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Meiotic behavior of aneuploid chromatin in mouse models of Down syndrome

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Abstract

Aneuploidy, which leads to unpaired chromosomal axes during meiosis, is frequently accompanied by infertility. We previously showed, using three mouse models of Down syndrome, that it is an extra chromosome, but not extra gene dose, that is associated with male infertility and virtual absence of post-meiotic germ cells. Here we test the hypothesis that aneuploid segments are differentially modified and expressed during meiosis, depending on whether they are present as an extra chromosome or not. In all three models examined, the trisomic region lacks a pairing partner, but in one case, spermatocytes have an extra (and unpaired) chromosome, while the two other models involve translocation of the trisomic region rather than an extra chromosome. An extra unpaired chromosome was always modified by phosphorylation of histone H2AX and lacked RNA PolII. But in the case of trisomic regions attached to a paired chromosome, assembly of these protein modifications was affected by the position of a trisomic region relative to a centromere and the physical extent of the unpaired chromatin. Analysis of gene expression in testes revealed that extra copy number alone was not sufficient for meiotic up-regulation of genes in the trisomic interval. Additionally and unexpectedly, presence of meiotic gene silencing chromatin modifications was not sufficient for down-regulation of genes in unpaired trisomic chromatin. Thus the meiotic chromatin modifications that are cytologically visible are unlikely to be directly involved in sterility versus fertility of DS models. Finally, the presence of an extra, unpaired chromosome, but not the presence of extra (trisomic) genes, caused global deregulation of transcription in spermatocytes. These results reveal mechanisms by which an extra chromosome, but not trisomic gene dose, impact on meiotic progress and infertility.

Keywords

aneuploidy; trisomy; meiosis; spermatogenesis; MSUC; MSC1; XY body

Introduction

The relationship between meiotic chromosome pairing and fertility is an intriguing problem; in many organisms meiotic asynapsis leads to spermatogenic arrest, but the mechanisms are unknown (Davisson et al. 2007; Peters et al. 1997). The most notable, and instructive, exception to this detrimental meiotic effect of unpaired chromosomal axes is the XY bivalent, which is only partially paired and occupies a heterochromatic nuclear territory in spermatocytes known as the sex body or XY body. Transcription is suppressed in the XY body and a host of proteins normally associated with heterochromatin, transcriptional silencing, DNA repair, protein turnover and RNA-mediated silencing specifically localize to the XY body. The process by which the unpaired axes of the sex chromosomes accumulate these modifications and become

transcriptionally suppressed is commonly referred to as MSCI (meiotic sex chromosome inactivation), and may be informative about the meiotic fate of unpaired chromatin in general. MSCI shares features with meiotic silencing of unpaired DNA (MSUD), first described in *Neurospora crassa*, a mechanism for transcriptional silencing of unpaired DNA sequences and/or additional copies of genes, as well as their endogenous homologs (Shiu et al. 2001). In mammals, analyses of mice carrying small regions of unpaired autosomal DNA or X:autosome translocations provide evidence that unpaired autosomal chromatin can be transcriptionally silenced and accumulate proteins that are normally restricted to the XY body (Baarends et al. 2005; Ferguson et al. 2008; Turner et al. 2005), a process now generally referred to as meiotic silencing of unpaired chromatin or MSUC (Schimenti 2005). However, chromosomal regions that lack a pairing partner do not always acquire MSUC modifications (Baarends et al. 2005); and, in general, the features that drive meiotic modification of unpaired autosomal axes are not well understood.

The meiotic fate and consequences of unpaired aneuploid chromatin are clinically relevant because human trisomy, most notably Trisomy 21, or Down syndrome (DS), is associated with infertility. The germ line effects of trisomy have been difficult to examine in human patients, but mice bearing segmental trisomies provide unique models to study the fate of unpaired autosomal axes in male meiosis and their impact on fertility. We previously investigated fertility in three mouse models for Trisomy 21 in a study revealing that the presence of an extra chromosome, but not trisomic gene content, was the determining factor for successful spermatogenesis and fertility (Davisson et al. 2007).

Here we use the same three trisomy models to determine mechanisms for this phenomenon. We tested the hypothesis that aneuploid chromosomal segments are differentially modified and expressed during meiosis, depending on whether they are present as an extra chromosome or not. The first known mouse model of DS, Ts(17¹⁶)65Dn (hereafter Ts65Dn), arose from a translocation of distal Chr 16 onto the centromere of Chr 17 (Davisson et al. 1993; Reeves et al. 1995) to form the Ts65 chromosome (Fig. 1). This intact chromosome contains ~13.4 Mb of distal Chr 16, from *Mrpl39* to *Zfp295* (Kahlem et al. 2004), a region that shares conserved synteny with human Chr 21. Male mice carrying this extra chromosome are sterile, and while female Ts65Dn mice are fertile, they produce smaller and fewer litters than chromosomally normal females (Davisson et al. 2007). The other two mouse DS models do not involve an independent chromosome, but are examples of translocation trisomy, differing in both extent and proximity of the trisomic segment to the chromosomal centromere. Like Ts65Dn mice, Ts(16C-tel)1Cje (hereafter Ts1Cje) mice are partially trisomic for much of the region of mouse Chr 16, but unlike Ts65Dn mice, the trisomic segment of Chr 16 is not present as an extra chromosome, but is translocated to the distal end of Chr 12 (Fig. 1) (Huang et al. 1997; Sago et al. 1998). Furthermore, the trisomic segment of Chr 16 in Ts1Cje mice is approximately 8 Mb, ~6 Mb smaller than the trisomic segment present in Ts65Dn. Both male and female Ts1Cje mice are fertile and produce normal litter sizes (Davisson et al. 2007). The third mouse DS model, another example of translocation trisomy, bears the Rb(12.T17¹⁶65Dn)2Cje chromosome (herein called Rb2Cje, but sometimes referred to as Ts2Cje), a Robertsonian fusion of the centromeric end of the Ts65 chromosome with the centromeric end of Chr 12 (Fig. 1). These mice are identical in gene content to the Ts65Dn mice, but do not bear an intact trisomic chromosome (Villar et al., 2005). Similar to Ts1Cje, Rb2Cje mice of both sexes are fertile.

Analysis of these three mouse DS models showed that meiotic modifications and transcriptional silencing of unpaired chromatin is influenced by both the position of the unpaired DNA relative to a paired centromere and by the physical extent of unpaired DNA. Furthermore, the presence of trisomic DNA as an unpaired chromosome causes global

disruption of meiotic gene expression that was not detected in trisomy not involving an extra chromosome.

Materials and Methods

Animals and Genotyping

Trisomy mice were obtained from the Cytogenetic Models Resource at The Jackson Laboratory. Colonies were maintained as described by the Cytogenetics Models Resource (<http://www.jax.org/cyto/>), and all animal care and procedures were approved by the Animal Care and Use Committee of The Jackson Laboratory. To produce mice for this study, animals were maintained on the same background that is used by the Cytogenetics Models Resource. Specifically, B6EiC3Sn-*a/A*-Ts(17¹⁶)65Dn (Ts65Dn) females were mated with [C57BL/6Jei x C3H/HeSnJ] F1 (B6EiC3SnF1) males and B6EiC3Sn-Ts(12<16C-tel>)1Cje (Ts1Cje) and B6EiC3Sn-Rb(12.T17¹⁶65Dn)2Cje/CjeDn (Rb2Cje) mice of both sexes were mated with B6EiC3SnF1.

Ts65Dn and Rb2Cje mice were genotyped using quantitative PCR as described previously (Liu et al. 2003). Ts1Cje animals were genotyped by touchdown PCR using a protocol for detection of the neomycin gene: (http://jaxmice.jax.org/pubcgi/protocols/protocols.sh?objtype=protocol&protocol_id=701).

Immunocytology and FISH

Spread chromatin from spermatocyte nuclei was prepared as previously described (Peters et al. 1997; Reinholdt et al. 2004). Briefly, testes from 16–17 dpp (days post partum) or 19–20 dpp males were detunicated in a drop of minimal essential medium (MEM) on parafilm. Single cell suspensions were prepared in the presence of protease inhibitors (Complete Protease Inhibitor Cocktail, Roche). Cells were pelleted by centrifugation, resuspended in 0.1M sucrose and 20 µl of each cell suspension was immediately dispersed onto a glass slide coated with fixative (1% paraformaldehyde, pH=9.2, 0.1% Triton-X). Nuclei were allowed to settle in a humid slide chamber for 2 hours and then slides were gently washed in a 1:250 dilution of Photo-Flo (Kodak). Slides were air dried and stored at –20°C for up to 1 month.

Immunolabeling of spread chromatin was performed as previously described ((Peters et al. 1997; Reinholdt et al. 2004)). Briefly, slides were washed 3 × 10 min. in 10% ADB (antibody dilution buffer: 10% horse serum, 3% BSA and 0.05% Triton-X in PBS) and incubated with primary antibody in a humid slide chamber for 1 hour at 37°C or overnight at 4°C. Primary antibodies were mouse anti-SYCP3 (Novus, 1:500), rabbit anti-SYCP3 (Novus, 1:500), rabbit anti-γH2AX (Upstate Biotechnology, Lot # 20284, 1:800), or mouse anti-phospho RNAPol II (AbCam, clone H14, phosphoserine 5, 1:250) diluted in ADB. Slides were then washed 3 × 10 min. with 10% ADB and incubated with secondary antibody conjugates. Secondary antibodies were donkey anti-mouse IgG or donkey anti-rabbit IgG, conjugated with Alexa 488, Alexa 594 or Alexa 647 (all at 1:1000, all from Molecular Probes, Invitrogen). Final washes were 3 × 10 min. each in PBS.

For DNA FISH, immunolabeled slides were post-fixed in fresh 1% paraformaldehyde, rinsed with 2X SSC, denatured in pre-warmed 75 – 80°C 70% formamide / 2XSSC for 8 minutes, and hybridized overnight at 37°C with a Cy3 labeled BAC genomic clone. To prepare DNA FISH probes, the Chr 16 genomic clone (2 µg) RPCI-23–212H10 was labeled with Cy3-dUTP by Nick Translation (Amersham) and precipitated with 10 µg salmon sperm DNA and 3 µg of 1 µg/µl mouse Cot-1 DNA by adding 3 volumes of ice cold, 100% ethanol. The precipitated probe was pelleted by centrifugation at 14,000 RPM for 10 minutes. The pellet was washed with 70% ethanol, centrifuged again at 14,000 RPM for 10 minutes, air dried for 20 minutes

and then resuspended in 50 μ l of 100%. During slide denaturation, 10 μ l of labeled probe (~400 ng) was added to 10 μ l of 100% formamide and 5 μ g mouse of 1 μ g/ μ l Cot-1 DNA. To denature the probe, the probe mixture was heated to 70°C in a thermal cycler for 10 minutes. During the last minute of probe denaturation, an equal volume of hot (70°C) 20% dextran sulfate, 2X SSC was added to the denaturing probe with thorough mixing. The denatured probe was placed on ice and then quickly added to hot, denatured slides. The slides were covered with parafilm or coverslips and incubated overnight at 37°C in a dark humid chamber. Post hybridization washes were 10 minutes in 50% formamide/2X SSC at 37°C, 10 minutes in 2X SSC at 37°C and 10 minutes in room temperature 1X SSC. The slides were then dried, mounted with an anti-fade reagent (SlowFade, Invitrogen S2828). Slides were imaged using a Leica DMRXE upright fluorescent microscope, a Micromax cooled charge-coupled device camera, and Metamorph image acquisition software.

Germ cell enrichment

To isolate germ cell RNA, preparations of enriched germ cells were made from the testes of pre-pubertal males (16–17 dpp and 19–20 dpp). Detunicated testes were transferred to a flask containing 10 ml of Krebs-Ringer Buffer (KRB) with dissolved collagenase (0.5 mg/ml). After a 20 min. incubation at 32°C in the presence of 5% CO₂, the tubules were washed 3 times with KRB to eliminate the interstitial cells. After the last wash, 10 ml of fresh KRB containing 0.5 mg/ml trypsin and 1 mg/ml DNase I was added to the tubules. After a 13 min. incubation at 32°C in the presence of 5% CO₂, germ cells were released from the tubules by repeated pipeting with a wide bore transfer pipet. Resulting cell suspensions were filtered through Nitex mesh into a 15 ml conical tube. Cells were washed three times with KRB, 1 mg/ml DNase I. After the last wash, cells were resuspended in 1 ml of KRB (without DNase I), counted on a hemocytometer, pelleted and then frozen at –80°C. For each genotype (Ts65Dn, Ts1Cje and diploid, diploid littermate controls (wild type), three biological replicates were collected for each of two time points: 16–17 dpp and 19–20 dpp. Equivalent numbers of cells from each sample were used for RNA extraction.

RNA extraction and Quantitative RT-PCR

Total RNA was extracted from frozen cell pellets using RNeasy mini columns with DNase treatment according to the manufacturer's instructions (Qiagen). RNA was quantitated using a Nanodrop spectrophotometer (Thermo Fisher Scientific), diluted to 100 ng or 10 ng / μ l, aliquoted and stored at –80°C.

The expression of select genes in enriched germ cell preparations was determined by quantitative RT-PCR with SYBR Green and the standard curve method (Bustin 2002) according to a method previously described (La Salle and Trasler 2006). Briefly, one step RT-PCR and gene specific primers were used to amplify *Mlh1* (*Mlh1*_exon3f; AACTGCAGACTTTTGAGGATTTA and *Mlh1*_exon4r; CATTTCCCATCAGCTGTTTT), *Rbmy1a1* (*Rbmy1a1*_exon4f; CAAGAAGAGACCACCATCCT and *Rbmy1a1*_exon5r; CTCCCAGAAGAACTCACATT), *App* (*App*_exon7f; CCATTTCAGAAAGCCAAAGA and *App*_exon8r; TGGATAACGGCCTTCTTGTCa), *Wrb* (*Wrb*_exon4f; CCTGGTAGCCTTTCCTACTC and *Wrb*_exon5r; ATCCGCTCTTTACCCTATCA) and 18S RNA (18S–F; GCCCTGTAATTGGAATGAGTCCACTT and 18S–R; GTCCCCAAGATCCAACTACGAGCTTT (La Salle and Trasler 2006)) in the presence of SYBR Green according to manufacturers instructions (Quantitect SYBR Green RT-PCR kit, Qiagen). Reaction volumes were 25 μ l and an ABI 7500 Real Time PCR system was used for amplification and detection. Reactions were run in 96 well optical plates that included triplicate reactions for each RNA sample. For test genes, 10 ng of RNA was used as template and for 18S RNA, 100 pg was used. Each 96 well plate included reactions for one test gene and 18S RNA. Each plate also included RNA samples from 19 dpp whole testes to generate standard

curve data for each primer set (test gene or 18S RNA). As mentioned above, three biological replicates were used: the genotypes were Ts1Cje, Ts65Dn and diploid (from wild type littermate controls), and samples collected at 16–17 dpp and 19–20 dpp, for a total of 6 RNA samples for each biological replicate. All results were normalized according to 18S RNA content. Results were expressed as n-fold difference relative to the expression of the test gene in littermate, diploid controls, or the normalized expression value from each representative biological replicate was plotted directly. In each figure, data from one representative biological replicate are shown. For each gene, pairwise comparisons of normalized expression values between each trisomy sample and the diploid control sample were made and JMP software (SAS Institute, Cary, NC) was used to calculate p values by a Dunnett's test.

Microarray analysis

RNA from enriched germ cell preparations was reverse transcribed followed by second strand cDNA synthesis. An *in vitro* transcription (IVT) reaction was carried out incorporating biotinylated nucleotides according to the manufacturer's protocol for the Illumina® Totalprep RNA amplification kit. (Ambion, Austin TX). 5.0 µg biotin-labeled cRNA was then hybridized onto an Illumina Mouse-6 v1.1 BeadArray (Illumina, San Diego CA) for 16 hours at 55°C. Post-hybridization staining and washing were performed according to manufacturer's protocols (Illumina). BeadChips were then scanned using Illumina's BeadStation 500 scanner. Images were checked for grid alignment and quantified using the BeadStudio software. A first pass read of the control summary graphs generated by BeadStudio was the quality control for hybridization, washing stringency and background.

Probe intensity levels for each bead type were generated by the Illumina Bead Studio software. Intensity histograms, box plots, scatter plots, and MA plots were generated with the Bioconductor R/BeadArray package (<http://www.bioconductor.org>) to detect anomalies in the array data. After these preprocessing diagnostics were performed, expression values for each probe were computed by log transforming the intensities and then quantile normalizing the transformed values to equalize their distribution across all arrays. All statistical tests for detecting differentially expressed probes between sample groups were performed with a modified *t* statistic incorporating shrinkage estimates of variance components from within the R/MAANOVA package (Cui et al. 2005; Yang and Churchill 2007). Statistical significance levels for each test were calculated by permutation analysis and were adjusted for multiple testing using the false discovery rate method, *q*-value (Storey 2002). Differentially expressed probes were declared at a false discovery rate threshold of 0.1 for each comparison, unless otherwise noted. Probes were annotated to genes using information provided by Illumina and to chromosome locations by mapping each probe to the National Center for Biotechnology Information Build 36 genome using BLAT (Kent 2002). Both Ingenuity Pathways Analysis software (Ingenuity Systems Inc.) and the DAVID Bioinformatics Resource Functional Annotation tool (NIAID, NIH) (Dennis et al. 2003) were used for pathways analysis and functional annotation of gene lists. All microarray data have been deposited to the Gene Expression Omnibus, accession number GSE13123, and are also available at The Jackson Laboratory's instance of caArray (<http://caarray.jax.org:38080/carray>).

Results

Meiotic modifications to unpaired trisomic chromatin

We first determined if unpaired trisomic autosomal chromatin is subject to chromatin modifications associated with MSUC. We studied this in the first wave of spermatogenesis, which is synchronous; in the strains we used, at 16–17 dpp, the majority of spermatocytes are in the early pachytene stage, while at 19–20 dpp, the majority of spermatocytes are in the late pachytene and diplotene stages of meiotic prophase. Microspread chromatin was

immunolabeled with an antibody raised against histone H2AX phosphorylated on Ser139 (γ H2AX), a protein required for MSCI in mammalian spermatocytes (Fernandez-Capetillo et al. 2003; Turner et al. 2004) (Fig. 2A). Like the XY chromatin, the unpaired Ts65 chromosome was modified by γ H2AX in pachytene spermatocytes (Fig. 2C and 2E). Additionally, the Ts65 chromosome was often in spatial proximity to the XY body, as previously observed (Table 1 and (Davisson et al. 2007)). Modification of the Ts65 chromosome with γ H2AX was observed whether or not it was associated with XY body (Fig. 2C and 2E). To test whether modification of the Ts65 chromosome reflects trisomy (gene content) or chromosomal context (unpaired intact autosome), microspread Ts1Cje (Fig. 1) nuclei from age-matched males were also examined. In Ts1Cje spermatocytes, the trisomic segment of Chr 16 at the distal end of Chr 12 does not accumulate γ H2AX (Fig. 2G). Localization of RAD51 and SUMO1, proteins / modifications that are normally restricted to the XY body during pachynema (Barlow et al. 1997) (Rogers et al. 2004), behaved similarly to γ H2AX in these models (data not shown). Although possible that modification might not be visible on the smaller trisomic segment of Ts1Cje spermatocytes, this observation suggests that meiotic modification of Ts65 chromatin is not an inevitable response to trisomy.

To determine if γ H2AX modification of the Ts65 chromosome is associated with cytological evidence for transcriptional silencing, spermatocytes from Ts65Dn and Ts1Cje males were immunolabeled with an antibody against the active phosphorylated form of RNA Pol II, a transcription enzyme. As expected, the XY chromatin of Ts65Dn, Ts1Cje and wild type, diploid spermatocytes was depleted of RNA Pol II (Fig. 2B–H). In addition, the Ts65 chromosome was also depleted of RNA Pol II when in proximity to the XY body (Fig. 2D, F), but not consistently depleted of RNA Pol II when not in proximity to the XY body. Possibly as a result of its frequent association with the Ts65 chromosome, the normal Chr 16 bivalent in 19–20 dpp Ts65Dn spermatocytes was often close to or associated with the XY body (75%, n=50), and in these cases the distal end of the Chr 16 bivalent (as determined by the cytological position of the BAC used in this study) was always depleted of RNA Pol II (Fig. 1D, inset). In wild type diploid spermatocytes, the Chr 16 bivalent was rarely associated with the XY body (<1% of nuclei, n=50), suggesting that the presence of the Ts65 chromosome in Ts65Dn spermatocytes disrupts the spatial organization of bivalent chromosomes during pachynema. Finally, in Ts1Cje spermatocytes, where the trisomic segment of Chr 16 is attached to a synapsed autosome, no depletion of RNA Pol II was observed (Fig. 2H). Together, these observations provide evidence that the unpaired intact Ts65 chromosome is subject to meiotic modification and transcriptional repression, while a similar, but smaller, unpaired trisomic segment attached to a fully paired autosome (Chr 12) in Ts1Cje spermatocytes is not.

Association with a fully paired autosome is not sufficient to suppress structural modification of unpaired trisomic chromatin

The lack of γ H2AX localization and normal distribution of RNA Pol II in the unpaired trisomic segment in Ts1Cje spermatocytes may be related to the size of the region, to its association with a fully paired autosome, and/or to its position with respect to a paired centromere. To test these possibilities, we examined a third trisomy mouse model of Down syndrome, Rb2Cje, which carries a Robertsonian fusion between the Ts65 chromosome and Chr 12 (Fig. 1). In this case, the unpaired segment of Chr 16 is identical in size and gene content to that of Ts65Dn (as determined by quantitative PCR and DNA FISH by Villar et al., 2005), but, importantly, the unpaired trisomic segment in Rb2Cje spermatocytes is translocated to proximal end of the Chr 12 bivalent, near the paired centromeres (in contrast to Ts1Cje spermatocytes, where the trisomic segment is at the distal end of the Chr 12 bivalent). Unlike the smaller trisomic segment in Ts1Cje spermatocytes, the ~16 Mb unpaired segment in Rb2Cje spermatocytes was readily visible by light microscopy (Fig. 3A and 3B) and frequently associated with the XY body, an association more frequent in 19–20 dpp spermatocytes (Table 1, Fig. 3D). The unpaired Rb2Cje

trisomic segment was always modified by γ H2AX regardless of its position in the nucleus (Fig. 3C and 3D), but was associated with RNA Pol II only when not adjacent or within the XY body (Fig. 3E and 3F). Therefore, lack of modification of the trisomic region in Ts1Cje spermatocytes is likely related either to its distal position with respect to the Chr 12 centromere and/or its smaller size.

Molecular evidence for both transcriptional silencing and gene expression from trisomic chromatin

Our cytological data provided evidence that the unpaired trisomic chromatin in Ts65Dn spermatocytes and in Rb2Cje spermatocytes are transcriptionally silenced, especially when in proximity to the XY body, while the Ts1Cje trisomic segment, which is smaller, attached to the distal end of a paired autosome and never in proximity to the sex body, is not silenced. However, because cytological data are not quantitative, we tested for silencing at the molecular level by using quantitative RT-PCR (qRT-PCR) to compare expression of trisomic genes in wild type spermatocytes (2 copies of the trisomic region) versus age matched Ts65Dn (3 copies, with one exhibiting cytological evidence of silencing) and Ts1Cje (3 copies, with no cytological evidence of silencing). Germ cells were isolated from 16–17 dpp (enriched for cells in early pachynema) and 19–20 dpp (enriched for cells in late pachynema) testes. The expression of *Wrb*, a gene in the Ts65Dn and Ts1Cje trisomic intervals, was compared to that of two control genes, *Rbmyl1a1* (a gene on the Y chromosome that is normally transcriptionally repressed during MSCI at 19–20 dpp) and *Mlh1* (a gene on Chr 9 required for meiotic recombination and expressed in germ cells throughout zygonema and pachynema). Transcriptional repression of the trisomic Ts65 chromosome would be revealed if *Wrb* expression in Ts65Dn spermatocytes was equivalent to its expression in diploid wild type littermate controls, and indeed, expression of *Wrb* in Ts65Dn spermatocytes was not significantly different from that in wild type spermatocytes ($p > 0.05$) (Fig. 4A and B). This molecular evidence therefore supports the cytological evidence for silencing of the Ts65 chromosome. In contrast, the expression of *Wrb* was significantly higher (~ 1.5 fold, $p < 0.05$) in Ts1Cje spermatocytes at 19–20 dpp relative to wild type, diploid spermatocytes (Fig. 4B), evidence that supports the cytological observation of active RNA Pol II localization to trisomic chromatin in Ts1Cje. However, in contrast, and as expected, expression of the non-trisomic, germ cell specific *Mlh1* gene was not significantly different among Ts65Dn, Ts1Cje and wild type, diploid spermatocytes spermatocytes ($p > 0.05$) (Fig. 4A and B). Interestingly, expression of *Rbmyl1a1* in both Ts65Dn and Ts1Cje spermatocytes was significantly lower than in wild type, diploid spermatocytes at 16–17 dpp ($p > 0.05$), but not at 19–20 dpp (Fig. 4C), which could suggest suppression of *Rbmyl1a1* in the trisomic spermatocytes compared to wild type at 16–17 dpp.

Presence of an extra, unpaired autosome, but not trisomy *per se*, causes global misregulation of meiotic gene expression

Previous work with trisomy mouse models has shown that genes present in three copies are generally upregulated by 1.5 fold in somatic tissues; however, there are exceptions to this generalization (Kahlem et al. 2004; Lyle et al. 2004; Prandini et al. 2007). Our qRT-PCR data supported this generalization for Ts1Cje spermatocytes, but not in Ts65 spermatocytes where the extra chromatin is subject to modification. Yet, our qRT-PCR data shed light on only one gene in the trisomy interval. Thus microarray analysis was conducted on germ cells isolated from 16–17 dpp testes (enriched for cells in early pachynema) and 19–20 dpp testes (enriched for cells in late pachynema). Three biological replicas for each genotype (Ts65Dn, Ts1Cje and diploid (wild type) littermate controls) were analyzed at each time point (16–17 dpp and 19–20 dpp).

Microarray analysis revealed global misregulation of gene expression in aneuploid Ts65Dn spermatocytes compared to both diploid (wild type) littermate spermatocytes (Supplemental

Fig. 1) and trisomic Ts1Cje spermatocytes (see below). At 16–17 dpp, 179 genes were differentially expressed between Ts65Dn spermatocytes and wild type spermatocytes (1.5 fold or more, $q \leq 0.1$; Supplemental Fig. 1A). Among these genes, six (*Nbam1*, *Tiam1*, *Mrap*, *Donson*, *App* and *Ifngr2*) are located in the Ts65Dn trisomic region and all six were up-regulated 1.5 fold or more compared to expression in wild type spermatocytes. Because this was unexpected in view of the cytological and qRT-PCR data above, this result was validated by qRT-PCR to determine the amount of *App* transcript in wild type and Ts65 spermatocytes at both time points, which confirmed significant up-regulation of *App* in Ts65Dn spermatocytes (Supplemental Fig. 2). This result suggests that despite evidence of MSUC-promoting modifications, some trisomy genes escape silencing in Ts65Dn spermatocytes, although alternative possibilities are that some trisomy transcripts synthesized prior to MSUC could be stabilized, or endogenous Chr 16 loci may be up-regulated.

Among the 179 genes that were significantly differentially expressed at 16–17 dpp, genes associated with cell stress (specifically endoplasmic reticulum stress) were over-represented. In addition, among the most significantly up-regulated genes were *Casp9* (8 fold), a known pro-apoptotic gene, as well as *Psmc1* (13.8 fold) and *Gabbr1* (3.2 fold), also associated with pro-apoptotic pathways (Cheng et al. 2007; Huang et al. 1997). These data indicate that cellular responses to trisomy (reflected by the presence of transcripts related to cell stress and cell death) occur before any observable defects in spermatogenesis or apoptosis (which are not histologically apparent at 16–17 dpp in Ts65Dn testes; results not shown).

At 19–20 dpp, approximately 2,907 genes were significantly differentially expressed between Ts65Dn and wild type spermatocytes from littermates (1.5 fold or more, $q \leq 0.1$; Supplemental Fig. 1B). Pathways analysis revealed that among up-regulated genes, there was over-representation of genes known to be associated with apoptosis and cell death. In addition, among down-regulated genes, over-representation of genes known to be associated with spermatogenesis, gametogenesis and reproduction was observed. These data are consistent with histological evidence for lower numbers of germ cells in 19–20 dpp testes and subsequent failure of spermatogenesis (Davisson et al. 2007). In addition to global mis-regulation of gene expression, 43 of the ~200 genes in the Ts65Dn trisomic interval were significantly differentially regulated by 1.3 fold or more at 19–20 dpp ($q \leq 0.1$, Fig. 5), providing evidence that over-expression of trisomy genes in these spermatocytes occurs despite cytological evidence for MSUC.

In contrast to the striking and global misregulation of gene expression in Ts65Dn spermatocytes, analysis of translocation trisomy Ts1Cje samples revealed only a few genes (24 genes, $q \leq 0.1$) with significant expression differences compared to wild type samples at either 16–17 dpp (Fig. 6A) or 19–20 dpp (Fig. 6B). No genes in the trisomic region were found among these. However, when the statistical cutoff was set at $q < 0.25$, 8 Chr 16 trisomy genes (including *Dyrk1a*, *Zfp367*, *Chaf1b*, *Sfrs15*, *Setd4*, *2610039C10Rik*, *Mrap*) were up-regulated by 1.2 fold or more and one (*Son*) was down regulated by approximately 1.5 fold at 19–20 dpp. These data were for the most part consistent with the qRT-PCR data showing that a gene in the trisomic region, *Wrb*, was up-regulated in Ts1Cje spermatocytes (the microarray data for *Wrb* also support up-regulation by 1.2 fold, although the q value was too high to be considered significant). However, because no cytological meiotic modifications to the unpaired segment in Ts1Cje spermatocytes were observed, it was surprising to find a down-regulated gene. It is possible that chromatin modifications of the trisomy region in Ts1Cje spermatocytes were simply below the detection threshold for immunocytology. However, if the trisomy region in Ts1Cje spermatocytes is subject to silencing (as the microarray data for *Son* would suggest), its effects are limited. Taken together, these microarray data provide evidence for gross misregulation of gene expression in Ts65Dn spermatocytes with an intact extra (trisomic) chromosome. However, copy number and absence of immunocytologically detectable meiotic

chromatin modifications do not accurately predict trisomic gene expression levels in Ts1Cje translocation trisomy, where the trisomic segment is part of another, paired, chromosome.

Discussion

This analysis of mouse trisomy models for DS has revealed mechanisms by which an extra chromosome, but not trisomy for the same region, adversely impacts on spermatogenesis and male fertility (Davisson et al. 2007). Although in all three models examined, the trisomic region of Chr 16 lacks a pairing partner, the important distinction among the models is that Ts65Dn cells have an extra (and unpaired) chromosome, while the two models of translocation trisomy (Ts1Cje, Rb2Cje) do not involve an extra chromosome. These features allowed an assessment of requirements for meiotic modification of unpaired chromatin. Like the unpaired chromatin in the XY body, γ H2AX is always immunocytoologically detectable in association with an extra chromosome (Ts65) at pachynema, however the extra chromosome not always depleted of RNA Pol II, especially when it is not associated with the XY body, and, consistent with this observation, some trisomic genes are expressed, while others are silenced. In contrast, when the same or a smaller region of trisomy is incorporated into another autosome, the chromatin is not always meiotically modified or silenced. Thus pairing status alone does not always predict chromatin modifications or gene silencing, and these data suggest that the meiotic modification and gene silencing processes are more effective at the whole-chromosome level. Additionally, the presence of an extra chromosome leads to global deregulation of meiotic gene expression not detected when the trisomic region is present on another chromosome that is otherwise fully paired.

Modification of unpaired chromatin in trisomy spermatocytes

During male meiosis, unpaired autosomal chromosome segments are subject to protein modifications and transcriptional silencing by MSUC, and similarly the unpaired regions of the X and Y chromosomes are subject to modifications and silencing by MSCI. While it is not known if MSUC and MSCI proceed by the same mechanism, chromatin modifications characteristic of each process are similar. The trisomy models examined here allowed us to determine how these epigenetic modifications can be influenced by 1) presence of an extra unpaired chromosome, 2) nuclear domain organization (proximity of the unpaired chromatin to the XY domain), 3) size of the unpaired region, and finally, 4) relationship of the unpaired region to a paired centromere.

The Ts65Dn model allowed us to test the first two issues. First, the MSUC-related protein modification we analyzed was a consistent feature of the extra, unpaired chromosome (Ts65) chromosome regardless of its sub-nuclear location. Is this always the case? This is a difficult question to answer because of lack of appropriate genetic models, but a recent study (Mahadevaiah et al. 2008) sheds some light on this; interestingly, when an intact unpaired chromosome is derived from another species, meiotic modification may not occur. In this study, a transchromosomal DS model (O'Doherty et al. 2005) carrying one copy of human chromosome 21 was examined. In contrast to our observation of consistent MSUC-like modifications on the Ts65 chromosome, the human Chr 21 in mouse spermatocytes was not always modified; it showed evidence of MSUC only when it was associated with the sex body. Does this mean that the epigenetic modification process is in some way sensitive to species-specific features of the chromosome (e.g., the centromere)? This is not yet known. However, this intriguing observation raises the second issue of nuclear domain influence. We found that although the Ts65 chromosome was consistently modified by γ H2AX, cytological evidence for transcription (presence of active RNA Pol II) depended on the proximity of the unpaired chromosome to the XY body (nuclear domain organization). This XY-association was particularly evident by late pachynema (Table 1). Similar co-localization of aberrant

chromosomes to the repressive XY domain has been observed in mouse chromosomal rearrangements causing infertility (de Boer and de Jong 1980), including another autosomal translocation between Chrs 16 and 17 (Homolka et al. 2007), as well as in azoospermic human translocation carriers (Sciurano et al. 2007). Thus, an intact and unpaired chromosome acquires proteins modifications characteristic of MSUC, but its transcriptional status may also depend on nuclear domain organization. Indeed, chromosomal aberrations may themselves modify nuclear three-dimensional organization (Garagna et al. 2001).

The possibility that MSUC-related chromatin modification of unpaired chromatin is related to size of the unpaired chromatin region and/or its relationship to contiguous paired chromosomal segments was tested by analysis of the two DS models that involve chromosomal translocations rather than the presence of an extra chromosome. The structural modification always characteristic of the Ts65 chromosome were not found on the smaller trisomic segment of Chr 16 in Ts1Cje spermatocytes, but were consistently present on the trisomic segment in Rb2Cje spermatocytes, where the segment is the same size as the Ts65Dn chromosome. Thus size of the unpaired chromosomal segment could be important in determining its recognition for epigenetic modifications. Work of Baarends et al. also supports the idea that the size of an unpaired chromosome segment could influence modification, showing that in double translocation heterozygous mice, T(1;13)70H/T(1;13)1Wa, the potentially unpaired region of approximately 10 cM (roughly corresponding to 20 Mb) shows varying degrees of synapsis and modification in spermatocyte nuclei (Baarends et al. 2005). Generally, when the unpaired region is visible by standard light microscopy as an unpaired SYCP3-positive axis, it is modified and transcriptionally repressed. But when the unpaired DNA in T(1;13)70H/T(1;13)1Wa spermatocytes undergoes heterologous synapsis or partial heterologous synapsis and is not visible as an unpaired SYCP3-positive axis (as in the case of Ts1Cje), it does not show evidence of MSUC-promoting modifications (Baarends et al. 2005). However, countering this argument, the size of the trisomic segment in Ts1Cje (approximately 8 Mb) is considerably larger than the unpaired region of the Y chromosome (approximately 1.5 Mb), where MSC1-promoting modifications are consistently observed, rendering it unclear that size plays a significant role in meiotic modification of unpaired chromatin. Nonetheless, it should also be noted that the small unpaired region of the Y chromosome is associated with the XY body and may be subject to modification by a spreading effect. However, as suggested by analysis of double translocation heterozygous mice (Baarends et al. 2005), the relationship of the unpaired segment to the adjacent paired chromatin and/or opportunity for heterologous synapsis may influence epigenetic modification of the unpaired region. In the translocation trisomy models analyzed, the small trisomic region in Ts1Cje is located distal to the centromere, but the trisomic region in Rb2Cje is adjacent to the paired Chr 12 centromeres. Thus, the unpaired Ts1Cje trisomic segment, by virtue of its association with the distal telomeric end of the fully paired Chr 12, may undergo heterologous synapsis and not be modified. Alternatively, it may be subject to self synapsis and therefore escape modification. In contrast, the association of the unpaired segment in Rb2Cje spermatocytes with the proximal end of the Chr 12 bivalent places this segment adjacent to paired centromeres, which could act as a barrier to heterologous or self synapsis (which is thought to occur by spreading of synaptonemal complex assembly), thus leading to MSUC-related chromatin modification on this un-synapsed trisomic region.

Trisomic and global gene expression in trisomy spermatocytes

The DS models studied here allowed us also to assess transcriptional effects as a possible mechanism for puzzling trisomy-related fertility effects: infertility caused by an extra chromosome in contrast to fertility of mice with trisomic gene content. We determined meiotic gene expression, both at the level of trisomic genes and globally.

Analysis of genes in the Chr 16 trisomy region revealed that even when the trisomic chromatin is subject to cytologically visible MSUC-related protein modification, transcriptional silencing in the unpaired region is not complete. Still, the expression of genes in the region never consistently reached levels predicted by the trisomic condition. This is not unexpected since recent work with tissues from Ts65Dn mice and human DS individuals has showed that many trisomy-region genes are under-expressed in somatic cells compared to diploid levels, while others are over-expressed, above the predicted 1.5 fold (Kahlem et al. 2004; Lyle et al. 2004; Prandini et al. 2007). In Ts65Dn spermatocytes, at least one trisomic gene (*Wrb*) was expressed at a level equivalent to diploid, as would be expected from cytological evidence of MSUC. However, in unexpected contrast, several genes were up-regulated by 1.5 fold or more, e.g., the *App* gene in the trisomic region was up-regulated by ~2.5 fold. This could be due solely to the presence of the extra copy or to transcriptional up-regulation of the normal Chr 16 loci; these two mechanisms could not be discriminated in our analysis. Furthermore, up-regulation of specific loci could be part of a generalized physiological response to low germ cell numbers or arrested spermatogenesis (although germ cell loss and histological evidence of arrested spermatogenesis is not apparent until 19–20 dpp). For example, microarray analysis of gene expression in *Spo11*-deficient testes, where germ cells arrest in meiotic prophase, showed that *App* is up-regulated (Smirnova et al. 2006). This interpretation is reinforced by the observation that in contrast to Ts65Dn spermatocytes, there are no significant changes in expression levels of trisomy genes compared to the diploid level of expression in Ts1Cje translocation trisomy spermatocytes. Although this is not consistent with the lack of modification of the trisomic chromatin (which would predict up-regulation), it may reflect that fact that spermatogenesis is not disrupted. From these observations, it is clear that the presence of MSUC-related modifications is not sufficient to cause silencing of all trisomic region genes and that an additional copy of a gene is not sufficient to cause 1.5 fold up-regulation of trisomic genes. Additionally, meiotic gene expression patterns may reflect physiological aspects of meiotic arrest.

This concept of aberrant gene expression may be better appreciated in the context of the observed genome-wide mis-regulation of gene expression that was observed in Ts65Dn spermatocytes, but not in Ts1Cje spermatocytes (Fig. 6 and Supplemental Fig. 1). Thus, copy number may be an important determinant of gene expression in trisomic cells, but cell physiology is also. Evidence suggests that the physiology of aneuploid cells is aberrant; for example, a systematic survey of aneuploid yeast strains revealed consistent up-regulation of cell-stress pathways, independent of the identity of the extra chromosome in each different strain (Torres et al. 2007). This generalization is true across many species and may reflect loss of cellular fitness due to protein stoichiometry imbalances (Torres et al. 2008). These data on meiotic gene expression support a growing body of evidence that changes in gene expression resulting from copy number alone in aneuploid cells or tissues is not sufficient to fully explain the phenotypic consequences of trisomy (Prandini et al. 2007; Torres et al. 2007). However, whether mis-regulated gene expression is a cause or consequence of impaired spermatogenesis is not yet clear. Clearly, mis-regulation of even a few genes, especially key transcription factors, could lead to drastic effects on expression of down-stream genes and by extension, spermatogenesis. Alternatively, it is possible that the presence of an unpaired chromosome may interfere with nuclear epigenetic events, such as histone transitions (van der Heijden et al. 2007), it is also possible that physiology of Ts65Dn spermatocytes might be profoundly modified, reflected by the elevated expression of cell stress and cell death genes.

In conclusion, these useful DS models reveal that copy number alone is not sufficient for up-regulation, and that MSUC-promoting modifications are not sufficient for down-regulation of genes that reside in unpaired trisomic chromatin during meiosis. Because cytologically detectable meiotic modifications (γ H2AX and absence of active RNA Pol II) are not consistently observed on trisomic chromatin and do not fully predict gene expression, these

alone probably do not determine fertility or sterility of trisomic males. Global disruption of gene expression, caused by the presence of an extra chromosome (but not by trisomic gene content) may instead be the mechanism underlying infertility of trisomic males bearing an extra chromosome versus the fertility of trisomic males not bearing an extra chromosome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Diagrammatic representation of trisomy in three Down syndrome mouse models: Ts(17¹⁶)65Dn (Ts65Dn), Rb(12.T17¹⁶65Dn)2Cje (Rb2Cje) and Ts(16C-tel)1Cje (Ts1Cje). Each of these models is trisomic for the portion of mouse Chr 16 that shares conserved synteny with human Chr 21. Chr 12 and the trisomic segments of Chr 16 are shown in black and red, respectively. Blue represents the Chr 17 centromere. Ts65Dn mice have a small (13.4 Mb) supernumerary chromosome that is the product of a translocation between the Chr 17 centromere and the distal end of Chr 16, which shares conserved synteny with human Chr 21. Rb2Cje mice carry the product of a Robertsonian fusion between the small Ts65 chromosome and the centromeric end of Chr 12. Ts1Cje mice carry the product of a serendipitous fusion between a smaller region (compared to the Ts65 chromosome) of distal Chr 16 (8 Mb), with the distal end of Chr 12.

Figure 2.

Localization of γ H2AX (blue) and RNA POLII (green) in pachytene stage spermatocytes from trisomy mice (Ts65Dn and Ts1Cje) and from diploid littermates (WT). DNA FISH with a Chr 16 BAC probe (red) was used to visualize Chr 16, the Ts65 chromosome and the trisomic Chr 16 segment (16C-tel) in Ts1Cje. In Ts65Dn spermatocytes, the Ts65 chromosome is always associated with the Chr 16 bivalent (E, arrows) and/ or with the XY body (C, D, F). In diploid pachytene spermatocytes, γ H2AX (blue) specifically localizes to the XY body (A). In Ts65Dn pachytene spermatocytes, γ H2AX (blue) localizes to the Ts65 chromosome whether or not the Ts65 chromosome is associated with the sex body (C, E). In diploid pachytene spermatocytes, the XY body and the chromocenters are depleted of RNA POLII (green, B). The Ts65

chromosome is also depleted of RNA POLII (green, D, E). In Ts1Cje spermatocytes, γ H2AX (blue) does not associate with the trisomic segment of Chr 16 (G) and the trisomic segment is not depleted of RNA POLII (green, H). 100X objective.

Figure 3.

Modification of the unpaired, trisomic segment of Chr 16 in Rb2Cje spermatocytes. Inverted anti-SYCP3 images of a Ts1Cje and Rb2Cje spermatocyte nuclei (A, B respectively). The unpaired segment (16C-tel) in the Ts1Cje spermatocyte nucleus is not visible by standard light microscopy (DNA FISH was used to identify 16C-tel in this spermatocyte) (A). In contrast, the unpaired segment (Ts17¹⁶) in an Rb2Cje spermatocyte nucleus is visible (B). The unpaired segment in Rb2Cje spermatocytes is modified by γ H2AX regardless of its association with the sex body (C, D, arrows) and is associated with RNA Pol II only when it is not associated with the sex body (E, F, arrows). 100X objective.

Figure 4.

Quantitative RT-PCR profiling of *Mlh1* *Wrh* (A, B) and *Rbmy1a1* (C) in enriched spermatocyte preparations from diploid and trisomy testes. The normalized expression value of each gene in Ts65Dn, Ts1Cje and diploid spermatocytes (WT) is plotted directly. The expression was determined for each gene in triplicate samples for each of three biological replicates for each genotype and time point. Values represented here are mean values from triplicate samples from one representative biological replicate. Mean $\pm$ SD (n = 3 experimental replicates). Bars within a panel marked with the same letter represent expression values that are not significantly different, while those marked with different letters are different represent expression values that are significantly different ($p < 0.05$).

Figure 5. Expression of genes within the Ts65Dn trisomy interval (as defined by (Kahlem et al. 2004); Chr 16: ~84,700,000 bp – 98,100,000 bp; or *Mrpl39* – *Znf295*) that showed significant ($q \leq 0.1$) differential expression compared to that of wild type, diploid (WT) enriched spermatocyte preparations from 19–20 dpp testes.

Figure 6. Expression of all genes that showed differential expression ($q \leq 0.1$) in Ts1Cje enriched spermatocyte preparations compared to that from wild type, diploid (WT) littermates at 16–17 dpp and 19–20 dpp

Table 1

Distribution of γ H2AX and DNA FISH signals in 16–17 dpp and 19–20 dpp pachytene spermatocytes from trisomy mice. In Ts65Dn and Rb2Cje spermatocytes, the unpaired trisomic segments were modified by γ H2AX. In both cases, when the trisomic segment (as visualized by DNA FISH) was not associated with the XY body, 2 γ H2AX regions were found. Likewise, when the unpaired trisomic segment was associated with the XY body, only one γ H2AX region was found. In Ts1Cje and wild type diploid spermatocytes (WT), one γ H2AX region (the XY body) was consistently observed. The trisomic segment in Ts1Cje spermatocytes, (as visualized by DNA FISH), was not modified by γ H2AX and was not found in association with the XY body.

	16–17 dpp (early pachynema, n = 50)	19–20 dpp (late pachynema, n = 50)
Ts65Dn		
1 γ H2AX region	54%	96%
2 γ H2AX regions	46%	4%
Rb2Cje		
1 γ H2AX	48%	90%
2 γ H2AX regions	51%	10%
Ts1Cje and B6EiC3Sn		
1 γ H2AX region	100%	100%
2 γ H2AX regions	0%	0%