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Metabolic epistasis among apoptosis-inducing factor and the mitochondrial import factor CHCHD4

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Hypomorphic mutation of apoptosis-inducing factor (AIF) in the whole body or organ-specific knockout of AIF compromises the activity of respiratory chain complexes I and IV, as it confers resistance to obesity and diabetes induced by high-fat diet. The mitochondrial defect induced by AIF deficiency can be explained by reduced AIF-dependent mitochondrial import of CHCHD4, which in turn is required for optimal import and assembly of respiratory chain complexes. Here we show that, as compared to wild type control littermates, mice with a heterozygous knockout of CHCHD4 exhibit reduced weight gain when fed with a Western style high-fat diet. This finding suggests widespread metabolic epistasis among AIF and CHCHD4. Targeting either of these proteins or their functional interaction might constitute a novel strategy to combat obesity.

Keywords: Apoptosis, diabetes, metabolism, obesity, programmed cell death

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Introduction

Apoptosis-inducing factor (AIF, official protein name: AIFM1) is a flavoprotein that is tethered to the outer side of the inner mitochondrial membrane.[1] Upon cell death-associated permeabilization of the outer membrane[2] and calpain-mediated cleavage of its membrane anchorage,[3] AIF is released from mitochondria to the cytosol and translocates to the nucleus. AIF has the property to directly interact with DNA.[4,5] However, its contribution to nuclear apoptosis and chromatin degradation requires the interaction with additional protein factors including cyclophilin A.[6,7]

Beyond its implication in developmental cell death,[8,9] AIF also contributes to pathological cell death, in particular in the brain and in the retina, where it may mediate caspase-independent cell loss of neurons and photoreceptors, respectively.[10-14] In addition, AIF plays a major role in normal mitochondrial pathogenesis.[15] Thus, yeast, mouse or human cells lacking AIF exhibit a reduced abundance of respiratory chain protein complexes (in particular complexes I and IV), which compromises oxidative phosphorylation.[16-18] In humans, several mutations of AIF have been described. Such mutants cause X-linked pathologies that resemble mitochondriopathies with regard to their clinical manifestations ranging from deafness and cognitive impairment to severe encephalomyopathy and cardiomyopathy.[19-23]

The mechanisms accounting for mitochondrial defect induced by deficient AIF expression have recently been elucidated.[24] Thus, AIF is required for the translational import of a mitochondrial intermembrane protein called coiled-coil-helix-coiled-coil-helix domain containing 4 (CHCHD4). CHCHD4, which is the human homolog of yeast Mia40,[25,26] in turn plays a major role in the import, folding and oxidative maturation (due to the introduction of intramolecular disulfide bonds) of other intermembrane proteins. Knockdown or knockout of CHCHD4 results in a

Table 1. Genotype frequency of adult *Chchd4*$^{+/-}$ mice

Total number	WT genotype	*Chchd4*$^{+/-}$
845	372 (44%)	473 (56%)

Mice arising from multiple crosses of female or male *Chchd4*$^{+/-}$ mice with wild type (WT) control mice were genotyped at weaning.

mitochondrial defect that is similar to that observed in AIF-deficient cells.[24] Moreover, transfection of cells with a CHCHD4 variant whose mitochondrial import does not rely on AIF can repair the respiratory defect of AIF-deficient cells, demonstrating that AIF and CHCHD4 are epistatic with respect to mitochondrial biogenesis and respiratory function.[24] Here, we addressed the question as to whether AIF and CHCHD4 also are epistatic with respect to their broad metabolic effects.

Results and Discussion

Mice bearing a hypomorphic mutation of apoptosis-inducing factor (AIF) exhibit the so-called Harlequin phenotype with cerebellar ataxia as a prominent hallmark.[27] Before such mice develop signs of neuropathology, they exhibit a progressive reduction in the abundance of respiratory chain complexes I and IV in multiple organs. At this stage, such mice are resistant against the induction of obesity and type 2 diabetes by high fat diet.[28] More convincingly, mice bearing a muscle-specific AIF knockout (which causes an age-dependent dilated cardiomyopathy and skeleton muscle degeneration) or a hepatocyte-specific AIF knockout (which does not cause any detectable phenotype) results in increased glucose tolerance, reduced fat mass, and increased insulin sensitivity. Such mice also resist weight gain and metabolic syndrome induced by a Western style high fat diet.[28] Thus, even a partial AIF defect may have positive effects on whole body metabolism.

Driven by these considerations, we decided to investigate the metabolic phenotype of CHCHD4-deficient mice. Homozygous knockout of *Chchd4* (*Chchd4*$^{-/-}$) results in embryonic lethality around E8, presumably due to a severe mitochondrial deficiency.[24] However, mice bearing a heterozygous knockout of *Chchd4* (*Chchd4*$^{+/-}$) are viable and have a life expectancy of at least 1.5 years (contrasting with mice bearing the Harlequin mutation or a muscle-specific AIF knockout) without any obvious phenotypic or behavioral alterations.

Both male and female *Chchd4*$^{+/-}$ mice were fertile, and *Chchd4*$^{+/-}$ pups resulting from the cross of WT and *Chchd4*$^{+/-}$ parents were born at an expected Mendelian frequency of close-to-50% (**Table 1**). Heterozygous knockout of *Chchd4* resulted in a reduction of CHCHD4 expression by approximately 50% (as compared to wild type mice), yet failed to affect the expression of AIF (**Fig. 1**) and failed to manifest major respiratory chain defects (not shown). Importantly, when fed with a Western style high-fat diet, female *Chchd4*$^{+/-}$ mice exhibited a markedly reduced weight gain as compared to their age- and sex-matched WT littermates (**Fig. 2**).

The aforementioned results indicate that defects in AIF and CHCHD4 result in similar metabolic phenotypes with respect to the resistance to diet-induced obesity. While AIF deficiency causing such a phenotype is linked to a major respiratory chain defect that is pathogenic (at least in the case of Harlequin mice and the muscle-specific deletion of AIF), the partial CHCHD4 deficiency linked to the *Chchd4*$^{+/-}$ genotype does not cause any obvious pathology, yet renders mice partially resistant against diet-induced obesity. This suggests that the beneficial effects of the AIF deficiency on whole body metabolism may be uncoupled from the negative effects of the mitochondriopathy.

At this stage, it remains to be determined through which molecular mechanisms the *Chchd4*$^{+/-}$ genotype confers resistance against obesity. As a possibility, *Chchd4*$^{+/-}$ mice might manifest a subclinical mitochondrial defect that affects particular cell types, hence explaining its beneficial effects. As an alternative, a partial defect in mitochondrial import might activate subtle homeostatic pathways (such as the mitochondrial unfolded protein response)[29] that avoid the manifestation of obesity (or other age-associated changes in metabolism) without any negative impact on mitochondrial respiration.[30] Finally, it is possible that *Chchd4* deficiency affects non-mitochondrial signaling pathways by virtue of its capacity to regulate the subcellular localization of p53,[31] a master transcription factor that controls the differentiation of both white and brown fat cells.[32,33] Future work must distinguish between these possibilities.

Irrespective of these possibilities, CHCHD4 emerges as a new putative target for therapeutic interventions on obesity and metabolic syndrome. A peptide derived from the N-terminus of CHCHD4 can competitively disrupt the interaction between AIF and CHCHD4,[24] suggesting the possibility of

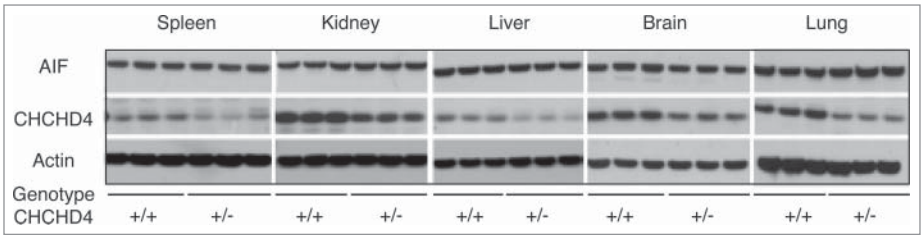

Figure 1. Impact of heterozygous knockout of CHCHD4 on the abundance of CHCHD4 protein and that of AIF. Levels of AIF and CHCHD4 expression were determined in various organs of adult mice. Triplicate samples from wild type (*chchd4*$^{+/+}$) and heterozygous (*chchd4*$^{+/-}$) were analyzed by immunoblot for the abundance of the indicated proteins. Actin expression was determined as a loading control.

creating small molecules that affect the AIF-CHCHD4 axis. It will be interesting to explore whether such molecules might be used for the avoidance or treatment of obesity.

Materials and Methods

Antibodies

Antibodies against the following proteins were used: actin (mouse mAb; Millipore); AIF (mouse mAb; Santa Cruz and rabbit pAB; Cell Signaling); CHCHD4 (rabbit pAB; Santa Cruz).

Animals

Mutant heterozygous *Chchd4* animals were constructed by Texas Institute for Genomic Medicine (TIGM) using a gene-trapping strategy.[24] All procedures and animal experimentation protocols were reviewed and deemed acceptable by the registered ethical Committee n°26 and carried out in the animal facility of Gustave Roussy.

Genotyping of *Chchd4* heterozygous mice

3 to 4 weeks after weaning, tail snip DNA was extracted using the Maxwell16 mouse tail DNA purification kit (Maxwell). Using the AmpliTaq Gold master Mix (Applied Biosystems), PCR was performed for the detection of the wt or mutant *Chchd4* allele interrupted by a gene trap vector. The wt allele was amplified using the Primers IST11943B12-F (TGGGCTGGTTAGTCAGTGATTGG) and IST11943B12-R (GTGCTCCTCATAGGGATCATTGG) and the mutant allele was amplified using IST11943B12-R and LTR2 (AAATGGCGTTACTTAAGCTAGCTTGC).

Tissue extract preparation for immunoblot

Wild type (*Chchd4*$^{+/+}$) or heterozygous (*Chchd4*$^{+/-}$) adult female mice were anesthetized and killed by decapitation. All the dissected organs were snap-frozen and then homogenized, using Precellys homogenizer (Bertin), in an ice-cold RIPA 1X buffer (Sigma Aldrich), supplemented with protease (EDTA- free protease inhibitor tablet - Roche Applied Science) and phosphatase inhibitors (PhosSTOP phosphatase inhibitor tablet - Roche Applied Science). The proteins present in the extracts were quantified (Bio-Rad DC protein assay) and samples were finally resolved directly by SDS-PAGE (NUPAGE; Invitrogen) after boiling in 1xSB (2% SDS, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol). After electrophoresis, the gel was subjected to immunoblot analysis to visualize specific protein bands.

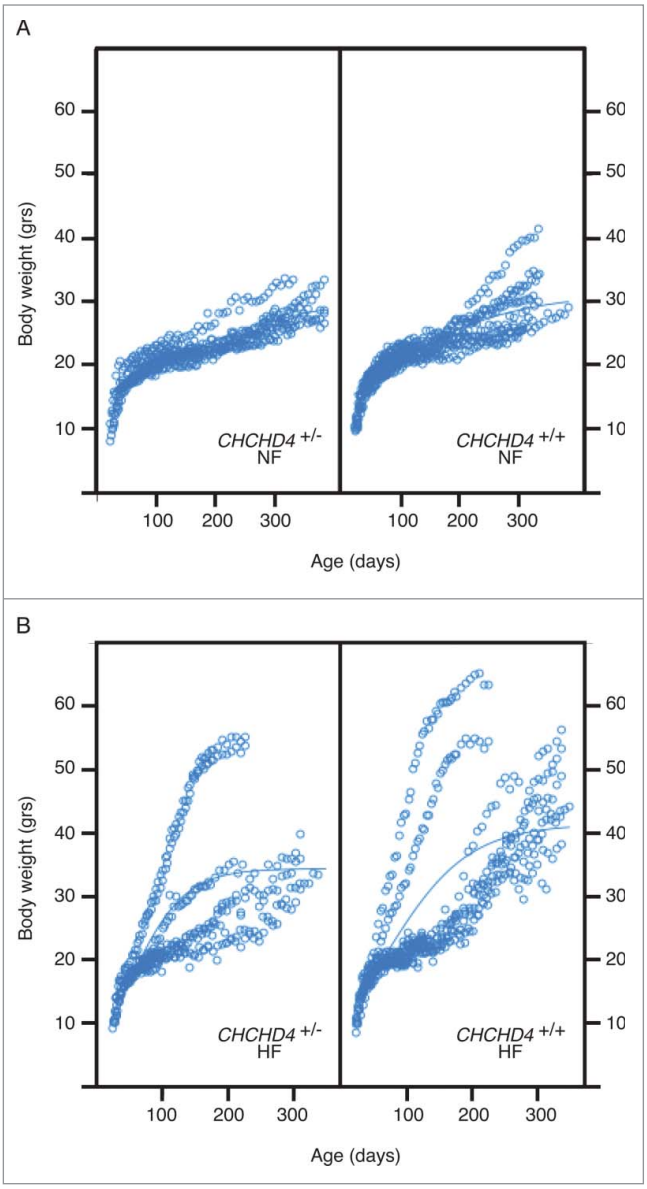

Figure 2. Impact of CHCHD4 on weight gain induced by high-fat diet. Weight gain was measured for wild type (*chchd4*$^{+/+}$) and heterozygous (*chchd4*$^{+/-}$) mice fed normal chow (**A**; NF: normal feeding) or high-fat diet (**B**; HF: high fat feeding). Maximum fitted values at age 300 to 310 days were: 28.2g ± 0.29 for *chchd4*$^{+/-}$/NF mice; 30.5g ± 0.68 for *chchd4*$^{+/+}$/NF; 33.6g ± 0.84 for *chchd4*$^{+/-}$/HF mice; 44.6g ± 1 for *chchd4*$^{+/+}$/HF. Values were significantly different for mice fed with high fat (HF) diet ($p < 0.00001$), while those for animals fed with normal (NF) diet were similar. Values are means ± SEM for animals of more than 300 days age in each category.

Regimens

Normal control Chow diet (A03) and high fat diet (A03 supplemented with 30% porcine fat, and 5% soya oil) were prepared by SAFE (Augy, France). Wild type (*chchd4*$^{+/+}$) and heterozygous

(*chchd4*$^{+/-}$) mice were fed normal chow or high-fat diet starting from 4 weeks of age until the termination of the experiment. Animals were kept under 12h light/dark cycle and weighted every 3 to 4 days.

Statistics

Growth curves were analyzed by means of R statistical software [R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.] by fitting a nonlinear model (by logistic regression, R package nlme). Data are expressed as mean ± SEM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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