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A Subcortical Maternal Complex Essential for Pre-implantation Mouse Embryogenesis

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SUMMARY

We have identified a **s**ub**c**ortical **m**aternal **c**omplex (SCMC) that assembles during oocyte growth and is essential for zygotes to progress beyond the first embryonic cell divisions. At least four maternally encoded proteins contribute to this MDa complex: FLOPED, MATER and TLE6 interact with each other while Filia binds independently to MATER. Although the transcripts encoding these proteins are degraded during meiotic maturation and ovulation, the SCMC proteins persist in the early embryo. The SCMC, located in the subcortex of eggs, is excluded from regions of cell-cell contact in the cleavage-stage embryo and segregates to the outer cells of the morulae and blastocyst. *Floped*$^{tm/tm}$ and/or *Mater*$^{tm/tm}$ eggs lack the SCMC, but can be fertilized. However, these embryos do not progress beyond cleavage stage development and female mice are sterile. The proteins are conserved in humans and similar maternal effect mutations may result in recurrent embryonic loss.

Keywords

maternal effect genes; **s**ub**c**ortical **m**aternal **c**omplex (SCMC); *Floped*; *Mater*; *Tle6*; *Filia*; pre-implantation mouse development

INTRODUCTION

During oocyte growth and maturation, mammalian eggs accumulate proteins required for successful fertilization and early embryogenesis. After ovulation into the oviduct, fusion of egg and sperm results in calcium-mediated egg activation (Swann et al., 2006) and establishment of the diploid embryo required for development (Aronson and Solter, 1987). In mice, the first cell division is complete within ~24 hours and the second, ~12 hours later (Bolton et al., 1984). At fertilization, each gamete is transcriptionally inert and robust embryonic gene expression is not detected until the two-cell stage (Latham and Schultz, 2001). During the hiatus between maternal and zygotic nuclear transcription, early development relies on post-transcriptional gene regulation. It has been long appreciated that proteins required for the transition from the maternal to zygotic program of development must be maternally encoded, but specific constituents have been elucidated only recently.

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To identify maternal effect genes, we have searched among targets of regulatory cascades operative during oogenesis. *Figla* encodes an oocyte-specific **b**asic **h**elix-**l**oop-**h**elix (bHLH) transcription factor that was first identified by its role in the coordinate activation of zona pellucida genes encoding an extracellular matrix that surrounds ovulated eggs and mediates fertilization (Liang et al., 1997). Genetic ablation of *Figla* not only affects zona gene expression, but also prevents formation of primordial follicles which suggests regulation of additional genetic pathways (Soyal et al., 2000). To uncover potential targets of FIGLA that might function as maternal effect genes, the transcriptomes of normal and *Figla* null newborn ovaries were compared by microarray and SAGE (Joshi et al., 2007).

The success of these screens was confirmed by the identification of *Mater* (***M***aternal ***a***ntigen ***t***hat ***e***mbryos ***r***equire; official name *Nlrp5*) one of the first molecularly characterized maternal effect genes in mice that encodes a 125 kDa protein, the absence of which precludes embryonic progression beyond two-cell embryos (Tong et al., 2000). Recently, an oocyte-specific binding partner of MATER was identified and designated Filia (Ohsugi et al., 2008). Transcripts of each gene accumulate during oogenesis and, although the mRNA is degraded during meiotic maturation, the cognate proteins persist until the early blastocyst stage of pre-implantation embryogenesis. Filia and MATER proteins physically interact with one another and co-localize in the subcortex of eggs and early embryos. Both proteins are reversibly excluded from regions of cell-cell contact with subsequent restriction to the subcortex of ‘outer’ cells and exclusion from ‘inner’ cells, the precursors of the embryonic epiblast.

Analysis of the aforementioned SAGE libraries identified two additional maternal effect genes, *Dppa3* (Payer et al., 2003) and *Padi6* (Esposito et al., 2007) that were present with ≥10 SAGE tags in normal and 0 tags in null ovaries (Joshi et al., 2007). We now characterize a fourth maternal effect gene (2410146L05Rik) from this screen which we designate *Floped* (***F***actor ***l***ocated in ***o***ocytes ***p***ermitting ***e***mbryonic ***d***evelopment). FLOPED interacts with MATER, TLE6 (a putative transcriptional co-repressor) and Filia with MATER to form a **s**ub**c**ortical **m**aternal **c**omplex (SCMC). Genetic ablation of individual components provides evidence that this subcortical maternal complex is required for normal cleavage stage mouse development.

RESULTS

Expression of *Floped*

Floped expression was detected in mouse ovaries, but not in eleven other tissues including male testes (Figure 1A), and, within the ovary, expression was restricted to growing oocytes (Figure 1B). *Floped* transcripts were first detected at embryonic day 15.5 (E15.5) and peaked 1 week after birth, an expression profile consistent with regulation by *Figla* which is first expressed beginning at E13.5 (Figure 1C). FLOPED protein was present in the subcortex of eggs where it overlapped with cortical F-actin, but extended further into the cytoplasm (Figure 1D). Beginning at the two-cell stage, FLOPED was excluded from regions of cell-cell contact, a phenomenon that was readily reversible upon disaggregation of blastomeres in the absence of calcium (Supplemental Figure 1A). The continued exclusion from cell-cell contact during pre-implantation embryogenesis resulted in the apparent absence of FLOPED in the ‘inner’ cells of the morula and from the ‘inner cell mass’ of the blastocyst (Supplemental Figure 1B)

Generation and Analysis of *Floped*$^{tm/tm}$ Mice

Floped null mouse lines were established from a gene-trapped embryonic stem cell line and disruption of the locus was confirmed by PCR and DNA sequencing (Supplemental Figure 2A, data not shown). Mating *Floped*$^{+/tm}$ females and males produced 18 litters with 157 offspring of which 33 (21%) were *Floped*$^{+/+}$, 90 (57%) were *Floped*$^{+/tm}$ and 35 (22%) were *Floped*$^{tm/tm}$ mice, consistent with expected Mendelian ratios of 1:2:1. *Floped*$^{+/tm}$ and

Floped$^{tm/tm}$ mice were viable, grew to adulthood and appeared grossly normal. Both normal and mutant *Floped* transcripts were present (Figure 1E), but using an anti-FLOPED antibody that recognizes both normal and mutant FLOPED proteins, no mutant FLOPED-β-galactosidase fusion protein was observed in mutant mice (Figure 1F). Immunoblots using anti-β-galactosidase antibodies confirmed the absence of a fusion protein in the transgenic mice (data not shown). Thus, homozygous *Floped* mutant female mice are functionally null with no detectable FLOPED protein in their ovaries or oocytes.

Both *Floped*$^{tm/tm}$ and control mice (Figure 1G) had normal ovarian histology in which follicles of all stages were present, including corpora lutea resulting from past ovulations. FLOPED was detected by immunofluorescence in growing oocytes within control heterozygous, but not homozygous *Floped* null ovaries (Supplemental Figure 2C). The number of eggs recovered after superovulation with gonadotrophins was similar (average ± s.e.m., number of animals) in *Floped*$^{tm/tm}$ (28.2 ± 2.5, n=12) null and *Floped*$^{+/tm}$ control mice (31.1 ± 2.8, n=13) and were morphologically indistinguishable (Figure 1H). *Floped*$^{+/tm}$ female controls produced normal sized litters with *Floped*$^{tm/tm}$ (8.3 ± 1.9 pups/litter, n=30); *Floped*$^{+/tm}$ (9.0 ± 2.4 pups/litter, n=29); and *Floped*$^{+/+}$ (8.0 ± 2.1 pups/litter, n=16) males. In each case, the mutant *Floped* allele was inherited with normal Mendelian ratios (data not shown). In contrast, 19 *Floped*$^{tm/tm}$ females never became visibly pregnant and produced no offspring despite mating for three months with either *Floped*$^{+/+}$, *Floped*$^{+/tm}$ or *Floped*$^{tm/tm}$ males.

Maternal Effect on Pre-implantation Embryogenesis

To resolve the discrepancy between the sterile phenotype and seemingly normal ovarian physiology, embryos were recovered from *Floped*$^{tm/tm}$ female mice at E0.5 (embryonic day 0.5), E1.5, E2.5 and E3.5. Eggs from *Floped*$^{tm/tm}$ females were fertilized and comparable numbers of normal appearing one-cell zygotes were recovered from oviducts of control and mutant female mice at E0.5 (Figures 2A,2B). However, progression from one- to two-cell embryos was delayed 6–8 hours (Figure 2C) and blastomeres derived from *Floped*$^{tm/tm}$ mice were often (57.6%, 74/128) unequal in size with attenuated contact regions compared to normal embryos (Figure 2A, E1.5). Fewer embryos (<20%) derived from *Floped*$^{tm/tm}$ mice progressed beyond the two-cell stage (Figure 2B) and by E2.5 those had formed cytoplasmic blebs and fragmented (Figure 2A). Normal morula and blastocysts were recovered from control females as expected at E2.5 (Figures 2A,2B) and E3.5 (data not shown).

Identification of Potential Binding Partners of FLOPED

Using anti-FLOPED antibodies, ovarian lysates of *Floped*$^{tm/tm}$ and normal mice were precipitated and analyzed by SDS-PAGE (Figure 3A). Bands that co-precipitated in normal but not *Floped* were digested with trypsin and analyzed by microscale tandem mass spectrometry. As anticipated, FLOPED was present in the immunoprecipitate and was identified based on two peptides covering 17% of the 164 amino acid protein. MATER (1163 amino acids), present in the subcortex of eggs with a null phenotype similar to *Floped*$^{tm/tm}$ (Tong et al., 2000), was also detected based on 23 unique peptides (24% coverage) as was Filia (346 amino acids), a recently described binding partner of MATER (8 peptides, 22% coverage). Unexpectedly, TLE6 (**T**ransducin-**L**ike **E**nhancer of Split **6**), a mammalian homologue of Drosophila Groucho, was identified as a co-precipitate with 8 peptides covering 17% of its 581 amino acids (Figure 3A).

Tle6 belongs to the *Groucho/Tle* super family of transcriptional co-repressors that plays critical roles in a range of developmental processes (Bajoghli, 2007; Buscarlet and Stifani, 2007). *Tle6* was expressed predominantly in ovaries (Figure 3B) where, similar to *Floped* and *Mater*, its transcripts accumulated in growing oocytes (Figure 3B, inset). The developmental expression of *Tle6*, which began at E15.5 and peaked in the first week after birth (Figure 3C),

was similar to *Floped* (Figure 1A) and consistent with co-regulation of the two genes. *Floped*, *Mater* and *Tle6* transcripts accumulated during oogenesis and their abundance was greatest in fully grown (80 μm) oocytes. During meiotic maturation and ovulation, the vast majority of FLOPED, MATER and TLE6 transcripts was degraded and virtually none was detected by the two-cell stage of embryogenesis (Figure 3D). However, the cognate proteins, first observed in growing oocytes, persisted during pre-implantation embryogenesis up to the blastocyst stage of development (Figure 3E).

FLOPED, MATER, TLE6 and Filia Form a Complex

Physical interactions of FLOPED, MATER, TLE6 and Filia were investigated by co-immunoprecipitation (Figure 4A). All four proteins were present in normal, but only residual amounts of MATER, TLE6 and Filia were observed in *Floped* null ovarian lysates. Using antibodies to either FLOPED or TLE6, all four proteins were immunoprecipitated from normal, but none from *Floped* null ovarian lysate. The ability of antibodies to TLE6 to immunoprecipitate itself, but not the other three proteins, suggested that TLE6, and by extension MATER and Filia, were present in *Floped* null oocytes (albeit at lower abundance), but not in complex with one another.

To further investigate the physical interactions among the SCMC components, pairs of the four proteins were expressed in COS cells. Cells lysates co-transfected with FLOPED-EGFP and either MATER-myc, TLE6 or Filia-HA were incubated with anti-EGFP to immunoprecipitate FLOPED and potential binding partners were detected by immunoblots of the precipitate. FLOPED interacted with MATER and TLE6, but not Filia (Figure 4B). Co-transfections with MATER-myc and each of the other three expression vectors confirmed FLOPED-MATER binding and demonstrated interactions between MATER and TLE6 as well as MATER and Filia (Figure 4C). Co-transfection of TLE6 and each of the other three expression vectors confirmed FLOPED/TLE6 and MATER/TLE6 interactions, but did not detect binding of TLE6 and Filia (Figure 4D). In reciprocal co-transfections with Filia-HA and each of the other three expression vectors, Filia did not bind to FLOPED or TLE6, but did bind to MATER (Figure 4E).

To explore the possibility that FLOPED, MATER, TLE6 and Filia might participate in a common supramolecular complex, extracts from eggs were separated by FPLC gel filtration and individual fractions were analyzed by immunoblot (Figure 4F). The peak fraction containing all four proteins fell between the void volume (M_r ~2000 kDa) and 669 kDa. The presence of Filia (50 kDa) in later fractions suggests either self oligomerization or interactions with other cytoplasmic proteins.

Localization of the Subcortical Maternal Complex in Pre-implantation Embryos

Using antibodies to FLOPED, MATER and TLE6, the three proteins co-localized in the subcortex of eggs and preimplantation embryos (Figure 5A). The fluorescent signals from each protein were attenuated where the zygotic membrane was in contact with polar bodies and each protein was seemingly excluded from the region of cell-cell contact at the two-cell stage. With subsequent cell division, this exclusion affected the entire surface of some cells and resulted in the absence of co-localizing signals in the ‘inner’ cells of morulae and in the ‘inner cell mass’ of blastocysts (Figure 5A). The similar expression pattern of the four genes, the physical interactions of the four cognate proteins and their co-localization in conjunction with the sterile phenotype of *Floped*$^{tm/tm}$ and *Mater*$^{tm/tm}$ female mice define a **S**ub**c**ortical **M**aternal **C**omplex (SCMC) required for progression through early cleavage stages of pre-implantation development.

The localization of the complex to cells of the trophectoderm in blastocysts (Figure 5A) raised the possibility that the presence of the SCMC triggers, either directly or indirectly, a commitment of the 'outer' cell progeny to become trophectoderm (Ohsugi et al., 2008). *Tead4* encodes a transcription factor with an evolutionarily conserved TEA domain that binds to DNA. *Tead4* null mice do not form a trophectoderm during pre-implantation development which results in embryonic lethality (Yagi et al., 2007; Nishioka et al., 2008). To determine if cell lineage or external location of a free cell surface dictated the presence of the SCMC, E3.5 *Tead4* null embryos were stained with antibodies to TLE6 and FLOPED. As reported (Yagi et al., 2007; Nishioka et al., 2008), null embryos did not form blastocysts, but remained as a clump of cells all of which were OCT4 positive, indicative of epiblast lineage. Immunolocalization of the SCMC with antibodies to TLE6 (Figure 5B) and FLOPED (Figure 5C) was restricted to 'outer' cells of E3.5 *Tead4* null embryos.

The SCMC Depends on the Presence of FLOPED and MATER

Embryos derived from *Floped*$^{tm/tm}$ and *Mater*$^{tm/tm}$ females had a similar phenotype which was indistinguishable from that observed in embryos derived from double mutant mice (Figure 2B, data not shown). Because of the strong sterile phenotypes observed in embryos derived from homozygous null females from each these three lines, the ability to form the SCMC in the absence of either protein product was investigated. Eggs from control, *Floped*$^{tm/tm}$ or *Mater*$^{tm/tm}$ females were incubated with antibodies specific to FLOPED, MATER, TLE6 or Filia while F-actin was detected with phalloidin (Figures 6A-D). Normal subcortical F-actin localization was not significantly affected by the absence of FLOPED or MATER. However, the SCMC was not observed and there was no subcortical localization of MATER, TLE6 or Filia in the *Floped*$^{tm/tm}$ or of FLOPED, TLE6 or Filia in *Mater*$^{tm/tm}$ eggs. Signals from MATER, TLE6 and Filia were faint, but diffusely detected in the cytoplasm of *Floped*$^{tm/tm}$ eggs as were FLOPED, TLE6 and Filia in *Mater*$^{tm/tm}$ eggs.

These decreases were confirmed by immunoblots of lysates of *Floped*$^{tm/tm}$ and *Mater*$^{tm/tm}$ eggs (Figure 6E). In *Floped*$^{tm/tm}$ eggs, MATER was 4.8% and TLE6 was 5.0% of normal levels. Neither FLOPED nor Filia was detected. In *Mater*$^{tm/tm}$ eggs, there was residual MATER protein as previously observed (Ohsugi et al., 2008) and FLOPED, TLE6 and Filia were 10.3%, 9.9% and 5.5% of normal, respectively. Although below the level of detection in these assays of 10 eggs, Filia were still faintly visible in the confocal microscopic images of *Floped*$^{tm/tm}$ eggs (Figure 6D). Levels of *Mater*, *Tle6* and *Filia* transcripts in *Floped*$^{tm/tm}$ or of *Floped*, *Tle6*, and *Filia* transcripts in *Mater*$^{tm/tm}$ was similar to that in normal ovaries (data not shown), suggesting instability of component proteins in the absence of the SCMC.

To determine if replacement of FLOPED would stabilize the other components and reform the SCMC, a bicistronic vector expressing *Floped* and GFP was injected into *Floped*$^{tm/tm}$ oocytes. Expression was not observed in all oocytes (Figure 6F, insets), but those that expressed GFP as a control for successful injection, also expressed FLOPED and reformed the SCMC (Figure 6F). The signal from the subcortical co-localization of FLOPED and Filia in the SCMC was comparable, albeit more diffuse, to that observed in control oocytes (Figures 6A,6D). Taken together, these results suggest that FLOPED, MATER and TLE6 directly interact each and that Filia directly interacts only with MATER in forming the SCMC, the stability of which is dependent on FLOPED and MATER (Figure 6G).

DISCUSSION

The interregnum of nuclear transcription between meiotic maturation in oocytes and activation of the embryonic genome ensures critical roles for pre-existing stores of RNA and proteins (Seydoux and Braun, 2006). Through gene targeting studies, individual maternal proteins have been implicated as essential for cleavage-stage development in mouse (Zheng and Dean,

2007). We have now identified a MDa complex of proteins composed of at least four components (FLOPED, MATER, TLE6, Filia) located in the subcortex of eggs and required for pre-implantation mouse development. Although the absence of the SCMC significantly impedes development beyond the two-cell embryo, it seems likely that defects arise earlier as progression from one- to two-cells is delayed and the initial cell division is often asymmetrical. This could result from abnormalities in syngamy, mitotic spindle formation, cytokinesis or cell cycle progression and could affect later events in embryonic development including axes formation, a subject of intense investigative interest (Rossant and Tam, 2004; Motosugi et al., 2005; Louvet-Vallee et al., 2005; Kurotaki et al., 2007; Bischoff et al., 2008).

Although genetic ablation of either *Floped* or *Mater* (or both) does not materially affect intraovarian folliculogenesis, ovulation or fertilization, the failure of mutant embryos to complete cleavage stage development results in a striking female sterile phenotype. FLOPED is the smallest (18 kDa, 164 amino acids) component, but its absence disrupts complex formation as effectively as MATER, the largest (125 kDa, 1163 amino acids). Genetic ablation of the genes encoding Filia (38 kDa, 346 amino acids) and of TLE6 (65 kDa, 581 amino acids) has not been reported. Three of the proteins have motifs associated with protein-protein interactions: MATER has 13 leucine-rich repeats (Kobe and Kajava, 2001) near its carboxyl terminus; TLE6 has 5 WD (~31 amino acid tryptophan-aspartate) repeats (Li and Roberts, 2001); and Filia has a novel 23 amino acid 10 fold repeat not described in other proteins. FLOPED contains a single ~70 amino acid KH (hnRNA K homology) domain, multimers of which associate with single-strand (RNA or DNA) nucleic acid (Valverde et al., 2008).

The SCMC has an M_r between 669 and 2000 kDa that is considerably in excess of the total mass (~250 kDa) of the four identified proteins. In addition, FLOPED, MATER, TLE6 or Filia (by themselves or paired with each other) fail to localize to the subcortex in heterologous cells (data not shown) which suggests the presence of additional components to anchor the SCMC near the membrane. In the current studies, PADI6 was identified by tandem mass spectrometry after immunoprecipitation with anti-FLOPED antibodies (6 peptides, 11% coverage). *Padi6* encodes an oocyte-specific, 77 kDa peptidylarginine deiminase that is preferentially located in the cortex of eggs and preimplantation embryos (Wright et al., 2003). *Padi6*$^{tm/tm}$ females ovulate eggs that can be fertilized, but do not progress beyond cleavage stage embryogenesis and females are sterile (Esposito et al., 2007). Similar to embryos derived from *Mater* null females (Tong et al., 2000), embryos from *Padi6* null females have impaired embryonic transcription and dysregulation of protein translation (Yurttas et al., 2008), a recapitulation which implicate PADI6 as a fifth member of the SCMC. The enzymatic activity of peptidylarginine deiminase converts arginine to citrulline in proteins and clarification of its role in early embryogenesis should provide additional insights into the role of the SCMC in early development.

The expression of the genes that encode at least four of these proteins (*Floped*, *Mater*, *Tle6*, *Padi6*) is regulated by FIGLA (**F**actor **i**n the **g**erm**l**ine, **a**lpha), a bHLH transcription factor. A role in activation of *Filia* remains indeterminate as transcripts were not represented in the screened microarrays and SAGE libraries used to identify potential downstream targets (Joshi et al., 2007). FIGLA was initially implicated as a transcription factor in the coordinate expression of the zona pellucida genes (*Zp1*, *Zp2*, *Zp3*) that encode three proteins that form an extracellular matrix surrounding eggs and pre-implantation embryos. Each zona gene contains a canonical E-box (CANNTG) within 250 bp of its transcription start site which when ablated affects reporter gene expression in heterologous cells (Liang et al., 1997). Zona transcripts were more abundant in normal than in *Figla* null ovaries (Soyal et al., 2000; Joshi et al., 2007). Thus, it appears that FIGLA regulates at least two multi-component complexes critical for early development, the extracellular zona pellucida and the intracellular SCMC. As observed with other tissue-specific bHLH master regulators (e.g. myoD, Tapscott, 2005), it is

likely that FIGLA does not act in isolation and that other transcription factors are critical modulators of genetic hierarchies required for successful fertilization and the onset of development. Recently, heterozygous mutation of *Figla* have been observed in two patients with premature ovarian failure (Zhao et al., 2008) raising the possibility that genetic pathways affected by FIGLA are causal in disease.

Impaired progression beyond the first embryonic cleavage has been observed in a number of experimental settings including defects in cell cycle, cytokinesis, and activation of the embryonic genome. Maternal ablation of the *Brg1* (official name: *Smarca4*), the catalytic subunit of SWI/SNF remodeling complex (Bultman et al., 2006) or of *Ube2a*, a ubiquitin-conjugating DNA repair enzyme (Roest et al., 2004), effectively arrests development at two-cells as does treatment with α-amanitin, an inhibitor of RNA polymerase (Golbus et al., 1973; Warner and Versteegh, 1974; Flach et al., 1982). Embryos derived from *Mater*$^{tm/tm}$ and *Padi6*$^{tm/tm}$ females also have a pronounced decrease in de novo transcription in two-cell embryos (Tong et al., 2000; Yurttas et al., 2008) which raises the possibility that the SCMC plays a role in activation of the embryonic genome. However, it remains perplexing how a subcortical complex would affect nuclear events, although disruption of TCL1 (T cell leukemia/lymphoma 1) mediated shuttling of Akt (protein kinase B) into the nucleus inhibits embryonic progression (Narducci et al., 2002; Pekarsky et al., 2000). Perhaps more likely, the observed decrease in transcription and deregulation of translation reflects impeding death of early embryos derived from *Mater* and *Padi6* null females. To further investigate the role of SMCM in cell cycle progression and cytokinesis, we are establishing suitable reagents for in vivo investigations using time-lapse confocal microscopy.

The persistence of the SCMC in the early blastocyst raises the possibility of a role(s) beyond ensuring embryonic progression through cleavage stage development. Following the third cell division, the blastomeres compact to form the morula and individual embryonic cells are polarized with the SCMC remaining at the apical cortex. Subsequent cell division, orthogonal to the axis of polarity results in ‘outer’ cells that contain the SCMC and ‘inner’ cells that do not. Outer cells contribute progeny to the trophectoderm (precursor to the placenta) whereas ‘inner’ cells form the embryonic inner cell mass (Tarkowski and Wroblewska, 1967; Johnson and Ziomek, 1981). The plasticity in the reformation of the SCMC in ‘inner cells’ after release from cell-cell contacts may reflect the regulative nature of early mouse embryogenesis. Embryos lacking TEAD4 do not form a trophectoderm (Yagi et al., 2007; Nishioka et al., 2008) and the persistence of the SCMC in the outer cells of E3.5 *Tead4* null embryos indicates that topology rather than cell lineage determines the presence of the complex. Thus, the SCMC does not appear to be sufficient to establish trophectoderm lineage.

Other maternal proteins adopt a polarized location during pre-implantation development, some as early as the two-cell stage including TCL1 and UCH-L1 (ubiquitin carboxylterminal hydrolase L1). Although *Tcl1* and *Uchl1* homozygous null female are fertile, both have marked decreases in fecundity with litter sizes about half the number of normal (Narducci et al., 2002; Sekiguchi et al., 2006). Additional embryonic proteins such as EZRIN and PAR3/aPKC also become polarized, albeit not until the 8-cell stage of embryogenesis. *Ezr* null mice die perinatally (Saotome et al., 2004) and EZRIN is an early marker of blastomere polarization and of trophoblast precursor cells (Louvet et al., 1996; Dard et al., 2001). The PAR3/aPKC complex is asymmetrically located in the 8-cell embryo and experimental disruption of either protein in individual blastomeres in vitro, partially redirects their cell fate toward that of an ‘inner’ cell after the fourth embryonic cleavage (Plusa et al., 2005). Whether these proteins interact with the SCMC beginning at the 8-cell stage of development has not been ascertained, but their apical localization occurs after the initial formation of the complex.

In earlier screens looking for oocyte-specific proteins, 2410146L05Rik (FLOPED) was identified as cat, dog or mouse oocyte expressed protein (C/D/MOEP) and proposed as an RNA binding proteins based on the presence of a KH domain (Pierre et al., 2007; Herr et al., 2008). However, staining RNA in normal embryos with acridine orange did not co-localize with the SCMC and treatment with RNase did not disrupt the complex as determined by immunoprecipitation (data not shown). Nevertheless, specific transcripts could bind to the SCMC and given the prominent role of RNA localization in development (Strome and Lehmann, 2007), this possibility warrants further investigation. Human homologues can be identified for FLOPED (149 amino acids, 39% identity), MATER (1200 amino acids, 46% identity), TLE6 (449 amino acids, 44% identity), Filia (217 amino acids, 41% identity) and PADI6 (694 amino acids, 67% identity). Thus, further elucidation of the molecular role of the SCMC in the arrested development observed with embryos derived from *Floped*$^{tm/tm}$ and *Mater*$^{tm/tm}$ female mice may provide insights into clinical infertility or recurrent spontaneous abortion.

EXPERIMENTAL PROCEDURES

Experimental Animals and Isolation of Eggs and Embryos

Mice were maintained in compliance with the guidelines of the Animal Care and Use Committee of the National Institutes of Health under a Division of Intramural Research, NIDDK approved animal study protocol. A gene-trapped ES cell line (139A2-3, Centre for Modeling Human Disease, University of Toronto, Canada) was microinjected into C57BL/6 blastocysts (Li et al., 1999) and germline transmission was confirmed by PCR genotyping using Extract-N-Amp Tissue PCR Kits (Sigma-Aldrich). *Floped* null (Supplemental Data), *Mater* null (Tong et al., 2000), *Tead4* null (Yagi et al., 2007) and control CF1, C57BL/6J and FVB female mice (4–5 week old) were stimulated with gonadotrophins to obtain ovulated eggs or embryos (Rankin et al., 2003). Oocytes, eggs and embryos were collected in M2 medium (Chemicon) and embryos were cultured in EmbryoMax® KSOM medium (Millipore) at 37°C in 5% CO_2.

RNA Isolation and Quantitative Real-time RT-PCR

Total RNA or mRNA was isolated from mouse tissues with RNeasy Mini Kit (Qiagen) and Dynabeads® mRNA DIRECT™ Micro Kit (Dynal), respectively. Transcript abundance was assessed by qRT-PCR using TaqMan® probes and gene-specific primers (Applied Biosystems). Each data point was the average of duplicate assays performed on three independently obtained biological samples and expressed as a percent of GADPH abundance (average, ± s.e.m.). The abundance of normal and mutant *Floped* transcripts was assayed by semi-quantitative PCR with OneStep RT-PCR Kit (Qiagen) and *in situ* hybridization was performed as described (Ohsugi et al., 2008).

Photomicroscopy

Rabbit antisera were raised to FLOPED (1–19 aa) and TLE6 (172–191 aa) peptide antigens (Covance). Using these and antisera specific to MATER and Filia (sources of other antibodies are listed in Supplemental Data), confocal and immunofluorescence images were obtained on LSM 510 and Axioplan Zeiss microscopes, respectively (Ohsugi et al., 2008). For triple staining, individual rabbit antibodies were directly labeled by Monoclonal Antibody Labeling Kits (Invitrogen). Fixed, plastic embedded tissue was stained and imaged as described (Rankin et al., 1999).

Immunoblots, Co-immunoprecipitation and Mass Spectrometry

SDS-PAGE separated samples were transferred to PVDF membrane (Invitrogen) and incubated with primary antibodies, second antibodies and developed with SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology). Results were analyzed with a LAS-3000 using Multi Gauge software (FujiFilm Medical Systems). Ovarian lysates were precipitated using ProFound™ Mammalian Co-Immunoprecipitation Kit (Pierce Biotechnology) and, after SDS-PAGE, protein bands of interest were identified by the NIDDK Proteomics and Mass Spectrometry Facility. In addition, *Floped* cDNA cloned in pEGFP-C2 (Clontech), *Tle6* in pCMV-SPORT6 (Invitrogen), *Mater* in pCMV-myc and *Filia* pCMV-HA (Clontech) were transfected into COS cells (Yi et al., 2007) and immunoprecipitated using antibody to EGFP, Myc, HA and TLE6, prior to analysis by immunoblot.

Gel Filtration Chromatography

Using an AKTApurifier FPLC System (GE Healthcare), egg lysates were chromatographed on a Superose 6 10/300 GL column pre-equilibrated with PBS, 100 μM KCl, 1 μM EDTA and 1 μM DTT, pH 7.4. The column was calibrated with protein standards from Gel Filtration Calibration Kits and fractions (1.0 ml) were analyzed by immunoblots.

Microinjection of Expression Plasmids into Floped$^{tm/tm}$ Oocytes

Floped cDNA was cloned into pIRES2-ZsGreen1 (Clontech) and injected into the nucleus of oocytes isolated from 3 week old *Floped*$^{tm/tm}$ mice. Injected oocytes were incubated in KSOM medium containing dibutyryl cyclic AMP (250 μM) at 37°C, 5% CO_2 prior to imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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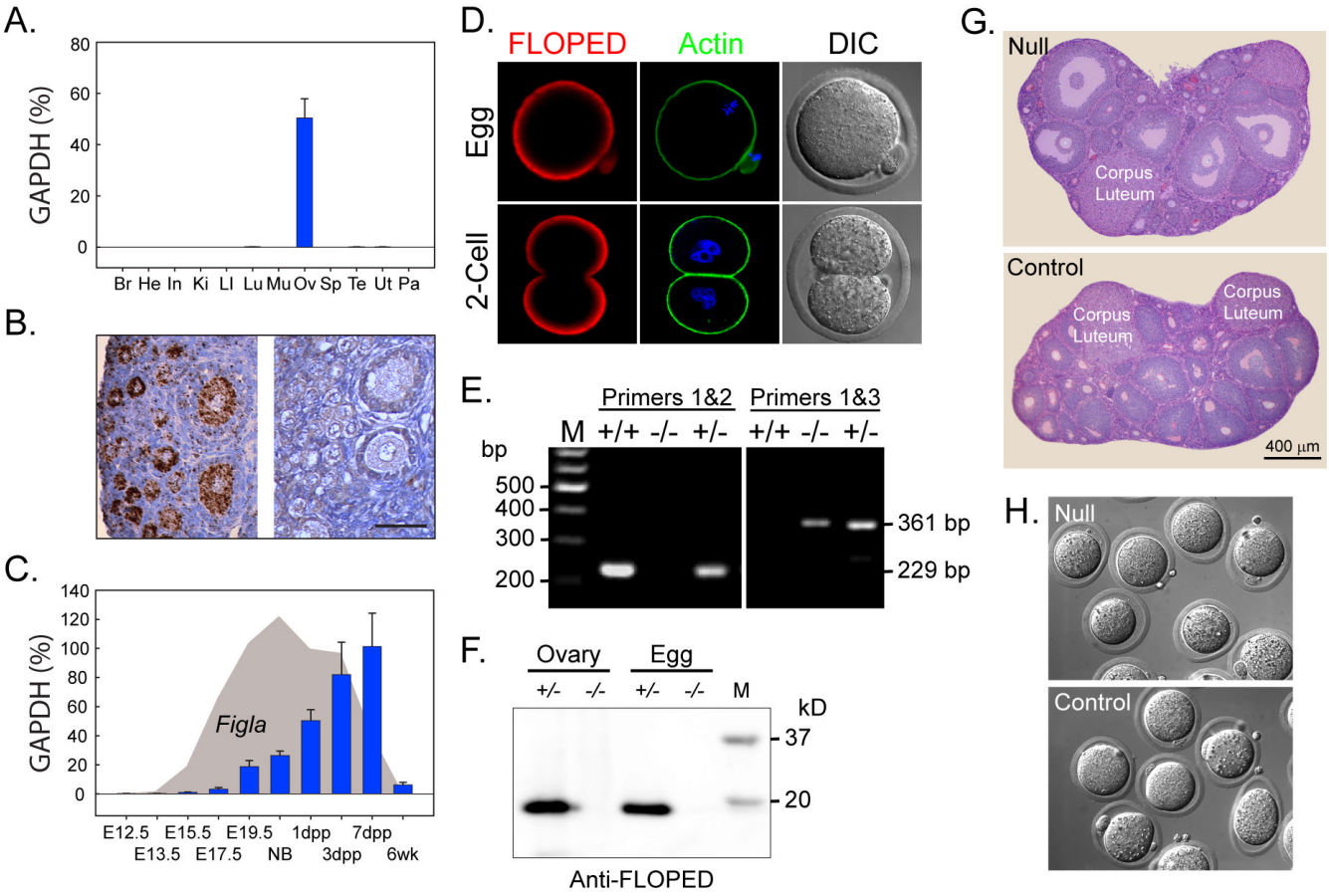

Figure 1. Expression of *Floped* and *Floped*$^{tm/tm}$ mouse lines

(A). Quantitative reverse-transcription, polymerase chain reaction (qRT-PCR) with total RNA extracted from newborn brain (Br), heart (He), intestines (In), kidney (Ki), liver (Li), lung (Lu), muscle (Mu), ovary (Ov), spleen (Sp), testis (Te), uterus (Ut) and pancreas (Pa) expressed as a percent of GAPDH. (B). In situ hybridization of fixed, paraffin-embedded 4 μm ovarian sections probed with DIG-labeled antisense (left) or sense (right) synthetic *Floped* oligonucleotides. Scale bar, 50 μm. (C). qRT-PCR of *Floped* (blue bars) and *Figla* expression (grey background) using total RNA isolated at embryonic day 12.5 (E12.5) to E19.5, newborn (NB), 1–7 days post-partum (dpp) and at six weeks (6wk). (D). Eggs and two-cell embryos were isolated, fixed and stained with peptide-purified antibodies to FLOPED or with Hoechst and phalloidin which bind to DNA and F-actin, respectively. Morphology of eggs and early embryos was observed with differential interference contrast (DIC). (E). Total ovarian RNA was primed with oligo dT and PCR with P1 and P2 primers (Supplemental Data) produced a 229 bp band in normal (+/+) and heterozygote (+/−), but not in *Floped* null (−/−) mice (left). RT-PCR with P1 and P3 primers produced a 361 bp band in null (−/−) and in heterozygote (+/−), but not in normal mice (right). M, molecular mass markers. (F). Immunoblots of total ovarian extract (20 μg) and 10 ovulated eggs from heterozygous (+/−) or homozygous (−/−) *Floped* null mice were probed with anti-FLOPED antibody. (G). Plastic embedded ovarian sections from homozygous (top) and control heterozygous (bottom) *Floped* null mice. (H). Ovulated eggs from hormonally stimulated homozygous (top) and control heterozygous (bottom,) *Floped* null mice were imaged by DIC.

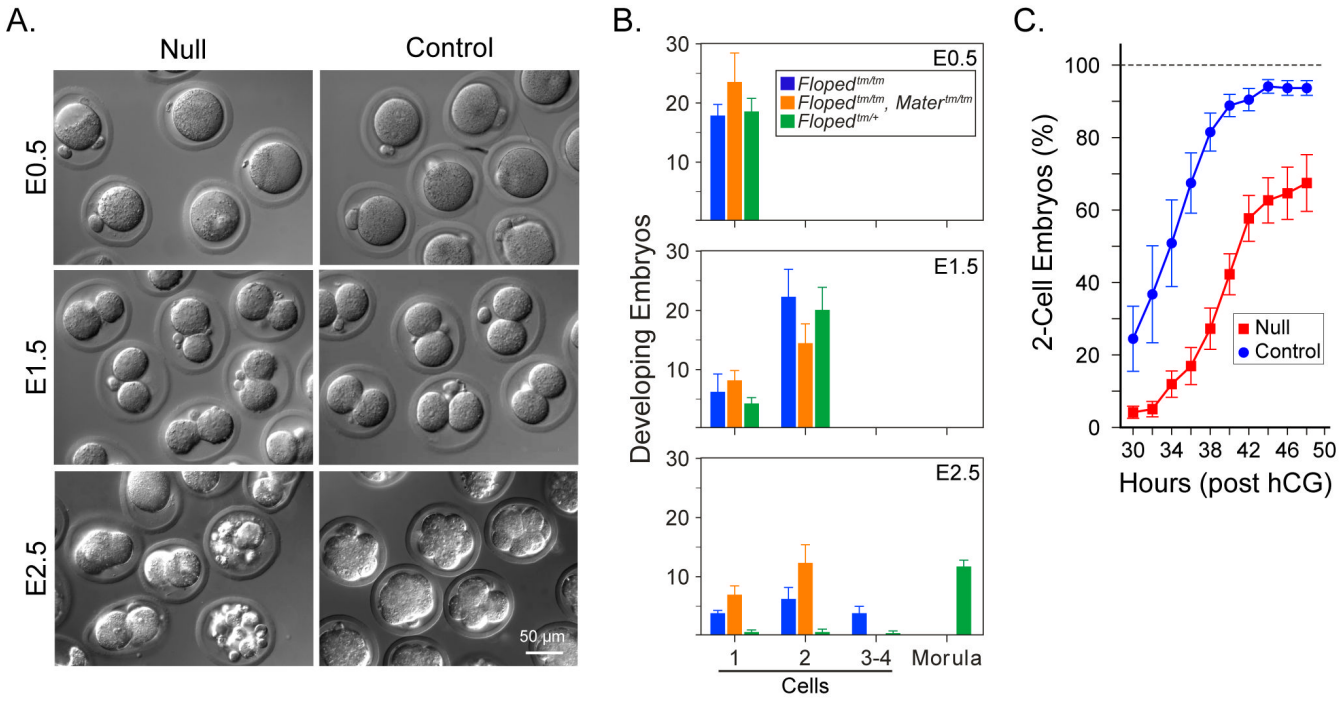

Figure 2. Phenotype of embryos derived from *Floped*$^{tm/tm}$ female mice

(A). Embryos were flushed from the oviducts of *Floped* null and heterozygous controls at E0.5, E1.5 and E2.5. (B). *In vivo* progression of pre-implantation embryos from (A) was quantified from at least five females and expressed as the average ±s.e.m. Embryos derived from nine *Floped*$^{tm/tm}$, *Mater*$^{tm/tm}$ and double mutant females were isolated at E0.5 and cultured in vitro for one (E1.5) or two (E2.5) days. (C). Homozygous and heterozygous (control) *Floped* null females were mated with normal males to isolate one-cell embryos 30 hours after hCG administration. Embryos (174 from *Floped*$^{+/tm}$ and 132 from *Floped*$^{tm/tm}$ females) were cultured an additional 18 hours and progression to two-cell embryos was assessed morphologically. Data is the percent of two-cell embryos (average ± s.e.m.) observed at two hour intervals.

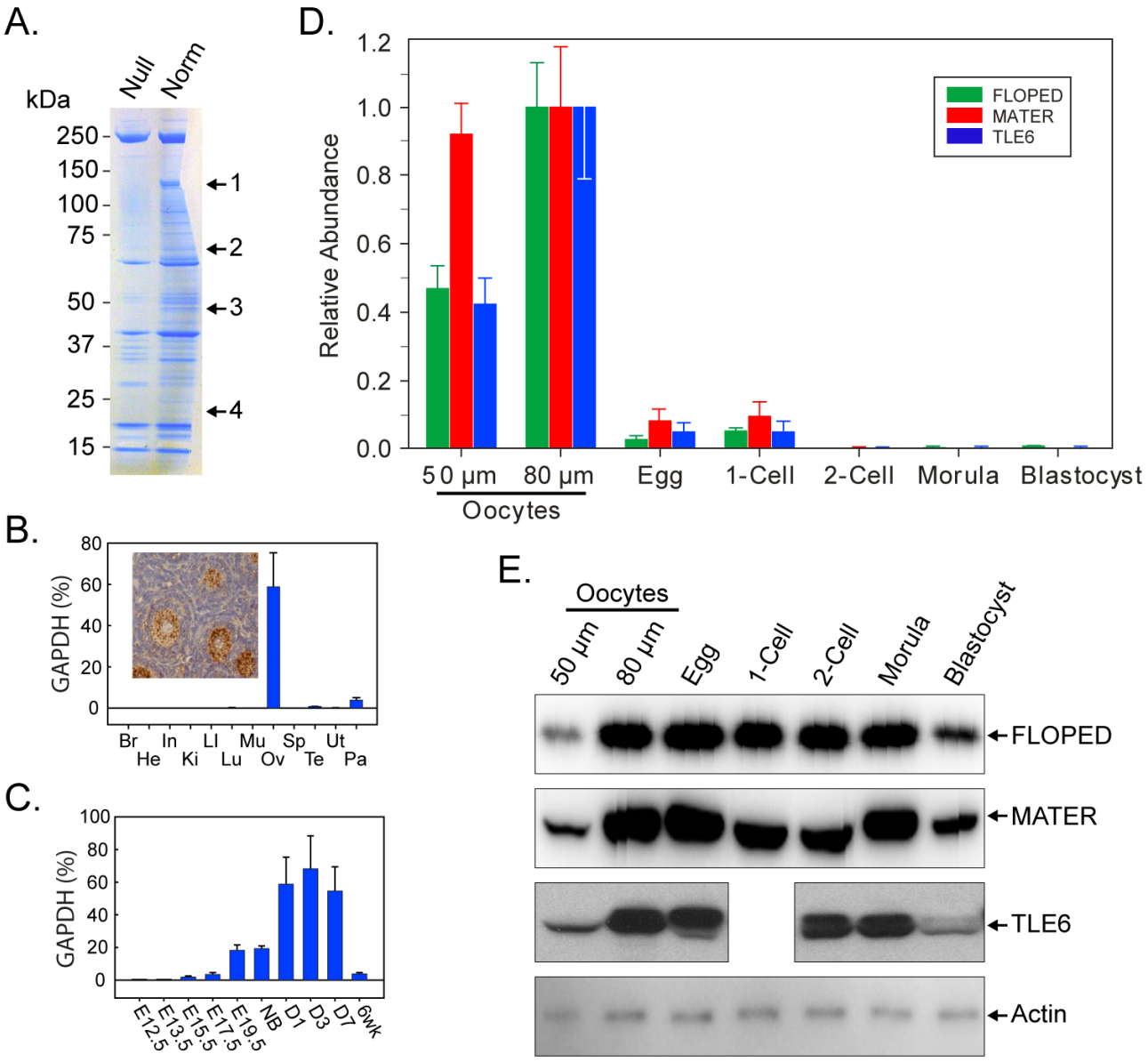

Figure 3. Identification of FLOPED binding partners

(A). Ovarian lysates from normal and *Floped* null mice were precipitated with peptide-purified, rabbit anti-FLOPED antibody. Immunoprecipitates were separated by SDS-PAGE and stained with colloidal blue. Arrows indicate protein bands present in normal, but not null extracts and represent the relative mobility of MATER (1), TLE6 (2), Filia (3) and FLOPED (4). Molecular mass (kDa) indicated on left. (B). Tissue-specific expression of *Tle6* determined by qRT-PCR of total RNA using TLE6 specific primers and probes. Inset, in situ hybridization with DIG-labeled antisense synthetic *Tle6* oligonucleotides. (C). Developmental expression profile of *Tle6*. (D). Poly(A)$^+$ RNA was isolated from oocytes/eggs/embryos, reversed transcribed with oligo dT and aliquots were analyzed by qRT-PCR using synthetic oligonucleotide primers and TaqMan® probes specific to *Floped*, *Tle6*, and *Mater* transcripts. Results were normalized to the abundance in full grown (80 µm) oocytes. (E). Immunoblot of lysates isolated from 10

growing oocytes, eggs and pre-implantation embryos were probed with antibodies specific to FLOPED, MATER, TLE6 or β-actin as a load control.

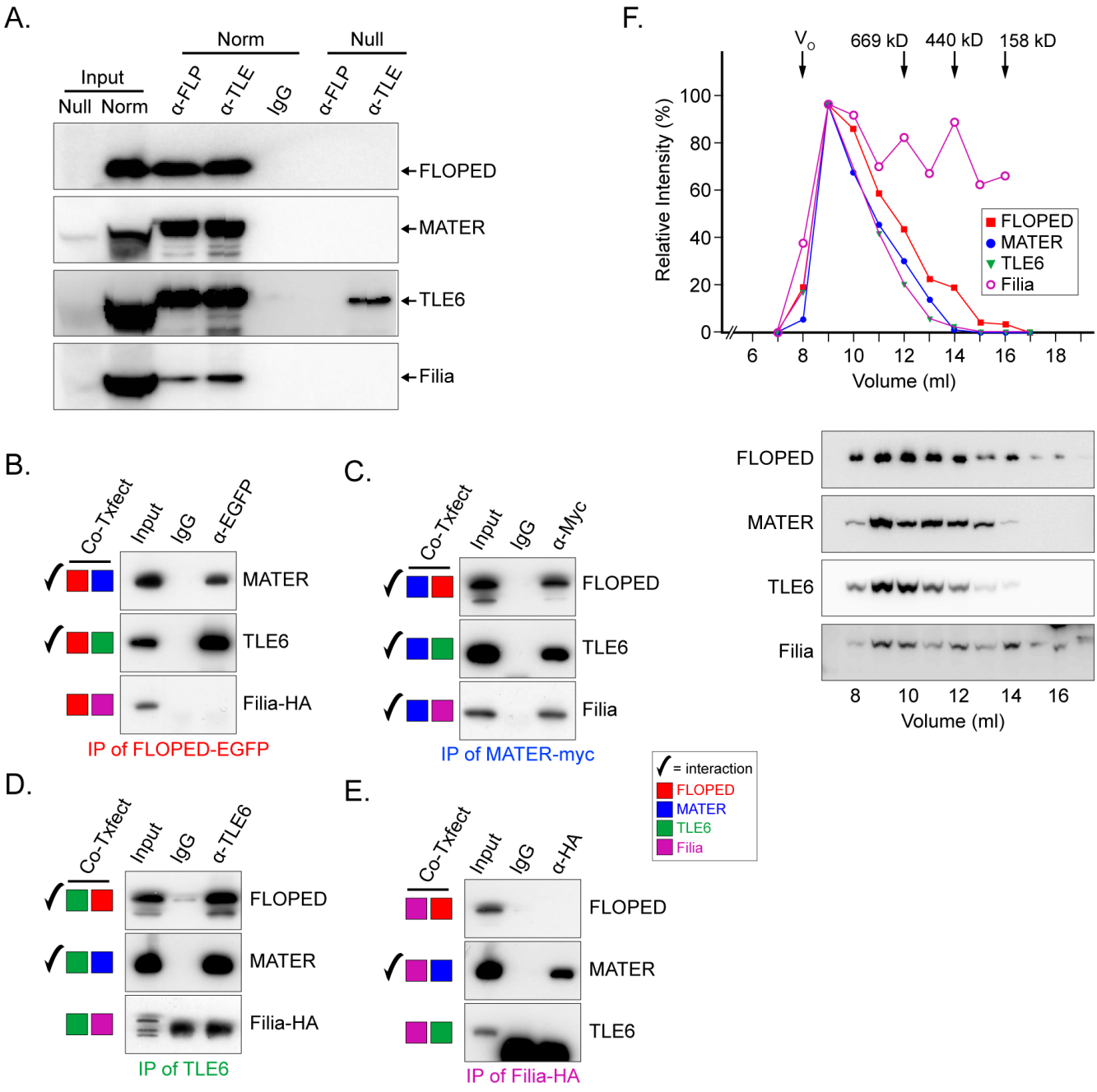

Figure 4. Interactions of FLOPED, MATER, TLE6 and Filia

(A). Ovarian lysates, before (Input) or after immunoprecipitation with antibodies to FLOPED (α-FLP) or TLE6 (α-TLE) were immunoblotted and probed with antibodies to FLOPED, MATER, TLE6 and Filia. Abbreviations include: Norm (normal) and Null (*Floped*$^{tm/tm}$) ovarian lysates; α-FLP (rabbit anti-FLOPED antibody); α-TLE (rabbit anti-TLE6 antibody); IgG (normal rabbit immunoglobulin), as a negative control. (B). FLOPED-EGFP and either MATER-myc, TLE6 or Filia-HA expression vectors were co-transfected (Co-Txfect) into COS cells. Lysates before (Input) or after immunoprecipitation with control immunoglobulin (IgG) an antibody to EGFP (α-EGFP) were used to isolate FLOPED and associated proteins. Immunoblots were probed with antibodies to myc, TLE6 and HA (hemaglutinin) to detect

MATER, TLE6 and Filia, respectively. (C). Same as (B) except that MATER with either FLOPED, TLE6 or Filia were co-transfected into COS cells; antibodies to myc (α-myc) were used to immunoprecipitate MATER and associated proteins. Immunoblots were probed with appropriate antibodies to detect FLOPED, TLE6 and Filia. (D). Same as (B) except that TLE6 with either FLOPED, MATER or Filia were co-transfected into COS cells; antibodies to TLE6 (α-TLE6) were used to immunoprecipitate TLE and associated proteins. Immunoblots were probed with appropriate antibodies to detect FLOPED, MATER and Filia. (E). Same as (B) except that Filia with either FLOPED, MATER or TLE6 were co-transfected into COS cells; antibodies to HA (α-HA) were used to immunoprecipitate Filia and associated proteins. Immunoblots were probed with appropriate antibodies to detect FLOPED, MATER and TLE6. (F). Normal egg lysates (150) were chromatographed by FPLC on a Superose 6 10/300 GL column with a void volume (V_0) of ~2×10^6 Da (upper). Immunoblots of individual 1 ml fractions (lower) detected FLOPED (fractions 8–16), MATER (fractions 8–14), TLE6 (fractions 8–14) and Filia (fractions 8–16). The data (average of two experiments) was quantified by fluorescent image analysis and the peak values in fraction 9 were set at a relative intensity of 100%. Elution of protein standards are indicated by arrows.

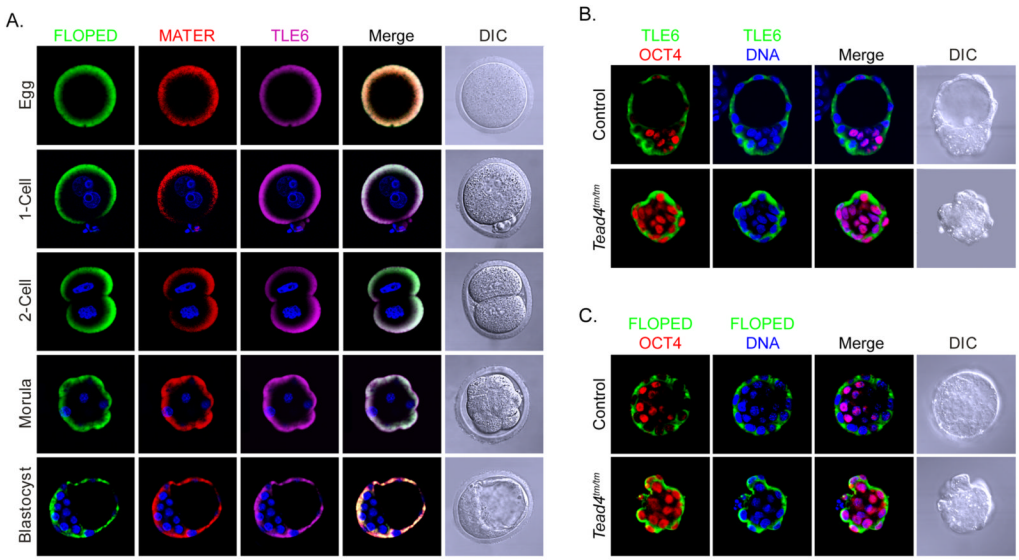

Figure 5. Localization of the SCMC in eggs and pre-implantation embryos

(A). Confocal microscopic images of eggs and pre-implantation embryos after permeabilization and incubation with antibodies to FLOPED, MATER, and TLE6. Co-localization of FLOPED, MATER and TLE6 was observed in the merge. (B). At E3.5, control (*Tead4*$^{+/tm}$) and *Tead4*$^{tm/tm}$ embryos were isolated from the oviduct of mated mice, permeabilized and incubated with antibodies to TLE6 and OCT4 (epiblast marker) or (C) with antibodies to FLOPED and OCT4. In all eggs and embryos, DNA was visualized with Hoechst and morphology was determined by DIC.

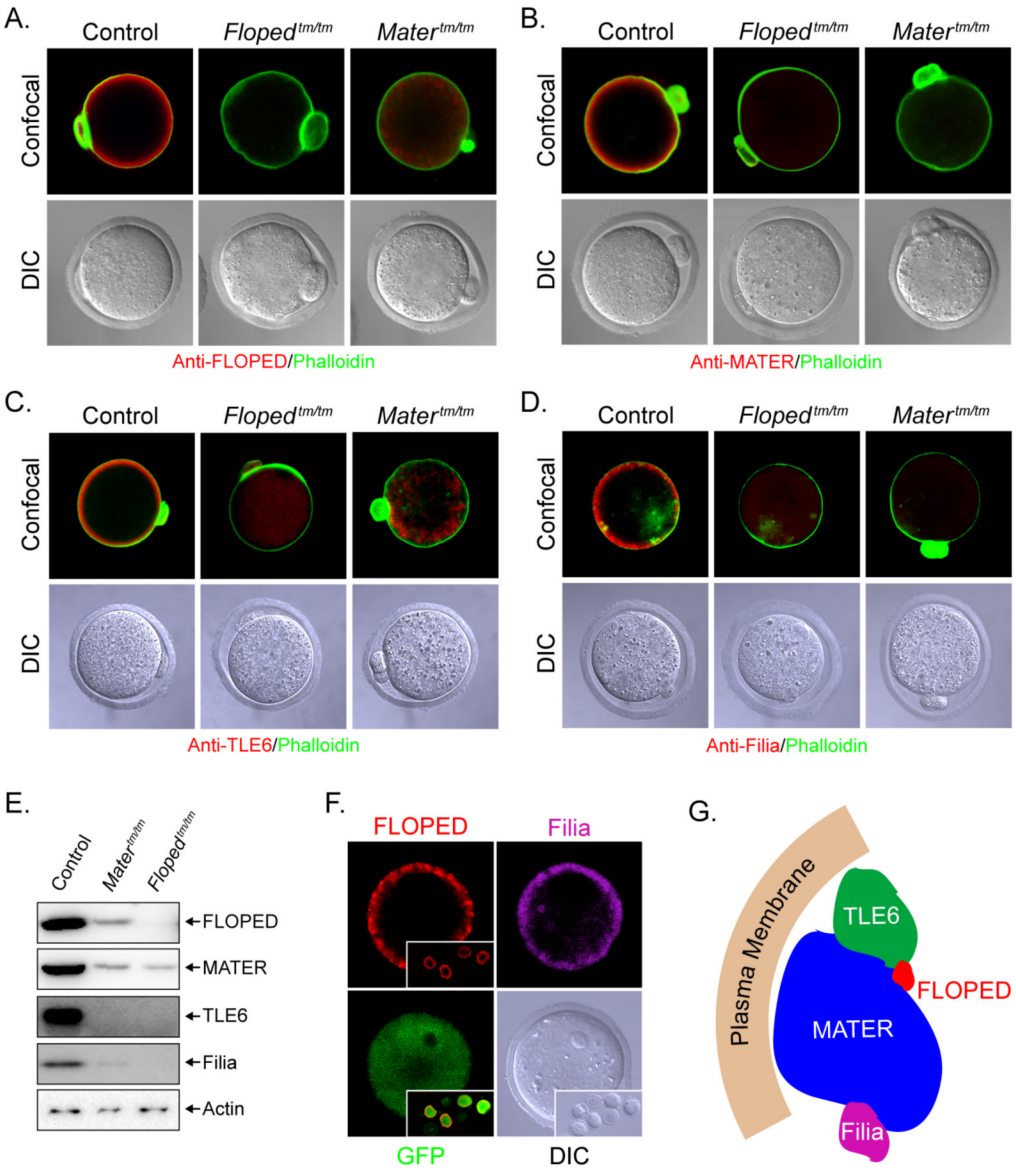

Figure 6. Formation of the SCMC depends on FLOPED and MATER

(A). Eggs isolated from normal, *Floped*$^{tm/tm}$ or *Mater*$^{tm/tm}$ mice were fixed, permeabilized and incubated with antibodies to FLOPED and phalloidin to detect F-actin. (B) Same as (A) except with antibodies to MATER. (C) Same as (A) except with antibodies to TLE6. (D) Same as (A) except with antibodies to Filia. (E). Egg lysates (10) from control (normal), *Floped*$^{tm/tm}$ or *Mater*$^{tm/tm}$ mice were immunoblotted and probed with antibodies specific to FLOPED, MATER, TLE6, Filia and β-actin. (F). *Floped*$^{tm/tm}$ oocytes were injected with a plasmid independently expressing FLOPED and GFP (positive transcription/translation control). After ~2 days in culture, oocytes were fixed and imaged by confocal microscopy for FLOPED, Filia and GFP as well as by DIC. (G). A model of the subcortical maternal complex (SCMC) formed during oogenesis that is required for cleavage stage embryogenesis. The SCMC is composed of FLOPED, MATER, TLE6 and Filia. The first three proteins (FLOPED, TLE6, MATER) interact directly with one another and Filia interacts with MATER, but not FLOPED or TLE6. The size of each protein corresponds to its predicted molecular mass.