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5 **TGF- β family ligands exhibit distinct signaling dynamics**
6 **that are driven by receptor localization**

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25 trafficking

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29 **Abstract**

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31 Growth factor-induced signal transduction pathways are tightly regulated at multiple
32 points intracellularly, but how cells monitor levels of extracellular ligand and translate
33 this information into appropriate downstream responses remains unclear.
34 Understanding signaling dynamics is thus a key challenge in determining how cells
35 respond to external cues. Here, we demonstrate that different TGF- β family ligands,
36 namely Activin A and BMP4, signal with distinct dynamics, which differ profoundly
37 from those of TGF- β itself. The distinct signaling dynamics are driven by differences
38 in the localization and internalization of receptors for each ligand, which in turn
39 determine the capability of cells to monitor levels of extracellular ligand. Using
40 mathematical modeling, we demonstrate that the distinct receptor behaviors and
41 signaling dynamics observed may be primarily driven by differences in ligand-
42 receptor affinity. Furthermore, our results provide a clear rationale for the different
43 mechanisms of pathway regulation found *in vivo* for each of these growth factors.

44 **Introduction**

45 The transforming growth factor β (TGF- β) family of ligands plays diverse roles in
46 embryonic development and adult tissue homeostasis, and moreover, their signaling is
47 deregulated in a range of human diseases, including cancer (Massague, 2008, Pickup
48 et al., 2017). The mammalian family consists of 33 members, which signal via the
49 same conserved mechanism (Moses et al., 2016). Two classes of cell surface
50 serine/threonine kinase receptors, termed type I and type II, recognize TGF- β family
51 ligands. Ligand binding brings the receptors together, allowing the constitutively
52 active kinase of the type II receptor to phosphorylate the type I receptor. This both
53 activates the type I receptor, and provides a binding site for the intracellular effectors
54 of the pathways, the SMADs (Heldin and Moustakas, 2016). The receptor-regulated
55 SMADs (R-SMADs) become phosphorylated at their extreme C-termini by the type I
56 receptor, which drives the formation of complexes with the common mediator
57 SMAD, SMAD4. These complexes accumulate in the nucleus where they regulate the
58 transcription of a battery of target genes in conjunction with specific co-factors. The
59 TGF- β family has traditionally been split into two pathways, with the TGF- β s,
60 NODAL and Activin leading to the phosphorylation of SMAD2/3, whereas the BMPs
61 and some of the GDFs induce phosphorylation of SMAD1/5/9 (Schmierer and Hill,
62 2007). This, however, is a simplification, as some ligands, in particular TGF- β and
63 Activin, can activate both signaling arms (Daly et al., 2008, Hatsell et al., 2015,
64 Ramachandran et al., 2018).

65 TGF- β receptors are known to internalize in the absence and presence of
66 ligand, and once activated, to signal from early endosomes (Di Guglielmo et al., 2003,
67 He et al., 2015, Miller et al., 2018, Mitchell et al., 2004). A proportion of internalized
68 receptors have been shown to recycle constitutively back to the cell surface, while the
69 remainder are targeted for degradation (Le Roy and Wrana, 2005, Yakymovych et al.,
70 2018). Although the mechanisms underlying the immediate cellular response to TGF-
71 β family ligands is relatively well understood, the response to longer durations of
72 ligand exposure, and the resulting dynamics of signaling, have been much less
73 studied. All the mammalian TGF- β family ligands signal through just seven type I
74 and five type II receptors, so the wide range of cell behaviors seen in response to
75 different ligands are likely to involve additional levels of complexity, some of which
76 will be at the level of signaling dynamics. Because cells are exposed to the continuous

77 presence of TGF- β family ligands during embryonic development and in disease
78 states (Hill, 2017, Miller and Hill, 2016, Schier and Talbot, 2005), as well as in the
79 context of regenerative medicine (Pagliuca et al., 2014), it is crucial to understand
80 how long-term exposure to ligands is regulated. This will be essential for identifying
81 potential novel points of intervention in each pathway, both experimentally and for
82 the development of therapeutic strategies. Moreover, as all TGF- β family ligands
83 result in the phosphorylation of just two classes of R-SMAD, understanding whether
84 particular ligands lead to different dynamic patterns of SMAD phosphorylation, and
85 how these are regulated, is critical for our understanding of how these pathways
86 evolved and diverged.

87 We have previously shown that in response to the continuous presence of
88 TGF- β , cells enter a refractory state where they no longer respond to acute TGF- β
89 stimulation. This is due to the rapid depletion of receptors from the cell surface in
90 response to ligand (Vizan et al., 2013). This means that intracellular signaling
91 downstream of TGF- β (as read out, for example, by levels of phosphorylated R-
92 SMADs) is not proportional to the duration of signaling, neither is it sensitive to the
93 presence of ligand antagonists in the extracellular milieu. This type of behavior would
94 clearly be incompatible with the ability of ligands like BMPs, NODAL and Activin to
95 act as morphogens that signal over many cell diameters in the context of embryonic
96 development and tissue homeostasis (Langdon and Mullins, 2011, Hedger and de
97 Kretser, 2013). We thus postulated that these other TGF- β family ligands might
98 respond to prolonged ligand exposure in a different manner to TGF- β .

99 We set out to directly test this hypothesis by fully characterizing the response
100 of cells to prolonged Activin and BMP4 stimulation. Our results show that in contrast
101 to TGF- β , cells integrate their response to BMP4 and Activin over time, and do not
102 enter a refractory state when stimulated with these ligands. Moreover, we observe an
103 oscillatory SMAD1/5 phosphorylation in response to BMP4 stimulation, which we
104 show is driven by the transient expression of the I-SMADs, SMAD6 and SMAD7,
105 which leads to a temporary depletion of receptors from the cell surface. By combining
106 our experimental insights with mathematical modeling we can explain these distinct
107 behaviors of Activin, BMP4 and TGF- β by differences in trafficking of their cognate
108 receptors, and differential affinities of ligands for their receptors. This in turn may
109 explain the distinct functional roles these ligands play *in vivo*.

110 **Results**

111 **BMP4 and Activin exhibit distinct patterns of signaling dynamics**

112 We have previously shown that when cells are stimulated with TGF- β , SMAD2
113 phosphorylation peaks after 1 hr, before attenuating to lower levels. After an initial
114 acute response, cells are refractory to further acute stimulation due to an almost
115 complete depletion of receptors from the cell surface (Vizan et al., 2013). To
116 understand whether this was a common feature of all TGF- β family ligands, we
117 characterized the response of cells to other members of the TGF- β family, namely
118 Activin A and BMP4, and compared and contrasted them with each other and with
119 TGF- β . For the Activin responses we have predominantly used the P19 mouse
120 teratoma cell line, as SMAD2 is robustly phosphorylated in response to Activin in this
121 cell line (Coda et al., 2017). Activin signaling in these cells is mediated by ACVR1B
122 as the type I receptor, and either ACVR2A or ACVR2B as the type II receptors, as
123 demonstrated by the abrogation of signaling when these receptors are knocked down
124 by siRNA (Figure 1 – figure supplement 1). These cells also produce and secrete the
125 TGF- β family ligands NODAL and GDF3, resulting in a relatively high level of basal
126 level of SMAD2 phosphorylation (Coda et al., 2017). To characterize the BMP4
127 responses we have predominantly used the human breast cancer cell line, MDA-MB-
128 231 and the mouse fibroblast cell line NIH-3T3, both of which induce robust
129 SMAD1/5 phosphorylation in response to BMP4. In addition, we have used HaCaTs,
130 the cell line we previously used to characterize TGF- β signaling dynamics (Vizan et
131 al., 2013).

132 In response to continuous stimulation with BMP4, SMAD1/5 phosphorylation
133 in MDA-MB-231 cells peaks after 1 hr, then drops down to a lower level after 4 hr,
134 before increasing back up to its maximal level after 8 hr of stimulation (Figure 1A).
135 This is strikingly different to the dynamics of signaling seen in response to TGF- β ,
136 where chronic exposure of cells to ligand leads to signal attenuation resulting in a low
137 level of SMAD2 phosphorylation (Vizan et al., 2013). A similar single oscillation is
138 evident when NIH-3T3 cells (Figure 1 – figure supplement 2A) or human
139 keratinocyte HaCaT cells (Figure 1 – figure supplement 2B) are stimulated with
140 BMP4, although NIH-3T3s reach their low point of signaling after 2 hr of stimulation,
141 rather than 4 hr, and in neither of these cell types does the signal return to the
142 maximal level, as in does in the MDA-MB-231 line. The long term response to

143 Activin is different. P19 cells stimulated with Activin exhibit maximal levels of
144 PSMAD2 after 1 hr, which modestly attenuates down to the basal level over the next
145 24 hr (Figure 1B). Basal PSMAD2 is completely abolished by overnight incubation
146 with the type I receptor inhibitor SB-431542 (Inman et al., 2002a) (Figure 1B). P19s
147 can also be induced by Activin from the SB-431542-inhibited baseline, and in this
148 case, show a very sustained response, due to the autocrine production of NODAL and
149 GDF3 (Coda et al., 2017). In HaCaTs, in contrast, the baseline of PSMAD2 is low
150 and the Activin response is more transient, likely because HaCaTs do not exhibit
151 autocrine signaling (Figure 1C)

152

153 **Activin and BMP4 signaling is integrated over time**

154 We next sought to determine whether signaling by Activin and BMP4 is integrated
155 over time after stimulation, and compared the behaviors with that TGF- β . Cells were
156 therefore stimulated for increasing periods of time with Activin, BMP4, or TGF- β ,
157 and then chased for the remainder of the 1 hr with saturating doses of the natural
158 ligand antagonists, Follistatin (Nakamura et al., 1990) or Noggin (Zimmerman et al.,
159 1996) for Activin and BMP4 respectively, or, in the case of TGF- β , the neutralizing
160 antibody 1D11 (Nam et al., 2008) (Figure 2A). All cells were harvested together at
161 the 1 hr time point. TGF- β induced a maximal PSMAD2 response after just 5 mins of
162 exposure to ligand (Figure 2B), which we have previously demonstrated is due to the
163 rapid depletion from the cell surface of the type II TGF- β receptor TGFBR2 within
164 this time frame, so that little to no new signaling is induced over the remainder of the
165 first hour of signaling (Vizan et al., 2013). In contrast, the cellular response to Activin
166 is integrated over the first hour of signaling, with a greater induction of PSMAD2
167 resulting from longer exposure to ligand (Figure 2C and D). A similar pattern was
168 observed with SMAD1/5 phosphorylation resulting from BMP4 stimulation in MDA-
169 MB-231 cells (Figure 2E) and HaCaT cells (Figure 2F). We conclude that cells
170 continuously monitor the presence of BMP4 and Activin in their extracellular
171 environment, such that the R-SMAD phosphorylation observed after 1 hr in response
172 to BMP and Activin is an integration of all of the signaling that has occurred in the
173 first hour. This behavior is distinct from that of TGF- β , where the SMAD
174 phosphorylation seen after 1 hr of stimulation is the result of the first 5 minutes of
175 ligand exposure.

176

177 **Stimulation with Activin and BMP4 does not induce refractory behavior**

178 We have previously shown that cells enter a refractory state in response to TGF- β
179 treatment, where they are unable to respond to acute stimulation with the same ligand.
180 To determine whether the same state is induced in response to Activin and BMP4,
181 cells were stimulated with these ligands for 1 hr, followed by ligand antagonists for 2
182 hr to reduce R-SMAD phosphorylation levels down to basal. The ligand antagonists
183 were then washed out and cells re-stimulated with ligand for 1 hr. The efficacy of the
184 ligand antagonists and their wash-out was confirmed (Figure 3A and B). For both
185 BMP4 (Figure 3A) and Activin (Figure 3B), re-stimulation to maximal PSMAD
186 levels was observed after just 2 hr treatment with ligand antagonists, indicating that
187 cells do not enter a refractory state in response to these ligands. This contrasts with
188 the behavior of cells stimulated with TGF- β . In this case, where cells take 12–24 hr
189 after the removal of external ligand to recover the ability to fully respond again to
190 ligand (Vizan et al., 2013).

191

192 **The distinct signaling dynamics of TGF- β , Activin and BMP4 are not explained**
193 **by the intracellular lifetimes of their receptors**

194 TGF- β family receptors can signal from internal cellular compartments (Itoh et al.,
195 2002), and we have shown that the lifetime of receptors in these compartments is
196 likely to be an important factor for regulating the dynamics of signaling (Vizan et al.,
197 2013). We therefore determined whether the distinct signaling dynamics observed in
198 response to each ligand could be driven by the duration for which activated receptors
199 signal from internal compartments.

200 To address this, cells were stimulated for 1 hr with TGF- β , BMP4 or Activin,
201 then chased over a time course of 8 hr with the cognate ligand antagonists 1D11,
202 Noggin and Follistatin respectively, and the levels of R-SMAD phosphorylation were
203 assayed (Figure 4). Because there is no new signaling induced by the activation of
204 receptors with external ligands once antagonists are added, any on-going PSMAD
205 signal must arise from the combined activities of the receptors signaling from
206 internalized compartments, and cellular R-SMAD phosphatases. To control for the
207 latter, the decay in R-SMAD phosphorylation due to the action of R-SMAD
208 phosphatases was assayed directly by chasing stimulated cells with receptor kinase

209 inhibitors SB-431542 (for TGF- β and Activin) or LDN-193189 (for BMP4; Cuny et
210 al., 2008) over the same time course. By comparing the decay in signal seen with
211 ligand antagonists versus receptor kinase inhibitors, the duration of signaling from
212 internal compartments can be determined. In the presence of the kinase inhibitors,
213 maximal R-SMAD dephosphorylation occurred within around 30 mins in all cases
214 (Figure 4B–D), with a half-life of approximately 15 mins, measured by fitting an
215 exponential decay curve to the data. In contrast, in the presence of ligand antagonists,
216 the signal in response to TGF- β decayed with a half-life of approximately 52 mins,
217 the signal from BMP4 in approximately 44 mins and that from Activin in
218 approximately 42 mins (Figure 4B–D). Thus, signaling persists for around 2 hr in all
219 cases, suggesting that receptors signal from endosomes for approximately 90 mins,
220 with no obvious differences seen between the different ligands.

221

222 **Receptor trafficking behaviors drive distinct signaling dynamics for the different** 223 **ligands**

224 We reasoned that differences in signaling dynamics could be driven by differences in
225 the behavior of the receptors for each ligand. Antibodies for Western blot were
226 validated against one of the BMP4 type II receptors, BMPR2 (Daly et al., 2008), the
227 Activin type I receptor ACVR1B (formerly known as ALK4) and ACVR2B
228 (Tsuchida et al., 2009). In all cases, PNGase treatment, which removes N-linked
229 sugars, resulted in an increased mobility of the receptors, and siRNA knockdown was
230 used to confirm the specificity of the antibodies and the identity of the correct band
231 (Figure 5 – figure supplement 1A–C).

232 We next determined the half-life of each receptor species to which we had
233 antibodies. Cycloheximide chase time courses were performed, which showed that
234 BMPR2 has a half-life of approximately 4 hr (Figure 5 – figure supplement 1D), and
235 ACVR1B approximately 1 hr (Figure 5 – figure supplement 1E). ACVR2B was not
236 noticeably degraded at all over the time course in either P19s (Figure 5 – figure
237 supplement 1F) or HaCaTs (Figure 5 – figure supplement 1G), indicating that it has a
238 much longer half-life than the other receptors tested. The half-lives of BMPR2 and
239 ACVR1B are of the same order as those previously calculated for the TGF- β
240 receptors (\sim 2 hr for TGFBR2 and \sim 4 hr for TGFBR1; Vizan et al., 2013).

241 We previously showed that TGFBR1 and TGFBR2 become rapidly depleted
242 from the surface of cells in response to TGF- β stimulation (Vizan et al., 2013). We
243 therefore wanted to know whether BMP and Activin stimulation similarly drives
244 receptor depletion, and used surface biotinylation assays on cells treated with BMP4
245 or Activin to test this. In MDA-MB-231 cells, BMPR2 was depleted from the cell
246 surface after 2 hr of BMP4 treatment, before re-accumulating at later time points
247 (Figure 5A). Although receptors re-accumulated, they did not appear to fully reach
248 their level in unstimulated cells. Receptor depletion and re-accumulation occurs with
249 similar dynamics to the oscillation in PSMAD1/5 levels seen in response to signal.
250 Despite the transient depletion of BMPR2, in response to long-term stimulation, it
251 remains present at the cell surface. This explains why cells do not become refractory
252 to further acute stimulation after treatment with BMP4.

253 By contrast, using P19 cells, we could show that neither ACVR1B nor
254 ACVR2B deplete from the cell surface in response to Activin or in the presence of the
255 receptor inhibitor, SB-431542 (Figure 5B). As a control for visualization of a cell
256 surface protein, whose levels change in response to signal, we assessed the cell
257 surface levels of the NODAL/GDF co-receptor TDGF1, whose expression is up-
258 regulated in response to Activin signaling. TDGF1 robustly accumulated in response
259 to Activin both at the cell surface and in whole cell lysates (Figure 5B). Again, the
260 constant presence of Activin receptors at the cell surface during ligand stimulation
261 explains why cells do not enter a refractory state after an acute Activin induction.
262 Cells thus remain competent to respond to acute doses of ligand in their extracellular
263 environment, even after an initial stimulation with Activin.

264

265 **The oscillatory response to BMP4 depends on the continuous presence of BMP4**
266 **in the extracellular milieu and requires new protein synthesis**

267 Stimulation with BMP4 leads to an oscillatory PSMAD1/5 response driven by
268 receptor depletion and re-accumulation. This oscillatory behavior is visible in
269 multiple cell lines from different species, including NIH-3T3s, MDA-MB-231s and
270 HaCaTs, although they show slightly different time points at which PSMAD1/5
271 reaches its nadir, and recover to different extents (Figure 1, Figure 1 – figure
272 supplement 2). Because NIH-3T3 cells exhibited the most robust oscillation, they
273 were used for subsequent experiments. To determine if the second wave of signaling

274 after the dip in PSMAD1/5 is a result of new receptor activation at the cell surface or
275 a second wave of signaling from internalized receptors, cells were stimulated for 1 hr
276 with BMP4, which was subsequently washed out (Figure 6 – figure supplement 1A)
277 or neutralized with Noggin (Figure 6 – figure supplement 1B). In both cases, no
278 second wave of signaling was seen, indicating that the continuous presence of BMP4
279 in the media is necessary for the second increase in PSMAD1/5 observed after an
280 initial decrease.

281 One possible explanation for these oscillatory dynamics is that another TGF- β
282 family ligand, such as TGF- β itself, could be playing a role, possibly as a feedback
283 target of the pathway that could be negatively regulating SMAD1/5 phosphorylation
284 (Gronroos et al., 2012). To exclude this possibility, at least for a large subset of
285 ligands that signal through SMAD2/3, BMP4 time courses were performed in the
286 presence and absence of the TGF- β /Activin/Nodal receptor inhibitor, SB-431542
287 (Figure 6 – figure supplement 1C). However, no differences in PSMAD1/5 dynamics
288 in response to BMP4 were seen in the presence or absence of SB-431542, ruling out
289 such a feedback mechanism.

290 We also investigated whether protein synthesis was required for the oscillatory
291 behavior. Time courses of BMP4 treatment were performed in the presence or
292 absence of the translation inhibitor, cycloheximide. In the presence of cycloheximide,
293 no oscillation in PSMAD1/5 levels was observed and levels remained high throughout
294 the time course (Figure 6 – figure supplement 2A). This indicates that a negative
295 regulator of the pathway must be expressed in response to signaling, and that this
296 factor is responsible for oscillatory PSMAD1/5 dynamics. To confirm this, time
297 courses of BMP4 treatment were performed in the presence of the transcriptional
298 inhibitor Actinomycin D (Figure 6 – figure supplement 2B). Again, the dip in
299 PSMAD1/5 levels seen in control cells is abrogated in the absence of new
300 transcription.

301

302 **The oscillatory response to BMP4 requires the inhibitory SMADs, SMAD6 and** 303 **SMAD7**

304 Two of the most likely candidates to be feedback inhibitors of BMP signaling are the
305 inhibitory SMADs (I-SMADs), SMAD6 and SMAD7. Both I-SMADs have long been
306 known to be targets of BMP signaling (Takase et al., 1998) and are negative

307 regulators of the pathway. Several mechanisms for their inhibitory activity have been
308 proposed, including interfering with SMAD complex formation (Hata et al., 1998),
309 inhibiting R-SMAD phosphorylation (Hayashi et al., 1997, Nakao et al., 1997,
310 Imamura et al., 1997), targeting receptors for degradation (Ebisawa et al., 2001,
311 Kavsak et al., 2000) or blocking the DNA binding and transcriptional activity of the
312 SMADs (Lin et al., 2003). In NIH-3T3s, qPCR revealed that in response to BMP4
313 stimulation, *Smad6* and *Smad7* mRNAs are both induced in a transient manner that is
314 the exact inverse of the PSMAD1/5 signal for *Smad6*, and in phase with PSMAD1/5
315 signal for *Smad7* (Figure 6A). siRNA-mediated knockdown of *Smad6* and *Smad7*
316 together abrogated the oscillation in PSMAD1/5 levels seen with control, non-
317 targeting (NT) siRNAs (Figure 6B). Individual siRNA pools against *Smad6* and
318 *Smad7* both abolish oscillations in PSMAD1/5, although knockdown of *Smad7* leads
319 to a weaker PSMAD1/5 response and a reduction in total SMAD1 levels (Figure 6 –
320 figure supplement 2C).

321 To confirm that these results apply across cell lines from different species, the
322 dynamics of expression of *SMAD6* and *SMAD7* in response to BMP4 in MDA-MB-
323 231 cells were also examined. *SMAD6* is induced after 2 hr of BMP4 stimulation and
324 stays elevated over the duration of an 8-hr time course, while *SMAD7* shows a
325 transient peak of expression after 2 hr, then declines down to a lower level (Figure 6 –
326 figure supplement 3A). Knockdown of *SMAD6* and *SMAD7* together in MDA-MB-
327 231 cells abrogates the PSMAD1/5 oscillation in a similar way to that observed in
328 NIH-3T3 cells (Figure 6 – figure supplement 3B), indicating that this mechanism is
329 conserved across species.

330

331 **SMAD6 and 7 are required for the transient depletion of BMPR2 from the cell** 332 **surface**

333 SMAD6 and SMAD7 have been described to target TGF- β superfamily receptors for
334 degradation (Ebisawa et al., 2001, Goto et al., 2007, Kavsak et al., 2000). We
335 therefore reasoned that the transient peak in their expression in response to BMP4
336 could be responsible for the transient depletion of BMP receptors from the cell
337 surface, leading to the subsequent dip in SMAD1/5 phosphorylation. To test this,
338 surface biotinylation assays were performed in NIH-3T3 cells transfected with either
339 control NT siRNAs or siRNAs against *Smad6* and *Smad7*. The BMPR2 receptor also

340 transiently depletes and re-accumulates in this cell line in response to BMP4
341 stimulation, indicating that this mechanism is conserved across species (Figure 6C).
342 With knockdown of SMAD6 and SMAD7, BMPR2 was no longer transiently
343 depleted from the cell surface in response to BMP4, but remained at high levels
344 throughout the time course, indicating that a failure to deplete receptors from the cell
345 surface in the absence of SMAD6 and SMAD7 underlies the lack of oscillation in
346 SMAD1/5 phosphorylation in this condition (Figure 6C).

347

348 **Using mathematical modeling to find the key parameters that dictate specific** 349 **signaling dynamics**

350 Finally, we used mathematical modeling to obtain clues as to key parameters that
351 might explain the distinct signaling dynamics of the different ligands. We previously
352 built a mathematical model of the TGF- β pathway that simulated the refractory
353 behavior of the TGF- β ligand (Vizan et al., 2013). Using this model as a starting
354 point, we used our experimental findings, as well as the published literature, to
355 determine whether, by changing some key parameters, we could simulate the
356 signaling dynamics of Activin and BMP4 that we observe experimentally.

357 A striking difference between TGF- β itself and the other TGF- β family
358 ligands is that TGF- β binds its receptors cooperatively, whilst there is no evidence for
359 cooperativity in receptor binding for BMP4 and Activin (Hinck, 2012, Groppe et al.,
360 2008). This likely explains the higher affinity measured for TGF- β 1 and TGF- β 3 for
361 their receptors ($K_d = 5\text{--}30$ pM) (De Crescenzo et al., 2003, Massague, 1990),
362 compared with the lower affinities measured for BMP4 and Activin with their cognate
363 receptors ($K_d = 110$ pM for BMP4 and $100\text{--}380$ pM for Activin) (Attisano et al.,
364 1992, Luyten et al., 1994).

365 Starting first with the Activin pathway, we used our model to investigate
366 whether lowering the affinity of Activin for its receptors would result in the distinct
367 behaviors we have measured for Activin signaling versus TGF- β signaling. We found
368 that implementing a K_d of 365 pM for Activin, and making minor adjustments to
369 several other parameters (see Methods section) resulted in the model converting from
370 simulating the characteristic behaviors of TGF- β signaling to those of Activin. The
371 modified model faithfully captures the long-term Activin dynamics both in cells with
372 no basal signaling, like HaCaTs, or with basal signaling, like P19s (Figure 7A). The

373 simulations also reproduced the observed integration of signaling over time (Figure
374 7B), the behavior of the pathway when receptors are inhibited with a small molecule
375 inhibitor, or when ligand is neutralised with Follistatin (Figure 7C), and also the
376 ability of the pathway to be re-stimulated after ligand removal (Figure 7D).

377 BMP4 signaling dynamics are similar to Activin's in the long term, but
378 additionally show oscillatory behavior in the short term. We have shown that SMAD6
379 and SMAD7 are required for the oscillation, likely due to their role in inducing
380 activated receptor degradation (Ebisawa et al., 2001, Kavsak et al., 2000). Their effect
381 is transient, because expression of *Smad6* and *Smad7* in response to BMP4 is transient
382 (Figure 6A). We implemented a K_d of 365 pM for BMP4 binding to its receptors, and
383 additionally included the induction of SMAD6/7 by nuclear PSMAD1–SMAD4
384 complexes. This was implemented with an RNA intermediate and a non-linear
385 dependency of *Smad6/7* expression on activatory PSMAD1–SMAD4 complexes. The
386 SMAD6/7 is then assumed to act on the stability of activated receptors (see Methods
387 section for the parameters and details of the modeling). This model captured all the
388 main behaviors of BMP signaling that we observe experimentally, including the
389 oscillation, signal integration over time, the behavior of the pathway when receptors
390 are inhibited, or when ligand is neutralised with Noggin, and also the ability of the
391 pathway to be re-stimulated after ligand removal (Figure 7E–H).

392 **Discussion**

393 **Receptor trafficking and degradation dictates signaling dynamics for different** 394 **TGF- β family ligands**

395 In both physiological and pathological contexts *in vivo*, cells are frequently exposed
396 to extracellular ligands for prolonged periods, yet little is currently understood about
397 how cells respond to sustained ligand exposure, or about how signaling dynamics are
398 modulated over time. In this study we have addressed these questions for members of
399 the TGF- β family of ligands. We have shown that the signaling dynamics differ
400 considerably between Activin, BMP4 and TGF- β and that they are dependent on the
401 localization and behavior of cell surface receptors. In contrast to the behavior of cells
402 treated with TGF- β , cells monitor the presence of Activin and BMP4 in the
403 extracellular milieu during signaling, and as a result, signaling is integrated over time.
404 Cells also do not enter a refractory state after an acute stimulation with Activin and
405 BMP4, as they do in response to TGF- β . However, while continuous Activin
406 stimulation leads to fairly stable SMAD2/3 phosphorylation in P19 cells, due to the
407 continuous presence of receptors at the cell surface and autocrine signaling, BMP4
408 stimulation in a number of different cell lines leads to a transient depletion of the
409 receptors from the cell surface due to the transient up-regulation of the I-SMADs,
410 SMAD6 and SMAD7. This in turn results in an oscillatory signaling response to
411 BMP4, where the response as read out by R-SMAD phosphorylation transiently dips
412 and then recovers.

413 We therefore propose a model where the dynamics of signaling observed in
414 response to different ligands of the TGF- β superfamily are determined by the
415 localization and trafficking of cell surface receptors, specifically their rates of
416 internalization from the cell surface and degradation, and their rates of renewal by
417 recycling and/or new synthesis. At steady state prior to ligand induction, for all
418 receptors, the rate of renewal matches the rate of depletion (Figure 8A). For TGF- β ,
419 ligand addition increases the rate of receptor internalization and degradation, so
420 receptors become depleted from the cell surface and signaling attenuates (Figure 8B)
421 (Vizan et al., 2013). For Activin, upon ligand addition, depletion is matched by
422 renewal, such that receptors are not depleted from the cell surface. Moreover, the
423 response to ligand is integrated until maximal R-SMAD phosphorylation is reached,
424 and cells do not become refractory to acute stimulation (Figure 8C). For BMP4,

425 receptor behavior over the first hour and in the longer term is similar to Activin, but a
426 transient peak of SMAD6 and SMAD7 expression means that the rate of depletion
427 and/or degradation is greater than the rate of renewal, leading to a transient dip in
428 SMAD1 phosphorylation (Figure 8D).

429 Our mathematical modeling approach has suggested for the first time the
430 importance of ligand affinity for receptors in shaping the signaling dynamics. We
431 have shown that we can convert our mathematical model from simulating the
432 refractory behavior observed for TGF- β to the non-refractory, integrated signaling
433 behavior observed for Activin and BMP, by reducing the affinity of receptors for their
434 ligand. This suggests that it is the high affinity that TGF- β has for its receptors,
435 (which is likely, at least in part, to be due to the cooperative interaction between TGF-
436 β and the TGF- β type I and type II receptors (Hinck, 2012, Groppe et al., 2008)), that
437 explains how TGF- β binding leads to a dramatic depletion in surface receptors, and
438 the subsequent refractory behavior. In contrast, Activin, which binds its receptors
439 with lower affinity, may not saturate the cell surface receptors, and thus does not
440 cause obvious cell surface receptor depletion. In the case of BMP4, our experimental
441 and modeling results indicate that it essentially functions like Activin, but the activity
442 of the induced SMAD6/7 causes a transient depletion of receptors from the surface
443 and a subsequent dip in PSMAD1/5 levels, giving the characteristic single oscillatory
444 behavior.

445 The differences in surface receptor depletion seen in response to TGF- β ,
446 BMP4 and Activin also explains the differences in the integration of signaling
447 observed over the first hour after stimulation. The constant presence of BMP and
448 Activin receptors at the surface results in a continuous increase in receptor activation
449 over the first hour, such that a longer duration of ligand exposure leads to more
450 receptors being activated. Because the R-SMADs monitor receptor activity as a result
451 of their nucleocytoplasmic shuttling, accumulation of activated receptors results in
452 accumulation of phosphorylated R-SMADs (Schmierer et al., 2008). In the case of
453 TGF- β , receptor activation is maximal after 5–10 min and does not continue to
454 increase with time of ligand exposure.

455

456 **Distinct TGF- β family signaling dynamics may account for the different *in vivo***
457 **roles for these ligands**

458 The differences in signaling dynamics that we have uncovered may account for the
459 distinct roles these ligands play during embryonic development and tissue
460 homeostasis. Activin, and the related ligand NODAL, as well as the BMPs, are well
461 known to form gradients to pattern tissues, and are thought to act as morphogens
462 (Wharton et al., 1993, Gurdon et al., 1994). Crucially, these ligands are all regulated
463 by soluble extracellular ligand antagonists, such as Chordin or Noggin for BMPs,
464 Follistatin for Activin, and Lefty1/2 for NODAL, among others (Brazil et al., 2015,
465 Hedger and de Kretser, 2013, Schier, 2009). The formation of morphogen gradients
466 requires cells to be sensitive to ligand levels at all times and both the BMP and
467 NODAL gradients formed in early zebrafish embryos have been shown to be shaped
468 by the action of ligand antagonists (Schier, 2009, Pomreinke et al., 2017, Ramel and
469 Hill, 2013, van Boxtel et al., 2015, Zinski et al., 2017).

470 In contrast to Activin, NODAL and BMPs, TGF- β itself has never been shown
471 to act in a gradient during embryonic development. The main roles of TGF- β during
472 early stages of development are in facial morphogenesis (Dudas et al., 2006), heart
473 valve formation (Mercado-Pimentel and Runyan, 2007) and in the development and
474 maintenance of the vascular system (ten Dijke and Arthur, 2007), and graded ligand
475 activity is not apparent in any of these processes. Furthermore, unlike Activin,
476 NODAL and the BMPs, TGF- β has no known natural ligand antagonists. Like all the
477 TGF- β family ligands, TGF- β is synthesized as a precursor, with a large prodomain
478 and a C-terminal mature domain. The mature domain is then cleaved from the
479 prodomain by proteases of the subtilisin-like pro-protein convertase (SPC) family
480 (Miller and Hill, 2016). This pro-mature complex forms a latent complex with latent
481 TGF- β binding proteins (LTBPs), and a further activation step is required to release
482 mature TGF- β protein (reviewed in (Miller and Hill, 2016). Activin and BMPs are
483 also secreted as pro-mature complexes, but their pro and mature domains are only
484 weakly associated (Mi et al., 2015, Wang et al., 2016). It has been demonstrated for
485 Activin that the pro and mature domains have a dissociation constant of ~ 5 nM and
486 thus will be mostly dissociated at the concentrations required for full bioactivity
487 (Wang et al., 2016). Thus, active TGF- β is only generated when and where it is
488 required, while Activin and BMPs are essentially secreted as active ligands. We
489 speculate that in the absence of any natural antagonists, the refractory behavior

490 exhibited by TGF- β after stimulation may be a defence against deregulated signaling,
491 such as occurs in cancer and fibrosis (Akhurst and Hata, 2012).

492 Morphogen gradients have been shown to be gradients, not just of ligand
493 concentration, but also of time (Kutejova et al., 2009). In the current paradigm, both
494 the amount and the duration of ligand exposure determines the fate of a cell in a
495 gradient. For the Activin, NODAL and BMP pathways, where signaling receptors
496 accumulate over time while ligand is present, the levels of PSMAD are proportional
497 to signal duration and ligand dose. In contrast, a cell in a TGF- β gradient would be
498 unable to measure the duration of its exposure to ligand, as almost signaling is
499 initiated within the first few minutes. Moreover, a putative ligand antagonist would be
500 unable to neutralize TGF- β , as most of the signaling occurs from internal
501 compartments. Thus, TGF- β is regulated at the level of ligand production and release
502 from the latency complex, and does not form signaling gradients.

503

504 **BMP exhibits an oscillatory behavior**

505 We have demonstrated an oscillation in signaling downstream of BMP4 in multiple
506 cell lines. This behavior depends on the transient upregulation of SMAD6 and
507 SMAD7, which are required for the transient depletion of BMPR2 from the cell
508 surface, that in turn correlates with the transient attenuation of signaling. The next
509 step will be to investigate whether oscillations downstream of BMP signaling are
510 observed in *in vivo* systems and what their function is. An attractive possibility is that
511 they could be involved in periodically providing competence for cell fate decisions. It
512 has been hypothesized that oscillatory behavior of both BMP and Notch signaling is
513 required for vascular patterning, in particular, in sprouting angiogenesis, to determine
514 the selection of tip versus stalk cells (Moya et al., 2012, Beets et al., 2013). This idea
515 was based on the scattered expression of *Id1/2/3* (prominent BMP target genes) in the
516 mouse angiogenic epithelium, which was postulated to reflect a snapshot of non-
517 synchronized oscillatory gene expression. It will be very interesting in the future to
518 directly monitor BMP signaling live in this system, to determine whether such
519 oscillations occur.

520

521

522

523 **Materials and Methods**

524

525 **Cell lines, and treatments**

526 The human keratinocyte cell line, HaCaT, the human breast cancer line MDA-MB-
527 231, the mouse fibroblast cell line NIH-3T3 and the mouse teratoma cell line P19
528 were used throughout this study. All cells were maintained in DMEM (Thermo Fisher
529 Scientific), supplemented with 10% FCS. Ligands and reagents were used at the
530 following concentrations: TGF- β (Peprotech), 2 ng/ml; BMP4 (Peprotech), 20 ng/ml;
531 Activin A (PeproTech), 20 ng/ml; Noggin (PeproTech), 500 ng/ml; Follistatin
532 (Sigma), 500 ng/ml; LDN-193189 (Gift from Paul Yu), 1 μ M; SB-431542 (Tocris),
533 10 μ M; Cycloheximide (Sigma), 20 μ g/ml; Actinomycin D (Sigma) 1 μ g/ml. The
534 TGF- β neutralizing antibody, 1D11, and isotype-matched IgG1 monoclonal control
535 antibody raised against Shigella toxin (13C4) were as described (Nam et al., 2008),
536 and used at 30 μ g/ml. All stimulations were performed in full serum. Where ligands
537 or drugs were washed out, cells were washed three times with warm media. Whole
538 cell extracts were prepared as previously described (Inman et al., 2002b). Where
539 required, cell lysates were treated with PNGase F (New England Biosciences), 500 U
540 per 100 μ g of protein.

541

542 **Surface biotinylation and immunoblotting**

543 Surface biotinylation assays were as previously described (Vizan et al., 2013).
544 Immunoblotting were performed using standard techniques with the following
545 antibodies: anti-PSMAD2 (Cell Signaling Technology, Cat. # 3108), anti-SMAD2/3
546 (BD Biosciences, Cat. # 610843), anti-PSMAD1/5 (Cat. # 13820), anti-SMAD1
547 (Invitrogen, Cat. # 38-5400), anti-ACVR1B (Abcam, Cat. # Ab133478), anti-
548 ACVR2B (Aviva Systems Biology, Cat. # ARP45041), anti-BMP2 (BD Biosciences,
549 Cat. # 612292), anti-TGFBR1 (Santa Cruz, Cat. # sc-398), anti-TGFBR2 (Santa Cruz,
550 Cat. # 17792), anti-TDGF1 (Cell Signaling Technology, Cat. # 2818), anti-MCM6
551 (Santa Cruz, Cat. # sc-9843), anti-Tubulin (Abcam, Cat. # Ab6160). Western blots
552 were visualized on film or using an ImageQuant LAS 4000 mini (GE Healthcare) and
553 quantified with ImageJ. For quantifications, densitometry measurements were

554 normalized to loading controls and are shown relative to levels in cells stimulated
555 with ligand for 1 hr, except where indicated.

556

557 **qPCR and siRNA knockdown**

558 qRT-PCR was performed as previously described (Gronroos et al., 2012). Primer
559 sequences are given in Supplementary file 1. For siRNA experiments, cells were
560 plated, and 24 hr later transfected with 30 nM siRNA/3 μ l RNAiMax (Thermo Fisher
561 Scientific) for NIH-3T3 cells and P19 cells or 5nM siRNA/8 μ l INTERFERin
562 (PolyPlus) for MDA-MB-231s and 200 μ l Opti-MEM (ThermoFisher Scientific) in
563 fresh media. Volumes are given for a 6-well plate. Experiments were performed 72 hr
564 after siRNA transfection. siRNAs were purchased from Dharmacon and sequences are
565 given in Supplementary file 1. They were used as SMARTpools.

566

567 **Statistical analysis**

568 Student's t-tests were performed where appropriate using GraphPad Prism 7 software.

569

570 **Mathematical modeling**

571 The mathematical models of Activin and BMP signaling are based on our previously
572 published model of TGF- β signaling (Vizan et al., 2013), with the following key
573 modifications.

574 Ligand binding to competent surface receptors is now treated as a reversible
575 process. In the original TGF- β model, the dissociation rate of the ligand/receptor
576 interaction was considered negligible compared to the activation of the receptor
577 complex by the ligand, and ligand binding was treated as irreversible for simplicity. In
578 the new model, this reaction is made reversible to allow modeling of different binding
579 affinities of different ligands. An off-rate k'_{off} was thus introduced.

580 In addition, a negative feedback mechanism mediated by I-SMADs was
581 included to model the behavior of cells in response to BMP4. I-SMADs were
582 assumed to be synthesized in response to ligand, and to promote the degradation of
583 signaling competent receptors, as well as the ligand-induced increase in degradation
584 of active receptors.

585 I-SMADs are transcriptional targets of nuclear R-SMAD–SMAD4 complexes.
586 Both I-SMAD RNA and protein were included to capture the time delay between

587 ligand addition and I-SMAD expression. The two new equations for *I-SMAD* RNA
 588 and I-SMAD protein read:

589

$$590 \quad \frac{dS_i^{RNA}}{dt} = k_{synbas}^{Ri} + k_{syn}^{Ri} S_{24n}^4 - k_{deg}^{Ri} S_i^{RNA}$$

591

$$592 \quad \frac{dS_i}{dt} = k_{syn}^{Si} S_i^{RNA} - k_{deg}^{Si} S_i$$

593

594

595 With these modifications, equations 2–5 from (Vizan et al., 2013) now read (new
 596 terms indicated in bold):

597

598

599

$$600 \quad \frac{1}{k_d} \frac{dR_{com}}{dt} = \alpha R - \frac{K_{Si} + S_i}{K_{Si}} R_{com}^l + \mathbf{k'_{Toff} R_T} - k'_T TGF R_{com}^S$$

601

602

$$603 \quad \frac{1}{k_d} \frac{dR_T}{dt} = k'_T TGF R_{com}^S - \left(k'_{act} + \mathbf{k'_{Toff}} + D \frac{K_{Si} + S_i}{K_{Si}} \right) R_T$$

604

$$605 \quad \frac{dR_{act}}{dt} = k'_{act} R_T - D \frac{K_{Si} + S_i}{K_{Si}} R_{act}$$

606

607

$$608 \quad \frac{1}{k_d} \frac{dTGF}{dt} = \mathbf{k'_{Toff} R_T} - (k'_T R_{com}^S + k'_{cc}) TGF$$

609

610

611

612 The following parameters were used to model the behavior of the I-SMADs.

613

Parameter	Value
k_{synbas}^{Ri}	0
k_{syn}^{Ri}	4
k_{deg}^{Ri}	1
K_{Si}	0.02
k_{syn}^{Si}	1
k_{deg}^{Si}	1

614

615 We have implemented these changes into a single model that can capture the
 616 dynamics of each ligand simply by changing the parameters in each case. The

617 following parameters were changed to model each ligand, with key parameter
 618 changes indicated in bold:

619

Parameter	Value			
Model	BMP4	Activin (HaCaT)	Activin (P19s)	TGF- β
Ligand in ng/ml	20	20	20	2
$TSca$	1	0.5	0.5	2
k'_{Toff}	2	2	2	2
k'_T	0.2	0.2	0.2	100
D	2	2	2	4
k'_{cc}	0.05	0.35	0.35	0.35
k_d	1	0.67	0.67	0.32
k^{bas}_{synT}	0	0	0.8	0
K_{SBI}	0.001	0.001	0.001	0.196565
Y/N Feedback	1	0	0	0

620

621 The key parameters changed are as follows:

- 622
- 623 • The on-rate of ligand to receptor binding, k'_T , was chosen such that the
 624 dissociation constant of the ligand/receptor interaction, which is given by $\frac{k'_{Toff}}{k'_T}$,
 625 is very small for TGF- β (reflecting the high affinity of this ligand for its
 626 receptors), and is much larger for the other ligands. This is the only critical
 627 change necessary to alter the overall behavior of the model in response to each
 628 ligand.
 - 629 • k'_{cc} is the constitutive clearance of the ligand from the medium. Assuming that
 630 BMP4 is cleared from the medium at the same speed as the other ligands does
 631 not model the data well; it seems to be more persistent in the medium.
 - 632 • k^{bas}_{synT} is the basal ligand production, which is required for modeling Activin
 633 dynamics in P19s, which secrete ligand in an autocrine fashion.
 - 634 • K_{SBI} is the dissociation constant of SB from the receptors.
 - 635 • Y/N Feedback is a toggle switch that allows us to switch on and off I-SMAD
 636 production in response to ligand.

636

637 In addition, alterations to the following parameters were necessary to accurately
 638 capture the experimental data:

- 639
- 640 • k_d is the half-life of receptors in the absence of ligand.
 - D is the ligand induced increase in degradation of active receptors

641 • *TSca* scales the relative amounts of ligand to receptor

642

643 The model was implemented in the freely available software packages COPASI

644 (<http://www.copasi.org>) and XPP (<http://www.math.pitt.edu/~bard/xpp/xpp.html>). All

645 simulations and parameter fitting were performed in COPASI (Hoops et al., 2006). The

646 model has been deposited in the Biomodels database (Chelliah et al., 2015) and

647 assigned the identifier *MODEL1810160001* and will be made publicly available after

648 curation.

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650

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658

659 **Author contributions:**

660 CSH and DSJM designed the study, DSJM performed all the experiments and BS
661 performed the mathematical modeling. CSH and DSJM wrote the manuscript with
662 input from BS.

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872

873 **Figure Legends**

874

875 **Figure 1. BMP4 and Activin signal with distinct dynamics.**

876 (A) MDA-MB-231 cells were treated with BMP4 for the times indicated. (B and C)
877 P19 cells (B) or HaCaT cells (C) were treated with Activin A for the times indicated
878 or SB-431542 (SB) overnight. Western blotting for PSMAD1/5, SMAD1, PSMAD2,
879 SMAD2/3 and Tubulin as a loading control was performed. Quantifications are the
880 normalized means and standard deviations (SDs) of densitometry measurements from
881 three independent experiments.

882

883 **Figure 2. Activin and BMP4 signals are integrated over time, whilst TGF- β** 884 **signals are not.**

885 (A) Experimental scheme. Cells were untreated (a), or treated with ligand for 5 (b), 10
886 (c), 20 (d), 30 (e), or 60 (f) minutes, followed by the cognate ligand antagonist for the
887 remainder of 60 min. To ensure that inhibitors were working as expected, cells were
888 pre-treated with inhibitor for 5 mins, followed by ligand for 60 mins (g). (B) MDA-
889 MB-231 cells were treated as in (A) with TGF- β and the blocking antibody, 1D11.
890 (C) P19 cells were treated as in (A) with Activin and Follistatin, and additionally
891 overnight with SB-431542 (SB). (D) HaCaT cells were treated as in (A) with Activin
892 and Follistatin. (E) MDA-MB-231 cells were treated as in (A) with BMP4 and
893 Noggin. (F) HaCaT cells were treated as in (A) with BMP4 and Noggin. Western
894 blotting for PSMAD1/5, SMAD1, PSMAD2, SMAD2/3 and Tubulin as a loading
895 control was performed. Quantifications are the normalized means and SDs of
896 densitometry measurements from three independent experiments.

897

898 **Figure 3. BMP4 and Activin do not induce refractory behavior.**

899 (A) Left, a schematic of experimental set-up. NIH-3T3 cells were untreated (a) or
900 treated with BMP4 for 1 hr (b) or 3 hr (c). After 1 hr of BMP4 stimulation, signal was
901 brought down to baseline with Noggin for 2 hr (d), which was then washed out and
902 cells re-stimulated with BMP4 for 1 hr (e). The efficacy of Noggin washout was
903 confirmed (f), as was its inhibitory ability by adding the ligand and antagonist
904 simultaneously (g). To confirm that BMP4 was not depleted from the media in the
905 time period of these experiments, cells were stimulated with BMP4 for 3 hr, then the

906 media transferred to naïve cells for 1 hr (h). Western blotting for PSMAD1/5,
907 SMAD1 and Tubulin as a loading control was performed. Quantifications are the
908 normalized means and SDs of densitometry measurements from three independent
909 experiments. **(B)** Left, a schematic of experimental set-up. P19 cells were untreated
910 (a), treated overnight with SB-431542 (b) or treated with Activin for 1 hr (c) or 3 hr
911 (d). After 1 hr of Activin stimulation, signal was brought down to baseline with
912 Follistatin for 2 hr (e), which was then washed out and cells re-stimulated with
913 Activin for 1 hr (f). The efficacy of Follistatin washout was confirmed (g), as was its
914 inhibitory ability (g). To confirm that Activin was not depleted from the media in the
915 time period of these experiments, cells were stimulated with Activin for 3 hr, then the
916 media transferred to naïve cells for 1 hr (i). Western blotting for PSMAD2, SMAD2
917 and Tubulin as a loading control was performed. Quantifications are the normalized
918 means and SDs of densitometry measurements from three independent experiments.

919
920

921 **Figure 4. The distinct TGF- β , Activin and BMP4 signaling dynamics are not**
922 **explained by the intracellular lifetimes of their receptors**

923 **(A)** Experimental scheme. Cells were untreated (a), or treated for 1 hr with ligand
924 (b), then with ligand antagonist or receptor kinase inhibitors for 30 mins (c), 1 hr (d),
925 2 hr (e), 4 hr (f) or 8 hr (g), or with ligand and receptor kinase inhibitor together for 8
926 hr (h). **(B)** MDA-MB-231 cells were treated as in (A) with TGF- β , 1D11 or SB-
927 431542 (SB). **(C)** MDA-MB-231 cells were treated as in (A) with BMP4, Noggin
928 (Nog) or LDN-193189 (LDN). **(D)** P19 cells were treated as in (A) with Activin A,
929 Follistatin (Foll) or SB-431542 (SB). Western blotting for PSMAD1/5, SMAD1,
930 PSMAD2, SMAD2/3 and Tubulin as a loading control was performed.
931 Quantifications are the normalized means and SDs of densitometry measurements
932 from three independent experiments.

933

934 **Figure 5. BMP4 and Activin drive distinct receptor trafficking behaviors.**

935 **(A)** MDA-MB-231 cells were treated with BMP4 for the times indicated. **(B)** P19
936 cells were treated with Activin for the times indicated or SB-431542 overnight (SB).
937 Whole cell extracts were Western blotted for BMPR2, PSMAD1/5, SMAD1,
938 ACVR1B, ACVR2B, TDGF1, PSMAD2, SMAD2/3 with Tubulin as a loading

939 control (Inputs). Surface biotinylation assays were performed to isolate surface
940 receptor populations, which were Western blotted for BMPR2, ACVR1B, ACVR2B
941 and TDGF1. For the lanes marked -Biotin unstimulated cell extracts were treated
942 identically to the other samples, but without the addition of Biotin. In A, the lane
943 marked PNG corresponds to a 0 time point where the sample was treated with
944 PNGase to remove N-linked sugars from the receptors prior to gel electrophoresis.
945 Quantifications are the normalized means and SDs of densitometry measurements
946 from three independent experiments, relative to the levels in untreated cells.

947

948 **Figure 6. SMAD6 and SMAD7 are required for the oscillatory signaling response**
949 **to BMP4.**

950 (A) NIH-3T3 cells were treated with BMP4 for the times indicated. Levels of *Smad6*
951 and *Smad7* mRNA were assayed by qPCR. Shown are the normalized means and SDs
952 from three independent experiments, expressed as fold change in mRNA level relative
953 to untreated cells, overlaid with SMAD1/5 phosphorylation data from Figure 2 –
954 figure supplement 2. (B) NIH-3T3 cells were transfected with non-targeting control
955 siRNAs (NT) or siRNA SMARTpools targeting *Smad6* and *Smad7*, and were then
956 treated with BMP4 for the times indicated. Western blotting for PSMAD1/5, SMAD1
957 and Tubulin was performed. Quantifications are the normalized means and SDs of
958 densitometry measurements from three independent experiments. * indicates $p < 0.05$.
959 The extent of knockdown was determined by qPCR. Shown are the normalized means
960 and SDs from three independent experiments, expressed as fold change in mRNA
961 level relative to NT controls. (C) NIH-3T3 cells were transfected with non-targeting
962 control siRNAs (NT) or siRNA SMARTpools targeting *Smad6* and *Smad7*, and were
963 then treated with BMP4 for the times indicated. A biotinylation assay was performed
964 to isolate surface receptor populations, which were Western blotted for BMPR2. Input
965 cell lysates were also Western blotted for BMPR2, PSMAD1/5, SMAD1 and Tubulin
966 as a loading control. For the lane marked -Biotin unstimulated cell extracts were
967 treated identically to the other samples, but without the addition of Biotin.
968 Quantifications are the normalized means and SDs of densitometry measurements
969 from three independent experiments, relative to the levels in untreated cells. *
970 indicates $p < 0.05$. The extent of knockdown was determined by qPCR. Shown are the
971 normalized means and SDs from three independent experiments, expressed as fold
972 change in mRNA level relative to NT controls.

973 **Figure 7. Mathematical models of the Activin and BMP pathways can simulate**
974 **the experimentally-observed behaviors of these ligands.**

975 **(A–D)** The mathematical model was used to simulate the response of cells to Activin.
976 In all cases, responses in cells with no baseline (e.g. HaCaTs) are shown on the left
977 and responses in a cell line that has a basal level of PSMAD2 signaling (e.g. P19
978 cells) are shown on the right. **(A)** Simulation of a long-term Activin response;
979 compare with experimental results in Figure 1C (HaCaTs) or Figure 1B (P19s). **(B)**
980 Simulation of the signal integration experiments; compare with Figure 2D and Figure
981 2C respectively. **(C)** Simulation of the experiment shown in Figure 4D, which shows
982 that signaling occurs from intracellular compartments, presumed to be endosomes.
983 **(D)** Simulation of repeated Activin stimulation; compare Figure 3B. **(E–H)**
984 Equivalent simulations were performed for the BMP4 responses. Compare **(E)** with
985 Figure 1A; **(F)** with Figure 2E; **(G)** with Figure 4C and **(H)** with Figure 3A. In all
986 cases concentrations of the indicated species are plotted in arbitrary units. In **(B)** and
987 **(C)**, PSMAD2 concentration is plotted, and in **(F)** and **(G)**, PSMAD1 concentration is
988 plotted.

989
990

991 **Figure 8. TGF- β family signaling dynamics are determined by a balance between**
992 **receptor depletion and renewal at the cell surface.**

993 **(A)** In untreated cells, the internalization and degradation of receptors is balanced by
994 the synthesis and maturation of new receptors, and their renewal at the cell surface.
995 Arrow size indicates relative rate. **(B)** In the presence of TGF- β , internalization and
996 degradation is faster than renewal, so receptors become depleted from the cell surface.
997 **(C)** In the presence of Activin, internalization and degradation are matched by
998 renewal, so no depletion is seen. **(D)** In the presence of BMP4, the balance is
999 transiently tipped towards internalization and degradation due to the up-regulation of
1000 SMAD6/7, depleting receptors from the cell surface. In the presence of longer
1001 durations of BMP4, SMAD6 and SMAD7 are down-regulated and internalization and
1002 degradation are again matched by renewal. Receptors re-accumulate at the cell
1003 surface.
1004

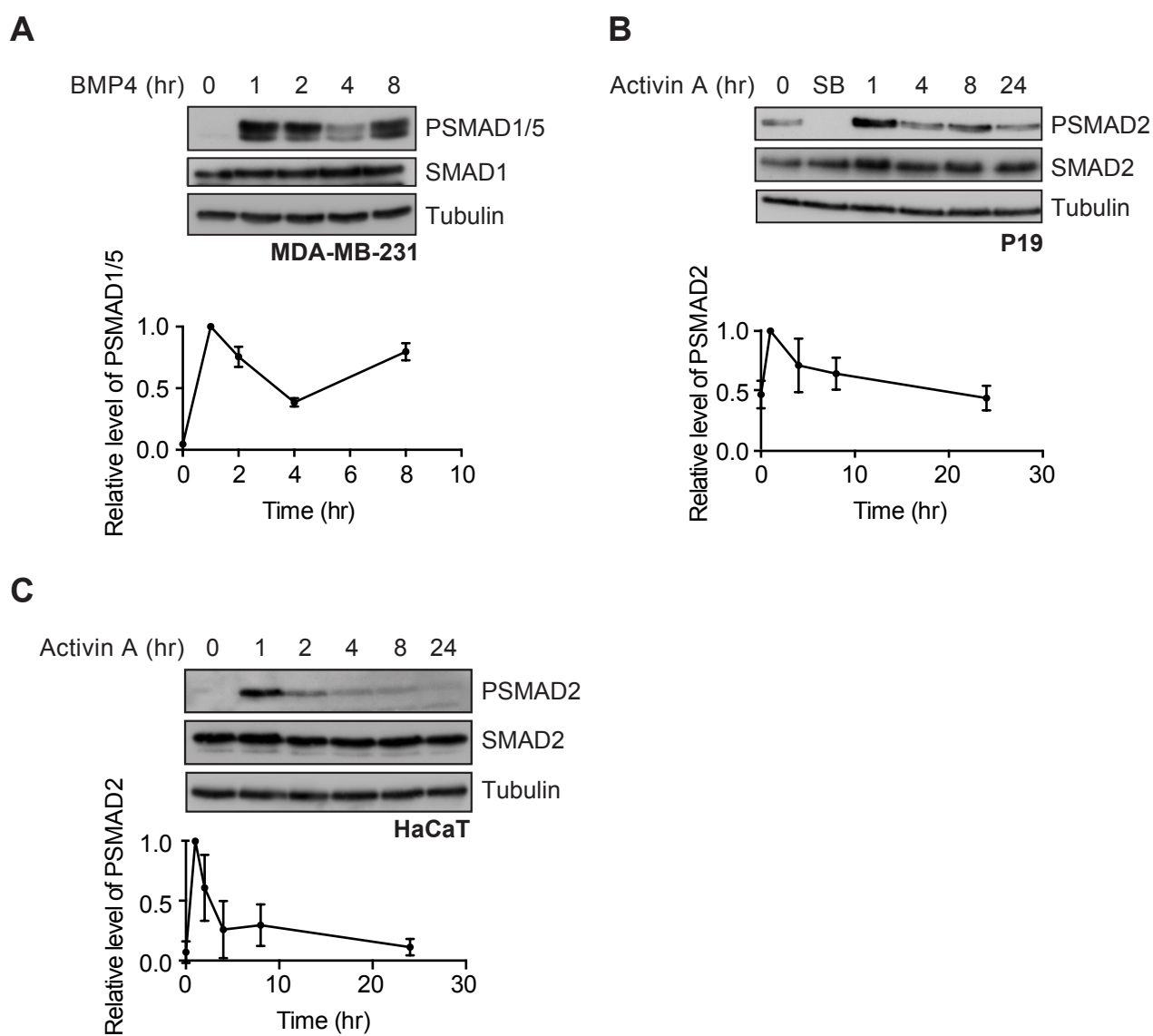


Figure 1

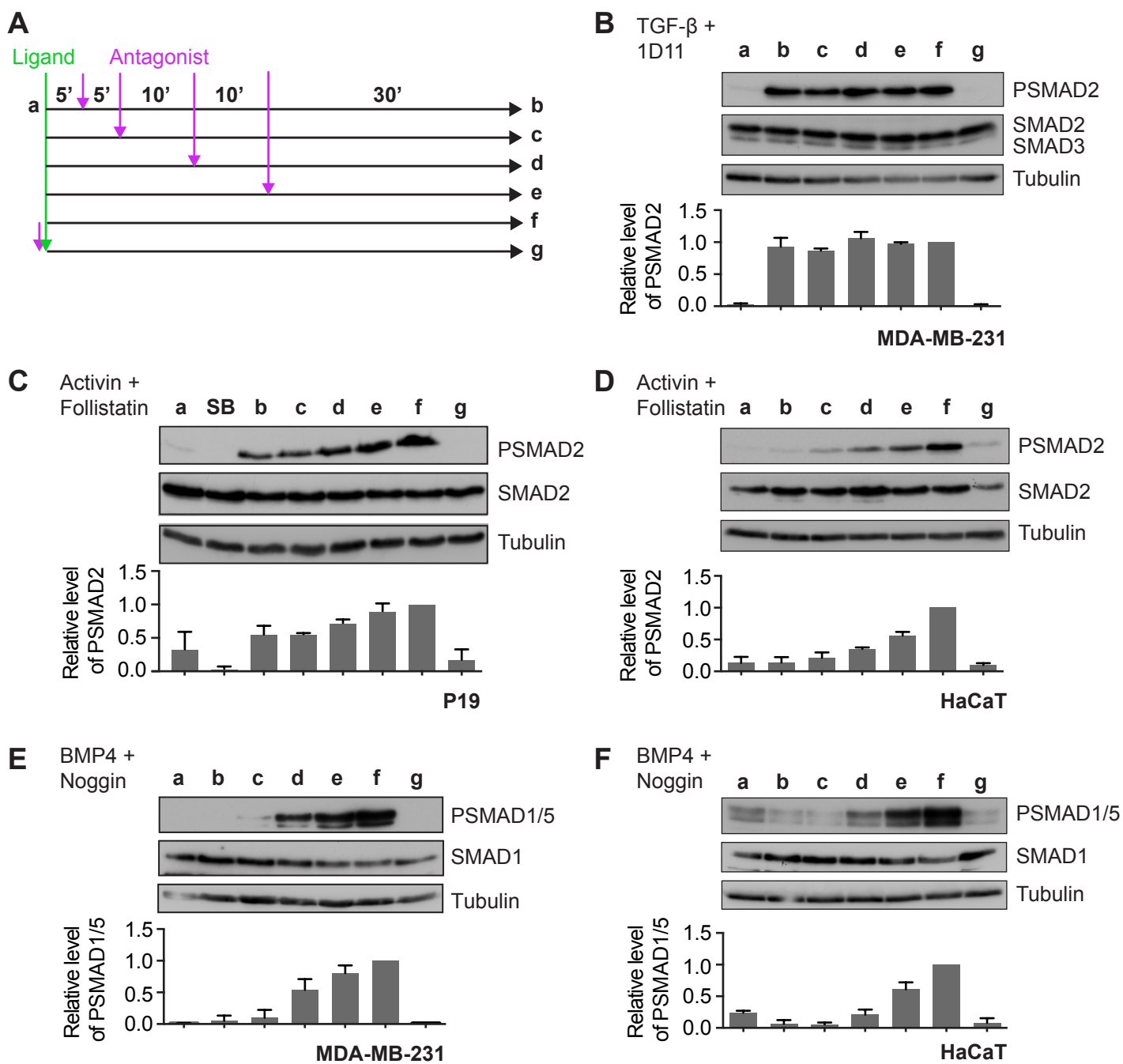
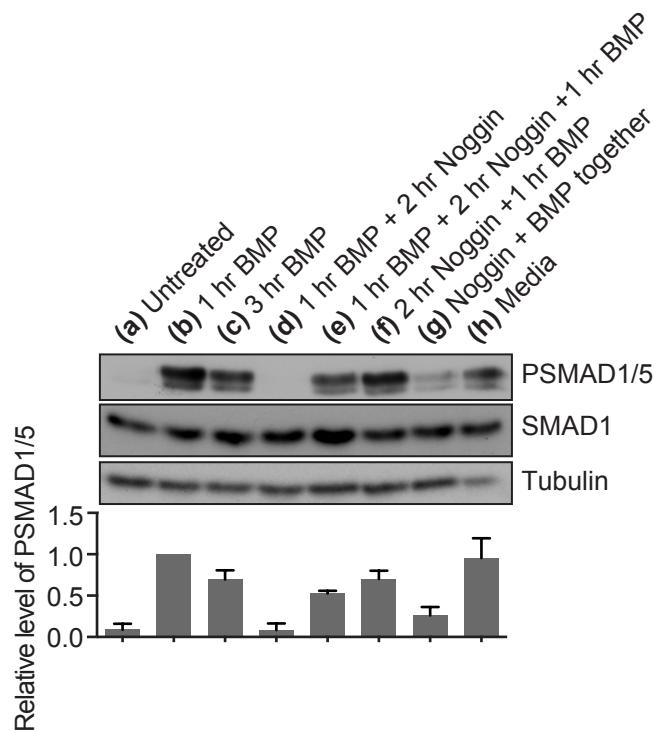
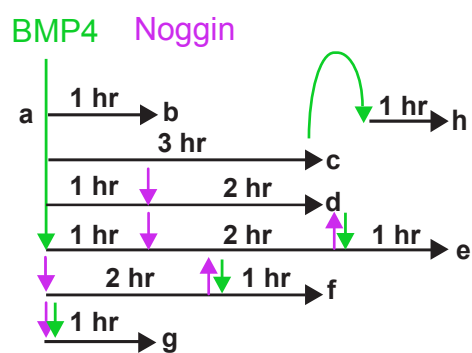


Figure 2

A



B

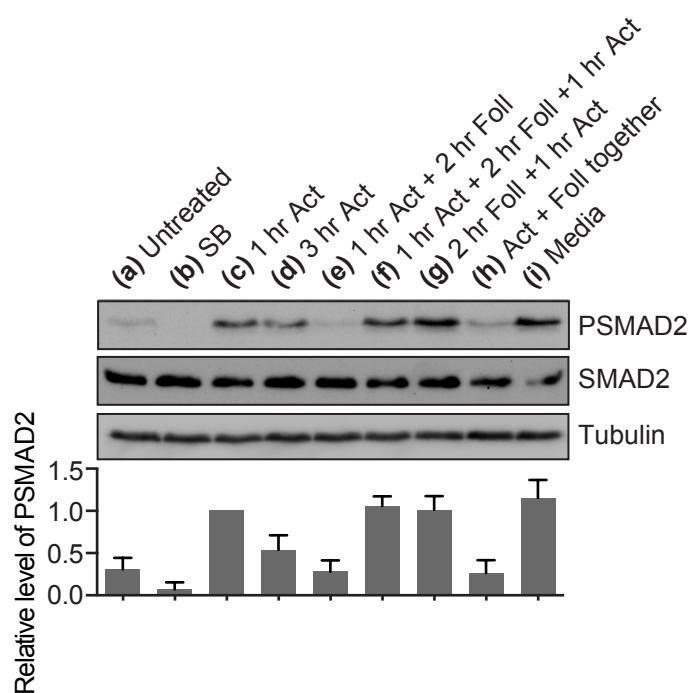
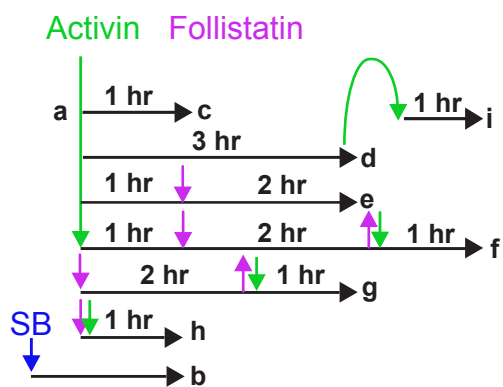
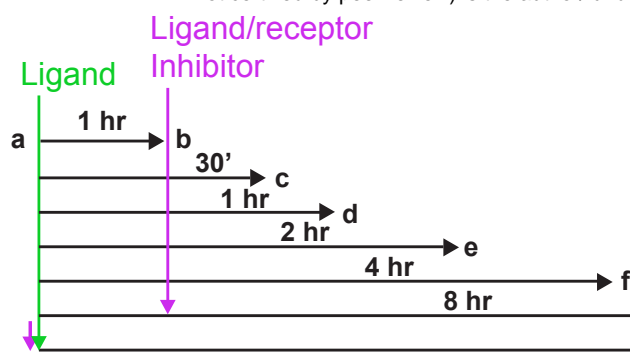


Figure 3

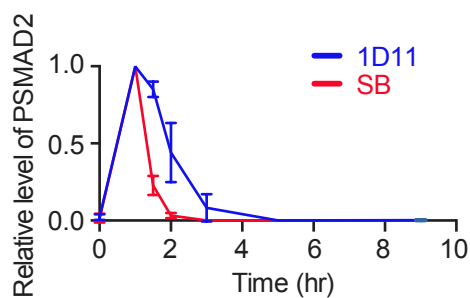
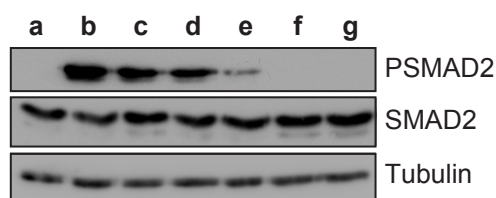
A



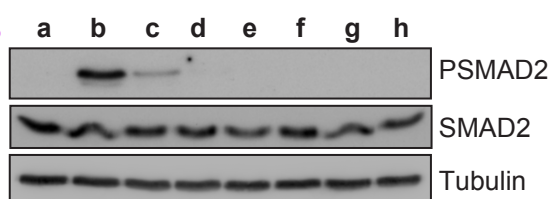
B

TGF- β

1D11



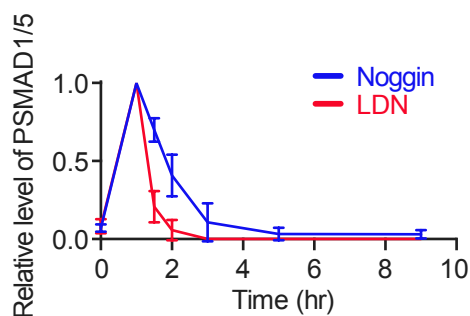
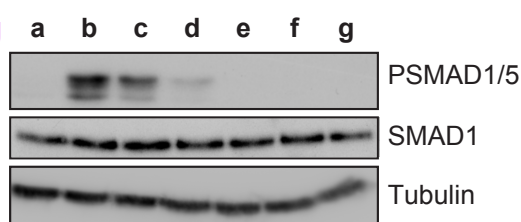
SB



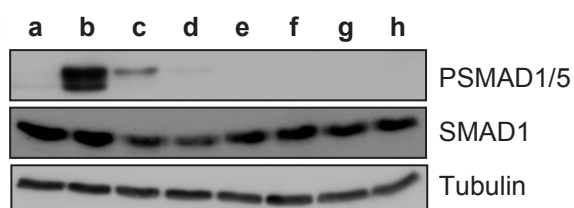
C

BMP4

Nog



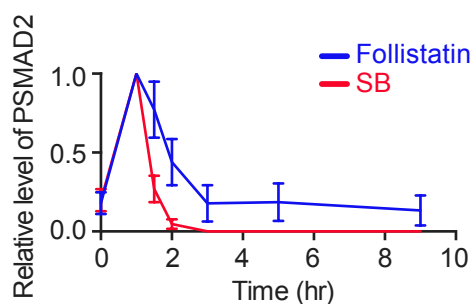
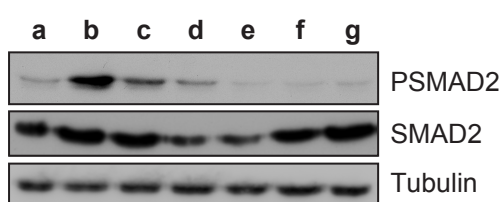
LDN



D

Activin

Foll



SB

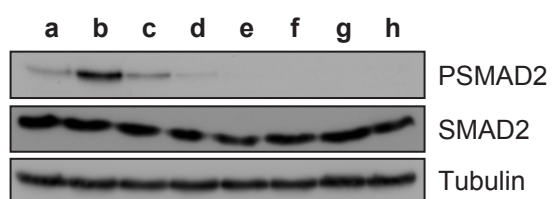


Figure 4

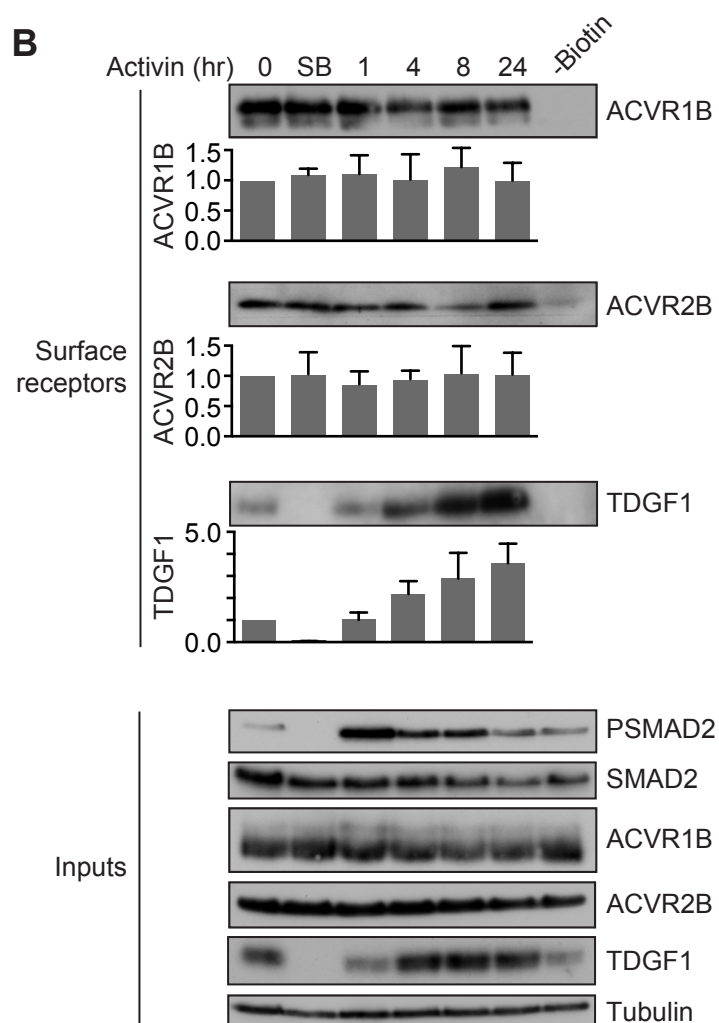
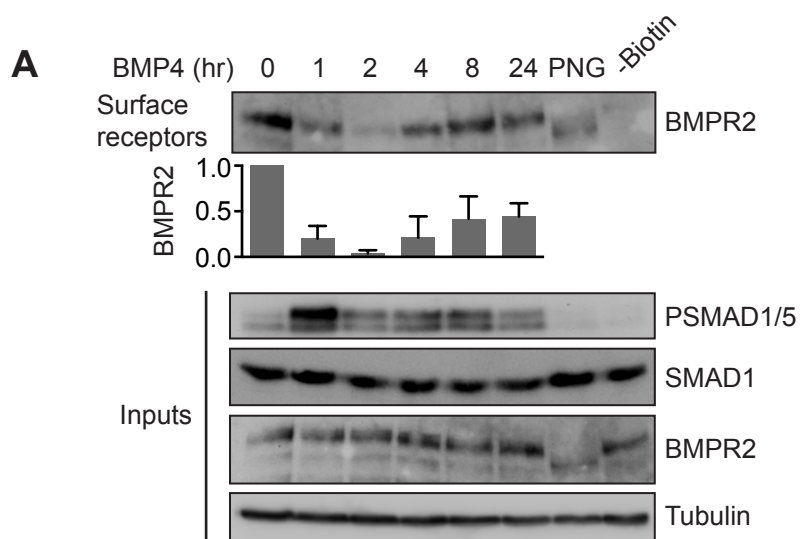
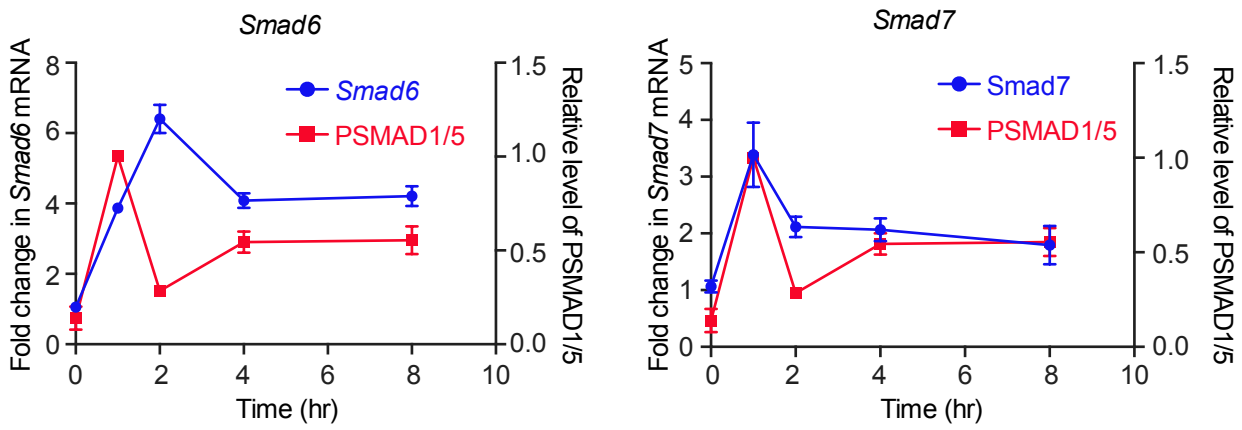
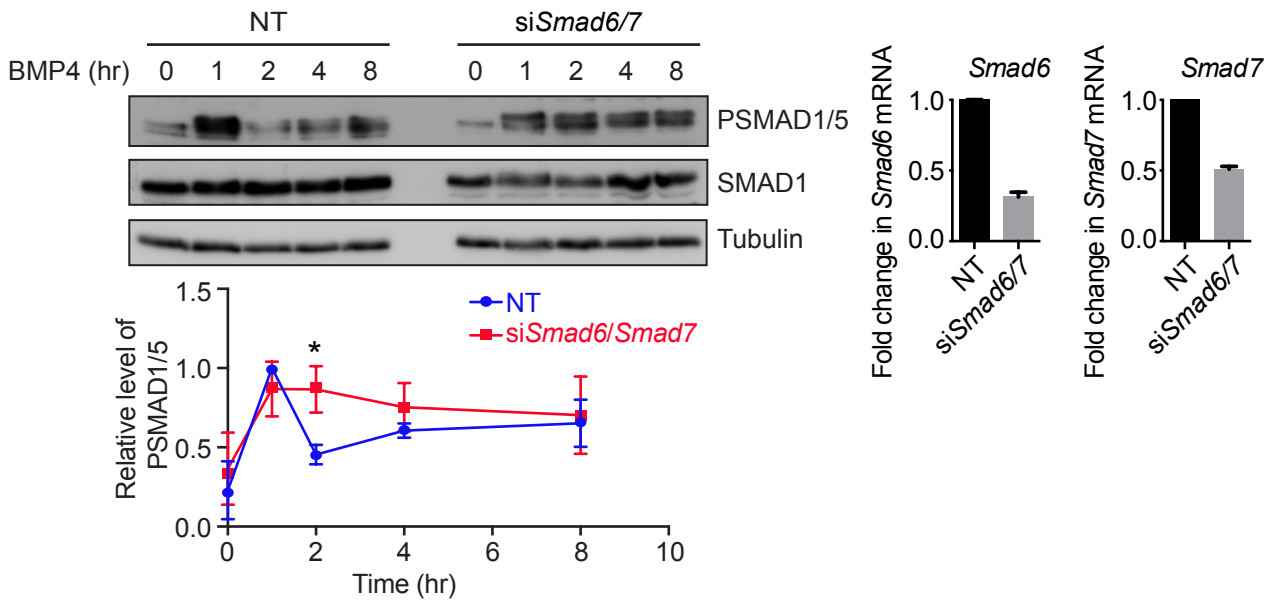


Figure 5

A



B



C

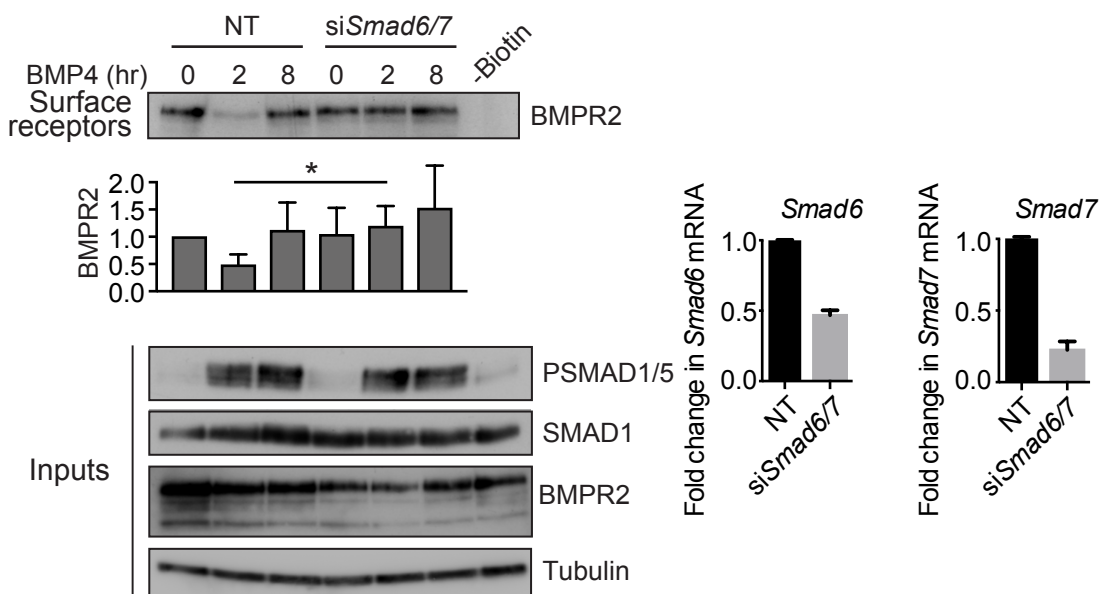
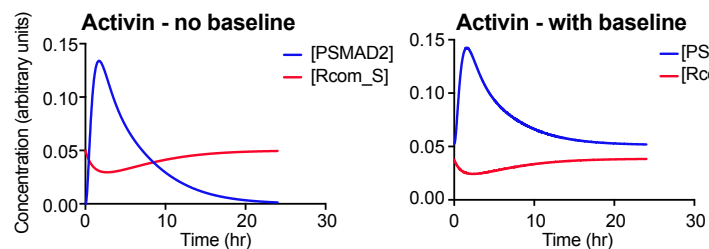
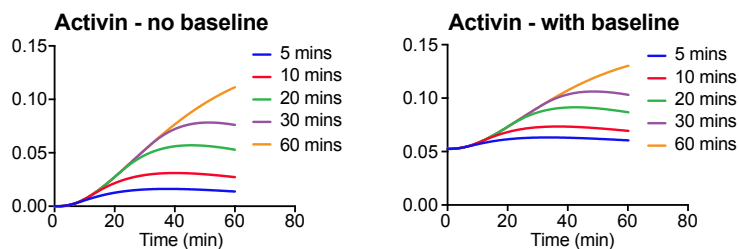


Figure 6

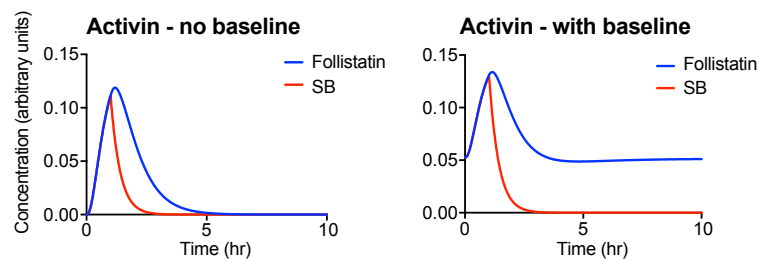
A Long term signaling



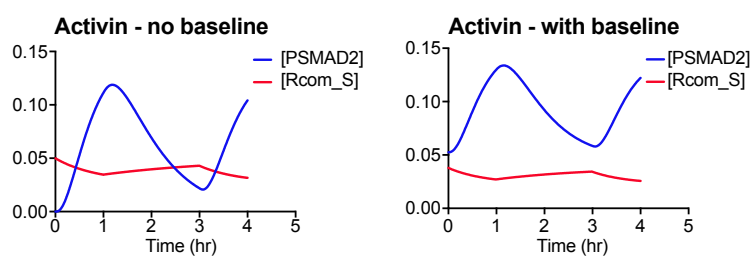
B Signal integration



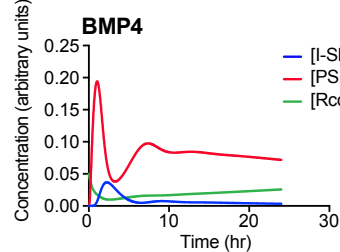
C Signaling from endosomes



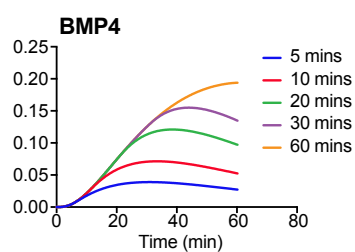
D Repeated stimulation



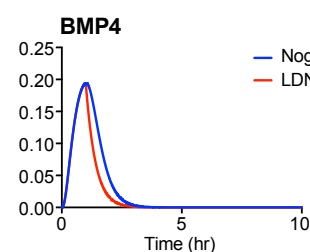
E Long term signaling



F Signal integration



G Signaling from endosomes



H Repeated stimulation

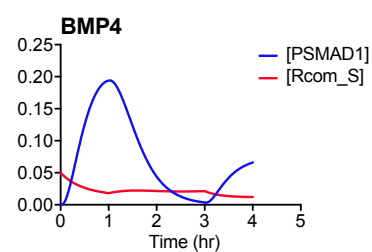


Figure 7

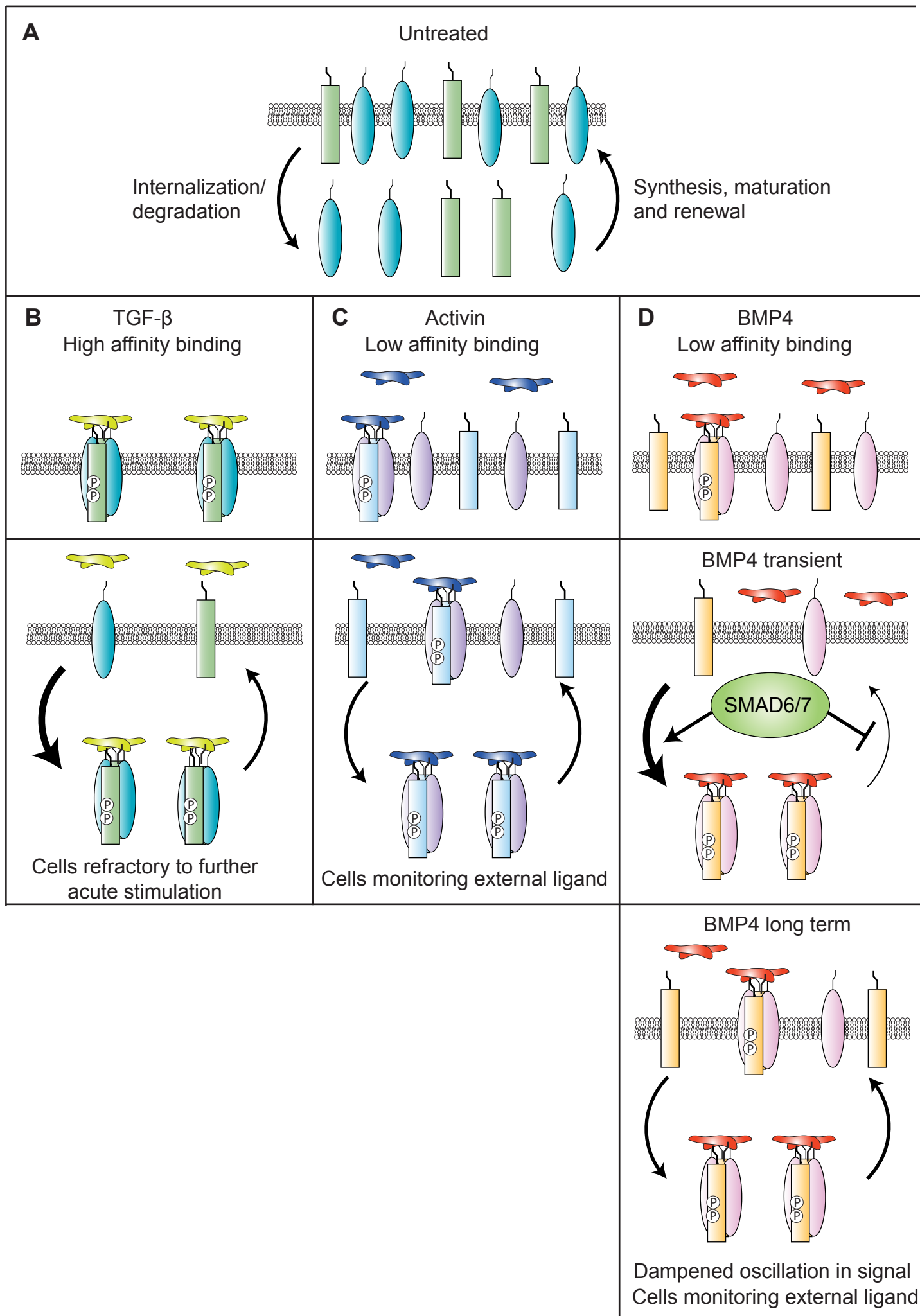


Figure 8

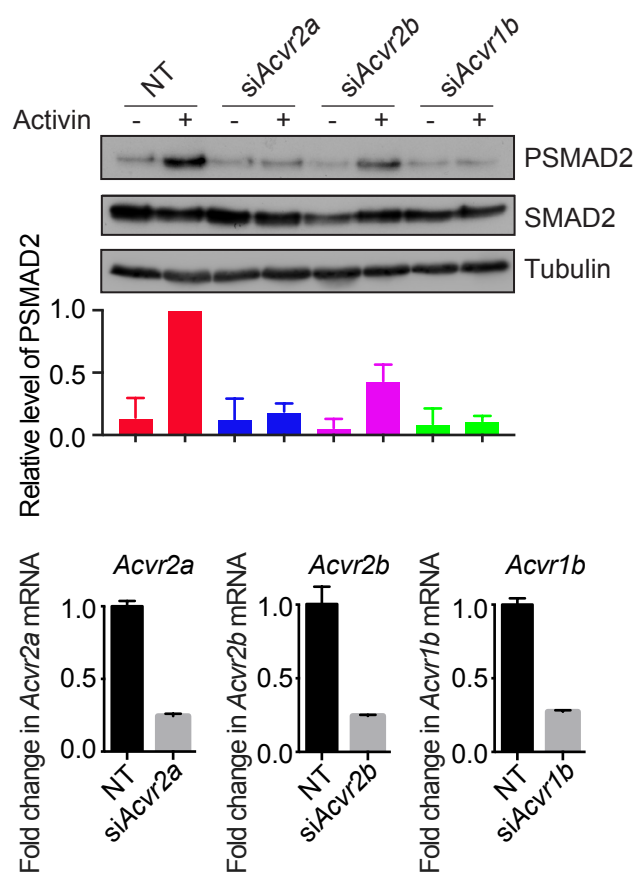


Figure 1 - figure supplement 1

Figure 1 – figure supplement 1. Characterization of Activin receptors in P19 cells.

P19s were transfected with siRNAs against *Acvr2a*, *Acvr2b* or *Acvr1b*, then treated or not with Activin for 1 hr. Western blotting for PSMAD2, SMAD2 and Tubulin as a loading control was performed. Quantifications are the normalized means and SDs of densitometry measurements from two independent experiments. Below, the extent of knockdown was determined by qPCR. Shown are the normalized averages and SDs from two independent experiments, expressed as fold change in mRNA level relative to NT controls.

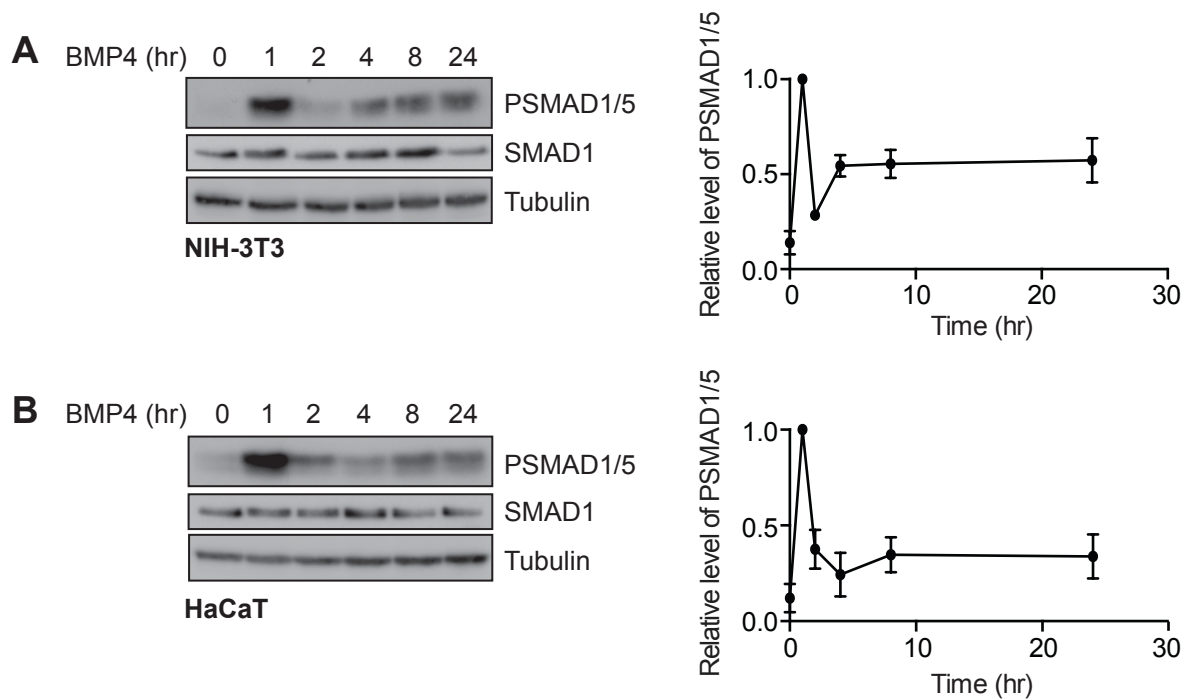


Figure 1 - figure supplement 2

Figure 1 – figure supplement 2. BMP4 exhibits oscillatory signaling in NIH-3T3 cells and in HaCaTs.

(A) NIH-3T3s or (B) HaCaTs were treated with BMP4 for the times indicated. Western blotting for PSMAD1/5, SMAD1 and Tubulin as a loading control was performed. Quantifications are the normalized means and SDs of densitometry measurements from three independent experiments.

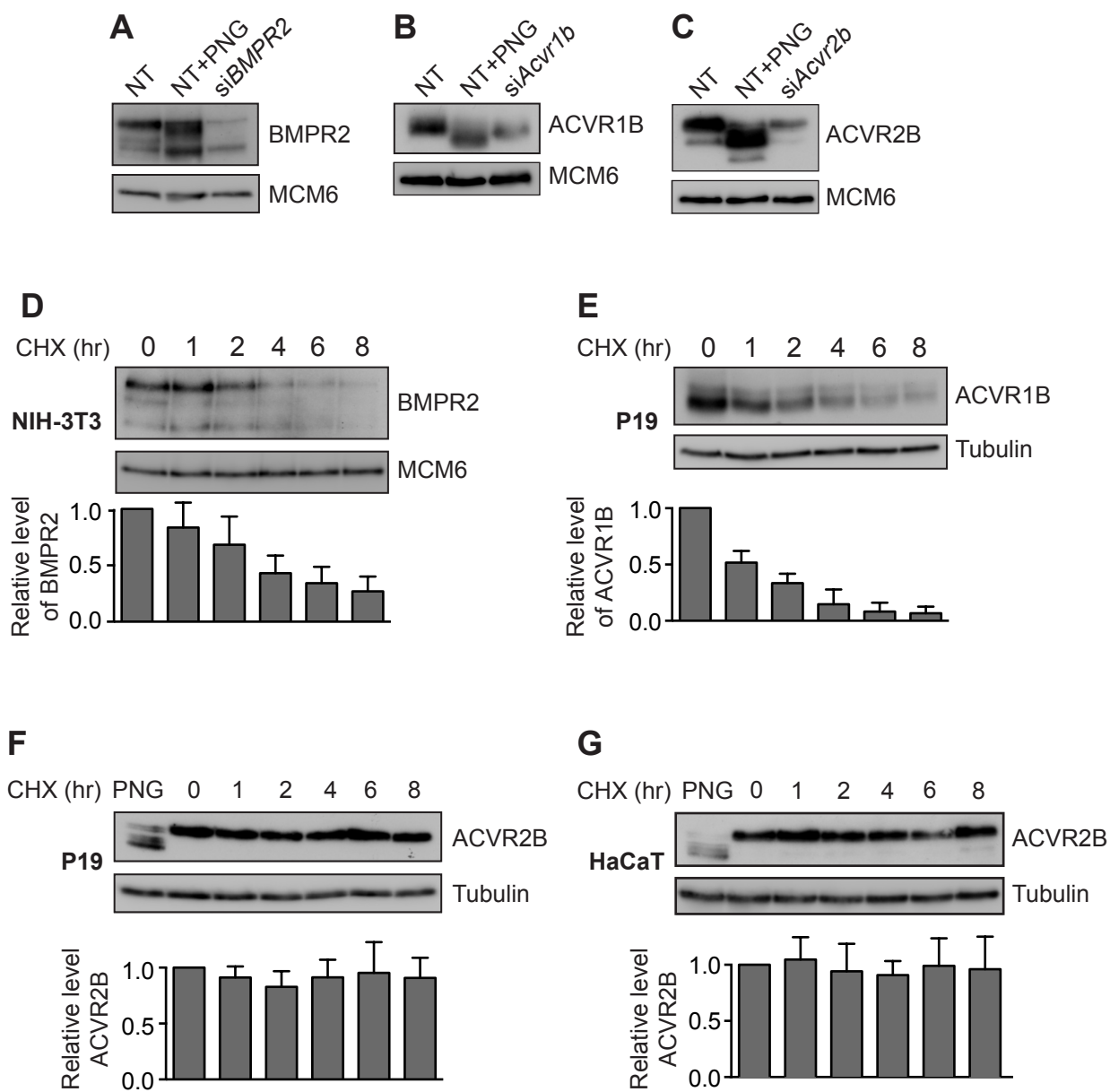


Figure 5 - figure supplement 1

Figure 5 – figure supplement 1. Characterization of receptor stabilities.

(A) MDA-MB-231 cells were transfected with siRNAs against NT controls or BMPR2. Lysates were treated or not with PNGase (PNG). (B) P19 cells were transfected with siRNAs against NT controls or *Acvr1b*. Lysates were treated or not with PNGase (PNG). (C) P19 cells were transfected with siRNAs against NT controls or *Acvr2b*. Lysates were treated or not with PNGase (PNG). (D-G) NIH-3T3, P19 cells or HaCaTs as indicated were treated with cycloheximide (CHX) for the times indicated. Western blotting for BMPR2, ACVR1B, ACVR2B and Tubulin or MCM6 as a loading control was performed. In all cases, quantifications are the normalized means and SDs of densitometry measurements from three independent experiments relative to levels in untreated cells.

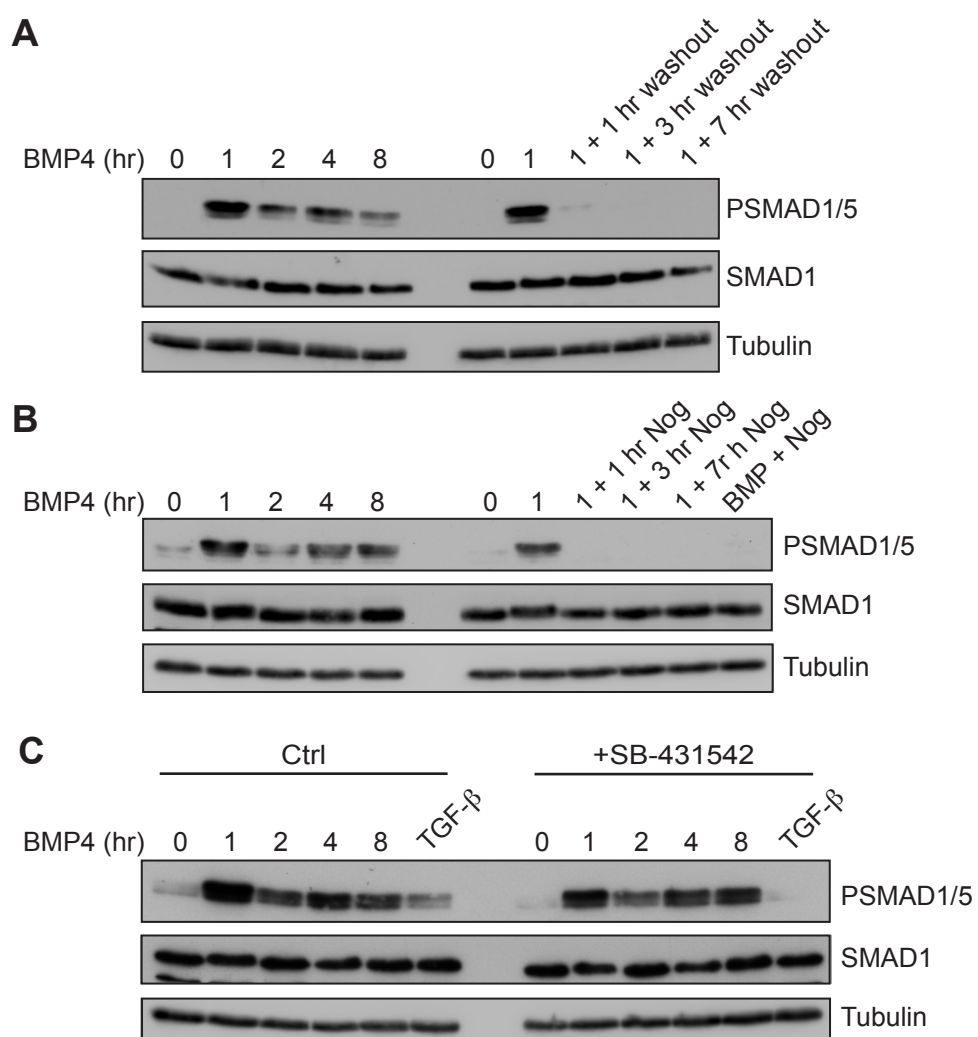
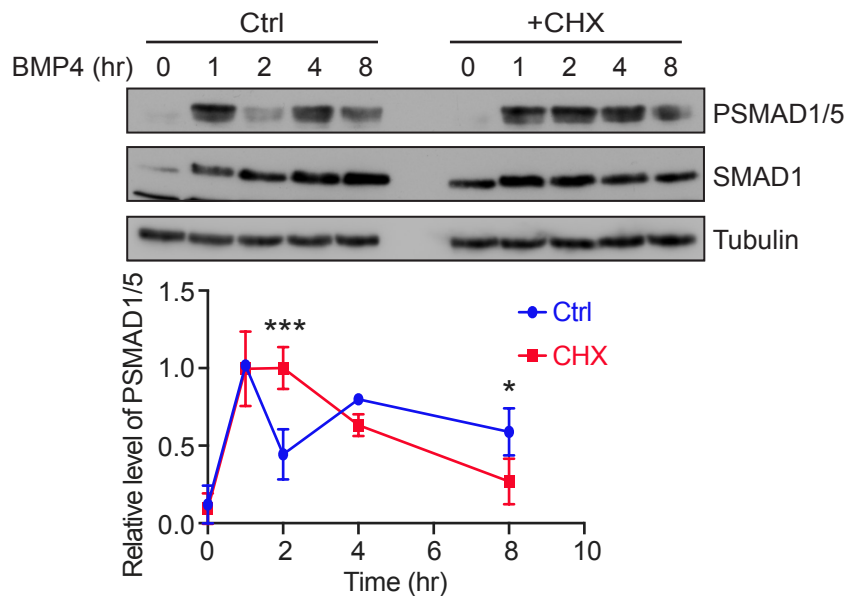


Figure 6 - figure supplement 1

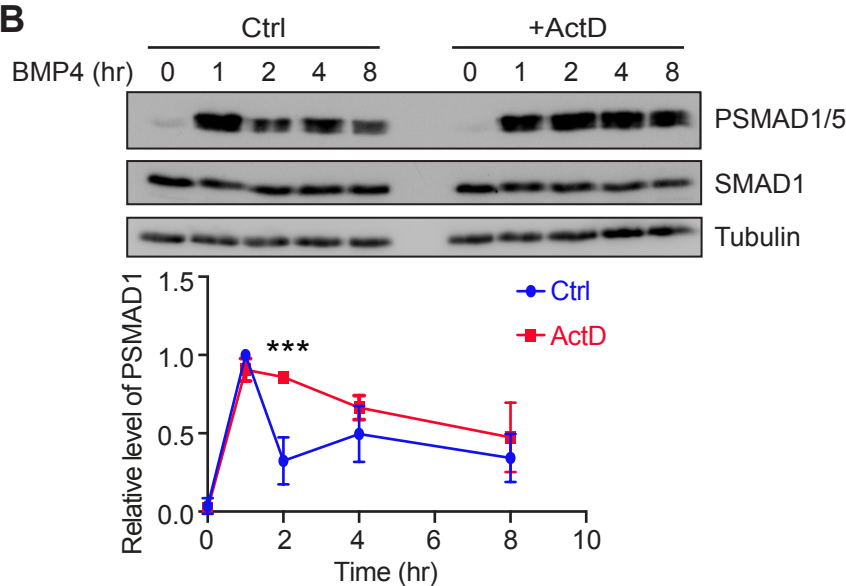
Figure 6 – figure supplement 1. The BMP4 oscillation requires persistent exposure to BMP4 and is not mediated indirectly via SMAD2/3 signaling.

(A) NIH-3T3 cells were treated with BMP4 for the times indicated, or for 1 hr with BMP4, before washout and incubation for the times indicated. (B) NIH-3T3s were treated for BMP4 for the times indicated, or for 1 hr with BMP4 before addition of Noggin for the times indicated. In the final lane, BMP4 and Noggin were added simultaneously and cells incubated for 1 hr. (C) NIH-3T3 cells were stimulated with BMP4 for the times indicated or with TGF- β for 1 hr, in the absence (Ctrl) or presence (+SB) of SB-431542. Western blotting for PSMAD1/5, SMAD1 or Tubulin as a loading control was performed. Representative blots from two independent experiments are shown.

A



B



C

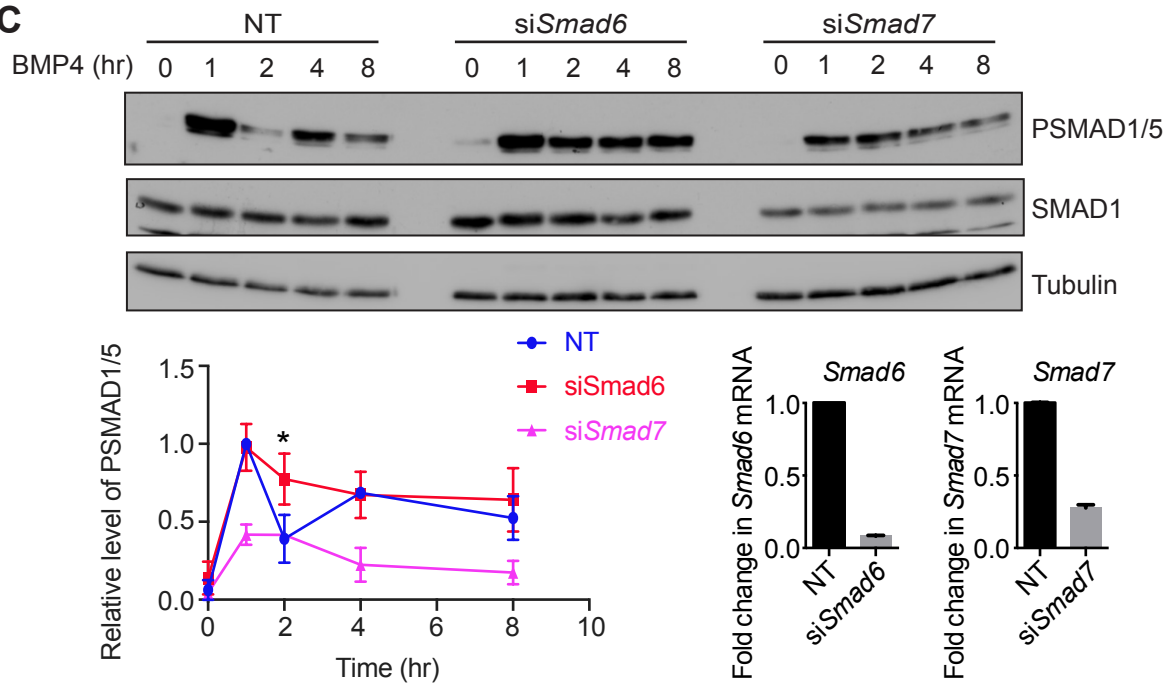


Figure 6 – figure supplement 2. The BMP4 oscillation requires new protein synthesis.

(A) NIH-3T3 cells were pre-treated or not with cycloheximide (CHX) for 5 mins, followed by BMP4 for the times indicated. **(B)** NIH-3T3 cells were pre-treated or not with Actinomycin D (Act D) for 5 mins, followed by BMP4 for the times indicated. In both cases, Western blotting for PSMAD1/5, SMAD1 and Tubulin as a loading control was performed. Quantifications are the normalized means and SDs of densitometry measurements from three independent experiments. *** indicates $p < 0.0005$ **(C)** NIH-3T3 cells were transfected with siRNAs against NT controls, *Smad6* or *Smad7* and stimulated with BMP4 for the times indicated. Western blotting for PSMAD1/5, SMAD1 and Tubulin as a loading control was performed. Quantifications are the normalized averages and SDs of densitometry measurements from three independent experiments. * indicates $p < 0.05$. Below right, the extent of knockdown was determined by qPCR. Shown are the normalized averages and SDs from two independent experiments, expressed as fold change in mRNA level relative to NT controls.

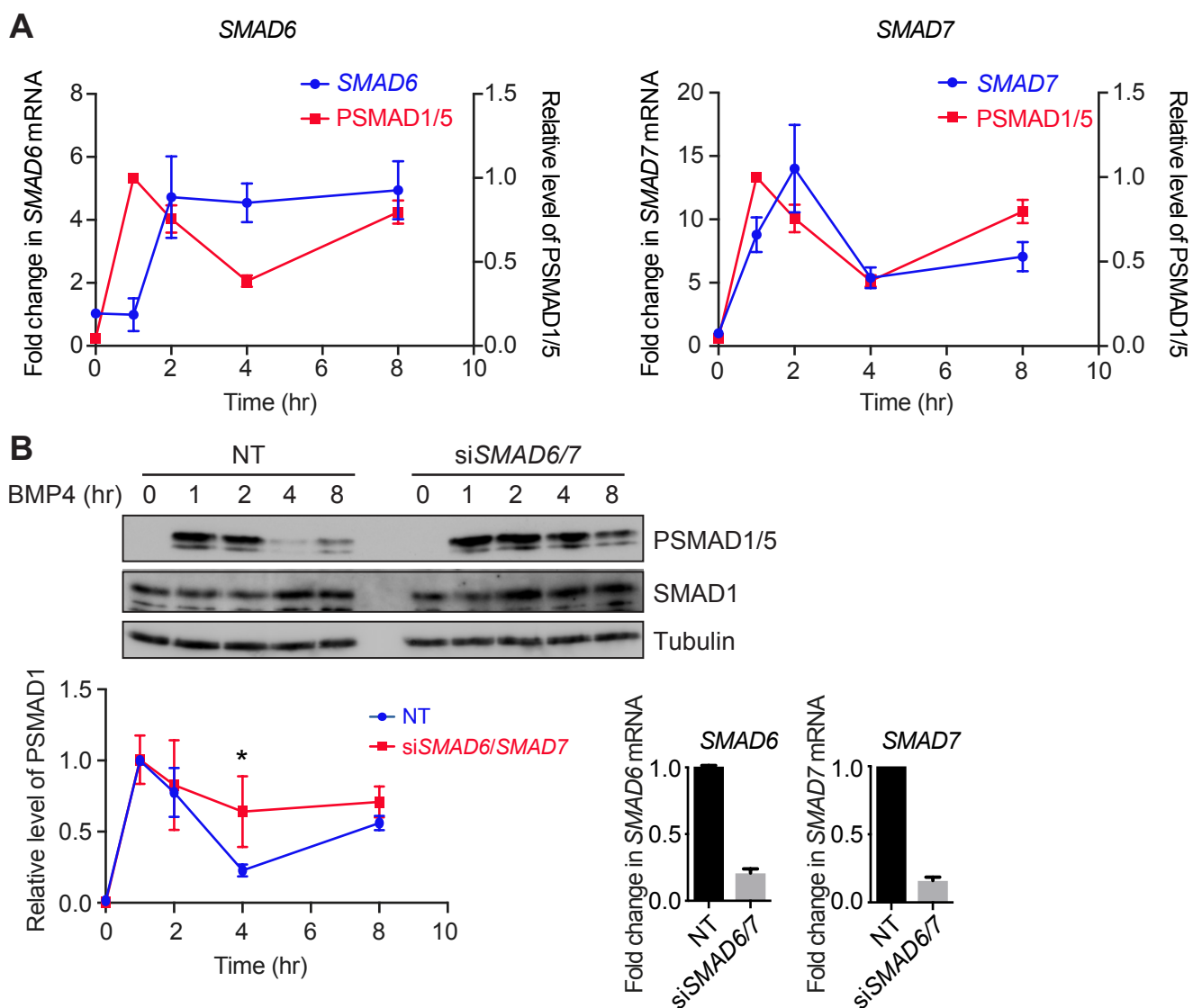


Figure 6 - figure supplement 3

Figure 6 – figure supplement 3. The BMP4 oscillation requires SMAD6/SMAD7 in MDA-MB-231 cells.

(A) MDA-MB-231 cells were treated with BMP4 for the times indicated. Levels of *SMAD6* and *SMAD7* mRNA were assayed by qPCR. Shown are the normalized averages and SDs from three independent experiments, expressed as fold change in mRNA level relative to untreated cells. The PSMAD1/5 levels are from the data shown in Figure 1A. **(B)** MDA-MB-231 cells were transfected with non-targeting control siRNAs (NT) or siRNA SMARTpools targeting *SMAD6* and *SMAD7*, and were then treated with BMP4 for the times indicated. Western blotting for PSMAD1/5, SMAD1 and Tubulin was performed. Quantifications are the normalized means and SDs of densitometry measurements from three independent experiments. * indicates $p < 0.05$. The extent of knockdown was determined by qPCR. Shown are the normalized means and SDs from three independent experiments, expressed as fold change in mRNA level relative to NT controls.