\(\beta\)-Catenin regulates Cripto- and Wnt3-dependent gene expression programs in mouse axis and mesoderm formation

Markus Morkel\(^1\), Joerg Huelsken\(^1\), Maki Wakamiya\(^2\), Jixiang Ding\(^3\), Marc van de Wetering\(^4\), Hans Clevers\(^4\), Makoto M. Taketo\(^5\), Richard R. Behringer\(^2\), Michael M. Shen\(^2\) and Walter Birchmeier\(^1\), *

\(^1\)Max Delbrueck Center for Molecular Medicine, Robert-Roessle-Strasse 10, 13125 Berlin, Germany
\(^2\)Department of Molecular Genetics, The University of Texas, M. D. Anderson Cancer Center, Houston, TX 77030, USA
\(^3\)Center for Advanced Biotechnology and Medicine and Dept. of Pediatrics, UMDNJ-Robert Wood Johnson Medical School, 679 Hoes Lane, Piscataway, NJ 08854, USA
\(^4\)Department of Immunology, University Hospital Utrecht, NL-3584 CX Utrecht, The Netherlands
\(^5\)Department of Pharmacology, Kyoto University Graduate School of Medicine, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan

*Author for correspondence (e-mail: wbirch@mdc-berlin.de)

Accepted 10 September 2003

Summary

Gene expression profiling of \(\beta\)-catenin, Cripto and Wnt3 mutant mouse embryos has been used to characterise the genetic networks that regulate early embryonic development. We have defined genes whose expression is regulated by \(\beta\)-catenin during formation of the anteroposterior axis and the mesoderm, and have identified Cripto, which encodes a Nodal co-receptor, as a primary target of \(\beta\)-catenin signals both in embryogenesis as well as in colo carcinoma cell lines and tissues. We have also defined groups of genes regulated by Wnt3/\(\beta\)-catenin signalling during primitive streak and mesoderm formation. Our data assign a key role to \(\beta\)-catenin upstream of two distinct gene expression programs during anteroposterior axis and mesoderm formation.

Introduction

Tissue and organ development of vertebrates is tightly regulated by signalling cascades that act in a spatially and temporally controlled manner (for reviews, see Beddington and Robertson, 1999; Jessell, 2000; Capdevila and Izpisua Belmonte, 2001). Prominent examples of these tightly controlled signalling pathways include the canonical Wnt/\(\beta\)-catenin and the Tgf\(\beta\)/Nodal signalling pathways, both of which play multiple essential roles in early embryogenesis.

The formation of the primary body axes during early embryogenesis is controlled in many organisms by canonical Wnt/\(\beta\)-catenin signal transduction (reviewed by Sokol, 1999; De Robertis et al., 2000). The expression of Wnt target genes is regulated by nuclear \(\beta\)-catenin that is bound to transcription factors of the Lef/Tcf family (Behrens et al., 1996; Molenaar et al., 1996; He et al., 1998; Tetsu and McCormick, 1999). In Xenopus, depletion of \(\beta\)-catenin mRNA interferes with the formation of the dorsal-anterior axis, while injection of Wnt mRNA promotes axis formation (Smith and Harland, 1991; Sokol et al., 1991; Heasman et al., 1994). In zebrafish, misexpression of Wnt receptors, or mutations in Axin or Ichabod, which encode a \(\beta\)-catenin scaffolding protein and a regulator of \(\beta\)-catenin localisation, modulate the function of the dorsal organiser and impair the formation of the anteroposterior axis (Nasevicius et al., 1998; Kelly et al., 2000; Heisenberg et al., 2001). In the mouse, ablation of \(\beta\)-catenin results in failure to orient the anteroposterior axis (Huelsken et al., 2000), i.e. the movement of an anterior signalling centre from the distal to the anterior visceral endoderm does not occur (Thomas and Beddington, 1996). Studies employing chimeras between mutant and wild-type embryos indicate that \(\beta\)-catenin acts in the embryonic ectoderm at this developmental step.

Signals provided by the TGF-\(\beta\)/Nodal pathway are also essential for the formation of the primary body axes (reviewed by De Robertis et al., 2000; Whitman, 2001). Nodal signals are transduced via Smads, which accumulate in the nucleus, interact with other transcription factors, and regulate gene expression (Chen et al., 1996; Liu et al., 1996; Zhang et al., 1996; Watanabe and Whitman, 1999). Cripto, an essential co-receptor of Nodal, acts together with activin receptors to transmit the Nodal signal via Smad2 and Smad3 (Gritsman et al., 1999; Yeo and Whitman, 2001; Kumar et al., 2001; Yan et al., 2002). Previous studies have shown a requirement for Nodal signalling in anteroposterior axis formation in both zebrafish and mice. In particular, zebrafish that carry double mutations in the cyclops and sqint genes, which encode Nodal-related ligands, display a mispositioned anteroposterior axis (Feldman et al., 1998). In the mouse, Nodal mutants lack a proximodistal axis as indicated by the absence of the signalling centres in extra-embryonic ectoderm and visceral endoderm, and as a consequence, do not form an anteroposterior axis (Conlon et al., 1994; Varlet et al., 1997;
Brennan et al., 2001). Mutants of Cripto establish a proximodistal axis, but fail to position the nascent anteroposterior axis in the correct orientation (Ding et al., 1998). Thus, in early mouse embryogenesis, Nodal signals are required for the formation of the anteroposterior axis, but its co-receptor Cripto is required only for the proper positioning of the anteroposterior axis. Whether β-catenin and Nodal/Cripto act in the same or in parallel pathways during the formation and positioning of the anteroposterior axis has been unclear.

Subsequently, Wnt/β-catenin and TGF-β signals regulate the formation of the vertebrate mesoderm. In Xenopus, ectodermal expression of factors of the TGFβ family induces mesoderm (reviewed by De Robertis et al., 2000; Kimelman and Griffin, 2000), and TGFβ and Wnt signals cooperate to induce expression of brachyury that specifies mesoderm (Vonica and Gumbiner, 2002). In the mouse, Wnt3 mutants lack a primitive streak and mesoderm, but form the anteroposterior axis (Liu et al., 1999). In cultured epithelial cells, Wnt3 signals are transmitted via β-catenin, suggesting that Wnt3 uses the canonical Wnt/β-catenin pathway (Shimizu et al., 1997). Brachyury is expressed in the mesoderm, and is a direct transcriptional target for canonical Wnt/β-catenin signals (Yamaguchi et al., 1999; Arnold et al., 2000; Galceran et al., 2001). Nodal mutant mice lack a primitive streak and only rarely form patches of mesoderm (Conlon et al., 1994), and experiments employing chimeric embryos indicate that these essential Nodal signals are generated in the epiblast (Varlet et al., 1997). By contrast, Cripto mutants do form mesoderm that arises at an ectopic position (Ding et al., 1998). Thus, the comparison of Wnt3 and Cripto mutants indicates that formation of the anteroposterior axis and of mesoderm can occur independently of each other. However, β-catenin is required in both processes.

In the adult, deregulation of both Wnt/β-catenin and TGF-β signals play important roles in the establishment and progression of cancer. Mutations in Apc (Groden et al., 1991; Kinzler et al., 1991), which encodes a regulator of β-catenin stability, or in β-catenin (Morin et al., 1997; Rubinfeld et al., 1997) are found in the majority of human colorectal cancers (reviewed by Polakis, 2000). Mutations in Axin and conductin (now known as Axin2), which encode scaffolding proteins of the β-catenin degradation complex, have been identified in medulloblastomas and colorectal carcinomas (Liu et al., 2000; Satoh et al., 2000; Dahmen et al., 2001). Genes that encode proteins of the TGFβ signalling pathway act as tumour suppressor genes: Smad2 and Smad4 in human colorectal cancer (Eppert et al., 1996; Thiagalingam et al., 1996), and Tgfb2 in colon tumours with microsatellite instability (Markowitz et al., 1995). The Nodal co-receptor Cripto is overexpressed in many colon carcinomas and can transform fibroblasts and epithelial cells (Niemyer et al., 1998; Salomon et al., 2000).

Target genes of β-catenin during formation of the anteroposterior axis and the mesoderm have not been analyzed in a systematic and genome-wide manner. Using microarray technology, we identify Cripto, which encodes a Nodal co-receptor, as a primary target of β-catenin. This defines the basis of a novel molecular interaction between Nodal and β-catenin signalling pathways in early embryogenesis. Interestingly, Cripto expression in the early embryo and in tumours depends on β-catenin signals. Comparison of the genome-wide expression profiles of β-catenin, Cripto and Wnt3 mutant embryos results in the identification of novel genes that are active during formation of the anteroposterior axis and of the mesoderm.

**Materials and methods**

**Mouse mutants**

β-catenin-, Cripto- and Wnt3-deficient mice have been described previously (Ding et al., 1998; Liu et al., 1999; Huelsken et al., 2000). Mouse embryos were staged according to standard criteria: E6.0 was defined as strictly pre-streak, and embryos displaying signs of streak formation were not used for analysis. E6.5 was defined as early streak stage: only early streak stage wild type embryos and mutant littermates were used for analysis. Mice carrying an allele of β-catenin that containsloxP sites flanking exon 3 (Harada et al., 1999) were crossed with mice expressing Cre recombinase under the control of the CMV promoter (Nagy et al., 1998) to obtain gain-of-function mutants. All mouse embryos were genotyped by PCR (Ding et al., 1998; Liu et al., 1999; Huelsken et al., 2000; Soshnikova et al., 2003). In situ hybridisation was performed as previously described (Huelsken et al., 2000). To generate gene expression profiles of isolated embryonic tissues, wild-type embryos were dissected at mid-streak stage, using a combination of mechanical and enzymatic means (Harrison et al., 1995).

**Microarray analysis**

RNA and genomic DNA were isolated from single mouse embryos, using Trizol (GibcoBRL), according to the manufacturer’s instructions. RNA was reverse transcribed using a T7-promoter tagged polyT primer, and antisense RNA was produced using T7 RNA polymerase. Antisense RNA was examined by gel electrophoresis, and equivalent amounts of RNA from three to five age-matched embryos of the same genotype were pooled to generate a microarray probe. Probes were biotin-labeled in a second round of T7 amplification, as previously described (Luo et al., 1999). Profiles of β-catenin-, Cripto- and Wnt3-deficient mice were compared with profiles of wild-type littermates of the same genetic background. Microarray profiling was performed in triplicate from independent preparations of embryonic RNA using Affymetrix Mu11k GeneChips (11,000 sequences). Isolated tissue domains were profiled in duplicate using MG-U74A GeneChips (8,000 sequences).

GeneChip image analysis, data normalisation and comparative analysis were performed using the statistical algorithm of Affymetrix Microarray Suite (MAS) 5.0 software following the manufacturer’s guidelines. Ranking and filtering of genes was performed using Microsoft Excel. β-Catenin-responsive genes were defined as genes that changed expression more than twofold, with a Change p-Value limit of 0.1 in cross-wise comparative analyses. Genes commonly deregulated in β-catenin- and either Cripto or Wnt3 mutants were defined as genes that changed expression greater than twofold, or with a Change p-Value limit of 0.1. GeneChip data of isolated tissues were normalised and visualised using Silicon Genetics GeneSpring software. The significance of the overlap between genes deregulated in both β-catenin and Cripto or Wnt3 mutants was calculated using the hypergeometric distribution function (Feller, 1968). The microarray data set is available on our website http://www.mdc-berlin.de/~zelldiff

**Tissue culture**

Ls174T cells that express tetracycline-inducible, dominant-negative Tcf4 have been described before (van de Wetering et al., 2002). For northern blot analysis, RNA was isolated using Trizol (GibcoBRL) before and 12 hours after induction with 1 µg/ml doxycyclin. For β-catenin-Tcf4-dependent reporter assays, 293T cells were transfected
by calcium phosphate/DNA co-precipitation with 1 µg reporter constructs, 0.1 or 0.4 µg Tcf4-β-catenin-fusion-construct, 1 µg pSV-β-Gal-Plasmid, and empty pCMVneo to 4 µg DNA per well. Transfections and measurements were performed in triplicate.

Plasmid construction

CRIPTO enhancer sequences were amplified from a cosmid clone using the primer sequences 5′-AAC TAC TTT GCC ACC CTG TC-3′ and 5′-ACT GCC CAG AAG CTA TG G-3′ and cloned into the Sall-sites of FOPFlash (Molenaar et al., 1996) to replace the FOP sites. Putative Lef/Tcf-binding sites were mutated using PCR mutagenesis and the primer sequences 5′-GGG CGA TAA ATC AAC TGC GTT TGT GTC CTC TCT TGG-3′, 5′-GTC CTC CGG AAT CCT GCG ATT CCT TCG AGA GGA C-3′, 5′-GTC TCC TCT ATG TGC TGG GAT GGC TGG CTA GAT-3′ (mutated nucleotides are underlined). CRIPTO 5′ flanking sequences were amplified using the primer sequences 5′-GCC AGT GTG GAC AAG TCC TG-3′ and 5′-GCC AGT GTG GAC AAG TCC TG-3′ (mutated nucleotides are underlined). CRIPTO 5′ flanking sequences were amplified using the primer sequences 5′-GCC AGT GTG GAC AAG TCC TG-3′ and 5′-GCC AGT GTG GAC AAG TCC TG-3′ (mutated nucleotides are underlined).

Results

Expression profiling of β-catenin mutant embryos

To define the roles of β-catenin in the formation of the anteroposterior axis and the mesoderm, we compared gene expression profiles of wild-type and β-catenin mutant embryos. To this end, litters from β-catenin heterozygous intercrosses were dissected, and RNA from pools of wild-type and β-catenin mutant embryos was examined using Affymetrix GeneChips.

We found that 106 and 60 genes are deregulated in β-catenin−/− embryos at E6.0 and E6.5, respectively, corresponding to the stages at which the anteroposterior axis and the mesoderm are formed (for selected genes see Table 1, for a complete list see Table S1 at http://dev.biologists.org/). The profiling data do not provide information whether these genes are modulated directly or indirectly by β-catenin. Many genes that are modulated in β-catenin−/− embryos encode signalling molecules or transcription factors. Remarkably, CRIPTO is the most prominently downregulated gene at E6.0, and we provide evidence below that CRIPTO may be a direct transcriptional target of β-catenin. We also find deregulated expression of Mxl1, Tm7SF1, Tssc3 (PhilDA2 – Mouse Genome Informatics) Apf, Sfrp1, Fasl (Tnfsf6 – Mouse Genome Informatics), Sim2, Neurod1, Frz7 and other genes at this stage. Among these, Mxl1 and Frz7 have previously been identified to be regulated by β-catenin, but it is not known whether this regulation is direct or indirect (Willert et al., 2002; Kielman et al., 2002).

At E6.5, we find downregulation of brachyury, Eomes, Fgf8 and Evx1 in β-catenin−/− embryos; these genes have known functions in mesoderm development (Wilkinson et al., 1990; Dush and Martin, 1992; Sun et al., 1999; Russ et al., 2000). Brachyury has been shown to be a direct transcriptional target of β-catenin (Yamaguchi et al., 1999; Arnold et al., 2000; Galceran et al., 2001). Other genes are also downregulated or absent. In particular, we noted the downregulation of the Nanog homeobox gene, which is essential for maintenance of embryonic stem cells (Chambers et al., 2003; Mitsu et al., 2003). We do not see changes in the expression of regulators of cell proliferation and the cell cycle, which have previously been shown to be β-catenin targets, i.e. Cnd1 (the gene encoding cyclin D1) or Myc (He et al., 1998; Tetsu and McCormick, 1999; Shtutman et al., 1999). However, we find increased expression of p57Kip2 (Cdkn1c – Mouse Genome Informatics) in β-catenin mutants at E6.5, which codes for a negative regulator of cell proliferation (Lee et al., 1995). It is unlikely that upregulated genes are directly controlled by β-catenin signalling, as loss of the transcriptional activator β-catenin should lead to downregulation of direct target genes. We find little overlap of β-catenin controlled genes in the embryo and those regulated by Wnt3a in teratocarcinoma cells (Willert et al., 2002).

In control studies, we also examined transcriptional changes that occur between E6.0 and E6.5 in wild-type embryos during normal development. We found that 130 genes are downregulated at E6.5 in wild type, and of these, 127 were also downregulated in β-catenin mutants (Fig. 1A). In addition, 673

<table>
<thead>
<tr>
<th>GenBank ID</th>
<th>Name</th>
<th>Suggested molecular function</th>
<th>Fold change</th>
<th>Confidence (Change p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>m87321</td>
<td>CRIPTO (Tdgf1)</td>
<td>Signal transducer</td>
<td>−3.8</td>
<td>0.001</td>
</tr>
<tr>
<td>s95251</td>
<td>Mxl1</td>
<td>Signal transducer</td>
<td>−2.7</td>
<td>0.05</td>
</tr>
<tr>
<td>a177300</td>
<td>Tm7SF1</td>
<td>Signal transducer</td>
<td>−2.3</td>
<td>0.005</td>
</tr>
<tr>
<td>a002708</td>
<td>Tssc3 (Ipl)</td>
<td>Signal transducer</td>
<td>−2.1</td>
<td>0.001</td>
</tr>
<tr>
<td>a023873</td>
<td>Sim2</td>
<td>Transcription factor</td>
<td>+6.2</td>
<td>0.1</td>
</tr>
<tr>
<td>x12761</td>
<td>Jun</td>
<td>Transcription factor</td>
<td>+5.4</td>
<td>0.001</td>
</tr>
<tr>
<td>a031896</td>
<td>Cer1</td>
<td>Transcription factor</td>
<td>+3.8</td>
<td>0.001</td>
</tr>
<tr>
<td>u43320</td>
<td>Fzd7</td>
<td>Transcription factor</td>
<td>+3.4</td>
<td>0.005</td>
</tr>
<tr>
<td>v00743</td>
<td>Afp</td>
<td>Fetal serum protein</td>
<td>+3.0</td>
<td>0.001</td>
</tr>
<tr>
<td>m55512</td>
<td>Wt1</td>
<td>Transcription factor</td>
<td>+2.6</td>
<td>0.001</td>
</tr>
<tr>
<td>u88566</td>
<td>Sfrp1</td>
<td>Signal transducer</td>
<td>+2.6</td>
<td>0.01</td>
</tr>
<tr>
<td>u63146</td>
<td>Rhp4</td>
<td>Signal transducer</td>
<td>+2.4</td>
<td>0.001</td>
</tr>
<tr>
<td>u06948</td>
<td>Fax (Tnfsf6)</td>
<td>Signal transducer</td>
<td>+2.4</td>
<td>0.005</td>
</tr>
<tr>
<td>u28068</td>
<td>Neurod1</td>
<td>Transcription factor</td>
<td>+2.3</td>
<td>0.1</td>
</tr>
<tr>
<td>x70298</td>
<td>Sox4</td>
<td>Transcription factor</td>
<td>+2.0</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Genes were annotated using the Affymetrix NetAffx database, Mouse Genome Informatics database, NCBI Unigene database and PubMed searches. The average fold change and the upper limit of the Change p-Values are given, as calculated by Affymetrix MAS 5.0 software. Selected genes with a role in signalling or proposed function in embryonic development are shown. Genes whose differential expression was confirmed by in situ hybridisation are indicated in bold.
genes were upregulated in wild-type embryos at E6.5, and of these, 591 were also upregulated, and only 82 were downregulated in β-catenin mutants (green and red in Fig. 1A, respectively). Thus, despite the severe phenotypic defects in β-catenin mutant embryos, the general program of gene expression is overall highly similar to wild type at these developmental stages.

Expression patterns of β-catenin responsive genes

The 106 and 60 genes deregulated in β-catenin−/− embryos at E6.0 and E6.5 were further characterised by their expression patterns in normal development, using both in situ hybridisation and gene expression profiling of isolated tissue domains. For the latter, wild-type primitive streak stage embryos were dissected into four tissue domains, i.e. extraembryonic tissues, visceral endoderm, embryonic ectoderm and mesoderm (Fig. 1B), and the corresponding gene expression profiles were determined. To assess the quality of the dissection, we analyzed the distribution of transcripts for Pou5f1, brachyury, Cer1 and Bmp4. The expression patterns of these genes have been previously determined, and their transcripts displayed the expected tissue distribution in our profiling (Fig. 1B, lower panel). We found that the majority of genes that were deregulated in β-catenin mutants were expressed in a restricted manner in the wild type, i.e. their transcripts were observed in one or two, but not all tissues (Fig. 1C).

Expression patterns of β-catenin responsive genes

The 106 and 60 genes deregulated in β-catenin−/− embryos at E6.0 and E6.5 were further characterised by their expression patterns in normal development, using both in situ hybridisation and gene expression profiling of isolated tissue domains. For the latter, wild-type primitive streak stage embryos were dissected into four tissue domains, i.e. extraembryonic tissues, visceral endoderm, embryonic ectoderm and mesoderm (Fig. 1B), and the corresponding gene expression profiles were determined. To assess the quality of the dissection, we analyzed the distribution of transcripts for Pou5f1, brachyury, Cer1 and Bmp4. The expression patterns of these genes have been previously determined, and their transcripts displayed the expected tissue distribution in our profiling (Fig. 1B, lower panel). We found that the majority of genes that were deregulated in β-catenin mutants were expressed in a restricted manner in the wild type, i.e. their transcripts were observed in one or two, but not all tissues (Fig. 1C). β-Catenin has previously been found to be required in the embryonic ectoderm for anteroposterior axis formation (Huelsken et al., 2000), and Cripto, the most strongly downregulated gene at E6.0, is indeed expressed in the embryonic ectoderm of the wild type (Fig. 1C). β-Catenin has previously been found to be required in the embryonic ectoderm for anteroposterior axis formation (Huelsken et al., 2000), and Cripto, the most strongly downregulated gene at E6.0, is indeed expressed in the embryonic ectoderm of the wild type (Fig. 1C). β-Catenin has previously been found to be required in the embryonic ectoderm for anteroposterior axis formation (Huelsken et al., 2000), and Cripto, the most strongly downregulated gene at E6.0, is indeed expressed in the embryonic ectoderm of the wild type (Fig. 1C). β-Catenin has previously been found to be required in the embryonic ectoderm for anteroposterior axis formation (Huelsken et al., 2000), and Cripto, the most strongly downregulated gene at E6.0, is indeed expressed in the embryonic ectoderm of the wild type (Fig. 1C).
in the formation of the anteroposterior axis (Kimura et al., 2000; Perea-Gomez et al., 2001), is expressed appropriately in β-catenin mutants at E6.0, but is absent during later developmental stages (Fig. 2G,H) (Huelsken et al., 2000).

At E6.5, a large proportion of genes that are downregulated in β-catenin−/− embryos, are expressed in the embryonic ectoderm and the mesoderm of control embryos (Fig. 1C). It should be noted that mesodermal structures are newly generated in control embryos at E6.5, but they are absent in β-catenin mutants. Again, we have no information on whether these genes are regulated directly or indirectly by β-catenin. Fgf8 is expressed in the posterior embryonic ectoderm and mesoderm of control embryos (Sun et al., 1999), and absent in β-catenin mutants (Fig. 2I,J). We find that the Nanog homeobox gene is expressed in the proximal embryonic ectoderm prior to streak formation, and in the distal and posterior embryonic ectoderm at E6.5 in control embryos, but is absent in β-catenin mutants (Fig. 2K,L). We find that the Nanog homeobox gene is expressed in the proximal embryonic ectoderm prior to streak formation, and in the distal and posterior embryonic ectoderm at E6.5 in control embryos, but is absent in β-catenin mutants (Fig. 2K,L). Many genes whose expression is changed in β-catenin mutants are expressed in the visceral endoderm and in extra-embryonic tissues (Fig. 1C), indicating that these tissues also require β-catenin activity for normal development. For example, the genes Tm7Sf1 and Afp are expressed in subdomains of the visceral endoderm in wild type, but are not expressed or are expressed in the entire visceral endoderm in β-catenin−/− embryos, respectively (Fig. 2Q-R). Again, we do not have information on whether these genes are regulated directly or indirectly by β-catenin, and we have not monitored possible ectopic expression of genes in the mutant. Taken together, the combination of previous analysis (Huelsken et al., 2000) and the microarray and in situ hybridisation experiments presented here, show the following major changes in β-catenin-deficient embryos: (1) loss of the expression of Cripto and of other genes in the embryonic ectoderm, (2) downregulation of genes expressed in the primitive streak and/or in the mesoderm, and (3) modified expression of genes in the visceral endoderm.

Comparison of expression profiles of β-catenin, Cripto and Wnt3 mutant embryos

To define groups of genes regulated by signals that direct the formation of the anteroposterior axis and the mesoderm, we determined the expression profiles of Cripto and Wnt3 mutant embryos at E6.0 and E6.5 and compared them with those of β-catenin−/− embryos. We found 30 (out of 106) genes commonly deregulated in β-catenin and Cripto mutants at E6.0 (see Table S1 at http://dev.biologists.org/supplemental/). The number of
positioned genetically downstream of Cripto, and thus may not upregulated. The commonly deregulated genes may be Sox1/2 embryos are typically expressed in developing neural tissues ectoderm, but also in other tissue domains (Fig. 3A, upper panel). We find downregulated expression of these genes in wild-type embryos shown. Genes were selected from all genes that are deregulated in the respective mutants (see http://www.mdc-berlin.de/~zelldiff). (A) Genes commonly deregulated in both β-catenin and Cripto mutants at E6.0. Upper panel, commonly downregulated genes with putative signalling or developmental functions; lower panel, commonly upregulated genes of which ESTs have been preferentially isolated from neural tissues. (B) Genes commonly downregulated in both β-catenin and Wnt3 mutants at E6.5. Upper panel, genes downregulated specifically at E6.5 (late genes); lower panel, genes deregulated at both E6.5 and E6.0 (early genes); in brackets, fold change at E6.0, nd, not determined. Red, high relative expression; blue, low relative expression; bright area, genes with consistent measurements; dark area, genes with variable or low measurements in certain tissues.

When β-catenin and Wnt3 mutants were compared at E6.5, we found 16 (of 60) genes commonly deregulated (see Table S1 at http://dev.biologists.org/supplemental/). This number is also larger than expected to occur randomly (P<0.005, as determined by the hypergeometric distribution function). Two groups of genes can be distinguished: the first group is deregulated at E6.5, but not at E6.0 (here called late genes, upper panel in Fig. 3B), and contains genes strongly expressed in the mesoderm of control embryos, e.g. Evx1, Fgf8 and Acrv2. The second group is downregulated at E6.5 and at E6.0 (here called early genes, lower panel in Fig. 3B), and includes genes expressed prior to primitive streak and mesoderm formation in the proximal embryonic ectoderm like brachyury and Nanog (Fig. 2K-N) (Wilkinson et al., 1990). We expect direct targets of β-catenin rather among the early than the late genes. These results are consistent with the established role for a Wnt3/β-catenin-dependent gene expression program for primitive streak and mesoderm formation (Liu et al., 1999).
A gain-of-function mutation of β-catenin promotes Cripto expression and disturbs anteroposterior axis formation

As loss of β-catenin led to a loss of Cripto, we asked whether increased β-catenin signals may promote Cripto expression. Constitutively active β-catenin can be produced in mice by a gain-of-function mutation in the β-catenin gene by using the Cre-loxP technology (Harada et al., 1999; Soshnikova et al., 2003). We introduced this conditional mutation in the early embryo by employing a mouse strain that expresses Cre recombinase early and ubiquitously under the control of the CMV promoter (Nagy et al., 1998). Using this technique, the floxed exon 3 of β-catenin is removed, which leads to constitutively active β-catenin that cannot be phosphorylated at the N terminus and cannot be degraded. We examined the expression of essential genes in embryos at E6.5 by immunohistochemistry and in situ staining of transversal sections. In wild-type embryos, β-catenin is produced asymmetrically, i.e. enriched in the cytoplasm on the posterior side, as is expression of conductin, which is a direct target of β-catenin signals (Lustig et al., 2002; Jho et al., 2002). In the gain-of-function mutants, β-catenin and conductin were highly expressed, but now symmetrically in the entire epiblast (Fig. 4A-D). Remarkably, Cripto expression that is also increased on the posterior side in the wild type, was as well highly expressed symmetrically (Fig. 4E,F). These data indicate that the asymmetric expression of Cripto is a result of asymmetric β-catenin function, strongly suggesting that Cripto is a β-catenin target. Moreover, in β-catenin gain-of-function mutants, we also found loss of asymmetry of the expression of Nanog, brachyury and Cerberus, which mark the posterior ectoderm, posterior mesoderm and anterior visceral endoderm, respectively, and we found downregulation of the ectodermally expressed gene Pou5f1/Oct4 (Fig. 4G-N).

β-catenin regulates Cripto expression

To determine whether Cripto is regulated by β-catenin in other tissue contexts, we first assessed its regulation in the human colon adenocarcinoma cell line LS174T, which carries an Apc mutation. In these cells, β-catenin is stabilised and can be detected in the cytoplasm and the nucleus, and Lef/Tcf/β-catenin responsive promoters are active (van de Wetering et al., 2002). We found that Cripto is strongly expressed in these cells, while induction of dominant-negative Tcf4 abolishes Cripto expression (Fig. 5A), and blocks the expression of other β-catenin responsive genes (van de Wetering et al., 2002). We next examined expression of Cripto in intestinal adenomas of Min mice, which carry an Apc mutation, exhibit increased β-
Fig. 5. Cripto is regulated by β-catenin/Tcf in colon cancer cells and tumours. (A) Northern blot analysis of Cripto expression in Ls174T colon adenocarcinoma cells. Expression of Cripto in control Ls174T cells and in cells that express dominant-negative Tcf4 in a tetracycline-inducible manner is shown. Cripto expression was assessed before (−) and 12 hours after (+) induction with doxycyclin. RNA-loading control, 28s rRNA. (B) In situ hybridisation on consecutive sections of intestinal epithelium of Min (APC mutant) mice that contain an adenoma. The β-catenin target gene conductin marks the crypts and the adenoma. Cripto and Tssc3 (see Table 1) are also upregulated in the adenoma. (C) Identity plot of murine and human genomic sequences in the region of the Cripto locus. The mouse sequence is indicated on the horizontal axis. The transcribed region of Cripto is indicated by a horizontal arrow, the position of Cripto exons by black boxes. The Cripto enhancer (8 kb upstream) and immediate 5′ flanking regions are indicated by red and green boxes, respectively. Conserved sequence stretches between mouse and human sequences are indicated by the short horizontal lines (50% to 100% identity). Consensus sequences for Lef/Tcf binding sites (WWCAAAAG) in the murine genome are indicated by vertical red lines. Sequences were aligned using the Pipmaker program (Schwartz et al., 2000). (D,E) Identification of β-catenin/Tcf responsive elements in the Cripto enhancer by luciferase reporter gene assay, using a Tcf4-β-catenin hybrid effector plasmid. β-Catenin-responsive TOPflash and inactive FOPflash have been described previously (Molenaar et al., 1996). Mutations in the three Lef/Tcf-binding sites (3×MUT) are described in the Materials and methods.

Catenin signalling and develop multiple intestinal adenomas (Moser et al., 1992; Smits et al., 1999). Cripto expression was indeed upregulated in the adenomas of Min mice (Fig. 5B), as was the expression of the known Wnt target gene conductin (Lustig et al., 2002; Jho et al., 2002). We also analyzed the expression of other β-catenin responsive genes identified in the embryo, and found Tssc3 to be upregulated in adenomas of Min mice (Fig. 5B, see also Table 1).

We identified three closely linked Lef/Tcf consensus binding sites in the mouse genome 8 kb upstream of the Cripto transcription start site; this region, including one of the Lef/Tcf consensus sites, is highly conserved between mouse and human genomes (Fig. 5C). A 0.4 kb mouse genomic fragment containing the putative Lef/Tcf-binding sites conferred responsiveness to Tcf4 when inserted into a reporter gene construct (Fig. 5D), comparable with the artificial Wnt reporter TOPflash (Molenaar et al., 1996). Mutation of the Lef/Tcf site consensus sequences strongly reduced responsiveness of the reporter to Tcf4 (Fig. 5E). By contrast, the immediate 5′ flanking region of the Cripto gene (green in Fig. 5C) did not activate gene expression in a Tcf4-dependent manner (Fig. 5D). These results suggest that expression of Cripto may be modulated directly by Lef/Tcf/β-catenin complexes.

Discussion

β-Catenin mutant embryos fail to undergo two crucial developmental steps: (1) the distal visceral endoderm does not become positioned at the anterior side at E6.0, and (2) primitive streak and mesoderm formation does not occur at E6.5 (see scheme in Fig. 6A) (Huelsken et al., 2000). These changes can be interpreted as the sum of the phenotypes observed in Cripto and Wnt3 mutant mice. Criptoβ−/− embryos fail to re-orient the anteroposterior axis at E6.0, but generate extra-embryonic mesoderm from the proximal epiblast at E6.5, whereas Wnt3β−/− embryos correctly position the anteroposterior axis at E6.0, but fail to generate mesoderm at E6.5 (Fig. 6A) (Ding et al., 1998; Liu et al., 1999). Using expression profiling, we have identified Cripto and other genes whose expression is absent in β-catenin mutants at E6.0, when the anteroposterior axis is normally re-oriented in wild-type embryos. We also identified brachyury, Nanog and other genes whose expression depends on β-catenin at E6.5, when the primitive streak and mesoderm are formed.
Furthermore, we find a significant overlap of genes whose expression is deregulated in β-catenin and Cripto, and in β-catenin and Wnt3 mutant embryos at E6.0 and E6.5, respectively. Our profiling data thus support our model of two distinct β-catenin dependent steps (Fig. 6B). In the first step, β-catenin is essential for the expression of the Nodal co-receptor gene Cripto in the epiblast, which is required for translocation of the distal visceral endoderm to the anterior side, and thus the correct orientation of the anteroposterior axis at E6.0. In the second step, β-catenin is required for Wnt3 signalling and thus regulates the expression of target genes in the proximal/posterior epiblast that are essential for mesoderm formation.

Although the transcriptional activity of β-catenin is generally thought to be Wnt dependent, the Wnt ligand that regulates β-catenin activity during formation of the anteroposterior axis is unknown (Fig. 6B). Notably, mutant mice in which canonical Wnt signal transduction is completely blocked upstream of β-catenin by mutation of a chaperone of the Wnt co-receptors LRP5/6 display defective mesoderm formation, but form a normal anteroposterior axis (Hsieh et al., 2003). These studies suggest the existence of a Wnt-independent requirement for β-catenin during mouse anteroposterior axis positioning. Perhaps consistent with this idea, the development of the dorsoorientation axis in Xenopus is dependent on β-catenin and additional components of the Wnt signal transduction machinery such as GBP/Gsk3-β/Axin, but cannot be inhibited by dominant-negative Wnt molecules (Yost et al., 1998; De Robertis et al., 2000).

We have demonstrated an unexpected mechanism underlying the genetic interaction between β-catenin and Nodal/Cripto signalling during early mouse embryogenesis. Previous studies in Xenopus have demonstrated an interaction between the β-catenin and Nodal pathways through the coordinate transcriptional regulation of downstream targets such as Twin, a homeobox gene, which is essential for the formation of the Spemann organiser (Nishita et al., 2000; Labbe et al., 2000). We show that β-catenin signalling in the mouse embryo impinges upon the Nodal pathway through regulation of the Nodal co-receptor Cripto. Cripto expression is absent in β-catenin-deficient embryos, while it is enforced in β-catenin gain-of-function mutants. Nodal+/- mice do not express Cripto (Brennan et al., 2001), and thus the regulation of Cripto is not solely dependent on β-catenin, but requires Nodal activity as well. We find that Nodal is expressed appropriately in β-catenin mutants, indicating that loss of Cripto is not due to the absence of Nodal.

We have identified genes that are commonly downregulated in both Cripto- and β-catenin-deficient embryos at E6.0, e.g. Sox11, Ppp2ca and Plk. These latter genes are expressed in the embryonic ectoderm and may be targets of Cripto. Alternatively, Cripto may signal to associated tissue domains, e.g. the visceral endoderm and the extra-embryonic ectoderm.
during this developmental stage. It is known that developmental signalling pathways interact across tissue borders (Struhl and Basler, 1993; Brennan et al., 2001). We have also identified genes that are commonly upregulated in both Cripto and β-catenin-deficient embryos at E6.0, e.g. Sim2, Neurod1 and Aqp4. Interestingly, these genes are often expressed in neural tissues (Kimura et al., 2001). We also found loss of expression of the ectodermally expressed gene Pou5f1/Oct4 in β-catenin gain-of-function mutants. Consistent with these findings, recent studies indicate that β-catenin-mediated signals can block ectodermal differentiation of embryonic stem cells, whereas the suppression of such signals induces neuroectodermal differentiation (Kielman et al., 2002; Aubert et al., 2002). Moreover, precocious expression of the neurally expressed genes Hesx and Six3 has previously been noted in Cripto mutants (Ding et al., 1998). The increased expression of neurally expressed genes in β-catenin and Cripto mutants may be indicative of premature neuroectodermal differentiation, and may imply that these genes act downstream of Cripto.

The comparison of genes deregulated in β-catenin and Wnt3 mutants at E6.5 and E6.0 allowed us to dissect the sequence of events that leads to primitive streak and mesoderm formation. In control embryos, genes like brachyury (Wilkinson et al., 1990), Nanog and Eomes (Russ et al., 2000) are expressed early in the embryonic ectoderm before the primitive streak and mesoderm are formed, and these genes are deregulated at both E6.0 and E6.5 in β-catenin and Wnt3 mutants (early genes). Subsequently, genes like Fgf8 (Sun et al., 1999) and Evx1 (Dush and Martin, 1992) are expressed in the developing mesoderm of control embryos, and these are deregulated at E6.5, but not at E6.0 in β-catenin and Wnt3 mutants, owing to absence of mesoderm (late genes). Among the set of early genes, we have identified the homeobox gene Nanog, which is required for maintenance of pluripotency of epiblast cells prior to implantation (Chambers et al., 2003; Mitsu et al., 2003). At E6.0, Nanog is expressed in the proximal epiblast, and this expression domain shifts to more distal and posterior positions in the E6.5 embryo. Nanog expression is thus similar to the expression pattern of Wnt3 (Liu et al., 1999), suggesting that Nanog may be regulated by Wnt3/β-catenin signals during gastrulation. Our analysis therefore indicates that Nanog may have additional functions in the post-implantation mouse embryo.

Gene expression profiling revealed that the vast majority of genes that change expression between E6.0 and E6.5 in wild-type embryos are regulated in a similar manner in β-catenin mutants, i.e. they are either commonly up- or commonly downregulated. This finding suggests that the loss of β-catenin affects specific, and not general developmental programs. Moreover, most genes that are deregulated in β-catenin mutant embryos are expressed in specific domains of the wild-type embryo, suggesting tissue-specific functions. Surprisingly, a large group of β-catenin-modulated genes are expressed in extra-embryonic tissues of control embryos, and only a fraction of these are also deregulated in Cripto mutants. It is thus possible that additional β-catenin-dependent developmental programs regulate gene expression in visceral endoderm and extra-embryonic ectoderm. In accordance with this, a loss of regionalisation of the visceral endoderm in β-catenin mutants was previously observed by electron microscopy (Huelsken et al., 2000). A recent study using conditional inactivation of β-catenin has indicated a requirement of β-catenin in the visceral endoderm (Lickert et al., 2002). Together with our previous results, i.e. that β-catenin function is required in the embryonic ectoderm (Huelsken et al., 2000), these data may indicate that β-catenin has multiple essential roles in early embryogenesis.

We also observed regulation of Cripto expression by β-catenin during tumourigenesis. Cripto expression is regulated by Lef/Tcf in colon cancer cells, and by β-catenin in colon adenomas of Min mice. Cripto was previously found to be overexpressed in a wide range of epithelial tumours, including colon, gastric and endometrial carcinomas, which also frequently harbour activating mutations in the Wnt/β-catenin signalling pathway (Salomon et al., 2000). Tissue culture studies have implicated Cripto in cell survival, proliferation control, and oncogenic transformation (Ciaraldiello et al., 1991; Niemeyer et al., 1998), indicating that Cripto expression contributes to malignancy. Our work suggests that activation of β-catenin signalling in tumours may be responsible for Cripto overexpression. Cripto may thus be a direct and critical target gene of Lef/Tcf transcription factors in tumorigenesis and could potentially represent a suitable extracellular target for future tumour therapy.

We thank Carmen Birchmeier for helpful discussions and valuable comments on the manuscript, Frauke Kosel for excellent technical assistance and Marta Rosario for critical reading of the manuscript. This research was supported by awards from the American Heart Association (J.D.) and the Leukemia and Lymphoma Society (J.D.), NIH grants HD42837 and HD38766 (M.M.S.), and Helmholtz Society Strategic Fund Initiative ‘DNA Chips’ (M.M. and W.B.).

References
β-Catenin in mouse embryogenesis 6293
	nodal in the formation and maintenance of the primitive streak in the mouse. Development 120. 1919-1928.


