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## Mosaic type-1 NF1 microdeletions as a cause of both generalized and segmental neurofibromatosis type-1 (NF1)

Ludwine M Messiaen, Julia Vogt, Kathrin Bengesser, Chuanhua Fu, Fady Mikhail, Eduard Serra, Carles Garcia-Linares, David N. Cooper, Conxi Lázaro, Hildegard Kehrer-Sawatzki

► **To cite this version:**

Ludwine M Messiaen, Julia Vogt, Kathrin Bengesser, Chuanhua Fu, Fady Mikhail, et al.. Mosaic type-1 NF1 microdeletions as a cause of both generalized and segmental neurofibromatosis type-1 (NF1). *Human Mutation*, 2011, 32 (2), pp.213. 10.1002/humu.21418 . hal-00612010

**HAL Id: hal-00612010**

**<https://hal.science/hal-00612010>**

Submitted on 28 Jul 2011

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**Mosaic type-1 NF1 microdeletions as a cause of both generalized and segmental neurofibromatosis type-1 (NF1)**

|                               |  |
|-------------------------------|--|
| Journal:                      | <i>Human Mutation</i>  |
| Manuscript ID:                | humu-2010-0440.R1  |
| Wiley - Manuscript type:      | Research Article   |
| Date Submitted by the Author: | 19-Oct-2010  |
| Complete List of Authors:     | Messiaen, Ludwine; University of Alabama at Birmingham, Department of Genetics<br>Vogt, Julia; University of Ulm, Human Genetics<br>Bengesser, Kathrin; University of Ulm, Human Genetics<br>Fu, Chuanhua; University of Alabama at Birmingham, Department of Genetics<br>Mikhail, Fady; University of Alabama at Birmingham, Department of Genetics<br>Serra, Eduard; IMPPC, Hereditary Cancer Program<br>Garcia-Linares, Carles; IMPPC, Hereditary Cancer Program<br>Cooper, David; Cardiff University, Institute of Medical Genetics, College of Medicine<br>Lázaro, Conxi; Catalan Institute of Oncology (ICO), Translational Research Laboratory<br>Kehrer-Sawatzki, Hildegard; University of Ulm |
| Key Words:                    | Neurofibromatosis type 1 , mosaicism, microdeletion, recombination hotspots, segmental neurofibromatosis   |
|                               |  |

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Manuscripts

1 | **Mosaic type-1 *NFI* microdeletions as a cause of both generalized and segmental**  
 2 | **neurofibromatosis type-1 (NF1)**  
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32 | **Key Words:** Neurofibromatosis type 1 (*NFI*) gene, microdeletion, recombination hotspots,  
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**Abstract**

Mosaicism is an important feature of type-1 neurofibromatosis (NF1) on account of its impact upon both clinical manifestations and transmission risk. Using FISH and MLPA to screen 3500 NF1 patients, we identified 146 individuals harbouring gross *NF1* deletions, 14 of whom (9.6%) displayed somatic mosaicism. The high rate of mosaicism in patients with *NF1* deletions supports the postulated idea of a direct relationship between the high new mutation rate in this cancer predisposition syndrome and the frequency of mosaicism. Seven of the 14 mosaic *NF1* deletions were type-2, whereas 4 were putatively type-1, and three were atypical. Two of the 4 probable type-1 deletions were confirmed as such by breakpoint-spanning PCR or SNP analysis. Both deletions were associated with a generalized manifestation of NF1. Independently, we identified a third patient with a mosaic type-1 *NF1* deletion who exhibited segmental NF1. Together, these three cases constitute the first proven mosaic type-1 deletions so far reported. In two of these three mosaic type-1 deletions, the breakpoints were located within PRS1 and PRS2, previously identified as hotspots for non-allelic homologous recombination (NAHR) during meiosis. Hence, NAHR within PRS1 and PRS2 is not confined to meiosis but may also occur during postzygotic mitotic cell cycles.

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## Introduction

Neurofibromatosis type 1 (NF1; MIM #162200) is a common neurocutaneous disorder with an estimated birth incidence of 1/3000 [Friedman et al., 1999; Lammert et al., 2005]. Characteristic clinical symptoms include multiple café-au-lait spots, skinfold freckling, Lisch nodules and neurofibromas. Large deletions of the entire *NF1* gene and its flanking regions (also termed *NF1* microdeletions) are observed in ~5% of all patients with neurofibromatosis type 1 (NF1) [Kluwe et al., 2004]. Four types of large *NF1* deletion (type-1, type-2, type-3 and atypical) have been identified, which differ with respect to the extent of the deleted region and the location of the respective breakpoints. The most frequent *NF1* deletion is type-1 which encompasses 1.4-Mb and includes 14 genes. The breakpoints of type-1 deletions are invariably located within low-copy repeats, termed NF1-REP A and NF1-REP C, which flank the *NF1* gene region [Dorschner et al., 2000; Jenne et al., 2001; López-Correa et al., 2001]. Most type-1 *NF1* deletions are caused by non-allelic homologous recombination (NAHR) occurring within two recombination hotspots, termed paralogous recombination sites 1 and 2 (PRS1 and PRS2) located within NF1-REPs A and C [Forbes et al., 2004; De Raedt et al., 2006]. By contrast, type-2 *NF1* deletions encompass 1.2-Mb; their breakpoints are located within the *SUZ12* gene and its pseudogene *SUZ12P*, located adjacent to the NF1-REPs [Petek et al., 2003; Kehrer-Sawatzki et al., 2004; Steinmann et al., 2007; Roehl et al., 2010a]. The major mechanism underlying type-2 *NF1* deletions is NAHR [Roehl et al., 2010b]. In addition to type-1 and type-2 deletions, a third recurrent type of *NF1* deletion has recently been identified which is also mediated by NAHR. These type-3 deletions span 1.0-Mb, with breakpoints located within NF1-REP B and NF-REP C [Bengesser et al., 2010; Pasmant et al., 2010]. In contrast to the aforementioned recurrent *NF1* deletions mediated by NAHR, the so called “atypical” *NF1* deletions are neither mediated by homology-based mechanisms nor are they recurrent with respect to the localization of their breakpoints. Consequently, atypical

1 *NFI* deletions can differ quite markedly from each other in terms of the number of deleted  
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3 genes [Kehrer-Sawatzki et al., 2008 and references therein; Pasmant et al., 2008, 2010].

4  
5 *NFI* microdeletions are often associated with severe disease manifestations including a high  
6  
7 tumour load, facial dysmorphism and mental retardation [Mensink et al., 2006; Venturin et  
8  
9 al., 2006; Mautner et al., 2010; Pasmant et al., 2010].

10  
11 Somatic mosaicism is an important issue in NF1 because it influences the phenotypic  
12  
13 expression of the disease, [its recurrence](#) as well as its transmission risk [Erickson, 2010;  
14  
15 Gottlieb et al., 2001; Kehrer-Sawatzki and Cooper, 2008; Youssoufian and Pyeritz, 2002].

16  
17 Most patients with mosaic NF1, [in whom](#) causative *NFI* mutations have been identified,  
18  
19 possess large deletions of the *NFI* gene region. This excess of deletions is probably due to an  
20  
21 ascertainment bias since mosaic *NFI* microdeletions are relatively easy to identify at the  
22  
23 individual cell level using FISH analysis. By contrast, the detection of mosaicism for a  
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25 nucleotide substitution within the *NFI* gene is much more difficult, particularly if cells  
26  
27 bearing the mutation are present at low levels [Maertens et al., 2007]. So far, the majority of  
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29 reported mosaic *NFI* microdeletions have been either atypical or type-2 [reviewed in Kehrer-  
30  
31 Sawatzki and Cooper, 2008].

32  
33 Although ~80% of *NFI* deletions are type-1 [Dorschner et al., 2000; Pasmant et al., 2010],  
34  
35 only one solitary example of a putative mosaic type-1 *NFI* deletion has so far been reported  
36  
37 [De Luca et al., 2007]. This deletion was not unequivocally shown to be type-1 since the  
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39 extent of the deletion was determined solely by multiplex ligation-dependent probe  
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41 amplification (MLPA). The MLPA-kit available at that time, and used by De Luca et al.

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43 [2007], did not include any MLPA probes located outside the 1.4-Mb type-1 *NFI* deletion  
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45 region. Consequently, MLPA could not differentiate between a 1.4-Mb deletion and a larger,  
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47 atypical deletion. Likewise, the currently available MLPA-kit (P122, version C1) cannot  
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49 provide the degree of resolution required to distinguish between type-1 *NFI* deletions (with  
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51 breakpoints in the NF1-REPs) and atypical deletions with breakpoints located close to, but

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1 outside of, the NF1-REPs A and C as outlined previously [Mautner et al., 2010]. Such  
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3 atypical deletions could also encompass the *GOSRI*, *TBC1D29*, *RHOT1*, *RHBDL3* and  
4  
5 *CI7orf75* genes in addition to the 14 genes present within the type-1 deletion interval (Figure  
6  
7 1A). Our current knowledge of atypical deletions should potentiate the improved positioning  
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9 of probes in future MLPA-kits, an important issue since atypical deletions are present in  
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11 ~10% of patients with large *NF1* deletions [Pasmant et al., 2010; this study].  
12

13 The erstwhile absence of confirmed mosaic type-1 *NF1* deletions raised the question as to  
14  
15 whether type-1 deletions might be confined to the germline. This prompted us to undertake an  
16  
17 extensive search for this type of deletion and to determine its relative frequency. Beyond the  
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19 intrinsic importance of mosaicism itself, the existence of mosaic type-1 deletions would serve  
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21 to demonstrate that NAHR between the highly homologous NF1-REPs A and C is not  
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23 confined to the germline but might also occur during postzygotic mitotic cell cycles.  
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## 26 **Patients and Methods**

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28 In this study, we identified 5 putative mosaic type-1 *NF1* deletions and then proceeded to  
29  
30 analyse the extent of each deletion in detail. This study was approved by the Institutional  
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32 Review Boards of the participating institutions. Four of these mosaic deletions (those in  
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34 patients UAB-M1, UAB-r3302, UAB-r7332 and UAB-r3222) were identified in a cohort of  
35  
36 3500 unrelated NF1 patients investigated by comprehensive *NF1* mutation analysis performed  
37  
38 at the Medical Genomics Laboratory, Department of Genetics, University of Alabama at  
39  
40 Birmingham, as described [Messiaen and Wimmer, 2008]. The fifth patient, P067, was  
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42 identified during the course of a loss of heterozygosity (LOH) analysis that included 518  
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44 dermal neurofibromas from 113 patients with NF1 [Garcia-Linares et al., 2010].  
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1 *Individual case reports*

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3 Patient UAB-M1

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5 This 14-year-old white American boy exhibited ~~three~~ café-au-lait spots and freckling/  
6 hyperpigmentation on the left upper thigh. Freckling in the left inguinal region was also  
7 noted. Since only the left upper thigh/inguinal region was affected, the patient was considered  
8  
9 to exhibit segmental NF1. No dermal, subcutaneous or plexiform neurofibromas were  
10  
11 observed. The presence of Lisch nodules or asymptomatic optic gliomas were not  
12  
13 investigated. Developmental delay was not observed. Melanocytes and fibroblasts were  
14  
15 successfully cultured from a biopsy of one of the patient's café-au-lait spots, as described  
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17 [Maertens et al., 2007]. cDNA and genomic DNA derived from both cell types were  
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19 investigated by sequencing of *NF1* exons and MLPA, respectively. A blood sample from this  
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21 patient could not however be obtained for further testing.  
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27 Patient UAB-r3302

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29 This 15-year-old boy of Asian origin had >6 café-au-lait spots, axillary freckling and  
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31 cutaneous neurofibromas on the left neck, arm and trunk region. Further~~more~~, he had a  
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33 plexiform neurofibroma on the left arm (with bluish overlying skin). Despite the fact that he  
34  
35 was affected mainly on his left body side, a localized segmental manifestation was deemed  
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37 unlikely since freckling and a few café-au-lait spots were also noted on the right arm and  
38  
39 hand. Bilateral Lisch nodules were also diagnosed. Spinal neurofibromas and optic gliomas  
40  
41 were absent as determined by MRI. Skeletal abnormalities were also absent. cDNA and  
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43 genomic DNA isolated from venous blood was investigated by MLPA, FISH and PCR to  
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45 assess heterozygosity.  
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49 Patient UAB-r7332

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51 This 10-year-old white American boy exhibited multiple (>6) café-au-lait spots, bilateral  
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1 axillary and inguinal freckling, bilateral Lisch nodules, 2-6 cutaneous and 2-6 subcutaneous  
2 neurofibromas. Plexiform neurofibromas were not observed. The boy had a symptomatic  
3 optic glioma of the left optic nerve and optic chiasm. Scoliosis was also noted. His  
4 development was not delayed and his school education was appropriate to his age. cDNA and  
5 DNA isolated from venous blood was investigated by MLPA, FISH and PCR.  
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#### 13 Patient UAB-r3222

14 This 27-year-old white American woman had two café-au-lait spots but no axillary or inguinal  
15 freckling. Bilateral Lisch nodules were observed as well as >100 cutaneous neurofibromas but  
16 no plexiform neurofibromas. No symptomatic optic gliomas were present, although no MRI  
17 data were available. Skeletal abnormalities were not detected. The patient's development was  
18 normal. cDNA and DNA isolated from venous blood was investigated by MLPA, FISH and  
19 PCR.  
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#### 29 Patient P067

30 This 45-year-old white Spanish female patient exhibited a rather localized manifestation of  
31 symptoms compatible with a diagnosis of segmental NF1. She had ~20 small dermal  
32 neurofibromas as well as freckling on her upper right back and below the right breast (Supp.  
33 Figure S1). Inguinal but not axillary freckling was noted. Café-au-lait spots and Lisch nodules  
34 were not observed. The patient suffered from severe hypertension. Facial dysmorphic  
35 features, developmental delay or learning disabilities were not noted. The patient had an IQ of  
36 99. DNA samples were isolated from blood, saliva, two neurofibromas and the skin overlying  
37 these neurofibromas in order to perform deletion breakpoint-spanning PCRs. FISH analysis  
38 was not performed.  
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#### 50 *Methods*

1 Multiplex Ligation-dependent Probe Amplification (MLPA)

2 DNA was isolated from venous blood samples of the patients using the Qiamp kit (Qiagen,  
3 Valencia, CA, USA). Large deletions of the *NF1* gene and its flanking regions were identified  
4 using the SALSA P122 C1 MLPA assay (MRC-Holland, Amsterdam, Netherlands) according  
5 to the manufacturer's instructions and as described by Wimmer et al., [2006].  
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10 Microsatellite marker analysis

11 In addition to MLPA, four microsatellite markers located within the *NF1* gene region (JVs27-  
12 GXAlu, IVs27TG24.8, IVs27AC28.4, JVs38TG53.0) were analysed by PCR in all 3500 *NF1*  
13 patients in order to investigate heterozygosity and in particular the occurrence of skewed  
14 marker peaks (reduced in peak height and area as compared to the peak of the other allele)  
15 indicative of mosaicism.  
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26 Fluorescence *in situ* hybridization (FISH)

27 Where MLPA or microsatellite marker analysis gave any hint as to the presence of a deletion  
28 with or without mosaicism with normal cells, interphase FISH was performed on 200  
29 uncultured blood cells using probes RP5-1002G3 (detected by red fluorescent signals) and  
30 RP5-926B9 (green). If the proportion of normal cells was very low (>0% and >5%), 400  
31 interphase nuclei were evaluated. Probe RP5-1002G3 (GenBank accession no.: CG786083) is  
32 located in the telomeric region of the *NF1* gene, whereas FISH probe RP5-926B9 spans the  
33 centromeric part of the *NF1* gene [De Raedt et al., 2004]. The genomic region encompassed  
34 by these FISH probes is ~150-kb. Thus, two red/green fusion FISH signals should be obtained  
35 from an interphase nucleus with two normal chromosomes 17 lacking the *NF1* deletion. The  
36 centromere-specific alpha satellite probe D17Z1 was used as a reference FISH probe from  
37 outside of the *NF1* deletion region (CEP17 Spectrum-Aqua, Vysis, Abbott Molecular Inc.,  
38 Illinois, USA). Owing to the use of two test probes located within the *NF1* gene region, our  
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1 assay is well suited to exclude consideration of wrongly assigned normal cells because it is  
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 3 highly unlikely that both of the two test probes would yield false positive signals. In no case  
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 5 was the occurrence of only one normal cell (versus 199 cells with the deletion) observed in  
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 7 our assays.  
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#### 11 Breakpoint-spanning PCR (deletion junction assays)

12 To detect type-1 *NF1* deletions with breakpoints located within the PRS1 and PRS2 hotspots,  
 13 breakpoint-spanning PCRs were performed using the Expand Long Template PCR system  
 14  
 15 (Roche, Penzberg, Germany) with 400 ng genomic DNA isolated from whole blood samples  
 16  
 17 as template. The primers used for these deletion junction PCRs are listed in Supp. Table S1.  
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19 Positive PCR products of 2.9-kb and 3.5-kb were sequenced from both ends. The analysis of  
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 21 the paralogous-specific sequence variants that distinguish NF1-REP A and NF1-REP C  
 22  
 23 indicated whether the PCR products were true positive recombinant deletion junction  
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 25 fragments. In addition to PRS1 and PRS2, we also investigated the breakpoint region  
 26  
 27 identified in a single patient (UWA160-1) as reported by Forbes et al., [2004] using primers  
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 29 160P1-F (5'-GGCTCATGTGTAATGATCTTTAACGCG-3') and MD1-R(v2) (5'-  
 30  
 31 CAAAGATTATCACTGATGGAGTTGG-3') (Supp. Figure S2).  
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33 All DNA samples were pre-tested by PCR to determine if the DNA was of sufficient  
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 35 quality for robust amplification of large DNA fragments. For this purpose, we used  
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 37 primers17s1 (5' CAAGGCAACAACTGAAGCA 3') and tg5 (5'  
 38  
 39 TCCATTTGCTGTCCCATTTT 3') to amplify a 5-kb fragment from 17q12. Positive PCR  
 40  
 41 products using these primers confirmed that long PCR products could be amplified from the  
 42  
 43 respective DNA sample and that negative breakpoint-spanning PCRs were not due to low  
 44  
 45 template DNA quality.  
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#### 50 Single nucleotide polymorphism (SNP) analysis

1 The extent of those deletions that were negative in the deletion junction assays was further  
 2 investigated by SNP analysis using PCR (primers listed in Supp. Table S2). The respective  
 3 PCR products were sequenced and analysed by BLAT sequence alignments  
 4 (<http://genome.ucsc.edu/>) to avoid confusion between paralogous sequence differences and  
 5 SNPs.  
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## 13 Results

14 MLPA, microsatellite marker analysis and FISH were used in concert to analyze a cohort of  
 15 3500 unrelated NF1 patients for gross *NF1* deletions. A total of 146 individuals (4.2%) were  
 16 identified as harbouring gross *NF1* deletions. Of these 146 *NF1* deletions, 116 were type-1, 16  
 17 were type-2, two were type-3 and 12 were atypical (Table 1). Somatic mosaicism was  
 18 identified in 14 (10%) of the 146 patients with *NF1* deletions including 4 individuals with  
 19 putative type-1 *NF1* deletions (Table 1), as suggested by MLPA performed with DNA from  
 20 whole blood samples (patients UAB-r3302, UAB-r7332 and UAB-r3222) or with DNA  
 21 derived from café-au-lait spot melanocytes (patient UAB-M1). With the currently available  
 22 MLPA-kit, it is however impossible to distinguish type-1 *NF1* deletions (with breakpoints in  
 23 the *NF1*-REPs) from atypical deletions with breakpoints located close to the *NF1*-REPs A and  
 24 C. Consequently, further analyses were needed in order to narrow down the extent of the  
 25 deletions in these 4 patients, as outlined in the following paragraphs.  
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38 In patient UAB-M1, comprehensive *NF1* mutation analysis revealed a splice site mutation  
 39 (c.889-2A>G) at the splice acceptor site of exon 7 (according to the traditional exon  
 40 nomenclature, or exon 9 according to the numbering from sequence NM\_000267.3) in  
 41 addition to a type-1 *NF1* deletion in melanocyte-derived DNA from this patient. The presence  
 42 of two hits in the café-au-lait spot melanocytes of this patient is in line with our previous  
 43 findings [Maertens et al., 2007; De Schepper et al., 2008]. Remarkably, neither the *NF1*  
 44 deletion nor the splice site mutation were identifiable in DNA from café-au-lait spot-derived  
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1 fibroblast cell cultures from patient UAB-M1. Unfortunately, neither a blood sample nor a  
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 3 biopsy from a second café-au-lait spot from this patient was available. Thus, it remains  
 4  
 5 unclear whether or not the *NF1* deletion represented the first-hit mutation in this patient.

6  
 7 Mosaicism in patients UAB-r3302, UAB-r7332 and UAB-r3222, from whom blood samples  
 8  
 9 were available, was ascertained by interphase FISH as summarized in Table 2.

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 11 Since the currently available MLPA-kit does not allow an unambiguous distinction to be  
 12  
 13 made between type-1 *NF1* deletions and atypical deletions characterized by breakpoints  
 14  
 15 located close to the NF1-REPs (Figure 1A), it could not be formally confirmed that the 4  
 16  
 17 putative mosaic deletions (detected in patients UAB-M1, UAB-r3302, UAB-r7332 and UAB-  
 18  
 19 r3222) were indeed type-1. To characterize the extent of these deletions, we performed  
 20  
 21 breakpoint-spanning PCRs or SNP analysis as outlined below.

#### 22 *Breakpoint identification by PCR*

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 27 Employing breakpoint-spanning PCR, we next investigated whether the breakpoints of the  
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 29 deletions in patients UAB-r3302, UAB-r3222 and UAB-r7332 were located within the PRS1  
 30  
 31 and PRS2 hotspots. In patient UAB-r3222, a PRS2 breakpoint-spanning fragment was  
 32  
 33 obtained and subsequent sequence analysis confirmed that the deletion breakpoint was indeed  
 34  
 35 located within the PRS2 hotspot. However, patients UAB-r3302 and UAB-r7332 were both  
 36  
 37 negative by breakpoint-spanning PCR analysis for PRS1 and PRS2. Therefore, we  
 38  
 39 investigated whether the deletion breakpoints in these two patients might be located in the  
 40  
 41 same genomic region within which the deletion breakpoint of patient UWA160-1 had been  
 42  
 43 previously identified [Forbes et al., 2004]. These authors mapped the deletion breakpoints of  
 44  
 45 patient UWA160-1 some 23-kb centromeric to the PRS1 region (Supp. Figure S2). However,  
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 47 PCR assays with primers designed to amplify across these deletion breakpoints (those noted  
 48  
 49 in patient UWA160-1) were negative in both patients UAB-r3302 and UAB-r7332.

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1 Breakpoint-spanning PCR assays could not be performed with DNA isolated from melanocyte  
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3 cultures of patient UAB-M1 on account of the paucity of the DNA obtained.

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5 Deletion junction PCRs were also performed to analyse DNA samples from patient P067.  
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7 Previously performed microsatellite marker analysis had indicated loss of heterozygosity  
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9 (LOH) in DNA isolated from a neurofibroma from patient P067 (tumour P067-2N) as  
10  
11 described by Garcia-Linares et al., [2010]. MLPA analysis of *NF1* -/- Schwann cells from this  
12  
13 tumour was suggestive of the presence of a type-1 *NF1* deletion [Garcia-Linares et al., 2010].

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15 In this study, we investigated whether the deletion breakpoints were located within the PRS1  
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17 and PRS2 regions. A PRS1-spanning PCR product was successfully amplified from DNA of  
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19 neurofibroma P067-2N. Surprisingly, another neurofibroma (tumour P067-1N) from this  
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21 patient also proved positive in the PRS1 deletion junction assay even although LOH was not  
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23 detected in this tumour. Additionally, the PRS1 breakpoint-spanning fragment was detected in  
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25 DNA samples isolated from the skin overlying both neurofibromas. These findings strongly  
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27 implied that patient P067 is mosaic for a type-1 *NF1* deletion and that the deletion is not  
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29 tumour-specific but rather occurred during embryonic development resulting in somatic  
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31 mosaicism. Remarkably, the PRS1 breakpoint-spanning PCR was not positive using DNA  
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33 isolated from blood and saliva containing up to 74% leucocytes [Thiede et al., 2000]. Thus,  
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35 the type-1 *NF1* deletion is either absent from the blood cells of patient P067 or alternatively  
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37 occurs at a level that is below the detection limit of the breakpoint-spanning PCR assays. We  
38  
39 therefore concluded that patient P067, who manifests segmental NF1, is mosaic for a type-1  
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41 deletion that is probably confined to specific cell lineages.

#### 42 43 44 *SNP analysis*

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46 Since patients UAB-r3302 and UAB-r7332 were negative for the breakpoint-spanning PCRs  
47  
48 designed to detect NAHR events within the PRS1 and PRS2 hotspots, it was unclear whether  
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50 the respective deletion breakpoints were located within NF1-REPs A and C, as is  
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1 characteristic of type-1 *NF1* deletions. To determine the extent of these deletions and that of  
2 patient UAB-M1, we performed SNP analysis (Supp. Table S2). Heterozygosity for SNPs  
3 located between the MLPA probes and the NF1-REPs would indicate that the region tagged  
4 by these SNPs is not deleted. However, this method is not without its limitations, i.e. the  
5 availability of heterozygous (and hence informative) SNPs. Further, the majority of SNPs  
6 assigned to the NF1-REPs by the HapMap Genome Browser are not well genotyped and  
7 hence could represent paralogous sequence variants rather than SNPs. Despite these  
8 limitations, the SNP analysis indicated the presence of a heterozygous type-1 deletion in  
9 patient UAB-r3302. Since heterozygosity for SNPs rs216440 and rs7218060 was observed,  
10 we conclude that the deletion breakpoints in patient UAB-r3302 are probably located within  
11 NF1-REPs A and C (Figure 1B). By contrast, the centromeric deletion breakpoint in patient  
12 UAB-r7332 could not be unequivocally assigned to within NF1-REP A, since the distance  
13 between the heterozygous SNP rs216455 and the centromeric boundary of NF1-REP A was  
14 found to be ~37-kb (Figure 1C). In patient UAB-M1, the extent of the deletion could not be  
15 narrowed down any further by SNP analysis. Hence, a type-1 *NF1* deletion could not be  
16 unequivocally demonstrated in either patient UAB-M1 or UAB-r7332.

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## Discussion

36 The type-1 *NF1* deletion is the most frequently encountered type of *NF1* microdeletion.  
37 Indeed, previous studies have suggested that ~80% of all *NF1* deletions are type-1 [Dorschner  
38 et al., 2000; Forbes et al., 2004; Pasmant et al., 2010]. The results of the analysis presented  
39 here, the largest performed to date, provides independent confirmation of this estimate since  
40 116 of the 146 *NF1* deletions identified by MLPA were deemed likely to be type-1 (79%;  
41 Table 1). Only 16 of these 116 cases with type-1 deletions were familial, the rest occurred *de*  
42 *nov*. FISH analysis indicated that 4 of these 116 type-1 deletions were mosaic cases.  
43 However, some doubt remained as to the deletion type since MLPA is not well suited to

1 distinguish unequivocally between type-1 deletions and atypical deletions with breakpoints  
2 located outside of (but close to) the NF1-REPs (Figure 1A). Therefore, we reanalysed these 4  
3 putative mosaic type-1 deletions using methods capable of providing a higher level of  
4 resolution of the breakpoint positions than MLPA. By these means, we were able to confirm  
5 that two of these four deletions, those harboured by patients UAB-r3222 and UAB-r3302,  
6 were indeed type-1. Thus, at least 2% (up to 4%) of all patients with *de novo* type-1 *NF1*  
7 deletions exhibit mosaicism.  
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9 In the cohort of 146 *NF1* deletions investigated here, 16 type-2 deletions were detected.  
10 Ten of these 16 patients with type-2 deletions were known sporadic cases, whereas three,  
11 patients with type-2 deletions were familial. For a further three cases, it was unknown  
12 whether they were sporadic or familial. Remarkably, 7 of the 10 known sporadic patients with  
13 type-2 deletions exhibited mosaicism with normal cells (70%) and all seven patients were  
14 females. This supports previous findings of a preponderance of females among patients with  
15 type-2 *NF1* deletions [Steinmann et al., 2007]. The actual proportion of mosaic type-2  
16 deletions within this cohort could however have been underestimated since the parents of the  
17 non-founder type-2 deletion patients were not investigated by FISH. Thus, mosaicism in the  
18 founders of such families could not be unequivocally excluded. Indeed, in a previous analysis,  
19 we observed three cases of the transmission of a type-2 *NF1* deletion from a mosaic parent,  
20 exhibiting no (or only minor) disease manifestations, to a child [Steinmann et al., 2007].  
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22 Furthermore, 16 of 18 patients with type-2 *NF1* deletions investigated previously exhibited  
23 somatic mosaicism [Roehl et al., 2010b]. These studies also indicated that in the majority of  
24 patients with type-2 deletions, the proportion of cells bearing the deletion is very high (>90%)  
25 in blood but considerably lower in buccal smears. Importantly, although such deletions will  
26 have been identified using aCGH or MLPA analysis, these techniques would have been  
27 inadequate to the task of identifying their mosaic nature, especially when the fraction of non-  
28 deleted alleles was low. Hence the use of FISH analysis, on blood or other cell types (e.g.  
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1 from buccal smears, urine samples etc), is advisable as an additional detection technique in  
2  
3 potential cases of mosaicism.  
4

5 Even if we underestimated the frequency of mosaic type-2 deletions in this study, the  
6 analysis of the 146 *NFI* deletions served to indicate that somatic mosaicism occurs much  
7 more frequently in association with type-2 deletions than with type-1 deletions (cf. at least  
8 70% vs. 2%). We have proposed that the relative frequencies of meiotic vs. mitotic  
9 (postzygotic) NAHR events within the *NFI* gene region are likely to correlate with specific  
10 sequence features at the breakpoints which necessarily differ between the two deletion types.  
11 These features could include GC-content as well as the presence of recombination-promoting  
12 sequence motifs [Roehl et al., 2010b and references therein].  
13  
14

15 In addition to the confirmed mosaic type-1 *NFI* deletions observed in patients UAB-r3222  
16 and UAB-r3302 from the cohort of 146 deletion patients, we identified a third case of a  
17 mosaic type-1 *NFI* deletion in patient P067, who was initially investigated during the course  
18 of an LOH study in neurofibromas [Garcia-Linares et al., 2010]. The mosaic type-1 deletion  
19 in this patient was observed in two different neurofibromas and overlying skin samples only  
20 after breakpoint identification by PCR. Taken together, these three cases represent the first  
21 examples of mosaic type-1 deletions that have been unequivocally confirmed by high  
22 resolution techniques. Two of the three mosaic type-1 deletions, those identified in patients  
23 P067 and UAB-r3222, had breakpoints within the PRS1 and PRS2 regions, respectively.  
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26 Previous studies have shown that 70-80% of all germline type-1 *NFI* deletions exhibit  
27 breakpoints within these hotspots of NAHR [López-Correa et al., 2001; Forbes et al., 2004].  
28 Since the majority of germline type-1 *NFI* deletions occur during maternal meiosis I [López-  
29 Correa et al., 2000], PRS1 and PRS2 have long been considered to represent NAHR hotspots  
30 that were specific to maternal meiosis. However, our discovery that the breakpoints of mosaic  
31 type-1 deletions may sometimes also be located in PRS1 and PRS2 indicates that NAHR is  
32 not confined exclusively to maternal meiosis but may also occur within these recombination  
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1 hotspots during postzygotic (mitotic) cell cycles, even although the frequency of occurrence  
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 3 of NAHR is much lower than during meiosis,  
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5 We have previously proposed that a high frequency of mosaicism observed in several  
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 7 cancer predisposition syndromes may be related to a high new mutation rate [Kehrer-

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 9 Sawatzki and Cooper, 2008]. Our current analysis demonstrates that mosaicism represents an  
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 11 important consideration in the analysis and diagnosis of all types of gross *NF1* deletion (Table  
 12  
 13 1). Mosaicism in *NF1* can be associated either with a generalized but often mild disease  
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 15 manifestation or alternatively a regionally limited distribution of *NF1* disease features, termed  
 16  
 17 segmental *NF1* [Ruggieri and Huson, 2001]. Estimates of the prevalence of segmental *NF1* in  
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 19 the general population range from 0.0006% [Wolkenstein et al., 1995] to 0.0018% [Ingordo et  
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 21 al., 1995]. Although unusual, many patients with this condition have been described in the  
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 23 literature [Ruggieri, 2001; Ruggieri and Huson, 2001; Listernick et al., 2003 and references  
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 25 therein; Redlick and Shaw, 2004]. So far, mutations have only been reported in 5 cases of  
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 27 segmental *NF1*, three of these carrying atypical *NF1* deletions (Supp. Table S3) [Tinschert et  
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 29 al., 2000; Consoli et al., 2005; Maertens et al., 2007]. Remarkably, patient P067 identified in  
 30  
 31 this study is the first individual described who not only has a mosaic type-1 *NF1* deletion but  
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 33 also segmental *NF1*. The breakpoints of this patient's somatic deletion are both located within  
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 35 PRS1, a recombination hotspot previously shown to harbour breakpoints of germline type-1  
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 37 *NF1* deletions [Forbes et al., 2004; De Raedt et al., 2006]. We may however infer that the  
 38  
 39 NAHR event responsible for the deletion in patient P067 must have occurred relatively late on  
 40  
 41 during embryonic development, giving rise to the highly localized manifestation of the  
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 43 disease (Supp. Figure S1). Importantly, the type-1 deletion was detected only in tumour  
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 45 samples and skin samples overlying the tumours from patient P067 but not in either her blood  
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 47 or saliva. Similarly, Consoli et al., [2005] described a patient with segmental *NF1* and a  
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 49 mosaic R1947X *NF1* gene mutation that was only detectable in keratinocyte and fibroblast  
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1 cell lines isolated from a café-au-lait spot but not in unaffected skin samples or peripheral  
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3 blood of the patient.

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5 By contrast, the remaining two patients shown to [harbour](#) a mosaic type-1 *NF1* deletion  
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7 (UAB-r3222 and UAB-r3302) exhibited a generalized manifestation of the disease. Many of  
8  
9 the mosaic *NF1* cases identified to date have been associated with a generalized (albeit mild)  
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11 disease manifestation, but without typical symptoms [being](#) restricted to only a few regions of  
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13 the body [Ruggieri and Huson, 2001; reviewed in Kehrer-Sawatzki and Cooper, 2008;  
14  
15 Bottillo et al., 2010; Kaplan et al., 2010; Muram-Zborovski et al., 2010]. From the clinical  
16  
17 point of view, it is very difficult to distinguish mosaic *NF1* patients with generalized  
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19 manifestations from those classical patients with germline *NF1* mutations. Phenotypically,  
20  
21 patients UAB-r3222 and UAB-r3302 gave no outward and visible clue that their type-1  
22  
23 deletions might be mosaic. This implies that a comprehensive *NF1* screening protocol, which  
24  
25 should also include FISH analysis, will be necessary to assess mosaicism in all types of gross  
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27 *NF1* deletion.

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#### 30 Acknowledgements

31  
32 This work was supported by the [Deutsche Forschungsgemeinschaft \[DFG grants KE 724/9-1;](#)  
33  
34 [KE 724/7-1; H. K.-S.\]](#), [by the](#) Deutsche Krebshilfe ([grant #108793, H.K.-S.](#)) and from the  
35  
36 Spanish Health Research Fund, the Carlos III Health Institute, the Catalan Health Institute and  
37  
38 Autonomous Government of Catalonia, the Asociación Española de Afectados de  
39  
40 Neurofibromatosis and the Asociación Española contra el Cáncer (ISCIIRETIC:  
41  
42 RD06/0020/1051 and RD06/0020/1050, PI081871 and 2009SGR290). [J. V. holds a](#)  
43  
44 [fellowship from the International Graduate School in Molecular Medicine, Ulm.](#)  
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## Legend

**Figure 1:** Schema of the *NF1* gene region indicating the genes located therein, the relative positions of the MLPA probes employed and the SNPs investigated in the characterization of the extent of the gross *NF1* deletions in patients UAB-r3302 and UAB-r7332. (A) The functional genes located within the *NF1* microdeletion region at 17q11.2 are indicated by black bars. *LRRC37B2* and *SUZ12P* are pseudogenes. The positions of the MLPA probes deleted in the case of type-1 *NF1* deletions are given by grey arrows, whereas those not deleted are denoted by black arrows. The name and genomic position of each MLPA probe are as follows: arrow no. 1: MLPA probe CPD 9628-L9913 (25,795038); no. 2: CPD 9629-L9914 (25,813,548); no. 3: SUZ12p before ex1 11798-L12590 (26,082,519); no. 4: SUZ12p ex1 11801-L12591 (26,082,967); no. 5: SUZ12p ex6 11801-L12592 (26,109,273); no. 6: CRLF3 3780-L3290 (26,148,508); no. 7: ATAD5 3781-L3290 (26,186,172); no.8: CENTA2 3782-L3291 (26,278,001); no. 9: RNF135 3783-L3292 (26,335,816); no. 10: NF1 Ex. 1 2491-L1922 (26,445,726); no. 11: NF1 Ex. 12B 2507-L1938 (26,576,330); no. 12: NF1 Ex. 23-2 2512-L1943 (26,600,151); no. 13: NF1 Ex. 40 2525-L1943 (26,700,280); no. 14: NF1 Ex. 48 5220-L3309 (26,711,704); no. 15: UTP6 3785-L3294 (27,226,463); no. 16: JAZ1 3786-L3295 (27,339,525); no. 17: LRRC37B 3787-L3296 (27,372,684); no. 18: ZNF207 9637-L9949 (27,717,868). (B) Positions of the heterozygous SNPs (rs216440 and rs7218060) in patient UAB-r3302 relative to NF1-REP A and NF1-REP C. Numbers indicate the nucleotide positions of SNPs, MLPA probes and the extent of NF1-REPs A and C according to the sequence of the Human Genome Assembly, 18 March 2006, NCBI Build 36.1. The MLPA probes within *SUZ12P* and *LRRC37B*, located within the deletion region, are identified by red arrows, whereas those MLPA probes not deleted are denoted by black arrows. SNP rs216440, located at nucleotide position 25,952,435, is located within NF1-REP A, which begins at position 25,951,642. Heterozygosity for this SNP was observed in patient UAB-r3302. The telomeric end of NF1-REP A (at 26,079,631) is separated from the MLPA probe within *SUZ12P* (at 26,082,519) by ~2.8-kb. The distance between SNP rs7218060 and the telomeric end of NF1-REP C is ~3.9-kb. (C) Positions of heterozygous SNPs (rs216455 and rs7218060) in patient UAB-r7332 relative to NF1-REP A and NF1-REP C. The distance between SNP rs216455 (at nucleotide position 25,914,329) and the centromeric boundary of NF1-REP A is ~37-kb. The distance between SNP rs7218060 and the telomeric end of NF1-REP C is ~3.9-kb.

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Table 1: Frequency of type-1, type-2, type-3 and atypical *NF1* deletions among the 146 deletions identified by MLPA in a cohort of 3500 *NF1* patients

|  | <i>NF1</i> deletion |          |          |           | Total |
|--|---------------------|----------|----------|-----------|-------|
|  | type-1              | type-2   | type-3   | atypical  |       |
| Number of deletions with this type among the total of 146 <i>NF1</i> deletions       | 116 (79.4%)         | 16 (11%) | 2 (1.4%) | 12 (8.2%) | 146   |
| Frequency of deletion of a given type among all 3500 <i>NF1</i> patient investigated | 3.3%                | 0.5%     | 0.01%    | 0.3%      | –     |
| Number of mosaic cases with the given deletion type                                  | 4 <sup>a</sup>      | 7        | 0        | 3         | 14    |

a: Two of these 4 putative type-1 *NF1* deletions were confirmed as such by means of breakpoint-spanning PCR and SNP analysis.

Table 2: Characterization of the 5 mosaic *NF1* microdeletions

|   | Patient |           |                  |           |      |
|---|---------|-----------|------------------|-----------|------|
|   | UAB-M1  | UAB-r3302 | UAB-r7332        | UAB-r3222 | P067 |
| Type-1 <i>NF1</i> deletion as determined by MLPA                      | +       | +         | +                | +         | +    |
| Proportion of cells with the <i>NF1</i> deletion <sup>a</sup>         | n.d.    | 80%       | 97% <sup>b</sup> | 50%       | n.d. |
| Breakpoint localization according to <a href="#">PCR</a> <sup>c</sup> | n.d.    | negative  | negative         | PRS2      | PRS1 |
| <i>NF1</i> deletion type according to SNP analysis                    | n.i.    | type-1    | n.i.             | n.d.      | n.d. |

a: FISH was performed on 200 interphase nuclei from whole blood samples.

b: [In this case FISH was performed on 400 interphase nuclei from whole blood for this patient.](#)

c: The paralogous recombination regions PRS2 and PRS1 are hotspots of recombination within NF1-REPs A and C. Breakpoint localization within PRS1, PRS2 and the region within which the deletion breakpoint of patient UWA160-1, identified previously by Forbes et al., [2004], was determined by deletion junction PCRs. n.d.: not determined; n.i.: not informative

Deleted: PCR<sup>b</sup>

Deleted: b

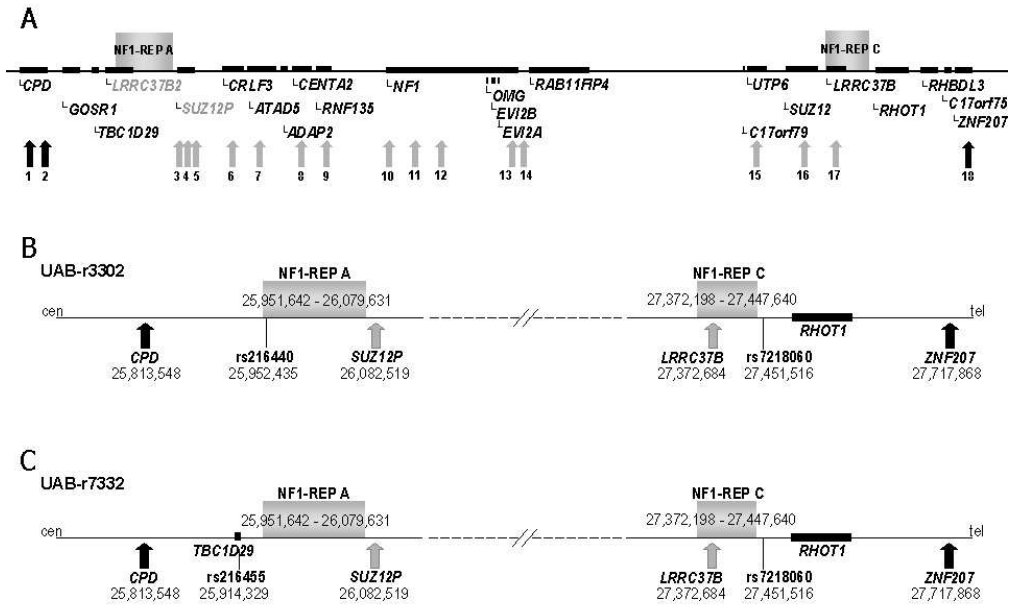


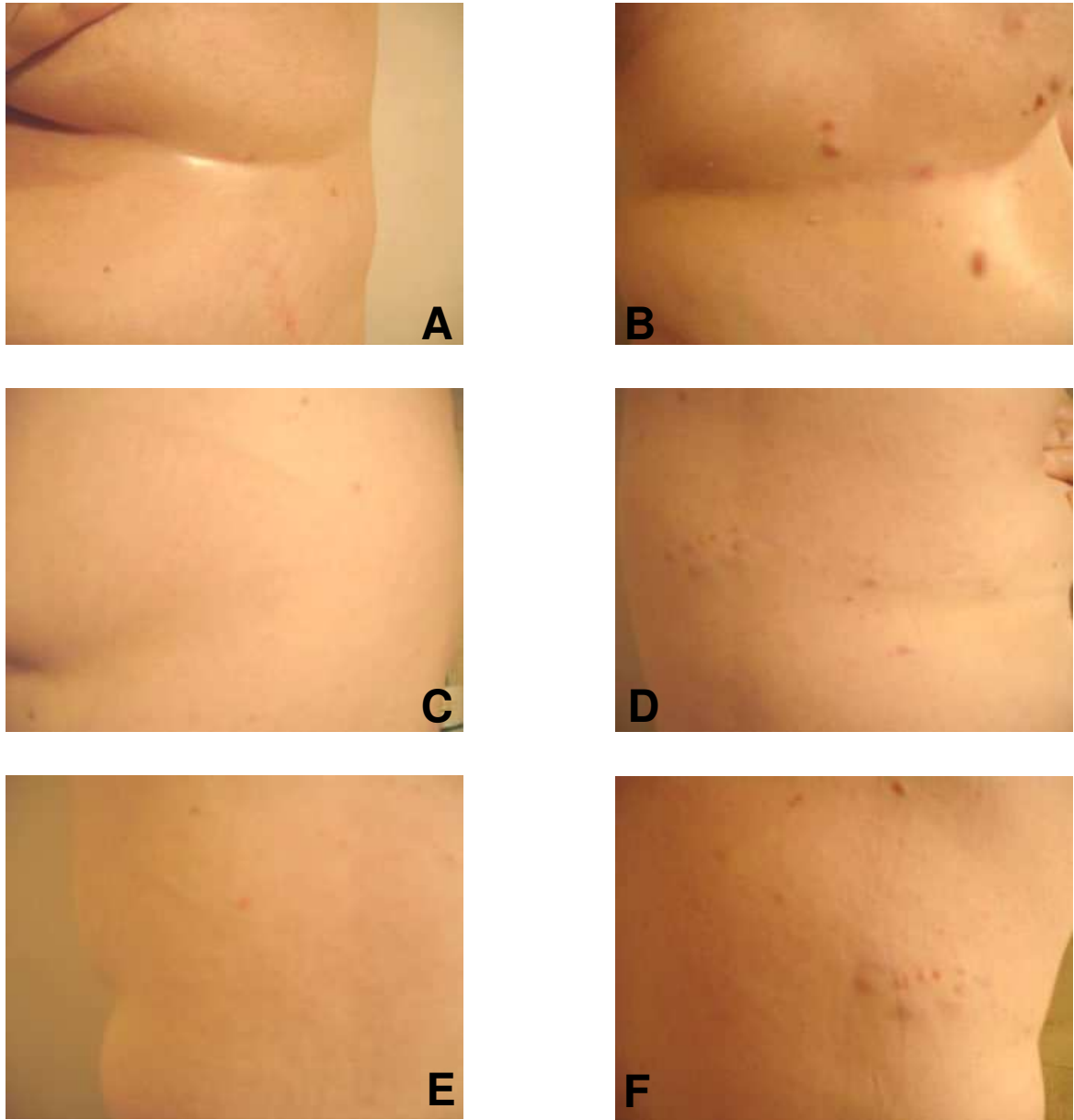
Figure 1

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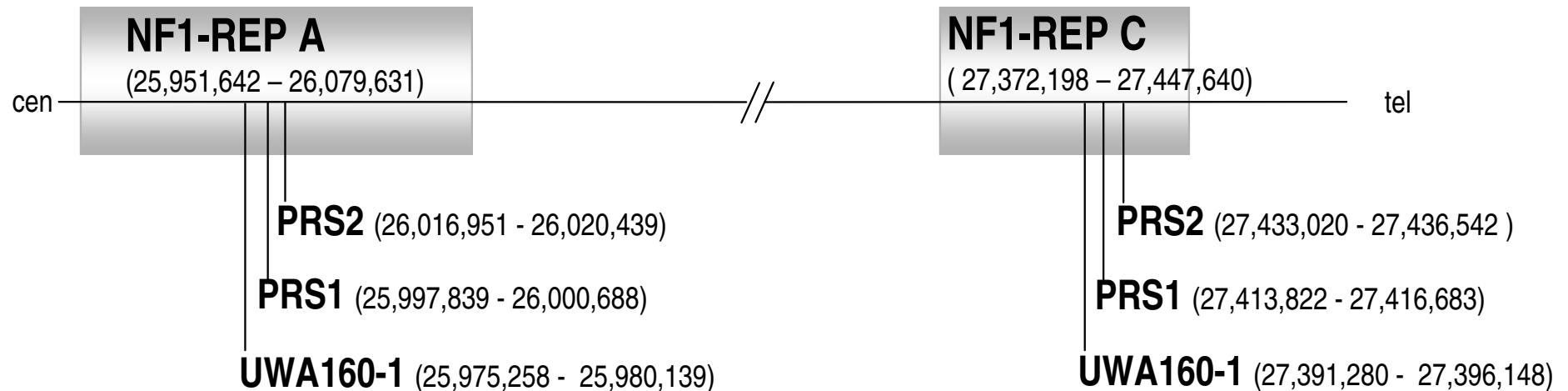
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**Supplementary Figure S1:** Segmental NF1 in patient P067. The patient manifested disease features (small neurofibromas and freckling) which were confined to the region below the right breast (B), on the right upper flank (D) and back (F). In A, C and E, the corresponding unaffected regions on the left side are depicted.



**Supplementary Figure S2:** Schematic diagram of the genomic region of the NF1-REPs and the relative positions of the deletion breakpoint hotspot regions PRS1 and PRS2. The deletion breakpoint region of patient UWA160-1, identified previously by Forbes et al., [2004], is also indicated. The numbers in parentheses refer to genomic positions according to the base-pair numbering of chromosome 17 in the Human Genome Assembly, 18 March 2006, NCBI Build 36.1, database version 44.36f.



**Supplementary Table S1: Primers used for breakpoint-spanning PCRs to amplify type-1 *NF1* deletion breakpoints located within the paralogous recombination sites 1 and 2 (PRS1 and PRS2) according to López-Correa et al., [2001] and Forbes et al., [2004]**

| Primer            | Sequence 5'→3'                                     | Size of PCR product | Position on chromosome 17 <sup>a</sup>         | Paralogous recombination site detected within the NF1-REPs |
|-------------------|--|---------------------|--|--|
| DJ2290<br>DJ2290r | TCAACCTCCCAGGCTCCCGAA<br>AGCCCCGAGGGGATGAAAAGC     | 3.5-kb              | 26,016,951-26,016,971<br>27,436,522-27,436,542 | PRS2   |
| ext-f<br>ext-r    | CAACCTCCCAGGCTCAAGTG<br>GGCAGGGTTAGGAGCTCTGGAA     | 7-kb                | 26,016,952-26,016,971<br>27,440,231-27,440,252 |  |
| md4f<br>m30r      | ATCCTGTAGCCCAGTTCTCCTC<br>CAGTGAACCGAGATTGTACTACCA | 2.9-kb              | 25,997,839-25,997,860<br>27,416,683-27,416,706 | PRS1   |

<sup>a</sup>: According to the nucleotide numbering in the Human Genome Assembly, 18 March 2006, NCBI Build 36.1, database version 44.36f.

**Supplementary Table S2: SNP analysis in NF1 patients UAB-r3302, UAB-r7332, and UAB-M1 who were negative for the PCRs designed to detect deletion breakpoints in the paralogous recombination sites PRS1 and PRS2**

| PCR-Primer | SNP        | SNP position <sup>a</sup> | Heterozygosity frequency in CEUs | Primer sequence 5' → 3'                              | Alleles detected in patient <sup>b</sup> |           |        |
|------------|------------|---------------------------|----------------------------------|--|--|-----------|--------|
|            |            |                           |                                  |  | UAB-r3302                                | UAB-r7332 | UAB-M1 |
| 45f/46r    | rs216466   | 25,884,080                | C/T 0.504                        | AGATGCATGGAGTTCGAAGAG<br>AGCTGCACAGGTCACCTTT         | C/T                                      | C/T       | —      |
| 55f/56r    | rs216455   | 25,914,329                | C/T 0.400                        | GGAACCCCATCCCTACTACC<br>GCTCAAGGCTTGTGTCTCTG         | T/C                                      | T/C       | —      |
| 1f/2r      | rs170053   | 25,917,209                | C/T 0.491                        | AGAGTCCCTGGGGGAGAAC<br>GGTTTCCCGAGACTCACACA          | n.d.                                     | T         | n.d.   |
| 29f/30r    | rs216462   | 25,920,023                | C/T 0.212                        | CGCAGCTTGCTGAGGAGT<br>AGGGTCAGACAGAGCAGAGG           | n.d.                                     | C         | n.d.   |
| 7f/8r      | rs17606460 | 25,923,150                | C/T 0.416                        | ATGGGCCACCTGCTAGCTC<br>ACCTGACACTGCAACCCATT          | n.d.                                     | C         | —      |
| 81f/82r    | rs17669584 | 25,923,740                | A/G 0.300                        | CTTGAGACCAACCCACATC<br>TCGGCAGTCTGAAACATGAC          | n.d.                                     | A         | n.d.   |
| 31f/32r    | rs216412   | 25,927,520                | A/G 0.207                        | GCAGATCTTTGCCTCCCTTT<br>GAAGCTTCGCCTGCACCT           | A/G                                      | G         | —      |
| 31f/32r    | rs170052   | 25,927,544                | A/C 0.230                        | GCAGATCTTTGCCTCCCTTT<br>GAAGCTTCGCCTGCACCT           | C/A                                      | C         | —      |
| 31f/32r    | rs216411   | 25,927,619                | C/T 0.200                        | GCAGATCTTTGCCTCCCTTT<br>GAAGCTTCGCCTGCACCT           | T/C                                      | C         | —      |
| 83f/84r    | rs216410   | 25,927,957                | C/G 0.200                        | CTTCTGAGCTGTGGTCGTCA<br>AAGACGCAAAGGCCAATAAAA        | C/G                                      | G         | —      |
| S102/103   | rs426434   | 25,949,479                | C/T 0.183                        | CCTCTGCATGTTAACCCCTGTAT<br>GGCTGAACAGTGGCAAAAA       | C/T                                      | C         | C      |
| 85f/86r    | rs9890961  | 25,952,308                | A/C 0.018                        | GCGGGGTACCTGTTGACTT<br>TTCAATAATCCCTTCACGAG          | C  | C         | C      |
| 87f/88r    | rs216440   | 25,952,435                | A/T 0.422                        | TTGTGGAGGGGAGGATACAC<br>ACAGGCACATGGGAAAAGTC         | T/A                                      | A         | A      |
| 13f/14r    | rs7218060  | 27,451,516                | G/T 0.257                        | TCATTATGAATTTTCATGTGATAGAAC<br>GAACACATTGTGGCCTAAAGC | G/T                                      | G/T       | —      |
| 17f/18r    | rs8081372  | 27,461,882                | A/G 0.265                        | AGGGGAAGACAGTGGACTTG<br>TAATGTCGTCTGTGCCCTTG         | G/A                                      | G/A       | G      |
| 37f/38r    | rs3809786  | 27,463,437                | A/G 0.271                        | GGCCAGGAGGGAGTTGTTAC<br>TGGAAAATGGATTCCGAGTCT        | n.d.                                     | G/A       | —      |
| 21f/22r    | rs7226186  | 27,468,787                | A/G 0.252                        | TAAGCGAAGTGGGATCTTGG<br>CTTGCCTCCAGCCTAGAA           | G/A                                      | G/A       | —      |
| 25f/26r    | rs9915569  | 27,490,232                | A/C 0.274                        | CACGGCGGCTTAATTCTTTA<br>GCACTTTCAGTGAGCAATGG         | n.d.                                     | n.d.      | A      |
| 57f/58r    | rs7220511  | 27,504,797                | A/G 0.300                        | GGATGAGAATATGGAGATATTTGAA<br>ACAACCTACCACGATGGA      | n.d.                                     | n.d.      | G      |

a: according to the Human Genome Assembly, hg18 March 2006, NCBI Build 36.1

n.d.: not determined

b: Lymphocyte DNA from patients UAB-r3302 and UAB-r7332 was investigated. In the case of patient UAB-M1, DNA isolated from melanocyte cultures was analysed. Due to the low amount of DNA obtained from the melanocyte cultures, only a subset of SNPs could be analysed. —: denotes SNP not investigated owing to the limited amount of DNA available. CEU: Utah residents with Northern and Western European ancestry from the CEPH collection

SNPs marked in yellow are located proximal to or at the beginning of NF1-REP A, whereas SNPs marked in blue are located distal to NF1-REP C.

**Supplementary Table S3: Patients with segmental NF1 and identified mosaic *NF1* gene mutations**

| Patient/ Reference                 | <i>NF1</i> gene mutation identified (first hit) and breakpoint localization of deletions  |
|------------------------------------|---|
| Tinschert et al., [2000]           | large <i>NF1</i> deletion as determined by FISH but the type of deletion was not further specified  |
| SNF1-3/<br>Maertens et al., [2007] | atypical <i>NF1</i> deletion of 1.8 – 2.8-Mb. The proximal deletion breakpoint is located 174 – 353-kb centromeric to NF1-REP A whereas the distal breakpoint is located between the telomeric end of NF1-REP C and position 27,598,975 (151-kb breakpoint interval). |
| SNF1-2/<br>Maertens et al., [2007] | atypical <i>NF1</i> deletion of ~ 1.3-Mb with the proximal breakpoint located within NF1-REP A and the distal deletion breakpoint located within the <i>SUZ12</i> gene  |
| SNF1-1/<br>Maertens et al., [2007] | mutation c.2041C>T (p.R681X)  |
| Consoli et al., [2005]             | mutation c.5839C>T (p.R1947X)   |
| P067<br>This study                 | type-1 <i>NF1</i> deletion with breakpoints located within the PRS1 region of NF1-REPs A and C  |