



## Golgi apparatus casein kinase phosphorylates bioactive Ser-6 of bone morphogenetic protein 15 and growth and differentiation factor 9

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### ABSTRACT

**Bone morphogenetic protein-15 (BMP-15) and growth and differentiation factor-9 (GDF-9) are oocyte-secreted factors that play essential roles in human folliculogenesis and ovulation. Their bioactivity is tightly regulated through phosphorylation, likely to occur within the Golgi apparatus of the secretory pathway. Here we show that Golgi apparatus casein kinase (G-CK) catalyzes the phosphorylation of rhBMP-15 and rhGDF-9. rhBMP-15, in particular, is an excellent substrate for G-CK. In each protein a single residue is phosphorylated by G-CK, corresponding to the serine residue at the sixth position of the mature region of both rhBMP-15 and rhGDF-9, whose phosphorylation is required for biological activity.**

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### 1. Introduction

Oocyte-secreted factors, such as bone morphogenetic protein-15 (BMP-15) and growth and differentiation factor-9 (GDF-9) play critical roles in regulating folliculogenesis and ovulation in mammals [1]. BMP-15 and GDF-9 are synthesized as precursors composed of a signal peptide, a proregion, and a biologically active mature protein [2–4]. Recently, we have shown that both BMP-15 and GDF-9 are phosphorylated and that phosphorylation is essential for bioactivity of the proteins [5–7]. Moreover, de-phosphorylated rhBMP-15 and rhGDF-9 exhibit antagonistic activity toward not only their phosphorylated counterparts but also toward each other, as well as rhBMP-7, a member of the TGF- $\beta$  superfamily that shares the same receptors. Thus, phosphorylation appears to be a means of regulating the bioactivity of rhBMP-15 and rhGDF-9, as well as other members of the TGF- $\beta$  superfamily. BMP-15

and GDF-9 are secreted proteins, indicating that phosphorylation is likely to occur in the Golgi apparatus of the secretory pathway. Based on the probable location of phosphorylation in the secretory pathway and the known phosphorylation site of rhBMP-15 at Ser<sup>6</sup>, we postulated that Golgi apparatus casein kinase (G-CK) could be the kinase implicated [8–10]. The consensus sequence targeted by G-CK, (Ser)-(Xaa)-(Glu or previously phosphorylated Ser) [8–10], is present in the hBMP-15 mature region coinciding with the phosphorylated residue at Ser<sup>6</sup>. Moreover, hGDF-9 contains a similar consensus sequence: (Ser<sup>6</sup>-Ser<sup>7</sup>-Glu<sup>8</sup>). Thus, both rhBMP-15 and rhGDF-9 are potential substrates for G-CK.

Identification of the kinase responsible for phosphorylating rhBMP-15 and rhGDF-9 is essential for further understanding the role of phosphorylation in regulation of bioactivity of the proteins. In the present study, we show that G-CK is able to phosphorylate rhBMP-15 and rhGDF-9. Additionally, we identified the residues phosphorylated by G-CK using mass spectrometry (MS), and we show that they are those required for bioactivity.

### 2. Materials and methods

#### 2.1. Production and purification of rhBMP-15 and rhGDF-9

rhBMP-15 and rhGDF-9, both tagged with a Flag epitope at the C-terminus were prepared as described earlier [5,7].

**Abbreviations:** BMP-15, bone morphogenetic protein-15; GDF-9, growth and differentiation factor-9; G-CK, Golgi apparatus casein kinase; MALDI TOF/TOF, matrix-assisted laser-desorption/ionization time-of-flight/time-of-flight; MS, mass spectrometry; MS/MS, tandem mass spectrometry

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## 2.2. Protein kinase

G-CK was purified from the Golgi fraction of lactating mammary gland according to [11–13] and as described in [Supplementary data](#).

## 2.3. Phosphorylation assays

rhBMP-15 and rhGDF-9 phosphorylations were performed as described in [10–13] and in [Supplementary data](#).

## 2.4. Trypsin digestion

After in vitro phosphorylation by G-CK, the proteins rhBMP-15 and rhGDF-9 were purified by SDS-PAGE and the bands corresponding to the different forms (precursor and mature proteins) were excised and subjected to in-gel reduction/alkylation followed by overnight digestion with trypsin (Promega) according to [14].

## 2.5. Phosphopeptides enrichment and MS

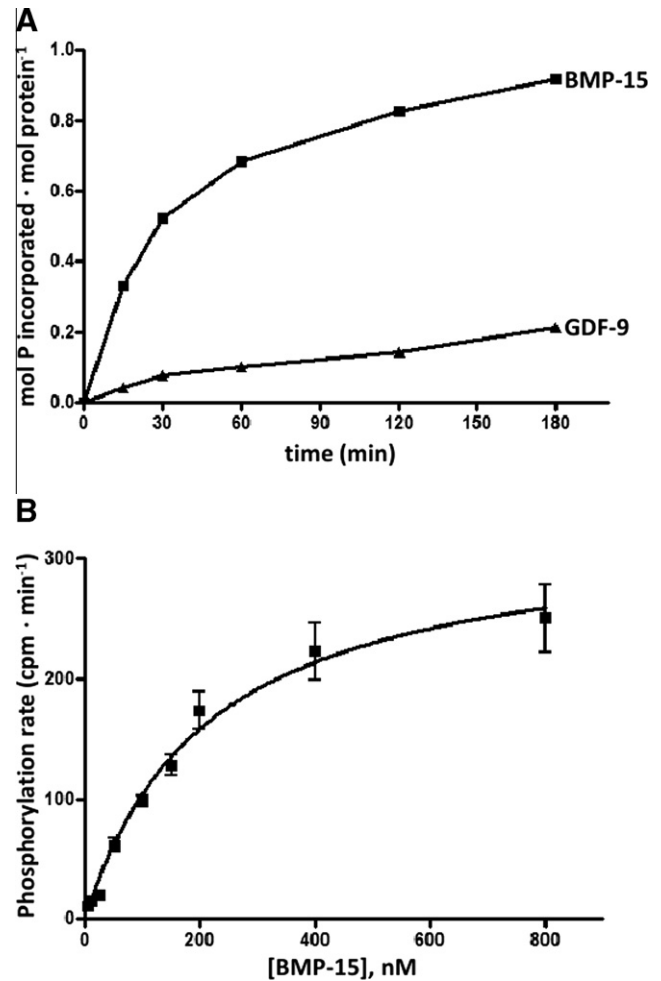
Phosphopeptides were enriched according to [15] using in-house made microcolumns of  $\text{TiO}_2$  and analyzed using a LCQ XP mass spectrometer (Thermo Scientific) coupled to a nano-HPLC system 1100 (Agilent Technologies) for the rhGDF-9 samples, whereas the rhBMP-15 samples were analyzed with a 4800 Plus matrix-assisted laser-desorption/ionization time-of-flight/time-of-flight (MALDI TOF/TOF) (Applied Biosystems). Tandem mass spectrometry (MS/MS) data were then searched using Mascot search engine (Matix Science). For further details see [Supplementary data](#).

## 3. Results

### 3.1. rhBMP-15 and rhGDF-9 are phosphorylated by G-CK with good kinetics parameters

In order to evaluate whether rhBMP-15 and rhGDF-9 are susceptible to phosphorylation by G-CK, the proteins, each at 200 nM, were subjected to phosphorylation assays with highly purified G-CK from rat mammary gland [11]. The time course of phosphorylation (Fig. 1A) shows that rhBMP-15 was readily phosphorylated by the kinase, while rhGDF-9 was phosphorylated to a lesser extent. In particular, after 3 h of incubation, up to 0.92 pmol and 0.13 pmol of phosphate/mol protein was incorporated into rhBMP-15 and rhGDF-9, respectively. Kinetic analysis allowed us to estimate an apparent  $K_m$  value of 0.196  $\mu\text{M}$  for rhBMP-15 phosphorylation (Fig. 1B). In the case of rhGDF-9 initial phosphorylation rates were too low and the kinetic constants could not be calculated.

To rule out the possibility that protein kinase(s) other than G-CK are involved in the phosphorylation of BMP-15, we took advantage of the unique insensitivity of G-CK to staurosporine [11], a potent broad specificity inhibitor of protein kinases in general. Furthermore the  $\beta$ -casein 28–40 peptide that is phosphorylated only by G-CK was used as a competitor for BMP-15 phosphorylation by G-CK [10–11]. We found that 100  $\mu\text{M}$  staurosporine, a concentration suppressing the activities of any other known protein kinase, has no effect on BMP-15 phosphorylation by G-CK. All our experiments were performed in the presence of 100  $\mu\text{M}$  staurosporine unless differently indicated. Furthermore, as shown in Fig. 2, the intensity of rhBMP-15 phosphoradiolabeling was decreased by adding in the reaction the specific peptide substrate (500  $\mu\text{M}$ ) indicating that it competes with rhBMP-15 for phosphorylation by G-CK. Taken together, these results support

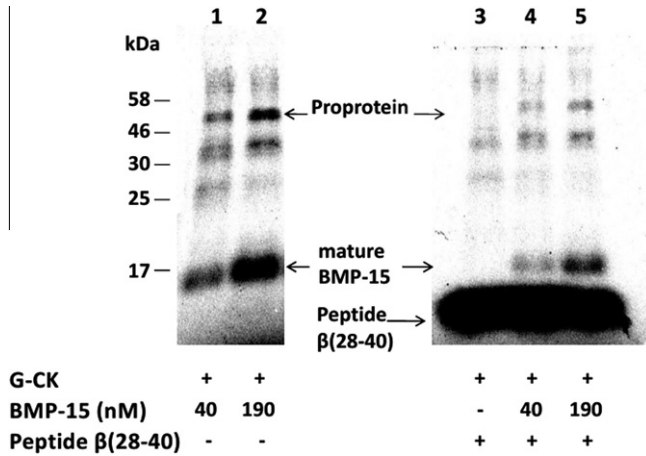


**Fig. 1.** (A) Time course of phosphorylation of rhBMP-15 and rhGDF-9 (200 nM) by G-CK. Phosphorylation was performed as described in [Supplementary data](#). At different times reactions were stopped and phosphate incorporated was evaluated by SDS-PAGE and autoradiography. Its amount is expressed as mol P mol prot<sup>-1</sup>. (B) Representative kinetic curve obtained by incubating G-CK with 50  $\mu\text{M}$  [ $\gamma$ -<sup>32</sup>P]ATP and increasing concentrations of rhBMP-15 under conditions detailed in [Supplementary data](#). Values, expressed as cpm·min<sup>-1</sup>, are means of at least three determinations, with S.E.M. The  $K_m$  value calculated by this experiment was 0.196  $\mu\text{M}$ .

the view that the kinase responsible for rhBMP-15 and rhGDF-9 phosphorylation is indeed G-CK.

### 3.2. Identification of the phosphorylation sites by MS

To identify the residues phosphorylated by G-CK, rhBMP-15 and rhGDF-9 were exhaustively phosphorylated by G-CK, and the phosphorylated residues were identified by trypsin digestion followed by LC-MS/MS analysis as detailed in Section 2. MS analysis allowed us to detect several phosphopeptides belonging to rhBMP-15 and rhGDF-9, but only one phosphorylation site was identified for each of the proteins. All the MS<sup>2</sup> spectra featuring loss of 98, 49 or 32.6 Da from the parent mass (strongly indicating that the peptide contains a phosphate group) and the relative MS<sup>3</sup> spectra were manually inspected and interpreted to identify the peptide sequence and the phosphorylation sites. The high number of phosphopeptides identified was due to partial tryptic cleavages around the same phosphoresidue and to chemical modifications of the peptides. As shown in Fig. 3, for the mature form of rhGDF-9 we could clearly identify Ser<sup>6</sup> as phosphorylated. The same phosphorylated residue was also identified in the mature



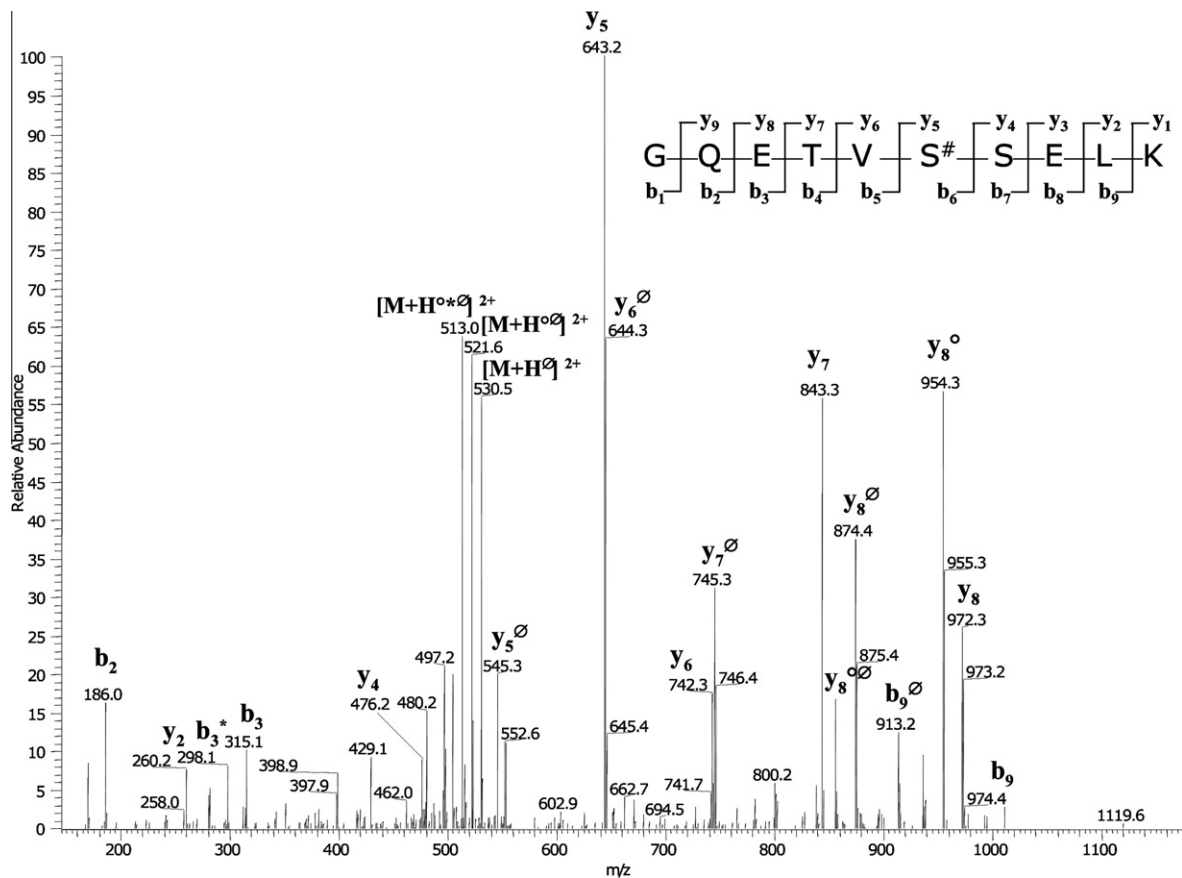
**Fig. 2.** Effect of staurosporine and of the specific  $\beta$ -casein-derived peptide substrate  $\beta$  (28–40) on BMP-15 phosphorylation. BMP-15 either 40 nM (lanes 1 and 4) or 190 nM (lanes 2 and 5) was phosphorylated in the absence (lanes 1 and 2) or presence of 0.5 mM specific  $\beta$ -casein-derived peptide substrate  $\beta$  (28–40) (lanes 4 and 5) and in the presence of staurosporine 100  $\mu$ M. After 10 min incubation reactions were stopped, samples resolved by SDS–PAGE and phosphorylated bands were identified by direct scanning of the gel on a Cyclone Storage Phosphor System (Perkin–Elmer), as described in Supplementary data. Controls without staurosporine displayed identical radiolabeling intensity of the phosphorylated bands (not shown).

form of rhBMP-15 (Fig. 4). Both proteins are therefore phosphorylated at a Ser residue that matches the consensus sequence for G-CK [8–10]. The same analysis was also performed on the full

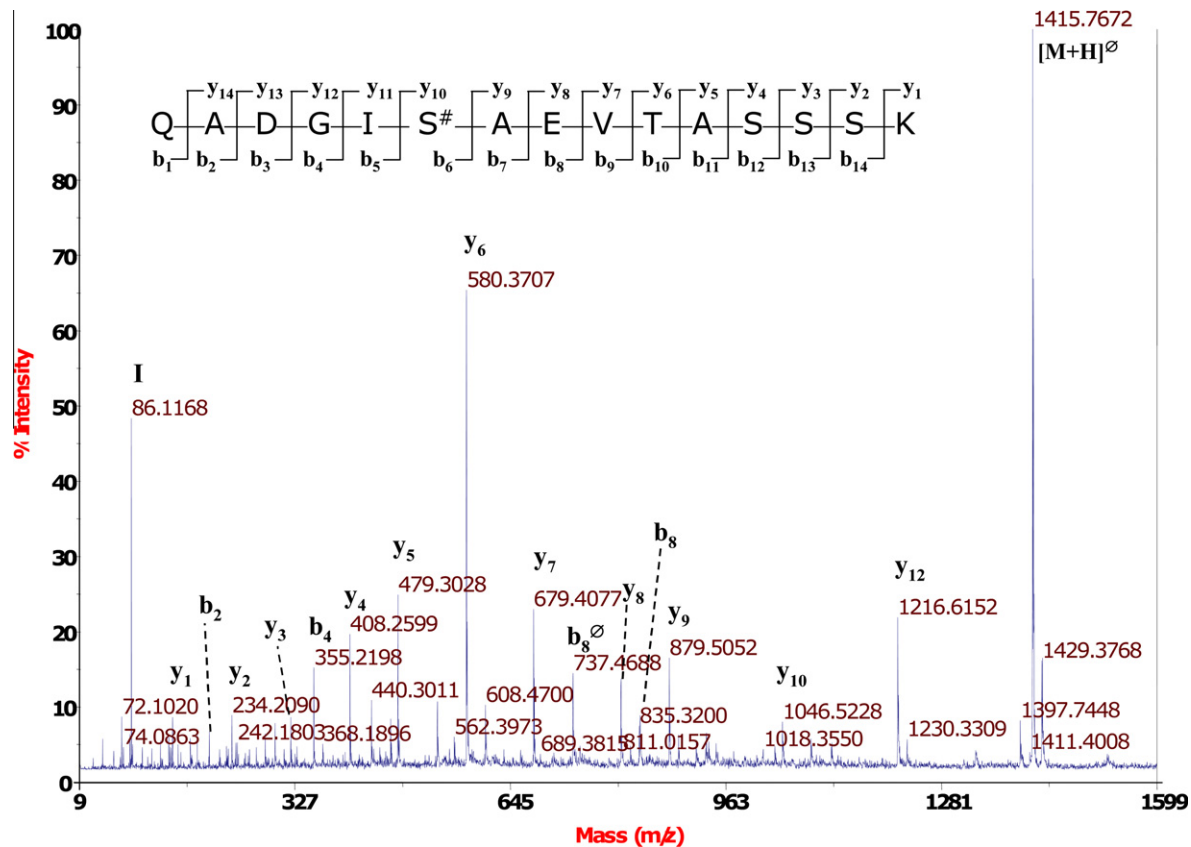
length proteins (i.e., the proteins that have not been cleaved to give the mature form), obtaining similar results, since the same phosphopeptides were identified. Finally we searched for possible phosphorylated residues present in the proregion of the proproteins but we could not identify any phosphopeptides in these regions of either rhBMP-15 or rhGDF-9. Altogether, our data indicate that rhBMP-15 and rhGDF-9 are phosphorylated by G-CK at the same main residue (Ser<sup>6</sup> of the mature forms) and this seems to be the only target of the kinase. Moreover, G-CK appears to be equally able to phosphorylate the proteins, at least in vitro, regardless of whether they are full-length or in their mature form.

#### 4. Discussion

Previously, we have shown that rhBMP-15 and rhGDF-9 are phosphorylated [6], and that phosphorylation is essential for bioactivity of the proteins [7]. In the present study, we identified G-CK as the kinase responsible for phosphorylating rhBMP-15 and rhGDF-9. These proteins are secreted proteins; thus, phosphorylation is likely to occur in the secretory pathway, specifically, within the Golgi apparatus. The 16 kDa mature region of rhBMP-15 was previously shown to be phosphorylated at a single site, Ser<sup>6</sup> [5]. Based on these phosphorylation conditions, G-CK emerged as a candidate kinase [8–10]. G-CK has been detected in the Golgi apparatus of a variety of tissues [12], and has been shown to phosphorylate substrates such as progastrin [16–18], ACTH [19,20], IGF binding protein-1 [21] and osteopontin [22,23], in addition to  $\beta$ -casein [12,24]. G-CK targets proteins with a consensus sequence



**Fig. 3.** MS/MS spectrum of the phosphopeptide GQSTVS#SELK from GDF-9. After isolation of the phosphopeptides by TiO<sub>2</sub> affinity chromatography, the analysis was carried out with an Ion trap mass spectrometer coupled to a nano-HPLC. In the spectrum are evident the intense losses of phosphate from the precursor ion and from the daughter ions (peaks marked with  $\Delta$ ). The y and b ion series give a clear indication of the peptide sequence and of the site of phosphorylation.



**Fig. 4.** MS/MS spectrum of the phosphopeptide QADGIS#AEVTASSK from rhBMP-15. After isolation of the phosphopeptides by TiO<sub>2</sub> affinity chromatography, the analysis was carried out with a MALDI TOF/TOF mass spectrometer. The base peak of the spectrum is due to the loss of phosphate ( $\Delta$ ) from the precursor ion. The y and b ion series give a clear indication of the peptide sequence and of the site of phosphorylation. The total mass of the peptide (1513.76 Da) and the fragmentation pattern indicate that the peptide was subjected to the loss of ammonia from the N-terminus. This loss was probably due to an in-source fragmentation process.

of (Ser)-(Xaa)-(Glu or previously phosphorylated Ser) [8–10]. The amino acids surrounding the rhBMP-15 phosphorylation site form the consensus sequence (Ser<sup>6</sup>-Ala<sup>7</sup>-Glu<sup>8</sup>), while the mature region of rhGDF-9 contains a similar consensus sequence: (Ser<sup>6</sup>-Ser<sup>7</sup>-Glu<sup>8</sup>). Thus, both rhBMP-15 and rhGDF-9 conform to the G-CK consensus.

Here, we show that rhBMP-15 is an excellent substrate for G-CK. A low apparent  $K_m$  value of 0.196  $\mu$ M was estimated from the phosphorylation reaction of rhBMP-15 by G-CK, indicating that the phosphorylation is physiologically relevant. While rhGDF-9 is also a substrate for G-CK, it was phosphorylated much more slowly, so kinetic analysis was not possible. We next used MS to identify the residues phosphorylated by G-CK and found that Ser<sup>6</sup>, i.e., the residue found phosphorylated in BMP-15 [6] is the one readily phosphorylated by G-CK in the mature form of BMP-15. Also the mature form of rhGDF-9 was phosphorylated at Ser<sup>6</sup>. Both their residues conform to the consensus sequence of G-CK (S-X-E/Ps) as they display the sequences Ser<sup>6</sup>-Ala-Glu and Ser<sup>6</sup>-Ser-Glu in rhBMP-15 and rhGDF-9, respectively. Phosphorylation of the full-length rhBMP-15 and rhGDF-9 proteins, which have not been cleaved to yield the mature forms, with G-CK also revealed phosphorylation albeit weaker at the Ser<sup>6</sup> residue of both proteins. No phosphorylation sites were detected in the proregion of either rhBMP-15 or rhGDF-9 proprotein. Thus, both rhBMP-15 and rhGDF-9 contain a single phosphorylation site located at Ser<sup>6</sup> of the mature region of the proteins.

Our data add a new facet to the multifarious repertoire of functions of one of the most enigmatic protein kinases, G-CK. Believed for a long time to be a highly dedicated enzyme committed only to the phosphorylation of casein within lactating mammary gland,

G-CK was later shown to be a pleiotropic protein kinase present in the Golgi apparatus of many tissues where it is presumably implicated in the phosphorylation of a wide variety of phosphoacceptor substrates displaying the motif pS-X-E/pS [12]. As unambiguously shown many years ago this motif is similar to but definitely distinct from the one recognized by another highly pleiotropic protein kinase, CK2 (S/T-X-X-E/D) [9]. This has made possible the development of an extremely selective peptide substrate unaffected by any other kinase [10], whose phosphorylation still represents the strongest criterion for G-CK identification, since the gene(s) expressing G-CK are still unknown, despite many efforts in different labs.

Recently G-CK activity has been also linked to the autism spectrum disorders as revealed by defective phosphorylation of salivary proteins at sites having the stigmata for being G-CK targets [25]. It appears therefore more and more urgent to fill this gap and disclose the gene encoding for this as yet orphan enzyme, responsible for the phosphorylation among many others substrates, of casein which was the first phosphoprotein to be described as early as in 1883 [26].

In conclusion, we have identified G-CK as the kinase responsible for phosphorylating rhBMP-15 and rhGDF-9. Interestingly, both rhBMP-15 and rhGDF-9 are phosphorylated at the same serine residue in the mature form of the proteins, Ser<sup>6</sup>. rhBMP-15 and rhGDF-9 each contain only a single phosphorylation site, further underscoring the potential importance of this post-translational modification in regulation of bioactivity. These findings provide a new understanding of BMP-15 and GDF-9 regulation and may result in the identification of novel causes of human ovarian dysfunction.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.12.052.

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