

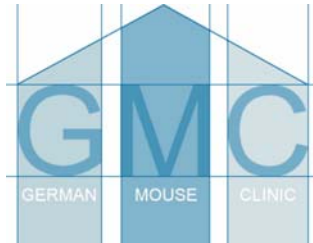
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# GERMAN MOUSE CLINIC

## Report for Arl4

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# The German Mouse Clinic



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

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

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## Content

1	Summary.....	1
1.1	Primary Screening .....	1
1.2	Recommendations for Secondary Screening.....	1
2	General Part.....	4
2.1	The Role of the Gene.....	4
2.2	Known Phenotypes .....	4
2.3	Expected Phenotypes .....	4
2.4	Suggested Human Disease Model.....	4
2.5	Mice .....	4
2.5.1	Number and kind of mice .....	4
2.5.2	Housing conditions .....	5
2.6	Workflow .....	5
2.6.1	Standardized workflow for the primary screen in the German Mouse Clinic.....	5
2.6.2	Applied screens .....	8
2.6.3	Quality Management.....	8
2.7	Statistical Analysis of Data.....	8
2.8	References.....	8
3	Specific part .....	10
3.1	Behavior Screen .....	10
3.1.1	Summary .....	10
3.1.2	Mice .....	10
3.1.3	Material and Methods .....	10
3.1.4	Results.....	12
3.1.5	Discussion .....	12
3.1.6	References .....	13
3.2	Dysmorphology, Bone and Cartilage .....	18
3.2.1	Summary .....	18
3.2.2	Mice .....	18
3.2.3	Material and Methods .....	18
3.2.4	Results and Discussion.....	19
3.2.5	References .....	20
3.3	Neurology Screen .....	25
3.3.1	Summary .....	25
3.3.2	Mice .....	25
3.3.3	Material and Methods .....	25
3.3.4	Parameters .....	27
3.3.5	Results.....	27
3.3.6	Discussion .....	28
3.3.7	References .....	28

3.4	Eye Screen .....	32
3.4.1	Summary .....	32
3.4.2	Mice .....	32
3.4.3	Materials and Methods.....	32
3.4.4	Parameters .....	33
3.4.5	Results.....	33
3.4.6	References .....	34
3.5	Clinical-Chemical Screen .....	37
3.5.1	Summary .....	37
3.5.2	Mice .....	37
3.5.3	Materials and Methods.....	37
3.5.4	Parameters .....	39
3.5.5	Results.....	39
3.5.6	Discussion .....	40
3.5.7	References .....	41
3.6	Immunology Screen .....	45
3.6.1	Summary .....	45
3.6.2	Mice .....	45
3.6.3	Material and Methods .....	45
3.6.4	Parameters .....	46
3.6.5	Results and Discussion.....	46
3.6.6	References .....	46
3.7	Allergy Screen.....	49
3.7.1	Summary .....	49
3.7.2	Mice .....	49
3.7.3	Material and Methods .....	49
3.7.4	Results and Discussion.....	49
3.7.5	References .....	50
3.8	Nociceptive Screen .....	51
3.8.1	Summary .....	51
3.8.2	Mice .....	51
3.8.3	Material and Methods .....	51
3.8.4	Parameters .....	52
3.8.5	Results.....	52
3.8.6	Discussion .....	53
3.8.7	References .....	53
3.9	Cardiovascular Screen.....	55
3.9.1	Summary .....	55
3.9.2	Mice .....	55
3.9.3	Material and Methods .....	55
3.9.4	Parameters .....	58
3.9.5	Results.....	58
3.9.6	Discussion .....	58
3.9.7	Reference .....	59
3.10	Lung Function Screen.....	64
3.10.1	Introduction .....	64
3.10.2	References .....	64

3.11	Expression Profiling .....	65
3.11.1	Introduction .....	65
3.11.2	Methods and Materials.....	65
3.11.3	Upcoming Experiments.....	65
3.11.4	References .....	65
3.12	Metabolic Screen .....	66
3.12.1	Summary .....	66
3.12.2	Mice .....	66
3.12.3	Material and Methods .....	66
3.12.4	Parameters .....	67
3.12.5	Results and Discussion.....	67
3.12.6	References .....	68
3.13	Pathology Screen.....	70
3.13.1	Summary .....	70
3.13.2	Mice .....	70
3.13.3	Materials and Methods.....	70
3.13.4	Results.....	71
3.13.5	Discussion .....	72
3.13.6	References .....	73

# 1 Summary

## 1.1 Primary Screening

In a primary screen 52 mice (30 mutants, 22 wild-type control littermates) of the *Arl4* mutant mouse line have been analyzed in the German Mouse Clinic (GMC) in the screens Behavior, Dysmorphology, Bone and Cartilage, Neurology, Eye, Clinical Chemistry, Immunology, Allergy, Cardio-Vascular System, Nociception, Energy Metabolism, and Pathology.

**Behavior Screen:** *Arl4* mutants exhibited reductions in arousal and explorative behaviour, mainly rearing.

**Dysmorphology Screen:** In the DXA analysis pBMD (partial bone mineral density) and body weight were significantly decreased in male mutants compared to male control animals.

**Neurology Screen:** Transfer arousal and tail elevation was different in male mutant mice. The mutants appeared less active than their control littermates since locomotor activity was also slightly reduced in the mutant males. But the changes observed are within the normal range. All other tested SHIRPA parameters as well as grip strength analysis were without significant findings.

**Clinical Chemical Screen:** The hematological investigations revealed a slightly reduced mean cell volume in the mutant mice suggesting that hematopoiesis is influenced by the *Arl4* knockout.

**Nociception Screen:** We found a significant genotype difference in the first reaction to pain between the wild-type control and mutant animals: thermal latencies were decreased in mutant mice, mutant mice exhibit hyperalgesia.

**Cardiovascular Screen:** The blood pressure measurement revealed a potential hypertension phenotype in the mutant mice, seen as increased levels of systolic, diastolic and mean arterial blood pressure in both sexes.

In the screens **Eye, Immunology, Allergy, Energy Metabolism, and Pathology**, no genotype-specific differences were detected.

## 1.2 Recommendations for Secondary Screening

Secondary screening is suggested from the screen Neurology, Nociception, Clinical Chemistry, Energy Metabolism and Pathology. We would recommend analyzing:

**Neurology Screen:** To evaluate whether the reduced rearing seen in the behavioral screen is not caused by a motor coordination deficit we recommend a rotarod analysis in our screen with the next batch of mice.

**Clinical-Chemical Screen:** Findings concerning the red blood cell count are very subtle; we do not recommend any additional investigations before having it confirmed in another batch of mice. It might get more pronounced in older animals. Therefore we would test a second batch of mice at least at two time points. In aged mice it might be additionally useful to repeat the clinical chemistry screen to assess secondary defects that might develop due to chronic hypertension.

**Nociception Screen:** We recommend performing an in-depth analysis of the pain phenotype. More detailed pain related studies would include:

1. Base studies e.g.
  - von Frey filament test to study the reaction of animals to mechanical pain,
  - Hargreaves test to study the reaction of the mice to another type of thermal pain,
  - acetic acid test to study the reaction to visceral inflammation (optional),
2. Tail flick test, to study whether the hypoalgesia has a spinal or supraspinal origin.

If we find in the above mentioned tests further difference in pain phenotype between wild-type control and mutant animals, we can perform the following chronic pain related studies

1. Chronic pain tests:
  - Formalin test to study the acute, nociceptive (early) and tonic, inflammatory (late) pain reaction of the same animals (optional);
  - Carrageenan test to study the reaction to inflammation (optional);
2. Neuropathy test: Total ligation of the sciatic nerve on the left side, weekly measurement of the pain sensitivity with von Frey filament test and with planar test.

**Cardiovascular Screen:** We suggest a secondary screening of animals on high-fat diet at the age of the normal screening, and again in aged mice repeated determination of blood pressure as well as echocardiography. In addition, we suggest investigating the activity of phospholipase D and angiotensin II in tissues, such as heart, lung, kidney and liver to obtain information about involved pathomechanisms.

**Metabolic Screen:** Both food restriction, which could not be performed due to limited capacities, and feeding a high caloric diet might produce more specific effects in this mutant line. Therefore, we suggest to repeat the primary screen with a group of mice fed the standard chow and in parallel a second group fed a high caloric (or high fat) diet which still has to be specified. Subsequently, a secondary screen focussing on energy balance features (daily energy expen-

diture, basal metabolic rate, and respiratory exchange quotient), spontaneous locomotor activity and body temperature should be conducted.

**Pathology Screen:** Since hypertension was observed in the cardiovascular screen, it would be interesting to look for pathological changes secondary to hypertension (vascular remodeling changes, hypoxic tissue damage, hemorrhage, infarction, left ventricular hypertrophy, glomerulosclerosis), which could be developed in elderly mice.

Therefore we suggest performing an secondary screening with a workflow as outlined below.

<b>Standard Chow</b>				
1. Group: six animals of each sex and genotype				
Neurology Screen	Clinical-Chemical Screen	Lung Function Screen		
2. Group: six animal of each sex and genotype				
			Metabolic Screen incl. Fasting	
3 Group: 10 - 12 male animals of each genotype				
	Expression Profiling (10 weeks)			Expression Profiling (15 weeks)
<b>High Fat Diet</b>				
1. Group: 1 animals of each sex and genotype				
Neurology Screen	Clinical-Chemical Screen	Cardiovascular Screen	Metabolic Screen incl. Fasting	
2. Group: 10 - 12 male animals of each genotype				
	Expression Profiling (10 weeks)			Expression Profiling (15 weeks)
<b>Aging</b>				
		Cardiovascular Screen		Pathology Screen

## 2 General Part

### 2.1 The Role of the Gene

ARL4 (ARF-like 4) is a 22 kDa GTP-binding protein which belongs to the family of ADP-ribosylation factors (ARFs). It is assumed that ARL4 acts as a GTP-operated molecular switch that regulates intracellular vesicle and/or protein transport (Jacobs *et al.*, 1999)

*Arl4* is highly expressed in testis (spermatocytes and spermatides), lower expression was detected in spleen, intestine, brain, heart, fat, liver, lung, and thymus. In adult testis, ARL4 was detected in pre- and postmeiotic cells, in spermatocytes, and spermatides, but not in spermatogonia and mature spermatozoa. ARL4 is required for progression through meiosis, but not for the division of spermatogonia or the function of mature spermatozoa (Schürmann *et al.*, 2002).

*Arl4* plays a role in adipocyte function (Schürmann *et al.*, 1994) and in embryonic development (upregulated in the somites; Buttitta *et al.*, 2003).

### 2.2 Known Phenotypes

*Arl4*-knockout mice exhibited a significant reduction in testis weight and in the number of spermatozoa with no effect on their mobility or reproductive function (Schürmann *et al.*, 2002).

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

### 2.3 Expected Phenotypes

Since an induction of ARL4 expression during differentiation of the adipocyte cells has been found, the provider expects to see differences in fat cell development of the knockout. It is possible that such a phenotype is only detectable after forced condition for instance under a high fat diet.

### 2.4 Suggested Human Disease Model

No disease model has been addressed to a mutation in the *Arl4* gene so far.

### 2.5 Mice

#### 2.5.1 Number and kind of mice

As described by the owner, exon 2 was replaced with a neo cassette.

**Table 1: Arl4 mice provided for analysis.**

Numbers in brackets indicate animals which were kept in reserve.

<b>Genotype / Sex</b>	<b>Number of Animals</b>
Mutant female	15 (+5), one animal died
Mutant male	15 (+5), one animal died
Control female	10
Control male	11, one animal died

The mice analyzed were an 11<sup>th</sup> backcross generation on a C57BL/6 background.

## 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.

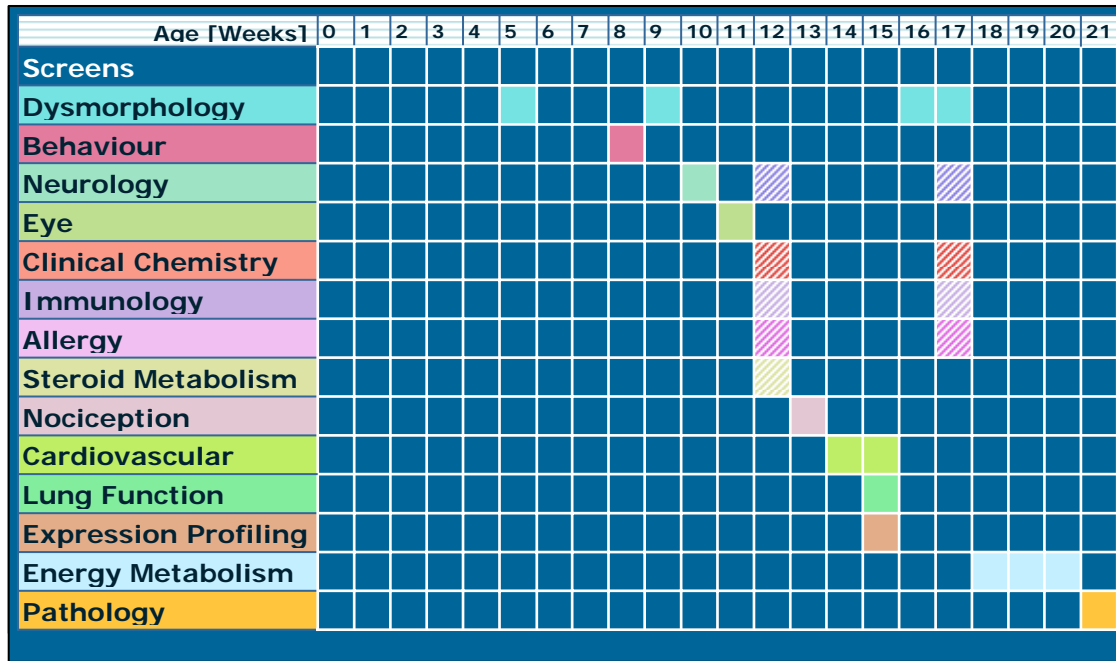
## 2.6 Workflow

### 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.

**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 alterations of the eye	funduscopy laser interference biometry slit lamp biomicroscopy histological analysis
<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>Cardio Vascular</b>	assessment of functional cardio-vascular parameters	non-invasive tail-cuff blood pressure measurement, surface limb ECG
<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



**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. One week later the mice were passed to the Cardiovascular Screen wherein the mice stay two weeks. 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. Five weeks after testing of the first blood sample, a second sample is taken to confirm the findings. Then the mice stay three weeks in the Metabolic Screen. After completion of the primary screen, all animals end up in the Pathology.

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

the specific number of analyzed animals from the sections of the applied screen.

## **2.6.2 Applied screens**

The GMC standard workflow for the primary screen as described above was applied to analyze the Arl4 mice. As the demanded number of 60 animals (15 mice per sex per genotype) could not be delivered, the workflow was adapted to the available number of animals: The screens Lung Function and Expression Profiling were skipped. 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. A few animals died during the primary screen after blood withdrawal and thus could not be analyzed for all parameters (Table 1). If possible, these animals were substituted by reserve animals.

## **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.

## **2.8 References**

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## Abbreviations and Wording

ARL4	ARF-like 4 (GTP-binding protein belonging to ADP-ribosylation factors)
<i>Arl4</i>	gene
GMC	German Mouse Clinic
IVC	individually ventilated cage
control	<i>Arl4</i> <sup>+/+</sup> , homozygous wild type control
mutant	<i>Arl4</i> <sup>-/-</sup> , homozygous mutant
wt	wild type
KO	knockout
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>

# 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 (See Ohl *et al.*, 2001).

Mutant Arl4 mice demonstrated reduced arousal, indicated by a reduced latency to grooming, an increased number of grooming and a tendential increase of maximum board entry duration. They also displayed altered explorative behavior, mainly a reduction of rearings.

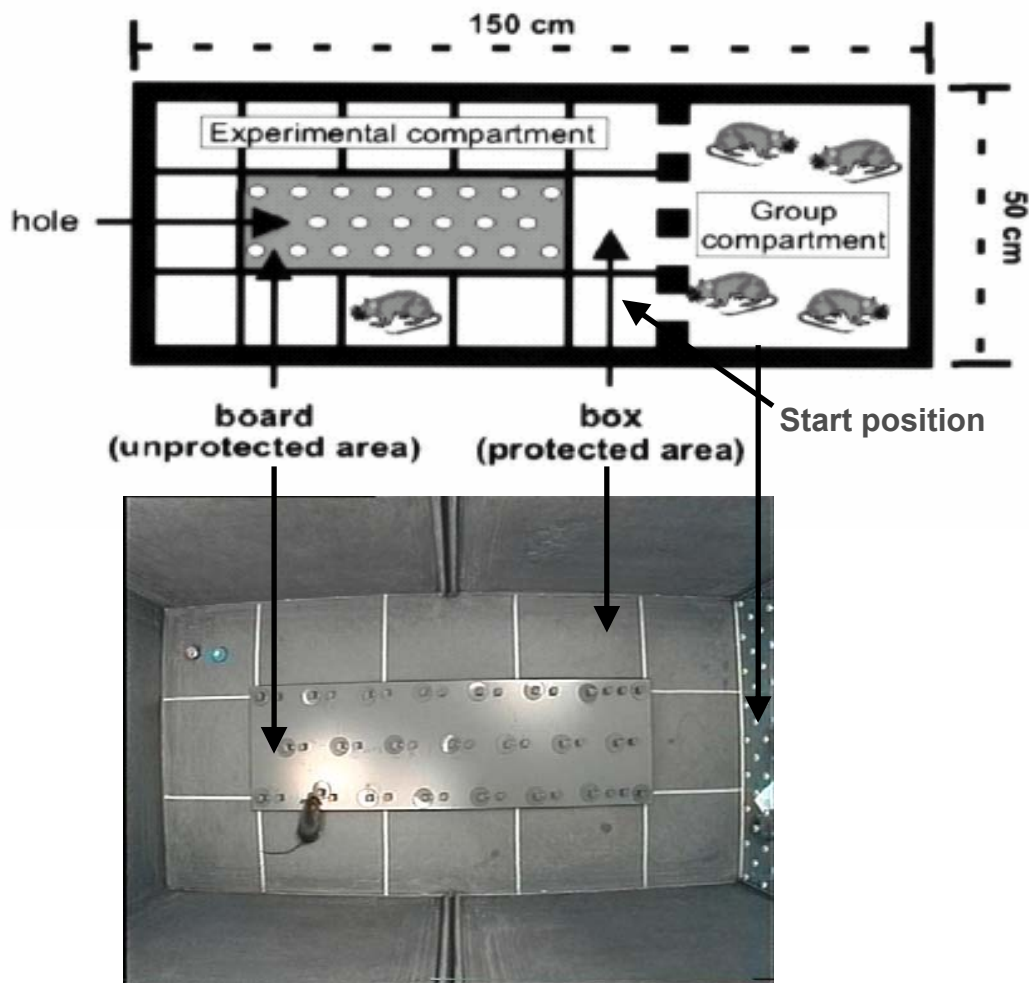
### 3.1.2 Mice

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, 25 female mice (10 controls, 15 mutants) and 25 male mice (11 controls, 14 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

Behavioral analysis of spontaneous activity in a novel environment, as measured by the modified Hole Board test, revealed reduced exploratory behavior in mutants, indicated by a reduced number of rearings and an increased latency to rearing (Table 4), and a tendential ( $p = 0.08$ , n.s.) decrease of maximum velocity (Table 5).

Additionally, mutants displayed altered grooming behavior, indicated by a reduced latency to grooming and a sex-specific increase in grooming frequency in male mutants only (Table 4). In both sexes, the maximum duration of a board entry was tendentially ( $p = 0.06$ , n.s.) increased, while all other board entry parameters were unaltered (Table 4).

There were no genotype effects detected in any other parameter.

<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 (number), Total distance travelled
Vertical exploratory behavior	Rearings in the box (number, latency), Rearings on the board (number)
Speed of movement	Mean and maximum velocity
Immobility	Time spent immobile
Risk assessment	Stretched attends
Anxiety-related behavior	Latency until first board entry, Time spent on board, Board entries
Horizontal exploratory behavior	Hole exploration, object exploration (obi);
Grooming behavior	Latency to grooming, Time spent grooming, Number of groomings
Defecation	Latency to defecation, Number of boli
Social affinity	Group contacts (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.

### 3.1.5 Discussion

Behavioral analysis in the modified Hole Board demonstrated an effect of the *Arl4* mutation mainly on rearing and grooming activity: rearing was reduced, grooming increased in mutants.

This behavioural pattern suggests a reduced arousal phenotype (Table 3), as at least in males, latency to grooming is positively correlated with the level of arousal and therefore considered an arousal indicator. This interpretation is supported by the increase in grooming frequency in male mutants and the tendential increase of maximum board entry duration, which both also suggest that mutants are tendentially “more relaxed” while exploring the test arena.

The reduction in rearing behaviour could be a (secondary) behavioural consequence of the reduced arousal phenotype. However, since rearing behaviour can also be related to balance and motor coordination, this interpretation would need to be corroborated by an exclusion of a motor coordination defect by the neurological screen.

At present it is unclear how these results are related to those of the neurological and the cardiovascular screen (3.3.5 and 3.9.5). The link could be the sympathetic nervous system, particularly adrenal function, since they are involved in the regulation of both behavioural arousal and blood pressure.

### **3.1.6 References**

Ohl, F., Sillaber, I., Binder, E., Keck, M.E. and 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.

<b>Table 4: Results of behavioral observation in the modified Hole Board Test</b>									
Data are presented as mean ± standard error of mean. Parameters which are altered only in one sex are marked in yellow									
Parameter	Control		Mutant		Male + Female		ANOVA		
	male (n=11)	female (n=10)	male (n=14)	female (n=15)	control (n=21)	mutant (n=29)	sex	genotype	Interaction
Line crossing [frequency]	146.09 ± 8.03	154.2 ± 6.12	148.33 ± 4.72	142.64 ± 5.47	149.95 ± 5.07	145.59 ± 4.97	ns	ns	ns
Line crossing [latency]	1.08 ± 0.1	0.96 ± 0.13	0.93 ± 0.07	1.09 ± 0.07	1.02 ± 0.08	1 ± 0.07	ns	ns	ns
Rearings in box [frequency]	38.45 ± 2.51	35.4 ± 2.05	31.13 ± 1.88	26.21 ± 1.72	37 ± 1.63	28.76 ± 1.86	<0.05	<0.001	ns
Rearings in box [latency]	26.25 ± 2.8	19.32 ± 2.94	32.1 ± 3	35.89 ± 3.54	22.95 ± 2.12	33.93 ± 3.19	ns	<0.01	ns
Hole exploration [frequency]	50.73 ± 5.52	44.9 ± 6.25	46.87 ± 2.47	49.93 ± 4.79	47.95 ± 4.1	48.34 ± 3.63	ns	ns	ns
Hole exploration [latency]	19.22 ± 2.71	16.3 ± 3.32	11.41 ± 2.06	17.23 ± 4.71	17.83 ± 2.09	14.22 ± 3.51	ns	ns	ns
Hole visit [frequency]	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	ns	ns	ns
Hole visit [latency]	300 ± 0	300 ± 0	300 ± 0	300 ± 0	300 ± 0	300 ± 0	ns	ns	ns
Board entry [frequency]	8 ± 1.42	7.1 ± 1.85	5.27 ± 0.74	7.71 ± 1.05	7.57 ± 1.13	6.45 ± 0.92	ns	ns	ns

Board entry [latency]	74.04 ± 21.16	107.6 ± 24.8	94.01 ± 17.22	89.16 ± 13.35	90.02 ± 16.22	91.67 ± 32.6	ns	ns	ns
Board entry [total duration %]	6.35 ± 1	6.16 ± 1.48	5.74 ± 0.76	7.98 ± 1.11	6.26 ± 0.86	6.82 ± 0.95	ns	ns	ns
Rearing on board [frequency]	1 ± 0.5	0.8 ± 0.42	0.4 ± 0.21	1.07 ± 0.35	0.9 ± 0.32	0.72 ± 0.29	ns	ns	ns
Rearing on board [latency]	252.39 ± 21.28	268.03 ± 17.47	289.98 ± 5.27	258.36 ± 11.77	259.84 ± 13.68	274.72 ± 9.55	ns	ns	ns
Risk assessment [frequency]	0 ± 0	0 ± 0	0.07 ± 0.07	0 ± 0	0 ± 0	0.03 ± 0.05	ns	ns	ns
Risk assessment [latency]	300 ± 0	300 ± 0	300 ± 0	300 ± 0	300 ± 0	300 ± 0	ns	ns	ns
Group contact [frequency]	19.18 ± 0.95	17.2 ± 0.92	18.07 ± 0.73	17.64 ± 0.84	18.24 ± 0.68	17.86 ± 0.76	ns	ns	ns
Group contact [latency]	15.99 ± 1.78	20.64 ± 2.56	16.02 ± 1.39	18.59 ± 3.49	18.2 ± 1.58	17.26 ± 2.52	ns	ns	ns
Group contact [total duration %]	20.19 ± 2.73	17.56 ± 1.02	17.15 ± 0.69	19.41 ± 0.89	18.94 ± 1.5	18.24 ± 0.82	ns	ns	ns
Grooming [frequency]	0.82 ± 0.23	1.7 ± 0.3	2.13 ± 0.4	1.57 ± 0.33	1.24 ± 0.21	1.86 ± 0.36	ns	ns	<0.05
Grooming [latency]	242.58 ± 16.75	232.79 ± 15.84	172.86 ± 16.48	217.78 ± 20.48	237.92 ± 11.33	194.54 ± 18.77	ns	<0.05	ns
Grooming [total duration %]	1.28 ± 0.39	1.57 ± 0.39	1.28 ± 0.23	1.35 ± 0.36	1.42 ± 0.27	1.31 ± 0.29	ns	ns	ns

<b>Defecation [frequency]</b>	0.73 ± 0.49	0.1 ± 0.1	0.73 ± 0.36	0.21 ± 0.11	0.43 ± 0.26	0.48 ± 0.27	<b>ns</b>	<b>ns</b>	<b>ns</b>
<b>Defecation [latency]</b>	260.41 ± 27.24	270.24 ± 29.76	259.62 ± 19.2	244.86 ± 29.66	265.09 ± 19.63	252.5 ± 23.86	<b>ns</b>	<b>ns</b>	<b>ns</b>
<b>Unfamiliar object exploration [frequency]</b>	7.27 ± 0.68	8.3 ± 1.26	7.2 ± 0.61	6.43 ± 0.91	7.76 ± 0.69	6.83 ± 0.75	<b>ns</b>	<b>ns</b>	<b>ns</b>
<b>Familiar object exploration [frequency]</b>	8.36 ± 0.95	9.3 ± 1.48	7.8 ± 0.62	7.57 ± 0.73	8.81 ± 0.85	7.69 ± 0.65	<b>ns</b>	<b>ns</b>	<b>ns</b>
<b>Unfamiliar object exploration [latency]</b>	21.55 ± 8.56	37.57 ± 11.64	21.98 ± 4.46	48.41 ± 20.69	29.18 ± 7.17	34.74 ± 14.4	<b>ns</b>	<b>ns</b>	<b>ns</b>
<b>Familiar object exploration [latency]</b>	21.83 ± 11.53	18.15 ± 4.75	27.9 ± 5.96	23.78 ± 5.55	20.08 ± 6.31	25.91 ± 5.61	<b>ns</b>	<b>ns</b>	<b>ns</b>
<b>Unfamiliar object exploration [total duration %]</b>	1.58 ± 0.18	2.86 ± 0.47	1.37 ± 0.17	1.95 ± 0.37	2.19 ± 0.28	1.65 ± 0.28	<b>&lt;0.01</b>	<b>ns</b>	<b>ns</b>
<b>Familiar object exploration [total duration %]</b>	1.29 ± 0.14	1.51 ± 0.23	1.37 ± 0.24	1.14 ± 0.12	1.4 ± 0.13	1.26 ± 0.19	<b>ns</b>	<b>ns</b>	<b>ns</b>
<b>Object Index</b>	0.11 ± 0.07	0.28 ± 0.09	0.04 ± 0.09	0.18 ± 0.09	0.19 ± 0.06	0.11 ± 0.09	<b>ns</b>	<b>ns</b>	<b>ns</b>

**Table 5: Video-tracking results regarding locomotor behavior**

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

Parameter	Control		Mutant		Male + Female		ANOVA		
	male (n=11)	female (n=10)	male (n=14)	female (n=15)	control (n=21)	mutant (n=29)	sex	genotype	Interaction
<b>Total Distance Moved [cm]</b>	3664.57 $\pm$ 214.35	3807.76 $\pm$ 139.82	3629.88 $\pm$ 116.58	3574.81 $\pm$ 126.76	3732.76 $\pm$ 128.37	3603.3 $\pm$ 117.52	ns	ns	ns
<b>Mean Velocity [cm/sec]</b>	21.67 $\pm$ 0.73	22.52 $\pm$ 0.7	21.55 $\pm$ 0.57	20.58 $\pm$ 0.43	22.08 $\pm$ 0.5	21.08 $\pm$ 0.51	ns	ns	ns
<b>Maximum velocity [cm/sec]</b>	67.95 $\pm$ 2.91	61.91 $\pm$ 2.14	63.9 $\pm$ 2.89	56.81 $\pm$ 1.62	65.07 $\pm$ 1.91	60.47 $\pm$ 2.49	<0.01	ns	ns
<b>Turns [frequency]</b>	1807.18 $\pm$ 68.71	1847.8 $\pm$ 41.27	1815.13 $\pm$ 27.79	1873.5 $\pm$ 50.43	1826.52 $\pm$ 40.28	1843.31 $\pm$ 39.33	ns	ns	ns
<b>Mean Turn Angle [degrees]</b>	23.88 $\pm$ 0.95	20.99 $\pm$ 0.41	22.78 $\pm$ 0.61	20.2 $\pm$ 0.33	22.51 $\pm$ 0.61	21.53 $\pm$ 0.59	<0.001	ns	ns
<b>Angular Velocity [degrees/sec.]</b>	166.37 $\pm$ 4.7	140.88 $\pm$ 2.72	161.85 $\pm$ 7.07	138.23 $\pm$ 2.41	154.23 $\pm$ 3.93	150.45 $\pm$ 6.1	<0.001	ns	ns
<b>Absolute Meander [degrees/sec.]</b>	16.81 $\pm$ 0.83	14.65 $\pm$ 0.37	15.82 $\pm$ 0.36	14.26 $\pm$ 0.28	15.78 $\pm$ 0.52	15.07 $\pm$ 0.38	<0.001	ns	ns
<b>Board entry [maximum duration. sec.]</b>	5.8 $\pm$ 0.44	7.32 $\pm$ 1.15	8.11 $\pm$ 1	8.47 $\pm$ 0.79	6.48 $\pm$ 0.59	8.28 $\pm$ 0.88	ns	ns	ns
<b>Mean distance to wall [cm]</b>	6.73 $\pm$ 0.24	6.53 $\pm$ 0.47	6.64 $\pm$ 0.22	6.77 $\pm$ 0.33	6.64 $\pm$ 0.25	6.7 $\pm$ 0.27	ns	ns	ns
<b>Mean distance to board [cm]</b>	8.87 $\pm$ 0.19	8.97 $\pm$ 0.32	8.79 $\pm$ 0.15	8.89 $\pm$ 0.24	8.92 $\pm$ 0.18	8.84 $\pm$ 0.19	ns	ns	ns

## 3.2 Dysmorphology, Bone and Cartilage

### 3.2.1 Summary

In the Dysmorphology, Bone and Cartilage Screen of the German Mouse Clinic mice are analyzed for morphological abnormalities in different organ systems with special focus on bone and cartilage development and homeostasis. We adapted the successful dysmorphology screening protocol from the Munich ENU-Mutagenesis Screen (Hrabé de Angelis *et al.* 2000) for use in the German Mouse Clinic. The nomenclature of the parameters was adapted according to the 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 51 animals of *Arl4* mutant mouse line were analyzed in the Dysmorphology, Bone, and Cartilage module of the German Mouse Clinic. In the morphological investigation via visual inspection and X-ray analysis a few minor phenotypes were found, which were present in both *Arl4*-mutant mice and wild-type littermate controls. In the DEXA analysis pBMD and body weight were significantly decreased in male mutants compared to control animals.

### 3.2.2 Mice

Twenty-six male (11 controls, 15 mutants) and 25 female (10 controls, 15 mutants) mice were analyzed by morphological inspection at the age of 9 weeks. 16-week-old mutants (20 animals) and controls (19 animals) entered the bone density and X-ray analysis.

### 3.2.3 Material and Methods

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

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

#### Morphological Observation

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

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

### **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**

Fifty-one animals of the *Arl4* mutant mouse line were analyzed in the Dysmorphology, Bone, and Cartilage module of the German Mouse Clinic. In the morphological investigation via visual inspection and X-ray analysis a few minor phenotypes were found, which were present in both *Arl4*-mutant mice and wild-type control littermates (Tables 6 and 7). In the clickbox test (Table 8) to test the hearing ability of the mice, we observed a normal reaction in mutants and controls.

In the bone densitometry using DEXA analysis (Table 9) pBMD (partial bone mineral density, whole body excluding skull) and body weight were significantly decreased in male mutants compared to control animals. No significant difference was observed when BMD was related to the body weight

(sBMD). Therefore the difference in pBMD might be due to changes in body weight and thus be a secondary effect. 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 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	11	15	10	15
<b>Weight</b>				
normal	11	15	10	15
<b>Body size</b>				
normal	11	14	10	14
smaller	-	1	-	1
<b>Eye</b>				
normal	11	15	10	15
<b>Coat hair growth</b>				
normal	11	15	10	15
<b>Coat hair texture</b>				
normal	11	15	10	15
<b>Coat color</b>				
agouti	11	13	7	13
white tail	-	2	3	2
<b>Hair follicle structure / orientation</b>				
normal	11	15	10	15
<b>Skin pigmentation</b>				
normal	11	15	10	15
<b>Skin texture / condition</b>				
normal	11	15	10	15
<b>Vibrissae</b>				
normal	11	15	10	15
<b>Limbs</b>				
normal	11	15	10	15
<b>Digits</b>				
normal	10	15	10	15
bent toe (right)	1	-	-	-
<b>Tail</b>				
normal	10	14	10	15
kinked tail	1	1	-	-
<b>Teeth</b>				
normal	11	15	10	14
small (down)	-	-	-	1
<b>Ear morphology</b>				
normal	11	15	10	15
<b>Musculature</b>				
normal	11	15	10	15
<b>Seizures / epilepsy</b>				
no	11	15	10	15

<b>Motor capabilities / coordination</b>				
normal	11	15	10	15
running in cycles	-	-	-	1
<b>Movement</b>				
normal	11	15	10	15
<b>Feeding / drinking behavior</b>				
normal	11	15	10	15
<b>Respiratory system</b>				
normal	11	15	10	15
<b>Reproductive system</b>				
normal	11	15	10	15
<b>Other abnormalities</b>				
no	11	15	10	15
<b>Animals analyzed</b>	<b>11</b>	<b>15</b>	<b>10</b>	<b>15</b>

<b>Table 7: 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	9	10	10	10
<b>Mandibles</b>				
normal	9	10	10	10
<b>Maxilla</b>				
normal	9	10	10	10
<b>Teeth</b>				
normal	9	10	10	10
<b>Orbit</b>				
normal	9	10	10	10
<b>Number of cervical vertebrae</b>				
normal	9	10	10	10
<b>Number of thoracic vertebrae</b>				
normal	9	10	10	10
<b>Number of lumbar vertebrae</b>				
normal	9	10	10	10
<b>Number of pelvic vertebrae</b>				
normal	9	10	10	10
<b>Number of sacral vertebrae</b>				
normal	9	10	10	10
<b>Vertebrae shape</b>				
normal	9	10	10	10
<b>Number of ribs</b>				
26	9	10	10	10
<b>Rib shape</b>				
normal	9	10	10	10

<b>Scapulas</b>				
normal	9	10	10	10
<b>Clavicle</b>				
normal	9	10	10	10
<b>Pelvis</b>				
normal	9	10	10	10
<b>Femur shape</b>				
normal	9	10	10	10
<b>Tibia</b>				
normal	9	10	10	10
<b>Fibula</b>				
normal	9	10	10	10
<b>Humerus</b>				
normal	9	10	10	10
<b>Ulna</b>				
normal	9	10	10	10
<b>Radius</b>				
normal	9	10	10	10
<b>Number of digits</b>				
normal	9	10	10	10
<b>Completeness of digits</b>				
yes	9	10	10	10
<b>Joints</b>				
normal	9	10	10	10
<b>Animals analyzed</b>	<b>9</b>	<b>10</b>	<b>10</b>	<b>10</b>

<b>Table 8: 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	-	1	-	-
2	1	6	4	1
3	10	5	6	14
4	-	3	-	-
<b>Mean Score</b>	<b>2.91</b>	<b>2.67</b>	<b>2.60</b>	<b>2.93</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

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

Parameter	Control (A)		Mutant (B)		A ~ B	A ~ B	ANOVA		
	Male	Female	Male	Female	Male	Female	p – value genotype	p – value sex	p – value interaction
	(n=9)	(n=10)	(n=10)	(n=10)	p – value	p – value			
<b>BMD [mg/cm<sup>2</sup>]</b>	61 $\pm$ 2	59 $\pm$ 2	57 $\pm$ 1	60 $\pm$ 2	n.s.	n.s.	n.s.	n.s.	n.s.
<b>pBMD [mg/cm<sup>2</sup>]</b>	51 $\pm$ 1	47 $\pm$ 2	47 $\pm$ 1	48 $\pm$ 2	< 0.01	n.s.	n.s.	n.s.	n.s.
<b>sBMD [10<sup>-3</sup> x cm<sup>-2</sup>]</b>	1.94 $\pm$ 0.10	2.62 $\pm$ 0.11	1.92 $\pm$ 0.06	2.61 $\pm$ 0.11	n.s.	n.s.	n.s.	< 0.0001	n.s.
<b>BMC [mg]</b>	675 $\pm$ 51	506 $\pm$ 32	651 $\pm$ 28	538 $\pm$ 38	n.s.	n.s.	n.s.	< 0.001	n.s.
<b>Body Length [cm]</b>	9.61 $\pm$ 0.11	9.15 $\pm$ 0.08	9.75 $\pm$ 0.08	9.2 $\pm$ 0.08	n.s.	n.s.	n.s.	< 0.0001	n.s.
<b>Body Weight [g]</b>	31.48 $\pm$ 0.77	22.67 $\pm$ 0.35	29.54 $\pm$ 0.46	23.15 $\pm$ 0.61	< 0.05	n.s.	n.s.	< 0.0001	< 0.05
<b>Lean mass [units]</b>	24.14 $\pm$ 0.88	18.03 $\pm$ 0.50	23.25 $\pm$ 0.38	17.25 $\pm$ 0.44	n.s.	n.s.	n.s.	< 0.0001	n.s.
<b>Fat mass [units]</b>	4.42 $\pm$ 0.85	2.05 $\pm$ 0.53	3.23 $\pm$ 0.33	3.06 $\pm$ 0.83	n.s.	n.s.	n.s.	n.s.	n.s.
<b>Bone Content [%]</b>	2.13 $\pm$ 0.13	2.22 $\pm$ 0.13	2.20 $\pm$ 0.08	2.31 $\pm$ 0.12	n.s.	n.s.	n.s.	n.s.	n.s.
<b>Lean Content [units x 100/g]</b>	76.85 $\pm$ 2.72	79.74 $\pm$ 2.62	78.77 $\pm$ 1.07	75.12 $\pm$ 3.12	n.s.	n.s.	n.s.	n.s.	n.s.
<b>Fat Content [units x 100/g]</b>	13.86 $\pm$ 2.67	8.90 $\pm$ 2.27	10.86 $\pm$ 1.07	12.58 $\pm$ 3.14	n.s.	n.s.	n.s.	n.s.	n.s.
<b>Femur span<sup>1</sup> [mm]</b>	1.38 $\pm$ 0.04	1.23 $\pm$ 0.02	1.40 $\pm$ 0.03	1.25 $\pm$ 0.02	n.s.	n.s.	n.s.	< 0.0001	n.s.
<b>Subcutaneous fat<sup>1</sup> [mm]</b>	4.51 $\pm$ 1.0	3.98 $\pm$ 0.2	3.60 $\pm$ 0.1	4.17 $\pm$ 0.2	n.s.	n.s.	n.s.	n.s.	n.s.
<b>Vertebrae hight<sup>2</sup> [mm]</b>	3.11 $\pm$ 0.05	3.02 $\pm$ 0.05	3.12 $\pm$ 0.04	2.97 $\pm$ 0.08	n.s.	n.s.	n.s.	< 0.05	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 *Arl4*-deficient mice (15 males / 15 females) and 21 control mice (11 males / 10 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.*, 1997). We carried out 23 of 38 designed 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. Moreover, we measured forelimb grip strength to evaluate muscle function.

The comparison of *Arl4*-mutant mice to controls revealed a significant difference in body weight of males only. Transfer arousal and tail elevation were different in male mutant mice, too. All other tested parameters were without significant findings.

### 3.3.2 Mice

Fifteen 10-week-old male mutant and 10 control mice entered the neurological screen at the beginning of the 31<sup>st</sup> calendar week. Fifteen female mutant and 11 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

#### 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 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, defecation, and vocalization were recorded. Between testing of each mouse, faecal pellets and urination were removed from the viewing jar and arena. All experimental equipment was thoroughly cleaned with Pursept-A and dried prior to testing.

### **Additional 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).

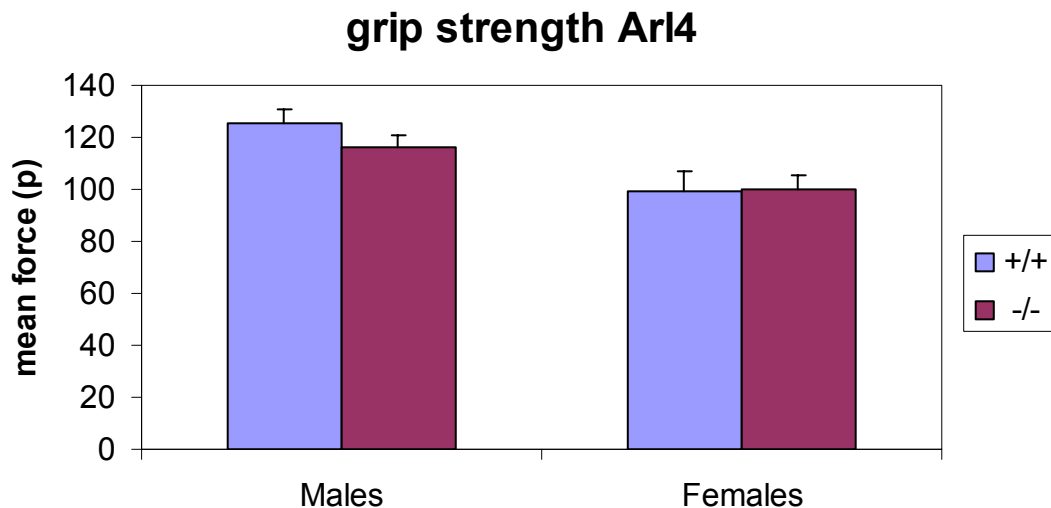
**Statistical analysis of the grip strength trial results:** Grip strength trial results are compared between genotypes, controlling for the effects of gender and weight, by fitting linear mixed effect models (Pinheiro and Bates, 2000). A linear mixed effect model is a modified analysis of variance/covariance approach allowing for dependencies in the data. In our case, dependencies arise from repeated trials within each mouse. Genotype, gender and weight are modelled as fixed effects; mouse-specific intercepts are modelled 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, abdominal tone, 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
<b>Neurological reflexes</b>
Righting reflex (pons), contact righting reflex, pinna reflex
<b>General appearance</b>
Body weight, body position, transfer arousal, touch escape, vocalization, positional passivity, aggression, spontaneous activity, locomotor activity, skin color

### 3.3.5 Results

Body weight of male mutant mice was significantly reduced (Table 9). Other parameters with significant findings for male mutant mice were **tail elevation** and **transfer arousal**. All other SHIRPA parameters were without pathological findings. In addition we analyzed forelimb grip strength of mutants versus controls and detected also no differences between the genotypes (Fig. 3).



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

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

### 3.3.6 Discussion

In our neurological screening, *Arl4*-mutant mice showed only few altered parameters. Body weight was slightly reduced in males only; tail elevation and transfer arousal were altered in males, too. Male control mice moved faster than the mutants after transferred into the arena, a behavior often seen in the C57BL/6-background. The mutants appeared less active than their control littermates since locomotor activity was also slightly reduced in the mutant males. But the changes observed are within the normal range.

Taken together the *Arl4*-knockout mice did not show any pathological alterations in our primary neurological screen.

To evaluate whether the reduced rearing seen in the behavioral screen is not due to a motor coordination deficit we recommend a rotarod analysis in our screen with the next batch of mice.

### 3.3.7 References

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Rogers D. C., E.M. Fisher, S.D. Brown, J. Peters, A.J. Hunter and 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 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)

<b>Table 10: Recording of body weight</b>							
Data are presented as mean ± standard error of mean.							
Parameter	Male			Female			both
	Control (n=11)	Mutant (n=15)	<i>p</i> -value	Control (n=10)	Mutant (n=15)	<i>p</i> -value	<i>p</i> -value
<b>Body Weight [g]</b>	28.5±0.6	26.8±0.5	<i>p</i> <0.05	20.8±0.3	21.7±0.4	<i>n.s.</i>	<i>n.s.</i>

<b>Table 11: Behavior recorded in the viewing jar</b>							
Statistical analysis: chi-squared test; significance <i>p</i> <0.05							
Parameter	Male			Female			both
	Control (n=11)	Mutant (n=15)	<i>p</i> -value	Control (n=10)	Mutant (n=15)	<i>p</i> -value	<i>p</i> -value
<b>Body Position</b>							
Inactive	0	0		0	0		
Active	11	15		10	15		
Excessive Activity	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
<b>Tremor</b>							
Absent	11	15		10	15		
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
<b>Palpebral closure</b>							
Eyes open	11	15		10	15		
Eyes closed	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
<b>Coat Appearance</b>							
Tidy and well groomed	11	15		10	15		
Irregularities	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
<b>Whiskers</b>							
Present	11	15		8	15		
Absent	0	0	<i>n.s.</i>	2	0	<i>n.s.</i>	<i>n.s.</i>
<b>Lacrimation</b>							
Absent	11	15		10	15		
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
<b>Defecation</b>							
Present	11	15		10	15		
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<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			both
	Control (n=11)	Mutant (n=15)	<i>p-value</i>	Control (n=10)	Mutant (n=15)	<i>p-value</i>	<i>p-value</i>
<b>Transfer arousal</b>							
Extended freeze	0	0		1	1		
Brief freeze	5	14		9	14		
Immediate movement	6	1	<i>p&lt;0.05</i>	0	0	<i>n.s.</i>	<i>p&lt;0.05</i>
<b>Locomotor activity</b>	29.9 $\pm$ 2.5	25.8 $\pm$ 1.3	<i>n.s.</i>	32.2 $\pm$ 2.1	33.7 $\pm$ 1.8	<i>n.s.</i>	<i>n.s.</i>
<b>Gait</b>							
Fluid movement	11	15		9	7		
Lack Fluidity	0	0	<i>n.s.</i>	1	8	<i>n.s.</i>	<i>n.s.</i>
<b>Tail Elevation</b>							
Dragging							
Horizontally extension	0	0		0	0		
	11	8		9	12		
Elevated/Straub tail	0	7	<i>p&lt;0.05</i>	1	3	<i>n.s.</i>	<i>p&lt;0.05</i>
<b>Touch Escape</b>							
No response	0	0		2	1		
Response to touch	11	15		8	14		
Flees prior to touch	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
<b>Positional Passivity</b>							
Struggles when held by tail	11	15		10	15		
No struggle	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>

<b>Table 13: Behavior recorded in or above the arena</b>							
Statistical analysis: chi-squared test; significance $p < 0.05$							
Parameter	Male			Female			both
	Control (n=11)	Mutant (n=15)	<i>p-value</i>	Control (n=10)	Mutant (n=15)	<i>p-value</i>	<i>p-value</i>
<b>Skin color</b>							
Blanched	0	0		0	0		
Pink	11	15		10	15		
Bright deep red	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
<b>Trunk curl</b>							
Absent	0	0		0	1		
Present	11	15	<i>n.s.</i>	10	14	<i>n.s.</i>	<i>n.s.</i>
<b>Limb Grasping</b>							
Absent	11	15		10	15		
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
<b>Pinna Reflex</b>							
Present	11	15		10	15		
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
<b>Corneal Reflex</b>							
Present	11	15		10	15		
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
<b>Righting Reflex</b>							
Rights itself	11	15		10	15		
Fails to right when released	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
<b>Contact Righting</b>							
Present	11	15		10	15		
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
<b>Evidence of biting</b>							
None	10	15		10	14		
Biting in response to handling	1	0	<i>n.s.</i>	0	1	<i>n.s.</i>	<i>n.s.</i>
<b>Vocalization</b>							
None	5	12		9	10		
Vocal	6	3	<i>n.s.</i>	1	5	<i>n.s.</i>	<i>n.s.</i>

## 3.4 Eye Screen

### 3.4.1 Summary

In the primary screen, different methods were employed to analyze the eyes of mutant mouse line in comparison to their control littermates. Mice were examined for anterior segment abnormalities by slit lamp biomicroscopy (Favor, 1983), as well as for posterior segment abnormalities by funduscopy. The axial eye length was measured by laser interference biometry (LIB). If required, the retinal function can be tested with a high throughput electroretinography (ERG; Dalke *et al.*, 2004) in a secondary screen.

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

No genotype-specific differences between control and mutant *Arl4* mice were detected.

### 3.4.2 Mice

Twenty-one control (11 male, 10 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 funduscopy, on the following day the laser interference biometry was performed. Mice were kept under standard laboratory conditions with food and water *ad libitum*. When the mice were killed for pathological examinations the eyes of some mice were fixed for histological analysis in the eye screen.

### 3.4.3 Materials and Methods

**Funduscopy (Ophthalmoscopy):** The posterior parts of both eyes were examined by funduscopy. After pupil dilation with one drop of atropine (1%), the mouse is grasped firmly in one hand and clinically evaluated using a head-worn indirect ophthalmoscope (Sigma 150 K, Heine Optotechnik, Herrsching, Germany) in conjunction with a condensing lens (90D lens, Volk, Mentor, OH, USA) mounted between the ophthalmoscope and the eye.

**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.

**Laser Interference Biometry (LIB)** was performed using the “AC Master” (Meditec, Carl Zeiss, Jena, Germany) equipped with a new technique, optical low coherence interferometry (OLCI), adapted for short measurement distances (Schmucker and Schaeffel, 2004). Mice were anaesthetized with 137 mg Ketamine and 6.6 mg Xylazine per kg body weight and placed in front of the ACMaster.

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

**Statistical Analysis:** Laser interference biometry 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>Funduscopy</b>
(qualitative) abnormalities of the retinal fundus and optic disc, vessel alterations and development disorders
<b>Slit lamp biomicroscopy</b>
(qualitative) abnormalities of lens and cornea like opacity and development disorders
<b>Laser Interference Biometry (LIB)</b>
axial eye length abnormalities
<b>Histology</b>
(qualitative) retinal lamination and morphology of cell layers and lens
<b>Morphology</b>
(qualitative) like size and degree of closure

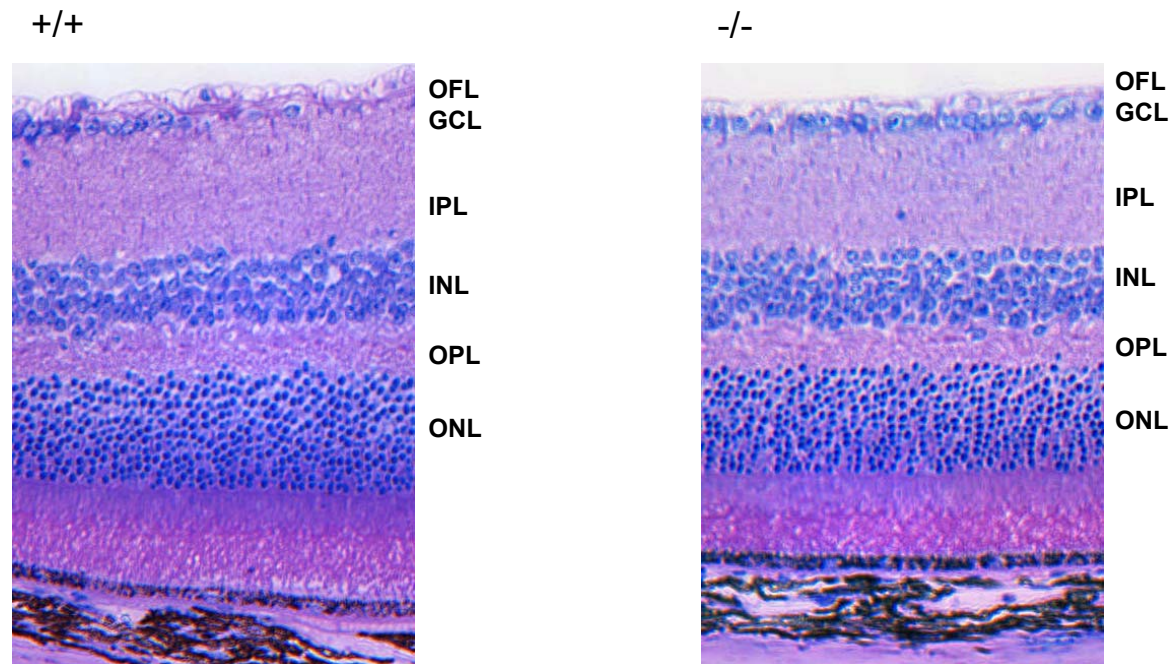
### 3.4.5 Results

**Laser Interference Biometry** data were recorded from the groups of Arl4 (control and mutant) mice. A comparison of the axial eye length of left and right eye was performed for each group. Since no differences were observed between the left and right eye, data of both eyes were averaged for further evaluation. The mean value and standard error was calculated for each group of mice, male and female, control and mutant (Table 14). The comparison of the axial eye lengths of males and females revealed significant differences in the control group, but not in the mutant group. Differences were found between the groups of mutant and control male and female mice, which are due to the different size of the control and mutant mice. When normalized with the body length, no significant differences were observed between the groups of

mutant and wild-type control littermates mice. Only sex differences were found, which is a frequent observation.

All *Arl4* mice were examined by **funduscopy**. No consistent abnormalities were detected in the wild- type control and mutant groups (Table 15).

**Histological analysis** revealed no obvious abnormalities in the retinal morphology (Fig. 4).



**Figure 4: Histological analysis of the retina.**

No morphological abnormalities were observed in the retinæ of *Arl4* mice. OFL-outer fiber layer, GCL-ganglion cell layer, IPL-inner plexiform layer, INL-inner nuclear layer, OPL-outer plexiform layer, ONL-outer nuclear layer.

A total of 51 mice were examined ophthalmologically by **slit lamp biomicroscopy** (Table 16). All animals expressed minor flecks in the nucleus of the lens, which is likely due to the genetic background (C57BL/6) of the mutant line. No anterior segment phenotype was shown to be associated with the *Arl4* mutation.

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### **Abbreviations**

ERG	electroretinography
n.s.	not significant
NAD	no abnormality detected

**Table 14: Axial eye length**

Mean ± standard error

Parameter	Control (A)			Mutant (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=11)	(n=10)	<i>p</i> -value	(n=15)	(n=15)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
<b>Axial length [mm]</b>	3.541 ± 0.007	3.510 ± 0.006	<0.01	3.568 ± 0.006	3.562 ± 0.008	n.s.	<0.01	<0.001
<b>Axial length / body length</b>	0.369 ± 0.004	0.384 ± 0.003	<0.02	0.367 ± 0.003	0.387 ± 0.003	<0.001	n.s.	n.s.

**Table 15: Results from funduscopy**

Genotype	NAD	small white dot (unilateral)	not tested due to opacity of the lens	Vessel attenuations
<b>male +/+</b> (n=11)	11	-	-	-
<b>female +/+</b> (n=10)	10	-	-	-
<b>male -/-</b> (n=15)	14	1	-	-
<b>female -/-</b> (n=15)	13	-	2*	-

\* please compare with Table 16

**Table 16: Results from slit lamp biomicroscopy**

Genotype	NAD	Microphthalmia, total opacity	Anterior polar opacity	Minor flecks in the lens nucleus
<b>male +/+</b> (n=11)	-	-	-	11
<b>female +/+</b> (n=10)	-	-	-	10
<b>male -/-</b> (n=15)	-	-	-	15
<b>female -/-</b> (n=15)	-	1	1	15

**Abbreviations**

n.s. not significant

NAD no abnormality detected

## 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 functions, 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, twenty (10 males/10 females) control mice and thirty (15 males /15 females) *Arl4*-mutant mice were analyzed. Twenty different clinical-chemical parameters were measured including various enzyme activities, as well as plasma concentrations of specific substrates and electrolytes. Additionally, we measured ten basic hematological parameters. Selected parameters showing genotype related deviations were retested in a subset of mice five weeks after the first investigation.

Concerning the clinical chemical parameters we detected several slight differences between mutant and control mice in the first test. However, none of the findings were reproducible in the second investigation of a reduced number of mice. Therefore we could not find a clear clinical-chemical phenotype. The hematological investigations revealed a slightly reduced mean cell volume in the mutant mice suggesting that hematopoiesis is influenced by the *Arl4* knockout.

### 3.5.2 Mice

Ten 12-week-old control and fifteen 12-week-old mutant males entered the clinical-chemical screen at the beginning of the 33<sup>rd</sup> calendar week 2005. Ten control and 15 mutant females entered the screen one week later. Additionally, groups of male and female mutant and control mice with ten individuals each were tested at the end of the 38<sup>th</sup> (males) and of the 39<sup>th</sup> (females) calendar week 2005 for a subset of the parameters investigated previously.

### 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 an

EDTA-coated tube (KABE, Art.No 078035). Each tube was immediately inverted five times to achieve a homogeneous distribution of the anticoagulant. After removal of 40 µl blood for the Neurology Screen, the Li-heparin-coated tubes were stored in a rack at room temperature for two hours. 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 µl), the Allergy Screen (30 µl), the Clinical Chemical Screen (130 µl) and the Steroid Screen (residual), while the cell pellet was given to the Immunology Screen for FACS-analysis. The plasma sample for the clinical chemical analysis was transferred into an Eppendorf tube and diluted 1:2 with aqua dest. The solution was mixed for a few seconds (Vortex genie, Scientific Industries, New York, America) to prevent clotting and then centrifuged again for 10 min at 4656 x g. Additionally the Clinical Chemical Screen received the EDTA-blood sample for hematological investigations.

### **Clinical Chemistry**

The 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, 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). Red blood cells, white blood cells, and platelets are measured by electrical impedance, and hemoglobin by spectrophotometry. Mean corpuscular volume (MCV), mean platelet volume (MPV) and red blood cell distribution width (RDW) are calculated directly from the cell volume measurements. The hematocrit (HCT) is assessed by multiplying the MCV with the red blood cell count. Mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) are calculated from hemoglobin/red blood cells count (MCH) and hemoglobin/hematocrit (MCHC) respectively.

### **Second sample analysis**

In the second sample collected from a subgroup of the previously tested mice at the age of 17 weeks only parameters showing genotype specific differences of unclear relevance or unusual values in individual mice were retested to check the reproducibility of these findings.

### **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), Red blood cell distribution width (RDW), Platelet count (PLT) and Mean platelet volume (MPV)

### 3.5.5 Results

Most values obtained for the clinical chemical and hematological parameters were situated within the normal ranges usually found in C57BL/6 mice at the age of three months as supported by previously published data with only few exceptions as described below (Hough *et al.*, 2002; Quimby and Loeb, 1999; Kile *et al.* 2003; Klempt *et al.*, 2006; own unpublished results). Sex differences were detected for many clinical chemical and hematological parameters in the control animals as well as in the *Arl4* mutant mice reflecting the physiological differences usually found in C57BL/6 mice.

#### Clinical Chemistry

We found several genotype-specific differences which were different for each sex: in the female group the mutants displayed slightly decreased sodium and uric acid concentrations compared to the control group, while the mean potassium concentration, creatine kinase and amylase activities were increased. In the male mutants the mean sodium, chloride, inorganic phosphorous and uric acid concentrations were increased and the mean cholesterol level was slightly decreased compared to the control group (Table 17).

Analysing a second sample obtained from ten mice per group five weeks later, we were able to confirm most of the sex-specific differences, but did not detect any of the genotype-specific findings again (Table 18).

#### Hematology

Concerning the white and red blood cell count we detected a reduced mean corpuscular volume and mean corpuscular hemoglobin content in mutant mice of both sexes compared to the control mice. In the female mutants the

haemoglobin concentration and hematocrit were additionally reduced (Table 19).

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

### **3.5.6 Discussion**

#### **Clinical Chemistry**

Significant differences between mutant and control mice have been detected for several parameters in the first sample. However, the findings were inconsistent and partly (sodium, uric acid) contradictory for the two genders. There were no differences of means judged to be of clinical relevance except for creatine kinase (CK) activity in the female mice. Furthermore, none of the findings were reproducible in a second sample collected five weeks after the first one. CK activity in plasma is a very sensitive measure for muscle injury. It is massively increased in the case of muscle injury or dystrophy, but also reacts to increased muscle activity muscle compression during mouse handling. Differences in CK activities were caused by a few individual outliers, which were generated most likely by the handling during blood sample collection. It is well known that CK activity reacts very sensitively to differences in manipulation.

Taken together, we were not able to detect a genotype-related phenotype for the clinical-chemical parameters.

#### **Hematology**

The hematological investigation detected a slightly decreased mean corpuscular volume in the mutant mice of both genders compared to the control mice. An empiric study in humans has shown that mean corpuscular volume and blood pressure are negatively correlated in individuals with hypertension (Sharp *et al.* 1996). Therefore the slight differences might be based on regulatory effects resulting from the increased blood pressure detected by the Cardio-Vascular Screen (3.9.5).

#### **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, with urea levels of the female mice being situated in the upper region of the physiologic range and hemoglobin and hematocrit values of the male mice being situated below the range we usually see in C57BL/6 mice. One male mutant mouse displayed severely increased  $\alpha$ -amylase and lipase activities in the first blood sample. This is a finding we also see sometimes in C57BL/6 wild-type mice, suggesting that this strain might have a disposition to develop temporary pancreatitis, since the values are mostly normal, when the animals are retested three weeks later. Beside this, several mice showed increased CK- and aspartate-aminotransferase activities, which is most likely an effect of the blood collecting procedure, as discussed above.

### **Secondary screen recommendations**

Since the finding concerning the red blood cell count are very subtle, we do not recommend any additional investigations before having it confirmed in another batch of mice. It might get more pronounced in older animals. Therefore we would test a second batch of mice at least at two time points. In aged mice it might be additionally useful to repeat the clinical chemistry screen to assess secondary defects that might develop due to chronic hypertension.

### **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	Control (A)			Mutant (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=10)	(n=10)	<i>p</i> -value	(n=15)	(n=15)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
<b>Sodium [mmol/l]</b>	160.8 $\pm$ 0.61	156.2 $\pm$ 0.36	<0.001	162.3 $\pm$ 0.27	154.9 $\pm$ 0.47	<0.001	<0.05	<0.05
<b>Potassium [mmol/l]</b>	3.8 $\pm$ 0.12	3.6 $\pm$ 0.05	n.s.	3.9 $\pm$ 0.06	3.9 $\pm$ 0.08	n.s.	n.s.	<0.02
<b>Calcium [mmol/l]</b>	2.2 $\pm$ 0.02	2.1 $\pm$ 0.03	n.s.	2.1 $\pm$ 0.03	2.0 $\pm$ 0.02	<0.02	n.s.	n.s.
<b>Chloride [mmol/l]</b>	111.7 $\pm$ 0.74	113.1 $\pm$ 0.34	n.s.	114.9 $\pm$ 0.56	112.2 $\pm$ 0.47	<0.01	<0.01	n.s.
<b>Inorganic Phosphate [mmol/l]</b>	2.0 $\pm$ 0.09	1.7 $\pm$ 0.05	<0.02	2.2 $\pm$ 0.06	1.6 $\pm$ 0.07	<0.001	<0.05	n.s.
<b>Total Protein [g/dl]</b>	4.68 $\pm$ 0.10	4.72 $\pm$ 0.09	n.s.	4.45 $\pm$ 0.06	4.67 $\pm$ 0.07	<0.05	n.s.	n.s.
<b>Creatinine [mg/dl]</b>	0.396 $\pm$ 0.008	0.355 $\pm$ 0.004	<0.001	0.374 $\pm$ 0.008	0.358 $\pm$ 0.005	n.s.	n.s.	n.s.
<b>Urea [mg/dl]</b>	62.0 $\pm$ 1.60	81.5 $\pm$ 3.22	<0.001	60.8 $\pm$ 2.23	74.5 $\pm$ 2.06	<0.001	n.s.	n.s.
<b>Uric acid [mg/dl]</b>	0.70 $\pm$ 0.07	2.22 $\pm$ 0.14	<0.001	0.98 $\pm$ 0.11	1.51 $\pm$ 0.05	<0.001	<0.05	<0.001
<b>Cholesterol [mg/dl]</b>	118.8 $\pm$ 4.28	85.1 $\pm$ 1.33	<0.001	106.1 $\pm$ 1.80	82.6 $\pm$ 1.88	<0.001	<0.02	n.s.
<b>Triglyceride [mg/dl]</b>	147.4 $\pm$ 16.40	91.7 $\pm$ 6.24	<0.01	127.0 $\pm$ 7.77	91.4 $\pm$ 6.00	<0.01	n.s.	n.s.
<b>Creatine Kinase [U/l]</b>	118.2 $\pm$ 29.08	154.2 $\pm$ 3.06	n.s.	142.3 $\pm$ 20.33	370.8 $\pm$ 78.51	<0.02	n.s.	<0.05
<b>Alanine-Amino-transferase (ALAT,GPT) [U/l]</b>	22.8 $\pm$ 1.74	17.6 $\pm$ 0.78	<0.02	29.7 $\pm$ 3.86	17.2 $\pm$ 0.97	<0.01	n.s.	n.s.
<b>Aspartate-Amino-transferase (AST,GOT) [U/l]</b>	24.0 $\pm$ 1.58	26.0 $\pm$ 2.19	n.s.	29.2 $\pm$ 2.33	32.5 $\pm$ 2.39	n.s.	n.s.	n.s.
<b>Alkaline Phosphatase [U/l]</b>	106.8 $\pm$ 6.18	154.6 $\pm$ 3.06	<0.001	107.2 $\pm$ 7.06	152.9 $\pm$ 5.27	<0.001	n.s.	n.s.
<b><math>\alpha</math>-Amylase [U/l]</b>	2509 $\pm$ 48	2033 $\pm$ 22	<0.001	2556 $\pm$ 76	2229 $\pm$ 41	<0.01	n.s.	<0.001
<b>Glucose [mg/dl]</b>	205.5 $\pm$ 12.48	188.9 $\pm$ 8.11	n.s.	193.9 $\pm$ 9.05	174.8 $\pm$ 7.35	n.s.	n.s.	n.s.
<b>Ferritin [ng/ml]</b>		28.7 $\pm$ 1.72			29.9 $\pm$ 2.45			n.s.
<b>Transferrin [mg/dl]</b>	148.7 $\pm$ 1.35	151.3 $\pm$ 0.51	n.s.	151.3 $\pm$ 2.50	151.3 $\pm$ 0.98	n.s.	n.s.	n.s.
<b>Lipase [U/l]</b>	83.6 $\pm$ 2.98	56.0 $\pm$ 1.53	<0.001	91.1 $\pm$ 4.83	59.9 $\pm$ 1.69	<0.001	n.s.	n.s.

**Table 18: Clinical-chemical parameters at the age of 17 weeks.**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=10)	(n=10)	<i>p- value</i>	(n=10)	(n=10)	<i>p-value</i>	<i>p-value</i>	<i>p-value</i>
<b>Sodium [mmol/l]</b>	158.4 $\pm$ 1.78	152.6 $\pm$ 0.79	<0.02	158.0 $\pm$ 1.03	153.2 $\pm$ 0.68	<0.01	n.s.	n.s.
<b>Potassium [mmol/l]</b>	4.5 $\pm$ 0.10	3.6 $\pm$ 0.11	<0.001	4.3 $\pm$ 0.08	3.7 $\pm$ 0.08	<0.001	n.s.	n.s.
<b>Chloride [mmol/l]</b>	113.3 $\pm$ 1.30	111.4 $\pm$ 0.69	n.s.	113.3 $\pm$ 0.63	111.7 $\pm$ 0.40	<0.05	n.s.	n.s.
<b>Uric acid [mg/dl]</b>	1.9 $\pm$ 0.33	1.0 $\pm$ 0.10	<0.05	2.0 $\pm$ 0.28	1.0 $\pm$ 0.06	<0.01	n.s.	n.s.
<b>Creatine Kinase [U/l]</b>	431.2 $\pm$ 103.47	113.0 $\pm$ 34.97	<0.02	320.6 $\pm$ 108.7 4	60.4 $\pm$ 12.2	<0.05	n.s.	n.s.
<b>Alanine-Amino-transferase (ALAT,GPT) [ U/l]</b>	39.0 $\pm$ 5.97			29.0 $\pm$ 4.21			n.s.	
<b>Aspartate-Amino-transferase (AST,GOT) [U/l]</b>	37.0 $\pm$ 3.92	25.0 $\pm$ 1.59	<0.02	36.6 $\pm$ 6.92	21.4 $\pm$ 0.73	n.s.	n.s.	n.s.
<b><math>\alpha</math>-Amylase [U/l]</b>		2252 $\pm$ 153			2261 $\pm$ 67			n.s.
<b>Ferritin [ng/ml]</b>	27.1 $\pm$ 2.43			31.1 $\pm$ 2.56			n.s.	

**Table 19: Hematological parameters at the age of 12 weeks.**

Data are presented as mean ± standard error of mean.

Parameter	Control (A)			Mutant (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=10)	(n=10)	<i>p</i> - value	(n=15)	(n=15)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
White blood cell count [10 <sup>3</sup> /μl]	3.62 ±0.34	5.05 ±0.28	<0.01	3.88 ±0.27	4.85 ±0.24	<0.05	n.s.	n.s.
Red blood cell count [10 <sup>3</sup> /μl]	9.1 ±0.28	10.3 ±0.08	<0.01	9.4 ±0.15	10.1 ±0.15	<0.01	n.s.	n.s.
Hemoglobin [g/dl]	13.7 ±0.39	15.4 ±0.13	<0.01	13.9 ±0.23	14.6 ±0.24	<0.05	n.s.	<0.02
Hematocrit [%]	42.4 ±1.26	47.5 ±0.35	<0.01	43.0 ±0.66	45.7 ±0.64	<0.01	n.s.	<0.05
Mean corpuscular volume [fl]	46.8 ±0.20	46.1 ±0.18	<0.02	45.7 ±0.16	45.3 ±0.12	n.s.	<0.001	<0.01
Mean corpuscular hemoglobin [pg]	15.2 ±0.15	14.9 ±0.07	n.s.	14.8 ±0.07	14.5 ±0.12	n.s.	<0.05	<0.02
Mean corpuscular hemoglobin concentration [g/dl]	32.4 ±0.26	32.4 ±0.08	n.s.	32.4 ±0.12	32.0 ±0.22	n.s.	n.s.	n.s.
Red blood cell distribution width [%]	15.2 ±0.21	14.3 ±0.07	<0.01	15.2 ±0.16	14.5 ±0.08	<0.001	n.s.	n.s.
Platelet count [10 <sup>3</sup> /μl]	816 ±59	811 ±25	n.s.	879 ±29	759 ±26	<0.01	n.s.	n.s.
Mean platelet volume [fl]	5.3 ±0.07	5.1 ±0.07	<0.05	5.3 ±0.10	5.1 ±0.06	n.s.	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, no immunological phenotype was known for the *Arl4* mutant line. The analysis in the Immunology Screen did not reveal significant differences between mutant mice and their littermate controls.

### 3.6.2 Mice

We analyzed 31 mutant *Arl4* animals (15 females and 16 males) and 20 age- and sex-matched littermate controls (10 females and 10 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 (BectonDickinson, 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, Bir-

mingham, 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 first analysis of *Arl4* mice in the Immunology Screen revealed decreased frequency of B cells (CD19<sup>+</sup>) and IgA in mutant males, as well as increased percentages of NK cells (CD49b<sup>+</sup>) and B1 B cells (CD19<sup>+</sup>CD5<sup>+</sup>) in *Arl4*-deficient females (Table 20). To verify this phenotype we tested blood samples from the same cohort of mice at a later time point (see workflow) and selectively repeated the measurements for the altered parameters. However, we could not confirm the described significant alterations (Table 21). Most likely, the initially detected differences were due to physiological variations and did not represent a real phenotype.

### 3.6.6 References

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**Table 20: Basic parameters analyzed in the Immunology Screen.**Data are presented as mean  $\pm$  standard error of mean.

Parameter	Mutants (A)			Control (B)			A ~ B	
	Male	Female		Male	Female		Male	Female
	(n=16)	(n=15)	<i>p</i> -value	(n=10)	(n=10)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
CD19 <sup>+</sup> [%]	29.2 $\pm$ 1.2	23.9 $\pm$ 0.8	<0.01	35.5 $\pm$ 1.5	21.7 $\pm$ 0.6	<0.01	<0.01	n.s.
CD19 <sup>+</sup> CD5 <sup>-</sup> [%]	93.8 $\pm$ 0.2	95.9 $\pm$ 0.3	<0.001	92.6 $\pm$ 0.4	97.2 $\pm$ 0.6	<0.001	n.s.	<0.01
CD19 <sup>+</sup> CD5 <sup>+</sup> [%]	6.9 $\pm$ 0.3	4.0 $\pm$ 0.3	<0.001	7.1 $\pm$ 0.4	2.6 $\pm$ 0.6	<0.001	n.s.	<0.01
CD3 <sup>+</sup> [%]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
$\gamma/\delta$ TCR <sup>+</sup> [%]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Gr-1 <sup>+</sup> [%]	17.7 $\pm$ 1.9	17.2 $\pm$ 0.7	n.s.	18.2 $\pm$ 1.7	19.3 $\pm$ 1.0	n.s.	n.s.	n.s.
CD49b <sup>+</sup> [%]	40.9 $\pm$ 2.0	33.9 $\pm$ 2.1	n.s.	40.4 $\pm$ 2.2	22.8 $\pm$ 1.0	<0.001	n.s.	<0.001
CD4 <sup>+</sup> [%]	32.3 $\pm$ 1.3	18.9 $\pm$ 1.2	<0.001	29.3 $\pm$ 1.3	21.0 $\pm$ 0.6	<0.001	n.s.	n.s.
CD8 $\beta$ <sup>+</sup> [%]	21.0 $\pm$ 0.8	17.8 $\pm$ 0.7	<0.01	19.5 $\pm$ 0.7	19.9 $\pm$ 0.7	n.s.	n.s.	n.s.
IgG <sub>1</sub> [ $\mu$ g/ml]	139 $\pm$ 12	201 $\pm$ 20	n.s.	196 $\pm$ 27	203 $\pm$ 29	n.s.	n.s.	n.s.
IgG <sub>2a</sub> [ $\mu$ g/ml]	211 $\pm$ 26	133 $\pm$ 12	<0.02	185 $\pm$ 24	144 $\pm$ 18	n.s.	n.s.	n.s.
IgG <sub>2b</sub> [ $\mu$ g/ml]	229 $\pm$ 27	385 $\pm$ 43	<0.05	240 $\pm$ 37	439 $\pm$ 46	<0.01	n.s.	n.s.
IgG <sub>3</sub> [ $\mu$ g/ml]	120 $\pm$ 16	170 $\pm$ 19	n.s.	100 $\pm$ 9	246 $\pm$ 107	n.s.	n.s.	n.s.
IgM [ $\mu$ g/ml]	109 $\pm$ 15	161 $\pm$ 36	n.s.	113 $\pm$ 17	110 $\pm$ 20	n.s.	n.s.	n.s.
IgA [ $\mu$ g/ml]	301 $\pm$ 44	308 $\pm$ 33	n.s.	620 $\pm$ 128	334 $\pm$ 30	n.s.	<0.05	n.s.
Anti-DNA Ab [%]	0	0	n.s.	0	0	n.s.	n.s.	n.s.
Rheuma- toid factor [%]	0	0	n.s.	0	0	n.s.	n.s.	n.s.

<b>Table 21: Basic parameters analyzed in a second screen.</b>								
Data are presented as mean $\pm$ standard error of mean.								
Parameter	Mutants (A)			Control (B)			A ~ B	
	Male	Female		Male	Female		Male	Female
	(n=11)	(n=10)	<i>p - value</i>	(n=9)	(n=10)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
<b>CD19<sup>+</sup></b> [%]	46.5 $\pm$ 2.6			48.5 $\pm$ 2.9			n.s.	
<b>CD19<sup>+</sup>CD5<sup>-</sup></b> [%]		11.2 $\pm$ 2.5			13.3 $\pm$ 2.5			n.s.
<b>CD19<sup>+</sup>CD5<sup>+</sup></b> [%]		88.8 $\pm$ 2.5			86.7 $\pm$ 2.5			n.s.
<b>CD49b<sup>+</sup></b> [%]		31.5 $\pm$ 2.7			23.6 $\pm$ 3.0			n.s.
<b>IgA</b> [ $\mu$ g/ml]	922 $\pm$ 201			755 $\pm$ 171			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 *Arl4*, mice 18 control and 25 mutant animals at the age of 12 weeks and 20 control and 20 mutant animals at the age of 17 weeks were screened. The analysis of *Arl4* mice in the allergy screen did not reveal any profound differences between mutant and control mice.

### 3.7.2 Mice

Two age- and sex-matched group of 18 control (10 females, 8 males) and 25 mutant (15 females, 10 males) mice aged 12 weeks and 20 control (10 females, 10 males) and 20 mutant (10 females, 10 males) at the age of 17 weeks were analysed 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 *Arl4*-mutant mice and their sex- and age-matched control littermates at the age of 12 weeks revealed no statistically significant differences (Table 22). We analyzed the animals again five

weeks later at the age of 17 weeks (Table 23). In both mutant and control animals the mean concentration of total IgE was higher in females than in males with significant differences in both group. Again no statistically significant difference between mutant and control mice was found.

Taken together, under standard screening conditions for the primary allergy screen, *Arl4*-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 22: Total plasma IgE in <i>Arl4</i> mice (12 weeks old)</b>								
Data are presented as mean ± standard error of mean.								
	Control (A)			Mutant (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=8)	(n=10)	<i>p</i> - value	(n=10)	(n=15)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
<b>Total IgE [ng/ml]</b>	16.9± 1.7	16± 2	n.s.	16.9± 1.7	25.1± 4.7	n.s.	n.s.	n.s.

<b>Table 23: Total plasma IgE in <i>Arl4</i> mice (17 weeks old)</b>								
Data are presented as mean ± standard error of mean.								
	Control (A)			Mutant (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=10)	(n=10)	<i>p</i> - value	(n=10)	(n=10)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
<b>Total IgE [ng/ml]</b>	24.1± 2	32.3± 1.7	<0.01	25.3± 5.7	43.5± 5.7	<0.01	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 supra-threshold stimuli. Hyperalgesia can occur both at the site of tissue damage (primary hyperalgesia) and in the surrounding undamaged areas (secondary hyperalgesia; Wall and Melzak, 1984). By means of different inbred mouse strains it could be demonstrated that rodents display large and heritable differences in both nociceptive and analgesic sensitivity (Mogil, 1999; Mogil *et al.*, 1999)

In the Primary Screen the responsiveness of the intact somatosensory system to thermal pain was tested in the *Arl4* mutant mouse line by means of the hot plate test (nociceptive pain).

We found a significant genotype difference in the first reaction to pain between the wild-type control and mutant animals, thermal latencies were decreased in mutant mice; therefore mutant mice might exhibit hyperalgesia. We recommend making further pain related studies in the *Arl4* mutant mouse line to confirm the detected phenotype.

### 3.8.2 Mice

Thirty *Arl4*-knockout mice (15 male, 15 female), and 20 control animals (10 male, 10 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  $50\pm 0.2^{\circ}\text{C}$  (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.



**Figure 5: Hot plate system**

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

### **Statistical analysis**

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

### **3.8.4 Parameters**

<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**

The first nociceptive response observed in these mice was hind paw shaking. In mutant mice this response was observed earlier than in the controls. The other nociceptive responses hind paw licking and jumping were monitored

with comparable latencies for both genotypes and sexes (Table 24). The third examined response was the jumping of animals. We could not record any significant differences in the latencies.

Raw data will be available on demand.

### 3.8.6 Discussion

We found a genotype-specific difference in pain sensitivity of the *Arl4*-knockout animals in the parameter hind paw shaking. Thermal latencies are decreased in *Arl4* mutant mice; mutant mice might exhibit hyperalgesia.

We recommend performing an in-depth analysis of the pain phenotype. More detailed pain related studies would include:

1. Base studies e.g.
  - von Frey filament test to study the reaction of animals to mechanical pain,
  - Hargreaves test to study the reaction of the mice to another type of thermal pain,
  - acetic acid test to study the reaction to visceral inflammation (optional),
2. Tail flick test, to study whether the hypoalgesia has a spinal or supraspinal origin.

If we can find in the above mentioned tests further difference in pain phenotype between wild type and knock out animals, we can perform the following chronic pain related studies

3. Chronic pain tests:
  - Formalin test to study the acute, nociceptive (early) and tonic, inflammatory (late) pain reaction of the same animals (optional);
  - Carrageenan test to study the reaction to inflammation (optional);
4. Neuropathy test: Total ligation of the sciatic nerve on the left side, weekly measurement of the pain sensitivity with von Frey filament test and with planar test.

For the secondary screen we need 10-15 mice per group and sex aged at least 10 weeks. After treatment with acetic acid, formalin or carrageenan we have to kill the mice. These experiments have to be performed at the University of Bonn.

### 3.8.7 References

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## Abbreviations

h.p. hind paw

<b>Table 24: Nociceptive Screen</b>									
Data are presented as mean $\pm$ standard error of mean.									
							ANOVA		
							genotype		sex*genotype
Parameter	Control (A)			Mutant (B)			A~B	A~B	ANOVA
	Male	Female		Male	Female		Male	Female	
Latency [s]	(n=10)	(n=10)	<i>p</i> - value	(n=15)	(n=15)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
<b>H.p. licking</b>	20.3 $\pm$ 2.2	19.5 $\pm$ 2.2	n.s.	20.9 $\pm$ 1.8	20.5 $\pm$ 1.8	n.s.	n.s.	n.s.	n.s.
<b>H.p. shaking</b>	18.8 $\pm$ 1.6	18.9 $\pm$ 1.6	n.s.	14.6 $\pm$ 1.3	15.2 $\pm$ 1.3	n.s.	0.046	n.s.	n.s.
<b>Jumping</b>	53.8 $\pm$ 2.4	60 $\pm$ 2.4	n.s.	55.1 $\pm$ 1.95	53.4 $\pm$ 1.95	n.s.	n.s.	n.s.	n.s.

ANOVA

H.p. shaking: genotype:  $p < 0.009$ ; sex:  $p < 0.829$ ; genotype\*sex:  $p < 0.856$

H.p. licking: genotype:  $p < 0.677$ ; sex:  $p < 0.757$ ; genotype\*sex:  $p < 0.933$

Jumping: genotype:  $p < 0.231$ ; sex:  $p < 0.303$ ; genotype\*sex:  $p < 0.079$

## 3.9 Cardiovascular Screen

### 3.9.1 Summary

Blood pressure (BP) analysis provides insights into functions of the vascular system including the regulation of vascular tone and left ventricular pump function. BP is strongly influenced by defects in many organ systems (heart, kidney, lung, liver) and metabolic or (neuro)endocrine pathways. Imbalances in one or, usually several organs and pathways, result in changes of this sensitive global parameter (Krege *et al.*, 1995; Lorenz, 2002; Deschepper *et al.*, 2004).

The ECG measures the electrical activity, rate and rhythm of the heart beat, supplying information about the conductive properties (function of ion channels), the excitable myocardial mass and the propagation of excitation within the heart tissue. Almost all types of cardiac pathologies will eventually cause also distinct ECG changes. Therefore, the ECG provides a comprehensive overview on cardiac function (Doevendans *et al.*, 1998; Ehmke, 2003; Royer *et al.*, 2005).

The blood pressure measurement revealed a hypertension phenotype in the mutant mice, seen as increased levels of systolic, diastolic and mean arterial blood pressure in both sexes. Therefore, we suggest performing a secondary screening of these animals.

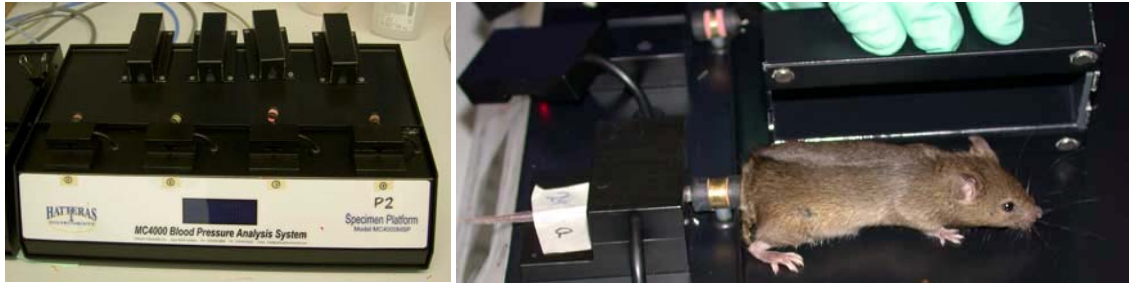
### 3.9.2 Mice

The mice reached the Cardiovascular Screen at the age of 14 weeks. Twenty female mice (10 controls, 10 mutants) and 19 male mice (9 controls, 10 mutants) underwent blood pressure and ECG analysis.

### 3.9.3 Material and Methods

#### Tail-cuff blood pressure measurement

Blood pressure was measured in unanesthetized mice with a non-invasive tail-cuff method using the MC4000 Blood Pressure Analysis Systems (Hatteras Instruments Inc., Cary, North Carolina, USA). Four animals were restrained on a pre-warmed metal platform in metal boxes. The tails were looped through a tail-cuff and fixed in a notch containing an optical path with a LED light and a photosensor.



**Figure 6: Blood pressure set up**

(A) Platform with four measurement slots, (B) mouse fixed in a tail-cuff underneath a restrainer box.

The blood pulse wave in the tail artery is detected transformed into an optical pulse signal by measurement of light extinction. Pulse detection, cuff inflation and pressure evaluation are automated by the system software. After five initial inflation runs for habituation, 12 measurement runs are performed for each animal in one session. Runs with movement artifacts are excluded.

After one day of training, in which the animals are habituated to the apparatus and protocol, the measurements are performed on four consecutive days between 8:30 and 11:30 AM.

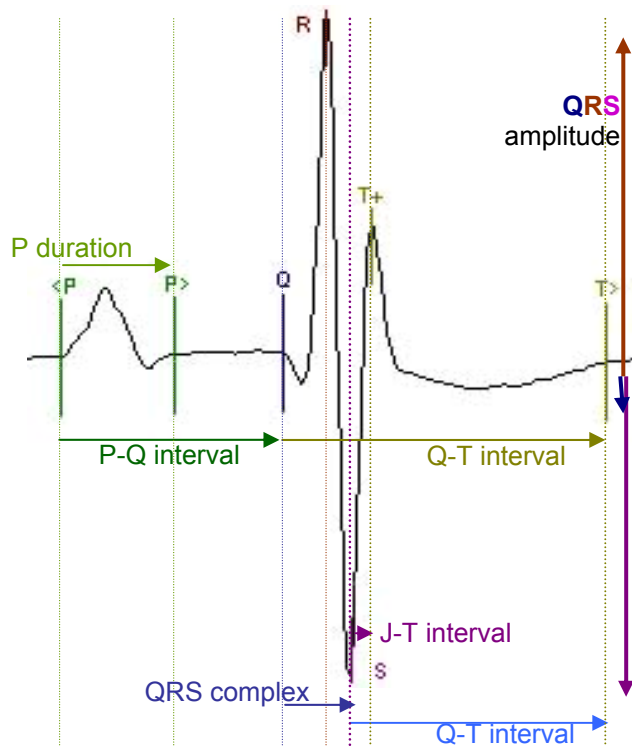
### Surface limb ECG

ECG is performed in anesthetized (isoflurane/pressured air inhalation) mice by use of three metal bracelets that are put on the joints of the feet together with electrode gel. The complete setup is located in a faraday cage. The electrodes are positioned on the front-paws and the left hind-paw, resulting in the bipolar standard limb leads I, II and III and the augmented unipolar leads AVF, AVR, AVL. ECG is recorded for about seven minutes.



**Figure 7: ECG-setup**

Left: ECG-setup in the faraday cage; right: mouse with bracelet electrode under anesthesia.



**Figure 8: Example of ECG trace with analyzed parameters.**

A shape analysis of the ECG traces is performed with the software ECG-auto (EMKA technologies, Paris, France). For each animal, intervals and amplitudes are evaluated from five different sets of averaged beats (usually lead II). The parameter Q-T interval is also corrected for the RR interval. In addition, the recordings are screened for arrhythmias, including supraventricular and ventricular extrasystoles and conduction blockages.

### **Analysis of data**

For blood pressure analysis, at least 20 to 48 individual measurements are pooled to obtain a mean over the four measurement days for each animal. In the quantitative ECG analysis sets of five analyzed beats are averaged for one animal.

The data were analyzed statistically using Statistica. Analysis of variance (ANOVA) tests are used for multi-factorial analysis of sex and genotype. Post hoc analysis for multiple comparisons included a Duncan's Multiple Range Test & Critical Ranges.

### 3.9.4 Parameters

<b>Blood Pressure Analysis</b>
Systolic Pressure, Diastolic Pressure, Mean Arterial Pressure (MAP), Pulse
<b>ECG Quantitative Analysis</b>
PQ Interval, P-Wave Duration, QRS-Complex Duration, QT Interval, QT <sub>corrected</sub> Interval, RR Interval, Heart Rate, JT Interval, ST Interval, Q Amplitude, R Amplitude, S Amplitude, QRS Amplitude
<b>ECG Qualitative Analysis</b>
Events of Supraventricular Extrasystoles, Ventricular Extrasystoles, AV I Blockage, AV II Blockage, AV III Blockage, AV Dissociation

### 3.9.5 Results

**Blood pressure** results are summarized in Table 25. Highly significant genotype-specific differences were detected in all blood pressure parameters. The levels of systolic, diastolic and mean arterial pressure were increased in mutant mice of both sexes. Sex-specific differences were comparable in mutant animals and controls.

**ECG** results are shown in Tables 26 and 27 and did not reveal a specific phenotype. Subtle differences detected in the parameters P-wave duration and J-T interval were inconsistent between groups and sexes.

Raw data are available on request.

### 3.9.6 Discussion

The results of different blood pressure parameters revealed a hypertensive phenotype of the *Arl4*-mutant mice. Hypertension can be caused by several changes in metabolic and endocrine pathways or organ systems. A major player for blood pressure regulation is the Renin-Angiotensin-Aldosterone-System (RAAS) affecting the vaso-constriction and dilatation as well as the blood volume.

A potential physiological connection of ARL4 to this regulatory system may be seen in the function of ARFs for the regulation of angiotensin II and phospholipase D (PLD). ARFs were shown to mediate the angiotensin II induced activation of phospholipase D (Shome *et al.*, 2000; Hong *et al.*, 1998) found an activation of the PLD similar to ARFs in human ARL1 under certain conditions. In addition, spontaneously hypertensive rats exhibited an increased and differentially expressed angiotensin-dependent PLD activity (Andresen *et al.*, 2001; Min *et al.*, 2001).

Interestingly, blood analysis (Clinical Chemistry Screen, 3.5.5) showed a difference in the Mean Corpuscular Volume (MCV) with slightly smaller volumes for the mutants. Empirical human studies found that hypertensive individuals have a lower MCV than normotensive persons (Sharp *et al.*, 1996).

We would suggest a secondary screening (including blood pressure analysis and echocardiography) of animals on high-fat diet: a first analysis at the age of the primary screening (repetition) and again in aged mice (12 month). In addition, we suggest investigating the activity of phospholipase D and angiotensin II in tissues, such as heart, lung, kidney and liver.

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<b>Table 25: Blood pressure parameters</b>								
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 = 9 )	( n = 10 )	<i>p - value</i>	( n = 10 )	( n = 10 )	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
<b>Systolic pressure [mm Hg]</b>	116.1 $\pm$ 1.7	119.8 $\pm$ 1.7	n.s.	124.7 $\pm$ 1.6	127.2 $\pm$ 1.8	n.s.	p<0.01	p<0.01
<b>Diastolic pressure [mm Hg]</b>	99.5 $\pm$ 1.7	107.8 $\pm$ 1.8	p<0.01	108.0 $\pm$ 1.9	116.3 $\pm$ 1.9	p<0.01	p<0.01	p<0.01
<b>Mean arterial pressure [mm Hg]</b>	104.7 $\pm$ 1.7	111.5 $\pm$ 1.7	p<0.01	113.2 $\pm$ 1.7	119.6 $\pm$ 1.8	p<0.05	p<0.01	p<0.01
<b>Pulse [bpm]</b>	629.5 $\pm$ 11.6	607.5 $\pm$ 14.3	n.s.	610.7 $\pm$ 14.2	567.6 $\pm$ 16.9	n.s.	n.s.	n.s.

**Table 26: ECG parameters**Data are presented as mean  $\pm$  standard error of mean.

Parameter	Control (A)			Mutant (B)			A~B	A~B
	Male	Female	<i>p</i> - value	Male	Female	<i>p</i> - value	Male	Female
	( n = 9 )	( n = 10 )		( n = 10 )	( n = 10 )		<i>p</i> - value	<i>p</i> - value
PQ interval [ms]	38.2 $\pm$ 1.3	38.8 $\pm$ 0.7	n.s.	39.3 $\pm$ 1.1	41.2 $\pm$ 0.9	n.s.	n.s.	n.s.
P-wave duration [ms]	21.0 $\pm$ 1.3	22.0 $\pm$ 0.4	n.s.	19.1 $\pm$ 0.6	22.0 $\pm$ 0.7	<b>p&lt;0.05</b>	n.s.	n.s.
QRS-complex duration [ms]	9.7 $\pm$ 0.3	10.2 $\pm$ 0.2	n.s.	10.0 $\pm$ 0.3	10.1 $\pm$ 0.2	n.s.	n.s.	n.s.
QT interval [ms]	40.8 $\pm$ 0.9	38.2 $\pm$ 1.1	n.s.	42.1 $\pm$ 1.4	40.3 $\pm$ 1.5	n.s.	n.s.	n.s.
QT <sub>corrected</sub> [ms]	36.9 $\pm$ 0.9	34.8 $\pm$ 0.5	n.s.	37.2 $\pm$ 0.8	36.5 $\pm$ 1.4	n.s.	n.s.	n.s.
RR interval [ms]	123.5 $\pm$ 6.3	122.3 $\pm$ 3.6	n.s.	129.9 $\pm$ 5.2	122.3 $\pm$ 3.4	n.s.	n.s.	n.s.
Heart rate [bpm]	496.4 $\pm$ 23.3	495.2 $\pm$ 13.6	n.s.	469.4 $\pm$ 19.0	496.1 $\pm$ 13.5	n.s.	n.s.	n.s.
JT interval [ms]	3.1 $\pm$ 0.1	3.5 $\pm$ 0.1	<b>p&lt;0.05</b>	3.6 $\pm$ 0.1	3.4 $\pm$ 0.2	n.s.	<b>p&lt;0.05</b>	n.s.
ST interval [ms]	31.1 $\pm$ 0.9	28.0 $\pm$ 1.0	n.s.	32.1 $\pm$ 1.2	30.2 $\pm$ 1.4	n.s.	n.s.	n.s.
Q amplitude [mV]	0.03 $\pm$ 0.01	0.03 $\pm$ 0.00	n.s.	0.03 $\pm$ 0.00	0.04 $\pm$ 0.01	n.s.	n.s.	n.s.
R amplitude [mV]	2.54 $\pm$ 0.15	2.72 $\pm$ 0.16	n.s.	2.27 $\pm$ 0.14	2.83 $\pm$ 0.25	n.s.	n.s.	n.s.
S amplitude [mV]	-1.02 $\pm$ 0.15	-1.13 $\pm$ 0.16	n.s.	-0.91 $\pm$ 0.08	-0.74 $\pm$ 0.14	n.s.	n.s.	n.s.
QRS amplitude [mV]	3.56 $\pm$ 0.20	3.84 $\pm$ 0.17	n.s.	3.17 $\pm$ 0.18	3.59 $\pm$ 0.31	n.s.	n.s.	n.s.

<b>Table 26: ECG parameters</b>								
Data are presented as mean ± standard error of mean.								
Parameter	Control (A)			Mutant (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	( n = 9 )	( n = 10 )	<i>p - value</i>	( n = 10 )	( n = 10 )	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
<b>Arrhythmias</b> [number of animals]	0	0		0	0			
<b>Regular</b> [number of animals]	9	10		10	10			

## 3.10 Lung Function Screen

### 3.10.1 Introduction

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). By use of genetically engineered mice, candidate genes for human developmental disorders of breathing have been identified (Katz, 2003). Neural and mechanical processes that control breathing frequency have been investigated in man for a long time (Mead, 1960; Otis *et al.*, 1959). Baseline breathing patterns are genetically regulated in man and mice (Tankersley *et al.*, 1997; Tankersley, 1999). Several mouse models for human ventilation disorders are described.

In the primary screen, the parameters listed below are measured:

<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)

Due to the low number of animals, no mice of the first batch could be analyzed in the Lung Function screen. Therefore the measurements will be performed with the second batch of young mice on regular diet. Additional secondary screening will be done with aged mice on high fat diet.

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## 3.11 Expression Profiling

### 3.11.1 Introduction

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

### 3.11.2 Methods and Materials

The molecular phenotyping screen archives organs of mutant mice for subsequent DNA-chip expression profiling analysis. Due to the low number of animals, no male mice of the *Arl4* mutant mouse line were provided to the molecular phenotyping screen.

Organs are collected at the age of 15 weeks. To minimize the influence of circadian rhythm on gene expression, mice are killed between 9 am and 12 am by carbon dioxide asphyxiation. The following 13 organs are collected and archived in liquid nitrogen following our established SOPs (Standard operation procedure): bulbourethral gland, spleen, kidney, seminal vesicles, testis, liver, heart, lung, thymus, skin/cartilage (outer ear), skeletal muscle, salivary gland and brain. Organs were immediately frozen and stored in liquid nitrogen until isolation of total RNA. The 130 organ samples collected in this collaboration may either be used for further expression profiling analysis in the GMC or, alternatively may be transferred to the collaborator.

### 3.11.3 Upcoming Experiments

Kidney and adrenal glands are selected for analysis in our screen. We will do these experiments with mice of a second batch of animals (age: ~15 weeks).

Additionally we offer the further experiments (if there are enough mice):

Expression profiling analysis of kidney and adrenal glands of

- a) 15-week-old mice, high fat diet
- b) older mice, normal food
- c) older mice, high fat diet

When further examination is considered necessary, please contact Johannes Beckers, ([beckers@gsf.de](mailto:beckers@gsf.de)) to discuss this option.

### 3.11.4 References

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## 3.12 Metabolic Screen

### 3.12.1 Summary

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

The primary metabolic screen focuses on investigation of metabolic demands of mice determining daily body weight, energy uptake, metabolizable energy and body temperature and adaptive capacity of metabolic processes. Mice are first fed under *ad libitum* conditions for two weeks, followed by two days of acute fasting to analyze adaptive responses of metabolism. In the primary metabolic screen 14 animals of the *Arl4* mutant mouse line were analyzed. Thirteen control mice (six males/ seven females) were available and seven mutants of each sex were analyzed.

Comparing the sexes within both genotypes indicated that males are heavier than females with significantly higher food and energy uptake. Genotype-specific differences could not be found – hence, no metabolic phenotype was monitored.

### 3.12.2 Mice

Six adult control males and seven adult *Arl4*-mutant males entered the Metabolic Screen at the beginning of calendar week 39 in 2005. The females (seven controls and seven mutants) entered the metabolic laboratory one week later. The mice were single caged on grid panels (0.5°cm grid hole diameter). Deviating from our above described SOP *Arl4* mice were only fed *ad libitum* during their stay in the Metabolic Screen and were not food restricted to analyze adaptive responses of metabolism.

### 3.12.3 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 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.

## 3.12.4 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 ( $F_{ass}$ )
--

## 3.12.5 Results and Discussion

Common sex-specific differences were found in both groups, indicating males heavier than females, with higher food and energy intake of male mice. Taking the body weight into account, the energy uptake and metabolized energy was slightly increased in females indicating higher energetic demands (Table 27). A comparison by genotype revealed no statistical differences.

In conclusion, we could not detect significant differences between genotypes so the results of the primary metabolic screen do not indicate a metabolic phenotype of *Arl4*-mutant mice. However, both food restriction, which could not be performed due to limited capacities, or feeding a high caloric diet might produce more specific effects in this mutant line.

Therefore, we suggest to repeat the primary screen with a group of mice fed the standard chow and in parallel a second group fed a high caloric (or high fat) diet which still has to be specified. Subsequently, a secondary screen focussing on energy balance features (daily energy expenditure, basal metabolic rate, respiratory exchange quotient), spontaneous locomotor activity and body temperature should be conducted.

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

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### 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 27: Metabolic parameters recorded in the primary screen**

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

Parameter	Control		Mutant		2 – Way - ANOVA		
	<i>ad libitum</i>		<i>ad libitum</i>		<i>p-value genotype</i>	<i>p-value sex</i>	<i>p-value interaction</i>
	male (n=6)	female (n=7)	male (n=7)	female (n=7)	<i>ad libitum</i>	<i>ad libitum</i>	<i>ad libitum</i>
Body weight [g]	32.1 $\pm$ 0.97	22.3 $\pm$ 0.43	30.8 $\pm$ 0.5	23.2 $\pm$ 0.9	n.s.	< 0.001	n.s.
Rectal body temperature [°C]	36.2 $\pm$ 0.18	36.6 $\pm$ 0.1	36.5 $\pm$ 0.06	36.8 $\pm$ 0.06	n.s.	0.01	n.s.
Food consumption [g day <sup>-1</sup> ]	4.38 $\pm$ 0.28	3.71 $\pm$ 0.23	4.62 $\pm$ 0.12	3.72 $\pm$ 0.19	n.s.	< 0.001	n.s.
Energy uptake [kJ day <sup>-1</sup> ]	78.3 $\pm$ 5.05	68.5 $\pm$ 4.2	82.5 $\pm$ 2.14	66.4 $\pm$ 3.4	n.s.	< 0.001	n.s.
Energy uptake BW <sup>-1</sup> [kJ g <sup>-1</sup> day <sup>-1</sup> ]	2.43 $\pm$ 0.1	2.97 $\pm$ 0.19	2.68 $\pm$ 0.08	2.87 $\pm$ 0.12	n.s.	< 0.05	n.s.
Feces production [g day <sup>-1</sup> ]	0.86 $\pm$ 0.04	0.66 $\pm$ 0.02	0.94 $\pm$ 0.04	0.68 $\pm$ 0.04	n.s.	< 0.001	n.s.
Energy content feces [kJ g <sup>-1</sup> ]	15.3 $\pm$ 0.07	15.1 $\pm$ 0.07	15.3 $\pm$ 0.07	15.2 $\pm$ 0.08	n.s.	n.s.	n.s.
Metabolized energy [kJ day <sup>-1</sup> ]	63.8 $\pm$ 4.34	55.1 $\pm$ 4.03	66.7 $\pm$ 1.92	54.9 $\pm$ 3.15	n.s.	< 0.01	n.s.
Metabolized energy [kJ g <sup>-1</sup> day <sup>-1</sup> ]	1.98 $\pm$ 0.09	2.47 $\pm$ 0.19	2.16 $\pm$ 0.07	2.37 $\pm$ 0.11	n.s.	< 0.05	n.s.
Food assimilation coefficient [%]	81.5 $\pm$ 0.37	82.8 $\pm$ 1.16	80.8 $\pm$ 0.66	82.6 $\pm$ 0.99	n.s.	n.s.	n.s.

## 3.13 Pathology Screen

### 3.13.1 Summary

The Pathology screen performed a complete morphological analysis with standard stains. We did not find any pathological alteration. Although the testes were lighter in the mutant mice than in the control mice, the difference is not statistically significant. Therefore, the expected significant abnormalities in the testes weight and the testes morphology could not be confirmed. This could be explained by the pure genetic background and the younger age of the analyzed mice in our screen compared to the analyses described by Schürmann *et al.* (2002).

### 3.13.2 Mice

A total of 44 mice, 25 mutants (15 females, 10 males), and 19 control animals (10 females, 9 males) were analyzed. The mice were received from the different screens at the age of 21 weeks.

Table 28: Arl4 mice and their control littermates analyzed.					
Origin	Controls		Mutants		Number of Animals
	Female	Male	Female	Male	
Dysmorphology Screen	3	3	3	3	12
Metabolic Screen	7	6	7	7	27
Other Screens			5		5
Total Number of Animals	10	9	15	10	44

### 3.13.3 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 body length and the testes weight were measured in all mice received from the metabolic screen (six controls and seven mutants). 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, 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).

### 3.13.4 Results

#### Overview

<b>Table 29: Genotype-specific alterations of Arl4 mice:</b>			
<b>Parameters</b>	<b>Alteration</b>	<b>Parameters</b>	<b>Alteration</b>
Body weight	No	Mamma	No
Skin	No	Pancreas	No
Musculoskeletal system	No	Cervical lymph node	No
Eyes	No	Thymus	No
Cerebrum	No	Spleen	No
Cerebellum	No	Thyroid	No
Heart	No	Parathyroid	No
Trachea	No	Adrenal gland	No
Lung	No	Kidneys	No
Teeth	No	Urinary bladder	No
Salivary gland	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

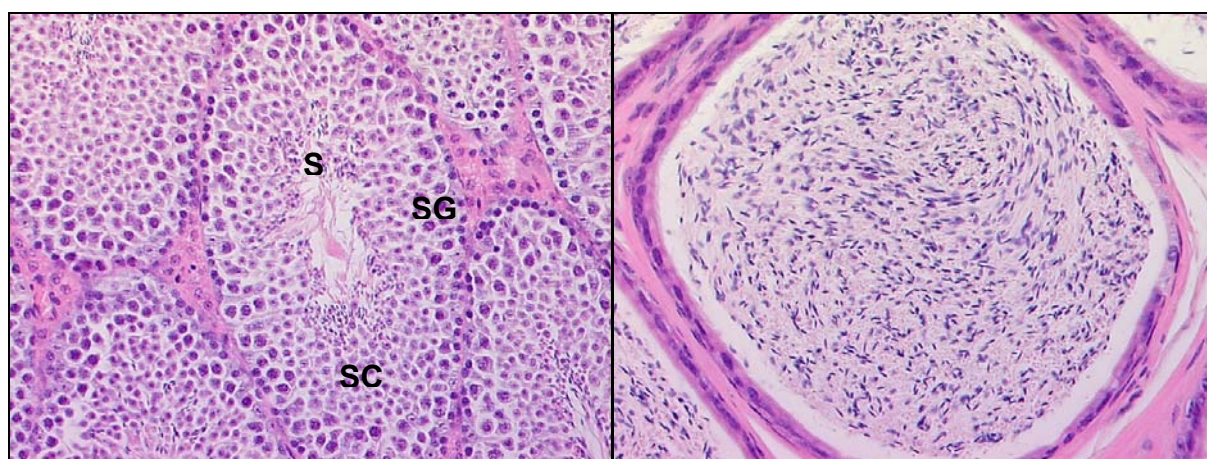
The measurement of the body weight did not reveal any significant differences between control and mutant mice (see Table 30 for more details).

<b>Table 30: Mean body [g] weight <math>\pm</math> standard deviation of Arl4 mice and their control littermates.</b>				
<b>Origin</b>	<b>Females</b>		<b>Males</b>	
	<b>Controls</b>	<b>Mutants</b>	<b>Controls</b>	<b>Mutants</b>
<b>Dysmorphology Screen</b>	22.84 $\pm$ 1.16	23.72 $\pm$ 1.07	31.00 $\pm$ 2.16	29.33 $\pm$ 1.89
<b>Other Screen</b>	-	25.40 $\pm$ 1.36	-	-
<b>Metabolic Screen</b>	22.86 $\pm$ 0.99	23.36 $\pm$ 2.05	32.67 $\pm$ 2.82	30.97 $\pm$ 1.02

## Testes

The data of the testes weight of 13 animals (six controls and seven mutants) did not show a significant difference between mutant and control mice at the age of 21 weeks, although the testes of the mutants were less heavy (Table 31). The p-value measured by t-test accounts for 0.0595.

Table 31: Testes weight				
	Body weight [g]	Body length [g]	Testes weight [g]	Testes weight % of body weight
Controls	32.67 ± 2.82	0.98 ± 0.02	229 ± 19.36	0.70%
Mutants	30.97 ± 1.02	0.97 ± 0.06	209 ± 7.05	0.67%



**Figure 9: Histological analysis of testis**

The left panel shows a normal testis with normal spermatogenesis: SG: spermatogonia, SC: spermatocytes, S: spermatides (H&E 200x). The right panel demonstrates that the ductus deferens is filled with a normal amount of spermatozoa (H&E 320x).

### 3.13.5 Discussion

The expected abnormalities in the testes weight and the testes morphology could not be confirmed. Although the mutants showed lower testes weight compared to the control mice (209 vs. 229 mg), the difference was not statistically significant (p-value = 0.0595 measured by t-test). Due to the young age of 21 weeks, we can not exclude that atrophic changes may occur with the age. However, the morphology of the testes and the spermatogenesis of all *Arl4*-mutant mice were within physiological range.

The fact that we could not confirm the results described by Schürmann *et al.* (2002) concerning the significant reduce in testes weight and the altered spermatogenesis, is partially explained by the different genetic background, and additionally, the mice were analyzed at a different age: We received mice of pure C57BL/6 background (backcrossed more than 11 times, N11), and analyzed them at the age of

21 weeks. In contrast, the F1-hybrids described by Schürmann *et al.* (2002) were analyzed at the age of 18 month.

In general, we did not find any pathological alteration. In general, we did not find any pathological alteration. However, as hypertension was observed in the cardiovascular screen, it would be interesting to look for pathological changes secondary to hypertension (vascular remodeling changes, hypoxic tissue damage, hemorrhage, infarction, left ventricular hypertrophy, glomerulosclerosis), which could be developed in elderly mice.

### **3.13.6 References**

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## Appendix: Tables

Table	1: Arl4 mice provided for analysis.....	5
Table	2: Primary Screen at GMC.....	6
Table	3: Evaluation of the behavioral phenotype.....	12
Table	4: Results of behavioral observation in the modified Hole Board Test.....	14
Table	5: Video-tracking results regarding locomotor behavior.....	17
Table	6: Results from the morphological inspection.....	21
Table	7: Results from the X-ray analysis.....	22
Table	8: Results from Clickbox Test (hearing test).....	23
Table	9: Bone- and weight-related quantitative parameters.....	24
Table	10: Recording of body weight.....	29
Table	11: Behavior recorded in the viewing jar.....	29
Table	12: Recording of locomotor activity and behavior in the arena.....	30
Table	13: Behavior recorded in or above the arena.....	31
Table	14: Axial eye length.....	36
Table	15: Results from funduscopy.....	36
Table	16: Results from slit lamp biomicroscopy.....	36
Table	17: Clinical-chemical parameters at the age of 12 weeks.....	42
Table	18: Clinical-chemical parameters at the age of 17 weeks.....	43
Table	19: Hematological parameters at the age of 12 weeks.....	44
Table	20: Basic parameters analyzed in the Immunology Screen.....	47
Table	21: Basic parameters analyzed in a second screen.....	48
Table	22: Total plasma IgE in Arl4 mice (12 weeks old).....	50
Table	23: Total plasma IgE in Arl4 mice (17 weeks old).....	50
Table	24: Nociceptive Screen.....	54
Table	25: Blood pressure parameters.....	61
Table	26: ECG parameters.....	62
Table	27: Metabolic parameters recorded in the primary screen.....	69
Table	28: Arl4 mice and their control littermates analyzed.....	70
Table	29: Genotype-specific alterations of Arl4 mice:.....	71
Table	30: Mean body [g] weight $\pm$ standard deviation of Arl4 mice and their control littermates.....	71
Table	31: Testes weight.....	72

## Figures

Figure	1: Workflow of the primary screen.....	7
Figure	2: Test arena for modified Hole Board test.....	11
Figure	3: Results from grip strength testing.....	27
Figure	4: Histological analysis of the retina.....	34
Figure	5: Hot plate system.....	52
Figure	6: Blood pressure set up.....	56
Figure	7: ECG-setup.....	56
Figure	8: Example of ECG trace with analyzed parameters.....	57
Figure	9: Histological analysis of testis.....	72

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## **Pathology Screen**

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