Title:

PGRMC1 localization and putative function in the nucleolus of bovine granulosa cells and oocytes

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Abstract

Progesterone Receptor Membrane Component-1 (PGRMC1) is a highly conserved multifunctional protein that is found in numerous systems, including reproductive system. Interestingly, PGRMC1 is expressed at several intracellular locations, including the nucleolus. The aim of this study is to investigate the functional relationship between PGRMC1 and nucleolus. Immunofluorescence experiments confirmed PGRMC1's nucleolar localization in bovine granulosa cells (bGC) and oocytes. Additional experiments conducted on bGC revealed that PGRMC1 co-localizes with nucleolin, a major nucleolar protein. Furthermore, small interfering RNA (RNAi) mediated gene-silencing experiments showed that when PGRMC1 expression was depleted, nucleolin translocated from the nucleolus to the nucleoplasm. Although PGRMC1 influenced the localization of nucleolin, a direct interaction between these two proteins was not detected using in situ proximity ligation assay. This suggests the involvement of additional molecules in mediating the co-localizaton of PGRMC1 and nucleolin. Since nucleolin translocates into the nucleoplasm in response to various cellular stressors, PGRMC1's

ability to regulate its localization within the nucleolus is likely an important component of mechanism by which cells response to stress. This concept is consistent with PGRMC1's well-described ability to promote ovarian cell survival and provides a rationale for future studies on PGRMC1, nucleolin and the molecular mechanism by which these two proteins protect against the adverse effect of various cellular stressors.

Introduction

Progesterone Receptor Membrane Component 1 (PGRMC1) is a widespread and multifunctional protein that is highly conserved in eukaryotes. It belongs to the membrane associated progesterone receptor (MAPR) family and is expressed in several mammalian organs and tissues (Runko *et al.* 1999, Raza *et al.* 2001, Sakamoto *et al.* 2004, Bali *et al.* 2013a, Bali *et al.* 2013b), including those of the reproductive system (Zhang *et al.* 2008, Luciano *et al.* 2010, Aparicio *et al.* 2011, Luciano *et al.* 2011, Keator *et al.* 2012, Saint-Dizier *et al.* 2012, Tahir *et al.* 2013, Kowalik *et al.* 2016). Specifically, granulosa and luteal cells of human, rodent, bovine and canine ovaries (Engmann *et al.* 2006, Peluso 2006, Aparicio *et al.* 2011, Luciano *et al.* 2011, Tahir *et al.* 2013, Griffin *et al.* 2014, Terzaghi *et al.* 2016) as well as oocytes express PGRMC1 (Luciano *et al.* 2010, Luciano *et al.* 2013, Terzaghi *et al.* 2016).

Multiple functions are attributed to PGRMC1 (reviewed in (Cahill 2007, Brinton *et al.* 2008, Neubauer *et al.* 2013, Peluso & Pru 2014, Cahill *et al.* 2016, Ryu *et al.* 2017)) as reflected by it being localized to numerous sub-

cellular compartments. As predicted by the presence of a transmembrane domain, PGRMC1 localizes in several membranous compartments, such as the endoplasmic reticulum, the Golgi apparatus, the nuclear and plasma membranes, the endosomes, and the secretory vesicles (Meyer *et al.* 1996, Raza *et al.* 2001, Bramley *et al.* 2002, Hand & Craven 2003, Shin *et al.* 2003, Sakamoto *et al.* 2004, Min *et al.* 2005, Peluso *et al.* 2006, Zhang *et al.* 2008, Neubauer *et al.* 2009, Ahmed *et al.* 2010, Roy *et al.* 2010, Wu *et al.* 2011, Xu *et al.* 2011, Mir *et al.* 2012, Mir *et al.* 2013, Thomas *et al.* 2014). Interestingly, PGRMC1 is also detected in the nucleus (Beausoleil *et al.* 2004, Peluso *et al.* 2008, Zhang *et al.* 2008, Ahmad *et al.* 2009, Peluso *et al.* 2009, Luciano *et al.* 2010, Peluso *et al.* 2010a, Peluso *et al.* 2012), specifically to the nucleolus (Ahmad *et al.* 2009, Luciano *et al.* 2010, Boisvert *et al.* 2012, Thul *et al.* 2017)] http://www.proteinatlas.org]. This high compartmentalization suggests that at each site PGRMC1 participates in the control of precise cellular processes.

In order to shed light into the intricate story of PGRMC1's biological significance, it is important to dissect the function of PGRMC1 at each sub-cellular compartment. To this end, we have started to address whether PGRMC1 has a role in regulating the nucleolar function. Although the nucleolus' main function involves ribosome subunits production, recent advances describe it as a multifunctional subnuclear compartment. It appears that the nucleolus is a dynamic structure, which disassembles during mitosis and responds to signaling events during interphase. As such, it is involved in cell cycle control, especially regulating protein modifications such as sumoylation and phosphorylation or sequestrating specific proteins (Boisvert

et al. 2007). Furthermore, it acts as a stress sensor mediating p53 stabilization in order to arrest cell cycle progression (Boisvert *et al.* 2007, Boulon *et al.* 2010). Thus, the overall goal of the present study is to examine the role of PGRMC1 on nucleolar function, particularly its relationship with nucleolin (NCL), a well-characterized nucleolar protein (Boisvert *et al.* 2007, Tajrishi *et al.* 2011).

Material and methods

Reagents

All the chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO) except for those specifically mentioned. Gene silencing was performed by using the Stealth RNAi™ siRNA technology from Life Technologies as previously described (Terzaghi *et al.* 2016) using PGRMC1 Stealth RNAi (PGRMC1 RNAi: (RNA)-GAG UUG UAG UCA AGU GUC UUG GUC U) within the coding region of the bovine PGRMC1 sequence (RefSeq: NM_001075133). Negative control (cat n. 12935-200) was chosen among the Stealth RNAi negative control (CTRL RNAi) duplexes available from Life Technologies, designed to minimize sequence homology to any known vertebrate transcript. Primary antibodies used in this study are listed in Table 1.

Sample collection

Bovine samples:

Ovaries from Holstein dairy cows were recovered at the abattoir (INALCA S.p.A., Ospedaletto Lodigiano, LO, IT 2270M CE, Italy) from pubertal females subjected to routine veterinary inspection and in accordance to the specific health requirements stated in Council Directive 89/556/ECC and subsequent modifications. Ovaries were transported to the laboratory within 2 hours in sterile saline (NaCl, 9 g/l) maintained at 26°C and all subsequent procedures, unless differently specified, were performed at 35-38°C. Bovine granulosa cells (bGC) were collected as previously described (Terzaghi *et al.* 2016). Briefly the content of 2-8 mm ovarian follicles, which typically contain fully-grown oocytes, was aspirated and cumulus-oocyte complexes (COCs) were collected and processed for further immunofluorescence analysis (see below). Remaining follicular cells were washed in M199 supplemented with HEPES 20 mM, 1,790 units/L Heparin and 0.4% of bovine serum albumin (M199-D). The cell pellet was re-suspended in 1ml of Dulbecco's modified growth medium supplemented with 10% of bovine calf serum, 100 U/ml penicillin G, 100 µg/ml, Streptomycin and Glutamax 100U/ml (Gibco). The cell suspension was plated in a 25 cm^2 flask with 6 ml of growth medium and incubated in humidified air at 37°C with 5% CO_2. After 24 hours cells were gently washed with PBS and the growth medium was changed. Cells were incubated until confluence, then collected after trypsinization and re-plated according to the experimental design (see below).

Oocytes in their growing phase, characterized by a diffuse filamentous pattern of chromatin in the nuclear area and the presence of an active nucleolus were collected as previously described from 0.5 to <2 mm early antral follicles by rupturing the follicle wall under the stereomicroscope (Lodde

et al. 2008); Both COCs collected from 0.5 to <2 mm and 2-8 antral follicles were mechanically denuded using the vortex and fixed for further immunofluorescence analysis.

RNAi treatment

RNA interference (RNAi) experiments on bGC were conducted as previously described (Terzaghi *et al.* 2016). Cells were plated in a total number of 2 X 10^5 bGC cells in 2 ml of medium on cover glasses in 35-mm culture dishes and incubated in humidified air at 37°C with 5% CO_2. After 24 h, cells at 50-70% of confluence were transfected with 6 µl of 20 µM PGRMC1 Stealth RNAi or CTRL RNAi combined with 10 µl of Lipofectamine RNAi MAX (Life Technologies) in a final volume of 2 ml OPTIMEM (Life Technologies), according to the manufacturer protocol, and cultured for 48 h.

Western blot analysis

The levels of PGRMC1 protein expression in CTRL and PGRMC1 RNAi treated bGC were assessed by Western blotting assay as previously described (Terzaghi *et al.* 2016). PGRMC1 or CTRL RNAi treated bGC were lysed in radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 (NP-40), and 0.25% sodium deoxycolate], supplemented with protease inhibitors and phosphatase inhibitors. All procedures were conducted on ice. Total amount of protein was determined using the Qubit® Protein Assay Kit and Qubit® fluorometer (Thermo Fisher Scientific). 20 µg of total protein/lane were used for Western blottings. After the run, samples were transferred to nitrocellulose membrane

(Bio-Rad), which was then incubated with 5% dry milk powder in TBS containing 0.1% tween (TBS/T) for 2 hours at room temperature. PGRMC1 immunodetection was conducted using the rabbit polyclonal antibodies (see table 1) in 5% dry milk TBS/T. PGRMC1 was revealed using a stabilized goat anti rabbit IgG peroxidase conjugated antibody and detected using the Super Signal West Dura Extended Duration Substrate (Thermo Fisher Scientific). The nitrocellulose membrane was stripped in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl [pH 6.7]) at 50°C for 30 min and re-probed with the anti beta tubulin antibody at dilution 1:1000, which was revealed using a stabilized goat anti mouse IgG peroxidase conjugated antibody as loading control.

Immunofluorescence

Immunofluorescence staining was performed on bGC as previously described (Lodde & Peluso 2011, Terzaghi *et al.* 2016). Briefly, cells were fixed in 4% paraformaldehyde in PBS for 7 minutes and permeabilized with 0.1% triton-X in PBS for 7 minutes. Samples were blocked with 20% normal donkey serum in PBS and incubated overnight at 4°C with the rabbit anti PGRMC1 antibody (see table1). Double immunostaining was performed on bGC by incubating the samples with the rabbit anti PGRMC1 or the mouse anti NCL antibodies or a combination of the two. After incubation with secondary antibodies for 1 h at room temperature, samples were washed and finally mounted on slides in the antifade medium Vecta Shield (Vector Laboratories) supplemented with 1 µg/ml 40,6-diamidino-2-phenylindole (DAPI). Immunofluorescent analysis on bovine oocytes were performed as

previously described (Luciano *et al.* 2010) on 4% paraformaldehyde fixed oocytes. Immunofluorescent staining was performed as described for bGC with the exception that oocytes were fixed for 30 min at room temperature followed by 30 min and permeabilized with 0.3% Triton-X 100 for 10 minutes.

bGC and oocytes were analyzed on an epifluorescence microscope (Eclipse E600; Nikon) equipped with a 40 X and a 60X objective, a digital camera (Nikon digital sight, DS-U3) and software (NIS elements Imaging Software; Nikon). Immunofluorescence negative controls, which were performed by omitting one or both the primary antibodies, did not show any staining under the same exposure settings. Images that were used for image quantification analysis were captured under the same settings.

In situ Proximity Ligation Assay (PLA)

In situ Proximity Ligation Assay (PLA; Duolink® SIGMA) was used to assess the interaction between PGRMC1 and nucleolin in bGC following following the manufacturer protocol. Primary antibodies for PGRMC1 and nucleolin were the same used for immunofluorescence, while anti-rabbit PLUS and anti-mouse MINUS PLA probes were used as secondary antibodies. Negative controls were performed omitting one of the two primary antibodies. Cells were mounted with Duolink mounting medium.

Image analysis

Quantification of Fluorescent Intensity (FI) signal was performed using the ImageJ software (https://imagej.net). The nucleolar signal of PGRMC1 in bGC was quantified calculating the integrated density of PGRMC1 signal

selecting the whole PGRMC1 positive areas in the nucleus of a total of 50 cells for each treatment (CTRL RNAi and PGRMC1 RNAi treated cells) at 48 h after RNAi treatment. Data were polled and the mean FI of the CTRL RNAi treated group was set at 100%. FI values of the CTRL RNAi and PGRMC1 RNAi treatments were expressed as a percentage of the mean CTRL RNAi value. For image quantification of the NCL nucleolar and nucleoplasmic signals, threshold was selected by choosing a cutoff value such that all the nucleolar areas with an intense NCL signal within each cell. Then, the NCL total nuclear FI was assessed by selecting the whole nuclear area and calculating the integrated density of the corresponding regions of interests (ROI) of a total of 50 randomly selected nuclei in CTRL RNAi and PGRMC1 RNAi treated cells. The NCL nucleolar signal was calculated by analyzing the integrated density of the threshold area in each nucleus, while the NCL nucleoplasmic signal was calculated by subtracting the total nucleolar FI to the total nuclear FI of each nucleus. Data were polled and the mean nucleolar and nucleoplasmic NCL FI of the CTRL RNAi treated group were set at 100%. FI values of the CTRL RNAi and PGRMC1 RNAi treatments were expressed as a percentage of the mean CTRL RNAi value. Background signals did not change significantly among treatments

Statistical analysis

Experiments were run in triplicates, unless otherwise specified. All statistical analysis was done using Prism software (GraphPad Prism v. 6.0e, La Jolla, CA, USA). Data from the replicate experiments were pooled and the

data expressed as a mean ± SEM. Student's t test was used to determine differences between two groups.

Results

PGRMC1 localization

Immunofluorescence analysis indicated that PGRMC1 localized to areas of the interphase nucleus that were not stained by DAPI. PGRMC1's nuclear localization in bGC was the same regardless of which PGRMC1 antibody was used (Figure 1). However, nuclear staining for PGRMC1 with the Sigma Prestige antibody displayed a diffuse signal with the staining associated with DAPI-negative areas and only slightly more intense than that observed for the overall nucleus. In contrast, the non-DAPI stained areas within the nucleus were more intensely stained using the PGRMC1 antibody provided by Proteintech (Figure 1). These non-DAPI stained areas typically correspond to areas of the interphase nucleus where the nucleoli resided.

PGRMC1 co-localization with NCL

To further characterize PGRMC1 localization in the nucleus, we evaluated its co-localization with the nucleolar marker, nucleolin (NCL), in both bGC and bovine growing and fully-grown oocytes. Immunofluorescence data indicated that the two proteins co-localized in the nucleolus of bGC as shown in Figure 2; PGRMC1 signal appeared as a dotted pattern in the area corresponding to the nucleolus compared to NCL signal, which fully covered the nucleolus space (i.e. nuclear areas not stained by DAPI). Although co-

localized, in situ proximity ligation assay did not detect an interaction between PGRMC1 and NCL, indicating the absence of a direct interaction between the two proteins in bGC (Data not shown).

In growing bovine oocytes, which are characterized by the presence of an active nucleolus (Fair *et al.* 1996, Lodde *et al.* 2008), NCL marked the nucleolus and showed a light diffuse staining pattern in the nucleoplasm as previously described (Fair *et al.* 2001, Baran *et al.* 2004, Maddox-Hyttel *et al.* 2005). In particular, NCL nucleolar signal was intense and slightly more concentrated at the periphery of the nucleolus. In these oocytes PGRMC1 localized in the nucleolus showing a dotted staining pattern, similar to that observed in bGC nucleoli (Figure 2). In fully-grown oocytes (Figure 2), which typically displayed inactive nucleolar remnants (Fair *et al.* 1996, Lodde *et al.* 2008), NCL was mainly dispersed in the nucleoplasm with a faint staining in the nucleolar remnants as previously described (Fair *et al.* 2001, Baran *et al.* 2004, Maddox-Hyttel *et al.* 2005). In these oocytes PGRMC1 concentrated in one or multiple dots where it co-localized with NCL.

Assessment of PGRMC1 and nucleolin functional interaction

In order to establish the possible functional relationship between PGRMC1 and NCL, we silenced PGRMC1 expression in bGC by using RNAi. The RNAi protocol was previously validated by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) showing a significant reduction of PGRMC1 mRNA levels compared to CTRL RNAi treated group (Terzaghi *et al.* 2016). That PGRMC1 expression was reduced after 48h PGRMC1-RNAi treatment was confirmed by Western blot analysis, regardless

of which PGRMC1 antibody was used. As shown in Figure 3A, PGRMC1 was present in multiple bands, whose intensity decreased after 48h of PGRMC1 RNAi treatment. Moreover, quantification of PGRMC1 nucleolar immunofluorescent signal in PGRMC1 and CTRL-RNAi treated bGC revealed an approximate 40% decrease in PGRMC1 abundance in the nucleolus, which also gives confirmation of the specificity of PGRMC1's nucleolar localization (Figure 3B and 3C).

In order to assess the putative functional relationship between PGRMC1 and NCL, the effect of depleting PGRMC1 on the localization of NCL was evaluated. As shown in Figure 4, when PGRMC1 was depleted, a significantly higher quantity of nucleolin was present in the nucleoplasm when compared to the CTRL-RNAi treated group.

Discussion

The present findings demonstrate that PGRMC1 localizes to the nucleolus of both bovine granulosa cells and oocytes, suggesting that PGRMC1 has a role in regulating the function of the nucleolus of these two cell types. The prominent nucleolar localization of PGRMC1 as revealed using the Protein Tech antibody is consistent with investigations of non-ovarian cells that detect PGRMC1 within the nucleolus by either immunohistochemistry (http://www.proteinatlas.org) or mass spectrometric analysis (Ahmad *et al.* 2009, Luciano *et al.* 2010, Boisvert *et al.* 2012, Thul *et al.* 2017). However, the rabbit polyclonal antibody to PGRMC1 provided by Sigma Prestige detects PGRMC1 not only within the nucleolus but also in other interchromatin regions that resemble the nuclear speckles (Spector &

Lamond 2011). The reason for this discord likely relates to the two antibodies detecting different molecular weight forms of PGRMC1. Western blots using either the Proteintech or the Sigma Prestige antibody detect PGRMC1 as bands at ≈ 25 and ≈ 55 kDa, while the Sigma antibody also detects an additional band at 37 kDA and two bands greater than 55 kDA. All the bands detected by either antibody are specific since their intensity is decreased in PGRMC1 RNAi-treated cells. The different size forms of PGRMC1 are due to dimerization and post-translational modifications such as phosphorylation and sumoylation (Neubauer *et al.* 2008, Peluso *et al.* 2010b, Peluso *et al.* 2012, Kabe *et al.* 2016). Therefore, it is not surprising that polyclonal antibodies obtained using different immunogens may preferentially recognize one or multiple forms of PGRMC1, which in turn might preferentially localize in different subcellular compartment.

Because the Proteintech antibody precisely localizes PGRMC1 to the nucleolus, it was used to determine whether PGRMC1 co-localizes with the nucleolar protein, NCL. This approach reveals that PGRMC1 and NCL co-localizes to the nucleolus in bGC. Moreover, depletion of PGRMC1 results in NCL within the nucleolus redistributing to nucleoplasm in these cells. Thus, localization of NCL is likely dependent in part on PGRMC1. This observation is biologically relevant since NCL mobilization from the nucleolus into the nucleoplasm is induced by different types of cellular stress. For example, heat shock, ionizing radiation, and hypoxia all promote the translocation of nucleolin into the nucleoplasm (Daniely & Borowiec 2000, Daniely *et al.* 2002). In particular, NCL redistribution is induced by heat stress in HeLa cells and accompanied by an increase in the formation of a complex between NCL

and Replication Protein A (RPA) (Daniely & Borowiec 2000), which exerts important functions during DNA replication (Iftode *et al.* 1999). NCL-RPA interaction in turn strongly inhibits DNA replication, likely by sequestering RPA away from sites of ongoing DNA synthesis (Daniely & Borowiec 2000). Other studies in U2-OS and U2-OS p53-depleted cells demonstrate that NCL redistribution occurs when stress is induced by γ-irradiation and treatment with the radiomimetic agent, camptothecin. Under these stress conditions, NCL binds p53, which facilitates its transit into the nucleoplasm (Daniely *et al.* 2002). These stress-induced changes in NCL's localization suggest that various stressors change PGRMC1 function, altering its ability to retain NCL, which allows NCL to transit to the nucleoplasm. This concept merits further investigation.

Although PGRMC1 and NCL often co-localize to the same sub-region of the nucleolus of bGC, they do not seem to directly interact, since we were not able to detect a positive signal by means of PLA assay. This might suggest that their functional interaction could involve the participation of other yet to be identified protein. Interestingly a known PGRMC1 binding protein, Plasminogen Activator Inhibitor 1 RNA-Binding Protein (PAIRBP1) (Peluso *et al.* 2006, Peluso *et al.* 2008, Peluso *et al.* 2013) (also known as SERPINE1 mRNA Binding Protein 1), which is typically found in the cytoplasm, localizes to the nucleolus under specific experimental stress induced conditions in Hela cells, such as treatment with arsenite and the methylation inhibitor adenosine periodate (Lee *et al.* 2014). Therefore, it is possible that PAIRBP1 competes with this putative intermediary protein for binding to PGRMC1. The stress-induced formation of the PGRMC1: PAIRBP1 complex could potentially

interfere with PGRMC1' ability to retain NCL within the nucleolus and account for the translocation of nucleolin from the nucleolus into the nucleoplasm under stress condition.

Finally, the present study reveals the relationship between PGRMC1 and NCL in bovine oocytes. PGRMC1 is present in the nucleolus of growing oocytes and the signal is retained to some extent in the nucleolar remnants of fully-grown bovine oocytes. During growth, the oocyte's nucleus is characterized by the presence of a diffuse filamentous transcriptionally active chromatin and by a functional fibrillogranular nucleolus, which is gradually disassembled forming the so called 'nucleolar remnants', along with the progressive inactivation of rRNA synthesis that occurs at the end of oocyte growth (Fair *et al.* 1996, Lodde *et al.* 2008). Ultrastructurally, the nucleolar remnants appear as electron dense spheres often showing a semilunar fibrillar center-like structures attached (Fair *et al.* 1996, Lodde *et al.* 2008). In bovine oocytes, proteins such as RNA polymerase I and UBF remain associated to the inactive nucleolar remnants, while others, such as NCL and nucleophosmin mostly disperse in the nucleoplasm (Fair *et al.* 2001, Baran *et al.* 2004, Maddox-Hyttel *et al.* 2005). Upon meiotic resumption and during oocyte maturation the nucleolar remnant further disassembles and nucleolar proteins are probably dispersed in the cytoplasm. After fertilization the so called 'nucleolar precursor bodies' (NPBs) appear as electron dense compact spheres in the male and female pronuclei reviewed in (Maddox-Hyttel *et al.* 2005). The NPBs serve for the re-establishment of a functional fibrillogranular nucleolus, which in bovine occurs at the time of major embryonic genome activation (at the 8-16 cell stage). It has been proposed that proteins engaged

in late rRNA processing of maternal origin, including NCL, are to some extent re-used for nucleologenesis in the embryo while others need to be de-novo transcribed before being incorporated in the nucleolus (reviewed in (Maddox-Hyttel *et al.* 2005)). In this scenario, PGRMC1 localization in growing and fully-grown oocytes and in the NPBs of bovine zygotes (Luciano *et al.* 2010) suggests a role in both the disassembly and the reassembly of the nucleolus during meiosis and early embryogenesis. Interestingly, in growing oocytes (as in bGC) PGRMC1 and NCL showed a different localization pattern, with PGRMC1 showing a dotted localization. A similar pattern in growing bovine oocytes has been reported for the RNA polymerase I-specific transcription initiation factor, Upstream Binding Factor (UBF) (Baran *et al.* 2004). In future studies, it will be important to assess whether a specific functional interaction between NCL or other nucleolar proteins and PGRMC1 exists during early embryonic development and thereby influences the embryogenesis.

Tables

Table 1: list of antibody used

Cell type	Technique	Primary antibody	Secondary antibody

Bovine Granulosa Cells (bGC)	Immunofluorescence	- Rabbit polyclonal anti-**PGRMC1** (1:50; Protein tech, 12990-1-AP) - Rabbit polyclonal anti-**PGRMC1** (1:50; Sigma, HPA002877) - Mouse monoclonal anti-**nucleolin** (1:2000; Thermo scientific, MA1-20800)	- TRITC-labeled donkey anti-rabbit (1:100; Vector Laboratories, Inc.) - Alexa Fluor 488-labeled donkey anti-mouse (1:1000;Life Technologies)
Oocytes	Immunofluorescence	- Rabbit polyclonal anti-**PGRMC1** (1:200; Protein tech, 12990-1-AP) - Mouse monoclonal anti-**nucleolin** (1:2000; Thermo scientific, MA1-20800)	- TRITC-labeled donkey anti-rabbit (1:100; Vector Laboratories, Inc.) - Alexa Fluor 488-labeled donkey anti-mouse (1:1000;Life Technologies)
Bovine Granulosa Cells (bGC)	In situ proximity ligation assay (PLA)	- Rabbit polyclonal anti-**PGRMC1** (Protein tech, 12990-1-AP -1:50) - Mouse monoclonal anti-**nucleolin** (1:2000, Thermo scientific, MA1-20800)	Anti-rabbit PLUS and anti-mouse MINUS PLA probes (Duolink® In Situ PLA®)
Bovine Granulosa Cells (bGC)	Western blot	- Rabbit polyclonal anti-**PGRMC1** (1:200; Protein tech, 12990-1-AP) - Rabbit polyclonal anti-**PGRMC1** (1:50; Sigma, HPA002877) - Mouse monoclonal anti-**beta tubulin** (1:1000; Sigma, T8328)	- Goat anti rabbit IgG peroxidase conjugated (1:1000; Thermo scientific) - Goat anti mouse IgG peroxidase conjugated (1:1000; Thermo scientific)

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430 **Figure caption**

Figure 1: PGRMC1 immunofluorescent localization (red) in bGC obtained using SIGMA Prestige (A) and the Proteintech (B) rabbit polyclonal antibodies. DNA is stained with DAPI (blue). Insets show a single magnified nucleus. Note that both antibodies show intense staining in DAPI negative areas (arrows).

Figure 2: PGRMC1 (red) and NCL (green) immunofluorescence localization in bGC, growing oocytes and fully-grown oocytes. DNA is stained with DAPI (blue). Merged images shows partial PGRMC1-nucleolin co-localization (yellow). Insets represent 3X magnification.

Figure 3: Effect of PGRMC1 RNAi mediated gene silencing on PGRMC1 expression. (A) Representative Western blotting analysis showing PGRMC1 protein levels in PGRMC1 and CTRL-RNAi treated bGC after 48 h of treatment using the SIGMA Prestige and the Proteintech rabbit polyclonal antibodies. Beta tubulin was used as loading control. (B) Representative images showing PGRMC1 immunofluorescent staining in PGRMC1 and CTRL-RNAi treated bGC. (C) Graph showing analysis of PGRMC1 immunofluorescence intensity in the nucleolus of PGRMC1 and CTRL-RNAi treated bGC after 48h of treatment; * indicates significant difference (t-test, $p<0.05$)

Figure 4: Effect of PGRMC1 RNAi mediated gene silencing on NCL localization. (A) Representative images showing NCL immunofluorescent staining in PGRMC1 and CTRL-RNAi treated bGC; note the increased

nucleoplasmic signal in PGRMC1 RNAi treated cell. (C) Graph showing analysis of PGRMC1 immunofluorescence intensity in the nucleoplasm of PGRMC1 and CTRL-RNAi treated bGC after 48h of treatment; * indicates significant difference (t-test, $p<0.05$)

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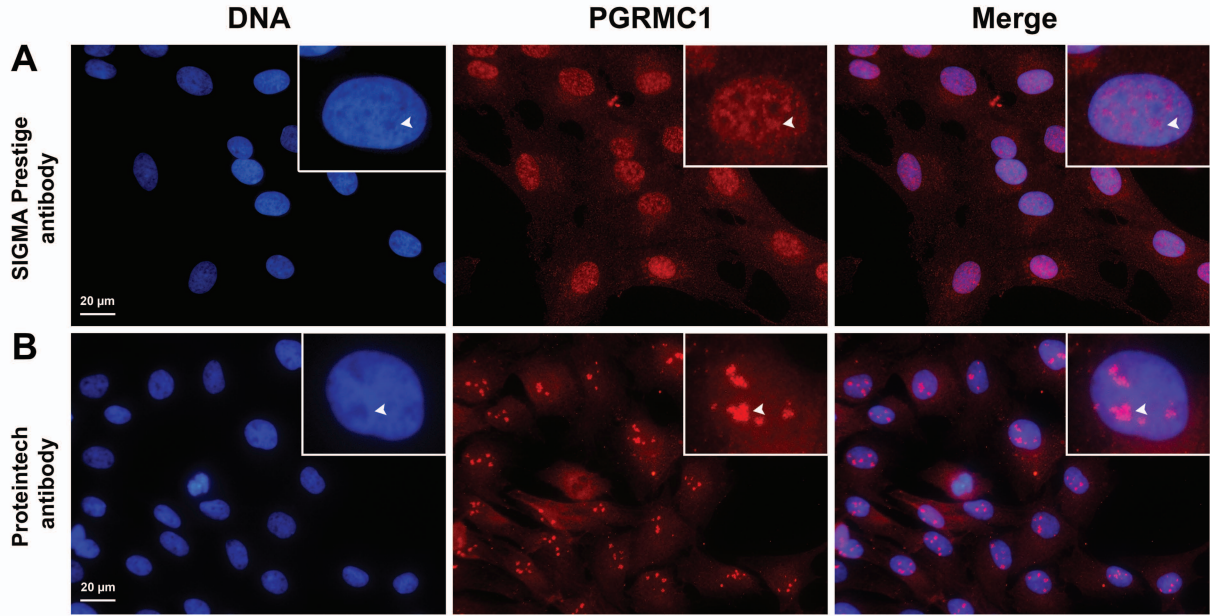
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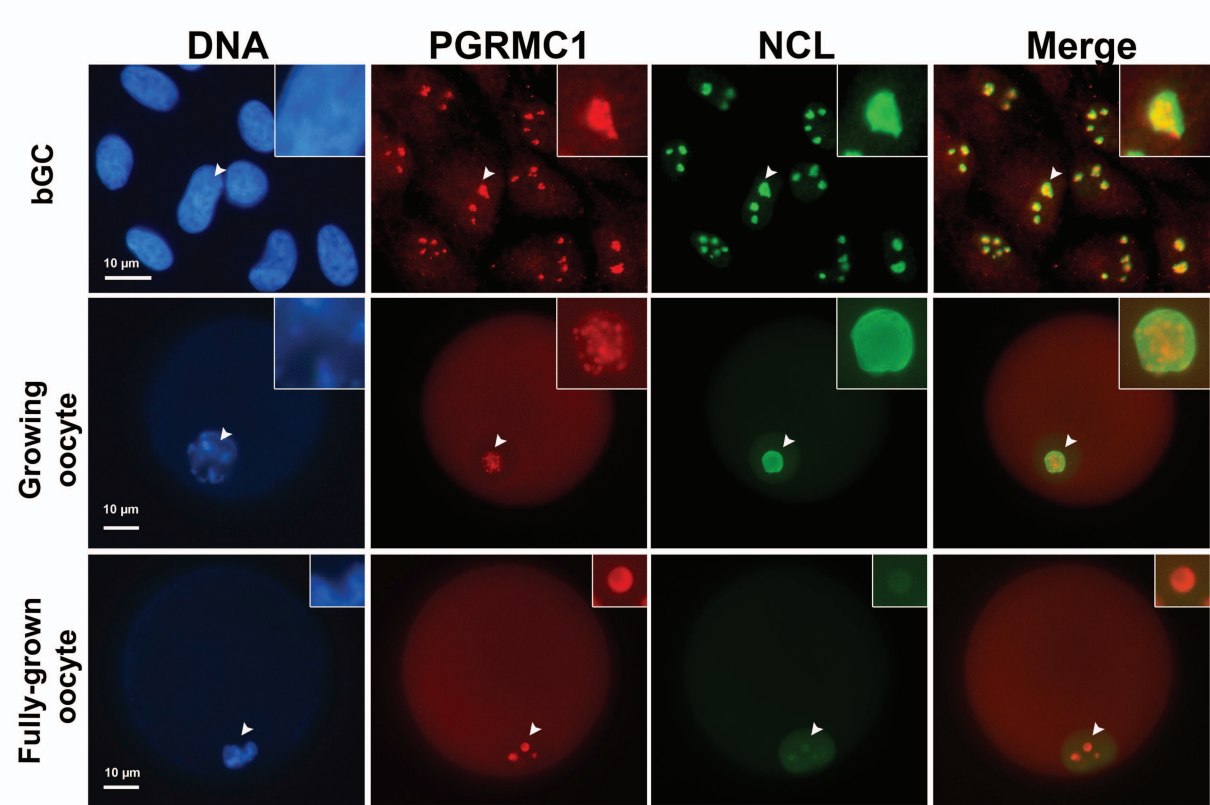
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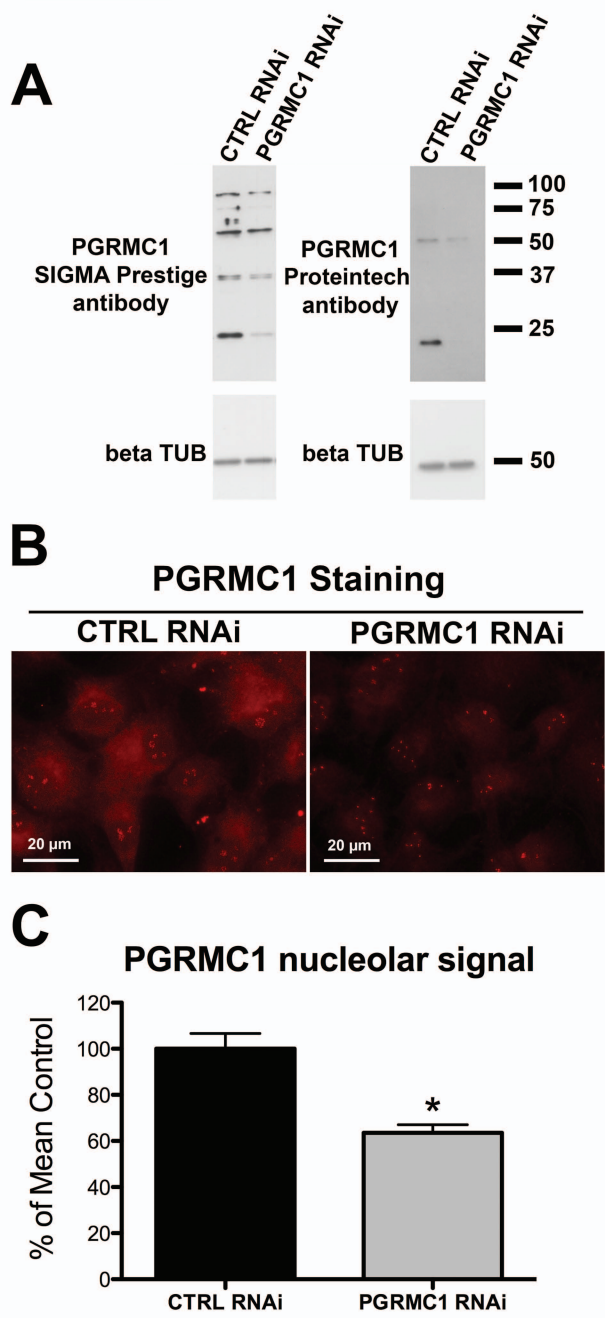
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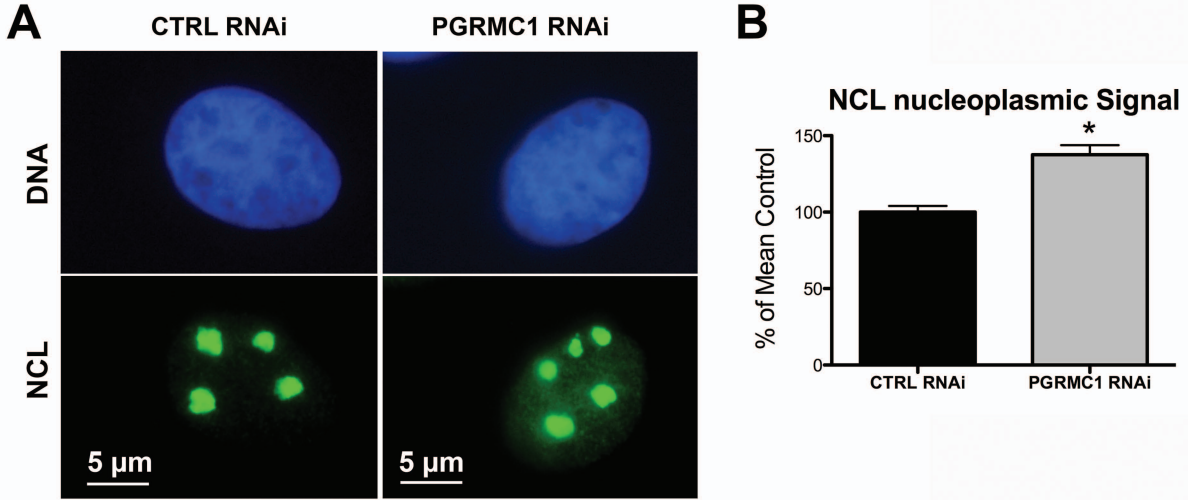
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