Cumulin, an oocyte-secreted heterodimer of the transforming growth factor-β family, is a potent activator of granulosa cells and improves oocyte quality*

David G. Mottershead[1,2], Satoshi Sugimura[1,3], Sara L. Al-Musawi[4], Jing-Jie Li[1,5] , Dulama Richani[6], Melissa A. White[1], Georgia A. Martin[1], Andrew P. Trotta[1], Lesley J. Ritter[1,7], Junyan Shi[1], Thomas D. Mueller[8], Craig A. Harrison[4] , Robert B. Gilchrist[1,6,#]

[1]Robinson Research Institute, and Discipline of Obstetrics and Gynaecology, School of Paediatrics and Reproductive Health, The University of Adelaide SA 5005, Australia.
[2]Mottasis Oy Ltd, Helsinki, Finland.
[3]Institute of Agriculture, Department of Biological Production, Tokyo University of Agriculture and Technology, Tokyo, Japan.
[4]Hudson Institute of Medical Research, Clayton, Victoria, Australia.
[5]Center of Reproductive Medicine, the Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou, China.
[6]Discipline of Obstetrics & Gynaecology, School of Women's & Children's Health, University of New South Wales, Sydney, Australia.
[7]Australian Research Council Centre of Excellence in Nanoscale BioPhotonics, The University of Adelaide, Adelaide, SA 5005, Australia.
[8]Department of Plant Physiology and Biophysics, Julius-von-Sachs Institute of the University Wuerzburg, Julius-von Sachs Platz 2, Wuerzburg, Germany.

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[#]To whom correspondence should be addressed: Robert B Gilchrist, Discipline of Obstetrics & Gynaecology, School of Women's & Children's Health, Royal Hospital for Women, University of New South Wales, Sydney, Australia, Tel: +61 (0)2 93852562, Fax: +61 (0)2 93852573, e-mail: r.gilchrist@unsw.edu.au

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Background: Cumulin is a newly identified heterodimeric member of the TGF-β family.
Results: Mature cumulin potently stimulates granulosa cell signaling and function, while pro-cumulin promotes oocyte quality.
Conclusion: Formation of cumulin and its potent actions are likely to be central to oocyte paracrine signaling and mammalian fecundity.
Significance: The discovery of cumulin provides unique opportunities to improve female fertility in mammals.

ABSTRACT
Growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are oocyte-specific growth factors with central roles in mammalian reproduction, regulating species-specific fecundity, ovarian follicular somatic cell differentiation and oocyte quality. In the human, GDF9 is produced in a latent form, the mechanism of activation being an open question. Here, we produced a range of recombinant GDF9 and BMP15 variants, examined their *in silico* and physical interactions, and their effects on ovarian granulosa cells (GC) and oocytes. We found that the potent synergistic actions of GDF9 and BMP15 on GC can be attributed to the formation of a heterodimer, which we have termed cumulin. Structural modelling of cumulin revealed a dimerization interface identical to homodimeric GDF9 and BMP15, indicating likely formation of a stable complex. This was confirmed by

generation of recombinant heterodimeric complexes of pro/mature domains (pro-cumulin) and covalent mature domains (cumulin). Both pro-cumulin and cumulin exhibited highly potent bioactivity on GC, activating both SMAD2/3 and SMAD1/5/8 signaling pathways, and promoting proliferation and expression of a set of genes associated with oocyte-regulated GC differentiation. Cumulin was more potent than pro-cumulin, pro-GDF9, pro-BMP15 or the two combined on GC. However, on cumulus-oocyte complexes, pro-cumulin was more effective than all other growth factors at notably improving oocyte quality as assessed by subsequent day 7 embryo development. Our results support a model of activation for human GDF9 dependant on cumulin formation through heterodimerization with BMP15. Oocyte-secreted cumulin is likely to be a central regulator of fertility in mono-ovular mammals.

In this study we consider the unique interaction of two members of the TGF-β family: growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15). These two closely related proteins are oocyte-secreted factors essential for fertility in mammals (1). The primary role of GDF9 and BMP15 is to act on the cells surrounding the oocyte, known as granulosa cells (GC), which in turn supply the oocyte with the support necessary for future healthy embryo/fetal development (2). As with all members of the TGF-β family, GDF9 and BMP15 are produced as pro-proteins, consisting of an N-terminal proregion and a receptor binding C-terminal mature region. Activation of these factors requires proteolytic processing by a furin-like protease (3,4). For most TGF-β members, the processed mature domains signal as covalently linked homodimers, although covalent heterodimers are also known (inhibin A and B (5)). GDF9 and BMP15 are unusual within the TGF-β family in that they (together with GDF3 and Lefty1/2) lack the cysteine residue involved in forming the mature region inter-subunit disulfide bond (6). Despite this, mature GDF9 and BMP15 are thought to maintain the canonical dimer architecture of other TGF-β ligands and signal as non-covalent dimers.

As with all members of the TGF-β family, GDF9 and BMP15 signal via cell surface receptors, which activate intracellular *Sma-* and *Mad-related* (SMAD) proteins by phosphorylation of critical residues, which subsequently regulate the transcription of various genes (1). The receptors are Ser/Thr kinases and are, on the basis of distinct features in the cytoplasmic domain, referred to as either type I (activated by the relevant type II receptor) or type II (constitutively active) receptors. Following prodomain displacement, BMP15 binds to complexes of BMP receptor type II (BMPRII) and activin receptor-like kinase 6 (ALK6), thereby leading to activation of the SMAD1/5/8 intracellular pathway (7,8). Mature GDF9 binds BMPRII and ALK5 and signals through the SMAD2/3 pathway (9-11).

GDF9 and BMP15 are also functionally unusual within the TGF-β family, in that they show remarkable species differences in their activity. Notably, human GDF9 is latent, as it remains associated with its prodomain, whereas mouse GDF9 is particularly potent, due to its prodomain being readily displaced (3,12,13). By contrast, human BMP15 is recombinantly produced in an active form in mammalian cell culture, whereas mouse BMP15 is not expressed under the same conditions (4). The species-specific differences in expression and activity of these oocyte-secreted growth factors is principally due to alterations in a small number of amino acids in the pro- (BMP15) or mature (GDF9) domains, which affect complex formation or stability, respectively (3,4,13). Hence, the relative levels of oocyte expression of BMP15 and GDF9 vary markedly across species, whereby the mono-ovular phenotype (i.e. low fecundity) is characterised by balanced BMP15:GDF9 ratios, whereas poly-ovular species (high fecundity) express predominately GDF9 (14). As such, GDF9, but not BMP15, is essential for fertility in the mouse (15,16), whereas both are required in mono-ovular species, such as sheep (17,18).

Interactions at the genetic level between these two proteins have been observed in both mice (16) and sheep (1,18). Evidence for GDF9/BMP15 physical interactions have also been provided by co-immunoprecipitation results (19,20), and a number of functional studies have demonstrated marked synergistic interactions between these two proteins (20-23). Recently,

Peng et al (24) have provided evidence that heterodimerization of GDF9 and BMP15 gives rise to a more potent ligand that signals solely via the SMAD2/3 pathway.

In the current study we hypothesized that human GDF9 and BMP15 form a stable heterodimer that has potent actions on mural GC, cumulus GC and oocyte development. We provide the first evidence of a purified BMP15:GDF9 heterodimer. We find that both the promature form and a covalent mature form of the heterodimer are particularly potent and are dual pathway activators, signaling via the SMAD2/3 and SMAD1/5/8 pathways. Interestingly, only the promature heterodimer is capable of improving oocyte quality, indicative of the crucial function that the prodomain plays in the functioning of these proteins. In deference to its potent activation of cumulus cells, and to align with the existing nomenclature for the other major reproductive TGF-β superfamily heterodimer, inhibin, we have called this new heterodimer cumulin.

EXPERIMENTAL PROCEDURES

Expression plasmids, stable cell lines and protein production - Expression cassettes encoding wild type human GDF9 or BMP15, or mouse GDF9 (mGDF9) were synthesized (Genscript USA, Inc., Piscataway, NJ) incorporating the rat serum albumin signal sequence at the 5' end followed by sequences encoding for His8 and StrepII affinity tags at the N-terminus of the respective proregion. The various human GDF9 and BMP15 Ser-Cys mutants (Ser^{418} in *GDF9* and Ser^{356} in *BMP15*) used in this study were derived from expression cassettes (Genscript USA, Inc., Piscataway, NJ) incorporating the glutamate receptor GluR-D subunit signal sequence (MRIICRQIVLLFSGFWGLAMG) at the 5' end followed by sequences encoding for the FLAG tag (a design based on Kuusinen *et al*. (25)) and His6 affinity tag at the N-terminus of the respective proregion (as used previously (8)). All the cDNA fragments were cloned into the pEF-IRES expression vector (26).

HEK293T cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified eagle medium (DMEM; Invitrogen, Auckland, New Zealand) containing 10% fetal calf serum (FCS), 2mM L-glutamine, 100IU/ml penicillin (Sigma, St Louis, USA) and 100μg/ml streptomycin. The different expression plasmids were transfected into HEK293T cells using lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Stable cell lines were established via puromycin selection. For production of cells producing pro-cumulin, HEK293T cells were co-transfected with both an expression plasmid for human GDF9 of wild type sequence, incorporating no epitope tags, along with the expression plasmid for wild type human BMP15 (His8/StrepII tagged), as described above. For production of cells producing cumulin (as a covalently stabilized form), HEK293T cells were co-transfected with expression plasmids encoding human GDF9 and BMP15 as the Ser-Cys mutant forms, as described above.

The mature regions of GDF9 (12), BMP15 (27) and the BMP15 Ser-Cys mutant (8) were produced and purified as previously described. Cells producing pro-GDF9, pro-mGDF9, pro-BMP15, pro-cumulin or covalent cumulin were grown to near confluence and growth media was then replaced by production media containing DMEM/F12 Glutamax, 0.1mg/ml BSA (Sigma), 25 IU/ml Fragmin (Pfizer, NSW, Australia), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma). After 48h incubation, the conditioned culture medium was collected.

Chromatography - Production medium containing pro-GDF9, pro-BMP15 or pro-cumulin was modified to include 10mM imidazole, 500μL protease inhibitor cocktail (Thermo Scientific, Rockford, USA), 500μL phosphatase inhibitor cocktail (Calbiochem, Alexandria, Australia) and subsequently adjusted to pH 7.4. Invitrogen Ni-NTA agarose resin was incubated with conditioned medium on a rotary agitator at 4°C for 2 hours, centrifuged at 900rpm for 5 minutes and the supernatant removed. The His-tagged target protein was eluted from the Ni-NTA agarose using elution buffer (PBS/500mM imidazole) and stored in LoBind tubes (Eppendorf, Hamburg, Germany) at -80°C. Cumulin (the covalently stabilized form) was produced and purified as above, with the exception that the mature region was eluted from the IMAC resin via a 7 M urea wash. This material was then loaded onto a reverse phase HPLC column equilibrated with 0.1% TFA and the different mature region forms (i.e. GDF9, cumulin and finally BMP15) eluted via a linear gradient of

acetonitrile. The fractions were stored in LoBind tubes at -80°C.

Silver staining, Western blotting and protein quantification - Fractions from the cumulin reverse phase HPLC were prepared for electrophoresis by lyophilizing 5 µl and resuspending in SDS-PAGE loading buffer +/- DTT. All samples were heated at 95°C for 5 minutes and then loaded onto a Mini PROTEAN TGX 4-15% gel (BioRad Laboratories, Hercules, USA). After 40 minutes at 150V, the gels were used for silver staining or Western blotting. Silver staining was carried out following the manufacturer's instructions using a silver quest staining kit (Invitrogen). For Western blotting, proteins were transferred to a Hybond-ECL membrane (GE Healthcare, Waukesha, USA) for 75 minutes at 100V. Membranes were blocked with 2% blocking reagent (supplied in an ECL Advance kit; GE Healthcare) diluted in TBS containing 0.1% (v/v) Tween 20 for 1 hour at room temperature. Subsequently the membranes were incubated with anti-GDF9 antibody (mAb53; (28)) or anti-BMP15 antibody (mAb28; (27)) diluted 1:5000 at 4°C overnight, followed by incubation with goat anti-mouse IRDye ™ 800 CW antibody diluted 1:10000, for one hour in the dark. Fluorescence intensity was determined using an Odyssey infrared imaging system (Licor Bioscience, Lincoln, NE). The various purified forms of GDF9 or BMP15 were quantified via Western analysis relative to recombinant mGDF9 or hBMP15 standards, respectively (R&D Systems, Minneapolis, USA).

Granulosa cell bioassays - Bioactivities of pro- and/or mature forms of BMP15, GDF9 and cumulin were compared using primary mouse mural GC and a human granulosa tumour cell line (COV434). The mouse granulosa cell ^{3}H-thymidine incorporation bioassay was performed using standard procedures as previously described (28,29). In brief, mural GC were collected from 129/Sv mice 44-46h after gonadotropin priming. Cells were cultured at 37 °C in 5% CO_2 in protein-free medium at 2 x 10^5 cells/ml with treatments for 18 hours followed by a further 6 hours with 15.4kBq ^{3}H-thymidine (Perkin Elmer, MA, USA). Granulosa cell ^{3}H-thymidine incorporation was assessed using a Wallac Microbeta plate counter as an indicator of cell DNA synthesis. Each treatment was performed in duplicate and experiments were replicated 3-6 times.

The relative capacity of BMP15, GDF9 and cumulin to activate SMAD signaling was assessed with luciferase assays using human COV434 cells (3,30). In brief, cells were plated at 33 × 10^3 cells/well in 96-well plates in complete medium (DMEM supplemented with 10% FCS) at 37 °C in 5% CO_2. After 24 h incubation, cells were transfected (130 ng/ well) with a BMP responsive reporter, BRE-luciferase, or an activin/TGF-β responsive reporter, A3-luciferase, using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions for 24 h. The cells were treated with BMP15, GDF9 or cumulin variants for 16 h. The medium was then aspirated, and the cells were solubilized in solubilisation buffer [25 mM glycylglycine (pH 7.8), 15 mM $MgSO_4$, 4 mM EGTA, 1% (v/v) Triton X-100, and 1 mM dithiothreitol], and luciferase reporter activity was measured as previously described (30).

RT-PCR - Mural GC were collected from 21- to 26-day-old C57BL/6 x DBA F1 hybrid female mice 46 h after intraperitoneal administration of gonadotropin priming. Mice were maintained in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes and with the approval of the University of New South Wales Animal Ethics Committee. Mural GC ($1x10^5$ live cells per 24-plate well) were cultured for 18 h in bicarbonate-buffered αMEM (Gibco Life Technologies, Grand Island, NY) supplemented with 3 mg/ml bovine serum albumin (BSA; Sigma Aldrich, St. Louis, MO). Cells were then treated for 6 h with fresh media containing recombinant human FSH (50 mIU/ml, Puregon; Organon, Oss, The Netherlands) plus the following treatments, as indicated: 100 ng/ml cumulin; 100 ng/ml pro-cumulin; 100 ng/ml pro-mature GDF9; 100 ng/ml pro-mature BMP15; or 50 ng/ml each of pro-mature GDF9 + pro-mature BMP15 when added together. RNA extraction, reverse transcription, and qPCR were performed as previously described (31) using their reported primers, however PCRs were performed on a Roche LightCycler 480 and results are expressed as raw gene expression levels relative to the geometric mean of two housekeeping genes (*Mrpl19* and *Ppia*) using the $2^{-(\Delta\Delta CT)}$ method. Data are means ± SEM of 4 replicate experiments.

Oocyte quality assay - The effects of treatments on oocyte quality (oocyte developmental competence) were assessed by treating cumulus-oocyte complexes with the different growth factors during the oocyte in vitro maturation (IVM) phase, followed by in vitro fertilisation (IVF) and embryo culture and assessment of subsequent blastocyst yield and quality. We chose to use a well-established porcine model of low oocyte developmental competence, as this experimental model better approximates the complexities of human oocyte developmental competence, than that of murine oocytes. This model uses oocytes from small antral follicles collected from abattoir-derived ovaries from gilts (peri-pubertal female pig) (13,32-34). The methods used were as per recently described in detail (13,34). In brief, cumulus-oocyte complexes were aspirated from small antral follicles (2-4 mm) and matured in protein-free porcine oocyte maturation (POM) basic IVM medium (35), supplemented for the first 22 hours of IVM with 1 mM dibutyryl-cAMP, 100 ng/ml amphiregulin (R&D Systems), and treated with either vehicle (control), 20 or 100 ng/ml pro-cumulin, 20 or 100 ng/ml mature cumulin, 100 ng/ml pro-GDF9, 100 ng/ml pro-BMP15, or 100 ng/ml of each of pro-GDF9 + pro-BMP15. Cumulus expansion was assessed at 22 h of IVM and scored as per standard criteria (36,37). At 22 hours, cumulus-oocyte complexes were washed and matured for a further 22 hours in POM without supplements or treatments (13,33,34). Thereafter IVF, in vitro embryo production and assessment of blastocysts was performed as previously described (13). Five replicate experiments were performed with approximately 180 oocytes/replicate.

Molecular modelling – To obtain 3D structure models for mature human GDF9, mature human BMP15 and cumulin, homology modelling was performed using either the software SWISS-Modeller or a manual approach. To identify the most suitable structure template, the sequences of GDF9 and BMP15 were blasted against the protein sequences present in the PDB databank revealing that both oocyte factors exhibit the highest sequence homology (32 – 33 % identity) to either BMP2 or GDF5. For manual modelling the sequences of the mature region of GDF9 and BMP15 were then aligned to the amino acid sequence of the identified templates, i.e. BMP2 (PDB structure entry, 3BMP, 1REW (chains A and B) and 3BK3 (chains A and B), GDF5 (PDB entry 1WAQ) using the software CLUSTALW. A model of human GDF9 comprising residues Asn327 to Arg430 was then built manually by replacing amino acid residues in the BMP2 template (chains A and B of PDB entry 3BK3) employing the tool ProteinDesign of software package Quanta2008 (MIS Accelrys, San Diego). A single amino acid insertion at the end of helix α1 found in GDF9 (when compared with the template BMP2) was rebuilt manually. For modelling of human BMP15 (comprising residues Asn289 to Arg392) the same approach was applied but using the structure of human GDF5 (PDB entry 1WAQ) as BMP15, similar to GDF5, binds specifically the BMP receptor BMPR1b. Both initial models were subsequently refined first by performing rotamer searches using the tool X-BUILT of the software Quanta2008 to identify side chain conformations not leading to van der Waals clashes. Then energy minimization runs were performed employing only geometrical but no electrostatic energy terms using Quanta2008 and the forcefield CHARMm27. First all backbone atoms were kept fixed and only side atoms were allowed to move, but restrained by harmonic positional restraints (5 kcal mol^{-1} $Å^{-2}$) to minimize atom movements. After energy minimization of side chain atoms converged these restraints were removed and the position of the backbone atoms were refined by applying a strong harmonic potential (25 kcal mol^{-1} $Å^{-2}$). The final models of GDF9 and BMP15 exhibit good backbone and side chain geometry. The structure models for both homodimeric factors are highly similar yielding a root mean square deviation of only 1.1Å for 164 Cα carbons. Only 40 residues, which are located either in the fingertips or the loop N-terminal of helix α1, exhibit differing conformations, which may be due to selecting different template structures for the models and does not reflect inherent structural differences between GDF9 and BMP15.

The model of cumulin was then built by superimposing the 3D models of homodimeric GDF9 and BMP15 and selecting one chain of each structure to form a heterodimeric assembly. Due to the almost identical amino acid composition in the dimer interface the model of cumulin required very minor minimization, which was performed by rotamer searches for side chains with close

contacts. The heterodimeric non-covalent form of cumulin was then converted into the covalent form by exchanging the serine residues Ser356 in BMP15 and Ser394 in GDF9 against cysteines and connecting both sulphur atoms to form the thioether bond. The disulfide bond was subsequently refined by a short energy minimization run to obtain a thio-ether bond of correct length.

Statistical analysis - For mRNA expression data, differences between means were assessed by 1-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Similarly, effects of treatments on embryo yield were assessed by 1-way ANOVA on arc-sine transformed data followed by a least significant difference post-hoc test. Cumulus expansion data were assessed by a Kruskal-Wallis 1-way ANOVA on ranks followed by a Dunnett's post-hoc test. $P<0.05$ was considered statistically significant.

RESULTS

The various forms of GDF9, BMP15 and cumulin analysed in this study were:

(1) Pro-forms: non-covalent homo- and heterodimers of the promature regions of BMP15 and GDF9 (the form in which these proteins are secreted from the cell).

(2) Mature forms: non-covalent homo- and heterodimers of the mature regions of BMP15 and GDF9 (the form in which these proteins bind to their signalling receptors).

(3) Covalent forms: covalent homo- and heterodimers of the mature regions of BMP15 and GDF9 (stabilised versions of the mature growth factors).

GDF9/BMP15 synergism is mediated by heterodimer (i.e. cumulin) formation - We have consistently observed GDF9 and BMP15 interacting in a synergistic manner, whether in their pro-forms (i.e. as isolated pro/mature complexes; Fig. 1) or as purified mature proteins (23,38). The extracellular formation of a BMP15/GDF9 heterodimer, which we have termed cumulin, could underlie this potent synergism. To directly test this possibility, we generated a covalent BMP15 homodimer (by replacing Ser^{356} with the corresponding cysteine found in other TGF-β proteins), which would be incapable of forming cumulin in the presence of wild type GDF9.

Covalent human BMP15 mature region homodimer ($BMP15_{S:C}$) was purified and tested for its ability to synergise with human GDF9 (mature region homodimer) in a mural granulosa cell proliferation assay. At the low doses tested, GDF9, BMP15 and $BMP15_{S:C}$ did not promote proliferation, however, the GDF9+BMP15 combination showed potent synergism (Fig 2A). In contrast, this synergism was not observed with the GDF9+$BMP15_{S:C}$ combination. As we have previously shown that GDF9/BMP15 synergism is dependent upon Smad2/3 activation (23,38), we examined the ability of our BMP15 variants to affect this response in COV434 cells. While wild type BMP15 promoted GDF9-dependent Smad2/3 transcriptional activity in a dose-dependent manner, the $BMP15_{S:C}$ variant could not potentiate a GDF9 effect (Fig 2B). To ensure the $BMP15_{S:C}$ variant had bioactivity, we tested its ability to stimulate the SMAD1/5/8 pathway in COV434 cells transfected with a BMP-responsive luciferase reporter. In this system, the $BMP15_{S:C}$ protein was equally potent as wild-type BMP15 (Fig 2C). As expected, GDF9 did not activate the SMAD1/5/8 pathway. Together, these results indicate that extracellular heterodimerization of mature GDF9 and BMP15 is possible, as simple co-addition of GDF9+BMP15, but not of GDF9+$BMP15_{S:C}$, resulted in potent synergism.

To further explore extracellular associations between GDF9 and BMP15, we mixed conditioned media from cells expressing untagged pro-GDF9 with conditioned media from cells expressing N-terminally poly-His tagged pro-BMP15, and carried out IMAC purification of the pro-BMP15. Under the mild conditions used, the untagged GDF9 mature region was capable of associating and co-purifying with pro-BMP15, as evidenced by GDF9 immunoreactivity co-eluting off the IMAC resin (Fig 3A). No GDF9 immunoreactivity was detected when BMP15 was purified alone (*data not shown*) and the untagged GDF9 did not bind to the IMAC resin in the absence of the poly-His tagged BMP15 (Fig. 3B). It is clear from these results that, like the mature proteins, the pro forms of GDF9 and BMP15 are capable of associating extracellularly to form pro-cumulin.

Intracellular formation of cumulin - GDF9 and BMP15 are co-expressed in the oocyte throughout folliculogenesis and so do not naturally

exist in isolation (reviewed; (39)). Thus, heterodimerization of these two TGF-β family members to form cumulin is likely to occur during synthesis within the oocyte, as well as after secretion into the extracellular space. In the *in vivo* context, cumulin will be in a form where the mature regions are non-covalently associated, and will most likely act as a pro/mature complex. To produce such a form (i.e. pro-cumulin), we co-expressed N-terminally poly-His tagged pro-BMP15 with untagged pro-GDF9, and purified the non-covalent pro-cumulin complex by IMAC. Theoretically, this approach could lead to the purification of a mixture of pro-BMP15 homodimers and pro-cumulin. Quantification of the levels of both the processed GDF9 and BMP15 mature regions following IMAC purification indicated a 3:1 ratio of pro-BMP15 homodimer:pro-cumulin heterodimer (*data not shown*).

Pro-cumulin is a potent activator of granulosa cells via both SMAD pathways - We next examined the potency of pro-cumulin in ovarian GC bioassays. We compared pro-cumulin to pro-GDF9, pro-BMP15 and pro-GDF9+pro-BMP15, after quantitating each protein form relative to the level of the GDF9 mature region (*data not shown*). Pro-GDF9 alone was inactive in promoting GC DNA synthesis, as shown previously (3), as was pro-BMP15 alone (Fig. 4A). In contrast, pro-cumulin was exceptionally potent on primary mouse mural GC, stimulating a 3.8-fold increase in [^{3}H]-thymidine incorporation at the lowest dose tested (1 ng/ml, Fig. 4A). Pro-cumulin was notably more potent (6-12-fold) than pro-GDF9+pro-BMP15 at all doses examined.

To examine SMAD signaling by pro-cumulin, we conducted transcriptional reporter assays using the human granulosa cell line, COV434, as previously reported (3). Pro-cumulin dose-dependently activated SMAD2/3 signaling in GC, producing high levels of A3-Lux reporter activity at 50 and 100 ng/ml (Fig. 4B). Pro-GDF9+pro-BMP15 also dose-dependently activated SMAD2/3, but did not reach the same levels of stimulation as pro-cumulin. As expected, pro-GDF9 did not activate SMAD2/3, as it is latent (3,12). Interestingly, pro-cumulin and pro-GDF9+pro-BMP15 promoted similar levels of BRE-luciferase activation (reflecting the level of SMAD1/5/8 phosphorylation) as pro-BMP15 (Fig 4C). As expected, GDF9 did not activate the SMAD1/5/8 pathway (Fig 4C) and BMP15 did not activate the SMAD2/3 pathway (Fig 4B).

Engineering covalent mature cumulin - The pro-cumulin preparation described above and that previously produced by Peng *et al* (24,40) contain a significant amount of contaminating pro-BMP15 homodimer. This was due to the almost equal propensity of monomeric BMP15 to homodimerize or heterodimerize (with GDF9) and the fact that the purification strategies were directed against tagged BMP15 prodomains. In order to overcome these limitations and to more conclusively examine the biological function of cumulin, we undertook to engineer a covalent form of cumulin, which could be fully purified from contaminating BMP15 and GDF9. In order to produce such a protein, we replaced Ser^{418} in *GDF9* and Ser^{356} in *BMP15* with the corresponding cysteine found in other TGF-β proteins, and co-expressed the resultant cDNAs in HEK293T cells. We have used this approach before to produce human covalent mature BMP15 (8). Both the pro-BMP15$_{S:C}$ and pro-GDF9$_{S:C}$ mutants were produced with N-terminal poly-His tags, hence IMAC purification of conditioned media from cells co-expressing these proteins theoretically could result in the co-purification of pro-GDF9 or pro-BMP15 homodimers, as well as the sought after pro-cumulin. We decided to target the GDF9 and BMP15 mature regions for purification in order to avoid any potentially misfolded disulfide linked pro/mature complexes. This was accomplished by a two-step procedure of IMAC chromatography followed by RP-HPLC. This approach succeeded in resolving three distinct covalent species: mature GDF9, mature cumulin and mature BMP15 (Fig 5A). This can be concluded as Western analysis of the fractions from the RP-HPLC purification indicated that fractions 31-32 contained GDF9, but no BMP15; fractions 33-34 contained both GDF9 and BMP15 (i.e. cumulin), whereas fractions 36-39 contained only BMP15 (Fig 5B). The silver stained gels confirmed the presence of disulfide-linked dimers, as the reduced bands at 15-18 kDa (Fig 5C, fractions 32-34 and 37), migrated at 25-35 kDa without reduction (Fig 5D, fractions 32-34 and 37).

Covalent mature cumulin is potently bioactive - As purified mature cumulin has not

previously been produced, it was important to determine its bioactivity, especially given its form as a stabilised disulfide linked covalent mature region heterodimer. We initially tested the fractions across the RP-HPLC profile for bioactivity in the mural GC assay. The peak of bioactivity (Fig 5E, fraction 34) coincided with the fraction containing the peak of covalent cumulin protein (Fig 5B-D, fraction 34).

We next directly compared the bioactivity of covalent mature cumulin to the previously characterised non-covalent pro-cumulin on GC. Mature cumulin was highly potent in the range of different granulosa cell assays performed; in most cases, more potent than pro-cumulin (Fig 6). In the primary mouse mural granulosa cell assay of [^{3}H]-thymidine incorporation, covalent mature cumulin had an ED_{50} of 0.6 ng/ml, whereas pro-cumulin had an ED_{50} of 4 ng/ml (Fig. 6A). Mature covalent cumulin was also more effective than pro-cumulin at promoting SMAD2 signaling in human COV434 cells, as assessed by A3-Lux activity (Fig 6B), while the two cumulin forms were equipotent at promoting SMAD1/5/8 signaling (Fig 6C). Together, our analysis of non-covalent pro-cumulin and covalent mature cumulin, allow us to propose a model whereby cumulin activates both SMAD pathways, but SMAD2/3 in particular (see Fig 7).

One of the physiological roles of oocyte-secreted GDF9 and BMP15 is to direct granulosa cell differentiation towards the cumulus cell phenotype (41), which can be characterised by expression of genes required for cumulus cell mucification (*Ptx3, Has2, Tnfaip6, Ptgs2*) (42). Pro-cumulin and mature covalent cumulin were notably more effective at promoting mRNA expression of these genes in primary mouse mural GC than pro-GDF9, pro-BMP15 or these combined (Fig 6D). Mature cumulin was more effective than pro-cumulin, particularly at promoting *Ptx3* mRNA expression ($P<0.05$).

Pro-cumulin, but not mature covalent cumulin, is a potent stimulator of oocyte developmental competence - To test our human proteins in a model closer to the *in vivo* situation, we isolated porcine COCs from small unstimulated ovarian follicles and treated them for 24h with our different proteins, and examined cumulus cell expansion and oocyte developmental competence (capacity of the oocyte to support embryo development to day 7). With best practice IVM and embryo production, this model of low oocyte developmental competence typically yields 10-20% blastocysts (13,32). Interestingly, when porcine COCs are used as the target, rather than cells in a monolayer (either mural granulosa or COV434 cells), the most bioactive protein was non-covalent pro-cumulin and not covalent mature cumulin (Fig 8). For cumulus expansion, the only protein that caused a significant increase was pro-cumulin (Fig 8A). Exposure of oocytes to 20 ng/ml pro-cumulin for 24h improved oocyte quality as assessed by a substantial improvement in capacity to support embryo development to the blastocyst (2.3-fold; 28% to 63%; $P<0.05$; Fig 8B) and hatched blastocyst stages (3.8-fold; 5% to 19%; $P<0.05$; Fig 8C). There was no significant difference in blastocyst rates when oocytes were treated with 20 or 100 ng/ml pro-cumulin (63% and 55%, respectively), or mature cumulin (32% and 39%, respectively). No other treatment improved oocyte developmental competence, although pro-GDF9+pro-BMP15 tended to increase blastocyst and hatched blastocyst rates ($P>0.05$; Fig 8C). There was no effect of any treatments on subsequent blastocyst quality as assessed by blastocyst cell numbers (Fig 8D).

Molecular modelling and analysis of cumulin assembly - To provide molecular insights into cumulin assembly, stability and potential receptor-binding properties we performed homology modelling to obtain structural models of homo- and heterodimeric forms of GDF9 and BMP15. Structure templates were selected on the basis of sequence homology to BMPs and GDFs with known structure in the RCSB databank. The models of mature GDF9 (Fig. 9A,B) and BMP15 (Fig. 10A,B) exhibit the canonical butterfly-shaped architecture of TGF-β ligands. The structure of the monomeric subunits is defined by the cysteine-knot, which results in three loops emanating from this central motif. The two loops running in parallel form β-sheets, whereas the third loop, which emanates from the cysteine core in the opposite direction, folds into an α-helix. The monomer architecture is also often compared to an open hand, with the β-sheets forming fingers 1 and 2 and the α-helix forming the wrist (Figs. 9A,B and 10A,B). The dimer assembly can then be

envisioned as the inner sides of the two hands coming together at palm and wrist.

Unlike most TGF-β ligands, GDF9 and BMP15 lack the cysteine residue that forms an intermolecular disulfide bond, locking the dimer assembly (this cysteine is replaced by an isosteric serine in both GDF9 and BMP15). This unique feature, together with the very high sequence conservation across the GDF9 and BMP15 dimer interfaces (Figs. 9C and 10C), enables these growth factors to heterodimerize to form cumulin (Fig. 11A,B). Importantly, the cumulin model shows a dimerization interface (Fig. 11C) identical to homodimeric GDF9 and BMP15 (Figs. 9C and 10C, respectively), indicating that formation of the heterodimer is possible and that the stability of such a complex is likely indistinguishable from the homodimeric forms. Production of covalent cumulin also appears likely based upon our modelling, as *in silico* exchange of the two serine residues (Ser^{418} in GDF9 and Ser^{356} in BMP15; Fig. 11B) locates both sulfur atoms sufficiently close to form a thioether bond without requiring conformational or large structural rearrangements of the dimer architecture (Fig. 11D).

Finally, analysis of our homology models indicates that the binding sites for type I receptors within the "wrist" regions are distinct in cumulin, relative to its homodimeric ancestors (Fig 11B, Figs 9B and 10B). This is due to the fact that, in contrast to the type II receptor epitopes, the wrist or type I receptor-binding site is built from both monomer subunits. In cumulin this epitope therefore presents a chimera with unique amino acid composition not found in either homodimeric growth factor ancestors. These differences may alter type I receptor affinity and/or specificity for cumulin and contribute to its enhanced signalling capacity, particularly in comparison to latent human GDF9.

DISCUSSION

In females, GDF9 and BMP15 are essentially only expressed in oocytes where they are co-expressed throughout most of oogenesis and so do not naturally exist in isolation. Moreover, given their high sequence homology, these key growth factors can be expected to interact with each other and hence should generally be considered as acting together. There is ample evidence for interactions between GDF9 and BMP15; at genetic (1,16,18), biochemical (19,20) and functional levels (20-23). A putative BMP15:GDF9 heterodimer was first modelled by McNatty *et al*., (1) in 2004. Most recently, Peng *et al*. (24) produced GDF9:BMP15 heterodimers with potent bioactivity. We have pointed out a number of potential methodological and interpretation deficiencies in that study (43), including that the preparation contained other protein forms and the only evidence presented for formation of a heterodimer was co-immunoprecipitation of the two proteins. In the current study, we have conducted detailed molecular modelling of heterodimer assembly and succeeded in producing two heterodimeric forms; pro-cumulin and mature covalent cumulin. Both forms are particularly potent on GCs acting via both the SMAD2/3 and SMAD1/5/8 pathways, and show differential actions on mural GC versus cumulus GC.

The co-addition of separately expressed GDF9 and BMP15 homodimers elicits a potent synergistic response on GC (20-23). The results of the current study provide strong evidence that the basis for such synergism is formation of the cumulin heterodimer. Such GDF9/BMP15 synergism has been described by others as the action of two homodimers (24). Our results do not support this contention. We deduce this from the observation that the $BMP15_{S:C}$ covalent homodimer, which cannot heterodimerize with GDF9, does not synergize with wild-type GDF9, in contrast to wild-type BMP15. The $BMP15_{S:C}$ mutant in all other aspects resembles wild-type BMP15. Furthermore, both pro-cumulin and mature covalent cumulin elicited a more potent response than the synergistic effects of co-addition of wild-type GDF9 and BMP15 homodimers. Using the novel covalent cumulin alleviates any concerns arising from studying solely a non-covalent heterodimer, which may exist in an equilibrium between homodimers and heterodimers, as we previously discussed (43). This leads us to hypothesize that the process of heterodimerization is the mechanism of activation of the otherwise latent GDF9 in the human.

While inhibin A and B are the other major naturally occurring TGF-β heterodimers (5), it is possible to co-express other TGF-β ligands and force their dimerization, and in so doing generate more biologically potent proteins. Within the BMP

sub-group, BMP2/7 (44,45), BMP2/6 (46) and BMP7/GDF7 (47) heterodimers are all reported as more potent or as exhibiting altered bioactivity, in comparison to a combination of homodimers. There are a number of possible mechanisms to account for greater potency of heterodimers. Firstly, a heterodimer may have a decreased binding affinity for extracellular BMP binding proteins, which commonly act as antagonists. Many of these antagonists act themselves as obligate or non-obligate dimers and thus require a symmetrical homodimer for maximal binding affinity. Another explanation is that TGF-β ligands tend to have a high affinity for either a type I or for a type II receptor, often leading to a strict sequential binding mechanism. A heterodimer however, could combine a high affinity-binding site for a type I and a type II receptor within the one dimeric ligand, thereby greatly enhancing the overall affinity to its cell surface receptors. Finally, due to its inherent asymmetry, a heterodimeric ligand is more prone to recruit two different type I receptors into the signalling complex. For example, the cumulin receptor complex (Fig. 7) is predicted to comprise two BMPRII receptors, one ALK4/5/7 receptor, which is necessary for the activation of the SMAD2/3 pathway, and one ALK6 receptor, needed for SMAD1/5/8 signaling, as originally hypothesized by McNatty *et al*., (1).

An open question prior to this study was whether cumulin could only form inside the oocyte or whether it might also spontaneously assemble from homodimeric GDF9 and BMP15 present in the extracellular space. The latter seemed feasible as GDF9 and BMP15 naturally form transient dimers, whose dimer architecture is not fixed by an intermolecular disulfide bond. Indeed, we observed that the two wild-type homodimeric proteins, separately produced and purified, synergise via a process of heterodimer formation. High-resolution structure studies of different BMPs provide a possible explanation for GDF9 and BMP15 disassembly and subsequent cumulin formation. Specifically, the structures show that the interface between the monomeric halves of TGF-β proteins is considerably hydrated, which is unusual compared to the water-free hydrophobic core of soluble proteins (48-51). The localization of these conserved water molecules in the dimerization interface around the central cysteine-knot likely limits stability of the dimer, however, in the case of covalent BMPs, a full disassembly of the dimer is prevented due to the covalent linkage via the intermolecular disulfide. But since GDF9 and BMP15 lack this bond, both factors potentially undergo full dis- and re-assembly, which, when both factors are simultaneously present (usual situation in most mammals), would yield an equilibrium-based mixture of homo- and heterodimers unless steric restraints specifically interfere with heterodimer formation. The latter seems highly unlikely though given the fact that the interface residues are highly conserved between GDF9 and BMP15. Together, this evidence suggests that the formation of cumulin *in vivo* might, at least in part, occur post-secretion from the oocyte in the extra-cellular matrix of the granulosa and cumulus cells. If so, it seems plausible that the nature and composition of the extra-cellular matrix, which in turn is affected by *in vivo* and *in vitro* context, is likely to affect cumulin assembly and bioactivity.

Two remarkable features of cumulin bioactivity is its capacity to activate both the SMAD2/3 and SMAD1/5/8 signaling pathways, as well as its enhanced signalling potency, in particular when compared to the GDF9 and BMP15 homodimers. On isolated mural GCs, mature covalent cumulin was even more potent than pro-cumulin, particularly in terms of GC proliferation, which is consistent with its enhanced SMAD2/3 signaling (29). Dual SMAD pathway activation by cumulin is in disagreement with Peng *et al*. (24), who claimed that the GDF9:BMP15 heterodimer binds to, but does not activate, the ALK6 receptor. The form of heterodimer that Peng *et al*. (24) produced has an epitope tag (either FLAG or myc) incorporated into the N-terminus of the mature region. Although this appears to not impede BMP15 homodimer bioactivity (52), the effects of the GDF9 (myc) and BMP15 (FLAG) tags on heterodimer activity are unknown. In contrast, covalent mature cumulin does not contain any tags, and pro-cumulin incorporates a tag only at the N-terminus of the BMP15 prodomain. Hence, in all forms of GDF9, BMP15 or cumulin used in the current study, the amino acid sequence of the mature region is identical to the wild-type sequence (apart from the Ser-Cys mutation to form covalent dimers). The lack of any modifications to our GDF9 and

BMP15 mature regions may also explain why we are able to detect synergism between separately expressed and purified pro-GDF9 and pro-BMP15 (current study), whereas others do not (24,53).

An intriguing and important observation from the current study is that when cumulus-oocyte complexes were treated, only the pro-cumulin form improved oocyte quality, as assessed by subsequent embryo yield, whereas mature covalent cumulin did not, despite being exceptionally potent on mural GC. Clearly, pro-cumulin contains the prodomains of GDF9 and BMP15 as part of a complex, whereas mature cumulin is an isolated mature region heterodimer (Fig 3). These results are entirely consistent with our recent observation that pro-BMP15, but not mature domain BMP15 nor mature domain mouse GDF9, enhances oocyte developmental competence (54,55). It is well established that prodomains of TGF-β superfamily growth factors have important roles in protein folding and regulation of bioactivity (56,57). In particular, interactions of the prodomains of cumulin with the specialised extracellular matrix of cumulus GC may facilitate presentation of the heterodimeric mature domain to its receptors. In support of these concepts, we have shown that pro-GDF9 binds strongly to heparin, suggesting that certain heparan sulfate proteoglycans (HSPG) may act as co-receptors for pro-GDF9, pro-BMP15 and pro-cumulin (58). Hence, the differential activities of pro-cumulin and mature cumulin on mural GC versus cumulus GC, may be attributed to differential expression of HSPG and/or type I or II receptors in these GC.

Finally, the current study also has significant practical implications for reproductive medicine and advanced breeding in domestic animals. The development of purified and highly potent pro-cumulin, is likely to prove an important additive for oocyte *in vitro* maturation (IVM) (59). IVM is an assisted reproductive technology that generates viable embryos using reduced or no exogenous ovarian hormone stimulation of the patient. IVM is thus an important adjunct technology to *in vitro* fertilisation. To make IVM more clinically viable, including in fertility preservation in cancer survivors, an additive such as pro-cumulin is needed to improve the efficiency of IVM (60). We and others have shown that addition of "native" oocyte-secreted factors (e.g. pro-BMP15 or pro-GDF9) to IVM enhances oocyte quality, having profound effects on embryo development and fetal survival (13,54,55,61-64). This study is the first report on the effects of cumulin on oocyte quality. We carefully chose to use an established porcine model of low oocyte developmental competence, using oocytes from growing antral follicles, to better recapitulate the human scenario. Addition of pro-cumulin led to a major improvement in oocyte quality, more than doubling embryo yield, which, if translatable to human oocytes, would have a large impact on clinical IVM and oncofertility.

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Conflict of interest: RBG is listed as an inventor on the patent US20140073052A1 owned by the University of Adelaide. RBG and DGM are listed on a distribution of benefits agreement in connection with patent US20140073052A1.

Author contributions: DGM and RBG designed the study. DGM, CAH and RBG secured funding for the project. DGM oversaw all aspects of recombinant protein design, production and purification, which were performed by JJL, MAW, GAM, AT, JS and DGM. SS developed methodology for and performed the porcine oocyte quality experiments. Additional experiments were performed and analysed by the following authors: DR, RT-PCR; SLM, COV434 SMAD reporters; LJR, mouse GC DNA synthesis;

TDM, molecular modelling. DGM, CAH and RBG wrote the manuscript, which was edited and approved by all authors.

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The abbreviations used are: ALK, activin receptor-like kinase; ANOVA, analysis of variance; BMP15, bone morphogenetic protein 15; BMPRII, BMP receptor type II; ECM, extra-cellular matrix; GC, granulosa cell; GDF9, growth differentiation factor 9; IVM, oocyte in vitro maturation; SMAD; *Sma-* and *Mad-related* proteins;

FIGURE 1. Pro-forms of BMP15 and GDF9 synergise. Pro-hBMP15 synergizes with pro-mouse GDF9 and also with pro-human GDF9 which is naturally latent. Bioactivity was assessed by [^{3}H]-thymidine incorporation in primary mouse mural GC. The various pro-proteins were produced in HEK-293T cells with N-terminal His-tags attached to their prodomains allowing purification by IMAC.

FIGURE 2. GDF9/BMP15 synergism is mediated by heterodimer formation. The BMP15 (Ser^{356}Cys) covalent homodimer ($BMP15_{S:C}$) does not synergise with GDF9, in contrast to wild type BMP15, where

clear synergism is observed. Bioactivity was assessed by; **A)** [^{3}H]-thymidine incorporation in primary mouse mural GC, or **B)** Smad2/3-responsive luciferase reporter activity, or **C**) Smad1/5/8 luciferase reporter activity, in COV434 human GC. The various proteins were produced in HEK-293T cells with N-terminal His-tags attached to their prodomains allowing purification by IMAC, followed by isolation of mature domains by reverse-phase HPLC. BMP15 = HPLC purified human BMP15 mature region homodimer, GDF9 = HPLC purified human GDF9 mature region homodimer, $BMP15_{S\text{-}C}$ = HPLC purified human BMP15 (Ser-Cys) mature region covalent homodimer.

FIGURE 3. GDF9 associates and co-purifies with BMP15 to form pro-cumulin. Conditioned media containing untagged pro-GDF9 and N-terminally His8 tagged pro-BMP15 were combined and subjected to IMAC chromatography. **A)** Western blot of resin load probed with Mab28 (specific for human BMP15), or the resin load, unbounded material, wash and eluted peak fraction, probed with Mab53 (specific for GDF9). **B)** Western blot for IMAC chromatography of conditioned media containing only untagged pro-GDF9. Shown are the resin load, unbound material, wash and eluate, probed with Mab53.

FIGURE 4. Bioactivity and SMAD signaling of pro-cumulin. Dose response curves of the different pro-protein forms of GDF9, BMP15 and cumulin. Bioactivity was assessed by; **A)** [^{3}H]-thymidine incorporation in primary mouse mural GC, or **B)** Smad2/3-responsive luciferase reporter activity, or **C**) Smad1/5/8 luciferase reporter activity, in COV434 human GC. The various pro-proteins were produced in HEK-293T cells with N-terminal His-tags attached to their prodomains allowing purification by IMAC.

FIGURE 5. Engineering and bioactivity of covalent cumulin. Conditioned media from cells co-expressing GDF9 and BMP15, both harbouring the appropriate Ser-Cys mutations, was subjected to IMAC chromatography followed by reverse phase HPLC. **A)** Absorbance profile at 280 nm from the HPLC purification. **B)** Western blot of HPLC fractions probed for GDF9 with Mab53 or for BMP15 with Mab28. Silver stained SDS-PAGE gel of the HPLC fractions, run under reduced **C)** or non-reduced **D)** conditions. **E**) Bioactivity of the fractions (0.25 µl of each 1 ml fraction) across the HPLC profile, as measured by [^{3}H]-thymidine incorporation into primary cultures of murine mural GC.

FIGURE 6. The mature domain of covalent cumulin is more potent than pro-cumulin on GC. The pro-cumulin preparation was produced in HEK-293T cells with a N-terminal His-tag in the prodomain allowing purification by IMAC. To generate purified mature cumulin, the IMAC product was subsequently subjected to reverse-phase HPLC, generating a highly pure mature domain protein with fixed heterodimer architecture (mature covalent cumulin). Bioactivity was assessed by; **A)** [^{3}H]-thymidine incorporation in primary mouse mural GC, **B)** Smad2/3-responsive luciferase reporter activity and **C)** Smad1/5/8 luciferase reporter activity, in COV434 human GC, and by **D)** mouse primary mural GC mRNA expression of genes associated with cumulus cell differentiation, *Ptx3, Tnfaip6, Has2* and *Ptgs2*, (all proteins at 100 ng/ml, except pro-GDF9+pro-BMP15, which were added at 50 ng/ml each). Bars with different letters indicate significant differences at $P < 0.05$ within the same graph.

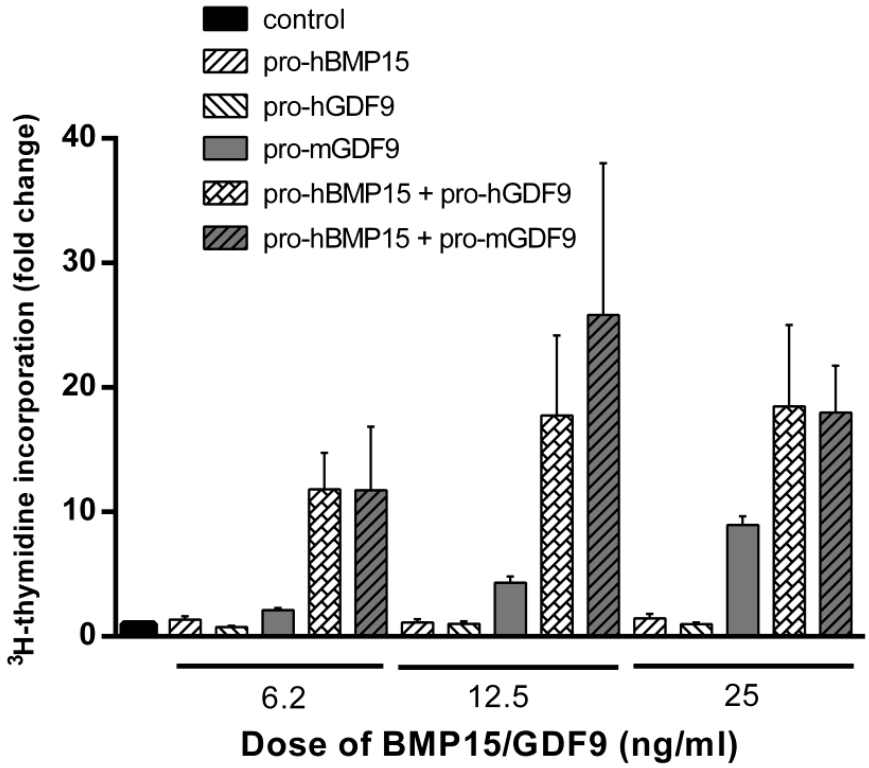
FIGURE 7. Hypothesized model of human cumulin formation and signaling. BMP15 and GDF9 are co-expressed in the oocyte throughout most of oogenesis. **A)** During synthesis, the prodomains of BMP15 and GDF9 direct folding and dimerization of their respective mature domains. **B)** Dimeric precursors are cleaved by furin-like proteases then BMP15 and GDF9 are secreted from the oocyte non-covalently associated with their prodomains, forming their respective pro-forms. The pro-cumulin heterodimer is likely to assemble within the oocyte, but can also form in the extracellular space (ECM). In isolation, human pro-GDF9 is latent and activation is dependent upon heterodimerization with BMP15 to form cumulin. **C)** Following prodomain displacement, **E)** cumulin activates both SMAD2/3 and SMAD1/5 transcription factors, **D)** presumably through a receptor complex involving two BMPRII receptors, an ALK6 receptor and an ALK5/4 receptor, on cumulus GC and mural GC. Pro-cumulin and mature cumulin exhibit differing potencies on different granulosa cell lineages.

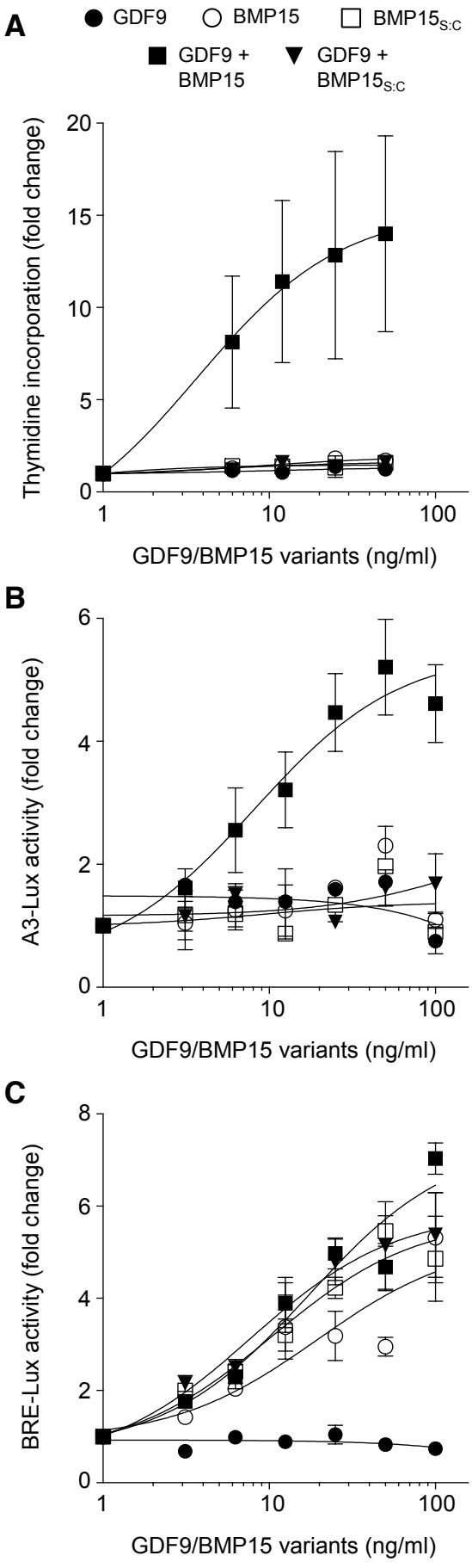
FIGURE 8. Pro-cumulin, but not mature cumulin, is a potent stimulator of oocyte developmental competence. A porcine *in vitro* experimental model of low oocyte developmental competence was used to examine the effect of the various growth factors on oocyte quality. Porcine COCs were treated with vehicle, 20 ng/ml pro-cumulin, 20 ng/ml mature covalent cumulin, 100 ng/ml pro-GDF9, 100 ng/ml pro-BMP15, or 100 ng/ml of each of pro-GDF9 + pro-BMP15 for the first 22 h of oocyte *in vitro* maturation. **A)** Morphological cumulus expansion was assessed and scored at 22 h according to the Vanderhyden criteria. Effects of the treatments on oocyte development competence were examined by assessing embryo development on day 7: **B)** blastocyst rate and **C)** hatching blastocyst rate [hatching or hatched blastocysts], both expressed as a percentage of the number of cleaved embryos, and **D)** average blastocyst cell number. All values are represented as mean ± SEM from 5 replicate experiments. Bars with different letters indicate significant differences at $P < 0.05$ within the same graph.

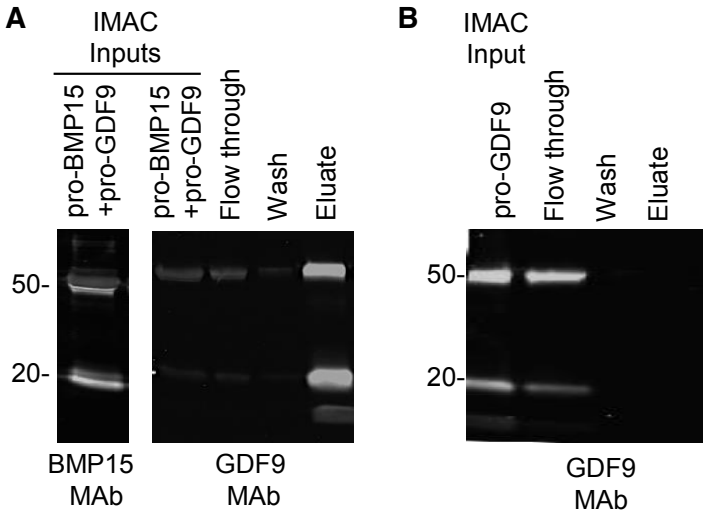
FIGURE 9. Homology model of mature GDF9. **A)** Ribbon plot of human mature GDF9 highlighting the canonical dimer architecture. The two monomer subunits of the homodimer are coloured in red and orange. The potential binding epitopes for the type I (wrist) and type II (knuckle) receptors are marked. The six cysteine residues forming the characteristic cystin-knot motif as well as the serine residues replacing the cysteine residues, which are involved in the intermolecular disulfide bond found in other TGF-β members, are shown as sticks. **B)** As in (A) but rotated around the x-axis by 90°. **C)** Detail of the GDF9 dimer interface showing that interfacial amino acid residues are highly conserved between GDF9 and BMP15 (see also Figure 10) suggesting that heterodimer formation (see Figure 11) is very likely not impaired by steric or chemical restraints resulting from differences in the dimer interface.

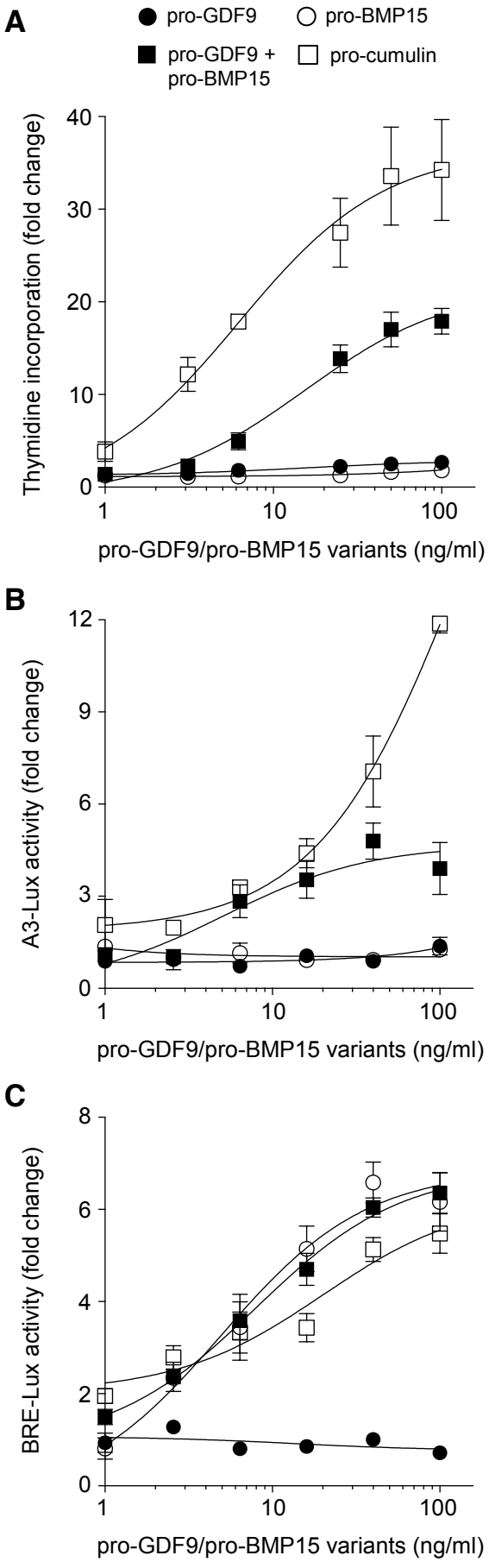
FIGURE 10. Homology model of mature BMP15. **A)** Ribbon plot of human mature BMP15 showing the canonical dimer architecture. The two monomer subunits of the homodimer are coloured in light and dark green. The potential binding epitopes for the type I (wrist) and type II (knuckle) receptors are indicated. The six cysteine residues (coloured in yellow) forming the characteristic cystin-knot motif as well as the serine residues replacing the cysteine residues, which are involved in the intermolecular disulfide bond found in other TGF-β members, are shown as sticks. **B)** As in (A) but rotated around the x-axis by 90°. **C)** Detailed view of the BMP15 dimer interface showing that interfacial amino acid residues are highly conserved with those found in GDF9 (see also Figure 9) suggesting that cumulin formation (see Figure 11) is very likely not impaired by steric or chemical restraints by differences in the dimer interface.

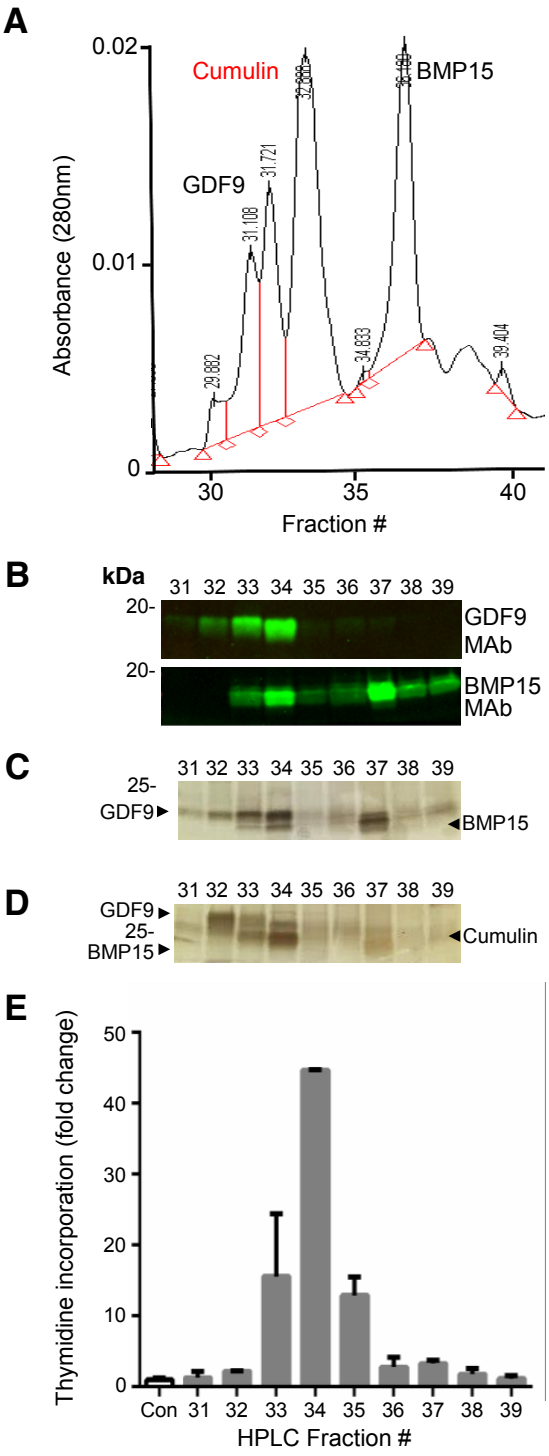
FIGURE 11. Molecular modelling of mature cumulin. **A)** Ribbon plot of the homology model of human mature cumulin showing the butterfly-shaped dimer architecture and with the monomer subunits coloured in red and green. Structural features and the potential binding epitopes for the type I (wrist) and type II (knuckle) receptors are indicated. The six cysteine residues forming the characteristic cystin-knot motif are shown as sticks. **B)** As in (A) but rotated around the x-axis by 90°. **C)** Detail of the cumulin dimer interface showing that, despite lacking the intermolecular disulphide bond present in most other TGF-β ligands, similar polar and hydrophobic interactions stabilize dimer assembly. **D)** Production of covalent cumulin appears possible, as *in silico* exchange of the two serine residues (Ser418 in GDF9 and Ser356 in BMP15) with cysteines locates both sulphur atoms sufficiently close to form a thio-ether bond without requiring conformational or large structural rearrangements of the dimer architecture.

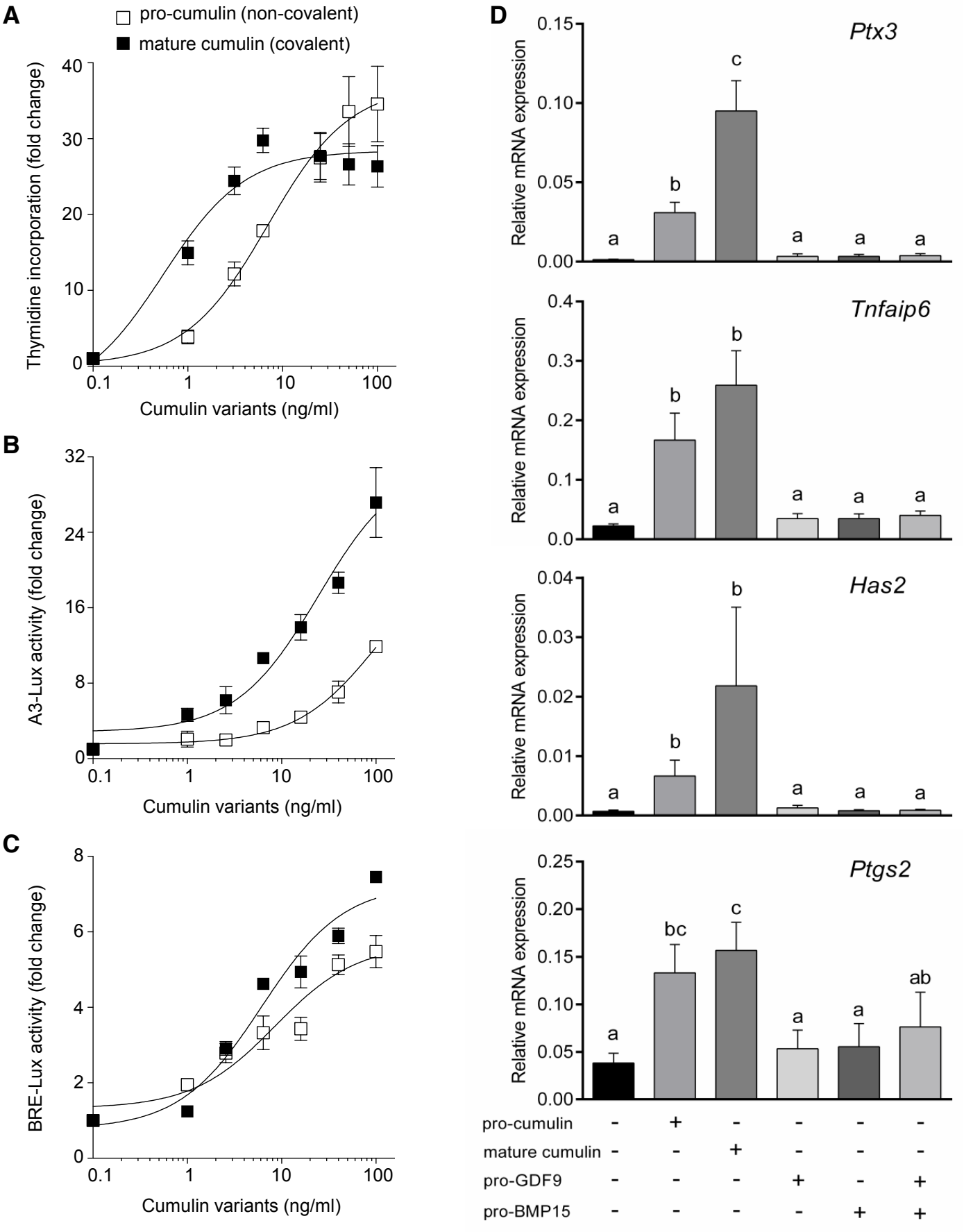

control
pro-hBMP15
pro-hGDF9
pro-mGDF9
pro-hBMP15 + pro-hGDF9
pro-hBMP15 + pro-mGDF9
^{3}H-thymidine incorporation (fold change)
40
30
20
10
0
6.2
12.5
25
Dose of BMP15/GDF9 (ng/ml)

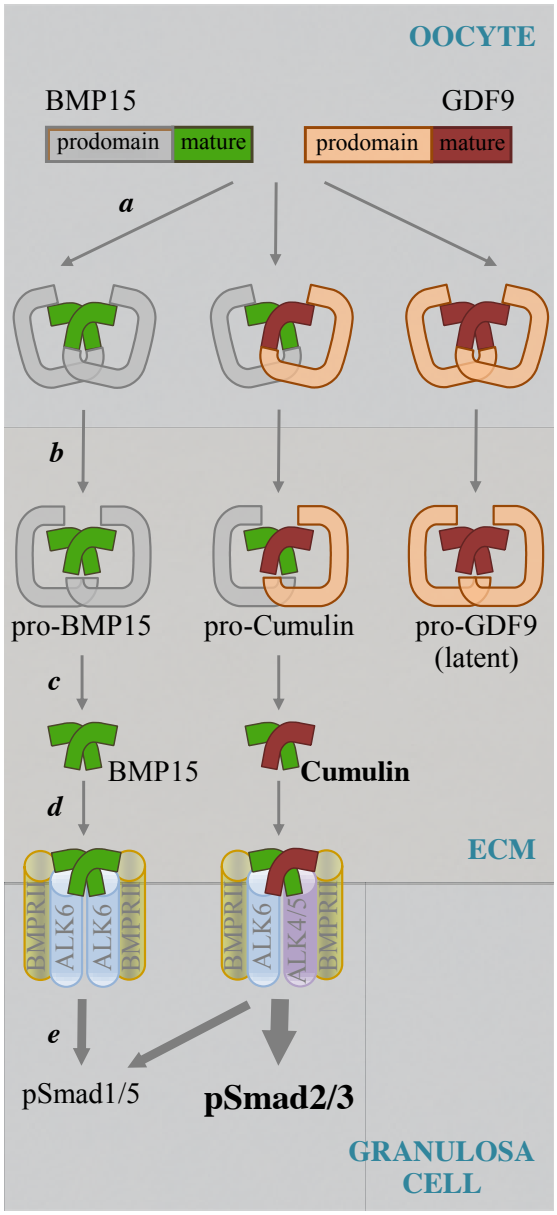

A
GDF9
BMP15
BMP15S:C
GDF9 + BMP15
GDF9 + BMP15S:C
Thymidine incorporation (fold change)
GDF9/BMP15 variants (ng/ml)
B
A3-Lux activity (fold change)
GDF9/BMP15 variants (ng/ml)
C
BRE-Lux activity (fold change)
GDF9/BMP15 variants (ng/ml)

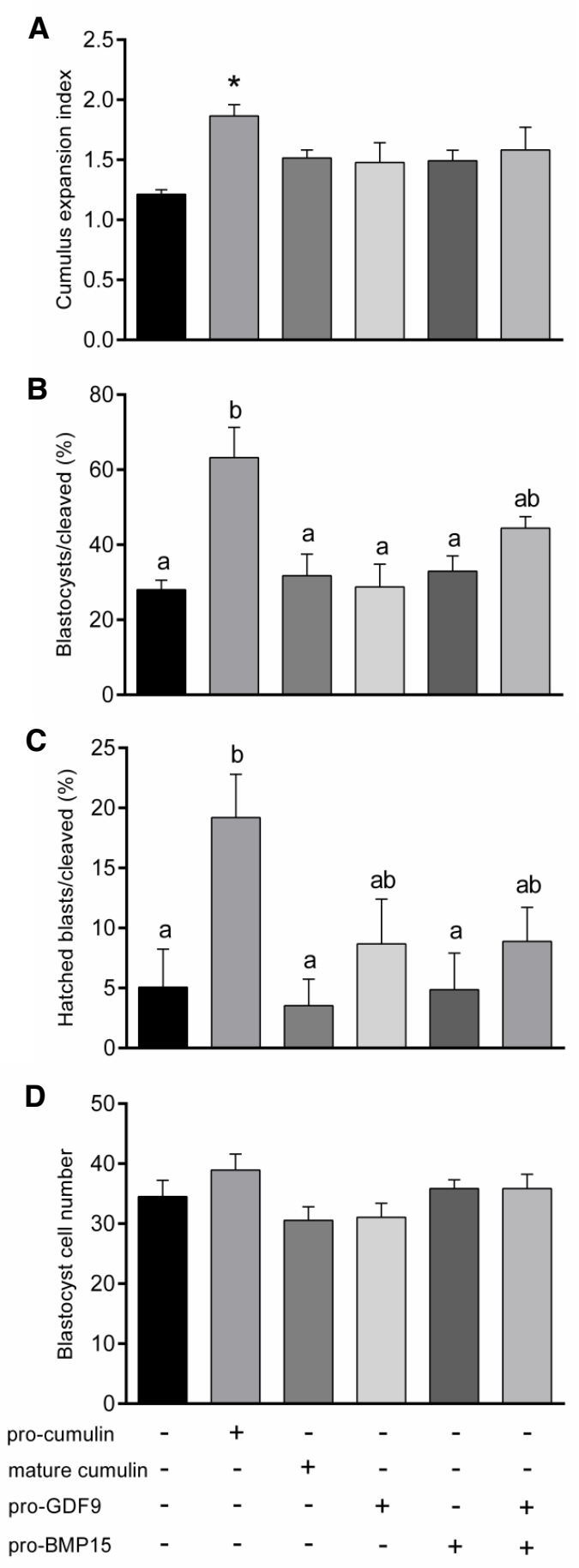

A
IMAC
Inputs
pro-BMP15
+pro-GDF9
pro-BMP15
+pro-GDF9
Flow through
Wash
Eluate
50-
20-
BMP15
MAb
GDF9
MAb
B
IMAC
Input
pro-GDF9
Flow through
Wash
Eluate
50-
20-
GDF9
MAb

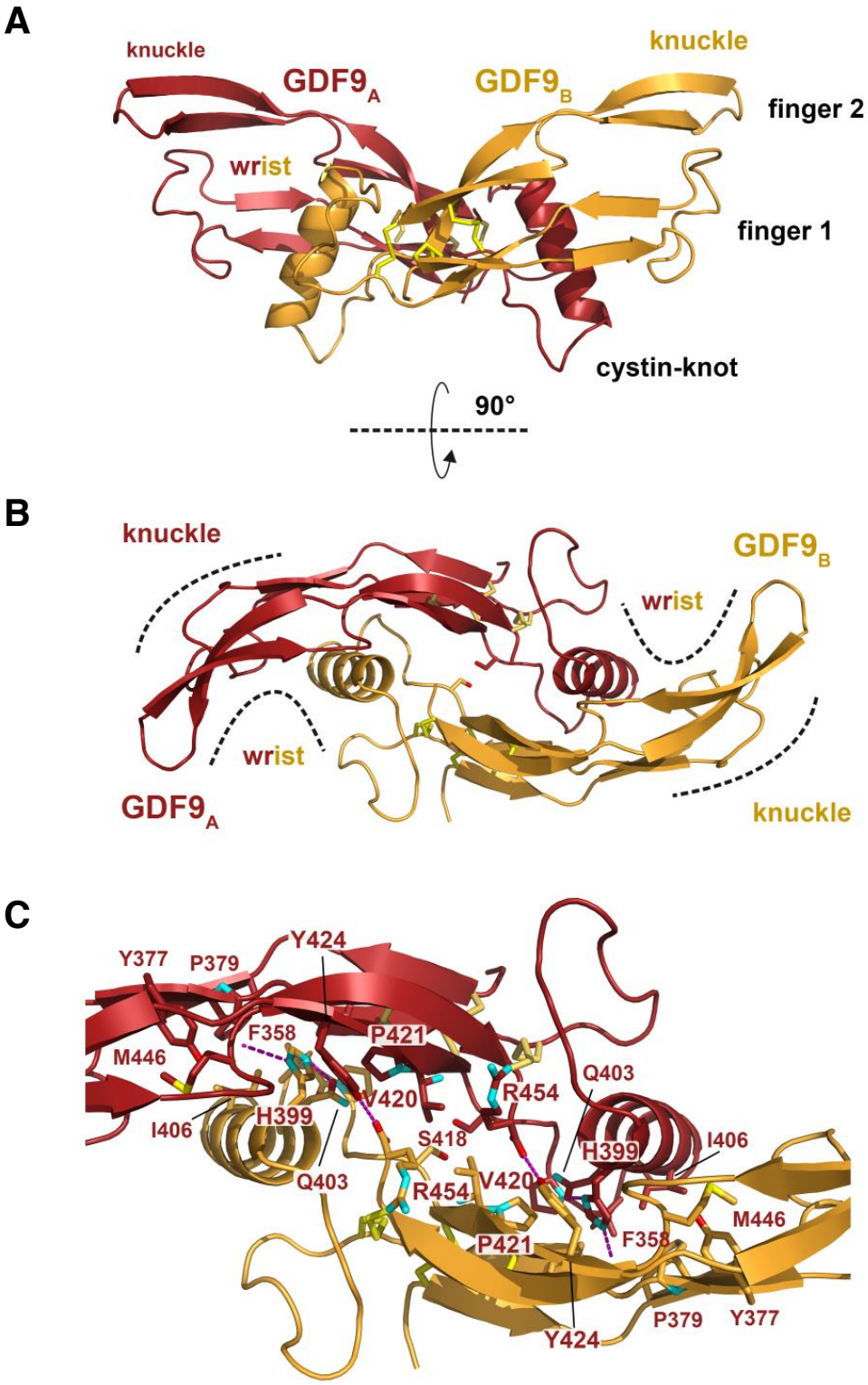

A
pro-GDF9
pro-BMP15
pro-GDF9 + pro-BMP15
pro-cumulin
Thymidine incorporation (fold change)
40
30
20
10
0
1
10
100
pro-GDF9/pro-BMP15 variants (ng/ml)
B
A3-Lux activity (fold change)
12
9
6
3
0
1
10
100
pro-GDF9/pro-BMP15 variants (ng/ml)
C
BRE-Lux activity (fold change)
8
6
4
2
0
1
10
100
pro-GDF9/pro-BMP15 variants (ng/ml)

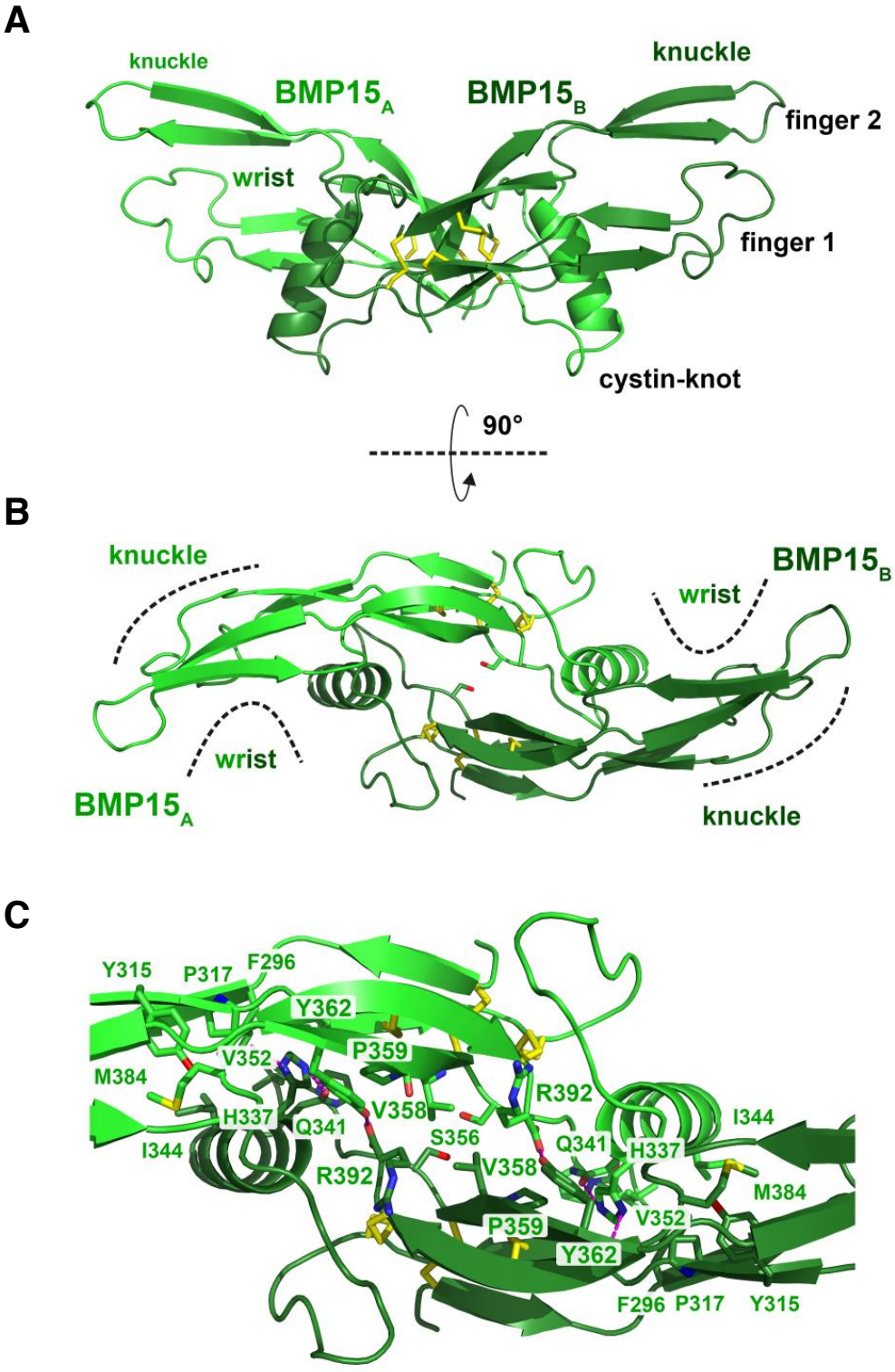

A
0.02
0.01
0
Absorbance (280nm)
Cumulin
BMP15
GDF9
29.882
31.108
31.721
34.833
39.404
30
35
40
Fraction #
B
kDa
31 32 33 34 35 36 37 38 39
20-
GDF9 MAb
20-
BMP15 MAb
C
31 32 33 34 35 36 37 38 39
25-
GDF9
BMP15
D
31 32 33 34 35 36 37 38 39
GDF9
25-
BMP15
Cumulin
E
Thymidine incorporation (fold change)
50
40
30
20
10
0
Con 31 32 33 34 35 36 37 38 39
HPLC Fraction #


A
pro-cumulin (non-covalent)
mature cumulin (covalent)
Thymidine incorporation (fold change)
Cumulin variants (ng/ml)
B
A3-Lux activity (fold change)
Cumulin variants (ng/ml)
C
BRE-Lux activity (fold change)
Cumulin variants (ng/ml)
D
Ptx3
Tnfaip6
Has2
Ptgs2
Relative mRNA expression
pro-cumulin
mature cumulin
pro-GDF9
pro-BMP15


OOCYTE
BMP15
prodomain
mature
GDF9
prodomain
mature
a
b
pro-BMP15
pro-Cumulin
pro-GDF9
(latent)
c
BMP15
Cumulin
d
ECM
BMPRII
ALK6
ALK6
BMPRII
BMPRII
ALK6
ALK4/5
BMPRII
e
pSmad1/5
pSmad2/3
GRANULOSA
CELL


A
Cumulus expansion index
2.5
2.0
1.5
1.0
0.5
0.0
*
B
Blastocysts/cleaved (%)
80
60
40
20
0
a
b
a
a
a
ab
C
Hatched blasts/cleaved (%)
25
20
15
10
5
0
a
b
a
ab
a
ab
D
Blastocyst cell number
50
40
30
20
10
0
pro-cumulin - + - - - -
mature cumulin - - + - - -
pro-GDF9 - - - + - +
pro-BMP15 - - - - + +


A
knuckle
GDF9A
GDF9B
knuckle
finger 2
wrist
finger 1
cystin-knot
90°
B
knuckle
GDF9B
wrist
wrist
GDF9A
knuckle
C
Y377
P379
Y424
F358
P421
M446
Q403
R454
V420
H399
I406
S418
Q403
R454
V420
H399
I406
M446
P421
F358
P379
Y377
Y424


A
knuckle
BMP15A
BMP15B
knuckle
finger 2
wrist
finger 1
cystin-knot
90°
B
knuckle
BMP15B
wrist
wrist
BMP15A
knuckle
C
Y315
P317
F296
Y362
P359
V352
M384
R392
H337
Q341
V358
I344
S356
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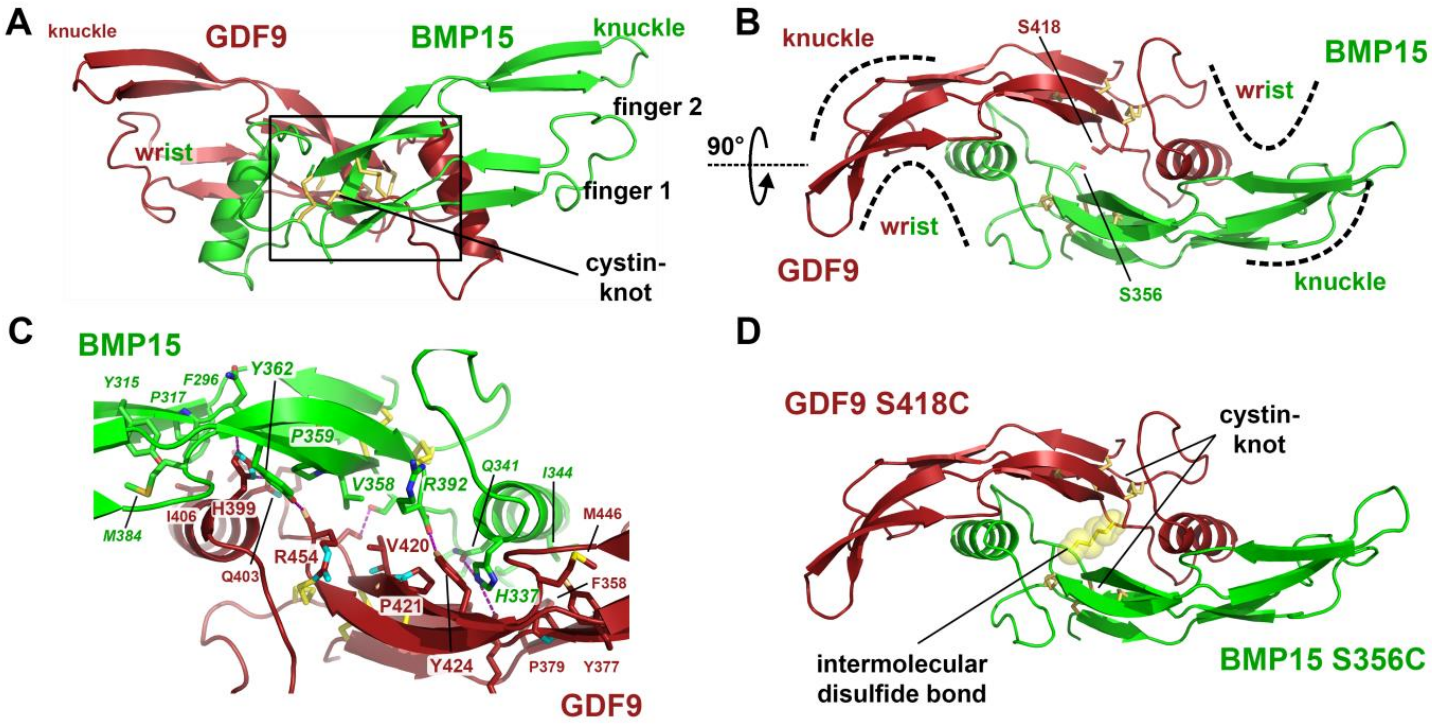
H337
I344
R392
V358
M384
P359
V352
Y362
F296
P317
Y315


A
knuckle
GDF9
BMP15
knuckle
finger 2
wrist
finger 1
cystin-
knot
B
knuckle
S418
BMP15
wrist
90°
wrist
GDF9
S356
knuckle
C
BMP15
Y315
F296
Y362
P317
P359
V358
R392
Q341
I344
I406
H399
M446
M384
V420
R454
Q403
F358
H337
P421
Y424
P379
Y377
GDF9
D
GDF9 S418C
cystin-
knot
intermolecular
disulfide bond
BMP15 S356C