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

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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, Mγat1–/– 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-synF) 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-synF/ZP3Cre females in which T-synF was deleted at the beginning of oogenesis generated eggs lacking core-1-derived O-glycans. Nevertheless, T-synF/F:ZP3Cre females were fertile and their eggs bound sperm similarly to controls. In addition, T-syn–/– embryos generated from T-syn null eggs developed up to ~E12.5. Thus, core-1-derived O-glycans are not required for blastogenesis, implantation, or development prior to midgestation. Moreover, T-syn–/–Mγat1–/– 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 pellucida, 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, 1984). These glycoproteins are heterogeneously glycosylated with N- and O-glycans (Philpott et al., 1987) but only ZP1 and ZP3 appear to possess heterogeneously glycosylated with N- and O-glycans (Philpott et al., 1987). All three ZP proteins are expressed on mouse eggs from ZP glycoproteins ZP1, ZP2, ZP3 during the 2-3 weeks of oogenesis prior to ovulation (Green, 1984). These glycoproteins are 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, eggs 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 LewisX and LewisA determinants (Johnston et al., 1998; Kerr et al., 2004). However, fertility in α1,3-fucosyltransferase 9 (Fut9)–/– mice whose eggs lack the LewisX determinant, is normal (Kudo et al., 2004). Finally, mannosyl present on N-
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 mannose-6-phosphate receptor (M6P/IGF2R) has been shown to affect sperm-egg binding in mouse sperm-egg binding but do not lead to a unified hypothesis.

Results

Generation of the T-syn floxed allele and T-syn+/−:ZP3Cre females
To allow oocyte-specific deletion of the T-syn gene, mice carrying the T-synF 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-synF) and the Neo gene was deleted as described in the Materials and Methods (Fig. 1A). ES cells with a T-synF allele were identified by Southern analysis following BamHI digestion of genomic DNA. An extra BamHI site introduced immediately upstream of exon 1 of the T-synF allele gave a 5.9 kb band following Cre-mediated deletion. Thus analysis of T-synF+ 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-synF allele were used to generate T-synF+/− heterozygotes. T-synF+/−:ZP3Cre males were mated to T-synF+/−:ZP3Cre females to obtain T-synF+/−:ZP3Cre 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-synF+/−:ZP3Cre females were mated with C57BL/6 males. T-synF+/−:ZP3Cre and T-synF+/−:ZP3Cre females were also mated as controls. The ZP3Cre 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 (Phibb et al., 1987). To confirm that T-syn had been deleted in ovulated eggs, pups from T-synF+/−:ZP3Cre and T-synF−/−:ZP3Cre 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-synF+/−:ZP3Cre females had deleted both T-syn alleles, and 52% of pups from T-synF+/−:ZP3Cre 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-synF+/−:ZP3Cre 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-synF+/−:ZP3Cre females in the same period as by control females, and litter size was actually larger in T-synF+/−:ZP3Cre females than controls, an interesting finding that is under further investigation. The ZP3Cre transgene had no effect on fertility with a litter size of 8.0±2.4 in T-synF+/−:ZP3Cre females (n=34) compared with 8.0±2.7 for T-synF+/−:ZP3Cre females (n=29).

Core-1-derived O-glycans are not required for development before E12.5
Mutant T-synF−/− embryos lacking T-synthase generated from T-synF−/− 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...
in heterozygous eggs (Su et al., 2004). To investigate potential roles for T-synthase in pre-implantation development, timed matings between T-synFr/ZP3Cre females and T-synFr–/– 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-synFr–/– eggs**

T-synFr/ZP3Cre females were as fertile as controls (Table 1). However, fertilization requires only one sperm to bind and traverse the zona. The zona on T-synFr–/– 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-synFr–/– 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-synFr/ZP3Cre females binds sperm equivalently to wild-type ZP. In three experiments using eggs ovulated from two to three control or T-synFr/ZP3Cre females in each experiment, sperm binding to wild-type and T-synFr–/– eggs was always equivalent.

**T-synFr–/– eggs lack core-1-derived O-glycans**

Considering the fertility of T-synFr/ZP3Cre 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-synFr/ZP3Cre females stained brightly with PNA-FITC (Fig. 5A), whereas eggs from T-synFr/ZP3Cre 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-synFr–/– 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-synFr–/– eggs. However, as expected, complex N-glycans were not affected, as shown by the fact that T-synFr–/– 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 \( T-syn^{F/F}:ZP3Cre \) females mated with C57BL/6 males

<table>
<thead>
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<th>( T-syn^{F/+}:ZP3Cre )</th>
<th>( T-syn^{F/F}:ZP3Cre )</th>
<th>( T-syn^{F/F}:ZP3Cre )</th>
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<td>Number of females</td>
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<td>6</td>
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<tr>
<td>Days to first litter</td>
<td>21.1±1.6</td>
<td>24.3±2.8</td>
<td>21.7±1.4</td>
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<tr>
<td>Number of pups/number genotyped</td>
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<td>6.5±2.8 ( ^b )</td>
<td>10.4±3.1 ( ^c )</td>
</tr>
<tr>
<td>Pup genotype/arrowheads</td>
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<td>86</td>
<td>170</td>
</tr>
<tr>
<td>Pup genotype/arrowheads</td>
<td>NA</td>
<td>T-( syn^{F/+} ) T-( syn^{F/+} )</td>
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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 \( T-syn^{F/F}:ZP3Cre \) 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 \( T-syn^{F/F}:ZP3Cre \) 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 oocytes migrated with an apparent molecular mass of ~95-155 kDa whereas ZP1 from mutant oocytes in a tight band of ~60 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 \( T-syn^{F/F}:ZP3Cre \) 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 \( M g a t 1 \)

The absence of Gal and GlcNAc residues on core-1-derived O-glycans of \( T-syn^{F/-} \) eggs did not alter sperm binding or fertility. However, complex and hybrid N-glycans generated by GlcNAc-T1 encoded by the \( M g a t 1 \) 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, \( T-syn^{F/F}M g a t 1^{F/F}:ZP3Cre \) females were generated and mated with C57BL/6 males. \( T-syn^{F/+}M g a t 1^{F/+}:ZP3Cre \) and \( T-syn^{F/F}M g a t 1^{F+/+}:ZP3Cre \) females were used as controls. The genotypes of their respective oocytes are shown in Fig. 2D. The results in Table 2 show that \( T-syn^{F/F}M g a t 1^{F/F}:ZP3Cre \) 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 \( M g a t 1 \) alleles in all pups from \( T-syn^{F/F}M g a t 1^{F/F}:ZP3Cre \) and \( T-syn^{F/+}M g a t 1^{F/+}:ZP3Cre \) females (see Fig. 2B). This demonstrates that expression of the ZP3Cre transgene was able to delete two floxed alleles as efficiently as a single floxed allele such as \( T-syn \) or \( M g a t 1 \) (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.
Complex glycans are dispensable for fertilization in mice 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 oligomannosy1 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-synF/F:Mgat1F/F:ZP3Cre 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-synF/F:Mgat1F/F:ZP3Cre females, including those that had given birth (Fig. 7A). The complete absence of ZP lectin staining in T-synF/F:Mgat1F/F:ZP3Cre ovaries before and after digestion with N-glycanase.

Discussion
The O-glycans on ZP3 have been implicated in fertilization of
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<sup>F</sup>-Cre<sup>F</sup> allele and used a ZP3Cre 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<sup>F</sup>-Cre<sup>F</sup>:ZP3Cre 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<sup>F</sup>-Cre<sup>F</sup> 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, the ZP serves as a receptor for sperm (Williams et al., 2006). 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 solubilized 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-synthase, 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 midgestation. Eggs that lack both core-1-derived O-glycans and

## Table 2. Fertility of T-syn<sup>F</sup>F<sup>F</sup> Mgat1<sup>F</sup>F<sup>F</sup>:ZP3Cre females mated with C57Bl/6 males

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<th>T-syn&lt;sup&gt;F&lt;/sup&gt;F&lt;sup&gt;F&lt;/sup&gt; Mgat1&lt;sup&gt;F&lt;/sup&gt;F&lt;sup&gt;F&lt;/sup&gt;:ZP3Cre</th>
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Values are the mean ± s.d.; a versus c: P=0.009, b versus c: P=0.005. NA, not applicable.
Complex glycans are dispensable for fertilization in mice

Fig. 6. Eggs from T-syn\textsuperscript{F/F}Mgat1\textsuperscript{F/F}:ZP3Cre 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\textsuperscript{F/F}Mgat1\textsuperscript{F/F} eggs after hyaluronidase treatment. (C,D) Higher magnification of T-syn\textsuperscript{F/F}Mgat1\textsuperscript{F/F} eggs with less cumulus attached revealed a thin fragile zona. (E,F) PNA-FITC did not bind to T-syn\textsuperscript{F/F}Mgat1\textsuperscript{F/F} eggs. Similar data were obtained in four experiments with eggs from 12 T-syn\textsuperscript{F/F}Mgat1\textsuperscript{F/F}:ZP3Cre females. (G,H) L-PHA-FITC did not bind to T-syn\textsuperscript{F/F}Mgat1\textsuperscript{F/F} eggs. Similar data were obtained in three experiments from 16 T-syn\textsuperscript{F/F}Mgat1\textsuperscript{F/F}:ZP3Cre females. (I,J) Con A-Rho staining was enhanced in T-syn\textsuperscript{F/F}Mgat1\textsuperscript{F/F} eggs consistent with increased oligomannosyl N-glycans.

Materials and Methods

Generation of T-syn\textsuperscript{F/F} mice

To establish mice in which the T-syn gene was flanked by loxP sites (T-syn\textsuperscript{F/F}), mouse embryonic stem (ES) cells previously targeted to contain three loxP 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 BamHI, 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\textsuperscript{F/F} mice were bred to generate homozygous T-syn\textsuperscript{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 BamHI and hybridization using exon 2 of the T-syn gene.

Mice with floxed T-syn and Mgat1 alleles and a ZP3Cre transgene

Female mice with a T-syn\textsuperscript{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\textsuperscript{F/F}:ZP3Cre females and T-syn\textsuperscript{F/F}:ZP3Cre 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\textsuperscript{F/F} mice, described previously (Shi et al., 2004), were crossed with females in which inactivation of the T-syn gene is induced (Shi et al., 2004). DM females lack core-1-derived O-glycans and complex and hybrid N-glycans.

Fig. 7. Oocytes from T-syn\textsuperscript{F/F}Mgat1\textsuperscript{F/F}:ZP3Cre double mutant (DM) females lack core-1-derived O-glycans and complex and hybrid N-glycans. (A) The ZP of oocytes in T-syn\textsuperscript{F/F}Mgat1\textsuperscript{F/F}:ZP3Cre 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<sup>−/−</sup>Mgat1<sup>−/−</sup>:ZP3Cre and T-syn<sup>−/−</sup>Mgat1<sup>−/−</sup>:ZP3Cre females. T-syn<sup>−/−</sup>:ZP3Cre and T-syn<sup>−/−</sup>Mgat1<sup>−/−</sup>:ZP3Cre females were generated as controls.

To distinguish between mice carrying floxed or deleted T-syn alleles, and the ZP3Cre transgene, separate PCR genotyping was performed using tail genomic DNA. Primers TS-1 (5'-ctcctcagacgagcagc-3') and TS-2 (5'-cgagctcctgctgggtc-3') detected the wild-type (T-syn<sup>+</sup>) and the floxed T-syn<sup>−</sup> alleles, and primers TS-1 and TS-2 primers, PCR reactions of 25 µl contained 2.5 µl 10× PCR buffer (not containing MgCl<sub>2</sub>) (Invitrogen, Carlsbad, CA), 1.5 µl 50 mM MgCl<sub>2</sub> (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-2 primers, reactions of 25 µl contained 2.5 µl of 10× PCR buffer already containing 20 mM MgCl<sub>2</sub>, 0.5 µl 50 mM MgCl<sub>2</sub>, 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 detected using primers Mgt1 Del 1 (5'-ctcctcagacgagcagc-3') and Mgt1 Del 2 (5'-cgagctcctgctgggtc-3'). These reactions of 25 µl contained 2.5 µl of 10× PCR buffer containing 20 mM MgCl<sub>2</sub>, 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 were performed as described above with an annealing temperature of 65°C and 35 cycles. Mgat1 genotyping for the floxed allele and PCR reactions for the ZP3Cre transgene were performed as described previously (Shi et al., 2004).

**Fertility of T-syn<sup>−/−</sup>:ZP3Cre and T-syn<sup>−/−</sup>Mgat1<sup>−/−</sup>:ZP3Cre females**

To determine fertility, T-syn<sup>−/−</sup>:ZP3Cre, T-syn<sup>−/−</sup>Mgat1<sup>−/−</sup>:ZP3Cre and T-syn<sup>−/−</sup>Mgat1<sup>−/−</sup>Cre females were mated with C57BL/6 males. Time to first litter and litter size were determined, and all pups born from mothers carrying a T-syn<sup>−/−</sup> or Mgat1<sup>−/−</sup> 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 from the oviduct and treated with 0.3 mg/ml hyaluronidase (Sigma-Aldrich) to remove cumulus cells (denuded eggs) in the CO2 incubator with pre-equilibrated IVF-30 medium. Two-cell embryos were used for superovulation.

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, Beverly, MA). Protein was separated on a 7% Tris gel using SDS-PAGE under reducing conditions and transferred to a polyvinylidene fluoride 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).

**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 and embedded in paraffin. Sections of 5 µm 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; Vectorstain 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 with 3 minutes of TBST, sections were incubated with rabbit anti-rat immunoglobulin G biotinylated secondary antibody (Vectorstain Elite ABC kit; 50 µl in 10 ml of TBS-15% NRS) for 30 minutes, washed, and incubated with ABC solution (Vectorstain 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 dehydroxy 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 ZP3Cre 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.

**References**


Complex glycans are dispensable for fertilization in mice


