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Grb10 and developmental programming: evaluation of a maternal diet restriction model during gestation

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Grb10 and developmental programming: evaluation of a maternal diet restriction model during gestation

Grb10 och perinatal programmering: utvärdering av en maternell restriktionsdiet under dräktighet

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SAMMANFATTNING

Den här studien genomfördes på University of Bath i England från september till december 2014 som del av ett pågående forskningsprojekt som syftar till att utröna vad för roll genen *Grb10* har i att mediera långsiktiga hälsoeffekter (som t ex övervikt, diabetes och hjärt-kärlsjukdomar) som orsakas av miljöfaktorer under det tidiga utvecklingsstadiet.

Det primära syftet med den här uppsatsen var inte att svara på några av de större frågor som projeketet inbegriper eftersom det skulle kräva mycket mer data än vad som vore rimligt att samla in under loppet av några månader, utan snarare att utvärdera de metoder som används i forskningsprojektet och klarlägga huruvida de fungerar på det sätt de är avsedda att göra. I projektet, som innefattar möss där *Grb10*-genen har slagits ut samt vildtypsmöss som kontroll, används en dietär restriktionsmodell som syftar till att generera avkommor med lägre födelsevikt och i förlängningen skadliga hälsoeffekter under vuxenlivet. Detta skall åstadkommas genom att begränsa proteininnehållet i vissa av mödrarnas diet till 9 % (jämfört med kontrolldieten på 20 %) under dräktigheten. Det är av största vikt att den dietära restriktionsmodellen fungerar korrekt för att få de önskade "programmeringseffekter" på ett tidigt stadie i livet som krävs för att sedan kunna genomföra meningsfulla tester i vuxen ålder, så som t ex blodtrycksmätning, glukostoleranstest, mätning av fett/muskelsammansättning osv.

Alla eventuella effekter av proteinrestriktionen skall manifesteras endast hos avkomman och inte förloras genom att orsaka oönskade tillväxteffekter på modern själv. Även om dieternas proteininnehåll skiljer sig åt så har de samma kaloriinnehåll och förväntas därför inte ha någon signifikant effekt på mödrarnas tillväxt under dräktigheten. För att validera detta så vägdes 27 mödrar beroende på genotyp och diet dagligen under dräktigheten. Genotypen fastställdes med hjälp av PCR med öron- eller svansbiopsier som material. Födointag mättes också. Detta upplägg gjorde det dessutom möjligt att observera eventuella hittills okända effekter av *Grb10* på tillväxt under perioder av metabola och endokrinologiska förändringar i vuxenlivet så som dräktighet. En tidig indikation på den dietära restriktionsmodellens effekter på avkommorna kunde också ges genom vägning av 60 foster på embryodag 18.5 (dvs en dag innan födsel).

Studien visade att det inte fanns någon signifikant skillnad i tillväxten hos dräktiga möss beroende på genotyp eller diet. Inte heller födointaget skiljde sig åt beroende på diet. Dock gavs en preliminär indikation på att de önskade tillväxtbegränsande effekterna av dieten på avkommorna inte erhålls. Utöver detta upptäcktes en avvikelse från den förväntade 50/50-fördelningen mellan vildtyp- och maternella Grb10-knock-outavkommor i kullar från vildtypsfäder korsade med Grb10KO-mödrar. Orsaken till avvikelsen är ej känd men skulle kunna vara perinatal mortalitet till följd av kvävning hos maternella Grb10-knock-outs.

SUMMARY

This study was conducted at the University of Bath, UK, from September to December 2014 as part of an ongoing research project aimed at elucidating how the *Grb10* gene might act as a mediator of long-term health effects (such as predisposition to obesity, diabetes, coronary heart disease and hypertension) caused by environmental factors during development. This phenomenon is known as developmental programming.

The purpose of this thesis was not primarily to answer any of the broader questions posed by this research project at large since this would require much more data than is reasonable to acquire over the course of a few months, but rather to evaluate the methods used in this project and reveal whether they are working the way they are presumed to. In the research project, which employs mice with the *Grb10* gene knocked out as well as wild-type control mice, a dietary restriction model is utilized during gestation, which is supposed to generate offspring with lower birth weight and subsequent detrimental health effects in adulthood. This is supposed to be achieved by restricting the protein content of the pregnant mothers' diet to 9 % (as opposed to the control diet of 20 %) throughout gestation. It is pivotal that the maternal dietary restriction model is functioning properly in order to gain developmental programming effects and perform worthwhile further testing later on in this project, such as blood pressure measurements, glucose tolerance tests and body composition analyzing.

Any effects of the dietary restriction of the mothers during gestation are supposed to be shunted directly to the offspring and not to be lost by ”leaking off” and eliciting any adverse effects on the mothers themselves. Although the maternal diets differ in protein content, they are isocalorific and therefore not supposed to have any substantial effect on maternal growth during pregnancy. To validate this, 27 pregnant mice were weighed daily throughout gestation depending on genotype and diet. Genotype was determined via PCR of ear or tail clips of the mice. Food intake was also recorded. This set-up also allowed to record any hitherto unknown effects of *Grb10* on growth induced by metabolic and endocrinological changes in adulthood such as pregnancy. An early indication of the dietary restriction effects on the offspring was also provided by weighing 60 pups at embryonic day 18.5 (e.g. one day before birth).

The study revealed that there was no significant difference in the growth of pregnant mice according to genotype and diet, nor was the food intake affected by the dietary regime. However, it also gave a preliminary indication that there could potentially be problems with not achieving the desired lower birth weight effects on the offspring. Additionally, a deviation from the expected 50/50 birth ratio of maternal *Grb10* knock-out offspring versus wild-type offspring when breeding a wild-type father with a Grb10 knock-out mother was discovered. The cause of the deviation is not known, but the findings indicate a perinatal mortality due to suffocation in maternal *Grb10* knock-outs.
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INTRODUCTION

The concept of developmental programming refers to the notion that altered environmental factors during development can have long-term consequences on adult health, including predisposition to obesity, insulin resistance/glucose intolerance and diabetes. (Segovia et al, 2014; Langley-Evans, 2006; Li, et al 2011) Moreover, aberrant growth during development is associated with an increased risk for coronary heart disease and hypertension. (Barker, 1995)

Although it is established that health status in adult life is influenced by growth during prenatal and postnatal development, no study has thus far verified a specific gene as being involved in developmental programming. Since previous experiments have shown that *Grb10* acts to restrict fetal and placental growth in mice (Charalambous et al, 2003), and adult mice lacking *Grb10* have an increased ability to clear glucose from the blood stream (Smith et al, 2007), *Grb10* is a potential candidate for being a developmental programming gene.

In this project, *Grb10* knock-out mice were generated and along with wild-type control mice subjected to a dietary restriction model throughout gestation. Some of the mothers were kept on a low-protein diet (9 %) and another cohort of mothers on a regular protein diet (20 %) during gestation. Pups born to low-protein diet mothers are expected to be born smaller with a higher risk of detrimental health effects in adulthood. Cross-fostering immediately upon birth to a regular diet mother will then induce catch-up growth in the pup and subject it to developmental programming effects, such as increased risk for diabetes and obesity.

This project will thus ascertain whether *Grb10* knock-out mice are protected from these effects, which will be tested by differential dietary regimes for the offspring and a variety of tests such as blood pressure measurements, body composition analyzing and glucose tolerance in adult life. If successful, this research project will answer fundamental questions about the role of *Grb10* in developmental programming, including: does ablation of the *Grb10* gene counteract the long-term harmful health effects of suboptimal environmental circumstances in early life?

This thesis will focus on specific parts of this project, including analyzing the growth of mothers during gestation depending on genotype and dietary regime, comparing the embryonic growth when the mothers are fed a low-protein diet versus a regular diet during gestation and assessment of breeding success. This data is important in order to confirm the validity of the generated results and conclusions drawn thereof at the end of the project.

LITERATURE REVIEW

Grb10 imprinting and epigenetic regulation

Grb10 (growth factor receptor-bound protein 10) is a cellular adapter protein belonging to a family of structurally similar adapter proteins which participate in a wide range of cellular processes such as cell growth, metabolism, apoptosis and cell migration. It can interact with most tyrosine kinase receptors, but binds mainly to the insulin receptor (Insr) and the IGF1 receptor (IGF1R). Signaling through Insr regulates metabolism whereas signaling through IGF1R regulates growth. *Grb10* acts to inhibit signaling pathways utilized by these receptors. (Holt & Siddle, 2005).

Like many other genes regulating fetal and placental growth, *Grb10* is one of approximately 100 imprinted genes discovered in the mouse thus far. (Wilkins, 2014). An imprinted gene is a gene that is expressed predominantly or exclusively from one of the two parental alleles. In the case of *Grb10*, the expression is predominantly maternal, with paternal expression only occuring in CNS.

Since the discovery of gene imprinting in 1984 (Surani et al, 1984), imprinted genes have been the subject of a large number of studies concerning fetal growth and development as well as postnatal metabolism and behaviour. The expression of imprinted genes is regulated by epigenetic modifications of the imprinted loci. (Abramowitz et al, 2012). Epigenetics is a phenomenon whereby heritable changes in gene expression are not due to alteration of the DNA sequence or the amount of DNA. (Bird, 2007) New mechanisms of epigenetic regulation are continuously being discovered, however there are currently three well-established epigenetic mechanisms by which gene expression can be regulated.

1. DNA Methylation

The addition of a methyl group to nucleobases of eukaryotic DNA. In mammals, the only nucleobases to be affected by methylation are cytosine residues in CpG dinucleotides. The bulky methyl group prevents binding of transcription factors to the particular gene or region thereof, and is often associated with gene silencing. This mechanism is always deactivating. (Robertson, 2005)

2. (Posttranslational) histone modifications

Histones are proteins located in the cell nucleus, where they package DNA into nucleosomes. They have tails which can be modified by undergoing covalent modifications such as methylation and acetylation at certain amino acids. Most commonly, the amino acid lysine is subjected to these modifications, which alter the histone-DNA-interaction. Lysine

methylation is generally deactivating while acetylation is exclusively activating (Berger, 2007).

3. Non-coding RNAs

Non-coding RNAs include microRNA (miRNA), small-interfering RNA (siRNA) and long non-coding RNAs (lncRNAs). Although the mechanism is thus far poorly understood, it is hypothesized that these RNAs perform gene regulating activities, the most prominent example being X-chromosome inactivation in female mammals where lncRNAs are said to coat the inactivated X-chromosome. (Chuang & Jones, 2007)

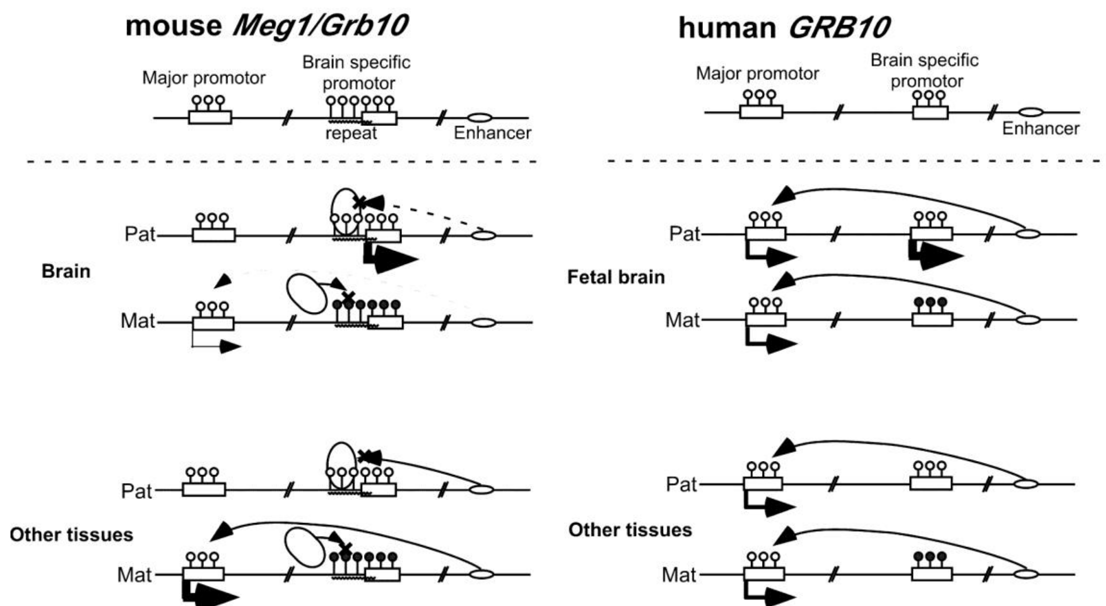


Figure 1. The epigentic regulation of Grb10. *(Hikichi et al, 2003). These schematic illustrations depict how expression of Grb10 is regulated in the mouse and humans respectively. White lollipops represent unmethylated CpG motifs, black lollipops methylated CpG motifs. Small oval figures represent downstream enhancers, large oval figures a transcriptional repressor named CTCF.*

Grb10 expression is generally thought to be regulated by DNA methylation. In this proposed model (Figure 1), methylation of CpG motifs between the *Grb10* promotor and its downstream enhancer regulates the binding of transcription factors and subsequent transcription of the *Grb10* gene.

The paternal expression of *Grb10* stems from paternally-expressed gene promotors which are regulated by methylation of DNA and brain-specific activators (not shown in figure). The CTCF insulator blocks interaction between the downstream enhancer and the upstream maternally-expressed gene promotor. Since the activators are exclusively located in the brain, this is the only site of paternal *Grb10* expression. Similarly, the paternal expression in other tissues is blocked by the CTCF insulator.

In the maternal alleles, DNA methylation prevents CTCF from binding and blocking enhancer-promotor interaction. Thus, the gene is expressed. Although this is the case for brain as well as other tissues, maternal *Grb10* expression in the brain is comparatively low, raising the possibility that the downstream enhancer is to some extent tissue specific, rendering a biallelic but overwhelmingly paternal expression in the brain and exclusively maternal expression in other tissues.

Grb10 expression

The expression pattern of *Grb10* in the mouse is predominantly maternal during embryonic development, with maternal alleles being expressed in tissues of mesodermal and endodermal origin, whereas paternal expression is limited mainly to the CNS i.e. the diencephalon, ventral midbrain and the medulla oblongata. (Garfield et al, 2011).

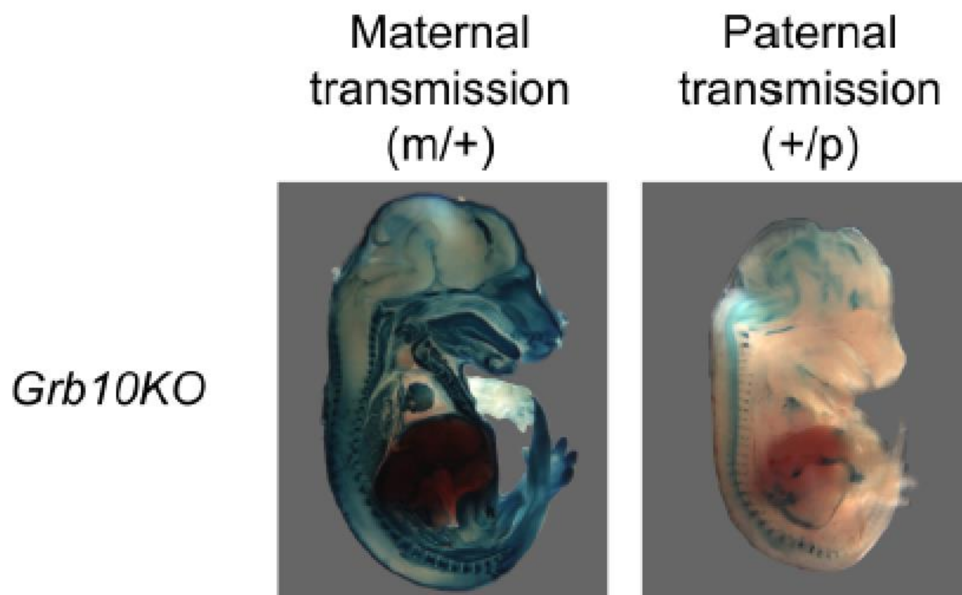


Figure 2. *LacZ staining of Grb10KO embryos at e14.5, showing abundant maternal Grb10 expression in peripheral tissues and paternal Grb10 expression confined to the brain and CNS. (Cowley et al, 2014).*

Typically, imprinted genes show biallelic expression (i.e. non-imprinted) at some expression sites. The degree of reciprocal imprinting exhibited by *Grb10* is however unprecedented and prompts questions about the evolution of *Grb10* and its imprinting pattern.

It appears as if the mother and the father are utilizing two distinctly different strategies, one targeted at the brain and the other at the body, in order to maximize the success of their offspring.

A number of theories including the ”parental-conflict hypothesis” (Trivers, 1974) and the maternal-offspring co-adaptation theory (Wolf & Hager, 2006) can to some degree explain the evolution of the imprinting of *Grb10* but none of them adequately. Most of the data on

Grb10 thus far is consistent with the parental-conflict hypothesis, but recent discoveries, such as the fact that *Grb10* elicits different biological responses when expressed in the mother versus the offspring; controlling postnatal supply of nutrients in the mother and demand of nutrients in the offspring (Cowley et al, 2014) fit better with the co-adaptation theory. Moreover, *Grb10* expressed in the mother influences fat mass in the offspring, while *Grb10* expressed in the offspring influences its lean mass. Together, these combined effects establish proportionate growth and body composition of the offspring. Nevertheless, more research is needed in order to confidently attribute *Grb10* to the co-adaptation theory.

Adult expression of *Grb10* in the mouse is less characterized than the embryonic expression. A 2007 study revealed that adult expression of *Grb10* is imprinted and confined to a restricted set of tissues. β-galactosidase staining was performed and β-galactosidase activity was displayed from the maternal allele in skeletal muscle, adipose tissue, endocrine pancreas, oviduct, uterine horns and Leydig cells in the testes. β-galactosidase was expressed from the paternal allele in the hypothalamus. (Smith et al, 2007).

Grb10 function

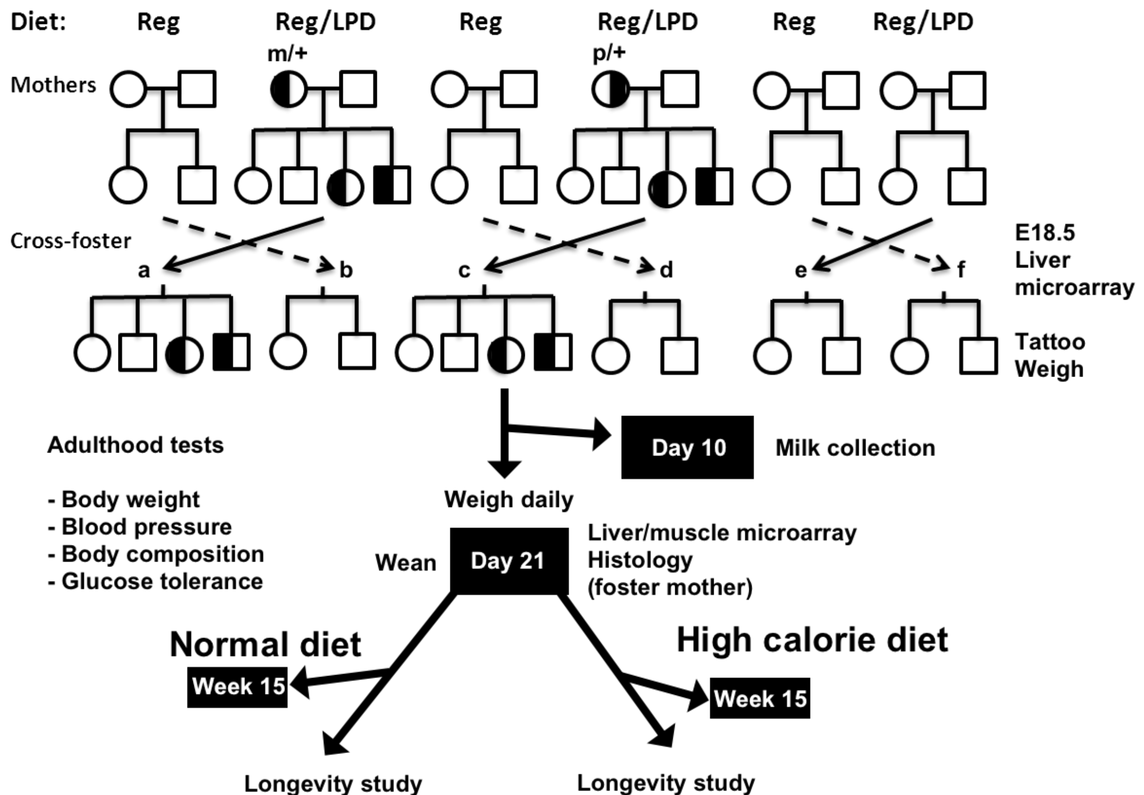
Consistent with the pattern of expression, *Grb10* has proved to have different functions depending on which allele is knocked out. Maternal-specific expression of *Grb10* restricts fetal and placental growth, as mouse maternal-specific *Grb10* knock-outs ($Grb10KO^{m/+}$) are born ~30 % heavier than wild-type littermates (Charalambous, 2003) . Together with the discovery that *Grb10* knock-outs in adulthood have an increased ability to clear glucose from the blood circulation (Smith et al, 2007), the concept of *Grb10* functioning as a link between fetal growth and adult health was conceived.

In addition to increased glucose uptake, adult $Grb10KO^{m/+}$ display a leaner body constitution (i.e. elevated muscle mass and decreased adipose mass) and thus a phenotype that might be deemed ”anti-diabetic”. Moreover, several of the tissues displaying *Grb10* adult expression, including muscle, white adipose tissue, pancreas and brain fit the description ”insulin-responsive”. Pancreas-specific knock-out of *Grb10* results in significantly elevated pancreas weight (Zhang et al, 2012), indicating that *Grb10* inhibits tissue growth and acts to control growth locally, which is in accordance with its intracellular signaling function.

On the contrary, paternal expression of *Grb10* influences social behaviour. In a 2011 study, mice with paternal-specific ablation of *Grb10* ($Grb10KO^{+/p}$) were significantly less likely to back down in a ”tube test” than their wild-type counterparts. (Garfield et al, 2011). A tube test forces an encounter between in this case two mice of different genotypes. Additionally, this change in behaviour was found to correlate with an increased incidence of facial barbering, so called allogrooming, in cages containing a $Grb10KO^{+/p}$ mouse. Hitherto, *Grb10* is the only imprinted gene that has been shown to affect social behaviour. It is still unclear how these findings fit in with the proposed evolutionary theories for the imprinting of *Grb10*.

MATERIALS AND METHODS

Project background and experiment setup



Genetic crosses, cross-fostering strategy and project plan. *Legend: white square=wild-type father, white circle=wild-type mother, half-filled circle=Grb10KO mother (can be Grb10KO$^{m/+}$ or Grb10KO$^{+/p}$, as denoted in the figure).*

Wild-type mothers give birth to only wild-type pups, while Grb10KO mothers give birth to mixed litters of wild-type and Grb10KO$^{m/+}$ pups. In cross "a", pups are from mothers fed a low-protein or regular diet during gestation. Pups from low-protein diet mothers are expected to be born small. Cross-fostering them to foster mothers exposed to a regular diet during gestation will induce catch-up growth in the pup, important for eliciting developmental programming effects, which predispose the pup for harmful health effects in adult life. Grb10KO$^{m/+}$ pups are expected to be protected from these health effects, since they are born larger and exhibit an "anti-diabetic" phenotype with lean body proportions and increased glucose metabolism in adulthood. Crosses b-f address questions which are not covered by this degree project. Offspring are then followed into adulthood and recruited into longevity or week 15 cohorts on different dietary regimes, where they will be subject to a variety of tests including body weight, blood pressure, body composition and glucose tolerance.

A *Grb10* knock-out mouse strain (Grb10KO) that had previously been generated by incorporating a LacZ (β-geo) gene-trap cassette into exon 7 of the *Grb10* gene was utilized in this experiment. Transmission of the Grb10KO allele from the mother (Grb10KO$^{m/+}$) and the father (Grb10KO$^{+/p}$) allowed allele-specific observation of the *Grb10* expression.

The knock-out offspring were then derived from a breeding strategy including Grb10KO$^{m/+}$, Grb10KO$^{+/p}$ and wild-type mice. Each breeding cross included a female or male wild-type mouse paired with a Grb10KO$^{m/+}$ or Grb10KO$^{+/p}$. If the Grb10KO genotype is inherited by the offspring from the mother, the offspring will always be Grb10KO$^{m/+}$ regardless of whether the mother was a Grb10KO$^{m/+}$ or Grb10KO$^{+/p}$. Similarly, if the Grb10KO genotype is inherited from the father, the offspring will be Grb10KO$^{+/p}$.

On the morning following mating, examination of vaginal plugs was performed on the females. The vaginal plug consists of secretions from the vesicular glands of the male. Vaginal plug checking is a blunt tool for determining pregnancy since the existence of a vaginal plug does not confirm pregnancy itself, only that sexual activity has occurred (The Jackson Laboratory, 2006). Full confirmation of pregnancy was therefore not obtained until the end of the expected gestation period, which in the mouse lasts for on average 19-21 days.

If presence of a vaginal plug was noted, the mice were then moved to a separate cage. During the gestation period, the mothers were separated into two cohorts, one being fed a regular diet (20 %) and the other a low-protein diet (9 %). Daily weighing (except weekends) of the pregnant mice as well as their food to determine how much they had eaten was performed. The pregnant mice were each kept in separate cages, ensuring that the food had been consumed by the respective mouse. Food was continuously added *ad libitum*.

Immediately after birth, tattooing of the pup paws was done to enable individual identification. Pups were then cross-fostered to nurses that had also given birth on the same day. Offspring from mothers on a low-protein diet during gestation were cross-fostered to nurses that had been fed a regular diet during gestation.

When necessary, litter size was adjusted (to a maximum of 7). In case of large litters, spare pups were euthanized and dissected due to the unsuitability of maintaining very large litters in one cage and in order to provide material for other coinciding studies. In case cross-fostering was not possible due to lack of receiving foster mothers, the pups were dissected at E18.5.

Mice used in the study

Table 1. *Maternal genotypes and diets in the pregnant mother analysis*

Genotype	Diet	Number of mice
Grb10KO$^{m/+}$	Regular	4
Grb10KO$^{m/+}$	Low protein	1
Grb10KO$^{+/p}$	Regular	7
Grb10KO$^{+/p}$	Low protein	4
Wild type	Regular	6

Genotype	Diet	Number of mice
Wild type	Low protein	5
Total		27

Factoring out the diet yielded 5 Grb10KO$^{m/+}$ mothers (4+1) for the genotype analysis, 11 Grb10KO$^{+/p}$ mothers (7+4) and 11 wild type mothers (6+5). The diet analysis included 10 mothers in the low protein cohort (1+4+5) and 17 in the regular diet cohort (4+7+6).

Table 2. *Mother-offspring and maternal diet combinations in the embryonic growth analysis*

Embryo genotype	Mother genotype	Maternal diet	Legend	Number of mice
Wild type	Wild type	Low protein	BwtLPDwt	6
Wild type	Wild type	Regular	BwtREGwt	19
Grb10KO$^{m/+}$	Grb10KO$^{+/p}$	Low protein	BJmpLPDJm	2
Wild type	Grb10KO$^{+/p}$	Low protein	BJmpLPDwt	7
Grb10KO$^{m/+}$	Grb10KO$^{m/+}$	Low protein	BJmmLPDJm	6
Grb10KO$^{m/+}$	Grb10KO$^{m/+}$	Regular	BJmmREGJm	7
Wild type	Grb10KO$^{m/+}$	Low protein	BJmmLPDwt	6
Wild type	Grb10KO$^{m/+}$	Regular	BJmmREGwt	7
Total				60

PCR genotyping

Ear clip or tail biopsies from the mice were used as starting material. If tail biopsies were used, a small part of the tail was cut off with a scalpel blade on a petri dish and the rest of the tail saved for eventual further use. The ear clips/tail clips were placed into to a 1,8 ml eppendorf tube each, and 600 µl of 100 mM sodium hydroxide (NaOH) was added to each tube. The lids were then pierced with a needle and placed in a floatable rack. The mixture was boiled for 10 minutes and then left to cool to room temperature. Then 100 µl of 1M Tris-HCl (pH 8) was added to each sample, before they were stored in -20°C for later use.

To amplify the 500 bp βgeo DNA region in Grb10KO mice, 1 µl of the mix acquired as previously described was added to a master mix consisting of 7,5 µl PCR BIO Taq mix

(BioTaq, Bioline) containing buffer, Taq polymerase, dNTPs, red dye and primers, 0,75 µl of a primer mix containing 6 µM βgeo F2 and 6 µM βgeo R2 primers, as well as 5,75 µl MQ water. In total, each tube then contained 15 µl of the final mix.

For amplification, the PCR profile was programmed to a pre-denaturation of 95 °C for 2 minutes, 35 cycles of 95 °C, 60 °C and 72 °C (all for 15 seconds) and finally 72 °C for 5 minutes. For each 40 samples run on electrophoresis, 70 ml of Agarose gel was made by making a 1 % agarose gel (consisting of 1 % agarose powder in TAE). The jar was then heated until the agarose had fully dissolved in the liquid. Ethidium bromide at a concentration of 0,5 µl/10 ml was pipetted into the mixture, which was poured into a plate rack and left to set for 20 minutes.

6 µl of 100 bp DNA ladder was then pipetted into the first lane and then 15 µl (i.e. the total content) from each tube into the subsequent lanes. The gel was run at 90 V for 35 minutes. Finally, the gel was visualized under UV light and the presence of PCR products was checked for.

Subsequent points not covered in this thesis

This degree project does not involve any of the points introduced in the study beyond the steps explained above, however these are reviewed nevertheless in order to provide a full understanding of the study.

The remaining pups were then weighed daily until weaning (at day 21), at which point they were randomly recruited into either the 15 week cohort or the longevity cohort. In conjunction with the weaning, the pups were ear clipped and genotyped. At weaning, the foster mother was dissected. Additionally, milk was collected from the foster mothers 10 days after giving birth, since Cowley and co-workers (2014) report that the maternal genotype affects the nutrient provisioning capacity of the mother and it is thought that this effect is somehow mediated via the milk. At the time of writing, the exact analyses to be performed on the milk had not yet been decided but may include screening for growth factors.

Offspring recruited into the 15 week cohort were maintained on either a high fat (45 %) or a control diet *ad libitum*, food weighed daily. Again, this will test whether Grb10KO$^{m/+}$ pups are protected from developmental programming effects, since they exhibit a leaner body constitution in adulthood. Feeding them a high fat diet will show whether they stay leaner than wild-type littermates on the same diet. During this period, the mice were weighed weekly. Mice in the longevity study were kept alive for essentially as long as possible and subjected to testing of blood pressure, glucose tolerance testing and serum collection.

At the time of dissection, brain, heart, lungs, pancreas, liver, spleen, testes/ovaries, brown adipose tissue and white adipose tissue was removed, individually weighed and stored for further histological analysis.

Offspring ratio

Sheets containing various information on the mice utilized in the study including offspring genotype (as determined at 3 weeks of age) and their parents genotype were reviewed and the total number of offspring of wild-type and Grb10KO genotype born to Grb10KO mothers were counted up. Chi-square tests were then performed on the data to determine if there was a deviation from the expected 50/50 ratio of wild-type and Grb10KO offspring.

Additionally, a record of deaths between birth and 3 weeks of age which was kept by the technical staff at the animal research facility was also used. Although these records did not contain any genotype information, they stated the number of total deaths of offspring born to Grb10KO mothers.

RESULTS

Pregnancy weight and dietary effects during gestation

As previously described, the project included pregnant mice of three different genotypes: wild- type, Grb10KO$^{m/+}$ and Grb10KO$^{+/p}$. Taking into account the fact that they were kept on two different diets, regular and low-protein diet (LPD), this arrangement yielded six different comparable groups: wild-type regular, wild-type LPD, Grb10KO$^{m/+}$ regular, Grb10KO$^{m/+}$ LPD, Grb10KO$^{+/p}$ regular and Grb10KO$^{+/p}$ LPD. The assignment of mice of different genotypes to the regular and LPD cohorts was done in a random manner.

In total, 27 mice were weighed daily during gestation. Because of the small sample size of certain groups in particular, all six of them were not used in the final report. Instead, when examining growth according to genotype, LPD and regular diet mice were bundled together for each genotype. A median value was calculated in order to minimize the effect of potential outliers.

Table 3. *Maternal genotypes and number of mice analyzed*

Genotype	Number of mice
Grb10KO$^{m/+}$	5
Grb10KO$^{+/p}$	11
Wild type	11
	27

Weight of mothers during gestation by genotype

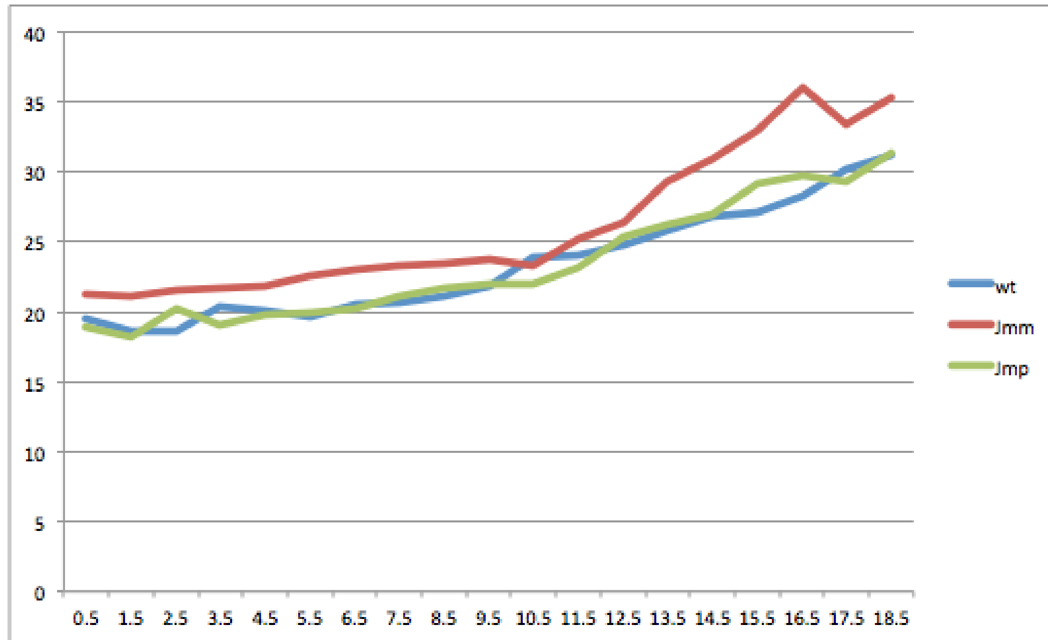


***Figure 3. Weight of mothers during pregnancy by genotype**. Legend: wt (n=11) Grb10KO$^{m/+}$ (n=5), Grb10KO$^{+/p}$ (n=11). E2.5: p=0.17, E8.5: p=0.66, E14.5: p=0.29 (one-way ANOVA test).*

One-way ANOVA tests were performed on three points throughout the gestation period (E2.5, E8.5, E14.5) and all resulted in $p>0.05$ ($\alpha=0.05$), showing that there was no statistically significant difference between any of the three groups.

While Figure 3 shows that Grb10KO$^{m/+}$ mice maintain a slightly higher weight throughout gestation, it does not clearly reveal whether this is due to the higher starting weight or increased growth.

Relative weight of mothers during gestation by genotype

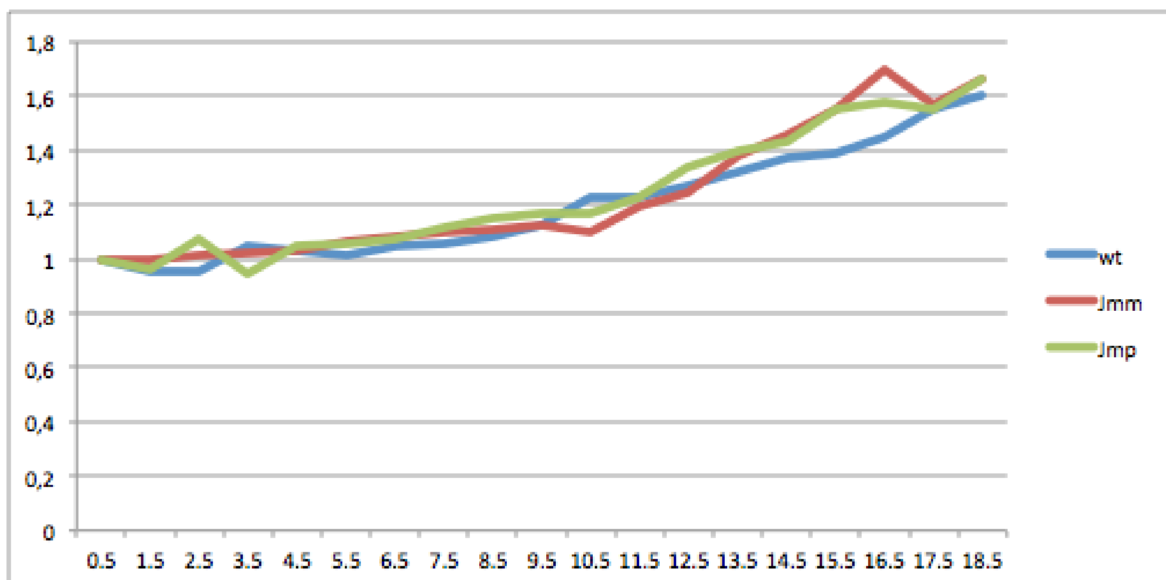


Figure 4. Relative weight of mothers by genotype. *wt (n=11), Grb10KO$^{m/+}$ (n=5), Grb10KO$^{+/p}$ (n=11). E2.5: p=0.58, E8.5 p=0.25, E14.5 p=0.43 (one-way ANOVA test)*

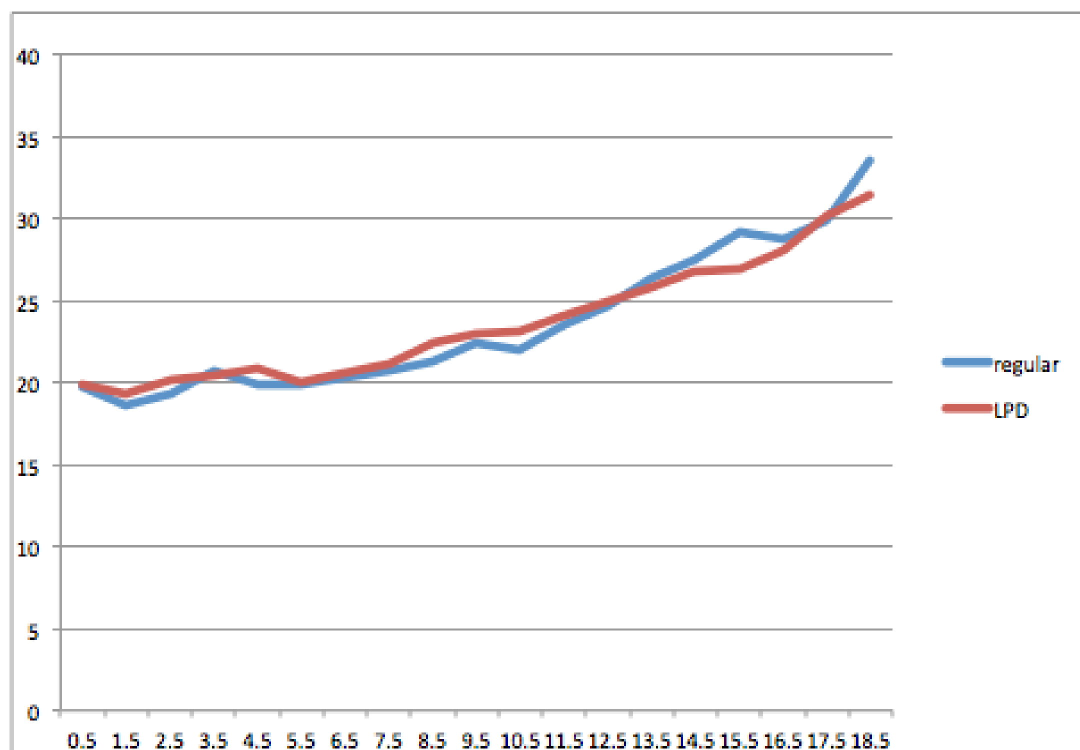
Figure 4 accounts for the higher starting weight of Grb10KO$^{m/+}$ offspring by factoring out the starting weight of all mice (covered in the Discussion part). This data was analyzed with an ANOVA test performed exactly as for Figure 3, again none of them resulting in statistically significant difference between the groups.

To see if there was any significant difference in maternal growth based solely on dietary regime, the different genotypes were factored out and weight data was plotted (Figure 5) according to regular or low-protein diet.

Table 4. *Maternal diets and number of mice analyzed*

Diet	Number of mice
Regular	17
LPD	10
	27

Weight of mothers during gestation by diet



5. *Weight of mothers during pregnancy by diet.* *Regular diet (n=17), LPD (n=10). E2.5: p=0.54 T-test (two-tailed distribution, two-sample equal variance)*, *E8.5: p=0.76 T-test (two-tailed distribution, two-sample equal variance)*, *E14.5: p=0.98 T-test (two-tailed distribution, two-sample equal variance)*

For Figure 5, a Student's t-test (suitable for comparing two groups) was applied to the three selected points instead of ANOVA. All tests returned $p>0.05$, confirming the hypothesis that dietary regime would not impact the growth of the mothers during pregnancy.

Food intake of mothers by diet

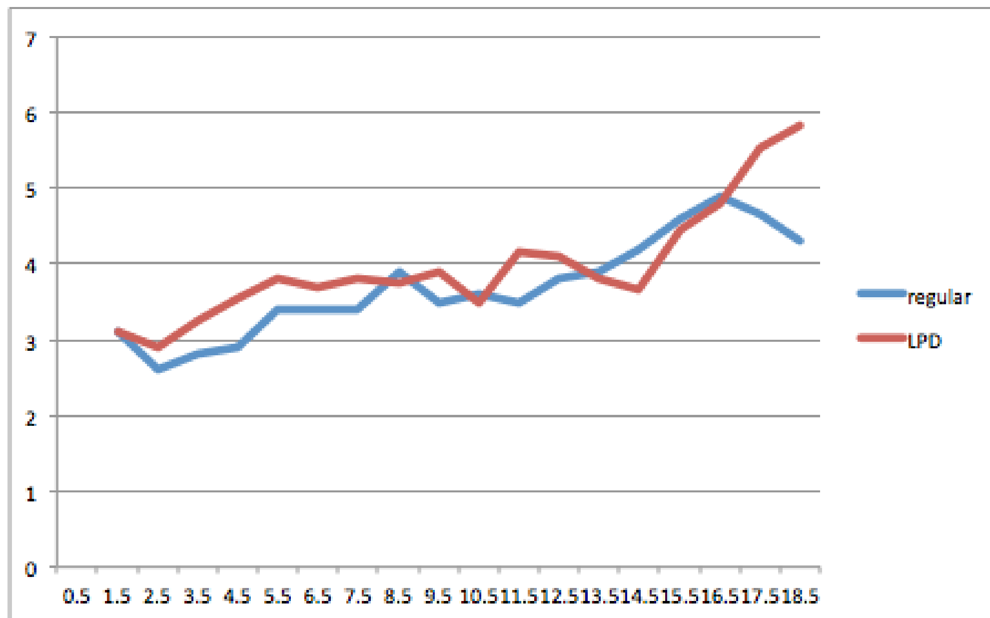


Figure 6. Daily food intake of mothers by diet. *Regular diet (n=17), LPD (n=10), E2.5: p=0.45 T-test (two-tailed distribution, two-sample equal variance), E8.5: p=0.77 T-test (two-tailed distribution, two-sample equal variance), E14.5: p=0.91 T-test (two-tailed distribution, two-sample equal variance)*

Figure 6 depicts the daily food intake of pregnant mice according to dietary regime. A Student's t-test returned $p>0.05$ for all points tested, showing no statistically significant difference in food consumption between low-protein and regular diet mothers.

Embryonic growth

This experiment included in total 60 embryos, born to mothers of different genotypes and dietary regimes during gestation. See Table 2 for a full review of all the mother-offspring combinations used.

Embryonic weight at E18.5 by genotype in mother and pup dietary regime

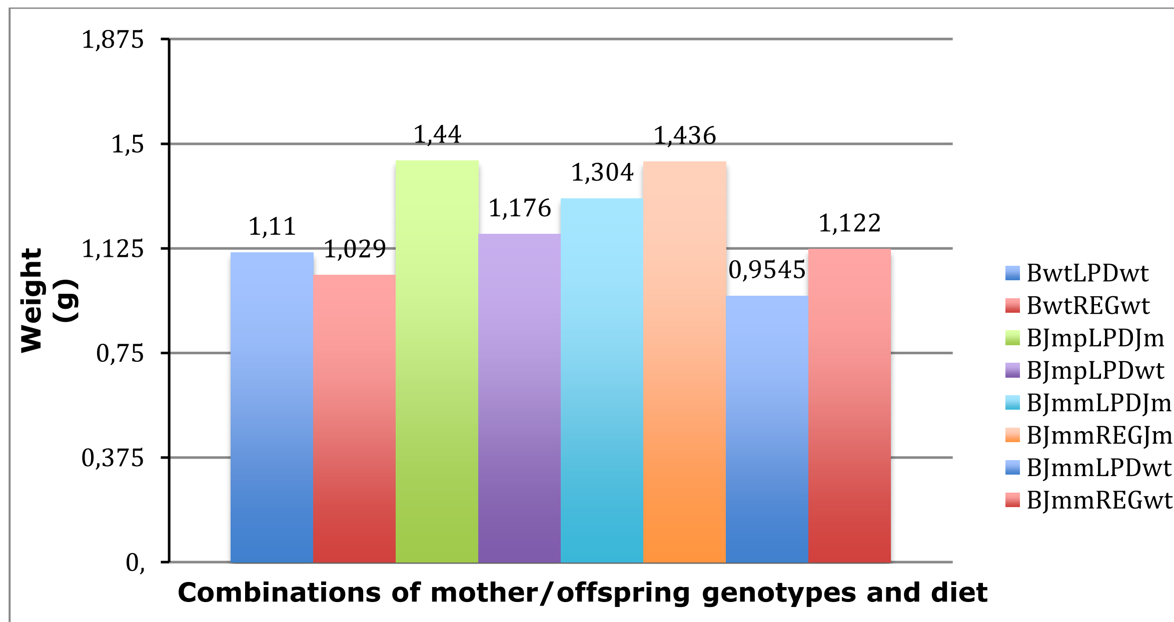


Figure 7. Embryonic weight at E18.5 by genotype in mother and pup and dietary regime.

Legend: wt = wild-type, Jmp = Grb10KO$^{+/p}$, Jmm = Grb10KO$^{m/+}$. B = born to, LPD = low-protein diet, REG = regular diet. BwtLPDwt (n=6), BwtREGwt (n=19), BJmpLPDJm (n=2), BJmpLPDwt (n=7), BJmmLPDJm (n=6), BJmmREGJm (n=7), BJmmLPDwt (n=6), BJmmREGwt (n=7).

The median weight of wild-type embryos at E18.5 when the mothers had been kept on a regular diet (BwtREGwt) was 93 % of that of wild-type embryos with mothers on a low-protein diet (BwtLPDwt) (1,11 g versus 1,029 g). p=0.29 (Student's t-test, one-tailed distribution, two-sample equal variance). A Student's t-test (one-tailed distribution, two-sample equal variance) on the two cohorts rendered p=0.42.

The embryos' genotypes were factored out respectively and new charts were plotted (Figure 8) in order to see if mum's genotype somehow could protect the offspring from the effects of protein restriction.

Effect of mum's diet on weight of E18.5 offspring

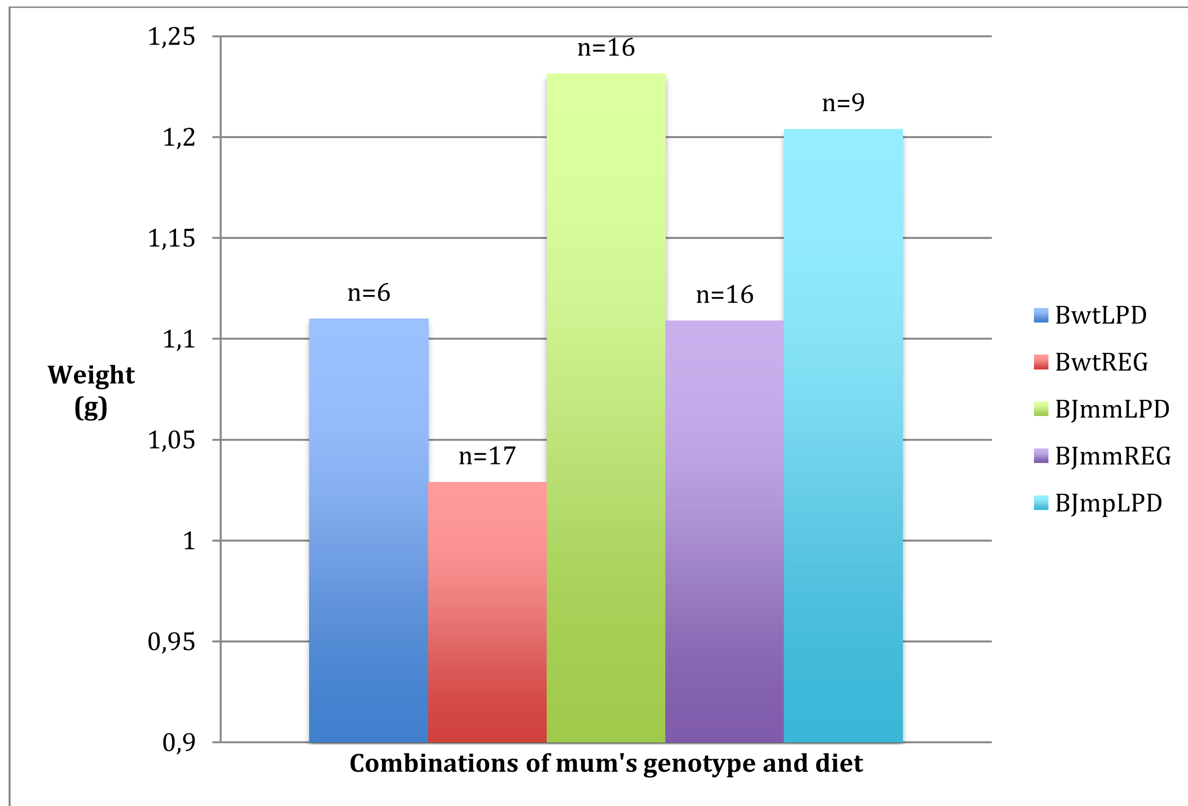


Figure 8. Effect of mum's genotype on weight of E18.5 offspring. *Litter sizes: BJmmLPD (7, 9), BJmmREG (8, 8). BwtLPD (n=6), BwtREG (n=19), BJmmLPD (n=15), BJmmREG (n=14), BJmpLPD (n=9).*

Interestingly, Grb10KO$^{m/+}$ mothers give birth to larger Grb10KO$^{m/+}$ offspring on a low-protein diet than on a regular diet, but not with any statistically significant difference (p=0.34, Student's t-test, one-tailed distribution, two-sample equal variance). The litter sizes are similar for both groups (7 and 9 for LPD mums, 8 and 8 for regular diet mums). No BJmpREG data was available, prohibiting Grb10KO$^{+/p}$ comparison.

Compared to Figure 7, this study also included four embryos in the BJmmREG group whose genotypes could not be identified and therefore could not be used in Figure 7 where the embryo genotype needs to be known.

Offspring ratio

During the course of the project, a lower than expected number of Grb10KO$^{m/+}$ mice was suspected when reviewing the birth records. It seemed as if the proportion of Grb10KO$^{m/+}$ offspring compared to wild-types in litters from Grb10KO mothers crossed with a wild-type father was skewed. To establish whether this was the result of a temporary shortage due to pure chance or if other factors were involved in the low supply, all crossing data since the beginning of the project was scrutinized and the number of offspring of different genotypes

counted.

In total, 182 mice born to a Grb10KO father and 205 mice born to a Grb10KO mother were counted. Out of the 182 mice derived from a Grb10KO father, 81 had inherited the knock-out gene (44,5 %) and 101 mice were wild-types (55,5 %). Out of the 205 mice derived from a Grb10KO mother, 81 were knock-outs (39,5 %) and 124 wild-types (60,5 %). These numbers were based on genotyping records of offspring at 3 weeks of age.

To establish whether these deviations from the expected ratio (50 %/50 %) were statistically significant, a chi-square test was performed on the data.

Ratio at 3 weeks of age

Table 5. *Offspring ratio at 3 weeks from Grb10KO fathers*

Grb10KO fathers

	+	-
Observed	81	101
Expected	91	91
Deviation	-10	-10
Deviation ^{2}	100	100
d ^{2}/e	1,099	1,099

□^{2} = □ d^2/e = 2,198

p = 0.138

Ho: Grb10KO fathers do not give rise to a litter of fewer Grb10KO offspring at 3 weeks than wild-type offspring when paired with a wild-type mother.

p=0.138. Null hypothesis not rejected ($p > 0.05$).

Table 6. *Offspring ratio at 3 weeks from Grb10KO mothers*

Grb10KO mothers

	+	-
Observed	81	124
Expected	102,5	102,5
Deviation	21,5	21,5
Deviation^2	462,25	462,25
d ^{2}/e	4,510	4,510

□^{2} = □ d^2/e = 9.02

p = 0.003

Ho: Grb10KO mothers do not give rise to a litter of fewer Grb10KO offspring at 3 weeks than wild-type offspring when paired with a wild-type father.

p=0.003. Null hypothesis rejected ($p < 0.05$).

Ratio at E18.5

A smaller cohort of embryos genotyped at E18.5 (n=36) was also analyzed. This cohort included only Grb10KO mothers.

Table 7. *Offspring ratio at E18.5 from Grb10KO mothers*

Grb10KO mothers

	+	-
Observed	19	17
Expected	18	18
Deviation	1	-1
Deviation^2	1	1
d^2/e	0.056	0.056

$\square^2 = \square\ d^2/e = 0.112$
p = 0.73

Ho: Grb10KO mothers do not give rise to a litter of fewer Grb10KO offspring at E18.5 than wild-type offspring when paired with a wild-type father.
p=0.73. Null hypothesis not rejected ($p > 0.05$).

Number of death records

22 deaths of offspring derived from a Grb10KO mother and a wild-type father were counted up using these records, although genotyping information was not available for these mice. However, assuming that they were all Grb10KO, this would account for 48,8 % of the deviation in table 2.

A new Chi-square test was performed, assuming that *all* of the perished offspring were Grb10KO$^{m/+}$.

Table 4. *Offspring ratio at 3 weeks from Grb10KO mothers, if all unidentified deaths in our records were Grb10KO*

Grb10KO mothers

	+	-
Observed	103	124
Expected	113,5	113,5
Deviation	10,5	10,5
Deviation^2	110,25	110,25
d^2/e	0,971	0,971

$\square^2 = \square\ d^2/e = 1.943$
p = 0.16

Ho: Grb10KO mothers do not give rise to a litter of fewer Grb10KO offspring at 3 weeks than wild-type offspring when paired with a wild-type father, provided that all unidentified deaths in our records were Grb10KO.
p=0.16. Null hypothesis not rejected.

PCR genotyping

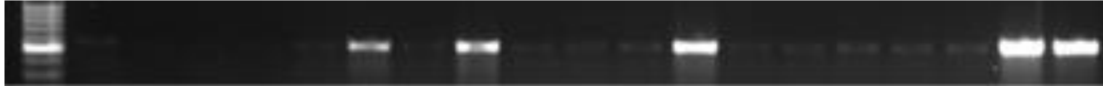


Figure 9. PCR analysis: example of genotyping results. Detection of the 500 bp β-geo gene-trap cassette (Grb10KO positive) in five samples represented by white bands.

Summary of the hypotheses tested in the study

Table 8. *Summary of the hypotheses tested by the data retrieved in this study*

Hypothesis	Source	Results	Interpretation
Knocking out *Grb10* does not affect the growth of mothers during pregnancy	Daily weighing of pregnant wt, m/+ and +/p mice	E2.5: p=0.58, E8.5 p=0.25, E14.5 p=0.43 (ANOVA one-way test)	No significant difference between the compared genotypes
Low-protein diet or regular protein diet does not affect the growth of pregnant mice	Daily weighing of LPD and regular diet mice	E2.5: p=0.54, E8.5: p=0.76, E14.5: p=0.98 (T-test, two-tailed distribution, two-sample equal variance)	No significant difference in growth between LPD and regular diet
Food intake is not affected by protein content of the diet	Daily weighing of food in LPD and regular diet cages	E2.5: p=0.45, E8.5: p=0.77, E14.5: p=0.91 (T-test, two-tailed distribution, two-sample equal variance)	No significant difference in food intake between the different dietary regimes
A Grb10KO mother/father crossed with a wild-type gives rise to a 50/50 litter of Grb10KO/wildtype	Review of dissection sheets	Grb10KO father: p=0.14 Grb10KO mother: p=0.003 (Chi square-test)	The shortage of $Grb10KO^{m/+}$ pups is not due to chance, but to some other underlying reason

offspring

Restricting the protein content of the wild-type mothers' diet restricts embryonic growth up to E18.5	Weighing of wildtype embryos at E18.5	p=0.29 (T-test, one-tailed distribution, two-sample equal variance)	Could not be proved, at least not with the provided sample size
Restricting the protein content of the Grb10KO mothers' diet restricts embryonic growth up to E18.5	Weighing of Grb10KO embryos at E18.5	p=0.42 (T-test, one-tailed distribution, two-sample equal variance)	Could not be proved, at least not with the provided sample size

DISCUSSION

All findings in this thesis must be treated as preliminary since they were gathered at an early point of a lengthy project when the availability of samples was limited. More time and opportunity to perform further testing would have added further substance to the findings.

Genotyping

Obviously, correctly performed genotyping is essential in order to attribute the data to the correct group of mice and gain accurate results. There are several steps in the process of genotyping that can provide faulty results, such as cross-contamination at any point during the preparation of the samples, pipetting, loading of the wells. To minimize the risk of cross-contamination, careful measurements were made such as cutting up the tail ends at different parts of the petri dish, cleaning the forceps between any handling of the tissues and changing tips in-between pipetting the different samples. The assessment of the final PCR product is subjectively made by ocular inspection, but in case of ambiguous results (”faint” lines on the gel), these samples were genotyped a second time using the same tissue samples. No ambiguous results were obtained after the second genotyping.

Pregnant mother data

The inherent length of scientific projects involving mouse breeding and gestation imposes a natural limit on the availability of mice over a relatively short period of time. This study could have been significantly improved with more time. Firstly, and most importantly, the design of this experiment allowed for six comparable groups. In this preliminary report however, low-protein diet and regular diet groups of the pregnant mothers were bundled together such that

only three of the groups were used, since the Grb10KO$^{m/+}$ LPD (n=2) and Grb10KO$^{m/+}$ regular diet (n=4) cohorts were deemed too small to allow statistically relevant analysis. If more samples would have been available, this would have enabled more refined examination of the joint effects of diet and genotype. From this data, it cannot be excluded that a certain combination of diet and genotype might impact the growth of the mother during pregnancy.

The data was collected during a 12 week visit. To achieve the same statistical power as for the three cohorts analyzed in this study for all six cohorts, perhaps at least twice as much time would thus have been needed.

Secondly, pregnancy in mice cannot be accurately determined until late in the gestation period, rendering some of the acquired data unusable. Thirdly, in order to attain true comparability between the pregnant mother weights, they would have to be allocated into different groups according to litter size. Ideally, there would have been one separate group for each individual number of offspring (i.e. one group for litter size=1, one for litter size=2 and so on), since each fetus obviously will contribute to the total weight of the mother. Alternatively, a rough distribution into ”small” and ”large” litters could have been made, with for instance small litters defined as ≤4 and large litters as >4. On the other hand, it was decided that further breakdown of the groups would not necessarily have resulted in more accurate results and thus it was not performed.

When interpreting the ”raw” weight data in Figure 3, it is important to note that the Grb10KO$^{m/+}$ mothers started out at an approximately 2 grams higher weight (21.2 g compared to 19.4 g for wild-types and 18.9 g for Grb10KO$^{+/p}$). Although Grb10KO$^{m/+}$ mice are born significantly larger than Grb10KO$^{+/p}$ and wild-types, it has recently been shown that they adjust to wild-type size such that they are not statistically different from wild-types as early as 8 days after birth, but only when suckling from a Grb10KO$^{m/+}$ mother (Cowley et al, 2014). The aforementioned study does not track the offspring weights beyond day 15 and it is therefore not known whether Grb10KO$^{m/+}$ offspring suckling from a wild-type mother adjust to statistical correlation beyond that point.

The average age at vaginal plug checking (i.e. the day after mating) in this experiment was 59 days. The mothers utilized here will come from a mixed background of wild-type and Grb10KO$^{m/+}$ mothers and it is therefore impossible to conclude whether the higher starting weight is due to chance or lingering effects of the genotypically different birth weights. Whatever the underlying reason for this discrepancy could be, the effects of the higher starting weights were nullified by generating Figure 4 where the starting weights were factored out.

Embryonic growth

As this data was gathered during a short period at an early point of the project, certain potential cohorts were absent from the final report. No Grb10KO$^{+/p}$ or wild-type offspring born to a Grb10KO$^{+/p}$ mother (BJmpREGJm and BJmpREGwt) on a regular diet were

available. Furthermore, the BJmpLPDJm cohort was too small (n=2) for statistical analysis. Therefore, the effect of the mother's diet on Grb10KO$^{+/p}$ embryonic growth could not be assessed.

The fact that wild-types born to regular diet wild-type mothers are smaller at E18.5 than wild-types born to LPD mothers is somewhat puzzling. Wild-types are not supposed to be protected from the effects of restricting the protein for the mothers so this data opposes our prediction that the low-protein diet during gestation will result in lower birth weight. However, it is here important to consider three things; litter size, number of samples and growth during the final day of gestation. Sex has proved to be a non-existant or very slight factor in influencing birth weight in mice (Grüneberg, 1944) whereas it is has been consistently shown that smaller litters will result in a higher average birth weight for each individual born (Enzmann & Crozier, 1935). The litter sizes for wild-type mice on a low-protein diet were 2 and 4, compared to 4, 6 and 8 for the regular diet mice. The notion that the data is at least partly skewed by difference in litter size therefore holds some merit. This will remain merely an assumption though since with so few litters, it would not be fruitful to break the data down according to litter size.

As always, more samples would result in more reliable statistics. This data was culled during a very early stage of the project. The growth during the final embryonic day can obviously not be derived from this data and to draw firmer conclusions, P0 pups (pups at the day of birth) would have to be analyzed. There were two main reasons for not doing so in this project however. Although all born pups are weighed daily, they are not biopsied and genotyped until 3 weeks of age according to the setup of this study, meaning that it was possible to collect more data from E18.5 embryos during the short time span of the author's visit. Secondly, this data is intended for publication by the research lab at a later point in time and considered too valuable to divulge at a preliminary stage.

It has been speculated that upregulation of *Grb10* expression in the offspring protects it from some of the detrimental health effects of feeding the mother a restricted diet. A recent study (Ivanova et al, 2012) reports a significantly elevated *Grb10* expression in the liver of 3-week-old offspring from mothers on a low-protein diet during gestation. In similar fashion, Radford et al (2012) report significant upregulation of *Grb10* in the liver of E16.5 embryos of mothers fed a calory-restricted diet late in gestation. They speculate that the significance of this finding is that *Grb10* upregulation in the liver suppresses the hepatic response to insulin and IGF2, preserving blood glucose for development of cardinal organs. Moreover, they show growth restriction at birth in the offspring following maternal dietary restriction, attributed to elevated *Grb10* in these individuals. Another theory that supports this biological response is the thrifty phenotype hypothesis, proposing that poor nutrition during gestation causes the mother to modify the development of the fetus in order to prepare it for a probable life of meager resources. (Hales & Barker, 1992). Long-term adult detrimental health effects such as obesity, diabetes and hypertension might in that case be an unwanted side-effect of life-saving regulations during early development.

These findings are however difficult to reconcile with the data gathered in this study. The

most obvious expected result that would be in accordance with the findings presented in the two aforementioned studies would be that wild-type pups born to LPD mothers (wtLPDwt) are significantly smaller than wild-type pups born to regular diet mothers (wtREGwt). As mentioned previously in this discussion, this is however not the case. Comparison of the BJmmLPDJm and BJmmLPDwt cohorts in this study offer results that might be consistent with Radford, et al (2012) and Ivanova, et al (2012), since wild-type embryos of Grb10KO$^{m/+}$ LPD mothers, thus expressing *Grb10*, are significantly smaller than Grb10KO$^{m/+}$ embryos of the same mothers (p=0.005, T-test, two-tailed distribution, two-sample equal variance). Nevertheless, this must be interpreted in the context of Grb10KO$^{m/+}$ offspring being ~30 % larger than wild-type littermates at birth, so the difference in this study could not be solely the result of a response to dietary restriction. Comparing wtLPDwt and wtREGwt with a larger number of samples and comparable litter sizes at P0 instead of E18.5 would be a much more accurate and efficient way of validating the findings of Radford, et al (2012) and Ivanova, et al (2012).

Presupposing that *Grb10* expression in the offspring reduces the fetal growth in response to maternal undernourishment, we are presented with the question whether Grb10KO$^{m/+}$ offspring are resistant to this adaptation, thus counteracting the growth-restricting effects of protein restriction. In theory, comparison of the BJmmREGJm and BJmmLPDJm cohorts could have shed some light on this question, since their weights would be expected to correlate with statistical significance if there is some sort of resistance mechanism in play. In the provided data, there is a statistical correlation between Grb10KO$^{m/+}$ offspring born to LPD versus regular diet Grb10KO$^{m/+}$ mothers (p=0.42), but with the low number of samples analyzed, this study provides very little indication either way. Furthermore, comparing the two groups does not reveal whether the presupposed resistance is mediated by the mother, the offspring or a joint effect of mother/offspring.

Another way of approaching the question is by examining Figure 8, where it is evident that Grb10KO$^{m/+}$ mothers on a low-protein diet on average give rise to heavier embryos at E18.5 than Grb10KO$^{m/+}$ mothers on a regular diet. Although this finding makes it tempting to speculate that Grb10KO$^{m/+}$ in the mother protects the offspring from the restricted growth effects of a LPD mother, it is very difficult to draw definitive conclusions about this because although the litter sizes are similar, the number of wild-types in the litters vary and since wild-type embryos are generally smaller than Grb10KO embryos, the number of wild-type littermates may skew the data. Adding further to the complexity of this issue, wild-type pups in a litter also containing Grb10KO$^{m/+}$ are larger than wild-type pups in an all-wild-type litter (Cowley et al, 2014). More litters with the same distribution of wild-types/Grb10KO embryos would be needed to make a congruent comparison.

Factoring out the embryos' genotypes in a similar way as the mothers' genotypes were factored out in Figure 8 would not have helped us determine whether Grb10KO$^{m/+}$ in the offspring mediates resistance to maternal undernourishment, since this would have yielded the same data as in Figure 7 (a Grb10KO$^{m/+}$ embryo can only ever be derived from a Grb10KO$^{m/+}$ mother).

The data presented by Ivanova, et al (2012) shows a significant increase in liver expression of *Grb10* of 3-week-old offspring from mothers on a LPD during lactation, but not from mothers on a LPD during gestation. However, they were both assessed at the same time (3 weeks after birth), allowing the possibility that *Grb10* levels rise similarly during gestation but drop off significantly in time for the measurement. *Grb10* upregulation may thus be a short-lasting response to maternal undernourishment.

Offspring ratio

The acquired results strongly suggest that other factors than chance account for the fact that a higher proportion of wild-types than knock-outs are being born to Grb10KO mothers. At 3 weeks of age, this discrepancy is very clear (p=0.003). However, this does not reveal anything about the point of time at which the disproportionate distribution of wild-type versus knock-out offspring is caused. Analysis of maternally-derived knock-out offspring at E18.5 narrows the time window, since there is no statistically significant difference between knock-out and wild-type offspring born to Grb10KO mothers at E18.5. Presumably this means that the majority of the Grb10KO$^{m/+}$ offspring losses are most likely not at the prenatal stage, but somewhere along the line from birth to 3 weeks. Analysis of the record of deaths between birth and 3 weeks of age however yields perplexing results, since the total number of deaths is too small to account for even half of the deviation from the expected 50/50 ratio, even if all the recorded deaths hypothetically were of Grb10KO$^{m/+}$ genotype. Thus, there must be an additional point of time not covered by any of the analyzed records at which a considerable proportion of the Grb10KO$^{m/+}$ offspring are lost. The most plausible explanation is that the deficiency is caused predominantly or at least partially in so close proximity to death that the litter has not yet been inspected by staff and thus not been noted in any records. It is well-known that mouse mothers are inclined to consume stillborn or weak pups shortly after birth (Grüneberg, 1944). The actual delivery of the pups is very rarely observed by staff, and perinatal mortality of a different strain of Grb10KO$^{m/+}$ offspring has been described before (Charalambous, 2003). The cause was speculated to be suffocation due to blood-filled alveoli and trachea, possibly caused by abnormal lung development. It is conceivable that the mice in this study perish for the same reason, but surveillance of their birth would have been needed in order to comment further on this theory.

Final remarks

Ideally, the author would have liked to have enough time and funding to study the long-term effects on offspring from LPD mothers versus regular diet mothers. It would have been interesting to observe whether Grb10KO$^{m/+}$ offspring are protected from developmental programming effects, such that they do not exhibit increased predisposition to obesity and diabetes (which would be tested by glucose tolerance tests) even if they are born to LPD mothers.

However, considering the set-up of the experiment: mice are mated at approximately 7 weeks of age, followed by 3 weeks of gestation (if the mating turns out successful), cross-fostering,

3 weeks until weaning of the pups and then 15 weeks in the high fat study, it becomes obvious that collecting enough samples to perform any sort of relevant analysis would require much more time than available and would go far beyond the scope of this student project.

Glucose testing and blood pressure testing on a smaller scale was considered, but ultimately rejected for several reasons. Although equipment for glucose tolerance tests was installed, a Home Office licence issue prevented it from being available for use in sufficient time. Blood pressure testing was also considered as a part of this project, but ultimately decided against due to the need of secluded testing areas, environmental acclimation of the mice, repeated measurements and supervising while operating the machine. Moreover, since the number of samples available at the time would not have permitted any statistically relevant analysis, it was decided not to be feasible within the framework of this study. Still, the data gathered and analysed in this thesis will be of assistance in further testing of this sort.

Conclusions

The findings indicate that the maternal restriction diet during gestation utilized in the project works well in the sense that no secondary effects are spilled over on the pregnant mothers. Ablation of *Grb10* has no significant impact on the growth of pregnant mice, irrespective of which allele is knocked out.

Furthermore, protein-restricted diet did not impact the growth of the pregnant mice analyzed in this study, nor was the food intake affected by which diet the mice were subjected to. These findings are linked in such a way that higher intake of a particular forage presumably will lead to increased growth. However, the fact that the food intake did not differ significally between the dietary regimes suggests that the protein content of the food does not have any significant impact on the growth of the mother. In turn, since the diet did not affect the growth of the mothers significantly, it seems that any hypothetical effect of genotype on growth during gestation is not counteracted and thus ”silenced” by a confounding dietary effect. Because of the limited amount of material available, it is important to stress the fact that these interpretations refer to the mice utilized in this study only and are not general conclusions.

It is also important to point out that although the protein content of the two diets differ, they have the same content of calories.

It is too early to tell whether the desired growth-restricting effects on offspring are achieved. These preliminary findings suggest that there may be some sort of problem in transmitting the developmental programming effects to the wild-type and/or Grb10KO$^{m/+}$ offspring. A previously unknown protecting effect of Grb10KO$^{m/+}$ pups and/or mothers could account for the lack of clear-cut effects. It could also be that more time is needed for the effects to show in the data.

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