

# Integration of TGF- $\beta$ /Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition

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**Epithelial-to-mesenchymal transitions (EMTs) underlie cell plasticity required in embryonic development and frequently observed in advanced carcinogenesis. Transforming growth factor- $\beta$  (TGF- $\beta$ ) induces EMT phenotypes in epithelial cells *in vitro* and has been associated with EMT *in vivo*. Here we report that expression of the hairy/enhancer-of-split-related transcriptional repressor Hey1, and the Notch-ligand Jagged1 (Jag1), was induced by TGF- $\beta$  at the onset of EMT in epithelial cells from mammary gland, kidney tubules, and epidermis. The HEY1 expression profile was biphasic, consisting of immediate-early Smad3-dependent, Jagged1/Notch-independent activation, followed by delayed, indirect Jagged1/Notch-dependent activation. TGF- $\beta$ -induced EMT was blocked by RNA silencing of HEY1 or JAG1, and by chemical inactivation of Notch. The EMT phenotype, biphasic activation of Hey1, and delayed expression of Jag1 were induced by TGF- $\beta$  in wild-type, but not in Smad3-deficient, primary mouse kidney tubular epithelial cells. Our findings identify a new mechanism for functional integration of Jagged1/Notch signalling and coordinated activation of the Hey1 transcriptional repressor controlled by TGF- $\beta$ /Smad3, and demonstrate functional roles for Smad3, Hey1, and Jagged1/Notch in mediating TGF- $\beta$ -induced EMT.**

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## Introduction

During embryogenesis, epithelial cell layers undergo epithelial-to-mesenchymal transitions (EMTs) characterized by disassembly of cell–cell contacts, reorganization of actin cytoskeleton, and cell–cell separation resulting in fibroblast-like cells with mesenchymal marker expression and migra-

tory properties (Hay, 1995). Extracellular cues, including cell adhesion molecules and growth factors, have been shown to induce various phenotypic aspects of EMT *in vitro* and *in vivo* (reviewed in Thiery, 2002). In addition, members of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily have been identified as important inducers of EMT in cardiac, palatal and hair follicle development, and in cutaneous wound repair (Sanford *et al.*, 1997; Sun *et al.*, 1998; Romano and Runyan, 2000; Camenisch *et al.*, 2002). Importantly, the metastatic potential of certain carcinomas is associated with cooperative signalling by activated Ras and autocrine TGF- $\beta$  inducing an irreversible EMT phenotype (Janda *et al.*, 2002).

Members of the TGF- $\beta$  superfamily are multifunctional cytokines that control cell fates, including cell cycle arrest, differentiation, and apoptosis (Roberts and Sporn, 1990). TGF- $\beta$  signals through complexes of heteromeric transmembrane type I (TbRI) and type II (TbRII) receptors (Massague, 2000), activating Smad2 and/or Smad3, two signalling mediators of the SMAD protein family (Heldin *et al.*, 1997; Hoodless and Wrana, 1998), which associate with the shared partner Smad4 and translocate to the nucleus. Nuclear SMAD protein complexes bind specific DNA sequence motifs and participate in transcriptional regulation of target genes (Derynck *et al.*, 1998; Yang *et al.*, 2003). TGF- $\beta$  controls a context-dependent spectrum of epithelial cell plasticity, ranging from cell scattering phenotypes to partial and reversible EMT, or complete and persistent EMT, in nontransformed epithelial cells, indicating an important role for TGF- $\beta$ -dependent signals in inducing EMT phenotypes. Extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K), RhoA and Rac1 small GTPases, and Smads have all been implicated as mediators of some or all phenotypic aspects of TGF- $\beta$ -induced EMT (Miettinen *et al.*, 1994; Piek *et al.*, 1999; Bakin *et al.*, 2000; Bhowmick *et al.*, 2001; Zavadil *et al.*, 2001). Zinc-finger transcriptional repressors Snail and Slug, and the two-handed E-box-binding zinc-finger protein SIP1 are targets of TGF- $\beta$  signalling (Romano and Runyan, 2000; Comijn *et al.*, 2001; Zavadil *et al.*, 2001) with putative roles in EMT (Nieto, 2002). These findings together emphasize our currently incomplete understanding of the complexity of TGF- $\beta$  signalling in orchestrating EMT phenotypes.

We have previously reported that TGF- $\beta$  induction of an EMT phenotype in cultured human keratinocytes was associated with early upregulation of components and target genes of Notch receptor signalling, including transcriptional repressors related to *Drosophila* hairy and enhancer-of-split (H/E(spl)) proteins (Zavadil *et al.*, 2001). Binding of transmembrane Notch receptors with membrane-bound ligands of the Delta, Serrate/Jagged, or *Caenorhabditis elegans* Lag-2 (DSL) family initiates proteolytic processing of Notch receptors mediated by accessory protein complexes and  $\gamma$ -secretase activity, followed by nuclear translocation of the intracellular domain of Notch (NICD) (Mumm and Kopan, 2000).

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Activation (derepression) of Notch target genes involves NICD binding with constitutive repressor complexes anchored at CBF-1-binding elements, converting them into transcriptional activator complexes. The basic helix-loop-helix (bHLH) DNA-binding transcriptional repressors of the H/E(Spl) protein family are direct targets of Notch. To date, H/E(Spl) proteins have not been implicated in EMT and/or TGF- $\beta$  signalling. Although both TGF- $\beta$ /Smad and Notch pathways are widely involved in cell fate decisions in development and pathobiology, functional and molecular interactions of these pathways have not been reported to date.

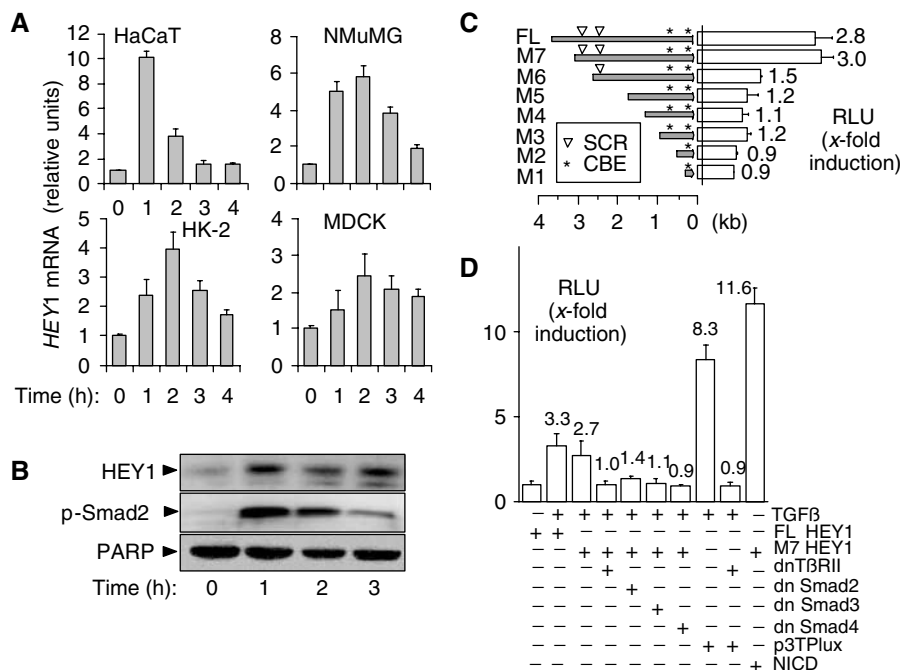
Here we report a new signalling mechanism involved in EMT activated by TGF- $\beta$ . We show that TGF- $\beta$  stimulates two kinetically coordinated waves of expression of *Hey1*. Immediate-early activation of *Hey1* by TGF- $\beta$  is mediated directly by Smad3/4 binding at distinct Smad-binding element core repeats (SCRs) in their distal promoter regions and does not require Notch signalling. The immediate-early activation cycle is followed by a second cycle of gene activation that requires classical Notch-dependent signalling, which is activated by TGF- $\beta$  through Smad3- and ERK MAPK-dependent synthesis of Jagged1. When either *Hey1*, Jagged1, Notch, or Smad3 is inactivated, TGF- $\beta$  fails to induce key EMT phenotypes, including disassembly of E-cadherin adherens junctions, disassembly of cortical actin, and cell-cell separation. Our results suggest that functional integration of TGF- $\beta$  and Notch pathways is mediated by Smad3/ERK MAPK-dependent synthesis of Jagged1 and has important roles in regulation of epithelial cell plasticity by TGF- $\beta$ .

## Results

### *The Notch-regulated transcriptional repressor Hey1 is a new direct target gene of TGF- $\beta$ /Smad signalling in epithelial cells*

Our previous work indicated a potential role for Notch and Notch target genes of the H/E(*spl*) family of bHLH transcriptional repressors in TGF- $\beta$ -induced EMT (Zavadil *et al*, 2001). Here we found that TGF- $\beta$  induced expression of the H/E(*spl*) gene *Hey1* in established cell culture models of TGF- $\beta$ -induced EMT (Figure 1A), including normal murine mammary gland epithelial cells (NMuMGs), kidney proximal tubular epithelial (HK-2), and distal tubular epithelial cell lines Madin-Darby Canine Kidney (MDCK). Induction was RNA synthesis dependent and protein synthesis independent (data not shown), indicating that *Hey1* is a new immediate-early target gene of TGF- $\beta$ . Consistent with mRNA expression, *Hey1* protein synthesis was induced upon stimulation by TGF- $\beta$  (Figure 1B).

To investigate molecular mechanisms of *HEY1* activation by TGF- $\beta$ , we isolated a 3.9 kb fragment of the 5' flanking region of human *HEY1* and tested its response to TGF- $\beta$  in transiently transfected keratinocytes using a luciferase reporter construct. We observed a significant  $\sim$ 3-fold increase in luciferase activity upon TGF- $\beta$  treatment (Figure 1C). Sequence analysis of the *HEY1* promoter identified five putative consensus binding elements for Smad3/Smad4 (Dennler *et al*, 1998). Four of the Smad-binding elements (SBE) were arranged in two GTCTnGTCT repeats at positions



**Figure 1** *HEY1* is a direct target gene of TGF- $\beta$ /Smad signalling. **(A)** Bar graphs show averages of relative *HEY1* mRNA abundance determined by quantitative RT-PCR analysis in keratinocytes (HaCaT), mouse mammary gland epithelial cells (NMuMG), human kidney proximal tubular epithelial cells (HK-2), and canine kidney distal tubular epithelial cell line (MDCK). **(B)** Immunoblots show *HEY1* and C-terminally phosphorylated Smad2 p-Smad2 proteins in keratinocytes stimulated with TGF- $\beta$ . PARP, loading control. **(C)** Bars show average (N=3) fold induction by TGF- $\beta$  of relative luciferase units (RLUs) in keratinocytes transfected with reporter constructs of human *HEY1* promoter deletions (full-length FL, M7 to M1). (▽) positions of SCRs; (\*) positions of NICD-responsive CBEs. **(D)** Bars indicate average (N=3) fold induction by TGF- $\beta$  of luciferase activity (RLU) in keratinocytes cotransfected with luciferase reporter constructs (FL-*HEY1*, M7 *HEY1*, or 3TPLux) and plasmids expressing dominant-negative mutants of type II TGF- $\beta$  receptor (dnTbRII), Smad2, 3 and 4 (dn Smad2, 3 and 4), or NICD. p3TPLux, positive control for TGF- $\beta$ /Smad; NICD, positive control for activation of CBE sites.

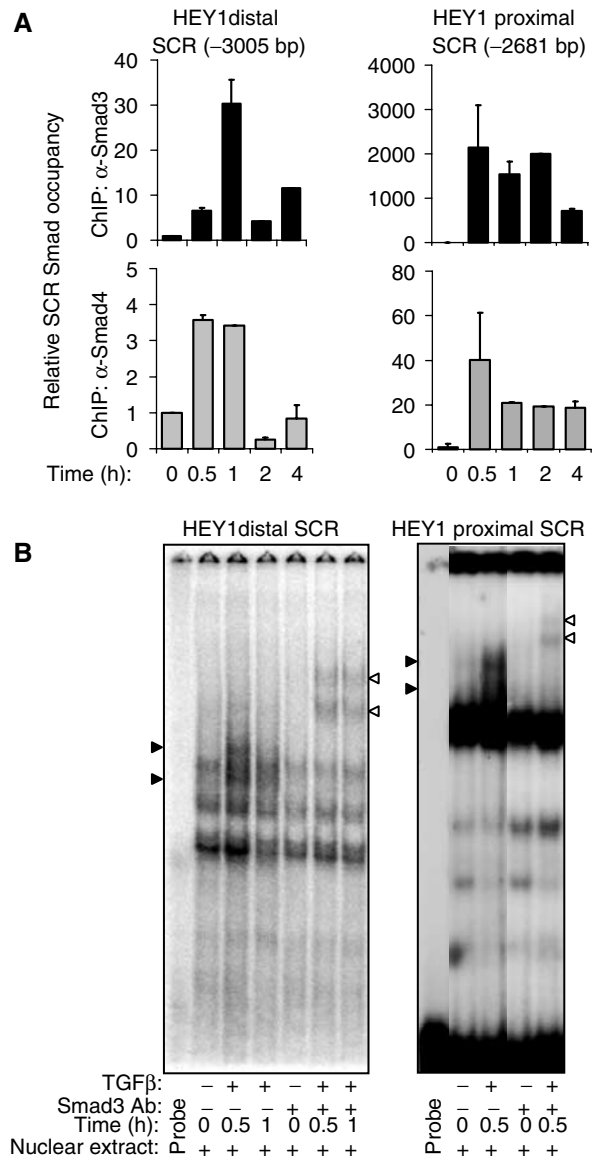
–2681 (proximal) and –3005 (distal) relative to the translation initiation codon (Figure 1C). Such SCRs have been shown to mediate high-affinity binding of Smad3/Smad4 protein complexes (von Gersdorff *et al*, 2000), and are characteristic elements of TGF- $\beta$ -responsive immediate-early genes (Yang *et al*, 2003). We used a series of *HEY1* promoter deletion constructs to confirm that promoter fragments containing both proximal and distal SCRs were required for the activation by TGF- $\beta$  signals, while constructs lacking the SCRs (M1–M5) were unresponsive (Figure 1C). Dominant-negative mutant interference with TGF- $\beta$  signals by cotransfection with expression plasmids for either T $\beta$ RII-DN-K277R, Smad3 $\Delta$ exon8, or Smad4 $\Delta$ M4 reduced the TGF- $\beta$ -inducible activity of *HEY1* full-length (FL) or M7 reporter constructs to baseline levels, while Smad2 3S $\rightarrow$ A had a partial effect (Figure 1D). These results indicate that key components of the TGF- $\beta$ /Smad pathway were required for induction of the *HEY1* promoter. Two Notch-responsive CBEs are located in the proximal part of the promoter in constructs FL, M2–M7 while construct M1 contains only a proximal CBE (Figure 1C). When the reporter constructs were cotransfected with a construct expressing active Notch intracellular domain (NICD), all promoter fragments except for M1 were sufficient to mediate induction of luciferase activity by NICD (data not shown). TGF- $\beta$ /Smad and Notch signals thus use physically distinct promoter regions to activate *HEY1*.

**TGF- $\beta$  induces rapid and transient binding of endogenous Smad3/Smad4 complexes at SCRs in the *HEY1* promoter**

*In vivo* binding of endogenous Smad3- and Smad4-containing complexes with both *HEY1* promoter SCRs at positions –2681 and –3005, respectively, was analyzed by chromatin immunoprecipitation with anti-Smad3 and anti-Smad4 antibodies, followed by quantitative PCR analysis of the SCR loci (ChIP-QPCR). TGF- $\beta$  induced rapid and transient increases of SCR binding with Smad3/Smad4-containing complexes (Figure 2A). In contrast, we could not detect TGF- $\beta$ -inducible SCR binding of endogenous Smad2 (data not shown). To confirm our *in vivo* results, conventional electrophoretic mobility shift assays (EMSAs) and antibody interference studies also demonstrated TGF- $\beta$ -induced binding of Smad3-containing protein complexes to synthetic oligonucleotide probes containing either the distal or the proximal *HEY1* SCR (Figure 2B). Together, these experiments define two separate TGF- $\beta$ -inducible, Smad3/4 response elements at positions –3005 and –2681 bp relative to the translation initiation site of the human *HEY1* gene.

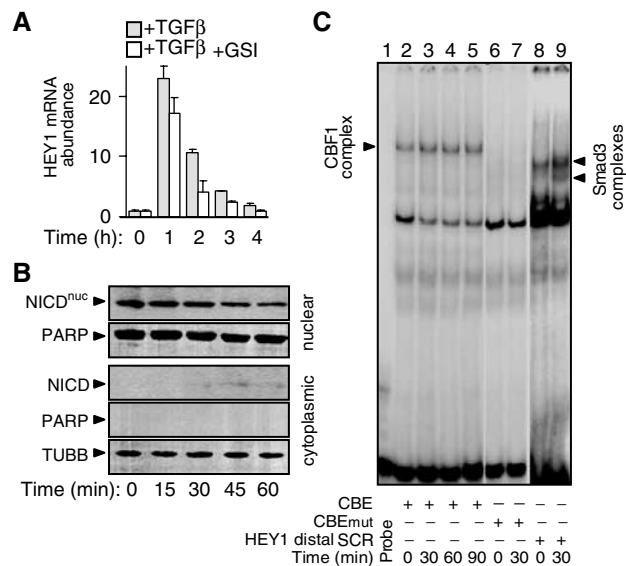
**Notch receptor activation and function is not required for Smad3/4-dependent, immediate-early induction of *HEY1* by TGF- $\beta$**

To investigate whether TGF- $\beta$ /Smad3 and Notch pathways may cooperate in the immediate-early induction of *HEY1*, we examined classical features of Notch receptor signalling. Chemical inhibition (GSI) of  $\gamma$ -secretase activity, typically required for proteolytic activation of Notch (Mumm and Kopan, 2000), did not block induction of *HEY1* by TGF- $\beta$  (Figure 3A). We did not detect nuclear accumulation of transcriptionally active NICD within 60 min of TGF- $\beta$  treatment (Figures 3B and 5B). TGF- $\beta$  had no effect on the



**Figure 2** TGF- $\beta$  induces rapid *in vivo* binding of endogenous Smad3/Smad4 protein complexes with SCR elements in the *HEY1* promoter. (A) Bar graphs show the relative amount of distal *HEY1* SCR (–3005 bp), or proximal SCR (–2681 bp), respectively, determined by quantitative, sequence-specific PCR that is bound with immunoprecipitated Smad3 (black bars) or Smad4 (gray bars) in untreated keratinocytes (time 0 h; baseline unit set at 1) and TGF- $\beta$ -treated keratinocytes. (B) EMSAs demonstrate binding of oligonucleotide probes containing the distal (–3005) SCR or proximal (–2681) SCR, respectively, and nuclear protein extracts from keratinocytes left untreated (–) or treated with TGF- $\beta$  (+) for 0.5 and 1 h. Specific DNA-binding protein complexes are depicted by block arrowheads, and are supershifted by preincubation with anti-Smad3 antibody (open arrowhead).

constitutive CBF-1 protein complex binding with oligonucleotide probes containing proximal (Figure 3C) or distal CBE sites of the *HEY1* promoter, respectively. Furthermore, the level of 300 kDa transmembrane form of Notch1, as assessed by immunoblotting, was unchanged within the first hour of TGF- $\beta$  treatment (see Figure 5B). Together, our results indicate that Notch receptor function was not required for the immediate-early activation of *HEY1* mediated by Smad3/4 in TGF- $\beta$ -treated HaCaT cells.



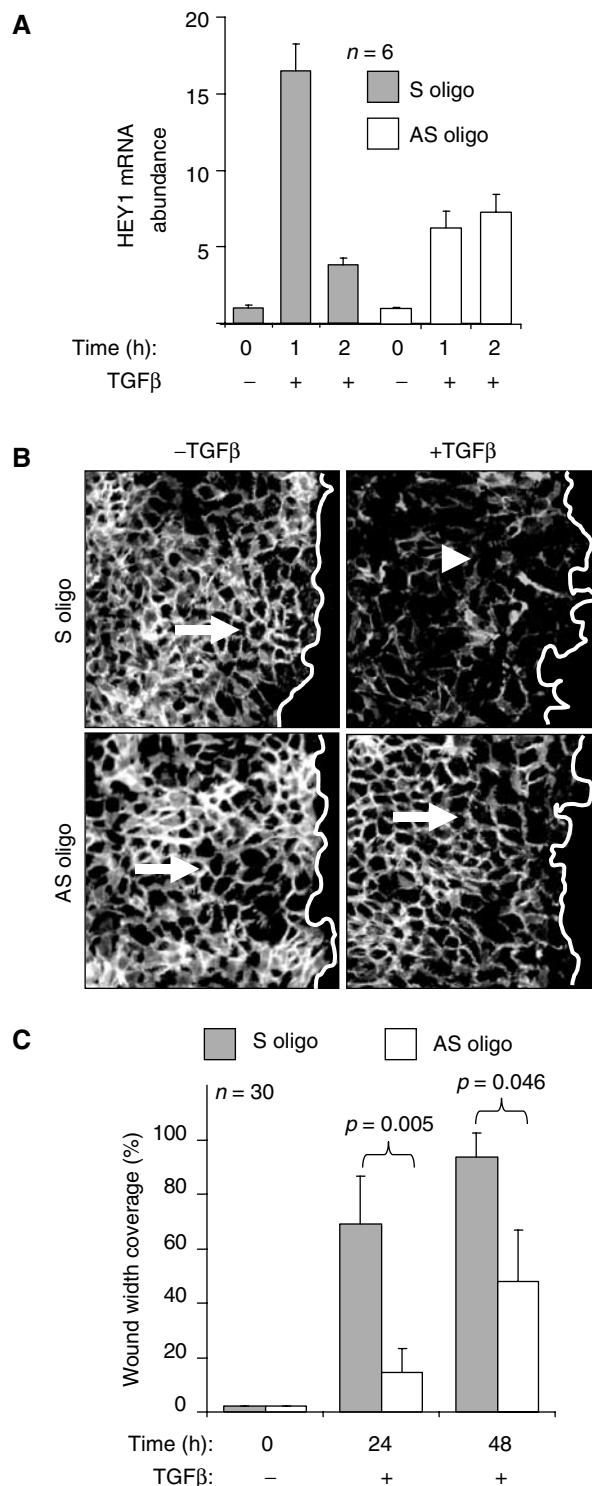
**Figure 3** Activation of the Notch receptor is not involved in TGF- $\beta$  induction of *HEY1*. **(A)** Histograms show the relative abundance of *HEY1* mRNA expression as determined by quantitative RT-PCR in keratinocytes treated with TGF- $\beta$  in the absence or presence of  $\gamma$ -secretase inhibitor GSI. **(B)** Immunoblot showing NICD in nuclear and cytoplasmic protein fractions of keratinocytes treated with TGF- $\beta$ . PARP (poly- $\alpha$ -ADP-ribosyltransferase) and TUBB (tubulin  $\beta$ ) demonstrate purity of cytoplasmic and nuclear protein lysate fractions, respectively. **(C)** EMSA demonstrates binding of CBF-1 protein complex with oligonucleotide probe containing the distal CBE of the *HEY1* promoter (lanes 1–5). Lanes 6 and 7: EMSA using mutated CBE oligonucleotide probe (CBE<sup>mut</sup>) with single bp exchange (cgtggGaaa  $\rightarrow$  cgtggCaaa). Lanes 8 and 9: EMSA using probe containing the distal (–3005 bp) *HEY1* SCR sequence (*HEY1* distal SCR) demonstrates TGF- $\beta$ -inducible Smad protein complexes.

**Disassembly of E-cadherin adherens junctions, cell-cell separation, and cell motility induced by TGF- $\beta$  in keratinocytes require *HEY1* function**

We used antisense oligonucleotides to inactivate *HEY1* function. *HEY1* induction by TGF- $\beta$  at 1 h was significantly reduced in antisense oligotransfected, but not in sense oligotransfected, cells (Figure 4A). In confluent keratinocyte cultures transfected with control sense oligonucleotide, TGF- $\beta$  treatment for 24 h induced typical features of EMT, including disassembly and disappearance of E-cadherin adherens junctions, cell-cell separation, and conversion to fibroblastoid

**Figure 4** *HEY1* is required for EMT and cell motility induced by TGF- $\beta$  in keratinocytes. **(A)** Histograms show the relative abundance of *HEY1* mRNA after treatment with TGF- $\beta$  in keratinocytes transfected with the control S (sense) and AS (antisense) oligonucleotides, respectively. **(B)** *In vitro* scratch-wound assay of the cells transfected with S and AS oligonucleotide and stimulated by TGF- $\beta$  for 24 h and immunostained for E-cadherin (white staining). The empty space right of the white line indicates the wound area. The arrow depicts E-cadherin-positive cell adherens junctions in untreated cells, and the arrowhead indicates loss of E-cadherin-positive adherens junctions in TGF- $\beta$ -treated cells. **(C)** Histograms indicate the average coverage of scratch wound widths in % relative to baseline wound width (0 h) at 24 and 48 h after TGF- $\beta$  treatment in keratinocyte cultures transfected with S (gray bars) or AS (open bars) oligonucleotides. Significance of the motility change was determined by Student's *t*-test. Wound width coverage at 24 and 48 h in untreated control cells was not significantly different from baseline (not shown).

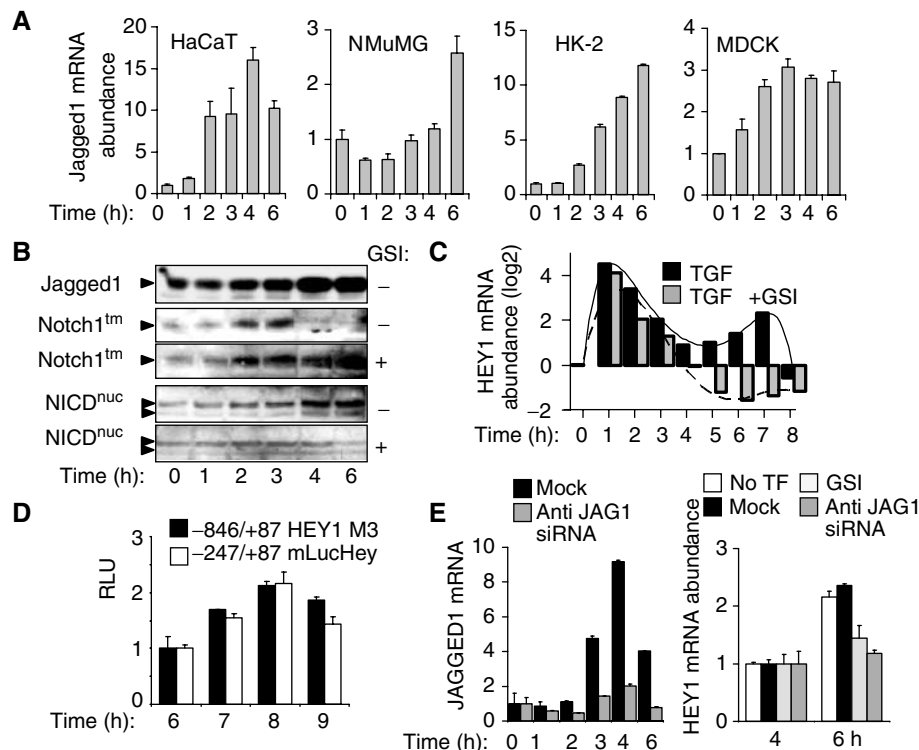
cell morphology (Figure 4B). In contrast, transfection of *HEY1* antisense oligonucleotide blocked these manifestations of TGF- $\beta$ -induced EMT as monitored after 24 h (Figure 4B). In addition, transfection of *HEY1* antisense, but not sense oligonucleotide, significantly reduced TGF- $\beta$ -induced cell motility associated with EMT at 24 and 48 h (Figure 4C), as measured by *in vitro* scratch-wound assays. These results demonstrate that activation of *HEY1* by TGF- $\beta$ /Smad3 is required for TGF- $\beta$ -induced EMT phenotype manifestations and cell motility in human keratinocytes.



**TGF- $\beta$  induces delayed Jagged1 synthesis to activate kinetically coordinated Notch signalling required for a second, delayed phase of HEY1 induction**

We reported previously that stimulation of HaCaT keratinocytes with TGF- $\beta$  indirectly activates ERK MAPK between 1 and 2 h, and that ERK function was required for delayed induction of the Notch ligand JAG1 after 2 h of TGF- $\beta$  treatment (Zavadil *et al*, 2001). Here we show that TGF- $\beta$  increased the expression of Jagged1 mRNA between 2 and 6 h in all four epithelial cell lines examined (Figure 5A). Moreover, TGF- $\beta$  treatment strongly induced Jagged1 protein levels beginning at 2 h in keratinocytes. Jagged1 expression was associated with a dramatic decrease of the levels of intact 300 kDa transmembrane Notch protein (Notch<sup>tm</sup>) and accumulation of NICD in the nucleus (NICD<sup>nuc</sup>) by 4 h (Figure 5B). The reduction of transmembrane Notch protein and the accumulation of NICD in the nucleus were prevented by chemical inhibitor of  $\gamma$ -secretase activity, suggesting that classical Notch receptor activation was associated with increased Jagged1 synthesis induced by TGF- $\beta$ . As Notch is known to activate transcription of several *H/E(spl)* genes directly, including *Hey1* (Nakagawa *et al*, 2000), we examined the expression of HEY1 mRNA over an extended time period (0–8 h) of TGF- $\beta$  stimulation. The immediate-early, TGF- $\beta$ /Smad-dependent wave of activation (1–3 h) was followed by

a second, delayed induction wave of HEY1 (5–8 h) (Figure 4C). Inhibition of  $\gamma$ -secretase had no effect on the immediate-early activation, but completely blocked the delayed induction of HEY1 mRNA (Figure 4C), indicating that it was mediated by Notch receptor activation. Interestingly, we observed that TGF- $\beta$  induced biphasic activation profiles of additional *H/E(spl)* genes HEY2, HES1, and HES5, similar to HEY1 (Supplementary Data Figure S1). Immediate-early regulation of all four TGF- $\beta$ -induced *H/E(spl)* genes was Notch independent and associated with inducible Smad3/Smad4 protein complex binding at SCR Smad complex response elements conserved in their promoters (Supplementary Data Figure S2). Similar to HEY1, a delayed activation phase of HEY2, HES1, and HES5 with peak activation between 5 and 8 h was either completely blocked (HEY2, HES5) or diminished (HES1) by GSI  $\gamma$ -secretase inhibitor (Supplementary Data Figure S1). In addition, the delayed Jagged1-mediated activation of Notch was associated with activation of Notch/NICD-responsive and TGF- $\beta$ /Smad-unresponsive HEY1 promoter regions, as assayed by luciferase reporter constructs M3 HEY1 (human) and mLucHey (mouse) (Maier and Gessler, 2000) (Figure 5D). Together, these results indicate that delayed activation of Notch by TGF- $\beta$  leads to classical NICD signalling and



**Figure 5** Jagged1-induced Notch receptor activation initiates a second, delayed activation of HEY1. (A) Histograms show the mean of relative mRNA abundance determined by quantitative RT-PCR of Notch ligand JAGGED1 in response to TGF- $\beta$  in epithelial cells ( $N=3$ ). (B) Immunoblotting depicts JAGGED1 protein, intact 300 kDa transmembrane form (Notch1<sup>tm</sup>) of Notch in the absence or presence of  $\gamma$ -secretase inhibitor GSI, and nuclear accumulation of NICD (NICD<sup>nuc</sup>) in the absence or presence of GSI in TGF- $\beta$ -treated keratinocytes. (C) Bar graphs indicate the relative abundance of HEY1 mRNA as determined by quantitative RT-PCR analysis in keratinocytes treated with TGF- $\beta$  in the absence or presence of GSI. (D) Histogram shows RLU in keratinocytes transfected with reporter constructs containing proximal human and mouse HEY1 promoter fragments, as indicated, after TGF- $\beta$  stimulation ( $N=3$ ). (E) Left histogram: Bars indicate a representative experiment showing the relative abundance of JAGGED1 mRNA determined by quantitative RT-PCR in keratinocytes, either control transfected (mock) or transfected with anti-JAGGED1 siRNA (anti-JAG1 siRNA). Right histogram: Bars show the mean relative abundance of HEY1 mRNA at 4 and 6 h after TGF- $\beta$  treatment in untransfected keratinocytes (no TF), control-transfected keratinocytes (mock), control-transfected keratinocytes pretreated with GSI (GSI), or in anti-JAGGED1 siRNA-transfected keratinocytes (anti-JAG1 siRNA).

transcriptional activation of a broader complement of classical Notch target genes.

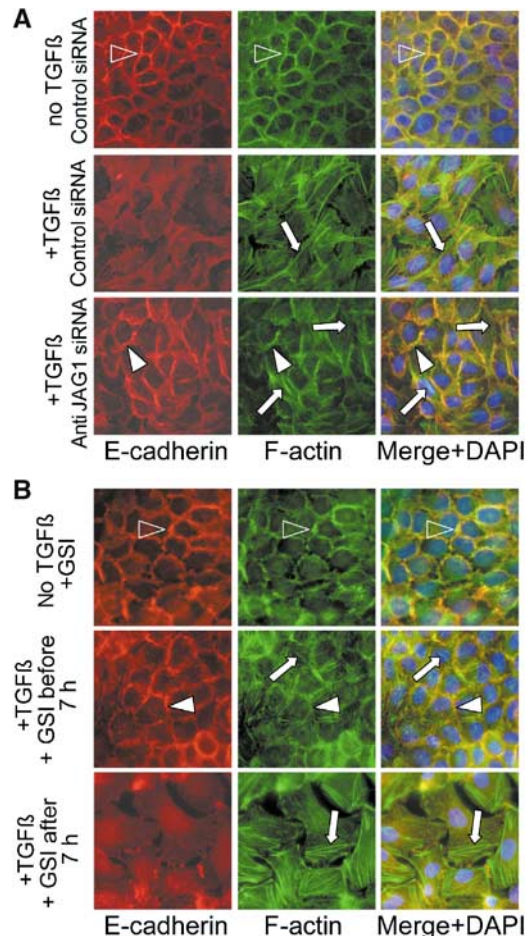
To examine directly whether Jagged1 was required for the delayed, Notch-dependent induction wave of *HEY1*, we used RNA interference to silence TGF- $\beta$ -inducible Jagged1 expression. We confirmed that anti-Jagged1 short-interfering RNA (siRNA) blocked induction of Jagged1 mRNA synthesis by TGF- $\beta$  (Figure 5E). The delayed increase of *HEY1* mRNA observed at 6 h after TGF- $\beta$  treatment in untransfected and control oligo (Mock)-transfected cells was completely blocked in cells transfected with anti-Jagged1 siRNA (Figure 5E), similar to cells pretreated with  $\gamma$ -secretase inhibitor (Figure 5E). Taken together, our results demonstrate that TGF- $\beta$  stimulates two kinetically coordinated waves of activation of *HEY1*. Immediate-early activation of *HEY1* by TGF- $\beta$  is mediated directly by Smad3/4 binding at two distinct SCR sequences in the distal *HEY1* promoter region and does not require Notch signalling. The Smad-dependent, Notch-independent immediate-early activation is followed by a second wave of *HEY1* activation mediated by classical Notch-dependent signalling induced through indirect and delayed induction of Jagged1 synthesis by TGF- $\beta$ .

**Functional inactivation of Jagged1 or Notch inhibits disassembly of E-cadherin adherens junctions and cell-cell separation induced by TGF- $\beta$**

Next, we examined the effects of Jagged1 silencing on EMT phenotypes. In cells transfected with control siRNA, disassembly of E-cadherin adherens junctions and cortical actin bundles, cell-cell separations, and stress fiber formation were induced by TGF- $\beta$  within 24 h (Figure 6A). In contrast, cells transfected with anti-Jagged1 siRNA retained epithelial morphology and cells failed to separate, although E-cadherin adherens junctions appeared widened, and limited stress fiber formation was detectable (bottom row panels) when compared with untreated, control-transfected cells (upper row panels) (Figure 6A). To further delineate the timeframe for functional requirement of Jagged1-induced Notch signalling in EMT, we used timed pharmacological inhibition of  $\gamma$ -secretase activity (Figure 5). When  $\gamma$ -secretase inhibitor GSI was applied before or, up to 7 h, after treatment with TGF- $\beta$ , the cells retained epithelial morphology during TGF- $\beta$  exposure while some stress fiber formation was observed (Figure 6B), similar to cells transfected with anti-Jagged1 siRNA (Figure 6A). In contrast, addition of the inhibitor at 8 h or later time points failed to prevent TGF- $\beta$ -induced EMT (not shown). Thus, the timeframe for functional requirement of Notch signalling in TGF- $\beta$ -induced EMT phenotypes coincided with the delayed phase of Jagged1- and  $\gamma$ -secretase-dependent synthesis of *HEY1*. These results demonstrate that the TGF- $\beta$ -induced Jagged1-dependent activation of Notch signalling is required for disassembly of E-cadherin adherens junctions and cortical actin bundles, and for cell-cell separation induced by TGF- $\beta$  in keratinocytes.

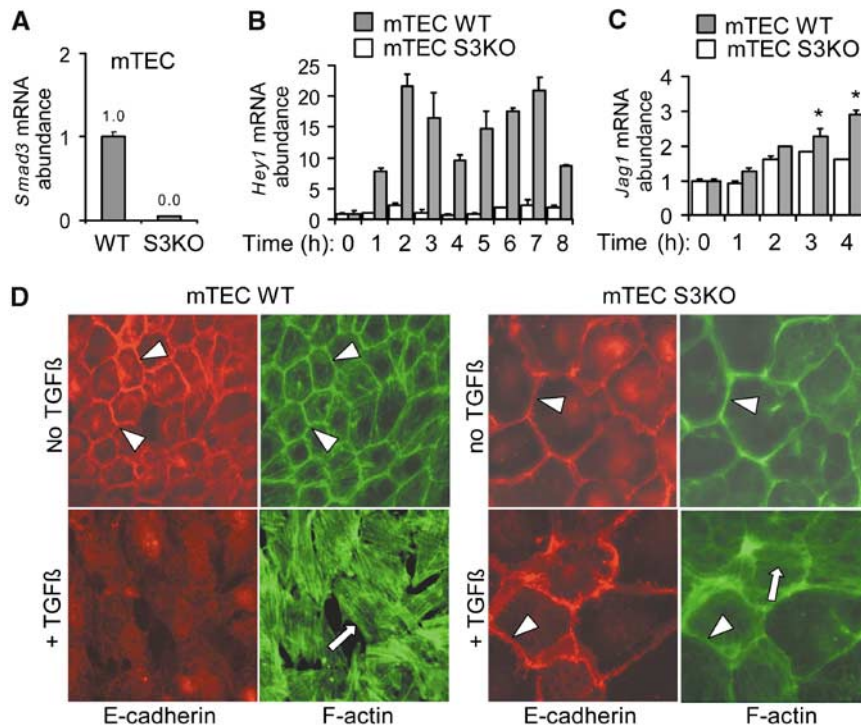
**Smad3 is required for induction of Snail family repressors, Hey1, and Jagged1 expression by TGF- $\beta$ , and is essential for TGF- $\beta$ -induced EMT phenotypes in primary mouse kidney tubular epithelial cells**

To examine the role of Smad proteins in TGF- $\beta$  signalling in EMT, we established primary tubular epithelial cell cultures (mTEC) from mice with homozygous deletion of Smad3



**Figure 6** Jagged1 and Notch activation is required for TGF- $\beta$ -induced EMT. (A) Immunofluorescence labelling for E-cadherin (red), F-actin (green), or both merged with nuclear DAPI staining, as indicated, of control-transfected, untreated (no TGF- $\beta$ , control siRNA), or TGF- $\beta$ -treated (+ TGF- $\beta$ , control siRNA) keratinocytes, and keratinocytes transfected with anti-JAGGED1 siRNA and treated with TGF- $\beta$  (+ TGF- $\beta$ , anti-JAGGED1 siRNA). Immunostaining assays were performed 24 h after TGF- $\beta$  induction. Open arrowheads show E-cadherin-positive adherens junctions and cortical actin bundles, respectively. White arrowheads depict widened E-cadherin-positive adherens junctions. Arrows denote actin stress fibers. (B) Experiments shown as in (A) in untransfected keratinocytes treated with TGF- $\beta$  in the absence or presence of  $\gamma$ -secretase inhibitor (GSI) added either within 0–7 h of TGF- $\beta$  treatment (+ GSI before 7 h) or after 7 h (+ GSI after 7 h).

(Smad3 <sup>$\Delta$ ex8/ $\Delta$ ex8</sup>) and wild-type control littermates (Yang *et al*, 1999). Quantitative real-time PCR (RT-PCR) analysis confirmed that Smad3 mRNA was present in wild-type (mTEC WT) control cells, but not detectable in Smad3 knockout (mTEC S3KO) (Figure 7A). Please note that mTEC S3KO appeared on average larger than mTEC WT. TGF- $\beta$  strongly induced a biphasic pattern of *Hey1* mRNA expression in mTEC WT, including an immediate-early induction (1–3 h) and a delayed induction (5–7 h) (Figure 7B). In contrast, both phases of *Hey1* induction were dramatically reduced in mTEC S3KO (Figure 7B). Compared with a progressive and sustained increase of Jagged1 mRNA in mTEC WT after 2 h of TGF- $\beta$  stimulation, induction of Jagged1 mRNA levels was significantly lower and not sustained in mTEC S3KO (Figure 7C). Next, we analyzed patterns of E-



**Figure 7** Smad3 is required for induction of *Hey1* and *Jagged1*, and for EMT, in primary mouse kidney tubular epithelial cells (mTEC). (A) Quantitative RT-PCR analysis of Smad3 in wild-type (mTEC WT) and Smad3-deficient (mTEC S3KO) primary tubular epithelial cells of the mouse kidney. (B, C) Quantitative RT-PCR analysis of *Hey1* and *Jagged1* inductions by TGF- $\beta$  in wild-type (mTEC WT) and Smad3-deficient (mTEC S3KO) primary mTEC cultures. (D) Immunofluorescence labelling for E-cadherin (red), and phalloidin labelling for F-actin (green) in wild-type (mTEC WT) and Smad3-deficient (mTEC S3KO) primary, left untreated or treated with TGF- $\beta$  for 24 h. Arrowheads indicate E-cadherin adherens junctions and cortical actin, respectively. Arrows depict actin stress fibers.

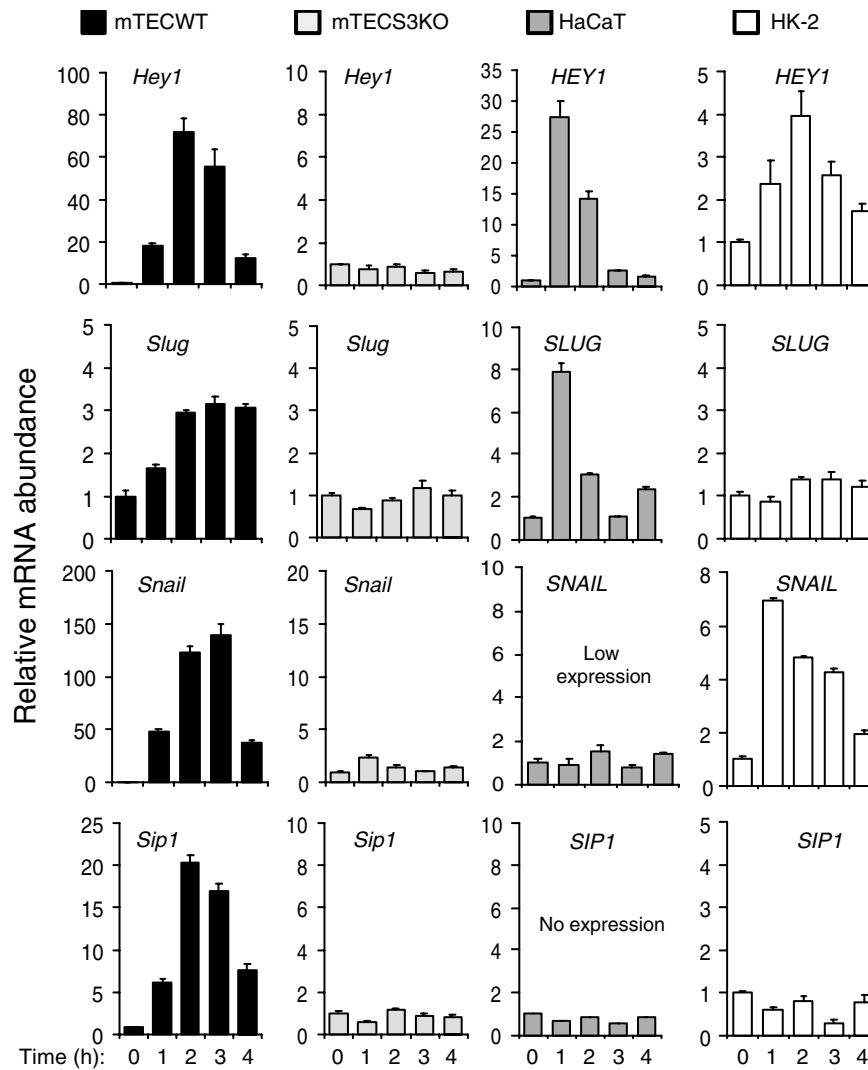
cadherin and actin cytoskeleton in mTEC WT and mTEC S3KO in response to TGF- $\beta$ . E-cadherin and cortical actin patterns were characteristic of epithelial cell phenotype in untreated mTEC WT and mTEC S3KO cultures (Figure 7D). TGF- $\beta$  treatment induced disassembly of E-cadherin adherens junctions and cortical actin bundles, cell separation, and actin stress fiber formation in mTEC WT (Figure 7D). In contrast, E-cadherin cell junctions and cortical actin remained intact but appeared wider and cells did not separate in TGF- $\beta$ -treated mTEC S3KO cultures, while some stress formation was observed (Figure 7D). Together, these findings suggest that Smad3 is required for biphasic induction of HEY1 expression, for progressive increase of Jagged1 expression, and for manifestation of EMT phenotypes induced by TGF- $\beta$  in primary mouse tubular epithelial cells.

To characterize further the role of Smad3 in EMT induced by TGF- $\beta$ , we examined responsiveness of previously identified TGF- $\beta$ -regulated genes with functional roles in EMT and repression of E-cadherin, including *Slug* (Romano and Runyan, 2000), *Snail* (Peinado *et al*, 2003), and *Sip1* (Comijn *et al*, 2001). Strong, immediate-early gene inductions by TGF- $\beta$  were observed for *Hey1*, *Snail*, and *Sip1* in mTEC WT, but not in mTEC S3KO (Figure 8). Induction of *Slug* was more modest and persistent in mTEC WT, but also absent in mTEC S3KO (Figure 8). Interestingly, TGF- $\beta$  strongly induced *HEY1* and *SLUG*, but not *SNAIL* and *SIP1*, in human HaCaT keratinocytes; however, in HK-2 tubular epithelial cells, TGF- $\beta$  induced *HEY1* and *SNAIL*, but not *SLUG* and *SIP1*, expression. These findings indicate that TGF- $\beta$  may induce the bHLH transcriptional repressor HEY1 in a broad spectrum of epithelial

cell types, while regulation of zinc-finger transcriptional repressors *Snail*, *Slug*, and *Sip1* may be more restricted, perhaps depending on the tissue origin of the epithelial cell type.

## Discussion

We report a new mechanism of functional integration between TGF- $\beta$  and Notch signalling leading to coordinated, biphasic activation of bHLH transcriptional repressor *Hey1* as an essential mediator for initiation of EMT by TGF- $\beta$ . First, TGF- $\beta$  directly activates *HEY1* transcription through inducible interaction of Smad3/Smad4 transcriptional activators with two distinct SCRs. Immediate-early activation of *HEY1* is observed across a spectrum of epithelial cells known to undergo EMT in response to TGF- $\beta$ , requires Smad3, and is independent of Notch signalling. Second, a delayed wave of *HEY1* activation between 4 and 7 h of TGF- $\beta$  stimulation is distinct from the immediate-early wave and is also observed in all epithelial cells examined. It is mediated indirectly through delayed synthesis of Jagged1, activating classical Notch signalling. The delayed and sustained activation of Jagged1 by TGF- $\beta$  requires ERK MAPK, as previously shown (Zavadil *et al*, 2001), and is significantly reduced in Smad3-deficient tubular epithelial cells. Thus, Smad3 and ERK MAPK may cooperate to integrate the Jagged1/Notch module as part of the TGF- $\beta$  signalling network. Third, selective inactivations of either *Smad3*, *Hey1*, *Jagged1*, or *Notch1*, respectively, consistently prevent disassembly of E-cadherin adherens junctions and of cortical actin bundles, and cell-cell



**Figure 8** Histograms demonstrate the effects of TGF- $\beta$  treatments, as indicated, on the relative abundance of Hey1/HEY1, Slug/SLUG, Snail/SNAIL, and Sip1/SIP1 mRNAs, respectively, in primary mouse kidney tubular epithelial cultures derived from wild-type control (mTEC WT) and Smad3 knockout (mTEC S3KO) mice, and in human keratinocytes (HaCaT) and human kidney tubular epithelial cells (HK-2). Graphs show representative results from triplicate experiments.

separation, but appear to permit some widening of cell-cell contacts and limited stress fiber formation in keratinocytes and kidney tubular epithelial cells treated with TGF- $\beta$ . These findings are consistent with our previous gene expression profiling study where inhibition of ERK MAPK prevented activation of genes with roles in cell adhesion and motility, but had little effect on genes involved in actin cytoskeleton remodelling (Zavadil *et al*, 2001). Finally, in primary kidney tubular epithelial cells, Smad3 is not only required for immediate-early gene induction of the bHLH gene *Hey1*, but also of zinc-finger transcriptional repressors (*Slug*, *Snail*, *Sip1*) with known important roles in disassembly of various cell-cell contacts and/or repression of E-cadherin by TGF- $\beta$ .

Roles of Smad-dependent signalling mechanisms in TGF- $\beta$ -induced EMT have been examined with conflicting results by overexpressing Smads (Piek *et al*, 1999), or dominant-negative mutant Smads (Bhowmick *et al*, 2001). Our findings presented here, and recent reports by others (Yu *et al*, 2002; Itoh *et al*, 2003), strongly support an important role for Smads. For example, TGF- $\beta$  type I receptor (TbRI) mutants

with intact kinase function sufficient to activate MAPK pathways, but inability to bind and activate Smads due to mutations in the critical L45 loop, were not sufficient to induce stress fiber formation and/or EMT (Yu *et al*, 2002; Itoh *et al*, 2003). Our own results demonstrate that Smad3 function is at a minimum required for key aspects of EMT, including disassembly of E-cadherin adherens junctions and separation of cells induced by TGF- $\beta$ . This conclusion is consistent with our finding that Smad3 is essential for immediate-early regulation of Snail family transcriptional repressors known to be required for downregulation of E-cadherin and disassembly of various cell-cell junctions. However, TGF- $\beta$  was able to induce a widening of cell-cell contacts and cortical actin bundles, and to stimulate a limited degree of stress fiber formation in Smad3-deficient tubular epithelial cells, indicating that either Smad2- and/or Smad-independent pathways may be sufficient to mediate these aspects of EMT, which remain to be defined at a molecular level. For example, RhoA and its target p160<sup>ROCK</sup> are rapidly activated by TGF- $\beta$ : RhoA has been shown to be necessary, but not sufficient for cell



contact disassembly, while p160<sup>ROCK</sup> may signal specifically to activate programs leading to formation of stress fibers (Bhowmick *et al*, 2001). Activation of p38 MAPK appears at least to some extent Smad independent (Yu *et al*, 2002; Itoh *et al*, 2003) and contributes important signals to EMT, stress fiber formation, and cell migration induced by TGF- $\beta$  in NMuMG (Bakin *et al*, 2002; Yu *et al*, 2002). We (Zavadil *et al*, 2001) and others (Peinado *et al*, 2003) have shown that delayed, indirect activation of ERK MAPK by TGF- $\beta$  provides important signals for cell migration and turnover of cell-matrix adhesion complexes. However, in contrast with Smad-independent p38 activation, the relationship and hierarchy of Smad and ERK MAPK activation is more complicated and will require further study. Since the outcomes of Jagged1/Notch inhibition overlap largely with the effects of Smad3 deficiency and inactivation of Hey1 in our study, and include the effects of ERK inhibition previously shown (Zavadil *et al*, 2001), we conclude that Smad3/ERK-dependent coactivation of Jagged1/Notch constitutes an important module of the increasingly complex and differentiated network of signalling pathways that cooperate to mediate the full spectrum of EMT phenotypes induced by TGF- $\beta$  in epithelial cells.

Our model entails a number of new observations with implications for several active fields of research. First, we identify the H/E(spl) gene *Hey1* as a new immediate-early target of TGF- $\beta$  and define molecular elements of the transcriptional activation of *Hey1* by Smad3/Smad4. Interestingly, activation of Notch signalling targets by TGF- $\beta$ /Smad may be conserved across species as indicated by recent observations on *Drosophila* organogenesis, which identified *decapentaplegic* (*Dpp*) and *Mothers-against-decapentaplegic* (*Mad*) (respective fly orthologs of TGF- $\beta$ /BMP and Smads) as positive regulators of *hairy* expression in the developing eye and leg sensory organ patterning (Greenwood and Struhl, 1999; Hays *et al*, 1999). Furthermore, gene knockout experiments in mice indicate that *Tgfb2* and *Hey2* may be components of a functional pathway directing EMT in endothelial cushion remodelling during cardiogenesis (Camenisch *et al*, 2002; Donovan *et al*, 2002; Sakata *et al*, 2002). In both vertebrates and invertebrates, the members of the H/E(spl) subfamily of bHLH proteins restrict the differentiation of precursor cells in a variety of tissues (Davis and Turner, 2001). Therefore, direct induction of *Hey1* by TGF- $\beta$ /Smad provides a new potential mechanism for TGF- $\beta$  to regulate and/or switch cellular differentiation programs. In addition, the expression patterns of H/E(spl) genes observed in the developing nervous system, muscle, lymphocyte, blood vessels, and presomitic mesoderm may now be explained by TGF- $\beta$ /Smad signalling in addition to, or instead of, Notch signalling.

Finally, functional integration of TGF- $\beta$ /Smad and Notch pathways mediating biphasic activation of *Hey1*, and likely other yet unknown target genes, may have implications for the disease pathogenesis of multiple sclerosis and injury of kidney tubular epithelium (John *et al*, 2002; Morrissey *et al*, 2002). Recent reports indicate that Notch1 may exert a dual role in skin carcinogenesis, similar to TGF- $\beta$ . While *Tgfb1* and *Notch1* may function as tumor suppressors in skin by maintaining epidermal homeostasis (Cui *et al*, 1996; Nicolas *et al*, 2003), both may also exert overlapping oncogenic activities in skin cancer progression that is characterized by EMT (Cui *et al*, 1996; Weijzen *et al*, 2002). In light of these findings, and

our observations reported here, *in vivo* co-analyses of TGF- $\beta$ /Smad, Jagged/Notch, and H/E(spl) transcriptional repressors in chronic degenerative and malignant diseases seem warranted. Further molecular investigations will be required to differentiate target genes of TGF- $\beta$ /Smad-induced immediate-early activation of *Hey1*, and of the Jagged1/Notch pathway in epithelial cell plasticity.

## Materials and methods

### Cell culture

HaCaT keratinocytes, NMuMG normal mouse mammary gland epithelium, HK-2 human kidney proximal tubule epithelium, and MDCK canine kidney distal tubule epithelium were expanded to 90% confluency followed by serum deprivation in 0.2% FBS for 24 or 48 h prior to stimulation with 10 pM rhTGF- $\beta$ 1 (R&D Systems) in the absence or presence of 5  $\mu$ M  $\gamma$ -secretase inhibitor X or L-685, 458 (Calbiochem) added 15 min before TGF- $\beta$ .

### Primary cell culture of mouse tubular epithelium

Kidneys were collected from 8- to 12-week-old C57BL/6 wild-type and C57BL/6 Smad3 knockout (Smad3<sup>ex8/ex8</sup>) mice (Yang *et al*, 1999). Kidneys were minced and passed through serial sieves (180, 106, 75  $\mu$ m) in PBS. Tubular fragments were digested for 30 min with 1 mg/ml of collagenase I (Worthington Biochemical) at 37°C in an incubator shaker. Cells were seeded in collagen I-coated plates maintained in culture at 37°C with 5% CO<sub>2</sub> in DMEM/F12 medium (Gibco, cat# 11320-033) containing 5% fetal calf serum, penicillin/streptomycin antibiotic, the ITS supplement of 0.5% insulin, transferrin, selenium (Sigma-Aldrich), and 5 nM dexamethasone. Cells were analyzed at passages one to three.

### Transfections and gene silencing

Human *HEY1* promoter fragment (full-length FL, 3.9 kb) isolated from human BAC clone RP11-27N21 was subcloned into pGL3 luciferase construct (Promega). Deletion mutants M1-M7 were generated by sequential restriction digests of FL. The NICD expression construct was a kind gift of Dr R Kopan. Dominant-negative clones of TbrII (TbrII-DN K277R), Smad2 (Smad2 3S $\rightarrow$ A, serine residues of SSXS motif changed to alanine), Smad3 (Smad3  $\Delta$ exon8), and Smad4 (Smad4  $\Delta$ M4) were provided by Dr E Piek, and the 3TPlux construct was a gift of Dr J Wrana. Antisense oligonucleotides against human *HEY1* were designed as previously described (Henderson *et al*, 2001). Transfections were carried out in triplicate for using Superfect reagent (Qiagen) and 3  $\mu$ g of total plasmid DNA per 4  $\times$  10<sup>5</sup> cells seeded. TGF- $\beta$ -induced versus uninduced (baseline control) triplicate cultures of identical transfection background were compared in all transfection experiments. The target sequence of the JAG1 siRNA duplex (Dharmacon Research) was AAGGUGUGUGGGCCUCGGGU. High-efficiency (>90% cells) oligonucleotide and siRNA transfections were carried out in growing cells at 50–60% confluence using the recommended protocol for Oligofectamine (Life Technologies, Invitrogen). In all, 4  $\mu$ M anti-HEY1 oligonucleotide (AS) or 200 nM anti-JAG1 siRNA concentrations were used. The cells were recovered post-transfection by overnight culture in 10% serum, then serum deprived for 24 h (0.2% serum) and stimulated with TGF- $\beta$ . The efficiency of transfection in RNAi experiments was monitored by using Cy3-labelled siRNA duplex of GL2 luciferase sequence (Dharmacon Research).

### Quantitative RT-PCR

Total RNA was prepared using TRIZOL reagent, and reverse transcribed with SuperScript II reverse transcriptase (Life Technologies, Invitrogen). The cDNA was amplified using SYBR-Green PCR Master Mix (Applied Biosystems) and gene-specific exon-exon junction spanning primers (sequences available upon request) in ABI 7900HT sequence detection system (Applied Biosystems) or iCycler (BioRad). Normalizations across samples were performed using geometric average of constitutive gene expression of *GLUD1*, *HPRT1*, and *B2M*.

### Protein extraction

Whole-cell protein extracts from keratinocyte cultures were prepared as described (Zavadil *et al*, 2001). For cytoplasmic/nuclear cell fractionation, the cells were lysed in 300  $\mu$ l of sucrose buffer per 100 mm dish (0.32 M sucrose, 10 mM Tris-HCl pH 8.0, 3 mM CaCl<sub>2</sub>, 2 mM magnesium acetate, 0.1 mM EDTA, 0.5% NP-40), and cytoplasmic fraction was cleared by centrifugation (2500 rpm, 5 min). The nuclei were washed twice with sucrose buffer and resuspended in 40  $\mu$ l of low-salt buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl<sub>2</sub>, 20 mM KCl, 0.2 mM EDTA, 25% glycerol, 1% NP-40 (v/v)) and mixed with 40  $\mu$ l of high-salt buffer (low-salt buffer with 800 mM KCl) and incubated for 40 min at 4°C. Nuclear protein extracts were cleared by centrifugation (13 000 rpm, 20 min).

### Quantitative chromatin immunoprecipitation

The formaldehyde-crosslinked protein-DNA complexes in the cell protein lysates were sonicated to the size range of 30–500 bp using Fisher Sonic Dismembrator 500 with a Branson 450 microtip, submerged in the mixture of dry ice in methanol. Protein-DNA complexes were precleared and immunoprecipitated using protein A/G agarose beads (Santa Cruz) and specific antibodies or isotype controls, reverse crosslinked by proteinase K treatment and overnight incubation at 65°C. The enrichment of specific promoter DNA contents was measured by quantitative RT-PCR (primer sequences available upon request) and adjusted relative to background contents of unrelated promoter sequences, to immunoprecipitation by isotype IgG controls and to the relative contents of target sequences in the initial sonicated chromatin input.

### Electrophoretic mobility shift assay

In all, 2.5 pmol of <sup>32</sup>P-labelled oligonucleotides were incubated with 15  $\mu$ g of nuclear proteins in binding buffer (4% glycerol, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl pH 7.5, 0.1 mg/ml dl-dC) for 30 min at room temperature. For supershift analysis, the nuclear proteins were preincubated for 2 h at 4°C with 3  $\mu$ g of anti-Smad3 antibody (Santa Cruz, sc-6202X) or 3  $\mu$ g of nonrelated goat IgG. Protein-DNA complexes were resolved on 5% polyacrylamide gel in Tris-borate buffer.

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### Immunodetection of proteins

Immunohistochemical and immunoblot analyses were performed as described (Zavadil *et al*, 2001), using anti-poly (ADP-ribose) polymerase (anti-PARP) (Enzyme systems, C2.10), anti- $\beta$  tubulin (Sigma, T-4026), anti-Notch1 (Santa Cruz, sc-6014), anti-HEY1 (Santa Cruz, sc-16424), anti-Jagged1 (Santa Cruz, sc-6011), anti-Smad2 (Santa Cruz, sc-6200), and anti-Smad3 (Santa Cruz, sc-6202X and ZYMED, 51-1500). Phosphospecific anti-Smad2 serum was kindly provided by Dr P ten Dijke. The Q-ChIP immunoprecipitations were performed using anti-Smad3 (FL-425, Santa Cruz), anti-Smad2/3 (Upstate Biotechnology), anti-Smad4 (C-20, Santa Cruz), and anti-Smad4 (Upstate Biotechnology).

### Cell motility

Confluent serum-deprived monolayer cultures of HaCaT keratinocytes transfected with AS or S oligonucleotides were 'wounded' by scratching the layer using 200  $\mu$ l pipet tip in the presence or absence of TGF- $\beta$  and monitored by phase contrast microscope photography as described (Cano *et al*, 2000). Motility of the cells was quantified by 30 measurements in each experimental category (AS versus S) and expressed as a percentage of distance coverage by cells moving into the scratch wound area 24 and 48 h after wounding. Student's *t*-test was applied to assess the significance of the AS versus S effect on cell motility.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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