Fundamental role of BMP15 in human ovarian folliculogenesis revealed by null and missense mutations associated with primary ovarian insufficiency

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ABSTRACT

BMP15 encodes an oocyte factor with a relevant role for folliculogenesis as homodimer or cumulin heterodimer (BMP15-GDF9). Heterozygous BMP15 variants in the precursor or mature peptide had been associated with primary ovarian insufficiency (POI), but the underlying mechanism remains elusive and a double dose of BMP15 was suggested to be required for adequate ovarian reserve. We uncovered two homozygous BMP15 null variants found in two girls with POI and primary amenorrhea. Both heterozygous mothers reported physiological menopause. We then performed western blot, immunofluorescence and reporter assays to investigate how previously reported missense variants, p.Y235C and p.R329C, located in the precursor or mature domains of BMP15, may affect protein function. The p.R329C variant demonstrates an impaired colocalization with GDF9 at confocal images and diminished activation of the SMAD pathways at western blot and reporter assays in COV434 follicular cell line. In conclusion, BMP15 null mutations cause POI only in the homozygous state, thus discarding the possibility that isolated BMP15 haploinsufficiency can cause evident ovarian defects, Alternatively, heterozygous BMP15 missense variants may affect ovarian function by interfering with cumulin activity. Our data definitely supports the fundamental role of BMP15 in human ovarian folliculogenesis.

Key words

BMP15 – GDF9 – cumulin – Primary Ovarian Insufficiency – Ovarian Dysgenesis – Ovarian folliculogenesis

Introduction

BMP15 (MIM# 300247) was indicated as the first ovarian determining gene on the X chromosome (Layman, 2006). It encodes a protein known to act as homo- or hetero-dimer, together with the product of *GDF9* (MIM# 601919) gene. The Bone morphogenetic protein 15 (BMP15) and the Growth/differentiation factor 9 (GDF9) are members of the Transforming growth factor beta (TGF-β) superfamily and factors of oocyte origin playing apparently variable fundamental roles in the ovary of mono- or poly-ovulating species (Persani, 2014). Like all TGFβ-like ligands, BMP15 and GDF9 are synthetized as large pre-proproteins composed of a signal peptide, a pro-region that directs their dimerization (Hashimoto, 2005; McIntosh, 2008) and a mature domain (Shimasaki, 2004). As processing proceeds, the dimeric proproteins are cleaved at RXXR site by furin-like proteases to generate the dimeric mature proteins, then secreted in the follicular fluid (Simpson, 2012; Al-Musawi, 2013). BMP15 and GDF9 are part of the few TGFβ members with the fourth cysteine of the "cystine knot" domain, which is involved in the formation of intersubunit disulfide bond, substituted by a serine (McDonald and Hendrickson, 1993; McPherron and Lee, 1993). Nevertheless, they are known to be able to form noncovalently linked homodimers (Liao, 2003), as well as noncovalently linked heterodimer, which was named cumulin (Mottershead, 2015), able to exhibit higher potent bioactivity on granulosa cells (GCs) (Mottershead, 2012; Peng, 2013). The action of BMP15 homodimer and/or BMP15-GDF9 heterodimer include: i) the promotion of early follicle growth and maturation, closely linked to GCs proliferation, in rats, humans and ruminants; ii) the regulation of follicular GCs sensitivity to the Follicle-Stimulating Hormone (FSH) action in rats and sheeps and the determination of ovulation quota in sheeps and mice; iii) the prevention of GCs apoptosis and iv) the promotion of oocyte developmental competence in ruminants (for review, Persani, 2014). As stated earlier, BMP15 and GDF9 influence fundamental female reproductive processes. In Humans, non-synonymous variants in the *BMP15* and *GDF9* genes have been associated with different ovarian phenotypes, such as Primary Ovarian Insufficiency (POI; MIM# 300510) (Di Pasquale, 2004 and 2006; Dixit, 2006; Laissue, 2006; Rossetti, 2009; Tiotiu, 2010; Wang, 2010 and 2013; Zhang, 2018), dizygotic twinning (MIM# 276400) (Montgomery, 2004; Palmer, 2006; Zhao, 2008), polycystic ovary syndrome (PCOS; MIM# 184700) (Wang, 2010; Liu, 2011; Mehdizadeh, 2016) and ovarian hyperstimulation syndrome (OHSS; MIM# 608115) (Morón, 2006; Hanevik, 2011). POI represents one major cause of female infertility before 40 years of age. The premature loss of

ovarian function may be due to a wide range of pathogenic causes, among which the genetic component seems to play a considerable role (Rossetti, 2017). *BMP15* and *GDF9* gene variants were found associated with significant prevalence with POI phenotype, respectively 1.5-12% for *BMP15* (Persani, 2014) and 1-4% for *GDF9* (Qin, 2015). Recently, this calculation has been confirmed with all nonsynonymous *BMP15* variants identified by March 2017 (Belli and Shimasaki, 2018). Since the first identified human BMP15 p.Y235C mutation associated with hypergonadotropic ovarian failure and 46,XX gonadal dysgenesis (Di Pasquale, 2004), other 25 POI-related variants have been reported (Zhang, 2018; Belli and Shimasaki, 2018), mostly at the heterozygous state and located in the pro-region of the protein. So far, a few variants have been functionally tested showing either effects of negative dominance (Di Pasquale, 2004) or haploinsufficiency, due to altered protein processing and impaired bioactivity on target GCs (Rossetti, 2009; Inagaki and Shimasaki, 2010). However, the degree of functional impairment and the molecular mechanisms underlying most of the identified variants remain largely unexplained. Recently, novel *BMP15* deletions have been identified in compound heterozygosity in a family with two sisters affected with POI, yielding a complete lack of mature BMP15 and representing the first example of a human "knockout-like" effect. Notably, the mother was heterozygous for only one of these mutations and had a normal fertility. Therefore, this family did not support previous reports of *BMP15* haploinsufficiency and gene dosage in humans (Mayer, 2017). Here we obtain insights on these open questions by: a) the description of two *BMP15* null homozygous variations in two girls with POI and primary amenorrhea confirming that *BMP15* haploinsufficiency is not sufficient to cause POI; b) comprehensive molecular studies on previously described variants in the precursor or mature BMP15 peptide (Wang, 2010), suggesting that heterozygous missense variants can predispose to POI by interfering with the cumulin activity. These data give further support to the fundamental role of BMP15 in human ovarian folliculogenesis.

Materials and Methods

Patients

Patient A (Fig. 1A, III.1) entered spontaneous menarche at 14 years of age but amenorrhea occurred few months later. She presented at 15 years with hypergonadotropic secondary amenorrhea (FSH; 63.2-73.1 U/L;

LH: 28.1 U/L), low estradiol levels (14.84 pg/ml) and a normal 46,XX karyotype. Prolactin and TSH levels were normal (respectively: 8.4 µg/L and 3.0 mU/L). Both ovaries were small (left: 26x10x16mm; right: 25x7x16mm) and without follicle on pelvic ultrasound. *FMR1* analysis revealed a normal CGG trinucleotide expansion. Parents are first-degree cousins. The mother (Fig. 1A, II.2) entered menarche at 15 years of age, reported normal pubertal development, and entered physiological menopause at 56 years of age. Proband's sister (Fig. 1A, III.2) never presented spontaneous menses and menarche was induced at 19 years of age. The hormone levels were similar to those of her sister. Unfortunately, the genomic DNAs (gDNA) of both mother and sister of the proband were not available for genetic analysis.

Patient B (Fig. 1C, III.5) was included in a recent study (Bestetti, 2019). She presented at 16 years old with hypergonadotropic primary amenorrhea (FSH: 141-135 U/L; LH: 43.0 U/L) and hypoestrogenism (17betaE2 27 pg/ml), while prolactin and TSH levels were normal (respectively: 12.2 µg/L and 1.4 mU/L). Both ovaries were small (left: 17x6mm; right: 19x10mm) and without follicle on pelvic ultrasound. Additionally, this patient presented glucose-6-phosphate dehydrogenase deficiency (locus Xq28) but no *FMR1* premutation. Karyotype was 46,XX. Likewise to patient A, parents (Fig. 1C, II.6 and II.7) were first-degree cousins and the mother (Fig. 1C, II.7) entered physiological menopause at 50 years of age.

Ethical approval

Written informed consent for blood sampling and genetic investigations, approved by the Ethical Clinical Research Committees of IRCCS Istituto Auxologico Italiano, was obtained from patients and families.

Next Generation Sequencing

Genomic DNA (gDNA) was isolated from leukocytes of peripheral blood by using automatic DNA extractor Tecan Freedom Evo (Tecan Group Ltd, Männedorf, Switzerland). Mutations were sought in the coding exons and flanking splice sites of five POI candidate genes (*BMP15*, *GDF9*, *NOBOX*, *FSHR*, *NR5A1*). Primers were designed using DesignStudio software (Illumina, San Diego, CA, USA). Library preparation was performed according to the manufacturer protocol using 50 ng of gDNA. Samples were prepared for sequencing using the TruSeq Custom Amplicon Library Preparation Kit (Illumina). Amplicon libraries were sequenced on Illumina MiSeq system. The MiSeq Reagent Kits v2 was used for all sequencing reactions.

After sequencing, reads were automatically aligned to the human Genome Assembly hg19 (GRCh37, UCSC Genome Browser) using the MiSeq Reporter software and visualized using the Amplicon Viewer software (both from Illumina). Variants with Minor Allele Frequency >0.01 were filtered out and IGV software was used for the real-time visualization and annotation of single nucleotide variants (SNVs) and small insertions/deletions (indels) (Robinson, 2011; Thorvaldsdóttir, 2013). The identified mutation was confirmed using Sanger sequencing.

Array-CGH

Patient B's gDNA was processed by 400K Human Genome CGH Microarray, spanning approximately 411,056 probes with an average spatial resolution of 5.3 kb (4.6 kb in RefSeq genes), as recently reported (Bestetti, 2019).

Real-Time qPCR

For copy number quantification using real-time quantitative PCR, 20 ng of gDNA from Patient B and her parents were used as target templates for the TaqMan Copy Number Assay (Thermo Fisher Scientific, Waltham, MA, USA), following the protocol previously described (Castronovo, 2014). A 46,XX gDNA known to have two copies of the target sequences was chosen as calibrator whereas a 46,XY gDNA was chosen as internal control with one copy of the target sequences. Four replicates for each sample were analyzed twice. Reactions were run on a 7900HT Real-Time PCR instrument (Thermo Fisher Scientific), SDS software 2.4, performing the Absolute Quantitation method.

Constructs

The NM_005448.2(BMP15_v001):c.462del variant (from Patient A) has been introduced by site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis kit (Agilent Technologies) and a specific pair of primers (Supp. Table S1) into the pcDNA4 vector (Thermo Fisher Scientific) containing a full-length human *BMP15* wt cDNA (NM_005448.2) in frame with a myc-histidine tag at C-term, thus leading to the pcDNA4-BMP15-W155Gfs*22 construct, which expresses a prematurely truncated form of BMP15 within the proregion domain. Similarly, the missense mutant NM_005448.2(BMP15_i001):p.R329C was obtained by site-directed mutagenesis of the wt pcDNA4-BMP15 construct with the

NM_005448.2(BMP15_v001):c.985C>T variant, as control (Supp. Table S1). As further control, we used the pcDNA4-BMP15-Y235C vector expressing the NM_005448.2(BMP15_i001):p.Y235C variant (NM_005448.2(BMP15_v001):c.704A>G), already described (Di Pasquale, 2004). The analysis of SMAD2 pathway activation was obtained by the cotransfection of pCS2 constructs for *BMP15* (wt or variants) with the wt pCS2-GDF9, after subcloning the corresponding pcDNA4 vectors with *ClaI* and *XbaI* restriction enzymes (New England Biolabs, Ipswich, MA, USA) (Rossetti, 2009). For the reporter assay, it was used the pCS2-BMP15-R329C construct.

The coding sequence of human *GDF9* (NM_005260) was also cloned into the vector pCDNA3.1 with V5-histidine tag at C-term by using *HindIII* and *XhoI* FastDigest restriction enzymes (all by Thermo Fisher Scientific). The eGFP-tagged plasmid containing the cDNA of human *BMPR1b* (NM_001203) was obtained by cloning the vector peGFP-N1 (Takara Bio USA, Mountain View, CA, USA) by using *EcoRI* and *BamHI* enzymes (New England Biolabs).

All the plasmid sequences were verified by means of direct sequencing to exclude unwanted substitutions.

Cell cultures and transfection

Both HEK293T and COV434 cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) with Glutamax (Thermo Fisher Scientific) supplemented with 1% penicillin-streptomycin and 10% foetal bovine serum (all from Sigma Aldrich, Saint Louis, MO, USA) at 37°C and 5% CO_2, and transiently transfected using Fugene HD (Promega, Madison, WI, USA) according to the manufacturer's protocol in either multiwell-6 for immunoblotting or in multiwell-12 for immunofluorescence microscopy.

Treatments

The broad-spectrum Matrix MetalloProteinase (MMP) inhibitor GM6001 (Merck, Darmstadt, Germany), or Ilomastat, was used at the final concentration of 20 μM, as previously reported (Panigone, 2008), and was added to HEK293T cells respectively 5 hours, 24 hours or 30 hours post-transfection in Optimem + Glutamax (Thermo Fisher Scientific) with 1% FBS. Culture media from treated and untreated cells from the three time points were finally recovered 48 hours post-transfection for purification and subsequent immunoblot.

Antibodies

Commercial primary antibodies were mouse monoclonal anti-myc (Thermo Fisher Scientific), rabbit monoclonal anti-Phospho-Smad2 (Ser465/467) (Cell Signaling Technology, Leiden, The Netherlands), mouse monoclonal anti-Smad2/3 (BD Biosciences, Franklin Lakes, New Jersey, U.S.A.), anti-actin (BD Biosciences) and rabbit polyclonal anti-V5 (Cell Signaling Technology, Danvers, MA, U.S.A.). For immunofluorescence experiments, double stainings were carried out with 555/488-tagged mouse or rabbit secondary antibodies (Thermo Fisher Scientific). Horseradish Peroxidase-conjugated secondary antibodies were purchased from Jackson Laboratories (Jackson ImmunoResearch Europe, Cambridge, UK).

Immunoblotting

Cultured HEK293T cells were lysed 48 hours post transfection in RIPA buffer (Radio-Immune Precipitation Assay: 50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 1% NP-40, 0.5% Sodium Deoxycholic acid, 0.1% sodium dodecylsulfate) with protease inhibitor cocktail (Roche, Basilea, Switzerland). Besides, culture media were recovered from the same wells and processed for the purification of recombinant human BMP15 proteins wt or mutated by using the Dynabeads His-Tag Isolation & Pulldown kit (Thermo Fisher Scientific). Eluted samples were further precipitated with 4 volumes of Acetone (Sigma Aldrich) and then solubilized in sodium dodecyl sulphate denaturation buffer. Cultured COV434 cells were lysed 48 hours post-transfection in RIPA buffer with protease inhibitor cocktail and phosphatase inhibitor cocktail 3 (Sigma Aldrich). In each representative blots, equal amounts of extracted proteins were separated on NuPage 4–12% BIS-TRIS gels (Thermo Fisher Scientific) and transferred onto nitrocellulose membranes (Thermo Fisher Scientific). Signals were visualized by means of ECL Westar Supernova (Cyanagen, Bologna, Italy) after incubation with primary and secondary antibodies. Densitometry analysis was performed by using the NIH ImageJ software.

Immunofluorescence microscopy

Fortyeight hours after transfection, HEK293T cells were fixed for 20 min in 4% paraformaldehyde (Santa Cruz Biotechnology, Dallas, TX, USA) and immunostained in either permeabilizing or non-permeabilizing conditions, as follows: a) Permeabilizing conditions. Fixed cells were permeabilized in GDB solution (0.1%

gelatine, 0.3% Triton-X100, 20 mM PO_4 buffer pH 7.4, 0.45 mM NaCl). Primary and secondary antibodies were diluted in GDB. b) Non-Permeabilizing conditions. After blocking for 1 hour with 5% goat serum (Thermo Fisher Scientific) in PBS, cells were incubated with primary and secondary antibodies diluted in PBS + 1% BSA (Bovine Serum albumin, Sigma Aldrich).

After immunostaining, cells were mounted using ProLong Gold Antifade reagent (Thermo Fischer Scientific) containing DAPI for nuclei staining. Images were acquired using a Nikon EclipseTi-E inverted microscope with IMA10X Argon-ion laser System by Melles Griot (Nikon, Tokyo, Japan); all images were acquired with CFI Plan Apo VC 60X Oil (Nikon), with a 2.1X digital magnification.

Image Analysis

The black and white magnifications in Fig. 7A were obtained by inverting the signal of the red channel (boxed areas only) using Adobe Photoshop (Adobe Systems, San Jose, CA, USA). The color-coded surface plots from the same areas were obtained using the NIH ImageJ software plugin 'Interactive 3D Surface Plot' using the 'Fire LUT' option; the scale on the right indicates fluorescence intensities expressed in Relative Light Units (RLUs). The Plot Profile graphs in Fig. 5B, Fig. 6B and Supp. Figure S4 were obtained using ImageJ plot profile.

The degree of extracellular BMP15 clusterization in transfected cells was quantified on confocal images as follows: cell outlines (deduced from the GFP-BMPR1b signal) were drawn using ImageJ 'segmented line' tool (line width= 5 pixels). For the BMP15 signal alone, a plot profile (similar to the ones presented in Supp. Figure S4) was generated for each cell perimeter. To determine the 'Number of peaks per cell', numerical data used to generate the plot profiles (corresponding to the florescence intensity of each pixel of the cell perimeter, expressed in RLUs) were exported in Microsoft Office Excel and manually analysed. A string of numbers higher than 5 RLUs and separated from the other strings by at least 5 black pixels (RLU=0) was defined as a single peak. The 'Mean peak's length' was inferred from the length of the numerical strings. At least 45 cells from 3 independent experiments were quantified for each transfectant.

The percentage of colocalization between BMP15 and GDF9 in permeabilizing conditions was obtained by calculating the Manders' colocalization coefficient (MCC) using ImageJ plugin JACoP (Bolte and

Cordelières, 2006) in at least 15 cells for each transfectant. Similarly, the percentage of colocalization between each BMP15 variant and GDF9 along the cell membrane was obtained by calculating the MCC using ImageJ plugin JACoP (Bolte and Cordelières, 2006). Thresholds were calculated automatically by the plugin.

Reporter assay

The bioactivity of NM_005448.2(BMP15_i001):p.R329C variant was performed as previously described (Rossetti, 2009) by using the Dual Luciferase reporter Assay kit (Promega) and measuring the emitted luminescence in relative light units (RLU).

Statistical analysis

Real-Time qPCR: data files containing four replicates per sample CT values for each reporter dye were imported into the Copy Caller software analysis tool (Thermo Fisher Scientific) to calculate sample copy number values by relative quantification, using the comparative CT (ΔΔCT) method. The method measured the CT difference (ΔCT) between target and reference sequences and then compared the ΔCT values of each sample to the ΔCT of calibrator sample (a *BMP15* wt 46,XX female), known to have two copies of the target sequence. The assay was repeated twice. Results are indicated as median, specifying the range of max and min values obtained.

Image Analysis of immunofluorescence microscopy: all quantitative data are presented as mean ± s.e.m. The box and whiskers plot in Fig. 5C summarizes the data of three independent experiments, and the Tukey method was applied to identify outlier values. Multiple comparisons among groups were carried out with One-way Anova using Prism software (GraphPad Prism software, La Jolla, CA, USA), *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Immunoblot densitometry: the immunoblots of P-SMAD2 and SMAD2 were performed in duplicate, a representative blot is shown in Fig. 7A (left). The densitometry analysis was carried out with One-way Anova using Prism software, *$p<0.05$ (right).

Reporter assay: the bioassay was performed four times in triplicate wells. All values represent the mean ± s.e.m. A Student's t-test was used for statistical evaluation and a P value <0.05 was considered statistically significant, **p<0.01.

Results

Identification of two homozygous *BMP15* variants

Genomic DNAs extracted from lymphocytes of peripheral blood samples from Patient A and B were analyzed by Next Generation Sequencing (NGS) for 5 candidate genes known for having a role in the pathogenesis of POI: *BMP15*, *GDF9*, *FSHR*, *NOBOX*, *NR5A1*, we routinely screen for diagnostic purpose in patients with idiopathic POI. The NGS analysis of Patient A revealed the c.462del variant in the *BMP15* gene in homozygosis (Fig. 1A, III.1), further validated by Sanger sequencing (Fig. 1B, left). No signal of *BMP15* probes were instead detected by NGS in Patient B (Fig. 1C, III.5; Fig. 2A), nor by direct sequencing, suggesting a biallelic deletion of this gene. The subsequent evaluation by high-resolution array-CGH (aCGH) analysis confirmed the homozygous deletion of 7 spots spanning 35.6 kb in Xp11.22 (Fig. 2B), consistent with the biallelic deletion of *BMP15* in Patient B. Any other rare copy number variant in Patient B's whole genome has been excluded (Bestetti, 2019). Patient B and her parents were further investigated for *BMP15* copy number by real-time quantitative PCR (Fig. 2C). As expected, no copy of *BMP15* was detected in both the proband and her father (Fig. 1C, II.6; Fig. 2C) whereas her mother (Fig. 1C, II.7; Fig. 2C) showed one *BMP15* allele, resulted wild-type (wt) at direct sequencing. No variants were identified in the other screened candidates for both patients.

The c.462del variant generates an aberrant precursor

The c.462del variant causes a frameshift of *BMP15* coding sequence leading to a premature stop codon at position 177 in the pro-region which ultimately results in a prematurely truncated aberrant precursor (here and thereafter named p.W155Gfs*22) of approximately 31KDa, as demonstrated by the immunoblot assay on total cell lysates obtained by HEK293T cells transfected with myc/his-tagged constructs (Fig. 3A). We included in the study two previously reported variants. One variant is p.R329C, located in the mature

peptide, which was reported in a Chinese patient with POI and secondary amenorrhea, but no further molecular or functional characterizations have been performed yet (Wang, 2010). The other variant is p.Y235C, the first described BMP15 variant, which is associated with POI and 46,XX gonadal dysgenesis (Di Pasquale, 2004). Particularly, the p.Y235 was characterized as a residue under positive evolutive selection, thus suggesting its key importance in the function of the protein (Auclair, 2013). In the immunoblot of cell lysates with the monoclonal anti-myc antibody, the p.R329C variant shows a precursor band comparable to the wt and a faint band corresponding to the mature peptide, in contrast to the profile expression pattern of wt and the other variant p.Y235C (Fig. 3A). As expected, the secretion profile of recombinant human BMP15 proteins myc/his tagged, which were purified by cell culture media from the same transfections, confirmed the absence of the mature protein of p.W155Gfs*22 variant (Fig. 3B). Moreover, we excluded translation re-initiation downstream of the premature stop codon by transfecting HEK293T cells with the pcDNA-BMP15-462del mutant construct, where the nonsense codon generated by the single-base deletion was in frame with the myc-tag (Supp. Figure S1). Taking into account the two additional bands 1 and 2 (respectively at approximately 20 and 10 KDa) of possible degradation of the mature peptide, we noticed that the p.R329C lacks the second lower band (band 2 in Fig. 3B and 4), differently from wt and the p.Y235C variant.

The p.R329C variant shows a peculiar processing

In Fig. 4, we then evaluated the time course of production of p.R329C protein. At 24 hours post-transfection (pt), it is visible the precursor at 55 KDa, similarly to wt, and it appears a faint upper band of approximately 70 KDa of uncertain origin (band 3). This band results more accentuated at 30 hours pt and completely disappears 48 hours pt. Band 3 is completely absent in the wt immunoblot. In contrast, band 2 never appears in presence of the variant at any time points. To further investigate if this band could be the result of the activity of a metalloproteinase of membrane (MMP), which it is known can modulate many bioactive molecules at the cell surface (Stefanidakis and Koivunen, 2006), and if the presence of p.R329C variant could eventually abolish the MMP activity, we treated HEK293T cells expressing wt or BMP15 p.R329C with GM6001, a broad-spectrum MMP inhibitor, at different time points (added at 5, 24 and 30 hours pt). However, as shown in Supp. Figure S2, no difference between treated and untreated cells was revealed. We

cannot exclude that the protein degradation might still occur by other MMPs (not sensitive to GM6001), we have not tested here, or directly by the furin protease itself, which is able to translocate from trans-Golgi network to the cell membrane and here cut protein precursors (Molloy, 1994).

All BMP15 variants are able to bind the external surface of cell membrane to different extents

Given the differences in the extent of BMP15 variants secretion, we assessed whether the morphology of the organelles belonging to the secretion pathway might be macroscopically altered. For this purpose, 48 hours pt with either wt or mutant V5-tagged *BMP15* constructs, HEK293T cells were processed for immunofluorescence staining under permeabilizing conditions. As shown in Fig. 5A, no macroscopical changes are visible in the organelles of the secretory pathway. Contrary to the p.W155G*fs22 variant, the wt and variants p.R329C and p.Y235C showed additional enrichments spots at the cell periphery (see boxed areas and their digitally modified magnifications in Fig. 5A). Such spots were localized at the plasma membrane (PM), as demonstrated by cotransfection with GFP-BMPR1b, which we used as a PM marker (Supp. Figure S3, see boxed areas and their magnifications). To discriminate if such spots were either i) BMP15-filled secretion vescicles docked at the intracellular side of the PM or ii) secreted BMP15 molecules bound on the extracellular face of the PM, we performed immunofluorescence stainings in HEK293T cells under non-permeabilizing conditions (Fig. 5B). The construct encoding for GFP-BMPR1b was again cotranfected with either wt or mutant *BMP15* as a PM marker. As shown by the representative images in Fig. 5B, the punctuate profile of wt, p.R329C and p.Y235C was still evident under non-permeabilizing conditions (see areas indicated by arrows, their magnifications and plot profiles). Surprisingly, the p.W155G*fs22 variant also showed an extracellular signal, which was however diffusely distributed along the PM (see area indicated by arrow, its magnification and plot profile). The different distribution of the wt and p.W155G*fs22 variant along the PM is particularly evident when comparing the plot profiles generated from the confocal images of two representative cells, as shown in Supp. Figure S4. To better characterize the behavior of all the variants at the level of PM, additional plot profiles were generated and analyzed to determine the number of peaks per cells and their relative mean lengths. As demonstrated by the graphs in Fig. 5C, the reduced number of peaks per cell observed in the p.W155G*fs22 variant compared to the other

transfectants inversely correlates with an increased peak's length, collectively suggesting a decreased ability of this variant to clusterize.

BMP15 variants do not impair GDF9 trafficking and secretion

Given the importance of the BMP15-GDF9 heterodimer (cumulin) in mono-ovulatory species (Monestier, 2014), we sought to analyze whether any of the BMP15 variants under study could affect GDF9 trafficking and secretion in cotransfected HEK293T cells. Immunofluorescence experiments under permeabilizing conditions conducted 48 hours pt showed that GDF9 protein is evenly distributed along the organelles of the secretory pathway without any morphological changes, regardless of the cotransfected BMP15 variant (Fig. 6A). Indeed, the percentage of colocalization between any BMP15 variant and GDF9 along the secretory pathway was around 62-65%, calculated with ImageJ plugin JACoP.

Lastly, immunofluorescence stainings under non-permeabilizing conditions in BMP15 and GDF9 cotransfected HEK293T cells were performed to evaluate if GDF9 could: i) be secreted and ii) colocalize with BMP15 along the extracellular side of the PM. GDF9 could indeed be secreted and localize in a punctuated manner along the extracellular side of the PM, albeit with a substantial lower intensity of the GDF9-expressing cells found on the coverslip (see the magnified green channels in Fig. 6B), regardless of the cotransfected BMP15 variant. Nonetheless, for all BMP15 variants, except the frameshift, it was possible to assess an overlap, at least partial, with GDF9, by calculating the levels of colocalization (see the percentages in magnifications and the red and green peaks of the representative plot profiles in Fig. 6B). Notably, the p.W155G*fs22 variant maintained its diffusely distributed signal along the PM, even in the presence of clusters of GDF9 (see the magnified single channels and the representative plot profile in Fig. 6B).

p.R329C variant impairs cumulin signaling and BMP15 bioactivity

With the purpose of functionally evaluating the heterodimer signaling in the presence of wt or mutated BMP15, COV434 cells were cotransfected with equal amounts of BMP15 (wt or mutated) and GDF9 constructs. The pathway of SMAD2/3, which is known to be preferentially activated by the heterodimer

(Peng, 2013), resulted significantly impaired in presence of the p.R329C variant ($p<0.05$, Fig. 7A on the right).

The luciferase reporter assay study of the p.R329C variant in COV434 granulosa cells stably expressing the BMP responsive element, further demonstrated a significant decrease ($p<0.01$) of luciferase activity upon transfection with the mutant construct compared to the wt, which was not restored even upon cotransfection with an equal amount of the wt construct (Fig. 7B).

Discussion

In this work we report two null mutations of *BMP15* identified in homozygous condition in two patients with POI and primary amenorrhea. In one patient, the c.462del variant causes a frameshift with the inclusion of a premature stop codon at position 177 in the pro-region, resulting in a prematurely truncated protein (p.W155Gfs*22) and leading to the complete absence of the mature BMP15. When a stop codon is introduced more than 50 nucleotides upstream the final splice junction of an mRNA, the stop codon is classified as a premature termination codon and the corresponding mRNA isoform undergoes Nonsense Mediated Decay (NMD) (Lewis, 2003; Chang, 2007). However, the p.W155Gfs*22 variant occurs in the second and last exon of *BMP15*, downstream of the final exon-junction complex, therefore the nonsense transcript is predicted to be normally translated. Moreover, the possibility of a translation re-initiation downstream of the premature stop codon and therefore the presence of the mature form of BMP15 have been further excluded. Collectively, the experiments of confocal microscopy showed that the p.W155G*fs22 aberrant precursor is secreted extracellularly, but with a diffusely distributed signal along the PM, thus suggesting a decreased ability of this variant to bundle, even in the presence of GDF9 clusters. Since receptor clustering within microdomains upon ligand binding is known to lead to spatially restricted activation of a signaling pathway (Stauffer and Meyer, 1997), we hypothesize that the BMP15 p.W155G*fs22 variant could still bind the receptor, but might prevent its activation. Further studies should be performed to validate this hypothesis. We also report a biallelic *BMP15* whole-gene deletion. Also in this case, the parents were first-degree cousins and the mother entered physiological menopause. Collectively, NGS, array-CGH and

Taqman copy number analysis confirmed the proband and her father have no copy of *BMP15* whereas the fertile mother has one wt allele.

Taking into account the physiological menopause occurred in mothers of both patients, *BMP15* haploinsufficiency cannot cause POI. Alternatively, we propose as possible explanation the disruption of the functional interaction of BMP15 with its paralogue GDF9. Indeed, accumulating evidences indicate that cumulin, the heterodimer BMP15-GDF9, is more bioactive than the homodimers (Mottershead, 2012; Peng, 2013) and this cooperation would be regulated by the BMP15 pro-region (Hashimoto, 2005; McIntosh, 2008). As supporting evidence, in this work we also show that the heterozygous arginine to cysteine substitution of residue 329 of the mature peptide of BMP15 (Wang, 2010) alters protein production and significantly decreases its function *in vitro*. During synthesis, the BMP15 prodomain interacts noncovalently with its mature peptide, maintaining the molecule in a conformation competent for dimerization and secretion (Harrison, 2011). The band of approximately 70 KDa, which appears 24 hours pt and then disappears 48 hours pt, could be the result of a prolonged unstable binding between the precursor and the mature peptides which might confer latency to the growth factor. This variation, located next to cysteines involved in the cystine knot domain (i.e. C324 to C391), could alter pro-protein intracellular integrity and modify the intra-chain disulfide bridges (Darling, 2000), however, further experiments should be performed to validate these hypothesis. It has been reported that individual bonds of cystine knot are essential for secretion and heterodimer formation but are not a prerequisite for *in vitro* bioactivity (Sato, 1997). As shown by immunofluorescence staining, the p.R329C variant, as well as wt and p.Y235C, show specific extracellular punctuate profile, which indicates the capacity of the variants to cluster. As several receptors have been reported to re-locate from a uniform distribution along the PM to punctuate microdomains in response to ligand binding, BMP15 extracellular clusterization on the PM could be the result of its ability to bind and activate its receptor (Stauffer and Meyer, 1997; Holowka, 2000). Nevertheless we can see only a faint clustering of GFP-BMPR1b corresponding to BMP15 peaks in our experiments (Fig. 5B, absent in plot profiles, but appreciate the green channels of Supp. Figure S3). Since HEK293T cells naturally express the BMPR1b (Human Protein Atlas) (Thul, 2017), the lack of visible receptor clustering in our experiments might be due to the preferential BMP15 binding with the endogenous BMPR1b, which does not bear the GFP molecule. Despite this, in the presence of p.R329C change we observed a severe reduction of luciferase

reporter activation with respect to the wt, which was not recovered by cotransfection of the same amount of wt and mutant constructs, suggesting an aberrant binding and/or activation of its receptor, maybe due to a prolonged latent form of the protein, as other Authors already described for TGF β-1 (Sengle, 2011). This result correlates with the finding that the p.R329 residue confers the high activity of the protein at the binding interface with the receptor BMPR1b (Al-Musawi, 2013). Moreover, in immunofluorescence experiments, the levels of colocalization of p.R329C variant with GDF9 seemed much lower than either BMP15 wt or p.Y235C, thus suggesting also a minor ability of this variant to activate the BMP15-GDF9 heterodimer signaling. That is reflected in the significant impairment of the phosphorylation of SMAD2 upon cotransfection of BMP15 p.R329C variant and GDF9 constructs in COV434 cells. As consequence, the impaired signaling pathways of both BMP15 homodimer and BMP15-GDF9 heterodimer suggests a dominant negative effect of this variant as possible pathogenic cause of an augmented rate of ovarian follicles expenditure, correlating with a late POI onset.

In our hypothesis, heterozygous missense variants in BMP15 pro-region and, as here demonstrated, in the mature peptide would interfere with the heterodimer formation, thus predisposing to a variable reduction of ovarian reserve. In contrast, this interaction would be grossly unaffected in presence of only one copy of *BMP15*. A similar underlying mechanism has been observed in case of naturally occurring *BMP15* mutations in the "Grivette" and "Olkuska" sheeps, which would mainly have an effect on the BMP15-GDF9 heterodimer signaling (Demars, 2013). Since GDF9 is pivotal for the initiation of primordial follicle growth (Vitt, 2000) and no *GDF9* variants have been found in our patients, the recruitment of primordial follicles from the resting pool into the growth phase would still be possible even in the absence of *BMP15*, as demonstrated by the occurrence of spontaneous menarche in Patient A, and recently suggested by other Authors (Mayer, 2017). However, the absence of the concurrent action of the heterodimer would then lead to a dysregulated progression of folliculogenesis and a premature complete exhaustion of the impaired ovarian reserve. As previously stated, the short arm on X chromosome and, in particular, the proximal region from Xp11.1 to Xp21 containing the *BMP15* gene is of greatest importance for ovarian function, including several traits of Turner Syndrome. Notably, we previously reported the case of a patient with Turner syndrome 45,X who harbors a duplication of the entire *BMP15* gene and had spontaneous menarche (Castronovo, 2014). In this patient, the double dose of *BMP15* may have contributed to the maintenance of the ovarian reserve for

several years but it was lost few years after puberty, probably due to the absence of other X-linked ovarian determinants. Therefore, *BMP15* mutation/deletion might contribute to POI in a context of a polygenic-multifactorial origin of the disease (Bouilly, 2016). Accordingly, only the combination of *BMP15* haploinsufficiency with the loss of other still unknown X-linked genes might cause the ovarian phenotype of Turner syndrome.

In conclusion, the two *BMP15* null mutations identified in our patients with POI and primary amenorrhea unveil a knockout-like effect with a recessive mode of inheritance, which further confirms the critical role of *BMP15* and *GDF9* for human female fertility. Altogether these findings suggest that cumulin heterodimer is required for adequate ovarian folliculogenesis in humans.

Author's roles

All authors contributed to the intellectual development of this paper. R.R. designed the work, performed the sequencing, western blot experiments and luciferase assay, performed and supervised the data analysis, and wrote the manuscript. I.F. performed the confocal experiments, collected and analyzed the data, and wrote the manuscript. I.B. carried out aCGH experiments, collected and analyzed the data. S.M. carried out experiments for the revision. F.B and L.Pet. recruited patients and their families and revised the manuscript. P.F. analyzed the aCGH data and revised the manuscript. L.Per. conceived the work and revised the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interests.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. All the variants reported in this manuscript have been submitted to ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/) with the Submission ID SUB6688985.

References

Al-Musawi S. L., Walton K. L., Heath D., Simpson C. M., Harrison C. A. (2013). Species differences in the expression and activity of bone morphogenetic protein 15. *Endocrinology, 154,* 888-899. *doi*:10.1210/en.2012-2015

Auclair S., Rossetti R., Meslin C., Monestier O., Di Pasquale E., Pascal G., Persani L., Fabre S. (2013). Positive selection in bone morphogenetic protein 15 targets a natural mutation associated with primary ovarian insufficiency in human. *PLoS One, 8,* e78199. *doi:*10.1371/journal.pone.0078199

Belli M., & Shimasaki S. (2018). Molecular Aspects and Clinical Relevance of GDF9 and BMP15 in Ovarian Function. *Vitamins and Hormones, 107,* 317-348. *doi*:10.1016/bs.vh.2017.12.003

Bestetti I., Castronovo C., Sironi A., Caslini C., Sala C., Rossetti R., Crippa M., Ferrari I., Pistocchi A., Toniolo D., Persani L., Marozzi A., Finelli P. (2019). High-resolution array-CGH analysis on 46,XX patients affected by early onsetprimary ovarian insufficiency discloses new genes involved in ovarian function. *Human Reproduction, 34,* 574-583. *doi*:10.1093/humrep/dey389

Bolte S., & Cordelières F. P. (2006). A guided tour into subcellular colocalization analysis in light microscopy. *Journal of Microscopy, 224,* 213-232. *doi*:10.1111/j.1365-2818.2006.01706.x

Bouilly J., Beau I., Barraud S., Bernard V., Azibi K., Fagart J, Fèvre A., Todeschini A. L., Veitia R. A., Beldjord C., Delemer B., Dodé C., Young J., Binart N. (2016). Identification of Multiple Gene Mutations Accounts for a new Genetic Architecture of Primary Ovarian Insufficiency. *The Journal of Clinical Endocrinology & Metabolism, 12,* 4541-4550. *doi*:10.1210/jc.2016-2152

Castronovo C., Rossetti R., Rusconi D., Recalcati M. P., Cacciatore C., Beccaria E., Calcaterra V., Invernizzi P., Larizza D., Finelli P., Persani L. (2014). Gene dosage as a relevant mechanism contributing to the determination of ovarian function in Turner syndrome. *Human Reproduction, 29,* 368-379. *doi*:10.1093/humrep/det436

Chang Y. F., Imam J. S., Wilkinson M. F. (2007). The nonsense-mediated decay RNA surveillance pathway. *Annual Review of Biochemestry, 76*, 51-74. *doi*:10.1146/annurev.biochem.76.050106.093909

Darling R. J., Ruddon R. W., Perini F., Bedows E. (2000). Cystine knot mutations affect the folding of the glycoprotein hormone alpha-subunit. Differential secretion and assembly of partially folded intermediates. *Journal of Biological Chemistry*, *275*, 15413-15421. *doi*:10.1074/jbc.275.20.15413

Demars J., Fabre S., Sarry J., Rossetti R., Gilbert H., Persani L., Tosser-Klopp G., Mulsant P., Nowak Z., Drobik W., Martyniuk E., Bodin L. (2013). Genome-wide association studies identify two novel BMP15 mutations responsible for an atypical hyperprolificacy phenotype in sheep. *PLoS Genetics*, *9*, e1003482. *doi*:10.1371/journal.pgen.1003482

Di Pasquale E., Beck-Peccoz P., Persani L. (2004). Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. *American Journal of Human Genetics*, *75*, 106-111. *doi*:10.1086/422103

Di Pasquale E., Rossetti R., Marozzi A., Bodega B., Borgato S., Cavallo L., Einaudi S., Radetti G., Russo G., Sacco M., Nelson L. M., Persani L. (2006). Identification of new variants of human BMP15 gene in a large cohort of women with premature ovarian failure. *The Journal of Clinical Endocrinology and Metabolism*, *91*, 1976-1979. *doi*:10.1210/jc.2005-2650

Dixit H., Rao L. K., Padmalatha V. V., Kanakavalli M., Deenadayal M., Gupta N., Chakrabarty B., Singh L. (2006). Missense mutations in the BMP15 gene are associated with ovarian failure. *Human Genetics*, *119*, 408-415. *doi*:10.1007/s00439-006-0150-0

Hanevik H. I., Hilmarsen H. T., Skjelbred C. F., Tanbo T., Kahn J. A. (2011). A single nucleotide polymorphism in BMP15 is associated with high response to ovarian stimulation. *Reproductive Biomedicine Online*, *23*, 97-104. *doi*:10.1016/j.rbmo.2011.02.015

Harrison C. A., Al-Musawi S. L., Walton K. L. (2011). Prodomains regulate the synthesis, extracellular localisation and activity of TGF-β superfamily ligands. *Growth Factors*, *29*, 174-186. *doi*:10.3109/08977194.2011.608666

Hashimoto O., Moore R. K., Shimasaki S. (2005). Posttranslational processing of mouse and human BMP-15: potential implication in the determination of ovulation quota. *Proceedings of the National Academy of Sciences U.S.A., 102*, 5426-5431. *doi*:10.1073/pnas.0409533102

Holowka D., Sheets E. D., Baird B. (2000). Interactions between Fc(epsilon)RI and lipid raft components are regulated by the actin cytoskeleton. *Journal of Cell Science, 113*, 1009-1019.

Inagaki K., & Shimasaki S. (2010). Impaired production of BMP-15 and GDF-9 mature proteins derived from proproteins WITH mutations in the proregion. *Molecular and Cellular Endocrinology, 328*, 1-7. *doi*:10.1016/j.mce.2010.05.017

Laissue P., Christin-Maitre S., Touraine P., Kuttenn F., Ritvos O., Aittomaki K., Bourcigaux N., Jacquesson L., Bouchard P., Frydman R., Dewailly D., Reyss A. C., Jeffery L., Bachelot A., Massin N., Fellous M., Veitia R. A. (2006). Mutations and sequence variants in GDF9 and BMP15 in patients with premature ovarian failure. *European Journal of Endocrinology, 154*, 739-744. *doi*:10.1530/eje.1.02135

Layman L. C. (2006). Editorial: BMP15--the first true ovarian determinant gene on the X-chromosome? *The Journal of Clinical Endocrinology & Metabolism*, 91, 1673-1676.

Lewis B. P., Green R. E., Brenner S. E. (2003). Evidence for the widespread coupling of alternative splicing and nonsense-mediated mRNA decay in humans. *Proceedings of the National Academy of Sciences U.S.A., 100*, 189-192. *doi*:10.1073/pnas.0136770100

Liao W. X., Moore R. K., Otsuka F., Shimasaki S. (2003). Effect of intracellular interactions on the processing and secretion of bone morphogenetic protein-15 (BMP-15) and growth and differentiation factor-9. Implication of the aberrant ovarian phenotype of BMP-15 mutant sheep. *The Journal of Biological Chemistry, 278*, 3713-3719. *doi*:10.1074/jbc.M210598200

Liu J., Wang B., Wei Z., Zhou P., Zu Y., Zhou S., Wen Q., Wang J., Cao Y., Ma X. (2011). Mutational analysis of human bone morphogenetic protein 15 in Chinese women with polycystic ovary syndrome. *Metabolism*, 60, 1511-1514. *doi*:10.1016/j.metabol.2010.10.006

544 Mayer A., Fouquet B., Pugeat M., Misrahi M. (2017). Bmp15 "Knockout-Like" effect in familial premature
545 ovarian insufficiency with persistent ovarian reserve. *Clinical Genetics, 92,* 208-212. *doi*:10.1111/cge.12970

546 McDonald N. Q., Hendrickson W.A. (1993). A structural superfamily of growth factors containing a cystine
547 knot motif. *Cell, 73* ,421-424. *doi*:10.1016/0092-8674(93)90127-c

548 McIntosh C. J., Lun S., Lawrence S., Western A. H., McNatty K. P., Juengel J. L. (2008). The proregion of
549 mouse BMP15 regulates the cooperative interactions of BMP15 and GDF9. *Biology of Reproduction, 79,*
550 889-896. *doi*:10.1095/biolreprod.108.068163

551 McPherron A. C., & Lee S. J. (1993). GDF-3 and GDF-9: two new members of the transforming growth
552 factor-beta superfamily containing a novel pattern of cysteines. *Journal of Biological Chemistry, 268,* 3444-
553 3449.

554 Mehdizadeh A., Sheikhha M. H., Kalantar S. M., Aali B. S., Ghanei A. (2016). Mutation analysis of exon1
555 of bone morphogenetic protein-15 gene in Iranian patients with polycystic ovarian syndrome. *International*
556 *Journal of Reproductive Biomedicine* (Yazd), *14*, 527-532.

557 Molloy S. S., Thomas L., VanSlyke J. K., Stenberg P. E., Thomas G. (1994). Intracellular trafficking and
558 activation of the furin proprotein convertase: localization to the TGN and recycling from the cell surface.
559 *EMBO Journal, 13,* 18-33.

560 Monestier O., Servin B., Auclair S., Bourquard T., Poupon A., Pascal G., Fabre S. (2014). Evolutionary
561 origin of bone morphogenetic protein 15 and growth and differentiation factor 9 and differential selective
562 pressure between mono- and polyovulating species. *Biology of Reproduction,* 91: *83,* 1-13.
563 *doi*:10.1095/biolreprod.114.119735

564 Montgomery G. W., Zhao Z. Z., Marsh A. J., Mayne R., Treloar S. A., James M., Martin N. G., Boomsma D.
565 I., Duffy D. L. (2004). A deletion mutation in GDF9 in sisters with spontaneous DZ twins. *Twin Research, 7,*
566 548-555. *doi*:10.1375/1369052042663823

567 Morón F. J., de Castro F., Royo J.L., Montoro L., Mira E., Sáez M. E., Real L. M., González A., Mañes S.,
568 Ruiz A. (2006). Bone morphogenetic protein 15 (BMP15) alleles predict over-response to recombinant

follicle stimulation hormone and iatrogenic ovarian hyperstimulation syndrome (OHSS). *Pharmacogenetics and Genomics*, *16*, 485-495. *doi*:10.1097/01.fpc.0000215073.44589.96

Mottershead D. G., Ritter L. J., Gilchrist R. B. (2012). Signalling pathways mediating specific synergistic interactions between GDF9 and BMP15. *Molecular Human Reproduction*, *18*, 121-128. *doi*:10.1093/molehr/gar056

Mottershead D. G., Sugimura S., Al-Musawi S. L., Li J. J., Richani D., White M. A., Martin G. A., Trotta A. P., Ritter L. J., Shi J., Mueller T. D., Harrison C. A., Gilchrist R. B. (2015). Cumulin, an Oocyte-secreted Heterodimer of the Transforming Growth Factor-β Family, Is a Potent Activator of Granulosa Cells and Improves Oocyte Quality. The *Journal of Biological Chemistry*, *290*, 24007-24020. *doi*:10.1074/jbc.M115.671487

Palmer J. S., Zhao Z. Z., Hoekstra C., Hayward N.K., Webb P.M., Whiteman D. C., Martin N. G., Boomsma D. I., Duffy D. L., Montgomery G. W. (2006). Novel variants in growth differentiation factor 9 in mothers of dizygotic twins. *The Journal of Clinical Endocrinology & Metabolism*, *91*, 4713-4716 *doi*:10.1210/jc.2006-0970

Panigone S., Hsieh M., Fu M., Persani L., Conti M. (2008). Luteinizing hormone signaling in preovulatory follicles involves early activation of the epidermal growth factor receptor pathway. *Molecular Endocrinology*, *22*, 924-936. *doi*:10.1210/me.2007-0246

Peng J., Li Q., Wigglesworth K., Rangarajan A., Kattamuri C., Peterson R. T., Eppig J. J., Thompson T. B., Matzuk M. M. (2013). Growth differentiation factor 9: bone morphogenetic protein 15 heterodimers are potent regulators of ovarian functions *Proceedings of the National Academy of Sciences U.S.A.*, *110*, E776-E785. *doi*:10.1073/pnas.1218020110

Persani L., Rossetti R., Cacciatore C. (2010). Genes involved in human premature ovarian failure. *Journal of Molecular Endocrinology*, *45*, 257-279. *doi*:10.1677/JME-10-0070

592 Persani L., Rossetti R., Di Pasquale E., Cacciatore C., Fabre S. (2014). The fundamental role of bone
593 morphogenetic protein 15 in ovarian function and its involvement in female fertility disorders. *Human*
594 *Reproduction Update, 20,* 869-883. *doi*:10.1093/humupd/dmu036

595 Qin Y., Jiao X., Simpson J. L., Chen Z. J. (2015). Genetics of primary ovarian insufficiency: new
596 developments and opportunities. *Human Reproduction Update, 21,* 787-808. *doi*:10.1093/humupd/dmv036

597 Robinson J. T., Thorvaldsdóttir H., Winckler W., Guttman M., Lander E. S., Getz G., Mesirov J. P. (2011).
598 Integrative genomics viewer. *Nature Biotechnology, 29,* 24-26. *doi*:10.1038/nbt.1754

599 Rossetti R., Di Pasquale E., Marozzi A., Bione S., Toniolo D., Grammatico P., Nelson L. M., Beck-Peccoz
600 P., Persani L. (2009). BMP15 mutations associated with primary ovarian insufficiency cause a defective
601 production of bioactive protein. *Human Mutation, 30,* 804-810. *doi*:10.1002/humu.20961

602 Rossetti R., Ferrari I., Bonomi M., Persani L. (2017). Genetics of primary ovarian insufficiency. *Clinical*
603 *Genetics, 91,* 183-198. *doi*:10.1111/cge.12921

604 Sato A., Perlas E., Ben-Menahem D., Kudo M., Pixley M. R., Furuhashi M., Hsueh A. J., Boime I. (1997).
605 Cystine knot of the gonadotropin alpha subunit is critical for intracellular behavior but not for in vitro
606 biological activity. *Journal of Biological Chemistry, 272,* 18098-18103. *doi*:10.1074/jbc.272.29.18098

607 Sengle G., Ono R. N., Sasaki T., Sakai L. Y. (2011). Prodomains of transforming growth factor beta
608 (TGFbeta) superfamily members specify different functions: extracellular matrix interactions and growth
609 factor bioavailability. *The Journal of Biological Chemistry, 286,* 5087-5099. *doi*:10.1074/jbc.M110.188615

610 Shimasaki S., Moore R. K., Otsuka F., Erickson G. F. (2004). The bone morphogenetic protein system in
611 mammalian reproduction. *Endocrine Reviews, 25,* 72-101. *doi*:10.1210/er.2003-0007

612 Simpson C. M., Stanton P. G., Walton K. L., Chan K. L., Ritter L.J., Gilchrist R. B., Harrison C. A. (2012).
613 Activation of latent human GDF9 by a single residue change (Gly 391 Arg) in the mature domain.
614 *Endocrinology, 153,* 1301-1310. *doi*:10.1210/en.2011-1632

615 Stauffer T. P., & Meyer T. (1997). Compartmentalized IgE receptor-mediated signal transduction in living
616 cells. *Journal of Cell Biology, 139,* 1447-1454. *doi*:10.1083/jcb.139.6.1447

Stefanidakis M., & Koivunen E. (2006). Cell-surface association between matrix metalloproteinases and integrins: role of the complexes in leukocyte migration and cancer progression. Blood, 108, 1441-1450. doi:10.1182/blood-2006-02-005363

Thorvaldsdóttir H., Robinson J. T., Mesirov J. P. (2013). Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Briefing in Bioinformatics, 14,* 178-192. *doi*:10.1093/bib/bbs017

Thul P. J., Åkesson L., Wiking M., Mahdessian D., Geladaki A., Ait Blal H., Alm T., Asplund A., Björk L., Breckels L. M., Bäckström A., Danielsson F., Fagerberg L., Fall J., Gatto L., Gnann C., Hober S., Hjelmare M., Johansson F., Lee S., Lindskog C., Mulder J., Mulvey C. M., Nilsson P., Oksvold P., Rockberg J., Schutten R., Schwenk J. M., Sivertsson Å., Sjöstedt E., Skogs M., Stadler C., Sullivan D. P., Tegel H., Winsnes C., Zhang C., Zwahlen M., Mardinoglu A., Pontén F., von Feilitzen K., Lilley K.S., Uhlén M., Lundberg E. (2017). A subcellular map of the human proteome. *Science, 356*. *doi*:10.1126/science.aal3321

Tiotiu D., Alvaro Mercadal B., Imbert R., Verbist J., Demeestere I., De Leener A., Englert Y., Vassart G., Costagliola S., Delbaere A. (2010). Variants of the BMP15 gene in a cohort of patients with premature ovarian failure. *Human Reproduction*, *25*, 1581-1587. *doi*:10.1093/humrep/deq073

Vitt U. A., McGee E. A., Hayashi M., Hsueh A. J. (2000). In vivo treatment with GDF-9 stimulates primordial and primary follicle progression and theca cell marker CYP17 in ovaries of immature rats. *Endocrinology, 141,* 3814-3820 *doi*:10.1210/endo.141.10.7732

Wang B., Wen Q., Ni F., Zhou S., Wang J., Cao Y., Ma X. (2010). Analyses of growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) mutation in Chinese women with premature ovarian failure. *Clinical Endocrinology* (Oxf), *72,* 135-136. *doi*:10.1111/j.1365-2265.2009.03613.x.

Wang B., Zhou S., Wang J., Liu J., Ni F., Yan J., Mu Y., Cao Y., Ma X. (2010). Identification of novel missense mutations of GDF9 in Chinese women with polycystic ovary syndrome. *Reproductive Biomedicine Online, 21,* 344-348. *doi*:10.1016/j.rbmo.2010.04.013

Wang T. T., Ke Z. H., Song Y., Chen L. T., Chen X. J., Feng C., Zhang D., Zhang R. J., Wu Y. T., Zhang Y., Sheng J. Z., Huang H. F. (2013). Identification of a mutation in GDF9 as a novel cause of diminished ovarian reserve in young women. *Human Reproduction, 28,* 2473-2481. *doi*:10.1093/humrep/det291

Zhang W., Wang J., Wang X., Li L., Pan H., Chen B., Zhu Y., Li T., Cao Y., Wang B. (2018). A novel homozygous mutation of bone morphogenetic protein 15 identified in a consanguineous marriage family with primary ovarian insufficiency. *Reproductive Biomedicine Online, 36,* 206-209. *doi*:10.1016/j.rbmo.2017.10.104

Zhao Z. Z., Painter J. N., Palmer J. S., Webb P. M., Hayward N. K., Whiteman D. C., Boomsma D. I., Martin N. G., Duffy D. L., Montgomery G. W. (2008). Variation in bone morphogenetic protein 15 is not associated with spontaneous human dizygotic twinning. *Human Reproduction, 23,* 2372-2379. *doi*:10.1093/humrep/den268

652

653 **Figure Legends**

654 **Figure 1. A) Family pedigrees of Patients A.** Patient A (III.1) presented with early secondary amenorrhea
655 and harbors the c.462del variant in the *BMP15* gene in homozygosis, likely inherited from her first-degree
656 cousins parents (II.1 and II.2). Her mother (II.2) experienced a physiological menopause and proband's sister
657 never had menses (III.2). They could not be genetically investigated due to gDNAs unavailability. **B) Sanger**
658 **sequencing (left) and the alignment results (right).** DNA sequence of *BMP15* gene exon 2 of Patient A
659 and the alignment with the reference sequences are shown. **C) Family pedigrees of Patients B.** Patient B
660 (III.5) presented with primary amenorrhea and the biallelic deletion of the *BMP15* gene (highlighted as red
661 dots in the representation of sex chromosomes) (Bestetti, 2019). Parents are consanguineous. The father
662 (II.6) has no *BMP15* allele, while the fertile mother (II.7) has one wt *BMP15* allele.

663 **Figure 2. A) NGS alignment of *BMP15* sequences of Patient B and a wt control DNA sample.** Patient B
664 and a control sample sequences of *BMP15* gene are displayed through the Integrative Genomics Viewer
665 software (Robinson, 2011; Thorvaldsdóttir, 2013). The main window of IGV displays four tracks from top to
666 bottom: 1) the *BMP15* gene region (highlighted by a red line) on the X chromosome 2) the track of .bam files
667 of Patient B; 3) the track of .bam files of the wt control sample; 4) the track of the reference genome Human
668 hg19. No signal sequence of *BMP15* gene has been detected in Patient B. **B) High-resolution array-CGH**
669 **analysis.** The genome scan of Patient B was performed using the 400K Human Genome CGH Microarray
670 (Bestetti, 2019). Pooled gDNA from peripheral blood of 10 healthy donors, sex-matched to the samples, was
671 used as a reference DNA. The profile output derived from a competitive hybridization of Patient B and
672 reference gDNA is represented for chrX and *BMP15* gene region. Positive and negative values are shown as
673 blue and red dots, respectively. The analysis confirmed the deletion of 7 spots spanning 35.6 kb in Xp11.22
674 in homozygosity, compatible with the biallelic deletion of *BMP15* in Patient B. **C) Real-time quantitative**
675 **PCR.** The Taqman Copy Number assay has been performed on gDNAs from patient B and her parents. All
676 samples were compared with a 46,XX control gDNA, known to have two copies of the target sequences
677 (calibrator), and a 46,XY gDNA, with one copy of the target sequences (internal control). Four replicates for
678 each sample were analyzed twice. Results are indicated as median, specifying the range of max and min

values obtained. No *BMP15* copy number was detected in both the Patient B and her father. Only one *BMP15* copy number was detected in proband's mother.

Figure 3. Molecular analysis of BMP15 variants. Representative Western blots of: **A)** HEK293T cells lysates (20µg of loaded protein each) and **B)** equal amounts of their relative purified and precipitated culture media, transiently transfected for 72 hours with the indicated myc-his tagged BMP15 constructs. The pcDNA4-myc-his empty vector was transfected as control (MOCK). The p.W155Gfs*22 variant generates an aberrant precursor in total cell lysates and no mature protein is detected in the culture medium.

Figure 4. The p.R329C variant shows a peculiar processing. Representative Western blots of the time course of production of p.R329C protein compared with the BMP15 wt counterpart from purified and precipitated culture media of HEK293T cells transiently transfected for 72 hours with the indicated myc-his tagged BMP15 constructs. Time points were set at 24, 30 and 48 hours post transfection. Differently from wt, a faint upper band of approximately 70 KDa (band 3) characterizes the p.R329C variant after 24 hours from transfection, but completely disappears after 48 hours. The p.R329C variant also lacks band 2 of approximately 10KDa that is instead present in the wt lane.

Figure 5. BMP15 variants bind to the extracellular surface of the plasma membrane. A) Representative confocal images of HEK293T cells transiently transfected with V5-tagged wt and mutant BMP15. Cells were stained under permeabilizing conditions with a polyclonal antibody against the V5 tag (red); nuclei were stained with DAPI (blue). The 2X magnifications of the boxed areas (red channel only) are presented both as inverted staining (on the left) and as color-coded interactive 3D surface plots (on the right); the scale on the right indicates fluorescence intensities expressed in Relative Light Units (RLUs). Bar, 10 µm. **B)** Laser confocal images of HEK293T cells transiently co-transfected with GFP-BMPR1b (green) and V5-tagged BMP15, wt or mutants. Cells were probed under non-permeabilizing conditions with a polyclonal antibody against the V5 tag (red); nuclei were stained with DAPI (blue). Merged images and individual stainings of the areas indicated by arrows are shown below (2X magnifications). The corresponding plot profiles were obtained with NIH ImageJ Software. Bar, 10 µm. **C, left)** Box and Whiskers plot representing the number of peaks per cell, obtained from at least 45 cells from three different experiments for each transfectant. The Tukey method was applied to identify outlier values. The red lines represent the median values. **C,**

right) Histogram representing the mean peaks' length ($n > 45$ cells from three different experiments from each transfectant). P-values*: **:* $p < 0.01$*; ***:* $p < 0.001$.

Figure 6. BMP15 variants do not impair GDF9 trafficking and secretion. A) Representative confocal images of HEK293T cells transiently co-transfected with V5-tagged wt or mutant BMP15 and myc-tagged GDF9. BMP15 and GDF9 localization was assessed under permeabilizing conditions with commercial antibodies against the V5 (red) and myc (green) tags, respectively. Nuclei were stained with DAPI (blue). The 2X magnifications of the boxed areas represent merged and individual stainings, as well as the colocalization signal (yellow) obtained with NIH ImageJ function 'Image Calculator'. Bar: 10 µm. **B)** Laser confocal images of HEK293T cells transiently co-transfected with V5-tagged wt or mutant BMP15 and myc-tagged GDF9. Cells were stained under non-permeabilizing conditions with commercial antibodies against the V5 (red, BMP15) and myc (green, GDF9) tags. Nuclei were stained with DAPI (blue). Merged images and individual stainings of the boxed areas are shown below (2X magnifications). The corresponding plot profiles were obtained with NIH ImageJ Software. Bar, 10 µm. The values reported in 2X magnifications represent the percentage of colocalization between each BMP15 variant and GDF9 along the PM obtained by calculating the MCC with NIH ImageJ Software.

Figure 7. Functional evaluation of cumulin in presence of BMP15 variants. A, left) Representative western blot of cell lysates (15µg of loaded protein each) of COV434 cells transiently cotransfected for 48 hours with equal amounts of pCS2-BMP15 (wt or mutated) and pCS2-GDF9 constructs. The pCS2 empty vector was transfected as control (MOCK) and not transfected cells (NT) control was also included. The p.R329C variant significantly impaired the phosphorylation of SMAD2. **A, right)** Densitometric analysis of the phosphorylation state of SMAD2/3 pathway. Results are expressed as the mean (± SEM) on two independent experiments and statistics were obtained by using One-way Anova, *$p<0.05$ (Prism software). **B)** The transcriptional activity of the p.R329C variant located in the mature peptide of BMP15 was investigated in the granulosa-derived COV434 cell line stably expressing the BMP responsive element luciferase-reporter. Cells transfected with empty vector were used as negative control (MOCK). A positive control of exogenous commercial rhBMP15 (100 ng/ml) was included. Co-transfections with equal amounts (250ng) of wt and mutant or mock constructs were performed to mimic heterozygous situations. Results are

733 expressed as the mean (± SEM) of Relative Light Units (RLU) of four separate experiments each performed

734 in triplicates. **$p<0.01$.

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