The guanine nucleotide exchange factor Gea1 rescues an isoform-specific 14-3-3 phenotype

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Abstract
14-3-3 proteins are abundant modulators of cellular processes, in particular signal transduction. They function by binding to a broad spectrum of client proteins, thus affecting client protein localisation or function [1] [2]. Animals and plants express 14-3-3 proteins encoded by several genes, which has made it difficult to study their unique rather than shared functions. The yeast Saccharomyces cerevisiae possesses only two highly homologous 14-3-3 genes, BMH1 and BMH2. Using this model system we now uncover novel aspects of functional specificity between the two yeast 14-3-3s. We show that bmh1 but not bmh2 cells display an altered morphology of the endomembrane system and specific trafficking defects under glucose starvation. This but not a second phenotype specific to the bmh1 strain, that is, the accumulation of glycogen, was rescued by overexpression of the nucleotide exchange factor Gea1, suggesting a role for Bmh1 in Gea1’s function or regulation.

Objective
Here, we aimed at dissecting specific cellular roles of Bmh1 using the YFP reporter assay and morphological analysis by transmission electron microscopy.

Introduction
Substantial progress has been made in understanding the interaction of 14-3-3 proteins with individual client proteins at a molecular level and in elucidating the functional consequences of individual interactions [4] [5]. However, in many experiments addressing the cellular roles of 14-3-3, they are treated as a generic entity although they are always present as a complex mixture of different homo- and heterodimers, and individual isoforms have been shown to bind with different affinities to a single substrate [6]. Since mammals have seven different isoforms and plants have thirteen, it has been challenging to study their functional divergence in these organisms, and our understanding of the unique functions of the different 14-3-3 isoforms is limited. This problem is beginning to be tackled in different model organisms - Xenopus laevis [7] and Arabidopsis thaliana [8] - and several instances of 14-3-3-specific interactions with well-studied clients such as the plant plasma membrane proton pump [9] or the ERK1/2 scaffold KSR1 [10] have been described and mechanistically dissected. The genome of Saccharomyces cerevisiae encodes only two highly homologous 14-3-3 proteins, Bmh1 and Bmh2 (93% sequence identity), and either deletion strain is viable. In most laboratory strains the double deletion strain is not viable, illustrating the existence of overlapping functions of Bmh1 and Bmh2 and consistent with a largely overlapping interactome of the yeast 14-3-3 [11]. However, distinct phenotypes have been described in the individual deletion strains [12] [13] [14]. We set out to use yeast as a simple model system to further dissect isoform-specific 14-3-3 functions. It has been shown that a yeast multimeric membrane reporter protein that exposes arginine (R)-based COPI binding motifs (Pmp2-cc-YFP-LRKRS, from now on referred to as YFP reporter) is mislocalised specifically in bmh1 deletion cells [13]. Importantly, overexpression of BMH2 in a bmh1 strain did not rescue the specific trafficking phenotype of the bmh1 strain, consistent with the notion that the phenotype is not merely due to changes in 14-3-3 protein abundance in the cell, but that it indeed represents functional specificity between the two isoforms.
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Figure Legend

Gea1 rescues bmh1-specific phenotypes, that is, alterations in protein trafficking along the secretory pathway and endomembrane morphology.

(a) Localisation of a YFP reporter (Pmp2-cc-YFP-LRKRS) in log-phase cultures before and 90 min after glucose withdrawal (plus D, minus D) in the indicated strains. FM4-64 [3] labels vacuolar (vac) membranes, to assign staining patterns to either the vacuole or the perinuclear ER for quantification. Scale bar 5 μm.

(b) Quantification of subcellular YFP-reporter localisation as in (a). Cells showing predominantly ER, equal ER and vacuole, or predominantly vacuole staining were counted. Between 422 and 491 cells were counted from 6 biological replicates. Error bars are shown only in the negative direction and are standard error of the mean (S.E.M), p values were calculated using a two-tailed Student’s test.

(c-h) bmh2 shows reduced vesicular traffic, and increased glycogen accumulation under glucose starvation. Representative electron micrographs of (c) wild type cells showing multivesicular bodies, (e) bmh2 showing vesicle clusters, (g) bmh1 showing glycogen accumulation. Scale bars, black=1,000 nm, white=500 nm. CW: cell wall, ER: endoplasmic reticulum, MVB: multivesicular body, VC: vesicle cluster, LD: lipid droplet, G: glycogen, V: vacuole. For quantification, electron micrographs of each strain were examined and several morphological features were quantified: (d) MVBs, (f) VC (>3 vesicles) and (h) glycogen accumulation. Wild type n=391, bmh1 n=342, bmh2 n=361. *** indicates p<0.001, * indicates p<0.005 as calculated using the chi-squared test.

(i) Photograph of the indicated strains spotted onto SC plates and exposed to iodine vapour reveals increased glycogen content in bmh1 strain.

(j, k, l) Quantification of electron micrographs obtained as in (c), from bmh1 strains expressing GEA1 and bmh1 and wild type strains transformed with an empty vector as indicated, (j) MVBs, (k) VC (>3 vesicles), (l) glycogen accumulation. wt + empty vector n=405, bmh1 + empty vector n= 409, bmh1 + GEA1 n=401. *** indicates p<0.001, * indicates p<0.005 as calculated using the chi-squared test.

(m) Localisation of YFP reporter in log phase cultures expressing HA-GEA1 from the strong PGK promoter in either wt, bmh1 or bmh2 cells. FM4-64 marks vacuole membranes.

Results & Discussion

Bmh1-specific function in protein transport along the secretory pathway

To better understand the specific role of Bmh1 in membrane transport of the YFP reporter we assessed this phenomenon quantitatively by determining the subcellular localisation of the reporter in wild type, bmh1 and bmh2 knockout strains (Fig. 1a, b). In logarithmic phase, the reporter was predominately localised to the vacuole of wild type and bmh2 cells (left panels (a)) in ca. 80% of cells, quantification in (b). In contrast, the reporter localised to the vacuole of only 30% and was visible in the ER of 65% of bmh1 cells, suggesting that trafficking of our YFP reporter along the secretory pathways was compromised. These phenotypes became even more pronounced during stationary phase [13]. Since stationary yeast cultures typically experience glucose deprivation we assayed whether this was the relevant parameter determining the Bmh1 requirement for reporter localisation. Indeed, upon glucose starvation, the requirement for Bmh1 was even more pronounced and the reporter only fully reached the vacuole in 10% of cells, as opposed to 50% in the wild type and bmh2 deletion strains (Fig. 1a, b). We concluded that the role of the 14-3-3 Bmh1 in targeting the YFP reporter to the vacuole is particularly relevant under conditions of glucose starvation.

Bmh1 but not Bmh2 is required to maintain the global morphology of the endomembrane system.

To assess the global morphology of the endomembrane system, we investigated the wild type, bmh1 and bmh2 deletion strains after glucose starvation using transmission electron microscopy (TEM) (Fig. 1c, e, g). Qualitative assessment revealed three different subcellular structures whose abundance varied with the genotypes, that is, multivesicular bodies (MVBs, Fig. 1c), vesicle clusters (VC, Fig. 1e) and glycogen granules (Fig. 1g). Quantification revealed that the bmh1 strain possessed less MVBs (Fig. 1d) and less VC's (Fig. 1f), consistent with global alterations in vesicular traffic. Furthermore, we
observed substantial accumulation of glycogen granules in the \textit{bmh1} strain (Fig. 1h), which was independently confirmed by iodine staining (Fig. 1i).

The guanine nucleotide exchange factor Gea1 rescues Bmh1-specific alterations in the endomembrane system/vesicular transport but not glycogen accumulation

Guanine nucleotide exchange factor (GEF) proteins activate the GTPase Arf1 by conversion of GDP-Arf1 to active GTP-Arf1. In consequence, the N-terminal myristoylation moiety is exposed and is able to anchor Arf1 in the Golgi membrane, where it recruits COPI coat components and initiates the process of COPI budding \[15\]. Gea1 is one of four GEFs found in yeast \[16\]. Together with mammalian GBF1, Gea1 and Gea2 are part of a subfamily of GEFs distinct from the Sec7 subfamily. Different functionalities of the subfamilies are highlighted by the fact that \textit{GEA1} cannot suppress the temperature sensitivity of a \textit{sec7} mutant and vice versa. Phosphorylation of GBF1 was shown to have a critical role in Golgi disassembly \[17\] and \textit{gea1/2} mutants showed disrupted Golgi in yeast \[18\], supporting the notion that they have similar roles in the two systems. Furthermore, Gea1 has been shown to increase the rate of GTP\(\gamma\)S binding to Arf1 \textit{in vitro} \[16\] suggesting that it may affect COPI-dependent processes \textit{in vivo}.

In mammalian cells, AMPK kinase is known to phosphorylate the GEF GBF1 \[17\] \[19\]. It is thought that this regulation attenuates the function of GBF1 when the intracellular concentration of ATP drops. Therefore, yeast AMPK may regulate Gea1 function in response to glucose availability. Hence, we tested whether we could suppress Bmh1-specific phenotypes by overexpression of \textit{GEA1}, to date the better studied of the two yeast homologues of GBF1 \[19\], to supply the cell with additional GEF activity and thus counteract a putative down-regulation of GEF function in the \textit{bmh1} deletion strain (Fig. 1j, k, l, m). Indeed, overexpression of \textit{GEA1} restored the localisation of the YFP reporter to the vacuole in the \textit{bmh1} strain (Fig. 1m), just as observed for the wild type and \textit{bmh2} strains. Compare Fig. 1a right, without \textit{GEA1} overexpression and Fig. 1m showing a cell in which \textit{GEA1} was overexpressed. Similarly, \textit{GEA1} overexpression in the \textit{bmh1} strain increased the abundance of MVBs (Fig. 1j) and VCs (Fig. 1k) to levels even higher than in the wild type strain. In contrast, the accumulation of glycogen granules in the \textit{bmh1} background was unaffected by overexpression of the Arf-GEF \textit{GEA1} (Fig. 1l). These results are consistent with the notion that the global alterations in vesicular traffic observed in the \textit{bmh1} strain (reduction of reporter trafficking to the vacuole, reduction in MVBs and VCs) are downstream of Gea1 function and that Gea1 is less functional in the absence of this 14\-3\-3 protein. However, overexpression of \textit{GEA1} did not suppress glycogen accumulation in the \textit{bmh1} strain. This indicates that Bmh1 action is upstream of Gea1 in a pathway that culminates in changes in vesicular trafficking, yet Bmh1 function is independent of Gea1 in the pathway leading to glycogen accumulation.

\textit{GEA1} overexpression rescues the isoform-specific protein trafficking defect seen in \textit{bmh1} strains, suggesting that Bmh1 has a regulatory effect on the activity of Gea1. Gea1 did not abrogate the glycogen build-up in \textit{bmh1} cells, indicating that Bmh1 has additional effects on pathways distinct from the protein-trafficking processes involving Gea1.

It would be interesting to test whether the effect of Bmh1 on Gea1 is direct or via some upstream regulatory factor and to identify this factor. This could then delineate the various pathways specifically affected by Bmh1 and enable elucidation of the underlying molecular mechanisms.

Although \textit{GEA1} and \textit{GEA2} are redundant in some functions, and the double deletion is inviable \[16\], the genes have only 51\% sequence identity, and independent functions have been demonstrated. Spang \textit{et al.} showed that \textit{GEA1} and \textit{GEA2} differentially rescue COPI mutant strains, and that the corresponding mutants have differing synthetic lethality with \textit{arf1} \[20\]. Only \textit{gea2} is lethal in combination with \textit{arf1}, indicating it has roles other than Arf1 activation. It has been linked to Arf3 for example as having a role in actin organisation \[21\]. It remains unknown whether \textit{GEA2} would also rescue the Bmh1-specific phenotypes seen here; if not it would further demonstrate the differential specificity of the GEFs, and beg the question whether or not both Gea1 and Gea2 are regulated in a Bmh1-mediated manner.

Snf1, the yeast AMPK kinase, and Reg1, a regulatory subunit of the phosphatase Glc7,
which is known to regulate Snf1 and may also act on Snf1 substrates [22], may provide a further link between Bmh1 and Gea1. Reg1 is one of the best-characterised binding partners of Bmh1 [11] [23] [24]. The reg1 deletion strain also accumulates glycozen [25], similar to the bmh1 strain. Snf1’s main function is to adapt the cell to changes in glucose availability [26] [27] [28]. It is tempting to propose that Bmh1 may impact on a phosphorylation-controlled activation mechanism targeting Gea1, thus providing another layer of regulation of Gea1’s GEF activity. The first step in testing this hypothesis would be to demonstrate differential phosphorylation of endogenous Gea1 in different bmh deletion strains.

Additional Information

Methods and Supplementary Material
Please see https://scien mematters.io/articles/201609000004.

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Ethics Statement
Not Applicable.

Citations


Dombek K. M., Kacheryovsky N., and Young E. T. "The Reg1-interacting Proteins, Bmh1, Bmh2, Ssb1, and Ssb2, Have Roles in Maintaining Glucose Repression in Saccharomyces cerevisiae". In: Journal of Biological Chemistry 279.37 (June 2004), pp. 39165-39174. DOI: 10.1074/jbc.m400433200. URL: http://dx.doi.org/10.1074/jbc.m400433200.