

1 Summary

1.1 Primary Screening

In a primary screen 48 mice (25 heterozygous mutants, 23 wild-type control littermates) of the *FOXP2_delta_ex7* mutant mouse line have been analyzed in the German Mouse Clinic (GMC) in the screens Behavior, Dymorphology, Fertility, Neurology, Eye, Clinical Chemistry, Immunology, Allergy, Steroid Metabolism, Cardiovascular Function, Nociception, Lung Function (primary and secondary), Energy Metabolism, and Pathology. The screening started on May 28, 2007. The results are summarized by screen briefly below.

Behavior: The behavioral pattern in the mHB suggests decreased locomotion in heterozygous mutants and less neo-phobic behavior in both sexes. Furthermore, the performance in the object recognition task supports that learning and memory is not impaired in mutants as already stated by Shu *et al.*, (2005).

Fertility: The *FOXP2*-mutant mice showed a higher sperm velocity and progressivity, and a higher amplitude of the lateral head displacement, indicating that the sperm swim with a stronger tail movement. The biological significance of these findings, however, should be clarified in further studies addressing the actual *in vivo* and/or *in vitro* fertilizing potential of the knockout sperm.

Neurology: The only significant finding in our neurological screening of male and female heterozygous *FOXP2*-mutant mice was a decreased rotarod performance hinting towards a defect in motor coordination.

Immunology: The Immunology Screen found statistically significant differences between female mutant mice and their littermate controls concerning the frequencies of CD4+ T-cells [↑] in peripheral blood. In male mutant mice we found a higher proportion of CD62L expressing cells within the CD8+ T-cells compartment. Furthermore, slightly higher blood plasma levels of IgM immunoglobulin have been measured in female mutant mice.

Cardiovascular: The comparison of the *FOXP2*-mutant to control mice in blood pressure and ECG analysis revealed only a subtle difference seen as slightly decreased pulse in male mutants.

Lung Function: The comparison of the breathing patterns between wild-type and mutant *FOXP2* mice of both sexes exhibited no differences at all, with the exception of *FOXP2*-mutant males suggested to be somewhat more active during study period as indicated by significantly higher mean respiratory rates (12%). In addition, the assessment of lung volumes, respiratory mechanics, intrapulmonary and alveolar-capillary gas transport (secondary screen) in male mutants did not reveal a phenotype associated with the *FOXP2* mutation. We therefore do not suggest the *FOXP2_delta_ex7* mutant mouse line to be a phenotype as far as respiratory function is concerned.

Energy Metabolism: The analysis for genotype-specific differences revealed a strong body temperature reduction during fasting in mutant mice.

Pathology: The Pathology Screen performed a complete pathological analysis. Using macroscopic and histological criteria, heterozygous FOXP2 knock-out mice exhibited no morphological differences from their control littermates. The results of the performed immunohistochemistry for FOXP2 protein expression are showed and discussed. Special interest is paid to the FOXP2 protein expression detected in **testis** and ovary due to its possible role in sperm and ovarian follicle development.

In the screens **Dysmorphology, Eye, Clinical Chemistry, Allergy, Steroid Metabolism, Nociception,** and **Pathology**, no genotype-specific differences could be found.

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

2 General Part

2.1 The Role of the Gene

Currently there are no confirmed targets or pathways known, except for CC10, a lung-specific gene that is repressed by FOXP2 (Shu *et al.*, 2001).

2.2 Known Phenotypes

Homozygous *FOXP2*-knockout mice have severe motor deficits, cerebellar abnormalities, especially in Purkinje cells and die around P21. As pups, heterozygous knockout mice have a slightly lower weight and vocalize less than wild-type littermates.

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

2.3 Expected Phenotypes

Since *FOXP2* is expressed in connection with lung development (Shu *et al.*, 2001) and is expressed in many other tissues, an effect in other areas could also likely be.

2.4 Suggested Human Disease Model

This mouse line is a disease model for developmental verbal dyspraxia (OMIM 602081), a form of specific language impairment. The condition is characterized by impaired intellectual, linguistic, and orofacial praxic functions (Vargha-Khadem *et al.*, 2005).

2.5 Mice

2.5.1 Number and kind of mice

Exon 7 of the *FOXP2* gene has been deleted in this mutant mouse line. The mutant line is on C57BL/6 background.

Table 1: FOXP2_delta_ex7 mice provided for analysis.
Numbers in brackets indicate animals which were kept in reserve.

Genotype / Sex	Number of Animals
Heterozygous mutant female	15 (+2) -6
Heterozygous mutant male	10 (+1) -1
Control female	13 -2
Control male	10 (+9)

Nine mice died during primary screening (please see 2.6.2).

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

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

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

2.6 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 *et al.*, 2005/2009; Fuchs *et al.*, 2009). Analyzed parameters are listed in Table 2.

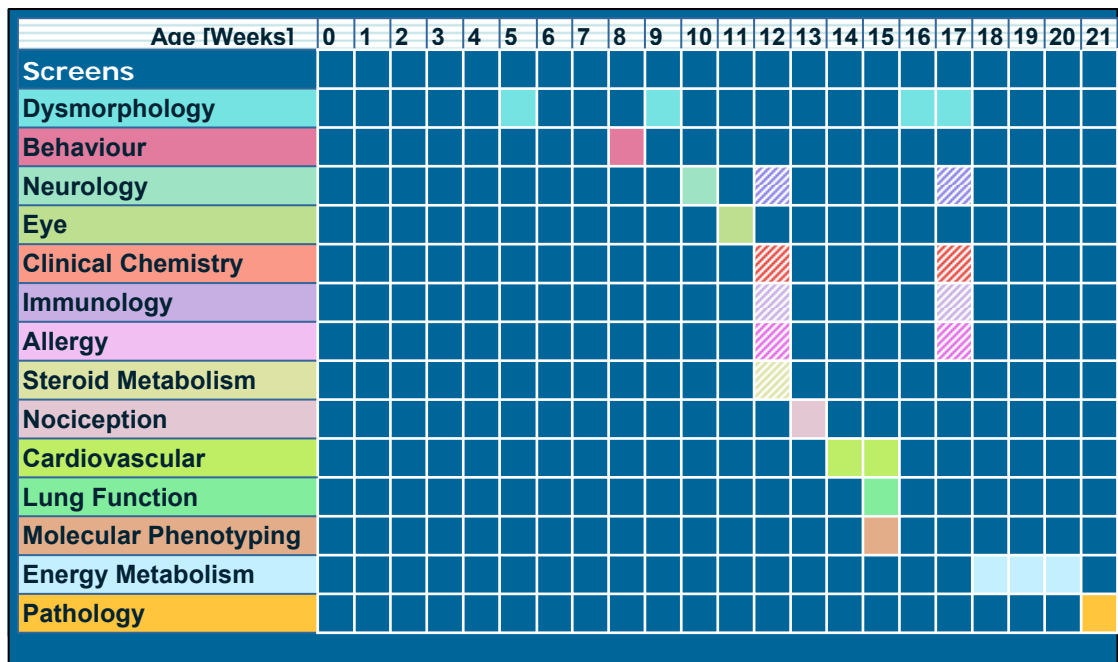



Figure 1: Workflow of the primary screen

Explanation below,  Analysis of blood-based parameters.

After the mice arrive at the GMC, they are acclimatized in the new environment for one week. The males then start in the Behavior Screen. There they stay for three weeks. Directly after the behavior tests, the anatomical inspection of the Dysmorphology Screen is performed. In the next week, the Neurology Screen is applied. One week later the mice go through the tests of the Eye Screen. When the mice were 12 weeks old, blood is taken, and samples are distributed to the blood-based screens for Clinical Chemistry, Immunology, and Allergy. 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 molecular phenotyping on request (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 are analyzed macro- and microscopically in the Pathology.

The screening of female animals starts one week later and follows the same workflow (with the exception of Molecular Phenotyping 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 heterozygous *FOXP2*-knockout 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: *female* mice have been delivered to lung function and molecular expression screen. 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 (7) and for unknown reason (2) and thus could not be analyzed for all parameters. In addition to the primary screen, the mice were subjected to a secondary Lung Function, Sperm Motility, and Pathology screening.

2.6.3 Quality Assurance

The Quality Assurance as part of the Quality Management at the GMC consists of the following elements: standardized analyses via Standard Operating Procedures (SOP) and validation of analysis protocols by different institutions within the EUMORPHIA program, standardized data and project management supported by the central database system MausDB (Maier *et al.*, 2008) and the GMC coordination tool CoordDB as well as Quality Control and continuous training of the staff.

Coordination of the GMC's operations

The GMC management team (Core Facility) coordinates the scientific issues, logistics and administration of the GMC. The coordination software tool CoordDB supports the GMC management team in handling the incoming phenotyping requests and managing the complex phenotyping workflow of the primary and secondary screening. Besides the operational business activities the management team organizes the expansion of the screening services in collaboration with its partners. Additionally, the management arranges regular training of the staff members and the clinic's quality assurance.

Standardized Operation Procedures (SOP) and Validation of Protocols

The GMC developed a set of SOPs which cover all steps from mouse import and handling to phenotyping and data analysis. These SOPs are strictly followed during the whole screening process in the GMC and all procedures are documented.

The GMC is one of the major partners of the EUMODIC consortium that emerged from the EUMORPHIA program (Brown *et al.*, 2005), a consortium for the selection, establishment, and standardization of phenotyping protocols for mice as models for human diseases and for mouse husbandry. Cross-validation of protocols by EUMORPHIA is performed by the different institutions. A collection of the protocols (EMPreSS) is posted on the EUMORPHIA web site at (<http://www.eumorphia.org/EMPreSS/>).

Central Database System

Another tool for quality assessment is the central database system which ensures full traceability of samples and documentation of all data. All mouse data is entered into the system (e.g. date of birth, sex, cage) and all screening results linked to the corresponding SOP as well as any changes of the mouse conditions are immediately put in.

Quality Control

In addition to routinely screen-specific quality control tests, control animals of selected strains (e.g. C57BL/6 and C3HeB/FeJ) are analyzed through the standard protocol for all phenotypes at regular intervals. This data is reviewed by the coordination team.

A tissue archive has been established for the storage of tail and blood plasma samples taken from all mice that have ever been analyzed in the GMC. The tail clips can be used for post-hoc genotyping in case of doubtful genotype information. The sanitary status of every mouse completing the screening can be tested by means of these plasma samples.

Continuous Training

Regularly specific training courses are held at the GMC. Specialists are invited to give lectures and to offer practical training at special days. Staff training is documented and maintained by the management team.

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. Raw data are available on request.

2.8 References

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Abbreviations and Wording

<i>FOXP2</i>	gene coding for the transcription factor FOXP2
GMC	German Mouse Clinic
IVC	individually ventilated cage
control	homozygous wild-type control, <i>FOXP2</i> ^{+/+}
mutant	heterozygous mutant, mutant <i>FOXP2</i> ^{+/-}
wt	wild type
KO	knockout
FELASA	F ederation of E uropean L aboratory A nimal S cience A ssociations, 25 Shaftesbury Avenue, London W1D 7EG, UK, www.felasa.org

Table 2: Primary Screen at GMC

Screens	Goal	Methods
Dysmorphology, Bone and Cartilage	morphological analysis of body, skeleton, bone and cartilage	morphological observation, bone densitometry, X-ray, micro-computed tomography
Behavior	locomotor, exploratory, emotional and social behavior, object recognition memory	modified hole board
Neurology	assessment of muscle, spinocerebellar, sensory, and autonomic function	modified SHIRPA protocol
Eye	assessment of morphological alterations of the eye	funduscopy laser interference biometry slit lamp biomicroscopy histological analysis
Clinical Chemistry and Hematology	determination of clinical-chemical and hematological parameters in blood	blood autoanalyzer, ABC-animal blood counter
Immunology	analysis of peripheral blood samples for immunological parameters	flow cytometry, Multiplex Bead Array
Allergy	analysis of total plasma IgE	ELISA
Steroid Metabolism	analysis of plasma testosterone and DHEA	ELISA
Nociception	detection of altered pain response	hot plate assay
Cardiovascular	assessment of functional cardio-vascular parameters	non-invasive tail-cuff blood pressure measurement, surface limb ECG
Lung Function	assessment of alterations in breathing patterns	whole body plethysmography (Buxco®)
Molecular Phenotyping	RNA expression profiling	DNA-chip technology
Energy Metabolism	measurement of altered body weight regulation, body temperature and energy balance	bomb calorimetry
Pathology	microscopic and macroscopic examination	histology, immunochemistry

3 Specific part

3.1 Behavior Screen

3.1.1 Introduction

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

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

3.1.2 Summary

The behavioral pattern in the mHB suggests decreased locomotion in heterozygous mutants and less neo-phobic behavior in both sexes. Furthermore, the performance in the object recognition task supports that learning and memory is not impaired in mutants as already stated by Shu *et al.* (2005).

3.1.3 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, 28 female mice (13 controls, 15 heterozygous mutants) and 20 male mice (10 controls, 10 heterozygous mutants) were available for analysis.

3.1.4 Material and Methods

The modified Hole Board test (Ohl *et al.*, 2001) was carried out as previously described (Kallnik *et al.*, 2007). The test apparatus consisted of a test

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

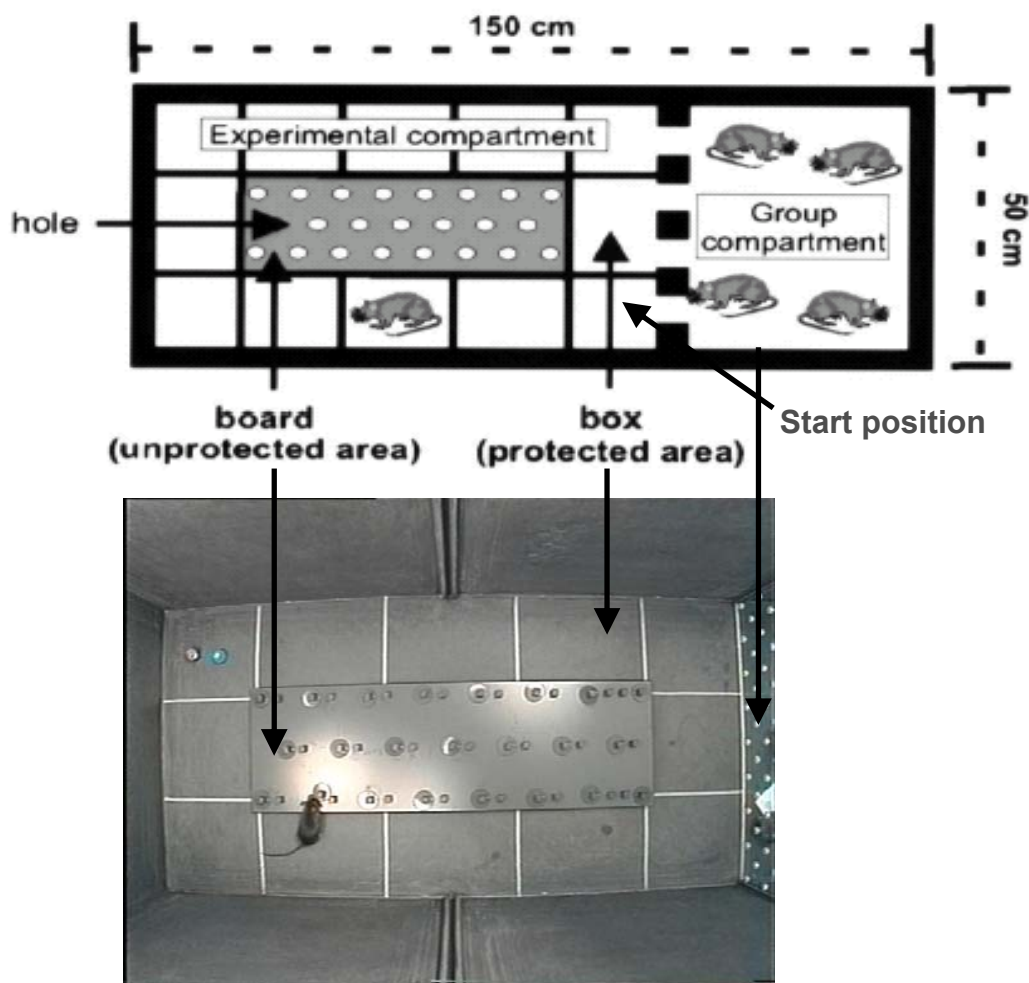


Figure 2: Test arena for modified Hole Board test.

For testing, each animal was placed individually into the test arena and allowed to explore it freely for 5 min. The animals were always placed into the test arena in the same corner next to the partition, facing the board diagonally. The two objects were placed in the corner quadrant diametrical to the starting point. During the 5 min trial, the animal's behavior was recorded by a trained observer with a hand-held computer. Data were analyzed by using the Ob-

server 4.1 Software (Noldus, Wageningen). Additionally, a camera was mounted 1.20 m above the center of the test arena, and the animal's track was videotaped and its locomotor path analyzed with a video-tracking system (Ethovision 2.3, Noldus, Wageningen). After each trial, the test arena was cleaned carefully with a disinfectant.

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

3.1.5 Results

Behavioral analysis of spontaneous activity in a novel environment, as measured by the **modified Hole Board** test, revealed that mutants of both sexes travelled less distance (Table 5), and exhibited reduced line crossing (Table 4) and turning frequency (Table 5). Additionally, the maximum speed of movement was *tendentially* increased in mutants, without any changes in the mean speed of movement (Table 5). Moreover, the path shape of mutant mice was less straight forward since they made more changes in direction between two samples (mean turn angle) and more turns per unit distance (absolute meander, Table 5).

Concerning social affinity, mutants of both sexes approached the partition less frequently, but the duration of group contact was not significantly reduced as compared to controls (Table 4).

Regarding anxiety-related behavior, mutants entered the board as frequently as controls, and with the same latency and duration (Table 4). However, sex-specifically mutant males exhibited a *slightly* increased maximum duration of board entry and kept a bigger distance to the wall, and consequently a shorter one to the board (Table 5).

With respect to exploration, sex-specifically mutant females reared more frequently than control females, but exhibited delayed hole exploration while exploring the same amount of holes (Table 4). The analysis of vertical exploration on board (rearings on board, Table 4) *tendentially* indicated a different genotype effect in males than in females. However, *post hoc* analysis did not show significant differences between the genotypes within a sex.

Although mutants of both sexes explored the unfamiliar object more intensively and mutant females also more frequently than controls, the object recognition index did not differ between the genotypes (Table 4).

There were no genotype effects on any other observed parameter (Table 3).

3.1.6 Discussion

The **primary behavioral observation** in the modified Hole Board demonstrated reduced locomotion in heterozygous mutants of both sexes. Mutants

traveled less distance (total distance, line crossings, turns). The reduction of forward locomotion was expected since homozygous KO mutants exhibit severe motor impairments and for heterozygous mutants cerebellar abnormalities have been reported (Shu *et al.*, 2005). The mHB result of a less straight forward path shape in heterozygous mutants supports the findings of impaired movements in *FOXP2*-KO mice. This is also supported by the findings of the Neurology Screen in the Rotarod, although the genotype-related differences on the Rod were small ones.

The reduced social contacts were most likely secondary to reduced forward locomotion since the duration of stay in social contact did not differ from controls.

Table 3: Evaluation of the behavioral phenotype	
Behaviors which are considered affected in mutants due to the pattern of significantly altered parameters are marked in red.	
Behavior	Parameters
forward locomotion	distance moved, line crossings (latency, frequency)
speed of movement	velocity (mean, maximum, angular)
exploration	vertical: females rearings (box, board; latency, frequency) horizontal: females holes (latency, frequency), objects (novel, familiar; latency, females frequency, duration)
risk assessment	stretched attends (latency, frequency)
anxiety-related	board entries (latency, frequency, males maximum duration); distance to the wall
grooming	grooming (latency, frequency, duration)
defecation	boli (latency, frequency)
social affinity	exploration of the partition (latency, frequency, duration)
object memory	object recognition index

Reduction in anxiety-related behavior towards the board was only evident in mutant males and has to be considered as a subtle effect, because the major anxiety-related parameters (entries on board, latency to first entry and total duration of stay) did not significantly differ from control males. However, together with the increased exploration of the unfamiliar object in mutants – evident in both sexes – one may interpret the behavioral pattern as less neophobic behavior in heterozygous knock-out mice.

The object recognition memory in the mHB, indicated by the object index, was the same in both genotypes. This finding is in line with observations from Shu *et al.*, (2005) reporting that learning and memory appear to be normal in heterozygous *FOXP2* animals, at least in the Morris Water Maze.

The behavioral pattern for general exploration was rather unspecific: On the one hand mutant females reared more in the box, but on the other hand they exhibited hole exploration later. Therefore, a genotype effect on general exploration can not be concluded from the mHB analysis.

Taken together, the behavioral pattern in the mHB suggests decreased locomotion in heterozygous mutants and less neo-phobic behavior in both sexes. Furthermore, the performance in the object recognition task supports that learning and memory is not impaired in mutants as already stated by Shu *et al.*, (2005).

Comparing *FOXP2*-KO mice to both batches of *FOXP2*-KI mice, the mHB analyses revealed an opposite effect of the mutation depending on the targeting strategies: While *FOXP2* heterozygous KO mutants showed less neo-phobic behavior in the mHB, both batches of *FOXP2*-KI mutants were considered more neo-phobic/anxious during the test situation. The reduced locomotion was true for both, the *FOXP2* heterozygous KO and the *FOXP2*-KI mice. Therefore, reduced locomotion could be considered an unspecific finding. However, there are two arguments against this hypothesis. Firstly, the reduced locomotion in *FOXP2* heterozygous KO mice is an analogous finding to severe motor impairments in *FOXP2*-KO mice from Shu and co-workers (2005). Secondly, the reduced locomotion for the *FOXP2* knock-in mouse line was only evident in the batch still containing the neo cassette, whereas alterations in locomotion were not evident in *FOXP2*-KI mutants lacking the neo cassette. This suggests that in *FOXP2*-KI mice the changed locomotion might reflect an unspecific effect of the neo cassette, if not the additional band.

At the moment, we have no suggestions for further analysis, but we are open for discussions.

3.1.7 References

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

yellow indicate significant alterations only in one sex

Parameter	Control		Mutant		Male + Female		ANOVA		
	Male	Female	Male	Female	Control	Mutant	sex	genotype	Interaction
	(n=10)	(n=13)	(n=10)	(n=15)	(n=23)	(n=25)			
Line crossing [frequency]	115.5 \pm 6.64	121.31 \pm 8.08	94.3 \pm 5.29	106.73 \pm 4.46	118.78 \pm 5.32	101.76 \pm 3.57	n.s.	p<0.01	n.s.
Line crossing [latency]	1.98 \pm 0.81	1.06 \pm 0.08	1.35 \pm 0.16	0.97 \pm 0.04	1.46 \pm 0.36	1.12 \pm 0.08	n.s.	n.s.	n.s.
Rearings in box [frequency]	26.1 \pm 1.74	22.46 \pm 1.48	22.6 \pm 2.16	27.4 \pm 1.39	24.04 \pm 1.17	25.48 \pm 1.27	n.s.	n.s.	p<0.05
Rearings in box [latency]	20.58 \pm 3.88	27.94 \pm 3.57	17.76 \pm 3.35	24.35 \pm 6.12	24.74 \pm 2.68	21.72 \pm 3.9	n.s.	n.s.	n.s.
Hole exploration [frequency]	48.1 \pm 5.16	54.54 \pm 4.8	58.4 \pm 5.13	55.47 \pm 4.72	51.74 \pm 3.51	56.64 \pm 3.44	n.s.	n.s.	n.s.
Hole exploration [latency]	19.24 \pm 4.44	7.65 \pm 1.55	16.12 \pm 3.64	16.87 \pm 2.7	12.69 \pm 2.4	16.57 \pm 2.13	n.s.	n.s.	p=0.05
Hole visit [frequency]	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	n.s.	n.s.	n.s.
Hole visit [latency]	300 \pm 0	300 \pm 0	300 \pm 0	300 \pm 0	300 \pm 0	300 \pm 0	n.s.	n.s.	n.s.
Board entry [frequency]	9.2 \pm 1.26	10.62 \pm 1.31	10.1 \pm 1.34	10.27 \pm 1.26	10 \pm 0.91	10.2 \pm 0.91	n.s.	n.s.	n.s.

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

yellow indicate significant alterations only in one sex

Parameter	Control		Mutant		Male + Female		ANOVA		
	Male	Female	Male	Female	Control	Mutant	sex	genotype	Interaction
	(n=10)	(n=13)	(n=10)	(n=15)	(n=23)	(n=25)			
Board entry [latency]	46.97 \pm 7.9	58.17 \pm 15.18	53.33 \pm 15.5	44.13 \pm 3.74	53.3 \pm 9.14	47.81 \pm 6.46	n.s.	n.s.	n.s.
Board entry [total duration %]	9.61 \pm 1.48	11.65 \pm 1.75	14.95 \pm 1.76	11.63 \pm 1.34	10.76 \pm 1.18	12.96 \pm 1.1	n.s.	n.s.	n.s.
Rearing on board [frequency]	1.1 \pm 0.62	1.92 \pm 0.65	2.7 \pm 1.07	0.93 \pm 0.34	1.57 \pm 0.45	1.64 \pm 0.49	n.s.	n.s.	p=0.06
Rearing on board [latency]	234.05 \pm 27.71	208.18 \pm 26.8	178.32 \pm 32.91	216.75 \pm 24.56	219.43 \pm 19.12	201.38 \pm 19.71	n.s.	n.s.	n.s.
Risk assessment [frequency]	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	n.s.	n.s.	n.s.
Risk assessment [latency]	300 \pm 0	300 \pm 0	300 \pm 0	300 \pm 0	300 \pm 0	300 \pm 0	n.s.	n.s.	n.s.
Group contact [frequency]	24.4 \pm 2.32	25.08 \pm 3.54	17.5 \pm 1.88	18.87 \pm 1.7	24.78 \pm 2.2	18.32 \pm 1.25	n.s.	p<0.05	n.s.
Group contact [latency]	12.37 \pm 2.72	17.36 \pm 3.12	14 \pm 2.66	17.43 \pm 2.33	15.19 \pm 2.14	16.06 \pm 1.76	n.s.	n.s.	n.s.
Group contact [total duration %]	16.16 \pm 2.31	15.85 \pm 2.52	12.39 \pm 1.86	12.65 \pm 1.54	15.99 \pm 1.71	12.54 \pm 1.16	n.s.	n.s.	n.s.

Table 4: Results of behavioral observation in the modified Hole Board test

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

yellow indicate significant alterations only in one sex

Parameter	Control		Mutant		Male + Female		ANOVA		
	Male	Female	Male	Female	Control	Mutant	sex	genotype	Interaction
	(n=10)	(n=13)	(n=10)	(n=15)	(n=23)	(n=25)			
Grooming [frequency]	2.1 \pm 0.53	3 \pm 0.57	3.4 \pm 0.98	3 \pm 0.67	2.61 \pm 0.4	3.16 \pm 0.55	n.s.	n.s.	n.s.
Grooming [latency]	172.91 \pm 28	183.54 \pm 17.99	163.89 \pm 17.32	179.87 \pm 21.09	178.92 \pm 15.51	173.48 \pm 14.25	n.s.	n.s.	n.s.
Grooming [total duration %]	1.9 \pm 0.43	2.29 \pm 0.27	1.87 \pm 0.41	1.62 \pm 0.44	2.12 \pm 0.24	1.72 \pm 0.31	n.s.	n.s.	n.s.
Defecation [frequency]	1 \pm 0.45	0.54 \pm 0.33	1.2 \pm 0.55	0.4 \pm 0.19	0.74 \pm 0.27	0.72 \pm 0.26	n.s.	n.s.	n.s.
Defecation [latency]	233.56 \pm 31.13	275.53 \pm 14.89	249.09 \pm 22.41	270.08 \pm 17.18	257.28 \pm 16.14	261.68 \pm 13.53	n.s.	n.s.	n.s.
Unfamiliar object exploration [frequency]	8.2 \pm 1.32	3.54 \pm 0.37	6.8 \pm 0.9	5.4 \pm 0.64	5.57 \pm 0.77	5.96 \pm 0.53	p<0.001	n.s.	p=0.05
Familiar object exploration [frequency]	8.5 \pm 1.06	5.23 \pm 0.71	6.5 \pm 0.72	6.8 \pm 0.66	6.65 \pm 0.69	6.68 \pm 0.48	n.s.	n.s.	p<0.05
Unfamiliar object exploration [latency]	23.59 \pm 8.94	34.81 \pm 10.89	20.96 \pm 4.47	32.93 \pm 10.9	29.93 \pm 7.22	28.14 \pm 6.78	n.s.	n.s.	n.s.

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

yellow indicate significant alterations only in one sex

Parameter	Control		Mutant		Male + Female		ANOVA		
	Male	Female	Male	Female	Control	Mutant	sex	genotype	Interaction
	(n=10)	(n=13)	(n=10)	(n=15)	(n=23)	(n=25)			
Familiar object exploration [latency]	19.46 \pm 5.06	54.14 \pm 21.98	23.45 \pm 5.8	25.32 \pm 6.72	39.06 \pm 12.92	24.57 \pm 4.57	n.s.	n.s.	n.s.
Unfamiliar object exploration [total duration %]	1.78 \pm 0.3	0.62 \pm 0.09	1.94 \pm 0.26	1.33 \pm 0.17	1.13 \pm 0.18	1.57 \pm 0.16	p<0.001	p<0.05	n.s.
Familiar object exploration [total duration %]	1.21 \pm 0.15	0.72 \pm 0.12	1.24 \pm 0.23	1.21 \pm 0.13	0.94 \pm 0.1	1.22 \pm 0.12	n.s.	n.s.	n.s.
Object Index	0.13 \pm 0.08	-0.02 \pm 0.11	0.23 \pm 0.05	0.01 \pm 0.07	0.05 \pm 0.07	0.1 \pm 0.05	n.s.	n.s.	n.s.

Table 5: Video-tracking results regarding locomotor behavior

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

yellow indicate significant alterations only in one sex

Parameter	Control		Mutant		Male + Female		ANOVA		
	Male	Female	Male	Female	Control	Mutant	sex	genotype	Interaction
	(n=10)	(n=13)	(n=10)	(n=15)	(n=23)	(n=25)			
Total Distance Moved [cm]	2912.87 \pm 132.7	3135.65 \pm 150.27	2604.53 \pm 114.61	2879.14 \pm 84.44	3038.79 \pm 103.19	2769.3 \pm 72.25	p<0.05	p<0.05	n.s.
Mean Velocity [cm/sec]	18.78 \pm 0.62	19.22 \pm 0.63	17.27 \pm 0.54	18.69 \pm 0.47	19.02 \pm 0.44	18.12 \pm 0.38	n.s.	n.s.	n.s.
Maximum Velocity [cm/sec]	56.75 \pm 1.54	53.63 \pm 1.84	58.01 \pm 2.36	61.49 \pm 2.83	54.98 \pm 1.26	60.1 \pm 1.94	n.s.	p=0.06	n.s.
Turns [Frequency]	1559.4 \pm 42.54	1668.08 \pm 48.46	1462.2 \pm 48.96	1543.6 \pm 25.86	1620.83 \pm 34.32	1511.04 \pm 25.69	p<0.05	p<0.05	n.s.
Mean Turn Angle [degrees]	24.52 \pm 0.8	23.4 \pm 0.53	26.16 \pm 0.59	24.82 \pm 0.79	23.89 \pm 0.46	25.36 \pm 0.54	n.s.	p<0.05	n.s.
Angular Velocity [degrees/sec.]	154.92 \pm 3.53	150.58 \pm 3.04	159.02 \pm 3.82	152.51 \pm 4.78	152.47 \pm 2.3	155.12 \pm 3.26	n.s.	n.s.	n.s.
Absolute Meander [degrees/sec.]	17.06 \pm 0.69	16.21 \pm 0.44	18.55 \pm 0.49	17.49 \pm 0.65	16.58 \pm 0.39	17.92 \pm 0.44	n.s.	p<0.05	n.s.
Board entry [maximum duration. sec.]	6.63 \pm 0.95	8.42 \pm 1.34	11.34 \pm 2.01	8 \pm 0.77	7.65 \pm 0.87	9.33 \pm 0.96	n.s.	n.s.	p=0.05
Mean distance to wall [cm]	7.2 \pm 0.27	8.09 \pm 0.4	8.5 \pm 0.35	8.04 \pm 0.26	7.7 \pm 0.27	8.22 \pm 0.21	n.s.	p=0.07	p<0.5
Mean distance to board [cm]	8.48 \pm 0.18	7.82 \pm 0.29	7.66 \pm 0.24	7.86 \pm 0.18	8.11 \pm 0.19	7.78 \pm 0.14	n.s.	n.s.	p=0.07

3.2 Dymorphology, Bone and Cartilage

3.2.1 Introduction

In the Dymorphology, Bone and Cartilage Screen of the German Mouse Clinic mice are analyzed for morphological abnormalities in different organ systems with special focus on bone and cartilage development and homeostasis. The aim of the screen is to establish mouse models for human skeletal diseases like osteoporosis (McLean & Olsen, 2001; Rosen *et al.*, 2001), scoliosis (Giampietro *et al.*, 2003), limb defects (Mariani & Martin, 2003), osteogenesis imperfecta (Rauch & Glorieux, 2004; Chipman *et al.*, 1993) or osteoarthritis (Abe *et al.*, 2006). We adapted the successful dymorphological screening protocol from the Munich ENU-Mutagenesis Screen (Hrabé de Angelis *et al.* 2000) for use in the German Mouse Clinic. The nomenclature of the parameters was adapted according to the Mammalian Phenotype Ontology wording (www.informatics.jax.org/searches/MP_form.shtml). Further tests for defects in bone development and homeostasis were taken over from human diagnosis, and were adapted for the use in mice analysis. Such tests include: X-ray analysis, bone densitometry, and, in special cases, micro computed tomography. Detailed protocols for screening for bone and cartilage phenotypes in mice are described in Fuchs *et al.* (2006).

3.2.2 Summary

A total of 48 animals of FOXP2_delta_ex7 mutant mouse line were analyzed in the Dymorphology, Bone and Cartilage module of the German Mouse Clinic. In the morphological investigation via visual inspection and X-ray analysis, no genotype-specific differences were found. In the dual-energy X-ray absorptiometry (DXA), no significant differences could be detected.

3.2.3 Mice

Twenty male (10 controls, 10 mutants) and 28 female (13 controls, 15 mutants) mice were analyzed by morphological inspection at the age of 9 weeks. 17-18-week-old mutants (16 animals) and controls (17 animals) entered the bone density and X-ray analysis.

3.2.4 Material and Methods

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

1. At the age of five weeks, i.e. when the mice entered the facility, the general physical condition and health were checked,
2. at the age of nine weeks, a morphological observation as a whole-body checkup was performed; and
3. 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).

3.2.5 Results and Discussion

Forty-eight animals of FOXP2_delta_ex7 mutant mouse line were analyzed in the Dysmorphology, Bone and Cartilage module of the German Mouse Clinic. In the morphological investigation via visual inspection and X-ray analysis no genotype-specific differences were found (Tables 6 and 7). In the Clickbox test (Table 8) to test the hearing ability of the mice, we observed a significantly retarded reaction in female mutants (same tendency in males) but not

in controls. A retarded reaction is not an abnormal phenotype because the C57BL/6 strain exhibits severe sensorineural hearing loss at an early age (Turner *et al.*, 2005; The Jackson Laboratory: <http://www.informatics.jax.org/external/festing/mouse/docs/C57BL.shtml>). It should be investigated if control mice also exhibit hearing loss at an older age. In the bone densitometry using DXA analysis (Table 9), no significant differences could be detected. Fat mass and fat content were slightly increased (not significant) in mutants compared to controls whereas lean mass and lean content were slightly decreased (not significant). The sex differences we observed are common in many mouse strains, and thus are not abnormal (unpublished data).

3.2.6 References

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Abbreviations

BMC	bone mineral content
BMD	bone mineral density
DXA	dual-energy X-ray absorptiometry
μ CT	micro computed tomography
pQCT	peripheral quantitative computed tomography
pBMD	partial bone mineral density (excluding skull)
sBMD	specific bone mineral density

Table 6: Results from the morphological inspection (nine-week old mice)				
Parameter	Male		Female	
	Control	Mutant	Control	Mutant
Body appearance				
normal	10	10	13	15
Craniofacial / head morphology				
normal	10	10	13	15
Limbs				
normal	10	10	13	15
Digits				
normal	10	9	13	13
missing digit	-	1	-	1
bent digit	-	-	-	1
Tail				
normal	10	10	13	15
Eyes				
normal	10	10	13	15
Ears				
normal	10	10	13	15
Teeth				
normal	10	10	13	15
Vibrissae				
normal	10	10	13	15
Coat appearance				
normal	10	10	13	15
Coat / hair growth				
normal	10	10	13	15
Coat / hair texture				
normal	10	10	13	15
Hair follicle structure / orientation				
normal	10	10	13	15
Skin pigmentation				
normal	10	10	13	15
Skin condition / texture				
normal	10	10	13	15
Muscle morphology				
normal	10	10	13	15
Seizures / epilepsy				
no	10	10	13	15
Motor capabilities / coordination				
normal	10	10	13	15
Movement				
normal	10	10	13	15

Eating / drinking behavior				
normal	10	10	13	15
Respiratory system				
normal	10	10	13	15
Reproductive / urinary system				
normal	10	10	13	15
Other abnormalities				
no	10	10	13	15
Animals analyzed	10	10	13	15

Table 7: Results from the X-ray analysis (17-18-week old mice)

Parameter	Male		Female	
	Control	Mutant	Control	Mutant
Skull				
normal	10	10	7	6
Mandibles				
normal	10	10	7	6
Maxilla				
normal	10	10	7	6
Teeth				
normal	10	10	7	6
Orbit				
normal	10	10	7	6
Spine				
normal	10	10	7	6
Number of cervical vertebrae				
normal (7)	10	10	7	6
Number of thoracic vertebrae				
normal (13)	10	10	7	6
Number of lumbar vertebrae				
normal (6)	10	10	7	6
Number of sacral vertebrae				
normal (4)	10	10	7	6
Number of caudal vertebrae				
normal	10	10	7	6
Vertebrae				
normal	10	10	7	5
abnormal tail	-	-	-	1
Ribs				
normal (26)	9	10	7	6
24	1	-	-	-
Scapulas				
normal	10	10	7	6
Clavicle				

normal	10	10	7	6
Pelvis				
normal	10	10	7	6
Femur				
normal	10	10	7	6
Tibia				
normal	10	10	7	6
Fibula				
normal	10	10	7	6
Humerus				
normal	10	10	7	6
Ulna				
normal	10	10	7	6
Radius				
normal	10	10	7	6
Digits				
normal (20)	10	9	7	6
digit missing	-	1	-	-
Joints				
normal	10	10	7	6
Animals analyzed	10	10	7	6

Table 8: Results from clickbox test (hearing test; nine-week old mice)

Phenotype	Male		Female	
	Control	Mutant	Control	Mutant
0	-	-	-	-
1	-	2	-	2
2	1	3	-	10
3	8	5	12	3
4	1	-	1	-
5	-	-	-	-
Mean Score	3.00	2.30	3.08	2.07

Kruskal-Wallis ANOVA on Ranks: females significant, P < 0.001

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

Table 9: Bone- and weight-related quantitative parameters (17-week old mice)

(data presented as mean ± standard error of mean)

Parameter	FOXP2- delta_ex7 Control		FOXP2- delta_ex7 Mutant		C ~ M		ANOVA		
	Male	Female	Male	Female	Male	Female	<i>p</i> – value genotype	<i>p</i> – value sex	<i>p</i> – value interaction
	(n=10)	(n=7)	(n=10)	(n=6)	<i>p</i> – value	<i>p</i> – value			
BMD [mg/cm ²]	65 ± 2	62 ± 3	62 ± 2	66 ± 3	n.a.	n.a.	n.s.	n.s.	n.s.
pBMD [mg/cm ²]	54 ± 1	50 ± 2	51 ± 1	52 ± 2	n.a.	n.a.	n.s.	n.s.	n.s.
sBMD [10 ⁻³ x cm ⁻²]	2.21 ± 0.07	2.55 ± 0.12	2.13 ± 0.10	2.63 ± 0.20	n.a.	n.a.	n.s.	< 0.01	n.s.
BMC [mg]	567 ± 39	529 ± 45	618 ± 47	534 ± 56	n.a.	n.a.	n.s.	n.s.	n.s.
Bone Content [%]	1.90 ± 0.11	2.17 ± 0.18	2.09 ± 0.13	2.09 ± 0.18	n.a.	n.a.	n.s.	n.s.	n.s.
Body Length [cm]	9.80 ± 0.08	9.50 ± 0.00	9.85 ± 0.08	9.50 ± 0.00	n.a.	n.a.	n.s.	< 0.0001	n.s.
Body Weight [g]	29.74 ± 0.53	24.44 ± 0.30	29.41 ± 0.95	25.33 ± 0.75	n.a.	n.a.	n.s.	< 0.0001	n.s.
Fat mass [units]	5.71 ± 0.79	4.27 ± 0.66	7.23 ± 0.85	6.49 ± 0.99	n.s.	n.s.	< 0.05	n.s.	n.s.
Fat Content [units x 100/g]	18.99 ± 2.40	17.50 ± 2.71	24.14 ± 2.55	25.19 ± 3.37	n.s.	n.s.	< 0.05	n.s.	n.s.
Lean mass [units]	20.91 ± 0.66	16.99 ± 0.73	18.91 ± 0.72	15.83 ± 0.49	n.s.	n.s.	< 0.05	< 0.0001	n.s.
Lean Content [units x 100/g]	70.51 ± 2.57	69.51 ± 2.78	64.68 ± 2.62	62.89 ± 3.24	n.s.	n.s.	< 0.05	n.s.	n.s.

3.3 Fertility Screen

3.3.1 Introduction

The Fertility Screen is not part of the standard primary workflow of the German Mouse Clinic, but is performed as a secondary screening for mouse lines with a supposed fertility phenotype.

There is a strong relationship between sperm quality and fertility in humans and animals including the mouse (Dunbar and O'Rand 1991, Lovercamp *et al.*, 2007). For example, reduced sperm concentration and/or motility are primary causes of infertility. Many mutations have been identified which cause a change of sperm characteristics and consequently lead to subfertility and sterility (Ballester *et al.*, 2004; Braden and Gluecksohn-Waelsch 1958; Buaas *et al.*, 2004; Cleary *et al.*, 2001; Laing *et al.*, 2000; Lyon 1987; Paisley *et al.*, 2004). According to Mouse Genome Database (MGD, <http://www.informatics.jax.org/>) more than 300 genes causing abnormal spermatogenesis have been identified using the gene knockout technology.

3.3.2 Summary

To clarify if the absence or presence of a functional FOXP2 gene has an impact on the testis function and the sperm characteristics, a Sperm Motility Assay (SMA) was performed in *FOXP2*-mutant compared to wild type control mice.

In the SMA, in total 10 sperm parameters, namely concentration and 9 motility parameters, were measured using a Hamilton-Thorne Sperm Analyzer (HTM-IVOS). The use of these parameters for the estimation of sperm motion is well documented for rodent sperm analysis, for example in toxicologic studies (Horimoto *et al.*, 2000; Kato *et al.*; 2001a, Kato *et al.*, 2001b; Kawaguchi *et al.*, 2004).

The *FOXP2*-KO mice showed a higher sperm velocity and progressivity, and a higher amplitude of the lateral head displacement, indicating that the sperm swim with a stronger tail movement. The biological significance of these findings, however, should be clarified in further studies addressing the actual *in vivo* and/or *in vitro* fertilizing potential of the knockout sperm.

3.3.3 Mice

Before the mice entered the Fertility Screen, they were housed with food and water *ad libitum* under standard laboratory conditions. Fifteen male mice (seven control, eight mutants) at an age of 22 weeks were analyzed.

3.3.4 Material and Methods

Collection of epididymal sperm

Males were sacrificed humanely by cervical dislocation and both *caudae epididymidis* and *vasa deferentia* were dissected and washed briefly in 0.9% NaCl at room temperature. Fat and blood vessels were removed using spring scissors and watchmaker forceps. After transferring the organs to 500 µl of

HTF medium (Quinn et al. 1985), both *caudae epididymides* and *vasa deferentia* were cut several times. Sperm were allowed to swim out at room temperature for 4 minutes and were dispersed by gently shaking the dish. The resulting homogenous sperm suspension was used for the Sperm Motility Assay.

Sperm Motility Assay

Three 10 µl aliquots of the sperm suspension from every male were transferred each into a Petri dish containing a 500 µl drop of HTF medium, covered by mineral oil (Sigma-Aldrich, Deisenhofen, Germany) and prewarmed in the incubator at 37°C. Thus 3 dishes per male were prepared and incubated for 10 min at 37°C and 5% CO₂. After this time, 2 15 µl aliquots of the diluted sperm were pipetted into both halves of a sperm analysis chamber (2X-Cel, height 80 µm; Hamilton Thorne Research, Beverly, USA). Hence, 6 measurements were made for every male.

Sperm quality was determined at 37°C using an IVOS Sperm Analyzer (Version 12.1c; Hamilton Thorne Research, Beverly, USA) (Douglas-Hamilton 1995). Briefly, the analyzer uses a microscope with stroboscopic illumination at 662 nm and a 4X phase-contrast objective to produce a dark field image of the sperm. The microscopic image is focused onto an electronic CCD camera, digitized and analyzed by processing algorithms, which determine the properties of sperm motion. The resulting values for the parameters are strongly dependent on the setup parameters used for the analysis. The image capture setup parameters were: frames per second, 60 Hz; number of frames, 30. Cell detection parameters were: minimum contrast, 70; minimum cell size, 1 pixel. For every cell identified as sperm the parameters as listed in Table 1 were measured and calculated. At least 6 fields per analysis chamber were randomly selected and mean values of the sperm were saved for statistical analysis.

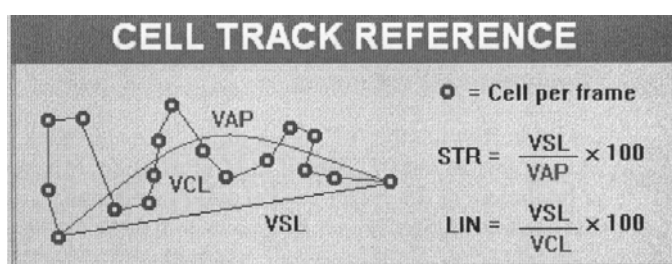


Figure 3: Diagram illustrating the definitions of sperm velocities and motion parameters

(from the Technical Guide of the IVOS, Hamilton Thorne Research, Beverly, USA)

Parameters of the Sperm Motility Assay			
Abbreviation	Meaning	Unit	Explanation
MOT	Motility	%	Ratio of the motile cells to the total cell concentration
PROG	Progressivity	%	Ratio of the progressive cells (VAP>60 $\mu\text{m/s}$; STR>50%) to the total cell concentration
CONC	Concentration	Mill./ml	Total cell concentration, motile and static
VAP	Velocity of the average path	$\mu\text{m/s}$	Smoothed average position of the sperm head to reduce the effect of the ALH (see Fig. 3)
VSL	Velocity of the straight line	$\mu\text{m/s}$	Straight line distance between the beginning and the end of the track divided by the time elapsed (see Fig. 3)
VCL	Velocity of the curvilinear path	$\mu\text{m/s}$	Point-to-point velocity, total distance between each sperm head position, divided by the time elapsed (see Fig. 3)
ALH	Average head displacement	μm	Mean width of the head oscillation as the cell swims
BCF	Beat cross frequency	Hz	Frequency with which the cell track crosses the cell path in either direction
STR	Straightness	%	Ratio of VSL/VAP (see Fig. 3)
LIN	Linearity	%	Ratio of VSL/VCL (see Fig. 3)

Statistical analysis:

Data were statistically analyzed using S-PLUS® 6.2 for Windows, PROFESSIONAL EDITION (Insightful Corp., Seattle, USA). Besides summary statistics an Analysis of Variance (ANOVA) was conducted using the animal, genotype, dish and analysis chamber as fixed effects on each of the 10 sperm parameters. The chosen level of significance was $p < 0.05$.

3.3.5 Results

As shown in Table 10, genotype had a highly significant effect on the percentage of progressive cells (PROG), all three velocity parameters (VAP, VSL, VCL) and the amplitude of the lateral head displacement (ALH). In all these parameters the mutant mice were superior to the control mice. This means, the *FOXP2*-mutant mice had sperm with a higher progressivity, VAP, VSL, VCL and ALH. The other parameters were not significantly different between the two genotypes. Additionally, there existed a significant effect of the factors animal, chamber and dish on nearly all parameters besides CONC and VAP.

3.3.6 Discussion

The higher velocity values of the *FOXP2*-mutant sperm are particularly manifest in the parameters VAP (148.93 vs. 140.13 $\mu\text{m/s}$) and VCL (296.53 vs. 278.84 $\mu\text{m/s}$), and less in the VSL (103.26 vs. 97.23). A higher speed could theoretically be achieved by a higher beat cross frequency (BCF) or more vigorous and stronger movement of the flagellum, leading to a higher amplitude of the lateral head displacement. The highly significant ALH values of the *FOXP2*-mutant sperm indicate that this second possibility is the case. The BCF values do not differ significantly between genotypes.

The percentage of motile cells (MOT) has a tendency to be higher in the *FOXP2*-mutant mice. A significance level could possibly be reached if a higher number of animals would be analyzed.

The partly significant influence of the 'coincidence factors' animal, chamber and dish on some parameters demonstrates that the chosen strategy of examining several dishes and chambers of one male was necessary and appropriate to avoid sampling errors which would occur, if for example only one dish or one chamber would be examined. When examining mouse sperm it has to be kept in mind that mouse spermatozoa are very sensible to mechanical forces and osmotic changes and have therefore to be treated with extreme care. Extensive pipetting and shaking of a dish or vial containing a sperm suspension – which would be necessary to get an absolutely homogeneous sperm suspension – as it is performed for the examination of the sperm of other species, is therefore not possible.

Until now only little is known about the role of the *FOXP2* gene for sperm quality, but several findings from evolutionary anthropology point to a significance of the gene for testis and sperm function. When comparing the gene expression in several tissues between man and chimpanzee, testis was - besides brain- the most striking tissue showing the largest interspecific divergence and lowest intraspecific diversity. About 32% of the measured gene activity varied between man and chimpanzee (Khaitovich *et al.*, 2006; Khaitovich *et al.*, 2005). The authors regard this as evidence for a positive selection. The *FOXP2* gene is a good candidate for a positively selected gene because of its role in the human ability of speech and language (Enard *et al.*, 2002). Human expression data from the Human Protein Atlas (www.proteinatlas.org) show that there is a strong expression of *FOXP2* in the ductus seminiferous and a moderate expression in the Leydig cells (Persson *et al.*, 2006, Uhlen *et al.*, 2005). Expression data for the mouse are not yet published.

The biological significance of the higher progressivity and velocity parameters can hardly be estimated on the basis of these results. A potential role of the *FOXP2* gene in the testis is not known. A further screening especially for in-vitro and in-vivo fertility parameters would be necessary to clarify if the superior sperm quality would lead to an experimentally verifiable advantage in the reproduction of a mouse with or without a functional *FOXP2* gene.

3.3.7 References

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Table 10: Results of Sperm Motility Assay

Data are presented as mean \pm standard deviation of the mean;
ANOVA model: Parameter ~ Genotype + Animal + Chamber + Dish

Parameter	Control	Mutant	ANOVA			
	(n=7)	(n=8)	<i>genotype</i>	<i>animal</i>	<i>chamber</i>	<i>dish</i>
MOT	75.42 \pm 10.57	78.58 \pm 12.15	ns	p<0.05	p<0.05	p<0.05
PROG	42.59 \pm 11.72	48.77 \pm 9.23	p<0.01	p<0.001	ns	p<0.001
CONC	61.69 \pm 34.58	70.67 \pm 57.44	ns	ns	ns	ns
VAP	140.13 \pm 9.32	148.93 \pm 7.49	p<0.0001	ns	ns	ns
VSL	97.23 \pm 8.67	103.26 \pm 6.77	p<0.001	ns	ns	p<0.05
VCL	278.84 \pm 17.84	296.53 \pm 14.81	p<0.0001	p<0.05	p<0.05	p<0.001
ALH	13.27 \pm 0.78	14.06 \pm 0.81	p<0.0001	ns	ns	p<0.01
BCF	31.00 \pm 1.95	30.45 \pm 2.05	ns	ns	p<0.05	p<0.05
STR	67.09 \pm 4.75	67.43 \pm 3.40	ns	p<0.01	ns	p<0.001
LIN	36.33 \pm 3.81	36.22 \pm 2.60	ns	p<0.01	ns	p<0.001

3.4 Neurology Screen

3.4.1 Introduction

Neurological dysfunction results in a wide variety of disorders ranging from impaired movement to severe mental illness. Studying the neurobehavioral phenotype of mutant mice is a powerful tool to understand the neural basis of behavior and the pathophysiology of neurological disorders. Comparison of the mouse and human brain transcriptomes shows a good correlation for highly expressed genes in both transcript identity and abundance (Fougerousse *et al.*, 2000). Therefore, screening of mice with respect to neurological disorders potentially offers an understanding of etiology and pathogenesis of the human nervous system (Hafezparast *et al.*, 2002).

The 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). Dependant upon results of this primary screen and due to specific questions, additional tests can be carried out for further assessment of neurological functions in a hierarchical way (Schneider *et al.*, 2006).

3.4.2 Summary

In the primary neurological screen 25 heterozygous *FOXP2*-knockout mice (10 males/15 females) and 23 control mice (10 males/13 females) were analyzed according to our modified SHIRPA protocol where a battery of behavioral tests is carried out. We examined the mice using designed 23 test parameters (See web page: http://www.mgu.har.mrc.ac.uk/facilities/mutagenesis/mutabase/shirpa_summary.html) to detect phenotypic differences between knockout and control mice. The test parameters contribute to an overall assessment of muscle, motor neuron, spinocerebellar, sensory and autonomic functions. The primary neurological screen is focused on investigating neurological signs to determine the neurological functioning of a mouse. We also examine forelimb grip force for the assessment of muscular function.

In addition, upon special request of the provider, we tested the mice on the accelerating rotarod on 3 consecutive days in order to evaluate motor coordination as well as motor learning abilities and to be able to compare these results with earlier analysis of *FOXP2*-knockin mice. The single significant finding in our neurological screening of male and female heterozygous *FOXP2*-knockout mice was a decreased rotarod performance hinting towards a defect in motor coordination.

3.4.3 Mice

Ten 10-week-old male *FOXP2*-knockout and ten 10-week-old male control mice entered the neurological screen at the beginning of the 23th calendar week. Fifteen 10-week-old female *FOXP2*-knockout and thirteen 10-week-old female 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.

3.4.4 Material and Methods

Primary screening 1: 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, fecal pellets and urination were removed from the viewing jar and arena. All experimental equipment was thoroughly cleaned with Pursept-A and dried prior to testing (Schneider *et al.*, 2006).

Primary screening 2: grip strength

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

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

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

PLUS (The R Project for Statistical Computing, 2004). The p-value for the genotype effect within the specific model found for the data indicates the significance of the statistical test of interest; a confidence interval for the genotype effect can also be extracted.

Secondary Screening: Rotarod test

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



Figure 4: The rotarod apparatus

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

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

Statistical analysis of the Rota Rod performance results: The Rotarod data contain dependencies which are more complex than the grip strength

data. Repeated measurements arise from four different trials with a break in between. To compare the performance results between genotypes, linear mixed-effect models are fitted, that allow for the dependencies of genotype and trial and for the effects of sex and weight. The latter are modelled as fixed effects. Interaction effects are considered and included in the model, if necessary.

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

3.4.5 Parameters

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

3.4.6 Results

In the first neurological check of the mice, no alterations were detected. All **SHIRPA parameters** were without pathological findings (Tables 11 – 14).

Comparing the **forelimb grip strength** revealed no genotype-related differences (Fig. 5).

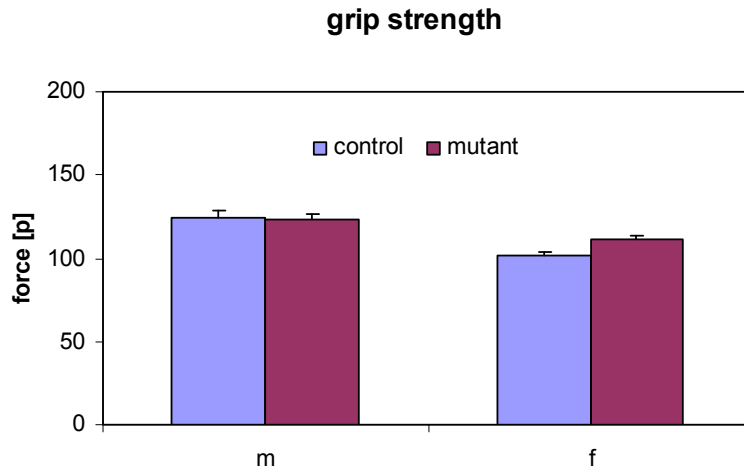


Figure 5: Results from grip strength testing

Motor coordination on the accelerating rotarod showed small differences between mutants and controls. Mutant mice fell of the rod earlier though this was only significant for the interaction of trial number and genotype at day 1 (Fig. 6).

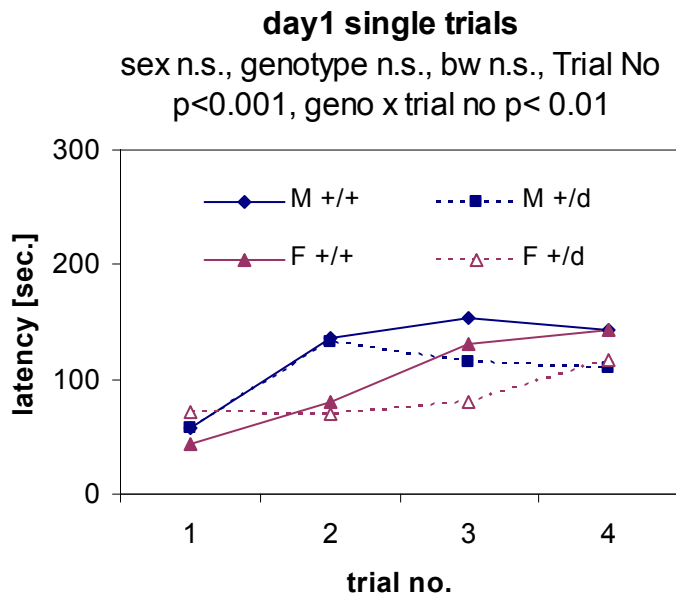


Figure 6: Results from rotarod single trials day 1.

The graphs for males and females over the three days tested shows that in all trials the mutants performed slightly worse but this was not significant (Fig. 7).

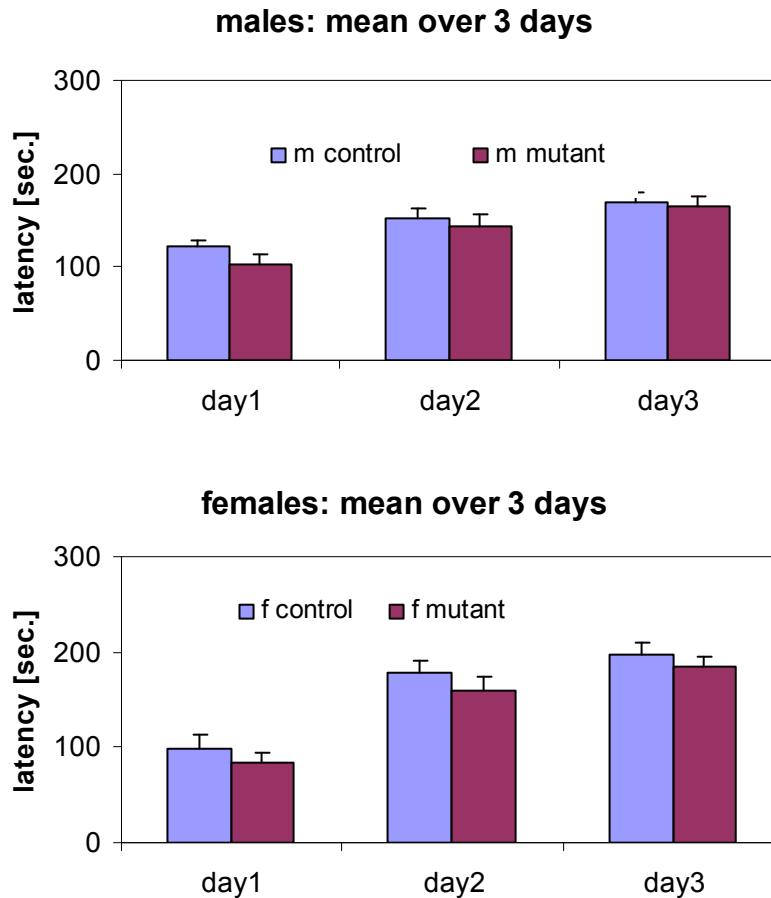


Figure 7: Results from rotarod of days 1-3 of *FOXP2*-KO mice

3.4.7 Discussion

The single significant finding in our neurological screening of male and female heterozygous *FOXP2*-knockout mice was a decreased rotarod performance hinting towards a defect in motor coordination.

The *FOXP2* gene was shown to be expressed especially in motor related circuits (Lai *et al.*, 2003) and the knockout of the gene was described to cause severe motor impairment (Shu *et al.*, 2005).

The detected differences confirm the role of *FOXP2* in motor coordination. We could demonstrate that some motor impairment could also be detected in heterozygous mice although they do not show overt neurological signs.

3.4.8 References

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Abbreviations

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

Table 11: Recording of body weight							
Data are presented as mean ± standard error of mean.							
Parameter	Male			Female			both
	Control (n=10)	Mutant (n=10)	<i>p-value</i>	Control (n=13)	Mutant (n=15)	<i>p-value</i>	<i>p-value</i>
Body Weight [g]	27.5±0.4	26.8±0.8	<i>n.s.</i>	21.8±0.3	22.4±0.4	<i>n.s.</i>	<i>n.s.</i>

Table 12: Behavior recorded in viewing jar							
Statistical analysis: chi-squared test; significance $p < 0.05$							
Parameter	Male			Female			both
	Control (n=10)	Mutant (n=10)	<i>p-value</i>	Control (n=13)	Mutant (n=15)	<i>p-value</i>	<i>p-value</i>
Body Position							
Inactive	0	0		0	0		
Active	10	10		13	15		
Excessive Activity	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Tremor							
Absent	10	10		13	15		
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Palpebral closure							
Eyes open	10	10		13	15		
Eyes closed	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Coat Appearance							
Tidy and well groomed	10	10		13	15		
Irregularities	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Whiskers							
Present	10	10		12	13		
Absent	0	0	<i>n.s.</i>	1	2	<i>n.s.</i>	<i>n.s.</i>
Lacrimation							
Absent	10	10		13	15		
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Defecation							
Present	10	10		13	15		
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>

Table 13: 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=10)	Mutant (n=10)	<i>p</i> -value	Control (n=13)	Mutant (n=15)	<i>p</i> -value	<i>p</i> -value
Transfer arousal							
Extended freeze	0	0		0	0		
Brief freeze	10	10		13	15		
Immediate movement	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Locomotor activity	19.2 \pm 1.8	21.4 \pm 1.7	<i>n.s.</i>	23.1 \pm 1.4	21.5 \pm 1.9	<i>n.s.</i>	<i>n.s.</i>
Gait							
Fluid movement	10	10		13	15		
Lack Fluidity	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Tail Elevation							
Dragging							
Horizontally extension	0	0		0	0		
	10	10		8	12		
Elevated/Straub tail	0	0	<i>n.s.</i>	5	3	<i>n.s.</i>	<i>n.s.</i>
Touch Escape							
No response	0	0		0	0		
Response to touch	10	10		13	15		
Flees prior to touch	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Positional Passivity							
Struggles when held by tail	10	10		13	15		
No struggle	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>

Table 14: Behavior recorded in or above the arenaStatistical analysis: chi-squared test; significance $p < 0.05$

Parameter	Male			Female			both
	Control (n=10)	Mutant (n=10)	<i>p-value</i>	Control (n=13)	Mutant (n=15)	<i>p-value</i>	<i>p-value</i>
Skin color							
Blanched	0	0		0	0		
Pink	10	10		13	15		
Bright deep red	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Trunk curl							
Absent	10	10		13	15		
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Limb Grasping							
Absent	10	10		13	15		
Present	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Pinna Reflex							
Present	10	10		13	15		
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Corneal Reflex							
Present	10	10		13	15		
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Righting Reflex							
Rights itself	10	10		13	15		
Fails to right when released	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Contact Righting							
Present	10	10		13	15		
Absent	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Evidence of biting							
None	10	10		13	15		
Biting in response to handling	0	0	<i>n.s.</i>	0	0	<i>n.s.</i>	<i>n.s.</i>
Vocalization							
None	6	9		11	14		
Vocal	4	1	<i>n.s.</i>	2	1	<i>n.s.</i>	<i>n.s.</i>

Table 15: Lactate levelsData shown represent the results of the mean blood lactate concentrations, value (\pm SEM)

	Male			Female			both
	Control (n=10)	Mutant (n=10)	<i>p</i> - <i>value</i>	Control (n=13)	Mutant (n=15)	<i>p</i> - <i>value</i>	<i>p</i> - <i>value</i>
Lactate (mmo/l)	11.6 \pm 0.4	11.1 \pm 0.4	<i>n.s.</i>	10.3 \pm 0.2	10.4 \pm 0.3	<i>n.s.</i>	<i>n.s.</i>

3.5 Eye Screen

3.5.1 Introduction

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; Puk *et al.*, 2006). 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).

3.5.2 Summary

No genotype-specific differences between wild-type control and mutant *FOXP2_delta_ex7* mice were detected.

3.5.3 Mice

Twenty-three *FOXP2*-control (10 male, 13 female) and 25 *FOXP2*-mutant mice (10 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.

3.5.4 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).

3.5.5 Parameters

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

3.5.6 Results and Discussion

Laser Interference Biometry data were recorded from the groups of control and *FOXP2*-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, wild type control and mutant (Table 13). The comparison of the axial lengths between males and females revealed only sex-specific differences. Comparing *FOXP2*-mutant mice with their littermate controls no significant difference was found, neither in the male nor in the female group.

All *FOXP2_delta_ex7* mice were examined by **funduscopy**. No abnormalities associated with the *FOXP2* mutation were detected (Table 14).

A total of 48 mice were examined ophthalmologically by **slit lamp biomicroscopy** (Table 15). No anterior segment phenotype was shown to be associated with the *FOXP2* mutation, either.

3.5.7 References

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Abbreviations

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

Table 16: Axial eye length								
Mean \pm standard error								
Parameter	Control (A)			Mutant (B)			A-B	A-B
	Male	Female		Male	Female		Male	Female
	(n=10)	(n=13)	<i>p</i> -value	(n=10)	(n=15)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
Axial length [mm]	3.575 \pm 0.009	3.538 \pm 0.008	<0.01	3.556 \pm 0.011	3.521 \pm 0.009	<0.02	n.s.	n.s.
Axial length / body length	0.038 \pm 0.0002	0.039 \pm 0.0003	<0.01	0.038 \pm 0.0003	0.040 \pm 0.0003	<0.001	n.s.	n.s.

Table 17: Results from Funduscopy				
Genotype	NAD	Pale cloudy fundus, unilateral	Light-coloured patches, unilateral	Vessel alterations
male control (n=10)	10			
female control (n=13)	12		1	
male mutant (n=10)	9	1		
female mutant (n=15)	15			

Table 18: Results from Slit Lamp Biomicroscopy				
Genotype	NAD	Nuclear flecks	Posterior pole opacity	Anterior pole
male control (n=10)	6	1	3	
female control (n=13)	11		2	
male mutant (n=10)	9		1	
female mutant (n=15)	12		1	2

3.6 Clinical Chemistry and Hematology

3.6.1 Introduction

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).

3.6.2 Summary

In the primary clinical-chemical screen, twenty-three (10 males/ 13 females) control mice and twenty-five (10 males /15 females) heterozygous *FOXP2*-knockout mice on C57BL/6J background were analyzed. Twenty different clinical-chemical parameters were measured including various enzyme activities, as well as plasma concentrations of specific substrates and electrolytes. Additionally, we measured eight basic hematological parameters.

We did not detect any significant differences between control and mutant mice. All values were situated within the physiological ranges of C57BL/6 wild-type mice. Only the physiological sex-related differences were found. Therefore we can state that the mutation neither influences the metabolic pathways and organ functions investigated nor regulation of hematopoiesis.

3.6.3 Mice

Ten 12-week-old wild-type and ten 12-week-old mutant males entered the clinical-chemical screen at the beginning of the 25th calendar week in 2007. In addition thirteen 13-week-old wild-type and fifteen 13-week-old mutant females entered the screen at the beginning of the 26th calendar week of 2007.

3.6.4 Materials and Methods

Blood Withdrawal and Storage

For the clinical-chemical Screen of the German Mouse Clinic blood samples were taken from isoflurane-anesthetized mice by puncturing the retro-orbital sinus with non-heparinized capillaries (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. Each blood sample was divided into two portions. The major portion was collected in a heparinized tube (Li-heparin, KABE; Nümbrecht, Germany; Art.No. 078028). The smaller portion 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.

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, 9503 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 9503 x g. Additionally the Clinical-Chemical Screen received the EDTA-blood sample for hematological investigations.

Clinical Chemistry

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

Hematology

A volume of 50 µl EDTA-blood was used to measure basic hematological parameters with a blood analyzer, which has been carefully validated for the analysis of mouse blood (ABC-Blutbild-Analyzer, Scil Animal Care Company GmbH; Viernheim, Germany). Number and size of red blood cells, white blood cells, and platelets are measured by electrical impedance and hemoglobin by spectrophotometry. Mean corpuscular volume (MCV), 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 concentrations (MCHC) are calculated from hemoglobin/ red blood cells count (MCH) and hemoglobin/ hematocrit (MCHC), respectively.

Second sample analysis

A second sample was collected from a subgroup of the previously tested mice at the age of 13 (males) and 18 (females) weeks. A subset of parameters which had shown subtle differences in the first test of clinical-chemistry and hematology were retested.

Analysis of Data

Data were statistically analyzed using Excel and Sigma Stat 3.1 with the level of significance set at $p < 0.05$, by an ANOVA test on the influence of genotype and sex and subsequent pairwise comparisons of the means by T-test.

3.6.5 Parameters

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

3.6.6 Results

Clinical Chemistry

Mutant mice significantly decreased values for inorganic Phosphorus and significantly increased values for Ferritin (Table 19). These findings could not be confirmed in the second clinical-chemical test (Table 20). Sex-related differences were detected for many parameters, mainly reflecting the physiological differences usually seen in C57BL/6J mice.

Hematology

In the first test, significantly increased values for the red blood cell count (RBC), hemoglobin and hematocrit were determined (Table 21). These findings could not be confirmed in the second hematology test (Table 22). Sex-related differences have been detected for some parameters, reflecting the physiological differences usually detected in C57BL/6 wild type mice.

3.6.7 Discussion

Clinical Chemistry

Only subtle differences were observed in *FOXP2*-knockout mice. However, these differences were not reproducible and thus most likely findings by chance due to physiological variation. Already in the GMC screen of *FOXP2*-knockin mice, no influence of the genotype on metabolic pathways or organ functions could be seen in the clinical-chemistry data (see GMC report of the *FOXP2*-knockin mice).

Hematology

Also concerning the hematological parameters no genotype-related differences have been detected. This corresponds to the GMC screen of *FOXP2*-knockin mice, where no influence of the *FOXP2* mutation on hematopoiesis had been found (see GMC report of the *FOXP2*-knockin mice). The significantly elevated hematocrit percentage of the first test might be derived from the elevated Red blood cell count, which also corresponds to the elevated hemoglobin concentration (Table 21).

Comparison to baseline data

Almost all values for all parameters were situated within the normal variation range of normal C57BL/6 mice as supported by previously published data (Hough *et al.*, 2002; Loeb and Quimby, 1999; Rathkolb *et al.*, 2000; Kile *et al.*, 2003; own unpublished results) with the exception of CK and ASAT activity in some individual mice belonging to different groups. Additionally the blood glucose level was moderately increased in some of the mice of both genotypes. The former finding is most likely an effect of blood collection procedure, since CK activities are well known to react very sensitive to differences in mouse handling. The latter finding is most likely due to the genetic background, since C57BL/6 mice have a disposition to develop diabetes.

A secondary screen is not recommended.

3.6.8 References

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Table 19: Clinical-chemical parameters at the age of 13 weeks.

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

Parameter	Control (A)			Mutant (B)			A~B	A~B	A~B
	Male	Female		Male	Female		Male	Female	All
	(n=10)	(n=13)	<i>p</i> -value	(n=10)	(n=15)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
Sodium [mmol/l]	149.8 \pm 2.59	146.5 \pm 0.61	n.s.	148.2 \pm 0.63	145.7 \pm 0.87	p<0.05	n.s.	n.s.	n.s.
Potassium [mmol/l]	4.2 \pm 0.2	4.23 \pm 0.05	n.s.	4.3 \pm 0.05	4.03 \pm 0.08	p<0.05	n.s.	p<0.05	n.s.
Calcium [mmol/l]	2.32 \pm 0.04	2.22 \pm 0.02	p<0.05	2.34 \pm 0.03	2.2 \pm 0.03	p<0.01	n.s.	n.s.	n.s.
Chloride [mmol/l]	111.3 \pm 1.94	110 \pm 0.51	n.s.	110.6 \pm 0.55	109.9 \pm 0.6	n.s.	n.s.	n.s.	n.s.
Inorganic Phosphate [mmol/l]	1.26 \pm 0.09	1.34 \pm 0.09	n.s.	1 \pm 0.08	1.16 \pm 0.07	n.s.	p<0.05	n.s.	p<0.05
Total Protein [g/dl]	5.18 \pm 0.17	5.08 \pm 0.09	n.s.	5.18 \pm 0.09	4.95 \pm 0.07	n.s.	n.s.	n.s.	n.s.
Creatinine [mg/dl]	0.344 \pm 0.006	0.348 \pm 0.006	n.s.	0.34 \pm 0.006	0.328 \pm 0.008	n.s.	n.s.	p<0.05	n.s.
Urea [mg/dl]	70.6 \pm 3.69	63.4 \pm 3.35	n.s.	65.5 \pm 1.91	67.1 \pm 2.78	n.s.	n.s.	n.s.	n.s.
Uric acid [mg/dl]	2 \pm 0.18	1.98 \pm 0.14	n.s.	2.18 \pm 0.13	1.74 \pm 0.09	p<0.05	n.s.	n.s.	n.s.
Cholesterol [mg/dl]	114.1 \pm 4.91	78.8 \pm 3.53	p<0.001	113.1 \pm 3.66	81.8 \pm 2.8	p<0.001	n.s.	n.s.	n.s.
Triglyceride [mg/dl]	159 \pm 13.1	73 \pm 5.2	p<0.001	159 \pm 12.5	97 \pm 7.6	p<0.001	n.s.	p<0.05	n.s.
Creatine Kinase [U/l]	266.6 \pm 100.94	121.2 \pm 26.35	n.s.	318.6 \pm 73.6	233.1 \pm 63.2	n.s.	n.s.	n.s.	n.s.
Alanine-Amino-transferase (ALT) [U/l]	46.4 \pm 12.86	27.7 \pm 2.51	n.s.	32.2 \pm 5.35	29.6 \pm 2.63	n.s.	n.s.	n.s.	n.s.
Aspartate-Aminotransferase (AST) [U/l]	63.4 \pm 9.5	56.3 \pm 3.64	n.s.	67.8 \pm 6.55	64.4 \pm 5.83	n.s.	n.s.	n.s.	n.s.
Alkaline Phosphatase [U/l]	99 \pm 2.99	132.9 \pm 7.29	p<0.001	104.4 \pm 3.84	132.8 \pm 4.26	p<0.001	n.s.	n.s.	n.s.
α -Amylase [U/l]	2585 \pm 116.8	2188 \pm 97.9	p<0.05	2752 \pm 235.5	2158 \pm 144.8	p<0.05	n.s.	n.s.	n.s.
Glucose [mg/dl]	157.6 \pm 11.5	137.6 \pm 7.3	n.s.	158.4 \pm 10.3	140.8 \pm 5.8	n.s.	n.s.	n.s.	n.s.
Ferritin [ng/ml]	24.3 \pm 1.7	27.2 \pm 2.07	n.s.	29.9 \pm 4.39	31.9 \pm 1.63	n.s.	n.s.	n.s.	p<0.05
Transferrin [mg/dl]	144.6 \pm 2.86	154.8 \pm 2.01	p<0.01	143.3 \pm 3.51	153.5 \pm 2.05	p<0.05	n.s.	n.s.	n.s.
Lipase [U/l]	77.7 \pm 5.22	75.1 \pm 7.23	n.s.	73.2 \pm 3.82	71.2 \pm 7.71	n.s.	n.s.	n.s.	n.s.

Table 20: Clinical-chemical parameters at the age of 18 weeks.Data are presented as mean \pm standard error of mean.

Parameter	Control (A)			Mutant (B)			A~B	A~B	A~B
	Male	Female		Male	Female		Male	Female	All
	(n=10)	(n=7)	<i>p-value</i>	(n=10)	(n=6)	<i>p-value</i>	<i>p-value</i>	<i>p-value</i>	<i>p-value</i>
Sodium [mmol/l]	149 \pm 0.37	147.1 \pm 0.4	p<0.01	149.2 \pm 0.29	145.7 \pm 1.5	n.s.	n.s.	n.s.	n.s.
Potassium [mmol/l]	4.73 \pm 0.08	4.63 \pm 0.09	n.s.	4.72 \pm 0.07	4.63 \pm 0.08	n.s.	n.s.	n.s.	n.s.
Chloride [mmol/l]	110.7 \pm 0.43	111.5 \pm 0.62	n.s.	111.5 \pm 0.34	111.3 \pm 0.88	n.s.	n.s.	n.s.	n.s.
Inorganic Phosphate [mmol/l]	1.47 \pm 0.03	1.51 \pm 0.07	n.s.	1.34 \pm 0.06	1.4 \pm 0.13	n.s.	n.s.	n.s.	n.s.
Total Protein [g/dl]	5.55 \pm 0.05	5.49 \pm 0.12	n.s.	5.7 \pm 0.05	5.57 \pm 0.12	n.s.	n.s.	n.s.	n.s.
Creatine Kinase [U/l]	58.4 \pm 9	28 \pm 1.95	p<0.01	131.7 \pm 73.8	89.7 \pm 18.38	n.s.	n.s.	p<0.05	n.s.

Table 21: Hematological parameters at the age of 13 weeks.Data are presented as mean \pm standard error of mean.

Parameter	Control (A)			Mutant (B)			A~B	A~B	A~B
	Male	Female		Male	Female		Male	Female	All
	(n=10)	(n=13)	<i>p</i> -value	(n=10)	(n=15)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
White blood cell count [10 ³ /μl]	6.2 \pm 0.42	5.4 \pm 0.32	n.s.	5.6 \pm 0.47	4.7 \pm 0.27	n.s.	n.s.	n.s.	n.s.
Red blood cell count [10 ³ /μl]	10 \pm 0.08	9.8 \pm 0.13	n.s.	10.2 \pm 0.08	10 \pm 0.08	n.s.	n.s.	n.s.	p<0.05
Platelet count [10 ³ /μl]	1068 \pm 18.8	923 \pm 32.2	p<0.01	1042 \pm 18.7	917 \pm 18.9	p<0.001	n.s.	n.s.	n.s.
Hemoglobin [g/dl]	14.9 \pm 0.08	14.7 \pm 0.13	n.s.	15.1 \pm 0.08	14.9 \pm 0.1	n.s.	p<0.05	n.s.	p<0.05
Hematocrit [%]	46.9 \pm 0.28	45.8 \pm 0.44	n.s.	47.7 \pm 0.26	46.8 \pm 0.38	n.s.	p<0.05	n.s.	p<0.05
Mean corpuscular volume [fl]	46.8 \pm 0.2	46.6 \pm 0.4	n.s.	46.8 \pm 0.2	46.7 \pm 0.28	n.s.	n.s.	n.s.	n.s.
Mean corpuscular hemoglobin [pg]	14.9 \pm 0.06	15 \pm 0.12	n.s.	14.8 \pm 0.07	14.9 \pm 0.11	n.s.	n.s.	n.s.	n.s.
Mean corpuscular hemoglobin concentration [g/dl]	31.7 \pm 0.12	32.1 \pm 0.09	p<0.05	31.7 \pm 0.07	31.9 \pm 0.2	n.s.	n.s.	n.s.	n.s.
Red blood cell distribution width [% of MCV]	14.1 \pm 0.05	14.2 \pm 0.08	n.s.	14.2 \pm 0.04	14.3 \pm 0.06	n.s.	n.s.	n.s.	n.s.
Mean Platelet Volume [fl]	5.22 \pm 0.05	5.24 \pm 0.04	n.s.	5.26 \pm 0.04	5.24 \pm 0.02	n.s.	n.s.	n.s.	n.s.

Table 22: Hematological parameters at the age of 18 weeks.Data are presented as mean \pm standard error of mean.

Parameter	Control (A)			Mutant (B)			A~B	A~B	A~B
	Male	Female		Male	Female		Male	Female	All
	(n=10)	(n=7)	<i>p</i> -value	(n=10)	(n=6)	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
White blood cell count [10 ³ /μl]	7.1 \pm 0.59	6 \pm 0.28	n.s.	6.5 \pm 0.33	5.6 \pm 0.39	n.s.	n.s.	n.s.	n.s.
Red blood cell count [10 ³ /μl]	10.1 \pm 0.08	10 \pm 0.12	n.s.	10.2 \pm 0.17	9.9 \pm 0.19	n.s.	n.s.	n.s.	n.s.
Platelet count [10 ³ /μl]	1079 \pm 27	903 \pm 58.3	p<0.05	1030 \pm 17.4	959 \pm 41.3	n.s.	n.s.	n.s.	n.s.
Hemoglobin [g/dl]	15 \pm 0.14	15.1 \pm 0.13	n.s.	15.1 \pm 0.26	15 \pm 0.19	n.s.	n.s.	n.s.	n.s.
Hematocrit [%]	46.6 \pm 0.43	46.1 \pm 0.37	n.s.	46.9 \pm 0.74	46.1 \pm 0.51	n.s.	n.s.	n.s.	n.s.
Mean corpuscular volume [fl]	46.2 \pm 0.13	45.9 \pm 0.26	n.s.	46 \pm 0.26	46.5 \pm 0.43	n.s.	n.s.	n.s.	n.s.
Mean corpuscular hemoglobin [pg]	15 \pm 0.06	15 \pm 0.09	n.s.	14.9 \pm 0.08	15.1 \pm 0.13	n.s.	n.s.	n.s.	n.s.
Mean corpuscular hemoglobin concentration [g/dl]	32.3 \pm 0.11	32.7 \pm 0.15	p<0.05	32.3 \pm 0.1	32.5 \pm 0.11	n.s.	n.s.	n.s.	n.s.
Red blood cell distribution width [% of MCV]	14.4 \pm 0.14	14.9 \pm 0.11	p<0.05	14.2 \pm 0.12	14.9 \pm 0.24	p<0.05	n.s.	n.s.	n.s.
Mean Platelet Volume [fl]	5.35 \pm 0.04	5.4 \pm 0.04	n.s.	5.24 \pm 0.04	5.45 \pm 0.06	p<0.05	n.s.	n.s.	n.s.

3.7 Immunology Screen

3.7.1 Introduction

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). In primary screen we measure leukocyte populations in peripheral blood and immunoglobulin levels in blood plasma.

The proportions of leukocyte populations in peripheral blood are genetically regulated (e.g., Mice: Chen and Harrison 2002; Men: Hall *et al.*, 2000). As a consequence, inbred strains differ in the frequency of leukocyte subsets in the lymphoid organs and in peripheral blood. Moreover, several CD antigens are restricted to specific mouse strains (e.g., Carlyle *et al.*, 2006) or interstrain differences occur concerning the level of expression of certain CD antigens (e.g., Haegel and Ceredig 2005). Strain specific differences in the immune response are further reflected in different susceptibilities to infectious agents (e.g., Medina *et al.*, 2001).

In individual mice, the number of circulating leukocytes and the proportions of subpopulations show daily rhythmic variations (Yellon and Tran 1992) and depend further on homeostatic proliferation and/or retraction (Freitas and Rocha 2000), as well as on activation through environmental and/or microbial factors (e.g., Grewal *et al.*, 1997), which might be related to subtle behavioral characteristics (e.g., Kim *et al.* 1999). Furthermore, sex-dependent factors are documented to influence the immune status (Krzych *et al.*, 1978) and have an impact on infection susceptibility (Pasche *et al.*, 2005).

Under baseline conditions, the levels of Ig classes and IgG isotypes are characteristic of a special inbred mouse strains, and under genetic control (Sant'Anna *et al.*, 1985). The profile of Ig subclasses interests further, because it reflects the direction of T-cell help; e.g. Th2-directed switching is to IgG1 and IgE. In mice, antibodies of the IgG2a subclass are the predominant isotype produced to viral infections (e.g., Coutelier *et al.*, 1987) and in cytokine-induced Th1-type responses (e.g., Snapper and Paul, 1987; Finkelman *et al.*, 1988; Stevens *et al.*, 1988). The levels of Ig classes and IgG isotypes are characteristic of a special inbred mouse strains and seem to underlie genetic control mechanisms (Sant'Anna *et al.*, 1985).

3.7.2 Summary

Under the baseline conditions of the primary immunology screen, we found statistically significant differences between female mutant mice and their littermate controls concerning the frequencies of CD4⁺ T-cells [↑]. In male mutant mice we found a higher proportion of CD62L expressing cells within the

CD8+ T-cells compartment. Furthermore, slightly higher blood plasma levels of IgM immunoglobulins have been measured in female mutant mice.

3.7.3 Mice

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

3.7.4 Material and Methods

Peripheral blood leukocytes (PBLs) were isolated from 500 μ l blood by erythrocyte lysis with NH₄Cl (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 Fc block (clone 2.4G2, PharMingen, San Diego, USA). Cells were then stained with fluorescence-conjugated monoclonal antibodies (PharMingen). After the antibody incubation, propidium iodide was added for the identification of dying/dead cells (Zamei *et al.*, 1996), which might bind antibodies unspecifically, and/or loose specific antigens upon apoptosis (Diaz *et al.*, 2004).

Samples were acquired automatically from 96 well plates with an HTS on a LSRII flow cytometer (Becton Dickinson, USA) until a total number of 30000 living CD45+ cells is reached for each well. For analysis, intact cells are first identified by their FSC/SSC profile. These cells were gated on the basis of their propidium iodide/PE signal (compensated parameters), allowing the dead cells to be gated out. Living cells were then gated using their SSC/CD45 signal, gating out remaining erythrocytes, thrombocytes and debris (Weaver and Broud 2002). CD45+ cells are subsequently analyzed by software based analysis (Flowjo, TreeStar Inc, USA). In former experiments, FMO (Fluorescence minus one) controls from wt mice have been used to define 'positive' and 'negative' regions (Baumgarth and Roederer, 2000).

The plasma levels of IgM, IgG₁, IgG_{2a}, IgG_{2b}, IgG₃, and IgA were determined simultaneously in the same sample using a bead-based assay (Fulton *et al.*, 1997) with monoclonal anti-mouse antibodies conjugated to beads of different regions (Biorad, USA), and acquired on a Bioplex reader (Biorad). The presence of rheumatoid factor and anti-DNA antibodies was evaluated by indirect ELISA with rabbit IgG (Sigma-Aldrich, Steinheim, Germany) and calf thymus DNA (Sigma-Aldrich), respectively, as antigens and AP-conjugated goat anti-mouse secondary antibody (Sigma-Aldrich). Serum samples from MRL/MpJ-Tnfrsf6^{lpr} mice (Jackson Laboratory, Bar Harbor, USA) were used as positive controls in the autoantibody assays.

3.7.5 Parameters

Flow cytometry
Main lineages:
<i>Staining 1:</i> T-cells (CD3 ⁺), CD4 ⁺ T-cells, CD8 ⁺ T-cells, γ/δ T-cells (CD3 ⁺ γ/δ TCR ⁺), T reg cells (CD4 ⁺ CD25 ⁺)
<i>Staining 2:</i> B-cells (CD19 ⁺), B1 B-cells (CD19 ⁺ CD5 ⁺), mature B-cells (CD19 ⁺ IgD ⁺), granulocytes (CD11b ⁺ Gr1 ⁺⁺), NK cells (NK1.1 ⁺ CD5 ⁻) and NK T-cells (NK1.1 ⁺ CD5 ⁺)
Subpopulations:
Further subpopulations are identified by bi-variate gating with the following markers:
Staining 1: CD25, CD62L, Ly-6C, CD44.
Staining 2: CD19 ⁺ cells: IgD, B220, CD11b, MHC-II(I-A, I-E), CD5, Gr1; CD19 ⁻ cells: Gr1, B220, CD5, MHCII, CD11b.
Bioplex/ELISA
IgM, IgG ₁ , IgG _{2a} , IgG _{2b} , IgG ₃ , IgA; anti-DNA antibodies, rheumatoid factor

Note: in C57/BL6 mice the IgG2a gene is deleted, and instead, the equivalent isotype IgG2c is produced. Anti-IgG2a antibodies might underestimate the levels of IgG2c (Martin *et al.* 1998).

Used antibodies

Fluochrome	blood staining 1	blood staining 2	Blood Staining 3	Extra staining for females (2 nd bleeding)
	Parameter	Parameter	Parameter	
FITC	CD45RB	IgD/ 11-26c2a	gdTCR/(Armenian Hamster clone)	
PE	CD103/	CD5/ RB6-8C5	Cd8b/H35-17.2	
	Propidium Iodide	Propidium Iodide		
PerCPCy55	CD4/ RM4-5	Gr1/ RB6-8C5	CD4/ RM4-5	
PECy7	CD62L/ MEL-14	CD19/ ID3	CD62L/ MEL-14	
Pacific Blue	CD3/ 17A2	CD3/ 17A2	CD3/ 17A2	
Cascade Yellow	Ly6C/ AL-21		DX5	
APC	CD44/ IM7	CD11b/ M1/70	CD25/ PC61	
AlexaFluor 700	CD45/ 30F11	CD45/30F11	CD45/30F11	
APC-Alexa750	CD8a/ 53-6.7	B220/RA3-6B2	CD8a/ 53-6.7	

Fluochrome	special staining Parameter
FITC	CD4/H129.19
PE	CD8b/ H35-17.2
	Propidium Iodide
PECy7	CD19/ 1D3
Pacific Blue	CD3/ 17A2
TRICOLOR	CD19/caltag, CatRM7706
AlexaFluor 700	CD45/ 30F11

3.7.6 Results

The analysis of *FOXP2*-mutant mice in the primary Immunology Screen revealed

- 1.) A statistically significant higher frequency of CD4+ T-cells in female mice. Furthermore, there was a sex-specific difference in both control and mutant mice with higher frequencies of granulocytes and lower frequencies of B-cells occurring in female mice (Tables 23-25).
- 2.) We found a higher proportion of CD62L expressing cells in the CD8+ population of peripheral blood from male mutant mice (Tables 23-24).
- 3.) Analysis of the plasma levels of immune globulins revealed slightly lower levels of IgM in female mutant mice (Table 26).

3.7.7 Discussion

1.) Sex-specific differences have been reported for several different inbred mouse strains. However, the observed difference in the proportions of granulocytes and B-cells is not consistent with usual sex-dependent changes. The influence of sex hormones on immune cells has been described by others (Gaunt and Pierce 1985; Ahmed *et al.*, 1985; Krzych *et al.*, 1978), and in women a consistent drop of neutrophil counts at menstruation has been shown (Bain and England 1975). In recent experiments (unpublished data), we do observe a negative correlation between the proportion of granulocytes and B-cells in peripheral blood of wild-type mice of both sexes, and a similar correlation for B and T-cells. Interestingly, Domínguez-Gerpe and Rey-Méndez (2001) observed this trend (granulocytes \uparrow , B-cells \downarrow , and T-cells \uparrow) in stressed animals; in the latter case, the changes in the proportions went together with a markedly decrease of the cell number. In the analyzed batch of *FOXP2*-mutant and control mice, the white blood cell count (see: Clinical Analysis), does not show statistically significant differences between male and female mice.

Taken together, the sex-specific difference in the proportions of leukocyte subsets might hint to a different immune homeostasis in the analyzed female mice compared to male mice. This difference might be due to behaviour/environmental/gender-related conditions, but cannot easily be explained as sex-dependent. The observed genotype-specific difference in the frequency of CD4+T-cells in female mice could also fit to the potential homeostatic dysregulation'. However, whether the observed differences between mutant and control mice are *FOXP2* gene-dependent has to be elucidated with further experiments.

2.) CD62L (L-Selectin) is an adhesion molecule important to lymph node homing of leukocytes. On CD8+ T-cells it is expressed on naïve cells and a subset of memory cells, referred to as 'central memory cells' (Wherry *et al.*, 2003; Huster *et al.* 2004). These memory cells co-express CD44 (Judge *et al.*, 2002, Boyman *et al.*, 2006). In the CD8+CD62L+ compartment we measured 50% of CD44 co-expressing cells and no significant difference between control and *FOXP2* mutant mouse. We conclude that the difference in CD62L expression between control and mutant male mice is unlikely to be determined by differences in the central memory cell compartment. In our screening experiences, some variability in the expression of CD62L on T-cells is a frequently occur-

ring phenomenon. In wild-type mice from different strains we observe a correlation of T-cell frequency and CD62L expression within the T-cell compartment, which reflects that the number of T-cells in the periphery under baseline conditions depends on the production of naïve T-cells in the thymus. High CD62L expression in T-cells from human peripheral blood has been further correlated to panic disorder (Manfro *et al.*, 2000) and nephropathy (De March *et al.*, 1999).

In summary, the lower frequency of CD62L high expressing cells in control compared to FOXP2 mice might be due to a lower proportion of 'naïve' CD8 T-cells. The question whether FOXP2-dependent mechanisms are involved can only be addressed by further experiments.

The levels of IgM observed in all tested mice are high, whereas immunoglobulins of the IgG class are considerable lower. This finding might hint to an 'acute' status of antibody immune response (like through an ongoing infection). Therefore, the statistically significant differences between control and mutant female mice should be interpreted with caution, especially since they are in a similar range of antibody levels in both groups.

In order to test the hypothesis whether the phenotype of *FOXP2* mutant mice depend on genetic differences affecting immune functions, further tests, as for example analysis of the immune cells in other compartments (e.g. spleen, lymphnodes, thymus, bone marrow), or in response to immunization/ infection might be of further help. However, prior to any more in depth analyses a confirmation of our findings with an independent cohort of mice should be performed.

3.7.8 References

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Table 23: Basic parameters analyzed in the Immunology Screen I: Antibodies.Data are presented as mean \pm standard error of mean.

Parameter	Control (A)			Mutant (B)			A ~ B	
	Male	Female	<i>p</i> - value	Male	Female	<i>p</i> - value	Male	Female
	(n=9)	(n=15)		(n=10)	(n=13)		<i>p</i> - value	<i>p</i> - value
IgG ₁ [μ g/ml]	219 \pm 53.3	288 \pm 40.7	n.s.	246 \pm 46.4	208 \pm 21.2	n.s.	n.s.	n.s.
IgG _{2a} [μ g/ml]	60.4 \pm 11.69	62.2 \pm 9.07	n.s.	54.2 \pm 8.27	61.2 \pm 12.67	n.s.	n.s.	n.s.
IgG _{2b} [μ g/ml]	507 \pm 34.39	778.8 \pm 61.99	p<0.01	537.5 \pm 31.36	889.1 \pm 76.71	p<0.001	n.s.	n.s.
IgG ₃ [μ g/ml]	236.9 \pm 76.42	308.3 \pm 41.88	n.s.	297.9 \pm 48.4	281.2 \pm 28.53	n.s.	n.s.	n.s.
IgM [μ g/ml]	1701.1 \pm 164.13	1959.8 \pm 85.36	n.s.	2015.9 \pm 154.28	1680.9 \pm 95.93	n.s.	n.s.	p<0.05
IgA [μ g/ml]	563 \pm 79.9	757 \pm 114.6	n.s.	690 \pm 125.5	776 \pm 225.8	n.s.	n.s.	n.s.
Anti-DNA Ab [%]	0.5 \pm 0	0.6 \pm 0	p<0.05	0.6 \pm 0	0.7 \pm 0.1	n.s.	p<0.05	n.s.
Rheuma- toid factor [%]	0.3 \pm 0.01	0.4 \pm 0.06	n.s.	0.3 \pm 0.02	0.3 \pm 0.02	n.s.	n.s.	n.s.

Table 24: Basic parameters analyzed in the Immunology Screen II: FACS analysis.Frequencies of leukocyte populations from peripheral blood, taken at the age of 13 weeks (% of CD45+ cells, respectively of parent gate, as indicated). Data are presented as mean \pm standard error of mean.

Parameter	Control (A)			Mutant (B)			A ~ B	
	Male	Female	<i>p</i> - value	Male	Female	<i>p</i> - value	Male	Female
	(n=10)	(n=14)		(n=10)	(n=15)		<i>p</i> - value	<i>p</i> - value
CD19+IgD +	66 \pm 1.31	52.7 \pm 1.28	p<0.001	63.9 \pm 0.69	52.7 \pm 1.59	p<0.001	n.s.	n.s.
CD4+/CD2 5+	3.7 \pm 0.2	4.8 \pm 0.32	p<0.01	3.7 \pm 0.28	4.6 \pm 0.21	p<0.05	n.s.	n.s.
CD4+	9.05 \pm 0.59	8.93 \pm 0.35	n.s.	9.7 \pm 0.61	10.47 \pm 0.5	n.s.	n.s.	p<0.05
CD8a+	8.05 \pm 0.36	8.23 \pm 0.29	n.s.	8.07 \pm 0.39	9.74 \pm 0.38	p<0.01	n.s.	p<0.01
CD8a+/62L +	64.51 \pm 3.846	70.336 \pm 2.208	n.s.	74.17 \pm 1.926	71.833 \pm 2.383	n.s.	p<0.05	n.s.
GR-1+	9.9 \pm 0.94	17 \pm 1.58	p<0.01	10.3 \pm 0.63	14.1 \pm 0.96	p<0.01	n.s.	n.s.
gdTCR+	0.63 \pm 0.06	0.95 \pm 0.08	p<0.01	0.99 \pm 0.34	0.86 \pm 0.05	n.s.	n.s.	n.s.
DX5	2.7 \pm 0.31	4.4 \pm 0.36	p<0.01	2.5 \pm 0.28	4.2 \pm 0.24	p<0.001	n.s.	n.s.

Table 25: Basic parameters analyzed in the Immunology Screen II: FACS analysis.
 Frequencies of leukocyte populations from peripheral blood, taken at the age of 18 weeks (% of CD45+ cells, respectively of parent gate, as indicated). Data are presented as mean \pm standard error of mean.

Parameter	Control (A)			Mutant (B)			A ~ B	
	Male	Female	p - value	Male	Female	p - value	Male	Female
	(n=10)	(n=7)		(n=10)	(n=6)		p - value	p - value
CD19+	61.9 \pm 1.48	56.2 \pm 1.17	p<0.01	61.2 \pm 2.3	54.3 \pm 2.26	n.s.	n.s.	n.s.
CD19+/IgD+B220+	96.32 \pm 0.14	95.11 \pm 0.26	p<0.01	95.43 \pm 0.2	95.17 \pm 0.27	n.s.	p<0.01	n.s.
CD19+/CD5+	2.68 \pm 0.09	2.82 \pm 0.17	n.s.	2.68 \pm 0.13	2.82 \pm 0.2	n.s.	n.s.	n.s.
Gr1++	9.3 \pm 0.9	14.7 \pm 0.87	p<0.001	10.2 \pm 1.18	15.3 \pm 1.91	n.s.	n.s.	n.s.
CD4+	9.83 \pm 0.27	10.62 \pm 0.3	n.s.	9.54 \pm 0.5	11.72 \pm 0.33	p<0.01	n.s.	p<0.05
CD4+/CD25+	5.17 \pm 0.3	6.55 \pm 0.33	p<0.01	5.71 \pm 0.28	6.43 \pm 0.25	n.s.	n.s.	n.s.
CD8a+	8.561 \pm 0.242	9.35 \pm 0.257	p<0.05	8.019 \pm 0.476	9.485 \pm 0.517	n.s.	n.s.	n.s.
CD8a+/CD62L+	78.9 \pm 1.52	81.7 \pm 2.37	n.s.	83.5 \pm 1.55	83 \pm 1.39	n.s.	p<0.05	n.s.
gdTCR+	0.53 \pm 0.02	0.63 \pm 0.07	n.s.	0.57 \pm 0.03	0.69 \pm 0.08	n.s.	n.s.	n.s.

Table 26: Frequencies of B-cells (CD19+) and CD4+, resp. CD8+ cells, in peripheral blood of female mice

Taken at the age of 17 weeks (% of CD45+ cells). Same samples as in Table 25, stained with a different panel of antibodies, including two different mAb, directed against CD19, and antibodies directed against CD4, CD8b, CD3, and CD45 within the same panel.
 Data are presented as mean \pm standard error of mean.

Parameter	Control (A)	Mutant (B)	A~B
	7	5	
I/45+,Count	30376.1 \pm 30.63	30538 \pm 57.67	n.s.
I/45+/CD19+CD19+	64.46 \pm 1.17	63.2 \pm 1.66	n.s.
nonBcells/CD4+/CD3+Freq.45+	9.9 \pm 0.32	11.23 \pm 0.41	p<0.05
nonBcells/CD8b+/CD3+Freq.45+	6.4 \pm 0.37	6.8 \pm 0.32	n.s.

3.8 Allergy Screen

3.8.1 Introduction

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. IgE-mediated atopic disorders such as allergic asthma, allergic rhinitis, and atopic dermatitis are now considered as environmentally and exposure driven immune disorders leading to the expression of various clinical phenotypes in individuals with defined genetic risk profiles (Arruda, *et al.*, 2005; Kabesch 2006). Genome screens with classical linkage and fine mapping approaches suggest that susceptibility to asthma is determined by multiple genes with each gene having a moderate dose effect (Wjst, *et al.*, 1999). In this respect, the development of phenotypically and genotypically defined animal models will be an important step (Bochner and Busse 2005). To detect allergy prone mouse mutants in systematic screening efforts, total plasma IgE was established as a powerful screening parameter (Alessandrini, *et al.*, 2001; Jakob, *et al.*, 2007).

3.8.2 Summary

In the primary allergy screen of FOXP2_delta_ex7 mice, 23 control and 25 mutant animals at the age of 13 weeks were screened. Their analysis did not reveal profound differences in plasma IgE between knockout and control mice.

3.8.3 Mice

An age- and sex-matched group of 23 control (10 females, 10 males) and 25 mutant (15 females, 10 males) mice aged 12 weeks was analyzed in the Allergy Screen.

3.8.4 Material and Methods

Male and female mice were screened for alterations in plasma total IgE concentrations. Blood samples were taken from animals by puncturing the retro-orbital plexus under isoflurane anesthesia.

Plasma was analyzed for total IgE, using a classical immunoassay isotype-specific sandwich ELISA. In brief, microtiter plates (96-well) were coated with 10 µg/ml anti-mouse-IgE rat monoclonal IgG (clone-PC284, The Binding Site) to detect total IgE. Serum samples were diluted 1:10 and standards for murine IgE (Mouse IgE, k clone C38-2 BD Pharmingen™) were appropriately diluted. As secondary antibodies, biotinylated rat anti-mouse IgE (clone R35-118, BD Pharmingen™) were used followed by incubation with BD OptEIA Reagent Set B (Cat. No. 550534 BD Pharmingen™) Plates were analyzed using a standard micro well ELISA reader at 450 nm. Total murine IgE data are reported in ng/ml, based on a standard curve of purified murine IgE (Alessandrini *et al.*, 2001).

3.8.5 Results and Discussion

The analysis of total IgE levels in plasma (Median \pm Standard deviation) of FOXP2_delta_ex7 mice revealed no statistically significant differences between the groups (Table 27). In mutant animals, the median concentration of total IgE was higher in females than in males but no significant difference between groups were found. This is a common finding for many mouse inbred strains (Allessandrini *et al.*, 2000; Corteling *et al.*, 2004; Seymour *et al.*, 2002; Melgert *et al.*, 2005).

Additionally we obtain two outliers samples (one in a male control and the other in a female mutant) that remarkable influences the standard deviation.

Taken together, under standard screening conditions for primary Allergy screen, FOXP2-mutant mice did not show changes in total plasma IgE levels that would reveal a major allergy phenotype.

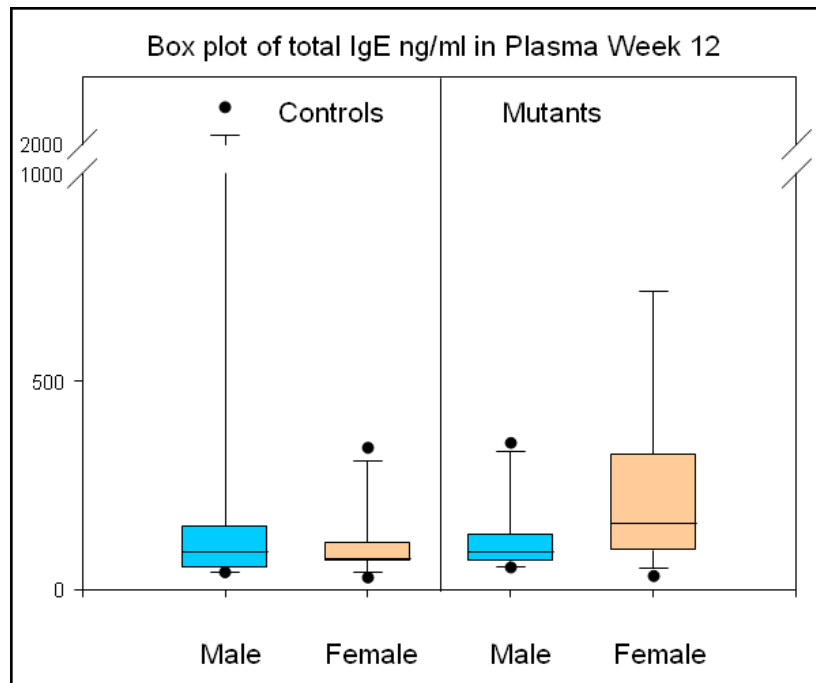


Figure 8: Total plasma IgE in FOXP2_delta_ex7 mice ng/ml

Table 27: Total plasma IgE in FOXP2_delta_ex7 mice (12 weeks old)								
Data are presented as median values \pm standard deviation.								
	Control (A)			Mutant (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=10)	(n=13)	<i>p</i> - value	(n=10)	(n=15)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
Total IgE [ng/ml]	90 \pm 691	75.3 \pm 89	n.s.	91.7 \pm 87	158 \pm 254	n.s.	n.s.	n.s.

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3.9 Steroid Metabolism Screen

3.9.1 Introduction

Steroids control differentiation and proliferation processes of cells and tissues. They participate in the regulation of apoptosis (Bansal *et al.*, 1991), bone remodeling (Jerome, 2004) and neuroregeneration (Chowen *et al.*, 2000). Severe diseases are caused by monogenic mutations with loss of function of steroid pathway proteins. But defects in steroid metabolism contribute as well to the pathogenesis of many different multifactorial diseases like cancer, diseases of cartilage and bone or neurological diseases (Mindnich *et al.*, 2007; Möller *et al.*, 2006; Prehn *et al.*, 2007). The main focus of the Steroid Screen is the identification of new animal models for human steroid-related diseases therewith supporting the development of their future medical treatment. For primary screening the key steroids dehydroepiandrosterone (DHEA) and testosterone (Labrie *et al.*, 1995) are extracted from plasma and quantified by ELISA.

3.9.2 Summary

In the primary steroid screening of FOXP2_delta_ex7 mice, 23 control and 25 mutant animals at the age of 13 weeks have been screened. Their analysis did not reveal profound differences between mutants and control mice.

3.9.3 Mice

Two age- and sex-matched groups of 23 control (10 males, 13 females) and 25 mutant (10 males, 15 females) mice aged 13 weeks have been analyzed in the Steroid Metabolism Screen.

3.9.4 Material and Methods

Male and female mice were screened for alterations in plasma concentrations of DHEA and testosterone. Blood was collected retro-bulbar from 12 weeks old narcotized mice (isoflurane) and plasma was prepared by centrifugation and stored at -20°C.

Since no steroid ELISA kits are available for mouse samples, human ELISA kits have to be used, but analysis is disturbed by the mouse plasma matrix. Therefore, the steroids have to be extracted from the matrix by liquid/liquid-extraction. 40 µl of plasma were extracted three times in each case with 400 µl *tert*-butylmethylether (TBME). The combined organic extracts were evaporated, dissolved *de novo* in TBME, subdivided for the two ELISA tests (15:25 respectively for DHEA and testosterone) and evaporated again. The material was reconstituted for the respective kit, DHEA in assay puffer, testosterone in steroid free serum.

The steroids are quantified by competitive ELISA according to the manufacturer's protocols. The plates were read in a standard microplate reader at a wavelength of 405 nm (DHEA) and 450 nm (testosterone). The concentrations are reported in pg/ml (DHEA) and ng/ml (testosterone) based on the respec-

tive kit standard curve. The sensitivity of the tests is 2.9 pg/ml for DHEA and 0.083 ng/ml for testosterone respectively.

We used the following ELISA kits:

Testosterone ELISA: DRG Instruments GmbH, Catalog No. EIA-1559

DHEA ELISA: AssayDesigns, Catalog No. 901-093

3.9.5 Results and Discussion

At the age of 13 weeks, the analysis of DHEA and testosterone concentrations in plasma of FOXP2_delta_ex7 mice revealed no statistically significant differences between mutants and controls (Table 28). Additionally, in both groups of mutant and control animals, the testosterone levels were significantly higher in males than in females. These are the typical sex-specific differences in the concentration of testosterone.

For this reason, we do not recommend a secondary screening.

Table 28: Plasma levels of DHEA and testosterone of FOXP2_delta_ex7 mice (13 weeks old)								
Data are presented as median (25 %/75 % - interquartile range)								
	Control (A)			Mutant (B)			A~B	A~B
	Male	Female		Male	Female		Male	Female
	(n=10)	(n=13)	<i>p</i> - value	(n=10)	(n=15)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
DHEA [pg/ml]	2.9 (<2.9 / 6.8)	<2.9 (<2.9 / <2.9)	n.s.	<2.9 (<2.9 / <2.9)	<2.9 (<2.9 / <2.9)	n.s.	n.s.	n.s.
Testosterone [ng/ml]	0.321 (<0.083 / 1.566)	<0.083 (<0.083 / <0.083)	<0.05	0.817 (0.173 / 4.118)	<0.083 (<0.083 / <0.083)	<0.05	n.s.	n.s.

3.9.6 References

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3.10 Nociceptive Screen

3.10.1 Introduction

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)

3.10.2 Summary

In the Primary Screen the responsiveness of the intact somatosensory system to thermal pain was tested in the *FOXP2_delta_ex7* mutant mouse line by means of the hot plate test (nociceptive pain).

We found a moderate but significant difference in the jumping reaction. This reaction is not the first sign of pain. We consider this difference in jumping as a diverse behavior pattern in pain response. To conclude, the *FOXP2*-knockout mice showed no difference in pain reaction compared to wild-type control animals.

3.10.3 Mice

Thirty *FOXP2*-knockout mice (15 male, 15 female), and 30 wild type control animals (15 male, 15 female) were tested in our first screen.

3.10.4 Material and Methods

Hot plate test

The mice were placed on a metal surface maintained at $52 \pm 0.2^\circ\text{C}$ (Hot plate system was made by TSE GMBH, Germany; Eddy and Leimbach, 1953). Locomotion of the mouse on the hot plate was constrained by 20 cm high Plexiglas wall to a circular area with a diameter of 28 cm (Fig. 9). Mice remained on the plate until they performed one of three behaviors regarded as indica-

tive of nociception: hind paw lick (h.p. licking), hind paw shake/flutter (h.p. shaking) or jumping.

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

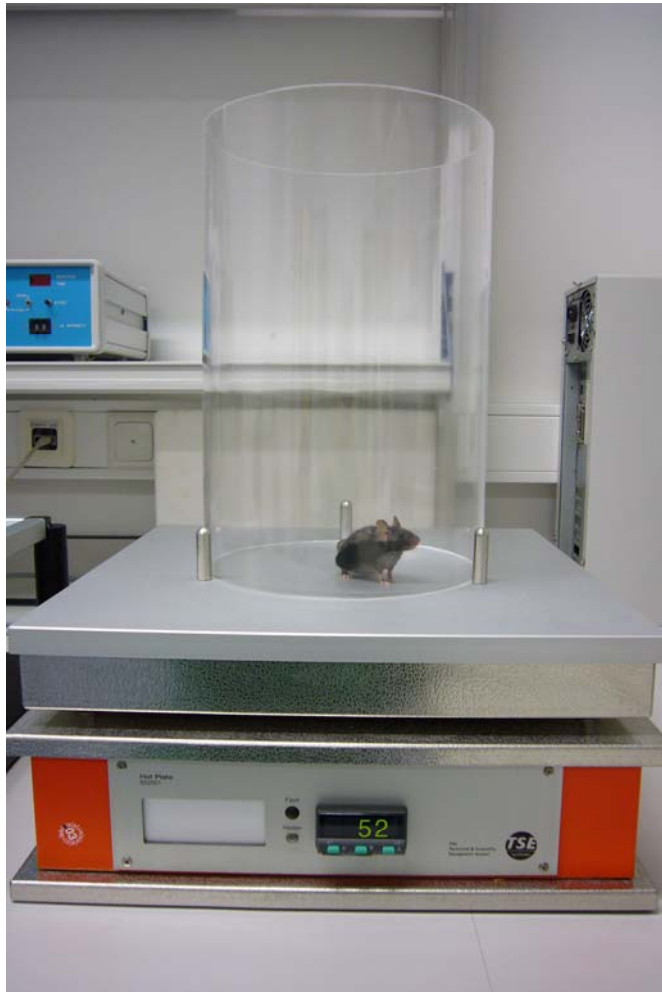


Figure 9: Hot plate system

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 $p < 0.05$.

3.10.5 Parameters

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

3.10.6 Results and Discussion

The first nociceptive response observed in these mice was 'hind paw shaking', the second one 'hind paw licking', however, the wild-type control and knockout animals showed these reactions after similar reactions periods. The third response was 'jumping', the knockout animals presented with significantly shorter latencies compared to wild-type control mice. We found a significant difference between the sexes in the shaking reaction, namely the female animals had longer reaction times.

To conclude, the *FOXP2*-knockout animals showed no significant difference in pain reaction compared to wild-type control mice, because 'jumping' is not the first response to thermal stimulation. Therefore we do not recommend making more pain-related studies in these mice.

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Abbreviations

h.p. hind paw

Table 29: Nociceptive Screen									
Data are presented as mean ± standard error of mean.									
						ANOVA			
						genotype		sex*genotype	
Parameter Latency [s]	Control (B)			Mutant (A)			A~B	A~B	ANOVA
	Male	Female		Male	Female		Male	Female	
	(n=15)	(n=15)	p-value	(n=15)	(n=15)	p-value	p-value	p-value	p-value
h.p.shaking	8.7± 0.96	12.9± 0.92	0.003	6.6± 0.96	13.7± 0.92	<0.0001	n.s.	n.s.	n.s.
h.p.licking	21.3± 2.6	17.2± 2.5	n.s.	14.6± 2.6	18.9± 2.5	n.s.	n.s.	n.s.	n.s.
jumping	50.6± 2.9	55.2± 2.8	n.s.	48.9± 2.9	43.7± 2.8	n.s.	0.02	0.02	n.s.

ANOVA:

SHAKING: genotype; p = 0.484, sex; p < 0.001, genotype*sex; p = 0.133

LICKING: genotype; p = 0.339, sex p = 0.966, genotype*sex; p = 0.109

JUMPING: genotype; p = 0.026, sex p = 0.919, genotype*sex; p = 0.089

3.11 Cardiovascular Screen

3.11.1 Introduction

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).

3.11.2 Summary

The comparison of the FOXP2-delta_ex7 mutant to control mice in blood pressure and ECG analysis revealed only a subtle difference seen as slightly decreased pulse in male mutants.

3.11.3 Mice

The mice reached the Cardiovascular Screen at the age of 15 weeks, 13 female mice (7 controls, 6 mutants) and 20 male mice (10 controls, 10 mutants) underwent blood pressure and ECG analysis.

3.11.4 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 10: Blood pressure set up

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

The blood pulse wave in the tail artery is detected as 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 11: ECG-setup

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

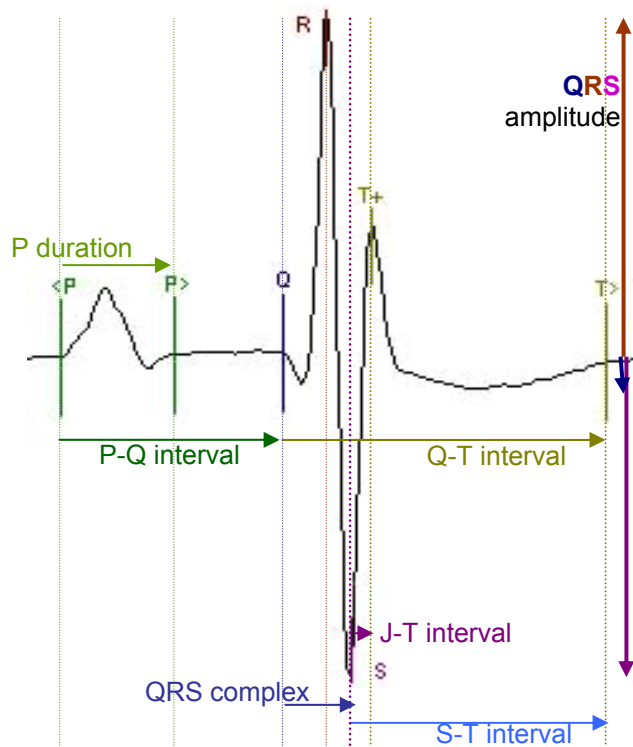


Figure 12: Example of ECG trace with analyzed parameters.

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.

3.11.5 Parameters

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

3.11.6 Results and Discussion

In **Blood pressure analysis** (Table 30) a genotype-specific difference was found for the parameter pulse. The *post hoc* analysis showed that the pulse was significantly decreased only in the male mutants. The origin of this difference is not clear and probably does not reflect a relevant effect on cardiac function.

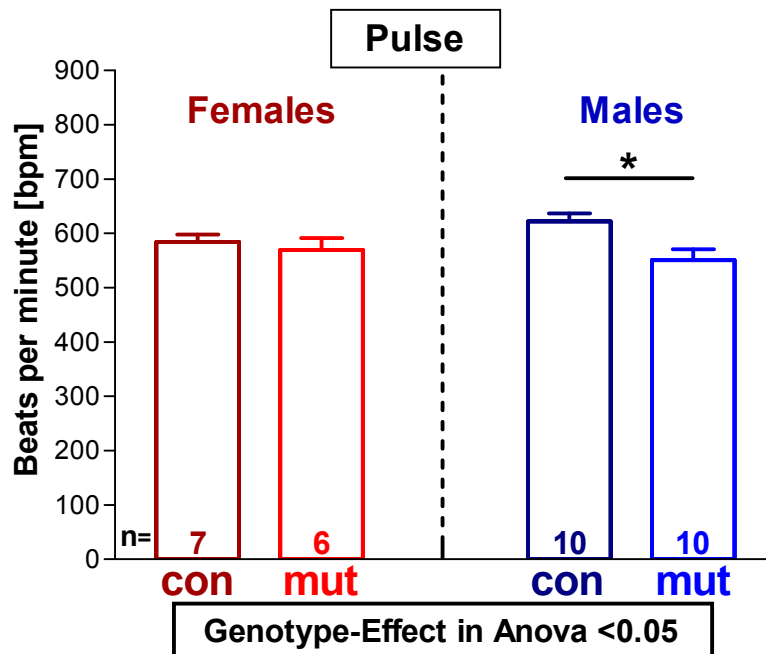


Figure 13: Blood pressure parameter Pulse

A genotype specific difference is found only in male mutants showing a slightly higher pulse than the controls.

The **ECG analysis** (Table 31) revealed no genotype-specific differences between the groups.

Since the subtle changes are of unknown relevance and no other alterations in cardiovascular parameters are found, we would not recommend a secondary screening for cardiovascular functions.

3.11.7 References

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Table 30: Blood Pressure Parameters

Data are presented as mean +/- standard error of mean.

Parameter	Control (A)		Mutant (B)		ANOVA			Post hoc test	
	Male	Female	Male	Female	Sex	Genotyp	Interact.	A~B Male	A~B Female
	(n = 10)	(n = 7)	(n = 10)	(n = 6)	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value	<i>p</i> - value
Systolic pressure [mm Hg]	117.9 +/- 2.6	123.1 +/- 5.9	116.5 +/- 3.0	114.5 +/- 2.7	n.s.	n.s.	n.s.	n.a.	n.a.
Diastolic pressure [mm Hg]	100.9 +/- 1.9	107.0 +/- 6.4	100.1 +/- 1.0	102.5 +/- 2.7	n.s.	n.s.	n.s.	n.a.	n.a.
Mean arterial pressure [mm Hg]	106.2 +/- 2.1	112.1 +/- 6.2	105.2 +/- 1.4	106.1 +/- 2.7	n.s.	n.s.	n.s.	n.a.	n.a.
Pulse [bpm]	622.2 +/- 14.4	584.4 +/- 13.3	550.5 +/- 20.2	569.5 +/- 21.9	n.s.	p<0.05	n.s.	p<0.05	n.s.

Abbreviations

SVES Supraventricular Extrasystoles
VES Ventricular Extrasystoles

Table 31: ECG Parameters

Data are presented as mean +/- standard error of mean.

Parameter	Control (A)						Mutant (B)						ANOVA			Post hoc test	
	Male			Female			Male			Female			Sex	Genotyp	Interact.	A~B Male	A~B Female
	(n = 10)			(n = 7)			(n = 10)			(n = 6)			p - value	p - value	p - value	p - value	p - value
PQ interval [ms]	40.8 +/- 0.8			40.0 +/- 0.5			40.5 +/- 0.8			40.1 +/- 0.8			n.s.	n.s.	n.s.	n.a.	n.a.
P-wave duration [ms]	19.7 +/- 0.4			20.2 +/- 0.6			19.9 +/- 0.5			20.8 +/- 0.3			n.s.	n.s.	n.s.	n.a.	n.a.
QRS-complex duration [ms]	9.5 +/- 0.3			10.1 +/- 0.3			9.5 +/- 0.2			9.8 +/- 0.3			n.s.	n.s.	n.s.	n.a.	n.a.
QT interval [ms]	43.4 +/- 0.7			43.0 +/- 1.2			44.7 +/- 1.1			42.5 +/- 1.2			n.s.	n.s.	n.s.	n.a.	n.a.
QT _{corrected} [ms]	36.3 +/- 0.5			38.1 +/- 1.0			37.6 +/- 0.9			36.8 +/- 0.8			n.s.	n.s.	n.s.	n.a.	n.a.
RR interval [ms]	143.2 +/- 2.7			128.4 +/- 4.8			142.7 +/- 5.6			133.9 +/- 6.1			p<0.05	n.s.	n.s.	n.a.	n.a.
Heart rate [bpm]	423.1 +/- 8.4			472.8 +/- 16.9			428.2 +/- 16.3			455.2 +/- 19.7			p<0.05	n.s.	n.s.	n.a.	n.a.
JT interval [ms]	3.0 +/- 0.1			3.3 +/- 0.3			3.4 +/- 0.2			3.2 +/- 0.2			n.s.	n.s.	n.s.	n.a.	n.a.
ST interval [ms]	33.9 +/- 0.8			32.9 +/- 1.1			35.2 +/- 1.0			32.7 +/- 1.2			n.s.	n.s.	n.s.	n.a.	n.a.
Q amplitude [mV]	0.02 +/- 0.01			0.02 +/- 0.01			0.03 +/- 0.00			0.02 +/- 0.00			n.s.	n.s.	n.s.	n.a.	n.a.
R amplitude [mV]	2.77 +/- 0.14			3.10 +/- 0.42			2.58 +/- 0.15			3.32 +/- 0.33			p<0.05	n.s.	n.s.	n.a.	n.a.
S amplitude [mV]	-0.50 +/- 0.18			-0.86 +/- 0.25			-0.56 +/- 0.13			-0.75 +/- 0.27			n.s.	n.s.	n.s.	n.a.	n.a.
QRS amplitude [mV]	3.29 +/- 0.26			4.02 +/- 0.51			3.13 +/- 0.10			4.10 +/- 0.44			p<0.05	n.s.	n.s.	n.a.	n.a.
Arrhythmias [# of animals]	SVES	VES	other	SVES	VES	other	SVES	VES	other	SVES	VES	other					
	1	0	0	1	0	0	0	0	1	0	1	2					
Regular [# of animals]	9			6			9			4							

3.12 Lung Function Screen

3.12.1 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; Reinhard *et al.*, 2002; Reinhard *et al.*, 2005). By use of genetically engineered mice, candidate genes for human developmental disorders of breathing have been identified (Katz, 2003).

3.12.2 Summary

Spontaneous breathing patterns during rest and activity were studied in 15-week-old male and female mutant and wild-type control mice of the *FOXP2_delta_ex7* mutant mouse line.

The comparison of the breathing patterns between wild-type and *FOXP2*-knockout mice of both sexes exhibited no differences at all, with the exception of *FOXP2*-knockout males suggested to be somewhat more active during study period as indicated by significantly higher mean respiratory rates (12%).

In addition, the assessment of lung volumes, respiratory mechanics, intrapulmonary and alveolar-capillary gas transport (secondary screen) in male mutants did not reveal a phenotype associated with the *FOXP2* mutation. We therefore do not suggest the *FOXP2_delta_ex7* mutant mouse line to be a phenotype as far as respiratory function is concerned.

3.12.3 Mice

Wild-type control and mutant mice of both sexes were studied at the age of 12 weeks. Mean body weights did not differ between the groups (Table 32) but showed the typical sex differences. A second cohort of five male mutant and control mice were subjected to lung function analysis (Secondary screen).

3.12.4 Material and Methods

Whole Body Plethysmography

A commercially available system from Buxco[®] Electronics (Sharon, Connecticut) was used to assess breathing patterns in unrestrained animals according to the principle described by Drorbaugh and Fenn (1955). It measures the pressure changes which arise from inspiratory and expiratory temperature and humidity fluctuations during breathing (Figs. 14 and 15).

Calibration of the system allows to transform these pressure swings into flow and volume signals so that automated data analysis provides tidal volumes (TV), respiratory rates (f), minute ventilation (MV), inspiratory and expiratory times (Ti, Te), as well as peak inspiratory and peak expiratory flow

rates (PIF, PEF). These data were stored online as mean values at 10 s intervals.



Figure 14: System used at GMC to assess breathing patterns.

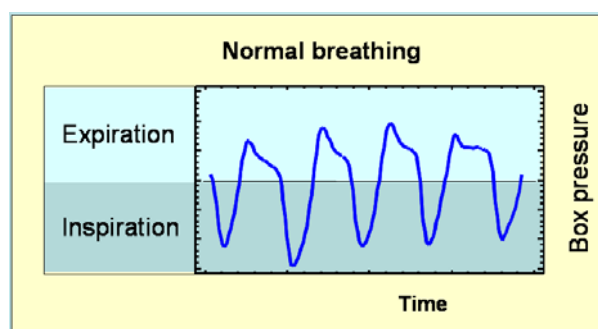


Figure 15: Recorded data used to calculate the breathing parameters.

Measurements were always performed between 8 a.m. and 11 a.m. to account for potential diurnal variations in breathing. The system was set up in a quiet room where temperature and humidity were kept constant throughout the measurements. Before each measurement, the system was calibrated and the actual barometric pressure, temperature, and humidity were supplied to warrant adequate calculations of flow rates and volumes. After placing the animals into the chamber, data recording was immediately started and was continued for 40 min. Mice underwent typical phases during the measuring period. Primarily, the animals were stressed so that the respiratory rate was highest at the beginning. Usually after 5 min. the animals became calmer, they slightly reduced their respiratory rate, and began to explore the chamber and start cleaning themselves – *phase of activity*. Later activity was more and more interrupted by phases of rest or even short periods of snoozing – *resting phase*. Some of the animals even went to *phases of sleep*, which resulted in a further marked decrease in respiratory rate. The frequency histogram of the respiratory rates was determined for each individual, and breathing was analyzed for the above mentioned parameters during the phases of activity and rest. In addition to the directly recorded parameters, mean inspiratory and expiratory flow rates (MEF, MIF) were calculated offline from the ratio of tidal volume and the respective time interval. The relative duration of inspiration (T_i/TT) was determined from the ratio of inspiratory time to total time required for the breathing cycle. Specific tidal volumes and minute ventilations (sTV, sMV) were calculated by relating the absolute values to the body weight of the animal. Furthermore, the mean of all breathing frequencies ($mean_f$) measured during the 40-minute-period was calculated as a rough and ready pa-

parameter to assess whether the duration of rest and activity was similar in all mouse strains.

Lung function

Lung function studies were conducted in male mutants and controls at an age of 12 weeks. Experimental setup: A schematic diagram of the experimental setup is shown in Figure 16. The intubated mouse was connected to a custom made computer-controlled piston-type servo ventilator. In principle, this ventilator is a miniaturized version of the ventilator applied for lung function studies in dogs (Schulz *et al.*, 1992a, 1992b) and exhibits corresponding functional characteristics. The ventilator allows for positive pressure ventilation at preselected tidal volumes and flow rates for the performance of reproducible breathing maneuvers for lung function testing.

To adjust the test maneuvers to the individual lung size, the system provides for independent respiratory settings of tidal volume, in- and expiratory flow as well as end-inspiratory and end-expiratory pauses. Furthermore, the operator has the choice to apply a released or a constant flow exhalation. For the instantaneous switch between different gas compositions for ventilation, e.g., for single-breath maneuvers, a set of four valves is connected to the piston. Care was taken to minimize the instrumental dead space volume (70 μ l including tracheal cannula). A miniaturized pressure transducer (EPE-L21, Entran Sensoren GmbH, Ludwigshafen, Germany) located close to the end of the tracheal cannula allows for continuous measurement of airway opening pressure (P_{ao}). A second pressure transducer, located to the end of a thin-walled, water-filled tube which is connected to an esophageal cannula allows monitoring of the esophageal pressure (P_{oe}). Concentrations of oxygen, carbon dioxide, labeled carbon monoxide ($C^{18}O$), and helium are measured by a magnetic sector field mass spectrometer (modified M3, Varian MAT). Gas samples were taken close to the end of the tracheal tube through a 1-m heated inlet capillary with an inner diameter of 165 μ m at a rate of 0.1 ml per second.

The sampling rate as well as the delay and 5-95% response times of the mass spectrometer were controlled before and after each lung function measurement. The values for the delay times at different days ranged between 230-250 ms, those for the 5-95% response times between 35-40 ms. The volume, pressure, and gas concentration signals were amplified and continuously recorded on a multichannel recorder (RS 3800, Gould). During lung function measurements, the signals of interest were also digitized and stored in a personal computer at a rate of 100-500 Hz. Before data analysis, the output signals of the mass spectrometer were corrected for the lag times and the volume signal of the ventilator for the suction rate of the mass spectrometer in order to obtain real time and volume data, respectively.

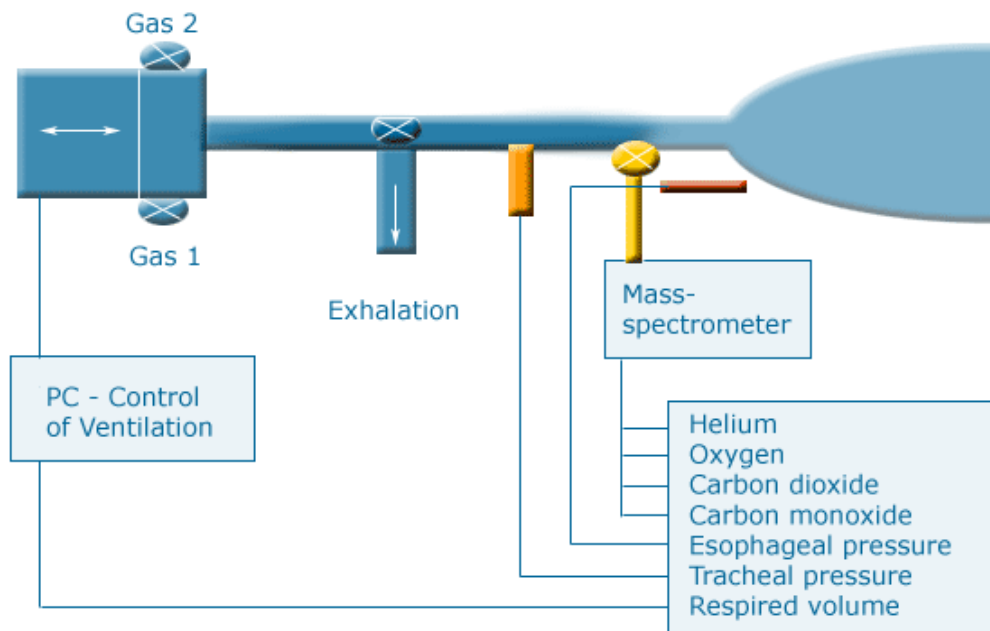


Figure 16: Experimental set-up for the analysis of lung function

Statistical Analysis of Data

Statistical analyses were performed using a commercially available statistics package (Statgraphics®, Statistical Graphics Corporation, Rockville, MD). Differences between strains were evaluated by Student's t-test. Statistical significance was assumed at $p < 0.05$. Data are presented as mean values \pm standard error of the mean (SEM).

1.1.5 Parameters

Spontaneous breathing pattern (Primary Screen)

Directly recorded data

Tidal volumes (TV), respiratory rates (f), minute ventilation (MV), inspiratory and expiratory times (T_i , T_e), as well as peak inspiratory and peak expiratory flow rates (PIF, PEF).

Calculated data

mean inspiratory flow rates (MEF), expiratory flow rates (MIF), relative duration of inspiration (T_i/TT), specific tidal volumes (sTV), minute ventilations (sMV), mean of all breathing frequencies (mean_f)

Lung function (Secondary Screen)

Lung volumes
TLC (μl) total lung capacity; TLC/bw ($\mu\text{l/g}$) specific total lung capacity; IC (μl) inspiratory capacity; IC/TLC specific inspiratory capacity; FRC (μl) functional residual capacity; FRC/TLC specific functional residual capacity; ERV (μl) expiratory reserve volume; VD (μl) conducting airway volume, i.e. series (Fowler) dead space volume from helium exspiogram; VD/TLC specific conducting airway volume
Respiratory mechanics: Compliance and resistance
C _{dyn} ($\mu\text{l/cmH}_2\text{O}$) dynamic compliance of respiratory system; C _L dyn ($\mu\text{l/cmH}_2\text{O}$) dynamic lung compliance; C ($\mu\text{l/cmH}_2\text{O}$) static compliance of respiratory system; C/TLC specific compliance of the respiratory system ($\mu\text{l/cmH}_2\text{O/ml TLC}$); C _L ($\mu\text{l/cmH}_2\text{O}$) static lung compliance; C _L /TLC specific lung compliance ($\mu\text{l/cmH}_2\text{O/ml TLC}$); R ($\text{cmH}_2\text{O/ml/s}$) respiratory system resistance; sR ($\text{cmH}_2\text{O/s}$) specific respiratory system resistance (R x TLC)
Intrapulmonary gas mixing
S _{He} (mmHg/ml) slope of the alveolar plateau (phase III) from helium expiogram
Alveolar-capillary gas transfer
D _{CO} ($\mu\text{mol/min/hPa}$) diffusing capacity for carbon monoxide; D _{CO} /TLC specific diffusing capacity ($\text{mol/min/hPa/ml TLC}$)

3.12.5 Results and Discussion

Spontaneous breathing pattern – Primary Screen

Tables 32-35 summarize the results obtained for spontaneous breathing under resting, and active conditions. Mutant males were suggested to be somewhat more active since mean respiratory rates were higher (12%) compared to wild-type males. Further we found no statistically significant difference in any of the directly recorded or calculated data concerning spontaneous breathing pattern – neither between wild-type and mutant males, nor between respective females. This lack of differences indicates that the mutation does not affect spontaneous breathing of *FOXP2*-knockout mice during rest and activity.

Lung function - Secondary Screen

Table 36 summarizes the results obtained by lung function measurements. *FOXP2* males had a significant lower functional residual capacity (FRC, 7%, $p < 0.05$) and a lower inspiratory capacity (IC, 4%, ns) causing total lung capacity (TLC) to be also somewhat smaller in *FOXP2*-knockout males (5%, ns). However, TLC related to body weight (TLC/bw) as well as IC/TLC and FRC/TLC were not significant different suggesting that difference in lung volumes are largely due to slight differences in body size. Similarly, the conducting airway volume (VD) was significantly lower in male mutants (7%, $p < 0.01$) but when related to TLC (VD/TLC) a significant difference was no longer detectable. Parameters characterizing respiratory mechanics, i.e. static and dynamic compliance and resistance of either the respiratory system or the lung were not significantly affected by the mutation. The slope of the alveolar pla-

teau of the helium expirogram (S_{He} , phase III) was less steep suggesting a somewhat improved intrapulmonary gas transport and mixing efficiency in mutants. The gas transfer across the alveolar-capillary barrier (DCO), either as an absolute value or related to lung size, was not affected by the mutation.

Overall, lung function measure, i.e. spontaneous breathing patterns at different levels of activity and lung volumes, respiratory mechanics, intrapulmonary and alveolar-capillary gas transport do not provide evidence for a respiratory phenotype in FOXP2 mutants.

3.12.6 References

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Abbreviations

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

Table 32: Characterization of studied FOXP2 mice, primary screening

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=7)	(n=5)	<i>p - value</i>	(n=7)	(n=2)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
Bw [g]	28.5 \pm 0.4	19.4 \pm 0.1	< 0.001	27.4 \pm 0.4	22.0 \pm 0.0	< 0.001	n.s.	< 0.001
Age [d]	83.1 \pm 0.7	85.0 \pm 0.8		83.0 \pm 0.7	80.0 \pm 0.0			
Mean_f [1/min]	431.6 \pm 14.4	450.3 \pm 10.6	n.s.	484.4 \pm 7.3	459.2 \pm 43.6	n.s.	< 0.01	n.s.

Table 33: Respiratory rate and timing at rest and activity

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=7)	(n=5)	<i>p - value</i>	(n=7)	(n=2)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
Rest				n=4				
f [1/min]	335.0 ± 9.6	318.0 ± 9.0	n.s.	345.6 ± 5.5	326.0 ± 13.6	n.s.	n.s.	n.s.
Ti [ms]	54.4 ± 1.9	52.3 ± 0.9	n.s.	52.5 ± 1.0	53.3 ± 0.9	n.s.	n.s.	n.s.
Te [ms]	125.6 ± 3.6	136.9 ± 4.4	n.s.	121.3 ± 2.3	131.1 ± 8.6	n.s.	n.s.	n.s.
Ti/TT	0.30 ± 0.00	0.28 ± 0.00	< 0.01	0.30 ± 0.00	0.29 ± 0.02	n.s.	n.s.	n.s.
Activity								
f [1/min]	463.0 ± 9.2	481.8 ± 6.8	n.s.	484.0 ± 8.1	478.8 ± 22.8	n.s.	n.s.	n.s.
Ti [ms]	43.0 ± 1.0	41.4 ± 0.7	n.s.	39.7 ± 0.7	39.4 ± 0.5	< 0.05	n.s.	n.s.
Te [ms]	86.9 ± 1.7	83.2 ± 1.4	n.s.	84.4 ± 1.4	86.2 ± 5.5	n.s.	n.s.	n.s.
Ti/TT	0.33 ± 0.00	0.33 ± 0.00	n.s.	0.32 ± 0.00	0.31 ± 0.01	n.s.	n.s.	n.s.

Table 34: Tidal volume and flow rates at, rest and activity

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=7)	(n=5)	<i>p - value</i>	(n=7)	(n=2)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
Rest				n=4				
TV [ml]	0.23 ± 0.01	0.17 ± 0.01	< 0.01	0.21 ± 0.01	0.19 ± 0.01	n.s.	n.s.	n.s.
PIF [ml/s]	7.3 ± 0.2	5.6 ± 0.4	< 0.01	7.0 ± 0.2	6.0 ± 0.4	n.s.	n.s.	n.s.
PEF [ml/s]	3.7 ± 0.2	2.7 ± 0.2	< 0.01	3.5 ± 0.1	3.3 ± 0.1	n.s.	n.s.	n.s.
MIF [ml/s]	4.3 ± 0.1	3.3 ± 0.2	< 0.01	4.0 ± 0.1	3.5 ± 0.3	n.s.	n.s.	n.s.
MEF [ml/s]	1.9 ± 0.1	1.2 ± 0.1	< 0.001	1.7 ± 0.0	1.4 ± 0.0	n.s.	n.s.	n.s.
Activity								
TV [ml]	0.23 ± 0.01	0.17 ± 0.01	< 0.01	0.21 ± 0.01	0.20 ± 0.01	n.s.	n.s.	n.s.
PIF [ml/s]	8.9 ± 0.3	6.8 ± 0.6	< 0.01	8.9 ± 0.2	8.4 ± 0.5	n.s.	n.s.	n.s.
PEF [ml/s]	5.1 ± 0.3	4.2 ± 0.4	n.s.	4.8 ± 0.2	4.8 ± 0.2	n.s.	n.s.	n.s.
MIF [ml/s]	5.3 ± 0.2	4.1 ± 0.3	< 0.01	5.2 ± 0.1	5.0 ± 0.3	n.s.	n.s.	n.s.
MEF [ml/s]	2.6 ± 0.1	2.0 ± 0.2	< 0.05	2.5 ± 0.1	2.3 ± 0.0	n.s.	n.s.	n.s.

Table 35: Minute ventilation and body size/weight related parameters at rest and activityData 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=7)	(n=5)	<i>p - value</i>	(n=7)	(n=2)	<i>p - value</i>	<i>p - value</i>	<i>p - value</i>
Rest				n=4				
sTV [μl/g]	8.2 \pm 0.5	8.8 \pm 0.6	n.s.	7.6 \pm 0.1	8.5 \pm 0.5	n.s.	n.s.	n.s.
MV [ml/min]	74.9 \pm 3.2	52.3 \pm 3.5	< 0.001	69.5 \pm 1.0	57.2 \pm 1.0	n.s.	n.s.	n.s.
sMV [ml/min/g]	2.6 \pm 0.1	2.7 \pm 0.2	n.s.	2.5 \pm 0.0	2.6 \pm 0.0	n.s.	n.s.	n.s.
Activity								
sTV [μl/g]	8.1 \pm 0.4	8.7 \pm 0.7	n.s.	7.6 \pm 0.3	9.0 \pm 0.6	n.s.	n.s.	n.s.
MV [ml/min]	104.4 \pm 4.7	81.6 \pm 7.1	< 0.02	98.5 \pm 3.1	92.5 \pm 1.8	n.s.	n.s.	n.s.
sMV [ml/min/g]	3.7 \pm 0.2	4.2 \pm 0.4	n.s.	3.6 \pm 0.1	4.2 \pm 0.1	n.s.	n.s.	n.s.

Abbreviations

BW (g) body weight
TLC (μ l) total lung capacity;
TLC/bw (μ l/g) specific total lung capacity
IC (μ l) inspiratory capacity;
IC/TLC specific inspiratory capacity
FRC (μ l) functional residual capacity;
FRC/TLC specific functional residual capacity

ERV (μ l) expiratory reserve volume;
VD (μ l) conducting airway volume, i.e. series (Fowler) dead space volume from Helium exspiogram;
VD/TLC specific conducting airway volume;

Phase I from Heliumexspiogram

C (μ l/cmH₂O) static compliance of respiratory system;
C/TLC (μ l/cm/H₂O/ml) specific compliance of respiratory system
C_{dyn} (μ l/cmH₂O) dynamic compliance of respiratory system;
C_L (μ l/cmH₂O) static lung compliance;
C_Ldyn (μ l/cmH₂O) dynamic lung compliance;
R (cmH₂O/ml/s) respiratory system resistance;
sR (cmH₂O/s) specific respiratory system resistance (R x TLC);
S_{He} (mmHg/ml) slope of the alveolar plateau (phase III) from helium exspiogram;
D_{CO} (mol/min/hPa) diffusing capacity for carbon monoxide;
D_{CO}/TLC (mol/min/hPa/ml) specific diffusing capacity for carbon monoxide;

Table 36: Lung function measurementsData are presented as mean \pm standard error of mean.

Parameter	Control male		Mutant male		<i>p</i> - value
	mean	SD	mean	SD	
FOXP2-KO	(n=5)		(n=5)		
bw	28.1	1.0	27.3	1.1	n.s.
TLC (μl)	1303	55	1243	80	n.s.
TLC/bw (μl/g)	46.4	2.1	45.6	4.2	n.s.
IC (μl)	934	44	897	61	n.s.
IC/TLC	71.7	1.1	72.3	0.8	n.s.
FRC	369	20	344	12	p<0.05
FRC/TLC	28.32	1.14	27.72	0.81	n.s.
ERV (μl)	173	21	156	8	n.s.
VD (μl)	257	6	241	7	p<0.01
VD/TLC	19.73	0.43	19.49	0.82	n.s.
Cdyn (μ l/cmH ₂ O)	40.9	1.1	39.5	2.9	n.s.
CLdyn (μ l/cmH ₂ O)	48.0	4.8	46.9	7.9	n.s.
C (μ l/cmH ₂ O)	53.9	2.8	54.0	3.0	n.s.
C/TLC (μ l/cm/H ₂ O/ml)	41.4	2.0	43.5	1.7	n.s.
CL (μ l/cmH ₂ O)	72.5	18.0	67.0	10.9	n.s.
CL/TLC	55.5	12.1	53.9	7.7	n.s.
R (cmH ₂ O/ml/s)	0.74	0.09	0.70	0.10	n.s.
sR (RxTLC) (cmH ₂ O/s)	0.97	0.15	0.86	0.11	n.s.
S_{He} (mmHg/ml)	0.76	0.06	0.70	0.04	p<0.05
DCO (mol/min/hPa)	12.9	1.5	12.4	0.9	n.s.
DCO/TLC (mol/min/hPa/ml)	9.9	1.0	10.0	0.7	n.s.

3.13 Molecular Phenotyping

3.13.1 Introduction

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

In a first discussion no organ was selected for analysis.

3.13.2 Methods and Materials

Organ Collection

The molecular phenotyping screen archives organs of mutant mice for subsequent DNA-chip expression profiling analysis. Ten female mice of the FOXP2_delta_ex7 mutant mouse line were provided to the molecular phenotyping screen.

Organs were collected at the age of 16 weeks. To minimize the influence of circadian rhythm on gene expression, mice were killed between 9 am and 12 am by carbon dioxide asphyxiation. The following 10 organs were collected and archived in liquid nitrogen following our established SOPs (Standard operation protocols): spleen, kidney, testis, liver, heart, lung, thymus, skin/cartilage (outer ear), skeletal muscle and brain. Organs were immediately frozen and stored in liquid nitrogen until isolation of total RNA. The 100 organ samples collected either may be used for further expression profiling analysis in the GMC or, alternatively may be transferred to the collaborator.

Methods of expression profiling analysis in the GMC

Total RNA is isolated from the selected organs according to the manufacturer's protocol using the RNeasy Midi kits (Qiagen, Germany). For reverse transcription 15µg total RNA of each sample is used and indirectly labeled with Cy3 or Cy5 fluorescent dyes according to a modified TIGR protocol as previously described (Seltmann *et al*, 2005; Greenwood *et al.*, 2005).

Gene specific DNA probes were PCR amplified from the Lion Bioscience 20k mouse arrayTAG clone set (Heidelberg, Germany) as recently described (Drobyshev *et al.*, 2003). Amplified probes are dissolved in 3xSSC and spotted on aldehyde-coated slides (CEL Associates, Pearland, TX/USA) using the Microgrid TAS II spotter (Genomic Solution Ltd., Huntingdon, UK). Spotted slides are rehydrated, blocked, denatured and dried as recently described (Drobyshev *et al.*, 2003; Beckers *et al.*, 2005; Greenwood *et al.*, 2005). The hybridization mixture is placed on prehybridized microarrays, hybridized at 42°C for 16h and then successively washed in decreasing SSC concentration (3x, 1x, 0.5x and 0.1x) and dried with nitrogen using HS4800 hybstation

(Tecan). Slides are scanned with a GenePix 4000A scanner and analyzed using the GenePix Pro3.0 image processing software (Axon Instruments, Burlingame, CA/USA).

Samples of each mutant individual is hybridizes against a pool of wild types. Each sample is analyzed in two experimental replicates including a dye swap experiment. Gene expression data are normalized independently for each DNA chip. Identification of significantly differentially expressed genes is performed using TIGR MultiExperiment Viewer (Mev; Saeed *et al.*, 2003; Tusher *et al.*, 2001).

3.13.3 Selected organs

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

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3.14 Metabolic Screen

3.14.1 Introduction

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.

3.14.2 Summary

In the primary metabolic screen 11 animals (7 males/ 4 females) of the *FOXP2_delta_7* mutant mouse line were analyzed. Twelve control mice (7 males/ 5 females) were available. They were fed under *ad libitum* conditions for two weeks followed by two days of acute fasting. The primary metabolic screen focuses on investigation of metabolic demands of mice determining daily body weight, energy uptake, metabolizable energy and body temperature. The analysis for genotype-specific differences revealed a strong body temperature reduction during fasting in mutant mice.

3.14.3 Mice

Seven adult control males and seven adult *FOXP2-KO* males entered the Metabolic Screen at the beginning of calendar week 31 in 2007. The females (five controls and four mutants) entered the metabolic laboratory one week later. The mice were single caged on grid panels (0.5°cm grid hole diameter). They were fed *ad libitum* for a period of 14 days, followed by two days of acute fasting to analyze adaptive responses of metabolism.

3.14.4 Material and Methods

Recorded Data

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

Analysis of Feces

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

Statistical Analysis

All values are presented as means \pm SEM. Two-way-ANOVA (SigmaStat, Jandel Scientific) was used to test for effects of the factors strain and sex (ANCOVA with body mass as covariate). The Fisher 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.

3.14.5 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})
--

3.14.6 Results and Discussion

FOXP2-KO animals showed significantly increased absolute and mass specific food consumption and energy assimilation. Assimilation efficiency was also slightly but significantly increased in mutant *FOXP2*-KO mice.

Food restriction had a similar effect on body mass and body temperature both in mutant and control mice. No other effects of the mutation could be detected.

Prior the metabolic screening in the GMC no phenotype related to energy regulation was described for *FOXP2*-KO mice. Mutant and control mice did not differ in body mass; therefore, differences in energy assimilation might indicate higher energy demands in *FOXP2* mutants which could be investigated in a secondary screen using indirect calorimetry. However, as body temperature was similar in both groups and the compensatory mass loss and body temperature reduction during food restriction did not differ, the results remain of unclear relevance.

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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 37: 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>2 days acute fasting</i>		<i>Ad libitum</i>		<i>2 days acute fasting</i>		<i>p</i> <i>genotype</i>	<i>p</i> <i>sex</i>	<i>p</i> <i>interaction</i>
	male (n=7)	female (n=5)	male (n=7)	female (n=5)	male (n=7)	female (n=4)	Male (n=7)	female (n=4)	<i>ad libitum</i> <i>food reduced</i>	<i>ad libitum</i> <i>food reduced</i>	
Body weight [g]	29.2 \pm 0.4	24.7 \pm 0.5	24.2 \pm 0.5	20.3 \pm 0.6	28.9 \pm 1.0	25.0 \pm 1.1	24.5 \pm 1.0	20.8 \pm 0.9	n.s. n.s.	<0.001 <0.001	n.s. n.s.
Rectal body temperature [°C]	36.8 \pm 0.2	37.6 \pm 0.2	34.7 \pm 0.2	33.3 \pm 0.8	36.7 \pm 0.1	37.6 \pm 0.1	34.2 \pm 0.4	34.3 \pm 0.8	n.s. n.s.	<0.001 n.s.	n.s. n.s.
Food consumption [g day ⁻¹]	2.6 \pm 0.1	2.7 \pm 0.1	no food for two days		3.0 \pm 0.1	2.8 \pm 0.2	no food for two days		<0.05	n.s.	n.s.
Energy uptake [kJ day ⁻¹]	44.62 \pm 1.11	45.99 \pm 2.19			51.95 \pm 1.08	47.45 \pm 3.51			n.a.	n.a.	n.a.
Feces production [g day ⁻¹]	0.65 \pm 0.01	0.68 \pm 0.03			0.71 \pm 0.02	0.69 \pm 0.06			n.a.	n.a.	n.a.
Energy content feces [kJ g ⁻¹]	15.53 \pm 0.03	15.46 \pm 0.05			15.45 \pm 0.04	15.45 \pm 0.06			n.s.	n.s.	n.s.
Metabolized energy [kJ day ⁻¹]	33.81 \pm 0.92	34.72 \pm 1.79			40.11 \pm 0.90	36.12 \pm 2.55			<0.05	n.s.	n.s.
Metabolized energy [kJ g ⁻¹ day ⁻¹]	1.16 \pm 0.03	1.41 \pm 0.06			1.40 \pm 0.05	1.45 \pm 0.10			<0.05	<0.05	n.a.
Food assimilation coefficient [%]	75.8 \pm 0.3	75.5 \pm 0.7			77.2 \pm 0.4	76.2 \pm 0.3			<0.05	n.s.	n.s.

3.15 Pathology Screen

3.15.1 Introduction

The anatomic pathological evaluation (gross and histological analysis) plays a vital role in the final analysis of mutant mouse models and provides insights into the understanding how genetic alterations influence the development of human diseases that the mutant mice are intended to model (Brayton *et al.*, 2001). The morphological phenotyping of the mutant mice can identify anatomical changes introduced by the mutation of interest. In addition, it can detect unexpected phenotypes inherent to the mouse strain or related to environmental influences and simultaneously facilitate to establish a correlation between the anatomical abnormalities and the functional abnormalities found in other screens.

Furthermore, in cases in which the morphological data alone are not enough to reach a conclusive diagnosis, immunohistochemistry is a crucial technique to identify specific proteins on paraffin- or frozen- processed tissue sections. The positive immunostaining can be then combined with image analysis software in order to quantitatively analyze the data.

Transmission electron microscopy is another important tool in the pathology screen to examine the substructure and ultrastructure of individual cells in scenarios where conventional light microscopy identifies minor abnormalities despite clear evidence of disease (Stirling *et al.*, 1999, Mattila *et al.*, 2009).

Recently, the pathology screen has implemented the virtual slide microscopy, which enables the diagnosis and the archiving of tissue sections for discussion both remotely or in online conferences, facilitating worldwide consultations and discussions.

3.15.2 Summary

The Pathology Screen performed a complete pathological analysis. Using macroscopic and histological criteria, heterozygous FOXP2-KO mice exhibited no morphological differences from their control littermates. The results of the performed immunohistochemistry for FOXP2 protein expression are showed and discussed. Special interest is paid to the FOXP2 protein expression detected in **testis** and ovary due to its possible role in sperm and ovarian follicle development.

3.15.3 Mice

The mice were received at the age of 18-20 weeks. Macroscopical and histological analysis of all organs was performed in a total of 39 mice of the C57BL/6J background strain, 19 FOXP2-mutant mice and 20 control littermates (Table 38).

Table 38: Analyzed *FOXP2*-mutant mice and their control littermates.

Control		Mutant		Number of Animals	Age [weeks]
Males	Females	Males	Females		
11	9	10	9	39	18-20

No genotype-specific alterations were found in the mutant mice when compared to their control littermates. Because *FOXP2*-gene is highly expressed in brain and cerebellum, representative pictures of these organs are shown. Table 39 shows an overview of all the analyzed organs.

Table 39: Morphological alterations of *FOXP2*-mutant mice compared to their control littermates.

Organ	Alteration	Organ	Alteration
Body weight	No	Stomach	No
Musculoskeletal system	No	Small intestine	No
Cerebrum	No	Cecum and Colon	No
Cerebellum	No	Liver	No
Heart	No	Pancreas	No
Thymus	No	Spleen	No
Lung	No	Urinary bladder	No
Thyroid gland	No	Testes	No
Parathyroid gland	No	Epididymis	No
Trachea	No	Funiculus spermaticus	No
Kidneys	No	Ovaries	No
Adrenal glands	No	Uterus	No
Salivary glands	No	Vagina	No
Cervical lymph nodes	No	Skin	No
Esophagus	No	External lesions	No

Brain.

No differences between mutant mice and their controls were observed in the principal neurons of the CA1 cortex and/or of the hippocampus (Figure 17 A, B) and in the morphology of the Purkinje cells (Figure 17 C, D, arrows). The Figure 17 shows also the overall morphology of the cerebrum and the similar distribution of the neurons of the granular and molecular layer at the level of the cerebellum.

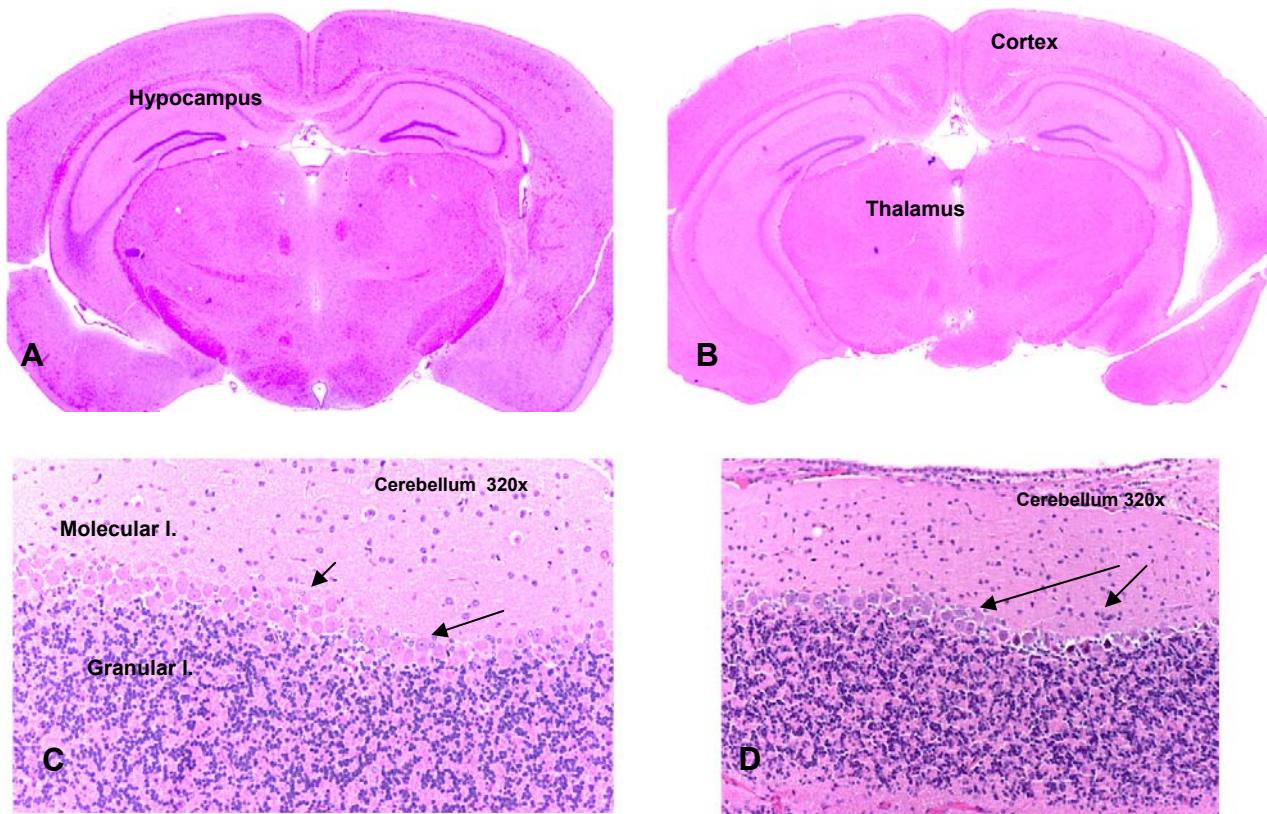


Figure 17: H&E staining of selected regions of the brain

Superior panels: H&E staining of the coronal section at the level of the middle brain 125x. A: Control mouse, B mutant mouse. Inferior panels: H&E staining of the cerebellum 320x. C Control mouse, D mutant mouse.

The analysis of the **testes** was performed in the animals received from the GMC-primary screen plus the animals analyzed by the GMC-fertility group. A total of 33 males (17 mutant and 18 control mice) were analyzed at the age of 18-22 weeks. Mild tubular testicular degeneration characterized by the reduction in the number of germinal cells was observed in both: 3 / 18 (16,6%) control mice and 5 / 17 (29,4-%) of mutant mice. With few exceptions, the degenerated tubules represented less than 10% of the total tubules (Figure 18). The difference was statistic not significant (p-value = 0.443, Fisher's exact test).

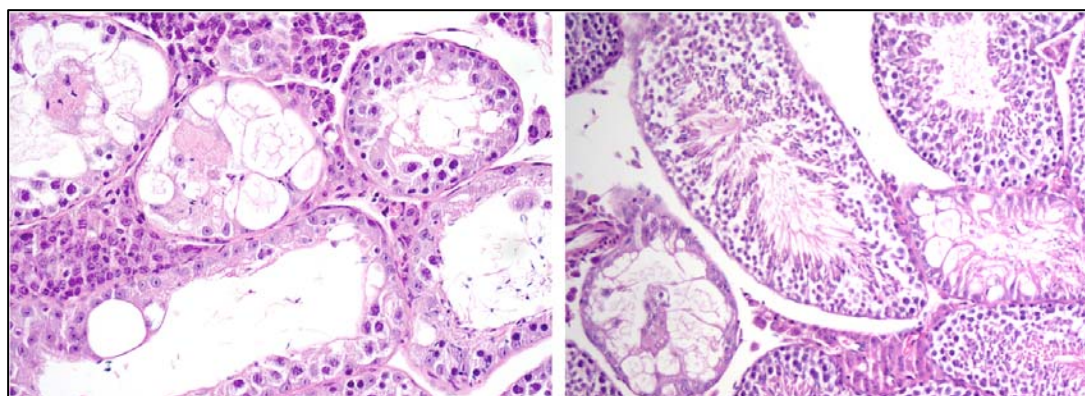


Figure 18: H&E staining of testis with testicular tubular degeneration

3.15.4 Secondary Screen Results

No differences were observed in the *FOXP2* expression between control mice and *FOXP2* delta_ex7 heterozygous mice in all organs analyzed. Table 40 shows all the organs analyzed for the reactivity to the *FOXP2* antibody. Figures 19-21 show in a comparative form the expression observed in the different organs.

Table 40: Overview of FOXP2 protein expression pattern			
Organ	Reactivity	Organ	Reactivity
Cerebrum	Yes	Stomach	No
Cerebellum	Yes	Small intestine	No
Heart	No	Cecum and Colon	No
Thymus	No	Liver	No
Lung	No	Pancreas	Yes
Thyroid gland	No	Spleen	No
Trachea	No	Urinary bladder	No
Kidneys	No	Testes	Yes
Adrenal glands	Yes	Epididymis	No
Salivary glands	No	Ovaries	Yes
Cervical lymph nodes	No	Uterus	No
Esophagus	No	Vagina	No

In **Brain** strong nuclear expression of FOXP2 was detected in the neurons of the cortex, thalamus and in the Purkinje cells of the cerebellum (marked by arrows in Figure 19).

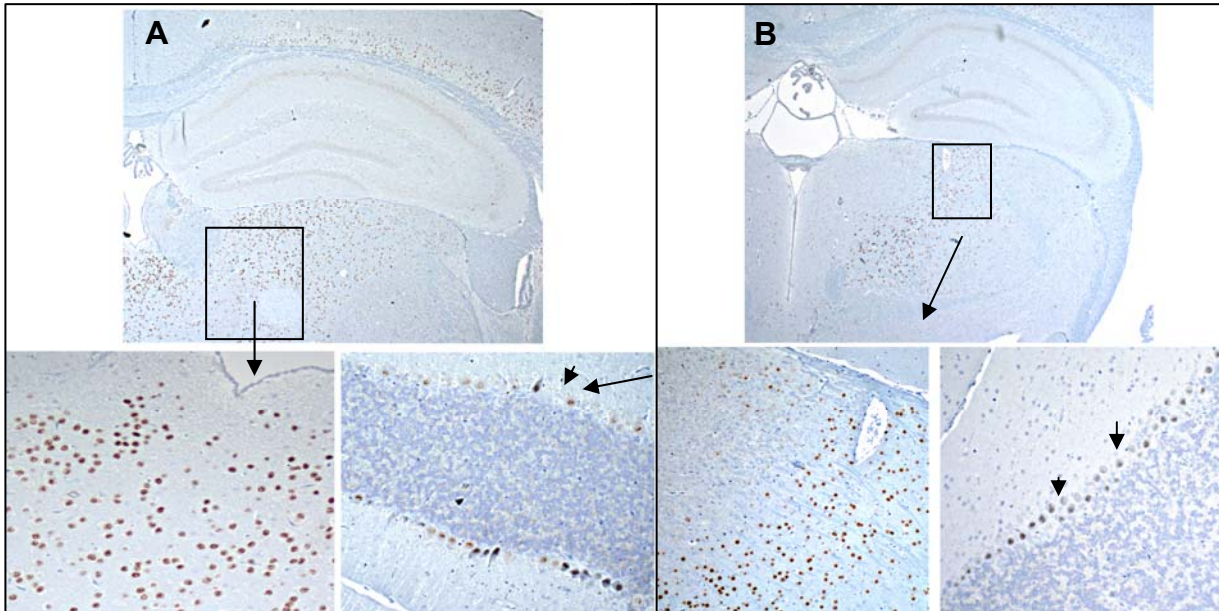


Figure 19: FOXP2 immunostaining of the brain to compare the expression by the control mouse in A. and by the mutant mice in B.

In **testis**, FOXP2 was expressed in the seminiferous tubules (spermatids and Sertoli cells). In contrast, Leydig cells were not reactive. The expression of FOXP2 appeared to be circumscribed to specific steps of maturity. Apparently only the spermatids in the intermediate stages of maturity (stages 8-12) were reactive. Germ cells that lie close to the basal membrane of the tubule and the mature spermatids (from stage 15) in the epididymis showed no immunoreactivity for FOXP2 (Figure 20, next page).

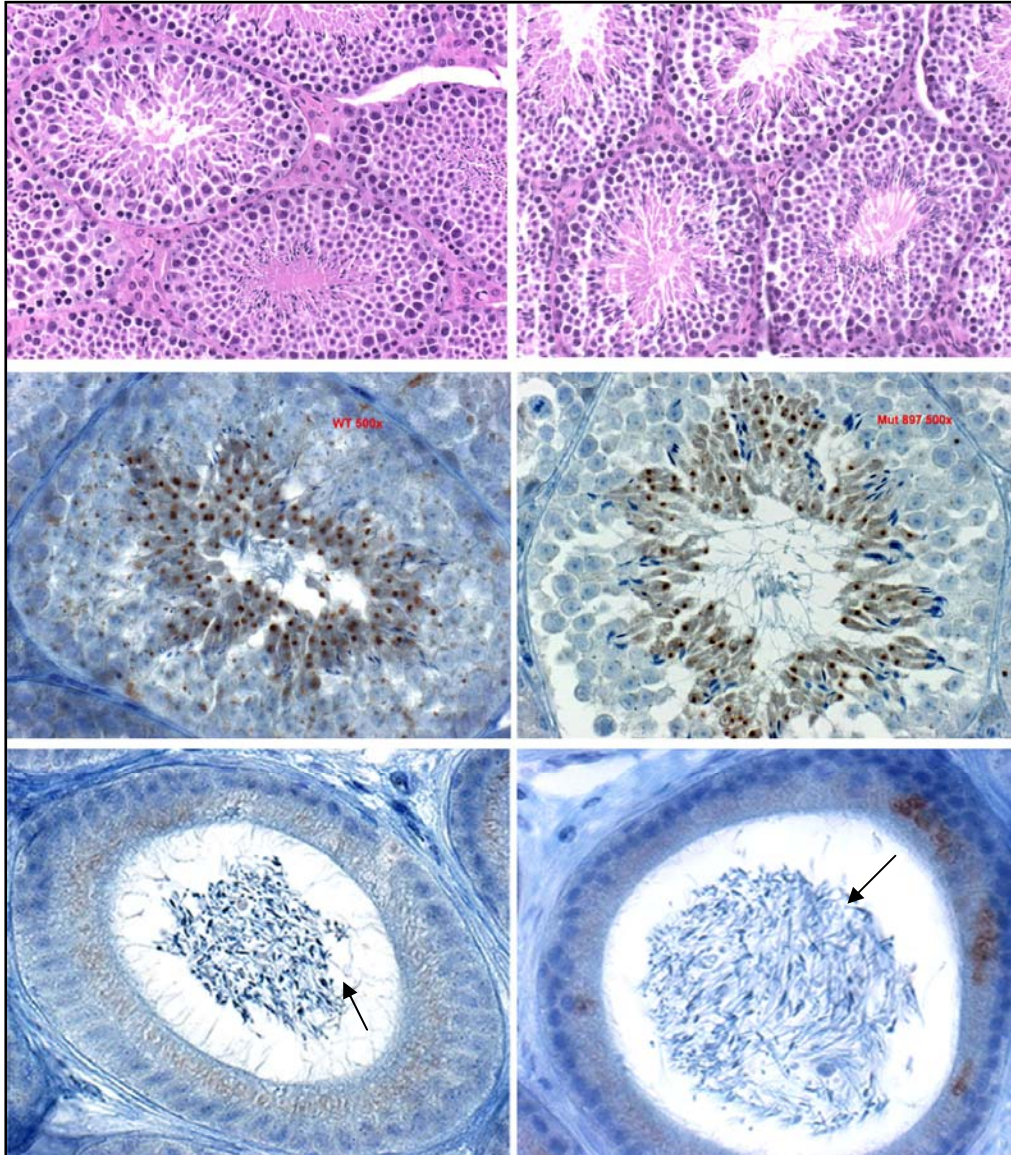


Figure 20: H&E staining of testicular seminiferous tubules

(A) control and (B) FOXP2 mutant mouse. FOXP2 immunostaining of seminiferous tubules in testis of control (C, E) and mutant mice (D, F). The mature spermatids of the epididymal tubules (arrows) presented no FOXP2 immunoreactivity and are depicted in panels (E, F).

In the **ovary**, the expression of FOXP2 protein is dependent of follicular development and circumscribed to specific stages of maturity. It appears that FOXP2 immunoreactivity was expressed from medium follicles onwards. Antral follicles were also positive (Figure 21, arrows, next page). The small follicles with one or two layers of granulosa cells and the corpora lutea (c. lut.) were not reactive

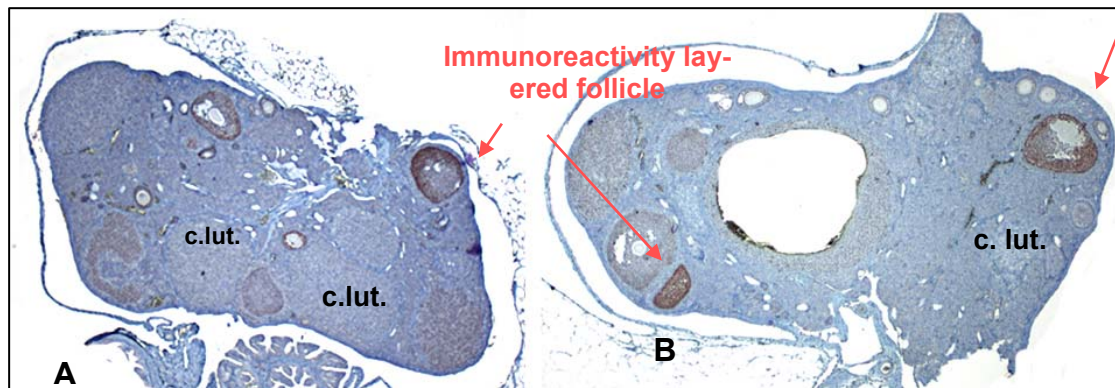


Figure 21: FOXP2 immunostaining of the ovary of a control (A) and of a mutant mouse (B).

In the **lung**, no expression of FOXP2 protein was observed (Figure 22). The acinar cells of the **exocrine pancreas** showed strong expression of FOXP2 protein. However, FOXP2 expression in the islets of the endocrine pancreas was very weak (Figure 22).

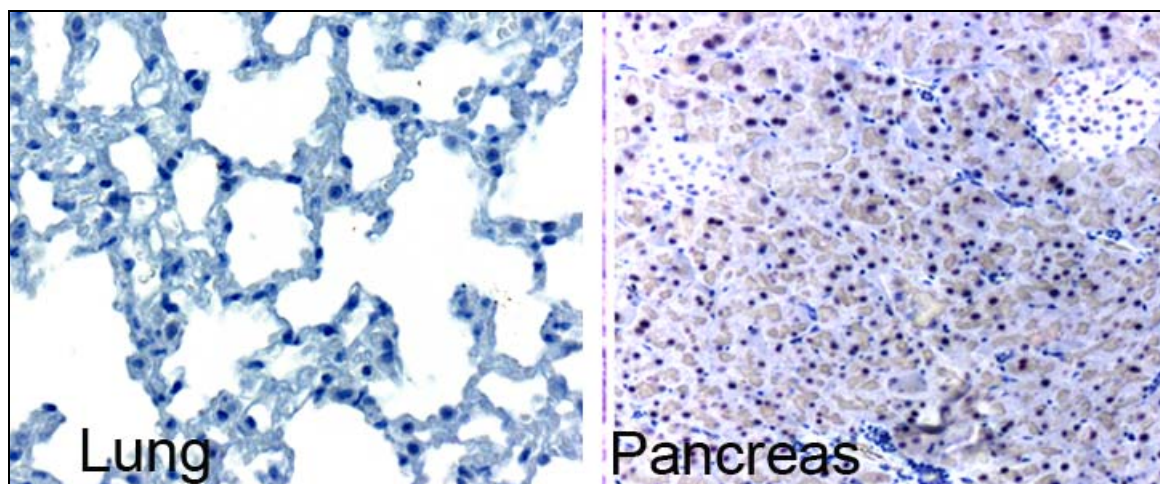


Figure 22: FOXP2 staining in lung and in pancreas tissues from a control mouse.

3.15.5 Discussion

It is known that FOXP2 is a putative transcription factor with nuclear expression in the neurons. Shu *et al.* (2005) reported that **homozygous** FOXP2 knock-out mice revealed cerebellar abnormalities such as thinner molecular layer and changes in the Purkinje cells. These consisted in the lack of alignment in one row and short and simple dendritic processes when compared to the wild-type counterpart animals.

In the primary screen, the pathological analysis revealed no genotype-specific morphological differences between the **heterozygous** knock-out mice when compared to their control littermates. It is suggested that the loss of a single FOXP2 allele does not influence the morphology of inner organs.

It is known that FOXP2 is expressed in many organs with the strongest expression seen in neural circuits mediating the motor learning (striatum, cerebellum, thalamus, inferior olive) where it remains throughout adulthood in mice (Lai *et al.*, 2001, 2003). The expression in **thalamus and cerebellum** depicted in our analysis confirmed these data.

Shu *et al.* 2001, 2007 identified *FOXP2* and *Foxp1* as crucial regulators of lung and esophageal development. The author performed immunohistochemistry for Foxp protein expression in airway and gastrointestinal epithelia using non commercial antisera, showing immunoreactivity in lung, esophagus and pancreas. We detected *FOXP2* expression in the pancreas but no reactivity in lung and esophagus, probably due to the use of a different type of antibody.

In addition, the pathological analysis revealed no differences in the FOXP2-expression pattern between the **heterozygous** knock-out mice when compared to their control wild-type littermates in all analyzed organs. We conclude that a single FOXP2 allele is enough for immunoreactivity but only the complete loss of FOXP2 function results in morphological defects and disease.

Concerning the FOXP2 protein expression detected in **testis** and ovary, and despite no clear pathological changes found in the reproductive organs, it is possible that the *FOXP2* gene plays a role in sperm and ovarian follicle development, as previously reported by Persson *et al.*, 2006; Uhlen *et al.*, 2005

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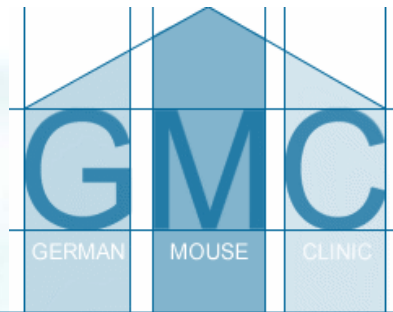
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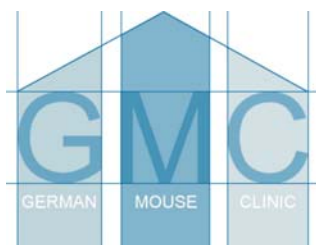
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Report for FOXP2_delta_ex7

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The German Mouse Clinic



The German Mouse Clinic (GMC) was founded January 2002 at the Helmholtz Zentrum München - German Research Center for Environmental Health (GmbH) in Munich 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 allergology, behavior, bone and cartilage, cardiovascular diseases, clinical chemistry, energy metabolism, eye development and vision, immunology, lung function, molecular phenotyping, neurology, nociception, pathology, and steroid metabolism. Additional screens for host-pathogen interaction can be performed at the HZI Braunschweig. Secondary and tertiary screening for in-depth analysis is offered by the different screens and is available on request.

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