

## COLON CANCER

Rescue of human *RET* gene expression by sodium butyrate: a novel powerful tool for molecular studies in Hirschsprung disease

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**Background:** The *RET* gene encodes a tyrosine kinase receptor involved in different human neurocristopathies, such as specific neuroendocrine tumours and Hirschsprung disease (HSCR). Gene expression is developmentally regulated and the *RET* transcript is undetectable in most adult cells, including lymphocytes. The impossibility of performing functional studies on *RET* mRNA has to date limited the detection and characterisation of an indefinite proportion of gene anomalies that cannot be identified by conventional DNA genomic screening in HSCR cases.

**Aims:** Development of a protocol suitable to activate *RET* expression in *RET* negative cell lines and therefore to investigate directly *RET* mRNA, extending the conventional gene mutation analysis to detection of splicing anomalies and impaired expression of the *RET* gene.

**Methods:** The effect of sodium butyrate (NaB), a histone deacetylase inhibitor, on rescuing *RET* expression was tested by one round of reverse transcription-polymerase chain reaction from total RNA of treated lymphoblasts from both HSCR patients and control individuals.

**Results:** Analysis of *RET* expression was possible by NaB treatment of *RET* negative cells, such as lymphoblasts. This treatment allowed us to detect impaired *RET* expression as well as a splicing defect in two HSCR patients previously believed to be devoid of any gene abnormality.

**Conclusions:** The full application of the proposed protocol in most of the unexplained HSCR cases will allow us to establish the precise role of *RET* not only in causing but also in predisposing to HSCR pathogenesis.

The *RET* proto-oncogene (REarranged during Transfection) encodes a tyrosine kinase receptor predominantly expressed during neural crest development and involved in different human neurocristopathies.<sup>1</sup> Germline *RET* mutations have been found in association with both inherited cancer syndromes, including multiple endocrine neoplasia type 2A (MEN2A), type 2B (MEN2B), and familial medullary thyroid carcinoma, and Hirschsprung disease (HSCR), a developmental disorder characterised by the absence of enteric neurones in variable lengths of the distal gastrointestinal tract.<sup>2</sup> Loss of function, low penetrant *RET* mutations have been identified in only 10–40% of total HSCR cases while a small proportion of patients (5–10%) show alterations in other genes, generally related to the developmental programme of neural crest cells.<sup>3,4</sup> By using sibpair analysis, a recent study has recognised three HSCR susceptibility loci at 3p21, 10q11, and 19q12 which may clarify the molecular basis of a great proportion of unexplained HSCR cases.<sup>5</sup> The locus in 10q11 probably corresponds to *RET*, confirming the central role played by this gene in HSCR pathogenesis. Taking into account the persistent failure to detect *RET* coding sequence mutations in a proportion of familial cases found to be linked to the gene, it has recently been suggested that genetic defects in introns and in the promoter region can contribute in some cases to the HSCR phenotype.<sup>5–7</sup>

Moreover, several common polymorphisms of the *RET* proto-oncogene have been associated with a variable risk of HSCR development. In particular, in different populations *RET* single nucleotide polymorphism (SNP) alleles are present with different frequencies in HSCR patients with respect to controls,<sup>8,9</sup> while specific haplotypes have been demonstrated to display either protective or predisposing effects, and/or to modulate the severity of the resulting phenotype.<sup>10,11</sup>

To date, no *RET* SNP allele has been proved to have any effect on Ret function; on the other hand, the functional variants responsible for the predisposing or protective effect displayed by specific *RET* haplotypes, and expected in linkage disequilibrium with them, have not yet been identified. These latter variants may be represented by genetic defects in regulatory regions, contributing to HSCR by decreasing the expression level of correct and active Ret receptor.

Unfortunately, the search for non-coding *RET* mutations associated with HSCR disease has been hampered by: (i) the size of the gene (52.4 kb); (ii) the presence of large introns (intron 1 spans more than 20 kb); (iii) the abundance of DNA repeats; and (iv) the lack of mutational hotspots. As already proved for other genes such as ATM (ataxia-telangiectasia mutated) and NF1 (neurofibromatosis type 1),<sup>12,13</sup> direct investigation on splicing and expression would be very useful to detect uncovered *RET* molecular defects. At present, such a study is not possible as the transcript is undetectable in the majority of adult human tissues, including peripheral blood lymphocytes.<sup>14,15</sup> Indeed, it is well known that *RET* expression is mostly confined to specific stages during embryogenesis when the gene is transcribed in tissues derived from embryonic neuroectoderm and the developing urogenital system.<sup>16,17</sup>

**Abbreviations:** ATM, ataxia-telangiectasia mutated; DHPLC, denaturing high performance liquid chromatography; G3PDH, glucose 3-phosphate dehydrogenase; HSCR, Hirschsprung disease; MEN, multiple endocrine neoplasia; NaB, sodium butyrate; NF1, neurofibromatosis type 1; RET, REarranged during Transfection; RT-PCR, reverse transcription-polymerase chain reaction; SNP, single nucleotide polymorphism; SSCP, single strand conformation polymorphism.

In the past few years, we and others have approached the study of the functional effect of *RET* splicing mutations (synonymous mutations or splice site alterations) by using RNA obtained from illegitimate transcription coupled with nested reverse transcription-polymerase chain reaction (RT-PCR).<sup>18–20</sup> Indeed, in immortalised lymphoblastoid cells a minimal amount of *RET* product can be detected after two rounds of PCR (for a total of 70 cycles).<sup>21</sup> However, illegitimate transcription does not reflect physiological gene expression and false positive products can be obtained as a result of its use. These considerations compelled us to confirm results from RT-PCR with mini gene constructs, cloning genomic fragments of the *RET* gene in the expression vector pSPL3.<sup>18–20</sup> Unfortunately, such a time consuming and expensive approach cannot easily be used for rapid screening of *RET* splicing anomalies or to assess defective allelic expression.

In a previous report, we demonstrated that sodium butyrate (NaB), a histone deacetylase inhibitor, upregulated *RET* transcription in *RET* positive cell lines.<sup>22</sup> In particular, NaB treatment increased *RET* transcription in IMR32 cells, already displaying low levels of its mRNA, while it had no effect on MTC-TT cells, characterised by a plateau level of *RET* mRNA.<sup>22</sup> Accordingly, we also demonstrated that NaB treatment increased the endogenous level of *RET* histones acetylation (generally associated with transcriptionally active chromatin) in IMR32 but not in MTC-TT cells. These data strongly suggest that NaB treatment mimics a physiological regulatory mechanism of the *RET* gene.

In the present study, we show that NaB also stimulates a detectable level of transcription in two *RET* negative cell lines, SK-N-MC and lymphoblastoid cells. We started from such an observation to develop an original methodological approach to study the molecular basis of HSCR and to perform functional tests on *RET* mRNA obtained from cell sources easily available and routinely used for genomic DNA extraction, such as lymphocytes or lymphoblasts. The full application of the proposed NaB treatment protocol to unexplained HSCR cases (still representing the majority of all cases) will allow us to establish the precise role of *RET* in the pathogenesis of HSCR.

## METHODS

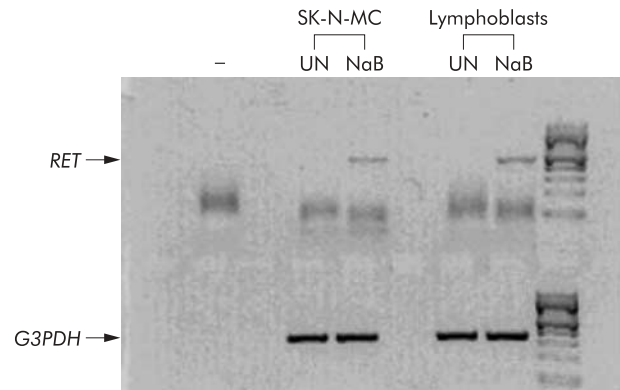
### Cells and NaB treatment

The human neuroblastoma cell line SK-N-MC and lymphoblasts from HSCR patients and controls were grown in RPMI medium (Hyclone Laboratories, Logan, Utah, USA), supplemented with 10% fetal calf serum (Gibco BRL, Life Technologies Italia Srl, Milano, Italy), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. NaB (B 5887; Sigma-Aldrich Srl, Milano, Italy) was dissolved in water (1 M stock solution) and delivered to cells at a final concentration of 5 mM. Treatment was performed overnight. The day after, cells were given fresh medium and NaB was delivered for another night. RNA extraction was performed using commercially available TRIzol (Gibco BRL).

### RNA analysis

Random hexamer primed cDNA was prepared from 500 ng of total RNA. The human housekeeping *G3PDH* gene (glucose 3-phosphate dehydrogenase) was amplified with forward primer 5'-TGAAGGTCGGAGTCAACGGATTGGT-3' and reverse primer 5'-GCAGAGATGATACCCTTTGGCTC-3'. The human *RET* gene was amplified with forward IP18F primer 5'-GGATTTCGGCTTGCCGAG-3' and reverse IP20R primer 5'-CCATGTGGAAGGGAGGGCTC-3'.

We analysed RNA from NaB treated lymphoblasts of eight individuals (both HSCR patients and controls). Individuals 1–4 carry different exon 14 genotypes. Random hexamer primed total cDNA (3 µl) and 200 ng of genomic DNA of each individual were used to perform PCR reactions with the exonic



**Figure 1** Appearance of *RET* transcript in *RET* mRNA negative cells after sodium butyrate (NaB) treatment. Results obtained after a single round of *RET* amplification (38 cycles) from retrotranscribed diluted samples of total RNA obtained from untreated (UN) and NaB treated SK-N-MC and lymphoblastoid cell cultures. Glucose 3-phosphate dehydrogenase (*G3PDH*) expression was used as an internal standard and the minus symbol indicates the reaction blank control.

primers 14F (5'-CTCCTCATCGTGGAGTACGC-3') and 14R (5'-CTCGGCCAGATACTGCATCC-3') (38 cycles). Restriction enzyme and single strand conformation polymorphism (SSCP) analysis were performed, as previously described.<sup>11,23</sup> Individual No 5 is a normal control while individual No 6 is a HSCR patient carrying an intronic mutation, IVS12+19 C>T. RT-PCR from NaB induced lymphoblast RNA of these two latter individuals was performed as previously described.<sup>20</sup>

Individuals belonging to the family reported below were genotyped using the intragenic polymorphisms of *RET* exons 2, 13, and 19, as described by Griseri and colleagues.<sup>11,23</sup> Individuals 7 (control) and 8 (HSCR patient) were both heterozygous for the 2307T>G SNP of exon 13. RT-PCR was performed from total cDNA with primers 4F (5'-GAAAAGTGGTCAAGGCAA-3') and 18R (5'-GAATCTTCATCTCCGCC-3') (38 cycles), and products digested with *TaqI* to verify the presence of the two alleles in the transcripts.

## RESULTS

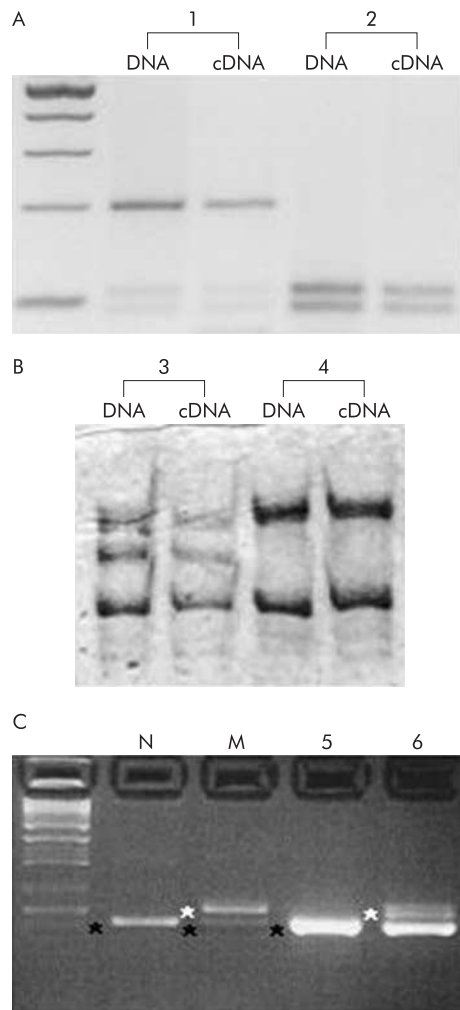
### NaB treatment: rescue of gene expression in *RET* negative cells

*RET* upregulation obtained with NaB treatment of *RET* positive cell lines compelled us to also test *RET* negative cells, such as lymphoblasts and neuroblastoma SK-N-MC cells, for NaB dependent expression of the gene.

As shown in fig 1, in total RNA from untreated cells, in agreement with previous data,<sup>24,25</sup> *RET* expression was not detectable while in NaB treated cell lines, both lymphoblasts and neuroblastoma cells, we were able to detect *RET* transcript after a single round of PCR.

### Comparison of *RET* analysis between genomic DNA and NaB induced cDNA

As a first application of the NaB treatment to lymphoblasts, we assessed whether *RET* cDNA analysis could reproduce results from genomic DNA. In fig 2A we report the migration pattern of endonuclease digestion of a known polymorphism in exon 14 of the *RET* gene, 2508C>T (S836S), from both cDNA and genomic DNA of a heterozygote for the common T variant (individual No 1), and of a CC control (individual No 2). Moreover, fig 2B shows the results of SSCP analysis of *RET* exon 14 from both cDNA and genomic DNA in two additional individuals, a normal control (individual No 4) and a HSCR patient (individual No 3). In individual No 4, only wild-type alleles are present, while individual No 3 carries the 2436C>T



**Figure 2** Analysis of RNA from patients with Hirschsprung disease (HSCR) and controls. Lymphoblastoid cells from six individuals were used for cDNA synthesis and transcripts analysis. (A) Restriction analysis of *RET* exon 14 from genomic DNA and cDNA is shown. Individual No 1 is heterozygous for the 2508C>T polymorphic variant while only wild-type alleles are present in individual No 2. (B) Single strand conformation polymorphism profiles of individuals carrying the 2436C>T mutation (individual No 3) and the wild-type control (individual No 4) are compared. (C) The intronic *RET* mutation IVS12+19C>T was analysed from two RNA sources. In the first two lanes, cDNA was obtained from COS7 cells transfected with pSPL3 constructs carrying the wild-type (N) and mutated (M) alleles.<sup>20</sup> Lanes 5 and 6 refer to reverse transcription-polymerase chain reaction samples from sodium butyrate treated cells of one control (individual No 5) and the patient carrying the splicing variant (individual No 6). Both correctly processed and aberrant transcripts are shown. An additional band corresponding to the heteroduplex product is present in the last lane.

missense mutation (R813W), previously described in an unrelated HSCR patient.<sup>18</sup> In all of these samples both the amount and migration pattern of PCR products from genomic DNA were comparable with those obtained from NaB induced cDNA. This result represents the basis of developing a rapid primary screening of *RET* unknown mutations in which the entire cDNA is used as a direct substrate for traditional mutation detection methods such as denaturing high performance liquid chromatography (DHPLC), SSCP, etc.

#### Detection of *RET* abnormal splicing in a HSCR patient

To determine if NaB treatment allowed for efficient detection of *RET* anomalous transcripts, we performed RT-PCR analysis on total RNA from a HSCR patient carrying the intronic *RET*

mutation IVS12+19C>T, already demonstrated to cause aberrant RNA processing.<sup>20</sup> We previously showed, using mini gene constructs, that such an intronic mutation introduces a novel splice donor site, recognised and actively used during RNA splicing, leading to an alternative transcript bearing 17 additional nucleotides retained from the 5' end of intron 12. In the present work, we assessed whether lymphoblastoid cells treated with NaB could maintain the same pattern of alternative splicing previously demonstrated in vitro. As shown in fig 2C, an expected fragment of 187 bp, corresponding to the normally spliced transcript, was present in both the patient and control cDNA samples while an additional larger product of 204 bp, corresponding to the aberrantly spliced transcript, was detected only in the HSCR individual, in agreement with the splicing pattern obtained from the use of mini gene constructs (see legend to fig 2C). This result suggests that our approach can be used for primary screening of unknown *RET* splicing anomalies, undetectable with standard genomic analysis.

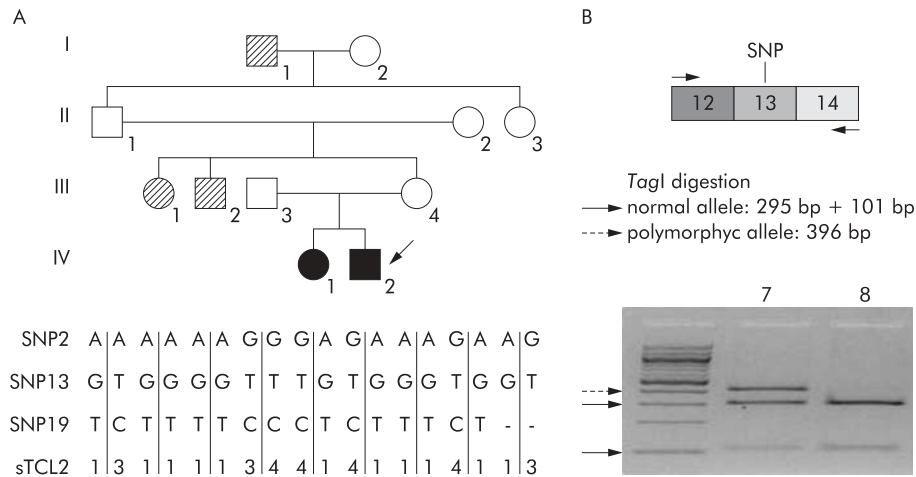
#### Loss of heterozygosity of the *RET* cDNA in a familial case of HSCR disease

In HSCR disease, *RET* coding mutations account for only a small proportion of cases, suggesting the existence of non-coding variations in the *RET* gene and/or additional genetic risk factors.<sup>5</sup>

In order to identify *RET* expression defects otherwise undetectable, we performed NaB treatment on lymphoblastoid cells from a HSCR patient affected with a familial long form of congenital megacolon. Linkage analysis previously showed that the disease phenotype recurring in this family was closely linked to *RET* gene markers (fig 3).<sup>26</sup> However, screening of all 21 exons and the intronic junctions did not show any type of mutation. To investigate the possible presence of a *RET* deletion, we characterised genomic DNA for three SNPs distributed along the whole gene. However, the heterozygous status of the proband for SNPs 2, 13, and 19 excluded the presence of genomic deletions involving these polymorphic loci. Lymphoblasts from the proband and a control individual, both heterozygous for a known polymorphism in exon 13 (2307G>T), were treated with NaB. Endonuclease digestion of RT-PCR products was used to identify the two alleles in RNA: the transversion G>T eliminates the *TaqI* recognition site leading to a variant fragment of 396 bp. As shown in fig 3, the different products expected from digestion of variant and normal alleles (396 bp+295 bp+101 bp) were present in the transcript obtained from the control sample (individual No 7) while only the bands corresponding to the normal allele (295 bp+101 bp) were observed in the patient (individual No 8). These data clearly indicate loss of expression of one *RET* allele, which segregates with the disease in the pedigree, and confirm the hypothesis that non-coding mutations can significantly contribute to *RET* haplo insufficiency and therefore to the pathogenesis of HSCR.

#### DISCUSSION

It is well established that histone acetylation is an important mechanism that modulates gene expression by altering accessibility of transcription factors to DNA. Switch from inactive to active chromatin is often accompanied by histone hyperacetylation of critical sites in gene regulatory regions.<sup>27</sup> In a previous paper, we demonstrated that the histone deacetylase inhibitor NaB increased *RET* transcription in cells displaying low mRNA levels while it had no effect in cells already expressing high mRNA levels.<sup>22</sup> Moreover, we identified two *RET* upstream regions sensitive to NaB treatment, the SOX10/Pax3 enhancer and the minimal promoter, supporting the view that acetylation of *RET* histones is a crucial and physiological regulatory mechanism of *RET* transcription.<sup>22</sup> Here we have shown that NaB treatment induces the appearance of *RET* transcript in *RET* negative cells, such as lymphoblasts and



**Figure 3** Detection of a *RET* expression defect. (A) Haplotype reconstruction at the *RET* locus in a family with Hirschsprung disease (HSCR); filled symbols represent histologically confirmed long segment HSCR patients while hatched symbols represent individuals showing a disease status, not studied by histological examination but clinically documented, as previously described.<sup>26</sup> The two alleles of single nucleotide polymorphisms (SNP2, SNP13, and SNP19, and alleles of the microsatellite sTCL2 (previously reported as corresponding to RET by Yin and colleagues<sup>26</sup>) are indicated. (B) cDNA analysis from the proband (individual No 8, corresponding to individual IV-2 of the pedigree) and a control (individual No 7), both heterozygous for a known polymorphism in exon 13 (2307G>T). A schematic representation of the polymerase chain reaction products and the subsequent *TaqI* digestion is shown. Arrows indicate the normal and polymorphic alleles.

the SK-N-MC cell line. Starting from such an observation we have developed a novel and useful approach to analyse the *RET* transcription product. In particular, we have demonstrated that, after NaB treatment, *RET* cDNA can be analysed directly through either restriction enzyme digestion (normally used for typing *RET* polymorphisms) or SSCP analysis (a common method to investigate *RET* mutations). Indeed, we found a similar intensity and migration pattern of RT-PCR products obtained after NaB treatment compared with PCR products from genomic DNA. These data clearly indicate that *RET* analysis normally used for genomic DNA assay (SSCP, DHPLC, sequencing, and others) can now also be applied to the full length cDNA.

Splicing alterations are believed to represent 15% of all gene defects but they are rarely detected when located far from the splice canonical sites.<sup>28</sup> For this reason, it has been generally suggested that gene mutation analysis should also cover introns, an approach applicable only to a small number of genes characterised by reduced number and size of intronic regions. Analysis of gene transcripts is a useful alternative to detect the effect of splicing mutations, as suggested by the high number of such anomalies demonstrated in genes normally expressed in lymphoblasts, such as *ATM* and *NE*.<sup>12-13</sup> Another important achievement of the present pilot study is the demonstration that NaB treatment allows direct detection of *RET* anomalous transcripts in mRNA obtained from lymphoblasts of HSCR patients, without inducing aberrant splicing per se, as shown in fig 2B. Lymphoblasts from both HSCR patients and controls were treated with NaB but only patient carrying the IVS12+19C>T mutation showed an aberrant spliced product. These data confirm our method as a rapid primary screening for splicing mutations, provided positive results obtained by NaB treated lymphoblasts are followed by specific genomic analysis to avoid the possibility of false positives such as, for instance, those derived from tissue specific patterns of splicing.

The potency of NaB treatment combined with the fact that it seems to reproduce a physiological mechanism of transcription convinced us to undertake a NaB based approach for the study of possible *RET* expression defects.

As hypothesised, we demonstrated defective *RET* expression in a familial case of HSCR showing no mutation in the coding region of the gene. The observed loss of heterozygosity in the cDNA might be caused by an as yet unidentified defect in the

*RET* transcription regulatory region (promoter and 5' region) and/or in post-transcriptional control (splicing defect or instability of allele specific *RET* mRNA), both impairing correct *RET* expression. Characterisation of the functional variant responsible for this effect is currently under investigation. This result is in agreement with the hypothesis that non-coding *RET* variations have been underestimated and this could explain the discrepancy between the proportion of HSCR families linked to *RET* (88%) and the proportion of identified mutations (<40%).<sup>5</sup> Hence NaB treatment could allow the detection of a wide spectrum of *RET* defects which we were unable to identify by standard genomic analysis.

As a further useful application, NaB treatment could be used to investigate additional elements possibly involved in HSCR susceptibility such as the potential functional effect of *RET* polymorphic variants and the amount of the two terminal isoforms, RET9 and RET51.<sup>10-11, 29-30</sup> Indeed, the full application of the proposed NaB treatment protocol may allow dissection of the possible functional contribution of SNPs, such as the 135G>A in exon 2 of the gene or the newly identified SNPs of the *RET* promoter.<sup>9-11</sup> In addition, the expression pattern of the RET9 and RET51 isoforms, recently demonstrated to have different biological and signalling properties, may be correlated with the development of HSCR disease and allied related pathologies such as MEN2A and MEN2B.<sup>29-30</sup>

In conclusion, our observation that NaB treatment activates *RET* expression in cultured cells represents a novel powerful tool to test the relative amount and quality of the *RET* transcript in cell sources easily available, such as lymphocytes or lymphoblasts. The full application of the proposed NaB treatment protocol to unexplained HSCR cases (still representing the majority of all cases) and other *RET* related pathologies will allow the establishment of the precise role the *RET* proto-oncogene plays in their development.

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