Repulsive guidance molecule is a structural bridge between neogenin and bone morphogenetic protein

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Repulsive guidance molecules (RGMs) control crucial processes including cell motility, adhesion, immune-cell regulation and systemic iron metabolism. RGMs signal via the neogenin (NEO1) and the bone morphogenetic protein (BMP) pathways. Here, we report crystal structures of the N-terminal domains of all human RGM family members in complex with the BMP ligand BMP2, revealing a new protein fold and a conserved BMP-binding mode. Our structural and functional data suggest a pH-linked mechanism for RGM-activated BMP signaling and offer a rationale for RGM mutations causing juvenile hemochromatosis. We also determined the crystal structure of the ternary BMP2–RGM–NEO1 complex, which, along with solution scattering and live-cell super-resolution fluorescence microscopy, indicates BMP-induced clustering of the RGM–NEO1 complex. Our results show how RGM acts as the central hub that links BMP and NEO1 and physically connects these fundamental signaling pathways.

RGMs are glycosphatidylinositol (GPI)-anchored glycoproteins. There are three mammalian family members: RGMA, RGMB (also known as DRAGON) and RGMC (also known as hemojuvelin, HFE2). RGM dysfunction is linked to regenerative failure, inflammation, multiple sclerosis, cancer and blood diseases. RGMs were initially discovered as a repulsive axon-guidance cue. They signal by binding to the cell-surface receptor NEO1 (refs. 7,8), which belongs to the immunoglobulin superfamily and shares homology with the receptor deleted in colorectal cancer (DCC). We previously showed that this process is triggered by two RGM molecules that act as a molecular staple by bringing together the juxtamembrane regions of two NEO1 receptors, thus resulting in downstream signaling and actin cytoskeleton rearrangements. All RGM family members have also been identified as co-receptors for the BMP morphogen pathway, a process that was previously suggested to be modulated by NEO1 (refs. 13,14).

BMPs compose the largest subgroup of the transforming growth factor β (TGFβ) superfamily and are key signaling players in embryonic development and in adult organisms. The active BMP signaling complex consists of the BMP ligand, a constitutive disulfide-linked dimer, which concomitantly binds to the BMP type I and type II receptors. Four different BMP type I receptors (ALK1, ACVR1, BMPR1A and BMPR1B) and three BMP type II receptors (ACVR2A, ACVR2B and BMPR2) have been identified. Ligand binding triggers intracellular phosphorylation and activation of the type I receptor kinase domain by the constitutively active type II receptor kinase. Subsequent downstream signaling occurs either via the SMAD signaling cascade or via less well characterized alternative pathways.

The cellular localization and the site of action of TGFβ and BMP receptors are still under debate, and endocytosis has been shown to be important for TGFβ and BMP signaling. BMP receptors (type I and II) undergo constitutive clathrin-mediated endocytosis even in the absence of the BMP ligand, thus resulting in a potentiation of SMAD-dependent BMP signaling upon BMP-ligand exposure. Moreover, BMPR2 is also internalized through caveolae, and the balance between caveola- and clathrin-mediated endocytosis has been suggested to modulate the patterns of gene transcription initiated by BMP signaling. In addition to endocytosis of the BMP receptors, internalization of the BMP ligand has also been observed, and components of the SMAD signaling cascade are recruited to endosomal structures for activation.

Multiple effector proteins act to regulate and fine-tune spatiotemporal BMP signaling at the membrane. These include soluble secreted antagonists (such as Noggin, Chordin and the DAN or Cerberus protein family), transmembrane proteins (for example, BAMBI and Endoglin) and the membrane-attached RGM family.

RGMs are important co-receptors and activators for BMP signaling. RGMs are important co-receptors and activators for BMP signaling. RGMs can bind directly to BMPs with nanomolar affinities; however, the molecular mechanism by which RGMs activate BMP signaling and the role of NEO1 in these processes have remained unclear.

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Received 17 November 2014; accepted 31 March 2015; published online 4 May 2015; doi:10.1038/nsmb.3016
To elucidate the molecular mechanism of RGM-activated BMP signaling, we solved crystal structures of BMP2 in complex with all human RGMs. Together with biophysical and cellular experiments, these structures suggest a mechanism for RGM-mediated activation of BMP signaling that is potentially linked to subcellular localization, and they offer a molecular rationale for JHH caused by RGM mutations. To address the role of NEO1 in these processes, we determined the crystal structure of a ternary BMP–NEO1–RGM complex, which, along with X-ray solution scattering and quantitative super-resolution microscopic clustering analyses, provides direct evidence of a physical link between the NEO1 and BMP pathways bridged by RGMs, thus putting forward a new mechanism for cellular signaling.

RESULTS

The structure of the BMP–RGM complex

We solved the crystal structure of human BMP2 in complex with the N-terminal domain of human RGMC (RGMCND) to 2.35 Å resolution (Fig. 1, Supplementary Figs. 1 and 2 and Table 1). In the complex, two molecules of RGMC bind to one disulfide-linked BMP2 dimer (Fig. 1a–c and Supplementary Fig. 2). The RGMC molecules are related by a noncrystallographic pseudo-two-fold axis and have an r.m.s. deviation (r.m.s.d.) of 0.73 Å for 66 equivalent Cα positions. RGMCND adopts a new fold composed of a triple-helix bundle stabilized by three disulfide bonds (Fig. 1b–d and Supplementary Fig. 3). RGMCND binds to the ‘finger’ region of BMP2, interacting with both BMP2 molecules (Fig. 1d–f). The RGM interface on BMP2 is highly conserved in all vertebrate BMP2, BMP4, BMP6 and BMP7 family members (Fig. 1c and Supplementary Fig. 1). RGMC contacts both molecules of the disulfide-linked BMP2 dimer (total buried surface area of 1,690 Å²), an interaction that displays mixed electrostatics (6 hydrogen bonds and 105 nonbonded contacts; Fig. 1f).

RGMA and RGMC contain a conserved RGD motif (traditionally known to be important in integrin-fibronectin–mediated adhesion33,34). This motif, containing RGMC residues R98, G99 and D100, and located in a loop region between helices α2 and α3, forms a major interaction site with BMP2 (Fig. 2a,b). Specifically, RGMC residues R98 and G99 provide several hydrogen bonds and nonbonded contacts (Fig. 2b), thereby positioning RGMC H104 to allow a π-stacking interaction with BMP2 W313 (Fig. 2c). This arrangement is further stabilized by a T-shaped, orthogonal π-stacking interaction between BMP2 residues W313 and W310. Intriguingly, mutations of RGMC residues G99, of the proposed RGD motif, and L101, located in the center of the BMP-binding interface (Fig. 2b), cause the severe iron-overload disease JHH5–28,35.

To validate our structural data, we carried out surface plasmon resonance (SPR) equilibrium binding experiments (Fig. 2d–g). Our SPR analysis revealed a level of nonspecific interaction between RGMs and BMP2, an effect that was markedly decreased for the N-terminal-domain RGM constructs. Both the full-length RGM ectodomain constructs (RGMA, RGMB and RGMC) and the RGM N-terminal-domain constructs (RGMA ND, RGMB ND and RGMC ND) bound to BMP2 with nanomolar affinities (the tightest being RGMBND, with a Kd of 88 nM), thus demonstrating that the RGM N-terminal domain is sufficient for interaction with BMP2 (Fig. 2d,e and Supplementary Fig. 4). In addition, mutation of RGMC H104 to alanine impaired binding to BMP2 (from a Kd of 124 nM to a Kd of 280 nM) (Fig. 2e and Supplementary Fig. 4f,g). This confirmed that the conserved RGMC H104 is important for efficient complex formation through a π-stacking interaction with BMP2 W313; we observed a similar behavior for the RGMB H106R mutation (Supplementary Fig. 4i).

We also solved crystal structures of BMP2 in complex with the N-terminal domains of human RGMA and RGMB (RGMA ND and RGMB ND, respectively) (Fig. 3 and Table 1). A structural superposition revealed that the overall complex architecture (Fig. 3a) and BMP2 binding mode (Fig. 3b–e) is highly conserved across all RGM family members and across species (Supplementary Fig. 1), demonstrating a common mode for the BMP-RGM interaction.

Figure 1 Structure of the BMP2–RGM complex. (a) Schematic of human (h)BMP2 and RGMC. SP, signal peptide; GPI, glycosylphosphatidylinositol anchor; WfD, von Willebrand Factor D-like domain. The potential integrin-interaction motif (RGD) and the autocatalytic-cleavage-site residues (GDPH) of RGMC are shown. (b) Cartoon representation of the BMP2–RGMCND complex. BMP2 is shown in blue and cyan; RGMCND is shown in orange and violet. (c) View rotated 90° around the x axis relative to b. Disulfide bonds (black sticks) are labeled with Roman numerals. (d–f) Surface representation of the BMP2 finger region, showing the RGM-binding site. RGMC is in ribbon representation. Disordered regions are shown as dotted lines. Orientation is as in b. (d) The BMP dimer, color coded as in b. (e) Amino acid residue conservation (from nonconserved in white to conserved in black), mapped onto the BMP2 dimer surface on the basis of alignments containing sequences from all available vertebrate BMP2, BMP4, BMP6 and BMP7 family members. (f) Electrostatic potential from red (−8 kT/e) to blue (8 kT/e), mapped onto the BMP2 dimer surface.
The RGMC–BMP2 structure offers a rationale for JHH mutations
JHH is an autosomal-recessive iron-overload disorder that results in cardiomyopathy and diabetes. JHH is caused by a deficiency in the levels of hepcidin expression and control of serum iron levels, and mutations of hepcidin are a major cause of JHH. Most of these mutations are located in the C-terminal domain of RGMC, the region responsible for interacting with BMP2 and reduce the affinity of the RGMC-BMP2 interaction. Here, we show that two of these, RGMC G99R and L101P, are located in the interface with BMP2 and reduce the affinity of the RGMC-BMP2 interaction (K_{d} of 910 nM and 1.5 µM respectively) (Fig. 2f and Supplementary Fig. 4h,i).

Figure 2 Interaction determinants of the BMP2–RGM complex. (a) Surface representation of the BMP2 dimer showing the RGM-binding site. Two regions involved in key RGM-BMP interactions are highlighted by boxes. (b,c) Close-up views of the BMP2-RGM interface. Color coding is as in Figure 1b. Selected interface residues are shown in stick representation. Hydrogen bonds are depicted as dotted lines. (d) The RGD motif, highlighted by asterisks. (e) The α, β, γ (°) of hepcidin expression and control of serum iron levels, and mutations of hepcidin are a major cause of JHH. Most of these mutations are located in the C-terminal domain of RGMC, the region responsible for interacting with BMP2 and reduce the affinity of the RGMC-BMP2 interaction (K_{d} of 910 nM and 1.5 µM respectively) (Fig. 2f and Supplementary Fig. 4h,i).

Table 1 Data collection and refinement statistics
|                      | RGM_{ND}–BMP2 | RGMB_{ND}–BMP2 form 1 | RGMB_{ND}–BMP2 form 2 | RGMC_{ND}–BMP2 | eRGMB–BMP2–NEO1_{ND} |
|----------------------|---------------|-----------------------|-----------------------|----------------|----------------------|
| **Data collection**  |               |                       |                       |                |                      |
| Space group          | P_{3_{1}}2_{1} | I_{2_{1}}3             | P_{3_{1}}2_{1}         | P_{2_{1}}2_{1} | P_{4_{1}}2_{1}       |
| Cell dimensions      |               |                       |                       |                |                      |
| a, b, c (Å)          | 83.9, 83.9, 114.8 | 129.0, 129.0, 129.0  | 85.0, 85.0, 115.2     | 69.0, 76.3, 81.7 | 120.1, 120.1, 204.1 |
| α, β, γ (°)          | 90, 90, 90    | 90, 90, 90            | 90, 90, 90            | 90, 90, 90     | 90, 90, 90          |
| Resolution (Å)       | 45.00–3.20    | 50.00–2.85            | 45.00–2.80            | 45.00–2.35     | 103.50–3.15         |
| R_{merge}            | 0.15 (0.74)   | 0.05 (0.81)           | 0.10 (0.59)           | 0.14 (0.79)    | 0.08 (1.64)         |
| R_{free}             | 0.06 (0.29)   | 0.03 (0.50)           | 0.04 (0.39)           | 0.05 (0.71)    | 0.03 (0.62)         |
| CC_{1/2}             | 0.994 (0.970) | 0.998 (0.550)         | 0.999 (0.934)         | 0.998 (0.589)  | 0.999 (0.815)       |
| I / σ(I)             | 9.1 (3.0)     | 15.1 (1.5)            | 14.7 (2.7)            | 11.7 (2.0)     | 20.2 (1.5)          |
| Completeness (%)     | 100 (100)     | 99.4 (98.9)           | 99.6 (99.4)           | 99.9 (100.0)   | 98.6 (96.1)         |
| Redundancy           | 7.1 (7.3)     | 4.2 (4.0)             | 9.6 (9.0)             | 5.7 (5.1)      | 8.7 (8.69)          |

Each structure was determined from one crystal. *Values in parentheses are for highest-resolution shell.

The RGMC–BMP2 structure offers a rationale for JHH mutations
JHH is an autosomal-recessive iron-overload disorder that results in cardiomyopathy and diabetes. JHH is caused by a deficiency in the levels of hepcidin, whereas an excess of hepcidin is linked to anemia of inflammation. RGM-activated BMP signaling is crucial for upregulation of hepcidin expression and control of serum iron levels, and mutations in RGMC are the major cause of JHH. Most of these mutations are located in the C-terminal domain of RGMC, the region responsible for interacting with BMP2 and reduce the affinity of the RGMC-BMP2 interaction (K_{d} of 910 nM and 1.5 µM respectively) (Fig. 2f and Supplementary Fig. 4h,i).
The mode of RGM-BMP2 interactions is conserved in RGMA, RGMB and RGMC. (a) Superposition of structures of the BMP2–RGMA<sub>ND</sub>, BMP2–RGMB<sub>ND</sub> and BMP2–RGMC<sub>ND</sub> complexes, with the BMP2 dimer as reference. BMP2 dimer is shown as solvent-accessible surface. RGMA (blue), RGMB (yellow) and RGMC (orange) are depicted as ribbons. Disulfide bonds are labeled with Roman numerals. Dashed asparagine in RGMB. Disulfide bonds are indicated by sticks. The RGD motif is indicated by a s. The RGD motif is indicated by asterisks in b and c. In c, the aspartate in the RGD motif is replaced with an asparagine in RGMB. Disulfide bonds are labeled with Roman numerals. Dashed lines indicate hydrogen bonds.

This not only validates the interface observed in our RGM<sub>ND</sub>–BMP2 structures but also suggests that disruption of the BMP-RGMC interaction is the molecular mechanism for HHH disease pathology. When taken together, our analyses may provide a basis for the structure-guided design of new therapeutics for the treatment of iron-related disorders such as hemochromatosis and anemia of inflammation.

RGM competes with the BMP type I receptor for BMP2 binding

Crystal structures of BMP ligands with their respective receptor ectodomains have revealed a common mode of binding in which two BMP type I and II receptor molecules bind independently to a BMP dimer in a symmetric arrangement<sup>39</sup>–<sup>41</sup>. In our RGM–BMP2 structures, RGM<sub>ND</sub> unexpectedly shares an overlapping BMP2-binding interface with the ectodomain of the BMP type I receptor BMP<sub>PR1A</sub> (eBMPR1A; Fig. 4a, b); however, the BMP2-binding site of the BMP type II receptor (eBMP2 or eACVR2A) does not overlap (Fig. 4a). To confirm this observation drawn from our structural analyses, we carried out a series of SPR experiments. The secreted ectodomain of BMP<sub>PR1A</sub> (eBMPR1A) bound to BMP2 with a <i>K<sub>d</sub></i> of 280 ± 10 nM (Fig. 4c), a result in agreement with those from previous studies<sup>42</sup>.

**Figure 3** The mode of RGM-BMP2 interactions is conserved in RGMA, RGMB and RGMC. (a) Superposition of structures of the BMP2–RGMA<sub>ND</sub>, BMP2–RGMB<sub>ND</sub> and BMP2–RGMC<sub>ND</sub> complexes, with the BMP2 dimer as reference. BMP2 dimer is shown as solvent-accessible surface. RGMA (blue), RGMB (yellow) and RGMC (orange) are depicted as ribbons. Disulfide bonds are labeled with Roman numerals. Dashed asparagine in RGMB. Disulfide bonds are indicated by sticks. The RGD motif is indicated by asterisks in b and c. In c, the aspartate in the RGD motif is replaced with an asparagine in RGMB. Disulfide bonds are labeled with Roman numerals. Dashed lines indicate hydrogen bonds.

**Figure 4** RGMs and BMP<sub>PR1A</sub> share a common binding site on BMP2. (a) Superimposition of BMP2–RGMC<sub>ND</sub> and BMP2–eBMPR1A–eACVR2A (PDB 2G0O<sup>39</sup>) complexes. BMP2 surface (light and dark blue) and RGMC<sub>ND</sub> (orange), eBMPR1A (green) and eACVR2A (pink) ribbons are shown. (b) Close-up view of the BMP2-binding region of RGMC and BMPR1A. (c) SPR equilibrium binding experiment of eBMPR1A to BMP2. <i>K<sub>d</sub></i> is shown as the best-fit value of the model ± s.d., fitted to one data set representative of 2 repeat experiments. (d) SPR competition binding experiment between eBMPR1A and eGMB to BMP2. IC<sub>50</sub> half-maximal inhibitory concentration; <i>R<sup>2</sup></i> goodness of fit; error bars, s.e.m. (n = 3 technical replicates). (e,f) MALS of the BMP2–RGMB<sub>ND</sub> and the BMP2–eBMPR1A complexes at pH 7.5 (black), pH 6.5 (dark gray) and pH 5.5 (light gray). Absorbance curves (at 280 nm) are plotted, and lines indicate the molecular weights (kDa) of the peaks. The BMP2–RGMB<sub>ND</sub> complex (46.2 ± 2.7 kDa) is marked by an asterisk, and the BMP2–eBMPR1A complex (53.0 ± 0.1; 53.2 ± 0.3; and 54.2 ± 0.5 kDa) is marked by a plus sign. (g) SMAD-mediated BMP2 response with cotransfected GPI-anchored GMB constructs. Data are from three independent experiments. Error bars, s.e.m. (n = 40 cell cultures). ****P < 0.0001; not significant (NS), P = 0.992 (RGMB<sub>ND</sub>) and 0.861 (eRGMB) by one-way ANOVA with Dunnett multiple comparison test. R.U., relative luciferase units.
and comparable to the RGMB-BMP2 interaction (Supplementary Fig. 4b-e). In this experimental setup, we did not detect specific binding between eRGMB and BMP-receptor ectodomain constructs (eBMPR1A, eBMP2, and eACVR2A; Supplementary Fig. 4f–r), in contrast to previously reported pulldown experiments with full-length BMP receptors.11. Next, we tested the ability of eBMPR1A to compete with eRGMB for BMP2 binding (Fig. 4d). We observed that a 1.7-fold molar excess of eBMPR1A was required to displace eRGMB in solution, a result suggesting that eRGMB effectively competes with eBMPR1A for BMP binding, as expected from our structural data.

The RGMB-BMP2 interaction is pH dependent
Although the structures of eBMPR1A and RGMBND are distinct, both share a common helix located at the interface with BMP2 (Fig. 4a,b), which is part of the BMPR1A site previously identified to be crucial for BMP2 interaction. In this key helix, RGMB H106 (corresponding to RGMC H104) occupies the equivalent position to BMPR1A F108. Both residues are involved in π-stacking interactions with BMP2 residues W310 and W313 (Figs. 3c and 4b). With the hypothesis that the protonation state of RGMB H106 might affect the BMP2 W313 π-stacking, we performed multangle light-scattering (MALS) measurements of both BMP2-RGMBND and BMP2-eBMPR1A complexes at different pH values (Fig. 4e,f). For BMP2-RGMBND, we observed a major species at neutral pH with a molecular weight corresponding to the 2:2 BMP2-RGMBND complex, whereas at pH 6.5 or lower dissociation of the complex occurred (Fig. 4e). Our data showed that the BMP2-RGMBND interaction is pH dependent, whereas the BMP2-eBMPR1A interaction is not (Fig. 4f).

RGMB and BMPR1A differentially alter BMP signaling
How does RGMB activate BMP signaling when it competes with the canonical BMP binding mode for BMP type I receptors? In order to answer this, we conducted a BMP-responsive luciferase reporter (BRE-Luc) assay in LLC-PK1 cells.11,44. Stimulation with 6 nM purified BMP2 increased BRE-Luc activity to approximately five-fold (n = 40, P < 0.0001) over the control (Fig. 4g). When we transfected cells with full-length (GPI-anchored) RGMB, BRE-Luc activity was further enhanced to approximately three-fold (n = 40, P < 0.0001) (Fig. 4g), comparably to previously published results,11 whereas transfection with a GPI-anchored RGMB construct lacking the RGMBND domain (RGMBND) had no effect (n = 40, P = 0.0509) on the BMP-induced response (Fig. 4g). This confirmed that the N-terminal domain is necessary for this activation, results concordant with our structural and SPR analyses (Fig. 3d,e and Supplementary Fig. 4j).

Next, we investigated the effects of soluble proteins (lacking the membrane-attachment sites) in the same luciferase reporter assay (Fig. 4h and Supplementary Fig. 5). Stimulation with 6 nM purified BMP2 increased BRE-Luc activity to approximately ten-fold (n = 72, P < 0.0001) over the control (n = 37) (Fig. 4h). When we transfected cells with soluble eBMPR1A, BRE-Luc activity decreased to ~75% (n = 22, P < 0.0001). In contrast, transfection with soluble eRGMB and RGMBND did not reduce BRE-Luc activity (n = 22, P = 0.8606; n = 24, P = 0.992, respectively) (Fig. 4h and Supplementary Fig. 5), results contrasting with those from previous studies using an RGMB-Fc fusion construct11. To further validate our observation, we performed similar experiments but added purified eBMPR1A or eRGMB proteins directly to LLC-PK1 or C2C12 cells (Supplementary Fig. 5).
Figure 6 BMP2-mediated clustering of RGM–NEO1 complex. (a,b) Live-TIRF (a) and live-TIRF-dSTORM (b) images of a COS7 cell coexpressing NEO1-mVenus and RGMB, treated with BMP2. Scale bars, 10 μm. Inset of b, cluster maps generated from a 5 × 5 μm region of interest (ROI) (red square in b), with scale bars of 1.5 μm. Bottom inset, cluster heat map colored in blue (low clustering) to red (high clustering).

(c,d) Ripley’s K function (L(t)), calculated from an ROI of a single cell treated with BMP2 (c) or control (d). Time-gated window = 7 min (2,000 frames). (e) Plot of the maxima of Ripley’s K function (L(t)max) from the experiments shown in c and d at the indicated time points. BMP2-treated (black dots, from c) and control (white dots, from d) cells are labeled. (f) Mean L(f)max calculated for all analyzed live cells treated with BMP2 (black dots, n = 8 cells) or control (white dots, n = 6 cells). BMP2 addition was at time 0 min. Error bars, s.d. P < 0.001 by two-tailed Student’s t-test. 

Again, we observed inhibition of BMP signaling by eBMPR1A, whereas eRGMB did not inhibit BMP2 signaling in either cell type, even at concentrations (2.5 μM) three times the Kᵣ for the eRGMB–BMP2 interaction (Supplementary Fig. 5). In summary, soluble BMPR1A ectodomain acts as a ‘ligand trap,’ competing with endogenous BMP type I receptors and inhibiting signaling, as expected. Surprisingly, however, we found that this behavior does not extend to soluble RGMB proteins that nonetheless have similar binding affinities and can compete with the BMPR1A–BMP2 interaction. This finding, linked with the pH dependence of the RGMB–BMP2 interaction, may imply an endocytosis-linked mechanism of RGM-activated BMP signaling.

Structure of the ternary RGM–RGM–NEO1 complex

To place the RGM–BMP2 interaction into the context of the RGM–NEO1 signaling hub⁶, we next determined the crystal structure of the ternary complex composed of BMP2, eRGMB and the juxtaembrane region of NEO1 including the fifth (FN5) and sixth (FN6) fibronectin type III domains (Fig. 5a,b). In the complex, a disulfide-linked BMP2 dimer binds to two molecules of RGMB in a very similar arrangement to that observed in the binary BMP2–RGMB complex (r.m.s.d. of 0.799 Å for 328 equivalent Cα positions) (Supplementary Fig. 6a). Each RGMB is connected to the BMP2 C-terminal domain (RGMBCD) via a disordered 15-residue linker not visible in the electron density map. RGMBCD interacts with NEO1 via a similar mode to that observed for the major interaction site in the previously determined structure of the eRGMB–NEO1 complex⁷ (r.m.s.d. of 0.511 Å for 368 equivalent Cα positions) (Supplementary Fig. 6a). In the ternary complex, the FN5 domain of NEO1 also contacts BMP2 (Fig. 5a). However, analysis of the observed interfaces (Supplementary Fig. 6b) suggests that this NEO1-BMP2 interaction is due to crystal packing rather than to an important biological interface; this would also be consistent with the similar binding affinities of BMP2 to eRGMB or the purified eRGMB–NEO1 complex and the lack of increase in affinity contributed by NEO1 (Supplementary Fig. 4k).

To test whether a similar arrangement of the RGM–NEO1–BMP2 complex exists in solution, we carried out small-angle X-ray scattering (SAXS) experiments of the eRGMB–NEO1–BMP2 complex and its components (Fig. 5c and Supplementary Fig. 7). The eRGMB–NEO1–BMP2 complex, prepared via size-exclusion chromatography (Supplementary Fig. 7ab), resulted in a particle with a mass consistent with the ternary complex in a 2:2:2 stoichiometry. Starting from the crystal structure, we generated ensembles of RGM–NEO1–BMP2 models by molecular dynamics sampling and selected these against the SAXS data. The solution structure can be accurately described (χ² = 1.9) as a mixture of two models that have architectures similar to the crystal structure but that show structural variation only at the level of the linker that connects RGMB and RGMBCD and thereby dislocates the NEO1–FN5 domain away from BMP2 (Fig. 5c); this supports our analysis of the observed interfaces in this complex interaction network (Supplementary Fig. 6b).

BMP mediates clustering of RGM–NEO1 at the cell surface

In the ternary complex, the two RGM and NEO1 molecules are oriented in such a way that the C termini (which, in the context of the full-length proteins, are connected with the lipid bilayer of the plasma membrane) point in the same direction (Fig. 5b). This arrangement, also observed in solution, combined with the active signaling conformation of the 2:2 complex between NEO1 and the C-terminal domain of RGM, suggests a mode of clustering in which RGM bridges the dimers of BMP and NEO1 (Fig. 5d). Indeed, total internal reflection
fluorescence microscopy (TIRFM) combined with direct stochastic optical reconstruction microscopy (dSTORM)\textsuperscript{45} in COS7 cells (Fig. 6a,b) revealed an increase in the clustering of fluorescently tagged NEO1 molecules in a time-dependent manner after the addition of 20 nM BMP2 with live (Fig. 6a–f) and fixed (Fig. 6g,h) cells. This clustering was dependent on the presence of full-length RGMB and reached a maximum ~15 min after addition of BMP2 (Fig. 6e–h).

DISCUSSION
RGMs can signal through both trans (intercellular)\textsuperscript{9} and cis (same-cell) interactions. cis Signaling occurs in a BMP-dependent manner, such as in chondrocytes\textsuperscript{46} and hepatocytes\textsuperscript{14}, when both NEO1 and RGM are expressed on the same cell surface. Although the role of NEO1 in BMP signaling is still unclear and cell-type dependent\textsuperscript{47}, multiple lines of evidence point toward a central role for the NEO1-RGM interaction in controlling BMP ligand-receptor localization. In hepatocytes, NEO1 inhibits RGMC shedding, thus enhancing BMP signaling and hepcidin expression in the liver\textsuperscript{13,14}. This is in agreement with our results in which high local RGM concentrations were required for activation of BMP in a luciferase reporter assay (Fig. 4g,h). In vivo, such concentrations are probably provided only by membrane attachment of RGMs to BMP-responsive cells. Furthermore, SMAD-dependent BMP signaling is reduced in chondrocytes in NEO1-deficient mice, and NEO1 regulates BMP-receptor localization in membrane microdomains, an interaction that is potentially mediated by RGMs\textsuperscript{46}. It is interesting to note that NEO1 is located in membrane microdomains in the growth cones of axons, a process that is dependent on the presence of both RGM and BMP signaling\textsuperscript{48}. Our structural and functional data identify the RGM N-terminal domain as being the site of direct interaction for the BMP ligand. This interaction is accommodated in the multidomain architecture of our NEO1–RGM–BMP2 ternary-complex structure, which allows simultaneous binding of RGMs to NEO1 and BMP ligands and results in RGM-mediated clustering by bridging dimers of NEO1 and BMP2.

Our luciferase reporter data, together with the pH dependence of the RGMB-BMP2 interaction, suggest a potential mechanism for RGM-mediated activation of BMP signaling. We showed that soluble eBMPR1A acts as an inhibitor of BMP signaling, whereas eRGMB does not. We propose that this difference is linked to the pH dependence of the RGMB-BMP2 interaction and the subcellular localization of the BMP signaling complexes. Upon clathrin-mediated endocytosis, BMP2–RGMB complexes might be targeted into endosomes, which are enriched with BMP type I receptors\textsuperscript{22}. The acidification of the endosomes might then promote dissociation of RGMB from the complex and replacement by the BMP type I receptor, thus leading to enhanced BMP signals due to potentiation of SMAD signaling provided by the endosomal environment compared to the cell surface\textsuperscript{21,22}. In this scenario, the RGM–NEO1 complex could act as a shuttle for the BMP ligand (and potentially BMP type II receptors, which can be accommodated in our RGM–NEO1–BMP complex). The RGM–NEO1 complex potentially sequesters the BMP ligand at the membrane, priming it for transport via endosomal pathways. Future work will be required to test our hypothesis linking RGMs to BMP endocytosis and to characterize this in different biological contexts. Translocation of the signaling machinery through established pathways to place it in proximity to the nucleus, and therefore to downstream effector targets, is a very efficient way of effecting changes in gene expression. This mechanism has been suggested for other signaling pathways including the closely related TGFβ signaling\textsuperscript{24} as well as signaling by epidermal growth factor\textsuperscript{49} and glial cell–derived neurotrophic factor\textsuperscript{50}. The molecular mechanism of RGM-mediated BMP activation, based on endocytosis of the entire signaling complex, may provide a paradigm for many ‘cell surface’ signaling events.

METHODS
Methods and any associated references are available in the online version of the paper.

Accession codes. Atomic coordinates and structure factors for RGM\textsubscript{BND}–BMP2 (PDB 4UHY), RGM\textsubscript{BND}–BMP2 form 1 (4UHZ), RGM\textsubscript{BND}–BMP2 form 2 (4U10), RGM\textsubscript{CND}–BMP2 (4U11) and eRGMB–BMP2–NEO1\textsubscript{FN56} (4U12) have been deposited in the Protein Data Bank.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS
We thank the staff of beamlines I03, I04 and I04-1 at the Diamond Light Source (X-ray diffraction data, proposal MX-10627), BM29 at the European Synchrotron Radiation Facility (SAXS data) and the Cellular Imaging Core at the Wellcome Trust Centre for Human Genetics (TIRF and dSTORM data) for assistance; T. Walter and K.哈尔斯 for help with crystallization; R. Robinson and G. Sutton for help with MALD; and A.R. Aresicu and D.I. Stuart for reading the manuscript. This work was supported by Cancer Research UK (C20724/A14414 (C.S.) and the Wellcome Trust (097301/Z/11/Z (E.G.H.))). Further support from the Wellcome Trust core award grant 090532/Z/09/Z (C.S.) and the Wellcome Trust multi-user equipment grant 101584/MA (S.P.-P. and C.S.) are acknowledged. E.G.H. is supported by a Marie-Curie Postdoctoral Fellow (FP7-328531). S.P.-P. is supported as a Nuffield Department of Medicine Leadership Fellow. C.S. is supported as a Cancer Research UK Senior Research Fellow.

AUTHOR CONTRIBUTIONS
C.S. designed and supervised the project. E.G.H. and C.H.B. cloned all RGM, NEO1 and BMP constructs. E.G.H., B.B. and C.H.B. performed protein expression and purification, and E.G.H. crystallized the proteins. E.G.H. and C.S. collected the data and solved and refined the crystal structures. E.G.H. and B.B. carried out SPR and luciferase experiments, and E.G.H. performed the MALD experiments. SAXS data were collected by J.E. and E.G.H., and J.E. conducted all subsequent SAXS data processing. E.G.H., B.B. and S.P.-P. collected the imaging data, and S.P.-P. completed the dSTORM data processing. C.S. and E.G.H. wrote the paper, and all authors discussed the results and commented on the paper.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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Expression and purification of RGMs, NEO1 and BMP-receptor constructs.

Constructs of the extracellular domain of human RGM (GenBank AL136826; RGMAND aa 45–139), human RGMB (GenBank AK074887; eRGMB aa 53–412, RGMNBND aa 53–136, and RGM-BAN aa 37–412) human RGMC (GenBank AY372521; eRGMC aa 36–400 and RGMCMN aa 36–147), human BMPRIA (GenBank AK291764; eBMPRIA aa 49–141), human ACVR2A (GenBank X63128; eACVR2A aa 27–118), human BMP2 (GenBank Z48923; eBMP2 aa 33–132) and mouse NEO1 (GenBank Y09555; NEO1 aa 37–149, NEO1FNS6 aa 883–1134 (ref. 51), and NEO1FNS6M aa 883–1134 (ref. 51)), C-terminally fused with a hexahistidine, a BirA-recognition sequence, or an mVenus tag, were cloned side chain. The proteins were concentrated and further purified by size-exclusion chromatography with TALON beads (Clontech) and, for crystallization, treated with endoglycosidase F1 (75 μg·mg⁻¹ protein, 12 h, 21 °C) to cleave glycosidic bonds of N-linked sugars and result in only one N-acetylgalactosamine moiety bound to the corresponding asparaginyl side chain. The proteins were concentrated and further purified by size-exclusion chromatography (SEC) (Superdex 200 100/60 column, GE Healthcare) in buffer containing 10 mM HEPES, pH 7.5, and 150 mM NaCl. The production of NEO1FNS6 and NEO1FNS6M followed a protocol described previously51.

Expression and purification of BMP2 and formation of protein complexes.

BMP2 was expressed as inclusion bodies and purified as follows (protocol adapted from ref. 55). After cell lysis, inclusion-body pellets were washed four times with 20 mM EDTA, 2% (v/v) Triton X-100, and 500 mM NaCl, pH 7, and then solubilized in 6 M guanidinium hydrochloride (GdmHCl), 0.1 M Tris·HCl, pH 8.5, 1 mM EDTA and 100 mM DTT. The pH was dropped to 3–4 to inhibit disulfide-bond formation. Residual insoluble material was removed by centrifugation (10 min, 10,000 g, 4 °C). DTT was removed by dialysis four times against a 10- to 20-fold volume of 6 M GdmHCl, pH 3–4. Refolding was carried out by incubation of the reduced and solubilized inclusion-body preparation (24 h, 4 °C, —200 μg·ml⁻¹ concentration) in 100 mM Tris·HCl, 5 mM EDTA, 1 M L-arginine, pH 8.3, 100 μM oxidized glutathione (GSSG), and 100 μM reduced glutathione (GSH). The sample was concentrated to 1 mg/ml with Amicon pressure filtration (Millipore) and the reaction split into two halves, one of which was oxidized with 25 mM GSSG (3 h, 4 °C). Excess GSSG was removed by dialysis, and the two reaction halves were combined. This sample was concentrated and applied to a heparin column (5 ml HiTrap Heparin HP, GE Healthcare) and then subjected to SEC in 4 M urea, 100 mM Tris·HCl, pH 6.0, and 5 mM EDTA. SEC fractions were analyzed by nonreducing SDS-PAGE, and those with a purity >95% of the dimeric species were pooled. BMP2–RGMAD complexes were formed by mixture of the proteins in a 1:1 molar ratio. For the ternary BMP2–eRGMB–NEO1FNS6 complex, BMP2 was mixed in equimolar amounts with a previously SEC-purified eRGMB–NEO1FNS6 complex. The complex mixtures were incubated for 1 h at room temperature before crystallization and concentrated to the appropriate concentration.

Site-directed mutagenesis.

Site-directed mutagenesis of RGM proteins to test the specificity of protein–protein interactions was carried out by two-step overlap-extension PCR with Pyrobest Polymerase (Takara). PCR products were cloned into the pHSec vector52 and expressed by transient transfection in HEK-293T with a hexahistidine, a BirA-recognition sequence, or an mVenus tag, were cloned. The proteins were concentrated and further purified by size-exclusion chromatography with TALON beads (Clontech) and, for crystallization, treated with endoglycosidase F1 (75 μg·mg⁻¹ protein, 12 h, 21 °C) to cleave glycosidic bonds of N-linked sugars and result in only one N-acetylgalactosamine moiety bound to the corresponding asparaginyl side chain. The proteins were concentrated and further purified by size-exclusion chromatography (SEC) (Superdex 200 100/60 column, GE Healthcare) in buffer containing 10 mM HEPES, pH 7.5, and 150 mM NaCl. The production of NEO1FNS6 and NEO1FNS6M followed a protocol described previously51.

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Small-angle X-ray scattering (SAXS).

Data were collected at beamline BM29 of the European Synchrotron Radiation Facility (ESRF, Grenoble, France) at 293 K within a momentum transfer (q) range of 0.01 Å⁻¹ < q < 0.45 Å⁻¹, where q = 4πsin(θ)/λ, and θ is the scattering angle. The X-ray wavelength was 0.0995 nm, and data were collected on a Pilatus 1M detector. Protein samples were measured at the following concentrations: NEO1FNS6M 1.06 and 4.94 mg/ml; eRGMB, 0.96 and 5.48 mg/ml; NEO1FNS6M–eRGMB, 0.51 and 1.01 mg/ml; and BMP2–eRGMB–NEO1FNS6M, 0.68 and 1.14 mg/ml. Data reduction and calculation of invariants was carried out with the ATSAS suite76. A merged data set was calculated with ROSETTA3.5 (ref. 78), constrained by imposing α-helical secondary structure parameters on the high-angle part of the high-concentration data set. Molecular-weight determination was performed with the volume of correlation metric Vc with Scatther77. The BMP2–eRGMB–NEO1FNS6M solution structure was modeled starting from the crystal structure of the ternary complex. A structural model for C-terminal eRGMB residues His335–Ser410 (not observed in the electron density) was calculated with ROSETTA3.5 (ref. 78), constrained by imposing α-helical secondary structure and a disulfide bond between Cys358 and Cys372. Missing loops and N and C termini were added in extended conformations with Modeller77. Starting models for NEO1FNS6M–eRGMB, and the eRGMB–NEO1FNS6M binary subcomplex were then extracted from the completed BMP2–eRGMB–NEO1FNS6M model. All-atom ensemble modeling of NEO1FNS6M–eRGMB, eRGMB–NEO1FNS6M...
and BMP2–eRGMB–NEO1FN56M was performed with AllosMod80, and in each case 50 independent ensembles of 100 models were generated. From this pool, automated selection of the minimal set of models satisfying the scattering data was performed with MES85, and calculation and fitting of scattering patterns were performed with FoXS85. This procedure was automated with the AllosMod-FoXS web server86. The solution structures of NEO1FN56M, eRGMB–NEO1FN56M and BMP2–eRGMB–NEO1FN56M are described by one, one, three and two models, respectively.

Multifluorescence light scattering (MALS). MALS experiments were carried out with a DAWN HELEOS II (Wyatt Technology), equipped with a K5 flow cell and a 30 mW linearly polarized GaAs laser with a wavelength of 690 nm. Proteins used for MALS contained wild-type sugars. Proteins were purified by SEC, and the BMP2–RGMBND and BMP2–eBMPR1A complexes were formed by mixture of the components in a 1:1 molar ratio. Complexes were dialyzed against buffers generated with the malic acid, MES, Tris (MMT) buffer system (Molecular Dimensions): 10 mM MMT, pH 5.5/6.5/7.5, and 150 mM NaCl. Complexes were concentrated to 2 mg/ml before MALS analysis. Data were analyzed with ASTRAA (Wyatt Technologies), and molecular weights were calculated with the Debye fit method. Molecular weights were calculated as: BMP2–RGMBND, pH 7.5, 46.2 ± 2.7 and 22.4 ± 0.9; BMP2–RGMBND, pH 6.5, 20.1 ± 1.4 and 13.4 ± 0.4; BMP2–RGMBND, pH 5.5, 24.0 ± 1.2 and 15.8 ± 0.3; BMP2–eBMPR1A, pH 7.5, 53.5 ± 0.1 and 15.0 ± 0.2; BMP2–eBMPR1A, pH 6.5, 53.2 ± 0.3 and 18.8 ± 0.1; and BMP2–eBMPR1A, pH 5.5, 54.2 ± 0.5 and 19.7 ± 0.4. These molecular weights correspond to the following calculated masses: BMP2–RGMBND, 47 kDa; BMP2, 26 kDa; RGMBND, 11 kDa; BMP2–eBMPR1A, 53 kDa; and eBMPR1A, 14 kDa. Graphs were produced with GraphPad Prism Version 6.04 (GraphPad Software).

Surface plasmon resonance (SPR) binding studies. SPR experiments were performed with a BLAcore T200 machine (GE Healthcare) at 25 °C in SPR running buffer (10 mM HEPES, pH 7.5, 150 mM NaCl and 0.005% (v/v) polyorbate 20). All experiments were performed with direct protein immobilization by amine coupling to CM5 biosensor chips except for those in Supplementary Figure 5m–o, in which biotinylated eRGMB was immobilized onto streptavidin-coupled CM5 biosensor chips84. Analytes were dialyzed against SPR running buffer before use, and 1:2 dilution series were prepared. For the competition experiment, a 1:2 dilution series of a mixture of 20 mM eBMPR1A and 2.5 mM eRGMB was prepared in buffer containing 2.5 μM eRGMB, to result in a dilution series of eBMPR1A in a constant (2.5 μM) concentration of eRGMB. BMP2 surface concentrations were 150, 500 and 1,000 response units. Surfaces coupled with BMP2 were regenerated by bursts of 4 M urea, 50 mM Tris, pH 8.0, and 150 mM NaCl (120 s, 20 μl/min) and the experimental trace returned to baseline. In all experiments, error range is ±s.e.m. The signal from experimental flow cells was corrected by subtraction of the nearest blank injection and the reference signal from a blank flow cell. All data were analyzed with SCRUBBER2 (Biologic) and GraphPad Prism Version 6.04 (GraphPad Software). Best-fit binding curves were calculated for BMP2–BMP receptor interactions with nonlinear curve fitting of a ‘one-site specific binding’ model (Y = Bmax × X/(Kd + X); X, analyte concentration; Bmax, maximum analyte binding). For BMP2–RGMB binding, best-fit curves were calculated with nonlinear curve fitting of a ‘one-site total binding’ model (Y = Bmax × X/(Kd + X) + NS × X + background; X, analyte concentration; background = 0 because data were already referenced). Nonspecific binding is proportional to analyte concentration, and therefore NS is the slope of nonspecific binding. Bmax and Kd values were determined for the specific binding component only. For the eBMPR1A–eRGMB competition experiment, a best-fit binding curve was calculated with a ‘log(agonist) versus response variable slope’ model (Y = bottom + (top − bottom)/(1 + 10(−logEC50−logX))); X, analyte concentration; top, Ymax−IC50, concentration of agonist that gives a response halfway between bottom and top). The HillSlope parameter was constrained to 1.0. An R2 value to quantify goodness of fit (range 0–1.0) and an IC50 value were reported.

Luciferase reporter assay. LLC-PK1 cells, or C2C12 cells stably transfected with a BREF-luciferase reporter plasmid85, were plated in complete DMEM supplemented with 10% FBS at a density of 5 × 104 cells/ml in a 96-well plate (100 μl/well) (Nunc-Immuno MicroWell 96-well polystyrene plates, Sigma-Aldrich). After 24 h, LLC-PK1 cells were transfected with Lipofectamine 2000 transfection reagent (Life Technologies) according to the manufacturer’s protocol, with 40 ng pGL3 BRE-Luc plasmid86, 30 ng Renilla control plasmid and, where indicated, 20 ng of empty pHHsc vector control or test constructs as indicated. Eight hours after transfection, the cells were washed with PBS (100 μl) and serum starved overnight in complete DMEM supplemented with 0.1% FBS. Cells were stimulated with 6 nM, 10 nM or 25 nM BMP2, as indicated, or buffer. When soluble proteins were directly added to the cells, BMP2 was preincubated with a dilution series (from 0.4 to 100 times the molar concentration of BMP2) of eBMPR1A or eRGMB. After 48-h incubation, cells were washed with PBS and lysed, and luciferase activity was measured with a dual luciferase assay system (Promega) according to the manufacturer’s instructions. Luminescence was quantified with a luminometer (Tecan, Infinite 200 PRO). Graphs were produced and statistical tests carried out with GraphPad Prism Version 6.04 (GraphPad Software).

Sample preparation for microscopy. Prior to imaging, 40 nM BMP2 solution was dialyzed against phenol red–free complete DMEM supplemented with 0.1% FBS to remove 10 mM ammonium acetate buffer. COS7 cells were seeded at a density of 1 × 105 cells/ml in glass-bottomed dishes (2 ml) (MatTek). After 24 h, cells were transfected with 1.5 μg NEO1-mVenUS and 1.5 μg full-length RGMB with Lipofectamine 2000 transfection reagent (Life Technologies) according to the manufacturer’s protocol. Eight hours after transfection, the cells were serum-starved overnight in phenol red–free complete DMEM supplemented with 0.1% FBS. Prior to imaging, cell medium was replaced with either 20 nM BMP2–containing medium or blank medium. Live-cell imaging was carried out immediately. For fixed samples, the BMP2 or blank medium was removed after the indicated amount of time, and cells were fixed with 4% paraformaldehyde (PFA) (10 min, room temperature) and washed thoroughly in PBS to remove excess PFA before being stored in PBS at 4 °C.

Imaging. Images were acquired on a total internal reflection fluorescence microscope (TIRFM) (ELYRA; Zeiss) with a 100× oil-immersion objective with a numerical aperture of 1.46. For illumination/photoconversion, 30% transmission of the 488-nm laser and 1% transmission of the 405-nm laser were used, and 5,000–10,000 images were acquired per sample with a cooled, electron-multiplying charge-coupled-device camera (iXon DU-897D; Andor). Exposure time was 300 ms. Recorded images were analyzed with Zeiss ZEN software.

dSTORM data processing. Raw fluorescence-intensity images were analyzed with Zen 2010D (Zeiss MicroImaging). A Gaussian and Laplace filter was applied to each frame, and overlapping events were excluded. An event was classified as originating from a single molecule when F – M > 65 (with F representing event intensity, M mean image intensity, and S the s.d. of image intensity). The center of each point-spread function was then calculated by fitting to a two-dimensional Gaussian distribution, and a table containing the x-y-particle coordinates of each molecule was extracted. Regions of interest, 5 μm × 5 μm or 3 μm × 3 μm in area, containing the two-dimensional molecular coordinates, were cropped for analysis with Origin, and events with localization precision worse than 60 nm were discarded. Areas containing ~300 molecules for live-cell imaging and ~1,000 events for the fixed samples were selected. To analyze the spatial point pattern, we used Ripley’s K function, calculated with SpPack87 and the Spatstat package for R software (http://www.R-project.org/), and plotted the L function as described88,89. Briefly, Ripley’s K function is a measure of the number of points encircled by concentric circles of radius r centered on each point. K values from the Ripley function therefore scale with circle area and so are transformed into the L function. With this equation, scaling is linear with the radius. Random distributions have an L(r) value of r for all r values. Therefore, to analyze levels of clustering we plotted L(r) against r and positive values at a given r indicate clustering at that spatial scale. Quantitative cluster maps were generated with Getis and Frankin’s analysis as previously described88,89. Briefly, L(r) values at a spatial scale of 50 nm (L(50)) were computed for each point with R and interpolated with Origin to produce a quantitative cluster map. This was then pseudocolored with ImageJ (http://imagej.nih.gov/ij/) to highlight regions of high clustering. Live-cell dSTORM images were constructed with a time-gated window approach90,91. On the basis of the frame number from the raw-data acquisition, cluster maps were generated from 5,000 continuous frames, with each cluster map shifted by 2,000
frames relative to the previous one. This corresponded to a time-gated window of ~7 min with the clustering analyzed over a total of 30 min.

Illustrations. Figures were produced with PyMOL (http://www.pymol.org/), Adobe Photoshop (Adobe Systems), ImageJ (http://image.nih.gov/ij/), and Corel Draw (Corel Corporation). Sequence alignments were conducted with MULTALIN (http://bioinfo.genotoul.fr/multalin/multalin.html) and formatted with ESPRRIPT (http://esprirt.ibcp.fr/ESPr ipt/ESPr ipt/).

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