

The collapsin response mediator protein 1 (CRMP-1) and the promyelocytic leukemia zinc finger protein (PLZF) bind to UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE), the key enzyme of sialic acid biosynthesis

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Received 29 September 2006; revised 3 November 2006; accepted 3 November 2006

Available online 14 November 2006

Edited by Gianni Cesareni

Abstract Sialic acids (Sia) are expressed as terminal sugars in many glycoconjugates. They are involved in a variety of cell–cell interactions and therefore play an important role during development and regeneration. UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE) is the key enzyme in the de novo synthesis of Sia and it is a regulator of cell surface sialylation. Inactivation of GNE in mice results in early embryonic lethality. Mutations in the GNE gene are of clinical relevance in hereditary inclusion body myopathy, but these mutations do not necessarily decrease the enzymatic activity of GNE. In this study, we searched for novel function of the GNE protein beside its enzymatic function in the Sia biosynthesis. We here report the identification of novel GNE-interacting proteins. Using a human prey matrix we identified four proteins interacting with GNE in a yeast two-hybrid assay. For two of them, the collapsin response mediator protein 1 and the promyelocytic leukemia zinc finger protein, we could verify protein–protein interaction with GNE. © 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Yeast two-hybrid screen; Sialylation; Protein–protein interaction; GNE; PLZF; CRMP-1

1. Introduction

Sialic acids (Sia) [1], which constitute a family of aminosugars, are the most abundant terminal monosaccharides on glycoconjugates of eukaryotic cell surfaces. Sia are involved in a variety of cellular functions, such as cell–cell interaction and virus infection, and they confer stability on glycoproteins [2–4]. Sia are synthesized in the cytosol from UDP-*N*-acetylglucosamine by four consecutive reactions. The first two steps in Sia biosynthesis are catalyzed by a single bi-functional enzyme, UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE) [5,6]. GNE is the key enzyme of Sia synthesis and it is a regulator of cell surface sialylation [7]. Only the hex-

americ form of the enzyme catalyzes both enzymatic reactions [6]. Recently, we showed that the carboxy-terminal kinase domain of GNE is responsible for its multimerization [8]. The sequence of GNE is very conserved between rat, mouse and human tissues [5,9,10]. Using northern-blot analysis and in situ hybridization, the highest expression of GNE was found in the liver. The enzyme was expressed to a lesser extent in all other investigated organs. It is fully expressed at all the stages of mouse development investigated so far [9]. GNE is mainly found in the cytosol but Krause et al. [11] very recently demonstrated that GNE is also present in the nucleus.

The enzymatic activity of GNE is regulated by phosphorylation and several protein kinase C isoforms have been coimmunoprecipitated together with GNE from rat liver homogenates [12]. Inactivation of GNE by gene targeting causes early embryonic lethality in mice, thereby emphasizing the fundamental role of this bifunctional enzyme and sialylation in vivo [13]. The clinical relevance of GNE is revealed by a binding defect of the feedback inhibitor CMP-Sia. This leads to a Sia storage disease termed sialuria, in which free Sia accumulates in the cytoplasm, resulting in severe mental retardation of the surviving patients [14]. Further biological significance of this enzyme is illustrated by the observation that in hepatoma the low expression of Sia is correlated with a dramatically reduced activity of GNE [15].

Recently, it was demonstrated that mutations in the human GNE-gene are responsible for hereditary inclusion body myopathy (HIBM), an unique group of neuromuscular disorders characterized by adult onset, slowly progressive weakness and typical muscle pathology [16].

However, the different mutations result in different enzymatic activities but not in different disease phenotypes and, therefore, do not suggest a direct role of the enzymatic function of GNE in the disease mechanism during HIBM [17]. The aim of this study was to find novel functions of GNE, independent of its function as a key enzyme of Sia biosynthesis. For this purpose, we searched for GNE-interacting proteins by using a yeast two-hybrid system. We identified four proteins interacting with GNE in a yeast two-hybrid matrix screen and demonstrated direct protein–protein interaction of GNE with two of these, i.e. the collapsin response mediator protein 1 (CRMP-1) and the promyelocytic leukemia zinc finger protein (PLZF).

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2. Material and methods

2.1. Cell culture

PC12 and HL-60 cells were cultured in suspension. PC12 cells were cultured in RPMI supplemented with 10% horse serum, penicillin (100 U/ml) and streptomycin (100 µg/ml), 2 mM L-glutamine. HL-60 cells were cultured in DMEM supplemented with 20% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml), 2 mM L-glutamine. Mouse embryonic stem cells were grown on gelatin-coated flasks in DMEM supplemented with 15% fetal calf serum, leukemia inhibitory factor (2000 U/ml), 0.1 mM β-mercaptoethanol, 0.2 mM non-essential amino acids, 2 mM L-glutamine and nucleosides. All cells were incubated at 37 °C with 5% CO₂ in a humidified atmosphere and were passaged every 2–3 days. Sf900 insect cells were cultured in suspension at 27 °C in Sf900 II Medium (GibcoBRL) supplemented with 2 mM L-glutamine on a rotation shaker. Cells were passaged every 2–3 days.

2.2. Antibodies

Recombinant His-tagged rat GNE was used as an antigen for immunizing rabbits (Pineda Antibody Services, Berlin, Germany). Anti-GNE antibody was purified from rabbit serum using His fusion protein (His-GNE) bound to CNBr-activated Sepharose® 4B (Pharmacia) according to the manufacturer's instructions.

Antibodies to CRMP1 were purchased from Upstate (clone Y21) and immunoglobulins conjugated to HRP were obtained from Diagnostics.

2.3. Yeast two hybrid assay

The yeast two hybrid assaying was performed in an automated matrix format. The LexA DNA binding domain fusion protein ("bait", GNE-fragment) was expressed in the yeast strain L40ccua [MATa] and screened against a non-redundant set of ~5500 of human activation domain (AD) fusion proteins in L40ccuz [MATα] in an interaction mating matrix approach as described previously [18]. Interactions were detected by assaying the simultaneous activation of the (lexAop)₄-HIS3 and (lexAop)₈-URA3 reporter genes on SDIV selection medium. The activity of the (lexAop)₈-lacZ reporter was measured using a β-galactosidase assay after growth on SDIV agar in an additional experiment [19].

2.4. Construction of the GNE domains

The construction of the GNE and the GNE-domains needed for the two-hybrid screen was described in Blume et al. [8]. Fig. 1 summarizes all domains and constructs used in this study.

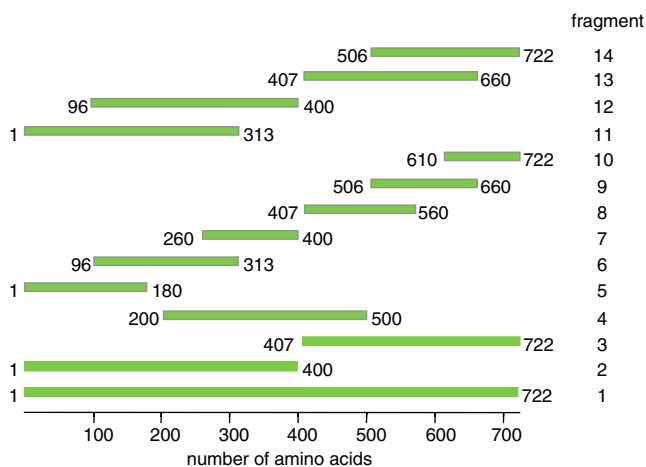


Fig. 1. GNE constructs used for the two-hybrid assay. Fragment 1 contains the entire sequence of the GNE. Fragment 2 represents the epimerase domain of GNE, whereas fragment 3 represents the kinase domain. Numbers represent the amino acids.

2.5. Preparation of cell extracts

Cells were harvested by centrifugation at 900 rpm for 3 min and washed once in PBS. The pellet was resuspended in lysis buffer (PBS, 1 mM PMSF, 1:500 proteinase inhibitory cocktail (Sigma), and cells were sonicated and centrifuged (13000 rpm, 10 min, 4 °C). The supernatant was used for coimmunoprecipitation. Sf900 insect cells were harvested by centrifugation (1000 rpm, 15 min). The pellet was resuspended in lysis buffer (10 mM NaP_i pH 7.5, 1 mM EDTA, 1 mM DTT, 1 mM PMSF), then disrupted in a French press, followed by centrifugation (13000 rpm, 15 min).

2.6. Transfection

HL60-cells expressing or not expressing GNE were transiently transfected with pcDNA3.1/Zeo-GNE plasmid DNA using the Nucleofektor™-Technology of Amaxa Biosystems (transfection program T-19), using 2 × 10⁶ cells per transfection. Cells were resuspended in 100 µl Nucleofektor™ solution V supplemented with 5 µg Plasmid-DNA, then transferred to 1.5 ml pre-warmed medium and incubated for 24 or 48 h at 37 °C and 5% CO₂ prior to further investigation.

2.7. Generation of baculoviruses and protein expression in insect cells

Baculovirus for expression of His-GNE was obtained as described [8]. For generation of a recombinant baculovirus for the expression of GST, the GST-cDNA was amplified by a PCR using the primers 5'-GGA TCC GGA TCC ATG TCC CCT ATA CTA G-3' and 5'-GGA TCC GGA TCC ATG TCC CCT ATA CTA G-3', and the pGEX4T-2 vector (GE Healthcare) as a template. The PCR was performed using the Accuprime Pfx SuperMix (Invitrogen) following the manufacturer's instructions. The PCR product was first cloned into the pCR-Blunt vector (Invitrogen), and then into the pFastBac1 vector (Invitrogen) using the restriction enzymes *Bam*HI and *Eco*RI. After verifying the correct cDNA sequence, baculovirus was generated by the Bac-To-Bac method as described [8].

For generation of a recombinant baculovirus for the expression of GST fusion protein (GST-PLZF), the PLZF-cDNA was amplified by a PCR using the degenerated primers 5'-GTC GAC GTC GAC AGA TGG ATC TGA CAA AAA TGG-3' and 5'-AAG CTT AAG CTT TCA CAC ATA GCA CAG GTA-3', and the cDNA clone IR-AKp961P0229QQ2 (Deutsches Ressourcenzentrum für Genomforschung, Berlin, Germany) as a template. The PCR was performed using the Accuprime Pfx SuperMix (Invitrogen). The PCR product was first cloned into the pCR-Blunt vector (Invitrogen), and then into the pFastBac1-GST vector (see above) using the restriction enzymes *Sal*I and *Hind*III. After verifying the correct cDNA sequence, baculovirus was generated by the Bac-To-Bac method.

Protein expression in insect (Sf9) cells was performed as described [8]. For pilot expression insect cells were infected with baculoviruses at an MOI of 1 for 3 days. Cells were analyzed by SDS-PAGE and revealed similar expression levels for His-GNE, GST and GST-PLZF. Co-expressions were performed by infection of insect cells with the indicated combinations of two baculoviruses under the same conditions, and the cells were then applied to GST pull-down assays.

2.8. Coimmunoprecipitation

Protein A Sepharose (Amersham Biosciences) was washed three times with PBS then incubated with the cell extracts for 1 h at room temperature on a rotator. The Sepharose was centrifuged (900 rpm, 3 min) and washed three times with PBS. The resulting pellet was resuspended in 50 µl SDS loading buffer and incubated at 95 °C for 5 min. Coimmunoprecipitation was analyzed by separating the resulting mixture on SDS-PAGE and by Western blotting using anti-GNE and anti-CRMP1 (Upstate) antibodies.

2.9. GST pull-down assay

GST-PLZF and His-GNE were coexpressed in Sf900 insect cells [8]. Cells were collected by centrifugation (1000 rpm, 15 min) and disrupted in a French press. After centrifugation (13000 rpm, 10 min, 4 °C) the supernatant was used for pull-down assays. Glutathione Sepharose™ 4 Fast Flow (Amersham Biosciences) was washed three times with PBS then incubated with the supernatant from the cell extracts for 1 h at room temperature on a rotator. The Sepharose was centrifuged (900 rpm, 3 min) and washed three times with PBS. The resulting pellet was resuspended in 50 µl SDS loading buffer and

incubated at 95 °C for 5 min. The pull-down assay was analyzed by separating the resulting mixture on SDS-PAGE and by Western blotting using anti-GST (Sigma) and anti-His (Qiagen) antibodies.

2.10. Immunoblotting

Samples were separated on SDS-PAGE and transferred to nitrocellulose filters by Western blotting. The blots were blocked with 5% fat-free dry milk in TBS-Tween for 1 h at room temperature with shaking. Blots were washed three times in TBS-Tween, and then incubated with primary antibody in 5% BSA in TBS-Tween overnight at 4 °C with shaking. After washing, blots were incubated with secondary antibody in TBS-Tween for 1 h at room temperature with shaking. Blots were washed three times with TBS-Tween and proteins were detected by enhanced chemiluminescence (Amersham Buchler) according to the manufacturer's instructions and visualized by exposing the blots to a Fuji imager system (LAS) for time periods between 3 and 10 min.

3. Results

3.1. Characterization of GNE-specific antibodies

Our many attempts to generate GNE-specific antibodies showed a low success rate. However, by immunizing rabbits with GNE expressed in Sf900 cells we generated highly specific GNE antibodies. With these antibodies we were able to detect GNE after immunoprecipitation in mouse embryonic stem cells but not in mouse embryonic stem cells derived from GNE-deficient mice (Fig. 2A). When overexpressing rat GNE in HL60-cells we could detect strong signal after immunoprecipitation (Fig. 2B). We also were able to detect GNE in untransfected HL60- or PC12-cells after immunoprecipitation, indicating that our antibodies do recognize at least mouse (embryonic stem cells), rat (PC12-cells) and human (HL60-cells) GNE (data not shown). The fact that we did not detect GNE in direct immunoblotting experiments indicates the very low expression of GNE in non-transfected cells (data not shown). The low expression of GNE was already shown for all tissues investigated during the purification and cloning of GNE in 1997 [5]. The activity of GNE in most organs includ-

ing skeletal muscle was below the detection limit of 100 μ U/mg tissue [5]. The lowest expression of GNE m-RNA was found in human skeletal muscle [6]. However, our antibodies have been shown to detect both native and denatured GNE from mouse, rat and human GNE.

3.2. GNE-interacting proteins

Sialylation seems to be a prerequisite for life since inactivation of the key enzyme of Sia-biosynthesis, GNE, causes embryonic lethality [13]. However, it is not clear at present whether the decrease in sialylation, the absence of the GNE protein or both are responsible for the embryonic lethality in GNE-deficient mice. Heterozygous GNE-deficient mice possessing only 50% GNE-activity are viable, although they express 25% less Sia on their cell surfaces compared to wild-type animals [20]. This is of special interest because mutations in the GNE cause HIBM, but no other defect in any organ due to missing or reduced sialylation has been detected in HIBM-patients so far. Since it is well accepted that protein-protein interactions regulate the behavior of cells, we tried to identify proteins that directly interact with the GNE.

We constructed 14 fragments of the rat GNE (Fig. 1), which were used as baits in a yeast two-hybrid assay, and screened a prey matrix containing more than 5500 human cDNAs [18]. The matrix was assembled by individual subcloning of a non-redundant set of cDNA fragments from a human fetal brain library (HEX1 [21]) and by "GATEWAY recombinational cloning" of full-length human open reading frames from entry vectors into activation domain (AD) yeast two-hybrid assay vectors. The yeast two-hybrid assay protein matrix is an unbiased, representative subset of the human genome with respect to ORF length and Gene Ontology annotation [18]. In the matrix screening procedure, all prey proteins have been tested for interaction with the GNE baits individually by interaction mating so that each interacting pair has the same probability of being identified [22]. The interactions found in the screening procedure were retested in an independent, second yeast two-hybrid assay. Yeast two-hybrid interactions between four different prey proteins and bait fragment 1 (representing the entire GNE) or bait fragment 3 (representing the kinase domain of the GNE) were positive in both assays. Identified prey sequences are the promyelocytic leukemia zinc finger protein (PLZF), the (RIF1), two independent clones of the collapsin response mediator protein 1 (CRMP-1) and KIAA 1549 (Table 1).

3.3. GNE interacts directly with PLZF

To verify GNE-PLZF interactions, we expressed PLZF fused to GST and GNE with a His-tag. Both fusion proteins were further used for pull-down assays (Fig. 3). The GST-fused PLZF was coexpressed together with the His-tagged

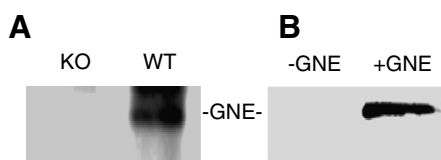


Fig. 2. Characterization of GNE-specific antibodies. A. GNE was immunoprecipitated from cytosol of mouse embryonic stem cells (WT) and GNE-deficient mouse embryonic stem cells (KO). Precipitates were analyzed by Western blot. Note that in GNE-deficient mouse embryonic stem cells no GNE is detectable. B. HL60 cells were transfected with cDNA encoding rat GNE. Lysates of transfected (+GNE) and untransfected cells (-GNE) were analyzed after immunoprecipitation by Western blot.

Table 1
GNE-interacting molecules identified in the yeast two-hybrid assay

Bait	Prey clone (protein)	Swiss-Prot entry	Selection medium	β -Galactosidase-assay	Amino acid number of the Swiss-Prot entry
1	299b (PLZF)	ZBT16_human	+	+	380–673
1	452b (RIF1)	RIF1_human	+	+	455–739
3	132a (CRMP1)	DPYL1_human	+	+	390–572
3	419b (CRMP1)	DPYL1_human	+	+	345–572
3	16E09 (KIAA1549)	Q5BJD6_human	+	+	1–110

The numbering of the baits is according to the numbering of the GNE fragments in Fig. 2.

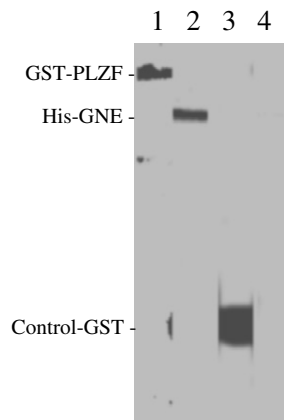


Fig. 3. Pull-down of his-tag GNE with GST-PLZF. GST fusion protein (GST-PLZF) and His fusion protein (His-GNE) were coexpressed in Sf900 insect cells. Cells were lysed and lysate (2 mg/ml) containing both his-tag GNE and GST-PLZF or GNE and GST-control were incubated for 1 h. Mixtures of molecules were incubated with GST-coupled to Sepharose. Precipitated proteins were separated by SDS-PAGE and immunoblotted using anti GST-antibodies (lane 1) or anti his antibodies (lane 2). For control we used a control GST-protein instead of GNE. Precipitated proteins were again separated by SDS-PAGE and immunoblotted using anti GST-antibodies (lane 3) or anti-his antibodies (lane 4).

GNE in Sf900 cells and the GST-fusion portion was then precipitated. In this precipitate we identified both PLZF (lane 1) and GNE (lane 2). As a control, GNE with a His-tag was incubated with the GST-fusion protein without the PLZF domain. In these precipitates we found only the GST fusion domain (lane 3) but not GNE (lane 4), demonstrating that GNE with a His-tag does not unspecifically bind to the GST fusion portion.

Coimmunoprecipitation is also one of the accepted methods for verifying protein–protein interactions at the cellular level. Unfortunately, we could not detect PLZF-specific bands by Western blot analysis after coimmunoprecipitations using several cell lines including GNE-overexpressing HL60 cells, PC12 cells or ES cells (data not shown).

3.4. CRMP-1 can be coimmunoprecipitated together with GNE

To verify the interaction between GNE and CRMP1 in a cellular environment, we performed coimmunoprecipitation experiments using rat PC12-cells. CRMP1 is highly expressed in PC12 cells (Fig. 4A). After enrichment of GNE by immunoprecipitation we were able to detect GNE in PC12-cells



Fig. 4. Coimmunoprecipitation of GNE with CRMP1. A. Cytosol of PC12 cells was analyzed using anti-CRMP1 antibodies. B. Cytosol of PC12 cells was prepared and GNE was precipitated using polyclonal anti-GNE antibodies and detected by Western blot (lane 1). For control a precipitation was performed using a mouse anti-rabbit antibody and no GNE could be detected (lane 2). C. GNE precipitates were analyzed using a CRMP1-specific antibody. CRMP1 could be detected in the GNE-precipitates (lane 1), but not in the control precipitate (lane 2).

(Fig. 4B, lane 1). As a negative control, a precipitation was performed with a mouse anti-rabbit antibody and no GNE could be detected (Fig. 4B, lane 2). When analyzing this sample with a CRMP1-specific antibody, we detected CRMP1 in the GNE-precipitates (Fig. 4C, lane 1), but no CRMP1 in the control precipitate (Fig. 4C, lane 2). Our results demonstrate that CRMP1 was coimmunoprecipitated with GNE from PC12 cells.

4. Discussion

Sia is the most common naturally occurring terminal carbohydrate of a variety of glycoconjugates [2]. The aim of our study was to find novel functions of GNE, independent of its function as a key enzyme of Sia biosynthesis. For this purpose, we searched for GNE-interacting proteins by performing a yeast two-hybrid screen. We identified CRMP1 and PLZF as interacting molecules and could verify the interactions between CRMP1 or PLZF with GNE using pull-down assays or coimmunoprecipitations. Furthermore, we generated highly specific antibodies to GNE and found that GNE is expressed at very low levels in several cell lines.

CRMP1 belongs to a family of proteins (CRMP1-5) and is very similar to the Ulip protein (mouse), TOAD-64 (rat) or Unc-33 (*C. elegans*) [23,24]. The human members of the CRMP family are represented by the family of the dihydropyrimidinase-related proteins [25]. The first indication that Sia biosynthesis and CRMP proteins are linked was provided by experiments in which the incubation of PC12 cells with Sia precursors resulted in reduced expression of TOAD-64 [26]. CRMP1 is mainly expressed within the nervous system [24], but also in a variety of lung tumors with high invasive potential [27,28]. The interaction of GNE and CRMP1 might therefore be of interest in the progression of (lung)-cancer. Furthermore, CRMP1 has been shown to be involved in the organization of the cytoskeleton by interacting with the rho kinase α [29], but is not known whether the interaction of GNE with CRMP1 modulates its interaction with the rho kinase α , in which case GNE could be involved in the organization of the cytoskeleton.

Whether CRMP1 is participating in the onset or progression of HIBM or other muscle defects is not known. But it is generally accepted that CRMP1 and related proteins are involved in cell differentiation. Therefore, one could speculate that a disturbed interaction of CRMP1 with a mutated GNE plays a role in onset or progression of HIBM.

PLZF is a transcription factor notable for its BTB (BR-C, ttk, bab/Pox) domain [30]. It regulates the expression of Hox genes, which play a crucial role during development [31,32]. Very recent studies suggest that BTB domains interact with cullin3 [33,34]. Cullin3 is a component of the E3-ligases, which are involved in the ubiquitinylation of proteins. Ubiquitinylation is a very important modification of proteins. Monoubiquitinylation is involved in cellular trafficking of proteins [35] and protein phosphorylation [36]. In contrast, polyubiquitinylation is a signal for degradation by the proteasome [37]. Binding of PLZF to GNE might therefore have consequences for the localization of GNE or for protein degradation. The localization of GNE is still not understood. Sia is biosynthesized in the cytosol and GNE is also mainly expressed in the cytosol. However, the activation of Sia with CMP occurs within the nucleus

and Krause and coworkers [11] found GNE also in the nucleus. Nevertheless, it is possible to speculate about the interaction between PLZF and GNE and their role during protein degradation. HIBM patients show abnormal accumulation of proteins in muscles [38]. Misrouted GNE or PLZF could interact with each other thus disturbing the balance between polyubiquitinylation and degradation via the proteasome and thereby be responsible for the accumulation of proteins. Research on PLZF focuses on acute promyelocytic leukemia. Unfortunately, nothing is known on muscle disorders and a possible role of PLZF. However, PLZF knockout mice display distinct musculoskeletal defects [39] and myogenesis in mice is accompanied by increased expression of *LAZ3*, a protein very similar to PLZF [40]. Finally, Inoue and coworkers could demonstrate, very recently a role of PLZF in early osteoblastic differentiation [41].

Taken together, we here propose that GNE might not only be the key enzyme of Sia synthesis, but it might also regulate fundamental cellular functions by interacting during development with key proteins such as CRMP1 or PLZF.

Acknowledgements: The authors acknowledge Ilona Danßmann and Christiane Kilian for technical assistance and Dr. T.A. Scott for improving the English of the manuscript. This work received financial support by the Deutsche Forschungsgemeinschaft (RH), the Mizutani-Foundation (RH), the Fonds der Chemischen Industrie (RH) and the German-Israeli Foundation for Scientific Research & Development (SH). The work in the AG Neuroproteomics (MDC Berlin) was supported by the NGFN (KB-P4T03, 01GR0471).

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