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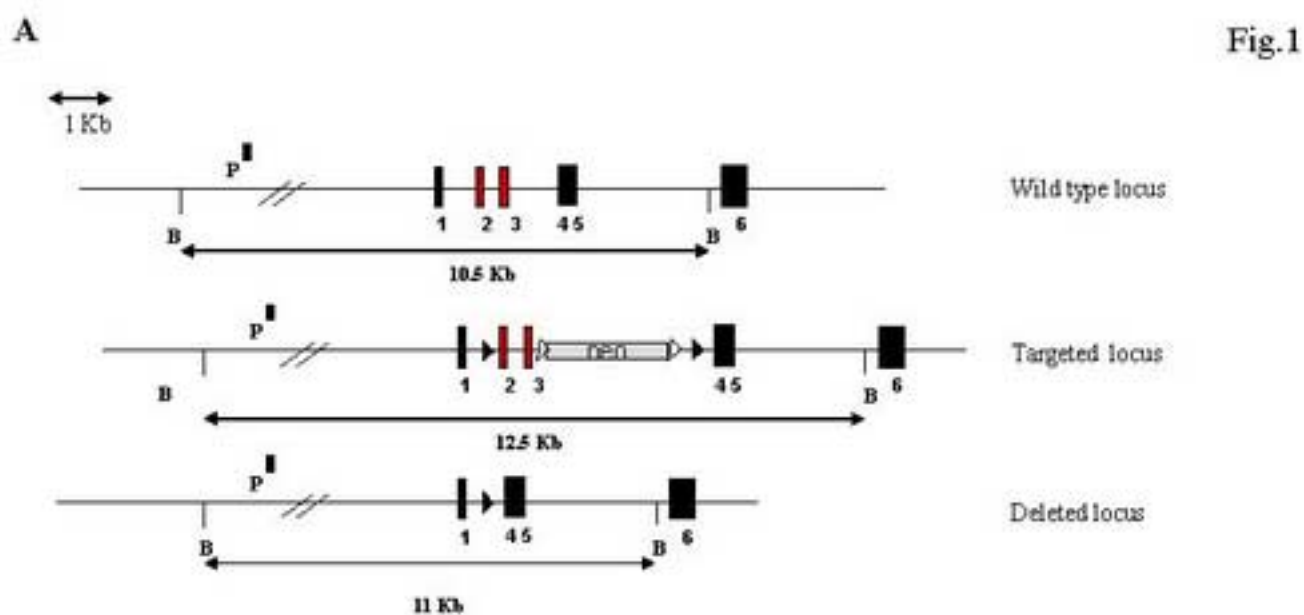
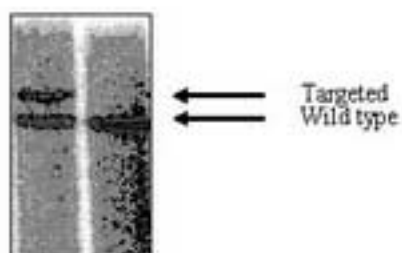


Fig.1

B



C

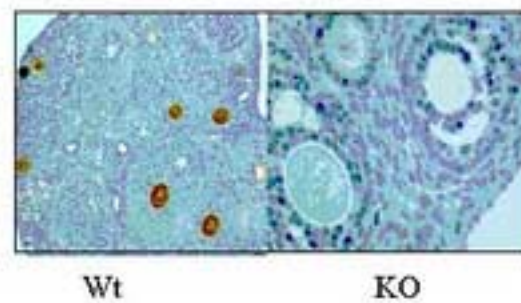


Fig 3

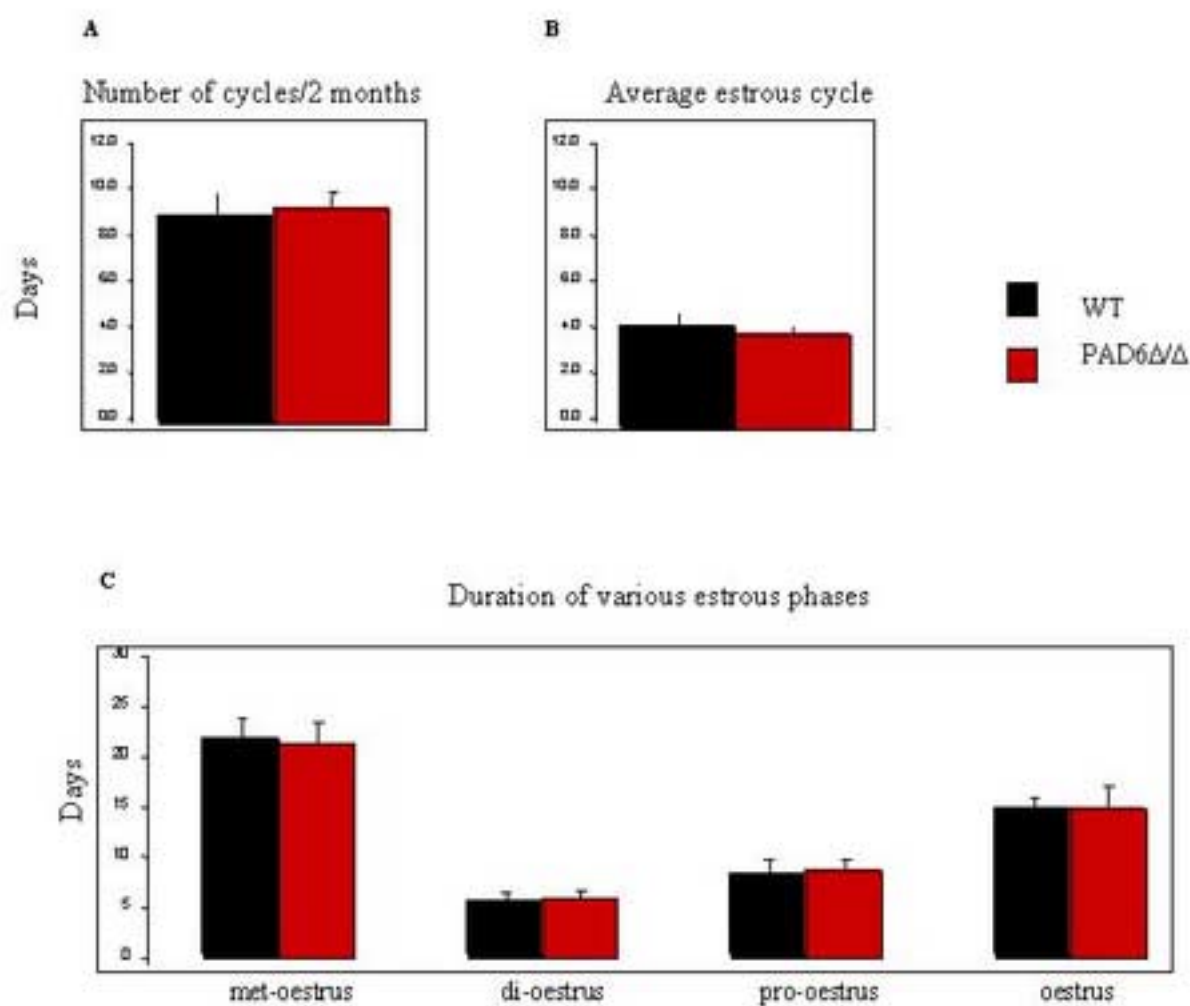


Fig 4

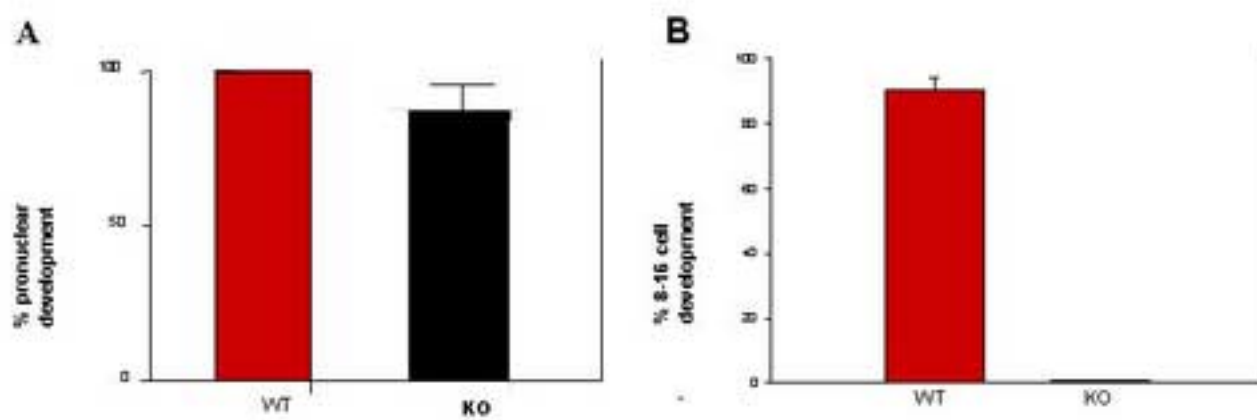
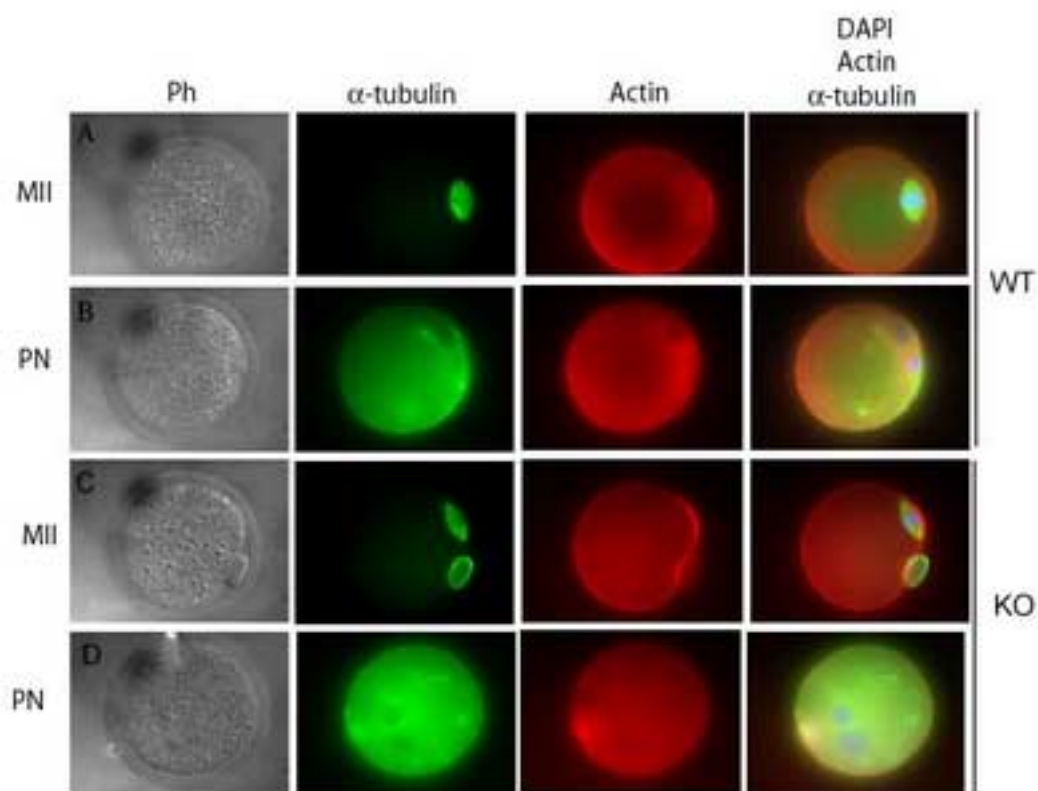


Fig 6



(MII = metaphase II arrest) (PN = pronuclear)

Fig.2

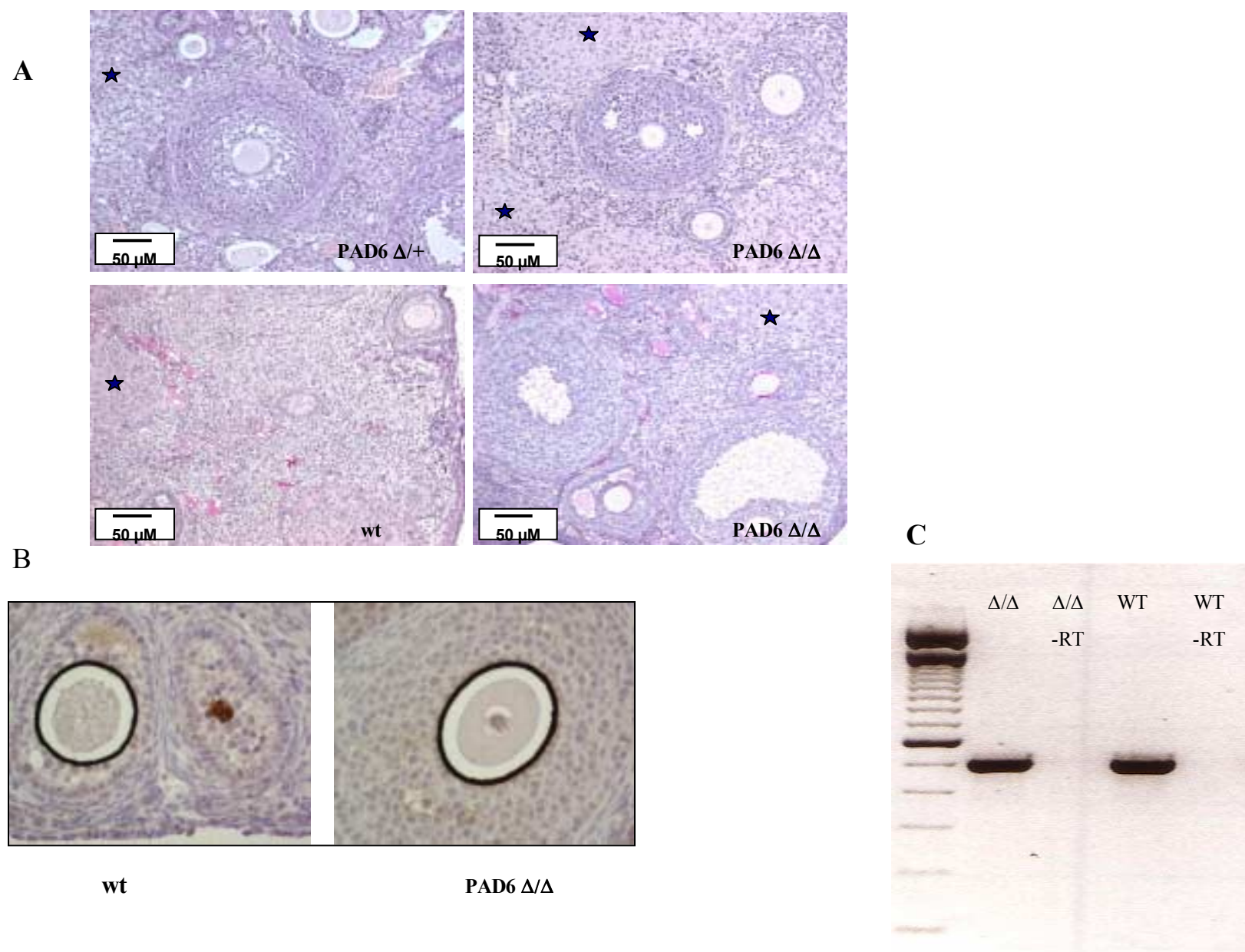


Fig.5

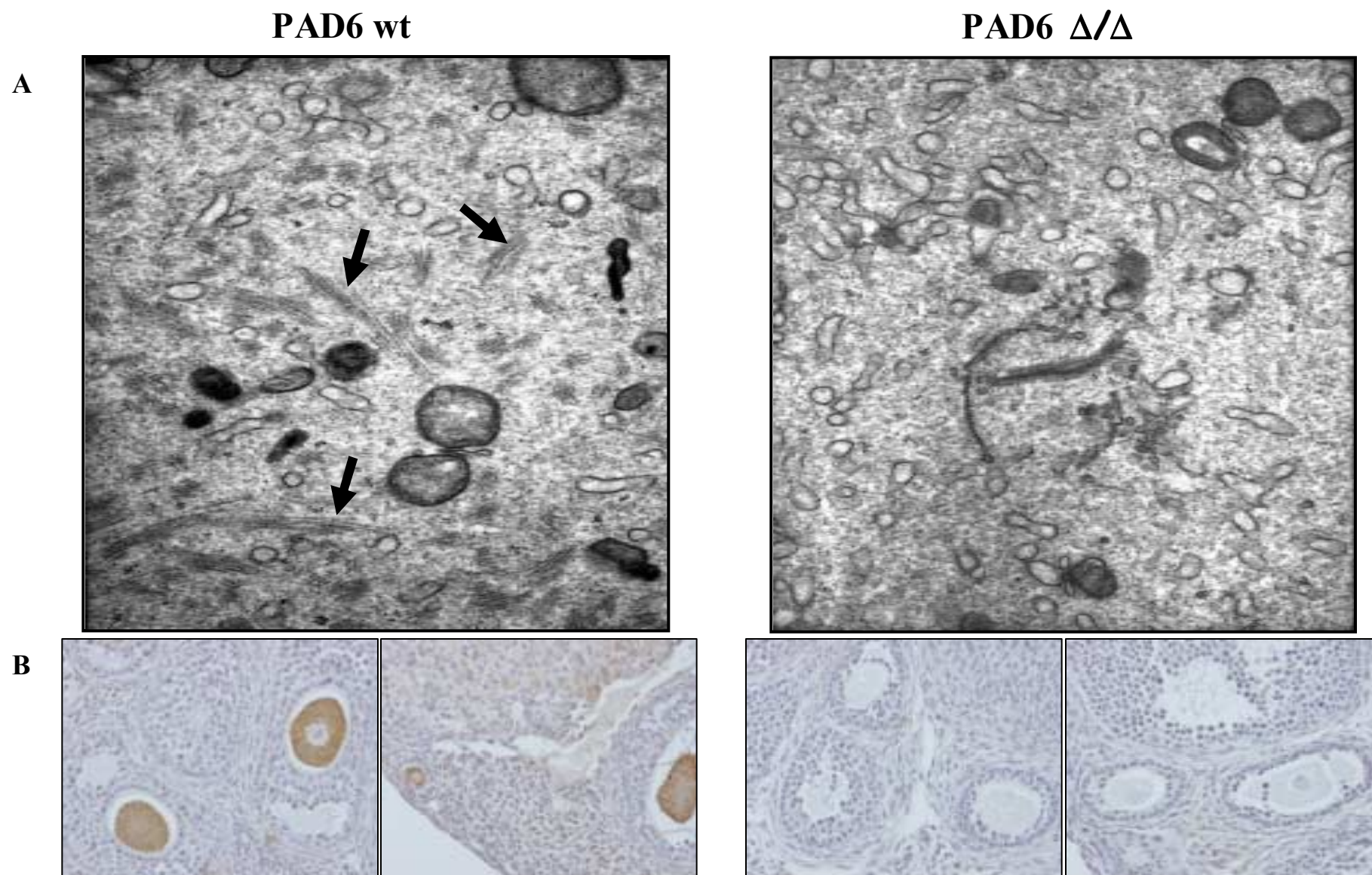
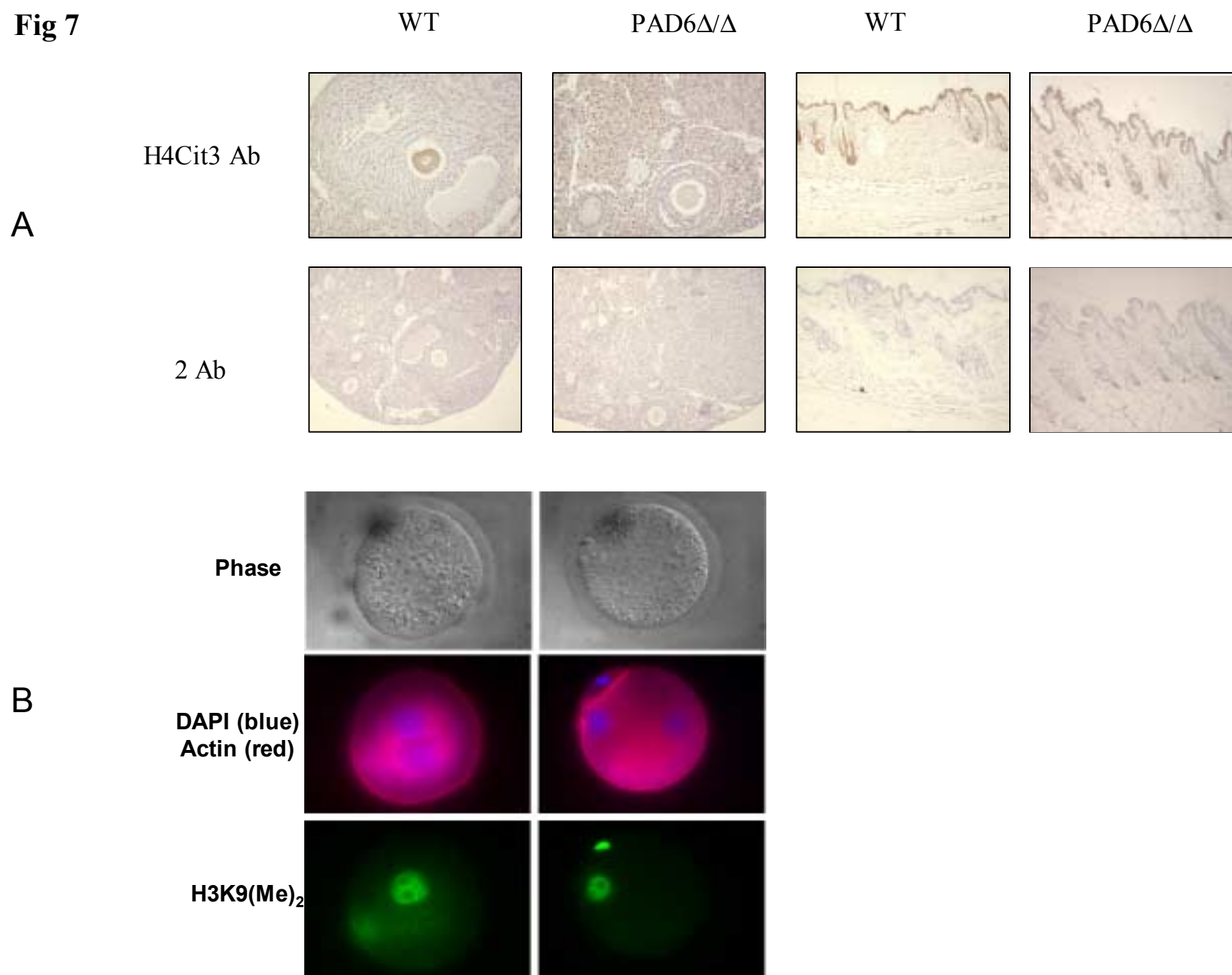


Fig 7

Peptidylarginine deiminase (PAD) 6 is essential for oocyte cytoskeletal sheet formation and female fertility

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Abstract

Peptidylarginine deiminase 6 (PAD6) is an enzyme that is uniquely expressed in male and female germ cells. To study the function of this enzyme *in vivo* we generated mice deficient for PAD6. Here we show that inactivation of the PAD6 gene in mice leads to female infertility whereas male fertility is not affected. The absence of the PAD6 protein and consequently absence of citrullination activity in oocytes results in dispersal of the cytoskeletal sheets in oocytes, indicating an essential role of these germ cell-specific structures in zygote/embryo development. PAD6 deficient mice do not show any other overt phenotype. Thus, we identify citrullination as a new regulator of fertility.

1. Introduction

The peptidylarginine deiminase family (PAD, E3.53.15)(Vossenaar et., 2003) is a class of Ca^{2+} -dependent enzymes widely distributed in mammals that introduces post-translational modifications to arginine residues through a process called citrullination (Rothnagel and Rogers, 1984). These modifications have a profound effect on protein stability and degradation and play a crucial role during development and cell differentiation (rev. in György et al., 2006 and Akiyama and Senshu, 1999). PAD enzymes show specificity to different protein substrates (Lamensa and Moscarello, 1993; Kubilis et al., 1980; Wang et al., 2004) and among them keratins represent a major target for citrullination (Senshu et al., 1999). The egg and embryo cytoskeletal sheets have been described as a major component of mammalian oocyte and early embryo cytoplasm (Schwarz et al., 1995; McGaughey et al., 1989) and have not been observed in somatic cells. They are highly dynamic structures containing various keratins (Schwarz et al., 1995) that undergo a series of complex reorganization processes starting at the onset of fertilisation and finally dissociate into filaments at the blastocyst stage of embryogenesis. PAD6 is the most recent member of the PAD family (Wright et al., 2003; Chavanas et al., 2004) and is expressed uniquely in mammalian oocytes, sperm cells and early embryo and appears to be associated to the cytoskeletal sheets (Wright et al., 2003).

In the present study, we further studied the role of PAD6 in folliculogenesis and spermatogenesis by generating mice deficient for PAD6. Here we show that female mice deficient for PAD6 do ovulate and can be fertilized but are infertile due to an early

zygote/embryo developmental defect. The absence of cytoskeletal sheet formation in the early embryo due to lack of citrullination indicates an essential role for PAD6 in female fertility.

2. Material and Methods

2.1 Mice

Animals were housed in macrolon cages (4-6 animals/cage) at 19-21°C; the relative humidity was 50-60% and an artificial light cycle of 12 hours alternating with 12 hours darkness was offered. Standard pelleted food (SDS, supplied by Special Diet Services, Witham, Essex, England) and water was provided *ad libitum* during the in-life period. The animals were observed for morbidity and mortality daily during the in-life period. Additionally, the animals were regularly observed for any behavioural and physical abnormalities.

All animal experiments were approved by the Organon Animal Ethics Committee.

2.2 Immunohistochemistry

Immunohistochemical staining was performed using an indirect immunoperoxidase technique. Mouse tissue sections (4 µm) were deparaffinised and hydrated and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in 100% methanol for 15 minutes. Antigen retrieval was performed by microwave processing at 500 Watt for 10 minutes in 0.01 M (pH 6.0) citrate buffer (Immunotech, Beckman Coulter Company) for all antibodies except for the H4Cit3.

Slides were incubated with pre-block (PBS-Tween) solution (2% BSA, Albumin bovine serum, Sigma, 2% mouse and 2% normal goat sera, Vector Laboratories) for 30 minutes at room temperature in a moisture chamber, then rinsed in PBS-Tween (Wash buffer 25x, 1WC, Biomerieux bv). PAD6 and ZP3 antibodies were incubated overnight at 4°C, while the H4Cit3 antibody (anti-Histone H4 citrulline 3, UpState Cell signalling solutions) was incubated for 2 hours at RT. Rinsed with PBS-Tween. Then the biotinylated secondary antibody (1:200, Vector Laboratories, BA-1000) was applied for 45 minutes. Slides were then treated with avidin-biotinylated peroxidase complex (Vectastain ABC reagent PK-6100, Vector Laboratories) for 30 minutes and developed in a solution containing 3, 3_

diaminobenzidine in Stable Peroxide Substrate Buffer (10X). (Immuno Pure metal enhanced DAB substrate (DAB, Pierce)

All sections were counterstained with hematoxylin, dehydrated, mounted and observed by light microscopy.

2.3 Superovulation

19-21 days old female mice (PAD6 Δ/Δ , wt littermates and B6CBAF1 control animals), were super ovulated as follow: the mice were injected subcutaneously with 7.5 IU urinary FSH (Humegon, N.V. Organon, The Netherlands), 5 IU hCG (Pregnyl, N.V. Organon) was administrated 46 hours after Humegon for ovulation induction.

2.4 RNA isolation and RT-PCR analysis

Total RNA was isolated from testes from wild-type, heterozygous and homozygous littermates using Trizol (GibcoBRL). First strand synthesis was performed using Oligo(dT)₁₅ (Promega) and pd(N)₆ (Amersham Pharmacia Biotech Inc) primers and Superscript II Rnase H-Reverse Transcriptase (Invitrogen) according to manufacturer instructions. Reverse transcribed products were then PCR amplified for 30 cycles with primers mMaterF GCTGGGCACAGGGTAAACTCT and mMaterR GGCCTGGCATTGGTCAAA. PCR products were analysed on a 1% agarose gel.

3. Results and discussion

PAD6 protein is detected in mouse oocytes from the primordial to the ovulatory follicle stage of development and persists in embryo up to the blastocyst stage (Wright et al., 2003). To define the role of PAD6 in oocyte and embryo development we disrupted the PAD6 gene in ES cells by gene targeting. We flanked with LoxP sites exon 2 and 3 of the PAD6 gene (Fig.1a-b) and these conditional knockout mice were subsequently crossed with a CMV-Cre transgenic line (Schwenk et al., 1995). CMV-Cre transgenics are characterized by ubiquitous deletion of LoxP-flanked gene sequences including the germ cells and were successfully used

to generated a null allele (PAD6 Δ/Δ) as shown by the absence of PAD6 gene product in mutant oocytes (Fig.1c). Homozygous PAD6 mutants are viable and present at the expected Mendelian ratio. They do not exhibit any overt abnormalities or histopathological abnormalities in any of the organs and tissues studied when comparing 6-week-old homozygous PAD6 knockout (KO) mice with wild-type (wt) littermates. Whereas male mutant mice were normally fertile, prolonged matings (up to two months) of female PAD6 Δ/Δ mice with wt or heterozygous males did not produce any offspring. 16 females were bred with either wt or heterozygous males and all females were evaluated for the presence of vaginal plugs during the first 3 days of mating (of the 16 PAD6 Δ/Δ females, 6 underwent two rounds of breeding and 4 three rounds always changing males. 30 control PAD6 $\Delta/+$ female mice produced 28 litters). Thus PAD6 gene disruption leads to female infertility. To determine the causes of infertility we examined the ovaries from control and mutant mice in greater detail by histopathology. Ovaries collected at different ages appeared to be normal in terms of morphology and physiology (Fig.2a). All stages of follicular development were present and no differences in numbers of follicles were detected between mutant and control ovaries from mice up to 10 months of age. These observations indicate that PAD6 is not required for oocyte growth, maturation or ovulation. Moreover, *ex vivo* follicle cultures were performed to assess the ability of PAD6 Δ/Δ follicles to undergo normal development and maturation. No differences were noticed between follicles obtained from mutant and littermate or control B6CBAF1 animals (79 PAD6 Δ/Δ , 40 wt littermate and 34 control follicles were isolated and cultured as described by Rose et al., 1991). Furthermore, the capability of the PAD6-deficient oocytes to reach complete maturation was assessed by analyzing the expression of marker genes essential for zygote development (Tong et al., 2004) or sperm binding (Liu et al., 1996). No changes in the expression of either MATER or ZP3 were observed (Fig 2b and 2c).

Previous studies have shown that oocytes are able to influence the function of follicular cells and in particular to alter hormone levels and oestrous cycle (Lan et al., 2003; Matzuk et al., 2002). To test the hypothesis that the PAD6 mutants fertility defect is due to endocrine dysfunction, we first evaluated steroid synthesis in the ovaries from mutant mice versus control littermates by performing semi-quantitative analysis of mRNAs encoding proteins that directly regulate steroid synthesis (aromatase, Star, 3 β HSD1, (Lan et al., 2003)) or for secreted factors that indirectly influence hormone levels (GDF9 and BMP15; Lan et al., 2003). The data obtained did not show differences in expression of the above mentioned genes (results not shown). Accordingly, the oestrous cycle of the PAD6 Δ/Δ female mice was

found to be similar to wild type littermates in terms of cycle duration, number of cycles per month and duration of the different phases (Fig. 3). These results indicate that PAD6 deficiency results either in a fertilization or early embryonic development defect. To further assess the time point at which PAD6 deficiency affects fertility, we isolated and analyzed eggs and early embryos from female PAD6 Δ/Δ and wt littermate mice at 24 and 72 hours after mating with a stud male. When eggs were collected from stimulated females 24 hours after mating, we found that the percent of pronuclear stage eggs isolated from wt (100% following normalization) and PAD6 Δ/Δ (95%) females was not statistically different. To confirm that the PAD6 Δ/Δ zygotes were diploid and contained both a male and a female pronucleus, we stained the zygotes with an antibody generated against dimethylated histone H3 lysine 9 (H3K9(Me)₂) which only stains maternal chromatin (Liu et al., 2004). Results showed that, as with the wild type zygotes, the antibody is only reactive with the maternal pronucleus adjacent to the extruded polar body (Fig. S3b). This result suggests that sperm-egg binding and fusion, sperm decondensation, and pronuclear formation occurs normally in PAD6 Δ/Δ eggs. Strikingly, when embryos were collected from PAD6 Δ/Δ females 72 h after mating, no zygotes were found to have progressed beyond the two-cell stage of development while 82% of wt zygotes progress to the 8-16 cell stage. As shown in Fig. 4a, there was no difference in pronuclear formation in wt and PAD6 Δ/Δ eggs collected 24 hours after mating. Thus, PAD6 appears not to play a role in sperm-egg binding and fusion, sperm decondensation, or pronuclear formation. However, when oviducts were flushed 72 hours after mating, all PAD6 Δ/Δ oocytes appeared to be arrested at the pronuclear to 2-cell stage while the zygotes from wt littermates proceeded to the 8-16 cell stage (Fig 4b). This result indicates that the maternally expressed PAD6 is required for embryonic development identifying PAD6 as one of the few known oocyte restricted genes that exert maternal effects in mammals.

Since we previously showed that PAD6 localizes to the cytoskeletal sheets (Wright et al., 2003), this defect would be consistent with a role for PAD6 in organizing these structures or activating them at the onset of fertilization and egg division. We therefore, performed ultrastructural analysis of oocytes from PAD6 Δ/Δ and wt littermate mice by transmission electron microscopy. To our surprise metaphase II arrested PAD6 Δ/Δ oocytes completely lacked cytoplasmic sheets (fig 5a) confirming the crucial role of PAD6 in the organization of these unique structures. We also investigated whether microfilament or microtubule networks might be affected by PAD6 deficiency. Metaphase II-arrested oocytes from PAD6 Δ/Δ and wt

littermates were probed with phalloidin and anti-tubulin antibodies. The results obtained show that loss of PAD6 did not dramatically affect other cytoskeletal structures (Fig.6).

Sequence homology and chromosomal localization (Chavanas et al., 2004) identify PAD6 as a citrullinating enzyme, although it seems to have undergone substantial sequence diversification from the other members of the family (Wright et al., 2003). Importantly, PAD6 seems to have lost the ability to bind Ca^{2+} as critical amino acids required for binding of Ca^{2+} (Arita et al., 2004) are missing in the PAD6 protein. In general, PAD enzymes are known to be promiscuous in terms of substrate specificity and can citrullinate a wide range of substrates including keratins and histones. It is therefore, tempting to speculate that due to the risk of PAD6 to interact and modify histones during GVB and male genome decondensation, Ca^{2+} -mediated activation had to be counter-selected and a new and yet unknown modification may be responsible for PAD6 activation.

To test if PAD6 may be regulating fertility through citrullination, we stained ovarian sections from PAD6 Δ/Δ and control littermate mice with an antibody that was raised against a synthetic histone H4 N-terminal peptide containing citrulline at the 3rd position as opposed to arginine (SGCitGKGGK). We previously found that this Ab recognizes citrullinated histone H4 in several somatic cell lines (Wang et al., 2004). Here we show that, while the anti-H4Cit3 was not reactive with nuclear protein. Instead, the cytoplasm of wt oocytes from primary to ovulatory follicles show intense staining while PAD6 Δ/Δ oocytes are devoid of staining confirming that in oocytes a cytoplasmic protein is citrullinated by PAD6 (Fig.5b). The exact nature of this substrate remains to be determined. However, specific staining of keratinocytes in the corneal layer of the epidermis and of the hair follicles with the H4Cit3 Ab (Fig. 7), would sustain the notion that a cytokeratin is the target of PAD6 activity. Indeed, keratins and in particular cytokeratins 18, 5 and 6 (Schwarz et al., 1995; McGaughey et al., 1989) have been described as the major constituents of cytoskeletal sheets. Also, keratins are known to be targets of citrullinating enzymes in keratinocytes (Nachat et al., 2005), we may therefore suggest that PAD6 through citrullination of oocyte-specific keratins is able to organize the formation and accumulation of cytoskeletal sheets whose function will be essential only at later stages of oocyte development and early zygote formation.

In conclusion we report here, for the first time, the phenotype of a mouse deficient for a peptidylarginine deiminase enzyme, the oocyte-specific PAD6. We demonstrate that PAD6 plays a crucial role in the organization of the cytoskeletal sheets and is required for development beyond the two-cell stage. The fertility defect observed identifies citrullination as a novel regulator of female fertility.

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Figure legends

Fig.1 Generation of PAD6-deficient mice. (A) Schematic representation of the gene targeting in the PAD6 locus by homologous recombination. Only exon 1 to 6 of the wild-type locus are shown. Rectangles represent coding DNA. Triangles represent LoxP sites. Only the relevant BamHI sites are indicated. B, BamHI site; P, 5' external probe. (B) Targeted ES cells. A Southern blot of BamHI-digested DNA from targeted ES and wild-type cells, respectively, is shown. A 5' external probe was used. The wild-type fragment migrates at 10.5 Kb and the fragment from the targeted locus migrates at 12.5 Kb. Chimeric mice were generated with ES cells carrying the correctly recombined targeted allele. The mutation was transmitted into the germ line. Crossing of the mutant mice to a Cre-expressing transgenic line a PAD6 Δ/Δ locus was generated. (C) Immunohistochemical localisation of PAD6 protein in ovaries from wt and mutant mice confirmed PAD6 gene disruption.

Fig.2 Ovarian development is normal in PAD6 Δ/Δ mice. (A) Formalin-fixed and paraffin-embedded sections of the ovaries of 6-month-old females (top, HE-stained sections) and 10-month-old females (bottom, PAS-stained sections). All ovaries show normal developing follicles and corpora lutea (asterisks). Obj. 20x. (B) Shown is the unaffected expression of ZP3 in wt as compared to mutant oocytes. (C) RNA analysis of the Mater gene in PAD6 Δ/Δ and wt ovary samples.

Fig.3 The oestrous cycle of the PAD6 Δ/Δ mice is not altered. Oestrous cycle was evaluated by vaginal smear daily for 53 days in 8 PAD6 Δ/Δ and 6 wt littermate control mice (shown are the results of one of two experiments that gave comparable results). (A) Number of cycles in 53 days; (B) Average duration of cycle; (C) Duration of cycle phases. The graphs represent mean values $\pm$ SEM.

Fig. 4 Embryos from wild-type and PAD6 Δ/Δ superovulated females were isolated from the oviducts at 24 and 72 hours post mating. (A) The rate of pronuclear development at 24 h post mating between wt (100% following normalization) and PAD6 Δ/Δ (95%) females was not different ($P < 0.05$ by t-test). (B) At 72 hours post mating, however, no zygotes from PAD6 Δ/Δ females progressed beyond the two-cell stage of development, while 82% of PAD6 wild type littermates progress to the 8-16 cell embryos ($*P < 0.05$ by t-test.).

Fig.5 PAD6 plays a crucial role in cytoplasmic sheet organisation through citrullination. (A) Electron micrograph images (19,000 X) of wt and PAD6 Δ/Δ oocytes. The cytoplasmic sheets, highlighted by arrows in the wt sample, are not visible in the PAD6 Δ/Δ oocyte, while other cytoplasmic structures appear unaltered. (B) Ovarian sections from wt and PAD6 Δ/Δ mice were stained with the H4Cit3 Ab. The presence of citrullinated protein(s) in the wt oocyte cytoplasm is visible from the primary stage on, whereas mutant oocytes are devoid of citrullinated products due to lack PAD6 enzymatic activity.

Fig. 6 The microtubule and microfilament networks do not appear to be affected by loss of PAD6 and disruption of the cytoskeletal sheets. wt and PAD Δ/Δ metaphase II-arrested oocytes and pronuclear-stage zygotes were treated with phalloidin and stained with anti-tubulin antibodies and visualized by fluorescence microscopy in order to determine whether loss of PAD6 and the sheet structure leads to gross defects in other cytoskeletal elements. Results show that both cytoskeletal the microtubule (anti-tubulin) and microfilament (phalloidin) appear normal in both wt and PAD6 Δ/Δ metaphase II-arrested oocytes and pronuclear-stage zygotes.

Fig. 7 (A) PAD6 may citrullinate oocyte-specific cytokeratin(s). Shown are ovarian and skin sections from PAD6 Δ/Δ and wt mice stained with the H4Cit3 Ab (upper panel). The specific staining of the corneal layer of the skin and wt oocytes indicates that PAD6 citrullinates oocyte-specific keratin(s). In the lower panel is shown the secondary Ab control staining. (B) PAD6 Δ/Δ embryos arrest at the two-cell stage of development. (A) To investigate the ploidy of PAD6 Δ/Δ embryos, wt and PAD6 Δ/Δ zygotes were isolated 24 hours after mating and stained with a maternal pronucleus-specific histone modification antibody (histone H3 lysine 9 dimethyl [H3K9(Me)₂]). As with wild type zygotes, the H3K9(Me)₂ antibody only reacted with the pronucleus adjacent to the extruded polar body in PAD6 Δ/Δ eggs, indicating that PAD6 Δ/Δ embryos are diploid and are not parthenogenetically activated.