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Generation of mice lacking DUF1220 protein domains: effects on fecundity and hyperactivity

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Abstract

Sequences encoding DUF1220 protein domains show the most extreme human lineage-specific copy number increase of any coding region in the genome and have been linked to human brain evolution. In addition, DUF1220 copy number (dosage) has been implicated in influencing brain size within the human species, both in normal populations and in individuals associated with brain size pathologies (1q21-associated microcephaly and macrocephaly). More recently, increasing dosage of a subtype of DUF1220 has been linked with increasing severity of the primary symptoms of autism. Despite these intriguing associations, a function for these domains has not been described. As a first step in addressing this question we have developed the first transgenic model of DUF1220 function by removing the single DUF1220 domain (the ancestral form) encoded in the mouse genome. In a hypothesis generating exercise, these mice were evaluated by 197 different phenotype measurements. While resulting DUF1220-minus (KO) mice show no obvious anatomical peculiarities, they exhibit a significantly reduced fecundity (χ^2= 19.1, df = 2, p = 7.0×10^{-5}). Further extensive phenotypic analyses suggest hyperactivity ($p < 0.05$) of DUF1220 mice and changes in gene expression levels of brain associated with distinct neurological functions and disease. Other changes that met statistical significance include an increase in plasma glucose concentration (as measured by Area Under the Curve, AUC 0-30 and AUC 30-120) in male mutants, fasting glucose levels, reduce sodium levels in male mutants, increased levels of the liver functional indicator ALAT/GPT in males, levels of alkaline phosphatase (also an indicator of liver function), mean R and SR amplitude by electrocardiography, elevated IgG3 levels, a reduced ratio of CD4:CD8 cells, and a reduced frequency of T cells; though it should be noted that many of these differences are quite small and require further examination. The linking of DUF1220 loss to a hyperactive phenotype is consistent with separate findings in which DUF1220 over expression results in a down-regulation of mitochondrial function, and potentially suggest a role in developmental metabolism. Finally, the substantially reduced fecundity we observe associated with KO mice argues that the ancestral DUF1220 domain provides an important biological function that is critical to survivability and reproductive success.

INTRODUCTION

DUF1220 protein domains exhibit the greatest human lineage-specific increase in copy number of any protein coding sequence in the human genome and DUF1220 dosage has been linearly linked to brain size among primates and within the human population. While non-primate mammals and prosimians have few copies of DUF1220 (1–8), a large burst in copy number has occurred in anthropoid primates (monkeys, apes and humans) (O'Bleness *et al*. 2012). Within this suborder DUF1220 copy number increases generally as a function of a species evolutionary proximity to humans: monkeys (25–35 copies), apes (90–120) and humans (270) (Popesco *et al*. 2006). DUF1220 copy number strongly correlates with overall brain size and cortical neuron number among primate lineages (Dumas *et al*. 2012), as well as gray matter volume, white matter volume, gyrification and surface area (Keeney *et al*. in press). DUF1220 has also been linked to differences in human brain size in both normal and disease-associated (microcephaly and macrocephaly) populations (Dumas *et al*. 2012). Recently, in individuals with autism, increase in copy number of a DUF1220 subtype has been shown to be linearly associated with increasing severity for each of the primary

symptoms of autism (Davis *et al*. 2014). While these findings point to an important role in brain function, a functional role for these domains has not yet been described.

DUF1220 protein domains are approximately 65 amino acids in length and can be divided into two general types: 1) an ancestral form that is low copy number in all mammals and found only as a single copy in the gene encoding phosphodiesterase 4D interacting protein (*PDE4DIP* or *myomegalin*), and 2) a derived form that is found in almost all mammals and that underwent the extreme copy number amplification in anthropoid primates (O'Bleness *et al*. 2012), primarily within the *NBPF* gene family (Vandepoele *et al.* 2005). Mice contain only one copy of DUF1220, which exists within the *Pde4dip* gene. Pde4dip is thought to act as a scaffold protein (Uys GM *et al*. 2011), and is known to co-localize with Pde4d (Verde I *et al*. 2001), a protein involved in the cAMP cascade. Several isoforms have been described for *Pde4dip*. The full length isoform, isoform 1, contains a DUF1220 domain and is expressed at the golgi/centrosome region in many cell types (Verde I *et al*. 2001). It is known to be expressed in the developing mouse neocortex (http://www.informatics.jax.org/assay/MGI:5421053) and, although an association between *Pde4dip* and microcephaly has not yet been described, a well-established association exists between human microcephaly and a homolog of *PDE4DIP*, *CDK5RAP2* (Bond *et al*. 2005).

In the present study, we removed the sequence encoding the single DUF1220 domain from the mouse genome, (i.e. from the *Pde4dip* gene) and fused the remaining gene back together, such that the open reading frame was retained. This resulted in an otherwise functional *Pde4dip* gene missing only the DUF1220 domain. Here we report on fecundity effects and the results of a systematic analysis of the resulting DUF1220-KO mice involving 197 phenotypic parameters related to behavior, physiology and anatomy. Additionally, genome-wide transcriptome analysis of brain was performed to identify molecular changes related to the lack of the DUF1220 domain. This is the first living animal model of DUF1220 domain function and we report that removal of the single DUF1220 domain from the mouse genome confers significant effects related to hyperactivity and fecundity.

MATERIALS AND METHODS

Generation of KO mice

A *PDE4DIP* construct lacking a DUF1220 domain was generated by amplifying the upstream ("left arm") and downstream ("right arm") regions of *Pde4dip*, such that the DUF1220 region was not amplified (figure 1A). An HpaI site was also introduced into the right arm primer. The two arms were ligated together, producing a construct that was identical to *Pde4dip* upstream and downstream of DUF1220, and only lacked the DUF1220 domain. A PGK/neo cassette was inserted into the HpaI site for drug selection. The construct was linearized by NotI digestion and was used to transfect mouse embryonic stem (ES) cells by electroporation. G418 was applied to select integrants, in which the homologous recombination event was confirmed by long-ranged PCR at both the left and right arm. Targeted ES cells were injected into mouse blastocysts, which were implanted into pseudo pregnant mothers who carry the embryos as surrogates, to generate chimeras. The mouse ES cell line used was EC7.1. which is an F1 hybrid of C57BL/6 × 129X1/SvJ. Hybrid ES cells were used because of robustness for germline transmission. Transgenic

mice were bred with C57BL/6 for more than 10 generations, and therefore are considered a C57BL/6 congenic.

Genotyping of wild type, heterozygous and knock out mice was performed using a PCR based gel-sizing assay. A forward PCR primer (Sequence: CTTTGTGGGACCTGTGCTTT) was designed to Exon 31 and the reverse primer (GGATCCCAAGGTCTCCTCTC) was designed to Exon 32 the mouse PDE4DIP gene. A 25ul PCR mix contained 2.5ul 10X High Fidelity PCR Buffer (Invitrogen), 2ul 10uM mixed forward and reverse primers (IDT), 0.5ul 2mM dNTPs, 0.75ul 50mM $MgSO_4$ (Invitrogen), 18ul dH2O, 0.2ul Platinum Taq Hi-Fidelity (Invitrogen) and 1ul 50ng/ul genomic DNA. PCR was performed using a gradual reduction in annealing temperature PCR. PCR parameters were as follows: Hold 95 °C 5min. 2 cycles of 94 °C 1 min; 65°C 1 min; 72 °C 3min. 3 cycles of 94 °C 1 min; 63°C 1 min; 72 °C 3min. 4 cycles of 94 °C 1 min; 61 °C 1 min; 72 °C 3min. 4 cycles of 94 °C 1 min; 58 °C 1 min; 72 °C 3min. 4 cycles of 94 °C 1 min; 56 °C 1 min; 72 °C 3min. 4 cycles of 94 °C 1 min; 54 °C 1 min; 72 °C 3min. 4 cycles of 94 °C 1 min; 52 °C 1 min; 72 °C 3min. 15 cycles of 94 °C 1 min; 58 °C 1 min; 72 °C 3min. Hold 70°C 10min. Hold 4 °C indefinitely. PCR products were run out on a 1% agarose gel and analyzed for size variation. Wild-type products are 1,378 bp, knock-out products are 207 bp, and heterozygous mice have one of each size band (figure 1B).

Verification of Expression

RT-PCR, using primers designed to the left arm, right arm, and DUF1220 region, were used to verify that no aberrant truncation had taken place (figure 2). Both the left arm and right arm are expressed normally in the KO and heterozygous animals. Only the DUF1220 domain is missing from the KO animal, indicating the removal of the DUF1220 domain has not negatively impacted downstream gene expression and confirming that any phenotypes observed are due exclusively to the removal of the DUF1220 domain. Western Blot analysis of protein using a commercially available antibody against Pde4dip (GeneTex) revealed no detectable difference in protein level.

Neuroanatomy

Adult brains from each genotype (3 WT, 2 heterozygous and 6 KO) were obtained by transcardial perfusion with 4% PFA. Whole brains were sectioned coronally and reacted with the acidophilic dye thionine to reveal general brain structure. Stained sections were imaged at 10X and automatically scanned and stitched together using Surveyor software. Images were compared using GeneSpringGX software.

Association Memory

Tests of association memory were carried out in olfactometers as described in (Slotnick and Restrepo 2005). The training paradigm was a “go-no go” task as described in (Hellier *et al*. 2010). Mice were kept in static caging during training, and odors were prepared fresh weekly.

Phenotyping in the German Mouse Clinic (GMC)

15 male and 15 female each of WT and homozygous mice, all born within one week of each other, were shipped to the German Mouse Clinic (GMC) (www.mouseclinic.de) for extensive, standardized phenotyping. GMC testing evaluated dysmorphology, cardiovascular health, energy metabolism, clinical chemistry, eye, lung function, molecular phenotyping, behavior, neurology, nociception, immunology, and pathology. Phenotypic screens at the GMC were performed according to standardized methods. (Gailus-Durner *et al*. 2008, Gailus-Durner *et al*. 2009, Fuchs *et al*. 2011). Mice were tested between the ages of 9 and 21 weeks terminated with transcriptome analysis of brain from male mice (3 mutants, 4 wild type) as recently described (Kugler *et al*. 2013) (Horsch *et al*. 2008). Differential gene regulation was identified comparing single mutant values with the mean of four wild types (FDR < 10%, fold change > 1.5). Overrepresented functional annotations were provided as GO (Gene Ontology) terms of the category ‘Disease and Function Annotations’ (Ingenuity Pathway Analysis, IPA). Expression data were available at the public repository database GEO (Edgar et al (GSE58308). At the GMC mice were maintained in IVC cages with water and standard mouse chow (Altromin no. 1314) according to the GMC housing conditions and German laws. All tests performed at the GMC were approved by the responsible authority of the Government of the district government of Upper Bavaria, Germany.

RESULTS

KO mice have reduced fecundity

Genotyping of 964 mice indicates that homozygous KO mice have significantly reduced fecundity, yielding only about 68% of expected numbers (Table 1). Whether KO pups are dying *in utero* or are dying shortly after birth, is unknown, however, removal of the DUF1220 protein coding domain appears to confer a survival disadvantage.

KO mice show no differences in neuroanatomy

In order to evaluate potential structural differences in the neuroanatomy of KO mice, brains from littermates of each genotype (WT, heterozygous and KO) were fixed, removed, sectioned and reacted with thionine. The entire brain was evaluated for general differences in cellular organization, and particular attention was given to the amygdaloid nucleus, as these are thought to be dysmorphic in *Pde4d* KO mice (Rutten K *et al*. 2008). No structural or anatomical differences were apparent in this analysis, including brain size and cortical thickness. It should be noted that structural elements such as neuron number and connectivity are not visible with this technique. Other anatomical features were also compared for size and gross morphology, including skeletal muscle, heart, liver, spleen, skeleton and others (see supplemental report), and these also showed no consistent differences between genotypes.

KO mice have normal association memory

In order to assess association memory, mice were tested in a go-no go task using an olfactometer as described previously (Hellier *et al*. 2010). Water deprived mice were

evaluated for their ability to associate one odor out of a pair with a water reward, but no consistent difference was found between genotypes.

KO mice are hyperactive

15 homozygous KO male mice, 15 homozygous KO female mice, 15 WT male mice and 15 WT female mice were sent to the German Mouse Clinic to undergo exploratory behavioral and other phenotypic analyses. These consisted of 197 measures of several phenotypes, including anxiety, grip strength, pain response, gross anatomy, cardiovascular function and several others (see supplemental report). This is treated as a hypothesis generating exercise and no correction for multiple comparisons was conducted. ANOVA was utilized to test differences between phenotypes. We identified an association ($p<0.05$) with two hyperactive phenotypes, rearing counts and distance with genotype (figure 3). Both male and female KO mice were more active and reared more than their WT counterparts in their home cage during the 21 hours indirect calorimetry measurement (figure 4, supplemental table 1). Locomotor and rearing activity was particularly increased during nighttime with highest levels during the first half of the night. Hyperactivity did not markedly affect energy expenditure determined from oxygen consumption and carbon dioxide production. During the indirect calorimetry, daily energy expenditure, resting metabolic rate, and metabolic fuel utilization did not differ between genotypes. There were no genotype-related differences in spontaneous locomotor activity in a novel environment during a 20 minute Open Field test (see supplemental data).

Other results revealed slightly significant associations with genotype or gentype + sex, including plasma glucose levels, sodium levels, alanine aminotransferase (ALAT/GPT) enzyme levels (indicating potential liver differences), and concentration of CD45+ T cells. Borderline significance was also found in a genotype:sex association for fasting glucose, alkaline phosphatase (ALP) enzyme levels (involved in many physiological roles), electrocardiography, acoustic startle reactivity and IgG3 concentration (see supplemental report).

Transcriptome profiles of brain

Despite the lack of structural differences in brain of KO mice we performed transcriptome profiles of brain to identify differential gene regulation according to the missing DUF1220 domain in *Pde4dip*. Statistical analysis revealed 491 significantly down-regulated genes in brain (supplemental table 2) including moderately reduced expression levels of *Pde4dip* (fold change: -1.64 ± 0.13). Functional classification of the regulated genes give strong evidence for changes in brain and neurological functions indicated e.g. by the following over-represented GO terms: Behavior (conditioning, memory, and learning), cell morphology (axonogenexis), cell-to-cell signaling (synaptogenesis and neurotransmission), hereditary disorder (schizophrenia and Huntington's disease) and neurological disease (epileptic seizure, ataxia, epilepsy and neurodegeneration) (Table 2). In addition, gene expression (transcription), cellular growth (cell proliferation), reproductive, infectious and metabolic disease (diabetes mellitus) were over-represented functions in the dataset of regulated genes in brain of DUF1220KO mice. These functional annotations might be of

particular interest with regards to the possible functions of the DUF1220 domain and the phenotypical alterations identified so far.

DISCUSSION

We have generated DUF1220-minus mice, the first successful animal model of DUF1220 domain function. Because the copy of DUF1220 that has been removed is the ancestral form that is shared across animal taxa, including human, and is expressed in multiple tissues, this mouse model represents an invaluable tool for investigating general cell mechanisms in which the ancestral form of DUF1220 may be functioning. Our analysis of several hundred DUF1220 KO mice has indicated a strong reduction in fecundity compared to their wild-type littermates. Although the nature of this reduction is thus far unclear, this result suggests an evolutionary advantage for this 65 amino acid domain alone.

These mice were also evaluated for 197 other physical and behavioral phenotypes. Although effects not directly related to metabolism were detected, such as the frequency of certain immune system cells and liver functionality, most of the results that were near or surpassed significance are related to metabolism. It should also be noted that the liver is known to be among the most energetically expensive tissues in the body (Wang *et al*.), and a metabolic phenotype may therefore still explain these differences. Although not corrected for multiple comparisons, the association found with the hyperactive phenotypes is consistent with the recent finding that up-regulation of DUF1220 domains in vitro is associated with down-regulation of mitochondria-related genes (Keeney, unpublished data). If the hyperactivity associations discovered here were due to chance alone, they would not be expected to cluster on a specific phenotype, but would be expected to be randomly dispersed. Indeed, expression profiling analysis of brain identified only down-regulation and a few genes were annotated with mitochondrial functions. While *Ppargc1* and *Dhtkd1* play roles in mitochondrial biogenesis (Dumont *et al*. 2014, Xu *et al*. 2013) *Kif1b* is responsible for the movement of mitochondria along the axon (Conforti *et al*. 1999). Further, reduced expression of *Gsk3b* which has an impact on mitochondrial trafficking, was annotated with reduced motility of mitochondria (Llorens-Martin *et al*. 2011).

Although mice showed increased hyperactivity and rearing behavior in the homecage, there was no detectable difference in locomotor activity in a novel environment, in neurological parameters or in associative memory, suggesting a specific effect. However, those 13 genes differentially expressed in brain annotated with memory and another 25 with progressive motor neuropathy indicate at least moderate alterations on molecular level related to these function. Conversely, though no difference in body mass or food intake was observed, significant differences in fasting levels of glucose, sodium and potassium were detected by genotype, indicating that the observed hyperactivity may potentially be metabolically driven. Elevated ALAT and lactate dehydrogenase (LDH) levels seen in KO males may support this hypothesis, though these results were not apparent in KO female mice.

These results hint towards a down-regulation of metabolism by DUF1220 domains supported by decreased expression levels in brain of a group of genes associated with lipid and carbohydrate metabolism. Though presently untestable in a mouse model, these results

are consistent with recent observations that primate brains have expanded by a mechanism that restricts cell size to a near constant value across all primates, putting an emphasis on cell number, rather than cell size. Like the burst in genomic copy number of DUF1220, this phenomenon is restricted to anthropoid primates (O'Bleness *et al*. 2012), and there is a strong correlation between DUF1220 and brain size across these species. These observations raise the possibility that down-regulation of mitochondria-related proteins may therefore be acting to restrict metabolism – and therefore cell size – during neural development.

A metabolic suppression during development would also be consistent with the Warburg Effect: the observation that cycling cells (whether stem cells or cancerous cells) tend to prefer glycolysis followed by lactic acid fermentation, rather than mitochondrially mediated oxidative phosphorylation, as is used in other cells, despite ample oxygen availability (Wargburg *et* al. 1927). Consistent with this, many cancers are known to elevate LDH levels, presumably for this reason (see Granchi C *et* al. 2010 for a review). These results may point to a role for DUF1220 in cell number and/or cell cycle control via a metabolic mechanism.

Finally, while this study has provided insight into understanding the cell biology of the ancestral form of DUF1220 *in vivo*, it should be noted that the ancestral DUF1220 domain may have a function independent of the derived form of DUF1220 that has been markedly amplified in copy number in the anthropoid primate lineage. This could be addressed through the development of a "humanized" mouse line, in which the amplified, primate form of DUF1220 is transgenically integrated into the mouse genome.

In addition to linking the ancestral DUF1220 domain with fecundity, hyperactivity and regulation of genes involved in distinct neurological functions and disease, the results also provide evidence for the possibility that DUF1220 plays a role in down regulating metabolism that involves mitochondrial pathways. Further work will be required to investigate these hypotheses, evaluate the mechanistic nature of this effect, and determine if this effect is contributing to the exceptional brain growth observed in anthropoid primates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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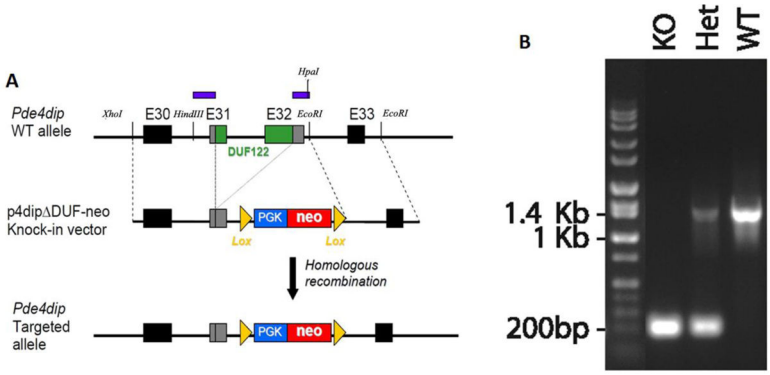

Figure 1.

Strategy for generating domain knockout mice and verification of genotype. A: The DUF1220 domain is encoded by parts of exons 31 and 32 (green boxes). Primers (purple boxes) designed to upstream (“left arm”) and downstream (“right arm”) regions were used to amplify PDE4DIP without DUF1220. The two arms were ligated together. An HpaI site was introduced into the right arm, and this was used to insert PGK/neo cassette for later selection of positive transformants by neomycin resistance. This vector was transfected into mouse ES cells and incorporated into the mouse genome by homologous recombination. B: Genotyping gel. Primers designed to span exons 31 and 32 produce a heavy band in WT mice of approximately 1.4 Kb, a small band of 199 bp in KO mice, and one of each band in heterozygous mice.

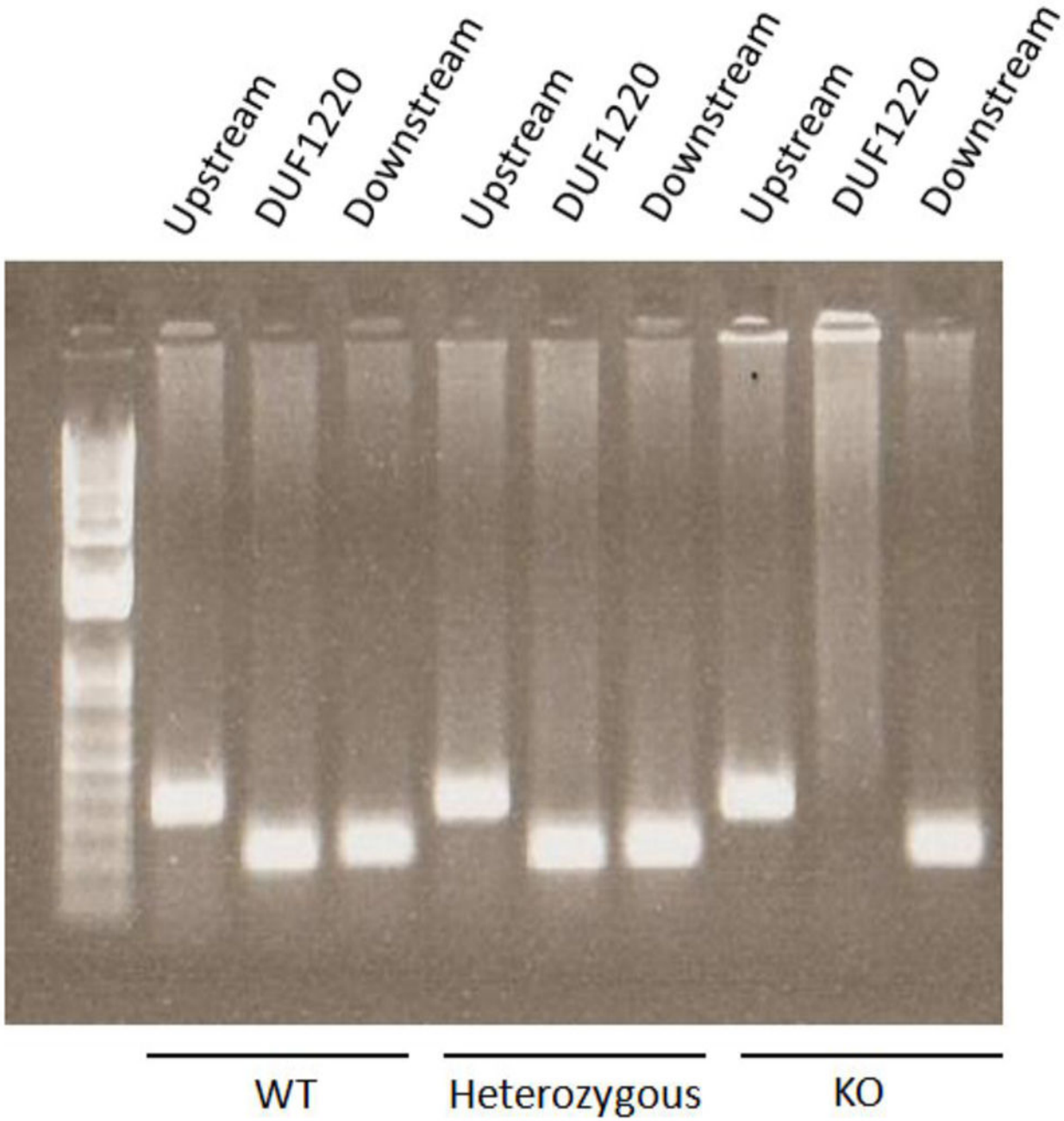

Figure 2.

DUF1220 domains are not expressed in KO mice, and do not affect full length protein expression. RT-PCR Primers designed to the region upstream of DUF1220, the DUF1220 region itself, and the downstream region were used to test PDE4DIP expression in the mice generated by this method. The knockout mouse shows no disruption of either the upstream or downstream regions of PDE4DIP.

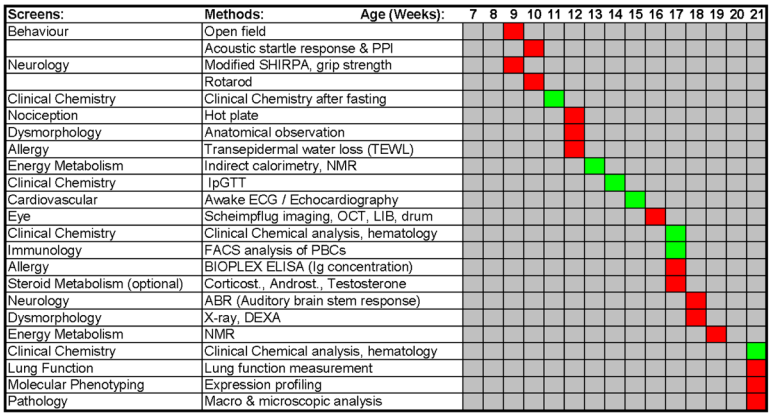

Figure 3.

Overview of tests performed by the German Mouse Clinic and summary of results. Anatomical and behavioral evaluations are arranged according to the age at which the tests were performed and color coded red for negative results and green for positive results, based on criteria of significant difference between WT and KO mice, with $p \leq 0.05$. Significant differences were found related to metabolic differences in Clinical Chemistry and Energy Metabolism categories.

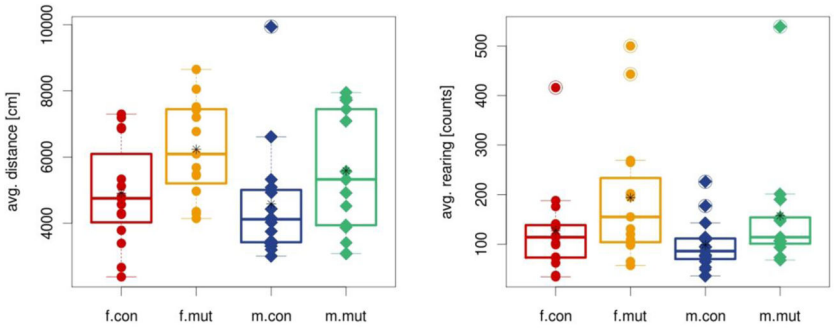

Figure 4.

Results of indirect calorimetry measurements. **A:** Average distance traveled in centimeters per 20 minutes is plotted by sex and genotype. KO mice tend to travel farther distances than their WT counterparts. **B:** Average number of rearing events per 20 minutes observed is plotted by sex and genotype. KO mice of either gender tend to rear more frequently than their WT counterparts.

Table 1

Mice lacking a DUF1220 domain show significantly reduced fecundity. Mendelian ratios predict 241 mice given a total of 964, but only 165 have been born (χ^2= 19.1396, df = 2, p = 6.981e-05).

Total Mice = 964		
	Observed	**Expected**
WT	287 (30%)	241 (25%)
HET	512 (53%)	482 (50%)
KO	165 (17%)	241 (25%)

Table 2

Overrepresented functional annotations among regulated genes in brain of DUF1220 knockout mice mainly associated with distinct neurological functions and disease

Category	Diseases or Functions Annotation	p-Value	# genes
behavior	conditioning	1.22E-03	12
	memory	1.80E-03	13
	emotional behavior	3.54E-04	15
	learning	4.26E-03	18
cell death and survival	apoptosis of brain cells	2.25E-04	11
cell morphology	morphology of neurites	2.31E-03	10
	formation of neurites	1.38E-05	15
	axonogenesis	3.80E-06	16
cell-to-cell signaling	synaptogenesis	1.20E-03	11
	neurotransmission	6.09E-06	26
cellular development	proliferation of neuronal cells	2.51E-03	11
	differentiation of neurons	8.99E-04	20
	neuritogenesis	9.41E-05	26
developmental disorder	mental retardation	8.78E-04	12
embryonic development	development of cerebellum	1.31E-05	11
hereditary disorder	autosomal dominant disease	1.84E-03	22
	Schizophrenia	1.07E-05	30
	Huntington’s Disease	2.56E-06	35
nervous system development and function	nociception	2.52E-03	10
	abnormal morphology of hippocampus	2.00E-05	11
	quantity of brain cells	3.90E-07	14
	myelination	1.90E-05	14
	morphology of cerebral cortex	1.32E-05	16
	coordination	2.50E-05	16
	synaptic transmission of cells	2.03E-04	16
neurological disease	gait disturbance	6.26E-04	10
	neurodegeneration	4.35E-03	11
	epileptic seizure	1.18E-04	13
	ataxia	9.01E-04	14
	bipolar disorder	2.47E-03	16
	epilepsy	1.83E-05	20
	progressive motor neuropathy	6.79E-04	25
	seizures	1.59E-07	29
psychological disorders	depressive disorder	3.48E-04	16
	Mood Disorders	7.93E-04	23