9-O-Acetylation of sialic acids is catalysed by CASD1 via a covalent acetyl-enzyme intermediate

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Sialic acids, terminal sugars of glycoproteins and glycolipids, play important roles in development, cellular recognition processes and host-pathogen interactions. A common modification of sialic acids is 9-O-acetylation, which has been implicated in sialoglycan recognition, ganglioside biology, and the survival and drug resistance of acute lymphoblastic leukaemia cells. Despite many functional implications, the molecular basis of 9-O-acetylation has remained elusive thus far. Following cellular approaches, including selective gene knockout by CRISPR/Cas genome editing, we here show that CASD1—a previously identified human candidate gene—is essential for sialic acid 9-O-acetylation. In vitro assays with the purified N-terminal luminal domain of CASD1 demonstrate transfer of acetyl groups from acetyl-coenzyme A to CMP-activated sialic acid and formation of a covalent acetyl-enzyme intermediate. Our study provides direct evidence that CASD1 is a sialate O-acetyltransferase and serves as key enzyme in the biosynthesis of 9-O-acetylated sialoglycans.
Sialic acids (Sias) are acidic nine-carbon sugars that commonly cap the glycan chains of cell surface glycoproteins and glycolipids. Sialylglycopes are key determinants for numerous cell recognition events and play fundamental roles in development and host–pathogen interactions. Of note, Sias show a pronounced structural diversity that arises from N- and O-substitutions of the parent compound neuraminic acid and this diversity is of critical importance for Sia recognition and subsequent cellular processes. For example, the prevalent human Sia 5-N-acetylneuraminic acid (Neu5Ac) can be substituted by 9-O-acetylation, a modification that affects the binding of several Sia-specific lectins and has functional implications in particular in the immune system. 9-O-Acetylation impairs host cell binding of influenza A and B viruses, but at the same time creates cellular receptors for influenza C and several nidoviruses. Another prominent example for the impact of 9-O-acetylation is given by CD22, a B-cell-restricted member of the Sia-binding immunoglobulin-type lectin (siglec) family. On binding to N2,6-sialylated N-glycans, CD22 regulates B-cell antigen receptor signalling by setting a threshold for receptor activation. In vitro data showed that the CD22-ligand interaction is blocked by 9-O-acetylation, a process that is in vivo tightly controlled by two counteracting enzymatic activities: sialate O-acetyltransferase (SOAT) and sialate 9-O-acetyleraseterase (SIAE). Ablation of SIAE function by targeted gene knockout in mice resulted in increased 9-O-acetylation and defective CD22 signalling in B-cells and caused defects in peripheral B-cell development and tolerance. Moreover, catalytically defective rare variants of SIAE were associated with autoimmune diseases in humans.

Sia O-acetylation is also a developmentally regulated modification of gangliosides, implicated in neural precursor cell migration, peripheral nerve regeneration and Mycobacterium leprae infection of Schwann cells. Best studied is the 9-O-acetylation of GD3, a disialo ganglioside that acts as a potent inducer of apoptosis on translocation to mitochondrial membranes. 9-O-Acetylation of the terminal 2,8-linked Sia residue blocks the pro-apoptotic activity of GD3 and promotes survival of cancer cells. In acute lymphoblastic leukaemia (ALL), survival and drug resistance of lymphoblasts critically depend on 9-O-acetylation, which was found on both GD3 and sialoglycoproteins. Enzymatic removal of 9-O-acetyl groups from internal and cell surface-bound Sias was lethal to ALL cells, opening new perspectives for therapeutic concepts. The use of monoclonal antibodies recognizing the carbohydrate epitopes of non-O-acetylated GD3 (CD60a), 7-O-acetylated GD3 (CD60c) or 9-O-acetylated GD3 (CD60b) revealed a stimulatory or co-stimulatory effect of anti-C60c and anti-C60b antibodies on the proliferation of human lymphocytes and implicated distinct roles of 7-O- and 9-O-acetylated GD3 during activation and apoptosis of tonsillar B and T lymphocytes.

Biochemical studies demonstrated that O-acetylation of Sia is a postsynthetic modification that takes place in the Golgi apparatus, probably in concert with Golgi-resident sialyltransferases and coupled with the import of acetyl groups from cytosolic acetyl-coenzyme A (acytetyl-CoA), the presumptive acetyl donor. However, the genes encoding eukaryotic SOATs have remained unknown. Despite many attempts, expression cloning was not successful and isolation of SOAT activity by demonstrating CASD1-mediated transfer of acetyl groups from acetyl-CoA to CMP-activated Neu5Ac.

To analyse the subcellular localization and topology of CASD1, a full-length variant, containing an N-terminal V5 and a C-terminal Myc epitope, was expressed in the murine fibroblast cell line LM-TK araC. The orientation of the epitope tags was determined by selective membrane permeabilization and immunofluorescence analysis (Fig. 1b,c). Under conditions that allowed antibody access to all cellular compartments (0.2% Triton X-100), both tags were detectable and showed co-localization with the Golgi marker α-mannosidase II (α-Man II). Selective permeabilization of the plasma membrane with low concentrations of digitonin (5 μg ml⁻¹) allowed visualization of the N-terminal V5-tag, but not the C-terminal Myc-tag, demonstrating cytosolic and luminal orientation of N- and C-terminus, respectively. The integrity of the Golgi membrane was demonstrated by parallel staining with an antibody specific for the luminal domain of α-Man II. Staining was observed exclusively in Triton X-100, but not in digitonin-permeabilized cells. Identical results were obtained on staining with an antibody against the SNHG-like domain of CASD1 (pAb 506), demonstrating that this domain faces the Golgi lumen (Fig. 1c). On the basis of these results and on our predictions for the CASD1 transmembrane domains (Supplementary Fig. 1), we propose a topology model encompassing a luminal catalytic domain flanked by one N-terminal and 12 C-terminal transmembrane domains (Fig. 1a).

CASD1 forms a covalent acetyl-enzyme intermediate. To analyse the enzymatic activity of CASD1 in vitro, we generated a construct that allowed the expression of a soluble secreted form of CASD1 (termed sCASD1), which encompassed the SNHG-like luminal domain and a C-terminal Myc-His₆-tag (Fig. 2a–c).
This form was produced in baculovirus-infected Sf9 insect cells, either as wild-type variant (sCASD1-wt) or as active site serine mutant (sCASD1-S94A). Affinity purified proteins were then used as an enzyme source for in vitro assays. First, we analysed whether sCASD1 possesses esterase activity, since the GDSL/SGNH fold is usually found among hydrolytic enzymes\(^3\), including the sialate 9-O-acetylesterase of \textit{Escherichia coli} O157:H7 and the sialate 9-O-acetylesterase domains of the haemagglutinin-esterase (HE) proteins of influenza C and bovine coronavirus (BCoV)\(^4\)–\(^6\). However, under conditions that displayed high O-acetyltransferase activity for BCoV-HE, no activity was observed for sCASD1-wt, neither towards the synthetic acetylesterase substrate para-nitrophenylacetate (pNP-acetate; Fig. 2d) nor towards natural O-acetylated sialoglycoconjugates (not shown). Thus, while the N-terminal luminal domain of CASD1 displays the characteristic fold of an esterase\(^9\), it does not appear to function as such. We therefore tested whether sCASD1 might catalyse the reverse reaction and act as a SOAT. Indeed, in the presence of \(^3\)H]acetyl-CoA, sCASD1-wt showed acetyltransferase activity, resulting in the formation of radiolabelled protein, even in the absence of acceptor substrates (Fig. 2e). No radioactive incorporation was seen in parallel experiments with the mutant sCASD1-S94A, demonstrating that the observed acetyl transfer was enzyme catalysed. SOAT activity of sCASD1 towards its own N-glycans was improbable. Although sCASD1 is N-glycosylated (Fig. 2c), glycoproteins that are produced in Sf9 cells typically bear pauci mannosidic N-glycans lacking penultimate galactose and terminal Sia residues\(^4\). Consequently, we asked whether sCASD1 can catalyse the formation of a covalent acetyl-enzyme intermediate that could be trapped in the absence of acceptor substrate. Therefore, purified sCASD1 was incubated in the presence or absence of acetyl-CoA, digested with trypsin and analysed by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). After incubation of sCASD1-wt with acetyl-CoA, the peptide containing the active site residue S94 (87-HIAFIGD\(_9\)AR-95) occurred as a mixed population of unmodified and modified peptide with a mass shift of 42 Da (shift by \(m/z = 21\) for the doubly charged ion), consistent with the formation of an acetyl adduct (Fig. 2f; note that the sequence of the peptides was confirmed by tandem MS/MS; see Supplementary Fig. 2). Analysis of the peak areas in the extracted ion chromatograms revealed that about 19% of the peptide population was acetylated (Supplementary Fig. 3). In parallel experiments with sCASD1-S94A, only the respective unmodified peptide (87-HIAFIGD\(_5\)AR-95) was found (Fig. 2f;...
9-O-acetylation of CMP-Neu5Ac. To test SOAT activity, purified sCASD1 was incubated with acetyl-CoA and Sia-containing acceptor substrates. After the reaction, Sia was released by acidic hydrolysis to allow derivatization with 1,2-diamino-4,5-methylenedioxy-benzene (DMB) at the free reducing end. The obtained fluorescent Sia derivatives were analysed by reversed-phase high-performance liquid chromatography (HPLC; Fig. 3a–e). SOAT activity was clearly detected towards CMP-Neu5Ac (as indicated by an additional peak in the HPLC profile, which eluted at the same retention time as DMB-derivatized 9-O-acetylated Neu5Ac (Neu5,9Ac2) of the reference panel; see Fig. 3a,c). The additional peak was not detected in samples obtained from parallel reactions without enzyme (Fig. 3b) or from reactions with sCASD1-S94A (Fig. 3d).

Compared with free Neu5Ac or sialoglycoconjugates, CMP-Neu5Ac was by far the best acceptor substrate (Fig. 3e). The identity of the product peak obtained with CMP-Neu5Ac was confirmed by LC-ESI-MS, which showed the expected parent ion mass of 508.25, m/z [M+2H]^2+ = 254.12 (ref. 45; Fig. 3f,g). Together, these data demonstrate that the isolated SGNH-like domain of CASD1 displays SOAT activity in vitro and transfers acetyl groups from acetyl-CoA to CMP-Neu5Ac, resulting in the formation of CMP-Neu5,9Ac2.

CASD1 mediates 9-O-acetylation of cellular sialoglycans. To study the enzymatic function of CASD1 in a cellular context, we first screened for a mammalian cell line that lacks O-acetylated sialoglycans. As a detection tool, we made use of the haemagglutinin-esterase (HE) protein of BCoV. BCoV uses 9-O-acetylated sialic acids as host cell receptors and the HE contains...
Figure 3 | In vitro SOAT activity of sCASD1 monitored by DMB-HPLC and LC-ESI-MS. (a) HPLC elution profile of the DMB-derivatized Sia reference panel, which included also two non-human Sia species, that is, Neu5Gc (5-N-glycolyl neuramic acid) and 9-O-acetylated Neu5Gc (Neu5Gc9Ac). Asterisks denote that the indicated Sia species are labelled with DMB. (b-d) HPLC elution profiles of DMB-derivatized samples obtained after incubation of CMP-Neu5Ac and acetyl-CoA without enzyme (b), with sCASD1-wt (c) or with sCASD1-S94A (d). In all profiles, the retention time corresponding to DMB-Neu5,9Ac2 of the reference panel is highlighted by a grey box. (e) SOAT activity of sCASD1 towards different acceptor substrates. Purified sCASD1-wt and sCASD1-S94A were incubated with acetyl-CoA and the indicated acceptor substrates. Product analysis was performed by DMB-HPLC analysis and the amount of DMB-Neu5,9Ac2 was quantified by integrating the corresponding peak areas. Parallel HPLC runs of reactions without CASD1 were used to measure the background, and data are presented as background subtracted data with the values obtained for CMP-Neu5Ac in the presence of sCASD1-wt and acetyl-CoA set to 100% (mean ± s.d., n = 3). (f) Scheme showing sCASD1-catalysed 9-O-acetylation of CMP-Neu5Ac, subsequent DMB labelling of the reaction product, and the calculated m/z value ([M + H]^+) for DMB-derivatized Neu5,9Ac2. Structures and calculated m/z values ([M + H]^+) of the fragmentation compounds of DMB-Neu5,9Ac2 are given according to Klein et al.55. (g) LC-ESI-MS/MS analysis. In the HPLC runs shown in (b-d), the peak material eluting at the retention time of DMB-Neu5,9Ac2 was collected and subjected to LC-ESI-MS/MS. Ions at m/z 468.16 ([M + H]^+), indicating DMB-Neu5,9Ac2, were obtained only for the material collected from c and subsequent fragmentation revealed the three ions (m/z 313, 295 and 229) that are characteristic for DMB-Neu5,9Ac2 (ref. 45). Numbers in brackets above the spectrum refer to the structures given in f. DMB, 1,2-diamino-4,5-methylenedioxy-benzene.

both a lectin domain and a receptor-destroying sialate 9-O-acetylesterase domain32,46. Inactivation of the esterase activity by S40A mutation (HE0) and fusion to the Fc part of human IgG gave rise to a soluble vireolectin (BCoV-HE0-Fc) that selectively recognizes 9-O-acetylated Sias42, as confirmed by glycan array screening47. Immunofluorescence analysis with BCoV-HE0-Fc revealed that several mammalian cell lines express 9-O-acetylated sialoglycotopes exclusively in the Golgi apparatus (Supplementary Fig. 4a). In Chinese hamster ovary (CHO) cells, the underlying carrier structures were N- and O-glycans, since blocking O-glycosylation sufficiently abrogated 9-O-acetylation only in cells that additionally lacked complex and hybrid N-glycans (Supplementary Fig. 5).

Importantly, all cell lines that were positive for 9-O-acetylation were also positive for CASD1 transcripts (Supplementary Fig. 4b). In fact, we found only a single cell line, the murine fibroblast cell line LM-TK−, that was devoid of both (Supplementary Fig. 4). Transfection of this naturally occurring O-Ac-negative cell line with CASD1 complementary DNA (cDNA), but not empty vector, led to the formation of Golgi-resident 9-O-acetylated...
sialoglycotopes as shown by virolectin staining (Fig. 4a). De-O-acetylation, by either alkali treatment or the sialate 9-O-acetylsterase activity of BCoV-HE, completely abolished the virolectin signals and was used as specificity control (Fig. 4a). After stable expression of wild-type CASD1, 9-O-acetylated sialoglycotopes were detected in all cells and co-localized with the Golgi marker α-Man II and Golgi-localized V5-tagged CASD1 (Fig. 4b). Introduction of the S94A exchange into full-length CASD1 (CASD1-S94A) resulted in a loss of function. Although the mutant protein was expressed and localized to the Golgi, virolectin staining was seen only in <1% of the cells (Fig. 4c). Taken together, these experiments demonstrate that CASD1 mediates sialate 9-acetylation in LM-TK− cells and that this function is impaired by alanine exchange of S94.

**CASD1 knockout abolishes Sia O-acetylation in HAP1 cells.** To confirm that CASD1 plays a crucial role in the formation of 9-O-acetylated sialoglycans, we generated a CASD1 knockout in the near-haploid human cell line HAP1 using the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated 9) system48. The obtained knockout cell line (HAP1ΔCASD1) harbors a 16-bp frame shift mutation in exon 3 of CASD1, allowing only the production of a truncated polypeptide that already lacks S94, the most N-terminally located residue of the catalytic triad (Fig. 5a-c). Whereas parental HAP1 cells expressed 9-O-acetylated sialoglycans in the Golgi apparatus, 9-O-acetylation was completely lost in HAP1ΔCASD1 cells (Fig. 5d). This was further confirmed by DMB-HPLC analysis revealing the presence of DMB-derivatized Neu5,9Ac2 in parental but not in HAP1ΔCASD1 cells (Supplementary Fig. 6).

**Figure 4** | CASD1 mediates 9-O-acetylation of cellular sialoglycoconjugates. (a) LM-TK− cells were transfected with empty vector (mock) or a plasmid encoding epitope-tagged CASD1 (+ CASD1-wt). After fixation and permeabilization with Triton X-100, cells were stained with the virolectin BCoV-HE0-Fc, which recognizes 9-O-acetylated Sia. The specificity of the virolectin stain was controlled using human Fc fragments instead of virolectin (Fc) or by de-O-acetylation before the virolectin staining. De-O-acetylation was performed by alkali treatment (+ alkali) or incubation with BCoV-HE-Fc harbouring an enzymatically active esterase domain (+ esterase). Nuclei were counterstained with DAPI (blue). (b,c) LM-TK− cells stably expressing V5-tagged wild-type CASD1 (+ CASD1-wt) or CASD1 carrying the mutation S94A (+ CASD1-S94A) were co-stained with BCoV-HE0-Fc (red) and anti-α-Man II pAb (green) or BCoV-HE0-Fc (red) and anti-V5 mAb (green) as shown in the upper and lower panel, respectively. Nuclei were stained with DAPI (blue). Insets in c show a representative cell of a minor cell population (<1%), which stained positively with BCoV-HE0-Fc. Scale bar, 10 μm. DAPI, 4,6-diamidino-2-phenylindole.

Transfection with CASD1 cDNA, but not empty vector, resulted in successful complementation of the loss-of-function defect and restored 9-O-acetylation of Golgi-localized sialoglycotopes (Fig. 5e). Of note, a genetic knockout carried out in parallel in human embryonic kidney (HEK) 293T cells showed the same outcome (Supplementary Fig. 7) and thus provided independent evidence for the essential role of CASD1 in Sia 9-O-acetylation.

**CASD1 mediates 9-O-acetylation of gangliosides.** On the basis of HAP1 and HAP1ΔCASD1 cells, we next explored the role of CASD1 in the 9-O-acetylation of gangliosides such as GD3. To do so, we made use of a set of monoclonal antibodies (mAbs) that distinguish between GD3 (CD60a) and 9-O-acetylated GD3 (CD60b). HAP1 cells lack GD3 but can generate the disialo ganglioside after expression of the sialyltransferase ST8Sia I (Fig. 6a; mAb R24). ST8Sia I converts GM3 to GD3 by adding an α2,8-linked Sia residue, which in turn can carry an O-acetyl group in C9 position (Fig. 6c)49. In HAP1 wild-type cells, expression of ST8Sia I resulted in the formation of both CD60a and CD60b antigens, as shown by additional staining with mAb UM4D4 (Fig. 6a). As described for other anti-CD60b antibodies, UM4D4 recognizes 9-O-acetylated GD3 and similar gangliosides possessing a terminal 9-O-acetylated disialo group such as 9-O-acetylated disialo neolactotetraosylceramide and 9-O-acetylated disialo neolactohexaosylceramide (see Supplementary Fig. 8c for details on ganglioside structures)50. However, binding of mAb UM4D4 strictly relies on the O-acetylation of the carbohydrate epitope and no cross-reactivity towards the unmodified epitope is seen (see refs 50,51 and Supplementary Fig. 8b). Newly synthesized CD60a and CD60b epitopes were both detected at...
the cell surface, as demonstrated by respective signals on non-permeabilized cells. In HAP1ΔCASD1 cells, expression of ST8Sia I alone led to the formation of GD3 (Fig. 6b; mAb R24) but not 9-O-Ac-GD3 or other 9-O-acetylated gangliosides of the neolacto series (Fig. 6b; mAb UM4D4). Ganglioside modification was only detected after co-expression of ST8Sia I with CASD1, demonstrating that CASD1 is crucial for the formation of 9-O-acetylated CD60b epitopes (Fig. 6b; middle images). Again, alanine exchange of the active site residue S94 drastically impaired CASD1 function (Fig. 6b; right images).

The critical role of CASD1 for the formation of 9-O-acetylated CD60b epitopes was further confirmed in HEK293T wt and HEK293TΔCASD1 cells by immunofluorescence and thin-layer chromatography (TLC) analysis, whereas no indication for the presence of 7-O-acetylated disialo gangliosides was found (Supplementary Fig. 8a,b).

**Discussion**

Here we define CASD1 as the key factor for Sia 9-O-acetylation, the most common Sia modification in humans. Pivotal to our study was the observation that mammalian cell lines frequently express 9-O-acetylated sialoglycans in the Golgi apparatus, even if 9-O-acetylation of cell surface glycans is non-detectable. This phenomenon, described only by one previous study37, highlights that 9-O-acetylation is much more common than anticipated from cell surface staining. The finding that Golgi-confined 9-O-acetylated Sias were attached to N- and O-glycans suggests Golgi-resident glycoproteins as underlying carriers and interestingly, 9-O-acetylated Sia was recently found on the N-glycans of a human sialyltransferase52.

As shown for HAP1 and HEK293T cells, CASD1 is essential and sufficient for Golgi-confined 9-O-acetylation. However, 9-O-acetylation of cell surface molecules such as GD3 and similar
Figure 6 | CASD1 mediates 9-O-acetylation of gangliosides. (a) Parental, wild-type HAPI cells (HAPI wt) were analysed for the expression of GD3 and 9-O-Ac-GD3 before (Ø) and after transient expression of V5-tagged ST8Sia I (+ ST8SIA1). Non-permeabilized cells were stained with anti-CD60a mAb R24 (upper panel) or anti-CD60b mAb UM4D4 (middle panel). Triton-permeabilized cells (lower panel) were co-stained with mAb UM4D4 (red) and anti-V5 mAb (green). Nuclei were counterstained with DAPI (blue). Scale bar, 10 μm. (b) HAPIΔCASD1 cells were transiently transfected with ST8SIA1 cDNA either alone (+ ST8SIA1), together with wild-type CASD1 cDNA (+ ST8SIA1+CASD1-wt) or together with mutated CASD1 cDNA (+ ST8SIA1+CASD1-S94A). Non-permeabilized cells were stained with mAb R24 (upper panel) and mAb UM4D4 (middle panel) and Triton-permeabilized cells (lower panel) were co-stained with mAb UM4D4 (red) and anti-V5 mAb (green). Nuclei were counterstained with DAPI (blue). Scale bar, 10 μm. (c) Scheme showing the conversion of GM3 to GD3 and the formation of 9-O-Ac-GD3, CD60a and 9-O-Ac-SOAT activity. Arming et al.35 observed a role of CASD1 in 7-O-acetylation but not in 9-O-acetylation of GD3 as shown by FACS analysis with anti-CD60c and anti-CD60b mAbs.35 The stability of O-acetyl groups at C7 might thus be cell-type-specific and could explain the discrepancy with our data.

Although our data demonstrates that CASD1 mediates 9-O-acetylation, we cannot rule out the possibility that the enzyme initially transfers the acetyl group to the C7 position of Sia followed by migration to position C9 in a non-enzymatic step. Even under physiological conditions, O-acetyl groups at the Sia side chain can spontaneously migrate from C7 to C8 to an unsubstituted hydroxyl group at position C9, where the O-acetyl group is more stable.57,58 Moreover, 7-O-Ac-specific SOAT activity has been detected in several tissues31,59,60 and studies on microsomal fractions from bovine submandibular glands provided evidence for an enzyme that accelerates the migration of O-acetyl groups from C7 to C8. Arming et al.35 observed a role of CASD1 in 7-O-acetylation but not in 9-O-acetylation of GD3 as shown by FACS analysis with anti-CD60c and anti-CD60b mAbs.35 The stability of O-acetyl groups at C7 might thus be cell-type-specific and could explain the discrepancy with our data.

By identification of a covalent acetyl-enzyme intermediate that involves the active site residue S94, we provide first insight into the catalytic mechanism of CASD1. Transfer of an acetyl group from acetyl-CoA to a serine placed in a Ser–His–Asp catalytic triad is reminiscent of what we found for the polysialic acid-specific O-acetyltransferase OatC of Neisseria meningitidis,64 and suggests a ping–pong mechanism as described for other members of the SGNH-hydrolase family.62,63 Consistently, we propose a mechanism in which the catalytic triad of CASD1 forms a charge relay network to activate S94, allowing nucleophilic attack of the carbonyl carbon of the acetyl donor. This leads to the transfer of 9-O-acetylated Sia from the donor to the acceptor in a ping–pong mechanism as described for other members of the SGNH-hydrolase family.62,63
the acetyl group to S94, followed by transfer to CMP-Neu5Ac during the second half reaction. Alanine exchange of S94 in sCASD1 prevented the formation of the acetyl-enzyme intermediate and resulted in a complete loss of SOAT activity in vitro.

The soluble sCASD1 variant used for in vitro experiments encompassed only the SGNH-like domain, whereas in vivo, this domain is fused to a C-terminal multi-TM domain. Detection of residual virolectin staining in cells expressing full-length CASD1-S94A may indicate that the C-terminal part contributes to CASD1 activity in vivo. First indication for this scenario is given by a bioinformatics analysis of the cryptococcal Cas1 protein (Cas1p)39. This study highlighted that the multi-TM domain found in Cas1p and its animal orthologue CASD1 shares homology with bacterial multi-TM O-acetyltansferases that are involved in the modification of cell surface or periplasmic biopolymers such as polysaccharides and peptidoglycan 39. Of note, several of the bacterial multi-TM proteins work in concert with an SGNH-like O-acetyltansferase, with the latter being expressed either as separate protein or fused to the multi-TM part as in CASD1 (refs 62, 64, 65).

In conclusion, we characterized human CASD1 as key enzyme for Sia 9-O-acetylation and provided first insight into the catalytic mechanism. This opens new experimental avenues for the development of effective therapeutic strategies targeting CASD1 to regulate pathological changes in the 9-O-acetylation status and to combat drug-resistant cancer cells in ALL, whose survival depends on this 9-O-acetylation23.

Methods

Homology modelling. The three-dimensional structure of the catalytic domain of CASD1 was modelled using the Phyre protein fold recognition server46. On the basis of the structure of the isoamy acetate-hydrolysing esterase from Saccharomyces cerevisiae (PDB accession code 3m1l), which was identified as top rank template, residues 83-290 of human CASD1 were modelled with 99.3% confidence.

Generation of mammalian expression plasmids. To generate a construct encoding full-length CASD1 with a N-terminal V5 and a C-terminal Myc epitope (V5-CASD1-Myc), the coding region of human CASD1 (accession no. NM_022900) was amplified by PCR using the primers 5′-GCTCGAGATGCGGACGCTGGTCCTGCCTACAACTGTG-3′ and 5′-GTCGCTCGAAGATGTTATTTATGAGTAGTATGTCATGATG-3′ containing BamHI and XhoI restriction sites (underlined), respectively, and the resulting PCR product was ligated into the corresponding restriction sites of the vector pcDNA3 (Invitrogen). Sequences encoding the epitope tags were inserted as N-terminal V5 or C-terminal Myc (Polysciences) instead of Lipofectamine Max (Life Technologies) were added 72 h after start of the transfection. Colonies were picked and cloned by limiting dilution.

CRISPR/Cas-mediated genome editing. CASD1 knockout cells were generated by Hublegen Genomics (Austria) based on the near-haploid human cell line HAPI and CRISPR/Cas-mediated genome editing58. In brief, HAPI cells were used to generate retroviral vector encoding the V5 epitope (for HEK293T cells). Site-directed mutagenesis was performed by PCR using the QuikChange site-directed mutagenesis kit (Stratagene) and pcDNA3-V5-CASD1(-myc) as template. The mutated oligonucleotide pair 5′-AGCTCGAGATGTTATTTATGAGTAGTATGTCATGATG-3′ and 5′-CAATGAGGATTATTGGGGTG-3′ was inserted into the XhoI and XbaI sites of pcDNA3, resulting in the plasmid pcDNA3-V5-CASD1(-myc). Site-directed mutagenesis was performed by PCR using the QuikChange site-directed mutagenesis kit (Stratagene) and pcDNA3-V5-CASD1(-myc) as template. The mutated oligonucleotide pair 5′-AGCTCGAGATGTTATTTATGAGTAGTATGTCATGATG-3′ and 5′-CAATGAGGATTATTGGGGTG-3′ was inserted into the XhoI and NotI sites of pcDNA3, resulting in the plasmid pcDNA3-V5-CASD1(-myc).

Transfection of mammalian cells. Transfection of LM-TK− and CHO cells was performed with Lipofectamine (Invitrogen). Briefly, cells were grown in six-well plates (70-80% confluence), washed twice with PBS and transfected with 1 μg of plasmid DNA and 6 μl Lipofectamine 1 ml of OptiMEM (Gibco). After 7 h at 37 °C and 5% CO2 the transfection was stopped by adding 1 ml of culture medium supplemented with 10% FCS. For HAPI and HEK293T cells, the protocol was varied by using 2 μl TurboFectin 80 (Origene Technologies) or 2 μl poly–L-lysine (Solarbio). Transfection efficiency was determined by selection of stable transfecants, 750 μg ml−1 G418 (Calbiochem) or 750 μg ml−1 zeocin (Life Technologies) were added 72 h after start of the transfection. Colonies were picked and cloned by limiting dilution.

Reverse transcription–PCR analysis. RNA was prepared from mammalian cell lines using TRizol reagent (Invitrogen) according to the manufacturer’s instructions. cDNA was generated from 1 μg of RNA using Revert Aid premium reverse transcriptase (Fermentas) and random hexamer primers according to the manufacturer’s recommendations. Gene-specific cDNA was amplified using the following primer pairs: 5′-GGTGGCAACCTCACAGTTGTATGATG-3′ and 5′-GGTTTTGCAACCTCACAGTTGTATGATG-3′ (for HEK293T cells). PCR products were purified from the tissue culture supernatant as described42. As an alternative source of recombinant proteins, CHO cells stably transfected with plasmids encoding BCoV-HE-Fc or BCoV-HE0-Fc were cultured in DMEM/Ham’s F-12 1:1 (Biochrom) supplemented with 1 mM sodium pyruvate and 10% FCS. All cell lines were routinely screened for mycoplasma contamination using the PlasmoTest detection kit (InvivoGen).

Production and purification of BCoV-HE-Fc and BCoV-HE0-Fc. BCoV-H5-EF-Fc and BCoV-H5-EF-Fc were produced by transient expression in HEK293T cells and purified from the tissue culture supernatant as described52. As an alternative source of recombinant proteins, CHO cells stably transfected with plasmids encoding BCoV-HE-Fc or BCoV-HE0-Fc were cultivated in DMEM/Ham’s F-12 1:1 (Biochrom) containing 1 mM sodium pyruvate and 5% low Ig FCS (Gibco).
Expression and purification of sCASD1.

Plasmids enabling the secreted expression of sCASD1 in insect cells were based on a modified pFastBac1 vector (Invitrogen) carrying sequence stretches encoding an N-terminal honeybee melittin signal peptide (HBM) and a C-terminal Myc-His6-tag. The sequence encoding residues 39–304 of human CASD1 was amplified by PCR using the primers 5′-GCTCGAGGTCCGATCCGCTTACCAGGAG-3′ and 5′-GCTGCTGCT AATTTGACCATCACCAGG-3′, containing SacI and XbaI restriction sites (underlined), and either pDNA-V5-CASD1(wt)-Myc or pDNA-V5-CASD1(S94A)-Myc as template. The resulting PCR products were ligated into the restriction sites of the modified pFastBac1 vector, resulting in the plasmids pFastBac1-HBM-sCASD1(wt)-Myc-His6 and pFastBac1-HBM-sCASD1(S94A)-Myc-His6.

Spodoptera frugiperda (SB) cells (Gibco) were grown at 27 °C in shaking cultures at 80 r.p.m. in protein-free Insect-Xpress medium (Lonza) and maintained at a density of 0.5 × 10⁶ to 6 × 10⁶ viable cells per ml. Transient expression of sCASD1-wt and sCASD1-S94A was performed on infection with recombinant baculovirus particles generated by the Bac-to-Bac system (Invitrogen) and the pFastBac1-based plasmids described above. Conditions medium was collected 96 h after infection, concentrated 10-fold by tangential flow ultrafiltration (UltraFres, cutoff of 10 kDa, Pall Corporation), dialyzed against 50 mM sodium phosphate buffer pH 7.5 containing 100 mM NaCl and passed over a HisTrap HP (Ultrasette, cutoff of 10 kDa, Pall Corporation), diafiltered against 50 mM sodium phosphate pH 7.5 containing 10% glycerol, bound protein was eluted by an imidazole gradient. The imidazole was subsequently removed by gel filtration using a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with 20 mM MES pH 6.8 containing 100 mM NaCl.

Enzymatic removal of N-glycans.

To remove N-glycans, 2 µg of sCASD1-wt were incubated with two units of PNGaseF (Alexis Biochemicals) in a total volume of 30 µl containing 50 mM phosphate buffer pH 6.5, 50 mM KCl and 50 mM NaCl to remove cell surface Sia. Cells were pelleted by centrifugation and the supernatant was lyophilized, derivatized with Glyco sialic acid reference standard (Proryme) and used for further analysis.

In vitro SOAT activity assay using DMB-HPLC analysis.

Enzyme assays were performed for 3 h at 37 °C in 20 µl containing 50 mM MES pH 6.5, 10 mM MnCl₂, 1 mM acetyl-CoA with or without 5 µg sCASD1-wt or sCASD1-S94A. As acceptor substrate, 1 µg sCASD1-wt or sCASD1-S94A was mixed with the indicated protein and 2.5 µl of 100 mM sodium phosphate buffer pH 6.5 and 50 mM NaCl. After storage at -20°C for at least 18 h, the aliquot of 10 µl was spotted on Whatman grade 3MM chromatography paper and free radioactivity was removed by descending paper chromatography using 300 mM ammonium acetate pH 7.5/70% ethanol as mobile phase. Radioactivity incorporated into protein remained at the origin and was quantified by scintillation counting.
Ganglioside analysis by thin-layer chromatography. Gangliosides were extracted as described. Briefly, 2 × 10⁶ cells were resuspended in chloroform/methanol (1:2, v/v), lysed for 15 min by sonication and centrifuged for 10 min at 3,000 r.p.m. Water was added to this 1.5-fold final ratio of chloroform/methanol/H₂O of 4:85 (v/v/v). After vortexing and centrifugation at 5 min for 5,400 r.p.m., the upper phase was recovered. The obtained ganglioside fraction was desalted on a Chromabond C18 cartridge (Macherey-Nagel) and evaporated to dryness under nitrogen gas.

Gangliosides were resuspended in chloroform/methanol (1:2, v/v), spotted on high-performance TLC plates (Nano-DURASIL-20, 10 × 10 cm, Macherey-Nagel) and chromatographed for 30 min in chloroform/methanol/H₂O (120:70:7, v/v/v) containing 0.02% CaCl₂ (ref. 5). Plates were treated with 0.3% polyisobutyl methacrylate (Sigma Aldrich) in hexane and dried. After incubation in PBS followed by blocking with 1% BSA in PBS for 1 h at RT, immunostaining with enhanced as standards were purified from bovine buttermilk by Bernhard Kniep and have been generously provided by Reinhard Schwartz-Albiez.

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Author contributions
A.-M.T.B. and M.M. designed the experiments, analysed the data and wrote the manuscript. A.-M.T.B. performed the homology modelling, biochemistry, cell culture, in vitro assays and fluorescence microscopy. F.F.R.B. performed the mass spectrometry. M.G. and M.H. assisted in plasmid construction, cell culture and microscopy. M.H. performed the TLC experiments. M.J.G.B., M.A.L. and R.J.d.G. contributed new reagents/methods to the project and Hans Bakker for help with TLC. This work was supported by grant MU 1774/4 (to M.M.) from the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation), by the Hannover Biomedical Research School (HBRS) and by the DFG funded Cluster of Excellence REBIRTH (From Regenerative Biology to Reconstructive Therapy).

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