Supplementary information

Expression and reporter constructs
A 7 kb genomic region upstream of the Msgn1 translational start site was obtained by genomic PCR and cloned into a reporter vector containing the lacZ gene and a SV40 polyadenylation sequence. For luciferase reporter constructs, the promoter fragments were inserted in the pGL3-Basic Vector (Promega). Lef/Tcf and T/Tbx6 binding sites were modified using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene). To disrupt the Lef/Tcf binding sites, L1 (position -54/-46) was replaced by (ACTCTATC), L2 (position -89/-81) by (CCTCTAAA), L3 (position -148/-140) by (ATTAGAGA), and L4 (position -296/-288) by (TCTCTAGC). To disrupt the T/Tbx6 binding sites, T1 (position -363/-351) was replaced by (GTAGATAAGGGAGT), T2 (position -661/-648) by (AAA-TACAGAAATA), T3 (position -839/-827) by (AAGTATAAAAT), and T4 (position -951/-940) by (AAATACACGGT). The transcriptional start site of Msgn1 was determined by 5'-RACE PCR (Invitrogen).

DNA microinjection and staining for β-galactosidase activity
Linearized lacZ reporter constructs were injected into pronuclei of fertilized mouse eggs of the strains CD1 or NMRI according to standard techniques; transient transgenic embryos were prepared at the indicated stages and stained for β-galactosidase activity as described (Beckers et al. 2000). Numbers of transient transgenic embryos at E9.5-10.5 showing β-galactosidase activity or lacZ transcripts are: 7kb-wt-lacZ (7); 1.2kb-wt-lacZ (7); 1.2kb-mut T-lacZ (8); 1.2kb-Mut L1,3,4-lacZ (5); 1.2kb-mut L1-4-lacZ (5); 1.2kb-mut T/L1,3,4-lacZ (5); 1.2kb-wt-lacZ (WISH: 2).

In situ hybridization and Histology
Whole mount in situ hybridization analyses and preparation of histological sections were done by standard procedures.

Cell culture and reporter gene assays
HEK 293-T cells were grown in DMEM (GibcoBRL) supplemented with 10% FCS, 1% Glutamin and 1x Penicillin-Streptomycin at 37°C and 5% CO2. Cells were seeded at a density of 2x10^5 cells/well in 24-well plates 20h before transfection. Cells were cotransfected with 180 ng luciferase reporter vector and 100 ng of the transactivator constructs respectively. Total amounts of transfected DNA were kept equal by adding pBluescript plasmid DNA where necessary. 20ng pRL-TK (Pro-
mega) were additionally co-transfected as an internal control for normalization of the transfection efficiency. Cloning of Tbx6-VP16 and T-VP 16 trans-activator plasmids is described in Hofmann et al., 2004. The Lef-beta-catenin fusion construct consists of the C-terminal part of the mouse β-Ca-
tenin ORF (aa 572-694) fused to the C-terminus of the full length ORF of murine Lef1, under the control of the human UBC promoter (Joerg Huelsken, unpublished). The activity of the Lef-beta-catenin fusion protein was tested by a transactivation assay with the TOP-flash and FOP-flash reporter system (see supplementary material). Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturers protocol. 24h after transfection cells were lysed and luciferase activity was measured using the Dual-Luciferase Assay System (Promega) according to the manufacturers protocol. The reporter gene activities show average values obtained from four independent experiments.

**Bioinformatic Analysis**

Genomic sequence data was retrieved from genome releases Ensembl 32 Mouse assembly NCBI 36 and UCSC mm8, March 2006 (Karolchik et al. 2003). Sequence data was searched with Fuzznuc (EMBOSS; Rice et al. 2000) using a consensus motif and with custom PERL programs using the TFBS PERL library and a position weight matrix calculated from the sequences described (Hofmann et al. 2004). In addition evolutionary conserved regions between mouse, rat, human, dog, opossum and chick where identified from multiple alignments (UCSC) (rat: Nov 2004, rn4; human: Feb 2006, hg18; dog: May 2005, canFam2; opossum: Jan 2006, monDom4; chicken: Feb 2004, galGal2). The experimental analysis was focusing on the putative binding sites in these conserved regions.

**Chromatin Immunoprecipitation Assays**

Tissue from 80-100 dissected embryos were cross-linked in 1% formaldehyde in PBS for 15 minutes. Adding glycin to a final concentration of 0.125 M stopped cross-linking. After washing with PBS, ChIP lysis buffer was added (50 mM HEPES-KOH pH7.5, 140 mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, Roche Complete), tissues were sonicated to fragment the genomic DNA to an average size of 500bp. After pre-clearing the lysate with an unspecific antibody (normal rabbit IgG, Santa Cruz #2027) and magnetic ProteinG beads (Invitrogen), the lysate was incubated with two anti-Tbx6 antibodies (1.5µg each; White and Chapman 2005). Tbx6 comple-
xes were isolated with magnetic Protein G beads (Invitrogen), the protein/DNA complexes were reverse cross-linked and the isolated DNA amplified using the QIAGEN Repli-g Kit (#150043). Quantitative PCR analyses on amplified DNA was performed using the ABI PRISM 7900. Briefly, randomly amplified DNA from pre-enrichment (Input) sample, unspecific antibody (IgG) enriched sample and specific (anti-Tbx6) antibody enriched sample was analyzed for enrichment of specific regions compared to a control region using real time PCR analysis. The ratio of specific to control amplicon in the Input sample was used as reference and set to 1-fold enrichment with a correction factor. This correction factor allows accounting for amplicon specific effects in the real time PCR analysis. The ratios of specific to control region in the two antibody enriched samples were determined, normalized with the correction factor and compared to the reference.

References


Supplementary Figure 1

In vitro transactivation assay showing that the TOP-flash WNT reporter is specifically and strongly activated by a Lef-beta-catenin fusion protein.