

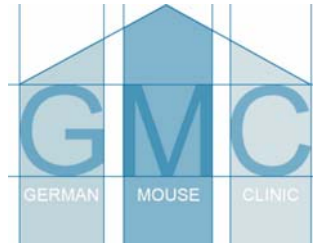
The

GERMAN MOUSE CLINIC

Report for DII1 129SViso

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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.

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1 Summary

1.1 Primary Screening

In a primary screen, 60 animals of the *Dll1* 129SViso (*Dll1*) mutant mouse line (30 *Dll1* heterozygous mutant and 30 *Dll1* control littermates) have been analyzed in the German Mouse Clinic (GMC) in the screens Behavior, Dymorphology, Bone and Cartilage, Neurology, Eye, Clinical Chemistry, Immunology, Allergy, Nociception, Lung Function, Metabolism, Molecular Phenotyping, and Pathology. The screening started on January 12, 2004.

Behavior: The behavioral observation demonstrated a sex-specific effect of the *Dll1* mutation on locomotion in mutant females indicating a hyperactive phenotype.

Dymorphology: In all mutants we observed a significant decreased body weight, fat mass, fat content and an increased lean content compared to control animals.

Neurology: The comparison of mutant mice to controls revealed no obvious neurological phenotype. Female mutant mice showed a significantly higher locomotor activity compared to controls (see also Behavior Screen).

Eye: We detected differences in the ERG response between mutant and control mice, however, they were judged to be without any relevance because the control mice exhibited exceptionally high values compared to our baseline data.

Clinical Chemistry: In the male mutant mice we detected a slightly higher mean inorganic phosphorus concentration compared to the control group. This could give a hint of an imbalance of phosphate homeostasis that might be caused by skeleton disease, kidney disease or impaired endocrine regulation of calcium and phosphorus metabolism. Additionally we detected an elevated white blood cell and platelet count in female mutants, with the same tendency in the males. This might indicate an influence on the immune system (see also Immunology Screen).

Immunology: The analysis revealed differences in the relative frequencies of B cells, cytotoxic T cells, and IgM levels between mutants and their wild-type control littermates.

Nociception: There was a significant difference in pain reactivity between mutant and control animals, namely thermal latencies were longer in mutants pointing at hypoalgesia. The pain phenotype should be determined in additional experiments.

Molecular Phenotyping: The data analysis and various statistical methods detected a number of genes differentially regulated between mutant and wild-type tissues in all experiments. We detected genes involved, for example in tumorigenesis, immune response, left-right development, cell adhesion, and associated with Delta-Notch signalling, wnt and Eph pathways.

Energy Metabolism: Mutant mice showed lower body weights than the control mice, but no differences could be measured in food intake. Hence, mutant mice had higher values in energy uptake and ratio of metabolized energy when calculated per unit body weight (statistically significant in females, slightly elevated in males). This indicates a metabolic phenotype, possibly caused by an increased basal metabolic rate.

In the screens **Allergy**, **Lung Function**, and **Pathology**, no genotype-specific differences could be found.

1.2 Recommendations for Secondary Screening

Secondary screening is suggested from the screens Behavior, Immunology, Allergy, Nociception, and Energy Metabolism.

Behavior Screen: Mutants displayed a different familiar object exploration pattern, further investigation involving learning and memory tests would be interesting. Given the overall phenotypic pattern, we suggest analysis of the dopaminergic system, which is known to be involved in motor function, attention, cognition and reward.

Dysmorphology Screen: To confirm the observed differences especially in the bone parameters more data should be collected in secondary analysis (pQCT) with another batch of mice.

Immunology Screen: Since the Notch signalling pathway plays an important role in lymphocyte differentiation, we would suggest re-testing another batch of mutant mice and littermate controls in order to confirm our initial findings. If similar differences are observed, further investigations for in-depth characterization of this immunological phenotype should be undertaken.

Allergy Screen: Since *Dll1* has a presumed function in Delta-Notch cell-cell signal-transduction pathway, further allergological investigations using an allergen challenge screen should be considered.

Nociception Screen: We would suggest making additional pain-related studies to specify the pain sensitivity of this line. More detailed pain-related studies would include:

1. Base studies e.g.,
 - von Frey filament test to study the reaction of animals to mechanical pain,
 - acetic acid test to study the reaction to visceral inflammation. (optional)

2. Tail flick test, to study whether the hypoalgesia has a spinal or supraspinal origin.
3. Chronic pain tests:
 - Formalin test to study the acute, nociceptive (early) and tonic, inflammatory (late) pain reaction of the same animals, (optional)
 - Carrageenan test to study the reaction to inflammation. (Optional)
4. Neuropathy test: Total ligation of the sciatic nerve on the left side, weekly measurement of the pain sensitivity with von Frey filament test and with plantar test.

For the secondary screen, we need 10-15 mice per group and sex. We can analyze the mice at age of 10 weeks or even older. The results of the whole set of experiments will provide a complete picture of the pain phenotype of this mutant mouse line.

Metabolic Screen: We would suggest investigating the basal metabolic rate in more detail.

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

2 General Part

2.1 The Role of the Gene

The evolutionarily conserved Delta-Notch cell-cell signal-transduction pathway regulates the determination of various cell fates, which are important for the morphogenesis and development of numerous organs and tissues in many vertebrates and invertebrates. Cell-cell signaling in this pathway involves the binding of the transmembrane ligands Delta or Serrate (Jagged in vertebrates) to the transmembrane Notch receptor expressed on neighboring cells. In mammals, 4 different Notch receptor proteins (Notch1-4) and 5 different DSL-ligands (Delta-like [Dll] 1, 2, 4 and Jagged1, 2) are known (Baron, 2003).

2.2 Known Phenotypes

Dll1^{-/-} homozygous mutant mice are lethal due to haemorrhagic phenotype around E11.5. Homozygous mutants show defects in somitogenesis, neurogenesis and L/R-axis formation. Heterozygous mutants are fully fertile and do not show obvious phenotypes (Hrabé de Angelis *et al.*, 1997; Przemeck *et al.*, 2003).

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

2.3 Expected Phenotypes

It is thinkable that the precise analysis of *Dll1*^{lacZ/+} heterozygous animals by μ CT and X-ray uncover **mild skeletal defects** especially in the cervical region of the vertebral column. Recently, Costa *et al.* (2003) reported about **learning** and **memory deficits** in heterozygous *Notch1* mutant mice. Therefore, similar defects or other behavioral anomalies in heterozygous *Dll1*^{lacZ/+} mice could be detected in the neurological and/or behavioral screen.

2.4 Possible disease models

The Delta/Notch signal transduction pathway is associated with several human syndromes such as Alagille Syndrome (*Jag1*; OMIM #118450), CADASIL (*Notch3*; OMIM #125310), Acute T Cell Lymphoblastic Leukemia (OMIM *190198), and Spondylocostal Dysostosis (*Dll3*, OMIM #277300). In some mammary tumors (OMIM *164951) the *Notch4* gene is activated by integration of MMTV (mouse mammary tumor virus). Human *dll1* appears to mediate cell fate decisions during hematopoiesis. A soluble form of the protein has been shown to delay the acquisition of differentiation markers by murine hematopoietic progenitor cells cultured in vitro with cytokines. This soluble protein also promotes expansion of the primitive hematopoietic precursor cell population (Han *et al.*, 2000; OMIM *606582).

2.5 Mice

2.5.1 Number and kind of mice

Sixty animals of the Dll1 mutant mouse line arrived in week 3 in 2004 (Table 1). All animals were used for the tests. As described by the sender, the Dll1 gene was mutated by replacement of amino acid 2-116 with an in-frame fusion of the *lacZ* gene of *E.coli* (Hrabé de Angelis *et al.*, 1997). Heterozygous F1-animals were backcrossed several times to the 129Sv/J wild type. The mutant line is maintained by matings between sibling or crosses between heterozygous males and 129Sv/J females.

Table 1: Dll1 mice provided for analysis.		
Numbers in brackets indicate animals which were kept in reserve.		
Genotype / Sex	Number of Animals	
Mutant female	15 (+2)	1 died
Mutant male	15 (+1)	1 died
Control female	15	
Control male	15 (+5)	3 died

A first batch of mice in a mixed 129/Sv x C57BL/6 background was analyzed in the blood-based screens Clinical Chemistry, Immunology and Allergy in March 2003. More information is given these chapters.

2.5.2 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 car-

ried 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.

2.6 Workflow

2.6.1 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.

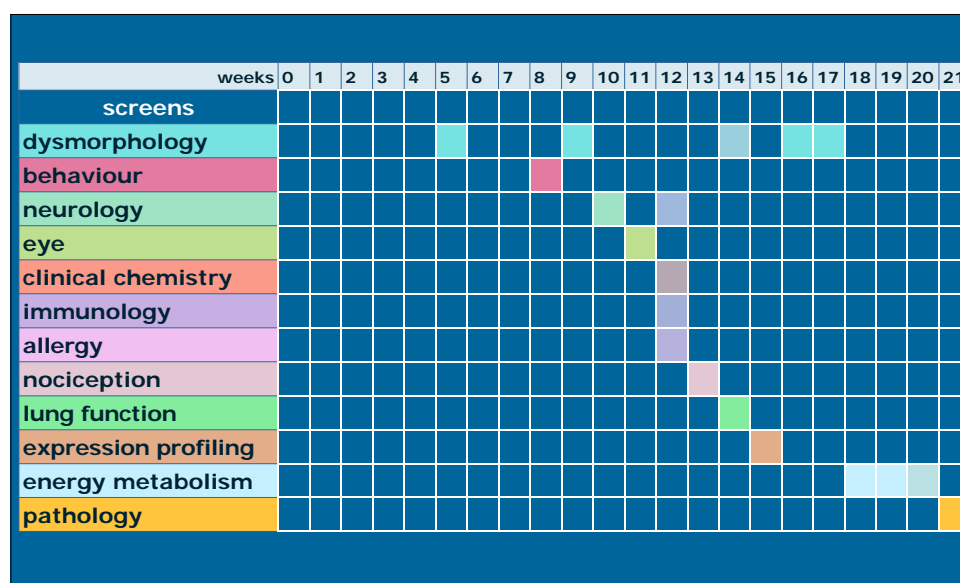



Figure 1: Workflow of the primary screen

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 Dymorphology Screen, and then stay three weeks in the Metabolic Screen. After completion of the primary screen all animals analyzed macro- and microscopically 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.

2.6.2 Applied screens

The GMC standard workflow for the primary screen as described above was applied to analyze the Dll1 mutant mouse line. 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. Five animals did not recover from narcosis and thus could not be analyzed for all parameters.

2.6.3 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.

2.7 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.

2.8 References

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Abbreviations and wording

Dll1	Delta like 1 (protein and mutant mouse line Dll1 129SViso)
<i>Dll1</i>	gene
GMC	German Mouse Clinic
IVC	individually ventilated cage
CADASIL	C erebral A utosomal D ominant A rteriopathy with S ubcortical I nfarcts & L eukoencephalopathy
control	homozygous wild-type control, <i>Dll1</i> ^{+/+}
mutant	heterozygous mutant, <i>Dll1</i> ^{lacZ/+}
FELASA	F ederation of E uropean L aboratory A nimal S cience A ssociations, 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 (Ohl *et al.*, 2001).

The behavioral observation demonstrated a sex-specific effect of the *Dll1* mutation on locomotion in mutant females indicating a hyperactive phenotype.

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, 30 female mice (15 controls, 15 heterozygous mutants) and 30 male mice (15 controls, 15 heterozygous mutants) were available for analysis. For video-track analysis, 15 controls and 14 mutant females and 15 controls and 15 mutant males were available.

3.1.3 Material and Methods

The modified Hole Board test was carried out according to the procedures described by Ohl *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.

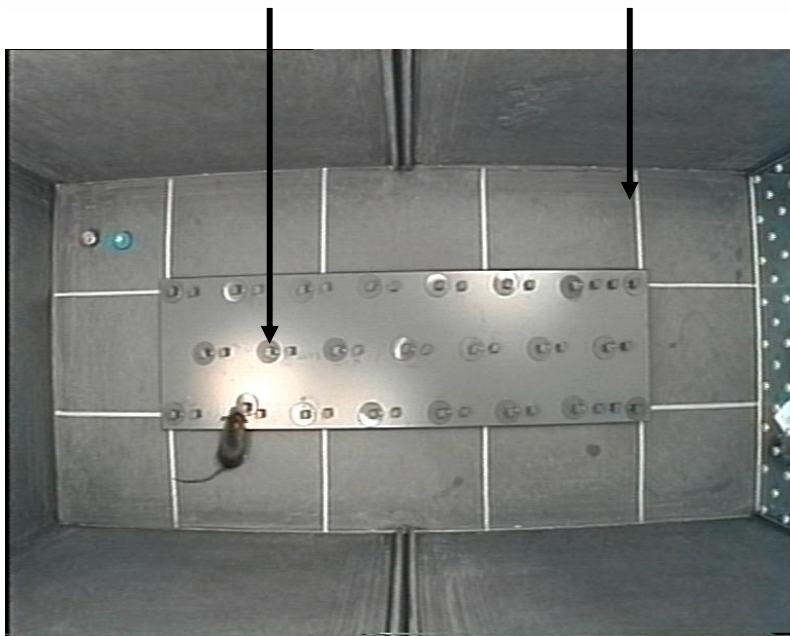
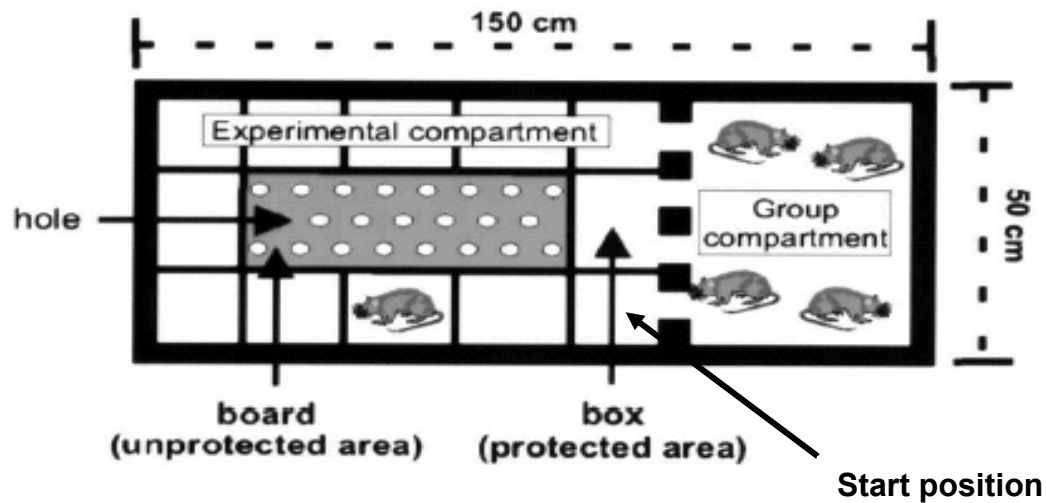


Figure 2: Test arena for modified Hole Board test (Ohl *et al.*, 2001).

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 alterations in locomotion in mutant females as compared to control females. Mutant females demonstrated enhanced forward locomotor activity indicated by increases in the number of line crossings (Table 4) and total distance travelled (Table 5), whereas speed of movement (mean and maximum velocity, Table 5) remained unchanged. Additionally, mutant females did more turns (Table 5).

Concerning exploratory behavior, mutant females were more interested in exploring the familiar object as evident from enhanced familiar object exploration frequencies and total duration (Table 4). As a consequence, the object recognition index was tendentially reduced in mutant females (Table 4). The activity-related latency to familiar object exploration (Table 4) did not differ from control females. In contrast to females, male mutants investigated the familiar object later than control males (latency to familiar object exploration, Table 4), but at similar intensity (familiar object exploration frequency and total duration, Table 4). There were no genotype effects on any other observed parameter.

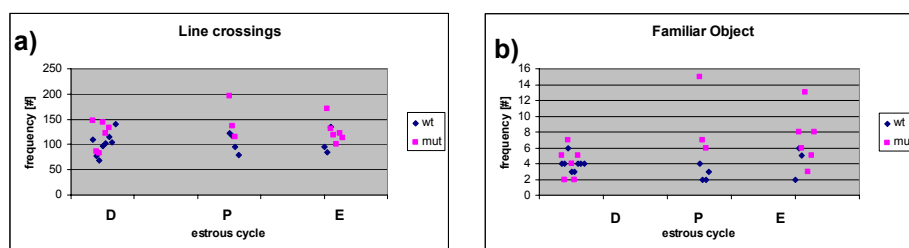


Figure 3: Activity levels of Dll1 females in different estrous cycle phases. Frequency of (a) line crossings and (b) familiar object exploration in control (wt) and mutant (mut) females during diestrous (D), proestrous (P) and estrous (E).

Possible dependencies of the monitored behaviors of female mice on the estrous cycle (e.g. Farr *et al.*, 1995; Frick & Berger-Sweeney, 2001 and literature cited therein) were tested by plotting latencies against cycle stage. Analysis of the vaginal smears (Frick & Berger-Sweeney, 2001) revealed that simi-

lar numbers of control and mutant females were in each of the three different cycle stages (diestrous, proestrous, estrous) at the time point of testing (Fig. 3). The increase in locomotor activity in mutant females compared to controls was not related to any specific cycle stage (Fig. 3a).

This was slightly different for familiar object exploration activity (Fig. 3b). Whereas familiar object exploration activity of control females was similar in all three cycle stages, there was a trend in mutant females to explore the familiar object more often in proestrous and estrous than in diestrous. In diestrous mutant values were within the range of control values, but in proestrous and estrous mutant values tended to be higher than control values.

3.1.5 Discussion

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 (females) and blue (males).	
Behavior	Measured parameters
Forward locomotor activity	Line crossings, Total distance travelled
Vertical locomotor activity	Rearings in the box , Rearings on the board
Speed of movement	Mean and maximum velocity
Immobility	Time spent immobile
Risk assessment	Stretched attends
Anxiety-related behavior	Board entries, Latency until first board entry, Time spent on board
Exploratory behavior	Directed: Hole exploration, object exploration; Undirected: Rearings, activity levels
Grooming behavior	Latency to grooming, Time spent grooming, Number of groomings
Defecation	Latency to defecation, Number of boli
Social affinity	Group contacts (frequency, latency), 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.

The behavioral observation in the modified Hole Board demonstrated an effect of the *Dll1* mutation on locomotion in mutant females. Mutant females moved more (line crossings, total distance travelled) than control females, but did not move faster and did not generally explore more (vertical exploration, hole exploration), indicating a specific increase in forward locomotor activity. Increased number of turns without changes in path shape parameters (mean turn angle, angular velocity and absolute meander, Table 3) fit to the conclusion that the hyperactivity is not a general one, but limited to forward locomotor activity.

In addition, *Dll1*-mutant females showed increased frequency and duration of familiar object exploration resulting in a *tendency* towards an object

recognition deficit as compared to control females. As also mutant males displayed a slightly different familiar object exploration pattern, further investigation involving learning and memory tests would be interesting (Costa, et al., 2003).

In females, analysis of the behavioral pattern in respect to the estrous cycle suggested that locomotion was independent of the cycle in both genotypes, whereas investigation of the familiar object was increased in proestrous and estrous when compared to diestrous in mutant females, but not in controls. Therefore we can not exclude an impact of the Dll1 mutation on regulatory processes affecting exploratory behavior in dependency of the estrous cycle in females.

Given the phenotypic pattern, we suggest analysis of the dopaminergic system, which is known to be involved in motor function, attention, cognition and reward (for reviews see Jay, 2003 and Zeiss, 2005). Taking the phenotypic pattern into account, we speculate that the behavioral alterations observed in Dll1 mutants are rather related to changes in attentional, cognitive and reward-related processes (mesocorticolimbic DA system) than to changes in basic motor functions (nigrostriatal DA system). Testing of this working hypothesis and evaluation of the validity of such a prediction would be interesting.

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=15)	(n=15)	<i>p</i> - value	(n=15)	(n=15)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
Line crossing [frequency]	120.13 \pm 5.47	103 \pm 5.39	N.A.	138.17 \pm 15.91	128.13 \pm 7.66	N.A.	n.s.	p<0.05
Line crossing [latency]	1.35 \pm 0.26	1.63 \pm 0.45	N.A.	2.58 \pm 1.13	2.2 \pm 1.17	N.A.	n.s.	n.s.
Rearings in box [frequency]	20.73 \pm 2.83	18.4 \pm 1.98	N.A.	14.17 \pm 3.27	17.07 \pm 2.55	N.A.	n.s.	n.s.
Rearings in box [latency]	44.55 \pm 8.08	31.62 \pm 3.89	N.A.	83.11 \pm 23.5	47.07 \pm 7.08	N.A.	n.s.	n.s.
Hole exploration [frequency]	23 \pm 2.84	18.2 \pm 2.19	N.A.	17.42 \pm 3.06	23.93 \pm 2.36	N.A.	n.s.	n.s.
Hole exploration [latency]	43.35 \pm 10.62	37.84 \pm 9.88	N.A.	49.28 \pm 14.25	43.59 \pm 10.84	N.A.	n.s.	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]	10.27 \pm 1.38	9.27 \pm 1.3	N.A.	7.83 \pm 1.74	10.87 \pm 1.27	N.A.	n.s.	n.s.
Board entry [latency]	48.16 \pm 8.9	58.02 \pm 13.92	N.A.	94.63 \pm 28.58	85.18 \pm 11.57	N.A.	n.s.	n.s.
Board entry [total duration %]	9.63 \pm 1.29	9.4 \pm 1.34	N.A.	12.2 \pm 5.18	10.37 \pm 0.99	N.A.	n.s.	n.s.
Rearing on board	0.07 \pm 0.07	0.13 \pm 0.09	N.A.	0.33 \pm 0.22	0.27 \pm 0.21	N.A.	n.s.	n.s.

[frequency]								
Rearing on board [latency]	294.9 ± 5.1	284.67 ± 12.02	N.A.	281.07 ± 16.03	289.76 ± 7.05	N.A.	n.s.	n.s.
Risk assessment [frequency]	0 ± 0	0 ± 0	N.A.	0 ± 0	0.13 ± 0.09	N.A.	n.s.	n.s.
Risk assessment [latency]	300 ± 0	300 ± 0	N.A.	300 ± 0	263.86 ± 24.68	N.A.	n.s.	n.s.
Group contact [frequency]	9.93 ± 0.57	10.33 ± 0.69	N.A.	12.25 ± 1.57	12.27 ± 0.69	N.A.	n.s.	n.s.
Group contact [latency]	19.46 ± 2.4	19.11 ± 2.25	N.A.	15.65 ± 3.19	17.87 ± 1.93	N.A.	n.s.	n.s.
Group contact [total duration %]	18.32 ± 1.95	23.92 ± 2.21	N.A.	17.92 ± 3.25	21.29 ± 1.47	N.A.	n.s.	n.s.
Grooming [frequency]	0.6 ± 0.19	1.27 ± 0.3	N.A.	1.17 ± 0.44	1 ± 0.35	N.A.	n.s.	n.s.
Grooming [latency]	275.77 ± 10.43	223.87 ± 19.44	N.A.	238.43 ± 20.64	241.48 ± 16.8	N.A.	n.s.	n.s.
Grooming [total duration %]	0.96 ± 0.37	2.84 ± 1.25	N.A.	3.62 ± 2	2.55 ± 1.01	N.A.	n.s.	n.s.
Defecation [frequency]	0.07 ± 0.07	0.53 ± 0.29	N.A.	0 ± 0	0.8 ± 0.28	N.A.	n.s.	n.s.
Defecation [latency]	281.46 ± 18.54	272.29 ± 13.89	N.A.	300 ± 0	189.37 ± 33.03	N.A.	n.s.	n.s.
Unfamiliar object exploration [frequency]	4.73 ± 0.78	4.07 ± 0.43	N.A.	5.5 ± 0.82	4.67 ± 0.56	N.A.	n.s.	n.s.
Familiar object exploration [frequency]	5.4 ± 0.6	3.73 ± 0.33	N.A.	4.42 ± 0.66	6.4 ± 0.94	N.A.	n.s.	p<0.05
Unfamiliar object exploration [latency]	53.47 ± 16.89	48.04 ± 14.06	N.A.	57.33 ± 24.82	43.25 ± 13.72	N.A.	n.s.	n.s.

Familiar object exploration [latency]	39.09 ± 8.05	61.38 ± 12.07	N.A.	73.91 ± 18.95	43.52 ± 10.83	N.A.	p<0.05	n.s.
Unfamiliar object exploration [total duration %]	1.58 ± 0.26	1.09 ± 0.12	N.A.	1.31 ± 0.19	1.31 ± 0.12	N.A.	n.s.	n.s.
Familiar object exploration [total duration %]	1.26 ± 0.12	0.79 ± 0.08	N.A.	0.99 ± 0.15	1.43 ± 0.2	N.A.	n.s.	p<0.05
Object Index	0.1 ± 0.05	0.14 ± 0.07	N.A.	0.15 ± 0.08	-0.01 ± 0.05	N.A.	n.s.	p=0.07

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=15)	(n=14)	<i>p</i> -value	(n=15)	(n=15)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
Total Distance Moved [cm]	3232.67 \pm 136.48	2831.98 \pm 154.03	N.A.	3162.76 \pm 194.33	3434.81 \pm 177.66	N.A.	n.s.	p<0.05
Mean Velocity [cm/sec]	19.02 \pm 0.71	18.72 \pm 0.47	N.A.	18.41 \pm 0.98	20.11 \pm 0.64	N.A.	n.s.	n.s.
Maximum velocity [cm/sec]	56.98 \pm 2.4	59.38 \pm 4.22	N.A.	52.74 \pm 2.85	56.56 \pm 3.39	N.A.	n.s.	n.s.
Turns [Frequency]	1755 \pm 38.8	1549.93 \pm 68.19	N.A.	1693.21 \pm 82.12	1800.4 \pm 71.24	N.A.	n.s.	p<0.05
Mean Turn Angle [degrees]	23.18 \pm 0.37	22.98 \pm 1.23	N.A.	23.91 \pm 1.43	22.71 \pm 0.86	N.A.	n.s.	n.s.
Angular Velocity [degrees/sec.]	150.78 \pm 2.56	147.5 \pm 12.57	N.A.	151.3 \pm 6.72	153.96 \pm 6.32	N.A.	n.s.	n.s.
Absolute Meander [degrees/sec.]	16.7 \pm 0.33	16.39 \pm 0.75	N.A.	17.21 \pm 1.15	15.98 \pm 0.63	N.A.	n.s.	n.s.
Board entry max. duration [sec]	5.73 \pm 0.73	5.86 \pm 0.64	N.A.	12.56 \pm 7.39	6.91 \pm 0.8	N.A.	n.s.	n.s.
Distance to Wall [cm]	6.88 \pm 0.37	6.97 \pm 0.41	N.A.	6.83 \pm 0.52	7.14 \pm 0.29	N.A.	n.s.	n.s.
Distance to Board [cm]	8.93 \pm 0.25	9.13 \pm 0.29	N.A.	8.42 \pm 0.42	8.84 \pm 0.19	N.A.	n.s.	n.s.

3.2 Dysmorphology, Bone and Cartilage

3.2.1 Summary

In the Dysmorphology, Bone and Cartilage Screen of the German Mouse 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 Mammalian Phenotype Ontology wording (www.informatics.jax.org/searches/MP_form.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 60 animals of Dll1 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 a hardened or kinked tail tip was observed in some mutant mice, but also in two wild-type control littermates. In the DXA analysis we detected slightly reduced bone mineral density (BMD) and pBMD (BMD without skull) in male mutants, but no significant difference was observed when BMD was related to the body weight (sBMD). In female mutants increased sBMD and decreased bone mineral content and body length was measured. In all mutants we observed a significantly decreased body weight, fat mass, fat content and an increased lean content compared to control animals.

3.2.2 Mice

Fifteen animals of each genotype and sex 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 19 mutant and 20 control animals, and 16-week-old mutants (19 animals) and controls (19 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

Sixty animals of *Dll1* 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 a hardened or kinked tail tip (fusions of tail vertebrae, Fig. 4) was observed in some mutant mice, but also in two wild-type control littermates. Single individuals showed some minor phenotypes like brighter upper teeth, which were present in both mutant and control animals (Tables 7 and 8).

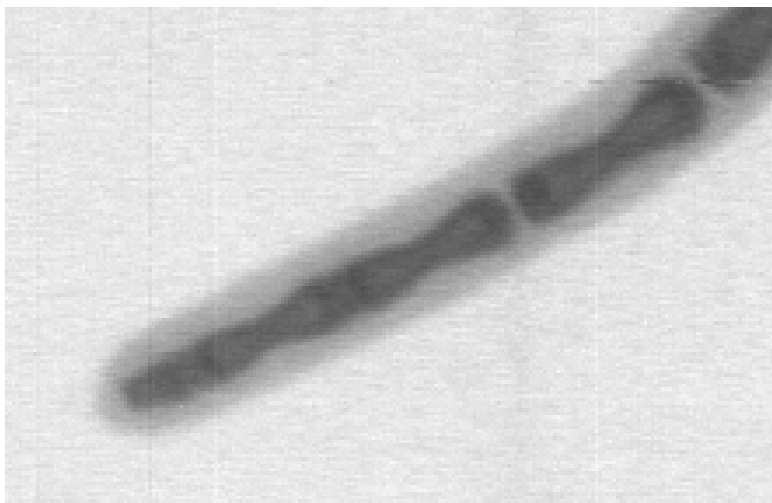


Figure 4: Fusions of tail vertebrae

(Control: one animal, mutant: two animals and one with strong kink)

Bone densitometry using DXA analysis revealed significantly reduced bone mineral density (BMD) and pBMD (BMD without skull) values in male mutants, but no significant difference was observed when BMD was related to the body weight (sBMD). In female mutants increased sBMD and decreased bone mineral content and body length was measured. In all *Dll1*-mutants we observed a significant decreased body weight, fat mass, fat content and an increased lean content compared to control animals. Differences between mutants and

controls in bone parameters 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 (t-test) between mutants and controls (Table 9).

To confirm the observed differences especially in the bone parameters more data should be collected in secondary analysis (pQCT) with another batch of mice.

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 clickbox test (hearing test)				
Phenotype	Male		Female	
	Control	Mutant	Control	Mutant
0	3	3	-	-
1	5	5	5	3
2	1	5	4	7
3	6	1	6	5
4	-	1	-	-
Mean Score	1.67	1.47	2.07	2.13

Kruskal-Wallis Anova on Ranks: n.s. (P = 0.229)

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

Table 7: Results from the morphological inspection				
Parameter	Male		Female	
	Control	Mutant	Control	Mutant
Growth				
normal	15	15	15	15
Weight				
normal	15	15	15	15
Body size				
normal	15	15	15	15
Eye				
normal	15	15	14	15
red	-	-	1	-
Coat hair growth				
normal	15	15	15	15
Coat hair texture				
normal	15	15	15	15
Coat color				
agouti	15	15	12	13
agouti bright	-	-	2	2
yellow	-	-	1	-
white belly	-	-	1	2
Hair follicle structure / orientation				
normal	15	15	15	15
Skin pigmentation				
normal	-	-	15	15
Dark skin	15	15	-	-

Skin texture / condition				
normal	15	15	15	15
Vibrissae				
normal	15	15	15	15
Limbs				
normal	15	15	15	15
Digits				
normal	15	15	15	15
Tail				
normal	13	11	15	14
hard end	1	2	-	-
kinked tail	1	2	-	1
Teeth				
normal	10	14	12	14
maxillary teeth white/brighter	5	1	3	1
Ear morphology				
normal	15	15	15	15
Musculature				
normal	15	15	15	15
Seizures / epilepsy				
no	15	15	15	15
Motor capabilities / coordination				
normal	15	15	15	15
Movement				
normal	15	15	15	15
Feeding / drinking behavior				
normal	15	15	15	15
Respiratory system				
normal	15	15	15	15
Reproductive system				
normal	15	15	15	15
Other abnormalities				
no	15	14	15	15
hanging behavior weird	-	1	-	-
Animals analyzed	15	15	15	15

Table 8: Results from the X-ray analysis

Parameter	Male		Female	
	Control	Mutant	Control	Mutant
Skull shape				
normal	9	9	10	9
nose contorted	-	-	-	1
Mandibles				
normal	9	9	10	10
Maxilla				
normal	9	9	10	10
Teeth				
normal	9	9	10	10
Orbit				
normal	9	9	10	10
Number of cervical vertebrae				
normal	9	9	10	10
Number of thoracic vertebrae				
normal	9	9	10	10
Number of lumbar vertebrae				
6	8	7	9	9
5	1	2	1	1
Number of pelvic vertebrae				
normal	9	9	10	10
Number of sacral vertebrae				
normal	9	9	10	10
Vertebrae shape				
normal	9	9	9	8
kinked tail	-	-	-	1
tail vertebrae connected	-	-	1	1
thoracic vertebrae kinky	-	-	1	-
Number of ribs				
26	9	8	9	10
25	-	1	1	-
Rib shape				
normal	9	9	10	10
Scapulas				
normal	9	9	10	10
Clavicle				
normal	9	9	10	10
Pelvis				
normal	9	9	10	10
Femur shape				
normal	9	9	10	10

Tibia				
normal	9	9	10	10
Fibula				
normal	9	9	10	10
Humerus				
normal	9	9	10	10
Ulna				
normal	9	9	10	10
Radius				
normal	9	9	10	10
Number of digits				
normal	9	9	10	10
Completeness of digits				
yes	9	9	10	10
Joints				
normal	9	9	10	10
Animals analyzed	9	9	10	10

Table 9: Bone- and weight-related quantitative parameters
(data presented as mean \pm standard error of mean)

Parameter	Control (A)		Mutant (B)		A~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=9)	(n=10)	(n=9)	(n=10)	<i>p</i> – value	<i>p</i> – value			
BMD [mg/cm ²]	71.33 \pm 2.21	65.81 \pm 1.66	65.10 \pm 1.60	67.84 \pm 2.29	< 0.05	n.s.	n.s.	n.s.	< 0.05
pBMD [mg/cm ²]	60.29 \pm 1.75	54.62 \pm 1.69	54.21 \pm 1.58	55.02 \pm 1.76	< 0.05	n.s.	n.s.	n.s.	n.s.
sBMD [10 ⁻³ x cm ⁻²]	2.36 \pm 0.09	2.64 \pm 0.08	2.43 \pm 0.05	3.08 \pm 0.13	n.s.	< 0.01	< 0.05	< 0.0001	n.s.
BMC [mg]	841 \pm 48.55	736 \pm 30.64	745 \pm 35.84	631 \pm 28.82	n.s.	< 0.05	< 0.01	< 0.01	n.s.
Body Length [cm]	9.28 \pm 0.09	9.10 \pm 0.10	9.11 \pm 0.07	8.70 \pm 0.08	n.s.	< 0.01	< 0.01	< 0.01	n.s.
Body Weight [g]	30.42 \pm 0.74	25.10 \pm 0.75	26.87 \pm 0.55	22.10 \pm 0.42	< 0.01	< 0.01	< 0.0001	< 0.0001	n.s.
Lean mass [units]	18.50 \pm 0.64	15.60 \pm 0.68	19.33 \pm 0.70	15.43 \pm 0.58	n.s.	n.s.	n.s.	< 0.0001	n.s.
Fat mass [units]	7.89 \pm 0.88	5.88 \pm 0.70	4.22 \pm 0.81	3.53 \pm 0.43	< 0.01	< 0.05	< 0.001	n.s.	n.s.
Bone Content [%]	2.77 \pm 0.15	2.93 \pm 0.05	2.77 \pm 0.10	2.85 \pm 0.11	n.s.	n.s.	n.s.	n.s.	n.s.
Lean Content [units x 100/g]	61.13 \pm 2.74	62.29 \pm 2.40	72.15 \pm 2.79	69.77 \pm 2.13	< 0.05	< 0.05	< 0.001	n.s.	n.s.
Fat Content [units x 100/g]	25.69 \pm 2.63	23.21 \pm 2.43	15.49 \pm 2.79	15.95 \pm 1.95	< 0.05	< 0.05	< 0.01	n.s.	n.s.
Femur span¹⁾ [mm]	1.37 \pm 0.02	1.31 \pm 0.03	1.40 \pm 0.02	1.31 \pm 0.02	n.s.	n.s.	n.s.	< 0.01	n.s.
Subcutaneous fat¹⁾ [mm]	4.20 \pm 0.15	3.96 \pm 0.09	4.10 \pm 0.16	4.49 \pm 0.15	n.s.	< 0.01	n.s.	n.s.	n.s.
Vertebrae height²⁾ [mm]	3.47 \pm 0.05	3.53 \pm 0.07	3.54 \pm 0.07	3.31 \pm 0.095	n.s.	n.s.	n.s.	< 0.05	n.s.
	Male	Female	Male	Female	A~B	A~B	ANOVA		
	(n=10)	(n=10)	(n=9)	(n=10)	<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]	1.23 \pm 0.02	1.25 \pm 0.02	1.18 \pm 0.02	1.19 \pm 0.02	n.s.	n.s.	< 0.05	n.s.	n.s.

1: mean value of the two hind limbs

2: third lumbar vertebra

Raw data will be available on demand.

3.3 Neurology Screen

3.3.1 Summary

In the primary neurological screen, 30 heterozygous *Dll1*-deficient mice (15 males/ 15 females) and 30 control mice (15 males/15 females) were screened. Animals were analyzed according to our modified SHIRPA protocol where a battery of behavioral tests is carried out. This primary observation screen is a modification of the Irwin procedure (Irwin, 1968) and was proposed as a rapid, comprehensive and semi-quantitative screening method for qualitative analysis of abnormal phenotypes in a mouse strain (Rogers *et al.*, 1994). We carried out 37 of 40 designed test parameters (See web page: http://www.mgu.har.mrc.ac.uk/facilities/mutagenesis/mutabase/shirpa_summary.html) to detect phenotypic differences between *Dll1* mutant and control mice. Each test parameter contributes to an overall assessment in muscle, lower motor neuron, spinocerebellar, sensory and autonomic function. The primary neurological screen is focused on investigating neurological reflexes to determine the neurological functioning of a mouse. We also examine lactate levels in the blood of mice to draw conclusions about energy metabolism.

The comparison of mutant mice to controls revealed no obvious neurological phenotype. Female mutants showed a significantly higher locomotor activity as compared to controls. Additionally we found significant differences in body weight between mutant and control mice.

3.3.2 Mice

Fifteen 10-week-old male mutant and 15 control mice entered the neurological screen at the beginning of the 6th calendar week in 2004. The females (15 of each genotype) 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

At the age of 10 weeks assessment of each animal started 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. In this arena, locomotor activity and motor behavior was observed (*Behavior recorded in the Arena*). This was followed by a sequence of manipulations testing reflexes, grip strength, toe pinch and wire maneuver (*Behavior recorded on or above the arena*). For the wire maneuver test, a rigid horizontal wire (3 mm in diameter) is secured across the rear right corner of the arena. For grip strength testing, a grid (270 x 275 mm) is secured across the width of the arena. In the last part of the observation (*Behavior recorded during Supine Restraint*), the animals were restrained in a supine position to record autonomic responses such as salivation. Measurements were completed with the recording of limb tone, provoked biting, and body length. The last part of the primary screen also involves the analysis of right-

ing reflex, negative geotaxis and contact righting reflex. A glass cylinder (35 mm diameter, 135 mm length) is used for testing the contact-righting reflex. Throughout the entire procedure, abnormal behavior, irritability, fear, aggression and vocalization were recorded. Between testing of each mouse, faecal pellets and urination were removed from the viewing jar and arena. All experimental equipment is 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.

3.3.4 Parameters

Muscle/lower motor neuron function
Body position, gait, Positional passivity, wire maneuver, tail elevation, limb tone, body tone, abdominal tone, grip strength, urination, defecation
Spinocerebellar function
Body position, gait, righting reflex, tail elevation, visual placing, limb tone, body tone, abdominal tone, grip strength
Sensory function
Transfer arousal, touch escape, gait, visual placing, toe pinch, pinna reflex, righting reflex
Autonomic function
Palpebral closure, urination, salivation, respiration rate, defecation
Neurological reflexes
Righting reflex (pons), contact righting reflex, visual placing, toe pinch/flexion reflex (cerebellar/spinal cord), negative geotaxis, corneal reflex (medulla), pinna reflex (hearing test)
Physiological parameters
Body weight, body length
General appearance
Body weight, body length, body position, transfer arousal, fear, touch escape, irritability, vocalization, positional passivity, aggression, spontaneous activity, locomotor activity, skin color

3.3.5 Results

Female mutants showed a significantly **increased locomotor activity** (Table 12). A similar but not significant increase in locomotor activity was found in male mutant mice, too. Furthermore, **body weight** of female and male mutant mice was decreased when compared to control mice (Table 10). Blood lactate level did not differ between mutants and controls (Table 15).

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

3.3.6 Discussion

In our neurological screen, an obvious finding was the significantly increased locomotor activity of the female mutant mice as compared to their littermate controls (see Behavior Screen, 3.1.5). Locomotor activity is an indicator for explorative behavior and is controlled by a wide range of brain regions and transmitter systems. In the modified SHIRPA protocol we measure a basic locomotor activity to assess elementary neurological functions and fundamental locomotive abilities. Quantitative and more complex evaluation of locomotor activity is measured with a video tracking system in the Behavior Screen (3.1.5). Therefore, we recommend a further examination of the locomotor activity in the Behavior Screen.

The decreased body weight observed in our screen may be caused by alterations in the metabolism of these mutants (please compare with the results of the Energy Metabolism Screen, 3.12.5) or are related to the increased locomotor activity and hyperactivity, respectively, seen also in the Behavior Screen.

Since we do consider the hyperactivity phenotype to be due to behavioral abnormalities, we conclude that the comparison of mutant mice to controls revealed no obvious neurological phenotype.

3.3.7 References

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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/mutabase/shirpa_summary.html

s.a. Sub-maxillary area

Table 10: Recording of body length and body weightData are presented as mean \pm standard error of mean.

Parameter	Male			Female		
	Control (n=15)	Mutant (n=15)	<i>p-value</i>	Control (n=15)	Mutant (n=15)	<i>p-value</i>
Body Length [g]	8.55 ± 0.03	8.53 ± 0.02	<i>n.s.</i>	8.4 ± 0.03	8.3 ± 0.02	<i>n.s.</i>
Body Weight [g]	26.5 ± 0.18	24.7 ± 0.1	0.03	22.4 ± 0.1	20.2 ± 0.9	0.005

Table 11: Behavior recorded in viewing jarData shown represents the results of test parameters from major tests where a behavioral response was observed. Test parameters which did not elicit any response were excluded from this data. Statistical analysis: chi-squared test; significance $p < 0.05$

Parameter	Male			Female		
	Control (n=15)	Mutant (n=15)	<i>p-value</i>	Control (n=15)	Mutant (n=15)	<i>p-value</i>
Body Position						
Sitting or standing	15	15	<i>n.s.</i>	15	15	<i>n.s.</i>
Spontaneous Behavior						
Slow movement	0	0		1	0	
Moderate movement	15	13		14	15	
Vigorous	0	2	<i>n.s.</i>	0	0	<i>n.s.</i>
Respiration rate						
Normal	15	15	<i>n.s.</i>	15	15	<i>n.s.</i>
Tremor						
None	14	12	<i>n.s.</i>	15	15	<i>n.s.</i>

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

Locomotor activity data are shown as mean (\pm SEM). Data from behavior recorded in the Arena represent the results of test parameters from major tests where a behavioral response was observed. Test parameters, which did not elicit any response, were excluded from this data. Statistical analysis: chi-squared test; significance $p < 0.05$

Parameter	Male			Female		
	Control (n=15)	Mutant (n=15)	<i>p-value</i>	Control (n=15)	Mutant (n=15)	<i>p-value</i>
Locomotor Activity	24.2 ± 0.4	29.9 ± 0.7	<i>n.s.</i>	18.8 ± 0.35	23.6 ± 0.4	0.035
Transfer arousal						
Extended freeze	0	1	<i>n.s.</i>	1	1	<i>n.s.</i>
Brief freeze	12	7		12	7	
Momentary freeze	3	7		2	7	
Palpebral Closure						
Eyes wide open	15	15	<i>n.s.</i>	15	15	<i>n.s.</i>
Piloerection						
None	15	15	<i>n.s.</i>	15	15	<i>n.s.</i>
Gait						
Normal	15	14		15	13	
Fluid but abnormal	0	1	<i>n.s.</i>	0	2	<i>n.s.</i>
Pelvic Elevation						
Normal	15	13		15	15	
Elevated	0	2	<i>n.s.</i>	0	0	<i>n.s.</i>
Tail Elevation						
Horizontally extended	15	15		15	15	
Elevated/Straub tail	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Touch Escape						
Mild	0	1		2	2	
Moderate	15	14	<i>n.s.</i>	13	13	<i>n.s.</i>
Positional Passivity						
Struggles when held by tail	15	15		15	15	
Struggles when held by neck	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>

Table 13: Behavior recorded in or above the arena

Data shown represent the results of test parameters from major tests where a behavioral response was observed. Test parameters, which did not elicit any response, were excluded from this data. Statistical analysis: chi-squared test; significance $p < 0.05$

Parameter	Male			Female		
	Control (n=15)	Mutant (n=15)	<i>p-value</i>	Control (n=15)	Mutant (n=15)	<i>p-value</i>
Trunk Curl						
Absent	15	15	<i>n.s.</i>	13	9	<i>n.s.</i>
Present	0	0		2	6	
Limb Grasping						
Absent	15	15	<i>n.s.</i>	15	15	<i>n.s.</i>
Present	0	0		0	0	
Visual Placing						
Upon nose contact	6	5	<i>n.s.</i>	2	3	<i>n.s.</i>
Upon vibrassee contact	9	10		13	12	
Grip strength						
None	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Moderate	15	15		15	15	
Body Tone						
Flaccid	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Slight resistance	15	15		15	15	
Pinna reflex						
None	1	1	<i>n.s.</i>	0	1	<i>n.s.</i>
Active retraction	14	14		15	14	
Corneal Reflex						
Active single eye blink	15	15	<i>n.s.</i>	15	15	<i>n.s.</i>
Toe Pinch						
Slight	0	0	<i>n.s.</i>	1	0	<i>n.s.</i>
Moderate withdrawal	15	15		14	15	
Wire maneuver						
Active grip	0	0	<i>n.s.</i>	4	1	<i>n.s.</i>
Difficulty to grasp	15	15		11	14	
Unable to grasp	0	0		0	0	

Table 14: Behavior during supine restraint

Data shown represent the results of test parameters from major tests where a behavioral response was observed. Test parameters, which did not elicit any response, were excluded from this data. Statistical analysis: chi-squared test; significance $p < 0.05$.

Parameter	Male			Female		
	Control (n=15)	Mutant (n=15)	<i>p-value</i>	Control (n=15)	Mutant (n=15)	<i>p-value</i>
Skin Color						
Pink	15	15	<i>n.s.</i>	15	15	<i>n.s.</i>
Limb Tone						
Slight resistance	15	15	<i>n.s.</i>	15	15	<i>n.s.</i>
Abdominal Tone						
Slight resistance	15	15	<i>n.s.</i>	15	15	<i>n.s.</i>
Lacrimation						
None	15	15	<i>n.s.</i>	15	15	<i>n.s.</i>
Salivation						
Slight margin of s.a.	15	15		15	15	
Wet zone entire of s.a	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Provoked biting						
Absent	15	15	<i>n.s.</i>	15	15	<i>n.s.</i>
Righting reflex						
No impairment	15	15	<i>n.s.</i>	15	15	<i>n.s.</i>
Contact righting reflex						
Present	15	15	<i>n.s.</i>	15	15	<i>n.s.</i>
Negative Geotaxis						
Turns and climb the grid	14	14		15	15	
Turns but then freezes	0	1		0	0	
Moves, but fails to turn	1	0	<i>n.s.</i>	0	0	<i>n.s.</i>
Irritability						
None	15	15	<i>n.s.</i>	15	15	<i>n.s.</i>
Aggression						
None	15	15	<i>n.s.</i>	15	15	<i>n.s.</i>
Vocalization						
None	12	12		12	14	
Provoked during handling	3	3	<i>n.s.</i>	3	1	<i>n.s.</i>

Table 15: Lactate levels

Data shown represent the results of the mean blood lactate concentrations, value (\pm SEM). Statistical analysis: Chi-squared test; significance $p < 0.05$.

	Male			Female		
	Control (n=15)	Mutant (n=15)	<i>p-value</i>	Control (n=15)	Mutant (n=15)	<i>p-value</i>
Lactate [mmo/l]	4.45 \pm 0.4	4.4 \pm 0.4	<i>n.s.</i>	3.9 \pm 0.4	4.0 \pm 0.2	<i>n.s.</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 slitlamp 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).

We could not address a phenotype to the Dll1 mutation. Concerning ERG, all groups of Dll1 mice showed strong variability. Results from the slit lamp examination indicated that both, mutant mice and littermate controls, occasionally express nuclear and zonular opacity.

3.4.2 Mice

Fifteen mice of each sex and genotype entered the Eye Screen at the age of 11 weeks. Mice were first examined by slitlamp biomicroscopy and on the following day, an ERG was performed. Mice were kept under standard laboratory conditions with food and water *ad libitum*.

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.

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

ERG responses were recorded from the groups of Dll1 (control – mutant) mice with light pulses at two different light intensities. These two luminance levels were chosen because at 500 cd/m² a well discernable b-wave amplitude (nearly no a-wave) mainly stemming from the rod system is induced, while light pulses at 12,500 cd/m² induce a maximally developed b-wave response and an a-wave, coming presumably from rods and cones. At first, a comparison of the left and right eyes for each group was performed on the amplitudes of a- and b-wave for both luminance intensities (data not shown). Since no major differences were observed between the left and right eye, ERG amplitudes of both eyes were averaged for further evaluation. The mean value and standard error was calculated for each group of mice, male and female, wild type and mutant (Table 16).

In all Dll1 groups some mice with very low ERG amplitudes were found. The other animals showed ERG responses with normal developed a- and b-waves, but variable amplitudes. PCR-analysis revealed that the animals with nearly no ERG responses are homozygous carriers of the *Pde6b*^{rd1} allele (Table 17). Therefore these animals were excluded from statistical evaluation. Mutant mice showed lower values compared to the controls, the differences were significant only for the females.

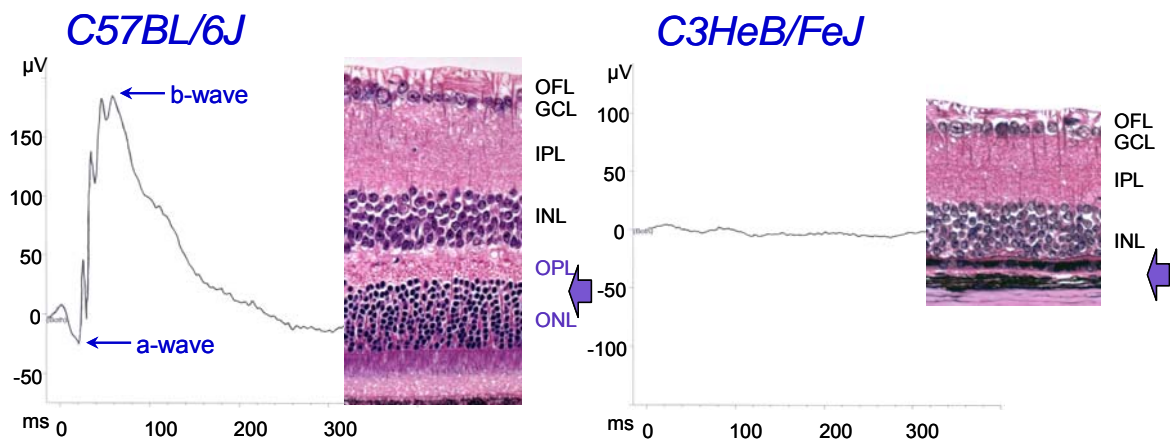


Figure 5: Comparison of retina structure and ERG response

Left: Histological examination of the retina of C57BL/6J mice, which illustrates wild-type conditions/organization. The recorded ERG response to light pulses displays well discriminable a- and b-waves.

Right: Due to the recessive *Pde6b^{rd1}* allele in C3HeB/FeJ mice, the photoreceptor cell layer is missing (bold arrow) and upon light pulses, no a- and b-waves can be recorded.

OFL: outer fibre layer
 GCL: ganglion cell layer
 IPL: inner plexiforme layer
 INL: inner nuclear layer
 OPL: outer plexiforme layer
 ONL: outer nuclear layer

Examination by **slit lamp biomicroscopy** indicated that both wild-type control and mutant mice express nuclear and zonular opacity (Table 18) with no differences in the frequencies between groups ($P_{\text{male}} = 0.365$; $P_{\text{female}} = 0.191$; $P_{\text{combined}} = 0.133$, Fisher's exact test, one-tailed)

3.4.6 Discussion

ERG screening is a quick, robust and reproducible in-vivo method to detect functional retinal impairment in mice. The comparison of a- and b-wave amplitudes of males and females, mutant and control revealed no consistent differences between the groups. Most of the p-values (T-test) calculated were not significant. In both groups of mutant and control mice, animals with nearly no ERG response were found. In a PCR analysis we showed that these animals are homozygous carriers of the *Pde6^{brd1}* allele, which is known to cause retinal degeneration and at last result in blindness (Pittler and Baehr, 1991; Fig. 5 for C3HeB/FeJ mice). The controls exhibited high values compared to our baseline data (results not shown), whereas all other mutant mice exhibited

ERG responses within non-pathologic ranges although the ERG amplitudes showed a strong variability.

Results from slit lamp biomicroscopy indicated there was no association of anterior segment abnormality and genotype. Therefore, further examination of *Dll1*-mutant mice for eye defects is not recommended.

3.4.7 References

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Abbreviations

cd/m ²	candela per square meter
ERG	electroretinography
Hz	hertz
NAD	no abnormality detected

Table 16: Comparison of ERG-responses at illumination levels of 500 and 12,500 cd/m².Only mice without the *Pde6*^{brd1} allele were incorporated in the calculations (see Table 17).

Mean ± standard error is calculated for a- and b-wave amplitudes.

Parameter	Control (A)			Mutant (B)			A~B	A~B
	Male	Female	<i>p</i> - value	Male	Female	<i>p</i> - value	Male	Female
	(n=5)	(n=7)		(n=7)	(n=8)		<i>p</i> - value	<i>p</i> - value
a-wave 500 cd/m ²	-9 ± 2.7	-19 ± 2.7	<0.05	-9 ± 2.5	-11 ± 1.9	n.s.	n.s.	<0.05
b-wave 500 cd/m ²	205 ± 19.9	259 ± 13.1	<0.05	179 ± 13.9	214 ± 12.9	n.s.	n.s.	<0.05
a-wave 12,500 cd/m ²	-34 ± 5.9	-43 ± 3.5	n.s.	-28 ± 4.1	-34 ± 4.0	n.s.	n.s.	n.s.
b-wave 12,500 cd/m ²	292 ± 40.8	336 ± 22.7	n.s.	218 ± 16.5	254 ± 16.8	n.s.	n.s.	<0.01

Table 17: Results from PCR analysis		
Sex / Genotype	homozygous <i>Pde6b</i> ^{rd1} (blind)	normal phenotype
M <i>DII1</i> ^{+/+}	10	5
M <i>DII1</i> ^{+/-}	8	7
F <i>DII1</i> ^{+/+}	8	7
F <i>DII1</i> ^{+/-}	7	8

Table 18: Results from slit lamp biomicroscopy		
Sex / Genotype	Control	Nuclear-zonular cataract
M <i>DII1</i> ^{+/+}	9	6
M <i>DII1</i> ^{+/-}	9	6
F <i>DII1</i> ^{+/+}	9	6
F <i>DII1</i> ^{+/-}	5	10

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, thirty (15 males/15 females) control mice and thirty-one (16 males /15 females) mutant mice were analyzed. Twenty 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.

We found a higher inorganic phosphorus concentration in mutant males. This could give a hint at an imbalance of phosphate homeostasis that might be caused by skeleton disease, kidney disease or impaired endocrine regulation of calcium and phosphorus metabolism. Additionally we detected an elevated white blood cell and platelet count in the female mutant mice with the same tendency in the male mice. This might indicate an influence on the immune system (see also Immunology screen, 3.6). However, all parameters of both mutants and control mice were within normal ranges.

3.5.2 Mice

Fifteen 12-week-old wild-type and sixteen 12-week-old mutant males entered the clinical-chemical screen at the beginning of the 8th calendar week in 2004. Fifteen 12-week-old wild-type and fifteen 12-week-old mutant females entered the screen at the beginning of the 9th calendar week. Out of these, thirteen (six males, seven females) Dll1-mutant mice and fourteen (seven males, seven females) control mice were analysed a second time for 11 energy metabolism related parameters after food restriction at the age of 20 weeks

3.5.3 Materials and Methods

Blood Withdrawal and Storage

The Clinical-chemical Screen of the German Mouse Clinic routinely analyzed 12-week-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. 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

EDTA-coated tubes (KABE, Art.No 078035). The tube was immediately inverted five times to achieve a homogeneous distribution of the anticoagulant.

After removal of 40 μ l blood for the Neurology Screen, the Li-heparin-coated tubes were stored in a rack at room temperature for two hours. Afterwards, 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 μ l), the Allergy Screen (30 μ l), the Clinical Chemical Screen (130 μ l) 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 μ l 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). 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 $MCV \times$ red blood cell count. Mean corpuscular hemoglobin (MCH) and mean concentration of corpuscular hemoglobin (MCHC) are calculated from hemoglobin/red blood cells count (MCH) and 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

Differences between mutants and controls were seen only in inorganic phosphorus concentrations in males (Table 19). We detected sex differences in many clinical chemical parameters in the control animals as well as in the mutant mice. Most values obtained for the clinical chemical parameters were within the normal ranges usually found in 129SV mice at the age of three months and were supported by previously published data (Suckow *et al*, 2001; Quimby, 1999 and publications cited therein).

Hematology

In the primary screen for hematological parameters, significant differences between mutants and controls were detected for white blood cell count and platelets count only in females (Table 20). The same tendency was observed in the males. All parameters of both controls and mutants were within normal ranges.

Results from the first analysis in 2003

The mutants showed reduced potassium and glucose concentrations and an elevated platelet count (data not shown). The latter was confirmed in the second batch.

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

3.5.6 Discussion

Clinical Chemistry. We detected a slightly higher inorganic phosphorus concentration in mutant males compared to the control group. This could give a

hint at an imbalance of phosphate homeostasis that might be caused by skeleton disease, kidney disease or impaired endocrine regulation of calcium and phosphorus metabolism. Nevertheless, all clinical chemical values were situated within the normal ranges.

In a second blood sampling procedure (at age 20 weeks), which was performed after six days of food restriction in the Metabolic Screen, no significant differences between mutant and control animals were detected (Table 21). However, the inorganic phosphorus concentration was not measured in this group. Taken together, the results indicated no major influence of the loss of one *Dll1* allele on organ functions and metabolic pathways investigated.

The **haematological findings** might reflect minimal differences concerning immunological status or regulation of hematopoiesis due to the known influence of the Delta/Notch signal transduction pathway on these systems. However, since all values were within the normal range of 129/Sv mice and genotype-specific differences were observed only in one sex, the findings might be considered as being without biological relevance.

It might be useful to confirm the findings concerning bone metabolism related parameters and blood cell count in a second batch of mice to insure that they really are genotype-dependent differences. If the haematological findings were confirmed, we could additionally investigate blood smears to determine differential white blood cell counts.

3.5.7 References

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Table 19: Clinical-chemical parameters.								
Data are presented as mean ± standard error of mean.								
Parameter	Mutant (A)			Control (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=16)	(n=15)	<i>p- value</i>	(n=15)	(n=14)	<i>p-value</i>	<i>p-value</i>	<i>p-value</i>
Calcium [mmol/l]	2.0 ±0.02	2.0 ±0.01	n.s.	2.0 ±0.00	2.0 ±0.02	n.s.	n.s.	n.s.
Inorganic Phosphate [mmol/l]	1.5 ±0.07	1.0 ±0.08	<0.001	1.3 ±0.06	1.0 ±0.05	<0.01	<0.05	n.s.
Total Protein [g/dl]	4.8 ±0.08	5.0 ±0.08	n.s.	4.9 ±0.05	4.9 ±0.10	n.s.	n.s.	n.s.
Creatinine [mg/dl]	0.275 ±0.01	0.319 ±0.00	<0.01	0.267 ±0.01	0.317 ±0.01	<0.01	n.s.	n.s.
Urea [mg/dl]	46.4 ±1.71	52.9 ±2.33	<0.05	47.6 ±2.2	50.6 ±2.8	n.s.	n.s.	n.s.
Uric acid [mg/dl]	5.5 ±0.62	1.4 ±0.19	<0.001	4.6 ±0.4	1.3 ±0.2	<0.001	n.s.	n.s.
Cholesterol [mg/dl]	130.3 ±5.73	97.0 ±3.63	<0.001	134.8 ±4.1	100.8 ±5.1	<0.01	n.s.	n.s.
Triglyceride [mg/dl]	161.2 ±13.5	127.0 ±8.09	<0.001	183.9 ±11.8	125.3 ±12.7	<0.01	n.s.	n.s.
Creatine Kinase [U/l]	57 ±16.52	54 ±8.45	n.s.	50 ±9.00	63 ±13.00	n.s.	n.s.	n.s.
Alanine-Amino-transferase (ALAT,GPT) [U/l]	29 ±5.91	14 ±0.86	<0.05	22 ±4.00	16 ±3.00	n.s.	n.s.	n.s.
Aspartate-Amino-transferase (AST,GOT) [U/l]	28 ±2.31	27 ±0.93	n.s.	26 ±2.00	28 ±1.00	n.s.	n.s.	n.s.
Alkaline Phosphatase [U/l]	61 ±1.66	100 ±5.21	<0.001	62 ±3.00	86 ±4.00	<0.001	n.s.	n.s.
α-Amylase [U/l]	2561 ±101.48	2309 ±53.18	<0.05	2615 ±66.00	2339 ±49.00	n.s.	n.s.	n.s.
Glucose [mg/dl]	176.5 ±8.01	128.4 ±4.67	<0.001	176.2 ±10.3	140.5 ±6.50	<0.01	n.s.	n.s.
Ferritin [ng/ml]	31.5 ±2.68	39.2 ±2.68	n.s.	37.0 ±2.3	36.7 ±2.50	n.s.	n.s.	n.s.
Transferrin [mg/dl]	169.3 ±1.82	172.3 ±1.36	n.s.	167.9 ±2.1	174.9 ±2.1	<0.05	n.s.	n.s.
Lipase [U/l]	71.5 ±3.49	57.7 ±2.46	<0.01	64.8 ±1.6	55.7 ±1.4	<0.001	n.s.	n.s.

Table 20: 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=16)	(n=15)	<i>p</i> - value	(n=15)	(n=15)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
White blood cell count [10 ³ / μ l]	7.61 \uparrow \pm 0.48	7.46 $\uparrow\uparrow$ \pm 0.54	n.s.	6.65 \pm 0.30	5.77 \pm 0.40	n.s.	n.s.	<0.02
Red blood cell count [10 ³ / μ l]	10.08 \downarrow \pm 0.32	10.93 \pm 0.12	<0.05	10.82 \pm 0.16	10.96 \pm 0.17	n.s.	<0.05	n.s.
Hemoglobin [g/dl]	16.03 \pm 0.39	17.39 \pm 0.21	<0.01	16.87 \pm 0.13	17.43 \pm 0.28	n.s.	n.s.	n.s.
Hematocrit [%]	50 \pm 1.31	54 \pm 0.61	<0.02	52 \pm 0.50	54 \pm 0.84	n.s.	n.s.	n.s.
Mean corpuscular volume [fl]	49.56 \pm 0.68	48.87 \pm 0.26	n.s.	48.53 \pm 0.58	48.87 \pm 0.24	n.s.	n.s.	n.s.
Mean corpuscular hemoglobin [pg]	16.00 \pm 0.30	15.91 \pm 0.09	n.s.	15.62 \pm 0.17	15.90 \pm 0.10	n.s.	n.s.	n.s.
Mean corpuscular hemoglobin concentration [g/dl]	32.28 \pm 0.27	32.49 \pm 0.11	n.s.	32.20 \pm 0.15	32.56 \pm 0.16	n.s.	n.s.	n.s.
Platelet count [10 ³ / μ l]	688 \uparrow \pm 30.27	570 $\uparrow\uparrow$ \pm 16.46	<0.05	633 \pm 13.29	501 \pm 11.24	<0.001	n.s.	<0.01

Table 21: Clinical-chemical parameters after food restrictionData are presented as mean \pm standard error of mean.

Parameter	Mutant (A)			Control (B)			A~B	A~B
	Male	Female	<i>p</i> -value	Male	Female	<i>p</i> -value	Male	Female
	(n=6)	(n=7)		(n=7)	(n=7)		<i>p</i> -value	<i>p</i> -value
Total Protein [g/dl]	5.6 \pm 0.09	5.5 \pm 0.14	NA	5.7 \pm 0.14	5.4 \pm 0.11	NA	n.s.	n.s.
Creatinine [mg/dl]	0.291 \pm 0.03	0.273 \pm 0.01	NA	0.257 \pm 0.03	0.287 \pm 0.01	NA	n.s.	n.s.
Urea [mg/dl]	36.6 \pm 3.6	33.9 \pm 2.50	NA	31.1 \pm 2.77	39.6 \pm 4.97	NA	n.s.	n.s.
Cholesterol [mg/dl]	122.1 \pm 9.1	122.5 \pm 4.10	NA	127.9 \pm 4.56	120.6 \pm 8.32	NA	n.s.	n.s.
Triglyceride [mg/dl]	53.2 \pm 4.7	61.5 \pm 3.70	NA	73.6 \pm 4.59	61.9 \pm 2.82	NA	<0.02	n.s.
Alanine-Aminotransferase (ALAT/GPT) [U/l]	23 \pm 5.00	11 \pm 1.00	NA	20 \pm 3.69	15 \pm 2.17	NA	n.s.	n.s.
Aspartate-Aminotransferase (AST/GOT) [U/l]	38 \pm 4.00	21 \pm 1.00	NA	36 \pm 7.03	23 \pm 0.84	NA	n.s.	n.s.
Alkaline Phosphatase [U/l]	42 \pm 2.00	69 \pm 6.00	NA	39 \pm 2.57	64 \pm 2.81	NA	n.s.	n.s.
α-Amylase [U/l]	2068 \pm 145.00	2020 \pm 72.00	NA	2179 \pm 132.54	2014 \pm 89.95	NA	n.s.	n.s.
Glucose [mg/dl]	62.9 \pm 5.00	71.9 \pm 5.30	NA	70.0 \pm 9.55	65.0 \pm 3.73	NA	n.s.	n.s.
Lipase [U/l]	41.4 \pm 1.80	38.5 \pm 2.3	NA	59.1 \pm 17.53	40.1 \pm 1.58	NA	n.s.	n.s.

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 (Blüethmann 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 in the Dll1 mutant mouse line. Its analysis in the Immunology Screen revealed differences in the relative frequencies of B cells, cytotoxic T cells, and IgM levels between mutants and their control littermates.

3.6.2 Mice

We analyzed 31 mutant animals (15 females and 16 males) and the 30 age- and sex-matched littermate controls (15 females and 15 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

Second batch

The analysis of standard immunological parameters measured in the primary screen (Table 22) has revealed: increased percentage of B cells (CD19⁺) in female mutant mice; increased frequency of cytotoxic T cells (CD8 β ⁺) in male mutants; decreased level of IgM in female mutants. Although sex-specific, tendencies similar to the described lymphocyte differences could be observed in the opposite sex as well.

First batch

Only minor changes were detected in immunological baseline values of Dll1-mutant mice on C%/BL/6x129/Sv genetic background (data not shown):

- slight increase of Gr-1+ cells in heterozygous animals
- no changes in AB titers or other cell populations detectable

3.6.6 Discussion

Under standard screen conditions, mutant mice showed changes in the percentage of certain lymphocyte subsets, as well as in immunoglobulin levels. Although statistically significant, the observed changes do not represent ab-

normally altered values. Nevertheless, since the Notch signalling pathway plays an important role in lymphocyte differentiation (Hozumi *et al.*, 2004), we would suggest re-testing another batch of Dll1 mice in order to confirm our initial findings. If similar differences are observed, further investigations for in-depth characterization of this immunological phenotype should be undertaken.

Due to the fact that more changes in *Dll1*-mutant mice were detectable on a pure 129iso background (IgM \downarrow & B cells \uparrow females; CD8b \uparrow males), the genetic background seems to be important for analysis of Dll1 function as observed by the Clinical Chemical Screen (3.5.5).

3.6.7 References

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Table 22: 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 (n=16)	Female (n=15)		Male (n=15)	Female (n=15)		Male	Female
CD19 ⁺ [%]	32.4 \pm 1.2	26.5 \pm 1.1	<0.01	28.9 \pm 2	20.4 \pm 2	<0.01	n.s.	<0.05
CD19 ⁺ CD5 ⁻ [%]	88.7 \pm 1.1	91.8 \pm 0.5	<0.02	90.2 \pm 1	90 \pm 0.8	n.s.	n.s.	n.s.
CD19 ⁺ CD5 ⁺ [%]	11.3 \pm 1	8 \pm 0.5	<0.02	9.8 \pm 1	9.7 \pm 0.8	n.s.	n.s.	n.s.
CD3 ⁺ [%]	45.6 \pm 1.6	51.3 \pm 1	n.s.	44.7 \pm 1.9	49 \pm 3.6	n.s.	n.s.	n.s.
γ/δ TCR ⁺ [%]	1.2 \pm 0.1	n.a.	n.a.	1 \pm 0.1	n.a.	n.a.	n.s.	n.a.
Gr-1 ⁺ [%]	20.3 \pm 1.3	15 \pm 0.9	<0.01	22 \pm 1.1	21.2 \pm 5	n.s.	n.s.	n.s.
CD49b ⁺ [%]	18.2 \pm 2.0	2.9 \pm 0.6	<0.001	18.9 \pm 1.3	3.2 \pm 1.4	<0.001	n.s.	n.s.
CD4 ⁺ [%]	37.2 \pm 1.4	36.5 \pm 1	n.s.	38.8 \pm 2.1	34.9 \pm 3	n.s.	n.s.	n.s.
CD8 β ⁺ [%]	5.2 \pm 0.3	8.1 \pm 0.4	<0.001	4.2 \pm 0.3	7.7 \pm 0.6	<0.001	<0.05	n.s.
IgG ₁ [μ g/ml]	386.5 \pm 18.4	464.3 \pm 66.4	n.s.	424.7 \pm 23.8	445.8 \pm 48.6	n.s.	n.s.	n.s.
IgG _{2a} [μ g/ml]	279.5 \pm 36.7	277.7 \pm 46.3	n.s.	194 \pm 19.4	356.8 \pm 50.2	n.s.	n.s.	n.s.
IgG _{2b} [μ g/ml]	354.2 \pm 26.8	195.6 \pm 14	<0.001	376.3 \pm 36.7	194.3 \pm 15.7	n.s.	n.s.	n.s.
IgG ₃ [μ g/ml]	186.8 \pm 17.1	160.9 \pm 16.3	n.s.	182.2 \pm 10.8	202.6 \pm 20.1	n.s.	n.s.	n.s.
IgM [μ g/ml]	122.8 \pm 14.9	105.1 \pm 11.1	n.s.	148.8 \pm 19.5	210.3 \pm 34.8	n.s.	n.s.	<0.01
IgA [μ g/ml]	18.6 \pm 2.2	32.5 \pm 5.5	<0.01	24.4 \pm 2.1	39.1 \pm 4.5	n.s.	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 the DII1 mutant mouse line, 24 control and 29 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 24 control (11 females, 13 males) and 29 mutant (14 females, 15 males) mice aged 12 weeks was analyzed in 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 DII1 mice revealed no statistically significant difference between mutant and control mice. We detected higher mean IgE concentrations in female animals. This sex-difference was statistically significant in both mutant and control mice (Table 23) and is known for many inbred strains (Alessandrini *et al.*, 2000; Corteling *et al.*, 2004; Seymour *et al.*, 2002).

Taken together, under standard screening conditions for the primary Allergy screen, *Dll1*-mutant mice did not show changes in total plasma IgE levels that would reveal a major allergy phenotype. However, since the Delta-Notch pathway has a presumed function in the immune response (e.g., Hoyne *et al.*, 2000), further allergological investigations using an allergen challenge screen should be considered.

Raw data will be available on demand.

Table 23: Total plasma IgE [ng/ml]								
Data are presented as mean ± standard error of mean.								
Batch	Control (A)			Mutant (B)			A~B	A~B
	Female	Male		Female	Male		Female	Male
	(n=10)	(n=7)	<i>p</i> - value	(n=11)	(n=12)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
2003 BL6x129	53.5 ±15.1	16.4 ±2.5	0.06	31 ±10.2	26.5 ±10.5	n.s.	n.s.	n.s.
	(n=11)	(n=13)	<i>p</i> - value	(n=14)	(n=15)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
2004 129/Sv	313 ±36.3	168 ±26.5	<0.01	389 ±102	170 ±23.6	<0.05	n.s.	n.s.

Raw data will be available on demand.

3.7.5 References:

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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 Dll1 mutant mouse line by means of the hot plate test (nociceptive pain). We detected significant differences in pain reactivity between mutant animals and their control littermates: Thermal latencies of mutant mice were longer for the first two parameters. Therefore, mutant animals might exhibit hypoalgesia. In conclusion we suggest performing additional pain related studies.

3.8.2 Mice

Thirty mutant mice (15 male, 15 female), and 28 control animals (13 male, 15 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. 6). 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.

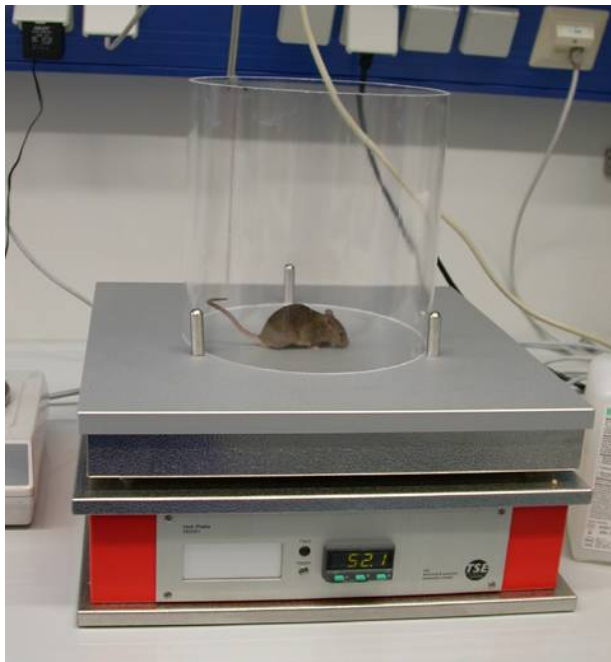


Figure 6: Hot plate system

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

The results are shown in Table 24. In general, the first nociceptive response observed in mice is hind paw shaking. Both genotypes also showed hind paw licking, another typical nociceptive response. The third examined response was jumping. We detected a significant difference in the hind paw shaking behavior between the mutants and their control littermate: mutant animals had longer latencies. The same tendency was observed for hind paw licking, too. Therefore, the mice might show hypoalgesia. Both sexes reacted almost uniformly to thermal pain.

Raw data will be available on demand.

3.8.6 Discussion

We found a significant difference in the first reaction to thermal pain of *D111*-mutant animals. We could not clearly determine the type of pain phenotype of this mutant mouse line on the base of the hot plate test, mutant animals might show hypoalgesia. We would suggest performing further pain related studies to specify the pain sensitivity of this mutant mouse line in more detail.

Further pain related studies would include:

1. Base studies e.g.,
 - von Frey filament test to study the reaction of animals to mechanical pain,
 - 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:
 - Formalin test to study the acute, nociceptive (early) and tonic, inflammatory (late) pain reaction of the same animals;
 - 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.

3.8.7 References

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Wall P.D. and R. Melzack (Eds.) Textbook of Pain, Churchill Livingstone, London, 1984

Abbreviations

h.p. hind paw

Table 24: Nociceptive Screen								
Data are presented as mean ± standard error of mean.								
Parameter Latency [s]	Mutant (A)			Control (B)			A~B	A~B
	Female	Male		Fe- male	Male		Fe- male	Male
	(n=15)	(n=15)	<i>p</i> - <i>value</i>	(n=15)	(n=13)	<i>p</i> - <i>value</i>	<i>p</i> - <i>value</i>	<i>p</i> - <i>value</i>
h.p. licking	27.8↑ ±2.57	32.87↑ ±2.57	n.s.	23.54 ±2.57	28.8 ±2.75	n.s.	n.s.	n.s.
h.p. shaking	23.57↑↑ ±1.65	24.22↑↑ ±1.65	n.s.	17.8 ±1.65	14.4 ±1.77	n.s.	0.161	0.0001
jumping	58.57 ±0.73	60	n.s.	60	60	n.s.	n.s.	n.s.

ANOVA:

Licking: sex; p=0.0545, genotype; p=0.1162

Shaking: sex; p=0.4233, genotype; **p=0.000028**

Jumping: sex; p=0.33953, genotype; p=0.33953

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 sleep were studied in 15-week-old male and female heterozygous $Dll1^{lacZ}$ and control mice. Neither differences between mutant and control mice nor sex differences were found for the absolute values indicating that the mutation does not affect the breathing pattern. The most striking finding was the low level of activity (sleep) in both control and mutant mice reflected by low mean respiratory rates.

3.9.2 Mice

Control and mutant mice of both sexes were studied at the age of 15 weeks. Mean body weights are listed in Table 25.

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 (Figs. 7 and 8).

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 (Fig. 7), data recording was immediately started and was continued for 40 min.



Figure 7: System used at GMC to assess breathing patterns.

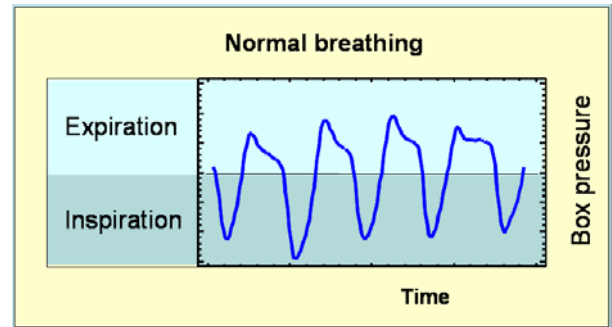


Figure 8: Recorded data used to calculate the breathing parameters.

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 and Discussion

Table 26 summarizes the results obtained for spontaneous breathing under sleeping conditions. In contrast to the behavior typically observed in other strains or mutant mouse lines as outlined above (3.9.3), the Dll1 mice rapidly came to rest and fall asleep for short periods or even for periods of up to 15 minutes. Hence, data evaluation is restricted to these phases of sleep. Neither sex differences nor differences between control and mutant mice were found for the absolute values.

The specific tidal volume was significantly higher in mutant females compared to control females and compared to their male counterparts. Since between the groups also significant differences in body weights were found, the differences in specific tidal volumes are related to the differences in body weight.

The most striking finding was the low level of activity in both control and mutant mice reflected by low mean respiratory rates. To date, we have no explanation for this finding, since the locomotor activity (Behavior Screen, 3.1.5) of these mice compared to 129/Sv mice .

In summary, no evidence was found that the mutation affects the breathing pattern.

3.9.6 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 25: 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=5)	<i>p - value</i>	(n=5)	(n=5)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
Bw [g]	23.4 \pm 1.1	24.5 \pm 0.9	n.s.	24.3 \pm 0.2	20.2 \pm 0.5	< 0.001	n.s.	< 0.01
Mean_f [1/min]	286.5 \pm 26.3	271.7 \pm 5.3	n.s.	289.9 \pm 15.8	291.1 \pm 16.1	n.s.	n.s.	n.s.

Table 26: Spontaneous breathing pattern during sleepData 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=5)	<i>p</i> - value	(n=5)	(n=5)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
Sleep								
f [1/min]	209.8 \pm 16.0	196.6 \pm 9.5	n.s.	200.0 \pm 12.4	191.3 \pm 2.7	n.s.	n.s.	n.s.
TV [ml]	0.26 \pm 0.01	0.26 \pm 0.01	n.s.	0.27 \pm 0.01	0.24 \pm 0.01	n.s.	n.s.	n.s.
sTV [μl/g]	10.4 \pm 0.4	10.6 \pm 0.3	n.s.	11.0 \pm 0.2	12.1 \pm 0.3	< 0.02	n.s.	< 0.01
MV [ml/min]	54.2 \pm 1.6	50.7 \pm 3.7	n.s.	52.8 \pm 3.3	46.4 \pm 0.8	n.s.	n.s.	n.s.
sMV [ml/min/g]	2.1 \pm 0.1	2.1 \pm 0.1	n.s.	2.2 \pm 0.1	2.3 \pm 0.1	n.s.	n.s.	n.s.
Ti [ms]	95.3 \pm 5.2	94.2 \pm 7.0	n.s.	100.3 \pm 4.9	98.8 \pm 2.2	n.s.	n.s.	n.s.
Te [ms]	195.3 \pm 15.4	213.7 \pm 11.3	n.s.	203.8 \pm 12.9	215.2 \pm 6.1	n.s.	n.s.	n.s.
Ti/TT	0.33 \pm 0.01	0.31 \pm 0.02	n.s.	0.33 \pm 0.01	0.32 \pm 0.01	n.s.	n.s.	n.s.
PIF [ml/s]	4.5 \pm 0.1	4.6 \pm 0.5	n.s.	4.4 \pm 0.3	4.3 \pm 0.2	n.s.	n.s.	n.s.
PEF [ml/s]	3.1 \pm 0.2	2.8 \pm 0.2	n.s.	2.8 \pm 0.1	2.5 \pm 0.1	n.s.	n.s.	n.s.
MIF [ml/s]	2.8 \pm 0.1	2.8 \pm 0.3	n.s.	2.7 \pm 0.1	2.5 \pm 0.1	n.s.	n.s.	n.s.
MEF [ml/s]	1.4 \pm 0.1	1.2 \pm 0.1	n.s.	1.3 \pm 0.1	1.1 \pm 0.1	n.s.	n.s.	n.s.

3.10 Expression Profiling

3.10.1 Summary

In this report, we describe the results of using close to genome-wide 21K cDNA microarrays for the RNA expression profiling of thymus, liver, spleen, and brain of the *Dll1* mutant mouse line. In total 50 chip hybridizations were performed. The data analysis and various statistical methods detected a number of genes differentially regulated between mutant and wild-type control tissues in all experiments. We detect genes involved, for example in tumorigenesis, left-right development, cell adhesion, and associated with Delta-Notch signalling, wnt, and Eph pathways.

3.10.2 Mice

The molecular phenotyping screen archives organs of mutant mice for subsequent DNA-chip expression profiling analysis. Nine male mice (five mutants and four controls) of the *Dll1* mouse mutant line were provided to the molecular phenotyping screen (Table 27).

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 a.m. and 12 a.m. by carbon dioxide asphyxiation. The following 17 organs were collected and archived in liquid nitrogen following our established standard operating protocols: bulbourethral gland, spleen, kidney, seminal vesicles, testis, white fat, liver, heart, lung, thymus, skin/cartilage (outer ear), bone (femur), skeletal muscle, salivary gland, brain, brown fat, and eye.

Table 27: Organs of *Dll1*-mutant and control mice stored for expression profiling.

Mouse ID	Strain	Sex	Date of Birth	Genotype	Date of Collection
30015729	<i>Dll1</i>	m	18.11.2003	+/-	9.03.2004
30015730	<i>Dll1</i>	m	18.11.2003	+/-	9.03.2004
30015734	<i>Dll1</i>	m	17.11.2003	+/-	9.03.2004
30015738	<i>Dll1</i>	m	18.11.2003	+/-	9.03.2004
30015740	<i>Dll1</i>	m	18.11.2003	+/-	9.03.2004
30015724	<i>Dll1</i>	m	17.11.2003	+/+	9.03.2004
30015727	<i>Dll1</i>	m	18.11.2003	+/+	9.03.2004
30015728	<i>Dll1</i>	m	18.11.2003	+/+	9.03.2004
30015731	<i>Dll1</i>	m	18.11.2003	+/+	9.03.2004

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 homogenized using a Polytron homogenizer. 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 were amplified from the mouse arrayTAG library from Lion Bioscience comprising approximately 20,200 clones (Heidelberg, Germany). PCR products were 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 were rehydrated overnight in a humid chamber containing 50-70% aqueous solution of glycerol. Rehydrated slides were 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. Slides were pre-hybridized for 1 hour in pre-hybridization buffer (6x SSC, 1% BSA, 0.5% SDS) rinsed in water, dried and hybridized the same day (Seltmann *et al*, 2005).

Reverse Transcription and Fluorescent Labeling

For labeling 20µg of total RNA were used for reverse transcription and indirectly labeled with Cy3 or Cy5 fluorescent dye according the TIGR protocol (http://pga.tigr.org/sop/M004_1a.pdf). Labeled cDNA was dissolved in 30µl hybridisation 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 was placed on a pre-hybridized microarray, under a cover slip, placed into a hybridization chamber (Genetix) and immersed in a thermostatic bath at 42°C for at least 16 hours. After hybridization slides were washed in 40 ml of 3x SSC, 40 ml of 1x SSC and 40 ml of 0.25x SSC at room temperature. For drying slides were placed in an empty 50 ml Falcon tube (Becton Dickinson, USA) and centrifuged at 4000 m/s². Dried slides were scanned with a GenePix 4000A microarray scanner and the images were analyzed using the GenePix Pro3.0 image processing software (Axon Instruments, USA). All data

were 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) was used.

Chip Hybridization

Depending on the amount of RNA available for hybridization, in general four chip hybridizations were performed with RNA from all organs of each four individual mutant mice (in total 16 hybridizations). Each chip hybridization was performed against the identical pool of each organ of control RNA (reference RNA pool; wt). For each individual the chip experiments included two color-flip experiments. For detailed explanation of the raw data provided on the CD-ROM and the analysis of data, see Figure 9.

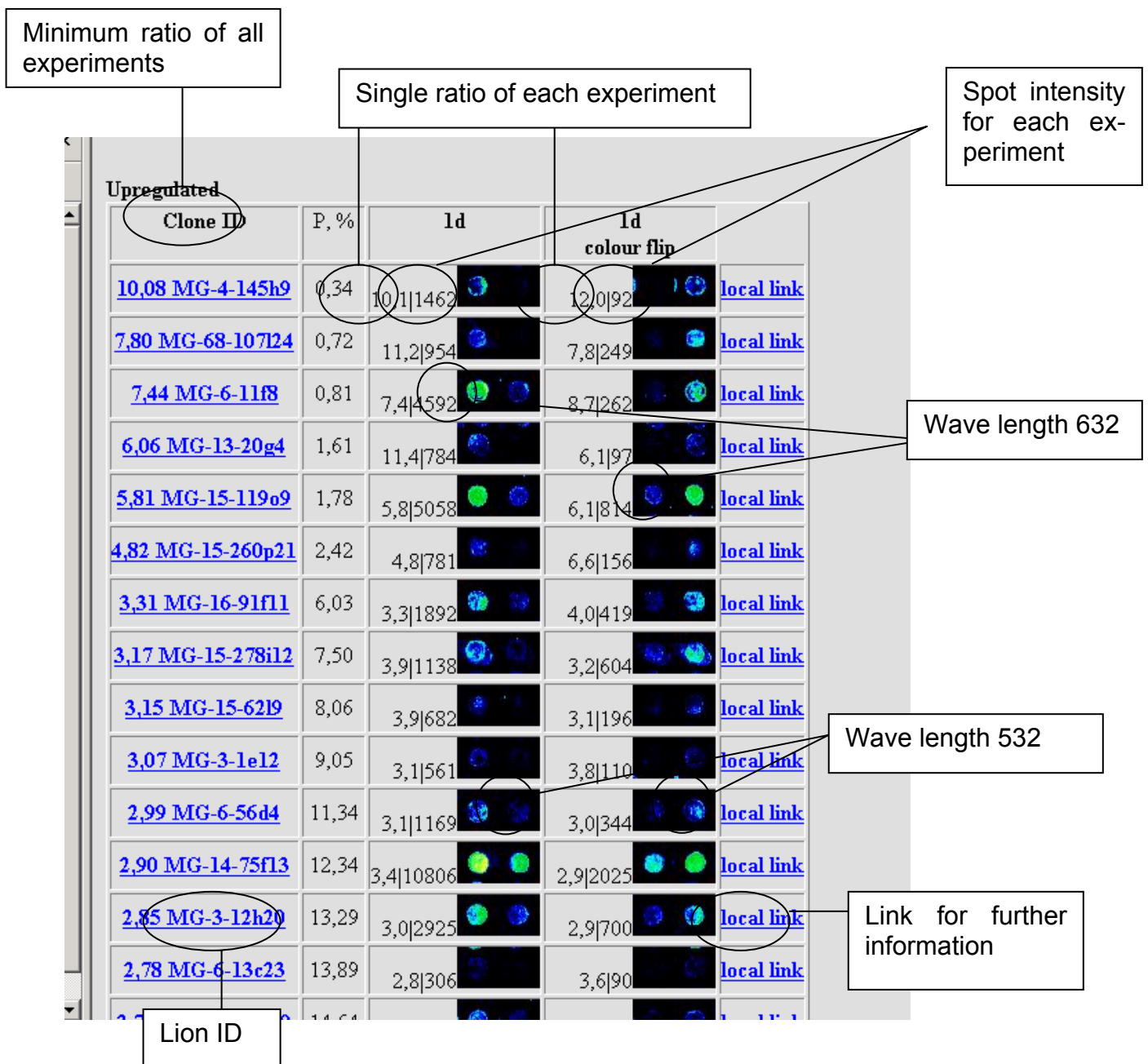


Figure 9: Screenshot and analysis of data.

3.10.4 Results

Selected Organs and Isolated RNA

Thymus, liver, spleen and brain were selected as organs for expression profiling analysis based on data from other GMC-screens. We isolated total RNA of these organs of five mutant mice and four control individuals (Table 28).

Table 28: Amount of total RNA [μg] isolated from different organs.				
Mouse ID	Thymus	Liver	Spleen	Brain
30015729	43	2137	211	10
30015730	19	1340	348	169
30015734	28	324	232	163
30015738	28	150	320	82
30015740	8	650	152	148
30015724	90	36	255	243
30015727	37	65	162	237
30015728	36	159	160	180
30015731	67	820	136	192

Analysis of Gene Expression in Thymus

Due to a small amount of RNA we hybridized pooled RNA of mutant mice against a pool of control RNA of thymus. Table 29 summarizes the results of six chip hybridizations performed with RNA from Thymus. In total, 856 probes show signals in all six chip hybridizations.

Table 29: Chip hybridization of thymus: labeling and number of detected spots		
Chip ID	Cy5/Cy3	Detected Spots
#1	Wt/ Dll1 pool	3202
#2	Wt/ Dll1 pool	1996
#3	Wt/ Dll1 pool	2310
#4	Dll1 pool /Wt	1848
#5	Dll1 pool /Wt	2394
#6	Dll1 pool /Wt	1739
		856 overlap

These genes were evaluated for the significance of differential gene expression. Genes were ranked according the lowest absolute ratio of signal intensities (Dll1 versus control) in six 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 levels $p < 0.01$ and $p < 0.05$.

For example, the selection of the top 150 ranked genes with reproducible up- or down-regulation contains seven genes with non-reproducible chip data. The remaining 43 genes with reproducible up- or down-regulation contain nine or more non-differentially expressed genes (NDE) with the significance level $p < 5\%$, or 10 or more non-differentially expressed genes with the significance level $p < 0.01$. The minimal ratios of expression for this selection ranged from 11.32 to 1.37 fold induction/repression.

Table 30: Chip hybridization of thymus: evaluation of data				
Ranked Genes (According Lowest of 6 Ratios)	Non-uniform Patterns	NDE (False Positives)		Fold Induction (Minimum of 6 Chips)
		$p < 0.01$	$p < 0.05$	
1 - 20	0	≥ 2	≥ 1	11.32 – 3.39
1 - 40	0	≥ 2	≥ 1	11.32 – 2.33
1 - 60	1	≥ 3	≥ 3	11.32 – 1.98
1 - 100	3	≥ 5	≥ 5	11.32 – 1.55
1 - 150	7	≥ 10	≥ 9	11.32 – 1.37
1 - 300	37	≥ 43	≥ 42	11.32 – 1.16
1 - 600	225	≥ 241	≥ 239	11.32 – 1.04

Genes that may be of interest for the *Dll1* mutant line: for details see *Dll1_thymus-Folder* on the CD-ROM. The selected genes are reproducibly up- or down-regulated in all six experiments; genes were ranked according to the minimum of (6) ratios (minimum of maximum). Only signals with intensity above 200 were selected.

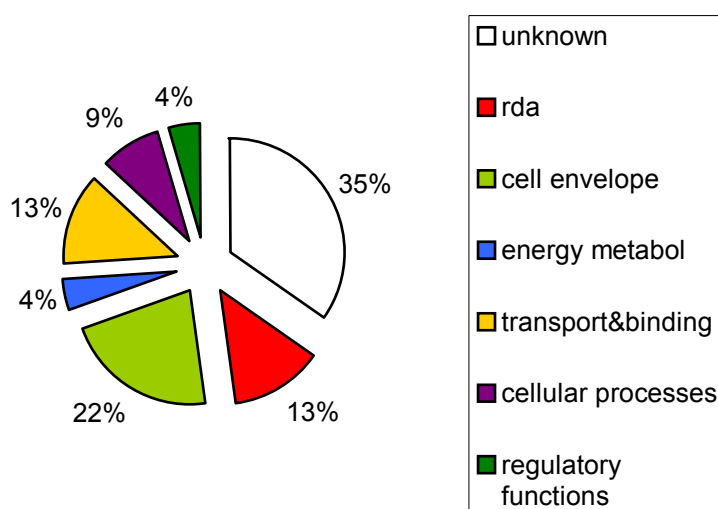


Figure 10: Gene expression in thymus in Dll1 mice
Top 23 up-regulated genes sorted by functional classes.

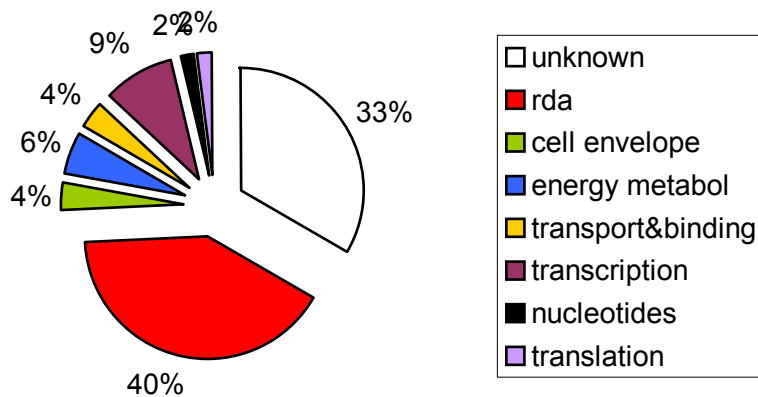


Figure 11: Gene expression in thymus in Dll1 mice
 Top 54 **down-regulated** genes sorted by functional classes.

Analysis of Gene Expression in Liver

Table 31 summarizes the results of 13 chip hybridizations performed with RNA from liver. In total, 391 probes show signals in all 13 chip hybridizations.

Table 31: Chip hybridization of liver: labeling and number of detected spots		
Numbers indicate the ID of mutant mice.		
Chip	Cy5/Cy3	Detected Spots
#7	Wt/5734	1943
#8	5734/Wt	1470
#9	Wt/5729	3251
#10	Wt/5729	3429
#11	5729/Wt	2116
#12	5729/Wt	2689
#13	Wt/5740	2949
#14	Wt/5740	2326
#15	5740/Wt	1758
#16	5740/Wt	2210
#17	Wt/5730	3803
#18	5730/Wt	4313
#19	5730/Wt	3716
		391 overlap

These genes were evaluated for the significance of differential gene expression (Table 32). Genes were ranked according the lowest absolute ratio of signal intensities (mutant versus control mice) in 13 micro array 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 levels $p < 0.01$ and $p < 0.05$.

For example, the selection of the top 20 ranked genes contains eight genes with non-reproducible chip data. The remaining 12 genes with reproducible up- or down-regulation contain nine or more non-differentially expressed genes with the significance level $p < 0.05$, or nine or more non-differentially expressed genes with the significance level $p < 0.01$. The minimal ratios of expression for this selection ranged from 2.85 to 1.13 fold induction/repression.

Table 32: Chip hybridization of liver: evaluation of data				
Ranked Genes (According Lowest of 13 Ratios)	Non-uniform Patterns	NDE (False Positives)		Fold Induction (Minimum of 13 Chips)
		p<0.01	p<0.05	
1 - 20	8	≥ 9	≥ 9	2.85 – 1.13
1 - 40	28	≥ 29	≥ 29	2.85– 1.08
1 - 60	47	≥ 48	≥ 47	2.85– 1.06
1 - 100	87	≥ 88	≥ 87	2.85– 1.04
1 - 150	136	≥ 139	≥ 137	2.85– 1.03
1 - 300	286	≥ 289	≥ 288	2.85– 1.01

Genes that may be of interest for the Dll1 mutant line: for details see *Dll1_liv_Crit1-Folder*. The selected genes are reproducibly up- or down-regulated in all at least 10 of 13 experiments; genes were ranked according to the minimum of (10) ratios (minimum of maximum). Only signals with intensity above 200 were selected.

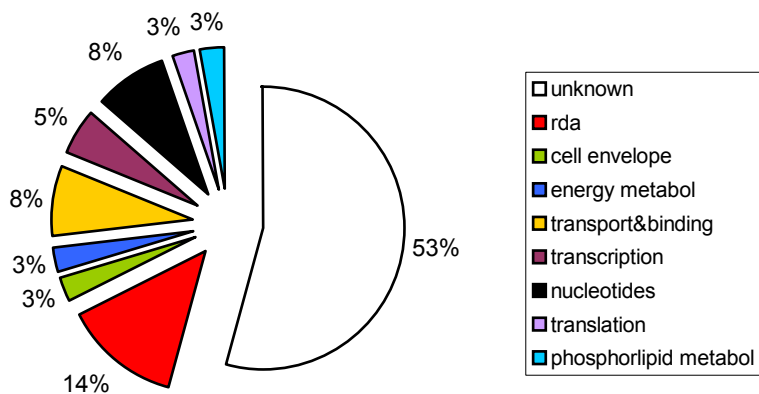


Figure 12: Gene expression in liver in Dll1 mice

Top 37 **up-regulated** genes sorted by functional classes.

Analysis of Gene Expression in Spleen

Table 33 summarizes the results of 16 chip hybridizations performed with RNA from liver. In total, 329 probes show signals in all 16 chip hybridizations.

Table 33: Chip hybridization of spleen: labeling and number of detected spots		
Numbers indicate the ID of mutant mice.		
Chip	Cy5/Cy3	Detected Spots
#20	Wt/5734	1378
#21	Wt/5734	1750
#22	5734/Wt	3435
#23	5734/Wt	1030
#24	Wt/5740	1729
#25	Wt/5740	2482
#26	5740/Wt	1788
#27	5740/Wt	2911
#28	Wt/5729	4623
#29	5729/Wt	2758
#30	Wt/5738	1951
#31	Wt/5738	934
#32	5738/Wt	570
#33	5738/Wt	1422
#34	Wt/5730	3150
#35	5730/Wt	2292
		329 overlap

These genes were evaluated for the significance of differential gene expression (Table 34). Genes were ranked according the lowest absolute ratio of signal intensities (mutant versus control mice) in 13 micro array 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 levels $p < 0.01$ and $p < 0.05$.

For example, the selection of the top 20 ranked genes contains six genes with non-reproducible chip data. The remaining 14 genes with reproducible up- or down-regulation contain eight or more non-differentially expressed genes with the significance level $p < 0.05$, or eight or more non-differentially expressed genes with the significance level $p < 0.01$. The minimal ratios of expression for this selection ranged from 4.45 to 1.83 fold induction/repression.

Table 34: Chip hybridization of spleen: evaluation of data				
Ranked Genes (According Lowest of 16 Ratios)	Non-uniform Patterns	NDE (False Positives)		Fold Induction (Minimum of 16 Chips)
		$p < 0.01$	$p < 0.05$	
1 - 20	6	≥ 8	≥ 8	4.45 – 1.83
1 - 40	6	≥ 8	≥ 8	4.45 – 1.52
1 - 60	8	≥ 10	≥ 10	4.45 – 1.44
1 - 100	20	≥ 23	≥ 22	4.45 – 1.37
1 - 150	35	≥ 38	≥ 37	4.45 – 1.31
1 - 300	95	≥ 99	≥ 99	4.45 – 1.23

Genes that may be of interest for the *Dll1* mutant mouse line: for details see *Dll1_spl_Crit1-Folder*. The selected genes are reproducibly up- or down-regulated in at least 12 of 16 experiments; genes were ranked according to the minimum of (12) ratios (minimum of maximum). Only signals with intensity above 200 were selected.

Inspection of expression data from individual mice revealed non-correlation of the expression patterns in all five samples. There was no correlation of up- or down-regulated genes found between single individuals in addition to those shown in *Dll1_spl_Crit1-Folder*.

Analysis of Gene Expression in Brain

Table 35 summarizes the results of 15 chip hybridizations performed with RNA from brain. Due to a small amount of RNA (see Table 28) no experiments could be performed of individual 5929. In total, 833 probes show signals in all 15 chip hybridization experiments.

Table 35: Chip hybridization of brain: labeling and number of detected spots		
Numbers indicate the ID of mutant mice.		
Chip	Cy5/Cy3	Detected Spots
#36	Wt/5734	2273
#37	Wt/5734	3483
#38	5734/Wt	2457
#39	5734/Wt	6543
#40	Wt/5738	4660
#41	Wt/5738	2035
#42	5738/Wt	2388
#43	5738/Wt	4042
#44	Wt/5740	2925
#45	Wt/5740	2447
#46	5740/Wt	2936
#47	Wt/5730	3011
#48	Wt/5730	2181
#49	5730/Wt	2094
#50	5730/Wt	2593
		833 overlap

These genes were evaluated for the significance of differential gene expression.

Table 36: Chip hybridization of brain: evaluation of data				
Ranked Genes (According Lowest of 15 Ratios)	Non-uniform Patterns	NDE (False Positives)		Fold Induction (Minimum of 15 Chips)
		p<0.01	p<0.05	
1 - 20	20	≥ 20	≥ 19	1.14 – 1.05
1 - 40	40	≥ 40	≥ 19	1.14 – 1.04

Genes were ranked according the lowest absolute ratio of signal intensities (mutant versus control mice) in 15 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 levels $p < 0.01$ and $p < 0.05$.

For example, the selection of the top 20 ranked genes contains 20 genes with non-reproducible chip data. There is no gene with reproducible expression in liver in all four individuals. The minimal ratios of expression for

this selection ranged from 1.14 to 1.05 fold induction/repression. To sum up: There was no differential gene expression in all experiments.

Inspection of expression data from individual mice revealed a stronger correlation of down-regulated genes in samples 5738 and 5740. Expression pattern of sample 5734 and 5730 shows anti-correlation to the other two samples in up-regulated genes. Genes down-regulated in individual 5738 and 5740 show no differential gene expression in sample 5734 and 5730. May be genes down-regulated in individual 5738 and 5740 are of interest for the *Dll1* mutant line: for details see *Dll1_brn_Crit1-Folder*. The selected genes are reproducibly down-regulated in at all 7 experiments of the two samples; genes were ranked according to the minimum of (7) ratios (minimum of maximum). Only signals with intensity above 200 were selected.

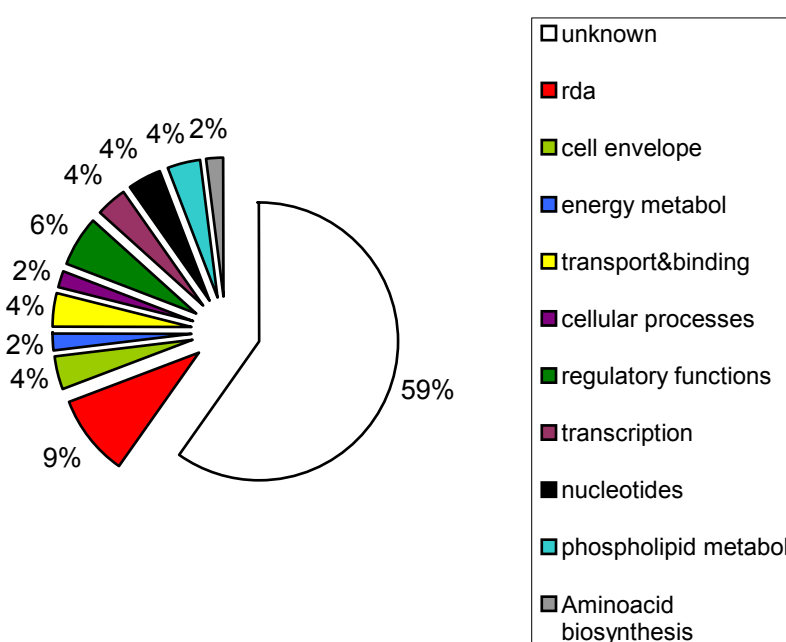


Figure 13: Gene expression in brain *Dll1* mice

Top 52 **down-regulated** genes sorted by functional classes.

3.10.5 Discussion

In **thymus**, we observed only a few genes involved in immune response: *Fth* showed over-expression, whereas *Idh1* and *Ppib* are down-regulated. Another group of genes down-regulated in thymus are involved in **tumorigenesis**: *Mfl2*, *Hnrpu*, *Ddx5* and *Coro1a*. Attendance to **energy metabolism** was found for four up-regulated genes (*Ipl*, *Fabp4*, *Scd1*, and *Sorbs1*). *Adipsin* (*Adn*), a gene with metabolic functions, showed over-expression in thymus. The *Adipsin* proximal promoter contains a putative binding site for the Notch-induced transcriptional regulator *Hes-1* (Searfoss *at al.*, 2003). In these studies, over-expression of *Hes-1* was able to down-regulate *adipsin* expression. So maybe *Adn* is regulated by *Hes-1*. There was no differentially gene expression observed for *Hes-1* in thymus.

The number of detected genes in **spleen** was unusually low. We found only three genes that were differentially expressed. Inspection of expression data from individual mice revealed non-correlation of the expression patterns in all five samples. Also in brain, a stronger correlation of down-regulated genes was found in individual 5738 and 5740. Maybe biological variability in genes oscillation and stress-responsive genes are potential reasons for anti-correlation in the expression patterns between single individuals. In addition, several recent publications have provided evidence for biological variability of expression levels for particular genes (Oishi *et al.*, 2003; Pritchard *et al.*, 2001; Drobyshev *et al.*, 2003a; Drobyshev *et al.*, 2003b; Churchill *et al.*, 2002).

In **liver** up-regulated genes (**Nras**, **Spi1-4** & **Laptm4a**) are involved in tumorigenesis. **Accn1** (cation channel) and **Aplp2** (amyloid beta precursor-like protein2) are up-regulated in liver. For these genes, down-regulation in thymus (Accn1) respectively brain (Aplp2) was observed.

A proposed function of **Aplp2** is the promotion of neurite outgrowth (Adlerz 2003). An accumulation of the amyloid beta protein peptide could be observed in Alzheimer's disease brain (Eggert, 2004).

Kif5c belong to the kinesin family that act mainly as intracellular transport proteins. **Kif5c** is expressed in a variety of developing tissues including neuronal and mesodermal tissues and is involved in the formation of the left-right body axis. **Kif5c** plays a role during different morphogenetic processes (Dathe *et al.*, 2004).

Fto is a candidate gene involved in processes such as programmed cell death, craniofacial development, and establishment of left-right asymmetry (Peters *et al.*, 1999).

Daam1, dishevelled protein 1, are implicated in the WNT-beta-catenin pathway. WNT signals play key roles in embryogenesis through the specification of cell fate and polarity (Katho 2003).

Kif5c, *Fto*, and *Daam1*, all involved in determination of the left-right body axis(!), showed down-regulation in brain.

The **Eph-related receptor tyrosine kinase** has been implicated in intercellular communication during embryonic development. It is known that the carboxyl terminus of Eph-related EphB3 interacts with the PDZ domain of the ras-binding protein AF6. This PDZ domain also interacts with C-terminal sequences derived from other transmembrane receptors like the Notch ligand Jagged (Hock *at al.* 2003). It is also indicated that EphB receptor cell autonomous forward signalling is responsible for dendritic spine formation and synaptic maturation in hippocampal neurons (Henkemeyer, 2000). Down-regulation of *Ephb3* was detected in brain.

Gtl2, down-regulated in brain, encodes for a non-translated RNA on mouse chromosome 12. Delta-like (Dlk) was identified 80kb upstream of Gtl2. *Gtl2* and *Dlk* were found to be co-expressed in the same tissues throughout development (Takada *et al.*, 1998; Wylie *et al.*, 2000).

Cadherins are cell adhesion molecules that play important roles in development of a variety of organs, including the vertebrate limb (Liu 2003). In our gene expression analysis down-regulation for **cadherin11** in brain was observed.

Using the selection criteria described above, we could identify a number of genes that are differentially expressed in thymus, spleen, liver, and brain of *Dll1*-mutant mice. The relevance of these genes should be evaluated in terms of the studied allele. This may be done by a detailed inspection of the functional annotations for each of these genes, as initiated here in the discussion. We would be grateful for any feedback on this and would be glad to support you in this process. Please, contact us if you have questions concerning this analysis.

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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 focused 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.

Mutant mice showed lower body weights than the control mice, but no differences could be measured in food intake. Hence, mutant mice had higher values in energy uptake and ratio of metabolized energy when calculated per unit body weight (statistically significant in females, slightly elevated in males). This indicates a metabolic phenotype, possibly caused by an increased basal metabolic rate.

3.11.2 Mice

Seven adult control males and six adult mutant males entered the Metabolic Screen at the beginning of calendar week 14 in 2004. The females (seven control and seven mutant mice) 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 (F_{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

Mutant mice had significantly lower body weight than the control littermates. Female mutant mice showed higher ratios of food assimilation due to lower energy content of feces and simultaneously lower feces production. The same tendency was observed for the males. During *ad libitum* feeding, body temperature did not differ between the genotypes and both got hypothermic during food restriction, reflecting a metabolic reaction to reduced food offer.

Mutant and control mice showed common sex differences in almost all parameters with higher values in males which are known for many inbred strains (own unpublished data).

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

3.11.6 Discussion

No information about metabolic parameters were available prior to the metabolic screening of the Dll1 mutant mouse line. We found a body weight phenotype characterized by significantly lower values in the mutants. The lower body weight but approximately the same food intake resulted in a higher energy uptake and ratio of metabolized energy per unit body weight. This could be determined only in mutant females, but there was a tendency also for the mutant males.

Higher ratios of metabolized energy and higher values of food assimilation in combination with lower body weight suggest increased levels of daily energy expenditure possibly due to high levels of spontaneous locomotor activity as observed in other screens (Neurology, Behavior) or elevated basal metabolic rate of mutant mice. The later aspect was supported by the de-

crease of body temperature during food restriction. Both findings could be followed up in a secondary screen to exactly evaluate energy metabolism and the factors affecting energy balance.

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 37: Metabolic parameters recorded in the primary screen												
Data are presented as mean ± standard error of mean.												
Parameter	Control (A)					Mutant (B)					A~B	
	<i>ad libitum</i>			food reduction, 7 days to 60%		<i>ad libitum</i>			food reduction, 7 days to 60%			
	Male	Female		Male	Female	Male	Female		Male	Female	Male	Female
	(n=7)	(n=7)	<i>p</i> -value	(n=7)	(n=7)	(n=6)	(n=7)	<i>p</i> -value	(n=6)	(n=7)	<i>p</i> -value	<i>p</i> -value
Body weight [g]	30.5 ± 0.78	23.7 ± 0.51	< 0.001	25.1 ± 0.55	20.8 ± 0.49	27.9 ± 0.59	21.7 ± 0.51	< 0.001	21.9 ± 0.61	18.8 ± 0.54	0.024	0.019
Rectal body temperature [°C]	36.6 ± 0.22	36.5 ± 0.11	n.s.	34.6 ± 0.15	34.1 ± 0.3	36.5 ± 0.07	36.4 ± 0.09	n.s.	33.7 ± 0.35	33.8 ± 0.3	n.s.	n.s.
Food consumption [g day ⁻¹]	3.54 ± 0.18	3.01 ± 0.09	0.021	60% of <i>ad libitum</i>		3.46 ± 0.24	3.05 ± 0.09	n.s.	60% of <i>ad libitum</i>		n.s.	n.s.
Energy uptake [kJ day ⁻¹]	65.2 ± 3.32	55.4 ± 1.64	0.021	39.1 ± 1.99	33.2 ± 0.98	63.8 ± 4.35	56.3 ± 1.66	n.s.	38.3 ± 2.61	33.7 ± 0.99	n.s.	n.s.
Energy uptake BW ⁻¹ [kJ g ⁻¹ day ⁻¹]	2.14 ± 0.08	2.34 ± 0.05	n.s.	1.55 ± 0.06	1.59 ± 0.04	2.28 ± 0.12	2.58 ± 0.04	0.051	1.74 ± 0.08	1.79 ± 0.01	n.s.	0.002
Feces production [g day ⁻¹]	0.76 ± 0.03	0.58 ± 0.02	< 0.001	0.4 ± 0.03	0.33 ± 0.02	0.73 ± 0.04	0.56 ± 0.02	0.002	0.38 ± 0.05	0.33 ± 0.02	n.s.	n.s.
Energy content feces [kJ g ⁻¹]	16.22 ± 0.08	15.84 ± 0.1	0.013	16.02 ± 0.06	15.97 ± 0.12	16.18 ± 0.07	15.69 ± 0.08	0.001	15.97 ± 0.08	15.76 ± 0.09	n.s.	n.s.
Metabolized energy [kJ day ⁻¹]	53.2 ± 2.91	46.5 ± 1.44	n.s.	32.8 ± 1.62	28.1 ± 0.81	52.2 ± 3.7	47.6 ± 1.47	n.s.	32.3 ± 1.88	28.7 ± 0.08	n.s.	n.s.
Metabolized energy [kJ g ⁻¹ day ⁻¹]	1.74 ± 0.08	1.96 ± 0.04	0.026	1.3 ± 0.05	1.35 ± 0.04	1.87 ± 0.1	2.19 ± 0.03	0.002	1.47 ± 0.06	1.53 ± 0.03	n.s.	0.001
Food assimilation coefficient [%]	81.4 ± 0.35	83.8 ± 0.33	< 0.001	83.8 ± 0.49	84.6 ± 0.53	81.7 ± 0.3	84.6 ± 0.34	< 0.001	84.6 ± 0.97	85.1 ± 0.75	n.s.	n.s.

3.12 Pathology Screen

3.12.1 Summary

The Pathology screen performed a complete morphological analysis with standard staining methods. Our analysis in the primary screen did not reveal any *Dll1*-specific morphological phenotype. Therefore, there is not any morphological change that suggests the presence of an Alagille Syndrome.

3.12.2 Mice

A total of 51 mice, 25 heterozygous mutant mice (10 males, 15 females) and 26 control mice (11 males, 15 females) were analyzed. Due to the workflow in the GMC, mice of different ages were received from different screens, and thus at different ages (Table 38).

Table 38: <i>Dll1</i> mice analyzed.						
Origin	Control		Mutant		Number of Animals	Age [days]
	Males	Females	Males	Females		
Lung Screen	0	5	0	5	10	15
Dysmorphology Screen	3	3	3	3	12	20- 21
Metabolic Screen	7	7	7	7	28	20 - 21
Other Screens		0	1	0	1	20
Total Number of Animals	10	15	10	15	51	

3.12.3 Materials and Methods

Mice received in the laboratory of pathology were sacrificed with CO₂. The animals were analyzed macroscopically and weighed. 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, muscle, 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.

3.12.4 Genotype-specific Results

Overview

Table 39: Morphological alterations of <i>Dll1</i>-mutant mice compared to their litter mates.			
Organ:	Alteration	Organ:	Alteration
Skin	No	Pancreas	No
Musculoskeletal system	No	Cervical lymph node	No
Eyes	No	Thymus	No
Brain	No	Spleen	No
Cerebellum	No	Thyroid gland	No
Heart	No	Parathyroid	No
Trachea	No	Adrenal gland	No
Lung	No	Kidneys	No
Teeth	No	Urinary bladder	No
Salivary glands	No	Testes	No
Esophagus	No	Epididymis	No
Stomach	No	Funiculus spermaticus	No
Small intestine	No	Ovaries	No
Large intestine	No	Uterus	No
Liver	No	Vagina	No

No specific changes were identified in the mutant mice when compared to their control littermates.

X-rays analyses

Cadavers of four mutant and four control animals were radiologically examined. However, bone abnormalities could not be confirmed by conventional radiological examination (see also Dysmorphology Screen, 3.2.5).

Body Weight

The *Dll1*-mutant mice coming from the Metabolic Screen (after one week of food restriction), and their control littermates had similar average body weight. The mean body weight in the mutant animals coming from the Dysmorphology Screen was lower compared to that of the controls (Table 41). Because of the small number of animals received, no statistical analysis was performed.

Table 40: Mean body weight \pm standard deviation of Dll1 mice and their control littermates.					
Number of animals in brackets.					
Origin	Control		Mutant		Age [weeks]
	Female	Male	Female	Male	
Lung Screen	20.80 \pm 3.19 (5)	--	21.60 \pm 1.85 (5)	--	15
Dysmorphology Screen	28.67 \pm 3.40 (3)	29.33 \pm 5.79 (3)	23.00 \pm 1.63 (3)	24.67 \pm 1.25 (3)	21
Metabolic Screen	23.86 \pm 1.98 (3)	30.29 \pm 1.67 (3)	22.29 \pm 1.25 (3)	28.57 \pm 1.68 (3)	21

3.12.5 Secondary, Non-genotype-specific Results

Pancreas

Lipomatosis, regarded as deposition of fat cells in pancreas was observed in both controls and mutants animals (Table 40). In one case, we observed total replacement of exocrine lobules by fatty tissue (Fig. 14).

Table 41: Lipomatosis in Dll1 mice.		
	Control	Mutant
Minimal or moderate lipomatosis	11	8
Severe lipomatosis	0	1 female
Number of mice with fat cells depot in pancreas	11 of 25 (7 females, 4 males)	8 of 25 (4 females, 4 males)

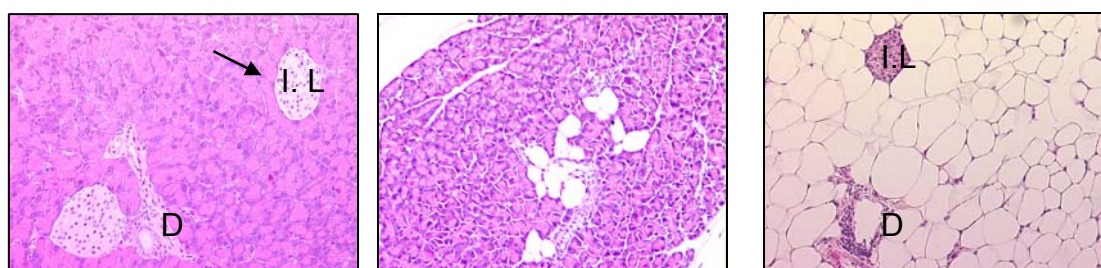


Figure 14: Histology of pancreas

The left panel shows the normal pancreas. The right panel demonstrates the total replacement of exocrine lobules by fat. Note that only the islets of Langerhans (I.L) and the exocrine ducts (D) were spared. The central panel shows mild fat deposit in the pancreas. H&E 160x

3.12.6 Discussion

According to the preliminary information received from the provider, the Delta/Notch signal transduction pathway is associated with Alagille and CADASIL syndrome (JAG1, OMIM 601920; NOTCH3, OMIM 600276, respectively). The first is characterized by cardiac defects, chronic liver disease, skeletal defects, and exocrine pancreatic insufficiency (Alagille *et al.*, 1975; Chong *et al.*, 1989). CADASIL leads to strokes and dementia at an early age. In addition, the Notch signaling pathway controls pancreatic cell differentiation (Apelquist *et al.*, 1999) and lymphocyte development (Hozumi *et al.*, 2004).

We did not observe any genotype-specific changes in the liver, heart, and other organs, which are frequently affected in the Alagille syndrome. We observed minimal to moderate pancreas lipomatosis (both controls and affected littermates). In our experience, this is a relative frequent finding in mice, and does not convey clinical symptoms or impaired pancreatic function. Only the severe lipomatosis present in one mutant female mouse has been associated with pancreatic insufficiency (Olsen, 1978). The relevance of this change and its association with a phenotype has to be determined.

In the *Dll1* mutant, mild disturbances in the cervical region of the vertebral column were identified in a previous screen by the provider. In order to corroborate this finding, we analyzed a group of affected animals radiologically and compared them to their control littermates. However, we could not confirm the skeletal malformations previously observed. The mutant mice coming from Metabolic Screen (after one week food restriction), had a similar average body weight as their control littermates. There is no correlation between high body weight and the lipomatosis of pancreas.

In conclusion, our analysis in the primary screen did not reveal any *Dll1*-specific morphological phenotype.

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