TGFβ (transforming growth factor β) superfamily signalling is critical both for early embryonic development and later for tissue homeostasis in adult organisms. The use of gene-disruption techniques in mice has been essential to understanding the functional roles of the components of the pathways downstream of TGFβ superfamily ligands, in particular, the receptors and the Smads that transduce signals from the plasma membrane to the nucleus. Smad2 functions downstream of TGFβ, Activin and Nodal, and a number of Smad2 mutant mice have been generated by different laboratories. Although in all cases these Smad2-deficient mice were embryonic lethal, those created by deletion of the first coding exon survived longer than those generated by replacing part of the MH (Mad homology) 1 domain or deleting all or part of the MH2 domain. Moreover, they displayed a less severe phenotype, as they were capable of transiently inducing mesoderm. In the present study, we show that embryonic fibroblasts taken from the mutant mice created by deletion of the first coding exon express a small amount of an N-terminally truncated Smad2 protein. We show this protein results from internal initiation at Met241 and encodes the entire MH2 domain and the C-terminal part of the linker. We demonstrate that this protein is incorporated into Smad heteromeric complexes, can interact with DNA-binding transcription factors and thereby can mediate TGFβ-induced transcriptional activation from a number of TGFβ-responsive elements. We propose that this functional truncated Smad2 protein can partially compensate for the loss of full-length Smad2, thereby providing an explanation for the differing phenotypes of Smad2 mutant mice.

Key words: hypomorph, mouse knockout, Smad2, transforming growth factor β (TGFβ) signalling, transcription.

INTRODUCTION

Ligands of the TGFβ (transforming growth factor β) superfamily have a wide variety of functional roles, including embryonic patterning and organogenesis, wound healing, tissue homeostasis, cell migration, proliferation and differentiation. The effects that the TGFβ superfamily ligands exert depend on the cellular context; the cell type, the age of the cell and the competence of the cell to respond to signals [1]. Additionally, the strength and duration of the signal has important roles in the specificity of the biological consequences of this signalling pathway [2]. Members of this superfamily of ligands signal to the nucleus via complexes of activated Smads. These complexes are composed of phosphorylated R-Smads (receptor-regulated Smads), specifically Smad2 and Smad3, in response to TGFβ/Activin/Nodal signalling, in combination with the Co-Smad (common mediator Smad) Smad4 [3].

Sequence alignment revealed that the R- and Co-Smad proteins have two highly conserved domains, the N- and C-terminal MH (Mad homology) 1 and 2 domains, which are separated by a less well-conserved proline-rich linker. The MH1 domain is responsible for auto-inhibition of the MH2 domain in the basal unphosphorylated state and, additionally, in the case of Smad3 and Smad4, but not Smad2, has some weak intrinsic DNA-binding activity. In contrast, the MH2 domain is commonly considered to be the ‘effector’ domain of Smad proteins, mediating multiple protein–protein interactions, including those with other Smads, and with receptors, transcription-factor partners, co-activators and co-repressors [3].

Owing to their poor affinity for DNA, Smad complexes are primarily recruited to TGFβ-responsive promoter elements by interactions with site-specific transcription factors. During the development of the early Xenopus embryo, Smad2 interacts with a number of transcription factors, such as those of the Mix family, namely Mixer, Milk and Bix3 [4,5], and of the Fast/FoxH1 family, XFoxH1a and XFoxH1b [6,7], resulting in transcriptional activation of, for example, the goosecoid gene via the DE (distal element), and the Mix.2 gene via the ARE (Activin-responsive element) respectively. The Smad-transcription factor interaction is mediated by the highly conserved proline-rich SIM (Smad-interaction motif) present in the C-terminus of these transcription factors, which binds to a hydrophobic pocket in the Smad2 MH2 domain [5]. Additionally, the FoxH1s contain a FM (Fast/FoxH1 motif), which allows them to interact specifically with phosphorylated Smad2-containing activated Smad complexes [8].

Targeted gene disruption in mice has hugely advanced our understanding of how and when the Smads function. Unlike Smad2 deficient mice, which are viable and survive to full term [9–11], Smad2-deficient mice that were generated by replacing part of the MH1 domain or inserting a LacZ reporter gene in the MH2 domain [12], or by deleting part of the MH2 domain [13], are embryonic lethal (E7.5–8.5 (embryonic day 7.5–8.5)) and exhibit severe gastrulation defects. These mice fail to form an egg cylinder and lack all mesoderm, as demonstrated by the complete

Abbreviations used: ARE, Activin-responsive element; ARF, Activin-responsive factor; Co-Smad, common mediator Smad; DE, distal element; FM, Fast/FoxH1 motif; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Grb2, growth-factor-receptor-bound protein 2; HA, haemagglutinin; HEK-293T cell, human embryonic kidney cell expressing the large T-antigen of simian virus 40; Luc, luciferase; MEF, mouse embryonic fibroblast; MH, Mad homology; PARP, poly(ADP-ribose) polymerase; R-Smad, receptor-regulated Smad; RT-PCR, reverse transcription-PCR; S2 KO, Smad2 knockout; SIM, Smad-interaction motif; siRNA, small interfering RNA; TGFβ, transforming growth factor β.

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lack of Brachyury expression [12,13]. A proportion of Smad2 heterozygous embryos also exhibit gastrulation defects, and lack mandibles and eyes later in development, indicating that signalling through Smad2 may function in a dose-dependent fashion [12,13], confirming studies performed previously in Xenopus [14,15]. Interestingly, Smad2 mutant mice generated by two other laboratories by disruption of the first coding exon displayed a transient induction of mesoderm [16,17], suggesting that Smad2 was dispensable for mesoderm induction. We demonstrated previously that MEFs (mouse embryonic fibroblasts) taken from such homozygous mice [16,18] express a low level of truncated Smad2 protein that is phosphorylated in response to TGFβ [8]. Since the phenotype of the mice expressing this N-terminally truncated form of Smad2 [16,17] was less severe than that of the other Smad2 mutant mice [12,13], we wanted to characterize this truncated protein fully to determine if it is functional. In the present study, we show that this truncated Smad2 protein results from internal initiation at Met241 and encodes the entire MH2 domain as well as the C-terminal region of the linker. We demonstrate that, as observed for wild-type Smad2, this protein forms heteromeric complexes with Smad4 in response to TGFβ that are able to mediate ligand-induced transcriptional activation. We also show that truncated Smad2 is incorporated into stable DNA-binding complexes with FoxH1a/b and Smad4 or FoxH1a/b, Smad3 and Smad4. Therefore the truncated Smad2 protein expressed in these MEFs is functional, and we propose that it could partially compensate for the loss of full-length Smad2 in the Smad2-deficient mice, thereby accounting for the observed differences in phenotype of the Smad2 mutant mice described previously [12,13,16,17].

MATERIALS AND METHODS

Plasmids

The following plasmids have been described previously: pEF-HA Mixer [8], XFoxH1a and XFoxH1b in pEF-HA and pEF-FLAG [4,7], pEF-HA Smad3 [19], (DE)2-Luc, (ARE)2-Luc and pEF-LacZ [20] and CAGA2-Luc [21], pEF and pEF-HA Smad2(241–467) were constructed by PCR. Oligonucleotides were designed to amplify the region of mouse Smad2 which corresponds to amino acids 241–467, and the resulting sequence was cloned into pEF-plank [22] and pEF-HA. The M241A point mutation was generated in the context of pEF-Smad2(241–467) by PCR. All constructs were verified by DNA sequencing.

Transfections, reporter assays and RT-PCR (reverse transcription-PCR)

Maintenance of NIH 3T3 cells, transfection and reporter assays (reverse transcription-PCR)

When antibodies were used in Western blots, immunoprecipitations and bandshift assays were performed as described previously [4,7,25]. Immunoprecipitations were performed as described previously [20]. Bandshifts probes corresponding to ARE were synthesized as described previously [19], and bandshift assays were performed as described previously [4], except that 50 μg of whole-cell extract was used per lane. The antibodies used in Western blots, immunoprecipitations and bandshift assays were anti-(phospho-Smad2) (Cell Signalling Technology), anti-Smad4 (B8; Santa Cruz Biotechnology), anti-FLAG M2 antibody (Sigma), anti-(Smad2/Smad3) antibody and anti-Grb2 antibody (BD Biosciences) (where Grb2 is growth-factor-receptor-bound protein 2), anti-Smad3 antibody (Zymed) and anti-PARP antibody (BD Biosciences) (where PARP is poly(ADP-ribose) polymerase), anti-HA antibody (where HA is haemagglutinin) and anti-HA-HRP antibody (Roche) (where HRP is horseradish peroxidase). When antibodies were used in Western blots, they were used at 1:1000 dilution.

RESULTS

Smad2-deficient MEFs express a truncated form of Smad2 that is localized to the nucleus and can be phosphorylated in response to TGFβ

In response to TGFβ, Smad2 is phosphorylated on the last two serine residues of the C-terminal SSXS motif and, in complex with Smad4, accumulates in the nucleus, where it regulates gene expression. The truncated Smad2 protein expressed by the S2 KO MEFs [18] is phosphorylated when cells are stimulated with TGFβ [8]. In order to test whether this protein acts like full-length Smad2, we compared its behaviour with Smad2 protein from wild-type MEFs in a number of assays. Nuclear and cytoplasmic extracts were prepared from wild-type and S2 KO MEFs cultured in the presence or absence of TGFβ and were Western blotted for the phosphorylated form of Smad2. As expected, wild-type MEFs express Smad2, and this is phosphorylated and accumulates in the nucleus upon the addition of TGFβ. In response to TGFβ, Smad2 protein in the S2 KO MEFs was phosphorylated when cells are stimulated with TGFβ [8]. Differences for phosphorylated Smad2 protein that can be phosphorylated upon TGFβ addition. This protein was localized to the nucleus when the cells were stimulated with ligand (Figure 1). As antibodies specific for unphosphorylated Smad2 cannot recognize this truncated Smad2 protein, it is not possible to determine its cellular localization in

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Figure 1 Truncated Smad2 is phosphorylated and localized to the nucleus in response to TGFβ signalling

Cytoplasmic (Cyt) and nuclear (Nuc) extracts were prepared from wild-type and S2 KO MEFs that had been treated with or without TGFβ for 1 h. Extracts were then analysed by Western blotting and probed with an anti-(phospho-Smad2) antibody (α-P-Smad2) and with anti-PARP and anti-Grb2 antibodies (which acted as controls for nuclear and cytoplasmic proteins respectively). The positions of the molecular-mass markers are shown on the left-hand side (in kDa). P-Smad2, phosphorylated Smad2; Trunc. P-Smad2, phosphorylated truncated Smad2(241–467).

the absence of signal. Antibodies against PARP and Grb2 were used as loading controls for nuclear and cytoplasmic extracts respectively.

Internal initiation of translation at Met241 gives rise to the truncated Smad2 protein

We next determined the identity of the initiating amino acid in truncated Smad2. It was shown previously that mice with the first coding exon of Smad2 deleted expressed an RNA transcript which was 300 bp shorter than wild-type [26]. This transcript arises by splicing around the PGK (phosphoglycerate kinase) neomycin cassette replacing the first coding exon. There are only three in-frame methionine residues from which the truncated Smad2 protein could be initiated: Met241, Met327 and Met411. Moreover, we know that the protein contains the extreme C-terminus of Smad2, as it is phosphorylated in response to TGFβ. From its size, which is approx. 28 kDa as determined by SDS/PAGE, the truncated Smad2 protein would be predicted to start at the first internal in-frame methionine residue, Met241, and would therefore encode the entire MH2 domain along with the extreme C-terminal 33 amino acids of the linker (Figure 2A). To test this, a mouse Smad2 construct starting at Met241, Smad2(241–467), was constructed. This plasmid was transfected into NIH 3T3 cells, and whole-cell extracts from cells treated with or without TGFβ were compared with extracts from wild-type and S2 KO MEFs by Western blotting using an antibody against phosphorylated Smad2. The endogenous truncated Smad2 protein migrated identically with the transfected Smad2 construct when resolved by SDS/PAGE (Figure 2B). Full-length endogenous Smad2 is present and is phosphorylated upon TGFβ stimulation in both the NIH 3T3 cells and the wild-type MEF cells. To confirm that Met241 was indeed the initiation codon, we generated a construct in which Met241 was mutated to an alanine residue. This plasmid was transfected, along with the wild-type truncated Smad2(241–467) expression plasmid, into human HEK-293T cells, in order to be able to distinguish between mouse and human Smad2 transcripts. Mutation of Met241 to an alanine residue resulted in complete loss of the truncated Smad2 protein (Figure 2C), despite significant mRNA levels being detected.

Figure 2 The truncated Smad2 protein expressed by S2 KO MEFs is the result of an internal initiation at Met241

(A) Schematic diagram of Smad2 and truncated Smad2(241–467) (Trunc. Smad2), with the position of Met241 (M241) indicated. (B) Whole-cell extracts prepared from uninduced (−) or TGFβ-induced (+) wild-type and S2 KO MEFs, and from NIH 3T3 cells overexpressing Smad2(241–467), were assayed by Western blotting with the anti-(phospho-Smad2) antibody (α-P-Smad2). (C) HEK-293T cells were transfected with plasmids expressing truncated Smad2(241–467) or the truncated Smad2 mutant Smad2(M241A–467), or were untransfected. Cells were then treated with the ALK5 (Activin-receptor-like kinase 5) inhibitor SB-431542 to inhibit autocrine signalling (−). After 15 h, SB-431542 was washed out in half the samples, and these were treated with Activin for 1 h (+). Whole-cell extracts were assayed by Western blotting using antibodies against phospho-Smad2 (α-P-Smad2), and Smad2/3 and Grb2 used as loading controls. Note that although the 15 h treatment with SB-431542 was sufficient to cause dephosphorylation of endogenous full-length Smad2 in HEK-293T cells (top panel), it was not sufficient to cause dephosphorylation of truncated Smad2 (second panel). (D) Total RNA was isolated from HEK-293T cells (untransfected), or HEK-293T cells overexpressing wild-type truncated Smad2 [Smad2(241–467)] or M241A truncated Smad2 [Smad2(M241A–467)] constructs, and RT-PCR was performed using primers specific for mouse Smad2 (mSmad2) and GAPDH used as a control (+RT). To ensure that plasmid DNA was not being detected, RT-PCR reactions were also performed in the absence of reverse transcriptase (−RT). Trunc. P-Smad2/P-Smad2(241–467), phosphorylated truncated Smad2(241–467).
Smad4 can form a complex with the truncated Smad2 protein

Whole-cell extracts were prepared from wild-type or S2 KO MEFs that had been incubated with (+) or without (−) TGFβ for 1 h. Whole-cell extracts were then assayed by immunoprecipitation (IP) of complexes with an anti-Smad4 antibody, followed by Western blotting (WB) with an anti-(phospho-Smad2) antibody (top panel). Western blotting of whole-cell extracts (Input) with anti-(phospho-Smad2) antibody (α-P-Smad2) (middle panel) and anti-Smad4 antibody (bottom panel) acted as loading controls. A non-specific background band is marked by an asterisk (*). P-Smad2, phosphorylated Smad2.

Truncated Smad2 forms a heteromeric complex with Smad4 in response to TGFβ

Upon TGFβ stimulation, Smad oligomerization occurs in order to form transcriptionally active R-Smad–Smad4 heterodimers and heterotrimers [19,27–30]. These complexes are the result of extensive protein–protein interactions between the MH2 domains of the Smads and are stabilized by binding of the phosphorylated C-terminus of the R-Smad to the L3 loop/B8 β-strand of the adjacent monomer [27]. Since the Smad2 protein expressed by S2 KO MEFs is N-terminally truncated and therefore has an intact MH2 domain, we wanted to determine whether it could form heteromeric complexes with Smad4. Whole-cell extracts from un-induced or TGFβ-induced wild-type and S2 KO MEFs were immunoprecipitated using an anti-Smad4 antibody and then Western blotted to detect endogenous phosphorylated Smad2. Smad4 immunoprecipitated full-length phosphorylated Smad2 from wild-type MEFs, and truncated phosphorylated Smad2 from S2 KO MEFs (Figure 3). Thus internal initiation from Met231 produces a Smad2 protein that is appropriately folded and is capable of forming heteromeric complexes with Smad4.

TGFβ-induced transcriptional activation is supported by truncated Smad2

Smad2 cannot bind DNA directly and thus forms transcriptionally active complexes through its interaction with a number of different transcription factors. The best characterized Smad2-interacting transcription factors are found in the early Xenopus embryo. Here, Smad2–Smad4 complexes interact with paired-like homeodomain transcription factors of the Mix family, such as Mixer, Milk and Bix3, in order to be recruited to the DE of the goosecoid promoter, and with the forkhead/winged-helix transcription factors FoxH1a and FoxH1b at the Mix.2 ARE [4–7]. Since the Smad complex interacts with these transcription factors through the Smad2 MH2 domain [4], and we have demonstrated that the truncated Smad2 protein can form Smad2–Smad4 complexes (Figure 3), it is conceivable that this protein can also interact with its transcription-factor partners and thereby mediate TGFβ-induced transcriptional activation. Indeed, it has been demonstrated in in vitro transcription assays from chromatinized templates that this portion of Smad2 possesses transcriptional activity [31]. In order to test whether the truncated Smad2 protein can form active Smad–transcription-factor complexes and is therefore functional, wild-type and S2 KO MEFs were transfected with Mixer and the (DE)4–Luc (where Luc is luciferase) reporter, or with FoxH1a or FoxH1b and the (ARE)3–Luc reporter construct, and then treated with or without TGFβ. Additionally, the two cell lines were transfected with the Smad3-dependent reporter CAGA12–Luc, which acts a TGFβ-responsive but Smad2-independent control. The results indicate that there is no significant difference in the ability of full-length or truncated Smad2 to mediate TGFβ-induced transcriptional activation. In the wild-type MEFs, treatment with TGFβ resulted in an average 3.1-fold induction of (DE)4–Luc when Mixer was also transfected (Figure 4A and Table 1) and, in the same way, this reporter was induced 3.8-fold in the S2 KO MEFs. Similarly, (ARE)3–Luc was activated comparably in both cell types when FoxH1a/FoxH1b and TGFβ were present. As expected for a Smad3-dependent reporter, induction of the CAGA12–Luc reporter was not significantly different in the S2 KO MEFs when compared with the wild-type cells, being induced 12.3-fold and 10.1-fold respectively (Figure 4A and Table 1).

Since the S2 KO MEFs contain levels of Smad3 which are comparable with those in wild-type MEFs, it remained a possibility that Smad3 could be mediating the TGFβ-induced transcriptional activation of the reporters. In order to address this directly, S2 KO MEFs were transfected with siRNAs specific for mouse Smad2 or Smad3, or a non-target control siRNA, to knockdown the remaining levels of these proteins (Figure 4C), and then TGFβ-induced activation of the (ARE)3–Luc reporter in combination with FoxH1b was assayed. Interestingly, knockdown of either Smad2 or Smad3 not only reduced the background level of activity of the reporter in the absence of FoxH1b, but also significantly inhibited TGFβ-induced transcriptional activation (Figure 4B). Therefore the truncated Smad2 acts like full-length wild-type Smad2 in its ability to interact with its transcription-factor partners and thereby mediate TGFβ-induced activation via either the DE–Luc or the ARE–Luc reporters.

XFoxH1a and XFoxH1b form stable complexes on DNA with endogenous truncated Smad2 and Smad4 in vitro

Having established that the truncated Smad2 protein found in S2 KO MEFs mediates TGFβ-induced transcriptional activation...
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Figure 4 Truncated Smad2 can mediate TGFβ-induced transcriptional activation via the DE and ARE comparable with wild-type full-length Smad2

(A) Wild-type (top) and S2 KO (bottom) MEFs were transfected with (DE)4–Luc, (ARE)3–Luc or CAGA12–Luc reporters along with (+) or without (−) plasmids expressing Mixer, FoxH1a or FoxH1b as indicated. Cells were incubated in the presence (+) or absence (−) of TGFβ for 8 h, and luc was quantified relative to the β-galactosidase internal control. Results are from one representative experiment performed in triplicate. See Table 1 for details of means ± S.D. from four independent experiments. (B) S2 KO MEFs were transfected with the control non-target siRNA SMARTpool or with a single duplex siRNA targeting mouse Smad2 (mS2) or an siRNA SMARTpool targeting mouse Smad3 (mS3), followed by transfection with (ARE)3–Luc with (+) or without (−) a plasmid expressing FoxH1b and treatment with (+) or without (−) TGFβ as in (A). Results are means ± S.D. from one representative experiment performed in triplicate. (C) Western blotting to show the specificity of the knockdown of mouse Smad2 and mouse Smad3 using the siRNAs stated in (B) in S2 KO MEFs. Blots were probed with anti-(Smad2/Smad3) antibody, anti-(phospho-Smad2) antibody and anti-Grb2 antibody, which was used as a control. α-P-Smad2, anti-(phospho-Smad2) antibody; P-Smad2, phosphorylated Smad2. NT, non-target.

from two different reporter constructs, we wanted to investigate whether it can form stable Smad2–Smad4–FoxH1 DNA-bound complexes on the ARE. Here, Smad2–Smad4 heterotrimers interact with FoxH1a and FoxH1b to form ARF (Activin-responsive factor) 1 and ARF2 complexes respectively [7,19]. Therefore wild-type and S2 KO MEFs were transfected with either FLAG-tagged FoxH1a or FoxH1b, and treated with TGFβ for 1 h before whole-cell extracts were prepared and assayed for ARF1/2 formation by bandshift assays. As expected, in the wild-type MEFs, transfection of FoxH1a and FoxH1b resulted in the production of strong TGFβ-dependent ARF1 and ARF2 complexes respectively, and these fully shifted with antibodies against the FLAG tag, Smad2/Smad3 and Smad4, representing each of the components of the complexes (Figure 5A). Addition of an antibody against Smad3 also shifted a small amount of the ARF1 and ARF2 complex. Interestingly, in the S2 KO MEFs, the ARF complexes migrated as a triplet of bands, the faint top band of which possessed the same mobility as the ARF complexes formed in wild-type MEFs. The two other bands exhibited higher mobility. These complexes were again fully shifted using antibodies against the FLAG tag, Smad2/Smad3 and Smad4, representing each of the components of the complexes (Figure 5A). Addition of an antibody against Smad3 also shifted a small amount of the ARF1 and ARF2 complex. Interestingly, in the S2 KO MEFs, the ARF complexes migrated as a triplet of bands, the faint top band of which possessed the same mobility as the ARF complexes formed in wild-type MEFs. The two other bands exhibited higher mobility. These complexes were again fully shifted using antibodies against the FLAG tag and against Smad4, indicating that all three complexes contained FoxH1 and Smad4. The complexes were only partially shifted by anti-(Smad2/Smad3) and anti-Smad3 antibodies (Figure 5A). ARF complexes formed in S2 KO MEFs appeared to be slightly weaker than those from wild-type MEFs, probably resulting from the lower amount of truncated Smad2 protein in these mutant cells compared with full-length Smad2 in wild-type MEFs.

One possible explanation for the formation of the three complexes on the ARE when whole-cell extracts from S2 KO MEFs were used is that the composition of the Smad heterotrimer that binds to FoxH1 [19] is different in the three bands. We reasoned that the faint top band could be an ARF complex composed of FoxH1 with a Smad3–Smad4 heterotrimer, the middle band could contain a truncated Smad2–Smad3–Smad4 trimer with FoxH1, and the highest-mobility complex could contain a trimer composed of two truncated Smad2 proteins complexed with one Smad4 and FoxH1. In order to investigate the composition of the three ARF1 complexes formed in S2 KO MEFs, the cells were transfected with FLAG-tagged FoxH1a, either alone or with plasmids expressing HA-tagged versions of Smad2(241–467) or Smad3. As demonstrated above, when cells expressing FoxH1a were induced with TGFβ, three complexes formed on the ARE probe, and these complexes shifted with antibodies against the FLAG tag, Smad2/Smad3 and Smad4. As expected, no shift was observed when the complexes were incubated with the anti-HA antibody (Figure 5B, lane 6). However, when HA-tagged Smad2(241–467) was overexpressed, the lower two complexes of the native trimer were enhanced at the expense of the upper complex, as would be expected if they contain truncated Smad2,
and these complexes shifted with the addition of the anti-HA antibody (Figure 5B, compare lanes 8 and 12). In contrast, exogenous HA-tagged Smad3 increased the amount of the top two complexes, supporting the theory that these contain Smad3–Smad4 and truncated Smad2–Smad3–Smad4 heterotrimers respectively. These complexes were shifted using an anti-Smad3 antibody and also an anti-HA antibody (Figure 5B, compare lanes 14, 16 and 18). Extracts were Western blotted to check the levels of expression of the HA-tagged Smads (Figure 5C).

Thus taking all of our results together we conclude that truncated Smad2 can form stable complexes either with Smad4 and FoxH1 or with Smad3, Smad4 and FoxH1 on the ARE, and that these complexes are transcriptionally active.

**DISCUSSION**

In the present study, we show that MEFs lacking expression of full-length Smad2 [18] express a small amount of a truncated form of Smad2 that starts at Met241 and encodes the entire MH2 domain and the extreme C-terminal end of the linker. This protein is functional in terms of its ability to be phosphorylated by the receptor, to form complexes with transcription factors and other Smads, and consequently it mediates ligand-induced transcriptional activation of a number of TGFβ-responsive reporter constructs. In fact, further N-terminal deletion of Smad2 to amino acid residue 245 results in loss of the ability to be phosphorylated by the receptor [8], indicating that this truncated form of Smad2 is the minimal fragment that can be activated by TGFβ. The mice from which these embryonic fibroblasts were taken were created by disruption of the first coding exon of *Smad2* [16]. Mice created using this strategy [16,17] displayed a less severe phenotype than *Smad2*−knockout mice generated differently by two other laboratories [12,13], displaying a transient induction of mesoderm as well as surviving for an additional day. We propose that this is the result of the incomplete loss of all functional Smad2 protein, and that there is sufficient Smad2 activity in the form of this N-terminally truncated Smad2 protein to account for the less severe phenotype.

Studies in *Xenopus* demonstrate that Activin, Nodal and Smad2 all act in a dose-dependent manner to induce mesoderm [14,15,32,33], the result of different transcriptional activation thresholds of specific Smad2-responsive genes. Since the genetic response of a cell to TGFβ superfamily ligands is a consequence of the strength and duration of the signal, and this itself is a function of the concentration of active Smad complexes in the nucleus, decreased cellular Smad levels would be expected to affect TGFβ/Activin/Nodal-responsive transcriptional regulation considerably.

A previous study investigated the expression of a number of TGFβ-dependent genes in Smad2- and Smad3-deficient...
fibroblasts [18]. In this previous study, the authors identified a number of Smad3-dependent genes, including c-fos, Smad7 and TGFβ1, whereas loss of Smad2 alone had no significant effect on the expression of all but one of the genes analysed [MMP-2 (matrix metalloproteinase 2)]. However, the Smad2-knockout MEFs used previously [18] were the same ones as those described in the present study and therefore express truncated functional Smad2 protein. The small number of Smad2-dependent target genes identified previously [18] is thus expected to be artificially low. It is therefore also likely that a number of the ‘Smad3-dependent’ genes identified are also additionally regulated by Smad2, and that these two R-Smads have considerable overlapping but non-redundant roles in TGFβ-mediated gene expression.

The ability of Smad3 to interact with FoxH1 family members and thus compensate for the loss of Smad2 has been investigated previously. Experiments using overexpressed or recombinant purified Smad3 have supported the proposed interaction between these proteins [34–37]. However, unless Smad3 is overexpressed, an ARF1 complex formed in HaCaT cells does not contain Smad3 when analysed by bandshift assays [8]. This suggests that Smad2 is preferentially recruited over Smad3 into ARF complexes. FoxH1a and FoxH1b are thought to interact with a Smad heterotrimer composed of two Smad2s and one Smad4 through their two distinct Smad-interaction motifs; the SIM contacts one Smad2 subunit, and the FM contacts the second [8]. The FM allows FoxH1a/FoxH1b to discriminate between Smad2/Smad4 heterotrimeric and Smad3/Smad4 heterodimeric complexes. This has been attributed to the fact that the FM recognizes the R-Smad–R-Smad interface in the Smad heteromer and, since the Smad3/Smad4 complex is dimeric, at least when bound to the c-Jun Smad-binding region [19], it therefore lacks this binding groove. However, in vitro purified activated Smad3 can form heterotrimeric complexes in association with Smad4, with a stoichiometry of two Smad3s and one Smad4 [27,38,39].

In the present study, we have demonstrated that when endogenous levels of Smad2 protein are low, as in wild-type MEFs, or when Smad2 is truncated and expressed at a low level, as in S2 KO MEFs, Smad3 can become incorporated into ARF complexes and mediate ligand-induced activation of the (ARE)3–Luc reporter. In the S2 KO MEFs, TGFβ-induced transcriptional activation is thus likely to be mediated by complexes containing Smad4 and truncated Smad2, either alone or in combination with Smad3. Indeed, our knockdown experiment (Figure 4B) confirms that both truncated Smad2 and Smad3 contribute to transcriptional activation via the ARE. Thus the truncated Smad2 protein has transcriptional activity and can clearly partially compensate for the loss of full-length Smad2, since the mice that express this protein [16,17] have a less severe phenotype than those generated by methods other than deletion of the first coding exon [12,13]. However, it is important to note that, since the mice expressing truncated Smad2 still fail to develop normally and survive to full term, this truncated Smad2 protein cannot fully compensate for the loss of the full-length protein. This could either be the result of the reduced level of expression of the truncated protein, which could mean that it is simply not at a high enough threshold to activate certain developmental genes, or might indicate an as yet unidentified role for the MH1 domain of Smad2. This second possibility seems likely in the light of previous work showing that directed expression of Smad2 lacking exon 3, but not full-length Smad2, can rescue the ability of Smad2-deficient ES (embryonic stem) cells to contribute to descendants of definitive endoderm in wild-type host embryos [40]. In addition, mice that exclusively express Smad2 lacking exon 3 develop normally and are viable and fertile. The difference in the ability of Smad2 lacking exon 3 compared with full-length Smad2 to rescue the loss of Smad2 suggests a critical function of the Smad2 MH1 domain, which lacks exon 3. Since the truncated Smad2 that we have examined in the present study has no MH1 domain, it cannot therefore fully rescue Smad2-null embryos.

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