Fertilization in mouse does not require terminal galactose or N-acetylglucosamine on the zona pellucida glycans

Suzannah A. Williams[1,*], Lijun Xia[2,3,*], Richard D. Cummings[3,‡], Rodger P. McEver[2,3] and Pamela Stanley[1,§]

[1]Department of Cell Biology, Albert Einstein College of Medicine, New York, NY 10461, USA
[2]Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104, USA
[3]Department of Biochemistry and Molecular Biology, Oklahoma Center for Medical Glycobiology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA

*These authors contributed equally to this work
‡Present address: Department of Biochemistry, Emory University, Atlanta, GA 30322, USA
§Author for correspondence (e-mail: stanley@aecom.yu.edu)

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Summary

Fertilization in mammals requires sperm to bind to the zona pellucida (ZP) that surrounds the egg. Galactose (Gal) or *N*-acetylglucosamine (GlcNAc) residues on the glycans of ZP protein 3 (ZP3) have been implicated as mouse sperm receptors. However, *Mgat1*$^{-/-}$ eggs with modified *N*-glycans lacking terminal Gal and GlcNAc residues are fertilized. To determine if Gal and GlcNAc on *O*-glycans of the ZP are required for fertilization, a conditional allele of the T-synthase gene (*T-syn*^{F}) was generated. *T-syn* encodes core 1 β1,3-galactosyltransferase 1 (T-synthase), which initiates the synthesis of core-1-derived *O*-glycans, the only *O*-glycans on mouse ZP3. *T-syn*$^{F/F}$:ZP3*Cre* females in which *T-syn*^{F} was deleted at the beginning of oogenesis generated eggs lacking core-1-derived *O*-glycans. Nevertheless, *T-syn*$^{F/F}$:ZP3*Cre* females were fertile and their eggs bound sperm similarly to controls. In addition, *T-syn*$^{-/-}$ embryos generated from *T-syn* null eggs developed until ~E12.5. Thus, core-1-derived *O*-glycans are not required for blastogenesis, implantation, or development prior to midgestation. Moreover, *T-syn*$^{-/-}$*Mgat1*$^{-/-}$ eggs lacking complex and hybrid *N*-glycans as well as core-1-derived *O*-glycans were fertilized. The combined data show that mouse ZP3 does not require terminal Gal or GlcNAc on either *N*- or *O*-glycans for fertilization.

Key words: Fertilization, Zona pelucida, O-glycans, N-glycans, T-synthase

Introduction

Sperm binding to the egg zona pellucida (ZP) is critical for fertilization to occur in mammals. The ZP extracellular matrix that surrounds mouse oocytes and ovulated eggs is generated from ZP glycoproteins ZP1, ZP2, ZP3 in the ratio 1:4:4 (Green, 1997). ZP proteins are synthesized and secreted exclusively by the oocyte during the 2-3 weeks of oogenesis prior to ovulation (Philpott et al., 1987). All three ZP proteins are heterogeneously glycosylated with *N*- and *O*-glycans (Philpott et al., 1987) but only ZP1 and ZP3 appear to possess *O*-glycans attached to Ser or Thr residues (Boja et al., 2003; Chalabi et al., 2006; Nagdas et al., 1994). ZP1 is not required to generate a functional ZP or for fertility in the mouse (Rankin et al., 1999). Soluble ZP3, but not soluble ZP1 or ZP2, competitively inhibits sperm binding to eggs in a dose-dependent manner, suggesting that ZP3 carries sperm receptor(s) (Bleil and Wassarman, 1980). The glycans of ZP3 were subsequently implicated in sperm binding using in vitro assays with ZP3 glycopeptides generated by Pronase digestion (Florman et al., 1984). These glycopeptides were almost devoid of amino acids suggesting that only ZP3 glycans are recognized by mouse sperm. Consistent with a potential direct or indirect role for glycans in sperm-egg binding in vivo is the finding that mouse eggs with a humanized zona containing human ZP2 and ZP3 in place of mouse ZP2 and ZP3 bind mouse sperm but do not bind human sperm (Rankin et al., 2003).

Support for specific roles for *N*- or *O*-glycans in mouse sperm-egg binding has also been obtained from glycosidase digestions. In vitro removal of terminal galactose (Gal) residues from *O*-glycans of mouse eggs by α-galactosidase abolished the ability of ZP3 to inhibit sperm binding (Bleil and Wassarman, 1988; Florman and Wassarman, 1985). However, mice lacking α1,3-galactosyltransferase and thus Galα1→3Gal termini on ZP glycans are fertile (Thall et al., 1995). Removal of terminal *N*-acetylglucosamine (GlcNAc) from the zona by digestion of eggs with β-*N*-acetylglucosaminidase also inhibited sperm binding (Shur and Hall, 1982), and terminal GlcNAc was thus proposed as a sperm receptor recognized by β1,4-galactosyltransferase 1 (β4GalT-1) on the sperm head (Lopez et al., 1985; Miller et al., 1992). However, sperm lacking β4GalT-1 are able to fertilize ovulated eggs (Asano et al., 1997) with sperm binding actually increased (Lu and Shur, 1997). Fucose has also been proposed to play a role due to the inhibition of sperm binding by the Lewis^{X} and Lewis^{A} determinants (Johnston et al., 1998; Kerr et al., 2004). However, fertility in α1,3-fucosyltransferase 9 (*Fut9*)-null (*Fut9*$^{-/-}$) mice whose eggs lack the Lewis^{X} determinant, is normal (Kudo et al., 2004). Finally, mannose present on *N*-

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glycans has been implicated in mouse sperm-egg recognition (Cornwall et al., 1991). However, treatment with *N*-glycanase, which should remove all *N*-glycans, did not affect sperm binding to mouse eggs (Florman and Wassarman, 1985).

The combined biochemical data implicate sugar recognition in mouse sperm-egg binding but do not lead to a unified hypothesis. Indeed in most instances, genetic ablation in vivo of sugars identified as critical determinants of sperm-egg binding by in vitro biochemical assays, does not lead to infertility. Most recently, oocyte-specific deletion of the mannoside acetylglucosaminyltransferase 1 (*Mgat1*) gene revealed that mouse eggs lacking terminal Gal and GlcNAc residues on *N*-glycans are efficiently fertilized, although they have a fragile, thin zona (Shi et al., 2004), and bind fewer sperm than control eggs using a classic in vitro sperm binding assay (Hoodbhoy et al., 2005). Whereas this genetic approach ruled out terminal Gal or GlcNAc on *N*-glycans as necessary for fertilization or sperm binding, *O*-glycans remained unaltered. Mouse ZP3 has at least five *O*-glycans and mouse ZP1 has multiple *O*-glycans (Boja et al., 2003). The predominant form of *O*-glycan on mouse ZP3 has the core 2 structure (Dell et al., 2003) that is generated from a core 1 *O*-glycan. Core-1-derived *O*-glycans arise from the extension of the Tn antigen (GalNAcα1-Ser/Thr) by the action of core 1 β1,3-galactosyltransferase (T-synthase) which adds Gal to generate the T antigen or core 1 *O*-glycans (Galβ1-3GalNAcα1-Ser/Thr) (Ju et al., 2002a; Ju et al., 2002b). The addition of a branching β1,6-linked GlcNAc to the GalNAc (*N*-acetylgalactosamine) of a core 1 *O*-glycan generates core 2 which may be extended with Gal and *N*-acetyllactosamine. Mice lacking core 2 *N*-acetylglucosaminyltransferase L (C2GnT-L) are fully fertile (Ellies et al., 1998), but two other core 2 GlcNAcTs exist (Bierhuizen et al., 1993; Yeh et al., 1999) and may rescue the C2GnT-L deficiency in eggs. No core 3, core 4 or sialyl-GalNAc *O*-glycans were detected on mouse ZP1 or ZP3 by mass spectrometry (Chalabi et al., 2006; Dell et al., 2003; Easton et al., 2000).

In this paper we investigate whether terminal Gal or GlcNAc residues on *O*-glycans of the ZP are required for fertilization in the mouse using a genetic approach. A logical choice for inhibiting *O*-glycan synthesis would be to prevent the addition of the initiating GalNAc, but UDP-GalNAc:polypeptide GalNAc-transferases are encoded by many different genes. On the other hand, T-synthase acts after GalNAc-transferase to generate core-1-derived *O*-glycans and has no obvious homologs in mammalian genomes (Ju et al., 2002a; Ju et al., 2002b). It is, therefore, a suitable target for producing eggs lacking extended *O*-glycans. However, *T-syn*$^{-/-}$ (also known as *C1galt1*$^{-/-}$; MGI) embryos die by embryonic day (E)14 from angiogenic defects (Xia et al., 2004), and thus oocyte-specific deletion of the *T-syn* gene was performed to generate mouse eggs lacking core-1-derived *O*-glycans by inhibition of their synthesis rather than biochemical removal post ovulation. This results in the generation of an intact mutant zona on oocytes and eggs, allowing functional analysis in a biologically relevant environment. We show here that mouse eggs lacking core-1-derived *O*-glycans are efficiently fertilized and bind sperm well. Additional removal of complex and hybrid *N*-glycans in double mutant *T-syn*$^{F/F}$*Mgat1*$^{F/F}$:ZP3*Cre* females also resulted in eggs that were fertilized. Thus neither Gal nor GlcNAc on *N*- or *O*-glycans of the mouse ZP function as essential sperm receptors.

Results

Generation of the *T-syn* floxed allele and *T-syn*$^{F/F}$:ZP3*Cre* females

To allow oocyte-specific deletion of the *T-syn* gene, mice carrying the *T-syn*^{F} allele were generated. Embryonic stem (ES) cells targeted previously at the *T-syn* locus (Xia et al., 2004) were transfected with an expression vector carrying Cre recombinase to obtain ES cells in which the *T-syn* allele was floxed (*T-syn*^{F}) and the *Neo* gene was deleted as described in the Materials and Methods (Fig. 1A). ES cells with a *T-syn*^{F} allele were identified by Southern analysis following *Bam*HI digestion of genomic DNA. An extra *Bam*HI site introduced immediately upstream of exon 1 of the *T-syn*^{F} allele gave a 5.9 kb band following *Cre*-mediated deletion. Thus analysis of *T-syn*$^{F/+}$ ES cells showed the 5.9 kb band and a 10 kb wild-type band, whereas analysis of parental ES cells showed the 10 kb wild-type band and a 7.7 kb band corresponding to the tri-*loxP* *T-syn* allele containing the *Neo* gene (Fig. 1B). Chimeras that transmitted the *T-syn*^{F} allele were used to generate *T-syn*$^{F/+}$ heterozygotes. *T-syn*$^{F/+}$ females were mated to *T-syn*$^{F/+}$:ZP3*Cre* males to obtain *T-syn*$^{F/F}$:ZP3*Cre* females and control females.

Fertility of females with oocyte-specific deletion of *T-syn*

To determine if eggs lacking core-1-derived *O*-glycans could be fertilized, *T-syn*$^{F/F}$:ZP3*Cre* females were mated with C57BL/6 males. *T-syn*$^{F/+}$:ZP3*Cre* and *T-syn*$^{+/+}$:ZP3*Cre* females were also mated as controls. The ZP3*Cre* transgene causes deletion of floxed gene fragments specifically in the oocyte (Lewandoski et al., 1997) when the ZP3 promoter becomes active 2-3 weeks prior to ovulation (Philpott et al., 1987). To confirm that *T-syn* had been deleted in ovulated eggs, pups from *T-syn*$^{F/F}$:ZP3*Cre* and *T-syn*$^{F/+}$:ZP3*Cre* females were genotyped using the primers described in Fig. 2A that identify the wild-type, floxed and deleted *T-syn* alleles (Fig. 2B). The reaction catalyzed by T-synthase to form core 1 and core 2 *O*-glycans is shown in Fig. 2C and the genotypes of females carrying floxed alleles and their oocytes after gene deletion are shown in Fig. 2D.

All pups in all litters from *T-syn*$^{F/F}$:ZP3*Cre* females had deleted both *T-syn* alleles, and 52% of pups from *T-syn*$^{F/+}$:ZP3*Cre* females carried one deleted *T-syn* allele (Table 1). Therefore the ZP3Cre recombinase was 100% efficient at the *T-syn* locus. More importantly, the data in Table 1 show that *T-syn*$^{F/F}$:ZP3*Cre* females were fertile, indicating that core-1-derived *O*-glycans on oocyte or egg glycoproteins are not essential for oogenesis, ovulation or fertilization. Litters were produced by *T-syn*$^{F/F}$:ZP3*Cre* females in the same period as by control females, and litter size was actually larger in *T-syn*$^{F/F}$:ZP3*Cre* females than controls, an interesting finding that is under further investigation. The ZP3*Cre* transgene had no effect on fertility with a litter size of 8.0±2.4 in *T-syn*$^{+/+}$ females (n=34) compared with 8.0±2.7 for *T-syn*$^{+/+}$:ZP3*Cre* females (n=29).

Core-1-derived *O*-glycans are not required for development before E12.5

Mutant *T-syn*$^{-/-}$ embryos lacking T-synthase generated from *T-syn*$^{+/-}$ matings have hemorrhages in the brain and spinal region at E11-E13 and die by E14 (Xia et al., 2004). Such embryos might be rescued during blastogenesis and implantation by maternal T-synthase transcripts that are expected to be present

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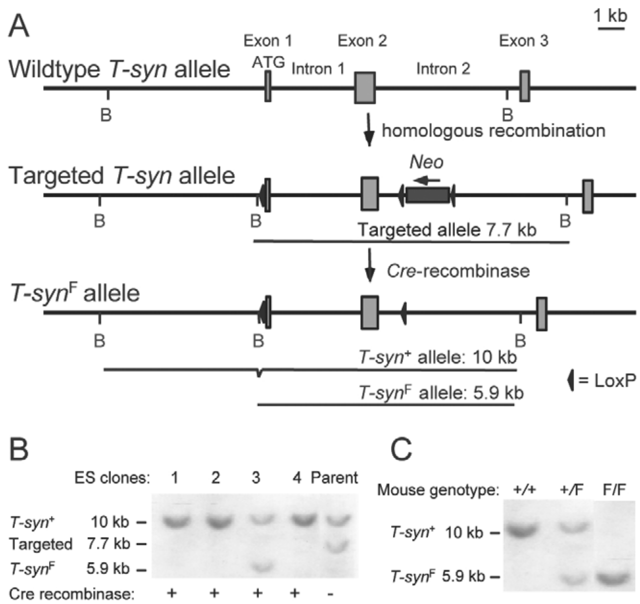


Fig. 1. Generation of the *T-syn*^{F} allele. (A) Gene targeting scheme. B, *Bam*HI. (B) Southern analysis of *Bam*HI-digested genomic DNA from ES clones after Cre-recombinase-mediated deletion of the *Neo* selection marker. (C) Southern blot analysis of *Bam*HI-digested genomic DNA from mouse tails identifying *T-syn*^{F} mice. The probe for each Southern analysis was exon 2 of the *T-syn* gene.

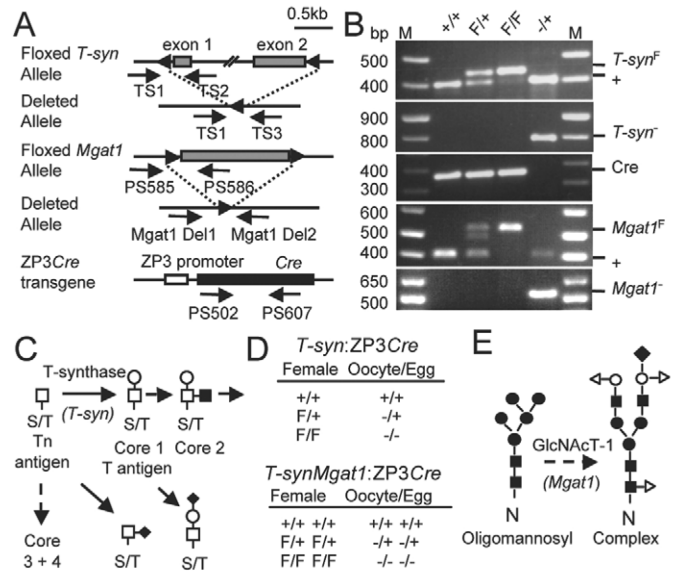


Fig. 2. Generation of the *T-syn* allele and oocyte-specific deletion. (A) Diagram of *T-syn* and *Mgat1* floxed and deleted alleles, and the ZP3*Cre* transgene with positions of primers used in genotyping. The *T-syn-*, *Mgat1-* and *Cre*-coding regions are shaded, and the promoter region for ZP3 is an open box. (B) Mouse genotype was determined using PCR of genomic tail DNA. Lane M, 1 kb plus markers. (C) *O*-glycan synthesis. T-synthase transfers Gal to GalNAc on Ser/Thr generating core 1. Subsequently GlcNAc may be added to the GalNAc to generate core 2. Core 3 and 4 structures have not been detected on mouse ZP3 (Boja et al., 2003; Chalabi et al., 2006). (D) Relationship of genotype of mutant females to genotype of eggs. (E) Diagram of complex *N*-glycan structure showing that the deletion of the *Mgat1* gene generates oligomannosyl *N*-glycans. Open square, GalNAc; open circle, Gal; black square, GlcNAc; black diamond, sialic acid; triangle, fucose; black circle, mannose.

in heterozygous eggs (Su et al., 2004). To investigate potential roles for T-synthase in pre-implantation development, timed matings between *T-syn*$^{F/F}$:ZP3*Cre* females and *T-syn*$^{+/-}$ males were performed. Two females were dissected at E11.5 and five females at E12.5. Mutant embryos had the same overall phenotype of hemorrhaging and defective angiogenesis at E11.5 (data not shown), which was more severe at E12.5 (Fig. 3A) as described previously (Xia et al., 2004). In addition, mutant embryos were obtained at the expected ratio of 1:1 (Fig. 3B) providing strong evidence that blastocysts lacking both maternal and zygotic T-synthase are able to develop, implant and progress to ~E12.5.

Sperm bind efficiently to *T-syn*$^{-/-}$ eggs

T-syn$^{F/F}$:ZP3*Cre* females were as fertile as controls (Table 1). However, fertilization requires only one sperm to bind and traverse the zona. The zona on *T-syn*$^{-/-}$ eggs was marginally thinner and slightly looser in appearance than the ZP of wild-type eggs (Fig. 4A,B). To determine if sperm binding to mutant zona was altered by the removal of terminal Gal and GlcNAc residues on *O*-glycans, classic in vitro assays of sperm binding were performed. Ovulated eggs denuded of cumulus cells by hyaluronidase treatment were incubated with sperm in the presence of two-cell embryo controls that do not bind sperm. Sperm binding of *T-syn*$^{-/-}$ eggs was indistinguishable from that of wild-type eggs, under conditions in which two-cell embryos showed no sperm binding (Fig. 4C-F). Thus, the ZP surrounding the eggs of *T-syn*$^{F/F}$:ZP3*Cre* females binds sperm equivalently to wild-type ZP. In three experiments using eggs ovulated from two to three control or *T-syn*$^{F/F}$:ZP3*Cre* females in each experiment, sperm binding to wild-type and *T-syn*$^{-/-}$ eggs was always equivalent.

T-syn$^{-/-}$ eggs lack core-1-derived *O*-glycans

Considering the fertility of *T-syn*$^{F/F}$:ZP3*Cre* females and the robust binding of sperm to their eggs, it was important to confirm that core-1-derived *O*-glycans were absent. T antigen (Galβ3GalNAcα1Ser/Thr) generated by T-synthase (Fig. 2C) was detected by binding of fluoresceinated peanut agglutinin (PNA-FITC). The Tn antigen (GalNAcα1Ser/Thr), the precursor of the T antigen, was detected using anti-Tn antibody (Fig. 5A-D). Eggs from *T-syn*$^{+/+}$:ZP3*Cre* females stained brightly with PNA-FITC (Fig. 5A), whereas eggs from *T-syn*$^{F/F}$:ZP3*Cre* females bound only background levels of PNA-FITC (Fig. 5B). Consistent with the absence of the T antigen and a lack of T-synthase activity in mutant eggs, *T-syn*$^{-/-}$ eggs bound anti-Tn antibody, whereas wild-type or heterozygous eggs did not (Fig. 5C,D). Thus core-1-derived *O*-glycans were essentially absent from *T-syn*$^{-/-}$ eggs. However, as expected, complex *N*-glycans were not affected, as shown by the fact that *T-syn*$^{-/-}$ eggs bound *Phaseolus vulgaris* leukoagglutinin-FITC (L-PHA-FITC) equivalently to wild-type eggs (Fig. 5E,F). The combined data provide strong evidence that T-synthase was expressed in wild-type eggs and was not active in *T-syn* null eggs. Further evidence of this was obtained by western analysis of mutant oocytes.

Table 1. Fertility of *T-syn*$^{F/F}$:ZP3*Cre* females mated with C57BL/6 males

	T-syn$^{+/+}$:ZP3*Cre*	*T-syn*$^{F/+}$:ZP3*Cre*		*T-syn*$^{F/F}$:ZP3*Cre*	
Number of females	7	6		6	
Days to first litter	21.1±1.6	24.3±2.8		21.7±1.4	
Number of pups	8.0±2.7[a]	6.5±2.8[b]		10.4±3.1[c]	
Pups genotyped	NA	86		170	
	NA	*T-syn*$^{+/+}$	*T-syn*$^{+/–}$	*T-syn*$^{+/+}$	*T-syn*$^{+/–}$
Pup genotype	NA	41	45	0	170

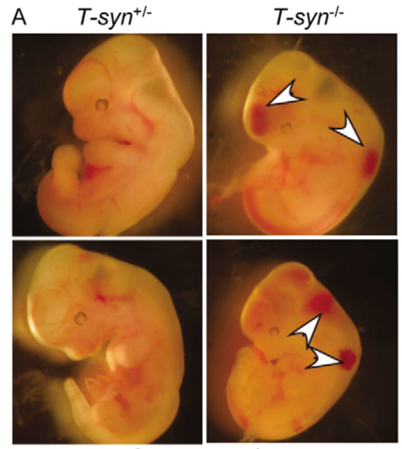
Values are the mean ± s.d.; a versus c: P=0.005, b versus c: P=0.0002. NA, not applicable.

ZP1 and ZP3 from mutant oocytes lack core-1-derived *O*-glycans

To confirm that the two proteins of the mouse zona that contain *O*-glycans, ZP3 and ZP1, had been modified from the beginning of oogenesis, western analyses of ovarian homogenates containing oocytes at all stages of development were performed using monoclonal antibodies to ZP3 and ZP1. ZP3 from wild-type ovarian homogenate migrated with an apparent molecular mass of ~65-80 kDa, reflecting glycan heterogeneity, whereas ZP3 from *T-syn*$^{F/F}$:ZP3*Cre* ovaries migrated with an apparent molecular mass of ~63-72 kDa, consistent with the loss of *O*-glycans from mutant oocytes (Fig. 5G). Prolonged exposure of the membrane did not reveal any ZP3 bands of higher molecular masses from *T-syn*$^{F/F}$:ZP3*Cre* ovaries indicating that all of the ZP3 was affected by the loss of T-synthase. Removal of *N*-glycans using *N*-glycanase reduced the molecular mass of wild-type ZP3 to ~35-45 kDa and of mutant ZP3 to ~32 kDa, which is the predicted mass of ZP3 with no *N*-glycans and only five GalNAc residues at *O*-glycan sites (Boja et al., 2003). The lack of heterogeneity of ZP3 from mutant ovaries and its molecular mass after *N*-glycanase treatment provides strong evidence that no *O*-glycans were extended by T-synthase in mutant oocytes. Similar conclusions were obtained from western analyses of ZP1. ZP1 from wild-type ovaries migrated with an apparent molecular mass of ~95-155 kDa whereas ZP1 from mutant ovaries migrated from ~75-140 kDa (Fig. 5H). Prolonged exposure of the membrane did not reveal any ZP1 from mutant ovary of the highest molecular mass of wild-type ZP1. Removal of *N*-glycans by digestion with *N*-glycanase caused wild-type ZP1 to migrate in a band of ~65-90 kDa and ZP1 from mutant oocytes in a tight band of ~60 kDa (Fig. 5H), consistent with the predicted molecular mass of ZP1 lacking *N*-glycans and having only GalNAc at *O*-glycan sites (Boja et al., 2003). Three females of each genotype gave similar western results. ZP2 from oocytes or eggs of wild-type and *T-syn*$^{F/F}$:ZP3*Cre* females had the same apparent molecular mass (data not shown) consistent with the predicted absence of *O*-glycans on ZP2 (Boja et al., 2003). The combined data show that inactivation of the gene encoding T-synthase gives rise to oocytes with glycoproteins (exemplified by ZP1 and ZP3) that are essentially devoid of core 1 and core 2 *O*-glycans.

Fertility of females with oocyte-specific deletion of *T-syn* and *Mgat1*

The absence of Gal and GlcNAc residues on core-1-derived *O*-glycans of *T-syn*$^{–/–}$ eggs did not alter sperm binding or fertility. However, complex and hybrid *N*-glycans generated by GlcNAc-T1 encoded by the *Mgat1* gene also have Gal and GlcNAc residues which may compensate for their loss of core-1-derived *O*-glycans (Fig. 2E). To investigate this question, *T-syn*$^{F/F}$*Mgat1*$^{F/F}$:ZP3*Cre* females were generated and mated with C57BL/6 males. *T-syn*$^{F/+}$*Mgat1*$^{F/+}$:ZP3*Cre* and *T-syn*$^{+/+}$*Mgat1*$^{+/+}$:ZP3*Cre* females were used as controls. The genotypes of their respective oocytes are shown in Fig. 2D. The results in Table 2 show that *T-syn*$^{F/F}$*Mgat1*$^{F/F}$:ZP3*Cre* double mutant (DM) females were fertile but their fertility was severely reduced compared to control females. Only three of 10 DM females produced a litter (Table 2) and no DM female produced more than a single litter, despite being with a male for up to 6 months. These single litters were produced at the same time after mating as the first litters of control females, suggesting that ovulation was initially unaffected. However, the number of pups produced by DM females was smaller (Table 2). Genotyping using the primers shown in Fig. 2A confirmed the absence of floxed *T-syn* and *Mgat1* alleles in all pups from *T-syn*$^{F/F}$*Mgat1*$^{F/F}$:ZP3*Cre* and *T-syn*$^{F/+}$*Mgat1*$^{F/+}$:ZP3*Cre* females (see Fig. 2B). This demonstrates that expression of the ZP3*Cre* transgene was able to delete two floxed alleles as efficiently as a single floxed allele such as *T-syn* or *Mgat1* (Shi et al., 2004). Superovulation of 4-week-old females resulted in ~50% fewer eggs from DM females compared with wild-type females. Moreover, superovulation of the DM females (Table 2) after the termination of mating resulted in no eggs from any of the 10 females, whereas wild-type and heterozygous females ovulated 49.8±9.8 eggs (n=6) and 44.0±12.8 eggs (n=4), respectively. This strongly suggests that the reduced fertility of DM females was not due to defective fertilization



B	E11.5		E12.5	
Females	2		5	
Embryos	21		46	
T-syn	+/-	-/-	+/-	-/-
	12	9	25	21

Fig. 3. Embryonic development from eggs lacking T-synthase. (A) *T-syn*$^{–/–}$ embryos were obtained from mating *T-syn*$^{F/F}$:ZP3*Cre* females to *T-syn*$^{+/–}$ males. At E12.5 mutant embryos exhibited angiogenic defects with hemorrhaging in the spinal and brain area (open arrowheads). (B) Embryos of each genotype were generated in approximately equal proportions from *T-syn*$^{–/–}$ eggs.

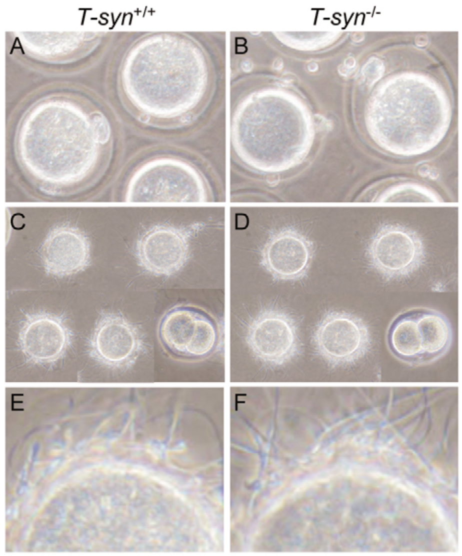


Fig. 4. Sperm binding to *T-syn*$^{-/-}$ eggs. (A,B) The zona of *T-syn*$^{-/-}$ eggs was marginally thinner and less uniform than wild-type zona. (C,D) Sperm binding to *T-syn*$^{-/-}$ and wild-type eggs was equivalent. Two-cell embryo controls had no sperm binding under these conditions. (E,F) Higher magnification shows comparable numbers of sperm bound to eggs of each genotype.

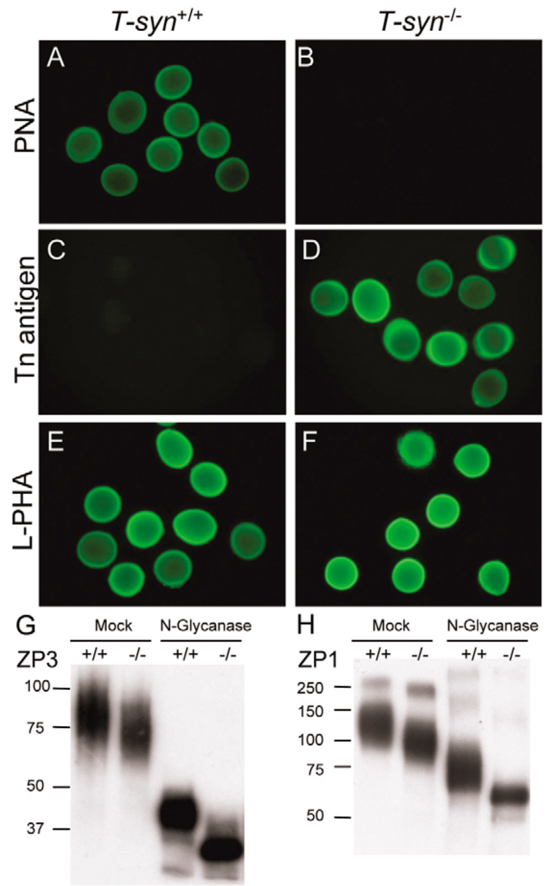


Fig. 5. Eggs from *T-syn*$^{F/F}$:ZP3*Cre* females lack core-1-derived *O*-glycans. (A,B) PNA-FITC bound to wild-type but not *T-syn*$^{-/-}$ eggs. Similar data were obtained in five experiments with eggs from 12 *T-syn*$^{F/F}$:ZP3*Cre* females. (C,D) Anti-Tn antibodies bound to the Tn antigen on *T-syn*$^{-/-}$ eggs, but poorly to wild-type eggs. Similar results were obtained in four experiments from 16 *T-syn*$^{F/F}$:ZP3*Cre* females. (E,F) L-PHA-FITC bound to mutant and wild-type eggs equivalently. Similar results were obtained in three experiments from seven *T-syn*$^{F/F}$:ZP3*Cre* females. (G,H) Western analyses of ZP1 and ZP3 from control and *T-syn*$^{F/F}$:ZP3*Cre* ovaries before and after digestion with *N*-glycanase.

but to compromised oogenesis, a result confirmed by finding a reduced number of developing follicles in ovaries from these DM females at 3-6 months of age.

Eggs from DM females lack core-1-derived *O*-glycans and complex and hybrid *N*-glycans

To confirm that both the *O*- and *N*-glycans of the ZP had been altered in DM females, lectins were used to detect the T antigen and complex *N*-glycans on ovulated eggs. PNA-FITC bound well to wild-type eggs whereas eggs from DM females bound little PNA-FITC, confirming the absence of the T antigen (Fig. 6E,F), as observed with *T-syn*$^{-/-}$ eggs (Fig. 5A,B). L-PHA-FITC binding was also absent (Fig. 6G,H), and concanavalin A-Rhodamine (Con A-Rho) binding to DM eggs was enhanced (Fig. 6I,J). This confirmed the lack of complex and hybrid *N*-glycans and the consequent increase in oligomannosyl *N*-glycans on DM eggs. Therefore, the *O*- and *N*-glycans of eggs from DM females were altered as expected. However, properties of the zona of DM eggs were also altered. Cumulus cells remained attached to most DM eggs (Fig. 6B,D) but not wild-type eggs (Fig. 6A,C) after 3-5 minutes of hyaluronidase digestion. Prolonged incubation, of up to 20 minutes, in hyaluronidase did not remove the cumulus cells from DM eggs. Mild agitation by pipetting resulted in the zona tearing away from DM eggs without cumulus cells being removed, and revealed the thinness of the DM zona (Fig. 6D). A fragile, thin, zona was to be expected in eggs lacking *Mgat1*, as previously described (Shi et al., 2004).

Oocytes from DM females lack core-1-derived *O*-glycans and complex and hybrid *N*-glycans

T-syn$^{F/F}$*Mgat1*$^{F/F}$:ZP3*Cre* females were either infertile or gave birth to small litters (Table 2). To confirm that this was not due to inconsistent deletion of the two floxed genes in oocytes, lectin staining was performed on ovarian sections from these mice (Fig. 7). The absence of the T antigen was demonstrated by a lack of PNA-FITC staining of the zona in ovarian sections from DM females (Fig. 7A) and the absence of complex *N*-glycans was demonstrated by a lack of L-PHA-FITC staining in the zona of ovaries from all *T-syn*$^{F/F}$*Mgat1*$^{F/F}$:ZP3*Cre* females, including those that had given birth (Fig. 7A). The complete absence of ZP lectin staining in *T-syn*$^{F/F}$*Mgat1*$^{F/F}$:ZP3*Cre* ovaries was not due to the lack of a zona on mutant oocytes because all of them bound antibodies to ZP1, ZP2 and ZP3 (Fig. 7B). However, compared with controls, the amount of each ZP was reduced in DM oocytes as seen previously in ovarian sections from *Mgat1*$^{F/F}$:ZP3*Cre* females (Shi et al., 2004).

Discussion

The *O*-glycans on ZP3 have been implicated in fertilization of

Table 2. Fertility of *T-syn*$^{F/F}$ *Mgat1*$^{F/F}$:ZP3*Cre* females mated with C57Bl/6 males

	T-syn$^{+/+}$ *Mgat1*$^{+/+}$:ZP3*Cre*	*T-syn*$^{F/+}$ *Mgat1*$^{F/+}$:ZP3*Cre*		*T-syn*$^{F/F}$ *Mgat1*$^{F/F}$:ZP3*Cre*	
Number of females	6	6		10	
Fertile females	6	5		3	
Days to first litter	23.7±5.3	22.4±3.9		23.0±2.7	
Number of pups	8.8±2.3[a]	8.4±2.0[b]		4.0±2.7[c]	
Pups genotyped	NA	82		12	
	NA	*T-syn*$^{-}$	*Mgat1*$^{-}$	*T-syn*$^{-}$	*Mgat1*$^{-}$
Pup genotype	NA	46	48	12	12

Values are the mean ± s.d.; a versus c: P=0.009, b versus c: P=0.005. NA, not applicable.

mouse eggs for over 20 years on the basis of in vitro glycosidase treatments, inhibition of sperm-egg binding by glycopeptides, and transgenic mutant ZP experiments (Clark and Dell, 2006; Shur et al., 2006; Wassarman, 2005; Wassarman et al., 2005). In this paper we addressed this question by targeted inactivation of T-synthase, the glycosyltransferase that initiates the synthesis of the core 1 and core 2 *O*-glycans, the only mucin *O*-glycans detected on mouse ZP3 by mass spectrometry (Boja et al., 2003; Chalabi et al., 2006). T-synthase is essential for embryonic development beyond E14 (Xia et al., 2004). Therefore, we generated the conditional *T-syn*^{F} allele and used a ZP3*Cre* transgene for oocyte-specific deletion to obtain eggs lacking core-1-derived *O*-glycans. This approach enabled direct assessment of the in vivo function of mutant ZP in fertilization.

Given the predicted importance of *O*-glycans on ZP3 for sperm binding, it was surprising to find that fertilization was not in the least impaired in *T-syn*$^{F/F}$:ZP3*Cre* females. We eliminated the possibility that another glycosyltransferase substituted for T-synthase by showing that mutant eggs did not bind PNA, which recognizes the product of T-synthase, but did bind anti-Tn antibody, which recognizes the GalNAcα1Ser/Thr substrate of T-synthase. More importantly, western analyses showed that the full complement of ZP1 and ZP3 glycoproteins was affected by the mutation in *T-syn*$^{-/-}$ eggs. It has been proposed that, because of the heterogeneous nature of the zona matrix that leads to differences in the glycans present on the outer surface of the ZP compared to the inner (Aviles et al., 2000), only ZP3 proteins on the outer surface of the ZP need to have *O*-glycans at Ser 332 and 334 in order for them to function as sperm receptors (Williams et al., 2006); a model that we were able to test. Inactivation of the *T-syn* gene early in oogenesis left no ZP1 or ZP3 with a wild-type complement of core-1-derived *O*-glycans and thus ovulated eggs had no core-1-derived *O*-glycans on the outer layer of their ZP. These results provide convincing evidence that Gal (Bleil and Wassarman, 1988), GlcNAc (Miller et al., 1992; Shur and Hall, 1982) and/or fucose in the context of Lewis antigens (Johnston et al., 1998; Kerr et al., 2004) on *O*-glycans are dispensable for fertilization and thus for sperm binding. Gal or GlcNAc on *N*-glycans did not compensate for their loss from *O*-glycans because, when complex and hybrid *N*-glycans were also removed by deletion of the *Mgat1* gene, DM females were fertile. However, oogenesis was severely compromised since none of the DM females ovulated after stimulation with exogenous gonadotrophins at 3-6 months of age. The defective oogenesis in DM females was much more severe than the partially compromised developmental competence of ovulated eggs previously observed in *Mgat1*$^{F/F}$:ZP3*Cre* females that lack only complex and hybrid *N*-glycans during oogenesis (Shi et al., 2004). Nevertheless, the fact that a proportion of DM females with eggs shown to possess the expected glycosylation-defective phenotype gave rise to at least one litter leads to the conclusion that terminal Gal or GlcNAc residues on ZP glycoproteins are not essential for fertilization in the mouse. Indeed, there may not be a single sperm receptor. The recently described ZP3-independent sperm-binding ligand (Rodeheffer and Shur, 2004) may play a significant role in fertilization and, may compensate for the lack of complex *O*- and *N*-glycans in DM females.

The results described in this paper also have implications for a second model of the molecular basis of the specificity of mouse sperm-egg recognition which is based on the supramolecular structure of the mouse zona (Dean, 2004). This model proposes that the overall conformation of the ZP is different in different species and is responsible for taxon-specific sperm binding. Such a ZP conformation would need to be quite robust to account for the fertilization of mouse eggs with a thin, fragile ZP lacking both core-1-derived *O*-glycans and complex and hybrid *N*-glycans.

Whereas the genetic ablation strategy clearly demonstrates that complex *O*- and *N*-glycans terminating in Gal or GlcNAc are superfluous for fertilization, considerable in vitro biochemical evidence indicates a requirement for these glycans for sperm-egg binding. Purified solublized zona proteins were used for the competitive in vitro binding studies. However, solubilizing zona glycoproteins alters their conformation from the structure assembled into the zona matrix, potentially exposing protein or glycan determinants which may function in vitro but not in vivo. In addition, the zona matrix is heterogeneously glycosylated (Aviles et al., 2000) and purified ZP proteins generated from whole zona will contain glycoproteins that are not on the zona surface and thus would be unavailable for sperm binding in the oviduct. The data presented here demonstrate the importance of performing in vivo modifications using genetic deletion analysis to arrive at definitive conclusions regarding biological functions.

In summary, the mouse models we describe allow analyses of tissue-specific roles for core-1-derived *O*-glycans and complex *O*- and *N*-glycans. Following oocyte-specific deletion of *T-syn*, females were fully fertile even though their eggs lacked core-1-derived *O*-glycans. In addition, embryos lacking maternal and zygotic T-synthase progressed at a normal rate through blastogenesis and early embryonic development to E12.5. Thus, core-1-derived *O*-glycans are dispensable for sperm binding, fertilization, and for development to mid-gestation. Eggs that lack both core-1-derived *O*-glycans and

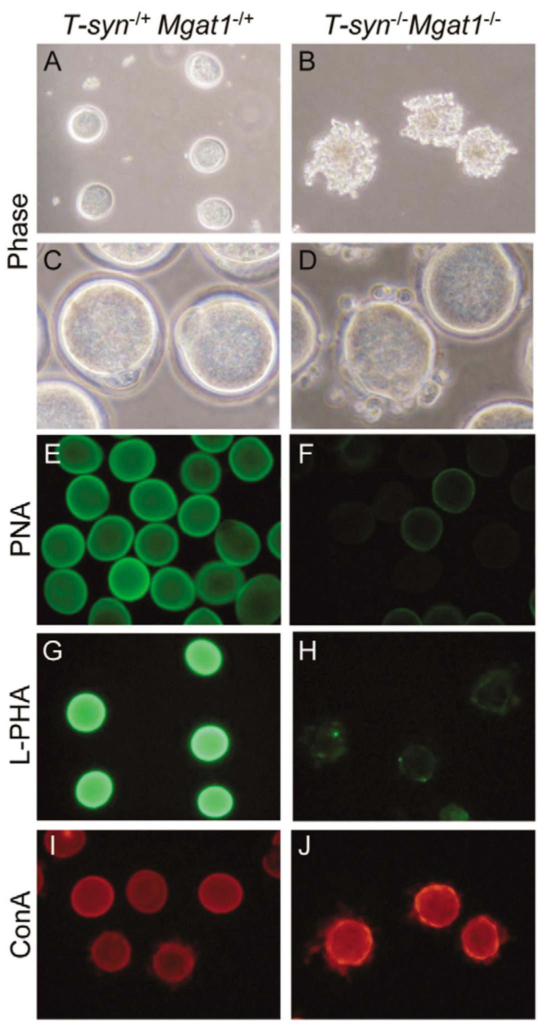


Fig. 6. Eggs from *T-syn*$^{F/F}$*Mgat1*$^{F/F}$:ZP3*Cre* DM females lack core-1-derived *O*-glycans and complex and hybrid *N*-glycans. (A,B) Phase-contrast micrographs showing persistent cumulus cells attached to *T-syn*$^{-/-}$*Mgat1*$^{-/-}$ eggs after hyaluronidase treatment. (C,D) Higher magnification of *T-syn*$^{-/-}$*Mgat1*$^{-/-}$ eggs with less cumulus attached revealed a thin fragile zona. (E,F) PNA-FITC did not bind to *T-syn*$^{-/-}$*Mgat1*$^{-/-}$ eggs. Similar data were obtained in three experiments with eggs from 12 *T-syn*$^{F/F}$*Mgat1*$^{F/F}$:ZP3*Cre* females. (G,H) L-PHA-FITC did not bind to *T-syn*$^{-/-}$*Mgat1*$^{-/-}$ eggs. Similar data were obtained in four experiments from 16 *T-syn*$^{F/F}$*Mgat1*$^{F/F}$:ZP3*Cre* females. (I,J) Con A-Rho staining was enhanced in *T-syn*$^{-/-}$*Mgat1*$^{-/-}$ eggs consistent with increased oligomannosyl *N*-glycans.

complex and hybrid *N*-glycans and thus have no terminal Gal or GlcNAc on their ZP are also fertilized, demonstrating that these residues are not essential for functional sperm-egg recognition in the mouse.

Materials and Methods

Generation of *T-syn*$^{F/F}$ mice

To establish mice in which the *T-syn* gene was flanked by *loxP* sites (*T-syn*$^{F/F}$), mouse embryonic stem (ES) cells previously targeted to contain three *lox*P sites flanking exons 1 and 2 of the *T-syn* gene and a neomycin (*Neo*) cassette in the *T-syn* locus (Xia et al., 2004) (Fig. 1A) were transiently transfected with an expression vector encoding Cre recombinase (a gift from Brian Sauer, Stowers Institute for Medical Research, Kansas City, MO) to delete the *Neo* cassette. To screen for ES cells with *Cre*-mediated recombination, individual clones were transferred into a 96-well plate after transfection. When the clones were confluent, cells were frozen, and also divided into two 96-well plates in medium with and without G418. After 3 days, *Cre*-mediated deletion of the *Neo* gene was considered to have taken place in clones that did not survive G418 selection. PCR genotyping was used for further screening as described previously (Xia et al., 2004). About 15% of ES clones had deleted only the *Neo* gene. Genomic DNA from these ES cells was digested with *Bam*HI, subjected to Southern analysis and probed with exon 2 DNA from the *T-syn* gene. ES cells with a floxed *T-syn* gene shown to also have a normal karyotype were microinjected into C57BL/6J blastocysts, which were implanted into pseudopregnant mice. Six of ten chimeras transmitted the floxed *T-syn* allele to their offspring. *T-syn*$^{F/+}$ mice were bred to generate homozygous *T-syn*$^{F/F}$ mice in a mixed 129/SvlmJ and C57BL/6J genetic background. Southern analysis of genomic DNA from mouse tail biopsies was performed following digestion with *Bam*HI and hybridization using exon 2 of the *T-syn* gene.

Mice with floxed *T-syn* and *Mgat1* alleles and a ZP3*Cre* transgene

Female mice with a *T-syn*^{F} allele were crossed with male mice of a mixed background carrying a *Cre* recombinase transgene under the control of the ZP3 promoter (Shi et al., 2004). Subsequent matings generated *T-syn*$^{F/F}$:ZP3*Cre* females and *T-syn*$^{F/+}$:ZP3*Cre* females in which inactivation of the *T-syn* gene occurs at the start of oogenesis when ZP3 is expressed (Lewandoski et al., 1997; Philpott et al., 1987) (Fig. 2A). To obtain homozygote and heterozygote double mutant (DM) females carrying floxed *Mgat1* allele(s) (Shi et al., 2004) in addition to floxed *T-syn* allele(s), *Mgat1*$^{F/F}$ mice, described previously (Shi et al., 2004), were crossed

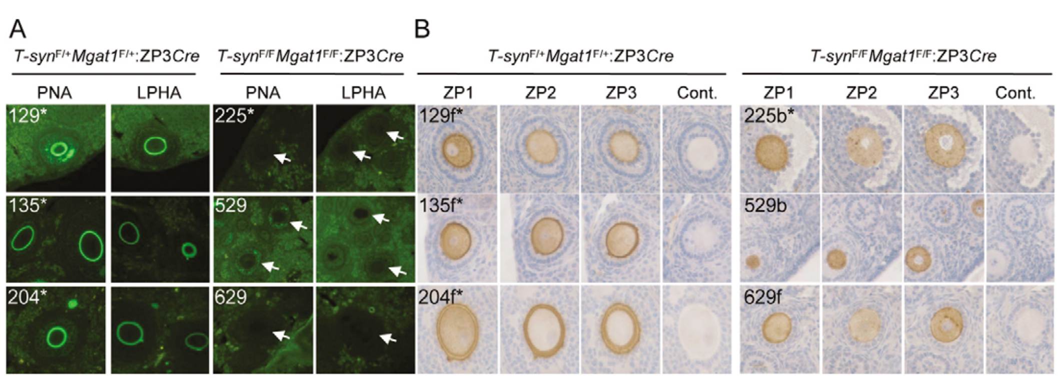


Fig. 7. Oocytes from *T-syn*$^{F/F}$*Mgat1*$^{F/F}$:ZP3*Cre* double mutant (DM) females lack core-1-derived *O*-glycans and complex and hybrid *N*-glycans. (A) The ZP of oocytes in *T-syn*$^{F/+}$*Mgat1*$^{F/+}$:ZP3*Cre* control ovaries bound both L-PHA-FITC and PNA-FITC whereas the ZP on oocytes of DM ovaries (arrows) did not bind either lectin. Images are overexposed to reveal unstained oocytes present in DM ovaries and follicles are size-matched for developmental stage. (B) Monoclonal antibodies to ZP1, ZP2 and ZP3 bound to control and DM ZP. The ovaries were from three previously mated control and 3 DM females and represent six control and ten DM females. Asterisks indicate females that gave birth. Formalin fixation was used for lectin staining whereas both formalin (f) and Bouins (b) fixed ovaries were used for ZP staining.

to obtain *T-syn*$^{F/F}$*Mgat1*$^{F/F}$:ZP3*Cre* and *T-syn*$^{F/+}$*Mgat1*$^{F/+}$:ZP3*Cre* females. *T-syn*$^{+/+}$:ZP3*Cre* and *T-syn*$^{+/+}$*Mgat1*$^{+/+}$:ZP3*Cre* females were generated as controls.

To distinguish between mice carrying floxed or deleted *T-syn* allele(s), and the ZP3*Cre* transgene, separate PCR genotyping was performed using tail genomic DNA. Primers TS-1 (5′-gataaatgtcttacagaagg-3′) and TS-2 (5′-ttatgttggctggaatctgc-3′) detected the wild-type (*T-syn*$^+$) and the floxed *T-syn*^{F} alleles, and primers TS-1 and TS-3 (5′-aatactgtcctgggctatactacagtg-3′) detected the deleted *T-syn* allele. For TS-1 and TS-2 primers, PCR reactions of 25 μl contained 2.5 μl 10× PCR buffer (not containing $MgCl_2$) (Invitrogen, Carlsbad, CA), 1.5 μl 50 mM $MgCl_2$ (Invitrogen), 0.5 μl 10 mM dNTPs (Invitrogen), 1 μl 10 mM primers, 3 IU Taq polymerase (Roche, Indianapolis, IN) and 1.5 μl DNA. For TS-1 and TS-3 primers, reactions of 25 μl contained 2.5 μl of 10× PCR buffer already containing 20 mM $MgCl_2$, 0.5 μl 50 mM $MgCl_2$, 0.5 μl 10 mM dNTPs, 0.5 μl 10 mM primers, 3 IU Taq polymerase (Roche), and 0.5 μl DNA. Deleted *Mgat1* was detected using primers Mgat1 Del 1 (5′-ctgctccaggacaagagcca-3′) and Mgat1 Del 2 (5′-gagacctgcttactgcagcc-3′). These reactions of 25 μl contained 2.5 μl of 10× PCR buffer containing 20 mM $MgCl_2$, 0.5 μl 10 mM dNTPs, 0.5 μl 10 mM primers, 1.5 IU Taq polymerase (Roche), and 1 μl DNA. All PCR reactions except for deleted *Mgat1* were performed as follows: preheating (94°C, 2 minutes), 40 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute, followed by one cycle of 72°C for 5 minutes. Deleted *Mgat1* PCR reactions were as described above with an annealing temperature of 65°C and 35 cycles. *Mgat1* genotyping for the floxed allele and PCR reactions for the ZP3*Cre* transgene were performed as described previously (Shi et al., 2004).

Fertility of *T-syn*$^{F/F}$:ZP3*Cre* and *T-syn*$^{F/F}$*Mgat1*$^{F/F}$:ZP3*Cre* females

To determine fertility, *T-syn*$^{F/F}$:ZP3*Cre*, *T-syn*$^{F/+}$:ZP3*Cre* and *T-syn*$^{+/+}$:ZP3*Cre* females and *T-syn*$^{F/F}$*Mgat1*$^{F/F}$:ZP3*Cre*, *T-syn*$^{F/+}$*Mgat1*$^{F/+}$:ZP3*Cre* and *T-syn*$^{+/+}$*Mgat1*$^{+/+}$:ZP3*Cre* females were mated to C57BL/6 males. Time to first litter and litter size were determined, and all pups born from mothers carrying a *T-syn*^{F} or *Mgat1*^{F} allele were genotyped to determine deletion of the floxed gene. At the termination of breeding (3-6 months of age), double mutant females were superovulated by intraperitoneal injection of 5 IU pregnant mare serum gonadotrophin (Sigma-Aldrich, St Louis, MO) followed 46-48 hours later by 5 IU human chorionic gonadotrophin (Sigma-Aldrich). Fourteen hours later, eggs were collected and counted, and the ovaries were fixed for 7-10 hours in Bouins or 10% buffered formalin (Sigma-Aldrich) for subsequent immunohistochemistry. All fixations were carried out at room temperature.

Preimplantation development in embryos lacking core-1-derived *O*-glycans

In order to generate *T-syn*$^{-/-}$ embryos from eggs that lacked core-1-derived *O*-glycans and *T-syn*$^-$ sperm, *T-syn*$^{F/F}$:ZP3*Cre* females were mated with *T-syn*$^{+/-}$ males and embryonic development was assessed at E11.5 and E12.5. Noon of the following day after pairs were placed together at 4 pm was taken as 0.5 days post-coitum. Hind leg genomic DNA was used to genotype the embryos, as described previously (Xia et al., 2004).

Sperm binding

To examine sperm binding, 3-week-old female mice were superovulated as described above. Eggs were collected from the oviduct and treated with 0.3 mg/ml hyaluronidase (Sigma-Aldrich) to remove cumulus cells (denuded eggs) in the presence of protease inhibitors (Roche). Cauda epididymi from a male of proven fertility were dissected, minced in 1 ml IVF-30 medium (Vitrolife, Denver, CO) and allowed to ‘swim out’. After 15 minutes, 10 μl sperm were added to a 250 μl droplet of IVF-30 medium and capacitated for 1 hour. Denuded eggs (20-30) and 2-cell embryo controls (3-5) were added and incubated for 30 minutes at 37°C in a 5% CO_2 incubator with pre-equilibrated IVF-30 medium. Two-cell embryos were used as negative controls because they have a ZP that is modified at fertilization to prevent further sperm binding. They were generated by IVF the previous day with superovulated wild-type eggs and incubated with capacitated sperm for 6 hours as described. Sperm were allowed to bind to eggs and two-cell embryos for 30 minutes, after which they were gently washed by pipetting until less than five sperm were bound per two-cell embryo, fixed in 50 μl of 2% paraformaldehyde for 1 hour, washed again, and photographed.

Egg collection and cytochemistry

Three-week-old females were superovulated as described above. Eggs were collected into M2 medium (Sigma-Aldrich) and cumulus cells removed with 0.3 mg/ml hyaluronidase with protease inhibitors (Roche). Eggs were fixed in 2% paraformaldehyde in M2 medium for 1 hour. All blockings, washings and lectin dilutions were in phosphate-buffered saline (PBS) pH 7.2 containing 2% bovine serum albumin (BSA) and performed at room temperature. After blocking for 1 hour, T antigen was detected with 20 μg/ml peanut agglutinin conjugated to fluorescein isothiocyanate (PNA-FITC; Vector Labs, Burlingame, CA) by incubation for 1 hour followed by washing and photography. Tn antigen was detected by incubation in anti-Tn monoclonal antibody (a generous gift from Henrik Clausen, University of Copenhagen, Denmark) for 1 hour. Eggs were then washed, and incubated with goat anti-mouse FITC-conjugated antibody (Zymed, San Francisco, CA), washed and photographed. Binding of *Phaseolus vulgaris* leukoagglutinin-FITC (L-PHA-FITC; Vector Labs) at 20 μg/ml was also determined as described above. Double mutant eggs obtained with deleted *T-syn* and *Mgat1* were examined for binding of LPHA-FITC and concanavalin A-Rhodamine (Con A-Rho, 2.5 μg/ml; Vector Labs) in addition to PNA-FITC.

N-Glycanase digestion and western analysis

Ovaries were isolated in dissection buffer [40 mM Tris, 150 mM NaCl, complete protease inhibitors (Roche)] and immediately homogenized using a pestle in a 1.5 ml microcentrifuge tube containing 400 μl dissection buffer with 0.1% SDS. Protein concentration was determined using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) with BSA standards. Ovary samples (2.5 μg protein) were digested for 12 hours at 37°C with 1000 units of *N*-glycanase (New England Biolabs, Beverley, MA). Protein was separated on a 7% Tris gel using SDS-PAGE under reducing conditions and transferred to a polyvinylidenefluoride membrane which was probed with monoclonal antibodies to ZP1 (Rankin et al., 1998), ZP2 (East et al., 1984) or ZP3 (East et al., 1985) as previously described (Shi et al., 2004).

Lectin staining of ovarian sections

Ovaries were fixed in 10% buffered formalin (Sigma-Aldrich) for 6-8 hours, washed in 70% ethanol and embedded in paraffin. Sections of 5 μm were dewaxed with Histoclear (Sigma-Aldrich) and rehydrated. For lectin staining, sections were preincubated for 1 hour in PBS-2% BSA and incubated with 20 μg/ml PNA-FITC or L-PHA-FITC 20 μg/ml. After 1 hour, sections were washed with PBS-2% BSA and photographed.

ZP immunohistochemistry

ZP immunohistochemistry was performed as previously described (Shi et al., 2004). Briefly, ovaries were fixed in 10% buffered formalin or Bouins for 6-8 hours, washed in 70% ethanol, embedded in paraffin, and 5 μm sections cut. Sections were dewaxed, rehydrated and incubated in methanol containing 0.3% hydrogen peroxide for 30 minutes. Slides were washed for 3 minutes in water, 3 minutes in Tris-buffered saline [TBS; 0.1 M Tris (pH 7.5) and 0.3 M NaCl] with 0.05% Tween 20 (TBST), and incubated in TBS containing 15% normal rabbit serum (NRS; Vectastain Elite ABC kit, Vector Labs) for 30 minutes in a humidified chamber. Sections were incubated with undiluted hybridoma medium containing monoclonal antibodies to ZP1, ZP2 and ZP3 for 1 hour or TBS-15% NRS as a control. After washing three times for 3 minutes with TBST, sections were incubated with rabbit anti-rat immunoglobulin G biotinylated secondary antibody (Vectastain Elite ABC kit; 50 μl in 10 ml of TBS-15% NRS) for 30 minutes, washed, and incubated with ABC solution (Vectastain Elite ABC kit) for 30 minutes. After three washes with PBS containing 0.05% Tween 20, sections were stained using a DAB kit (Vector Labs) and counterstained with Hematoxylin before dehydration and mounting.

Statistical analyses

Statistical analyses were determined using two-tailed *t*-tests using Microsoft Excel Data Analysis package.

L.X. generated the *T-syn* floxed mouse and embryology data. S.A.W. generated all other data. We gratefully acknowledge the excellent technical assistance of Wen Dong and Michael McDaniel, advice from Radma Mahmood, and discussions with Mark Stahl. Mice with the ZP3*Cre* transgene were originally obtained from Jamey Marth. This work was supported by grant R01 CA30645 to P.S. and by grants P20 RR18758 and P01 HL85607 to R.P.M., L.X. and R.D.C. from the National Institutes of Health.

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