9, we used pTRE-SOX9WT cells, which are stably transfected with a SOX9 complementary DNA (cDNA) under the control of a Dox-inducible promoter (Fig. 1E). To increase the chances of identifying relevant SOX9 targets, we also generated mutations in the transcription domains of SOX9, in the hope of finding a separation-of-function mutant that is compromised for activation of some SOX9 targets, but still retained the ability to repress β-catenin signaling. Mutations in the TAC domain (e.g., Fig. 3A) rendered SOX9 equally defective in the activation of SoxFlash and repression of TopFlash. However, substitution of three amino acids (SOX9WT C293EFD to C293AAA) known to be essential for the TAM transcriptional domain (13), generated a suitable SOX9 variant. SOX9EFD-AAA displayed little ability to activate the SoxFlash reporter (Fig. S4A) but retained the ability to suppress TopFlash (Fig. S4B). We established an inducible stable cell line expressing this SOX9 mutant (pTRE-SOX9EFD-AAA). Dox induction resulted in the inhibition of TopFlash, albeit to a slightly lesser extent than pTRE-SOX9WT cells (Fig. S4C). We reasoned that having two distinct cell lines with the ability to repress the Wnt reporter would increase the chances of identifying common SOX9 targets that contribute to Wnt repression.

Transcriptome analysis of pTRE-SOX9WT and pTRE-SOX9EFD-AAA cells revealed a large number of genes whose expression was altered following a 13.5-hour treatment with Dox (data file S1 for complete list of genes). As surmised from the SoxFlash data (Fig. S4A), the number of regulated genes was less for SOX9EFD-AAA than for SOX9WT (Fig. 4, A and B). Because SOX9EVP16 (which should behave as a dedicated transcriptional activator) was competent for repressing TopFlash and β-catenin levels (Fig. 3, C to E), we focused our search for Wnt antagonists on the 275 genes that were up-regulated by both pTRE-SOX9WT and pTRE-SOX9EFD-AAA (Fig. 4A).

One of the genes activated by SOX9WT and SOX9EFD-AAA that drew our attention was MAML2 (Fig. 4, C and D). MAML2 gets its name from the Drosophila gene mastermind (mam), identified as an essential component of the Notch signaling pathway (41). Vertebrates have three MAML homologs, all of which have an N-terminal domain that forms a trimeric complex with CSL transcription factors and the ankyrin repeats of intracellular Notch (ICN) (42). C-terminal to this domain, Mam/MAMLs share similar protein architecture with two acidic domains and stretches of polyglutamine (polyQ) (Fig. 4E) that contribute to the ability of Mam/MAMLs to activate transcription of Notch target genes (42, 43). Construction of a phylogenetic tree of fly Mam and the human MAMLs revealed that MAML2 is most similar in primary sequence to Mam (fig. S5). In addition to their role in Notch signaling, all three MAMLs have been shown to potentiate Wnt signaling when coexpressed with β-catenin in cultured cells (44). While the fly Mam protein also has this Wnt potentiation activity in mammalian cell culture, genetic interaction studies in flies suggested that Mam represses Wingless/Wnt signaling (45).

Consistent with Mam acting as a repressor of Wg/Wnt signaling, we identified mamm in a genetic screen for suppressors of a small eye phenotype caused by overexpression of a destruction complex–insensitive, stabilized version of the fly β-catenin* (Armadillo* or Arm*). Expression of wg or arm* in the developing fly eye via the GMR promoter results in a marked reduction in eye size (Fig. 4, F and G) (46). Using GMR-arm* flies as the parental line, we screened for insertions of a P-element transposon containing Gal4 binding sites (UAS) and a minimal promoter (P[EP]). Insertions of P[EP] places adjacent genes under Gal4 control (47). Previously, similar screens using a GMR-wg stock uncovered several conserved regulators of Wg/Wnt signaling (46, 48–50). Here, we identified a P[EP] insertion in the first intron of the mam locus that suppressed both the GMR-wg and GMR-arm* phenotypes (Fig. 4, I, J, and L). As a control, a small eye caused by GMR-mediated expression of the proapoptotic factor head involution defective (HID) (51) was minimally suppressed by the mam insertion (Fig. 4, H, K, and L). These results support and extend the previous findings that Mam represses Wg/Wnt signaling in Drosophila. Our finding that Mam can suppress Arm* is notably reminiscent of SOX9’s ability to down-regulate β-catenin though a mechanism independent of the destruction complex.
Further analysis in HEK293T cells indicated that MAML2 is required for SOX9 repression of Wnt/β-catenin signaling. While all three MAML genes are expressed in these cells, only MAML2 is activated by SOX9WT and SOX9 EFD-AAA (Fig. 5A and data file S1). The activation of MAML2 was further validated using quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Fig. 5B) and Western blotting, where MAML2 was only detectable after SOX9 induction (Fig. 5C). Two distinct small interfering RNAs (siRNAs) directed against the second exon of MAML2 suppressed its expression. Notably, MAML2 depletion blocked the down-regulation of CHIR-induced β-catenin by SOX9 (Fig. 5C). MAML2 depletion also disrupted the ability of SOX9 to repress TopFlash (Fig. 5D). To extend these results, we generated two independent, Cas9/CRISPR-induced MAML2 mutant cell lines in the pTRE-SOX9 WT background (see fig. S6 and Materials and Methods for details). Both cell lines displayed no repression of TopFlash after SOX9 induction (Fig. 5E). These results convincingly demonstrate that MAML2, which is activated by SOX9, is required for SOX9-dependent repression of the Wnt pathway.

**MAML2 promotes β-catenin turnover independently of its role in Notch signaling**

To address whether MAML2 regulates the Wnt/β-catenin signaling in the absence of SOX9, we examined parental and MAML2 knockout (KO) cells in the absence of SOX9 induction, with or without...
activating the Wnt pathway by CHIR. Without CHIR, the absence of MAML2 did not result in elevated Wnt reporter activity; however, the MAML2 mutants were significantly more sensitive to CHIR activation than parental cells (Fig. 6A). Thus, MAML2 is distinct from destruction complex components, whose loss of function leads to elevated Wnt signaling in the absence of Wnt ligand (52–55). In contrast, MAML2 acts to limit the response of the pathway when Wnt signaling has been activated.

That Mam/MAML proteins are required for Notch signaling (41, 42) raises the possibility that the effect of MAML2 on Wnt signaling is through the Notch pathway. The Notch receptor has been reported to repress Wnt/β-catenin signaling in Drosophila (56–58) and mammalian cells (59, 60). To address the possibility that SOX9 activation of MAML2 inhibited the Wnt pathway through Notch, we used the 4xCSL-luciferase reporter, which has four CSL/RBP-J binding sites upstream of a minimal promoter. This Notch reporter had a high basal activity in HEK293T cells, which was repressed by SOX9 expression (fig. S7A). Reporter activity was also reduced in MAML2-KO cells or by a MAML2 dominant negative (MAML2DN, expressing the first 334 amino acids of MAML2) in WT cells (fig. S7, B and C). SOX9 still repressed the Wnt pathway when the Notch pathway was inhibited by MAML2DN (fig. S7D). Activation of Notch signaling reduced MAML2 mRNA, while inhibition of the Notch pathway had no effect on MAML2 transcript levels (fig. S7E). Together, these data are inconsistent with models where SOX9-MAML2 antagonism of the Wnt pathway occurs through changes in Notch signaling. Rather, the failure of SOX9 to block Wnt/β-catenin signaling when MAML2 is depleted/deleted (Figs. 5, C to E, and 6A) is evidence of a Notch-independent role for MAML2 in regulating the Wnt pathway.

MAML2 is required for SOX9-dependent down-regulation of β-catenin (Fig. 5C), and SOX9 down-regulates β-catenin by increasing its turnover (Fig. 2, F and G). This suggests that MAML2 regulates β-catenin by promoting its degradation. To test this, we examined the half-life of β-catenin stabilized by CHIR (Fig. 6B) or by mutation of phosphorylation sites, i.e., β-catenin* (Fig. 6C). In both cases, we found that the two forms of “stabilized” β-catenin were unexpectedly labile in parental cells, but their half-lives were significantly lengthened in MAML2-KO cells (Fig. 6, B and C). The individual blots shown were exposed for varying times to best illustrate the decay of β-catenin during the CHX chase, e.g., longer exposures were used for the parental cells to highlight the rapid turnover. When protein extracts from parental and MAML2-KO cells treated with CHIR were analyzed side by side, there was a slight increase in overall β-catenin levels in the cells lacking MAML2 (fig. S8, A to C).

MAML1 has been shown to directly associate with β-catenin and acts as a coactivator (44), prompting us to examine whether MAML2 also associates with β-catenin. We were unable to express full-length recombinant MAML2 (1156 amino acids) but could obtain recombinant proteins of the N and C termini (1 to 612 amino acids and 551 to 1156 amino acids, termed MAML2-N and MAML2-C, respectively) (Fig. 6D). Both halves of MAML2 were found to be specifically pulled down by a glutathione S-transferase (GST)–β-catenin fusion protein (Fig. 6E). The purity of the MAML2-N and MAML2-C preparations (fig. S8) does not allow us to rule out the possibility that other Escherichia coli–derived proteins (e.g., chaperones binding to the unstructured domains of MAML2) are involved in MAML2’s association with β-catenin. This caveat aside, our data support a model where MAML2 directly associates with β-catenin and promotes its turnover independent of the destruction complex, proteasomes, or lysosomes.

**DISCUSSION**

This study provides evidence that MAML2 is an essential component of a pathway that acts to inhibit the steady-state levels of β-catenin. MAML2 promotes the turnover of β-catenin under conditions where GSK3 is inhibited (Fig. 6B) or when β-catenin is rendered insensitive to the destruction complex by mutation of regulatory phosphorylation...
sites (Fig. 6C). This indicates that MAML2 acts in parallel to the destruction complex to degrade β-catenin. Under conditions of low Wnt signaling and low SOX9 levels, MAML2 expression is relatively low, the destruction complex is the primary mechanism for β-catenin turnover (Fig. 6F). In cells with high levels of Wnt and SOX9, the MAML2-dependent mechanism predominates and is a major factor in limiting Wnt/β-catenin signaling (Fig. 6G).

While inhibition of the destruction complex results in activation of Wnt/β-catenin signaling independent of Wnt stimulation (52–55), loss of MAML2 by itself is not sufficient to activate the pathway. Rather, MAML2 acts to restrict the level of Wnt responsiveness when the destruction complex is partially inhibited (Fig. 6A). In this way, MAML2’s relationship to Wnt signaling is similar to ZNRF3 and RNF43, E3 ligases that down-regulate the number of Wnt receptors on the cell surface. Like MAML2, loss of ZNRF3/RNF43 activity (through mutation or via R-spondin treatment) heightens the ability of cells to respond to Wnt stimulation (61). However, MAML2 exerts its effect further downstream in the pathway than ZNRF3/RNF43, acting directly at the level of β-catenin turnover.

We identified MAML2 as a β-catenin antagonist through our investigation into the mechanism of how SOX9 inhibits the Wnt pathway and down-regulates β-catenin levels. Through mutagenesis of the SOX9 protein, we provide compelling evidence that the Wnt/β-catenin inhibitory activity of SOX9 was tightly linked to its ability to activate transcription (Fig. 3, A to D). Transcriptome profiling revealed many genes activated by SOX9 in HEK293T cells, and MAML2 was identified as a relevant candidate because its fly homolog Mam was identified in a genetic screen for Wg/Wnt antagonists in the Drosophila eye (Fig. 4). Notably, depletion of MAML2 by siRNA or CRISPR/Cas9 mutagenesis abolished the ability of SOX9 to block Wnt signaling and down-regulate β-catenin (Fig. 5, C to E). Given that we found that SOX9 inhibits Wnt/β-catenin independent of proteasomal and lysosomal activity (Fig. 2, D and E, and fig. S1D), we conclude that MAML2’s promotion of β-catenin turnover is also independent of these systems. We also provide evidence for a direct association between MAML2 and β-catenin, likely through distinct binding sites in its N- and C-halves (Fig. 6, D and E). A more precise identification of the interaction sites on MAML2 and β-catenin will allow testing of whether this binding is required for MAML2’s ability to promote β-catenin degradation.

Our results, while providing the most complete picture of how SOX9 interferes with Wnt/β-catenin signaling, are at odds with previous literature. Several reports have argued that SOX9 blocks Wnt signaling by directly binding to β-catenin, diverting it away from TCFs (19, 23–25). The β-catenin binding domain and Wnt inhibitory activity were both localized to the C terminus of SOX9 (19). While our results do not rule out SOX9 repressing β-catenin activity at the posttranslational level, previous publications did not take into account that the C terminus also contains two transactivation domains (PQA and TAC) (13). Our data demonstrating that replacing the C terminus of SOX9 with three distinct transactivation domains restores SOX9’s ability to inhibit Wnt signaling and down-regulate β-catenin (Fig. 3, A to D) provide a compelling argument that SOX9’s repression of the Wnt pathway requires its ability to act as a transcriptional factor.

Another major discrepancy between our results and the literature is the role of the proteasome in SOX9-mediated repression of Wnt signaling. Previous reports indicated that proteasome inhibition blocked the ability of SOX9 to down-regulate β-catenin in HEK293T cells (19) and in CHO (Chinese hamster ovary) cells (26). In contrast, our studies convincingly demonstrate that SOX9 can repress a Wnt reporter and β-catenin levels under conditions where the proteasome inhibited. While difficult to definitively resolve these differences, proteasome inhibitors are very deleterious to cell health (62), and we took care to limit the time of drug exposure (6 to 8 hours) while ensuring that the treatment markedly elevated Wnt/β-catenin signaling (Fig. 2D). The report using CHO cells argued for a mechanism where the N terminus of SOX9 interacted with components of the destruction complex, increasing its ability to target β-catenin for degradation (26). The possibility remains that this discrepancy is due to differences in cell type.

One previous report argued that SOX9 inhibited Wnt/β-catenin signaling through a transcriptional intermediate in CRC cells (21). This group suggested that SOX9 activation of Groucho-related (Gro/TLE/Grg) family members could be responsible for the Wnt inhibitory effect but did not provide functional data. It will be interesting to determine whether MAML2 is required for SOX9-mediated Wnt antagonism in CRC and other cell types.

The Mam/MAML family, including MAML2, plays an essential role in Notch signaling (41, 42). Notch has been reported to antagonize Wnt signaling in flies and mammalian cell culture (56–58), raising the possibility that SOX9 induction of MAML2 expression antagonized Wnt/β-catenin signaling through elevation of Notch signaling. However, while SOX9 repression of the Wnt pathway was dependent on MAML2, blocking MAML2’s Notch signaling activity (via a dominant negative) had no effect on SOX9-mediated Wnt inhibition. In addition, SOX9 represses the activity of a Notch reporter, inconsistent with the notion that SOX9 blocks Wnt signaling through MAML2-mediated elevation of Notch signaling (fig. S5). Notch’s ability to antagonize β-catenin is thought to occur by binding to the uncleaved Notch receptor, via interaction between the Notch intracellular domain (NICTD) and β-catenin. In flies, it has been proposed that intact Notch titrates β-catenin away from the nucleus (57, 58). In mammalian cells, the Notch receptor directs β-catenin toward autophagic degradation (59, 60). These results are not consistent with MAML2’s role as a coactivator for Notch target genes (41, 42) and our findings that SOX9 inhibition of Wnt/β-catenin signaling is independent of lysosomal activity (fig. S1D). Rather, we propose that MAML2 has an evolutionarily conserved function in limiting Wnt/β-catenin signaling that is distinct from its role in Notch target gene regulation.

Presumably, MAML2 acts with other factors to facilitate β-catenin degradation. TRIM33, a RING domain containing ubiquitin ligating enzyme, has been shown to bind the C-terminal region of β-catenin and promote its degradation in a GSK3- and βTRCP-independent manner. TRIM binding and subsequent ubiquitination require phosphorylation of β-catenin (S763) (8). It is unlikely that TRIM33 is involved with MAML2-induced turnover of β-catenin, given its dependence on the proteasome and our results that β-catenin lacking the C-terminal TRIM33 binding site is still down-regulated by SOX9 (Fig. 3E and fig. S3B). SENP2 is a hydrolase belonging to the SENP family of de-SUMOylases, which has been shown to promote down-regulation of β-catenin in several systems (63–65). The mechanism is not clear, e.g., proteasome dependence has not been tested. Whether SENP2 plays a role in the Wnt/MAML2 axis we have identified remains to be explored.

SOX9’s repression of the Wnt pathway is an important regulatory circuit in several contexts, including chondrogenesis/skeletogenesis, homeostasis in the intestine, and sex determination in mammals (17–21).
Our findings raise the possibility that MAML2 activation by SOX9 may be involved in these processes. Deletion of MAML1 and MAML3 in mice results in phenotypes that can be explained by general reduction in Notch signaling (66), but there are no reports on loss of MAML2 in any system beyond the KO lines described in this report. Further elucidation of the mechanism of MAML2’s role in repressing β-catenin will provide tools to understand the importance of this regulatory mechanism at the organismal level, allowing uncoupling of its roles in Notch and Wnt signaling.

MATERIALS AND METHODS

Study design and statistical analyses

The purpose of this study was to understand and broaden our knowledge of SOX9 repression of Wnt/β-catenin signaling. Once we identified SOX9’s ability to activate transcription was an important part of its Wnt-inhibitory mechanism, we identified MAML2 as a SOX9 transcriptional target that is required for Wnt repression. This work was carried out using controlled laboratory experiments that are described in detail below. All cell culture experiments were performed at the same temperature, under the same conditions using age-matched (in terms of passages) cells. Transfections were carried out using plasmids prepared similarly, and all biological replicates received DNA from the same transfection pool for the same condition. Luciferase experiments were performed at least three times with biological triplicates condition/independent cell line, and graphs were plotted on Microsoft Excel. Statistical analysis was performed using a two-tailed Student’s t test, and differences with $P < 0.05$ were considered significant. All the Western blots were performed at least three times, and the figures show representative blots for each experiment. Similarly, the GST pull-down assay shows the representative Western blot from three independent biological replicates. qRT-PCR experiments were performed in triplicates for at least three biological replicates. Statistical analysis was performed using a two-tailed Student’s t test, and differences with $P < 0.05$ were considered significant. For Drosophila experiments, the sample size (mentioned in the figure legends) was determined from previous experiments and end point for data collection was reached when we found statistical significance between groups. Eye size was quantified using ImageJ software. Graphs were plotted using GraphPad Prism 8. Data comparisons of more than two groups were done using one-way analysis of variance (ANOVA) followed by Dunnett’s test. Differences with $P < 0.05$ were considered significant.

Cell lines

HEK293T, TM4, and NT2/D1 cells were obtained from the American Type Culture Collection. HEK293 cells were a gift from G. Dressler (University of Michigan). HeLa cells were a gift from Y. Wang (University of Michigan). NIH3T3 cells were a gift from H. Xu (University of Michigan). HEK293 Tet-On 3G stable cells, constitutively expressing viral Tet-On 3G protein under a cytomegalovirus (CMV) promoter (Clontech, 631182), were a gift from N. E. Marsh (University of Michigan). d4TCF cells and the parental controls were a gift from K. Basler (University of Zurich).

Plasmids

pCDNA3.1 (Thermo Fisher Scientific), with 2×Flag-tag 5’ of the multiple cloning site (MCS), was used as the standard vector backbone for all the mammalian expression plasmids unless otherwise mentioned. Restriction enzymes were purchased from New England Biolabs, and all the gblocks and primers (listed in table S1) were ordered from Integrated DNA Technologies. The human SOX9 open reading frame (ORF) was cloned via PCR amplification of the SOX9 ORF (OriGene, NM_000346) and inserted in frame into the Bam HI/Xho I sites of 2×Flag pcDNA3.1. SOX9 ORF (OriGene; NM_000346) was cloned into Bam HI/Xho I sites, and all the mutated versions of SOX9 (Fig. 3A) were subsequently generated using gBlocks. β-Catenin* ORF was subcloned into the Bam HI/Xba I sites of pcDNA3.1 from pCDNA3-S33Y (a gift from E. Fearon, University of Michigan), and the three mutated versions (fig. S3B) were generated using gBlocks. pCDNA3-S45F β-catenin* was a gift from O. MacDougald (University of Michigan). The Viperin ORF, cloned into the MCS of pTRE-3G-Bi (Takara Bio, a gift from N. Marsh, University of Michigan), was replaced with SOX9WT or SOX9EFD-AAA ORF along with the 2×Flag-tag after it was amplified from pCDNA3.1-SOX9WT or pCDNA3.1-SOX9EFD-AAA using specific primers containing Sal I/Eco RV sites. MAML2 ORF was amplified from pMSCV-Flag-HA-MAML2 (a gift from J. Rual, University of Michigan) using specific primers and was cloned into pcDNA3.1 between Kpn I and Xba I sites. MAML2DN (1-334) was generated by Gibson assembly using a NEBuilder HiFi DNA assembly kit (New England Biolabs). All the reporter plasmids are based on pGL4.23 (Promega), where specific transcription cofactor binding sites were cloned upstream of a minimal TATA box promoter driving the expression of a firefly luciferase gene. TopFlash has 6× TCF-specific binding sequence and was a gift from E. Fearon (6× CTTTGTAC). SoxFlash has 3× SOX9-specific binding sequence (3× AACAATGTGTTCTTTGT), while the human Axin2 WRE [+45 kb downstream of the TSS (transcription start site)] was cloned to generate Axin reporter construct. CSL-Luc was a gift from M. Chiang (University of Michigan) and has 4× RBP binding sites (4× CGTGGGAA). In all the cases, Renilla luciferase (Promega) was used as an internal control. Two single-guide RNAs, sgRNA1 (5′-CACCGGaatgagtcaagcagac-3′) and sgRNA2 (5′-CACCGtaaactgcgacagcagc-3′), targeting two different areas of the MAML2 second exon (fig. S4D), were cloned separately in pX458 (Addgene, 48138) between Bbs I sites.

Wnt agonists and pharmacological agents

Wnt3a was purchased from R&D Systems. CHIR99021 was purchased from Apex-Bio. MG132 and bortezomib were purchased from Calbiochem. CHX, NH4Cl, Dox, and puromycin (Puro) were purchased from Sigma-Aldrich. Bafilomycin A1 (BafA1) and CQ were a gift from Y. Wang (University of Michigan).

Cell culture, transfection, treatment, generation of stable cells, and luciferase assay

HEK293T, HeLa, TM4, NT2/D1, NIH3T3, and d4TCF cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals) and 1× penicillin-streptomycin-glutamine (Invitrogen). HEK293 Tet-On 3G stable cells were grown in the same conditions, except Tet-screened FBS (HyClone) was used instead of regular FBS. Cells were grown at 90% confluency before being passaged in a humidified chamber at 37°C with 5% CO2. For transfection, 50,000 cells were plated in a well of a 48-well plate (cell numbers were scaled up accordingly when the dish size changed) and grown until they reached 70 to 80% confluency. Cells were transfected using either polyethylenimine-MAX (PEL-MAX, Polyscience) or Lipofectamine
2000 (Invitrogen) following standard procedures. Total DNA amount used for transfection was kept the same using an empty pCDNA3.1 vector throughout all the experiments. For all the activator and inhibitor treatment, cells were plated and transfected for 24 hours, and the chemical reagents were added to the media unless otherwise mentioned. To activate Wnt signaling, Wnt3a (500 ng/ml) or CHIR99021 (1 to 10 μM) was added for 16 hours before being assayed unless otherwise mentioned. To inhibit proteasome complex, 20 μM MG132 or 10 μM bortezomib was added to the cells for 8 hours before lysis. Autophagy was blocked using 400 nM bafilomycin A1, 50 μM CQ, or 20 mM NH4Cl for 8 hours before being lysed for Western blot. For the β-catenin turnover assay, cells were either transfected or treated with or without Dox (250 ng/ml) for 24 hours total with 5 μM CHIR for the last 16 hours before being treated with CHX (100 μg/ml) and subjected for Western blot. For knockdown experiments, MAML2 silenced select siRNAs s39004 (GGGUUGAAUCAGUC- GAGGAtt) and s39006 (GGUAAUCCACAACAUUGAtt) and control siRNA (silencer select negative #1 siRNA) were obtained from Thermo Fisher Scientific. siRNAs were transfected in cells at a concentration of 10 μM per well of a 48-well plate using Lipofectamine 2000 for a total period of 80 hours, where cells were transfected and chemically treated for the last 24 hours before being assayed. To generate stable HEK293 cells inducibly expressing SOX9WT and SOX9EFD-AAA, HEK293 Tet-On 3G cells were plated on 24-well plates, pTRE-SOX9WT and pTRE-SOX9EFD-AAA plasmids were linearized with Sca I and cotransfected separately with pCAG-Puro (a gift from J. Nandakumar, University of Michigan), which was linearized with Bam HI, using Lipofectamine 2000. Cells were subjected for puromycin selection at 0.75 μg/ml in DMEM containing Tet (Thermo Fisher Scientific). Cells were blocked in 4% FBS, stained with anti-rabbit–Alexa488 (Molecular Probes), washed, and counter-stained with primary antibodies (Cell Signaling Technology, 1:1000), washed, stained with secondary antibody running at 75 kDa on Western and being able to suppress TopFlash induction using primers specific for the transgene (table S1). Selected and Western blot was done at every step selecting the ones fitting the criteria of expression, i.e., expressing a Flag-tagged SOX9 protein running at 75 kDa on Western and being able to suppress TopFlash activation strongly upon Dox induction. These clones were also checked by qRT-PCR for expression of SOX9 transcript upon Dox induction using primers specific for the transgene (table S1). Selected clones were grown in Tet-free media supplemented with puromycin (0.2 μg/ml). To generate CRISPR KO cells lacking MAML2 expression, pTRE-SOX9WT stable cells were transfected with pX458 vectors carrying either sgRNA1 or sgRNA2 (fig. S4D) using Lipofectamine 2000 following the manufacturer’s recommendation. Cells were sorted after 4 days on the basis of high enhanced green fluorescent protein (EGFP) expression and were plated onto 96-well plates at a single-cell density. Cells were allowed to grow into clones, and they were expanded and screened by Western blot. Genomic DNA was isolated from clones missing MAML2 expression using a Genomic DNA Isolation kit (Invitrogen) and PCR-amplified using primers specifically designed for the second exon (table S1). These amplicons were cloned into a pMiniT 2.0 vector using a PCR cloning kit (New England Biolabs) and sequenced. Clones with deletion were further screened by TopFlash assay. Two independent clones (MAML2 KO-1 and MAML2 KO-2) were chosen for further studies. Luciferase assays were carried out according to the manufacturer’s recommendation using a dual luciferase kit (Promega). Briefly, cells were lysed using 1× PLB (passive lysis buffer) (65 μl per well of a 48-well plate) at room temperature for 20 to 30 min with gentle shaking. Cell lysate was trituted, and 20 μl of the lysate was transferred to one well of a black 96-well microplate. The enzymatic reaction was allowed to happen inside a GloMax luminometer (Promega), and the readings were collected. Ratios of Firefly luciferase versus Renilla luciferase were calculated, and minimum of three biological replicates were used to plot a graph with SDs. Individual experiments were repeated at least three times, and any experiment showing a significant variation in Renilla numbers was discarded to maintain higher standards.

### Western blot and immunofluorescence

Cells were lysed in hot 2× SDS loading buffer (75 μl per well of a 48-well plate). Lysate was clarified using centrifugation, and 20 μl was loaded on an 8.5% SDS gel and separated for 2 hours at 100 V. Separated protein samples were transferred onto a polyvinylidene difluoride membrane (Bio-Rad) and nonspecifically blocked using 5% bovine serum albumin (BSA; DOT Scientific) for 1 hour at room temperature with shaking. Protein blots were incubated in primary antibody (diluted in 5% BSA) overnight at 4°C, washed three times with 1× phosphate-buffered saline (PBS) carrying 1% Tween 20 (Thermo Fisher Scientific) (PBST), incubated for 1 hour with secondary antibody at room temperature, washed again in 1× PBST for three times, and prepared for imaging by adding a 1:1 mixture of H2O2 and Luminol (SuperSignal West Pico PLUS Kit, Thermo Fisher Scientific). Blots were imaged in Odyssey CLX (LiCOR), and images were processed in Adobe Photoshop CC. The following antibodies were used: anti–Flag–horseradish peroxidase (HRP) (clone M2, Sigma-Aldrich, 1:5000), anti–β-catenin (BD, 1:5000), anti–α-tubulin (Cell Signaling Technology, 1:5000), anti-HIS (GE Healthcare, 1:1000), anti-MAML2 (Cell Signaling Technology, 1:1000), and anti-mouse HRP/anti-rabbit HRP (Jackson Immunochemicals, 1:2000). For subcellular localization studies, HEK293T cells were grown on coverslips (Thermo Fisher Scientific) in a 24-well plate. Twenty-four hours after transfection, cells were fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich) and permeabilized in Triton-X (Thermo Fisher Scientific). Cells were blocked in 4% PBS, stained with anti-SOX9 antibodies (Cell Signaling Technology, 1:1000), washed, stained with anti-rabbit–Alexa488 (Molecular Probes), washed, and counter-stained with DAPI (4′,6-diamidino-2-phenylindole). They were then imaged in a Leica SP5 confocal microscope, and images were processed in Adobe Photoshop CC.

### qRT-PCR and RNA sequencing

For qRT-PCR, total RNA was isolated using TRIzol LS reagent (Thermo Fisher Scientific) and 1 μg of RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was set up in a 96-well plate and carried out in a 7500 Fast RT system (Applied Biosystems). G6PD (glucose-6-phosphate dehydrogenase) was used as a baseline control. All the primer sequences are listed in table S1. For RNA sequencing (RNA-seq), total RNA was purified using a Direct-Zol RNA kit (Zymo Research). Eighty-one–base pair single-end reads were performed by the UG DNA Sequencing Core, using the Illumina Next-Seq 500 platform with a depth of ~40 million reads per sample. Reads were aligned to the UCSC hgg19 reference genome using TopHat and Bowtie2 (67, 68). HTSeq was used to quantify non-ambiguously mapped reads, and the DESeq2 package was used to identify differentially expressed genes (69). Differentially expressed genes were defined as genes that satisfied the criteria FDR (false discovery rate) ≤ 0.05 and log2 (fold change) ≥ 1.5 or log2 (fold change) ≤ −1.5.
Protein expression, purification, and in-vitro binding assay

S33Y ORF was cloned into pGEX-6P-1 (Sigma-Aldrich) between Bam HI and Not I sites. MAML2-N-term and C-term were PCR-amplified from pCDNA3.1-MAML2 and cloned into pET52B between Kpn I and Not I sites. These plasmids were transformed into Arctic Express (DE3, Agilent Technologies, a gift from A. Vecchiarelli, University of Michigan), grown in LB media at 37°C supplemented with carbenicillin (100 μg/ml) (Sigma-Aldrich) until the OD (Optical density) reached 0.6, induced with 100 μM IPTG (isopropyl-β-d-thiogalactopyranoside) (Sigma-Aldrich), grown subsequently at 16°C for 16 to 18 hours, pelleted, and lysed. GST- or HIS-tagged proteins were purified using a GST Sepharose or Ni-NTA beads (Qiagen) and eluted in elution buffers containing 10% glycerol (Thermo Fisher Scientific). Quality of the purified proteins was checked by Coomassie blue staining and Western blotting. Briefly, different amounts of proteins were run on polyacrylamide gel electrophoresis (PAGE), the gel was washed in distilled water for 15 min with gentle shaking, and then the gel was stained in Coomassie blue for 2 hours after baking in microwave for 40 s. The gel was destained overnight with three to four changes and baking it in microwave for 40 s every time the solution was changed. Last, the gel was washed in distilled water for a day before scanning it. For Western blot, different amounts of purified HIS-tagged MAML2 proteins were loaded on gel, blotted, and probed with anti-HIS and anti-β-catenin antibodies, developed, and imaged.

Neighbor-joining tree analysis of Mam and MAML proteins

The possible evolutionary relationships between invertebrate Mam and vertebrate MAMLs were inferred using the neighbor-joining method with MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 (70). One thousand replicates were analyzed and clustered together in a bootstrap test.

Drosophila genetics

All Drosophila stocks were reared on yeast/glucose food, and experiments were performed at 25°C. The P[MamEP] line, inserted in the first intron of the mam locus, was identified in a screen where P[EP, white+] transposons were mobilized from the X chromosome with P[Δ2-3] transposase as described previously (46), except that the new autosomal P[EP] insertions were balanced and screened for suppression of a P[GMR-Gal], P[GMR-Arm-] small eye phenotype. This GMR-arm* stock, balanced over Sm5-TM6B, as well as P[GMR-Gal], P[UAS-wg]; +/+;Sm5-TM6B and P[GMR-Gal], P[GMR-hid]; +/+;Sm5-TM6B are as previously described (46). Flies carrying P[MamiEP] combined with GMR-arm*, GMR-wg, and GMR-hid were frozen overnight and then photographed with Leica Stereo Dissecting Scope (Leica DM16000B) attached to a digital camera.

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SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/8/eabe0849/DC1

View request a protocol for this paper from Bio-protocol.
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Repression of Wnt/β-catenin signaling by SOX9 and Mastermind-like transcriptional coactivator 2

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