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► To cite this version:

Dominique Heymann, Muñoz-Garcia Javier, Vargas-Franco Jorge W., Schiavone Kristina, Keatinge Marcus T., et al.. Interleukin-34 orchestrates bone formation through its binding to Bone Morphogenic Proteins. *Theranostics*, In press, 10.7150/thno.107340 . hal-04901466

HAL Id: hal-04901466

<https://hal.science/hal-04901466v1>

Submitted on 20 Jan 2025

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1 **Title:**
2 Interleukin-34 orchestrates bone formation through its binding to Bone Morphogenic Proteins

3 **Authors:**
4 Javier Muñoz-Garcia^{1,2,†}, Jorge W. Vargas-Franco^{3,†}, Kristina Schiavone⁴, Marcus T.
5 Keatinge⁵, Robin Young⁶, Jérôme Amiaud⁷, Laurie Fradet¹, Jean-François Jégou⁸, Hideo
6 Yagita⁹, Claudine Blin-Wakkach¹⁰, Abdelilah Wakkach¹⁰, Denis Cochonneau², Emilie
7 Ollivier², Martine Pugièrè¹¹, Corinne Henriquet¹¹, Marie Legendre¹², Irina Giurgea¹², Serge
8 Amselem¹², Marie-Françoise Heymann^{1,2}, Stéphane Télétchéa¹, Frédéric Lézot^{12,‡*},
9 Dominique Heymann^{1,2,4,7,‡*}.

10 **Affiliations:**

11 ¹ Nantes University, CNRS, US2B, UMR 6286, Nantes, France, 44300.
12 ² Institut de Cancérologie de l'Ouest, Saint-Herblain, France, 44805.
13 ³ University of Antioquia, Department of Basic Studies, Faculty of Odontology,
14 Medellín, Colombia, 1225.
15 ⁴ Université of Sheffield, School of Medicine and Population Health, Sheffield, UK, S10
16 2TN.
17 ⁵ University of Edinburgh, Centre for Discovery Brain Sciences, Edinburgh, UK, EH8 9XD.
18 ⁶ Sheffield Teaching Hospitals NHS Foundation Trust, Sheffield, UK, S10 2JF.
19 ⁷ Nantes University, Department of Histology and Embryology, Medical School, Nantes,
20 France, 44000.
21 ⁸ University of Poitiers, LITEC, UR15560, Poitiers, France, 86073.
22 ⁹ Juntendo University, Department of Immunology, School of Medicine, Tokyo, Japan, 113-
23 8421.
24 ¹⁰ University Côte d'Azur, CNRS, UMR7370, LP2M, Nice, France, 06107.
25 ¹¹ University of Montpellier, INSERM, UMR1194, IRCM, Montpellier, France, 34298.
26 ¹² Sorbonne University, INSERM, UMR933, Hospital Armand-Trousseau (AP-HP), Paris,
27 France, 75012.

28 †,‡ These authors contributed equally to this work

29 ***Corresponding authors:**

30 Dominique Heymann	Frédéric Lézot
31 Institut de Cancérologie de l'Ouest	INSERM UMR933
32 Boulevard Professeur Jacques Monod	Hôpital Armand-Trousseau
33 Saint-Herblain, France, 44805	26 Avenue du Dr Arnold Netter
34 dominique.heyman@univ-nantes.fr	Paris, France, 75012
35 00 33 (0)2 40 67 9841	frederic.lezot@inserm.fr
	00 33 (0)1 44 73 52 39

36
37 **Author contributions:**

38 Conceptualization: JM-G, JWV-F, ST, FL, DH.
39 Methodology: JM-G, JWV-F, KS, MTK, RY, JA, LF, J-FJ, HY, CB-W, AW, DC, EO, MP,
40 CH, ML, IG, SA, M-FH, ST, FL, DH.

41 Investigation: JM-G, JWV-F, KS, MTK, RY, JA, LF, J-FJ, CB-W, AW, DC, EO, MP, CH,
42 ML, IG, SA, M-FH, ST, FL, DH.
43 Visualization: JM-G, JWV-F, KS, MTK, RY, JA, LF, J-FJ, CB-W, AW, DC, EO, MP, CH,
44 ML, IG, SA, M-FH, ST, FL, DH.
45 Funding acquisition: FL, CB-W, DH.
46 Project administration: FL, DH.
47 Supervision: FL, DH.
48 Writing – original draft: JM-G, JWV-F, FL, DH.
49 Writing – review & editing: JM-G, JWV-F, KS, MTK, RY, JA, LF, J-FJ, HY, CB-W, AW,
50 DC, EO, MP, CH, ML, IG, SA, M-FH, ST, FL, DH.
51

52 **Abstract**

53 **Rationale:** During development, the contribution of IL34, a ligand of macrophage colony
54 stimulating factor receptor (MCSFR), has not been fully defined. Together with its twin
55 cytokine MCSF, they display an essential role in macrophage differentiation and activation,
56 including tissue specialized macrophages. The mechanism of action of each molecule
57 involves the phosphorylation of MCSFR in varying intensity and kinetics. Furthermore, IL34
58 can interact with other receptors and cofactors, opening a wide range of modulations during
59 development. The aim of this work was to investigate these effects through the suppression of
60 IL34 in different animal models and study molecular interactions, with a particular focus on
61 osteoclast / osteoblast regulation.

62 **Methods:** Two different and unique models of *IL34*^{-/-} were generated in zebrafish and mouse.
63 The skeleton of both species was analyzed and compared by histological and morphometric
64 (Micro-CT) approaches. The role of IL34 and new partners in osteoclast and osteoblast
65 differentiation was analyzed by multiple techniques including mineralization assays, tartrate
66 resistant acid phosphatase (TRAP) staining, receptor phosphorylation and activation assays,
67 and gene expression (real-time quantitative PCR) studies. Furthermore, protein interactions
68 were studied by surface plasmon resonance approach and protein-protein docking ClusPro
69 analysis.

70 **Results:** Significant growth delay and hypo-mineralization of skeletal elements were
71 observed in both *IL34*^{-/-} models, as well as craniofacial dysmorphoses in mice. With regard to
72 bone cells, an unexpected increase in the number of osteoclasts and an accumulation of pre-
73 osteoblasts were observed in mice lacking IL34. For the first time, *in vitro* analyses
74 complemented by protein binding and molecular docking studies established that IL34
75 interacts directly with certain Bone Morphogenetic Proteins (BMPs), modulating their
76 various activities such as the stimulation of osteoblast differentiation.

77 **Conclusions:** A new mechanism of action for IL34 through BMPs has been characterized.
78 IL34 interactions with MCSFR and BMPs appear crucial for both osteoclastogenesis and
79 osteoblastogenesis, impacting bone tissue homeostasis and development. The potential
80 interaction of IL34 with different members of the BMP family and their functional impact,
81 including pathological situations such as cancer, should be further explored, opening new
82 therapeutic perspectives.

83

84 **Keywords:** development, bone homeostasis, osteoclastogenesis, osteoblastogenesis, protein
85 docking.

86

87

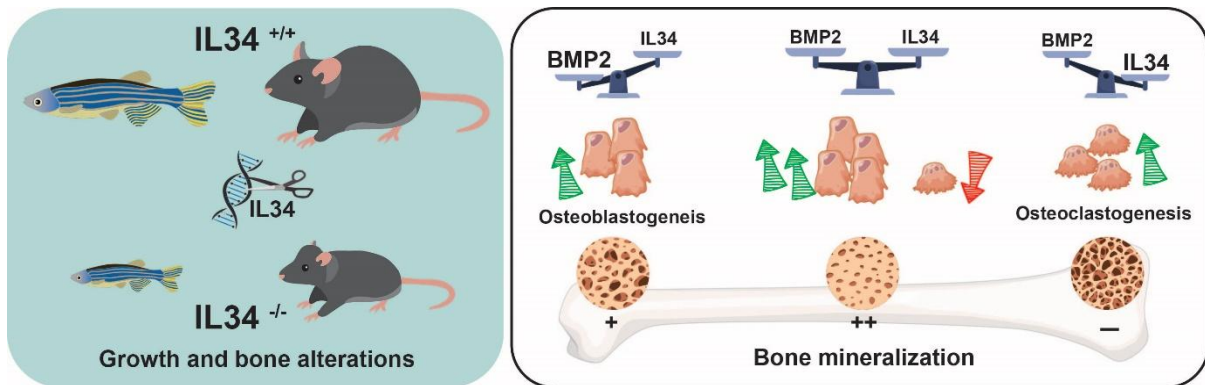
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91 **Graphical Abstract**

92



93

94

95 **Introduction**

96 Interleukin-34 (IL34) is a soluble cytokine discovered in 2008 by its ability to bind to
97 macrophage colony-stimulating factor receptor (MCSFR), also known as c-
98 FMS/CSF1R/CD115 [1]. This work has rekindled interest in the MCSFR signaling pathway
99 and in the roles of the twin cytokines MCSF/IL34 in the differentiation and activation of
100 myeloid cell lineage, such as macrophages, Langerhans cells, microglia cells and osteoclasts
101 [2–5]. IL34 binding to MCSFR can occur as a homodimer or heterodimer with MCSF/CSF1,
102 depending on the relative amounts of the two cytokines [6]. The twin cytokines induce
103 similar patterns of phosphorylation of MCSFR but with variable intensity and kinetics,
104 raising the question of their functional redundancy and specific functions. Their functional
105 redundancy is confirmed by the greater severity of the bone phenotype associated with
106 *MCSFR* versus *MCSF* invalidation in mice [7,8]. As far as the implications of IL34 during
107 bone development are concerned, the data currently available are scarce, and focus mainly on
108 its pro-osteoclastic action via its binding to the M-CSFR receptor (the binding that led to its
109 identification [1]). Similarly to MCSF, IL34, by binding to MCSFR on the surface of
110 osteoclastic precursors of myeloid origin, induces their engagement in the osteoclastic
111 differentiation pathway which will then be completed by RANKL stimulation [2,9–14].
112 Regarding the source of IL34, osteoblastic expression [15,16] and chondroblastic expression
113 [17] have been reported, suggesting that IL34 may be involved in communications between
114 bone forming cells and osteoclasts. In the absence of a detailed description of the skeletal
115 phenotype associated with IL34 invalidation during growth, the roles of IL34 in this growth
116 process are still unclear. Additional receptors of IL34 have been identified and include
117 Protein-Tyrosine Phosphatase β/ζ receptor (PTP β/ζ) [18], Triggering Receptor Expressed on
118 Myeloid cells-2 (TREM2) [19] and syndecan-1 [20]. PTP β/ζ is mainly expressed by neuronal
119 progenitors and glial cells and known as pleiotrophin/heparin-binding growth-associated
120 molecule receptor [21]. TREM2 is a lipid-binding receptor [22], carried by myeloid lineage
121 cells, whose differentiation and migratory capacities it modulates [23]. Finally, IL34 binds to
122 Syndecan-1 (CD138) and this binding modulates IL34-mediated activation of MCSFR [20].
123 The diversity of IL34 receptors and co-ligands suggests that this cytokine plays an important
124 role in the differentiation and activation of myeloid, neural and glial cells. In this context, the
125 existence of other partners for IL34 must not be excluded. To analyze these functions, IL34
126 was suppressed in zebrafish and mouse and the phenotypes of these mutants was fully
127 deciphered during development. New partners have been identified and their functional and
128 biological implications have been analyzed.

129

130

131 **Results**

132 **Zebrafish and mouse IL34 null models show significant alterations of the skeleton** 133 **during development**

134 *IL34* invalidation was genetically achieved in zebrafish and mouse using respectively
135 CrispR/Cas9 technology on one-cell stage embryos and conventional homologous
136 recombination in embryonic stem cells.

137

138 Two zebrafish loss of function lines were generated for the single *Il34* allele, corresponding
139 to a 23 bp deletion (mutant #1) and a 50 bp deletion with a 6 bp insertion (mutant 2) in exon
140 3 (**Figure 1A; Figure S1B-E**). In both zebrafish mutant lines, individuals at homozygous (-/-
141) status presented a severe growth alteration as shown in adult fish (**Figure 1B**). At 5 days
142 post fertilization, both null mutations resulted in a poorer craniofacial skeletal mineralization
143 (Von Kossa and alcian blue staining) comparatively to the control (+/+) but no evident
144 dysmorphism (**Figure 1C**, showed example for mutation #1).

145
146 The IL34 invalidated mouse line was obtained by CRE-recombinase activation on genetically
147 modified *Il34* gene (**Figure 1D and Figure S2**) with LoxP sites in introns 2 and 5 enabling to
148 remove exons 3 to 5 while maintaining a lacZ reporter sequence located in the 5' part of
149 intron 2 (*Il34^{LacZ}* allele in **Figure S2**). The functionality of the generated *Il34^{LacZ}* allele was
150 validated in the skin (**Figure S3**), a well-known site of IL34 expression (for instance [24,25]),
151 with in mice homozygous for this allele (thereafter called *Il34^{-/-}* mice), the expected absence
152 of IL34 expression (**Figure S3A**) associated to a significant reduction of CD207⁺ Langerhans
153 cells (**Figures S3B-D**). The LacZ reporter was also functional as attested by the β-
154 galactosidase staining on skin section of mice heterozygous for the *Il34^{LacZ}* allele (**Figure**
155 **S3E**). *Il34^{-/-}* mice were phenotypically altered. Indeed, 15 days-old IL34 invalidated mice
156 exhibited a severe growth delay and dysmorphoses in whole skeleton elements, specifically
157 in the craniofacial skeleton associated with hydrocephaly (**Figure 1E**). MicroCT scan 3D
158 reconstructions of skull and tibia enabled visualization of these growth defects in *Il34^{-/-}* mice
159 (**Figure 1F**, red arrowheads). Morphometric analysis evidenced significant reduction in the
160 skull growth in all planes (sagittal, vertical and transversal) and of the long bone growth in
161 the length and width dimensions in *Il34^{-/-}* mice compared to wild type (WT) (red vs black
162 values in **Figure 1H and Figure S4A**). However, a significant augmentation was observed
163 for the middle cranial vault and no impact was reported on the cranial vault length and the
164 inter-zygomatic root width. Interestingly, the use of a murine IL34 blocking antibody
165 (Sheff.5 clone) during the first post-natal week in WT mouse pups (protocol described in
166 **Figure S4B**) similarly induced skull growth alterations in all planes but to a lower extent
167 when compared to WT mice (brown vs black values in **Figure 1H**). MicroCT scans were also
168 used to determine bone structure parameters and bone mineral density (BMD) in various
169 anatomical sites, namely the mandibular, the vertebral, the cranial and the tibial bones. No
170 significant difference in the trabecular thickness (Tb.Th), the trabecular space (Tr. Sp) or the
171 percentage of bone volume (BV/TV) was observed between *Il34^{+/+}* and *Il34^{-/-}* mice
172 whichever bone was considered (**Figure S4C and Figure 2C**, red vs black values). On the
173 contrary, the trabecular number (Tb.N) was significantly increased only for the vertebral bone
174 in *Il34^{-/-}* (**Figure S4C**). Injections of the Sheff.5 blocking antibody had no impact on the bone
175 structure parameters (**Figure S5**). Regarding the bone mineral density, a significant reduction
176 in bone mineralization was observed in the cranial and the tibial bones of *Il34^{-/-}* mice
177 compared to *Il34^{+/+}* mice (**Figures 2A-B and D**). The Sheff.5 antibody transitory treatment
178 was insufficient to induce a similar bone mineral reduction in WT mice (**Figure 2A, Figure**
179 **S5**).

180 Taken together, all those data demonstrated that IL34-invalidation during development
181 induces important bone modifications.

182

183

184 **The absence of IL34 alters the osteoclast-osteoblast balance and bone homeostasis.**

185 Histological analyses on tibia sections performed at the level of the proximal epiphysis
186 (Safranin-O staining **Figure 2E**; Masson's trichrome staining **Figure S6**) revealed an
187 important reduction in the growth plate hypertrophic chondrocytes area ($Il34^{+/+}$ $0.277 \pm$
188 0.021 mm^2 and $Il34^{-/-}$ $0.146 \pm 0.094 \text{ mm}^2$). Tartrate resistant acid phosphatase (TRAP) and
189 Osterix (Osx/SP7) dual staining carried out by histoenzymology and immunohistochemistry
190 respectively (**Figure 2F** and top panel **Figure S7** for higher magnification) outlined an
191 increase of both staining corresponding to osteoclastic (red stain) and pre-osteoblastic cells
192 (brown stain) in the null mutant comparatively to the wild-type ($Il34^{+/+}$) littermate (**Figure**
193 **2F**). Interestingly, the RUNX2 immunohistochemistry staining, which enables identification
194 of cells of the osteoblastic lineage (**Figure S8**, top panels), showed no difference in the
195 number of stained cells between $Il34^{-/-}$ and $Il34^{+/+}$ mice, suggesting a slowdown of the
196 osteoblast differentiation process with an accumulation of Osterix-positive pre-osteoblasts in
197 the null mutant and without reduction of the total number of cells committed in this process.

198

199 To identify the part of the $Il34^{-/-}$ mouse skeleton phenotype linked to the increased number of
200 osteoclastic cells, a RANKL blocking antibody (IK22.5) was injected during the first
201 postnatal week to totally block the osteoclastogenesis (protocol described in **Figure S4B**).
202 Such blockade had no consequence on the morphometric parameters in the null mutant (green
203 vs red in **Figure 1G**) but impacted the trabecular parameters, the BV/TV and the BMD with
204 significant differences for cranial and tibial bones (green vs red in **Figure S4C** and **Figures**
205 **2C-D**). Histological analyses on tibia sections performed at the level of the proximal
206 epiphysis enabled visualization in the null mutant treated with the IK22.5 blocking antibody
207 of a massive reduction of the TRAP positive cells (**Figure 2F** and bottom panels in **Figure**
208 **S7**) associated with an apparent normalization of the growth plate hypertrophic chondrocytes
209 area from $0.146 \pm 0.094 \text{ mm}^2$ to $0.250 \pm 0.033 \text{ mm}^2$ (**Figure 2E**) whereas no impact was
210 noticed on the number of Osterix-positive (**Figure 2F** and **Figure S7**) and RUNX2-positive
211 (**Figure S8**) cells.

212 Overall, these results suggested that IL34 may directly impact osteoblastic differentiation
213 during development.

214

215 **IL34 improves BMP2 activity in osteoblastic differentiation**

216 BMPs and TGF β s proteins are direct involved in skeletal development and bone homeostasis
217 (nicely reviewed in [26]). Interestingly, conditionally, conditional knockout BMP2 and BMP4
218 mice described in the literature have a phenotype similar to our $Il34^{-/-}$ mouse model, with
219 small body size and cranial and growth plate defects [27]. BMPs and TGF β s signaling
220 pathways are crucial for proper osteoclast and osteoblast differentiation and maturation by
221 regulating key transcriptional factors as NF- κ B in bone marrow monocytes and RUNX2 in
222 mesenchymal stem cells. In order to see if those phenotypic and histological defects observed
223 in $Il34^{-/-}$ models could be due to unknown interactions between IL34 and BMPs / TGF β s

224 members we performed *in vitro* studies to evaluate the impact of IL34 in BMPs / TGFβs
225 signaling during osteoblastogenesis and osteoclastogenesis.

226 *In vitro* human mesenchymal stem cell differentiation into osteoblasts was induced by a
227 standard osteoblastic differentiation medium (composition described in the Methods section).
228 This differentiation, quantified by the phosphocalcic mineral deposition (alizarin red
229 staining). and the expression levels of differentiation markers (*RUNX2*, *ALP* and *OCN*), was
230 accelerated by addition of the bone morphogenic protein 2 (BMP2) at 10 ng/mL as shown in
231 **Figure 3A-B**, **Figure S9** and **Figure S10**. The addition of IL34 alone (20 ng/mL) to the
232 differentiation medium had no impact on the rate of osteoblastic differentiation, but
233 interestingly it was able to potentiate the effect of BMP2 when added in combination with an
234 optimal IL34/BMP2 concentration ratio (ng/mL) of two (**Figure 3A**). This concentration ratio
235 corresponded to an equal amount in molarity of the two cytokines (**Figure S11A**). The
236 combination of both molecules resulted in an earlier formation of calcium phosphate crystals
237 (identifiable by alizarin red staining) (**Figure 3A** and **Figure S9**). No mineralization was
238 observed in osteoblasts cultured in basic culture medium (CT-) neither in the presence alone
239 or in combination of BMP2 and IL34 (data not shown). The expression levels of osteoblast
240 differentiation markers were in agreement with the alizarin red staining. Indeed, the
241 combination of both molecules impacted the expression of early (*RUNX2*) and late (*ALP* and
242 *OCN*) markers of osteoblastogenesis in the way of a more rapid differentiation clearly visible
243 from day 3 for *RUNX2* and at day 14 for *ALP* and *OCN* (**Figure 3B** and **Figure S10**). These
244 data suggest a potentiation of BMP2 functions induced by IL34.

245
246 To validate this hypothesis, the canonical BMP signaling pathway was analyzed by Western
247 blots in human mesenchymal stem cells. IL34 treatment resulted in an increased and earlier
248 phosphorylation of the SMAD1/5 proteins observed in the presence of BMP2 compared to
249 each molecule alone (Figure 3C). Due to the amount of human mesenchymal stem cells
250 required for Western blot analysis, we decided to use an osteoblastic human osteosarcoma
251 cell line (MNNG-HOS). This cell line recapitulated the same effect in SMAD1/5
252 phosphorylation of IL34 treatment in the presence of BMP2 (**Figure 4A**), BMP4 and BMP7
253 (**Figure S11B-E**), whereas IL34 has no similar impact on the phosphorylation of the SMAD2
254 protein induced by the TGFβ (**Figure 4A**, **Figure S12**). Interestingly, the potentiation effect
255 of IL34 was blocked by the use of a specific human-IL34 blocking antibody named BT34
256 (**Figure 4B**, **Figure S12**), and the blocking of BMP2 pro-differentiation signaling with its
257 natural inhibitor NOGGIN was annihilated by the presence of IL34 (**Figure 4C**, **Figure S12**).
258 The potentiation effect of IL34 was moreover rapid (**Figure 4D**, **Figure S12**) and as
259 previously mentioned sensitive to the ratio between the two cytokines (**Figure 4E-G**, **Figure**
260 **S12**). The combination of BMP2 at 10 ng/mL to IL34 at 20 or 40 ng/mL induced higher
261 SMAD1/5 phosphorylation than those observed with BMP2 alone, while the combination of
262 BMP2 at 10 ng/mL and IL34 at 80 or 100 ng/mL significantly reduced SMAD1/5
263 phosphorylation (**Figure 4E**, **Figure S12**). The addition of IL34 at 20 ng/mL to BMP2 at 5,
264 10 or 20 ng/mL induced higher SMAD1/5 phosphorylation than those observed with BMP2
265 alone, while the combination of IL34 at 20 ng/mL and BMP2 at 40 or 80 ng/mL decreased
266 SMAD1/5 phosphorylation (**Figure 4G**, **Figure S12**). This observation supported the

267 existence of a physical and strong functional interaction between IL34 and some proteins of
268 the BMP family.

269

270 To complete the evidence on a physical interaction between IL34 and members of the BMP
271 protein family, the potential impact of BMP2 addition on the IL34-induced
272 osteoclastogenesis was evaluated *in vitro*. Differentiation of human CD14⁺ cells into
273 osteoclasts can be achieved by a two-step protocol corresponding to a 3-days culture period
274 in the presence of MCSF (25 ng/mL) or IL34 (100 ng/mL) to the culture medium, followed
275 by an 8-days period in presence of MCSF or IL34 combined to RANKL (100 ng/mL).
276 Osteoclasts were identified in the culture by their expression of the TRAP activity (TRAP
277 histoenzymology: purple staining). As previously shown [28], BMP2 addition to the cell
278 cultures (concentrations from 1 to 50 ng/mL) may replace either MCSF or IL34 during the
279 second period. However, while the combined addition of BMP2 (concentrations from 1 to 50
280 ng/mL) and MCSF to RANKL had no impact on the osteoclastogenesis, the combined
281 addition of BMP2 and IL34 to RANKL induced a reduction of the number of osteoclasts
282 formed (**Figure 3D-E**). Furthermore, the phosphorylation of MCSFR in response to IL34 was
283 inhibited in presence of BMP2 and this inhibition was reversed by addition of NOGGIN
284 supporting the existence of a functional physical link between IL34 and some members of the
285 BMP protein family (**Figure 4H, Figure S12**).

286

287

288 **Physical interactions between IL34 and some members of BMPs and receptors**

289 To definitively establish the physical interaction between IL34 and BMPs, surface plasmon
290 resonance experiments were performed and demonstrated effective binding of IL34 to BMP2,
291 BMP4 and BMP7 with KD values of 3.63E-07 M, 4.26E-07 M and 9.22E-07 M respectively
292 (**Figure 5A and Figure S13**).

293

294 A molecular modelling approach (see Materials and Methods), corresponding to a protein-
295 protein docking study, established that IL34 binding to BMP2 occurred at the “Knuckle” sites
296 of the BMP2 dimers known to correspond to the binding sites of BMP type 1 receptors and
297 did not impinge on the “Wrist” sites that correspond to the binding sites of BMP type 2
298 receptors (**Figure 5B-D; Figure S14**). The amino acids of BMP2 and IL34 involved in
299 binding were identified (**Figure 5E and Figure S15**), with BMP2 involving a pocket (formed
300 by F305, W310, W313, Y385 and M388) in which the phenylalanine in position 85 for
301 BMPR1A (F85) or the arginine in position 48 for IL34 (R48) are positioned during their
302 respective interactions (Figure 5F). It is important to note that the amino acids involved are
303 phylogenetically highly conserved in both BMP2 and IL34 and that, in addition, the amino
304 acids of BMP2 implicated are also found conserved in several members of the BMP family
305 (**Figure S16**). It was therefore possible to model the binding to IL34 of certain BMPs for
306 which crystallographic structures were available, such as BMP3, BMP6 and BMP7 (**Figure**
307 **S17**). The existence of direct physical links between BMP proteins and IL34 having been
308 established, the question of the consequences of these links on the binding of BMPs to their
309 receptors on the one hand and the binding of IL34 to MCSFR on the other was raised.
310 Binding of BMP2 to the type 1 BMP receptor is hindered by IL34, which binds to the same

311 site as shown above. It should be noted that this “Knuckle” site is also the binding site for co-
312 receptor proteins of the RGM family (**Figure S14C**) and that it is partially masked by
313 NOGGIN binding (**Figure S14D**). With regard to the binding of BMP2 to type 2 BMP
314 receptors, modelling shows that binding of the ACVR2A receptor, for example, is entirely
315 possible on a BMP2 dimer with two IL34 binders (**Figure 6A**), the “Wrist” sites not being
316 masked by the presence of IL34. These different possibilities for binding type 1 and 2
317 receptors and IL34 to a BMP2 dimer are shown in 3D in **Movie S1**. Concerning the binding
318 of IL34 to MCSFR, the binding of IL34 to BMP2 occurs at a site that overlaps with the
319 binding site of MCSFR to IL34 (**Figure 6B**), preventing the simultaneous binding of MCSFR
320 and BMP2 to IL34.

321

322

323 **Discussion**

324 IL34, one of the latest cytokines identified [29], has been shown to bind to a variety of
325 receptors with consequences for the differentiation and activation of myeloid, neural and glial
326 cells (For review [4,5]). Surprisingly, the implications of this cytokine during development
327 and growth had not been addressed in detail, unlike those of its receptor MCSFR (for review
328 [30]), although its ability to stimulate differentiation of osteoclasts, cells important for
329 skeletal growth, had been established via binding and activation of this receptor [13,14,31].
330 The primary aim of the work presented here was to determine these implications by
331 generating two *in vivo* models of IL34 invalidation, one in zebrafish and the other in mice,
332 and to characterize the associated skeletal phenotypes. Both models showed significant
333 growth retardation, with reductions in cartilage mineralization in zebrafish and bone
334 mineralization in mice. Concerning bone mineral density (BMD), the observation in *IL34*^{-/-}
335 mice of a significant reduction of this parameter only in certain bones raises questions.
336 Interestingly, a dichotomy was observed between bones with endochondral mineralization
337 (mandible, vertebrae, tibia trabecular zone (metaphyseal)) and those with intramembranous
338 mineralization (cranial bone, tibia periosteal zone (diaphyseal)), the latter being the only ones
339 to show a reduction in BMD. To decipher the molecular basis of such a difference, further
340 studies will be required, focusing on the expression of all the factors involved in these two
341 types of mineralization (matrix proteins, nucleation factors, enzymes, etc.).

342 In mice invalidated for IL34, obtained at an expected frequency (Mendelian inheritance) but
343 with a reduced life expectancy (3 weeks), significant craniofacial dysmorphoses were
344 observed with the presence of hydrocephalus. Such defects in craniofacial development are
345 consistent with the previously established implications of IL34 in neural and microglial cells
346 differentiation and activation [24,32–38]. This model should therefore provide a useful
347 additional tool for deciphering the precise functions of IL34 during normal and pathological
348 development of the central nervous system.

349 Histological study of bone tissue from *IL34*-invalidated mice revealed a marked increase in
350 the number of osteoclasts in the growth plate, in contrast to the phenotype envisaged for the
351 loss of a factor known to stimulate osteoclastogenesis [10,12,13]. As this increase in
352 osteoclast numbers was associated with an accumulation of pre-osteoblasts (OSX-positive)

353 and a reduction in the hypertrophic zone of the growth plate, the question of a role for IL34 in
354 the differentiation of osteoblasts and chondroblasts was raised. To check whether these two
355 points were simply not secondary to the increase in osteoclastogenesis, the consequences of
356 inhibiting RANKL (a factor essential to osteoclastogenesis) during the first week of life in
357 *Il34*^{-/-} mice were analyzed. No impact was observed on the accumulation of OSX-positive
358 cells, while normalization of the size of the hypertrophic zone was observed. These results
359 suggest that IL34 may directly regulate osteoblastic differentiation and probably indirectly
360 that of chondroblasts via osteoclasts, bearing in mind that in inflammatory situations, both
361 mature osteoblasts and hypertrophic chondrocytes can become important sources of pro-
362 osteoclastic IL34 [16,39,40]. Interestingly, a relationship has already been observed between
363 the level of osteoclastic activity and the size of the hypertrophic zone of the growth plate, and
364 vice versa. Thus, a decrease in the hypertrophic zone goes hand in hand with an increase in
365 osteoclastic activity [41,42] and an increase in this zone with a decrease in osteoclastic
366 activity [43–47]. It should then be noted that the disruption of one or other of the elements in
367 this relationship, over and above the repercussions on the other, induces growth retardation in
368 all cases, as has been reported in patients with disorders of osteoclastogenesis (for example,
369 in patients suffering from juvenile osteoporosis [48] or osteopetrosis [49]) as well as in those
370 with chondrodysplasia (for review [50]). The increase in osteoclasts observed in *Il34*^{-/-} mice,
371 appropriately associated with a reduction in the hypertrophic zone of the growth plate, could
372 therefore explain the growth retardation. Establishing the origin of the increase in osteoclasts
373 in relation to the absence of IL34 was not immediately obvious. Osterix expression marks
374 pre-osteoblasts, which are known as an important source of RANKL during growth [51,52].
375 The accumulation of OSX-positive cells at the subchondral level in *Il34*^{-/-} mice could explain
376 the increased number of osteoclasts, taking into account that an analysis of the number
377 CD11b-positive cells (osteoclast precursors) in the bone marrow and spleen of *Il34*^{-/-} mice
378 revealed a marked increase (**Figure S18**). The question then arose as to the origin of the
379 accumulation of these OSX-positive pre-osteoblasts in *Il34*^{-/-} mice, given that the total
380 number of cells committed to osteoblastic lineage according to RUNX2 labeling did not
381 appear to be affected. An impact of IL34 absence on osteoblastogenesis was therefore
382 strongly suspected.

383 Members of the TGF β -BMP family, in particular BMP2, are major stimulators of
384 osteoblastogenesis (for review [26]). The co-addition of IL34 with BMP2 in the culture
385 medium of mesenchymal stem cells undergoing osteoblastic differentiation has shown, for
386 certain ratios, a potentiation of the effect of BMP2 on this differentiation. Protein binding
387 studies showed that IL34 could bind directly to BMP2, and 3D modeling identified the amino
388 acids involved in this binding in the sequences of IL34 and BMP2. With regard to the BMP
389 family, the amino acids involved in IL34 binding were found to be highly conserved, and the
390 veracity of the direct binding of BMP4 and BMP7 to IL34 was established, suggesting that
391 IL34 may potentiate the effects of several family members. IL34 binds to the "Knuckle" site
392 of BMP2, which is also the binding site for type 1 receptors to BMPs, without obscuring the
393 "Wrist" binding site for type 2 receptors. Protein binding studies have also shown that IL34
394 can directly bind type 2 receptors to BMPs (**Figure S19**), enabling it to occupy the "Knuckle"

395 site of a BMP and transform it into a "Wrist"-like site. A biphasic mechanism of action
396 associated with IL34 binding to BMP dimers can then be proposed (**Figure 6C-D**),
397 corresponding to the progressive modification of the ratio between type 1 and 2 BMP
398 receptors. Thus, in the absence of IL34, basal activity is observed with a receptor ratio of 2/2.
399 Then with an amount of IL34 equivalent to that of BMP2, maximum activity is observed
400 corresponding to a receptor ratio of 1/3. Finally, with an excess of IL34, zero activity is
401 observed with a receptor ratio of 0/4. Interestingly, several studies have reported that in an
402 inflammatory context, BMP2 could inhibit IL34 expression [53–55], suggesting the possible
403 existence of a feedback loop of IL34 potentiation of BMP2 activity. The ratio of IL34 to
404 BMP2 has also been shown to impact IL34 binding to the MCSFR so the osteoclastogenesis
405 in bone. IL34 thus appears to play a key role in bone formation, modulating both
406 osteoclastogenesis via its direct binding to the MCSFR and osteoblastogenesis via its binding
407 to BMPs.

408 In a more general context, IL34's ability to directly control MCSF receptor activation and
409 indirectly BMP receptors activation defines it as a major player in the development, growth,
410 homeostasis and function of most organs. Further studies will obviously be needed to
411 determine which members of the BMP family are IL34 partners in each organ, in normal
412 physiology and pathological situations (for review [56]) including cancers for which IL34 is
413 already presented as a therapeutic target of major interest [57–59].

414

415 **Materials and Methods**

416 ***In vivo* experiments**

417 All zebrafish (*Danio rerio*) used for this project were located in the aquaria at the Bateson
418 Centre, at the University of Sheffield (UK). Zebrafish were present in tanks at a density of no
419 more than four zebrafish per liter, with 14 hours light and 10 hours dark cycle, at a
420 temperature of 28 °C. All experimental procedures were carried out in accordance with the
421 UK Home Office Project License PPL70/8178 and personal license IO6008638. All
422 transgenic mice (*Mus Musculus*) used for this project were housed under pathogen-free
423 conditions at the Experimental Therapy Unit at the Faculty of Medicine of the University of
424 Nantes, France (Agreement D44015 and DUO 6781). All protocols applied in the present
425 study were first validated by the French ethical committee of the "Pays de la Loire" (CEEA-
426 PdL-06) and authorized by the French ministry of agriculture and fisheries (authorization #
427 18415-201901101823350 v2).

428

429 **Generation of IL34 mutant zebrafish**

430 The zebrafish *Il34* gene (ENSDARG00000091003.2 or ZDB-GENE-050419-150) contains
431 seven exons as human and mouse genes (**Figure 1**). IL34 mutant zebrafish was generated
432 using the CRISPR-Cas9 technology as previously described [60,61]. Exon 3 was targeted
433 using the sequence shown in **Figure S1A** and the corresponding 20 bp spacer region was
434 placed into a guide RNA template for *in vitro* transcription. The gRNA was then transcribed
435 using the MEGAshortscript T7 kit (Life Technologies, UK) and microinjected with Cas9

436 protein (NEB, UK) into the yolk of zebrafish embryos the one cell stage. F0 adult fish were
437 crossed with wild-type fish to identify founder with germline transmission. Primers used for
438 genotyping were (Fw 5'-TCA GCC AAT AAA TAT CAG ATC CA-3' and Rv 5'-CGT CTC
439 CTG GTT GCA TTT-3') which amplify a 300 bp fragment of the WT sequence of zebrafish
440 IL34 exon3 covering the chosen CRISPR target sequence. Obtained fragments of shorter
441 sizes were sequenced to identify mutations induced in the different founders. Two mutations
442 corresponding to a 23 bp deletion (mutant #1 in **Figure 1**) and a 50 bp deletion combined to a
443 6bp insertion (mutant #2 in **Figure 1**) were obtained. Phenotypes of zebrafishes homozygous
444 for each of these mutations (*Il34*^{-/-} from F3 or following generations) were compared to
445 ensure for link to *Il34* deficiency and not from potential background mutations. Genotyping
446 was performed on DNA extracted from the caudal fins by PCR using same primers as those
447 used to identify founders. Fragments of 300 bp, 277 bp and 256 bp were amplified
448 respectively for *Il34* exon 3 WT, mutant #1 and mutant #2 sequences. Animal were studied at
449 5 days post fertilization or at 3 months after birth.

450

451 **Van Kossa and Alcian Blue staining of zebrafish skeleton**

452 For Von Kossa staining, samples were fixed in 4% PFA for 2 h at room temperature, rinsed
453 in water containing 0.01% tween 20, and left to incubate in a solution of silver nitrate under a
454 60 W light bulb for 1 h. After rinsing with water containing 0.01% tween 20, samples were
455 fixed in 2.5% sodium-thiosulfate for 10 min, rinsed and again fixed in 4% PFA for 30 min at
456 room temperature. Preservation was done in glycerol, and samples were kept at room
457 temperature in dark until images were taken.

458 For Alcian Blue Staining, samples were fixed overnight in 4% PFA at 4 °C. After several
459 washes in a phosphate buffer solution containing 0.1% tween 20 (PBS-T) and dehydration
460 using methanol, samples were transferred into Alcian blue staining solution (0.1% Alcian
461 Blue, 70% ethanol, 1% concentrated hydrochloric acid) and left to stain overnight at room
462 temperature. Samples were then rinsed in PBS-T and bleached in 30% hydrogen peroxide
463 for 10 min at 37 °C. A 30% saturated borate solution was then used to eliminate all residues
464 of bleaching solution before putting the samples into a trypsin digestion solution for 30 min
465 at 37 °C until brains and eyes appeared translucent. A rehydration was performed, and
466 samples were put in glycerol for preservation until images were taken.

467 Zebrafish were imaged for both stains using the SMZ1500 stereomicroscope, with a DS-Fi1
468 camera (both Nikon, Japan), at 20 X magnification and Nikon Elements software.

469

470 **Generation of *Il34* mutant mouse**

471 The *Il34* mutant mouse was generated at the Mouse Clinical Institute (IGBMC, Illkirch,
472 France; Project IR00004258 / K4258) by classical embryonic stem cells (ES) injection in
473 blastocyst stage embryo. Three JM8.N4 ES cell clones carrying the targeted
474 *Il34*^{tm1a(EUCOMM)Wtsi} allele were purchased at the European Conditional Mouse Mutagenesis
475 Consortium (EUCOMM) and the clone EPD0146_4_F02 (embryonic stem line JM8.N4;
476 C57BL/6) that was confirmed by PCR and Sanger sequencing (**Figure S2**) as being correctly
477 targeted was used to generate the *Il34* conditional mutant mouse line. Breeding with ERT2-
478 Cre mice (B6.Cg- Tg(UBC- cre/ERT2)1Ejb/J, JR#8085, Jackson Laboratory, Bar Harbor,
479 Maine, USA) enabled to (Tamoxifen dependently) delete exons 3–5 of *Il34* and the

480 neomycin-resistance cassette generating the $Il34^{+/LacZ}$ mice (**Figure S2**). Breeding with CAG-
481 FLPe mice (C57BL/6-Tg(CAG-flpe)16It0, RBRC10707, RIKEN BRC, Tsukuba, Ibaraki
482 305-0074, Japan) allowed to delete the whole LacZ–NeoR cassette and generate mice
483 carrying a loxP-flanked *Il34* allele ($Il34^{+/f}$). Homozygous $Il34^{LacZ/LacZ}$ mice (called $Il34^{-/-}$ in
484 the manuscript) were used for analysis. Mice were genotyped by PCR (**Figure S2**) with the
485 primers Il34-S2: 5'-GTC AGT ATC GGC GGA ATT-3', Il34-S3: 5'-GTT TGG CCG ATG
486 CTG GCA AAG G-3' and Il34-AS2: 5'-CTG TCT TAT GAA GAT GGC ATG CC-3'. Il34-
487 S2 and Il34-AS2 primers enable to amplify a 440 bp fragment in presence of $Il34^{LacZ}$ allele,
488 and Il34-S3 and Il34-AS2 primers fragments of 240 bp and 290 bp respectively in presence
489 of wild type (WT) and $Il34^f$ alleles (**Figure S2**).

490

491 **Alizarin Red and Alcian Blue double staining of mouse skeleton**

492 The whole-mount skeletal staining protocol used is derived from the protocol of Rigueur and
493 Lyons [62]. Briefly, after euthanasia, all skin, internal organs, adipose tissue and as much as
494 possible muscle were removed before fixation in a PBS 1X pH 7.4 solution containing 2% of
495 paraformaldehyde and 0.2% glutaraldehyde. Skeletons were then dehydrated in ethanol and
496 placed in acetone for permeabilization. Cartilage staining was then realized by submerging
497 the skeletons in the Alcian blue stain (Alcian blue 8GX 0.03% (w/v), 80% EtOH, 20% glacial
498 acetic acid). After washes in 70% and 95% ethanol, a pre-clear of the tissue was realized in a
499 1% KOH solution. Bone staining was then carried out in Alizarin red stain (Alizarin red
500 0.005% (w/v) in 1% (w/v) KOH). The Alizarin red solution was then replaced with a v/v mix
501 of glycerol and 1% KOH to remove the excess red color. Skeleton were transferred to 100%
502 glycerol for long-term storage and imaging.

503

504 **New-born mice treatment with blocking antibodies**

505 The protocol used to treat newborn mice with blocking antibodies was previously described
506 [46]. Briefly, newborn C57BL/6 mice from naïve and transgenic $Il34^{+/LacZ}$ mothers received
507 four subcutaneous injections (25 mg/kg of body weight) of respectively Sheff-5 (rat anti-
508 mouse IL34 blocking IgG1 antibody, Diaclone, Besançon, France) and IK22-5 rat anti-
509 mouse RANKL blocking IgG2a antibody [63] or isotopic corresponding control every 2 days
510 beginning at day 1 after birth (**Figure S4B**). The mice were finally sacrificed at postnatal day
511 15 for phenotyping.

512

513 **Micro-CT analysis**

514 A Skyscan 1076 micro-CT scanner (Skyscan, Kontich, Belgium) was used to analyze and
515 compare between the different groups of mice (at 15 days postnatal and n=8 for each group
516 except for $Il34^{-/-}$ + IK22, n=4) the bone morphometric, structural and mineral parameters at
517 different anatomical sites namely the tibia, the mandible, the vertebra and the cranium. All
518 samples were scanned using the same parameters (pixel size 9 μ m, 50 kV, 0.5 mm Aluminum
519 filter, 20 min of scanning). The scanner reconstruction was carried out using the NRecon
520 software and the analyses were performed using CTAn, CTVox, and DataViewer software
521 (Skyscan). In order to obtain the different measurements, the IMAGE-J software (National
522 Institutes of Health, Bethesda, MD, USA) was used. In this way, the acquisition of the image

523 in CTVox was systematically calibrated with a phantom of 5 mm (known size) and all
524 measurements were finally sized using the analysis scale in the IMAGE-J software.

525 Bone morphometric parameters including tibia total length and width were sized using
526 specific reference marks (**Figure 1C** and **Figure S4A**), and for the cranium measurements
527 were made using the method previously described [64]. Briefly, seven measurements
528 regarding the sagittal, vertical and transversal planes of craniofacial growth were made
529 (**Figure 1C** and **Figure S4A**).

530 Bone mineral and structural parameters including the bone mineral density (BMD), the
531 percentage of bone volume (BV/TV), the trabecula thickness (Tb.Th), the trabecula
532 separation (Tb.Sp) and the trabecula number (Tb.N) were analyzed for each bone at different
533 anatomical sites using a volume of interest (VOI) measuring 2.0 mm x 1.1 mm x 1.1 mm.
534 The VOI was sectioned using the Data Viewer software and analyzed using the CTAn
535 software. The different points chosen for the analysis are presented in **Figure S4**. To facilitate
536 the identification of changes in the different structures, a “color density range” was used in
537 the CTAn software that made it possible to adjust the correspondence of color and brightness
538 values using image gray scales. For tibia and head images, a brightness level of -32 and a
539 contrast level of 6 from the color density range of the CTAn software were systematically
540 used.

541

542 **Histology, histoenzymology and immunohistochemistry**

543 Histology, histoenzymology and immunohistochemistry were performed on 3 µm thickness
544 paraffin embedded sections of the different samples prepared as previously described [65].
545 Masson's trichrome and Safranin-O stains were performed following classical protocols and
546 tartrate-resistant acid phosphatase (TRAP) histoenzymology was carried out as previously
547 described [66]. Immunohistochemistry was performed by using the protocol as previously
548 described [67] and the following antibodies: rabbit monoclonal anti-RUNX2 (Abcam,
549 ref#ab192256, 1/1000), rabbit polyclonal anti- osterix (OSX) (Abcam ref#ab22552, 1/1000),
550 anti-CD207 (eBioscience, ref# 14-2073-80, 1/100).

551

552 **LacZ staining**

553 Sections (12µm) of *IL34^{+LacZ}* mice epidermis embedded in OCT were cut using Cryostat
554 Leica CM3050S. Slices were fixed with PFA 1% 5 min, rinsed with PBS 1x and incubated in
555 Xgal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) solution overnight at 37 °C.
556 Sections were rinsed with PBS 1X, left to dry and mounted with EUKITT® medium.

557

558 **In vitro experiments**

559 **Reagents**

560 Recombinant human Macrophage-Colony Stimulating Factor (MCSF), human interleukin-34
561 (IL34), human M-CSF receptor (MCSFR/CD115), human TGF-β1, human bone
562 morphogenetic protein 2 (BMP2), human bone morphogenetic protein 4 (BMP4), human
563 bone morphogenetic protein 7 (BMP7), human Noggin, Activin RIIA receptor (ActRIIA),
564 human Activin RIIB receptor (ActRIIB), human TRANCE (RANKL) and antibody anti-
565 human M-CSFR, Anti-Phosho-M-CSFR (Y723) were obtained from R&D Systems
566 (Abingdon, UK). Anti-human IL34 (BT-34) mouse IgG1 monoclonal antibody was produced

567 by Diaclone (Besançon, France) under patent (Heymann D, Ségaliny A, Brion R. University
568 of Nantes /Nantes Hospital/INSERM, “Anti-IL-34 antibodies”. WO/2016/097420 A1, 2016).
569 Antibodies directed against human Smad1 (D59D7), human Smad2 (D43B4), anti Phospho-
570 Smad1/5 (ser463/465) (41D10), anti-phospho-Smad2 (Ser465/467) (138D), β -Actin
571 (8H10D10) and HRP-conjugated secondary antibodies were purchased from Cell Signalling
572 (Ozyme, Saint Quentin Yvelines, France). AlphaLISA® SureFire® Ultra Total SMAD1 and
573 p-SMAD1 (Ser463/465) Assay kits were purchase from PerkinElmer (Villebon-sur-Yvette,
574 France).

575

576 **Cell cultures**

577 The cell lines used in the present study were purchased from the American Tissue Cell
578 Collection (ATCC, Molsheim, France). HEK293 (HEK) transfected with the pCDNA3 empty
579 plasmid or the pCDNA3 plasmid containing the MCSFR gene as described by Segaliny et al.,
580 [6]. Human Mesenchymal Stem Cells-Bone Marrow (HMSC-BM) (CLS catalog number
581 300665, Lot.071222P2) and human MNNG/HOS osteosarcoma cell line (ATCC, catalog
582 number CRL-1547) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Lonza,
583 Levallois-Perret, France) supplemented with 10% fetal bovine serum (FBS; Hyclone Perbio,
584 Bezons, France) and 2 mmol/L of L-glutamine. All the experiments using HMSC-BM cells
585 were done at passage 2. For the Human MNNG/HOS osteosarcoma cell line experiments
586 were performed between passage 2 and 4. All cell lines were regularly tested for the absence
587 of mycoplasma.

588

589 **Human osteoclast differentiation**

590 CD14⁺ monocytes were isolated from peripheral blood of 3 healthy donors CD14⁺ cells were
591 initially isolated from human peripheral blood donors provided by the French blood bank
592 institute (Etablissement Français du Sang, Nantes, France, authorization number: NTS 2000-
593 24), by using MACS microbeads (MiltenyiBiotec, Bergisch Gladbach, Germany) as
594 previously described [68]. For osteoclast differentiation, CD14⁺ cells were cultured in alpha-
595 MEM (Lonza) supplemented with 10% human serum (Invitrogen, France) and in the
596 presence of human MCSF (25 ng/mL) or human IL34 (100 ng/mL) +/- human BMP2 (40 or
597 100 ng/mL) for 3 days. Then cells were treated with same molecules in the presence of
598 human RANKL (100 ng/mL) for 11 days. Medium was renewed every 3 days. After 11 days
599 of treatment, osteoclasts were analyzed by Acid Phosphatase (TRAP) staining kits (Sigma
600 Aldrich, Saint-Quentin Fallavier, France). TRAP⁺ multinucleated cells with 3 nuclei and
601 more were considered as osteoclasts and were manually enumerated.

602

603 **Human osteoblastic differentiation**

604 Human Mesenchymal Stem Cells-Bone Marrow (HMSC-BM) (CLS catalog number 300665)
605 were purchased from CLS (Germany). Osteoblast differentiation assays were performed as
606 previously described [67,69]. Briefly, HMSC-BM were cultured in DMEM was
607 supplemented 10% of FBS, vitamin D3 (10^{-8} M; Sigma) and dexamethasone (10^{-7} M;
608 Sigma). After 3 days, ascorbic acid (50 ng/mL; Sigma) and β -glycerophosphate (10 mM;
609 Sigma) were added to allow mineralization detected by alizarin red-S staining for three
610 weeks. Images were captured using a stereomicroscope (Nikon), and mineralized surfaces

611 were quantified using Image J software. Mineralization process was carried out in the
612 presence or absence of human cytokine IL34 (25 ng/mL), BMP2 (10 ng/mL) or combination
613 of both molecules for 3 weeks. RNA samples were collected at days 3, 4, 14 and 21 after the
614 induction of differentiation.

615

616 **Flow cytometry**

617 FACS analysis of CD11b monocytic bone marrow and spleen cells were performed as
618 previously described [70]. Briefly, after red blood cell lysis (Sigma-Aldrich), bone marrow
619 and spleen cells were labelled with anti-CD11b (clone M1/70; BD Bioscience, Le Pont de
620 Claix, France). Data were acquired using a FACS Canto-II (BD Biosciences).

621

622 **Western blot**

623 The cells were collected in a RIPA buffer (10 mM Tris pH 8, 1 mM EDTA, 150 mM NaCl,
624 1% NP40, 0.1% SDS containing a cocktail of protease and phosphatase inhibitors Halt™
625 (Thermo Fisher, Waltham, MA, USA). The protein concentration was determined using a
626 BCA (bicinchoninic acid) method by BC Assay Protein Quantitation Kit (Interchim,
627 Montluçon, France). 50 µg of protein extracts were prepared in a Laemmli buffer (62.5 mM
628 Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol
629 blue) and then separated by SDS-polyacrylamide gel electrophoresis. After electrophoretic
630 transfer, the immobilon-P membranes (Millipore, Molsheim, France) were blotted with the
631 antibodies referenced in the “Reagents” section. The membranes were then probed with
632 secondary antibodies coupled with horseradish peroxidase. Antibody binding was visualized
633 with an enhanced chemiluminescence (ECL) kit Clarity™ Western ECL Substrate (Bio-Rad,
634 Marnes-la-Coquette, France). The luminescence was detected with a ChemiDoc MP Imaging
635 System (Bio-Rad). Blots images and semi-quantitative analysis were done using ImageJ
636 software (USA). Each experiment was repeated at least 3 times.

637

638 **SMAD1/5 signaling measured by Alpha SureFire® Technology**

639 Direct quantification analysis of cell signaling was performed by using Alpha SureFire®
640 Technology from PerkinElmer in a Victor® Nivo™ multimode microplate reader (ALSU-
641 PSM1; PerkinElmer, Villebon-sur-Yvette, France).

642

643 **RNA isolation and real-time PCR**

644 Total RNA was extracted using NucleoSpin® RNA Plus (Macherey-Nagel, Duren,
645 Germany). 1 µg of total RNA was used for first strand cDNA synthesis using the OneScript®
646 RT Mix (Ozyme). Real-time PCR was performed on 20 ng of reverse transcribed total RNA
647 (cDNA), 300 nM of primers (QuantiTect Primer® Assays, Qiagen) and PowerUp™ SYBR™
648 Master Mix from Applied Biosystems™ (Thermo Fisher) in a CFX96 Touch Deep Well
649 Real-Time PCR Detection system from Bio-Rad. Thermal cycle conditions were perform by
650 following manufacture protocol. The analysis was performed with CFX Manager Software
651 (Bio-Rad) using human glyceraldehyde 3-phosphate dehydrogenase (GAPDH),
652 Hypoxanthine Phosphoribosyl transferase 1 (HPRT1) and TATA box binding protein (TBP)
653 as invariant controls (QuantiTect Primer® Assays, Qiagen). Oligonucleotides were designed
654 with Primer-Blast software (NCBI) and purchased from Eurogentec (Eurogentec, Angers,

655 France). The $2^{-\Delta\Delta C_t}$ (cycle threshold) method was used to calculate expression levels. List of
 656 primers and gene name symbols with corresponding full names are indicated in Tables S1
 657 and S2 below.

658

659 **Table S1 Qiagen q-PCR murine primers**

Official full name; Alias	Official symbol	Gene Globe Id
Alkaline phosphatase, liver/bone/kidney	ALPL	QT00012957
Osteocalcin or bone gamma-carboxyglutamate protein	BGLAP	QT00232771
Runt-related transcription factor 2; <i>CBFA1</i>	RUNX2	QT00020517
Tumor necrosis factor receptor superfamily, member 11b	TNFRSF11B	QT00014294
Macrophage Colony Stimulating Factor	MCSF	QT00035224
Macrophage Colony Stimulating Factor Receptor	MCSFR	QT00073276
Bone Morphogenetic Protein Receptor 1A	BMPR1A	QT00085358
Bone Morphogenetic Protein Receptor 2	BMPR2	QT00226065
Activin Receptor 2A	ACVR2A	QT00077749

660

661 **Table S2: Eurogentec q-PCR murine primers**

Official full name; Alias	Official symbol	Gene Globe Id
Interleukin-34	IL-34	Fwd 5'-GGA CAC ACT TCT GGG GAC A-3' Rev 5'-CCA AAG CCA CGT CAA GTA GG-3'
Langerin	CD207	Fwd 5'-TCA CCT CCA TTG TGC TTC AG-3' Rev 5'-ATC GTC CAC ACG ACC TCT TT-3'
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Fwd 5'-TGC GAC TTC AAC AGC AAC TC-3' Rev 5'-CTT GCT CAG TGT CCT TGC TG-3'

662

663 **Surface plasmon resonance (SPR) assays**

664 All SPR experiments were performed on a T200 apparatus (Cytiva) at 25 °C in PBS pH 7.4
 665 containing 0.05% of surfactant P20. Human recombinant BMP2, BMP4 and BMP7 proteins
 666 were immobilized (1500- 2300 RU) at pH 4.5 on CM5-S sensor chip by amine coupling
 667 following the manufacturer's instructions (Cytiva, Velizy-Villacoublay, France). IL34
 668 kinetics were measured using one cycle titration, for these five increasing concentrations of
 669 recombinant human IL34 (12.5, 25, 50, 100, 200 nM) were injected during 60 s at 100
 670 μ L/min on coated BMPs. The last injection was followed by a 600 s dissociation time in
 671 running buffer. The KD values were evaluated using a bivalent fitting model (T200
 672 Evaluation software 3.2.1, Cytiva). All sensorgrams were corrected by subtracting the low
 673 signal from the control reference surface (without any immobilized protein) and blank buffer
 674 injections before fitting. For KD evaluation of IL34 on human recombinant receptors

675 BMPRIIA, Act RIIA and Act RIIB, these receptors were captured on immobilized anti-
676 human Fc (Cytiva), four increasing concentrations of IL34 (18.75, 37.5, 75, 150, 300 nM)
677 were injected. The KD values were evaluated by using a steady-state fitting model. The
678 binding responses of IL34 (50 nM) alone, Noggin (50 nM) alone and a mixing of IL34 and
679 Noggin were measured by 180 s injection on different coated BMP proteins (BMP2, BMP4,
680 BMP7) at a flow rate of 30 μ L/min followed by a dissociation time of 400 s in running
681 buffer.

682

683 **Protein-Protein docking and analysis**

684 Structures of M-CSFR, BMPR1 and IL-34 were extracted from their bound crystallographic
685 forms (1REW for BMPR1A + BMP2 [71], 4WRL for M-CSF:M-CSFR1 [72] and 4DKD for
686 IL-34:M-CSFR1 [73]). Docking experiments were performed using either BMP-2 fixed and
687 the partner protein mobile, or the reverse, as previously published [71]. ClusPro analysis [74]
688 was performed in balanced mode, only the first 10 binding modes clusters were considered
689 for analysis, the best modes were selected by visual inspection. Interface analysis was
690 performed using the PISA web server [75]. Visualization and superimposition of docking
691 poses and crystallographic structures were done using PyMOL (The PyMOL Molecular
692 Graphics System, Version 2.5 Schrödinger, LLC; Schrödinger, LLC 2015).

693

694 **Statistical analysis**

695 All experiments were repeated at least three times in independent experiments. The
696 differences between the experimental conditions were assessed with Student's t test or a one-
697 way ANOVA followed by the Mann-Whitney test or Kruskal-Wallis test (in the case of more
698 than two independent samples of equal or different sample size). The results are given as a
699 mean \pm SD. Results were considered significant at p-values of ≤ 0.05 , p-values of ≤ 0.01 and
700 p-values of ≤ 0.001 . GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA)
701 and Real Statistics Resource Pack Software (Release 8.91), copyright (2013-2023) Charles
702 Zaiontz (www.real-statistics.com) were used for statistical analyses.

703

704 **Supplementary Material**

705 Supplementary information is available in Supplementary File.

706

707 **Acknowledgments**

708 The authors wish to thank members from the Therapeutic Experimental Unit (Nantes, France)
709 for their technical assistance.

710

711 **Funding**

712 *Il-34*^{-/-} mice were generated at the "Institut Clinique de la Souris" (Illkirch-Graffenstaden,
713 France) and their development was supported by an INSERM grant (Project IR00004258 /
714 K4258). The work was supported by a maturation grant funding program of the Ouest
715 Valorisation Technology Transfer Office (SATT Ouest Valorisation, Nantes, France; ref.
716 DV2439) and by Agence Nationale de la Recherche (ANR-20-CE14-0037).

717

718 **Data sharing**

719 The authors of this investigation declare that all the data, analytical methods, and study
720 materials are available to the researchers. All the details information is available in
721 Supplemental Data.

722

723 **Conflict of interests**

724 Authors declare that they have no competing interests.

725

726

727

728

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730

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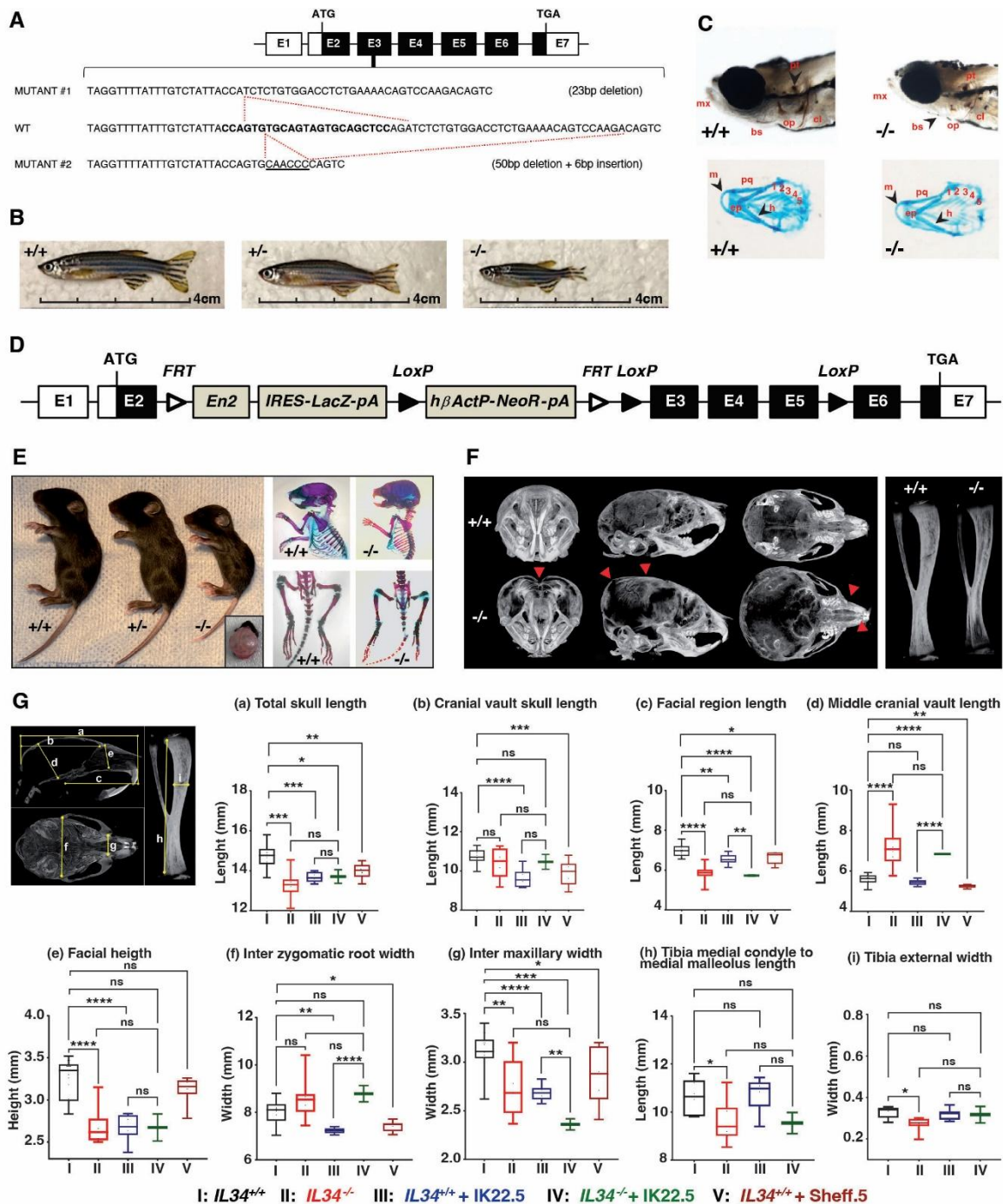
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Figure 1



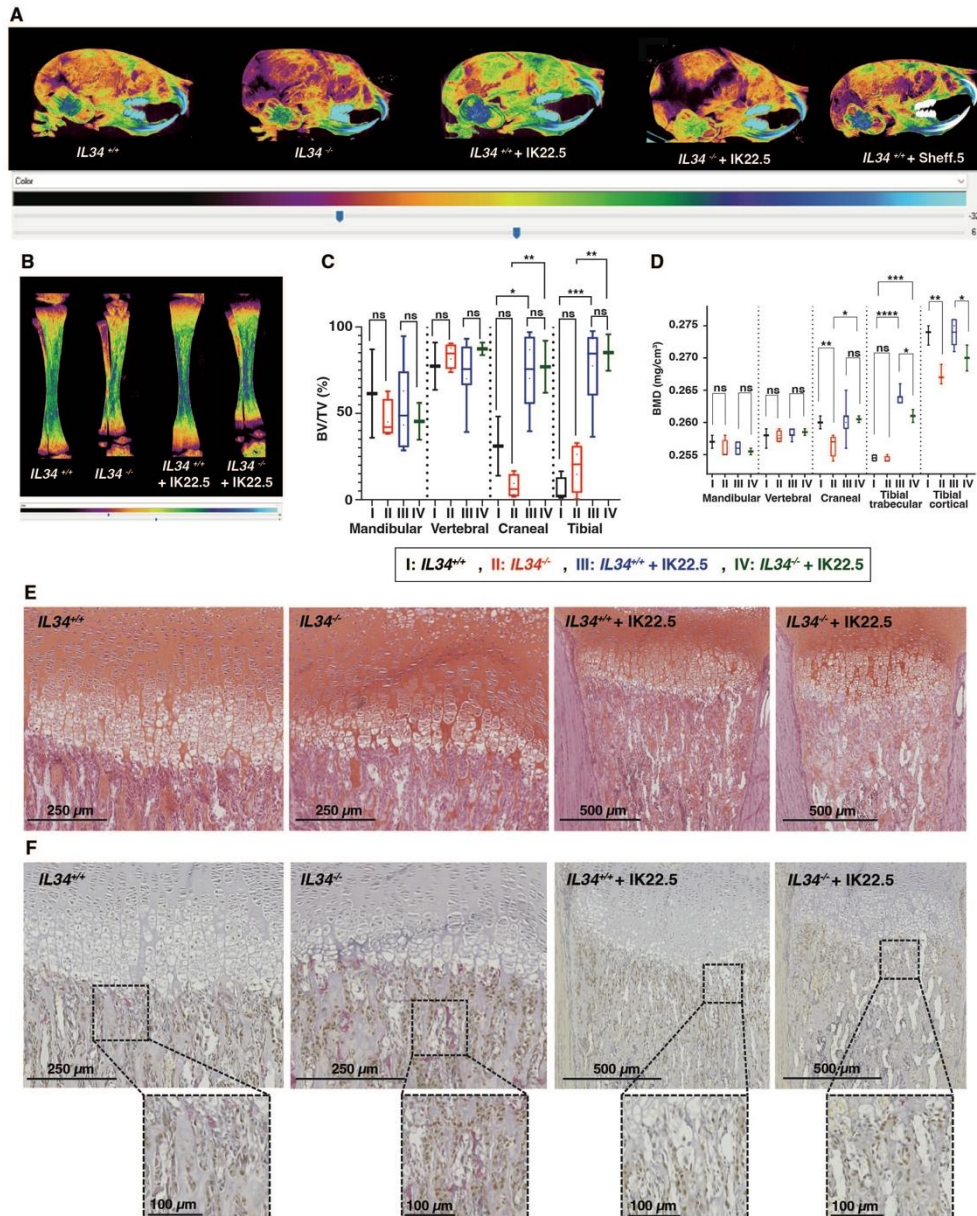
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940 **Figure 1. Growth alterations associated with *IL34* genetic invalidations in zebrafish and mouse.** (A)
941 Scheme of *IL34* Exon3 genetic alterations induced by CrispR/Cas9 technology in zebrafish. (B) Images of
942 zebrafish mutants compared to the control at age of 3 months. (C) Mineralization of craniofacial skeleton by
943 Von Kossa and Acian Blue staining of embryos at 5 days post fecundation. Abbreviations: mx - branchio
944 maxilla, bs - branchistegal ray, op - opercle, cl - cleithrum, pt - pharyngeal teeth, m - Meckel's cartilage, pq -
945 palatoquadrate, ch - ceratohyal, ep - ethmoid plate, marked 1-5 - different arches. (D) Scheme of *IL34* floxed
946 allele used to obtain constitutive invalidation of *IL34* in mouse by removing exons 3 to 5 under CRE
947 recombinase activity. (E) Images at 15 days after birth of consequences of the constitutive invalidation of *IL34*
948 with detail of hydrocephaly in *IL34*^{-/-} mouse (left panel). And comparative of skeletons at 15 postnatal days
949 visualized by Alizarin red / Alcian blue double staining (right panel). (F) MicroCT scan 3D reconstructions of
950 skull and tibia enable to visualize growth defects (red arrowheads). (G) Quantification of growth defects in the
951 different morphometric planes (a to i) in wile type (black box) vs. *IL34*^{-/-} mice (red box), both treated with

952 IK22.5 RANKL blocking antibody (blue and green boxes), or wild type mice with Sheff.5 IL34 blocking
 953 antibody (brown box). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. The differences between the
 954 experimental conditions were assessed one-way ANOVA test. $n = 8$ except for $IL34^{-/-} + IK22.5$ ($n = 4$).

955 **Figure 2**

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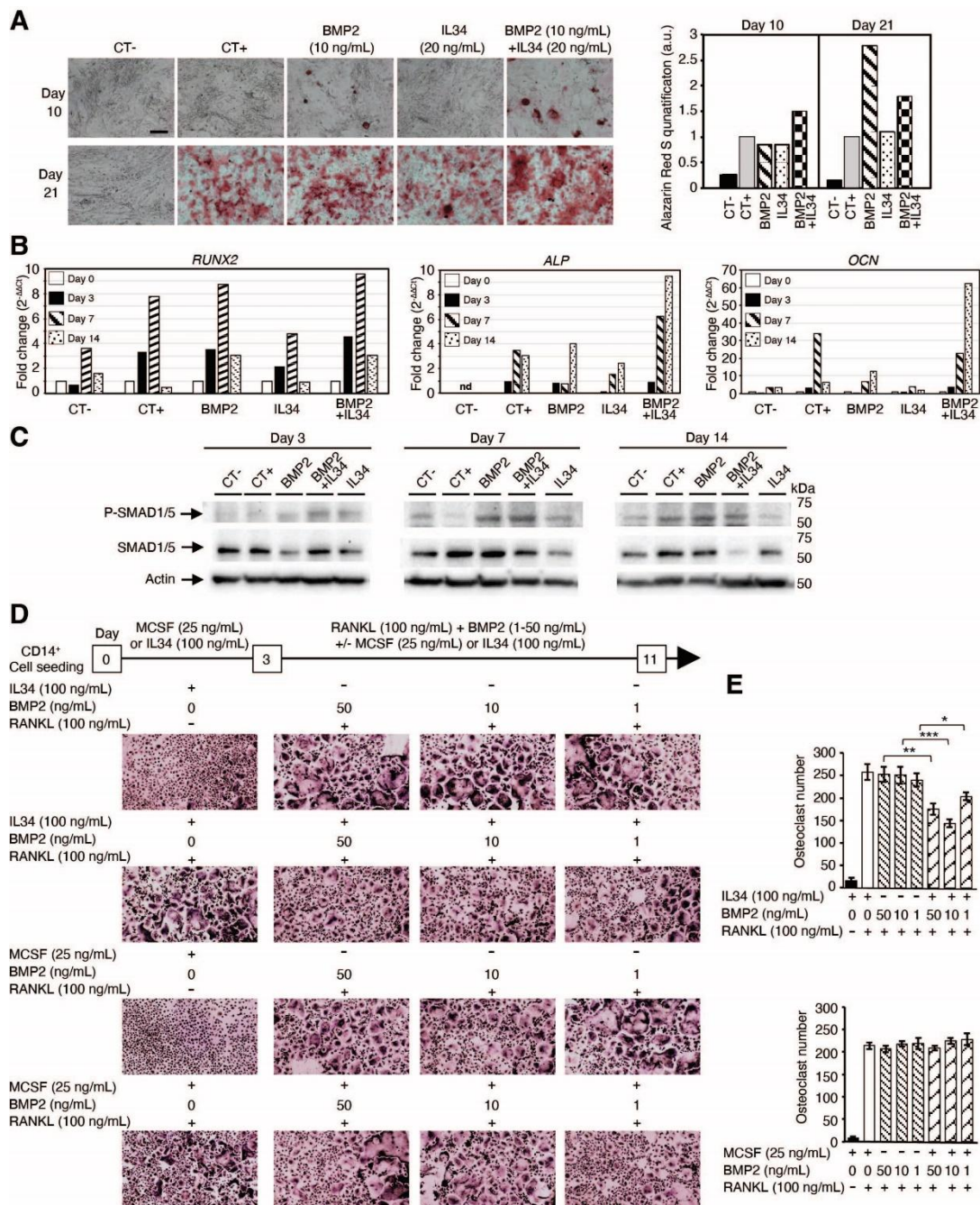
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958 **Figure 2. Bone mineral and histologic alterations associated with $IL34$ genetic invalidation in mouse. (A)**
 959 Comparative analyses of skull bones mineralization levels between $IL34^{+/+}$, $IL34^{-/-}$, $IL34^{+/+}$ injected with IK22.5
 960 antibody, $IL34^{-/-}$ injected with IK22.5 antibody and $IL34^{+/+}$ injected with Sheff.5 antibody mice at age of 15 days,
 961 using profile views of the microCT scan 3D reconstructions. The color density ranges from black (lower
 962 mineralization) to clear blue (higher mineralization). **(B)** Comparative analyses of tibias mineralization levels
 963 between $IL34^{+/+}$, $IL34^{-/-}$, $IL34^{+/+}$ treated with IK22.5 antibody and $IL34^{-/-}$ treated with IK22.5 antibody mice at age
 964 of 15 days, using longitudinal views of the microCT scan 3D reconstructions. **(C)** Comparative analysis of the
 965 bone volume (BV)/total volume (TV) ratio between $IL34^{+/+}$, $IL34^{-/-}$, $IL34^{+/+}$ treated with IK22.5 antibody and $IL34^{-/-}$
 966 treated with IK22.5 antibody in bone of different anatomical sites: the mandible, the vertebra, the skull and the
 967 tibia. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns: not significant. $n = 8$ except for $IL34^{-/-} + IK22.5$ ($n = 4$).
 968 **(D)** Comparative analysis of the bone mineral density (BMD) between $IL34^{+/+}$, $IL34^{-/-}$, $IL34^{+/+}$ treated with IK22.5
 969 antibody and $IL34^{-/-}$ treated with IK22.5 antibody in bone of different anatomical sites: the mandible, the

vertebra, the skull and the tibia. Two areas were considered for the tibia, the trabecular and the cortical.
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant. n=8 except for *Il34*^{-/-} + IK22.5 (n=4). (E) Chondrocytes stained by safranin-O staining of tibia longitudinal sections at the level of the proximal epiphysis performed for *Il34*^{-/-} and *Il34*^{+/+} mice injected or not with the IK22.5 antibody. (F) Tartrate resistant acid phosphatase (TRAP) and Osterix (Osx/SP7) dual-staining of tibia longitudinal sections at the level of the proximal epiphysis performed for *Il34*^{-/-} and *Il34*^{+/+} mice injected or not with the IK22.5 antibody. TRAP red staining for osteoclast cells. OSX brown staining for pre-osteoblasts cells. The scales are given as bars with the corresponding values in the lower part of each histological view.

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Figure 3



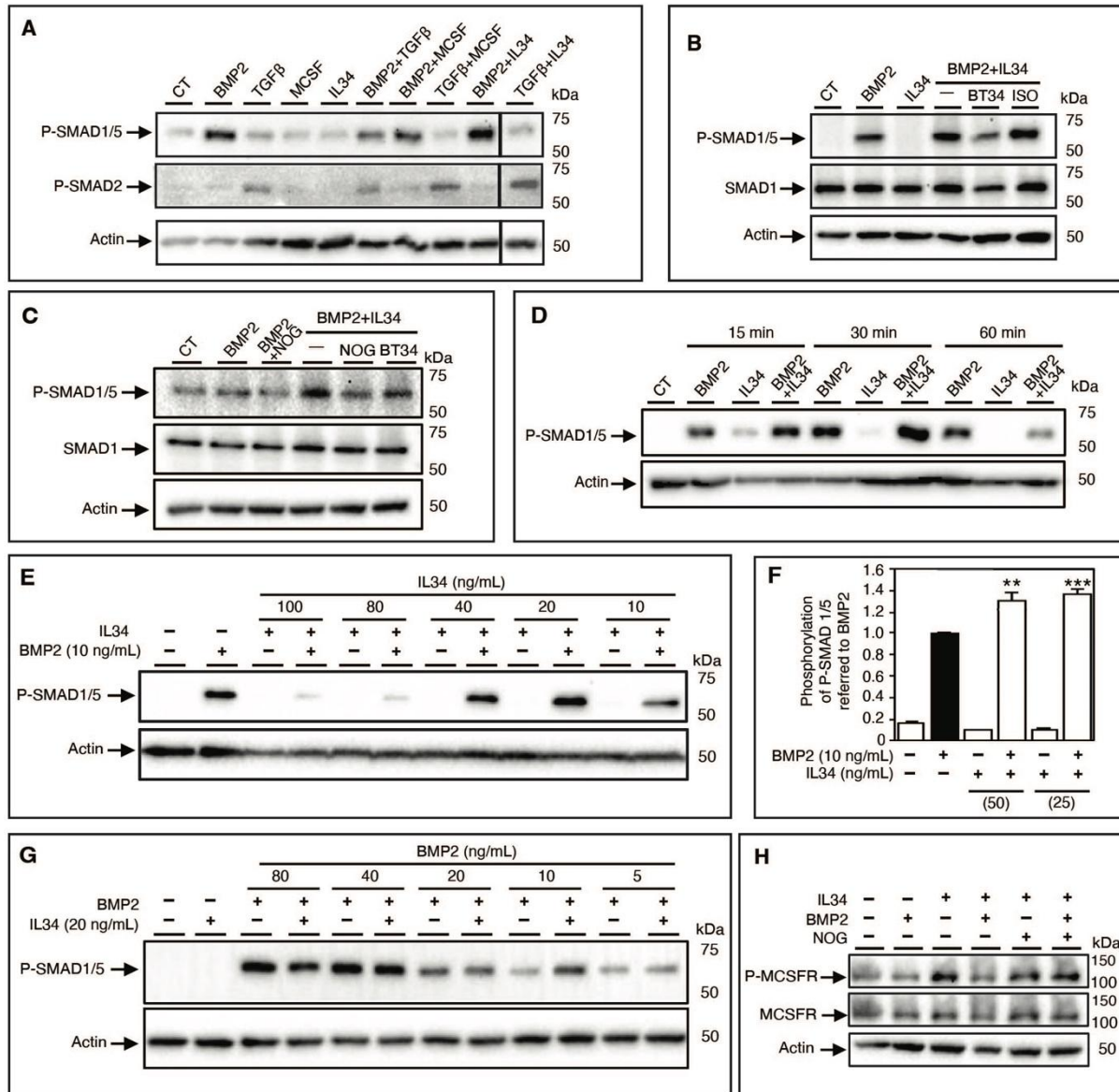
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1024 **Figure 3. IL34 regulates BMP2-associated osteoblastic and osteoclastic differentiation.** (A) Images of
 1025 human mesenchymal stem cells differentiated into osteoblasts cultured in basic culture medium (CT-) or in
 1026 osteogenic culture medium (CT+) in the absence or presence of BMP2 (10 ng/mL), IL34 (20 ng/mL) or
 1027 combination of both at 10 and 21 days. Right panel: quantification of alizarin red staining. Magnification was
 1028 similar for all views and the bar in CT- view at day 10 corresponds to 500 μ m. (B) Real-time PCR
 1029 quantification of early (*RUNX2*) and late (*ALP* and *OCN*) markers of osteoblastogenesis at days 0, 3 7 and 14.
 1030 Data correspond to fold increase by $2^{-\Delta\Delta Ct}$ (cycle threshold) method. A representative experiment is shown. nd:
 1031 non detected. (C) Western blot analysis of SMDA1/5 phosphorylation at different times of human mesenchymal
 1032 stem cells differentiated into osteoblasts in basic culture medium (CT-) and in osteogenic culture medium (CT+)
 1033 in the absence or presence of BMP2 (10 ng/mL), IL34 (20 ng/mL) or combination of both. (D) Differentiation
 1034 of human CD14⁺ cells into osteoclastic cells analyzed by Tartrate Resistant Acid phosphatase activity (TRAP
 1035 histoenzymology: purple staining) after 3-day culture period in the presence of MCSF (25 ng/mL) or IL34 (100
 1036 ng/mL), followed by an 8-day period of maturation with the addition of RANKL (100 ng/mL) and /or BMP2

1037 addition (concentrations from 1 to 50 ng/mL) (E) Quantification of the different experiments repeated in
1038 triplicate and presented in D. At least two independent experiments have been carried in triplicate. *p<0.05,
1039 **p<0.01, ***p<0.001.

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Figure 4

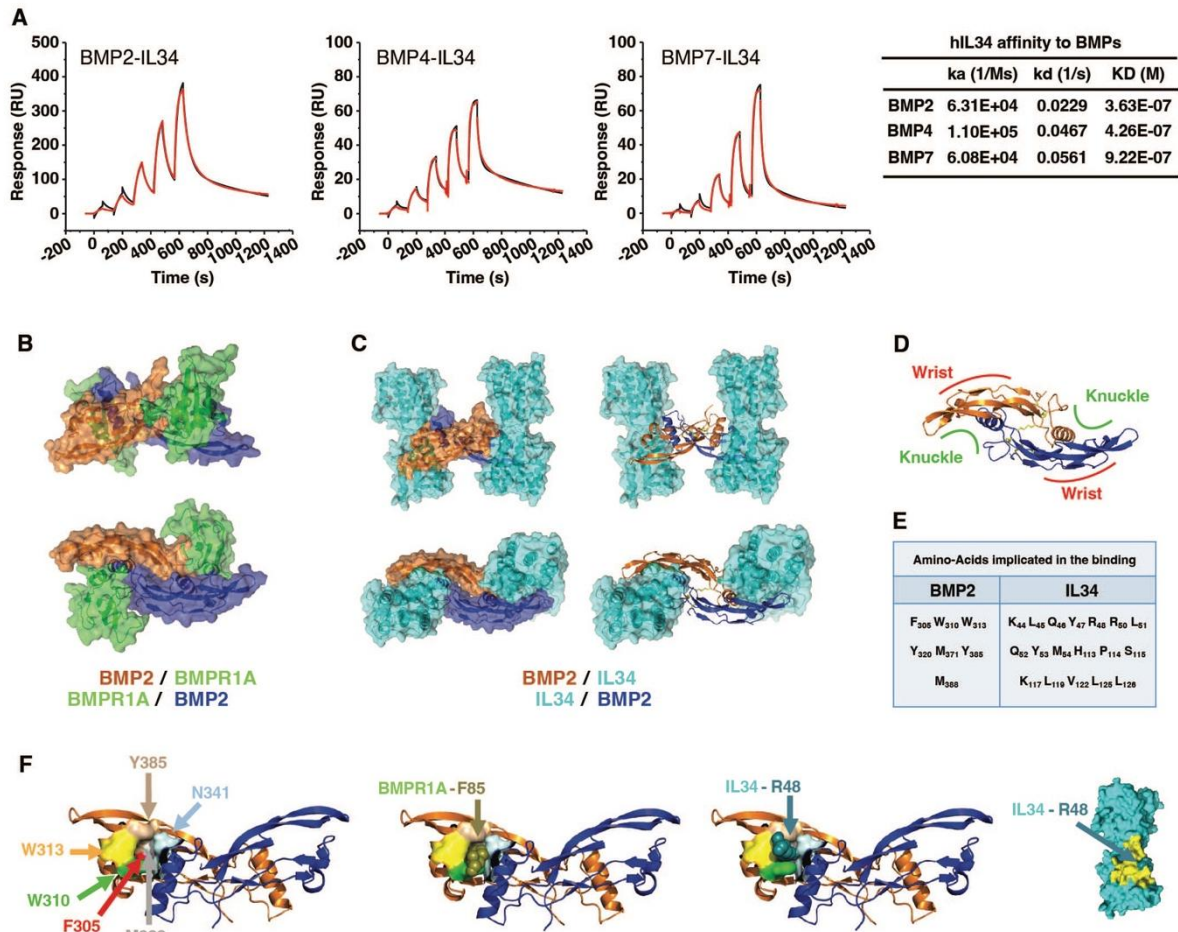


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 1090 **Figure 4. The interaction IL34-BMP2 modulates SMAD1/5 as well as MCSF receptor (MCSFR)**
 1091 **phosphorylation and related signaling.** (A) Western blot analysis of SMDA1/5 and SMAD2 phosphorylations
 1092 of human MNNG-HOS osteosarcoma cells in the presence of BMP2 (10 ng/mL), TGFβ (10 ng/mL), MCSF (20
 1093 ng/mL), IL34 (20 ng/mL) alone or in corresponding combination. A representative experiment is shown. CT:
 1094 basic culture medium. (B) Western blot analysis of SMDA1/5 phosphorylation of human MNNG-HOS
 1095 osteosarcoma cells in the presence of BMP2 (10 ng/mL), IL34 (20 ng/mL) alone or in combination
 1096 (BMP2+IL34, -) plus the human IL34 blocking antibody (BT34) (100 μg/mL) or its irrelevant isotypic control
 1097 antibody (ISO) (100 μg/mL). CT: basic culture medium. (C) Western blot analysis of SMDA1/5, similar
 1098 conditions used in B in the presence of the human IL34 blocking antibody (BT34) (100 μg/mL) or the natural
 1099 inhibitor of BMP2 called NOGGIN (NOG) (200 ng/mL). (D) Kinetic analysis by Western blot of the
 1100 potentiating effect of IL34 on BMP2-induced SMAD1/5 phosphorylation at 15 min, 30 min and 60 min with
 1101 similar corresponding molecules concentrations described in B. (E) Western blot analysis of SMDA1/5 as
 1102 described in B in the presence of a single concentration of BMP2 (10 ng/mL) in combination with gradual
 1103 quantities of IL34 (10, 20, 40, 80 and 100 ng/mL). (F) The Alpha SureFire technology (Revvity) was used to
 1104 quantitatively validate the potentiation effect of IL34 on BMP2 activation of SMAD1/5 phosphorylation. Co-
 1105 additions of 25 or 50 ng/mL of IL34 increased significantly the phosphorylation of SMAD1/5 induced by the
 1106 addition of BMP2 at 10 ng/mL. **p<0.01, ***p<0.001. (G) Western blot analysis of SMDA1/5 as described in
 1107 B with a single concentration of IL34 (20 ng/mL) in combination with gradual quantities of IL34 (5, 10, 20, 40

1108 and 80 ng/mL). (H) Western blot analysis of MCSFR phosphorylation expressed in HEK293 transfected cells in
1109 the presence or absence of BMP2 (10 ng/mL), IL34 (20 ng/mL) or in combination (BMP2+IL34, -) plus
1110 NOGGIN (NOG) (200 ng/mL). Quantifications of all the Western blots presented in this figure are shown in
1111 Figure S12. All experiments have done at least three times independently.

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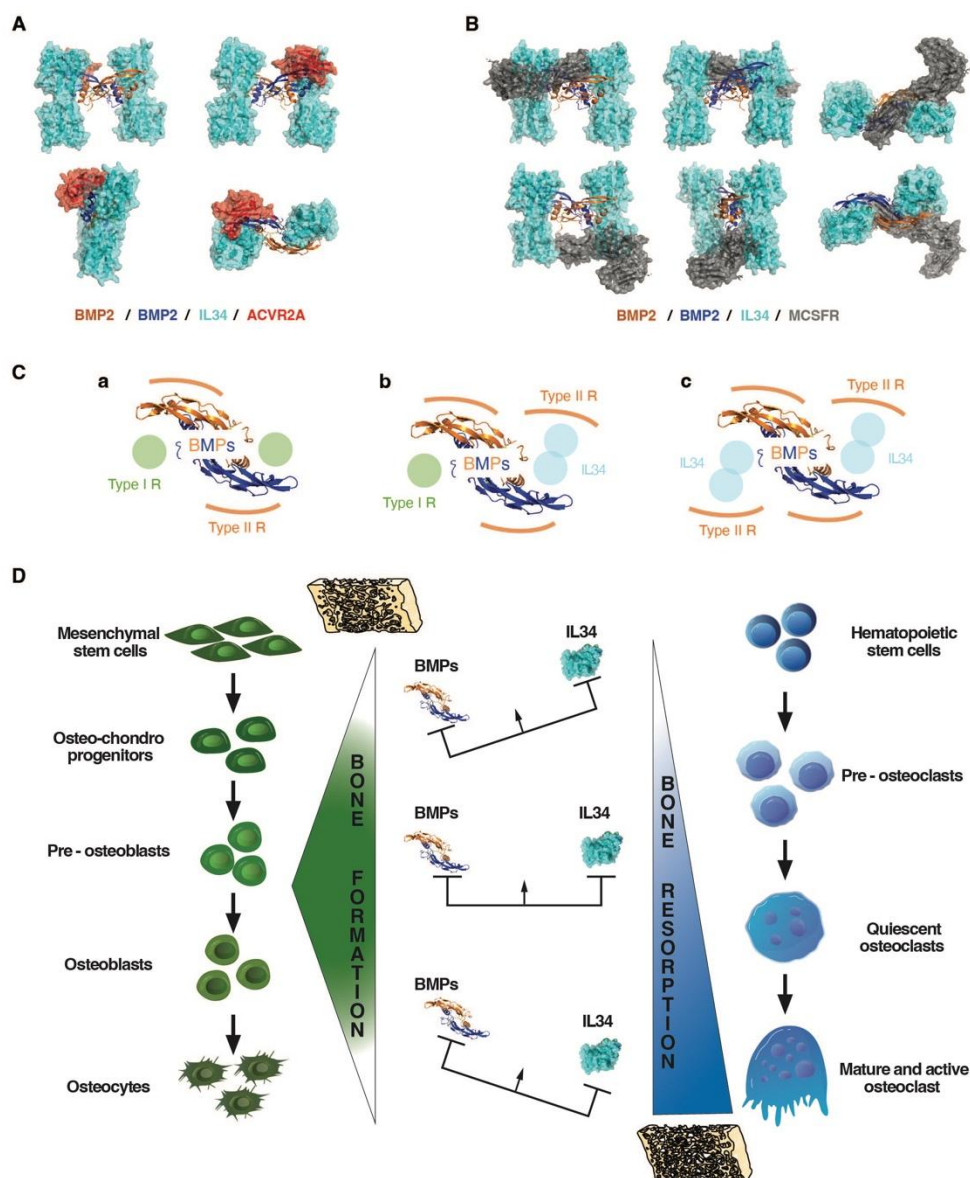
Figure 5



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Figure 5. Demonstration and deciphering at the molecular level of the physical interaction between the IL34 protein and proteins of the BMP family. (A) Surface plasmon resonance experiments (described in Materials and Methods section) and values of proteins interaction parameters between IL34 and BMPs. ka: association rate constant, kd: dissociation rate constant, KD: affinity constant. (B) Molecular modelling of the binding of two BMPR1A receptors (green) to a BMP2 dimer (brown and dark blue) by using PyMOL. (C) Molecular modelling of the binding of two IL34 proteins (cyan) to a BMP2 dimer (brown and dark blue) seen in profile (top) and from above (bottom) with a representation of the BMP2 proteins in surface (left) and in structure (right) by using PyMOL. (D) Structural representation of a BMP2 dimer seen from above with the location of the “Knuckle” and “Wrist” binding sites to the type 1 and type 2 receptors respectively as described by Sebald and collaborators [76,77]. (E) Main amino acid of BMP2 and IL34 identified as being involved in binding. In addition, hydrogen bonds and salt bridges were found between BMP2 and IL34, more specifically between residues K383-D190, D312-K55 and E376-R73. (F) Localization on the representation of the BMP2 protein in structure of amino acids important for partner binding: F305 in red, W310 in bright green, W313 in yellow, Y385 in light brown and M388 in grey. These amino acids delineate the pocket in which residues F85 of BMPR1A and R48 of IL34 are positioned during their interaction with BMP2. The amino acid N341 presented in light blue, despite is localization in the most outside part of the pocket, was not identified as important for the binding to IL34. IL34 is displayed in surface representation with the entire binding region colored in yellow, and the important intercalating residue R48 is indicated in duck blue.

Figure 6



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1187 **Figure 6. Impacts of the binding between BMP2 and IL34 on the ability of BMP2 and IL34 to bind to**
 1188 **ACVR2A and MCSFR receptors respectively: importance of stoichiometry and functional consequences**
 1189 **on bone formation and resorption.** (A) ACVR2A receptor binding to BMP2 (“Wrist” site) does not appear to
 1190 be affected by IL34 binding to the “Knuckle” sites of a BMP2 dimer. (B) MCSFR receptor binding to IL34
 1191 occurs at a site overlapping the BMP2 “Knuckle” site binding site. Simultaneous binding of BMP2 and MCSFR
 1192 to IL34 is therefore impossible. (C) BMP receptor binding stoichiometry to a BMP2 dimer. The standard
 1193 binding of two type 1 and two type 2 receptors per dimer (a), is gradually modified by the amount of IL34
 1194 present with potential transformation of a “Knuckle” site into a “Wrist”-like site at intermediate concentrations
 1195 (b), then a second at high IL34 concentrations (c), bearing in mind that IL34 can bind type 2 BMP receptors. (D)
 1196 Schematic representation of the impact of different ratios of BMPs and IL34 on bone formation and resorption.

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Supplementary Figure Legends

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Figure S1. Tools to *IL34* genetic invalidation in zebrafish using CrispR/Cas9 technology and functional confirmation. (A) Schematic representation of the targeted sequence in *il34* exon 3 including the protospacer adjacent motif (PAM) and the use guide RNA template sequence containing a T7 promoter sequence and the reverse targeted sequence and PAM. (B-E) Functional confirmation of *il34* invalidation was achieved showing the loss of the known IL34 effects on the differentiation and survival of monocytes and macrophages, [6,78] using the caudal fin amputations strategy on 3 day embryos from a heterozygous (+/-) in-cross of the double transgenic line *tg(fms: GFP)*. This line contained the heterozygous mutant *il34* allele, and GFP expression of macrophages was driven by the *MCSFR* promoter. To visualize these inflammatory cells throughout injury, live images of the amputated caudal fins were taken at 8 hours post fin injuries. The 8 hours-time-point was chosen as this is the optimal time for recruitment of macrophages to the site of injury. At this time, despite no variation of *MCSF* and *MCSFR* transcript expression levels induced by IL34 invalidation B-C, an important reduction of the number of macrophages recruited D-E was observed in the *il34*^{-/-} comparatively to the control (*il34*^{+/+}).

Figure S2. *IL34* genetic invalidation in mouse. *Il34*^{Flox} allele (Genbank: JN962093.1) and the derived *Il34*^{LacZ} allele obtained after recombination between its 5' and 3' LoxP sites are graphically represented. Primers used to genotype the different alleles are given in the table and positioned on the different graphical representations. Primers S3 and AS2 enable to discriminate the *Il34*^{Flox} allele from the WT allele with respectively amplification of 240 bp and 290 bp fragments. Primers S2 and AS2 enable to identify the *Il34*^{LacZ} allele corresponding to the amplification of a 440 bp fragment. LacZ and NeoR primer pairs were used to check the integrity of the *Il34*^{Flox} allele. Fragments amplified with S1-AS1 and S2-AS2 primer pairs from genomic DNA extracted from *Il34*^{LacZ/LacZ} mouse tail were Sanger sequenced to confirm recombination terminals (sequences corresponding to the construct cassette are underlined). The disruption of IL34 has been also analyzed by western blot. Briefly, whole protein extracts were obtained from frozen spleens from WT and IL34 null mice and specific antibody targeting IL34 was used. As showed, IL34 was only detected in WT and not in IL34 null sample.

Figure S3. Functional confirmation of *IL34* invalidation and *IL34 LacZ* reporter in mouse. (A-D) In order to confirm the effective loss of IL34 function in *Il34*^{-/-} mouse, skin well-known as an IL34 expression site [25] was used. Correlated to IL34 invalidation, a significant reduction of CD207 expressing cell (Langerhans cells) was observed. (E) β -galactosidase staining (blue) performed on section of *Il34*^{+/-LacZ} mouse enable to confirm previously described expression of IL34 [24,25,79] in some keratinocytes and cells from the hair follicle.

Figure S4. Graphical representation of morphometric parameters used for mouse head and tibia analyses, blocking antibodies injections protocol and results of bone trabecular structure analyses at different anatomical sites. (A) The seven morphometric parameters (a-g) measured for the head (craniofacial skeleton) and the two parameters (h-i) measured for tibia (appendicular skeleton) are listed and represented on 2D microCT scan views. (B) The blocking antibodies (IK22.5 and Sheff5) injections protocol was characterized by four subcutaneous injections at postnatal days 1, 3, 5 and 7 of 25 mg/kg of antibody following by a sacrifice at day 15. (C) The trabecular thickness (Tb. Th), the trabecular space (Tb. Sp) and the trabecular number (Tb. N) were determined at four anatomical sites, the mandible, the vertebra (C2) the skull and the tibia. *Il34*^{+/+} are represented in black, *Il34*^{-/-} in red, *Il34*^{+/+} treated with IK22.5 in blue, and *Il34*^{-/-} treated with IK22.5 in green. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant. n=8 except for *Il34*^{-/-} + IK22.5 (n=4).

Figure S5. Analyses of the impact of Sheff.5 antibody injections in WT pups on the bone trabecular structure (Tb. Th, Tb. Sp and Tb. N), the percentage of BV/TV and BMD. No significant variation was observed comparatively to pups injected with a control irrelevant antibody from the same idiotype whatever the parameter considered. ns: not significant. CT: control group. n=8.

Figure S6. Histological comparative analysis of the tibia proximal epiphysis area of 15-day-old *Il34*^{-/-} and *Il34*^{+/+} mice using Masson's trichome stained longitudinal sections. An important reduction of the thickness of the hypertrophic chondrocytes area was observed in the null mutant. The scales are given as bars with the corresponding values in the lower part of each histological view.

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Figure S7. High magnification views of tartrate resistant acid phosphatase (TRAP) and Osterix dual-staining of tibia longitudinal sections at the level of the proximal epiphysis performed for 15-day-old *Il34*^{-/-} and *Il34*^{+/+} mice treated or not with the IK22.5 antibody. An important increase of both staining (TRAP in red and OSX in brown) was observed in the null mutant mouse. The IK22.5 injections drastically reduced the number of TRAP-positive cells in both *Il34*^{-/-} and *Il34*^{+/+} mice, whereas no evident variation of the number of cells stained for OSX was evidenced. The scale is given as a bar corresponding to 50 μm in the lower part of each histological view.

Figure S8. Comparative analysis of RUNX2 expression in the tibia proximal epiphysis area of 15-day-old *Il34*^{-/-} and *Il34*^{+/+} mice treated or not with the IK22.5 antibody using immunohistochemistry applied to longitudinal sections. No variation of the number of stained cells was evidenced between *Il34*^{-/-} and *Il34*^{+/+} mice as between *Il34*^{-/-} and *Il34*^{+/+} injected with IK22.5 antibody. The scale is given as a bar corresponding to 250 μm in the lower part of each histological view.

Figure S9. Functional impact of BMP2, IL34 or combined BMP2+IL34 addition in the culture medium onto osteoblastic differentiations. (A) BMP2 addition (10 ng/mL) to the osteogenic differentiation medium (CT+) induced an acceleration of the differentiation as evidenced by the higher alizarin red staining corresponding to phosphocalcic crystal deposition at 7, 14 and 21 days. IL34 addition (20 ng/mL) to the osteogenic differentiation medium had no effect on the differentiation (view not shown but the quantification is presented in B, right panel). Co-addition of IL34 (10, 20 or 40 ng/mL) with BMP2 (10 ng/mL) potentialized the acceleration of the osteoblastic differentiation observed with BMP2 alone with an optimal concentration ratio (ng/mL) of 2 between BMP2 and IL34. Magnification is similar for all views and the bar in CT- view at day 7 correspond to 500 μm. (B) Quantification of the alizarin red staining corresponding to independent experiments from different donors. BIL: BMP2+IL34 addition; Numbers (10, 20 or 40) correspond to the used concentrations of IL34 in ng/mL.

Figure S10. Impacts of the additions of BMP2 (10 ng/mL), IL34 (20 ng/mL) or BMP2+IL34 to the CT+ medium onto the expression of early (*RUNX2*) and late (*ALP* and *OCN*) markers of osteoblast differentiation, onto osteoprotegerin (*OPG*, a major regulatory factor of osteoclastogenesis), and onto type 1 and type 2 receptors of the BMPs (*BMPRIA*, *BMPR2* and *ACVR2A*). Results of three different experiments (Exp. 1 to 3) carried out with three different batches of mesenchymal stem cells (obtained from different donors) are presented in order to deal with the inter-batches variabilities considering only results similarly observed in the three experiments. BMP2 addition accelerated the osteoblastic differentiation and the co-addition of IL34 potentialized this effect as evidenced for *RUNX2* expression at days 3 and 7, and for *ALP* and *OCN* at day 14. BMP2 addition alone or in combination with IL34 reduced the *OPG* expression at days 3 and 7. Regarding the different receptors of the BMPs, no reproducible effects were observed between experiments, but all three receptors are expressed at all stages (days) of differentiation whatever the culture conditions used. nd: not detected.

Figure S11. Impacts of addition of different combinations of BMP4+IL34 or BMP7+IL34 onto the activation of BMP receptors evidenced by SMAD1-5 phosphorylation in human MNNG-HOS osteosarcoma cell line. (A) Correspondence table between concentrations used in ng/mL and in pM or nM for IL34, BMP2, BMP4 and BMP7. (B) Joint addition of BMP4 (graded concentrations from 5 to 80 ng/mL) and IL34 (20 ng/mL) induced SMAD1-5 phosphorylation, with greater effects observed with 20 and 80 ng/mL BMP4. (C) Joint addition of BMP4 (20 ng/mL) and IL34 (graded concentration from 10 to 80 ng/mL) induced SMAD1-5 phosphorylation, with an optimal effect observed with 20 ng/mL IL34. (D) Joint addition of BMP7 (graded concentrations from 5 to 80 ng/mL) and IL34 (20 ng/mL) induced SMAD1-5 phosphorylation, with greater effects observed with 10 and 80 ng/mL BMP7. (E) Joint addition of BMP7 (10 ng/mL) and IL34 (graded concentration from 10 to 80 ng/mL) induces SMAD1-5 phosphorylation, with greater effects observed with 20 and 40 ng/mL IL34.

1306 **Figure S12. Quantitative analyses of Western blots presented in Figure 4.** Histograms corresponding to the
1307 control condition, the BMP2-only condition and the IL34-only condition appear in red, black and blue
1308 respectively. * $p < 0.05$, ** $p < 0.01$.

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1310 **Figure S13. Curves obtained during the surface plasmon resonance experiments with immobilization of**
1311 **the BMP proteins (2, 4 and 7) and additions of IL34 and NOGGIN alone or in combination.** Whatever the
1312 BMP protein considered an effective binding of IL34 as NOGGIN was observed with an additive effect of the
1313 two factors.

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1315 **Figure S14. Representative structures of BMP2 dimer bound proteins resolved experimentally.** BMP-2
1316 dimers are displayed in blue and orange surface. (A) BMPRII structure (green) as found in PDB ID 1ES7 [80].
1317 State that binding the knuckle site. (B) BMPRII (green) and Activin Receptor IIA (red) occupy each wrist and
1318 knuckle epitopes (PDB ID 2GOO [81]). (C) RGMA (yellow) as found in PDB ID 4UHY [82]. State that binding
1319 the knuckle site. (D) NOGGIN was determined to occupy both epitopes in structure PDB ID 7AG0 [83]. Left:
1320 side view of protein complexes, right: top view of protein complexes.

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1322 **Figure S15. Representative structures of the BMP2 dimer and IL34 in electrostatics surface view with the**
1323 **hydrophobic (white) and hydrophilic sites (red for negative patches, blue for positive patches) and**
1324 **identification of their respective binding sites.** (A) Representation of the BMP2 dimer with the knuckle site
1325 pocket underlined (arrow). (B) BMP2 dimer and IL34 position in cartoon representation. (C) IL34 with the
1326 BMP2 binding site indicated by the arrow. (D) IL34 and BMP2 position in cartoon representation.

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1328 **Figure S16. Analysis in the BMP-TGF β family of the conservation of the amino acids implicated in the**
1329 **binding to IL34 using COBALT alignment tool (NCBI).** Alignment of the human protein sequences of the 29
1330 BMP-TGF β family members was first given with amino acids implicated in the binding to IL34 conserved
1331 comparatively to BMP2 overlined in green, those changed by another with close physical-chemical properties
1332 overlined in blue and those changed by another with different physical-chemical properties overlined in yellow.
1333 Secondly for each BMP-TGF β family members the phylogenetic conservation in mammalian (15 species) of the
1334 amino acids implicated in the binding to IL34 was analyzed and given. Finally, the phylogenetic conservation of
1335 the amino acids of IL34 implicated in the binding to BMPs was also analyzed in the same 15 mammalian
1336 species and presented.

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1338 **Figure S17. Representative structures of IL34 binding to different BMP proteins.** IL34 is displayed in cyan
1339 surface. (A) IL34 binding to BMP3 structure (lime color) as found PDB ID 2QCQ [84]. (B) IL34 binding to
1340 BMP6 structure (wheat color) as found PDB ID 2R52 [85]. (C) IL34 binding to BMP7 structure (purple color)
1341 as found PDB ID 1BMP [86]. Left: side view of protein complexes, right: top view of protein complexes.

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1343 **Figure S18. FACS comparative analysis of the populations of CD11b monocytic cells in both the bone**
1344 **marrow and the spleen of $Il34^{-/-}$ versus $Il34^{+/+}$ mice.** In $Il34^{-/-}$ (IL34-KO) mice compared with $Il34^{+/+}$ (WT)
1345 mice, significant increases in the percentage of CD11b-positive cells were observed in both spleen (22.7% vs.
1346 2.54%) and bone marrow (15.2% vs. 9.83%).

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1348 **Figure S19. Demonstration at the molecular level of the physical interaction between the IL34 protein and**
1349 **proteins of the BMP receptor type II family.** The surface plasmon resonance experiments showed that IL34
1350 bound efficiently to BMPRII, ACTRIIA and ACTRIIB with KD values of 1.2E-07 M, 3.6E-07 M and 1.1E-07
1351 M respectively.

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Supplementary movie (S1)

1354 **Movie S1. Movie evidencing the simultaneous binding of one IL34 monomer (cyan), one BMPRII**
1355 **monomer (green) and one ACVR2A monomer (red) on a dimer of BMP2 (blue and orange).**

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