A polymorphism in the Irisin-encoding gene (FNDC5) associates with hepatic steatosis by differential miRNA binding to the 3'UTR

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Lay summary: Irisin, a novel protein produced mainly by muscle and is known to be released into the circulation, with an unclear role in liver fat deposition. This study demonstrates that genetic variants in the gene encoding the irisin protein modulates the risk of liver fat in patients with fatty liver disease. Interesting, these effects are independent of, but additive to that of other recently described genetic variants that contribute to liver fat. In functional studies, we have deciphered the detailed molecular mechanisms by which the genetic variant mediates its effects.

Abstract (N= 248 words)

Background & Aims: Irisin, the cleaved extra-cellular fragment of the Fibronectin type III domain-containing protein 5 (FNDC5) is a myokine with proposed favorable metabolic activity. The role of variants in the *FNDC5* gene in non-alcoholic fatty liver disease (NAFLD) is not defined.

Methods: We prioritized SNPs in *FNDC5* on the basis of their putative biological function and identified rs3480 in the 3′ untranslated region (3′UTR). We studied the association of rs3480 with liver disease severity and the metabolic profile of 987 Caucasian patients with NAFLD. Functional investigations were undertaken using luciferase reporter assays of the 3'UTR of human FNDC5, pyrosequencing for allele-specific expression of FNDC5 in liver, measurement of serum irisin, and bioinformatics analysis.

Results: The rs3480 (G) allele was associated with advanced steatosis (OR: 1.29, 95: CI: 1.08-1.55, p=0.004), but not with other histological features. This effect was independent but additive to *PNPLA3* and *TM6SF2*. The rs3480 polymorphism influenced FNDC5 mRNA stability and the binding of miR-135a-5P. Compared with controls, hepatic expression of this microRNA was upregulated while FNDC5 expression was downregulated. Elevated serum irisin was associated with reduced steatosis, and an improved metabolic profile.

Conclusions: Carriage of the *FNDC5* rs3480 minor (G) allele is associated with more severe steatosis in NAFLD through a microRNA-mediated mechanism controlling FNDC5 mRNA stability. Irisin is likely to have a favourable metabolic impact on NAFLD.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a principal liver disorder in Western countries and is on trajectory to become the leading cause of end-stage disease and liver transplantation [1-3]. Notably, NAFLD is part of a multisystem metabolic disturbance as its impact is not limited to the liver but also affects extra-hepatic sites such as the cardiovascular system and kidneys [4]. In NAFLD, there is pathological hepatic accumulation of fat that over time can lead to inflammation and progress to cirrhosis, end-stage liver disease and hepatocellular carcinoma. The mechanisms underlying the accumulation of liver fat are complex and gene x environment interactions play a critical role [5-7].

The heritable component of hepatic steatosis has been estimated at ~50% based on a prospective twin study [8]. To date, the major inherited determinants of hepatic fat accumulation based on genome wide association studies are the patatin-like phospholipase domain-containing 3 (*PNPLA3*) I148M and the transmembrane 6 superfamily member 2 (*TM6SF2*) E167K gene variants [9, 10]. However, these polymorphisms explain only 10−20% of the heritability [6, 11]. Thus, other as yet unidentified genetic and epigenetic variations likely exist to explain the missing heritability.

Adipose-derived hormones, collectively termed adipokines are established drivers/contributors to the pathogenesis of NAFLD [12-14]. More recently, the myokine irisin was isolated from muscle by Boström et al. 2012 [15]. Irisin is a 12 kD, 112 amino acid fragment that is proteolytically processed from the fibronectin type III domain-containing protein 5 (FNDC5) and released into the circulation [16]. The functions of irisin and even its existence however has been a matter of debate [16, 17], with some reports suggesting that the irisin polypeptide is a ''myth'' [17]. Human *FNDC5* has an atypical ATA translation start codon rather than the ATG sequence. Hence, it has been argued that the human ATA codon represents a null mutation without irisin production and that reports measuring human irisin are an artefact from using an FNDC5 antibody with poor specificity [17]. However, recent work using quantitative mass spectrometry has confirmed the existence of circulating irisin [18]. The three

4

published reports have shown inconsistent associations of NAFLD with irisin levels [19-21]; in addition, the role of genetic variants in *FNDC5* and the involved mechanisms have not been well defined.

In this work, after SNP prioritisation, we explored the role of the *FNDC5* polymorphism rs3480 on hepatic steatosis and other histological features in a cohort of 987 Caucasian patients with NAFLD and investigated the mechanisms underlying the association.

Methods

Patient cohort

The study comprised 987 Caucasian patients with biopsy-proven NAFLD. The details of the cohort and their clinical and laboratory assessment are provided in **supplementary methods**. Ethics approval was obtained from the Human Research Ethics Committees of the Western Sydney Local Health District and the University of Sydney. All other sites had ethics approval from their respective ethics committees.

Prioritization of *FNDC5* SNPs.

To prioritize SNPs for genotyping, we followed the scheme illustrated in **Supplementary Figure 1**. In brief, common SNPs (MAF > 1%) within the FNDC5 gene were tested for their status as eQTLs using the GTEx (Genotype-Tissue Expression) project [22], as also eQTLs from the MuTHER study using the exSNP database [23]. SNPs that were eQTLs for FNDC5 were then investigated for p-values for the eQTL. Following this filter, two SNPs (rs3480 and rs10753269) in tight LD (r^2=0.99) were found; the latter is upstream of *FNDC5*, while rs3480 is located in the 3′UTR. From this scheme, rs3480 was determined to be the SNP with the highest priority for genotyping in our cohort.

Genotyping

Genotyping for *FNDC5* rs3480, *PNPLA3* rs738409 and *TM6SF2* rs58542926 was undertaken using the TaqMan SNP genotyping allelic discrimination method (Applied Biosystems, Foster City, CA, USA). All genotyping was blinded to clinical variables. Some of the *PNPLA3* rs738409 and *TM6SF2* rs58542926 genotyping data were extracted from a recent report [24].

Liver Histopathology

Liver biopsies were scored by an expert liver pathologist in each participating centre unaware of clinical or genetic data. Histological scoring was based on the system proposed by Kleiner et al.[25]

Steatosis was graded from 0 to 3, lobular inflammation from 0 to 3 and hepatocellular ballooning from 0 to 2. Fibrosis was staged from 0 to 4 with 4 representing cirrhosis. The NAFLD Activity Score (NAS) was calculated to quantify disease activity [25]. The inter-observer agreement between pathologists was studied previously and was excellent for steatosis ($\kappa = 0.85$) and good for fibrosis ($\kappa = 0.78$) [26].

Allele specific expression

Allelic ratios quantified by pyrosequencing was performed at the Australian Genome Research Facility. Full details are provided in supplementary methods, supplementary table 1 and figure 2.

Enzyme-linked immunosorbent assay (ELISA)

Serum irisin level was measured using a recently released irisin kit from Phoenix Pharmaceuticals (EK-067-29) [27] in a sub-cohort of 152 patients with available serum samples and with characteristics similar to the entire cohort.

Bioinformatics analyses

We queried the online miRdSNP database which is based on Tragetscan and PicTar databases [28] to determine whether rs3480 (the SNP derived from our prioritisation approach) in the 3'UTR of FNDC5 could be in miRNA target sites. Secondary structures of the mRNA and minimum folding energy (MFE) of FNDC-G and FNDC5-A were extrapolated using the RNAfold online server (Vienna RNA package, version 2.0.0, with option '-p')[29].

Gene set enrichment analysis

The target genes of MiR135a predicted by TargetScan. gene set enrichment analysis used the Database for Annotation, Visualization and Integrated Discovery (DAVID)[30] to ask which Kyoto Encyclopedia of Genes and Genomes (KEGG) were enriched within the genes that contained binding sites for miR-135a.

Luciferase reporter assay

7

The full-length 3′UTR of human *FNDC5,* with genetic variation corresponding to A or G (of rs3480) was cloned into the PmeI/SalI restriction site downstream of the gene encoding firefly luciferase in the pmirGLO reporter vector (Promega). Details of Cell transfection and reporter assays are provided in supplementary methods and supplementary CTAT Table.

Human liver sample miRNA extraction and Droplet Digital PCR

RNA was extracted from 5 µm of liver tissue (obtained from resections and stored at –80 °C) using the miRNeasy kit (Qiagen) from 20 subjects (n = 10 controls and n = 10 with NAFLD). The first-strand complementary synthesis reaction (cDNA) was performed using the TaqMan MicroRNA Reverse Transcription kit (Life Technologies) for miRNAs.

For miRNAs and FNDC5 copy number quantification, droplet digital PCR was performed, as previously described [31, 32]; details are provided in supplementary methods.

Statistical methods

Results are expressed as mean ± SD (standard deviation), median and interquartile range or number (percentage) of patients. All tests were two-tailed and p values <0.05 were considered significant, the details are provided in supplementary methods.

Results

FNDC5 rs3480 and the degree of hepatic steatosis

Baseline characteristics of the cohort are summarized in **supplementary table 2.** After SNP prioritization (described in detail in **Supplementary Fig. 2** and in Methods), we found that rs3480 satisfied our criteria. *FNDC5* rs3480 was confirmed to be in Hardy–Weinberg equilibrium and showed a minor allele frequency (MAF) of 0.41; 0.403, 0.41 and 0.404 in Australian, Italian and Spanish samples, respectively, similar to that observed in other European populations (MAF= 0.41), http://browser.1000genomes.org). No differences in clinical, anthropometric and biochemical indices including aminotransferases were found between the *FNDC5* rs3480 genotypes (**Supplementary Table 3**).

Fixed-effects meta-analysis of the association of rs3480 with steatosis (S2-S3) was significant (OR 1.20 (95% CI 1.01–1.3), meta-P = 0.0001; **Figure 1A**). In multivariate logistic regression analysis incorporating age, gender, BMI, T2DM, recruiting centre and *PNPLA3* rs738409 and *TM6SF2* rs58542926 genotype, the *FNDC5* rs3480 (G) allele was associated with an increased risk of steatosis (S2-S3) (OR: 1.29, 95% CI: 1.08-1.55, p=0.004) (**Supplementary Table 4**).

PNPLA3, *TM6SF2* and *FNDC5* polymorphisms have an additive effect

We next examined the proportion of attributable risk conferred by the *FNDC5*, *PNPLA3*, and *TM6SF2* genetic variants. As expected, the *PNPLA3* I148M variant had the largest impact on steatosis (S2-S3); the estimated population-attributable risk (PAR) was 25.1%. Of interest, the *FNDC5* rs3480 variant had a greater PAR compared to the E167K *TM6SF2* variant (10.6% vs. 6.7%, respectively).

We then examined for interaction between the three variants but did not observe an impact on hepatic steatosis (P > 0.05). Instead, the 3 variants appeared to have an additive impact with a stepwise increase in odds ratio for steatosis (S2-S3) for each additional risk allele (p<0.0001 for trend) (**Figure 1**). In total, *FNDC5* rs3480 is a novel risk variant for steatosis, independent, but additive to the known risk variants, *PNPLA3* and *TM6SF2*.

FNDC5 rs3480 and the severity of hepatic inflammation and fibrosis

We tested the association of the gene variants with the severity of lobular inflammation and ballooning. *FNDC5* rs3480 was not associated with more severe lobular-inflammation (A2-A3) (OR: 1.3, 95% CI: 0.9-1.87, p=0.1) and remained the same after adjustment for age, sex, BMI, diabetes, and severity of steatosis (p=0.2). Similarly, no association with fibrosis (F2-F4) was observed (OR: 1.05, 95% CI: 0.74-1.47, p=0.7) and remained the same after adjustment for the variables above (p=0.79).

Impact of SNP at rs3480 on the 3′ UTR of FNDC5

To investigate the mechanisms for the genetic association, we evaluated the influence of the 3′ UTR SNP rs3480 on the post-transcriptional regulation and stability of FNDC5 mRNA. To examine whether mRNA folding of FNDC5 could be altered by rs3480, bioinformatic analysis using the RNAfold web server was used. This demonstrated that rs3480 SNPs altered mRNA structure, suggesting that it may affect the stability of FNDC5 mRNA, with a marginal variation in the MFE, from $\Delta G = -1102.20$ to -1102.10 kcal mol^{-1} (**Supplementary Figure 2**).

To validate these findings, we generated full-length 3′ UTRs of FNDC5 containing either the G or the A allele and cloned them into the pmirGLO vector (**Figure 2A**), then transfected human hepatoma (Huh7) cells with the constructs and measured luciferase activity. The FNDC5-G 3′ UTR conferred significantly lower luciferase activity compared to the FNDC5-A 3′ UTR (**Supplementary**

Figure 3). We next assessed the effect of rs3480 on mRNA stability. Analysis of the luciferase–encoding mRNA that remained in Huh7 cells after treatment with actinomycin D revealed that mRNA bearing the FNDC5-G 3′ UTR decayed significantly faster than that bearing the FNDC5-A 3′ UTR (**Figure 2B**). This implies that rs3480 alleles affect the stability of the mRNA transcript.

Next, we performed allele-specific expression analysis using pyrosequencing of gDNA (blood) and cDNA from 10 human liver samples from rs3480 heterozygote subjects. As demonstrated in **Supplementary Figure 4**, modest but significant allelic differences in gene expression were observed, with lower expression in the G-allele (P = 0.003, nonparametric Wilcoxon's paired test). Together these data demonstrated that rs3480 influences the stability and expression of FNDC5.

Regulation of FNDC5 by miRNA

We investigated whether the rs3480 SNP could influence recruitment of miRNAs to the 3′ UTR of FNDC5. Bioinformatics analysis of the 3′ UTR sequence around the site of rs3480 identified potential binding sites for miR-135a-5P and miR-135b-5P (**Figure 2C**). To investigate the preferential binding of the miRNAs to the rs3480 alleles, we co-transfected Huh7 cells with miR-135a-5P and miR-135-5P and negative control mimics with luciferase reporter constructs containing FNDC-A or FNDC5-G. Luciferase assay showing that miR-135a-5p binds to both A and G alleles, but preferentially to the G allele, demonstrated by significantly lower luciferase activity between G+ miR-135a-5p compared to A+ miR-135a-5p (**Figure 2D**). Notably, the difference between alleles was blunted in the presence of the miR-135a-5P inhibitor (**Figure 2E**), suggesting the specificity of decreased luciferase activity by miR-135a-5P. In contrast, miR-135b-5P binds to both alleles with no preferential binding (**Supplementary figure 5**).

Gene set enrichment analysis

We then undertook pathway analysis of the common predicted targets of miR-135a-5P using KEGG pathway mapping. Collectively miR-135a-5P targets mapped onto different pathways (**Figure 2F and Supplementary Table 5**). Notably, they included overrepresentation of insulin signalling and type 2 diabetes pathways.

Hepatic miRNA and FNDC5 levels in NAFLD

We next interrogated hepatic levels of miR-135a-5P, miR-135b-5P and FNDC5 in liver from NAFLD patients and controls by ddPCR. Compared to controls, patients with NAFLD had significantly higher levels of miR-135a-5P but not miR-135b (P<0.05, **Figure 3 A and supplementary Figure 6**). FNDC5 levels tended to be lower in NAFLD, but not significantly (**Figure 3B**). A statistically significant (r^2=0.80 p ≤ 0.002) positive correlation was observed between miR-135a-5P and FNDC5 in liver from normal control subjects, while they were negatively correlated in NAFLD (r^2=-0.36) (**Supplementary Figure 7**). There was no difference in hepatic miR-135a and miR-135b levels according to rs3480 genotype (data not shown).

Elevated circulating irisin is associated with reduced steatosis and a favorable metabolic profile

Finally, we examined which clinical parameters were associated with serum irisin level in a sub-cohort of patients with NAFLD (n=152) having available serum. Irisin levels correlated positively with high-density lipoprotein (HDL) cholesterol levels (P<0.001), and negatively with HOMA-IR (p=0.04). No correlations were observed with other clinical parameters (**Supplementary table 6**). Median irisin levels were higher in patients less than 40 years compared to those ≥ 40 years ($P < 0.01$) (**Figure 4A**) and in subjects with HOMA-IR <3 compared to those with HOMA-IR ≥3 (p<0.001) (**Figure 4B**). Irisin serum levels were similar between females and males (p=0.5). Finally, serum irisin levels (**Figure 4C**) were lower in those with greater steatosis compared to those with mild steatosis (*P*

< 0.03), but did not differ by other histological features. Patients with the GG genotype had a trend towards lower median irisin levels compared with individuals carrying at least one A allele, though it was not significant (p=0.07, **Supplementary figure 7**). No difference in irisin level according to PNPLA3 or TM6SF2 genotypes was observed. Collectively, elevated serum irisin levels are associated with reduced steatosis and an improved metabolic profile.

Discussion

In this study, we demonstrate that *FNDC5* rs3480 is a novel additional and possibly hitherto overlooked genetic variant affecting the risk of hepatic steatosis in NAFLD. The influence of the risk genotype was independent of, but additive to *PNPLA3* and *TM6SF2* in Caucasian patients. We show a 1.4- to 6.8-fold increase in hepatic steatosis risk based on the cumulative association of the three variants among subjects who had at least one copy of the risk allele at each of the three regions, compared with subjects without any risk alleles. The G allele associated with greater FNDC5 mRNA degradation compared to A allele, had a modest but significant effect on liver FNDC5 allele-specific expression and also created a binding site for miR-135a-5P. Compared with controls, NAFLD patients had significantly higher levels of hepatic miR-135a-5p and lower FNDC5. Concomitantly, this miRNA can regulate several pathways linked to liver disease including that for insulin signaling and type 2 diabetes. Lastly, elevated serum irisin levels were associated with reduced steatosis and with improved indices of the circulating metabolic profile. Collectively, these data suggest that miRNA binding to a SNP in the 3'UTR, impairs FNDC5 expression and thereby increases the severity of hepatic steatosis (**Figure 5**).

While carriage of the *FNDC5* rs3480 (G) minor allele was associated with an increased risk of steatosis, it did not impact other histologic features. The influence on steatosis was also independent of known steatosis risk confounders including age, gender, BMI, T2DM and recruitment centre. Since no interaction was observed between *FNDC5* rs3480, *PNPLA3* rs738409 and *TM6SF2* rs58542926 genotypes, our data suggests that the three genes regulate different pathways in steatosis pathogenesis.

Serum irisin levels were reduced in subjects with greater hepatic steatosis (S2-S3). Both in a study of 296 obese Chinese adults in whom hepatic triglyceride content was determined by ^{1}H MRS spectroscopy [21] and another of 84 Koreans with NAFLD in whom steatosis was measured by

14

abdominal ultrasonography [19], irisin levels declined with increased liver fat. However, in another small study (n = 31) of patients with biopsy-proven NAFLD, no difference in serum irisin according to steatosis grade was observed [20]. These studies used the same ELISA kit and thus differences are likely due to sample size considerations. While there has been scepticism on the quality of commercial ELISAs to quantify irisin, in the present work it was measured using the recently released Phoenix Pharmaceuticals (EK-067-29) kit that has been validated by comparison with data from Western blot and MALDI-TOF mass spectrometry [27].

The mechanisms for the protective effects of irisin on hepatic steatosis in NAFLD are not clear, but suggests both direct and indirect beneficial effects. In humans, *FNDC5* is expressed in skeletal muscle and adipose tissues serving as both a myokine and adipokine, though the principle source appears to be muscle [33]. In liver, hepatocyte-released irisin functions as a paracrine/autocrine hormone [34]. It has been reported that adipose tissue *FNDC5* mRNA and circulating irisin levels negatively correlate with hyperglycaemia, elevated triglycerides, visceral adiposity and extramyocellular lipid deposition, features that are concordant with the metabolic abnormalities observed in NAFLD [35]. Moreover, treating muscle cells *in vitro* with palmitate and glucose lowered *FNDC5* mRNA suggesting peripheral effects on metabolic and glucose homeostasis [35]. This data is supported by animal studies that FNDC5 improves hepatic steatosis, protects mice against genetic- and diet-induced obesity, and improves hepatic and peripheral insulin resistance via regulating AMPK signaling in myocytes and hepatocytes [34, 36-38].

miRNAs are small non-coding RNA transcripts of ~22 nucleotides that regulate gene expression at the posttranscriptional level [39]. They do so by targeting sites of complementarity in the 3`UTR of mRNAs, thereby mediating mRNA decay or translational repression. miRNAs can repress multiple targets within the same pathway resulting in amplification of their biological effects. SNPs can

create, destroy or modify sites for miRNA binding. Our results reveal that rs3480 with an A-to-G substitution in the 3′ UTR of FNDC5 results in decreased expression of FNDC5 through two post-transcriptional mechanisms. Firstly, FNDC5-G suffered more degradation than did FNDC5-A; secondly, it also differentially bound mir-135a-5P. Hepatic levels of mir-135a-5P and FNDC5 as measured by ddPCR were upregulated and downregulated in NAFLD patients compared with controls, respectively. Not surprisingly, a positive correlation was present between mir-135a-5P and FNDC5 in normal subjects, while this correlation was reversed in NAFLD. This suggests that miRNA modulation of FNDC5 might be a form of "compensatory adaptation" in normal liver, whereby the miRNAs do not downregulate the expression of FNDC5 and might indicate a signal for transcript downregulation. The correlations turn to be negative with failure of adaptive mechanisms when fatty liver develops. The more profound modulation of mir-135a-5P compared to FNDC5 with NAFLD suggests that induction of mir-135a-5P might be an early event in NAFLD development. Consistently, miR-135a levels were elevated in serum, plasma, renal and skeletal muscle from diabetic patients compared to controls [40-42]. Notably, our pathway analysis also suggested that miR-135a maps to different pathways, including an overrepresentation of insulin signaling and type 2 diabetes pathways. In this regard, mir-135a has been shown to inhibit insulin signaling and glucose uptake by targeting Insulin receptor substrate 2 (IRS2). Of relevance, we demonstrated that serum irisin level was negatively correlated with HOMA-IR.

We found no association between *FNDC5* rs3480 genotype or serum irisin levels with histological disease activity or fibrosis, or with liver tests as indices of liver injury. According to our data, genotypic variation at rs3480 has a modest effect size on steatosis and thus is likely insufficient to influence the transition to steatohepatitis and fibrosis. Our data however do not exclude the possibility of effects on inflammation and fibrosis in larger cohorts powered to detect such effects. This is relevant, as we have shown that serum irisin levels demonstrate an inverse correlation with measures of

16

insulin resistance and dyslipidaemia that are associated with inflammation and fibrosis [12, 43]. However, the alternate possibility is that irisin disentangles steatosis from fibrosis. In this context, we have previously shown that serum leptin correlates with steatosis but not fibrosis in NAFLD [14], suggesting both shared and differential pathways between susceptibility to steatosis and risk of disease progression.

A recent smaller study (n=593) reported that the rs3480 (G) allele was associated with the severity of hepatic steatosis only in univariate analysis, consistent with our finding, while the association was lost after multiple logistic regression [44]. Surprisingly, in that study, the same allele was associated with less severe hepatic fibrosis. The reasons for this discrepancy are unclear.

In conclusion, in this study we characterized a functional *FNDC5* variant as an additional risk variant associated with hepatic steatosis independent of *PNPLA3* and *TM6SF2*. This polymorphism influenced transcript stability, as well as the binding of miRNAs, suggesting a model whereby rs3480 modulates steatosis by regulating *FNDC5* expression.

Figures:

Figure 1: FNDC5 association the severity of hepatic steatosis. (A) Forest plot of associations of rs3480 with the severity of hepatic steatosis in individual samples and in the combined, fixed-effects meta-analysis (n=987) **(B)** Association between the number of *PNPLA3*, *TM6SF2*, and *FNDC5* risk alleles and the severity of hepatic steatosis. The bar plots show the ORs (95% CI) for each number of risk alleles using zero risk alleles as the reference (right y axis). The histogram denotes the proportion of individuals in each genotype score category (left y axis).

Figure 2: Allele-specific regulation of FNDC5 by miRNA-135a-5P. (**A**) FNDC5 dual luciferase reporter constructs. The 3'UTR of FNDC5 was cloned downstream of luciferase, which is driven by the constitutive Pgk promoter. (**B**) Stability of luciferase mRNA in Huh7 cells transfected with the FNDC5-A or FNDC5-G luciferase construct and treated with actinomycin D (ActD) to arrest new transcription, presented as mRNA remaining over time relative to that at 0 h, set as 100%. Half-lives (50% mRNA remaining (dashed line)): FNDC5-A 3′ UTR, 6.2 h; FNDC5-G 3′ UTR, 3.7 h. (**c**) Bioinformatics analyses showing that rs3480 is the predicted target gene for miR-135a-5p and miR-135b-5p. (**d**) The miRNA mimics or miR inhibitor was co-transfected with the reporter gene constructions into Huh7 cells. Luciferase assay showing that miR-135a-5p binds preferentially to the G allele, while there was less binding to the A allele. (**e**) Depletion of miR-135a-5p by miR inhibitor abrogated the difference in luciferase activity between the two alleles. (**f**) Gene set enrichment analysis of pathways targeted by miR-135a-5p showing −log P-value (**Supplementary Table 5**). * P<0.05, **P<0.01, *** P<0.001 by two-tailed Student's t test. Error bars represent standard error of the mean.

Figure 3: Absolute quantification of hepatic miR-135a-5p levels and FNDC5 levels. (**a**) miR-135a-5p levels are upregulated in NAFLD compared to controls in liver samples. (**i**) FNDC5 levels are

18

downregulated in NAFLD compared to controls in liver (n=10 controls, n=10 NAFLD). * P<0.05, **P<0.01, *** P<0.001 by Mann–Whitney U test. Boxplots are median, minimum and maximum.

Figure 4: Serum irisin levels (A), HOMA-IR (B) and steatosis (C) in a sub-cohort of 152 NAFLD patients. Boxplots are median, minimum and maximum. Statistical significance was analyzed by the Mann-Whitney U test. The number of samples tested in each group is shown.

Figure 5. Proposed model for the regulation of FNDC5 and hepatic steatosis. miR-135a-5p binds preferentially to the G allele of rs3480 in the 3'untranslated region (UTR) of the Fibronectin type III domain-containing protein 5 (FNDC5) gene. Consequently, miRNA mediated decay of FNDC5 is enhanced and this results in less FNDC5 mRNA. As FNDC5 has favorable metabolic impacts, this results in greater hepatic steatosis.
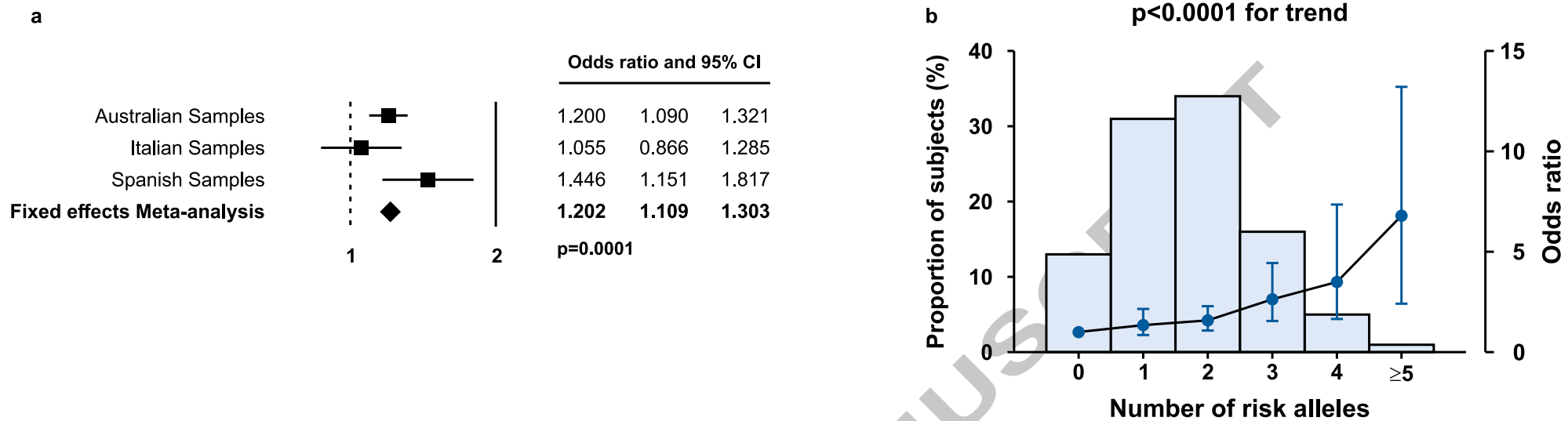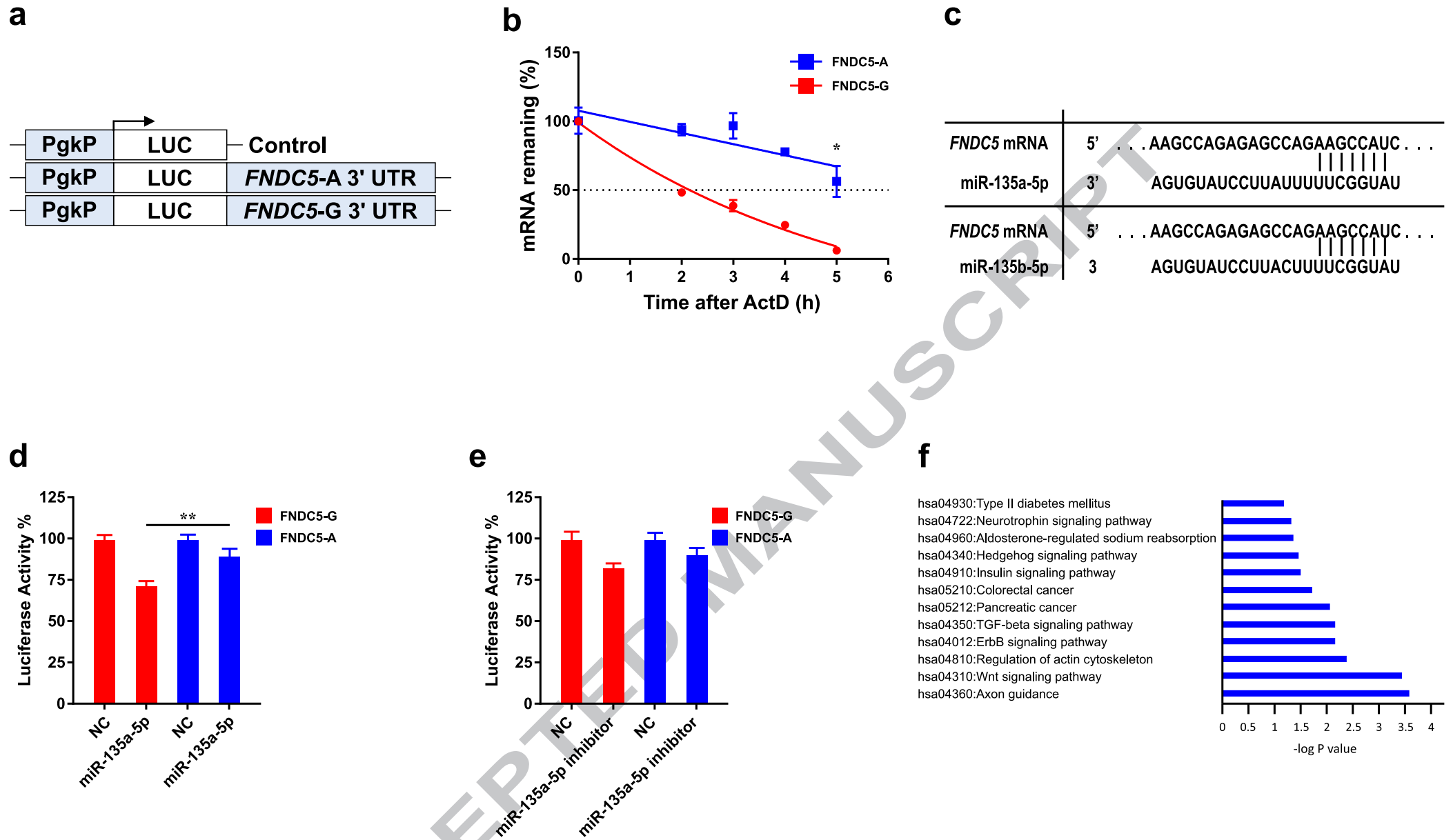
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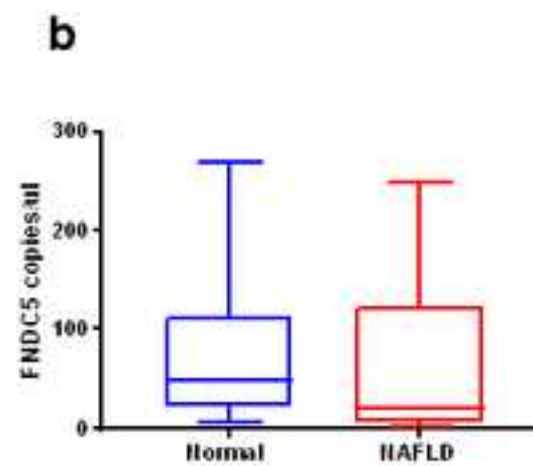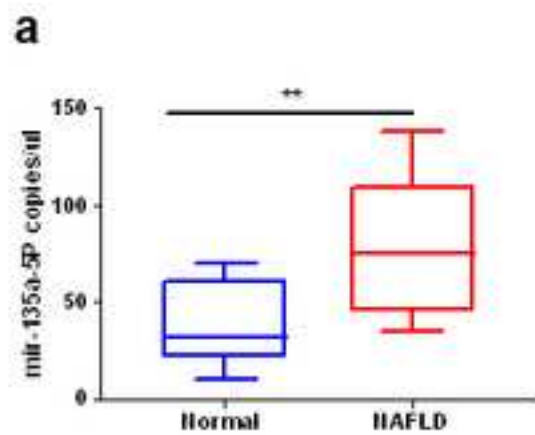
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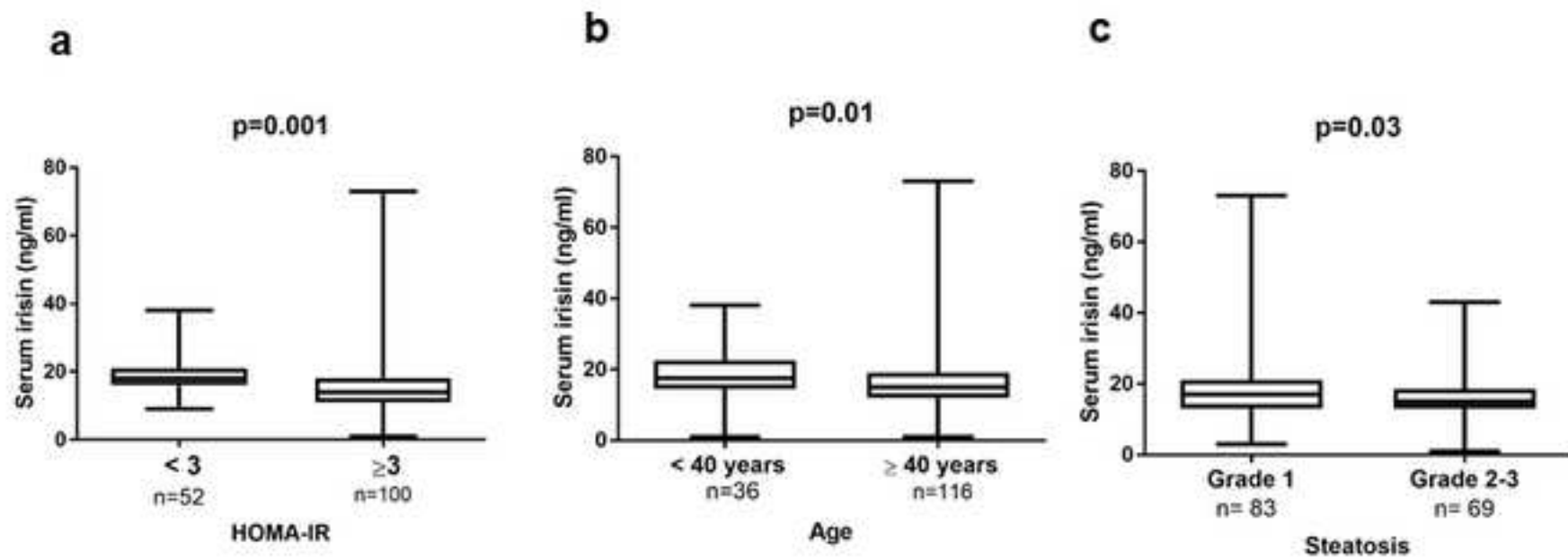
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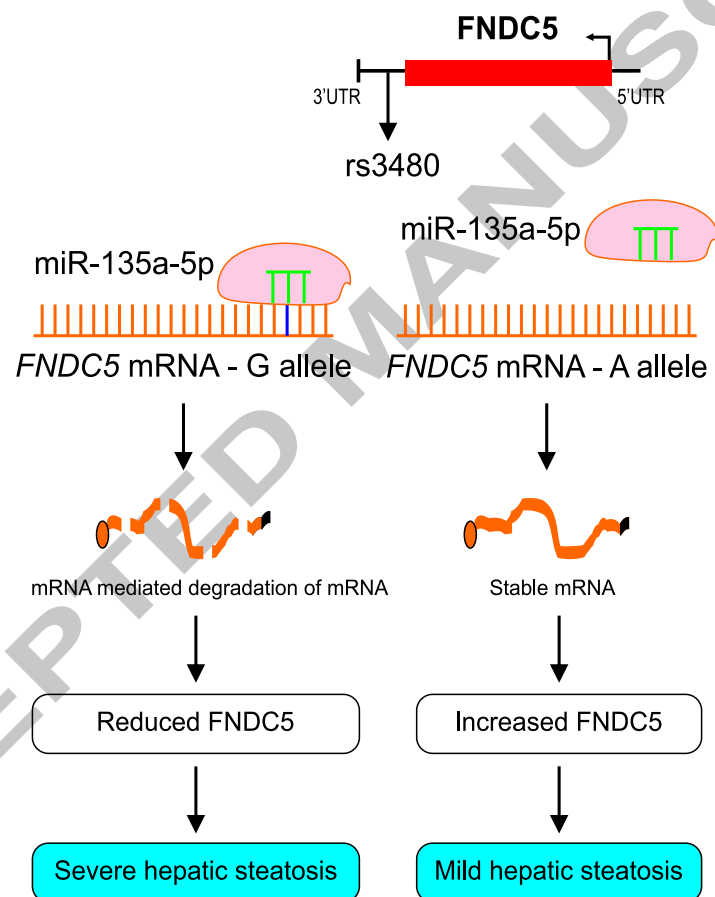
Figure 1

a

	Odds ratio and 95% CI		
Australian Samples	1.200	1.090	1.321
Italian Samples	1.055	0.866	1.285
Spanish Samples	1.446	1.151	1.817
Fixed effects Meta-analysis	**1.202**	**1.109**	**1.303**
	p=0.0001		

b p<0.0001 for trend

Figure 2

Figure 3

Figure 4

Figure 5

FNDC5

3'UTR   5'UTR

rs3480

miR-135a-5p

miR-135a-5p

FNDC5 mRNA - G allele   *FNDC5* mRNA - A allele

mRNA mediated degradation of mRNA   Stable mRNA

Reduced FNDC5   Increased FNDC5

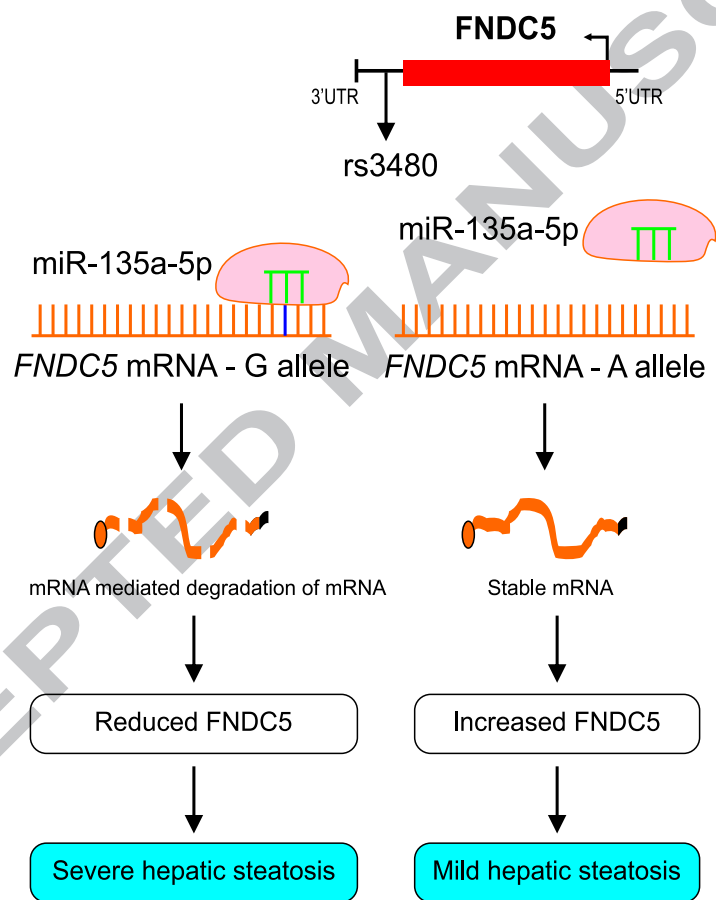Severe hepatic steatosis   Mild hepatic steatosis

*Graphical Abstract

Highlights

- Irisin, the cleaved extra-cellular fragment of the Fibronectin type III domain-containing protein 5 (FNDC5) is a myokine with proposed favorable metabolic activity.
- The role of variants in the FNDC5 gene in non-alcoholic fatty liver disease (NAFLD) is not defined.
- Genetic variants in FNDC5 confer risk of human severe hepatic steatosis.
- Functional studies reveal that this variant mediates this effect through a microRNA-mediated mechanism controlling FNDC5 mRNA stability.
- Irisin is likely to have a favourable metabolic impact on NAFLD.