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# Analysis of relative gene dosage and expression differences of the paralogs *RABL2A* and *RABL2B* by Pyrosequencing

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ABSTRACT

The paralogous genes *RABL2A* (chr2) and *RABL2B* (chr22) emerged by duplication of a single gene in the human–chimpanzee ancestor and share a high degree of sequence similarity. In Phelan–McDermid-Syndrome microdeletions of 22q13 often also affecting*RABL2B* are of clinical importance but their incidence is still unknown. We analyzed a German population (190 individuals) for such aneuploidies and the paralogs' expression in cell lines by RABL2 paralogous sequence quantification. For determination of the genomic and transcriptional ratios of *RABL2A* and *RABL2B* a Pyrosequencing protocol was introduced as a high-throughput method. During PCR the 3′ end of the biotinylated strand is engineered by a backfolding oligonucleotide to hybridize in the Pyrosequencing reaction to an internal site near the sequence to be analyzed. In human samples no deviations of the euploid genomic state could be detected indicating that 22q13 microdeletions involving *RABL2B* are rare. However, despite equal gene dosage a preferential expression of *RABL2B* in human tissues and lymphoblastoid cell lines was detected which is most pronounced in brain and placenta. This renders a complete functional complementation of one paralog by the respective other unlikely and hints to a functional and clinical importance, in particular with respect to the 22q13 chromosomal deletion syndrome. Remarkably and in contrast to human, expression levels of the two paralogs in a chimpanzee cell line are equal. This finding is discussed in view of the relocation of *RABL2A* from its ancestral telomeric to its pericentromeric location in human.


## 1. Introduction

Two closely related human paralogous genes, *RABL2A* and *RABL2B* (RAB-LIKE 2A and 2B), have been identified at chromosomal locations 2q13 and 22q13.3 (Wong et al., 1999). They result from a very recent duplication (Wong et al., 1999; Martin et al., 2002) and share a high degree of sequence identity (>98%). The putatively coded proteins exhibit similarity with the RAS oncogene family (RAB). Although this large protein family of small GTPases attracts considerable interest for their manifold of functions and involvement in disease (Cheng et al., 2005; Wu et al., 2008) the *RABL2* genes and their putative products have not been studied in detail since their original description by Wong et al. (1999). This is the more surprising for *RABL2B* often falls within microdeletions associated with complex phenotypes such as mental retardation, impairment of speech and language development and autistic behavior (chromosome 22q13 deletion syndrome, Phelan–McDermid-Syndrome, OMIM #606232). The incidence of 22q13 microdeletions in the general population is still unknown (Phelan, 2008) mainly due to technical inconvenience of laborious methods such as FISH applied for their detection. A genetic screening for this cryptic subtelomeric chromosome rearrangement is routinely done for patients with mental retardation only. In a large population of 11,688 individuals with developmental inabilities Ravnan et al. (2006) detected 15 patients carrying 22q deletions. Two of them (13%) were de novo deletions underscroring the need for a routine screening independent and ahead of clinical conspicuousness. Although *RABL2B* is often affected in reported deletions showing phenotypic consequences, its contribution to patients' suffering has been questioned. Wong et al. (1999) argued that as both *RABL2A* and *RABL2B* are expressed and very close in sequence it seems to be unlikely that *RABL2B* is dosage sensitive. Also, Anderlid et al. (2002) consider *RABL2A* expression sufficient to compensate for a monosomic *RABL2B* deficiency. However, in their view, the high expression in fetal brain and the identification of a

*Abbreviations:* EBV, Epstein-Barr-Virus; ECACC, European Collection of Cell Cultures; FCS, fetal calf serum; IUPAC, International Union of Pure and Applied Chemistry; SD, standard deviation; TAE buffer, Tris-acetat-EDTA buffer.

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muscle-specific splice isoform could implicate a connection to the cognitive deficits and the muscular hypotonia found in patients with 22q13 deletions. A comparison of the relative expression of *RABL2A* and *RABL2B* in five tissues (Wong et al., 1999) revealed expression of both genes across all tissues tested. However, not all reported differences in expression levels of the two genes could be replicated in that study using paralog discrimination by restriction digest.

Another interesting aspect of the genetics of the *RABL2* paralogs is their evolutionary history. The duplication of the ancestral gene on chromosome 22 took place during hominid evolution only recently. Orangutan (*Pongo pygmaeus*) still represents the original situation with a single *RABL2* gene in its haploid genome (Martin et al., 2002). Thus, the gene duplication must have occurred after divergence of orangutan but before the split of human (*Homo sapiens*) and chimpanzee (*Pan troglodytes*). Chimpanzee retained the ancestral situation with two paralogs in the subtelomeric region of chromosomes 2b and 22. In human, the fusion of ancestral chromosomes 2a and 2b formed chromosome 2, finally placing *RABL2A* to its current pericentromeric position at 2q13. It is unknown whether the different localization of the human and chimpanzee paralogs affect their function and if so, contribute to phenotypic differences among the two primates.

The close relationship of the two paralogs offers the opportunity to use a paralog ratio test to evaluate both relative gene dosage as well as their relative expression. Gene dosage will be informative for unbalanced structural changes affecting the *RABL2B* locus within the subtelomeric region of the long arm of chromosome 22 or the *RABL2A* locus at chromosome 2, respectively. We aimed to develop a convenient high-throughput method for determination of RABL2A/B ratios by means of Pyrosequencing. Pyrosequencing has been widely used for sequencing short stretches of DNA and for the quantitative determination of nucleic acids (Ronaghi et al., 2007). On a genomic level it allows the evaluation of allele dosage/genotypes determined by single nucleotide polymorphisms or structural variations such as copy number variants (Langaee and Ronaghi, 2005; Huse et al., 2008) and it is also used to characterize gene expression (Wittkopp et al., 2008). In Pyrosequencing pyrophosphate is stoichiometrically split off from the deoxynucleoside triphosphates during polymerase reaction and initiates a reaction cascade leading to quantifiable light emission. Recently, Pyrosequencing was exploited for next-generation sequencing, too (Margulies et al., 2005).

For Pyrosequencing, templates must be single-stranded. After PCR amplification this is routinely achieved by avidin/streptavidin-mediated purification of the biotinylated strand by means of a biotin-labeled primer oligonucleotide. Subsequently, a sequencing primer is hybridized close to the sequence to be analyzed. The iterative addition of deoxynucleoside triphosphates then allows sequence detection.

Pyrosequencing is relatively straightforward but is also a laborious methodology. Purification of the single-stranded template and annealing of the sequencing primer are consecutive steps which, because intermittent washing is necessary each time, decelerate the assay. We therefore made an attempt to simplify the procedure of sample preparation by a specially designed backfolding oligonucleotide as PCR primer, thereby including the sequencing primer already during PCR amplification. Such a simplification is especially valuable for high-throughput assays for the evaluation of many samples, typically in biomedical screenings.

The ameliorated Pyrosequencing-based paralog ratio test was applied to the *RABL2* paralogs by exploiting sequence variations in order to determine genomic dosage and relative expression in human and chimpanzee lymphoblastoid cell lines and human tissues.

## 2. Materials and methods

### 2.1. Cell culture

Human EBV transformed lymphoblastoid cell lines GM10847, GM12760, GM12864, GM12870, GM12871, GM15215, GM15324, GM15386, GM18502, GM18552, GM18858, GM18972, GM19140, and GM19204 were obtained from the Coriell Cell Repository (Camden, USA). The chimpanzee cell line EB176(JC) was from ECACC (Salisbury, UK).

Cells were cultured at 37 °C in RPMI 1640 medium (Gibco, Eggenstein, Germany) supplemented with 15% FCS (Gibco) and 2 mM L-glutamine (Gibco) in a 5% $CO_2$ atmosphere at 95% humidity.

### 2.2. Nucleic acids

Genomic DNA was isolated from blood of healthy, unrelated Caucasian volunteers (99 female and 91 male subjects) or from the indicated cell lines using the Blood & Cell Culture DNA Mini Kit (Qiagen, Hilden, Germany) according to standard protocols. Blood was collected at the University Hospital Kiel (Krawczak et al., 2006), and written consent was obtained from each participant. A genomic DNA reference sample of a patient with established 22q13 micro-deletion (22q13del) was obtained from the University of Berlin (patient characteristics as Supplementary material). Pooled genomic DNA originating from human blood of about 100 individuals was purchased from Roche (Mannheim, Germany). The 1 kb Plus DNA Ladder™ was purchased from Invitrogen (Karlsruhe, Germany).

Total RNA was isolated from the cell lines using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. cDNA first strand synthesis was performed with "Sprint RT Complete-Random Hexamer" cDNA synthesis kit (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France) following the manufacturer's recommendations. Five milligram total RNA was used for reverse transcription.

Human tissue cDNAs were from Clontech (MTC Multiple Tissue cDNA Panels).

### 2.3. PCR amplification

Primers were designed using genomic reference sequences from the human (NCBI Build 36.1) and chimpanzee (Build 2 Version 1, Oct. 2005) assemblies to match completely both sequences.

Primer used for the classical Pyrosequencing approach: btRABL2ex89r (biotin-CTCAGCTGTGGGGAGAGG), and RABL2ex89f (CAATTCGATTAGCTGTGTC) for PCR, RABL2ex9fs (CAGCAGCATCGAGACCCCA) as sequencing primer.

Primer (bfOligo) used for the simplified Pyrosequencing approach: RABL2ex89f+s (TGGGGTCTCGATGCTGCAATTCGATTAGCTGTGTC) instead of RABL2ex89f.

Primers were from Metabion (Martinsried, Germany). Amplification was performed in 96-well PCR plates (ABgene, Epsom, UK) containing 25-µl reaction/well using the complete ready-to-use 2× reaction mix BioMix (Bioline, Taunton, USA) according to the manufacturer's recommendation. Template amounts were in the range of 10–20 ng. One hundred pmol of biotinylated primer (btRABL2ex89r) were always used as was RABL2ex89f in the classical Pyrosequencing approach. The simplified protocol was tested with 100–500 pmol of RABL2ex89f+s and 300 pmol was routinely used. Initial denaturation was at 95 °C for 1 min, cycling was done 25 times at 95 °C (30 s)/59 °C (30 s)/72 °C (1 min).

### 2.4. Amplicon verification

Amplicon sizes were characterized by 1% (w/v) agarose gel electrophoresis using Ultra Pure™ agarose (Invitrogen) in TAE buffer. Sequence integrity was checked by BigDye Terminator v3.1 chemistry and 3730xl DNA Analyzer (ABI, Foster City, USA) according to the manufacturer's instructions.

### 2.5. Pyrosequencing

Biotin-labeled PCR products were immobilized on Streptavidin Sepharose™ (Qiagen/Biotage, Uppsala, Sweden) by mixing 20 µl of a

PCR product with 6 µl Streptavidin Sepharose suspension, 10 µl water and 40 µl Binding Buffer (Qiagen/Biotage) followed by shaking at room temperature for 15 min. To remove the unbiotinylated DNA strand, the samples were sequentially washed with 70% ethanol and 0.5 M NaOH using the PyroMark Vacuum Prep Tool (Qiagen/Biotage). Immobilized ssDNA was then washed with 1× Washing Buffer for 10 s, transferred to 40 µl 1× Annealing Buffer plus 8 µl target specific sequencing primer (10 pmol/µl in water), or, for the modified protocol, with 48 µl annealing buffer. Using a thermal cycler, the mixture was equilibrated to 81.5 °C and subsequently cooled down to ambient temperature with 0.2 °C/s. After equilibration to room temperature, the sequencing reaction was performed using the Pyro Gold Reagent Kit (Qiagen/Biotage) in the PSQ 96MA Pyrosequencing™ instrument according to the manufacturer's instructions. Consecutive addition of dNTPs was done by six repetitive cycles of T, A, C, and G.

## 3. Results and discussion

### 3.1. One paralog ratio test suitable for the determination of relative gene dosage and expression of RABL2 paralogs

There are several reasons to determine relative gene dosage and relative expression of the paralogous genes *RABL2A* and *RABL2B*. Although there is accumulated evidence for *SHANK3* being the most important gene in chromosome 22q13 deletion syndrome (Wilson et al., 2003; Durand et al., 2007; Delahaye et al., 2009) it is not yet excluded that also *RABL2B* monosomy contributes to the complex and variable phenotypes observed. Moreover, for *RABL2B* is localized less than 40 kb downstream of *SHANK3* its gene dosage may serve as a surrogate marker for many microdeletions involving both SHANK3 and *RABL2B*. This gene dosage relative to its paralog is easily determined by a paralog ratio test.

It is widely assumed that a *RABL2B* deficiency can be compensated by *RABL2A* expression because the two paralogs are very close in sequence (Wong et al., 1999). However, gene duplicates which are not pseudogenized may be required to simply increase the gene dosage or must have adopted distinct functions (Lynch and Conery, 2000; Conrad and Antonarakis, 2007). Acquisition of new functions or subfunctionalization would separate *RABL2A* and *RABL2B* and would render a reciprocal functional compensation less likely. To date, this has not been systematically studied. The expression profiles of the two paralogs might provide a first hint for definite biological roles of the individual gene products. Wong et al. already demonstrated widespread expression in different human tissues for both paralogs and provided a first indication for their differential expression (Wong et al., 1999).

Accordingly, we developed a RABL2B/A ratio test to screen for RABL2B gene dosage as indicator for 22q13 microdeletions and for the paralogs' transcription ratio as well as to extend the expression analysis in human and to compare it to that of the chimpanzee paralogs. As a quantitative method we have chosen a Pyrosequencing-based approach.

Pyrosequencing has been widely used to quantify nucleic acids by comparing nucleotide differences with the advantage of neighboring sequence validation in parallel. Such exploitable sequence variations are allele-discriminating single nucleotide polymorphisms (SNP) or paralog-discriminating sequence variations (PSV). For instance, Deutsch et al. (2004) have used Pyrosequencing for a Paralog Ratio Test (PRT) to detect aneuploidies by paralogous sequence quantification (a similar problem as assessed here) and Huse et al. (2008) to determine copy numbers of the ß-defensin locus at 8p23. The analytical power of Pyrosequencing analyses has also been shown with the quantification of SNPs across a wide range of allele frequencies in pooled DNA samples (Gruber et al., 2002). Therefore, Pyrosequencing should be suitable for the PSV-based determination of *RABL2A*/*RABL2B* ratios on both the genomic level to detect aneuploidies as well as on the transcript level to characterize the relative expression of both genes in human and chimpanzee.

There are several prerequisites for a PRT that works on genomic DNA as well as cDNA for the two species: (i) primers hybridize to identical sequences in both paralogs of human and chimpanzee, (ii) allow amplification of genomic as well as cDNA, and (iii) each amplicon must harbor a PSV in both species. Note that the paralogs' differences are species-specific. The homologous genomic loci of *RABL2A* and *RABL2B* which span about 16 kb contain a sufficient number of nucleotide differences but most of these are located in introns. For our assay only PSVs in exons were considered. Within the nine exons per gene just 17 and 25 single nucleotide variations are annotated as polymorphisms (SNP) in human and chimpanzee SNP databases, respectively. To assure that the assay works with genomic DNA and cDNA, the annealing sites for the PCR and sequencing primers have to be fully exonic. Furthermore, primer positions on cDNA are critical with respect to alternative splicing (e.g. cassette exons should be avoided). Inspection of the more than 40 *RABL2* transcript sequences (mRNAs and ESTs) deposited in the NCBI databases indicates multiple alternative splicing events for human *RABL2A* and *RABL2B*. However, exons 8 and 9 were present in all of these transcripts suggestive of constitutive joining of both exons. As a control, we performed an RT-PCR by using primers located in exon 1 and exon 9 of the human paralogs and confirmed by cloning and sequencing the inclusion of the entire exon 8 in front of exon 9 in a multitude of splice isoforms in all inspected tissues and in the chimpanzee cell line (Supplementary information). Besides transcripts exhibiting yet unknown alternative last exons, all transcripts should therefore be covered by primers having their hybridization sites in exons 8 and 9. Because these two exons are spaced apart by a small intron (IVS8) of only 166 nt, such primers also allow amplification of genomic DNA. PSVs discriminating *RABL2A* and *RABL2B* in human are located at position +80 (dbSNP rs2519478) and in chimpanzee at +77 in exon 9. These PSVs are suited for the PRT because they are flanked immediately downstream by 160 nt identical sequence and upstream by exon 8 which is identical in all four paralogs. Fig. 1 depicts the principle of our PRT to determine the relative gene dosage as well as relative expression of *RABL2A* and *RABL2B*. In case of euploidy, a balanced ratio of the two discriminating nucleotides in genomic DNA is indicative for the equal ratio of both genes. Deletion of one *RABL2B* allele would be detectable by a ratio $RABL2B/A = 0.5$. In contrast to genomic ratios, transcript ratios are unpredictable but may cover a wide range.

### 3.2. Modified Pyrosequencing protocol for high-throughput screening

For a simplified high-throughput test, we decided to use a chimerical oligonucleotide serving as PCR as well as sequencing primer. This will save the addition of the sequencing primer from the standard protocol, thereby reducing costs and efforts. This is achieved by attaching the sequencing primer in reverse complementary orientation to the 5′ end of the unbiotinylated PCR primer (Fig. 2). In the resulting amplicon, the 3′ end of the biotinylated strand anneals to the internal site and can be elongated during the sequencing steps. In addition, the 3′ end of the template, if used directly for sequencing, will not be accessible for side reactions. This is often a problem in Pyrosequencing, especially if longer templates are used (Utting et al., 2004). However, concern arises to elongate the unbiotinylated primer by a substantial number of nucleotides matching the target at an internal site. In general, overhang primers are routinely used in specialized PCR protocols, for instance to incorporate restriction sites at the ends of amplicons. This allows restriction digests of PCR products in order to ease downstream cloning steps. However, in our approach this overhang is designed to backfold and hybridizes to an internal site of the amplicon and could be elongated unintentionally during PCR. We therefore tested different ratios of the biotinylated vs. the bfOligo (Fig. 3). A surplus of biotinylated primer

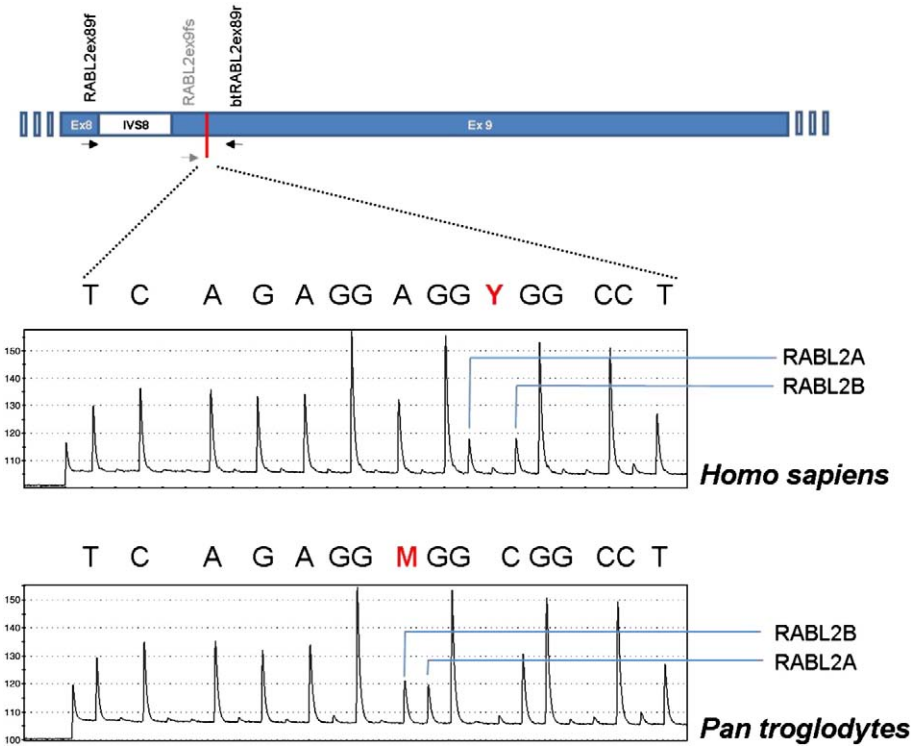


**Fig. 1.** Pyrosequencing strategy to discriminate *RABL2A* and *RABL2B*. At the top, the genomic structure around the PSVs in exon 9 (red line) is given with the respective relative primer positions. Pyrograms obtained with the standard protocol for human and chimpanzee DNA is presented underneath. Red letters in the sequence readouts indicate the informative PSVs.

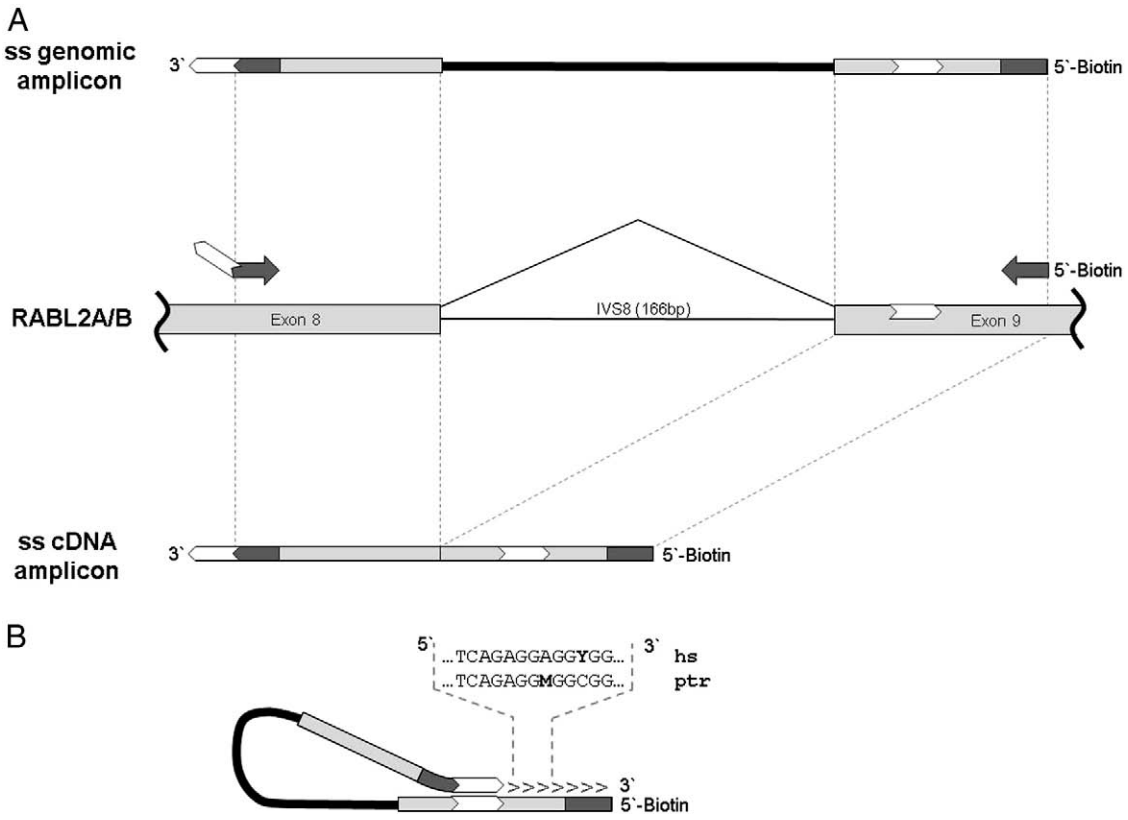


**Fig. 2.** Principle of the simplified Pyrosequencing protocol. (A) The genomic structure of *RABL2A* and *RABL2B* (RABL2A/B, center) and the resulting single-stranded genomic (above) and cDNA (below) Pyrosequencing templates are shown. Boxes indicate exon fragments and full lines intron 8 and the respective splice event. Dotted lines give the respective relations of exon–intron, intron–exon and exon–exon borders in genomic and cDNA. Primers are shown as black arrows; those sequences of the chimeric primer and the internal sequence which are complementary and allow self-priming of the single-stranded (ss) amplicons as white arrows. (B) Self-priming is sketched for the genomic template. The sequences analyzed are given for human (hs) and chimpanzee (ptr) with the paralogs discriminating nucleotides in bold letters (IUPAC code, Y: C/T, M: A/C).

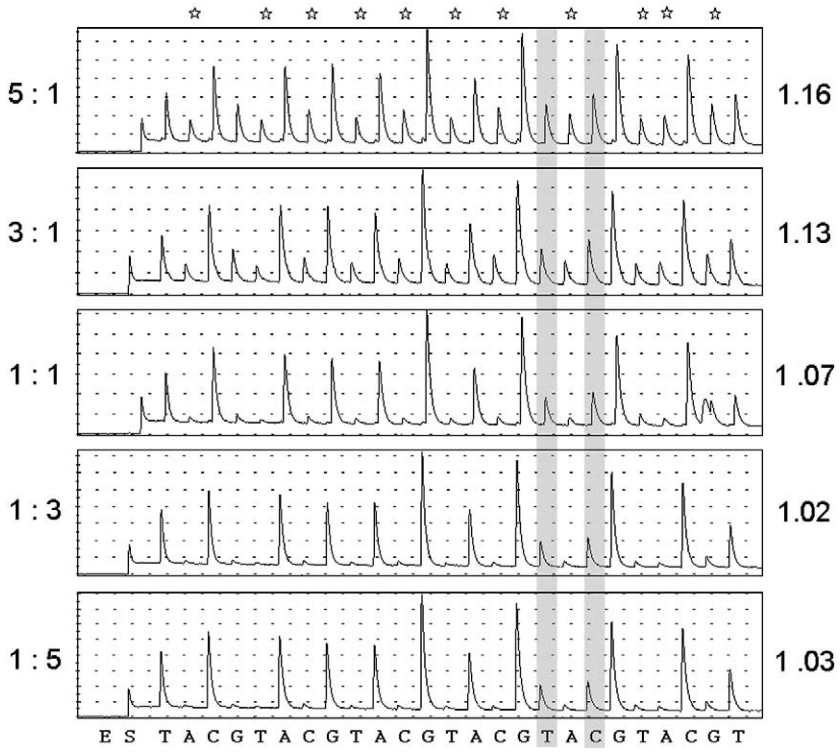


**Fig. 3.** PCR primer ratios influence the Pyrosequencing outcome. Pyrograms obtained with genomic DNA of cell line GM12864 when different primer ratios (left, biotinylated: bfOligo) are used during PCR are shown. High ratios (5:1 and 3:1) cause ghost peaks which disappear at low ratios (1:3 and 1:5). Positions of background signals are marked by stars. The positions of informative nucleotides discriminating the paralogs are shaded and the calculated *RABL2B*/*RABL2A* ratios are listed at the right of the respective Pyrogram.

causes the appearance of ghost peaks in the pyrograms. Under these conditions of an asymmetric PCR the strand carrying the bfOligo sequence at its 3′ end is preferentially amplified. Due to the shortage of the bfOligo its 3′ end cannot be effectively blocked by primer hybridization and free 3′ ends will tend to backfold and elongate to different sizes. In contrast, the ghost peaks of Pyrosequencing disappear when the relative amount of the bfOligo is increased in the PCR. Due to its higher concentration and longer size the bfOligo out-competes the internal hybridization site and allows correct PSV quantification. Therefore, all subsequent analyses used a three-fold surplus of the bfOligo. The reliability of this assay was tested with 12-fold complete replicates (PCR as well as Pyrosequencing) using genomic DNA of cell lines GM12864 (human) and EB176 (chimpanzee). Mean *RABL2B*/*A* ratios were determined at 1.01 (SD 0.027) and 0.98 (SD 0.049) which is in good agreement with the results of the classical PRT approach of 1.01 (0.024) and 0.99 (0.046), respectively. The genomic ratio of the patient carrying a 22q13 deletion was determined with 4-fold replicates at 0.62 (SD 0.02). Although clearly indicating a *RABL2B* deficiency, the value is different from the expected ratio of 0.5. Whether this reflects a mosaic 22q13 deletion known to occur (Bonaglia et al., 2009) remains to be elucidated. Data are summarized in Table 1.

### 3.3. Monosomy of RABL2B is not common in a German population

Next we analyzed a commercially available genomic DNA pool and 190 genomic DNAs from blood of German individuals as well as genomic DNAs obtained from EBV transformed lymphoblastoid cell lines (10 human, one chimpanzee; Table 1). For the genomic pool the mean *RABL2B*/*A* ratio was determined at 1.03 (SD 0.023, 15 replicates) indicating a low incidence of numerical abnormalities of the paralogs. In the 190 individual human samples the RABL2A/B ratios are also balanced suggestive of a low frequency of 22q13 microdeletions involving *RABL2B* in the German population. Furthermore, although *RABL2B* is localized in a subtelomeric region known for an exceptional level of instability, a common *RABL2B* copy number variation is unlikely and as expected in the view of the observed low frequency of 0.13% in patients with mental retardation (Ravnan et al., 2006).

**Table 1**
Genomic and transcript ratios of *RABL2A* and *RABL2B* determined by Pyrosequencing-based rs2519478 quantification.

| Genomic DNA | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Human blood[a] | | | | 22q13del[b] | | Human LCLs[c] | | Human LCL GM18858[d] | Chimpanzee LCL[d] |
| *RABL2B*/*A* | 1.03 (0.92–1.14) | | | | 0.62 ± 0.02 | | 0.98–1.06 | | 3.01 ± 0.03 | 0.98 ± 0.05 |
| cDNA[c] | | | | | | | | | | |
| | Brain | Liver | Placenta | Kidney | Heart | Prostate | Leucocytes | Human LCLs[c] | Human LCL GM18858 | Chimpanzee LCL |
| *RABL2B*/*A* | 3.9 ± 0.3 | 3.1 ± 0.1 | 4.2 ± 0.4 | 3.3 ± 0.1 | 2.8 ± 0.1 | 2.5 ± 0.2 | 2.6 ± 0.5 | 1.8–3.0 | 3.8 ± 0.3 | 1.0 ± 0.2 |

[a] Mean (and range) from single determinations of 190 human individuals.
[b] Means ± SD from 4 determinations.
[c] LCL: EBV transformed lymphoblastoid cell lines, range of the means of 13 LCLs from two determinations, GM18858 not included.
[d] Means ± SD from 12 determinations.

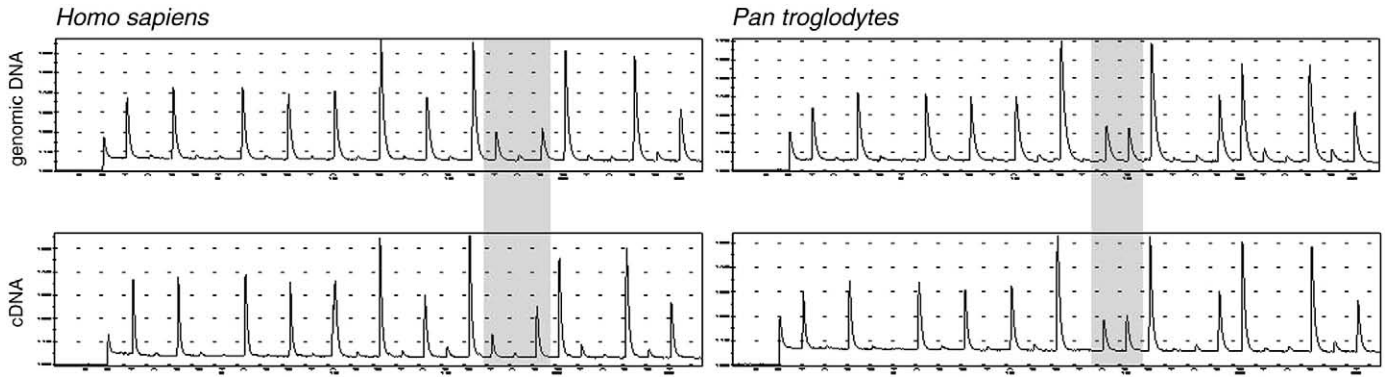


**Fig. 4.** Expression of *RABL2B* is higher than that of *RABL2A* in human lymphoblastoid cells but not in a corresponding chimpanzee cell line. Cell line GM12864 derived from a male Caucasian (left) is compared to cell line EB176 derived from a male chimpanzee (right). Although both genomes have *RABL2A* and *RABL2B* at equal gene dosages only in the chimpanzee their expression is also equal.

In the cell lines analyzed all but one sample showed ratios around 1 (0.98–1.06). The exception was cell line GM18858, derived from a Yoruba individual, showing a skewed *RABL2B* to *A* ratio of 3:1. This is inconsistent with a simple deletion event of *RABL2A* or a gain of an additional *RABL2B* copy because those would result in 2:1 or 3:2 ratios, respectively. To elucidate this finding we amplified and sequenced a fragment of exon 9 containing this variation together with a second PSV (rs73433419) downstream of rs2519478. In contrast to the rs2519478 ratio of 3:1 the rs73433419 alleles were found at a 1:1 ratio. The most parsimonious explanations of this observation are that: (i) the rs73433419 ratio of 1:1 is consistent with a normal biallelic state of both *RABL2A* and *RABL2B* in GM18858; (ii) in this cell line rs2519478 is not a PSV but has to be considered as a heterozygous *RABL2A* SNP with an ancient homozygous *RABL2B* allele, thus resulting in a ratio of B/A = 3:1; and (iii) in contrast to the African population, in the Caucasian population the novel *RABL2A* allele T is fixed or the ancient allele C is extremely rare. Wong et al. (1999) list this variation as one of only three nucleotide differences causing an exchange in the predicted amino acid sequences of RABL2A (valine) and RABL2B (alanine). Now, our finding sets the two paralogs' sequences even closer to each other. Unfortunately, this result limits the application of rs2519478 in the screening for microdeletions in the *RABL2B* locus to populations like Caucasians where it represents most likely a true PSV. Nevertheless, this and the result of the patient with 22q13 deletion show that our Pyrosequencing method is sensitive enough to discriminate between subtle gene or allele dosage differences.

### *3.4. Expression levels of RABL2A and RABL2B differ in human but not in chimpanzee*

Finally we tested the paralogs' relative expression (Table 1 and Fig. 4). In the chimpanzee cell line expression of *RABL2A* and *RABL2B* is equal. Accordingly, RT-PCR derived Pyrograms are indistinguishable from those obtained with genomic DNA. In human tissues and human cell lines, however, the two paralogs are differentially expressed with *RABL2B* being preferred in all cases. Expression ratios in GM18858 must be considered separately because its genomic situation with the C-allele of rs2519478 does not derive exclusively from *RABL2B*.

Interestingly, expression of *RABL2B* in the brain and placenta is about four times higher than that of *RABL2A*. Therefore, in case of *RABL2B* monosomy, a substantial loss of transcript levels can be expected having functional consequences especially in tissues like brain and placenta.

The different expression patterns in human and chimpanzee are especially noteworthy. It is one of the challenges of evolutionary neurogenomics to describe those human lineage specific changes which shaped the unique capabilities of the human brain. As King and Wilson pointed out more than 30 years ago (King and Wilson, 1975), the macromolecules of humans and chimpanzee are so similar that regulatory mutations may account for biological differences. Expression differences as described here may constitute such a regulatory effect. In both species, the paralogs share a high degree of homology as is the case for their available promoter sequences. It might be speculated that it is not sequence disparity but the translocation of *RABL2A* in the human lineage from the ancestral telomeric to a pericentromeric position which is responsible for the differential expression in the human lineage.

## 4. Conclusion

Genomic ratios of *RABL2A* and *RABL2B* were analyzed in 190 German blood samples using a novel Pyrosequencing assay. Identical gene dosage of both paralogous genes in all samples indicates euploidy and a low incidence of 22q13 microdeletions. The functional complementation of both paralogs proposed earlier is questioned by the finding of significant higher *RABL2B* expression, notably in brain. Chimpanzee shows equal expression of its paralogs, and the differential expression also suggests a possible contribution of *RABL2B* deletions in neurological complications in telomeric 22q13 monosomy syndrome.

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