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Original Article

ORIGINAL ARTICLE

Early hypermethylation of hepatic *Igfbp2* results in its reduced expression preceding fatty liver in mice

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Abstract

Obesity and ectopic fat disposition are risk factors for metabolic disease. Recent data indicate that *IGFBP2* expression in liver is epigenetically inhibited during hepatic steatosis. The aim of this study was to investigate if epigenetic de-regulation of hepatic *Igfbp2* occurs already early in life and is associated with increased risk for diet-induced obesity (DIO) during adolescence. Male C57BL/6J mice received a high-fat diet. After 3 weeks on this diet (age of 6 weeks), DIO-susceptible (responder, Resp) and DIO-resistant (non-responder, nResp) mice were identified by early weight gain. At the age of 6 weeks, Resp mice exhibited elevated blood glucose ($p < 0.05$), plasma insulin ($p < 0.01$), HOMA-IR and leptin/adiponectin ratio, whereas liver triglycerides were identical but significantly increased ($p < 0.01$) in Resp mice at 20 weeks of age. *Igfbp2* expression was reduced in young Resp compared with nResp mice ($p < 0.01$), an effect that correlated with elevated DNA methylation of intronic CpG_{2605} ($p < 0.01$). The epigenetic inhibition of *Igfbp2* was stable over time and preceded DIO and hepatosteatosis in adult mice. *In vitro* studies demonstrated that selective methylation of CpG_{2605} significantly reduced reporter activity by ~85%, indicating that *Igfbp2* expression is modulated by methylation. In human whole blood cells, methylation of *IGFBP2* at the homologous CpG site was increased in obese men with impaired glucose tolerance. In conclusion, our data show that increased methylation of hepatic *Igfbp2* during infancy predicts the development of fatty liver later in life and is linked to deterioration of glucose metabolism.

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Introduction

Obesity has become a major health threat, affecting more than half a billion people worldwide in 2014 (1). In particular, obesity increases the risk for insulin resistance, hypertension, coronary heart disease and type 2 diabetes (T2D). The risk of obesity is largely influenced by the family background, indicated by heritability rates of up to 40% for body mass index (BMI) (2). However, genetic variants identified so far only explain <5% of the observed heritability, a phenomenon referred to as "missing heritability" (3,4).

Epigenetics is suggested to explain parts of the missing heritability. Epigenetic alterations (e.g. DNA methylation at CpG dinucleotides or histone modifications) modify gene expression without altering genomic sequences and are therefore missed in conventional genome-wide association studies (5,6). Intriguingly, even in inbred mouse strains like C57BL/6J, individual mice largely differ in their response to diet-induced obesity (DIO), rendering these strains a suitable model to unravel non-genetic mechanisms in body weight regulation (7). In this context, Koza *et al.* (7) identified three genes (*Sfrp5*, *Mest* and *Bmp3*) in adipose tissue, which are differentially expressed in DIO-responders already at 7 weeks of age and associated with increased susceptibility to DIO.

In addition to adipose tissue, the liver plays a major role in the regulation of energy metabolism and hepatic insulin sensitivity, and consequently in the pathogenesis of obesity and T2D (8,9). Accordingly, hepatic genes involved in glucose and lipid metabolism have been shown to be altered in mice prone to DIO, including insulin-like growth factor 2 (*Igf2*) (10). The IGF-axis appears to play a crucial role in the regulation of body weight early in life. IGF-2 is involved in embryonic growth, and altered methylation of the human *IGF2* gene is associated with childhood obesity (11–13). This is of particular interest, since plasma IGFBP-2 levels have been found to be decreased in obese males and females (14,15). Furthermore, reduced circulating IGFBP-2 levels in obese subjects are associated with increased bioavailability of IGF-1, and hyperinsulinemia (14). Furthermore, *Igfbp2* transgenic mice show reduced body weight gain on a standard diet and are protected against the development of a DIO and insulin resistance (16,17). Recently, Ahrens *et al.* (18) reported that hepatic expression of *IGFBP2*, a modulator of IGF-1/2 bioavailability, is epigenetically inhibited in patients with different grades of hepatic lipid accumulation.

In this study, we investigated whether the variance in DIO and hepatic steatosis of genetically identical C57BL/6J mice is reflected by epigenetic modulation of hepatic *Igfbp2* early in life. The establishment of weight prediction parameters allowed us to isolate liver tissues before the onset of fatty liver, and to study differences between mice prone to DIO and mice resistant against it. We found *Igfbp2* to be hypermethylated and transcriptionally repressed in young mice prone to DIO, already before the onset of hepatic steatosis. These transcriptional and epigenetic differences of *Igfbp2* that are present early in life and linked to obesity and insulin resistance in adolescence underline the significance of IGFBP-2 for body weight development and fatty liver. Interestingly, differential methylation occurs at the CpG site homologous to the human site in the *IGFBP2* gene published by Ahrens *et al.* (18). In whole blood cells of human subjects exhibiting an impaired glucose tolerance the IGFBP2 methylation was significantly increased.

Results

Prediction of DIO by early weight parameters

In order to investigate early modulators of *Igfbp2* expression, it was necessary to discriminate between mice prone and resistant to DIO at the earliest time point possible. Therefore, we established early predictors of obesity in a larger cohort of 324 male C57BL/6J mice fed a high-fat diet (HFD, 60 kcal% from fat) for 20 weeks. In this cohort, body weights varied in a range of 27.2–52.7 g (Supplementary Material, Fig. S1). DIO responders (Resp) were defined as individuals that reached body weights >38 g, while non-responders (nResp) reached <38 g at 20 weeks of age. Analysis of the individual weight courses indicated that weight gain during the first 3 weeks after weening (life week 3–6) in combination with the absolute body weight at week 6 is the strongest predictor for DIO susceptibility. In detail, nResp mice were characterized by an early weight gain of <0.9 g/day plus body weights <22 g in week 6, resulting in a probability for correct prediction of $p = 0.81$. Resp mice were characterized by an early weight gain of >0.7 g/day plus body weights >24 g in week 6 ($p = 0.80$). Next, the quality of prediction was confirmed in a second cohort of mice for which the prediction parameters were applied in week 6 (Fig. 1A). Low-fat diet (LFD)-fed mice served as a lean control. The early difference in body weight between Resp and nResp mice was reflected by both lean and fat mass, and increased over time (Fig. 1B, Table 1). Early body weight differences between both groups were not because of differences in body length, or food and water intake that were detected in single caged mice (Table 1).

Impaired glucose homeostasis precedes the onset of hepatosteatosis

Next, we investigated whether differences in body weight gain were associated with changes in blood glucose, insulin or leptin levels. Surprisingly, blood glucose levels in Resp mice were elevated compared with nResp mice already at 6 weeks of age (8.5 ± 0.5 versus 6.8 ± 0.4 mM, $p < 0.05$) (Fig. 1C). Furthermore, plasma insulin (0.88 ± 0.10 versus 0.44 ± 0.06 µg/l, $p < 0.01$) and leptin (0.54 ± 0.08 versus 1.62 ± 0.31 ng/ml, $p < 0.01$) levels were also increased in Resp mice at this early time point (Fig. 1D and E). The differences of these blood parameters were stable and further increased over time (Fig. 1C–E). Accordingly, HOMA-IR indicated that Resp mice already at 6 weeks of age were less insulin sensitive compared with nResp mice (8.3 ± 1.1 versus 3.4 ± 0.6, $p < 0.001$) (Fig. 1F). The leptin per adiponectin ratio (LAR) is a second measure of systemic insulin resistance (19) and showed the same differences between Resp and nResp mice (Fig. 1G). Therefore, the impaired insulin sensitivity observed at the age of 20 weeks in Resp mice (Fig. 1F and G) was already manifested at 6 weeks of age and preceded the obese phenotype. Importantly, liver triglyceride levels were not different at 6 weeks of age but increased in Resp mice at 20 weeks of age (0.82 ± 0.08 versus 1.21 ± 0.07 $\mu g/\mu g_{protein}$) (Fig. 1H). Absolute liver weight was not different between Resp and nResp mice at 6 (42.2 ± 1.3 versus 39.3 ± 1.2 $mg/g_{body\ weight}$) and 20 (33.3 ± 1.1 versus 31.8 ± 0.9 $mg/g_{body\ weight}$) weeks of age, respectively.

Reduced *Igfbp2* expression in young DIO responders

Recently, it has been shown that human hepatic steatosis is associated with reduced expression of hepatic *IGFBP2* (18). In Resp mice, expression of hepatic *Igfbp2* was reduced by 35.55 ± 10.20% ($p < 0.01$) and 40.68 ± 8.11% ($p < 0.001$) at weeks 6 and 20, respectively, as compared with nResp mice (Fig. 2A and B). This decrease of gene expression was paralleled by reduced IGFBP-2 protein level in liver tissue (Fig. 2C). In order to investigate whether plasma levels of other IGFBP isoforms are also altered

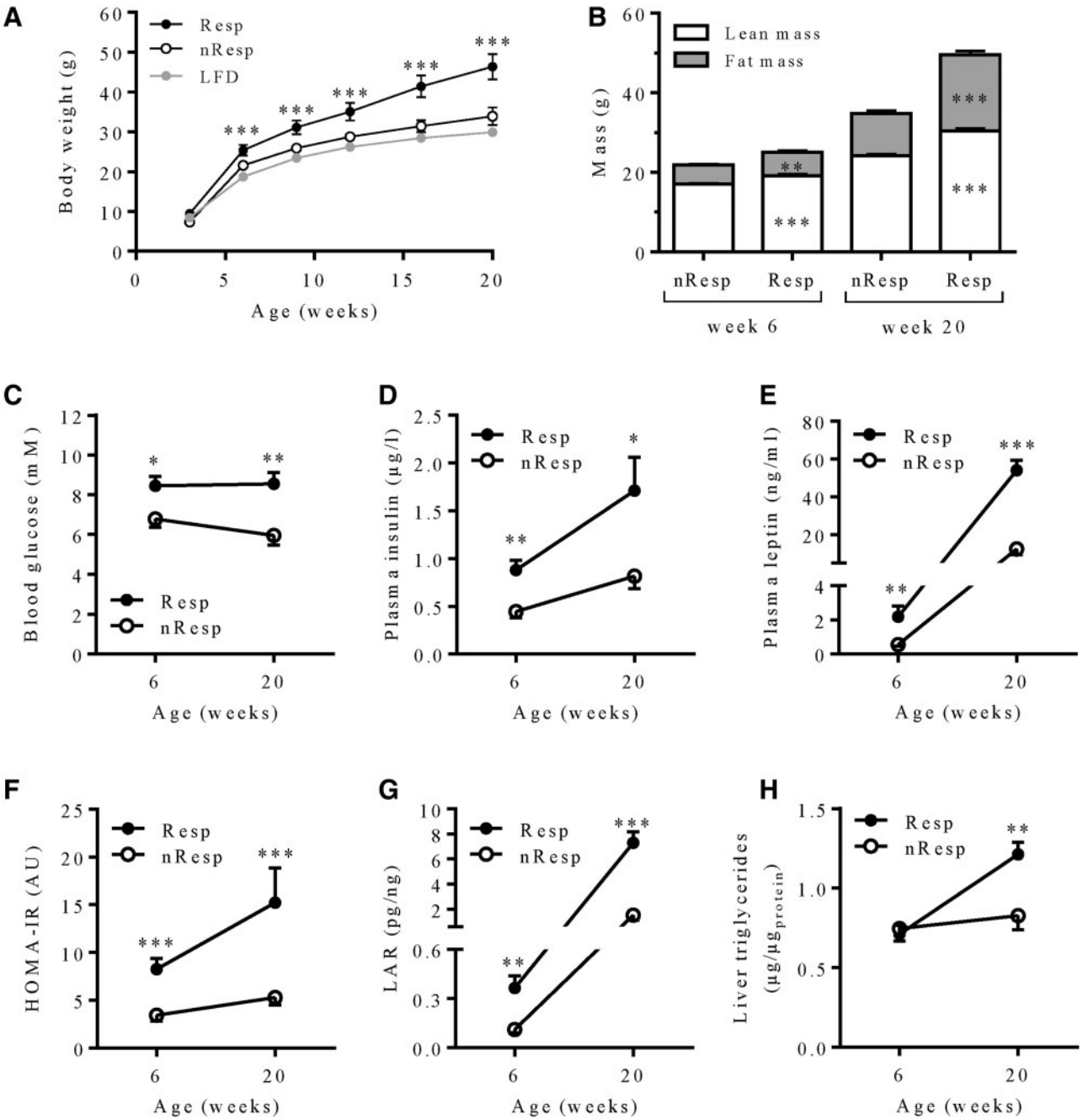

Figure 1. Impaired insulin sensitivity precedes liver fat accumulation. (**A**) Body weight development of DIO-responders (Resp, $n = 9$) and DIO-non-responders (nResp, $n = 11$) upon identification in week 6. Mice receiving an LFD served as control group. Body composition (**B**), fasted blood glucose (**C**), plasma insulin (**D**) and leptin (**E**) levels. (**F**) HOMA-IR calculated from fasted blood glucose and insulin levels. (**G**) LAR. (**H**) Liver triglyceride levels. All data represented as mean ± SEM of $n = 10$–15 animals per group. Differences between DIO-responder (Resp, black circle) and DIO-non-responder (nResp, white circle) mice were calculated by Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

in Resp mice, we performed quantitative western ligand blots against IGF-2 (20). IGFBP-2 levels were decreased in Resp mice ($p < 0.01$) (Fig. 2D), whereas IGFBP-3 levels were increased at week 6 ($p < 0.01$) (Fig. 2E). Expression of IGFBP-4 was below the limit of quantification of the analytical system. In the circulation, IGFBP-2 binds to IGF-1 and decreases its bioavailability (21). In obese subjects, reduced expression of *IGFBP2* results in increased serum levels of free IGF-1 (14). Accordingly, serum levels of total IGF-1 were increased in Resp mice compared with nResp mice (Fig. 2F).

Increased *Igfbp2* methylation detected early in life of Resp mice

Reduced expression of *IGFBP2* in NAFLD and NASH patients was associated with increased methylation of one CpG (cg11669516) in the first intron of the human *IGFBP2* gene at position 2965 bp downstream the transcription start site (TSS) (18). Because the genomic region around cg11669516 is highly conserved between humans and mice (Fig. 3A), we investigated whether methylation at this site also occurs in young obesity-prone mice, and

Table 1. Physiological parameters of nResp and Resp mice

	Week 6					Week 20				
	nResp		Resp		*p*-value	nResp		Resp		*p*-value
	Amount	SEM	Amount	SEM		Amount	SEM	Amount	SEM	
Body weight (g)	21.3	0.2	24.9	0.3	<0.001	33.4	0.6	45.7	1.0	<0.001
Lean mass (g)	17.1	0.2	19.1	0.3	<0.001	24.1	0.3	30.4	0.5	<0.001
Fat mass (g)	4.8	0.1	6.2	0.3	<0.01	10.8	0.7	19.8	1.0	<0.001
Body length (cm)	9.0	0.1	9.5	0.2	0.071	10.3	0.1	10.8	0.1	0.008
				Week 10						
Food intake (g/day/g BW)	0.087	0.002	0.082	0.004	0.205					
Water intake (g/day/g BW)	0.098	0.006	0.090	0.006	0.354					

whether this methylation is paralleled by early differences in *Igfbp2* expression. Indeed, methylation at the corresponding mouse CpG (CpG_{2605}, 2605 bp downstream the TSS) was increased by 6.2 ± 1.2% ($p < 0.001$) in Resp mice at 6 weeks of age (Fig. 3B). In addition, we used direct bisulfite sequencing PCR (dBSP) to study CpG methylation of the proximal promoter region and identified three additional CpG sites (CpG_{-842}, CpG_{-738} and CpG_{884}) that were methylated differentially in nResp and Resp mice (Supplementary Material, Fig. S2A and B). However, as these sites could not be validated by pyrosequencing (Supplementary Material, Fig. S2C–E), we concentrated on CpG_{2605} for further analyses. Methylation of the intronic CpG_{2605} in the young animals was inversely correlated with expression of *Igfbp2* ($R^2 = 0.551$, $p < 0.001$) and positively correlated with body weight ($R^2 = 0.686$, $p < 0.001$) (Fig. 3C and D). Neither blood glucose nor HOMA-IR was correlated with CpG_{2605} methylation; however, the LAR was positively correlated with CpG_{2605} methylation (Supplementary Material, Fig. S3A–C). The methylation difference between both groups persisted, as Resp mice displayed increased CpG_{2605} methylation compared with nResp mice also at 20 weeks of age (6.0 ± 1.3%, $p < 0.001$) (Fig. 3E). Again, methylation of CpG_{2605} was negatively correlated with gene expression ($R^2 = 0.392$, $p < 0.01$) and positively with body weight ($R^2 = 0.339$, $p < 0.01$) (Fig. 3F and G). At this later time point, methylation of CpG_{2605} was also correlated with blood glucose and HOMA-IR but not with LAR (Supplementary Material, Fig. S3D–F). In summary, these data indicate that susceptibility to DIO and insulin resistance is associated with increased *Igfbp2* methylation and reduced expression early in life.

CpG_{2605} methylation modulates expression of the *Igfbp2* gene

In order to investigate whether methylation of CpG_{2605} and expression of *Igfbp2* are causally linked, we generated a luciferase reporter construct. It carried a 700-bp fragment of intron 1 in a CpG-free backbone vector (pCpGL) and was *in vitro* methylated by methyltransferase HpaII (CCGG) and M.SssI (CG), respectively (Fig. 4A). Of note, CpG_{2605} is embedded into an HpaII recognition site (Fig. 3A) that is unique in pCpGL-*Igfbp2*. Therefore, *in vitro* methylation of pCpGL-*Igfbp2* by HpaII allowed us to selectively study the impact of CpG_{2605} methylation on *Igfbp2* expression. After *in vitro* methylation, luciferase activity was assessed in AML12 hepatocytes. While full methylation by M.SssI almost completely abolished luciferase activity (−91.3 ± 3.9%, $p < 0.001$), selective methylation of CpG_{2605} with HpaII led to a reduction of luciferase activity by 84.8 ± 4.7% ($p < 0.001$) (Fig. 4B), indicating that *Igfbp2* expression is indeed modulated by methylation of CpG_{2605}. Because the differences in body weight are rather small within HFD-fed C57BL/6J mice, we also compared male C57BL/6J mice with male New Zealand Obese (NZO) mice fed a standard diet at the age of 6 weeks. NZO mice develop severe obesity and are, in contrast to C57BL/6J, susceptible to T2D (22,23). Already at the age of 6 weeks, NZO mice show increased body weight mainly because of increased fat mass compared with C57BL/6J mice which is also reflected by the significantly elevated plasma leptin concentrations. Accordingly, the young NZO mice are insulin resistant as fasted insulin levels and LAR are significantly higher than in C57BL/6 mice. However, both strains do not differ significantly in their fasted blood glucose levels (Table 2). Indeed, NZO mice displayed decreased hepatic *Igfbp2* expression levels and increased methylation of CpG_{2605} compared with C57BL/6J mice (Fig. 4C and D). Again, the degree of CpG_{2605} methylation correlated with body weight (Fig. 4E) as well as with insulin concentration (Fig. 4F) and LAR (Fig. 4G).

Plasticity of *Igfbp2* methylation

In humans, *IGFBP2* expression is increased by caloric restriction and exercise, two mechanisms known to reduce liver fat content (24,25). In order to investigate the plasticity of *Igfbp2* methylation under conditions that modulate *Igfbp2* gene expression, we performed a second set of experiments with obese mice that had received HFD for 18 weeks. The first set of mice was killed directly after the HFD feeding (HFD group); the second group of mice was subjected to 25% weight loss by restrictive feeding of an LFD (10 kcal% from fat, 16.1 kJ/g, D12450B, Research Diets, Inc.) for 3 weeks (HFD-CR group). The third group of mice was also caloric restricted for 3 weeks but received *ad libitum* HFD again afterwards for another 3 weeks (HFD–CR–HFD group). Caloric restriction (CR) led to the desired weight loss, and re-feeding with HFD led to a complete re-gain of body weight (Fig. 5A). Weight loss and re-gain were because of changes in both lean and fat mass (Fig. 5B). In parallel to the weight loss, expression of *Igfbp2* increased in mice of the HFD–CR group (+52.2 ± 15.7%, $p < 0.05$) and decreased again upon HFD re-feeding (Fig. 5C). Accordingly, methylation of CpG_{2605} revealed the opposite trends, with a decrease during CR (−2.6 ± 2.2%, $p = 0.25$) and increase (+5.8 ± 2.7%, $p = 0.04$) during HFD re-feeding (Fig. 5D). To test if changes in *Igfbp2* expression correlate with alterations in blood glucose or plasma insulin we measured these two parameters in the indicated groups. Blood glucose concentrations did not change in response to weight loss but increased significantly after the re-feeding period (Fig. 5E). As expected caloric restriction resulted in a decrease of insulin levels which rose after re-feeding, however, without reaching the initial concentration (Fig. 5F). These data indicate the

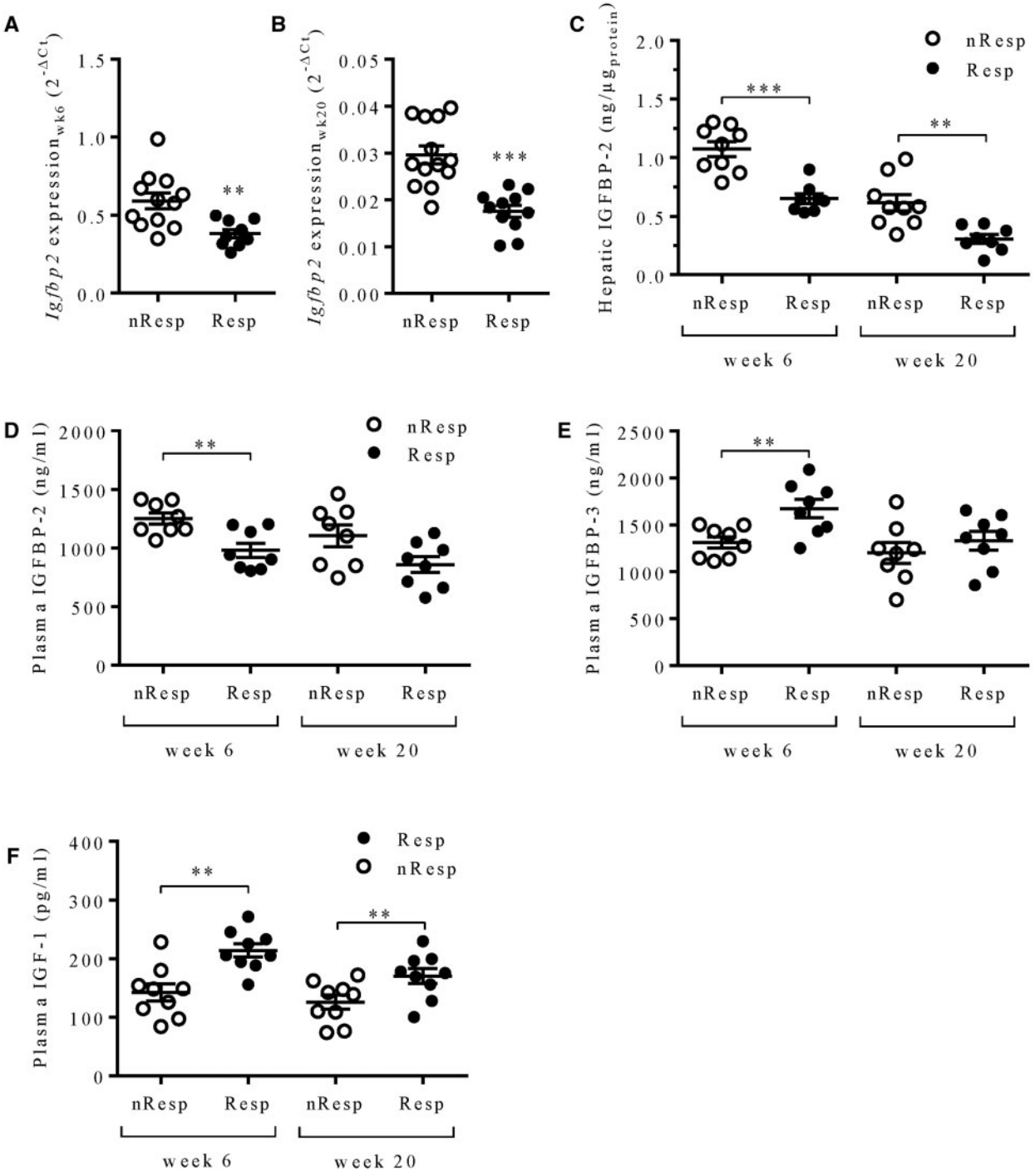

Figure 2. Modulation of the IGF axis. Hepatic *Igfbp2* expression in 6 (**A**) and 20 (**B**) weeks old mice and the corresponding protein levels (**C**). Plasma IGFBP-2 (**D**) and IGFBP-3 (**E**) levels of the same animals determined by quantitative western ligand blot against IGF-2. (**F**) Plasma IGF-1 levels. All data represented as mean ± SEM of $n = 8$–12 animals per group. Differences between DIO-responder (Resp, black circle) and DIO-non-responder (nResp, white circle) mice were calculated by Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

changes in methylation of CpG_{2605} occur in a similar manner as that of insulin levels.

Increased methylation of human CpG_{2965} in whole blood cells of obese and glucose intolerant men

Our data indicate that methylation of CpG_{2605} is a marker for obesity and linked to changes of glucose tolerance or insulin sensitivity. Therefore, we next measured methylation of human CpG_{2965} in whole blood cells of men with different stages of metabolic disease. Subjects were grouped according to their glucose tolerance obtained during an oral glucose tolerance test (oGTT) as well as BMI into normal glucose tolerant subjects with a BMI <27 (NGT.BMI <27) or BMI >30 (NGT.BMI >30) as well as impaired glucose tolerant with a BMI <27 (IGT.BMI <27) or BMI >30 (IGT.BMI >30). Compared with the NGT.BMI <27 group,

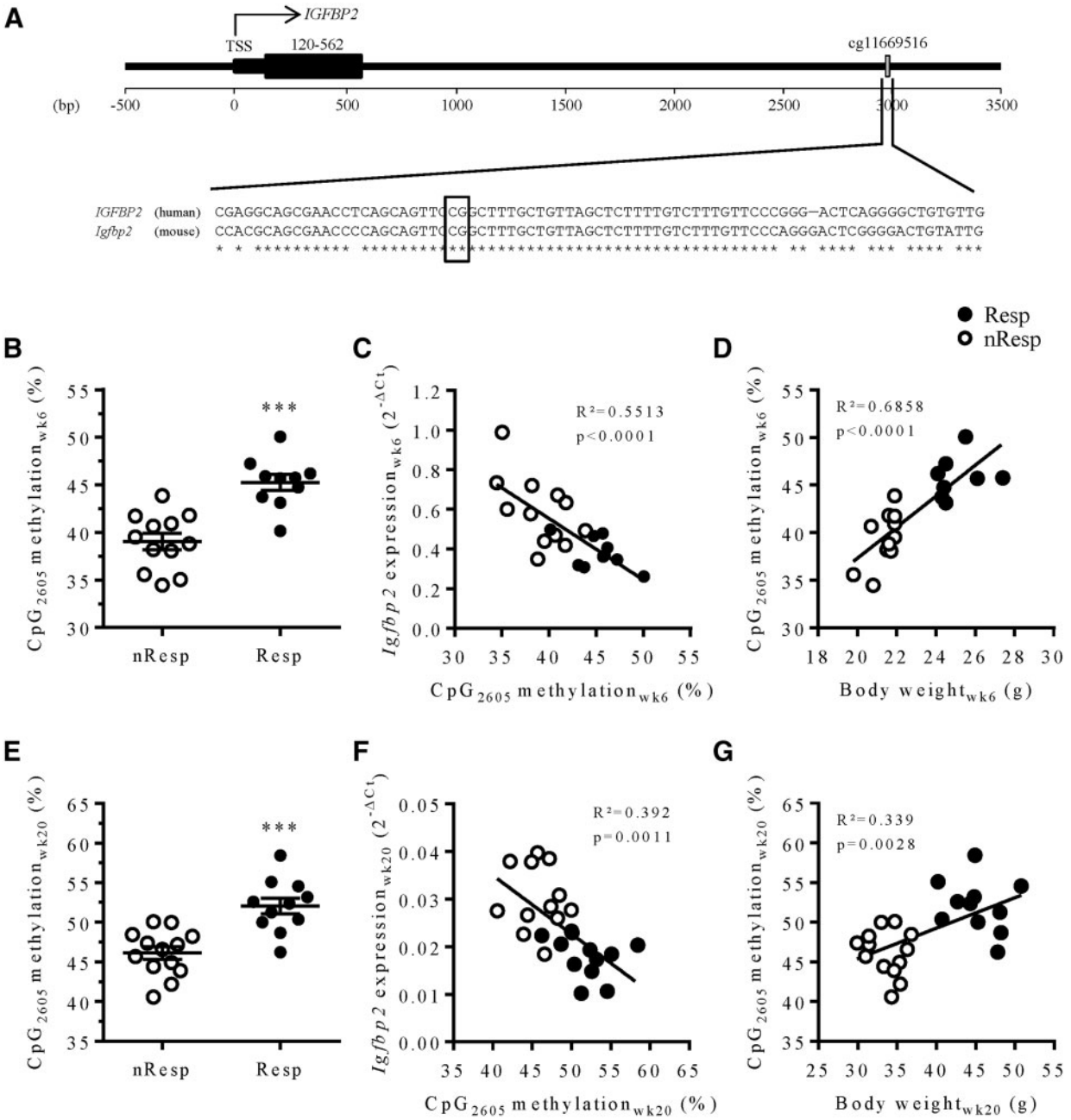

Figure 3. Differential methylation of hepatic *Igfbp2* at CpG_{2605}. (**A**) Genomic localization of cg11669516 in the human *IGFBP2* gene (region −500 bp and +3500 bp relative to the transcriptional start site). Methylation of the mouse homologue CpG_{2605} in week 6 (**B**) and in week 20 (**E**), respectively. Correlation of *Igfbp2* expression and CpG_{2605} methylation at 6 (**C**) and 20 (**F**) weeks of age. Correlation of CpG_{2605} methylation and body weight at 6 (**D**) and 20 (**G**) weeks of age. All data represented as mean ± SEM of $n = 10$–14 animals per group. Differences between Resp (black circle) and nResp (white circle) mice were calculated by Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$. R^2 = Pearson's correlation coefficient.

methylation of CpG_{2965} was not different in the NGT.BMI >30 and IGT.BMI <27 groups, but increased significantly ($p < 0.05$) by 5.3 ± 2.2% in the IGT.BMI >30 group (Fig. 6A). Furthermore, methylation of CpG_{2965} correlated with fasted blood glucose levels ($R^2 = 0.077$, $p < 0.01$) (Fig. 6B), but neither with BMI ($R^2 = 0.00023$, $p = 0.888$) nor age ($R^2 = 0.002$, $p = 0.667$).

Discussion

In this study, we investigated early inter-individual differences in the epigenetic regulation of hepatic *Igfbp2* that determine DIO susceptibility in inbred C57BL/6J mice. Already at a very young age hepatic *Igfbp2* expression was decreased and methylation at CpG_{2605} was increased in DIO responders. This epigenetic repression of hepatic *Igfbp2* was stable over time and, most importantly, preceded the development of fatty liver. Selective *in vitro* methylation of CpG_{2605} decreased luciferase reporter activity, indicating a direct influence of methylation at this site on *Igfbp2* expression levels. Transcriptional regulation of *Igfbp2* largely followed body weight dynamics, as caloric restriction and HFD feeding conversely affected *Igfbp2* expression and methylation. Furthermore, methylation of *Igfbp2* was

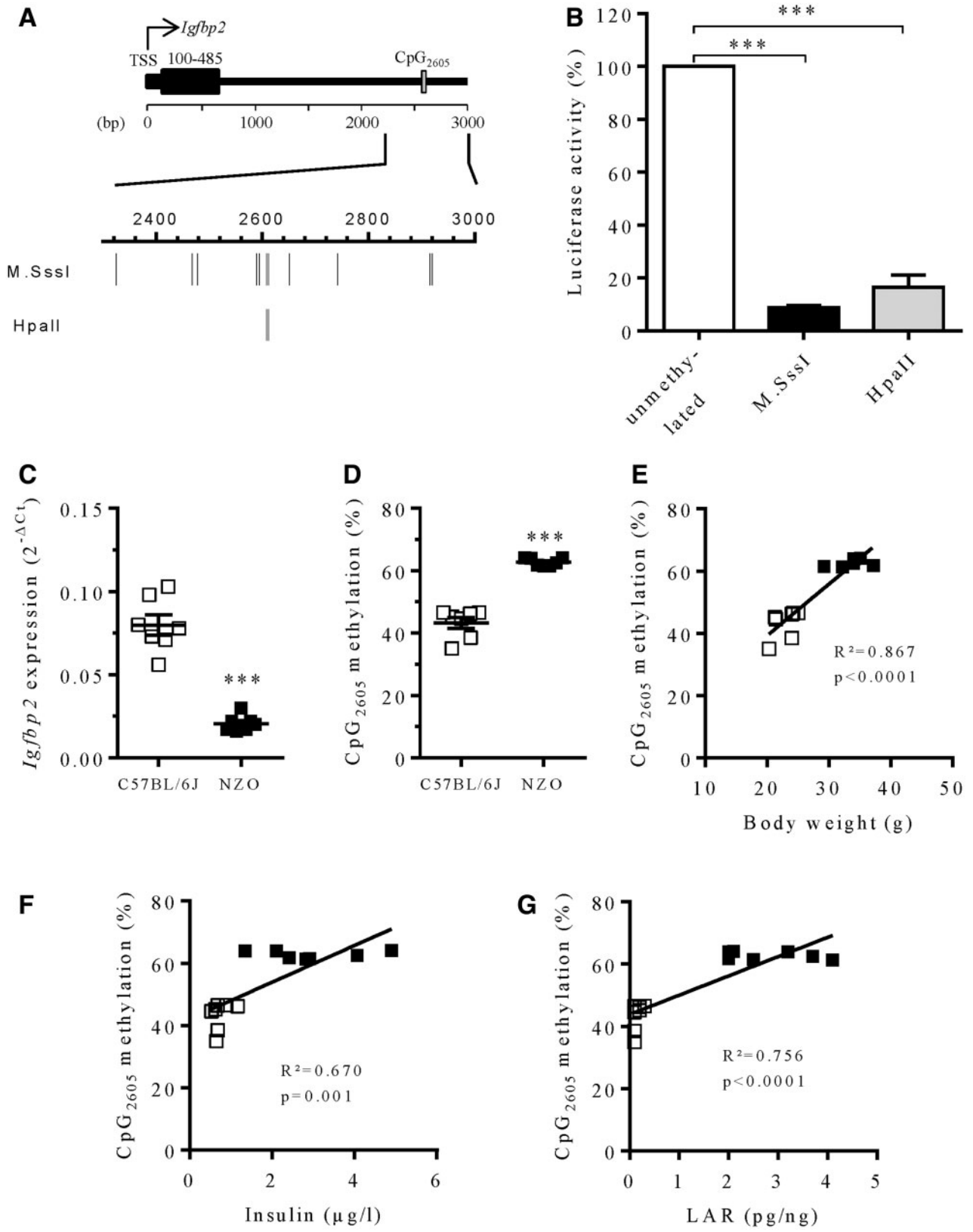

Figure 4. pCpGL–*Igfbp2* luciferase reporter assays. (**A**) Intragenic region of murine *Igfbp2* gene spanning from 2320 to 2997 bp relative to TSS. Recognition sites for methyltransferases HpaII and M.SssI, CpG_{2605} depicted in grey. (**B**) Luciferase activity upon selective methylation of pCpGL-*Igfbp2*. *Igfbp2* expression (**C**) and CpG_{2605} methylation (**D**) in NZO mice. Correlation of CpG_{2605} methylation and body weight (**E**), insulin concentrations (**F**) and LAR (**G**) of 6 weeks old HFD-fed C57BL/6J and NZO mice. All data represented as mean ± SEM of $n = 3$–4 independent experiments and $n = 7$ animals per group, respectively. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with untreated control group.

Table 2. Physiological parameters of male C57BL/6J and NZO mice

	C57BL/6J ($n = 7$)		NZO ($n = 7$)		p-value
	Amount	SEM	Amount	SEM	
Body weight (g)	22.9	0.69	33.7	0.93	<0.001
Lean mass (g)	20.5	0.57	27.2	0.46	<0.001
Fat mass (g)	1.2	0.14	6.3	0.24	<0.001
Fasted BG (mM)	6.3	0.21	7.5	0.78	0.175
Fasted insulin (μg/L)	0.7	0.08	2.9	0.45	<0.001
HOMA-IR (AU)	5.2	0.62	24.5	4.46	0.001
Leptin (ng/ml)	1.1	0.23	16.67	1.24	<0.001
Adiponectin (ng/ml)	6131	430	6227	458	0.881
LAR (pg/ng)	0.157	0.028	2.800	0.328	<0.001

associated with blood glucose, a finding that was confirmed in human whole blood cells of obese men. In young individuals, enhanced *Igfbp2* methylation reflects early deteriorations of glucose metabolism, which in turn represents an enhanced risk for fatty liver and insulin resistance later in life.

Serum levels of insulin-like growth factor 1 (IGF-1) are tightly regulated to ensure proper body weight development and growth in the neonatal period (26,27). IGF-1 binding proteins (IGFBPs) control the bioavailability of circulating IGF-1 (28). For example, the repression of *IGFBP1* and *IGFBP2* during chronic hyperinsulinemia increases the bioavailability of IGF-1 (free IGF-1) and supports body weight gain (14,29). Our present finding of reduced hepatic *Igfbp2* expression in mice with elevated plasma insulin levels is in line with these published observations. Recently, we presented data of mouse lines selected for either high or low body weight gain for 58 generations (20). We showed that the sub-population of high body weight gainers exhibited a reduced hepatic *Igfbp2* expression (20), similar to the data presented here. Importantly, the differential *Igfbp2* expression and methylation reported in this study occurred in a single generation of mice, and differences were observed even between littermates.

We could show that repression of *Igfbp2* in livers of obesity-prone mice is linked with increased methylation of CpG_{2605} in the first intron of the gene, already at a very young age. However, whether increased CpG_{2605} methylation is a result of elevated plasma insulin and glucose levels or independently established during development could not be resolved by our experiments. Nevertheless, our data indicate that epigenetic alterations of *Igfbp2* occur early in life and precede the development of fatty liver.

In line with our observation, low plasma IGFBP-2 levels have also been found in obese children (30,31). Increased IGF-1 bioavailability at least partially explains the accelerated growth observed in children when reaching the pre-pubertal period (30). At this age, decreased IGFBP-2 serum levels were associated with increased body weight, body fat mass, serum insulin and leptin levels (32,33). Again, the phenotypic characteristics of our obesity-prone mice are in line with the observations in young humans. In addition, our study in inbred mice indicates that epigenetic variability early in life might contribute to the observed differences in circulating IGFBP-2 levels. In contrast to IGFBP-2, IGFBP-3 levels were increased in young mice prone to weight gain. In humans, serum levels of IGFBP-3 are decreased in children born small for gestational age, and tend to be increased in obese children (34,35). Furthermore, mice transgenic for *Igfbp3* display increased serum levels of both IGF-1 and IGFBP-3 as well as impaired glucose tolerance and insulin

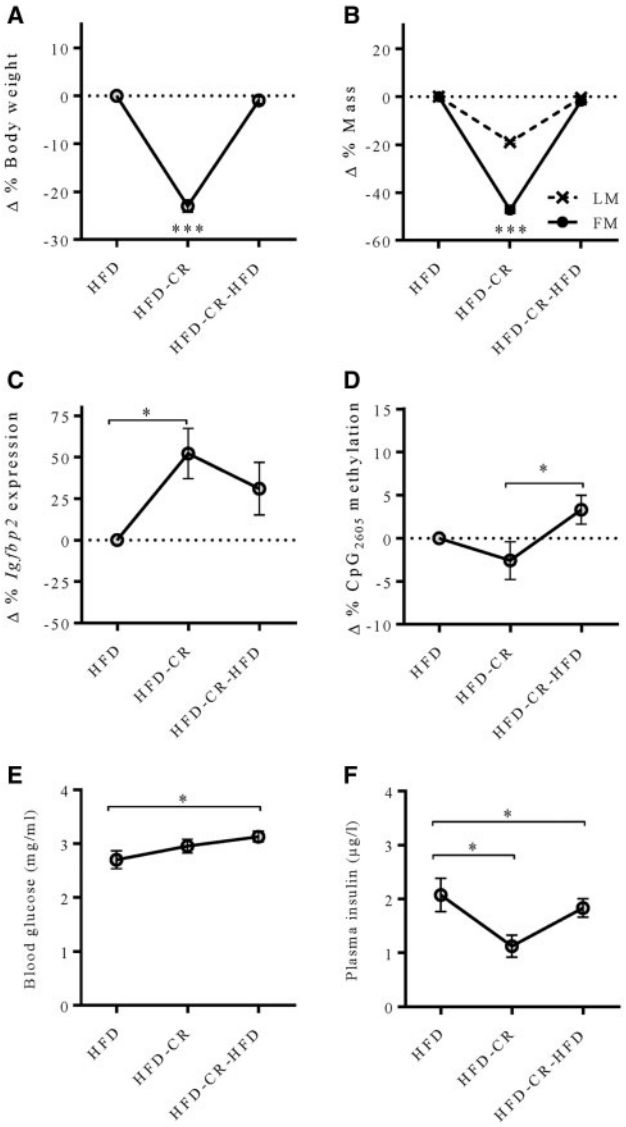

Figure 5. *Igfbp2* expression and methylation during weight loss and regain. (**A**) Body weight development of mice fed an HFD for 20 weeks (HFD) which were then caloric restricted for 3 weeks (HFD–CR) and again fed an HFD for 3 weeks (HFD–CR–HFD). (**B**) Modulation of lean mass (LM) and fat mass (FM) during weight cycling. Hepatic *Igfbp2* expression (**C**) and methylation of CpG_{2605} (**D**) during weight cycling. Blood glucose (**E**) and plasma insulin concentrations (**F**) of the indicated groups. All data represented as mean ± SEM ($n = 10$ animals per group). Differences between HFD, HFD–CR and HFD–CR–HFD mice were calculated by Student's t-test. $^{*}p < 0.05$, $^{***}p < 0.001$. R^2 = Pearson's correlation coefficient.

resistance (36). Therefore, the increased IGFBP-3 levels found in young Resp mice might also contribute to the metabolic phenotype.

There are several possible mediators of *Igfbp2* methylation and expression in obesity-prone individuals. Besides glucose, we found circulating insulin and leptin to be increased in obesity-prone mice compared with obesity-resistant mice. As mentioned above, insulin is a potent inhibitor of *Igfbp2* expression (37,38). Therefore, it can be speculated that low insulin concentration in obesity-resistant mice may cause an increase in *Igfbp2* expression. In contrast to insulin, leptin increases *Igfbp2* expression in a dose-dependent manner (39). In DIO, however, *Igfbp2* expression is rather low despite high levels of circulating

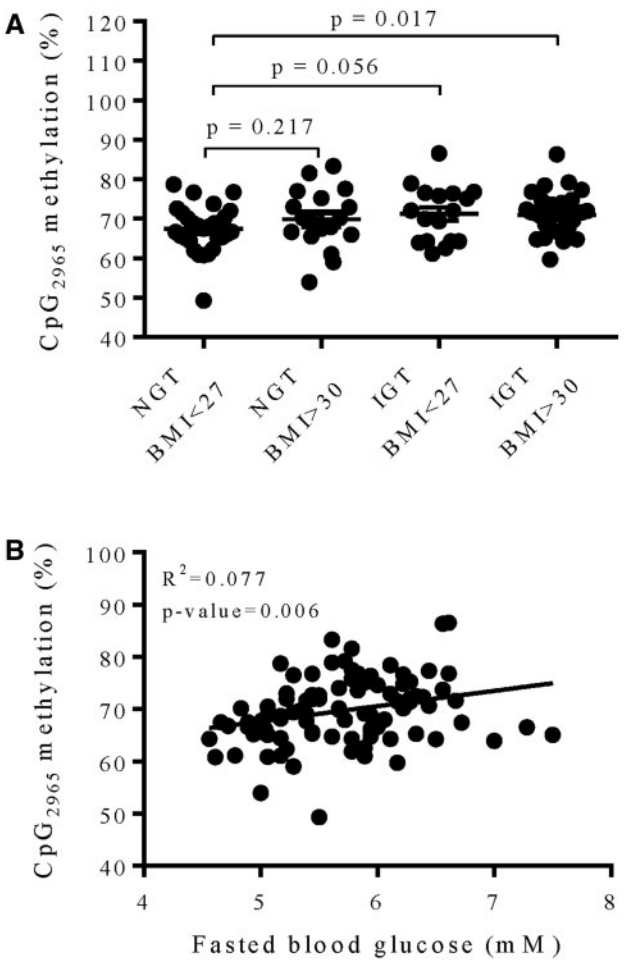

Figure 6. Methylation of CpG_{2965} in human whole blood cells. (**A**) DNA methylation of CpG_{2965} in whole blood cells from human subjects classified into normal glucose tolerant with BMI <27 (NGT.BMI <27) or BMI >30 (NGT.BMI >30) or impaired glucose tolerant with BMI <27 (IGT.BMI <27) or BMI >30 (IGT.BMI >30). Correlation of CpG_{2965} methylation and fasted blood glucose (**B**). All data represented as mean ± SEM. Differences between groups were calculated by Student's *t*-test. $^*p < 0.05$. R^2 = Pearson's correlation coefficient.

leptin (39). This could be a result of the concomitant hyperinsulinemia or of the leptin resistance. The correlation of CpG_{2605} methylation and plasma leptin levels in our young animals might therefore reflect early metabolic disturbances induced by the HFD (39). .As overnutrition is known to suppress *Igfbp2* expression (14) it is possible that the observed alterations in *Igfbp2* mRNA levels and DNA methylation are just an epiphenomenon of elevated dietary intake. However, as food intake that was measured in week 10 on the individual basis was not different between responder and non-responder mice, we believe that alterations in *Igfbp2* expression and methylation are rather a consequence of intrauterine and/or early postnatal programming.

As mentioned earlier, lifestyle interventions that improve insulin sensitivity and liver fat content, e.g. caloric restriction and exercise, have been shown to increase *IGFBP2* expression (24,25). Caloric restriction also resulted in increased expression of hepatic *Igfbp2* in our obese mice. De-methylation of CpG_{2605} under these conditions is apparently slower and did not reach statistical significance in our experiment. However, induction of CpG_{2605} methylation by re-feeding the HFD significantly increased methylation again. Together, these data indicate that long-term caloric intake can alter the methylation of CpG_{2605}. The differential *Igfbp2* expression as well as methylation associated with changes in body weight before the onset of liver fat accumulation might already play an important role during fetal programming. Human studies found an association of high IGFBP-2 cord serum concentrations with inter-uterine growth retardation in newborns (40), indicating an effect of IGFBP-2 on fetal growth.

In conclusion, our data suggest that increased methylation of hepatic *Igfbp2* during infancy indicates an increased risk to develop obesity and ectopic fat accumulation in adolescence. Furthermore, our data suggest that early deteriorations of glucose metabolism parallels epigenetic deregulation of *Igfbp2*.

Materials and Methods

Animals

C57BL/6J breeding pairs (Charles River, Germany) received a standard chow (ssniff) and were housed in temperature-controlled room (22 + 1°C) on a 12:12 h light dark cycle. After weaning male mice were fed an HFD (60 kcal% fat, 21.9 kJ/g, D12492, Research Diets, Inc., USA) *ad libitum* in groups of two to six animals per cage. The mice were housed and handled in accordance with the 'Principles of laboratory animal care' (41). The animal welfare committees of the DIfE as well as the local authorities (LUGV, Brandenburg, Germany) approved all animal experiments. Weekly body weight measurements were performed in the morning (8–10 am) from 3 to 20 weeks of age. Body composition was analyzed by nuclear magnetic resonance (NMR, Minispec LF50, Bruker Biospin). Blood glucose was measured from tail blood with a Glucometer Elite (Bayer, Germany). At 10 weeks of age, food intake was monitored with an automatic drinking and feeding monitor system (TSE Systems, Germany). Mice were housed in individual cages and adapted to food baskets connected to weight sensors for 2 days. During the measurement period of 3 days, the baskets contained HFD pellets to which mice had *ad libitum* access. At the age of 6 and 20 weeks, respectively, mice were killed after 6 h fasting. All tissues were directly frozen in liquid nitrogen and stored at −80°C until further processing.

Plasma analysis

Plasma insulin was measured using the Mouse Ultra-sensitive insulin ELISA kit from ALPCO (USA). Insulin-like growth factor binding protein 2 (IGFBP-2) as well as insulin-like growth factor 1 (IGF-1) plasma levels were quantified using an enzyme-linked immunosorbent assays from R&D Systems (mouse-IGFBP-2, DY797; mouse IGF-1, DY791; R&D Systems, USA). The assays were performed according to the manufacturers' instructions.

Hepatic triglycerides

Liver triglycerides were quantified using a commercial kit (RandoxTR-210, UK). Livers were homogenized in 10 mmol/l sodium dihydrogen phosphate, 1 mmol/l EDTA and 1% (vol./vol.) polyoxyethylene-10-tridecyl ether and incubated for 5 min at 37°C. After centrifugation, the triacylglycerols in the supernatant fraction could be detected.

Quantitative real-time PCR (qPCR)

Total RNA from liver tissue of mice was extracted, and cDNA synthesis as well as TaqMan gene expression assays were performed as described previously (42).

Quantitative western ligand blot analysis of IGFBP

Insulin-like growth factor binding proteins were analyzed in plasma by quantitative western ligand blot analysis, as described previously (43,44). Plasma as well as recombinant human IGFBP standards (R&D Systems, USA) were diluted 1:20 in artificial serum matrix (Biopanda) and boiled in sample buffer (312.5 mM Tris (pH 6.8), 50% (wt/vol) glycerol, 5 mM EDTA (pH 8), 1% (wt/vol) SDS and 0.02% bromophenol blue) for 5 min. Proteins were separated by electrophoreses [12% polyacrylamide gel (Peqlab "Perfect Blue")] and transferred to polyvinylidene fluoride membrane (Millipore Corp., USA), followed by immunoblotting. The blots were incubated with biotin-labeled human IGF-2 (1:500; BioIGF-2-10; Ibt GmbH, Germany). IGFBP were detected by enhanced chemiluminescence using the reagent Luminata Forte (Millipore Corp., USA) and bands were visualized on KODAK Image Station 4000 MM (Molecular Imaging Systems; Carstream Health Inc., USA) and afterwards semi-quantified by using ImageQuant 5.2 software (Molecular Dynamics, USA). For each IGFBP, curve fitting was done by a 7-parametric nonlinear regression (Hill equation).

Isolation of genomic DNA and bisulfite treatment

Genomic DNA (gDNA) from liver tissue was isolated using the Invisorb Genomic DNA Kit II (Stratec, Germany) according to manufacturer's instructions. Bisulfite conversion of 500 ng gDNA was performed using the EpiTect Fast Bisulfite Kit (QIAGEN, Germany). Bisulfite-converted DNA (bsDNA) was purified as recommended and eluted in a total volume of 15 µl.

dBSP and pyrosequencing

Bisulfite-specific primers were designed using MethPrimer (45). PCRs (2.5 µl bsDNA) were performed using TaKaRa EpiTaq™ HS (Takara Bio Inc., Japan) and amplicons purified with the QIAquick PCR Purification Kit (QIAGEN, Germany). Sanger sequencing of the samples was carried out by GATC Biotech (Germany). Quantification of dBSP was done as described previously (46). In order to avoid over-representation of cytosine peaks after normalization, the raw sequencing data were used for quantification (CodonCode Aligner Software, version 5.1.5, CodonCode Corporation, USA). Primers for pyrosequencing were designed using the PyroMark Assay Design software (QIAGEN, Germany). Pyrosequencing was performed on a PyroMark Q96 Pyrosequencer according to the manufacturer's instructions and quantified using PyroMark Q96 software 2.5.7 (QIAGEN, Germany).

Construction of CpG-free reporter gene plasmid

A fragment of the murine *Igfbp2* gene, 2320–2997 bp downstream of the TSS, was PCR amplified using primers generating restriction sites for *Bam*HI (forward primer 5′-GATC GGATCC GAGC CTG GTT TTG CCA GGT TG) and *Nco*I (reversed primer 5′-GATC CCATGG GCC TGG CCT GAA TTC TGA AAG G) with JumpStart™ REDAccuTaq® LA DNA Polymerase (Sigma-Aldrich, USA). The purified PCR amplificates were cloned into the promoter-less CpG-free pCpGL-Basic vector (kindly provided by Prof. Rehli, Regensburg, Germany) upstream of the luciferase reporter gene. For plasmid amplification, the construct (pCpGL-*Igfbp2*) was transformed into One Shot® PIR1 Competent *Escherichia coli* (Invitrogen, USA).

In vitro methylation of pCpGL-*Igfbp2* was performed using either 4 U/µg DNA M.SssI or 2 U/µg DNA HpaII (New England Biolabs, USA) in the presence of 160 µM S-adenosylmethionine and incubated at 37°C for 1.5 h. After plasmid purification using QIAGEN QIAquick PCR Purification Kit (QIAGEN, Germany) concentration was measured with the NanoDrop Spectrophometer (ND-1000, PeqLab, Germany).

AML12 cell culture and luciferase activity assay

The murine AML12 cell line (ATCC® CRL-2254™) was cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (PAN-Biotech, Germany) with 0.005 mg/ml insulin (Roche Life Science, USA), 0.005 mg/ml transferrin (Sigma-Aldrich, USA), 5 ng/ml selenium (Sigma-Aldrich, USA) and 40 ng/ml dexamethasone (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (Biochrom, Germany). Cells were maintained in humidified air replenished with 5% CO_2. In a 48-well plate, 4×10^4 AML12 cells/well were co-transfected using Lipofectamine®2000 (Invitrogen, USA) with 500 ng/well unmethylated or methylated pCpGL-*Igfbp2* and 5 ng/well pRL-TK *Renilla* control vector (Promega, USA). After 48 h, cells were harvested and analyzed for firefly and renilla luciferase activity using the dual-luciferase reporter assay system (Promega, USA). Firefly luciferase activity was normalized to its renilla luciferase transfection control.

Human participants and whole blood collection

The study population consisted of 109 German individuals recruited from the ongoing Tübingen Family Study. The study currently comprises >2500 non-related individuals at increased risk for T2D, i.e. non-diabetic subjects with family history of type-2 diabetes, BMI ≥27 kg/m^2, impaired fasting glycaemia, and/or previous gestational diabetes and healthy controls. All

Table 3. Characteristics of normal and impaired glucose tolerant men

	NGT.BMI <27 (n = 29)		NGT.BMI >30 (n = 18)		IGT.BMI <27 (n = 17)		IGT.BMI >30 (n = 31)	
	Amount	SEM	Amount	SEM	Amount	SEM	Amount	SEM
Patient age (years)	57.8	1.73	60.2	1.27	61.2	1.37	57.2	1.59
BMI	24.5	0.33	33.5	0.73	25.43	0.21	34.3	0.65
Fasted BG (mM)	5.4	0.08	5.6	0.11	5.7	0.14	6.0	0.11
oGTT (mM)	5.9	0.14	6.0	0.27	9.3	0.20	9.2	0.19

participants underwent assessment of medical history, smoking status and alcohol consumption habits, physical examination, routine blood tests and oral glucose tolerance tests (oGTTs). The study population consisted of individuals with complete oGTT and documented absence of medication known to influence glucose tolerance, insulin sensitivity or insulin secretion. The study adhered to the Declaration of Helsinki and each participant signed a written informed consent. The Ethical Committee of the Medical Faculty of the University of Tübingen approved the study protocol. Subjects are grouped into normal glucose tolerant with BMI <27 (NGT.BMI <27, $n = 29$) or BMI >30 (NGT.BMI >30, $n = 18$) and impaired glucose tolerant with BMI <27 (IGT.BMI <27, $n = 17$) or BMI >30 (IGT.BMI >30, $n = 31$). Participant characteristics are shown in Table 3. A standardized 75 g oGTT was performed following a 10-h overnight fast. For the determination of plasma glucose venous blood samples were drawn at baseline and at time-points 30, 60, 90 and 120 min of the oGTT. For DNA methylation analysis, DNA was isolated from whole blood using a commercial kit (NucleoSpin, Macherey & Nagel, Düren, Germany).

Statistical analysis

Statistical analysis was performed by Student's *t*-test, one-way ANOVA or linear regression analysis using the software Prism6 from Graph Pad Software (La Jolla, USA). Significance levels were set for *p*-values <0.05 (*), 0.01 (**) and 0.001 (***).

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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