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Research

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**LRFN5 locus structure is influenced by the individual's sex and associated with autism**

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ABSTRACT

Background: *LRFN5* is a brain-specific gene needed for synaptic development and plasticity. It is the only gene in a large 5.4 Mb topologically associating domain (TAD) on chromosome 14, which we term the *LRFN5* locus. This locus is highly conserved, but has extensive copy number variation.

Methods: Locus structure was studied by chromatin immunoprecipitation (chIP-on-chip) in fibroblasts from individuals with autism and controls, supplemented with a capture-HiC determination of TAD structures in a family trio. *LRFN5* expression was studied in foetal brain cell cultures. In addition, locus interaction was studied in four large and independent cohorts by measuring deviations from Hardy-Weinberg equilibrium of a common deletion polymorphism.

Results: We found that locus structural changes are associated with developmental delay (DD) and autism spectrum disorders (ASD). In a large family, ASD in males segregated with a chromosome 14 haplotype carrying a 172 kb deletion upstream of *LRFN5*. In a fibroblast capture-HiC study on an ASD-patient-parent trio, the ASD-susceptible haplotype (in the mother and her autistic son) had a TAD pattern different from both the father and a female control. When the trimethylated histone-3-lysine-9 chromatin (H3K9me3) profiles in fibroblasts from control males (n=6) and females (n=7) were compared, a male-female difference was observed around the *LRFN5* gene itself (p<0.01). Intriguingly, in three cohorts of individuals with DD (n=8757), the number of heterozygotes of a common deletion polymorphism upstream of *LRFN5* was 20-26% lower than expected from Hardy-Weinberg equilibrium. This indicates early allelic interaction, and the genomic conversions from heterozygosity to wild-type or deletion homozygosity were of equal magnitudes. In a control group of medical students (n=1416), such conversions were three times more common than in
the DD-patient cohorts (p=0.00001). Hypothetically, such allelic interaction is needed
to establish monoallelic expression, which we found in foetal brain cell cultures.

Limitations: The male-female difference in H3K9me3 profiles was based on
fibroblast data from a small number of individuals, and the monoallelic expression
data on a single experiment.

Conclusions: Taken together, allelic interaction, monoallelic expression and sex-
dependent differences make the LRFN5 locus attractive for exploring the genetic basis
of synaptic memory and high-functioning male autism.

Keywords: LRFN5, SALM5, developmental delay, chromatin, topologically
associating domains, TAD, autism spectrum disorder, gene conversion

BACKGROUND

Autism spectrum disorders (ASD) are 3-4 times more frequent in males than in
females, and this sex difference is even more prominent in the high-functioning
ASD group (previously called Asperger syndrome). The cause of this sex
discrepancy is unknown and not X-linked. Despite high heritability, the genetic
cause of mild ASD is mostly unknown in the absence of pathogenic genomic copy
number gains or losses. Genome-wide association studies (GWAS) have not
identified any major autism loci, but have linked ASD, intellectual disability (ID) and
schizophrenia to an overlapping set of low-risk variants. The *LRFN5* locus has only been identified in a family-based GWAS study from 2009, a finding not replicated in more recent population-based or meta-GWAS analyses. There are, however, other results that link the *LRFN5* locus to autism. A copy number variation (CNV) study of an ASD cohort found that nearly 1% of all ASD-linked rare CNVs were in the *LRFN5* locus and none were reported *de novo*. Even more relevant is a homozygous-haplotype-sharing-in-autism-study that identified both *LRFN5* and the flanking gene *FBXO33* as ASD candidate genes in at least two population clusters.

*LRFN5*, called SALM5 (synaptic adhesion-like molecule-5) in mice, belongs to a family of five small transmembrane protein genes involved in synaptic development, organization and plasticity. *LRFN5* acts as a dimer, and induces presynaptic differentiation through binding to the LAR family of receptor tyrosine phosphatases. *LRFN5* is the only family member located in the middle of a gene desert that can also form a large TAD of 5.4 Mb. The single-gene-in-a-TAD structure is not unique, but this TAD is unusually large, the average TAD size being about 0.9 Mb. This suggests complex gene regulation at the structural level. It is also the only LRFN-gene with a large 5’UTR (of 1.9 kb), encoded by the first 2 (of 6) exons. This 5’UTR can potentially form a complex hairpin with an estimated ΔG of -760 kcal/mol (http://www.unafold.org/). This suggests gene regulation also at the translational level.

Here we show that structural differences in this region are related to the sex of the individual and associated with autism in males, that monoallelic expression do seem to occur, and that locus homogenization by frequent allelic conversion could both be a mechanism of structure maintenance and of *LRFN5* expression regulation.
METHODS

Aim of study

Inspired by rare cytogenetic findings in individuals with translocations or copy number aberrations affecting the LRFN5 locus, in-depth investigation of locus structure was done to find autism-associated changes, and to explain sex-biased inheritance of autism susceptibility in a large family. Unexpectedly, violations of rules of inheritance were also found, indicating frequent locus interactions with allelic conversions.

Patients and DNA samples

DNA samples were obtained from the diagnostic genetic service in Bergen (Norway) and in Nijmegen (the Netherlands). Patients were recruited from the clinical genetic service in Bergen (Norway). Consent was obtained after genetic counselling of the two families having boys with autism. They have also been counselled about the results presented here. The senior author (GH) met these four males with autism 10-20 years ago and again now recently. They are all unable to hold regular jobs because they lack social/communication skills. Most have finished regular school and high school, but not without difficulties (especially in mathematics). Language skills are generally good (also in English). Typically, a digital clock display was easier to understand than a classical clockface. Their range of daily-life problems is strikingly similar. We have also counselled the families of the female with a de novo translocation and the male with the de novo 6;14 translocation.

ChIP-on-chip experiments

Chromatin immunoprecipitation (chIP) was done on primary fibroblast cultures obtained from skin biopsies from patients, family members and controls. Fibroblasts
were frozen in several batches after primary culture, and a single batch was thawed
for an experiment. This was done to avoid major differences in number of passages
(cell culture doubling times) between experiments. For each sample, a total of 4x10^7
fibroblast cells were cultured to ~90% confluence. Cells were cross-linked for 15 min
with 1% formaldehyde at 37°C before 125 mM glycine was added, followed by
incubation for 5 min at room temperature (RT). Next, cells were washed 3 times with
ice-cold phosphate-buffered saline (PBS) before harvesting by scraping, and cell
pellet were collected by centrifugation. Chromatin from cell pellet was isolated as
follows: Incubation for 10 min on ice in 4 ml cell lysis buffer [5 mM PIPES pH 8; 85
mM KCl; 0,5% NP-40; 1x protease inhibitors], centrifugation and incubation of cell
pellet for 30 min on ice in another lysis buffer [50 mM Tris pH 8; 10 mM EDTA; 1%
SDS; 1x protease inhibitors]. Chromatin was sheared on a S220 ultra-sonicator
(Covaris, MA, USA) according to the protocol “Chromatin shearing with SDS
detergent buffers” from the manufacturer. The level of fragmentation was examined
on a 1% agarose gel, and shearing was continued until the major part of the DNA
fragments had a size of ~600 bp. The cell solution was centrifuged, and the lysate was
frozen in batches of 100 µl at -80°C for later use, except for 50 µl lysate which was
used for determination of the chromatin concentration (=input DNA) by standard
DNA isolation procedures.

Chromatin immunoprecipitation (chIP) was done as follows: Per chIP, 50 µl
chromatin (on average 15 µg), 25 µl protein A/G PLUS beads (Santa Cruz
Biotechnology, TX; USA), and 425 µl incubation buffer [0,2% SDS, 1% Triton, 150
mM NaCl, 2 mM EDTA, 0,5 mM EGTA, 10 mM Tris pH 8,5, 1x protease inhibitors]
were incubated with rotation for 1 hour at 37°C. Protein-DNA complexes were
immunoprecipitated with a chIP-validated antibody at 4°C. The antibodies used were
against histone H3 (histone H3, #4620; Cell signalling, MA, USA), histone H3 trimethylated at lysine 4 (H3K4me3, ab8580; Abcam, Cambridge, UK), histone H3 trimethylated at lysine 27 (H3K27me3, ab6002; Abcam), histone H3 trimethylated at lysine 9 (H3K9me3, ab8898; Abcam), histone H3 acetylated at lysine 9 (H3K9ac, ab4441; Abcam), and rabbit IgG (#2729; Cell signalling). The amount of antibody added was according to manufacturers’ recommendations. Next day, 25 µl protein A/G PLUS beads were added to each chIP and incubated for 2 hours at 4°C. Subsequently, the protein-DNA complexes underwent a series of washes: 2x [0,1% SDS, 0,1% DOC, 1% triton, 150 mM NaCl, 1 mM EDTA, 0,5 mM EGTA, 10 mM Tris pH 8,5], 1x [0,1% SDS, 0,1% DOC, 1% triton, 500 mM NaCl, 1 mM EDTA, 0,5 mM EGTA, 10 mM Tris pH 8,5], 1x [0,25% LiCl, 0,5% DOC, 0,5% NP-40, 1 mM EDTA, 0,5 mM EGTA, 10 mM Tris pH 8,5], and 2x [1 mM EDTA, 0,5 mM EGTA, 10 mM Tris pH 8,5]. The formaldehyde-induced crosslinks were reversed by incubation for 30 min at RT with SDS-solution [0,1 M NaHCO3 + 1% SDS ], and genomic DNA was recovered by standard DNA isolation procedures. Validation of the chIPs were done by PCR on non-amplified chIP samples with primers recognizing the \textit{FBXO33} and \textit{LRFN5} genes. In the PCR, rabbit IgG-chIP was used as a pre-immune control and 2% of input DNA was used as a control of the immunoprecipitation efficiency. For the chIP-on-chip analysis, 60% of each of two replicate chIP samples were concentrated using microcon YM-30 spin columns (Merck Millipore, MA, USA) and amplified using the GenomePlex Complete Whole Genome Amplification kit (Sigma-Aldrich, MO, USA). The chIP-on-chip hybridizations were done on a custom designed high resolution NimbleGen 3x 720K array (Roche NimbleGen, WI, USA). The array probes (50-mers, positions according to Chr37/hg19) covered unique
regions of chromosome 2 (168,500–178,500 Mb), chromosome 13 (94,000–113,000 Mb), chromosome 14 (16,475–70,975 Mb), and chromosome 17 (41,370–81,195 Mb) uniformly, with a median probe spacing of ~150 bp. DNA labelling, array hybridization, post-hybridization washes and scanning were performed according to the manufacturer's protocol: “NimbleGen Arrays User´s Guide, ChIP-chip Array”, v6.2 (Roche NimbleGen). In short, the chIP and input DNA (DNA from non-precipitated chromatin) samples were labelled with Cy5- and Cy3-conjugated random nonamers, respectively. The labelled samples were purified, combined, denatured and hybridized to the array for 16 hours at 42°C. After stringent washing, the array was scanned using an Axon 4200AL Scanner (Molecular Devices, CA, USA) at 5-μm resolution. The acquired images were analysed by DEVA v1.2 software (Roche NimbleGen) creating pair reports, including raw intensities for each probe and per image. From these data, ratio files were generated. For data visualization, the average ratios of two replicate experiments were binned per kb, each adjusted for the number of probes per bin. These data were transferred to in Excel spreadsheets for further calculations and generation of plain text files in .bedgraph format, and then the data was plotted against chromosomal position using the UCSC browser’s custom track option.

**Capture HiC-based LRFN5 locus TAD-structure determination**

**LRFN5 locus selection**: The capture Hi-C (CHiC) SureSelect library was designed over the genomic interval (chr14:539,000,000-47,000,000, GRCh37) using the SureDesign tool from Agilent (Agilent Technologies, Santa Clara, CA). The coverage was 70,5% by 159698 probes of total size 4,668 Mb and 5x tiling density.

**Fixation of fibroblast nuclei**: Capture HiC experiments were performed on dermal fibroblasts from a family trio (parents and child with ASD) and a control female of
the same age as the mother. Trypsinised fibroblasts were washed in PBS and then transferred to a 50-ml Falcon tube and complemented with 10% FCS/PBS. 37% formaldehyde was added to a final concentration of 2% and cells were fixed for 10 min at room temperature. Crosslinking was quenched by adding glycine (final concentration; 125 mM). Fixed cells were washed twice with cold PBS and lysed using fresh lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 5 mM MgCl₂, 0.1 mM EGTA with protease inhibitor) to isolate nuclei. Cell lysis was assessed microscopically after 10-min incubation in ice. Nuclei were centrifuged for 5 min at 480g, washed once with PBS and snap frozen in liquid N₂.

**Chromosome conformation capture library preparation and sequencing:**

3C libraries were prepared from fixed nuclei as described previously.²² Briefly, lysis buffer was removed by centrifugation at 400 g for 5 min at 4 °C, followed by supernatant aspiration, snap-freezing, and pellet storage at -80 °C. Later, nuclei pellets were thawed on ice, resuspended in 520 μl 1× DpnII buffer, and then incubated with 7.4 μl 20% SDS shaking at 900 rpm. at 37 °C for 1 hour. Next, 75 μl 20% Triton X-100 was added and the pellet was left shaking at 900 rpm. at 37°C for 1 hour. A 15-μl aliquot was taken as a control for undigested chromatin (stored at -20°C). The chromatin was digested using 40 μl 10 U/μl DpnII buffer shaking at 900 rpm. at 37°C for 6 h; 40 μl of DpnII was added and samples were incubated overnight, shaking at 900 rpm. at 37°C. On day three, 20 μl DpnII buffer was added to the samples followed by shaking for 5 more hours at 900 rpm. at 37 °C. DpnII subsequently was inactivated at 65°C for 25 min and a 50-μl aliquot was taken to test digestion efficiency (stored at -20°C). Next, digested chromatin was diluted in 5.1 ml H₂O, 700 μl 10× ligation buffer (Thermo Fisher Scientific), 5 μl 30 U/μl T4 DNA ligase and incubated at 16°C for 4 h while rotating. Ligated samples were incubated for a further
30 min at room temperature. Chimeric chromatin products and test aliquots were de-
crosslinked overnight by adding 30 µl and 5 µl proteinase K, respectively, and
incubated at 65°C overnight. On the fourth day, 30 µl or 5 µl of 10 mg/ml RNase was
added to the samples and aliquots, respectively, and incubated for 45 min at 37°C.
Next, chromatin was precipitated by adding 1 volume phenol-chloroform to the
samples and aliquots, vigorously shaking them, followed by centrifugation at 4,000
rpm. at room temperature for 15 min. To precipitate aliquoted chromatin, 1 volume
100% ethanol and 0.1 volume 3M NaAc, pH 5.6 was added and the aliquots placed at
-80°C for 30 min. DNA was then precipitated by centrifugation at 5,000 rpm. for 45
min at 4°C followed by washing with 70% ethanol, and resuspension in 20 µl with 10
mM Tris-HCl, pH 7.5. To precipitate samples, extracted sample aqueous phases were
mixed with 7 ml H2O, 1 ml 3M NaAc, pH 5.6, and 35 ml 100% ethanol. Following
incubation at -20°C for at least 3 h, precipitated chromatin was isolated by
centrifugation at 5,000 rpm. for 45 min at 4 °C. The chromatin pellet was washed
with 70% ethanol and further centrifuged at 5,000 rpm. for 15 min at 4°C. Finally, 3C
library chromatin pellets were dried at room temperature and resuspended in 10 mM
Tris-HCl, pH 7.5. To check the 3C library, 600 ng were loaded on a 1% gel together
with the undigested and digested aliquots. The 3C library was then sheared using a
Covaris sonicator (duty cycle: 10%; intensity: 5; cycles per burst: 200; time: 6 cycles
of 60 s each; set mode: frequency sweeping; temperature: 4-7°C). Adaptors were
added to the sheared DNA and amplified according to the manufacturer’s instructions
for Illumina sequencing (Agilent). The library was hybridized to the custom designed
SureSelect beads and indexed for sequencing (150 bp paired-end) following the
manufacturer’s instructions (Agilent).

**Capture-HiC analysis**: Raw reads were pre-processed with cutadapt v1.15 to trim
potential low-quality bases (-q 20 -m 25) and any remaining sequencing adapters
(-a and -A option with Illumina TruSeq adapter sequences according to
the cutadapt documentation) at the 3’ ends of the reads. Mapping, filtering and
deduplication of the short reads were performed with the HiCUP pipeline v0.7.04
(https://doi.org/10.12688%2Ff1000research.7334.1) (no size selection, Nofill: 1,
Format: Sanger). The pipeline employed Bowtie2 v2.2.6
(https://doi.org/10.1038%2Ffnmeth.1923) for mapping short reads to the hg19 human
reference genomes. Juicer tools 0.7.5 (https://doi.org/10.1016%2Fj.cels.2016.07.002)
was used to generate binned-contact maps from valid and unique read pairs with
MAPQ ≥ 30 and to normalize contact maps by Knight and Ruiz (KR) matrix
balancing (https://doi.org/10.1016%2Fj.cell.2014.11.021
https://doi.org/10.1093%2Fimanum%2Fdrs019). For the generation of capture-HiC
contact maps, only reads pairs mapping to the enriched genomic region were
considered and shifted by the offset of the enriched genomic region. For the import
with Juicer tools, we used a custom chrom.sizes file containing only the size of the
enriched part of the genome. Afterward, KR-normalized maps were exported at 10-kb
resolution, and coordinates were shifted back to their original values.

DNA methylation studies
CpG methylation of the _LRFN5_ locus was initially interrogated by the Illumina 450K
DNA methylation array (Infinum HumanMethylation450 BeadChip), following
manufacturer’s protocol. No methylation differences were found between male and
female controls. Specific positions in the _LRFN5_ locus (namely the region
corresponding to the peak of the locus-TAD and the _LRFN5_ promoter) were analysed
by both DNA sequencing after bisulphite treatment, and custom designed MLPA tests
(using the P-300 kit from MRC-Holland, and following the manufacturer’s protocol).
Still, no sex differences were found. All these methylation studies were done on leukocyte DNA from peripheral blood samples.

**LRFN5 expression in foetal brain cell cultures**

Human neural stem cell lines were established from 12-week foetal brain cell cultures using a conditionally immortalization methodology, as described. STROC05, STROC08 and STROC11 are striatal cell lines that are identical and from the same dissection, just different isolated clones. An Illumina SNP array was used to identify heterozygous SNPs in *LRFN5*, and the *LRFN5* cDNA sequence of polyclonal brain cells was then compared to the cDNA sequence of these cell lines.

**SNP array analysis of a deletion polymorphism upstream of LRFN5**

The allele frequencies of a common 60 kb deletion, chr14(GRCh37):g.41609383-41669664, detected by at least 12 oligonucleotides on the Affymetrix 6.0 SNP array and by at least 18 oligonucleotides on the Affymetrix CytoScan SNP array (Affymetrix, ThermoFischer Scientific, USA), were determined in four cohorts of individuals: three ascertained due to developmental disorders, and one group of mostly medical students (Table 2). All cohorts were anonymized. Three of the cohorts (patient cohort I and III and the student cohort) had their genomic copy number status determined by the Affymetrix 6.0 array, while patient cohort II was tested by the Affymetrix CytoScan array.

**Statistical analysis**

Basic and on-line statistical tools were used to investigate the statistical significance of the following findings: the segregation of the A-haplotype in the autism family: Fisher Exact Test (p = 0.0476); the female-male comparison in the table of Figure 3: Student two-sided t-test (p = 0.005); the difference between Hardy-Weinberg
equilibrium and observed allelic distribution in the patient cohorts in Table 2: Chi-square test (p < 0.00001); and the difference between the allelic distribution in the patient cohorts and the student cohort in Table 2: Chi-square test (p < 0.00001).

**RESULTS**

*LRFN5 locus structural changes can be associated with developmental delay and autism*

Our attention was drawn to the *LRFN5* locus in 2007 when a girl with intellectual disability (ID) that gave no social contact was found to have two *de novo* chromosome changes: a small 2q31.1 deletion and a balanced 14;21-translocation. One translocation breakpoint was in the *LRFN5* locus at 14q21.1, and the other on the acrocentric 21p arm. To explore the consequences of these chromosome aberrations for chromatin structure, we performed ChIP-on-chip experiments on patient skin fibroblasts. The results were compared to a non-autistic boy with another *LRFN5* locus translocation: a *de novo* t(6;14)(q26;q21.1) causing Coffin-Sirin syndrome because *ARID1B* was disrupted on chromosome 6 (Supplementary Figure 1). He speaks well and has good social function. A clear difference in the *LRFN5* locus chromatin profiles was only seen in the autistic girl. Possibly this is because her translocation fused the *LRFN5* locus to an acrocentric p-arm, and a trimethylated histone-3-lysine-9 (H3K9me3) spreading effect from *FBXO33* and centromeric could be seen on derivative chromosome 14, while more trimethylated histon-3-lysine-4 (H3K4me3) and less trimethylated histon-3-lysine-27 (H3K27me3) was seen in the *LRFN5* locus on derivative chromosome 21 (Supplementary Figure 1). In addition, an apparent “compensatory” H3K4me3-effect corresponding to the 2.6 Mb 2q31.1 deletion could also be seen. However, this unexpected and unprecedented effect could
be 2q31.1-locus specific as it was not found in an individual with a 2.2 Mb chromosome 17q23.1 deletion (Supplementary Figure 2). These two unique 14;21-translocation patients initiated our LRFN5 research, and we noted that autistic behaviour was only seen in the individual with chromatin changes to this locus.

Of more general relevance for LRFN5 function is the number of individuals with DD/ASD and copy number variants (CNVs) in the LRFN5 locus in the DECIPHER database (n=30) and our own records (n=3) (Supplementary Figures 3 and 4, pluss reference 25). Only one small deletion containing LRFN5 itself was registered as de novo. Because many CNVs are inherited from a seemingly unaffected parent, most have been regarded as non-pathogenic. Nevertheless, if this truly is an ASD susceptibility locus, even reduced penetrance should not have prevented that from being discovered. However, if sex-of-origin also matters for autism susceptibility, an ASD link could easily be missed.

**Autism in males segregated with a specific LRFN5 locus haplotype inherited from their mothers**

High-functioning ASD was diagnosed in two pairs of brothers from two families from the same geographical region (Table 1; fam #1 and fam #2). These four males have been seen by the senior author both as children and adults, and their autism phenotype is strikingly similar. They all had a 172 kb deletion just upstream of the LRFN5 promoter and the same locus haplotype (called the A-haplotype in Table 1), inherited from their normal mothers. The families were too distant to know about any relatedness. The A-haplotype was also found in a normal maternal uncle, inherited from his mother. Of the nine individuals sharing the A-haplotype in these families, 4/5 males had ASD, and 4/4 SRY-negatives (three females and one XX-male) were
normal. This may suggest that the A-haplotype increased ASD susceptibility in males in this family (Fisher Exact Test p=0.0476). We also found overlapping deletions on different haplotypes in four other individuals or families, ascertained by copy-number high-resolution SNP-array testing because of developmental delay, ID, ASD or schizophrenia (Table 1). This could indicate that the A-haplotype is linked to male ASD-susceptibility, not the 172 kb deletion \textit{per se}. An effect of the deletion is not excluded, but then it must be haplotype dependent. The 172 kb deletion does not contain enhancer-like chromatin profiles or lncRNAs.

The structure of the \textit{LRFN5} locus is influenced by the sex of the individual

To explore if the A-haplotype influenced TAD-structure of the \textit{LRFN5} locus, we performed a capture HiC-experiment on skin fibroblasts from a family trio (boy with ASD and his unaffected parents), with a normal unrelated female as control (Figure 1). Three patterns emerged: In the autistic boy and his mother, both sharing the A-haplotype, the whole-locus 5.4 Mb mega-TAD had three sub-TADs with boundaries at the \textit{LRFN5} promoter (arrow A in Figure 1) and the middle of the \textit{LRFN5}-downstream gene desert (arrow B in Figure 1). The small red diamond just centromeric to the \textit{LRFN5} promoter marks the 172 kb deletion. In the father, only two TADs could be discerned inside the mega-TAD. No distinct “B-junction” could be seen. In the control female, only the mega-TAD was distinct; an “A-junction” was diffuse if at all present (Figure 1).

These subtle capture-HiC differences point to variation in TAD structure and that the autistic boy inherited his mother’s structure. Hypothetically, this specific structure (with a “B-junction”) could be A-haplotype dependent and the one linked to autism in
this family. We also noted that the father and the control female had TAD differences (Figure 1).

To explore if this could reflect differences in their chromatin profiles, chIP-on-chip-generated chromatin data (H3K4me3, H3K27me3 and H3K9me3) on fibroblasts from four family members, five control males and seven control females, were compared. A sex difference was found for the H3K9me3 profiles, i.e. heterochromatin protein-1 (HP1)-associated constitutive heterochromatin, but only corresponding to the LRFN5 gene itself and the region downstream to the H3K4me3/H3K27me3 signals marking the peak of the mega-TAD (Figure 2). In this area, males had more heterochromatin than females. This difference was significant, but individual differences in the degree of this effect should be noted (Figure 2). In fact, the groups are overlapping (Figure 3). In contrast, the sex difference was zero in a region 1 Mb upstream (Figure 3).

The four family members, including the mother and the SRY-negative brother of the index boy that shared the A-haplotype (Table 1), all had a “male-type” of chromatin pattern (Figure 2). Possibly, this pattern is SRY-dependent, but not if one inherits the A-haplotype. The control male and female with the largest H3K9me3-associated chromatin differences are also shown at the bottom of the locus overview figure (Figure 4), where their H3K9me3-chromatin profiles are aligned to the ASD-associated A-haplotype TAD structure, CTCF-binding sites, and open-chromatin neuronal single-cell ATAC sites.

To explore if we could test large numbers of individuals for sex-related or autism-related differences in this region, we performed a pilot MLPA-based study on anonymized blood leukocyte DNA to see if these sex difference in chromatin profiles was also reflected in methylation differences of a CpG located at the conserved CTCF
binding site in the *LRFN5* promoter (chr14:42,069,895-42,069,949, hg19). An average methylation degree of 0.19 (CI 0.13-0.25) in control males (n=16) and control females (n=6) were found with no sex difference, and the same average level (0.20) was found in patients investigated because of non-ID associated autism (n=14). In a separate experiment, using the Illumina 450K BeadChip methylation test, we found an average degree of CpG methylation of 0.14 at the *LRFN5* promoter (19 CpGs were interrogated) and 0.52 in the *LRFN5* gene itself (8 CpGs were interrogated) in 10 other control individuals, still without sex difference. This shows that the difference in fibroblast H3K9me3 profiles at the *LRFN5* gene is not reflected in differences in CpG methylation of leukocyte DNA in the same region.

**Allelic interaction must be frequent in the *LRFN5* locus**

If the *LRFN5* locus structure is critical for brain function, how is it maintained when copy number changes are so common? A possible answer to this question was unexpectedly found upon examining the allelic distribution of a common 60 kb deletion in the middle of the *LRFN5*-upstream gene desert (chr14:41,609,383-41,669,664 (hg19); Table 2 (position of deletion indicated in Figure 4). We examined three large and independent cohorts of individuals (two from Norway and one from the Netherlands) who had been investigated with a high-density SNP-array because of a developmental disorder, usually including variable degrees of developmental delay (DD). In addition, one Dutch cohort (n=1,416) of mainly medical students served as a control. The deletion’s minor allele frequency (MAF) was around 8.5% in the Dutch population, 15% in the Norwegian population, and 14% in gnomAD (gnomad.broadinstitute.org). When comparing the observed allelic distribution in these cohorts with the expected distribution as per Hardy-Weinberg equilibrium, there
were too few heterozygotes in all cohorts. We found that the loss of heterozygote wild-type (wt) / deletion (del) was of equal magnitude in both directions, i.e. to wt/wt and to del/del. In the three patient cohorts, this loss was 20%, 24% and 26%, respectively, but in the student cohort, it was 80% (Table 2). This surprising difference was not a technical artefact. 12-18 SNP-array oligonucleotides covered the 60 kb deletion, and ≥5 in a row is usually sufficient for correct calling. Furthermore, if this was due to missed wt/del calling, the high number of del/del homozygotes would still be incompatible with Hardy-Weinberg equilibrium. Also, when examining SNP array results from our diagnostic routine, we found that there was an excess of homozygosity regions >1 Mb in the LRFN5 locus compared to many other regions in the genome. We are not aware of any other potential explanations for these observations than an early mitotic allelic conversion event with a frequency of at least 1 in 5 individuals in the DD group and as high as 4 in 5 individuals in the student group. This is far beyond expectation. Maybe this allelic interaction is needed to establish monoallelic expression. In human fetal brain cell cultures from the striatum, we did indeed find evidence indicating monoallelic LRFN5 expression (Supplementary Figure 5).

DISCUSSION

Despite extensive research, it has remained elusive why autism, and especially the higher-functioning variants, is more common in males than in females. Here we show that the synaptic regulation and maintenance gene LRFN5, situated in the middle of a conserved gene desert capable of forming a 5.4 Mb mega-TAD, may be part of the answer to this question. The three main reasons for this are the large family with remotely related pairs of brothers with a very similar form of higher-functioning
autism sharing the same maternally inherited *LRFN5* locus haplotype (Table 1), the sex-influenced differences in locus chromatin 2D structure in fibroblasts from normal males and females (Figure 2 and 3), and the allelic interaction that must take place in early embryonic development, as evidenced by the striking deviation from Hardy-Weinberg equilibrium of a 60 kb deletion polymorphism (Table 2).

There are eight putative SOX9 and SRY binding sites flanking the *LRFN5* locus; 3+2 SOX9 sites and 1+2 SRY sites (Figure 2 and Supplementary Figure 6; TFBS_conc track in the UCSC browser). This could have importance for generation of the observed male/female chromatin difference (Figures 2 and 3). The mechanism behind the early locus interaction is more difficult to explain. We are unaware of such frequent allelic interaction and locus conversion in any other part of the genome, e.g. the frequency of inter-chromosomal gene conversion in gene families with more than two alleles has been estimated to be around 0.2%.\textsuperscript{27} Gene conversion events are more common towards the 3’UTR end of protein-coding genes, and this is believed to be due to RNA transcription aiding the process.\textsuperscript{27} Maybe *LINC02315*, an RNA gene upstream of *LRFN5* ending in the common 60 kb deletion polymorphism, has a similar role (Figure 4).

The most interesting question is why frequent locus homozygotisation by gene conversions occurs. We hypothesize that this process is needed to establish stochastic monoallelic expression, advantageous for fine-tuning *LRFN5* expression. Given the high conversion frequencies, allelic interaction is probably the rule, and allelic conversion a consequence. Exploratory foetal brain expression data suggests that monoallelic expression does occur in humans (Supplementary Figure 5), but we do not know if this is always the case or if it is stochastic. The next question is why fine-
tuning of *LRFN5* expression is so critical that the gene, on top of having a 1.9 kb 5'UTR encoded by exons 1 and 2, needs to be framed by 2-3 Mb of conserved, presumed regulatory, gene deserts. Maybe the difference in locus conversion frequencies between a student cohort (4 in 5 were converted to homozygosity) and three DD-cohorts (1 in 4 were converted to homozygosity) provides a clue (Table 2). Hypothetically, if the *LRFN5* locus is a genetic basis for synaptic memory, then both allelic interaction and conversion could be advantageous for this function. This fits well with *LRFN5*’s role as a protein involved in synapse strength and dynamics,15,20,28 and it could have relevance for the photographic memory of details that some autistic individuals may have.

The concept of autism-related risk haplotypes of the *LRFN5* locus, variable locus structure influenced by the individual’s sex, and early allelic homozygotisation, fits well with the complex pattern of ASD inheritance.4 In the families with the ASD-susceptible A-haplotype described here, all ASD males were born to carrier mothers, and one non-penetrant male was also recorded (Table 1). Of note, this male was hemi-methylated at rs144497930, i.e. at the major TAD-peak around the CTCF binding site in Figure 4 (Supplementary Table 1). Other family members and control individuals were fully methylated at this CpG. Maybe the epigenetic pattern predisposing to autism was not established during his early embryogenesis. In a “locus-interaction-determines-ASD-susceptibility” model, this makes sense and would also explain why rare deletions recorded in DECIPHER may be pathogenic despite inheritance from a presumed normal male or female parent (Supplementary Figure 3). Such deletions could interfere with allelic interaction and epigenetic regulation. It should also be noted that while many different deletions may be seen in the *LRFN5* locus (Supplementary Figure 6), duplications are rare, also in DECIPHER (Supplementary
Finally, the whole *LRFN5* locus or the *LRFN5* gene itself can be disrupted or deleted, apparently without (additional) phenotypic consequences, as indicated from the DECIPHER database, a gnomAD pLI of 0.56, and our own diagnostic genomic copy-number records. This is as expected if stochastic monoallelic expression is the rule.

**LIMITATIONS**

The capture-HiC and chromatin chIP-on-chip experiments were all done on human skin fibroblasts, not brain cells, which is the relevant tissue for *LRFN5* expression. As the TAD (3D) structure and probably also the chromatin (2D) structure can be activity dependent, the relevance of these finding for *LRFN5* locus structure in brain cells is yet unknown, but TAD structure tends to be conserved between cell types. The size of the control cohort exploring sex-related differences in H3K9me3 profiles of the *LRFN5* locus is small (6 males +7 females), and these data need to be confirmed in an independent and preferably larger cohort to be certain of a sex difference. Hopefully, an easier method to investigate such changes will be developed. Finally, the monoallelic expression data (Supplementary Figure 5) is from a single experiment.

**CONCLUSIONS**

Our work suggests that *LRFN5* regulation could be Y-chromosome-dependent and complex with a strikingly high frequency of early embryonic allelic interaction with locus conversions. Structural variants in this locus are linked to autism, and we speculate that this locus is a significant part of the genetic basis of synaptic memory.
DECLARATIONS

Ethical board approvals

The methylation study on the anonymized ASD cohort was approved by the Ethical Review Board of Northern Norway, REK-Nord# 2013/965. The study on sex-dependent differences in the epigenetic profile of the LRFN5 locus was approved by the Ethical Review Board of Western Norway, REK-Vest# 2016/25.

Consent for publication

All involved members of the two families with two autistic brothers, including the brothers themselves, have been counselled about these results and have given consent to publication, recorded in the hospital’s journal. Other individuals participating in this study were anonymised.

Availability of data and materials

Data generated during this study are available from the corresponding author on reasonable request. The chIP-on-chip data can be shared through custom tracks in the UCSC browser.

Competing interest

The authors have no conflicts of interest to declare.

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Author contributions

HL did all chromatin profiling experiments, MR did the capture-HiC experiments with data-analysis and figure preparation, NdL and JYH-K delivered the Dutch
deletion frequency data, AJ did the human foetal brain cell \textit{LRFN5} expression test, BIH, SB, DdB, SM and MS gave valuable input during the experimental and scientific process and participated in analysing the data. GH counselled the patients and families, designed the research project, wrote research applications, plotted and analysed chromatin and allele frequency data, made most of the figures and all tables, and wrote the manuscript. All authors have reviewed and approved the final version.

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**Tables**

**Table 1**

LRFN5 locus haplotypes in affected individuals and their families, all having overlapping deletions on different haplotypes (A-E) just upstream of the LRFN5 promoter.

| Fam # | Individual       | DD/ASD | Deletion haplotype¹ |
|-------|------------------|--------|---------------------|
| 1     | Father           | -      | -                   |
| 1     | Mother           | -      | A                   |
| 1     | Son²             | -      | A                   |
| 1     | Son              | ASD    | A                   |
| 1     | Son              | ASD    | A                   |
| 1     | Son              | -      | -                   |
| 1     | Daughter         | -      | -                   |
| 1     | Daughter         | -      | -                   |
| 2     | Mat grandfather  | -      | -                   |
| 2     | Mat grandmother  | -      | A                   |
| 2     | Mat uncle        | -      | A                   |
| 2     | Father           | -      | -                   |
| 2     | Mother           | -      | A                   |
| 2     | Son              | ASD    | A                   |
| 2     | Son              | ASD    | A                   |
| 2     | Son              | -      | -                   |
| 2     | Daughter         | -      | -                   |
| 3     | Father           | -      | B                   |
| 3     | Mother           | -      | -                   |
| 3     | Son              | DD     | B                   |
| 4     | Father           | -      | -                   |
| 4     | Mother           | -      | C                   |
| 4     | Son              | DD/ASD | C                   |
| 5     | Father           | -      | -                   |
| 5     | Mother           | -      | D                   |
| 5     | Daughter         | Rett-like | D               |
| 6     | Adult male       | Mild ID/ schizophrenia | E     |

¹ Deletion sizes (kb, hg19): A: 41846-42020, B: 41846-42023, C: 41867-41979, D: 41822-41933, E: 41836-42004. The haplotypes were also determined based on LRFN5 locus SNP pattern.

² This individual was a SRY-negative 46,XX-DSD male.
### Table 2

Allelic distribution of a common 60 kb deletion* (from 41608 to 41657 kb in GRCh37 based on SNP-array data) in three patient cohorts and one student cohort.

The D-column shows the fold difference of allele frequencies between the observed del/del numbers and expected del/del numbers from Hardy-Weinberg equilibrium.

|                    | n   | wt/wt | wt/del | del/del | Deletion MAF | wt/wt   | wt/del   | del/del   | Δ       | % loss of wt/del to wt/wt and del/del |
|--------------------|-----|-------|--------|---------|--------------|---------|----------|-----------|--------|---------------------------------------|
| Patient cohort I (NO) expected if HWE | 850 | 649   | 168    | 33      | 0.138        | 0.760   | 0.200    | 0.040     | 2.1    | 10% + 10%                             |
| Patient cohort II (NO) expected if HWE | 4843| 3582  | 1025   | 236     | 0.155        | 0.740   | 0.212    | 0.049     | 2.0    | 12% + 12%                             |
| Patient cohort III (NL) expected if HWE | 3064| 2600  | 389    | 75      | 0.088        | 0.850   | 0.130    | 0.020     | 2.5    | 13% + 13%                             |
| Student cohort (NL) expected if HWE      | 1416| 1247  | 114    | 55      | 0.079        | 0.881   | 0.081    | 0.039     | 6.5    | 40% + 40%                             |

*Size based on gnomAD v3.1.1 whole-genome sequencing data is 41609383-41669664 (60.3 kb) with MAF 0.139 (2990/21518 alleles).

#### Figure legends

- **Figure 1**: Capture-HiC results of a family trio (index male with ASD, his mother and father) and a control female (of the same age as index’ mother). Arrows indicate TAD junctions (A and B). The small diamond just centromeric to TAD junction A (corresponds to the LRFN5 promoter) marks the 172 kb familial deletion.

- **Figure 2**: Top: LRFN5 locus H3K9me3 chIP-on-chip profiles from index with ASD, his 46,XX-DSD non-autistic brother, his normal mother and his normal father (top four lanes).
Middle: Profiles from 5 control males. Bottom: Profiles from 7 control females. Note the profile variability around the \textit{LRFN5} gene in contrast to the flanking H3K9me3-enriched domains.

\textbf{Figure 3}

Quantification of data from Figure 2: \textit{LRFN5} locus H3K9me3 chromatin levels (relative to genomic average) in fibroblasts from control males (M1-M6) and females (F1-F7). The investigated positions are indicated in the table below, and the bar diagram above illustrates the individual male/female distribution. Note that the groups are overlapping.

\textbf{Figure 4}

Top: the capture-HiC result of the index male with ASD, showing three subTADs inside the 5.4 Mb mega-TAD. The small diamond marked with an asterisk indicates the 172 kb deletion, and the position of the common 60 kb deletion polymorphism is also shown. Middle part shows CTCF sites (marking e.g. TAD boundaries and the peak of the mega-TAD) and single-cell ATAC sites in selected cell types (indicating areas of open chromatin). On the bottom is the H3K9me3 chromatin profiles of the two individuals with the most pronounced male and female pattern from the control profiles displayed in Figure 2.
SUPPLEMENTARY DATA

Supplementary Figure 1
14q21 chIP-on-chip chromatin profiles from a male control (Ctr), a male with Coffin-Siris syndrome and a de novo 6;14 translocation disrupting ARID1B (6;14), and a female with a de novo 14;21 translocation and severe ID/ASD (14;21). The arrows indicate approximate (FISH verified) translocation breakpoints. A: H3K4me3 profiles, B: H3K27me3 profiles, C: H3K9me3 profiles. Only the 14;21-chromatin pattern is markedly different from the Ctr and 6;14 cases: The H3K4me3 and H3K27me3 profiles for the LRFN5 locus on derivative chromosome 21 show a more “open” chromatin pattern, and the H3K9me3 profiles upstream of the FBXO33 gene on derivative chromosome 14 (upstream of 14;21 arrow marking the translocation breakpoint) show a more “closed” chromatin pattern.

Supplementary Figure 2
ChIP-on-chip chromatin profiles from the female with a de novo 14;21 translocation (same individual as in Supplementary Figure 1) as well as a de novo 2q31.1 deletion (panel A), compared to a male with a de novo 17q23.1 deletion (panel B). Black lines indicate deletions. The “compensatory” increase in H3K4me3 signal corresponding to the 2q31.1 deletion (panel A, top lane) was not reproduced by the 17q23.1 deletion.

Supplementary Figure 3
Overview of LRFN5 locus deletions registered in DECIPHER. The registered phenotype is shown above the deletion: ID = intellectual disability, DD = developmental delay, ASD = autism spectrum disorder, incid? = likely incidental finding (no phenotype recorded), mat = maternally inherited, pat = paternally inherited. On the bottom, three deletions found in own patient records are shown (red
lines framed in black). The vertical green shades indicate LRFN5 locus regions were
CNVs appear to be of no clinical consequence. The vertical red shades indicate
LRFN5 locus regions where CNVs could increase susceptibility for DD/ASD.

Supplementary Figure 4
Overview of LRFN5 locus duplications registered in DECIPHER. The registered
phenotype is shown above the deletion: ID = intellectual disability, DD =
developmental delay, epil = seizures, mat = maternally inherited, pat = paternally
inherited. Note that duplications are rarer than deletions in this region, which is also
the case in the DGV database (database of genomic variants; dgv.tcag.ca, see
Supplementary Figure 6).

Supplementary Figure 5
Cells from 12-week human foetal brain were examined for allelic balance of LRFN5
expression. Three SNP found to be informative (heterozygous) by an Illumina SNP
array were interrogated by cDNA Sanger sequencing: rs1137567 in exon 1 (5’UTR of
mRNA), rs6572117 in exon 4 (protein-coding sequence) and rs7148658 in intron 4. In
four (polyclonal) foetal brain samples (FB 11189/11242/11196/11192), both alleles
were expressed. In three striatal neuron cell cultures (STRO 05/08/11), only one allele
was expressed, suggesting monoallelic expression. Since the same allele was
expressed in all three cell lines, one cannot tell if the monoallelic expression was
stochastic.

Supplementary Figure 6
Overview of LRFN5 locus, taken from the UCSC browser (version GRCh37), with a
transparent overlay of the capture-HiC result of the ASD index boy. Lane on top
shows computer predicted SOX9 and SRY binding sites, in the middle CTCF binding
sites in three different cell types can be found (note that junction B also corresponds
to CTCF signals), and on the bottom DECIPHER and DGV copy number variants are
found. In both, deletions are more common than duplications. Red vertical bars
indicate areas were copy number variants have been found in patients with ID/ASD,
and green areas where this so far is not the case.
**Supplementary Table 1**

Targeted MLPA-based DNA methylation results in the genomic area underlying the mega-TAD-peak in a small group of selected individuals. Please note that the normal male with the A-haplotype was hemi-methylated in a position fully methylated in other individuals.

| Individual         | Haplo-type | TAD-peak lncRNA CpG | TAD-peak lncRNA CpG | TAD-peak conserved CpG | TAD-peak conserved CpG |
|--------------------|------------|---------------------|---------------------|------------------------|------------------------|
| ASD male           | A          | ~100%               | ~100%               | ~10%                   | ~10%                   |
| ASD male           | A          | ~100%               | ~100%               | ~5%                    | ~5%                    |
| Normal male        | A          | ~100%               | ~50%**              | ~5%                    | ~5%                    |
| Normal female      | A          | ~100%               | tech. failure       | ~20%                   | ~20%                   |
| ASD male           | -          | ~100%               | ~100%               | ~10%                   | ~10%                   |
| ASD male           | -          | ~100%               | ~100%               | ~10%                   | ~10%                   |
| Normal male        | -          | ~100%               | ~100%               | ~10%                   | ~10%                   |
| Normal female      | -          | ~100%               | ~100%               | ~10%                   | ~10%                   |
| Normal female      | D          | ~100%               | ~100%               | ~50%                   | ~50%                   |
| Normal female      | C          | ~100%               | ~100%               | ~20%                   | ~20%                   |

*CpG positions corresponding to the mega-TAD peak on chromosome 14 are according to GRCh37. Haplotypes are according to Extended Data Table 1, i.e. all from individuals with a small locus deletion. ** Sanger sequencing verified that this finding was not due to a SNP, i.e. a C to T transition.
Capture-HiC results of a family trio (index male with ASD, his mother and father) and a control female (of the same age as index’ mother). Arrows indicate TAD junctions (A and B). The small diamond just centromeric to TAD junction A (corresponds to the LRFN5 promoter) marks the 172 kb familial deletion.
Figure 2

Top: LRFN5 locus H3K9me3 chIP-on-chip profiles from index with ASD, his 46,XX17 DSD non-autistic brother, his normal mother and his normal father (top four lanes). Middle: Profiles from 5 control males. Bottom: 1 Profiles from 7 control females. Note the profile variability around the LRFN5 gene in contrast to the flanking H3K9me3- enriched domains.
Display of the individual *LRFN5* chromatin data from males (M1-M6) and females (F1-F7) based on the table below

H3K9me3 chromatin scores (relative to genomic average) in fibroblasts from control males (M’s) and females (F’s).

| Genomic position | M1  | M2  | M3  | M4  | M5  | M6  | F1  | F2  | F3  | F4  | F5  | F6  | F7  | Average males | Average females | Diff       |
|------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----------------|----------------|-----------|
| *LRFN5* (42050-42400 kb) | 3   | 11  | 4   | 9   | 26  | -5  | -5  | -19 | -5  | 5   | -24 | -26 | -19 | 8 (0 - 16)     | -13 (-22 - -4) | 21*       |
| Reference pos. (40550-40900 kb) | 54  | 57  | 51  | 38  | 68  | 60  | 46  | 58  | 60  | 61  | 43  | 60  | 55  | 55 (47 - 63)   | 55 (49 - 61)    | 0         |

The chromatin scores are based on 673-1907 measurement points (= one oligo on the array), on average around 700 measurements per position. The reference position was chosen to be in the middle of the heterochromatin stretch upstream of *LRFN5*. 95% confidence intervals are shown in parentheses. *p = 0.005 (Student two-sided t-test).

Figure 3

Quantification of data from Figure 2: *LRFN5* locus H3K9me3 chromatin levels (relative to genomic average) in fibroblasts from control males (M1-M6) and females (F1-F7). The investigated positions are indicated in the table below, and the bar diagram above illustrates the individual male/female distribution. Note that the groups are overlapping.
Figure 4

Top: the capture-HiC result of the index male with ASD, showing three subTADs inside the 5.4 Mb mega-TAD. The small diamond marked with an asterisk indicates the 172 kb deletion, and the position of the common 60 kb deletion polymorphism is also shown. Middle part shows CTCF sites (marking e.g. TAD boundaries and the peak of the mega-TAD) and single-cell ATAC sites in selected cell types (indicating areas of open chromatin). On the bottom is the H3K9me3 chromatin profiles of the two individuals with the most pronounced male and female pattern from the control profiles displayed in Figure 2.

Supplementary Files
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