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# Role of complement regulatory proteins CD46, CD55 and CD59 in reproduction

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**Abstract.** In humans, CD46 has been detected on the acrosomal membrane in sperm, in contrast to widespread surface expression on somatic cells where it plays a key role in the protection from complement attack. In rodents, CD46 is expressed solely on the acrosomal membrane of mature sperm and their immediate precursors, spermatids. A monoclonal antibody against the short consensus repeat (SCR1) ectodomain of CD46 blocks binding of human sperm to zona-free oocytes *in vitro*. However, CD46-knockout mice are fertile and have an accelerated spontaneous acrosome reaction. Wild-caught field mice (*Apodemus*) also exhibit a rapid acrosome reaction and CD46 is not expressed in *Apodemus* sperm. CD46 may, therefore, play a role in stabilization of the acrosomal membrane. Two other complement regulatory proteins, CD55 and CD59, are localized on the plasma membrane of mammalian sperm. In human sperm, CD55 and CD59 are expressed also on the inner acrosomal membrane. It remains to be clarified what is the role of CD46, CD55 and CD59 during fertilization and what are the advantages of not expressing CD46 in field mice sperm.

**Key words:** CD46, CD55, CD59, sperm, rodent, human

## Introduction

CD46 (membrane cofactor protein) is a membrane-associated glycoprotein that is present in human cells, including sperm, and in cells of other mammals. In somatic cells, CD46 is a cofactor facilitating the degradation of C3b convertase and, therefore, plays key role in protecting host cells from complement attack (Seya & Atkinson 1989, Liszewski et al. 1991, Liszewski et al. 1996). Another role of the CD46 protein is activation of several intracellular pathways in different types of cells, including T-lymphocytes (Wong et al. 1997, Wang et al. 2000, Liszewski et al. 2005). On the other hand, expression of CD46 protein was demonstrated solely on sperm in rat and mouse (Mizuno et al. 2004, Johnson et al. 2007), with the exception of field mice (*Apodemus*) where spermatozoal CD46 protein is not expressed (Johnson et al. 2007, Clift et al. 2009a). Several pathogens, such as measles virus (Dorig et al. 1993, Naniche et al. 1993), human herpes virus-6 (Santoro et al. 1999), group B and D adenoviruses (Gaggar et al. 2003, Wu et al. 2004), group A *streptococcus* (Okada et al. 1995) and *Neisseria* (Kallstrom et al. 1997), bind to CD46 and use it as receptor enabling them to enter the cell. The loss of sperm CD46 may be an advantage in the case of promiscuous field mice that are exposed to

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a large amount of genital pathogens. The aim of this mini-review is to summarize present knowledge about CD46, as well as two other complement regulatory (CReg) proteins CD55 and CD59, and discuss their role in fertilization.

**CD46 and its isoforms**

In humans, CD46 protein is expressed by all cells except erythrocytes and exists predominantly in four different isoforms – these isoforms are the products of alternative splicing of a single gene (Liszewski et al. 1994). They consist of four extracellular domains on the amino-terminus called short concensus repeats (SCRs), a serine-threonine-proline (STP)-rich area, transmembrane domain (TM), intracytoplasmic anchor and one of two forms of cytoplasmic tail (CYT-1 of 16 amino acids and CYT-2 of 23 amino acids) that arise by alternative splicing at the carboxyl-terminus (Liszewski & Atkinson 1992). The SCR1, two and four domains are sites of N-linked glycosylation. The STP region is a site of extensive O-linked glycosylation and is composed of 14 or 29 amino acids. The size of the STP region depends on the presence of STP exon B that is often spliced out. The absence of STP exon B leads to lower molecular weight CD46 isoforms of CD46, while its presence leads to higher molecular weight CD46 isoforms (Liszewski et al. 1991, Post et al. 1991). However, an even lower molecular weight hypoglycosylated isoform of CD46 is expressed in mammalian sperm. In humans, polymorphisms of the *Cd46* gene were detected in normal individuals using *Hin*dIII and *Eco*RI restriction enzymes (Risk et al. 1991).

Mizuno et al. (2004) demonstrated that CD46 protein is expressed exclusively on the acrosomal membrane of mature sperm and on their immediate precursors, spermatids, in rodents. There are only 11 functional exons in the murine *Cd46* gene compared to the human which contains 14. The murine *Cd46* gene contains exons encoding four SCRs domains, an STP-rich area, intracytoplasmic anchor, transmembrane domain and cytoplasmic tail (Miwa et al. 1998, Tsujimura et al. 1998). Differences in tissue-specific mRNA expression of rat *Cd46* are rather quantitative than qualitative, reaching the highest level in testicular tissue (Mead et al. 1999). Our results in mice confirm the presence of *Cd46* in various tissues (Fig. 1) but the significantly highest expression was

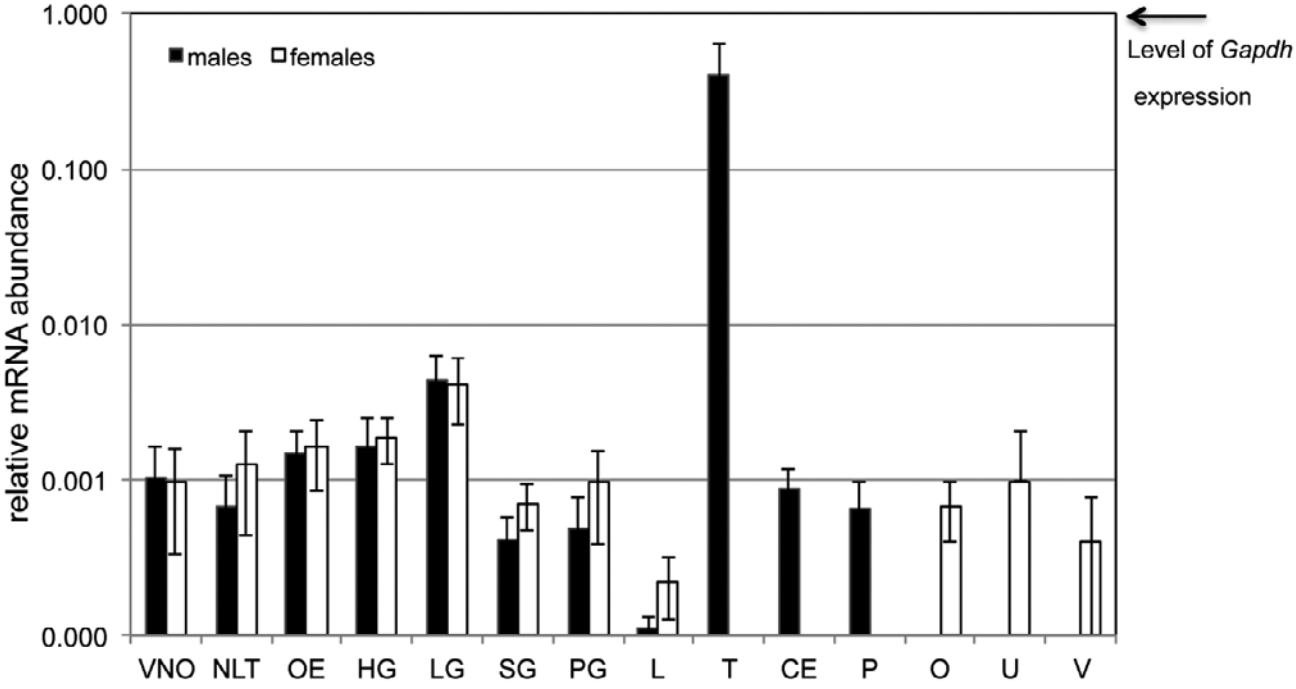


***Fig. 1.*** *The level of mRNA abundance of Cd46 in three male and three female* Mus musculus domesticus *was studied in the following tissues: vomeronasal organ (VNO), nasal-associated lymphoid tissue (NLT), main olfactory epithelium (OE), Harderian gland (HG), extraorbital lacrimal gland (LG), submandibular salivary gland (SG), preputial gland (PG), liver (L), ovary (O), uterus (U), vagina including cervix (V), testes (T), cauda epididymis (CE) and prostate (P). An efficiency-sensitive model with Gapdh as a reference gene was used for the calculation of mRNA abundance (Pffafl 2001). Low expression was detected in all tested tissues; only in testes did the amount of Cd46 mRNA reach the level of housekeeping gene.*

detected in testes. The testicular mRNA abundance of *Cd46* is elevated such that the expression level is almost 4-fold higher than the level typical for other tissues, where expression is far below the level of the common house-keeping gene *Gapdh*. Johnson et al. (2007) demonstrated that field mice of three species, *Apodemus sylvaticus*, *A. flavicollis* and *A. microps*, produce alternatively spliced transcripts of testicular *Cd46* mRNA lacking exons 5-7 or 6-7, together with a 3'-untranslated region of 54nt sequence extension and an often truncated a 5'-terminal sequence deletion (Fig. 2), resulting in failure to express spermatozoal CD46 protein. Similar results were described in the case of *A. agrarius* that produces two alternatively spliced transcripts of testicular *Cd46* mRNA, both lacking exon 7. However, these transcripts differ from transcripts found in other *Apodemus* species. The larger *A. agrarius Cd46* transcript has an insert between exons 10 and 11 which, if translated, would result in a novel cytoplasmic tail (Clift et al. 2009b).

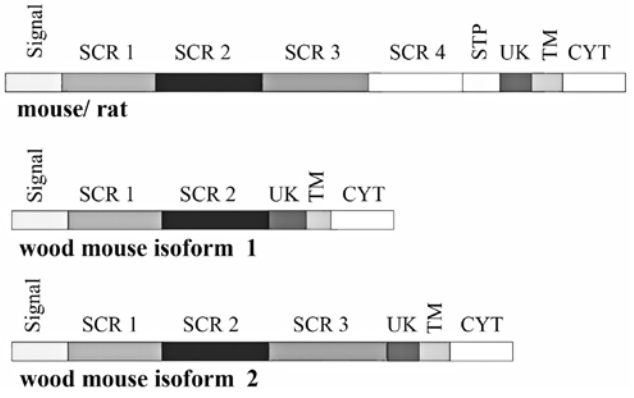


***Fig. 2.*** *Comparison of CD46 protein structure in mouse and rat, with possible CD46 protein structure in wood mouse in case the protein was translated.*

## CD46 in sperm

CD46 is exposed to the extracellular environment only after the acrosomal reaction due to its localization to the acrosomal membrane (Anderson et al. 1989, Riley et al. 2002a) (Fig. 3). The SCR1 ectodomain of CD46 especially plays an important role in fertilization. This is indicated also by the absence of the SCR1 ectodomain in all cell types of New World monkeys except sperm (Riley et al. 2002b). A monoclonal antibody against the SCR1 ectodomain of CD46 blocks binding of human sperm to zona-free oocytes *in vitro* (Okabe et al. 1990, Taylor et al. 1994). This finding suggested the possibility that CD46 could be a candidate fusion protein in sperm binding to the complementary egg surface binding protein. Further, Miyado et al. (2000) and Kaji et al. (2000) revealed in CD9 knock-out mice

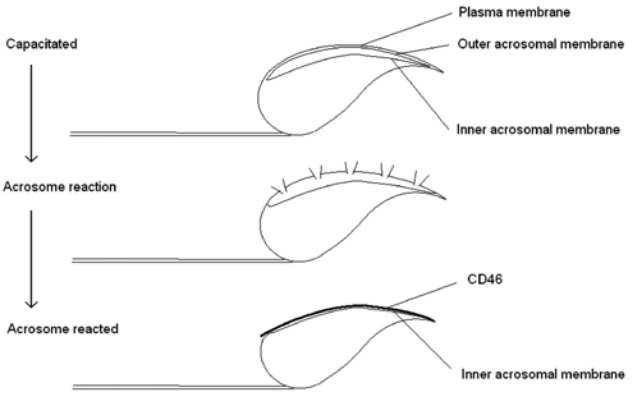


***Fig. 3.*** *Localization of CD46 protein in sperm.*

that it is the egg CD9 protein that plays a key role in sperm-egg fusion. In rodents, CD46 was discovered exclusively on the acrosomal membrane of mature sperm and their immediate precursors, spermatids (Mizuno et al. 2004). Surprisingly, antibodies against CD46 did not block fertilization in rat (Mizuno et al. 2007). Inoue et al. (2003) produced CD46-knockout mice and showed that the males had normal testes and, moreover, fertile sperm. However, in contrast with the wild-type sperm, sperm of CD46-deficient males showed an accelerated spontaneous acrosome reaction. The protein IZUMO was later discovered in mouse and human sperm by Inoue et al. (2005) and described as the first sperm membrane protein shown to be essential for sperm-egg fusion.

## CD46 in field mice

As well as CD46 knockout mice, wild-caught field mice (*Apodemus*) also exhibit a more rapid acrosome reaction and, surprisingly, it was discovered that field mice do not translate CD46 protein during spermiogenesis; therefore, CD46 protein is not expressed in *Apodemus* sperm (Johnson et al. 2007). Minor nucleotide sequence polymorphism was detected between *A. sylvaticus*, *A. flavicolis* and *A.microps*, and polymorphisms both in noncoding and coding regions were identified between individuals of *A. sylvaticus* mice. These changes in the coding region lead to non-synonymous changes and may be the basis for the unique CD46 splicing (Johnson et al. 2007).

Although CD46 plays a key role in protecting the host cells from complement attack in somatic cells, data published by Inoue et al. (2003) and Johnson et al. (2007) suggested a role for CD46 in sperm relating to integrity of the acrosome vesicle or regulation of the acrosome reaction rather than protection of sperm from complement attack. This led to questions as to what is the role of CD46 during fertilization and what

are the advantages/disadvantages of not expressing CD46 in field mice sperm?

**Sperm trains in field mice**

Field mice are highly territorial and there is pronounced sperm competition, which is reflected by their disproportionately large testis (Breed & Taylor 2000, Bryja & Stopka 2005). Relative testis mass is associated with the shape of the apical hook in the falciform head of murine rodent sperm (Moore et al. 2002). Sperm of *A. sylvaticus* have a very long apical hook and Moore et al. (2002) demonstrated that these sperm form sperm "trains" with significantly increased motility in comparison with an individual spermatozoon. Dissociation of sperm trains is a prerequisite for fertilization and it is believed that the spontaneous acrosomal reaction of part of the involved sperm plays a role in this process (Moore et al. 2002). A significantly faster spontaneous acrosome reaction rate in field mice compared to house mice (Johnson et al. 2007) may be important for the dissociation of sperm trains and, moreover, may help bypass the primary binding of acrosome-unreacted sperm to zona pellucida (Myles et al. 1987, Yamagata et al. 2002). It is possible that a release of the acrosomal content of many sperm at the same time may affect the zona pellucida (ZP) structure and help sperm to passage faster through the ZP, especially since the acrosome reaction is a time-consuming process lasting for up to an hour (Shur et al. 2006). It has been shown that acrosomal exocytosis specifically induced by ZP3-dependent GalT aggregation is not critical for fertilization (Talbot et al. 2003). Huang et al. (1981) showed that only acrosome-reacted sperm can bind to and penetrate the ZP in the guinea pig. These data suggest that acrosomal instability may provide a novel genus-specific strategy to favour rapid fertilization and competitive advantage in the promiscuous reproductive behaviour of wild field mice (Johnson et al. 2007).

**CD46 and cytoskeleton**

The way in which CD46 may stabilize the acrosomal membrane is still unclear. One of the possibilities is an interaction of CD46 with β1-integrins. Integrins are transmembrane receptors involved in cell-cell adhesion and cell migration, proliferation and differentiation as well as cellular apoptosis. These receptors may mediate a signal both into and out of the cell (Liu et al. 2000). CD46 binding to β1-integrins (Lozahic et al. 2000, Kurita-Taniguchi et al. 2002, Rezcallah et al. 2005), as well as β1-integrin interaction with the actin cytoskeleton (Calderwood et al. 2000), has been demonstrated. β1-integrin interaction with the actin cytoskeleton is realized by direct and indirect binding of integrin β-tails and actin-binding proteins (Critchley 2000, Liu et al. 2000). Actin and actin polymerization play a key role during sperm capacitation and acrosome reaction (Brener et al. 2003, Dvořáková et al. 2005). Filamentous F-actin, part of cortical cytoskeleton, is localized between the plasma and acrosomal membrane where it protects these two membranes from connection. Moreover, this cytoskeleton plays a pivotal role in relocalization of proteins during the acrosome reaction, e.g. the primary fusogenic protein IZUMO (Sosnik et al. 2009). It appears that CD46 stimulation may activate protein kinase signalling leading to actin reorganization (Wong et al. 1997, Zaffran et al. 2001). These facts may lead to the concept that the absence of CD46 enables faster F-actin breakdown and thus rapid sperm acrosome reaction.

**Role of CD46 in sperm protection**

Complement components are extensively present in the female reproductive tract, where they protect host cells against pathogens. Hence, it is expected that sperm should have some kind of protection against complement attack in female reproductive tract. This protection is provided by complement regulatory proteins, of which CD46 is one of them.

CD46 is localized on the acrosomal membrane (Anderson et al. 1989, Riley et al. 2002a) in mammalian sperm. For this reason, it may only protect sperm against complement attack after the acrosome reaction when the sperm plasma membrane and other plasma membrane-associated CReg proteins are lost and the inner acrosome membrane is exposed to the surrounding of the female reproductive tract. However, the inner acrosome membrane and CD46 are exposed for a very short time before the actual sperm-egg fusion. On the other hand due to the fact that the huge majority of sperm never get to the egg, and CD46 continues to be expressed, CD46 could have a role in the phagocytic clearance of redundant time-expired sperm. The primary role of sperm CD46 may be expected to be different from protection of sperm against complement attack. Therefore, other plasma membrane-associated Creg proteins are likely to play the main role in protecting sperm against complement-mediated damage prior to fertilization (Clift et al. 2009b).

**CD55 and CD59**

Besides the presence of CD46 in the acrosome membrane of mammalian sperm, there are two

other CReg proteins (CD55 and CD59) localized in the plasma membrane of mammalian sperm. CD55, termed decay accelerating factor (DAF), inhibits the assembly of C3 convertases (Fujita et al. 1987) similarly to CD46. However CD59 inhibits the assembly of the membrane attack complex on the cellular plasma membrane (Davies et al. 1989).

In humans, in contrast to CD46, CD55 is present only as glycosylphosphatidylinositol (GPI)-anchored molecule. However, the expression of both glycosylphosphatidylinositol (GPI)-anchored and transmembrane (TM)-anchored isoforms of CD55 has been shown in mice (Spicer et al. 1995). CD55 isoforms are the products of two different but highly homologous genes (Spicer et al. 1995), compared to CD46 isoforms which are the products of alternative splicing of a single gene (Liszewski et al. 1994). Lin et al. (2001) showed in mice that GPI-CD55 protein is expressed in most of tissues, while TM-CD55 protein is mainly present in the testis and dendritic cells in spleen. The same distribution of GPI- and TM-CD55 protein was demonstrated in rats, with the difference that GPI- and TM-CD55 are products of alternative splicing of a single copy gene (Hinchliffe et al. 1998, Miwa et al. 2000), such as in case of CD46. CD55 isoforms are also generated by alternative splicing of a single copy gene in *Apodemus sylvaticus* (Clift et al. 2009b).

CD59 is another GPI-anchored protein protecting host cells from complement attack and may also be involved in fertilization. The gene encoding CD59 is duplicated in the mouse and two CD59 isoforms, named CD59a and CD59b, are expressed (Qian et al. 2000). It was shown that CD59a is distributed in most tissues, in contrast to CD59b which is exclusively expressed on mature sperm and their immediate precursors, spermatids (Baalasubramanian et al. 2004, Donev et al. 2008). Only one CD59 isoform was found in the rat (Hughes et al. 1992, Rushmere et al. 1994).

Crry, a rodent-specific membrane CReg protein, takes much of the role of protecting host cells from complement attack because, in contrast to humans, no CD46 isoform is expressed in rodent somatic cells (Hosokawa et al. 1996, Molina 2002).

Overall, the exact role of CD55 and CD59 in sperm is still unclear. In mice and field mice sperm, the presence of these proteins has been shown on the plasma membrane in the acrosome, neck and tail regions (Clift et al. 2009b). The presence of these two proteins in the main domains of rodent sperm plasma membrane indicates that CD55 and CD59 could play the key role in the protection of sperm from complement attack in female reproductive tract. On the other hand, Donev et al. (2008) published that a monoclonal antibody against CD59b inhibits mouse sperm motility, and it has also been demonstrated that CD55 and CD59 are expressed on the inner acrosomal membrane in human sperm (Cummerson et al. 2006). These facts suggest that CD55 and CD59, as well as CD46, may play a more complex role in process of reproduction.

**Complement regulatory proteins and the tetraspanin web**

As indicated, the primary function of sperm CD46 is probably different from protection of sperm against complement attack. One of the possibilities is that CD46 mediates specific binding between the spermatozoa and egg. However, CD46 may not be involved directly in initial fusion events, as fusion begins in the equatorial or post-acrosomal regions where the membrane remains intact after the acrosome reaction, rather than the inner acrosomal membrane region where CD46 is expressed (Taylor et al. 1994). In relation to sperm-egg fusion studies, it is relevant that there may be the ability of CD46 to bind to CD9 antigen, which was originally shown in HeLa cell lines (Lozahic et al. 2000) as well as in macrophages (Kurita-Taniguchi et al. 2002). The crucial role of CD9 in sperm-egg fusion was demonstrated by the inability of CD9 knock-out mouse oocytes to fuse with sperm (Le Naour et al. 2000, Miyado et al. 2000, Kaji et al. 2000) and by the ability of anti-CD9 antibodies to inhibit this fusion (Chen et al. 1999, Le Naour et al. 2000, Miller et al. 2000). While the CD9 molecule belongs to the tetraspanin family of proteins forming complexes with each other and with various membrane proteins within a network of molecular interactions called the "tetraspanin web" (Boucheix & Rubinstein 2001, Berditchevski 2001, Hemler 2003), it could be proposed that the association with CD46 mediates binding between spermatozoa and the egg. However, the fact that recombinant proteins including the large extracellular loop of CD9 partially inhibit fusion when preincubated with eggs, but not with sperm, indicates that CD9 is probably not a receptor for a sperm molecule and has drawn attention to molecules that associate with CD9 at the oocyte surface and thus to the tetraspanin web (Zhu et al. 2002). The preferred binding partner of CD9 within the tetraspanin web is CD81. Human oocytes express CD81 and CD151, as well as alpha6 and beta1 integrin subunits (Ziyyat et al. 2006). The expression of CD81 has also been demonstrated on mouse eggs (Takahashi et al. 2001),

but these three tetraspanins behave differently in the mouse with respect to sperm-egg fusion than in human. In any event, CD9 controls the formation cluster that contains the tetraspanin CD151 and the alpha6beta1 integrin (Ziyyat et al. 2006).

Treatment of mouse oocytes with phosphatidylinositol-specific phospholipase C (an enzyme cleaving GPI-anchored structures) during *in vitro* fertilization studies has shown a dramatically reduced ability of the oocyte to bind and fuse with a sperm (Coonrod et al. 1999). CD55 and CD59 have been identified as egg GPI-anchored proteins (Taylor & Johnson 1996), but mice that have targeted deletions in these genes are fertile (Sun et al. 1999, Holt et al. 2001). These data suggest that sperm-egg interactions depend on the presence of GPI-linked egg surface proteins, and possibly on the presence of other proteins on the plasma membrane. Lefevre et al. (2010) assume the lipid-modulated participation of GPI-anchored proteins and/or the CD9 tetraspanin with other necessary proteins in the formation of a particular constitution making the membrane adequate for fusion; the absence of one of them disrupting the membrane organization.

The majority of tetraspanin CD9 protein interactions in the oolema occur in the *cis* configuration: however, it has been suggested that CD9 may additionally bind in *trans* configuration to a PSG17-related ligand (Ellerman et al. 2003) present on the sperm surface which is an immunoglobulin superfamily (IgSF) member, such as IZUMO. Sutovsky (2009) assume, therefore, that sperm IZUMO may bind to oocyte tetraspanins CD9 and/or CD81 to mediate adhesion and fusion. Within the tetraspanin web on the oolema, CD9 and CD81 may interact with each other and also with integrins that are able to bind to CD46 and interconnect the actin cytoskeleton. Ellerman et al. (2009), in co-immunoprecipitation, studies showed the presence of other sperm proteins associated with IZUMO. They favour the model that, rather than directly mediating interaction between sperm and the egg, IZUMO is essential for formation of complexes with other sperm proteins required for gamete fusion. Miyado et al. (2008) reported that sperm-egg fusion is mediated by vesicles containing CD9 proteins that are released from the egg and interact directly with sperm. CD9 is transferred from the oocyte membrane to the spermatozoon present in the pervitelline space, and it may induce a sperm membrane molecular reorganization (Lefevre et al. 2010). Furthermore, CD9 molecules in the inner acrosomal membrane and also on the plasma membrane covering the equatorial segment of mouse sperm have been detected by Ito et al. (2010). Therefore, the existence of a tetraspanin web on sperm may be envisaged. It could be that the protein IZUMO, as well as CD46, are part of it. Moreover, CD46 stimulation may activate protein kinase signalling which leads to actin reorganization. CD9 is an organizer of membrane functionality through association with EWI-2, a member of IgSF8, as recently reported (Glazar & Evans 2009). EWI- 2 and EWI-F are major partners of CD9 and CD81 and, through their direct interaction with ERM (ezrin/radixin/moesin) proteins, act as linkers to connect tetraspanin-associated microdomains to the actin cytoskeleton (Sala-Valdes et al. 2006). Thus, according to the current state of knowledge, the tetraspanin web may play a role in acrosome stabilization.

## Conclusion

The present knowledge about the complement regulatory proteins CD46, CD55 and CD59 suggests that role of these proteins in process of reproduction is not only in protection of sperm against complement attack, but that it is more complex. Moreover, reported data suggest that the role of CD46 protein in rodents may be different compared to humans and other mammals. The absence of CD46 protein in field mice is surprising and leads to the question as to what are the advantages of not expressing CD46 in sperm? There is experimental data suggesting that CD46 may play a role in stabilization of the acrosome vesicule due to its interaction with the cytoskeleton and the fact that CD46 may be a part of the predicted tetraspanin web in sperm. The stability of the acrosome vesicle is believed to be an important feature of sperm quality since only an intact sperm is generally able to bind to the zona pellucida of the egg and consequently fertilize it. On the other hand, in highly promiscuous species such as field mice, the simultaneous release of the acrosomal content of a majority of the sperm population, occurring independently of the zona pellucida but in its immediate surrounding, could weaken the zona pellucida structure. In this case, not having the acrosome would represent for sperm an advantage bypassing the primary zona pellucida binding, therefore accelerating the actual process of fertilization. Another advantage of not expressing sperm CD46 protein in this highly promiscuous environment could be result of selective pressure towards protection of sperm against a certain pathogens that use CD46 as a cell surface receptor. However, field mice sperm lacking CD46 are still protected against complement-mediated attack by expressing

on their surface the CD55 and CD59 complement regulatory proteins (Clift et al. 2009b). It has been also shown that CD59 may not only be involved in sperm protection but may also play a role in sperm motility (Donev et al. 2008). It appears that all three complement regulatory proteins, CD46, CD55 and CD59, are not involved in only one particular process but their contribution to sperm fertilization ability is more general and needs yet to be further clarified. Detailed investigation of this broad field is needed to understand fully and comprehend the complexity of the subject.


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***Appendix.*** *Methods: real-time PCR analysis of Cd46 expression.*

Although this is a review-type of article, we present here original data on *Cd46* expression to demonstrate the prevalence of *Cd46* mRNA in testicular tissue. Total RNA was extracted from 11 different tissues of three male and three female *M. m. domesticus*. Tissues included in the expression analysis were as follows: vomeronasal organ (VNO), nasal-associated lymphoid tissue (NLT), main olfactory epithelium (OE), Harderian gland (HG), extraorbital lacrimal gland (LG), submandibular salivary gland (SG), preputial gland (PG), liver (L), ovary (O), uterus (U), vagina including cervix (V), testes (T), cauda epididymis (CE) and prostate (P). RNA isolation was performed using TriReagent from Sigma Life Science according to the manufacturer's protocol. The purity of RNA was assessed from the ratio of the optical densities at 260 and 280 nm, and the integrity was controlled by electrophoresis on a 1 % agarose gel containing ethidium bromide. Prior to cDNA synthesis, the RNA was cleaned with a DNase protocol (Fermentas) to avoid potential DNA contamination. One microgram of total RNA was used for the synthesis of single-stranded cDNA according to a first-strand cDNA synthesis protocol (Fermentas UAB, Vilnius, Lithuania) with RevertAid™M-MulV Reverse Transcriptase and oligo$(dT)_{18}$ primer. Real-time PCR was performed on a Light Cycler 480 (Roche Applied Sciences) using the specific dual hydrolization probe method (Universal Probe – Roche Applied Sciences) with the appropriate Probe Master kit (Roche) and protocol according to the manufacturer's protocol. Primers and specific probe number 17 for the *Cd46* sequence were designed by Universal probe library software (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp) using the NCBI reference sequence NM_010778.3, intron-spanning assay condition and multiplex PCR with reference gene (*Gapdh*) requirement. The resulting primers were: *Cd46* F: 5'-AGCCCTCCGGAGTGTAAAGT-3' on the 4$^{th}$ exon and *Cd46* R: 5'-ACATCACTGTTGATTGATAGGAAAAT-3' on the 5$^{th}$ exon. Correct amplification of the primer product was validated by sequencing. PCR amplification was performed with the following conditions: initial denaturation at 95 °C for 10 min, followed by 40 cycles consisting of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s where fluorescence was acquired, and elongation at 72 °C for 5 s. The data used for calculation are the means of Cp values of triplicate samples. Variation of triplicates did not exceed 0.5 CP. The level of *Cd46* mRNA in each sample was calculated relative to the mRNA level of the reference gene (*Gapdh*) amplified in the same well. A calibration curve was generated for both the target and reference gene, and values for PCR efficiency (E; in both cases, E was equal to 0.99, i.e. 99 % efficiency of PCR reaction) were used in a formula calculating normalized mRNA abundance (Efficiency sensitive model): Normalized amount of mRNA = $(1 + E_{reference})^{CP\ reference\ gene}/(1 + E_{target})^{CP\ target\ gene}$

Non-template and non-RT reactions were performed as a control samples.