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A family of putative metalloproteases in the salivary glands of the tick *Ixodes ricinus*

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Ticks are obligate blood-feeding arachnids. During their long-lasting blood meal, they have to counteract the protective barriers and defense mechanisms of their host. These include tissue integrity, pain, hemostasis, and the inflammatory and immune reactions. Here, we describe a multigene family coding for five putative salivary metalloproteases induced during the blood meal of *Ixodes ricinus*. The evolutionary divergence inside the family was driven by positive Darwinian selection. This came together with individual variation of expression, functional heterogeneity, and antigenic diversification. Inhibition of the expression of some of these genes by RNA interference prevented completion of the tick blood meal and affected the ability of the tick saliva to interfere with host fibrinolysis. This family of proteins could therefore participate in the inhibition of wound healing after the tick bite, thereby facilitating the completion of the blood meal.

Keywords
fibrinolysis; *Ixodes ricinus*; metalloprotease; tick; tick saliva

Ticks are blood-feeding acarines composed of three families [1]. They infest a large variety of vertebrates, ranging from amphibians to mammals, including domestic animals and humans. As they threaten the health of their hosts through anemia subsequent to blood loss, toxicosis or paralysis by saliva injection or transmission of pathogens, they are of prime medical and veterinary interest [2]. For example, *Ixodes ricinus* is the vector of *Babesia bovis*, *Ehrlichia* sp., tick-borne encephalitis virus, and *Borrelia burgdorferi*, the respective agents of babesiosis, ehrlichiosis, tick-borne encephalitis and Lyme disease in Europe [3,4].

In order to obtain their blood meal, ticks anchor onto their hosts, take up blood, and then fall off upon repletion. Unlike many other blood-feeding arthropods, ticks of the Ixodidae family, including *I. ricinus*, remain attached to their hosts for long time periods (sometimes reaching several weeks) [5]. Therefore, to

Abbreviations
dN, number of nonsynonymous changes per nonsynonymous site; dS, number of synonymous changes per synonymous site; ECLT, euglobulin clot lysis time; SGE, salivary gland extract; siRNA, small interfering RNA.

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complete the whole process, they have to counteract various barriers and defense mechanisms of the host: they disrupt tissues and fight against processes such as hemostasis, immune reaction, or pain [6–10]. Although part of this action is performed at the physical level by the mouthparts, most of it is ensured by the chemical effects of the salivary components injected during the entire length of the meal. Thus, salivary gland extracts (SGEs) or saliva have both been reported to inhibit different host defenses [11], and several of their components that are able to inhibit pain sensation, counteract the immune system, or interfere with various pathways of hemostasis, have been characterized. For example, Salp15 and Iris [12,13] suppress the immune reaction, a metallo-dipeptidyl-carboxypeptidase [14] and histamine-binding proteins (the RaHBP family) [15] decrease inflammation and inhibit pain and itching respectively, and hemaphysalin [16] and Ixolaris or the tick carboxypeptidase inhibitor [17,18] have been found to interfere with hemostasis, as anticoagulant agent and fibrinolysis modulators, respectively; in addition, an aspartic protease able to hydrolyze hemoglobin has been characterized in *Haemaphysalis longicornis* [19].

Proteases or protease inhibitors are putative weapons of the parasite. To date, metalloproteases have been found to be involved in (among other roles) tissue remodeling or disruption through digestion of structural components [20]. Inoculation of metalloproteases found in the saliva of various biting animals also participates in disrupting the balance of hemostasis [21,22]. For example, the toxicity of snake venom is partly due to several metalloproteases that interfere with hemostasis, disrupt basal laminae or extracellular matrices, and trigger hemorrhages [23]. Metalloproteases could perform similar functions in the saliva of blood-feeding arthropods.

We previously described the vaccinating potential of two closely related metalloproteases in *I. ricinus* (termed Metis, for metalloprotease *I. ricinus*). The expression of the corresponding genes was induced in salivary glands during a tick blood meal. Vaccination with one of these metalloproteases interfered with the completion of the tick developmental cycle. The other metalloprotease was essential for completion of the blood meal, as gene knockdown interfered with fibrinolysis *in vitro* and induced a high mortality rate in feeding ticks [24]. Here, we describe three new, more distant, members of this metalloprotease family that were also induced during the blood meal. These were found to be highly divergent, as they showed only between 24% and 34% identity with the founding member. Our data suggest that the evolutionary divergence within this family was driven by Darwinian positive selection and that the five diverging members are endowed with different antigenic specificities and are differentially expressed during the blood meals of individual ticks. Knockdown of the expression of *metis* genes by RNA interference *in vivo* suggests a variable requirement for the different members of this family for completion of the blood meal. Accordingly, RNA interference *ex vivo* in tick SGEs suggests a variable ability to modulate fibrinolysis. On the basis of these results, we speculate that some members of the Metis family could interfere with correct wound repair and thereby facilitate the blood meal.

Results

A multigene family encoding putative metalloproteases

We previously reported that a differential screening between salivary gland cDNA libraries from fed and unfed *I. ricinus* female ticks allowed the cloning of 27 partial cDNAs (called *seq1* to *seq27*) corresponding to transcripts induced during the blood meal of female *I. ricinus*. One of them, termed *seq16*, was homologous to snake venom metalloproteases of the reprolysin family [25]. Oligonucleotides were designed from these 27 partial sequences and then pooled to screen a full-length expression library. We reported that this allowed the isolation of two sequences closely related to *seq16*: *seq16* itself, which was renamed Metis1 for metalloprotease *I. ricinus*, and a new sequence that was called Metis2. Vaccination with one of the encoded proteins interfered with the proper completion of the ticks' parasitic cycle, as it decreased their reproductive capability. Gene knockdown by RNA interference *in vivo* affected the viability of the ticks, an effect probably related to the decreased ability of their SGEs to interfere with fibrinolysis [24]. The 44 clones obtained during the secondary screening also contained three other sequences found to be more distantly related to *seq16*. Primers were designed to clone the corresponding full-length cDNAs using 5′-RACE and/or 3′-RACE PCR, and the three new sequences were named Metis3 to Metis5.

A bioinformatics analysis led to the following observations, illustrated in Fig. 1. All sequences contained a putative signal peptide (as determined by SIGNAL P3.0, which also allowed the locatation of putative cleavage sites). Sequence analysis and comparison with an already described *Ixodes scapularis* sequence [21] indicated the presence of a propeptide. The cleavage site of this propeptide was tentatively located between

Fig. 1. Alignment of the five *metis* amino acid sequences (*metis1* AM747806, *metis2* AM747807, *metis3* AM747808, *metis4* AM747809, and *metis5* AM747810). Amino acid sequences were predicted by *in silico* translation of the five ORFs found in the sequenced cDNAs. The predicted signal peptides are indicated by a reversed background. The predicted prosequences of *metis1* and *metis5* are highlighted in gray. The zinc-binding motifs HEXXHXXGXXHD are boxed. The putative methionine turn found after the zinc-binding domain is in bold. Asterisks indicate identities. Dots indicate similarities.

position 169 and position 170 for the Metis1 and Metis2 proteins. A zinc-binding domain was detected, which is characteristic of the M12B reprolysin family of the metzinin group. The placement of Metis proteins in this family was further supported by a Met turn encoded 3′ of the catalytic site.

Phylogenic analysis of the Metis family indicated the existence of three subgroups

The nucleotide sequences and the corresponding translated amino acid sequences of the fiveis members were used to construct a distance tree using CLUSTALNJ. The tree was then submitted to bootstrap analysis. We also included in this tree all the putative metalloproteases from *Ixodes* species found to date. This tree, presented in Fig. 2, was supported by high bootstrap values. Inside this tree, metalloprotease proteins clustered in four sub-branches. The first contained Metis3 and Metis4, which shared 84.7% identity. The second contained Metis1 and Metis2, which shared 84% identity. Metis5 and AAO85918 from *I. scapularis* are not related to any cluster. The identity levels between members of the different subgroups were found to be only between 13.7% and 36.4%.

Further analysis, detailed in Table 1, indicated that the identity level between the different sub-branches of Metis was higher when nucleotide sequences were considered than when amino acid sequences were considered. Therefore, the Nei–Gojobori method was used to calculate the number of synonymous changes

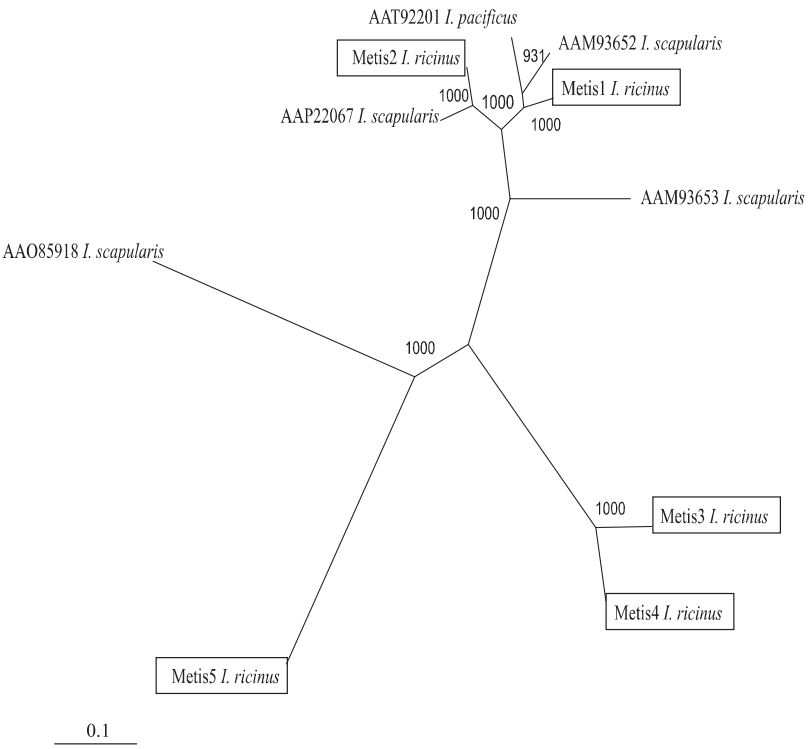

Fig. 2. Phylogenic analysis of the metalloproteases from *I. ricinus*, *I. scapularis* and *I. pacificus*. Amino acid sequences predicted by *in silico* translation of the corresponding ORFs were analyzed by the distance and neighbor-joining methods. The validity of the tree was verified by bootstrap analysis, and the obtained bootstrap values are indicated at the branch nodes. Members of the Metis family are boxed.

Table 1. Percentage of amino acid or nucleotide identity.

	Amino acid									
Nucleotide	Metis1	Metis2	Metis3	Metis4	Metis5	AAM93653	AAT92201	AAM93652	AAP22067	AAO85918
Metis1		84	35	35	26.5	54.8	63	66.7	83.8	22.7
Metis2	87.4		36.4	36.2	27.2	54.5	57	61.3	91.4	22.6
Metis3	52.3	52.5		84.7	25.2	28.1	25	26.6	34.3	19.3
Metis4	53	52.6	87.5		25.7	28.5	24.5	26.2	34.3	18.6
Metis5	50.1	49.7	48.8	49.9		21.5	18.1	18.8	27.6	23
AAM93653	58.7	58.4	47	47.1	44.9		57.8	60.8	54.1	14.5
AAT92201	65.9	59.9	42.5	42.3	42	62.3		83.1	55.7	13.7
AAM93652	69.1	64.3	44.1	44.3	43.2	66.2	87.3		60.5	15
AAP22067	86.9	92.2	51.7	52.6	49.4	57.7	59.6	63.8		21.2
AAO85918	47.2	46.6	45.4	45.3	46.1	46.9	44.7	45.2	46.9	

Table 2. Divergence among *Ixodes* species. Pairwise estimates of synonymous (dS) and nonsynonymous (dN) substitutions per site by the modified Nei–Gojobori method [38].

	Metis1	Metis2	AAM93653	AAT92201	AAM93652	AAP22067	Metis3	Metis4	Metis5	AAO85918
dN										
Metis1		0.102	0.216	0.059	0.063	0.1	0.774	0.486	0.566	0.649
Metis2			0.215	0.126	0.113	0.05	0.48	0.487	0.56	0.573
AAM93653				0.226	0.23	0.225	0.502	0.511	0.568	0.686
AAT92201					0.064	0.129	0.495	0.489	0.579	0.717
AAM93652						0.114	0.492	0.496	0.573	0.694
AAP22067							0.489	0.496	0.56	0.589
Metis3								0.083	0.583	0.601
Metis4									0.573	0.604
Metis5										0.535
AAO85918										
dS										
Metis1		0.187	0.45	0.105	0.121	0.201	0.619	0.594	0.559	0.583
Metis2			0.455	0.238	0.231	0.144	0.609	0.612	0.588	0.603
AAM93653				0.43	0.437	0.449	0.632	0.633	0.611	0.626
AAT92201					0.437	0.449	0.618	0.653	0.613	0.6
AAM93652						0.242	0.638	0.64	0.598	0.603
AAP22067							0.616	0.592	0.575	0.628
Metis3								0.224	0.645	0.591
Metis4									0.615	0.606
Metis5										0.594
AAO85918										
dN/dS										
Metis1		0.545	0.480	0.562	0.521	0.498	0.774	0.818	1.013	1.113
Metis2			0.473	0.529	0.489	0.347	0.788	0.796	0.952	0.950
AAM93653				0.526	0.526	0.501	0.794	0.807	0.930	1.096
AAT92201					0.610	0.510	0.801	0.749	0.945	1.195
AAM93652						0.471	0.771	0.775	0.958	1.151
AAP22067							0.794	0.838	0.974	0.938
Metis3								0.371	0.904	1.017
Metis4									0.932	0.997
Metis5										0.901
AAO85918										

per synonymous site (dS) and the number of nonsynonymous changes per nonsynonymous site (dN). Analysis of the values indicated a higher ratio between nonsynonymous and synonymous substitutions when members of different phylogenic groups were compared than in the same phylogenic group (Table 2).

Thus, the calculated dN/dS ratio was between 0.371 and 0.61 between members of the same cluster and 0.774 and 1.151 between members of different clusters. This bias towards nonsynonymous substitutions strongly suggests that positive Darwinian selection is at play and is driving this evolutionary divergence.

Antigenic variability among the Metis family

The five *metis* ORFs were cloned into the pcDNA3.1/V5–His mammalian expression vector. Constructs were transfected in COS-1 cells for transient expression. The ability of these vectors to drive the expression of whole V5–His fusion proteins was checked by western blot analysis of culture supernatants using an antibody to V5 (Fig. 3A). These five constructs were then used for genetic immunization of mice. The resulting sera were tested by western blot analysis of the corresponding Metis proteins expressed in COS cells. The results showed that antibodies were raised only in mice immunized with pcDNA3.1/V5–His *metis1*, *metis3* and *metis4* and not in mice immunized with pcDNA3.1/V5–His *metis2* and *metis5* (results not shown). Each of these sera was then used in western blot analysis of each of the five recombinant proteins. As shown in Fig. 3B–D, both anti-Metis3 and anti-Metis4 sera recognized both Metis3 and Metis4, whereas the anti-Metis1 serum reacted with Metis1 and also crossreacted with Metis2. None of the immune sera recognized Metis5. These results suggest that the three Metis phylogenic groups display epitopes

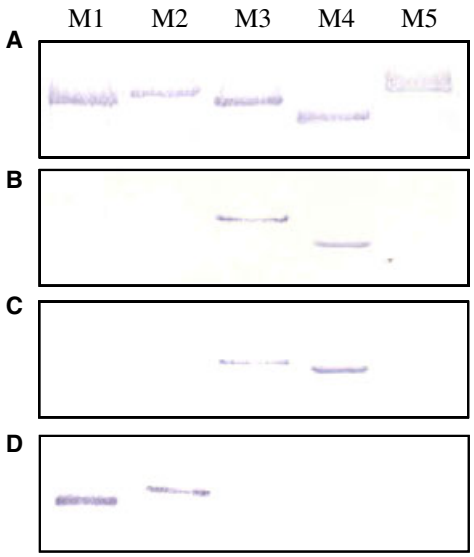

Fig. 3. Antigenic specificity of the Metis proteins. The five pCDNA3.1/V5–His expression vectors for Metis1 to Metis5 (M1–M5) were used for protein expression in COS-1 cells and genetic immunization of mice. Standardized amounts of the five Metis proteins harvested from culture supernatants were subjected to western blot analysis using antibodies to V5 or sera from the immunized mice. Mice immunized with *metis2* and *metis5* did not produce detectable antibodies (not shown). (A) Analysis with antibodies to V5 demonstrated equal loading of the five Metis proteins. (B) The anti-Metis3 serum only detected recombinant Metis3 and Metis4. (C) The anti-Metis4 serum only detected recombinant Metis3 and Metis4. (D) The anti-Metis1 serum only recognized recombinant Metis1 and Metis2.

that are shared inside each of them but different between them.

Time-specific, tissue-specific and individual-specific expression of Metis

Our previous RT-PCR analysis of poly(A+) RNA confirmed that the levels of *metis1* and *metis2* mature RNA were higher in salivary gland extracts of females fed for 5 days than in those of unfed female ticks [24].

In order to extend this analysis to all the members of the Metis family at different blood meal steps and parasitic stages, and in different individuals, specific pairs of primers were designed that were able to discriminate between the different members of the family and amplify only one of them at a time (data not shown). A pair of primers allowing actin mRNA amplification was used in order to verify the integrity and correct loading of the samples. In order to monitor time-specific, tissue-specific and individual-specific expression, these pairs of primers were used to analyze polyA(+) RNA extracted from various sources: whole pooled unfed or fed larvae, nymphs, males and females before or after removal of salivary glands, salivary glands from unfed females and females fed for 1, 3 or 5 days, and finally from salivary glands of 10 individual females fed for 5 days. Although the RT-PCR illustrated in Fig. 4 was only semiquantitative, it allowed us to draw the following conclusions: (a) expression of the five *metis* genes was induced upon blood feeding in larvae, nymphs, and females; (b) none of the *metis* transcripts was detectable in the adult males; (c) expression of the five *metis* genes was not detectable in unfed ticks; and (d) expression of the five *metis* genes was undetectable in females when the salivary glands were dissected out, indicating that it was restricted to the salivary glands (at least in female ticks). A finer analysis of the females indicated that the expression was induced specifically in salivary glands as early as the first day of a blood meal. It also indicated that, among a tick population, the pattern of expression of the five metalloproteases varied among different individuals. These results suggest that the *metis* genes are induced and expressed specifically in salivary glands during a blood meal.

Metis proteins contributed to completion of the blood meal

In order to investigate the role of the Metis family, we decided to observe ticks upon *in vivo metis* gene knockdown by RNA interference. Small interfering RNAs (siRNAs) specific for each member of the family were designed, and their specificity was assessed by cotransfection with each of the pcDNA3.1 expression vectors in COS-1 cells. Supernatants of all the cotransfections were harvested and subjected to western blot analysis using antibodies to V5 epitope. Figure 5 shows that the expression of a given Metis protein was suppressed only when its expression vector was cotransfected with the matching siRNA but not in any other cotransfection. These results showed that each designed siRNA was functional, as it knocked down the expression of the corresponding protein, and highly specific, as it did not affect the expression of other family members.

We then proceeded to analyze the *in vivo* effect of each of these siRNAs. For that purpose, groups of 30 adult female ticks were microinjected with different siRNAs, alone or pooled, in the ventral torso of the idiosoma. As shown in Fig. 6, injection of the buffer alone or of an irrelevant siRNA as control caused a maximum of 30% mortality rate. Injection of each of the five siRNAs targeting Metis1 to Metis5 alone had a different effect on the basal mortality rate, ranging from a total lack of effect (Metis1 and Metis5:

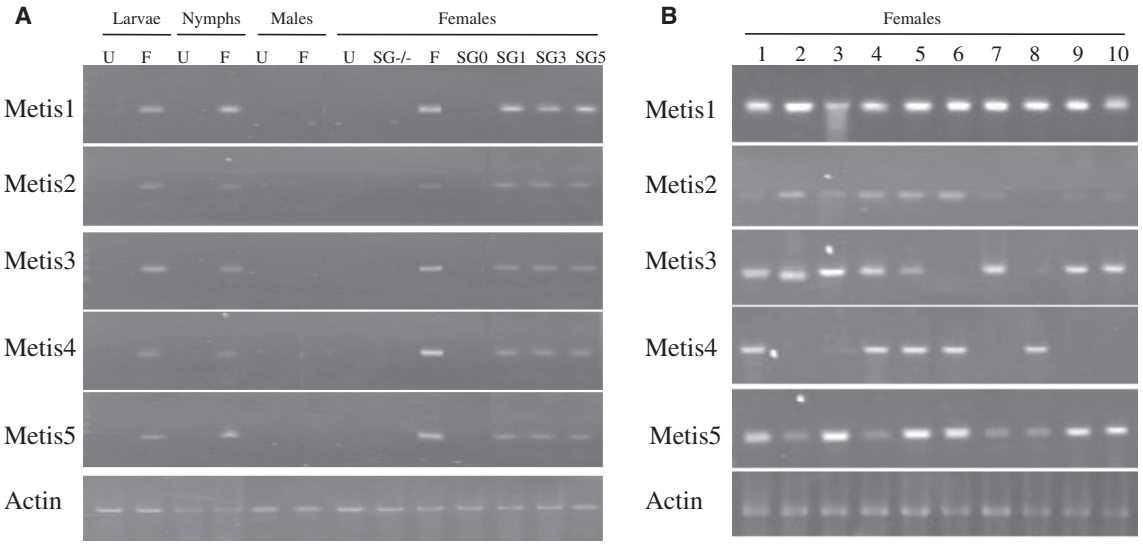

Fig. 4. Stage, time and individual specific expression of *metis* genes. Poly A(+) RNAs were extracted from different developmental stages, at different times of the blood meal, from different individuals, and analyzed by RT-PCR using pairs of primers specific for *metis1* to *metis5* and actin. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining, and sequenced. (A) Analysis of different tissues from different developmental stages at different steps of the blood meal from pooled populations. Whole unfed (U) or fed (F) ticks, whole fed females without salivary glands (SG−/−), salivary glands of unfed females (SG0), and females fed for 1 day (SG1), 3 days (SG3), or 5 days (SG5) females. (B) Analysis of RNA extracted from salivary glands of 10 individual females (numbered 1–10) fed for 5 days.

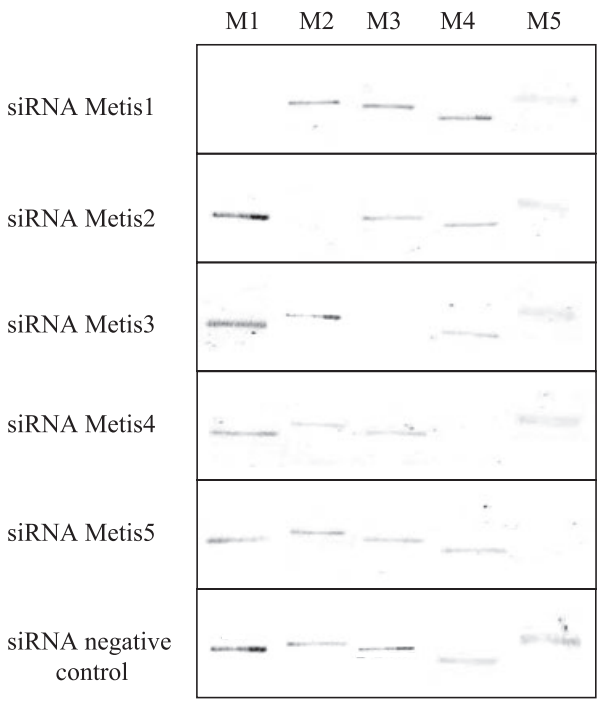

Fig. 5. Specific siRNA silencing of *metis* gene expression. All possible combinations of each of the pCDNA3.1/V5–His vectors expressing Metis1 to Metis5 (M1–M5) and each of the siRNAs targeting *metis1* to *metis5* were cotransfected in COS-1 cells, and Metis protein expression was analyzed by western blot using antibodies to V5.

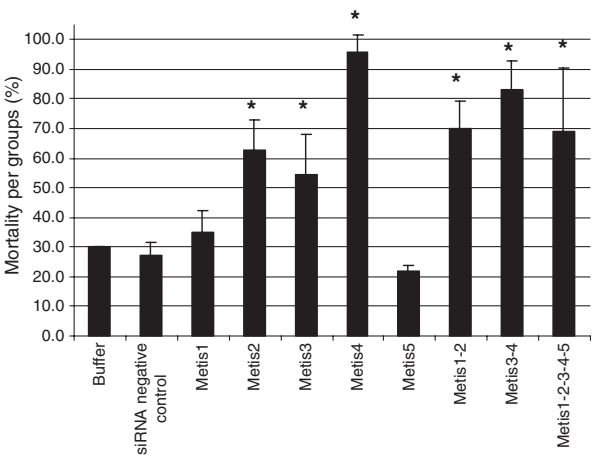

Fig. 6. Tick viability after Metis knockdown by RNA interference. *I. ricinus* adult ticks (30 ticks per experimental group) were injected into the idiosoma with buffer alone or siRNAs targeting the five *metis* genes individually, grouped by two (e.g. *metis1* and *metis2*, and *metis3* and *metis4*), or all five *metis* genes, as well as siRNA targeting an irrelevant sequence (siRNA negative control). Ticks were followed daily until day 5 postinfestation. The percentage of dead ticks and the standard deviation for three independent experiments are shown. *$P < 0.05$ as compared to control (ANOVA one-way analysis of variance and Student–Newman–Keuls test).

35.0 ± 7.1% and 21.7 ± 2.4% respectively) to a dramatic effect (Metis4: 96 ± 5.7%), with intermediate effects for Metis2 and Metis3 (62.5 ± 10.6% and

54.5 ± 13.4%, respectively). This mortality was observed to be an early effect (within 16 h). The absence of effect of some individual siRNAs (together with the organization of the family in divergent phylogenic subgroups) suggested a possible functional redundancy. In order to check this hypothesis, the siRNAs targeting all the members of one phylogenic branch at a time were pooled and injected. Whereas we confirmed that the Metis5 siRNA had no effect, pooled Metis1 and Metis2 and pooled Metis3 and Metis4 induced mortality in injected ticks of 70.0 ± 9.4% and 83.3 ± 9.4%, respectively. The latter value was not significantly different from the value obtained after injection of Metis4 siRNA alone, which was already nearly completely lethal on its own. The former suggested that the two members of the Metis1–2 group had redundant actions, as the combined action of the two siRNAs together was significantly stronger than that of each one alone. Finally, injection of a pool of the five siRNAs gave a mortality rate of 68.9 ± 21.4%. Although not significantly different, this lower value could be related to the fact that the amount of each siRNA in the cocktail is five times lower than in individual injections. Finally, no difference between control ticks and ticks surviving the microinjection could be observed according to any of the analyzed criteria (weight gain, meal duration).

These results suggest that some Metis proteins but not others are essential for the survival of ticks during a blood meal. They also suggest some cases of redundancy inside the family.

Metis proteins interfered with fibrinolysis

We then decided to investigate the mechanism affected by the action of Metis. Despite repeated attempts, we failed to express an active form of any of the Metis proteins (see Discussion). This is a common problem encountered when studying metalloproteases, and unfortunately prevents a direct assessment of enzymatic activity. In order to bypass this problem, we set up an *ex vivo* siRNA interference assay. SGEs or purified proteins have been shown to interfere with several physiological processes *in vitro*. In particular, an effect on fibrinolysis has been observed, and it has been proposed to be mediated by metalloproteases [21,26]. siRNAs were therefore mixed with the SGEs from dissected ticks, and their influence was monitored in a fibrinolysis assay. In the assay that we set up, various quantities of SGEs from female ticks fed for 5 days were mixed with a preformed fibrin clot, and the fibrinolysis was monitored by euglobin clot lysis time. SGEs from fed ticks decreased the fibrinolysis time; this effect was dose-dependent, with a maximal reduction of 301 ± 10 min (corresponding to 60%) being observed upon addition of 30 μg of SGEs (Fig. 7). As shown in Fig. 8A, none of the individually added *metis* siRNAs had an effect on this reduction, and nor did the combination *metis3–4* siRNA. In contrast, the combination *metis1–2* siRNA added to SGEs interfered with their ability to decrease fibrinolysis time, as they raised it by half. That is to say, SGEs supplemented with buffer or an siRNA negative control lowered the fibrinolysis time by 201 ± 14 min (40.1%) and by 208 ± 6 min (41.5%), respectively. Addition of pooled *metis1* and *metis2* siRNA reduced this action to 112 ± 16 min (22.4%). Addition of an equivalent amount of buffer or siRNA targeting other Metis proteins or siRNA negative control did not influence the measured values. The specific action of the *metis1–2* siRNA was strongly supported by an observed dose-dependent effect (Fig. 8B), which, however, plateaued at high concentrations.

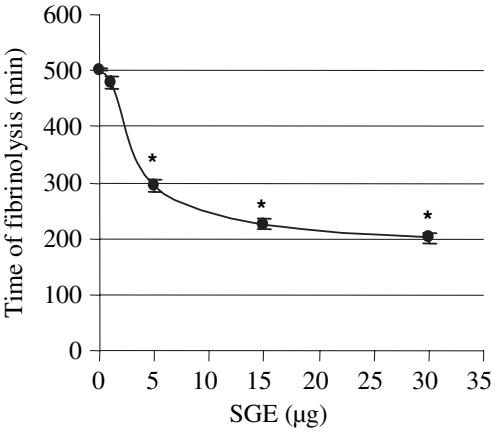

Fig. 7. Fibrinolysis in the presence of SGEs. SGEs were incubated with a preformed fibrin clot. The time necessary for clot reduction was measured using the ECLT assay. The indicated amounts of SGE from adult females fed for 5 days were added to the fibrin clot. *$P < 0.001$ as compared to values observed in the absence of SGE (ANOVA one-way analysis of variance and Student–Newman–Keuls test).

These results suggest some redundancy in the fibrinolytic action of Metis1 and Metis2; they are compatible with a lack of interference of some other Metis family members with fibrinolysis.

Discussion

We describe a family of five putative metalloproteases from the salivary glands of the tick *I. ricinus*. Several reports on the sialome of *I. scapularis* and *Ixodes pacificus* [26–29] indicated that organization into large

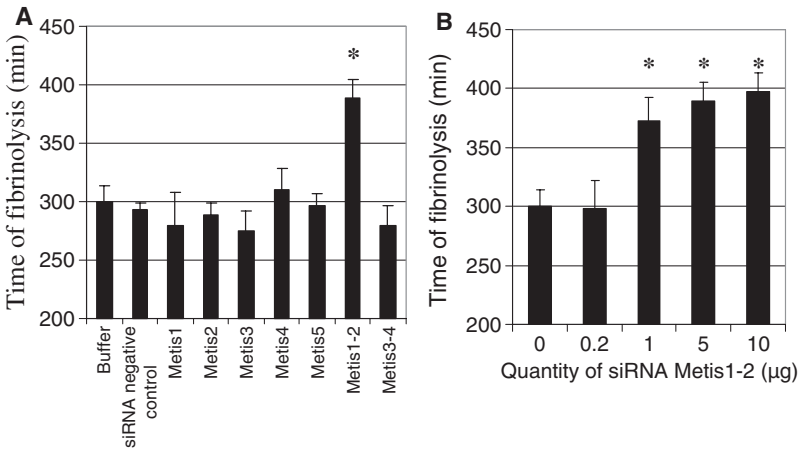

Fig. 8. Metis knockdown interfered with the stimulation of fibrinolysis by SGEs. (A) SGEs were incubated with buffer or the indicated siRNA for 6 h prior to incubation with a preformed fibrin clot. The time necessary for clot reduction was measured using the ECLT assay. (B) SGEs were incubated with an increasing quantity of a combination of *metis1* and *metis2* siRNA prior to incubation with a preformed fibrin clot. The time necessary for clot reduction was measured using the ECLT time assay. $*P < 0.05$ as compared to the control (ANOVA one-way analysis of variance and Student–Newman–Keuls test).

gene families is a recurrent theme in the salivary glands of these organisms. Metalloproteases constituted one of the important groups of salivary proteins of *I. scapularis*, containing at least four members. The relative abundance of Edman degradation peptides suggested that they were highly expressed. A more extensive interrogation of the databases allowed us to detect one, one, five and seven sequences from *I. pacificus*, *Rhipicephalus haemaphysalidoides*, *Boophilus microplus* and *Haemaphysalis longicornis*, respectively. Scant information is available about some of these tick metalloproteases. For instance, members of a family demonstrate differential salivary expression during the blood meal in *H. longicornis* [30], and another one is a male-specific enzyme in *I. scapularis* [31]. None has been expressed as an active recombinant form. Therefore, the metal-dependent fibrino(geno)lytic or anti-angiogenic activities [21,22] of the saliva in *I. scapularis* could not be attributed to any of them. Here we contribute five new genes from *I. ricinus* to this family, and take this opportunity to report one of the first in-depth analyses focused on one gene family coding for tick metalloproteases.

Among the reported roles of metalloproteases is the alteration of tissues, and in particular the remodeling of the basal lamina and extracellular matrix. This is a useful function for biting animals to acquire in their saliva. Also, the toxicity of some snake venoms is due to the action of metalloproteases.

Our analysis of the amino acid sequences of the Metis protein family reveals several characteristics (a signal peptide, a zinc-binding domain, a conserved critical methionine, and a pattern of C-terminal cysteines) indicating that they are members of the metzinin group, which includes the snake venom proteases. Our biochemical analysis is also in agreement with a putative protease activity, as the knockdown of some *metis* genes interferes with tick blood meal completion *in vivo* and modulates fibrinolysis by SGEs *ex vivo*. These observations suggest that Metis stimulates fibrinolysis, an action that counteracts hemostasis. Metis could thereby interfere with wound healing of the endothelial epithelium and therefore facilitate the blood meal. This is in agreement with a previous report that the salivary compounds of ticks affect tissue repair [32]. Wound repair is divided into several phases, including coagulation, inflammation, cell proliferation, and tissue remodeling. Ticks are able to prevent the first two phases, as a fibrin clot is not formed at the bite site, and therefore fibrinolysis should not be necessary. Clotting prevention is attributed to salivary anticoagulant proteins. Furthermore, it could also be partly due to a redundant action of profibrinolytic agents (e.g. members of the Metis family), in the unlikely event of coagulation being initiated. On the other hand, we cannot rule out the possibility that at least some Metis proteins (e.g. Metis4) are proteases with more widespread roles, such as digestion of food. This hypothesis is supported by the lack of effect of Metis4 knockdown on fibrinolysis. As Metis4 is nevertheless essential for tick survival, this argues for the acquisition of a new function by this member of the family. In any case, several observations suggest a function that is strictly related to efficient accomplishment of

the blood meal. The *metis* genes are not detectable in males, which take only occasional and very short blood meals. Their expression is induced during the blood meal in the feeding stages, and is strictly specific to the salivary glands.

Although the saliva of some tick species has been shown to display a fibrino(geno)lytic action [21], only a few molecules of tick saliva have been reported to interfere with the physiological process of fibrinolysis. One of the only examples is tick carboxypeptidase inhibitor from *Rhipicephalus bursa*, which binds and inhibits thrombin-activable fibrinolysis inhibitor to stimulate fibrinolysis [18]. In addition, we have already mentioned two cDNAs coding for homologs of snake venom metalloproteases. These snake venom metalloproteases have been found to have saliva calcium-dependent hydrolytic activity [23]. We now describe five different genes coding for the Metis family of salivary putative metalloproteases. Our sequence comparisons and phylogenetic tree indicate that the Metis proteins belong to three subgroups. The observed bias towards nonsynonymous substitutions strongly suggests that positive Darwinian selection is at play, driving apart the members of these different Metis subgroups. This indicates an evolutionary pressure towards functional diversification between these different subgroups. Several important evolutionary problems, such as functional, developmental or tissue specialization, host range extension, and antigenic variation, can be solved by diversification.

Regarding the functional specialization, our experiments, however, indicate that there could be some redundancy inside given subgroups (e.g. Metis1 and Metis2, involved in blood meal completion, only of which seems to be necessary). At least one member (Metis4) seems to be essential for tick survival, although the lack of this protein does not interfere with fibrinolysis at all. Sequence diversification could therefore provide this protein with new unrelated functions, thus driving functional diversification. These observations are reminiscent of the few other reports on similar tick salivary proteins that are either involved in the same function (madanin) [33] or not (Salp14 and Salp9pac) [34].

In addition, we show that the observed divergence inside the Metis family probably generates antigenic diversification, as antibodies raised against members of different subgroups do not crossreact with members of other subgroups. In natural conditions, this would allow some Metis family members to escape a pre-existing immune reaction to the other members of the Metis subgroups in previously infested hosts. This would only be efficient if different individuals expressed different sets of family members. This is indeed what we observe.

The analysis of paralogs always raises the issue of whether they are alleles or different gene copies. We do not favor the hypothesis that the genes encoding the five described Metis family members are alleles, for the following reasons: First, they are extensively divergent, as members of different Metis subgroups do not share more than 36% identity, and even members of the same subgroups are at most 85% identical. Second, the *metis* cDNAs have been characterized within a very secluded population that has been inbred for years, restricting the number of alleles propagated. Third, more than two copies have been observed to be expressed in a same individual. Finally, sequencing of *metis1–5* PCR products revealed one, three, one, three and 11 paralogs respectively (results not shown). The Metis2, Metis4 and Metis5 paralogs shared 93–96%, 99% and 94–99% identity, respectively. It is likely that it is this low variability that suggests the existence of alleles encoding each of the Metis family members. In any case, a definite decision about the nature of the Metis family members as being encoded by alleles or divergent genes will await genome sequencing.

The Metis family proteins are unlikely to be the only tick saliva proteins able to modulate fibrinolysis, as half of the SGE activity still remains after Metis knockdown. This indicates the presence of other salivary proteins with fibrinolytic activity.

A definitive validation of the Metis family's role as metalloproteases awaits the production of functional recombinant proteins. Unfortunately, to date, our attempts to perform such an assay have been unsuccessful, a common feature in the study of metalloproteases. Thus, Metis1 was expressed with or without its propeptide in *Escherichia coli*, yeast, COS cells and, baculovirus. It was soluble in all systems except bacteria. Nevertheless, it was never found to be active, irrespective of the presence or the absence of the prosequence, the activator used *p*-aminophenylmercuric acetate (AMPA), or the substrate tested (including fibronectin, lamin, casein, collagen, elastin, gelatin, fibrinogen, and fibrin).

Experimental procedures

Ticks, SGEs and saliva

I. ricinus ticks were bred and maintained at the Institute of Biology, University of Neuchâtel (Switzerland). Founders of the colony were initially collected in the woodland near Neuchâtel, and have been maintained on rabbits or Swiss mice for over 20 years. For the experiments described in

this article, pairs of adult (one female and one male) ticks were allowed to anchor and feed on rabbits for the indicated periods. The larvae and the nymphs were fed on mice until repletion.

For the preparation of SGEs, unfed female ticks and engorged female ticks fed for 1, 3 and 5 days were dissected under the microscope. Salivary glands were harvested in ice-cold NaCl/P_i. Tissue was then disrupted and homogenized using a dounce homogenizer. Samples were centrifuged for 5 min at 10 000 ***g***. Supernatants were recovered and stored at −20 °C. For the preparation of larvae, nymph, male and female extracts, whole fully engorged individuals were crushed and prepared as above. For the preparation of female extracts without salivary glands, female ticks were dissected and the salivary glands were removed under the microscope.

cDNA library screening and RACE

Analysis of a cDNA subtractive library from *I. ricinus* salivary glands fed for 5 days and unfed originally identified 27 distinct sets of sequences specific to the fed state [25]. Specific oligonucleotides (Table 3) were designed from these sequences, pooled, and used to screen a full-length cDNA library by conventional hybridization procedures. This screening identified 44 clones. These clones were sequenced using M13 forward and reverse primers. Sequences were BLASTed against the original 27 clones, and five sequences showed homology with a snake venom metalloprotease.

Table 3. Primer sequences used for the screening of the full-length cDNA library.

Clone	Probe (5′- to 3′)
seq1	GTCCTCTTCTAAATAAGACCCATCC
seq2	AAGTCACTTGCACTTATCAAGCTCC
seq3	TTATGCTGCCGCCTACTTTTCCTTC
seq4	AGTACCCCTGTGAACTCTGGCTTTG
seq5	ATTGCCCTTGACGTACTCTCTCAAC
seq6	GAAGGAACAGGCACAAATATACTAC
seq8	GACCGATTCCACATTGTAGTACACC
seq9	TGTGACCATATCTTTGTTTCCCCTG
seq10	ACATATCATTTGGAGGAAGGCGTAG
seq11	GTGATAACCATATCCATTCCTCACC
seq12	TGGTTTACCGTAACAAGTACACCAG
seq13	CTGCCTCTACAAAGTCAATGCCAAG
seq14	CTCACCAAACACATCAAATACCCCC
seq15	CATGCCTTCGTCGTACATATACACC
seq17	TCGAATTGCACTTCGGAACTTCCAC
seq18	TCCCCGCCCCTTGACAATCGTCCGA
seq19	ATCCGAATGAGTTGTCAAATGACAT
seq20	AGAAGAGTAAGGTTTTCACCGACAG
seq21	TGTTGCTACAGACTCGACGTTTCGA
seq22	TGAAACTTGAAATACTCCCACAGTC
seq23	GACCACCCCGTCCGAACTTGCTAAA
seq25	TCCAATCTACAATCTTTCCTCGCAC
seq26	AGAAGACTGGGAAGATAAGAAGCAC
seq27	TCACCTGCTATTCCAGAAGTACACC

In cases where full-length cDNA clones were not recovered, the missing ends were generated by RACE, using the GeneRacer Kit (Invitrogen, Merelbeke, Belgium). In these cases, gene-specific primers used for 5′-RACE were as follows: *metis3*, 5′-TGACGTCCTCTCAAGGCTGAGG GTAA-3′; *metis4*, 5′-TTGTTATGGTCCGAGCCGTCGA CATAA-3′; and *metis5*, 5′-GGGGCTCAGTGCTCCT CTCACTTCTAA-3′. The primer used for 3′-RACE was as follows: *metis2*, 5′-GCTAATCTTAGGTATGCCAGC TTCGTAT-3′. Amplification was performed using ThermoZyme (Invitrogen) DNA polymerase according to the manufacturer's instruction. Products were cloned into the pCR4-TOPO vector (Invitrogen) and sequenced.

RNA extraction and RT-PCR analysis

mRNAs were isolated by oligo-dT chromatography (Micro-FastTrack 2.0 mRNA Isolation Kit; Invitrogen) from various tissues at different developmental stages or from whole individuals after tissue disruption using a dounce homogenizer and clearing by centrifugation. Reverse transcription was routinely performed in a 20 μL standard RT reaction mixture, according to the instructions of the manufacturer (First-Strand cDNA Synthesis System; Invitrogen), using the oligo-dT primer. The RT product was then used as a template in 50 μL of a standard PCR reaction mixture with the gene-specific primers described in Table 4 to generate products of the indicated size. Thus, the PCR was routinely performed in a 50 μL volume of Takara buffer containing 2.5 U of *Taq* polymerase (Takara Ex Taq; Takara, Shiga, Japan), 10 pmol of each primer, and 2.5 nmol of each dNTP (Takara). PCR conditions were as follows: 30 cycles of 30 s at 95 °C, 30 s at 58 °C, and 1 min at 72 °C, preceded by an initial 4 min denaturation step at 95 °C and followed by a final 10 min extension step at 72 °C. A pair of primers designed to amplify a 1131 bp sequence from the actin complete ORF (sense primer, 5′-ATGTGTGACGACGAG GTTGCC-3′, and antisense primer, 5′-TTAGAAGCAC TTGCGGTGGATG-3′) was used as control. Ten microliter volumes of the PCR reactions were analyzed on a 2% agarose gel. PCR products were then cloned into the pCRII-TOPO vector (Invitrogen) and sequenced.

Expression of recombinant proteins in mammalian cells

Pairs of primers were designed to amplify by RT-PCR the five *metis* complete ORFs from mRNA from salivary glands of ticks fed for 5 days used as a template (Table 5). Unique restriction sites were added 5′ and 3′ of the *metis* ORFs by PCR in order to subclone them into the mammalian expression vector pCDNA3.1/V5–His, coding for the His and V5 epitope tags. The PCRs were performed in a

Table 4. Primers used for specific amplification of the *metis* fragments.

Gene	Direct primer (5′- to 3′)	Reverse primer (5′- to 3′)	Size (pb)
metis1	GTCAAAACATTTTTATCGTACTGCC	GATTGTTTTTTTCGCTTTTCGTTCAG	423
metis2	ATCAAAAAAATTCTATAATACCTCCGAT	TATTGTTTTTGTCGTGTATCGTTCTC	423
metis3	GCTTGACATAGTTGGGGTAGCGA	TTCCTCCTGTTTTTGTTGTCGCAA	588
metis4	GCTTGATATAGTTGGGGTAGCGA	TTCTTGGGGTTTCTCTTGTCGCAA	588
metis5	ATGATGAATATACTATGGCGCCTGT	GTCCATTATTACGCGTCGGGGTT	456

Table 5. Primers used for amplification of the *metis* genes.

Gene	Direct primer (5′- to 3′)	Reverse primer (5′- to 3′)
metis1	GGATCCATGTCGGGACTCAGCCTGAAA	TTCGAAGTCCTTCTTGCTTATTTTGATTTT
metis2	GGTACCATGTCGGAACTCAGCCTGAAAT	TCTAGAGTCCGTTCTGCTTATTTTGATCG
metis3	GGTACCATGACTATCATTTTGCGGCTCC	TCTAGATTCCCGGTACGTTCTCTCCG
metis4	GGTACCATGACTACCATTTTGCGGCTCC	TCTAGATTCCCGGTACGTTCTCTGGG
metis5	GGTACCATGTTGTGGCTCTATGTGTTGGT	TCTAGAGCCACTTCGACGTCCCCAGT

50 μL reaction volume containing 2.5 U of *Taq* polymerase (Takara Ex Taq; Takara), 10 pmol of specific primers for each target region amplified and 2.5 nmol of each dNTP (Takara) in a standard buffer supplied by the manufacturer (Takara). The PCR conditions were as follows: one cycle at 95 °C for 4 min, followed by 30 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 2 min, followed by a final extension step at 72 °C for 10 min. The PCR products were then purified by PAGE followed by electroelution. The PCR products were cloned into the pCDNA3.1/V5–His *Kpn*I and *Xba*I (for *metis2* to *metis4*) or *Bam*HI and *Sfu*I (for *metis1*) restriction sites. The *metis*/pCDNA3.1/V5–His constructs were then transfected into COS-1 cells by means of Fugene 6 (Roche, Vilvoorde, Belgium), according to the manufacturer's recommendations. The expression of proteins recovered in the culture supernatant was confirmed by western blot analysis.

SDS/PAGE and western blot analysis

Tissue samples or culture supernatants containing recombinant proteins were mixed with one volume of 2 × sample buffer, loaded on a 12.5% polyacrylamide gel, and electrophoresed. Proteins were then analyzed by western blotting, using the indicated primary antibodies [anti-V5 (Invitrogen) or antibodies raised against various Metis proteins as previously described], and anti-rabbit IgG alkaline phosphatase conjugate (Promega, Leiden, the Netherlands). Membranes were developed using Nitro Blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

DNA immunization

Intramuscular injections of 100 μg of *metis*/pCDNA3.1/V5–His in 100 μL of NaCl/P_i were given into the quadriceps of mice and repeated five times at 3-week intervals. They were immediately preceded by a local injection of 100 μL of cardiotoxin (10 μM).

siRNA

siRNAs (Table 6) were designed to target specifically each of the *metis* mRNAs and were synthesized by Eurogentec (Liège, Belgium).

siRNA silencing in COS-1 cells

COS-1 cells were cotransfected with all possible combinations of 500 ng of each of *metis*/pCDNA3.1/V5–His and 100 ng of each of the specific siRNAs using the X-tremeGENE siRNA transfection Reagent (Roche) according to the manufacturer's recommendations. Forty-eight hours post-transfection, culture supernatant was harvested and protein expression was analyzed by western blot.

siRNA interference in live ticks

One microliter of 1 mM EDTA/10 mM Tris/HCl (pH 7.5) buffer only or containing 650 ng of specific *metis* siRNA or siRNA negative control duplexe (Eurogentec) was injected into the ventral torso of the idiosoma, away from the anal

Table 6. siRNA used for silencing of the *metis* genes.

Gene	Direct siRNA (5′- to 3′)	Reverse siRNA (5′- to 3′)
metis1	ACACUCAGAUGAAGUCAAA	UUUGACUUCAUCUGAGUGU
metis2	GCACGACAGAGGGAAAUGA	UCAUUUCCCUCUGUCGUGC
metis3	CAACAUGCGCUGCUUACAA	UUGUAAGCAGCGCAUGUUG
metis4	CGAGGAGGCUACCAUUCAU	AUGAAUGGUAGCCUCCUCG
metis5	CUACUGCGGAUAUAAUGAU	AUCAUUAUAUCCGCAGUAG

opening, of adult *I. ricinus* females. The injections were carried out using Hamilton Microliter syringes with 33-gauge needles. Thirty ticks were used per group. The ticks were allowed to recover for 1 day before infestation (together with uninjected male ticks) of the ears of New Zealand White rabbits. The ticks were monitored daily. Female ticks that fell off upon repletion were collected and weighed on a digital balance.

Ex vivo siRNA silencing in salivary glands

The salivary glands from 10 partially (5 days) fed female ticks were incubated for 6 h at 37 °C in the presence of 5 μg of siRNA negative control duplexe (Eurogentec) or *metis* siRNA (individually or in combination) or buffer alone in a total volume of 200 μL of incubation buffer TC-199 (Sigma, Bornem, Belgium) containing 20 mM Mops (pH 7.0).

Fibrinolysis assay – euglobulin clot lysis time (ECLT)

The euglobulin fraction was prepared as described by Zouaoui Boudjeltia *et al.* [35]. Briefly, 75 μL of acetic acid (0.025%) and 900 μL of de-ionized water were added to 100 μL of human plasma. The sample was centrifuged at 4000 ***g*** for 10 min at 4 °C. The pellet was resuspended in 100 μL of Owren buffer. Euglobulin clot formation was started by adding 25 μL (1.5 $U{\cdot}mL^{-1}$) of thrombin. After 10 min, 10 μL of SGE in Owren buffer was added to the reaction, and the lysis time was measured by a semiautomatic method using a 'Lysis Timer' device.

Bioinformatics

For sequence collection, the GenBank databases were interrogated online at the NCBI server using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Nucleotide and amino acid sequences of *metis* were used as baits to screen the species databases or genome project preliminary releases. Translation of the ORF was performed using the TRANSLATE tool program from Expasy (http://www.expasy.ch/tools/dna.html). Alignments were performed and visualized using the program CLUSTALW 1.8 (http://search-launcher.bcm.tmc.edu/multi-align/multi-align.html).

Signal peptide and its cleavage site were determined on the amino acid sequences using the program SIGNALP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). Calculations of molecular weights were performed using the PEPSTAT software from wEMBOSS (https://oryx.ulb.ac.be/wEMBOSS/).

The inframe alignment of the codons was obtained by aligning the coding sequences using CLUSTALW under the MEGA3 package [36] (http://www.megasoftware.net/). Manual adjustments were made. The GENEDOC package was then used to visualize alignments. Identity levels were also calculated using the GENEDOC package (http://www.psc.edu/biomed/genedoc). For phylogenetic analysis by the distance method, we used the program CLUSTALNJ from wEMBOSS (https://oryx.ulb.ac.be/wEMBOSS/). We performed the bootstrap analysis of 1000 replicates of the original datasets; the tree was visualized using the TREEVIEW software [37] (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The dN and dS values were calculated using the Nei–Gojobori method [38] as implemented in the MEGA3 package.

Statistical analyses

The significance of the data was evaluated using one-way ANOVA and the Student–Newman–Keuls test implemented in MEDCALC for Windows, version 8.2.0.1 (MedCalc Software, Mariakerke, Belgium).

Animal use

Animal care and experimental procedures were carried out in accordance with local institutional guidelines (laboratory licence no. LA 1500474) and the Belgian law of 14 August 1986 as well as the Royal decree of 14 November 1993 relating to the protection of laboratory animals.

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