

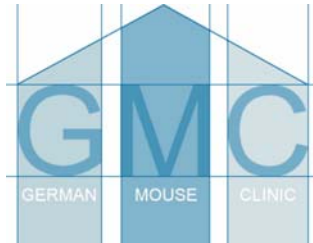
The

GERMAN MOUSE CLINIC

Report for Eyl

Helmut Fuchs, Valérie Gailus-Durner, Christoph Lengger, Beatrix Naton, Lore Becker, Ines Bolle, Markus Brielmeier, Julia Calzada-Wack, Claudia Dalke, Nicole Ehrhardt, Ralf Elvert, Tobias Franz, Elisabeth Grundner-Culemann, Wolfgang Hans, Sabine M. Hölter, Gabriele Hölzlwimmer, Marion Horsch, Anahita Javaheri, Svetoslav Kalaydjiev, Magdalena Kallnik, Martina Klempt, Eva Kling, Sandra Kunder, Thomas Lisse, Holger Maier, Ildikó Rácz, Claudia Reinhard, Ilka Schneider, Ralf Steinkamp, Johannes Beckers, Heidrun Behrendt, Dirk H. Busch, Jochen Graw, Gerhard Heldmaier, Heinz Höfler, Jack Favor, Thilo Jacob, Martin Klingenspor, Thomas Klopstock, Markus Ollert, Leticia Quintanilla-Martinez, Jörg Schmidt, Holger Schulz, Eckhard Wolf, Wolfgang Wurst, Andreas Zimmer, and Martin Hrabé de Angelis

The German Mouse Clinic



The German Mouse Clinic (GMC) was founded January 2002 at the GSF research center in Munich/Neuherberg to provide an open access platform for standardized mouse phenotyping. The GMC is supported by the National Genome Research Network (NGFN, <http://www.ngfn.de/>) and is a partner of the EUMORPHIA research program (<http://www.eumorphia.org/>).

In the GMC, experts from various fields of mouse genetics, physiology and pathology in close collaboration with clinicians work side by side at one location. We offer a primary phenotypic analysis of mouse mutants (more than 240 parameters/mouse) in the areas of allergy, behavior, bone and cartilage, cardiovascular diseases, clinical chemistry, energy metabolism, eye development and vision, immunology, lung function, molecular phenotyping, neurology, nociception, and pathology. Additional screens for host-pathogen interaction can be performed at the GBF Braunschweig. Secondary and tertiary screening for in depth analysis is offered by the different screens and is available on demand.

Director

Prof. Dr. Martin Hrabé de Angelis
Institute of Experimental Genetics
GSF National Research Center for
Environment and Health
Ingolstädter Landstraße 1
D-85764 Neuherberg / Munich
Tel.: 089-3187-3302
Fax: 089-3187-3500



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1 Summary

1.1 Primary Screening

In a primary screen 45 animals (26 controls, 19 mutants) of the Eyl mutant mouse line were analyzed in the German Mouse Clinic (GMC) in the screens Behavior, Dymorphology, Bone and Cartilage, Neurology, Eye, Clinical Chemistry, Immunology, Allergy, Nociception, Lung Function, Energy Metabolism, Molecular Phenotyping, and Pathology.

1.1.1 Overall Assessment of the Results

In several screens we detected genotype-specific differences which were more pronounced in females (Dymorphology and Immunology) or showed different expression in both sexes (Behavior, Energy Metabolism, and Pathology). We confirmed the eye phenotype and detected several new phenotypes which have not been described before for this mutant mouse line or the Aphakia mutant. The results are summarized briefly below.

1.1.2 Results by Screen

Behavior: The behavioral observation in the modified Hole Board demonstrated a sex-specific hypoactivity and an increase in anxiety as well as social affinity parameters in mutant females, whereas mutant males displayed less anxiety-related behaviour, increased arousal and unaltered locomotor activity levels.

Dymorphology, Bone and Cartilage: In the DEXA analysis all bone and body composition parameters were significantly changed in female mutants compared to control animals, but not in male mutants.

Neurology: In the primary screen (SHIRPA), the only distinctive feature beneath the palpebral closure was a decreased body weight in mutant females. In the secondary screen, male and female mutant mice showed reduced forelimb grip strength. This reduction could hint to a neuromuscular phenotype or to an alteration in central motor coordination.

Eye: All mutant mice expressed bilateral extreme microphthalmia-anophthalmia indicating that the mutated gene is critical for normal eye development.

Clinical Chemistry and Hematology: The creatine kinase activity of seven mutant males and of two mutant females was extremely high; additionally the AST concentrations of these animals were elevated. In combination with the result of grip strength testing this is a strong hint at a muscle disorder.

Immunology: We were able to detect some minor, but statistically significant differences affecting predominantly female mutants: increased frequencies of

T cells (both CD4⁺ and CD8⁺) and decreased Gr-1⁺ cells, decreased levels of IgG₃ and IgM. In addition, the level of IgG₃ was lower in male mutants as well.

Nociception: We found significantly different pain reactivity in mutant animals, hot plate latencies (parameter hind paw licking) were longer. This might hint at hypoalgesia.

Lung Function: These data suggest that male mutant mice have an altered spontaneous breathing pattern with more frequent but less effective breathing compared to control mice. Female mice have not been tested so far.

Energy Metabolism: Present data showed heavier females compared to males in the control group, while mutant mice exhibited lighter females. Genotype-specific differences originate from this inverted relation. From our experience body weight of C3H control males in the present batch is too low to serve as control group. However, there is also the possibility that body weight of control females is too high. The consequence is that due to the body weight differences in control mice, a reasonable interpretation concerning a metabolic phenotype is very difficult and present findings should be taken carefully.

Pathology: We confirmed the congenital microphthalmia in all mutant females and the reduced expression of dopamine transporter 1 in the midbrain in all mutants. In addition, we found congenital anophthalmia in all mutant males, an increased extramedullary hematopoiesis in the spleen in all mutants, and hepatosteatosis in seven of eleven analysed mutants. These latter findings suggest a possible metabolic phenotype.

In the screens **Allergy** and **Molecular Phenotyping**, no genotype-specific differences could be found.

1.2 Recommendations for Secondary Screening

Secondary screening is suggested from the screens Behavior, Dysmorphology, Neurology, Eye, Clinical Chemistry, Immunology, Nociception, and Lung Function. We would recommend analyzing:

Behavior Screen: To differentiate whether the hypoactivity is independent or due to a particular kind of anxiety, further analysis in secondary tests would need to be performed. In this case, it would be essential to know if mutants and controls are equally visually impaired. Furthermore, for detailed analysis of the neuropathological phenotype it would be essential to know which neurotransmitter systems are affected in which brain regions and whether alterations are similar in both sexes.

After clarification of these aspects, it might make sense to test Eyl mice in specific tests for anxiety-related behavior (elevated plus-maze, light/dark box, social interaction). In addition, continuous monitoring of home cage behavior might be useful to test for general alterations in activity levels and diurnal patterns.

Dysmorphology Screen: To confirm the observed differences in female mutants especially in the bone parameters more data should be collected in secondary analysis (μ CT, three-point bend test, pQCT) with another batch of mice.

Neurology Screen: We suggest performing our staircase test for skilled reaching and our gait analysis, as these tests are sensitive for extrapyramidal motor syndromes.

Eye Screen: In a secondary screening more detailed analysis could be done to confirm the microphthalmia and anophthalmia phenotype in female and male mutants.

Clinical Chemical Screen: For secondary studies, it would be interesting to confirm the primary findings of elevated CK activity and AST concentration in combination with a histological investigation of the muscles, which could be performed by the Pathology or Neurology Screen.

Immunology Screen: As the detected alterations might represent an interesting sex-specific immunological phenotype, we would like to repeat the primary screen with a smaller batch of mice. If the phenotype is confirmed, possibilities for secondary screening will be discussed with the provider.

Nociceptive Screen: We would suggest making further pain related studies to specify the pain sensitivity of this mutant mouse line in more detail. More detailed pain related studies would include:

- 1) Base studies e.g.,
 - a) von Frey filament test to study the reaction of animals to mechanical pain,
 - b) acetic acid test to study the reaction to visceral inflammation.
 - c) Tail flick test, to study whether the hypoalgesia has a spinal or supraspinal origin.
- 2) Chronic pain tests:
 - a) Formalin test to study the acute, nociceptive (early) and tonic, inflammatory (late) pain reaction of the same animals,
 - b) Carrageenan test to study the reaction to inflammation.

The results of the whole set of experiments will provide a complete picture of the pain reactivity of this mutant mouse line.

Lung Function Screen: Due to the low number of animals available we suggest analyzing a second batch to confirm the findings in the males and to investigate the breathing pattern of females.

Please contact Valérie Gailus-Durner to discuss further steps and details.

2 General Part

2.1 The Role of the Gene

The *Eyl* mutant mouse line was generated by a spontaneous mutation. Due to its resemblance of the "aphakia" mutant line which is characterized by small eyes that lack a lens, the *Pitx3* (Pituitary homeobox 3, OMIM 602669) gene was sequenced. *Pitx3* is a transcription factor involved in organ development and tyrosine hydroxylase expression. The *Eyl* mutation is caused by a single base insertion in the coding region which results in a frame shift. As a consequence the OAR domain is abolished. The OAR domain is important for transactivation of *Pitx3* target genes.

Expression analysis of *Pitx3* in the mouse supported a unique role in early ocular development (Graw, 2003), with later expression extending to the midbrain (mesencephalic dopaminergic neurons), tongue, incisors, sternum, vertebrae, and limbs (Gage *et al.*, 1999). The findings strongly suggested the role of PITX3 in ASMD (anterior segment mesenchymal dysgenesis; OMIM 107250) and cataracts and provided new evidence of the contribution of the RIEG/PITX gene family to the developmental program underpinning normal eye formation. The allelic mutant Aphakia (*Pitx3*^{-/-}) shows progressive degeneration in dopaminergic neurons of the substantia nigra and at about 9 month of age, some features of a Parkinson-like behavior (van den Munckhof *et al.*, 2003).

2.2 Known Phenotypes

- Homozygote mutants show complete absence of lens induction at the placode state.
- Preliminary results show distorted structure of substantia nigra.
- Slightly reduced fertility

All further findings which will be shown in this report we consider as new.

2.3 Possible disease models

- Anterior segment mesenchymal dysgenesis (ASMD) *Pitx3* on chromosome 10q25 (Semina *et al.*, 1998),
- Parkinson-like disorders (van den Munckhof *et al.*, 2003).

2.4 Mice

2.4.1 Number and kind of mice

As described by the owner, a spontaneous mutant arose during repeated selective inbreeding of a chromosome 7 fragment from 102-strain onto C3H/Nhg

strain. Since the PITX3 gene maps to chromosome 19, however, it is unlikely that the selective ingression of the 102 allele onto chromosome 7 of this line has any causative relation with the phenotype.

Table 1: Eyl mice provided for analysis.	
Numbers in brackets indicate animals which were kept in reserve.	
Genotype / Sex	Number of Animals
Mutant female	7 1 died
Mutant male	12
Control female	15 (+3) 1 died
Control male	11

As described by the sender, the mice analyzed were a 6th backcross generation to C3H.

2.4.2 Housing conditions

In the GMC mice are housed in type II polycarbonate cages in individually ventilated caging (IVC) systems (VentiRack Bioscreen TM, Biozone, Margate, UK) on wood fibre (Altromin, Lage, Germany). The IVCs operate with positive pressure. Mice are transferred in weekly intervals to new cages with forceps in Laminar Flow Class II changing stations. Mice are fed with irradiated standard rodent high energy breeding diet (Altromin 1314) and given semidemineralized filtered (0.2 µm) water *ad libitum*. Light is adjusted to a 12h/12h light/dark cycle; temperature and relative humidity are regulated to 22 ± 1°C and 55 ± 5%, respectively. In specified modules husbandry conditions are adjusted according to the experiment requirements (See corresponding sections). All people attending the facility completely change their garment (jackets and trousers autoclaved) and shoes and wear caps and masks before entering the GMC (Brielmeier *et al.*, 2002).

Outbred 8-week-old male SPF Swiss mice are used as sentinels and kept on a mixture of new bedding and aliquots of soiled bedding (50:50) from all cages of the IVC rack. In addition, the sentinels were also exposed to soiled air from all “upstream” cages of the IVC rack. Health monitoring is carried out by on-site examination of the sentinel mice by certified laboratories according to FELASA recommendations (www.felasa.org).

Mice are kept according to the German laws. Tests were carried out by authority of the Regierung von Oberbayern.

2.5 Workflow

2.5.1 Standardized workflow for the primary screen in the German Mouse Clinic

Mouse mutants entering the GMC are examined in a primary screen according to the following standard workflow (Fig. 1; Gailus-Durner, Fuchs *et al.*, 2005). Analyzed parameters are listed in Table 2.

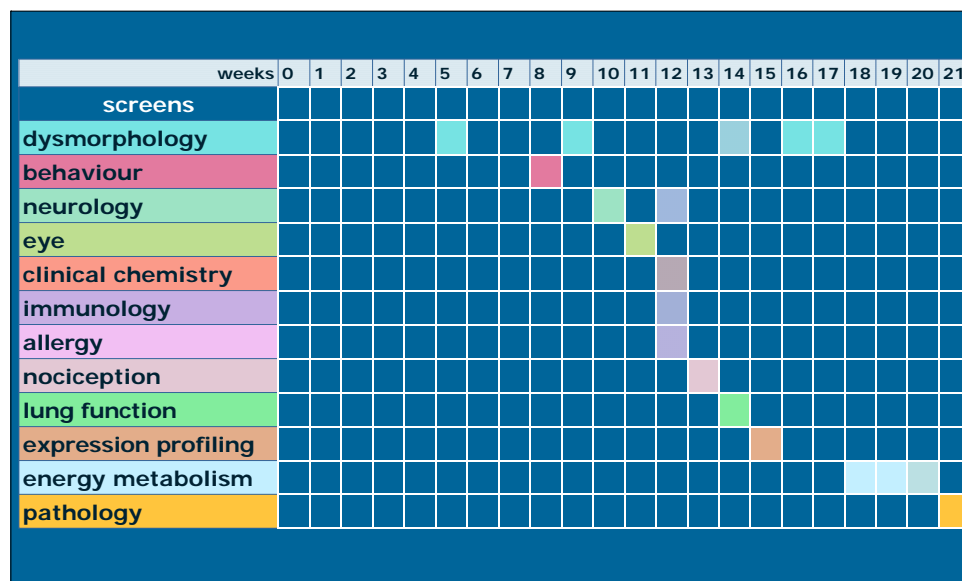



Figure 1: Workflow of the primary screen

Explanation below,  Analysis of blood-based parameters.

After the mice arrive at the GMC, they are acclimatized in the new environment for one week. The males then start in the Behavior Screen. There they stay for three weeks. Directly after the Behavior Tests, the anatomical inspection of the Dysmorphology Screen is performed. In the next week, the Neurology Screen is applied. One week later the mice go through the tests of the Eye Screen. When the mice were 12 weeks old, blood is taken, and samples are distributed to the blood-based screens for Clinical Chemistry, Immunology, Allergy and the Lactate test. One week later, the animals are tested in the Nociceptive Screen. Two weeks after testing of the first blood sample, a second sample is taken to confirm outliers, and to supply the Dysmorphology Screen with material for determination of blood-based bone-related parameters. In parallel, 10 mutant animals (five males / five females) and 10 controls (five males / five females) leave the animal facility for the Lung Function Analysis, which for technical reasons is located elsewhere. These animals are, for hygienic reasons, not allowed to re-enter the German Mouse Clinic. The females go directly to Pathology. The males are used to freeze organs for future expression profiling on demand (remaining organs from those animals are analyzed by the Pathology). All other animals go through the bone and

cartilage tests of the Dysmorphology Screen, and then stay three weeks in the Metabolic Screen. After completion of the primary screen, all animals end up in the Pathology.

The screening of female animals starts one week later and follows the same workflow (with the exception of Expression Profiling sampling). Deviations from our Standard operation procedure (SOP) are listed below; please take the specific number of analyzed animals from the sections of the applied screen.

2.5.2 Applied screens

The GMC standard workflow for the primary screen as described above was applied to analyze the Eyl mice. As the demanded number of 60 animals (15 mice per sex per genotype) could not be delivered, the workflow was adapted to the available number of animals. Therefore the lung function analysis was performed only with male animals. Some parameters measured in the blood based screens could not be determined in all animals, as it was not possible to get the needed amount of blood from these animals. Two animals died during the primary screen after blood withdrawal (Table 1) and thus could not be analyzed for all parameters. One control female could be replaced by an animal kept in reserve for this purpose.

2.5.3 Quality Management

As a routine quality control, we take blood samples from all animals for serological tests of the sanitary status of all mice after completing the GMC primary screen. The serum is tested for MHV (BioDoc, Hannover). We chose MHV as a "sentinel" pathogen, as it is one of the most common viruses in mouse facilities worldwide and it is transmitted easily. To be open for collaboration for as many partners as possible, we allow MHV positive animals to enter our facility.

Additionally, tail clips were taken of all mice, which were delivered to the Pathology Screen (3.12.) and Expression Profiling Screen (3.10.). All samples were immediately frozen and stored in liquid nitrogen. Tail clips collected may be used for re-genotyping in any case of doubt.

2.6 Statistical Analysis of Data

If not otherwise stated, data of males and females was analyzed separately comparing mutant and control data using a Student's t-test. Sex differences within the mutant or the control group also were determined with a t-test. Tables summarizing the data will show mean \pm standard error of the mean. Significant differences are indicated stepwise from 0.05, 0.02, 0.01, 0.001 to 0.0001.

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Abbreviations and Wording

Pitx3	Pituitary homeobox 3
Eyl	eyeless (mutant mouse line) caused by a mutation in the Pitx3 gene
ASMD	anterior segment mesenchymal dysgenesis
GMC	German Mouse Clinic
IVC	individually ventilated cage
control	homozygous wild-type control littermate, <i>Pitx3</i> ^{+/+}
mutant	homozygous mutant, <i>Pitx3</i> ^{-/-}
FELASA	Federation of European Laboratory Animal Science Associations, 25 Shaftesbury Avenue, London W1D 7EG, UK, www.felasa.org

Table 2: Primary Screen at GMC

Screens	Goal	Methods
Dysmorphology, Bone and Cartilage	morphological analysis of body, skeleton, bone and cartilage	morphological observation, bone densitometry, X-ray, AVL analyzer, micro-computer tomography
Behavior	locomotor, exploratory, emotional and social behavior, object recognition memory	modified hole board
Neurology	assessment of muscle, spinocerebellar, sensory, and autonomic function	modified SHIRPA protocol
Eye	assessment of morphological and functional alterations of the eye	electroretinography, slit lamp biomicroscopy
Clinical Chemistry	determination of clinical-chemical and hematological parameters in blood	blood autoanalyzer, ABC-animal blood counter
Immunology	analysis of peripheral blood samples for immunological parameters	flow cytometry, ELISA
Allergy	analysis of total plasma IgE	ELISA
Nociception	detection of altered pain response	hot plate assay
Lung function	assessment of alterations in breathing patterns	whole body plethysmography (Buxco®)
Expression Profiling	RNA expression profiling	DNA-chip technology
Energy Metabolism	measurement of altered body weight regulation, body temperature and energy balance	bomb calorimetry
Pathology	microscopic and macroscopic examination	histology, immunochemistry

3 Specific part

3.1 Behavior Screen

3.1.1 Summary

Genetic studies in the mouse are important for the elucidation of molecular pathways underlying behavior. The goal of this endeavor is not only the identification of genes that control brain function and influence behavior, but also understanding of genetic factors involved in human psychiatric disorders (Tarrantino & Bucan, 2000; Bucan & Abel, 2002). These disorders are associated with quantitative phenotypes called “intermediate traits” or endophenotypes, some of which, in contrast to the full complex disorder, can readily be modeled in mice. These traits are risk factors which are considered to be closer to the genetic etiology than the full syndrome. Examples are anxiety in depression, prepulse inhibition and working memory deficits in schizophrenia, and social interaction deficits in autism and schizophrenia (Seong et al., 2002; Gottesman & Gould, 2003; Inoue & Lupski, 2003).

In the attempt to efficiently screen for candidate endophenotypes within a limited time frame, we use the modified Hole Board test as primary screen in the behavioral phenotyping module of the GMC. This test allows the comprehensive analysis of a range of parameters known to be indicative of behavioral dimensions such as locomotor activity, exploratory behavior, arousal, emotionality, memory and social affinity in a single short test (See Ohi *et al.*, 2001).

The behavioral observation in the modified Hole Board demonstrated a sex-specific hypoactivity and an increase in anxiety as well as social affinity parameters in mutant females, whereas mutant males displayed less anxiety-related behaviour, increased arousal and unaltered locomotor activity levels.

3.1.2 Mice

Mice were housed with food and water *ad libitum* under standard laboratory conditions. Animals were separated based on sex, but not genotype. They entered the laboratory at the age of six weeks, were given two weeks for acclimatization and were tested at the age of eight weeks. Three days before testing, an object (metal cube) was placed into the home cage and removed one day before testing. In this screen, 22 female mice (15 wt, 7 mutants) and 23 male mice (11 wt, 12 mutants) were available for analysis.

3.1.3 Material and Methods

The modified hole board test was carried out according to the procedures described by Ohi *et al.*, 2001. The test apparatus consisted of a test arena (100 x 50 cm), in the middle of which a board (60 x 20 x 2 cm) with 23 holes (1.5 x 0.5 cm) staggered in three lines with all holes covered by movable lids was placed, thus representing the central area of the test arena as an open

field. The area around the board was divided into 12 similarly sized quadrants by lines taped onto the floor of the box (See Ohl *et al.*, 2001). Both box and board were made of dark grey PVC. All lids were closed before the start of a trial. For each trial, an unfamiliar object (a blue plastic tube lid, similar in size to the metal cube) and the familiar object (metal cube) were placed into the test arena with a distance of 2 cm between them. The illumination levels were set at approximately 150 lux in the corners and 200 lux in the middle of the test arena.

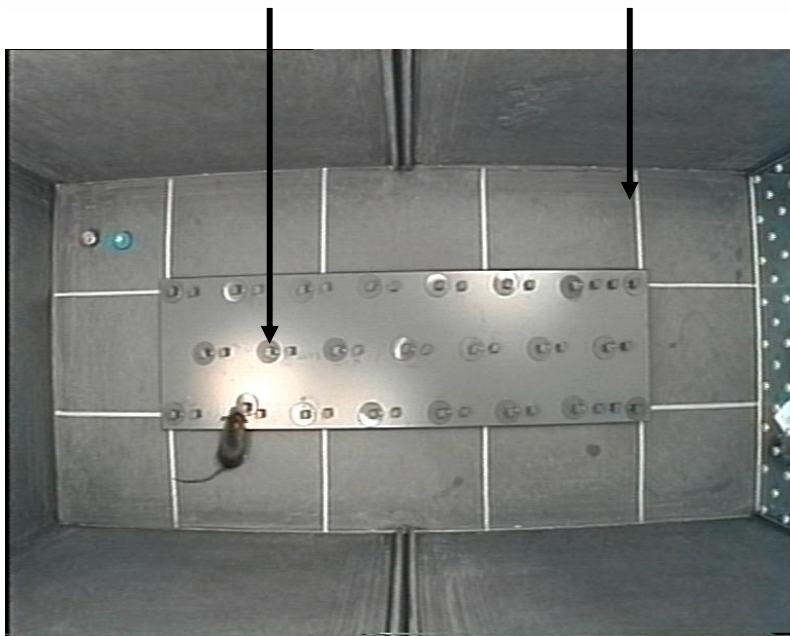
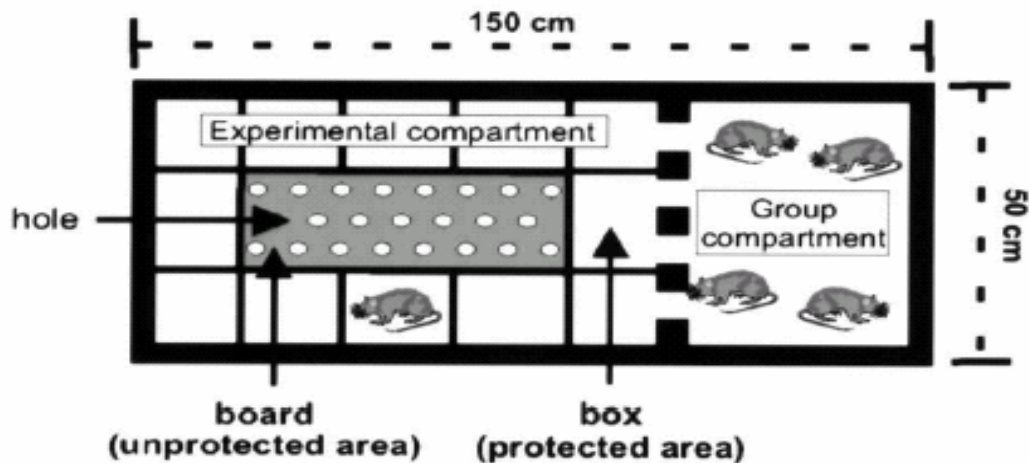


Figure 2: Test Arena for modified hole board test (Ohl *et al.*, 2001).

For testing, each animal was placed individually into the test arena and allowed to explore it freely for 5 min. The animals were always placed into the test arena in the same corner next to the partition, facing the board diagonally. The two objects were placed in the corner quadrant diametrical to the starting

point. During the 5 min trial, the animal's behavior was recorded by a trained observer with a hand-held computer. Data were analyzed by using the Observer 4.1 Software (Noldus, Wageningen). Additionally, a camera was mounted 1.20 m above the center of the test arena, and the animal's track was videotaped and its locomotor path analyzed with a video-tracking system (Ethovision 2.3, Noldus, Wageningen). After each trial, the test arena was cleaned carefully with a disinfectant.

Data were statistically analyzed using SPSS software (SPSS Inc, Chicago, USA). The chosen level of significance was $p < 0.05$.

3.1.4 Results

Behavioral analysis of spontaneous activity in a novel environment, as measured by the **modified Hole Board** test, revealed reduced forward locomotor activity in mutant females, indicated by reduced line crossings (Table 4), total distance travelled (Table 5), number of turns (Table 5) and travelling with lowered speed of movement (mean and maximum velocity, Table 5) as compared to control females. Additionally, mutant females did more changes in direction while travelling (enhanced mean turn angle, angular velocity and meander, Table 5).

Concerning anxiety, mutant females exhibited a reduction in locomotor related anxiety parameters (board entry latencies and frequency, Table 5), whereas the most specific anxiety-related parameter (board entry duration, Table 4) did not differ from control females. In contrast, mutant males displayed clearly less anxiety-related behaviours as measured by reduced latency to board entry, increased number of board entries and time spent on the board (Table 5). Accordingly, mutant males moved on average in greater distance to the wall and, consequently, in shorter distance to the board (Table 5).

Regarding horizontal exploration, exploration on the board was reduced in mutant females (hole exploration frequency, Table 4) and enhanced in mutant males (hole exploration latency, Table 4) compared to controls, corresponding to their behaviour towards the board as described above. Besides, mutant females explored the two objects within the mHB less intensive than control females, indicated by enhanced latency, reduced number and duration of unfamiliar as well as familiar object exploration (Table 4). Also vertical exploration was attenuated in mutant females. They reared less (rearings in box and on board, Table 4) and later (rearing in box latency, Table 4).

Furthermore, mutant females exhibited altered social affinity as they spent more time at the partition (group contact duration, Table 4) and did *tendentially* earlier social contacts (group contact latency, Table 4) and mutant males exhibited more arousal as compared to controls, indicated by higher latencies to first grooming behavior (Table 4).

3.1.5 Discussion

The **primary behavioral observation** in the modified Hole Board revealed hypoactivity in **mutant females** as indicated by the reduction in forward locomotor activity (line crossings, total distance moved, turns, velocity). Alterations in anxiety-related behaviour in mutant females could reflect this hypoactivity as they were limited to alterations in *locomotor related* anxiety parameters. This interpretation could be supported by the observation that reduced activity was also evident in horizontal (hole and object exploration) as well as vertical exploratory behaviour (rearing in the box) in mutant females.

In contrast, analysis of **male Eyl** mice revealed *less* anxiety related behaviour (earlier and more board entries, more time on board, more distance to wall and less to the board) and more arousal in mutant males. Earlier hole exploration in mutant males was secondary to their increased activity towards the board.

Table 3: Evaluation of the Behavioral Phenotype	
Behaviors which are considered affected in mutants due to the pattern of significantly altered parameters are marked in red.	
Behavior	Measured parameters
Forward locomotor activity	Line crossings (number), Total distance travelled
Vertical exploratory behavior	Rearings in the box (number, latency), Rearings on the board (number)
Speed of movement	Mean and maximum velocity
Immobility	Time spent immobile
Risk assessment	Stretched attends
Anxiety-related behavior	Latency until first board entry, Time spent on board, Board entries
Horizontal exploratory behavior	Hole exploration, object exploration (obj);
Grooming behavior	Latency to grooming, Time spent grooming, Number of groomings
Defecation	Latency to defecation, Number of boli
Social affinity	Group contacts, Time spent at partition
Familiar object exploration	Latency to obj. expl., Time spent in obj. expl., Number of obj. expl.
Unfamiliar object exploration	Latency to obj. expl., Time spent in obj. expl., Number of obj. expl.

It should be noted that behaviours towards the exposed area represented by the board and linked to anxiety are valid for mice with normal vision, but in blind animals, it is uncertain whether alterations in these parameters are specific. However, the observed hypoactivity in combination with travelling with increased hesitation, indicated by more changes in direction, and increased group contact in females might well reflect enhanced anxiety. As mutant males displayed behaviours indicating less anxiety, differences in behavioral

alterations between the sexes might reflect sex-specific differences in the expression of the same underlying physiological mechanism.

To differentiate whether the hypoactivity is independent or due to a particular kind of anxiety, further analysis in secondary tests would need to be performed. In this case, it would be essential to know if mutants and controls are equally visually impaired. Furthermore, for detailed analysis of the neuropathological phenotype it would be essential to know which neurotransmitter systems are affected in which brain regions and whether alterations are similar in both sexes.

After clarification of these aspects, it might make sense to test Eyl mice in specific tests for anxiety-related behavior (elevated plus-maze, light/dark box, social interaction). In addition, continuous monitoring of home cage behavior might be useful to test for general alterations in activity levels and diurnal patterns.

Since such an analysis would be very time-consuming, it would have to be planned well in advance if to be performed in collaboration with us.

3.1.6 References

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Table 4: Results of behavioral observation in the modified hole board testData are presented as mean \pm standard error of mean.

Parameter	Control (A)			Mutant (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=11)	(n=15)	<i>p - value</i>	(n=12)	(n=7)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
Line crossing [frequency]	60.45 \pm 12.99	97.73 \pm 6.97	N.A.	53.75 \pm 6.29	28.86 \pm 6.73	N.A.	n.s.	p<0.01
Line crossing [latency]	2.06 \pm 0.81	1.03 \pm 0.17	N.A.	0.88 \pm 0.13	2.84 \pm 2.06	N.A.	n.s.	n.s.
Rearings in box [frequency]	12.27 \pm 3.63	28.53 \pm 1.93	N.A.	8.08 \pm 1.67	5.43 \pm 1.38	N.A.	n.s.	p<0.01
Rearings in box [latency]	76.81 \pm 23.36	32.64 \pm 4.17	N.A.	83.64 \pm 20.11	80.7 \pm 17.38	N.A.	n.s.	p<0.01
Hole exploration [frequency]	13.45 \pm 4.31	17.87 \pm 2.33	N.A.	18.25 \pm 2.25	4.86 \pm 1.7	N.A.	n.s.	p<0.01
Hole exploration [latency]	117.27 \pm 38.87	49.48 \pm 11.17	N.A.	20.09 \pm 5.15	57.67 \pm 41.33	N.A.	p<0.05	n.s.
Hole visit [frequency]	0 \pm 0	0 \pm 0	N.A.	0 \pm 0	0 \pm 0	N.A.	n.s.	n.s.
Hole visit [latency]	300 \pm 0	300 \pm 0	N.A.	300 \pm 0	300 \pm 0	N.A.	n.s.	n.s.
Board entry [frequency]	4.27 \pm 1.58	5.4 \pm 1.05	N.A.	8 \pm 1.52	1.43 \pm 1.02	N.A.	n.s.	p<0.05
Board entry [latency]	161.21 \pm 40.44	91.66 \pm 19.65	N.A.	45.39 \pm 16.94	236.7 \pm 43.62	N.A.	p<0.05	p<0.01
Board entry [total duration %]	7.8 \pm 2.93	7.87 \pm 1.37	N.A.	22.15 \pm 2.88	5.33 \pm 3.53	N.A.	p<0.01	n.s.
Rearing on board [frequency]	1.27 \pm 0.62	0.8 \pm 0.37	N.A.	0 \pm 0	0 \pm 0	N.A.	n.s.	p<0.05
Rearing on board	250.03 \pm 23.4	258.28 \pm 16.6	N.A.	300 \pm 0	300 \pm 0	N.A.	n.s.	n.s.

[latency]								
Risk assessment [frequency]	0.55 ± 0.31	0.07 ± 0.07	N.A.	0.42 ± 0.19	2.57 ± 1.43	N.A.	n.s.	n.s.
Risk assessment [latency]	251.73 ± 27	281.11 ± 18.89	N.A.	219.32 ± 35.2	201.6 ± 46.97	N.A.	n.s.	n.s.
Group contact [frequency]	7.82 ± 1.02	6.8 ± 0.44	N.A.	6.67 ± 0.93	9 ± 1.41	N.A.	n.s.	n.s.
Group contact [latency]	21.1 ± 4.03	22.94 ± 4.3	N.A.	42.53 ± 10.06	45.06 ± 10.75	N.A.	n.s.	p=0.06
Group contact [total duration %]	20.45 ± 4.17	12.61 ± 1.54	N.A.	13.69 ± 2.4	37.32 ± 7.71	N.A.	n.s.	p<0.01
Grooming [frequency]	1.45 ± 0.43	0.87 ± 0.09	N.A.	0.67 ± 0.14	1.14 ± 0.26	N.A.	n.s.	n.s.
Grooming [latency]	173.36 ± 20.81	195.47 ± 17.67	N.A.	250.65 ± 12.56	168.54 ± 42.12	N.A.	p<0.01	n.s.
Grooming [total duration %]	3.99 ± 1.59	1.54 ± 0.27	N.A.	0.88 ± 0.25	1.47 ± 0.37	N.A.	n.s.	n.s.
Defecation [frequency]	0.45 ± 0.28	0.2 ± 0.11	N.A.	1.08 ± 0.29	1.14 ± 0.55	N.A.	n.s.	n.s.
Defecation [latency]	232.74 ± 34.99	282.23 ± 11.39	N.A.	180.94 ± 31.62	189.93 ± 48.95	N.A.	n.s.	n.s.
Unfamiliar object exploration [frequency]	2.82 ± 0.97	4.07 ± 0.46	N.A.	1.42 ± 0.47	0.71 ± 0.47	N.A.	n.s.	p<0.01
Familiar object exploration [frequency]	3.36 ± 1	4.47 ± 0.43	N.A.	1.33 ± 0.38	0.43 ± 0.43	N.A.	n.s.	p<0.01
Unfamiliar object exploration [latency]	149.65 ± 41.88	55.97 ± 19.43	N.A.	199.04 ± 29.38	257.37 ± 39.1	N.A.	n.s.	p<0.01
Familiar object exploration [latency]	133.45 ± 41.27	38.24 ± 9.39	N.A.	199.47 ± 27.34	289.8 ± 10.2	N.A.	n.s.	p<0.01

Unfamiliar object exploration [total duration %]	0.99 ± 0.35	1.44 ± 0.17	N.A.	1.01 ± 0.35	0.41 ± 0.27	N.A.	n.s.	p<0.01
Familiar object exploration [total duration %]	0.96 ± 0.35	1.1 ± 0.15	N.A.	1.27 ± 0.54	0.2 ± 0.2	N.A.	n.s.	p<0.01
Object Index	0.05 ± 0.13	0.13 ± 0.08	N.A.	-0.08 ± 0.17	0.52 ± 0.48	N.A.	n.s.	n.s.

Table 5: Video-tracking results regarding locomotor behavior

Data are presented as mean \pm standard error of mean.

Parameter	Control (A)			Mutant (B)			A-B	A-B
	Male	Female		Male	Female		Male	Female
	(n=11)	(n=15)	<i>p - value</i>	(n=12)	(n=7)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
Total Distance Moved [cm]	2009.83 \pm 324.35	2612.62 \pm 165.61	N.A.	1898.63 \pm 183.51	1141.06 \pm 176.05	N.A.	n.s.	p<0.01
Mean Velocity [cm/sec]	13.36 \pm 1.64	17.69 \pm 0.81	N.A.	12.72 \pm 1.01	9.59 \pm 1.04	N.A.	n.s.	p<0.01
Maximum velocity [cm/sec]	50.6 \pm 3.51	55.7 \pm 2.39	N.A.	46.02 \pm 3.19	44.72 \pm 3.4	N.A.	n.s.	p<0.01
Turns [Frequency]	1167.73 \pm 149.65	1415.5 \pm 54.83	N.A.	1155.87 \pm 93.9	770.14 \pm 107.16	N.A.	n.s.	p<0.01
Mean Turn Angle [degrees]	45.26 \pm 4.66	30.55 \pm 0.97	N.A.	37.57 \pm 2.81	55.29 \pm 5.59	N.A.	n.s.	p<0.01
Angular Velocity [degrees/sec.]	229.83 \pm 20.16	178.39 \pm 4.19	N.A.	198.19 \pm 13.51	256.87 \pm 25.14	N.A.	n.s.	p<0.01
Absolute Meander [degrees/sec.]	33.9 \pm 3.87	21.9 \pm 0.88	N.A.	28.18 \pm 2.27	42.78 \pm 4.58	N.A.	n.s.	p<0.01
Board entry max. duration [sec]	6.96 \pm 2.49	10.14 \pm 2.05	N.A.	17.37 \pm 2.55	8.97 \pm 5.4	N.A.	p<0.01	n.s.
Distance to Wall [cm]	5.52 \pm 0.82	6.4 \pm 0.24	N.A.	7.37 \pm 0.65	5.68 \pm 0.89	N.A.	p<0.01	n.s.
Distance to Board [cm]	10.65 \pm 0.78	9.39 \pm 0.21	N.A.	8.54 \pm 0.63	10.49 \pm 0.93	N.A.	p<0.01	n.s.

3.2 Dysmorphology, Bone and Cartilage

3.2.1 Summary

In the Dysmorphology, Bone and Cartilage Screen of the German Mouse Clinic mice are analyzed for morphological abnormalities in different organ systems with special focus on bone and cartilage development and homeostasis. We adapted the successful dysmorphology screening protocol from the Munich ENU-Mutagenesis Screen (Hrabé de Angelis *et al.* 2000) for use in the German Mouse Clinic. The nomenclature of the parameters was adapted according to the “Phenoslim” wording (<http://www.informatics.jax.org/userdocs/phenoslim.shtml>). Further tests for defects in bone development and homeostasis were taken over from human diagnosis, and were adapted for the use in mice analysis. Such tests include: X-ray analysis, bone densitometry and, in a limited number of animals, micro-computer tomography.

A total of 45 animals of Eyl mutant mouse line were analyzed in the Dysmorphology, Bone, and Cartilage module of the German Mouse Clinic. In the morphological investigation via visual inspection and X-ray analysis, no new genotype specific differences were detected between *Pitx3*-mutant mice and wild-type littermate controls. In the DXA analysis all bone and body composition parameters were significantly changed in female mutants compared to control animals, but not in male mutants.

3.2.2 Mice

Twenty-three male (11 controls, 12 mutants) and 22 female (15 controls, seven mutants) mice were analyzed by morphological inspection at the age of 9 weeks. Blood was taken at the age of 14 weeks for determination of ionic calcium from 16 mutant and 25 control animals, and 16-week-old mutants (12 animals) and controls (21 animals) entered the bone density and X-ray analysis.

3.2.3 Material and Methods

The Dysmorphology, Bone and Cartilage module of the German Mouse Clinic analyzed the mice in different phases:

1. At the age of 5 weeks, i.e. when the mice entered the facility, the general physical condition and health were checked;
2. At the age of 9 weeks, a morphological observation as a whole-body checkup was performed;
3. The ionized fraction of calcium in blood was analyzed in 14-week-old mice, and
4. At the age of 16 to 17 weeks, X-ray analysis and bone densitometry were performed.

Morphological Observation

The animals were screened using the protocol for morphological analysis from Fuchs *et al.* (2000) as adapted for the German Mouse Clinic.

Using a clickbox (supplied by the MRC Institute of Hearing Research, Nottingham, UK) we tested the mice's ability to hear a sound of 20 kHz. The reaction of the animals was classified into six categories (0=no reaction at all, 1=no Preyer reflex, 2= retarded reaction, 3= normal reaction, 4= strong reaction, 5= particularly strong reaction).

Ionized calcium Analysis

Equipment: AVL 9180 Electrolyte Analyzer (distributed by Roche Diagnostics GmbH, Mannheim, Germany)

cleaning solution and conditioning solution (Roche),

ISEtrol Quality Control Solutions (Roche),

lithium-heparin polypropylen tubes,

glass capillary (0.8 mm diameter, 32 mm length, without heparin; special product of Laborteam K+K, Munich).

Quality control: Calibration of the system and quality control were performed at intervals recommended and with solutions provided by the manufacturer. The results from the quality control were recorded by the system. Before blood measurement, daily cleaning, conditioning and calibration of the analyzer were performed.

Procedure: Blood (100 µl) was collected from anesthetized mice in lithium heparin tubes and transferred directly to the analyzer. Values were transferred directly to the database.

X-ray Images

Equipment: Faxitron X-ray Model MX-20 (Specimen Radiography System, Illinois, USA),

NTB Digital X-ray Scanner EZ 40 (NTB GmbH, Diepholz, Germany),

Quality control: Calibration of the system is done in monthly intervals,

Settings: Voltage 25 kV, integration time 40 ms,

Procedure: The anesthetized mouse was fixed on an X-ray-permeable plate and placed in the machine. Using iX-Pect software supplied by the manufacturer of the X-ray scanner, the image was taken and analyzed. Analysis was done qualitatively by visual inspection of the images as well as quantitatively by using the ruler tool of iX-Pect software.

Bone density analysis

Equipment: pDEXA Sabre X-ray Bone Densitometer (Norland Medical Systems. Inc., Basingstoke, Hampshire, UK; distributed by Stratec Medizintechnik GmbH, Pforzheim, Germany),

Quality control: Calibration of the system was done in daily intervals using the QC and the QA phantoms delivered by the manufacturer. Results from the quality control were recorded by the system.

Settings: Scan speed 20 mm/s, Resolution 0.5 mm x 1.0 mm, HAW 0.020

Procedure: After anesthesia, the weight and length of the mouse were recorded, and the mouse was placed in the analyzer. After a scout scan, the area of interest was optimized and the measure scan started.

Data-analysis: For analysis of the data, regions have to be defined. The standard analysis comprises a whole body analysis as well as a whole body analysis excluding the skull.

Statistical analysis of data

Analysis of quantitative data sets was carried out using StatView software package (SAS Corporation).

3.2.4 Results and Discussion

Forty-five animals of the Eyl mutant mouse line were analyzed in the Dymorphology, Bone, and Cartilage module of the German Mouse Clinic. In the morphological investigation via visual inspection and X-ray analysis no new genotype-specific differences were found between mutant mice and wild-type littermate controls (Tables 5 and 6). The abnormal eye phenotype could be confirmed (microphthalmia, anophthalmia). In quantitative X-ray data, the lumbar vertebrae height was significantly decreased in female mutants compared to controls (Table 8). In the Clickbox test (Table 7) to test the hearing ability of the mice, we observed a normal reaction in mutants and controls.

In the bone densitometry using DEXA analysis (Table 8), we detected significantly reduced BMD and pBMD, but significantly increased sBMD (BMD related to the body weight) values in female mutants compared to controls. BMC and bone content (BMC related to the body weight) were significantly decreased in female mutants compared to controls. Compared to our C3HeB/FeJ baseline data (data not shown) most values of male control animals were already lower. This might explain that we found no differences between male mutants and controls.

Body length, body weight, fat mass, fat content and subcutaneous fat were significantly decreased, lean mass and lean content were significantly increased in female mutants compared to control animals. Differences between female mutants and controls in bone mineral density might be due to changes in body composition and thus be secondary effects.

The analysis of ionic calcium in the blood at the age of 14 weeks revealed no significant difference between mutants and controls (Table 8).

To confirm the observed differences in female mutants especially in the bone parameters more data should be collected in secondary analysis (μ CT, three-point bend test, pQCT) with another batch of mice.

Raw data will be available on demand.

3.2.5 References

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Abbreviations

BMC	bone mineral content
BMD	bone mineral density
pBMD	partial bone mineral density (excluding skull)
sBMD	specific bone mineral density

Table 6: Results from the morphological inspection				
Parameter	Male		Female	
	control	mutant	control	mutant
Growth				
normal	11	12	15	7
Weight				
normal	11	12	15	7
Body size				
normal	11	11	15	7
smaller	-	1	-	-
Eye				
normal	11	-	15	-
microphthalmia, anophthalmia	-	12	-	7
Coat hair growth				
normal	11	12	15	7
Coat hair texture				
normal	11	12	15	7
Coat color				
agouti	11	12	15	7
Hair follicle structure / orientation				
normal	11	12	15	7
Skin pigmentation				
normal	11	12	15	7
Skin texture / condition				
normal	11	12	15	7
Vibrissae				
normal	11	12	15	7
Limbs				
normal	11	12	15	7
Digits				
normal	10	12	15	7
bent toe	1	-	-	-
Tail				
normal	11	12	15	7
Teeth				
normal	11	12	15	7
Ear morphology				
normal	11	12	15	7
Musculature				
normal	11	12	15	7
Seizures / epilepsy				
no	11	12	15	7
Motor capabilities / coordination				
normal	11	12	15	7

Movement				
normal	11	12	15	7
Feeding / drinking behavior				
normal	11	12	15	7
Respiratory system				
normal	11	12	15	7
Reproductive system				
normal	11	12	15	7
Other abnormalities				
no	11	12	15	7
Animals analyzed	11	12	15	7

Table 7: Results from the X-ray analysis

Parameter	Male		Female	
	control	mutant	control	mutant
Skull shape				
normal	5	4	14	4
abnormal snout/nose	2	2	-	2
Mandibles				
normal	7	6	14	6
Maxilla				
normal	7	6	14	6
Teeth				
normal	7	6	14	6
Orbit				
normal	7	6	14	6
Number of cervical vertebrae				
normal	7	6	14	6
Number of thoracic vertebrae				
normal	7	6	14	6
Number of lumbar vertebrae				
6	6	5	11	4
5	1	1	3	2
Number of pelvic vertebrae				
normal	7	6	14	6
Number of sacral vertebrae				
normal	7	6	14	6
Vertebrae shape				
normal	7	6	14	6
Number of ribs				
normal	7	6	14	6
Rib shape				
normal	7	6	14	5
fusions	-	-	-	1

Scapulas				
normal	7	6	14	6
Clavicle				
normal	7	6	14	6
Pelvis				
normal	7	6	14	6
Femur shape				
normal	7	6	14	6
Tibia				
normal	7	6	14	6
Fibula				
normal	7	6	14	6
Humerus				
normal	7	6	14	6
Ulna				
normal	7	6	14	6
Radius				
normal	7	6	14	6
Number of digits				
normal	7	6	14	6
Completeness of digits				
yes	7	6	14	6
Joints				
normal	7	6	14	6
Animals analyzed	7	6	14	6

Table 8: Results from clickbox test (hearing test)				
Phenotype	Male		Female	
	control	mutant	control	mutant
0	-	-	-	-
1	-	-	-	-
2	-	-	-	-
3	11	11	13	5
4	-	1	2	2
Mean Score	3.00	3.08	3.13	3.92

Kruskal-Wallis Anova on Ranks: n.s.

0: no reaction at all,
1: very slow reaction,
2: retarded reaction,
3: normal reaction,
4: strong reaction

Table 9: Bone- and weight-related quantitative parameters (data presented as mean \pm standard error of mean)									
Parameter	Control (A)		Mutant (B)		A ~ B		ANOVA		
	Male	Female	Male	Female	Male	Female	<i>p</i> – value genotype	<i>p</i> – value sex	<i>p</i> – value interaction
	(n=7)	(n=14)	(n=6)	(n=6)	<i>p</i> – value	<i>p</i> – value			
BMD [mg/cm ²]	67 \pm 2	74 \pm 1	62 \pm 2	70 \pm 2	n.s.	< 0.05	< 0.05	< 0.001	n.s.
pBMD [mg/cm ²]	55 \pm 2	64 \pm 1	52 \pm 2	58 \pm 2	n.s.	< 0.01	< 0.05	< 0.0001	n.s.
sBMD [10 ⁻³ x cm ⁻²]	2.22 \pm 0.12	2.12 \pm 0.06	2.22 \pm 0.05	2.69 \pm 0.06	n.s.	< 0.0001	< 0.01	< 0.05	< 0.01
BMC [mg]	782 \pm 53	1251 \pm 46	784 \pm 55	793 \pm 87	n.s.	< 0.0001	< 0.01	< 0.001	<= 0.001
Body Length [cm]	9.93 \pm 0.07	10.14 \pm 0.06	9.67 \pm 0.11	9.67 \pm 0.11	n.s.	< 0.001	< 0.001	n.s.	n.s.
Body Weight [g]	30.16 \pm 1.11	35.05 \pm 1.06	28.13 \pm 1.05	25.90 \pm 0.82	n.s.	< 0.0001	< 0.0001	n.s.	< 0.01
Lean mass [units]	18.31 \pm 0.97	9.37 \pm 0.82	17.08 \pm 0.64	13.82 \pm 1.28	n.s.	< 0.01	n.s.	< 0.0001	< 0.01
Fat mass [units]	7.99 \pm 0.80	21.17 \pm 1.43	7.48 \pm 1.18	8.39 \pm 1.68	n.s.	< 0.0001	< 0.001	0.0001	< 0.001
Bone Content [%]	2.60 \pm 0.16	3.57 \pm 0.09	2.78 \pm 0.13	3.05 \pm 0.29	n.s.	< 0.05	n.s.	< 0.001	< 0.05
Lean Content [units x 100/g]	60.73 \pm 2.52	27.45 \pm 3.04	61.17 \pm 3.42	54.05 \pm 5.93	n.s.	< 0.001	< 0.01	< 0.0001	< 0.01
Fat Content [units x 100/g]	26.45 \pm 2.39	59.60 \pm 2.93	26.09 \pm 3.41	31.73 \pm 5.65	n.s.	< 0.001	< 0.001	< 0.0001	< 0.01
Femur span¹ [mm]	1.17 \pm 0.03	1.09 \pm 0.01	1.13 \pm 0.02	1.08 \pm 0.02	n.s.	n.s.	n.s.	< 0.01	n.s.
Subcutaneous fat¹ [mm]	4.31 \pm 0.15	7.12 \pm 0.25	4.05 \pm 0.24	4.75 \pm 0.24	n.s.	< 0.0001	< 0.0001	< 0.0001	< 0.001
Vertebrae hight² [mm]	3.37 \pm 0.02	3.50 \pm 0.05	3.28 \pm 0.05	3.20 \pm 0.08	n.s.	< 0.01	< 0.01	n.s.	n.s.
	Male	Female	Male	Female	A ~ B Male	A ~ B Female	ANOVA		
	(n=11)	(n=14)	(n=9)	(n=7)	<i>p</i> – value	<i>p</i> – value	<i>p</i> – value genotype	<i>p</i> – value sex	<i>p</i> – value interaction
Ionized Calcium [mmol/l]	1.19 \pm 0.03	1.29 \pm 0.02	1.14 \pm 0.02	1.33 \pm 0.03	n.s.	n.s.	n.s.	< 0.001	n.s.

1: mean value of left and right
2: third lumbar vertebra

3.3 Neurology Screen

3.3.1 Summary

In the primary neurological screen, 19 *Eyl*-mutant mice (12 males / seven females) and 26 control mice (11 males / 15 females) were screened. These animals were analyzed according to our modified SHIRPA protocol, which includes a battery of behavioral tests. This primary observation screen is a modification of the Irwin procedure (Irwin, 1968) and is proposed as a rapid, comprehensive and semi-quantitative screening method for qualitative analysis of abnormal phenotypes in a mouse (Rogers et al., 1997). We carried out 23 test parameters to detect phenotypic differences between male and female mutant and age-matched control mice (www.mgu.har.mrc.ac.uk/facilities/mutagenesis/mutabase/shirpa_summary.html). Each test parameter contributes to an overall assessment of muscle and lower motor neuron function, spinocerebellar function and sensory and autonomic function. The primary neurological screen is thereby focused on the investigation of neurological reflexes to determine the overall neurological functioning of a mouse. In addition, we examined lactate in the blood of mice to draw conclusions about energy metabolism. Moreover, we measured forelimb grip strength to evaluate muscle function.

The comparison of mutant mice to controls revealed significant differences in the palpebral closure and in the body weight. Lactate levels differed between both sexes and genotypes. All other SHIRPA test parameters were without pathological findings. In addition, we found a significantly reduced grip strength of male mutant mice whereas performance on the rotarod was not altered.

3.3.2 Mice

Twelve 10-week-old male mutants and eleven 10-week-old male control mice entered the neurological screen at the beginning of the 34th calendar week. Seven 10-week-old female mutants and fifteen 10-week-old female controls entered the neurological laboratory one week later. All animals were fed *ad libitum* for a period of one week during their stay in the neurological screen.

3.3.3 Material and Methods

Primary screening: modified SHIRPA protocol

Assessment of each animal at age 10 weeks began with observation of undisturbed behavior (*Viewing Jar Behavior*) in a glass cylinder (11 cm in diameter). The mice were then transferred to an arena consisting of a clear Perspex box (420 x 260 x 180 mm) in which a Perspex sheet on the floor is marked with 15 squares. Locomotor activity and motor behavior was observed within this area (*Behavior recorded in the Arena*). This was followed by a sequence of manipulations testing reflexes (*Behavior recorded on or above the arena*). Measurements were completed with the recording of biting, and body weight. The last part of the primary screen also involved the analysis of righting reflex,

and contact righting reflex. A glass cylinder (35 mm diameter, 135 mm length) was used for testing of the contact righting reflex. Throughout the entire procedure, abnormal behavior, biting, and vocalization were recorded. Between testing of each mouse, fecal pellets and urination were removed from the viewing jar and arena. All experimental equipment was thoroughly cleaned with Pursept-A and dried prior to testing.

Values for body length, body weight and locomotor activity are presented as means \pm SEM. Kruskal-Wallis-test (S-PLUS, Insightful) was used to test for effects of genotype and sex factors on these parameters. The Chi-Squared test was applied for all other parameters.

Further screening: grip strength

The grip strength meter system determines the forelimb grip strength, i.e. muscle strength of a mouse. The device exploits the tendency of a mouse to grasp a horizontal metal bar while being pulled by its tail. During the trial set-up, the mouse grasps a special adjustable grip (2 mm) mounted on a force sensor. The sensor allows measurements of up to 600 Ponds. Five trials were undertaken for each mouse within one minute. The mean value is used to represent the grip strength of a mouse.

All experimental equipment was thoroughly cleaned with Pursept-A and dried prior subsequent tests. Values were presented as means \pm standard error of mean (SEM).

Statistical analysis of the grip strength trial results. Grip strength trial results are compared between genotypes, controlling for the effects of sex and weight, by fitting linear mixed effect models (Pinheiro and Bates, 2000). A linear mixed effect model is a modified analysis of variance/covariance approach allowing for dependencies in the data. In our case, dependencies arise from repeated trials within each mouse. Genotype, sex and weight are modelled as fixed effects; mouse-specific intercepts are modelled by including the intercept as random effect. Interaction effects are tested and included in the model if they show a significant contribution. A serial dependency on the trial number can be tested by including the trial number as random effect with an autoregressive correlation structure. Model fitting is performed by the nlme-Package in the open-source statistical software R, a close relative of S-PLUS (The R Project for Statistical Computing, 2004). The p-value for the genotype effect within the specific model found for the data indicates the significance of the statistical test of interest; a confidence interval for the genotype effect can also be extracted.

Secondary screening: rotarod test

The TSE-RotaRod 3375 apparatus (Accelerating Model, TSE, Bad Homburg) was used to measure fore limb and hind limb motor coordination, balance and motor learning ability (Jones and Roberts, 1968). The machine was set up in an environment with minimal stimuli such as noise and movement.



Figure 3: The rotarod apparatus

The rotarod device is equipped with a computer controlled motor-driven rotating rod. The unit consists of a rotating spindle and four individual lanes for each mouse (Fig. 4). The software allows pre-programming of session protocols with varying rotational speeds. Infrared beams are used to detect when a mouse falls onto the grids beneath the rotarod. The mouse is placed perpendicular to the axis of rotation, with head facing the direction of the rotation.

In general, the mouse is placed perpendicular to the axis of rotation, with head facing the direction of the rotation. All mice were placed on the Rotarod at an accelerating speed from 4 to 40 rpm for 300 sec with 20 min between each trial. In motor coordination testing, mice were given four trials at the accelerating speed at one day. The mean latency to fall off the Rotarod during the trials at each speed level was recorded and used in subsequent analysis. Before the start of the first trial, mice were weighed. The Rotarod automatically recorded the length of time that each mouse was able to spend on the rotating rod.

Statistical analysis of the Rotarod performance results. The Rotarod data contain dependencies, which are more complex than the grip strength data. Repeated measurements arise from different rotation velocities and, in addition, from the repetition of the trial on three consecutive days. To compare the performance results between genotypes, linear mixed-effect models are fitted, that allow for these dependencies and for the effects of sex and weight. The latter are modelled as fixed effects, as well as rotation speed. In addition, the effect of rotation speed is allowed to be mouse-specific, with an autoregressive correlation structure. Interaction effects are considered and included in the model, if necessary.

In each model, the parameter of interest is the coefficient of the genotype effect. A significance test or a confidence interval for this coefficient can be extracted from the model fitted.

3.3.4 Parameters

Muscle/lower motor neuron function
Body position, gait, Positional passivity, tail elevation, grip strength, defecation
Spinocerebellar function
Body position, gait, righting reflex, tail elevation, grip strength
Sensory function
Transfer arousal, touch escape, gait, pinna reflex, righting reflex
Autonomic function
Palpebral closure, defecation, lacrimation
Neurological reflexes
Righting reflex (pons), contact righting reflex, pinna reflex
General appearance
Body weight, body position, transfer arousal, touch escape, vocalization, positional passivity, aggression, spontaneous activity, locomotor activity, skin color

3.3.5 Results

A parameter with significant finding for the Eyl mutant mouse line was **palpebral closure** (Table 11; confirmation of the eye phenotype). Another parameter found was the significantly decreased **body weight** of female mutant mice when compared to control mice (see Table 10), whereas both control males and females had similar weight at the age of 10 weeks. **Blood lactate** screening (Table 14) showed significantly elevated mean levels in male mutant and female control mice. All other SHIRPA parameters were without pathological findings.

Furthermore, we did a secondary screening with special interest in **grip strength** testing and **motor coordination** (Rotarod). Within these examinations, we found significantly decreased grip strength in male knockout mice (Fig. 4). Rotarod testing revealed no significant difference between the genotypes (Fig. 5).

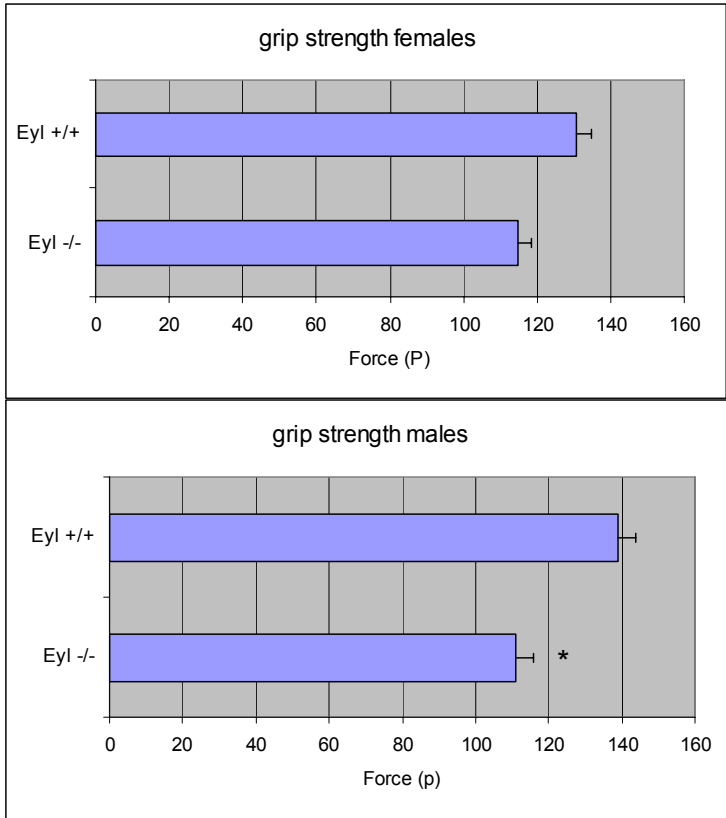


Figure 4: Results from grip strength testing
 Male mutants are significantly weaker, * $p < 0.05$

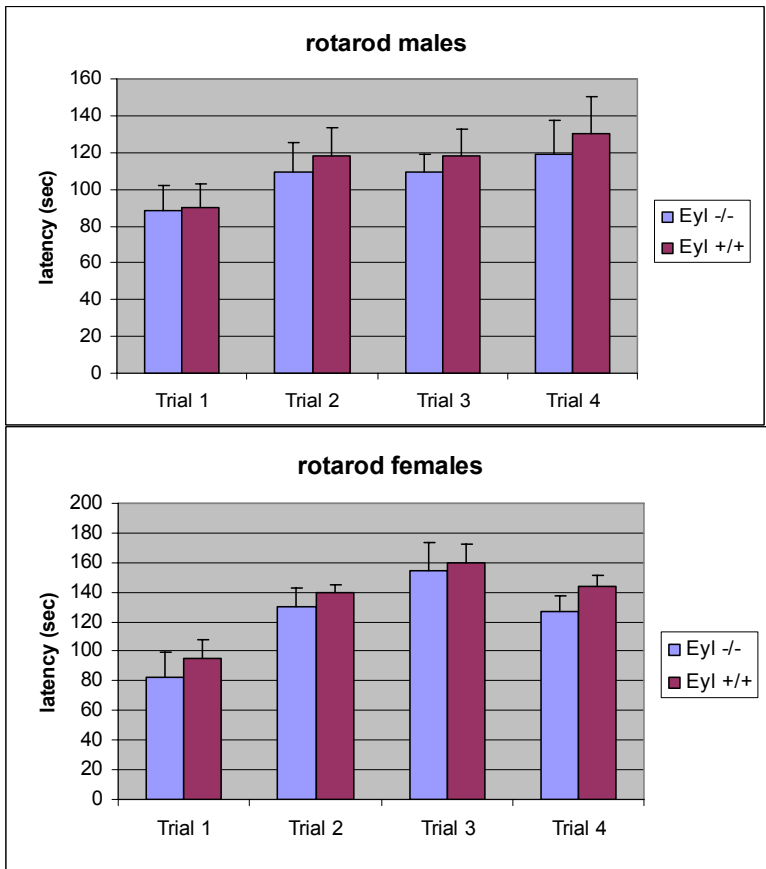


Figure 5: Results from Rotarod testing
 No genotype-specific differences

Raw data for each individual are available on demand in Excel sheets.

3.3.6 Discussion

In our neurological screening, male and female mutant mice showed an altered neurological behavior that was not obvious from first screening. In the primary screen (SHIRPA), the only distinctive feature beneath the palpebral closure was a decreased body weight in mutant females. The increased lactate level of male mutant mice is not in accord to the increase in female control mice. Since the weight of female littermate controls was as high as that of male controls (please see also 3.11.5, Metabolic Screen) there could already be a difference in the background of the mice concerning sex effects.

In the secondary screen, male and female mutant mice showed a phenotype in grip strength testing. The reduction of grip strength in mutant mice could hint to a neuromuscular phenotype or to an alteration in central motor coordination. The allelic mutant *aphakia* shows a progressive degeneration in dopaminergic neurons in the substantia nigra projecting to the basal ganglia and a Parkinson-like phenotype in older mutant mice. However, rotarod testing showed no difference and all non-eye-related SHIRPA parameter appeared normal in mice at the age of 10 weeks.

In addition, we suggest performing our staircase test for skilled reaching and our gait analysis, as these tests are sensitive for extrapyramidal motor syndromes. Since the Parkinson-like phenotype could be expected to be more severe in older animals, these tests as well as a second rotarod testing should be performed in older mice.

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Abbreviations

SHIRPA **S**mithKline Beecham Pharmaceuticals, **H**arwell, MRC Mouse Genome Centre and Mammalian Genetics Unit, **I**mperial College School of Medicine at St Mary's **R**oyal London Hospital, St Bartholomew's and the Royal London School of Medicine **P**henotype **A**ssessment
http://www.mgu.har.mrc.ac.uk/facilities/mutagenesis/mutabase/shirpa_summary.html

Table 10: Recording of body length and body weight						
Data are presented as mean ± standard error of mean.						
Parameter	Male			Female		
	Control (n=11)	Mutant (n=12)	<i>p-value</i>	Control (n=15)	Mutant (n=7)	<i>p-value</i>
Body Weight [g]	27.72± 0,7	25.40±0,8	0.06	27.95±0.8	23.13±0.7	<0.01

Table 11: Behavior recorded in viewing jar						
Statistical analysis: chi-squared test; significance $p < 0.05$						
Parameter	Male			Female		
	Control (n=11)	Mutant (n=12)	<i>p-value</i>	Control (n=15)	Mutant (n=7)	<i>p-value</i>
Body Position						
Inactive	0	0		0	0	
Active	11	10		14	5	
Excessive Activity	0	2	<i>n.s.</i>	1	2	<i>n.s.</i>
Tremor						
Absent	11	12		15	7	
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Palpebral closure						
Eyes open	11	2		15	0	
Eyes closed	0	10	<0.01	0	7	<0.01
Coat Appearance						
Tidy and well groomed	11	12		15	7	
Irregularities	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Whiskers						
Present	11	12		15	7	
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Lacrimation						
Absent	11	12		15	7	
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Defecation						
Present	7	10		12	4	
Absent	4	2	<i>n.s.</i>	3	3	<i>n.s.</i>

Table 12: Recording of locomotor activity and behavior in the arena

Statistical analysis: chi-squared test; significance $p < 0.05$. Locomotor activity data are shown as mean (\pm SEM)

Parameter	Male			Female		
	Control (n=11)	Mutant (n=12)	<i>p-value</i>	Control (n=15)	Mutant (n=7)	<i>p-value</i>
Transfer arousal						
Extended freeze	0	0		0	0	
Brief freeze	15	15		15	15	
Immediate movement	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Locomotor activity	14.4 \pm 1.1	17.3 \pm 1.3	<i>n.s.</i>	13.9 \pm 1.5	18.4 \pm 1.4	<0.05
Gait						
Fluid movement	11	12		13	6	
Lack Fluidity	0	0	<i>n.s.</i>	2	1	<i>n.s.</i>
Tail Elevation						
Dragging	2	0		1	0	
Horizontally extension	9	12		14	7	
Elevated/Straub tail	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Touch Escape						
No response	0	0		1	0	
Response to touch	11	12		14	7	
Flees prior to touch	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Positional Passivity						
Struggles when held by tail	11	12		15	7	
No struggle	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>

Table 13: Behavior recorded in or above the arena						
Statistical analysis: chi-squared test; significance $p < 0.05$						
Parameter	Male			Female		
	Control (n=11)	Mutant (n=12)	<i>p-value</i>	Control (n=15)	Mutant (n=7)	<i>p-value</i>
Skin color						
Blanched	0	2		0	0	
Pink	11	12		15	7	
Bright deep red	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Trunk curl						
Absent	11	12		14	7	
Present	0	0	<i>n.s.</i>	1	0	<i>n.s.</i>
Limb Grasping						
Absent	11	12		15	7	
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Pinna Reflex						
Present	11	12		15	7	
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Corneal Reflex						
Present	11	12		15	7	
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Righting Reflex						
Rights itself						
Fails to right when released	11 0	12 0	<i>n.s.</i>	15 0	7 0	<i>n.s.</i>
Contact Righting						
Present	10	12		14	7	
Absent	1	0	<i>n.s.</i>	1	0	<i>n.s.</i>
Evidence of biting						
None						
Biting in response to handling	11 0	12 0	<i>n.s.</i>	15 0	7 0	<i>n.s.</i>
Vocalization						
None	11	11		14	7	
Vocal	0	1	<i>n.s.</i>	0	0	<i>n.s.</i>

Table 14: Lactate levels						
Data shown represent the results of the mean blood lactate concentrations, value (\pm SEM)						
	Male			Female		
	Control (n=11)	Mutant (n=12)	<i>p-value</i>	Control (n=15)	Mutant (n=7)	<i>p-value</i>
Lactate [mmo/l]	5.37 \pm 0.32	7.25 \pm 0.24	<i><0.01</i>	7.08 \pm 0.35	5.3 \pm 0.33	<i><0.01</i>

3.4 Eye Screen

3.4.1 Summary

In the Eye Screen, a high throughput electroretinography method (ERG) was employed to examine mice for retinal impairment (Dalke *et al.*, 2004). Furthermore, mice were examined for anterior segment abnormalities by slit lamp biomicroscopy (Favor, 1983).

In humans blindness is caused by several different ocular diseases. Among these, the cataracts are responsible for half of all cases (Johnson and Foster, 2003). The retinal disorders cover a broad variety of clinical symptoms and many different genes are involved in the corresponding pathological conditions in humans. The two most important groups are retinitis pigmentosa (RP) and age-related-macular-degeneration (ARMD; for recent reviews, see Rivolta *et al.*, 2002 and Stone *et al.*, 2001). Mouse models are appropriate tools to understand the genetic and biochemical mechanisms of ocular disorders. There is a rapid increasing number of mouse mutants available suffering from various types of eye diseases (for recent reviews see Graw, 2003 and Dalke & Graw, 2005).

The Eyl mutant mouse line is bred on a C3H background carrying the *Pde6b*^{rd1} allele which causes retinal degeneration. In the primary screen usually the mice are examined ophthalmologically by slit lamp biomicroscopy and electroretinography (ERG). These methods could not be used for this mutant mouse line, because the mutation is associated with severe microphthalmia and both mutants and controls are blind.

3.4.2 Mice

Twenty-six control (11 male, 15 female) and 19 mutant mice (12 male, 7 female) entered the Eye Screen at the age of 11 weeks. Mice were kept under standard laboratory conditions with food and water *ad libitum*. Mice were not examined by standard primary screen methods. A histological analysis was done for some mice of each group.

3.4.3 Materials and Methods

Electroretinography (ERG) was used to examine the retinal function as described (Dalke *et al.*, 2004). Mice were dark-adapted for at least 12 hours and anaesthetized with 137 mg Ketamine and 6.6 mg Xylazine per kg body weight. After pupil dilation (1 drop Atropine 1%), individual mice were fixed on a sled with Velcro straps. Gold wires (as active electrodes) were placed on the cornea; care was taken not to obstruct the pupillary opening. The ground electrode was a subcutaneous needle in the tail; a reference electrode was placed subcutaneously between the eyes. The mice were introduced into an ESPION ColorBurst Handheld Ganzfeld LED stimulator (Diagnosys LLC, Littleton, MA, USA) on a rail to guide the sled (High-Throughput Mouse-ERG, STZ for Biomedical Optics and Function Testing, Tübingen, Germany). To minimize temperature influences on the ERG, body temperature was kept at 37°C using a warming plate. 10 ms light pulses were delivered at a frequency

of 0.48 Hz in two steps at 500 and 12,500 cd/m². Bandpass filter was set ranging from 0.15 to 1000 Hz. Responses were recorded simultaneously from both eyes with an ESPION Console (Diagnosys LLC, Littleton, MA, USA) and stored for offline analysis after averaging 10-40 individual measurements at each step.

Histology: Eyes were fixed 24 hours in Davidson solution, dehydrated and embedded in plastic medium. Transverse 2- μ m-thick sections were cut with an ultramicrotome, stained with methylene blue and basic fuchsin, and evaluated with a light microscope.

Slit Lamp Biomicroscopy: Mice were examined biomicroscopically for eye abnormalities as previously described (Favor, 1983). Briefly, pupils were dilated with a 1% atropine solution applied to the eyes at least 10 min prior to examination. Both eyes of the mice were examined by slit lamp biomicroscopy (Zeiss SLM30) at 48x magnification with a narrow beam slit lamp illumination at 25-30° angle from the direction of observation. Observed phenotypic variants of the eyes were carefully documented.

Statistical Analysis: ERG data were statistically analyzed using MS-Excel. Differences between mouse groups were evaluated with the Student's t-test. Statistical significance was set at p<0.05. Data are presented as mean values \pm standard error of the mean (SEM).

3.4.4 Parameters

Electroretinography (ERG)
a/b-wave, left/right eye at 500/12.50 cd/m ²
Slit lamp biomicroscopy
(qualitative) abnormalities of lens and cornea like opacity and development disorders
Histology
(qualitative) retinal lamination and morphology of cell layers and lens
Morphology
(qualitative) like size and degree of closure

3.4.5 Results and Discussion

ERG responses were not recorded from the Eyl mice, because of the severe microphthalmia in *Eyl*^{-/-} mice and the well known retinal degeneration in C3H mice, which is caused by a nonsense mutation in the *Pde6b* gene (Pittler and Baehr, 1991). The retinal degeneration caused by the recessive *Pde6b*^{rd1} allele is characterized by the total loss of photoreceptors until the age of 3 weeks. Therefore the *Eyl*^{+/+} mice are also blind.

The known eye phenotype of the mutant mice was confirmed by histological analysis of female mice (Fig. 7). In these mice no lens formation was

detected, the retina is degraded and the remaining eye ball is much smaller as in the wild-type littermates. Absence of the photoreceptor cell layer, due to the presence of the *Pde6b^{rd1}* allele was observed in histological sections of both mutant and control mice. Male mice were not analyzed.

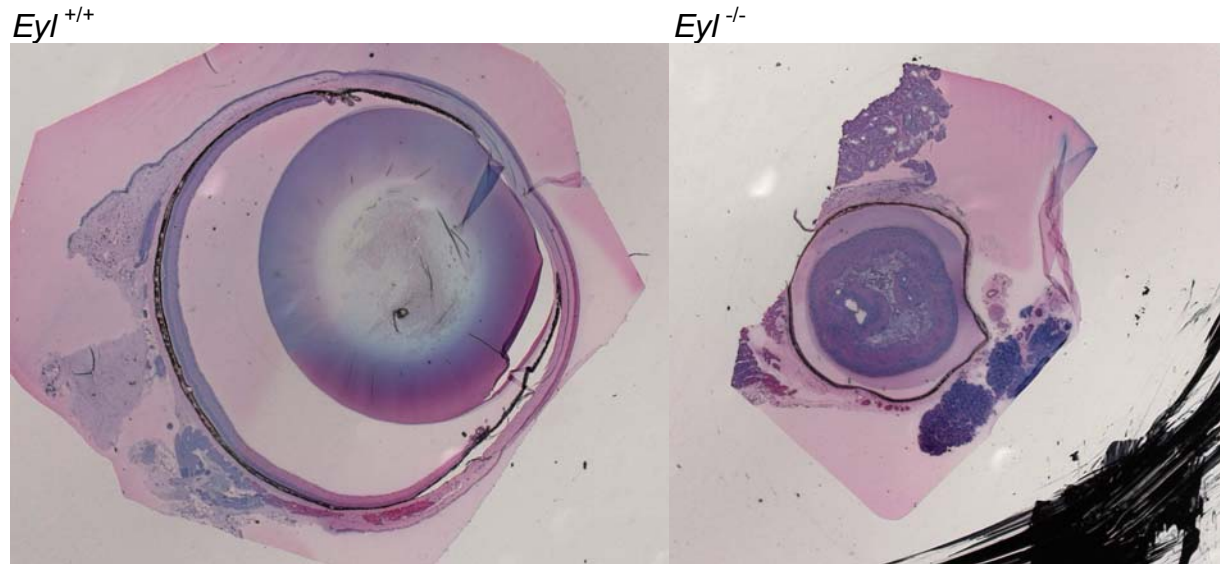


Figure 6: Histological examination of the eye.

The *Eyl^{+/+}* mice show the typically degenerated retina of mice homozygous for the *Pde6b^{rd1}* mutation. In addition to this phenotype, female *Eyl^{-/-}* mice do not develop a lens, the retina is folded and the eye is much smaller (microphthalmia) compared to *Eyl^{+/+}*.

A total of 44 mice were examined ophthalmologically by **slit lamp biomicroscopy**. All homozygous mutant mice expressed bilateral extreme microphthalmia-anophthalmia indicating that the gene mutated is critical for normal eye development (Table 14).

Table 15: Results from slit lamp biomicroscopy				
Genotype	NAD	Nuclear-zonular opacities	Corneal opacity	Microphthalmia/ Anophthalmia
+/+	25	-	-	-
-/-	-	-	-	19

In a secondary screening more detailed analysis could be done to confirm the microphthalmia and anophthalmia phenotype in female and male mutants.

Abbreviations

ERG	electroretinography
NAD	no abnormality detected

3.4.6 References

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3.5 Clinical-Chemical Screen

3.5.1 Summary

The aim of the Clinical-Chemical Screen is the detection of hematological changes, defects of various organ systems, and changes in metabolic pathways and electrolyte homeostasis by means of suitable laboratory diagnostic tools. Since most inherited metabolic disorders are known to lead directly or indirectly, via altered organ function, to changes in the parameters investigated, this screening process provides a comprehensive investigation of clinical phenotypes with counterparts in humans and animal species (Rathkolb *et al.*, 2000). The methods used are routine procedures, allowing the appropriate screen of large numbers of mice for a broad spectrum of clinical-chemical and hematological parameters (Champy *et al.*, 2004; Hough *et al.*, 2002).

In the primary clinical chemical screen, 20 different clinical-chemical parameters were measured including various enzyme activities, as well as plasma concentrations of specific substrates and electrolytes. Additionally, we measured eight basic hematological parameters.

All parameters of both mutant and control mice were within the normal ranges usually found in C3H mice with the exception of creatine kinase activity. It was noticeable that the creatine kinase activity of seven mutant males and of two mutant females was extremely high; additionally the AST concentrations of these animals were elevated. In combination with the result of grip strength testing this is a strong hint at a muscle disorder. All hematological parameters were without pathological findings.

3.5.2 Mice

Eleven 12-week-old control and nine 12-week-old mutant males entered the clinical-chemical screen at the beginning of the 36th calendar week. Fifteen 12-week-old control and five 12-week-old mutant females entered the screen at the beginning of the 37th calendar week in 2004.

3.5.3 Materials and Methods

Blood Withdrawal and Storage

The Clinical-chemical Screen of the German Mouse Clinic routinely analyzed 12-weeks-old mice. A blood sample was taken from an ether-anesthetized mouse by puncturing the retro-orbital sinus with a non-heparinized capillary (0.8 mm in diameter; Laborteam K&K; Munich, Germany; Art.No. 1.28.13.1.2). The time for sample taking was recorded in a work list. An amount of 500µl blood was collected in a heparinized tube (Li-heparin, KABE; Nümbrecht, Germany; Art.No. 078028). An additional smaller sample was collected (using the same capillary) in an EDTA-coated tube (KABE, Art.No 078035). The tubes were immediately inverted five times to achieve a homogeneous distribution of the anticoagulant.

After removal of 40 µl blood for the Neurology Screen, the Li-heparin-coated tubes were stored in a rack at room temperature for two hours. After-

wards, cells and plasma were separated by a centrifugation step (10 min, 4656 x g; Biofuge, Heraeus; Hanau, Germany). Plasma was distributed between the Immunology Screen (30 µl), the Allergy Screen (30 µl), the Clinical Chemical Screen (130 µl) and the Steroid Screen (residual), while the cell pellet was given to the Immunology Screen for FACS-analysis. The plasma sample for the clinical chemical analysis was transferred into an Eppendorf tube and diluted 1:2 with aqua dest. The solution was mixed for a few seconds (Vortex genie, Scientific Industries, New York, America) to prevent clotting and then centrifuged again for 10 min at 4656 x g. Additionally the Clinical Chemical Screen received the EDTA-blood sample for hematological investigations.

Clinical Chemistry

The screen was performed using an Olympus AU 400 autoanalyzer and adapted reagents from Olympus (Hamburg, Germany) and Roche (Mannheim, Germany). In the primary screen, 20 different parameters were measured including various enzyme activities, as well as plasma concentrations of specific substrates and electrolytes.

Hematology

A volume of 50 µl EDTA-blood was used to measure basic hematological parameters with a blood analyzer, which has been carefully validated for the analysis of mouse blood (ABC-Blutbild-Analyzer, Scil Animal Care Company GmbH, Viernheim). Number and size of red blood cells, white blood cells, and platelets are measured by electrical impedance, and hemoglobin by spectrophotometry. Mean corpuscular volume (MCV) is calculated directly from the cell volume measurements, the hematocrit (HCT) from $MCV * \text{red blood cell count}$. Mean corpuscular hemoglobin (MCH) and mean concentration of corpuscular hemoglobin (MCHC) are calculated from $\text{hemoglobin/red blood cells count}$ (MCH) and $\text{hemoglobin/hematocrit}$ (MCHC).

Analysis of Data

Data were statistically analyzed using Excel and Sigma Stat 2.0 with the level of significance set at $p < 0.05$.

3.5.4 Parameters

Proteins and plasma enzyme activities
Alkaline phosphatase (EC 3.1.3.1), α -Amylase (EC 3.2.1.1), Creatine kinase (EC 2.7.3.2), Aspartate-aminotransferase (AST/GOT; EC 2.6.1.1), Alanine-aminotransferase (ALT/GPT; EC 2.6.1.2), Ferritin, Transferrin, Lipase (EC 3.1.1.3), Total protein
Plasma concentrations of specific substrates
Glucose, Cholesterol, Triglycerides, Uric acid, Urea, Creatinine
Plasma concentrations of electrolytes
Potassium, Sodium, Chloride, Calcium, Inorganic phosphate
Basic hematology
White blood cell count (WBC), Red blood cell count (RBC) Hematocrit (HCT), Hemoglobin (HGB), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), and Platelet count (PLT)

3.5.5 Results

Clinical Chemistry

Comparison to baseline data. Most values obtained for the clinical chemical parameters (Table 18) were within the normal ranges usually found in C3HeB/FeJ mice at the age of three months as supported by previously published data (Hough *et al.*, 2002; Quimby 1999; Klempt *et al.*, 2006; own unpublished results). Some of the mutant animals (five males, two females) showed extremely high creatine kinase activity, ranging between 396 to 1024 U/l. Additionally the AST concentration in some of these animals were elevated.

Differences between mutants and controls were seen in the following parameters: mutant male mice showed significantly increased serum activity of creatine kinase compared to the control. Triglyceride concentration and amylase activity were significantly decreased in mutant mice for both sexes while the alkaline phosphatase activity was increased. Additionally in the females the mean cholesterol and transferrin concentrations were decreased while the mean uric acid concentration was above the level of the control animals. Furthermore there was a significant decrease of the inorganic phosphorus concentration in mutant male mice compared to their control littermates.

Sex differences were detected for many clinical chemical parameters in the control animals as well as in the mutant mice reflecting the physiological differences usually found in C3H mice.

Hematology

In the primary screen for hematological parameters all results of both control and mutant mice were within normal ranges (Table 19) with significant differences between mutant and control animals for white blood cell count (males) and the platelet numbers (females).

Raw data for each individual are available on demand in Excel sheets.

3.5.6 Discussion

Most values of mutant and control animals for all parameters were within the normal ranges typical for baseline C3H mice. All clinical chemical and hematological parameters were without pathological findings with the exception of **creatinine kinase** and **AST activity** in some mutant mice. The combination of elevated creatine kinase activity and AST concentration is a hint for muscular damage or disorders (please see also results from grip strength testing, 3.3.5). Significant differences were also found in alkaline phosphatase (both sexes). Alkaline phosphatase is found in osteoblast and liver ductules, an increase of alkaline phosphatase concentration is associated with skeletal and hepatobiliary disorders. The decrease of transferrin concentration as well as the reduced amylase activities can also be an effect of **liver dysfunction** (please also see Pathology Screen, 3.12.5), since the liver mainly produces transferrin and low amylase activities in mice are often seen in connection with liver diseases. The lower triglyceride concentration (in both sexes) and the lower cholesterol concentration in mutant female mice hint at additional changes in metabolic functions (3.11.5, Metabolic Screen).

For secondary studies, it would be interesting to confirm the primary findings of elevated CK activity and AST concentration in combination with a histological investigation of the muscles, which could be performed by the Pathology or Neurology Screen.

3.5.7 References

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Table 16: Clinical-Chemical Parameters.Data are presented as mean \pm standard error of mean.

Parameter	Mutant (A)			Control (B)			A~B	A~B
	Male	Female	p- value	Male	Female	p-value	Male	Female
	(n=11)	(n=6)		(n=11)	(n=15)		p-value	p-value
Sodium [mmol/l]	153 ± 0.86	163 ± 0.80	<0.001	151 ± 0.73	161 ± 0.47	<0.001	n.s.	n.s.
Potassium [mmol/l]	4.6 ± 0.08	4.6 ± 0.17	n.s.	4.6 ± 0.07	4.4 ± 0.05	<0.05	n.s.	n.s.
Calcium [mmol/l]	2.1 ± 0.03	2.3 ± 0.04	<0.05	2.1 ± 0.04	2.3 ± 0.03	<0.001	n.s.	n.s.
Chloride [mmol/l]	109.1 ± 0.87	118.8 ± 0.72	<0.001	107.5 ± 0.28	117.5 ± 0.45	<0.001	n.s.	n.s.
Inorganic Phosphorus [mmol/l]	1.8 ± 0.10	1.8 ± 0.00	n.s.	1.5 ± 0.06	1.7 ± 0.08	<0.05	<0.05	n.s.
Total Protein [g/dl]	5.0 ± 0.11	5.3 ± 0.08	<0.05	5.0 ± 0.08	5.5 ± 0.07	<0.001	n.s.	n.s.
Creatinine [mg/dl]	0.345 ± 0.00	0.356 ± 0.01	n.s.	0.342 ± 0.01	0.368 ± 0.01	<0.01	n.s.	n.s.
Urea [mg/dl]	57.6 ± 2.38	52.5 ± 4.8	n.s.	61.7 ± 1.61	59.5 ± 1.61	n.s.	n.s.	n.s.
Uric acid [mg/dl]	1.7 ± 0.29	3.7 ± 0.36	<0.001	1.2 ± 0.36	2.3 ± 0.24	<0.05	n.s.	<0.01
Cholesterol [mg/dl]	130.7 ± 2.88	116.6 ± 7.77	n.s.	139.1 ± 4.86	143.4 ± 4.71	n.s.	n.s.	<0.02
Triglyceride [mg/dl]	252.2 ± 13.7	223.3 ± 17.7	n.s.	313.9 ± 20.5	364.7 ± 15.3	n.s.	<0.05	<0.001
Creatine Kinase [U/l]	539 ± 106.9	282 ± 117	n.s.	91 ± 11.9	111 ± 26.7	n.s.	<0.01	n.s.
Alanine-Amino-transferase (ALAT,GPT) [U/l]	16 ± 1.14	15 ± 2.23	n.s.	16 ± 1.21	17 ± 1.24	n.s.	n.s.	n.s.
Aspartate-Amino-transferase (AST,GOT) [U/l]	51 ± 6.01	44 ± 10.2	n.s.	26 ± 0.92	31 ± 2.95	n.s.	<0.01	n.s.
Alkaline Phosphatase [U/l]	113 ± 4.39	141 ± 5.93	<0.01	90 ± 2.79	124 ± 4.05	<0.001	<0.001	<0.05
α-Amylase [U/l]	1962 ± 65.1	1781 ± 41.3	<0.05	2265 ± 28.7	2042 ± 25.6	<0.001	<0.001	<0.001
Glucose [mg/dl]	151.8 ± 10.7	144.2 ± 6.89	n.s.	144.1 ± 8.01	163.3 ± 5.40	n.s.	n.s.	n.s.
Ferritin [ng/ml]	52.0 ± 1.54	40.5 ± 3.34	<0.02	53.9 ± 1.92	45.3 ± 1.66	<0.01	n.s.	n.s.
Transferrin [mg/dl]	157.6 ± 1.53	171.6 ± 1.77	<0.001	163.9 ± 5.01	177.8 ± 1.55	<0.05	n.s.	<0.05
Lipase [U/l]	45.3 ± 6.44	46.7 ± 1.41	n.s.	45.7 ± 1.73	52.1 ± 2.57	n.s.	n.s.	n.s.

Table 17: Hematological Parameters.Data are presented as mean \pm standard error of mean.

Parameter	Mutant (A)			Control (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=11)	(n=6)	<i>p</i> - value	(n=11)	(n=15)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
White blood cell count [$10^3/\mu\text{l}$]	6.08 ± 0.65	6.55 ± 0.72	n.s.	8.24 ± 0.49	7.11 ± 0.18	n.s.	<0.02	n.s.
Red blood cell count [$10^6/\mu\text{l}$]	9.19 ± 0.29	9.60 ± 0.22	n.s.	9.84 ± 0.25	9.40 ± 0.07	n.s.	n.s.	n.s.
Hemoglobin [g/dl]	15.6 ± 0.44	15.8 ± 0.47	n.s.	16.7 ± 0.38	15.6 ± 0.12	<0.05	n.s.	n.s.
Hematocrit [%]	45 ± 1.43	47 ± 1.04	n.s.	48 ± 1.23	46 ± 0.33	n.s.	n.s.	n.s.
Mean corpuscular volume [fl]	48.7 ± 0.19	49.3 ± 0.29	n.s.	48.4 ± 0.15	49.1 ± 0.18	<0.01	n.s.	n.s.
Mean corpuscular hemoglobin [pg]	17.0 ± 0.13	16.5 ± 0.23	n.s.	17.0 0.11	16.7 ± 0.08	<0.05	n.s.	n.s.
Mean corpuscular hemoglobin concentration [g/dl]	35.0 ± 0.24	33.5 ± 0.36	n.s.	35.0 ± 0.21	34.0 ± 0.13	<0.001	n.s.	n.s.
Platelet count [$10^3/\mu\text{l}$]	583 ± 18.6	525 ± 65.4	n.s.	584 ± 18.8	700 ± 20.7	<0.0.001	n.s.	<0.05

3.6 Immunology Screen

3.6.1 Summary

Mouse models have been a primary source of information for understanding the intricate mechanisms of the immune system (Bluethmann and Ohashi, 1994; Mak *et al.*, 2001; Fischer 2002; Rogner and Avner, 2003). The Immunology Screen at the GMC was set up to conduct a broad immunological phenotyping of mouse mutant lines with the intention of identifying distinct gene functions, which play key roles in the immune defenses of the organism through a complex network of cellular and soluble components (Janeway *et al.*, 2004).

According to the data summary of what is already known about the mutant mouse line presented to the GMC by the mouse provider, no immunological phenotype was known for the Eyl mutant line. Their analysis in the Immunology Screen revealed alterations affecting exclusively female mutants.

3.6.2 Mice

We analyzed 19 mutant animals (7 females and 12 males) and 26 age- and sex-matched littermate controls (15 females and 11 males).

3.6.3 Material and Methods

Peripheral blood leukocytes (PBLs) were isolated from 500 μ l blood by erythrocyte lysis with NH_4Cl (0.17M) - Tris buffer (pH 7.45) directly in 96-well microtiter plates. After subsequent washing with FACS staining buffer (PBS, 0.5% BSA, 0.02% sodium azide, pH 7.45), PBLs were incubated for 20 min with 1 μ M ethidium monazide bromide (EMA, Molecular Probes, The Netherlands) and Fc block (clone 2.4G2, PharMingen, San Diego, USA). EMA bound to the DNA of dead cells was photocrosslinked by brief light exposure. Cells were then stained with fluorescence-conjugated monoclonal antibodies (PharMingen).

The following main cell populations were analyzed: B cells (CD19⁺ clone 1D3), B1 B cells (CD19⁺CD5⁺, clone 53-7.3), B2 B cells (CD19⁺CD5⁻), T cells (CD3⁺, clone 145-2C11), CD4⁺ T cells (clone RM4-5), CD8⁺ T cells (CD8 α , clone 53-6.7; CD8 β , clone H35-17.2), γ/δ T cells (clone GL3), granulocytes (Gr-1⁺, clone RB6-8C5), and NK cells (CD49b⁺, clone DX5). We also analyzed additional subpopulations based on the following surface antigens: IgD (clone 11-26c.2a), B220 (clone RA3-6B2), CD11b (clone M1/70), CD103 (clone 2E7), CD25 (clone PC61), CD62L (clone MEL-14), CD45RA (clone 14.8), Ly-6C (clone AL-21), and CD44 (clone IM7). Data were acquired on a FACS Calibur (Becton Dickinson, San Diego, USA) and were analyzed using FlowJo software (TreeStar Inc, USA). All samples were acquired until a total number of 25,000 cells was reached.

The plasma levels of IgM, IgG₁, IgG_{2a}, IgG_{2b}, IgG₃, and IgA were determined by standard sandwich ELISAs using goat anti-mouse immunoglobulin antibodies and alkaline phosphatase (AP) conjugates (SouthernBiotech, Birmingham, USA). The presence of rheumatoid factor and anti-DNA antibodies was evaluated by indirect ELISA with rabbit IgG (Sigma-Aldrich, Steinheim,

Germany) and calf thymus DNA (Sigma-Aldrich), respectively, as antigens and AP-conjugated goat anti-mouse secondary antibody (Sigma-Aldrich). Serum samples from MRL/MpJ-Tnfrsf6^{lpr} mice (Jackson Laboratory, Bar Harbor, USA) were used as positive controls in the autoantibody assays.

3.6.4 Parameters

Flow cytometry
B cells (CD19 ⁺), B1 B cells (CD19 ⁺ CD5 ⁺), B2 B cells (CD19 ⁺ CD5 ⁻), T cells (CD3 ⁺), CD4 ⁺ T cells, CD8 ⁺ T cells, γ/δ T cells, granulocytes (Gr-1 ⁺), and NK cells (CD49b ⁺). Furthermore, all potential subpopulations which can be identified by co-staining for other surface markers (IgD, B220, CD11b, MHC II, I-A ^k , CD25, CD8 β , CD62L, CD45RA, Ly-6C, CD44) using 6 parameter/5 color flow cytometry were analyzed.
ELISA
IgM, IgG ₁ , IgG _{2a} , IgG _{2b} , IgG ₃ , IgA; anti-DNA antibodies, rheumatoid factor

3.6.5 Results and Discussion

The analysis of the Eyl mutant mouse line in the primary Immunology Screen did not reveal profound alterations in the tested parameters. However, we were able to detect some minor, but statistically significant differences affecting predominantly female mutants: increased frequencies of T cells (both CD4⁺ and CD8⁺) and decreased Gr-1⁺ cells, decreased levels of IgG₃ and IgM. In addition, the level of IgG₃ was lower in male mutants as well.

As the detected alterations might represent an interesting sex-specific immunological phenotype, we would like to repeat the primary screen with a smaller batch of mice. If the phenotype is confirmed, possibilities for secondary screening will be discussed with the provider.

3.6.6 References

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Table 18: Basic Parameters Analyzed in the Immunology Screen.Data are presented as mean \pm standard error of mean.

Parameter	Mutants (A)			Control (B)			A - B	
	Male	Female	<i>p</i> - value	Male	Female	<i>p</i> - value	Male	Female
	(n=12)	(n=7)		(n=11)	(n=15)		<i>p</i> - value	<i>p</i> - value
CD19⁺ [%]	29.3 \pm 1.9	26.8 \pm 1.3	n.s.	27.9 \pm 1.3	28.2 \pm 1.5	n.s.	n.s.	n.s.
CD19⁺CD5⁻ [%]	91.2 \pm 1.0	86.1 \pm 1.4	<0.05	86.8 \pm 1.7	89.4 \pm 1.4	n.s.	n.s.	n.s.
CD19⁺CD5⁺ [%]	8.6 \pm 1.0	13.8 \pm 1.4	<0.05	13.0 \pm 1.7	10.5 \pm 1.4	n.s.	n.s.	n.s.
CD3⁺ [%]	37.5 \pm 1.7	49.7 \pm 1.7	<0.001	38.1 \pm 1.9	43.1 \pm 1.3	n.s.	n.s.	<0.02
γ/δ TCR⁺ [%]	0.6 \pm 0.06	0.5 \pm 0.04	n.s.	0.7 \pm 0.06	0.6 \pm 0.05	n.s.	n.s.	n.s.
Gr-1⁺ [%]	21.6 \pm 1.5	9.9 \pm 0.8	<0.001	24.4 \pm 1.7	15.5 \pm 0.2	<0.001	n.s.	<0.001
CD49b⁺ [%]	15.6 \pm 2.6	12.4 \pm 1.8	n.s.	12.6 \pm 1.2	13.6 \pm 1.5	n.s.	n.s.	n.s.
CD4⁺ [%]	26.5 \pm 1.4	38.6 \pm 1.2	<0.001	27.3 \pm 1.2	34.6 \pm 0.7	<0.001	n.s.	<0.05
CD8β⁺ [%]	13.5 \pm 0.4	16.3 \pm 0.5	<0.05	13.7 \pm 0.6	14.5 \pm 0.7	n.s.	n.s.	<0.01
IgG₁ [μ g/ml]	100.1 \pm 9.5	159.1 \pm 18.9	<0.05	114.9 \pm 15.3	184.4 \pm 11.3	<0.01	n.s.	n.s.
IgG_{2a} [μ g/ml]	502.3 \pm 90.3	383.8 \pm 42.0	n.s.	405.3 \pm 59.7	439.7 \pm 34.0	n.s.	n.s.	n.s.
IgG_{2b} [μ g/ml]	148.9 \pm 9.0	138.1 \pm 8.4	n.s.	134.7 \pm 12.0	158.4 \pm 10.1	n.s.	n.s.	n.s.
IgG₃ [μ g/ml]	96.7 \pm 6.4	240.0 \pm 17.6	<0.001	161.8 \pm 18.1	477.3 \pm 49.0	<0.001	<0.01	<0.001
IgM [μ g/ml]	224.8 \pm 29.7	334 \pm 21	<0.02	208.4 \pm 17.9	472.3 \pm 33.0	<0.001	n.s.	<0.01
IgA [μ g/ml]	67.0 \pm 9.1	27.1 \pm 3.6	<0.01	69.4 \pm 8.5	33.0 \pm 6.6	<0.01	n.s.	n.s.
Anti-DNA Ab [%]	0	0	n.s.	0	0	n.s.	n.s.	n.s.
Rheumatoid factor [%]	0	0	n.s.	0	0	n.s.	n.s.	n.s.

Raw data will be available on demand.

3.7 Allergy Screen

3.7.1 Summary

The goal of the Allergy screen within the German Mouse Clinic (GMC) is to search for IgE mutants in order to establish mouse models for allergic diseases and to find new strategies for antiallergic therapy. The increased production of IgE in response to common environmental antigens is the hallmark of atopic diseases in man (Hamelmann *et al.* 1999). Mouse mutants with phenotypic alterations in IgE production represent a valuable tool to study and characterize the molecular mechanisms of IgE-mediated allergic hypersensitivity (Zhang *et al.* 1997).

In the primary Allergy screen of Eyl mutant mouse line 26 control and 19 mutant animals were screened. Their analysis did not reveal any profound differences between mutant and control mice.

3.7.2 Mice

An age- and sex-matched group of 26 control (15 females, 11 males) and 19 mutant (seven female, 12 male) mice aged 12 weeks was analysed in the Allergy screen.

3.7.3 Material and Methods

Twelve-week-old male and female mice were screened for alterations in plasma total IgE concentrations. Blood samples were taken from animals by puncturing the retroorbital plexus under ether anesthesia. Plasma IgE concentrations were measured by isotype-specific sandwich ELISA technique with a lower detection limit of 1 ng/ml. briefly, microtiter plates were coated with the IgG fraction of sheep anti-mouse IgE in sodium bicarbonate buffer (pH 9.6). After incubation, plates were washed with Tris buffer (pH 7.4) and blocked with 3% (w/v) bovine serum albumin at room temperature. Diluted plasma samples and standard were added to the plates. After overnight incubation biotinylated rat anti-mouse IgE was added and plates were incubated at room temperature for 2 h. Then plates were incubated in the presence of peroxidase-labeled streptavidin. After washing, tetramethylbenzidine (TMB) substrate solution was added and after an appropriate incubation time the stop solution (sulphuric acid, 2M) was added. The plates were read in a standard microplate reader at a wavelength of 450 nm. Total murine IgE data are reported in ng/ml, based on a standard curve of purified murine IgE (Alessandrini *et al.*, 2001).

3.7.4 Results and Discussion

The analysis of total IgE levels in plasma of Eyl mice revealed no statistically significant differences between the mutant and control or between male and female mice (Table 18). The latter is a surprising finding because in general

IgE levels are higher in female mice (Alessandrini *et al.*, 2000; Corteling *et al.*, 2004; Seymour *et al.*, 2002).

Taken together, under standard screening conditions for primary Allergy screen, *Eyl*-mutant mice did not show changes in total plasma IgE levels that would reveal a major allergy phenotype.

Raw data will be available on demand.

Table 19: Total plasma IgE.								
Data are presented as mean ± standard error of mean.								
	Control (A)			Mutant (B)			A~B	A~B
	Female	Male		Female	Male		Female	Male
	(n=15)	(n=11)	<i>p</i>-value	(n=7)	(n=12)	<i>p</i>-value	<i>p</i>-value	<i>p</i>-value
Total IgE [ng/ml]	46± 20.6	37± 5.5	n.s.	53± 11.3	55± 4.3	n.s.	n.s.	n.s.

3.7.5 Reference

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3.8 Nociceptive Screen

3.8.1 Summary

Pain is the perception of an aversive or unpleasant sensation that originates from a specific region of the body. The highly subjective nature of pain is one of the factors that make it difficult to define and to treat clinically. Pain is more than a conspicuous sensory experience that warns of danger.

Nociceptors are activated by tissue injury but also by mechanical, thermal, or chemical stimuli. Harmful stimuli applied to the skin or to subcutaneous tissue, activate nociceptors, the peripheral endings of primary sensory neurons whose cell bodies are located in the dorsal root or in the trigeminal ganglia.

A noxious stimulus activates the nociceptor by depolarizing the membrane of the sensory ending. When peripheral tissues are damaged, the sensation of pain in response to subsequent stimuli is enhanced. This phenomenon termed hyperalgesia, may involve a lowering of threshold of the nociceptors or an increase in the magnitude of pain evoked by supra-threshold stimuli. Hyperalgesia can occur both at the site of tissue damage (primary hyperalgesia) and in the surrounding undamaged areas (secondary hyperalgesia; Wall and Melzak, 1984). By means of different inbred mouse strains it could be demonstrated that rodents display large and heritable differences in both nociceptive and analgesic sensitivity (Mogil, 1999; Mogil *et al.*, 1999)

In the Primary Screen the responsiveness of the intact somatosensory system to thermal pain was tested in the Eyl mutant mouse line by means of the hot plate test (nociceptive pain). We found significantly different pain reactivity in mutant animals in hind paw licking, namely thermal latencies were longer in mutant mice compared to controls. We found a significant sex difference in control animals in hind paw licking. It is strongly suggested making further pain related studies in this mutant mouse line.

3.8.2 Mice

Sixteen mutant mice (nine male, seven female), and 25 control animals (11 male, 14 female) were tested in our first screen.

3.8.3 Material and Methods

Hot plate test

The mice were placed on a metal surface maintained at $52 \pm 0.2^\circ\text{C}$ (Hot plate system was made by TSE GMBH, Germany; Eddy and Leimbach, 1953). Locomotion of the mouse on the hot plate was constrained by 20 cm high Plexiglas wall to a circular area with a diameter of 28 cm (Fig. 7). Mice remained on the plate until they performed one of three behaviors regarded as indicative of nociception: hind paw lick (h.p. licking), hind paw shake/flutter (h.p. shaking) or jumping.

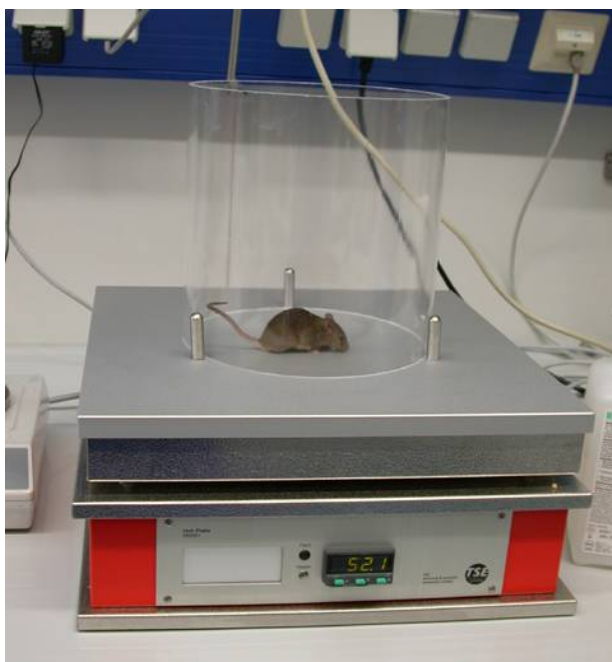


Figure 7: Hot plate system

We evaluated only hind paw but not the front paw responses, because fore paw licking and lifting are components of normal grooming behavior. Each mouse was tested only once since repeated testing leads to profound changes in response latencies. The latency was recorded to the nearest 0.1 s. To avoid tissue injury 60 s cut-off time was used. The data values are given in seconds.

Statistical analysis

Statistical analysis was performed using a statistical package Statgraphics® (Statistical Graphics Corporation, Rockville, MD). The differences between the groups were compared with ANOVA; LSD test was used as *post hoc*. Statistical significance was assumed at $p < 0.05$.

3.8.4 Parameters

Hind paw licking
Reaction with licking of hind paw to the thermal pain
Hind paw shaking
Reaction with shaking of hind paw to the thermal pain
Jumping
Jumping reaction to the thermal pain

3.8.5 Results

Typically, the first nociceptive response observed in mice is hind paw shaking, which control and mutant animals showed after comparable mean latencies (Table 20). Hind paw licking, another typical nociceptive response was shown

by both genotypes. A comparison by genotype revealed significantly longer latencies of mutant mice. The third examined response was jumping of the animals. The control animals did not jump at all (cut-off time of 60 s) whereas the mean reaction times of mutant animals were similar for both sexes (57.3 s (m) and 57.9 s (f) respectively). Additionally, there was a significant sex difference in control animals in hind paw licking, male animals had longer latencies.

Raw data will be available on demand.

3.8.6 Discussion

Hot plate latencies of mutant mice were longer concerning parameter 'hind paw licking'. *Eyl*-mutant mice had hypoalgesia compared to wild-type control mice despite the mutant and control mice reacted similarly to the thermal pain stimulus considering the other two signs of pain. Therefore we would suggest making further pain related studies to specify the pain sensitivity of this mutant mouse line in more detail, because the Neurology Screen (3.3.5) and Clinical Chemical Screen detected hints of muscular disorders (3.5.5).

More detailed pain related studies would include:

1. Base studies e.g.,
 - a. von Frey filament test to study the reaction of animals to mechanical pain,
 - b. acetic acid test to study the reaction to visceral inflammation.
2. Tail flick test, to study whether the hypoalgesia has a spinal or supraspinal origin.
3. Chronic pain tests:
 - a. Formalin test to study the acute, nociceptive (early) and tonic, inflammatory (late) pain reaction of the same animals,
 - b. Carrageenan test to study the reaction to inflammation.

The results of the whole set of experiments will provide a complete picture about the pain reactivity of this mutant mouse line.

3.8.7 References

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Abbreviations

h.p. hind paw

Table 20: Nociceptive Screen									
Data are presented as mean \pm standard error of mean.									
							ANOVA		
							genotype		sex*genotype
Parameter Latency [s]	Mutant (A)			Control (B)			A~B	A~B	ANOVA
	Female	Male		Female	Male		Female	Male	
	(n=7)	(n=9)	<i>p</i> -value	(n=14)	(n=11)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
H.p. shaking	19.6 \pm 4.3	21.2 \pm 3.77	n.s.	21.5 \pm 3.0	25.6 \pm 3.4	n.s.	n.s.	n.s.	n.s.
H.p. licking	52.5 \pm \uparrow 4.1	56.3 \pm \uparrow 3.6	n.s.	28.9 \pm 2.9	40.8 \pm \uparrow 3.3	<0.01	<0.001	<0.01	n.s.
Jumping	57.9 \pm 1.4	57.3 \pm 1.2	n.s.	60	60	n.s.	n.s.	n.s.	n.s.

\uparrow genotype-specific differences (delay)

\uparrow sex-specific differences (delay)

3.9 Lung Function Screen

3.9.1 Summary

Neural and mechanical processes that control breathing frequency have been investigated in man for a long time (Mead, 1960; Otis *et al.*, 1959), but only with the availability of mouse inbred strains the contribution of genetic determinants to differential baseline breathing patterns could be elucidated (Tankersley *et al.*, 1997; Tankersley, 1999; Reinhard *et al.*, 2002; Reinhard *et al.*, 2005). By use of genetically engineered mice, candidate genes for human developmental disorders of breathing have been identified (Katz, 2003).

Spontaneous breathing patterns during activity were studied in 15-week-old male EYL mice. Mutant mice showed slightly higher breathing rates accompanied by significantly lower values for tidal volume, minute ventilation, inspiratory timing, and mean inspiratory flow rate. These data suggest that the mutant mice have an altered spontaneous breathing pattern with more frequent but less effective breathing compared to wild-type mice. This could be either due to reduced lung size and/or compliance or simply due to the smaller body size of the mutants.

3.9.2 Mice

Four male control and three male mutant mice were studied at the age of 15 weeks. Female mice were not available. Mean body weights were significantly different with values in control mice of 30.4 ± 1.4 g and in mutant mice of 24.1 ± 1.7 g (Table 22).

3.9.3 Material and Methods

Whole Body Plethysmography

A commercially available system from Buxco[®] Electronics (Sharon, Connecticut) was used to assess breathing patterns in unrestrained animals according to the principle described by Drorbaugh and Fenn (1955). It measures the pressure changes which arise from inspiratory and expiratory temperature and humidity fluctuations during breathing (Fig. 8 and 9).

Calibration of the system allows to transform these pressure swings into flow and volume signals so that automated data analysis provides tidal volumes (TV), respiratory rates (f), minute ventilation (MV), inspiratory and expiratory times (Ti, Te), as well as peak inspiratory and peak expiratory flow rates (PIF, PEF). These data were stored online as mean values at 10 s intervals.

Measurements were always performed between 8 a.m. and 11 a.m. to account for potential diurnal variations in breathing. The system was set up in a quiet room where temperature and humidity were kept constant throughout the measurements. Before each measurement, the system was calibrated and the actual barometric pressure, temperature, and humidity were supplied to warrant adequate calculations of flow rates and volumes. After placing the animals into the chamber, data recording was immediately started and was continued for 40 min.



Figure 8: System used at GMC to assess breathing patterns.

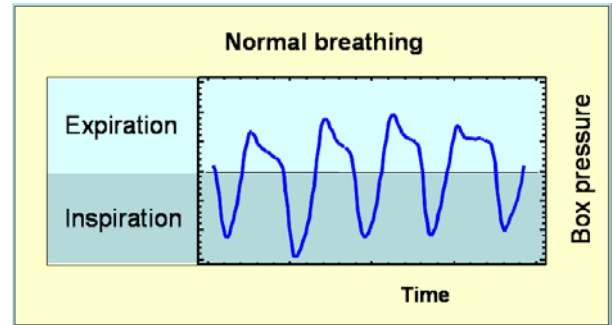


Figure 9: Recorded data used to calculate the breathing parameters.

Mice underwent typical phases during the measuring period. Primarily, the animals were stressed so that the respiratory rate was highest at the beginning. Usually after 5 min. the animals became calmer, they slightly reduced their respiratory rate, and began to explore the chamber and start cleaning themselves – *phase of activity*. Later activity was more and more interrupted by phases of rest or even short periods of snoozing – *resting phase*. Some of the animals even went to *phases of sleep*, which resulted in a further marked decrease in respiratory rate. The frequency histogram of the respiratory rates was determined for each individual, and breathing was analyzed for the above mentioned parameters during the phases of activity and rest. In addition to the directly recorded parameters, mean inspiratory and expiratory flow rates (MEF, MIF) were calculated offline from the ratio of tidal volume and the respective time interval. The relative duration of inspiration (T_i/TT) was determined from the ratio of inspiratory time to total time required for the breathing cycle. Specific tidal volumes and minute ventilations (sTV, sMV) were calculated by relating the absolute values to the body weight of the animal. Furthermore, the mean of all breathing frequencies (mean_f) measured during the 40-minute-period was calculated as a rough and ready parameter to assess whether the duration of rest and activity was similar in all mouse strains.

Statistical Analysis of Data

Statistical analyses were performed using a commercially available statistics package (Statgraphics®, Statistical Graphics Corporation, Rockville, MD). Differences between strains were evaluated by Students t-test. Statistical significance was assumed at $p < 0.05$. Data are presented as mean values \pm standard error of the mean (SEM).

3.9.4 Parameters

Directly recorded data
Tidal volumes (TV), respiratory rates (f), minute ventilation (MV), inspiratory and expiratory times (Ti, Te), as well as peak inspiratory and peak expiratory flow rates (PIF, PEF).
Calculated data
mean inspiratory flow rates (MEF), expiratory flow rates (MIF), relative duration of inspiration (Ti/TT), specific tidal volumes (sTV), minute ventilations (sMV), mean of all breathing frequencies (mean_f)

3.9.5 Results

Table 21 summarizes the results obtained for spontaneous breathing under resting and active conditions. Male mutant mice showed slightly higher breathing rates accompanied by significantly lower values for tidal volume, minute ventilation, inspiratory timing, and mean inspiratory flow rate compared to their wild-type counterparts. The mean breathing rate in mutant mice was significantly higher than in control mice and resting conditions were only seen in control mice.

Raw data are available on demand.

3.9.6 Discussion

The results suggest that mutant mice have an altered spontaneous breathing pattern with more frequent but less effective breathing compared to control mice (Fig. 10). This could be either due to reduced lung size and/or compliance or simply due to the smaller body size of the mutants.

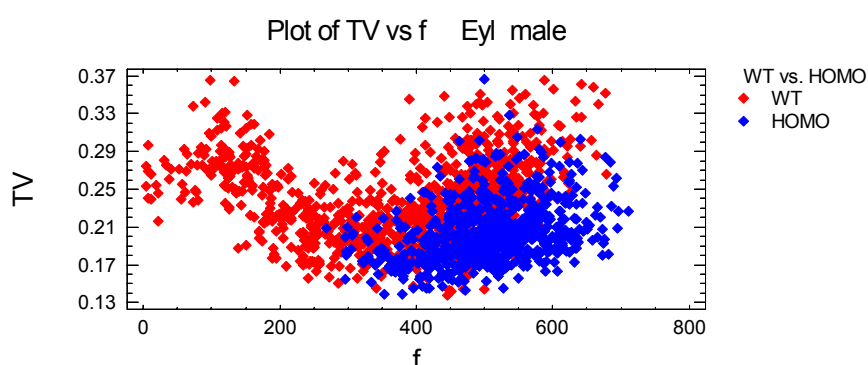


Figure 10: Plot tidal volume vs. respiratory rate.

Altered spontaneous breathing pattern of mutant mice (blue) with more frequent but less effective breathing compared to wild-type mice (red).

Mutant mice also seem to be more active than their littermate controls with respect to the mean breathing rate. Since the mean breathing rate is a rough

parameter to judge the level of activity, the results of the Behaviour screen (3.1.5) are to be carefully considered. Given the small number of mice, the data has to be interpreted carefully and confirmed by testing more and female mice.

3.9.7 References

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Abbreviations

bw	body weight (g)
mean_f	mean of all respiratory rates (1/min)
f	respiratory rate (1/min)
TV	tidal volume (ml)
sTV	specific tidal volume (μ l/g)
MV	minute ventilation (ml/min)
sMV	specific ventilation (ml/min/g)
Ti	inspiratory time (ms)
Te	expiratory time (ms)
Ti/TT	relative duration of inspiration
PIF	peak inspiratory flow rate (ml/s)
PEF	peak expiratory flow rate (ml/s)
MIF	mean inspiratory flow rate (ml/s)
MEF	mean expiratory flow rate (ml/s).

Table 21: Characterization of studied mice

Data are presented as mean \pm standard error of mean.

Parameter	Control (A)			Mutant (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=4)	(n=0)	<i>p - value</i>	(n=3)	(n=0)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
Bw [g]	30.4 \pm 1.4			24.1 \pm 1.7			< 0.05	
Age [d]	109.3 \pm 0.3			108.0 \pm 0				
Mean_f [1/min]	377.1 \pm 13.3			503.2 \pm 24.5			< 0.01	

Table 22: Spontaneous breathing pattern during rest and activity

Data are presented as mean \pm standard error of mean.

Parameter	Control (A)			Mutant (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=4)	(n=0)	<i>p</i> - value	(n=3)	(n=0)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
Rest								
f [1/min]	358.1 \pm 4.0							
TV [ml]	0.21 \pm 0.01							
sTV [μ l/g]	6.9 \pm 0.3							
MV [ml/min]	72.9 \pm 2.0							
sMV [ml/min/g]	2.4 \pm 0.1							
Ti [ms]	51.8 \pm 1.6							
Te [ms]	115.8 \pm 3.3							
Ti/TT	0.31 \pm 0.01							
PIF [ml/s]	7.8 \pm 0.6							
PEF [ml/s]	4.7 \pm 0.4							
MIF [ml/s]	4.1 \pm 0.2							
MEF [ml/s]	1.8 \pm 0.0							
Activity								
f [1/min]	483.1 \pm 6.8			502.1 \pm 24.1			n.s.	
TV [ml]	0.24 \pm 0.01			0.20 \pm 0.004			< 0.05	
sTV [μ l/g]	8.0 \pm 0.4			8.6 \pm 0.8			n.s.	
MV [ml/min]	115.4 \pm 3.6			102.1 \pm 3.1			< 0.05	
sMV [ml/min/g]	3.8 \pm 0.2			4.3 \pm 0.2			n.s.	
Ti [ms]	39.9 \pm 0.5			37.6 \pm 0.4			< 0.05	
Te [ms]	84.3 \pm 2.2			82.4 \pm 5.3			n.s.	
Ti/TT	0.32 \pm 0.008			0.31 \pm 0.01			n.s.	
PIF [ml/s]	10.9 \pm 0.7			9.7 \pm 0.3			n.s.	
PEF [ml/s]	6.9 \pm 0.4			5.9 \pm 0.3			n.s.	
MIF [ml/s]	6.1 \pm 0.3			5.4 \pm 0.1			< 0.05	
MEF [ml/s]	2.9 \pm 0.1			2.5 \pm 0.1			n.s.	

3.10 Molecular Phenotyping

3.10.1 Summary

Comparative genomewide expression profiling is a powerful tool in the effort to annotate the mouse genome with biological function. The analysis of RNA expression data of mouse lines might support the understanding of the molecular biology of such mutants and provide new insights into mammalian gene function. We demonstrated the feasibility to detect transcriptional affected organs employing RNA expression profiling as a tool for molecular phenotyping (Seltmann *et al*, 2005).

In this report, we describe the results of using close to genome-wide 20K cDNA microarrays for the RNA expression profiling of **liver** from seven animals of the Eyl mutant mouse line. In total only five chip hybridisations were performed. The data analysis and various statistical methods detected no differentially regulated genes between mutant and control liver tissue.

3.10.2 Organ Collection

The molecular phenotyping screen archives organs of mutant and control mice for subsequent DNA-chip expression profiling analysis.

Seven male mice (three mutants and four controls) of the Eyl mutant mouse line were provided to the molecular phenotyping screen.

Organs were collected at the age of 105-110 days. To minimize the influence of circadian rhythm on gene expression, mice were killed between 9 am and 12 am by carbon dioxide asphyxiation. The following 13 organs were collected and archived in liquid nitrogen following our established SOPs (Standard Operation Procedure): bulbourethral gland, spleen, kidney, seminal vesicles, testis, liver, heart, lung, thymus, skin/cartilage (outer ear), skeletal muscle, salivary gland and brain. Organs were immediately frozen and stored in liquid nitrogen until isolation of total RNA. The 130 organ samples collected in the collaboration either may be used for further expression profiling analysis in the GMC or, alternatively may be transferred to the collaborator.

Mouse ID	Strain	Sex	Date of Birth	Genotype	Date of Collection
30022000	Eyl	m	5.06.2004	-/-	22.09.2004
30022001	Eyl	m	5.06.2004	-/-	22.09.2004
30022002	Eyl	m	3.06.2004	+/+	22.09.2004
30021989	Eyl	m	4.06.2004	+/+	22.09.2004
30021988	Eyl	m	4.06.2004	+/+	22.09.2004
30021990	Eyl	m	4.06.2004	+/+	22.09.2004
30021998	Eyl	m	5.06.2004	-/-	22.09.2004

3.10.3 Material and Methods

Isolation of total RNA

Total RNA was isolated just before processing for expression profiling. For preparation of total RNA individual organs were thawed in buffer containing chaotropic salt (RLT buffer, Qiagen) and homogenised using a Polytron homogeniser. Total RNA from individual samples was obtained according to manufacturer's protocols using RNeasy Midi kits (Qiagen). 2 µg RNA aliquots were run on a formaldehyde agarose gel to check for RNA integrity and the concentration was calculated from OD_{260/280} measurement. The RNA was stored at -80°C in RNase free water (Qiagen).

Chip design

We use a glass-surface DNA-chip containing ≈ 21,000 probes. About 20,200 of these probes are from the commercial Lion mouse array-TAG clone set, which is mostly derived from 3'UTRs. All Lion probes have been sequenced. The remaining probes are genes associated with immune response. Mouse array-TAG clones have the general ID MG-VW-XYZ (e.g. MG-3-1a5, MG-12-190m5,...) and the other probes are named s0-geneID (e.g. s0-birk, s0-mark1...).

DNA Microarrays

PCR products with 5'-aminogroup are amplified from the mouse arrayTAG library from Lion Bioscience comprising approximately 20,200 clones (Heidelberg, Germany). PCR products are dissolved in 3x SSC buffer and spotted on aldehyde-coated slides (Telechem, USA) using a Microgrid TAS II spotter (Biorobotics) with 48 Stealth™ SMP3 pins (Telechem). Spotted slides are rehydrated overnight in a humid chamber containing 50-70% aqueous solution of glycerol. Rehydrated slides are immersed in blocking solution (0.1 M sodium borohydride in 0.75x PBS with 25% ethanol) for 5 minutes, boiled in water for 2 minutes, briefly immersed in 100% ethanol and air-dried (Seltmann *et al*, 2005). Slides are pre-hybridized for 45 minutes/42°C in pre-hybridization buffer (6x SSC, 1% BSA, 0.5% SDS) in a Hybstation (Tecan)

Chip Hybridization

Depending on the amount of RNA available for hybridization, in general two chip hybridizations are performed with RNA from all organs of each individual mutant mouse. Each chip hybridization is performed against the identical pool of the same organ of reference RNAs (reference RNA pool). For each individual the chip experiments include a color-flip experiment (in total 10 hybridizations). If differential gene expression will be detected between mutant and control mice, additional wt/wt_pool experiments will be performed. By this approach it would be possible to distinguish inter-individual variation of reference individuals that is relevant to a mutation from that which is not (Seltmann *et al*, 2005).

Reverse Transcription and Fluorescent Labeling

For labeling 20 µg of total RNA are used for reverse transcription and indirectly labeled with Cy3 or Cy5 fluorescent dye according the TIGR protocol (Hedge *et al.*, 2000). Labeled cDNA is dissolved in 30 µl hybridization buffer (6x SSC, 0.5% SDS, 5x Denhardt's solution and 50% formamide) and mixed with 30 µl of reference cDNA solution (pool from five control animals) labeled with the second dye. This hybridization mixture is injected on the pre-hybridized microarray in the Hybstation (Tecan). Hybridization (14-16h) at 42°C is performed automatically including wash steps with decreasing SSC concentrations and drying the slides with nitrogen. Slides are scanned with a GenePix 4000A microarray scanner and the images are analyzed using the GenePix Pro3.0 image processing software (Axon Instruments, USA). All data are normalized by adjusting the median of log-ratios of Cy5 to Cy3 intensities to 0. For data analysis, Pattern Analysis of Microarrays (PAM) (http://www.gsf.de/ieg/groups/exppro_cpt.html#PAM) is used.

Ranking of Genes

Genes are ranked according the lowest absolute ratio of signal intensities in all microarray experiments (mutant versus reference) for each organ. This ranking is independent of the reproducibility in terms of up- and down-regulation.

Significance of Regulation

Genes are evaluated for the significance of differential gene expression: The number of non-differentially expressed genes („NDE, false positives“) among genes with reproducible patterns is calculated for significance levels $p < 0.05$. This means: The selected top differentially expressed genes with reproducible up- or down-regulation include less than 30% non-uniform and less than 20% false positives (NDE) expressed genes with a probability $< 5\%$.

3.10.4 Results

Selected Organs and Isolated RNA

Liver was selected as organ for expression profiling analysis based on data from other GMC-screens (Clinical-Chemical Screen, 3.5.5). We isolated total RNA of these organ of three mutant mice and four wild-type control individuals (Table 24).

Table 24: Amount of total RNA [µg] isolated from liver.	
Mouse ID	Liver
30022000	97
30022001	235
30022002	285
30021989	183
30021988	31
30021990	360
30021998	128

Analysis of Gene Expression in Liver

Table 25 summarizes the results of five chip hybridizations performed with RNA from liver. In total, 2527 probes show signals in all five chip hybridization experiments.

Table 25: Chip hybridization of liver: labeling and number of detected spots	
Numbers indicate the ID of mutant mice.	
Cy5/Cy3	Detected Spots
Wt pool/ 000	4331
000/ Wt pool	5176
Wt pool / 001	3674
Wt pool/ 998	6420
998/ Wt pool	5329
	2171 overlap

These genes were evaluated for the significance of differential gene expression. Genes were ranked according to the lowest absolute ratio of signal intensities (mutant versus wt) in five microarray experiments. This ranking is independent of the reproducibility in terms of up- and down-regulation. The number of genes with non-reproducible up- or down-regulation („non-uniform patterns“) is given for different selections of genes in the ranking („ranked genes“). The number of non-differentially expressed genes („NDE, false positives“) among genes with reproducible patterns was calculated for significance level $p < 0.01$.

For example, the selection of the top 20 ranked genes contains 13 genes with non-reproducible chip data. The remaining seven genes with reproducible up- or down-regulation contain five or more non-differentially expressed genes with the significance level $p < 0.01$. The minimal ratios of expression for this selection ranged from 2.99 to 1.30 fold induction/repression.

Table 26: Chip hybridization of liver: evaluation of data			
Ranked Genes (According to Lowest of five Ratios)	Non-uniform Patterns	NDE (False Positives) $p < 0.01$	Fold Induction (Minimum of five Chips)
1 - 20	13	≥ 5	2.99 – 1.30
1 - 40	26	≥ 7	2.99 – 1.24
1 - 60	43	≥ 9	2.99 – 1.22
1- 100	75	≥ 12	2.99 – 1.18

According to 65% non-reproducible chip data of the top 20 ranked genes, no gene with differential expression in liver of *Ey1*-mutant mice was observed in all experiments.

3.10.5 Discussion

Using the selection criteria described above, we could identify no genes that are differentially expressed in liver tissue of *Eyl*-mutant mice.

3.10.6 References

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3.11 Metabolic Screen

3.11.1 Summary

The metabolic screening provides a comparative analysis of bioenergetic parameters in mice. Mechanisms which lead to disturbances in body weight regulation and energy metabolism are determined. Hence, the basal energetic demands are monitored during *ad libitum* feeding and under food restricted conditions. In humans unbalanced energy uptake and energy expenditure cause the development of obesity (Spiegelman and Flier, 2001) or anorexia nervosa with severe weight loss (Hebebrand *et al.*, 2003). Some rodent and other species tend to increase activity upon food restriction leading to weight loss when given access to an activity wheel (Exner *et al.*, 2000). Several studies described that fasting in mice results in transient depression of metabolic rate, heart rate, body temperature and locomotor activity (Duffy *et al.*, 1990; Williams *et al.*, 2002). Therefore the primary Metabolic Screening focuses on the determination of food and energy uptake under *ad libitum* conditions and metabolic adaptations during food restriction and serves as the origin for further investigations in the Secondary and Tertiary screening which go into details of energy expenditure and energy storage.

The primary metabolic screen focuses on investigation of metabolic demands of mice determining daily body weight, energy uptake, metabolizable energy and body temperature and adaptive capacity of metabolic processes.

Genotype-specific differences were found in body temperature of males with higher values in mutants. In females genotype-specific differences were found in body weight and body weight related parameters of females while mutant females consume more food and showed a higher ratio of metabolized energy. Females of wild-type littermate controls were heavier than males, while in mutants males showed higher body weights. Energy uptake per unit body weight of mutant females is higher than in males. In control mice, females showed less energy uptake per unit body weight than the corresponding males.

3.11.2 Mice

Seven adult control males and seven adult mutant males entered the Metabolic Screen at the beginning of calendar week 42 in 2004. The females (six control and six mutants) entered the metabolic laboratory one week later.

The mice were single caged on grid panels (0.5 cm grid hole diameter). They were fed *ad libitum* for a period of 14 days, followed by a period of food restriction to 60% of *ad libitum* for seven days to analyze adaptive responses of metabolism.

3.11.3 Material and Methods

Recorded Data

During the different feeding regimes body weight, food consumption (F_{con}), rectal temperature (T_{re}), daily feces production (Fec), energy uptake (E_{up}), energy content of the feces (E_{fec}), metabolizable energy (E_{met}) and the food assimilation coefficient (F_{ass}) were recorded.

Analysis of Feces

The separation of mice in single cages allowed collection of feces in three day intervals. Samples of lab chow and feces (~1 g) were dried at 60°C for two days, homogenized in a coffee grinder and squeezed to a pill for determination of energy content in a bomb calorimeter (IKA Calorimeter C7000) based on dry measurement principle. Energy uptake is determined as the product of food consumed and the caloric value of the food. To obtain metabolizable energy (E_{met}) the energy content of feces and urine (2% of E_{up} ; Drozd 1975) were subtracted from energy uptake.

Statistical Analysis

All values are presented as means \pm SEM. Two-way-ANOVA (SigmaStat, Jandel Scientific) was used to test for effects of the factors genotype and sex. The Tukey test was applied for post hoc multiple comparisons. The Mann-Whitney-Test for paired samples was used to analyze the effect of nutritional status on parameters of energy metabolism.

3.11.4 Parameters

Recorded Data during the different feeding regimes
body weight, food consumption (F_{con}), rectal temperature (T_{re}), daily feces production (Fec), energy uptake (E_{up}), energy content of the feces (E_{fec}), metabolizable energy (E_{met}), food assimilation coefficient (F_{ass})

3.11.5 Results

Genotype-specific differences in males were determined only in body temperature, showing higher values in mutant males. Mutant females were significantly lighter, but showed higher energy uptake and metabolized energy per unit body weight.

Comparing the **sexes** within both genotypes revealed differences in body weight as well. Control females were significantly heavier compared to males while mutant females were lighter than mutant males. Due to similar food consumption, the body weight related energy uptake and metabolized energy is lowered in control females. In mutant mice the food consumption between both sexes was similar, too, resulting in elevated energy uptake per unit body weight in mutant females.

Raw data for each individual are available on demand in Excel sheets.

3.11.6 Discussion

No information about metabolic properties were available prior the metabolic screening of Eyl mutant mouse line. This spontaneously aroused mutant line occurred during inbreeding from 102-strain on C3H strain. Usually body weights of both sexes are very similar in C3H mice. Presented data showed heavier females compared to males in the control group, while mutant mice exhibited lighter females.

Genotype-specific differences originate from this inverted relation. From our experience body weight of C3H control males in the present batch are too low to serve as control group. However, there is also the possibility that body weight of control females is too high. The consequence is that due to the body weight differences in control mice, a reasonable interpretation concerning a metabolic phenotype is very difficult and present findings should be considered carefully.

3.11.7 References

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Abbreviations

F_{con}	Food consumption
T_{re}	rectal temperature
F_{ec}	daily feces production
E_{up}	energy uptake
E_{fec}	energy content of the feces
E_{met}	metabolizable energy
F_{ass}	food assimilation coefficient

Table 27: Metabolic parameters recorded in the primary screen

Data are presented as mean \pm standard error of mean.

Parameter	Control (A)					Mutant (B)					A~B	
	<i>ad libitum</i>		<i>p</i> -value	food reduction, 7 days to 60%		<i>ad libitum</i>		<i>p</i> -value	food reduction, 7 days to 60%		<i>p</i> -value	<i>p</i> -value
	Male	Female		Male	Female	Male	Female		Male	Female		
	(n=7)	(n=7)		(n=7)	(n=7)	(n=7)	(n=6)		(n=7)	(n=6)		
Body weight [g]	30.7 \pm 1.18	35.8 \pm 1.57	<0.05	26.2 \pm 1.16	30.8 \pm 1.56	27.9 \pm 1.15	24.4 \pm 0.65	<0.05	21.8 \pm 1.41	18.3 \pm 0.83	n.s.	<0.01
Rectal body temperature [°C]	36.8 \pm 0.1	37.2 \pm 0.07	<0.05	35.9 \pm 0.24	37.0 \pm 0.14	37.8 \pm 0.31	37.8 \pm 0.26	n.s.	36.1 \pm 0.82	35.8 \pm 1.15	0.01	n.s.
Food consumption [g day⁻¹]	4.01 \pm 0.23	3.78 \pm 0.16	n.s.	60% of <i>ad libitum</i>		3.44 \pm 0.18	3.45 \pm 0.12	n.s.	60% of <i>ad libitum</i>		n.s.	n.s.
Energy uptake [kJ day⁻¹]	73.9 \pm 4.15	69.7 \pm 2.91	n.s.	44.3 \pm 2.49	42.0 \pm 2.36	63.5 \pm 3.36	63.5 \pm 2.14	n.s.	38.1 \pm 2.02	38.1 \pm 1.28	n.s.	n.s.
Energy uptake BW⁻¹ [kJ g⁻¹ day⁻¹]	2.4 \pm 0.09	1.95 \pm 0.06	<0.01	1.69 \pm 0.05	1.41 \pm 0.05	2.27 \pm 0.11	2.61 \pm 0.09	0.049	1.77 \pm 0.11	2.1 \pm 0.16	n.s.	<0.001
Feces production [g day⁻¹]	0.65 \pm 0.02	0.73 \pm 0.04	n.s.	0.45 \pm 0.04	0.44 \pm 0.03	0.6 \pm 0.04	0.63 \pm 0.03	n.s.	0.47 \pm 0.03	0.43 \pm 0.02	n.s.	<0.05
Energy content feces [kJ g⁻¹]	16.3 \pm 0.04	16.0 \pm 0.03	n.s.	16.3 \pm 0.07	16.2 \pm 0.04	16.4 \pm 0.05	15.9 \pm 0.11	<0.01	16.2 \pm 0.09	16.3 \pm 0.16	n.s.	n.s.
Metabolized energy [kJ day⁻¹]	61.4 \pm 3.75	56.6 \pm 2.3	n.s.	36.7 \pm 2.54	34.0 \pm 1.79	52.4 \pm 3.06	51.8 \pm 1.96	n.s.	29.7 \pm 1.51	30.3 \pm 1.05	n.s.	n.s.
Metabolized energy [kJ g⁻¹ day⁻¹]	2.01 \pm 0.08	1.58 \pm 0.05	0.001	1.4 \pm 0.06	1.14 \pm 0.05	1.88 \pm 0.11	2.12 \pm 0.07	n.s.	1.38 \pm 0.09	1.6 \pm 0.08	n.s.	<0.001
Food assimilation coefficient [%]	83.6 \pm 0.04	81.2 \pm 0.21	<0.001	81.3 \pm 1.19	80.9 \pm 0.56	82.3 \pm 1.19	81.5 \pm 0.71	n.s.	78.1 \pm 0.43	79.6 \pm 0.61	n.s.	n.s.

3.12 Pathology Screen

2.12.1. Summary

The Pathology Screen performed a complete morphological analysis with standard stains. We analyzed thirty-three mice. Mutant and control mice differ significantly in their body weight. A morphologic phenotype of the *Eyl*-mutant mice was seen in four organs. We confirmed the congenital anophthalmia in all mutant males and the reduced expression of dopamine transporter 1 in the midbrain in all mutants. In addition, we found congenital microphthalmia in all mutant females, an increased extramedullary hematopoiesis in the spleen in all mutants, and hepatosteatosis in seven of eleven analysed mutants. These latter findings suggest a possible metabolic phenotype.

2.12.2. Mice

A total of 33 mice, 15 mutant and 18 control mice, were analyzed. Due to the workflow in the GMC, mice were received from the metabolic screen at the age of 21-22 weeks. The term “other screens” is used when a mouse is received in the pathology from any other screen (Table 28).

Table 28: <i>Eyl</i> -mutant mice and their control littermates analyzed.						
Origin	Control		Mutant		Number of Animals	Age [weeks]
	Male	Female	Male	Female		
Metabolic Screen	7	10	5	6	28	21 - 22
Other Screens	0	1	3	1	5	12 - 16
Total Number of Animals	7	11	8	7	33	

3.12.1 Materials and Methods

Mice received in the laboratory of pathology were sacrificed with CO₂. The animals were analyzed macroscopically and weighed (www.eulep.org/Necropsy_of_the_Mouse/index_2004.php). The thymus and left lobe of the liver were measured. Blood samples were taken, centrifuged and the serum was saved at -20°C. Tails were preserved at -70°C for further genetic analysis. Following a complete dissection, an x-ray of the complete bone structure was taken, when indicated (Hewlett Packard, Cabinet X-Ray System Faxitron Series). All organs were fixed in 4% buffered formalin and embedded in paraffin for histological examination. Two-µm-thick sections from skin, heart, mus-

cle, lung, brain, cerebellum, thymus, spleen, cervical lymph nodes, thyroid, parathyroid, adrenal gland, stomach, intestine, liver, pancreas, kidney, reproductive organs, and urinary bladder were cut and stained with haematoxylin and eosin (H&E). Prussian's Blue staining was performed when indicated.

Immunohistochemistry was performed by Alessia Ivashkevich. Briefly, the slides were deparaffinized and rehydrated. The antigen retrieval was done in a microwave pressure cooker with 0.01 mol/l citrate buffer (pH 6) containing 0.1% Tween 20 at 600 W for 30 minutes. After cooling down the slides in Tris-buffered saline the sections were incubated in 3% goat serum for 20 minutes. Staining was carried out with an automated immunostainer (Ventana Medical Systems, Inc., Tucson AZ). The following primary antibody was used: polyclonal rabbit anti dopamine transporter 1 (Chemicon, Hampshire, GB; dilution: 1:5000).

When indicated samples for electron microscopy were fixed in glutaraldehyde and 0.5-µm-thick semifine sections were taken. For statistical analysis the t-test and the Fisher's exact test were performed. A p-value of less than 0.05 was assumed to be significant.

3.12.2 Results

Overview

Table 29: Eyl mice. Genotype-specific morphological alterations.					
Organ	Skin	Musculoskeletal System	Eyes	Brain	Cerebellum
Alteration	no	no	yes	yes	no
Organ	Heart	Trachea	Lung	Teeth	Salivary glands
Alteration	no	no	no	no	no
Organ	Esophagus	Stomach	Small Intestine	Large Intestine	Liver
Alteration	no	no	no	no	yes
Organ	Pancreas	Cervical Lymph Nodes	Thymus	Spleen	Thyroid
Alteration	no	no	no	yes	no
Organ	Parathyroid	Adrenal Gland	Kidneys	Urinary Bladder	Testes
Alteration	no	no	no	no	no
Organ	Epididymis	Funiculus spermaticus	Ovaries	Uterus	Vagina
Alteration	no	no	no	no	no

Our analysis in the primary screen did reveal an *Eyl*-associated morphologic phenotype in four organs (eye, midbrain, spleen and liver) and in the body weight of these mice.

Body Weight: Between mutant and control mice, differences in body weight were observed (see Table 29 for more details). The difference was significant for the females ($p < 0.001$) as well as for the males ($p < 0.01$). For a detailed discussion please see 3.11.6, Metabolic Screen).

Table 30: Mean body weight \pm standard deviation of mutant mice and their control littermates.					
Origin	Control		Mutant		Age [weeks]
	Male	Female	Male	Female	
Metabolic Screen	30 \pm 2.6	34.8 \pm 3.9	26.2 \pm 1.5	23.7 \pm 1.5	23 - 25

Eye phenotype. The pathology screen confirmed the eye phenotype. All mutants had congenital abnormalities in the eye development. All male mutants lacked both eyes. In contrast, a microphthalmia but not anophthalmia was observed in all mutant females. The eye phenotype is assumed to be genotype-specific (as no controls did show any alterations of the eyes) with 100% penetrance.

Liver phenotype. In seven out of eleven analyzed mutants (64%) and in one out of 17 control mice (6%) a micro- and macrovesicular steatosis of the liver was observed. That result was highly significant (Fisher's exact test: $p < 0.01$) Female mutants were gradually even more affected than male mutant mice (Fig. 10).

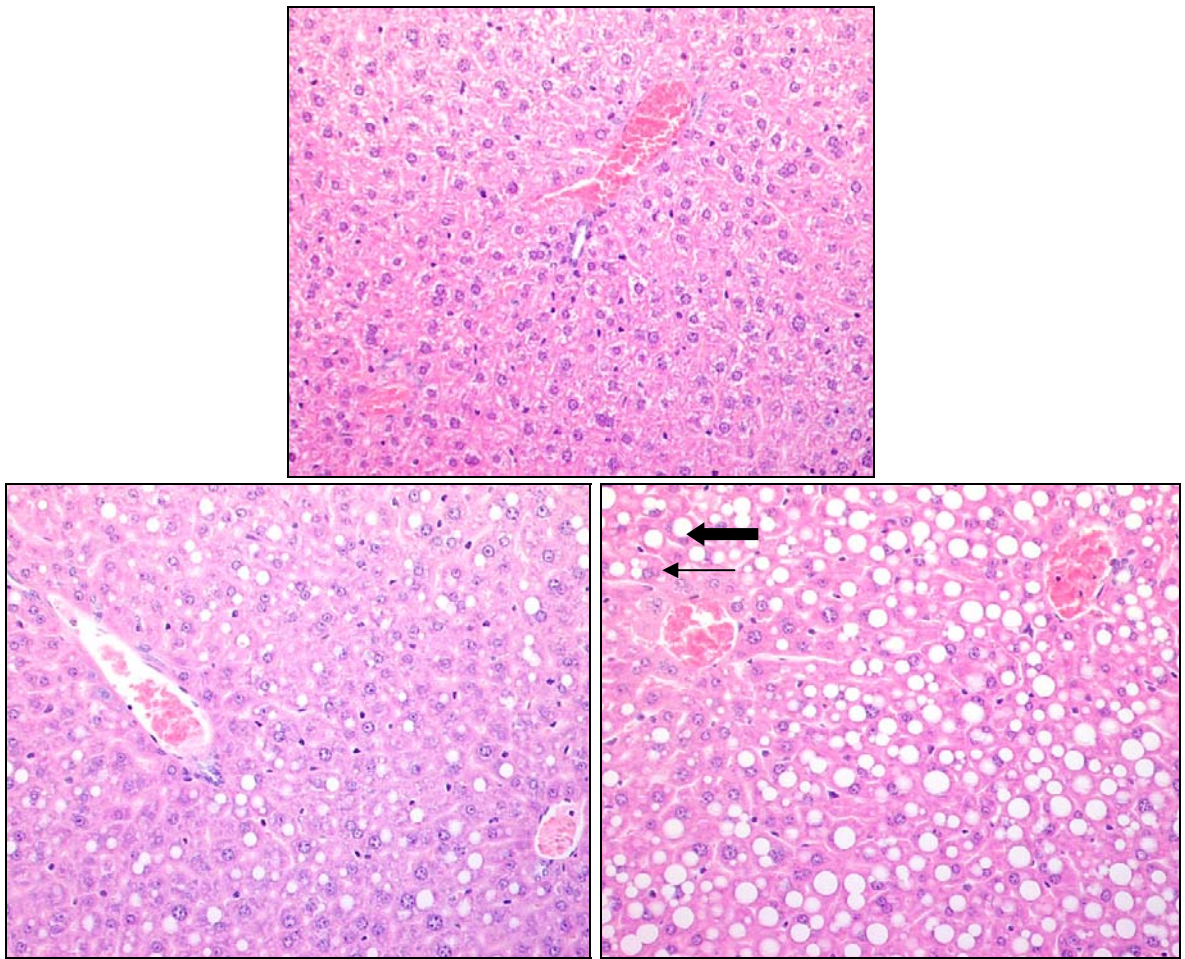


Figure 11: Steatosis of the liver.

The first picture (upper panel) is a photomicrograph of the liver of a control animal. The portal tract is shown in the center of the picture. The hepatocytes store a small amount of glycogen. Both lower panels depict livers of mutant mice. On the left (male mutant mouse), there are fat vacuoles within the cytoplasm of the hepatocytes. The steatosis is stronger on the right (female mutant mouse). The thick arrow indicates macrovesicular steatosis. In that condition the nucleus becomes dislocated towards the cell membrane. The thin arrow points to a hepatocyte with microvesicular steatosis. Here the nucleus is still located in the center of the cell.

Midbrain phenotype

The pathology screen confirmed the reduced expression of dopamine transporter 1 in the midbrain (Figs. 11 and 12).

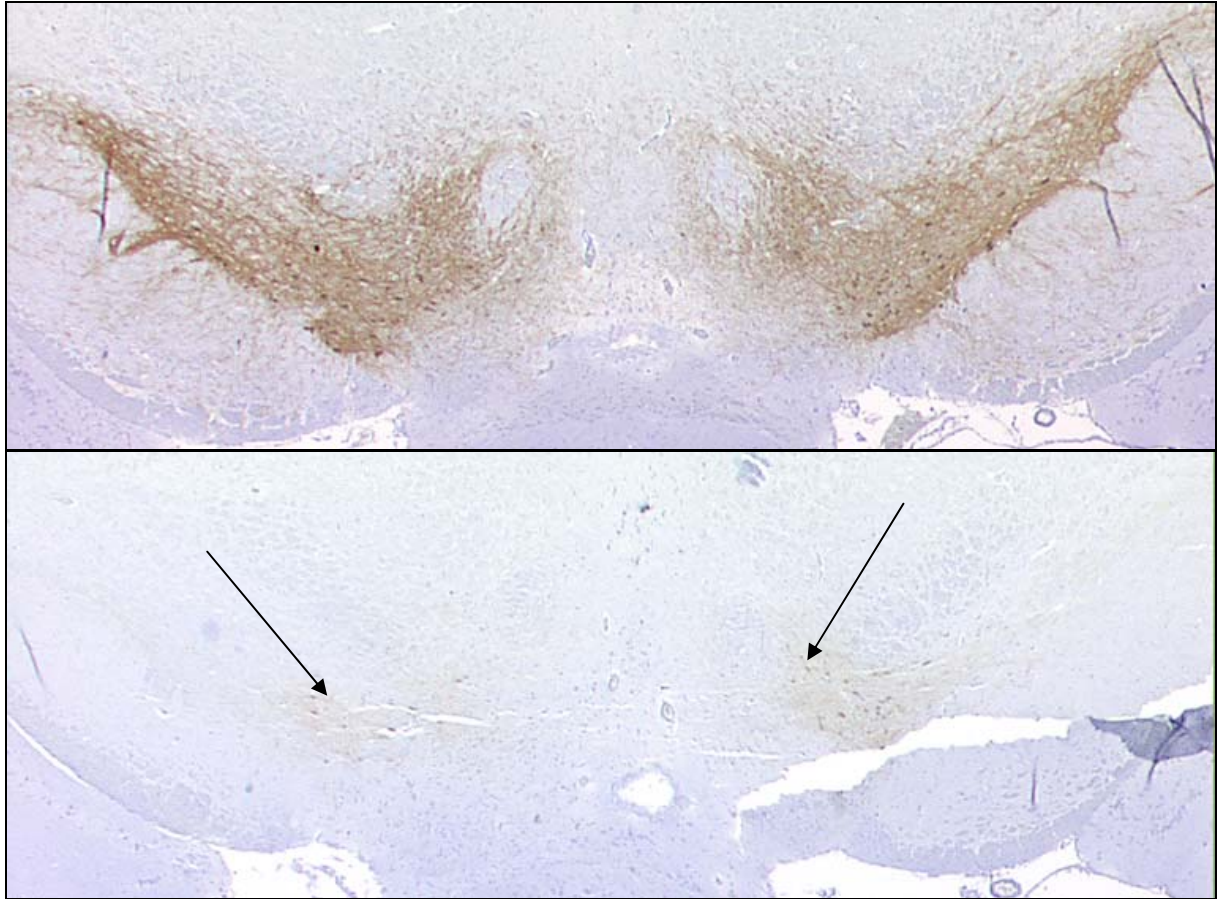


Figure 12: Reduced expression of dopamine transporter 1.

The upper panel shows the distribution of dopamine transporter 1 in the midbrain of a control mouse (25x). In the lower panel a photomicrograph of a mutant mouse is depicted. Note the weak tan color in the midbrain of the mutant mouse.

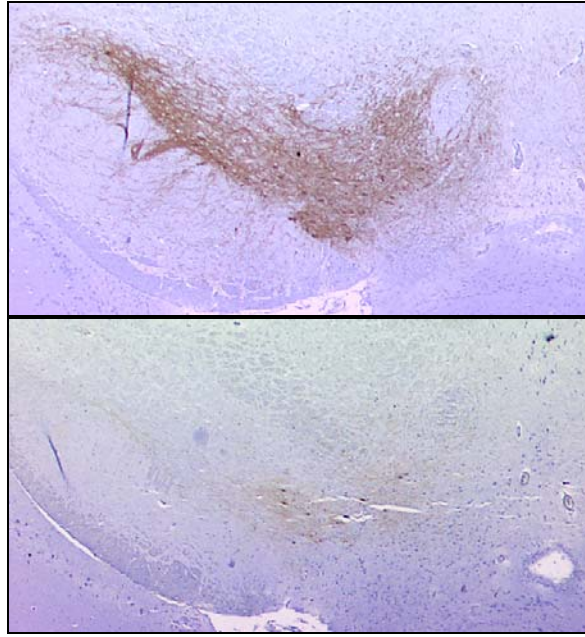


Figure 13: Higher magnification of dopamine transporter 1 expression.
Amount of positive stained neuronal cells in a control (40x, upper panel) compared to the corresponding neuronal cells in a mutant mouse (40x, lower panel).

Spleen phenotype

The analysis of the primary screen revealed a strong extramedullary hematopoiesis (Fig. 13) in the spleen of all mutant mice. Although four out of 17 analyzed control mice also showed the same pattern, the result was highly significant (Fisher's exact test: $p < 0.001$).

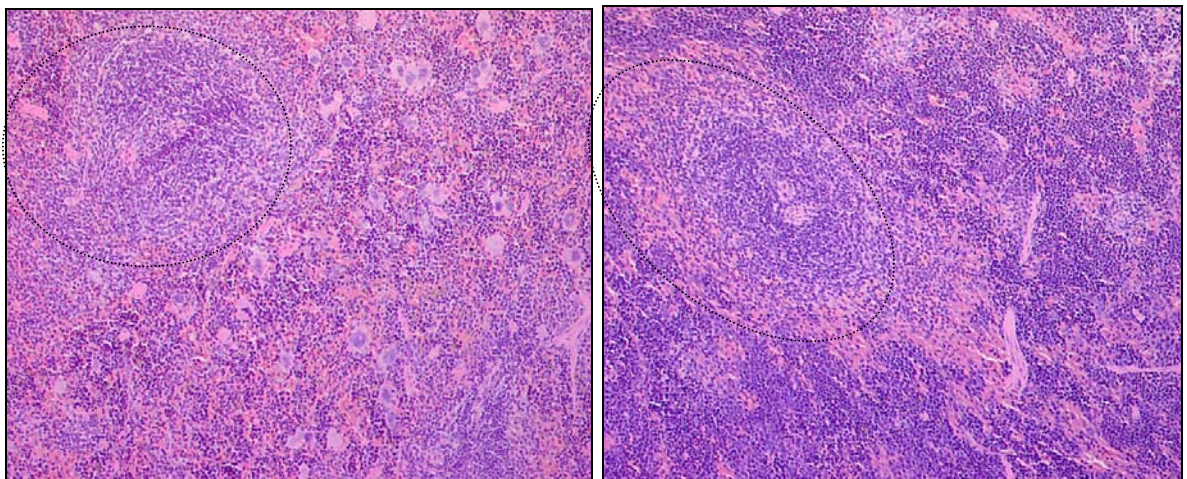


Figure 14: Extramedullary hematopoiesis in the spleen.
Both photomicrographs (100x) show the white (encircled) and red pulp (surrounding) of the spleen. Note the blue color of the red pulp on the right (mutant mouse) which is due to the strong proliferation of erythroblasts and myeloblasts.

3.12.3 Discussion

The complete morphological analysis in the primary screen confirmed two known phenotypes; the anophthalmia of male mutants and the reduced expression of dopamine reporter 1 in the midbrain. However, it was not described before for the allelic mutant *aphakia* that male mutants show anophthalmia (Semina *et al.*, 1997 and 2000). The reason for the variation of the eye phenotype between the sexes is not known; hence, hormonal differences cannot be excluded.

In addition, the morphological changes in the spleen and liver of the mutant mice suggest a possible metabolic phenotype (please see also 3.5.5, Clinical-chemical Screen). To our knowledge, these changes have not been described previously, and warrant further analysis.

3.12.4 References

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Semina EV, Murray JC, Reiter R, Hrstka RF, Graw J. (2000): Deletion in the promoter region and altered expression of Pitx3 homeobox gene in aphakia mice. *Hum Mol Genet.* 9(11): 1575-85.

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Addresses of screeners and modules

Coordinators

Dr. Valérie Gailus-Durner
Dr. Helmut Fuchs
Dr. Christoph Lengger
Dr. Beatrix Naton
Prof. Dr. Martin Hrabé de Angelis
Institute of Experimental Genetics
GSF National Research Center for Environment and Health
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-3613
Fax: 089-3187-3500
Email: gailus@gsf.de

Behavior Screen

Dr. Sabine M. Hölder
Magdalene Kallnik
Institute of Developmental Genetics
GSF National Research Center for Environment and Health
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-3674
Fax: 089-3187-3099
Email: hoelter@gsf.de

Dysmorphology Screen,

Dr. Helmut Fuchs
Dr. Elisabeth Grundner-Culemann
Dr. Wolfgang Hans
Thomas Lisse
Prof. Dr. Martin Hrabé de Angelis
GSF National Research Center for Environment and Health
Institute of Experimental Genetics
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-3151
Fax: 089-3187-3500
Email: hfuchs@gsf.de

Neurology Screen

Dr. Ilka Schneider
Dr. Lore Becker
Eva Kling
GSF National Research Center for Environment and Health
Institute of Experimental Genetics
German Mouse Clinic (GMC)/Neurology
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-3654
Fax: 089-3187-3500
Email: Lore.Becker@gsf.de

PD Dr. Thomas Klopstock
Department of Neurology
Klinikum Großhadern
LMU Ludwig-Maximilians-Universität
Marchioninistraße 15
D-81377 München
Tel.: 089-7095-5920
Fax: 089-7095-3677
Email: Thomas.Klopstock@nro.med.uni-muenchen.de

Eye Screen

Dr. Claudia Dalke
GSF-National Research Center for Environment and Health
Institute of Developmental Genetics
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-2910
Fax: 089-3187-2210
Email: dalke@gsf.de

Clinical-Chemical Screen

Dr. Martina Klempt
Institute of Experimental Genetics
GMC - German Mouse Clinic
Clinical-Chemical Screen
Institute for Experimental Genetics
GSF - National Research Center for Environment and Health
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-3282
Email: birgit.rathkolb@gsf.de

Prof. Dr. Eckhard Wolf
Dr. Birgit Rathkolb
Institute of Molecular Animal Breeding and Biotechnology
Genecenter
LMU München
Feodor Lynen-Straße 25
D-81377 München
Tel.: 089-21807-6800
Email: ewolf@imb.uni-muenchen.de
Email: b.rathkolb@gen.vetmed.uni-muenchen.de

Immunology Screen

Dr. Svetoslav Kalaydjiev
Dr. Tobias Franz
Prof. Dr. Dirk Busch
German Mouse Clinic
Institute for Experimental Genetics
GSF National Research Center for Environment and Health
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-3656
Fax: 089-3187-3500
Email: svetoslav.kalaydjiev@lrz.tum.de
Email: tobias.franz@gsf.de

Prof. Dr. Dirk Busch
Institute for Medical Microbiology,
Immunology and Hygiene
Technische Universität München (TUM)
Trogerstr. 9
D-81675 München
Tel.: 089-4140-6191
Fax: 089-4140-4139
Email: dirk.busch@lrz.tum.de

Allergy Screen

Anahita Javaheri, MSc
Prof. Dr. Markus Ollert
Klinik und Poliklinik für Dermatologie
und Allergologie am Biederstein
Technische Universität München (TUM)
Biedersteinerstraße 29
D-80802 München
Tel.: 089-4140-3551 (M.O.)
Tel.: 089-3187-2554 (A.J.)
Fax: 089-4140-3552
Email: ollert@lrz.tum.de

Nociceptive Screen

Dr. Ildikó Rácz
Laboratory of Molecular Neurobiology,
Department of Psychiatry
University of Bonn
Sigmund-Freud-Straße 25,
D-53105 Bonn
Tel.: 0228-287-9578
Fax: 0228-287-9125
Email: iracz@uni-bonn.de

Prof. Dr. Andreas Zimmer
Laboratory of Molecular Neurobiology,
Department of Psychiatry,
University of Bonn
Sigmund-Freud-Straße 25,
D-53105 Bonn
Tel.: 0228-287-9124
Fax.: 0228-287-9125

Lung Function Screen

Prof. Dr. Holger Schulz
Dr. Ines Bolle
Dr. Claudia Reinhard
GSF – National Research Center for Environment and Health
Institut für Inhalationsbiologie
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-4119
Fax.: 089-3187-2400
Email: schulz@gsf.de

Expression Profiling

Dr. Johannes Beckers
Dr. Marion Horsch
GSF – National Research Center for Environment and Health
Institute of Experimental Genetics
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-3513
Fax: 089-3187-4085
Email: beckers@gsf.de

Metabolic Screen

Dr. Ralf Elvert
Nicole Ehrhardt
Institute of Experimental Genetics
GMC - German Mouse Clinic
Metabolic Screen
GSF - National Research Center for Environment and Health
Ingolstädter Landstraße 1
D-85764 Neuherberg
Tel.: 089-3187-3648 or 3151
Fax: 089-3187-3500
Email: elvert@gsf.de

Pathology Screen

Dr. Julia Calzada-Wack

Sandra Kunder

Gabriele Hölzlwimmer

PD Dr. Leticia Quintanilla-Fend

GSF - National Research Center for Environment and Health

Institute of Pathology

Ingolstädter Landstraße 1

D-85764 Neuherberg

Tel.: 089-3187-2312

089-3187-3241

Fax 089-3187-3360

Email: calzada@gsf.de

sandra.kunder@gsf.de