Trypanosoma brucei and related organisms contain an organelle evolutionarily related to peroxisomes that sequesters glycolysis, among other pathways. We have previously shown that disruption of protein import into this organelle, the glycosome, can be accomplished through RNA interference (RNAi)-mediated knockdown of the peroxin PEX14. Decreased PEX14 in turn leads to cell death which, at least in the procyclic stage, can be triggered by the presence of glucose. Here we show that fructose, which is taken up and metabolized by procyclic form T. brucei, and glycerol, which interfaces with the glycosomal glycolytic pathway, are also toxic during PEX14 RNAi. Earlier computer modeling studies predicted that glycolysis would be toxic to T. brucei in the absence of glycosomal compartmentation due to the intrinsic lack of feedback regulation of the parasite hexokinase and phosphofructokinase. To further test this hypothesis, we performed double RNAi targeting hexokinase and PEX14. Knockdown of hexokinase rescued PEX14 knockdown cells from glucose toxicity, even though glycosomal proteins continue to be mislocalized to the cytosol. Knockdown of phosphofructokinase was benign in the absence of glucose but toxic in the presence of glucose. When PEX14 and phosphofructokinase mRNAs were jointly targeted for RNAi, glycerol remained toxic to the parasites. Taken together, these data indicate that the glycosome provides significant, but not complete, protection of trypanosomes from the dangerous design of glycolysis.
for each molecule of glucose that is metabolized, 2 ATPs are initially invested and then four are generated, for a net yield of 2 ATPs (see Fig. 1). The net ATP is generated in the cytosol, while ATP balance within the glycosome is maintained by multiple enzymes whose interplay has not been established (11). Hexokinase (HK) mediates the first reaction in glycolysis by phosphorylating glucose in an ATP-dependent manner, and, following isomerization, phosphofructokinase (PFK) generates fructose 1,6 bis-phosphate. In most organisms the activity of these enzymes is tightly regulated. However, this is not the case for the kinetoplastid homologues, and in particular HK is not inhibited by glucose-6-phosphate (12). This lack of regulatory control creates an automatic feed forward mechanism in kinetoplastids, sometimes referred to as ‘turbo glycolysis’ (13). Computer modeling studies, done on data derived from bloodstream form parasites, suggest that the role of compartmentation of glycolysis within the glycosome is to keep the activities of HK and PFK sequestered from cellular pools of substrates (e.g., ATP), and that this is essential to protect the cell (14;15). Mislocalization of these unregulated kinases to the cytosol might lead to runaway phosphorylation of hexoses (due to the availability of the cytosolic pool of ATP), ATP depletion, and cell death.

We have previously identified and examined the role of one of the *T. brucei* glycosome biogenesis proteins, PEX14 (4). This peroxin is an essential component of the receptor-docking complex, which is involved in the targeting of matrix proteins to the glycosome, and translocation of these matrix proteins across the glycosomal membrane. We demonstrated by RNA interference (RNAi) that PEX14 is essential for the proper localization of glycosomal matrix proteins, and its depletion results in death of both bloodstream and procyclic form parasites. In addition, we showed that one specific cause of this lethality in procyclic stage parasites was glucose. Here we extend our analysis to demonstrate that several sugars or components of the glycolytic pathway have a similar lethal phenotype in cells where PEX14 has been depleted by RNAi. In addition we demonstrate the specific role played by glycolysis in this phenotype by construction of *T. brucei* double RNAi cell lines that knock down both PEX14 and either HK or PFK expression.

Materials and Methods

Growth of Parasites—*T. brucei* strains Treu667 (16;17) and 29-13 (16) procyclic form parasites were maintained in the JRH Biosciences formulation of SDM-79 supplemented with 2 g/L sodium bicarbonate, 3.75 mg/L hemin and 10% FCS. G418, hygromycin and phleomycin were added to a final concentration of 15, 50 and 2.5 µg/mL respectively. This formulation does not contain glycerol, unlike our previous formulation of SDM-79. In addition, NaH₂PO₄·H₂O was increased from 126 to 954 mg/L, glutamine was increased from 320 to 513 mg/L and serine was reduced from 71 to 10 mg/L. We also obtained a special order medium from JRH Biosciences that further lacked glucose and glucosamine (SOGG medium). When cells were grown in SOGG, unless otherwise noted, we used serum that was dialyzed against a 40-fold volume of PBS (dialysis tubing was extensively washed to remove glycerol). RNAi experiments employed the strain 29-13 which contains integrated copies of T7 RNA polymerase and the Tet repressor, which allows Tet-regulated expression of introduced genes. Cell densities were determined using a Z1 Coulter counter and growth curves were performed using duplicate or triplicate cultures. Cultures in log phase were diluted to 10⁶ cells/mL to begin the experiment, and RNAi was induced by the addition of 4 µg/mL tetracycline (Tet). Tet was added every 24 to 48 hours, and cultures kept below 2 x 10⁷ cells/mL.

Plasmid Construction—RNAi constructs were based on the plasmid pZJM, which contains a cloning site between opposing T7 promoters under the control of the Tet operator (18). This plasmid can be integrated into the transcriptionally silent rDNA intergenic spacer. Amplification of the relevant fragments from *T. brucei* genomic DNA used primers containing HinDIII sites. For HK, the forward primer was 5'-AAGCTTCAATATCCTCGAACACATC-3', while the reverse primer was 5'-
AAGCTTCTCGAGGAAGCCCTTCGTCCAC CGG-3'. There are two HK loci in T. brucei (19), HK1 and HK2, which differ only by 1 bp in the 526 bp region used for RNAi (nt 18-543 of the 1416 nt ORF). For PFK, primers 5'-AAGCTTGAATCTCGCAGCCGTTACG-3' (forward) and 5'-AAGCTTGAAGAGGATGTTGACACCG-3' (reverse) were used to amplify nt 10-582 of the 1461 nt PFK ORF. The PCR fragments were cloned into pGEM-T easy and sequenced. Cloned fragments were then ligated into the vector pZJM-PEX14 (4) following digestion of both vector and insert with HindIII (see Fig. 3), yielding both orientations (tail to tail, A, or tail to head, B). The “A” orientation plasmid was digested with XhoI taking advantage of a site in the reverse primer (HK), or the naturally occurring site 5 bp upstream of the reverse primer (PFK), to release the PEX14 fragment. Religation then generated constructs targeting only the glycolytic enzyme.

**Determination of Transcript Levels by Real-Time PCR and Northern Analysis**—For the HK knockdown strains real-time PCR was used for analysis of HK1/HK2 and PEX14 transcript levels. Primers were specific for the endogenous genes and not the RNAi constructs. The HK primers matched both HK1 and HK2: GTCAACAGGCGCTGGAGAA (forward), 5'-CCGAGAGGCTGTTAAATAGAC-3' (reverse) and 5'-CCGGCAGCGCTTATT-3' (reporter). PEX14 primers were 5'-GGAGACTCAGGAAGCTTGAAG-3' (forward), 5'-GCAACTTCCGAGGTTGAT-3' (reverse) and 5'-CAAGGCTGAACTCTCC-3' (reporter). Samples (5x10⁷ cells) were taken after two days of RNAi induction and RNA prepared using Trizol (Invitrogen). Approximately 1 µg of RNA was converted to cDNA using the Taqman Reverse Transcriptase kit (Applied Biosystems). Approximately 50 ng of cDNA of each sample was loaded per well for PCR. RNA loading was normalized according to α-tubulin mRNA levels using the primers 5'-CGCTGTTAGACCAATGCG-3' (forward), 5'-ACCCTCAGCGATCTCCA-3' (reverse) and 5'-AAGCCGCGCTTCTTG-3' (reporter).

For the PFK strains, PFK and PEX14 transcript levels were determined by Northern analysis on RNA samples (10 µg) prepared as above. Samples were separated on a 1% formaldehyde-agarose gel (NuSieve: Agarose, 3:1) and transferred to nylon membranes (Nytran N, Schleicher and Schuell). Following prehybridization in ULTRAhyb (Ambion), RNA probes, prepared using the Promega Riboprobe-T7 kit, were added to 10⁶ cpm/mL. The probes detected both the native transcripts and those produced by the RNAi constructs. Blots were washed twice with 0.1X SSC (15 mM NaCl/1.5 mM sodium citrate, pH 7.0) and 0.1% SDS for one hr at 65°C.

**Western Analysis**—Cells were washed in 1X PBS three times and resuspended in SDS sample buffer. Approximately 5x10⁶ cell equivalents per sample were separated by electrophoresis on 10% acrylamide gels (Cambrex PAGEr). Samples were transferred to nitrocellulose membranes (Protran) and probed with rabbit anti-HK or anti-PFK (gift of Paul Michels) which were prepared against purified T. brucei HK1 or PFK (20). The second step was Protein A (1:5,000) coupled to horseradish peroxidase, detected using the ECL Plus system (Amersham, Piscataway, NJ). After quantitating the HK signal, blots were stripped and incubated with anti-PEX14 (1:100) (4) and anti-NOG1 (1:5000) (21) as a loading control. Image analysis was done using Molecular Dynamics Phosphorimager system and software.

**Immunofluorescence Analysis**—Immunofluorescence analysis was performed as previously described (4) using an antiserum directed against procyclic form glycosomes (1/100 dilution) (22) plus FITC-conjugated goat anti-rabbit IgG. After washing, slides were mounted using Prolong Antifade (Molecular Probes) for fluorescence microscopy.

**RESULTS**

PEX14 RNAi induction is lethal with various sugars or glycerol—In our previous work, we established an RNAi procyclic form cell line in which the expression of dsRNA corresponding to the 5' half of PEX14 was induced by Tet. We demonstrated that induced expression of PEX14 RNAi led to mislocalization of glycosomal matrix proteins and was lethal for parasites when glucose was present in the medium (4). This
phenotype was evident at 4 days following induction of RNAi in procyclic forms, apparently requiring the dilution of PEX14 through cell division and protein turnover. We wanted to determine what concentration of glucose was lethal, and if other sugars or other components of our medium related to glycosomal metabolism might be lethal as well. Although procyclic form cultures are normally maintained in SDM-79 medium, the previous experiments used a modified RPMI-based medium. We therefore obtained a special formulation of SDM-79 that lacked glycerol, glucose and glucosamine (SOGG medium).

When RNAi was induced, the PEX14 RNAi parasites in SOGG medium supplemented with 10% non-dialyzed FCS died after four days (results not shown). This concentration of FCS yielded a final concentration of 356 µM glucose. Growth of the PEX14 RNAi cell line was tested in SOGG medium using dialyzed serum (yielding a final concentration of 9 µM glucose) and supplemented with different concentrations of glucose (Fig. 2A). In the absence of additional glucose, induced cultures showed only a slight growth defect, with half the cell numbers of their uninduced counterparts by day 11. In the presence of 100 µM glucose, uninduced cultures grew somewhat better than the induced cultures. Higher levels of glucose (1 and 10 mM) were toxic to induced cultures. The difference in cumulative cell number by day 11 for these cultures was approximately two orders of magnitude. As we previously found in the RPMI-based medium, if cells were grown in the absence of glucose for several days during PEX14 RNAi induction, and then glucose was added back to 10 mM, the lag period was eliminated and the culture died within 24 hours (data not shown).

Galactose is not taken up by T. brucei (23;24) and as expected its presence had no effect on the growth of induced parasites (Fig. 2B). In contrast, a lethal phenotype was observed in induced cultures containing 10 mM fructose and 10 mM glycerol (Fig. 2B-C). Glycerol was additionally tested at various concentrations ranging from 100 µM to 10 mM. The addition of 100 µM glycerol to induced cultures reduced the number of viable parasites 10-fold, while 1 and 10 mM glycerol reduced them 100-fold (data not shown). 2-deoxyglucose is phosphorylated by HK but inhibits glucose-6-phosphate isomerase and is not further metabolized (see Fig. 1). It arrested growth almost immediately, both in the presence and absence of RNAi induction (Fig. 2C). This effect was also seen in another cell line, TREU667, and was ameliorated by increasing amounts of glucose in the medium (data not shown). 2-deoxyglucose may have other effects on the cell in addition to the futile consumption of ATP, such as inhibition of glycosylation. However, previous studies have shown that tunicamycin, an inhibitor of N-linked glycosylation, has little effect on the viability of procyclic forms (25).

Double RNAi Constructs are Effective in Knockdown of Multiple mRNAs—Computer modeling studies of bloodstream form parasites suggest that sequestration of glycolysis within the glycosome may be essential to protect the cell from the potentially lethal effects of unregulated HK and PFK in the cytosol (15). We tested this hypothesis with procyclic parasites in vivo by taking advantage of the mislocalization phenotype that arises when PEX14 is depleted. We created double RNAi constructs in T. brucei with portions of PEX14 and either HK1 or PFK. The 5' end of HK1 was amplified from T. brucei genomic DNA and cloned into the PEX14 RNAi construct in two orientations with respect to PEX14 (Fig. 3, orientations A and B). The dsRNA should target transcripts for both HK genes (HK1 and HK2), since the two differ by only one nt in the region employed. An RNAi construct targeting HK1/2 mRNA (but not PEX14) was constructed as well. These constructs were stably transfected into T. brucei 29-13.

The levels of HK1/2 and PEX14 mRNA were evaluated by reverse transcription and real-time PCR on day two after induction of RNAi (Fig. 4). HK1/2 mRNA levels in induced HK1 and HK1-PEX14 A and B RNAi cell lines were lowered to approximately 15% of the levels of the corresponding uninduced cell line. In the original PEX14 RNAi line, HK1/2 mRNA levels did not change upon induction. Similarly, PEX14 mRNA levels were reduced in the PEX14 and the two HK-PEX14 double RNAi
cell lines upon addition of Tet, but remained unchanged in the HK knockdown. In the PEX14 knockdown, PEX14 mRNA was approximately 25% of the uninduced control, and in the double RNAi mutants it was 35% of the control. Thus, knockdown of both mRNA target molecules was accomplished by induction of one RNAi construct with two gene fragments.

**HK knockdown rescues PEX14 knockdown**—To test whether depletion of HK could rescue the PEX14 knockdown, we examined the growth of cultures of HK, HK-PEX14 B and PEX14 RNAi cell lines in the presence of glucose. At the same time we assessed the levels of the relevant proteins as well as compartmentation of glycosomal proteins. Immunoblotting of cell lysates from several time points reveals that following induction of RNAi in the HK-PEX14B line, both PEX14 and HK protein levels were reduced to levels comparable to those seen by real-time PCR analysis (Fig. 5A, HK1 and HK2 both have molecular masses of 51 kDa). We also assessed when mislocalization of glycosomal proteins occurred since it is presumably linked to the detrimental phenotype. Immunofluorescence analysis was performed using an anti-glycosome antibody that recognizes three different glycosomal matrix proteins (pyruvate phosphate dikinase, aldolase, and glyceraldehyde phosphate dehydrogenase) (22). The HK RNAi knockdown showed the punctate pattern typical of uninduced cells at all time points (day eight is shown in Fig. 5B). In contrast, none of the PEX14 RNAi cells had the normal punctate pattern of staining four days after induction (Fig. 5B). By this time the population had stopped growing (Fig. 5C). The population begins to increase again around day 11. We have previously shown that this escape is accompanied by re-expression of normal levels of PEX14 (4). The more slow-growing HK-PEX14 double RNAi cell line showed a longer phenotypic lag for mislocalization. Little mislocalization of glycosomal proteins was seen at day four but strong a mislocalization pattern was observed at day eight, when only about 5% of the cells demonstrated the punctate phenotype (Fig. 5B). Of the remaining cells, approximately one-third showed a diffuse staining pattern while two-thirds had both diffuse and punctate characteristics. By 11 days post-induction, only 2% of cells had a punctate pattern of staining, two-thirds had diffuse, cytosolic staining, and one-third had both diffuse and punctate characteristics. During the time period when glycosomal proteins were accumulating in the cytosol of the HK-PEX14 RNAi cell line, there was no slowing of proliferation (Fig. 5C), and microscopic analysis showed no evidence of significant cell death. Taken together, these data show that a decrease in HK rescues the PEX14 knockdown from glucose toxicity.

**Effects of Silencing of PFK and PEX14**—The second ATP requiring step in glycolysis is mediated by PFK within the glycosome. We therefore generated RNAi constructs targeting PFK and PEX14 mRNA as well as one targeting PFK alone. mRNA levels in the corresponding single and double RNAi lines were evaluated by Northern analysis. As in the case of the HK mutants, significant reductions of both PFK mRNA and PEX14 mRNA (Fig. 6A) are seen in the double RNAi constructs, while only the expected knockdown was observed in the single RNAi lines. Immunoblot analysis of induced cultures of PFK and PFK-PEX14B showed that the proteins were similarly depleted (Fig. 6C,E). On day eight, PFK levels were reduced to 9% and 25% of control levels in the single and double knockdown lines respectively. On day 8, PEX14 was decreased to 10% of control levels in the double knockdown line.

We tested the effects of PFK depletion on growth in various media. In contradistinction to the result obtained with the knockdown of HK, knockdown of PFK mRNA resulted in sensitivity to glucose: growth ceased and cells began to die (Fig. 6B). RNAi induced knockdown of PFK would create a bottleneck in glycolysis at the same position as 2-deoxyglucose with respect to ATP consumption, which we demonstrated above to be toxic to the parasites. The effects of PFK RNAi take longer to appear than the effects of 2-deoxyglucose, at least in part because the RNAi induced phenotype requires that the pre-existing PFK be diluted to low levels. In contrast to glucose, the addition of glycerol to the medium was beneficial whether or not PFK RNAi was induced (Fig. 6B).
Because the knockdown of PFK alone was so deleterious in the presence of glucose, it is not surprising that knockdown of PFK did not rescue PEX14 RNAi for growth in glucose (data not shown). We predicted that PFK depletion would not rescue the PEX14 RNAi line for growth in glycerol, since glycerol interfaces with the glycolytic pathway well below the reaction mediated by PFK (see Fig. 1). The PFK-PEX14 double RNAi cell line was evaluated in the presence of 1 mM glycerol for growth (Fig. 6D). On day four, growth of the population ceased, and mislocalization of glycosomal proteins to the cytosol was observed by immunofluorescence (data not shown). These results suggest that the lethal phenotype caused by the loss of glycosomal compartmentation mediated by knockdown of PEX14, at least in the case of glycerol, occurs downstream of PFK.

DISCUSSION

The studies reported here shed additional light on the role of glycosomal compartmentation in T. brucei and the danger of unregulated glycolysis. The first step in the metabolism of glucose is phosphorylation by HK. Most reactions involving glucose flow from this initial event (e.g., glycolysis, pentose phosphate pathway, nucleotide sugar biosynthesis). The rescue of the otherwise lethal PEX14 RNAi by co-reduction of HK via the double RNAi construct suggests an important role for HK in inducing the lethal phenotype. It is possible that the unregulated activity of HK causes the lethal phenotype in the PEX14 RNAi cell line through a depletion of cellular ATP, and a concomitant accumulation of phosphorylated hexoses as suggested by the computer models of Bakker et al. (15). It is also possible that reduction in HK mRNA levels reduces flux through the glycolytic pathway preventing the accumulation of a toxic intermediate further downstream in the pathway. Other workers have shown that the removal of glucose from the medium or the knockdown of HK affects the expression of surface glycoconjugates (19) and resistance to the adenosine analog tubericidin (26), suggesting that glucose metabolism has more far-reaching effects on the cell. In any case, the rescue from PEX14 knockdown via depletion of HK appears essentially identical to deletion of glucose from the growth medium. The results for the PFK knockdown, while not entirely discordant with the suggestions of the computer modeling, are still a somewhat unexpected twist. Here it seems quite clear that a lethal phenotype results when glucose is present, even in the context of an intact glycosome. This phenotype could result because trypanosomal HK, unlike other HKs, is not inhibited by accumulating glucose-6-phosphate that would normally shut off glycolysis when PFK is depleted (see Fig. 1). One result would be the consumption of intraglycosomal ATP and increased glycosomal hexose-phosphates. The specific block in glycolysis caused by 2-deoxyglucose is at glucose-6-phosphate isomerase. With respect to ATP consumption in glycolysis and accumulation of hexose phosphates, this inhibition is at the same place as the PFK knockdown. Taken together, these results suggest that sequestration of the pathway within the glycosome does not provide complete protection from the dangerous design of glycolysis. These results highlight the consequences of futile initiation of glycolysis in the presence of an intact glycosome, even in a stage when the parasite does not even require glycolytic metabolism (27). How this knockdown of PFK impacts flux in other pathways in trypanosomes remains to be studied, although it could increase flow through the pentose phosphate pathway as it does in E. coli (28). The question remains as to whether the depletion of intraglycosomal ATP and accumulation of intraglycosomal hexose phosphates are sufficient to cause cell death or whether toxicity results from an interplay with other, possibly cytosolic, molecules. We hasten to point out that these results do not rule out the possibility that the effects of glucose toxicity result from a downstream process in the PEX14 RNAi mutant, which has lost glycosomal compartmentation.

The observation that the PEX14 knockdowns are not rescued from glycerol toxicity by co-reduction of PFK supports the idea that at least one problem with the PEX14 RNAi mutant lies downstream of HK and PFK. The parasites possess a glycerol kinase that is
normally sequestered within the glycosome (29) (see Fig. 1). The forward reaction for this enzyme (glycerol + ATP → glycerol-3-phosphate + ADP) is kinetically preferred (but normally opposed in glycosomal metabolism) and has the possibility of consuming cellular ATP, just as proposed for the hexose kinases. It will be interesting to determine whether this enzyme is involved in the lethal effect of glycerol during PEX14 RNAi.

We have found that upon prolonged growth in SOGG medium, growth of parasites induced for PEX14 RNAi begins to slow compared to their uninduced controls. These data indicate that while glucose and glycerol may mediate immediate toxic effects when PEX14 is reduced in this cell line, it is likely that the lack of a functional glycosome has other deleterious consequences. Examples of other pathways in the glycosome that might be impacted by loss of compartmentation include ether-lipid synthesis or β-oxidation of fatty acids (1). Additionally, protection against cellular stresses may also be affected since a member of the glutathione-dependent peroxidase family (30) and certain superoxide dismutases (31) appear to be glycosomal. The functions of these pathways in procyclic form T. brucei have received little attention. We expect that in other trypanosomatids such glycosomal pathways might prove to be more obviously important than in T. brucei. For example, knockout of ether-lipid biosynthesis in Leishmania causes a specific defect in the establishment of intracellular parasitism (32). Using RNAi in T. brucei we are now able to begin to experimentally address the importance of sequestering glycolysis within the glycosome. These insights allow us in turn to focus on a more general analysis of the role of compartmentation of other biological processes within this cellular compartment.

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FOOTNOTES

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1 The abbreviations used are: HK, hexokinase; PEX, peroxin; PFK, phosphofructokinase; RNAi, RNA interference; SOGG, special order SDM79 medium lacking glucose, glucosamine and glycerol; Tet, tetracycline.

FIGURE LEGENDS

Fig. 1. Schematic of glycolysis and related pathways in procyclic form T. brucei. Enzymes discussed in this manuscript are indicated: HK, hexokinase; GPI, glucose-6-phosphate isomerase; PFK, phosphofructokinase; GK, glycerol kinase. Also shown is the inhibition by 2-deoxyglucose-6-phosphate, 2-DOG-6P. The dotted line depicts the feedback inhibition of HK by glucose-6-phosphate, seen in most organisms except trypanosomatids. Other intermediates are abbreviated as follows: 1, 3-bisphosphoglycerate 1, 3 bPGA; 3-phosphoglyceric acid, 3-PGA; phosphoenolpyruvate, PEP; dihydroxyacetone phosphate, DHAP. The asterisk marks the glycerophosphate shuttle which recycles glycerol-3-phosphate back to dihydroxyacetone phosphate via the mitochondrial glycerol phosphate oxidase complex.

Fig. 2. Effect of various sugars and glycerol on growth of cells with impaired glycosomal matrix import. The X-axis indicates time after initiation of the experiment, with or without induction of RNAi. The Y-axis indicates the cumulative cell concentration. A, Growth on various concentrations of glucose as indicated. Solid lines, uninduced; dashed lines, induced for PEX14 RNAi. B, Growth of PEX14 RNAi line on various sugars. Solid lines, uninduced; dashed lines, induced. SOGG (no added sugars, □), 10
mM fructose (O); galactose (△). The arrow marks the induced culture in fructose. C, Growth of PEX14 RNAi line on various sugars. Solid lines, uninduced; dashed lines, induced. SOGG (no added sugars, ■), 1 mM glycerol (O), and 10 mM 2-deoxyglucose (△). The arrows mark the induced cultures for glycerol and 2-deoxyglucose.

Fig. 3. Construction of double and single RNAi knockdown plasmids. Nucleotides 10-582 of PEX14 and 18-543 of HK1 were used in the constructs. Arrows indicate the orientation of the native transcript. Constructs A and B differ in the orientation of the two gene fragments. Digestion of the A orientation with XhoI followed by religation created the construct that targets only HK mRNA.

Fig. 4. Transcript levels in HK and PEX14 knockdown lines. Transcript levels were measured by reverse transcription and real-time PCR. RNA loading was normalized by assessment of α-tubulin mRNA abundance. Target mRNA levels were compared in each case to the uninduced cell line. Relative mRNA abundance for PEX14 (light columns) and HK (dark columns) are shown for day 2 following induction of RNAi with Tet. The X-axis indicates the RNAi cell lines.

Fig. 5. Knockdown of HK rescues reduction in PEX14. The panels show the examination of different parameters during an RNAi induction experiment. A, Immunoblot analysis. HK and PEX14 protein levels were assessed after induction at the indicated timepoints by SDS-PAGE and immunoblot analysis in the HK-PEX14B double RNAi cell line. Protein levels were normalized to the nucleolar protein NOG1. B, Glycosomal compartmentation. Immunofluorescence analysis using an antiserum directed against glycosomal matrix proteins was carried out at various times after induction of RNAi in the indicated cell lines. Days following addition of Tet are indicated on each image. The pattern seen on the day 0 (uninduced) PEX14 image is identical to the parental line or to the HK-PEX14B double RNAi line prior to induction. All cultures were grown in SDM-79. All images are shown at the same scale. C, Growth analysis. Growth of the PEX14 RNAi strain (◊), the HK RNAi strain (×) and the HK-PEX14 double RNAi strain (●) in the complete SDM-79 medium with 10% FBS. Solid lines are uninduced cultures and dashed lines are induced. The cell lines corresponding to each induced culture are indicated.

Fig. 6. Analysis of PFK and PFK-PEX14 RNAi. A, Northern blot analysis of PFK and PEX14 transcript levels from induced (+Tet) and uninduced (-Tet) cultures. The cell lines are indicated above the images. The PFK-PEX14 RNA encoded by the RNAi construct is visible upon induction with Tet as a molecule migrating somewhat faster than the endogenous PEX14 mRNA. B, Growth of the PFK RNAi strain in SOGG only (▲), 10 mM glucose (O) and 10 mM glycerol (□). Solid lines are uninduced cultures and dashed lines are induced. Induced cultures are marked to indicate the medium used. C, Immunoblot analysis of PFK RNAi. PFK protein levels in the PFK RNAi cell line grown in SOGG were assessed at the indicated timepoints after induction. Protein levels were normalized using PEX14 abundance. D, Growth of the PFK-PEX14 double RNAi strain in SOGG in the presence of 1 mM glycerol. Solid lines are uninduced cultures and dashed lines are induced. E, Immunoblot analysis of PFK-PEX14B RNAi. PFK and PEX14 protein levels in the PFK-PEX14B double RNAi cell line grown in SOGG were assessed at the indicated timepoints after induction. Protein levels were normalized by abundance of the nucleolar protein NOG1.
Figure 1
Figure 3
Figure 6

A

B

C

D

E

Day:
0  4  8  10

PEX14

PFK

NOG1

PEX14

PFK

PEX14
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