

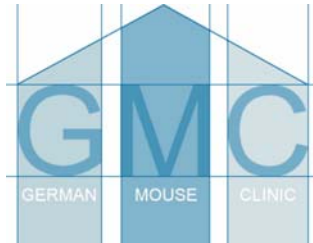
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# GERMAN MOUSE CLINIC

## Report for C57BL/6J-Ptdsr<sup>tm1.1</sup> Gbf

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

# The German Mouse Clinic



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

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

## Director

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



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# 1 Summary

## 1.1 Primary Screening

In a primary screen, sixty animals of the C57BL/6J-*Ptdsr*<sup>tm1.1 Gbf</sup> (*Ptdsr*) mutant mouse line (30 heterozygous mutants, 30 wild-type control littermates) have been analyzed in the German Mouse Clinic (GMC) in the modules Behavior, Dymorphology, Bone and Cartilage, Neurology, Eye, Clinical Chemistry, Immunology, Allergy, Nociception, Lung Function, Metabolism, Expression Profiling, and Pathology.

### 1.1.1 Overall Assessment of the Results

The genotype-specific differences detected in the screens listed below are subtle, and more pronounced in male mutants. One possibility is that the remaining amount of protein from the expression of the wild-type allele is sufficient to allow for maintenance of the pathways affected or compensatory mechanisms are involved.

### 1.1.2 Results by Screen

**Behavior Screen:** The behavioral observation in the modified Hole Board (mHB) test demonstrated an effect of the *Ptdsr* mutation on activity-related parameters of anxiety behavior, which was more pronounced in male mutants. Additionally, differences in exploratory behavior were detected.

**Immunology Screen:** The immunological investigations revealed some minor, but statistically significant differences in the frequencies of B1 B (CD19+CD5+) cells and  $\gamma/\delta$ T cells, which tended to be higher in male mutant mice. In addition, the level of IgG<sub>2b</sub> was higher in these mice.

**Expression Profiling:** The data analysis and various statistical methods detected differential gene expression between wild-type control and mutant tissues only in thymus. Expression of *Ptdsr* was detected in all organs but without regulation.

In the screens **Neurology, Dymorphology, Eye, Allergy, Nociception, Lung Function, Clinical Chemistry, Energy Metabolism, and Pathology**, no genotype-specific differences could be found.

## 1.2 Recommendations for Secondary Screening

Secondary screening is suggested from the **Behavior Screen**. To clarify the potential anxiety-related phenotype seen in the mHB, a new batch of mutants and littermate controls of both sexes would need to be analyzed for anxiety in more detail (Elevated plus maze, Light dark box, Open Field).

We would be happy also to support you in the evaluation of the relevance of genes identified in the **Molecular Phenotyping Screen**.

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

## 2 General Part

### 2.1 The Role of the Gene

The phosphatidylserine receptor (*Ptdsr*) on phagocytosis has been implicated in the recognition and engulfment of apoptotic cells and in anti-inflammatory signaling. Böse and coworkers (2004) demonstrated that *Ptdsr* in mice is essential for the development and differentiation of multiple organs during embryogenesis but not for apoptotic cell removal.

### 2.2 Known Phenotypes

*Ptdsr* heterozygous mutant animals display eye and coat color abnormalities of variable expressivity and penetrance whereas homozygous mutant animals show perinatal lethality due to the severe cardiopulmonary malformations (ventricular septal defects (VSD), double-outlet right ventricle (DORV), hypoplasia of the lung aorta; Schneider *et al.*, 2004). In-depth analysis of homozygous mutant embryos revealed (i) severe malformations of different organs (brain, eye, heart) and (ii) a significant delay in organ differentiation (lung, intestine, kidney, liver; Böse *et al.*, 2004).

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

### 2.3 Expected Phenotypes

Based on preliminary studies (Böse *et al.*, 2004; Schneider *et al.*, 2004) any phenotype related to defects in neural crest cell differentiation or migration is imaginable. Especially coat color abnormalities, cardiopulmonary dysfunction, impaired thymus differentiation or function (T-cell deficiency) or defects in craniofacial branchial arch derivatives (skeletal defects, hypothyroidism or hypoparathyroidism) can be expected. Furthermore, behavioural or neuropathological abnormalities cannot be excluded due to the high level and widespread expression of *Ptdsr* in the brain and neural tube during embryonic development.

### 2.4 Suggested Human Disease Model

So far, no human disease has been addressed to a mutation in the human phosphatidylserine receptor gene (OMIM [\\*604914](#)). It is suggested by the provider that the C57BL/6J-*Ptdsr*<sup>tm1.1 Gbf</sup> (*Ptdsr*) mutant mouse line could serve as an animal model to study congenital heart disease (CHD, especially Tetralogy of Fallot (OMIM [#187500](#), e.g. diGeorge Syndrome) and progressive ocular aberration.

## 2.5 Mice

### 2.5.1 Number and kind of mice

As described by the owner, a *Ptdsr* null allele was generated by gene targeting in Bruce4-ES cells (C57BL/6) and backcrossing of chimeric mice with C57BL/6J mice to maintain the mutation on an isogenic background.

Table 1: <i>Ptdsr</i> mice provided for analysis.		
Genotype / Sex	Number of Animals	
Mutant female	15	
Mutant male	15	
Control female	15	1 died
Control male	15	2 died

The heterozygous mutant mice and wild-type control littermates arrived in week 34 in 2004.

### 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).

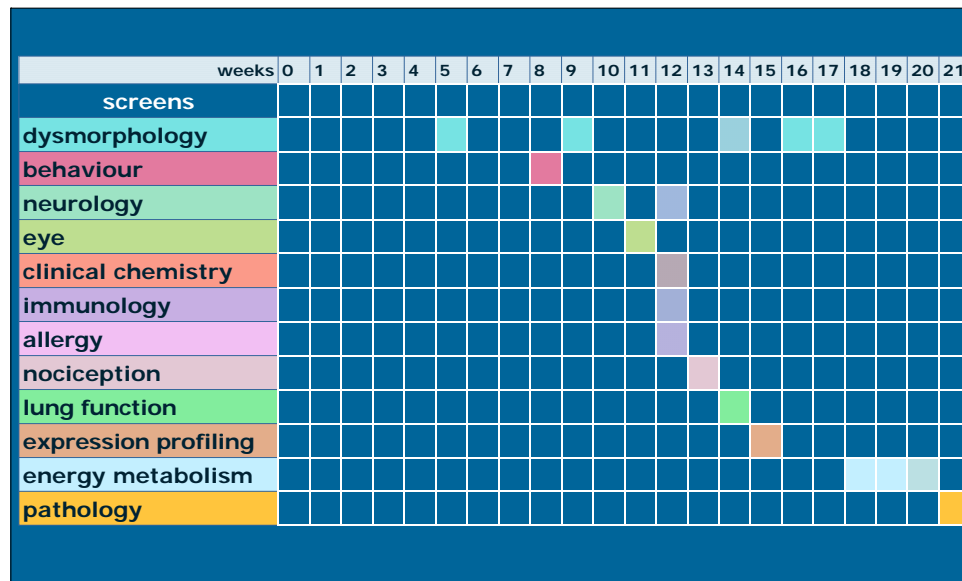
Outbred 8-week-old male SPF Swiss mice are used as sentinels and kept on a mixture of new bedding and aliquots of soiled bedding (50:50) from all cages of the IVC rack. In addition, the sentinels were also exposed to soiled air from all “upstream” cages of the IVC rack. Health monitoring is carried out by on-site examination of the sentinel mice by certified laboratories according to FELASA recommendations ([www.felasa.org](http://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 Dysmorphology 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 Ptdsr 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. Three animals died after blood withdrawal and thus could not be analyzed for all parameters (Table 1).

### **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.

## **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.

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### Abbreviations and Wording

Ptdsr	Phosphatidylserine (PS) receptor and C57BL/6J-Ptdsr <sup>tm1.1 Gbf</sup> mutant mouse line
<i>Ptdsr</i>	gene coding for phosphatidylserine receptor
GMC	German Mouse Clinic
IVC	individually ventilated cage
mutant	<i>Ptdsr</i> <sup>+/-</sup> , heterozygous mutant
control	<i>Ptdsr</i> <sup>+/+</sup> , homozygous wild-type control
FELASA	Federation of European Laboratory Animal Science Associations, 25 Shaftesbury Avenue, London W1D 7EG, UK, <a href="http://www.felasa.org">www.felasa.org</a>

**Table 2: Primary Screen at GMC**

<b>Screens</b>	<b>Goal</b>	<b>Methods</b>
<b>Dysmorphology, Bone and Cartilage</b>	morphological analysis of body, skeleton, bone and cartilage	morphological observation, bone densitometry, X-ray, AVL analyzer, micro-computer tomography
<b>Behavior</b>	locomotor, exploratory, emotional and social behavior, object recognition memory	modified hole board
<b>Neurology</b>	assessment of muscle, spinocerebellar, sensory, and autonomic function	modified SHIRPA protocol
<b>Eye</b>	assessment of morphological and functional alterations of the eye	electroretinography, slit lamp biomicroscopy
<b>Clinical Chemistry</b>	determination of clinical-chemical and hematological parameters in blood	blood autoanalyzer, ABC-animal blood counter
<b>Immunology</b>	analysis of peripheral blood samples for immunological parameters	flow cytometry, bead array, ELISA
<b>Allergy</b>	analysis of total plasma IgE	ELISA
<b>Nociception</b>	detection of altered pain response	hot plate assay
<b>Lung function</b>	assessment of alterations in breathing patterns	whole body plethysmography (Buxco®)
<b>Expression Profiling</b>	RNA expression profiling	DNA-chip technology
<b>Energy Metabolism</b>	measurement of altered body weight regulation, body temperature and energy balance	bomb calorimetry
<b>Pathology</b>	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 (mHB) test as primary screen in the behavioral phenotyping module of the GMC. This test allows the comprehensive analysis of a range of parameters known to be indicative of behavioral dimensions such as locomotor activity, exploratory behavior, arousal, emotionality, memory and social affinity in a single short test (See Ohi *et al.*, 2001).

We observed an effect of the *Ptdsr* mutation on activity-related alteration in anxiety behavior in mutants in the mHB, which was more pronounced in males. Additionally, slight differences in exploration pattern were detectable.

### 3.1.2 Mice

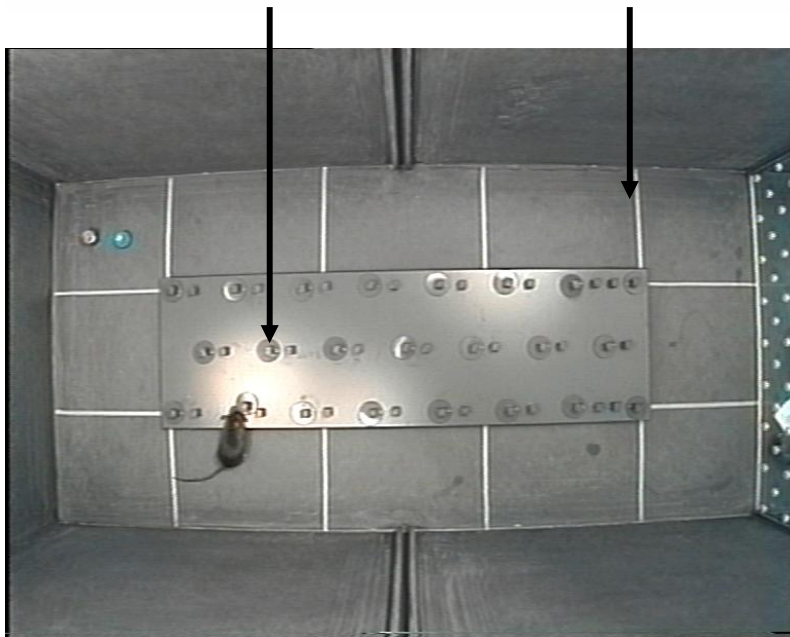
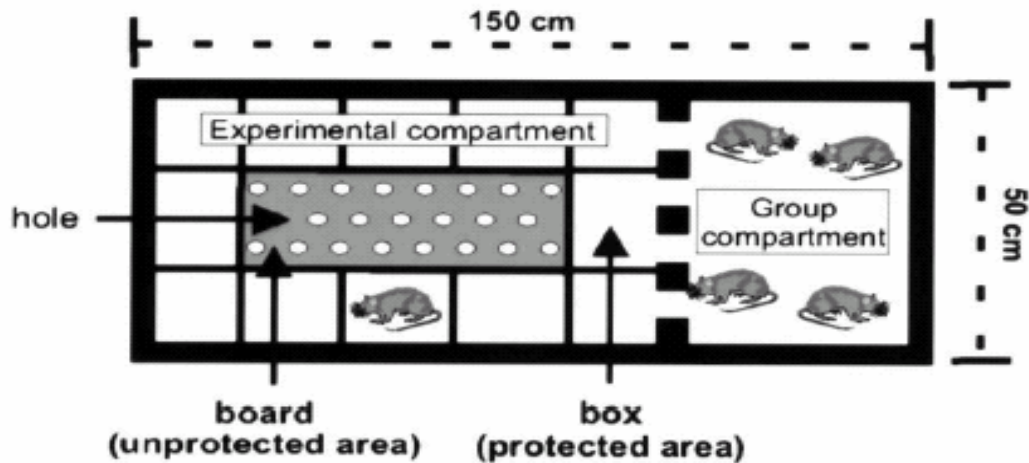
Mice were housed with food and water *ad libitum* under standard laboratory conditions. Animals were separated based on sex, but not genotype. They entered the laboratory at the age of six weeks, were given two weeks for acclimatization and were tested at the age of eight weeks. Three days before testing, 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 mutants) and 30 male mice (15 controls, 15 mutants) were available for analysis.

### 3.1.3 Material and Methods

**The modified hole board test** was carried out according to the procedures described by Ohi *et al.*, 2001. The test apparatus consisted of a test arena (100 x 50 cm), in the middle of which a board (60 x 20 x 2 cm) with 23 holes (1.5 x 0.5 cm) staggered in three lines with all holes covered by movable lids was placed, thus representing the central area of the test arena as an open field. The area around the board was divided into 12 similarly sized quadrants

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



**Figure 2: Test arena for modified Hole Board test.**

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 Ob-

server 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 an activity-related alteration in anxiety behavior in mutant males. Mutant males entered the board earlier and more often (board entry latency and frequency, Table 4) as compared to control males without spending more time on board (board entry total duration, Table 4). Mutant females showed a *tendency* towards more board entries (Table 4), but the reduction in latency to board entry was not significant (Table 4) in comparison to control females. In both sexes of mutant mice, the locomotion related parameters (line crossing, Table 4; total distance travelled, mean and maximum velocity, Table 5) remained unchanged.

Concerning exploration, mutant males did explore the board more often (hole exploration frequency, Table 4) and started undirected exploration earlier (rearing in the box latency, Table 4). Regarding the path shape, mutant females did more changes in direction of movement between two samples as indicated by enhanced numbers of turns (Table 5).

There were no genotype effects in all other observed parameters (Table 3).

### 3.1.5 Discussion

The behavioral observation in the modified Hole Board demonstrated an effect of the *Ptdsr* mutation on activity-related alteration in anxiety behavior that was more pronounced in mutant males. This interpretation of the data is based on the fact that even though mutants of both sexes did earlier and more board entries without resting longer on board, this observation was significant in mutant males and a tendency only in mutant females. However, general locomotor activity was unaltered in mutants of both sexes, excluding a locomotor phenotype.

Concerning exploratory behavior, the elevated horizontal exploration in mutant males (hole exploration) is most likely a consequence of their increased board entries. Whether changes in vertical exploration (rearing latency) in mutant males reflect general changes in exploratory behavior remains unclear, especially as other exploration related parameters were unchanged (rearing frequency, parameters of object exploration, exploration at the partition). As also mutant females displayed a slightly different exploration pattern than control females as they did more turns, an exploratory phenotype can not be excluded. At present, it is unclear whether the observed alterations

in anxiety-related parameters and in exploratory pattern are related or independent of each other.

<b>Table 3: Evaluation of the behavioral phenotype</b>	
Behaviors which are considered as affected in mutants due to the pattern of significantly altered parameters are marked in red.	
<b>Behavior</b>	<b>Measured parameters</b>
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 (number), object exploration; Undirected: Rearings (latency), 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.

Taken together, these results indicate less anxious and/or altered exploratory behavior in *Ptdsr*-mutants. To clarify the potential anxiety-related phenotype seen in the mHB, a new batch of mutants and littermate controls of both sexes would need to be analyzed for anxiety in more detail (Elevated plus maze, Light dark box, Open Field).

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**Table 4: Results of behavioral observation in the modified Hole Board test**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=15)	<i>p - value</i>	(n=15)	(n=15)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
Line crossing [frequency]	121 $\pm$ 4.12	119.93 $\pm$ 8.45	N.A.	123 $\pm$ 5.52	131.87 $\pm$ 6.85	N.A.	n.s.	n.s.
Line crossing [latency]	0.69 $\pm$ 0.06	0.85 $\pm$ 0.12	N.A.	0.66 $\pm$ 0.06	0.65 $\pm$ 0.09	N.A.	n.s.	n.s.
Rearings in box [frequency]	27.93 $\pm$ 2.12	21.87 $\pm$ 1.53	N.A.	29.87 $\pm$ 1.76	25.07 $\pm$ 1.47	N.A.	n.s.	n.s.
Rearings in box [latency]	29.27 $\pm$ 2.97	25.98 $\pm$ 3.28	N.A.	23.98 $\pm$ 1.81	31.75 $\pm$ 3.72	N.A.	<b>p&lt;0.05</b>	n.s.
Hole exploration [frequency]	20.33 $\pm$ 2.29	20.13 $\pm$ 2.2	N.A.	27.53 $\pm$ 2.12	20.4 $\pm$ 1.81	N.A.	<b>p&lt;0.05</b>	n.s.
Hole exploration [latency]	33.5 $\pm$ 4.74	41.35 $\pm$ 9.11	N.A.	23.1 $\pm$ 3.64	23.85 $\pm$ 3.64	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]	5.6 $\pm$ 0.87	5.8 $\pm$ 0.94	N.A.	8.53 $\pm$ 0.92	8.67 $\pm$ 1.2	N.A.	<b>p&lt;0.05</b>	n.s.
Board entry [latency]	102.37 $\pm$ 24.48	106.75 $\pm$ 20.56	N.A.	60.95 $\pm$ 16.19	75.18 $\pm$ 19.21	N.A.	<b>p=0.05</b>	n.s.
Board entry [total duration %]	7.01 $\pm$ 1.32	9.48 $\pm$ 1.76	N.A.	9.32 $\pm$ 1	8.89 $\pm$ 1.35	N.A.	n.s.	n.s.
Rearing on board [frequency]	0.27 $\pm$ 0.15	0.47 $\pm$ 0.29	N.A.	0.8 $\pm$ 0.24	0.53 $\pm$ 0.29	N.A.	n.s.	n.s.

Rearing on board [latency]	281.57 ± 11.82	275.27 ± 15.64	N.A.	260.45 ± 12.45	280.81 ± 11.17	N.A.	n.s.	n.s.
Risk assessment [frequency]	0 ± 0	0 ± 0	N.A.	0 ± 0	0.07 ± 0.07	N.A.	n.s.	n.s.
Risk assessment [latency]	300 ± 0	300 ± 0	N.A.	300 ± 0	282.25 ± 17.75	N.A.	n.s.	n.s.
Group contact [frequency]	11.67 ± 0.66	10.87 ± 0.75	N.A.	10.6 ± 0.67	12.33 ± 0.74	N.A.	n.s.	n.s.
Group contact [latency]	15.48 ± 3.38	12.55 ± 1.94	N.A.	13.53 ± 4.64	12.71 ± 1.53	N.A.	n.s.	n.s.
Group contact [total duration %]	20.65 ± 1.55	19.38 ± 1.84	N.A.	18.16 ± 1.48	20.48 ± 1.57	N.A.	n.s.	n.s.
Grooming [frequency]	1 ± 0.28	0.87 ± 0.24	N.A.	1.07 ± 0.25	1 ± 0.31	N.A.	n.s.	n.s.
Grooming [latency]	243.47 ± 15.97	244.03 ± 14.91	N.A.	221.51 ± 19.35	256.97 ± 13.71	N.A.	n.s.	n.s.
Grooming [total duration %]	1.35 ± 0.4	1.22 ± 0.37	N.A.	1.63 ± 0.43	1.28 ± 0.48	N.A.	n.s.	n.s.
Defecation [frequency]	0.27 ± 0.15	0 ± 0	N.A.	0.13 ± 0.09	0 ± 0	N.A.	n.s.	n.s.
Defecation [latency]	273.01 ± 16.01	300 ± 0	N.A.	281.46 ± 13.29	300 ± 0	N.A.	n.s.	n.s.
Unfamiliar object exploration [frequency]	6.53 ± 0.58	5.73 ± 0.44	N.A.	6.8 ± 0.71	5.33 ± 0.42	N.A.	n.s.	n.s.
Familiar object exploration [frequency]	7.67 ± 0.85	7.07 ± 0.94	N.A.	7.6 ± 0.65	6.47 ± 0.62	N.A.	n.s.	n.s.
Unfamiliar object exploration [latency]	35.87 ± 9.09	32.71 ± 7.66	N.A.	24.15 ± 7.59	35.24 ± 7.99	N.A.	n.s.	n.s.
Familiar object exploration [latency]	20.61 ± 6.01	28.38 ± 7.88	N.A.	28 ± 7.91	26.73 ± 5.44	N.A.	n.s.	n.s.

<b>Unfamiliar object exploration [total duration %]</b>	1.98 ± 0.18	1.65 ± 0.26	N.A.	2.3 ± 0.66	1.89 ± 0.25	N.A.	n.s.	n.s.
<b>Familiar object exploration [total duration %]</b>	1.4 ± 0.15	1.32 ± 0.17	N.A.	1.34 ± 0.14	1.22 ± 0.11	N.A.	n.s.	n.s.
<b>Object Index</b>	0.17 ± 0.06	0.09 ± 0.07	N.A.	0.14 ± 0.07	0.19 ± 0.06	N.A.	n.s.	n.s.

**Table 5: Video-tracking results regarding locomotor behavior**Data are presented as mean  $\pm$  standard error of mean.

Parameter	Control (A)			Mutant (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	3075.17 $\pm$ 88.42	3083.22 $\pm$ 157.85	N.A.	3070.74 $\pm$ 143.09	3455.32 $\pm$ 142.9	N.A.	n.s.	n.s.
Total Distance Moved [cm]	19.51 $\pm$ 0.48	19.4 $\pm$ 0.73	N.A.	19.11 $\pm$ 0.8	20.67 $\pm$ 0.63	N.A.	n.s.	n.s.
Mean Velocity [cm/sec]	57.16 $\pm$ 1.96	55.23 $\pm$ 2.3	N.A.	58.79 $\pm$ 2.83	59.06 $\pm$ 1.68	N.A.	n.s.	n.s.
Maximum velocity [cm/sec]	1636.27 $\pm$ 30.7	1640.86 $\pm$ 46.5	N.A.	1608.22 $\pm$ 68.48	1773.33 $\pm$ 39.04	N.A.	n.s.	p<0.05
Turns [Frequency]	22.39 $\pm$ 0.57	21.48 $\pm$ 0.46	N.A.	21.74 $\pm$ 0.86	20.44 $\pm$ 0.36	N.A.	n.s.	n.s.
Mean Turn Angle [degrees]	143.63 $\pm$ 2.15	137.07 $\pm$ 2.27	N.A.	139.6 $\pm$ 5.51	136.43 $\pm$ 2.18	N.A.	n.s.	n.s.
Angular Velocity [degrees/sec.]	15.93 $\pm$ 0.46	15.21 $\pm$ 0.42	N.A.	15.37 $\pm$ 0.63	14.26 $\pm$ 0.33	N.A.	n.s.	n.s.
Absolute Meander [degrees/sec.]	7.06 $\pm$ 0.7	10.62 $\pm$ 2.1	N.A.	7.5 $\pm$ 0.67	8.68 $\pm$ 1.34	N.A.	n.s.	n.s.
Distance to Wall [cm]	6.77 $\pm$ 0.22	7.1 $\pm$ 0.28	N.A.	6.66 $\pm$ 0.3	7.02 $\pm$ 0.28	N.A.	n.s.	n.s.
Distance to Board [cm]	8.76 $\pm$ 0.15	8.59 $\pm$ 0.16	N.A.	8.52 $\pm$ 0.34	8.72 $\pm$ 0.17	N.A.	n.s.	n.s.

## 3.2 Dymorphology, Bone and Cartilage

### 3.2.1 Summary

In the Dymorphology, 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 dymorphology 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](http://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 *Ptdsr* 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 few minor phenotypes were found, which were present in both *Ptdsr*-heterozygous mutant mice and wild-type control littermates. In the bone mineral density analysis no significant differences could be detected.

### 3.2.2 Mice

Thirty male (15 controls, 15 mutants) and 30 female (15 controls, 15 mutants) mice were analyzed by morphological inspection at the age of 9 weeks. Blood was taken at the age of 14 weeks for determination of ionic calcium from 18 mutant and 20 control animals, and 16-week-old mutants (20 animals) and controls (20 animals) entered the bone density and X-ray analysis.

### 3.2.3 Material and Methods

The Dymorphology, 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 Ptdsr 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 few minor phenotypes were found, which were present in both mutant mice and wild-type littermate controls (Tables 7 and 8). In the bone densitometry using DEXA analysis (Table 9), most values measured were in the range of our C57BL/6 baseline data (data not shown). The sex differences we observed are common in many mouse strains, and thus are not abnormal.

Raw data will be available on demand.

## **3.2.5 References**

Fuchs H, Schughart K, Wolf E, Balling R and Hrabé de Angelis M. (2000): Screening for dysmorphological abnormalities - a powerful tool to isolate new mouse mutants. *Mammalian Genome* 11(7): 528-30.

Hrabé de Angelis, M., H. Flaswinkel, H. Fuchs, B. Rathkolb, D. Soewarto, S. Marschall, S. Heffner, W. Pargent, K. Wuensch, M. Jung, A. Reis, T. Richter, F. Alessandrini, T. Jakob, E. Fuchs, H. Kolb, E. Kremmer, K. Schaeble, B. Rollinski, A. Roscher, C. Peters, T. Meitinger, T. Strom, T. Steckler, F. Holsboer, T. Klopstock, F. Gekeler, C. Schindewolf, T. Jung, K. Avraham, H. Behrendt, J. Ring, A. Zimmer, K. Schughart, K. Pfeffer, E. Wolf and R. Balling (2000): Genome-wide, large-scale production of mutant mice by ENU mutagenesis. *Nature Genetics* 25: 444 – 447

## **Abbreviations**

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

<b>Table 6: Results from clickbox test (hearing test)</b>				
<b>Phenotype</b>	<b>Male</b>		<b>Female</b>	
	<b>Control</b>	<b>Mutant</b>	<b>Control</b>	<b>Mutant</b>
0	-	-	-	-
1	-	-	-	-
2	4	2	1	1
3	11	13	13	11
4	-	-	1	3
<b>Mean Score</b>	<b>2.73</b>	<b>2.87</b>	<b>3.00</b>	<b>3.13</b>
Kruskal-Wallis Anova on Ranks: n.s.				

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

<b>Table 7: Results from the morphological inspection</b>				
<b>Parameter</b>	<b>Male</b>		<b>Female</b>	
	<b>Control</b>	<b>Mutant</b>	<b>Control</b>	<b>Mutant</b>
<b>Growth</b>				
normal	15	15	15	15
<b>Weight</b>				
normal	15	15	15	15
<b>Body size</b>				
normal	15	15	15	15
<b>Eye</b>				
normal	15	15	14	14
eye missing	-	-	1	1
<b>Coat hair growth</b>				
normal	15	15	13	11
hairless parts (esp. neck)	-	-	-	4
scanty parts (esp. croup)	-	-	2	-
<b>Coat hair texture</b>				
normal	15	15	15	15
<b>Coat color</b>				
agouti	15	15	15	15
<b>Hair follicle structure / orientation</b>				
normal	15	15	15	15
<b>Skin pigmentation</b>				
normal	15	13	6	3
dark skin (slight)	-	-	9	12

<b>Skin texture / condition</b>				
normal	15	15	15	15
<b>Vibrissae</b>				
normal	15	15	15	15
<b>Limbs</b>				
normal	15	15	15	15
<b>Digits</b>				
normal	14	13	15	15
stunted toe	1	2	-	-
<b>Tail</b>				
normal	15	15	15	15
<b>Teeth</b>				
normal	15	15	14	15
long	-	-	1	-
<b>Ear morphology</b>				
normal	15	15	15	15
<b>Musculature</b>				
normal	15	15	15	15
<b>Seizures / epilepsy</b>				
no	15	15	15	15
<b>Motor capabilities / coordination</b>				
normal	15	14	15	15
<b>Movement</b>				
normal	15	15	15	15
<b>Feeding / drinking behavior</b>				
normal	15	15	15	15
<b>Respiratory system</b>				
normal	15	15	15	15
<b>Reproductive system</b>				
normal	15	15	15	15
<b>Other abnormalities</b>				
no	15	15	15	15
<b>Animals analyzed</b>	<b>15</b>	<b>15</b>	<b>15</b>	<b>15</b>

<b>Table 8: Results from the X-ray analysis</b>				
<b>Parameter</b>	<b>Male</b>		<b>Female</b>	
	<b>Control</b>	<b>Mutant</b>	<b>Control</b>	<b>Mutant</b>
<b>Skull shape</b>				
normal	10	10	10	9
nose askew	-	-	-	1
<b>Mandibles</b>				
normal	10	10	10	10
<b>Maxilla</b>				
normal	10	10	10	10
<b>Teeth</b>				
normal	10	10	10	10
<b>Orbit</b>				
normal	10	10	10	10
<b>Number of cervical vertebrae</b>				
normal	10	10	10	10
<b>Number of thoracic vertebrae</b>				
normal	10	10	10	10
<b>Number of lumbar vertebrae</b>				
normal	10	10	10	10
<b>Number of pelvic vertebrae</b>				
normal	10	10	10	10
<b>Number of sacral vertebrae</b>				
normal	10	10	10	10
<b>Vertebrae shape</b>				
normal	10	9	9	10
knob in tail	-	1	1	-
<b>Number of ribs</b>				
normal	10	10	10	10
<b>Rib shape</b>				
normal	10	10	10	10
<b>Scapulas</b>				
normal	10	10	10	10
<b>Clavicle</b>				
normal	10	10	10	10
<b>Pelvis</b>				
normal	10	10	10	10
<b>Femur shape</b>				
normal	10	10	10	10
<b>Tibia</b>				
normal	10	10	10	10
<b>Fibula</b>				
normal	10	10	10	10

<b>Humerus</b>				
normal	10	10	10	10
<b>Ulna</b>				
normal	10	10	10	10
<b>Radius</b>				
normal	10	10	10	10
<b>Number of digits</b>				
normal	10	10	10	10
<b>Completeness of digits</b>				
yes	10	10	10	10
<b>Joints</b>				
normal	10	10	10	10
<b>Body fat</b>				
normal	10	10	10	10
<b>Growth</b>				
normal	10	10	10	10
<b><i>Animals analyzed</i></b>	<b>10</b>	<b>10</b>	<b>10</b>	<b>10</b>

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

Parameter	Control (A)		Mutant (B)		A~B Male	A~B Female	ANOVA		
	Male	Female	Male	Female	<i>p</i> – value	<i>p</i> – value	<i>geno</i> - <i>type</i>	<i>sex</i>	<i>interac</i> - <i>tion</i>
	(n=10)	(n=10)	(n=10)	(n=10)					
<b>BMD</b> [mg/cm <sup>2</sup> ]	63 $\pm$ 2	64 $\pm$ 3	66 $\pm$ 3	66 $\pm$ 2	n.s.	n.s.	n.s.	n.s.	n.s.
<b>pBMD</b> [mg/cm <sup>2</sup> ]	52 $\pm$ 2	52 $\pm$ 2	54 $\pm$ 2	53 $\pm$ 2	n.s.	n.s.	n.s.	n.s.	n.s.
<b>sBMD</b> [10 <sup>-3</sup> x cm <sup>-2</sup> ]	1.96 $\pm$ 0.01	2.70 $\pm$ 0.01	2.15 $\pm$ 0.01	2.78 $\pm$ 0.01	n.s.	n.s.	n.s.	< 0.0001	n.s.
<b>BMC</b> [mg]	699 $\pm$ 61	552 $\pm$ 47	548 $\pm$ 49	510 $\pm$ 53	n.s.	n.s.	n.s.	n.s.	n.s.
<b>Body Length</b> [cm]	10.15 $\pm$ 0.11	9.80 $\pm$ 0.08	10.15 $\pm$ 0.08	9.80 $\pm$ 0.08	n.s.	n.s.	n.s.	< 0.001	n.s.
<b>Body Weight</b> [g]	32.54 $\pm$ 1.03	23.68 $\pm$ 0.68	30.97 $\pm$ 0.77	24.09 $\pm$ 0.88	n.s.	n.s.	n.s.	< 0.0001	n.s.
<b>Lean mass</b> [units]	20.98 $\pm$ 0.82	16.43 $\pm$ 0.55	22.84 $\pm$ 0.92	17.29 $\pm$ 0.67	n.s.	n.s.	n.s.	< 0.0001	n.s.
<b>Fat mass</b> [units]	7.95 $\pm$ 1.25	4.36 $\pm$ 1.00	4.86 $\pm$ 0.85	3.79 $\pm$ 1.20	n.s.	n.s.	n.s.	< 0.05	n.s.
<b>Bone Content</b> [%]	2.13 $\pm$ 0.14	2.31 $\pm$ 0.15	1.76 $\pm$ 0.14	2.09 $\pm$ 0.16	n.s.	n.s.	n.s.	n.s.	n.s.
<b>Lean Content</b> [units x 100/g]	65.06 $\pm$ 3.18	70.24 $\pm$ 3.83	73.90 $\pm$ 2.92	72.87 $\pm$ 4.12	n.s.	n.s.	n.s.	n.s.	n.s.
<b>Fat Content</b> [units x 100/g]	23.81 $\pm$ 3.11	17.59 $\pm$ 3.63	15.52 $\pm$ 2.68	14.62 $\pm$ 4.04	n.s.	n.s.	n.s.	n.s.	n.s.
<b>Femur span<sup>1</sup></b> [mm]	1.30 $\pm$ 0.04	1.25 $\pm$ 0.02	1.31 $\pm$ 0.04	1.22 $\pm$ 0.04	n.s.	n.s.	n.s.	< 0.05	n.s.
<b>Subcutaneous fat<sup>1</sup></b> [mm]	4.07 $\pm$ 0.1	4.43 $\pm$ 0.1	3.80 $\pm$ 0.1	4.35 $\pm$ 0.1	n.s.	n.s.	n.s.	< 0.01	n.s.
<b>Vertebrae hight<sup>2</sup></b> [mm]	3.20 $\pm$ 0.06	2.98 $\pm$ 0.05	3.11 $\pm$ 0.04	2.88 $\pm$ 0.05	n.s.	n.s.	n.s.	< 0.0001	n.s.
	Male	Female	Male	Female	A~B Male	A~B Female	ANOVA		
	(n=10)	(n=10)	(n=8)	(n=10)	<i>p</i> – value	<i>p</i> – value	<i>geno</i> - <i>type</i>	<i>sex</i>	<i>interac</i> - <i>tion</i>
<b>Ionized Calcium</b> [mmol/l]	1.22 $\pm$ 0.02	1.08 $\pm$ 0.03	1.21 $\pm$ 0.03	1.09 $\pm$ 0.05	n.s.	n.s.	n.s.	< 0.001	n.s.

1: mean value of the two hind limbs  
2: third lumbar vertebra

## 3.3 Neurology Screen

### 3.3.1 Summary

In the primary neurological screen 30 heterozygous *Ptdsr*-mutant 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 23 test parameters (See web page: [http://www.mgu.har.mrc.ac.uk/facilities/mutagenesis/mutabase/shirpa\\_summary.html](http://www.mgu.har.mrc.ac.uk/facilities/mutagenesis/mutabase/shirpa_summary.html)) to detect phenotypic differences between mutant and control mice. The test parameters contribute to an overall assessment of muscle, motor neuron, spinocerebellar, sensory and autonomic functions. The primary neurological screen is focused on investigating neurological signs to determine the neurological functioning of a mouse. We also examine lactate levels in the blood of mice to draw conclusions about energy metabolism. Moreover, we measured forelimb grip strength to evaluate muscle function.

The comparison of mutant mice to controls revealed no significant differences. All SHIRPA test parameters were without pathological findings.

### 3.3.2 Mice

Fifteen 10-week-old male mutant and fifteen 10-week-old male control mice entered the neurological screen at the beginning of the 38<sup>th</sup> calendar week. Fifteen female animals 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

#### Primary screening: modified SHIRPA protocol

Assessment of each animal at age 10 weeks began with observation of undisturbed behavior (*Viewing Jar Behavior*) in a glass cylinder (11 cm in diameter). The mice were then transferred to an arena consisting of a clear Perspex box (420 x 260 x 180 mm) in which a Perspex sheet on the floor is marked with 15 squares. Locomotor activity and motor behavior within this area was observed (*Behavior recorded in the Arena*). This was followed by a sequence of manipulations testing reflexes (*Behavior recorded on or above the arena*). Measurements were completed with the recording of provoked biting, and body weight. The last part of the primary screen also involved the analysis of righting reflex, and contact righting reflex. A glass cylinder (35 mm diameter, 135 mm length) was used for testing of the contact righting reflex. Throughout the entire procedure, abnormal behavior, biting, and vocalization were recorded. Between testing of each mouse, fecal pellets and urination were re-

moved from the viewing jar and arena. All experimental equipment was thoroughly cleaned with Pursept-A and dried prior to testing.

#### **Further screening: grip strength**

The grip strength meter system determines the fore limb grip strength, i.e. muscle strength of a mouse. The device exploits the tendency of a mouse to grasp a horizontal metal bar while being pulled by its tail. During the trial set-up, the mouse grasps a special adjustable grip (2 mm) mounted on a force sensor. The sensor allows measurements of up to 600 Ponds. Five trials were undertaken for each mouse within one minute. The mean value is used to represent the grip strength of a mouse. All experimental equipment was thoroughly cleaned with Pursept-A and dried prior subsequent tests. Values were presented as means  $\pm$  standard error of mean (SEM).

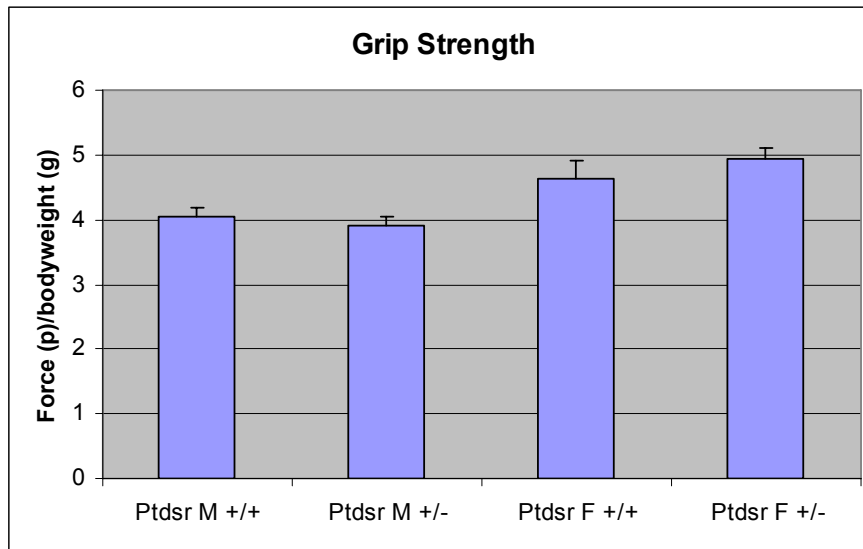
**Statistical analysis of the grip strength trial results:** Grip strength trial results are compared between genotypes, controlling for the effects of sex and weight, by fitting linear mixed effect models (Pinheiro and Bates, 2000). A linear mixed effect model is a modified analysis of variance/covariance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

### **3.3.4 Parameters**

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

### 3.3.5 Results

All SHIRPA parameters were without pathological findings (Tables 11-13). Mean blood lactate levels did not differ between the four groups (Table 14). In addition, we evaluated the grip strength of the fore limbs. We found no significant genotype-related differences between *Ptdsr*-mutant mice and controls (Fig. 3).



**Figure 3: Results from grip strength testing**

No significant genotype-related differences.

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

### 3.3.6 Discussion

In our primary neurological screening, male and female *Ptdsr*-mutant mice showed no altered neurological behavior. Within the SHIRPA parameters tested, no distinctive features were found. Grip strength of the fore limbs was not changed in these animals. Despite the expression of *Ptdsr* in brain during embryonic development and the fact that homozygous animals die perinatally and therefore a role in proper brain formation is suggested, no neurological differences were detected in the primary screen in heterozygous mutant animals. One possibility is that the remaining amount of protein from the expression of the wild-type allele is sufficient to allow for maintenance of the pathways affected or distinct effects or compensatory mechanisms are involved.

### 3.3.7 References

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Pinheiro and Bates (2000): *Mixed-Effects Models in S and S-PLUS*. Springer, New York.

The R Project for Statistical Computing (2004) <http://www.r-project.org/>

### 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](http://www.mgu.har.mrc.ac.uk/mutabase/shirpa_summary.html)

s.a. Sub-maxillary area

**Table 10: Recording of body weight**Data 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>
<b>Body Weight [g]</b>	28.2 $\pm$ 0.5	28.3 $\pm$ 0.6	<i>n.s.</i>	21.1 $\pm$ 0.4	21.6 $\pm$ 0.5	<i>n.s.</i>

**Table 11: Behavior recorded in viewing jar**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>
<b>Body Position</b>						
Inactive	0	0		0	0	
Active	15	15		15	15	
Excessive Activity	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
<b>Tremor</b>						
Absent	15	15		15	15	
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
<b>Palpebral closure</b>						
Eyes open	15	15		15	15	
Eyes closed	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
<b>Coat appearance</b>						
Tidy and well groomed	15	14		14	14	
Irregularities	0	1	<i>n.s.</i>	1	1	<i>n.s.</i>
<b>Whiskers</b>						
Present	15	15		6	6	
Absent	0	0	<i>n.s.</i>	9	9	<i>n.s.</i>
<b>Lacrimation</b>						
Absent	15	15		15	15	
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
<b>Defecation</b>						
Present	11	8		14	11	
Absent	4	7	<i>n.s.</i>	1	4	<i>n.s.</i>

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

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

Parameter	Male			Female		
	Control (n=15)	Mutant (n=15)	<i>p-value</i>	Control (n=15)	Mutant (n=15)	<i>p-value</i>
<b>Locomotor Activity</b>	22.7 $\pm$ 1.2	24.4 $\pm$ 1.5	<i>n.s.</i>	26.5 $\pm$ 1.2	25.4 $\pm$ 1.4	<i>n.s.</i>
<b>Transfer arousal</b>						
Extended freeze (over 5 sec)	0	1		0	0	
Brief freeze	14	14		15	15	
Immediate movement	1	0	<i>n.s.</i>	0	0	<i>n.s.</i>
<b>Gait</b>						
Fluid movement	15	14		15	15	
Lack fluidity	0	1	<i>n.s.</i>	0	0	<i>n.s.</i>
<b>Tail elevation</b>						
Dragging	0	0		0	0	
Horizontal extension	15	15		15	15	
Elevated/straub tail	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
<b>Touch Escape</b>						
No response	0	0		0	0	
Response to touch	15	15		15	15	
Flees prior to touch	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
<b>Positional Passivity</b>						
Struggles when held by tail	15	15		15	15	
No struggle	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>

**Table 13: Behavior recorded in or above the arena**Statistical analysis: chi-squared test; significance  $p < 0.05$ 

Parameter	Male			Female		
	Control (n=15)	Mutant (n=15)	<i>p-value</i>	Control (n=15)	Mutant (n=15)	<i>p-value</i>
<b>Skin color</b>						
Blanched	0	0		1	0	
Pink	15	15		14	15	
Bright deep red	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
<b>Trunk curl</b>						
Absent	15	13		15	14	
Present	0	2	<i>n.s.</i>	0	1	<i>n.s.</i>
<b>Limb grasping</b>						
Absent	15	15		15	15	
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
<b>Pinna reflex</b>						
Present	15	15		13	15	
Absent	0	0	<i>n.s.</i>	2	0	<i>n.s.</i>
<b>Corneal Reflex</b>						
Present	15	15		15	15	
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
<b>Righting reflex</b>						
Rights itself	14	15	<i>n.s.</i>	14	10	<i>n.s.</i>
Fails to right when released	1	0		1	0	
<b>Contact righting</b>						
Present	15	14		15	14	
Absent	0	1	<i>n.s.</i>	0	1	<i>n.s.</i>
<b>Evidence of biting</b>						
None	15	15		15	15	
Biting in response to handling	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
<b>Vocalization</b>						
None	15	15		15	15	
Vocal	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>

**Table 14: Blood lactate levels**

Data shown represent the results of the mean blood lactate concentrations, value ( $\pm$  SEM); 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>
<b>Lactate [mmo/l]</b>	6.9 $\pm$ 0.3	6.7 $\pm$ 0.3	<i>n.s.</i>	4.9 $\pm$ 0.3	5.3 $\pm$ 0.5	<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 slit lamp biomicroscopy (Favor, 1983).

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

No genotype-specific differences between wild-type control and mutant Ptdsr mice were detected.

### 3.4.2 Mice

Thirty control (15 male, 15 female) and 30 mutant mice (15 male, 15 female) 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/m<sup>2</sup>. 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

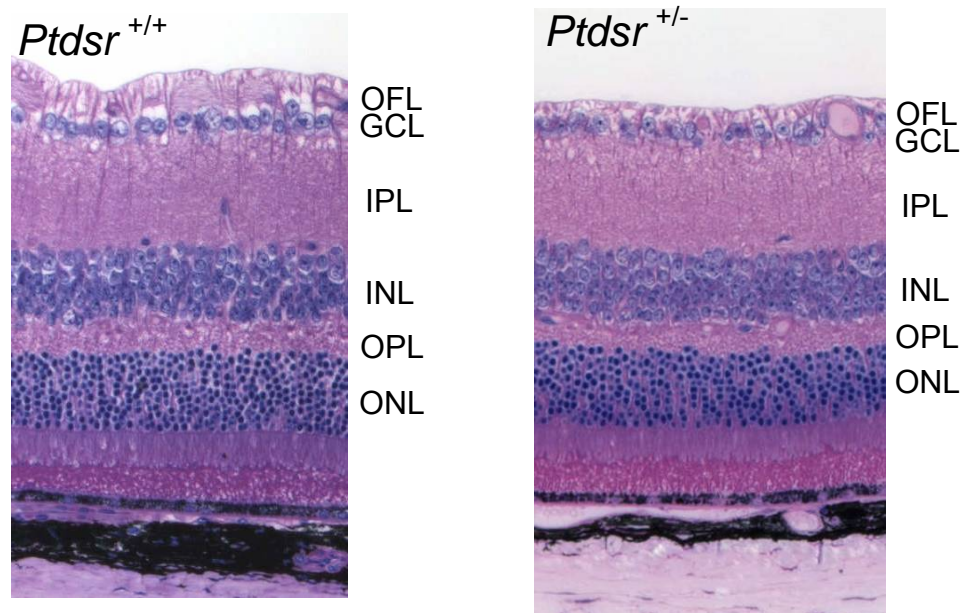
<b>Electroretinography (ERG)</b>
a/b-wave, left/right eye at 500/12.50 cd/m <sup>2</sup>
<b>Slit lamp biomicroscopy</b>
(qualitative) abnormalities of lens and cornea like opacity and development disorders
<b>Histology</b>
(qualitative) retinal lamination and morphology of cell layers and lens
<b>Morphology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

### 3.4.5 Results and Discussion

**ERG responses** were recorded from the groups of Ptdsr (control – mutant) mice with light pulses at two different light intensities. These two luminance levels were chosen because at 500 cd/m<sup>2</sup> 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/m<sup>2</sup> induce a maximally developed b-wave response and an a-wave, coming presumably from rods and cones (Dalke *et al.*, 2004).

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 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 15). The comparison of a- and b-wave amplitudes of males and females revealed no significant differences. Between the groups of mutant and control mice no consistent differences were found, neither in the male nor in the female group. Although there were significant differences in the means of the a-wave at 12,500 cd/m<sup>2</sup> between wild-type and mutant males, all values are not pathologic.

**Histological analysis** of the retinal structure revealed no obvious differences between mutant and control mice (Fig. 4).



**Figure 4: Histological analysis of the retinal structure**

No obvious differences between wild type and heterozygote.

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

A total of 60 *Ptdsr* mice (15 animals of each sex and genotype) were examined ophthalmologically by **slit lamp biomicroscopy**. No eye phenotype was shown to be associated with the *Ptdsr* mutation (Table 16) unlike to the known and expected phenotypes (2.2 and 2.3).

### 3.4.6 References

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### Abbreviations

cd/m <sup>2</sup>	candela per square meter
ERG	electroretinography
Hz	hertz
n.s.	not significant
NAD	no abnormality detected

Table 15: Comparison of ERG-responses at illumination levels of 500 and 12,500 cd/m <sup>2</sup> . Mean ± standard error is calculated for a- and b-wave amplitudes.								
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
<b>a-wave</b> [μV] 500 cd/m <sup>2</sup>	-8 ± 1.1	-7 ± 0.9	n.s.	-7 ± 1.4	-10 ± 1.1	n.s.	n.s.	n.s.
<b>b-wave</b> [μV] 500 cd/m <sup>2</sup>	130 ± 5.8	149 ± 7.3	n.s.	138 ± 6.4	139 ± 7.2	n.s.	n.s.	n.s.
<b>a-wave</b> [μV] 12,500 cd/m <sup>2</sup>	-42 ± 3.3	-38 ± 1.6	n.s.	-31 ± 2.2	-35 ± 1.9	n.s.	<0.02	n.s.
<b>b-wave</b> [μV] 12,500 cd/m <sup>2</sup>	178 ± 8.1	184 ± 7.1	n.s.	171 ± 8.1	172 ± 7.9	n.s.	n.s.	n.s.

Table 16: Results from slit lamp biomicroscopy				
Genotype/ Numbers	NAD	Nuclear opacity	Corneal erosions	Microphthalmia
30 +/-	27	2	-	1 (unilateral)
30 +/+	29	-	-	1 (unilateral)

## 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) *Ptdsr*-mutant and twenty-nine (14 males/ 15 females) control 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. All parameters of both mutant and control mice were within the normal ranges usually found in C57BL/6J mice. All hematological parameters were without pathological findings, too.

### 3.5.2 Mice

Fourteen 12-week-old wild-type and fifteen 12-week-old mutant males entered the clinical-chemical screen at the beginning of the 41<sup>st</sup> calendar week in 2004. Fifteen wild-type and mutant females entered the screen one week later. Additionally, groups of male and female mutant and control mice with six individuals each were analyzed again after food restriction at the end of the 49<sup>th</sup> (males) and of the 50<sup>th</sup> (females) calendar week.

### 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  $\mu$ 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  $\mu$ l), the Allergy Screen (30  $\mu$ l), the Clinical Chemical Screen (130  $\mu$ l) and the Steroid Screen (residual), while the cell pellet was given to the Immunology Screen for FACS-analysis. The plasma sam-

ple for the clinical chemical analysis was transferred into an Eppendorf tube and diluted 1:2 with aqua dest. The solution was mixed for a few seconds (Vortex genie, Scientific Industries, New York, America) to prevent clotting and then centrifuged again for 10 min at 4656 x g. Additionally the Clinical Chemical Screen received the EDTA-blood sample for hematological investigations.

### **Clinical Chemistry**

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

### **Hematology**

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

<b>Proteins and plasma enzyme activities</b>
Alkaline phosphatase (EC 3.1.3.1), $\alpha$ -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
<b>Plasma concentrations of specific substrates</b>
Glucose, Cholesterol, Triglycerides, Uric acid, Urea, Creatinine
<b>Plasma concentrations of electrolytes</b>
Potassium, Sodium, Chloride, Calcium, Inorganic phosphate
<b>Basic hematology</b>
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

Most values obtained for the clinical chemical parameters (Table 17) were within the normal ranges usually found in C57BL/6J mice at the age of three months as supported by previously published data (Hough, *et al.*, 2002; Quimby and Loeb 1999; Klempt *et al.*, 2006, own unpublished results). The unusually high standard error of the mean (SEM) of electrolytes in the male control animals and of lipase activity in the male mutant animals were due to exceptionally low electrolyte concentrations or lipase activity respectively measured in two individual plasma samples. If these outlier values are excluded from the statistical analysis of the affected parameters a significant increase is detected for sodium, chloride and calcium concentrations of the male mutant mice compared to the controls.

Sex differences were detected for many clinical chemical parameters in the mutant animals as well as in the control mice mainly reflecting the physiological differences usually found in this strain of mice (Kile *et al.*, 2003). Further differences between mutants and controls were seen in the following parameters: mutant male mice showed significantly decreased serum concentration of inorganic phosphorus compared to the wild type. Furthermore there was a significantly higher mean level of creatine kinase and GOT activity in mutant male mice. A tendency to higher activities of these enzymes in female mutant mice was also visible, although the differences were not significant. Beside that female mutant mice showed a significantly higher urea concentration and a higher GPT activity compared to the control animals.

#### Hematology

In the primary screen for hematological parameters all results of both mutant and control mice were within normal ranges (Table 19) without significant differences between mutant and control animals.

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

### 3.5.6 Discussion

#### Clinical Chemistry

The elevated urea concentration in female mutant mice could give a hint at subtle changes in kidney function, while the higher activity of muscle enzymes (CK, AST) might reflect an increased sensitivity of muscular tissues of mutant animals to mechanic injury. Kidney dysfunction might also affect electrolyte homeostasis leading to differences in electrolyte concentrations, as they were detected in the male mice. An elevation of ALT activity as it was found in the female mutant mice together with elevated levels of AST activity might also point at liver damage. However, the significant difference of mean ALT activity in the female mice is mainly caused by the high number of female control mice exhibiting an ALT activity value situated in the lower region of the ALT activity range of normal C57BL/6 mice. Therefore this finding is judged to be without pathological relevance. However, all differences found are subtle changes, which do not indicate a really pathological phenotype and might be due to secondary effects.

In a second blood sampling procedure, which was undertaken after six days of food restriction in the metabolic screen (Table 19), no differences between mutant and control mice were detected. But again two individuals with exceptionally high activities of ALT or ALT and AST respectively were detected within the male mutant mice investigated, indicating acute liver damage in these animals. However, exclusion of the values of these mice from the statistical analysis did not reveal any further significant differences.

### **Hematology**

Concerning the white and red blood cell count no differences were found between *Ptdsr*-mutant and control mice.

### **Comparison to baseline data**

Most values of mutant and control animals for all parameters were within the normal ranges typical for baseline C57BL/6 mice. All clinical chemical and hematological parameters were without pathological findings.

## **3.5.7 References**

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**Table 17: Clinical-chemical parameters (at the age of 12 weeks).**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=15)	(n=15)	<i>p</i> -value	(n=14)	(n=15)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
Sodium [mmol/l]	154 $\pm$ 0.81	152 $\pm$ 0.83	n.s.	154 $\pm$ 1.67	151 $\pm$ 0.67	n.s.	n.s.	n.s.
Potassium [mmol/l]	3.9 $\pm$ 0.06	3.5 $\pm$ 0.06	<0.001	3.9 $\pm$ 0.08	3.4 $\pm$ 0.05	<0.001	n.s.	n.s.
Calcium [mmol/l]	2.0 $\pm$ 0.02	2.0 $\pm$ 0.02	n.s.	2.1 $\pm$ 0.03	2.0 $\pm$ 0.01	n.s.	n.s.	n.s.
Chloride [mmol/l]	110.6 $\pm$ 0.68	112.6 $\pm$ 0.67	<0.05	111.5 $\pm$ 1.07	112.0 $\pm$ 0.58	n.s.	n.s.	n.s.
Inorganic Phosphate [mmol/l]	1.6 $\pm$ 0.04	1.7 $\pm$ 0.07	n.s.	1.8 $\pm$ 0.08	1.7 $\pm$ 0.06	n.s.	<0.05	n.s.
Total Protein [g/dl]	4.9 $\pm$ 0.07	4.9 $\pm$ 0.12	n.s.	5.0 $\pm$ 0.09	4.7 $\pm$ 0.07	<0.02	n.s.	n.s.
Creatinine [mg/dl]	0.356 $\pm$ 0.00	0.372 $\pm$ 0.01	<0.05	0.353 $\pm$ 0.01	0.367 $\pm$ 0.01	n.s.	n.s.	n.s.
Urea [mg/dl]	62.0 $\pm$ 1.51	61.7 $\pm$ 2.85	n.s.	58.1 $\pm$ 2.45	49.5 $\pm$ 2.14	<0.02	n.s.	<0.01
Uric acid [mg/dl]	1.0 $\pm$ 0.19	1.5 $\pm$ 0.10	n.s.	1.4 $\pm$ 0.12	1.5 $\pm$ 0.18	n.s.	n.s.	n.s.
Cholesterol [mg/dl]	99.2 $\pm$ 3.46	77.7 $\pm$ 1.73	<0.001	94.3 $\pm$ 3.91	77.4 $\pm$ 3.50	<0.01	n.s.	n.s.
Triglyceride [mg/dl]	144.1 $\pm$ 9.00	94.2 $\pm$ 5.84	<0.001	143.6 $\pm$ 9.75	87.3 $\pm$ 7.05	<0.001	n.s.	n.s.
Creatine Kinase [U/l]	163 $\pm$ 16.4	100 $\pm$ 16.2	<0.01	73 $\pm$ 16.3	70 $\pm$ 20.1	n.s.	<0.001	n.s.
Alanine-Amino-transferase (ALAT,GPT) [U/l]	22 $\pm$ 2.35	20 $\pm$ 1.76	n.s.	23 $\pm$ 1.76	13 $\pm$ 0.78	<0.001	n.s.	<0.01
Aspartate-Amino-transferase (AST,GOT) [U/l]	34 $\pm$ 1.83	30 $\pm$ 1.70	n.s.	25 $\pm$ 1.48	28 $\pm$ 2.04	n.s.	<0.001	n.s.
Alkaline Phosphatase [U/l]	83 $\pm$ 3.45	114 $\pm$ 2.32	<0.001	82 $\pm$ 3.77	117 $\pm$ 3.45	<0.001	n.s.	n.s.
$\alpha$ -Amylase [U/l]	2619 $\pm$ 97.0	2159 $\pm$ 62.0	<0.001	2591 $\pm$ 102	2117 $\pm$ 38.2	<0.001	n.s.	n.s.
Glucose [mg/dl]	165.4 $\pm$ 4.87	177.0 $\pm$ 3.81	n.s.	168.1 $\pm$ 5.70	174.3 $\pm$ 9.85	n.s.	n.s.	n.s.
Ferritin [ng/ml]	37.6 $\pm$ 1.38	43.1 $\pm$ 1.79	<0.05	36.8 $\pm$ 1.89	44.5 $\pm$ 1.81	<0.01	n.s.	n.s.
Transferrin [mg/dl]	137.0 $\pm$ 1.31	136.7 $\pm$ 1.17	n.s.	136.8 $\pm$ 1.29	136.7 $\pm$ 1.12	n.s.	n.s.	n.s.
Lipase [U/l]	83.2 $\pm$ 9.47	72.5 $\pm$ 2.08	n.s.	73.2 $\pm$ 4.22	70.8 $\pm$ 3.24	n.s.	n.s.	n.s.

**Table 18: Hematological parameters (at the age of 12 weeks).**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=15)	(n=15)	<i>p</i> - value	(n=14)	(n=15)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
White blood cell count [ $10^3/\mu\text{l}$ ]	5.98 $\pm 0.60$	4.78 $\pm 0.30$	n.s.	5.79 $\pm 0.45$	4.69 $\pm 0.31$	n.s.	n.s	n.s
Red blood cell count [ $10^3/\mu\text{l}$ ]	10.5 $\pm 0.15$	10.7 $\pm 0.14$	n.s.	10.59 $\pm 0.10$	10.43 $\pm 0.14$	n.s.	n.s	n.s
Hemoglobin [g/dl]	15.5 $\pm 0.19$	16.2 $\pm 0.16$	<0.02	15.9 $\pm 0.15$	15.9 $\pm 0.20$	n.s.	n.s	n.s
Hematocrit [%]	46 $\pm 0.54$	47 $\pm 0.51$	n.s.	46 $\pm 0.46$	46 $\pm 0.57$	n.s.	n.s	n.s
Mean corpuscular volume [fl]	43.3 $\pm 0.19$	43.7 $\pm 0.21$	n.s.	43.7 $\pm 0.15$	44.0 $\pm 0.24$	n.s.	n.s	n.s
Mean corpuscular hemoglobin [pg]	14.8 $\pm 0.07$	15.1 $\pm 0.08$	<0.01	15.0 $\pm 0.06$	15.3 $\pm 0.07$	<0.01	n.s	n.s
Mean corpuscular hemoglobin concentration [g/dl]	34.1 $\pm 0.11$	34.6 $\pm 0.08$	<0.01	34.4 $\pm 0.19$	34.7 $\pm 0.12$	n.s.	n.s	n.s
Platelet count [ $10^3/\mu\text{l}$ ]	768 $\pm 17.0$	716 $\pm 21.4$	n.s.	823 $\pm 24.4$	703 $\pm 23.3$	<0.001	n.s	n.s

**Table 19: Results of clinical-chemical parameters after food restriction**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=6)	(n=6)	<i>p</i> - value	(n=6)	(n=6)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
Inorganic Phosphorus [mmol/l]	1.5 $\pm 0.16$	1.7 $\pm 0.06$		1.6 $\pm 0.05$	1.6 $\pm 0.05$		n.s.	n.s.
Urea [mg/dl]	60.8 $\pm 4.8$	63.8 $\pm 3.8$		60.7 $\pm 5.03$	60.1 $\pm 3.20$		n.s.	n.s.
Creatine Kinase [U/l]	222 $\pm 48.7$	182 $\pm 7.39$		178 $\pm 50.3$	172 $\pm 46.7$		n.s.	n.s.
Alanine-Aminotransferase (ALAT,GPT) [U/l]	34 $\pm 11.9$	21 $\pm 4.90$		23 $\pm 4.55$	25 $\pm 5.55$		n.s.	n.s.
Aspartate-Aminotransferase (AST,GOT) [U/l]	35 $\pm 6.68$	40 $\pm 4.66$		27 $\pm 3.71$	30 $\pm 3.55$		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 (Bluethmann and Ohashi, 1994; Mak *et al.*, 2001; Fischer 2002; Rogner and Avner, 2003). The Immunology Screen at the GMC was set up to conduct a broad immunological phenotyping of mouse mutant lines with the intention of identifying distinct gene functions, which play key roles in the immune defenses of the organism through a complex network of cellular and soluble components (Janeway *et al.*, 2004).

According to the data summary of what is already known about the mutant mouse line presented to the GMC by the mouse provider, ablation of *Ptdsr* function results in reduced production of pro- and anti-inflammatory cytokines after stimulation of fetal liver-derived macrophages with LPS (or other PAMPs) or LPS together with apoptotic cells. The analysis *Ptdsr*-deficient mice in the Immunology Screen revealed subtle differences between the male mutants and their littermate controls.

### 3.6.2 Mice

We analyzed 30 mutant animals (15 females and 15 males) and 29 age- and sex-matched littermate controls (15 females and 14 males).

### 3.6.3 Material and Methods

Peripheral blood leukocytes (PBLs) were isolated from 500  $\mu$ l blood by erythrocyte lysis with  $\text{NH}_4\text{Cl}$  (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  $\mu$ 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<sup>+</sup> clone 1D3), B1 B cells (CD19<sup>+</sup>CD5<sup>+</sup>, clone 53-7.3), B2 B cells (CD19<sup>+</sup>CD5<sup>-</sup>), T cells (CD3<sup>+</sup>, clone 145-2C11), CD4<sup>+</sup> T cells (clone RM4-5), CD8<sup>+</sup> T cells (CD8 $\alpha$ , clone 53-6.7; CD8 $\beta$ , clone H35-17.2),  $\gamma/\delta$ T cells (clone GL3), granulocytes (Gr-1<sup>+</sup>, clone RB6-8C5), and NK cells (CD49b<sup>+</sup>, 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, IgG<sub>1</sub>, IgG<sub>2a</sub>, IgG<sub>2b</sub>, IgG<sub>3</sub>, 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<sup>lpr</sup> mice (Jackson Laboratory, Bar Harbor, USA) were used as positive controls in the autoantibody assays.

### 3.6.4 Parameters

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

### 3.6.5 Results and Discussion

The analysis of the Ptdsr mutant mouse line in the primary Immunology Screen did not reveal profound alterations in the tested parameters. However, we were able to detect some minor, but statistically significant differences in the frequencies of B1 B cells and  $\gamma/\delta$ T cells, which tended to be higher in male mutant mice. In addition, the level of IgG<sub>2b</sub> was higher in these mice.

Although we were able to detect these slight alterations, most likely they do not represent a major phenotype affecting the immune system, and are probably caused by physiological variation.

### 3.6.6 References

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<b>Table 20: Basic parameters analyzed in the Immunology Screen.</b>								
Data are presented as mean ± standard error of mean.								
Parameter	Mutants (A)			Control (B)			A ~ B	
	Male	Female		Male	Female		Male	Female
	(n=15)	(n=15)	<i>p</i> - value	(n=14)	(n=15)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
<b>CD19<sup>+</sup></b> [%]	62.9±2.7	60.1±1.2	n.s.	59.9±1.7	62.8±1.4	na	n.s.	n.s.
<b>CD19<sup>+</sup>CD5<sup>-</sup></b> [%]	91.2±0.5	96.1±0.1	<0.001	94.6±0.3	96.2±0.1	<0.001	<0.001	n.s.
<b>CD19<sup>+</sup>CD5<sup>+</sup></b> [%]	8.7±0.5	3.8±0.1	<0.001	5.3±0.3	3.4±0.1	<0.001	<0.001	n.s.
<b>CD3<sup>+</sup></b> [%]	25.6±1.0	30.9±0.7	<0.001	25.9±0.8	28.9±0.9	<0.05	n.s.	n.s.
<b>γ/δ TCR<sup>+</sup></b> [%]	3.4±0.1	1.0±0.06	<0.001	2.8±0.1	0.9±0.06	<0.001	<0.05	n.s.
<b>Gr-1<sup>+</sup></b> [%]	12.9±2.9	11.3±0.7	n.s.	11.9±1.6	10.0±0.6	n.s.	n.s.	n.s.
<b>CD49b<sup>+</sup></b> [%]	20.6±1.4	29.6±1.7	<0.001	20.9±0.8	23.2±2.5	n.s.	n.s.	n.s.
<b>CD4<sup>+</sup></b> [%]	17.3±0.7	22.0±0.4	<0.001	17.5±0.4	20.1±0.7	<0.001	n.s.	n.s.
<b>CD8β<sup>+</sup></b> [%]	11.9±0.3	13.9±0.4	<0.01	12.5±0.3	13.0±0.4	n.s.	n.s.	n.s.
<b>IgG<sub>1</sub></b> [μg/ml]	122.2±13.0	175.0±13.6	<0.02	145.5±24.5	141.9±7.5	n.s.	n.s.	n.s.
<b>IgG<sub>2a</sub></b> [μg/ml]	56.5±4.3	127.3±10.2	<0.001	53.0±4.2	146.4±12.6	<0.001	n.s.	n.s.
<b>IgG<sub>2b</sub></b> [μg/ml]	106.7±8.0	164.8±9.4	<0.001	83.9±4.7	187.9±7.1	<0.001	<0.05	n.s.
<b>IgG<sub>3</sub></b> [μg/ml]	156.2±17.4	162.1±19.0	n.s.	228.7±19.8	249.4±14.9	<0.05	n.s.	n.s.
<b>IgM</b> [μg/ml]	169.5±39.7	472.7±70.5.8	<0.01	209.7±21.4	412.3±56.2	<0.01	n.s.	n.s.
<b>IgA</b> [μg/ml]	58.7±4.7	88.1±6.9	<0.01	48.0±2.7	83.5±4.9	<0.001	n.s.	n.s.
<b>Anti-DNA Ab</b> [%]	0	0	n.s.	0	0	n.s.	n.s.	n.s.
<b>Rheumatoid factor</b> [%]	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 *Ptdsr* mutant mouse, line 29 wild-type control and 30 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 29 control (15 females, 14 males) and 30 mutant (15 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 *Ptdsr* mice revealed no statistically significant difference between mutant and control mice. We detected a higher mean IgE concentration in female mice compared to male animals. However, this difference was not statistically significant (Table 21). Taken together, under standard screening conditions for primary allergy screen, *Ptdsr*-

mutant mice did not show changes in total plasma IgE levels that would reveal a major allergy phenotype.

Raw data will be available on demand.

<b>Table 21: Total plasma IgE</b>								
Data are presented as mean $\pm$ standard error of mean.								
	<b>Control (A)</b>			<b>Mutant (B)</b>			<b>A~B</b>	<b>A~B</b>
	<b>Female</b>	<b>Male</b>		<b>Female</b>	<b>Male</b>		<b>Female</b>	<b>Male</b>
	<b>(n=15)</b>	<b>(n=14)</b>	<b><i>p</i> - <i>value</i></b>	<b>(n=15)</b>	<b>(n=15)</b>	<b><i>p</i> - <i>value</i></b>	<b><i>p</i> - <i>value</i></b>	<b><i>p</i> - <i>value</i></b>
<b>Total IgE [ng/ml]</b>	25 $\pm$ 3.5	16 $\pm$ 2.3	n.s.	24 $\pm$ 4.8	17 $\pm$ 1.6	n.s.	n.s.	n.s.

### 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 suprathreshold 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 *Ptdsr* mutant mouse line by means of the hot plate test (nociceptive pain). We found no significant differences in pain reactivity between the mutant and control animals. There was no sex difference in this mutant mouse line. We do not plan to perform further pain related studies in this mutant line.

### 3.8.2 Mice

Thirty mutant mice (15 male, 15 female), and 30 control animals (15 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. 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.

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

<b>Hind paw licking</b>
Reaction with licking of hind paw to the thermal pain
<b>Hind paw shaking</b>
Reaction with shaking of hind paw to the thermal pain
<b>Jumping</b>
Jumping reaction to the thermal pain

### 3.8.5 Results and Discussion

Typically, the first nociceptive response observed in *Ptdsr* mice was hind paw shaking (Table 22). Both genotypes performed also hind paw licking, another typical nociceptive response. The third examined response was jumping of the animal. The latencies of all three responses did not differ between the genotypes or the sexes.

To sum up, the analysis did not reveal significant differences between *Ptdsr*-mutant mice and their control littermates, nor an interaction between genotype and sex effects. We do not plan to perform further pain related studies.

### 3.8.6 References

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## Abbreviations

h.p. hind paw

<b>Table 22: Nociceptive Screen</b>									
Data are presented as mean $\pm$ standard error of mean.									
							ANOVA		
							genotype		sex*genotype
Parameter Latency [s]	Mutant (A)			Control (B)			A~B	A~B	ANOVA
	Female	Male		Female	Male		Female	Male	
	(n=15)	(n=15)	<i>p - value</i>	(n=15)	(n=15)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
<b>H.p. licking</b>	21.6 $\pm$ 1.76	20.6 $\pm$ 1.77	n.s.	24.1 $\pm$ 1.76	21.3 $\pm$ 1.77	n.s.	n.s.	n.s.	n.s.
<b>H.p. shaking</b>	15.1 $\pm$ 1.54	13.1 $\pm$ 1.54	n.s.	18.6 $\pm$ 1.54	17.4 $\pm$ 1.54	n.s.	n.s.	n.s.	n.s.
<b>Jumping</b>	58.7 $\pm$ 1.57	55.9 $\pm$ 1.57	n.s.	58.3 $\pm$ 1.57	56.2 $\pm$ 1.57	n.s.	n.s.	n.s.	n.s.

## 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 rest and activity were studied in 15-week-old *Ptdsr*-mutant and control mice. No physiologically relevant differences were detected between wild-type control and mutant mice. Typical sex differences with higher specific values for tidal volume and minute ventilation in female mice were seen in both wild type and mutant mice but were more pronounced in the mutants. These differences can be related to the significant differences in body weight between male and female mice.

### 3.9.2 Mice

Male and female mutant and control mice were studied at age 15 weeks. Body weight differed significantly between sexes but was comparable between wild-type and mutant mice in males as well as in females (Table 23).

### 3.9.3 Material and Methods

#### Whole Body Plethysmography

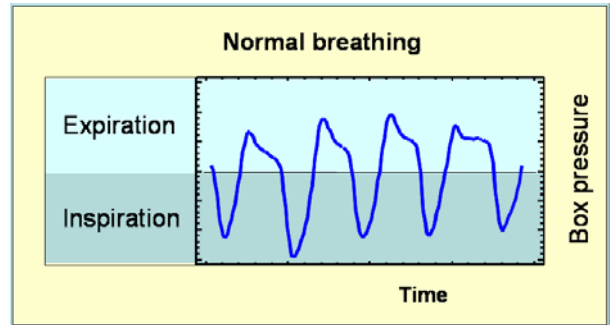
A commercially available system from Buxco<sup>®</sup> Electronics (Sharon, Connecticut) was used to assess breathing patterns in unrestrained animals according to the principle described by Drorbaugh and Fenn (1955). It measures the pressure changes which arise from inspiratory and expiratory temperature and humidity fluctuations during breathing (Figs. 5 and 6).

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

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



**Figure 5: System used at GMC to assess breathing patterns.**



**Figure 6: 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 Student's 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

<b>Directly recorded data</b>
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).
<b>Calculated data</b>
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 24 summarizes the results obtained for spontaneous breathing under resting and active conditions. No physiologically relevant differences were detected between wild type and mutant mice. The mutation does not seem to affect the spontaneous breathing pattern in mice since wild-type and *Ptdsr*-mutant mice did not reveal physiologically relevant differences.

Typical sex differences with higher specific values for tidal volume and minute ventilation in female mice were seen in both wild-type and mutant mice but were more pronounced in the mutants. These differences can be related to the significant differences in body weight between male and female mice that were also more pronounced within the mutants.

### 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 ( $\mu$ 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 23: 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=5)	(n=5)	<i>p - value</i>	(n=5)	(n=5)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
<b>Bw [g]</b>	29.6 $\pm$ 0.7	22.2 $\pm$ 0.5	< 0.001	32.0 $\pm$ 0.9	21.2 $\pm$ 0.8	< 0.001	n.s.	n.s.
<b>Age [d]</b>	111.2 $\pm$ 0.5	111.6 $\pm$ 0.2		110.8 $\pm$ 0.2	112.0 $\pm$ 0			
<b>Mean_f [1/min]</b>	417.3 $\pm$ 14.7	398.6 $\pm$ 14.5	n.s.	414.0 $\pm$ 17.6	414.7 $\pm$ 12.0	n.s.	n.s.	n.s.

**Table 24: Spontaneous breathing pattern during rest and activity**Data are presented as mean  $\pm$  standard error of mean.

Parameter	Control (A)			Mutant (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=5)	(n=5)	<i>p</i> - value	(n=5)	(n=5)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
<b>Rest</b>								
f [1/min]	353.9 $\pm$ 7.9	376.2 $\pm$ 20.7	n.s.	355.2 $\pm$ 13.9	363.4 $\pm$ 7.1	n.s.	n.s.	n.s.
TV [ml]	0.26 $\pm$ 0.02	0.24 $\pm$ 0.01	n.s.	0.31 $\pm$ 0.01	0.26 $\pm$ 0.01	n.s.	n.s.	n.s.
sTV [ $\mu$ l/g]	8.8 $\pm$ 0.8	10.9 $\pm$ 0.7	n.s.	9.7 $\pm$ 0.5	12.1 $\pm$ 0.4	< 0.01	n.s.	n.s.
MV [ml/min]	89.5 $\pm$ 6.9	88.2 $\pm$ 5.3	n.s.	107.2 $\pm$ 6.8	91.9 $\pm$ 4.9	n.s.	n.s.	n.s.
sMV [ml/min/g]	3.0 $\pm$ 0.2	4.0 $\pm$ 0.3	< 0.05	3.4 $\pm$ 0.3	4.3 $\pm$ 0.2	< 0.05	n.s.	n.s.
Ti [ms]	49.8 $\pm$ 1.5	50.2 $\pm$ 1.9	n.s.	51.1 $\pm$ 1.8	49.9 $\pm$ 2.5	n.s.	n.s.	n.s.
Te [ms]	120.0 $\pm$ 3.0	111.1 $\pm$ 6.7	n.s.	118.9 $\pm$ 5.4	115.5 $\pm$ 2.9	n.s.	n.s.	n.s.
Ti/TT	0.29 $\pm$ 0.01	0.31 $\pm$ 0.01	n.s.	0.30 $\pm$ 0.01	0.30 $\pm$ 0.01	n.s.	n.s.	n.s.
PIF [ml/s]	8.9 $\pm$ 0.5	8.4 $\pm$ 0.6	n.s.	10.4 $\pm$ 0.5	9.0 $\pm$ 0.6	n.s.	n.s.	n.s.
PEF [ml/s]	5.1 $\pm$ 0.4	5.0 $\pm$ 0.4	n.s.	6.0 $\pm$ 0.6	5.0 $\pm$ 0.5	n.s.	n.s.	n.s.
MIF [ml/s]	5.2 $\pm$ 0.3	4.8 $\pm$ 0.3	n.s.	6.1 $\pm$ 0.3	5.2 $\pm$ 0.3	n.s.	n.s.	n.s.
MEF [ml/s]	2.2 $\pm$ 0.2	2.2 $\pm$ 0.1	n.s.	2.6 $\pm$ 0.2	2.2 $\pm$ 0.1	n.s.	n.s.	n.s.
<b>Activity</b>								
f [1/min]	471.4 $\pm$ 6.9	477.2 $\pm$ 13.0	n.s.	465.0 $\pm$ 10.5	488.3 $\pm$ 5.0	n.s.	n.s.	n.s.
TV [ml]	0.27 $\pm$ 0.02	0.24 $\pm$ 0.01	n.s.	0.31 $\pm$ 0.01	0.25 $\pm$ 0.02	< 0.02	n.s.	n.s.
sTV [ $\mu$ l/g]	9.0 $\pm$ 0.6	10.9 $\pm$ 0.6	n.s.	9.8 $\pm$ 0.5	12.0 $\pm$ 0.7	< 0.05	n.s.	n.s.
MV [ml/min]	123.2 $\pm$ 6.9	113.5 $\pm$ 2.7	n.s.	141.9 $\pm$ 6.0	122.4 $\pm$ 8.0	n.s.	n.s.	n.s.
sMV [ml/min/g]	4.2 $\pm$ 0.3	5.1 $\pm$ 0.2	n.s.	4.5 $\pm$ 0.3	5.8 $\pm$ 0.4	< 0.05	n.s.	n.s.
Ti [ms]	42.3 $\pm$ 0.5	42.8 $\pm$ 0.6	n.s.	42.6 $\pm$ 0.6	40.7 $\pm$ 0.5	n.s.	n.s.	< 0.05
Te [ms]	85.1 $\pm$ 1.4	83.3 $\pm$ 2.9	n.s.	86.7 $\pm$ 2.6	82.2 $\pm$ 1.0	n.s.	n.s.	n.s.
Ti/TT	0.33 $\pm$ 0.01	0.34 $\pm$ 0.01	n.s.	0.33 $\pm$ 0.01	0.33 $\pm$ 0.003	n.s.	n.s.	n.s.
PIF [ml/s]	10.7 $\pm$ 0.6	9.7 $\pm$ 0.4	n.s.	12.2 $\pm$ 0.5	10.8 $\pm$ 0.8	n.s.	n.s.	n.s.
PEF [ml/s]	6.9 $\pm$ 0.6	6.4 $\pm$ 0.3	n.s.	8.1 $\pm$ 0.6	6.8 $\pm$ 0.7	n.s.	n.s.	n.s.
MIF [ml/s]	6.3 $\pm$ 0.3	5.6 $\pm$ 0.2	n.s.	7.3 $\pm$ 0.2	6.2 $\pm$ 0.4	0.0549	n.s.	n.s.
MEF [ml/s]	3.1 $\pm$ 0.2	2.9 $\pm$ 0.1	n.s.	3.6 $\pm$ 0.2	3.1 $\pm$ 0.2	n.s.	n.s.	n.s.

## 3.10 Expression Profiling

### 3.10.1 Summary

In this report, we describe the results of the RNA expression profiling of brain, liver, thymus, heart and spleen of male animals of the *Ptdsr* mutant mouse line. In total 40 chip hybridizations were performed. The data analysis and various statistical methods detected differential gene expression between wild-type and mutant tissues only in **thymus**. Expression of *Ptdsr* was detected in all organs but without regulation.

### 3.10.2 Mice

The molecular phenotyping screen archives organs of mutant and wild-type mice for subsequent DNA-chip expression profiling analysis. Ten male mice (five mutants and five controls) of the *Ptdsr* mutant mouse line were provided to the molecular phenotyping screen (Table 25).

To minimize the influence of circadian rhythm on gene expression, mice were killed between 9 am and 12 am by carbon dioxide asphyxiation. The following organs were collected and archived in liquid nitrogen following our established SOPs (Standard operation protocols): bulbourethral gland, spleen, kidney, seminal vesicles, testis, liver, heart, lung, thymus, skin/cartilage (outer ear), skeletal muscle, salivary gland and brain. Organs were immediately frozen and stored in liquid nitrogen until isolation of total RNA.

<b>Table 25: Organs of <i>Ptdsr</i>-mutant and control mice stored for expression profiling.</b>				
<b>Mouse ID</b>	<b>Strain</b>	<b>Sex</b>	<b>Genotype</b>	<b>Date of Collection</b>
30022135	<i>Ptdsr</i>	m	+/-	20.10.2004
30022136	<i>Ptdsr</i>	m	+/-	20.10.2004
30022137	<i>Ptdsr</i>	m	+/-	20.10.2004
30022138	<i>Ptdsr</i>	m	+/-	20.10.2004
30022139	<i>Ptdsr</i>	m	+/-	20.10.2004
30022157	<i>Ptdsr</i>	m	+/+	20.10.2004
30022151	<i>Ptdsr</i>	m	+/+	20.10.2004
30022152	<i>Ptdsr</i>	m	+/+	20.10.2004
30022153	<i>Ptdsr</i>	m	+/+	20.10.2004
30022154	<i>Ptdsr</i>	m	+/+	20.10.2004

### 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<sub>260/280</sub> 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<sup>TM</sup> 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 Labelling

For labeling 20 µg of total RNA were used for reverse transcription and indirectly labeled with Cy3 or Cy5 fluorescent dye according the TIGR protocol (Hedge *et al* 2000). Labeled cDNA was dissolved in 30 µl hybridization buffer (6x SSC, 0.5% SDS, 5x Denhardt's solution and 50% formamide) and mixed with 30 µl of reference cDNA solution (pool from five control animals) labeled with the second dye. This hybridization mixture 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/s<sup>2</sup>. 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/ieq/groups/exppro\\_cpt.html#PAM](http://www.gsf.de/ieq/groups/exppro_cpt.html#PAM)) was used.

### Chip Hybridization

In general two chip hybridizations were performed with RNA from all organs of each five individual mutant mice (in total 10 hybridizations) against the identical pool of the same organ of wild-type control RNAs (reference RNA pool; wt). For each individual the chip experiments included a color-flip experiment. If differential gene expression will be detected between mutant and wild-type mice, additionally wild-type/wild-type pool experiments will be done to confirm the differences between mutant and wild type.

## 3.10.4 Results

### Selected Organs and Isolated RNA

Brain, liver, thymus, heart and spleen were selected as organs for expression profiling analysis. We isolated total RNA of these organs of five *Ptdsr*-mutant mice and five wild-type control individuals (Table 26).

<b>Mouse ID</b>	<b>Brain</b>	<b>Liver</b>	<b>Thymus</b>	<b>Heart</b>	<b>Spleen</b>
30022135	286	50	151	151	147
30022136	400	37	145	110	186
30022137	396	21	110	145	209
30022138	552	19	110	110	168
30022139	308	22	81	81	210
30022157	357	37	142	103	170
30022151	278	52	289	202	318
30022152	469	26	182	75	118
30022153	272	25	182	208	192
30022154	325	86	82	112	248

## Analysis of Gene Expression in Brain Tissue

Table 27 summarizes the results of 10 chip hybridizations performed with RNA from brain of *Ptdsr*-mutant mice. In total, 2296 probes showed signals in all 10 chip hybridizations.

<b>Table 27: Chip Hybridization of Brain: Labeling and Number of Detected Spots</b>		
Numbers indicate the ID of mutant mice.		
<b>Chip ID</b>	<b>Cy5/Cy3</b>	<b>Detected Spots</b>
#1	135 / ref	8003
#2	ref / 135	8202
#3	136 / ref	9459
#4	ref / 136	8815
#5	137 / ref	10131
#6	ref / 137	9005
#7	138 / ref	8751
#8	ref / 138	6792
#9	139 / ref	8301
#10	ref / 139	7490
		<b>2296 overlap</b>

Genes were evaluated for the significance of differential gene expression. Genes were ranked according the lowest absolute ratio of signal intensities (*Ptdsr*-mutant versus reference) in 10 microarray experiments (Table 28). This ranking is independent of the reproducibility in terms of up- and down-regulation. The number of genes with non-reproducible up- or down-regulation („non-uniform patterns“) is given for different selections of genes in the ranking („ranked genes“). The number of non-differentially expressed genes („NDE, false positives“) among genes with reproducible patterns was calculated for significance level  $p < 0.05$ .

For example, the selection of the top 20 ranked genes with reproducible up- or down-regulation contains 9 genes with non-reproducible chip data. The remaining 11 genes with reproducible up- or down-regulation contains one or more non-differentially expressed with a significance level  $p < 0.05$ . The minimal ratios of expression for this selection ranged from 1.07 to 1.02 fold induction/repression.

<b>Table 28: Chip Hybridization of Brain Tissue: Evaluation of Data</b>			
<b>Ranked Genes (According Lowest of 10 Ratios)</b>	<b>Non-uniform Patterns</b>	<b>NDE (False Positives) <math>p &lt; 0.05</math></b>	<b>Fold Induction (Minimum of 10 Chips)</b>
1 - 20	9	$\geq 1$	1.07 – 1.02
1 - 40	38	$\geq 1$	1.07 – 1.01

According to the non-reproducibility of chip data of the top 40 ranked genes, no gene with differential expression in brain of *Ptdsr*-mutant mice was observed in all experiments.

#### Expression of *Ptdsr* in brain

In individual 136, 137 and 138 *Ptdsr* expression was detected without regulation. The fold induction ranged between 1.48 – 1.86. No expression for *Ptdsr* was found in sample 135 and 139. Signals on these spots in experiments of individual 135 and 139 were below detection thresholds.

#### **Analysis of Gene Expression in Liver Tissue**

The isolated total RNA of liver tissue showed degradation after gel electrophoreses and expression profiling experiments could not be performed for this organ.

#### **Analysis of Gene Expression in Thymus Tissue**

Table 29 summarizes the results of 10 chip hybridizations performed with RNA from thymus of *Ptdsr*-mutant mice. In total, 2624 probes showed signals in all 10 chip hybridizations.

<b>Table 29: Chip hybridization of thymus: labeling and number of detected spots</b>		
Numbers indicate the ID of mutant mice.		
<b>Chip ID</b>	<b>Cy5/Cy3</b>	<b>Detected Spots</b>
#1	135 / ref	10875
#2	ref / 135	9249
#3	136 / ref	9896
#4	ref / 136	11275
#5	137 / ref	10869
#6	ref / 137	7430
#7	138 / ref	14066
#8	ref / 138	14921
#9	139 / ref	6953
#10	ref / 139	7798
		<b>2624 overlap</b>

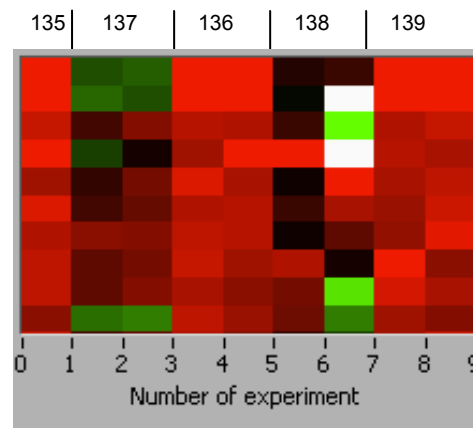
Genes were evaluated for the significance of differential gene expression. Genes were ranked according to the lowest absolute ratio of signal intensities (mutant versus reference) in 10 microarray experiments (Table 29). This ranking is independent of the reproducibility in terms of up- and down-regulation. The number of genes with non-reproducible up- or down-regulation („non-uniform patterns“) is given for different selections of genes in the ranking („ranked genes“). The number of non-differentially expressed genes („NDE, false positives“) among genes with reproducible patterns was calculated for significance level  $p < 0.05$ .

For example, the selection of the top 20 ranked genes with reproducible up- or down-regulation contains 17 genes with non-reproducible chip data. The remaining three genes with reproducible up- or down-regulation contains two or more non-differentially expressed with a significance level  $p < 0.05$ . The minimal ratios of expression for this selection ranged from 28.96 to 1.30 fold induction/repression.

Table 30: Chip hybridization of thymus tissue: evaluation of data			
Ranked Genes (According Lowest of 10 Ratios)	Non-uniform Patterns	NDE (False Positives) $p < 0.05$	Fold Induction (Minimum of 10 Chips)
1 - 20	17	$\geq 2$	28.96 – 1.30
1 - 40	36	$\geq 2$	28.96 – 1.24

According to 90% non-reproducible chip data of the top 40 ranked genes, no gene with differential expression in thymus of *Ptdsr*-mutant mice was observed in all experiments.

Inspection of expression data from individual mice revealed a stronger correlation of up-regulated genes in samples 135, 136 and 139. Expression pattern of sample 137 and 138 show anti-correlation to the other two samples in up-regulated genes (see Fig. 7).



**Figure 7: Biological variability between single individuals**

Individuals 135, 136 and 139 differ in the expression pattern of thymus from individual 137 and 138.

The selected genes are reproducibly up-regulated in all experiments of individual 135, 136 and 139; genes were ranked according to the minimum of six ratios. Maybe these genes are of interest for the *Ptdsr* mutant mouse line.

**Table 31: Genes up-regulated in thymus of *Ptdsr*-mutant mice (individual 135, 136 and 139)**

Rank	Lion ID	Gene Symbol	Gene name	Mean Ratio	Molecular Function	Chrom. Position
1	MG-14-61c20	Scd1	stearoyl-Coenzyme A desaturase 1	7,9 ± 1,19	iron ion binding, oxidoreductase activity	19
2	MG-14-3b19	Fabp4	fatty acid binding protein 4 adipocyte	5,2 ± 2,91	Lipid binding	3
3	MG-4-4h13	Grrp1	glycine/arginine rich protein 1	2,3 ± 0,12	-	4
4	MG-11-1i21	Thrsp	thyroid hormone responsive	3,6 ± 1,98	-	7
5	MG-4-146n10	Hbb-b1	hemoglobin beta adult major chain	2,2 ± 0,26	Heme binding	7
6	MG-8-40d4	Hbb-a1	hemoglobin alpha chain	2,3 ± 0,26	Heme binding	
7	MG-3-213n6	Sycp3	synaptonemal complex protein 3	1,9 ± 0,24	Protein binding, transferase activity	10
8	MG-4-86f7	Hbb-b1	hemoglobin beta adult major chain	2,3 ± 0,29	Heme binding	7
9	MG-8-86g2	11100 17116 Rik	-	2,1 ± 0,37	-	2
10	MG-4-145j12	Hbb-b1	hemoglobin beta adult major chain	2,2 ± 0,52	-	7
11	MG-3-23n11	Brp44	Brain protein 44	1,7 ± 0,05	-	
12	MG-4-3b2	Hbb-b1	hemoglobin beta adult major chain	2,7 ± 1,04	Heme binding	7
13	MG-8-21a18	Aspn	asporin	1,9 ± 0,22	Porine activity	13

## Expression of *Ptdsr* in Thymus

In individual 135 and 138 *Ptdsr* expression was detected without regulation. The fold induction ranged between 1.19 – 1.83. No expression for *Ptdsr* was found in sample 136, 137 and 139. Signals on these spots in experiments of these individuals were below detection thresholds.

## **Analysis of Gene Expression in Spleen Tissue**

Table 32 summarizes the results of 10 chip hybridizations performed with RNA from spleen. In total, 2388 probes showed signals in all 10 chip hybridizations.

<b>Table 32: Chip hybridization of spleen: labeling and number of detected spots</b>		
Numbers indicate the ID of mutant mice.		
<b>Chip ID</b>	<b>Cy5/Cy3</b>	<b>Detected Spots</b>
#1	135 / ref	6468
#2	ref / 135	5778
#3	136 / ref	8104
#4	ref / 136	15410
#5	137 / ref	5828
#6	ref / 137	9950
#7	138 / ref	12724
#8	ref / 138	10566
#9	139 / ref	14426
#10	ref / 139	12346
		<b>2388 overlap</b>

Genes were evaluated for the significance of differential gene expression. Genes were ranked according the lowest absolute ratio of signal intensities (*Ptdsr*-mutant mice versus reference) in 10 microarray experiments (Table 32). This ranking is independent of the reproducibility in terms of up- and down-regulation. The number of genes with non-reproducible up- or down-regulation („non-uniform patterns“) is given for different selections of genes in the ranking („ranked genes“). The number of non-differentially expressed genes („NDE, false positives“) among genes with reproducible patterns was calculated for significance level  $p < 0.05$ .

For example, the selection of the top 20 ranked genes with reproducible up- or down-regulation contains nine genes with non-reproducible chip data. The remaining 11 genes with reproducible up- or down-regulation contains one or more non-differentially expressed with a significance level  $p < 0.05$ . The minimal ratios of expression for this selection ranged from 1.07 to 1.02 fold induction/repression.

<b>Table 33: Chip hybridization of spleen tissue: evaluation of data</b>			
<b>Ranked Genes (According Lowest of 10 Ratios)</b>	<b>Non- uniform Patterns</b>	<b>NDE (False Positives) p&lt;0.05</b>	<b>Fold Induction (Minimum of 10 Chips)</b>
1 - 20	20	≥ 0	11.13 – 1.48
1 - 40	40	≥ 0	11.13 – 1.33

According to 100% non-reproducible chip data of the top 40 ranked genes, no gene with differential expression in spleen of mutant mice was observed in all experiments.

#### Expression of *Ptdsr* in Spleen

In individual 136, 137, 138 and 139 *Ptdsr* was detected without any regulation with a fold induction range between 0.94 – 1.63. No expression for *Ptdsr* was found in sample 135. Signals on these spots in experiments of individual 135 were below detection thresholds.

#### **Analysis of Gene Expression in Heart Tissue**

Table 34 summarizes the results of 10 chip hybridizations performed with RNA from heart. In total, 5336 probes showed signals in all 10 chip hybridizations.

<b>Table 34: Chip hybridization of heart: labeling and number of detected spots</b>		
Numbers indicate the ID of mutant mice.		
<b>Chip ID</b>	<b>Cy5/Cy3</b>	<b>Detected Spots</b>
#1	135 / ref	11763
#2	ref / 135	13451
#3	136 / ref	13105
#4	ref / 136	11918
#5	137 / ref	13107
#6	ref / 137	11442
#7	138 / ref	16694
#8	ref / 138	11379
#9	139 / ref	16267
#10	ref / 139	15304
		<b>5336 overlap</b>

Genes were evaluated for the significance of differential gene expression. Genes were ranked according the lowest absolute ratio of signal intensities (*Ptdsr*-mutant mice versus reference) in 10 microarray experiments (Table 35). This ranking is independent of the reproducibility in terms of up- and

down-regulation. The number of genes with non-reproducible up- or down-regulation („non-uniform patterns“) is given for different selections of genes in the ranking („ranked genes“). The number of non-differentially expressed genes („NDE, false positives“) among genes with reproducible patterns was calculated for significance level  $p < 0.05$ .

For example, the selection of the top 20 ranked genes with reproducible up- or down-regulation contains 19 genes with non-reproducible chip data. The remaining gene is non-differentially expressed with a significance level  $p < 0.05$ . The minimal ratios of expression for this selection ranged from 43.45 to 1.67 fold induction/repression.

<b>Table 35: Chip hybridization of heart tissue: evaluation of data</b>			
<b>Ranked Genes (According Lowest of 10 Ratios)</b>	<b>Non-uniform Patterns</b>	<b>NDE (False Positives) <math>p &lt; 0.05</math></b>	<b>Fold Induction (Minimum of 10 Chips)</b>
1 - 20	19	$\geq 1$	43.45 – 1.67
1 - 40	38	$\geq 2$	43.45 – 1.57
1-100	98	$\geq 2$	43.45 – 1.45

According to 98% non-reproducible chip data of the top 100 ranked genes, no gene with differential expression in heart of *Ptdsr*-mutant mice was observed in all experiments.

### Expression of *Ptdsr* in Heart

Expression of *Ptdsr* was detected in all individuals but without any regulation.

### **3.10.5 Discussion**

Inspection of expression data of thymus from individual mice revealed non-correlation of the expression patterns between the single individuals. A stronger correlation of up-regulated genes in samples 135, 136 and 139 was found in (Fig. 7). Anti-correlation of gene expression was found in the other samples. Maybe biological variability in gene expression, oscillation or stress responsive genes is a potential reason 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; Seltmann *et al.*, 2005).

In **thymus** expression data from individual 135, 136 and 139 revealed a stronger correlation of up-regulated genes. Several over-expressed genes with reproducible regulation in these individuals were selected; maybe they are of interest for the *Ptdsr* mutant mouse line.

Up-regulation of *Thrsp* and *Scp3* was detected in thymus. Over-expression of *Thrsp* was associated with a significant increase in cell death (Haas *et al.*, 2005). The *Scp3* gene is involved in apoptotic cell death during meiotic prophase (Yuan *et al.*, 2000).

Expression of the *Ptdsr* gene was detected in all analyzed organs but - except of heart - not in all of the individuals. For thymus only individual 135 and 138 and for brain 136, 137 and 138 showed expression of *Ptdsr*. For spleen no expression could be detected for individual 135. In all analyzed organs was no differential gene expression found for *Ptdsr*. Maybe these organs do not express the knock-out affected gene differently to the wild-type tissue; otherwise we would have expected a signal in the wild-type samples.

Using the selection criteria described above, we could identify a number of genes that are differentially expressed in **thymus** of the *Ptdsr*-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 the 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 focuses on the determination of food and energy uptake under *ad libitum* conditions and metabolic adaptations during food restriction and serves as the origin for further investigations in the Secondary and Tertiary screening which go into details of energy expenditure and energy storage.

In the primary metabolic screen mice were first fed under *ad libitum* conditions for two weeks, followed by a period of food restriction to 60% of *ad libitum* for seven days to analyze adaptive responses of metabolism. The primary metabolic screen focuses on investigation of metabolic demands of mice determining daily body weight, energy uptake, metabolizable energy and body temperature and adaptive capacity of metabolic processes. No genotype-specific difference was found which would indicate a metabolic phenotype. Common sex-specific differences concerning body weight were found in both genotype indicating males heavier than females. Furthermore females of both genotypes showed higher body temperature compared to males. Taking body weight into account energy uptake and metabolized energy were increased in females.

### 3.11.2 Mice

Seven adult control males and six adult mutant males entered the Metabolic Screen at the beginning of calendar week 46 in 2004. The females (seven control and seven mutants) entered the metabolic laboratory one week later. The mice were single caged on grid panels (0.5 cm grid hole diameter). They were fed *ad libitum* for a period of 14 days, followed by a period of food restriction to 60% of *ad libitum* for seven days to analyze adaptive responses of metabolism.

### 3.11.3 Material and Methods

#### Recorded Data

During the different feeding regimes body weight, food consumption ( $F_{\text{con}}$ ), rectal temperature ( $T_{\text{re}}$ ), daily feces production ( $F_{\text{ec}}$ ), energy uptake ( $E_{\text{up}}$ ),

energy content of the feces ( $E_{fec}$ ), metabolizable energy ( $E_{met}$ ) and the food assimilation coefficient ( $F_{ass}$ ) were recorded.

### Analysis of Feces

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

### Statistical Analysis

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

## 3.11.4 Parameters

Recorded Data during the different feeding regimes
--

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

## 3.11.5 Results

**Genotype-specific** differences were only determined in food assimilation indicating a slightly elevated energy extraction from chow in mutant males. This increase was linked to slightly lower feces production with similar energetic content of feces. Body weight, food and energy uptake, energy utilization and body temperature were not affected by the mutation.

Common **sex-specific** differences in body weight were found in wild-type control and mutant mice. Males were significantly heavier, although they were not consuming more food. Taking body weight into account, the females of both genotypes showed even higher energy uptake and higher ratio of metabolized energy. Also body temperature of females of both genotypes showed significantly elevated values.

During food restriction females of both genotypes increased food assimilation which is typically for C57BL/6J mice. In males the energetic content of feces increased, causing a slight decrease of food assimilation coefficient.

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

### 3.11.6 Discussion

No information about metabolic parameters were available prior the metabolic screening of the Ptdsr mutant mouse line. Beside some sex-specific differences, mice of both genotypes showed similar body weight, body temperature and food and energy uptake. Even food restriction to 60% of the *ad libitum* amount could not provoke a genotype-specific difference. Hence, we could not find a genotype-associated metabolic phenotype.

### 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 36: Metabolic parameters recorded in the primary metabolic screen**

Data are presented as mean  $\pm$  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)	p - value	(n=7)	(n=7)	(n=6)	(n=7)	p - value	(n=6)	(n=7)	p - value	p - value
<b>Body weight [g]</b>	32.8 $\pm$ 1.31	24.3 $\pm$ 1.05	< 0.001	26.9 $\pm$ 1.84	20.7 $\pm$ 0.91	30.5 $\pm$ 0.33	25.8 $\pm$ 1.18	< 0.01	25.1 $\pm$ 0.9	21.6 $\pm$ 1.21	n.s.	n.s.
<b>Rectal body temperature [°C]</b>	36.6 $\pm$ 0.09	37.2 $\pm$ 0.06	< 0.001	34.9 $\pm$ 0.3	34.7 $\pm$ 0.4	36.7 $\pm$ 0.09	37.2 $\pm$ 0.07	< 0.001	34.3 $\pm$ 0.55	34.9 $\pm$ 0.33	n.s.	n.s.
<b>Food consumption [g day<sup>-1</sup>]</b>	3.43 $\pm$ 0.3	3.44 $\pm$ 0.14	n.s.	60% of <i>ad libitum</i>		3.18 $\pm$ 0.32	3.59 $\pm$ 0.05	n.s.	60% of <i>ad libitum</i>		n.s.	n.s.
<b>Energy uptake [kJ day<sup>-1</sup>]</b>	63.3 $\pm$ 5.49	59.8 $\pm$ 4.24	n.s.	36.2 $\pm$ 3.32	35.9 $\pm$ 2.55	58.7 $\pm$ 5.89	62.5 $\pm$ 0.99	n.s.	35.2 $\pm$ 3.54	37.5 $\pm$ 0.59	n.s.	n.s.
<b>Energy uptake BW<sup>-1</sup> [kJ g<sup>-1</sup> day<sup>-1</sup>]</b>	1.91 $\pm$ 0.12	2.62 $\pm$ 0.22	< 0.001	1.33 $\pm$ 0.05	1.85 $\pm$ 0.13	1.93 $\pm$ 0.2	2.46 $\pm$ 0.12	< 0.05	1.39 $\pm$ 0.09	1.77 $\pm$ 0.09	n.s.	n.s.
<b>Feces production [g day<sup>-1</sup>]</b>	0.61 $\pm$ 0.06	0.64 $\pm$ 0.02	n.s.	0.33 $\pm$ 0.03	0.34 $\pm$ 0.01	0.52 $\pm$ 0.05	0.62 $\pm$ 0.01	n.s.	0.36 $\pm$ 0.04	0.32 $\pm$ 0.01	n.s.	n.s.
<b>Energy content feces [kJ g<sup>-1</sup>]</b>	15.9 $\pm$ 0.1	15.8 $\pm$ 0.71	n.s.	16.5 $\pm$ 0.19	15.7 $\pm$ 0.05	15.8 $\pm$ 0.12	15.84 $\pm$ 0.12	n.s.	16.4 $\pm$ 0.23	15.8 $\pm$ 0.03	n.s.	n.s.
<b>Metabolized energy [kJ day<sup>-1</sup>]</b>	52.4 $\pm$ 4.51	52.0 $\pm$ 2.1	n.s.	30.0 $\pm$ 2.82	32.0 $\pm$ 1.35	49.4 $\pm$ 5.02	51.5 $\pm$ 0.9	n.s.	28.7 $\pm$ 3.17	31.7 $\pm$ 0.67	n.s.	n.s.
<b>Metabolized energy [kJ g<sup>-1</sup> day<sup>-1</sup>]</b>	1.58 $\pm$ 0.09	2.15 $\pm$ 0.08	< 0.001	1.1 $\pm$ 0.05	1.55 $\pm$ 0.05	1.62 $\pm$ 0.17	2.02 $\pm$ 0.1	n.s.	1.13 $\pm$ 0.08	1.49 $\pm$ 0.09	n.s.	n.s.
<b>Food assimilation coefficient [%]</b>	82.9 $\pm$ 0.36	82.0 $\pm$ 0.24	n.s.	82.8 $\pm$ 0.24	84.0 $\pm$ 0.43	84.0 $\pm$ 0.36	82.3 $\pm$ 0.36	< 0.01	81.4 $\pm$ 0.54	84.5 $\pm$ 0.7	< 0.05	n.s.

## 3.12 Pathology Screen

### 3.12.1 Summary

The Pathology screen performed a complete morphological analysis with standard stains. Microphthalmia, lymphoblastic T-cell lymphoma, and focal testicular degeneration have been found in a few mutant mice; however, these findings are considered as secondary results. In general, we did not find any genotype-specific pathological alterations.

### 3.12.2 Mice

A total of 48 mice, 24 mutants (15 females, 9 males), and 24 control animals (15 females, 9 males) were analyzed. Due to the workflow in the GMC, mice of different ages were received from different screens (Table 37).

Table 37: <i>Ptdsr</i> -mutant mice and their control littermates analyzed.						
Origin	Control		Mutant		Number of Animals	Age [weeks]
	Female	Male	Female	Male		
Lung Screen	5	0	5	0	10	16
Dysmorphology Screen	3	3	3	3	12	19 - 22
Metabolic Screen	7	6	7	6	26	21 - 22
Total Number of Animals	15	9	15	9	48	

### 3.12.3 Materials and Methods

Mice received in the laboratory of pathology were sacrificed with CO<sub>2</sub>. The animals were analyzed macroscopically and weighed ([www.eulep.org/Necropsy\\_of\\_the\\_Mouse/index\\_2004.php](http://www.eulep.org/Necropsy_of_the_Mouse/index_2004.php)). The thymus and left lobe of the liver were measured. Blood samples were taken, centrifuged and the serum was saved at -20°C. Tails were preserved at -70°C for further genetic analysis. Following a complete dissection, an x-ray of the complete bone structure was taken, when indicated (Hewlett Packard, Cabinet X-Ray System Faxitron Series). All organs were fixed in 4% buffered formalin and embedded in paraffin for histological examination. Two-μm-thick sections from skin, heart, 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). Additionally, Masson's trichrome staining was used in four and Congo red in two cases.

### 3.12.4 Results

#### Overview

<b>Table 38: Morphological alterations of <i>Ptdsr</i>-mutant mice compared to their litter mates.</b>			
<b>Organ</b>	<b>Alteration</b>	<b>Organ</b>	<b>Alteration</b>
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

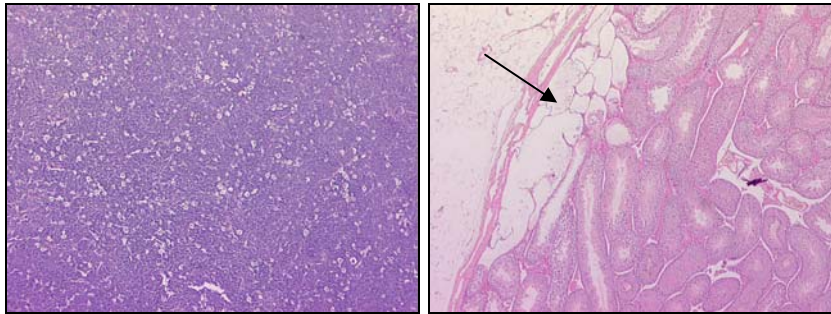
#### Body Weight

The body weight of *Ptdsr*-mutant mice does not differ significantly from that of their control littermates (see Table 39 for more details).

<b>Table 39: Mean body weight <math>\pm</math> standard deviation of <i>Ptdsr</i>-mutant mice and their control littermates.</b>					
<b>Origin</b>	<b>Control</b>		<b>Mutant</b>		<b>Age [weeks]</b>
	<b>Female</b>	<b>Male</b>	<b>Female</b>	<b>Male</b>	
<b>Lung Screen</b>	21.2 $\pm$ 1.0	-	20.6 $\pm$ 1.7	-	16
<b>Dysmorphology Screen</b>	25.0 $\pm$ 2.0	35.0 $\pm$ 2.0	23.3 $\pm$ 0.5	32.7 $\pm$ 1.7	19 - 22
<b>Metabolic Screen</b>	24.7 $\pm$ 1.5	30.5 $\pm$ 1.6	25.7 $\pm$ 3.0	30.7 $\pm$ 1.7	21 - 22

**Testes:** Two mutant mice had focal cystic tubular degeneration of the testes. This type of alteration does not affect the fertility.

**Thymus:** A lymphoblastic T-cell lymphoma was found in a mutant mouse.

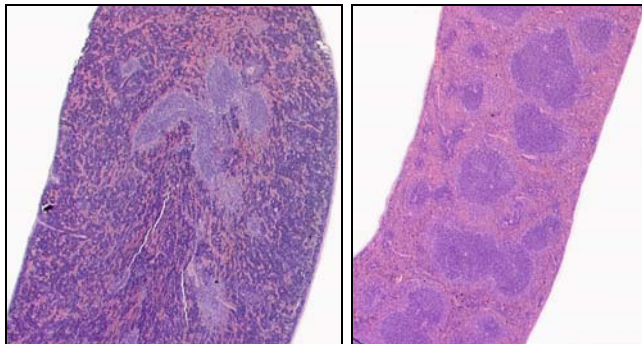


**Figure 8: Histological analysis of thymus and testis tissue.**

The left panel shows the lymphoblastic T-cell lymphoma in the thymus (40x H&E), and the right panel displays the focal degeneration (arrow) of the testis (31.25x H&E).

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**Spleen:** All mice received from the metabolic screen showed severe erythropoiesis in the spleen.



**Figure 9: Histological analysis of spleen tissue.**

The left panel demonstrates the severe erythropoiesis in the spleen, compared to a normal spleen on the right side (25x H&E).

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### 3.12.5 Discussion

The eyes and the coat color have been carefully examined during dissection. However, abnormalities could not be observed, with the exception of a single mutant mouse, which had microphthalmos (please see Eye Screen, 3.4.5). Microphthalmia and anophthalmia occur in 8-20% of the background strain C57BL/6 (Dagg, 1966); therefore, this finding lacks any genotype-related significance.

The lymphoblastic T-cell lymphoma of a mutant mouse and the focal tubular degeneration of the testes of two mutants are secondary results and not genotype-related ( $p=0.49$ , measured by Fisher's exact test). This kind of focal testicular alterations does not affect the fertility.

The severe erythropoiesis observed in the spleen is usually secondary to blood withdrawal several days before the mice are sacrificed and; therefore, represents reactive erythropoiesis.

In conclusion, despite the fact that three *Ptdsr*-mutant mice showed some interesting histological results, a clear phenotype could not be attested.

### **3.12.6 References**

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## Addresses of screeners and modules

### Coordinators

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

### Behavior Screen

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

### Dysmorphology Screen,

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

## Neurology Screen

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

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

## Eye Screen

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

## Clinical-Chemical Screen

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

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

## **Immunology Screen**

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

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

## **Allergy Screen**

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

## **Nociceptive Screen**

Dr. Ildiko Racz  
Laboratory of Molecular Neurobiology  
Department of Psychiatry  
University of Bonn  
Sigmund-Freud-Straße 25,  
D-53105 Bonn  
Tel.: 0228-287-9578  
Fax: 0228-287-9125  
Email: [iracz@uni-bonn.de](mailto:iracz@uni-bonn.de)

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

## **Lung Function Screen**

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

## **Expression Profiling**

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

## **Metabolic Screen**

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

## **Pathology Screen**

Dr. Julia Calzada-Wack

Sandra Kunder

Gabriele Hölzlwimmer

PD Dr. Leticia Quintanilla-Fend

GSF - National Research Center for Environment and Health

Institute of Pathology

Ingolstädter Landstraße 1

D-85764 Neuherberg

Tel.: 089-3187-2312

089-3187-3241

Fax 089-3187-3360

Email: [calzada@gsf.de](mailto:calzada@gsf.de)

[sandra.kunder@gsf.de](mailto:sandra.kunder@gsf.de)