REVIEW

Allosteric Activation of Yeast Enzyme Neutral Trehalase by Calcium and 14-3-3 Protein

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Summary

Neutral trehalase 1 (Nth1) from *Saccharomyces cerevisiae* catalyzes disaccharide trehalose hydrolysis and helps yeast to survive adverse conditions, such as heat shock, starvation or oxidative stress. 14-3-3 proteins, master regulators of hundreds of partner proteins, participate in many key cellular processes. Nth1 is activated by phosphorylation followed by 14-3-3 protein (Bmh) binding. The activation mechanism is also potentiated by Ca^{2+} binding within the EF-hand-like motif. This review summarizes the current knowledge about trehalases and the molecular and structural basis of Nth1 activation. The crystal structure of fully active Nth1 bound to 14-3-3 protein provided the first high-resolution view of a trehalase from a eukaryotic organism and showed 14-3-3 proteins as structural modulators and allosteric effectors of multi-domain binding partners.

Key words

14-3-3 protein • Trehalase • Calcium • Trehalose • Allostery • Conformation • Enzyme • Crystal structure

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Introduction

Trehalose (α-D-glukopyranosyl-α-D-glukopyranoside) is a non-reducing disaccharide first discovered in ergot of rye in 1832. Trehalose has been known as trehalose since 1858 when Marcellin Berthelot isolated this disaccharide as sweet trehala manna from weevil cocoons (reviewed in Elbein 1974, Nwaka and Holzer 1998). In the following decades, trehalose was discovered also in the yeast *S. cerevisiae* (Koch and Koch 1925) and in bacteria, plants, fungi, insects and other invertebrates but never in mammals (Elbein 1974, Thevelein 1984b, Nwaka and Holzer 1998). Because no trehalose synthesis has been shown in vertebrates, the trehalose pathway can be a target for the development of drugs against pathological fungi (Van Dijck *et al.* 2002, Foster *et al.* 2003, Petzold *et al.* 2006). The production of trehalose is induced by adverse living conditions, such as drought, extremely low or high temperatures, oxidative stress or toxic chemicals (Nwaka *et al.* 1995, Zahringer *et al.* 1997).

Trehalose can be synthetized from UDP-glucose and glucose-6-phosphate using two-step catalysis by a complex of trehalose-6-phosphate synthase (TPS1) and trehalose-6-phosphate phosphatase (TPS2) (Vandercammen *et al.* 1989, Londesborough and Varimo 1984). Trehalose accumulation protects cell membranes and proteins against damage, and trehalose can also act as a signaling molecule connecting its metabolism with glucose transport and with glycolysis (reviewed in Wiemken 1990).

In some bacterial species, trehalose was identified as a part of cell walls, especially in

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aktinomycetes (reviewed in Elbein *et al.* 2003). Cell trehalose levels depend on the cell cycle phase, nutrition and living environment. The content of trehalose is crucial for fungi spore germination (Rousseau *et al.* 1972, Thevelein 1984b). Moreover, trehalose is the main compound of the insect haemolymph and is crucial for insect flight. Thus, trehalase, the enzyme responsible for trehalose hydrolysis, is frequently used as a target for the development of new insecticides (Becker *et al.* 1996, Gibson *et al.* 2007). Trehalose accumulation is also a common feature of many anhydrobiotic organisms, which can survive decades without water (Crowe *et al.* 1984, Crowe *et al.* 1992).

Yeast with low trehalose concentrations are more sensitive to draught and heat damage, whereas yeast with high trehalose levels are more likely to survive (Hottiger *et al.* 1987). In the yeast *S. cerevisiae* trehalose is one from the main storage saccharides and form from 1 % to more than 23 % dry cell mass, depending on the living conditions and the cell cycle phase (Lillie and Pringle 1980). Many studies have confirmed the connection between trehalose accumulation in yeast cells and their tolerance to temperature extremes, dehydration or cyclic freezing and melting. These studies in yeast suggested that trehalose functions not only as carbohydrate storage or energy reserve but also as a membrane and protein protector during low levels of intracellular water (reviewed in Wiemken 1990, Nwaka and Holzer 1998). The ability of trehalose to protect proteins against denaturation and to protect other cellular components *in vitro* correlates with the protective function against various environmental stresses *in vivo* (De Virgilio *et al.* 1994, Hottiger *et al.* 1994).

Trehalase, the enzyme hydrolyzing trehalose, was first discovered in *Aspergillus niger*, in 1893, and Hermann Emil Fischer described a similar enzyme in *S. cerevisiae* two years later (Fischer 1895). Trehalases (α,α-trehalose-1-C-glukohydrolases) are part of the Glycoside hydrolase family 37 (EC 3.2.1.28) of O-Glycosyl hydrolases (EC 3.2.1.) which includes enzymes with mutual trehalase activity identified in many different organisms, from bacteria to fungi, plants and animals (Elbein 1974, App and Holzer 1989, Kopp *et al.* 1993). Trehalases are conserved enzymes that catalyze the hydrolysis of one of two glycoside bonds of trehalose (Kopp *et al.* 1993, Kopp *et al.* 1994, Nwaka and Holzer 1998, Bock *et al.* 1983). In the yeast *S. cerevisiae*, trehalose can be hydrolyzed by the neutral trehalases Nth1 and Nth2, which share 73 % identity, and by the acid trehalase Ath1 (Thevelein 1984a, Thevelein 1984b, Nwaka *et al.* 1995). Nth1 and Nth2 are located in the cytosol, whereas Ath1 is present in vacuoles. The pH optimum of phosphorylated Nth1 (pNth1) is 7.0, in contrast to Ath1 with a pH optimum 4.5 (Londesborough and Varimo 1984, Wiemken 1990, App and Holzer 1989). The molecular weight of Nth1 is 86 kDa, and this enzyme shows strict specificity for trehalose with no evidence of hydrolysis of other tested disaccharides, such as cellobiose, maltose, lactose, sucrose, raffinose or melibiose (App and Holzer 1989, Gibson *et al.* 2007).

Nth1 regulation of trehalose plays a key role in yeast metabolism and in the activities of important enzymes, including hexokinase (Nwaka and Holzer 1998). Disruption of the vegetative rest of yeast and fungi spores by heat shock or glucose addition sharply increases trehalase activity (Van Assche *et al.* 1972, Thevelein *et al.* 1982, van der Plaat 1974). Nth1 activation by glucose is a reversible process (Thevelein and Jones 1983). After heat shock, Nth1 expression increases with trehalose synthase expression and with trehalose concentration (Nwaka *et al.* 1995, Nwaka *et al.* 1996). These seemingly useless cyclic trehalose conversions during heat shock are likely necessary for maintaining the cytosolic glucose concentrations constant, which is essential for heat shock survival (Hottiger *et al.* 1987, Nwaka *et al.* 1995, Nwaka *et al.* 1996). Moreover, trehalose maintains proteins and membranes in their native state under high temperatures and decreases protein aggregation. However, the complete trehalose degradation is required for recovery after heat shock (Singer and Lindquist 1998). Nth1 expression is also induced by chemical stresses, such as $CuSO_4$ and $NaAsO_2$ (toxic chemicals), H_2O_2 (oxidative stress) or cycloheximide (proteosynthesis inhibition), thus suggesting that Nth1 participates in defense mechanism against oxidative stress or toxins. Conversely, osmotic stress-induced processes cause no changes in trehalose concentrations (Zahringer *et al.* 1997).

The yeast Nth1 has a unique N-terminal extension, in comparison with other trehalases from different organisms (Nwaka and Holzer 1998, Veisova *et al.* 2012), which can be phosphorylated by cAMP-dependent proteinkinase (PKA) or by cyclin dependent kinase 1 (Cdk1) (Uno *et al.* 1983, Ortiz *et al.* 1983, van der Plaat 1974, Panni *et al.* 2008, Veisova *et al.* 2012). Other structural domain features include a conserved catalytic domain (Nth1-CD, residues 180-751) and a Ca^{2+}-binding domain (Nth1-CaBD,

residues 96-176) with a so-called EF-hand-like motif (Fig. 1A). Nth1 can be activated by combination of Ca^{2+}-binding (Franco *et al.* 2003, Kopecka *et al.* 2014) and phosphorylation (Ortiz *et al.* 1983, Uno *et al.* 1983) followed by 14-3-3 protein binding (Panni *et al.* 2008, Veisova *et al.* 2012). Moreover, studies show that Nth1 activation is closely related to yeast cell cycle progression and metabolism (Zhao *et al.* 2016, Ewald *et al.* 2016) (Fig. 1B).

The same activation mechanism can be also expected for Nth2 whose primary structure differs only in the additional 32 amino acids within the N-terminal part but the PKA phosphorylation motifs and both Ca^{2+}-binding and catalytic domains remain highly conserved. The function of Nth2 was partially revealed by the deletion of *NTH2* gene in *S. cerevisiae* which did not influence the trehalose metabolism but increased the sensitivity to heat shock (Nwaka *et al.* 1995).

Trehalose and glycogen, the most important carbohydrate storages of yeast, are highly abundant in slow-growing cells and can serve as long-term carbon reserves. Moreover, they enable cell cycle progression during starvation (Ewald *et al.* 2016, Francois and Parrou 2001). Their levels are under strong cell cycle control, facilitated by a key cell cycle regulator, Cdk1, and by the metabolic regulator PKA (Guillou *et al.* 2004, Kuenzi and Fiechter 1969, Muller *et al.* 2003, Ewald *et al.* 2016, Zhao *et al.* 2016). The relationship between PKA- and Cdk1-dependent Nth1 activation has not been studied yet, but studies have already shown that the regulatory subunit of PKA (Bcy1) is a substrate for Cdk1, and three G1 cyclins have multiple PKA sites. Moreover, PKA activity peaks at the Start of the cell cycle, when Cdk1 activity is also high. Thus, these two kinases may potentiate each other while regulating Nth1 (Muller *et al.* 2003, Zhao *et al.* 2016, Holt *et al.* 2009, Ubersax *et al.* 2003). At the G1/S transition, Cdk1 and PKA phosphorylate and activate Nth1 and glycogen phosphorylase (Gph1), thereby causing trehalose and glycogen breakdown, respectively. In the early G1 phase, in slow-growing cells, carbohydrate storage levels rise, whereas in the late G1 phase, when cells enter the cell cycle, both main storage sugars are broken down into glucose. During the S/G2/M phases, Nth1 releases trehalose to fuel biosynthesis (Fig. 1B). Thus, catabolism can be coordinated with cell cycle progression and final cell division (Kuenzi and Fiechter 1969, Sillje *et al.* 1999, Muller *et al.* 2003, Zhao *et al.* 2016). The constant concentration of Nth1 during the cell cycle confirms that its catalytic activity, rather than its abundance, is regulated by phosphorylation (Ewald *et al.* 2016).

Yeast *S. cerevisiae* has two 14-3-3 protein-encoding genes (*BMH1* and *BMH2*), which are highly homologous and essential for most laboratory yeast

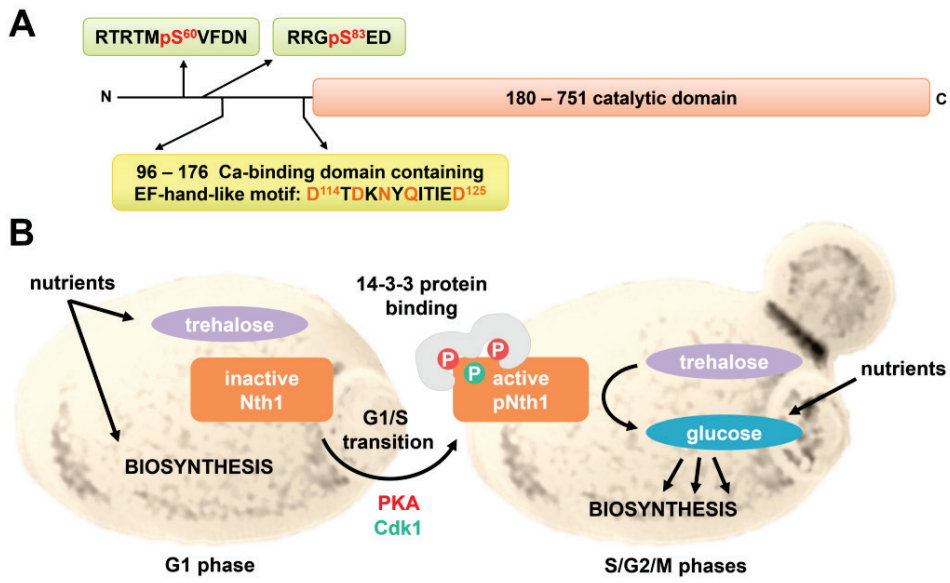


Fig. 1. Yeast neutral trehalase (Nth1) domain structure and function. (**A**) Domain structure of Nth1. The 14-3-3 binding motifs within the N-terminal extension, the calcium-binding domain (Nth1-CaBD) and the catalytic domain (Nth1-CD) are shown in green and yellow and salmon, respectively. The positions of PKA phosphorylation sites crucial for 14-3-3 binding are in red. Residues involved in calcium ion coordination are shown in orange, indicated within the sequence of the EF-hand-like motif. (**B**) Nth1 phosphorylation and activation is closely linked to yeast metabolism and to cell cycle progression. Intracellular trehalose levels are under strong cell cycle control facilitated by the cell cycle regulator Cdk1 and by the metabolic regulator PKA. In the G1 phase the excess nutrients can be stored in a form of storage carbohydrates trehalose and glycogen which can be utilized in following S, G2 and M phases and used for biosynthesis. The stored trehalose cannot be hydrolyzed until the Nth1 is phosphorylated and activated at the G1/S transition. Yeast 14-3-3 protein (Bmh) binding is essential for full Nth1 activation and trehalose breakdown. During the S/G2/M phases, Nth1 hydrolyzes trehalose into two molecules of glucose for biosynthesis. Thus, catabolism can be coordinated with cell-cycle progression and final cell division (Alblova *et al.* 2017, Ewald *et al.* 2016, Veisova *et al.* 2012, Zhao *et al.* 2016).

strains (van Heusden and Yde Steensma 2006, van Heusden 2009). Bmh proteins, originating from a conserved 14-3-3 protein family, interact with and affect the structure, localization, activity and thus the function of hundreds of different proteins. They play a key role in regulating several important cellular processes, such as metabolism, cell cycle, apoptosis and transcription or signal transduction (Aitken 1996, Fu *et al.* 2000). 14-3-3 proteins are expressed in all eukaryotic cells, their molecular weight is approximately 30 kDa, and they form rigid and highly stable homo- or heterodimers (Liu *et al.* 1995, Xiao *et al.* 1995). Each 14-3-3 protomer is formed by nine antiparallel α-helices, and the rigid structure of 14-3-3 dimer has the typical cup-like shape with a large central channel containing two amphipathic ligand-binding grooves (Obsil and Obsilova 2011). Thanks to their rigidity, 14-3-3 proteins can function as scaffolding molecules or platforms for their binding partners (Yaffe 2002). In addition, the presence of two ligand binding grooves in the 14-3-3 protein dimer allows the simultaneous binding of two different ligands or the binding of one ligand using two phosphorylated motifs. This review summarizes the state of the art on Nth1 activation and regulation by phosphorylation followed by 14-3-3 protein and Ca^{2+} binding.

Structural studies on trehalases

The first high-resolution trehalase structure reported was the crystal structure of prokaryotic trehalase Tre37A from *E. coli* (Gibson *et al.* 2007). The structure of Tre37A consists of a $(\alpha/\alpha)_6$ barrel, which is a typical structural feature of other toroidal glycosylases such as glukoamylase (Aleshin *et al.* 1994), chitobiose phosphorylase (Hidaka *et al.* 2004) or maltose phosphorylase (Egloff *et al.* 2001). All these enzymes originate from the six-hairpin glycosidase superfamily, and they catalyze the anomeric inversion of substrate configuration. Recently, our group reported the crystal structure of the yeast Nth1 (Alblova *et al.* 2017). The active site of these two trehalases is located in a deep pocket, buried within the enzyme structure (Fig. 2) (Gibson *et al.* 2007, Alblova *et al.* 2017). Therefore, a significant conformational change may be necessary for the substrate/trehalose entry and product/glucose

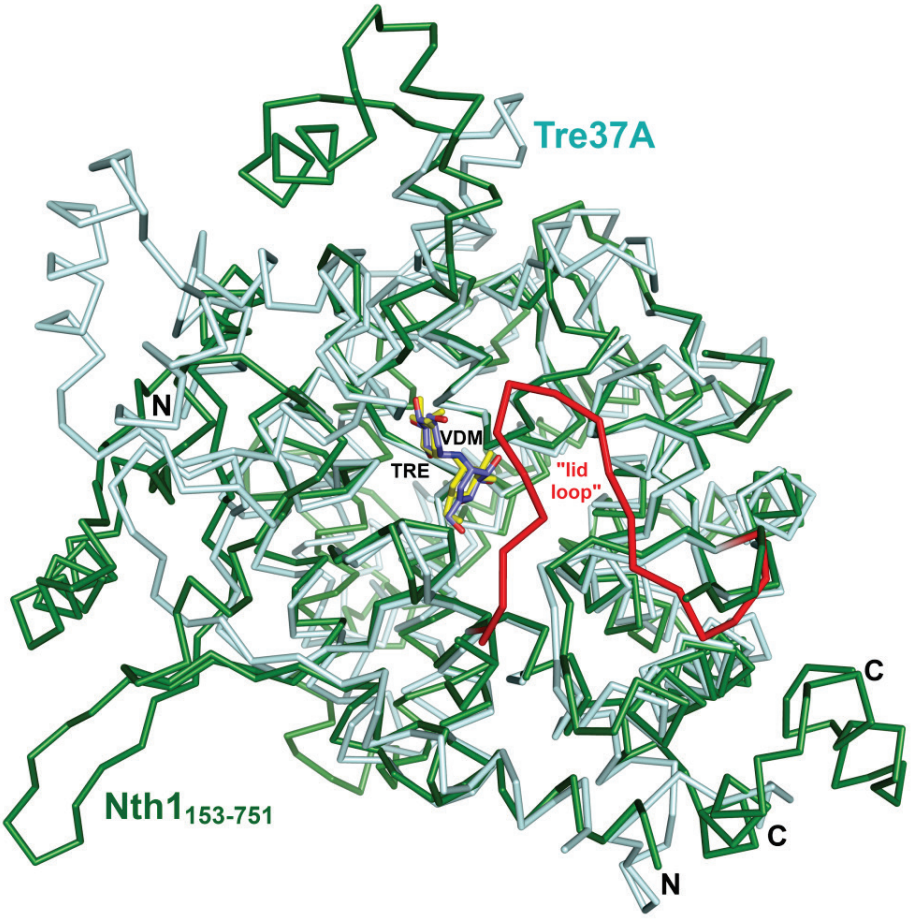


Fig. 2. Superimposition of the catalytic domains of yeast $Nth1_{153-751}$ (green, PDB: 5M4A) and periplasmic trehalase Tre37A from *E. coli* (cyan, PDB: 2JF4) (Alblova *et al.* 2017, Gibson *et al.* 2007). The Nth1-CD is shown in green, Tre37A is shown in light cyan and its "lid loop" in red. The active sites of Nth1 and Tre37A contain trehalose (TRE, in yellow) and validoxylamine A (VMD, in blue), respectively.

departure. In *E. coli* Tre37A, glycosidic hydrolysis proceeds according to the canonical mechanism, which includes the nucleophilic attack of the substrate by water molecule. In this case, the residue D312 plays the role of a catalytic acid and the residue E496 of a Brønsted base during the hydrolytic reaction (Gibson *et al.* 2007). The structure of the catalytic domain of $Nth1_{153-751}$ bound to trehalose (TRE) shows similar features to those of the Tre37A structure bound to the inhibitor validoxylamine A (VDM) (Fig. 2 - green versus cyan). In comparison with Tre37A, Nth1 residues D478 and E674 act as catalytic acid and base, respectively, as confirmed by site-directed mutagenesis (Alblova *et al.* 2017). Furthermore, the superimposition of both trehalase structures revealed a key difference: in the Tre37A:VDM complex, the active site is completely covered by a loop containing polyglycine stretch (Tre37A sequence S^{501}TTGTGGGGG**EY**PLQDGF^{518}, shown in red in Fig. 2). However, in the $Nth1_{153-751}$:TRE complex, this loop is disordered, likely due to its high flexibility (Nth1 sequence T^{679}RGTDPHRVEA**EY**GNQGADFKGAATE GF^{706}). However, this loop region contains a pair of EY residues critical for catalysis conserved among all trehalases. Differences in the length, sequence and flexibility of these loop regions suggest that the regulation of yeast neutral trehalases substantially differ from that of prokaryotic trehalases.

Regulation of yeast neutral trehalase

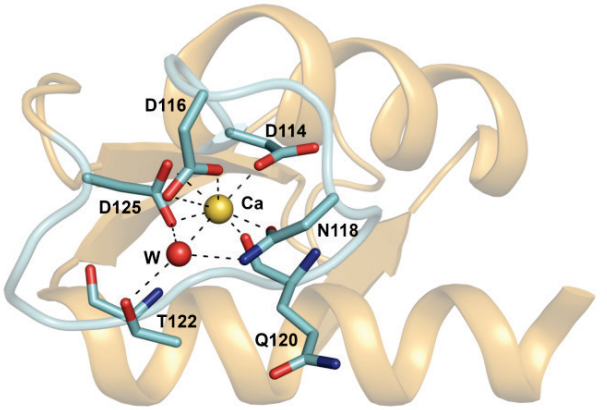
In the yeast *S. cerevisiae*, Nth1 activity is regulated at both transcriptional and posttranslational levels. *NTH1* expression is induced by different stress conditions, such as heat shock, chemicals or oxidative stress (Nwaka *et al.* 1995, Zahringer *et al.* 1997). Nth1 is regulated through phosphorylation by PKA, whose activity depends on cellular cAMP levels, and by Cdk1. The unique N-terminal extension of Nth1 contains four phosphorylation sites, namely Ser20, Ser21, Ser60 and Ser83, which are targets of PKA (Schepers *et al.* 2012, Panni *et al.* 2008, Veisova *et al.* 2012), and Ser66, which is phosphorylated by Cdk1 (Holt *et al.* 2009, Ewald *et al.* 2016, Zhao *et al.* 2016). The N-terminal extension is followed by a Ca^{2+} binding domain (Nth1-CaBD, residues 96−176) containing the EF-hand-like motif (sequence D^{114}TDKNYQITIED^{125}) and the catalytic domain (Nth1-CD, residues 180–751), which is conserved among trehalases from different species (Amaral *et al.* 1997) (Fig 1A). Sequence alignment of all trehalases available in UniProt showed that the N-terminal extension and the CaBD are distinct features of fungi trehalases.

Biochemical and biophysical characterization of the interaction between yeast 14-3-3 proteins and PKA phosphorylated Nth1 (pNth1) revealed that both Bmh1 and Bmh2 isoforms form stable complexes with pNth1 with 2:1 molar stoichiometry (Bmh dimer binds one Nth1 molecule) and dramatically increase the catalytic activity of pNth1. Moreover, site-directed mutagenesis revealed that the phosphorylation sites Ser60 and Ser83 function as 14-3-3 binding motifs and are essential for pNth1 activation both *in vitro* and *in vivo* (Veisova *et al.* 2012).

The regulation of Nth1 activity by PKA phosphorrylation is a good example of reciprocal regulation of degradation and synthesis. During this process, trehalose-6-phosphate synthase is inactivated by cAMP-dependent phosphorylation and reactivated by dephosphorylation (Panek *et al.* 1987). This is in line with trehalose degradation by Nth1 activated by cAMP-dependent phosphorylation and inactivated by dephosphorylation (Ortiz *et al.* 1983, App and Holzer 1989, Londesborough and Varimo 1984). Moreover, Dcs1, an mRNA decapping enzyme, inhibits 14-3-3 protein binding and acts as the negative regulator of trehalase activity. Thus, Dcs1 maintains Nth1 in the inactive form and prevents nonsense trehalose cyclic synthesis and degradation (Liu *et al.* 2002, De Mesquita *et al.* 2003, Schepers *et al.* 2012). Dcs1 deletion causes Nth1 activation but the deletion of both Bmh1 and Bmh2 proteins precludes Nth1 activation (Schepers *et al.* 2012).

Ca^{2+}-dependent Nth1 activation requires Ca^{2+} binding to the EF-hand-like motif within the Nth1-CaBD. Ca^{2+}- (or Mn^{2+}-) dependent Nth1 activation can be inhibited by chelating agents such as EDTA or EGTA (Londesborough and Varimo 1984, App and Holzer 1989, Amaral *et al.* 1997, Franco *et al.* 2003). Ca^{2+}-dependent Nth1 activation is much weaker than 14-3-3-dependent activation (Amaral *et al.* 1997, Franco *et al.* 2003, Veisova *et al.* 2012, Soto *et al.* 1999). However, the highest Nth1 activity was observed in the presence of both Ca^{2+} and 14-3-3 protein. The EF-hand-like motif was first discovered in neutral trehalase from *S. cerevisiae* and *K. lactis* (Amaral *et al.* 1997) and subsequently in other yeast strains, such as *S. pombe*, *Candida albicans*, *Aspergillus nidulans* and *Neurospora crassa* (Soto *et al.* 1998, d'Enfert *et al.* 1999, Eck *et al.*

1997). EF-hand-like motifs of neutral trehalases from different yeast strains contain aspartic acid residues at conserved positions 1, 3 and 12 and an asparagine residue at position 5 (Franco *et al.* 2003, Kopecka *et al.* 2014, Alblova *et al.* 2017). Mutational analysis of *S. cerevisiae* Nth1 revealed that the residues at positions 1, 5 and 12 of its EF-hand-like motif (D114, N118 and D125) are crucial for the Ca^{2+}-dependent enhancement of the catalytic activity in the presence of 14-3-3 (Fig. 3) (Kopecka *et al.* 2014). Taken together, these results show that Nth1 is activated by phosphorylation followed by 14-3-3 protein binding and that the entire process is potentiated in the presence of Ca^{2+} (Franco *et al.* 2003).



EF-hand-like motif of Nth1											
1	2	3	4	5	6	7	8	9	10	11	12
D^{114}	T	D	K	N	Y	Q	I	T	I	E	D^{125}

Fig. 3. Close-up view of Nth1-CaBD containing EF-hand-like motif (shown in blue) and residues crucial for Ca^{2+} coordination. The Ca^{2+} ion and the water molecule (W) are shown in yellow and red, respectively. The sequence of the motif is shown in lower panel.

14-3-3 proteins

The general mechanism of 14-3-3 protein action can be classified into three categories: 1) direct structural change of the bound partner, if the bound partner is an enzyme, this can affect its catalytic activity; 2) physical occlusion of sequence-specific or structural features of the target protein; and 3) stabilization of protein-protein interactions within multiprotein complexes (reviewed in (Aitken 1996, Fu *et al.* 2000, Tzivion and Avruch 2002, Bridges and Moorhead 2005, Obsil and Obsilova 2011)). Only few high-resolution crystal structures of 14-3-3 protein complexes have been reported until now. The first was the structure of the complex between 14-3-3ζ and serotonin N-acetyl-transferase (AANAT) (Obsil *et al.* 2001, Klein *et al.* 2003). Susbequently, structures of the 14-3-3:C-terminal part of the plasma membrane H^+-ATPase complex and the ternary complex of rice 14-3-3:florigen Hd3a:FD1 transcription factor peptide were reported (Ottmann *et al.* 2007, Taoka *et al.* 2011). Recently, the structure of two other 14-3-3 protein complexes with full-length binding partners were solved: 14-3-3:heat shock protein beta-6 (HSPB6) and 14-3-3:pNth1 (Alblova *et al.* 2017, Sluchanko *et al.* 2017a). Additional structural information was collected using low-resolution approaches, including studies on 14-3-3 complexes with transcription factor FOXO4 (Obsilova *et al.* 2005, Silhan *et al.* 2009), on the regulatory domain of human tyrosine hydroxylase (Obsilova *et al.* 2008), on the regulator of G-protein signalization 3 RGS3 (Rezabkova *et al.* 2010, Rezabkova *et al.* 2011), on protein kinases ASK1 and CaMKK2 (Kosek *et al.* 2014, Petrvalska *et al.* 2016, Psenakova *et al.* 2018), on Chibby (Killoran *et al.* 2015), on steroidogenic acute regulatory protein STARD1 (Sluchanko *et al.* 2017b) and on caspase-2 (Kalabova *et al.* 2017). The comparison of 14-3-3 protein structures in the absence and presence of ligands showed no significant structural changes in the 14-3-3 protein. This structural rigidity is caused by extensive hydrophobic interactions among α-helices of 14-3-3 protein. This enables 14-3-3 proteins to function as stable rigid platform on which the conformation of their binding partners can be shaped (Yaffe 2002).

14-3-3 protein-dependent activation of Nth1 is a bona fide example of mode 1 regulation (direct structural change of bound partners) and resembles 14-3-3 protein-dependent AANAT activation (Obsil *et al.* 2001, Alblova *et al.* 2017). In both cases, 14-3-3 protein enhances the catalytic activity of the bound enzyme by inducing structural changes in the close proximity of the active site. These structures also revealed that interactions between 14-3-3 and its ligand could extend beyond those involving phosphorylated motifs and the ligand-binding groove. The involvement of less conserved regions in these contacts may explain the observed isoform-binding specificity among 14-3-3 isoforms and their ligands.

14-3-3 proteins usually recognize one of three types of specific phosphoserine (pS) or phosphotreonine (pT) motifs, namely type I: R[S/Φ][+]pSXP, type II:RX[S/Φ][+]pSXP and type III:pS-X1-2-COOH, where Φ is an aromatic residue, + is a basic residue and X can be any amino acid residue, except cysteine (Rittinger

et al. 1999, Yaffe *et al.* 1997, Ganguly *et al.* 2005). However, these motifs vary widely because the number of 14-3-3 protein-binding partners is still increasing, and they may contain phosphorylated, unphosphorylated or even glycosylated motifs, which differ from the optimal motifs (Johnson *et al.* 2010, Masters *et al.* 1999, Toleman *et al.* 2018).

Structural basis of Ca^{2+}- and 14-3-3-dependent Nth1 activation

The Nth1-CaBD is placed within the 14-3-3 central channel bordering both phosphorylated motifs and interacting with 14-3-3 helices H1, H3 and H9 and with the loop between H1 and H2 (Fig. 4A)(Alblova *et al.* 2017). Nth1-CaBD consists of three α-helices and two antiparallel β-sheets, and the EF-hand-like is located between α3 and β2 and is responsible for the Ca^{2+} coordination (Fig. 3). The structure of the catalytic domain of the 14-3-3-bound full-length $pNth1_{1-751}$ (Fig. 4A in salmon) is very similar to that of the isolated catalytic domain $Nth1_{153-751}$, except for the "lid loop" located between helices α28 and α29. In complexed $pNth1_{1-751}$, this "lid loop" covers the active site of pNth1, as shown in the Tre37A structure (Fig. 2 in red). However, in the isolated catalytic domain $Nth1_{153-751}$, the "lid loop" is disordered, likely due to its high flexibility (Fig. 2 in green). Although the Tre37A "lid loop" is

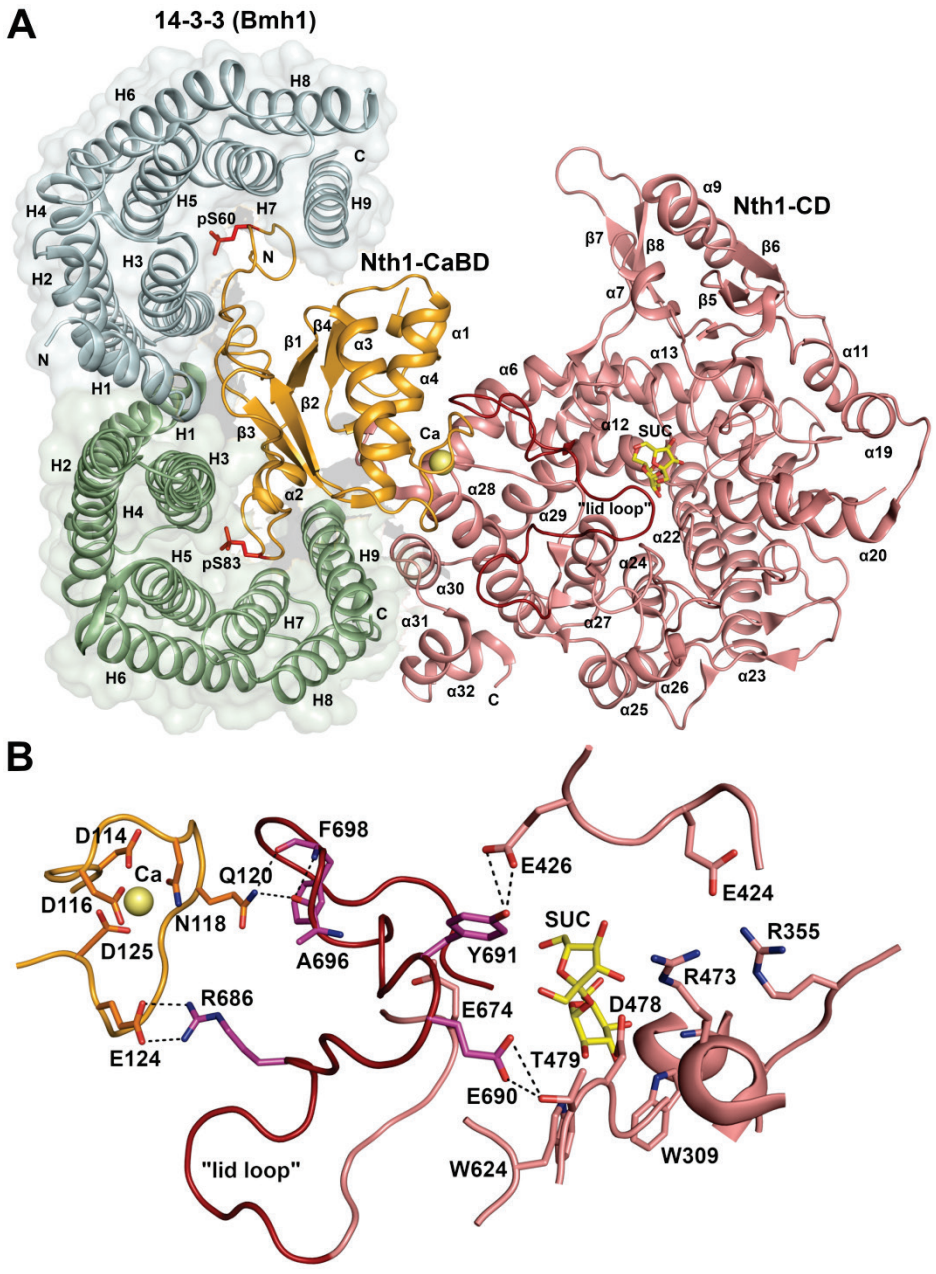


Fig. 4. Structure of the $pNth1_{1–751}$:14-3-3 complex. (**A**) $pNth1_{1–751}$: yeast 14-3-3 (Bmh1) crystal structure (PDB: 5N6N). The N-terminal extensions of $pNth1_{1–751}$ and the Ca-binding domain (Nth1-CaBD) are shown in orange. The catalytic domain of Nth1 (Nth1-CD) is shown in salmon. 14-3-3 protomers are shown in cyan and green. The C-terminal portion of Nth1 "lid loop" and phosphorylated serines 60 and 83 are highlighted in red. The Ca^{2+} ion within the EF-hand-like motif and sucrose (SUC) in the Nth1 active center are in yellow. (**B**) Close-up view of the "lid loop" interactions with the EF-hand-like motif of Nth1-CaBD and the Nth1-CD regions surrounding the active site. The Nth1 "lid loop" residues are labeled in purple, the Nth1-CaBD residues are labeled in orange and Nth1-CD residues in salmon (Alblova *et al.* 2017).

stabilized by contacts with bordering regions, the "lid loop" of complexed pNth1 is structured as a result of extensive interactions with the EF-hand-like motif (Fig. 4B). This motif is an integral part of Nth1-CaBD, which is held in the proper orientation with the respect to Nth1-CD by the 14-3-3 protein. Therefore, the "lid loop" of yeast Nth1 is stabilized by the proper orientation of Nth1-CaBD and Nth1-CD by the scaffolding protein 14-3-3. The tip of the stabilized lid loop then provides residues E690 and Y691 crucial for catalysis (Gibson *et al.* 2007, Alblova *et al.* 2017). Calcium-binding stabilizes the interface between Nth1-CaBD and Nth1-CD and thus potentiates 14-3-3-mediated pNth1 activation. This mechanism is consistent with the previously published results of the hydrogen-deuterium exchange coupled to mass spectrometry (HDX-MS) analysis, which suggested that both Nth1-CaBD (Fig. 5 in red) and several regions within Nth1-CD surrounding the active site and the "lid loop" (Fig. 5 in blue and "lid loop" in green) undergo significant structural changes in the presence of Ca^{2+} and/or 14-3-3 protein (Kopecka *et al.* 2014, Macakova *et al.* 2013). Moreover, the sequence alignment of *S. cerevisiae* neutral trehalases Nth1 and Nth2 and neutral trehalases from other yeast species such as *Candida albicans*, *Kluyveromyces lactis* or *Schizosaccharomyces pombe* revealed that all yeast neutral trehalases possess the combination of PKA phosphorylation sites, Ca^{2+}-binding and catalytic domains and distinct "lid loop" sequences indicating that the regulation in 14-3-3 dependent manner is a general feature for all yeast neutral trehalases (Alblova *et al.* 2017).

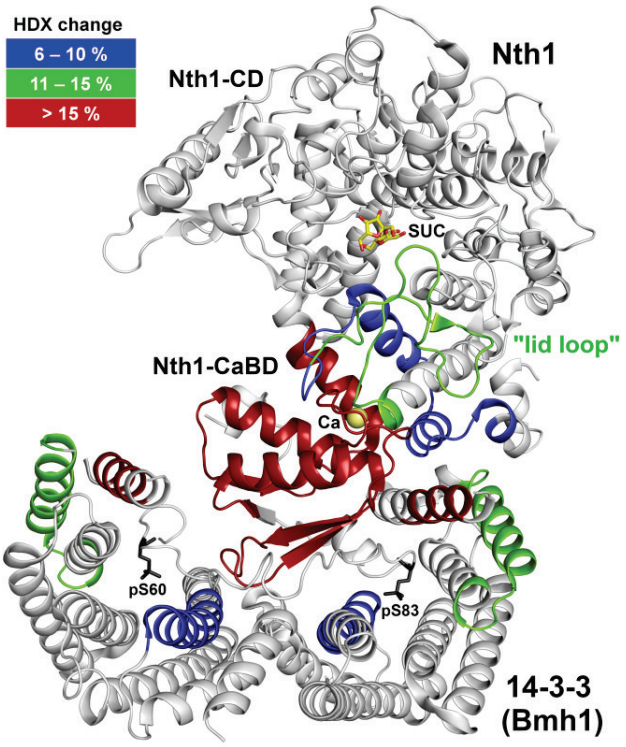


Fig. 5. HDX-MS reveals conformational changes of $pNth1_{1-751}$ or 14-3-3 protein (Bmh1). The regions that show slower deuterium exchange kinetics upon pNth1:14-3-3 complex formation mapped onto the crystal structure of $pNth1_{1-751}$:14-3-3 complex (PDB: 5N6N, shown in grey). Regions with 6-10 % HDX change after 10 min of deuteration are shown in blue, 11-15 % in green and higher than 15 % in red (Macakova *et al.* 2013, Kopecka *et al.* 2014, Alblova *et al.* 2017).

14-3-3 binding induces Nth1 disorder-to order-transition

Bioinformatic studies revealed that 90 % of 14-3-3 protein binding partners are partly or totally disordered and that their 14-3-3 binding motifs are mostly located within intrinsically disordered regions. These regions are most often present at their N- or C-termini or bordering their functional domains (Bustos and Iglesias 2006, Johnson *et al.* 2010). The plasticity and flexibility of disordered regions provide several functional advantages to the signaling proteins, such as binding to different binding partners and/or the sensitive control of their binding affinities (Dyson and Wright 2002, Wright and Dyson 2009). The next reason for the participation of disordered regions in binding interactions can be the improved ability to search for suitable binding partners, in contrast to compact, perfectly folded proteins with limited conformational flexibility (Shoemaker *et al.* 2000). Thus, the 14-3-3 protein binding to disordered segments can lead to significant disorder-to-order transitions in their binding partners. HDX-MS data suggested that this is also the case of pNth1, especially Nth1-CaBD, where a dramatic decrease in deuteration kinetics was observed upon the complex formation (slower HDX is consistent with a more structured state). 14-3-3-mediated pNth1 disorder-to-order transition was also shown by small-angle X-ray scattering (SAXS) measurements (Kopecka *et al.* 2014). The dimensionless Kratky plots, calculated from the scattering data of Nth1, yeast 14-3-3 (Bmh1) and their complex, suggest that 14-3-3 alone and the pNth1:14-3-3 complex exhibit substantially lower conformational flexibility than Nth1 alone. The scattering data of compact globular proteins in this plot show a maximum value of 1.104 at sRg ~1.73 (showed by grey lines in Fig. 6) (Receveur-Brechot and Durand 2012).

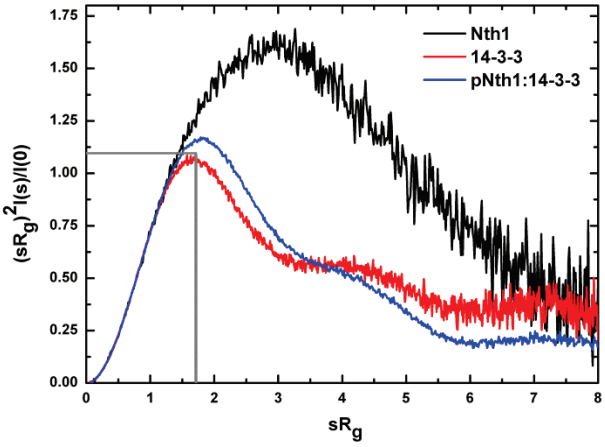


Fig. 6. Dimensionless Kratky plots. SAXS data collected at the corresponding protein concentrations are shown in black for Nth1 (1.8 mg/ml), in red for yeast 14-3-3 (Bmh1) (8.6 mg/ml) and in blue for the pNth1:14-3-3 complex mixed at 1:2 molar stoichiometry (15 mg/ml). Grey lines mark the maximum at a value of 1.104 for sR_g = 1.732, a typical attribute for the scattering data of compact globular proteins (Kopecka *et al.* 2014).

Conclusions

14-3-3 proteins serve as "readers" of phosphorylated motifs, and by these interactions they regulate the function of a number of biologically important signaling processes. Yeast neutral trehalases, crucial enzymes for yeast metabolism, differ from those of other prokaryotic and higher eukaryotic organisms because they have an N-terminal extension, with two 14-3-3 binding motifs and with an Nth1-CaBD, and a distinct "lid loop" sequence within Nth1-CD. 14-3-3 protein binding modulates Nth1 activity by enabling the proper three-dimensional configuration of Nth1-CD and CaBD domains relative to each other, thus stabilizing the flexible "lid loop" over the active site and providing residues important for catalysis.

Conflict of Interest

There is no conflict of interest.

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