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TGF- β uses a novel mode of receptor activation to phosphorylate SMAD1/5 and induce epithelial-to-mesenchymal transition

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Running title: The SMAD1/5 arm of TGF- β signaling

33 **Abstract**

34 The best characterized signaling pathway downstream of the transforming growth
35 factor β (TGF- β) pathway is through SMAD2 and SMAD3. However, TGF- β also
36 induces phosphorylation of SMAD1 and SMAD5, but the mechanism of this
37 phosphorylation and its functional relevance is not known. Here, we show that TGF-
38 β -induced SMAD1/5 phosphorylation requires members of two classes of type I
39 receptor, TGFBR1 and ACVR1, and establish a new paradigm for receptor activation
40 where TGFBR1 phosphorylates and activates ACVR1, which phosphorylates
41 SMAD1/5. We demonstrate the biological significance of this pathway by showing
42 that approximately a quarter of the TGF- β -induced transcriptome depends on
43 SMAD1/5 signaling, with major early transcriptional targets being the *ID* genes.
44 Finally, we show that TGF- β -induced epithelial-to-mesenchymal transition requires
45 signaling via both the SMAD3 and SMAD1/5 pathways, with SMAD1/5 signaling
46 being essential to induce ID1. Therefore, combinatorial signaling via both SMAD
47 pathways is essential for the full TGF- β -induced transcriptional program and
48 physiological responses.

49

50 **Introduction**

51

52 Members of the transforming growth factor β (TGF- β) family of ligands, which
53 includes the TGF- β s, Activins, NODAL, BMPs and GDFs, have pleiotropic effects on
54 cell behavior ranging from germ layer specification and patterning in embryonic
55 development, to tissue homeostasis and regeneration in adults (Massague, 2012,
56 Morikawa et al., 2016, Wu and Hill, 2009). TGF- β family signaling is also
57 deregulated in a number of human diseases through mutation or altered expression of
58 either the ligands or downstream signaling pathway components (Miller and Hill,
59 2016). In this context, the most widely studied pathology is cancer (Bellomo et al.,
60 2016, Massague, 2008, Meulmeester and Ten Dijke, 2011, Wakefield and Hill, 2013),
61 where TGF- β itself has both tumor suppressive and tumor promoting effects
62 (Massague, 2008). At early stages of cancer TGF- β 's tumor suppressive effects
63 dominate, such as its cytostatic and pro-apoptotic functions (Padua and Massague,
64 2009). As tumors develop, however, mutations in key components of the pathway or
65 downstream target genes allow the tumor to evade TGF- β 's tumor suppressive
66 effects, whilst remaining sensitive to its tumor promoting activities. TGF- β directly
67 promotes the oncogenic potential of tumor cells, for example by driving epithelial-to-
68 mesenchymal transition (EMT), a hallmark of cancer that enhances cell invasion and
69 migration, and also increases the frequency of tumor-initiating cancer stem cells
70 (Massague, 2008, Ye and Weinberg, 2015). TGF- β 's dual role in cancer thus provides
71 an excellent example of how diverse responses can be elicited by a single ligand.

72 The TGF- β family ligands all signal via a common mechanism, initiated by
73 ligand binding to two cell surface serine/threonine kinase receptors, the type II and
74 type I receptors. In the receptor complex, the type II receptors phosphorylate and
75 activate the type I receptors (Wrana et al., 1994). These in turn phosphorylate the
76 downstream effectors of the pathway, the receptor-regulated SMADs (R-SMADs) on
77 two serines in an SXS motif at their extreme C-terminus. Phosphorylated R-SMADs
78 form complexes with the common SMAD, SMAD4, which accumulate in the nucleus
79 and directly regulate the transcription of target genes, leading to new programs of
80 gene expression (Shi and Massague, 2003). In the classic view of TGF- β family
81 signaling there are two branches, characterized by distinct combinations of type II and
82 type I receptors, and the recruitment of specific R-SMADs to particular type I

83 receptors (Wakefield and Hill, 2013, Shi and Massague, 2003). One branch is
84 activated by TGF- β , Activins and NODAL and is mediated via the type I receptors
85 TGFBR1, ACVR1B and ACVR1C (also known as ALK5, ALK4 and ALK7
86 respectively), which phosphorylate SMAD2 and 3. The other is activated by BMPs
87 and GDFs, and is mediated via ACVRL1, ACVR1, BMPR1A and BMPR1B (also
88 known as ALK1, ALK2, ALK3 and ALK6 respectively), which phosphorylate
89 SMAD1, 5 and 9 (Miller and Hill, 2016).

90 In general, while this pairing between type I receptors and R-SMADs broadly
91 fits the assignment of specific ligands to the different branches of TGF- β family
92 signaling, it is an oversimplification. For example, ACVR1 is now described as a
93 BMP receptor, but early work indicated that it could bind Activin and TGF- β
94 (Massague, 1996, Miettinen et al., 1994), and very recently it has been shown to
95 signal downstream of Activin in the context of the disease, fibrodysplasia ossificans
96 progressiva (Hatsell et al., 2015, Hino et al., 2015). Furthermore, ACVRL1, a type I
97 receptor that recognizes BMP9 and 10, also transduces TGF- β signals in endothelial
98 cells (Pardali et al., 2010) by phosphorylating SMAD1/5 in parallel with the canonical
99 TGF- β -induced phosphorylation of SMAD2/3 (Goumans et al., 2002, Goumans et al.,
100 2003). This SMAD1/5 arm of TGF- β signaling has also been shown to occur in a
101 wide range of other cell types, including epithelial cells, fibroblasts and cancer cell
102 lines, which do not express ACVRL1 (Liu et al., 1998, Daly et al., 2008, Liu et al.,
103 2009, Wrighton et al., 2009).

104 Important questions concerning this noncanonical TGF- β -induced SMAD1/5
105 phosphorylation remain unanswered. First, the mechanism by which TGF- β induces
106 SMAD1/5 phosphorylation, in particular, the type I receptors involved, is not known.
107 Some studies have concluded that the canonical TGF- β receptors TGFBR1 and
108 TGFBR2 are sufficient for phosphorylation of both SMAD2/3 and SMAD1/5 (Liu et
109 al., 2009, Wrighton et al., 2009). In contrast, others demonstrated that one of the
110 classic BMP type I receptors (ACVR1 or BMPR1A), or in endothelial cells,
111 ACVRL1, is additionally required (Daly et al., 2008, Goumans et al., 2002, Goumans
112 et al., 2003). The second crucial issue concerns the biological relevance of TGF- β -
113 induced SMAD1/5 signaling. Nothing is known about the transcriptional program
114 activated by this arm of TGF- β signaling, or indeed, the specific SMAD complexes

115 involved. It is also not known to what extent this pathway is required for the
116 physiological responses to TGF- β .

117 Here we dissect the SMAD1/5 arm of TGF- β signaling and define the
118 underlying mechanism and its biological function. We show that TGF- β -induced
119 SMAD1/5 phosphorylation requires both TGFBR1 and ACVR1 and using biosensors,
120 and an optogenetic approach, we establish a new paradigm for TGF- β receptor
121 activation. We have mapped the binding sites on chromatin of nuclear phosphorylated
122 SMAD1/5 (pSMAD1/5) genome-wide, which led us to define the target genes
123 regulated by this arm of TGF- β signaling. We go on to show that this arm of signaling
124 is required for TGF- β -induced EMT. Our data reveal that the full transcriptional
125 programme activated in response to TGF- β requires integrated combinatorial
126 signaling via both the SMAD2/3 and SMAD1/5 pathways.

127

128 **Results**

129

130 **The kinetics of TGF- β -mediated SMAD1/5 phosphorylation**

131 To begin to dissect which receptors are required for TGF- β -induced SMAD1/5
132 phosphorylation, we compared the kinetics of SMAD1/5 and SMAD2
133 phosphorylation in response to TGF- β . Using the human breast cancer cell line MDA-
134 MB-231 and the mouse mammary epithelial cell line NMuMG as model systems we
135 found that TGF- β induced only transient phosphorylation of SMAD1/5 that peaked at
136 1 hr and then returned to baseline (Figure 1A). This was in contrast to a more
137 sustained TGF- β -induced SMAD2 phosphorylation, or SMAD1/5 phosphorylation in
138 response to BMP4. However, transient SMAD1/5 phosphorylation is not a defining
139 characteristic of this arm of TGF- β signaling, as another human breast cancer line,
140 BT-549, exhibited sustained SMAD1/5 phosphorylation that is still readily detectable
141 8 hr after TGF- β stimulation (Figure 1 – figure supplement 1A). Furthermore, when
142 BT-549 cells were grown as non-adherent spheres, TGF- β -induced SMAD1/5
143 phosphorylation did not attenuate at all in the first 8 hr of signaling (Figure 1 – figure
144 supplement 1A). Thus, the kinetics of TGF- β -induced SMAD1/5 phosphorylation is
145 cell type-specific and dependent on the culture conditions and are independent of the
146 kinetics of TGF- β -induced SMAD2/3 phosphorylation, suggesting a distinct receptor
147 complex may be involved.

148 To address whether new protein synthesis was required for the transient nature
149 of TGF- β -induced SMAD1/5 phosphorylation, cells were induced with TGF- β in the
150 presence of either a translation inhibitor, cycloheximide or a transcription inhibitor,
151 actinomycin D. Inhibition of translation was uninformative because it also led to a
152 very rapid loss of TGFBR2 and TGFBR1, due to their short half-lives (Vizan et al.,
153 2013). Use of actinomycin D, however, circumvented this problem, as *TGFBR2* and
154 *TGFBR1* mRNAs are relatively stable (Figure 1 - figure supplement 1B) and their
155 translation was unimpeded. In these conditions SMAD1/5 phosphorylation was
156 sustained (Figure 1B; Figure 1 - figure supplement 1C). Thus, the rapid loss of
157 pSMAD1/5 at later time points after TGF- β stimulation requires new transcription,
158 suggesting that it is mediated by a component whose expression is induced by TGF- β .

159 Acute TGF- β stimulation results in the rapid internalization of the receptors,
160 which is sufficient to deplete almost all of the type II receptor TGFBR2 from the cell
161 surface (Vizan et al., 2013). As a result, cells are refractory to further acute TGF- β
162 stimulation, read out by SMAD2 phosphorylation (Vizan et al., 2013). Cells in this
163 refractory state were also unable to induce SMAD1/5 phosphorylation in response to
164 TGF- β , although they remained responsive to BMP4 (Figure 1C, Figure 1 - figure
165 supplement 1D). This suggested that TGFBR2 is required for TGF- β -induced
166 SMAD1/5 activation.

167

168 **TGF- β -induced SMAD1/5 phosphorylation requires the kinase activity of two** 169 **different type I receptors**

170 The distinct kinetics of TGF- β -induced SMAD1/5 phosphorylation compared with
171 SMAD2/3 phosphorylation suggested that different receptor complexes are likely
172 involved. To explore this further, we used combinations of well-characterized small
173 molecule inhibitors of the type I receptor kinases in MDA-MB-231 cells. SB-431542,
174 a selective TGFBR1/ACVR1B/ACVR1C inhibitor (Inman et al., 2002), completely
175 inhibited the phosphorylation of both SMAD1/5 and SMAD2 in response to TGF- β
176 when used at 10 μ M (Figure 1D), indicating that the kinase activity of TGFBR1 is
177 essential for TGF- β -induced SMAD1/5 phosphorylation. Interestingly, a 40-fold
178 lower dose also substantially inhibited SMAD1/5 phosphorylation, whilst having less
179 effect on SMAD2 phosphorylation (Figure 1D). TGF- β -induced SMAD1/5
180 phosphorylation was also partially inhibited by the BMP type I receptor inhibitor
181 LDN-193189 (Cuny et al., 2008) (Figure 1D), establishing a requirement for a
182 member of this class of type I receptors. Strikingly, the combination of the low dose
183 SB-431542 and LDN-193189 completely inhibited TGF- β -dependent SMAD1/5
184 phosphorylation, without affecting phosphorylation of SMAD2 (Figure 1D).
185 Analogous results were obtained in NMuMG cells (Figure 1 – figure supplement 1E).

186 We conclude that the kinase activity of both classes of type I receptor is
187 required for maximal SMAD1/5 phosphorylation downstream of TGF- β . Taking these
188 results together with the receptor expression profiles of these cells and receptor
189 knockdown experiments (Daly et al., 2008), we deduce that the receptors involved are
190 TGFBR1, a canonical BMP type I receptor (ACVR1 and/or BMPR1A) and TGFBR2.

191

192 **SMAD1 is primarily phosphorylated by ACVR1**

193 We next used an *in vitro* approach to explore why TGF- β -induced phosphorylation of
194 SMAD1 requires two different type I receptors. We focused on ACVR1 as a
195 representative of the BMP type I receptor class, as it is the most homologous to
196 ACVRL1 that responds to TGF- β in endothelial cells (Chen and Massague, 1999).
197 Moreover, in some cell types, knockdown of ACVR1 was sufficient to block TGF- β -
198 induced pSMAD1/5 (Daly et al., 2008).

199 SMAD1 is known to be a poor substrate for TGFBR1 *in vivo* (Kretzschmar et
200 al., 1997, Hoodless et al., 1996). We demonstrated that SMAD1 is also a poor
201 substrate for TGFBR1 *in vitro*, although it is efficiently phosphorylated by both
202 ACVR1 and BMPR1A as expected (Figure 1 – figure supplement 2A, B). As a
203 control we showed that TGFBR1 could potently phosphorylate SMAD2, and
204 surprisingly, ACVR1 was also able to phosphorylate SMAD2 (Figure 1 – figure
205 supplement 2A, B).

206 Given that SMAD1 is a poor substrate for TGFBR1, it is intriguing that the
207 kinase activity of TGFBR1 is essential for TGF- β -induced SMAD1 phosphorylation.
208 We hypothesized that TGFBR1 might catalyze a priming phosphorylation on
209 SMAD1, which then serves as a substrate for ACVR1, or *vice versa*. To address this
210 we mapped the sites phosphorylated by ACVR1 on full length SMAD1. We identified
211 three species of C-terminal SMAD1 phosphorylation by ACVR1 – a dually
212 phosphorylated S[pS]V[pS] and the singly phosphorylated [pS]SVS and S[pS]VS
213 (Figure 1 – figure supplement 2C). From this it was clear that ACVR1 could
214 phosphorylate both serines in the critical SVS motif and we deduced that the order of
215 phosphorylation is the penultimate serine of the motif, followed by the terminal one.
216 Moreover, if the preceding serine was phosphorylated, it prevented the
217 phosphorylation of the other sites.

218 Taking all these results together, we conclude that in response to TGF- β , the
219 receptor kinase that phosphorylates SMAD1 is ACVR1 and not TGFBR1, and it does
220 so on both serines in the SVS motif in a defined order.

221

222 **ACVR1 is activated by TGFBR1 *in vitro* and *in vivo***

223 The absence of a role for the TGFBR1 kinase activity in phosphorylating SMAD1 left
224 open the question of why it is required *in vivo* for TGF- β -induced SMAD1/5

225 phosphorylation. We postulated that it might be necessary for ACVR1 activation, and
226 therefore investigated whether TGFBR1 could directly phosphorylate ACVR1. Both
227 TGFBR1 and ACVR1 exhibit significant autophosphorylation activity *in vitro*, which
228 was inhibited by SB-505124 (DaCosta Byfield et al., 2004) and LDN-193189
229 respectively (Figure 2A). Crucially, when TGFBR1 and ACVR1 were co-incubated,
230 ACVR1 was phosphorylated, even in the presence of LDN-193189, indicating that
231 ACVR1 *is* a substrate of TGFBR1 (Figure 2A).

232 To determine whether TGFBR1 could activate ACVR1 *in vivo* we used an
233 optogenetic approach. To this end we fused the light-oxygen-voltage (LOV) domain
234 of aureochrome1 from *Vaucheria frigida*, which dimerizes upon blue light stimulation
235 (Sako et al., 2016), to the C-terminal ends of the intracellular domains of a
236 constitutively-activated TGFBR1 (mutation T204D) (Wieser et al., 1995) and of wild
237 type ACVR1, along with an N-terminal myristoylation motif to anchor them to the
238 plasma membrane (Figure 2B; Supplementary Files 1 and 2). We refer to these
239 constructs as Opto-TGFBR1* and Opto-ACVR1, respectively. We tested their ability,
240 alone or in combination, to induce phosphorylation of SMAD1/5 in NIH-3T3 cells co-
241 transfected with FLAG-SMAD1 to increase the range of the assay. Transfection of the
242 Opto-ACVR1 alone resulted in no phosphorylation of co-transfected FLAG-SMAD1,
243 either in the absence or presence of blue light. However, when Opto-ACVR1 and
244 Opto-TGFBR1* were co-transfected, a robust light-inducible phosphorylation of
245 FLAG-SMAD1 was observed (Figure 2C). Importantly, this was inhibited by both
246 SB-505124 and LDN-193189, confirming the involvement of both receptors (Figure
247 2D). This directly demonstrates that TGFBR1 can activate ACVR1 *in vivo*. As a
248 control, we showed that Opto-TGFBR1* phosphorylated co-expressed GFP-SMAD3
249 in the presence of light, which was inhibited by SB-505124, but to a much lesser
250 extent by LDN-193189 (Figure 2E). As a further control to ensure that the activation
251 of ACVR1 by TGFBR1 required the kinase activity of the latter, we made a kinase-
252 dead version of Opto-TGFBR1. This construct was unable to induce the activity of
253 ACVR1 in a light inducible manner and was also unable to induce phosphorylation of
254 GFP-SMAD3 (Figure 2 – figure supplement 1).

255 To confirm that the light-inducible phosphorylation of FLAG-SMAD1
256 observed with the combination of Opto-ACVR1 and Opto-TGFBR1* genuinely
257 resulted from activation of Opto-ACVR1 by Opto-TGFBR1*, we generated a mutant
258 version of Opto-ACVR1, in which the serines and threonines of the GS domain were

259 mutated to alanine and valine respectively. Since phosphorylation of these serines and
260 threonines is required for type I receptor activation, we would expect this mutant to be
261 uninducible (Wieser et al., 1995). Indeed, we found that light-inducible
262 phosphorylation of FLAG-SMAD1 was inhibited when this GS domain mutant of
263 Opto-ACVR1 was used instead of the wild type Opto-ACVR1 (Figure 2F, G).

264 We therefore conclude that the requirement of the kinase activity of both
265 TGFBR1 and ACVR1 for TGF- β -induced phosphorylation of SMAD1/5 reflects a
266 requirement for activation of ACVR1 by TGFBR1 through phosphorylation of the
267 ACVR1 GS domain.

268

269 **TGF- β leads to clustering of ACVR1 and TGFBR1**

270 Having shown that both type I receptors are required, we next tested whether they
271 were components of the same tetrameric receptor complex, or whether they resided in
272 separate receptor complexes that clustered at the cell membrane in response to ligand
273 stimulation (compare model I and model II, Figure 3A). To distinguish between these
274 possibilities we used previously published recombinant versions of TGF- β 3,
275 designated WW and WD (Huang et al., 2011). TGF- β 3^{WW}, the wildtype TGF- β 3
276 dimer, is composed of two identical monomeric TGF- β 3 subunits, whereas TGF-
277 β 3^{WD} contains one wildtype subunit of TGF- β 3 and one mutated subunit that cannot
278 bind to either TGFBR2 or TGFBR1 (Huang et al., 2011). Thus, while the TGF- β 3^{WW}
279 ligand engages two type II:type I pairs in the tetrameric complex, the TGF- β 3^{WD}
280 ligand can only engage one pair. In addition, TGF- β 3^{WW} does not bind ACVR1, and
281 by inference, neither does TGF- β 3^{WD} (data not shown). It was previously
282 demonstrated that TGF- β 3^{WD} binding to a single type II:type I receptor pair is
283 sufficient to induce phosphorylation of SMAD3 (Huang et al., 2011). We therefore
284 reasoned that if model I was correct then only TGF- β 3^{WW} would induce
285 phosphorylation of SMAD1/5, as the heterotetrameric complex would not be able to
286 be assembled with TGF- β 3^{WD}. If model II was correct, however, then both TGF-
287 β 3^{WW} and TGF- β 3^{WD} would be competent to induce pSMAD1/5. Treatment of MDA-
288 MB-231 or NMuMG cells with either TGF- β 3^{WW} or TGF- β 3^{WD} led to a dose-
289 dependent increase in both SMAD1 and SMAD2 phosphorylation (Figure 3B; Figure
290 3 – figure supplement 1). Thus, TGF- β stimulation is unlikely to lead to formation of
291 a heterotetrameric complex comprising TGFBR2/TGFBR1/ACVR1, but instead,

292 leads to the formation of a higher order receptor cluster at the cell surface that
293 includes TGFBR2/TGFBR1 complexes and ACVR1.

294

295 **TGF- β induces ACVR1 activation *in vivo* in a TGFBR1-dependent manner**

296 To obtain direct evidence that TGF- β activates ACVR1, we generated an ACVR1
297 biosensor that fluoresces when activated. In this construct ACVR1 is fused to the
298 conformation-sensitive circularly permuted yellow fluorescent protein (cpYFP) core
299 of the InversePericam Ca²⁺ sensor and FKBP1A (formerly FKBP12) to make
300 ACVR1-InversePericam-FKBP1A (ACVR1-IPF) (Michel et al., 2011). When the
301 receptor is inactive, the FKBP1A moiety binds to the GS domain of the receptor,
302 which suppresses cpYFP fluorescence. Upon ligand induction, phosphorylation of the
303 GS domain releases FKBP1A, allowing the cpYFP to adopt a fluorescent
304 conformation (Michel et al., 2011). We first showed that ACVR1-IPF is functional in
305 that it is able to induce phosphorylation of SMAD1/5 when overexpressed (Figure 4 –
306 figure supplement 1A). We then stably expressed this biosensor in a number of cell
307 lines (Figure 4 – figure supplement 1B). In the polarized epithelial cell line, MDCKII
308 and in NIH-3T3 fibroblasts, ACVR1-IPF is readily detectable at the cell membrane,
309 as well as in internal structures, and had no adverse effect on the inducibility of these
310 cells in response to TGF- β or BMP4 (Figure 4 – figure supplement 1B–D). As a
311 control we showed that ACVR1-IPF was activated in response to FK506 which binds
312 FKBP1A and releases it from the GS domain of ACVR1 (Wang et al., 1994) (Figure
313 4 – figure supplement 1E). Treatment of the MDCKII ACVR1-IPF cells with TGF- β
314 resulted in a significant increase in fluorescence that was inhibited by SB-431542
315 (Figure 4A and B; Videos 1–3). Furthermore, using flow cytometry for a more
316 quantitative approach we demonstrated that the TGF- β -induced increase in
317 fluorescence was blocked by both SB-431542 and a TGF- β neutralizing antibody and
318 was independent of BMP signaling, as it was unaffected by the BMP antagonist
319 Noggin (Figure 4C). Similarly, TGF- β also activated ACVR1 in NIH-3T3 ACVR1-
320 IPF cells in a TGFBR1-dependent manner (Figure 4 – figure supplement 1F, G;
321 Videos 4–6).

322

323 **Mapping the binding sites on chromatin for TGF- β -induced pSMAD1/5 reveals** 324 **that *ID* genes are major transcriptional targets of this pathway**

325 Although the existence of TGF- β -induced pSMAD1/5 has been known for some time,
326 its transcriptional role has never been addressed. Earlier experiments had suggested
327 that TGF- β -induced pSMAD1/5 could only be found in complex with pSMAD2/3
328 (Daly et al., 2008), but using optimized immunoprecipitation conditions it was clear
329 that TGF- β -induced pSMAD1/5 can also be part of pSMAD1/5–SMAD4 complexes
330 (Figure 5A). We therefore used chromatin immunoprecipitation sequencing (ChIP-
331 seq) for pSMAD1/5 to explore where in the genome pSMAD1/5 binds in response to
332 TGF- β . We also wanted to determine which SMAD complexes were primarily
333 responsible for regulating transcription in addition to the canonical pSMAD2/3–
334 SMAD4 complexes (Figure 5A).

335 ChIP-seq in MDA-MB-231 cells for pSMAD1/5 and SMAD3 (as a control)
336 resulted in 2378 pSMAD1/5 peaks and 2440 SMAD3 peaks identified in response to
337 TGF- β after filtering (Figure 5 - Source data 1, sheet 1). The majority of the
338 pSMAD1/5 peaks (2287) were also bound by SMAD3. To identify binding sites
339 preferentially bound by pSMAD1/5 versus SMAD3 we calculated the ratio of the
340 number of tags in the pSMAD1/5 peaks versus the SMAD3 peaks, and focused on the
341 100 peaks with the highest pSMAD1/5:SMAD3 tag ratio (Figure 5 - Source data 1,
342 sheet 2). Interrogating the nearest genes to these peaks we found a significant
343 enrichment of both TGF- β and BMP target genes (Figure 5 - Source data 1, sheets 2
344 and 3). Strikingly, 8 of the top 10 peaks flanked known BMP target genes (*ID1*, *ID3*,
345 *ID4*, *ATOH8*, *BIRC3*) (Figure 5B; Figure 5 – figure supplement 1A; Figure 5 - Source
346 data 1, sheet 2) (Gronroos et al., 2012). In contrast, classical TGF- β target genes like
347 *JUNB*, *BHLHE40*, *PMEPA1*, *SERPINE1* (Levy and Hill, 2005) were not in this top
348 100 list, but were amongst those with the highest enrichment for SMAD3 (Figure 5B;
349 Figure 5 – figure supplement 1A; Figure 5 - Source data 1, sheet 1). Using ChIP-
350 qPCR, we validated these different binding patterns (Figure 5C; Figure 5 – figure
351 supplement 1A). For pSMAD1/5, the binding in response to TGF- β was transient,
352 peaking at 1 hr and thereafter decreasing, whilst SMAD3 binding at *JUNB* and
353 *PMEPA1* was sustained. A subset of the peaks were also validated in BT-549 cells
354 (Figure 5 – figure supplement 1B).

355 We performed motif enrichment analyses on the top 50 and 100 peaks with the
356 highest pSMAD1/5:SMAD3 tag ratio. In both cases a SMAD1/5 binding motif
357 GGCGCC was found (Figure 5D and E; Figure 5 – figure supplement 1C)

358 (Gaarenstroom and Hill, 2014). In addition, in the top 50 peaks the composite
359 SMAD1/5–SMAD4 site was clearly identified (GGCGCC(N₅)GTCT) (Gaarenstroom
360 and Hill, 2014, Morikawa et al., 2011) (Figure 5 – figure supplement 1C), with a
361 slightly more degenerate version being present in the top 100 peaks (Figure 5D). This
362 strongly suggests that TGF- β -induced SMAD1/5–SMAD4 complexes are responsible
363 for regulating the genes with the highest enrichment of pSMAD1/5.

364 The enrichment of pSMAD1/5 on the *ID* genes in response to TGF- β suggests
365 that they are *bona fide* target genes of this arm of TGF- β signaling. We confirmed this
366 using siRNAs to deplete specific SMADs. TGF- β induction of *ID1* and *ID3* in MDA-
367 MB-231 cells depended on SMAD1/5 and SMAD4, but not SMAD3 (Figure 5 –
368 figure supplement 2A and B). In contrast, the induction of *JUNB* required SMAD3
369 and SMAD4, but was independent of SMAD1/5 (Figure 5 – figure supplement 2A
370 and B). We further corroborated these observations using the drug dosing strategy that
371 selectively inhibits SMAD1/5 phosphorylation in response to TGF- β (Figure 1D). The
372 combination of low dose SB-431542 and LDN-193189 greatly decreased *ID* gene
373 induction without impacting on the induction of *JUNB* in both MDA-MB-231 and
374 NMuMG cells (Figure 5 – figure supplement 2C and D). The induction of target gene
375 expression was also examined after treatment of cells with TGF- β 3^{WW} or TGF- β 3^{WD}.
376 As expected both TGF- β ligands induced the expression of the *IDs* and *JUNB* (Figure
377 5 – figure supplement 2E).

378 The results in this section reveal that pSMAD1/5–SMAD4 complexes formed
379 in response to TGF- β are responsible for regulating the genes with the highest
380 enrichment of pSMAD1/5, and that the *IDs* are major early downstream targets.

381

382 **The SMAD1/5 arm of TGF- β signalling is required for TGF- β -induced EMT**

383 The *ID* proteins have been implicated in many processes involved in oncogenesis
384 (Lasorella et al., 2014), and importantly, *ID1* was shown to be upregulated by TGF- β
385 in tumor cells isolated from pathological pleural fluids from patients with ER- and
386 ER+ metastatic breast cancer, and also in patient-derived glioblastomas (Anido et al.,
387 2010, Padua et al., 2008). Since we have now shown that the pSMAD1/5 arm of TGF-
388 β signaling is responsible for TGF- β -induced *ID1* induction, this prompted us to
389 explore further the biological relevance of the pSMAD1/5 arm of TGF- β signaling in
390 oncogenic processes, and to gain a comprehensive view on the relative contribution of

391 this arm of signaling to longer term TGF- β responses. We decided to focus on the
392 process of EMT, as this is a key step in tumorigenesis that confers a migratory
393 phenotype, acquisition of stem cell properties and resistance to chemotherapeutic
394 agents (Ye and Weinberg, 2015). For these studies we primarily used the NMuMG
395 cell model, as we have shown above that these cells show a robust phosphorylation of
396 SMAD1/5 in response to TGF- β and are well known to undergo a TGF- β -induced
397 EMT within 48 hr (Piek et al., 1999).

398 CRISPR/Cas9 was used to generate clones of NMuMG cells deleted for
399 SMAD1 and SMAD5 (Figure 6A; Figure 6 – figure supplement 1A–C). We compared
400 the TGF- β -induced transcriptome at 48 hr of the parental clone with one deleted for
401 SMAD1/5 using RNA-sequencing (RNA-seq). Of the 5798 genes that are
402 significantly up- or down-regulated by TGF- β in this time frame we found that
403 approximately a quarter (1398) were dependent on the SMAD1/5 branch of signaling
404 (see Materials and Methods for the cut-offs used) (Figure 6 - Source data 1, sheets 1
405 and 2). This demonstrates that this arm of TGF- β signaling plays a crucial role in long
406 term downstream transcription responses. To corroborate the RNA-seq results we
407 validated a subset of them by qPCR, measuring levels of mRNA over time in
408 response to TGF- β (Figure 6 – figure supplement 2).

409 Gene set enrichment analysis revealed that the TGF- β target genes that depend
410 on this arm of signaling were involved in processes such as regulation of the
411 cytoskeleton, focal adhesions, adherens and tight junctions, as well as TGF- β
412 signaling in EMT (Figure 6 - Source data 1, sheet 3). We therefore next investigated
413 whether TGF- β -induced EMT required SMAD1/5 signaling. Using delocalization of
414 the adherens junction marker CDH1 (also called E-Cadherin) together with loss of the
415 tight junction marker TJP1 (also called ZO-1) as a measure of EMT, we could readily
416 demonstrate that signaling through SMAD1/5 was crucial for this process in two
417 separate Δ SMAD1/5 clones (Figure 6B; Figure 6 – figure supplement 1D). In
418 addition, we observed that two mesenchymal markers, *Acta2* (also called smooth
419 muscle actin) and *Fn1* were more weakly induced in the Δ SMAD1/5 clone compared
420 with the wild type (Figure 6 – figure supplement 2). We also used an siRNA
421 knockdown approach, and showed that EMT was dependent on SMAD1/5, SMAD4
422 and SMAD3, but independent of SMAD2 (Figure 6 – figure supplement 3A and B).
423 Furthermore, treatment of the cells with the BMP type 1 receptor inhibitor, LDN-

424 193189 also inhibited EMT either alone or when combined with low dose SB-431542
425 which we have shown is sufficient to inhibit TGF- β -induced SMAD1/5 signaling, but
426 not signaling through SMAD2/3 (Figure 6C and D; Figure 6 – figure supplement 3C).
427 Moreover DMH1, another BMP type 1 receptor inhibitor, had a similar effect (Figure
428 6 – figure supplement 3C and D). Finally, to confirm that that the dependence of
429 TGF- β induced EMT on SMAD1/5 signaling was not unique to NMuMG cells, we
430 used another mouse mammary cell line, EpRas that also undergoes a TGF- β -induced
431 EMT (Daly et al., 2010, Grunert et al., 2003). SMAD1/5 signaling in this line was
432 also essential for EMT (Figure 6E and F). Thus, we conclude that TGF- β -induced
433 EMT requires the SMAD1/5 arm of the signaling pathway, as well as the canonical
434 pathway through SMAD3.

435 Taking our ChIP-seq and RNA-seq analyses together, we found that the *ID*
436 genes are major early transcriptional targets of the SMAD1/5 arm of the TGF- β
437 pathway. Of these, ID1 was the prominent family member up regulated by TGF- β in
438 NMuMGs (Figure 7 – figure supplement 1A). We hypothesized that the dependency
439 on the SMAD1/5 arm of the TGF- β pathway could reflect a requirement of ID1 for
440 EMT. We tested this by knocking down *Id1* with siRNAs, both as a pool and as
441 individual siRNAs and found that cells depleted of ID1 were indeed unable to
442 undergo TGF- β -induced EMT (Figure 7A and B; Figure 7 figure supplement 1B and
443 C). Thus, we conclude that TGF- β -induced up-regulation of ID1 is essential for EMT.
444

445 **Discussion**

446

447 **Combinatorial signaling downstream of TGF- β**

448 Here we have defined both the mechanism whereby TGF- β induces the
449 phosphorylation of SMAD1/5, and its functional role. We have shown that two type I
450 receptors are required, the canonical TGF- β receptor TGFBR1, and additionally, one
451 of the classical BMP type I receptors, ACVR1. Using *in vitro* kinase assays, an
452 optogenetic approach and an ACVR1 receptor fluorescent biosensor, we have
453 uncovered a new mechanism for receptor activation whereby one type I receptor
454 activates another. We show that in response to TGF- β , TGFBR1 phosphorylates and
455 activates ACVR1, which phosphorylates SMAD1/5. To address the functional
456 significance of this arm of TGF- β signaling we used genome-wide ChIP-seq and
457 RNA-seq and show that approximately a quarter of the TGF- β -regulated
458 transcriptome is dependent on SMAD1/5, with major early targets being the ID
459 transcriptional regulators. Finally, we have also demonstrated that the SMAD1/5
460 pathway is essential for TGF- β -induced EMT, and this reflects a requirement for ID1.

461 Taking these results together with previous work (Liu et al., 2009, Daly et al.,
462 2008) we propose a model of combinatorial signaling that is essential for the TGF- β
463 cellular program (Figure 7C). In most cells tested the induction of pSMAD1/5 is more
464 transient than the pSMAD2/3 induction (Liu et al., 2009, Daly et al., 2008). Thus, the
465 initial transcriptional program is regulated by both SMAD pathways and is refined at
466 later time points by the SMAD2/3 pathway. Therefore, the full TGF- β -induced
467 transcriptional program requires combinatorial signaling via both SMAD pathways.
468 With respect to the functional relevance of TGF- β -induced SMAD1/5
469 phosphorylation, we have now shown that complete EMT requires both SMAD
470 pathways. TGF- β -induced anchorage-independent growth, migration and invasion
471 have also been shown to require SMAD1/5 signaling, whilst TGF- β -induced growth
472 arrest is only dependent on SMAD2/3 signaling (Liu et al., 2009, Daly et al., 2008)
473 (Figure 7C).

474 Since we have now demonstrated that TGF- β induces the formation of
475 SMAD1/5–SMAD4 complexes that regulate canonical BMP target genes, it is
476 important to ask what discriminates TGF- β signaling from BMP signaling as it is well

477 known that BMP and TGF- β functional responses are distinct (Itoh et al., 2014,
478 Miyazono et al., 2010). The answer lies in the combinatorial signaling, and likely also
479 in the signaling dynamics. In contrast to TGF- β , BMP stimulation leads to a sustained
480 phosphorylation of SMAD1/5 in the absence of SMAD2/3 activation (Gronroos et al.,
481 2012, Daly et al., 2008). As a result, although the gene expression program
482 downstream of BMP shares some common targets with that downstream of TGF- β at
483 early time points, it will be completely distinct at later time points as a result of the
484 sustained SMAD1/5 signaling and the absence of SMAD2/3-driven transcription
485 (Figure 7C).

486

487 **Receptor requirements for TGF- β -induced SMAD1/5 phosphorylation**

488 We have shown that two classes of type I receptors are necessary for TGF- β -induced
489 SMAD1/5 phosphorylation, the canonical TGF- β receptor, TGFBR1 and one of the
490 BMP type I receptors, of which we have focused on ACVR1. Our results demonstrate
491 that the kinase activity of TGFBR1 is essential for activation of ACVR1, whereas the
492 kinase activity of ACVR1 is necessary to phosphorylate SMAD1/5. Surprisingly, we
493 found that inhibition of TGF- β -induced SMAD1/5 phosphorylation by LDN-193189,
494 which inhibits the BMP type I receptors, is incomplete, even though the same LDN-
495 193189 concentration is sufficient to inhibit BMP-induced SMAD1/5
496 phosphorylation. This same result was also previously seen when the BMP type I
497 receptor inhibitor dorsomorphin was used (Daly et al., 2008). A complete inhibition
498 of TGF- β -induced pSMAD1/5 is achieved by combining LDN-193189 with a sub-
499 optimal dose of SB-431542. This is likely explained by the fact that LDN-193189-
500 inhibited ACVR1 is still able to efficiently recruit SMAD1/5, where it may be
501 inefficiently phosphorylated by TGFBR1, which is sensitive to the sub-optimal dose
502 of SB-431542. The requirement for two distinct type I receptors fits well with what
503 was shown for TGF- β responses in endothelial cells, where ACVLR1 and TGFBR1
504 were both required (Goumans et al., 2003, Goumans et al., 2002).

505 Our optogenetic experiments revealed that activated TGFBR1 phosphorylates
506 and activates ACVR1 *in vivo*. We previously hypothesized that TGFBR1 and
507 ACVR1 could be in the same receptor complex (Daly et al., 2008), but our use of the
508 mutant TGF- β 3 ligands here indicated that these two type I receptors are not part of
509 an obligate heterotetrameric receptor, but rather that TGFBR1, activated by TGFBR2

510 as a result of TGF- β stimulation can phosphorylate and activate ACVR1 in the
511 membrane as a result of receptor clustering (Figure 7C). We were surprised to see in
512 our optogenetic experiments that light-induced dimers of the activated kinase domain
513 of TGFBR1 were much more active than the monomeric domains, as this suggests
514 that TGFBR1 is able to autophosphorylate and auto-activate in the absence of type II
515 receptors, if brought into close proximity. In fact, a similar observation was made in
516 early studies using chimeric receptors with the extracellular domain of the
517 erythropoietin receptor and the intracellular domain of constitutively active TGFBR1
518 (Luo and Lodish, 1996). This chimeric receptor could only mediate a growth arrest
519 after stimulation with erythropoietin, indicating that clustering was important for
520 receptor activity *in vivo*.

521

522 **Dynamics of TGF- β -induced pSMAD1/5 signaling**

523 We and others have observed that in most cell types TGF- β -induced SMAD1/5
524 phosphorylation is transient compared with SMAD2/3 phosphorylation (Daly et al.,
525 2008, Liu et al., 2009, Wrighton et al., 2009). Using pSMAD2 as a readout, we
526 previously showed that pSMAD2 levels attenuate over time, and remain at a low
527 steady state level that depends on receptors replenishing the cell surface, for as long
528 as ligand is available (Vizan et al., 2013). Our demonstration that levels of
529 fluorescence of the ACVR1-IPF biosensor steadily increase over a number of hours
530 indicates that ACVR1 can also be continuously activated for as long as ligand is
531 present. We have shown that the transience of SMAD1/5 phosphorylation requires
532 new protein synthesis, indicating that SMAD1/5 phosphorylation is likely to be
533 actively terminated by an inhibitor induced by the pathway. Given the prolonged
534 activation of ACVR1-IPF in response to ligand, we hypothesize that such an inhibitor
535 is unlikely to target the receptors, but might be a TGF- β -induced phosphatase that
536 targets phosphorylated SMAD1/5 directly. The transience of SMAD1/5
537 phosphorylation is not a defining characteristic of this arm of TGF- β signaling as BT-
538 549 breast cancer cells exhibit a more sustained response, which is even more
539 pronounced when the cells are grown as spheres. Comparing TGF- β target genes in
540 BT-549s versus MDA-MB-231s where the response is transient, might shed light on
541 the identity of the putative inhibitor.

542

543 **TGF- β -induced pSMAD1/5 is transcriptionally active and required for a subset**
544 **of TGF- β -induced target genes**

545 Our ChIP-seq analysis demonstrated for the first time that TGF- β -induced
546 pSMAD1/5 accumulates in the nucleus and binds to chromatin. These experiments
547 revealed that the peaks with the highest pSMAD1/5 enrichment flanked classical
548 BMP target genes, such as *ID1*, *ID3* and *ATOH8*. Analysis of the binding sites led us
549 to the discovery that the SMAD complexes responsible for inducing these target
550 genes downstream of TGF- β were pSMAD1/5–SMAD4 complexes. The ChIP-seq
551 analysis also revealed widespread co-binding of pSMAD1/5 and SMAD3, which was
552 surprising. For the classical BMP targets, the ratio of pSMAD1/5:SMAD3 in the
553 peaks was high, whereas at classical TGF- β targets like *JUNB*, *PMEPA1*, *SERPINE1*
554 and *BHLHE40* (Kang et al., 2003, Levy and Hill, 2005), this ratio was less than 1. We
555 do not fully understand the functional significance of the pSMAD1/5 and SMAD3 co-
556 binding. We previously demonstrated that at least in some contexts, pSMAD3–
557 pSMAD1/5 complexes are inhibitory (Gronroos et al., 2012), and this is evident in the
558 work presented here for *ID3* induction. However, for *JUNB* we found that knockdown
559 of SMAD1/5 had no effect on TGF- β -induced transcription, suggesting that
560 pSMAD1/5 is not contributing to its transcriptional regulation. This may also be true
561 of other genes with a similar pattern of SMAD3/pSMAD1/5 binding.

562

563 **TGF- β -induced SMAD1/5 signaling is required for EMT through induction of**
564 **ID1.**

565 We have now shown that SMAD1/5 signaling in response to TGF- β is required for a
566 complete TGF- β -induced EMT in NMuMG cells and in EpRas cells. This accounts
567 for a previously unexplained observation that overexpression of dominant negative
568 ACVR1 in NMuMGs caused a partial loss of EMT in response to TGF- β (Miettinen
569 et al., 1994). In an earlier study using siRNAs we had concluded that the SMAD1/5
570 arm of the TGF- β pathway was not required for EMT in EpRas cells (Daly et al.,
571 2008). The likely explanation for this discrepancy is the poor SMAD1/5 knockdown
572 we achieved in those cells compared with the very effective strategy of inhibiting this
573 arm of TGF- β signaling using the combined small molecule inhibitors that we have
574 employed here.

575 We have gone on to show that TGF- β -induced ID1 is required for EMT.
576 Importantly, although ID1 is necessary for EMT, it is clearly not sufficient, as BMP
577 cannot induce EMT in NMuMGs (Kowanetz et al., 2004). Consistent with this we
578 have also shown that the SMAD3 pathway is essential for EMT. This arm of the
579 pathway is likely required for the induction of some or all of the so-called EMT-
580 associated transcription factors, most notably SNAI1, SNAI2, ZEB1, ZEB2 and
581 BHLH proteins such as TWIST1 and E47 (now called TCF3), some of which are
582 known direct TGF- β targets (Peinado et al., 2007, Diepenbruck and Christofori,
583 2016).

584 Our finding that EMT depends on TGF- β -induced ID1 expression has
585 implications for the role of SMAD1/5 and the IDs in cancer. The prevailing view is
586 that ID1 is downregulated by TGF- β in non-tumorigenic human epithelial lines, but
587 upregulated by TGF- β in established tumor cell lines, as we have observed here in
588 MDA-MB231 and BT-549s, and also in patient-derived tumor cells (Anido et al.,
589 2010, Padua et al., 2008, Lasorella et al., 2014). Furthermore, ID proteins are
590 overexpressed in many different tumor types, and are implicated in the maintenance
591 of tumor stem cells and for some cancer-related phenotypes (Lasorella et al., 2014).
592 ID1 was also found in a lung metastatic gene signature of breast cancer (Minn et al.,
593 2005). The role of ID1 in EMT is context dependent. In a recent study of breast
594 cancer, ID1 was shown to be expressed in tumor cells that had already undergone an
595 EMT, and it contributed to the growth of the primary tumor by inducing a stem cell-
596 like phenotype. At the metastatic site however, TGF- β -induced ID1 was proposed to
597 induce an mesenchymal-to-epithelial transition (MET) by interfering with the
598 activity of TWIST (Stankic et al., 2013). In light of our current data it will be
599 important to investigate in what tumor contexts ID1 is required for EMT, and more
600 broadly how the TGF- β -SMAD1/5 pathway contributes to different aspects of
601 tumorigenesis.

602

603 **Materials and Methods**

604

605 **Cell line origin, authentication and maintenance**

606 MDA-MB-231 cells were obtained from the ECACC/HPA culture collection, BT-549
607 cells were obtained from the Francis Crick Institute Cell Services, NMuMG cells
608 were obtained from ATCC, MDCKII cells were obtained from Sigma, NIH-3T3 cells
609 were obtained from Richard Treisman (Francis Crick Institute) and EpRas cells were
610 obtained from Martin Oft and Hartmut Beug (IMP, Vienna). All cell lines have been
611 banked by the Francis Crick Institute Cell Services, and certified negative for
612 mycoplasma. In addition, MDA-MB-231 and BT-549 cells were authenticated using
613 the short tandem repeat profiling, while MDCKII, NIH-3T3 and EpRas cells had
614 species confirmation at the Francis Crick Institute Cell Services. Their identity was
615 also authenticated by confirming that their responses to ligands and their phenotype
616 were consistent with published history.

617 MDA-MB-231, BT-549, EpRas, NIH-3T3 and MDCKII cells were maintained
618 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf
619 serum (FCS) and 1% penicillin/streptomycin. NMuMG cells were grown in the same
620 medium, but supplemented with 10 µg/ml insulin. MDA-MB-231 and MDCKII cells
621 were starved overnight in OptiMEM prior to ligand stimulation; NMuMG cells were
622 starved overnight in OptiMEM with 10 µg/ml insulin; NIH-3T3 cells were starved in
623 DMEM with 0.5% FCS. For ligand stimulation experiments, BT-549 cells were
624 plated in the mammosphere culture media (Dontu et al., 2003) (MEBM (PromoCell)
625 with B27 (Thermo Fisher), 20 ng/ml EGF (PeproTech), 20 ng/ml bFGF (PeproTech)
626 and 4 µg/ml heparin (Sigma)).

627

628 **Ligands and chemicals**

629 All recombinant ligands were reconstituted in 4.4 mM HCl supplemented with 0.1%
630 BSA. Cells were treated with recombinant TGF-β1 (PeproTech, 100-21C; 2 ng/ml),
631 BMP4 (PeproTech, 120-05ET; 20 ng/ml) and Noggin (PeproTech, 250-38; 300
632 ng/ml). TGF-β3^{WW} and TGF-β3^{WD} were as described (Huang et al., 2011). SB-
633 431542 (Tocris) was used at the concentrations indicated, SB-505124 (Tocris) at 10
634 or 50 µM, LDN-193189 (a gift from Paul Yu) at 1 or 0.5 µM, DMH1 (Selleck
635 Chemicals) at 1 µM, cyclohexamide (Sigma) at 20 µg/ml and actinomycin D (Sigma)

636 at 1 $\mu\text{g/ml}$. For TGF- β blocking experiments, the pan-TGF- β blocking antibody
637 (1D11) and the control antibody (13C4) were used at 30 $\mu\text{g/ml}$ (Nam et al., 2008).

638

639 **CRISPR/Cas9 knockout of SMAD1/5 and ACVR1/BMPR1A in NMuMG cells**

640 From the wildtype NMuMG cells, a parental clone was selected that expressed robust
641 junctional markers (TJP1 and CDH1) and underwent an efficient EMT in response to
642 TGF- β . Two guide RNAs (see Key Resources Table) targeting the MH1 domain
643 (SMAD1) and MH2 domain (SMAD5) were expressed from the plasmid
644 pSpCas9(BB)-2A-GFP (PX458) (Ran et al., 2013) and used to knockout SMAD1 and
645 SMAD5. NMuMG parental clone cells were simultaneously transfected with both
646 plasmids, sorted for GFP expression, plated as single cells in 96-well plates and
647 screened by sequencing to verify mutations in SMAD1 and SMAD5. Two knockout
648 clones, $\Delta\text{SMAD1/5}$ clone 1 and 2, were used in these studies. The same parental clone
649 of NMuMG cells was also used to generate a line knocked out for ACVR1 and
650 BMPR1A. The strategy was as described for the SMAD1/5 knockout and the guides
651 are given in the Key Resources Table.

652

653 **Generation of cell lines stably expressing ACVR1-IPF**

654 The inverse pericam FKBP1A (IPF) fusion protein was amplified by PCR from the
655 pCS2+zALK3-IPF (Michel et al., 2011) and cloned in-frame downstream of the
656 human ACVR1 cDNA sequence in the pcDNA3.1 Hygro + vector (Thermo Fisher).
657 MDCKII and NIH-3T3 cells were transfected with the ACVR1-IPF construct and
658 selected with 400 $\mu\text{g/ml}$ hygromycin or 40 $\mu\text{g/ml}$ hygromycin respectively. After
659 selection, cells were FACS sorted for GFP expression. MDCKII ACVR1-IPF cells
660 were maintained as a pool, while a single clone was isolated for NIH-3T3 cells. To
661 test the functionality of ACVR1-IPF, NMuMG cells knocked out for ACVR1 and
662 BMPR1A were transfected with empty pcDNA3.1 Hygro (+), ACVR1-IPF or FLAG-
663 ACVR1 (Daly et al., 2008) as a positive control, and activity was monitored by their
664 ability to induce phosphorylation of SMAD1/5.

665

666 **Generation and LED light photoactivation of Opto-receptors**

667 The general design of the Opto receptors was a previously described (Sako et al.,
668 2016). Opto-TGFBR1* and Opto-ACVR1 were generated by overlapping PCR

669 (Horton et al., 1990) to include a N-terminal myristylation domain, the intracellular
670 domain of either human TGFBR1 (residues 149-503) or human ACVR1 (residues
671 147-509), a light-oxygen-voltage (LOV) domain from *Vaucheria frigida* (Takahashi
672 et al., 2007) and a C-terminal HA-tag and cloned into the pCS2 expression plasmid
673 (see Supplementary Files 1 and 2). In the case of TGFBR1, the T204D point mutation
674 was introduced that renders the kinase constitutively active (Wieser et al., 1995), thus
675 generating the construct Opto-TGFBR1*. A kinase dead version of Opto-TGFBR1
676 was also generated in which K232 was mutated to R (Wrana et al., 1994).
677 Furthermore, the GS-domain of ACVR1 (¹⁸⁹TSGSGS¹⁹⁴) was mutated to VAGAGA
678 to generate Opto-ACVR1 GS-mut. NIH-3T3 cells were transfected with a total of 2
679 µg of plasmid DNA that included either 5 ng of GFP-SMAD3 (Nicolas et al., 2004)
680 or 25 ng of Flag-SMAD1 (Lechleider et al., 2001) alone or in combination with 25 ng
681 of Opto-TGFBR1* and/or 50 ng of Opto-ACVR1 (WT or GS-mut). We co-
682 transfected the SMADs with the Opto-receptors to increase the range of the assay.
683 Twenty-four hours post transfection, cells were starved overnight in DMEM with
684 0.5% FCS. Cells were then left untreated or pre-treated with 0.5 µM LDN-193189 or
685 50 µM SB-505124 and then exposed to blue light from an LED array for 1 hr at 37°C
686 in a humidified incubator. Control cells (i.e. in the dark) were wrapped in aluminium
687 foil and placed in the same incubator.

688

689 **siRNAs and transfections**

690 All siRNAs were purchased from Dharmacon/GE Health Care Life Sciences and are
691 listed in Supplementary file 3. MDA-MB-231 and NMuMG cells were transfected
692 with siRNAs at a final concentration of 20 nM with Interferin (Polyplus). Twenty four
693 hours post transfection, cells were starved overnight, and the following day cells were
694 treated with TGF-β or BMP-4 for 1 hr and RNA and/or protein extracted. NMuMG
695 cells were also treated with TGF-β for a further 24–48 hr to assess the effects of target
696 gene knockdown on EMT.

697

698 **EMT assay**

699

700 NMuMG or EpRas cells were plated on glass coverslips in 6-well plates (200,000 or
701 75,000 cells respectively). For NMuMG cells treated with small molecule inhibitors,
702 the media was changed the day after plating to OptiMEM with 10 µg/ml insulin and

703 the cells treated with 2 ng/ml TGF- β alone or in combination with 0.125 μ M SB-
704 431542, 1 μ M LDN-193189 or DMH1 for the durations indicated. For knockdown
705 experiments, NMuMG cells were transfected the day after plating with the indicated
706 siRNAs. Twenty-four hours after transfection, the media was changed to OptiMEM
707 with 10 μ g/ml insulin and the following day, cells were treated with TGF- β for the
708 durations indicated. For EpRas, cells were treated with 2 ng/ml TGF- β alone or in
709 combination with 0.125 μ M SB-431542 and 1 μ M LDN-193189 the day after plating.
710 EpRas cells were then split and re-plated at the initial splitting density in the presence
711 of 2 ng/ml TGF- β alone or in combination with SB-431542 and LDN-193189 every
712 three days.

713

714 **Antibodies, immunoblotting, immunoprecipitations and indirect** 715 **immunofluorescence**

716 All primary and secondary antibodies used are listed in the Key Resources Table.
717 Western blots using whole cell extracts and immunoprecipitations followed by
718 Western blotting were as previously described (Germain et al., 2000, Daly et al.,
719 2008). Indirect immunofluorescence of the ACVR1-IPF was performed after fixing
720 cells in 4% formaldehyde for 5 minutes. Indirect immunofluorescence for CDH1 and
721 TJP1 was performed after fixation in methanol:acetone (1:1) as previously described
722 (Nicolas and Hill, 2003). Nuclei were counter stained with DAPI (0.1 μ g/ml).
723 Imaging was performed on a Zeiss Upright 780 confocal microscope. Z-stacks were
724 acquired for all channels and maximum intensity projection images are shown.

725

726 **Live cell imaging**

727 Live cell imaging was performed for MDCKII ACVR1-IPF and NIH-3T3 ACVR1-
728 IPF cells on a Zeiss Invert 780 confocal microscope. Cells were plated on 35 mm
729 Matek dishes and starved overnight in phenol-free, HEPES-buffered DMEM with
730 0.5% FCS. During imaging, the temperature was maintained at 37°C. Data were
731 acquired every 15 minutes over a time course. At each time point, a z-stack was
732 acquired, and maximum intensity z-projections were quantified with ImageJ.

733

734 **Flow cytometry**

735 MDCKII ACVR1-IPF and NIH-3T3 ACVR1-IPF cells were treated with ligand ±
736 inhibitors. Twenty four hours post treatment, cells were trypsinized, washed and
737 analyzed for GFP/YPF fluorescence on a LSRII flow cytometer (BD Biosciences),
738 gated for viable, single cells. Treatment with FK506 (Sigma) was performed for 4 hr
739 prior to analysis.

740

741 **Recombinant proteins, *in vitro* kinase assays and mapping of phospho-sites**

742 Recombinant SMAD proteins were expressed in *E. coli* and purified as previously
743 described (Ross et al., 2006). Recombinant intracellular domains of ACVR1,
744 BMPR1A and TGFBR1 which were expressed in insect cells were purchased from
745 Carna Biosciences Inc (see Key Resources Table). Radioactive kinase reactions were
746 performed with varying amounts of receptor (25–200 ng) at 37°C for 1 hr in a 20 µl
747 reaction volume with 50 mM Tris-Cl (pH 7.5), 50 mM NaCl, 5 mM MnCl₂ (ACVR1
748 and TGFBR1) or MgCl₂ (BMPR1A), 16.5 nM ³²P-γ-ATP (Perkin Elmer;
749 NEG502A500UC) and either 200 µM or 50 µM cold ATP. Substrates were either the
750 receptors themselves (autophosphorylation) or 2 µg of recombinant SMAD proteins.
751 Reactions were stopped by adding Laemmli sample buffer and heating to 95°C for 5
752 minutes. Proteins were resolved on a NuPAGE Novex 4-12% Bis-Tris gradient gel
753 (Thermo Fisher) and stained with Colloidal Blue (Thermo Fisher). Gels were
754 destained, dried and radioactivity measured by autoradiography.

755 To map phosphorylated residues on SMAD1, radioactive kinase reactions
756 were performed in triplicate with 200 ng ACVRI, 2 µg recombinant SMAD1, 200 µM
757 cold ATP, 0.33 µM ³²P-γ-ATP. For phospho-residue mapping, ³²P-labelled SMAD1
758 was digested with trypsin, the peptides were resolved by HPLC with an acetonitrile
759 gradient and the ³²P-labelled peptides eluted. Edman sequencing and mass-
760 spectrometry (Orbitrap Classic, Thermo Fisher) were then used to confirm phospho-
761 residues, as described previously (Campbell and Morrice, 2002), with the addition of
762 multi-stage activation during the MS2 analysis.

763

764 **Chromatin immunoprecipitations, ChIP-Seq and motif enrichment**

765 Four million MDA-MB-231 or BT-549 cells were plated; 24 hr later, cells were
766 starved overnight and the following day treated with TGF-β or BMP-4. One 15 cm
767 plate was used per immunoprecipitation. Chromatin immunoprecipitations, ChIP-seq

768 library preparation, next generation sequencing and data analysis were performed in
769 biological duplicate essentially as previously described (Coda et al., 2017). In brief,
770 ChIP-seq was performed on an Illumina HiSeq2500 generating 50 bp single end
771 reads. Reads were aligned to the human GRCh37/hg19 genome assembly using BWA
772 version 0.6 (Li and Durbin, 2009) with a maximum mismatch of 2 bases. Picard tools
773 version 1.81 (<http://sourceforge.net/projects/picard/>) was used to sort, mark duplicates
774 and index the resulting alignment bam files. Normalized tdf files for visualization
775 purposes were created using IGVtools software (Robinson et al., 2011)
776 (<http://software.broadinstitute.org/software/igv/>) by extending reads by 50 bp and
777 normalizing to 10 million mapped reads per sample. Peaks were called by comparing
778 stimulated samples to the respective untreated samples using MACS version 1.4.2
779 (Zhang et al., 2008), using mfold change parameters of between 5 and 30. Peaks
780 called by MACS were annotated using the annotatepeaks command in the Homer
781 software (Heinz et al., 2010); <http://homer.salk.edu/homer/>).

782 Peaks with less than 20 tags in the pSMAD1/5 IP after TGF- β treatment or
783 less than 30 tags in the SMAD3 IP after TGF- β treatment were excluded from the
784 analysis. In addition, peaks that had less than 1 tag per 10 bp in either of the above
785 conditions were also excluded. Finally a ratio was taken between the number of tags
786 in the pSMAD1/5 IP and the number of tags in the SMAD3 IP after TGF- β treatment
787 to determine the top 100 peaks with preferential SMAD1/5 binding. Of these, the top
788 50 peaks with the highest density of tags per 10 bases in the pSMAD1/5 IP after TGF-
789 β treatment were used for more refined motif enrichment analysis and gene
790 annotation.

791 Motif enrichment was performed using MEME (<http://meme-suite.org/>) with
792 default parameters (zero or one occurrence per sequence, motifs between 6-50 bases
793 in width).

794

795 **RNA-sequencing analysis in the NMuMG parental clone and Δ SMAD1/5 clone 1**

796 NMuMG parental and Δ SMAD1/5 clone 1 were plated, starved the next day in
797 OptiMEM with 10 μ g/ml insulin and treated for a further 48 hr with 2 ng/ml TGF- β .
798 Total RNA was extracted as previously described (Gronroos et al., 2012), DNase I
799 (Qiagen) treated and cleaned up with RNeasy columns (Qiagen). Biological triplicate
800 libraries were prepared using the TruSeq RNA Library Prep Kit (Illumina) and were

801 single-end sequenced on Illumina HiSeq 2500 platform. Sequencing yield was
802 typically ~80 million strand-specific reads per sample. The RSEM package (version
803 1.2.31) (Li and Dewey, 2011) in conjunction with the STAR alignment algorithm
804 (version 2.5.2a) (Dobin et al., 2013) was used for the mapping and subsequent gene-
805 level counting of the sequenced reads with respect to Ensembl mouse GRCm.38.86
806 version genes. Normalization of raw count data and differential expression analysis
807 was performed with the DESeq2 package (version 1.10.1) (Love et al., 2014) within
808 the R programming environment (version 3.2.3) (R Development Core Team,
809 2009). Genes were first identified as differentially expressed in the parental clone if
810 they had more than 10 reads in either the untreated or TGF- β treated samples and a
811 fold change between untreated and TGF- β induced of > 1.5 or < 0.75 and FDR $<$
812 0.05 . An interaction contrast was then used to determine differentially regulated genes
813 after TGF- β treatment in the parental clone versus Δ SMAD1/5 clone 1. The resulting
814 gene lists ranked by the Wald statistic were used to look for pathway and biological
815 process enrichment using the Broad's GSEA Tool (Subramanian et al., 2005). Genes
816 with a fold difference between the two clones after TGF- β treatment of > 1.5 or $<$
817 0.75 and an FDR < 0.05 were judged to be dependent on SMAD1/5.

818

819 **Public availability of data**

820 The ChIP-seq data have been submitted to the NCBI Gene Expression Omnibus
821 (GEO) under the accession number GSE92443. The RNA-seq data has been
822 submitted to GEO under the accession number GSE103372.

823

824 **qPCR**

825 Oligonucleotides used are listed in Supplementary file 3. Total RNA extraction and
826 reverse transcription were performed as previously described (Gronroos et al., 2012).
827 The cDNA was diluted 10-fold and then used for quantitative PCR (qPCR). All
828 qPCRs were performed with Express Sybr Greener (Thermo Fisher) with 300 nM of
829 each primer and 2 μ l of diluted cDNA or eluted immunoprecipitated chromatin.
830 Fluorescence acquisition was performed on a 7500 FAST machine (Thermo Fisher).
831 Quantification for relative gene expression was done using the comparative Ct
832 method with target gene expression normalized to *GAPDH*. Quantification for ChIPs
833 was performed using a standard curve and presented normalized to input.

834

835 **Statistical analysis**

836 Western blots, immunofluorescence experiments and ChIP-PCRs are representative of
837 at least two biological replicate experiments. All qPCRs are the mean and SEM of
838 three independent biological experiments except gene expression after actinomycin D
839 treatment and stimulation with TGF- β ^{WW} and TGF- β ^{WD} and validation of RNA-
840 sequencing results that are a representative of two independent experiments.
841 Statistical analyses were performed with the unpaired Students T-Test, * $p < 0.05$, **
842 $p < 0.01$, *** $p < 0.001$, ns, non significant.

843

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1094
1095

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1110

1111 **Conflicts of Interest**

1112 The authors declare that they have no conflict of interest.

1113

1114 **Figure legends**

1115 **Figure 1. Characterization of SMAD1/5 phosphorylation by TGF- β .**

1116 (A) MDA-MB-231 and NMuMG cells were treated with TGF- β or BMP4 for the
1117 times indicated.

1118 (B) MDA-MB-231 cells were treated with TGF- β for the times shown either alone or
1119 after 5 minutes pre-treatment with cyclohexamide (CHX) or actinomycin D (Act D).

1120 (C) MDA-MB-231 cells were treated with TGF- β for 1 or 8 hr, and after 8 hr, cells
1121 were re-stimulated with TGF- β or BMP4 for 1 hr as shown in the scheme. For
1122 comparison, cells were stimulated for 1 hr with BMP4.

1123 (D) MDA-MB-231 cells were induced or not with TGF- β or BMP4 in the presence of
1124 either 0.25 μ M or 10 μ M SB-431542 (SB) or 1 μ M LDN-193189 (LDN) or a
1125 combination of 0.25 μ M or 10 μ M SB-431542 and 1 μ M LDN-193189. In all panels
1126 Western blots are shown probed with the antibodies indicated. B, BMP4, Un,
1127 unstimulated. In B, SERPINE1, whose expression is induced by TGF- β , provides a
1128 control for the efficacy of the CHX and Act D.

1129

1130 **Figure 2. ACVR1 is activated by TGFBR1 *in vitro* and *in vivo*.**

1131 (A) The kinase domains of TGFBR1 and ACVR1 were analyzed alone or together in
1132 an *in vitro* kinase reaction. SB-505124 and LDN-193189 were included as shown to
1133 inhibit the activity of TGFBR1 and ACVR1 respectively. The autoradiograph is
1134 shown in the top panel, with the Coomassie-stained gel below as a loading control.

1135 (B) Schematic to show the domain organization of the Opto receptors. In Opto-
1136 TGFBR1* and Opto-ACVR1, the kinase domains of TGFBR1 and ACVR1 are fused
1137 to the light sensitive LOV domain. At the N-terminus there is a myristylation domain
1138 (indicated by the red zig zag). At the C-terminus there is an HA tag. The kinase
1139 domain of TGFBR1 contains the activating mutation T204D. These Opto receptors
1140 dimerize in the presence of blue light.

1141 (C) NIH-3T3 cells were untransfected or transfected with FLAG-SMAD1 together
1142 with either Opto-TGFBR1*, Opto-ACVR1 or both receptors together. Post
1143 transfection, cells were either kept in the dark or exposed to blue light for 1 hr. Whole
1144 cell extracts were Western blotted using antibodies against pSMAD1/5 (which detects
1145 endogenous and FLAG pSMAD1/5), SMAD1 (which detects endogenous and FLAG
1146 SMAD1), HA (to detect the Opto receptors) and Tubulin as a loading control.

1147 (D) NIH-3T3 cells were untransfected or transfected with FLAG-SMAD1 together
1148 with either Opto-TGFBR1*, Opto-ACVR1 or both receptors together. Post
1149 transfection, cells were either kept in the dark or exposed to blue light for 1 hr. The
1150 inductions were performed in the absence or presence of 0.5 μ M LDN-193189 or 50
1151 μ M SB-505124 as indicated. Whole cell extracts were blotted as in (C).

1152 (E) The experimental set up was as in (D) except that GFP-SMAD3 was used instead
1153 of FLAG-SMAD1 to assess the activity of Opto-TGFBR1*.

1154 (F) As in (C), except that an ACVR1 mutant in which all the threonines and serines of
1155 the GS domain were mutated to valine or alanine respectively, was also assayed.

1156 (G) As in (F), except that GFP-SMAD3 was used instead of FLAG-SMAD1.

1157 Note that in all cases that the 1 hr induction with blue light led to reduced levels of the
1158 transfected receptors and substrates.

1159

1160 **Figure 3. TGFBR1 and ACVR1 are present in distinct receptor complexes upon**
1161 **TGF- β stimulation.**

1162 (A) Alternative models of receptor clustering mediated by TGF- β 3 derivatives
1163 capable of interacting with two pairs (TGF- β 3^{WW}) or one pair (TGF- β 3^{WD}) of type
1164 II:type I receptors. If an obligate heterotetramer of two type I:type II pairs is required
1165 for SMAD1/5 phosphorylation (Model I), then only TGF- β 3^{WW} would lead to
1166 SMAD1/5 phosphorylation. If TGF- β induces higher order receptor clustering at the
1167 cell surface (Model II), then both TGF- β 3^{WW} and TGF- β 3^{WD} would lead to SMAD1/5
1168 phosphorylation.

1169 (B) MDA-MB-231 cells were treated with different concentrations of TGF- β 3^{WW} or
1170 TGF- β 3^{WD} for 1 hr as indicated. As a control, cells were either untreated (Un) or
1171 treated with TGF- β 1 (T) or BMP4 (B) for 1 hr. Whole cell lysates were Western
1172 blotted using the antibodies shown.

1173

1174

1175 **Figure 4. ACVR1 is activated by TGF- β in a TGFBR1-dependent manner.**

1176 (A and B) MDCKII ACVR1-IPF cells were imaged at 15 min intervals for 60 min
1177 before the addition (arrow) of either media alone or media containing TGF- β \pm 10 μ M
1178 SB-431542 for a further 150 min. The panels in (A) are stills of the maximum
1179 intensity projections at the times shown. The quantifications are shown in (B). The
1180 fluorescence at the 60-min time point was taken as the reference that was subtracted
1181 from all other time points. Data presented are the mean \pm SD of three independent
1182 fields. Statistical significance is shown for the indicated pairs of conditions at the 210
1183 min timepoint.

1184 (C) Fluorescence in MDCKII ACVR1-IPF cells assayed by flow cytometry 24 hr
1185 after treatment. Each panel shows an overlay of the indicated treatment conditions.
1186 The black line indicates the median of the untreated (Un) sample. Quantifications are
1187 shown on the right. For each group, the percentage of cells greater than the median
1188 fluorescence intensity of the untreated sample was quantified. Data are the mean \pm
1189 SEM of three independent experiments. SB, SB-431542 at 10 μ M; Ab, antibody;
1190 Nog, noggin; C, control antibody; B, blocking antibody.

1191

1192 Figure 4 - Source data 1. Source data for ACVR1-IPF fluorescence (panel B)

1193 Figure 4 - Source data 2. Source data for ACVR1-IPF fluorescence by flow cytometry
1194 (panel C)

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1197

1198 **Figure 5. pSMAD1/5 is recruited to chromatin in response to TGF- β and is most**
1199 **highly enriched at GGCGCC motifs.**

1200 (A) MDA-MB-231 cells were either untreated (-) or treated with TGF- β (+) for 1 hr.
1201 Whole cell extracts were immunoprecipitated (IP) with the antibodies (Ab) indicated
1202 or beads alone (Be). The IPs were Western blotted using the antibodies shown. Inputs
1203 are shown on the left.

1204 (B) IGV browser displays over the *ID1*, *ID3* and *JUNB* loci after ChIP-Seq of MDA-
1205 MB-231 untreated (Un) and TGF- β -treated samples. IPs were performed with
1206 antibodies against pSMAD1/5 (pS1/5), SMAD3 (S3) or with beads alone as a
1207 negative control. Inputs are also shown. Red lines indicate regions validated in (C). U;
1208 upstream peak; D1, downstream peak 1.

1209 (C) Genomic regions were validated by ChIP-qPCR after treatment of MDA-MB-231
1210 cells with TGF- β (T) or BMP4 (B) for the times shown. IPs were as in (B). A
1211 representative experiment of two performed in triplicate is shown with means \pm SD.

1212 (D) The most enriched motif obtained from a MEME-ChIP analysis of the top 100
1213 pSMAD1/5 peaks.

1214 (E) Proportion of variants of the GGCGCC motif identified in the top 100 pSMAD1/5
1215 peaks.

1216

1217 Figure 5 - Source data 1. ChIP-seq datasets

1218 Figure 5 - Source data 2. ChIP-PCR data for graphs in panel C

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1223 **Figure 6. SMAD1/5 is required for TGF- β -induced EMT.**

1224 (A) The parental NMuMG clone and the Δ SMAD1/5 clone 1 were treated with TGF-
1225 β or BMP4 for the times shown. Whole cell extracts were immunoblotted with the
1226 antibodies indicated.

1227 (B) Parental NMuMG clone and the Δ SMAD1/5 clone 1 cells were left untreated or
1228 treated with TGF- β for 48 hr and imaged after indirect immunofluorescence (IF)
1229 using antibodies against TJP1 and CDH1. A merge of the two with DAPI in blue is
1230 also shown.

1231 (C) NMuMG cells were left untreated (Un) or treated with TGF- β alone or in
1232 combination with 1 μ M LDN-193189 (LDN) \pm 0.125 μ M SB-431542 (SB) for 48 hr.
1233 Panels show cells imaged under either phase contrast (left panels) or by indirect
1234 immunofluorescence (IF) using antibodies against TJP1 and CDH1. A merge of the
1235 two with DAPI in blue is also shown.

1236 (D) NMuMG cells were left untreated or treated with TGF- β alone or in combination
1237 with either 1 μ M LDN-193189 \pm 0.125 μ M SB-431542 for 48 hr. Whole cell lysates
1238 were immunoblotted with the indicated antibodies.

1239 (E) EpRas cells were left untreated (Un) or treated with TGF- β alone or in
1240 combination with 1 μ M LDN-193189 (LDN) \pm 0.125 μ M SB-431542 (SB) for 9 days,
1241 then imaged after indirect immunofluorescence (IF) using antibodies against TJP1
1242 and CDH1 or a merge of the two with DAPI in blue.

1243 (F) EpRas cells were left untreated or treated with TGF- β for 1 hr alone or with
1244 combinations of 1 μ M LDN-193189, 0.125 μ M SB-431542 or 10 μ M SB-431542 as
1245 indicated. Whole cell lysates were immunoblotted with the indicated antibodies

1246 In (B), (C) and (E) the indirect IF images are maximum intensity projections of a z-
1247 stack in each channel.

1248

1249 Figure 6 - Source data 1. RNA-seq datasets

1250

1251

1252

1253 **Figure 7. TGF- β -induced ID1 via pSMAD1/5 is required for EMT.**

1254 (A) NMuMG cells were transfected with siRNAs against *ID1* or NT control, then left
1255 untreated or treated with TGF- β for 24 hr. Cells were imaged after indirect IF with
1256 antibodies against TJP1 and CDH1 or a merge of the two with DAPI in blue. All
1257 indirect IF images are maximum intensity projections of a z-stack in each channel.

1258 (B) Western blots to show knockdown efficiency of the *ID1* siRNA. NMuMG cells
1259 were treated with TGF- β (T) or BMP4 (B) for 1 hr.

1260 (C) The model shows combinatorial signaling by TGF- β utilizing complexes
1261 containing two different type I receptors. Type II receptors are shown in blue,
1262 TGFBR1 in orange and ACVR1 in green as in Figure 3A. P denotes phosphorylation.
1263 S1/5, SMAD1/5; S2/3, SMAD2/3; S4, SMAD4. The question mark indicates that we
1264 do not yet know the function of the mixed R-SMAD complexes in the physiological
1265 responses. For discussion, see text.

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1268

1269 **Figure 1 – figure supplement 1. SMAD1 phosphorylation kinetics in response to**
1270 **TGF- β .**

1271 (A, C–E) Western blots are shown probed with the indicated antibodies.

1272 (A) BT-549 cells were grown as a monolayer on plastic (2D) or as spheres in low
1273 attachment plates (phase contrast images on the right) and treated with TGF- β or
1274 BMP4 for the times indicated. BT-549 cells show sustained SMAD1/5
1275 phosphorylation in response to TGF- β .

1276 (B) qPCR of the indicated genes in MDA-MB-231 cells treated with actinomycin D
1277 for the times shown. Data are presented as fold change relative to 0 hr. A
1278 representative experiment performed in triplicate is shown with means \pm SD.
1279 Transcripts of both *TGFBRI* and *TGFBRII* are relatively stable.

1280 (C) NMuMG cells were treated with TGF- β for the times shown either alone or after
1281 5 minutes pre-treatment with cyclohexamide (CHX) or actinomycin D (Act D). Act D
1282 prolongs, while CHX terminates both SMAD1/5 and SMAD2 phosphorylation in
1283 response to TGF- β . Un, untreated.

1284 (D) NMuMG cells were treated with TGF- β for 1 or 8 hr and after 8 hr, cells were
1285 restimulated with 10 or 20 ng/ml BMP4 as shown in the scheme. Cells were also
1286 treated for 1 hr with 10 or 20 ng/ml BMP4 as a control. Cells pre-treated with TGF- β
1287 can still be stimulated with BMP4.

1288 (E) NMuMG cells were left untreated or treated with TGF- β \pm SB-431542 (SB;
1289 0.125 μ M or 10 μ M) \pm 1 μ M LDN-193189 (LDN) or BMP4 \pm 1 μ M LDN-193189 for
1290 1 hr. The kinase activity of both classes of type I receptors is required for SMAD1/5
1291 phosphorylation by TGF- β .

1292

1293 Figure 1 – figure supplement 1 - Source data 1. Source data for qPCRs (panel B)

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1297 **Figure 1 - figure supplement 2. SMAD1 is efficiently phosphorylated by ACVR1**
1298 **and BMPR1A, but poorly phosphorylated by TGFBR1.**

1299 (A) *In vitro* kinase assays using the kinase domains of ACVR1, BMPR1A, and
1300 TGFBR1 at 200, 100, 50, 25 ng with recombinant SMAD1 (S1) or SMAD2 (S2) as
1301 substrates. Top panels, autoradiograph; bottom panels, Coomassie-stained gel.

1302 (B) Incorporation of ^{32}P into SMAD1 and SMAD2 catalysed by ACVR1 and
1303 TGFBR1 using different specific activities of $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$. A constant amount of
1304 $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ was added into the kinase reaction with either 200 or 50 μM cold ATP.
1305 Top panels, autoradiograph; bottom panels, Coomassie-stained gel. Numbers
1306 underneath indicate the fold changes relative to the ^{32}P incorporation in SMAD1
1307 (upper) or SMAD2 (lower) catalyzed by TGFBR1 using 200 μM cold ATP. The
1308 phosphorylation of SMAD1 and 2 by ACVR1 and TGFBR1 was dependent on the
1309 specific activity of the $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$, whilst the apparent phosphorylation of SMAD1
1310 by TGFBR1 is not, suggesting that it is non-specific.

1311 (C) Mapping ACVR1 phosphorylation sites on SMAD1. Full length SMAD1
1312 phosphorylated by ACVR1 was digested with trypsin. Peptides were resolved by
1313 reverse phase HPLC (left panel). The C-terminal peptide of SMAD1 existed in three
1314 different phosphorylation states (peptides a, b, and c); the three subsequent peaks are
1315 tryptic miscleavage products. The phosphorylation sites in the peptides were mapped
1316 using solid phase Edman sequencing (panels labelled a, b and c). The deduced
1317 phosphorylation sites in the SSVS motif in the individual peptides are shown in red.

1318

1319 **Figure 2 - figure supplement 1. Kinase dead Opto-TGFBR1 cannot activate**
1320 **Opto-ACVR1.**

1321 (A) NIH-3T3 cells were untransfected or transfected with FLAG-SMAD1 together
1322 with Opto-TGFBR1*, Opto-ACVR1 or both receptors together or a kinase-dead
1323 version of Opto-TGFBR1 (Opto-TGFBR1-KR) alone or together with Opto-ACVR1.
1324 Post transfection, cells were either kept in the dark or exposed to blue light for 1 hr.
1325 Whole cell extracts were Western blotted using antibodies against pSMAD1/5 (which
1326 detects endogenous and FLAG pSMAD1/5), SMAD1 (which detects endogenous and
1327 FLAG SMAD1), HA (to detect the Opto receptors) and Tubulin as a loading control.
1328 (B) The experimental set up was as in (A) except that GFP-SMAD3 was used instead
1329 of FLAG-SMAD1 to assess the activity of Opto-TGFBR1* and Opto-TGFBR1-KR.
1330 The band marked with an asterisk is a background band.

1331

1332

1333 **Figure 3 – figure supplement 1. NMuMG cells respond to both TGF- β ^{WW} and**
1334 **TGF- β ^{WD}.**

1335 NMuMG cells were untreated (Un) or treated with TGF- β 1 (T), BMP4 (B) or the
1336 indicated concentrations of TGF- β ^{WW} and TGF- β ^{WD} for 1 hr. Whole cell lysates
1337 were immunoblotted with the antibodies shown. Both TGF- β ^{WW} and TGF- β ^{WD}
1338 induce phosphorylation of pSMAD1/5, although the latter is less potent.

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1341

1342 **Figure 4 – figure supplement 1. Characterization of cells stably transfected with**
1343 **the ACVR1-IPF.**

1344 (A) NMuMG cells knocked out for ACVR1 and BMPR1A were transfected with
1345 either empty vector (pcDNA3.1 Hygro +), ACVR1-IPF or ACVR1-FLAG. Whole
1346 cell extracts were Western blotted for pSMAD1/5, SMAD1 and GFP and FLAG.
1347 Actin is a loading control.

1348 (B) MDCKII ACVR1-IPF or NIH-3T3 ACVR1-IPF cells imaged by indirect IF with
1349 an antibody against GFP (red) with nuclei stained with DAPI (blue). The controls
1350 were a secondary antibody only sample (left) and the matched empty vector-
1351 transfected cells stained with the GFP antibody (middle). Scale bar equates to 20 μ M.
1352 ACVR1-IPF localizes to the membrane in both cell types, with basolateral
1353 localisation in the MDCKII cells.

1354 (C and D) The MDCKII or NIH-3T3 cell lines shown in (B) were treated with TGF- β
1355 or BMP4 for the times indicated. Whole cell lysates were immunoblotted with the
1356 indicated antibodies. Stable transfection of ACVR1-IPF does not affect the
1357 phosphorylation kinetics of SMAD1/5 in response to TGF- β or BMP.

1358 (E) Median fluorescence intensity (y-axis) as measured by flow cytometry of
1359 MDCKII ACVR1-IPF cells treated with the indicated concentrations of FK506 for 4
1360 hr. FK506 activates ACVR1-IPF fluorescence in a dose-dependent manner.

1361 (F) Quantification of NIH-3T3 ACVR1-IPF cells imaged at 30-minute intervals for a
1362 total of 210 min after the addition of either media alone or media containing TGF- β \pm
1363 10 μ M SB-431542. Data presented are the mean \pm SD of three independent fields.
1364 Statistical significance is shown for the indicated pairs of conditions at the 210 min
1365 timepoint.

1366 (G) Fluorescence in NIH-3T3 ACVR1-IPF cells assayed by flow cytometry 24 hr
1367 after being treated with media alone or with TGF- β \pm 10 μ M SB-431542 (SB). The
1368 percentage of cells with fluorescence greater than the median fluorescence intensity of
1369 the untreated sample (-) was quantified. Data are presented as the mean \pm SEM of
1370 three independent experiments.

1371

1372 Figure 4 - figure supplement 1 - Source data 1. Source data for ACVR1-IPF
1373 fluorescence by flow cytometry (panel E)

1374

1375 Figure 4 - figure supplement 1 - Source data 2. Source data for ACVR1-IPF
1376 fluorescence (panel F)

1377 Figure 4 - figure supplement 1 - Source data 3. Source data for ACVR1-IPF
1378 fluorescence by flow cytometry (panel G)

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1382 **Figure 5 – figure supplement 1. Chromatin binding of pSMAD1/5 and SMAD3.**

1383 (A) IGV browser displays around the *ID4*, *ATOH8*, *PMEPA1*, *BHLHE40* and
1384 *SERPINE1* loci after ChIP-Seq for SMAD3 (S3) and pSMAD1/5 (pS1/5) in MDA-
1385 MB-231 cells. Untreated and TGF- β -treated samples and the inputs are shown. IPs
1386 were performed with beads as a negative control. Red lines indicate genomic regions
1387 validated by ChIP-qPCR after treatment of MDA-MB-231 cells with TGF- β (T) or
1388 BMP4 (B) for the times indicated (right panels). U, upstream peak; U1, upstream
1389 peak 1. In response to TGF- β , pSMAD1/5 bound transiently at the *ID4* and *ATOH8*
1390 loci while SMAD3 bound stably to *PMEPA1* over the same time course.

1391 (B) ChIP-qPCR of the indicated loci after treatment of BT-549 cells with TGF- β (T)
1392 or BMP4 (B) for 1 hr. IPs were as in (A). In response to TGF- β , pSMAD1/5 bound
1393 strongly around the *ID* loci while SMAD3 bound strongly to the *JUNB* upstream
1394 locus. In A and B a representative experiment of two performed in triplicate is shown
1395 with means \pm SD.

1396 (C) The most enriched motif obtained from a MEME-ChIP analysis of the top 50
1397 pSMAD1/5 peaks. The canonical SMAD1/5:SMAD4 binding element is strongly
1398 enriched in these peaks.

1399

1400 Figure 5 – figure supplement 1 - Source data 1. ChIP-PCR data for graphs in panel A

1401 Figure 5 – figure supplement 1 - Source data 2. ChIP-PCR data for graphs in panel B

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1407 **Figure 5 – figure supplement 2. *ID1* and *ID3* are TGF- β -induced target genes**
1408 **that require the pSMAD1/5 signaling arm.**

1409 (A) Western blots showing knockdown efficiency in MDA-MB-231s of the siRNAs
1410 shown. S3, SMAD3; S4, SMAD4; S1/5, SMAD1/5; NT, non targeting. Cells were
1411 untreated (U) or treated with TGF- β (T) or BMP4 (B) for 1 hr. Lysates were
1412 immunoblotted using the antibodies shown.

1413 (B) MDA-MB-231 cells were transfected with siRNAs against the indicated *SMADs*
1414 or a non-targeting control (NT) and then treated with TGF- β (T) or BMP4 (B) for 1
1415 hr. Un, untreated.

1416 (C) MDA-MB-231 cells were left untreated or treated with TGF- β \pm SB-431542 (SB;
1417 0.25 μ M or 10 μ M) \pm 1 μ M LDN-193189 (LDN) or BMP4 \pm 1 μ M LDN-193189 for
1418 1 hr. In B and C, gene expression was measured by qPCR. Data are presented as fold
1419 change relative to the untreated NT sample in B and to the (-) sample in (C) and are
1420 the means \pm SEM of three independent experiments. Statistical significance is shown
1421 for selected comparisons.

1422 (D) NMuMG cells were treated with TGF- β or BMP4 \pm the inhibitors indicated. Gene
1423 expression was measured by qPCR. The combination of 0.125 μ M SB-431542 (SB)
1424 and 1 μ M LDN-193189 (LDN) inhibited TGF- β -induced *Id1* and *Id3* expression
1425 without affecting *JunB* expression. The data are means \pm SEM of at least two
1426 independent experiments. Statistical significance is shown for selected comparisons.

1427 (E) MDA-MB-231 cells were treated with TGF- β 1 (T), BMP4 (B) or different
1428 concentrations of TGF- β 3^{WW} and TGF- β 3^{WD} for 1 hr as in Figure 3B. Gene
1429 expression was measured by qPCR. Both TGF- β 3^{WW} and TGF- β 3^{WD} led to the
1430 induction of *ID1*, *ID3* and *JUNB*, although the induction by TGF- β 3^{WD} was weaker.
1431 A representative experiment of two, performed in triplicate is shown with means \pm
1432 SD.

1433

1434 Figure 5 - figure supplement 2 - Source data 1. qPCR data for graphs in panel B

1435 Figure 5 - figure supplement 2 - Source data 2. qPCR data for graphs in panel C

1436 Figure 5 - figure supplement 2 - Source data 3. qPCR data for graphs in panel D

1437 Figure 5 - figure supplement 2 - Source data 4. qPCR data for graphs in panel E

1438

1439 **Figure 6 – figure supplement 1. Characterization of the NMuMG Δ SMAD1/5**
1440 **clones.**

1441 (A) Sequences of SMAD1 and SMAD5 in the regions around the guides in NMuMG
1442 Δ SMAD1/5 clone 1. From our sequencing we conclude that there are two alleles of
1443 SMAD1 and 3 alleles of SMAD5 in NMuMG cells. The protein sequence for the wild
1444 type (WT) is shown in red above the DNA sequence. Frame shifts are evident in both
1445 SMAD1 mutant alleles, and all three SMAD5 alleles.

1446 (B) The parental NMuMG clone and the Δ SMAD1/5 clone 2 were treated with TGF-
1447 β or BMP4 for the times shown. Whole cell extracts were immunoblotted with the
1448 antibodies indicated.

1449 (C) Sequences of SMAD1 and SMAD5 in the regions around the guides in NMuMG
1450 Δ SMAD1/5 clone 2. The protein sequence for the wild type (WT) is shown in red
1451 above the DNA sequence. This clone exhibits no pSMAD1/5 in response to either
1452 TGF- β or BMP4 (see panel B), despite having a single allele of SMAD1 and SMAD5
1453 with an in-frame deletion of a single amino acid. This is readily explained by the
1454 nature of those mutations, which likely lead to unfolded proteins. Mutant SMAD1
1455 allele 1 is deleted for the conserved amino acid Y125 in the MH1 domain, which is
1456 adjacent to H126, that is responsible for chelating a Zn ion in the zinc finger
1457 (BabuRajendran et al., 2010). Mutant SMAD5 allele 1 is deleted for the conserved
1458 amino acid V283 which is in β -sheet 2 of the MH2 domain, which is critical for
1459 folding of this domain (Wu et al., 2001, Qin et al., 2001). In SMAD1 allele 1 the
1460 insert length is 124 bp and in SMAD5 allele 3, it is 59 bp.

1461 (D) Parental NMuMG clone and the Δ SMAD1/5 clone 2 cells were treated with or
1462 without TGF- β for 48 hr, fixed and imaged following indirect immunofluorescence
1463 (IF) using antibodies against TJP1 and CDH1. A merge of the two with DAPI in blue
1464 is also shown. The indirect IF images are maximum intensity projections of a z-stack
1465 in each channel.

1466

1467

1468 **Figure 6 – figure supplement 2. Validation of SMAD1/5-dependent TGF- β**
1469 **induced genes.**

1470 NMuMG parental clone and NMuMG Δ SMAD1/5 clone 1 cells were untreated or
1471 treated with TGF- β for the times shown. Total RNA was extracted and qPCR was
1472 used to assay the levels of mRNA for the genes shown. The data shown are from a
1473 representative experiment (means \pm SD).

1474

1475 Figure 6 - figure supplement 2 - Source data 1. qPCR data for all graphs shown.

1476

1477 **Figure 6 – figure supplement 3. The pSMAD1/5 signaling arm is required for**
1478 **TGF- β -mediated EMT.**

1479 (A) NMuMG cells were transfected with non-targeting (NT) or siRNAs against the
1480 SMADs as indicated. Cells were then left untreated or treated with TGF- β for 24 hr.
1481 Cells were imaged after indirect IF with antibodies against TJP1 and CDH1. A merge
1482 of the two with DAPI in blue is also shown. All indirect IF images are maximum
1483 intensity projections of a z-stack in each channel. SMAD1/5, SMAD3 and SMAD4
1484 are all required for TGF- β -induced EMT.

1485 (B) Western blots to show knockdown efficiency of the siRNAs. NMuMG cells were
1486 untreated (Un) or treated with TGF- β (T) or BMP4 (B) for 1 hr. Lysates were
1487 immunoblotted using the antibodies shown.

1488 (C) NMuMG cells were treated with ligands or inhibitors as indicated. Cell lysates
1489 were immunoblotted using the antibodies shown. A combination of 0.125 μ M SB-
1490 431542 and 1 μ M LDN-193189 or 0.125 μ M SB-431542 and 1 μ M DMH1 was
1491 sufficient to abolish the TGF- β -induced phosphorylation of SMAD1/5.

1492 (D) NMuMG cells were either untreated (Un) or treated with TGF- β \pm 1 μ M DMH1 \pm
1493 0.125 μ M SB-431542 (SB) for 48 hr. Cells were fixed and stained for TJP1 and
1494 CDH1. In the merge, DAPI (blue) marks the nuclei. DMH1 is sufficient to inhibit
1495 TGF- β -induced EMT.

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1500 **Figure 7 – figure supplement 1. TGF- β -induced ID1 is required for EMT.**
1501 (A) NMuMG, BT-549 and MDA-MB-231s were treated with ligands as shown and
1502 lysates were immunoblotted using the antibodies indicated. The induction of ID1
1503 correlates with pSMAD1/5 phosphorylation.
1504 (B) NMuMG cells were transfected with non-targeting (NT) or individual siRNAs
1505 against the *Id1* as indicated. Cells were then left untreated or treated with TGF- β for
1506 48 hr. Cells were imaged after indirect IF with antibodies against TJP1 and CDH1. A
1507 merge of the two with DAPI in blue is also shown. All indirect IF images are
1508 maximum intensity projections of a z-stack in each channel. Knockdown of ID1 by
1509 any of the siRNAs inhibits TGF- β -induced EMT.
1510 (C) NMuMG cells were transfected with siRNAs as in (B). They were uninduced or
1511 induced with BMP4 for 1 hr to induce expression of ID1 in control-transfected cells.
1512 Whole cell extracts were immunoblotted for the proteins indicated.

1513

1514 **Supplementary Movie legends**

1515

1516 **Video 1. Fluorescence in MDCKII ACVR1-IPF cells treated with media alone.**

1517 MDCKII ACVR1-IPF cells were imaged for 1 hr prior to the addition of media alone
1518 followed by imaging for a further 2.5 hr. Very little increase in fluorescence was
1519 observed over the time course.

1520

1521 **Video 2. Fluorescence in MDCKII ACVR1-IPF cells treated with TGF- β .**

1522 MDCKII ACVR1-IPF cells were imaged for 1 hr prior to the addition of 2 ng/ml
1523 TGF- β followed by imaging for a further 2.5 hr. Significant increase in fluorescence
1524 was observed over the time course with intracellular puncta of fluorescence becoming
1525 more evident over time.

1526

1527 **Video 3. Fluorescence in MDCKII ACVR1-IPF cells treated with TGF- β and SB-**

1528 **431542.** MDCKII ACVR1-IPF cells were imaged for 1 hr prior to the addition of 2
1529 ng/ml TGF- β + 10 μ M SB-431542 to the cells followed by imaging for a further 2.5
1530 hr. Very little increase in fluorescence was observed over the time course.

1531

1532 **Video 4. Fluorescence in NIH-3T3 ACVR1-IPF cells treated with media alone.**

1533 NIH-3T3 ACVR1-IPF cells were imaged for 3.5 hr after the addition of media alone.
1534 A modest and gradual increase in fluorescence was observed over the time course.

1535

1536 **Video 5. Fluorescence in NIH-3T3 ACVR1-IPF cells treated with TGF- β .** NIH-

1537 3T3 ACVR1-IPF cells were imaged for 3.5 hr after the addition of 2 ng/ml TGF- β . A
1538 significant increase in fluorescence was observed over the time course with
1539 fluorescence becoming more evident on membrane projections and intracellular
1540 vesicles over time.

1541

1542 **Video 6. Fluorescence in NIH-3T3 ACVR1-IPF cells treated with TGF- β and SB-**

1543 **431542.** NIH-3T3 ACVR1-IPF cells were imaged for 3.5 hr after the addition of 2
1544 ng/ml TGF- β + 10 μ M SB-431542. A modest and gradual increase in fluorescence
1545 was observed over the time course.

1546 **Supplementary Files**

1547

1548 Supplementary File 1. Sequence of Opto-TGFBR1*

1549 Supplementary File 2. Sequence of Opto-ACVR1

1550 Supplementary File 3. List of oligonucleotides and siRNAs

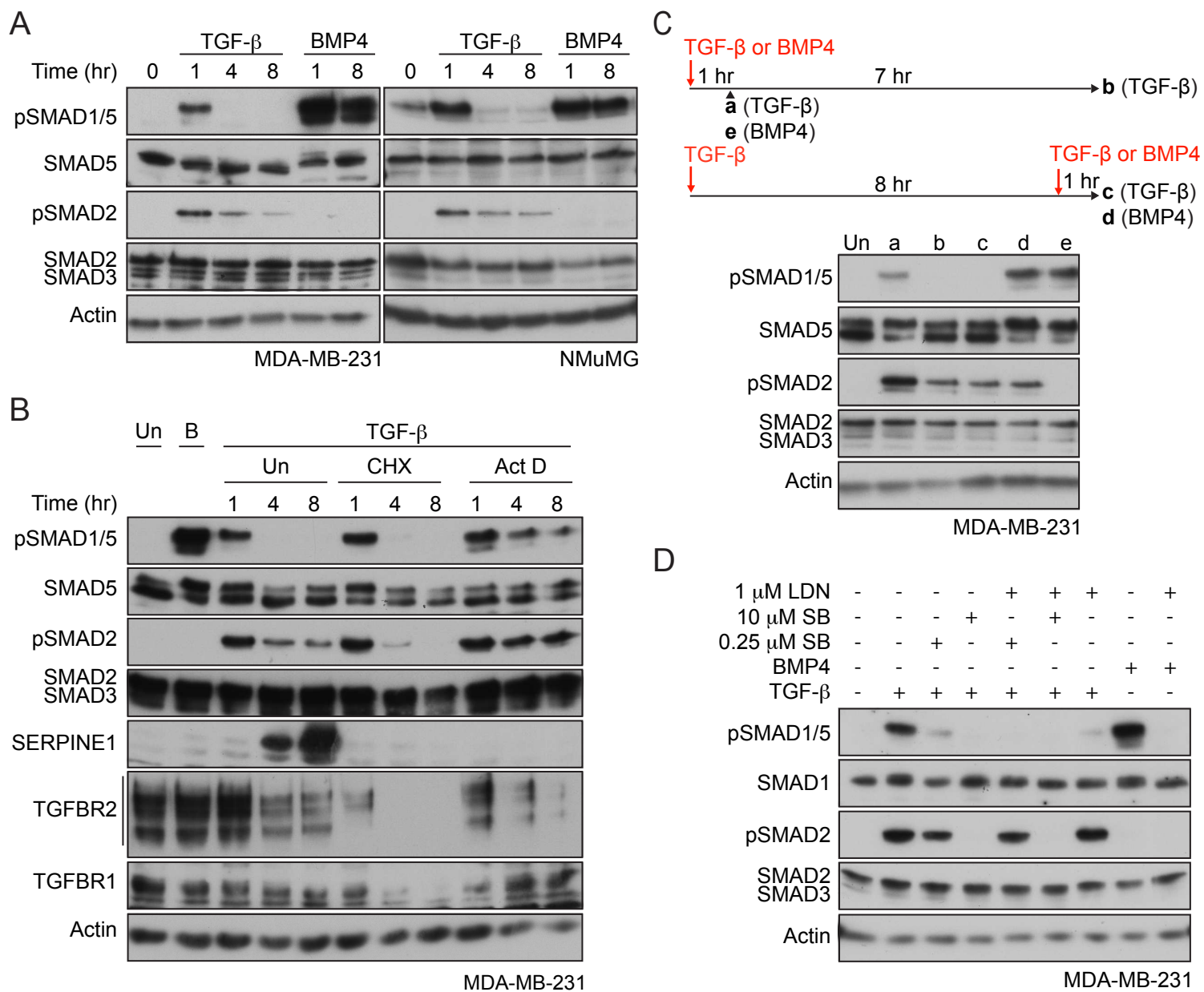


Figure 1

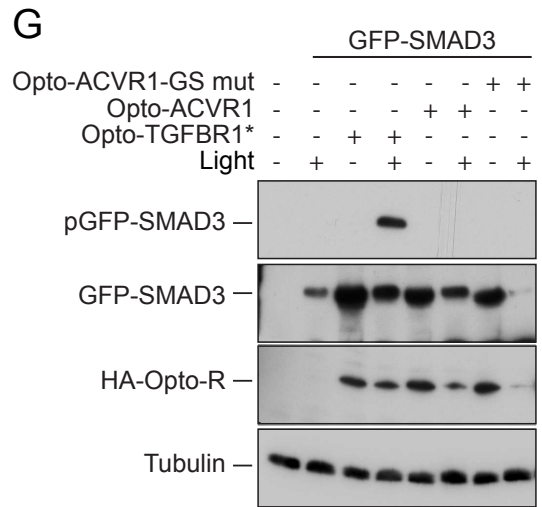
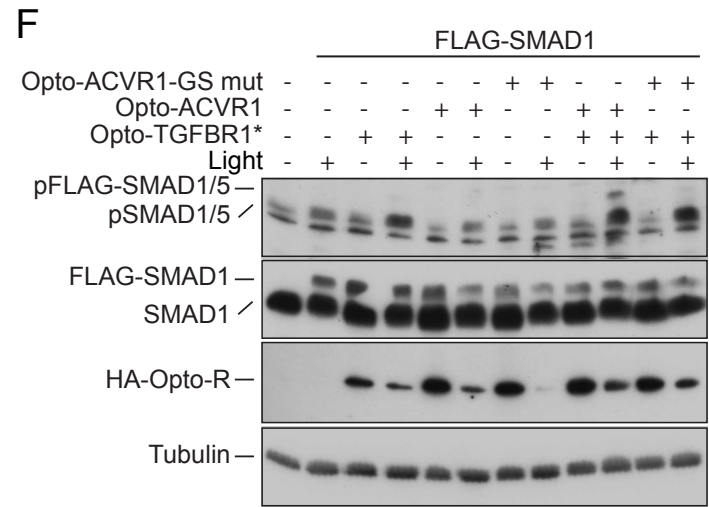
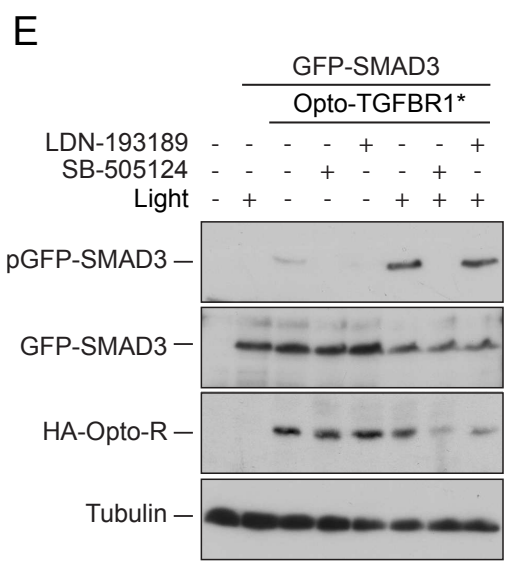
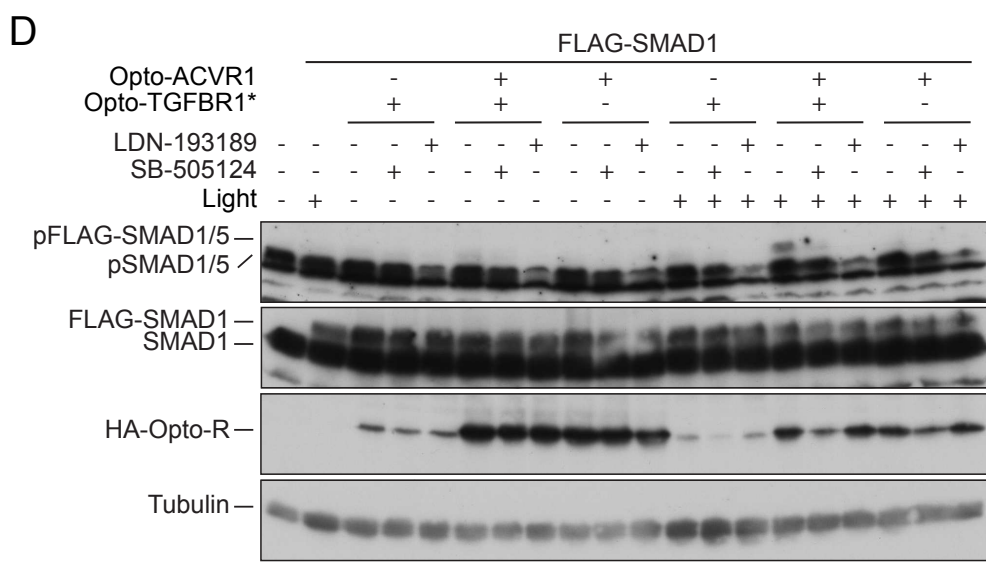
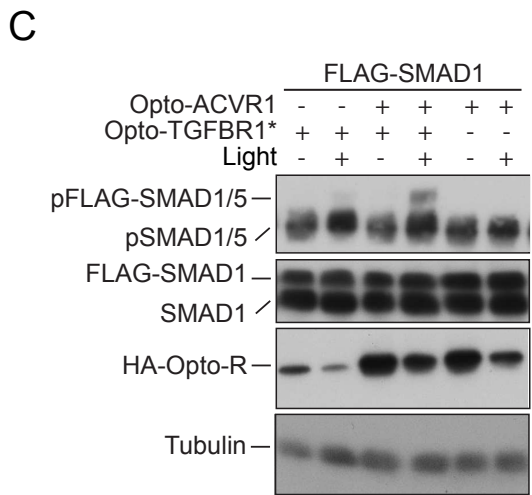
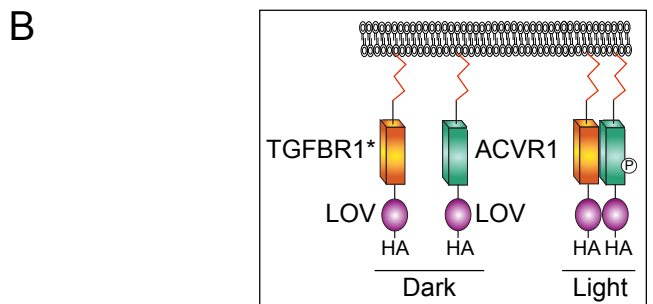
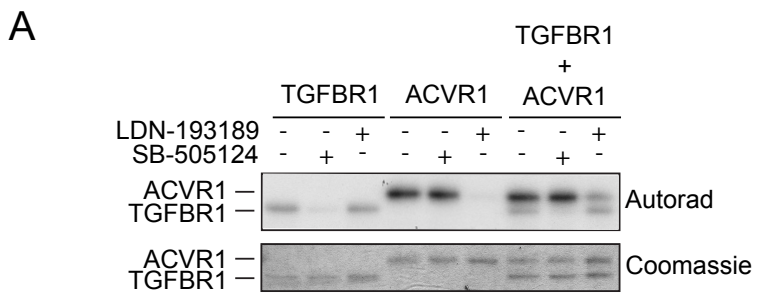
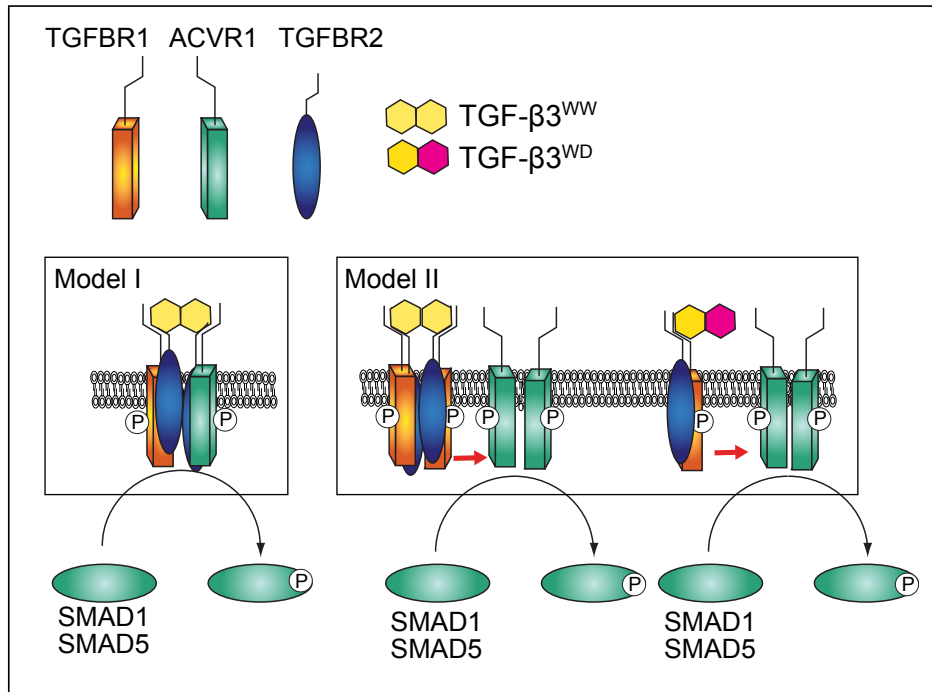


Figure 2

A



B

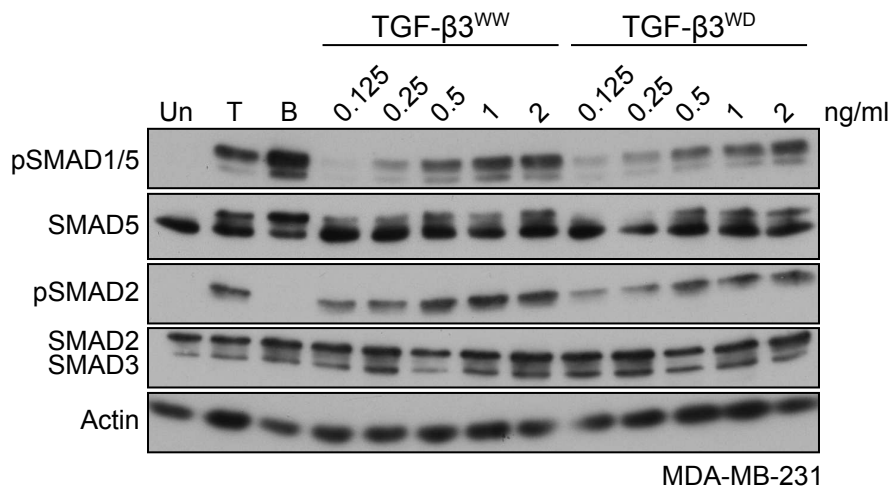


Figure 3

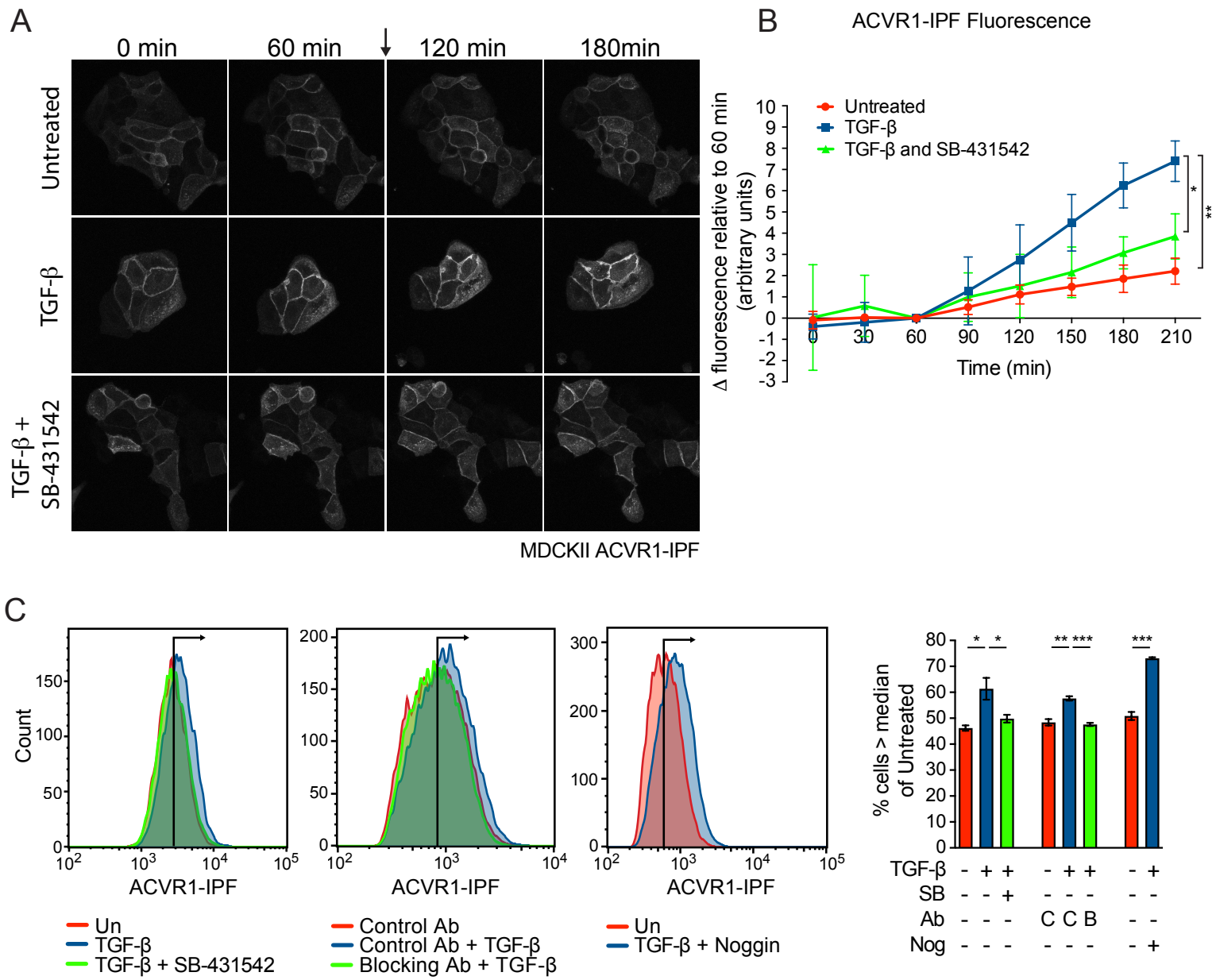


Figure 4

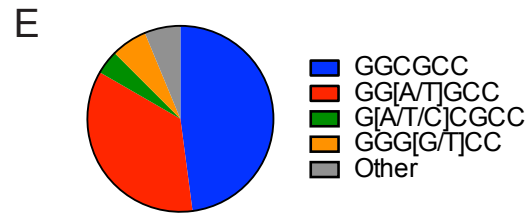
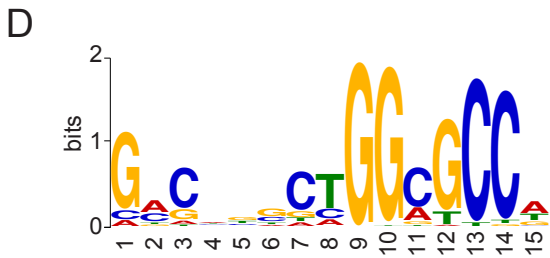
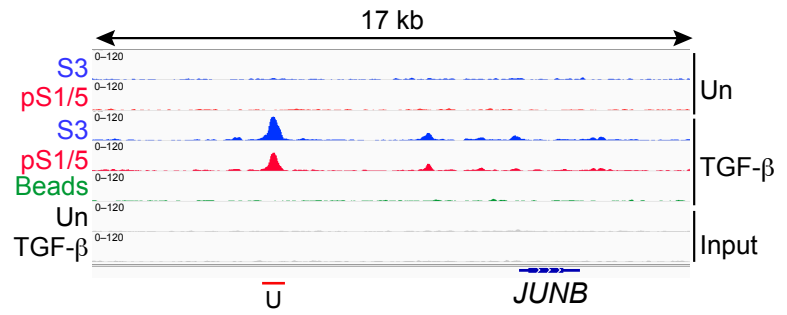
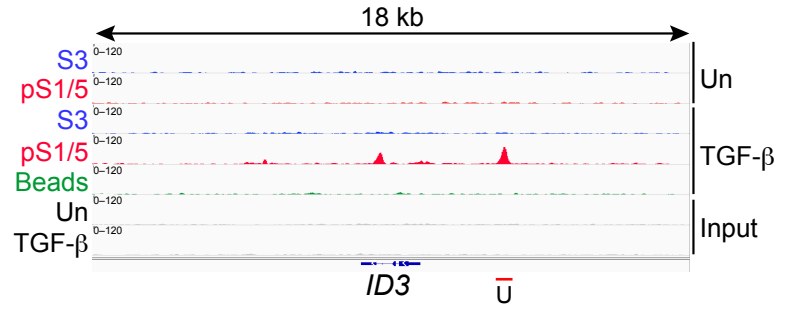
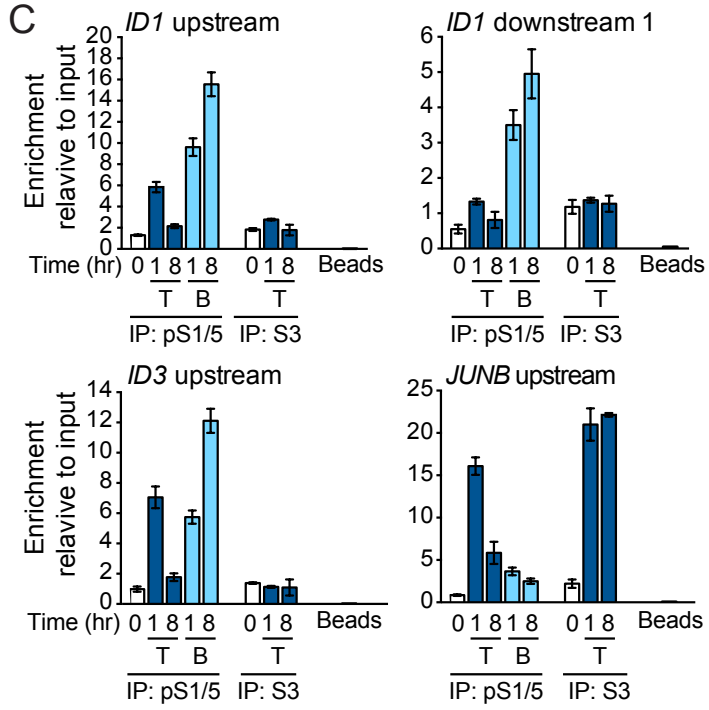
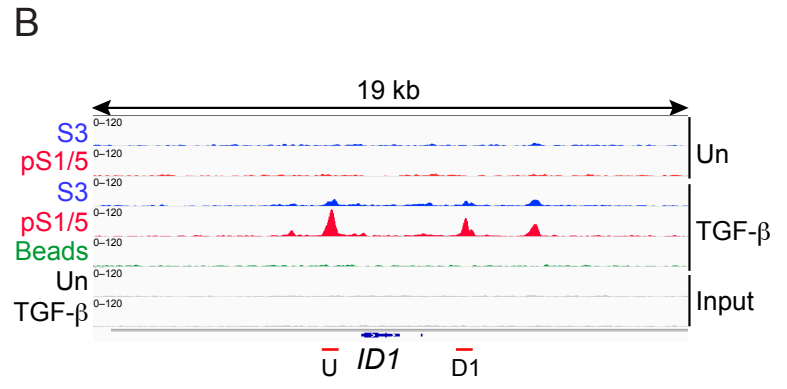
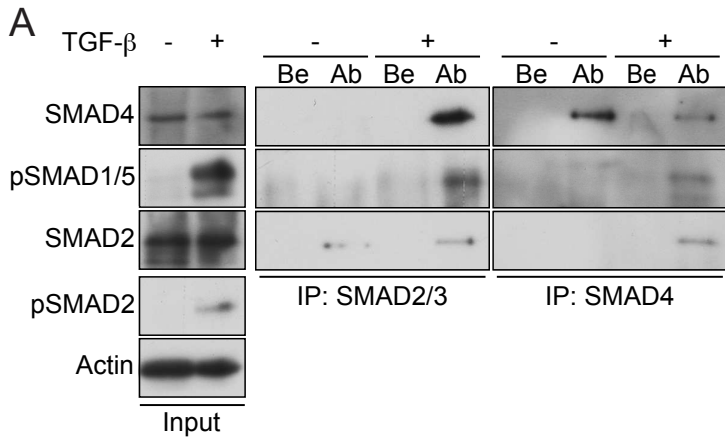


Figure 5

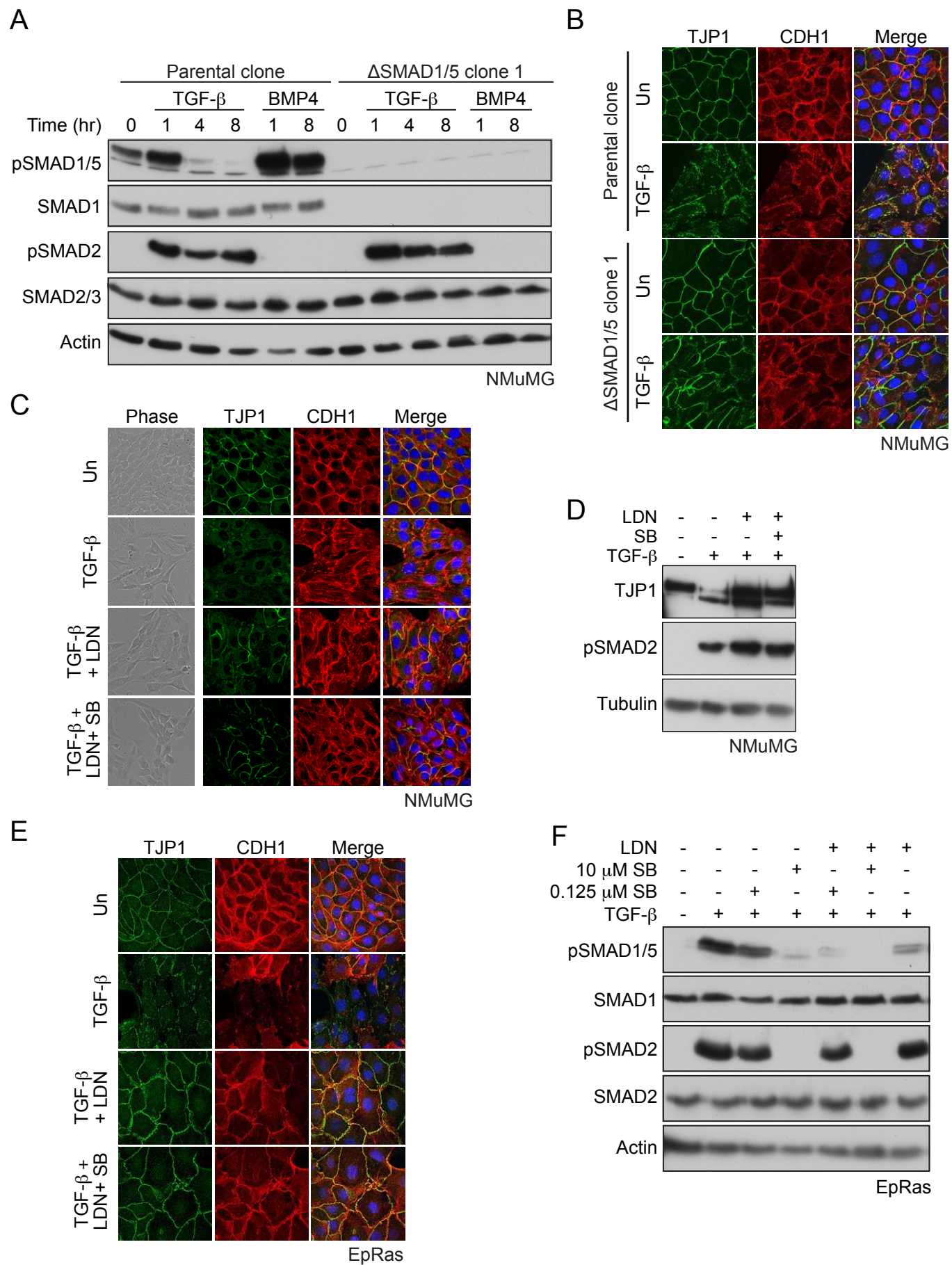


Figure 6

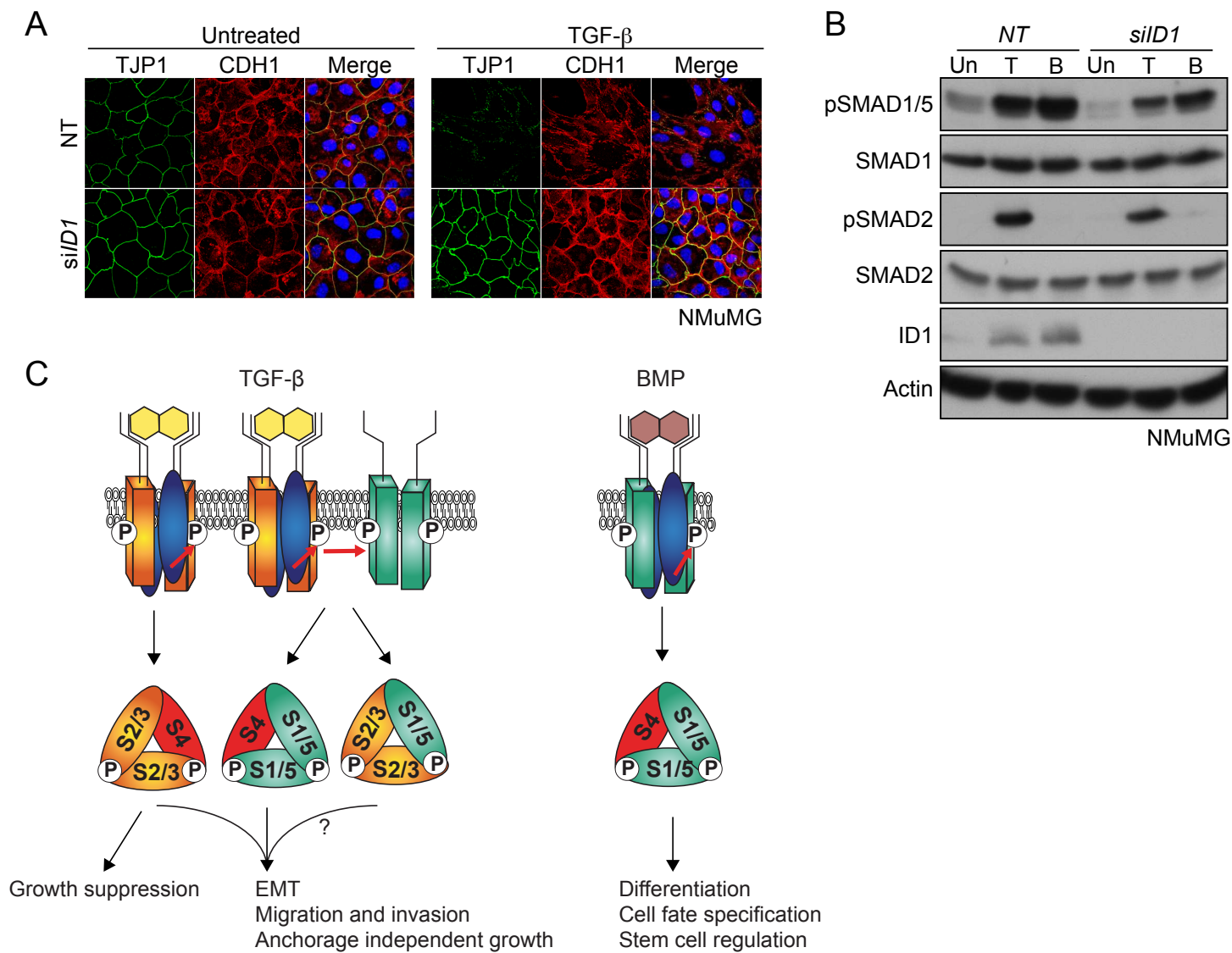


Figure 7

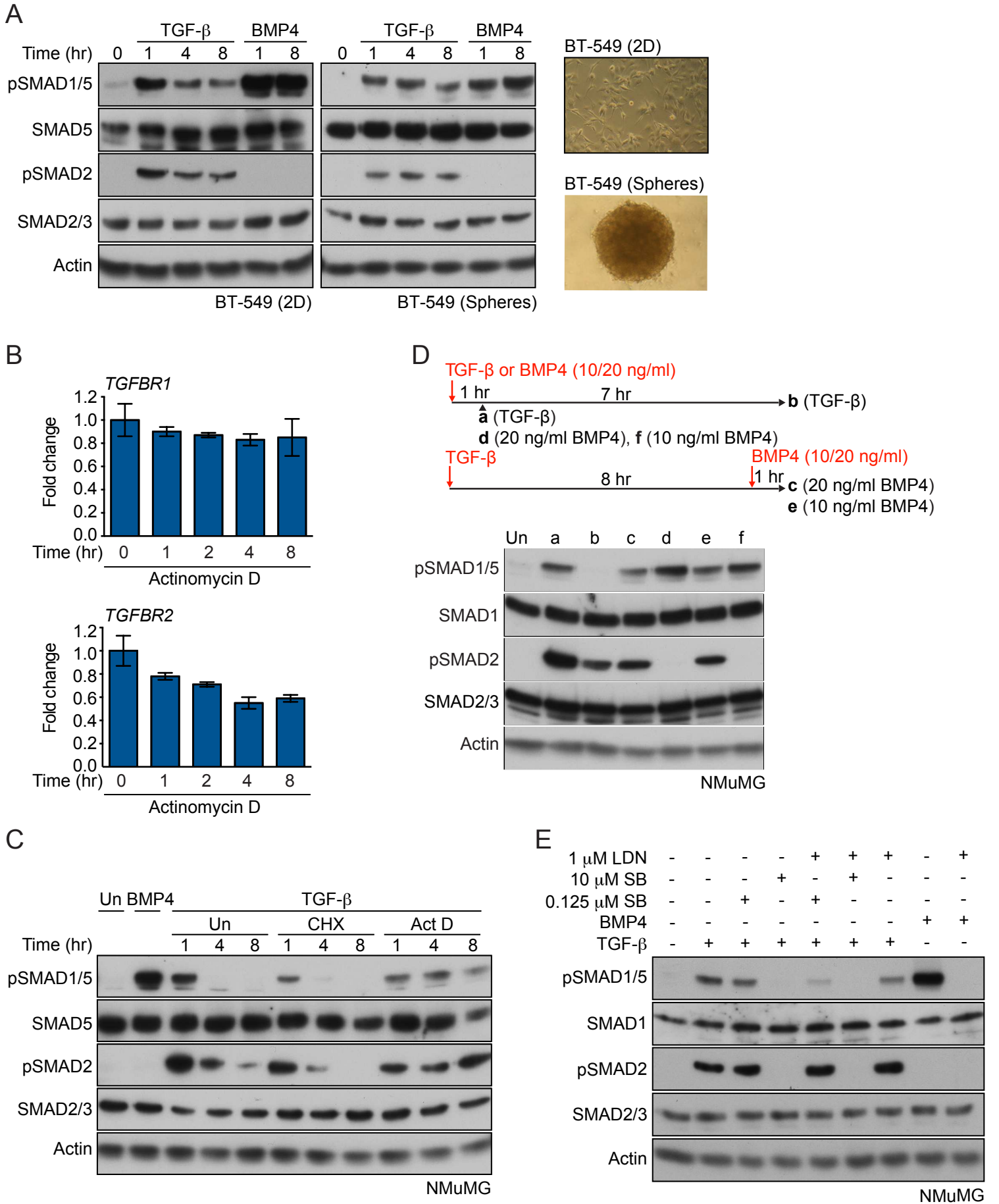


Figure 1 - figure supplement 1

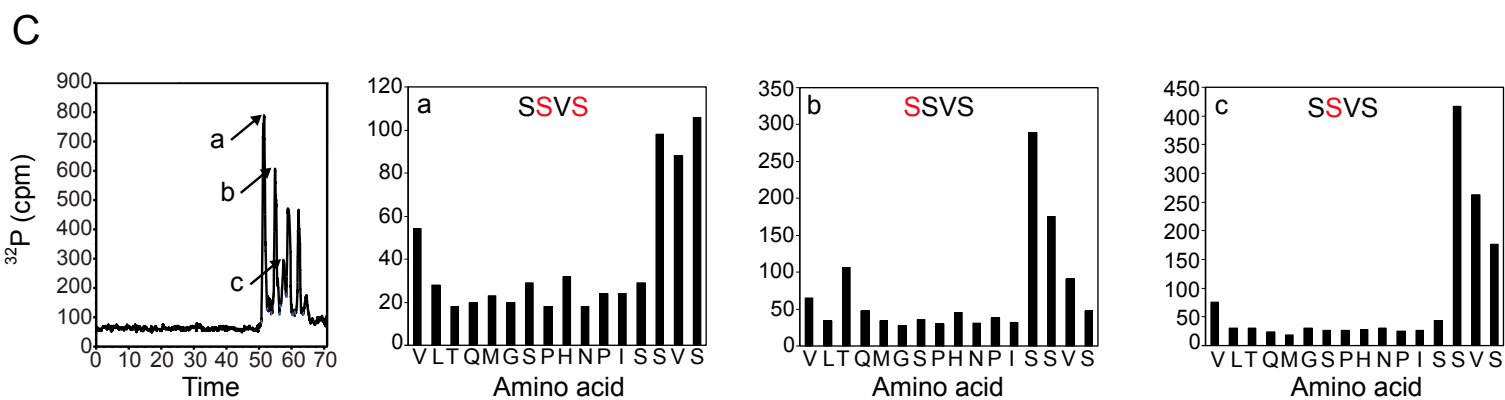
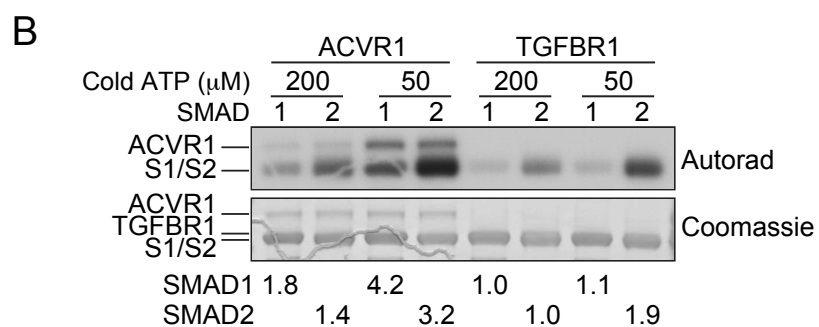
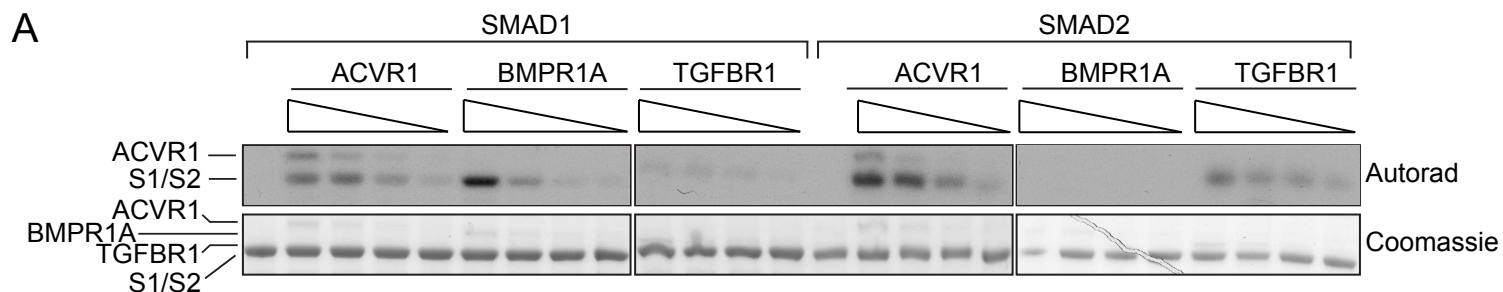


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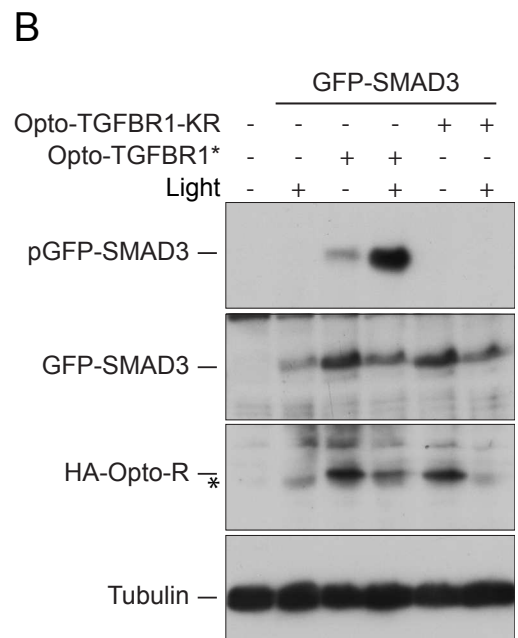
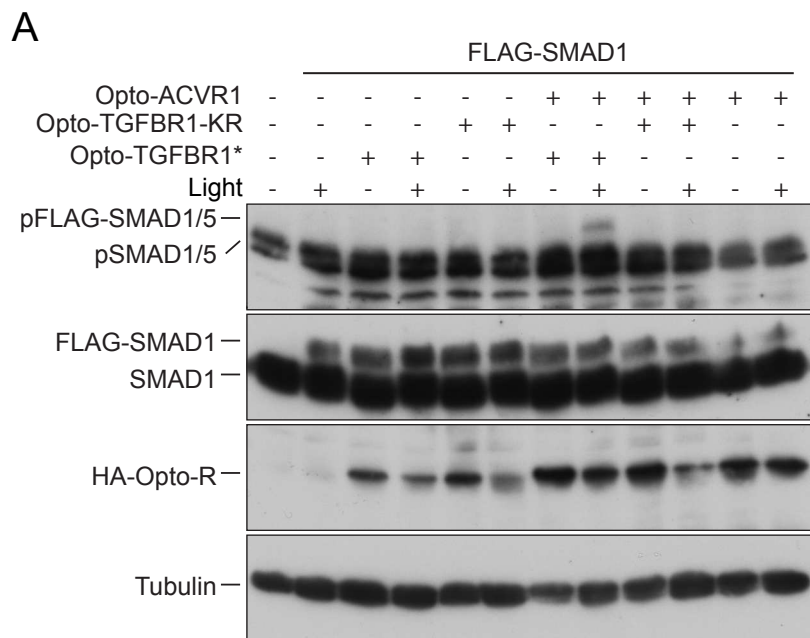


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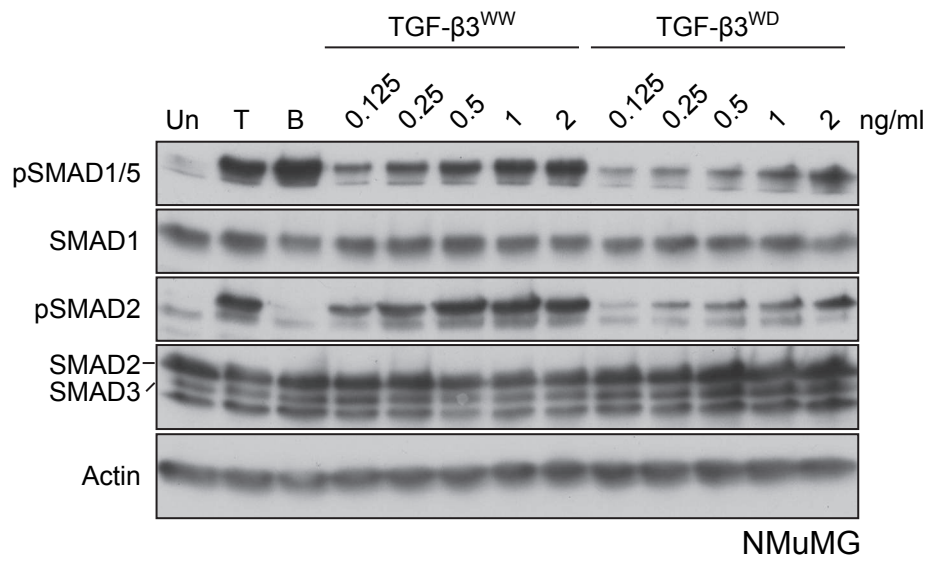


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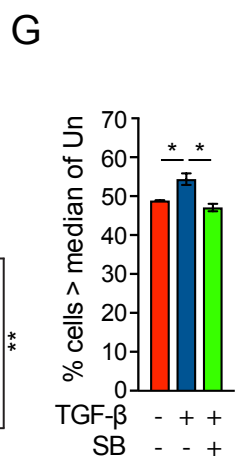
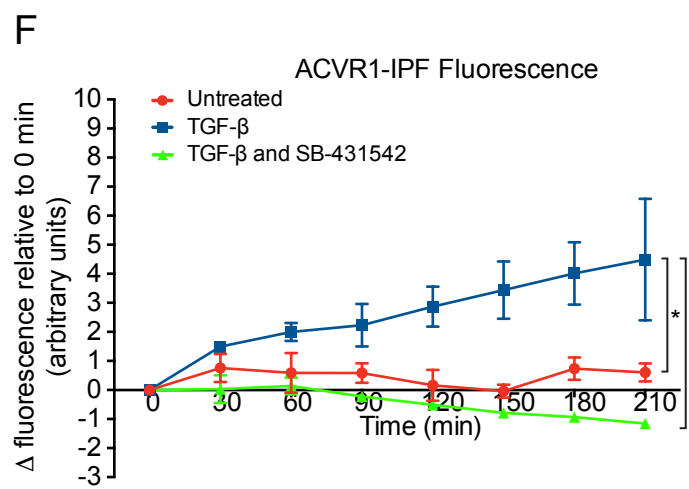
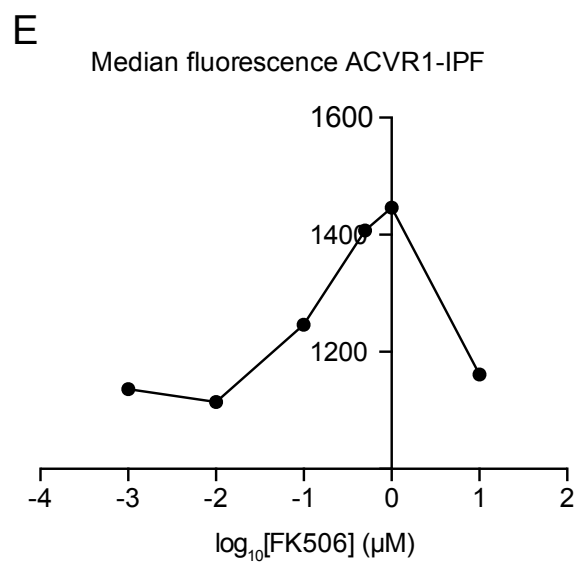
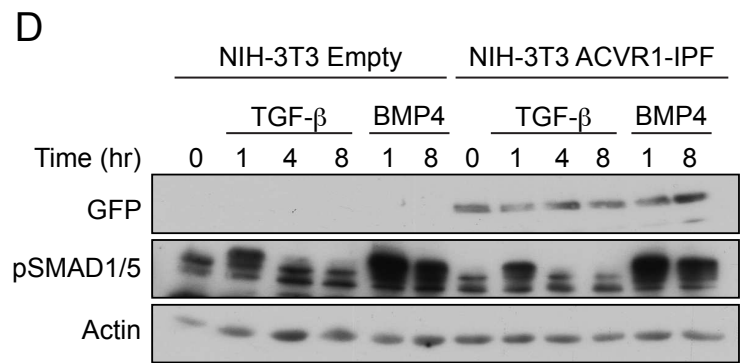
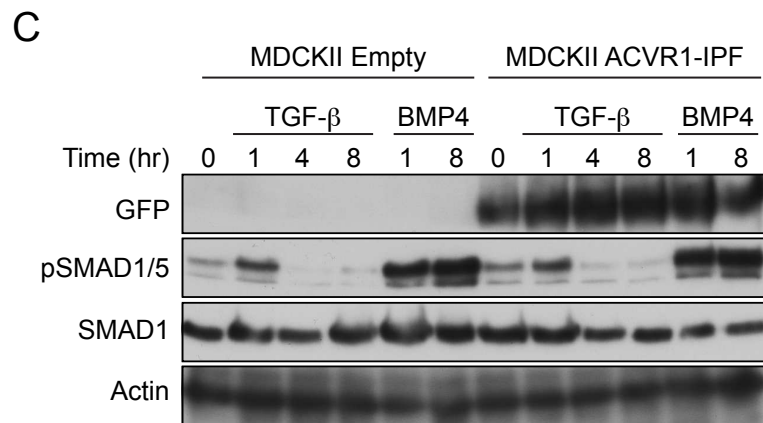
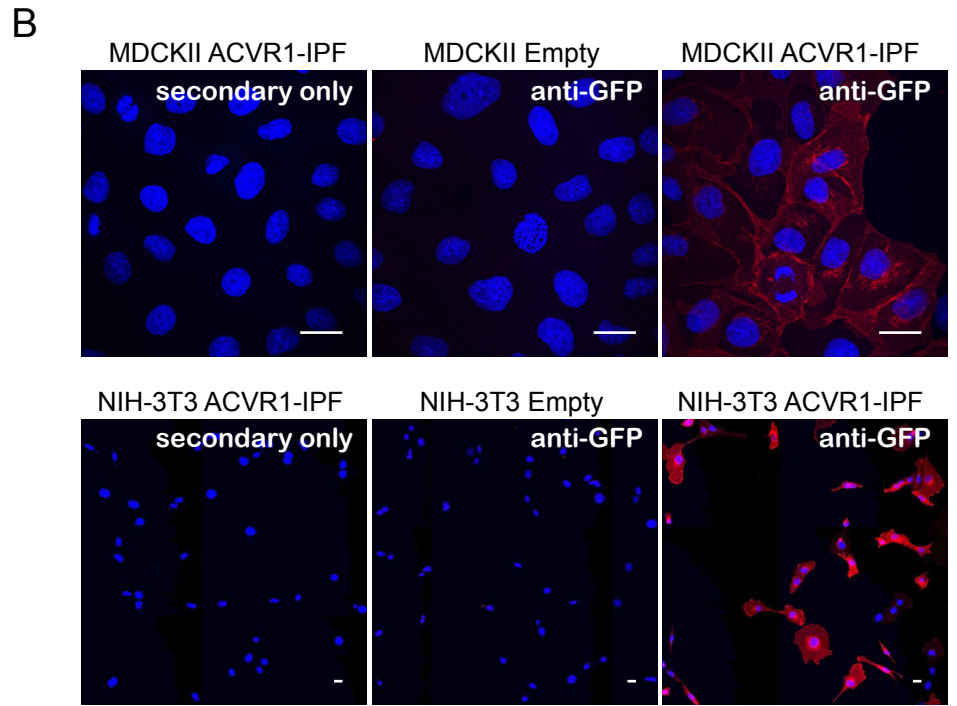
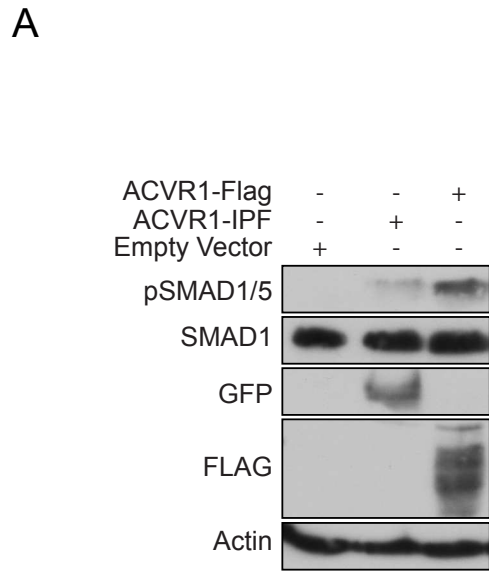
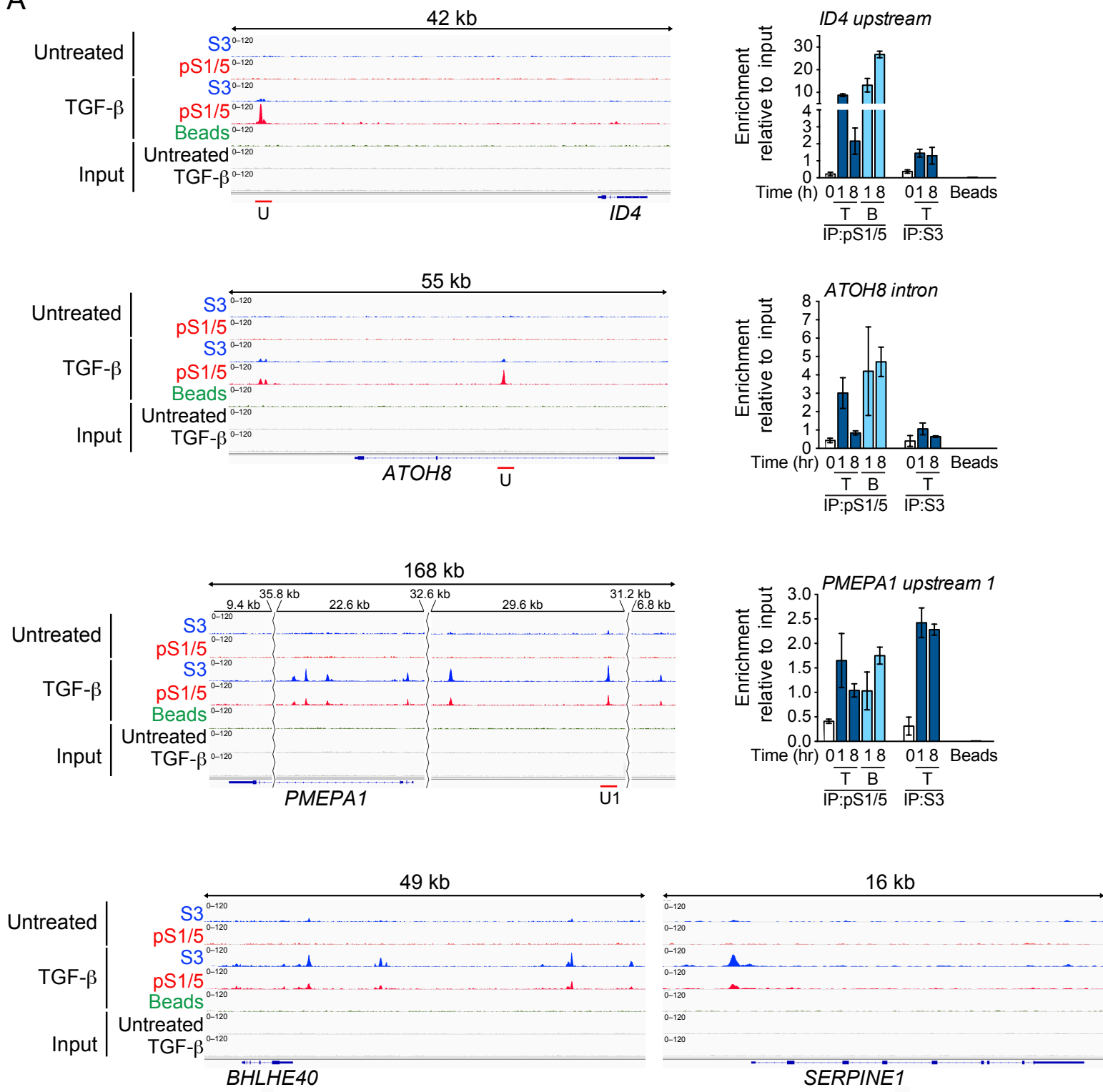
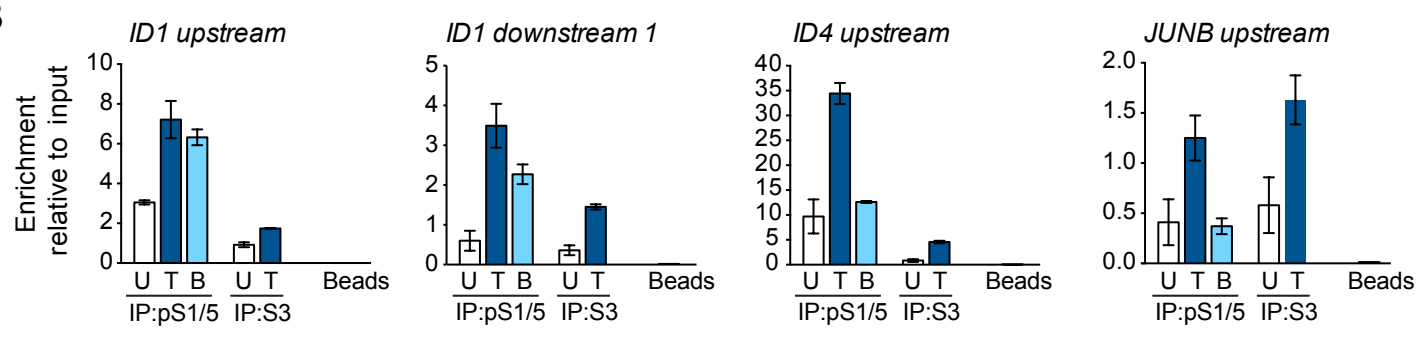


Figure 4 - figure supplement 1

A



B



C

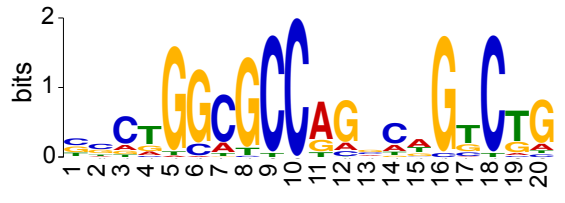


Figure 5 - figure supplement 1

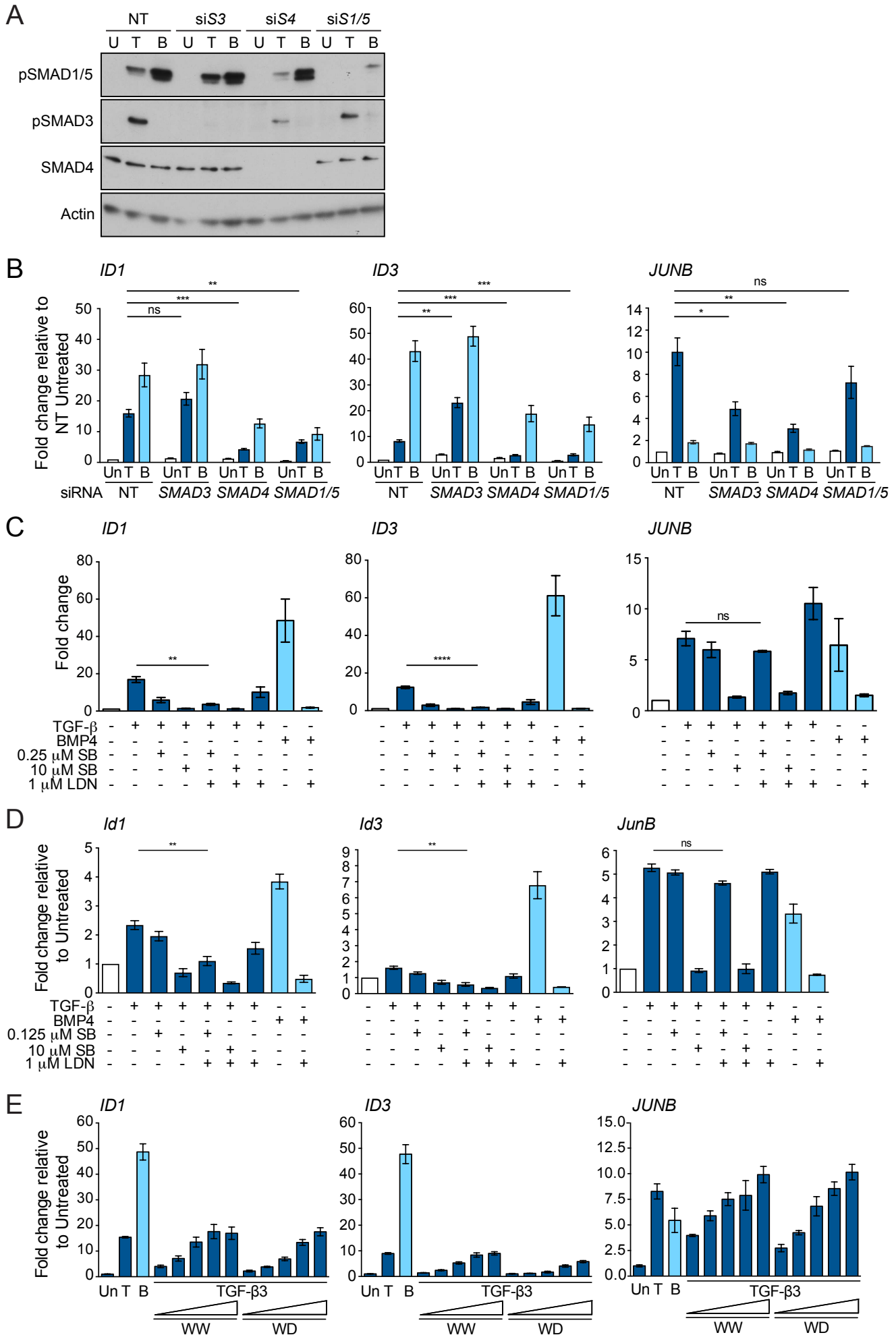


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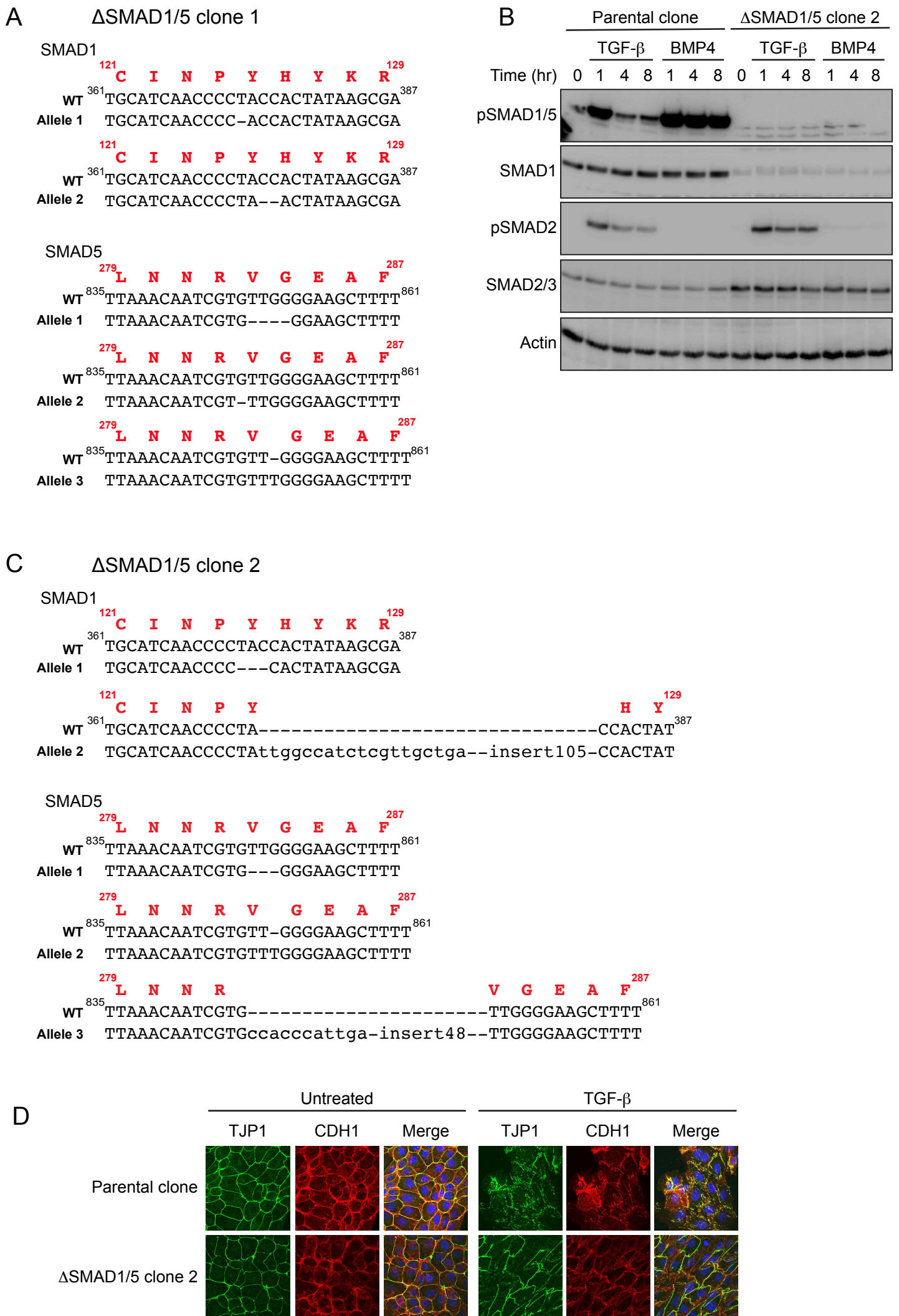


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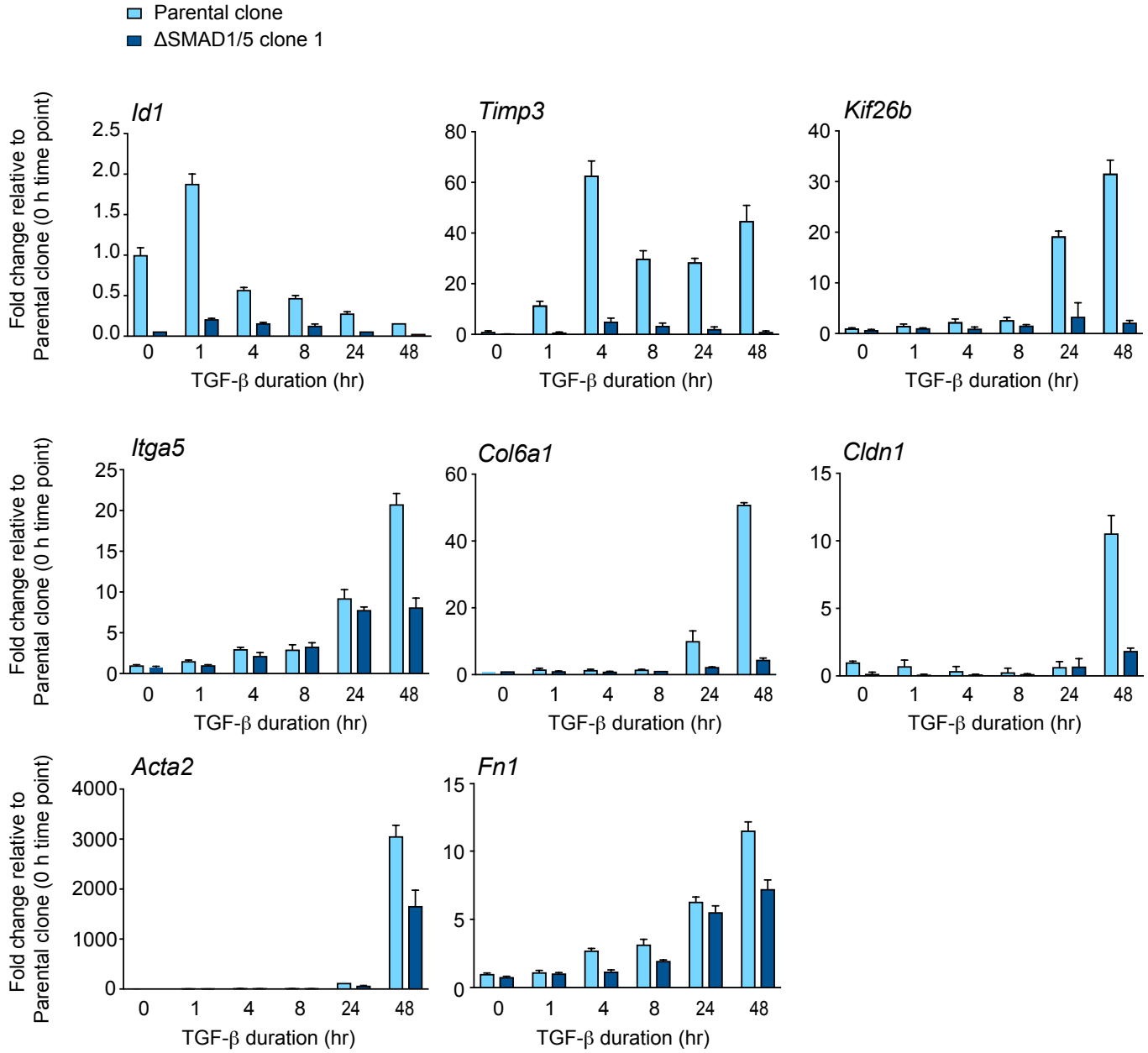
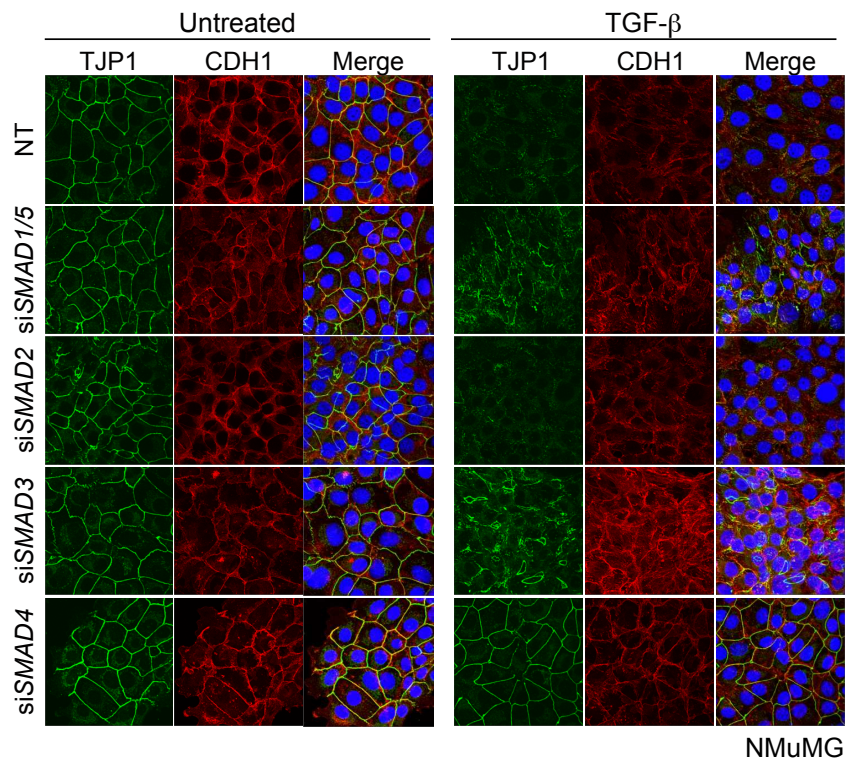
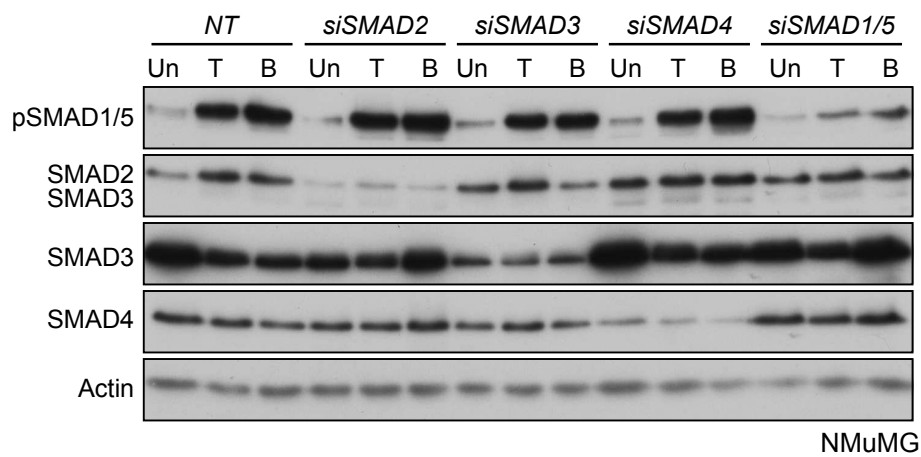


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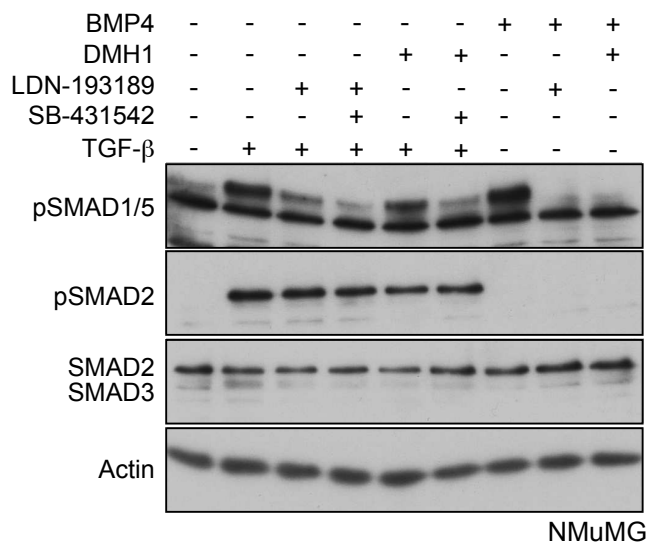
A



B



C



D

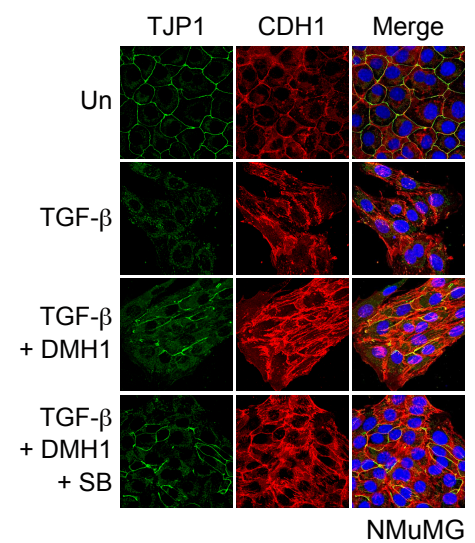


Figure 6 - figure supplement 3

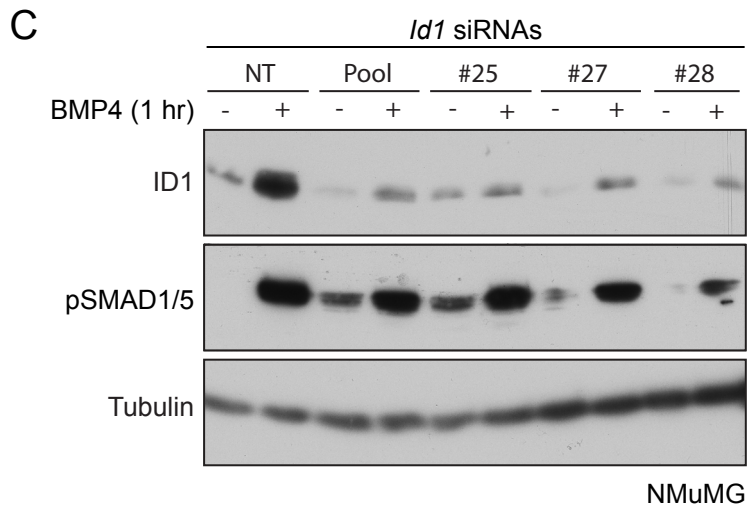
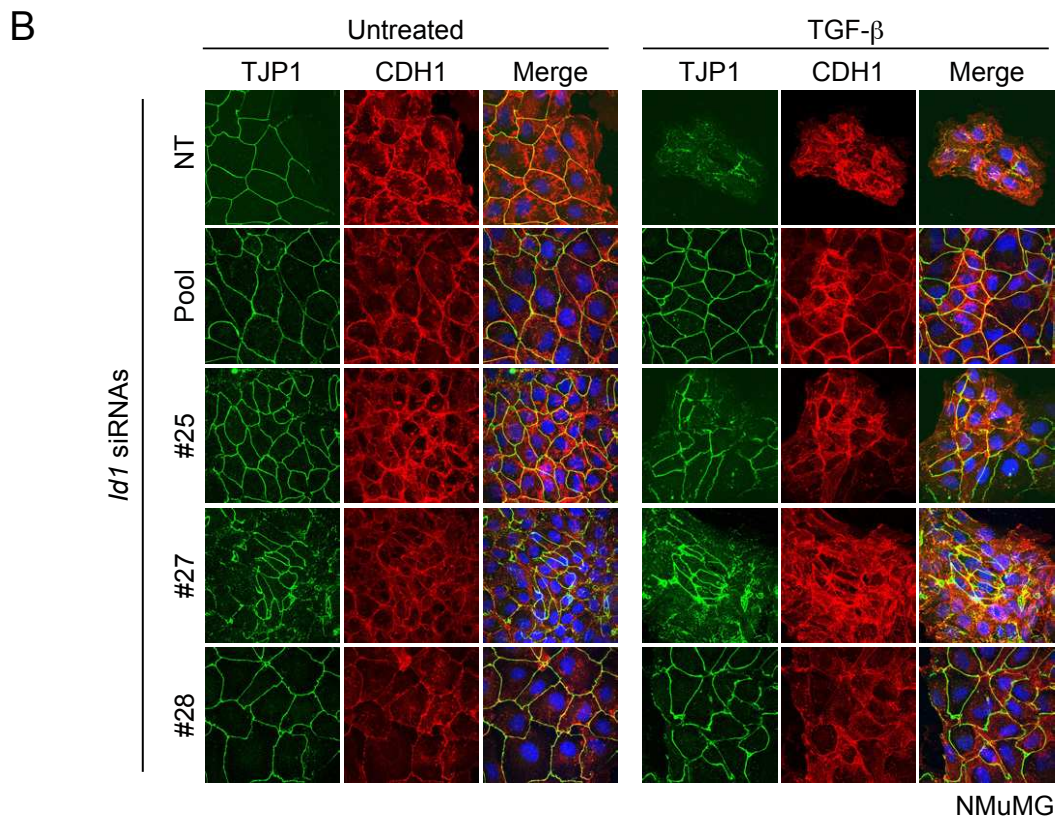
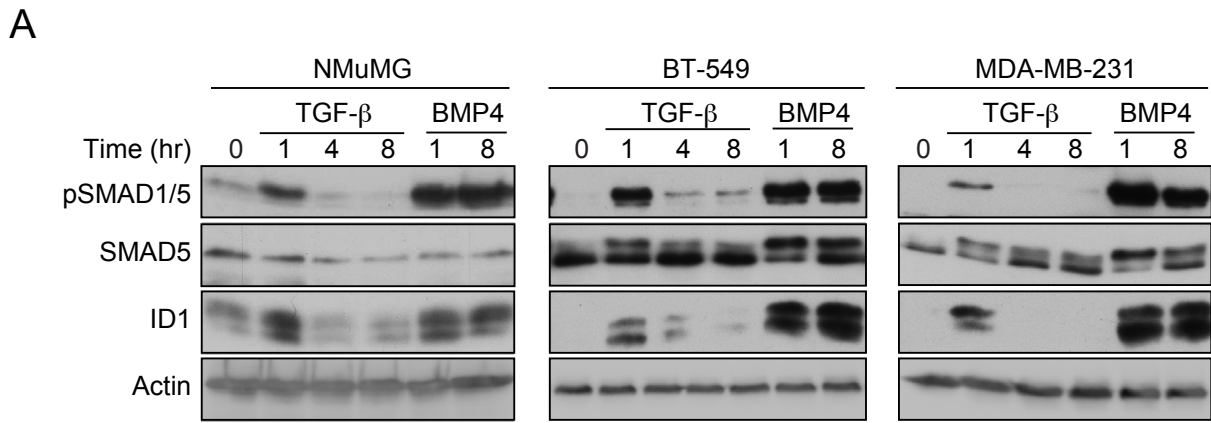


Figure 7 - figure supplement 1