

# Mutational spectrum of *NSDHL* in CHILD syndrome

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**C**HILD syndrome (congenital hemidysplasia with ichthyosiform nevus and limb defects, MIM 308050) is an X linked dominant, male lethal, multisystem birth defect characterised by an inflammatory epidermal nevus showing a unique lateralisation pattern and strict midline demarcation. Hypoplasia or aplasia of skeletal or visceral structures may be found ipsilateral to the major cutaneous involvement.<sup>1</sup> Owing to the highly characteristic clinical and histopathological features of the CHILD naevus,<sup>2</sup> a diagnosis can be established not only in classical cases (fig 1) but also in cases with minimal or atypical involvement.<sup>3</sup> In 2000, mutations in *NSDHL* (NAD(P)H steroid dehydrogenase-like protein) at Xq28 were identified by some of us to be the cause of this syndrome.<sup>4</sup> Four additional *NSDHL* mutations have subsequently been reported in individuals with CHILD syndrome.<sup>5-8</sup> Studies carried out on the murine *Nsdhl* mutants *bare patches* (*Bpa*) and *striated* (*Str*) have shown that this gene encodes a 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) that catalyses a step in the post-squalene cholesterol biosynthetic pathway and is localised within membranes of the endoplasmic reticulum and on the surface of intracellular lipid storage droplets.<sup>9-11</sup> Non-functional *NSDHL* might cause the CHILD phenotype through a lack of cholesterol or other sterols downstream of the block in biosynthesis, or by the accumulation of intermediates upstream of the product generated by *NSDHL*.

A related trait, X linked dominant chondrodysplasia punctata (CDPX2, MIM 302960),<sup>22</sup> is caused by mutations in *EBP* (emopamil binding protein) at Xp11.22-p11.23 that functions similarly in the late cholesterol biosynthesis, downstream of *NSDHL*.<sup>23, 24</sup> In the past, a case of X linked dominant chondrodysplasia punctata showing unilateral involvement was mistaken as an example of CHILD syndrome.<sup>25</sup> Because abnormal sterol patterns suggestive of an *EBP* mutation were found, the investigators erroneously concluded that CHILD syndrome is genetically heterogeneous. In fact, their case had all the clinical features of X linked dominant chondrodysplasia punctata but none of the morphological criteria of CHILD syndrome.<sup>1, 22</sup> Hence there is so far no case report of CHILD syndrome showing a mutation outside the *NSDHL* locus.

In this study we describe mutations of the *NSDHL* gene that are likely to affect the function of the encoded protein in 14 unrelated female patients with CHILD syndrome, extending the spectrum of human *NSDHL* mutations by nine alleles not previously observed. Known mutations A105V, G205S, and Y349C were found again in unrelated patients. Our molecular analysis confirms that CHILD syndrome is caused by loss of function of *NSDHL* and identifies highly conserved sites in the *NSDHL* protein, the mutation of which appears to be deleterious to this enzyme.

## METHODS

### Subjects

The study included 14 cases of CHILD syndrome (table 1). The diagnosis had been established before molecular analysis

### Key points

- The gene *NAD(P)H steroid dehydrogenase-like (NSDHL)* is mutated in CHILD syndrome (congenital hemidysplasia with ichthyosiform nevus and limb defects). Point mutations in the *NSDHL* gene have been detected in 14 unrelated female patients with CHILD syndrome. Brief clinical descriptions of the phenotypes and corresponding mutations are presented.
- As well as four stop mutations preventing the translation into full length protein, there were eight missense mutations, one mutation affecting a splice site, and one complete deletion of the gene. Two of the (unrelated) patients had an identical missense mutation, A105V.
- All *NSDHL* mutations known so far are discussed, including seven in the orthologous mouse gene *Nsdhl*. In four unrelated patients, A105 was mutated to V. In one mouse mutant the amino acid A94 of *NSDHL*, which takes a homologous position to A105 in the human enzyme, was mutated to T. Two patients showed G205S, and in three, Y349 was changed into C or H.
- Nonsense and missense mutations spreading along the gene, as well as a complete deletion of the gene, result in similar phenotypes, suggesting that loss of function of the *NSDHL* protein causes this birth defect.
- Many of the amino acids altered in missense mutations are highly conserved during evolution. Most, however, are not directly located at positions considered to be essential in functional domains, such as the cofactor binding site or the active site at the catalytic centre. These mutations apparently affect other important sites within the *NSDHL* protein, the function of which awaits elucidation.

in all patients on clinical and histopathological criteria as described elsewhere.<sup>1, 2, 4, 26</sup> Clinical features of 11 of these cases have been reported previously. Mutational analysis and examination of medical records were carried out with informed consent and under research protocols according to the Declaration of Helsinki. Table 1 shows whether CHILD syndrome occurred sporadically or whether other family members were affected. Of familial cases, only one affected individual was included in table 1.

**Abbreviations:** CHILD, congenital hemidysplasia with ichthyosiform naevus and limb defects; MAPH, measurement by amplifiable probe hybridisation; *NSDHL*, NAD(P)H steroid dehydrogenase-like protein; SSCA, single strand conformation analysis

**Table 1** Synopsis of clinical data and molecular analysis in patients with CHILD syndrome

| Family   | Clinical description (ref No) | Side    | Ipsilateral CHILD naevus  | Contralateral CHILD naevus                      | Ipsilateral extracutaneous defects   | NSDHL mutation                         |
|--|-------------------------------|---------|---|---|--|--|
| <b>Newly observed NSDHL mutations in CHILD syndrome patients</b>         |                               |         |   |   |  |  |
| 1  | – 12                          | R       | Diffuse involvement of trunk, linear lesions on thigh                                       | –   | Hypoplasia of leg and foot   | c.108+2T→G; splice site                |
| 2  | – 13                          | R       | Widespread linear lesions on trunk and leg, diffuse involvement of groin                    | Minor linear lesions on hand                    | Hypoplasia of pelvis and femur; 6th finger at birth  | c.208 C→T; p.Q70X                      |
| 3  | – 14                          | L       | Groin   | Minor lesions on scalp, neck, soles             | Aplasia of one finger  | c.314 C→T; p.A105V                     |
| 4  | + 15                          | L       | Diffuse widespread involvement of leg, trunk, and hand                                      | Linear lesions on fingers and toes              | Hypoplasia of pelvis and foot  | c.314 C→T; p.A105V                     |
| 5  | + –                           | L       | Patchy lesions on thigh, groin, and vulva, linear lesions on hand and foot                  | Nail dystrophy one finger, two toes             | Hypoplasia of leg, foot, and skull   | c.370 G→A; p.G124S                     |
| 6  | – 16                          | R       | Diffuse involvement of trunk, upper arm and thigh, linear lesions on forearm and lower leg  | Minor patchy and linear lesions on leg and foot | Hypoplasia of arm; dysplasia of hip and knee   | c.396 C→G; p.C132W                     |
| 7  | – 17                          | L       | Trunk and arm   | –   | Hypoplasia of upper arm  | c.613 G→A; p.G205S                     |
| 8  | + –                           | R       | Linear lesions on hand, foot, scalp, and forehead   | Linear lesion on one finger                     | Hypoplasia of leg and vertebral column   | c.894 G→A; p.W298X                     |
| 9  | – 18                          | L       | Diffuse and patchy involvement of neck and trunk, linear lesions on extremities             | Small patches on hand                           | Hypoplasia of leg and vertebral column; syndactyly of index and middle finger  | c.906 C→A; p.Y302X                     |
| 10   | – 14                          | R       | Patchy and linear lesions in body folds   | Minor lesions in body folds                     | Hypoplasia of arm and leg  | c.1018 T→C; p.C340R                    |
| 11   | – 19                          | R       | Patchy widespread involvement   | –   | Elongation of leg; hypoplasia of vertebrae; verrucous involvement of vaginal and gastric mucosae   | c.1041_42insCATG; p.G348fs; X358       |
| 12   | – 20                          | R       | Diffuse involvement of leg, linear lesions on abdomen and arm                               | –   | Hypoplasia of vertebral column and hand  | c.1045 T→C; p.Y349H; c.306C→T; p.F102F |
| 13   | – 21                          | R       | Diffuse involvement of trunk, forearm, and leg, extensive linear lesions on upper arm       | –   | Hypoplasia of arm and leg; liver lobe hypertrophy; spleen hypertrophy  | c.1046 A→G; p.Y349C                    |
| 14   | – –                           | L       | Patchy and linear lesions on trunk and extremities  | Patchy and linear lesions on groin and hand     | Hypoplasia of foot and vertebral column; ipsilateral deafness; ipsilateral vocal cord paralysis  | Complete deletion                      |
| <b>NSDHL mutations in CHILD syndrome patients as reported previously</b> |                               |         |   |   |  |  |
| 15   | – 4                           | R, male | Extensive linear lesions on trunk and limbs   | –   | Hypoplasia of leg  | c.262C→T; p.R88X                       |
| 16   | + 4                           | R       | Widespread patchy involvement with partial resolution                                       | Small linear lesions on face and arm            | Absence of kidney; dilated cerebral ventricles; hypoplasia of limbs and vertebral column   | c.314C→T; p.A105V                      |
| 17   | – 4                           | R       | Diffuse widespread unilateral involvement with partial resolution                           | Linear lesions on hand and thigh                | Absence of kidney; hypoplasia of limbs, mandible, ribs, and vertebrae  | c.314C→T; p.A105V                      |
| 18   | – 8                           | R       | Involvement of the right axilla, breast, trunk, upper and lower limb, vulva, and perineum   | –   | Hemidysplasia of right palm; valgity of right coxofemoral joint  | c.441T→A; p.S147R                      |
| 19   | – 6                           | L       | At birth, erythematous rash with minimal scaling over the chest wall, linear lesions on leg | –   | Hypoplasia of arm, leg, and foot; absence of ribs and hypoplasia of vertebrae; hypoplastic left lung with right shift of cardiac structures; absence of kidney | c.451G→T; p.E151X                      |
| 20   | – 5                           | R+L     | Almost symmetrical involvement of the body folds; right sided involvement of neck           | Almost symmetrical involvement of body folds    | Hypoplasia of leg and vertebral column; absence of facial muscles  | c.544G→C; p.A182P                      |
| 21   | – 4                           | R       | Widespread patchy unilateral involvement  | –   | Neurosensory hearing loss; absence of arm; hypoplasia of leg   | c.613G→A; p.G205S                      |
| 22   | – 4                           | R       | Large patch on the back, linear lesions on limbs  | Linear lesions on hand                          | Neurosensory hearing loss; hypoplasia of vertebral column and foot   | c.628C→T; p.Q210X                      |
| 23   | – 7                           | L       | Patchy involvement of neck and axillae, buttocks, lower back, and abdomen                   | –   | Shortening of toes 2, 3, and 4   | c.1046A→G; p.Y349C                     |

Family +/- = familial case, yes or no; R/L = right or left side preferentially affected.

Although case 13 shows the same mutation as observed by Murata and co-workers,<sup>7</sup> there is no familial relation to our knowledge between the two patients.

### Analysis of point mutations

Genomic DNA was extracted from blood lymphocytes or from cultured fibroblasts following standard procedures. The sequences of intron–exon boundaries and primers, as well as the polymerase chain reaction (PCR) conditions to amplify the eight exons of human *NSDHL*, have already been described.<sup>9</sup> Amplified coding exon sequences were analysed both by single strand conformation analysis (SSCA) at two temperatures and by genomic sequencing, as described previously.<sup>27–28</sup>

When a deviant fragment indicative of a mutation was detected in SSCA it was confirmed by a second amplification of the original template, and its presence was searched for in a sample of 100 unrelated individuals to exclude the possibility that it represented a polymorphism. Allele specific sequencing analysis was carried out following amplification of the appropriate gel fragments cut out of the dried SSCA gels. For extraction, gel pieces were incubated in 100 µl HPLC H<sub>2</sub>O overnight at 37°C.

Genomic sequence variants not associated with electrophoretic variation in SSCA were confirmed by the amplification refractory mutation system (ARMS) test.<sup>29</sup>

### Deletion screening

To search for minor deletions or duplications affecting *NSDHL* that would not be detected by either SSCA or genomic sequencing, the copy number of individual exons was determined by hybridisation with amplifiable probes (MAPH).<sup>30</sup> In this approach, locus copy number is measured by amplifying short probes of individual *NSDHL* exons quantitatively recovered after hybridisation to genomic DNA of a patient. The probes used for quantitative recovery after their hybridisation to immobilised patient DNA were exonic PCR products cloned into the pCR2.1TOPO vector (Invitrogen Inc, San Diego, California, USA). To generate these PCR products, exons 1–7 were amplified with the same primers and conditions as for SSCA,<sup>9</sup> except for novel reverse primers for exons 4 and 6 (N4br: CAC CCT TAG AAA GGG CCA TC and N6br: TCT CTG AAT GCG AGC ATG GAC). Exon 8 was amplified as two fragments by the use of primer pair N81f/N8-1cr: ACT GAT CAC CAT CAC CAG C and primer pair N8-4bf: CCT TCC CCT GTG GAT TGA TG/N8-2br: TGT ACA AGG ACA CTG GAA TG. The annealing temperature for the first of these two pairs was set to 59°C. All other conditions were as described previously.<sup>4</sup> One unique probe from chromosomes 5 (St) and 7 (C) each were taken as a control.

### Multiple sequence alignment

The predicted amino acid sequence of human *NSDHL* (Q15738) was aligned by the use of Clustal W<sup>31</sup> with homologous protein sequences identified by a BLAST search in the SWISSPROT database: human (*NSDHL\_Hs*; Q15738) and mouse (*Nsdhl\_Mm*; Q9R1J0) (NAD(P) dependent steroid dehydrogenases; *Saccharomyces cerevisiae* 3β-hydroxysteroid dehydrogenase (ERG26\_Sc; P53199); *Candida albicans* C-3 sterol dehydrogenase/C-4 decarboxylase (ERG26\_Ca; AAK69617); 3β-hydroxysteroid dehydrogenases from *Macaca mulatta* (3BHS\_Mam; P27365), *Mesocricetus auratus* (3BH1\_Mea; Q60555), and the mouse (3BH1\_Mm; P24815)

A putative transmembrane helix was predicted by application of a neural network system.<sup>32</sup>

## RESULTS

### Disease causing mutations in *NSDHL*

Mutations were searched for in the coding exons (2 to 8) of the *NSDHL* gene in 14 unrelated female patients with CHILD



**Figure 1** Thirteen year old patient with CHILD syndrome (case 9, table 1): ichthyosiform nevus showing lateralisation with unilateral distribution and midline demarcation; ipsilateral hypoplasia of arm and hand. Reproduced with permission.

syndrome. Brief clinical descriptions of the phenotypes as observed in individual patients and the detected mutations are listed in table 1. As an example of the phenotype, fig 1 shows the CHILD nevus and limb defects as observed in patient No 9.

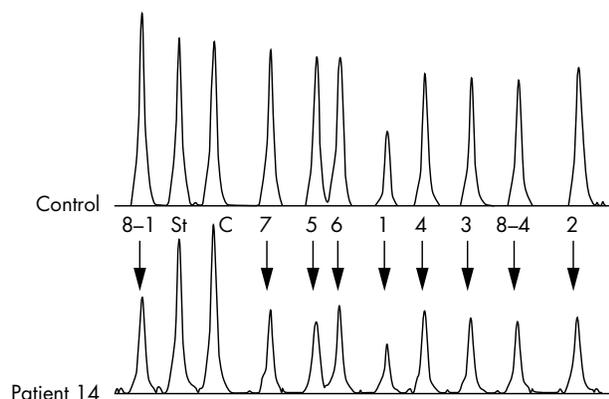
In these 14 patients, in addition to four mutations interrupting the reading frame by the introduction of stop codons, we found eight missense mutations, one mutation affecting a splice site, and one complete deletion of the gene. Two of the patients showed the previously known mutation A105V. Amino acid Y349 was substituted in two of the patients by C and H, respectively.

For most of these patients the mutations were detected by SSCA and confirmed by sequencing both strands of the relevant exons. For individuals 1 and 5 (table 1), the mutations were identified by genomic sequencing of all exons and subsequently confirmed by an ARMS test (data not shown). None of these mutations was observed in 100 unrelated control individuals.

To search for clustering of mutations in certain domains of the enzyme or genotype–phenotype correlations, the novel cases in table 1 were supplemented by all the other human *NSDHL* mutations known so far, from six patients previously described by us as well as three mutations identified by other groups. In all, seven stop mutations preventing the translation into full length protein, nine different missense mutations, one mutation affecting a splice site, and one complete deletion of the gene known in humans so far are listed in this table. Four unrelated patients showed the mutation A105V, two had G205S, and two had Y349C. In one case Y349 was mutated to H.

### A deletion of the coding region of *NSDHL*

No sequence variation could be detected in one patient (table 1, No 14) with a typical CHILD phenotype. The DNA of this patient was scrutinised by MAPH for microdeletions affecting the *NSDHL* gene. Locus copy number was measured by amplifying by PCR short probes of *NSDHL* exons 1–8 quantitatively recovered after hybridisation to genomic DNA of patient No 14. A reduction in the amplified product of all exons to half of the control amount (fig 2, arrows) indicated a deletion of the entire gene. Heterozygosity at three highly polymorphic microsatellite loci—DXS1193, DXS8043, and DXS8106—located on the centromeric side of *NSDHL* in chromosomal bands Xq28–q27 showed that the deletion did not affect the entire X chromosome (data not shown).



**Figure 2** Deletion of *NSDHL* detected in CHILD patient No 4 (listed in table 1) by the MAPH (measurement by amplifiable probe hybridisation) procedure. Polymerase chain reaction (PCR) products of all exons (labelled 1 to 7 and 8-1, 8-4) were separated on a sequencing gel and the signal intensities were quantified using the Genotyper software (Applied Biosystems). The reduction of the amplified product (arrows) in all exons to half of the amount observed in female control DNA indicates a deletion of the entire gene. St and C are MAPH-PCR products of unique loci on chromosomes 5 and 7, respectively, used as standards.

### Distribution of point mutations along *NSDHL*

The deduced amino acid sequences from human *NSDHL* and other selected  $\beta$ -HSDs were analysed with the CLUSTAL W program (fig 3) to demonstrate evolutionary conservation. Amino acids which are identical or show different levels of similarity from man to yeast were aligned.

Above the respective wild type amino acid positions, mutations observed in CHILD patients or in bare patches (*Bpa*) and striated (*Str*) mouse strains, which are mutated in the orthologous mouse gene *Nsdhl*,<sup>9, 11</sup> are depicted. Putative functional domains of the enzyme—such as the co-factor binding site, the catalytic site, and a transmembrane domain—are marked below their respective amino acid positions. One transmembrane helix was predicted by applying a neural network system.<sup>32</sup> The species of origin and the SwissProt database accession numbers of the protein sequences, compared in fig 3 with the human enzyme (*NSDHL\_Hs*), are listed in the Methods section. The point mutations in *NSDHL* are distributed along the coding sequence. Missense mutations preferentially affect highly conserved amino acids, but they are not clustered at known functional domains.

### Single nucleotide polymorphism in *NSDHL*

In addition to potentially deleterious mutations which were observed in patients only, the search for mutations within the exons revealed one polymorphism occurring in patients, non-affected family members, and among the non-affected control individuals: c.132G→T; p.G44G (data not shown). The frequency of the minor allele among 170 chromosomes from our German control group was 10.6%. This common polymorphism, and a second (c.306C→T) that was observed only in patient No 12 in addition to a missense mutation, are silent—that is, they do not result in an altered amino acid composition.

## DISCUSSION

### CHILD syndrome appears to be caused by loss of function

The phenotype of CHILD syndrome appears to be caused by loss of function of *NSDHL*, as all types of mutations, even the

deletion of the whole coding region, result clinically in the same types of defect.

The extent of the naevus on the skin and the developmental defects affecting other sites vary between individuals, most probably because of differences in the X inactivation pattern. Among the 23 patients in which *NSDHL* mutations have been identified so far<sup>4, 5-8</sup> (table 1), eight showed the symptoms preponderantly on the left side of the body, 14 on the right side, and one almost symmetrically on both sides. In several cases other family members were affected as well (table 1). Phenotypic variability appeared not to be associated with the type or site of the mutations. Thus we cannot detect allelic heterogeneity.

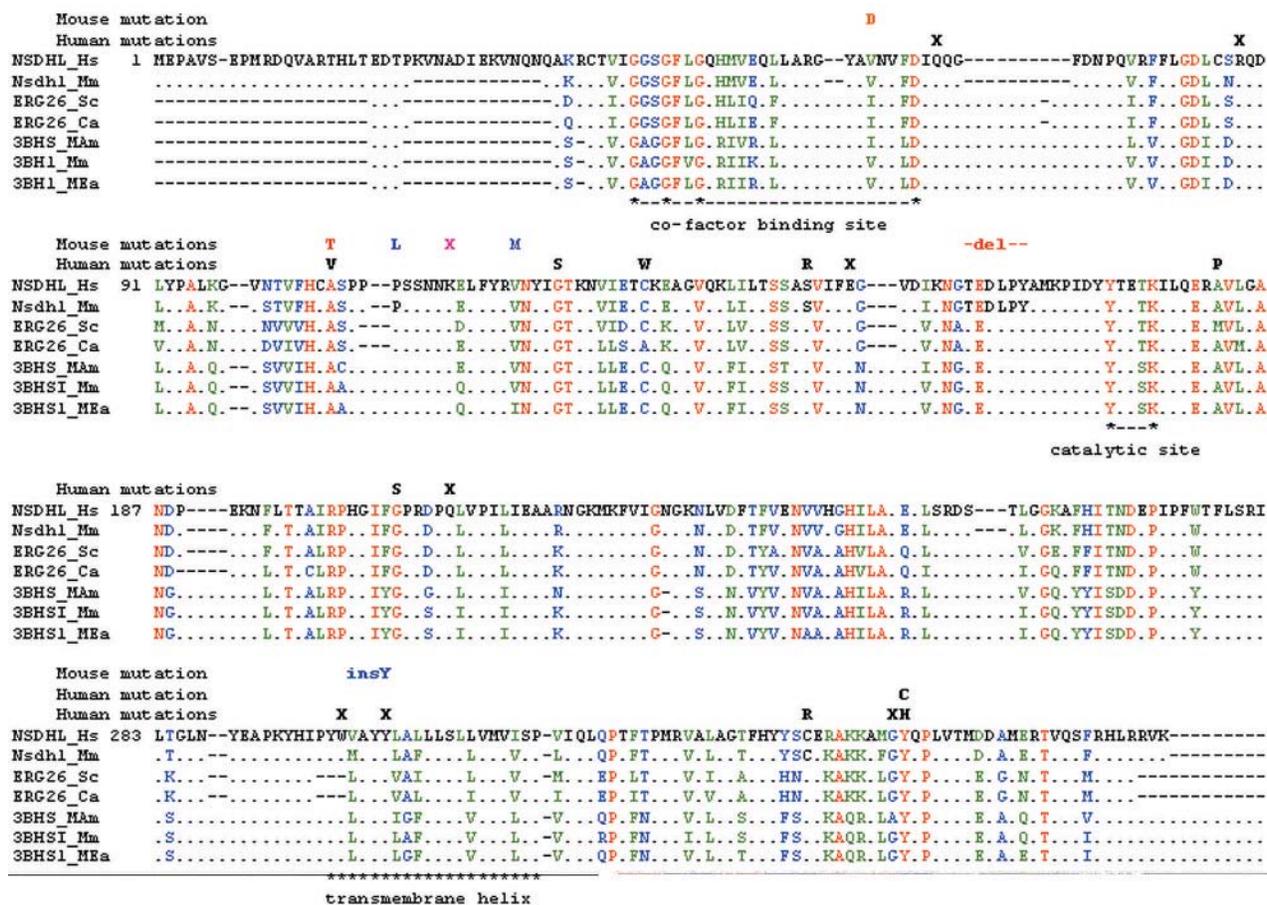
In the mouse homolog, however, on phenotypic grounds bare patches (*Bpa*) and striated (*Str*) had been described as different mutants, *Str* being less severely affected than *Bpa*.<sup>33, 34</sup> Genetic mapping had placed them to the same location on the X chromosome, suggesting that they might be allelic.<sup>35</sup> The identification of the sites in *Nsdhl* mutated in each of the known *Bpa* and *Str* strains confirmed that they represent allelic mutants.<sup>9, 36</sup> As in humans, the genotype in the mouse mutants does not allow one to predict the phenotypic outcome (fig 3). Lucas and coworkers<sup>36</sup> showed that the mouse *NSDHL* protein can rescue the lethality of *erg26* deficient cells of *Saccharomyces cerevisiae* that lack the yeast orthologue, thus substantiating the role of *NSDHL* as a C-3 sterol dehydrogenase. Using this yeast complementation assay, they have shown that two *Str* alleles function as hypomorphs, whereas three *Bpa* and one *Str* allele provide no complementation or rescue. The severity of the mouse phenotype, as defined by the timing of the loss of affected male embryos as well as the percentage and phenotype of surviving affected female mice, is not entirely reflected by the order of increased severity in the yeast assay, and neither phenotypic property correlates with the type or site of the underlying mutations.

### Missense mutations pinpoint conserved and possibly functionally relevant sites

Missense mutations associated with loss of function can pinpoint specific domains of particular functional relevance. The position of all *NSDHL* mutations known so far was correlated with the amino acid alignment of the derived protein sequence of human *NSDHL*, which has selected homologous protein sequences with C-3 sterol dehydrogenase activity from two yeast and three different mammalian species (fig 3). In addition, the amino acid changes in *Nsdhl* underlying the seven different *bare patches* or *striated* mutant mouse strains are indicated in fig 3.

The multiple sequence alignment shows that the missense mutations in human or murine *NSDHL* preferentially exchange conserved amino acids. The majority of changes affect amino acids conserved from yeast to man, while three mutations change amino acids conserved at least in human and murine *NSDHL*.

None of the observed changes directly alters the invariant amino acids that form two previously described conserved motifs, an N terminal cofactor binding site or the motif T-X-X-X-K characteristic for the active site of  $\beta$ -hydroxysteroid dehydrogenases, nor a sequence interval (aa 297–316) predicted to contain a transmembrane helix (fig 3). The high degree of sequence homology in various blocks outside the domains for which a function has been predicted suggests tight restraints on the amino acid composition well beyond the known motifs. The observation of missense mutations in CHILD patients or mouse mutants affecting conserved amino acids supports the view that they affect critical functions of the protein, although in a so far unknown way.



**Figure 3** Multiple sequence alignment shows that missense mutations in human or murine NSDHL preferentially exchange conserved amino acids. The deduced amino acid sequences from human NSDHL and other selected  $\beta$ -HSDs are aligned with CLUSTAL W. Numbers to the left indicate amino acid positions of human NSDHL, dashes indicate gaps. Identical amino acids are shown in red letters, strongly similar ones in green; weak similarity is indicated by blue colour, dots represent non-conserved positions. Mutations observed in CHILD patients (black, bold) (table 1) or Bpa (red, bold)/Str (blue, bold) mouse mutants<sup>9, 11</sup> are depicted above the respective positions. Putative functional domains are marked below the individual sites. The search for transmembrane helices by applying a neural network system<sup>32</sup> detects one major site. The protein sequences compared to the human enzyme (NSDHL\_Hs) are listed in the methods section.

### Frequent mutations

Three amino acids—A105, G205, and Y349—are repeatedly altered among apparently unrelated patients (A105V in four individuals, G205S in two, Y349C in two, and Y349H in one). In one mouse mutant the amino acid A94 of Nsdhl, which takes a homologous position to A105 in the human enzyme, is mutated to T. The repeated occurrence of mutations at these positions, even across species, could indicate that the gene is particularly prone to mutations at these genomic hotspots or that the function of the protein is specifically dependent on the presence of the proper amino acid at these sites. The amino acids A105 (A94 in mice) and Y349 could be indispensable, as the detrimental effect of their mutation appears to be independent of the amino acid change. However, as long as the role of the conserved domains of the protein and the functional impact of individual missense mutations have not been elucidated experimentally, the explanation for the occurrence of repeated mutation at specific positions remains speculative.

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