Bone (or body) morphogenetic proteins (BMPs) belong to the TGFβ superfamily and are crucial for embryonic patterning and organogenesis as well as for adult tissue homeostasis and repair. Activation of BMP receptors by their ligands leads to induction of several signaling cascades. Using fluorescence recovery after photobleaching, FRET, and single particle tracking microscopy, we demonstrate that BMP receptor type I and II (BMPRI and BMPRII) have distinct lateral mobility properties within the plasma membrane, which is mandatory for their involvement in different signaling pathways. Before ligand binding, BMPRII and a subpopulation of BMPRII exhibit confined motion, reflecting preassembled heteromeric receptor complexes. A second free diffusing BMPRII population only becomes restricted after ligand addition. This paper visualizes time-resolved BMP receptor complex formation and demonstrates that the lateral mobility of BMPRI has a major impact in stabilizing heteromeric BMPRI-BMPRII receptor complexes to differentially stimulate SMAD versus non-SMAD signaling.

Bone or body morphogenetic proteins (BMPs)⁷ comprise the largest subgroup of the transforming growth factor β (TGFβ) superfamily and play important roles in embryonic patterning and organogenesis (1) as well as in adult tissue homeostasis and regeneration (2, 3). BMPs are known to elicit diverse biological responses and regulate cell differentiation, migration, proliferation, and apoptosis (4). These secreted ligands transduce signals via transmembrane serine/threonine kinase receptors, of which four type I and three type II receptors are known (5). Both receptor types are required for signal transduction and form heteromeric complexes. Upon ligand binding, BMP receptor type II (BMPRII) transphosphorylates BMP receptor type I (BMPRI), which then phosphorylates the cytosolic signaling molecules (SMADs) and players of various non-SMAD pathways to initiate SMAD and non-SMAD signaling cascades. The non-SMAD pathways include signaling by mitogen-activated proteins kinases (MAPKs p38, ERK, and JNK) (6, 7), PI3K/AKT, and small Rho-like GTPases (8, 9) and are indispensable for osteogenic differentiation. However, the means for establishing the balance between SMAD and non-SMAD signaling are not yet fully understood.

One of the most intriguing questions in the field is how the same ligand can elicit such an immense diversity of biological responses through a limited set of receptors. To date, several regulatory mechanisms of signal specificity have been identified. For example, the mode of BMP/receptor oligomerization was shown to affect signal specificity. The ligand can either bind to a preformed type I-type II heteromeric receptor complex (PFC) or, alternatively, to its high affinity type I receptor, leading to recruitment of type II receptor and formation of a BMP-

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**Background:** BMP-2 signals via heteromeric complexes of transmembrane receptors (BMPRI and BMPRII) to induce SMAD and non-SMAD signaling.

**Results:** BMPRI and BMPRII show distinct lateral mobility behaviors, essential for signaling specificity.

**Conclusion:** SMAD and non-SMAD signaling is differentially affected by alterations in BMP receptor mobility.

**Significance:** This demonstrates a regulatory mechanism for fine-tuning BMP signaling through localization and biophysical properties of BMP receptors on the plasma membrane.
induced signaling complex (BISC) (10). Although PFCs were shown to initiate canonical SMAD signaling, BISCs activate the MAPK p38 pathway (11).

Moreover, the localization of BMP receptors in distinct plasma membrane domains was shown to have a major impact on signaling specificity (12, 13). Additionally, differing binding affinities of BMP ligands to type I and II receptors contribute to signaling outcome by creating a preference for a certain receptor subset. Although BMP-2 has a comparably high affinity for BMPRIa and BMPRIb ($K_D$ BMPRIa > BMPRIb $\gg$ BMPRII), growth differentiation factor 5 (GDF-5), another member of the BMP family, has a markedly higher affinity for BMPRIb than for BMPRIa ($K_D$ BMPRIb $\gg$ BMPRIa > BMPRII) (14).

However, most data regarding the mechanisms of ligand-induced initiation and specification of signaling pathways were obtained using biochemical assays. State of the art techniques, such as quantitative live cell imaging, can help to clarify signal initiation directly at the plasma membrane. Single particle tracking (SPT) is a technique with high spatiotemporal resolution that allows for detecting individual receptors and classifying their mobility in the context of their localization, assembly, and function on the plasma membrane of living cells. This technique can identify spatiotemporal aspects of a heterogeneous molecule population that might be obscured by fluorescence recovery after photobleaching (FRAP) (15). SPT has already provided valuable insights into the assembly and activation of receptors, such as EGF receptor (16). In several studies, changes in mobility of particular signaling molecules (e.g. the Ras molecule) were observed after their activation and linked to assembly of signaling complexes (17). Moreover, lateral mobility of GFP-tagged TGFβ receptor type 1 (TβR1) was shown to be reduced after ligand stimulation, reflecting its heteromeric complex formation with type II receptors (18).

In the present study, we use high resolution SPT, FRAP, and FRET microscopy combined with signaling studies to investigate the impact of lateral mobility of BMP receptors on their signaling capacity and specificity. Our data reveal for the first time that BMP receptor activation requires a distinct pattern of lateral movement of type I and type II receptors within the plasma membrane, which regulates the induction of SMAD versus non-SMAD signaling cascades.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfection, and Generation of Stable Cell Lines**—C2C12 and HEK293T cells were cultivated in Dulbecco’s modified Eagle’s culture medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM l-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin at 37 °C and 10% CO₂. For transient transfections, Lipofectamine™ 2000 (Invitrogen) was used according to the manufacturer’s instructions. Cells were seeded on plates or glass coverslips (24 mm; Invitrogen) was used according to the manufacturer’s instructions (Dual-Luciferase reporter gene assay) (20). For transient transfections, cells were co-transfected with Gateway®-based retroviral vector (Invitrogen) containing the sequence for HA-tagged BMPRIb WT or respective mutant and with vectors containing coding sequences for retroviral polymerase and viral envelope protein. Virus-containing supernatant from HEK293T cells was used to infect C2C12 cells. Transduced cells were selected using Hygromycin B and used for FACS sorting.

**Enzyme-mediated QuantumDot (QDot) Labeling of ACP-tagged Receptors**—Labeling was performed by incubating the cells on coverslips for 15–20 min at 37 °C in DMEM with 1% bovine serum albumin (BSA), 1.5 µM His₆-phosphopantetheinyl transferase, and 0.3 nM CdSe/ZnS quantum Dot-CoA molecules prepared as described previously (20). Before measurements, samples were washed three times and kept in DMEM (20).

**Antibody-mediated QDot Labeling of HA- and Myc-tagged Receptors**—Cells expressing epitope-tagged receptors were incubated with 0.6–2 µg/ml primary α-HA (clone H7, Sigma-Aldrich) or α-Myc (Cell Signaling) antibodies in growth medium for 10 min at 37 °C and repeatedly washed with DMEM plus 10% FCS. To avoid nonspecific binding, cells were incubated with growth medium supplemented with 5% goat serum for 5 min at 37 °C and washed with DMEM plus 10% FCS. Subsequently, cells were incubated with QDot655- or QDot585-conjugated secondary antibodies (α-mouse and α-rabbit IgG) (Invitrogen) for 25–30 min at room temperature and repeatedly washed with phenol red-free DMEM.

**Separation of Detergent-resistant Membranes (DRMs)**—Isolation of DRMs was performed as described (12) and in some cases combined with cell surface biotinylation. In short, C2C12 cells were lysed with buffer containing 20 mM CHAPS, 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 3 mM EDTA, and protease and phosphatase inhibitors in appropriate concentrations for 30 min at 4 °C, homogenized with a Potter apparatus, and cleared of cell debris by centrifugation. The lysate was adjusted to a concentration of 40% OptiPrep™ (Axis-Shield, Dundee, Scotland) and layered with a discontinuous OptiPrep gradient (30%, 5%). Following ultracentrifugation (20 h, 39,000 rpm, 4 °C), the gradient was fractionated by pipetting fractions of equal volume from the top, which were denaturated and analyzed by SDS-PAGE and Western blot.

**Dual Luciferase Reporter Gene Assay**—C2C12 cells (5 × 10³ cells/well of a 96-well plate) were transfected with 40 ng of BMP response element (BRE)-fused firefly luciferase construct (p(BRE)₄-luc) (21) and 30 ng of Renilla luciferase construct (pRLTK). 20 h post-transfection, cells were serum-starved and subsequently stimulated with 1–10 nM BMP-2 or GDF-5. Cell lysis and luciferase measurements were carried out according to the manufacturer’s instructions (Dual-Luciferase reporter assay system, Promega). Luciferase activity was measured by a Mithras plate luminometer (Berthold Technologies). All measurements were conducted in triplicate.

**Alkaline Phosphatase Activity Assay**—C2C12 cells (1.5 × 10⁴ cells/well of a 96-well plate) were grown for 20 h in normal growth medium, starved for 2 h in serum-reduced medium, and treated for 72 h with the indicated concentrations of BMP-2 or GDF-5 in serum-reduced medium. Cells were lysed, and alkaline phosphatase activity was determined at an absorption of 405 nm as described earlier (11).
Time-resolved Visualization of BMP Receptor Complexes

Western Blot Analysis of SMAD and Non-SMAD Signaling—C2C12 cells (1.5 × 10⁵ cells/well of a 6-well plate) were grown for 24 h in normal growth medium, starved in serum-reduced medium, and stimulated with 1–10 nM BMP-2 or GDF-5 for 30 min (SMAD1/5/8 and p38 phosphorylation). Stimulation was stopped by cell lysis. As a control for each time point, stimulation with PBS occurred for the same duration as in ligand-stimulated samples. After cell lysis and denaturation, samples were analyzed by SDS-PAGE and Western blotting.

Quantitative Reverse Transcription-PCR—C2C12 cells (3 × 10⁵ cells/well of a 6-well plate) were grown for 24 h in normal growth medium, starved for 2–5 h in serum-reduced medium, and stimulated for 12, 24, or 48 h with 10 nM BMP-2 or GDF-5. Total RNA was extracted using the NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. cDNA was synthesized from 1 μg of RNA and used in SYBR Green quantitative real-time PCR in 1:8 dilutions. The amount of Runx2, Osr (osterix), ALP (alkaline phosphatase), and OCN (osteocalcin) transcripts was determined relative to the housekeeping gene HPRT (hypoxanthine phosphoribosyltransferase). For each gene, real-time PCRs from BMPRIb WT versus mutant receptor-expressing samples under stimulated and unstimulated conditions were performed. All measurements were done in triplicates, and C(T) values were determined using StepOne Software version 2.2. Mean normalized expression and the corresponding S.E. were calculated according to Ref. 22.

Additional Methods—Constructs, antibodies, reagents, cell surface biotinylation, microscopy experiments, and data analysis (FRET, FRAP, and single particle tracking), cell treatments (ligand stimulation, cholesterol depletion, and microtubule disruption) are described in the supplemental Methods.

RESULTS

BMP Receptors Type I and II Have Different Lateral Mobilities—To investigate the mobility of BMP receptors in living cells, FRAP studies were performed on C2C12 cells expressing YFP-fused BMPRIa or BMPRII. FRAP revealed a 2-fold lower recovery of BMPRIa, implying a 2-fold greater immobile fraction for BMPRIa than for BMPRII (Fig. 1a). To analyze the lateral mobility of BMPRIa and BMPRII in more detail, we applied SPT on cells expressing HA-tagged BMP receptors. The receptors were visualized by antibody-mediated QDot labeling of epitope-tagged receptors. Trajectories (Fig. 1, b and c) and mean square displacement (MSD) analysis of QDot-labeled receptors from five independent experiments (Fig. 1d) revealed that BMPRIa and BMPRIib are confined to a region of <100 nm. This finding correlates with the predominant immobile fraction of BMPRIa in FRAP analysis.

Unlike BMPRI, for BMPRII, two populations with different mobilities were found. One subpopulation exhibited confined movement within regions of ≤200 nm (Fig. 1e, MSD analysis of receptors from five experiments in Fig. 1f (left)) much like BMPRI with diffusion coefficients of ~0.004 μm²/s (supplemental Table S1). Another subpopulation of BMPRII displayed higher mobility and free diffusion (Fig. 1, e and f(right) for MSD analysis) with a diffusion coefficient of 0.026 μm²/s (supplemental Table S1). The mobility of BMPRIa and BMPRII was also analyzed using another labeling technique, namely the enzyme-mediated QDot labeling of acyl carrier protein-tagged receptors, which gave results comparable with the results of antibody labeling (supplemental Fig. S1, a and b, and Table S1) (20). Taken together, the results from two different approaches imply confined mobility of BMPRI and BMPRII and the existence of a highly mobile BMPRII subpopulation, which agrees with the high recovery of BMPRII in FRAP experiments.

Ligand Binding Alters Lateral Mobility of BMP Receptors—Ligand stimulation was previously reported to modulate the mobility of various receptors, such as TJR (18), EGF receptor (23), and GPCRs (24). Therefore, we analyzed the effect of BMP stimulation on lateral mobility of BMP receptors (all tagged receptors used in this and later experiments have been tested for functionality; see supplemental Fig. S3, d and f). FRAP showed a reduction of BMPRII mobile fraction to BMPRI levels after BMP-2 stimulation (Fig. 2a), indicating that BMPRII becomes immobilized within minutes after the ligand addition.

SPT was performed on the same cell before and up to 30 min after stimulation with BMP-2 or GDF-5, the high affinity ligand for BMPRIb. The mobility of BMPRI remained confined with a slightly reduced confinement size (Fig. 2, b–d, and supplemental Fig. S1g). Interestingly, already 5 min after the BMP-2 addition, BMPRII exhibited solely confined movements and resembled BMPRII in diffusion coefficient and confinement size (Fig. 2, e and f). Further studies by FRET acceptor photobleaching (Fig. 3a) and time-resolved FRET ratio analysis (Fig. 3b) demonstrated the existence of ligand-independent PFCs and formation of additional ligand-induced BISCs, which supports earlier studies (10, 11). Acceptor photobleaching of YFP-BMPRIa in the absence of ligand increased the CFP signal originating from CFP-tagged BMPRII within the PFC (Fig. 3a). On the other hand, time lapse studies revealed an increased YFP signal after the BMP addition as a result of receptor hetero-oligomerization, reflecting the formation of BISCs (Fig. 3b).

To validate these findings biochemically, we analyzed cell surface receptors from DRMs. For this, cells were stimulated with the ligand, cell surface-biotinylated, and subjected to DRM isolation following sucrose gradient centrifugation. The co-fractionation of respective receptors with DRM markers, such as Caveolin1, implies an association of the receptor with membrane microdomains (12). We demonstrate that endogenous BMPRII is found on the plasma membrane in both DRM and non-DRM fractions and is redistributed toward DRM fractions 5 min after BMP-2 stimulation (Fig. 3, c and d), whereas BMPRIa is found exclusively in the DRM fractions as reported earlier (12) and shown in supplemental Fig. S2b. This agrees with FRAP and SPT data revealing high lateral mobility of BMPRII and its reduction upon ligand application.

Ligand Binding to Preformed Receptor Complexes Increases Complex Lifetime and Stabilizes the Tight Complex State—For time-resolved analysis of BMPRIb-BMPRII hetero-oligomerization in living cells, we established simultaneous two-color SPT. We observed that BMPRIb-BMPRII complexes change between a tight complex state, in which the receptors appear merged, and shorter intervals of loose complex state, during which the receptors appear separated (Fig. 4, a–c, and
supplemental material). This demonstrates very dynamic and transient binding between the BMP receptors and agrees with our recent studies (25). The unliganded heteromeric receptor complex has a life span of about 2 s before it separates. The addition of BMP-2 has three major effects on the receptor complex; it increases (i) the total lifetime of the heteromeric receptor complex, (ii) the number of changes between the tight and loose states, and (iii) the duration of the tight states (Fig. 4, c–f). This result suggests that the unliganded PFC has a much shorter lifetime as compared with a ligand-bound PFC or a ligand-induced heteromeric receptor complex. In order to address the question of whether confined motion of one of the receptors is required for efficient complex formation and signaling specificity, we modified the mobility of BMPRI by different approaches.

Modulating Lateral Mobility of BMPRI—BMPRIb was previously reported to have a strong biochemical association with cholesterol-rich membrane microdomains (12). Therefore, we used cholesterol depletion with methyl-β-cyclodextrin (MβCD) to investigate the impact of microdomains on the confined mobility of BMPRIb. MβCD induced a gradual increase of ~160 nm in confinement size of individual BMPRIb receptors 5 min after application and completely abrogated the confinement, resulting in free diffusion of BMPRIb after 60 min (Fig. 5, a–d). As a control for membrane integrity under cholesterol depletion, we analyzed the lateral mobility of transferrin receptor, a known non-DRM protein, with SPT and found it to be unaffected by MβCD treatment (supplemental Fig. S1, c and d).

To specify the microdomains in which BMPRIb might be localized, we investigated the role of caveolae, a subset of cholesterol-rich microdomains containing Caveolin1, which were reported to affect BMP signaling (12, 26). Therefore, siRNA-mediated knockdown of Caveolin1 was performed in C2C12 cells stably expressing HA-tagged BMPRIb (C2C12-
BMPRIB) before subjecting them to SPT (Fig. 5e). Caveolin1 knockdown in C2C12 cells abolished the confinement (Fig. 5f) and resulted in free diffusion of BMPRIB (Fig. 5g), demonstrating that specific localization of BMPRIB in caveolae is required to maintain the restricted mobility of this receptor.

Earlier studies have reported interactions of microdomains with the cytoskeleton (27–29) or direct interactions between receptors and cytoskeletal components, which can cause reduced receptor mobility (30, 31). For this reason, we addressed the contribution of the microtubule network on the immobility of BMPRIB. We treated C2C12-BMPRIB cells with the microtubule-disrupting agent nocodazole for 0–90 min and followed the receptors by SPT. Nocodazole application led to increased BMPRIB mobility already after 20 min and fully abolished the confinement of BMPRIB after 90 min (Fig. 5, h–k). This implies that the confined mobility of BMPRIB is dependent not only on receptor localization in caveolae but also on cytoskeletal interactions.

The Confined Mobility of BMPRIB Is Mediated by Its Transmembrane Domain—To identify the domain of BMPRIB mediating its immobility on the plasma membrane and to analyze its effect on signaling, we applied a mutagenesis approach. We investigated the contribution of the extracellular region, which was reported to mediate the microdomain association for EGF and TGFβ receptors (32, 33). However, mutation of the membrane-proximal L3 loop (34) (Fig. 6a) had only minor effects on lateral mobility and no effects on the signaling properties of BMPRIB (supplemental Fig. S1, e and f) (data not shown). However, we identified three mutations in the transmembrane region (TM2, TM5, and PKT) with reduced DRM association of the receptor (Fig. 6, b and c). This suggests that the transmembrane region is crucial for association of BMPRIB with cholesterol-rich membrane microdomains.

To analyze the properties of mutated receptors in living cells, stable C2C12 cell lines expressing respective HA-tagged BMPRIB mutants at comparable levels (C2C12-PKT, C2C12-TM2, and C2C12-TM5) were subjected to SPT. All tested mutants showed cell surface expression comparable with that of the wild type receptor (analyzed by flow cytometry; see supplemental Fig. S2b). Substitutions of three amino acids to alanines in the second turn of the transmembrane α-helix (PKT mutation) caused a 5-fold increase of the confinement area over BMPRIB WT (Fig. 6, d and e). Through introduction of four successive point mutations in the same helix turn (TM2 mutation), the lateral mobility of receptor increased even further (Fig. 6f and g). However, the mode of TM2 movement was still

**FIGURE 2. Lateral mobility of BMPRII is reduced upon ligand stimulation.** a, FRAP analysis of BMPRIa and BMPRII lateral diffusion upon BMP-2 stimulation. YFP-BMPRIa and YFP-BMPRII constructs were transiently expressed and imaged as in Fig. 1a. Fluorescence intensities normalized to the value before photobleaching are plotted versus time upon stimulation with BMP-2. The low recovery of BMPRII reflects the increased amount of immobilized BMPRII on the cell surface. b, c, and e, SPT traces of BMPRIa, BMPRIB, and BMPRII after 5-min BMP-2 stimulation. C2C12 cells expressing HA-tagged BMPRIa, BMPRIB, or BMPRII were QDot-labeled and imaged upon BMP-2 stimulation as described in the legend to Fig. 1b. Representative trajectories (from five independent experiments) of BMPRIa, BMPRIB, and BMPRII show confined movement for all receptor types after ligand stimulation. Scale bar, 100 nm. d and f, analysis of BMPRIa, BMPRIB, and BMPRII MSD versus time shows a comparable average mobility for all receptor types after BMP-2 stimulation. Data points from five independent experiments were pooled. Error bars, S.E. of each MSD data point for each particular lag time.

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confined, as revealed by MSD analysis, although the confinement area is 10-fold larger than of BMPRIb WT. In contrast, point mutations in the helix turn closest to the intracellular compartment (TM5) greatly enhanced the mobility and completely abolished the confinement, resulting in free diffusion (Fig. 6, \( h \) and \( i \)). The general mobility pattern of mutated receptors was not changed upon ligand stimulation (supplemental Fig. S1). Altogether, we demonstrate that the transmembrane domain is an essential structural component to confine the lateral mobility of BMPRIb on the cell surface.

Apart from mutating the transmembrane region, several intracellular truncation mutants of BMPRIb were generated to map possible sites for cytoskeletal interactions. Mobility analysis of these mutants suggested a possible contribution of the intracellular domain to the immobilization of BMPRIb (data not shown). However, low surface expression levels did not allow a valid comparison with BMPRIb WT.

Confined BMPRIb Mobility Is Dispensable for SMAD Signaling—BMPRIb mutants with increased lateral mobility were analyzed regarding activation of SMAD signaling, as assessed by Western blot analysis of C-terminal SMAD1/5/8 phosphorylation (Fig. 7, \( a \) and \( b \)). C2C12 cells express little to no endogenous BMPRIb (46, 47) and therefore are ideal for signaling experiments using exogenous BMPRIb and mutants thereof. To exclude signaling induced by the endogenous BMPRI (i.e. BMPRIa, which uses BMP-2 over GDF-5 as high affinity ligand) and to only follow alterations of signaling by the stably expressed BMPRI (i.e. BMPRIb, which uses GDF-5 over BMP-2 as high affinity ligand), we stimulated C2C12 cells with either ligands. C2C12-TM2, -TM5, and -PKT cells displayed no differences in ligand-induced activation of SMAD signaling as compared with C2C12-BMPRIb WT cells (Fig. 7, \( a \) and \( b \)). In addition, BMP/SMAD-responsive reporter gene assays showed that mutations in the transmembrane domain of BMPRIb do not interfere with SMAD-dependent transcriptional activation (Fig. 7c).

Confined BMPRIb Mobility Is Required for Efficient non-SMAD Signaling—To address the impact of BMPRIb transmembrane mutations on induction of non-SMAD signaling, we first analyzed ligand-induced p38 and AKT phosphorylation. No GDF-5-induced activation of p38 and AKT was observed in C2C12-TM2, -TM5, and -PKT cells, and only residual activation was observed upon stimulation with BMP-2 that utilizes both exogenous BMPRIb and endogenous BMPRIa (Fig. 7, \( d \) and \( e \), and supplemental Fig. S5, \( a \) and \( b \)).

To follow this, we measured the enzymatic activity of alkaline phosphatase (ALP), which is up-regulated during BMP-induced osteogenic differentiation (35). The induction of ALP is dependent on both the SMAD and non-SMAD pathways (7). Although C2C12 cells expressing BMPRIb WT induce ALP in response to GDF-5 stimulation, C2C12-TM2, -TM5, and -PKT cells fail to do so (Fig. 7f). However, BMP-2-induced ALP production was unimpaired in these cells, indicating that the mutated BMPRIb does not interfere with signaling by endoge-
nous BMPRIa, which uses BMP-2 and not GDF-5 as ligand (supplemental Fig. S3, a–c). The transcriptional effects of the altered signaling properties of BMPRIb mutants were assessed by expression analysis of BMP target genes Runx2, Osx, OCN, and ALP. Although the expression of the early and predominantly SMAD-dependent osteoblastic marker Runx2 was unimpaired, ALP expression, which requires both SMAD and non-SMAD signaling and is a later marker of osteogenesis, was strongly reduced in C2C12-TM2, -TM5, and -PKT cells (Fig. 7g). Furthermore, ligand-induced expression of Osx and OCN was compromised only in the C2C12-TM5 cell line, with TM5 being the mutation with the strongest increase of lateral mobility (Fig. 7g).

Confined BMPRI Mobility Is Prerequisite for Ligand-induced Stabilization of the BMPRI-BMPRII Receptor Complex—As previously shown, the different signaling pathways are initiated
by either ligand binding to PFCs (SMAD) or ligand-mediated recruitment of types I and II receptors into the BISC complex (non-SMAD). We therefore tested whether the BMPRIb mutants with defects in the non-SMAD pathway but normal SMAD signaling properties also differ in the dynamics of receptor assembly. Using the time-resolved simultaneous dual-color SPT measurements presented before (Fig. 4), we analyzed the number of oscillations (i.e. changes between the tight and loose complexes), the duration of tight complex state, and the total life-time of receptor complexes consisting of BMPRII and either PKT, TM2, or TM5 (Fig. 8, a–c). This quantification demonstrates that the increased lateral mobility of BMPRIb compromises the ligand-induced stabilization of the heteromeric complex (Fig. 8, a–c) but does not affect the duration of ligand-independent tight complexes (Fig. 8a). This implies that the confined mobility of BMPRI is required for both the stabilization of the BMP-bound tight receptor complex and the total complex lifetime. Because the non-SMAD pathway is strongly affected by enhanced BMPRI mobility, as is the stabilization and lifetime of the ligand-induced receptor complex, we propose that ligand-mediated receptor assembly is required for the non-SMAD pathway.

Taken together, we demonstrate from single receptor measurements confined lateral mobility of BMP type I receptors,
whereas BMP type II receptor is characterized by the existence of mobile and immobile receptor populations. The mobility of BMPRII is reduced upon ligand stimulation, reflecting complex formation with BMPRI. Moreover, we report that confined mobility of BMPRI is a prerequisite for stabilizing the ligand-bound BMPRI-BMPRII complex. These properties of BMPRI are mediated through its transmembrane region and require localization of the receptor in cholesterol-rich domains and interaction with cytoskeletal components. Finally we demonstrate that the confined mobility of BMPRIb is crucial specifically for mediation of non-SMAD responses and subsequent osteogenic differentiation.

DISCUSSION

The enrichment of signaling receptors in membrane microdomains was shown to be essential for the activation of downstream signaling cascades, in particular the MAPK pathway. This was demonstrated for platelet-derived growth factor (PDGF), nerve growth factor (NGF), and TGFβ receptors (36–38). The restricted lateral mobility of BMPRI is sustained by its localization in microdomains, specifically in caveolae, and by cytoskeletal interactions (Fig. 5). Enhancing lateral mobility of BMPRI by mutating essential residues in the transmembrane region strongly decreased the affinity of BMPRI to microdomains (Fig. 6) and selectively reduced non-SMAD signaling, whereas SMAD signaling was unaffected (Fig. 7). Because BMP-induced p38 signaling was shown to require recruitment of BMPRII into a signaling receptor complex (BISC) (11), we conclude from our data that immobilization of BMPRI is crucial to activate BISC-mediated non-SMAD pathways but dispensable for PFC-mediated SMAD activation.

Many components of the non-SMAD signaling cascade are associated with membrane microdomains, such as TRAF6, a TβRII-binding mediator of TGFβ-induced p38 activation (39), and the adaptor protein XIAP (40). Moreover, BMP co-receptors, such as Ror2 and the GPI-anchored repulsive guidance molecules (RGMs), are enriched in membrane microdomains (41, 42). RGMb, which directly interacts with BMP ligands and receptors (43), was recently reported to be required for BMP-induced MAPK signaling (42). This supports our earlier find-
ings that the localization of BMPRI in membrane microdomains is essential for activation of non-SMAD pathways (Fig. 7 and supplemental Fig. S5). Interestingly, Neogenin-linked RGM-BMPR complexes, which assemble in membrane microdomains, trigger SMAD rather than the p38 pathway in mature chondrocytes (13), suggesting cell context-specific effects by the expression of co-receptors. Although BMP-induced p38 activation was shown to be initiated from BISCs and dependent on microdomain association of signaling receptors, the mechanism of BMP-induced AKT signaling is not fully

FIGURE 6. The transmembrane region of BMPRIb is a determinant of the receptor’s lateral mobility. a, mutagenesis approach for identification of structural elements responsible for the immobility of BMPRIb. The transmembrane domain of BMPRIb was subjected to targeted mutagenesis by a polyalanine scan. The WT sequence of the transmembrane domain is depicted with the mutated amino acids shown in boldface letters. The respective constructs were stably transduced into C2C12 cells, and the generated stable cell lines were named C2C12-BMPRIb WT, -TM2, -TM5, and -PKT, respectively. b and c, reduced DRM association of BMPRI transmembrane domain mutants as assessed by co-fractionation of receptor with Caveolin1. C2C12-BMPRIb WT, -PKT, -TM2, and -TM5 cells were used for sucrose gradient fractionation, and fractions were subjected to Western blot analysis (WB) (b). The histogram (c) depicts the percentage of receptor present in DRM fractions obtained by quantification of the Western blot analysis. d–i, mutations of transmembrane domain increase lateral mobility of BMPRIb. C2C12-PKT, -TM2, and -TM5 cells were used for antibody-mediated QDot labeling of receptor and subjected to SPT. Representative trajectories and MSD analysis show a strong increase either in confinement size for the PKT (d and e) or in lateral mobility for TM2 (f and g) and TM5 (h and i) in comparison with BMPRIb WT. For MSD analysis, data points from five independent experiments were pooled. Scale bar, 100 nm. Error bars, S.E. of each MSD data point for each particular lag time.
understood. Our data suggest that microdomains are crucial for BMP-induced AKT signaling. This is in accordance with the reported microdomain association of upstream transducers of AKT signaling, such as PI3K and PDK1 (44, 45).

Remarkably, it is the high affinity receptor BMPRI that is restricted in lateral mobility and localized to microdomains essential for non-SMAD-signaling. In contrast, the low affinity BMPRII exhibits two distinct subpopulations prior to ligand addition: a confined moving and a free diffusing one, which becomes confined after ligand stimulation (Figs. 1 and 2). We hypothesize that the immobility of BMPRII reflects its hetero-oligomerization with BMPRI in a PFC or its ligand-induced

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**FIGURE 7.** BMPRIb mutants with enhanced lateral mobility fail to induce non-SMAD signaling and osteoblastic differentiation, whereas the SMAD pathway is not altered. a, Western blot analysis of SMAD1/5/8 in C2C12-BMPRIb WT, -TM2, -TM5, and -PKT cells. Cells were stimulated with BMP-2 or GDF-5 for 30 min. Cell lysates were subjected to Western blot analysis of the respective phosphorylated proteins. GAPDH was used as loading control. A representative experiment from three independent experiments is shown. Quantification of Western blot (b) depicts relative intensity of phospho-specific signals normalized to respective GAPDH. c, analysis of SMAD-mediated transcriptional activation by BMP-responsive reporter (BRE-LUC) in C2C12-BMPRIb WT, -TM2, -TM5, and -PKT cells. The respective cell lines were transiently transfected with BRE-LUC and RlTK and stimulated with GDF-5 for 6 h, and relative luciferase activity was measured. (Reporter gene assays stimulated with BMP-2 are shown in supplemental Fig. S3a.) A representative experiment from three independent experiments is shown. All measurements were conducted in triplicate. d, Western blot analysis of p38 and AKT phosphorylation in C2C12-BMPRIb WT, -TM2, -TM5, and -PKT cells. Cells were stimulated with BMP-2 or GDF-5 for 30–60 min. Cell lysates were subjected to Western blot analysis of the respective phosphorylated proteins. GAPDH was used as loading control. A representative experiment from three experiments is shown. Quantification of Western blot (e) depicts relative intensity of phospho-specific signals normalized to respective GAPDH. f, ALP activity assay. C2C12-BMPRIb WT, -TM2, -TM5, and -PKT cells were stimulated with the indicated concentrations of GDF-5 for 72 h. ALP production indicative of osteoblastic differentiation was assessed by a colorimetric assay of enzymatic activity of ALP. (ALP assays stimulated with BMP-2 are shown in supplemental Fig. S3b.) A representative experiment from three experiments is shown. All measurements were conducted in triplicate. g, expression analysis of osteoblastic differentiation markers in C2C12-BMPRIb WT or mutant cells. C2C12-BMPRIb WT, -TM2, -TM5, and -PKT cells were starved, stimulated with GDF-5 for 12 h to measure expression of Runx2 and Osterix or for 48 h to measure expression of ALP and OCN, and used for RNA isolation. Quantitative RT-PCR was performed to analyze gene expression of Runx2, ALP, Osterix, and OCN. Gene expression was normalized to HPRT expression and plotted as mean normalized expression (MNE). (Expression analysis upon BMP-2 stimulation is shown in supplemental Fig. S3c.) All measurements were conducted in triplicate. Error bars, S.E.
Whereas in the absence of ligand, a BMPRI-BMPRII complex is highly dynamic and transient (Fig. 4, a–c). In SPT analyses, BMPRII-TC1 exhibited a highly mobile, freely diffusing population (20) \((D = 0.02–0.05 \mu m^2/s)\) without the confined subpopulation observed for BMPRII. Moreover, the ligand addition induced only a transient confinement and immobilization of BMPRII-TC1 on the membrane (supplemental Fig. S4a). This result indicates that this mutant forms BISCs, which is in agreement with earlier studies (11). Thus, the confined movement of BMPRII reflects its ligand-independent (PFC) or ligand-induced (BISC) oligomerization with BMPRI.

By applying two-color SPT, we found that BMPRI-BMPRII complexes are highly dynamic and transient (Fig. 4, a–c), which is in accordance with our recent patch/FRAP studies (25). The restricted mobility of BMPRI ensures stabilization of this complex before and, more pronounced, after BMP-2 stimulation. Whereas in the absence of ligand, a BMPRI-BMPRII complex oscillates between tight and loose complex states for \(-1.6 s\), after the ligand addition, the complex persists for \(-8 s\), which means a 5-fold prolongation of the complex lifetime (Fig. 4, d–f). The prolonged complex lifetime is a consequence of the longer tight complex state (from 0.4 s to \(>0.8 s\)) and of the higher number of recombination events (on average from 4.1 to 10.3). The complex lifetime was strongly reduced when lateral mobility of BMPRII was enhanced by mutation and could not be prolonged upon ligand addition (Fig. 8). This indicates that the localization of BMPRI in membrane microdomains ensures the stability of heteromeric receptor complexes. Furthermore, we suggest that the BMP-mediated non-SMAD pathways require a greater stability of the heteromeric receptor complex as compared with the direct phosphorylation of SMADs. For this reason, the localization of heteromeric receptor complexes in specialized microdomains is crucial for efficient BMP-induced activation of distinct (i.e. non-SMAD) pathways. These studies demonstrate for the first time how cells direct different cytosolic BMP signaling pathways although initiated by the same ligand. Altering the mobility of cell surface receptors by changing membrane fluidity, association with cytoskeletal elements, scaffolding proteins, or membrane components allows for specific conductance of downstream signaling cascades and results in distinct cellular responses.

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