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Functional Notch signaling is required for BMP4-induced inhibition of myogenic differentiation

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Summary

The bone morphogenetic protein (BMP) and Notch signaling pathways are crucial for cellular differentiation. In many cases, the two pathways act similarly; for example, to inhibit myogenic differentiation. It is not known whether this inhibition is caused by distinct mechanisms or by an interplay between Notch and BMP signaling. Here we demonstrate that functional Notch signaling is required for BMP4-mediated block of differentiation of muscle stem cells, i.e. satellite cells and the myogenic cell line C2C12. Addition of BMP4 during induction of differentiation dramatically reduced the number of differentiated satellite and C2C12 cells. Differentiation was substantially restored in BMP4-treated cultures by blocking Notch signaling using either the γ-secretase inhibitor L-685,458 or by introduction of a dominant-negative version of the Notch signal mediator CSL. BMP4 addition to C2C12 cells increased transcription of two immediate Notch responsive genes, *Hes1* and *Hey1*, an effect that was abrogated by L-685,458. A 3 kb *Hey1*-promoter reporter construct was synergistically activated by the Notch 1 intracellular domain (Notch 1 ICD) and BMP4. The BMP4 mediator SMAD1 mimicked BMP activation of the *Hey1* promoter. A synthetic Notch-responsive promoter containing no SMAD1 binding sites responded to SMAD1, indicating that DNA-binding activity of SMAD1 is not required for activation. Accordingly, Notch 1 ICD and SMAD1 interacted in binding experiments in vitro. Thus, the data presented here provide evidence for a direct interaction between the Notch and BMP signaling pathways, and indicate that Notch has a crucial role in the execution of certain aspects of BMP-mediated differentiation control.

Supplemental data available online

Key words: SMAD, Delta, Serrate, TGFβ, skeletal muscle, γ-secretase inhibitor

Introduction

Notch signaling is an evolutionarily well-conserved system for cell-cell communication (Artavanis-Tsakonas et al., 1999). Perturbation of Notch signaling leads to dramatic effects for the differentiation of many organs, including muscle, the central nervous system, pancreas and the vascular system (Frisen and Lendahl, 2001; Zhong et al., 2000). The Notch receptor is a single transmembrane spanning protein that receives signals from cell-bound ligands of the Delta or Serrate type, and thus functions by direct cell-cell contact. The Notch receptor undergoes a complex series of proteolytic processing events that lead to the release of the intracellular domain of the receptor (Notch ICD) (for a review, see Ebinu and Yankner, 2002). Presenilins are important for the final proteolytic cleavage liberating the Notch ICD, and this cleavage can be blocked by γ-secretase inhibitors (Berezovska et al., 2000; Karlstrom et al., 2002). Notch ICD translocates to the nucleus, where it interacts with the DNA-binding protein CSL [also referred to as RBP-Jκ (Furukawa et al., 1992)] to regulate the transcription of target genes (Jarriault et al., 1995). The key downstream genes are the *Hes* and *Hey* genes encoding related basic helix-loop-helix transcription regulators (Iso et al., 2001; Jarriault et al., 1995; Maier and Gessler, 2000; Nakagawa et al., 2000).

It is an emerging concept that the response of a cell to extrinsic signals relies not only on the effect of a particular signaling pathway, but on the integration of signals from multiple pathways. This enables the cell to respond to a more complex repertoire of signals, and to integrate this information into the large number of physiological responses a cell can elicit. Despite the importance of Notch signaling for proper cellular differentiation in many tissues, little is known about the interaction of the Notch signaling pathway with other major signaling pathways. To begin to address this, we investigated the possibility of a signal integration between the Notch and BMP signaling pathways. The underlying rationale for this was that both Notch and BMP signaling block differentiation of certain cell types, including myogenic cells (Kopan et al., 1994; Takahashi et al., 1994). We wished to explore whether this differentiation block was mediated by distinct mechanisms or through cross-talk between the two pathways.

BMP is a member of the TGFβ superfamily of ligands and can elicit a large variety of cellular responses (Attisano and Wrana, 2002). In the case of BMP-mediated signaling, the ligand binds to a type II receptor, which phosphorylates the type I receptor in a heterotetrameric receptor complex at the plasma membrane (Attisano and Wrana, 2002). This leads to

phosphorylation of the cytoplasmic protein SMAD1, which is referred to as a receptor-regulated SMAD (R-SMAD). SMAD1, together with another SMAD protein (co-SMAD), SMAD4, translocates to the nucleus, where it controls the regulation of specific target genes (Attisano and Wrana, 2002; Miyazawa et al., 2002). SMADs bind DNA with low affinity and are thought to recruit tissue-specific factors to enhance DNA-binding and regulate cellular events. SMAD1 binds to GC-rich stretches in promoter sequences (Kusanagi et al., 2000). SMAD proteins are composed of two conserved domains, MH1 and MH2, which are separated by a linker sequence. SMADs have been shown to bind a number of proteins in the nucleus, including general transcription factors, co-activators and co-repressors (Miyazawa et al., 2002). The combination of bound factors influences DNA-binding specificity and the intensity of the transcriptional activation, which indicates that SMADs are crucial for signal integration. BMP signaling shares some principle features with Notch signaling, particularly that the transmission of the signal from the exterior of the cell involves only a few intermediates and requires the relocation of a signaling component from the cytoplasm to the nucleus. Furthermore, some of the factors important in modulating SMAD signaling, such as p300 and P/CAF, are also key proteins for regulating Notch signaling (Janknecht et al., 1998; Moustakas et al., 2001; Wallberg et al., 2002).

It has previously been demonstrated that both addition of BMP (Katagiri et al., 1994) and ligand induction of Notch (Kopan et al., 1994; Kuroda et al., 1999) cause a dramatic block in myotube formation in the myogenic cell line C2C12. In this report, we have addressed whether BMP- and Notch-mediated differentiation inhibition are distinct events, or whether they are in some way linked.

Materials and methods

Transient transfection and constructs

COS-7, C2C12 or 293T cells were transfected using the FuGENE6 reagent (ROCHE), according to the manufacturer's instructions. Each transfection contained 400 ng plasmid consisting of CMV-lacZ (50 ng), reporter plasmid (MH100, pHey1, 12×CSL; 100 ng) and various amounts of expression plasmids plus mock plasmid. Each experiment was repeated three times and a representative sample is shown. The plasmid constructs are described in the supplementary material available online (see Data S1 at http://dev.biologists.org/supplemental/).

RNA extraction, cDNA synthesis and quantitative PCR

RNA was extracted using RNeasy Miniprep (Qiagen), according to the manufacturer's instructions. For cDNA synthesis, 10 μl of RNA, together with 1 μl oligodT and 1 μl 10 mM dNTPs, was incubated at 65°C for 5 minutes and then chilled on ice. This was followed by the addition of 4 μl First Strand buffer, 2 μl 0.1 M DTT and 1 μl RNaseOut, and incubation at 42°C for 2 minutes. SuperscriptII (Invitrogen) was added and the reaction further incubated at 42°C for 50 minutes. The reaction was stopped by heat inactivation at 70°C for 15 minutes. Quantitative PCR was performed in accordance with the manufacturer's instructions, using a LightCycler rapid thermal cycler system (Applied Biosystems). A mastermix containing nucleotides, Taq polymerase, SYBR Green and buffer was mixed with primers and cDNA. A description of the primers used can be found online (see Data S2 at http://dev.biologists.org/supplemental/).

Differentiation assay

C2C12 cells were seeded at high density on gelatin-coated glass, transfected with the indicated constructs (2 μg/well in a 6-well plate) and incubated in differentiation medium (2% horse serum) for 2-6 days. Satellite cells were seeded onto pre-coated (Fibronectin) plates and subjected to similar differentiation conditions.

γ-secretase inhibitor treatment and ligand stimulation

L-685,458 (Bachem) was added to the cells for 1-12 hours at a concentration of 4 μM. For the differentiation assay, a concentration of 1 μM was used and the compounds were added fresh everyday. BMP4 (R&D) was added to the cells at a concentration of 25 or 50 ng/ml.

In vitro binding assays and western blot

GST-fusion proteins were produced in *E. coli* and purified on glutathione-conjugated beads (Pharmacia). The fusion proteins were incubated with cell lysates from cells transfected with the indicated plasmids overnight. Immunoprecipitation and westerns blots are described in the supplementary material available online (see Data S3 at http://dev.biologists.org/supplemental/).

Immunocytochemistry

C2C12 cells were fixed for 1-3 minutes in 2% paraformaldehyde, blocked for 20 minutes in blocking solution (5% BSA, 0.3% Triton X-100 and 10% goat serum in PBS) and incubated with primary antibody in blocking solution for 1 hour. The cells were extensively washed in PBS and incubated in the dark with secondary antibody for 40 minutes. Cells were then mounted in ProLong mounting medium (Molecular Probes). Primary antibodies were rabbit anti-Myc (Santa Cruz; diluted 1:20), rabbit anti-β-gal (ICN; diluted 1:200) and/or mouse anti-myosin heavy chain (MHC) (MF20, diluted 1:15; obtained from the Developmental Studies Hybridoma Bank). Secondary antibodies were goat anti-rabbit Alexa 488 and goat anti-mouse Alexa 546 (Molecular Probes).

Results

BMP4-induced inhibition of C2C12 differentiation requires functional Notch signaling

To investigate the possible interplay between BMP and Notch signaling during myogenic differentiation we used the myogenic C2C12 cell line. In culture, C2C12 cells differentiate 2-6 days after the addition of differentiation medium (Bains et al., 1984) and express the myosin heavy chain (MHC) protein, which identifies terminally differentiated myocytes (Fig. 1A). Typically 20% of the C2C12 cells are differentiated after 5 days in medium containing low serum. We show that the addition of 50 ng/ml BMP4 to confluent C2C12 cells during differentiation reduced the number of differentiated cells to 0.4% (Fig. 2, see table at top; Fig. 1A), which is in keeping with a previous report (Katagiri et al., 1994). To test the effect of blocking Notch signaling, we added the γ-secretase inhibitor L-685,458, which inhibits the final cleavage of the Notch receptor, and thus hinders Notch intracellular domain release (Karlstrom et al., 2002). Addition of L-685,458 to C2C12 cells results in an approximately 30% increase (from 21% to 27%) in the number of MHC-positive cells relative to non-treated cells (Fig. 2, table; Fig. 1A), which was as expected if low level endogenous Notch signaling was blocked. When L-685,458 was added to BMP4-stimulated cells, the differentiation block induced by BMP was substantially reversed and the number of MHC-positive cells increased 18-fold (from 0.4% to 7.3%) compared with when BMP alone was added (Fig. 2, table; Fig.

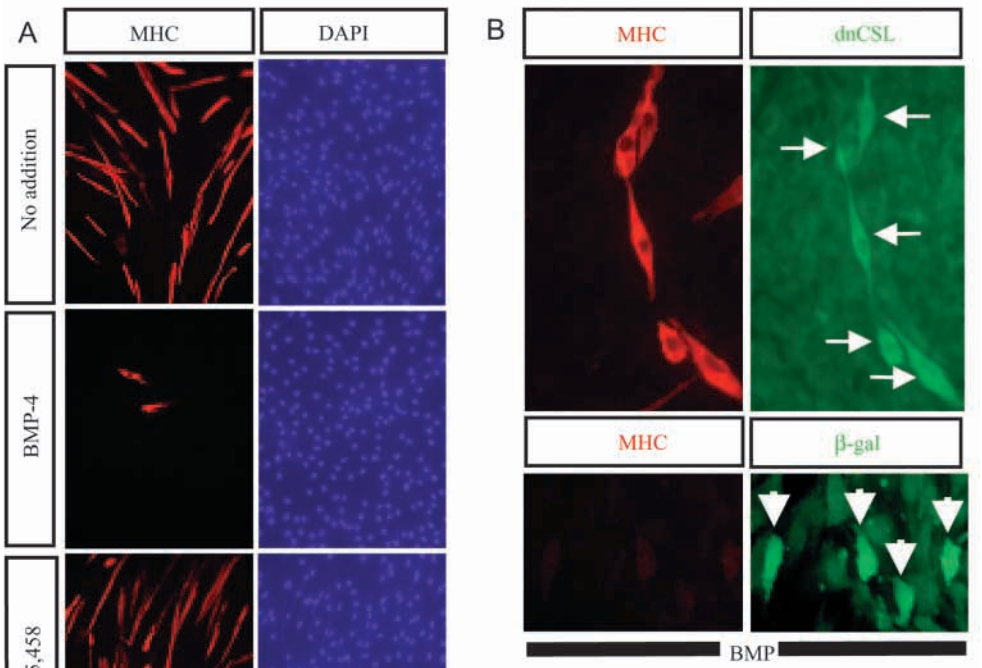

Fig. 1. Effects of BMP4 and Notch signaling on C2C12 differentiation. (A) C2C12 cells were analyzed 6 days post-addition of differentiation medium, and after BMP4 (50 ng/ml) and/or γ-secretase inhibitor (L-685,458; 1 μM) treatment. Myocyte differentiation was visualized by immunostaining for MHC (red; left panel) and DAPI staining (blue; right panel) was used to identify cell nuclei. Note that a considerable proportion of the cells were MHC-positive under normal differentiation conditions (no addition), and that the addition of BMP4 strongly reduced differentiation (BMP4). Treatment with L-685,458 (L-685,458) led to an increase in the number of differentiated cells compared with treatment with differentiation medium alone, and the combined treatment with BMP4 and L-685,458 (L-685,458 + BMP4) resulted in an increase in the number of MHC-positive cells, as compared with treatment with BMP4 alone. (B) C2C12 cells were analyzed for MHC expression 2 days post-addition of differentiation medium in the presence of BMP4 (50 ng/ml), and after transfection of CSL R218H (upper panel) or β-gal (lower panel).

1A). This strongly suggests that the BMP-mediated block of differentiation is, at least in part, mediated through Notch signaling, but it does not formally exclude the possibility that other proteins, whose processing is controlled by presenilins (Haass and Steiner, 2002), could mediate the effect. To inhibit Notch signaling in an independent manner, we transfected a dominant-negative version of the CSL protein (R218H) into the C2C12 cells. R218H CSL is thought to block the activation of genes downstream of Notch by forming a complex with Notch ICD that cannot bind to the promoter (Chung et al., 1994; Wettstein et al., 1997). We observed that 75% of R218H CSL-expressing C2C12 cells differentiated following addition of BMP4 (Fig. 1B, arrows), whereas only 4% of control (CMV-β-gal) transfected cells were MHC-positive. This demonstrates that the BMP-mediated block of myogenic differentiation in C2C12 cells requires functional Notch signaling.

We next tested whether this was also the case in primary muscle stem cells, so called satellite cells. Similar to C2C12 cells, addition of BMP4 led to a pronounced reduction in differentiation, from 21% to 3% MHC-positive mouse satellite cells (Fig. 2, table). Addition of L-685,458 had a dramatic effect with an increase in differentiation to 45% (Fig. 2, table). The combined addition of L-685,458 and BMP4 reversed the differentiation block exhibited by BMP4 alone, as 17% of the cells expressed MHC (Fig. 2, table).

BMP4 increases expression of genes downstream of Notch in C2C12 cells in a Notch-dependent manner

We next addressed whether BMP4 addition also induced expression of genes immediately downstream of Notch. To this end, we analyzed changes in expression levels of *Hes1* and *Hey1* following exposure to BMP4, and/or addition of L-685,458, using quantitative PCR. L-685,458 was added to the cells 12 hours before BMP4 to ensure that already cleaved Notch ICD was degraded prior to BMP stimulation. Addition of only L-685,458 to C2C12 cells led to a small decrease in the levels of both *Hes1* and *Hey1* mRNA (Fig. 2A,B), presumably by blocking low level endogenous Notch signaling. Addition of BMP4 led to a 2.8- and 7-fold increase in the levels of *Hes1* and *Hey1* mRNA, respectively (Fig. 2A,B). This increase was largely eliminated in cells simultaneously treated with BMP4 and L-685,458 (Fig. 2A,B). These data demonstrate that BMP4 increases the expression levels of the *Hey1* and *Hes1* genes in a Notch-dependent manner. We also tested whether the BMP induction could be observed at later stages in the differentiation process. *Hes1* and, in particular, *Hey1* expression in C2C12 cells was elevated at 5 days of differentiation in response to BMP4, whereas the expression levels under normal conditions were relatively similar (Fig. 2C). To rule out the possibility that L-685,458 affected BMP target genes by a more general mechanism not related to cleavage of the Notch receptor, we performed quantitative PCR on *Runx2*, which is a BMP4 target gene (Tsuji et al., 1998) that is known to be expressed in C2C12 cells. *Runx2* expression was moderately increased by BMP4, but L-685,458 did not alter *Runx2* levels (Fig. 2D). By contrast, *Hey1* expression was substantially increased and was blocked by L-685,458 in the same experiment (Fig. 2D).

To determine whether BMP also regulated transcription factors important for myogenesis, we analysed the effect of BMP4 stimulation on *MyoD* expression. First, we established the protein expression profiles of MyoD and MHC during

		No addition	L-685,458	BMP-4	BMP-4+ L-685,458	
Satellite	MHC-positive	91	231	16	108	α–MHC
	Total (DAPI-stained)	434	511	534	636	
	%	21	45	3	17	
C2C12	MHC-positive	87	104	3	27	α–MHC
	Total (DAPI-stained)	407	379	723	372	
	%	21	27	0.4	7.3	

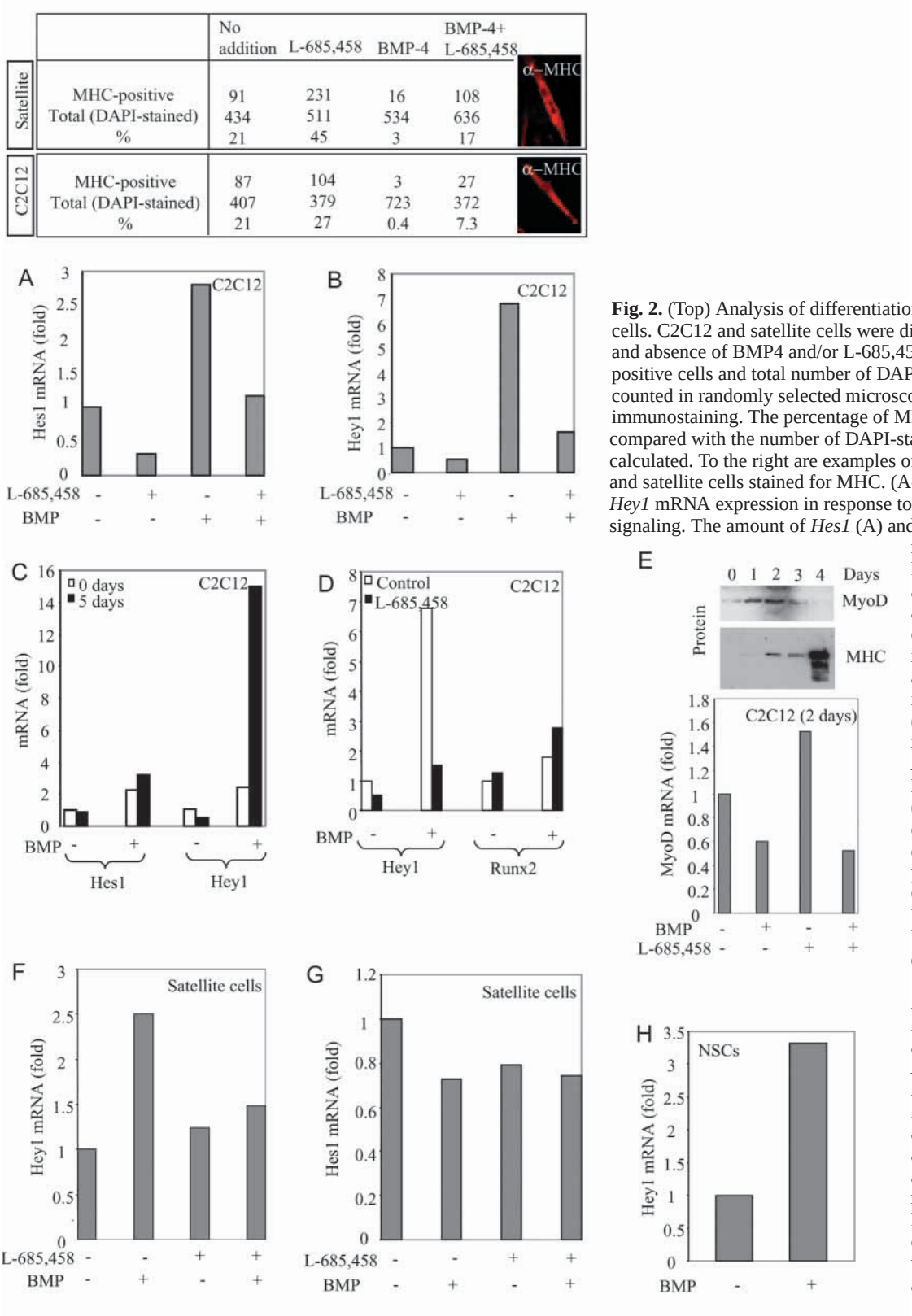

Fig. 2. (Top) Analysis of differentiation of C2C12 and satellite cells. C2C12 and satellite cells were differentiated in the presence and absence of BMP4 and/or L-685,458. The number of MHC-positive cells and total number of DAPI-positive cell nuclei were counted in randomly selected microscope fields after immunostaining. The percentage of MHC-positive cells compared with the number of DAPI-stained nuclei was calculated. To the right are examples of multinucleated C2C12 and satellite cells stained for MHC. (A-H) Analysis of *Hes1* and *Hey1* mRNA expression in response to BMP4 and Notch signaling. The amount of *Hes1* (A) and *Hey1* (B) mRNA, as measured by quantitative PCR, in C2C12 cells after addition of L-685,458 and/or BMP4. (C) An experiment similar to that in A and B, but measured at both 0 and 5 days after induction of differentiation. (D) Comparison of changes in expression of *Hey1* and *Runx2* in response to BMP4 and/or L-685,458. (E) Western blot analysis of MHC and MyoD protein expression at various time points after induction of C2C12 differentiation. Below is a quantitative PCR experiment demonstrating changes in *MyoD* expression in response to BMP4 and/or L-685,458. (F,G) The amount of *Hey1* (F) and *Hes1* (G) mRNA, as measured by quantitative PCR, in satellite cells after addition of L-685,458 and/or BMP4. (H) The amount of *Hey1* mRNA, as measured by quantitative PCR, in neural stem cells cultured as neurospheres under normal conditions or after addition of BMP4.

C2C12 differentiation by western blot analysis (Fig. 2E). In keeping with previous data (Dedieu et al., 2002), the expression of *MyoD* increased during the early phases of differentiation and reached a maximum two days after induction of differentiation (Fig. 2E). By contrast, MHC expression was seen first at two days after induction of differentiation and then increased to higher levels (Fig. 2E). We therefore analysed the regulation of *MyoD* by BMP4 after two days of differentiation, i.e. when *MyoD* expression should be maximal. Stimulation by BMP4 reduced *MyoD* mRNA

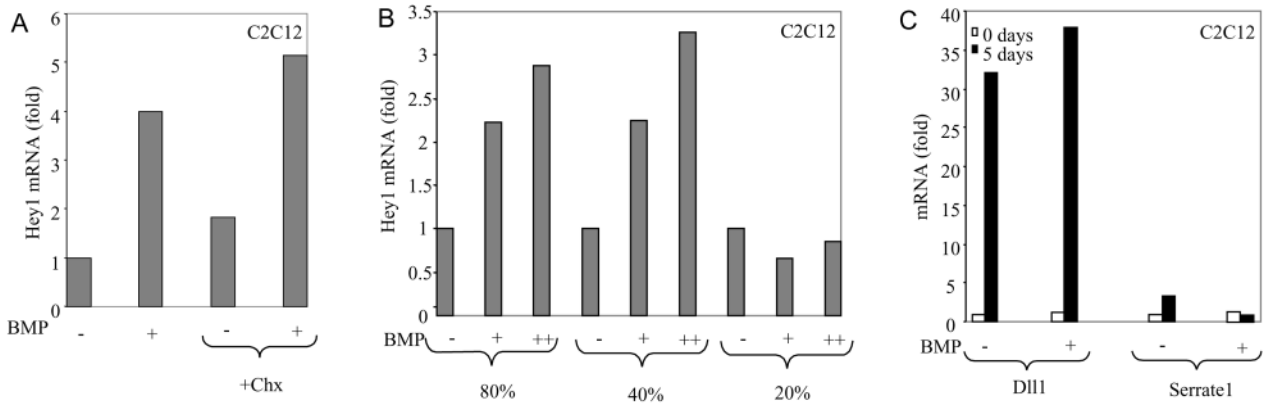

Fig. 3. The effect of Notch and BMP cross-talk is direct and requires cell-cell contact. (A) Quantitative PCR of *Hey1* mRNA expression in C2C12 cells exposed to BMP4 and/or cycloheximide (Chx). (B) Quantitative PCR of *Hey1* mRNA expression in C2C12 cells cultured at different densities (20, 40 or 80% confluent) and exposed to various concentrations of BMP4 [25 (+) or 50 (++) ng/ml BMP4]. (C) The amount of *Dll1* and *Serrate 1* mRNA, as measured by quantitative PCR, in C2C12 cells after addition BMP4.

expression, whereas L-685,458 significantly increased the level of expression (Fig. 2E). Simultaneous treatment by BMP4 and L-685,458 reduced *MyoD* expression to the same level as BMP4 alone (Fig. 2E). This suggests that BMP4 and Notch signaling can suppress expression of the myogenic transcription factor MyoD, but that the effect of BMP4 on *MyoD*, in contrast to the regulation of *Hes1* and *Hey1*, may not depend on Notch signaling.

We next investigated the effect of BMP4 and L685,458 on *Hes1* and *Hey1* expression in satellite cells. *Hey1* expression was induced by BMP4 in the absence, but not in the presence, of L-685,458 (Fig. 2F), whereas *Hes1* expression was unaffected (Fig. 2G). To address the effect of BMP4 in the regulation of *Hey1* in another, non-myogenic, primary cell type, we analyzed neural stem cells isolated from the lateral ventricle of the adult mouse brain and cultured as neurospheres. In these cells *Hey1* expression was increased 3-fold following BMP4 stimulation (Fig. 2H). Collectively, these results indicate that *Hey1* is a target gene for Notch and BMP stimulation in the three cell types tested.

BMP4-mediated induction of *Hey1* is direct and requires cell-cell contact

The fact that BMP4 was added to cells only one hour prior to analysis suggests a direct upregulation of *Hey1* expression that does not require intermediate protein synthesis. To test this more thoroughly, we stimulated cells with BMP4 in the presence of cycloheximide, which blocks protein synthesis. *Hey1* mRNA levels were upregulated in the presence of BMP both in the absence and presence of cycloheximide (Fig. 3A), in keeping with a direct effect. To test whether normal ligand-induced Notch signaling was required in the C2C12 cells for BMP to exert an effect on *Hey1* expression, we tested the effect of culturing the C2C12 cells at various densities. Cell populations at high density would be in direct cell-cell contact and therefore could have active Notch signaling, whereas sparsely seeded cells could not. At densities when the majority of C2C12 cells are in contact (80 and 40% confluency) a robust increase in *Hey1* expression was observed in response to BMP4 (Fig. 3B). By contrast, when cells were grown at 20% density, i.e. when there are few cell contacts, *Hey1* expression did not increase after BMP4 stimulation (Fig. 3B). Although this suggests that endogenous Notch signaling in C2C12 cells, mediated through ligand activation, is important for the BMP effect, it remained a possibility that BMP4 simply increased the amount of Notch ligand to induce elevated *Hey1* levels.

We therefore tested whether BMP4 altered the levels of mRNA for the two most commonly analyzed Notch ligands, *Dll1* and *Serrate 1*. Expression was analyzed at 0 and 5 days after the induction of differentiation, and in the absence and presence of added BMP4 (Fig. 3C). We observed that the level of *Dll1* mRNA increases during the differentiation period, but that BMP4 has no effect on the level of the mRNA (Fig. 3C). *Serrate 1* mRNA expression was very low at all time points (Fig. 3C). It therefore seems unlikely that the observed activation of genes downstream of Notch by BMP4 occurs as a consequence of upregulating Notch ligand.

BMP and Notch 1 ICD synergistically activate a *Hey1* promoter reporter construct

As discussed above, *Hey1* was upregulated by Notch and BMP in cells of both muscle and neural origin, This observation, combined with the fact that *Hey1* has been suggested to be important for inhibition of muscle development (Sun et al., 2001), led us to concentrate on *Hey1* to explore the interplay between Notch and BMP in more detail. The *Hey1* promoter contains both CSL-binding sites and a GC-rich domain comprising six GCCGnCGC sequences that are putative SMAD1 binding sites (see below) (Kusanagi et al., 2000). By contrast, no such elements were found in the 0.4 kb of the *Hes1* promoter that was functional in Notch response assays (data not shown).

We first tested the response of a 3 kb *Hey1* promoter-luciferase construct (Hey1-luc) to BMP4 and Notch stimulation in C2C12 cells. Addition of BMP4 to Hey1-luc reporter transfected cells resulted in a 2-fold activation (Fig. 4A). Transfection of a BMP receptor-regulated SMAD, SMAD1, resulted in a similar increase (Fig. 4A), and this could be further increased by the addition of BMP4, suggesting that endogenous SMAD1 may be present in too small amounts in the cells to mediate a full BMP response. Transfection of Notch 1 ICD into C2C12 cells led to a small induction of the *Hey1*

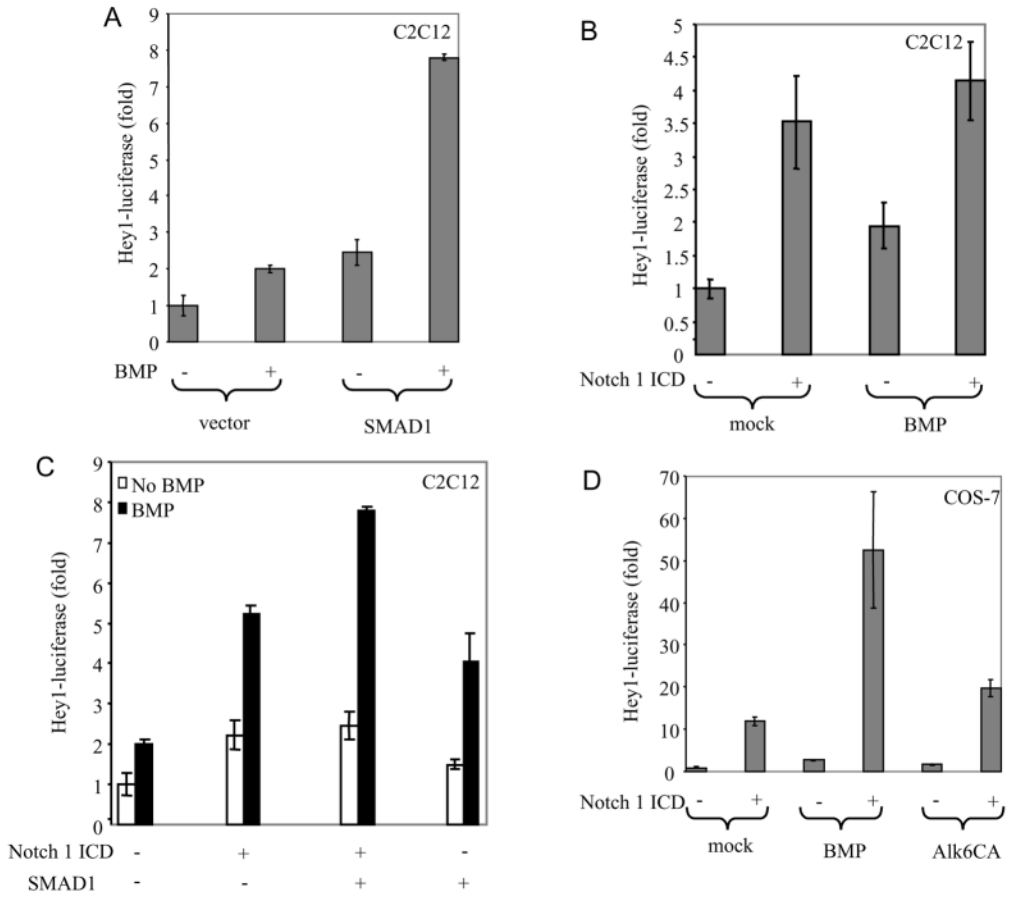

Fig. 4. BMP4 and Notch 1 ICD synergistically activate the *Hey1* promoter. (A) Transfection of C2C12 cells with SMAD1 in the absence (–) or presence (+) of exogenous BMP4 (50 ng/ml), measured by activation of a Hey1-luciferase reporter construct. Note the induction when SMAD1-transfected cells are exposed to BMP4. (B) Transfection with Notch 1 ICD combined with BMP4 stimulation (50 ng/ml). Note the effect of BMP4 on Notch 1 ICD-transfected cells. (C) Co-transfection with Notch 1 ICD and SMAD1, in the absence (white bars) or presence (black bars) of exogenous BMP4 (50 ng/ml). (D) Transfection of Cos-7 cells with Notch 1 ICD, combined with BMP4 stimulation (50 ng/ml) or transfection with Alk6CA.

reporter and addition of BMP4 further increased this (Fig. 4B). To test whether this increase could be enhanced with additional amounts of SMAD1, we transfected SMAD1 together with Notch 1 ICD in the presence or absence of BMP4. Introduction of SMAD1 together with Notch 1 ICD increased the transactivation about 8-fold in the presence of BMP4 (Fig. 4C). In the next set of experiments, we investigated the effects of Notch ICD and BMP4 signaling on the *Hey1* promoter in COS-7 cells. We chose COS-7 cells because the *Hey1* promoter can be robustly activated in this cell line (Nakagawa et al., 2000) and there is very little endogenous Notch signaling, which may provide an opportunity to see more pronounced effects in reporter gene activation. Transfection of Notch 1 ICD led to a 14-fold increase in activity, and this increase was elevated to 52-fold when Notch 1 ICD was introduced in cells exposed to BMP4 (Fig. 4D). Transfection of Notch 1 ICD together with a constitutively active form of the BMP type I receptor (Alk6CA) that mimics a ligand-activated BMP receptor (Moren et al., 2000) resulted in a 20-fold increase in activity (Fig. 4D). Taken together, these data indicate that stimulation of the BMP signaling pathway at three different levels, i.e. by ligand stimulation, by the constitutively activated Alk6CA receptor or by SMAD1, leads to increased *Hey1* promoter activity. Moreover, BMP addition can synergistically enhance the Notch ICD-mediated activation of the *Hey1* promoter.

To test whether the SMAD1 effect is dose-dependent, we transfected a fixed amount of Notch 1 ICD together with increasing amounts of SMAD1. This resulted in an increase in *Hey1* promoter activity proportional to the amount of SMAD1 in the presence (up to 200 ng SMAD1 plasmid) and absence (up to 300 ng SMAD1 plasmid) of exogenous BMP4 (Fig. 5A). To rule out the possibility that SMAD1 mediated the effect by stabilizing the Notch 1 ICD at the protein level, we determined the amounts of SMAD1 and Notch 1 ICD protein by western blot analysis from a similar experiment performed in the absence of exogenous BMP (Fig. 5B). The amount of SMAD1 protein increased proportionally to the concentration of SMAD1 plasmid used, whereas the levels of Notch 1 ICD remained approximately the same (Fig. 5B), which indicates that Notch 1 ICD protein levels are not affected by SMAD1 expression.

To investigate whether the SMAD1-dependent activation of the *Hey1* promoter requires the binding of Notch 1 ICD to CSL, we transfected the dominant-negative form of CSL R218H CSL with various combinations of Notch 1 ICD and SMAD1 (Fig. 5C). As expected R218H CSL reduced Notch 1 ICD-mediated activation in the presence and absence of exogenous BMP (Fig. 5C). However, R218H CSL did not reduce the effect mediated by SMAD1 alone (Fig. 5C). Cells transfected with both Notch 1 ICD and SMAD1 showed a pronounced activation of the *Hey1* promoter, in particular when BMP4 was added to the medium (Fig. 5C). This activation was strongly reduced by R218H CSL in the absence of exogenous BMP4 (Fig. 5C), but not in the presence of BMP4 (Fig. 5C). Collectively, these results suggest that at low levels of signaling the block of Notch signaling by a dominant-negative form of

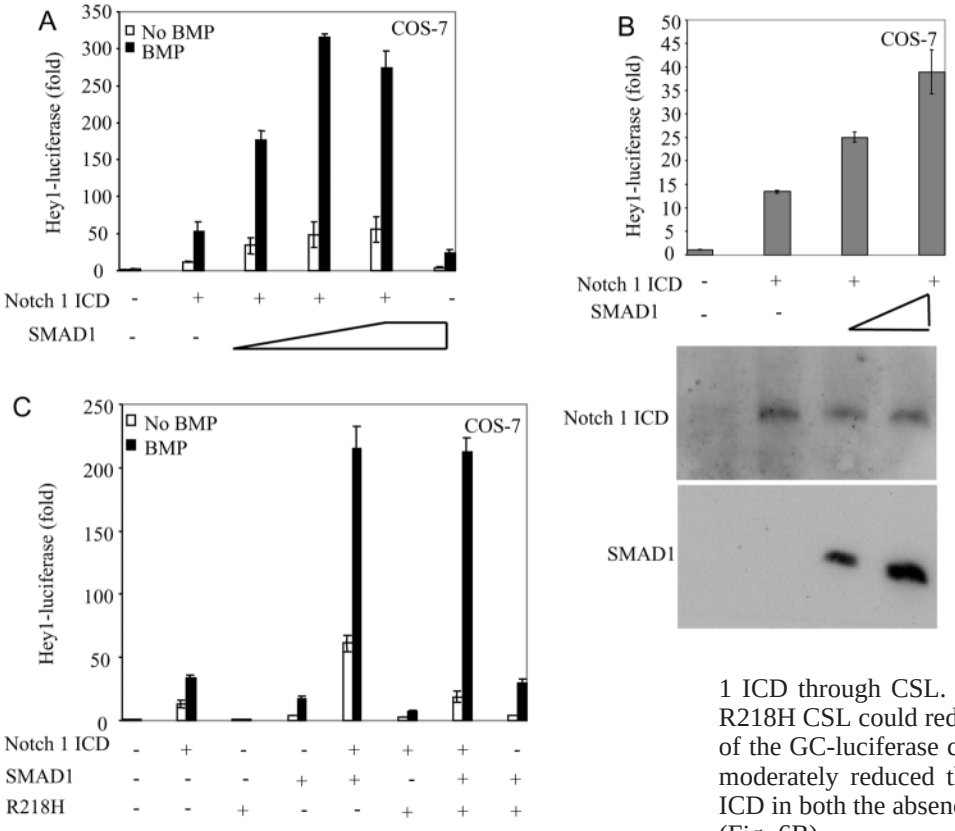

Fig. 5. The synergistic effect from Notch 1 ICD and SMAD1 is dose-dependent and partially CSL-dependent. (A) Transfection of Notch 1 ICD and variable amounts (100, 200 and 300 ng) of SMAD1 in COS-7 cells, in the absence (white bars) or presence (black bars) of exogenous BMP4 (50 ng/ml), measured by activation of a Hey1-luciferase reporter construct. (B) An experiment similar to that in A, carried out in the absence of exogenous BMP4, with 100 and 200 ng SMAD1. Shown below is a western blot for Notch 1 ICD and SMAD1. (C) Transfection of Notch 1 ICD (67 ng), SMAD1 (67 ng) and/or R218H CSL (67 ng) in COS-7 cells, in the absence (white bars) or presence (black bars) of exogenous BMP4 (50 ng/ml), measured by activation of a Hey1-luciferase reporter construct.

CSL is sufficient to reduce SMAD1-mediated activation, but that at higher levels of signaling this is not the case. This may indicate that SMAD1 acts both in a Notch-dependent and a Notch-independent manner to activate the *Hey1* promoter.

The GC-rich domain in the *Hey1* promoter is partially important for SMAD1 activation

To further test the notion that SMAD1 may act on the *Hey1* promoter in both a Notch-dependent and a Notch-independent manner, we analyzed different forms of the *Hey1* promoter for transcriptional activation. The 3 kb *Hey1* promoter used in the experiments described above contains a 500 bp GC-rich domain within 600 bp of the transcriptional start site (Fig. 6A). It has previously been shown that the sequence GCCGnCGC is a low-affinity binding site for SMAD1 (Kusanagi et al., 2000) and, as discussed above, the GC-rich domain contains six such sites (Maier and Gessler, 2000). There are also two bona fide CSL-binding sites in the promoter: one located within the GC-rich domain and another located immediately before the first codon of the *Hey1* gene (Fig. 6A). To discover whether the observed SMAD1-mediated effect on *Hey1* promoter activation is dependent on the GC-rich domain, we introduced a portion of the *Hey1* promoter containing five of the six GCCGnCGC motifs in front of the luciferase gene (referred to as GC-luciferase) (Fig. 6A). Transfection of the GC-luciferase construct into COS-7 cells resulted in a low level (5-fold) of activation by SMAD1 in the absence of exogenous BMP4 and a higher level (25-fold) if the cells were stimulated with BMP4 (Fig. 6B). A low level of induction was also observed with Notch 1 ICD alone (Fig. 6B), suggesting that the CSL-binding site in the GC-rich domain could bind Notch 1 ICD through CSL. This view is supported by the fact that R218H CSL could reduce the effect of Notch 1 ICD activation of the GC-luciferase construct. By contrast, R218H CSL only moderately reduced the activation by SMAD1 and Notch 1 ICD in both the absence and the presence of exogenous BMP4 (Fig. 6B).

We next tested the activity of a *Hey1* promoter construct lacking the GC-rich domain (Hey1-ΔGC, Fig. 6A), to investigate whether SMAD1 could exert an effect in the absence of five of the six putative SMAD1 binding sites. We observed that SMAD1 only marginally activated the ΔGC-reporter, whereas Notch 1 ICD was a more potent activator (Fig. 6C). The Notch 1 ICD response was augmented approximately 2-fold by the addition of SMAD1. This suggests that the GC-rich domain is of importance, but that it is not the sole determinant for activation by SMAD1.

SMAD1 increases transcription from promoters lacking SMAD1-binding sites

The SMAD1-mediated activation of the Hey1-ΔGC construct, from which most of the potential SMAD1 binding region had been removed, may suggest that SMAD1 exerts some effect without directly binding to DNA. To more stringently test this idea, we assessed in two different ways whether SMAD1 could mediate its effect on a promoter lacking SMAD1-binding sites. First, we used a previously established system with which a Gal4-Notch 1 ICD fusion has been shown to activate a reporter consisting of Gal4 binding sites and the luciferase gene (Beatus et al., 1999). Co-transfection of SMAD1 and Gal4-Notch 1 ICD led to increased transactivation, from 35-fold for Gal4-Notch 1 ICD alone, to approximately 175-fold (Fig. 7A). Addition of BMP4 further enhanced this increase (Fig. 7A). As the second approach, a minimal CSL-binding promoter construct composed of six dimeric CSL-binding sites (12×CSL-luc) (Kato et al., 1997) but lacking SMAD1-binding sites was used. Transfection of Notch 1 ICD resulted in a robust activation of the 12×CSL-luc construct (3000-fold) in the absence and presence of exogenous BMP (Fig. 7B), which is

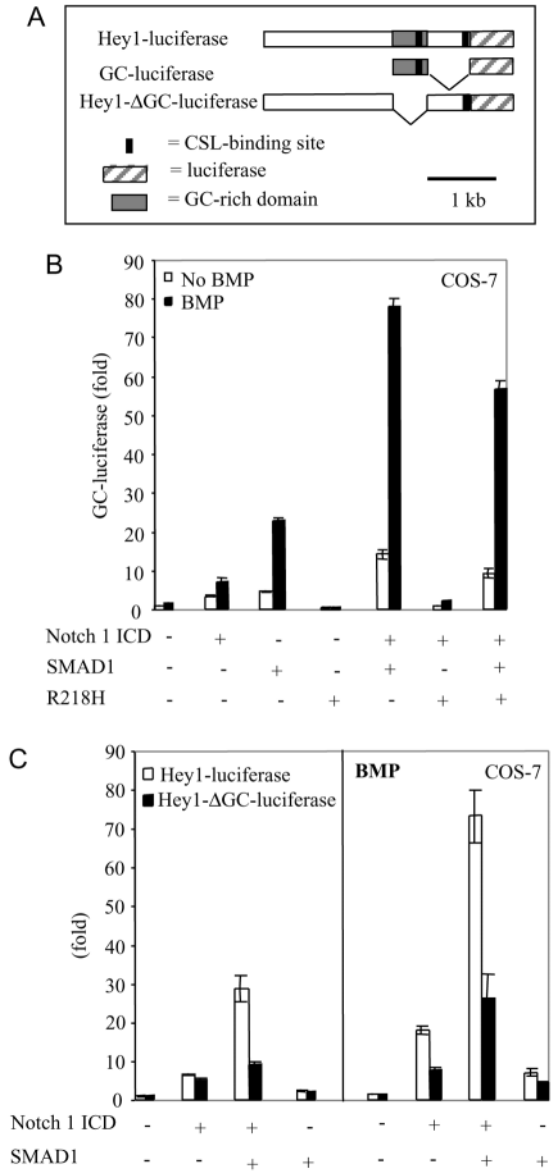

Fig. 6. Analysis of Notch 1 ICD and SMAD1-responsive elements in the *Hey1* promoter. (A) Schematic picture of the *Hey1* reporter construct with CSL-binding sites and the GC-rich domain (Hey1-luciferase), and the two mutants containing only the GC-rich domain (GC-luciferase) and comprising the *Hey1* promoter lacking the GC-rich domain (Hey1-ΔGC). (B) Transfection of Notch 1 ICD (67 ng), SMAD1 (67 ng) and/or R218H CSL (67 ng) in COS-7 cells, in the absence (white bars) or presence (black bars) of exogenous BMP4 (50 ng/ml), measured by activation of the GC-luciferase reporter construct. (C) Transfection of Notch 1 ICD (100 ng) and SMAD1 (100 ng) in COS-7 cells, in the absence (left) or presence (right) of exogenous (50 ng/ml) BMP4, measured by activation of Hey1-luciferase (white bars) or Hey1-ΔGC (black bars) reporter constructs.

in keeping with previous observations (Kato et al., 1997). SMAD1 alone did not increase transcription, but combined transfection of Notch 1 ICD and SMAD1 in the presence of BMP4 generated a 6000-fold increase in transcription, i.e. approximately twofold higher than for Notch 1 ICD alone (Fig. 7B). A similar increase was also observed in the absence of

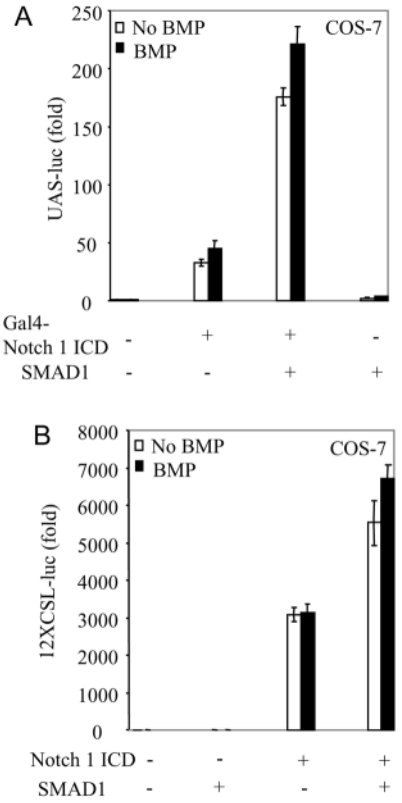

Fig. 7. SMAD1 potentiates Notch 1 ICD activation from promoters lacking SMAD1-binding sites. (A) Transfection of Gal4-Notch 1 ICD (100 ng) and SMAD1 (100 ng) in COS-7 cells, in the absence (white bars) or presence (black bars) of exogenous BMP4 (50 ng/ml), measured by activation of the UAS-luc reporter construct. (B) Transfection of Notch 1 ICD (100 ng) and SMAD1 (100 ng) in COS-7 cells, in the absence (white bars) or presence (black bars) of exogenous BMP4 (50 ng/ml), measured by activation of the 12×CSL-luc reporter construct.

exogenous BMP4. This demonstrates that SMAD1 can induce transcription from a Notch-responsive promoter that does not contain SMAD1-binding sites.

Interaction between SMAD1 and Notch 1 ICD

The lack of induction by SMAD1 alone, combined with the potentiation by SMAD1 of Notch1 ICD-mediated activation of the 12×CSL promoter and the Gal4 binding reporter (Fig. 7) led us to hypothesize that SMAD1 may interact with Notch 1 ICD, and may exert some of its effect through such an interaction. We performed GST-pulldown experiments in which GST-Notch 1 ICD was incubated with extracts from cells transfected with SMAD1. SMAD1 weakly interacted with GST-Notch 1 ICD but not with GST alone (Fig. 8A). The interaction was considerably weaker than the interaction between GST-Notch 1 ICD and CSL (Fig. 8A), which have been shown previously to interact strongly (Beatus et al., 1999). Co-immunoprecipitation experiments using COS-7 lysates from cells transfected with either Flag-tagged P/CAF, as a positive control, or SMAD1 together with Myc-tagged Notch 1 ICD, showed an equally strong interaction between SMAD1 and Notch 1 ICD as between P/CAF and Notch 1 ICD (Fig. 8B). These data suggest that there is an interaction

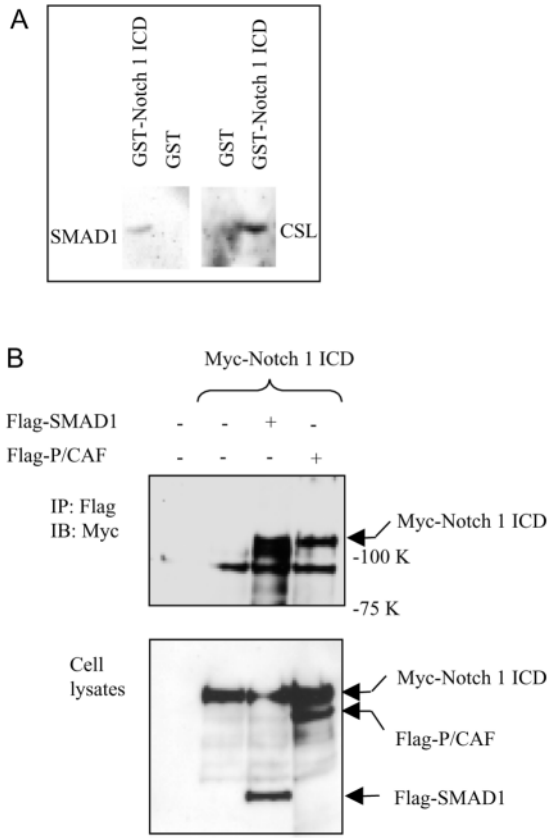

Fig. 8. Interaction between Notch 1 ICD and SMAD1. (A) Western blot of a pull-down with a GST-Notch 1 ICD fusion protein from lysates of cells transfected with CSL or SMAD1 proteins. The precipitated SMAD1 protein was visualized using a Flag antibody (the two lanes to the left), and the CSL protein using a Myc antibody (the two lanes to the right). Note that a small amount of SMAD1 was precipitated by GST-Notch 1 ICD, but not by GST.
(B) Immunoprecipitation from COS-7 cells transfected with Flag-tagged SMAD1 or Flag-tagged P/CAF in the presence of Myc-tagged Notch 1 ICD. Immunoprecipitation with an anti-Flag-antibody was followed by analysis by western blot using an anti-Myc antibody.

between SMAD1 and Notch 1 ICD, which potentially accounts for the ability of SMAD1 to activate a Notch responsive promoter that lacks SMAD1 binding sites.

Discussion

In this report, we provide evidence that the BMP4-mediated inhibition of myogenic differentiation of satellite and C2C12 cells, at least to some extent, requires functional Notch signaling. This was demonstrated by two independent means of blocking Notch signaling: inhibition of Notch receptor site 3 cleavage by the γ-secretase inhibitor L-685,458 and, in the case of C2C12 cells, by transfection of dominant-negative CSL. Both L-685,458 and R218H CSL were able to override the differentiation block by BMP4 and thus increase the number of MHC-expressing cells in the presence of BMP4. L-685,458 inhibits the activity of presenilins, which are pivotal for the site 3 proteolytic cleavage of the Notch receptor, and thus the release of the Notch ICD (Karlstrom et al., 2002). As presenilins control the cleavage of a number of proteins in addition to Notch, including N-cadherin, ERBB4 and CD44 (Ebinu and Yankner, 2002), it could be argued that L-685,458 is not exclusively specific for Notch signaling. However, R218H CSL, which produces a form of CSL that binds Notch ICD but does not produce an active complex on DNA (Chung et al., 1994; Wettstein et al., 1997), is most likely specific for the Notch signaling pathway. The observation that R218H CSL and L-685,458 caused C2C12 differentiation thus provides compelling evidence that the inhibitory effect of BMP4 on differentiation is alleviated, at least in part, by blocking Notch signaling. The ability of R218H CSL to promote differentiation of BMP4-stimulated C2C12 cells is also interesting in the light of previous data on CSL-dependent and -independent modes of myogenic differentiation following Notch stimulation. Activation of Notch receptors at least partly leads to a block of myogenic differentiation in a CSL-independent manner, as forms of Notch that do not bind CSL still inhibit C2C12 differentiation (Bush et al., 2001; Nofziger et al., 1999; Rusconi and Corbin, 1998; Shawber et al., 1996). However, our finding that the vast majority of BMP4-stimulated cells transfected with CSL R218H undergo differentiation indicates that the BMP-induced differentiation block is overridden predominantly in a CSL-dependent manner. This seems logical if the interaction between SMAD1 and Notch ICD (see below) is crucial, as the CSL-independent mode of Notch signaling does not require γ-cleavage of the Notch receptor, and presumably does not liberate Notch ICD (Bush et al., 2001).

Hey and Hes genes are important immediate downstream mediators of Notch signaling in many organs, including muscle and the vascular system (Donovan et al., 2002; Jarriault et al., 1995; Shawber et al., 1996; Zhong et al., 2000), and it is noteworthy that the BMP4-induced differentiation block in C2C12 cells coincides with increased expression of the two genes. The increase in both *Hes1* and *Hey1* was substantially reduced by L-685,458 in C2C12 cells, whereas satellite cells showed a BMP4-induced upregulation of *Hey1*, which was abrogated by L-685,458. This may indicate that *Hey1* plays an important role in maintaining myogenic cells in an undifferentiated state in response to the Notch-dependent BMP induction, but more work is needed to specifically address this issue.

To begin to decipher in more detail how Notch and BMP4 signals are integrated at a specific promoter, we analyzed the *Hey1* promoter. The BMP4 effect on *Hey1* transcription appears to be caused by canonical BMP signaling, as ligand stimulation, SMAD1 and a constitutively activated form of the receptor (Alk6CA) can activate the *Hey1* promoter. It seems likely that this activation is mediated both through the binding of SMAD1 to the promoter and through an interaction between SMAD1 and Notch ICD, an interaction not dependent on SMAD1 DNA binding. Evidence for SMAD1 binding to the *Hey1* promoter comes from the promoter deletion experiments, in which the GC-luciferase construct containing the GC-rich domain responded to SMAD1 activation. Furthermore, the Hey1-ΔGC and 12×CSL-luciferase constructs, which either have one putative SMAD1 binding site or which lack such sites, show little or no response to SMAD1 alone. Support for the view that SMAD1 activates transcription in a non-DNA-binding mode, and presumably through interaction with Notch ICD, comes from the experiments in which the 12×CSL-

luciferase construct was not activated by SMAD1 alone, but in which SMAD1 potentiated Notch ICD-induced activation 2-fold (Fig. 7B). Similarly, the activity of Gal4-Notch 1 ICD on an UAS-reporter gene construct was potentiated by SMAD1.

An interaction between Notch 1 ICD and SMAD1 was demonstrated by co-immunoprecipitation and GST-pulldown experiments. Co-immunoprecipitation revealed a robust interaction between SMAD1 and Notch 1 ICD. By contrast, the GST-pulldown experiments showed a weak interaction between Notch 1 ICD and SMAD1, when compared with, for example, the interaction between Notch 1 ICD and CSL. It should be noted that the GST-fused form of SMAD1 structurally mimics the phosphorylated form of SMAD1, thus eliminating the need for producing SMAD1 in mammalian cells to make it phosphorylated. The apparent differences in the strength of the interaction between the co-immunoprecipitation and GST-pulldown experiments raises the possibility that SMAD1 and Notch 1 ICD interact directly, but that the interaction may be stabilized by other Notch-interacting proteins, which may be more prominent under the co-immunoprecipitation conditions than under the GST-pulldown conditions. Precedence for such a stabilization comes from the Notch ICD and CSL interaction, where Maml or SKIP serve to strengthen the interaction (Wallberg et al., 2002; Wu et al., 2000; Zhou et al., 2000).

BMP signaling elicits a broad range of context-dependent cellular responses, despite an apparent simplicity in terms of proteins directly involved in the signaling cascade. The variety of different responses has, at least in part, been attributed to the ability of SMADs to act as signaling platforms through interactions with numerous proteins, such as p300/CBP, Runx2 and GATA3 (Bae et al., 2001; Blokzijl et al., 2002; Janknecht et al., 1998). The outcome of the different interactions of SMADs with other factors is context-dependent, where SMAD downstream genes are differentially regulated depending on cell type and/or BMP concentration. The data presented here widen the repertoire of BMP-inducible genes to include the Notch downstream genes *Hes1* and *Hey1*. The requirement for functional Notch signaling in this process, as well as for relieving the C2C12 and satellite cell differentiation block, brings a new facet to BMP signaling. Rather than acting as a strict integration platform, SMADs can also facilitate Notch signaling.

The Notch and BMP signaling pathways are evolutionarily highly conserved and influence differentiation processes in many organs. Skeletal muscle differentiation from somites is an example where BMP and Notch could co-operate in vivo. Absence of Notch results in enlarged clusters of mesodermal cells that behave as muscle founder cells (Corbin et al., 1991), and mice devoid of Notch 1 have disorganized somites (Conlon et al., 1995). Similarly, BMP4 acts in the lateral plate mesoderm to inhibit muscle formation (Pourquie et al., 1995; Pourquie et al., 1996). The proposed co-operation between Notch and BMP signaling may also extend to cell types other than muscle progenitors, such as neural stem cells. The anti-neurogenic effects of the two signaling pathways may act together to keep neural stem cells from differentiating into neurons.

In contrast to muscle and CNS development, in which Notch and BMP signaling appear to act largely synergistically, neural crest development provides an example of the two signaling pathways acting antagonistically. In neural crest stem cells, BMP induces neuronal differentiation, but this is irreversibly overridden by a short exposure of the cells to soluble Notch ligands (Morrison et al., 2000). It would be interesting to discover at what level Notch and BMP signaling interact in this case to produce an antagonistic effect. One possibility is that different SMAD combinations or the abundance of other SMAD-interacting proteins influence the differentiation outcome. Although much work remains to be done in order to understand these different interactions in vivo, this report provides the first evidence for signal integration between the Notch and BMP pathways, which may contribute to the ability of cells to decipher complex extracellular cues into meaningful responses.

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