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# The *NBPF* Gene Family

Vanessa Andries, Karl Vandepoele and Frans van Roy
*Department for Molecular Biomedical Research, VIB*
*Department of Biomedical Molecular Biology, Ghent University*
*Belgium*

## 1. Introduction

Neuroblastoma is one of the most intensely studied solid malignancies that affect children (Maris & Matthay, 1999). These tumours are heterogeneous biologically and clinically. One subset of neuroblastoma is susceptible to spontaneous apoptosis with little or no therapy and another subset differentiates over time, but most of these tumours are difficult to cure with current treatments. A relatively high proportion of affected children die due to resistance of the neuroblastoma to therapy (Van Roy *et al.*, 2009). Every step in the identification or functional understanding of the genes that play important roles in this cancer could bring us closer to understanding the molecular mechanism and could help to develop more effective therapy.

We will describe the novel *NBPF* gene family and its role in neuroblastoma. The *NBPF* gene family was originally identified by the disruption of one of its members in a neuroblastoma patient (Vandepoele *et al.*, 2008). This gene was named *NBPF1*, for Neuroblastoma Breakpoint Family, member 1. Several reports indicate that the *NBPF* gene family might play an important role in neuroblastoma and possibly in other cancers as well. Additionally, this evolutionarily recent gene family has been involved as an important player in human evolution.

## 2. Identification of *NBPF1* during the positional cloning of a constitutional translocation in a neuroblastoma patient

In 1983, karyotypic analysis of fibroblasts from a Belgian neuroblastoma patient revealed a *de novo*, constitutional translocation between chromosomes 1p36.2 and 17q11.2 (Laureys *et al.*, 1990; Laureys *et al.*, 1995). These two chromosomal regions are often rearranged in sporadic neuroblastoma tumours (Savelyeva *et al.*, 1994). So it was believed that the translocation had predisposed the patient to neuroblastoma by disrupting one or more genes. Although previous cytogenetic experiments indicated that the translocation was balanced, analysis of the breakpoint sequences showed greater complexity (Figure 1). First, in both derivative chromosomes, a few nucleotides of unknown origin were inserted between the chromosome 1 and 17 sequences (Vandepoele *et al.*, 2008). Such insertions are frequently observed in reciprocal constitutional translocations (Willett-Brozick *et al.*, 2001). Second, on chromosome 1, the translocation resulted in the deletion of a genomic fragment of 5,215 base pairs that is present in the human genome reference sequence (Vandepoele *et*

*al.*, 2008). However, numerous reports describe structural variation at this locus (see below), which makes it impossible to determine the precise size of the deletion in this neuroblastoma patient. No deletions or insertions of genomic regions were observed on chromosome 17, which means that the translocation at this level is indeed balanced. Notably, both translocation breakpoints are located in LINE repeats, classified as type L1-PA4 for chromosome 1 and as type L2 for chromosome 17, but no significant sequence homology between these two LINE repeats was found. Therefore, most probably these repeats do not participate in the mechanism of this translocation (Vandepoele *et al.*, 2008).

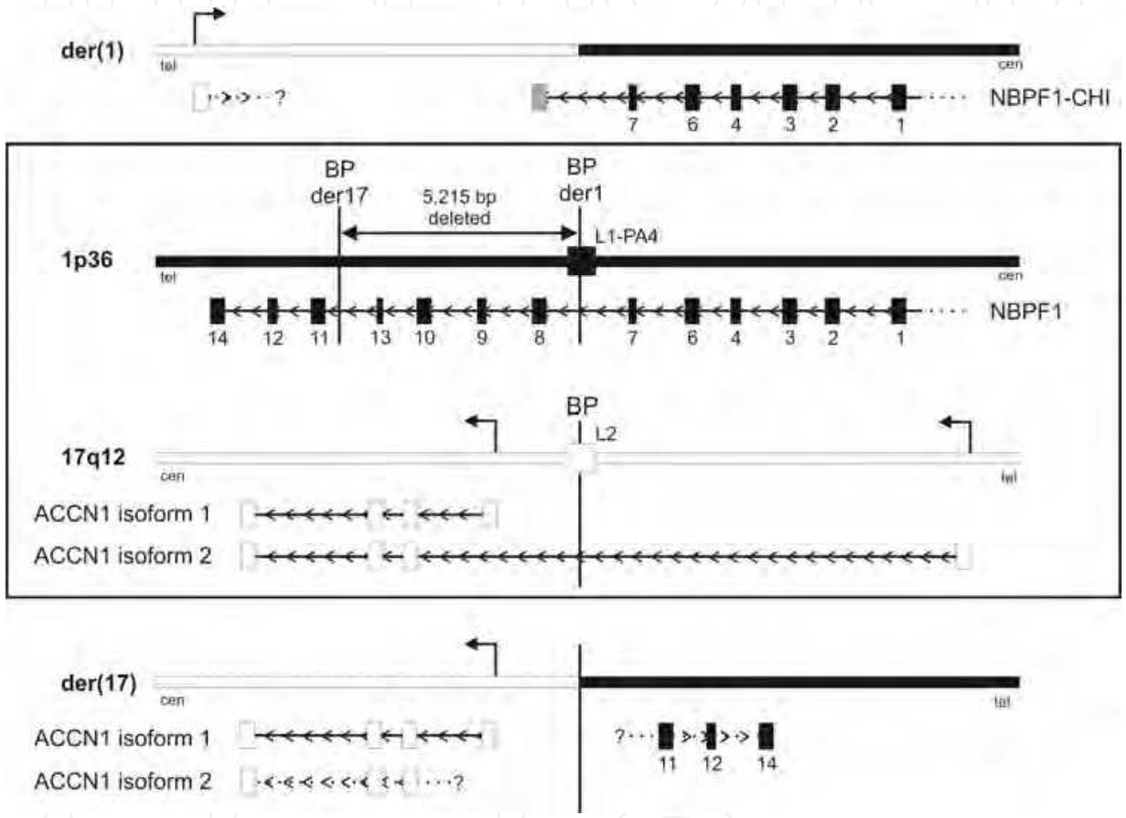



Fig. 1. Genomic overview of the translocation breakpoint of the der(1) and der(17) chromosomes.

**Central box:** Normal 1p36 and 17q12 chromosomes. Both translocation breakpoints (BP) on chromosome 1 are located in intronic regions of the *NBPF1* gene, and the translocation results in deletion of 5,215 bp. The translocation breakpoint on chromosome 17 is located in the *ACCN1* gene and disrupts only isoform 2. **Top**: On the der(1) chromosome, the translocation gives rise to two chimeric transcripts of *NBPF1* sequences (NBPF1-CHI) fused to sequences derived from chromosome 17 (grey box), thereby extending the open reading frame. Translocation of the promoter and the first exon of *ACCN1* isoform 2 might result in chimeric transcripts on der(1), but so far no transcripts have been isolated (question mark with dotted line). **Bottom**: On the der(17) chromosome, isoform 2 of the *ACCN1* gene is probably no longer expressed due to loss of its promoter and first exon (dotted line with question mark), whereas the last three *NBPF1* exons are translocated to the der(17) chromosome (exon types 11, 12 and 14) (Modified after Vandepoele *et al.*, 2008).

On chromosome 17, the translocation disrupts *ACCN1*, a potential glioma tumour suppressor gene (Vila-Carriles *et al.*, 2006). *ACCN1* encodes a member of the superfamily of amiloride-sensitive degenerin/epithelial sodium channels (DEG/ENaC) and is also known as MDEG (Waldmann *et al.*, 1996), BNaC1 (Garcia-Anoveros *et al.*, 1997) and ASIC2 (Waldmann *et al.*, 1997). *ACCN1* is expressed primarily in the brain, where it might play a role in neurotransmission (Garcia-Anoveros *et al.*, 1997). Alternative initiation of transcription results in two variants encoding distinct isoforms differing in their N-terminal domains (Lingueglia *et al.*, 1997). The translocation in the neuroblastoma patient disrupts only isoform 2 of the *ACCN1* gene. Translocation of the promoter and the first exon of *ACCN1* isoform 2 could result in chimeric transcripts from the derivative chromosome 1 (der(1)), but no such transcripts could be identified. Isoform 2 is probably not expressed from derivative chromosome 17 (der(17)) due to the loss of its promoter and first exon (Figure 1) (Vandepoele *et al.*, 2008).

Oncomine expression analysis (Rhodes *et al.*, 2007) of *ACCN1* showed that it is expressed at significantly lower levels in neuroblastoma tumours with *MYCN* amplification, in higher stage tumours, and in tumours with 1p36 loss of heterozygosity. These findings indicate that *ACCN1* plays a role in tumour aggressiveness (Vandepoele *et al.*, 2008). ACCN1 also has a role in oncogenesis of glioma cells, in which functional restoration of ACCN1 at the plasma membrane decreases cell migration (Vila-Carriles *et al.*, 2006). Gain of chromosome 17q is the most frequent genetic alteration observed in neuroblastoma, but the biological basis is complex because dosage alterations involve genes localised on both sides of the 17q breakpoints. It has been suggested that genes mapping between 17cen and 17q12 suppress tumour progression, whereas genes mapping between 17q23.1 and 17qter promote its progression (Lastowska *et al.*, 2002). These findings further stress the possible role of *ACCN1* as a tumour suppressor of neuroblastoma.

Cloning of the chromosome 1 breakpoint identified a novel gene that is disrupted by this translocation. This gene was named *NBPF1*, for Neuroblastoma Breakpoint Family, member 1. *NBPF1* is a member of a newly discovered gene family with an intricate genomic structure (see section 3.1). The translocation truncates *NBPF1* and gives rise to two chimeric transcripts of *NBPF1* sequences fused to sequences derived from chromosome 17, thereby extending the open reading frame with either 34 or 11 additional codons. The breakpoint of the der(1) chromosome is located in an intron between exon types 7 and 8 (Figure 1). The translocation breakpoint of the der(17) chromosome is also intronic, but it is located between exon types 13 and 11, which results in deletion of a part of the *NBPF1* gene, as described above. The three last *NBPF1* exons, of type 11, 12 and 14, are translocated to the der(17) chromosome, but so far no chimeric transcripts have been described (Figure 1) (Vandepoele *et al.*, 2008).

We determined the expression level of *NBPF1* in 32 neuroblastoma cell lines. This profiling showed that *NBPF1* expression varied widely between the different samples, in line with a previous expression analysis of the total *NBPF* family (Vandepoele *et al.*, 2008). Interestingly, we did not observe complete loss of *NBPF1* expression in any cell line, as has been reported for other genes located in the 1p36 region (Janoueix-Lerosey *et al.*, 2004; Fransson *et al.*, 2007). Statistical analysis of these data revealed a significantly lower expression level of *NBPF1* in cell lines with a heterozygous deletion of the *NBPF1* locus than in cell lines without *NBPF1* loss ($p<0.05$), indicating that *NBPF1* could be a tumour suppressor gene (TSG) in neuroblastoma. Although this decreased expression of *NBPF1* might be explained

by the lower gene copy number, it is striking that such downregulation was observed for only 15–20% of the genes affected by hemizygosity of this region (Janoueix-Lerosey *et al.*, 2004). Therefore, in addition to loss of heterozygosity, other mechanisms probably play a role in downregulation of some of these genes. It has been proposed that the combined decrease in the expression of these genes, rather than the inactivation of one single classical TSG, could cause the unfavourable outcome associated with 1p deletions in neuroblastoma (Fransson *et al.*, 2007). Oncomine analysis (Rhodes *et al.*, 2007) showed that in two studies there was no significant difference in *NBPF* expression between different tumour grades or INSS stages but one study showed decreased *NBPF* levels in neuroblastoma tumours that relapsed within five years, which also indicates that *NBPF1* might suppress the development of neuroblastoma. More importantly, induced *NBPF1* expression in human colon cancer cells decreased their clonal growth in a soft agar assay, demonstrating that NBPF1 can act as a TSG, at least in colon cancer (Vandepoele *et al.*, 2008). Like neuroblastoma, colon cancer is characterised by frequent deletions or translocations of 1p36 (Schwab *et al.*, 1996). Indeed several reports indicate that the same TSG might be implicated in both neuroblastoma and colon cancer (Kong *et al.*, 1997; Reyes-Mugica *et al.*, 1998).

## 3. The *NBPF* gene family

### 3.1 Structure of the *NBPF* gene family

The cloning of the breakpoints of the constitutional translocation in a neuroblastoma patient revealed that this translocation had disrupted *NBPF1* (see section 2). This gene is a member of the *NBPF* gene family, which is located primarily on duplicated regions of chromosome 1 (Figure 2A). In neuroblastoma, these regions frequently contain aberrations. For instance, one third of primary neuroblastoma tumours carry a deletion of the 1p36 region (White *et al.*, 2005). The *NBPF* genes have a repetitive structure with high intergenic and intragenic sequence similarity, both in coding and non-coding regions (Vinogradova *et al.*, 2002; Vandepoele *et al.*, 2005). The *NBPF1* gene consists of 25 coding exons, but on the basis of their high sequence identities, these exons can be classified into 14 different exon types (Figure 2B). All other *NBPF* genes contain variable numbers of some or all of the exon types present in *NBPF1*, but some genes also contain exons without significant homology to the *NBPF1* exons. The *NBPF* gene family consists of 24 members located primarily on three regions of chromosome 1, namely, 1p36, 1p12 and 1q21, but two members, *NBPF21P* and *NBPF22P,* are located on chromosomes 3p and 5q, respectively.

Several members of this gene family are abundantly expressed in different human cell lines and human tissues, but no discernable orthologs could be found in the genomes of mouse or rat. Nevertheless, the *NBPF* genes do not appear to be restricted to primates because sequences with low but significant homology to *NBPF* have been identified in bovine and canine genomes (Vandepoele *et al.*, 2005).

We previously analysed the genomic organisation of the *NBPF* gene family (Vandepoele *et al.*, 2005). For that analysis, we used the reference sequence of the human genome of the NCBI build 35, which is an improvement over the first release because it contains only 341 gaps (IHGSC 2004) instead of the 150,000 gaps that were present in the first draft version. However, the *NBPF* genes are located in regions that are refractory to all standard sequencing efforts. In this analysis, we obtained numerous small contigs that contained only partial *NBPF* genes. To provide an up-to-date description of the *NBPF* genes in the human genome reference sequence, we now report on the analysis of *NBPF* sequences in the current human genome release (Build 37.2).

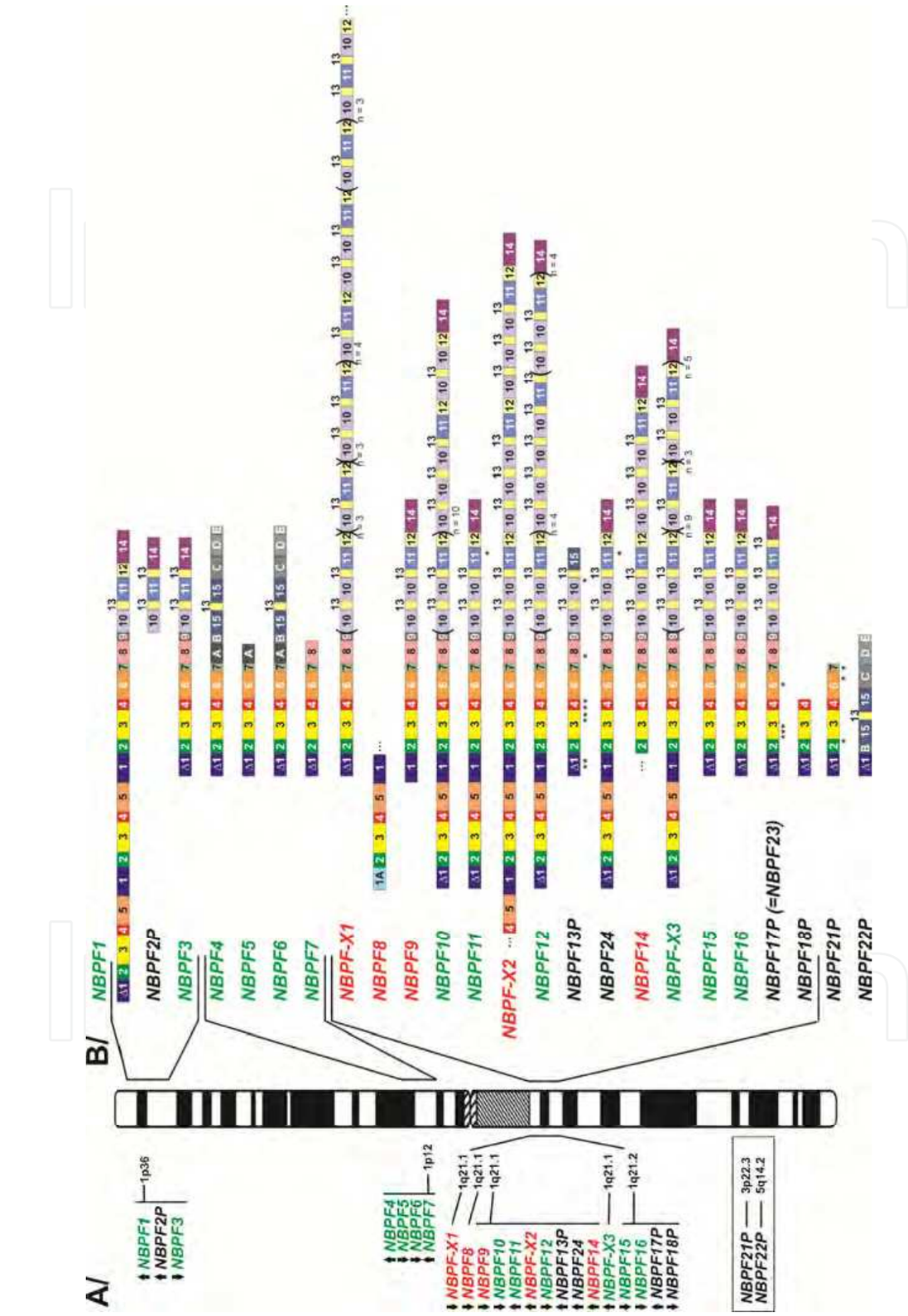



Fig. 2. Genomic overview of the *NBPF* gene family.

Legend to figure 2: Genomic overview of the *NBPF* gene family. A/ Ideogram of human chromosome 1 showing the localisation of the *NBPF* genes. The arrow in front of each gene indicates its orientation in the chromosome. The colour of the gene symbol indicates whether it is full-length (green; start and stop codons present in one continuous ORF in the predicted cDNA), partial (red; due to gaps in the genomic sequence), or a pseudogene (black, P). Vertical lines indicate whether genes are present in a single genomic contig of the human genome assembly (NCBI Build 37.2). The two genes not linked to the ideogram, *NBPF21P* and *NBPF22P*, are located on other chromosomes. B/ The open reading frames of the different *NBPF* genes were assembled *in silico* based on cDNA sequences. *NBPF4* to *NBPF6* and *NBPF22P* contain exons with no significant homology to exons present in *NBPF1*. These are shown as gray boxes with letters instead of numbers. *NBPF8* contains a deletion of two nucleotides in the first coding exon, which results in a frameshift mutation. Due to skipping of the type-2 exon in several *NBPF8* transcripts, this frameshift often does not result in a premature stop. *NBPF-X* sequences are new *NBPF* members that still lack an official gene symbol. Frameshift and nonsense mutations in pseudogenes are indicated by asterisks. Three consecutive dots indicate the presence of gaps in the current genomic assembly (Updated from Vandepoele *et al.*, 2005).

In addition to the 22 *NBPF* genes we identified before (Vandepoele *et al.*, 2005), two additional *NBPF* genes are currently present in GenBank: *NBPF23* and *NBPF24*. Copy number variations of *NBPF23* were recently shown to be involved in neuroblastoma (see below) (Diskin *et al.*, 2009). In our previous analysis, we only focused on the coding exons, and so the 5'UTR was not properly annotated in the databases. However, the *NBPF23* sequences are upstream of the *NBPF17P* encoding exons at a similar distance as the 5'UTR exons of *NBPF1* are from its coding sequences. We therefore believe that the *NBPF23* gene is not a novel gene but is the 5'UTR of *NBPF17P*. We described *NBPF17P* as a pseudogene because it contains nonsense mutations in the normal coding sequence (Vandepoele *et al.*, 2005). In view of that, it is interesting that Diskin *et al.* (2009) found that this sequence is strongly expressed in several tissues, which indicates that this "pseudogene" might have a function. The other new *NBPF* member in GenBank is *NBPF24*, but this is still a hypothetical sequence predicted from a fragment of genomic DNA (see below).

The short arm of chromosome 1 contains two regions that harbour *NBPF* genes. The 1p36 locus contains the *NBPF1* gene. Additionally, one functional gene (*NBPF3*) and a partial duplication of this gene (*NBPF2P)* are located proximal to *NBPF1*. Closer to the centromere on 1p12, there is a second cluster of *NBPF* genes (*NBPF4* to *NBPF7*). These genes are very similar to each other and make up a separate branch of the *NBPF* evolutionary tree (Vandepoele *et al.*, 2005). The sequences of these two clusters were not changed in the most recent human genome reference sequence. The updated version of the human genome reference sequence has undergone numerous changes in chromosome 1q. Overall, it now contains more *NBPF* sequences than the previously published sequence. The *NBPF8* gene was the most proximal *NBPF* gene in our old analysis, but it is now preceded by a novel *NBPF* member, which we call *NBPF-X1*. This is the longest *NBPF* gene we have observed so far, with a size of 77 kb and a cDNA of 11 kb. As there is still a gap in the genomic sequence on the 5' side of *NBPF-X1*, the gene and its derived cDNA are presumably even longer. Distal to *NBPF-X1*, *NBPF8* is an aberrant member of the *NBPF* gene family. Deletion of two nucleotides in its first coding exon causes a frameshift. However, this frameshift can be rescued by an alternative splicing event that excludes the second coding exon from the mature mRNA. *NBPF9*, *NBPF10* and *NBPF11* are identical to those in the old analysis. Like

*NBPF-X1*, *NBPF10* contains numerous repeats of the 3' exons. *NBPF11* contains a frameshift mutation in one of its last exons, but this gene is nevertheless expressed in a variety of tissues, and hence, we consider it a functional gene. *NBPF11* is followed by a second new *NBPF* gene, *NBPF-X2*. As the 5' end of this gene is located near a gap in the contig, only a partial sequence is available. In our previous analysis, the sequence of the *NBPF12* gene was incomplete, with only nine consecutive exons in an uninterrupted contig. In the current genome sequence, the complete *NBPF12* gene is present and contains 77 exons. This is followed by *NBPF13P*, a pseudogene with numerous nonsense mutations in different exons, and the new *NBPF24*. Interestingly, *NBPF24* is an almost identical copy of *NBPF11* and contains the same frameshift mutation in one of its last exons. The following gene, *NBFP14*, has not changed in the current assembly, but it is followed by the last new *NBPF* gene, *NBPF-X3*. Like other new members, *NBPF-X3* is also composed of many repetitions of the standard NBPF exon types. The identical *NBPF15* and *NBPF16* genes are contained in the last contig on chromosome 1q, together with the *NBPF17P* and *NBPF18P* pseudogenes. In our previous analysis, *NBPF19* and *NBPF20* were not positioned on chromosome 1. In the current assembly, these sequences are no longer represented. Since the 1q21 region is prone to copy number variation and the human genome reference sequence is derived from different haplotypes, it is possible that *NBFP19* and *NBPF20* are not true paralogs of the current *NBPF* genes, but simply variants of other *NBPF* genes in another haplotype.

In addition to the NBPF sequences on chromosome 1, two other regions in the human genome contain NBPF-like sequences: 3p22.3 (*NBPF21P*) and 5q14.2 (*NBPF22P*). These sequences have only limited homology to the *NBPF* genes on chromosome 1 but share a common origin and are hence included in the *NBPF* family.

Analysis of the predicted protein sequences showed that several pairs of exon types encode a novel protein domain, called the NBPF repeat. The NBPF repeat comprises a PFAM protein domain of unknown function, called DUF1220 (Vandepoele *et al.*, 2005), and we will refer to this repeat as the NBPF/DUF1220 repeat. It is always built of two exons and is present in multiple copies in the different NBPF proteins. It is also present as a single copy with lower homology in the human *PDE4DIP* gene encoding myomegalin (Figure 3) (Verde *et al.*, 2001). Myomegalin is a centrosomal protein and contains, besides its NBPF/DUF1220 domain, some regions that show homology to *CDK5RAP2*, a gene that lacks NBPF/DUF1220 sequences. Mutation of *CDK5RAP2* has been implicated in microcephaly (Bond & Woods, 2006). There is recent evidence for involvement of the NBPF/DUF1220 repeat in human evolutionary adaptation, cognitive function, and disease, including neuroblastoma (see below). Other than the novel NBPF/DUF1220 repeat, there are no known protein domains in the NBPF proteins.

### 3.2 The *NBPF* gene family and human evolution

Several regions on chromosome 1 consist of recently duplicated sequences, and the *NBPF* gene family is located primarily on such segmental duplications (Vandepoele *et al.*, 2005). Segmental duplications, also known as low copy repeats, are duplicated blocks of genomic DNA longer than 1 kbp and with high sequence identity, and they typically map to two or more locations in the genome (Marques-Bonet *et al.*, 2009b). The number of base pairs mapping to high-identity duplications are similar in the human and mouse genomes, but the segmental duplications in mouse are tandemly organised, whereas in humans most of them are interspersed (She *et al.*, 2008). Additionally, human segmental duplications tend to

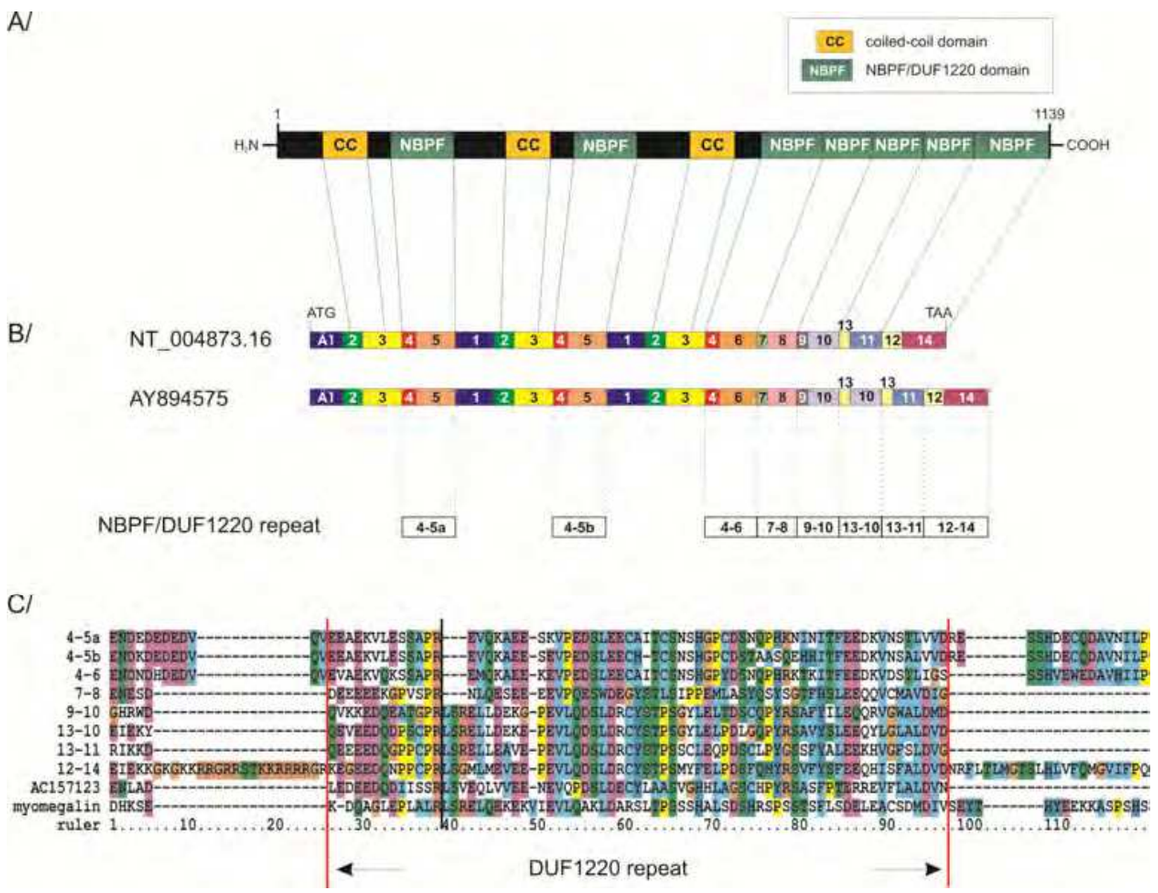


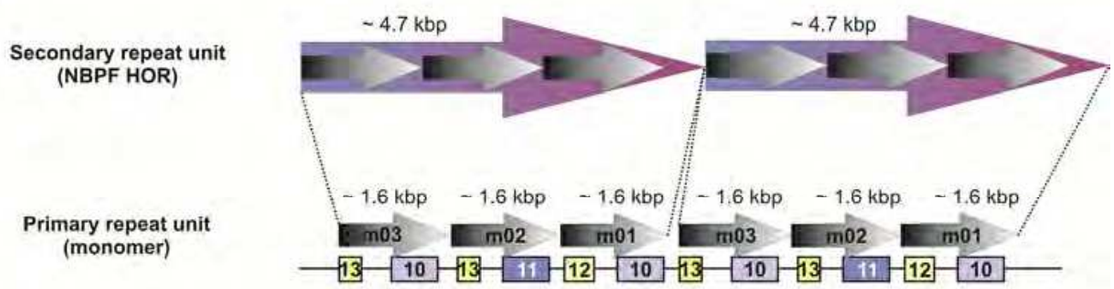

Fig. 3. Exon organisation of the *NBPF1* gene and the predicted domain structure of the translated NBPF1 protein.
A/ Analysis of the predicted NBPF protein sequences showed that each of several pairs of exon types encodes a novel protein domain, called the NBPF repeat. The NBPF repeat comprises a PFAM protein domain of unknown function, called DUF1220 (see C). It is always built of two exons and is present in multiple copies in the different NBPF proteins. Three coiled-coil regions were predicted in the N-terminal domain of the NBPF1 protein. Each coiled-coil is encoded by a combination of the type-2 exon and a part of the type-3 exon. B/ Schematic representation of the open reading frame of the *NBPF1* gene as predicted from the human genome sequence (NT_004873.16) and present in an isolated cDNA sequence (AY894575) derived from this gene. The isolated cDNA contains multiple copies of exon types 10, 11, 12 and 13, which are present only once in the genomic sequence. The several exon couples that constitute the NBPF/DUF1220 repeat are shown in white boxes at the bottom. C/ Amino acid alignment of the different NBPF repeats present in the NBPF1 protein. A homologous sequence was isolated from the draft version of the bovine genome and assembled *in silico* (depicted as AC157123). The bottom sequence is derived from one of the human homologs of rat myomegalin (AB007923; AA 1564-1654). The black line denotes the exon boundaries, whereas the red lines denote the PFAM protein domain of unknown function, DUF1220 (Adapted from Vandepoele *et al.*, 2005, Oxford Journals (2005), reproduced by permission).

be enriched in spliced transcripts (She *et al.*, 2008) and show a higher sequence identity (Marques-Bonet & Eichler, 2009). It has been shown that segmental duplications, together with retrotransposons, account for the large-scale structural variations in primate genomes. Humans and other great apes share interspersed duplications, but a burst of segmental duplications also occurred in the common ancestor of humans and African great apes (Marques-Bonet *et al.*, 2009a).

As segmental duplications appeared quite recently, it is presumed that genes residing in these regions played a role in evolution. The *morpheus* gene family, for example, is found only in humans and African apes and is located in segmental duplications on chromosome 16 (Johnson *et al.*, 2001). These genes were repeatedly duplicated and positively selected for during primate evolution and, therefore, presumably played a role in speciation events. Amino acid replacements in putative protein-encoding exons of this gene family occurred after the separation of human/great-ape lineages from orangutan and after the divergence of human and chimpanzee lineages. This points to adaptive evolution of the *morpheus* gene family during the emergence of humans and African apes (Johnson *et al.*, 2001). It is becoming clear that lineage-specific gains and losses in gene copy number have emerged as an important aspect of primate genetic variation (Cheng *et al.*, 2005). An array-based comparative genomic hybridisation that surveyed gene duplication and losses across 10 primate species identified over 4,000 genes that show lineage-specific copy-number gains and losses (Dumas *et al.*, 2007). Those genes exhibiting lineage-specific copy number changes are likely involved in the phenotypic differences that distinguish these primate lineages. Interestingly, NBPF/DUF1220 sequences show human lineage-specific copy number increases (Dumas *et al.*, 2007), consistent with previous reports (Fortna *et al.*, 2004; Vandepoele *et al.*, 2005; Popesco *et al.*, 2006). Different experiments indicated that the number of NBPF/DUF1220 repeats was very large in humans, reduced in African great apes, further reduced in orangutan and Old World monkeys, single-copy in non-primate mammals, and absent in non-mammalian species (Popesco *et al.*, 2006). The dramatically elevated copy number in humans indicates the importance of the NBPF/DUF1220 repeat in human evolution. Moreover, comparison of the human chromosome 1 with that of chimpanzee revealed a remarkable human 3-mer higher order repeat (HOR) organisation based on an ~1.6-kbp primary repeat unit fully embedded within the *NBPF* genes (Figure 4A). This HOR pattern is not found in chimpanzee and shows some peculiarities, namely that the repeat unit is much longer than most primary repeat units identified so far and that the HOR is fully embedded within a gene. Additionally, the total absence of tandem repeats of NBPF HOR copies in chimpanzees while 47 tandem repeat HOR copies are present in human genomes reflects a human accelerated HOR pattern that distinguishes humans from nonhuman primates (Figure 4B) (Paar *et al.*, 2011).

It has been shown that genes with an important contribution to human evolution, like the *NBPF* gene family, are located in human segmental duplications, which are frequently organised around core duplicons (Jiang *et al.*, 2007). Comparison of chimpanzee and man shows that new lineage-specific segmental duplications map preferentially near shared ancestral duplications, a phenomenon called duplication shadowing (Cheng *et al.*, 2005). Unique genes mapping near these duplication blocks have a ten-fold higher probability of becoming duplicated than other randomly distributed regions. The NBPF/DUF1220 domains are among the few core duplicons that exist in the human genome and appear to be responsible for much of the duplicated sequences in the pericentromeric region of chromosome 1 (Jiang *et al.*, 2007). These large, high-identity duplication blocks are prone to rearrangements that lead to the formation of both harmless and pathogenic copy number variations, as discussed in the following section.

A/




B/

| Species | NBPF monomers | Number of NBPF HOR copies | Number of tandem NBPF HOR copies |
|---|---|---|---|
| Human | 165 | 57 | 47 |
| Chimpanzee | 48 | 14 | 0 |
| Orangutan | 17 | 7 | 0 |
| Rhesus macaque | 7 | 2 | 0 |
| Mouse | 0 | 0 | 0 |

Fig. 4. Schematic illustration of the NBPF HOR copy.
A/ The NBPF HOR copy consists of three 1.6-kbp primary repeat units organised into ~4,770-bp secondary repeat units. The divergence between the three consensus monomers (m01, m02 and m03) is between 15 and 20%, whereas the average divergence between the 3-mer HOR copies is mostly below 0.5%, which is characteristic of a well-developed HOR pattern. Figure not drawn to scale. B/ The total number of NBPF monomers and the number of NBPF HOR copies (both tandemly organised and dispersed) gradually increases with evolutionary development, but the tandem repetition of the NBPF HOR copies is exclusive to humans (Modified after Paar *et al.*, 2011, Oxford Journals (2011), reproduced by permission).

### 3.3 The *NBPF* gene family shows frequent copy number variations that are sometimes associated with pathologies, including neuroblastoma

As previously discussed, primate segmental duplications are larger, more complex and more interspersed than those in other mammalian genomes. This pattern promotes genomic instability and leads to considerable copy number variation (Bailey & Eichler, 2006). A copy number variation (CNV) is a structurally variable region of DNA longer than 1 kbp present in the genomes of humans (Redon *et al.*, 2006), primates and many other species (Graubert *et al.*, 2007; Chen *et al.*, 2009). In these genomes, the DNA region is present in various copy numbers due to gains and losses of genomic DNA. Surprisingly, as much as 12% of the human genome is copy number variable, and despite efficient DNA repair, the rates of *de novo* CNVs are at least 100 to 10,000 fold greater than rates of point mutations (Lupski, 2007).

Nonallelic homologous recombination between low-copy repeats that flank unique genomic segments changes the organisation of the genome and is thought to be one of the main mechanisms that give rise to CNVs (Inoue & Lupski, 2002). Several studies indicate that the

*NBPF* gene family is polymorphic within the human population (Database of Genomic Variants). Analysis of the copy number differences between the genomes of 159 human individuals showed that the *NBPF* gene family is one of the most variable gene families in humans (Sudmant *et al.*, 2010). Sudmant *et al*. (2010) also showed that the *NBPF* gene duplicates are underrepresented in the human reference genome because no individual examined exhibited a copy number less than or equal to the copy number in the reference genome. Paralog-specific copy number genotyping distinguished two distinct classes of paralogs: one group is mostly copy-invariant in humans, and the other group is extensively copy number variable with some bias toward gain or loss. The *NBPF* gene family showed a paralog-specific copy number variation, and certain paralogs (*NBPF1*) were frequently amplified, extremely variable, and stratified by population, whereas other paralogs (*NBPF7*) were nearly fixed and diploid (Sudmant *et al.*, 2010). Taken together, these data clearly show that the *NBPF* gene family is polymorphic in the human population.

The functional implications of these structural variations in the human genome can be of great importance: not only are they an important source of genetic diversity between individuals, they can also affect an individual's susceptibility to disease. Several diseases, such as lupus with glomerulonephritis and Crohn's disease, have been linked to CNV of *FCGR3B* and *HBD-2*, respectively. It has been shown that a low copy number of *FCGR3B* (Fc fragment of IgG, low affinity IIIb, receptor) predisposes to glomerulonephritis, an immunologically mediated renal disease (Aitman *et al.*, 2006), whereas individuals with less than three copies of the human *beta-defensin 2* (*HBD-2*) gene have a significantly higher risk of developing Crohn's disease than individuals with four or more copies (Fellermann *et al.*, 2006). In neuroblastoma, an inherited copy number variation was shown to be associated with this childhood cancer. Indeed, Diskin *et al*. (2009) found that a common deletion polymorphism spanning less than 145 kbp at 1q21.1 is associated with neuroblastoma. Validation by quantitative PCR, fluorescent *in situ* hybridisation, and analysis of matched tumour specimens showed that this CNV is a heritable genetic variation. Interestingly, the 5'UTR of *NBPF17P* (*NBPF23*; see above) is located in this CNV. Quantitative PCR showed that this *NBPF* paralog is expressed in a variety of fetal and adult tissues, but the strongest expression was observed in fetal brain and fetal sympathetic nervous tissue. Importantly, these experiments showed that the expression level was strictly correlated with the CNV state in neuroblastoma cells (Diskin *et al.*, 2009). Recently, it has become clear that CNVs in the 1q21.1 region are associated not only with neuroblastoma, but also with an increasing number of other pathologies. Intriguingly, these copy number gains or losses either encompassed or flanked members of the *NBPF* gene family, emphasising the possible involvement of the *NBPF* gene family in human disease. CNVs at these genomic loci have been found in patients with mental retardation and congenital anomalies (Mefford *et al.*, 2008), autism (Sharp *et al.*, 2006; Szatmari *et al.,* 2007) and schizophrenia (Stefansson *et al.*, 2008). Autism and schizophrenia can be described as diametric conditions because reciprocal variants of the same genomic sequences, namely duplication and deletion, represent risk factors for these respective psychiatric conditions. Additionally, autism and schizophrenia are associated with developmentally enhanced and reduced brain growth, respectively (Crespi *et al.*, 2009). A study of 1q21.1 CNVs and brain size found that deletions of the 1q21 region are associated with microcephaly, which is characterised by an abnormally small head, whereas duplications of this region are associated with macrocephaly, which is characterised by an abnormally large head (Brunetti-Pierri *et al.*, 2008). Notably, the most striking trend in human evolution is the rapid increase in brain size

over the past 3–4 million years, and the associated increase in complexity and cognitive capacity (Mekel-Bobrov *et al.*, 2007). Because NBPF/DUF1220 is present in remarkably more copies in the human genome than in other primates (Vandepoele *et al.*, 2005; Popesco *et al.*, 2006), it was hypothesised that the NBPF/DUF1220 copy number is correlated with brain size (Dumas & Sikela, 2009). Moreover, these data support the hypothesis that NBPF/DUF1220 domains are involved in the difference in brain size between autistic and schizophrenic populations and between macrocephalic and microcephalic populations. Therefore, NBPF/DUF1220 repeats could have played an important role in human evolution and in the associated increase in brain size. On the other hand, the selective advantage conferred by the increased number of NBPF/DUF1220 domains might have favored retention of the highly unstable 1q21.1 region, resulting in many recurrent rearrangements and leading to a spectrum of disorders of the human brain and to developmental defects (Dumas & Sikela, 2009).

## 4. *NBPF* promoter analysis

Gene duplication has generally been viewed as a primary mechanism for the origin of evolutionary novelties (Ohno, 1970). In most cases, duplication is not limited to the coding sequence but encompasses the regulatory region as well. Alternatively, new genes can acquire a regulatory region from a nonhomologous gene (Usakin *et al.*, 2005). We found evidence that the regulatory region of the *NBPF1* gene was obtained from an unrelated gene, called *EVI5* (ecotropic viral integration site 5) (Vandepoele *et al.*, 2009). *EVI5* was originally identified through its involvement in a constitutional chromosome translocation in a patient with stage 4S neuroblastoma (Roberts *et al.*, 1998). EVI5 is a centrosomal protein during interphase, but it relocalises to the midbody during late phases of mitosis. Its disruption leads to incomplete cell division and formation of multinucleate cells (Faitar *et al.*, 2005). EVI5 also exists in a protein complex with GTP-bound Rab11 and functions as a GTPase-activating protein for this small GTPase (Dabbeekeh *et al.*, 2007). Additionally, EVI5 is critical during interphase for the regulation of cyclin accumulation. It functions by stabilising the anaphase-promoting complex inhibitor, Emi1. RNA interference with EVI5 levels resulted in cell-cycle arrest and abnormal centrosome numbers *in vivo*, suggesting that EVI5 might not only regulate cyclin accumulation during interphase but also contribute to timing mechanisms to ensure mitotic fidelity (Eldridge *et al.*, 2006).

The human *EVI5*, located on chromosome 1p22, is transcribed as two isoforms differing in their transcription initiation sites and first exons. Intriguingly, the promoter of *NBPF1* shows homology only to the promoter used to control the expression of human isoform 2 of *EVI5* (called $EVI5_{NBPF}$), but not the promoter of isoform 1 (called $EVI5_{ALT}$) (Figure 5) (Vandepoele *et al.*, 2009).

The extensive sequence homology in the promoter regions of different *NBPF* paralogs shows that the recruitment of the promoter from $EVI5_{NBPF}$ occurred before expansion of the *NBPF* gene family. Phylogenetic analysis of the NBPF and $EVI5_{NBPF}$ core promoter sequences showed that promoter duplication occurred after the split between simians and prosimians, but before divergence between Old World and New World monkeys, and that positive selection played a role in the evolution of the *NBPF1* promoter in simians (Vandepoele *et al.*, 2009).

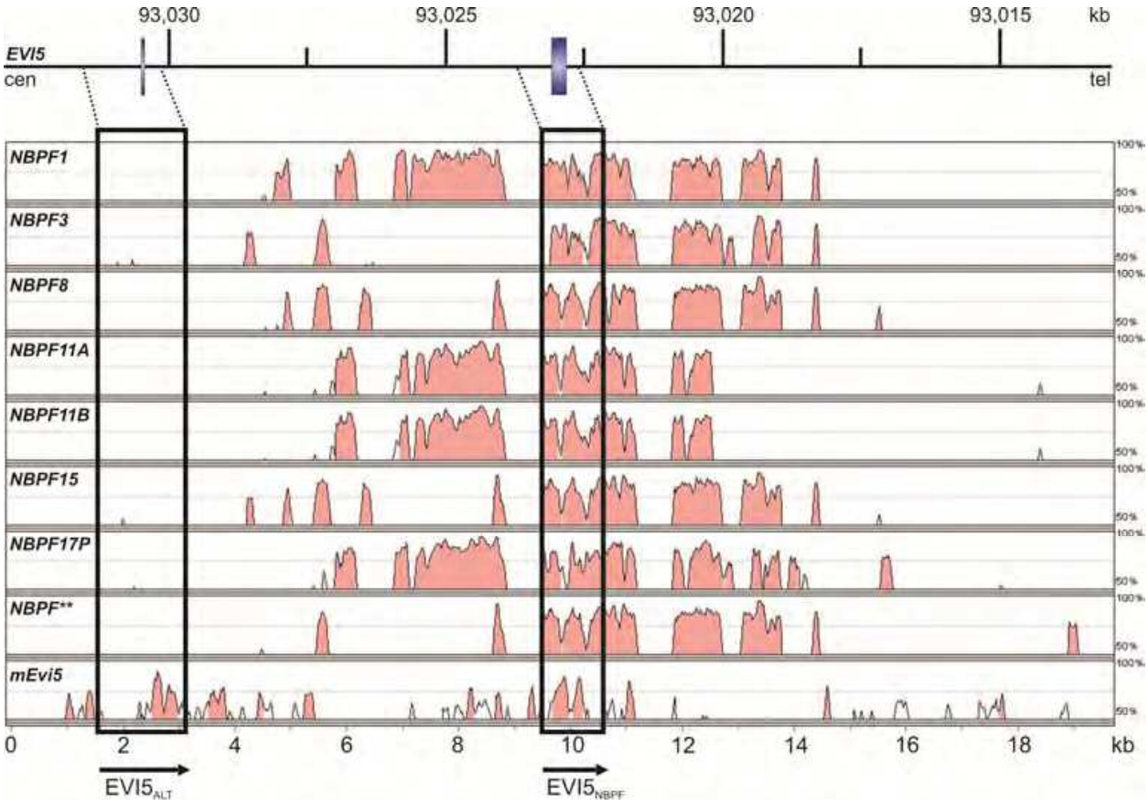



Fig. 5. Sequence homology between the promoter regions of different *NBPF* paralogs and the promoter regions of *EVI5*. Expression of the human EVI5 gene is controlled by two promoters: $EVI5_{ALT}$ controls the expression of isoform 1 and $EVI5_{NBPF}$ controls the expression of isoform 2. Arrows indicate the positions of these transcription initiation sites on the reverse strand of chromosome 1, and their first exons are represented by blue boxes on the top line. A region of 20 kbp flanking the transcription initiation site of human and mouse $EVI5_{NBPF}$ and different *NBPF* paralogs was aligned by the AVID algorithm in the Vista Genome browser. The promoter of *NBPF1* and its paralogs shows homology only with the promoter of human EVI5 that is used to control the expression of isoform 2 ($EVI5_{NBPF}$) but not the one for isoform 1 ($EVI5_{ALT}$). The homology between the human and mouse EVI5 sequences is confined mainly to the two regions corresponding to the putative promoter regions. No official gene symbol is available for NBPF** (Adapted from Vandepoele et al., 2009, Oxford Journals (2009), reproduced by permission).

The expression patterns of *NBPF* and *EVI5* in a panel of human cell lines are very similar, and the expression of both genes was decreased in neuroblastoma cell lines in which there is 1p36 loss of heterozygosity. Nevertheless, the mechanism of this decreased expression level was due to use of a different transcriptional and posttranscriptional regulation. Whereas *EVI5* was epigenetically silenced, treatment of the neuroblastoma cell lines with puromycin, an inhibitor of nonsense-mediated decay, indicated that mutation of the *NBPF* transcripts was probably responsible for decreasing the expression levels. These data together show that despite the almost identical promoter sequences and very similar expression patterns, regulation of *NBPF* and *EVI5* genes in neuroblastoma cell lines can occur by different mechanisms (Vandepoele *et al.*, 2009).

## 5. NBPF-interacting proteins

Many genes perform their functions by interaction of their encoded proteins with other proteins. The search for NBPF-interacting proteins revealed two hits so far: chibby and clusterin, which together can form a tri-molecular complex with NBPF1 (Vandepoele *et al.*, 2010). Interestingly, both NBPF1 and clusterin are candidate tumour suppressors for neuroblastoma (Vandepoele *et al.*, 2008; Chayka *et al.*, 2009). This suggests that the interactions between these three proteins are functionally important.

### 5.1 Chibby

Chibby (Cby) is a 15-kDa protein that has been strongly conserved throughout evolution from fly to human. It was originally identified as an inhibitor of the canonical Wnt-β-catenin pathway (Takemaru *et al.*, 2003). Cby interacts with the C-terminal activation domain of β-catenin and blocks its transcriptional activation potential by two mechanisms. First, it competes with T cell factor/lymphoid-enhancer factor (TCF/LEF) transcription factors for binding with β-catenin (Takemaru *et al.*, 2003). Second, in cooperation with 14-3-3 adaptor proteins, it facilitates nuclear export of β-catenin (Li *et al.*, 2008). Inhibition of the Wnt-β-catenin pathway is necessary for cardiomyocyte differentiation, proliferation and repair, and Cby has been implicated in facilitating cardiomyocyte differentiation (Singh *et al.*, 2007). Additionally, several molecules of the Wnt-β-catenin pathway play important roles in the inhibition of adipogenesis, and different findings indicate that Cby is required for adipocyte differentiation (Li *et al.*, 2007). Cby also plays a role in other processes. Reports on the binding of Cby to polycystin-2 (Hidaka *et al.*, 2004) and on the Cby knockout mouse (Voronina *et al.*, 2009; Love *et al.*, 2010) implicate Cby in the development of motile airway cilia. Germline inactivation of *Cby* resulted in complete absence of mucociliary transport due to scarcity of motile cilia in the nasal epithelium (Voronina *et al.*, 2009). In a follow-up study, characterisation of the lung morphology of Cby knockout mice showed that $Cby^{-/-}$ lungs were normal at birth, but alveolar airspace became enlarged, proliferation became reduced, and lung epithelial cells differentiated abnormally, which together affected pulmonary function (Love *et al.*, 2010). Both the lungs and nasal epithelia of Cby knockout mice showed increased expression of two direct β-catenin target genes, namely *Axin2* and *CyclinD1*, but it is not clear whether the ciliary phenotypes are related to the modest elevation of these Wnt related genes, since no change in the localisation of certain components of the Wnt-β-catenin pathway was observed. Presence of endogenous Cby at the base of cilia and the phenotypes related to the loss of Cby show that Cby is important in ciliogenesis (Voronina *et al.*, 2009; Love *et al.*, 2010).

Neuroblastoma is a disease of the sympathoadrenal lineage of the neural crest, and these tumours can develop anywhere in the sympathetic nervous system. At least three key pathways regulate the formation, migration and differentiation of the neural crest cells: the bone morphogenetic protein (BMP) pathway (Barembaum & Bronner-Fraser, 2005), the fibroblast growth factor pathway (Sauka-Spengler & Bronner-Fraser, 2008) and the Wnt signalling pathway (Crane & Trainor, 2006). Wnt signalling plays an important role in neural crest cells. For example, mouse embryos in which Wnt1 and Wnt3 were knocked out displayed severe loss of neural crest cells (Varga & Wrana, 2005). In addition, conditioned medium of cells overexpressing wingless (*Drosophila* homolog of Wnt1) could induce avian neural crest cells (Garcia-Castro *et al.*, 2002). Aberrant regulation of the embryonic developmental Wnt pathway has been implicated in many solid childhood tumours (reviewed in Koesters & Doeberitz, 2003), but a clear role for Wnt signalling in

neuroblastoma has remained elusive. Nevertheless, there is mounting evidence that the Wnt pathway is involved in neuroblastoma (Table 1).

The MSX1 homeobox transcription factor functions as an intermediate between the BMP and Wnt signalling pathways to ensure proper differentiation of the neural crest cells. Elevated expression of BMP in non-neuronal ectoderm switches on the neural crest differentiation program and induces the expression of MSX1, which leads to induction of the expression of BMP and Wnt in neural crest cells (Ramos & Robert, 2005). Msx genes might regulate *Wnt1* expression at the dorsal midline of the neural tube (Bach *et al.*, 2003). Additionally, MSX1 overexpression in the *MYCN*-amplified neuroblastoma cell line SJNB8 induced the expression of four Wnt inhibitor genes: Dickkopf 1 (DKK1), Dickkopf 2 (DKK2) and Dickkopf 3 (DKK3) and secreted frizzled-related protein 1 (SFRP-1) (Revet *et al.*, 2010). Analysis of the expression profiles of MSX1, DKK1, -2, -3 and SFRP-1 in a series of neuroblastic tumours showed a significant and positive correlation between MSX1 and DKK2 expression and between MSX1 and DKK3 expression. Additionally, stronger DKK2 and DKK3 expression correlated with a significantly better prognosis. Expression profiling of neuroblastic tumours and cell lines for the different Wnt family genes showed that both the canonical Wnt3a and the non-canonical Wnt5 were strongly expressed and that they could activate upstream Wnt signalling in neuroblastoma cells by phosphorylating the dishevelled co-receptor DVL3 (Revet *et al.*, 2010). The importance of DKK3 in neuroblastoma was already shown in previous reports, in which DKK3 was implicated as a marker for neuroblastic tumour maturation and was shown to be down-regulated by *MYCN* (Bell *et al.*, 2007; Koppen *et al.*, 2008).

Additionally, β-catenin has been shown to be strongly expressed and aberrantly localised in the nucleus in high-risk neuroblastoma cells without *MYCN* amplification (Liu *et al.*, 2008). Expression profiling of primary neuroblastoma tumours demonstrated increased expression of *WNT* ligands (*WNT1*, *WNT6*, *WNT7A*, *WNT10B*), *DVL1* and *TCF7* in high-risk neuroblastoma tumours without *MYCN* amplification. Also several β-catenin target genes,

| Gene | Function | Neuroblastoma |
|---|---|---|
| **DKK1** | interacts with co-receptor LRP | induced upon overexpression of Msx1 in NB cell line |
| **DKK2** | interacts with co-receptor LRP | |
| **DKK3** | interacts with co-receptor LRP | induced upon overexpression of Msx1 in NB cell line and downregulated by *MYCN* |
| **SFRP1** | secreted antagonist | induced upon overexpression of Msx1 in NB cell line |
| **Wnt3a** | ligand | strongly expressed in neuroblastic tumors and cell lines |
| **Wnt5** | ligand | |
| **B-catenin** | key mediator | localised in the nucleus in NB cells without MNA |
| **Wnt1** | ligand | increased expression in primary NB tumors without MNA |
| **Wnt6** | ligand | increased expression in primary NB tumors without MNA |
| **Wnt7A** | ligand | |
| **Wnt10B** | ligand | |
| **DVL1** | co-receptor | |
| **TCF7** | transcription factor | |
| **Myc** | target gene | |
| **CD44** | target gene | |
| **FZD1** | seven-transmembrane receptor | increased expression in chemoresistant NB cell lines and in relapsed patients |
| **MDR1** | target gene | |

Table 1. Wnt signalling is important in neuroblastoma tumourigenesis and chemoresistance.Schematic representation of different Wnt signalling components implicated in neuroblastoma. NB: neuroblastoma; MNA: *MYCN* amplification. See text for references.

*e.g. MYC* and *CD44*, were found to be coordinately upregulated in high-risk neuroblastomas without *MYCN* amplification in comparison to high-risk *MYCN*-amplified or intermediate-risk neuroblastomas (Liu *et al.*, 2008).

Another study implicated the Wnt-β-catenin pathway in neuroblastoma chemoresistance (Flahaut *et al.*, 2009). Gene expression profiling of two doxorubicin-resistant neuroblastoma cell lines identified the frizzled1 Wnt receptor (*FZD1*) gene as the most strongly overexpressed transcript in both cell lines in comparison with their sensitive parental cell lines. FZD1 silencing resulted in significant restoration of drug sensitivity and induced a parallel strong decrease in the expression of MDR1 (Flahaut *et al.*, 2009), another β-catenin target gene frequently associated with the resistance of neuroblastoma tumours to chemotherapeutic drugs (Haber *et al.*, 1997). Moreover, FZD1 and/or MDR1 expression was significantly enhanced in the group of relapsed patients after chemotherapy, whereas no significant increase in expression was measured in the group of non-relapsed patients (Flahaut *et al.*, 2009).

These data indicate that Wnt signalling is important in neuroblastoma tumourigenesis and chemoresistance (overview in Table 1). Therefore, we hypothesise that important players in the Wnt-β-catenin pathway might play important roles in neuroblastoma. But nothing is known yet about the role of Cby in neuroblastoma tumourigenesis or chemoresistance, and further investigation is needed.

### 5.2 Clusterin

Clusterin (CLU), also known as apolipoprotein J, is a heterodimeric sulfated glycoprotein that is expressed in most human tissues and body fluids and is associated with many biological activities, including regulation of apoptosis and cancer (Trougakos & Gonos, 2002). Research on *CLU* function and its relation to tumourigenesis has been intensive, but many contradictory data have been reported. These contradictions can be explained mainly by the existence of three protein isoforms with different sub-cellular localisations and biological functions (Rizzi & Bettuzzi, 2010).

The most extensively studied form of CLU is the secreted form (sCLU), which is a glycosylated heterodimer present in almost all physiological fluids. sCLU is exported from the cell and released in secretions, in which it acts as an extracellular chaperone (Humphreys *et al.*, 1999). Cytoplasmic clusterin has been shown to inhibit apoptosis by interfering with Bax activation in the mitochondria (Zhang *et al.*, 2005). Clusterin binds and stabilises the Ku70-Bax protein complex and serves as a cytosolic retention factor for Bax. Depletion of clusterin disrupts the Ku70-Bax complex and triggers Bax activation and its relocalisation to the mitochondria (Trougakos *et al.*, 2009). Cytoplasmic clusterin also promotes tumour cell survival by cooperating with c-Myc during transformation (Zhang *et al.*, 2005). Additionally, a large amount of CLU confers resistance to cytotoxic agents on different cancer cell types (Lourda *et al.*, 2006). However, a nuclear form of clusterin originating from an alternative translation start site has been shown to be proapoptotic in prostate cancer cells (Moretti *et al.*, 2007), breast cancer cells (Leskov *et al.*, 2003) and neuroblastoma (Yang *et al.*, 2000).

The main question is whether clusterin is a positive or negative modulator of mammalian tumourigenesis. It is becoming clear that *CLU* can act either as a tumour suppressor (Caporali *et al.*, 2004; Chayka *et al.*, 2009) or as a tumour promoter (Lourda *et al.*, 2006), but the time-course of the disease and the selection pressures imposed on the cancer by chemotherapy or other treatments have to be taken into account.

Clusterin knockout mice are more prone to autoimmune myocarditis (McLaughlin *et al.*, 2000) and to transformation of the prostate epithelium (Bettuzzi *et al.*, 2009). In homozygous knockout mice, the normal portion of prostate tissue is characterised by stronger expression of the proliferation marker Ki67 and shows activated NF-κB signalling, but this was not observed in wild type mice (Bettuzzi *et al.*, 2009). Additionally, in TRAMP mice, a transgenic animal model of prostate cancer (Greenberg *et al.*, 1995), the *CLU* gene is dramatically downregulated during onset and progression of prostate cancer (Caporali *et al.*, 2004). Crossing of these TRAMP mice with the clusterin knockout mice led to development of a more advanced invasive disease in the progeny, confirming the role of *CLU* as a negative tumour modulator in prostate cancer (Bettuzzi *et al.*, 2009).

On the contrary, overexpression of clusterin in LNCaP prostate cancer cells conferred resistance to both androgen ablation *in vivo* and cytotoxic chemotherapy (Miyake *et al.*, 2000c). Additionally, the administration of clusterin antisense oligonucleotides in prostate cancer xenograft models delayed progression to androgen independence and enhanced chemosensitivity (Miyake *et al.*, 2000b). A human clusterin antisense oligonucleotide (OGX-011) synergistically enhanced the cytotoxic effects of paclitaxel in human xenografts of prostate cancer (Miyake *et al.*, 2000a). This antisense oligonucleotide is currently undergoing clinical trials for tumour therapy. The phase I trial demonstrated that OGX-011 is well tolerated and that it inhibits clusterin expression in prostate cancer (Chi *et al.*, 2005). The phase II trial assessed the effects of combined therapy with androgen ablation and OGX-011 given prior to radical prostatectomy in patients with metastatic castration-resistant prostate cancer. This study showed that the combined therapy was associated with improved survival (Chi *et al.*, 2010).

The biological role of clusterin is controversial in both prostate cancer and neuroblastoma. Indeed, also in neuroblastoma it has been shown that clusterin can function both as a tumour suppressor gene and as an oncogene. Overexpression of clusterin impaired the invasion of neuroblastoma cell lines by inhibiting the NF-κB activity by stabilising IκBs, the inhibitors of NF-κB signalling (Santilli *et al.*, 2003). Additionally, Oncomine expression analysis (Rhodes *et al.*, 2007) showed a lower expression level of the *CLU* gene in primary human neuroblastomas with *MYCN* amplification, and further investigation demonstrated that MYCN acts as a negative regulator of clusterin expression by inducing transcription of the 17-5p~92 microRNA cluster (Chayka *et al.*, 2009). This polycistronic cluster encodes six microRNAs (miR-17-5p, -18a, -19a, -19b, -20a and -92) and is homologous to the miRNA 106a~92 cluster (Mestdagh *et al.*, 2010b). Most miRNAs encoded by this cluster are expressed at higher levels in neuroblastoma cell lines overexpressing *MYCN* or carrying *MYCN* amplification. MYCN transcriptionally induces the expression of the miRNA 17-5p~92 cluster by directly binding to its promoter. The therapeutic potential of this miRNA cluster was demonstrated by injecting a miR-17-5p-specific antagomir into nude mice that had been injected with a neuroblastoma cell line with *MYCN* amplification. Tumour growth *in vivo* was reduced substantially, and this reduction was associated with down-modulation of miR-17-5p activity and with a reciprocal increase of both p21 and BIM protein levels. Nevertheless, the expression pattern of miR-17-5p in primary tumours is complex, because high levels of miR-17-5p expression were detected in both *MYCN*-amplified and non *MYCN*-amplified tumours (Fontana *et al.*, 2008). Additionally, it has been demonstrated that miR-17-5p~92 dampens TGF-β signalling by acting both upstream and downstream of pSMAD2/SMAD4 (Mestdagh *et al.*, 2010a). This finding further underscores its ability to regulate multiple components of the same pathway.

When *MYCN*-transgenic mice were crossed with clusterin knockout mice, the progeny showed reduced tumour-free survival, active NF-κB signalling, and epithelial to mesenchymal transition (Chayka *et al.*, 2009); this resembles the phenotype of TRAMP mice in which CLU was homozygously deleted (Bettuzzi *et al.*, 2009). These data indicate that clusterin functions as a TSG for neuroblastoma, even though several reports indicate that it can promote neuroblastoma. Treatment with histone deacetylase inhibitors (HDACIs) resulted in upregulation of clusterin in different cancer cell lines, including neuroblastoma (Liu *et al.*, 2009; Subramanian *et al.*, 2011). Suppression of clusterin in combination with high-dose HDACIs synergistically enhanced HDACI-induced cell death through cytochome-c-mediated apoptosis in HDACI-resistant cancer cells. Additionally, combining OGX-011 with low-dose HDACIs enhanced HDACI-induced growth arrest in both HDACI-sensitive and -resistant cancer cell lines. Moreover, in mice xenografted with neuroblastoma cells, combining OGX-011 and valproate (the only HDACI proven to be safe in young children) synergistically inhibited tumour growth *in vivo*, whereas neither OGX-011 nor valproate alone had an effect on tumour progression (Liu *et al.*, 2009).

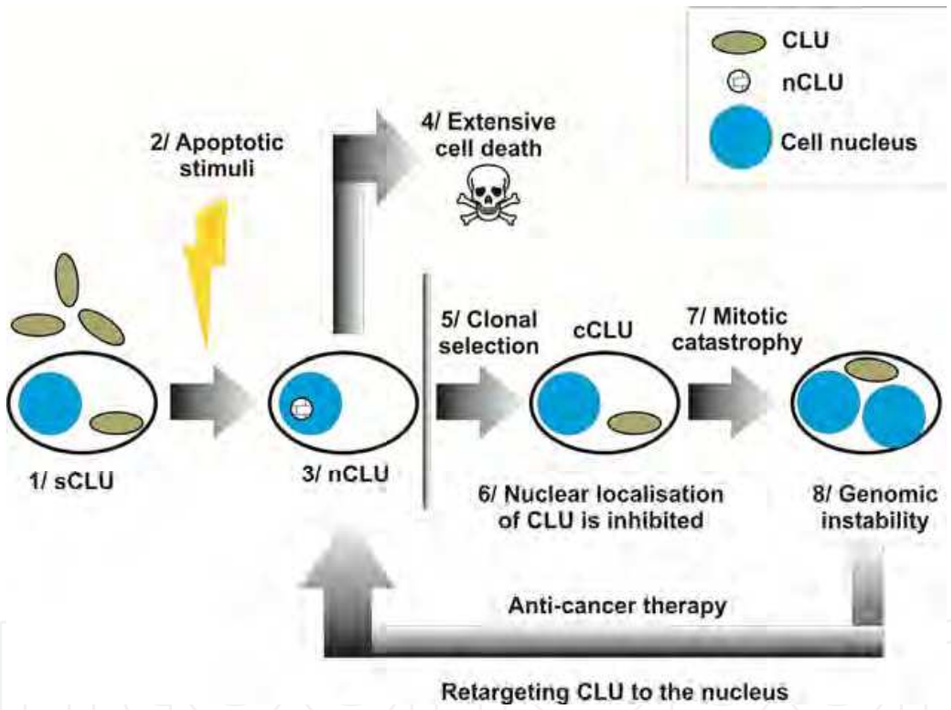



Fig. 6. Proposed model for CLU in tumourigenesis.
In normal cells and under physiological conditions the basal expression level of *CLU* in benign cells is low and confined to the secreted form of CLU (sCLU) (1). Several apoptotic stimuli (2) lead to the production of nuclear CLU (nCLU) (3), which in turn induces extensive cell death (4). In the early phases of tumourigenesis, genetic lesions lead to acquisition of survival advantages and consequent clonal selection (5). A key step in the transformation process is prevention of CLU from entering the nucleus. Cells with high levels of cytoplasmic CLU are then selected for, which results in acquisition of a death-resistant phenotype (6). This phenotype is associated with impaired mitosis (7), which leads eventually to genomic instability (8) (Modified after Rizzi & Bettuzzi, 2010, Society for Endocrinology (2010), reproduced by permission).

Based on these findings, which were observed in both prostate cancer and neuroblastoma, a role for clusterin in tumourigenesis has been proposed (Figure 6). This model proposes that clusterin acts as a tumour suppressor in the early stages of cancer but can become a tumour promoter in the more malignant and advanced stages, namely when tumours become resistant to therapy (Rizzi & Bettuzzi, 2010). Under physiological conditions, the basal expression level of *CLU* in benign cells is low and confined to the secreted form of CLU (sCLU). Several apoptotic stimuli lead to the production of nuclear CLU (nCLU), which in turn induces extensive cell death. At the onset of cancer, early genetic lesions disrupt or inactivate apoptotic pathways or lead to the activation of other survival pathways. Under these conditions of clonal selection, a key step in the transformation process would be the prevention of CLU from entering the nucleus in order to prevent induction of apoptosis. At this point, cancer cells are characterised by strong expression of cytoplasmic CLU (cCLU) and are therefore resistant to death by cytotoxic therapeutic agents. This resistant phenotype can promote cell transformation that leads to late cancer stages associated with impaired mitosis, which causes further genomic instability (Rizzi & Bettuzzi, 2010).

### 5.3 NBPF1, Chibby and clusterin can form a tri-molecular complex

A yeast two-hybrid screening with an N-terminal fragment of the NBPF11 protein identified Cby as a binding partner of NBPF11 (Vandepoele *et al.*, 2010). This interaction is not exclusive to NBPF11 but is common to other NBPF members. For instance, Cby can also interact with NBPF1. The interaction was confirmed by other techniques, such as co-immunoprecipitation and mitochondrial recruitment (Vandepoele *et al.*, 2010). It was shown that the coiled-coil region in the N-terminal domain of NBPF interacts with the coiled-coil region in the C-terminal domain of Cby. This C-terminal portion of Cby harbours not only the binding site for NBPF, but also the binding site for most other known interaction partners of Cby, such as β-catenin (Takemaru *et al.*, 2003), polycystin-2 (Hidaka *et al.*, 2004) and TC1 (Jung *et al.*, 2006).

Functional analysis of the interaction showed that NBPF did not influence Cby-mediated Wnt signalling, nor did it compete with β-catenin binding to Cby. This indicates the presence of two different pools of Cby, one binding to β-catenin and the other binding to NBPF, or alternatively, a dimeric Cby complex that can bind simultaneously to β-catenin and NBPF. Besides its repressor function in the Wnt pathway, Cby can also play an important role in ciliogenesis, as shown in the Cby knockout mouse (see section 5.1). The role of NBPF in the formation of cilia remains uncertain, as NBPF1 overexpression did not interfere with the formation of primary cilia (Vandepoele *et al.*, 2010). An additional yeast two-hybrid screen identified clusterin as a new binding partner for Cby and showed that the C-terminus of CLU, which contains a coiled-coil domain, interacts with both the N-terminal and C-terminal domains of Cby. Additionally, co-immunoprecipitation experiments in HEK293T cells demonstrated that NBPF1, Cby and clusterin can form a tri-molecular complex (Figure 7), because all three proteins were co-purified in every setting.

Since intracellular CLU can be found both in the nucleus and in the cytoplasm (see section 5.2), the effect of Cby or NBPF or their combination on the subcellular localisation of CLU was further investigated, but no difference was observed (Vandepoele *et al.*, 2010).

The identification of two proteins that interact with NBPF is a clue to the functions of NBPF, but further investigation is needed. Anyway, the identification within the complex of two proteins (NBPF1 and CLU) as candidate tumour suppressor genes linked to neuroblastoma (Vandepoele *et al.*, 2008; Chayka *et al.*, 2009) is intriguing and warrants further scrutiny.

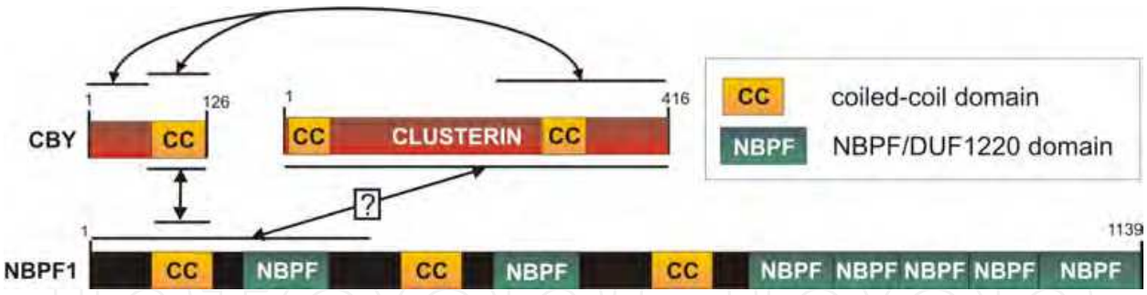



Fig. 7. NBPF1, Cby and CLU form a tri-molecular complex.

An overview of the mutual interactions between NBPF1, Cby and CLU. The coiled-coil region in the N-terminus of NBPF1 interacts with the coiled-coil region in the cytoplasmic domain of Cby. The C-terminus of CLU interacts with the full-length Cby protein, as interactions with both the N-terminus and C-terminus are observed. It is uncertain whether CLU and NBPF1 interact directly (boxed question mark) (Modified after Vandepoele *et al.*, 2010, Elsevier (2010), reproduced by permission).

## 6. Conclusion

In this chapter we summarise the growing evidence for the recent emergence of the *NBPF* gene family and for its role in human evolution. The NBPF/DUF1220 domains in particular seem to have been important in this context. This evidence is based on the large increase in copy number of the NBPF/DUF1220 domain in humans. On the other hand, *NBPF* genes seem to be involved in cancer and in brain and developmental disorders due to their location in unstable high-identity duplication blocks, which leads to recurrent chromosomal rearrangements. One case of particular interest is neuroblastoma. Evidence for the involvement of *NBPF* genes in this type of cancer comes not only from disruption of *NBPF1* in a neuroblastoma patient, but also from gene expression studies and the association with neuroblastoma of a copy number variation of an *NBPF* paralog. Additionally, the regulatory elements and the identification of NBPF-interacting proteins indicate that the *NBPF* gene family plays a role in neuroblastoma. Involvement of *NBPF* genes in neuroblastoma and possibly as well in other cancers points to the value of studying this gene family further. Every step in the functional understanding of these genes could bring us closer to unravelling the molecular mechanisms underlying neuroblastoma development and could help us to develop more effective therapy. Such studies could also shed light on the impact these novel genes had on recent primate evolution.

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## 8. References

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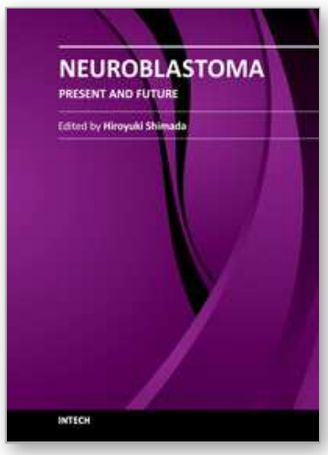


**Neuroblastoma - Present and Future**

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Neuroblastoma, once called "enigmatic", due to "unpredictable" clinical behaviors, is composed of biologically diverse tumors. Molecular/genomic properties unique to the individual tumors closely link to the clinical outcomes of patients. Establishing risk stratification models after analyzing biologic characteristics of each case has made a great success in patient management. However, the trend of improving survival rates in neuroblastoma over the last 30 years has started to level off, and currently available treatment modalities have almost reached to their maximized intensity. Furthermore, aggressive treatment causes significant long-term morbidities to the survivors. We really need to make the next step to the level of personalized medicine with more precise understanding of neuroblastoma biology. This book includes useful data and insights from the world's experts in this field. I believe this book can make an excellent contribution to all the investigators working hard and fighting for the children stricken by this disease.



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**InTech Europe**
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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Unit 405, Office Block, Hotel Equatorial Shanghai
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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
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