

Definition and characterization of a region of 1p36.3 consistently deleted in neuroblastoma

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Substantial genomic and functional evidence from primary tumors and cell lines indicates that a consistent region of distal chromosome 1p is deleted in a sizable proportion of human neuroblastomas, suggesting that this region contains one or more tumor suppressor genes. To determine systematically and precisely the location and extent of 1p deletion in neuroblastomas, we performed allelic loss studies of 737 primary neuroblastomas and genotype analysis of 46 neuroblastoma cell lines. Together, the results defined a single region within 1p36.3 that was consistently deleted in 25% of tumors and 87% of cell lines. Two neuroblastoma patients had constitutional deletions of distal 1p36 that overlapped the tumor-defined region. The tumor- and constitutionally-derived deletions together defined a smallest region of consistent deletion (SRD) between *DIS2795* and *DIS253*. The 1p36.3 SRD was deleted in all but one of the 184 tumors with 1p deletion. Physical mapping and DNA sequencing determined that the SRD minimally spans an estimated 729 kb. Genomic content and sequence analysis of the SRD identified 15 characterized, nine uncharacterized, and six predicted genes in the region. The RNA expression profiles of 21 of the genes were investigated in a variety of normal tissues. The *SHREW1* and *KCNAB2* genes both had tissue-restricted expression patterns, including expression in the nervous system. In addition, a novel gene (*CHD5*) with strong homology to proteins involved in chromatin remodeling was expressed mainly in neural tissues. Together, these results suggest that one or more genes involved in neuroblastoma tumorigenesis or tumor progression are likely contained within this region.

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Introduction

Neuroblastoma is a common pediatric malignancy of the peripheral sympathetic nervous system. Despite recent advances in therapy, a large proportion of neuroblastoma patients succumb to the disease. Thus, identification and characterization of the genetic events underlying neuroblastoma tumorigenesis and progression are important priorities for management of this malignancy. Cytogenetic and molecular analyses of neuroblastoma tumors and cell lines have identified several frequently occurring genetic abnormalities, including deletion of chromosomes 1p, 11q, and 14q; allelic gain of 11p and 17q; and amplification of the *MYCN* proto-oncogene (Brodeur, 2003). Deletion of distal 1p is highly correlated with both *MYCN* amplification and an adverse patient outcome, suggesting the presence of one or more tumor suppressor genes (TSGs) within this region (Maris *et al.*, 1995, 2000). Several lines of evidence support this hypothesis and further implicate 1p36 as the region most likely to contain a TSG. Numerous loss of heterozygosity (LOH) and molecular cytogenetic analyses of 1p in neuroblastoma have demonstrated allelic loss of 1p36 (Fong *et al.*, 1989; Takayama *et al.*, 1992; Schleiermacher *et al.*, 1994; Takeda *et al.*, 1994; White *et al.*, 1995; Martinsson *et al.*, 1997; Mora *et al.*, 2000; Bauer *et al.*, 2001; Maris *et al.*, 2001; Godfried *et al.*, 2002). Collectively, the incidence of LOH reported ranges from 25 to 35% of primary tumors. Furthermore, transfer of 1p chromosomal material into a neuroblastoma cell line has been shown to suppress tumorigenicity (Bader *et al.*, 1991).

However, progress in narrowing the region and defining candidate TSGs within 1p36 has been slow, due in part to the fact that most 1p deletions are large. In addition, while several neuroblastoma cell line and constitutional chromosomal rearrangements involving 1p36 have been identified, the affected chromosomal regions do not tightly cluster (Barker *et al.*, 1993; Savelyeva *et al.*, 1994; Amler *et al.*, 1995; van der Drift *et al.*, 1995; Casciano *et al.*, 1996; Ohira *et al.*, 2000; Spieker *et al.*, 2001; Satge *et al.*, 2003). Moreover, linkage analysis of familial cases has excluded 1p36 as

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containing a familial predisposition locus (Maris *et al.*, 1996).

We previously defined a region of allelic loss shared by virtually all primary neuroblastoma tumors with 1p deletions to 1p36.2–p36.3 by LOH analysis of 122 primary tumors (White *et al.*, 1995), a finding which has subsequently been confirmed by other groups (Martinson *et al.*, 1997; Mora *et al.*, 2000; Bauer *et al.*, 2001; Spieker *et al.*, 2001; Godfried *et al.*, 2002). Our defined smallest region of consistent deletion (SRD) partially overlapped a constitutional 1p36 deletion in a patient subsequently diagnosed with neuroblastoma. In the present study, we undertook a comprehensive and large-scale molecular genetic approach to further characterize our initially defined SRD, with the objective of further narrowing the region and number of candidate genes to characterize. We expanded the LOH analysis to 737 primary tumors, along with genotype analyses of 46 neuroblastoma cell lines and of a second neuroblastoma patient with a constitutional 1p36 deletion. These data have allowed definition of a precisely bounded SRD within 1p36.3 that is consistently deleted in at least 25% of primary neuroblastomas. We also describe the physical mapping, sequencing, sequence analysis, and transcriptional profiles of genes within the defined SRD.

Results

Paired primary tumor and normal DNA samples were collected from a cohort of 737 neuroblastoma patients. This cohort was generally representative of disease stage and age distributions, as well as for the frequency of *MYCN* amplification, with a slight bias towards tumors with a favorable outcome. Individual DNA pairs were genotyped for a subset of 61 polymorphic loci localizing to 1p (Table 1). The genotyping was performed in three phases. The first phase used a number of polymorphic loci throughout 1p in order to establish whether allelic loss was randomly distributed or concentrated within particular genomic regions of the chromosome arm. After establishment of 1p36 as the region of most consistent deletion, phase 2 primarily used polymorphic loci localized to distal 1p. As a distal 1p36 SRD was determined, phase 3 added 183 additional samples to the cohort ($n = 737$), and additional markers within 1p36.3 were added in order to identify tumors with breakpoints within or near the defined SRD. After each phase, samples with interesting breakpoints were genotyped at further loci to confirm and localize the breakpoints. A tumor sample was considered to have LOH only if one allele signal was reduced > 60% or more at two or more contiguous loci.

Within the entire primary tumor cohort, 184 of 737 tumors (25%) demonstrated allelic loss for two or more loci (Table 1). Of these, 117 cases showed LOH for all informative 1p loci surveyed, 65 cases showed partial 1p LOH that extended to the distal-most informative marker (partial terminal deletions), and two cases demonstrated interstitial deletion (Figure 1a). Of the

Table 1 Primary tumor cohort statistics

Total cases included	737
MYCN amplified	18.7%
Stage 1	15.6%
Stage 2	17.0%
Stage 3	22.8%
Stage 4	38.9%
Stage 4S	5.7%
Number of 1p loci	61
Marker density for 1p	1.9 Mb
Number of 1p36 loci	41
Marker density for 1p36	660 kb
Number of 1p36.3 loci	14
Marker density for 1p36.3	480 kb
Total genotypes	5335
Avg. per case	7.2
LOH genotypes	969
Avg. per case with LOH	5.3
Genotypes lacking	2668
Total informative genotypes	3637
Avg. per case	4.9
Uninformative genotypes	1698
Heterozygosity rate	68%
Cases with LOH	184
Entire surveyed region of 1p	117
Terminal deletions, partial 1p	65
Interstitial deletions	2
Cases without LOH	553
LOH %	25.0%
1p36.3 SRO	D1S2660 to D1S214
Genome build 34 maximal distance	2.20 Mb
Genome build 34 minimal distance	798 kb

cases with partial 1p deletions, the breakpoints were scattered throughout the chromosome arm, with no apparent clustering in specific regions. All but one of the 184 tumors with 1p LOH demonstrated LOH within a specific region of 1p36.3. This region was defined distally by tumor 670 with no LOH at *D1S2660* but LOH at the adjacent locus *D1S2795*, and proximally by cases 216 and 428, both of which demonstrated no LOH at *D1S214* but LOH distally (Figure 2). Case 216 showed LOH for the locus distally adjacent to *D1S214* (*D1S253*), whereas case 428 was not informative for *D1S253* but demonstrated LOH for the next adjacent locus distally (*D1S2870*). Together, these informative breakpoints defined a single SRD within 1p36.3.

Only one tumor demonstrated 1p LOH for a region other than the 1p36.3 SRD (Figure 1a). This tumor (case 222) demonstrated no allelic loss at 1p36.1 locus *D1S3720* and also for five loci distal to *D1S3720*. However, this case showed LOH for three closely spaced loci within 1p32 (*D1S1596*, *D1S1669*, and *D1S1643*). No other tumor was found to contain an interstitial deletion of 1p exclusively proximal to the 1p36.3 SRD in our cohort.

We also determined the extent of 1p deletion within two individuals with neuroblastoma and known constitutional monosomy of 1p36. Clinical manifestations and preliminary genetic analysis of these cases have been

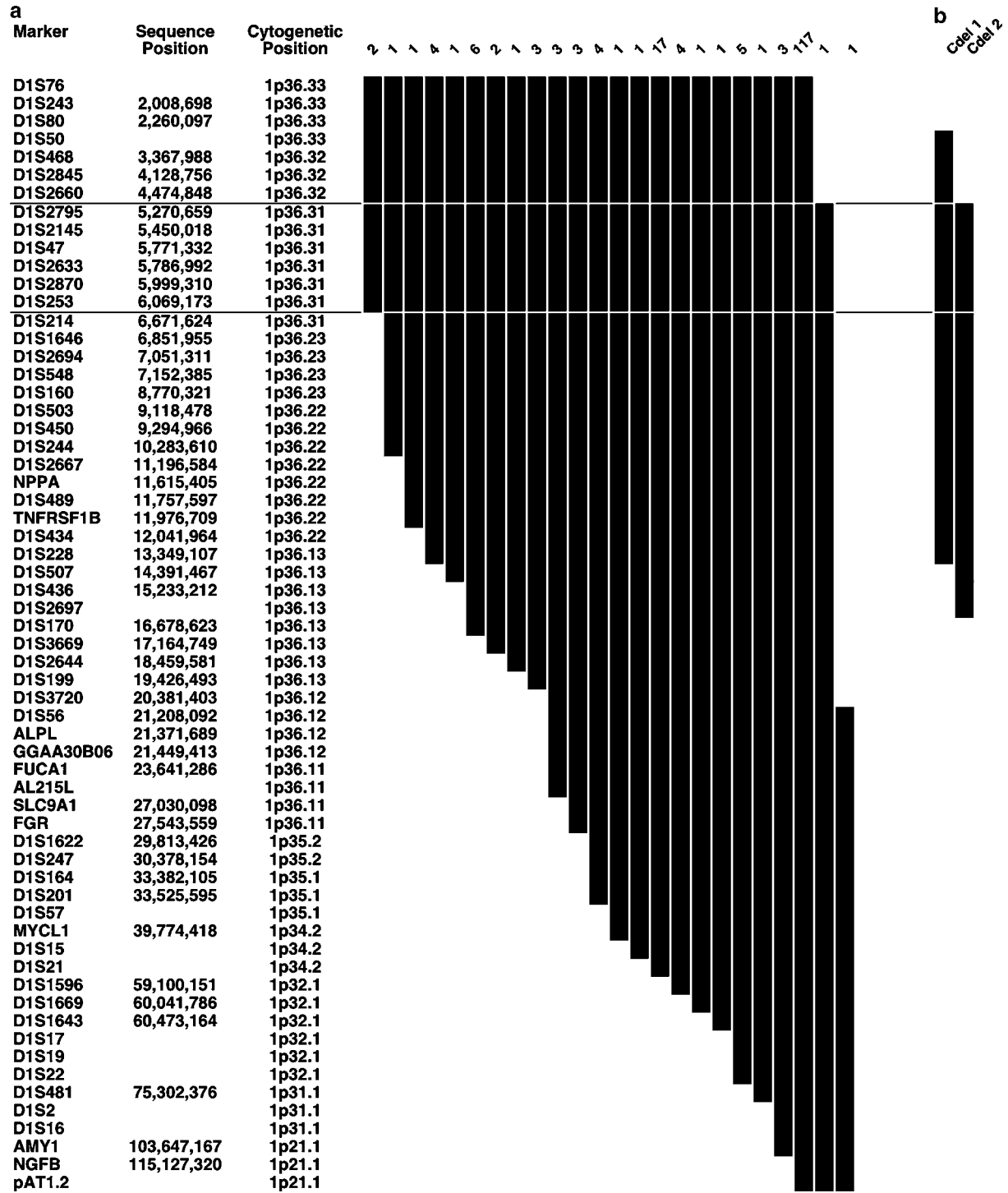


Figure 1 Summary of the 1p deletion studies. The extent of deletion or LOH identified in 184 primary neuroblastomas (a, left) and two neuroblastoma patients with constitutional 1p36 deletions (b, right) are shown. Vertical black bars indicate the maximal extent of LOH or deletion detected, with each individual bar representing an overall pattern. Numbers above each bar indicate the number of samples exhibiting the pattern shown. The 1p36.3 SRD, maximally located between *DIS2660* and *DIS214*, and minimally between *DIS2795* and *DIS253*, is indicated by horizontal lines. At left, the cytogenetic and (if known) DNA-sequence-based position from the p terminus for each marker is listed, as reported by the UCSC genome browser and eGenome, respectively

described previously (Biegel *et al.*, 1993; White *et al.*, 1997). To determine the precise extent of the distal 1p regions deleted in these patients, genotyping of DNA from blood samples of the patients and corresponding

parental samples was performed. Both 1p monosomy cases were found to have an interstitial deletion of the maternal copy of 1p36.2–1p36.3. Distally, case 1 showed heterozygosity for 1p36.3 locus *DIS80* but hemizyosity

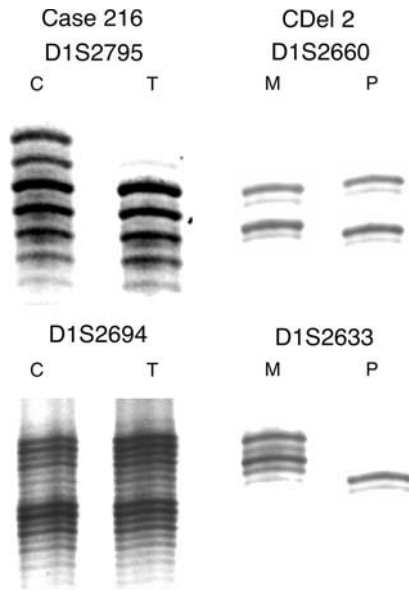


Figure 2 LOH in neuroblastoma cases defining the 1p SRD. Left, primary tumor (T) and corresponding constitutional (C) DNA samples from primary neuroblastoma case 216, which defines the proximal breakpoint of the 1p36.3 SRD. LOH for *DIS2694*, distal to the breakpoint, and retention of heterozygosity for *DIS2695*, proximal to the breakpoint, are shown. Right, maternal (M) and proband (P) DNA samples from case CDel2, a child with a constitutional deletion of 1p36.3 who subsequently developed neuroblastoma. This case defines the distal breakpoint of the 1p36.3 SRD. Genotypes of *DIS2660*, distal to the breakpoint, demonstrate heterozygosity in the proband (no deletion). Genotypes of *DIS2633* indicate absence of a maternal allele, consistent with deletion of this region

for the next most proximal marker surveyed, *DIS468*. Proximally, case 1 was deleted for 1p36.2 locus *DIS450*, but six markers proximal to this locus were retained in both copies. For case 2, heterozygosity was noted for 1p36.3 marker *DIS2660* and two informative loci distal to this locus (Figure 2). Proximally, case 2 was deleted for 1p36.2 locus *DIS507* but not for informative loci proximal to this marker. The distal deletion boundary of case 2 thus coincided with and confirmed the distal boundary of the SRD defined by the primary tumor LOH results. The extent of deletion for each constitutional case entirely overlapped the tumor-defined 1p36.3 SRD (Figure 1b).

We also studied 46 genotype-unique neuroblastoma cell lines for 1p36 deletion using fluorescence *in situ* hybridization (FISH), cytogenetics, and/or inference of deletion from observing homozygosity at ≥ 3 consecutive highly polymorphic 1p36 markers. In all, 40 of 46 (87%) cell lines demonstrated genomic abnormalities of 1p36 by these methods, either deletion (39 cases) or partial duplication and translocation (cell line NGP, t(1;15)(p36.1;q24),dup(1)(p36.2), see also Brodeur *et al.*, 1977; Amler *et al.*, 1995). All had relatively large deletions encompassing the entire SRD defined by the primary tumor results.

In combination, the primary tumor, constitutional case, and cell line deletion mapping results defined a

single SRD within 1p36.3, extending minimally from *DIS2795* to *DIS253* and maximally from *DIS2660* to *DIS214*. To create a physical map of the region, we first collected 381 PCR-formatted markers, including 227 representing unique transcripts that had been mapped previously to distal 1p. These were screened against a regional radiation-reduced hybrid cell line panel to determine the localization of each marker relative to the SRD. A total of 52 markers were localized within or near the SRD. These markers were then used to screen large-insert DNA clone libraries. Identified clones were assembled into contigs primarily by STS content mapping and endclone fingerprinting, yielding three contigs (Figure 3). A subset of clones representing minimal tiling paths for each contig was sequenced by the Sanger Institute, yielding 729 kb of finished sequence within the minimal SRD and 2.20 Mb within the maximal SRD. The two remaining gaps were cumulatively estimated to span 110 kb.

The consensus SRD was then analysed for the presence of transcriptional units by a variety of methods. First, our regional mapping work targeted all known genes and EST clusters previously localized to distal 1p. Besides the 52 markers used for large-insert clone identification, we assembled an additional 76 markers that localized to the SRD according to electronic-PCR or BLAST matches to the SRD DNA sequence, and also from localizations reported at eGenome and UCSC (White *et al.*, 1999; Karolchik *et al.*, 2003) (Figure 3). This set of 128 markers represented 11 characterized genes and five putative transcripts (Table 2). We also supplemented this transcriptional set by searching for additional genes and putative transcripts using a variety of approaches, including *ab initio*, transcript sequence homology, and comparative genomic homology-based gene prediction techniques, as well as incorporation and independent assessment of predictions made by other groups (e.g. Ensembl and UCSC). These efforts identified an additional 14 transcripts, for a total of 15 previously characterized genes and 15 uncharacterized and putative transcripts localized to the defined SRD (Table 2).

Finally, we determined the transcriptional expression profiles of 21 identified SRD genes in a panel of 17 normal human tissues, including four neural-derived tissues and adrenal gland, the latter of which is the site of origin for a large proportion of neuroblastomas. This analysis was initially performed both by our own experimentation, using a combination of semiquantitative (four genes) and real-time quantitative RT-PCR (17 genes) (Table 3). A total of 13 genes (*CAMTA1*, *KIAA0469*, *TAS1R1*, *KIAA0720*, *ESPN*, *HES2*, *BACH*, *GPR153*, *LOC284509*, *MGC40168*, *CHD5*, *KCNAB2*, and *SHREW1*) demonstrated some level of tissue specificity for the tissues surveyed. Of the genes, 17 were expressed in at least one neural-derived tissue; 10 of the genes (*CAMTA1*, *FLJ10737*, *MGC33488*, *HKR3*, *GPR153*, *ICMT*, *FLJ32096*, *CHD5*, *NPHP4*, and *SHREW1*) were expressed in all neural tissues surveyed; and 15 of the genes were expressed in the adrenal gland (Table 3). Of those genes with tissue specificity, six

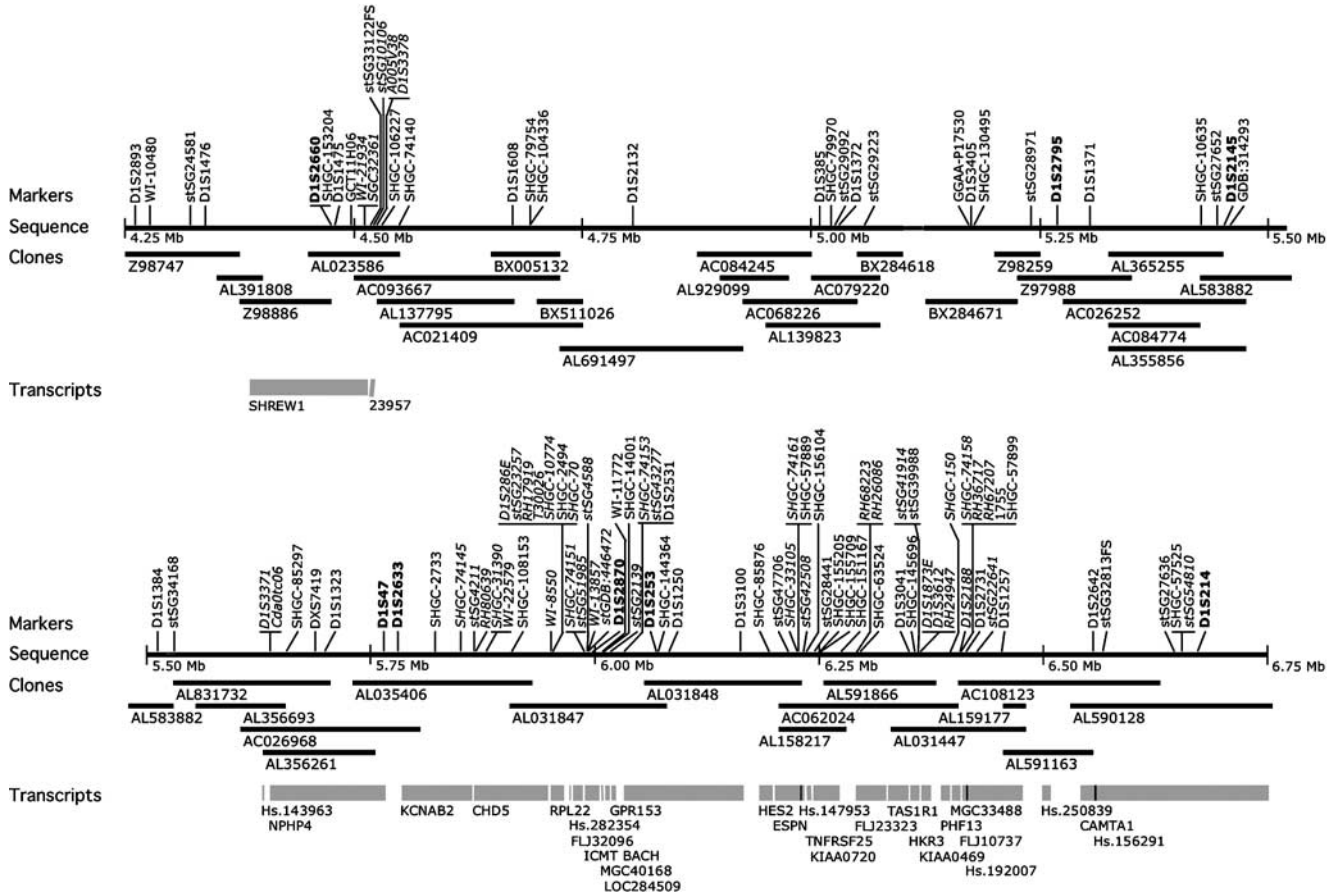


Figure 3 Genomic map of the 1p36.3 SRD. A representation of structural and functional genomic elements within the defined 1p36.3 SRD, between *DIS2660* and *DIS214*, is shown. The region is split into two continuous sections for clarity. At the top of each section (*markers layer*) are listed DNA markers used for mapping and annotation purposes in the study. Marker names in bold are polymorphisms used for LOH and genotyping of patient samples; marker names in italics are those representing transcribed segments. Positions of markers relative to the DNA sequence are indicated by vertical lines. The *sequence layer* represents the DNA sequence tract for this region. Gray segments represent gaps in the finished sequence determination. DNA sequence positions in base pairs, relative to the 1p terminus, are shown at regular intervals. The *clones layer* represents large-insert DNA clones identified and assembled, in collaboration with the Sanger Institute, to form contiguous clone arrays for DNA sequence determination. The *transcripts layer* indicates the presence and approximate positions of the 30 known and putative transcripts localized within the SRD

(*CAMTA1*, *BACH*, *GPR152*, *LOC284509*, *CHD5*, and *SHREW1*) were expressed at least partially in the nervous system, and only one (*SHREW1*) was found to be expressed preferentially in tissues of early development. Expression profiles for *CHD5*, *KCNAB2*, and *SHREW1* are shown in Figure 4.

Discussion

A sizable number of studies have independently identified and confirmed allelic loss within 1p for neuroblastoma cell lines and primary tumors (Fong *et al.*, 1989; Takayama *et al.*, 1992; Schleiermacher *et al.*, 1994; Takeda *et al.*, 1994; White *et al.*, 1995; Martinsson *et al.*, 1997; Mora *et al.*, 2000; Bauer *et al.*, 2001; Maris *et al.*, 2001; Godfried *et al.*, 2002). However, these studies have not identified a consistent region of deletion. Furthermore, these and other studies have proposed over 20 genes within these regions as

candidate neuroblastoma tumor suppressors, none of which have yet been proven to play a significant causative role in neuroblastoma tumor development (Ejeskar *et al.*, 2000; Judson *et al.*, 2000; De Toledo *et al.*, 2001; Huang *et al.*, 2001; Abel *et al.*, 2002; Cerignoli *et al.*, 2002; Krona *et al.*, 2003; Thompson *et al.*, 2003; Mathysen *et al.*, 2004). Our current work was designed to determine the location and extent of 1p deletion in a very large primary tumor cohort by extensive genotyping, sequencing, gene identification, and transcript characterization.

Our allelic loss studies initially targeted all of 1p. Subsequently, we increased the sample size and locus densities for distal 1p, and then for 1p36.3, as a single SRD emerged. Overall, allelic loss for 1p was identified in 184 of 737 (25%) primary neuroblastomas and 40 of 46 (87%) neuroblastoma cell lines, consistent with the frequencies of each that were identified in our earlier studies and those of other groups. Together, these results established a single SRD within 1p36.3, flanked

Table 2 Transcripts identified within the SRD

<i>Transcript name</i>	<i>Genomic position</i>	<i>Class</i>	<i>Putative function</i>
SHREW1	4384990–4514048	Characterized	Transmembrane protein
23957	4519835–4522776	Uncharacterized	
Hs.143963	5621649–5622924	Putative	
NPHP4	5632416–5762077	Characterized	Nephroretinin; causes nephronophthisis-4
KCNAB2	5795926–5870778	Characterized	Potassium voltage-gated channel
CHD5	5871399–5949729	Characterized	Chromodomain helicase DNA-binding protein
RPL22	5954637–5969201	Characterized	Ribosomal protein
Hs.282354	5974445–5975386	Putative	
FLJ32096	5978466–5990411	Uncharacterized	
ICMT	5990799–6005578	Characterized	Isoprenylcysteine carboxylmethyltransferase
MGC40168	6007417–6009046	Uncharacterized	
LOC284509	6013192–6015633	Uncharacterized	Signal transduction
GPR153	6016954–6023632	Characterized	G protein-coupled receptor
BACH	6033878–6163367	Characterized	Brain acyl-CoA hydrolase
HES2	6182046–6194276	Characterized	Hairy and enhancer of split homolog
ESPN	6194394–6230535	Characterized	Actin bundle assembly
Hs.147953	6225801–6226289	Putative	
TNFRSF25	6230774–6235781	Characterized	Tumor necrosis factor receptor superfamily member
KIAA0720	6235698–6266302	Uncharacterized	Intracellular signaling
FLJ23323	6290953–6324141	Uncharacterized	
TAS1R1	6324980–6349362	Characterized	Taste receptor
HKR3	6349657–6358885	Characterized	Transcription factor
KIAA0469	6360325–6372607	Uncharacterized	
PHF13	6383291–6393602	Characterized	Transcription factor
MGC33488	6394801–6403171	Uncharacterized	Transcription factor
FLJ10737	6403774–6471419	Uncharacterized	Heat shock protein
Hs.192007	6411144–6411685	Putative	
Hs.250839	6494243–6499595	Putative	
Hs.156291	6553498–6554571	Putative	
CAMTA1	6554930–6537449	Characterized	Calmodulin-binding transcription activator

Table 3 Tissue distribution patterns of SRD transcripts

<i>Transcript name</i>	<i>Tissue expression distribution</i>					
	<i>Distribution</i>	<i>Nervous system</i>	<i>Adrenal gland</i>	<i>Preferential in nervous system</i>	<i>Early development</i>	<i>Preferential in early development</i>
SHREW1	Differential	Yes	Yes	Yes	Yes	Yes
NPHP4	Ubiquitous	Yes	Yes	No	Yes	No
KCNAB2	Differential	Yes	No	No	No	No
CHD5	Differential	Yes	No	Yes	Yes	No
FLJ32096	Ubiquitous	Yes	Yes	No	Yes	No
ICMT	Ubiquitous	Yes	Yes	No	Yes	No
MGC40168	Differential	Yes	No	No	Yes	No
LOC284509	Differential	Yes	No	No	Yes	No
GPR153	Differential	Yes	Yes	No	Yes	No
BACH	Differential	Yes	Yes	Yes	No	No
HES2	Differential	Yes	No	No	Yes	No
ESPN	Differential	Yes	Yes	No	Yes	No
KIAA0720	Differential	Yes	Yes	No	No	No
FLJ23323	Differential	No	No	No	No	No
TAS1R1	Differential	No	Yes	No	No	No
HKR3	Ubiquitous	Yes	Yes	No	Yes	No
KIAA0469	Differential	No	Yes	No	No	No
PHF13	Differential	No	Yes	No	No	No
MGC33488	Ubiquitous	Yes	Yes	No	Yes	No
FLJ10737	Ubiquitous	Yes	Yes	No	Yes	No
CAMTA1	Differential	Yes	No	Yes	No	No

by markers *DIS2795* and *DIS253*, and spanning a minimum of 729 kb.

Our currently defined SRD confirms and more precisely defines the SRD found in our two previous studies, each of which used much smaller cohorts (Fong

et al., 1989; White *et al.*, 1995). All but one tumor with 1p LOH in our current study demonstrated allelic loss within this 1p36.3 SRD. The one exception was a tumor with a deletion extending proximally from the marker *DIS56* in 1p36.1. However, 21 cases with 1p36.3 LOH

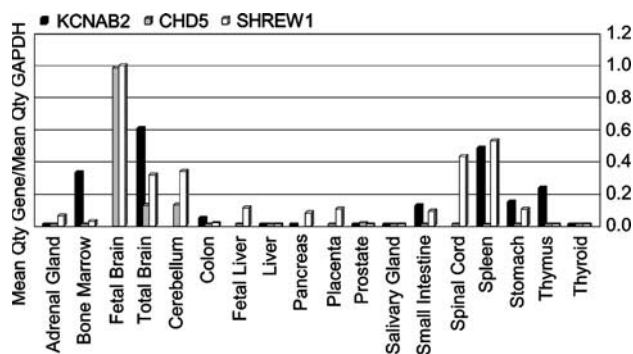


Figure 4 Tissue expression analysis of three SRD genes. The expression levels for three genes within the 1p36.3 SRD, determined for 18 normal tissues, are shown. The genes indicated are *KCNAB2* (black), *CHD5* (grey), and *SHREW1* (white), three surveyed genes within the SRD with interesting expression patterns from a neuroblastoma perspective. Expression levels are shown as mean quantity ratios relative to a GAPDH standard, performed in duplicate, as determined by real-time quantitative PCR (TaqMan, *CHD5* and *SHREW1*) or using a semiquantitative RT-PCR assay (*KCNAB2*)

did not have allelic loss extending to 1p36.1, so the exceptional case does not likely define a second SRD. The lack of additional SRDs is consistent with more recent LOH studies from other groups (Martinsson *et al.*, 1997; Bauer *et al.*, 2001; Caron *et al.*, 2001), although see Schleiermacher *et al.* (1994). Of perhaps more significance, our defined 1p36.3 SRD is either completely encompassed within (Schleiermacher *et al.*, 1994; Martinsson *et al.*, 1997; Caron *et al.*, 2001) or partially overlaps (Bauer *et al.*, 2001) the defined SRDs of other groups analysing smaller tumor cohorts by LOH analysis. The overlap between our SRD and that of Bauer *et al.* is at most 260 kb, between *DIS2731* and *DIS214*, and includes one characterized and three putative genes. Interestingly, although the Bauer Study surveyed only 49 primary neuroblastomas, three of 15 tumors with 1p36 LOH had interstitial deletions within 1p36.3, possibly indicating either a population variation or a difference in allelic loss assessment methodologies than with our current study, which identified only two interstitial deletions.

In contrast to recent LOH studies, there have been several investigations of individual neuroblastoma tumor- (Spieker *et al.*, 2001; Van Roy *et al.*, 2002) and cell-line-derived (Amler *et al.*, 1995; Casciano *et al.*, 1996; Ohira *et al.*, 2000) chromosomal rearrangements, including a balanced constitutional translocation within 1p36.2 and a 500 kb homozygous deletion (HD) in a neuroblastoma cell line. Comparison of these rearrangements with our findings shows that each rearrangement maps proximal to our defined SRD. This suggests the possibility of additional neuroblastoma tumor suppressor loci located more proximal within 1p36, and that disruption of two or more TSGs within 1p36 may further contribute to the tumorigenicity or progression of neuroblastoma in those tumors with larger 1p36 deletions. However, we previously screened a large panel of neuroblastoma cell lines at high genomic density for

homozygous deletions within 1p36 (Thompson *et al.*, 2001), including several markers within the 500 kb HD identified by Ohira *et al.* (2000), but found no evidence of HD supporting these findings.

Our analysis of neuroblastoma cell lines and two neuroblastoma patients with constitutional deletions of 1p36 found that these samples also contained deletions that completely encompassed the primary tumor-defined SRD. Constitutional deletion patient 2's distal deletion breakpoint corresponded with the breakpoint of the primary tumor defining the distal SRD boundary, thus providing supporting evidence for this location. Monosomy 1p36 has been recognized recently as a relatively frequent constitutional chromosomal abnormality (Shapira *et al.*, 1997; Heilstedt *et al.*, 2003). Approximately 40% of all characterized cases have deletions that include a portion of our defined neuroblastoma SRD, and 30% are monosomic within the entire SRD. However, none of these other patients are reported to have neuroblastoma. Thus, if a causal relationship exists between 1p36 monosomy and neuroblastoma development, constitutional deletion of the SRD is not sufficient for the latter.

Using a variety of techniques, including LOH, cytogenetic, and STS content-based molecular analyses, our group and others have now cumulatively surveyed a large cohort of primary neuroblastomas (Bader *et al.*, 1991; Schleiermacher *et al.*, 1994; Martinsson *et al.*, 1997; Caron *et al.*, 2001). Nevertheless, only a few rare cases with informative breakpoints within 1p36.3 have been identified to date, and no gene-specific or small regional genomic abnormalities have yet been detected within the collective 1p36.3 SRD. Given the propensity for large, hemizygous 1p36 deletions, alternative hypotheses for tumor suppression have been suggested. These include the possibility of an additional, *MYCN*-associated TSG in proximal 1p36 due to the observation that *MYCN*-amplified tumors invariably delete at least the majority of 1p36 (Takeda *et al.*, 1994; Caron *et al.*, 2001); haploinsufficiency-based suppression accounting for the rarity of 1p36 homozygous deletions (Janoueix-Lerosey *et al.*, 2004); the possibility of two or more nonoverlapping SRDs (White and Versteeg, 2000); and suppression of TSG expression from a hemizygous allele due to imprinting or other epigenetic modifications (Caron *et al.*, 2001; Hogarty *et al.*, 2002). Whether the lack of localized chromosomal abnormalities or HD is reflective of functional or structural mechanisms awaits additional molecular experimentation.

Genomic mapping, sequencing, and sequence analysis identified a total of 30 genes within the SRD, 15 of which were previously characterized to some extent. For 21 of these genes, we determined the expression patterns in 18 normal tissues. Of these, two genes are intriguing as candidate tumor suppressors. As we have reported previously (Thompson *et al.*, 2003), *CHD5* is a member of the chromodomain gene family, with high homology to *CHD3* and *CHD4*, both of which are thought to be functional components of nucleosome remodeling and histone deacetylation complexes (Woodage *et al.*, 1997). *CHD5* is of special interest due to its preferential

expression in the nervous system and the adrenal gland, as well as its lack of expression in 1p-deleted, high-risk neuroblastomas. The gene *SHREW1* was expressed preferentially in fetal brain, total brain, spinal cord, cerebellum, and spleen. *SHREW1* is predicted to encode a transmembrane-spanning protein and has recently been shown to interact specifically with E-cadherin/ β -catenin complexes (Bharti *et al.*, 2004), but little else is currently known regarding its function or that of its mouse homolog.

A number of additional SRD genes for which some function could be determined are plausible as having tumor suppressor roles, including the putative transcription factors *MGC33488*, *PHF13*, *KIAA0469*, *HES2*, *FLJ32096*, *LOC284509*, and *CAMTA1*. Of these, *CAMTA1* is particularly intriguing due to its expression being mainly restricted to neural tissues, and also that it lies within the region of overlap shared by the 1p36.3 neuroblastoma SRDs defined by all recent studies. *CAMTA1* is the first identified human homolog of a class of proteins whose members act as transcriptional activators, have a domain capable of binding calmodulin, and encode ankyrin repeats (Katoh, 2003). While *CAMTA1* has not yet been well characterized functionally, Nakatani *et al.* (2004) report that *CAMTA1* is highly expressed in N(euronal)-type neuroblastoma cell lines but absent in S(chwannian)-type cell lines.

HES2 is a member of a family of proteins with significant homology to the *Drosophila hairy* and *enhancer of split* transcription factors, both of which are required for sensory neurogenesis in late development (Katoh, 2004). *Hairy* also plays a crucial role as a pair-rule segmentation gene in early *Drosophila* development. Like other members of the human family, *HES2* contains a bHLH domain, suggesting the ability to act as a transcription factor. The mouse ortholog of *HES2* has been shown to bind to E-box and, with lower affinity, N-box regulatory sequences (Ishibashi *et al.*, 1993); however, unlike other *HES* family members, mouse *HES2* is not upregulated by the cellular differentiation factor Notch (Nishimura *et al.*, 1998). Our studies found *HES2* to be transcribed in most tissues surveyed, including brain and adrenal gland.

KIAA0720 encodes a gene with predicted pleckstrin and guanine nucleotide exchange factor domains, both of which indicate possible roles in intracellular signaling. *GPR153* encodes a seven-transmembrane-spanning G-protein-coupled receptor with both neural and adrenal gland expression. In addition, *NPHP4*, which has recently been identified as the gene causative for the kidney disorders nephronophthisis-4 and Senior-Loken syndrome (Mollet *et al.*, 2002), appears to be involved in early renal development but is also widely expressed. *KCNAB2* is a member of the *shaker* subfamily of voltage-gated potassium channels and serves an auxiliary role to the functional α subunit of the channel (McCormack *et al.*, 2002). Our analysis found this gene to be preferentially expressed in brain, which is consistent with previous findings indicating an association between deletion of *KCNAB2* and an epileptic phenotype in patients with monosomy 1p36.3 (Heilstedt

et al., 2001). Finally, the transcription factor *HKR3* and the death domain receptor *TNFRSF25* have previously been identified and extensively evaluated as candidate neuroblastoma tumor suppressor genes (Maris *et al.*, 1997; Grenet *et al.*, 1998), but no evidence to date implicates either in tumorigenesis.

In summary, our findings confirm, precisely refine, and characterize in detail a small region of allelic loss within 1p36.3 that is present in a substantial percentage of neuroblastoma primary tumors and cell lines. There is now a large body of evidence suggesting that one or more TSGs involved in neuroblastoma initiation and/or progression is localized to this region. A strong correlation between 1p36 deletion, *MYCN* amplification, and advanced stage disease indicates that this locus is an important contributor to neuroblastoma biology. Further characterization of the normal and neuroblastoma attributes of the genes within the SRD will assist in determining the gene(s) responsible for these biological effects.

Materials and methods

Biological samples

Paired tumor and constitutional samples were obtained from the Pediatric Oncology Group, the Children's Cancer Group, and the Children's Oncology Group. Cell lines were obtained from a variety of sources as previously described (Thompson *et al.*, 2001). Both constitutional deletion patients have been described previously (Biegel *et al.*, 1993; White *et al.*, 1997). Tumor and constitutional DNAs were isolated as described (White *et al.*, 1995). Tissue-specific RNAs were obtained from a commercial source (BD Biosciences; San Jose, CA, USA). The Children's Hospital of Philadelphia Institutional Review Board approved this research.

Genotyping and LOH analysis

Primers for PCR-formatted microsatellite and minisatellite polymorphisms *DIS76*, *DIS243*, *DIS80*, *DIS50*, *DIS468*, *DIS2845*, *DIS2660*, *DIS2795*, *DIS2145*, *DIS2633*, *DIS2870*, *DIS253*, *DIS214*, *DIS1646*, *DIS2694*, *DIS548*, *DIS160*, *DIS503*, *DIS450*, *DIS244*, *DIS2667*, *DIS489*, *DIS434*, *DIS228*, *DIS507*, *DIS436*, *DIS2697*, *DIS170*, *DIS3669*, *DIS2644*, *DIS199*, *DIS3720*, *GGAA30B06*, *DIS1622*, *DIS247*, *DIS164*, *DIS201*, *DIS1596*, *DIS1669*, *DIS1643*, and *DIS481* were obtained from the Genome Data Base and eGenome (Letovsky *et al.*, 1998; White *et al.*, 1999). Assays for the remaining polymorphic markers have been previously described (Dracopoli *et al.*, 1988; Fong *et al.*, 1989; White *et al.*, 1995). Samples were assayed using acrylamide gel electrophoresis and either radiolabeled PCR primers, autoradiography, and densitometric analysis (White *et al.*, 1995), or using fluorescently labeled PCR primers analysed with ABI GeneScan and ABI Genotyper software (Applied Biosystems, Foster City, CA, USA) (Maris *et al.*, 2000). Genotypes from all primary tumor pairs demonstrating LOH which defined tumor breakpoints were generated multiple times and confirmed by densitometric analysis with the threshold for LOH defined by an allelic intensity reduction of > 60% for one allele. Genotype analyses of 46 unique neuroblastoma cell lines, the two constitutional deletion cases, and parental samples for each constitutional case, were also performed by PCR using radiolabeled primers and autoradiographic detection. Results

from subsets of patients and loci used in this study have been reported elsewhere in detail (Fong *et al.*, 1989; White *et al.*, 1995) or preliminarily (White *et al.*, 1997; Hogarty *et al.*, 2000; White *et al.*, 2001).

Genomic mapping, sequencing, and sequence annotations

PCR-formatted genomic markers representing polymorphisms, transcripts, and random STSs previously localized to 1p36 were derived from eGenome (White *et al.*, 1999) and mapped by PCR against a distal 1p-specific radiation hybrid (RH) panel as described (Jensen *et al.*, 1997), or by direct string matching of primer and genomic sequences using me-PCR (Murphy *et al.*, 2004). Screening of large-insert clones and sequencing of PAC inserts, using a subset of the mapped genomic markers, has been described (Bentley *et al.*, 2001). Sequence analysis was performed using a variety of approaches, including use of the *in silico* gene prediction algorithms Genscan (Burge and Karlin, 1997) and Metagene (<http://www.goliath.ifrc.mcw.edu/MetaGene/>), and the sequence homology algorithms BLAST, BLASTP, and BLASTX (Altschul *et al.*, 1990), with the latest round of analyses performed using genome sequence build 34. In addition, gene predictions from the UCSC Genome Browser (Karolchik *et al.*, 2003), the NCBI (Wheeler *et al.*, 2004), and Ensembl (Birney *et al.*, 2004) were assessed, and candidate transcripts were added if substantial evidence from transcript/partial cDNA/EST sequence representation, gene- and/or transcript-level comparative homology, and *ab initio* prediction was present. As transcript prediction is a subjective task, it is possible that we have over- and/or underinterpreted the validity of the 30 genes presented.

Transcript profiling assays

Real-time RT-PCR (TaqMan) assays were generated for the genes *CAMTA1*, *FLJ10737*, *MGC33488*, *PHF13*, *KIAA0469*,

HKR3, *TAS1R1*, *FLJ23323*, *KIAA0720*, *ESPN*, *HES2*, *GPR153*, *MGC40168*, *FLJ32096*, *CHD5*, *NPHP4*, and *SHREW1*. A TaqMan assay for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), a ubiquitously expressed house-keeping gene, was multiplexed along with each target gene to provide an internal control and for quantification. Gene-specific primers and fluorescent probes were obtained from Applied Biosystems. Each amplification reaction contained 1 × Master Mix (Applied Biosystems), 50 ng of cDNA, 5 μM *GAPDH* detection primer, 5 μM target gene detection primer, 10 μM of each *GAPDH* amplification primer, and 10 μM of each target gene amplification primer. Samples were amplified in duplicate in 20 μl reaction volumes in a 96-well format, using conditions of one cycle at 50°C for 2 min; one cycle at 95°C for 10 min; 55 cycles at 95°C for 15 s and then 60°C for 1 min. Standards with known DNA concentrations were included in every run for reaction controls. Assays were performed on an ABI Prism 7700 sequence detection system. Signals were normalized and quantified relative to *GAPDH* with the associated program Sequence Detector v1.7, using the comparative method. Transcript levels for the genes *BACH*, *LOC284509*, *ICMT*, *CHD5*, and *KCNAB2* were assessed with a previously described semi-quantitative RT-PCR method (Eggert *et al.*, 2000). cDNAs for all transcript profiles were generated using a Superscript II RT[®] Kit (Invitrogen, Carlsbad, CA, USA).

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