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Choline-mediated hepatic lipid homoeostasis in yellow catfish: unravelling choline's lipotropic and methyl donor functions and significance of *ire-1α* signalling pathway

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Abstract

Choline plays a crucial role in hepatic lipid homeostasis by acting as a major methyl-group donor. However, despite this well-accepted fact, no study has yet explored how choline's methyl-donor function contributes to preventing hepatic lipid dysregulation. Moreover, the potential regulatory role of Ire-1*α*, an ER-transmembrane transducer for the unfolded protein response (UPRer), in choline-mediated hepatic lipid homeostasis remains unexplored. Thus, this study investigated the mechanism by which choline prevents hepatic lipid dysregulation, focusing on its role as a methyl-donor and the involvement of Ire-1*α* in this process. To this end, a model animal for lipid metabolism, yellow catfish (*Pelteobagrus fulvidraco*) were fed two different diets (adequate or deficient choline diets) *in vivo* for 10 weeks. The key findings of studies are as follows: 1. Dietary choline, upregulated selected lipolytic and fatty acid *β*-oxidation transcripts promoting hepatic lipid homeostasis. 2. Dietary choline ameliorated UPRer and prevented hepatic lipid dysregulation mainly through *ire-1α* signalling, not perk or *atf-6α* signalling. 3. Choline inhibited the transcriptional expression level of ire-1*α* by activating site-specific DNA methylations in the promoter of *ire-1α*. 4. Choline-mediated *ire-1α* methylations reduced Ire-1*α*/Fas interactions, thereby further inhibiting Fas activity and reducing lipid droplet deposition. These results offer a novel insight into the direct and indirect regulation of choline on lipid metabolism genes and suggests a potential crosstalk between *ire-1α* signalling and choline-deficiency-induced hepatic lipid dysregulation, highlighting the critical contribution of choline as a methyl-donor in maintaining hepatic lipid homeostasis.

Keywords: Choline: Hepatic lipid dysregulation: Unfolded protein response: Ire-1*α*: Methylation

Non-alcoholic steatohepatitis is a disease of emerging identity and importance and is now considered as one of the commonest liver diseases worldwide. It is frequently associated with severe obesity and is intimately related to various clinical and biological markers of hepatic lipid dysregulation[1]. Choline, as an essential dietary nutrient, plays a vital role in maintaining hepatic lipid homoeostasis[2]; thus, its deficiency has been closely linked to non-alcoholic steatohepatitis[3,4]. Additionally, choline, functioning as a lipotropic agent, has been commonly used in animal feeds[5]. Additionally, choline's role in lipid metabolism is of particular interest for aquaculture finfish species given its recognition as a lipotropic agent[5]. One of two major fates for choline are to be irreversibly oxidised by choline dehydrogenase (Chdh) and used as a donor of methyl groups[6]. Thus, emerging data support a role for dietary choline in modulating DNA methylation[7,8]. Furthermore, the close association between DNA methylation and lipid metabolic disorder has been investigated[9–11]. However, less research has been done on the contribution and mechanism of methyl donor function of choline in regulating hepatic lipid homoeostasis, although the crucial role of dietary choline in lipid metabolism and DNA methylation has been widely studied.

Endoplasmic reticulum (ER)-localised enzymes synthesise the vast majority of cellular lipids[12]. Hepatic lipid homoeostasis

Abbreviations: CHDH, choline dehydrogenase; ER, endoplasmic reticulum; Fas, fatty acid synthase; Ire1α, inositol-requiring enzyme 1α; LD, lipid droplet; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; UPRer, unfolded protein response.

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therefore depends on the ER function[13]. Naturally, as a conserved adaptive mechanism in response to ER dysfunction, ER unfolded protein response (UPRer) has been closely connected with hepatic lipid dysregulation[14]. Importantly, UPRer has an upstream and comprehensive role for regulating hepatic lipid metabolism. UPR is initially activated and transduced by three UPR branches: the *perk-eif2α* pathway, the *ire1α-xbp1* pathway and the *atf6-chop* pathway, and then induces translational inhibition followed by up-regulation of ER-resident chaperones, such as *grp78/bip*, *grp94* and *crt*[15]. Given the potential role of methylation in regulating UPRer has been investigated[16,17], UPRer should be a good and novel viewpoint from which to understand the underlying mechanism governing choline-mediated DNA methylation regulating hepatic lipid metabolism; however, the relevant regulatory mechanism still remains to be explored. On the other hand, some studies, including our previous study, have indicated the differential effects and mechanisms of three UPRer branches on regulating cell function and metabolism[18,19]. Accordingly, it is important to investigate the differential mechanism of three UPRer branches on choline regulating hepatic lipid metabolism.

Yellow catfish, *Pelteobagrus fulvidraco*, is an economically important freshwater teleost fish widely distributed in China and other countries[20]. Its genome has a high degree of similarity to that of humans[20,21] and is also a good model animal for studying lipid metabolism[22]. Our previous studies found UPRer was the major and most common inducements to hepatic lipid dysregulation in yellow catfish[18,23]. In addition, although choline's role in lipid metabolism is of particular interest for aquaculture finfish species given its recognition as a lipotropic agent[5], the mechanism of choline acting as methyl donor alleviating hepatic lipid dysregulation is still not fully understood. Thus, our present study reveals dietary choline addition down-regulated *ire1α* expression by activating specific CpG methylation sites and also found choline prevented hepatic lipid dysregulation *via* controlling Ire-1*α*/fatty acid synthase (Fas) interaction. These results emphasise the critical contribution of methyl donor function for choline acting as a lipotropic agent.

Materials and methods

Experimental treatments

Huazhong Agricultural University's (HZAU) institutional ethical guidelines for the care and use of laboratory animals were followed throughout all investigations and were approved by the Ethical Committee of HZAU (identification code: Fish-2020-07-21).

Expt. 1: Animals feeding, management and sampling in vivo *study.* Dietary formula and yellow catfish feeding were determined according to our previous studies[2]. We formulated two experimental diets, which are shown in online Supplementary Table S1. Fish oil and soyabean oil (1:1, w/w) were used as the lipid sources. The addition of choline chloride was 1658·4 (adequate choline) and 264·7 (choline deficiency) mg of choline per kg diet (≥ 99·0 % in purity, Sinopharm Chemical Reagent Co. Ltd.). One hundred and fifty uniform size juvenile yellow catfish, obtained from a local fish pond (Wuhan, China, mean initial weight: 3·83 (SEM 0·01) g, mixed sex), were randomly stocked in six tanks (300-l water volume), with triplicates for two treatment, twenty-five fish/tank. All fish were fed to the satiation twice daily at 08.00 and 16.30 hours. The experiment continued for 10 weeks. During the experiment, the parameters in water quality were as follows: water temperature 28 (SEM 0·6)°C; dissolved oxygen 6·53 (SEM 0·11) mg/l; pH 7·46 (SEM 0·17) and NH4-*N* 0·09 (SEM 0·02) mg/l.

At the end of the experiment, only before sampling, yellow catfish were fasted for 24 h to avoid the prandial effects. Yellow catfish were euthanised with MS-222 (tricaine methanesulfonate, 100 mg/l water). All yellow catfish were counted and weighed in bulk. The liver tissues from three fish of each tank were sampled for histological and ultrastructural observation, respectively. The liver samples from other fish were frozen immediately in liquid N_2 for other analysis, including the contents of TAG, methylation analysis, gene and protein expression.

Expt. 2: Cell culture and treatments in vitro *study.* Primary hepatocyte culture and treatment: yellow catfish (mean weight: 6·83 (SEM 0·24) g) were obtained from the *in vivo* experiment after 2 weeks of acclimation. Primary hepatocytes were isolated and cultured as previously described[24]. Briefly, hepatocytes were seeded in cell culture plates at a density of $1·4 \times 10^6$ cells/ml, and primary hepatocytes were cultured in RPMI 1640 media (Thermo Fisher Scientific) (supplemented with 10 % FBS, 1 mmol/l glutamine, 100 μg/ml streptomycin and 100 μg/ml penicillin) and adhered 4 h before treatment. To investigate the mechanisms of choline influencing hepatic lipid metabolism, we used choline to incubate yellow catfish primary hepatocytes. The total concentration of choline chloride was 0·6 mg/l for the adequate choline group and no extra addition for choline deficiency group based on our previous study and the cell viability in our pilot experiment. The cells were incubated at 28°C for 48 h. Each treatment was performed in triplicate. For each cell culture, a pool of cells from three fish was used.

If choline serves as a donor of methyl groups, it needed to be irreversibly oxidised by choline dehydrogenase (CHDH)[4]. Thus, in order to intercept the methyl donor function of choline, we designed and transfected siRNA against *chdh* to hepatocytes of yellow catfish. On the other hand, human embryonic kidney cells (HEK293T cells) have high transfection efficiency and have been widely used to explore genetic function in fish[25]. In the present study, HEK293T cells were used to explore the effect of special methylation sites on *ire1α* promoter activity.

Sample analysis

Oil red O, haematoxylin and eosin, Bodipy 493/503 staining and transmission electron microscopy observation. Oil red O and haematoxylin and eosin staining tests were conducted according to the manufacture's instruction and followed the description in our publication[22,24]. The ten fields of each sample were quantified by the software Image J (NIH) to get the statistics of the relative areas of lipid droplets (LD) in the Oil red O staining, the relative areas of vacuoles in the haematoxylin and eosin staining.

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Bodipy 493/503 staining for the LD and transmission electron microscopy (TEM) observation of hepatocytes were carried out according to the protocol, which was described in our previous publications[22,24]. For Bodipy 493/503 staining, briefly, hepatocytes were cultured in twelve well plates, treated with the corresponding treatments for 48 h, washed twice with PBS and incubated with 5 μg/ml Bodipy 493/503 (D3922; Thermos Fisher Scientific) for 30 min, next to thrice PBS washes. The hepatocytes were observed with a laser scanning confocal microscope (Leica DMI8) to visualise the intensity of fluorescence. The green dots were defined as lipophagic vacuoles, which were quantified with a CytoFlex flow cytometer (Beckman Coulter). The data analysis was conducted using FlowJo v.10 software. For transmission electron microscopy observation of hepatocytes, in brief, samples were fixed in 2·5% glutaraldehyde, followed by post-fixation in osmium tetroxide. The ultrathin sections were dehydrated in ethanol, embedded in resin, stained with uranyl acetate followed by lead citrate and then prepared for EM.

Cell viability and determination of lipid and TAG contents, and Fas activity. Cell viability was measured with the use of 3-(4,5-dimethylthia-zol-2-yl)-2, 5-diphenyltetrazolium bromide (V13154; Thermo Fisher Scientific) according to our previous publication[22,24]. Hepatic lipid content was determined by the ether extraction according to our previous publication[2]. The contents of TAG were determined by commercial kits (A110-1-1, Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's instructions. Soluble protein content was analysed, based on protocols by Bradford[22,24].

For Fas activity assays, the liver and cells samples were homogenised in three volumes of ice-cold buffer (0·02 M Tris–HCl, 0·25 M sucrose, 2 M MEDTA, 0·1 M sodium fluoride, 0·5 mM phenylmethylsulphonyl fluoride, 0·01 M *β*-mercapto-ethanol, pH 7·4) and centrifuged at 20 000 × ***g*** at 4°C for 30 min. The assay was carried out at 25°C on several dilutions of the supernatant fluid, in the final volume of 1 ml, and started by the addition of malonyl-CoA. The enzyme activity was calculated after subtraction of the non-specific oxidation of NADPH in the absence of malonyl-CoA. 2 mol NADPH was taken to correspond to 1 mol malonyl-CoA utilised. The reaction was started by addition of the tissue extract. The changes in absorbance at 340 nm were monitored at intervals of 15 s for 3 min.

RNA isolation and real-time quantitative PCR analysis. Total RNA was isolated by using the Trizol reagent and then transcribed into the cDNA by using the Reverse Transcription Kit. Analyses on gene transcript levels were conducted through the real-time quantitative PCR method described before[22,24]. The primer sequences used in this analysis are given in online Supplementary Table S3. A set of nine housekeeping genes (*gapdh, b2m, ef1α, 18s rRNA, tuba, β-actin, hprt1, ubc9, tbp*) were selected from our transcriptome database in order to test their transcription stability. Our pilot experiment indicated that *gapdh* and *β-actin* (M = 0·27) showed the most stable levels of expression across the experimental conditions, as suggested by geNorm[26]. Thus, the relative expression levels were normalised to the geometric mean of the combination of *gapdh* and *β-actin* and calculated using the $2^{-\Delta\Delta Ct}$ method.

Immunoprecipitation and western blot. Immunoprecipitation was performed to identify Fas–Ire1*α* interaction based on the protocols by Su *et al*[27].

Briefly, in order to conduct the immunoprecipitation analysis, we lysed the cells in NP-40 buffer (Beyotime, p0013F) with the addition of a protease inhibitor cocktail (Beyotime, P1010). The cell lysate was then mixed with anti-Ire1*α* (ab37073; Abcam), as the bait protein, at 4°C overnight. The incubation was sustained for 3 h, followed by the addition of protein A/G agarose (P2012; Beyotime). The immunocomplexes were washed five times by PBS supplemented with Phenylmethanesulfonyl fluoride (PMSF) protease inhibitor. Finally, the western blot analysis was performed with anti-Fas (ab133619; Abcam) or anti-Ire1*α* (ab37073; Abcam), as the prey protein. In addition, for the mutation analysis of Ire1*α*, the open reading frames of Fas and Ire1*α* sequences/or lacks interaction sequence (Ire1*α* Δ836–963) were subcloned into the pcDNA3·1 (+) vector with the Flag-tag and HA-tag sequences, respectively. Then, the cell lysate was then mixed with anti-HA-tag (ab236632; Abcam), as the bait protein, at 4°C overnight. The incubation was sustained for 3 h, followed by the addition of protein A/G agarose. Lastly, western blot analysis was performed with anti-HA-tag (ab236632; Abcam) or anti-Flag-tag (ab1162; Abcam), as the prey protein.

To identify the protein levels of Fas, Ire1*α*, Chdh, Dnmt1, Grp78 and Gapdh, western blot analysis was performed according to our previous study[22]. In brief, the protein was loaded onto the SDS-PAGE gel and then transferred to the Polyvinylidene fluoride (PVDF) membrane. Membranes were blocked with 5 % skimmed milk and then incubated overnight at 4°C with one of the following primary antibodies: anti-Fas, anti-Ire1*α*, anti-Gapdh (ab198233 1:10 000, 12 118, Cell Signaling Technology), anti-Chdh (1:1000, A16545, ABclonal), anti-Dnmt1 (1:1000, A5495, ABclonal) and anti-Grp78 (1:1000, A0241, ABclonal), respectively. The secondary antibodies, including an HRP-conjugated anti-rabbit IgG antibody (1:10 000, 7074, Cell Signaling Technology), were then incubated with the membranes. The membranes were seen by ECL (1 705 060, Bio-Rad) after additional washing. The membranes were seen using enhanced chemiluminescence, and Image J was used to measure the densitometry of these bands.

Bisulphite sequencing of ire-1α. To determine the methylation level of *ire-1α*, *ire-1α* gene was sequenced by bisulphite based on our published protocol[28]. Briefly, tissue DNA kit (Omega Biotek) was used to isolate genomic DNA from the intestine samples of yellow catfish and then the DNA Methylation-Gold Kit was used to modify them, based on the manufacturer's instructions. Then, PCR was used to amplify the bisulphite-modified DNA with specific primer pairs. The operation of PCR included 95°C denaturation for 3 min, then 35 cycles of 95°C for 30 s, 53°C for 30 s and 72°C for 40 s and end of 72°C extension for 5 min. The PCR products were purified and cloned into pMD19-T

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Vectors (TaKaRa). After cloning, nine clones were chosen from each sample and used for DNA sequencing. Sequencing analysis was performed by TsingKe Biological Technology.

RNAi and gene transfection. To generate a *chdh* knockdown cell, hepatocytes of yellow catfish were transfected with 103 nM of siRNA against *chdh* from Sigma-Aldrich based on our previous protocols[27]. Transfection was performed with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific). Target sequences for preparing the siRNA of yellow catfish *chdh* are shown in online Supplementary Table S3. The transfection of siRNA was performed using the Lipo2000 transfection reagent according to the supplier's protocol with transfection efficiencies of 42·9 (SEM 3·4) % with low levels of cell death (4·2 (SEM 1·0) %). Hepatocytes were transfected with indicated siRNA at 30 % confluence. The siRNA was added to one tube and mixed gently; 5 μl of Lipo2000 transfection reagent was added to the other tube and gently mixed. Then, the culture medium containing the siRNA was gently added to the culture solution containing the Lipo2000 transfection reagent, and the tube had a gently inverted mixture. A mixture of the Lipo2000 transfection reagent and siRNA was added to each well, and the culture medium was changed after 5 h.

Plasmid and cell transfection. To identify the role of Ire1*α*/Fas interaction on Fas activity, we constructed the Ire1*α* and Fas expression vectors. The open reading frames of Ire1*α* and Fas sequences were subcloned into the pcDNA3·1 (+) vector with the HA-tag and Flag-tag sequences inserted at the N-terminus of Ire1*α* and Fas sequences, respectively. Mutations of amino acid from 836 to 963, which are the interaction sequence for Ire1*α*, were produced in the HA-Ire1*α* plasmid by the Mut Express II Fast Mutagenesis Kit (Vazyme). The transient transfection of the plasmids into hepatocytes from yellow catfish was conducted using Lipofectamine 2000 (Invitrogen) based on the manufacturer's instructions.

Plasmid construction, transfections and luciferase assays. We constructed *perk* promoters into the pGl3 basic vector by using the ClonExpress™ II One Step Cloning Kit (C112, Vazyme) based on our published protocol[29]. CpG islands of *perk* were predicted by the MethPrimer (http://www.urogene.org/methprimer/). Mutations of CG sites in *ire-1α* promoter regions were performed according to the manufacturer' instructions of Mut Express II Fast Mutagenesis Kit (C214-01, Vazyme) and verified by sequencing. All these plasmids were transiently transfected into the HEK293T cells using the Lipofectamine 2000 (12566014, Invitrogen) at about 80 % confluence to measure the luciferase activity. Primers used for site-mutation analysis are presented in online Supplementary Table S3.

Protein docking for Fas and Ire-1α. Swiss-Model server (https://swissmodel.expasy.org/) was used to construct the protein structure of yellow catfish, from which the crystal structures were downloaded from the PDB protein data (Fas PDB template ID: 2vz9, Ire-1*α* PDB template ID: 6urc) and the appropriate templates were selected. The ZDOCK module of Discovery Studio 4.0 was used to perform Fas-Ire1*α* docking. Docking pose was picked up by using pymol from the given top models. The information for structural protein prediction model and Fas/Ire-1*α* protein interaction is presented in online Supplementary Figs S2, S3 and S4.

Statistical analysis

All data were expressed as mean values with their standard error of means. The normality of data distribution and the homogeneity of variances were analysed using the Kolmogorov–Smirnov test and Bartlett's test, respectively. Differences between adequate choline and choline deficiency groups or between si-NC and si-*chdh* groups were analysed using a Student's *t* test for independent samples using SPSS 19.0 software, and the minimum significance level was set at $P < 0·05$.

Results

Effects of dietary choline on growth performance and hepatic lipid metabolism

Weight Gain (WG) in the group fed the choline-deficient diet were higher than in the adequate choline diet group. FCR, HSI, CF and survival showed no significant differences between two treatments (online Supplementary Table S2). In the present study, when compared with adequate choline diet group, choline-deficient diet caused hepatic lipid dysregulation supported by increasing the vacuoles in haematoxylin and eosin and LD in Oil Red O (Fig. 1(a)–(c)). Meanwhile, at the transcription level, choline-deficient diet significantly up-regulated the expression of genes involved in lipogenesis (*srebp-1*, *pparγ*, *6pgd*, *accα* and *fas*) but down-regulated lipolysis and FA *β*-oxidation (*hsl, cpt-1α, echs1*) (Fig. 1(d)). Further study indicated, a key enzyme in lipogenesis, Fas was significantly up-regulated by choline-deficient diet at both protein expression and enzyme activity levels (Fig. 1(e)–(g)). All these results suggested choline-deficient diet caused hepatic lipid dysregulation in the livers of yellow catfish, which was further proved by the significant increased trend of TAG and lipid content (Fig. 1(h) and (i)).

Choline-deficient diet-induced hepatic unfolded protein response main via ire-1α signalling

We examined whether choline-deficient diet could cause UPRer in the liver of yellow catfish. As shown in Fig. 2(a), compared with adequate choline diet, transmission electron microscopy observation found severe swelling of ER in choline-deficient diet groups. Also, choline-deficient diet apparently up-regulated mRNA abundance of UPRer markers, including *grp78*, *grp94*, *crt*, *perk*, *eif2α*, *ire-1α*, *xbp-1*, *aft-6α* and *chop*. Furthermore, among these three UPRer signalling pathways, *ire-1α* signalling shown the most sensitive to choline-deficient diet (Fig. 2(b)). Meanwhile, this *ire-1α* signalling-mediated UPRer was further confirmed by the protein level of Grp78 and Ire-1*α* (Fig. 2(c)–(e)). All these indicated that choline-deficient

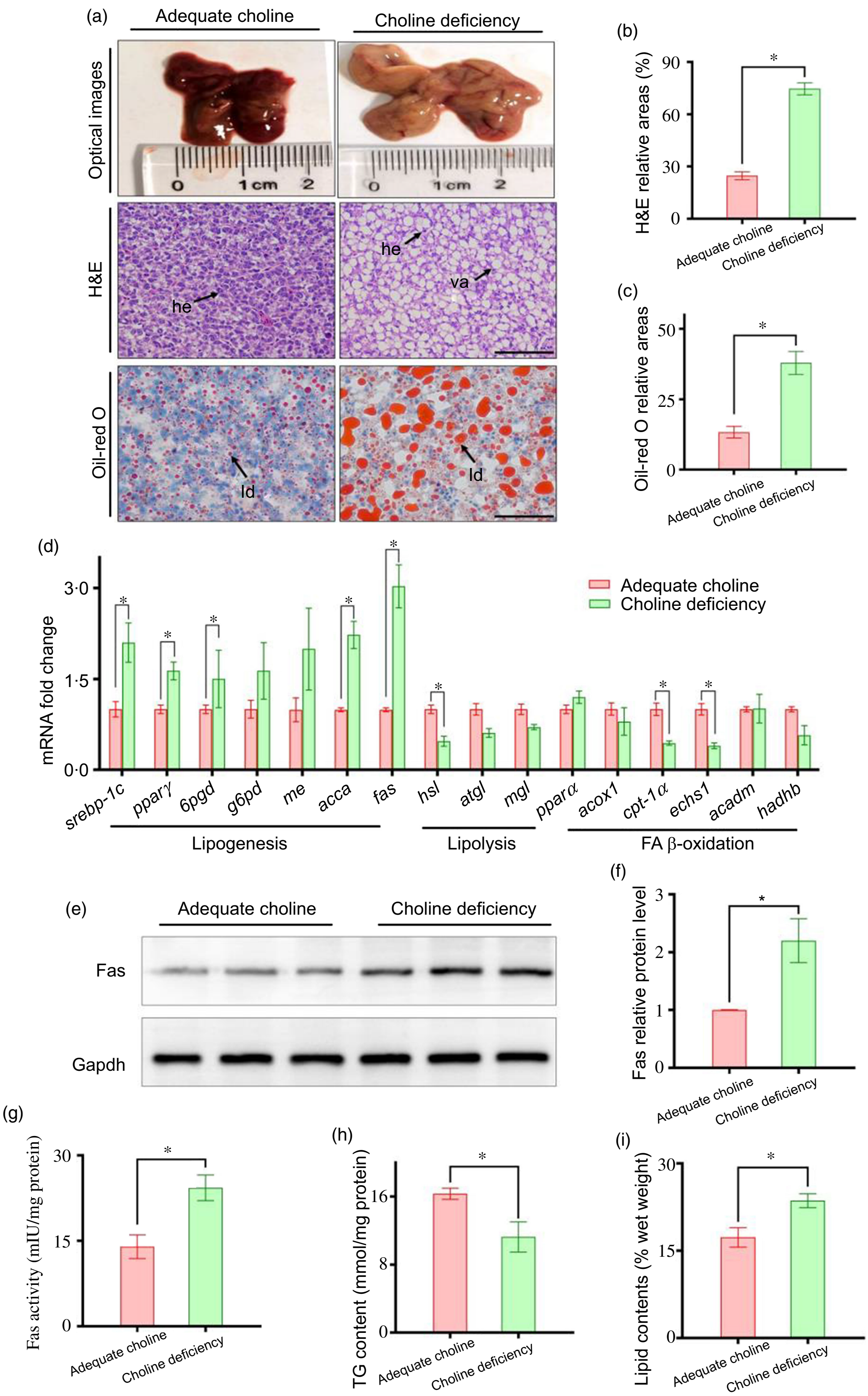

Fig. 1. Choline-deficient diet caused lipid dysregulation in the liver of yellow catfish. (a) Representative images of hepatic H&E and Oil red O stained. Scale bar, 30 μm; hepatocytes (he); vacuoles (va); lipid droplets (ld). (b)–(c) Relative areas for hepatic vacuoles in H&E staining and LD in Oil Red O staining. (d) mRNA levels of the genes related to hepatic lipid metabolism. (e)–(f) Western blot analysis and quantification analysis for Fas. (g) Fas activity. (h) TAG content. (i) Hepatic lipid contents. Data are mean values with their standard error of the means (n 3 replicate tanks). * indicates significant differences between adequate choline diet and choline-deficient diet group. H&E, haematoxylin and eosin; LD, lipid droplet.

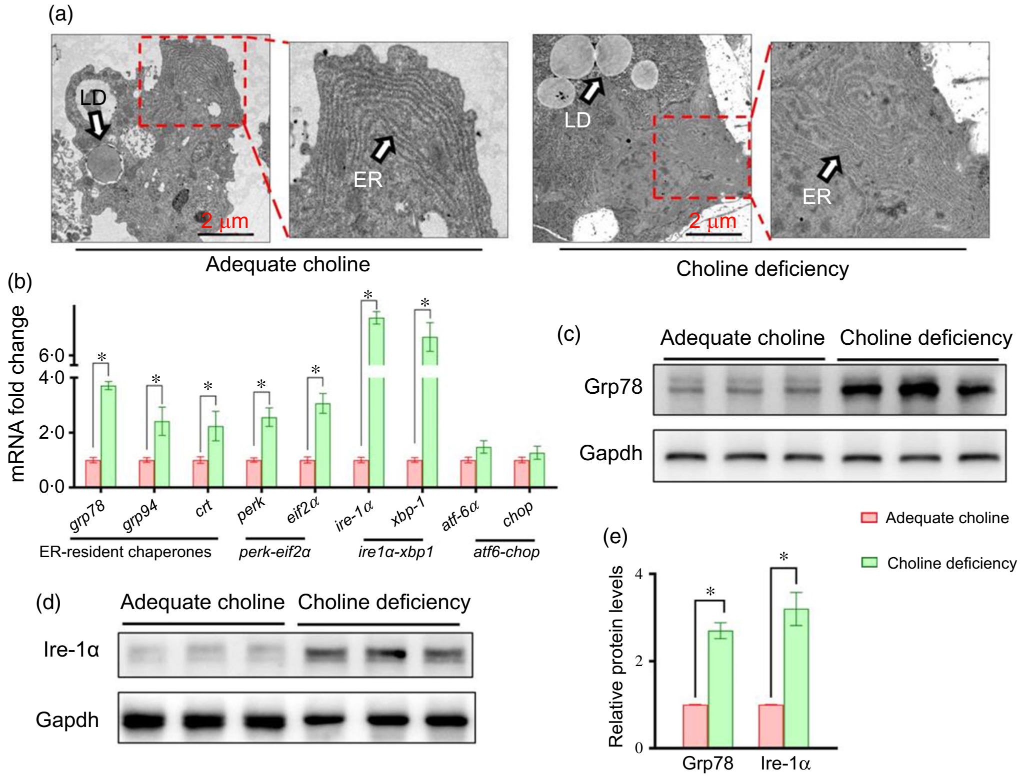

Fig. 2. Choline-deficient diet induced hepatic UPRer main *via ire-1α* signalling. (a) TEM structures of the liver, ER and LD; scale bars, 2 μm. (b) mRNA levels of the genes related to UPRer. (c)–(e) Western blot analysis and quantification analysis for Grp78 and Ire-1α. Data are mean values with their standard error of the means (*n* 3 replicate tanks). * indicates significant differences between adequate choline diet and choline-deficient diet group. UPRer, unfolded protein response; TEM, transmission electron microscopy; ER, endoplasmic reticulum; LD, lipid droplet.

diet caused severe hepatic UPRer main *via ire-1α* signalling in yellow catfish.

Dietary choline increased CpG methylation in the promoter of ire-1α

Choline can be irreversibly oxidised to yield betaine in a two-step process catalysed by CHDH and betaine aldehyde dehydrogenase mainly in the liver [4]. Betaine is an important methyl group donor. Thus, next we examined the dietary choline-induced alteration in DNA methylation reaction in the liver of yellow catfish. First, choline-deficient diet significantly reduced the concentration of choline and betaine and also the ratio of S-adenosylmethionine (SAM)/S-adenosylhomocysteine (SAH) (Fig. 3(a) and (b)). Second, the down-regulation of genes and/or protein involved in methionine cycle and methyltransferase (Fig. 3(c) and (d)). All these results suggested choline-deficient diet caused the inhibition of DNA methylation reaction. Since the main role of *ire-1α* signalling in choline-deficiency-induced up-regulation of UPRer markers was determined, and also given three CpG islands in the promoter of *ire-1α* from yellow catfish were predicted (Fig. 3(e)), we studied the effect of dietary choline on CpG methylation in the promoter of *ire-1α*. Compared with choline-deficient diet, adequate choline diet significantly up-regulated the levels of *ire-1α* promoter methylation (Fig. 3(f)). Importantly, we identified the significant choline-induced methylation sites, including the *−994*, *−1229*, *−1287 and −1497* sites in the promoter of *ire-1α* (Fig. 3(g)), implying the potential role of these methylation sites in regulating CpG methylation of *ire-1α*.

Choline down-regulated ire-1α expression by controlling site-specific DNA methylations

We further examined the mechanism of choline-mediated *CpG* methylation regulating *ire-1α* expression. By choline dehydrogenase (*chdh*), choline could be irreversibly oxidised and then serves as a donor of methyl groups. Thus, si-*chdh* could intercept the methyl donor function of choline. First, for adequate choline groups, si-*chdh* apparently up-regulated the concentration of choline but reduced the level of betaine, SAM and SAH; meanwhile, the ratio of SAM/SAH has also been decreased (Fig. 4(a) and (b)), suggesting the interception of choline oxidisation metabolism. In addition, in adequate choline groups, the si-*chdh* markedly down-regulated the mRNA abundance of genes involved in methionine cycle and methyltransferase (Fig. 4(c)). All these results suggested that si-chdh caused inhibition of choline-mediated methionine cycle and its follow-up DNA methylation reaction, which was further confirmed by the lower protein levels of Chdh and Dnmt1 (Fig. 4(d)).

Then si-*chdh* significantly up-regulated the expression of *ire-1α* at both protein and mRNA levels (Fig. 4(e) and (f)), suggesting

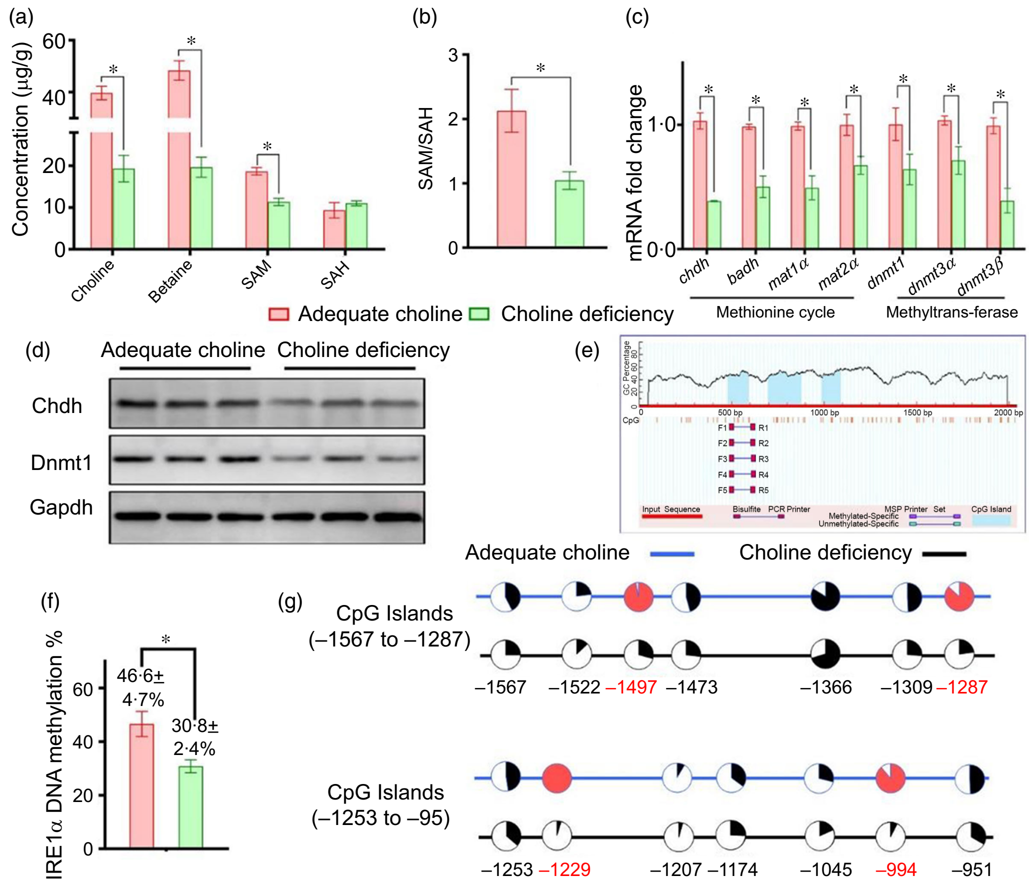

Fig. 3. Dietary choline increased CpG methylation in the promoter of *ire-1α*. (a) The concentration of choline, betaine, SAM and SAH. (b) The ratio of SAM/SAH. (c) mRNA levels of the genes related to methionine cycle and methyltransferase. (d) Western blot analysis for Chdh and Dnmt1. (e) Prediction analysis of CpG islands in the sequence range of 2000 bp upstream from the transcriptional start site in the *ire-1α* promoter region. (f) The methylation level of *ire-1α* promoter region. (g) The methylation level of specific sites in *ire-1α* promoter region. Data are mean values with their standard error of the means (*n* 3 replicate tanks). * indicates significant differences between adequate choline diet and choline-deficient diet group. SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

the potential regulatory role of DNA methylation on *ire-1α* expression. Thus, next we focus on the relationship between choline-mediated DNA methylation and *ire-1α* expression. As shown in Fig. 4(g), the levels of DNA methylation in the promoter of *ire-1α* were significantly down-regulated by si-*chdh*. In addition, using the site-specific CpG methylations analysis, we identified remarkable methylation sites, including the *−994, −1229 and −1497* sites in the promoter of *ire-1α* (Fig. 4(h)). Further, site mutation analysis shown only double mutation of −994 and −1229 methylation sites or triple mutation of *−994, −1229 and −1497* methylation sites significantly up-regulated the promoter methylation activity of *ire-1α*, suggesting the collaborative relationships of these specific methylation sites for regulating *ire-1α* expression. All these results indicated the key regulatory role of CpG methylation on *ire-1α* expression by controlling specific CpG methylation sites.

Choline prevents hepatic lipid dysregulation via reducing Ire1α–Fas interaction

The contribution and mechanism of choline acting as methyl donor in preventing hepatic lipid dysregulation were tested. First, transmission electron microscopy observation found si-*chdh* significantly aggravated swelling of ER in hepatocytes (Fig. 5(a)), suggesting the potential regulatory role of methyl donor function of choline mitigating hepatic UPRer. Further analysis with respect to protein–protein interaction prediction and immunoprecipitation of Fas and Ire-1*α* clearly demonstrated an interaction between these two proteins. This interaction was significantly down-regulated in the presence of adequate choline (Fig. 5(b) and (d)). In addition, si-*chdh* significantly inhibited the down-regulation for Ire1*α*–Fas interaction induced by choline addition (Fig. 5(c) and (d)). To determine whether Ire1*α*–Fas interaction is associated with Fas activity, the hepatocytes were co-transfected with Ire-1*α* that lacks the Ire1*α*–Fas interaction sequence (Ire1*α* Δ836–963) (Fig. 5(f) and (g)). The loss of Ire1*α*–Fas interaction sequence in ire-1*α* impaired Fas activity indicating a potential role of Ire-1*α* in regulating Fas activity. On the other hand, the content of LD in hepatocytes was significantly improved by si-*chdh* (Fig. 5(i)). These results indicated the contribution of choline acting as methyl donor in preventing hepatic lipid dysregulation, which was also further confirmed by the content of TAG in hepatocytes of yellow catfish (Fig. 5(j)).

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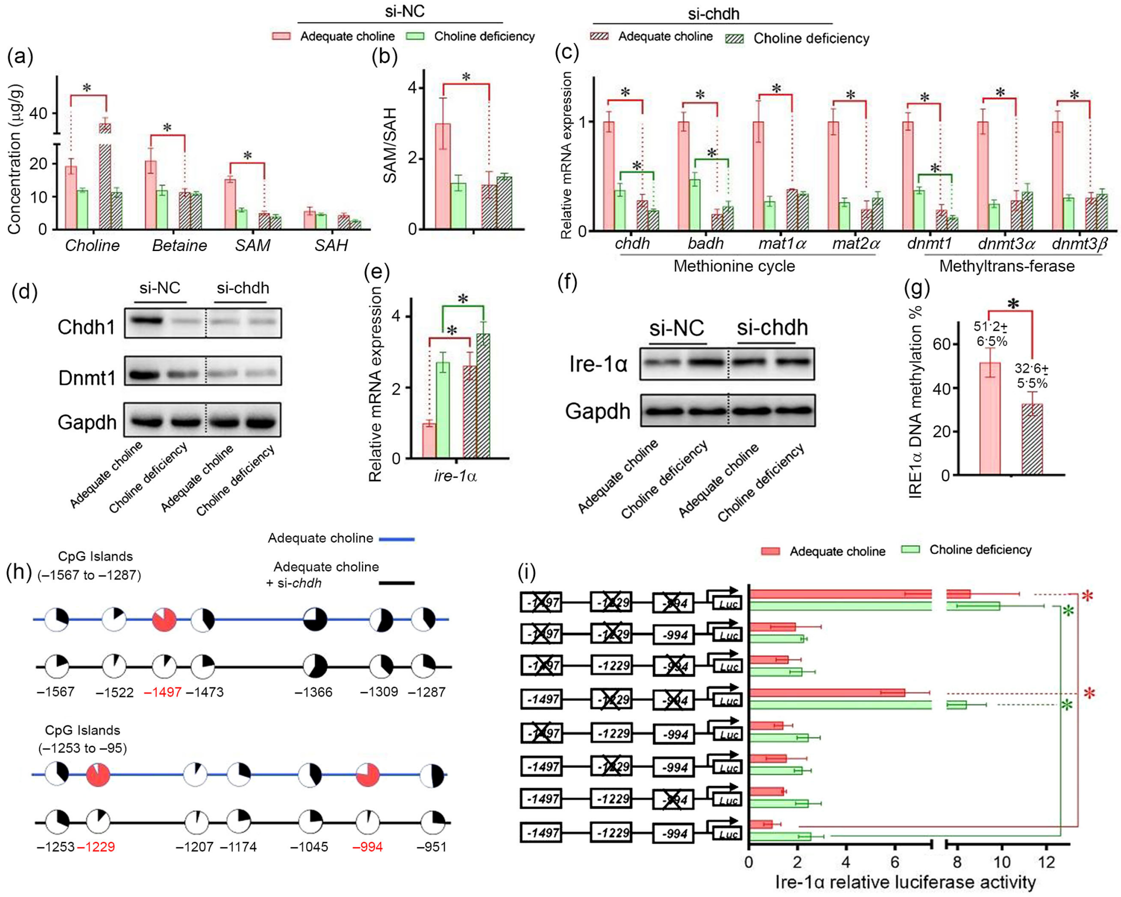

Fig. 4. Choline down-regulated ire-1*α* expression by controlling site-specific DNA methylations. (a) The concentration of choline, betaine, SAM and SAH. (b) The ratio of SAM/SAH. (c) mRNA levels of the genes related to methionine cycle and methyltransferase. (d) Western blot analysis for Chdh and Dnmt1. (e) mRNA levels of *ire-1α*. (f) Western blot analysis for Ire-1*α*. (g) The methylation level of *ire-1α* promoter region. (h) The methylation level of specific sites in *ire-1α* promoter region. (i) Site mutation analysis of *–994, –1229* and *–1497* methylation site on pGl3-*perk –1680/+116* vectors. Data are mean values with their standard error of the means (*n* 3 independent biological experiments). * indicates significant differences among same si-NC or si-*chdh* groups. SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

Discussion

Choline's role in hepatic lipid metabolism has been broadly investigated. Given choline's recognition as a lipotropic agent and an important component of several phospholipids, its role in lipid metabolism is of particular interest for aquaculture finfish species[5]. Although choline is primarily known for its function as a donor of methyl groups[4], the exact mechanisms by which it contributes to preventing excessive hepatic lipid deposition are not well understood. Furthermore, since UPRer serves as the key upstream pathway for lipid metabolism, it may have a regulatory role in choline-mediated hepatic lipid metabolism. However, the specific mechanism underlying this relationship has yet to be elucidated.

The present study indicated that choline-deficient diet caused lipid dysregulation in the liver of yellow catfish as expected, which has been confirmed in our previous studies[23]. The dietary choline deficiency markedly up-regulated the mRNA expression of genes involved in *de novo* lipogenesis, especially *fas*. This suggested the close association between lipogenesis disorders and choline-deficient model of hepatic lipid dysregulation, which also been proved by other study[30]. On the other hand, given ER is the main organelle for *de novo* lipogenesis and the crucial role of UPRer on hepatic lipid metabolism, we next evaluated the choline diet-induced alteration of UPRer. Choline plays a crucial role in the formation of several phospholipids and is essential for preserving the stability of the ER membrane structure. The choline deficiency has been linked to the activation of the UPRer in the ER[31]. Not surprisingly, our present study clearly indicated that choline-deficient diet generated severe swelling ER, indicating the inducement of UPRer in the liver of yellow catfish, in agreement with other studies[32,33]. Interestingly, mRNA expression analysis indicated that the choline-deficiency-induced UPRer is mainly originated from *ire-1α* signalling pathway compared with *perk* and *atf-6α* signalling pathways. This suggests a major role of *ire-1α* in choline-deficiency-induced UPRer. Similarly, the differential effects and mechanisms of three UPRer branches on regulating cell function and metabolism have been confirmed by other studies, including our previous study[18]. On the other hand, our *in vivo* studies indicated that WG was higher in choline-deficient diet. This may be attributed to lipid accumulation in whole body in response to dietary deficiency of choline.

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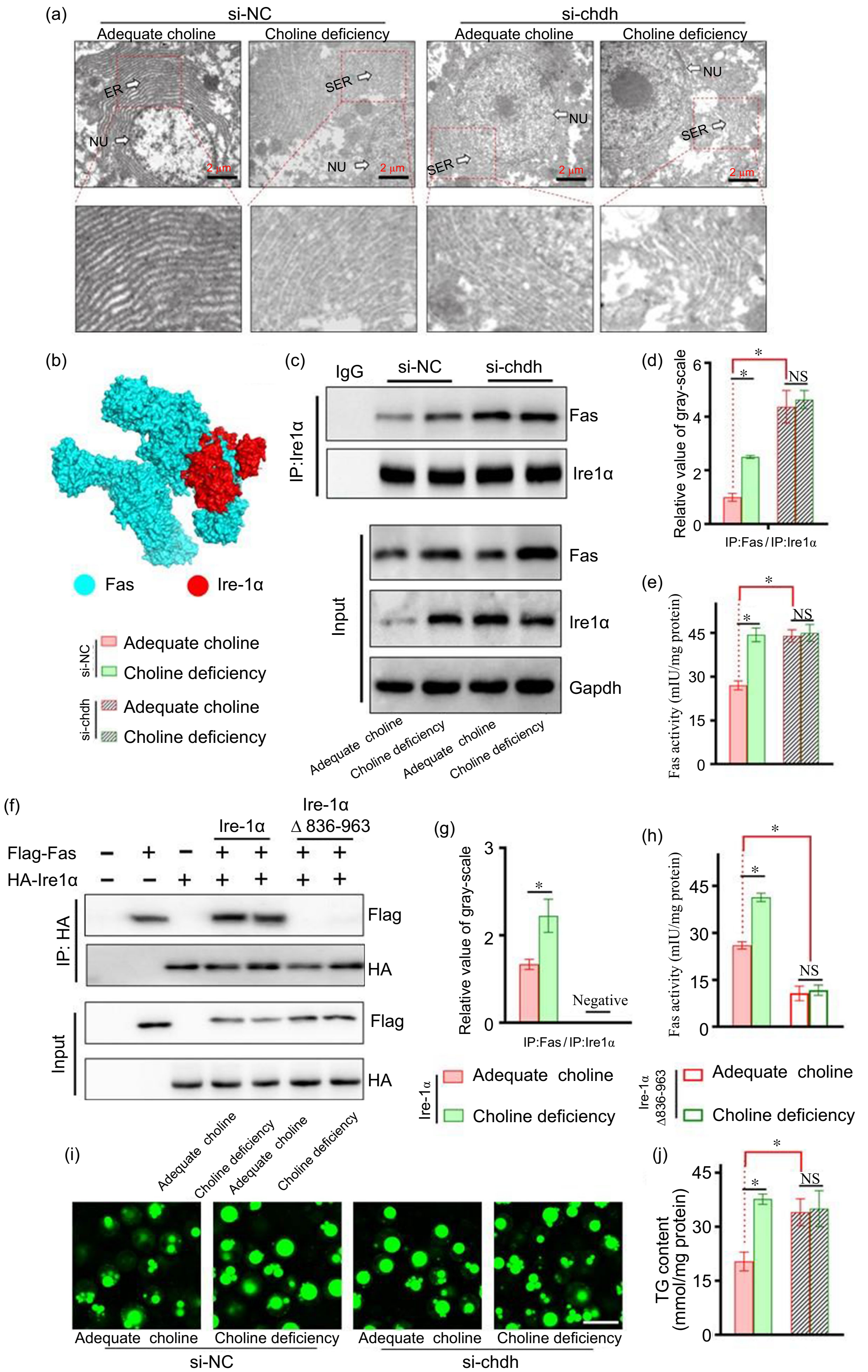

Fig. 5. Choline prevents hepatic lipid dysregulation *via* reducing Ire1α–Fas interaction. (a) TEM structures of the hepatocytes, ER, LD, SER and UN; scale bars, 2 μm. (b) The structural protein prediction model between Fas and Ire-1α. (c)–(d) IP analysis and quantification analysis for Fas-Ire1α complex. Flag-tag Fas and HA-tag Ire1α were transfected into hepatocytes from yellow catfish. The interaction between Fas and Ire1α was determined with IP and western blot. (e) Fas activity. (f)–(g) IP analysis and quantification analysis for Fas-Ire1α complex in hepatocytes transducing with the full-length Ire1α or Ire1α that lacks the Fas interaction sequence (Ire1α Δ836–963). (h) Fas activity in hepatocytes transducing with the full-length Ire1α or Ire1α that lacks the Fas interaction sequence (Ire1α Δ836–963). (i) Representative confocal microscopic image of lipid droplets (green) hepatocytes of yellow catfish (bar = 15 μm). (j) TAG content. Data are mean values with their standard error of the means (n 3 independent biological experiments). * indicates significant differences among same si-NC or si-*chdh* groups and also among same full-length Ire1α or Ire1α Δ836–963 groups. TEM, transmission electron microscopy; ER, endoplasmic reticulum; SER, swelling endoplasmic reticulum; UN, nucleus; IP, immunoprecipitation.

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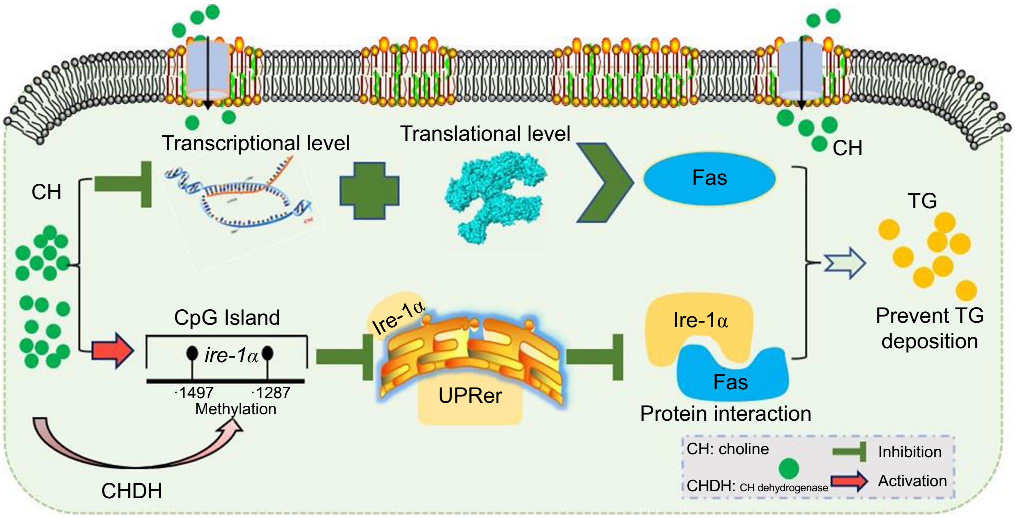

Fig. 6. Graphical conclusions for the mechanism of dietary choline prevent hepatic lipid dysregulation *via* specific CpG methylation of *ire-1α*. Choline (CH); dehydrogenase (CHDH); fatty acid synthase (Fas); TAG; unfolded protein response (UPR). Dietary choline serving as methyl donor, by controlling specific CpG methylation sites of *ire-1α* (–1497 and –1287), activated *ire-1α* promoter methylation, which down-regulated the *ire-1α* expression and alleviated UPRer, and then consequently prevented hepatic lipid dysregulation *via* Ire-1α/Fas interaction. Meanwhile, choline-prevented hepatic lipid dysregulation might also directly *via* reducing Fas expression.

DNA methylation, an important epigenetic modification, has been closely associated with hepatic lipogenesis and fatty liver[8,34]. In addition, diet intake of choline can modulate methylation because, *via* choline dehydrogenase (Chdh), this nutrient (and its metabolite, betaine) regulates the concentrations of SAM and SAH[8]. The present study found dietary choline addition increased the concentrations of choline and betaine and the ratio of SAM/SAH. Meanwhile, the process of DNA methylation is catalysed by a group of enzymes called DNA methyltransferases (Dnmts) mainly including Dnmt1, Dnmt3a and Dnmt3b[6,8]. Our current study found that dietary choline addition up-regulated the expression of genes and/or protein involved in methionine cycle and Dnmts. All these findings indicated that the dietary choline could activate DNA methylation in the liver of yellow catfish.

Given the major role of *ire-1α* for choline-deficient diet-caused UPRer and multiple CpG islands in *ire-1α* promoter region has been confirmed, we next explored the mechanism of choline-activated DNA methylation regulating *ire-1α* expression and their roles on UPRer and lipid metabolism. First, *in vivo*, dietary choline up-regulated the methylation level of *ire-1α* promoter region by activating specific CpG methylation sites. Then, *in vitro*, by using si-*chdh* transfection and mutation analysis of specific CpG methylation sites, we found *ire-1α* promoter methylation was inhibited by the interception of the methyl donor function of choline. Further, choline activated *ire-1α* promoter methylation, by controlling specific CpG methylation sites the in *ire-1α* promoter region. The activation of promoter methylation was closely correlated with down-regulation of gene expression[35]. Thus, we concluded that the decrease in *ire-1α* expression is attributed to choline-mediated CpG methylation. Importantly, *in vitro* experiments demonstrated the involvement of choline-mediated methylation of *ire-1α* promoter down-regulating UPRer pathway. Thus, our present study clearly showed that choline serving as methyl donor activated specific CpG methylation sites of *ire-1α*, which caused the down-regulation of *ire-1α* expression. Furthermore, *in vitro* experiments demonstrated that *si-chdh* counteracted the beneficial effect of choline on ER membrane structure. Collectively, the interception of the methyl donor function of choline could down-regulate CpG methylation of *ire-1α* promoter and then induce UPRer in liver. Similarly, studies have pointed out that alcohol-induced UPRer is manifested through altered DNA methylation, which subsequently regulates the expression of UPRer-associated factors[36]. Additionally, in rodents, dietary restriction of the methyl donors (methionine and/or choline) rapidly and reliably induces a spectrum of liver injury histologically[37].

On the other hand, we further found the interception of methyl donor function of choline caused the loss of choline-mediated prevention of hepatic LD deposition. This suggested choline serving as a lipotropic agent may depend on the methyl donor function, preventing the hepatic lipid dysregulation. Analogously, choline improved lipid homoeostasis in obesity through oxidative demethylation in murine model[38]. More interestingly, immunoprecipitation analysis showed that there is an interaction between Ire-1α and Fas, and this interaction was obviously down-regulated by choline, suggesting the involvement of Ire-1α/Fas interaction in choline mediating hepatic lipid metabolism. This also provided the first experimental evidence for direct crosstalk between Ire-1α signalling-mediated UPRer and hepatic lipid dysregulation. Importantly, the choline-induced down-regulation of Ire-1α/Fas interaction was lost along with si-*chdh* treatment, which further confirmed the contribution of choline acting as methyl donor. Our findings further revealed that, when Ire1α–Fas interaction was impaired, Fas activity was dwindled indicating novel regulatory role of Ire-1α protein in regulating Fas activity in choline-mediated lipid homoeostasis in hepatocytes. In a nut shell, by providing methyl group, choline activated CpG methylation of *ire-1α* promoter

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and then down-regulated *ire-1α* expression, which sequentially down-regulated the interaction with Fas and finally relieved hepatic lipid accumulation. On the other hand, along with the down-regulation of *ire-1α*, choline also improved ER membrane structure and alleviated hepatic UPRer, which also provided favourable assistance for hepatic lipid metabolism. Similarly, in fish, a potential causal relationship between UPRer-associated methylation and excessive lipid deposition was suggested in our previous study[28]. However, further investigation is necessary to elucidate the mechanisms underlying the improvement of the ER membrane integrity in response to adequate dietary choline levels, particularly considering the dual role of choline as both a methyl donor and a component of phospholipids.

It needs to be mentioned that the current study still has certain limitations. It is well established that dietary choline has direct role in promoting hepatic lipid homoeostasis by regulating the expression of many key enzymes and transcription factors[39], which also have been confirmed in the present study and our previous studies[2]. Thus, it is hard for us to remove this direct role of dietary choline regulating other critical enzymes and transcription factors, although the significance of choline as a methyl donor for hepatic lipid homoeostasis has been indicated in our current study.

In conclusion, the present study clearly demonstrated that dietary choline serving as methyl donor, by controlling specific CpG methylation sites of *ire-1α*, activated *ire-1α* promoter methylation, which led to the down-regulation of *ire-1α* expression and alleviated hepatic UPRer, and then consequently prevented hepatic lipid dysregulation *via* Ire-1α/Fas interaction. These results emphasise the critical contribution of methyl donor function of choline for its lipotropic agent role. Meanwhile, choline-prevented hepatic lipid dysregulation might also directly *via* reducing Fas expression, which need further investigation to confirm. The detail mechanism has been shown in Fig. 6.

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Z. Y. B. and Y. F. S. designed the experiment. Y. F. S. conducted the experiment and data analysis with the help of L. J. W. and H. Z. Y. F. S. drafted the manuscript. Z. L. revised the manuscript. All the authors reviewed and approved the manuscript.

The authors declared that they had no conflicts of interest with the contents of this article.

Supplementary material

For supplementary material referred to in this article, please visit https://doi.org/10.1017/S000711452300185X

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