Mice develop normally in the absence of Smad4 nucleocytoplasmic shuttling

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Smad4 in partnership with R-Smads (receptor-regulated Smads) activates TGF- β (transforming growth factor- β)-dependent signalling pathways essential for early mouse development. *Smad4* null embryos die shortly after implantation due to severe defects in cell proliferation and visceral endoderm differentiation. In the basal state, Smad4 undergoes continuous shuttling between the cytoplasm and the nucleus due to the combined activities of an N-terminal NLS (nuclear localization signal) and an NES (nuclear export signal) located in its linker region. Cell culture experiments suggest that Smad4 nucleocytoplasmic shuttling plays an important role in TGF- β signalling. In the present study we have investigated the role of Smad4 shuttling *in vivo* using gene targeting to engineer two independent mutations designed to eliminate Smad4 nuclear export. As predicted this results in in-

creased levels of Smad4 in the nucleus of homozygous ES cells (embryonic stem cells) and primary keratinocytes, in the presence or absence of ligand. Neither mutation affects *Smad4* expression levels nor its ability to mediate transcriptional activation in homozygous cell lines. Remarkably mouse mutants lacking the Smad4 NES develop normally. *Smad4* NES mutants carrying one copy of a *Smad4* null allele also fail to display developmental defects. The present study clearly demonstrates that Smad4 nucleocytoplasmic shuttling is not required for embryonic development or tissue homeostasis in normal, healthy adult mice.

Key words: embryonic, gene targeting, nuclear export, nucleocytoplasmic shuttling, Smad4, transforming growth factor- β signal.

INTRODUCTION

Members of the TGF- β (transforming growth factor- β) superfamily of growth and differentiation factors control many diverse processes, including cell proliferation, migration, adhesion, cell survival and differentiation during early embryogenesis and in adult tissues [1]. The ligands, comprising three subfamilies, namely the TGF- β s, the activin/nodal subfamily and the BMPs (bone morphogenetic proteins) and GDFs (growth and differentiation factors), signal by activating a complex of two serine/threonine kinase receptors at the plasma membrane. These receptor complexes subsequently phosphorylate and activate the downstream effectors of the TGF- β signalling pathways, the so-called R-Smads (receptor-regulated Smads) [2]. For the TGF- β , activin and nodal ligands, the R-Smads activated are Smad2 and 3; for the BMP and GDF ligands, the R-Smads are Smad1, 5 and 8. The common mediator Smad, or co-Smad, Smad4 shares overall structural features with the R-Smads namely the N-terminal conserved DNA-binding MH1 domain (Mad homology domain 1), a linker region, and the C-terminal MH2 domain (Mad homology domain 2). However, the Smad4 MH2 domain lacks the C-terminal SXS motif present in the R-Smads that is the target for receptor-mediated phosphorylation. Smad4 functions as a partner for the R-Smads in mediating TGF- β signals. Thus activated R-Smads form heteromeric complexes with Smad4 [3] that accumulate in the nucleus, where they regulate the trans-

cription of target genes both positively and negatively in conjunction with other transcription factors [2].

Smad behaviour has been extensively studied in tissue culture cell lines. In the basal steady-state R-Smads are predominantly concentrated in the cytoplasm, whereas Smad4 is distributed throughout the cell [4]. Upon ligand stimulation both R-Smads and Smad4 accumulate in the nucleus. Previous work indicates that in both unstimulated cells [5] and in the presence of a TGF- β signal [6], Smad2, 3 and 4 constantly shuttle between the cytoplasm and nucleus. Kinetic studies demonstrate that nuclear accumulation of activated Smad complexes is probably caused by a decrease in the rate of nuclear export. Thus R-Smad–Smad4 complexes are exported from the nucleus more slowly than monomeric Smads [7]. Shifts in nucleocytoplasmic distribution of Smads during TGF- β superfamily signalling reflect distinct steady states of a highly dynamic equilibrium. The continuous nucleocytoplasmic shuttling in the presence of a signal is thought to provide a sensing mechanism that allows R-Smads to constantly monitor receptor activity [6]. The activity of a nuclear phosphatase, recently identified as PPM1A [8] results in continuous dephosphorylation of R-Smads and dissociation of the Smad complexes. Monomeric R-Smads and Smad4 are exported from the nucleus independently [6,9]. In the absence of continued production of phosphorylated R-Smad by activated receptors, R-Smads accumulate in the dephosphorylated basal state in the cytoplasm. The shuttling behaviour of the R-Smads

Abbreviations used: BMP, bone morphogenetic proteins; CRM1, chromosomal region maintenance 1; d.p.c., days post-coitum; ES cell, embryonic stem cell; FBS, foetal bovine serum; GDF, growth and differentiation factor; LMB, leptomycin B; MEF, murine embryonic fibroblast; MH domain, Mad homology domain; NES, nuclear export signal; NLS, nuclear localization signal; RPA, ribonuclease protection assay; R-Smad, receptor-regulated Smad; SnoN, Ski-related novel protein N; TGF- β , transforming growth factor- β .

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in ligand-stimulated cells thus requires cycles of phosphorylation and dephosphorylation.

Smad4 contains an evolutionarily conserved NLS (nuclear localization signal) at its N-terminal MH1 domain [10] that is thought to function by binding to importin α . A well conserved NES (nuclear export signal) of the canonical CRM1 (chromosomal region maintenance 1)-dependent type lies just at the beginning of the linker domain [9,11]. Treatment with the CRM1 inhibitor, LMB (leptomycin B) causes a rapid accumulation of Smad4 in the nucleus, strengthening the argument that Smad4 continuously shuttles, even in the absence of a TGF- β signal [9,11]. In unstimulated cells, nuclear Smad4 has been proposed to act as a repressor of transcription together with the co-repressor, SnoN (Ski-related novel protein N) [9]. The fact that the Smad4 NLS and NES sequence motifs are highly conserved across species suggests that Smad4 nucleocytoplasmic shuttling plays an important role in TGF- β signalling. Interestingly, during early *Xenopus* development, the predominant *Smad4* species expressed is *XSmad4 β* , which does not contain an NES, and is exclusively nuclear [12,13]. However, at gastrulation, once the length of the cell cycle begins to increase, *XSmad4 β* is exchanged for *XSmad4 α* , which like mammalian Smad4 has both an NLS and NES [12,13]. Similarly, alternatively spliced mammalian *Smad4* transcripts lack the NES and encode a protein that is constitutively nuclear [9]. Comparative protein sequence alignments reveal that the NLS is present in all vertebrates [including human (BAB40977), mouse (AAM74472), rat (BAA83092), zebrafish (XM.682418) and *Xenopus* (4α ; BAA77514) as well as invertebrates including *Drosophila* (*Medea*; AAC62005), *Caenorhabditis elegans* *Daf3* (NM.075760) and *Sma4* (NP.498256)]. Similarly the sequences encoding an NES are also conserved in all of these species with the exception of *C. elegans*.

Gene-targeting experiments demonstrate that secreted TGF- β growth factors are essential for early mammalian development. Functional loss of the ligand nodal, cell surface receptors, or the downstream effector Smad2, disrupt specification of the A–P (anterior–posterior) axis, mesoderm patterning, definitive endoderm formation, and results in early embryonic arrest [14–16]. Similarly BMP ligands are crucial for mesoderm formation and patterning during gastrulation (reviewed in [17,18]). Dose-dependent Smad1 and 5 signals also regulate key events in early mouse development [19,20]. Consistent with its central role in mediating TGF- β signals in the early embryo, *Smad4* null mouse embryos die shortly after implantation due to a requirement for *Smad4* in the extra-embryonic lineages [21,22]. In the present study, to test whether Smad4 nucleocytoplasmic shuttling may influence its function *in vivo*, we engineered *Smad4*^{ΔNES/ΔNES} mutant mice that exclusively express the splice variant lacking the entire NES-encoding exon 4. [Due to recent inclusion of the 5'-UTR (untranslated region) as exon 1 for this gene (ENSMUSG00000024515), previously published work [9,11] refers to this third coding exon as exon 3.] Additionally we introduced a subtle mutation to create *Smad4*^{MEX/MEX} mice exclusively expressing *Smad4* in which two critical leucine residues in the NES were changed to glycine and alanine residues respectively. Substitutions with alanine residues at these positions have been shown to abolish NES activity *in vitro* [9]. As predicted, these mutant Smad4 proteins fail to shuttle and are predominantly localized to the nucleus in both the absence and presence of TGF- β signals. Surprisingly, embryos homozygous for these mutant *Smad4* alleles develop normally, are born in the expected Mendelian ratios and are post-natally viable. These experiments conclusively demonstrate that Smad4 nucleocytoplasmic shuttling is not required for dose-dependent TGF- β signals governing early devel-

opment, organogenesis or target gene expression in healthy adult mice, but leave open the question as to whether the NES plays an essential role under pathological and/or stress conditions.

EXPERIMENTAL

Generation of targeted ES cell (embryonic stem cell) lines

Two targeting vectors were constructed to target exon 4 of the murine *Smad4* gene using the Cre recombinase:loxP system. A *Smad4* exon 4 deletion allele (*Smad4*RobΔNES; referred to throughout as *Smad4*^{ΔNES}) and a mutant exon 4 allele (*Smad4*RobMEX4 referred to throughout as *Smad4*^{MEX}) were generated as shown in Figure 1. The parental pBluescriptII plasmid contained a 11.3 kb fragment of genomic *Smad4* sequence encompassing exons 2–4 and flanking intronic regions. Positive and negative selection cassettes [loxP-pgk-hygromycin-loxP (pgk-hygro) and pgk-diphtheria toxin antigen (pgk-dta)] were derived from the Universal Vector [23] using standard PCR and subcloning procedures. As shown in Figure 1(A), the *Smad4*^{ΔNES-TA} (where TA represents targeting allele) targeting vector contains a 5' pgk-dta cassette; a 5.6 kb (Sall-SmaI) 5' homology arm; the loxP-flanked pgk-hygro cassette inserted into intron 3 and a 5.7 kb (SmaI-XhoI) 3' homology arm containing a single loxP site introduced into the NsiI site in intron 4.

The *Smad4*^{MEX} targeting vector (Figure 1B) contains a 5' pgk-dta cassette; a 6.6 kb (Sall-XbaI) 5' homology arm and the loxP-flanked pgk-hygro cassette inserted into intron 4. A fragment containing a PCR mutagenized exon 4 was used to replace the endogenous XmaI-NsiI fragment within the 4.7 kb (XbaI-XhoI) 3' homology arm. The modified sequence (atctctcGgaGGCacaGCgcagagtaatg) substitutes a glycine and alanine residue respectively for the two endogenous leucine residues of the NES and introduces a diagnostic restriction enzyme recognition site change, substituting a BspEI site for the endogenous PstI site.

CCE ES cells [22a] (2×10^7) were electroporated with 15 μ g of NotI-linearized targeting vector. Drug-resistant colonies were screened by Southern blot analysis. Homologous recombinants were identified by BstZ17I digestion of DNA and screening with an intron 4 probe (XhoI-BstZ17I), external to the targeting vector. For the *Smad4*^{MEX} allele, targeted cell clones were also screened by PstI digestion using a second intron 4 probe (PstI-SacI) to identify clones that had also incorporated the mutated exon 4. Transient expression of Cre recombinase was used to remove the loxP-flanked regions. BstZ17I-digested DNA from the resulting sub-clones was screened with an intron 2 (PstI-AvrII) probe for the *Smad4*^{ΔNES} allele. For the *Smad4*^{MEX-TA/+} sub-clones, DNA was digested with PstI and screened with the intron 4 (PstI-SacI) probe.

Homozygous ES cells (*Smad4*^{ΔNES/ΔNES}) were generated by re-targeting the *Smad4*^{ΔNES/+} ES cells with the original targeting construct (*Smad4*^{ΔNES-TA/ΔNES}). Clones in which the remaining wild-type allele had been replaced by the targeting vector (including the intron 4 loxP site) were identified by screening with the intron 4 external probe. Subsequent *in vitro* Cre-mediated excision of representative clones yielded homozygous *Smad4*^{ΔNES/ΔNES} ES cells. Representative *Smad4*^{ΔNES/ΔNES} cell lines are herein referred to as NES[#]1, 5, 7 and 8.

Generation and genotyping of mutant mouse strains

Chimaeric animals were generated by injection of ES cells into C57BL/6J blastocysts. Chimaeric males were initially mated with C57BL/6J females, and the resulting agouti offspring were screened to identify those carrying the modified alleles. Animals

Figure 1 Generation of *Smad4*^{ΔNES} and *Smad4*^{MEX} alleles

Gene targeting strategies to (A) flank exon 4 containing the NES with loxP sites (large arrows) or (B) insert a mutated exon 4 into the endogenous *Smad4* locus are shown. For both constructs, hygromycin-resistant targeted clones were screened with a 3' external probe (probe a; grey box) to identify homologous recombinants. (A) ii, Following electroporation, targeted clones which had incorporated the targeting construct including the intron 4 loxP site (*Smad4*^{ΔNES-TA/+}) were distinguished from random integration event clones (*Smad4*^{+/+}) by screening DNA digested with BstZ171 with probe a. The positions of the 9.4 kb WT (wild-type) and 5.2 kb TA (targeted allele) bands are indicated. iii, For excision of the selection cassette, *Smad4*^{ΔNES-TA/+} clones were subjected to transient transfection with Cre recombinase. Screening with probe b [shown as a black box on (A, i)], confirmed excision of the hygro cassette and exon 4 (*Smad4*^{ΔNES/+}; 8.6 kb), the hygro cassette only (i.e. conditional allele *Smad4*^{ΔNES-CA/+}; 4.2 kb) or retention of the *Smad4*^{ΔNES-TA/+} allele (6.4 kb). iv, *Smad4*^{ΔNES/+} sub-clones were retargeted and screened with probe a, to detect either the *Smad4*^{ΔNES-TA} (5.2 kb), *Smad4*^{ΔNES} (8.6 kb) or *Smad4*^{+/+} (9.4 kb) alleles. Clones in which the remaining *Smad4* wild-type allele had been disrupted (i.e. *Smad4*^{ΔNES-TA/ΔNES}; 5.2 and 8.6 kb) were then subjected to *in vitro* Cre-mediated excision to generate homozygous mutant cell lines lacking both the pgk-hygro cassette and exon 4 (results not shown). (B) ii, Following electroporation, correctly targeted *Smad4*^{MEX-TA/+} clones were identified by screening BstZ171-digested DNA with probe a. The position of the 11.6 kb TA band is indicated. Further analysis via PstI digestion and screening with probe c was used to identify clones that had incorporated the mutant exon 4 and hence lost the endogenous PstI site (*Smad4*^{MEX-TA/+}; 1.3 kb or *Smad4*^{+/+}; 0.75 kb). iii, Screening of PstI digested DNA from *Smad4*^{MEX-TA/+} ES clones following transient transfection with pMC1-Cre. Probe c [shown as a black box on (B, i)] distinguishes *Smad4*^{MEX/+} (1.4 kb), *Smad4*^{MEX-TA/+} (1.3 kb) or *Smad4*^{+/+} (0.75 kb) clones. Bz, BstZ171; Bs, BspE1 and Ps, PstI. (C) PCR genotyping screen. i, Amplification of genomic DNA between primers Ex2F and Int3R (small arrows) detects three possible alleles, *Smad4*^{+/+}, *Smad4*^{ΔNES} and *Smad4*^{ΔNES-CA} (1.0, 0.4 and 1.1 kb respectively). ii, To detect the *Smad4*^{MEX} allele, PCR products generated using the same primers were digested with PstI to generate diagnostic banding patterns for wild-type (560, 270 and 175 bp), *Smad4*^{MEX/+} (830, 560, 270 and 175 bp) and *Smad4*^{MEX/MEX} (830 and 175 bp).

were housed in individually ventilated cages with a 12 h on/off light cycle and maintained on a Beekay Rat and Mouse Diet No. 2 (BK002P). All animal experimental procedures were performed in accordance with The Animals (Scientific Procedures) Act 1986.

For the *Smad4*^{ΔNES} allele, two mouse lines were created. The first line, *Smad4*^{ΔNES-TA/+}, was generated from an ES cell clone that retained the targeted allele (3E12-B6). These chimaeras were then bred for *in vivo* Cre-mediated excision of the selection cassette and exon 4 in the epiblast using the Sox2 Cre deleter strain to derive *Smad4*^{ΔNES/+} progeny [24,25]. Due to the complex genetic background, this line is referred to herein as *Smad4*^{ΔNES/+} (outbred). Additionally, chimaeric males derived from the ES cell clone 3E12-B9 in which both the pgk-hygro cassette and exon 4 were excised *in vitro* were crossed with C57BL/6J females to generate *Smad4*^{ΔNES/+} mice on a hybrid (129/Sv:C57BL/6J) background (*Smad4*^{ΔNES/+}; 129:C57). For the *Smad4*^{MEX} allele, two clones in which the selection cassette had been excised *in vitro* (1B4-C1 and 1B4-A10) were used to generate chimaeras on this background (*Smad4*^{MEX/+}; 129:C57). The *Smad4*^{ΔNES/+} (129:C57) animals were crossed with *Smad4* mutants carrying the null allele (*Smad4*^{RobN}) [22] to obtain offspring with a single functional *Smad4* gene lacking exon 4 coding sequences (i.e. *Smad4*^{ΔNES/N}).

Genotyping of ear biopsy-derived genomic DNA was performed in a 25 µl PCR reaction containing 2.5 mM MgCl₂, 4 mM dNTP mix, 2.5 units of AmpliTaq polymerase and 10 pmol each of the primers, *S4Ex2F* 5'-ATGTGATCTATGCCCGTCTGTGG-3' and *S4Int3R* 5'-ACAGGCAGCGACTCACATGATAC-3'. Cycling conditions were as follows: an initial denaturation step of 5 min at 94°C; 2 cycles of 30 s at 94°C, 1 min at 72°C and 90 s at 72°C; 2 cycles of 30 s at 94°C, 1 min at 70°C and 90 s at 72°C; 30 cycles of 30 s at 94°C, 1 min at 68°C and 90 s at 72°C; followed by a final extension cycle of 5 min at 72°C. For the *Smad4*^{ΔNES} allele genotyping, the PCR products were separated and visualized on a 1.5% agarose gel yielding 0.4, 1.1 and 1.0 kb products from the *Smad4*^{ΔNES/+}, *Smad4*^{ΔNES-CA/+} and wild-type alleles respectively. For the *Smad4*^{MEX} allele, the 25 µl of PCR product was digested with 1 unit of PstI enzyme in a total reaction volume of 50 µl, generating a banding pattern for wild-type (560, 270 and 175 bp), *Smad4*^{MEX/+} (830, 560, 270 and 175 bp) and *Smad4*^{MEX/MEX} (830 and 175 bp) alleles.

Generation of primary cell lines

To generate primary MEFs (murine embryonic fibroblasts) *Smad4*^{ΔNES/+} (outbred) offspring were intercrossed and embryos recovered at 12.5 d.p.c. (days post-coitum) and genotyped by PCR. Individual embryos were disaggregated with 0.25% trypsin/EDTA, plated in DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS (foetal bovine serum) and supplemented with 2 mM glutamine and 0.1 mg/ml gentamycin, and cultured to extreme confluency. At the next passage, primary fibroblast cultures were generated from surviving cells.

Primary keratinocytes were derived from dorsal surface skin biopsies from the lower back of adult (129:C57) *Smad4*^{ΔNES/ΔNES} mice, *Smad4*^{MEX/MEX} mice and *Smad4*^{+/+} control littermates as described [26]. Keratinocytes were seeded and cultured on a confluent feeder layer of mitotically inactivated J2-3T3 cells [27] as described previously [26]. For immunostaining, cells were plated on to collagen-coated coverslips and cultured overnight in keratinocyte culture medium. The cells were then washed in PBS and cultured for 24 h under growth-factor starvation conditions in medium lacking the HICE cocktail (0.5 µg/ml hydrocortisone, 5 µg/ml insulin, 10⁻¹⁰ M cholera enterotoxin and 10 ng/ml epidermal growth factor), containing only 0.5% FBS, and supplemented with 0.5 mM CaCl₂ to reduce levels of

autocrine TGF-β signalling. Cells were then treated with LMB or TGF-β as indicated in the Figure legends.

RPA (ribonuclease protection assays)

Total RNA was prepared using TRIzol[®] (Invitrogen) according to the manufacturer's instructions. RPAs were performed using 10 µg of total RNA (RPA III kit; Ambion). *Smad4*-specific antisense probes were designed to distinguish alternatively spliced mRNAs. Probe A spanned exons 4 and 5 and the first 3 nucleotides of exon 6. The protected fragments represent full length (243 bp) or short transcripts lacking exon 4 (ΔNES; 213 bp). Probe B spanned 43 nucleotides of exon 5, exons 6–8 and 21 nucleotides of exon 9 (353 bp). Probe C detected the 3' portion of *Smad4* transcripts. The 322 bp fragment spanned sequences of exons 9 and 10. For determination of comparative expression ratios against other probe signal intensities, the quantification of NES and full length transcripts are treated as additive.

Immunoprecipitation and Western blot analysis

Cell lysates were prepared from CCE (*Smad4*^{+/+}), BNN [22] (*Smad4*^{N/N}), NES#1 and NES#5 (*Smad4*^{ΔNES/ΔNES}) ES cell lines as described previously [28]. Western blot analysis was performed using standard techniques. The following primary antibodies were used: anti-Smad4 (B8; Santa Cruz), anti-Smad2/3 and anti-Grb2 mouse monoclonal antibodies (both from BD Biosciences) and anti-Smad1 (Zymed) and anti-Smad3 (Zymed) rabbit polyclonal antibodies followed by HRP (horseradish-peroxidase)-conjugated secondary antibodies (DAKO). Blots were developed by chemiluminescence using ECL[®] (Amersham).

For immunoprecipitations, *Smad4*^{ΔNES/ΔNES} or *Smad4*^{+/+} MEFs were treated overnight with 10 µM SB-431542 (Tocris) [29] to abolish autocrine signalling. SB-431542 was then washed out with PBS and cells were treated for 1 h with 2 ng/ml of TGF-β (PeproTech). Total protein extracts were prepared, and immunoprecipitations using the Smad4 B8 antibody were performed as described previously [9]. Immunoprecipitates were Western blotted and probed for phosphorylated Smad2 using a rabbit polyclonal antibody against phosphoserines 465 and 467 (Cell Signalling Technology) or Smad2/3 and Smad4 as described above.

Transfection assays

MEFs derived from *Smad4*^{ΔNES/ΔNES} or *Smad4*^{+/+} (outbred) mice were transfected using Lipofectamine[™] 2000 according to the manufacturer's instructions. For luciferase reporter assays, cells were transfected with either CAGA₁₂-luciferase [30] and EF-LacZ [31] as a transfection control, or ARE₃-luciferase and EF-Flag-XFoxH1a [9] with EF-LacZ. After transfection, cells were treated with 10 µM SB-431542 overnight to abolish autocrine signalling. The cells that were to be induced with TGF-β were then washed twice with PBS and stimulated for 8 h with 2 ng/ml of TGF-β. Luciferase assays and β-galactosidase assays were performed as previously described [9].

Indirect immunofluorescence microscopy

ES cells and primary keratinocytes were seeded on to coverslips (pre-treated with gelatin or collagen respectively) and cultured for 36–48 h in standard medium unless indicated otherwise. For TGF-β induction of ES cells, cells were treated with 10 µM SB-431542 overnight, washed twice with PBS and stimulated with 2 ng/ml of human recombinant TGF-β for 1 h at 37°C. Details

of TGF- β and LMB induction of keratinocytes are given in the legend to Figure 4.

Coverslips were washed with PBS, fixed in 4 % formaldehyde/PBS and rinsed again with PBS. Cells were then permeabilized with 0.3 % Triton X-100 in PBS and blocked in a mixture of 10 % FBS, 0.3 % BSA and 0.3 % Triton X-100 in PBS for 30 min at room temperature (22 °C). Coverslips were then incubated with primary antibodies [i.e. anti-Smad4 (1:30) or anti-Smad2/Smad3 (1:500)] in blocking solution for 1 h. Coverslips were washed three times with 0.1 % Triton X-100 in PBS and incubated with a goat anti-mouse IgG H + L secondary antibody (Alexa Fluor® 488, 1:500; Molecular Probes) for 1 h followed by a final wash. Coverslips were mounted with Vectashield mountant containing the nuclear counterstain DAPI (4',6-diamidino-2-phenylindole). Fluorescent images were captured with a Zeiss confocal LSM 510 microscope.

RESULTS

Generation and characterization of Smad4 mutant alleles

Previous studies have identified alternative splice variants of the *Smad4* linker region in human and mouse cell lines. The *Smad4* NES targeting motif mapped to exon 4, and transfection assays have shown that deletion of this exon disrupts Smad4 export from the nucleus [9]. However, to address the biological importance of the Smad4 (Δ NES) isoform and evaluate whether deletion of this sequence is essential for Smad4 shuttling and its ability to transduce TGF- β signalling *in vivo*, in the present study we tested the role of the Smad4 NES via targeted disruption in ES cells.

We engineered two different *Smad4* mutant alleles (Figure 1). The first allele (*Smad4* ^{Δ NES}) was designed to flank exon 4 of the *Smad4* gene with loxP sites, marking it for subsequent Cre-mediated deletion (Figure 1A). The second allele (*Smad4*^{MEX}) incorporated two point mutations within exon 4, replacing the two leucine residues essential to the nuclear export recognition sequence (Figure 1B).

ES cells carrying the targeted alleles were identified by Southern blot analysis [Figures 1A(ii) and 1B(ii)] with a final targeting frequency of 16 % (38/240) and 11 % (9/82) for the *Smad4* ^{Δ NES-TA} and *Smad4*^{MEX-TA} alleles respectively. These clones were then subjected to transient expression of Cre recombinase to excise the selection cassette to generate the *Smad4* ^{Δ NES-CA/+} and *Smad4*^{MEX/+} alleles [Figures 1A(iii) and 1B(iii)]. For the *Smad4* ^{Δ NES} allele, clones in which both the hygro cassette and exon 4 had been excised were also generated (*Smad4* ^{Δ NES/+}).

To generate homozygous mutant *Smad4* ^{Δ NES/ Δ NES} ES cell lines we retargeted heterozygous *Smad4* ^{Δ NES/+} ES cells to derive *Smad4* ^{Δ NES-TA/ Δ NES} clones [Figure 1A(iv)]. Approximately 5.5 % (30/541) of clones had incorporated the targeting construct, replacing the wild-type *Smad4* allele and including the additional loxP site. Selected clones were subsequently subjected to *in vitro* Cre-mediated excision of the hygro cassette and exon 4.

Splice variants lacking exon 4 have been detected in a range of cell lines and normal tissues [9]. RPA analysis (Figure 2) utilising a probe that spanned exons 4 and 5 (Figure 2A; Probe A), revealed that this short isoform was also expressed in murine ES cell lines. Thus alongside the full-length transcript (243 bp) we detected a small amount of *Smad4* ^{Δ NES} isoform (213 bp) (Figure 2B, lanes labelled CCE). The *Smad4* ^{Δ NES} isoform was also expressed at various embryonic stages (11.5 and 15.5 d.p.c.) and in adult tissues (thymus, kidney, spleen and brain; results not shown). To test whether the exon 4 deletion resulted in correctly spliced RNA transcripts, we examined RNA from mutant ES cells. Heterozygous *Smad4* ^{Δ NES/+} ES cells carrying one deleted allele

showed increased levels of the short isoform (Figure 2B, lanes labelled 3C11 and 3B8), whereas homozygous *Smad4* ^{Δ NES/ Δ NES} ES cells (Figure 2B, lanes labelled NES#1 and NES#5) produced only the truncated transcript. To confirm that the exon 4 deletion did not lead to aberrant splicing downstream, two additional RPA probes (Figure 2A; Probes B and C) encompassing exons 5–10 were also used. Probe B spanned exons 5–9, whereas Probe C was complementary to exons 9–10. Our RPA experiments demonstrated that the pattern of mRNA splicing was unaffected in *Smad4* ^{Δ NES/+} and *Smad4* ^{Δ NES/ Δ NES} cells (Figure 2C). Thus we concluded that removal of exon 4 had no deleterious effect upon downstream splicing events.

Next we examined Smad4 protein expression levels in Western blots to test whether protein stability might be influenced by deletion of exon 4. Cell lysates were generated from ES cells and Western blot analysis was performed using a mouse monoclonal antibody specific for Smad4 (Figure 2D). We detected a wild-type band of approximately 64 kDa present in the CCE *Smad4*^{+/+} control cell lines. As a control, the *Smad4* null cell line (*Smad4*^{N/N}; BNN) failed to give any signal. Selectively in mutant cell lines (NES#1 and #5) we observed a band corresponding to the Δ NES protein with slightly faster gel-mobility due to the deletion of 10 amino acid residues encoded by exon 4. Similarly deletion of the Smad4 NES failed to perturb R-Smad expression levels. Thus homozygous mutant ES cell lines expressed approximately wild-type levels of Smad1, 2 and 3 (Figure 2D; middle panels).

Smad4 localization in *Smad4* ^{Δ NES/ Δ NES} and *Smad4*^{MEX/MEX} cell lines

To examine possibly altered nucleocytoplasmic trafficking, we visualized subcellular Smad4 localization in *Smad4* ^{Δ NES/ Δ NES} ES cells using indirect immunofluorescence (Figure 3). In wild-type cells in the absence of TGF- β signalling, endogenous Smad4 primarily localized to the cytoplasm, although a small amount of protein was also detected in the nucleus [Figure 3A(i)]. In contrast, *Smad4* ^{Δ NES/ Δ NES} ES cells predominantly expressed nuclear Smad4 [Figure 3A(ii) and 3A(iii)].

ES cells were incubated overnight with the Alk (activin receptor-like kinase) 4/5/7 inhibitor SB-431542 to remove background autocrine TGF- β signalling and then, after washing, were treated with 2 ng/ml of recombinant human TGF- β for 1 h (Figure 3A; +TGF- β panels). As expected, full-length Smad4 rapidly accumulated in the nucleus and gave a predominantly nuclear staining pattern [Figure 3A(iv)]. In the Δ NES mutant ES cells, Smad4 was predominantly nuclear both in the absence and presence of TGF- β [Figures 3A(v) and 3A(vi)]. We also examined R-Smad localization following TGF- β stimulation (Figure 3B). We observed equal distribution of cytoplasmic and nuclear staining of Smad2/3 in unstimulated cells, in both the wild-type and *Smad4* ^{Δ NES/ Δ NES} mutant cell lines [Figures 3B(i)–3B(iii)]. Upon TGF- β signalling, Smad2/3 accumulated in the nucleus equally well in mutant (NES#1 and #5) and wild-type CCE cells. Thus we concluded that deletion of the Smad4 NES had no effect on R-Smad localization [Figure 3B(iv)–3B(vi)].

To further investigate Smad4 shuttling and to better visualize staining patterns, we also generated *Smad4*^{+/+}, *Smad4* ^{Δ NES/ Δ NES} and *Smad4*^{MEX/MEX} primary keratinocyte cell lines. In wild-type keratinocytes Smad4 was distributed throughout the cytoplasm and the nucleus [Figure 4A(i)]. In contrast, in *Smad4* ^{Δ NES/ Δ NES} and *Smad4*^{MEX/MEX} mutant keratinocyte cell lines, Smad4 was predominantly present in the nucleus [Figure 4A(ii) and 4A(iii)]. Thus cells carrying either targeted allele failed to export Smad4 to the cytoplasm. In *Smad4*^{+/+} keratinocytes TGF- β signalling resulted in Smad4 nuclear accumulation, whereas in the *Smad4* ^{Δ NES/ Δ NES} and *Smad4*^{MEX/MEX} mutant keratinocyte cell

Figure 2 Analysis of mRNA and protein expression in homozygous mutant ES cells

(A–C) RPA studies. (A) Total RNA was hybridized with probes specific for representative regions of the *Smad4* cDNA as illustrated. (B) Probe A (exons 4–5) distinguishes *Smad4* full-length and the Δ NES isoform. Both alternative splice variants are co-expressed in *Smad4*^{+/+} ES cells. Expression of Δ NES transcripts is up-regulated in heterozygous ES cells (*Smad4* ^{Δ NES/+}; 3C11 and 3B8) and the 243 full-length product is absent in homozygous mutant ES cells (*Smad4* ^{Δ NES/ Δ NES}; NES#1 and #5). The sizes of the protected fragments and expression ratios are indicated. (C) Analysis of downstream splicing events. Protected fragments detectable with probes B (exons 5–9) and C (exons 9–10) are present at the same levels irrespective of the genotype (lower panels). Thus the deletion has no detectable effect on splicing ratios within the linker or further downstream within the MH2 domain. (D) Homozygous mutant ES cells produce Smad4 protein lacking exon 4. Western analysis reveals that *Smad4* ^{Δ NES/ Δ NES} ES cells (NES#1 and #5) generate Smad4 protein at wild-type levels. In contrast to full-length protein detected in *Smad4*^{+/+} ES cell lines (CCE; upper panel), *Smad4*^{N/N} cells (BNN), lack Smad4 protein, whereas the *Smad4* Δ NES (NES#1 and #5) product shows a slightly faster mobility. Expression of R-Smads (i.e. Smad1, 2 and 3) is unaffected. Blots were probed with anti-Smad4 and anti-Smad2/3 mouse monoclonal antibodies and anti-Smad1 and anti-Smad3 rabbit polyclonal antibodies. Grb2 is included as a loading control (bottom panel).

lines, TGF- β had no effect on the localization of the already nuclear Smad4 [Figures 4A(iv), 4A(v) and 4A(vi)].

Continuous Smad4 nucleocytoplasmic shuttling operates via a CRM1-dependent mechanism [9] which can be blocked by the inhibitor LMB. LMB treatment leads to Smad4 nuclear accumulation due to loss of the NES nuclear export function. It was important to investigate whether treatment with LMB influenced the distribution of Smad4 in the mutant keratinocyte cell lines. As expected, in control wild-type keratinocytes, LMB treatment led to Smad4 localization within the nucleus [Figure 4A(vii)] generating a similar staining pattern to that of both of the constitutively nuclear mutants [Figures 4A(viii) and 4A(ix)]. Thus in primary keratinocytes and ES cells (results not shown), Smad4 is continuously shuttling between the cytoplasm and nucleus. LMB treatment had no effect on the distribution of Smad4 in the *Smad4* ^{Δ NES/ Δ NES} or *Smad4*^{MEX/MEX} cells [Figures 4A(vii), 4A(viii) and 4A(ix) respectively], indicating that the Smad4 in these cells does not shuttle between the cytoplasm and nucleus. Smad2/3 distribution was completely unaffected following LMB treatment of *Smad4*^{+/+}, *Smad4* ^{Δ NES/ Δ NES} or *Smad4*^{MEX/MEX} cells [Figures 4B(vii), 4B(viii) and 4B(ix) respectively] as expected, since R-Smad nuclear export is CRM1-independent [6].

Collectively these experiments confirm that under basal steady-state conditions, Smad4 is distributed throughout the cell. Inactivation of CRM1-mediated Smad4 nuclear export, via LMB treatment resulted in nuclear accumulation in wild-type cells. Similarly our homozygous NES mutant keratinocytes or ES cell lines lacked the ability to export Smad4 from the nucleus.

***Smad4* ^{Δ NES/ Δ NES} mutant protein associates with R-Smads and mediates TGF- β -induced transcriptional activation**

In response to TGF- β stimulation, phosphorylated R-Smads, in association with Smad4, accumulate in the nucleus. To further test the functional capabilities of Smad4 mutant proteins, we also generated MEF cell lines. Total protein extracts were prepared from uninduced and TGF- β -induced *Smad4*^{+/+} and *Smad4* ^{Δ NES/ Δ NES} MEFs and interactions with Smad2 were examined by co-immunoprecipitation with Smad4. To detect activated Smad2, Western blots were probed with a phosphorylated-Smad2 antibody. As a control, endogenous expression levels were also analysed (Figure 5A). As expected, in Δ NES mutant and wild-type MEF cell lines, SB-431542 effectively blocked autocrine signalling and there was no detectable Smad2 activation

Figure 3 Nuclear localization of *Smad4* in ES cells lacking the NES

Nucleocytoplasmic distribution of Smad proteins in ES cells. ES cells were grown on gelatin-coated coverslips under normal conditions (uninduced); treated with SB-431542 overnight followed by TGF- β induction for 1 h prior to fixation (+TGF- β). Cells were fixed and then processed for immunofluorescence using mouse monoclonal antibodies against either (A) Smad4 or (B) Smad2/3. (A) Smad4 shifts from a predominantly cytoplasmic localization in wild-type cells (i) to a largely nuclear localization in *Smad4*^{ΔNES/ΔNES} mutants (ii and iii). Following ligand stimulation, wild-type Smad4 rapidly accumulates in the nucleus and exhibits a predominantly nuclear staining pattern (iv), similar to that observed in the Δ NES mutants (v and vi). (B) Smad2/3 is broadly distributed in cells expressing all three genotypes. Responsiveness to ligand stimulation results in nuclear Smad2/3 accumulation (iv–vi). Indistinguishable localization patterns were observed for *Smad4*^{ΔNES/ΔNES} ES cell lines, NES #7 and #8 in independent experiments. Fluorescence was visualised using a Zeiss LSM 510 confocal microscope. Representative images are shown in green (upper panels) with DAPI nuclear counter-staining (blue) of the same cells provided in the lower panels. CCE, *Smad4*^{+/+} (i and iv); NES#1, *Smad4*^{ΔNES/ΔNES} (ii and v) and NES#5, *Smad4*^{ΔNES/ΔNES} (iii and vi).

(Figure 5A, upper panel; SB). Following TGF- β stimulation, we observed a dramatic increase in phosphorylated-Smad2 levels co-precipitated with Smad4 (Figure 5A, upper panel; TGF- β). These results demonstrated that the *Smad4*^{ΔNES} isoform efficiently interacts and forms complexes with phosphorylated Smad2 in the presence of ligand stimulation.

Next, to address whether constitutive nuclear Smad4 complexes retain the ability to activate target gene expression we transfected MEF cell lines with a CAGA₁₂-luciferase reporter construct (Figures 5B and 5C) containing Smad3/4-binding sequences responsive to TGF- β and activin (but not BMP) stimulation [30]. Alternatively, we also introduced an activin response element-luciferase reporter (ARE₃-luciferase) along with a plasmid expressing EF-Flag-XFoxH1a (Figures 5B and 5C) to study formation of the FoxH1–Smad2–Smad4 transcription factor complex [ARF (activin-responsive factor)] [9]. Cells were SB-431542-treated overnight, washed and induced with TGF- β for 8 h. As shown in Figure 5, the induction of luciferase activity in response to TGF- β in *Smad4*^{ΔNES/ΔNES} MEF cell lines was indistinguishable from that observed in wild-type *Smad4*^{+/+} cells for both reporters. Therefore the mutant Smad4 can effectively form complexes with activated Smad2 or 3 within the nucleus and retains the ability to mediate TGF- β -induced transcriptional activation.

***Smad4*^{ΔNES/ΔNES} and *Smad4*^{MEX/MEX} mutant embryos develop normally and are viable on multiple genetic backgrounds**

The results above demonstrate that the Smad4 mutant proteins we have engineered are constitutively located in the nucleus. Next, to evaluate whether Smad4 shuttling properties influence TGF- β signalling pathways in the developing embryo, germ line chimaeras were generated from the *Smad4*^{ΔNES- Δ TA/+} ES cell clone, 3E12-B6, and subsequently crossed to Sox2 Cre deleter mice [24,25] to excise both the selection cassette and exon 4 in the epiblast. The resulting mutant animals were considered effectively outbred i.e. *Smad4*^{ΔNES/+} (outbred). Matings between the heterozygous *Smad4*^{ΔNES/+} F₁ offspring were established to generate homozygous *Smad4*^{ΔNES/ΔNES} mice. From a total of 155 offspring we obtained both male and female homozygous progeny in the expected Mendelian ratios with an even sex distribution [Table 1(i)].

We also bred chimaeras from a *Smad4*^{ΔNES/+} ES cell clone (3E12-B9) in which both the pgk-hygro cassette and exon 4 had been excised *in vitro*. These males were crossed with C57BL/6J females to generate *Smad4*^{ΔNES/+} mice on a hybrid 129:C57 background. Additionally, chimaeras transmitting the *Smad4*^{MEX} allele were generated from two clones in which the selection cassette had been excised (1B4-C1 and 1B4-A10), to obtain *Smad4*^{MEX/+}

Figure 4 Targeted disruption of the *Smad4* nuclear localization signal restricts nucleocytoplasmic distribution in keratinocytes

Keratinocytes were grown on collagen-coated coverslips for 24 h in growth factor starvation medium (uninduced). Some cells were then treated for 1 h with TGF- β or LMB, as indicated, prior to fixation. Cells were then stained with either (A) Smad4 or (B) Smad2/3 monoclonal antibodies. (A) Smad4 shifts from a predominantly cytoplasmic localization in wild-type cells (i) to a largely nuclear localization in *Smad4* ^{Δ NES/ Δ NES} and *Smad4*^{MEX/MEX} mutants (ii and iii). Following ligand stimulation, wild-type Smad4 rapidly accumulates in the nucleus (iv), similar to that observed in the *Smad4* ^{Δ NES/ Δ NES} and *Smad4*^{MEX/MEX} mutants (v and vi). LMB treatment of wild-type cells results in accumulation of Smad4 in the nucleus (vii) in a similar pattern to cells lacking a functional NES i.e. *Smad4* ^{Δ NES/ Δ NES} and *Smad4*^{MEX/MEX} mutants (viii and ix). (B) For all three genotypes Smad2/3 was distributed throughout the cell (i–iii) and stimulation with TGF- β results in Smad2/3 nuclear localization. (iv–vi). LMB treatment has no effect on Smad2/3 nucleocytoplasmic distribution (vii–ix). Fluorescence was visualised using a Zeiss LSM 510 confocal microscope. Representative images are shown in green (upper panels) and DAPI nuclear counter-staining (blue) of the same cells is provided in the lower panels.

offspring on a 129:C57 background. Intercross mating pairs were established from both the *Smad4* ^{Δ NES/+} and *Smad4*^{MEX/+} F₁ progeny and, as above, homozygous mutant offspring were produced at the expected Mendelian ratios [Table 1(ii) and (iii)]. Thus we recovered viable adult homozygotes carrying both mutant alleles regardless of genetic background.

Finally, to test whether lowering the threshold of available Smad4 could potentially reveal any phenotypic disturbance(s), we also intercrossed the *Smad4* ^{Δ NES/+} (C57:129) line with the *Smad4* null line (*Smad4*^{RobN}) [22] to create mice carrying a single functional *Smad4* allele lacking exon 4 (*Smad4* ^{Δ NES/N}). As shown

in Table 1(iv), *Smad4* ^{Δ NES/N} offspring were recovered in the expected Mendelian ratio. Collectively these results demonstrate that mutant Smad4 proteins lacking the NES are able to function as effectively as wild-type Smad4 to promote normal mouse development.

DISCUSSION

Smad4 in partnership with R-Smads governs TGF- β -dependent target gene expression during early mouse development. Thus it

Figure 5 *Smad4*^{ΔNES} efficiently forms transcriptionally active complexes with phosphorylated Smad2 and Smad3

(A) *Smad4*^{ΔNES} co-precipitates with phosphorylated-Smad2 (P-Smad2). *Smad4* complexes were immunoprecipitated and Western blots subsequently probed with an anti-phosphorylated-Smad2 antibody. Control cell extracts from *Smad4*^{ΔNES/ΔNES} or *Smad4*^{+/+} MEFs were Western blotted for phosphorylated-Smad2, total Smad2 or Smad4 as indicated. Cells were cultured with 10 μM SB-431542 (SB) overnight to abolish autocrine signalling, and then washed twice with PBS and treated with 2 ng/ml of TGF-β (+ TGF-β) for 1 h. (B and C) *Smad4*^{ΔNES} efficiently functions as a transcriptional activator. *Smad4*^{ΔNES/ΔNES} or *Smad4*^{+/+} MEFs were transfected with CAGA₁₂-luciferase and EF-LacZ as a control, or with ARE₃-luciferase, EF-Flag-XFoxH1a and EF-LacZ. Cells pre-treated with SB-431542 overnight, as in (A), were washed and induced with TGF-β for 8 h or kept in SB-431542. Luciferase activity was quantified relative to β-galactosidase activity. The normalized luciferase values ± TGF-β are shown in (B) and the fold inductions are shown in (C). The data are the average of triplicates. The values for the *Smad4*^{ΔNES/ΔNES} MEFs are all lower than those for the *Smad4*^{+/+} MEFs. This is a result of the transfection efficiency being lower for the *Smad4*^{ΔNES/ΔNES} MEFs compared to the *Smad4*^{+/+} MEFs. However, the fold inductions are the same.

is known that *Smad4* null embryos die shortly after implantation, fail to form mesoderm, and exhibit defective visceral endoderm differentiation [21]. Studies analysing mosaic and conditional *Smad4* mutant embryos reveal a primary requirement for Smad4 in extra-embryonic tissues [21,22,32]. Smad4 function is clearly also required at later stages within the embryo proper. Thus conditional deletion in the epiblast results in selective loss of anterior streak derivatives such as prechordal plate, node, notochord and the definitive endoderm, and disrupts formation of primordial germ cells, distinctive cell lineages dependent on nodal and BMP

Table 1 Genotypes of offspring from *Smad4* heterozygous intercrosses

(i) *Smad4*^{ΔNES/+} x *Smad4*^{ΔNES/+} (outbred background), total animals = 155

Genotype	<i>Smad4</i> ^{+/+}	<i>Smad4</i> ^{ΔNES/+}	<i>Smad4</i> ^{ΔNES/ΔNES}
No. animals:	41 (27 %)	75 (48 %)	39 (25 %)
Expected:	39 (25 %)	77 (50 %)	39 (25 %)

(ii) *Smad4*^{ΔNES/+} x *Smad4*^{ΔNES/+} (129:C57 background), total animals = 152

Genotype	<i>Smad4</i> ^{+/+}	<i>Smad4</i> ^{ΔNES/+}	<i>Smad4</i> ^{ΔNES/ΔNES}
No. animals:	47 (31 %)	69 (45 %)	36 (24 %)
Expected:	38 (25 %)	76 (50 %)	38 (25 %)

(iii) *Smad4*^{MEX/+} x *Smad4*^{MEX/+} (129:C57 background), total animals = 154

Genotype	<i>Smad4</i> ^{+/+}	<i>Smad4</i> ^{MEX/+}	<i>Smad4</i> ^{MEX/MEX}
No. animals:	44 (29 %)	78 (50 %)	32 (21 %)
Expected:	38 (25 %)	78 (50 %)	38 (25 %)

(iv) *Smad4*^{ΔNES/+} x *Smad4*^{N/+} (outbred background), total animals = 79

Genotype	<i>Smad4</i> ^{+/+}	<i>Smad4</i> ^{ΔNES/+}	<i>Smad4</i> ^{N/+}	<i>Smad4</i> ^{ΔNES/N}
No. animals:	20 (25 %)	17 (22 %)	24 (30 %)	18 (23 %)
Expected:	20 (25 %)	20 (25 %)	20 (25 %)	20 (25 %)

signals respectively [22]. These striking tissue defects unequivocally demonstrate that Smad4 plays a central role as the common downstream mediator controlling cell growth and differentiation during embryogenesis.

In the basal state, Smad4 undergoes continuous shuttling between the cytoplasm and the nucleus which is governed by the relative activities of its NLS and NES [6,9,11]. According to current models, the association with phosphorylated R-Smads may mask the NES and favour accumulation of R-Smad–Smad4 complexes inside the nucleus [6,11,33]. Smad4 also interacts with an extensive array of transcription factors governing target gene selection (reviewed in [34,35]) and in unstimulated cells, nuclear Smad4 may act as a transcriptional repressor together with the co-repressor, SnoN [36]. R-Smad association with Smad4 has been assumed to occur in the cytoplasm and to precede nuclear accumulation (reviewed in [2]). However, R-Smad–Smad4 complexes could form equally well inside the nucleus. Indeed, NLSs have been identified universally on Smad proteins (reviewed in [4]). In the case of R-Smads, C-terminal phosphorylation seems to favour nuclear accumulation, whereas the cytoplasmic pool is predominantly de-phosphorylated. One possible scenario is that R-Smad phosphatases function inside the nucleus to promote R-Smad export, thus facilitating cycles of phosphorylation and dephosphorylation which acts as a mechanism by which the R-Smads monitor receptor activity [9]. In contrast, Smad4 lacks a C-terminal phosphorylation motif [9]. Continuous export of monomeric Smad4 molecules could in principle serve to enhance dose-dependent TGF-β signalling by increasing the formation of cytoplasmic R-Smad–Smad4 complexes downstream of activated receptors. This view of R-Smad–Smad4 partnerships at steady-state and in stimulated cells has come entirely from tissue culture experiments. In striking contrast, the present results strongly argue that R-Smad–Smad4 complexes are formed inside the nucleus.

In the present study, to test whether Smad4 nucleocytoplasmic shuttling contributes to TGF- β signalling pathways under physiological conditions *in vivo*, we introduced subtle mutations designed to selectively disrupt Smad4 nuclear export into the mouse germline. We engineered novel alleles at the *Smad4* locus carrying a deletion of exon 4 sequences or with defined leucine residue substitutions predicted to eliminate the NES [9]. The design of our targeting vectors was validated by analysing mutants independently created with both constructs. Homozygous mutant ES cells and keratinocytes were extensively characterized to verify that Smad4 nuclear export was completely abolished. As predicted, our cell staining experiments revealed markedly increased levels of Smad4 accumulation in the nucleus, in the presence or absence of signals. This phenotype resembles that induced by treatment with LMB [9], known to disrupt nuclear export by blocking CRM1 [37]. Nonetheless neither mutation had any noticeable effect on Smad4 stability nor the ability to mediate transcriptional activation. *In vivo* Smad4 functional requirements are reconstituted by these structural variants lacking NES activities. In the present study, in contrast to *Smad4* null embryos, we found that mutants lacking Smad4 NES function develop normally. Homozygous NES mutant strains created by two independent strategies are indistinguishable from wild-type littermates, on either an outbred or (C57BL/6 $\times$ 129) F₂ genetic background. Breeding studies demonstrate perfect Mendelian ratios of viable homozygous animals. The present work conclusively demonstrates that Smad4 nucleocytoplasmic shuttling is not required for visceral endoderm differentiation, anterior streak patterning, specification of germ cells, organogenesis or tissue homeostasis. We failed to observe any overt phenotype in normal, healthy adult mice. It is of course possible that exposure to pathological or stress conditions may reveal a crucial role for Smad4 NES signals.

The dose-dependent actions of *Smad4* as a tumour suppressor have been previously documented (reviewed in [38]). Thus heterozygous *Smad4* mutant mice carrying only one active copy of *Smad4* develop gastric polyps that can eventually become tumours [39]. Gene-dosage effects have also been described in the context of *Apc* (adenomatous polyposis coli) double heterozygous animals [40]. Recent work has shown that Smad4 function is dispensable for normal pancreas development but selective *Smad4* deletion in the pancreatic epithelium, in combination with the activated *K-Ras* (Kirsten rat sarcoma virus) mutant allele, enhances tumorigenesis [41]. We speculate that the *Smad4* NES mutations introduced on to a susceptible genetic background may influence the process of neoplastic transformation.

An extensive histological examination of adult mice up to 3 months of age failed to reveal any consistent evidence for tissue abnormalities. However, adult males of the *Smad4*^{NES} outbred strain occasionally develop a weakly penetrant kidney phenotype. By contrast, on the (C57BL/6J $\times$ 129) F₂ background none of the males show signs of ill health. Additional breeding studies are underway to further investigate genetic background contributions to this possibly late onset kidney phenotype. Interestingly, conditional deletion of *Smad4* in the developing kidney has been shown to cause focal defects [42]. Thus Smad4 function in the ureteric bud and collecting duct is dispensable but Smad4 loss in the metanephric mesenchyme disrupts morphogenesis of the progenitor population. We speculate that Smad4 nucleocytoplasmic transport may prove to be important for maintenance of adult stem cell populations in the kidney or possibly at other tissue sites. Consistent with this way of thinking, recent experiments demonstrate that *Smad4* conditional loss in T-lymphocytes has hardly any effect on T-cell lineage commitment or functional activities, but beginning at 9 months of age loss of Smad4 signalling in T-cells was found to be associated with the devel-

opment of spontaneous epithelial cancers throughout the gastrointestinal tract [43]. Future experiments aim to evaluate whether Smad4 nucleocytoplasmic shuttling may regulate tumour susceptibility, maintenance of adult stem cell microenvironments and/or tissue homeostasis late in life.

Mis-localized expression of key transcriptional regulators has been described in many different types of cancer cells [44]. Recent evidence suggests that the nuclear pore not only undergoes dynamic structural changes during cell-cycle transitions, but also its composition and architecture seem to be sensitive to tissue-specific growth conditions (reviewed in [45]). It has been reported that individual components of the transport machinery are selectively induced in response to cytokines. Thus Nup96 (nucleoporin 96) is involved in governing expression of several interferon-regulated target genes [46]. Changes in nuclear pore composition could influence Smad4 nucleocytoplasmic shuttling properties in discrete cell types in response to pathogenic infections. The present study describes novel mutant mice that selectively lack the Smad4 NES. As judged by RPAs, Western blot analysis and cell-staining experiments, these mice express wild-type levels of total Smad4 protein, and are thus identical in every respect except for Smad4 subcellular localization. In summary, our novel *Smad4* mutant mice should prove useful for dissecting the possible contribution of nucleocytoplasmic shuttling to diverse TGF- β responses *in vivo* such as those responsible for tumour surveillance, tissue remodelling and immune protection against infectious agents.

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