RESEARCH ARTICLE

A new MMP-mediated prodomain cleavage mechanism to activate bone morphogenetic proteins from the extracellular matrix

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Abstract

Since their discovery as pluripotent cytokines extractable from bone matrix, it has been speculated how bone morphogenetic proteins (BMPs) become released and activated from the extracellular matrix (ECM). In contrast to TGF-βs, most investigated BMPs are secreted as bioactive prodomain (PD)–growth factor (GF) complexes (CPLXs). Recently, we demonstrated that PD-dependent targeting of BMP-7 CPLXs to the extracellular fibrillin microfibril (FMF) components fibrillin-1 and -2 represents a BMP sequestration mechanism by rendering the GF latent. Understanding how BMPs become activated from ECM scaffolds such as FMF is crucial to elucidate pathomechanisms characterized by aberrant BMP activation and ECM destruction. Here, we describe a new MMP-dependent BMP-7 activation mechanism from ECM-targeted pools via specific PD degradation. Using Edman sequencing and mutagenesis, we identified a new and conserved MMP-13 cleavage site within the BMP-7

Abbreviations: BMP, bone morphogenetic protein; CD, circular dichroism spectroscopy; CPLX, complex; DPP, decapentaplegic; EBNA, Epstein-Barr virus nuclear antigen; ECM, extracellular matrix; FMF, fibrillin microfibrils; FRET, fluorescence resonance energy transfer; GDF, growth and differentiation factor; GF, growth factor; HEK, human embryonic kidney; LAP, latency-associated peptide; LLC, large latent complex; LTBP, latent TGF-β-binding protein; Mab, monoclonal antibody; MMP, matrix metalloproteinase; PD, prodomain; TEM, transmission electron microscopy; TGF, transforming growth factor; TLL2, tolloid-like protein 2; TMB, tetramethylbenzidine.

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1 | INTRODUCTION

Bone morphogenetic proteins (BMPs) belong to the TGF-β superfamily of growth factors (GFs) and play a central role in a multitude of cellular processes during embryogenesis and postnatal homeostasis by guiding cell differentiation, proliferation, survival, and apoptosis.\(^1\,^2\) Originally, BMPs were discovered as pluripotent cytokines extractable from bone matrix that are capable to induce ectopic bone formation.\(^3\) Further studies confirmed that BMPs are stored in embryonic and perinatal connective tissues such as kidney, skin, and blood vessels.\(^4\,^6\) These findings implicate that ECM-bound BMPs serve an important function and that mechanisms for their utilization must exist to control their release at the appropriate time points when their action is required. For instance, the importance of ECM-bound BMPs is not restricted to fetal osteogenesis but also for regeneration of adult bones as illustrated by limb-specific \(Bmp2\) null mice which presented with irreversible spontaneous fractures.\(^7\)

Among extracellular matrix (ECM) networks collagen fibers were first considered as the primary scaffold responsible for BMP sequestration.\(^8\,^9\) Thereby, it was shown that procollagen-2 is able to specifically bind BMP-2 and thereby influencing its bioactivity.\(^10\) In another prominent example it was found that collagen IV controls the bioavailability of the BMP homolog decapentaplegic (dpp) in drosophila by binding dpp directly or promoting the interaction with its receptor BMP homolog.\(^11\) The concept that proteolytic cleavage of LAP leads to the activation of TGF-β-1 GF has been explored in in vitro experiments using MMP-2 and MMP-9.\(^32\) Also a combined activation mechanism has been described, where αvβ8 integrin binds to TGF-β-1 via LAP and enables MMP-14 cleavage of LAP and release of TGF-β-1 GF.\(^33\) Another TGF-β-1 GF activation model proposed that BMP-1 cleavage of LTBP-1 at N- and C-terminal sites releases truncated LLC from the ECM which is followed by a second MMP-2-mediated cleavage event of LAP.\(^34\) GDF-8 and GDF-11 are activated by BMP-1/Tolloid (TLD) metalloproteinase-mediated cleavage of the PD.\(^35\,^36\) Recently, tolloid-like protein 2 (TLL2) was also shown to cleave the PD of GDF-8 and thereby activate it from the latent state.\(^37\) Similarly, our previous in vitro results
predicted a similar mechanism for BMP-10.\textsuperscript{12} Although much work has been undertaken to investigate the mechanisms of TGF-β activation, the regulatory pathways of other TGF-β superfamily members such as BMPs remain largely unknown.

In contrast to TGF-β, most BMPs are secreted as bioactive PD-GF complexes (CPLXs) to the extracellular space.\textsuperscript{12,38-41} Previously, we showed that the BMP-7 PD does not confer latency to the GF in solution, and that BMP type II receptors have free access to their binding sites on the GF. This suggestion arose from velocity sedimentation experiments in sucrose gradients showing that BMP type II receptor binding to the GF results in release of the free PD as a dimer from the CPLX.\textsuperscript{39} Recently, we could show that upon binding of the BMP-7 PD to the fibrillin-IN-terminal unique domain, a conformational change in the BMP-7 CPLX is induced which renders the GF inactive by locking the α2-helix of the PD in place, denying access to the BMP type II receptor site.\textsuperscript{42}

However, little is known about how BMPs are released and activated once they are targeted to the ECM. Therefore, the aim of this study was to investigate new BMP activation mechanisms from FMF-targeted pools.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

This study was carried out in strict accordance with German federal law on animal welfare, and the protocols were approved by the “Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen” (permit no. 84-02.04.2014.A397 for breeding and permit No. 84-02.05.40.14.115 for euthanasia).

2.2 | Antibodies

Previously described monoclonal anti-BMP-7 PD antibodies mab2 and mab33\textsuperscript{4} were kindly provided by Dr Lynn Sakai (Oregon Health and Science University). For western blots, mab33 was either used alone (1:1000 dilution), or in a mixture (1:1 molar ratio) together with mab2 (1:1000 dilution). The generation of polyclonal anti-fibrilllin-1 antibody was previously described.\textsuperscript{43} Polyclonal antibody against BMP-7 GF was purchased from PeproTech (#500-P198, Rocky Hill, NJ).

2.3 | Cell culture

Primary murine skin fibroblasts were isolated from newborn mice.\textsuperscript{44} Primary dermal fibroblasts and HEK 293 cells were cultured in Dulbecco’s Modified Eagle’s medium (DMEM GlutaMAX, Invitrogen, Carlsbad, CA) supplemented with 10% of fetal bovine serum and penicillin/streptomycin.

2.4 | mRNA expression analysis via quantitative real-time PCR

A total of $1 \times 10^5$ HEK 293 cells or primary murine skin fibroblasts cells were grown in 6-well plates prior to BMP GF stimulation at 100 ng/mL. After 24 hours of BMP stimulation, RNA extraction was performed by adding 1 mL of TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s protocol. A subsequent sample purification step was included using the RNeasy kit (Qiagen, Venlo, The Netherlands), and residual DNA contamination was removed from each sample using the Turbo DNA-free kit (Ambion, Austin, TX). RNA samples were quantified by photospectrometry, and 1.0 µg of RNA per sample was reverse-transcribed using the Bio-Rad iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative PCR was performed using SensiFAST SYBR Hi-ROX Kit in 25 µL reaction volume (Meridian Bioscience, Cincinnati, OH). PCR was conducted with the StepOnePlus system (Applied Biosystems, Thermo Fisher Scientific). The standard annealing temperature of 60°C was chosen for the selected primer pairs ($Mmp2F$: CAAAGTTCCCGGGCAGTTC, $Mmp2R$: TTCTGCTGTCAGTACCGCTGTC; $Mmp3F$: ACATGGAGACTTGTGCTCCTTTTG, $Mmp3R$: TTTGCTGAGTGGTAGAGTCCC; $Mmp13F$: TGTTTGCAAGAGCCTCAGG, $Mmp13R$: CAGTCACCTCCTAAGCAAG). Analysis of data was performed using the 2$^{-\Delta\Delta Ct}$ method\textsuperscript{45} and quantitated relative to the murine Arbp or human GAPDH gene. Gene expression was normalized to BSA-treated control samples, which provided an arbitrary constant for comparative fold expression. Primer pairs for human MMP genes were purchased from Qiagen.

2.5 | Protein expression and purification

BMP-7 CPLX was expressed and purified as described before.\textsuperscript{4} Briefly, the HEK 293 EBNA cell line stably transfected with N-terminally His\textsubscript{6}-tagged BMP-7 CPLX was kindly provided by Dr Lynn Sakai (Oregon Health and Science University). Cells were maintained in triple flasks, medium was collected, and affinity purified via nickel chelate affinity chromatography using the Ni-NTA resin (Cube Biotech, Germany). The highest purity fractions of BMP-7 CPLX were eluted with imidazole at a concentration of 50-250 mM. PDs of BMP-4, -5, -7, and -10 were expressed in E. coli with a C-terminally placed His\textsubscript{6}-tag, and purified by photospectrometry, and 1.0 µg of RNA per sample was reverse-transcribed using the Bio-Rad iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative PCR was performed using SensiFAST SYBR Hi-ROX Kit in 25 µL reaction volume (Meridian Bioscience, Cincinnati, OH). PCR was conducted with the StepOnePlus system (Applied Biosystems, Thermo Fisher Scientific). The standard annealing temperature of 60°C was chosen for the selected primer pairs ($Mmp2F$: CAAAGTTCCCGGGCAGTTC, $Mmp2R$: TTCTGCTGTCAGTACCGCTGTC; $Mmp3F$: ACATGGAGACTTGTGCTCCTTTTG, $Mmp3R$: TTTGCTGAGTGGTAGAGTCCC; $Mmp13F$: TGTTTGCAAGAGCCTCAGG, $Mmp13R$: CAGTCACCTCCTAAGCAAG). Analysis of data was performed using the 2$^{-\Delta\Delta Ct}$ method\textsuperscript{45} and quantitated relative to the murine Arbp or human GAPDH gene. Gene expression was normalized to BSA-treated control samples, which provided an arbitrary constant for comparative fold expression. Primer pairs for human MMP genes were purchased from Qiagen.
(MMP2: NP_032636.1, aa A30-C662; MMP7: NP_034940.2 aa L21-L267; MMP8: NP_032637.3, aa F21-S465; MMP9: NP_038627.1, aa A20-P370; MMP13: NP_032633.1, L19-C472) were expressed and purified as described previously.\(^{47}\) MMP-3, MMP-12, GDF-8 PD, and BMP-7 GF were purchased from R&D Systems (Minneapolis, MN).

### 2.6 | Proteolytic cleavage assays

MMPs were activated with 250 µM of amino-phenyl mercuric acetate (APMA) (Sigma-Aldrich, St. Louis, MO) for 2 hours at 37°C. For buffer exchange of solubilized BMP PDs to TC buffer (50 mM of Tris-HCl pH 7.5 and 1 mM of CaCl\(_2\)), Amicon ultra 0.5 mL centrifugal filters (Merck Millipore, Burlington, MA) were used. A total of 10 nM of each activated MMP was incubated with 1 µM of BMP PD in 50 µL for 2 hours at 25°C. Fragments were analyzed by western blotting and silver staining. For Edman sequencing, 6 µg of BMP-7 PDs were incubated with 60 ng of MMP-2, MMP-3, or MMP-13. Fragments were separated by 10%-20% SDS-PAGE and transferred to a PVDF membrane. After staining with Ponceau S, the cleavage products were excised, and subjected to N-terminal Edman degradation performed by Proteome Factory AG (Berlin, Germany). MMP activity was assessed through incubation with a quenched Omni-MMP fluorogenic substrate (#BML-P126-0001, Enzo Life Sciences, Lörrach, Germany) in black 96-well plates (Thermo Fisher Scientific, Waltham, MA). OD was read at 450 nm using a Sunrise microplate reader (Tecan).

### 2.7 | Circular dichroism spectroscopy

BMP-7 PD variants were dialyzed into 5 mM of HClO\(_4\). CD spectra were recorded using a Jasco J-715 spectropolarimeter at 260-170 nm in a 0.1-mm path length quartz cell (Hellma, Germany) at 20°C. After subtraction of the buffer contribution, data were converted to Δε.

### 2.8 | ELISA and sandwich ELISA

For ELISA, 100 ng/mL of BMP-7 CPLX was coated to microtiter plates (Nalge Nunc, Rochester, NY) in PBS overnight at 4°C. Wells were blocked with 5% nonfat dry milk/TBS for 1 hour at RT and washed twice with 0.025% TBS-tween, afterwards. Directly coated BMP-7 CPLX was incubated with MMPs at a molar ratio of 1:100 (MMP:BMP-7 CPLX) for 2 hours at RT in TC buffer. For sandwich ELISA detection, BMP-7 CPLX after MMP-13 cleavage was transferred to anti-BMP-7 GF antibody coated wells (10 µg/mL, PeproTech) and incubated for 1 hour. Wells were washed three times with 0.025% TBS-tween and incubated with detection antibody against BMP-7 PD (mab33 at 1:1000 dilution) in 2.5% nonfat dry milk/TBS for 2 hours at RT, followed by 1 hour incubation of HRP-conjugated anti-rabbit antibody in 2.5% nonfat dry milk/TBS at RT. Subsequently, wells were washed three times with TBS-tween, and incubated with 50 µL of 1-Step Ultra TMB-ELISA substrate solution for signal development (Thermo Fisher Scientific, Waltham, MA). OD was read at 450 nm using a Sunrise microplate reader (Tecan).

### 2.9 | MMP-13 cleavage assays on solid phase

For the generation of an assembled ECM fiber network, 1 × 10⁶ mouse skin fibroblasts were seeded on 6-well plates and cultivated for 4 days, followed by cell removal using deoxycholate.\(^{48}\) In brief, cell cultures were washed once with PBS and then, treated twice with 0.5% sodium deoxycholate in 10 mM of Tris-HCl buffer, pH 8.0, at 0°C for 10 minutes. The plates were then allowed to dry overnight at RT. Subsequently, wells were blocked in 5% BSA followed by incubation with BMP-7 CPLX. To assess colocalization between added BMP-7 CPLX and fibrillin-1 fibers by immunofluorescence, cells were grown on 24-well plates. To demonstrate a direct interaction between added BMP-7 CPLX and assembled ECM fibers by ELISA-style solid phase interaction assay, mouse fibroblasts were grown on 96-well plates. For this, 100 ng/mL of BMP-7 CPLX was titrated onto ECM-coated dishes following a 1:2 serial dilution in TBS buffer containing 1% of BSA at RT for 2 hours. BMP-7 CPLX immobilized to ECM fibers was submitted to MMP-13 cleavage (50 ng/mL) in 1 mL of TC buffer for 2 hours at 37°C. Afterwards, the supernatant was collected and subjected to TCA precipitation for western blot analysis or lyophilized to be subjected to BMP bioactivity assays.

### 2.10 | BMP bioactivity assay

Supernatant from 6-well plates containing GF released from ECM-targeted BMP-7 CPLX after MMP-13 cleavage was collected and dialyzed in mini dialysis tubes with a molecular weight cut-off of 2 kDa into 100 mM acetic acid overnight at 4°C. After dialysis, samples were shock-frozen in liquid nitrogen and lyophilized overnight. Subsequently, samples were resuspended in 10 µL of 4 mM
HCl and administered to BMP bioactivity assays. To measure BMP bioactivity murine C2C12 myoblasts were utilized as reporter cell line. For each measurement, 3.5 x 10⁴ cells/well were seeded onto 96-well plates. Stimulation was performed in eight wells per concentration in triplicates. Two or three microliters of the obtained supernatant after MMP-13 cleavage of ECM-bound BMP-7 CPLX and 2 or 3 µL of the supernatant without MMP-13 incubation were added for C2C12 cell stimulation. After 5 hours, the total mRNA content of cells was harvested, reverse-transcribed, and subjected to qPCR to measure the mRNA levels of BMP response gene Id3 (inhibitor of differentiation 3). ³⁹ Id3 mRNA levels were normalized to the mRNA expression of ARBP (“acidic ribosomal binding protein”) which served as housekeeping gene. A total of 10 ng/mL of BMP-7 GF (R&D systems) was added to the medium as positive control, and incubation of cells with 0.1% of BSA served as untreated negative control.

2.12 | Dynamic light scattering

BMP-7 CPLX was dialyzed overnight into 1 M urea and cleaved with MMP-13 as described above. DLS measurements of the cleaved and non-cleaved control BMP-7 CPLX were then taken using a Zetasizer Nano-S (Malvern, Herfordshire, UK) at a controlled temperature of 25°C.

2.13 | Molecular docking experiments

The generation of the BMP-7 CPLX closed-ring shape model (Figure 6), based on the TGF-β-1 LAP crystal structure (Protein Data Bank code 3RJR) as a template, with MODELLER ²² in UCSF Chimera ⁵³ was as described. ⁴² In this model, a break in the peptide chain was introduced at residue Pro ⁸⁰ to allow the PD to be rotated into an open conformation without moving the N-terminal region. Next, using the Chimera software a peptide bond with a phi torsional angle of −60° was introduced at the exact same position in order to re-join the polypeptide chains without moving the N-terminal PD region. The BMP-9 CPLX structure (Protein Data Bank code 4YCI) and the BMP-7 CPLX EM map were used to guide rotation of the PD into an open conformation. To obtain a structural model of BMP-7 PD in the open V-shape conformation, BMP-7 PD was modeled on the proactivin CPLX structure (Protein Data Bank code 5HLZ) using Swiss-model. To gain structural insight into the MMP-13 cleavage mechanism, BMP-7 PD in the open conformation or the closed-ring BMP-7 CPLX, inputted as the “receptors” were docked to the crystal structure of the activated MMP-13 (Protein Data Bank code 4fu4) inputted as the “ligand” in the ClusPro2.0 server ⁵⁴ after deleting the structure of the ActRII extracellular domain using UCSF chimera software. Subsequently, 30 molecular docking models were screened for each in silico experiment and models 21 and 16 were selected for closed and open BMP-7 PD conformations, respectively, due to excellent alignment of the MMP-13 catalytic site (His ²²², His ²²⁶, and His ²³²) to the prime region ⁸³MLD ⁸⁵ of the cleavage site. To obtain a theoretical model of MMP-13 cleaving the BMP-7 CPLX closed-ring, model 21 was structurally aligned to each BMP-7 PD monomer of the closed BMP-7 CPLX model at the ⁸³MLD ⁸⁵ site. Images were taken both in ribbon and surface representations. To pinpoint the exact molecular requirements for cleavage in the open BMP-7 PD conformation, model 16 was superimposed to the activated MMP-13 structure (Protein Data Bank code 4fu4) and the positioning of the co-crystallized peptide was compared to the ⁸³MLD ⁸⁵ site of BMP-7 PD. Ions and metals were visualized using the 4fu4 template and images were taken in ribbon representation. To generate the open V-shape BMP-7 CPLX model, BMP-7 PD was assembled into a dimer using Swiss-model and the proactivin CPLX was structurally aligned to the BMP-7 PD dimer. Next, the monomers of the BMP-7 GF crystal structure (Protein Data Bank code 1LX5) were structurally aligned to the monomers of the proactivin GF after deleting the structure of the ActRII extracellular domain using UCSF chimera. To understand how MMP-13 is cleaving the open V-shape BMP-7 CPLX, model 16 was structurally aligned to one BMP-7 PD of the CPLX and images were taken both in ribbon and surface representations.
2.14 | Statistical analysis

Data are expressed as mean ± SD. Statistical analyses were performed using GraphPad Prism software and the significance of differences between groups was determined by applying an unpaired two-tailed Student's t test. Values of \( P \leq .05 \) were considered significant.

3 | RESULTS

3.1 | BMP-7 GF stimulates metalloproteinase activity leading to specific cleavage of BMP-7 PD

Western blot analysis of HEK 293 cells transiently transfected with full length BMP-7 cDNA encoding for the entire BMP-7 PD-GF CPLX showed two bands for BMP-7 PD after 3 days of culture. After 1 day of transfection, only the expected size of full length BMP-7 PD at approximately 37 kDa was detected, while at day 3 an additional band of about 25 kDa could be observed (Figure 1A). This suggested that after secretion a specific proteolytic event occurred within the BMP-7 PD. To dissect whether BMP-7 PD cleavage may have been initiated by BMP autostimulation or by accumulation of general protease activity in the culture media over time, HEK 293 cells were incubated with BMP-7 GF only for 24 hours and the resulting supernatant was incubated with recombinantly expressed and affinity-purified BMP-7 CPLX protein (Figure 1B). Results from western blot analysis showed that only supernatant obtained from BMP-7 GF stimulated cell layers caused specific BMP-7 PD cleavage. This cleavage was inhibited upon addition of EDTA, suggesting that BMP-7 PD cleavage was caused by metalloproteinase activity (Figure 1B). Based on the findings of previous reports, we hypothesized that BMP-7 stimulation of HEK 293 cells may have induced upregulation of MMPs which in turn led to PD degradation of the BMP-7 CPLX. To
test this assumption, we measured the mRNA levels of representatives of MMP family subgroups after 24 hours of HEK 293 cell stimulation with BMP-7 GF (Figure 1C). We found that BMP-7 GF administration induced a significant upregulation of MMP-8, -9, and -13 mRNA expression suggesting that the BMP-7 PD was degraded by a BMP-induced MMP activity. Stimulation of ECM resident cells such as skin fibroblasts confirmed that BMP-7 GF is able to induce most robustly MMP-13 mRNA expression (Figure 1C). These experiments suggested that BMP-7 GF is capable to stimulate metalloproteinase expression which in turn leads to specific cleavage of BMP-7 PD.

3.2 BMP-7 PD as new substrate for MMPs

Previously, it was shown that active TGF-β is able to stimulate matrix metalloproteinase (MMP) expression.55 Furthermore, MMP cleavage of the TGF-β PD LAP was proposed as mechanism to release TGF-β GF from the ECM.31-33 To evaluate the existence of a potential similar MMP-driven ECM activation mechanism for BMPs, the hypothesis was tested whether the BMP-7 PD also serves as a substrate for MMPs. For this purpose, an in vitro cleavage screening assay was undertaken testing different recombinantly expressed and purified representatives of MMP family subgroups (Figure 2A) and BMP-7 PD and BMP-7 CPLX as substrates. In our screen, MMP-2 and -9 represented the gelatinases, MMP-3 the stromelysins, MMP-7 the matrysins, MMP-8 and -13 the collagenases, and MMP-12 the elastases.56 Prior to BMP-7 PD incubation, MMP activity was assessed through fluorescence increase after incubation with a specific MMP fluorogenic substrate. Cleavage of the substrate resulted in a fluorescence signal at 500 nm (Figure 2B). All tested MMPs were able to cleave the BMP-7 PD, either alone, or when complexed to its cognate GF (Figure 2C). Thereby, we observed varying BMP-7 PD cleavage efficiencies among MMPs, since MMP-7, and -9 only showed moderate activity under the chosen conditions. Interestingly, a considerable number of MMP-mediated cleavage events yielded in BMP-7 PD fragments of similar sizes approximately at 30, 25, and 20 kDa (Figure 2C).

3.3 Mapping of MMP cleavage site within the BMP-7 PD

To identify specific MMP cleavage sites within the BMP-7 PD, N-terminal Edman sequencing of cleavage products was performed after incubation with MMP-2, MMP-3, and MMP-13. Using this method, the N-terminal sequence of a consensus cleavage product at a size of 20 kDa for MMP-2, MMP-3, and MMP-13 was identified starting with 121LQDS124. In addition, the N-terminal amino acid sequence of two other peptides could be identified after cleavage by MMP-13: 83MLDL86, and 107YKA109 (Figure 3A). At 0.5 hours incubation time, a higher cleavage efficiency of MMP-13 was observed in comparison to MMP-2 and -3 (Figure 3A). After 0.5 hours incubation in presence of MMP-2 and -3, minor amounts of the first cleavage products could be also detected at 30 kDa similar to the fragment starting with 83MLDL86 identified after MMP-13 cleavage. After 2 hours of incubation, quantitative MMP-2 and -3 cleavage was observed yielding the 121LQDS124 fragment at 20 kDa as well as an additional fragment at about 17 kDa with the same N-terminal 121LQDS124 sequence most probably generated by a secondary cleavage event further downstream towards the C-terminus (Figure 3A).

Our assays revealed that MMP-13 cleavage activity was most effective in the presence of 0.05% Triton X-100 (Figure 3B). Therefore, the MMP-13-induced cleavage pattern of BMP-7 PD in the presence or absence of triton was assessed. In the presence of triton, full length BMP-7 PD, as well as the fragment starting with 83MLDL86 were not detectable, leading to a more prominent presence of fragments
starting with 121LQDS124 and 107YKA109 (Figure 3C). This finding suggests that fragments starting with 121LQDS124 and 107YKA109 are products of a secondary cleavage event derived from the fragment starting with 83MLDL86 produced in the primary cleavage event.

Overall, our data led us to the hypothesis that MMP-2, MMP-3, and the other tested MMPs initially process BMP-7 PD at the same cleavage sites as MMP-13. However, the cleavage products may be further processed by different secondary cleavage events. To further explore the possibility of a general MMP cleavage site within BMP-7 PD, the publicly available MMP cleavage prediction platform, CleavPredict,57 was utilized. With the help of this software which is based on Proteomic Identification of Protease Cleavage Sites (PICS) using human peptide libraries,57 potential cleavage sites within the BMP-7 PD for 11 representative MMPs could be predicted. Interestingly, all evaluated MMPs were predicted to cleave BMP-7 PD within the same sites identified by Edman degradation after MMP-13 incubation (Figure 3D).

### 3.4 MMPs specifically cleave PDs of TGF-β superfamily members

Sequence alignment of PDs showed that the identified MMP cleavage site 100PMFMLD85 within the BMP-7 PD is partially conserved among other members of the TGF-β superfamily, except for GDF-8 (Figure 4A). Subjecting PD sequences of BMP subgroup representatives to in silico cleavage by
CleavPredict revealed that most MMPs would also utilize this site (Figure S2). Previous studies had shown that Pro in P3 in the non-prime region before the scissile bond of the cleavage site (Figure 5A) is considered the most important amino acid required for MMP-13 recognition, followed by Leu or Met in position P1′, and acidic residues in P3′ of the prime region. The alignment showed that the corresponding residues identified in BMP-7 PD P80, M83, and D85 were conserved among BMP family members (Figure 4A). In addition, we found the motif 86LYN88 to be conserved for most PDs in positions P4′-P6′ after the scissile bond.

To experimentally validate that PDs of TGF-β family members serve as substrates for MMP-13 and other MMPs, an in vitro cleavage screen with representatives of TGF-β subgroups was performed. Under the chosen conditions, we observed that MMP-13 was able to process all tested PDs with varying efficiencies (Figure 4B). As expected, MMP-13 cleavage of the PD of BMP-5, which belongs to the BMP-5, -6, -7 subgroup, yielded the same cleavage pattern as seen for the BMP-7 PD (Figure 4B). After cleavage of the BMP-4 PD, a representative of the BMP-2, -4 subgroup, only the 20 kDa fragment could be detected (Figure 4B). A cleavage screen of MMP subgroup representatives with PDs of BMP-9 and -10 which both constitute their own BMP subgroup, revealed differences and similarities in resulting fragment patterns and cleavage efficiencies (Figure 4C). Similar cleavage results were observed after MMP-2, -7, and -13 incubation (Figure 4B,C), while BMP-10 PD processing by MMP-12 was more effective than that of BMP-9 PD. Interestingly, MMP-8 and -9 did not process PDs of this subgroup.

TGF-β-1 PD was efficiently cleaved by MMP-2 (Figure 4C) as already described. Also upon MMP-13 cleavage, a fragment at around 25 kDa could be observed in minor amounts (Figure 4B). In contrast, the TGF-β-2 PD was not susceptible to cleavage by the tested MMPs and only a minor degradation was found upon incubation with MMP-2, -12, and -13 (Figure 4B,C).

As GDF-8 lacks most of the residues of the identified, conserved MMP cleavage site, PMFMLD85 (Figure 4A), we expected that GDF-8 might be resistant against MMP
cleavage. However, in presence of MMP-7, -12 and -13, a cleavage product was detected around 19 kDa (Figure 4B,C).

3.5 | The 80PMFMLD85 motif is crucial for efficient MMP-13 cleavage of BMP-7 PD

To validate the relevance of the identified 80PMFMLD85 motif we attempted to inactivate MMP-13 processing of BMP-7 PD by introducing point mutations at critical positions (Figure 5A). Thereby, amino acid substitutions were guided by previous predictions.58 BMP-7 PD mutants were overexpressed in E coli and affinity purified to more than 95% purity as assessed by SDS-PAGE and Coomassie staining (Figure 5B). The generated BMP-7 PD mutant variants included the single point mutations P80E, M83E, and D85L, the double point mutations P80E/M83E, and P79E/D84L, as well as the triple point mutation P80E/M83E/D85L. All chosen point mutations did not result in secondary structure changes as assessed by circular dichroism (CD) spectroscopy (Figure 5B). All mutated BMP-7 PD variants were subjected to MMP-13 cleavage and the resulting fragments were analyzed via western blot analysis (Figure 5). In all mutant variants apart from M83E, production of the 25 kDa fragment was abolished and the presence of the 20 kDa fragment was significantly reduced (Figure 5C).

3.6 | Localization of the MMP-13 cleavage site within a three-dimensional structure model of BMP-7 CPLX

Binding of BMP-7 CPLX to fibrillin-1 induces a conformational change of the entire CPLX from an open bioactive V-shape to a closed latent ring-shape.42 This inactivation of BMP GF occurs due to a structural re-arrangement of the two PDs leading to blockage of the BMP type II receptor-binding site on the GF by the α2 helix of the PD42 (Figure 6A). By localizing the scissile bond of the identified MMP-13 cleavage site (between F82 and M 83) within the PD in a three-dimensional closed ring-shape structure model of BMP-7 CPLX (Figure 6A), we found that it resides within the α2 helix. Since molecular docking of MMP-13 and the closed BMP-7 CPLX suggested that this site was accessible (Figure 6B),

FIGURE 5 Characterization of MMP-13 cleavage site within BMP-7 PD. A, Introduced point mutations within identified MMP-13 cleavage site in BMP-7 PD. Identified MMP-13 cleavage motif within BMP-7 PD is indicated in red. The most efficiently cleaved motif and residues to be most inhibitory for MMP-13 cleavage were previously predicted.58 B, Evaluation of integrity, purity, and secondary structure of mutant BMP-7 PD variants by SDS-PAGE (12.5% gel stained with Coomassie), and circular dichroism. C, BMP-7 PD variants carrying point mutations: P80E, M83E, and D85L, double point mutations: P80E/M83E and P80E/D85L, as well as a triple point mutation: P80E/M83E/D85L were subjected to MMP-13 cleavage. Resulting fragments were separated by 10%-20% gradient SDS-PAGE and visualized by western blotting using anti-BMP-7 PD antibody (mab2/mab33, 1:1 mixture).
we hypothesized that MMP-13-mediated cleavage of latent fibrillin-1-bound BMP-7 CPLX leads to removal of the α2-helix resulting in release of bioactive BMP-7 GF.

### 3.7 | MMP-13 cleavage leads to release of active BMP-7 GF from ECM-bound BMP-7 CPLX pools

To test whether MMP-13 is able to cleave and activate BMP-7 CPLX from ECM-bound pools, BMP-7 CPLX was targeted to fibrillin fibers assembled by primary fibroblasts (Figure 7A). Potential release of bioactive GF after MMP-13 cleavage into the supernatant was monitored via western blotting and BMP bioactivity assays. First, efficient binding of BMP-7 CPLX to decellularized fibrillin-1 fibers was demonstrated by detecting co-localizing immunofluorescence signals as well as by ELISA showing a concentration-dependent signal increase (Figure 7A). In a subsequent step, wells with fibrillin fibers decorated with BMP-7 CPLX were incubated with MMP-13. Western blot analysis showed the presence of released BMP-7 GF into supernatant only when MMP-13 was added (Figure 7B). PD fragments could be only detected in the washed ECM layer, suggesting that upon cleavage they remain attached to the ECM. To assess whether released BMP-7 GF was bioactive, the supernatant was added to C2C12 cells and the mRNA expression of the endogenous BMP response gene *Id3* was measured (Figure 7B). Upon addition of MMP-13, an approximate fourfold increase in BMP activity was detected indicating that the BMP-7 GF was released in bioactive form (Figure 7B).

### 3.8 | MMP-13 cleavage of BMP-7 PD results in conformational change and CPLX disintegration

To investigate whether MMP-13 cleavage also leads to GF release when the CPLX is immobilized via hydrophobic residues to a non-ECM solid phase, we performed cleavage
studies with plastic-coated CPLX pools. For this purpose, BMP-7 CPLX was coated onto wells of microtiter plates, and was subsequently incubated with MMP-13 for 2 hours at RT in TC buffer (Figure 8). After MMP-13 cleavage, the supernatant was collected and subjected to SDS-PAGE and western blot analysis (Figure 8). In addition, BMP-7 CPLX remaining on the plate was stripped (in 300 mM NaCl, 200 mM acetic acid) and analyzed by western blotting. Similar to the release experiment from fibrillin-1 fibers, it was found that upon MMP-13 cleavage BMP-7 GF was released into the supernatant. However, in contrast to the ECM release experiment (Figure 7B), BMP-7 PD fragments were simultaneously released into the supernatant. Efficient release of BMP-7 GF could be also confirmed by ELISA detecting less than 40% of BMP-7 GF still immobilized to the plate (Figure 8).

To gain further insight into how BMP-7 PD cleavage by MMP-13 affects BMP-7 CPLX stability, we analyzed samples after in solution cleavage by SDS-PAGE, native-PAGE, sandwich ELISA, and single particle TEM. Interestingly, at 50% BMP-7 PD cleavage, as assessed by Ponceau and western blot analysis, the CPLX signal was not detectable by Coomassie staining on native gels (Figure 9A). To exclude the possibility...
that the loss of a distinct Coomassie band on native gels was not caused by major aggregation of the BMP-7 CPLX after PD cleavage, we performed dynamic light scattering (DLS) analysis before and after MMP-13 cleavage (Figure S3). Our analysis showed that MMP-13 cleavage did not lead to a decrease of the peak representing monomeric CPLX molecules at a particle size of 10 nm (Figure S3). The peak representing aggregated particles of an average size of 100 nm showed even a slight decrease suggesting that MMP-13-mediated cleavage of the PD led to a decrease rather than an increase of aggregation (Figure S3). To assess CPLX stability after MMP-13 cleavage we performed sandwich ELISA. However, when BMP-7 CPLX after in solution cleavage was transferred to wells pre-coated with anti-BMP-7 GF antibody, followed by incubation with anti-BMP-7 PD detection antibody, still 30% of signal could be detected compared to the non-cleaved control (Figure 9B).

Single particle TEM analysis of MMP-13-cleaved BMP-7 CPLX samples shown in Figure 9A revealed a conformational change characterized by a widened angle between the PD arms and a four-subparticle appearance. This appearance was similar to the conformational change induced by the addition of 1 M urea which results in partial CPLX unfolding leading to partial PD displacement from the GF.42 These findings suggested the possibility that processing of one PD per CPLX molecule leads to a conformational change in each molecule and, therefore, to an unfocused migration in native gels. The sandwich ELISA shows that less than 30% CPLX seems to be stable enough for detection. However, the unfolding leads to an unfocused migration in native-PAGE and, therefore, lack of staining intensity at the expected position.

### 3.9 Molecular docking suggests that BMP-7 CPLX cleavage by MMP-13 requires PD displacement

To understand how MMP-13 processing of BMP-7 CPLX may occur on a molecular level in silico docking experiments were conducted. The center of the MMP-13 catalytic site is...
composed of H^{222}, H^{226}, and H^{232} that capture divalent metal ions with their aromatic rings to polarize water molecules that subsequently attack peptide bonds to perform the proteolysis (Figure 10A). Our docking results revealed that positioning of the 83MLDL86 stretch of one PD at the MMP-13 active cleft allows for further processing of the BMP-7 PD at the subsequent cleavage sites 107YKA109 and 121LQD123 due to the interspacing loop regions that allow for flexibility of the PD structure at these sites during the cleavage. To validate the imaging approach, MMP-13 of our in silico cleavage model was aligned to MMP-13 structure co-crystallized with an N-terminal fragment of its activation peptide in its active cleft. Eight such co-crystallized peptides, originating from the MMP-13 activation peptide after cleavage, consisted of an α-helix or random coil. This agrees with our experimental data suggesting that the three MMP-13 cleavage sites of BMP-7 PD reside within the second and fourth α-helices at the N-terminal region of the PD (Figure 10A). The 83MLDL86 stretch aligns perfectly with the co-crystallized peptide used in this in silico experiment (Figure 10A).

Next, we aligned our generated MMP-13/BMP-7 PD cleavage model (Figure 10A) to PDs of our BMP-7 CPLX model in open V-shape conformation. Thereby, we found that in this open CPLX conformation MMP-13 would need to bend or even displace one PD to gain access for efficient processing of the other PD (Figure 10B).
DISCUSSION

Although many studies addressed the mechanisms of TGF-β activation,29,30,32,34,59,61,62 the required pathways for cellular utilization of other TGF-β superfamily members such as BMPs remain largely unknown. Since the discovery of BMPs as pluripotent cytokines extractable from bone matrix, it has been speculated how BMPs targeted to the ECM become released and activated.

In this study, we uncovered a new proteolytic PD cleavage mechanism involving MMPs to release BMP GFs from FMF-stored ECM pools. In this context, BMP PDs not only mediate efficient targeting and sequestration of BMP GFs upon ECM binding, but also allow controlled release of bioactive...
GF upon specific cleavage by MMPs. Our previous studies showed that binding of BMP-7 CPLX to fibrillin-1 induces a conformational change of the entire CPLX from an open bioactive V-shape to a closed latent ring-shape (Figure 6A). In this closed conformation, a structural re-arrangement of the two PDs leads to blockage of the BMP type II receptor-binding sites on the GF by the α2-helix of the PD (Figure 6).42 Here, in our proposed MMP-13-mediated BMP-7 activation mechanism, recognition of the 80PMFMLD85 motif followed by PD processing of the scissile bond between F82 and M83 leads to unfolding and disintegration of the entire CPLX resulting in release of the active GF dimer.

Our investigations revealed interesting molecular aspects of this BMP activation mechanism. Molecular docking experiments (Figure 6B) suggested the potential necessity of two MMP-13 monomers for efficient PD processing in the closed BMP-7 CPLX ring-shape conformation, to effectively release the active GF dimer. However, in the open V-shape CPLX, efficient scissile bond cleavage by MMP-13 at the 80PMFMLD85 site in one PD requires partial displacement of the other PD, suggesting a 1:1 stoichiometry (Figure 10).

In the BMP PD cleavage assays conducted, we found that presence of 0.05% Triton X-100 served as an optimal concentration for efficient MMP-13 cleavage (Figure 3B,C). It is known that MMP activity is dependent on the detergent concentration used. To avoid adverse effects, it is crucial to determine the optimal detergent concentration. At low concentrations, detergents act as monomers that may stabilize and activate MMPs. However, at high concentrations they form micelles that might sequester and inhibit MMP activity.35,63 In absence of Triton, BMP-7 PD processing by MMP-13 occurred with a reduced turn-over rate yielding three fragments starting with 83MLDL86, 121LQDS124, and 107YKA109 (Figure 3C). However, in presence of Triton the 83MLDL86 fragment disappeared leading to an increased presence of the other two fragments (Figure 3B). This finding suggests that the fragment starting with 83MLDL86 is produced during the primary cleavage event and 121LQDS124 and 107YKA109 are products of subsequent cleavage events. These experimental data are also in line with our in silico docking results which showed that the F82-M83 scissile bond was most surface accessible in comparison to P106-Y107, or S120-L121 in the closed ring-shape BMP-7 CPLX conformation. Our experimental data also suggest that the S120-L121 scissile bond is less accessible in absence of Triton in solid phase cleavage assays indicated by a less pronounced presence of the PD cleavage product at 20 kDa (Figures 8 and 9).

Using CleavPredict,58 a cleavage site composed of six amino acid residues was predicted, three non-prime (amino acids downstream to the cleaved scissile bond) and three prime (amino acids upstream to the cleaved scissile bond) (Figure 5A). For most efficient MMP-13 cleavage, a general cleavage site was proposed based on a pronounced presence of a rigid Pro in P3 (non-prime), and Leu, Ile, or Met in P1 (prime), followed by small or acidic residues in P3’ in the identified substrates.58 In all mutated BMP-7 PD variants, apart from M83E, production of the fragment starting with 83MLDL86 was abolished and the fragments starting with 121LQDS124 and 107YKA109 were significantly reduced (Figure 5C). This result is supportive of the hypothesis that F80 and P83 are required for PD cleavage by MMP-13 but disagrees with the assumption that M83 is crucial for MMP-13 processing, since mutation of this residue did not affect the cleavage pattern (Figure 5C).

Our sequence alignment analysis showed that the identified 80PMFMLD85 motif in the BMP-7 PD was conserved among other BMP PDs (Figure 4A, Figure S2), suggesting a general MMP-mediated BMP PD cleavage mechanism. Our BMP PD cleavage screen revealed similarities but also differences to BMP-7 PD processing. BMP-5 and -7 belong to the same BMP subgroup and showed an identical cleavage pattern for MMP-13 (Figure 4B). However, BMP-4 PD processing did not yield the band at 25 kDa suggesting more efficient secondary cleavage. This may be due to different amino acid residues present in the P1 and P2 positions of the non-prime region of the cleavage site. Also, in BMP-9 and 10 PDs the most significant changes of the cleavage motif are within this region resulting in less efficient processing
by MMP-13. More specifically, BMP-10 PD lacks an Asp (D) in the P3' position of the prime region that is predicted to be required for efficient cleavage (Figure 5A).58 This may explain the almost diminished MMP-13 cleavage of BMP-10 PD compared to BMP-9 PD (Figure 4B). However, our results also showed that most likely these changes protect BMP-9 and -10 PD from MMP-8 and -9 cleavage but make them more susceptible to MMP-12 processing in comparison to BMP-7 (Figure 4C). It is also conceivable that the different conformations of the BMP PDs impact MMP cleavage efficiency in the conducted assays. Based on the sequence homology, it can be assumed that BMP-9 and -10 PDs fold similarly but differently than BMP-7 PD.

In TGF-β-1 and -2 most critical residues of the identified MMP motif in BMP-7 PD are altered. However, maintaining a Pro in P3 and some conserved residues in the P4'-P6' prime region most probably allows MMP-13 processing of both TGF PDs to a minor extent. Interestingly, we observed only very limited MMP-2 cleavage of TGF-β-2 PD in comparison to TGF-β-1 (Figure 4C). This suggests that the presence of a second proline at the P2 position of the non-prime region is inhibitory for efficient MMP processing (Figure 4A). As GDF-8 lacks most residues predicted to be critical for MMP recognition and processing, we expected it to be inert against MMP cleavage. However, we observed that after MMP-7, -12, and -13 incubation, a cleavage product at around 19 kDa was detected. According to our predictions using CleavPredict, substitution of Pro to Leu in P1 which is found in the GDF-8 PD sequence giving rise to the BFP-2 fragment (V140-R154) lies further downstream beyond the 121LQD123 cleavage site.65 Interestingly, cleavage at the 107YKA109 site would split the BFP-1 peptide in two halves potentially leading to its inactivation. Our data suggest that MMP-13 processes BMP-7 PD first at the 83MLD85 site, followed by a secondary cleavage event at 107YKA109, which is succeeded by cleavage at the 121LQD123 site that will split BFP-1 into two smaller fragments. MMP-2 and MMP-3 seem to follow a different cleavage sequence that does not utilize the 107YKA109 cleavage site (Figure 3A). This implies that despite most MMP cleavage events yielded BMP-7 PD fragments of similar sizes, it is likely that PD processing by different MMPs yields PD fragments with different functional activity, which may add another layer of regulation of BMP activity in certain physiological conditions. For instance, in differentiation processes such as chondrogenic or osteogenic differentiation, where MMPs are known to play an important role,66,67 BMP bioactivity may be further modulated by the generation of functionally active PD fragments.

The concept that proteases serve as specific activators of TGF-β superfamily members through PD cleavage has been previously explored. TGF-β-1 PD could be cleaved in in vitro experiments by MMP-2 and MMP-9.32,59 In addition, MMP-14 activity, which depends on integrins, can promote TGF-β-1 activation.35 Furthermore, MMP cleavage of the TGF-β PD was proposed as a mechanism to release TGF-β GF from the ECM.27,31 In addition, it could be shown that the metalloproteinase BMP-1, a procollagen endopeptidase removing C-terminal collagen propeptides, serves as GF activating enzyme by PD cleavage of several TGF-β superfamily members. BMP-1 is capable to process the PDs of GDF-8, GDF-11, and BMP-10 PDs and thereby rendering the GF from latent to bioactive.12,35,36 Interestingly, in a previous cleavage screen with BMP-1 and PDs of BMP-4, -5, -7 no processing was observed,12 suggesting that
different TGF-β superfamily members are activated by different sets of metalloproteinases. This is probably reflecting the different cellular circumstances requiring activation of specific GFs. In the early stages of development where little ECM is present, active GFs are regulated by complexation to BMP antagonists such as chordin, which need to be also cleaved by BMP-1/tollloid-related metalloproteases to liberate the active GF. At later stages of development and certainly with the beginning of postnatal life when increasingly more ECM is present, PD-ECM interactions facilitate GF targeting and sequestration. In this phase, MMPs start to regulate the bioavailability of tissue resident BMPs such as BMP-7. However, for soluble BMPs such as BMP-10 it could be shown that the PD complexation facilitates GF specificity toward cellular surfaces. While the BMP-10 CPLX appeared to be latent toward C2C12 mouse myoblasts, it proved to be bioactive toward endothelial cells. BMP-9 and -10 have been suggested to have redundant functions and also form hetero GF dimers. Until now, the complex interplay of BMP-9 and -10 functions in development and disease are not fully understood. As MMPs are also present in blood, controlled MMP cleavage of BMP-9 and -10 PDs may represent a new mechanism to orchestrate their bioactivity under normal physiological or endothelial stress/disease conditions.

Our observation of BMP-dependent induction of MMP expression (Figure 1B,C) was also found to be implicated in developmental or disease mechanisms accompanied by ECM degradation. For instance, BMP-2 stimulation of the mouse myoblast cell line C2C12 initiated strong expression of MMP-13. Also, BMP-2 and -4 stimulation of primary human fibroblasts led to MMP-1, -2, -3, and -13 upregulation which was suggested to be a mechanism in melanoma invasion. Furthermore, other studies have shown that BMPs are drivers of tumor metastasis by inducing MMP expression and activity. For instance, BMP-stimulated MMP-2 and -9 activity was shown to be a relevant mechanism in breast cancer cell migration and invasion. Previous studies could associate BMP-7 expression with early bone metastasis development in breast cancer. MMP-13 was described to be overexpressed at the tumor-bone interface and abrogation of MMP-13 in this area inhibited bone metastasis. As cancer often times recapitulates embryonic programs, it is not surprising that similar mechanisms occur during cartilage development. During chondrogenesis, BMPs control terminal differentiation where chondrocytes become hypertrophic and remove the collagen matrix through the upregulation of MMP-13. Interestingly, during OA, chondrocytes in articular cartilage behave again as terminally differentiating chondrocytes. There, elevated BMP levels in damaged cartilage not only contribute to tissue repair by stimulating ECM synthesis but also promote cartilage degeneration by stimulating MMP-13 expression.

Overall, BMP-stimulated MMP production appears to be an established mechanism during development and disease with the goal to rapidly remodel ECM architecture. Thereby, a fine-tuned balance between BMP and MMP activity is crucial. In disease situations such as cancer or OA, small amounts of active BMP or MMP may initiate a vicious feed-forward cycle where MMP-mediated BMP release from ECM-targeted pools further promotes MMP production ultimately resulting in severe ECM destruction. Similarly, in connective tissue disorders such as Marfan syndrome, failed BMP sequestration due to ECM deficiency may also trigger MMP-mediated destruction cascade in tissues.

This study provides evidence for the existence of an MMP-dependent mechanism for BMP activation from ECM-targeted pools by PD cleavage. This knowledge may open up new therapeutic avenues, to impede pathomechanisms characterized by dysregulated BMP GF activity and ECM destruction.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
G. Sengle designed the research. A.G. Furlan, C.E.S. Spanou, A.R.F. Godwin, A.P. Wohl, and L.M.A. Zimmermann performed the research. T. Imhof and M. Koch contributed new reagents. A.G. Furlan, C.E.S. Spanou, A.R.F. Godwin, A.P. Wohl, L.M.A. Zimmermann, and C. Baldock analyzed the data. A.G. Furlan and G. Sengle wrote the manuscript.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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