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Novel dedicator of cytokinesis 8 mutations identified by multiplex ligation-dependent probe amplification

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Key words Dedicator of cytokinesis 8; novel mutations; multiplex ligation-dependent probe amplification

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Abstract

Dedicator of cytokinesis 8 (DOCK8) deficiency is an innate error of adaptive immunity characterized by recurrent infections with viruses, bacteria and fungi, very high serum IgE concentrations and a progressive deterioration of T- and B cell-mediated immunity. We studied the genetic and immunological features of two sisters (aged 11 and 6 years). Mutational analysis of genomic DNA and cDNA from the patients and their parents, by a combination of PCR and bidirectional targeted sequencing, failed to localize the mutation site. However, a multiplex ligation-dependent probe amplification (MLPA) assay revealed two novel large deletions, del1-14 exons and del8-18 exons, of *DOCK8* in both patients. Immunoblot analysis demonstrated that DOCK8 protein was absent from the peripheral blood lymphocytes of both patients. These data suggest that compound heterozygous del1-14 exons and del8-18 exons mutations result in a loss of function of DOCK8 protein and a typical DOCK8 deficiency phenotype.

Introduction

Dedicator of cytokinesis 8 (DOCK8) deficiency (OMIM #611432), caused by a biallelic loss-of-function mutation of *DOCK8,* is characterized by food allergy, asthma, atopic dermatitis, eosinophilia and high serum IgE levels. Affected individuals typically suffer from recurrent respiratory tract infections and are highly susceptible to cutaneous viral infections caused by herpesviruses, *Molluscum contagiosum* virus, and *Human papilloma* virus, and fungal diseases, such as chronic mucocutaneous candidiasis (CMC) (1-5). The less frequent and more unusual clinical signs observed in patients with DOCK8 deficiency include gastrointestinal infections, granulomatous soft tissue lesions in the central nervous system, leiomyosarcoma, lymphoma, squamous cell carcinoma and autoimmunity (3, 4, 6, 7). DOCK8

deficiency was initially thought to be the autosomal recessive form of hyper IgE syndrome (HIES) (2, 8, 9, 10), but it actually fulfills the criteria of severe combined immunodeficiency in most affected patients and only rarely presents with a mild phenotype, such as isolated atopic dermatitis (7). *DOCK8* mutations may be homozygous or compound heterozygous and typically result in a loss of function of DOCK8. The human *DOCK8* gene consists of 48 exons and is spread over 250 kb on chromosome 9p24.3 (1). DOCK8 is one of the 11 members of DOCK180 family of atypical guanine nucleotide-exchange factors, which function as activators of small G proteins and are involved in regulating the actin cytoskeleton (11-13).

Immunological studies in patients with DOCK8 deficiency have revealed T-cell depletion, impaired T-cell expansion *in vitro*, and secondary antibody responses to specific antigens (4, 14-16). Levels of B-cell proliferation and immunoglobulin production upon stimulation with Toll-like receptor (TLR)) 9 were considerably lower in DOCK8-deficient patients than in controls (16). DOCK8 may function as an adaptor linking TLR-MyD88 signaling pathways essential for TLR-9-driven B-cell activation and DOCK8-deficient patient have fewer CD27-positive memory B cells, potentially accounting for their low IgM level (17). DOCK8 plays a key role in CD8 T-cell survival and function in both humans and mice (14, 15). Impaired $CD4^{+}IL\text{-}17^{+}$ T cell-mediated immunity has also been reported, potentially accounting for the high level of susceptibility of affected patients to chronic mucocutaneous candidiasis (18). The identification of *DOCK8* mutations is challenging, because large deletions may overlap and the data obtained with Sanger sequencing technology may be misleading. We report here two patients who presented with a DOCK8 deficiency phenotype caused by previously unknown large deletions of *DOCK8*.

Materials and Methods

Patients

All the studies reported here were approved by the Regional Ethics Committee of the University of Debrecen Medical and Health Science Center. Informed consent was obtained from the parents. We analyzed two non consanguineous Hungarian girls, patient P1, who was 11 years old, and her sister, patient P2, who was six years old, both with recurrent viral and bacterial infections and CMC. The patients' mother is healthy; their father has had recurrent herpesvirus disease and allergic rhinitis. *Patient P1* was referred to our department at the age of nine years, with HIES diagnosed on the basis of high serum IgE concentration, eosinophilia and atopic dermatitis, which had begun at the age of two and a half years (Table 1). She received routine immunization with no complications. On admission, she had oral and nail candidiasis, gingivitis and bronchiectasis. She had had recurrent *Herpes simplex* virus skin infection since the age of two years, which had spread to both eyelids and caused keratitis, which was effectively treated with acyclovir. She was repeatedly treated for upper and lower respiratory tract infections and bacterial skin infections. She had marked T-cell lymphopenia and a moderately low number of natural killer cells, low serum IgM and high IgA concentrations and low levels of anti-pneumococcal antibodies (Table 1). She had suffered from severe allergy to various food allergens. At the age of 10 years, she was treated at another hospital for an intracerebral abscess of undefined origin, which resulted in a severe deterioration of her physical, mental and psychological condition. She is now receiving rehabilitation support. Cytogenetic studies her to have a normal karyotype. Here National Institutes of Health (NIH) score was only 35, suggesting that autosomal dominant (AD)-HIES was not likely, but the full-length *STAT3* (signal transducer and activator of transcription 3) gene was analyzed and found to have a wild-type sequence. Based on the patient's history, clinical signs and immunological findings, DOCK8 deficiency was suspected and the *DOCK8*

gene was analyzed. *Patient P2,* the sister of P1, was referred to us at the age of four years, with suspected HIES. She underwent routine vaccination with no complications. She had had atopic dermatitis since the age of 2.5 years, and had a high serum IgE level, and moderately high blood eosinophil counts. Recurrent respiratory tract infections, including pneumonia, began at the age of 10 months. Serum IgM level was low, IgA level was high and P2 had T-cell lymphopenia (Table 1). Her NIH score was 15, arguing against AD-HIES, and an analysis of the *STAT3* gene revealed a wild-type sequence with no mutations. At the age of five years, P2 underwent successful hematopoietic stem cell transplantation, with matched unrelated donor cells, at another hospital.

Immunological assays

Serum immunoglobulin isotypes M, G, A, and E were determined in routine laboratory assays. Lymphocyte subsets were analyzed with a Coulter 500 flow cytometer. Enzyme-linked immunosorbent assays were used to quantify anti-capsular IgG antibodies against pneumococcal polysaccharides.

DNA sequencing

Genomic DNA (gDNA) was isolated from peripheral blood mononuclear cells (PBMCs). Exons 1 to 48 of DOCK8 and the flanking intronic regions were amplified by polymerase chain reaction (PCR). The primer sequences are available on request. Total RNA was isolated from PBMCs and RNA was reverse-transcribed with the Superscript III first-strand synthesis supermix (Invitrogen). Complementary DNA (cDNA) sequencing of *DOCK8* and glyceraldehyde 3-phosphate dehydrogenase cDNAs was performed after PCR amplification across all exons. The

primer sequences are available on request. Mutational analysis was performed with the BigDye Terminator Cycle sequencing kit, and an ABI 3130 analyzer (AppliedBiosystems, Foster City, CA). The numbering of DNA and RNA mutations was based on the cDNA sequence (Ensemble: ENST00000453981), with the A of the translation initiation codon counted as the first nucleotide (19).

MLPA

MLPA (multiplex ligation-dependent probe amplification) analysis was carried out according to the kit manufacturer's instructions (MRC-Holland, Amsterdam, The Netherlands). Briefly, 300 ng DNA was denatured and hybridized overnight at 60°C with the SALSA probe mix P385-A1 *DOCK8* and P386-*A1DOCK8-STAT3*. Samples were then treated with DNA ligase for 15 min at 54°C. The reaction was stopped by incubation at 98°C for 5 min. PCR amplification was carried out with specific fluorescent labeled PCR primers.

Amplification products were subjected to electrophoresis on an ABI PRISM 3100 Genetic Analyzer. Fluorescence intensity was measured after inter-sample and intra-sample normalization.

Western blotting

Protein extracts of PBMCs were subjected to denaturing polyacrylamide gel electrophoresis and the bands obtained were transferred to PVDF membranes (Millipore Corp, Billericay, MA). For detection, the blot was incubated with polyclonal rabbit anti-DOCK8 or anti-actin antibodies (Sigma-Aldrich), and then with horse-radish peroxidase-conjugated anti-rabbit IgG (GE Healthcare, Buckinghamshire, UK). DOCK8 and actin were visualized by chemiluminescence (Termo Scientific, Rockford, IL).

Results

Analysis of the DOCK8 gDNA with PCR amplicons

PCR amplification of exons and flanking intronic fragments of the *DOCK8* gDNA gave normal bands for exons 1-8 and 14-48 (Fig. 1a). We sequenced the exon 8 and exon 14 fragments from P1 and found that we had actually amplified fragments from tubulin-tyrosine ligase-like protein 7 (TTLL7) rather than the *DOCK8*-specific exon 8 sequences observed in controls (Supplementary Fig. 1). Amplification of exon 14 fragments from the patients' DNA actually led to amplification of part of the PR domain-containing protein 11 (PRDM11) gene, rather than the *DOCK8*-specific exon 14 sequences obtained with the controls (Supplementary Fig. 1). Furthermore, we obtained single bands on the amplification of exon 12 from both patients. The sequencing of these bands showed them to be p21 protein (CDC42/Rac)-activated kinase 6 (PAK6)-specific sequences in the patients and *DOCK8*-specific sequences in the controls (Supplementary Fig. 1). These experiments suggested that the primers used to amplify exons 8, 12, and 14 were able to bind to the sequences of genes other than *DOCK8*. Amplification of gDNA from the mother gave normal bands for exons 8 to 14 (Fig. 1b) and for all the other exons (1-7 and 15-48) (not shown). Similar findings were obtained with the father: all of the exons from 1 to 48 could be amplified (Fig. 1c). These data suggest that both parents have biallelic or monoallelic wild-type *DOCK8* sequences.

Analysis of *DOCK8* cDNA with PCR amplicons

We then designed primers for the amplification of cDNA from the patients and the mother (the father was not available for cDNA isolation). We checked the quality of cDNA samples, by first amplifying the *GAPDH* cDNA as a control (Fig. 1b and 1c). Equivalent amplicons were obtained with samples from patients or controls. However, by contrast, no band was

obtained by amplification of the whole cDNA isolated from the patients (Fig.1c). We performed PCR amplifications on samples from the patients and the mother, with primers amplifying the cDNA sequences of exons 1-15 and 3-26. We detected a deletion of exons 1 and15 in the patients, but not in the mother (Fig. 1c). Further experiments with primers designed to amplify exons 3 and 26 clearly indicated the presence of the deletion in both patients, but not in the mother (Fig. 1c). Sequencing of the amplified exons 3-26 indicated that both patients carried homozygous deletions of exons 8-18 (Fig. 2). However, the sequencing of the sample from the mother presented us with a further challenge, because the electropherograms showed the sequence present to be wild-type (Fig. 2).

Identification of *DOCK8* mutation sites by MLPA

MLPA was performed for both patients and clearly showed that patient P1 had a compound heterozygous deletion mutation affecting exons 1-14 and exons 8-18 (Fig. 3a). The mother proved to be heterozygous for the deletion of exons 1-14, whereas the father was heterozygous for the deletion of exons 8-18 (Fig. 3b-d). These data suggest that the MLPA probe set used here is potentially valuable for the identification of large deletions of *DOCK8* and should used as a complement to Sanger sequencing analysis.

Immunoblotting

Figure 4 shows immunoblots for DOCK8 protein in the blood cells of the two patients and a healthy control. Lysates were obtained from freshly isolated PBMCs. Consistent with the clinical, immunological and genetic data, we were unable to detect DOCK8 protein on

western blots of the patients' PBMC extracts, whereas this protein was clearly detected on control blots (Fig. 4).

Discussion

The diagnosis of DOCK8 deficiency is challenging in clinical, immunological and genetic terms. In particular, the differential diagnosis of DOCK8 deficiency and AD-HIES caused by *STAT3* mutation on the basis of clinical signs is difficult because the signs of these two diseases overlap (Table 2). Most of the typical clinical findings of AD-HIES are due to connective tissue involvement occurring in children of school age (Table 2). Thus, these signs and other overlapping signs are not useful for clinical diagnosis. The suspicion of DOCK8 deficiency is increased by the presence of allergy, including life-threatening food allergy in some cases, recurrent viral infections, T-cell lymphopenia and dysgammaglobulinemia (Table 2). The higher rates of malignancy and mortality in patients with DOCK8 deficiency than in patients with AD-HIES requires close attention and the careful follow-up of patients from early childhood.

The identification of deletions in large genes, such as *DOCK8,* is important for the definitive diagnosis of the disease. The precise diagnosis of DOCK8 deficiency caused by point mutations or small deletions can be made by Sanger sequencing. MLPA has been introduced into DNA diagnostics for the detection of copy number variations due to deletions and duplications in several diseases (20-22). One of the objectives of this study was to confirm the clinical and immunological diagnosis of DOCK8 deficiency, by PCR amplification and Sanger sequencing. However, these traditional techniques failed to detect the *DOCK8* deletion, probably due to the overlap between the deletions identified in the two heterozygous parents. We therefore decided to use MLPA, a rapid and reliable assay for

detecting the large deletions commonly occurring in patients with *DOCK8* mutations. MLPA probes covering all 48 exons of *DOCK8* were available, making it possible to refine the definition of the breakpoints of exon deletions not clearly defined by PCR and targeted sequencing.

Deletions account for a large proportion of the mutations affecting the *DOCK8* gene. Previous studies have suggested that, of the 30 different genomic mutations identified in 32 DOCK8-deficient patients from 23 families, 63% were large deletions, 17% were point mutations that altered splicing, 10% were nonsense point mutations, and 10% were small indels (1, 2, 23, 24). An updated review of the current literature showed that 15 (40.5%) of the 37 *DOCK8* mutations reported up to datewere large deletions (1, 2, 5, 7, 23-30).
In summary, we diagnosed DOCK8 deficiency clinically and immunologically in two sisters and used MLPA analysis to detect deletions of the *DOCK8* gene in the patients and their heterozygous parents. The deletions in the patients resulted in a *DOCK8* frameshift at amino-acid position 276 and a stop codon leading to the termination of protein synthesis at amino-acid position 326. MLPA proved to be a powerful tool in our hands, making it possible us to confirm the clinical diagnosis of the disease. We suggest that MLPA could be used to screen for large deletions of *DOCK8* in routine genetic diagnosis and for the prenatal genetic screening of DOCK8 deficiency. This technique was informative, identifying two new deletions of *DOCK8*, the exons 1-14 and exons 8-18 deletions present in the compound heterozygous patients and individually in the heterozygous state in the parents (exons 1-14 deletion in the mother and exons 8-18 deletion in the father). This is the first report demonstrating confirmation of the clinical diagnosis of DOCK8 deficiency by MLPA.

Conflicts of interest

The authors have no competing financial interests to declare.

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Table 1 Clinical and laboratory features of the patients with DOCK8 deficiency

Patient	**Patient P1**	**Patient P2**
Age (yr)	11	6
Sex	female	female
Pneumonia	3 yr (3-4 per yr)[#]	10 mo (1-2 per yr)
Bronchitis	2 yr (1-2 per year)	2 yr (1-2 per year)
Sinusitis	2 yr (1-2 per year)	2 yr (1-2 per year)
Otitis media	2,5 yr (1-2 per year)	1.5 yr (1-2 per year)
Bacterial skin infection	2.5 yr	no
Mucocutaneous candidiasis	2.5 yr	no
Genital candidiasis	3.0 yr (recurrent)	2.5 yr (recurrent)
Herpes simplex virus infection	2 yr	no
Atopic dermatitis	2.5 yr	2.5 yr
Food allergy	2.5 yr*	2.5 yr (egg)

Asthma	3.0 yr	no	
Normal			
Eosinophils (G/l)	7.40	0.68	0.01-0.60
Lymphocytes (G/l)	1.83	2.12	0.9-4.44
$CD3^+$ (%)	30.2	34.1	55-78
$CD4^+$ (%)	13.2	16.4	27-53
$CD8^+$ (%)	17.4	10.5	19-34
$CD19^+$ (%)	64.3	48.4	10-31
$CD3\text{-}CD56^+$ (%)	2.30	13.5	4-26
Serum immunoglobulins			
IgM (g/l)	0.46	0.14	0.52-3.25
IgG (g/l)	12.9	10.1	5.4-15.1
IgA (g/l)	2.60	2.63	0.52-1.50
IgE (kU/l)	7071	604	<120
Specific antibodies			
Str. pneumoniae (μg/ml)[¶]	0,38	1.03	> 9.2

[#]Age at first episode (recurrence rate); *Patient P1 is allergic to soy bean, fish, milk, egg, hazelnut, tomato and liver. [¶]Antibodies were determined 6 months after the patients received 13-valent pneumococcus conjugate vaccine.

Table 2 Overlapping and specific signs of DOCK8 deficiency and HIES

DOCK8	DOCK8 & AD-HIES	AD-HIES
Asthma	Atopic dermatitis	Characteristic face
Allergies	Bacterial infections	Pneumatocele
Viral infections	CMC	Retention of primary teeth
Lymphopenia	Lymphoma	Pathologic fractures
IgM↓; IgG↑; IgA↑↓	Eosinophilia	Hyper-extensible joints
	High serum IgE level	Arched palate

DOCK8, dedicator of cytokinesis; AD-HIES, autosomal dominant HIES; CMC, chronic mucocutaneous candidiasis; Ig, immunoglobulin.

Figure legends

Fig. 1 PCR amplification of *DOCK8* gDNA and cDNA. (a) PCR amplification of *DOCK8* exons 8 to 14 from the two patients and a control. The visible bands for exons 8 and 14 in patients result from the nonspecific binding of the primers to other genes. The lack of PCR products from the patients's gDNA and the detection of such products with control gDNA suggested deletions of exons 8-14. (b) By contrast to the results obtained for the patients, *DOCK8* exons 8 to 14 could be amplified from the mother. (c) PCR amplification of the

full-length *GADPH* cDNA yielded a 244 bp band in both patients and the control (left). Amplification of the full-length *DOCK8* suggested an absence of the cDNA from the patients' samples and the presence of the 6249 bp *DOCK8* cDNA in the control (second from left). Amplification of *DOCK8* exons 1 to 15 suggested that this fragment was missing from the patients' cells but present in the control and the mother (second from right). PCR amplification of exons 3 to 26 of the *DOCK8* cDNA yielded a 1590 bp product for patients, corresponding to an aberrantly spliced mRNA (right), and a 2800 bp product for the control and the mother. *GADPH*, glyceraldehyde 3-phosphate dehydrogenase; bp, base pair; Mw, molecular weight; C, control; P1, Patient P1; P2, Patient P2; M, mother.

Fig. 2 Automated sequencing profiles of exons 3-26 of *DOCK8,* with cDNA as the template. Del8-18 exon mutations of *DOCK8* were found in both patients. P1, patient P1; P2, patient P2; aa, amino acid.

Fig. 3 MLPA analysis of the *DOCK8* gene. (Fig. 3a) Data for Patient P1 (black bars) and a healthy control (gray bars) are shown. Heterozygous deletions of exons 1-7 and exons 15-18, as indicated by a lower fluorescence intensity, and a homozygous deletion ofrexons 8-14 as indicated by the lack of fluorescence intensity in P1. Downstream from exon 19, equivalent fluorescence intensity peaks were found, indicating the presence of wild-type sequences of these allele segments in P1 and the healthy control. An analysis of samples from t P2 yielded similar results. (Fig. 3b and 3c) The heterozygous deletion of exons 8-18 in the father (3b) and of exons 1-14 in the mother (3c) was demonstrated by MLPA. (Fig. 3d) Schematic representation of the heterozygosity of the exons 1-14 deletion in the mother and the exons 8-18 deletion in the father, and

compound heterozygosity of the two new large deletions of *DOCK8* found in patients P1 and P2.

Fig. 4 Western blot analysis of DOCK8 protein in PBMC lysates. Experiments were performed with polyclonal antibodies against DOCK8 and PBMCs (50 μg of total lysate for control and 100 μg for the patients). A 230 kDa protein band, corresponding to the DOCK8 protein, was detected in the control sample. By contrast, DOCK8 was undetectable in samples from the patients, despite the use of double the amount of protein lysate. kDa, kiloDalton; C, control; P1, patient 1; P2, patient 2; M, molecular weight standard.

Fig. 1

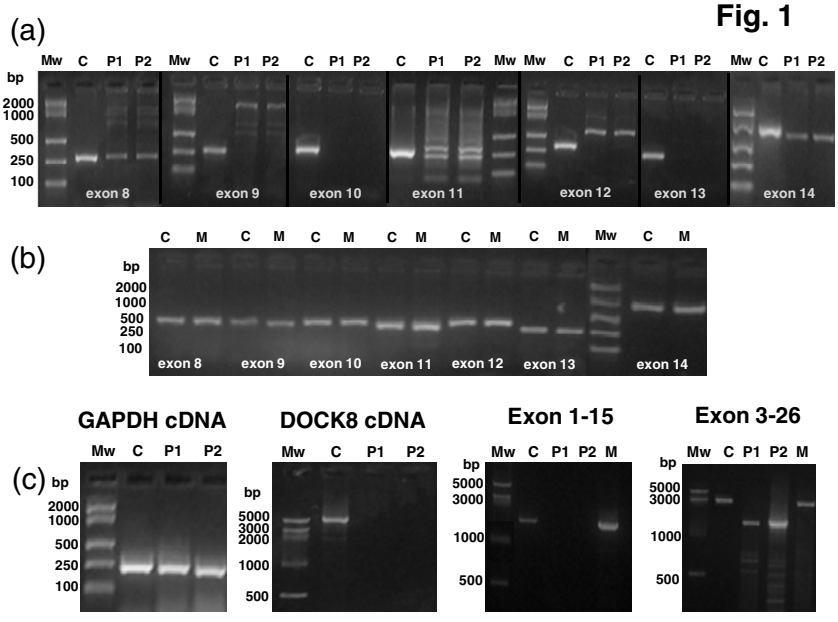

Fig. 2

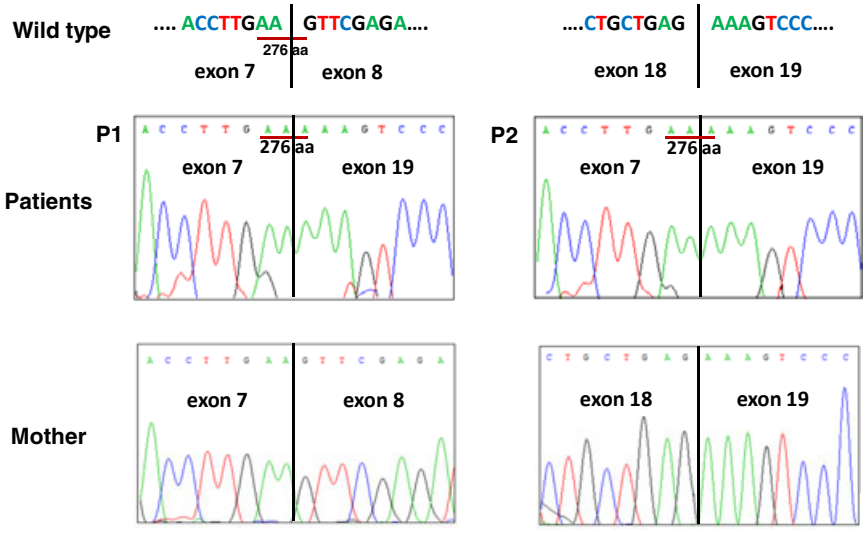

Fig. 3a

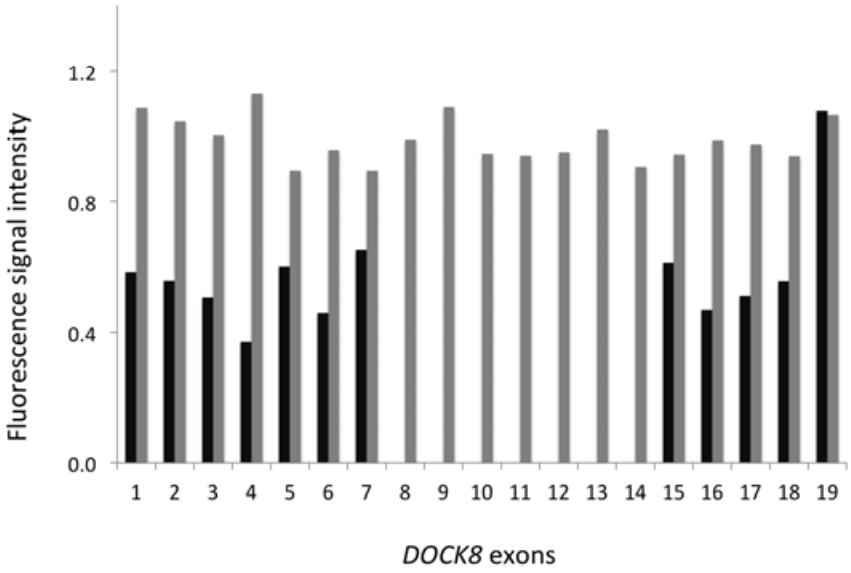

Fig. 3b

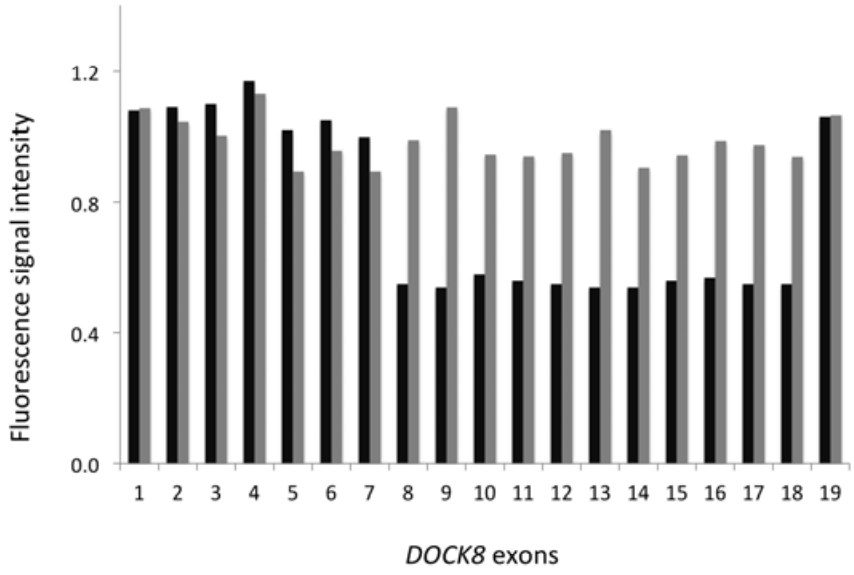

Fig. 3c

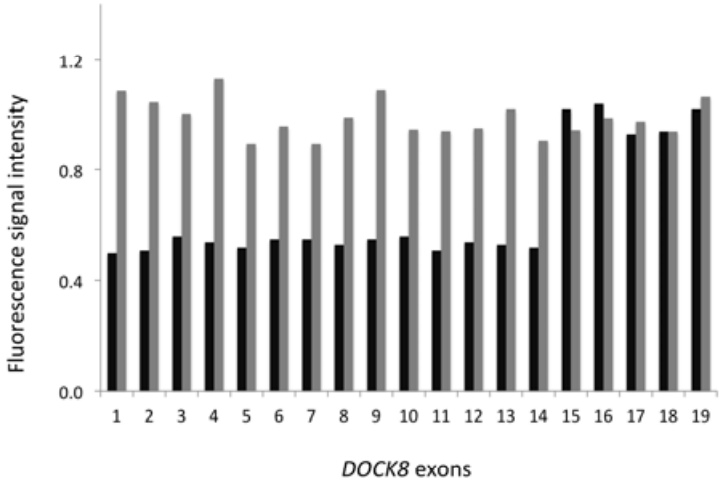

Fig. 3d

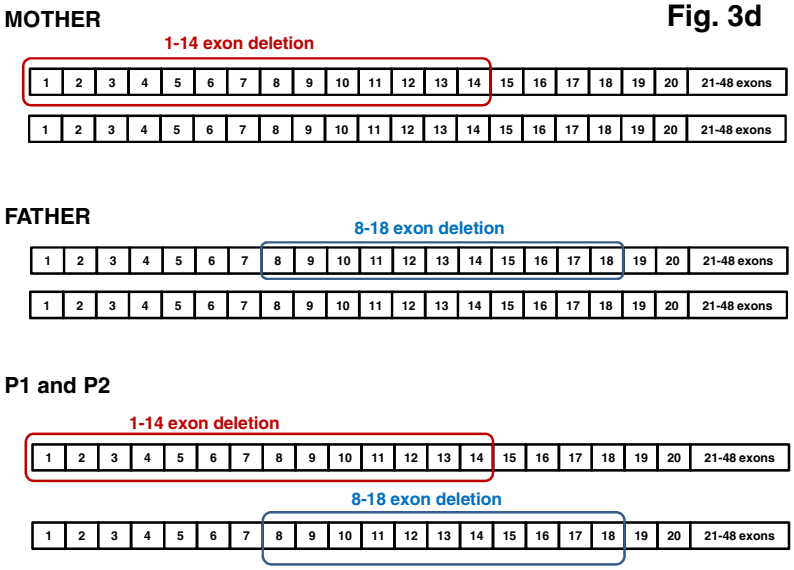

Fig. 4