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Human pancreatic β -cell glucokinase: subcellular localization and glucose repression signalling function in the yeast cell

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Running title: Glucose repression signalling by pancreatic glucokinase

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SUMMARY

Human pancreatic β -cell glucokinase (GK_{β}) is the main glucose phosphorylating enzyme in pancreatic β -cells. It shares several structural, catalytic and regulatory properties with hexokinase 2 (Hxk2) from *Saccharomyces cerevisiae*. In fact, it has been previously described that expression of GK_{β} in yeast could replace Hxk2 in the glucose signalling pathway of *S. cerevisiae*. In this study we report that GK_{β} exerts its regulatory role by association with the yeast transcriptional repressor Mig1; the presence of Mig1 allows GK_{β} to bind to the *SUC2* promoter, helping in this way in the maintenance of the repression of the *SUC2* gene under high-glucose conditions. Since a similar mechanism has been described for the yeast Hxk2, our findings suggest that the function of the regulatory domain present in these two proteins has been conserved throughout evolution. In addition, we report that GK_{β} is enriched in the yeast nucleus of high-glucose growing cells, whereas it shows a mitochondrial localization upon removal of the sugar. However, GK_{β} does not exit the nucleus in the absence of Mig1, suggesting that Mig1 regulates the nuclear exit of GK_{β} under low-glucose conditions. We also report that binding of GK_{β} to Mig1 allows the latter protein to be located at the mitochondrial network under low-glucose conditions.

Keywords: Pancreatic β -cell glucokinase; Hxk2; *Saccharomyces cerevisiae*; glucose repression; Mig1

Abbreviations: ChIP, chromatin immunoprecipitation; GAD, Gal4 activating domain; GBD, Gal4 DNA binding domain; GFP, green fluorescent protein; RFP, red fluorescent protein; GK_{β} , β -cell glucokinase; GK_L , liver glucokinase; GKRP, glucokinase regulatory protein; SD, synthetic complete medium containing glucose as carbon source;

INTRODUCTION

Glucokinase (GK), a member of the hexokinase family (hexokinase IV) [1], plays an essential role in glucose phosphorylation in the liver and endocrine β -cells of the pancreas [2]. GK also acts as a glucose sensor, integrating blood glucose levels and insulin release in pancreatic β -cells [3] and regulating glucose metabolism in the liver [2]. Hepatic GK shows dynamic nuclear-cytoplasm localization depending on the amount of glucose in blood. The nuclear localization of hepatic GK is regulated by its binding to a glucokinase regulatory protein (GKRP) under low-glucose conditions [4, 5]. In addition, it has been recently described that hepatic GK could also be associated with liver mitochondria [6, 7]. The GK subcellular distribution in the pancreatic β -cell is also regulated by glucose: low levels of glucose causes an association of GK with mitochondria [8], whereas high glucose levels prevents GK translocation to this organelle [8]. In β -cells, GK has also been localized on the secretory granules and, although it has been suggested that glucose causes dissociation of glucokinase from this location [9], later studies suggest that glucokinase is an integral component of the granule and does not translocate during glucose stimulation [10].

Pancreatic GK $_{\beta}$ and the yeast hexokinase 2 (Hxk2) proteins have several structural and functional similarities. For example, (i) pancreatic GK $_{\beta}$ is a monomer without allosteric regulation by glucose-6-P; (ii) yeast Hxk2, like pancreatic GK $_{\beta}$, has a glucose-regulated subcellular distribution [11, 12], in higher glucose conditions Hxk2 shows a nuclear enrichment that is absent under low-glucose conditions; (iii) both Hxk2 and GK $_{\beta}$ proteins play a vital role in glucose signalling in the yeast and the pancreatic β -cell respectively. Current evidence suggests that the main role of *S. cerevisiae* Hxk2 in the glucose signalling pathway is achieved by its interacting with both Mig1 and Snf1. It has been proposed that Hxk2 inhibits the phosphorylation of the Mig1 repressor, when the cells are growing in high glucose conditions, maintaining in this way the transcriptional repression of target genes [13, 14]. Since GK $_{\beta}$ plays a prominent role in pancreatic β -cell signalling, (inactivation of one glucokinase allele leads to maturity-onset diabetes of the young 2, whereas loss of both alleles is associated with permanent neonatal diabetes [15, 16]), GK $_{\beta}$ is considered as a promising drug target for diabetes therapy. Thus, the study of the regulatory properties of GK $_{\beta}$ is an important matter to be examined.

In this work, we took advantage of the facility to manipulate the *S. cerevisiae* genetic system and the similarity between the yeast Hxk2 and pancreatic GK β to study the role of GK β in the yeast glucose signalling pathway and also to study the regulation of the different subcellular localization that this enzyme presents in yeast cells.

MATERIALS AND METHODS

Strains and growth media

GFP and RFP-fluorescence experiments utilized yeast strain H250 (MAT α SUC2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mig1- δ 2::LEU2) [17], W303-1A (MATa ura3-52 trp1-289 leu2-3,112 his3- Δ 1 ade2-1 can1-100) [18], DBY1315 (MAT α ura3-52 leu2-3,2-112 lys2-801 gal2) [19], DBY2052 (MAT α hxk1::LEU2 hxk2-202 ura3-52 leu2-3,2-112 lys2-801 gal2) [19], Δ snf1 [20], Δ snf1 Δ hxk1 Δ hxk2 [20], Y03694 (MATa his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0 msn5::kanMX4) (euroscarf) and FMY388 (MAT α his3 Δ 1 ura3 Δ 0 leu2 Δ 0 met15 Δ 0 MIG1::gfp), the latter containing a gfp tagged *MIG1* ORF at its chromosomal location. Yeast two-hybrid experiments employed strain Y187 (MAT α ura3-52 his3-200 ade2-101 trp1-901 leu2-3,112 gal4 Δ gal80 Δ URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ) [21].

Escherichia coli DH5 α (*F* ϕ 80dlacZ Δ M15 *recA1 endA1 gyrA96 thi-1 hsdR17*(*r_k*-*r_k*-) *supE44 relA1 deoRA* (*lacZ* *YA-argF*)U169) was the host bacterial strain for the recombinant plasmid constructions.

Yeast cells were grown in the following media: YEPD, high-glucose (2% glucose, 2% peptone, and 1% yeast extract), YEPE, low-glucose (0.05% glucose, 3% ethanol, 2% peptone, and 1% yeast extract) and synthetic media containing the appropriate carbon source and lacking appropriate supplements to maintain selection for plasmids (2% glucose (SD); or 3% ethanol and 0.05% glucose; and 0.67% yeast nitrogen base without amino acids). Amino acids and other growth requirements were added at a final concentration of 20-150 μ g/ml. The solid media contained 2% agar in addition to the components described above.

Plasmids

The yeast expression plasmids YEp352-HXK2, YEp352-HXK2/gfp and pWS-GST/GK β were constructed as indicated previously [13, 22].

Plasmids YEp352-GK β and pWS93-GK β carried a 1398-bp DNA fragment with the complete coding region of human pancreatic glucokinase gene (*GK β*). Plasmid YEp352-GK β was generated by cloning 1398 bp DNA fragment, synthesized by PCR using plasmid pWS-GST/GK β [22] as the template and the primer pair OL1 (sense: CAGATAGGATCCATGCTGGACGACAGAGCCAGG) and OL2 (antisense: CTTCAGAATTCTCTACTGGCCCCAGCATACAGGC) into the *Bam*HI-*Eco*RI site of YEp352-HXK2. The YEp352-GK β plasmid expresses the complete coding region of *GK β* gene from the *HXK2* promoter. To make plasmid pWS93-GK β , an *Eco*RI-*Sal*I fragment obtained from pWS-GST/GK β was subcloned into the *Eco*RI-*Sal*I site of pWS93 vector.

Plasmid YEp352-GK β /gfp and YEp352-GK γ /gfp were constructed as follows: a 969-bp *Bam*HI-*Bgl*II fragment containing the *gfp* gene was subcloned into YEp352-HXK2 plasmid first cleaved with *Bam*HI. Then, a 1398-bp *Bam*HI PCR fragment (OL3: CGTAGGATCCATGGCTATGGATACTACAAG and OL4: GCTAGGATCCCGCCTGGGCCAGCATGCAAGCC) containing the complete coding region of *GK β* gene or a 1398-bp *Bam*HI PCR fragment (OL5: CGTAGGATCCATGGCTATGGATACTACAAG and OL6: GCTAGGATCCCGCCTGGGCCAGCATGCAAGCC) containing the complete coding region of *GK γ* gene were subcloned into a previously *Bam*HI digested YEp352-HXK2/gfp plasmid. The resulting plasmids express GK β or GK γ from the *HXK2* promoter as fusion proteins with GFP.

For two-hybrid analysis, plasmids pGBKT7-MIG1, pGBKT7-MIG1S311A and pGBKT7-HXK2 were constructed as indicated previously [13, 14, 23]. Plasmids pGBKT7-GK β and pGADT7-GK β carried a 1398-bp DNA fragment with the complete coding region of *GK β* gene. To make plasmid pGBKT7-GK β a *Eco*RI-*Sal*I fragment obtained from pWS93-GK β carrying the complete *GK β* gene was subcloned into the *Eco*RI-*Sal*I sites of pGBKT7. To make plasmid pGADT7-GK β , an *Eco*RI-*Sal*I fragment containing the complete coding region of *GK β* gene obtained from pWS93-GK β plasmid was subcloned into an *Eco*RI-*Xho*I previously cleaved pGADT7 vector. The DNA sequence of all PCR-generated constructs was verified by sequencing and all the clones used were verified by sequencing analysis of fusion points.

Fluorescence microscopy

Yeast strains expressing the GK β -GFP, GK γ -GFP, Hxk2-GFP, Mig1-GFP or

Su9-RFP fusion proteins were grown to early-log phase (OD_{600} of less than 0.8) in synthetic high-glucose medium (SD-ura). Half of the culture was shifted to synthetic low-glucose medium (SE-ura) for 1 h. The media contained the appropriate carbon source and lacked the appropriate supplements to maintain selection of plasmids. Cells (25 μ l) were loaded onto poly L-lysine-coated slides, and the remaining suspension was immediately withdrawn by aspiration. One microlitre of DAPI (2.5 μ g/ml in 80% glycerol) was added, and a covert slide was placed over the microscope slide. GFP, RFP and DAPI localization in live cultures was monitored by direct fluorescence using a Leica DM5000B microscope. To avoid the non-linear range of fluorescent signals, cells highly overexpressing GFP-tagged fusion protein were excluded from further analyses. The localization of proteins was monitored by visual inspection of the images. At least 100 cells were scored in each of at least three independent experiments. The distribution of fluorescence was scored in the following way: N, denotes a nuclear fluorescence signal; C, cytoplasmic fluorescence signal without nuclear or mitochondrial fluorescence signals; M, mitochondrial fluorescence signal.

To stain cells with Mitotracker® Red 580, yeasts were immobilized on poly L-lysine-coated slides. Then, they were incubated with 0.5 μ M MitoTracker® diluted in DMSO and glycerol 80% (v/v) for 20 min at room temperature. Finally, MitroTracker® solution was aspired and a glass cover slide was immediately placed over the microscope slide. Images were processed in Adobe Photoshop CS.

Yeast two-hybrid analysis

The yeast two-hybrid analysis [24] employed yeast vectors pGADT7 and pGBDKT7 and host strain Y187 (described above), in accordance with the Matchmaker two-hybrid system 3 from Clontech. Transformed yeasts were grown in high-glucose (SD/-Leu,Trp) medium. Assays for β -galactosidase activity followed protocols described elsewhere [25]. Expression levels of the GAD and GBD fusion proteins were controlled by Western blot analysis. Experiments were performed a minimum of three times.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed essentially as described previously [26]. Cells were harvested and disrupted by vortexing in the presence of glass beads, and the lysate was sonicated to generate DNA fragments that

ranged in size from 200 to 400 bp. To immunoprecipitate HA-tagged proteins, we incubated the extract overnight at 4°C with anti-HA antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). To immunoprecipitate Hxk2 protein, we incubated the extract overnight at 4°C with anti-Hxk2 antibodies [11]. The sequence primers for PCR to amplify the *SUC2* promoter region containing the MIG1 element were, 5'-TTATTACTCTGAACAGGA-3' (sense) and 5'-AAGTCGTCAAATCTTTCT-3' (antisense).

RESULTS

Subcellular localization of human pancreatic GK β in yeast cells

It has been previously described that human pancreatic glucokinase (GK β) complements the glucose signalling defect of *S. cerevisiae* $\Delta h x k 2$ mutants, indicating that GK β may substitute Hxk2 in its role of regulating glucose signalling in yeast [22]. To gain insight into the molecular mechanism by which GK β regulates this process in yeast, the glucose-dependent subcellular location of GK β was determined in *S. cerevisiae* cells.

To detect GK β in live yeast cells, a GK β -GFP protein fusion was expressed under the control of the *HXK2* gene promoter, and its subcellular location was determined by fluorescent microscopy. The GK β -GFP fusion protein maintains repression functions in the glucose signalling pathway (data not shown). In cells grown overnight in high-glucose, a fraction of GK β -GFP was enriched in the nucleus (Fig. 1A and C). Similar results were obtained with an Hxk2-GFP protein fusion, as previously described [13]. However, in cells grown overnight on low-glucose medium, GK β -GFP was found mainly associated to multiple organelles distributed through the cytoplasm, reminiscent of a typical mitochondrial network. In contrast, the Hxk2-GFP protein presented an even distribution between the nucleus and the cytoplasm, as previously described [13] (Fig. 1A). In order to determine whether the GK β -GFP protein presented a mitochondrial localization under low-glucose conditions, we labelled the mitochondrial network with a mitotracker probe and observed a similar pattern of subcellular distribution of GK β -GFP and the mitotracker (Fig. 1B), suggesting a mitochondrial distribution of GK β -GFP under these conditions. To confirm the mitochondrial localization of GK β -GFP protein under low-glucose conditions, a red fluorescent protein (RFP) fused with the mitochondrial targeting signal of *Neurospora* F₀F₁ ATPase subunit

9 (Su9) [27] was co-expressed with the GK $_{\beta}$ -GFP fusion protein in wild-type *S. cerevisiae* cells. As can be seen in Fig. 1B, the expressed proteins were associated with the mitochondria and the fluorescence signals completely overlapped.

Since liver and β -cell glucokinase isoforms differ only in the first 15 N-terminal residues, it was important to determine if the observed location of GK $_{\beta}$ was also shared by the liver isoform. As it can be seen in Fig. 1A and C, in cells grown overnight in high-glucose, a fraction of GK $_L$ -GFP was enriched in the nucleus and in cells grown overnight on low-glucose medium, GK $_L$ -GFP was found mainly associated to mitochondria (Fig. 1A-C). Therefore, our results suggest that in yeast, the subcellular localization of β -cell and liver glucokinase isoforms was similar and did not depend on their N-terminus.

The nuclear export of human pancreatic GK $_{\beta}$ in the yeast cell is Mig1-dependent

We have recently described that, under low-glucose conditions, Hxk2 exits the nucleus using a pathway in which Mig1 is involved [13, 14]. In order to determine whether Mig1 is also affecting the exit from the nucleus of GK $_{\beta}$ -GFP under the same conditions, we determined the subcellular distribution of GK $_{\beta}$ -GFP in $\Delta mig1$ mutant cells (Fig. 2A). The results indicated that GK $_{\beta}$ -GFP fusion protein was enriched in the nucleus both in high and low-glucose-grown cells, suggesting that in the absence of Mig1, GK $_{\beta}$ -GFP was not able to exit the nucleus and reach the mitochondria. The introduction in these cells of a plasmid expressing MIG1 under its own promoter (YEp351-MIG1), allowed GK $_{\beta}$ -GFP to exit the nucleus under low-glucose conditions (Fig. 2A), as in wild type cells (Fig. 1A), suggesting that in low-glucose conditions, Mig1 is necessary to export the GK $_{\beta}$ -GFP fusion from the nucleus to the mitochondria.

We also studied the subcellular distribution of GK $_{\beta}$ -GFP in a $\Delta h x k 1 \Delta h x k 2$ double mutant strain (Fig. 2B). The results demonstrated that in the absence of Hxk1 and Hxk2, GK $_{\beta}$ -GFP was targeted to the mitochondria both at high and low-glucose concentrations. Since, in the absence of Hxk1 and Hxk2, the protein kinase Snf1 is constitutively activated, we repeated the experiment in a triple $\Delta h x k 1 \Delta h x k 2 \Delta s n f 1$ mutant (Fig. 2C). In this case, no mitochondrial distribution of GK $_{\beta}$ -GFP was observed in any growth conditions (similar results were obtained with a single $\Delta s n f 1$ mutant, Fig. 2C). These results suggested that Snf1 played a major role in regulating the exit of GK $_{\beta}$ -GFP from the nucleus. When Snf1 was activated, either by growing the cells under low-

glucose conditions or by deleting negative regulators such as Hxk1 and Hxk2, GK β -GFP exited the nucleus and presented a mitochondrial distribution.

It is known that Snf1 is involved in the phosphorylation of Mig1 under low-glucose conditions, leading to its exit from the nucleus to the cytoplasm [28]. Since GK β -GFP followed the same pattern of distribution as Mig1 and, as we have described above, GK β -GFP did not exit the nucleus in the absence of Mig1, even under conditions where Snf1 is activated (low-glucose), we suggest that Mig1 could be involved in the exit of GK β -GFP from the nucleus under low-glucose conditions. In agreement with this suggestion, we observed that GK β -GFP fusion protein was enriched in the nucleus, in both high and low-glucose-grown cells, in the absence of Msn5 (Fig. 3A), a member of the importin family of nuclear transport proteins which is required to export Mig1 from the nucleus in low glucose conditions [29]. This result suggests that in the absence of Msn5, GK β -GFP was not able to exit the nucleus and reach the mitochondria. Thus, taken together, these data demonstrated that Mig1 and GK β -GFP could form part of a nuclear complex whose export to the cytoplasm was dependent on both, the phosphorylation of Mig1 by Snf1 and the exportin Msn5. The close relationship between Mig1 and GK β -GFP was confirmed when we observed that a Mig1-GFP protein fusion was dragged to the mitochondrial network, under low-glucose conditions, only if GK β was expressed in the same cells (Fig. 3B). These results suggested that Mig1 and GK β may form a complex in which Mig1 confers the determinants for nuclear export and GK β confers the determinants for mitochondrial localization under low-glucose conditions.

Human pancreatic GK β interacts with the yeast Mig1 transcriptional repressor

The experiments described above strongly suggest that Mig1 and GK β may form part of an *in vivo* complex. To confirm the possible physical interaction between Mig1 and GK β , we have used a yeast two-hybrid assay. Plasmid pGADT7-GK β (expressing a fusion of the Gal4 activating domain, GAD, to GK β) was co-transformed with a plasmid expressing a fusion of the Gal4 binding domain (GBD) with either Hxk2, Mig1, Mig1S311A or GK β , into an appropriate reporter strain. The interaction between the selected proteins was monitored by measurement of the β -galactosidase activity. As shown in Fig. 4, the GAD-GK β fusion protein produced a strong interaction with GBD-

Mig1. However, this interaction was absent in a GBD-Mig1S311A mutant, indicating that the Ser311 residue of Mig1, previously identified as essential for Hxk2 interaction [30], was also required for interaction with GK β . No interaction was observed between GAD-GK β and GBD-Hxk2 or GBD-GK β , indicating that these proteins did not form dimers. Similar levels of all protein fusions were detected in all the cases (not shown). To confirm the interaction we performed co-immunoprecipitation and GST pull-down assays but we were not able to observe any interaction between GK β and Mig1, probably because it was too weak or transient to be detected by these methods.

Human pancreatic GK β binds *in vivo* to the *SUC2*-Mig1 repressor complex

Previous reports have demonstrated that Hxk2 participates in the *SUC2*-Mig1 repressor complex by interaction with Mig1 protein and not by direct binding to DNA [14, 31, 32]. As GK β and Mig1 may form an *in vivo* complex (see above), we investigated the biological significance of this interaction by determining whether GK β localized in the *SUC2*-Mig1 repressor complex in a glucose dependent manner. To test this hypothesis, we used ChIP assays. Our results show that in cells grown in high glucose medium, both HA-GK β and Hxk2 proteins were recruited to a DNA fragment of the *SUC2* promoter containing the Mig1 binding site (Fig. 5A, lanes 1 and 3). Conversely, in low glucose medium, HA-GK β and Hxk2 binding to the *SUC2*-Mig1 complex was abolished (Fig. 5B, lanes 2 and 4). Binding of HA-GK β to the Mig1 site of the *SUC2* promoter was dependent on the presence of Mig1, since in Δ *mig1* cells, no binding of HA-GK β to the *SUC2* promoter was observed under any growth condition (Fig. 5B). No DNA amplification was observed when we used cells with untagged GK β or cells with HA-tagged Rgt1 protein (data not shown).

Taking all these results together we suggest that GK β forms a similar complex with Mig1 as Hxk2 does, and that this complex is involved in the glucose regulated expression of the *SUC2* gene. This complex exits the nucleus under conditions of Snf1 activation, being the presence of Mig1 and Msn5 necessary for the nuclear exit.

DISCUSSION

In this study, we have taken advantage of the facility to manipulate the *S. cerevisiae* genetic system to determine the mechanisms by which GK β regulates glucose

signalling in yeast cells and to examine the glucose-dependent localization of the GK $_{\beta}$ in yeasts.

Reports suggest that Hxk2, the yeast orthologue of GK $_{\beta}$, has a dual function in yeast cells, first initiating the intracellular metabolism of glucose by its enzymatic activity and secondly, signalling glucose repression by its interaction with nuclear Mig1 repressor, regulating its phosphorylation status [14]. Since Hxk2 protein mutations without phosphorylating activity but retaining activity in glucose repression signalling have not been achieved [33, 34], the idea that the sugar kinase activity and the sugar signalling properties are mediated through separate domains of Hxk2 is still controversial. In this study, we demonstrate that recombinant human pancreatic GK $_{\beta}$ is able to signal glucose repression in *S. cerevisiae*; both GK $_{\beta}$ and Hxk2 have a nuclear location during growth in high-glucose conditions and both are able to regulate the glucose-responsive *SUC2* gene in *S. cerevisiae*. We also report that GK $_{\beta}$ is physically associated with the Mig1 protein repressor and ChIP assays confirmed that both GK $_{\beta}$ and Hxk2 interacted with Mig1 in a cluster with DNA fragments containing the MIG1 site of the *SUC2* promoter. Therefore, our results suggest that GK $_{\beta}$ and Hxk2 use similar mechanisms to regulate glucose signalling in yeast.

The other important finding of this study regards the subcellular location of GK $_{\beta}$. We report that GK $_{\beta}$ moves between the nucleus and mitochondria in the yeast cell in response to glucose concentration in the medium: under high-glucose conditions, GK $_{\beta}$ is enriched in the nucleus and resides there in association with Mig1; however, under low-glucose conditions, GK $_{\beta}$ exits the nucleus and eventually reaches the mitochondria, either because it has uncharacterized mitochondrial determinants (GK $_{\beta}$, lacks the hydrophobic N-terminal sequence present in hexokinases I and II that allows their binding to the mitochondria [35]), or because it interacts with another protein that has these mitochondrial localization determinants, as in the case of mammalian pro-apoptotic factor BAD which is involved in the mitochondrial targeting of glucokinase in both liver and pancreatic beta-cells [6]. To reach GK $_{\beta}$ this subcellular location, first, Mig1 must be present in the cell and Snf1 must be active. Then, binding of Mig1 to GK $_{\beta}$ allows the Mig1-GK $_{\beta}$ complex to exit the nucleus by a mechanism dependent on the exportin Msn5. Finally, the complex is located in the mitochondrial network, location that has never been reported for either Mig1 or Hxk2.

Since the subcellular distribution of glucokinase in mammalian pancreatic β -cell is also regulated by glucose: low levels of glucose causes an association of GK with mitochondria [8], whereas, high glucose levels prevents GK translocation to this organelle [8], the yeast system offers a possibility to study the nature of the determinants present in GK $_{\beta}$ or the identification of interactive proteins that target GK $_{\beta}$ to the mitochondria under low-glucose conditions.

The role of Mig1 in regulating GK $_{\beta}$ exit from the nucleus could be compared to the role of the glucokinase regulatory protein (GKRP) in mammalian cells [4, 5]. However these two proteins present different domain structures and only 17% similarity (assessed by CLUSTAL analysis), suggesting that the relationship between the two proteins is only at the level of their interaction with GK.

Altogether, our results indicate that despite the strong phylogenetic difference between the human GK $_{\beta}$ and the yeast Hxk2 proteins, the mechanism of glucose signalling is maintained, suggesting that the function of the regulatory domain of these proteins has been conserved throughout evolution.

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FIGURE LEGENDS

Figure 1. Glucose regulates the subcellular localization of both Hxk2-GFP and GK β -GFP

(A) Yeast strain DBY1315 (WT) expressing Hxk2-GFP, GK β -GFP or GK β -GFP, from plasmids YEp352-HXK2/gfp, YEp352-GK β /gfp or YEp352-GK β /gfp respectively, was grown on high-glucose synthetic medium (H-Glc) until an OD 600nm of 1.0 was reached and then transferred to low glucose synthetic medium (L-Glc) for 60 min. Then, cells were stained with DAPI and imaged for GFP and DAPI fluorescence. (B) Cells growing under low-glucose conditions and expressing GK β -GFP were stained with Mitotracker® Red 580. Cells growing under low-glucose conditions and expressing both GK β -GFP and Su9-RFP were stained with DAPI and imaged for DAPI, GFP and RFP fluorescence. (C) The localization of fluorescent reporter proteins was determined in at least 100 cells in three independent experiments; N, denotes a nuclear fluorescence signal; C, cytoplasmic fluorescence signal without nuclear or mitochondrial fluorescence signals; M, mitochondrial fluorescence signal. Means and standard deviations are shown for at least three independent experiments.

Figure 2. Mig1 and Snf1 regulate the subcellular localization of GK β -GFP

(A) Yeast strain H250 ($\Delta mig1$), expressing GK β -GFP from a plasmid (YEp352-GK β /gfp) was transformed or not with plasmid YEp351-MIG1. Transformants were grown on high-glucose synthetic medium (H-Glc) until an OD 600nm of 1.0 and then transferred to medium with low-glucose synthetic medium (L-Glc) for 60 min. Then, cells were stained with DAPI and imaged for GFP and DAPI fluorescence. (B) Yeast strains DBY1315 (WT) and DBY2052 ($\Delta h x k 1 \Delta h x k 2$) and (C) yeast strains $\Delta snf 1$ and $\Delta snf 1 \Delta h x k 1 \Delta h x k 2$, expressing GK β -GFP from a plasmid YEp352- GK β /gfp, were analyzed in the same way.

Figure 3. Msn5 directs the nuclear export of GK β and GK β directs mitochondrial localization of Mig1.

(A) Yeast strain Y03694 ($\Delta msn5$), expressing Mig1-GFP or GK β -GFP from plasmids YEp352-MIG1/gfp or YEp352- GK β /gfp was grown on high-glucose synthetic medium (H-Glc) until an OD 600nm of 1.0 and then transferred to medium with low-glucose synthetic medium (L-Glc) for 60 min. Then, cells were stained with DAPI and

imaged for GFP and DAPI fluorescence. (B) Yeast strain FMY388 expressing a *gfp* tagged *MIG1* ORF at its chromosomal location, was transformed or not with plasmid YEp352-GK β . Transformants were analysed as indicated in part A.

Figure 4. Two-hybrid interaction between GK β and Mig1

Plasmids containing full-length Hxk2, Mig1, Mig1S311A and GK β fused to the Gal4 DNA binding domain (GBD) and the empty plasmid were co-transformed into yeast strain Y187 with a construct encoding the Gal4 activating domain (GAD) fused to full-length GK β . Transformants were grown on high-glucose synthetic medium (H-Glc) and protein-protein interactions were examined by measuring the β -galactosidase activity. Values are averages of β -galactosidase activities for three transformants, with a standard deviation of less than 10% in all the cases.

Figure 5. *In vivo* binding of GK β to the MIG1 element of the *SUC2* promoter

Wild type W303-1A (A) and $\Delta mig1$ mutant cells (B) containing a HA-tagged version of GK β (from plasmid pWS-GST/GK β) were grown in high (H-Glc) or low (L-Glc) glucose conditions. Cell extracts were prepared and immunoprecipitated with anti-HA or anti-Hxk2 antibodies and the DNA fragments were amplified by PCR using the combination of oligonucleotides indicated in Materials and Methods. The amplified fragments were resolved by agarose gel electrophoresis. Migration of standard markers is indicated on the left. A representative ChIP assay out of three independent experiments is shown.

FIGURE 1

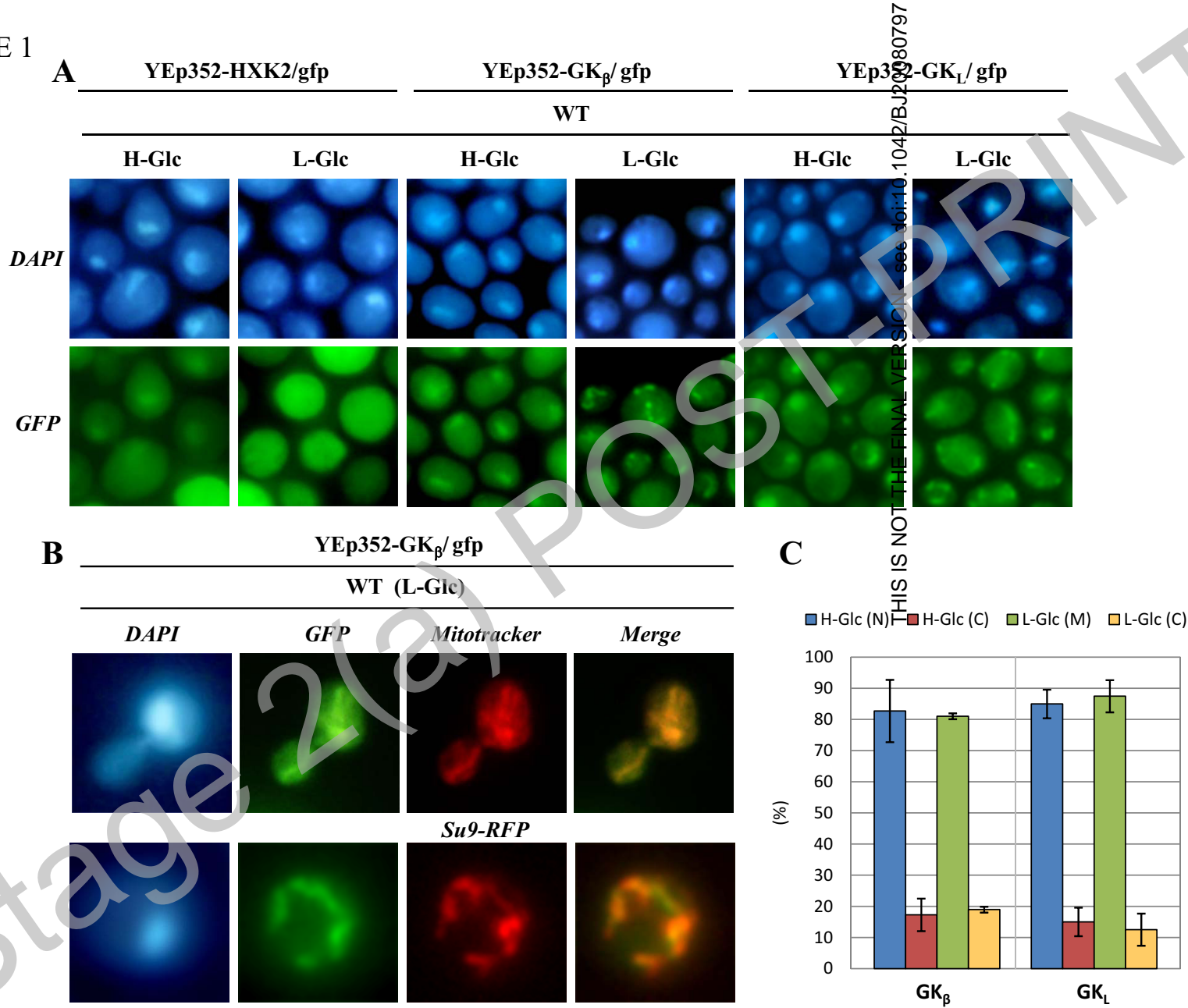


FIGURE 2

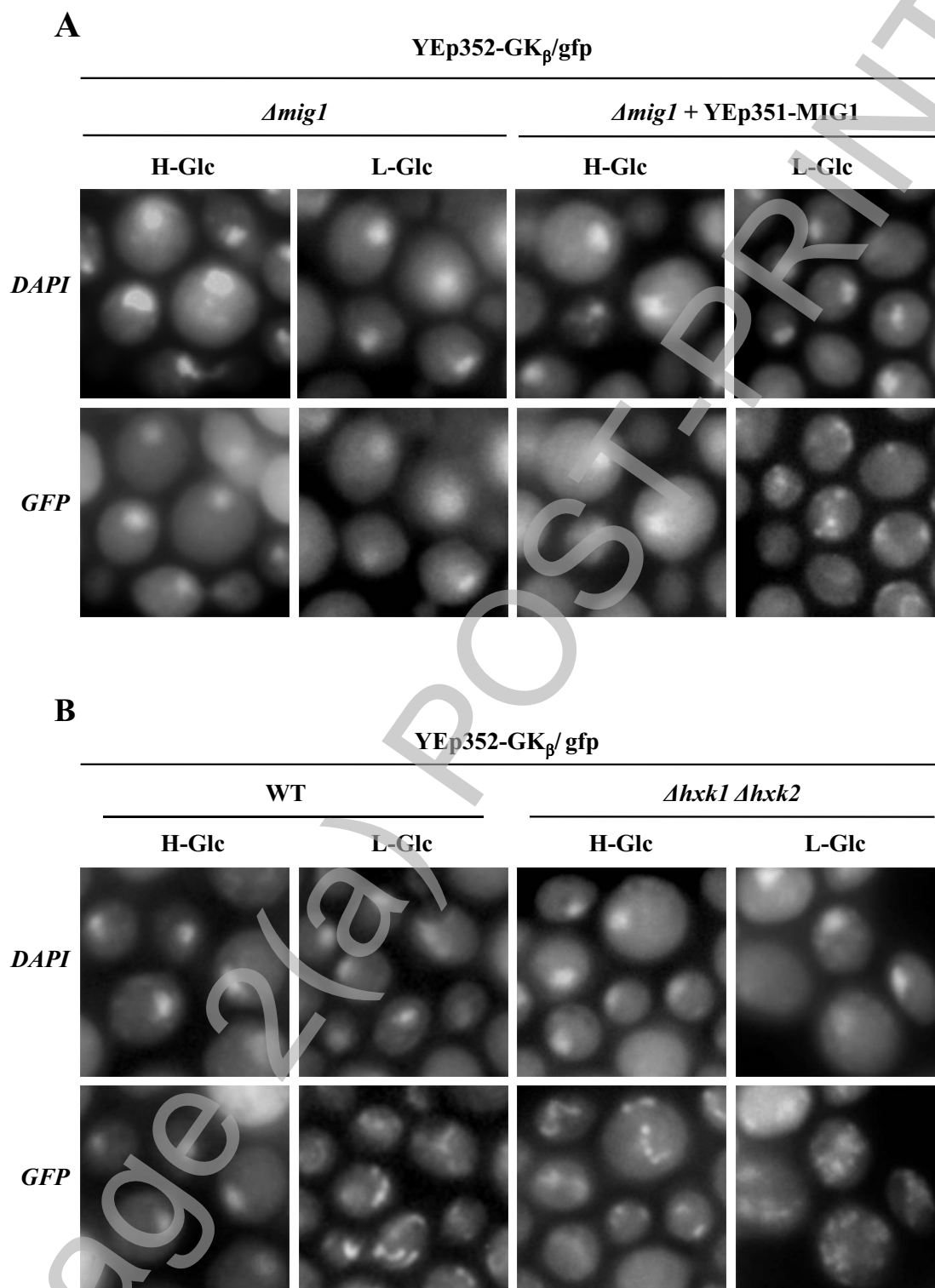


FIGURE 2

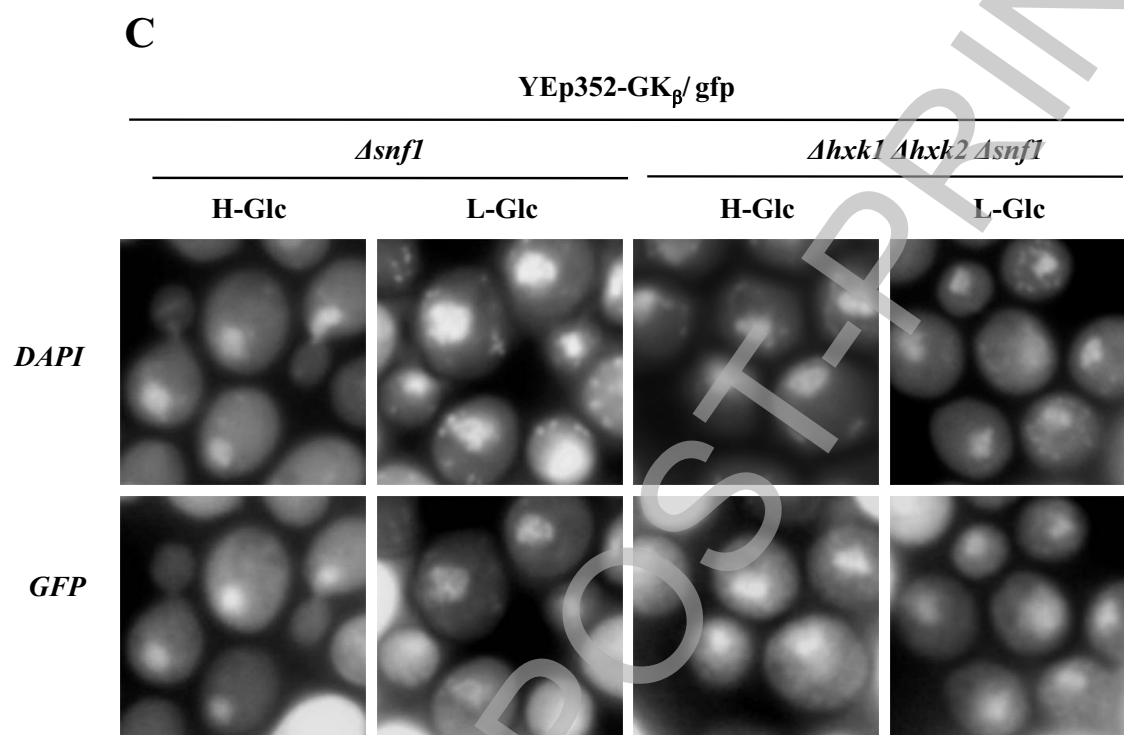


FIGURE 3

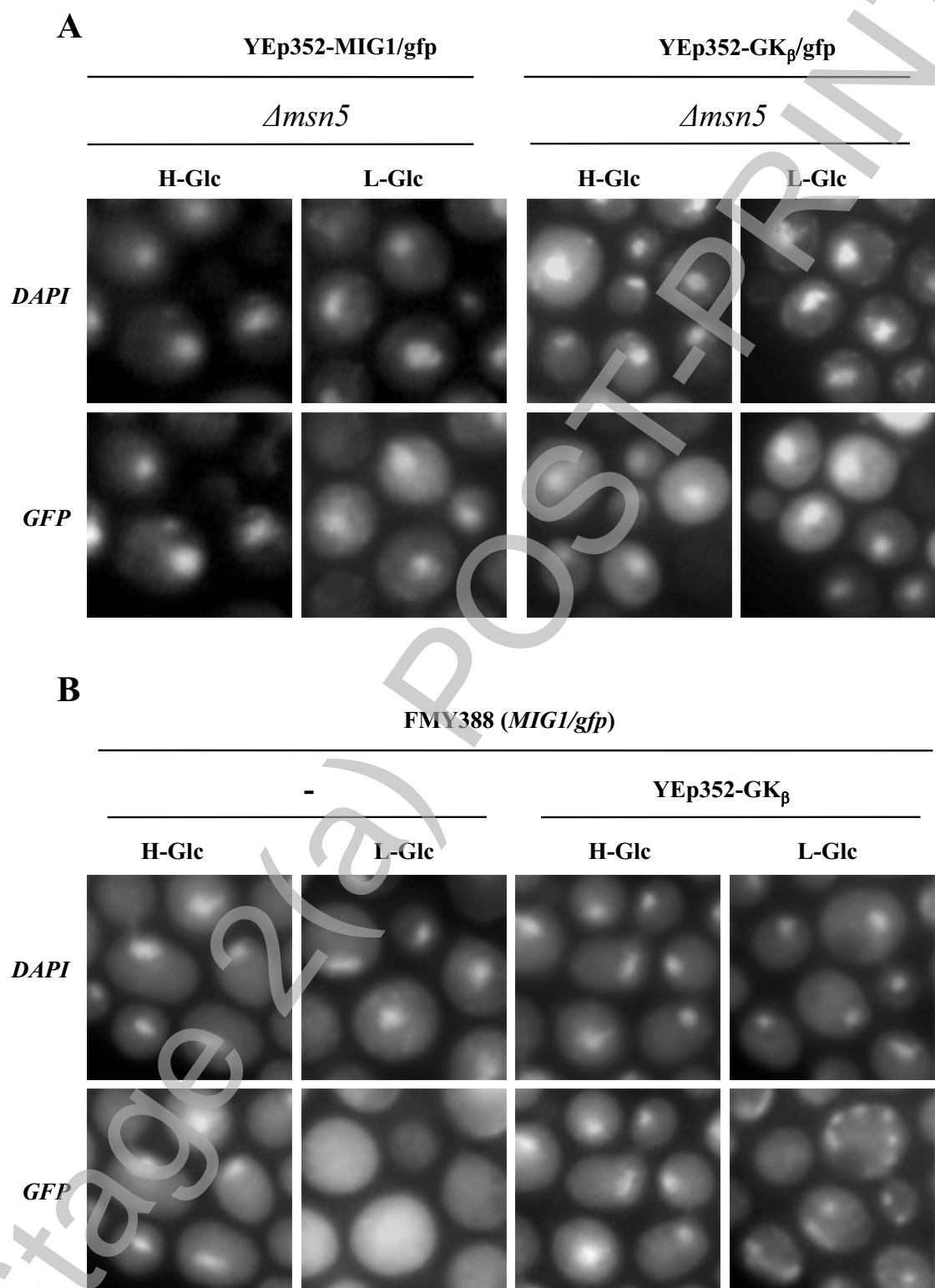


FIGURE 4

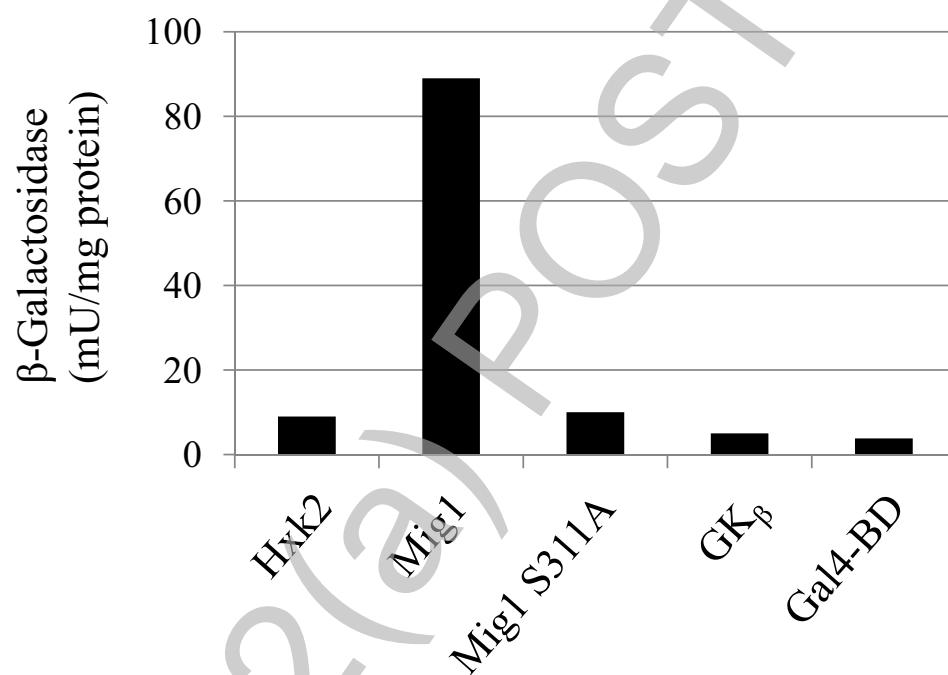


FIGURE 5

