Zinc alpha2 glycoprotein alleviates palmitic acid-induced intracellular lipid accumulation in hepatocytes

Xinhua Xiao a, 1, Han Li a, 1, Xiaoyan Qi a, Yadi Wang a, Canxin Xu b, Gexin Liu a, Gebo Wen a, Jianghua Liu a, *

a Department of Metabolism and Endocrinology, The First Affiliated Hospital of University of South China, 421001, People’s Republic of China
b Department of Pathology & Immunology, Developmental, Regenerative and Stem Cell Biology, Washington University in St. Louis, MO, 63110, United States

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Abstract
Zinc alpha2 glycoprotein (ZAG) plays an important role in stimulating fat mobilization and lipolysis in adipose tissue, but its role in hepatic lipid metabolism remains unclear. Palmitic acid (PA) was used to stimulate HepG2 cells with ZAG overexpression or ZAG knock down (shRNA). Overexpression of ZAG significantly inhibited lipogenesis, promoted lipolysis and fatty acid β-oxidation, and attenuated PA-induced intracellular fat accumulation. Moreover, ZAG overexpression dramatically stimulated adiponectin expression in HepG2 cells. In contrast, knockdown of ZAG notably inhibited fatty acid β-oxidation, increased lipogenesis and lipid accumulation. Collectively, these data suggest that ZAG has the potential to alleviate hepatosteatosis, making it a promising therapeutic target for fatty liver.

1. Introduction
Non-Alcoholic Fatty Liver Disease (NAFLD) appears to be one of the most frequent causes of liver dysfunction. The incidence of NAFLD has increased markedly over these years, and is associated with the development of type 2 diabetes, atherosclerosis, hypertension, and even coronary heart disease (Sanyal, 2005; Roden, 2006). Hepatosteatosis is an important manifestation of hepatic metabolic injury, which is characterized by aberrant hepatic triglyceride (TGs) accumulation, due to increased de novo lipogenesis, increased fatty acid uptake, reduced fatty acid oxidation and export of very low density lipid (VLDL) (Anstee et al., 2013; Fabbrini et al., 2010). Thus, identifying key molecular players in the regulatory network that governs hepatic lipid metabolism is an important step toward development of treatments for hepatosteatosis.

Zinc alpha2 glycoprotein (ZAG) is widely expressed in many different tissues including adipose tissue and liver, this soluble protein can be secreted into serum and other body fluids (Burgi and Schmid, 1961; Bing et al., 2004; Mracek et al., 2010; Sanchez et al., 1999; Hassan et al., 2008). Although first identified as a potential cancer marker for some malignant tumors (Tada et al., 1991; Díez-Itza et al., 1993; Abdul-Rahman et al., 2007), ZAG has also been reported as a lipid-mobilizing factor responsible for a loss of fat mass in patients with cancer cachexia (Todorov et al., 1998). More recent evidence has suggested that ZAG impacts lipolysis and increases fat utilization, which ultimately leads to decreased adipose tissue mass (Mracek et al., 2010; Russell and Tisdale, 2011a,b; Bing et al., 2010; Mracek et al., 2010). Interestingly, previous studies have shown that levels of hepatic ZAG expression level were remarkably down-regulated in obesity obese patients (Selva et al., 2009). Although the effects of ZAG in lipid metabolism within adipose tissue has been well characterized, the role of ZAG in the regulation of TG metabolism in the liver remains unknown.

The aim of the present study was to characterize the role of ZAG in the regulation of lipid metabolism in hepatocytes. We found that
manipulation of ZAG expression in hepatocytes altered hepatic TG accumulation and the expression of key fatty acid oxidation genes, lipogenesis genes and adiponectin. Overall, these data indicate that ZAG is a key player in hepatic lipid metabolism in cultured HepG2 cells.

2. Materials and methods

2.1. Cell cultures

HepG2 cells (American Type Culture Collection) were cultured in minimum Eagle's medium (low glucose, Invitrogen) supplemented with 10% fetal bovine serum (BI, Germany), 100 IU/ml penicillin (Invitrogen), and 100 µg/ml streptomycin (Invitrogen). Cells were incubated at 37 °C in a 5% CO2 incubator. Overexpression was accomplished by transfection with the pIRE2-ZsGreen1-hZAG plasmid vector for 24 h. Knockdown was accomplished by infection with LV-AZGP1-RNAi lentivirus for 48 h. Cells were then treated with 0.4 mM palmitic acid (PA) under serum-free conditions for 24 h.

2.2. Plasmids, lentivirus-RNAi transfection

Human ZAG full length DNA fragment cloned into the pIRE2-ZsGreen1 basic plasmid was obtained from Takara Bio (Dalian). Lentivirus expressing ZAG shRNA (ZAG-RNAi) was purchased from the manufacturer according to the manufacturer’s instructions.

2.3. Oil Red O staining and intracellular triglyceride assay

Lipid accumulation in HepG2 cells was evaluated by Oil Red O staining and the measurement of triglyceride (TG) content. Briefly, samples were fixed with 4% paraformaldehyde then stained with Oil Red O for 15 min. After washing in PBS repeatedly, staining was examined by light microscopy. Intracellular TG was extracted with chloroform/methanol (2:1) as described previously (Folch et al., 1957) and the TG concentration was determined using a triglyceride reagent (Sangon, Biotech Co., Ltd. Shanghai, China). cDNA was synthesized from 1 µg RNA in a total volume of 20 µl using random primer (Fermentas Life Sciences, EU) and reverse transcriptase (Invitrogen). Primers were designed using Primer5 input software (Fermentas Life Sciences, EU) and reverse transcriptase (Invitrogen). Primers were designed using Primer5 input software (Fermentas Life Sciences, EU) and reverse transcriptase (Invitrogen). The mRNA levels were normalized to β-actin. Primer sequences are listed in Table 1.

2.4. RNA isolation, quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA from HepG2 cell lysates was extracted with Trizol reagent (Sangon, Biotech Co., Ltd. Shanghai, China). cDNA was synthesized from 1 µl RNA in a total volume of 20 µl using random primer (Fermentas Life Sciences, EU) and reverse transcriptase (Invitrogen). Primers were designed using Primer5 input software and specificity of each primer was determined by a BLAST search. Quantitative real-time PCR was performed in a 25 µl reaction volume using Maxima SYBR Green/Rox qPCR Master Mix (Thermo Scientific) following the manufacturer’s instructions. Results were analyzed by using 2-ΔΔCT method described previously (Livak and Schmittgen, 2001). The mRNA levels were normalized to β-actin. Primer sequences are listed in Table 1.

2.5. Protein preparation and western blot

Total protein extracts from cultured hepatocytes were prepared as described previously, and protein concentrations were determined with the Mini Protein 3 Cell (Bio-Rad). Proteins were separated on 10–20% Tris-glycine gels (Invitrogen) and transferred to PVDF membrane (Millipore) for 1.5–2 h. Membranes were blocked in Tris-buffered saline with Tween (TBST; 20 mM Tris, 150 mM NaCl, pH 7.4, plus 0.1% Tween 20) containing 2.5% horse serum for 2 h at room temperature, and then incubated with primary antibodies at 4 °C overnight. Membranes were washed with 1% TBST (TBS containing 1% Tween-20) three times before incubation with horseradish peroxidase-conjugated secondary antibodies (Invitrogen Company, Shanghai, China) for 1 h at room temperature. Finally images were acquired using ECL Plus (Beyotime institute of Biotechnology, China). The optical density of the bands was analyzed with Quantity One software (Bio-Rhinnaer2200, Leica, Germany). Protein levels were normalized to β-actin.

2.6. Statistical analysis

Data are expressed as mean ± SEM. All experiments in vitro were carried out in triplicate and on three separate occasions. Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) with Student’s t-test was used to determine differences between groups. A value of p < 0.05 was taken as statistically significant.

3. Results

3.1. ZAG negatively regulates hepatic intracellular lipid accumulation

Evidence has suggested that ZAG may reduce lipid content in adipose tissue by increasing lipid utilization (Bing et al., 2010; Russell and Tisdale, 2011a,b). We examined the effect of ZAG on lipid content in liver cells by stimulating HepG2 cells that overexpressed (ZAG expression plasmid, Fig. S1) or are deficient in (lentivirus shRNA, Fig. S2) ZAG after exposure to PA. Cells transfected with either pIRE2-GFP or GFP RNAi served as controls for the overexpression and knock down, respectively. Oil Red O staining showed that exposure to PA led to intracellular lipid accumulation in pIRE2-GFP and GFP-RNAi transfected HepG2 cells, cellular TG content was obviously increased. While the cellular fat droplets and TG content were dramatically decreased especially in PA-induced HepG2 cells (Fig. 1A and B). On the other hand, knockdown ZAG by infection of lentivirus shRNA (LV-shRNA-ZAG) markedly increased triglyceride in hepatocytes compared to the control scrambled shRNA (LV-shRNA-scr) in both unstimulated and PA-stimulated HepG2 cells (Fig. 1C and D). These observations suggested that ZAG can negatively regulate unstimulated and PA-induced hepatic intracellular lipid accumulation.

<table>
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<th>Gene</th>
<th>Forward primer:5′–3′</th>
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<td>β-Actin</td>
<td>CATCCTGCGTCTGGACCTTG</td>
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3.2. ZAG negatively regulates lipogenic pathway

It is well established that lipogenesis, lipolysis and lipid uptake are three major factors that contribute to hepatic lipid accumulation. To determine how ZAG might regulate intracellular lipid metabolism in hepatocytes, we first tested whether alteration of ZAG expression levels in HepG2 cells would affect the lipogenesis pathway. To test our hypothesis, HepG2 cells were transfected with plasmid green fluorescent protein (pIRES2-GFP) and ZAG expression plasmid (pIRES2-hZAG). We found that ZAG overexpression led to a dramatic decrease of the metabolic nuclear receptors SREBP-1c and LXR, as well as the mRNA and protein levels of FAS, ACC and SCD-1 (lipogenic enzymes in the liver), this effect was enhanced in the presence of PA (Fig. 2A–E). Knockdown of hepatic ZAG, by transfection with siRNA oligos targeting ZAG, led to upregulation of SREBP-1c, LXR, FAS, ACC and SCD-1 (lipogenic enzymes in the liver), this effect was enhanced in the presence of PA (Fig. 2A–F). Knockdown of hepatic ZAG, by transfection with siRNA oligos targeting ZAG, led to upregulation of SREBP-1c, LXR, FAS, ACC and SCD-1 (lipogenic enzymes in the liver), this effect was enhanced in the presence of PA (Fig. 2A–F).

Fig. 1. ZAG alleviates intracellular lipid accumulation of palmitic acid (PA) induction in HepG2 cells in vitro. (A), (B) HepG2 cells were transfected with pIRES2-GFP or pIRES2-hZAG for 24 h, and then exposed to palmitic acid (PA) (0.4 mM) for 24 h. (A) Oil Red O staining of lipid droplets photographed at 200× magnification. (B) Intracellular TG content in HepG2 cells. Data are represented as mean ± SEM. **P < 0.01 versus pIRES2-GFP group, ###P < 0.01 versus palmitic acid treated pIRES2-GFP group. (C), (D) After 48 h of LV-GFP-RNAi or LV-ZAG-RNAi transfection, HepG2 cells were treated with palmitic acid (PA) (0.4 mM) for 24 h. (C) Oil Red O staining of lipid droplets and the image were photographed at 200× magnification. (D) Intracellular TG contents in HepG2 cells. Data are represented as mean ± SEM. *P < 0.05, **P < 0.01 versus GFP-RNAi group, ###P < 0.01 versus palmitic acid treated ZAG-RNAi group.

3.3. ZAG positively regulates FXR and PPARα

FXR and PPARα are nuclear receptors that mediate fatty acid β-oxidation in hepatocytes. To elucidate the underlying mechanisms by which ZAG affects lipid content in HepG2 cells, we transfected HepG2 cells with pIRES2-hZAG (ZAG overexpression) or LV-siRNA-ZAG (ZAG knock down) and then treated with PA. PA treatment suppressed FXR and PPARα expression in HepG2 cells. Overexpression of ZAG increased expression of FXR and PPARα (Fig. 3A–C). On the other hand, ZAG silencing decreased expression of FXR and PPARα (Fig. 3D–F). Together, these observations are consistent with the hypothesis that ZAG positively regulates FXR and PPARα in hepatocytes, which may contribute to increased lipolysis.

3.4. ZAG regulates lipid transport and fatty acid β-oxidation

mRNA and protein levels of fatty acid transport protein (FATP) and CPT-1A (which are related to lipid transport and fatty acid β-oxidation) in HepG2 cells were determined. There was no significant difference in FATP expression between pIRES2-hZAG-transfected (ZAG overexpression) and pIRES2-GFP-transfected (control) HepG2 cells in the absence of PA. However, after PA treatment, levels of FATP decreased, while CPT-1A levels decreased, in cells.
Fig. 2. ZAG suppresses transcripts and proteins that function in lipogenesis in HepG2 cells in vitro. (A–F) The mRNA and protein levels of nuclear receptors SREBP-1c, LXR, FAS, ACC and SCD-1 in HepG2 cells were determined by qRT-PCR and Western blotting. HepG2 cells were transfected with pIRES2-GFP or pIRES2-hZAG for 24 h, and then exposed to PA (0.4 mM) for 24 h. Data are represented as mean ± SEM. *P < 0.05, **P < 0.01 versus pIRES2-GFP group. #P < 0.05, ##P < 0.01 versus PA-treated pIRES2-GFP group. (G–L) The mRNA and protein levels of nuclear receptors SREBP-1c, LXR, FAS, ACC and SCD-1 in HepG2 cells were determined by qRT-PCR and Western blotting. HepG2 cells were transfected with LV-GFP-RNAi or LV-ZAG-RNAi for 48 h and then harvested after 24 h incubation with PA (0.4 mM). Data are represented as mean ± SEM. *P < 0.05, **P < 0.01 versus GFP-RNAi group, #P < 0.05, ##P < 0.01 versus PA-treated GFP-RNAi group.
Fig. 2. (continued)
that overexpressed ZAG (Fig. 4A–C). Similarly, the FATP expression level was not changed in HepG2 cells transfected with ZAG RNAi without PA induction. However, FATP and CPT-1A levels were increased significantly in the presence of PA in ZAG knockdown cells (Fig. 4D–F). Interestingly, the PA-treatment markedly decreased levels of CPT-1A in ZAG RNAi-transfected HepG2 cells (Fig. 4E and F). Collectively, these data suggest that ZAG can reduce lipid transport by decreasing expression of FATP and improve fatty acid β-oxidation by increasing expression of CPT-1A in PA-stimulated HepG2 cells.

3.5. ZAG regulates adiponectin expression

Adiponectin is an important adipokine that suppresses lipid accumulation in the liver (Musso et al., 2005; Hsieh et al., 2015; Xu et al., 2003). We tested whether ZAG could regulate adiponectin expression. Adiponectin mRNA and protein levels were decreased in PA-treated HepG2 cells (Fig. 5). Interestingly, adiponectin levels were markedly increased in ZAG-overexpressed HepG2 cells without PA treatment (Fig. 5A and B), and ZAG RNAi dramatically decreased adiponectin expression in HepG2 cells (Fig. 5C and D).

4. Discussion

Despite the strong evidence linking ZAG with lipolysis (Mracek et al., 2010; Russell and Tisdale, 2011a,b), the precise physiological function of ZAG in liver lipid metabolism in vitro remains unclear. The current study illustrates the importance of ZAG in hepatic lipid metabolism, using a HepG2 in vitro cellular steatosis model. This study represents the first report that overexpression of ZAG reduces the deposition of TG in unstimulated and PA-stimulated HepG2 cells. In contrast, knockdown of ZAG increases deposition of TG in unstimulated and PA-stimulated HepG2 cells. As expected, ZAG inhibits lipogenesis and promotes lipolysis by regulating metabolic nuclear receptors PPARα, FXR, SREBP-1c and LXR in hepatocytes. Unexpectedly, ZAG stimulates adiponectin in HepG2 cells. Therefore, we conclude that ZAG plays a significant role in lipid accumulation within hepatocytes.

Most available studies demonstrate that activation of ZAG is associated with lipolysis, specifically within adipose tissue (Russell and Tisdale, 2011a,b; Rolli et al., 2007). ZAG knockout mice are heavier than wild type mice, regardless of whether they are fed a standard diet or high fat diet (Shimano, 2000). Consistent with those data, this study demonstrates that ZAG silencing promotes deposition of TG, whereas overexpression of ZAG reduces TG deposition, all in response to PA stimulation.

Hepatic lipid accumulation results from an imbalance between lipid availability (from circulating lipid uptake or de novo fatty acid synthesis) and lipid disposal (via free fatty acid oxidation or triglyceride-rich lipoprotein secretion). Therefore, we examined key enzymes in these pathways to assess the impact of ZAG on these processes. FAS is a rate-limiting enzyme in de novo fatty acid biosynthesis (Munday, 2002). ACC is rate-limiting in the synthesis of malonyl-CoA, which is a crucial precursor for fatty acid biosynthesis. Furthermore, ACC could inhibit CPT-1A activity to prevent mitochondrial fatty acid oxidation (Shen et al., 2013; Ntambi, 1995). Finally, SCD-1 is a microsomal enzyme important for synthesis of mono-unsaturated fatty acids (Gong et al., 2010). Our results clearly demonstrate that ZAG downregulates each of these fatty-acid synthesis enzymes (FAS, ACC and SCD-1), which likely produced the observed decrease in hepatic TG synthesis. Downregulation of lipogenesis after ZAG-overexpression in HepG2 cells suggests that ZAG is a negative regulator of lipogenesis in hepatocytes. In addition, FATP levels were not altered with overexpression or knockdown of ZAG in untreated HepG2 cells, however it was dramatically increased by PA induction, consistent with another study in Huh-7 cells (Lópe-Celazquez et al., 2012).

Additionally, the fatty acid oxidation enzyme, CPT-1A, was upregulated by either ZAG overexpression or PA treatment. The PA induction of CPT-1A is supported by another study in rat primary hepatocytes (Thering et al., 2009). However, hepatic CPT-1A expression is decreased in mice fed with high fat diet (Park et al., 2014), which we have also observed (data not shown). We speculate that PA could take advantage of activate the lipogenesis pathway. Consequently, these results suggested that ZAG could downregulate FATP expression in the presence of PA to reduce lipid transport and upregulate CPT-1A expression to promote fatty acid β-oxidation, which will suppress cellular TG accumulation.

Metabolic nuclear receptors in the liver are important players in the pathogenesis of NAFLD, which has emerged as one of the most frequent chronic liver diseases across the globe (Pawlak et al., 2015). These receptors are involved in the processes of fat storage, export, uptake, oxidation, and lipolysis. Therefore, understanding the molecular mechanisms underlying the involvement of metabolic nuclear receptors in disease pathogenesis may offer targets for the development of new treatments for NAFLD. Of note, the bile acid-activated nuclear receptor FXR and PPARα have been shown to play a role in hepatic lipogenesis (Wojcik et al., 2007). Fatty acid synthesis in hepatocytes is positively regulated by the transcriptional factor SREBP-1c and LXR (Duran-Sandoval et al., 2004). Hepatic FXR expression is reduced in obesity, NAFLD patients and obese animal models (Yang et al., 2010; Lu et al., 2014; Watanabe et al., 2004). In addition, mice with FXR deletion demonstrate hyperlipidemia and even hepatic steatosis, whereas the overexpression of FXR improves serum lipids profiles and protects against hepatic fatty accumulation in obese mouse models, in part by repression of lipogenesis and promotion of lipolysis (Shimano, 2000; Zhang et al., 2006). SREBP-1c overexpression can increase cellular FFA and TG accumulation and produce hepatic steatosis (Zhang et al., 2013; Lefebvre et al., 2006). Furthermore, PPARα activation up-regulates gene expression associated with FFA oxidation (Rakhshandehrou et al., 2010; Luo and Tall, 2000). These two genes work together to maintain the balance of lipid metabolism in vivo. LXR may act to regulate SREBP-1c translation, which may promote TG generation through induction of FAS and ACC (Laffitte et al., 2001). Many studies have shown that FXR can reduce TG accumulation partly through preventing expression of SREBP-1c and its target gene FAS and by promoting PPARα activation and FFA oxidation, thereby alleviating fat deposition and improving insulin resistance (Musso et al., 2005; Zhang et al., 2006). In our studies, PA reduced expression of FXR and PPARα, enhanced expression of SREBP-1c and LXR in HepG2 cells. Overexpression of ZAG significantly increased expression of FXR and PPARα, decreased expression of SREBP-1c and LXR. Also, in our diet-induced obesity mouse model, ZAG, FXR and PPARα were decreased, whereas SREBP-1c and LXR were increased in the liver (Fig. S3). Tail vein injection of Ad-ZAG leading to ZAG overexpression in vivo markedly increased FXR and PPARα levels and reduced SREBP-1c and LXR (Fig. S4).

Finally, we found that ZAG is an important regulator of adiponectin in HepG2 cells. Adiponectin is also a promising target for the treatment of fatty liver (Hsieh et al., 2015; Xu et al., 2003). Adiponectin reportedly suppresses lipogenesis through down-regulation of FAS and ACC, activates fatty acid oxidation through up-regulation of CPT-1A and prevents FFA influx by reducing CD36 expression.
thereby alleviating fatty liver (Xu et al., 2003). ZAG can stimulate adiponectin secretion by human adipocytes (Rolli et al., 2007). Thus, we speculate that ZAG may also indirectly alleviate lipid accumulation in the liver by activation of adiponectin.

In summary, this report is the first to reveal a profound effect of ZAG on hepatocyte lipid homeostasis as well as on metabolic

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**Fig. 3.** ZAG upregulates FXR and PPARα in HepG2 cells. (A–C) The mRNA and protein levels of nuclear receptors FXR and PPARα in HepG2 cells were determined by qRT-PCR and Western blotting. HepG2 cells were transfected with pIRES2-GFP or pIRES2-hZAG for 24 h, and then exposed to PA (0.4 mM) for 24 h. Data are represented as mean ± SEM. *P < 0.05, **P < 0.01 versus pIRES2-GFP group. #P < 0.05 versus PA-treated pIRES2-GFP group. (D–F) The mRNA and protein levels of nuclear receptors FXR and PPARα in HepG2 cells were determined by qRT-PCR and Western blotting. HepG2 cells were transfected with LV-GFP-RNAi or LV-ZAG-RNAi for 48 h and then harvested after 24 h incubation with PA (0.4 mM). Data are represented as mean ± SEM. *P < 0.05, **P < 0.01 versus GFP-RNAi group, #P < 0.05 versus PA-treated GFP-RNAi group.
transcription factors involved in regulation of hepatic lipid content. Our data indicate that ZAG altered expression of several nuclear transcription factors. Therefore, we conclude that ZAG could play multiple significant roles in the maintenance of lipid metabolism and might be promising as a therapeutic candidate for the treatment of hepatic lipid metabolic disorders.

Fig. 4. ZAG regulates lipid transport protein and fatty acid β-oxidation in HepG2 cells. (A–C) HepG2 cells were transfected with pIRES2-GFP or pIRES2-hZAG for 24 h, and then exposed to PA (0.4 mM) for 24 h. The mRNA and protein levels of FATP and CPT-1A in HepG2 cells were determined by qRT-PCR and Western blotting. Data are represented as mean ± SEM. **P < 0.01 versus pIRES2-GFP group, #P < 0.05, ##P < 0.01 versus PA-treated pIRES2-GFP group. (D–F) 48 h after LV-GFP-RNAi or LV-ZAG-RNAi transfection, HepG2 cells were treated with PA (0.4 mM) for 24 h. The mRNA and protein levels of FATP and CPT-1A in HepG2 cells were determined by qRT-PCR and Western blotting. Data are represented as mean ± SEM. *P < 0.05, **P < 0.01 versus GFP-RNAi group, #P < 0.05, ##P < 0.01 versus PA-treated ZAG-RNAi group.
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Duality of interest

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2016.06.003.

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