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Deletion in the Y chromosome of B10.BR-Y^{del} mice alters transcription from MSYq genes and has moderate effect on DNA methylation.

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Abstract

B10.BR-Y^{del} male mice with large deletion in the male-specific region of the Y chromosome long arm (MSYq) are very useful experimental model which requires, however, more detailed characterization. In the present study the influence of the deletion on transcript levels of MSYq genes (*Ssty1*, *Ssty2*, *Sly*, *Srsy, Asty*,*Orly*) and homologous to them X-linked genes (*Sstx*, *Slx, Slxl1*, *Srsx*) was assessed. Quantitative PCR analysis showed that in testes of B10.BR-Y^{del} males activity of *Ssty1* is unchanged, but transcription from all other MSYq genes is highly reduced and reaches from 59% to only 5% of the control levels. The decrease in expression of MSYq genes is accompanied by the two-fold increase in expression of *Slx* and *Slxl1* genes. This is the first functional characterization of the deletion in B10.BR-Y^{del} strain. Another aim of the study was to reveal the mechanism through which deleted Y chromosome of B10.BR-Y^{del} males could alter phenotype of their female progeny, what was documented in our previous works. Epigenetic inheritance hypothesis was tested by microarray analysis of DNA methylation in B10.BR-Y^{del} and control B10.BR sperm. The assessment revealed moderate differences and allowed concluding that the mutated Y chromosome can influence traits of females from the next generation partially through altering sperm DNA methylation, but probably some additional mechanisms are engaged here. Breeding data indicate that feminization of pre- and neonatal environment in which next generation females develop is one of such additional mechanisms.

Keywords: sperm DNA methylation, spermiogenesis genes, transgenerational effects, X-Y conflict, Y chromosome long arm

1. Introduction

The male-specific region of the Y chromosome long arm (MSYq) contains genes that play an important role in spermatogenesis. Three multicopy protein-coding gene families were identified within the mouse MSYq: *Sly* (*Sycp3 like Y-linked*), *Ssty1/2* (*Spermiogenesis specific transcript on the Y*) and *Srsy* (*Serine-rich, secreted, Y-linked*) [1-6]. Recently, it has been demonstrated that *Srsy*-amplicon sequences lost their coding potential and produce several, separate, not-translated transcripts [7]. *Sly* as well as *Ssty1* and *Ssty 2* code functional proteins identified in round and early-elongating spermatids, where they co-localize with the post-meiotic sex chromatin and are involved in recruitment/maintenance of repressive epigenetic marks regulating X and Y gene expression [8-10]. *Sly* has been, additionally, shown as an important regulator of chromatin packaging, indispensable for maintaining DNA integrity in sperm [10]. Multicopy MSYq genes: *Sly*, *Ssty* and *Srsy* have their multicopy homologs on the X chromosome: *Slx (Sycp3 like X-linked)/Slxl1 (Slx-like1)*, *Sstx* and *Srsx (Serine-rich, secreted, X-linked)*, respectively [5, 12-13]. It is postulated that convergent acquisition and amplification of genes on the mouse X and Y chromosomes resulted from intragenomic conflict between sex chromosomes. According to this theory X- and Y-linked genes act antagonistically during sperm development and favor their own transmission to the next generation [5, 14-16]. The existence of competition between sex chromosomes has been proved for *Sly* and *Slx/Slxl1* genes. *Sly* and *Slx/Slxl1* have the opposite effects on X and Y gene expression in spermatids (repressive versus stimulatory) as well as on sex ratio in offspring (male versus female predominance) [8, 16-17].

Apart from *Sly, Ssty* and *Srsy*, within the mouse MSYq two other amplified genes: *Asty* (*Amplified spermatogenic transcripts Y encoded*) and *Orly* (*Oppositely-transcribed, rearranged locus on the Y*) were indicated. These genes produce poorly characterized

transcripts which are believed to belong to MSYq-derived non-coding RNAs regulating gene expression during sperm development. [4, 18]. *Asty* turned out to be a part of *Srsy*-amplicon named currently *Laidy* (*Large amplified intrinsically disordered protein-coding gene on the Y*) and including in addition to *Asty* also *Srsy* and *Gm28689* [7]. *Orly* remains an enigma but may relate to the piRNA clusters (*Pirmy*) identified recently on the mouse Y chromosome [19].

Mice with deletions in the Y chromosome long arm (Yq) are very useful models to study MSYq genes. Examination of their phenotypes allowed to discover that MSYq genes are indispensable for normal sperm development and male fertility [3, 20-25]. B10.BR and B10.BR-Y^{del} congenic inbred strains of mice belong to these precious models. Animals of both strains differ only in their Y chromosome, for which B10.BR-Y^{del} males have a broad deletion covering approximately 2/3 of the long arm. In cytological preparations the shortening of the mutated Y chromosome is clearly visible [20-21]. Despite the loss of the great part of MSYq sequences, B10.BR-Y^{del} males are fertile, but their sperm exhibits frequent morphological and ultrastructural abnormalities [20-21, 26], delay in epididymal maturation [26], deterioration of movement [27], difficulties in crossing uterotubal junction [28], aberrant expression of surface receptors [29], and lower fertilization efficiency [26, 30-31]. Additionally, in the offspring of mutant males, a characteristic sex ratio skew towards females is observed [29, 32]. It was demonstrated that the number of copies of *Ssty* genes is strongly reduced in the Y chromosome of B10.BR-Y^{del} males [33] and that these genes are still transcribed in their testes [27], but any comprehensive analysis of the deletion has not been done yet. Accordingly, in the first part of the present work we measured the extent to which the deletion reduces the transcription from MSYq genes: *Ssty1*, *Ssty2*, *Sly*, *Srsy, Asty* and *Orly*. Additionally, we assessed the influence of the deletion on transcript abundance levels of the X-linked genes related to MSYq genes: *Sstx*, *Slx/Slxl1*, *Srsx* and on expression of

one autosomal gene *Hsf2* which was shown to occupy MSYq region and to regulate its activity during sperm development [34].

Females sired by B10.BR-Y^{del} fathers, named B10.BR(Y^{del}) females, differ in some reproductive characteristics from B10.BR females (sired by control B10.BR males) [29, 31, 35]. Since Y chromosome is not inherited by female progeny, we hypothesized that MSYq genes are involved in establishment of not only temporary [8-11], but also stable and heritable epigenetic modifications of chromatin in male germ cells. These modifications, such as DNA methylation, transmitted via sperm to females from the next generation could influence their phenotypes. To verify the above hypothesis, we compared B10.BR and B10.BR-Y^{del} males in terms of total DNA methyltransferase (DNMT) activity in their testes as well as in terms of the whole-genome methylation pattern of their sperm.

Not only ours, but also many other studies showed that Y chromosomes of fathers can affect both physiological and behavioral traits of female offspring. In various mouse models different origins of paternal Y chromosomes lead to significant phenotypic divergence between genetically the same females [36-38]. The phenomenon was explained by the influence of the Y chromosome of male fetuses on the prenatal environment of female fetuses developing in their neighborhood. Contribution of H-Y antigens and *Sry* activity responsible for testosterone production were taken here into consideration [36-37]. In case of B10.BR(Y^{del}) females, their altered reproductive physiology could result from prenatal and early postnatal development in feminized environment (predominance of females in offspring of Y^{del} males). One of the effects of location of female between two other females in uterus is that in adulthood it gives birth to significantly more female than male pups [39]. In the last part of our study we assessed if such effect characterizes B10.BR(Y^{del}) females.

2. Materials and methods

2.1. Animals

Experiments were performed on 3-month-old male mice from the congenic B10.BR/SgSn and B10.BR-Y^{del} strains maintained in Institute of Zoology and Biomedical Research of the Jagiellonian University, Krakow, Poland. Males from the mutant B10.BR-Y^{del} strain are characterized by a partial deletion in the long arm of the Y chromosome (removing ~2/3 of Yq). Males from the control B10.BR strain have the intact Y chromosome. To avoid genetic divergence between the two congenic strains, in each generation B10.BR-Y^{del} males were backcrossed to B10.BR females. The mice were maintained under a 12 h light–dark cycle with free access to water and standard laboratory diet. Males at the appropriate age were killed by cervical dislocation. Their testes were used for RNA extraction or for isolation of nuclear proteins. Sperm released from vas deferens were used for DNA extraction. In total, 26 males from each strain were used in the experiments. The experiments were performed in accordance with Polish legal requirements, under the license of the First Local Ethical Committee on Animal Testing in Krakow (permission number: 88/2010).

2.2. RNA extraction from testes and reverse transcription

Testes of four males of each experimental group were released from tunica and homogenized. The total RNA was extracted using TRI reagent (Sigma-Aldrich, USA) and cleaned using RNeasy MinElute spin columns (Qiagen, Germany) in accordance with the manufacturers' protocols. The RNA purity (260/280 and 260/230 nm ratios) and concentration were assessed with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The RNA integrity was confirmed by electrophoresis on a denaturing agarose gel. 1 μg of the total RNA from each sample was digested with DNase I (Sigma-Aldrich) and reverse-transcribed into cDNA in a 20 μl reaction using High Capacity cDNA Reverse Transcription Kit (Applied

147 Biosystems, USA) and anchored oligo(dT)$_{23}$ primers (Sigma-Aldrich). Standard reaction
148 conditions were applied: 25ºC for 10 min, 37ºC for 120 min and 85ºC for 5 min. Reactions
149 without the addition of reverse transcriptase as well as without the addition of RNA were
150 introduced as negative controls that confirmed the lack of sample contamination with
151 genomic DNA and the purity of all reagents.
152
153 ***2.3. Quantitative PCRs***
154 Quantitative Polymerase Chain Reactions (qPCRs) were run on a StepOne-Plus Real-Time
155 PCR system (Applied Biosystems) using 96-well optical plates (Applied Biosystems). The
156 20-µl PCR reaction mixtures included: 5 µl of cDNA sample (diluted 1:20 in RNase-free
157 water), Power SYBR Green master mix (Applied Biosystems) and primers in a concentration
158 of 250 nM each. Primer sequences are listed in Table 1. β-actin (*Actb*) used as endogenous
159 reference gene was validated for its stable expression in all samples. The reactions were
160 incubated for 10 min at 95ºC followed by 40 cycles: 15 s at 95ºC and 1 min at 60ºC. Next, a
161 melt curve was drawn for each primer pair to ensure that there was no primer–dimer
162 formation. All PCRs were run in triplicate and the average threshold cycles (C_T) were then
163 calculated. For each sample, the C_T value of the endogenous reference gene was subtracted
164 from the C_T values of the target genes to obtain ΔC_T values and to normalize the results. For
165 graphical presentation and statistical analysis, relative mRNA level indexes of the all
166 examined genes were generated with the $2^{-\Delta CT}$ formula [40]. The fold changes of mRNA
167 levels in B10.BR-Y^{del} testes were calculated in relation to the wild type B10.BR testes.
168
169 ***2.4. Assessment of total DNMT activity in testes***
170 Nuclear proteins were isolated from testes of 7 B10.BR and 7 B10.BR-Y^{del} males using
171 EpiQuik Nuclear Extraction Kit (Epigentek, USA). Protein concentration in the obtained

extracts was measured with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). The nuclear extracts were immediately subjected to the assessment of total DNA methyltransferase (DNMT) activity with EpiQuik DNA Methyltransferase Activity/Inhibition Assay Kit (Epigentek) using the protocol recommended by the manufacturer. Results were calculated separately for each testis with the following formula: DNMT activity (OD/h/mg) = [(sample OD - blank OD) / (protein amount added into the reaction (μg) × time of sample incubation with cytosine-rich DNA substrate)] × 1000. The DNMT activity in B10.BR-Y^{del} testes was presented as percent of the activity in control B10.BR testes.

2.5. DNA extraction from sperm

The vas deferens of 3 B10.BR males or 3 B10.BR-Y^{del} males were carefully cleaned of fat and blood vessels. Next, sperm was gently squeezed out into 100 μl of PBS. During sperm releasing, the vas deferens were kept above the PBS surface to avoid contamination of the samples with somatic cells. Sperm suspensions were mixed with digestion buffer (600 μl; 100 mM Tris-HCl pH 8.0, 100 mM EDTA, 0.5% SDS, 100 mM NaCl), proteinase K (30 μl; 10 mg/ml) and DTT (20 μl; 1M), and incubated overnight at 56°C with shaking. Then phenol-chlorophorm-isopropanol DNA extraction protocol was applied. 5 independent extractions from pooled sperm of 3 individuals were done for each strain. Pelleted DNA was washed with 70% ethanol and suspended in 40 μl of sterile water. DNA concentration and its purity were assessed with a Nanodrop 2000 spectrophotometer.

2.6. MeDIP

Methylated DNA immunoprecipitation (MeDIP) was performed using a modified Weber's protocol [41]. 7 μg of genomic DNA of each sample was diluted with water to the volume of 150 μl and sheared by sonication to generate fragments between 300 and 1000 bp. A 3.5 μg

197 portion of sonicated DNA (test) was diluted in 450 µl of 10 mM Tris-HCl pH 8.0, heat-
198 denatured at 98°C for 10 min and immediately cooled on ice for 10 min. Second half of the
199 sonicated DNA was left to serve as the input control. The CpG methylated DNA fragments
200 were precipitated with anti-5-methyl-cytidine antibody (Eurogentec, Belgium). The antibody-
201 DNA complexes were captured with Dynabeads (Invitrogen, USA). The collected beads were
202 washed in order to remove non-specifically bound DNA, which was followed by the
203 treatment with proteinase K for 24 h at 50°C in order to remove proteins. The CpG
204 methylated DNA was extracted with phenol-chloroform method, precipitated with ethanol and
205 glycogen, and resuspended in 60 µl of 10 mM Tris-HCl pH 8.0.
206
207 ***2.7. DNA labeling and hybridization***
208 DNA labeling and hybridization were performed according to the NimbleGen protocol with
209 our slight modifications. The immunoprecipitated CpG methylated DNA (test) and the
210 untreated sonicated DNA (input control) were labeled by the random priming using the
211 NimbleGen Dual Color DNA Labeling Kit (Roche, Switzerland) with fluorescent dyes Cy3
212 (test) and Cy5 (input control). The combined (test and input DNA) samples were suspended in
213 hybridization buffer (Roche NimbleGen), cohybridized onto Mouse DNA Methylation
214 3x720K CpG Island Plus RefSeq Promoter Arrays for 20 h at 42°C, and washed with
215 NimbleGen Wash Kit (Roche).
216
217 ***2.8. Microarray data acquisition and processing***
218 Image acquisition was performed with Roche NimbleGen MS 200 Microarray Scanner at 2
219 µm resolution using high sensitivity and autogain settings. The data from scanned images
220 were extracted and processed with DEVA v. 1.0.2 software (Roche Nimblegen) using default
221 parameters (11 microarrays). After background subtraction, data was normalized using

quantile normalization. Data processing included obtaining log_2 ratios, P-scores and peak identification. P-score signifies value of positioning of identified methylation peak on chromosome. Log_2 ratios represent the ratios of the immunoprecipitated DNA signal to the input DNA signal. Methylation measurements were obtained by merging and averaging consecutive probes with P-scores over the assumed cut off (default 2.0) with maximum 500 bp spacing and minimum of two probes per peak. The methylation peaks were mapped to features (transcription start sites, primary transcripts, CpG islands and other tiled regions) using annotation file provided by the NimbleGen (MM9 CpG Refseq Prom MeDIP). The differences between B10.BR and B10.BR-Y^{del} animals were analyzed using the Student's *t* test. The level of false discovery rate was estimated using the Benjamini-Hochberg procedure.

2.9. Assessment of sex ratio in litters

Sex ratio in litters born to B10.BR and B10.BR(Y^{del}) females mated with B10.BR or B10.BR-Y^{del} males was calculated on the basis of breeding records maintained in our laboratory. In the case of B10.BR females all litters born to them within the last 10 years were analyzed. B10.BR(Y^{del}) females are not normally used for reproduction and they were maintained in pairs with males only for experimental purposes (30 pairs for each type of mating). All litters derived from these pairs were included into the assessment.

2.10. Statistical analysis

All data were evaluated with Shapiro-Wilk test of normality before selecting an appropriate method of statistical analysis (nonparametric or parametric). Sex ratio distortion towards females in litters sired by B10.BR-Y^{del} males were confirmed using chi-square test. Percentages of female pups in the examined types of mating were normalized by angular transformation (arcsin) and next compared using Kolmogorov-Smirnov test. For all other data

247 the Student's *t* test was applied. The P value below 0.05 was considered statistically
248 significant.
249
250 **3. Results**
251
252 ***3.1. Gene expression***
253 The transcript abundance levels of MSYq genes and homologous to them X-linked genes
254 were measured in B10.BR and B10.BR-Y^{del} testes (Figure 1). The qRT-PCR analyses
255 revealed that transcripts of all MSYq genes are present in gonads of mutant males. The
256 expression of *Ssty1* gene appeared to be unaffected by the deletion. Its mRNA abundance is at
257 the same level in testes of both B10.BR-Y^{del} and B10.BR males. However, the deletion causes
258 dramatic reduction of *Ssty2*, *Sly*, *Srsy*, *Asty* and *Orly* expression that fall in B10.BR-Y^{del}
259 gonads to respectively: 13% (P=0.00008), 5% (P=0.002), 39% (P=0.0098), 8% (P=0.0002)
260 and 59% (P=0.0092) of the control levels. The examined X-linked homologs, *Slx* and *Slxl1*
261 showed significantly higher expression in testes of B10.BR-Y^{del} males (P<0.05). The
262 tendency to reduced mRNA level of *Srsx* (P=0.06) in gonads of mutant animals was found.
263 The mRNA level of *Sstx* was unchanged (P=0.46). The broad deletion in the Y chromosome
264 long arm does not influence expression of autosomal gene *Hsf2* - mRNA abundance of this
265 transcription factor involved in regulation of MSYq genes is at the same level in testes of
266 B10.BR-Y^{del} and control B10.BR males (Figure 1).
267
268 ***3.2. DNA methylation***
269 Total DNMT activity was compared between B10.BR and B10.BR-Y^{del} testes (Figure 2).
270 DNMT activity in gonads of B10.BR-Y^{del} males attains only 64% of the control level. The
271 difference is statistically significant (P<0.05). The influence of testicular DNMT activity on

sperm whole genome methylation was further investigated using the methylated DNA immunoprecipitation microarrays (MeDIP-chips). In general, the reduced DNMT activity in gonads of B10.BR-Y^{del} males does not have a great impact on DNA methylation of their gametes. None of the analyzed DNA regions in B10.BR-Y^{del} spermatozoa showed difference in methylation level higher than 2-fold versus control. The differences between groups may have flattened subtly due to sperm contamination with extracellular somatic DNA, which was not eliminated from samples. However, at the particular threshold (*t* test $P<0.01$ and fold of difference >0.2) 93 microarray probe-sets were identified (Table S1). 33 genomic regions with the highest fold of difference (>0.3) were presented in Figure 3. Most of these regions (24) are hypomethylated in DNA of mutant males, which is consistent with the described above lower DNMT activity in B10.BR-Y^{del} testes. Among hypermethylated sequences only 2 are located on autosomes and the remaining 7 are located on sex chromosomes. The most hypermethylated genomic region in B10.BR-Y^{del} sperm has been assigned to *Ssty1* (fold of difference: 0.54). Hypermethylation of *Ssty1* is probably underestimated due to lower representation of MSYq sequences in DNA of B10.BR-Y^{del} males.

All genes annotated to the top differently methylated regions (Figure 3) have been checked in NCBI Gene base (https://www.ncbi.nlm.nih.gov/gene). 17 of these genes are recorded to have expression in mouse ovary: *Rab10os*, *Esam*, *Chd2*, *Epb41l3*, *Ccdc138*, *Gm29683*, *5430416N02Rik*, *Bbs10*, *Mkx*, *Chd8*, *Crybg1*, *Gm3055*, *Kiz*, *Ccnl1*, *Tmpo*, *Stat1* and *Zfp287*. 6 of them (*Chd2*, *Bbs10*, *Mkx*, *Chd8*, *Tmpo*, *Stat1*) are transcription factors.

3.3. Sex ratio in litters born to B10.BR and B10.BR(Y^{del}) females

In litters born to B10.BR females mated with B10.BR males the percentage of female pups amounts 48% which is very close to the normal 50:50 sex ratio. B10.BR females mated with B10.BR-Y^{del} males give litters with significantly higher percentage of females (59%). Sex

297 ratio distortion towards females is characteristic for litters sired by males with partial Yq
298 deletion [32, 42]. In order to check if females derived from such feminized litters are more
299 likely than control to give birth to female pups, we maintained them in pairs with B10.BR or
300 B10.BR-Y^{del} males and recorded sex of their offspring. Indeed, the percentage of females in
301 litters born to B10.BR(Y^{del}) mothers is higher than in control litters for both types of mating.
302 This tendency is more clear when only two first litters of B10.BR(Y^{del}) females are taken into
303 account (Table 2).
304
305 **4. Discussion**
306
307 Deletions on the mouse Y-chromosome long arm (Yq) lead to various sperm abnormalities
308 and fertility disorders with severity proportional to the extent of the mutation [3, 22, 25, 43].
309 B10.BR-Y^{del} males are the classic example of mice suffering from the partial Yq deletion.
310 They are fertile, but their sperm has low quality [20-21, 26-31], and their offspring show a
311 considerable sex ratio skew in favour of females [29, 32]. In the present study we demonstrate
312 that transcripts of the multicopy MSYq genes: *Ssty1*, *Ssty2*, *Sly, Srsy, Asty* and *Orly* are
313 present in testes of B10.BR-Y^{del} males, but abundance levels of the last five of these genes are
314 dramatically decreased in comparison with testes of the control B10.BR males. Our
315 quantitative RT-PCR analyses allowed comprehensively characterize the range of the
316 deletion, which has not been done before. This is indirect characterization based on mRNA
317 measurements, but it shows the real effect of the deletion on Y chromosome function in
318 B10.BR-Y^{del} strain, which is crucial for interpretation of all the past and future results.
319 The reduced transcription from the examined MSYq genes is presumably responsible
320 for the numerous irregularities of B10.BR-Y^{del} sperm. On the other hand, the fact that all
321 these genes are still active in gonads of B10.BR-Y^{del} males explains, why the mutants retained

their potential to produce competent spermatozoa, able to fertilize eggs [26, 30-31]. The mouse MSYq region consists predominantly of a half-megabase unit containing all spermiogenic genes. The unit is highly amplified, which causes the genes to be repeated in hundreds of copies along Yq [5]. Undoubtedly, this unique structure of the mouse MSYq minimizes negative consequences of partial Yq deletions. Thanks to the specific organization of MSYq, B10.BR-Y^{del} males saved functional copies of the all genes located in this region, but the diminution of their repeats had to cause tremendous lowering of transcription. *Ssty1* is the only MSYq gene which transcription is unchanged in the mutant strain. Analogical situation characterises XY^{RIII}qdel male mice with a very similar to B10.BR-Y^{del} deletion removing approximately 2/3 of MSYq: *Ssty1* is the only MSYq gene in that model, which expression is not statistically reduced [14]. Taking into consideration the organization of MSYq region, this phenomenon is surprising and difficult to explain without analysis on DNA level. It can be speculated that in the case of *Ssty1* gene, the deletion covered mostly its non-functional copies or that some compensation mechanism works here. The compensation mechanism may include methylation changes of regulatory sequences, as indicated by our microarray analysis. The most differently methylated genomic regions of B10.BR and B10.BR-Y^{del} spermatozoa include just *Ssty1*.

It is postulated that MSYq genes are engaged in intra-genomic conflict with their homologous genes located on X chromosome. This conflict was proved for *Sly* and its X-linked homologs *Slx* and *Slx-like1* (*Slxl1*). Deficiency of *Sly* transcript in gonads of transgenic shSLY males results in an increased level of *Slx/Slxl1* transcripts, various anomalies of sperm differentiation and predominance of females in progeny [8, 16]. Our study shows that reduction of *Sly* transcription in gonads of B10.BR-Y^{del} males to 5% of the control level is also associated with a significant over-expression of *Slx* and *Slxl1* genes. This explains the sex ratio distortion towards females in offspring of B10.BR-Y^{del} males and confirms that *Sly* and

Slx/Slx11 genes are opposite players in the postulated X-Y conflict. Our investigation does not allow extending this X-Y interaction onto *Ssty* and *Srsy* genes - their reduced activity in testes of B10.BR-Y^{del} males is not associated with significant change of transcription from their X-linked homologs. Such result is convergent with XY^{RIII}qdel model, where any dysregulation of *Sstx* were observed, while *Srsx* was not analysed [14].

Heat shock factor 2 (HSF2) is a transcription factor that binds to MSYq region and regulates activity of its multicopy genes. Knockout of *Hsf2* gene causes phenotype which is very similar to this characteristic for mice with 2/3 deletion of MSYq (i.e. decreased expression of *Ssty* and *Sly*, increased expression of *Slx*, production of sperm with frequent head abnormalities) [34]. Hence, we thought it would be interesting to check what happens with expression of *Hsf2* gene when 2/3 of MSYq is deleted. Transcript level of *Hsf2* gene appeared to be unchanged in testes of B10.BR-Y^{del} males showing that there is no relationship between its activity and the number of copies of MSYq genes. This is probably because HSF2 protein does not regulate exclusively Yq region, but also many other genes located on both sex chromosomes and autosomes [44]. Despite no change in the overall activity of *Hsf2* gene in B10.BR-Y^{del} testes, it is possible that some alterations could be detected in direct interaction of HSF2 protein with the reduced MSYq region and it is a promising direction of future studies.

In the second part of the study we focused on explaining origins of phenotypic divergence between genetically the same females sired by B10.BR or B10.BR-Y^{del} males. One of possible mechanisms through which paternal Y chromosome could affect reproductive traits of female progeny is its involvement in establishing epigenetic marks transferred via sperm to the next generation. MSYq genes are expressed predominantly in spermatids (after meiosis), but their products can reach X-bearing cells through intercellular bridges. It was proved that MSYq genes regulate chromatin state in spermatids being involved in such

processes as repression of sex chromosomes, DNA packaging and maintaining its integrity [8, 10-11, 23]. Since repression of sex chromosomes involves a set of epigenetic events, it cannot be excluded that these genes influence also epigenetic modifications at the level of DNA methylation. Sperm DNA methylation could theoretically serve as indirect link between paternal Y chromosome and daughter's phenotype and for that reason it was promising direction of our investigation. DNMT activity in testes of B10.BR-Y^{del} males appeared to be significantly reduced in comparison with testes of B10.BR males, which could indicate that MSYq genes actually participate in controlling DNA methylation process in male germ cells. Our whole-genome methylation analysis of B10.BR and B10.BR-Y^{del} spermatozoa revealed not large but considerable differences between both groups and showed that most of the differently methylated genomic regions (over 70%) are hypomethylated in gametes of mutant males. Majority of genes which were identified to have changed methylation in B10.BR-Y^{del} sperm are expressed in ovary fulfilling important functions, such as regulation of transcription. Since the revealed changes are moderate and not widely spread throughout the genome, we conclude that although the mutated Y chromosome probably influence phenotype of female offspring through DNA methylation pattern of X-bearing sperm, some additional mechanisms must be engaged here. Other chromatin modifications, such as histone retention and positioning [45], as well as non-coding RNA content [46] should be further studied in B10.BR-Y^{del} sperm to fully describe the way in which Yq deletion affects reproductive traits of females from the next generation.

The epigenetic inheritance is not the only factor that can explain the effect of mutated Y chromosome on females in B10.BR-Y^{del} strain. In the offspring of B10.BR-Y^{del} males there is a considerable sex ratio skew in favour of females [29, 32] caused by reduced efficiency of spermatozoa bearing Y^{del} chromosome [42]. From conception until weaning B10.BR(Y^{del}) females develop in feminized environment, which can influence their anatomy, physiology

and behaviour. It was proved, among others, that females developing *in utero* between two females produce first two litters with definite predominance of female pups. In the next litters this effect is weakening [39]. Our assessment revealed that B10.BR(Y^{del}) females, especially in the first two litters, show the significant tendency to have higher proportion of female pups then control B10.BR females (Table 2). This phenomenon indicates that Yq deletion of B10.BR-Y^{del} males can influence phenotype of females from the next generation not only through epigenetic alteration of X-bearing sperm, but also through feminization of pre- and neonatal environment in which they develop. Next studies are needed, however, to confirm this assumption.

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Author contributions

KK, JS conceived and designed the study; KK, AG collected the biological material; KK, AD, AG, PL performed the experiments and data acquisition; KK, MK, MP analysed the data; JS managed the project; KK wrote the paper. All authors read and approved the manuscript.

Declaration of competing interest

The authors report no declarations of interest.

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563

564 Table 1

565 Primer sequences used for quantitative RT-PCR.

gene name	primer sequence (5' to 3')	reference
Actb	forward: GGCACCACACCTTCTACAATG reverse: GTGGTGGTGAAGCTGTAGCC	[8]
Ssty1	forward: CCTCATGAAGAAGAGGAGGA reverse: GTGACAGGCTCATTACCTTC	[14]
Ssty2	forward: CAGGTGCCATTCTTACAGGACTAT reverse: ACCCAGGAACCTATTAAGAAGTCAT	[8]
Sly	forward: CATTTATAAGACGCTTCACATAAAG reverse: ATTCTCCATGATGGCTCTTTC	[8]
Srsy	forward: CTGACCAATGGGACACCAAA reverse: TGTGATGCCTGAGGCTGTCT	designed by authors
Asty	forward: GRGGAGTAGAACTCATCATC reverse: CAGGAGATGACTAACATAGCA	[8]
Orly	forward: TCCCAGTGGTGTATGAAAGG reverse: GCCATTGTCTGATGAAAGTACC	[8]
Sstx	forward: AGCATGGCTCTAAGGACGAG reverse: ACAAGCTCCAGGTCGATGTC	[13]
Slx	forward: TTCAGATGAAGAAGAAGAGCAGG reverse: TCCATATCAAACTTCTGCTCACAC	[8, 17]
Slxl1	forward: TTGGAGGAGGCTCATTCTGAAG reverse: ACGACTTGTTGTTGATCATCTCC	[8, 17]
Srsx	forward: CCTGTCACCCAAGAGGTCAT reverse: TGCCAAAGCTTCTGGAGTCT	designed by authors

Hsf2	forward: AGGGGAGT ACAACTGCATCG reverse: TTACTCTGGGTCGGTTCTGG	[47]

566

567

568 Table 2

569 Percentage of females in all litters born to B10.BR and B10.BR(Y^{del}) females mated with

570 B10.BR or B10.BR-Y^{del} males. For B10.BR(Y^{del}) females the sex ratio in their first two litters

571 was additionally calculated.

type of mating (female × male)	litters examined	no of litters	% of females (± SEM)
B10.BR × B10.BR	all	577	47.93 ± 1.02
B10.BR(Y^{del}) × B10.BR	all	130	52.52 ± 2.07 #
	two first	70	54.09 ± 2.90 #
B10.BR × B10.BR-Y^{del}	all	438	59.03 ± 1.21 **
B10.BR(Y^{del}) × B10.BR-Y^{del}	all	116	63.07 ± 2.30 **
	two first	70	64.31 ± 3.19 ** ^

572

573 ** Sex ratio distortion towards females (P<0.01; chi-square test)

574 # Significantly different from B10.BR × B10.BR (P<0.05; Kolmogorov-Smirnov test)

575 ^ Significantly different from B10.BR × B10.BR-Y^{del} (P<0.05; Kolmogorov-Smirnov test)

576

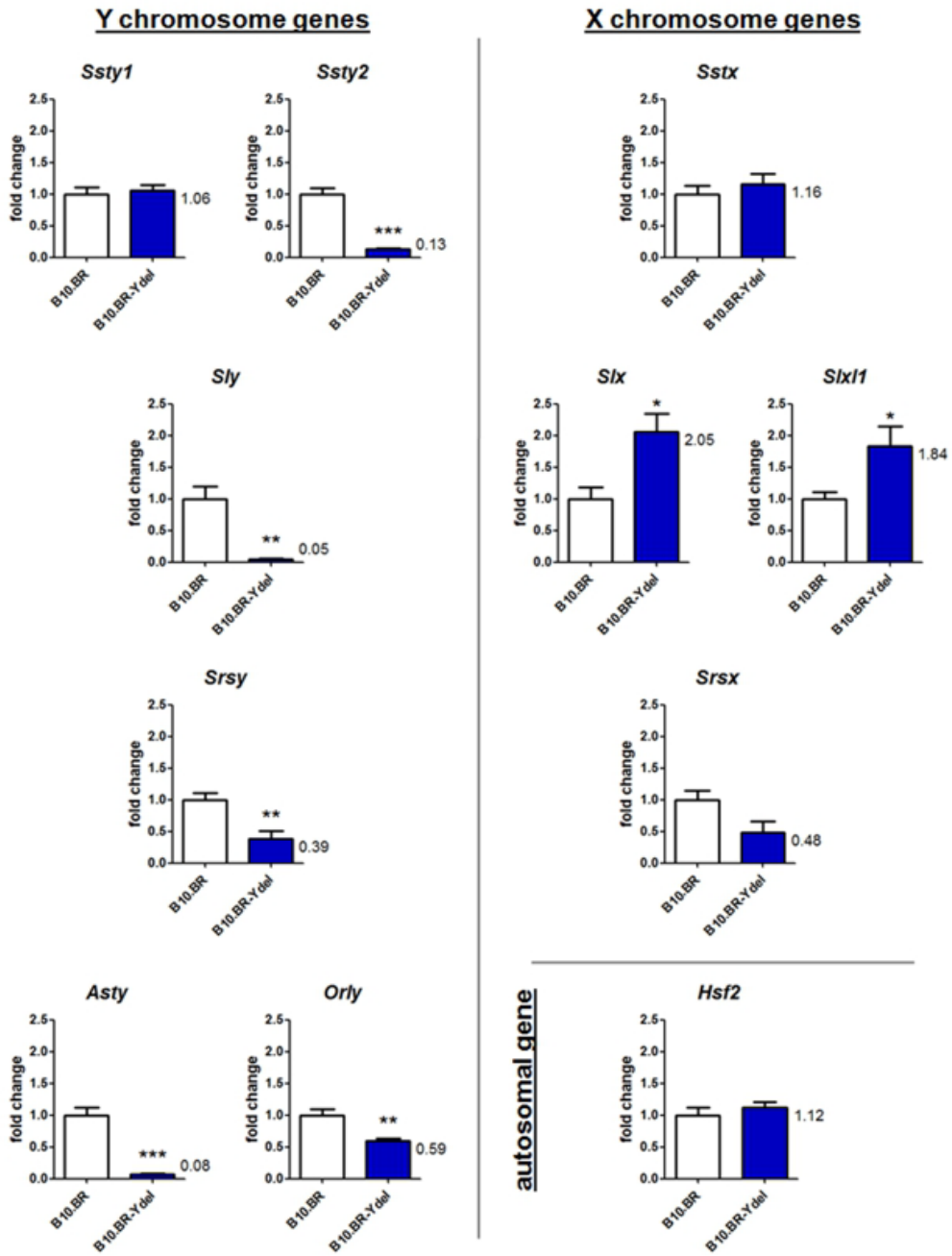

Figure 1

The differences between B10.BR-Y^{del} and wild type B10.BR testes in mRNA abundance levels of MSYq genes, their X-linked homologs and autosomal *Hsf2* gene. Bar graphs summarize the qPCR-based measurement of selected gene expression and present mean fold changes in mutant group over the wild type group with standard errors (n = 4). Results were normalized to *Actb* and analyzed with *t* test. Statistical analysis was performed on $2^{-\Delta CT}$ values. Significant differences are indicated by asterisks (*P<0.05; **P<0.01; ***P<0.001).

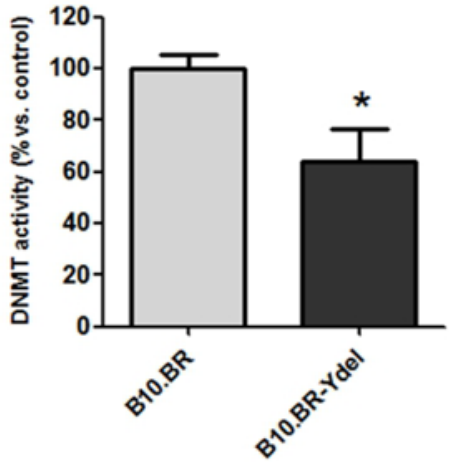

Figure 2

Total DNA methyltransferase (DNMT) activity in B10.BR-Y^{del} testes compared to wild type B10.BR testes (means ± standard errors, n=14 testes in each group). Results were calculated in OD/h/mg and presented as percent of activity of control B10.BR group.

* Significantly different from B10.BR ($P<0.05$; *t* test performed on OD/h/mg values).

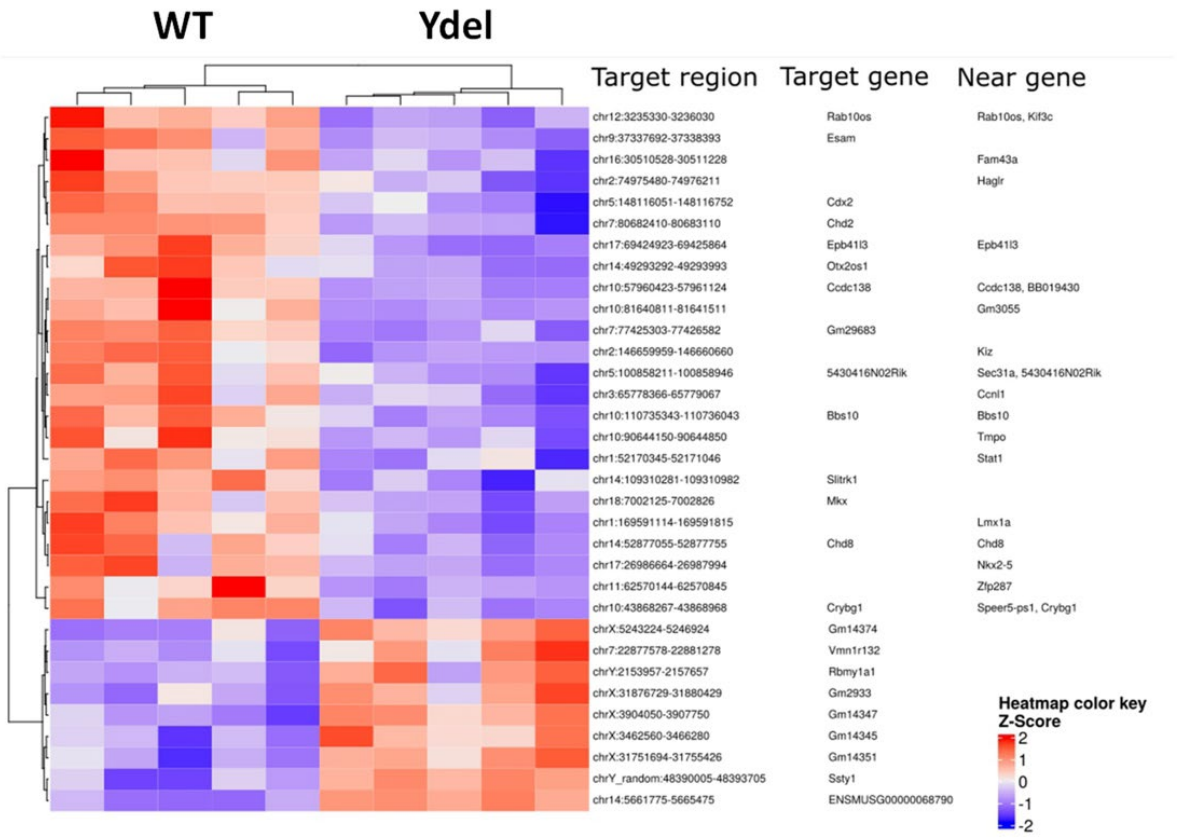

Figure 3

Hierarchical clustering presenting the most differently methylated regions in DNA of sperm produced by B10.BR-Y^{del} (Ydel) and control B10.BR (WT) male mice. Microarray results are shown as a heat map and include 33 genomic regions (*t* test $P<0.01$ and fold of difference >0.3). Colored rectangles represent level of methylation. The intensity of the color is proportional to the standardized values (between -2 and 2) from each microarray, as indicated on the bar below the heat map image. Clustering was performed using Euclidean distance.