CHAPTER 7
Generation and initial metabolic characterization of mice deficient in the C/EBPα upstream open reading frame (uORF)

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Abstract

Cis-regulatory elements in mRNA such as upstream open reading frames (uORFs) mediate gene-specific translational control of downstream coding sequences. The C/EBPα (CCAAT/enhancer binding protein α) transcription factor is critically involved in the regulation of proliferation, differentiation and organismal metabolism in vivo and is often mutated or otherwise deregulated in a number of cancers. The Cebpa-mRNA contains a uORF that is required for translation into a truncated C/EBPα-p30 isoform. C/EBPα-p30 lacks part of the transactivation domain and can therefore inhibit the transactivation function of the full-length C/EBPα isoform (C/EBPα-p42). However, the in vivo function of the C/EBPα-uORF and the role of the separate isoforms have not been extensively studied. Therefore, we generated the CebpaΔuORF mouse model through CRISPR/Cas9 targeting and homology directed repair which for the first time shows the in vivo requirement of the C/EBPα-uORF for p30 translation. CebpaΔuORF mice are viable, born to Mendelian ratios, display strongly reduced C/EBPα-p30/p42 isoform ratios and show gender-specific alterations in glucose metabolism. Using this model, we aim to study the effects of interfering with C/EBPα uORF-mediated translational control on organismal metabolism, health- and lifespan, hematopoiesis and potentially cancer development.
Generation and initial metabolic characterization of C/EBPα-ΔuORF mice

Introduction
Protein expression in mammalian cells is regulated by a variety of mechanisms ranging from transcription to translation and eventually protein degradation and all steps in between, both on a global and gene-specific level. During mRNA translation, regulation mainly takes place at the rate-limiting initiation stage (1). While the molecular architecture of protein synthesis allows for global inhibition in case of for example stress or nutrient deprivation, regulatory/structural elements in individual mRNAs control translation at the gene-specific level. One such element is the upstream open reading frame (uORF), a cis-regulatory element that codes for a small peptide and is found in the 5′ leader of mRNA. At least one uORF is found in 44% of mouse and 49% of human transcripts (2). In general, uORFs function to reduce translation into the downstream encoded protein however stimulating uORFs have also been described (3). The initial steps of mRNA translation are 5′-cap binding of eIF4E and recruitment of scaffold protein eIF4G and helicase eIF4A, forming the cap-bound eIF4F complex. eIF4F in turn recruits the 43S pre-initiation complex consisting of eIF1, eIF1A, eIF3, eIF5 and the small 40S ribosomal subunit bound to a ternary complex of eIF2, a methionine loaded initiator tRNA and GTP. This 48S complex scans the mRNA in a 5′ to 3′ direction until an initiation codon (AUG, but rarely also CUG or GUG) in a suitable sequence context (Kozak sequence) is encountered. Both the start codon and the Kozak sequence are paramount for start codon recognition by the 48S complex which is followed by GTP hydrolysis and 60S subunit joining leading to further decoding of the mRNA (1). The directionality of mammalian mRNA scanning (5′ → 3′) is the reason that most uORFs act as negative regulators of downstream protein synthesis; ribosomes that initiated at the uORF will terminate at its stop codon and may be recycled. On a global level, cells can regulate translation by for example integrating various nutrient sensing pathways in mTORC1 controlled 4E-binding protein (4E-BP) and S6 kinase 1 (S6K1) phosphorylation. Unphosphorylated 4E-BPs bind eukaryotic initiation factor 4E (eIF4E), preventing it from engaging with the mRNA 5′-cap and thereby inhibiting translation globally (1). Phosphorylation of eIF2α in response to oxidative stress, ER stress, ssRNA (in viral infection) or amino acid deprivation is another well-known mechanism of global protein synthesis inhibition. eIF2 needs to be recycled after each round of initiation as it has lost the GTP and initiator tRNA that together make up the ternary complex described above. Stress-induced phosphorylation of eIF2 subunit α (eIF2α) however prevents GTP loading of eIF2 and thereby depletes the amount of ternary complex available for translation initiation, resulting in the inhibition of translation of most mRNAs in the cell (1). For some genes such as ATF4 however, phosphorylation of eIF2α is the very mechanism that allows translation of its main ORF. Originally this type of mechanism was discovered for the yeast homolog GCN4, a transcription factor and master regulator of amino acid synthesis, and involves 4 uORFs upstream of the main coding sequence (4). As the first translated uORF is short, incomplete ribosome recycling can lead to re-scanning downstream of the first uORF. Under nutrient
rich conditions, there is plenty of new ternary complex (eIF2-GTP with methionine initiator tRNA) available to reload the scanning small ribosomal subunit before it reaches uORFs 2-4 to initiate translation there. Under conditions of nutrient starvation eIF2α is phosphorylated and the ternary complex required for translation initiation is less abundant, preventing the small ribosomal subunit from being reloaded prior to uORFs 2-4 are encountered. As scanning is continued, reloading may occur before encountering the AUG of the main ORF, resulting in GCN4 protein synthesis (5).

In addition to main ORF regulation, uORFs may also regulate synthesis of different protein isoforms from a single mRNA as is the case for the CCAAT/enhancer binding protein (C/EBP) α and β transcription factors. For C/EBPα and C/EBPβ respectively these isoforms are either extended (C/EBPα-extended and LAP*), full-length (p42 and LAP) or truncated (p30 and LIP) and arise through the use of different in frame initiation sites on the mRNA. Although slight differences between them exist, the general mRNA architecture is identical for both genes (reviewed in Chapter II & see Figure 1A) and it enables translation initiation at different sites on the same mRNA due to varying Kozak sequence context allowing certain start codons to be skipped (leaky scanning) in combination with uORF-mediated translation re-initiation (6). During scanning from the 5’-end on the C/EBPα mRNA a CUG codon is encountered first, but often skipped as CUG codons are inherently weaker initiation codons than AUG codons. The AUG initiation codon of a uORF in semi-favorable context is encountered next which, if used for translation initiation, results in synthesis of the small uORF-encoded peptide and, after translation termination, enables subsequent re-scanning and re-initiation at the downstream p30 start codon. As p30 is in frame with the other isoforms, an identical but truncated protein isoform is synthesized. Ribosomes that do not initiate at the uORF initiation site encounter two AUG codons closely together which allow synthesis of the full length p42 isoform, where the first AUG has significantly better Kozak sequence context and is therefore recognized more efficiently (Figure 1A). Translation of the shorter isoform is controlled by eIF4E and eIF2 and depends on the presence of the conserved uORF (7). When the C/EBPα uORF is mutated by removing the start codon, p30 isoform expression is abolished due to a lack of translation re-initiation (Figure 1B) (7). This truncated isoform is 117 amino acids shorter compared to p42 and thereby lacks two transactivation domains (TAD1 & TAD2) required for interaction with elements of the RNA polymerase II basal transcription machinery (8). On the C-terminus however, the DNA binding domain and leucine zipper domain are identical in all isoforms and ectopic p30 expression reduces p42 transcriptional activity (9), making the ratio between p30/p42 crucial for expression of C/EBPα targets.

C/EBPα is highly expressed in liver, white and brown adipose tissue (WAT and BAT respectively) and components of the hematopoietic system and profoundly affects normal homeostasis of these tissues. Homozygous full body knock-out of C/EBPα leads to postnatal lethality due to a lack of hepatic glycogen build up before birth which, together with impaired gluconeogenesis, leads to severe
Figure 1. Overview of murine Cebpa-mRNA translation into multiple protein isoforms

A) Schematic overview of M. Musculus Cebpa-mRNA structure and translation initiation sites. Optimal Kozak sequence context around an AUG start codon is depicted on the left. When ribosomes initiate at in frame sites A or B1 the extended and p42 isoforms are translated respectively. B2 has very poor sequence context but when used produces a slightly shorter version of p42. Upon initiation at site D ribosomes translate the uORF sequence and terminate, allowing re-initiation at the downstream initiation site C, resulting in p30 translation. As site C is downstream of and in frame with A/B1/B2, the resulting amino acid sequence is identical to the longer isoforms and contains a single transactivation domain (TAD) and DNA binding/dimerization domain, but misses part of the N-terminus containing 2 additional TADs.

B) Mutation of the uORF start codon (AUG -> UUG) abolishes translation of the uORF sequence and thereby prevents re-initiation events at site C, preventing translation of the p30 isoform.

hypoglycemia (10). The important role of C/EBPα in adipocyte differentiation together with the transcription factor PPARγ has been well described (reviewed in: (11, 12)). In mutant animals WAT does not store lipid droplets and BAT lacks characteristic uncoupling protein expression, essential for body temperature maintenance (10). Furthermore, animals display reduced expression of gluconeogenesis genes and ornithine (urea) cycle genes, leading to hyperammonemia (13). Liver-specific depletion of C/EBPα after birth in an adenoviral model results in hyperbilirubinemia and jaundice due to reduced bilirubin breakdown (14). In a genetic albumin-Cre driven liver-specific depletion model livers are enlarged, mice display hyperammonemia and develop steatosis and increased blood glucose levels over time (15). Thus, C/EBPα is a key factor in proper hepatocyte and adipocyte function and differentiation, thereby regulating organisinal energy homeostasis. However, although the importance of the C/EBPα-p30/p42 isoform ratio on target gene transcription is well established, studies on the effects of the different isoforms in relation to metabolism have not been performed so far.
C/EBPα is extensively involved in differentiation of myeloid cells and mutated in around 9% of de novo acute myeloid leukemia (AML) cases (16). Mutations are typically found in the C-terminal DNA binding and dimerization domain or cause a frameshift at the N-terminus of the p42 isoform; both of which cause reduction of C/EBPα-p42 driven transcription (albeit through distinct mechanisms) and inhibit differentiation and thereby promote AML (17). In agreement with a tumor-suppressive role of C/EBPα-p42, a mouse model in which the p42 isoform was deleted with maintained p30 expression resulted in AML development with complete penetrance (18). In addition to AML, C/EBPα has been reported to act as a tumor suppressor in amongst others liver, breast, lung and skin cancer (reviewed in (19)).

Here, we describe the successful generation of a mouse model with reduced C/EBPα-p30 expression through mutation of the uORF start codon: CebpΔuORF mice. The mutant mice are viable and born to Mendelian ratios, show no difference in body weight in both genders up to 5 months after birth and display a strongly decreased C/EBPα-p30/p42 ratio in liver. Initial metabolic characterization reveals alterations in glucose metabolism with differences between genders. We plan to use the CebpΔuORF model to study the role of uORF-mediated translational control and isoform-specific functions in the regulation of organismal metabolism, hematopoiesis, health- and lifespan and potentially cancer development.
Results

CRISPR/Cas9-mediated mutation of the Cebpa-uORF depletes C/EBPα-p30 in vivo

To establish the physiological relevance of uORF-directed C/EBPα translational control and the function of the p30 isoform in vivo, we generated a mouse model with a mutation of the uORF (for mRNA structure, see Figure 1) to abrogate p30 expression. The C/EBPα-uORF start codon (ATG) was mutated using CRISPR/Cas9 and a pronuclear injection strategy in C57BL/6N mice. Mosaic mutants were generated by embryo transplantation into pseudo pregnant mice following pronuclear injection of Cas9 mRNA, guideRNA targeting Cebpa and a single-stranded oligodeoxynucleotide (ssODN) repair template (Figure 2A, B, see Methods). To minimize secondary effects in C/EBPα function, in particular regarding the extended C/EBPα isoform as its coding sequence spans the uORF, only the uORF methionine was changed at the amino acid level and the additional genomic changes were silent mutations (Figure 2B). Successful genomic mutation was confirmed by genotyping PCR using specific primers recognizing the co-introduced BamHI site just 5’ of the mutated C/EBPα-uORF start codon to differentiate between heterozygous (Cebpa<sup>wt/uORF</sup>) and homozygous (Cebpa<sup>ΔuORF/ΔuORF</sup>) animals (Figure 2C). Offspring from heterozygous Cebpa<sup>wt/ΔuORF</sup> mating were born to Mendelian ratios (Supplementary Table 1) and Cebpa<sup>ΔuORF/ΔuORF</sup> mice displayed no obvious developmental defects, no increased postnatal death and no obvious changes in behavior or fur quality. Immunoblot analysis of liver tissue from Cebpa<sup>ΔuORF/ΔuORF</sup> mice revealed strongly decreased C/EBPα-p30 expression concomitant with a mild increase in C/EBPα-p42 leading to decreased C/EBPα-p30/p42 ratios (Figure 2D, E). Reduced p30 expression was anticipated and consistent with the C/EBPα-mRNA structure ((7) & Figure 1). The increased expression of p42 is likely a result from increased translation initiation at the p42 start codon, as ribosomes that would otherwise initiate at the uORF sequence now initiate at the downstream p42 start codon. Since C/EBP family members are known to heterodimerize with each other (20), we confirmed there was no compensation in the translation of C/EBPβ-LAP and -LIP isoforms in Cebpa<sup>ΔuORF/ΔuORF</sup> (from here on referred to as ‘C/EBPα-ΔuORF’) animals (Supplementary Figure 1).

C/EBPα-ΔuORF mice display altered glucose metabolism

Having established the mouse model expresses reduced C/EBPα-p30/p42 protein isoform ratios as intended, we examined body weight and glucose metabolism in male and female mice. C/EBPα-ΔuORF mice of both genders displayed normal weight gain up to 25 weeks after birth and no weight differences between wildtype and mutant mice were observed during this period (Figure 3A, B). We next performed intraperitoneal glucose tolerance tests (IPGTT) where starved mice are given a bolus of glucose and their blood glucose levels are monitored over time. In females we observed a significantly lower blood glucose level in C/EBPα-ΔuORF animals following starvation, but not under
Figure 2. Successful generation of C/EBPα-ΔuORF mice by CRISPR/Cas9 using an ssODN repair template

A) Pronuclear injection strategy of Cas9-mRNA, gRNA and a single-stranded oligodeoxynucleotide (ssODN) repair template to obtain mice with a mutated Cebpα upstream open reading frame start codon.

B) Partial Cebpα genomic sequence, gRNA sequence and gene targeting strategy using an ssODN template to introduce a point mutation in the Cebpα uORF start codon (red) and a BamHI site for genotyping (blue). Blue arrowhead indicates Cas9 cleavage site 3nt upstream of protospacer-adjacent motif (PAM) site.

C) Double PCR genotyping strategy to distinguish between wt (-/-), heterozygous (+/-) and homozygous (+/+ CebpαΔuORF) mutation. Successful mutation leads to loss (Rv wt) or gain (Rv ΔuORF) of genotyping primer binding to allow amplification, therefore amplification of the smaller product in both reactions must come from heterozygous mice. M: 100bp ladder.

D) Representative immunoblots of liver lysates from Cebpαwildtype and CebpαΔuORF mice. β-Actin was used as loading control.

E) Quantification of C/EBPα protein isoform ratio based on blots in D) and an additional blot per gender (data not shown), mean ± S.D., n = 8 per genotype per gender. ***: p < 0.001, Student’s T-test.
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ad libitum access to food (Supplementary Figure 2A). There was no difference in glucose clearance after injection (Figure 3C), indicating glucose disposal is unaffected in the mutant female mice. Also in i.p. Insulin Tolerance Test (IPITT) the mutant female mice displayed a similar drop and recovery in blood glucose compared to wildtype (Figure 3D). These results indicate the CebpaΔuORF mutation does not affect glucose clearance or insulin sensitivity in young female mice. Finally, we asked whether gluconeogenesis is affected. Mice were injected with pyruvate which under starvation conditions mainly gets processed to glucose in the liver via the gluconeogenesis pathway (21). Upon pyruvate injection, blood glucose levels reached a lower maximal value in C/EBPα-ΔuORF female mice compared to wildtype with similar kinetics (Figure 3E), indicating gluconeogenesis is negatively affected. The experiments were repeated in male mice with different outcomes. C/EBPα-ΔuORF male mice performed better in glucose tolerance test, as evidenced by a lower maximal blood glucose value and area under the curve (Figure 3F), while there were no basal differences in blood glucose levels in fed or fasted state (Supplementary Figure 2B). The increased glucose clearance is not caused by increased insulin sensitivity, as mutant male mice did not perform better than wild type in IPITT (Figure 3G). Finally, in contrast to the females, gluconeogenesis was not affected in the mutant males as measured by IPPTT (Figure 3H). In short, whole body glucose metabolism is significantly affected by the C/EBPα-ΔuORF mutation with differences between genders in glucose tolerance and gluconeogenesis.
Generation and initial metabolic characterization of C/EBPα-ΔuORF mice

Discussion
In this study we report the successful establishment of a mouse model with a loss-of-function mutation in a cis-regulatory element (uORF) in the Cebpa-mRNA leading to C/EBPα-p30 deficiency. To our knowledge, this is the first report showing that the C/EBPα-uORF regulates C/EBPα-p30 (truncated) isoform expression in vivo as had already been established in vitro (7). Homozygous Cebpa^ΔuORF/ΔuORF mice are viable, born to Mendelian ratios from heterozygous parents and show no differences in body weight compared to wild type up to 5 months after birth. We observed a clear improvement in glucose tolerance in males, no difference in insulin sensitivity in either gender and a decrease in gluconeogenesis in females while basal blood glucose levels after starvation in females were also lower.

The role of C/EBPα and C/EBPβ in regulating metabolism, differentiation, tissue homeostasis and ageing is well established (20, 22-24). For both factors, multiple protein isoforms are translated through a uORF cis-regulatory element in the mRNA and translational control of isoform production is paramount for organismal homeostasis. The truncated isoforms (p30 for C/EBPα and LIP for C/EBPβ) inhibit transcriptional activity of the full length isoforms (p42 for C/EBPα and LAP for C/EBPβ) (9, 25). To prove the function of the C/EBPβ uORF in vivo and to study isoform specific effects of C/EBPβ on metabolism and ageing, we previously generated C/EBPβ^ΔuORF mice with reduced C/EBPβ-LIP expression (26). These mice display several beneficial phenotypes similar to caloric restriction including lower fat accumulation, increased fatty acid oxidation and improved glucose clearance and insulin sensitivity (22). During ageing C/EBPβ-LIP levels increase (27), but when this is prevented in the mutant mice health span is improved as measured by for example body weight/fat, grip strength, motor coordination and glucose clearance (23). Furthermore, female C/EBPβ^ΔuORF mice have an increased median lifespan with reduced tumor incidence, highlighting the importance of proper C/EBP isoform regulation in vivo (23).

C/EBPα plays a major role in organismal metabolism by for example regulating proper adipocyte differentiation in cooperation with PPARy (11, 12). Whole body C/EBPα deficiency precludes postnatal survival and various models of C/EBPα deficiency in metabolic tissues display metabolic phenotypes such as hyperammonemia, hyperbilirubinemia, steatosis, reduced glycogen synthesis and impaired gluconeogenesis (10, 13-15). Here, we examined the role of C/EBPα-p30 specifically in organismal glucose metabolism. Male, but not female, Cebpa^ΔuORF mice displayed better glucose tolerance than their wild type counterparts. Postnatal liver-specific ablation of total C/EBPα is known to reduce glucose tolerance and glucokinase expression (15). It is therefore possible that depletion of p30 promotes transcription of (some) p42 targets including glucokinase and thereby facilitates increased glucose tolerance in males. An increase in insulin sensitivity might also explain improved glucose tolerance, although this seems unlikely as we did not find any difference between wild type
and C/EBPα-ΔuORF animals in i.p. insulin tolerance tests. In previous studies, C/EBPα was shown to be essential for insulin stimulated glucose uptake in in vitro differentiated adipocytes (28), arguing wild type C/EBPα activity is sufficient to induce maximal insulin stimulated glucose uptake. Basal blood glucose levels were unaffected by the mutation, which was also observed upon acute C/EBPα depletion in metabolic tissues (29). In female mice we did however notice a decrease in fasted blood glucose levels, which could have multiple causes. Fasted insulin levels might be elevated, basal tissue glucose clearance may be increased and/or gluconeogenesis might be inhibited. Glucose tolerance was unaffected and mutant mice displayed a lower increase in blood glucose levels in i.p. pyruvate tolerance tests, indicating gluconeogenesis is reduced. This latter finding was unexpected since C/EBPα is a known transactivator of gluconeogenesis genes phosphoenolpyruvate carboxykinase (Pepck) and glucose-6-phosphatase (G6pc) in neonatal mouse liver tissue (10). One might therefore expect increased expression of these genes in the absence of the inhibitory C/EBPα-p30 isoform. In addition to transcript levels of gluconeogenesis genes, it will be interesting to determine fasted insulin levels as insulin is well known to suppress gluconeogenesis (21). Further, since initial glucose clearance is mainly carried out by lean mass, determination of body composition will be paramount for proper interpretation of the results (30). We do not know why there are metabolic differences between the two genders in mutant mice. C/EBPα and other C/EBP transcription factors are known to interact with hormone receptors including estrogen, progesterone and glucocorticoid receptors (31-35). As the isoform ratios in wild type and mutant mice do not differ between genders (Figure 2E) and since males and females have obvious differences in hormone receptor regulation, availability of specific interaction partners may govern metabolic response to the shift in C/EBPα isoform ratio. Indeed, also in young C/EBPαΔuORF mice sex affected the differences between wild type and mutant mice for many phenotypes including body fat content and glucose tolerance (23). Future experiments on (metabolic) gene expression patterns in liver, body mass composition, fatty acid oxidation, circulating hormone levels in fed and fasted state and caloric intake will provide valuable insight into the C/EBPα isoform and gender specific regulation of organismal metabolism.

Finally, we plan to use the created mouse model to study the effects of C/EBPα-p30 expression on health- and lifespan and cancer development. In other mouse models constitutive C/EBPα-p30 expression results in AML development with complete penetrance (18), while enhanced C/EBPβ-LIP expression reduces life span and promotes lymphoma development (36). Mutation of C/EBPα is common in AML and prevents proper hematopoietic differentiation, in particular granulopoiesis (37). Mice with Cebpα knock-out fail to produce many cell types of the myeloid lineage (38), which is caused by a block in differentiation from common myeloid progenitors (CMPs) to granulocyte/monocyte progenitors (GMPs) (39). Bone marrow from these mice is filled with myeloblasts and their hematopoietic stem cells (HSCs) display increased transplantation potential (39), reminiscent of the
human AML situation and arguing a role for C/EBPα in both HSC differentiation and repopulation potential. For these reasons, it will be interesting to determine if C/EBPα-ΔuORF mice display altered HSC differentiation into myeloid lineages in particular. Function of C/EBPα as a tumor suppressor is however not limited to the hematopoietic system (40), and recently C/EBPα was shown to act as a gatekeeper for epithelial to mesenchymal transition (EMT) in breast cancer (40). As ageing is the main risk factor for tumor development, histology and necropsy from lifespan studies may indicate if p30 depletion protects from naturally occurring malignancies.

Taken together, our results suggest genetic ablation of C/EBPα-p30 expression by uORF mutation is compatible with life and affects glucose metabolism differently in males and females. In future studies we aim to mechanistically dissect these metabolic differences and establish whether mouse health- and lifespan are affected by interference with C/EBPα uORF-mediated translational control.
Chapter VII

MATERIALS & METHODS

Mouse model
Clustered regularly interspaced short palindromic repeats (CRISPR) / CRISPR associated 9 (Cas9) was used to generate knock-in mice (41) carrying a CebpaΔuORF mutation. A line carrying the GGCTGCCATGCCGG → GGAATCCCTTTGCCGG mutation at the C/EBPα uORF start codon was bred for subsequent experiments. Briefly, to generate super ovulated females, C57BL/6NHsd mice were i.p. injected with 5 IU Folligonan (0.2 ml) and 48h later with 5 IU Chorulon (0.2 ml). Zygotes were isolated the next day from the infundibulum and subjected to pronuclear injection with 100 ng/µl Cas9 mRNA, 50 ng/µl in vitro transcribed sgRNA (crRNA sequences 5’-GGCCGCGAGGCTCGCCATGC-3’ or 5’-TCTCCCGGCATGGCGAGCCT-3’ subcloned with T7 promoter primers from px459 to pZero Blunt) and 100 ng/µl ssODN repair template (5’-GC GCCGACGACGCCGCCACGCCACCAGCTGGAGGCGGCCGAGG ATCCCTTGGAGAAGACTCTAACTCCCCCATGGAGTCCGCGAATCCTACGAGG-3’). Injected zygotes were incubated overnight at 37°C in 5% CO2 before transfer to pseudopregnant female B6CBAF1/J mice. Offspring were genotyped by sequencing the amplicon of the targeted locus using sense (5’-AAAGTCACAGGAGAAGGCGG-3’) and antisense (5’-TCGATGTAGGCGCTGATGTC-3’) primers. Mosaic mice found to carry the mutation were subsequently crossed with wt C57BL/6NHsd. Cross-validation by BamHI digestion of the amplicon further confirmed successful mutation.

Mice
Mice heterozygous for the CebpaΔuORF mutation (C57BL/6NHsd) were crossed once with wt C57/BL6J mice. Offspring were obtained from heterozygous matings to obtain homozygous CebpaΔuORF/ΔuORF mice and wildtype littermates for experiments. For the first 3 months after birth mice were kept in individually ventilated cages (IVC), followed by conventional housing (group where possible). Mice were kept at 22°C on a 12hr light/dark cycle on a standard diet (V1554-703, Ssniff) at the Central Animal Facility (CDP), University Medical Center Groningen (UMCG), The Netherlands. For genotyping, ear clips were proteinase K digested to allow gDNA isolation for amplification using 34 cycles of 30s 95°C, 30s 58 °C, 60s 72 °C using FastStart Taq DNA Polymerase (Roche). Per reaction 1x PCR buffer + MgCl2, 1x GC enhancer, 0.5 µM primers (total), 100 µM dNTPs and 1 unit FastStart Taq in 25 µl total volume was used. Genotyping primer sequences are provided in Supplementary Table 2.

Mouse experiments
All animal experiments were carried out with approval from the Animal Ethical Committee, Rijksuniversiteit Groningen and the Central Authority for Scientific Procedures on Animals (CCD). To assess weight gain in different genders and genetic background, mice were weighed weekly. For tissue
harvest and subsequent RNA isolation or immunoblot analysis, 5-6 month old mice where anaesthetized with isoflurane and terminated by cervical dislocation. Harvested tissues were snap frozen in liquid nitrogen and stored at -80°C until further processing. I.p. glucose tolerance test (IPGTT), i.p. insulin tolerance test (IPITT) and tissue insulin sensitivity test were performed as described (22). For IPGTT and i.p. pyruvate tolerance test (IPPTT), mice were starved for 16h in fresh cages for next-day experiments. For IPPTT, mice were injected with 2g sodium pyruvate per kg body weight in 1xPBS at 200mg/ml. Blood glucose measurements were obtained from a small cut in the distal part of the tail using a glucometer (Accu Check Aviva, Roche). Mice without initial increase (IPGTT/IPPTT) or decrease (IPITT) in blood glucose levels upon injection were excluded from the analysis.

Immunoblot analysis
Tissues were lysed in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton-X 100 supplemented with protease and phosphatase inhibitors (Roche)) by glass mortar and pestle (Wheaton) and sonication (10 cycles 30s on, 30s off on high, Bioruptor). Equal amounts of protein were separated by gradient SDS-PAGE gels (Bio-rad) and transferred to PVDF membrane using Trans-Blot Turbo System (Bio-rad). The following antibodies were used for detection: C/EBPa D56F10 monoclonal (Cell Signalling; 1:1000; rabbit), C/EBPβ (E299) (Abcam; 1:1000; rabbit) and β-actin (clone C4) #691001 (MP Biomedicals; 1:5000; mouse). Secondary HRP-conjugated antibodies (Amersham Life Technologies) were used for chemiluminescence detection using ECL (Amersham Life Technologies) on an ImageQuant LAS 4000 mini (GE Healthcare). The supplied software was used for image quantification.

Authorship contributions
B.A.S., C.M., H.R.Z., J.H. and G.K. performed the experiments, C.M., H.R.Z. and C.F.C. analyzed the data, J.D. and B.S. generated the mouse model and C.F.C supervised the project. H.R.Z. wrote the manuscript with input from C.M.
Chapter VII

References
Generation and initial metabolic characterization of C/EBPα-ΔuORF mice


**Supplementary Figures**

**Supplementary Figure 1.** C/EBPα-ΔuORF mice display no difference in C/EBPβ protein isoform ratio  
A) Representative immunoblots of mouse liver samples from indicated gender and genotype in C57BL/6N background. ΔuORF indicates homozygous CebpaΔuORF/ΔuORF mice.  
B) Quantification of C/EBPβ protein isoforms based on blots in A) and an additional blot per gender (data not shown), mean ± S.D., n = 8 per genotype per gender. No significant differences were found by two-tailed Student’s T-test (p > 0.05).

**Supplementary Figure 2.** Blood glucose levels in fed and fasted C/EBPα-ΔuORF mice  
A) Basal glucose levels in female WT or C/EBPα-ΔuORF mice under ad libitum access to food or after overnight starvation. Mean ± S.D., n = 12 per genotype. *: p < 0.05, Student’s T-test.  
B) same as A) but for males. WT: n = 11 (fed & fasted), C/EBPα-ΔuORF n = 9 (fed) and n = 11 (fasted).
Supplementary Table 1. Offspring of heterozygous Cebpawt/ΔuORF mice are born to Mendelian ratios
In total 577 mice from heterozygous Cebpawt/ΔuORF parents were genotyped of which 293 (50.8%) males and 284 (49.2%) females. Gender distribution was not significantly different by binomial test (2-sided, p = 0.7391). Offspring genotype distribution is not significantly different from what would be expected based on Mendelian inheritance of the mutation (Cebpawt/wt : Cebpawt/ΔuORF : CebpaΔuORF/ΔuORF = 1:2:1) in both genders: p = 0.7268 and p = 0.3906 for males and females respectively, Chi-square goodness of fit test.

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<th>Genotype</th>
<th>Males</th>
<th>Females</th>
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<td>Cebpawt/wt</td>
<td>75 (25.6%)</td>
<td>68 (23.9%)</td>
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<td>Cebpawt/ΔuORF</td>
<td>140 (47.8%)</td>
<td>153 (53.9%)</td>
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<td>CebpaΔuORF/ΔuORF</td>
<td>78 (26.6%)</td>
<td>63 (22.2%)</td>
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<tr>
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Supplementary Table 2. Sequences of genotyping primers used

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