The longevity assurance homologue of yeast *lag1* (Lass) gene family (Review)

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Abstract. The Lass gene family contains a group of highly conserved genes that are found in eukaryotic species. The founding member, *lag1*, was discovered in a screen for yeast longevity genes. Subsequently, *lag1* homologs were discovered in other organisms including six mammalian paralogs. All Lass genes encode a highly conserved Lag1 domain and many also have an additional Hox domain. Lass proteins are ceramide synthases and therefore are critical for ceramide biosynthesis. Ceramide synthase is also a critical enzyme in the sphingolipid biosynthetic pathway. As ceramide and sphingolipids are key intermediates in diverse cellular processes such as cell growth, apoptosis, and stress response and may also play a role in cancer development, the function of Lass proteins is of great interest. In this review, we summarize the state of knowledge regarding Lass protein structure, biological function, and their emerging role in cancer development.

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1. Yeast *lag1* and *lac1*

The founding member of the Lass gene family was discovered in a yeast screen for genes that were preferentially expressed in younger cells (1). The gene was named longevity assurance gene (*lag1*), and its deletion increased the replicative capacity of yeast cells hence *lag1* became the first longevity gene to be identified in any species. A later study revealed that the effect of *lag1* is more complex with moderate expression increasing and high expression reducing life span. Two groups utilized a GenBank database search to identify a yeast paralog of *lag1* which was named *lac1*. The lag1 and lac1 proteins were shown to be multipass ER membrane proteins (2-4). *lac1 lag1* mutants were either inviable or had severe growth defects depending on the strain of cells used (3). The phenotype in the growth defective strain resulted from an inability to sustain ER-to-Golgi transport of GPI-anchored proteins. As this transport requires sphingolipid synthesis it was speculated that this pathway might be defective in *lag1 lac1* mutants. Additional biochemical studies confirmed this hypothesis and identified the synthesis of ceramide, which is a key intermediary in sphingolipid biosynthesis, as the specific defect in *lag1 lac1* mutants (Fig. 1) (5,6). Formal confirmation that *lag1* and *lac1* were components of the yeast ceramide synthase came when they were isolated to near homogeneity and co-purified with a fumonisin B1 (FB1) sensitive acyl-CoA-dependent ceramide synthase activity (6). FB1 is a compound produced by a fungal pathogen that specifically inhibits ceramide synthase and fatty acids are required for ceramide synthase catalyzed synthesis of ceramide. A third component of the lag1/lac1 complex, lip1, was identified by mass spectrometry. Lip1 is a single membrane spanning ER protein that is also required for FB1 sensitive, acyl-CoA-dependent ceramide synthesis (7). This series of experiments established that the yeast ceramide synthase complex consisted of at least a lag1/lac1/lip1 complex.

2. Non-yeast Lass homologues

GenBank database searches with the lag1 and lac1 sequences uncovered homologues in worms, mice, and humans (4,8,9). These proteins were more similar to lag1 than lac1 with a particularly highly conserved stretch of 52 amino acids that was termed the Lag1 domain (3). As more family members have been identified, the motif has been extended to 200 amino acids (5). The only other proteins with sequence similarity to lag1 are translocation chain associated membrane proteins (TRAM) but they lack the Lag1 domain. The evolutionary conservation between yeast lag1 and lac1 and their vertebrate

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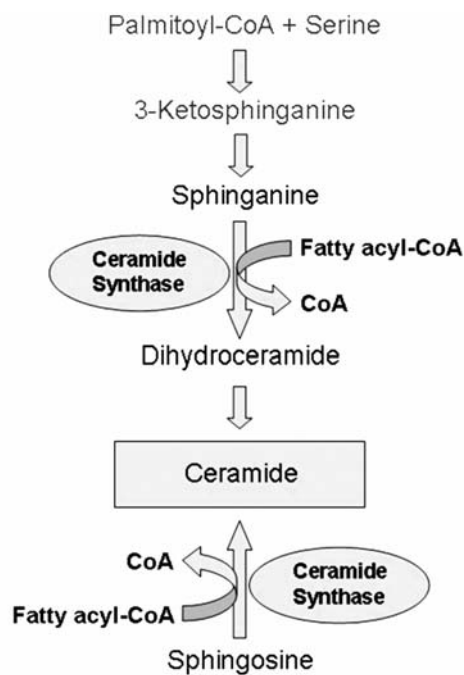E-mail: teufel@uni-mainz.de

Figure 1. Schematic drawing of ceramide synthesis. Ceramide is composed of a sphingoid long chain base to which a fatty acid is attached via an amide bond. The attached fatty acids may vary with respect to their length between C14 and C32.

and invertebrate counterparts, but not TRAM, was dramatically shown when *C. elegans* and human lag homologs rescued the phenotype of yeast *lag1 lac1* mutants (3). The name of the non-yeast *lag* genes was later changed to Lass (longevity assured homologue of yeast *lag1*). With the availability of complete genome sequences for many organisms it was found that all eukaryotes possess Lass genes. Interestingly, all non-Lass1 vertebrate Lass proteins contain a homeodomain motif which is normally found in nuclear transcriptional regulators (8,10).

3. Sequence and structure of Lass proteins

The Lag domain. The family of Lass proteins is characterized by a conserved lag1 domain, located at the C-terminal part of the protein [Pfam identifier: PF03798, (5,9)]. The Lag1 domain contains ~200 amino acids, with human Lass2, 3, 4, 5, and 6 lag1 domains containing 198 or 199 amino acids and the Lass1 Lag1 domain being somewhat longer with 213 amino acids. When aligning the *lag1* domains of all six human Lass homologues using the ClustalW algorithm (11), only 28% of all amino acids are at least 80% conserved among the homologues, and only 15% are completely conserved (Fig. 2). However, this comparably low conservation is enough to ensure ceramide synthase function for all members of the Lass family and may in contrast be the reason why these proteins promote ceramide synthesis with distinct fatty acid specificity for each member (discussed in a later section). Recently, targeted mutation experiments demonstrated that some of the conserved amino acids within the Lag1 domain were essential for ceramide synthesis (H182, H183, L189, D210, D213, L216), whereas others did not contribute to the biochemical process (S193, K220) (12).

This protein stretch containing the Lag1 domain has been described more broadly as part of the Tram-Lag-CLN8 (TLC) domain. The TLC domain is currently known to be shared by a group of 16 proteins, among them all Lass genes (Fig. 3). However, although TRAM has been identified to be structurally related to the Lag1 domain in yeast, the failure of TRAM proteins to complement the yeast *lag1 lac1* deficiency suggests that other TLC family members do not have a ceramide synthase activity (3,5). In addition, aligning all members of the TLC group, including all Lass proteins, the subgroup of Lass proteins is clearly distinct from other group members (Fig. 3). From the protein sequence alignments, it seems clear that the group of Lass genes although structurally related to TLC domain containing proteins comprises a distinct group of genes, both structurally and functionally. Alignment of all Lass orthologue proteins using ClustalW at EBI. The alignment clearly demonstrates the close relationship between Lass5 and 6 as well as Lass2, 3, and 4 (Fig. 4).

The homeodomain. All vertebrate Lass proteins except Lass1 contain a homeodomain. The Lass homeodomain is highly degenerate as only the very C-terminal 12 amino acids of the homeodomain, as recognized by SMART functional domain identification algorithm, are highly conserved among all members of the Lass family. By contrast most of the N-terminus is highly variable. However, this degree of conservation is sufficient for it to be classified as a homeodomain by several motif-detection algorithms (8,13).

Homeodomain-containing transcriptional regulators often operate differential genetic programs along the anterior-posterior axis of animal bodies (14). The domain binds DNA through a helix-turn-helix (HTH) structure. Thus, these proteins predominantly function as transcription factors important not only in embryonic but also cancer development (15). To date, there is no evidence of a transcriptional regulator function of Lass genes. The homeodomain must not be essential to ceramide synthase function as yeast *lag1* and *lac1* and human Lass1 both have ceramide synthase activity. A recent study demonstrated that for human Lass5 and 6 most of the homeodomain was dispensable for ceramide synthase activity but a 12 amino acid stretch of amino acids flanking the TLC and homeodomains is critical for ceramide synthase activity (16). This stretch includes the final eight amino acids of the homeodomain. It is still possible that the Lass proteins containing a homeodomain may have a novel function in addition to ceramide synthase activity in which the entire homeodomain plays a critical role.

Finally, it should be noted that all six genes in human (except, to date human Lass3) encode at least two isoforms, one of which lacks the homeodomain and part of the TLC domain; the relevance of these short isoforms is currently unknown but may imply similar mechanisms of transcriptional regulation and potentially unique isoform specific functions (17).

Transmembrane topology of Lass proteins. The transmembrane topology of yeast lac1 and lag1 was empirically determined by insertion of glycosylation sites and analysis of these modified proteins in yeast. This analysis revealed that lac1 and lag1 possess eight transmembrane spanning domains

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hLass5/1-392   MATAAQGPLSLLWGWLWSERFWLPENVSWADLEGPADGYGYPRGRHILSVFPLAAGIFFV
hLass6/1-384   --------MAGILAWFWNERFWLPHNVTWADLKNTEEAT-FPQAEDLYLAFPLAFCIFMV
hLASS2/1-380   -------MLQTLYDYFWWERLWLPVNLTWADLE-DRDGRVYAKASDLYITLPLALLFLIV
hLass3/1-383   -------MFWTFKEWFWLERFWLPPTIKWSDLE-DHDGLVFVKPSHLYVTIPYAFLLLII
hLASS4/1-394   -------MLSSFNEWFWQDRFWLPPNVTWTELE-DRDGRVYPHPQDLLAALPLALVLLAM
hLass1/1-350   ------------------------------MAAAGPAAGPTGPEP-----------MPSY

                                     Homeodomain
hLass5/1-392   RLLFERFIAKPCALCIGIEDSGPYQAQPNAILEKVFISITKYPDKKRLEGLSKQLDWNVR
hLass6/1-384   RLIFERFVAKPCAIALNIQANGPQIAPPNAILEKVFTAITKHPDEKRLEGLSKQLDWDVR
hLASS2/1-380   RYFFELYVATPLAALLNIKEKTRLRAPPNATLEHFYLTSGKQPKQVEVELLSRQSGLSGR
hLass3/1-383   RRVFEKFVASPLAKSFGIKETVR-KVTPNTVLENFFKHSTRQPLQTDIYGLAKKCNLTER
hLASS4/1-394   RLAFERFIGLPLSRWLGVRDQTRRQVKPNATLEKHFLTEGHRPKEPQLSLLAAQCGLTLQ
hLass1/1-350   AQLVQRGWGSALAAARGCTDCGWGLARRGLAEHAHLAPPELLLLALGALGWTALRSAATA

hLass5/1-392   KIQCWFRHRRNQDKPPTLTKFCESMWRFTFYLCIFCYGIRFLW--SSPWFWDIRQCWHNY
hLass6/1-384   SIQRWFRQRRNQEKPSTLTRFCESMWRFSFYLYVFTYGVRFLK--KTPWLWNTRHCWYNY
hLASS2/1-380   QVERWFRRRRNQDRPSLLKKFREASWRFTFYLIAFIAGMAVIV--DKPWFYDMKKVWEGY
hLass3/1-383   QVERWFRSRRNQERPSRLKKFQEACWRFAFYLMITVAGIAFLY--DKPWLYDLWEVWNGY
hLASS4/1-394   QTQRWFRRRRNQDRPQLTKKFCEASWRFLFYLSSFVGGLSVLY--HESWLWAPVMCWDRY
hLass1/1-350   RLFRPLAKRCCLQ-PRDAAKMPESAWKFLFYLGSWSYSAYLLFGTDYPFFHDPPSVFYDW

hLass5/1-392   PF-QPLSSGLYHYYIMELAFY-WSLMFSQFTDIKRKDFLIMFVHHLVTIGLISFSYINNM
hLass6/1-384   PY-QPLTTDLHYYILELSFY-WSLMFSQFTDIKRKDFGIMFLHHLVSIFLITFSYVNNM
hLASS2/1-380   PI-QSTIPSQYWYYMIELSFY-WSLLFSIASDVKRKDFKEQIIHHVATIILISFSWFANY
hLass3/1-383   PK-QPLLPSQYWYYILEMSFY-WSLLFRLGFDVKRKDFLAHIIHHLAAISLMSFSWCANY
hLASS4/1-394   PN-QTLKPSLYWWYLLELGFY-LSLLIRLPFDVKRKDFKEQVIHHFVAVILMTFSYSANL
hLass1/1-350   TPGMAVPRDIAAAYLLQGSFYGHSIYATLYMDTWRKDSVVMLLHHVVTLILIVSSYAFRY

                                      LAG1 Domain
hLass5/1-392   VRVGTLIMCLHDVSDFLLEAAKLANYAK---------YQRLCDTLFVIFSAVFMVTRLGI
hLass6/1-384   ARVGTLVLCLHDSADALLEAAKMANYAK---------FQKMCDLLFVMFAVVFITTRLGI
hLASS2/1-380   IRAGTLIMALHDSSDYLLESAKMFNYAG---------WKNTCNNIFIVFAIVFIITRLVI
hLass3/1-383   IRSGTLVMIVHDVADIWLESAKMFSYAG---------WTQTCNTLFFIFSTIFFISRLIV
hLASS4/1-394   LRIGSLVLLLHDSSDYLLEACKMVNYMQ---------YQQVCDALFLIFSFVFFYTRLVL
hLass1/1-350   HNVGILVLFLHDISDVQLEFTKLNIYFKSRGGSYHRLHALAADLGCLSFGFSWFWFRLYW

hLass5/1-392   YPFWILNTTLFESWEIIGPYASWWLLNGLLLTLQLLHVIWSYLIARIALKALIRGKVSKD
hLass6/1-384   FPLWVLNTTLFESWEIVGPYPSWWVFNLLLLLVQGLNCFWSYLIVKIACKAVSRGKVSKD
hLASS2/1-380   LPFWILHCTLVYPLELYPAFFGYYFFNSMMGVLQLLHIFWAYLILRMAHKFIT-GKLVED
hLass3/1-383   FPFWILYCTLILPMYHLEPFFSYIFLNLQLMILQVLHLYWGYYILKMLNRCIF-MKSIQD
hLASS4/1-394   FPTQILYTTYYESISNRGPFFGYYFFNGLLMLLQLLHVFWSCLILRMLYSFMKKGQMEKD
hLass1/1-350   FPLKVLYATSHCSLRTVPDIPFYFFFNALLLLLTLMNLYWFLYIVAFAAKVLTGQVHELK

hLass5/1-392   DRSDVE--SSSEEEDVTTC---------TKSPCDSSSSNGANRVNGHMG-GSYWAEE
hLass6/1-384   DRSDIE--SSSDEEDSEPP---------GKNPHTATTTNGTSGTNGYLLTGSCSMDD
hLASS2/1-380   ERSDRE--ETESSEGEEAA--------AGGG----AKSRPLANGHPILNNNHRKND
hLass3/1-383   VRSDDEDYEEEEEEEEEEA---------TKGKEMDCLKNGLRAERHLIPNGQHGH--
hLASS4/1-394   IRSDVE--ESDSSEEVAAAQEPLQLKNGAAGGPRPAPTDGPQSRVAGRLTNRHTTAT
hLass1/1-350   DLREYDTAEAQSLKPS------------------KAEKPLRNGLVKDKRF----
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Figure 2. Alignment of the Lag1 domains of the human Lass homologues using ClustalW at EBI. Homeo- and Lag1-domain are in grey, the conserved amino acids within the Lag1 domain essential for ceramide synthesis are black. The transmembrane domains are underlined.

(5,18). When an alignment of the protein sequences of Lass1 family member is made it appears that all non-yeast Lass (except Lass1) proteins have seven transmembrane domains (TMD) with the putative second TMD of the yeast proteins being absent in all other family members (Fig. 2). The TLC domain includes TMDs 2-8 of yeast Lag1 and Lac1 and TMDs 2-7 of the non-yeast Lass proteins. Most of the Lag1 domain encompasses TMD5-6 (yeast), TMD4-5 (non-yeast) with several amino acids in the ER lumen between these TMDs and some just N- and C-terminal of the first and
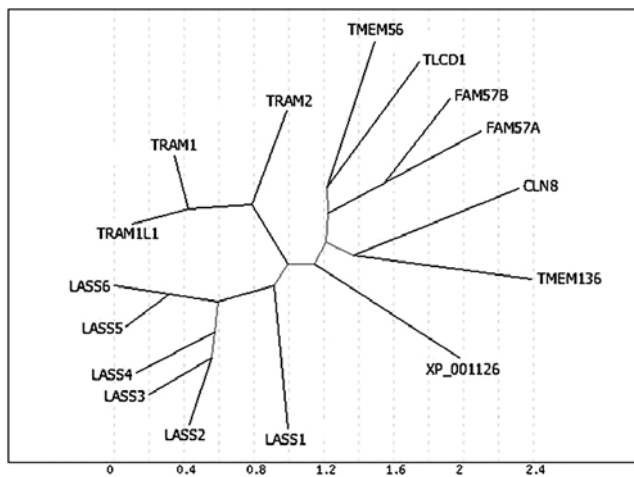
Figure 3. Alignment of all Lag1 domain containing proteins using ClustalW at EBI. The alignment clearly demonstrates the Lass genes being a distinct subgroup with Lass1 being more distantly related to the remaining family members as it does not contain a Hox domain (Ref-Protein IDs: Lass1: NP_067090, Lass2: NP_071358, Lass3: NP_849164, Lass4: NP_078828, Lass5: NP_671723, Lass6: NP_982288, TMEM56: NP_689700, TRAM1L1: NP_689615, TRAM2: NP_036420, CLN8: NP_001029233, TRAM1: NP_055109, TMEM136: NP_777586, FAM57B: NP_113666, FAM57A: NP_079068, XP_001126126, TLCD1: NP_612472).

second of these TMDs. The homeodomain faces the cytoplasmic side in between TMDs 1 and 2 of the vertebrate non-Lass1 proteins (5,18).

Subcellular localization of Lass proteins. As the ER is the site of ceramide synthesis Lass proteins would be expected to localize to this organelle and in fact the subcellular localization of yeast lag1 was the ER membrane (3). Yeast lag1 is the only yeast Lass protein for which subcellular localization studies have been performed and its Lag1 contains a C-terminal ER retention signal. Human Lass1 and 2, and the three *C. elegans* Lass proteins also have a C-terminal ER-retention signal and yeast lac1 has an N-terminal ER-retention motif. Although other Lass proteins lack an ER-retention motif such a motif is not required for ER localization and a recent study showed that Lass5 which lacks a recognizable ER-retention motif does in fact localize to the ER (16).

4. Function of non-yeast Lass proteins

Since human Lass1 rescued the yeast *lag1 lac1* mutant it was reasonable to assume that Lass1 was a component of the mammalian ceramide synthase. The first study showing Lass1 had ceramide synthase activity in mammalian cells came from observations that when the human embryonic kidney 293T cell line is transfected with Lass1 there is an FB1 sensitive, acyl-CoA-dependent increase in ceramide synthesis (19,20). Interestingly, the increase in ceramide synthesis occurred only in the presence of C18 acyl-CoA chains. Thus, this result suggested fatty acid selectivity by Lass paralogs/ceramide synthase in mammalian ceramide synthesis. Subsequently, similar results have been obtained for other members of the mammalian Lass protein family. Lass2
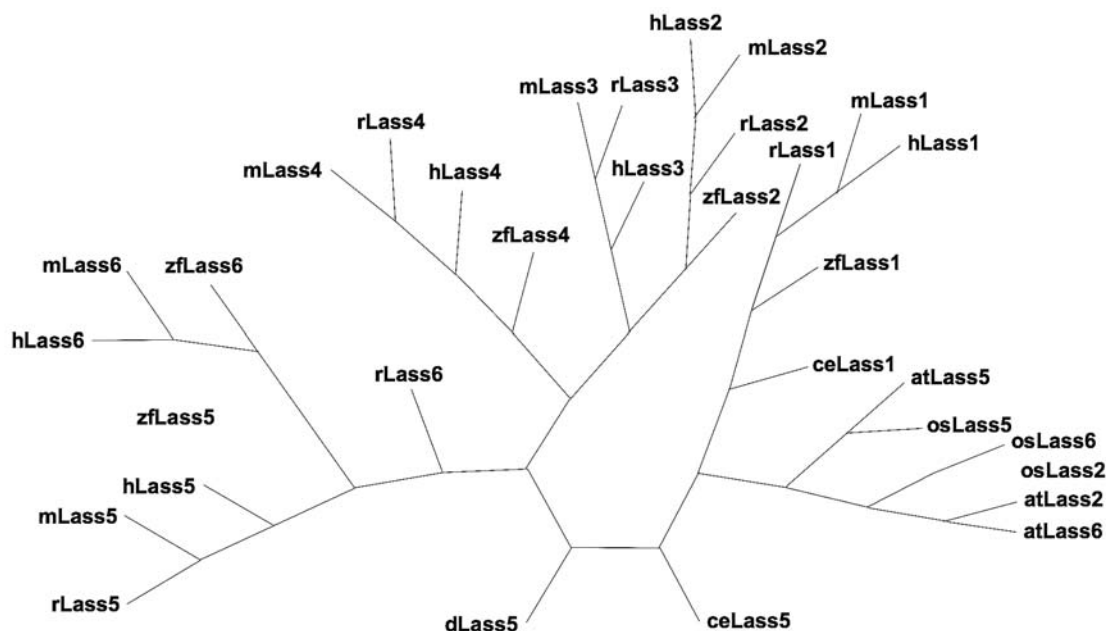

Figure 4. Alignment of all Lass orthologues proteins using ClustalW at EBI. The alignment clearly demonstrates the close relationship between Lass5 and 6 as well as Lass2, 3, and 4. The plant orthologues Lass sequences ('at' for *Arabidopsis thaliana* and 'os' for *Oryzias latipes*) were more distant assembling in an individual group most closely associated to Lass1/lag1. (Ref-Protein IDs: hLass1: NP_067090.1, mLass1: NP_619588.1, rLass1: XP_224733.4, zfLass1: XP_696339.2, ceLass: 1NP_493403.1, hLass2: NP_071358.1, mLass2: NP_084065.1, rLass2: NP_001028872.1, zfLass2: NP_705957.1, atLass2: NP_001031037.1, osLass2: NP_001047244.1, hLass3: NP_849164.2, mLass3: XP_620510.2, rLass3: XP_574469.2, hLass4: NP_078828.1, mLass4: NP_080334.2, rLass4: XP_221796.4, zfLass4: NP_705956.1, hLass5: NP_671723.1, mLass5: NP_082291.1, rLass5: XP_345871.3, zfLass5: NP_955922.1, dLass5: NP_652526.1, ceLass5: NP_501459.1, atLass5: NP_188557.1, osLass5: NP_001049639.1, hLass6: NP_982288.1, mLass6: NP_766444.1, rLass6: XP_345364.3, zfLass6: XP_693283.2, atLass6: NP_566769.1, osLass6: NP_001047999.1).

displayed a preference for long chain fatty acids (C22). Lass4 is involved in synthesis of longer ceramides such as C22:0- and C24:0-ceramides. Lass5 and 6 produce shorter ceramide species (C14:0- and C16:0-ceramides). By contrast, Lass3 exhibits relatively low substrate specificity toward fatty acyl-CoAs. Overexpression of Lass3 leads to increased levels of C18:0-, C22:0-, and C24:0-ceramides (6,13,21,22).

Overexpression of the mammalian Lass proteins in yeast *lag1 lac1* mutants can also result in some albeit a lower degree of specificity in regards to fatty acyl-CoA chain length. Lass1 generates C26:0, Lass2 and 4 C24:0, but Lass5 generates equal amounts of C12:0, C14:0, C16:0, C18:0, and C18:1 (7,23). The lower degree of specificity may be the result of species differences in ceramide synthase activity between yeast and mammals. As has been mentioned earlier that the yeast ceramide synthase consists of a lag1/lac1/lip1 complex. It should be noted that *lip1* is found only in yeast and the expression of Lass5 in a *lip1 lac1 lag1* yeast mutant rescues ceramide synthesis (7).

The only other species in which Lass function has been examined is the tomato (24). FB1 belongs to a cLass of natural toxins that are known as sphinanine-analog myco-toxins (SAMs). *Alternaria alternata f. sp. lycopersici* (AAL) is a pathogen that produces a toxin that is also a SAM. It was discovered that a homozygous mutation in a tomato Lass gene *(Asc-1)* that results in a stop codon in the second exon was responsible for sensitivity to AAL. Expression of the normal gene in mutant plants rescued AAL and FB1 sensitivity. FB1 sensitivity has been rescued by Lass in at least one other plant species, *Nicotiana*. It was established that *Asc-1* is a lag/Lass ortholog when it partially rescued the growth defect in *lag1 lac1* mutants (25).

5. Lass gene family in cancer development, a potential target for cancer therapy?

As Lass genes may have a function in longevity and one of the hallmarks of longevity is resistance to cancer, this family may be involved in cancer development. Additionally, ceramide signaling has previously been demonstrated to be essential to apoptosis, disturbance of which may result in cancer development. Thus, it seems reasonable to investigate the role of Lass family members in cancer development. At present little information is available in this area.

Lass2 expression displays an inhibitory effect on cell growth in hepatocellular carcinoma (HCC) cells. The number of colonies formed by Lass2-transformed HCC cells was reduced to ~20% of that of the vector control (26). In addition, an essential role for Lass1 and C18-ceramide in the regulation of growth of head and neck squamous cell carcinoma (HNSCC) was shown. Lass1 was shown to inhibit cell growth (70-80%), via modulation of telomerase activity and induction of apoptotic cell death by mitochondrial dysfunction (27).

Finally, Lass5 was found to play an essential role in the prevention of immune escape by cancer cells as a result of antigen presentation processing defects. Since elimination of tumor cells by cytotxic T-cells is triggered by MHC class I-restricted antigen presentation of the tumor cell, impairment of this antigen presentation is common in human cancers to evade an immune response (28-30). However, it was noted that a specific T-cell population is able to target these cells with impaired antigen presentation in part by presenting a Lass5 fragment as an epitope. This study shed light on a possible effective application of Lass5 peptides in peptide-based vaccines for CTL-mediated targeting of processing-deficient tumors (31).

In general, Lass proteins have been demonstrated to regulate ceramide synthesis and substantial evidence is accumulating for ceramides and ceramide synthesis in general with respect to cancer therapy. Multiple chemotherapeutic agents such as doxorubicin, irinotecan or paclitaxel and many others were demonstrated to increase the ceramide levels in cells (32). This increase of ceramides have been attributed to a stimulation of the *de novo* synthesis of ceramides but also speculatively to a disrupted ceramide catabolism (33). With this observation, the next steps would obviously be to manipulate and increase ceramide levels in cancer cells to induce apoptosis or to enhance cancer chemotherapy. To date, multiple agents modulating the sphingolipid and in particular the ceramide metabolism have been reported. Also many of these agents have demonstrated promising results in tumor growth inhibition (reviewed in ref. 33). However, most of these data were collected *in vitro* and have yet to be confirmed in *in vivo* models and also be evaluated with respect to possible side effects potentially limiting their therapeutic use.

Besides manipulating key regulatory enzymes in ceramide synthesis, direct intracellular delivery of antimitogenic and proapoptotic ceramides demonstrated potential therapeutic use in cancer therapy. Stover and Kester (35) first reported to have successfully challenged the limitations of intracellular delivery of ceramides due to its inherent lipid hydrophobicity and physicochemical properties. Using ceramide-formulated liposomes for intracellular delivery a significant inhibition of MDA-MB-231 cell proliferation was demonstrated, proof of principle for sufficient intracellular ceramide delivery and potential chemotherapeutic use. Furthermore, they were able to successfully transfer these results into a murine *in vivo* cancer model and reported that the systemic i.v. delivery of C6-ceramide (C6) in a pegylated liposomal formulation significantly limited the growth of solid tumors in a syngenic BALB/c mouse tumor model of breast adenocarcinoma (35). Evaluating ceramides in clinical cancer therapy in phase I studies evaluating the substance for toxicity and dose finding will therefore be of great interest and have yet to be performed.

Together, ceramides and therefore also ceramide synthases must be considered to be of high potential value in cancer therapy and substances regulating these family members have yet to be evaluated in clinical studies. A promising approach to regulate ceramide synthesis to achieve anti-cancer properties may come from regulating the Lass gene and protein family members. This approach has yet to be tested in *in vivo* models.

6. The Lass protein family: Unresolved questions

There are many questions remaining regarding vertebrate Lass proteins. These include the fundamental structure of the vertebrate ceramide synthase (Lass monomer, homodimer, heterodimer, other higher order structure with additional accessory proteins), loss and gain of function phenotypes in

mouse and other model species, the potential association of Lass genes with human diseases as has been found for the TRAM containing protein CLN8 in the neurodegenerative disorder neuronal ceroid lipoofuscinase (36), and as mentioned above the possibility that Lass genes could be part of future anti-cancer therapies. Answers to these questions are likely to have far-reaching consequences for the treatments of the many diseases caused by deregulation of ceramide.

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