

The

# GERMAN MOUSE CLINIC

## Report for Dmbt1

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# 1 Summary

**In a primary screen** 57 Dmbt1-deficient and control mice have been analyzed in the German Mouse Clinic (GMC) in the screens Behavior, Dymorphology, Bone and Cartilage, Neurology, Eye, Clinical Chemistry, Immunology, Allergy, Nociception, Lung Function, Metabolism, and a comprehensive Pathology.

In our primary screen, we could not find any profound differences between wild-type and Dmbt1-deficient mice. Since the observed effects were only subtle, no secondary screens are suggested at present.

Please contact Valérie Gailus-Durner to discuss details.

## 2 General Part

### 2.1 The Role of the Gene

According to the summary of the owner, Dmbt1 (Crp-ductin) codes for a large scavenger receptor cysteine-rich (SRCR) protein. It is thought to be multifunctional.

Possible functions in:

- the cellular immune response,
- gallstone formation,
- determination of cell polarity,
- epithelial differentiation.

Putative functions in:

- bacterial and viral pathogen-defense,
- epithelial protection,
- damage-response of tissues.
- candidate tumor suppressor.
- putative anti-caries factor.

Possible disease models are:

- Confirmed: epithelial protection, acute colitis,
- In progress: chronic colitis (Ulcerative colitis).

### 2.2 Known Phenotypes

DMBT1-KO mice seem to develop normally, are viable and fertile. It is assumed that they show increased susceptibility to infectious and noxious agents, and are potentially responsive for nutritional effects (sugar and/or lipid, especially cholesterol-content).

All further findings we consider as new.

### 2.3 Mice

#### 2.3.1 Number and kind of mice

One-hundred and three animals arrived in good conditions at 30.09.2003 in the German Mouse Clinic (GMC). As stated in the seminar which was held to introduce the mutant, the mice analyzed are on a 50% C57BL/6 and 50% SV129 genetic background. The mice arrived in good condition. To be able to analyze as many males as possible for all behavior parameters, heterozygous males were also delivered. All 57 animals (-/- mutant and +/+ wild-type littermates) were used for the tests.

### **2.3.2 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  $22 \pm 1^\circ\text{C}$  and  $55 \pm 5\%$ , respectively. In specified modules husbandry conditions are adjusted according to the experiment requirements (See corresponding sections).

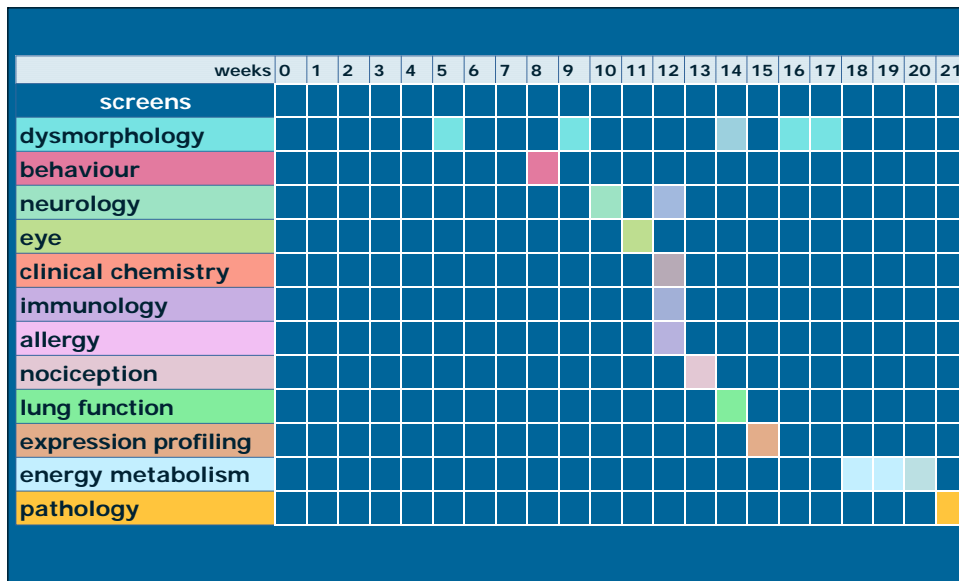
Mice are kept according to the German laws. Tests were carried out by authority of the Regierung von Oberbayern. All people attending the facility completely change their garment (jackets and trousers autoclaved) and shoes and wear caps and masks before entering the GMC (Brielmeier *et al.*, 2004).

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)).


## **2.4 Workflow**

### **2.4.1 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 workflow (Fig. 1). An overview about the screens of the GMC and the used techniques is given in Table. 1.



**Figure 1: Workflow of the primary screen**

Explanation below;  Analysis of blood-related 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 (5 males / 5 females) and 10 controls (5 males / 5 females) leave the animal facility for the Lung Function Analysis, which for technical reasons is located elsewhere. These animals are, for hygienic reasons, not allowed to re-enter the German Mouse Clinic. The females go directly to Pathology. The males are used to freeze organs for future expression profiling on demand (remaining organs from those animals are analyzed by the Pathology). All other animals go through the bone and cartilage tests of the Dysmorphology Screen, and then stay three weeks in the Metabolic Screen. After completion of the primary screen, all animals end up in the Pathology.

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

**Table 1: Primary Screen at GMC**

<b>Screens</b>	<b>Goal</b>	<b>Test</b>
<b>Dysmorphology, Bone and Cartilage</b>	morphological analysis of body, skeleton, bone and cartilage	morphological analysis, bone densitometry, X-ray, AVL analyzer, 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, 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
<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 histological examination	histology, immunochemistry

### 2.4.2 Applied screens

The GMC standard workflow as described above was applied to analyze the DMBT1 mice.

Additional wild-type mice were analyzed in the lung function screen. As the demanded number of 60 animals (15 mice per sex per genotype) could not be delivered, the workflow was adapted to the available number of animals. Some parameters from 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. A few animals died during the primary screen and thus they could not be analyzed for all parameters.

## 2.5 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 means  $\pm$  standard error of the mean. Significant differences are indicated stepwise from 0.05, 0.02, 0.01, 0.001 to 0.0001.

## 2.6 References

Brielmeier M., H. Fuchs, G. Przemeck, V. Gailus-Durner, M. Hrabé de Angelis, J. Schmidt (2004) The GSF – Phenotype Analysis Center (German Mouse Clinic, GMC): A sentinel-based health-monitoring concept in a multi-user unit for standardized characterization of mouse mutants. Lab Animals, in press.

### Abbreviations

Dmbt1	Crp-ductin, coding for a SRCR protein
GMC	German Mouse Clinic
IVC	individually ventilated cage
+/+	homozygote wild type
+/-	heterozygote mutant
-/-	homozygote mutant
KO	knockout
FELASA	European Laboratory Animal Science Associations, 25 Shaftesbury Avenue, London W1D 7EG, UK, <a href="http://www.felasa.org">www.felasa.org</a>

## 3 Specific part

### 3.1 Behavior Screen

#### 3.1.1 Summary

The modified hole board test is used as primary screen in the behavioral phenotyping module of the GMC, because it allows the comprehensive analysis of a range of behavioral parameters known to be indicative of behavioral dimensions such as locomotor activity, exploratory behavior, arousal, emotionality, memory and social affinity in a single short test (Ohl *et al.*, 2001).

In the case of *Dmbt1* KO mice, genotype-specific changes were only observed in males. We observed an increased response latency to initiate exploration, also an increased response latency to explore the familiar object, as well as non-significant tendencies towards increased board entry latency and higher object recognition index in *Dmbt1*-KO males.

These results suggest that *Dmbt1*-KO males are more hesitant or cautious than their controls, which in turn might result in tendentially improved object recognition memory. However, these effects are subtle.

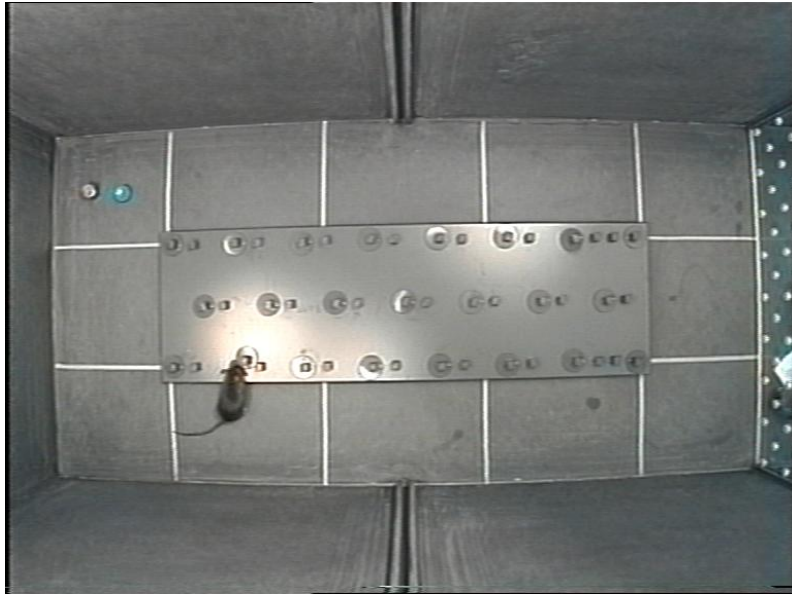
#### 3.1.2 Mice

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 wt, 15 mutants) and 27 male mice (17 wt, 10 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 Ohl *et al.*, 2001. The test apparatus consisted of a test arena (100 x 50 cm), in the middle of which a board (60 x 20 x 2 cm) with 23 holes (1.5 x 0.5 cm) staggered in three lines with all holes covered by movable lids was placed, thus representing the central area of the test arena as an open field. The area around the board was divided into 12 similarly sized quadrants by lines taped onto the floor of the box (See Ohl *et al.*, 2001). Both box and board were made of dark grey PVC. All lids were closed before the start of a trial. For each trial, an unfamiliar object (a blue plastic tube lid, similar in size to the metal cube) and the familiar object (metal cube) were placed into the test arena with a distance of 2 cm between them. The illumination levels were set at approximately 150 lux in the corners and 200 lux in the middle of the test arena.



**Figure 2: Test Arena for modified hole board test.**

For testing, each animal was placed individually into the test arena and allowed to explore it freely for 5 min. The animals were always placed into the test arena in the same corner next to the partition, facing the board diagonally. The two objects were placed in the corner quadrant diametrical to the starting point. During the 5 min trial, the animal's behavior was recorded by a trained observer with a hand-held computer. Data were analyzed by using the Observer 4.1 Software (Noldus, Wageningen). Additionally, a camera was mounted 1.20 m above the center of the test arena, and the animal's track was videotaped and its locomotor path analyzed with a video-tracking system (Ethovision 2.3, Noldus, Wageningen). After each trial, the test arena was cleaned carefully with a disinfectant.

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

### **3.1.4 Results**

Analysis of the observed behavioral parameters showed an increased latency to initiate exploration, also an increased latency to explore the familiar object, as well as non-significant tendencies towards increased board entry latency and higher object recognition index in Dmbt1 KO males only.

In general, females were more active (increased number of line crossings and total distance moved) and more anxious (reduced time spent on board) than males, but that was no genotype effect. All other parameters were not altered.

### **3.1.5 Discussion**

The observed alterations in Dmbt1 KO males were subtle, and indicate primarily that Dmbt1 KO males were more cautious than their wild-type littermates. It could well be that a similar difference in Dmbt1 KO females was just

not measurable with the methods applied here, because all females, independent of genotype, were more anxious. The increased cautiousness, which can reflect an enhanced perception and observation of environmental cues, is most likely the reason for the tendency towards improved object recognition in Dmbt1 KO males.

Since the observed effects were only subtle, no secondary screens for behavior are suggested at present.

### **3.1.6 Reference**

Ohl, F., Sillaber, I., Binder, E., Keck, M.E. & Holsboer, F. (2001) Differential analysis of behavior and diazepam-induced alterations in C57BL/6N and BALB/c mice using the modified hole board test. *J. Psychiatr. Res.* 35: 147-154.

**Table 2: Results of Behavioral Observation in the Modified Hole Board Test**

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

Parameter	Wild Type (A)			Mutant (B)			A-B	A-B
	Male	Female		Male	Female		Male	Female
	(n=17)	(n=15)	<i>p - value</i>	(n=10)	(n=15)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
Line crossing [frequency]	113.18 $\pm$ 8.4	142.33 $\pm$ 7.26	N.A.	109.8 $\pm$ 8.75	130.8 $\pm$ 8.03	N.A.	n.s.	n.s.
Line crossing [latency]	0.66 $\pm$ 0.1	1.28 $\pm$ 0.43	N.A.	2.88 $\pm$ 0.88	1.39 $\pm$ 0.46	N.A.	p<0.05	n.s.
Rearings in box [frequency]	25.47 $\pm$ 3.03	24.2 $\pm$ 2.49	N.A.	21.7 $\pm$ 3.48	20.67 $\pm$ 1.88	N.A.	n.s.	n.s.
Rearings in box [latency]	34.71 $\pm$ 5.58	31.12 $\pm$ 4.96	N.A.	44.4 $\pm$ 10.57	45.87 $\pm$ 6.15	N.A.	n.s.	n.s.
Hole exploration [frequency]	24.29 $\pm$ 3.64	1.79 $\pm$ 1.79	N.A.	20.6 $\pm$ 4.17	14.27 $\pm$ 1.71	N.A.	n.s.	n.s.
Hole exploration [latency]	32.15 $\pm$ 8.11	71.22 $\pm$ 12.79	N.A.	37.55 $\pm$ 10.12	61.24 $\pm$ 18.66	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]	7.76 $\pm$ 1.29	3.8 $\pm$ 0.6	N.A.	8.4 $\pm$ 3.02	4.33 $\pm$ 0.71	N.A.	n.s.	n.s.
Board entry [latency]	84.13 $\pm$ 29.1	107.03 $\pm$ 18.71	N.A.	138.66 $\pm$ 31.86	115.29 $\pm$ 25.6	N.A.	p=0.06	n.s.
Board entry [total duration %]	9.71 $\pm$ 1.62	5.17 $\pm$ 0.89	N.A.	9.53 $\pm$ 2.72	5.37 $\pm$ 0.87	N.A.	n.s.	n.s.
Rearing on board [frequency]	0.06 $\pm$ 0.06	0 $\pm$ 0	N.A.	0.5 $\pm$ 0.4	0 $\pm$ 0	N.A.	n.s.	n.s.
Rearing on board [latency]	288.82 $\pm$ 11.18	300 $\pm$ 0	N.A.	270.02 $\pm$ 24.9	300 $\pm$ 0	N.A.	n.s.	n.s.
Risk assessment [frequency]	0 $\pm$ 0	0 $\pm$ 0	N.A.	0.3 $\pm$ 0.3	0.07 $\pm$ 0.07	N.A.	n.s.	n.s.

Parameter	Wild Type (A)			Mutant (B)			A-B	A-B
	Male	Female		Male	Female		Male	Female
Group contact [frequency]	11.06 ± 1	13 ± 0.83	N.A.	11.7 ± 1.08	14.13 ± 0.88	N.A.	n.s.	n.s.
Group contact [latency]	18,6 ± 2,41	15,13 ± 2,63	N.A.	11,33 ± 3,38	16,4 ± 4,56	N.A.	n.s.	n.s.
Group contact [total duration %]	25,36 ± 2,77	25,39 ± 2,46	N.A.	20,49 ± 2,79	26,63 ± 3,32	N.A.	n.s.	n.s.
Grooming [frequency]	0.94 ± 0.22	1.13 ± 0.19	N.A.	0.8 ± 0.13	1.4 ± 0.39	N.A.	n.s.	n.s.
Grooming [latency]	220.25 ± 18.37	219.93 ± 14.68	N.A.	205.06 ± 0	246.29 ± 16.36	N.A.	n.s.	n.s.
Grooming [total duration %]	1,99 ± 0,62	2.47 ± 0.66	N.A.	2.49 ± 0.69	2.58 ± 1	N.A.	n.s.	n.s.
Defecation [frequency]	0 ± 0	0.13 ± 0.09	N.A.	0.1 ± 0.1	0.07 ± 0.07	N.A.	n.s.	n.s.
Defecation [latency]	300 ± 0	274.85 ± 19.94	N.A.	296.7 ± 3.3	289.77 ± 10.23	N.A.	n.s.	n.s.
Unfamiliar object exploration [frequency]	3.76 ± 0.48	4.27 ± 0.46	N.A.	3.8 ± 0.53	4.87 ± 0.62	N.A.	n.s.	n.s.
Familiar object exploration [frequency]	4.76 ± 0.56	4.73 ± 0.33	N.A.	3.8 ± 0.98	4.13 ± 0.68	N.A.	n.s.	n.s.
Unfamiliar object exploration [latency]	58.78 ± 22.1	26.16 ± 5.46	N.A.	59.16 ± 15.98	38.32 ± 11.46	N.A.	n.s.	n.s.
Familiar object exploration [latency]	36.69 ± 12.54	41.47 ± 6.39	N.A.	74.96 ± 18.93	74.63 ± 23.11	N.A.	p<0.05	n.s.
Unfamiliar object exploration [total duration %]	1.21 ± 0.15	1.57 ± 0.26	N.A.	0.89 ± 0.1	1.04 ± 0.08	N.A.	n.s.	n.s.
Familiar object exploration [total duration %]	1.43 ± 0.27	1.93 ± 0.27	N.A.	0.81 ± 0.19	1.07 ± 0.15	N.A.	n.s.	n.s.
Object Index	0.08 ± 0.07	0.12 ± 0.08	N.A.	0.29 ± 0.09	0.3 ± 0.08	N.A.	p=0.06	n.s.

**Table 3: Video-Tracking Results Regarding Locomotor Behavior**

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

Parameter	Wild Type (A)			Mutant (B)			A-B	A-B
	Male	Female		Male	Female		Male	Female
	(n=17)	(n=15)	<i>p</i> -value	(n=10)	(n=15)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
<b>Total distance moved [cm]</b>	3090,69 $\pm$ 206,51	3399,37 $\pm$ 150,3	N.A.	2923,85 $\pm$ 208,92	3280,92 $\pm$ 190,08	N.A.	n.s.	n.s.
<b>Mean velocity [cm/sec]</b>	19,02 $\pm$ 0,72	21,21 $\pm$ 0,73	N.A.	17,85 $\pm$ 0,98	20,03 $\pm$ 0,81	N.A.	n.s.	n.s.
<b>Maximum velocity [cm/sec]</b>	67,22 $\pm$ 4,99	65,17 $\pm$ 4,3	N.A.	60,65 $\pm$ 3,7	57,47 $\pm$ 2,63	N.A.	n.s.	n.s.
<b>Turns [Frequency]</b>	1632,59 $\pm$ 70,39	1700,31 $\pm$ 48,72	N.A.	1562,46 $\pm$ 88,91	1715,14 $\pm$ 56,36	N.A.	n.s.	n.s.
<b>Board entry [max. duration, sec.]</b>	7,03 $\pm$ 1,02	6,73 $\pm$ 1,12	N.A.	6,4 $\pm$ 0,9	6,79 $\pm$ 1,35	N.A.	n.s.	n.s.
<b>Mean distance to wall [cm]</b>	6,93 $\pm$ 0,4	6,38 $\pm$ 0,32	N.A.	6,51 $\pm$ 0,45	6,81 $\pm$ 0,36	N.A.	n.s.	n.s.
<b>Mean distance board [cm]</b>	9,07 $\pm$ 0,3	9,19 $\pm$ 0,25	N.A.	8,72 $\pm$ 0,45	8,88 $\pm$ 0,37	N.A.	n.s.	n.s.

## 3.2 Dysmorphology, Bone and Cartilage

### 3.2.1 Summary

A total of 54 animals of *Dmbt1* mice have been analyzed in the Dysmorphology, Bone and Cartilage module of the German Mouse Clinic. In the quantitative parameters, significant differences between male mutants and controls were detected in the parameters partial bone mineral density and lean mass. In the morphological investigation via visual inspection in single individuals some minor phenotypes were found, however there was no linkage of a phenotype to a certain genotype.

### 3.2.2 Mice

Twenty-four male (12 mutant, 12 wild-type) and 30 female (15 mutant, 15 wild-type) 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 22 knockout (KO) and 21 control animals, and 16-week-old KOs (17 animals) and controls (16 animals) entered the bone density and X-ray analysis.

### 3.2.3 Material and Methods

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

1. At the age of 5 weeks, when the mice entered the facility, the general physical condition and health were checked;
2. At the age of 9 weeks, a morphological whole-body checkup was carried out;
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 Test

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

#### 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.

### **Statistics**

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

## **3.2.4 Results and Discussion**

In the morphological inspection of the mice, some minor phenotypes were discovered, however, most of them were found in mutants and controls (Tab. 4), and therefore no linkage to a certain genotype could be detected.

In the quantitative parameters of the Dysmorphology Screen (Tab. 5), significant differences between male mutants and controls were detected in the parameters partial bone mineral density (pBMD), lean mass and femoral muscles span. The male mutants showed higher values in most bone mineral parameters. In BMD, pBMD and BMC their values exceeded the expected range for a standard background. The lean mass of male mutants was significantly reduced. However, variation was very high within the data sets, therefore we cannot assign any biological meaning to the findings. The significant

difference between male mutants and controls in femoral muscles span can be considered as a minor finding.

Raw data will be available on demand.

### **3.2.5 References**

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

### **Abbreviations**

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

<b>Table 4: Results from the Morphological Inspection</b>				
<b>Phenotype</b>	<b>Male</b>		<b>Female</b>	
	<b>wt</b>	<b>KO</b>	<b>wt</b>	<b>KO</b>
Weak reaction to click box	2	-	-	1
Deaf	-	-	1	-
Red Eyes	-	-	1	1
Small Eyes	-	-	-	1
One digit shorter	1	-	-	-
Abnormally thin skin	-	-	-	1
<b>Animals analyzed</b>	<b>12</b>	<b>12</b>	<b>15</b>	<b>15</b>

**Table 5: Bone-Related Quantitative Parameters**Data are presented as mean  $\pm$  standard error of mean.

Parameter	Wild Type (A)			Mutant (B)			Male A~B	Female A~B
	Male (n=6)	Female (n=10)	<i>p</i> -value	Male (n=7)	Female (n=10)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
<b>BMD [mg/ cm<sup>2</sup>]</b>	66 $\pm$ 2	69 $\pm$ 2	n.s.	73 $\pm$ 3	66 $\pm$ 2	n.s.	n.s.	n.s.
<b>pBMD [mg/ cm<sup>2</sup>]</b>	54 $\pm$ 2	56 $\pm$ 1	n.s.	61 $\pm$ 2	55 $\pm$ 2	n.s.	< 0.05	n.s.
<b>sBMD [10<sup>-3</sup>x cm<sup>-2</sup>]</b>	2.03 $\pm$ 0.09	2.67 $\pm$ 0.16	< 0.02	2.13 $\pm$ 0.19	2.40 $\pm$ 0.06	n.s.	n.s.	n.s.
<b>BMC [mg]</b>	707 $\pm$ 56	751 $\pm$ 102	n.s.	957 $\pm$ 184	830 $\pm$ 70	n.s.	n.s.	n.s.
<b>Lean mass [g]</b>	22.58 $\pm$ 1.22	14.98 $\pm$ 1.32	< 0.01	16.39 $\pm$ 2.31	15.40 $\pm$ 1.04	n.s.	< 0.05	n.s.
<b>Fat mass [g]</b>	6.81 $\pm$ 1.48	7.84 $\pm$ 2.26	n.s.	14.77 $\pm$ 4.29	8.84 $\pm$ 1.65	n.s.	n.s.	n.s.
<b>Body Length [cm]</b>	8.7 $\pm$ 0.1	8.7 $\pm$ 0.1	n.s.	8.8 $\pm$ 0.1	8.7 $\pm$ 0.1	n.s.	n.s.	n.s.
<b>Weight [g]</b>	32.73 $\pm$ 1.35	26.23 $\pm$ 1.23	< 0.01	35.15 $\pm$ 2.31	27.68 $\pm$ 0.98	< 0.01	n.s.	n.s.
<b>Bone Content [%]</b>	2.15 $\pm$ 0.13	2.77 $\pm$ 0.27	n.s.	2.59 $\pm$ 0.36	2.97 $\pm$ 0.16	n.s.	n.s.	n.s.
<b>Lean Content [%]</b>	69.25 $\pm$ 3.55	59.95 $\pm$ 7.02	n.s.	50.09 $\pm$ 9.42	56.90 $\pm$ 4.90	n.s.	n.s.	n.s.
<b>Fat Content [%]</b>	20.40 $\pm$ 3.87	27.10 $\pm$ 6.84	n.s.	38.50 $\pm$ 9.62	30.68 $\pm$ 4.71	n.s.	n.s.	n.s.
<b>Femur length [mm]</b>	14.2 $\pm$ 0.3	14.6 $\pm$ 0.2	n.s.	14.1 $\pm$ 0.4	14.8 $\pm$ 0.1	< 0.05	n.s.	n.s.
<b>Femur width [mm]</b>	1.3 $\pm$ 0.1	1.3 $\pm$ 0.1	n.s.	1.2 $\pm$ 0.1	1.3 $\pm$ 0.1	n.s.	n.s.	n.s.
<b>Lumbar vertebra height [mm]</b>	3.3 $\pm$ 0.1	3.4 $\pm$ 0.1	n.s.	3.2 $\pm$ 0.1	3.4 $\pm$ 0.1	< 0.05	n.s.	n.s.
<b>Lumbar vertebra width [mm]</b>	2.8 $\pm$ 0.1	2.8 $\pm$ 0.1	n.s.	2.7 $\pm$ 0.1	2.7 $\pm$ 0.1	n.s.	n.s.	n.s.
<b>Femoral muscles span [mm]</b>	11.1 $\pm$ 0.2	9.7 $\pm$ 0.1	< 0.001	10.4 $\pm$ 0.1	9.6 $\pm$ 0.1	< 0.001	< 0.05	n.s.
<b>Subcutaneous fat span [mm]</b>	3.8 $\pm$ 0.2	4.1 $\pm$ 0.3	n.s.	4.5 $\pm$ 0.2	4.1 $\pm$ 0.2	n.s.	n.s.	n.s.
	<b>(n=10)</b>	<b>(n=11)</b>		<b>(n=10)</b>	<b>(n=7)</b>			
<b>Ionic Calcium [mmol/l]</b>	1.14 $\pm$ 0.04	1.16 $\pm$ 0.02	n.s.	1.18 $\pm$ 0.03	1.17 $\pm$ 0.02	n.s.	n.s.	n.s.

## 3.3 Neurology Screen

### 3.3.1 Summary

In the primary neurological screen 27 Dmbt1-deficient mice (12 males/15 females), 27 Dmbt1 control mice (12 males/15 females) were screened. Animals were analyzed according to our modified SHIRPA protocol where a battery of behavioral tests is carried out. This primary observation screen is a modification of the Irwin procedure (Irwin, 1968) and was proposed as a rapid, comprehensive and semi-quantitative screening method for qualitative analysis of abnormal phenotypes in a mouse strain (Rogers *et al.*, 1994). We carried out 37 of 40 designed test parameters (See web page: [http://www.mgu.har.mrc.ac.uk/mutabase/shirpa\\_summary.html](http://www.mgu.har.mrc.ac.uk/mutabase/shirpa_summary.html)) to detect phenotypic differences between Dmbt1 knockout and control mice. Each test parameter contributes to an overall assessment in muscle, lower motor neuron, spinocerebellar, sensory and autonomic function. The primary neurological screen is focused on investigating neurological reflexes to determine the neurological functioning of a mouse. We also examine lactate levels in the blood of mice to draw conclusions about energy metabolism.

The comparison of Dmbt1 knockout to control mice revealed no obvious neurological phenotype.

### 3.3.2 Mice

Twelve 10-week-old male Dmbt1 knockout and twelve 10-week-old male Dmbt1 control mice entered the neurological screen at the beginning of the 44<sup>th</sup> calendar week. Fifteen 10-week-old female Dmbt1 knockout and fifteen 10-week-old female Dmbt1 control mice entered the neurological laboratory one week later. All animals were fed *ad libitum* for a period of one week during their stay in the neurological screen.

### 3.3.3 Material and Methods

At the age of 10 weeks assessment of each animal started with observation of undisturbed behavior (*Viewing Jar Behavior*) in a glass cylinder (11 cm in diameter). The mice were then transferred to an arena consisting of a clear Perspex box (420 x 260 x 180 mm) in which a Perspex sheet on the floor is marked with 15 squares. In this arena, locomotor activity and motor behavior was observed (*Behavior recorded in the Arena*). This was followed by a sequence of manipulations testing reflexes, grip strength, toe pinch and wire maneuver (*Behavior recorded on or above the Arena*). For the wire maneuver test, a rigid horizontal wire (3 mm in diameter) is secured across the rear right corner of the arena. For grip strength testing, a grid (270 x 275 mm) is secured across the width of the arena. In the last part of the observation (*Behavior recorded during Supine Restraint*), the animals were restrained in a supine position to record autonomic responses such as salivation. Measurements were completed with the recording of limb tone, provoked biting, and body length. The last part of the primary screen also involves the analysis of righting reflex, negative geotaxis and contact righting reflex. A glass cylinder

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

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

### 3.3.4 Results

All SHIRPA test parameters were without significant pathological findings (Tabs. 6-10). Blood lactate screening showed that both male and female *Dmbt1* knockout mice had no significant changes in their blood lactate level as compared to control mice (Tab. 11).

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

### 3.3.5 Discussion

In our neurological screen, male and female *Dmbt1* knockout mice did not show any significant SHIRPA parameter in comparison to controls. Since homozygous *Dmbt1* knockout mice seems to be more susceptible to brain damage (e.g. ischemia, infection, meningitis), defects in CNS connected with a neurological phenotypes might be only detectable after induction of a “challenged situation” for the mice.

### 3.3.6 Reference

Irwin S. (1968) Comprehensive observational assessment: Ia. A systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse. *Psychopharmacologia*. 13(3): 222-257.

Rogers D. C., E.M. Fisher, S.D. Brown, J. Peters, A.J. Hunter, J.E. Martin (1997) Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. *Mamm Genome*. 8(10): 711-713.

### Abbreviations

SHIRPA **S**mithKline Beecham Pharmazeuticals; **H**arwell, MRC Mouse Genome Centre and Mammalian Genetics Unit; **I**mperial Collegeschool of Medicineat St. Mary`s; **R**oyal London Hospital, **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 6: Recording of Body Length and Body Weight**Data are presented as mean  $\pm$  standard error of mean.

Parameter	Male			Female		
	Wild Type (n=12)	Mutant (n=12)	<i>p-value</i>	Wild Type (n=15)	Mutant (n=15)	<i>p-value</i>
<b>Body Length [g]</b>	8.18 $\pm$ 0.02	8.25 $\pm$ 0.02	<i>n.s.</i>	8 $\pm$ 0.01	8 $\pm$ 0.02	<i>n.s.</i>
<b>Body Weight [g]</b>	27.4 $\pm$ 0.19	27.9 $\pm$ 0.26	<i>n.s.</i>	23 $\pm$ 0.15	23 $\pm$ 0.19	<i>n.s.</i>

**Table 7: Behavior Recorded in Viewing Jar**Data shown represents the results of test parameters from major tests where a behavioral response was observed. Test parameters which did not elicit any response were excluded from this data. Statistical analysis: chi-squared test; significance  $p < 0.05$ 

Parameter	Male			Female		
	Wild Type (n=12)	Mutant (n=12)	<i>p-value</i>	Wild Type (n=15)	Mutant (n=15)	<i>p-value</i>
<b>Body Position</b>						
Sitting or standing	12	12	<i>n.s.</i>	15	15	<i>n.s.</i>
<b>Spontaneous Behavior</b>						
Slow movement	1	2		3	0	
Moderate movement	11	10	<i>n.s.</i>	12	15	<i>n.s.</i>
<b>Tremor</b>						
None	11	12		13	14	
Mild	1	0	<i>n.s.</i>	2	1	<i>n.s.</i>

**Table 8: Recording of Locomotor Activity and Behavior in the Arena**

Locomotor activity data are shown as mean ( $\pm$  SEM). Data from behavior recorded in the Arena represent the results of test parameters from major tests where a behavioral response was observed. Test parameters, which did not elicit any response, were excluded from this data. Statistical analysis: chi-squared test; significance  $p < 0,05$

Parameter	Male			Female		
	Wild Type (n=12)	Mutant (n=12)	<i>p-value</i>	Wild Type (n=15)	Mutant (n=15)	<i>p-value</i>
<b>Locomotor Activity</b>	9 $\pm$ 0.4	9 $\pm$ 0.29	<i>n.s.</i>	15.2 $\pm$ 0.4	11.2 $\pm$ 0.35	<i>n.s.</i>
<b>Transfer arousal</b>						
Prolonged freeze	0	0	<i>n.s.</i>	0	1	<i>n.s.</i>
Brief freeze	12	12		15	14	
<b>Palpebral Closure</b>						
Eyes wide open	12	12	<i>n.s.</i>	15	15	<i>n.s.</i>
<b>Piloerection</b>						
None	12	12	<i>n.s.</i>	15	15	<i>n.s.</i>
<b>Gait</b>						
Normal	12	12	<i>n.s.</i>	15	15	<i>n.s.</i>
<b>Pelvic Elevation</b>						
Normal	12	12	<i>n.s.</i>	15	15	<i>n.s.</i>
<b>Tail Elevation</b>						
Horizontally extended	7	6		15	15	
Elevated/Straub tail	5	6	<i>n.s.</i>	0	0	<i>n.s.</i>
<b>Touch Escape</b>						
Mild	4	2		2	1	
Moderate	8	10		12	14	
Vigorous	0	0	<i>n.s.</i>	1	0	<i>n.s.</i>
<b>Positional Passivity</b>						
Struggles when held by tail	12	12	<i>n.s.</i>	15	15	<i>n.s.</i>

**Table 9: Behavior Recorded in or above the Arena**

Data shown represent the results of test parameters from major tests where a behavioral response was observed. Test parameters, which did not elicit any response, were excluded from this data. Statistical analysis: chi-squared test; significance  $p < 0,05$

Parameter	Male			Female		
	Wild Type (n=12)	Mutant (n=12)	<i>p-value</i>	Wild Type (n=15)	Mutant (n=15)	<i>p-value</i>
<b>Trunk Curl</b>						
Absent	12	12	<i>n.s.</i>	15	15	<i>n.s.</i>
<b>Limb Grasping</b>						
Absent	12	12		15	14	
Present	0	0	<i>n.s.</i>	0	1	<i>n.s.</i>
<b>Visual Placing</b>						
Upon nose contact	9	12		14	15	
Upon vibrassee contact	3	0	<i>n.s.</i>	1	0	<i>n.s.</i>
<b>Grip strength</b>						
Moderate grip	11	11		12	12	
Active grip	1	1	<i>n.s.</i>	3	3	<i>n.s.</i>
<b>Body Tone</b>						
No return	2	1		0	0	
Slight resistance	10	11	<i>n.s.</i>	15	15	<i>n.s.</i>
<b>Pinna reflex</b>						
Active retraction	12	12	<i>n.s.</i>	15	15	<i>n.s.</i>
<b>Corneal Reflex</b>						
None	0	0		1	2	
Active single eye blink	12	12	<i>n.s.</i>	14	13	<i>n.s.</i>
<b>Toe Pinch</b>						
Moderate withdrawal	10	12		14	15	
Brisk	2	0	<i>n.s.</i>	1	0	<i>n.s.</i>
<b>Wire maneuver</b>						
Active grip	0	2		1	3	
Difficulty to grasp	7	6		8	7	
Unable to grasp	5	4		6	4	
Unable to lift	0	0		0	1	
Falls immediately	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>

**Table 10: Behavior during Supine Restraint**

Data shown represent the results of test parameters from major tests where a behavioral response was observed. Test parameters, which did not elicit any response, were excluded from this data. Statistical analysis: chi-squared test; significance  $p < 0,05$ .

Parameter	Male			Female		
	Wild Type (n=12)	Mutant (n=12)	<i>p-value</i>	Wild Type (n=15)	Mutant (n=15)	<i>p-value</i>
<b>Skin Color</b>						
Pink	12	12	<i>n.s.</i>	15	15	<i>n.s.</i>
<b>Limb Tone</b>						
No resistance	0	1		0	1	
Slight resistance	12	11	<i>n.s.</i>	15	14	<i>n.s.</i>
<b>Abdominal Tone</b>						
Slight resistance	12	12	<i>n.s.</i>	15	15	<i>n.s.</i>
<b>Lacrimation</b>						
None	12	12	<i>n.s.</i>	15	15	<i>n.s.</i>
<b>Salivation</b>						
None	9	7		7	7	
Slight margin of s.a.	3	5		8	8	
Wet zone entire of s.a.	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>
<b>Provoked biting</b>						
Absent	12	11		14	15	
Present	0	1	<i>n.s.</i>	1	0	<i>n.s.</i>
<b>Righting reflex</b>						
No impairment	12	12	<i>n.s.</i>	15	15	<i>n.s.</i>
<b>Contact righting reflex</b>						
Absent	2	1		0	2	
Present	10	11	<i>n.s.</i>	15	13	<i>n.s.</i>
<b>Negative Geotaxis</b>						
Turns and climbs the grid	12	11		15	15	
Moves, but fails to turn	0	1	<i>n.s.</i>	0	0	<i>n.s.</i>
<b>Irritability</b>						
None						
Struggles during supine restraint	11 1	11 1	<i>n.s.</i>	14 1	13 2	<i>n.s.</i>
<b>Aggression</b>						
None	12	12		12	15	
Provoked biting or attack	0	0	<i>n.s.</i>	3	0	<i>n.s.</i>
<b>Vocalization</b>						
None	2	2		9	9	
Provoked biting or attack	10	10	<i>n.s.</i>	6	6	<i>n.s.</i>
<b>Vocalization</b>						
None	4	7	<i>n.s.</i>	9	13	<i>n.s.</i>
Provoked during handling	10	5		6	2	

**Table 11: Lactate Levels**Data shown represent the results of the mean blood lactate concentrations, value ( $\pm$  SEM)

	Male			Female		
	Wild Type (n=12)	Mutant (n=12)	<i>p-value</i>	Wild Type (n=15)	Mutant (n=15)	<i>p-value</i>
Lactate (mmo/l)	5.9 $\pm 0.46$	6.8 $\pm 0.4$	<i>n.s.</i>	5.3 $\pm 0.34$	5.9 $\pm 0.32$	<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. Furthermore, mice were examined for anterior segment abnormalities by slitlamp biomicroscopy. No significant differences between wild-type and mutant *Dmbt1* mice were detected.

### 3.4.2 Mice

Twenty-seven *Dmbt1* wt (12 male, 15 female) and 27 *Dmbt1* mutant mice (12 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° angles 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 Results

**ERG responses** were recorded from the groups of *Dmbt1* (wild type – 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. At first, a comparison of the left and right eyes for each group was performed on the amplitudes of a- and b-wave for both luminance intensities (data not shown). Since no major differences were observed between the left and right eye, ERG amplitudes of both eyes were averaged for further evaluation. The mean value and standard error was calculated for each group of mice, male and female, wild-type and mutant (Tab. 12).

All animals were examined by **slit lamp biomicroscopy**. There were no major eye abnormalities observed and the minor opacities did not correlate with the genotype of animals. We conclude that there are no effects due to mutation of the *Dmbt1* gene.

### 3.4.5 Discussion

ERG screening is a quick, robust and reproducible *in-vivo* method to detect functional retinal impairment in mice. For the analysis of ERG data, the average amplitudes from left and right eyes was used, as no major differences between the eyes were detected in the ERG response.

Although the comparison of a- and b-wave amplitudes of males and females revealed significant differences, all ERG amplitudes varied in normal, non pathologic ranges and were observed in the group of wild-type and homozygous *Dmbt1* mice. No consistent differences were found between mutant and wild-type *Dmbt1* mice, neither in the male nor in the female group (Tab. 12). No effect due to loss of function of the *Dmbt1* gene could be observed in ERG.

Results from slit lamp biomicroscopy indicated there was no effect on eye phenotype due to loss of function of the *Dmbt1* gene.

### 3.4.6 References

- Favor, J. (1983) A comparison of the dominant cataract and recessive specific-locus mutation rates induced by treatment of male mice with ethylnitrosourea. *Mutation Research* 110: 367-382.
- Dalke C., J. Löster, H. Fuchs, V. Gailus-Durner, D. Soewarto, J. Favor, A. Neuhäuser-Klaus, W. Pretsch, F. Gekeler, K. Shinoda, E. Zrenner, T. Meitinger, M. Hrabé de Angelis, and J. Graw (2004) Electroretinography as a screening method for mutations causing retinal dysfunction in mice. *IOVS* 45: 601-609.

**Table 12: 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	Wild Type (A)			Mutant (B)			A-B	A-B
	Male	Female		Male	Female		Male	Female
	(n=12)	(n=15)	<i>p - value</i>	(n=12)	(n=15)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
<b>a-wave</b> 500 cd/m <sup>2</sup>	-12 ± 1,1	-11 ± 1,5	n.s.	-18 ± 2,2	-11 ± 1,3	<0,01	<0,02	n.s.
<b>b-wave</b> 500 cd/m <sup>2</sup>	192 ± 10,7	164 ± 6,2	<0,05	196 ± 8,8	152 ± 7,2	<0,001	n.s.	n.s.
<b>a-wave</b> 12,500 cd/m <sup>2</sup>	-46 ± 2,6	-32 ± 4,1	<0,001	-41 ± 3,1	-40 ± 2,4	n.s.	n.s.	n.s.
<b>b-wave</b> 12,500 cd/m <sup>2</sup>	249 ± 11,6	182 ± 10,0	<0,001	236 ± 12,9	184 ± 8,5	<0,01	n.s.	n.s.

**Abbreviations**

cd/m<sup>2</sup>      candela per square meter  
 ERG        electroretinography  
 Hz         hertz  
 n.s.        not significant

## 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. 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. In the primary clinical chemical screen, 24 (11 males/13 females) control mice and 25 (11 males /14 females) *Dmbt1*-KO mice were analyzed. Nineteen 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 mutants and control mice were within normal ranges. Differences between mutants and controls were seen in cholesterol level only in males.

### 3.5.2 Mice

Eleven 12-week-old wild-type and eleven 12-week-old mutant males entered the clinical-chemical screen at the beginning of the 46<sup>th</sup> calendar week. Thirteen 12-week-old wild-type and fourteen 12-week-old mutant females entered the screen at the beginning of the 47<sup>th</sup> calendar week.

Out of these, eleven (7 male, 4 female) *DMBT1*-KO mice and nine (5 male, 4 female) control mice were analysed a second time for 10 energy metabolism related parameters after food restriction at the age of 20 weeks.

### 3.5.3 Materials and Methods

#### Blood Withdrawal and Storage

The Clinical-chemical Screen of the German Mouse Clinic routinely analyzed 12-week-old mice. A blood sample was taken from an ether-anesthetized mouse by puncturing the retro-orbital sinus with a non-heparinized capillary (0.8 mm in diameter; Laborteam K&K; Munich, Germany; Art.No. 1.28.13.1.2). The time for sample taking was recorded in a work list. A total of 600 µl blood was collected in a heparinized tube (Li-heparin, KABE; Nümbrecht, Germany; Art.No. 078028). In addition, 50 µl blood 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.

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 (130 µl) 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. Blood samples were distributed to Clinical Chemical, Immunology, Allergy and Neurology (Lactate) Screens.

### **Clinical Chemistry**

The high-throughput of the screen was insured by the use of an Olympus AU 400 autoanalyzer and adapted reagents from Olympus (Hamburg, Germany) and Roche (Mannheim, Germany). In the primary screen, 17 different parameters were measured including various enzyme activities, as well as plasma concentrations of specific substrates.

### **Hematology**

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

### **Clinical Chemistry**

Most values obtained for the clinical chemical parameters were within the normal ranges usually found in C57BL/6 mice at the age of three months and were supported by previously published data (Tab. 13). In the control animals, we saw sex differences in potassium, uric acid, cholesterol, triglyceride, creatinine kinase, alanine-aminotransferase, alkaline phosphatase and amylase concentrations. In the Dmbt1 mice we found sex differences in potassium, chloride, uric acid, cholesterol, alanine-aminotransferase, alkaline phosphatase and amylase concentrations. Differences between mutants and controls were seen in cholesterol level only in males (Tab. 13).

### **Hematology**

In the primary screen for hematological parameters all parameters of both wild-type and KO mice were in normal ranges. We did not find any profound differences between wild-type and Dmbt1-KO mice (Tab. 14).

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

### **3.5.5 Discussion**

All clinical chemical and hematological parameters were within the normal ranges.

The cholesterol level was elevated in *Dmbt1* male mice. High cholesterol levels usually correlate with high body fat content, which was not elevated significantly (compare with results presented in chapter 3.2.4). In a second blood sampling procedure, which was taken after 6 days of food restriction in the metabolic screen, no significant differences between wild-type and *Dmbt1*-KO mice were detectable (Tab. 15).

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

<b>Table 13: Clinical-Chemical Parameters.</b>								
Data are presented as mean $\pm$ standard error of mean.								
Parameter	Mutant (A)			Wild Type (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=11)	(n=14)	<i>p- value</i>	(n=11)	(n=13)	<i>p-value</i>	<i>p-value</i>	<i>p-value</i>
<b>Sodium [mmol/l]</b>	156 $\pm 0.85$	155 $\pm 0.54$	n.s.	153 $\pm 1.55$	154 $\pm 0.88$	n.s.	n.s.	n.s.
<b>Potassium [mmol/l]</b>	4.3 $\pm 0.07$	4.0 $\pm 0.10$	<0.02	4.2 $\pm 0.11$	4.0 $\pm 0.06$	<0.02	n.s.	n.s.
<b>Calcium [mmol/l]</b>	1.9 $\pm 0.03$	2.0 $\pm 0.03$	n.s.	1.9 $\pm 0.03$	2.0 $\pm 0.03$	n.s.	n.s.	n.s.
<b>Chloride [mmol/l]</b>	111.2 $\pm 0.97$	113.9 $\pm 0.40$	<0.05	110.3 $\pm 1.08$	113.5 $\pm 0.59$	n.s.	n.s.	n.s.
<b>Inorganic Phosphate [mg/dl]</b>	1.6 $\pm 0.07$	1.5 $\pm 0.07$	n.s.	1.5 $\pm 0.07$	1.6 $\pm 0.06$	n.s.	n.s.	n.s.
<b>Total Protein [g/dl]</b>	5.0 $\pm 0.08$	5.2 $\pm 0.08$	n.s.	4.9 $\pm 0.07$	5.1 $\pm 0.13$	n.s.	n.s.	n.s.
<b>Creatinine [mg/dl]</b>	0.317 $\pm 0.01$	0.328 $\pm 0.01$	n.s.	0.316 $\pm 0.01$	0.323 $\pm 0.01$	n.s.	n.s.	n.s.
<b>Urea [mg/dl]</b>	61.1 $\pm 3.17$	59.2 $\pm 1.84$	<0.001	58.2 $\pm 2.7$	54.7 $\pm 3.3$	n.s.	n.s.	n.s.
<b>Uric acid [mg/dl]</b>	1.9 $\pm 0.28$	3.5 $\pm 0.31$	<0.001	2.9 $\pm 0.5$	3.7 $\pm 0.3$	<0.001	n.s.	n.s.
<b>Cholesterol [mg/dl]</b>	103.6 $\pm 4.77$	78.2 $\pm 4.56$	n.s.	82.2 $\pm 4.4$	67.7 $\pm 4.3$	<0.001	<0.01	n.s.
<b>Triglyceride [mg/dl]</b>	137.6 $\pm 17.67$	105.9 $\pm 12.18$	n.s.	110.8 $\pm 7.7$	87.7 $\pm 7.6$	<0.05	n.s.	n.s.
<b>Creatine Kinase [U/l]</b>	163 $\pm 54.80$	49 $\pm 8.22$	n.s.	66 $\pm 18.00$	35 $\pm 9.00$	<0.05	n.s.	n.s.
<b>Alanine-Amino-transferase (ALAT,GPT) [ U/l]</b>	24 $\pm 3.07$	14 $\pm 0.92$	<0.02	20 $\pm 1.00$	14 $\pm 1.00$	<0.02	n.s.	n.s.
<b>Aspartate-Amino-transferase (AST,GOT) [U/l]</b>	35 $\pm 4.74$	27 $\pm 1.35$	n.s.	27 $\pm 3.00$	26 $\pm 2.00$	n.s.	n.s.	n.s.
<b>Alkaline Phosphatase [U/l]</b>	112 $\pm 3.33$	152 $\pm 4.11$	<0.001	105 $\pm 7.00$	144 $\pm 6.00$	<0.001	n.s.	n.s.
<b><math>\alpha</math>-Amylase [U/l]</b>	2500 $\pm 75.40$	2221 $\pm 91.88$	<0.05	2501 $\pm 135.00$	2191 $\pm 64.00$	<0.01	n.s.	n.s.
<b>Glucose [mg/dl]</b>	173.1 $\pm 11.58$	163.3 $\pm 7.44$	n.s.	173.4 $\pm 10.2$	156.9 $\pm 6.9$	n.s.	n.s.	n.s.
<b>Ferritin [ng/ml]</b>	23.9 $\pm 1.73$	25.3 $\pm 1.29$	n.s.	23.3 $\pm 0.9$	24.6 $\pm 1.5$	n.s.	n.s.	n.s.
<b>Transferrin [mg/dl]</b>	139.4 $\pm 2.33$	142.7 $\pm 2.49$	n.s.	144.9 $\pm 3.9$	141.7 $\pm 1.7$	n.s.	n.s.	n.s.

**Table 14: Hematological Parameters.**Data are presented as mean  $\pm$  standard error of mean.

Parameter	Mutant (A)			Wild Type (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=12)	(n=15)	<i>p</i> - value	(n=11)	(n=14)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
White blood cell count [10 <sup>3</sup> /μl]	6.57 ±0.30	7.11 ±0.45	n.s.	7.68 ±0.77	7.74 ±0.62	n.s.	n.s.	n.s.
Red blood cell count [10 <sup>3</sup> /μl]	10.41 ±0.34	10.12 ±0.14	n.s.	10.04 ±0.26	9.96 ±0.13	n.s.	n.s.	n.s.
Hemoglobin [g/dl]	16.36 ±0.56	16.88 ±0.17	n.s.	16.00 ±0.30	16.88 ±0.24	<0.05	n.s.	n.s.
Hematocrit [%]	50 ±1.77	50 ±0.57	n.s.	50 ±1.08	50 ±0.59	n.s.	n.s.	n.s.
Mean corpuscular volume [fl]	47.92 ±0.43	49.13 ±0.40	n.s.	49.36 ±0.59	50.50 ±0.44	n.s.	n.s.	<0.05
Mean corpuscular hemoglobin [pg]	15.69 ±0.16	16.70 ±0.13	<0.001	15.98 ±0.22	16.95 ±0.18	<0.01	n.s.	n.s.
Mean corpuscular hemoglobin concentration [g/dl]	32.74 ±0.15	33.90 ±0.14	<0.001	32.34 ±0.32	33.58 ±0.33	<0.02	n.s.	n.s.
Platelet count [10 <sup>3</sup> /μl]	569 ±36.76	615 ±28.91	n.s.	681 ±15.15	571 ±34.14	<0.01	n.s.	n.s.

<b>Table 15: Clinical-Chemical Parameters after Food Restriction.</b>								
Data are presented as mean $\pm$ standard error of mean.								
Parameter	Mutant (A)			Wild Type (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=7)	(n=4)	<i>p-value</i>	(n=5)	(n=4)	<i>p-value</i>	<i>p-value</i>	<i>p-value</i>
Total Protein [g/dl]	5.3 $\pm 0.162$	5.6 $\pm 0.163$	NA	5.0 $\pm 0.07$	5.20 $\pm 0.183$	NA	n.s.	n.s.
Creatinine [mg/dl]	0.322 $\pm 0.02$	0.318 $\pm 0.01$	NA	0.328 $\pm 0.01$	0.318 $\pm 0.01$	NA	n.s.	n.s.
Urea [mg/dl]	59.6 $\pm 6.0$	43.80 $\pm 4.63$	NA	58.4 $\pm 5.61$	42.40 $\pm 3.74$	NA	n.s.	n.s.
Cholesterol [mg/dl]	79.66 $\pm 2.19$	74.50 $\pm 8.23$	NA	71.12 $\pm 4.73$	58.25 $\pm 10.99$	NA	n.s.	n.s.
Triglyceride [mg/dl]	51.40 $\pm 4.56$	41.70 $\pm 4.85$	NA	54.24 $\pm 3.28$	40.35 $\pm 2.01$	NA	n.s.	n.s.
Alanine-Amino-transferase (ALAT,GPT) [U/l]	12 $\pm 2.80$	11 $\pm 0.58$	NA	10 $\pm 1.83$	13.50 $\pm 2.75$	NA	n.s.	n.s.
Aspartate-Amino-transferase (AST,GOT) [U/l]	26 $\pm 1.82$	40 $\pm 7.96$	NA	26 $\pm 3.92$	31.50 $\pm 2.99$	NA	n.s.	n.s.
Alkaline Phosphatase [U/l]	54.29 $\pm 2.37$	78.50 $\pm 9.78$	NA	49.2 $\pm 3.14$	87.00 $\pm 17.18$	NA	n.s.	n.s.
$\alpha$ -Amylase [U/l]	2802 $\pm 201$	2360 $\pm 205$	NA	2497 $\pm 186$	2459 $\pm 366$	NA	n.s.	n.s.
Glucose [mg/dl]	77.00 $\pm 12.21$	96.55 $\pm 23.73$	NA	72.05 $\pm 8.07$	98.30 $\pm 1.88$	NA	n.s.	n.s.

## 3.6 Immunology Screen

### 3.6.1 Summary

According to the data summary presented to the GMC, no immunological phenotype was known in *Dmbt1* mice. Their analysis in the Immunology Screen could not reveal differences between mutants and their littermate controls.

### 3.6.2 Mice

We analyzed 27 KO animals (15 females and 12 males) and the 26 age- and sex-matched littermate controls (15 females and 11 males).

### 3.6.3 Material and Methods

Peripheral blood leukocytes (PBLs) were isolated from 500  $\mu$ 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 ( $\text{CD}19^+$  clone 1D3), B1 B cells ( $\text{CD}19^+\text{CD}5^+$ , clone 53-7.3), B2 B cells ( $\text{CD}19^+\text{CD}5^-$ ), T cells ( $\text{CD}3^+$ , clone 145-2C11),  $\text{CD}4^+$  T cells (clone RM4-5),  $\text{CD}8^+$  T cells ( $\text{CD}8\alpha$ , clone 53-6.7;  $\text{CD}8\beta$ , clone H35-17.2),  $\gamma/\delta$ T cells (clone GL3), granulocytes ( $\text{Gr-1}^+$ , clone RB6-8C5), and NK cells ( $\text{CD}49b^+$ , 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 Results

The analysis of all immunological parameters measured in the primary screen (Tab. 16) did not reveal significant differences between *Dmbt1*-deficient mice

and their littermate controls. We identified, however, some “unusually reversed” immunological sex differences, which did not comply with what is known from studies involving inbred mouse strains. For example, it is well established that males show higher percentage of B cells (CD19<sup>+</sup>) than females. Here we found exactly the opposite - increased frequency of B cells in females. Since such tendencies were observed also in control mice, we consider the reason for this observation to be the mixed genetic background of Dmbt1 mice.

### **3.6.5 Discussion**

Under standard screen conditions, Dmbt1-deficient mice do not show changes in the percentage of various cell subsets, or alterations in immunoglobulin levels. No further immunological investigations are recommended. Raw data will be available on demand.

**Table 16: Basic Parameters Analyzed in the Immunology Screen.**Data are presented as mean  $\pm$  standard error of mean.

Parameter	Mutants (A)			Wild Type (B)			A - B	
	Male	Female		Male	Female		Male	Female
	(n=12)	(n=15)	<i>p</i> - value	(n=11)	(n=15)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
<b>CD19<sup>+</sup></b> [%]	30.0 $\pm$ 2.1	47.4 $\pm$ 1.7	<0.001	30.3 $\pm$ 3.0	48.4 $\pm$ 2.1	<0.001	n.s.	n.s.
<b>CD19<sup>+</sup>CD5<sup>-</sup></b> [%]	96.0 $\pm$ 0.7	93.2 $\pm$ 0.5	<0.01	96.5 $\pm$ 0.7	92.6 $\pm$ 0.6	<0.01	n.s.	n.s.
<b>CD19<sup>+</sup>CD5<sup>+</sup></b> [%]	4.0 $\pm$ 0.7	6.8 $\pm$ 0.5	<0.01	4.1 $\pm$ 0.7	7.4 $\pm$ 0.6	<0.01	n.s.	n.s.
<b>CD3<sup>+</sup></b> [%]	38.5 $\pm$ 2.1	37.6 $\pm$ 1.5	n.s.	39.3 $\pm$ 2.6	39.2 $\pm$ 1.5	n.s.	n.s.	n.s.
<b><math>\gamma/\delta</math> TCR<sup>+</sup></b> [%]	3.2 $\pm$ 0.8	1.9 $\pm$ 0.2	n.s.	5.4 $\pm$ 1.8	1.9 $\pm$ 0.2	n.s.	n.s.	n.s.
<b>Gr-1<sup>+</sup></b> [%]	13.9 $\pm$ 1.0	11.0 $\pm$ 0.8	<0.05	21.4 $\pm$ 4.5	9.7 $\pm$ 0.8	<0.05	n.s.	n.s.
<b>CD49b<sup>+</sup></b> [%]	4.1 $\pm$ 1.8	21.5 $\pm$ 1.5	<0.001	2.8 $\pm$ 0.8	23.7 $\pm$ 1.6	<0.001	n.s.	n.s.
<b>CD4<sup>+</sup></b> [%]	26.8 $\pm$ 1.7	25.1 $\pm$ 1.4	n.s.	23.8 $\pm$ 1.6	17.1 $\pm$ 1.2	n.s.	n.s.	n.s.
<b>CD8<math>\beta</math><sup>+</sup></b> [%]	13.3 $\pm$ 0.6	11.8 $\pm$ 0.5	n.s.	13.3 $\pm$ 0.8	12.6 $\pm$ 0.6	n.s.	n.s.	n.s.
<b>IgG<sub>1</sub></b> [ $\mu$ g/ml]	237.6 $\pm$ 33.1	165.3 $\pm$ 36.1	n.s.	274.1 $\pm$ 43.6	157.8 $\pm$ 23.9	<0.05	n.s.	n.s.
<b>IgG<sub>2a</sub></b> [ $\mu$ g/ml]	191.4 $\pm$ 38.2	159.8 $\pm$ 43.2	n.s.	206.6 $\pm$ 41.5	106.9 $\pm$ 30.4	n.s.	n.s.	n.s.
<b>IgG<sub>2b</sub></b> [ $\mu$ g/ml]	140.6 $\pm$ 17.3	488.7 $\pm$ 18.7	<0.001	165.8 $\pm$ 25.1	496.1 $\pm$ 21.4	<0.001	n.s.	n.s.
<b>IgG<sub>3</sub></b> [ $\mu$ g/ml]	161.2 $\pm$ 19.6	170.2 $\pm$ 35.8	n.s.	201.4 $\pm$ 44.9	162.2 $\pm$ 27.7	n.s.	n.s.	n.s.
<b>IgM</b> [ $\mu$ g/ml]	1187.3 $\pm$ NA	NA	NA	1384 $\pm$ 27.5	NA	NA	NA	NA
<b>IgA</b> [ $\mu$ g/ml]	347.3 $\pm$ 28.3	261.7 $\pm$ 28.8	<0.05	326.7 $\pm$ 32.1	297.2 $\pm$ 36.1	n.s.	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.

In the primary Allergy screen, 24 wild-type and 21 Dmbt1-deficient mice were screened. The analysis of Dmbt1-deficient mice in Allergy screen did not reveal any profound differences between knockout and wild-type mice.

### 3.7.2 Mice

An age- and sex-matched group of 24 wild-type (14 females, 10 males) and 21 knockout (10 females, 11 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

The analysis of total IgE levels in plasma (mean  $\pm$  SE) of Dmbt1-KO mice and their sex- and age-matched wild-type littermates revealed higher mean IgE concentrations of male wild-type and knockout animals comparing to female mice. However, this difference was statistically not significant (Table 17).

Raw data will be available on demand.

**Table 17: Total plasma IgE in Dmbt1 mice**Data are presented as mean  $\pm$  standard error of mean.

	Mutant (A)			Wild Type (B)			A~B	A~B
	Female	Male		Female	Male		Female	Male
	(n=14)	(n=10)	<i>p - value</i>	(n=10)	(n=11)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
<b>Total IgE [ng/ml]</b>	17 $\pm$ 6.1	23 $\pm$ 15.1	n.s	18 $\pm$ 4.7	28 $\pm$ 16.2	n.s	n.s	n.s

### 3.7.5 Discussion

No statistically significant difference between Dmbt1-KO and wild-type mice was found. In both Dmbt1-KO and wild-type animals, the mean concentration of total IgE in males was higher than in females, but without statistical significance.

Taken together, under standard screening conditions for primary Allergy screen, Dmbt1-KO mice did not show changes in total plasma IgE levels that would reveal a major allergy phenotype.

### 3.7.6 Reference

Alessandrini, F., Jakob, T., Wolf, A., Wolf, E., Balling, R., Hrabé de Angelis, M., Ring, J., Behrendt, H. (2001): ENU mouse mutagenesis: Generation of mouse mutants with aberrant plasma IgE levels. *Int Arch Allergy Immunol.* 124: 25-28

## 3.8 Nociceptive Screen

### 3.8.1 Summary

In the primary screen we tested the responsiveness of the intact somatosensory system of the *Dmbt1* knockout mice and of the wild-type animals to the thermal pain by means of the hot plate test.

We did not find differences in pain reactivity either between the sexes or between the strains. We do not suggest to make further pain related studies.

### 3.8.2 Mice

Twenty-seven *Dmbt1* knockout mice (12 male, 15 female) and 25 control animals (11 male, 14 female) were tested in our first screen.

### 3.8.3 Material and Methods

#### Hot plate test

The mice were placed on a metal surface maintained at  $52 \pm 0.2^\circ\text{C}$  (Hot plate system was made by TSE GMBH, Germany; Eddy and Leimbach, 1953). Locomotion of the mouse on the hot plate was constrained by 20 cm high plexiglas wall to a circular area with a diameter of 28 cm. 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 Results

Typically, the first nociceptive response observed in these mice was **hind paw shaking**, which wild-type animals made after a 13.2 s (male) 11.5 s (female) latency. In knock out mice, this response was observed after 10.9 s (male) or 12.8 s (female). **Hind paw licking**, another typical nociceptive response showed both strains. This behavior was observed in wild-type animals after 17.2 s (male) and 14.7 s (female). In knock out animals this behavior was measured with a latency of 15.9 s (male) or 15.5 s (female). The third examined response was the **jumping** of animals. Wild-type animals jumped

after 58.6 s (male) and 53.2 s (female), knockout animals after 57.9 s (male) and 51.3 s (female).

We could find neither sex-specific nor strain-specific differences (Tab. 18).

Raw data will be available on demand.

<b>Table 18: Nociceptive Screen</b>								
Data are presented as mean ± standard error of mean.								
Parameter	Mutant (A)			Wild Type (B)			A~B	A~B
	Female	Male		Female	Male		Female	Male
	(n=15)	(n=12)	<i>p</i> -value	(n=14)	(n=11)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
<b>h.p. licking</b>	15,5± 1,38	15,9± 1,54	n.s.	14,7± 1,43	17,2± 1,51	n.s.	n.s.	n.s.
<b>h.p. shaking</b>	12,8± 1,2	10,9± 1,34	n.s.	11,5± 1,24	13,2± 1,4	n.s.	n.s.	n.s.
<b>jumping</b>	51,3± 2,83	57,9± 3,17	n.s.	53,2± 2,9	58,6± 3,3	n.s.	n.s.	n.s.

### 3.8.5 Discussion

We did not find any difference in pain sensitivity neither between Dmbt1 KO and wild-type animals nor between the sexes in this strain. We do not plan to perform further pain-related studies in this strain.

### 3.8.6 Reference

Eddy, N.B. Leimbach, D. (1953): Synthetic analgesics II. Diethienylbutenyl – and dithienylbutylamines. J. Pharmacol. Exp. Ther. 107: 385-393

### Abbreviations

h.p. hind paw

## 3.9 Lung function Screen

### 3.9.1 Summary

Spontaneous breathing pattern was studied in male and female wild-type and *Dmbt1* deficient mice. Neither during rest nor during activity, measurements revealed any statistically significant differences between groups.

### 3.9.2 Mice

The workflow of the screen provides male and female mice with a mean age of 15 weeks. Five wild-type males and 4 homozygous males with comparable body weights ( $28.5 \pm 1.1$  g;  $29.6 \pm 2.0$  g) were studied. Further, 8 wild-type (bw =  $24.6 \pm 0.9$  g) and 8 homozygous females (bw =  $25.6 \pm 2.0$  g), respectively, were analyzed (Tab. 20).

### 3.9.3 Material and Methods

#### Whole Body Plethysmography

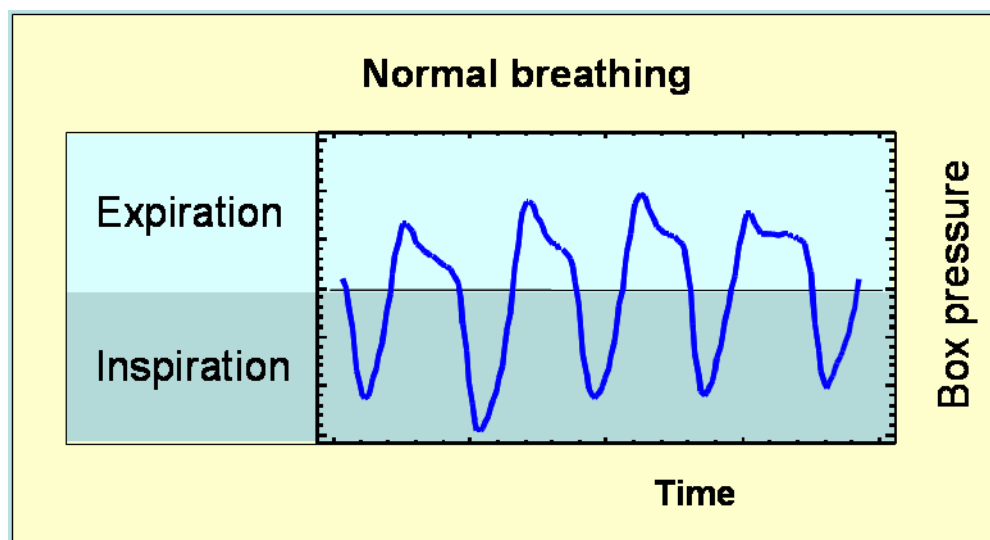
A commercially available system from Buxco<sup>®</sup> Electronics (Sharon, Connecticut) was used to assess breathing patterns in unrestrained animals according to the principle described by Drorbaugh and Fenn (1955). It measures the pressure changes which arise from inspiratory and expiratory temperature and humidity fluctuations during breathing (Figs. 3 and 4).



**Figure 3: System used at GMC to assess breathing patterns.**

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 ( $T_i$ ,  $T_e$ ), as well as peak inspiratory and peak expiratory flow rates (PIF, PEF). These data were stored online as mean values at 10 s intervals.



**Figure 4: Recorded data used to calculate the breathing parameters.**

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 (Fig. 4).

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 Results and Discussion**

Table 20 summarizes the results obtained for spontaneous breathing under resting and active conditions. Raw data are available on demand.

At the time of measurement, all animals were 15 weeks old and had comparable body weights. During both phases of activity, the comparison of 12 different parameters describing the spontaneous breathing pattern neither revealed any significant sex-specific differences nor any differences between wild-type mice and homozygous mutants. The changes in breathing pattern observed between the different levels of activity are typical for mice. All values are comparable between and within groups, which proves the high reproducibility of the measurements. The fact that ventilation and specific ventilation are comparable in all groups suggests that oxygen demand is similar and that oxygen uptake in the lungs is not affected by the mutation.

### **3.9.5 References**

Drorbaugh JE, Fenn, WO. (1955): A barometric method for measuring ventilation in newborn infants. *Pediatrics* 16: 81-87

## Abbreviations

bw	body weight (g)
mean_f	mean of all breathing frequencies
f	respiratory rate (1/min)
TV	tidal volume (ml)
sTV	specific tidal volume ( $\mu\text{l/g}$ )
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 19: Characterization of Studied Mice**

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

Parameter	Wild Type (A)			Mutant (B)			A-B	A-B
	Male	Female		Male	Female		Male	Female
	(n=5)	(n=8)	<i>p - value</i>	(n=4)	(n=8)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
<b>bw</b>	28.5 $\pm$ 1.1	24.6 $\pm$ 0.9	n.s.	29.6 $\pm$ 2.3	25.6 $\pm$ 2.0	<0.01	n.s.	n.s.
<b>mean_f</b>	389.4 $\pm$ 11.4	382.9 $\pm$ 27.9	n.s.	377.0 $\pm$ 23.8	371.6 $\pm$ 12.8	n.s.	n.s.	n.s.

**Table 20: Spontaneous Breathing Pattern during Rest and Activity**Data are presented as mean  $\pm$  standard error of mean.

Parameter	Wild Type (A)			Mutant (B)			A-B	A-B
	Male (n=5)	Female (n=8)		Male (n=4)	Female (n=8)		Male	Female
<b>rest</b>			<i>p - value</i>			<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
<b>f</b>	357.8 $\pm$ 8.0	372.6 $\pm$ 8.9	n.s.	341.2 $\pm$ 17.2	354.9 $\pm$ 9.0	n.s.	n.s.	n.s.
<b>TV</b>	0.26 $\pm$ 0.01	0.26 $\pm$ 0.02	n.s.	0.28 $\pm$ 0.02	0.25 $\pm$ 0.02	n.s.	n.s.	n.s.
<b>sTV</b>	9.1 $\pm$ 0.4	10.7 $\pm$ 0.9	n.s.	9.4 $\pm$ 0.5	9.8 $\pm$ 0.7	n.s.	n.s.	n.s.
<b>MV</b>	88.1 $\pm$ 2.8	92.9 $\pm$ 5.5	n.s.	91.2 $\pm$ 3.3	83.8 $\pm$ 4.7	n.s.	n.s.	n.s.
<b>sMV</b>	3.1 $\pm$ 0.1	3.8 $\pm$ 0.3	n.s.	3.1 $\pm$ 0.1	3.6 $\pm$ 0.2	n.s.	n.s.	n.s.
<b>Ti</b>	53.7 $\pm$ 3.4	51.7 $\pm$ 3.0	n.s.	58.4 $\pm$ 5.7	53.1 $\pm$ 1.8	n.s.	n.s.	n.s.
<b>Te</b>	114.3 $\pm$ 3.2	110.0 $\pm$ 3.1	n.s.	118.8 $\pm$ 3.5	116.7 $\pm$ 2.8	n.s.	n.s.	n.s.
<b>Ti/TT</b>	0.32 $\pm$ 0.008	0.32 $\pm$ 0.01	n.s.	0.33 $\pm$ 0.02	0.31 $\pm$ 0.01	n.s.	n.s.	n.s.
<b>PIF</b>	8.4 $\pm$ 0.4	8.9 $\pm$ 0.4	n.s.	8.4 $\pm$ 0.4	8.0 $\pm$ 0.3	n.s.	n.s.	n.s.
<b>PEF</b>	4.6 $\pm$ 0.3	5.6 $\pm$ 0.4	n.s.	5.5 $\pm$ 0.2	5.1 $\pm$ 0.2	n.s.	n.s.	n.s.
<b>MIF</b>	4.8 $\pm$ 0.3	5.0 $\pm$ 0.2	n.s.	4.8 $\pm$ 0.1	4.6 $\pm$ 0.2	n.s.	n.s.	n.s.
<b>MEF</b>	2.3 $\pm$ 0.06	2.4 $\pm$ 0.2	n.s.	2.3 $\pm$ 0.1	2.1 $\pm$ 0.1	n.s.	n.s.	n.s.
<b>activity</b>								
<b>f</b>	497.5 $\pm$ 7.8	503.7 $\pm$ 10.2	n.s.	455.1 $\pm$ 21.2	492.7 $\pm$ 9.5	n.s.	n.s.	n.s.
<b>TV</b>	0.25 $\pm$ 0.01	0.26 $\pm$ 0.01	n.s.	0.27 $\pm$ 0.02	0.25 $\pm$ 0.01	n.s.	n.s.	n.s.
<b>sTV</b>	8.7 $\pm$ 0.5	10.9 $\pm$ 0.8	n.s.	9.3 $\pm$ 0.4	9.8 $\pm$ 0.6	n.s.	n.s.	n.s.
<b>MV</b>	119.8 $\pm$ 4.4	131.2 $\pm$ 5.6	n.s.	121.1 $\pm$ 2.7	119.3 $\pm$ 6.2	n.s.	n.s.	n.s.
<b>sMV</b>	4.2 $\pm$ 0.2	5.4 $\pm$ 0.4	n.s.	4.1 $\pm$ 0.2	4.8 $\pm$ 0.3	n.s.	n.s.	n.s.
<b>Ti</b>	42.2 $\pm$ 0.7	42.5 $\pm$ 0.9	n.s.	45.9 $\pm$ 3.3	42.6 $\pm$ 0.8	n.s.	n.s.	n.s.
<b>Te</b>	78.5 $\pm$ 1.8	77.0 $\pm$ 2.2	n.s.	86.8 $\pm$ 2.9	79.5 $\pm$ 1.9	n.s.	n.s.	n.s.
<b>Ti/TT</b>	0.35 $\pm$ 0.01	0.36 $\pm$ 0.01	n.s.	0.35 $\pm$ 0.01	0.35 $\pm$ 0.01	n.s.	n.s.	n.s.
<b>PIF</b>	10.0 $\pm$ 0.5	10.8 $\pm$ 0.5	n.s.	10.4 $\pm$ 0.4	9.8 $\pm$ 0.5	n.s.	n.s.	n.s.
<b>PEF</b>	6.2 $\pm$ 0.4	7.5 $\pm$ 0.5	n.s.	7.1 $\pm$ 0.2	7.0 $\pm$ 0.4	n.s.	n.s.	n.s.
<b>MIF</b>	5.8 $\pm$ 0.3	6.2 $\pm$ 0.2	n.s.	6.0 $\pm$ 0.1	5.7 $\pm$ 0.3	n.s.	n.s.	n.s.
<b>MEF</b>	3.1 $\pm$ 0.1	3.5 $\pm$ 0.2	n.s.	3.1 $\pm$ 0.1	3.1 $\pm$ 0.2	n.s.	n.s.	n.s.

### 3.10 Expression Profiling

The molecular phenotyping screen archives organs of mutant mice for subsequent DNA-chip expression profiling analysis. Ten male mice of the *Dmbt1* strain were provided to the molecular phenotyping screen.

Organs were collected at the age of 105-110 days. The mice were killed by carbon dioxide asphyxiation. The following 17 organs were collected and archived in liquid nitrogen following our established SOPs (Standard operation protocols): bulbourethral gland, spleen, kidney, seminal vesicles, testis, white fat, liver, heart, lung, thymus, skin/cartilage (outer ear), bone (femur), skeletal muscle, salivary gland, brain, brown fat, and eye.

Organs were immediately frozen and stored in liquid nitrogen until isolation of total RNA. The 170 organ samples collected in this collaboration may either be used for further expression profiling analysis in the GMC or, alternatively may be transferred to the collaborator.

<b>Table 21: <i>Dmbt1</i>-Deficient and Wild Type Mice Stored for Expression Profiling</b>					
<b>Mouse ID</b>	<b>Strain</b>	<b>Sex</b>	<b>Date of Birth</b>	<b>Genotype</b>	<b>Date of Collection</b>
30012991	<i>Dmbt1</i>	m	21.08.2003	-/-	01.12.2003
30013037	<i>Dmbt1</i>	m	12.08.2003	-/-	01.12.2003
30013039	<i>Dmbt1</i>	m	12.08.2003	-/-	01.12.2003
30013046	<i>Dmbt1</i>	m	14.08.2003	-/-	01.12.2003
30013050	<i>Dmbt1</i>	m	11.08.2003	-/-	01.12.2003
30012972	<i>Dmbt1</i>	m	16.08.2003	+/+	01.12.2003
30012979	<i>Dmbt1</i>	m	14.08.2003	+/+	01.12.2003
30012986	<i>Dmbt1</i>	m	12.08.2003	+/+	01.12.2003
30012988	<i>Dmbt1</i>	m	12.08.2003	+/+	01.12.2003
30012993	<i>Dmbt1</i>	m	16.08.2003	+/+	01.12.2003

In a first discussion no organ was selected for analysis. When further examination is considered necessary, expression profiling analysis can be performed using our DNA-chip containing 21,000 probes. Please contact Johannes Beckers, ([beckers@gsf.de](mailto:beckers@gsf.de)) to discuss this option.

## 3.11 Metabolic Screen

### 3.11.1 Summary

In the primary metabolic screen 9 (5 male/4 female) wild-type mice and 11 (7 male/4 female) *Dmbt1*-KO mice were analyzed. They were first fed under *ad libitum* conditions for two weeks, followed by one week of food restriction to 60% of *ad libitum*. 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 significant genotype-specific differences have been found.

### 3.11.2 Mice

Five adult control males (*Dmbt1* wt) and 7 adult KO males (*Dmbt1* KO) entered the Metabolic screen at the beginning of calendar week 52/2003. The females (8 wt and 8 KO) 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 7 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 (Fec), energy uptake ( $E_{\text{up}}$ ), energy content of the feces ( $E_{\text{fec}}$ ), metabolizable energy ( $E_{\text{met}}$ ) and the food assimilation coefficient ( $F_{\text{ass}}$ ) 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 ( $E_{\text{met}}$ ) the energy content of feces and urine (2% of  $E_{\text{up}}$ ; 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 strain and gender. 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 Results

No strain specific differences between wt and KO mice could be found (Tab. 22). Only some general, sex-specific differences were detected: females of both strains are lighter than males, but with a p-value of 0.06 in KO mice. Body temperature did not differ between both strains. It was only slightly reduced during food restriction (in wt females  $p=0.045$ , in KO males  $p=0.017$  and KO females  $p=0.032$ ). Energy uptake and ratio of metabolized energy was not different between both strains. Even when taking the body weight into account, there was no statistical difference. While food assimilation was not affected by food restriction in wt mice and KO males, in KO females the assimilation coefficient increased during food restriction significantly ( $p=0.042$ ). Raw data for each individual are available on demand in Excel sheets.

### 3.11.5 Discussion

No information about metabolic parameters were available prior the metabolic screening of *Dmbt1* mice.

An appropriate evaluation of most of the metabolic parameters was difficult because of the heterogeneous background. No convincing differences between wt- and KO-mice have been found. Only KO females showed a slight significant increase of food assimilation, indicating an increase of energy extraction from food chow.

### 3.11.6 Reference

Drozdz (1975) A Food habits and food assimilation in mammals. In: Methods for Ecological Bioenergetics, edited by W. Grodzinski, RZ Klekowski and A Duncan. Oxford, UK: Blackwell, p: 23-47

### Abbreviations

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

Table 22: Metabolic Parameters Recorded in the Primary Screen												
Data are presented as mean ± standard error of mean.												
Parameter	Wild Type (A)					Mutant (B)					A~B	
	<i>ad libitum</i>		p-value	food reduction, 7 days to 60%		<i>ad libitum</i>		p-value	food reduction, 7 days to 60%		p-value	p-value
	Male	Female		Male	Female	Male	Female		Male	Female		
(n=5)	(n=4)	(n=5)	(n=4)	(n=7)	(n=4)	(n=7)	(n=4)	(n=7)	(n=4)			
Body weight [g]	34.0 ± 1.46	27.8 ± 2.19	<0.05	27.5 ± 1.05	22.1 ± 2.54	34.6 ± 2.2	27.6 ± 1.84	n.s.	27.4 ± 1.59	22.6 ± 2.36	n.s.	n.s.
Rectal body temperature [°C]	36.7 ± 0.19	36.9 ± 0.11	n.s.	36.1 ± 0.18	35.9 ± 0.35	36.4 ± 0.13	36.7 ± 0.13	n.s.	35.7 ± 0.22	35.6 ± 0.38	n.s.	n.s.
Food consumption [g day <sup>-1</sup> ]	3.8 ± 0.26	3.06 ± 0.17	n.s.	60% of <i>ad libitum</i>		3.28 ± 0.23	3.32 ± 0.27	n.s.	60% of <i>ad libitum</i>		n.s.	n.s.
Energy uptake [kJ day <sup>-1</sup> ]	70.3 ± 4.8	56.7 ± 1.52	n.s.	42.18 ± 2.88	34.03 ± 1.84	60.74 ± 4.19	61.49 ± 5.04	n.s.	36.44 ± 2.51	36.89 ± 3.03	n.s.	n.s.
Energy uptake BW <sup>-1</sup> [kJ g <sup>-1</sup> day <sup>-1</sup> ]	2.08 ± 0.16	2.06 ± 0.14	n.s.	1.53 ± 0.09	1.58 ± 0.13	1.79 ± 0.02	2.22 ± 0.08	n.s.	1.35 ± 0.12	1.64 ± 0.08	n.s.	n.s.
Feces production [g day <sup>-1</sup> ]	0.63 ± 0.05	0.55 ± 0.04	n.s.	0.37 ± 0.04	0.28 ± 0.03	0.54 ± 0.04	0.58 ± 0.06	n.s.	0.33 ± 0.03	0.29 ± 0.04	n.s.	n.s.
Energy content feces [kJ g <sup>-1</sup> ]	16.4 ± 0.04	16.0 ± 0.09	<0.02	15.69 ± 0.19	15.77 ± 0.99	16.62 ± 0.11	16.11 ± 0.07	<0.02	16.57 ± 0.13	16.05 ± 0.12	n.s.	n.s.
Metabolized energy [kJ day <sup>-1</sup> ]	60.18 ± 4.14	48.0 ± 2.69	0.054	36.34 ± 2.23	29.69 ± 1.71	51.99 ± 3.84	52.4 ± 3.84	n.s.	31.13 ± 2.16	32.28 ± 2.37	n.s.	n.s.
Metabolized energy [kJ g <sup>-1</sup> day <sup>-1</sup> ]	1.78 ± 0.14	1.74 ± 0.11	n.s.	1.32 ± 0.07	1.37 ± 0.11	1.53 ± 0.14	1.89 ± 0.07	n.s.	1.16 ± 0.11	1.44 ± 0.07	n.s.	n.s.
Food assimilation coefficient [%]	85.6 ± 0.71	84.6 ± 0.8	n.s.	86.3 ± 0.63	87.2 ± 1.16	85.4 ± 0.89	85.3 ± 0.48	n.s.	85.4 ± 0.69	87.7 ± 0.79	n.s.	n.s.

## 3.12 Pathology Screen

### 3.12.1 Summary

The Pathology screen performed a complete morphological analysis with standard stains. We did not find any line-specific morphological differences between the mutant and the knockout mice. Therefore, we can conclude that *Dmbt1* mice do not show a specific morphological phenotype

### 3.12.2 Mice

A total of 53 mice, 25 knockout mice (11 males, 14 females) and 28 control animals (14 males, 14 females) were analyzed. Due to the workflow in the GMC, mice of different ages were received from different screens, and thus from different ages (Table 24).

<b>Table 23: <i>Dmbt1</i>-Deficient Mice and their Control Littermates.</b>						
	<b>Mutant</b>		<b>Wild Type</b>		<b>Number of Animals</b>	<b>Age [weeks]</b>
	<b>Males</b>	<b>Females</b>	<b>Males</b>	<b>Females</b>		
<b>Lung Screen</b>	0	5	0	4	9	16
<b>Expression Profiling</b>	4	0	5	0	9	16
<b>Dysmorphology Screen</b>	0	4	3	3	10	23
<b>Metabolic Screen</b>	7	5	6	7	25	24
<b>Total Number of animals</b>	<b>11</b>	<b>14</b>	<b>14</b>	<b>14</b>	<b>53</b>	

### 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. 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- $\mu$ m-thick sections from skin, heart, muscle, lung, brain, cerebellum, thymus, spleen, cervical lymph nodes, thyroid, parathyroid, adrenal gland, stomach, intestine, liver, pancreas, kidney, reproductive organs, and urinary bladder were cut and stained with haematoxylin and eosin (H&E). Prussian's Blue staining was performed when indicated.

### **3.12.4 Results**

In both *Dmbt1* mice and their littermate controls, only non-specific inflammatory changes in different organs were observed. In 40 animals (22 wild-type and 18 KO) microgranulomas and small non-specific infiltrates in the liver were identified. In 25 animals (14 wild-type and 11 knockouts) non-specific mild chronic pyelonephritis was observed. In addition, 8 male animals (5 wild-type and 3 knockouts) and 3 female animals (1 wild-type and 2 knockouts) revealed urinary bladder dilatation and/or hydronephrosis. The term hydronephrosis used here signifies the combination of obstructive renal pelvic dilatation and obstructive renal disease. No specific changes were identified in the *Dmbt1* mice when compared to their control littermates.

### **3.12.5 Discussion**

An increased susceptibility to “obstructive nephropathy” was identified in this screen, including hydronephrosis and urinary bladder dilatation. However, renal alterations were present in both wild-type and knockout mice. Urinary bladder dilatation tended to occur more often in males, both wild types and knockouts. It also seems to correlate with age, since only older mice, approximately 18-22-week-old, developed urinary changes. This means that the renal phenotype found here is strain- and not line-specific. Interestingly, such renal alterations were never observed in the analysis of about 100 non-manipulated C57BL/6J mice. To our knowledge, the renal alterations observed have not been recognized as part of the normal phenotype in wild-type C57BL/6J. However, we have observed these renal changes in mice with a mixed background (C57BL/6J and 129/Ola).

Microgranulomas in the liver are very common for mice on a C57BL/6 genetic background. We observe these infiltrates practically in every C57BL/6J mouse. It is well known that also an infection with mouse hepatitis virus (MHV) would cause microgranulomas in the liver. As a routine quality control, we take blood samples from all animals for serological tests of the sanitary status of mice after they went through the GMC primary screen. All animals were serologically negative. Virus infections can be excluded as a source for the microgranulomas.

The complete morphological analysis indicates that *Dmbt1* mice do not show a specific phenotype.

## **Acknowledgements**

A large team consisting of scientists, technicians and animal caretakers all contribute to the success of the German Mouse Clinic. We want to thank Reinhard Seeliger, Elfi Holupirek, Christine Fürmann, Kerstin Kutzner, Mareike Maurer, Susanne Sommer, Rose Austin, Florian Schleicher, Gregor Pahnke, Susanne Wittich, Martin Taube, Claudia Zeller, Sandra Schädler, Elenore Samson, Nadine Kink, Jaqueline Müller, Sabine Holthaus for expert technical help and Daniela Kißling, Monika Katzbach, Uwe Drescher, Heiko Engelniederhammer, Manuela Krug, Tina Kohler, Petra Thalmeier, Aline Weingärtner, Daniela Elvert and Sven Korb for the care of the mice.

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