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Ngly1, a mouse gene encoding a deglycosylating enzyme implicated in proteasomal degradation: expression, genomic organization, and chromosomal mapping

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Abstract

In species as diverse as yeast and mammals, peptide:*N*-glycanase (*PNG1* in yeast; *Ngly1* in mouse) is believed to play a key role in the degradation of misfolded glycoproteins by the proteasome. In this study, we report the genomic organization and mRNA distribution of the mouse *Ngly1*. Mouse *Ngly1* spans 61 kb and is composed of 12 exons, the organization of which is conserved throughout vertebrates. Comparison of the mouse and human genomic sequence identifies a conserved gene structure with significant sequence similarity extending into introns. A 2.6 kb *Ngly1* message was detected in all mouse tissues examined, with the highest abundance in the testis. In addition, a lower molecular weight transcript of 2.4 kb was detected in the testis. From analysis of dbESTs the alternative transcript of *Ngly1* is predicted to be present in the human placenta. Given the key role *Ngly1* plays in glycoprotein degradation, we predict that *Ngly1* may be a contributing factor in "disease" susceptibility. To begin to address this question, we used radiation hybrid mapping to localize mouse *Ngly1* to chromosome 14 and the human orthologue to chromosome 3 with a strong link with known genes.

Keywords: Peptide:*N*-glycanase; ERAD; Quality control; Glycoprotein

The quality control machinery for secretory proteins and glycoproteins has the capacity to differentiate unfolded or misfolded proteins from correctly folded proteins in the endoplasmic reticulum (ER) [1]. Misfolded proteins are retained in the ER and eventually degraded by the mechanism called "ER-associated degradation" [2]. It is now clear that this degradation mechanism actually involves retro-translocation of the defective proteins or glycoproteins from the ER to the cytosol, followed by degradation by the proteasome. Further characterization of this quality control system may be medically important since human genetic diseases such as autosomal dominant neurohypophyseal diabetes insipidus, α-1 antitrypsin (A1Pi Z type) or polyglutamine diseases [spinobulbar muscular atrophy (SBMA)] are probably caused by toxic effects of the misfolded protein or aggregates, which accumulate because of inadequate degradation in the cytosol [3,4].

Recently the gene encoding a cytoplasmic PNGase (*PNG1*) was identified in yeast [5]. It is believed that the cytoplasmic peptide:*N*-glycanase (PNGase) activity plays a key role in the ER-associated degradation [6,7]. It is postulated that PNGase-mediated de-N-glycosylation of misfolded proteins facilitates their degradation by the proteasome. Biochemical studies show that Png1 binds to the 26S proteasome through its interaction with the DNA repair protein, Rad23 [8]. Moreover, a mouse Png1 homologue binds not only to mouse Rad23 orthologues, but also interacts with various proteins related to ubiquitin, including ubiquitin itself as well as with a proteasome subunit, by virtue of an extension on its N-terminus [9]. This domain includes the PUB/PUG domain [10,11] found in diverse proteins bearing UBA

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or UBX domains that have been implicated in the ubiquitin related pathway [12]. Moreover, quite recently the *Ngly1* gene product was proven to participate in the degradation of misfolded glycoproteins [13]. It is of note that the enzyme discriminates between non-native and folded glycoprotein [13], which is consistent with a proposed role for *N*-glycanase in the cytoplasmic turnover of glycoproteins [7]. In the current study, characterization of the mouse *PNG1* orthologue (*Ngly1*) is reported. Comparison of the mouse and human genomic sequence identified a conserved gene structure with significant sequence similarity extending into introns. Radiation hybrid mapping, as well as PCR of a BAC clone, identified the chromosomal location of *Ngly1* to chromosome 14, with a strong link with *Top2B* (type II DNA topoisomerase beta isoform) and *Rarb* (retinoic acid nuclear receptor isoform beta 2). A possible link of this gene to known genetic disorders is discussed.

Materials and methods

Isolation of BAC clones containing mouse PNG1 orthologue (Ngly1). The mouse BACs were isolated by hybridization using a 2.5-kb *Eco*RI/*Not*I fragment of EST clone No. 948982 (which includes the complete open reading frame [7] to a high-density gridded filter containing the E129/SvJ Mouse BAC (Incyte Genomics, Inc.). Five clones (19D22 (insert; ~250 kb), 26N7 (~200 kb), 40I15 (~200 kb), 70J14 (~200 kb), and 157J13 (~120 kb)) were purchased from Incyte and the inclusion of mouse PNGase gene in these clones was confirmed by colony PCR using the 3′ non-coding region. Hybridization and BAC electrophoresis conditions were described previously [14]. Clone No. 70J14 was used for genomic sequence analysis.

BAC 70J14 sequencing. BAC clone No. 70J14 was isolated and digested with *Bam*HI, *Pst*I, *Eco*RI, *Xba*I, and *Hin*dIII and the result of each digest was cloned into pBluescript SK (Stratagene). These clones were hybridized with mouse *Ngly1* cDNA (IMAGE No. 948982) and the sequence of each clone was determined by the primer-walking method. The sequence was assembled by the Phrap program (http://depts.washington.edu/ventures/uwtech/license/express/index.htm) and a gap (51061–56943) was isolated by PCR. The genomic sequence determined was deposited to NCBI database (Accession No. AY225417).

Comparison of genomic sequence. Repetitive element identification was performed essentially as described [15]. The MultiPIP Maker [16] was used to align the mouse BAC 70J14 sequence with human genome draft sequence (GenBank Accession No. NT_005762) as well as the *Fugu* genome sequence. The *Fugu* genome fragment containing the *Ngly1* orthologue was identified by tblastn analysis of JGI Fugu v3.0 (http://genome.jgi-psf.org/fugu6/fugu6.home.html) using the *Ngly1* amino acid sequence as query and the open reading frame was predicted using Genescan (http://genes.mit.edu/GENSCAN.html) as well as the comparison of EST sequence for the PNGase orthologue from Medaka fish (Gene Accession No. AJ457704).

Radiation mapping. The mouse *Ngly1* gene was mapped using the mouse/hamster radiation hybrid panel (Research Genetics, Catalog No. RH04.02). The human *Ngly1* gene orthologue was mapped using the G3 human–hamster radiation hybrid panel (Research Genetics, Catalog No. RH01.02). Primers used were the following: for mouse, 5′-GTTCTTTAAGAGCCTGTGGC-3′; 5′-TAGCAATATATCCTATGGCATC-3′ (to amplify 227 bp fragment corresponding to nts 64,485–63,504 in Accession No. AY225417); for human, 5′-CAAGGACTTACTGAAGTAGTC; 5′-ATCAGAAAATGTCAAATTACTATC-3′ (to amplify 327 bp fragment corresponding to nts 83,8692–83,9018 in NT 005762). Twenty-five nanograms of each hybrid DNA was used for PCR as recommended. For linkage analysis, PCR typing results were submitted to the following databases: for mouse, the Jackson Laboratory Mouse Radiation Hybrid Database (http://www.jax.org/resources/documents/cmdata/rhmap/RHIntro.html); for human, Stanford Human genome Center RH server (http://www-shgc.stanford.edu/RH/index.html).

Northern blot analysis. Identification of cDNA clones containing mouse *Ngly1* was reported previously [7]. We deposited the mouse cDNA sequences with NCBI database (Accession No. AF250927). A 1.4 kb hybridization probe for the mPng1p sequence was prepared by *Eco*RI–*Not*I fragment of IMAGE clone 719792 (Gene Accession No. AA260551) and labeled by the random priming method (Random Prime DNA labeling kit, Roche). The probe was hybridized to a mouse RNA blot (Mouse Multiple Tissue Northern Blot, Clontech) according to the manufacturer's protocol. For control, β-actin transcripts were detected using a probe provided by the manufacturer.

PCR for linkage analysis. To confirm the link between *Ngly1* and *Top2B* (type II DNA topoisomerase beta isoform) and *Rarb* (retinoic acid nuclear receptor isoform beta 2) in mouse chromosome 14, PCR using BAC clone 70J14 was performed. Primers were designed using their exon sequences of *Top2B* (D38046) and *Rarb* (S56660). The primers used and the length of amplified fragments were as follows—*Top2B*: 5′-GCGCGAGCAGCCCCTGG-3′ and 5′-CCCAGGTCAGCGCCCCG-3′ (83 bp); 5′-TGATGAAGACTTCTGAAGCC-3′ and 5′-AATCATATGCTCTTCTGGTC-3′ (144 bp); *Rarb*: 5′-CTTGGAGGCTATCATGACTG-3′ and 5′-CTTTCCCTTATGTTGCCCAG-3′ (207 bp).

Results

Genomic organization, localization, and expression of mouse Ngly1

It is likely that *Ngly1* plays a role in the degradation of misfolded glycoproteins or glycopeptides. As a first step towards understanding whether mutations in this gene contribute to the development of disease, we determined the genomic structure and chromosomal localization of the mouse *Ngly1* gene. We identified an approximately 200 kb BAC clone, 70J14, that contained the *Ngly1* coding sequence and determined the DNA sequence of a 70,991 bp segment spanning the *Ngly1* coding region. The *Ngly1* genomic sequence was deposited under Accession No. AY225417. Comparison of the mouse *Ngly1* cDNA sequence (AF250927) to the BAC 70J14 sequence demonstrated that the *Ngly1* coding region is divided into 12 exons spanning 70 kb of genomic DNA (Fig. 1). To determine the chromosomal location of mouse *Ngly1*, we carried out radiation hybrid analysis (Table 1). Mouse *Ngly1* gene had the highest anchor LOD of 14.5 to *D14Mit98.1* on chromosome 14. Statistical calculation placed the most probable location of *Ngly1* to 19 cR distal of *D14Mit98.1* (http://www.jax.org/resources/documents/cmdata/rhmap/RHIntro.html). We detected a 2.6 kb *Ngly1* transcript in all tissues tested (Fig. 2). The highest level of expression is found in the testis, where we

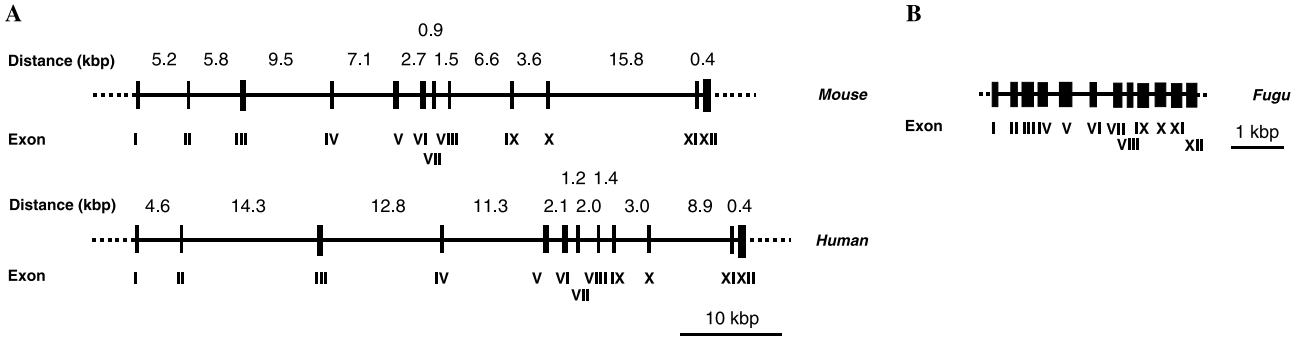

Fig. 1. Organization of *Ngly1* in various species. (A) Comparison of the exon organization of the mouse and human PNGase genes. Numbers represent a distance (kb) between exons. (B) Schematic representation of exon–intron organization of *Fugu Ngly1* orthologue.

Table 1
Human and mouse radiation hybrid mapping data

Gene/linkage marker	Panel scoring	LOD score	Chrom. No.	Distance (cRs)
Ngly1 (mouse)	0000000100 0111110000 0110001001 1101100110 1100100010 1010100100 1001101111 1010111101 0000101100 0001111110			
D14Mit98.1	0001110100 0?11110000 0110??1001 1011101111 110010?010 0010100100 ???1001101 1010111101 0000101100 0001??????	14.5	14	19.0
D14Mit220	0001111101 0?11100000 011??1100? 1011101111 1110?10010 0110101110 ??1101?101 1110111001 1000101100 0101??????	4.6	14	n/d
D14Mit132	0000110010 011?01?001 010000?001 100??01111 110?100?10 0010?00000 100?001101 1001101001 0100001100 0101100001	4.4	14	n/d
NGLY1 (human)	0000000000 0000001000 0000000000 0000000100 0010000100 0000000010 0001000001 0000001000 010			
SHGC-142206	0000000000 0000001000 0000000000 0000000000 0010000100 0000000010 0001000001 0000001000 010	9.78	3	7
SHGC-140862	0000000000 0000001000 0000000000 0000000000 0010000110 0000000010 0001000001 0000001000 011	7.85	3	20
SHGC-69364	0000000000 ?000001000 0000000000 0000000000 0010000100 0000000010 0000000001 0000001000 010	7.78	3	15

n/d, not determined.

observed a slightly faster migrating band (Fig. 2). This expression pattern is consistent with the earlier finding that PNGase enzyme activity can be found in all organs and tissues tested in the mouse [17].

Comparison of mouse, human, and Fugu (pufferfish) Ngly1

We identified the sequence containing the human (NT 005762) and *Fugu* (Scaffold 89 in the JGI Fugu v3.0 database (http://genome.jgi-psf.org/fugu6/fugu6.home.html) using a tblastn search. Although both mouse and human genes span approximately 70 kb we noted considerable variation in the size of several introns (Fig. 1). Integration of Line/L1 elements contributes much of the size variation between the human and the mouse second and 10th introns. To predict the exon–intron junction of *Fugu Ngly1*, Genescan as well as C-terminal EST sequence from Medaka fish (*Oryzias latipes* Gene Accession No. AJ457704) was utilized. Consistent with the compact *Fugu* genome, we determined that the *Fugu Ngly1* gene encoded by 12 exons was considerably smaller than mouse or human and spanned only 4 kb.

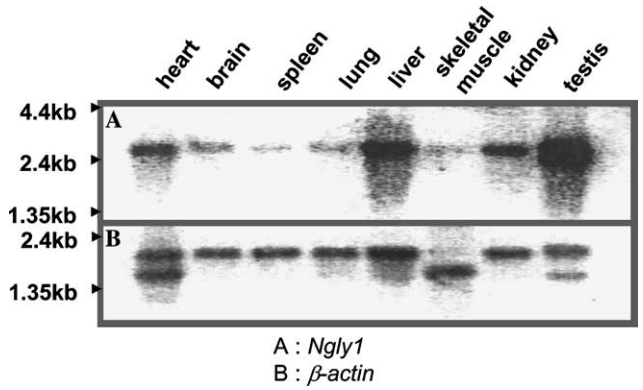

Fig. 2. Northern blot analysis. (A) Expression of *Ngly1* was evaluated on mouse Northern blots (Multiple Tissue Northern Blot, Clontech). (B) β-Actin was used as control for the quantitation of mRNA.

The predicted *Ngly1* protein sequences from mouse, human, and *Fugu* are aligned in Fig. 3. *Ngly1* is highly conserved throughout vertebrates (mouse vs. human; 84% identity, 6% similarity; mouse vs. *Fugu*; 56% identity, 14% similarity). Amino acid sequence conservation is greatest between residues 288 and 392 in mouse (mouse vs. human; 98% identity, 1% similarity; mouse vs. *Fugu*; 88% identity, 5% similarity), where the transglutaminase-like motif [18] containing the catalytic triad making up the active site in yeast Png1 is located [5,19]. This domain is conserved throughout eukaryotes (38% identity and 53% similarity between human and budding yeast; [5]. We also observed significant sequence conservation between residues 12 and 98 in mouse (mouse vs. human; 98% identical; mouse vs. *Fugu*; 77% identity, 12% similarity), where the PUB/PUG domain, a putative protein–protein interaction domain, is located [10,11]. This domain is conserved in *Ngly1* orthologues from vertebrates as well as from *Drosophila melanogaster*, but not from *Caenorhabditis elegans*, plants or fungi [7,10].

We utilized the MultiPIP [16] to evaluate the DNA sequence conservation between species (Fig. 4). Significant similarity between mouse and human was observed throughout the coding region as well as in the introns

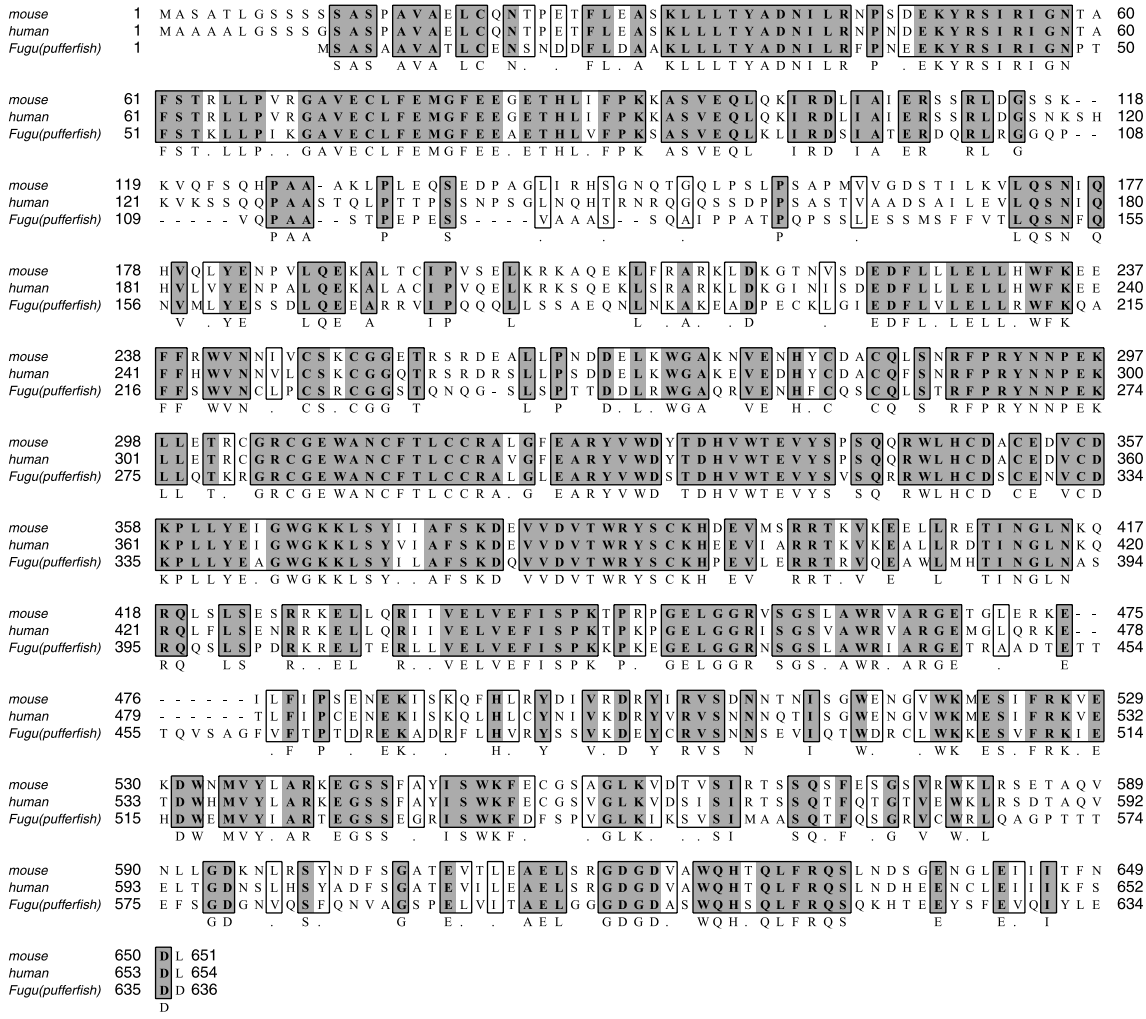

Fig. 3. Comparison of the mouse, human, and *Fugu Ngly1* predicted protein sequence.

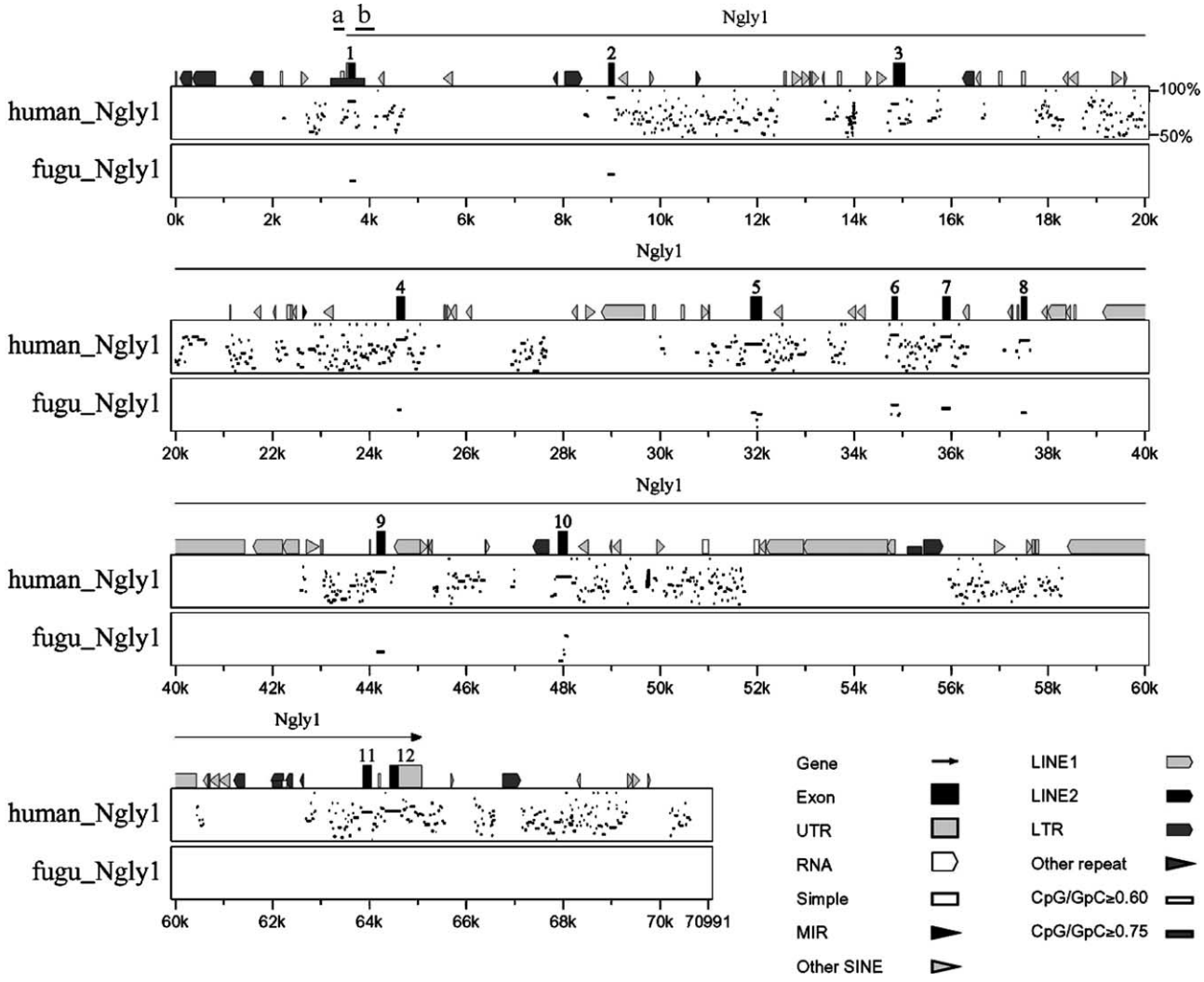

Fig. 4. MultiPIP Maker analysis of mouse *Ngly1* genome sequence derived from BAC 170J14. The nucleotide position corresponding to the mouse sequence is plotted on the *x*-axis, and the percentage sequence identity to the human draft sequence or *Fugu* genome Scaffold sequence (50–100%) is on the *y*-axis. Regions of significant sequence similarity appear as horizontal lines on the graph. Above the graph, the organization of the *Ngly1* gene is diagrammed. The legends identify specific elements. The exons (alternative UTR of the same gene) of the neighboring gene (a: AK016905 and b: BB655381) are also indicated in this figure.

and flanking regions. In contrast, sequence similarity between mouse and *Fugu* above 50% was not detected for exons 3, 11, and 12 and did not extend into introns. In addition, we noted that the sequence 286–423-bp upstream of the start of mouse *Ngly1* gene was highly conserved between mouse and human. Using blast analysis we determined that this conserved sequence represents the 5′ untranslated region of a gene related to 3-oxoacyl-[acyl-carrier-protein] synthase II (Gene Accession No. AK016905 for mouse) in a reverse direction. EST database analysis revealed that the neighboring gene has a number of different 5′ UTR regions, some of which are overlapping the *Ngly1* gene. For example, EST sequence Accession No. BB665381 has an exon equivalent to sequence 4012–3682, which overlaps with the first exon of *Ngly1* in a reverse direction (Fig. 4). Although an equivalent related 5′ untranslated sequence was not found in the *Fugu* genome, a segment with 84–93% identity at the nucleotide to AK016905 was observed 1.1–1.8 kb upstream of the *Fugu Ngly1* first exon in scaffold 89. Taken together, it is likely that the exon–intron organization of *Ngly1* as well as the close relationship to the neighboring gene is conserved throughout vertebrates.

Discussion

In this study we have characterized the genomic structure of the mouse gene (*Ngly1*) encoding the de-*N*-glycosylating enzyme implicated in proteasomal degradation of misfolded glycoproteins [5,7,13]. By comparing the genomic sequence between human, mouse, and *Fugu Ngly1* orthologues, it was found that

exon–intron organization was well conserved throughout vertebrates.

Northern blot analysis showed ubiquitous expression of an approximately 2.6 kb *Ngly1* mRNA in mouse tissue and organs consistent with the length of cDNA sequence identified previously [7]. The alternative splicing variant that is shorter in length (~2.4 kb) was found in the testis. The nature of this splice variant is currently unknown, but it should be noted that the human EST clone, which lacks exon 5 (225 bp), was found in placenta cDNA (ex. AU135833).

It is interesting to note that this gene is abundantly expressed in testis. Recently yeast Rad23 protein and its mouse orthologue (mHR23B) were found to bind to their corresponding PNGase in vivo [8,9]. Rad23 protein was originally identified to be involved in nucleotide excision repair (NER) [20,21]. Recently Rad23 protein was found to bind to the 26S proteasome through its ubiquitin-like domain at its N-terminus in both mammalian cells and yeast [22,23]. In yeast, Rad23p was found to mediate the physical interaction of PNGase and the 26S proteasome [8], suggesting that Rad23 protein may serve as an escort protein which links Png1p and the proteasome for reasons that are currently unknown. We also found that the Png1p–Rad23p complex [8] was distinct from Rad23p–Rad4p complex, which is involved in the nucleotide excision repair (NER) process [24–26]. An *mHR23B* knockout mice was recently reported [27]. It was shown to retain full NER activity presumably because of the presence of another Rad23 orthologue, *mHR23A*, although the homozygous animals showed significantly impaired embryonic development and a high rate (90%) of intrauterine or neonatal death. Surviving animals also displayed a variety of abnormalities including male sterility. These phenotypes may represent the functional importance of mHR23B in NER-independent cellular processes, possibly in the ubiquitin–proteasome pathway [27]. It would be interesting to see if the phenotype of male sterility can be related to the function of PNGase, since its gene is most highly expressed in testis.

Although the phenotypic consequence of a defect in PNGase in higher eukaryotes has not been determined, in *C. elegans* a mutation in *Ngly1* orthologue affects axon development and branching (Drs. A. Colavita and M. Tessier-Lavigne, personal communication). According to the NCBI OMIM gene map (http://www.ncbi.nlm.nih.gov/OMIM), both *Rarb* and *Top2B* map to 3p24 in human. There are several genetic disorders linked to this region for which the responsible gene has not yet been identified. For example, Familial Moyamoya disease, which is characterized by bilateral stenosis and/or occlusion of the terminal portion of the internal carotid artery, was recently mapped to chromosome 3p24.2-p26 [28]. Given the phenotypic consequence of *Ngly1* mutation in *C. elegans*, and the recent observation that sensory nerves determine the differentiation pattern of arteries and blood vessel branching [29], it would be interesting to see if *Ngly1* may be involved in this human genetic disorder.

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