function in transcriptional regulation, and that Gro acts in a repressor complex with dTcf. This dual regulatory role may be conserved in vertebrate Wnt signalling[9,19]. Therefore, we propose that the balance between the activity of Gro and Arm controls cell-fate choice by the Wnt pathway in both vertebrates and invertebrates. □

Methods

Fly stocks and crosses. Cuticle preparations and antibody stainings were performed as described[24]. In Figs 1, 2 and 4, genotypes were assigned by comparing the frequencies of phenotypic classes with expected genotypic frequencies; these data are summarized in Table 1. For *arm*, suppression was documented by ranking embryos in weak to strong phenotypic categories and calculating ratio of embryos in weak categories. wg^{CX4} is a molecular null allele[25]; *Df(2)DE*[18] is a *wg* hypomorph (A.B., unpublished observations); arm^{XP33} is a strong hypomorph; arm^{YD35} is a null allele[15]; both *dTcf* mutations used are molecular null alleles[8]; gro^{E48} is a putative null point mutation[21]; gro^{BX22} lacks *gro* and several neighbouring genes in the *Enhancer of split* complex[26]. Gal4 and UAS transgene stocks have been described[8].

Mammalian cell culture. Vector alone (pCDNA3), hTcf-1 or dTcf and Myc-epitope-tagged Gro(1–181) constructs (with a ratio of 10:1) were introduced into COS cells by diethyl aminoethyl-dextran transfections. Cells were prepared for immunohistochemistry using an anti-Myc-antibody. 2×10^6 IIAI.6 B cells were transfected by electroporation with 1 μg dTcf luciferase reporter plasmid (pTKTOP) or its negative control containing mutated dTcf sites (pTKFOP)[19]. These were co-transfected with 2 μg dTcf expression vector, 0.5 or 5.0 μg Gro expression plasmids and 0.5 μg Arm expression plasmid, balanced to equal plasmid amounts with pCDNA3. Luciferase activity was corrected by chloramphenicol acetyltransferase (CAT) activity[19]. Luciferase and CAT activities were determined as in ref. 8.

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The *Xenopus* Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors

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Tcf/Lef transcription factors mediate signalling from Wingless/Wnt proteins by recruiting Armadillo/β-catenin as a transcriptional co-activator[1–7]. However, studies of *Drosophila*, *Xenopus* and *Caenorhabditis elegans* have indicated that Tcf factors may also be transcriptional repressors[6,8–13]. Here we show that Tcf factors physically interact with members of the Groucho family of transcriptional repressors. In transient transfection assays, the *Xenopus* Groucho homologue XGrg-4 inhibited activation of transcription of synthetic Tcf reporter genes. In contrast, the naturally truncated Groucho-family member XGrg-5 enhanced transcriptional activation. Injection of *XGrg-4* into *Xenopus* embryos repressed transcription of *Siamois* and *Xnr-3*, endogenous targets of β-catenin–Tcf. Dorsal injection of *XGrg-4* had a ventralizing effect on *Xenopus* embryos. Secondary-axis formation induced by a dominant-positive Armadillo–Tcf fusion protein was inhibited by XGrg-4 and enhanced by XGrg-5. These data indicate that expression of Tcf target genes is regulated by a balance between Armadillo and Groucho.

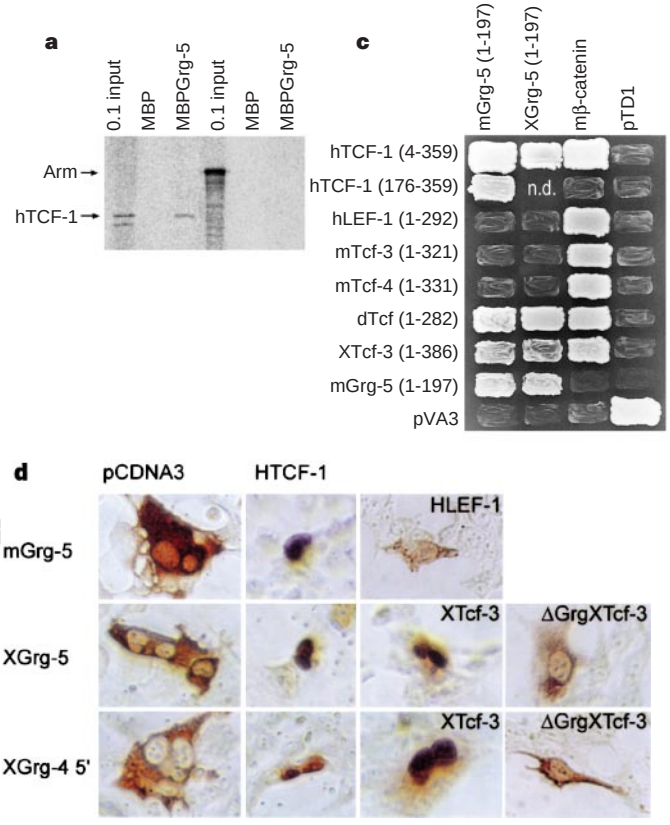
In our yeast two-hybrid screen for proteins interacting with human (h) TCF-1, which led to the identification of β-catenin[2], roughly 60 out of 300 clones encoded the murine (Gro)-related gene *Grg-5* (ref. 14). We confirmed independently that TCF-1 and Grg-5 interact in a binding assay using a recombinant maltose-binding protein (MBP)–Grg5 fusion protein and *in vitro*-translated hTCF-1 (Fig. 1a).

Groucho (*Gro*) is a broadly expressed *Drosophila* corepressor, and may be involved in segmentation, sex determination and neurogenesis[15–18]. Hairy and Enhancer of Split-like (HES) helix–loop–helix factors interact with the non-DNA-binding Gro protein to repress transcription of their target genes[16,19]. In mammals, multiple homologues with a similar overall domain structure have been identified. These are termed *TLE 1–4* in man, and *mGrg-1*, *-3* and *-4* in mouse (Fig. 1b). *Grg-5* encodes only the two amino-terminal domains of these proteins (Fig. 1b). In a search for maternally expressed Gro proteins in *Xenopus*, we cloned *XGrg-5* (or *XAES*[20]) and a *Grg-4* orthologue (Fig. 1b). Both were abundantly and ubiquitously expressed in oocytes and in embryos undergoing the pre-mid-blastula transition (results not shown).

In yeast two-hybrid assays, we found that mGrg-5 and XGrg-5 interacted with *Drosophila* (d) Tcf, XTcf-3 and, as reported previously[21], with mGrg-5 itself (Fig. 1c), but not with hLEF-1, mTcf-3 and mTcf-4. Deletion analysis defined a minimal region in hTCF-1 (amino acids 176–359) that was capable of binding to Grg-5; this domain was separable from the Armadillo (Arm)-interaction domain (amino acids 4–63; ref. 22). No conclusive data were obtained in yeast for the interactions between Tcfs and the 'long' Gro homologues, which we attribute to transcriptional repression of the selection gene (histidine). To circumvent this problem, tagged N-terminal fragments of Gro proteins (collinear with full-length Grg-5; Fig. 1b) were expressed in COS cells. Although the full-length proteins were nuclear (not shown), these N-terminal fragments localized to the cytoplasm. Co-transfection of such truncated complementary DNA clones with various *Tcf* expression constructs allowed us to visualize interaction between Tcfs and the long Gro homologues by nuclear translocation. In this assay, hTCF-1 interacted with mGrg-5, XGrg-4 and XGrg-5, whereas XTcf-3 interacted with XGrg-4 and XGrg-5. Removal of the Grg-interaction domain from XTcf-3 (Fig. 1b; ΔGrg-XTcf-3) abrogated the interaction of XTcf-3 with Gro homologues and nuclear translocation of the complexes (Fig. 1d).

In transient transfections using a previously established β-catenin–Tcf reporter gene assay[5], we found that XGrg-4 repressed the activation of transcription by Arm and XTcf-3 (Arm–XTcf3 complexes) (Fig. 2a). The repression was specific, as we did not observe effects on the mutant Tcf reporter gene (Fig. 2a), nor on the co-transfected control chloramphenicol acetyltransferase (CAT) vector. In contrast, XGrg-5, which lacks the carboxy-terminal WD40 repeats of the longer Grg proteins[14], enhanced the transcriptional activity of suboptimal amounts of Arm–XTcf-3 complexes (Fig. 2b). mGrg-5 had no intrinsic transactivation properties when fused to a Gal4 DNA-binding domain (not shown). The enhancement of transcription by XGrg-5 could probably be attributed to its interference with the repressive effects of endogenous Gro proteins. We note that each line in a large and diverse cell panel expressed multiple *Grg/TLE* genes (H.B., J.R. and H.C., unpublished observations); the B-cell line used in our reporter assay expressed moderate levels of both *Grg-1* and *Grg-4* (results not shown). A deletion mutant of XTcf-3 that lacked the Grg-interaction domain was a tenfold more potent transcriptional activator than its wild-type counterpart (Fig. 2c), confirming the activity of endogenous co-repressors of Tcf factors in our assay. As expected, this ΔGrg-XTcf-3 mutant was not subject to repression by XGrg-4 or anti-repression by XGrg-5 (Fig. 2d).

We then determined whether the Gro–Tcf interaction was involved in the *in vivo* regulation of β-catenin–Tcf target genes in *Xenopus* embryos. Tcf-binding sites in the *Siamois* promoter have



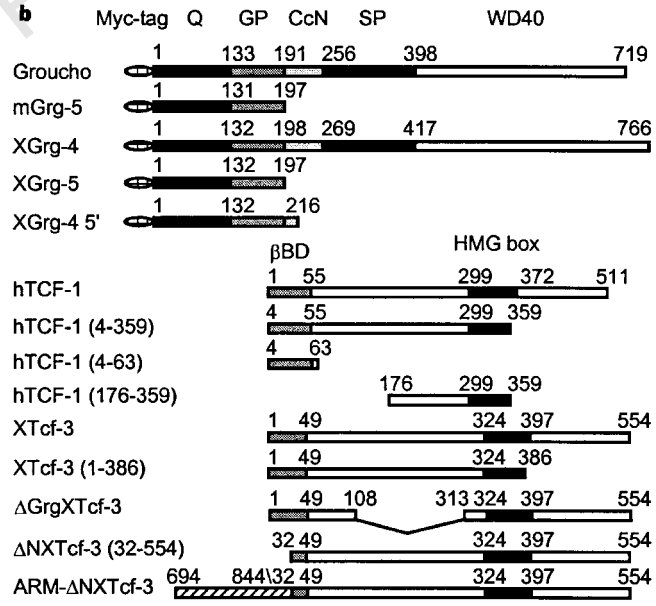


Figure 1 Physical interaction between Groucho (Gro)-related proteins and Tcf. **a**, *In vitro* binding assay for hTCF-1 and Grg-5. *In vitro*-transcribed and translated hTCF-1 binds to an MBP–Grg-5 fusion protein (lane 3), but not to control MBP protein (lane 2). *In vitro*-translated Armadillo does not bind to these MBP proteins (lane 5, 6). Input hTCF-1 protein is run in lane 1, and input Armadillo protein in lane 4. **b**, Domains of Gro and Tcf constructs. Like Gro, XGrg-4 contains five distinct domains: mGrg-5 and XGrg-5 contain only the Q and GP domains[14]. hTCF-1 and XTcf-3 contain a centrally located DNA-binding HMG box and the N-terminal β-catenin-binding domain (βBD). ΔGrg-XTcf-3 was constructed by deleting amino acids 109–312 of XTcf-3. A dominant-positive version of XTcf-3 (bottom) was created in which its N terminus is replaced by the C-terminal transactivation domain of Arm (amino acids 694–844). **c**, Two-hybrid assay for the interaction of Tcfs, Grgs and β-catenin. All tested Tcf-family members bind to β-catenin. In contrast, only hTCF-1, dTcf, and XTcf-3 interact with Grg-5. The Grg5-interaction domain of Tcf proteins (amino acids 176–359) is separable from the domain that interacts with β-catenin (amino acids 4–63 (ref. 22)). **d**, Tcf transports Gro to the nucleus. Tagged, truncated Gro proteins localize to the cytoplasm of COS cells. Introduction of hTCF-1 results in nuclear translocation of mGrg-5, XGrg-5 and XGrg-4 5′. In the same way, XTcf-3 interacts with XGrg-5 and XGrg-4 5′. ΔGrg-XTcf-3, however, did not cause nuclear translocation of XGrg-5 and XGrg-4 5′. Introduction of hLEF-1 does not result in nuclear translocation of mGrg-5.

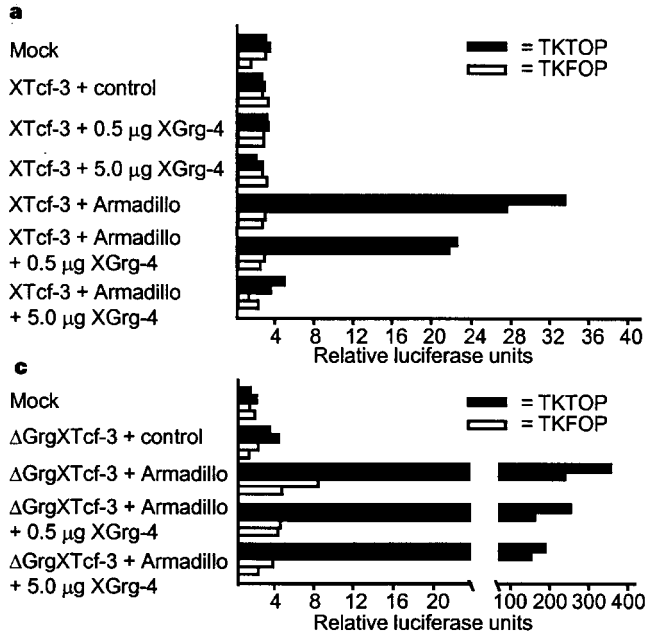
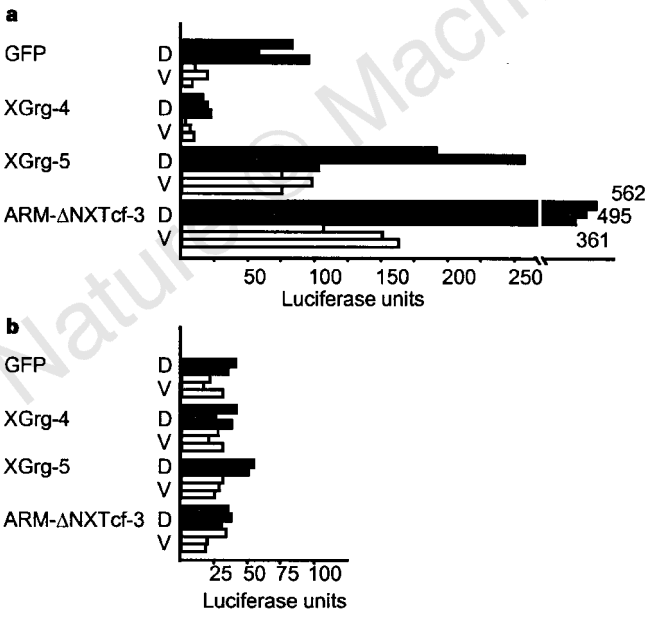
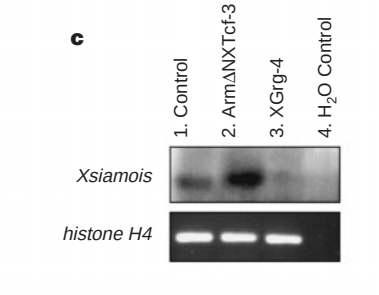


Figure 2 Gro represses Arm–Tcf-driven transactivation of a Tcf reporter. **a**, IIAI.6 B cells were transfected with optimal amounts of the indicated plasmids. XGrg-4 represses transactivation by Arm–XTcf-3. Tenfold transactivation is induced by 5 μg *Arm* plasmid. **b**, XGrg-5 enhances transcription in the presence of a suboptimal amount of Arm (0.5 μg). **c**, 5 μg *Arm* plasmid induces a 100-fold transactivation, with ΔGrg-XTcf-3 as an effector protein. The transactivation cannot be repressed by introduction of XGrg-4. **d**, XGrg-5 does not enhance transcription induced by ΔGrg-XTcf-3 and Arm (0.5 μg). In all figures, both values of duplicate transfections are given (corrected for transfection efficiency based on an internal CAT control). TKTOP, Tcf OPtimal reporter; TKFOP, Tcf Far-from-OPtimal reporter (see Methods).





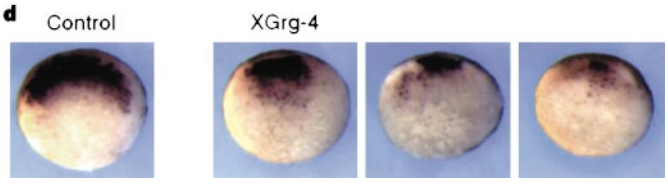


Figure 3 XGrg-4 represses *Siamois* promoter activity *in vivo*. **a**, **b**, For *Siamois* reporter assays two different constructs, S01234 and S24 (ref. 11), were injected in the equatorial region of the two dorsal (D) or the two ventral (V) blastomeres at the four-cell stage. S01234 is a luciferase reporter construct consisting of 0.8 kilobases of the wild-type *Siamois* promoter, containing three Tcf consensus sites that are β-catenin-responsive; these three sites are mutated in S24 (ref. 11). **a**, 265 pg S01234 were injected together with equimolar amounts of capped synthetic mRNA, encoding green fluorescent protein (GFP) (750 pg per embryo), ArmΔNXTcf-3 (500 pg), XGrg-4 (3,000 pg) or XGrg-5 (3,000 pg). Luciferase activities from two to three pools of five stage-10 embryos were determined for each combination. XGrg-4 represses dorsal *Siamois* promoter activation, whereas XGrg-5 enhances the activity. **b**, No effects are seen when the different RNAs are injected together with S24. **c**, RT-PCR analysis of *Siamois* expression. Embryos were injected at the four-cell stage in the equatorial region of each blastomere with either 500 pg *ArmΔNXTcf-3* (ventral) or 3,000 pg *XGrg-4* (dorsal). Twenty embryos were pooled for each data point. Dorsal injection of *XGrg-4* RNA results in a strong reduction of *Siamois* expression (lane 3), whereas *Siamois* expression is enhanced on injection of *ArmΔNXTcf-3* (lane 2). The amount of cDNA per sample was standardized for histone H4 expression (bottom). Nearly identical results were obtained in each of three independent experiments. Control embryos raised until stage 42 showed typical phenotypes (Fig. 4; ref. 2). **d**, *In situ* hybridization with *Xnr-3* for stage-9 embryos injected dorso-equatorially with 3,000 pg *XGrg-4* RNA at the four-cell stage. Left to right: non-injected embryo, in which expression of *Xnr-3* is seen at the future dorsal side, and three embryos injected with increasing amounts of *XGrg-4*, showing increasingly reduced levels of *Xnr-3* RNA.

Position	n	DAI	Percent
Animal	216	5	32
		4	60
		3	1
Equatorial	272	5	18
		4	37
		3	29
		2	1
		1	1
Vegetal	201	5	38
		4	50
		3	1

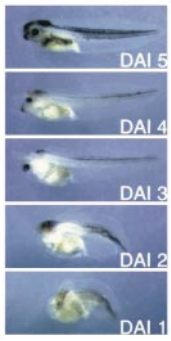


Figure 4 Dorsal injection of *XGrg-4* suppresses endogenous axis formation. Embryos were injected, at the four-cell stage, in each dorsal blastomere with 1,500 pg *XGrg-4* at the position indicated. The embryos were scored at stages 33–40 according to the standard dorso-anterior index scale (DAI[30]). A normal embryo is assigned DAI 5, whereas embryos lacking dorso-anterior structures are assigned DAI 0. A typical range of phenotypes found in one experiment is shown at the right. No effects were observed upon injection of *XGrg-5* or of control *GFP* or β-galactosidase mRNA.

been proposed to mediate ventral repression of this β-catenin-regulated gene in *Xenopus* embryos[10,11]. When we injected this promoter into *Xenopus* embryos, we obtained results that were nearly identical to those obtained when expressing the synthetic reporters in mammalian cells: XGrg-4 repressed promoter activity, whereas XGrg-5 enhanced the activity (Fig. 3a). These effects depended on the presence of the three Tcf-binding sites in the promoter (Fig. 3b). We then studied the consequence of injection of *XGrg-4* on the expression of the endogenous *Siamois* gene. Because it is expressed at low levels, we used the semiquantitative polymerase chain reaction (PCR) to amplify *Siamois* RNA purified from pooled, XGrg4-injected embryos[23]. Injection of *XGrg-4* at the four-cell stage led to a strong reduction of transcription of the endogenous *Siamois* gene at stage 10, whereas injection of dominant-positive XTcf-3 (see below) enhanced transcription of *Siamois* (Fig. 3c).

To test the effect of XGrg-4 on another direct target of XTcf-3, *Xnr-3* (ref. 24), we performed whole-mount *in situ* hybridization[25] on stage 9 embryos. As expected, dorsal injections of *XGrg-4* RNA at the four-cell stage markedly reduced the *Xnr-3* signal (Fig. 3d).

We have shown previously that XTcf-3 in *Xenopus* embryos mediates the axis-inducing β-catenin signal[2]. As XGrg-4 and XGrg-5 both interact with XTcf-3 and are expressed maternally, we analysed the effect of dorsal injections of these two proteins on axis formation. Injection of *XGrg-4* inhibited formation of the endogenous axis, resulting in ventralization of the embryos, whereas *XGrg-5* had no effect. Effects were strongest after injection in the equatorial region of the two dorsal blastomeres of four-cell-stage embryos. Phenotypes ranged from complete lack of a head in combination with a shortened tail, to microcephaly and cyclopia (Fig. 4).

We also tested the effects of Gro on axis duplication. To avoid perturbations of pools of endogenous Wnt cascade components, we designed a dominant-positive version of XTcf-3 in which the N terminus is replaced by the C-terminal transactivation domain of Arm[5] (Fig. 1b). Although ventrally injected *XTcf-3* is essentially inert and Δ*N-XTcf-3* is a potent dominant-negative mutant[2], the chimaeric protein potently induced secondary axes (Table 1). This showed that recruitment of the transactivating C terminus of Arm to Tcf sites constitutes the primary nuclear event upon signalling. Injection of the chimaeric protein with *XGrg-4* inhibited this activity, but injection of the chimaera with *XGrg-5* potentiated the activity (Table 1). This result concurred with the repressive effects of XGrg-4 and the enhancing effects of XGrg-5 on transcription in mammalian cells. Ventral injections of *XGrg-5* RNA alone had

Table 1 XGrg-4 suppresses, XGrg-5 potentiates axis duplication by Arm

RNA injected (pg)	Incomplete secondary axis (%)	Secondary axis incl. eye and cement gland (%)	Total number of embryos injected
500 Arm-ΔNXTcf-3 + 1,500 XGrg-4	17	0	334
500 Arm-ΔNXTcf-3 + 375 GFP	40	18	357
25 Arm-ΔNXTcf-3 + 1,000 XGrg-5	25	4	408
25 Arm-ΔNXTcf-3 + 1,000 GFP	9	2	384
100 XNoggin + 1,500 XGrg-4	46	6	282
100 XNoggin + 375 GFP	46	8	269

Embryos were injected at the four-cell stage in the equatorial region of one ventral blastomere and screened for secondary axis induction at stages 25–30. As a negative control, equimolar amounts of green fluorescent protein (GFP) mRNA were injected.

no effect on axis duplication (results not shown), indicating that derepression alone is not sufficient for the biological effect. Axis duplication induced by injection of *noggin* messenger RNA[26] could not be blocked by *XGrg-4* (Table 1).

We propose that the transcription of Tcf target genes is the result of a balance between the constitutive, repressive effects mediated by Gro (possibly counteracted by Grg5-like proteins) and the activating effects of Arm. In non-signalling cells, the repressive activities will dominate. Following Wnt activation, β-catenin will associate with Tcfs and will activate transcription of genes such as *Xnr-3* and *Siamois* in *Xenopus*, or *Engrailed* and *Ubx* in flies. The active repression by Gro secures tight control over the Wingless/Wnt-driven developmental decisions. The dual activities of Tcf factors may explain the puzzling observation that mutation of the *C. elegans Tcf* homologue *Pop-1* has opposite effects on E versus MS-cell specification to those resulting from mutation of Wnt and Arm[12]. Pop-1 probably functions mainly as a repressor of target gene transcription. Our results predict a role for Gro in Pop1-controlled cell-fate decisions.

Constitutive activation of Tcf-mediated transcription occurs in melanoma and colon carcinoma after loss of APC or gain-of-function mutations in β-catenin[27–29]. As Gro proteins repress Tcf activity, it will be interesting to study the status of *TLE* genes in human cancers in which Tcf transcription is inappropriately activated. □

Methods

Two-hybrid experiments were performed as described[2]. Plasmid pVA3 encodes a murine p53–Gal4 binding domain hybrid in pGBT9 (Clontech). Preys mGrg-5, XGrg-5, and β-catenin were inserted in frame with the Gal4 activation domain in pGADGH (Clontech) or pGADRX (Stratagene). pTD1 encodes SV40 large T antigen in pGAD3F (Clontech). Baits and preys were transformed into the *Saccharomyces cerevisiae* strain HF7C (Clontech).

***In vitro* binding assays.** Radiolabelled hTCF-1 and Armadillo were produced in the PROTEINscript kit (Ambion). Translated products were diluted in 0.5 ml ELB buffer (150 mM NaCl, 50 mM HEPES, pH 7.5, 5 mM EDTA, 0.1% NP40, 10 mM β-glycerophosphate, 5 mM NaF, 1 mM PMSF, 10 mg ml^{-1} trypsin inhibitor, 20 U ml^{-1} aprotinin and 1 mM sodium orthovanadate). Amylose-sepharose beads loaded with MBP–Grg5 fusion protein or control MBP were incubated at 4 °C for 2 h. Washed beads were analysed by gel electrophoresis and autoradiography.

Cloning of *XGrg-5* and *XGrg-4*. A *Xenopus* brain complementary DNA library in λgt11 (ref. 2) was screened at low stringency with murine *Grg-5* cDNA probes according to standard procedures. *XGrg-5* was previously described as *XAES*[20] (GenBank accession number U18776). The accession number of *XGrg-4* is AJ224945.

COS cell transfections. COS cells were transfected with *Tcf* and *XGrg* constructs in a ratio of 10:1 using standard diethyl aminoethyl (DEAE)-dextran transfection. After 24 h, cells were methanol-fixed and stained using an anti-Myc-tag antibody.

Luciferase assays and CAT assays. 2×10^6 IIAI.6 B cells were electroporated with a luciferase reporter plasmid containing three optimal Tcf sites upstream of the minimal HSV-TK promoter (1 μg pTKTOP) or its negative control containing mutated Tcf sites (pTKFOP), the internal transfection control (0.5 μg pSV40CAT), 20 μg *Tcf* expression vectors, and 0.5 or 5.0 μg Gro expression plasmids. For *XGrg-5* experiments, 0.5 μg *Arm* was used; for *XGrg-4* experiments, 5.0 μg pCDNA, was used as a stuffer when appropriate. cDNAs encoding tagged versions of *XTcf-3*, Δ*Grg-XTcf-3*, *XGrg-4* and *XGrg-5* were inserted into pCDNA3. Luciferase and CAT activities were separately determined 24 h after transfection as described[5].

Reverse transcription (RT)-PCR analysis. Total RNA for detection of endogenous *Siamois* mRNA by RT-PCR was isolated from stage 10 embryos[2]. Oligo-d(T)-primed cDNA from total RNA was prepared using standard techniques. cDNA was quantified by PCR analysis for histone H4 and products were compared after 12, 14, 16, 18 and 20 cycles. The product after 20 cycles is shown in Fig. 3c. RT-PCR was carried out as described[23].

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Androstane metabolites bind to and deactivate the nuclear receptor CAR-β

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The orphan receptor CAR-β (ref. 1) binds DNA as a heterodimer with the retinoid-X receptor and activates gene transcription in a constitutive manner. Here we show that, in contrast to the classical nuclear receptors, the constitutive activity of CAR-β results from a ligand-independent recruitment of transcriptional co-activators. While searching for potential ligands of CAR-β, we found that the steroids androstanol and androstenol inhibit the constitutive activity of CAR-β. This effect is stereospecific: only 3α-hydroxy, 5α-reduced androstanes are active. These androstanes do not interfere with heterodimerization or DNA binding of CAR-β; instead, they promote co-activator release from the ligand-binding domain. These androstane ligands are examples of naturally occurring inverse agonists[2,3] that reverse transcriptional activation by nuclear receptors. CAR-β (constitutive androstane receptor-β), therefore, defines an unanticipated steroidal signalling pathway that functions in a manner opposite to that of the conventional nuclear receptor pathways.

Unlike classical nuclear hormone receptors which are activated by their cognate ligands, mouse (m) CAR-β (ref. 1) is transcriptionally active in the absence of exogenous hormone (Fig. 1a). As the ligand-binding domain (LBD) of CAR-β contains sequence determinants characteristic of classical nuclear receptors, we determined whether CAR-β could respond to exogenous signalling molecules. Surprisingly, the constitutive activity of CAR-β was completely inhibited by the mammalian pheromone 5α-androst-16-en-3α-ol (ref. 4) (androstenol, 5 μM, Fig. 1a, b) and by 5α-androstan-3α-ol (androstanol, 5 μM, Fig. 1a, b). Both compounds exhibit half-maximal inhibition at concentrations of about 400 nM (Fig. 1c). Inhibition was specific for CAR-β, as no inhibition was observed with other receptors (data not shown).

Further studies showed that inhibition is stereospecific for 5α-reduced compounds with a 3α-hydroxy group. A compound lacking the 3-hydroxy moiety (5α-androstan) was entirely inactive, whereas compounds with a 3β-hydroxy group (5α-androstan-3β-ol, 5α-androst-16-en-3β-ol) or a 3-keto group (androstenone, 5α-androst-16-ene-3-one) had EC_{50} values (effector concentrations for a half-maximal response) of greater than 10 μM (Fig. 1c; data not shown). Similarly, potency is reduced by more than tenfold when a 5β-reduced compound (5β-androstan-3α-ol) is used. This pattern of response indicates that *in vivo* production of a CAR-β inhibitor may require the activity of steroid 5α-reductase.

The fact that both androstanol and androstenol reverse transcriptional activation by CAR-β indicated that other modifications might be tolerated at the 16 and 17 positions. However, many