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Truncating loss-of-function mutations of *DISP1* contribute to holoprosencephaly-like microform features in humans

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Abstract

Defective function of the Sonic Hedgehog (SHH) signaling pathway is the most frequent alteration underlying holoprosencephaly (HPE) or its various clinical microforms. We performed an extensive mutational analysis of the entire human *DISP1* gene, required for secretion of all hedgehog ligand (s) and which maps to the HPE 10 locus of human chromosome 1q41, as a HPE candidate gene. Here, we describe two independent families with truncating mutations in human *DISP1* that resemble the cardinal craniofacial and neuro-developmental features of a recently described microdeletion syndrome that includes this gene; therefore, we suggest that *DISP1* function contributes substantially to both of these signs in humans. While these clinical features are consistent with common HPE microforms, especially those linked to defective signaling by Sonic Hedgehog, we have insufficient evidence so far that functionally abnormal *DISP1* alleles will commonly contribute to the more severe features of typical HPE.

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Introduction

Holoprosencephaly (HPE) is the most common malformation of the developing brain and face, and its severity is often highly correlated with accompanying craniofacial findings (Muenke and Beachy 2000; Matsunaga and Shiota 1997; Cohen 2006; Ming and Muenke 2002). The latter are often indicative of abnormal midline development manifesting as hypotelorism, hypertelorism, midface hypoplasia, nasal anomalies, midline facial clefting and often extending towards microforms such as simple microcephaly with developmental delay, or solitary single maxillary incisor. While the etiology of HPE is highly heterogeneous and complex (Muenke and Beachy 2000; Cohen 2006), presumably including numerous genes and environmental factors (Ming and Muenke 2002; Krauss 2007), many of the key characteristics of both brain and facial anomalies can be experimentally demonstrated to result from defective function of the hedgehog-signaling pathway in these different structures (Cordero et al. 2004). Previous studies of HPE candidate genes had suggested that family-specific deleterious mutations of HPE genes could be a reliable indicator of the potential role of a gene in the overall pathogenetic process. All mutations thus far identified in components of the human HEDGEHOG pathway have been heterozygous variations in key pathway components including, most commonly, the SHH ligand itself (Roessler et al. 1996; Nanni et al. 1999; Traiffort et al. 2004; Maity et al. 2005), and less frequently the receptor PATCHED (Ming et al. 2002) and the transcription factor GLI2 (Roessler et al. 2003, 2005). Microdeletions have also been reported in HPE genes (Bendavid et al. 2005a, b) and affecting candidate HPE loci, including large microscopically visible deletions of chromosome 1q41q42 involving *DISP1* (Shaffer et al. 2007).Therefore, we considered the human *DISP1* [originally called *DISPA* based on its orthologous relationship to murine *DispA* (Ma et al. 2002)] gene as a strong candidate gene based on its location within the HPE 10 locus and its demonstrated requirement as a positive factor required for the efficient secretion of cholesterol-modified Shh from midline signaling structures. (Burke et al. 1999; Ma et al. 2002; Kawakami et al. 2002; Caspary et al. 2002; Nakano et al. 2004; Tian et al. 2004).

Subjects and methods

Study population

Our study consists of all available 463 unrelated cases from our HPE collection (that span the entire clinical spectrum of severity), 57 additional coded anonymous HPE samples from Rennes, France, as well as 95 unrelated individual normal controls (obtained as anonymous samples from the Coriell Institute for Medical Research that controlled primarily for common variants among the Caucasians of Northern European ancestry typical of the largest segment of our collection). All samples were investigated under an Institutional Review Board approved research protocol in accordance with the National Human Genome Institute, NIH guidelines. Recruitment of affected individuals over the past 17 years has been as inclusive as possible with an increasing fraction of diverse ethnicities (see Sect. “Discussion”).

The procedures for PCR amplification, mutation screening and DNA sequencing

The genomic organization of the human *DISP1* gene was characterized by using nucleotide homology searches with the murine DispA gene (Ma et al. 2002) in public databases (BLASTN program: http://www.ncbi.nlm.nih.gov/blast/) and comparing this with the UCSC Bioinformatics Site gene annotation (http://genome.ucsc.edu/). Oligo™4.1 was used to design primers for the seven coding exons as described in Table 2. Amplification of human genomic DNA was performed in a 30-μl reaction volume, using 60–100 ng DNA template, 50 μM each of deoxynucleotide triphosphate, 0.25 μM of each primer, 3 μl of 10× PCR Amplification buffer (Invitrogen), 1.5 μl 10× Enhancer buffer (Invitrogen) and 0.3 μl Taq polymerase. All reactions

were performed using a PTC-255 thermocycler (MJ Research, MA). Typical PCR cycling parameters were 95°C for 4 min followed by 30 cycles at 95°C, annealing at 62°C, extension at 72°C for 1 min, and a final extension step of 72°C for 5 min. One half of the PCR product was used for denaturing high-pressure liquid chromatography (dHPLC) analysis (WAVE™, Trangenomic, CA) and the remainder was retained for direct DNA sequencing. Amplicons displaying heterozygous profiles were purified using a high pure PCR purification kit (Roche, IN) and bi-directionally sequenced using the BigDye™version 3.1 terminator cycle sequencing kit according to the manufacturer's protocol (Applied Biosystems, CA) on an ABI 3100 automated sequencer.

Functional analysis of human *DISP1* alterations

The export of a Renilla luciferase-tagged Shh molecular probe in a transfected *Drosophila* S2R + cell line is dependent on the transporter activity of co-transfected murine DispA (or synthetic versions designed to mimic mutations in the human *DISP1* gene). Note that murine or human A genes are equivalent to the 1 genes, and are the only forms that are co-expressed with hedgehog genes in tissues producing the ligand. The murine or human B genes are indicative of the Disp2 orthologs; there is currently no known function for these $B = 2$ genes in vertebrate development. An export ratio index was measured essential as previously described (Ma et al. 2002), such that a ratio of 1 indicates that independent of the presence (or absence) of transfected Disp protein there is no effect on the measured export of the renilla-tagged Shh ligand; however, a positive ratio indicates that the export is enhanced by Disp bioactivity. Briefly, the S2R+ cells were maintained in Schneiders *Drosophila* medium supplemented with 10% fetal bovine serum and co-transfected with a modified Dispatched gene to be tested and a Shh-tagged reporter by the calcium phosphate technique. Three days after transfection the cells were collected by centrifugation and lysed directly in Passive Lysis Buffer (Promega Dual Luciferase, Promega). Conditioned media was further cleared by centrifugation at 21,000×*g* prior to analysis. Each Disp construct (murine or human) contained an N-terminal firefly luciferase tag to measure successful co-transfection (quantified on the *X*-axis as luciferase activity). Note that the ratio rapidly reaches a plateau value of approximately fourfold. A relative export efficiency index ratio was calculated using the following formula:

$$\frac{\{\text{Renilla luciferase activity in conditioned medium}/\text{activity in cell lysate}\}+\text{Disp}}{\{\text{Renilla luciferase activity in conditioned medium}/\text{activity in cell lysate}\}-\text{Disp}}$$

Results

Some *DISP1* genetic variants are found only among HPE patients

The entire coding region of the human *DISP1* gene was examined for genetic variants that might contribute directly towards defective signaling or potentially modify the effects of other factors influencing signaling strength. As illustrated in Table 1, we identified 12 alterations that were provisionally unique (i.e., not identified in healthy controls of Northern European ancestry or previously identified as a common variant in public databases) to the HPE patient group and served as candidate variants for more detailed analysis. Note that several of these variants were identified among self-identified Hispanic probands for whom ethnically matched samples were not studied. Since five of these changes occur in regions of the protein that are not conserved between the murine and human genes and the *Drosophila* disp (Burke et al. 1999) orthologs we focused our study on the remaining seven changes as suitable for functional analysis independent of the ethnicity of the subjects. Variants present equally in affected individuals and controls were not considered for detailed functional analysis (Fig. 1).

Despite being apparently patient-specific, most novel variants are benign

As shown in Fig. 2a, the export of a Renilla-tagged Shh biomarker into the culture medium is an effective method to estimate the function of Disp alleles (Ma et al. 2002). Unfortunately, when we tested the ability of the human *DISP1* gene to function in these cells we could not detect any enhancement of marker export. Presumably, the human gene product lacks some unknown feature that allows it to function efficiently in *Drosophila* cells. However, five of the mis-sense changes could readily be incorporated into the murine *Disp1* gene for testing. None of these alterations clearly diminished the ability of the murine gene to act normally and are likely to represent benign variants. These cases may well prove to be variants typical of ethnic groups not commonly studied in our control group or publicly funded databases. Alternatively, these may affect the function of the gene in ways not successfully measured in our system.

Loss-of-function is associated with the truncation alleles

As shown in Fig. 2b, both truncation alleles eliminate the effective transporter activity when introduced into the murine *Disp1* test construct, i.e., have an export ratio of less than unity. We conclude that individuals with similar truncations in the human gene would consequently have only a single functional copy of the *DISP1* gene.

In the first family, a clinically un-affected mother has transmitted the W475X allele to her daughter who has a history of seizures, developmental delay including speech, a midline cleft-lip/palate and mild decortication. She was evaluated in the genetics clinic and diagnosed as HPE sequence with a normal head CT scan. Neither the incomplete penetrance nor the apparent absence of brain findings is inconsistent with microforms of the HPE spectrum (Muenke and Beachy 2000; Cohen 2006; Ming and Muenke 2002). Molecular evaluations included normal chromosome analysis, no apparent uniparental disomy for D2S44 (HPE2/SIX3) or deletion of D7S22 (HPE3/SHH), and no known coding region mutations in SHH, SIX3, TGIF, ZIC2, PATCHED, SMOOTHENED, DKK1, SIL or *GLI2* genes.

The second family is quite similar with the presence of a clinically normal mother transmitting the Y734X mutation to her daughter who is of normal intelligence, has a normal karyotype, normal MRI, and normal molecular studies (vide supra). The proband has clear signs of microform HPE with a repaired bilateral cleft-lip/palate, hypotelorism, upslanting palpebral fissures and a solitary maxillary central incisor. All of these findings are typical of the mild end of the HPE spectrum and consistent with diminished signaling by Sonic Hedgehog as the likely mechanism.

Discussion

Our families with loss-of-function truncating mutations in human *DISP1* are highly reminiscent of the newly described deletion syndrome that encompasses this gene (Shaffer et al. 2007). In this study seven different de novo cases of deletion of chromosome 1q41q42 that were identified and confirmed by comparative genomic hybridization were compared (range 2.72–9.07 Mb with the smallest region of deletion overlap estimated to be ~1.17 Mb and encode 4 additional known genes, other than *DISP1*). Common features included significant developmental delay, seizures, craniofacial dysmorphisms, microcephaly, cleft palate, clubfeet, and short stature. Interestingly, the authors noted that none of their cases were diagnosed with HPE. At the extreme end of the clinical spectrum of this series of cases were two who carried the diagnosis of Fryns syndrome.

Our two independent families underscore that the craniofacial and neuro-developmental features resulting from a single functional copy of DISP1 are clearly independent of the brain anomalies associated with more typical HPE. Interestingly, although at the present time the

number of described cases are probably too small to generalize, we find it intriguing that only one of the five families with human *GLI2* haploinsufficiency have overt brain findings of HPE either. Thus, there are actually numerous instances known of a single functional gene in an essential component in the Sonic Hedgehog pathway yet only a mild phenotype detected.

We note a trend in studies of model organisms that often the best characterized phenotype(s) reflect the extremes with complete absence of Disp1 activity and are also unfortunately embryonic lethals. However, it is intriguing that when extrapolated from an initial and admittedly limited set of experiments in the mouse (Tian et al. 2008), it can be inferred that hypomorphic alleles of Disp can be generated and that craniofacial abnormalities manifest themselves before frank central nervous system (i.e., cyclopia) abnormalities as the levels of Disp1 function are progressively decreased. Therefore, we think that our cases of DISP1 truncations reflect the fact that the activity of the Sonic Hedgehog pathway is significantly impaired, but by no means eliminated. A model of HPE that depends on the concerted action of multiple genetic and/or environmental insults (Ming and Muenke 2002) (i.e., the “multiple hit hypothesis”) could provide the best framework for a more comprehensive understanding of the complex interplay among HPE predisposing factors.

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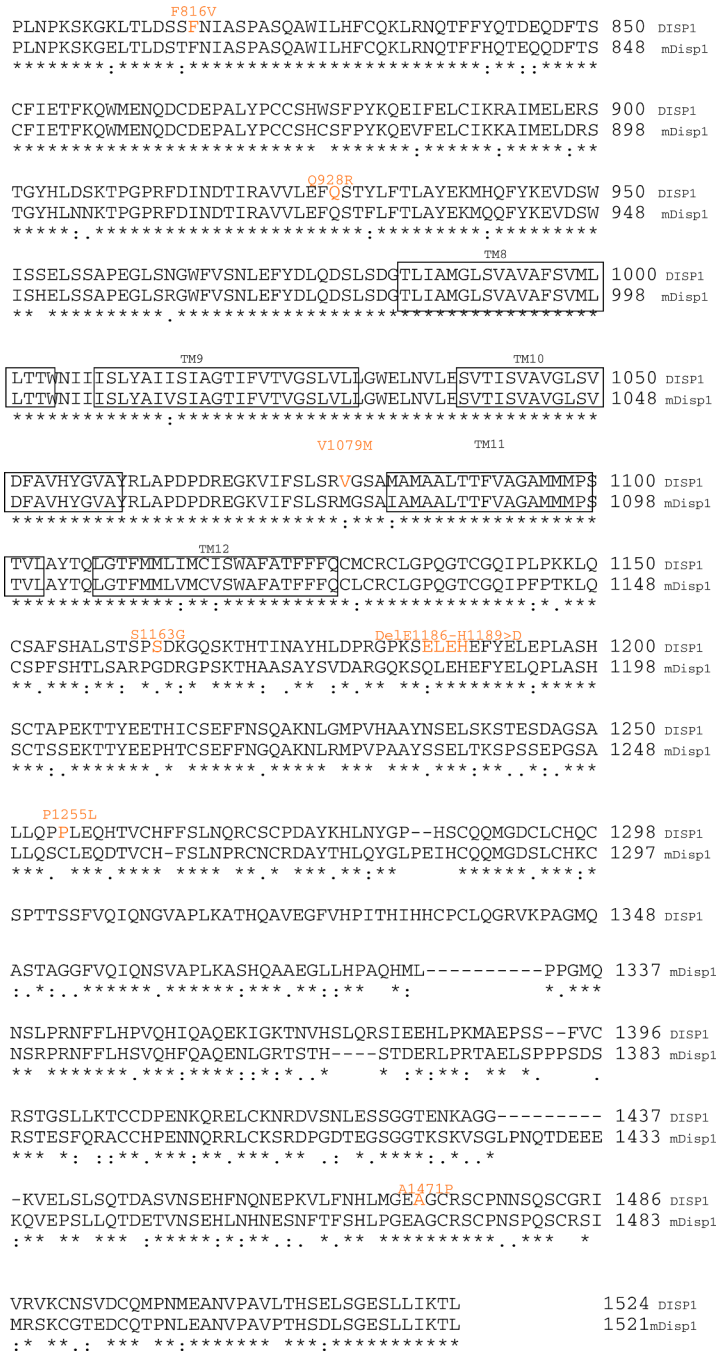
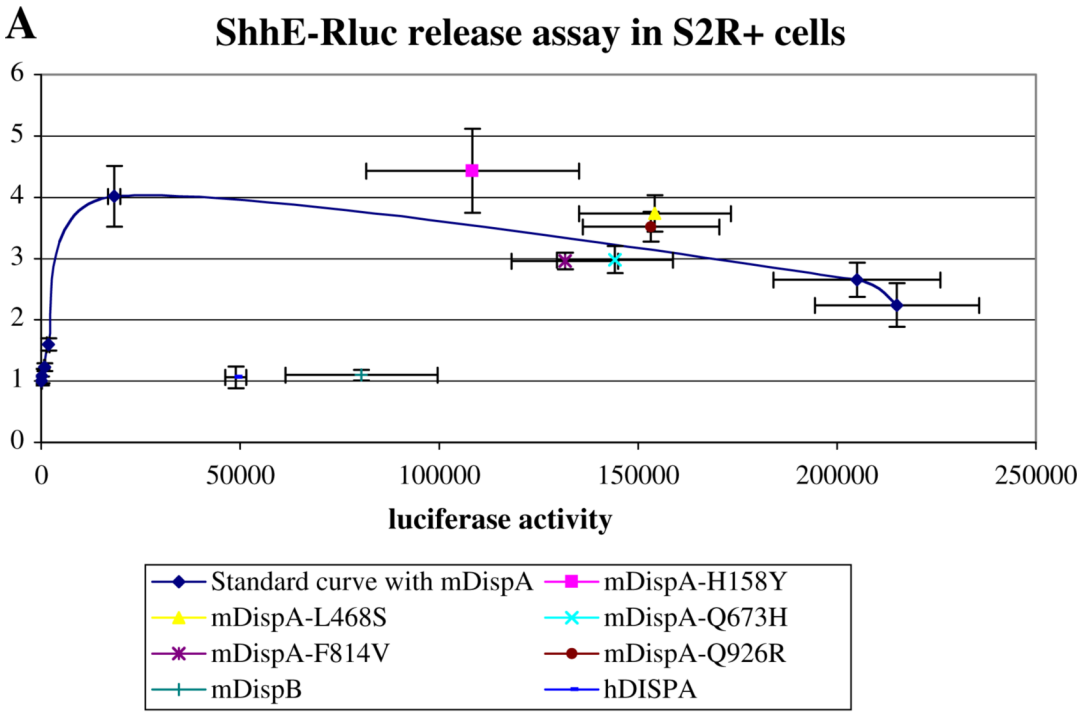


Fig. 1.

Protein alignment of human DISP1 and murine Disp1 proteins based on the Clustal W algorithm (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Both of these vertebrate *Disp* are orthologs of *Drosophila disp* (not shown) are predicted to share a common topology with a core 12 membrane-spanning structure (each transmembrane element is *boxed* to indicate the membrane embedded amino acids) including the segment between *boxed* transmembrane regions (TM) 2–5 comprising the sterol-sensing domain. Key differences between genetic variants of the human and murine proteins are highlighted in *orange* (mis-sense or in-frame deletion) or *red* (truncating mutations)



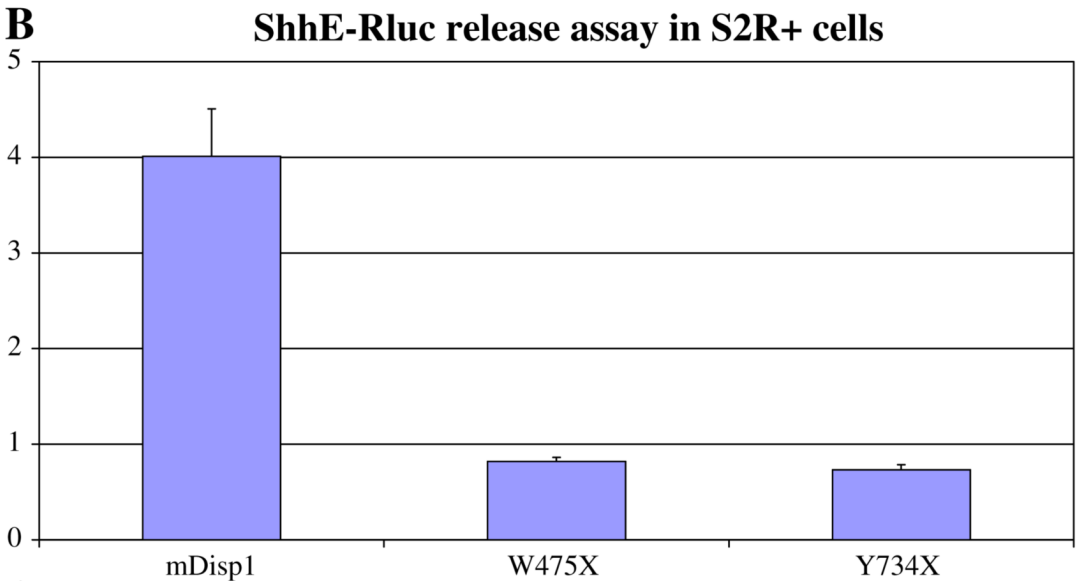


Fig. 2.

Determination of relative functional activity of genetic variants by assessment of the transporter function of Disp on a renilla-tagged Shh reporter in *Drosophila* S2R+ cells. **a** The standard dose–response curve with transfected murine DispA documents that increasing amounts of murine DispA (determined by measurement of the firefly luciferase activity on the *X*-axis) results in a maximum stimulation of ShhE-Rluc export of fourfold. In contrast, the murine DispB or the human DISPA fail to enhance ShhE-Rluc reporter export under these conditions (in spite of adequate expression of the N-firefly luciferase-tagged proteins). Five of the mis-sense changes incorporated within the murine DispA backbone cDNA could be studied; however, none of these variants appreciably affected the transporter-like function of the Disp

test protein. **b** Normally, a bioactive murine Disp1 construct will enhance the export of the tagged Shh ligand by 4- to 10-fold (Ma et al. 2002). Both truncation mutations (murine construct versions mimicking W475X and Y734X) abrogate the ability of the Disp1 test molecule to influence ShhE-Rluc export. Values reported are the maximal release values obtained at a fixed input of Disp1 construct used in all three examples

Table 1

Genetic variation at the human *DISP1* locus

Provisionally unique	Coding position	Suitable for testing	Comment
Yes (Hispanic Caucasian)	A19V	No	Not conserved or present in Disp
Yes (Hispanic Caucasian)	H159Y	Yes	N-terminal intracellular domain
Yes (Caucasian, USA)	L469S	Yes	N-terminal intracellular domain
Yes (Caucasian, Italy)	W475X	Yes	Termination between loops 1–2
Yes (Hispanic Caucasian, Mexico)	Q674H	Yes	Intracellular between loops 6–7
Yes (Hispanic Caucasian)	Y734X	Yes	Termination within transmembrane 7
Yes (Caucasian, USA)	F816V	Yes	Loop 7–8
Yes (Caucasian, USA)	Q928R	Yes	Loop 7–8
Yes (Caucasian, USA)	V1079M	No	Methionine normally present in murine Disp
Yes (Hispanic Caucasian)	Del(ELEH)>D	No	Not conserved
Yes (Caucasian, USA)	P1255L	No	Not conserved or present in Disp
Yes (Hispanic Caucasian)	A1471P	No	Not conserved or present in Disp
No	P25P	No	Common SNP including controls
No	E103N	No	Rs2789975
No	V514V	No	Common SNP including controls
No	K945K	No	Rs2609355
No	A1247T	No	Rs9441940
No	P1274P	No	Rs9441941
No	V1261M	No	Common SNP including controls
No	A637A	No	Synonymous change
Yes	D847D	No	Synonymous change
No	S1163G	No	Glycine the normal position in murine Disp
Yes	A182A	No	Synonymous change
No	IVS2-3T>G	No	Common SNP
No	IVS4+84T>C	No	Common SNP
No	IVS5-14T> C	No	rs2609359

Table 2

Primers for human *DISP1* mutational analysis

Exon	Primer	T(°C) PCR	Size (bp)	Tm(°C) dHPLC
1	5′ TCTTACTTAGAGTCAAGAAATTGG 3 5′ TGAATGCTAAAAGCAAAACTTTCG 3′	53	509	60.5
2	5′ ATGTTATGATGTTTATGATGCTCTG 3′ 5′ GAATTCCTCAAGCAGCCAACTCATG 3′	50	250	54
3	5′ AGTTATGCAGCTCTGATAGCCGAC 3′ 5′ CAATATTTGGAGATGATTTTAGGC 3′	54	216	59
4–5	5′ ACTAATGAGCACCTGTAATTTTGC 3′ 5′ TGGTTTGTTCATCTACAATGTCAC 3′	51	648	55
6	5′ TGAATTATTTCCAAATCCTGAGTC 3′ 5′ TAATACAACCTTATTTGTGCTAAG 3′	45	233	53
7A	5′ CCTTCTGCTTGTCTCTATCTCTGC 3′ 5′ AGTATCCATTAGAAGATAATCCTG 3′	53	525	58
7B	5′ GATTGAGTTTGGTATCAAACACAG 3′ 5′ TATATTAAGAAGATACCGCTCATG 3′	53	541	57.5
7C	5′ CATGGCTTCCAGCAGTTGTTGTGC 3′ 5′ GTTTGGTTTCTCAGTTTTTGACAG 3′	53	565	58
7D	5′ ACATCGCCAGCCCAGCTTCCCAGG 3′ 5′ CCAAGTTGTCAGCAGCATCACGCT 3′	58	563	57.5
7E	5′ CATGGGGCTGTCAGTTGCTGTTGC 3′ 5′ GTATGTGTTTTGCTTTGTCCCTTG 3′	58	556	59
7F	5′ TACAGTGCAGTGCCTTTTCCCATG 3′ 5′ AGGCAGGGACAGTGGTGGATGTGC 3′	58	568	59
7G	5′ TGAAGGCCACACACCAAGCTGTCG 3′ 5′ AGGCACTGGTTCTGAATTGAATGC 3′	58	632	59