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Sialylation in protostomes: a perspective from *Drosophila* genetics and biochemistry

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Abstract Numerous studies have revealed important functions for sialylation in both prokaryotes and higher animals. However, the genetic and biochemical potential for sialylation in *Drosophila* has only been confirmed recently. Recent studies suggest significant similarities between the sialylation pathways of vertebrates and insects and provide evidence for their common evolutionary origin. These new data support the hypothesis that sialylation in insects is a specialized and developmentally regulated process which likely plays a prominent role in the nervous system. Yet several key issues remain to be addressed in *Drosophila*, including the initiation of sialic acid *de novo* biosynthesis and understanding the structure and function of sialylated glycoconjugates. This review discusses our current knowledge of the *Drosophila* sialylation pathway, as compared to the pathway in bacteria and vertebrates. We arrive at the conclusion that *Drosophila* is emerging as a useful model organism that is poised to shed new light on the function of sialylation not only in protostomes, but also in a larger evolutionary context.

Keywords Sialic acid · Sialylation · *Drosophila* · Genetics · Evolution · Development · Insects

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Abbreviations

ATP	adenosine triphosphate
ADP	adenosine diphosphate
CST	CMP-sialic acid transporter
CTP	cytidine triphosphate
CMP	cytidine monophosphate
CNS	central nervous system
DmCSAS	*Drosophila melanogaster* CMP sialic acid synthetase
DmSAS	*Drosophila melanogaster* sialic acid synthetase
DSIAT	*Drosophila melanogaster* sialyltransferase
KDN	deaminoneuraminic acid or 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid
LacNAc	Galβ1–4GlcNAc
LacdiNAc	GalNAcβ1–4GlcNAc
ManNAc	*N*-acetylmannosamine
ManNAc-6-P	*N*-acetylmannosamine 6-phosphate
Man-6-P	mannose 6-phosphate
Neu5Ac	*N*-acetylneuraminic acid
NeuGc	*N*-glycolylneuraminic acid
Neu5Ac-9-P	*N*-acetylneuraminic acid 9-phosphate
P_i	phosphate
PP_i	pyrophosphate
PEP	phosphoenolpyruvate
Sia	sialic acid
Sia-9-P	sialic acid 9-phosphate
Sia transporter	sialic acid transporter
UDP	uridine 5′-diphosphate
UDP-GlcNAc	UDP-*N*-acetylglucosamine.

Introduction

Glycosylation is one of the most common forms of posttranslational protein modifications in eukaryotic cells. Sialylation, which occurs on glycolipids and N- and O-glycans, stands out among the other types of glycosylation as it is typically found on the terminal positions of the glycan chains. Unlike most other sugar residues, sialic acids are negatively charged, have a relatively large nine-carbon backbone, and they can carry a variety of side group modifications. Combination of these features makes sialic acids uniquely suited to participate in molecular recognition and to regulate cellular interactions.

Sialic acids can be found at almost all evolutionary levels of life (reviewed in [1]). Although most abundantly present and best studied in deuterostomes (echinoderms, ascidians, and vertebrates), sialylation is also found in a number of microorganisms (reviewed in [2]). Many of these microorganisms are pathogenic species able to synthesize sialic acids *de novo* or metabolize host sialic acids for their own cell surface epitopes to mimic the properties of host cells and to evade the host organism's immune response. Through specific receptors, they can also recognize host sialylated structures and use them in the pathogenic process. While it has been originally suggested that the presence of sialylation pathway enzymes in these microorganisms is a result of horizontal gene transfer, in many cases an independent evolutionary re-occurrence of this pathway is a more likely explanation, especially since the corresponding bacterial genes often do not show a close evolutionary relationship to their animal counterparts [3].

Great strides have been made over the past few decades in uncovering a large number of different sialylated structures and in cloning numerous sialyltransferases and other genes of sialylation machinery from different species (for reviews see [4–6]). However, our knowledge about the structural diversity and widespread occurrence of sialic acids is yet to be matched by the understanding of their biological function and underlying molecular mechanisms. Even though striking biological roles of sialic acids in key cellular and molecular interactions and developmental pathways have been revealed [7] (*e.g.,* virus-host interactions [8–10], cancer cell differentiation [11], immune [12] and nervous system functioning and development [13, 14], integrin-mediated adhesion [15], apoptosis [16], TrkA tyrosine kinase receptor signaling [17], *etc.*), these findings likely represent only the tip of the iceberg.

Gene targeting has recently become one of the most productive approaches for studying the biological functions of sialylation in animals, with sialyltransferase genes being the focus of these studies [18–23]. However, the genetic redundancy and limitations of genetic approaches can often interfere with the phenotypic analysis of mutations in mammalian organisms. Thus a simpler and more experimentally amenable organism like *Drosophila* represents an attractive model system to study the evolutionarily conserved functions of sialylation. However, sialylation in *Drosophila* and other protostomes (including annelids, arthropods, and mollusks) has not been extensively studied, and in fact some of these species appear to be devoid of biochemical potential for sialylation [1]. Therefore, the possibility of using *Drosophila* for studying sialylation poses two important questions, both still awaiting a more comprehensive consideration. First, does *Drosophila* indeed possess an efficient biosynthetic machinery to produce sialylated structures, or is it an "incomplete tool kit" (to paraphrase Angata and Varki [1])? Second, is the function of sialylation in *Drosophila* evolutionarily related to the roles of sialic acids in higher animals? In this review we have attempted to shed some light on these questions by discussing the current knowledge of insect and, more specifically, *Drosophila* sialylation pathway in relation to what is known about sialylation in other organisms, especially in mammals.

Biosynthetic pathways of sialic acids

The vertebrate sialic acid metabolic pathway comprises several enzymatic steps (Fig. 1). The biosynthetic branch specific for Neu5Ac begins with the synthesis of ManNAc-6-P from UDP-GlcNAc by a bifunctional enzyme, **UDP-GlcNAc 2-epimerase/ManNAc kinase**, or GNE [24]. Point mutations in human GNE are associated with hereditary inclusion body myopathy [25], while the lack of this enzyme leads to embryonic lethality in the mouse [26], clearly demonstrating the essential role of sialic acids in higher animals. In bacteria, the corresponding step is mediated by a homologous enzyme (BLAST E-value 2e-20 for *E. coli* NeuC and rat GNE proteins [27]), but it produces ManNAc instead of ManNAc-6-P, and the absence of the kinase activity is in agreement with the lack of the C-terminal kinase domain in the bacterial enzyme [28, 29]. At the same time, the bacterial enzymatic activity (GlcNAc-6-P 2-epimerase) that catalyzes the conversion of GlcNAc-6-P to ManNAc-6-P is not linked to sialic acid biosynthesis [30]. Interestingly, the genome of *Drosophila melanogaster* does not have a gene encoding a UDP-GlcNAc epimerase homologue, and only very low level of UDP-GlcNAc epimerase activity was detected in lepidopteran Sf9 cultured cells [31]. In addition, these cells were found to have ManNAc kinase activity, which however could come from an unrelated cytosolic kinase able to phosphorylate ManNAc (*e.g.*, GlcNAc kinase [32]). This poses an intriguing question about the initiation of sialic acid biosynthetic pathway in insects, since, as discussed

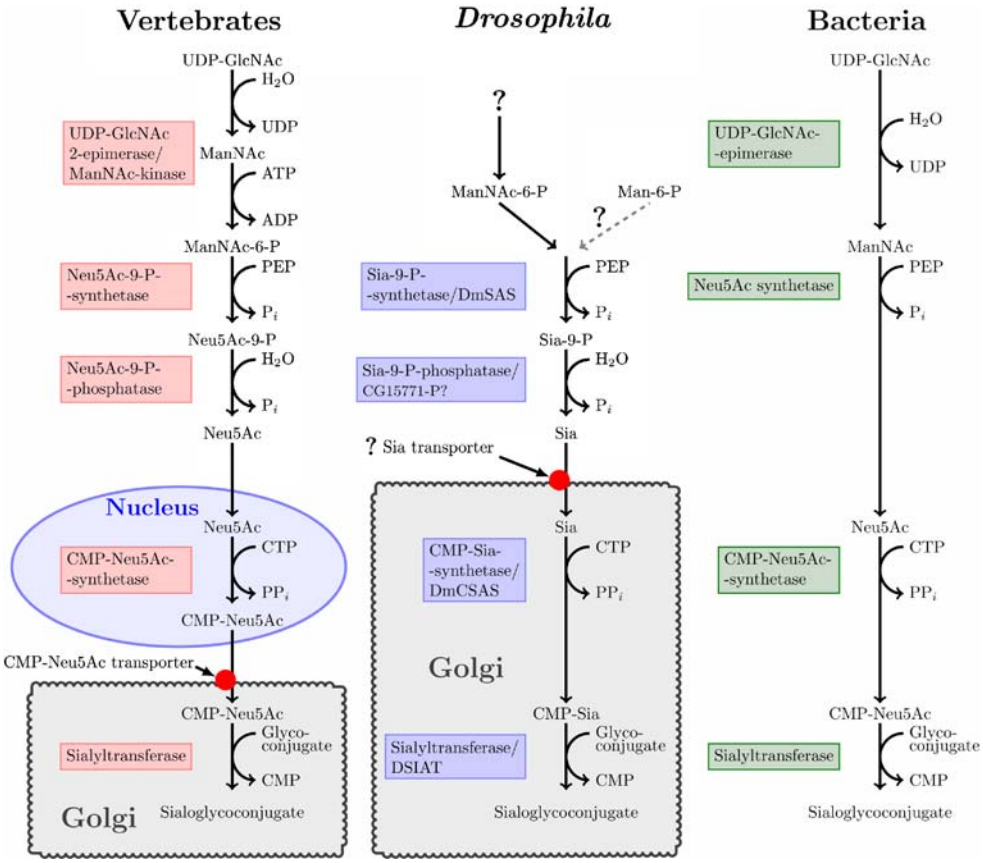

Fig. 1 Comparison between the *de novo* biosynthetic pathways of sialylation in vertebrates, *Drosophila*, and bacteria. *Question marks* indicate unknown/ hypothetical steps or enzymes. Thus, in *Drosophila*, given the apparent absence of UDP-GlcNAc 2-epimerase/ManNAc kinase gene from the genome, it is unclear how ManNAc-6-P is being synthesized. The *question mark* next to Man-6-P indicates the hypothetical possibility that in *Drosophila*, similar to lower vertebrates, the KDN pathway may be initiated at this step, thus bypassing the requirement for the epimerase/kinase enzyme. Therefore we use "Sia" as a general term for any of the possible sialic acids in this context, *e.g.* Neu5Ac or KDN. CG15771-P denotes the protein product of the *Drosophila melanogaster CG15771* gene. See text for discussion and Abbreviations section for the complete names of enzymes and other abbreviations

below, most other genes of the pathway are present and functional, which would suggest that insects are capable of *de novo* sialylation, but how they do it in the absence of UDP-GlcNAc epimerase remains to be revealed.

In the next step of the vertebrate biosynthetic pathway, ManNAc-6-phosphate is converted to Neu5Ac-9-phosphate through condensation with phosphoenolpyruvate (PEP) by **Neu5Ac-9-P synthetase** (Fig. 1). In addition to the Neu5Ac pathway, the biosynthesis of KDN (2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic or deaminated neuraminic acid) has been established as a prominent sialylation pathway in lower vertebrates and some pathogenic bacteria (reviewed in [33]). The *de novo* KDN biosynthesis is initiated by the condensation of Man-6-P with PEP to produce KDN-9-P, which is analogous to the condensation of ManNAc-6-P and PEP in the Neu5Ac pathway [34]. Since Man-6-P is presumably available within the cell from several biosynthetic sources [35], this initiation bypasses the step mediated by UDP-GlcNAc 2-epimerase/ManNAc-kinase in Neu5Ac pathway mentioned above, while the downstream part of KDN biosynthesis appears to follow the same steps as the biosynthesis of Neu5Ac in vertebrates (see below and [33]). The enzymes of the KDN biosynthetic pathway have not yet been well characterized, although some of them are possibly shared between the Neu5Ac and KDN pathways [33, 36–38]. Indeed, the human Neu5Ac-9-P synthetase can use Man-6-P instead of ManNAc-6-P as a substrate to produce KDN-9-phosphate [37]. The *E.coli* sialic acid synthetase, neuB [39], shows significant similarity to the human enzyme (35% identity, 56% similarity of amino acid sequences [37]), however instead of ManNAc-6-P, neuB uses ManNAc in the reaction with PEP to yield Neu5Ac. Many bacterial **Neu5Ac synthetases** have been cloned and characterized (*e.g.*, *E. coli K1* [40], *Streptococcus agalactiae* [41], *C. jejuni* [42], and *N. meningitidis* [43]). *Drosophila* has a single homologue of this enzyme, DmSAS, the activity of which was confirmed by *in vitro* and cell culture assays [44]. Interestingly, similar to the human but unlike the murine homologues [38, 45], DmSAS can also synthesize KDN-9-P using Man-6-P as the substrate [44]. The expression of *DmSAS* is present at every developmental stage examined [44], however the pattern of expression within the tissues has not been characterized in detail and as of now, no data on the phenotype of *DmSAS* mutants is available.

In the following biosynthetic step, Neu5Ac-9-P is dephosphorylated by ***N*-acetylneuraminate-9-phosphate phosphatase** [46] to yield Neu5Ac (Fig. 1). The enzyme has been localized to the cytosolic fraction in rat liver by subcellular fractionation and enzymatic assays, and presumably it can also use KDN-P as a substrate [47]. A *Drosophila* homologue is encoded by the CG15771 gene

on the X chromosome, but functional or phenotypic information is not yet available for this gene. Bacteria do not have this step in the pathway since Neu5Ac is synthesized directly, as opposed to its phosphorylated intermediate, in the upstream reaction.

The resulting Neu5Ac then becomes a substrate for **CMP-Neu5Ac synthetase** (CSAS) which produces the sugar donor molecule, CMP-Neu5Ac, necessary for the final enzymatic step of the pathway mediated by sialyltransferases. CMP-sialic acid synthetases were characterized and cloned from a number of bacterial and animal species ([40, 48, 49], reviewed in [50]). They all show significant amino acid sequence conservation within the five 'signature' domains of this enzyme family, with 41–47% overall protein sequence identity between the mammalian and bacterial enzymes (reviewed in [51]). Genetic and biochemical data from a number of vertebrate species indicate that CMP-Neu5Ac synthetases have a relatively broad substrate specificity and can also synthesize CMP-KDN, although their efficiency for KDN activation varies significantly between different species [33]. The enzyme localizes to the nucleus in vertebrate cells due to the presence of nuclear localization signal motifs, however the enzymatic activity and nuclear localization are separable features for the fish enzyme, and thus the functional importance of nuclear localization of vertebrate CMP-Neu5Ac synthetases is currently not known [52]. Nuclear localization of animal CMP-Neu5Ac synthetases originated relatively recently in evolution, early in the deuterostome lineage (in echinoderms), as the recently characterized *Drosophila* CMP-sialic acid synthetase, DmCSAS, unlike its vertebrate counterparts, localizes to the Golgi compartment [53]. This unusual localization of DmCSAS is likely due to the presence of an N-terminal hydrophobic domain that presumably functions as a signal peptide/anchoring domain. The Golgi localization of DmCSAS appears to be important for the functionality of this enzyme as a swap fusion of the *Drosophila* enzyme with the N-terminus (including nuclear localization signal) from the human counterpart was found to localize to the nucleus, while being enzymatically inactive [53].

In vertebrates, the CMP-Neu5Ac activated donor synthesized in the nucleus diffuses to the cytoplasm, and then it is pumped into the Golgi compartment by **CMP-Neu5Ac transporter** (CST) that belongs to the family of nucleotide sugar transporters (reviewed in [54]). The mammalian CMP-Neu5Ac transporter has 9 predicted membrane-spanning domains and localizes to the medial-trans Golgi [55, 56]. Two genes encoding close homologues of the mammalian CMP-Sia transporters are present in *Drosophila* genome, CG2675 and CG14040; their products have 40% identity/61% similarity and 25% identity/45% similarity to the human CST protein, respectively. However, predicting substrate specificities of nucleotide transporters from protein sequence alone can be misleading, since even transporters with ~50% identical amino acid sequence can prefer distinct substrates, while homologues from different species may have only 20% sequence identity and show the same substrate specificity [54, 57]. Thus, when assayed directly, the protein product of CG2675, the closest *Drosophila* homologue of mammalian CSTs, transported UDP-galactose but not CMP-sialic acid [58, 59]. The product of CG14040, the more distant *Drosophila* CST homologue, has never been functionally characterized; curiously, it shows a closer phylogenetic relationship to the recently characterized CST from *Arabidopsis* [60]. However, considering that *Drosophila* CSAS possibly works in the Golgi [53], perhaps the *Drosophila* sialylation pathway does not need a CMP-Neu5Ac transporter, since in this scenario the donor sugar would be synthesized directly within the Golgi compartment. However, the sialic acid would still need to be transported to the Golgi for the synthesis of the activated donor. Both bacteria and vertebrates have **sialic acid transporters**. In *E. coli*, it is the product of the *nanT* gene [61]; it has 14 membrane-spanning domains and shows little homology to any *Drosophila* protein. There are, however other, more general types of transporters in bacteria that are also involved in sialic acid uptake (for review see [2]), and homologues of these transporters were noted in *Drosophila* [53]. Human sialin, a well characterized lysosomal sialic acid transporter implicated in the lysosomal free sialic acid storage disorders [62], has multiple *Drosophila* homologues that have various developmentally regulated patterns of embryonic expression [63]. Thus, the possibility exists that some of these homologues function in the *Drosophila* sialylation pathway as transporters to deliver sialic acid to the Golgi.

A number of enzymes that are responsible for the generation of over 50 different sialic acid modifications (reviewed in [1]) have so far eluded molecular cloning in eukaryotic organisms, even though their biochemical properties have been assessed. The nature of some of these modifications can be subtle at the molecular level (*e.g.*, Neu5Gc *vs* Neu5Ac, or the presence of an extra *O*-acetyl group) yet they have far reaching biological consequences, affecting a wide variety of cellular processes, like apoptosis, erythrocyte survival, immune system function, cancer prognosis, susceptibility to influenza virus, *etc.* (reviewed in [1, 64]). In *Drosophila*, so far only Neu5Ac has been detected [65–67], while nothing is known about the existence of other varieties. Given their widespread presence in both prokaryotes and deuterostomes, it is likely that they will be also identified in protostomes in the future.

The final step in the biosynthetic pathway of sialic acids is the transfer of sialic acids from CMP-Sia to the acceptor glycoproteins or glycolipids by **sialyltransferases**. There

are currently 20 different sialyltransferases cloned from vertebrate organisms [4]. They are all type II transmembrane proteins localized in the Golgi. Their amino acid sequences are quite divergent at the N-terminal part including the short cytoplasmic tail, membrane-anchoring domain, and so-called 'stem region'. At the same time, their C-terminal part shows significant conservation and corresponds to a vaguely defined 'catalytic' domain. This domain contains highly conserved stretches of four "sialylmotifs", called *L*arge (L, or motif 1), *S*hort (S, or motif 2), motif III and *V*ery *S*hort (VS, or motif 4) [68–70]; these motifs are present in all known metazoan sialyltransferases. The functional significance of these motifs has been assessed through mutagenesis studies, and the L-sialylmotif is thought to be involved in the binding of CMP-Neu5Ac [71], whereas the S-sialylmotif binds both the donor and acceptor [72, 73]. The VS sialylmotif has been suggested to be part of the active site and shown to be required for catalytic activity [70, 74]. Motif III is thought to play an important conformational role and its integrity is also required for the enzymatic activity [70]. The molecular structure of animal sialyltransferases has not been solved, and so far detailed structural information is only available for some bacterial sialyltransferases [75, 76]. However, the bacterial enzymes show little sequence similarity to their animal counterparts, no sialylmotifs can be identified within their sequences (reviewed in [3]), and thus, they probably appeared independently during evolution. In addition, bacterial sialyltransferases have broader enzymatic specificities in comparison to the more strict enzymatic properties of most eukaryotic sialyltransferases.

In contrast to mammals, *Drosophila* has a sole gene encoding a vertebrate-type sialyltransferase, DSIAT. This enzyme has significant structural (38% and 41% amino acid sequence identity with human ST6Gal I and ST6Gal II, respectively, Fig. 2a) and functional (linkage and acceptor specificity, as assayed *in vitro*) similarity to ST6Gal family of vertebrate sialyltransferases [77]. This indicates that ST6Gal enzymes represent the most evolutionarily ancient family of animal sialyltransferases that gave rise to all other types of sialyltransferases in higher animals, with DSIAT closely relating to the ancestral 'prototype' of these enzymes [4, 77]. The recent phylogenetic analysis of fully sequenced genomes of 12 species from the *Drosophila* genus revealed that their evolutionary divergence exceeds the divergence of mammalian genomes, with a genetic distance between most diverged of the *Drosophila* genomes being comparable to that between lizards and humans [78]. We confirmed that a single orthologue of DSIAT is present in each of these 12 *Drosophila* species. Interestingly, despite the overall genetic divergence of the *Drosophila* genomes, phylogenetic distance between DSIAT proteins from a pair of most distant species, *Drosophila melanogaster* and *Drosophila mojavensis*, is significantly smaller than between corresponding pairs of orthologues from human and chicken (Fig. 2b). This remarkable conservation of DSIAT sequence in *Drosophila* evolution indicates the presence of a strong selection pressure to preserve the structure of DSIAT, which suggests an important biological function and close structure-function association for DSIAT protein. DSIAT orthologues are also significantly conserved in other insect species beyond the *Drosophila* genus (Fig. 3).

Sialidases, or neuraminidases, are responsible for the cleavage of sialic acids from glycoconjugates for catabolic purposes or for post-synthetic remodeling of glycan chains. Strictly speaking, they do not belong to the *de novo* biosynthetic pathway of sialic acids, but we mention them here because their functions can significantly contribute to the regulation of cellular sialylation. Mammalian sialidases fall into four general categories, based on their subcellular localization, enzymatic properties, and substrates; they have been implicated in the modulation of many important biological processes (reviewed in [5]). Sialidases are present in pathogenic bacteria and viruses where, besides catabolic functions, they are involved in pathogenesis (see reviews [2, 79]). Protein sequences of animal and bacterial sialidases show some similarity, including the presence of specific motifs (such as FRIP and Asp-box) [5, 80]. Somewhat surprisingly, similar to the GNE enzyme, sialidase homologues have not yet been identified in insects, including *Drosophila*.

Thus, recent studies have provided compelling evidence that *Drosophila* has the genetic potential for *de novo* sialylation. Most *Drosophila* homologues of key enzymes in sialylation pathway have been indentified and functionally confirmed *in vitro* and in cell culture assays. The components of the pathway characterized so far show close relationship to the sialylation pathway in vertebrates, suggesting their common evolutionary origin.

Occurrence of sialylation in *Drosophila*

Historically, the occurrence of sialic acids in protostomes, including insects, has been somewhat controversial. While some early studies confirmed the presence of sialylation (including, endogeneous sialyltransferase activity, or biochemical potential to provide sugar donor for sialylation [67, 81–84]), other reports failed to detect sialylated glycans, CMP-Neu5Ac donor, or sialyltransferase activity in insect cells [85–91]. This controversy has now been partially resolved, as several recent studies have provided crucial evidence that endogenous sialylation does occur in *Drosophila*. For example, advanced mass spectrometry approaches have unambiguously revealed the presence of

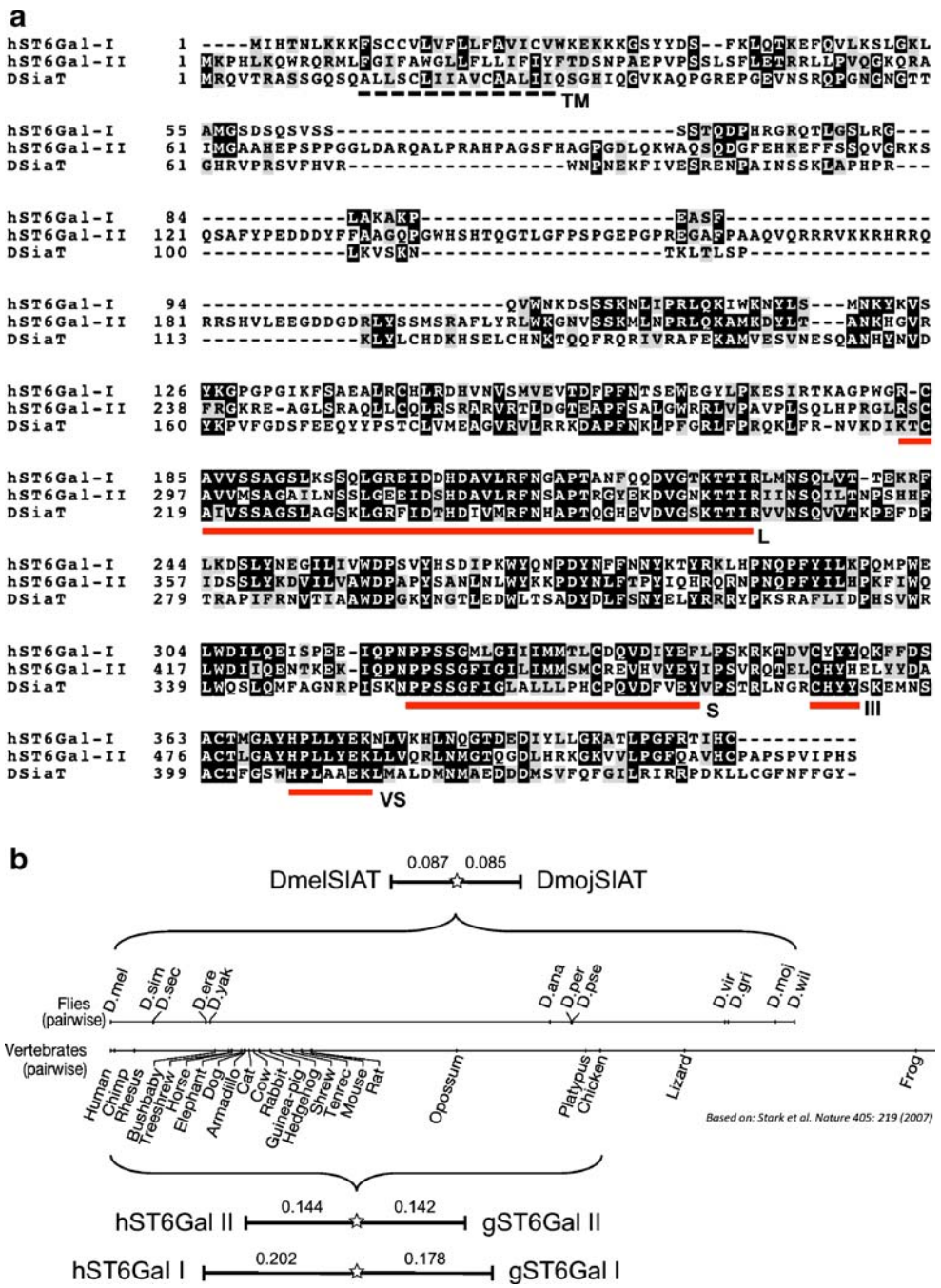

Fig. 2 **a** Multiple sequence alignment of protein sequences of human ST6Gal-I, ST6Gal-II, and *Drosophila melanogaster* DSIAT sialyltransferases (accession numbers X17247, XM038616, and AF397532, respectively). *Dashed underline* indicates transmembrane/anchoring domain of DSIAT, *solid underline* indicates L, S, III, and VS sialylmotifs. Alignment was performed by ClustalW2 program at http://www.ebi.ac.uk/Tools/clustalw2/index.html [101]. **b** Phylogenetic tree distance between sialyltransferase proteins from *Drosophila melanogaster* (DSIAT, referred here as DmelSIAT) and *Drosophila mojavensis* (DmojSIAT), as compared with that between human and chicken ST6Gal I and ST6Gal II sialyltransferases, respectively. The *middle part* of the figure illustrates the comparison of evolutionary distances spanned by fly and vertebrate trees based on comparison of their genome assemblies (modified from [78], not in scale with distances shown for sialyltransferase proteins). Phylogenetic distances from a common ancestor (indicated by *star*) are shown in substitutions per site (estimated by the neighbor-joining algorithm using ClustalW2 program). Abbreviations of *Drosophila* species: D.mel, *Drosophila melanogaster*; D.sim, *Drosophila simulans*; D.sec, *Drosophila sechellia*; D.ere, *Drosophila erecta*; D.yak, *Drosophila yakuba*; D.ana, *Drosophila ananassae*; D.per, *Drosophila persimilis*; D.pse, *Drosophila pseudoobscura*; D.vir, *Drosophila virilis*; D.gri, *Drosophila grimshawi*; D.moj, *Drosophila mojavensis*; D.wil, *Drosophila willistoni*

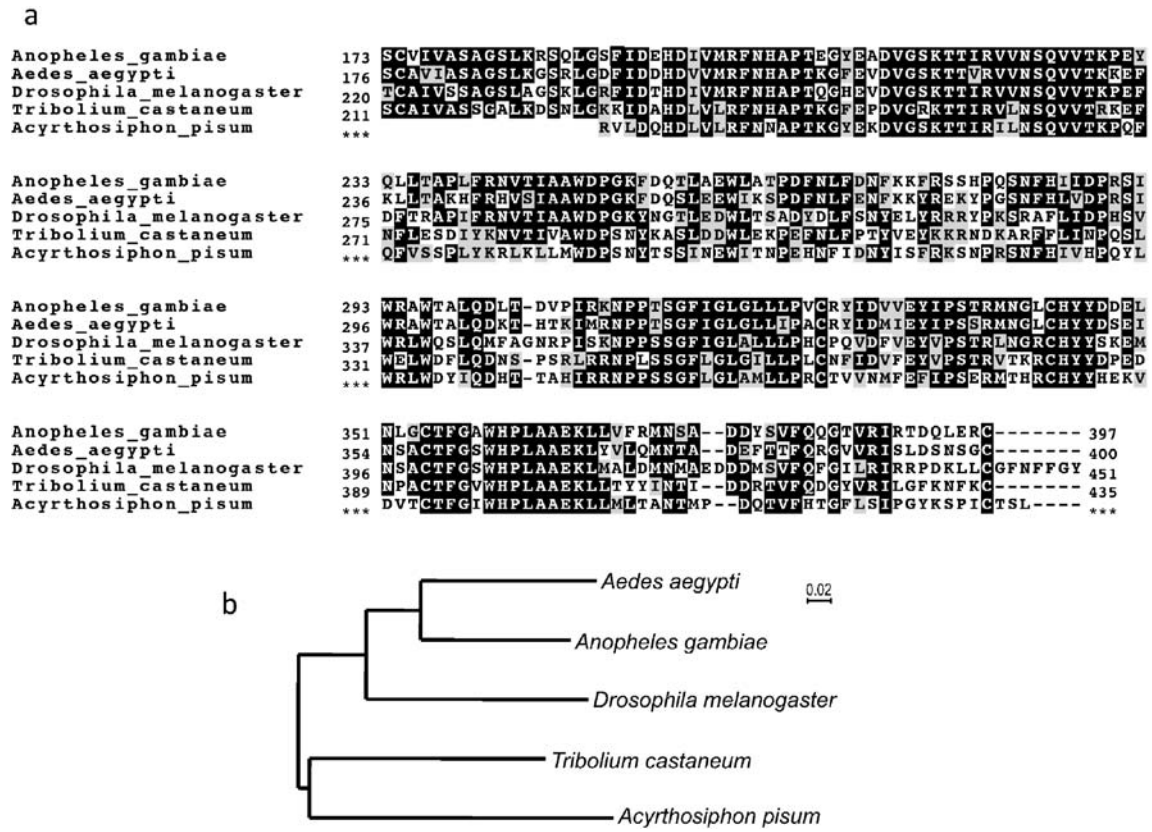

Fig. 3 **a** Multiple sequence alignment of protein sequences of the C-terminal domains of DSIAT orthologues from several insect species: African malaria mosquito (*Anopheles gambiae*, XP_308850), yellow fever mosquito (*Aedes aegypti*, XM_001649540), red flour beetle (*Tribolium castaneum*, XP_968750), and pea aphid (*Acyrthosiphon pisum*, reconstructed using SoftBerry prediction (www.softberry.com) and sequences from Pea Aphid Genome Project, http://www.hgsc.bcm.tmc.edu/projects/aphid/). *Triple asterisks* The complete sequence is not known because of a gap in genomic sequences. **b** Phylogenetic tree of insect orthologues of DSIAT. The *scale bar* shows relative units of evolutionary distances. Alignment and phylogenetic analysis was performed by ClustalW2 program at http://www.ebi.ac.uk/Tools/clustalw2/index.html

sialic acids in *Drosophila* at embryonic stages and in the adult heads [65, 66], although the detected amount of sialylation was significantly lower than reported previously [67]. In contrast to the previously reported presence of α2–8 linked polysialic acid in cicada *Philaenus spumarius* and *Drosophila* [67, 83], our experiments failed to detect this structure in *Drosophila* at any developmental stage (K. Koles and V. Panin, unpublished data). The only sialylated structure so far reliably detected in *Drosophila* is Neu5Ac residues in α2–6 linkage on LacNAc termini of N-linked glycans [65, 66], which is in agreement with the *in vitro* characterized acceptor specificity of DSIAT [77]. It is interesting to note that the acceptor preferred by DSIAT in *in vitro* assays, *i.e.* LacdiNAc, has not yet been identified on *Drosophila* glycoproteins [65, 66, 92], but it is abundantly present on *Drosophila* glycolipids [93, 94]. However, sialylation has not been detected on *Drosophila* glycolipids [94, 95], which is consistent with the absence of *in vitro* activity of DSIAT towards embryonic glycolipid acceptors [77].

Developmentally regulated pattern of expression of *DSiaT* and *DmCSAS*

The apparent inconsistency of observations about the presence of sialylation in insect cells has previously led to the hypothesis that sialylation in *Drosophila* is a specialized process restricted to certain types of cells, possibly in a developmentally regulated manner [1, 96]. The low level of sialylation detected in recent studies is also consistent with this hypothesis. Furthermore, it is supported by the data on the expression pattern of *DSiaT* and *DmCSAS* during development. The expression of both genes is not detectable early in embryogenesis, while they both become upregulated at later embryonic stages [53, 77]. This late embryonic expression correlates with the development of the embryonic CNS, and *DSiaT* was previously shown to be specifically expressed there [77]. To explore whether *DmCSAS* is expressed in the CNS, we assayed its pattern of embryonic expression by *in situ* hybridization and found that *DmCSAS* is also expressed within the developing CNS

in a pattern similar to that of *DSiaT* (Fig. 4, compare with 5a and [77]). It has to be noted though that the spatial pattern of *DmCSAS* expression within the CNS appears to be somewhat different from that of *DSiaT*, and a more precise mapping of their expression awaits further experimentation. Later in development, although detected in a broader domain of cells, the expression of *DSiaT* is still restricted to the CNS (Fig. 5b). In the adult head, *DSiaT* is expressed in a spatially restricted pattern in the optic lobes of the brain (Fig. 5c). Accordingly, the expression of *DmCSAS*, when assayed by RT-PCR, was also found to be elevated in the head of adult flies [53].

The CNS-specific expression of *DSiaT* and *DmCSAS* during development suggests that the function of sialylation in *Drosophila* is mainly limited to the nervous system. This conclusion is consistent with locomotion abnormalities and defects in brain development of *Mgat1* mutant flies [97]. These mutants are devoid of the activity of *Drosophila* homologue of GlcNAcT-I involved in the biosynthesis of hybrid and complex type N-glycans, and thus $Mgat1^{-/-}$ flies presumably also lack any sialylated N-linked structures.

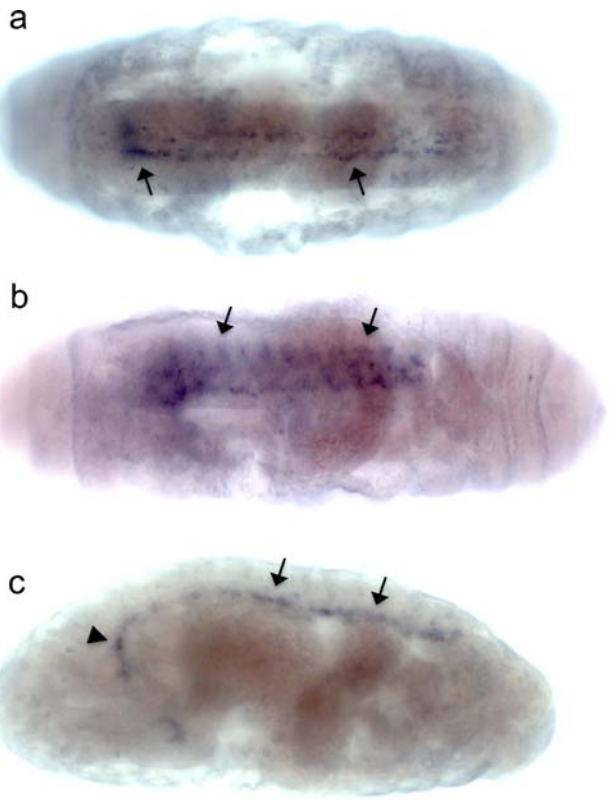

Fig. 4 *Drosophila CMP-Sia synthetase* embryonic expression pattern as revealed by *in situ* hybridization. RNA probe was synthesized using *DmCSAS* cDNA (RH11815) obtained from Drosophila Genomics Resource Center at Indiana University, Bloomington, IN. *Arrows* point to the expression within the ventral ganglion of the CNS; *arrowhead* indicates the expression in the brain hemispheres. **a** Embryonic stage 16; **b** embryonic stage 17; **c** embryonic stage 17. All images, anterior is to the left. **a**, **b** Ventral views; **c**, lateral view. Hybridization was performed as previously described [77]

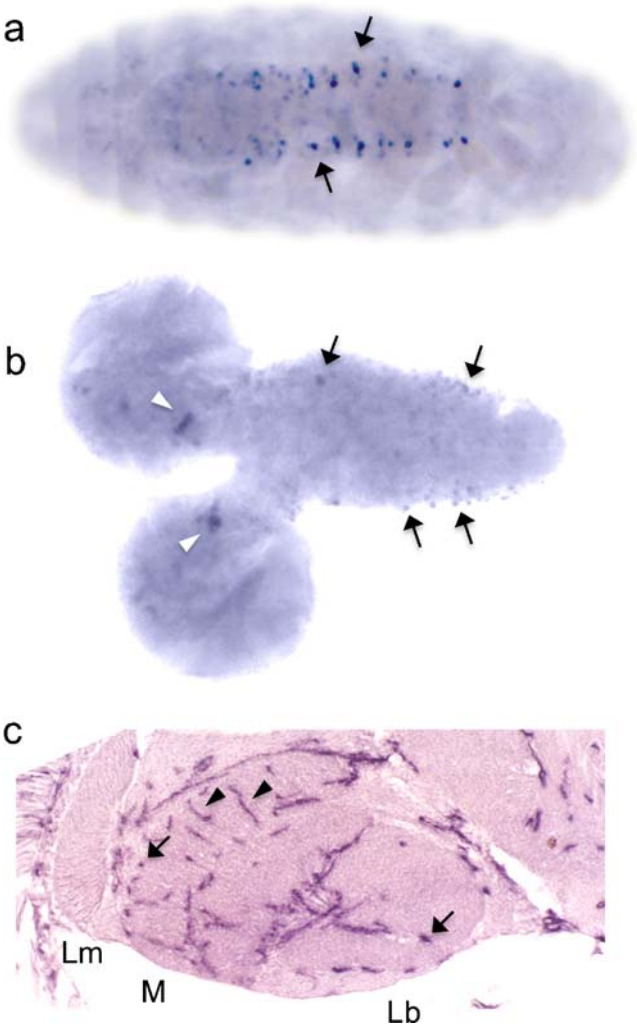

Fig. 5 *DSiaT* expression pattern at different developmental stages as revealed by *in situ* hybridization. **a** Embryonic stage 17; **b** third instar larval brain; **c** horizontal cryotome section of the adult head. The *DSiaT* transcript is detected in cell bodies (*arrows*), as well as in cell processes, presumably axons or dendrites (*black arrowheads*). *White arrowheads* in **c** indicate expression in the larval brain hemispheres. Regions of the optic lobe are labeled: *Lm* lamina (no expression); *M* medulla; *Lb* lobula. **a**, **b** Anterior is to the left. **c** Anterior is to the top. Hybridization was performed as previously described [77]

Humans, like other vertebrates, have two homologues of DSIAT, hST6Gal-I and hST6Gal-II, with hST6Gal-II being structurally more similar to DSIAT (Fig. 2). Interestingly, hSTGal-I is ubiquitously expressed in the organism, whereas hST6Gal-II has a more restricted pattern of expression with elevated levels in the fetal and adult brain [98, 99]. In addition, both DSIAT and ST6Gal-II (but not ST6Gal-I) exhibit a peculiar substrate specificity revealed by *in vitro* assays, *i.e.* they both efficiently sialylate GalNAcβ1–4GlcNAc (LacdiNAc) termini [77, 100]. These structural and functional similarities between DSIAT and

ST6Gal-II may reflect their common biological function, suggesting the existence of a conserved and ancient function for sialylation in a wide range of metazoan organisms, from *Drosophila* to humans.

Concluding remarks

Although studies on *Drosophila* sialylation provide strong evidence for the presence of a sialylation pathway that functions in a developmentally-regulated and tissue-specific manner, several important questions await further detailed investigation. First, given the experimentally confirmed *in vivo* presence of Neu5Ac, how is the *de novo* pathway of sialylation initiated in insects, *i.e.* what is the source of ManNAc or ManNAc-6-P? Three possibilities have been previously discussed as the potential sources of ManNAc/ManNAc-6-P, including a dietary source, symbiotic microorganisms (such as *Wolbachia*), and an as yet unknown GlcNAc epimerase present in insect cells [1]. All three possibilities are still viable and certainly require further thorough investigation. However, considering the high degree of overall conservation between *Drosophila* and vertebrate sialylation pathways (Fig. 1), each of these three scenarios would represent a somewhat exotic biological solution, from both biochemical and evolutionary standpoints.

On the other hand, in the light of available genome information from prokaryotes to humans, and the peculiar absence of UDP-GlcNAc 2-epimerase/ManNAc kinase and sialidase homologues in *Drosophila* genomes, an alternative scenario stands out, and that is the possibility of KDN as the predominant form of sialylation in insects. This attractive hypothesis has not been given adequate attention, however it appears consistent with most experimental observations, and it would provide a logical explanation for several unresolved problems. Indeed, the biosynthesis of KDN would not require UDP-GlcNAc 2-epimerase/ManNAc kinase, as Man-6-P, a presumably plentiful cell metabolite, would feed the pathway directly at the level of sialic acid synthetase (DmSAS; Fig. 1). This possibility is also consistent with the substrate specificities of DmSAS [44] and vertebrate homologues of Sia-P phosphatase, DmCSAS, and DSIAT [33, 46], as well as with the readiness of insect cultured cells to synthesize KDN in the absence of ManNAc supplementation [37, 44]. From an evolutionary perspective, this hypothesis correlates well with the more predominant presence of KDN in lower vertebrate species [33]. Furthermore, it might explain the absence of sialidase-like genes in the *Drosophila* genome, since KDNases, KDN-removing enzymes that still elude genetic identification, may have little structural similarity to sialidases [33]. However, KDN has so far not been detected in protostomes, and mass spectrometry analysis of N- and O-linked glycans failed to detect the presence of KDN in *Drosophila* (Michael Tiemeyer, personal communication). In addition, the possibility of KDN biosynthesis would still leave the presence of experimentally detected Neu5Ac-modified glycans in *Drosophila* unexplained, since to our knowledge, no precedent for the biosynthetic conversion of KDN into Neu5Ac has so far been reported. Thus, the KDN hypothesis calls for further investigation.

Another important question is related to the developmental regulation of sialylation pathway genes in *Drosophila*: do they have an overlapping expression pattern? The genes involved in the sialylation pathway are likely to be regulated in a coordinated manner. Although the expression of *DSiaT* and *DmCSAS* is similarly present within the developing embryonic CNS, this expression and the expression of other genes (*DmSAS*, putative *Sia-P phosphatase* and *Sia transporter* genes) has not been analyzed in detail at cellular resolution. Next, what are the mutant phenotypes of these genes involved in sialylation, do mutations in their loci show genetic interactions, and can they phenocopy each other? Finally, what are the *in vivo* target(s) of sialylation in *Drosophila*? Answers to these important questions could shed light on the evolutionarily ancient biological function of sialylation that is possibly conserved between *Drosophila* and higher animals, including humans; and from this perspective, *Drosophila* certainly represents a powerful and complete tool kit to address these and other related questions.

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