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ELECTRONIC LETTER

A novel susceptibility locus for Hirschsprung's disease maps to 4q31.3–q32.3

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We report on a multigenerational family with isolated Hirschsprung's disease (HSCR). Five patients were affected by either short segment or long segment HSCR. The family consists of two main branches: one with four patients (three siblings and one maternal uncle) and one with one patient. Analysis of the *RET* gene, the major gene involved in HSCR susceptibility, revealed neither linkage nor mutations. A genome wide linkage analysis was performed, revealing suggestive linkage to a region on 4q31–q32 with a maximum parametric multipoint LOD score of 2.7. Furthermore, non-parametric linkage (NPL) analysis of the genome wide scan data revealed a NPL score of 2.54 (p = 0.003) for the same region on chromosome 4q (D4S413–D4S3351). The minimum linkage interval spans a region of 11.7 cM (12.2 Mb). No genes within this chromosomal interval have previously been implicated in HSCR. Considering the low penetrance of disease in this family, the 4q locus may be necessary but not sufficient to cause HSCR in the absence of modifying loci elsewhere in the genome. Our results suggest the existence of a new susceptibility locus for HSCR at 4q31.3-q32.3.

Hirschsprung's disease (HSCR; OMIM #143623) is a congenital disorder characterised by the absence of enteric neurones, which are derived from the neural crest, in the digestive tract. Delayed passage of meconium is the cardinal symptom in neonates with HSCR. If untreated, bowel hypomotility leads to severe constipation, often associated with obstruction, gross distension of the bowel, and vomiting. The prevalence of HSCR is approximately 1 in every 5000 liveborn infants. In the majority of cases (75–80%) the aganglionosis typically involves the rectum and the sigmoid (short segment HSCR; SS-HSCR). In 20–25% of patients the aganglionosis extends proximally of the recto-sigmoid, and the disease is called long segment HSCR (LS-HSCR).[1] HSCR mostly presents as an isolated congenital malformation (non-syndromic HSCR), but can be found in association with other congenital abnormalities (syndromic HSCR).[2]

So far mutations in 10 genes (*RET*, *GDNF*, *EDNRB*, *EDN3*, *ECE1*, *SOX10*, *ZFHX1B*, *NTN*, *PHOX2B*, and *KIAA1279*) have been implicated in HSCR.[3–16] The *RET* gene, located at 10q11.2, is the major susceptibility locus in HSCR; 15%–35% of the sporadic patients have inactivating mutations in the coding sequence of *RET*,[17–20] and linkage analysis has shown that all autosomal dominant families but one are linked to *RET*.[21] High penetrance mutations in the coding sequence of the *RET* gene, however, are found in only 50% of the *RET* linked families. *RET* linked multigenerational families without a coding sequence mutation may have a mutation in the non-coding sequence of the *RET* gene, including alterations in intronic and promoter sequences, or may harbour (frequent) variants that change the function of the RET protein slightly.[21] Furthermore, similar haplotypes are found in the 5′ region of the *RET* locus in HSCR patient populations from all over the world, indicating the segregation of identical ancestral variant(s).[22–26] Evidence is accumulating that specific (common) non-coding low penetrance variants just before the gene and within intron 1 of *RET* are associated with susceptibility to HSCR.[22 25 27–30]

Single mutations leading to either isolated or syndromic HSCR have been found in the aforementioned 10 genes, although evidence is building up that HSCR is a multigenic congenital malformation in the majority of HSCR patients. A "multiplicative model" has been suggested, which assumes that additional loci are involved apart from the *RET* locus and that their individual effects can be multiplied.[31]

Furthermore, four HSCR susceptibility loci (9q31, 3p21, 19q12, and 16q23) have been identified, harbouring unidentified genes.[21 31 32] Linkage at 9q31 was reported in five families that also showed linkage with *RET*; however, no causative *RET* mutation could be identified. A sixth family that did not show linkage to *RET* was linked to the locus at 9q31.[21] Furthermore, susceptibility loci at 3p21 and 19q12 have been identified in affected nuclear families, suggesting that these two loci probably function as *RET* dependent modifiers. Non-random allele sharing was also found at 9q31 in nuclear families in which no *RET* mutation was identified, confirming the segregation of the 9q31 locus in multiplex families.[31 21]

In an inbred Mennonite population, *RET* not only interacts with *EDNRB*, but also with an unknown gene on chromosome 16q23. This locus is probably only of importance in this genetically isolated population,[32] in which HSCR can be associated with symptoms also found in Shah-Waardenburg syndrome.[7] Conversely, no linkage with the other susceptibility loci at 3p21, 9q31 and 19q12 was found in this Mennonite kindred.

Clearly, HSCR is a heterogeneous congenital malformation. It is estimated that only 30% of cases can be attributed to mutations of the known genes.[33] Thus, a considerable number of additional genes involved in enteric nervous system development could be identified in the future.[28] Here, we describe a five generation family with non-syndromic HSCR with suggestive evidence for a new susceptibility locus on chromosome 4.

Abbreviations: HSCR, Hirschsprung's disease; LS-HSCR, long segment Hirschsprung's disease; NPL, non-parametric linkage; SNP, single nucleotide polymorphism; SS-HSCR, short segment Hirschsprung's disease; STRP, short tandem repeat polymorphism

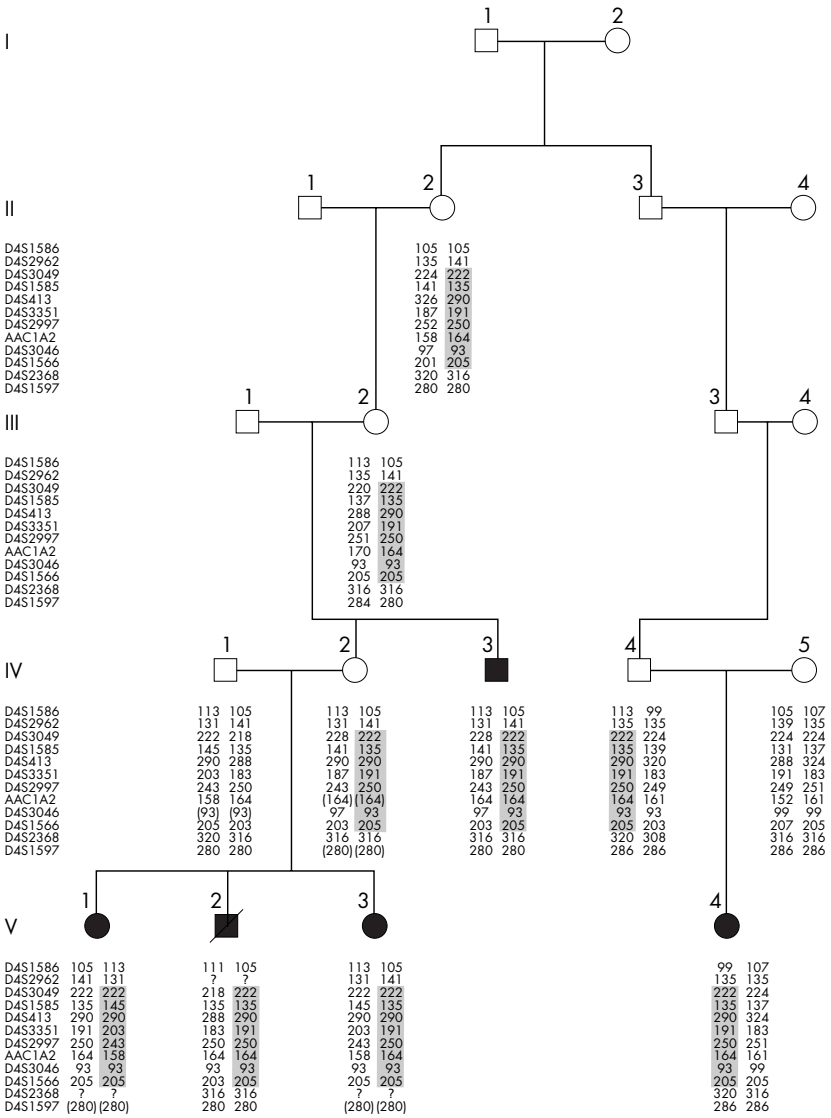

Figure 1 Pedigree structure and haplotypes. Patients are represented as filled symbols. The segregating 4q31-q32 haplotype is depicted. The minimum critical region at chromosome 4 spans 11.7 cM between D4S3049 and D4S1566.

METHODS

Patients

The family described here is a five generation pedigree of native Dutch origin with five cases presenting HSCR. The segregation pattern is compatible with an autosomal dominant mode of inheritance with incomplete penetrance. One branch of the family consists of a sibship with three affected children and an affected uncle. Two sisters (V-1 and V-3) have SS-HSCR. Their brother (V-2) was diagnosed with total intestinal aganglionosis, both large and small intestines were aganglionic. Given the poor prognosis, a joint medical and parental decision was made for conservative care of HSCR; he died at the age of 1 month. Delayed passage of meconium led to the suspected diagnosis of HSCR in all three children. Suction and/or full thickness biopsies were consistent with this diagnosis. Their maternal uncle (IV-3) had undergone surgery during childhood because of SS-HSCR. The other branch of the family contains one affected female member with SS-HSCR (V-4). Her paternal grandfather (III-3) is a cousin of the maternal grandmother (III-2) of the three siblings detailed above (fig 1). Congenital malformations indicative of syndromic HSCR were lacking in all five patients as confirmed by two dysmorphologists (ASB and IvL). Karyotyping was normal in patients V-1 and V-2. Brainstem evoked response audiometry, which we performed because of his expected early death and to exclude hearing loss consistent with Shah-Waardenburg syndrome, showed no abnormalities in patient V-2. Chronic severe constipation was not reported in II-2, III-2, or IV-4, although IV-2 and her father (III-1) experienced severe constipation in childhood. Informed consent was given by the parents of the children and by the adult patient (IV-3). Genomic DNA was isolated from peripheral blood obtained from II-2, III-2, IV-1, IV-2, IV-3, IV-4, IV-5, V-1, V-2, V-3, and V-4 using standard protocols.[34]

Analysis of markers encompassing the *RET* locus

The following markers D10S141 (−1 Mb to *RET*), *RET*int5, D10S1099 (1.4 Mb downstream to *RET*) and five single nucleotide polymorphisms (SNPs) (rs741763, rs2435362, rs2565206, rs2506004, and rs2435357) from the 5′*RET* region were analysed as reported by us and others.[26–28]

Mutational analysis of *RET*

Mutation analysis of the 21 exons of *RET* was performed in patients V-1 and V-4, as described previously.[20]

Genome wide linkage analysis

We performed a systematic genome scan using the ABI Prism MD-10 set (Applied Biosystems) consisting of 382 markers (short tandem repeat polymorphisms; STRPs), with an average spacing of 10 cM. Additional markers for further characterisation of candidate regions were selected from the sex average Marshfield genetic map or were newly designed. Genomic DNA (20 ng) was used as a template in 7.5 µl PCR reactions, with 5 pmol of oligonucleotides, 0.3 U Ampli*Taq* Gold polymerase in Gold buffer (Applied Biosystems) and 2.5 mmol/l $MgCl_2$. The thermal cycling consisted of an initial incubation at 95℃ for 5 minutes, followed by 10 cycles of 95℃ for 30 seconds, 55℃ for 15 seconds, and 72℃ for 30 seconds, then 25 cycles of 92℃ for 30 seconds, 55℃ for 15 seconds, and 72℃ for 30 seconds. PCR products were pooled and loaded on an automated sequencer (ABI3100; Applied Biosystems). Data were analysed using GeneMapper software (version 2.0). Mega2[35] was used to process the genetic data into the appropriate format and perform data validation checks. Simulation analysis to estimate the probability of detecting genetic linkage given the pedigree structure (statistical power) was performed with the SLINK program.[36] Because of uncertainties related to the correct genetic model in this pedigree, parametric and non-parametric linkage analyses were performed using SimWalk2 software (version 2.9).[37]

For the parametric analysis, we specified an autosomal dominant mode of inheritance, a mutant allele frequency of 0.01% with a penetrance of 40% and equal marker allele frequencies. Pedigree location scores were calculated; these location scores are directly comparable to multipoint LOD scores. For the non-parametric analysis, the maximum tree statistic (the largest number of affected members inheriting an allele from one founder allele) is reported. This statistic was designed for traits best modelled by dominant inheritance and was formerly known as STAT B. The NPL_ALL (STAT E) statistic, a measure of whether a few founder alleles are overly presented in affected memberss (suitable for an additive model) is reported as well. A large value of the statistic indicates a high degree of identity by descent allele sharing among the patients, and usually a result >2 can be considered significant. Empirical p values (10 000 simulations) were also obtained. This p value is the probability of obtaining a value for that statistic that is equal to or greater than the observed value, if the trait were not linked to the markers.

RESULTS

Linkage analysis to the *RET* locus and sequence analysis of the *RET* gene

The family we investigated is a five generation pedigree of native Dutch origin with five cases presenting HSCR (fig 1). Because the majority of the multigenerational families with HSCR show linkage to the *RET* gene,[21] we investigated the *RET* locus by haplotype and mutational analysis. We observed that not all five patients (IV-3, V-1, V-2, V-3, and V-4) shared the same haplotype at the *RET* locus (table 1), excluding linkage with the *RET* locus in both branches of this family. However, IV-2, IV-3, V-1, V-2, and V-3 from branch 1 did share the same haplotype encompassing the RET locus, which must be inherited from III-1. We also performed sequence analysis of the entire coding region of the *RET* gene. Direct sequencing revealed no mutations in patients V-1 (branch 1) and V-4 (branch 2).

Genome search

Simulation analysis (using SLINK) yielded an average LOD score of 1.54 and a maximum of 2.15. We performed a genome wide search using 382 STRPs. Results from the parametric linkage analysis excluded most of the genome (data not shown). Only two genomic regions displayed LOD scores >1, a region on chromosome 17 (D17S798, mLOD score = 1.15) and on chromosome 4 (mLOD = 1.84). We tested additional markers and performed haplotype analysis. The region on chromosome 17 was rapidly excluded because one patient (IV-3) was not sharing the haplotype observed in patients from branch 1.

The highest multipoint LOD score (mLOD = 1.84) was obtained for chromosome 4 between markers D4S424 and D4S413. This region fully segregated with the disease phenotype (fig 1). When we saturated the chromosome 4 region with additional markers, a maximum mLOD of 2.7 (fig 2) was reached between markers D4S1585 and D4S3351, which is consistent with suggestive linkage according to the Lander-Kruglyak guidelines for significance thresholds.[38]

We also performed non-parametric analysis for our genome wide scan data. We found non-parametric linkage (NPL) scores >1 only for this region on chromosome 4q. The maximum NPL was found for marker D4S413 (158.0 Mb, NCBI build 35.1) located between markers D4S1585 and

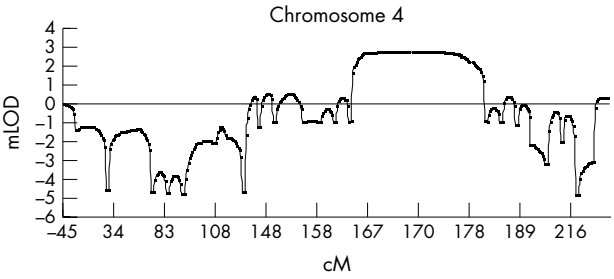

Figure 2 Multipoint LOD score analysis with several markers on chromosome 4. The *x* axis represents the chromosomal position of the markers. The *y* axis indicates the multipoint LOD score, showing a peak LOD score of 2.7.

Table 1 *RET* haplotypes

	II-2	III-2	IV-1	IV-2	IV-3	IV-4	IV-5	V-1	V-2	V-3	V-4
D10S141	3 1	3 2	2 2	4 3	**4 2**	3 4	2 2	**2 4**	**2 4**	**2 4**	**3 2**
rs741763	C G	C G	G G	C C	**C G**	C C	G G	**G C**	**G C**	**G C**	**C G**
rs2435362	C C	C C	A A	C C	**C C**	C C	C A	**A C**	**A C**	**A C**	**C C**
rs2435357	C C	C C	T T	C C	**C C**	C C	C T	**T C**	**T C**	**T C**	**C C**
rs2506004	C C	C C	A A	C C	**C C**	C C	C A	**A C**	**A C**	**A C**	**C C**
rs2565206	G T	G T	G G	G G	**G T**	G G	T G	**G G**	**G G**	**G G**	**G T**
Retint5	3 2	3 2	1 3	2 3	**2 2**	3 1	2 1	**1 2**	**1 2**	**3 2**	**3 2**
D10S1099	5 2	5 5	5 4	5 5	**5 5**	2 3	5 1	**5 5**	**5 5**	**4 5**	**2 5**

Affected family members are in **bold**.

Table 2 Results from the non-parametric analysis on chromosome 4q after fine mapping

Marker	Genetic position (cM*)	Physical position (Mb)†	Max tree‡	NPL_ All§	Empirical p value
D4S1575	132.1	135.1	0.35	0.38	0.418
D4S1644	143.3	142.1	0.80	0.83	0.148
D4S424	144.6	142.6	0.82	0.84	0.143
D4S1625	146.0	143.9	0.84	0.85	0.141
D4S1586	147.1	147.1	0.87	0.86	0.136
EDNRA	–	148.8	0.86	0.86	0.137
D4S2962	153.0	150.7	0.87	0.86	0.137
D4S3049	155.2	155.1	1.60	1.60	0.025
D4S1585	158.0	157.9	2.42	2.42	**0.004**
D4S413	158.0	158.7	2.54	2.55	**0.003**
D4S3351	158.7	159.9	2.54	2.54	**0.003**
D4S2997	158.7	160.0	2.52	2.51	**0.003**
AAC1A2	158.0	160.0	2.51	2.51	**0.003**
D4S3046	162.5	163.7	1.55	1.57	0.030
D4S1566	166.9	167.3	0.99	0.99	0.102
D4S2368	167.6	169.1	0.83	0.84	0.144
D4S1597	169.4	170.2	0.74	0.79	0.163
D4S2431	176.2	175.2	0.51	0.63	0.234
D4S415	181.4	179.1	0.10	0.11	0.776

*According to the Marshfield sex average genetic map; †according to the NCBI physical map, build 35.1; ‡the allele sharing statistic for traits best modelled as dominant inheritance; §the statistic for traits following an additive inheritance. Significant p values are shown in bold type.

D4S3351 (NPL = 2.55, p = 0.003), under both dominant and additive mode of inheritance (table 2).

Recombination events can be identified in individual IV-4 and show that marker D4S2962 at the centromeric site, and D4S2368 at the telomeric site limits the critical region. The maximum critical region between D4S2962 and D4S2368 spans approximately 16.4 cM (19.7 Mb according to NCBI physical map, build 35.1), the minimum shared region extend from marker D4S3049 until D4S1566 (11.7 cM or 12.2 Mb).

5′ *RET* common risk haplotype analysis

To test whether non-coding low penetrance variants just before or within intron 1 of *RET* were associated with HSCR susceptibility in a part of this family, we typed five SNPs all being part of an ancestral haplotype.[27] Spouse IV-1 (who had married into the family) was homozygous for the GATAG haplotype that is part of the core risk SNP haplotype detected in European, European American, and Asian American patients with sporadic HSCR.[27 28 39] The three affected siblings (V-1, V-2, and V-3) were thus carriers of the core risk haplotype. However, the other two patients (IV-3 and V-4) did not carry the 5′ *RET* common risk haplotype (table 1).

DISCUSSION

We identified a five generation family with five patients affected with HSCR. The patients were connected to a common ancestor within 3–4 generations. Two branches with HSCR patients within the family were identified. The inheritance pattern in the family is compatible with an autosomal dominant mode of inheritance with reduced penetrance of a single mutated gene.

Because *RET* is the major gene involved in HSCR susceptibility in multigenerational families,[21] linkage to the *RET* locus and mutations in the coding sequence of *RET* were first excluded in both branches. However, under the assumption that HSCR is caused by two separate genes in the two branches, an alternative hypothesis would be that the individuals of the main branch with four affected individuals (the core two generation family) carry a hitherto unidentified *RET* mutation (introduced into the family by III-1). As an oligogenic model with the contribution of two or more loci has been previously demonstrated for HSCR (9q31 and *RET*), we hypothesised that even if there is true linkage to *RET* in branch 1, this does not mutually exclude the existence of an additional locus in this family. Subsequently, we performed a genome wide scan to map additional disease gene(s) in this family. Model free or non-parametric linkage analysis methods are more robust than parametric or model dependent analysis when the mode of inheritance or the genetic model is uncertain, such as this pedigree. Consequently, we performed both parametric and non-parametric linkage analysis. Both methods highlighted the same region on chromosome 4q, and no other known HSCR susceptibility loci such as those at chromosomes 3, 9, and 19[21 31] showed positive LOD scores. When adjacent markers for chromosome 4 were tested, the evidence of linkage became stronger and we could observe a common haplotype, extending at least 11.7 cM, which was inherited by all the affected individuals from their common ancestor. Our results indicate the existence of a novel HSCR susceptibility locus on chromosome 4q.

Clearly, the chromosome 4q locus has incomplete penetrance. Does this 4q locus solely lead to HSCR or alternatively, is the phenotype only expressed in the presence of other susceptibility loci? Modifier loci either can increase susceptibility and severity of the phenotype or can act protectively to confer resistance to the disease in the face of a predisposing mutation.[40] Can variants within the *RET* gene explain the difference in penetrance observed in both branches? We investigated whether all patients shared a haplotype similar to the common risk haplotype defined by SNPs located in the 5′ region of the *RET* locus reported in Dutch HSCR patients.[26] We identified non-risk haplotypes in patients IV-3 and V-4. However, the spouse IV-1 and the three affected children (V-1, V-2, and V-3) were homozygous and heterozygous, respectively, for the GATAG haplotype, which contains the core risk haplotype detected in European, European American, and Asian American patients with sporadic HSCR.[27 28] The third and fourth SNPs (RET3+, rs2435357 and IVS1+9494, rs2506004) are particularly interesting as disease associated *RET* variants, because of the homology and evolutionary conservation between rodents and primates and the differences in allele/genotype frequencies among patients and controls.[27] Recent data show that RET3+ might lie within, and might compromise the activity of an enhancer-like sequence in the *RET* intron 1.[28] These findings make us hypothesise that the 5′ *RET* risk haplotype in combination with the identified chromosome 4 locus might play a role in the penetrance and severity observed in the sibship with three affected children.

We looked for candidate genes in the minimum 12.2 Mb linked region at 4q31.3–32.3 (between markers D4S3049 and D4S1566) in the human genome sequence. This region contains at least 57 genes, in accordance with the National Center for Biotechnology Information (build 35.1) of the human genome and the Ensemble Genome Browser. The maximum 20 Mb linked region between D4S2962 and D4S1597 encompasses 93 genes, including several interesting functional candidate genes that are proposed to be involved in neural crest development or neuronal development.

Unfortunately, the maximum genetic interval contains far too many candidates to begin functional evaluation of each gene individually; the best positional and functional candidate we could identify is *Mab21L2* (named after male abnormal 21 in *Caenorhabtidis elegans*). *Mab21L2* is expressed in the central nervous system and neural crest in midgestation embryogenesis in mice.[41] Furthermore, *Mab21L2* is linked to the transforming growth factor-β signalling pathway to which *ZFHX1,* the gene involved in Mowat-Wilson syndrome, a syndromic form of HSCR, also belongs.[13 42] *ZFHX1B*

ELECTRONIC DATABASE INFORMATION

Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.gov/Omim (for MIM 142623)

functions as a transcriptional repressor.[42] For these reasons we sequenced the complete coding region of the *Mab21L2* gene for mutations; however, we did not identify a sequence variant in *Mab21L2*. Besides *Mab21L2*, many other candidate genes are located in the region. Several proteins encoding neuropeptide Y receptors (NPY1R, NPY2R, and NPY5R) are located in the minimum 12.2 Mb linked region. In mammals, NPY, the ligand, is mainly found in cells derived from the neural crest, and is widely distributed in the central and peripheral nervous system.[43] Furthermore, the gene for the secreted frizzled related protein 2 (*SFRP2*) is located in this region.[44] Wnt-frizzled signalling is involved in neural crest formation.[45]

The 4q locus may be necessary but not sufficient to cause HSCR in the absence of modifying loci elsewhere in the genome. Linkage analysis in additional multigenerational HSCR families and association studies in sporadic patients with high density marker sets covering the entire interval will be necessary to confirm this suggestive linkage to chromosome 4q. Eventually, identification of the causative gene defect will specify the susceptibility to HSCR conferred by this novel locus at 4q31.3-q32.3.

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