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Review

Ceramide synthases in mammalians, worms, and insects: emerging schemes

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Abstract

The ceramide synthase (CerS) gene family comprises a group of highly conserved transmembrane proteins, which are found in all studied eukaryotes. The key feature of the CerS proteins is their role in ceramide synthase activity. Therefore, their original name 'longevity assurance gene (*Lass*) homologs', after the founding member, the yeast longevity assurance gene *lag1*, was altered to '*CerS*'. All CerS have high sequence similarity in a domain called LAG1 motif and a subset of CerS proteins is predicted to contain a Homeobox (Hox) domain. These domains could be the key to the multiple roles CerS have. CerS proteins play a role in diverse biological processes such as proliferation, differentiation, apoptosis, stress response, cancer, and neurodegeneration. In this review, we focus on CerS structure and biological function with emphasis of biological functions in the widely used model systems *Caenorhabditis elegans* and *Drosophila melanogaster*. Also, we focus on the accumulating data suggesting a role for CerS in lipid homeostasis.

Keywords: apoptosis; *Caenorhabditis elegans*; cancer; ceramide synthase; *Drosophila*; homeodomain; LAG1 motif; lipid homeostasis; longevity.

Introduction: ceramide *de novo* synthesis

All ceramide synthases (CerS) identified so far mediate the acylation of sphinganine to dihydroceramide, which is subsequently reduced to ceramide, the key intermediate of sphingolipid metabolism (1). Ceramide is the precursor of major sphingolipids such as sphingomyelin, glycosphingolipids, and ceramide-1-phosphate (2). There are two pathways where ceramide synthases are needed: a salvage pathway, where existing ceramide species are degraded to their components, e.g., sphingosine, which is then used by CerS to produce ceramide. The second pathway is the *de novo synthesis* (Figure 1). Here, ceramide is generated in the endoplasmic reticulum (ER) by condensing activated palmitate (palmitoyl-CoA) with L-serine through the activity of serine palmitoyl transferase (SPT); (3). The formed intermediate 3-ketosphinganine is then turned to sphinganine by the 3-ketosphinganine reductase (4). CerS now catalyze the CoA-dependent dihydroceramide synthesis. In this step, CoA activated fatty acids are used for aminoacylation of sphinganine carbon-2 (Figure 1). The generated dihydroceramide is further converted to ceramide by dihydroceramide desaturase. Initially, the molecular identity of enzymes and biochemical pathways of the *de novo* biosynthetic pathway have been identified and characterized, especially in mammalian cells and the budding yeast *Saccharomyces cerevisiae* (5–7). Thus, it became evident that these lipids and enzymes were conserved across species and phyla.

Identification of ceramide synthases

Although CerS activity was detected previously in 1970 (8), the genes encoding the CerS enzymes were first identified in yeast when *lag1* and *lac1* were shown to be required for ceramide synthesis in yeast (9, 10). *lag1* was originally isolated in a screen for genes that are preferentially expressed in yeast cells that underwent only a low number of cell divisions (11). It was named in accordance with the fact that when deleted it prolonged the replicative lifespan of yeast cells – longevity assurance gene (*lag1*). GenBank database searches then identified another yeast LAG1 paralog LAC1. Mutants for both *lag1* and *lac1* are either inviable or show poor growth (12). The first mammalian and plant (tomato) CerS proteins were originally described as upstream of growth and differentiation factor 1 (UOG1) and *Alternaria* stem canker resistance gene 1 (*Asc-1*), respectively (13, 14). Meanwhile, GenBank data searches in many organisms revealed that CerS homologs are present in all eukaryotes studied, e.g., worms (12), *Drosophila* (15), mice and humans (16–18). All CerS family members show a common transmembrane profile of four to seven predicted transmembrane domains (18), which is similar to that of translocating chain-associating membrane protein (TRAM1) and ceroid-lipofuscinosis, neuronal 8 (CLN8) proteins. This transmembrane profile, a region of 200 amino acids, is called TRAM Lag CLN8 (TLC) domain (17). A smaller, particularly high conserved stretch of 52 amino acids that was termed LAG1 motif (12) is only found among CerS homologs. It was later shown to be essential for ceramide synthesis (19, 20). In

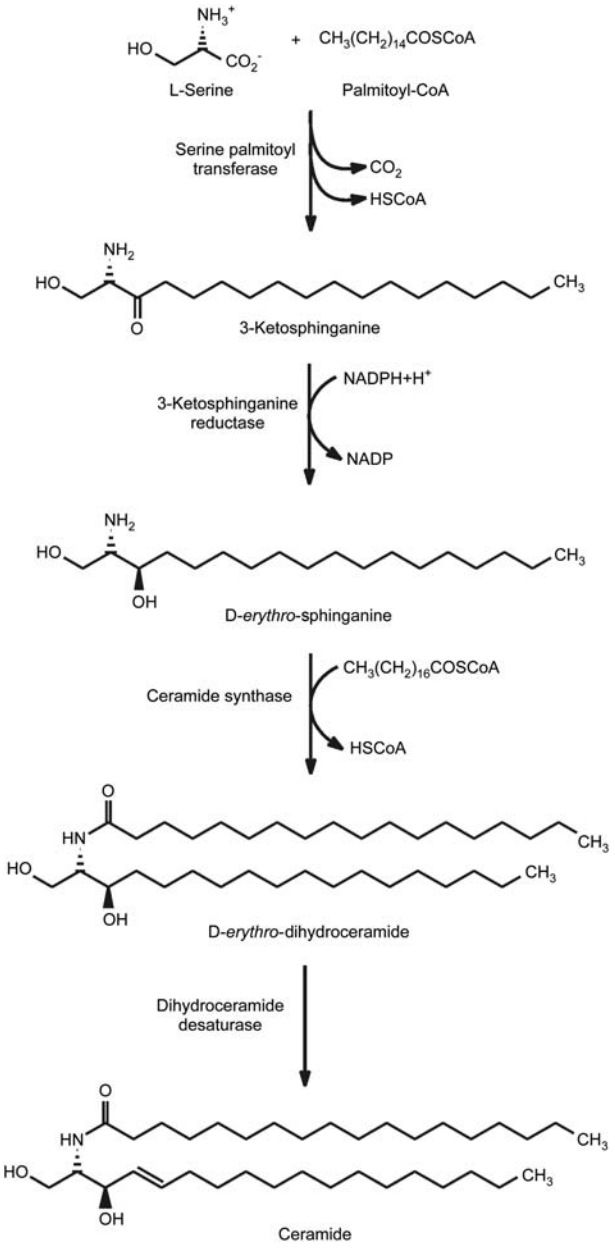


Figure 1 Ceramide *de novo* synthesis.
CerS catalyze the CoA-dependent *de novo* ceramide synthesis. CoA activated fatty acids with varying chain lengths are used for aminoacylation of sphinganine carbon-2 to yield dihydroceramide, which is reduced to ceramide by dihydroceramide desaturase.

addition, a subset of CerS genes contains a putative homeodomain, a DNA binding motif (18, 21).

Mammalian ceramide synthase gene family members

The first mammalian CerS for which ceramide synthase activity could be shown was UOG-1, the mammalian homolog of the yeast LAG1, which is also known as Lass1/CerS1. Human CerS1 could rescue *S. cerevisiae lag1/lac1* double mutants (12). For both proteins, LAG1p and LAC1p, a ceramide synthase activity was shown later (9, 10). Thus, mammalian CerS1 homologs were candidates for being ceramide synthases. And indeed, it could be shown further on that, when overexpressed in mammalian cells, mouse CerS1 led to an increase in C18-ceramides. This was the starting point for the identification of five additional ceramide synthases in mammalian species. All six CerS contain the TLC domain, which also harbors the highly conserved LAG1 motif (19) Each CerS has a preference for a specific subset of acyl-CoAs used for ceramide synthesis (22, 23). For instance, CerS1 exclusively uses C18-fatty acyl-CoA (24), CerS5 and CerS6 selectively add C16-fatty acyl-CoA, and CerS2, 3, and 4 have selectivity towards longer chain fatty acyl-CoAs, C22, C24 (25, 26). Thus, each CerS might have a specific functional role. This is also indicated by the fact that the expression patterns and mRNA expression levels of various CerS differ (26). There is also increasing evidence that CerS show intracellular differences in their distribution (27–30).

These differences in acyl-CoAs usage and specific expression patterns might be of importance in cancer development. For example, decreased C18-ceramide and increased C16-ceramide in head and neck squamous cell carcinoma (HNSCC) tumor tissues were associated with decreased and increased expression of CerS1 and 6, respectively (31, 32). The ratio of C16- and C18-ceramide could be related to the development of HNSCC tumors (33). Similarly, recent data suggest a function for CerS2 in breast cancer development (34, 35). Therefore, it might be possible to use CerS2 for diagnostic purposes in breast cancer. However, it is still unclear whether or not alterations in ceramide levels are the primary cause or a secondary effect of tumor formation. Interestingly, CerS2 expression also has an inhibitory effect on cell growth of hepatocellular carcinoma (HCC) cells (36). This is in good agreement with data from CerS2 *genetrap* mice, which are devoid of CerS2 transcript. These mice show noninvasive hepatocellular carcinoma (37, 38). In this context, it is noteworthy that ceramides with different fatty acid chain lengths, by themselves, have a function in the regulation of tumor growth and might be used for therapy (23, 39). A role for CerS1 in the regulation of cisplatin sensitivity has been demonstrated previously, indicating antiproliferative functions of CerS1-generated C18-ceramide in various cancers (40).

Very recently, it was shown that *de novo*-generated C18- and C16-ceramides by CerS1 and CerS6 can play opposing pro-apoptotic and prosurvival roles, respectively, in HNSCCs (29). But all in all, ceramides are usually thought to have a pro-apoptotic role. In adenocarcinoma cells (HCT116), cell viability strongly decreased in a time- and concentration-dependent manner after C16-ceramide treatment (41). RNAi against CerS6 in SW480 cells (isolated from primary colon carcinoma) resulted in a specific and significant decrease of the C16-ceramide species, which was sufficient to inhibit TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-induced apoptosis (29). Correspondingly, in SW620 TRAIL resistant cells elevation of CerS6 expression reversed

TRAIL resistance (29). However, contrary to the observed pro-apoptotic function, CerS6/C16-ceramide has also been shown to protect cells from ER stress and apoptosis in human head and neck cancer models via selective regulation of the ATF6/CHOP arm of ER-stress-response pathways (42).

A novel strategy for treatment of cancer could thus be the manipulation of CerS proteins and ceramide depending on the ceramide status of tumors (42, 43).

In addition to CerS6, a function in response to stress, which stimulates cells to generate ceramide, a second messenger in apoptotic signaling pathways, was also shown for other CerS (42, 44). Even more evidence for ceramide as a 'coordinator' of stress response is added by studies demonstrating even distinct roles for CerS2, 5, and 6 upon radiation induced apoptosis in HeLa cells (42). In HeLa cells, application of ionizing radiation induced *de novo* ceramide synthesis by CerS2, 5, and 6 in the mitochondrial-associated membrane (30). This resulted in opposing anti- and pro-apoptotic defects. One notion how specific roles of different ceramides in various cell types might be reconciled is discussed in the recent publication by Mesicek et al. (42) The fact that CerS2, 5, and 6 might exist as heterocomplexes in HeLa cells prompted them to speculate that the combined activation of these three CerS isoforms changes the result, depending on a balance between pro- and antiapoptotic ceramide species (42).

Until recently, most of the data, which show different roles of CerS, could only be collected *in vitro*. Animal models, which are now available such as CerS2 *transcriptional null* mice (37, 38, 45), will allow confirmation and evaluation of CerS functions during cancer development and apoptosis. Also, the roles of CerS in the nervous system and neurodegeneration can be analyzed [reviewed in (46)].

Invertebrate ceramide synthases

Ceramide synthases in *Caenorhabditis elegans*

Caenorhabditis elegans has long been a model in which genes specifying aging and longevity could be identified (47, 48). Thus, first analysis of a *C. elegans* homolog of yeast *lag1*, which was named *hyl-1*, aimed to address the question whether longevity effects might be found (49). Jiang and colleagues had already cloned the human yeast *lag1* homolog with the goal to examine its possible function in human aging and they also cloned two homologs from the nematode *C. elegans*, *hyl-1* and *hyl-2* (12). The cloned homologs from both human and *C. elegans* were able to functionally complement the yeast gene *lag1* (12, 50). Later, it became known that the yeast LAG1p is involved in ceramide biosynthesis (9, 10). Ceramide signaling has been shown to affect growth, differentiation, stress resistance, apoptosis, cell senescence, and insulin action, any of which could have an effect on lifespan (51–54). However, the observed effects on lifespan extension upon manipulation of the *hyl-1* gene were only moderate and in the case of a *hyl-1* null mutant were lost upon backcrossing into another genetic background (49). Interestingly, RNAi treatment with the LAG1p motif affecting both *hyl-1* and *hyl-2* transcripts did give a longevity response. Nonetheless, RNAi treatment for *hyl-2* or *lagr-1* alone did not increase longevity. Therefore, it was concluded that the level of gene expression of *hyl-1* and *hyl-2* that leads to increased mean lifespan is rather sensitive and apparently needs to be fine-tuned to achieve a long-life phenotype (49).

Almost at the same time in a screen for mutants resistant to radiation-induced germ cell apoptosis, it was found that *hyl-1* and *lagr-1* mutants, which are two of the three CerS proteins in *C. elegans*, showed apoptosis suppression (55). In contrast, a deletion mutant of the third *C. elegans* LAG1 homolog, *hyl-2*, displayed no defect in germ cell death (55). These results are also interesting in the above-mentioned context that *de novo* ceramide synthesis by CerS2, 5, and 6 in the mitochondrial-associated membrane resulted in opposing anti- and pro-apoptotic effects (44).

This supports the concept that, although functions of some CerS homologs such as HYL-1 and LAGR-1 are necessary for induction of radiation induced apoptosis, in the germ line of *C. elegans*, another CerS homolog, HYL-2, might not be necessary. Instead, HYL-2 CerS protein protects *C. elegans* from anoxia. HYL-1 could to some extent but not completely substitute for HYL-2 and thereby increase anoxia resistance (50). Analysis of *C. elegans* ceramides by ESI-MS and dihydroceramide synthase assay determined that HYL-1 and HYL-2 are involved in the generation of different ceramide species: C24–C26 and C20–C22. It was speculated that it is the chemical structure of ceramide species that is important for anoxia resistance (50). Evidence for an additional feature of a *C. elegans* CerS family member came again from a systematic screen of the *C. elegans* genome. In an RNAi screen for genes essential for fat storage, out of 16 757 downregulated genes, 305 were identified to be relevant in this context (56). Among these candidates, one ceramide synthase, LAGR-1, could be identified to be necessary for fat storage (56).

Ceramide synthases in *Drosophila*

Until recently, *Drosophila* Yeast LAG1 homologs have only been known from phylogenetic tree analyses (17, 18). Yet, in a screen for genes controlling larval growth in *Drosophila*, mutants have been identified affecting a gene, which was originally named *Drosophila longevity assurance gene 1 homolog* (*Dlag1*) due to its sequence homology to yeast LAG1 (11). Because the mutants displayed defects in larval growth and fat metabolism, this gene was renamed *schlank* ('slim' in German).

Analysis of ceramide synthase activity in *schlank* mutants, in RNAi *knockdown* animals, in tissue culture cells, and overexpressing experiments demonstrated that it is involved in the synthesis of a rather broad spectrum of ceramides (15). Interestingly, *schlank* mutants also showed a reduction of storage fat (15, 57), which is deposited as triacylglycerols (TAGs) in the *Drosophila* fat body. schlank can positively regulate fatty acid synthesis by promoting the expression of sterol responsive element binding protein (SREBP) and its target genes. The overexpression of schlank led to an increase of TAG (15, 57). Tissue culture studies in *Drosoph-*

ila S2 cells had already suggested a link between the sphingolipid pathway and SREBP (58). Furthermore, overexpression of schlank prevented lipolysis by downregulating TAG lipase expression. These results identified schlank as a new regulator of the balance between lipogenesis and lipolysis in *Drosophila*. Although the exact mechanisms by which lipid homeostasis is affected by schlank is not yet resolved, overexpression of a schlank variant, which contains a point mutation in the LAG1 motif shown to inhibit ceramide synthase function still caused an increase in TAG (16). These data indicate a mode of action, where schlank acts on TAG metabolism independent of its ceramide synthase function.

Homologs of yeast LAG1 have been identified in all eukaryotes examined until now and multiple homologs are the norm. However, in *Drosophila* only one homolog that conforms to the CerS consensus sequence could be identified. The only other *Drosophila* gene showing some sequence homology to CerS family members (59), the *CG11642* gene, is more homologous to members of the translocating chain-associated membrane (TRAM) protein family, which are not involved in ceramide synthesis (12, 17, 19). So far, there is also no experimental evidence for function of the *CG11642* gene as ceramide synthase in *Drosophila* (15). This could also explain the fact that a rather broad spectrum of ceramides is reduced in schlank mutants. In contrast, sphingolipid measurements after overexpression and *in vitro* assays suggest a preference of mammalian CerS for the generation of ceramides with specific fatty acid chain length (22, 23, 25, 26).

CerS gene family in body fat and lipid homeostasis regulation

During the process of *de novo* ceramide synthesis fatty acyl-CoAs are required twice: first, at the initiation step where serine-palmitoyltransferase catalyzes the condensation of serine and a long-chain fatty acyl-CoA leading to 3-ketosphinganine; and second, during transfer of CoA-activated fatty acids to (dihydro)sphingosine by CerS to form (dihydro)-ceramide (Figure 1). Therefore, CerS and the generated ceramides can be intimately linked to lipid homeostasis. Chronic dysregulation of the balance between lipolysis and lipogenesis can lead to metabolic abnormalities such as obesity, lipodystrophy syndromes or insulin resistance in humans (60, 61).

Lipidomic analysis of diet-induced obese mice revealed specific and dramatic increases in individual ceramide species. Administration of a specific inhibitor (myriocin) of SPT, the enzyme catalyzing the rate-limiting step of *de novo* ceramide synthesis (Figure 1), to obese mice, reduced circulating ceramide and improved multiple aspects of the metabolic syndrome. In addition, adipose expression of suppressor of cytokine signaling-3 (SOCS-3) and induced adipose uncoupling protein-3 (UCP3) was reduced (62). In contrast, administration of ceramide directly induced SOCS-3 and inhibited UCP3 mRNA in cultured adipocytes. This indicates a direct role for ceramide in the regulation of metabolism and energy expenditure (63). Obesity and type 2 diabetes are associated with insulin and leptin resistance and increased ceramide content in target tissues (64). Summers and colleagues have previously demonstrated that ceramide antagonizes insulin stimulation of glucose uptake and glycogen synthesis (65). They showed that inhibition of ceramide synthesis markedly improves glucose tolerance and prevents the onset of frank diabetes in obese rodents (66).

Administration of central leptin decreased total ceramide levels in white adipose tissue. This is consistent with the reduced expression of genes involved in *de novo* synthesis and/or recycling of ceramide, such as SPT, CerS2, and CerS4 (67). In addition, the activation of the lipogenic transcription factor, SREBP-1c, was also changed. It had been demonstrated that modification of ceramide synthesis can also contribute to SREBP regulation (68). Leptin-induced reduction of the expression of ceramide synthases CerS2 and CerS4 resulted in a downregulation of SREBP-1c mRNA and reduced lipogenesis in lean tissue (67, 69). In white adipose tissue proteolytic maturation of SREBP-1c was reduced (67). Similarly, in mutants for the *Drosophila* ceramide synthase, schlank, a reduced SREBP expression was observed. Moreover, schlank can positively regulate fatty acid synthesis by promoting the expression of SREBP and SREBP target genes. It further prevents lipolysis by downregulating TAG lipase expression (15). These data suggest that at least part of the effect of CerS proteins or its products might be mediated via the lipogenic transcription factor SREBP. How this occurs is not yet clear.

A role of CerS proteins in lipid homeostasis is most probably conserved. The *C. elegans* CerS family member *lagr-1* was identified in a genome-wide RNAi analysis of fat regulatory genes (52). In *Drosophila*, overexpression of murine CerS2 not only lead to increased TAG levels comparable to the increase seen when overexpressing schlank but also a rescue of the schlank phenotype was observed when expressed in schlank mutants (15). Interestingly, in CerS2 null mice the most prominent abnormalities observed were reduced body size and fat deposits (45). Reduced body fat and size was also observed in mutants for the *Drosophila* CerS schlank (15). Furthermore, as observed in schlank mutants, in CerS2 null mice, genes associated with lipid and fatty acid metabolism were downregulated (37).

How can all the different roles of CerS in different organisms be explained? Part of the answer is probably to know and understand the different CerS domains and their functions.

Ceramide synthase domains

CerS genes can be subdivided into different groups with CerS1 and CerS2–6 in different categories (24) consistent with the fact that CerS1 is much closer to the yeast protein variants (26). In all mammalian CerS proteins, with the exception of CerS1, three motifs seem to be conserved (in CerS1 only the first motif is conserved):

1. The LAG1 motif lying within the TLC domain is the defining motif for all CerS and can be found from CerS1

to CerS6 in all species that were studied so far or for which a prediction based on the sequencing of the genome was possible.
2. CerS2–6 homologs contain a Homeodomain/Hox domain-like motif that retains sequence similarity to the canonical Antennapedia/Bicoid (Antp/Bcd)-derived consensus motif. The homeodomain is conserved between the homologs of different species, but differs between CerS2 to CerS5 in a given species.
3. At the end of the homeodomain and the beginning of the TLC domain, there is a structural motif ([K/R]-P-x-x-x-x-[K/R]-F) that seems to be conserved in all Hox domain-containing CerS (70), except for *Gallus gallus* CerS4 (here, the first amino acid is exchanged for histidine; however, histidine can also be positively charged via its imidazole group). Mutations and deletions of the lysine or arginine residues in this motif were shown to inhibit the activity of CerS in terms of (dihydro)ceramide production. It remains to be seen whether these two positively charged amino acid side chain positions are really part of the catalytic domain (70) necessary for the acylation. Alternatively, mutations at those positions could just alter the overall protein topology and would therefore indirectly affect the secondary/tertiary structure of the LAG1 motif.

The LAG1 motif

The key structural element of CerS is the 52 amino acid catalytic LAG1 motif. It is common to all CerS and was described by Spassieva et al. in 2006 (19).

The motif (**R***K***D**xxxxxx**HH**xx*T*xx**L**xxx**S***Y*xxxxxxx**G**xx-xxx*LH***D**x*S***D**xx*L*xxx**K**xxx**Y**; conserved amino acids are depicted in bold font; conserved exchanges are depicted in italic font) shows a strong sequence conservation and can be found in nearly all CerS homologs, ranging from yeasts to humans (Figure 2B). Detailed mutational analysis of the LAG1 motif in mouse CerS1 and human CerS5 (19) showed that some of the amino acids are essential for catalytic activity. The first conserved histidine and the last conserved leucine are essential in both CerS1 and CerS5, whereas the second histidine and first leucine and both aspartates were essential for CerS1 activity. However, in *Bos taurus* CerS3 (ENSBTAP00000009181) the double histidine sequence is not conserved, but the last histidine is exchanged for glutamine (Figure 2B). One might speculate that the second histidine position might not be necessary for the catalytic activity, at least in this CerS3 variant. Also, the LAG1 motif of the *Xenopus* protein CerS 5 (ENSXETP00000029263) is one amino acid short.

Moreover, via BLAST, only one CerS homolog can be identified in *Apis mellifera*: XP_001123059.1. It contains a homeodomain, but the first half of the LAG1 motif is truncated and/or not conserved (Figure 2B, insect CerS). Additionally, there are some amino acid exchanges as well as varied spacing between conserved amino acids in the second part of the putative LAG1 motif (e.g., second aspartate of LAG1 motif). It will be rather interesting to see whether or not this protein can act as a ceramide synthase and which metabolic differences in the honeybee might lead to this strong alteration in the CerS catalytic domain. Also, there might be other CerS-like proteins and/or cofactors in this species that do not conform to the LAG1 motif and might therefore not be found via homology searches. For instance, the proteins encoded by the yeast genes, *lag1* and *lac1*, act in a complex with Lip1 (72), an integral ER membrane protein.

When comparing LAG1 motifs, there are conserved amino acids and stretches that clearly distinguish CerS1 proteins from other CerS classes (Figure 2B, highlighted in green). Also, a stretch exists ([Q/I/V/M]-MF-[V/I/L/A]-HH) that seems to be conserved in CerS5, CerS6, and insect CerS. CerS5 variants seem to prefer IMFVHH, except for the *Mus musculus* variant. Here, we find a conserved exchange of the first isoleucine for methionine (Figure 2B, highlighted in red). The CerS6 class shows an IMFLHH preference, but in *Bos taurus* isoleucine is exchanged for valine, and *Danio rerio* also deviates from this showing a LMFVHH motif, reminiscent of the CerS5 class. In insects, there is a WQMF-[V/I/A]-HH motif, except for WQ also similar to CerS5, especially with the MFLHH variants. Apart from this motif, there is a more or less conserved cysteine in CerS5 and CerS6 except for the CerS6 variant in *Xenopus tropicalis*. For the CerS2–4 class, it is rather difficult to find class specific differences. Either the class is phylogenetically not well-defined or the amino acid sequence strongly varies within one class. By contrast, there are many amino acids conserved between the classes (Figure 2A,B). As mentioned above, CerS proteins show preferences for a subset of acyl CoAs chosen for ceramide synthesis. For instance, CerS1, CerS5, and CerS6 group together and are specific for long-chain ceramide production. Otherwise, CerS2, CerS3, and CerS4 are specific for very long chain ceramide production. Therefore, it might be worthwhile to look closer whether these preferences are reflected and correlate with the amino acid sequence level. The LAG1 motif might be a worthwhile target.

Homeodomains in ceramide synthases: functional domains?

Homeodomains, stretches of approximately 60 amino acids in most cases, are thought to be involved in sequence specific DNA binding (21). All vertebrate CerS homologs, apart from CerS1 and all insect CerS homologs identified so far, contain a homeodomain. In our analysis, insect CerS are phylogenetically grouped due to their similarity (Figure 3A). *Caenorhabditis elegans* expresses three CerS homologs (HYL-1, HYL-2, and LAGR-1), but none contains a homeodomain. For this reason, they are excluded from the phylogenetic CerS homeodomain analysis (Figure 3A).

The homeodomains of vertebrate CerS2–6 and insect CerS are conserved to the homeodomain consensus (Figure 3B, *Drosophila* schlank and mouse CerS2–6 are shown as exam-

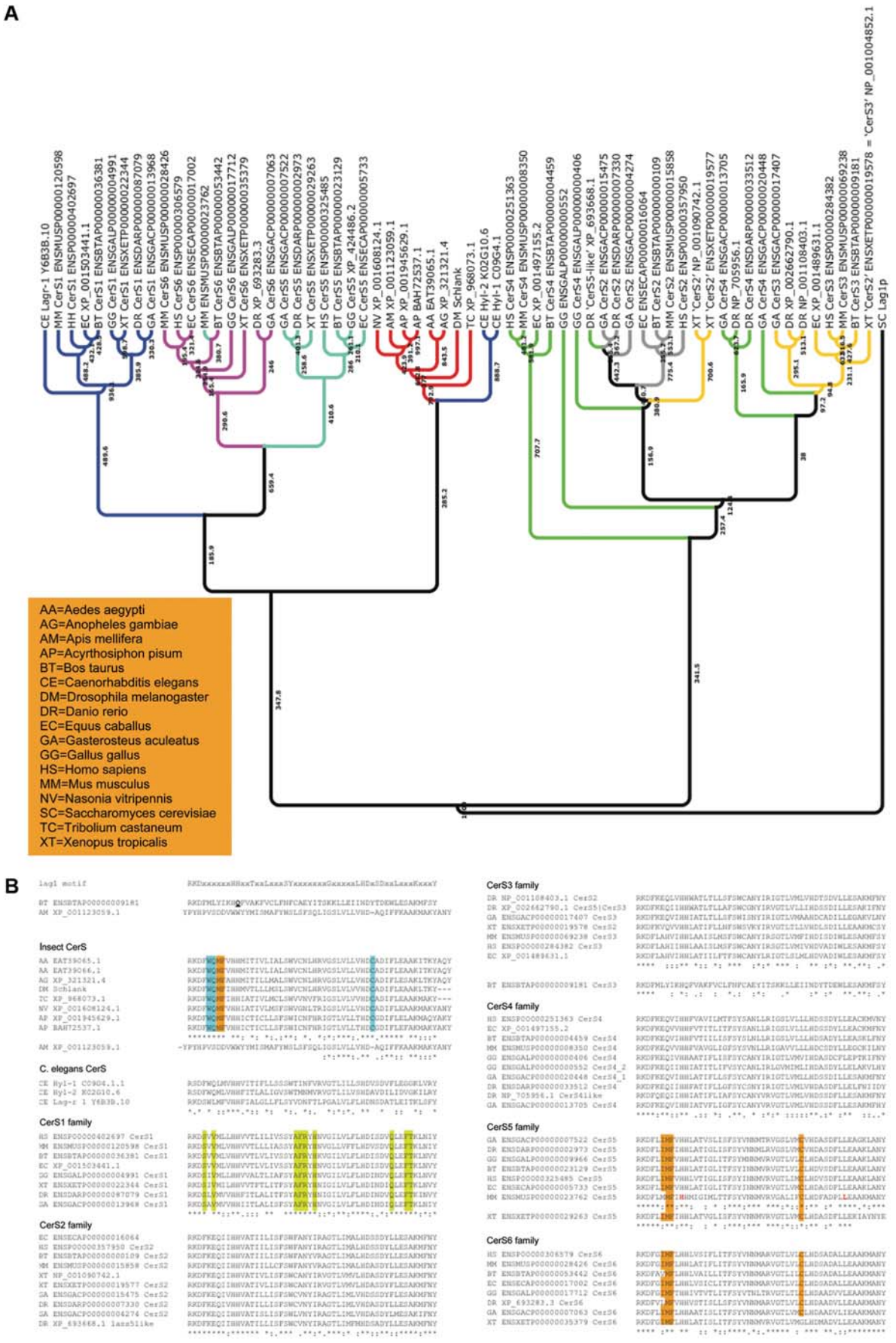

A
CE Lagr-1 Y6B3B.10
MM CerS1 ENSMUSP00000120598
HH CerS1 ENSP00000402697
EC XP_001503441.1
BT CerS1 ENSBTAP00000036381
GG CerS1 ENSGALP00000004991
XT CerS1 ENSXETP00000022344
DR CerS1 ENSDARP00000087079
GA CerS1 ENSGACP00000013968
MM CerS6 ENSMUSP00000028426
HS CerS6 ENSP00000306579
EC CerS6 ENSECAP00000017002
MM ENSMUSP00000023762
BT CerS6 ENSBTAP00000053442
GG CerS6 ENSGALP00000017712
XT CerS6 ENSXETP00000035379
DR XP_693283.3
GA CerS6 ENSGACP00000007063
GA CerS5 ENSGACP00000007522
DR CerS5 ENSDARP00000002973
XT CerS5 ENSXETP00000029263
HS CerS5 ENSP00000325485
BT CerS5 ENSBTAP00000023129
GG CerS5 XP_424486.2
EC CerS5 ENSECAP00000005733
NV XP_001608124.1
AM XP_001123059.1
AP XP_001945629.1
AP BAH72537.1
AA EAT39065.1
AG XP_321321.4
DM Schlank
TC XP_968073.1
CE Hyl-2 K02G10.6
CE Hyl-1 C09G4.1
HS CerS4 ENSP00000251363
MM CerS4 ENSMUSP00000008350
EC XP_001497155.2
BT CerS4 ENSBTAP00000004459
GG ENSGALP00000000552
GG CerS4 ENSGALP00000000406
DR 'CerS5-like' XP_693668.1
GA CerS2 ENSGACP00000015475
DR CerS2 ENSDARP00000007330
GA CerS2 ENSGACP00000004274
EC ENSECAP00000016064
BT CerS2 ENSBTAP00000000109
MM CerS2 ENSMUSP00000015858
HS CerS2 ENSP00000357950
XT 'CerS2' NP_001090742.1
XT 'CerS2' ENSXETP00000019577
GA CerS4 ENSGACP00000013705
DR NP_705956.1
DR CerS4 ENSDARP00000033512
GA CerS3 ENSGACP00000020448
GA CerS3 ENSGACP00000017407
DR XP_002662790.1
DR NP_001108403.1
EC XP_001489631.1
HS CerS3 ENSP00000284382
MM CerS3 ENSMUSP00000069238
BT CerS3 ENSBTAP00000009181
XT 'CerS2' ENSXETP00000019578 = 'CerS3' NP_001004852.1
SC Lag1p
AA=Aedes aegypti
AG=Anopheles gambiae
AM=Apis mellifera
AP=Acyrthosiphon pisum
BT=Bos taurus
CE=Caenorhabditis elegans
DM=Drosophila melanogaster
DR=Danio rerio
EC=Equus caballus
GA=Gasterosteus aculeatus
GG=Gallus gallus
HS=Homo sapiens
MM=Mus musculus
NV=Nasonia vitripennis
SC=Saccharomyces cerevisiae
TC=Tribolium castaneum
XT=Xenopus tropicalis
B
Lag1 motif
Insect CerS
C. elegans CerS
CerS1 family
CerS2 family
CerS3 family
CerS4 family
CerS5 family
CerS6 family

Figure 2 Phylogenetic analysis and alignments of LAG1 motifs.
(A) LAG1 motifs of various species were aligned using ClustalW (European Molecular Biology Laboratory – European Bioinformatics Institute Wellcome Trust Genome Campus, Hinxton, Cambridgeshire, UK), alignments were bootstrapped for 1000 datasets each and analyzed for parsimony using Protpars (Joseph Felsenstein, University of Washington, Seattle, WA, USA) from the PHYLIP package (71) with two random shuffles each. A consensus tree was built using Consense (Joseph Felsenstein, University of Washington, Seattle, WA, USA). *Saccharomyces cerevisiae* LAG1 was used as outgroup. Phylogenetic origin of the CerS2–4 families is poorly resolved. This is also reflected on the sequence level, when aligning the LAG1 motifs. (B) Alignments of LAG1 motifs within the CerS families and their conservation according to ClustalW are shown. *Bos taurus* and *Apis mellifera* variants that differ from the LAG1 motif consensus are shown in the first panel, compared to the consensus. Insect CerS are grouped, because they are most similar to one another and *C. elegans* CerS are also grouped, because they deviate in detail from other CerS families. Conserved amino acids that are specific for one or more CerS families are highlighted. Specific amino acids in CerS1 are highlighted in green, amino acids conserved in CerS5, 6, and a subset in insect CerS are shown in brown. Insect specific amino acids are shown in blue.

ples) to different degrees, but still contain the main features. CerS5 and CerS6 branch off early from the CerS2–4 subgroups. Among species, CerS6 orthologs and CerS5 orthologs are the most conserved ones. CerS2–4 subgroups cannot be told apart as clearly as the CerS5/6 subgroups when analyzing sequence variations phylogenetically (Figure 3A).

Although the homeodomains of vertebrate CerS2–6 and insect CerS are conserved to the homeodomain consensus (see exemplary alignments Figure 3B, also found as conserved domains via the NCBI database), doubt about the function of CerS homeodomains as sequence specific transcription factors continues to exist. The reason is two critical differences between CerS homeodomains (Hox) and other homeodomain containing proteins (70). First, the residue asparagin-51 was argued to be invariant in other homeodomains, but not in CerS homeodomains (except for one CerS – namely *Xenopus tropicalis* CerS4). Second, residue 58 would be a lysine or arginine in nearly all other homeodomains, which is replaced by a conserved proline in CerS. Interestingly, Holland and colleagues (75) demonstrate that human CerS homeodomains are closely related to ZEB and ZHX family homeodomains. In both families, neither asparagin-51 nor lysine-58 are conserved and yet human ZEB1 and ZEB2 were shown to be sequence specific transcriptional regulators (77, 78). Likewise, lysine-58 is exchanged in human PROX1, 2 and *Drosophila melanogaster* pros to isoleucine, and these transcription factors still retain their DNA-binding capabilities (78–81). The same is true for lysine-58 in *Drosophila* ct and the onecut, six and dbx family, where it is exchanged for glycine, aspartate, glutamate, asparagine, or alanine, respectively. In human TGIF1, 2, 2LX, 2LY, *Drosophila* achi and *Drosophila* vis homeodomains, lysine-58 is exchanged for proline as it is in the CerS family (75).

An additional point that questions CerS homeodomains as genuine transcription factors is the assumption that the first 15 amino acids of a canonical homeodomain would be missing in CerS homeodomains (70). In fact, rather than missing, the first 15 amino acids of CerS homeodomains are apparently not as much conserved to the Antp/Prd class homeobox consensus sequences. They are more similar to other homeodomains such as human ZEB1, ZHX3, and LHX4 (Figure 3C).

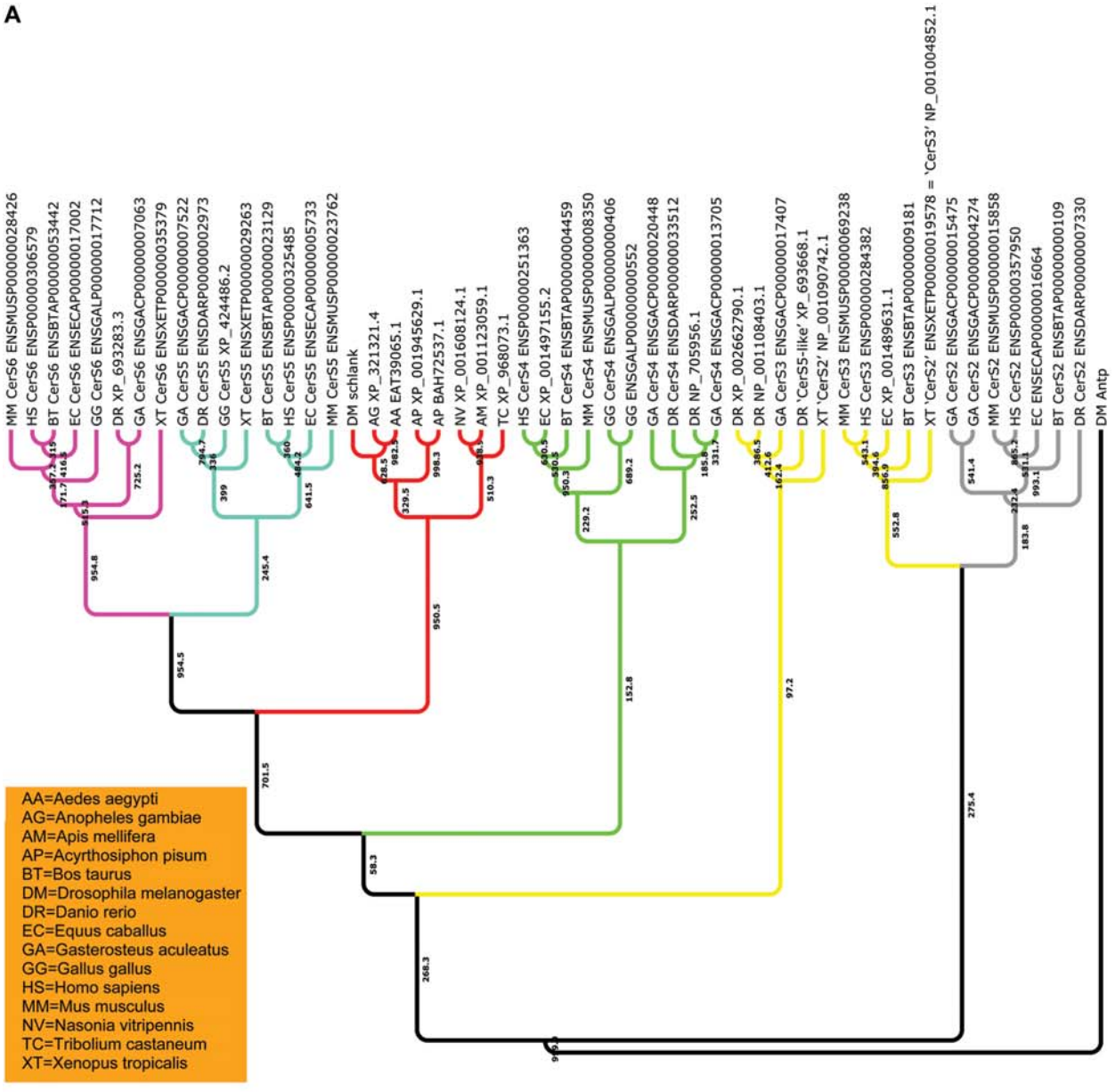
Another critical amino acid missing in CerS homeodomains is glutamine-50 (usually WFQ motif). Glutamine-50 has also been associated with DNA binding capabilities and is replaced by arginine in CerS homeodomains. However, *homo sapiens* (*Hs*) ZFHX3-I, ZFHX4-I, and *Drosophila* zfh2-I homeodomains show the same WFR stretch as mammalian CerS homeodomains. Additionally, homeodomains of *Hs* ADNP (HFS), *Hs* ADNP2 (FFG), the onecut family (FFM), Irx family (WFA), and *Hs* HDX (WIG) also diverge from this motif. Insect CerS at positions 49–50 show a conserved amino acid exchange. Most contain a WLR motif, whereas the *Drosophila* schlank homeodomain shows a WWR motif. Still, homeodomains that differ from the WFQ motif can nonetheless bind DNA (82–84).

All in all, on the sequence level, there are important differences to the homeodomain consensus, but none that would exclude a function as a homeodomain, because similar exchanges are found in other *de facto* DNA binding homeodomains. Essential for the binding properties of homeodomains is their tertiary structure. The major DNA interacting structure is the third helix, which is in contact with the major groove of DNA (85). Additionally, some amino acid side chains N-terminal of the first helix and/or C-terminal of the third helix contact the sugar-phosphate backbone or bases of DNA (85). NMR structure analysis of mouse CerS5 and Cers6 homeodomains by the RIKEN project [(86, 87); PDB file 2cqx for CerS5, 1x2m for CerS6, unpublished yet, but models released in RCSB Protein Data Bank)] revealed that those homeodomains, when expressed recombinantly, also had the typical 3-helical structure. For example, CerS6 NMR structure 1×2 m is, position-wise, very similar to Hop, Pdx1, and Ubx/Exd DNA binding conformations when compared via Vast Neighbor Summary (88). In summary, these data argue against a quick dismissal of DNA binding capabilities of the CerS homeodomains. Differences in the sequence of homeodomains among CerS2–6 of a given species might indicate different DNA binding specificities if this feature is indeed retained.

One peculiar issue, however, is that homeodomains with amino acid exchanges similar to those found in CerS homeodomains (TGIF family, ZEB family, and ZHX) are corepressors. Data for transcriptional misregulation in the fly CerS homolog schlank (15) also suggest a potential role as a repressor for lipase transcription, although the mechanism of action could not yet be shown.

Protein topology

Software-based analysis of membrane-spanning regions and their orientation usually predict multiple transmembrane



B

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consensus (Holland 2007)      KRRKRTTFTKEQLLELEKEFAKNPYPSREEREELAASLGLTERQVKVWFQNRRAKWKKQE
DM schlank                    GIRSSRPKKAANVPILEKTYAKSTRLDKKKLVPLSKQTDMSEREIERWWRLRRAQDKPST
                                *.  . .  ::  *** :**.. .:::   *: . .::**::: *:: ***: * .

consensus (Holland 2007)      KRRKRTTFTKEQLLELEKEFAKN-PYPSREEREELAASLGLTERQVKVWFQNRRAKWKKQE
MM CerS2 ENSMUSP00000015858  NVKEKTRLRAPPNATLEHFYQTSGKQPKQVEVDLLSRQSGLSGRQVERWFRRRRNQDRPSL
                              : :::* :       **: : ..   *.: * : *: . **: ***: **:.** : :.

consensus (Holland 2007)      KRRKRTTFTKEQLLELEKEFAKNP-YPSREEREELAASLGLTERQVKVWFQNRRAKWKKQE
MM CerS3 ENSMUSP00000069238  LGIKKTQHKIKPNAILENFFKHSTSKPSHTDIYGLAKKCNLTERQVERWLRIRQKQNKPCR
                                *:* .. :    **: * :..  **: :    ** . .******: *:: *: : *  .

consensus (Holland 2007)      KRRKRTTFTKEQLLELEKEFAKNPYPSREEREELAASLGLTERQVKVWFQNRRAKWKKQE
MM CerS4 ENSMUSP00000008350  VQDPIRRKIKPNPVLEKYFLRMKQCPEETQMVLLASQCGLTLRQTQRWFRRRRNQDRPSL
                               :       * : :  :  :  :  *.. :   **:. *** **.: **:.** : : .

consensus (Holland 2007)      KRRKRTTFTKEQLLELEKEFAKN-PYPSREEREELAASLGLTERQVKVWFQNRRAKWKKQE
MM CerS5 ENSMUSP00000023762  -IKDSPVNKVEPNDTLEKVFVSVTKYPDEKRLKGLSKQLDWSVRKIQCWFRHRRNQDKPPT
                               :. .. . *    *** *..   **..:. : *: .*. : *::: **::** : *

consensus (Holland 2007)      KRRKRTTFTKEQLLELEKEFAKNPYPSREEREELAASLGLTERQVKVWFQNRRAKWKKQE
MM CerS6 ENSMUSP00000028426  IQANGPQTAQPNAILEKVFTAITKHPDEKRLEGLSKQLDWDVRSIQRWFRQRRNQEKPST
                               : : .  :: : : :   * . :*..:. * *: .*.   *.:: **::** : * .
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consensus (Bürglin 1994)      RRRKRTAYTRYQLLELEKEFHFNRYLTRRRRIELAHSLNLTERQVKIWFQNRRHKWKKEN
DM schlank                    GIRSSRPKKAANVPILEKTYAKSTRLDKKKLVPLSKQTDMSEREIERWWRLRRAQDKPST
                                *.  . .  ::  *** :  .  * ::: : *::. :::**::: *:: ** : * ..

consensus (Bürglin 1994)      RRRKRTAYTRYQLLELEKEFHFNRYLTRRRRIELAHSLN-LTERQVKIWFQNRRHKWKKEN
MM CerS2 ENSMUSP00000015858  NVKEKTRLRAPPNATLEHFYQTSGKQPKQVEVDLLSRQSGLSGRQVERWFRRRRNQDRPSL
                              . :::*          **: :: .  .:: .::*    . *: ***: **:.**:: : .

consensus (Bürglin 1994)      RRRKRTAYTRYQLLELEKEFHFNR-YLTRRRRIELAHSLNLTERQVKIWFQNRRHKWKKEN
MM CerS3 ENSMUSP00000069238  LGIKKTQHKIKPNAILENFFKHSTSKPSHTDIYGLAKKCNLTERQVERWLRIRQKQNKPCR
                                *:* :.       **: *:..    ::      **:. *******: *:: *::: *  .

consensus (Bürglin 1994)      RRRKRTAYTRYQLLELEKEFHFNRYLTRRRRIELAHSLNLTERQVKIWFQNRRHKWKKEN
MM CerS4 ENSMUSP00000008350  VQDPIRRKIKPNPVLEKYFLRMKQCPEETQMVLLASQCGLTLRQTQRWFRRRRNQDRPSL
                               :       : : :  :  :::::   . : : ** . .** **.: **:.**:: : .

consensus (Bürglin 1994)      RRRKRTAYTRYQLLELEKEFHFNRYLTRRRRIELAHSLNLTERQVKIWFQNRRHKWKKEN
MM CerS5 ENSMUSP00000023762  IKDSPVNKVEPNDTLEKVFVSVTKYPDEKRLKGLSKQLDWSVRKIQCWFRHRRNQDKPPT
                               : . .  .. :    :  . ..:*  .:*   *::.*: : *::: **::**:: *  .

consensus (Bürglin 1994)      RRRKRTAYTRYQLLELEKEFHFNRYLTRRRRIELAHSLNLTERQVKIWFQNRRHKWKKEN
MM CerS6 ENSMUSP00000028426  IQANGPQTAQPNAILEKVFTAITKHPDEKRLEGLSKQLDWDVRSIQRWFRQRRNQEKPST
                               : : .  :: : :  :    :.:: .:*   *::.*:   *.:: **::**:: * ..
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C

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DM zfh1                       KVNVRTAINEEQQQQLKQHYSLNAR-PSRDEFRMIAARLQLDPRVVQVWFQNNRSRERKMQ
HS CerS2 ENSP00000357950      NIKEKTRLRAPPNATLEHFYLTSGKQPKQVEVELLSRQSGLSGRQVERWFRRRRNQDRPSL
                              ::: :* :.   :  *:::.*  ..: *.: *..::: :  *. * *: **:..*.::*

HS ZHX3-III                   -TPTKYKERAPEQLRALESSFAQNPLPLDEELDRLRSETKMTRREIDSWFSERRKKVNAEE
HS CerS2 ENSP00000357950      NIKEKTRLRAPPNATLEHFYLTSGKQPKQVEVELLSRQSGLSGRQVERWFRRRRNQDRPSL
                                 * : *** :   .  ::..  * : *:: *  :: :: *::: ** .**:: ...

HS LHX4                       AKRPRTTITAKQLETLKNAYKNSPKPARHVREQLSSETGLDMRVVQVWFQNRRAKEKRLK
DM Schlank                    GIRSSRPKKAANVPILEKTYAKSTRLDKKKLVPLSKQTDMSEREIERWWRLRRAQDKPST
                              . *.  . .* ::   *:::* :*.:  ::    **.:*.:. * :: *:: ***::*  .

HS ZEB1                       GNLGPSQPPLKNLLGLLKAYYALNAQPSAEELSKIADSVNLPLDVVKKWFEKMQAGQISVQ
DM Schlank                    G-IRSSRPKKAANVPILEKTYAKSTRLDKKKLVPLSKQTDMSEREIERWWRLRRAQDKPSTL
                              * : .*:*     :.:*:  ** .:: . ::*  ::...::.   :::*:.  :* : .
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Figure 3 Phylogenetic analysis of various CerS homeodomains.
(A) The tree shows a bootstrapped parsimony Consense (Joseph Felsenstein, University of Washington, Seattle, USA) tree. Numbers near the branches indicate number of branch occurrences in the parsimony trees of the bootstrapped datasets. Protein sequences were either derived from the ENSEMBL database or blasted for similarity via the NCBI blast tool. ENSEMBL IDs or accession numbers are given, where annotated, CerS classification, where annotated in ENSEMBL or NCBI, is also noted. Homeodomain sequences of various ceramide synthase homeodomains were aligned using ClustalW (European Molecular Biology Laboratory – European Bioinformatics Institute Wellcome Trust Genome Campus, Hinxton, Cambridgeshire, UK). Alignments were bootstrapped using SEQBOOT from the PHYLIP 3.69 package (71) creating 1000 datasets with two shuffles each. Protpars (Joseph Felsenstein, University of Washington, Seattle, USA) was used for parsimony analysis and Consense to build a consensus tree. *Drosophila melanogaster* Antp homeodomain was used as outgroup. The tree was visualized using Archaeopteryx Software (73, 74). CerS2 are shown in gray, CerS3 in yellow, Cers4 in green, CerS5 in blue and CerS6 in purple. Branching of the insect ceramide synthases is shown in red. (B) Exemplary ClustalW alignment of mouse CerS2–6 homeodomains and the fruit fly schlank protein to either the homeodomain consensus by Holland et al. (75) or the homeodomain consensus by Bürglin (76). (C) Exemplary alignment of CerS2 and schlank homeodomain compared to *Drosophila* zfh1, human ZHX3-III, LHX4, or ZEBI homeodomains. Note that there is some amino acid sequence similarity in the first 15 amino acids. Sequences were aligned using ClustalW, the output is shown and the first 15 amino acids of the homeodomain are highlighted in green.

domains in CerS proteins. First experiments to clarify the orientation of the N- and C-terminus were done by Kageyama-Yahara and Riezman (20) for the CerS1 orthologs in *Saccharomyces cerevisiae* (LAG1 and LAC1). It was shown that LAG1 contained eight transmembrane domains with the N- and C-terminus facing the cytosolic side with a potential ER-retention signal at the C-terminus.

N-glycosylation of the mammalian homeodomain-containing CerS2, 5, and 6 (26) provided evidence that their N-termini face the ER lumen. Furthermore, experiments using a C-terminally GFP-tagged CerS6 variant in combination with proteinase K digestion assays demonstrated cytosolic orientation of the C-terminus. From these data a model was deduced, where the N-terminus faces the ER lumen, the homeodomain is in the cytosol and after additional four transmembrane domains, finally the C-terminus would face the cytosol (26).

Conclusions/expert opinion

The most common feature of CerS proteins is their function in ceramide *de novo* synthesis, namely the N-acylation of sphinganine and in a salvage pathway reusing sphingosine by reacylation to form ceramide again. A major role in catalysis of this reaction has been attributed to the LAG1 motif, which is also the defining motif for all CerS. Although this motif is highly conserved among CerS, considerable differences can be detected. These differences can to some extent be reflected in the fatty acyl-CoA substrate chain length preference and in the different tissue specific expression patterns.

Recent studies reveal an emerging concept of different, even opposing roles of different ceramides and CerS in processes such as apoptosis and proliferation. The regulatory influence of CerS and their products would depend on the level and place of expression of the enzymes. Another aspect of regulation is added by the fact that CerS proteins most probably can interact with each other and form heterodimers or most probably even homodimers. Also, the interaction with other protein partners must be considered. Such arrangements could provide the molecular basis for selective and regulated manipulation of cellular processes. In insects, only one CerS can be identified so far. Here, the formation of homodimers or interaction partners other than CerS would be necessary to get in line with a concept of regulating cellular processes by fine-tuned expression of specific subsets of ceramide species by forming heterodimers. Otherwise, a regulatory potential can to some extent lie within the CerS proteins themselves via the homeodomain. Until now, only a loss of ceramide synthesis activity was described when specific positively charged amino acids at the end of the homeodomain were mutated. Yet, as revealed by our database analysis, the homeodomain still might bind DNA and thereby modulate directly or indirectly cellular processes even other than ceramide synthesis. A detailed structure-function characterization of the LAG1 motif and the homeodomain will be necessary. In this context, the combination of genetics and the analysis of ceramides generated in response to distinct stimuli will be very valuable to gain more information. Model systems such as *C. elegans* or *Drosophila melanogaster* could contribute tremendously. For instance, genomic engineering in *Drosophila* permits directed and highly efficient modifications of a chosen genomic locus into virtually any desired mutant allele, which could furthermore be used to test hypotheses about CerS domains and their *in vivo* functions. Results obtained from such approaches might then be used for targeted manipulation in mammalian models for verification and to understand more details.

Outlook

There are still several issues about CerS that require more research in the future. In the coming years, a detailed structure function analysis will help to unravel questions about the CerS domains and their functions. The selective deletion of all CerS in mammals will come and will yield additional animal models, which will then allow testing of different mutated CerS versions in the null background. Moreover, these models will provide information as to what extent gene products or mutated versions are associated with human diseases such as cancer, neurodegeneration, or the metabolic syndrome. CerS expression and their products, the cera-

mides, show correlations with the etiology of cancer or the metabolic syndrome. In this context, Cers are cited as very interesting targets for therapy. Therefore, to unravel the regulation of CerS during development and/or differentiation processes will be of great interest. In the coming years, we will see studies about CerS regulation and efforts to identify new interaction partners. The knowledge of all these studies will increase our understanding of CerS and give further insights how CerS can be used as therapeutic targets.

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