EVOLUTIONARY ANALYSIS OF THE B56 GENE FAMILY OF PP2A REGULATORY SUBUNITS

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

Sam Houston State University

In Partial Fulfillment

of the Requirements for the Degree of

Master of Science

by

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May, 2017

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ABSTRACT

Sommer, Lauren M., *Evolutionary analysis of the B56 gene family of PP2A regulatory subunits*. Master of Science (Biology), May, 2017, Sam Houston State University, Huntsville, Texas.

Protein phosphatase 2A (PP2A) is an abundant serine/threonine phosphatase that functions as a tumor suppressor in numerous cell-cell signaling pathways, including Wnt, myc, and ras. The B56 subunit of PP2A regulates its activity, and is encoded by five genes in humans. B56 proteins share a central core domain, but have divergent amino- and carboxy-termini, which are thought to provide isoform specificity. We performed phylogenetic analyses to better understand the evolution of the B56 gene family. We found that B56 was present as a single gene in eukaryotes prior to the divergence of animals, fungi, protists, and plants, and that B56 gene duplication prior to the divergence of protostomes and deuterostomes led to the origin of two B56 subfamilies, B56αβε and B56γδ. Further duplications led to three B56αβε genes and two B56γδ in vertebrates. Several nonvertebrate B56 gene names are based on distinct vertebrate isoform names, and would best be renamed. B56 subfamily genes lack significant divergence within primitive chordates, but each became distinct in complex vertebrates. Two vertebrate lineages have undergone B56 gene loss, *Xenopus* and *Aves.* In *Xenopus*, B56δ function may be compensated for by an alternatively spliced transcript, B56δ/γ, encoding a B56δ-like amino-terminal region and a B56γ core.

KEY WORDS: Protein phosphatase 2A; B56 regulatory subunit; Molecular phylogeny

ACKNOWLEDGEMENTS

This research was supported by grants from Sam Houston State University Enhancement Research Grant Program to Joni M. Seeling and National Institutes of Health Grant R24OD010435 to Ambystoma Sal-Site.

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CHAPTER I

Background

Cell Signaling

In order to respond to their environment, cells receive and process signals. They can receive many signals at the same time and integrate that information. These signals can be mechanical, such as sound, light, or touch, while others are chemical, such as growth factors, hormones, or neurotransmitters. Cell signaling occurs when a signaling molecule binds to a specific receptor protein. These receptors are generally transmembrane proteins which bind the signaling molecules outside the cell and then transmit the signal internally by undergoing a conformational change. This conformational change launches a series of biochemical reaction pathways, called signal transduction cascades. The cascades help to amplify the signaling message by producing multiple intracellular signals for every one receptor bound (NatureEducation, 2014).

Phosphorylation reactions control the activity of the enzymes involved in the intracellular signaling pathways. Protein kinases catalyze the transfer of phosphate groups from ATP molecules to either themselves or other proteins at either serine/threonine or tyrosine residues. The addition of a phosphate group causes a conformational change in the enzymes, either activating or inhibiting enzyme activity. Protein phosphatases remove the phosphate group form the enzymes, reversing the effect on enzymatic activity (NatureEducation, 2014).

Wnt Signaling

One type of a phosphorylation-regulated signal transduction pathway is the Wnt signaling pathway (Li, Yost, Virshup, & Seeling, 2001). The Wnt pathway regulates stem

cell pluripotency and cell fate specification and differentiation during development (NatureEducation, 2014). There are two different types of Wnt pathways, canonical and non-canonical. The non-canonical pathway function has not clearly been identified, however, it is believed to function in planar cell polarity. The canonical Wnt pathway activates gene transcription to control developmental processes (Berridge, 2014). A Wnt protein, a secreted glycoprotein, binds to the Frizzled and LRP5/6 coreceptors (Gilbert, 2013, and NatureEducation, 2014). This causes the LRP5/6 receptor to bind axin and glycogen synthase kinase 3β (GSK3β) and the Frizzled protein to bind to Disheveled. Disheveled is phosphorylated which stabilizes axin and prevents the GSK3β from phosphorylating β-catenin. β-catenin builds up and enters the nucleus where it binds to a LEF/TCF transcription factor. This converts the LEF/TCF repressor into a transcriptional activator (Gilbert, 2013). In the absence of Wnt, β-catenin is targeted by phosphorylation, and is then ubiquitinated and degraded by the proteasome, through the actions of axin, APC, and GSK3 (Wnt / β-Catenin Signaling Pathway, 2017).

The Wnt signaling pathway plays a major role in cancer development. If the Wnt pathway is overexpressed it leads to upregulation of cells, which is the basis of tumor formation. Research has shown that β-catenin point mutations in human tumors have prevented GSK3β phosphorylation and allows for accumulations. APC and axin mutations have also been documented in tumors supporting the pathway's role in cancer development (Wnt / β-Catenin Signaling Pathway, 2017).

Protein Phosphatase 2A

Protein phosphatase 2A (PP2A) is one of the most abundant serine/threonine protein phosphatases. It makes up about 0.3% of total cellular proteins. Its primary role is

to dephosphorylate many of the phosphoproteins that act in cell signaling pathways. PP2A has a trimeric structure consisting of a scaffolding A subunit, a regulatory B subunit, and a catalytic C subunit. Each subunit has multiple subunits within it. These subunits allow for many different combinations resulting in multiple heterotrimeric holoenzymes. The large family of B subunit proteins allows for much of the versatility of PP2A. The B subunits have slightly different properties allowing them to direct the holoenzyme to different cellular regions and substrates (Berridge, 2014). Some of the B subunits' roles are characterized, however, many are still unknown.

One of the roles of PP2A is to help regulate cell proliferation. It does this by reversing the protein phosphorylation that occurs in signaling pathways (Berridge, 2014). PP2A has been found to function as a tumor suppressor. One of the three families of the B subunit of PP2A, named B56, has been shown to regulate Wnt signaling. One of the three families of the B subunit of PP2A, named B56, has been shown to regulate Wnt signaling. B56 binds to APC, while the C subunit binds to axin to help regulate the Wnt signaling pathway (Gilbert, 2013). It is believed that PP2A acts in many different places in the Wnt cascade but not all have been determined.

Mutations of the A subunit of PP2A have been identified in various types of colon, lung, breast, skin, and ovarian cancer. Many of these mutations have been found to inhibit the A subunit from binding to the B and/or C subunits (Ruediger, Pham, & Walter, Alterations in protein phosphatase 2A subunit interactions in human carcinomas of the lung and colon with mutations in the Aß subunit gene, 2001). PP2A also contributes to Myc degradation, which regulates cell proliferation and is amplified in many human cancers. Research has also found that the SV40 small T antigen and polyoma virus small

T and middle T antigens bind to the scaffolding A subunit, resulting in decreased phosphatase activity (Berridge, 2014).

CHAPTER II

Evolutionary Analysis of the B56 Gene Family of PP2A Regulatory Subunits

Introduction

Although signal transduction cascades are intensely studied, relatively little is known about the role that serine/threonine phosphatases play in them. While there are over 400 serine/threonine kinase genes in the human genome, there are only around 40 serine/threonine phosphatase catalytic subunits to counter them. This was initially interpreted to mean that phosphatases have broad, constitutive activities, however, it was later found that phosphatases are highly specific and that the majority of phosphatases achieve diversity by forming numerous distinct multimeric protein complexes.

Two phosphatases, protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1), combined account for 90% of all serine/threonine phosphatase activity in the cell (Seshacharyulu, Pandey, Datta, & Batra, 2013 and Eichhorn, Creyghton, & Bernards, 2009). PP2A constitutes 1% of all cellular proteins (Eichhorn, Creyghton, & Bernards, 2009). PP2A is made up of three subunits: a scaffolding (A) subunit, a regulatory (B) subunit, and a catalytic (C) subunit (Seeling, et al., 1999). In the case of PP2A, there are at least three different B regulatory subunit gene families (B55/PR55/B, B56/PR56/B', and B72/PR72/B") that bind to the structural A subunit and the catalytic C subunits, each of which is encoded by two genes in humans. The A subunit consists of 15 HEAT repeats (Ruediger, Pham, & Walter, 2001). HEAT repeats are composed of two α helices connected by an inter-repeat loop. The B subunit of PP2A binds to repeat 1-10 of the A subunit while the C subunit binds to repeats 11-15 (Ruediger, Pham, & Walter, 2001). Through the combinatorial effects of the association of multiple subunits, and with the

inclusion of alternative splicing, PP2A could form as many as 200 different heterotrimers. As the B subunits are more diverse than the A and C subunits, they are the major contributors to substrate specificity and subcellular localization of the PP2A holoenzyme (McCright et al., 1996, and Cegielska et al., 1994).

PP2A carries out essential cellular functions, and therefore its subunits are encoded by one of the most highly conserved sets of genes. The C subunit is the most conserved, with 75% identity between human and yeast proteins; human and yeast A subunit proteins share 44% identity. In humans, B56 isoforms are encoded by five widely expressed genes, B56α, B56β, B56δ, B56γ, and B56ε. B56 proteins are highly conserved between species, sharing approximately 60% identity between human and yeast. Even though individual B56 isoforms have distinct functions, the five human B56 proteins share 66% to 81% identity. B56 genes encode proteins with a highly conserved core of about 400 amino acids and variable amino- and carboxy-termini ranging from approximately ten to one hundred amino acids in length in humans. The divergent amino- and carboxy-termini are thought to provide specificity to the different isoforms. Alternative splicing occurs at the B56γ locus to produce a transcript with either a B56γ amino-terminal extension (B56γ/γ) or a mixed-isoform transcript containing a B56δ-like amino-terminal extension (B56δ/γ) (Baek & Seeling, 2007). As the B56 amino- and carboxy-termini are proposed to determine substrate specificity, these alternative splice products are likely to have distinct roles in the cell.

B56 isoforms have roles in numerous cell-cell signaling pathways. B56 isoforms modulate canonical Wnt signaling; most B56 isoforms are inhibitory to Wnt signaling, however, B56ε is required for Wnt signaling (Seeling et al., 1999, Li et al., 2001, and

Yang et al., 2003). B56α inhibits Wnt signaling by acting in the ß-catenin degradation complex (Li, Yost, Virshup, & Seeling, 2001). The B56 family plays a major role in the PP2A complexes that act as tumor suppressors (Haesen, Sents, Ivanova, Lambrecht, & Janssens, 2012). B56 isoforms also have a role in ras signaling, as transgenic mice with an A subunit mutation unable to bind B56 and an activating ras mutation have a reduced lifespan when compared to those solely possessing activated ras (Walter and Ruediger, 2012). B56α inhibits Myc signaling by promoting Myc's proteasomal-mediated degradation (Arnold & Sears, 2006). B56γ inhibits cell spreading and metastasis by dephosphorylating paxillin (To et al., 2000). B56ε also has a role in hedgehog signaling (Rorick, et al., 2007).

Here we explored the evolution of the B56 gene family of PP2A regulatory subunits to provide us with a deeper understanding of B56 and how its evolution has resulted in five vertebrate genes that differentially regulate cell-cell signaling pathways. This characterization is especially important, as it will aid the integration of B56 studies in diverse organisms, especially when comparing functional analyses between species containing different complements of B56 genes. In addition, B56 isoforms can have antagonistic effects on signaling pathways, resulting in either growth inhibition or growth promotion. Understanding the origin of the antagonistic isoforms may be useful in understanding their disparate roles in signaling pathways. We performed a hierarchical clustering and a phylogenetic analysis to examine the highly conserved B56 isoforms. We traced the expansion of the B56 gene family from simple to complex organisms, and also found interesting patterns of gene duplication and deletion throughout the evolution of the B56 gene family.

Experimental Section

Identification of B56 Genes Homologs. All members of the B56 gene family (B56α, B56β, B56γ, B56δ, and B56ε) were identified from diverse species of animals, fungi, protists, and plants from the NCBI. Amino acid sequences of *Homo sapiens* B56 isoform proteins were used as queries to identify the corresponding target homologs of different species using Blastp (Altschul, Gish, Miller, Myers, & Lipman, 1990). A symmetrical similarity search scheme was employed to perform all pair-wise comparisons to confirm the homologs, and their accession numbers were then retrieved. The following stringency criteria were used for the identification of the best matches: percent query coverage ≥ 50, maximum score ≥ 100, percent identity ≥ 40, and E-value ≤ 10^{-3}.

Hierarchical Clustering. In this analysis, each of the B56 protein sequences was chosen in turn as the query sequence in a Blastp search. We collected the pair-wise amino acid identity values for all possible pairs of total 105 members of the B56 protein family, and used the resulting protein percent identity matrix for data visualization. We used agglomerative hierarchical clustering to visualize similarities within and between B56 isoforms. Hierarchical clustering constructs a hierarchical structure of input data and it has become a standard visualization method since its seminal application to microarray data (D'Haeseleer, 2005, and Eisen, Spellman, Brown, & Botstein, 1998). Particularly, agglomerative clustering method creates a hierarchical structure through a bottom-up approach, in which a pair of closest clusters is merged at each step. Agglomerative clustering takes an input of pair-wise similarities (or distances) among data items, from which cluster similarities (or distances) are inferred for grouping data items. We utilized

the clustergram function in the Bioinformatics Toolbox of a commercial software package MATLAB 7.11 (R2010b) (MathWorks, Natick, MA, USA) and generated the heat map with dendrograms as shown in Figure 1. Each row of the identity matrix was transformed so that its mean is 0 and the standard deviation is 1 for better visualization. Also, average linkage (i.e., UPGMA) was used to compute Euclidean distance between a data point and a cluster.

Phylogenetic Analysis. Phylogenetic analysis was run on the Geneious version 7.1.5 platform (Kearse, et al., 2012). B56 protein sequences from selected species were input into Geneious, and sequences were aligned using MUSCLE (MUltiple Sequence Comparison by Log-Expectation) (Edgar, 2004). A phylogenetic tree was inferred for these aligned protein sequences with FastTree version 2.1.5 with default settings (Price, Dehal, & Arkin, 2010). The resulting phylogeny was rooted by using the plant B56 genes as an out-group. FastTree 2 is an approximately maximum-likelihood phylogenetic method which efficiently uses alignment with a large number of genes or protein sequences (Price, Dehal, & Arkin, 2010). It is openly available software and it produces phylogenetic trees in a short amount of time that are as accurate as trees constructed by other maximum-likelihood methods such as PhyML 3.0 or RAxML 7.0. FastTree2 uses the CAT (category) approximation (Stamatakis, 2006) to account for variation in rates across sites and also implements the Shimodaira-Hasegawa (SH) test (Shimodaira & Hasegawa, 1999) to estimate the reliability of each split in the phylogeny, which is the same as PhyML3's SH-like local support values (Guindon, Delsuc, Dufayard, & Gascuel, 2009). A species phylogenetic tree was constructed based on the Tree of Life (Maddison & Schulz, 2007).

Identification of B56 Gene Family Homologs. We analyzed B56 sequences from thirty-three species, each possessing between one and nine B56 genes, for a total analysis of 105 B56 sequences (Table 1). The best match of each vertebrate B56 protein sequence to the corresponding human B56 ortholog is shown in Table 2. We examined B56 genes from sixteen diverse species of mammals, birds, reptiles, amphibians, and fish. Each of the vertebrate B56 isoforms matched the corresponding human ortholog, as can be seen from their low expected values, and their high maximum scores, percent query coverages, percent identities, and percent similarities. Their amino acid identities ranged from 75% to 100%, while their similarities ranged from 80% to 100%. The B56ε gene is highly conserved, as the B56ε protein from Homo sapiens is identical to that in six species: *Macaca mulatta, Bos taurus, Ovis aries, Canis lupus familiaris, Gallus,* and *Falco peregrinus*; and 97.2%–99.8% identical to that in seven species: *Mus musculus, Rattus norvegicus, Felis catus, Ambystoma mexicanum, Chrysemys picta bellii, Xenopus laevis*, and *Xenopus tropicalis*. This suggests that B56ε orthologs experienced a strong selective pressure to maintain their function.

Table 1

Summary of B56 Sequences

Groups	Species	Isoforms/Species	Total Sequences
Plants	3	1-9	17
Protists	4	1	4
Fungi	5	1-2	6
Diploblasts	2	2	4
Protostomes	2	2	4
Echinoderm	1	2	2
Gnathostomes	16	4-5	68
Total	33	1-9	105

Table 2

Blast Summary of Vertebrate B56 Sequence Alignment

Query	Species	Class	Isoform	Accession #	E	M	Q	% I	% S
α	*Mus musculus*	Mammalia	α	NP_659129.2	0	976	100	97.7	98.8
β			β	NP_937811.1	0	1006	100	98.8	99.2
γ			γ	XP_006515983.1	0	1014	100	92.2	93.1
δ			δ	BAB62015.1	0	1178	100	96.0	97.3
γ			δ/γ	XP_006515987.1	0	978	96	91.9	93.1
ε			ε	NP_036154.1	0	955	100	99.8	100.0
α	*Felis catus*	Mammalia	α	XP_003999369.2 ^	0	820	100	99.5	99.5
β			β	XP_003993624.1	0	1012	100	99.2	99.6
γ			γ	XP_006933193.1 ^	0	905	90	88.2	89.6
δ			δ	XP_003986198.1	0	1217	88	98.7	99.0
ε			ε	XP_003987771.1	0	954	100	99.8	99.8
α	*Macaca mulatta*	Mammalia	α	NP_001244568.1	0	998	100	99.8	100.0
β			β	XP_001118226.1	0	692	88	83.6	86.1
γ			γ	XP_002805259.1	0	1052	100	99.2	99.4
δ			δ	XP_001087636.2	0	1177	100	95.3	95.8
γ			δ/γ	XP_001112240.1 ^	0	1020	96	98.6	99.2
ε			ε	NP_001253672.1	0	956	100	100.0	100.0
α	*Ovis aries*	Mammalia	α	XP_004013951.1	0	978	99	98.4	98.8
β			β	XP_004019707.1	0	953	100	94.6	95.6
γ			γ	XP_004018041.1 ^	0	888	96	99.5	99.5

(continued)

Query	Species	Class	Isoform	Accession #	E	M	Q	% I	% S
δ			δ	XP_004019269.1	0	1164	98	96.5	96.8
ε			ε	XP_004010759.1	0	956	100	100.0	100.0
α	*Bos taurus*	Mammalia	α	NP_001075197.1	0	978	99	98.4	98.8
β			β	NP_001068700.1	0	1006	100	99.0	99.4
γ			γ	NP_001076845.1	0	1024	98	96.9	97.9
δ			δ	NP_001193287.1	0	1214	100	98.8	99.2
γ			δ/γ	XP_005222201.1	0	962	99	94.7	96.5
ε			ε	NP_001076937.1	0	956	100	100.0	100.0
α	*Canis lupus*	Mammalia	α	XP_005622406.1 ^	0	820	100	99.5	99.5
β	*familiaris*		β	XP_540876.1	0	1013	100	99.2	99.6
γ			γ	XP_854560.1	0	1031	100	97.5	97.7
δ			δ	XP_005627428.1	0	1195	100	97.5	98.0
γ			δ/γ	XP_005623875.1	0	996	96	97.2	97.8
ε			ε	XP_537472.2	0	956	100	100.0	100.0
α	*Rattus*	Mammalia	α	NP_001101361.1	0	980	100	97.9	98.8
β	*norvegicus*		β	NP_852044.1	0	1008	100	99.0	99.4
γ			γ	NP_001178041.1	0	1028	100	96.9	97.5
δ			N/A	AAH99800.1	0	1195	95	97.5	98.2
γ			δ/γ	XP_001077680.1	0	994	96	96.4	97.2
ε			ε	XP_006240284.1	0	955	100	99.8	100.0
α	*Falco*	Aves	α	XP_005238767.1	0	874	97	95.4	97.9
γ	*peregrinus*		γ	XP_005243013.1	0	980	100	94.7	96.7

(continued)

Query	Species	Class	Isoform	Accession #	E	M	Q	% I	% S
δ			δ	XP_005242599.1	0	984	85	94.8	97.8
γ			δ/γ	XP_005243015.1 ^	0	848	87	94.2	96.4
ε			ε	XP_005241867 ^	0	956	100	100.0	100.0
α	*Gallus*	Aves	α	XP_419432.2	0	872	97	95.0	97.7
γ			γ	NP_001072950.1	0	977	99	94.9	96.9
δ			δ	XP_419321.3	0	1086	98	90.9	93.9
ε			ε	XP_421412.2	0	956	100	100.0	100.0
α	*Alligator*	Reptilia	α	XP_006268191.1	0	858	96	94.9	97.7
β	*mississippiensis*		β	XP_006265023.1	0	806	92	90.5	94.8
γ			γ	XP_006270473.1	0	978	99	95.1	97.1
δ			δ	XP_006268832.1	0	1082	91	94.7	98.2
γ			δ/γ	XP_006270474.1 ^	0	847	87	94.6	96.9
ε			ε	XP_006264307.1 ^	0	739	100	78.3	80.2
α	*Chrysemys picta*	Reptilia	α	XP_005306377.1	0	874	97	95.2	97.7
β	*bellii*		β	XP_005307965.1	0	876	100	88.0	94.4
γ			γ	XP_005285849.1	0	977	99	94.3	96.7
δ			δ	XP_005293566.1	0	1088	100	91.1	94.4
γ			δ/γ	XP_005285851.1 ^	0	845	87	94.0	96.4
ε			ε	XP_008166448.1	0	937	100	98.7	98.7
α	*Xenopus laevis*	Amphibia	α	NP_001108316.1	0	838	88	92.3	97.2
β			β	rXL259o17ex °^	3×10^{-20}	70.5	9	88.9	100.0
γ			γ	[3] ^	0	875	89	97.7	99.3

(continued)

Query	Species	Class	Isoform	Accession #	E	M	Q	% I	% S
γ			δ/γ	NP_001087638.1 ^	0	840	85	97.6	99.5
ε			ε	NP_001088245.1	0	937	100	97.2	98.9
α	*Xenopus*	Amphibia	α	NP_001072157.1	0	844	97	90.3	95.6
β	*tropicalis*		β	NP_001093749.1	0	830	96	85.8	93.5
γ			δ/γ	Q6P3P7 [2]	0	942	96	93.3	97.0
ε			ε	NP_989253.1	0	929	100	97.4	98.5
α	*Ambystoma*	Amphibia	α	contig314980 *^	0	743	82	92.6	96.8
β	*mexicanum*		β	contig328022 *^	0	655	72	87.2	94.4
γ			γ	contig314598 *^	0	950	99	91.6	94.9
δ			δ	contig133764 *^	0	739	64	95.9	99.2
ε			ε	contig190869 *^	0	865	92	97.7	99.3
α	*Danio rerio*	Actino-pterygii	α	XP_690932.3	0	791	97	82.1	91.4
β			β	XP_690770.2	0	787	96	81.2	89.7
γ			γ	XP_005160957.1	0	950	99	89.3	94.4
δ			δ	NP_998483.1	0	1043	97	84.9	91.0
δ			δ/γ	A4QP33 [2]	0	914	98	75.3	84.4
ε			ε	NP_919396.1	0	871	100	90.2	95.3

Homo sapiens B56α, B56β, B56γ, B56δ, and B56ε were used as queries in Blastp searches against the NCBI database. The highest-ranking chordate B56 isoform hits from fifteen species are provided along with their protein accession number (Accession #), E-value (E), maximum score (M), percent query coverage (Q), percent identity (% I), and percent similarity (% S). The superscript ^ denotes sequences that were not used in the phylogenetic analysis due to the short length of the sequence. The superscript ° denotes a sequence retrieved from Xenopus Database 3.2 (XB3.2) (XDB3.2, 2014). The superscript * denotes a sequence that came from Sal-Site (Sal-Site, 2014), whereas the superscript [2] denotes a sequence that came from Uniprot (UniProt Consortium, 2015). The high level of conservation of the vertebrate B56 isoforms can be seen through the low E-values, high maximum scores, and high query coverages.

B56 is also well conserved in simple chordates, nonchordate animals, fungi, protists, and plants. The amino acid identities between both simple chordate and nonchordate animals versus human B56 proteins were 59% to 84%, while their similarities were 77% to 94% (Table 3). The identities and similarities between fungi and protists versus human B56 proteins ranged from 51% to 62% and 69% to 80%, respectively (Table 3). The identities and similarities between plant and human B56 proteins were slightly less than those observed with fungi and protists, and ranged from 47% to 57% and 61% to 77%, respectively (Table 4). The high conservation of B56 proteins between animals, fungi, protists, and plants suggest that B56 plays a key role in basic cellular functions. The details of the protein similarities of vertebrates; simple animals, fungi, and protists; and plants, including data from all B56 pair-wise comparisons with human B56 isoforms.

Hierarchical Clustering. A hierarchical clustering was undertaken to gain insight into the relationship among the 105 B56 genes from animal, fungal, protist, and plant species. This analysis is based on sequence identity obtained through BLAST hits. The identity matrix was populated with the percent identity values, where rows correspond to the queries of the 105 genes, and columns correspond to the target database of the 105 genes. The identity matrix was then visualized using hierarchical clustering (Figure 1). The dendrograms and heat maps clearly delineate separate gene clusters for animal and plant B56 genes, with the animal cluster further subdivided into two clusters, B56αβε and B56γδ.

Table 3

Blast Summary of Simple Chordate/Nonchrodate Animal/Fungi/Protist B56 Sequence Alignment

Query	Species	Class	Isoform	Accession #	E	M	Q	% I	% S
α	*Petromyzon*	Cephalaspidomorphi	N/A	S4RHV1 [2]	0	706	93	74.6	89.0
γ	*marinus*		N/A	S4RN43 [2]	0	830	95	81.3	88.6
ε			N/A	S4RGA7 [2]	9×10^{-138}	388	56	81.9	94.4
γ	*Branchiostoma*	Leptocardii	N/A	BF237525 [1]	0	775	94	78.6	87.4
γ	*floridae*		N/A	BF252487 [1]	0	691	95	70.1	81.1
ε			N/A	BF112597 [1]	1×10^{-55}	171	36	59.1	77.3
ε			N/A	BF284583 [1]	0	645	88	77.3	89.0
ε			N/A	BF237518 [1]	0	685	81	84.4	93.9
δ	*Strongylocentrotus*	Echinoidea	δ	XP_003730246.1	0	797	90	72.2	81.9
ε	*purpuratus*		α	XP_780697.2	0	713	97	75.3	87.7
α	*Hydra vulgaris*	Hydrozoa	α	XP_002154794.2	0	725	94	74.0	88.1
γ			δ	XP_004208357.1	0	687	78	83.0	92.7
γ	*Amphimedon*	Demospongiae	β	XP_003386538.1	0	558	87	65.5	79.0
γ	*queenslandica*		δ	XP_003384582.1	0	678	93	67.0	79.0
δ	*Caenorhabditis*	Chromadorea	PPTR-2	NP_505808.1	0	741	88	65.6	79.4
ε	*elegans*		PPTR-1	NP_507133.4	0	641	95	69.0	82.7
α	*Drosophila*	Insecta	wdb	NP_733219.1	0	689	86	78.1	87.7
γ	*melanogaster*		B56-1	CAB86364.1	0	701	79	79.5	92.2
γ	*Aspergillus niger*	Eurotiomycetes	N/A	EHA23297.1	0	599	90	61.4	77.8
γ	*Aspergillus nidulans*	Eurotiomycetes	parA	XP_868849.1	0	608	94	62.1	79.5
γ	*Ashbya gossypii*	Saccharomycetes	RTS1	NP_984527	0	521	83	56.5	72.2

(continued)

Query	Species	Class	Isoform	Accession #	E	M	Q	% I	% S
γ	*Saccharomyces cerevisiae*	Saccharomycetes	RTS1	AAB38372.1	3×10^{-176}	508	80	56.0	72.4
γ	*Schizosaccharomyces*	Schizosaccharomycetes	par1	NP_588206.1	1×10^{-178}	506	79	60.6	78.2
γ	*pombe*		par2	NP_593298.1	1×10^{-167}	481	79	54.8	76.3
δ	*Dictyostelium discoideum*	Dictyostelia	psrA	XP_641193.1	9×10^{-147}	431	75	51.3	68.9
γ	*Dictyostelium purpureum*	Dictyostelia	N/A	XP_003290675.1	8×10^{-149}	430	75	53.1	72.9
γ	*Dictyostelium fasciculatum*	Dictyostelia	N/A	XP_004360558.1	2×10^{-149}	433	77	53.1	72.1
γ	*Polysphondylium pallidum*	Dictyostelia	N/A	EFA76858.1	1×10^{-149}	431	76	53.9	74.0

Error! Reference source not found.*Homo sapiens* B56 isoforms were used as queries in Blastp searches against the NCBI database. Each of the five *H. sapiens* B56 isoforms was similar in its identity and similarity to each of the hits, and therefore no specific B56 isoform orthologs could be identified. However, the NCBI hits are listed with the *H. sapiens* query with which they had the lowest E-value. The NCBI hits are provided along with their protein accession number (Accession #), E-value (E), maximum score (M), percent query coverage (Q), percent identity (% I), and percent similarity (% S). The superscript [1] denotes sequences retrieved from JGI (Grigoriev, et al., 2012), while the superscript [2] denotes sequences from Uniprot (UniProt Consortium, 2015). The high level of conservation of the B56 isoforms in distant species can be seen through the low E-values, high maximum scores, and high query coverages. Identities range from 51% to 84% and similarities range from 69% to 94%.

Table 4

Blast Summary of Plant B56 Sequence Alignment

Query	Species	Class	Isoform	Accession #	E	M	Q	% I	% S
γ	*Chlamydomonas reinhardtii*	Chlorophyceae	wdb	XP_001693445.1	0	775	94	57.1	76.5
γ	*Arabidopsis thaliana*	Dicot	θ	NP_973816.1	1×10^{-170}	484	82	54.8	72.2
γ			ζ	NP_188802.1	2×10^{-165}	473	83	53.2	70.3
γ			N/A	NP_197933.1	7×10^{-154}	441	80	50.2	70.7
δ			α	NP_195967.1	2×10^{-161}	464	71	54.5	74.9
δ			β	NP_187599.1	5×10^{-166}	475	68	55.4	75.3
δ			δ	AAD02810.1	2×10^{-165}	473	72	51.6	70.2
δ			γ	NP_849390.1	2×10^{-168}	483	70	54.0	72.4
ε			ε	NP_191053.1	1×10^{-155}	444	96	50.7	67.0
ε			η	NP_001154648.1	1×10^{-140}	407	88	47.0	60.6
α	*Oryza sativa*	Monocot	N/A	NP_001059361.1	9×10^{-161}	459	81	52.0	71.6
γ			N/A	AAP68376.1	3×10^{-160}	459	79	52.1	71.7
γ			κ	CAC85920.1	5×10^{-149}	429	80	49.8	68.8
δ			ζ	CAC85921.1	1×10^{-171}	491	68	56.4	73.8
δ			θ	CAC85922.1	1×10^{-166}	478	68	53.8	73.8
δ			N/A	NP_001054799.1	8×10^{-136}	398	67	49.3	69.9
ε			η	NP_001053130.1	5×10^{-171}	484	88	55.3	72.8

Homo sapiens B56 isoforms were used as queries in Blastp searches against the NCBI database. Each *H. sapiens* B56 isoform was similar in its identity and similarity to each of the hits, and therefore no specific B56 isoform orthologs could be identified. However, the NCBI hits are listed with the *H. sapiens* query with which they had the lowest E-value. The NCBI hits are provided along with their protein accession number (Accession #), E-value (E), maximum score (M), percent query coverage (Q), percent identity (% I), and percent similarity (% S). The relatively high level of conservation of the B56 isoforms in plant species can be seen through the low E-values, high maximum scores, and high query coverage.

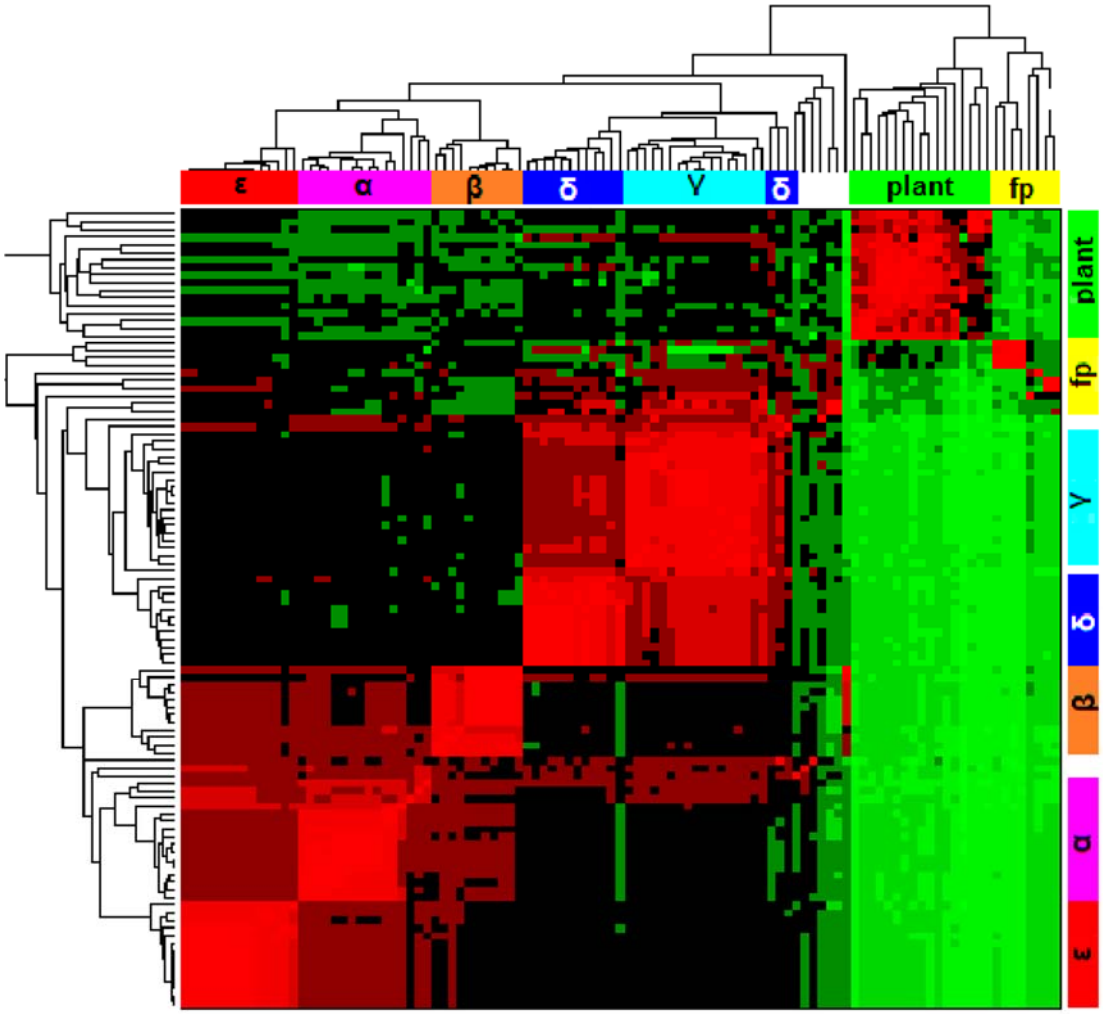


Figure 1. B56 Hierarchical Cluster Based on Percent Identity. Each B56 protein sequence was chosen in turn as the query sequence in Blastp search. The resultant pair-wise percent identities were plotted. The identity is indicated by color, ranging from the highest to lowest identity, progressively colored light red, red, maroon, black, dark green, medium green, and light green. The B56 isoform designation refers to the vertebrate isoforms; fp refers to fungal and protist B56 genes; plant refers to plant B56 genes.

Within the animal B56 genes, the B56αβε cluster has clearly grouped into its three isoforms and the B56γδ cluster has segregated into its two isoforms. The increased heterogeneity in the B56αβε cluster may suggest that the duplicate copies were retained because they acquired novel functions. The plant B56 genes do not segregate into distinct families, suggesting that plant B56 family genes underwent duplication later than in animal lineages. However, we only examined three plant species, and a broader analysis may reveal additional information. Species possessing a single B56 gene of each B56 subfamily (*Amphimedon queenslandica, Hydra vulgaris, Drosophila melanogaster, Caenorhabditis elegans*, and *Strongylocentrotus purpuratus*) generally fall in line with either the B56αβε or B56γδ subfamilies. Although this data visualization clearly delineates B56 subfamilies and suggests relationships between the B56 genes, it provides only an overview of B56 gene family divergence and evolution. A phylogenetic analysis was performed to trace the diversification of the B56 family.

B56 Gene Family Phylogeny

B56 was present as a single gene in eukaryotes prior to the divergence of animals, fungi, protists, and plants (Appendix A). Subsequently, four separate B56 clades evolved, mirroring species divergence. The plant B56 clade displayed the deepest division, followed by the protist B56 clade, with a local support value of 1.0, and then the fungal and animal B56 clades, with a local support value of 0.91. The B56αβε and B56γδ clades separated with a local support value of 0.93. Because of the structure of the B56 gene products, which are comprised of an approximately 400 amino acid conserved core domain and variable amino- and carboxy-termini, this global analysis included the core domain but the termini were excluded.

This was a consequence of the lack of significant identity between the amino- and carboxy-termini of distant B56 isoforms, as the algorithm used for these analyses eliminates any region in the alignment displaying a gap in any sequence in the phylogenetic tree construction. To study the evolution of B56 genes in more detail, we examined individual B56 gene clades separately from the remaining B56 gene family, thereby reducing the exclusion of the less-conserved termini.

Plants. Two different sets of nomenclature were initially used to describe the B56 genes, B56 and B', as several laboratories concurrently isolated the genes; the B' designations have been retained to describe the plant B56 genes (Latorre, Harris, & Rundle, 1997). The separate analysis of B56 plant genes yielded a phylogenetic tree with more sequence coverage than the global B56 analysis, as fewer sequence gaps reduced the extent of the sequences excluded in the FastTree phylogenetic tree construction. As *Chlamydomonas reinhardtii*, a unicellular green algae, is believed to be a representative of a terrestrial plant progenitor, the single B56 gene present in *C. reinhardtii* likely represents the B56 progenitor of multicellular plants (Willis & McElwain, 2002). The *C. reinhardtii* B56 gene is named wdb, which is a misnomer. It is not more highly related to its namesake, which was initially identified in *D. melanogaster*, than to the other B56 isoforms, and would more appropriately be renamed B56, without an isoform designation (Hannus, Feiguin, Heisenberg, & Eaton, 2002). The B56 gene was duplicated numerous times within multicellular plant species, as *Arabidopsis thaliana* has nine B56 genes while *Oryza sativa* (Japanese rice) has seven (Figure 3). A previous report proposed a B56 family tree composed of eight *A. thaliana* and five *O. sativa* genes based on a

neighbor-joining algorithm UPGMA (Unweighted Pair Group Method with Arithmetic Mean).

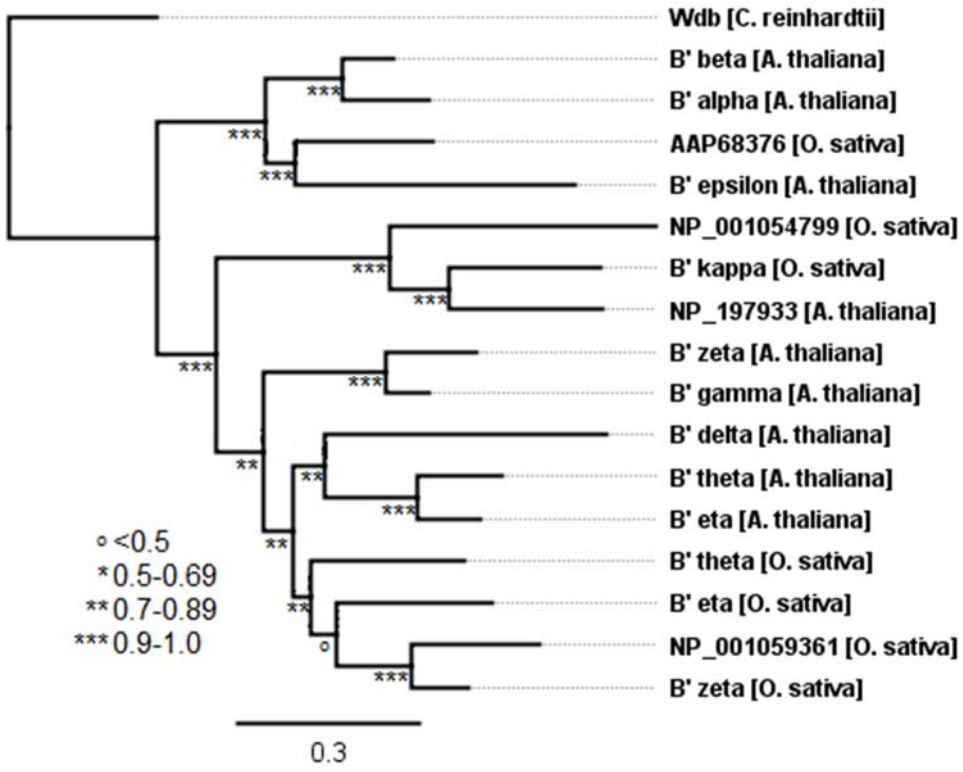


Figure 2. The Evolution of B56 Genes in Plants. A plant B' phylogenetic tree was built using FastTree 2. The tree was rooted with *C. reinhardtii* wdb. Horizontal lines are proportional to the substitution rate. The bar represents 0.3 changes per amino acid. Local support values are marked with ***, **, and º for 0.9–1.0, 0.7–0.89, and <0.5, respectively.

The tree consisted of three B56 subfamilies named B'α, B'η, and B'κ, with two A. thaliana genes, B'γ and B'δ, placed outside of the defined subfamilies (Terol, Bargues, Carrasco, Perez-Alonso, & Paricio, 2002). Our analysis employed several multiple sequence alignment algorithms and maximum likelihood methods for phylogenetic tree construction, and differs from that previously proposed (Figure 3 and data not shown). Three distinct clades were resolved. Each of these clades was present in both *A. thaliana*

and *O. sativa*, and therefore likely present prior to the divergence of monocots (*O. sativa*) and dicots (*A. thaliana*). One clade consists of B'β, B'α, and B'ε from *A. thaliana* and an unannotated gene from *O. sativa*, AAP68376, and was supported with a local support value of 1.0. The other two clades diverged with a local support value of 0.96. One of these clades consists of *O. sativa* (B'κ and NP_001054799) and *A. thaliana* (NP_197933) genes. The other clade consists of three subgroups: *A. thaliana* B'ζ and B'γ; *A. thaliana* B'δ, B'θ, and B'η; and *O. sativa* B'θ, B'η, B'ζ, and NP_001059361. As each of these subfamilies was either *A. thaliana* or *O. sativa* specific, they likely resulted from duplications occurring within each species. In summary, plants express a unique set of B56 gene orthologs and paralogs that have undergone both pre-speciation and post-speciation duplications.

Protists and Fungi. The protists, *Dictyostelium discoideum, Dictyostelium purpureum, Dictyostelium fasciculatum*, and *Polyspondylium pallidum*, each contain a single B56 gene, as do the fungi *Saccharomyces cerevisiae, Ashbya gossypii, Aspergillus nidulans*, and *Aspergillus niger* (Figure 4). In contrast, the fungus *Schizosaccharomyces pombe* possesses two B56 genes, likely resulting from a gene duplication occurring after the divergence of Aspergillus and S. pombe.

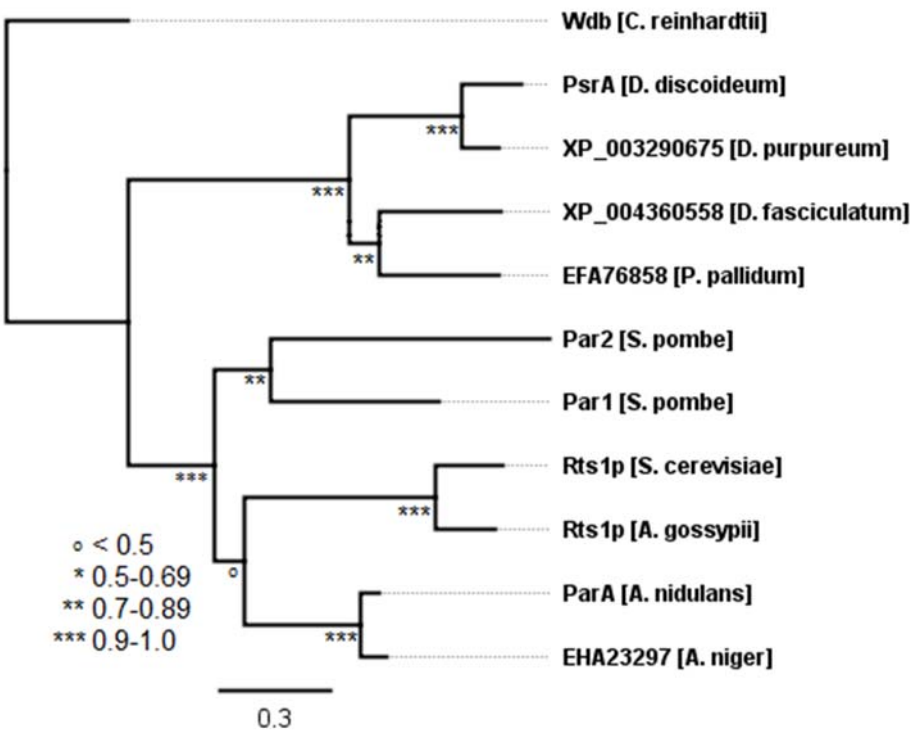


Figure 3. The Evolution of B56 Genes in Fungi and Protists. A B56 protist and fungal phylogenetic tree was built using FastTree 2. Each tree was rooted with *C. reinhardtii* wdb. Horizontal lines are proportional to the substitution rate. The bar represents 0.3 changes per amino acid. Local support values are marked with ***, **, and ° for 0.9–1.0, 0.7–0.89, and <0.5, respectively.

The lineage of the B56 gene does not precisely follow that of the fungal species. With regard to species divergence, *S. cerevisiae* and *A. gossypii* form a clade separate from *S. pombe* and *Aspergillus* species, whereas with the B56 gene, *S. cerevisiae, A. gossypii,* and *Aspergillus* form a clade separate from *S. pombe*. This is not uncommon, as many fungal species have acquired genes by horizontal gene transfer from not only distantly related fungal species, but also from bacteria and plants (Slot & Rokas, 2011, and Fitzpatrick, 2012).

Animals. A duplication of the B56 gene prior to the divergence of diploblastic and triploblastic species, animals with two or three germ layers, respectively, led to the

formation of two animal B56 clades, B56αβε (B56-1) and B56γδ (B56-2), with a local support value of 0.93 (Appendix A). The diploblasts *Amphimedon queenslandica* (sponge) and *Hydra vulgaris* (fresh water polyp) maintained one representative from each B56 subfamily (*A. queenslandica*: B56β and B56δ; H. vulgaris: B56α and B56δ). Within the triploblasts, protostomes *D. melanogaster* and *Caenorhabditis elegans* retained a single B56 gene from each subfamily: wdb and PPTR-1 from B56αβε, and B56-1 and PPTR-2 from B56γδ, respectively. In deuterostomes, *Strongylocentrotus purpuratus* (sea urchin) possesses a B56 gene from each subfamily, named B56α and B56δ. Although current nomenclature suggests that these genes may be more closely related to an individual isoform within the subfamilies, *A. queenslandica, H. vulgaris, D. melanogaster, C. elegans*, and *S. purpuratus*, B56αβε and B56γδ subfamily genes are derived from branches that diverged prior to divergence within the B56αβε and B56γδ subfamily clades. Consequently, *A. queenslandica* B56β, *H. vulgaris* B56α, *D. melanogaster* wdb, *C. elegans* PPTR-1, and *S. purpuratus* B56α should be more appropriately named; we suggest B56-1. In addition, *A. queenslandica* B56δ, *H. vulgaris* B56δ, *D. melanogaster* B56-1, *C. elegans* PPTR-2, and *S. purpuratus* B56δ should be more appropriately named to signify that they diverged prior to divergence of the B56γδ subfamily clade, perhaps with the name B56-2. In congruence with this nomenclature, the B56αβε subfamily would become the B56-1 subfamily and the B56γδ subfamily would become the B56-2 subfamily. Two rounds of whole-genome duplications occurred after the divergence of urochordates (e.g., sea squirt) and cephalochordates (e.g., lancelets) but prior to the divergence of cyclostomes (e.g., lamprey) and gnathostomes (jawed vertebrates) (Kuraku, Meyer, & Kuratani, 2009). Many paralogous genes present on

duplicated genomes were lost, but some remain. Not surprisingly then, chordates contain higher copy numbers of B56 genes than simpler organisms. *B. floridae* (lancelet, a chordate containing a neural cord and notochord but lacking vertebrae), whose genome sequence was first reported in 2008, has a full complement of five B56 genes (Putnam, et al., 2008). Three of these genes share 70%–90% identity and 82%–96% similarity with one another and fall into the B56αβε subfamily, but have not separated into distinct B56α, B56β, and B56ε isoforms; the other two B56 genes share 88% identity and 90% similarity and are within the B56γδ subfamily (Figure 5). This suggests that B. floridae branched off from vertebrate progenitors after two rounds of whole genome duplication, but prior to the time at which the B56αβε or B56γδ subfamilies evolved into the five vertebrate isoforms. In addition, the presence of three B56αβε genes and two B56γδ genes suggests that one B56αβε gene and two B56γδ genes were lost after the whole-genome duplications (or one B56γδ gene was lost after the first genome-wide duplication).

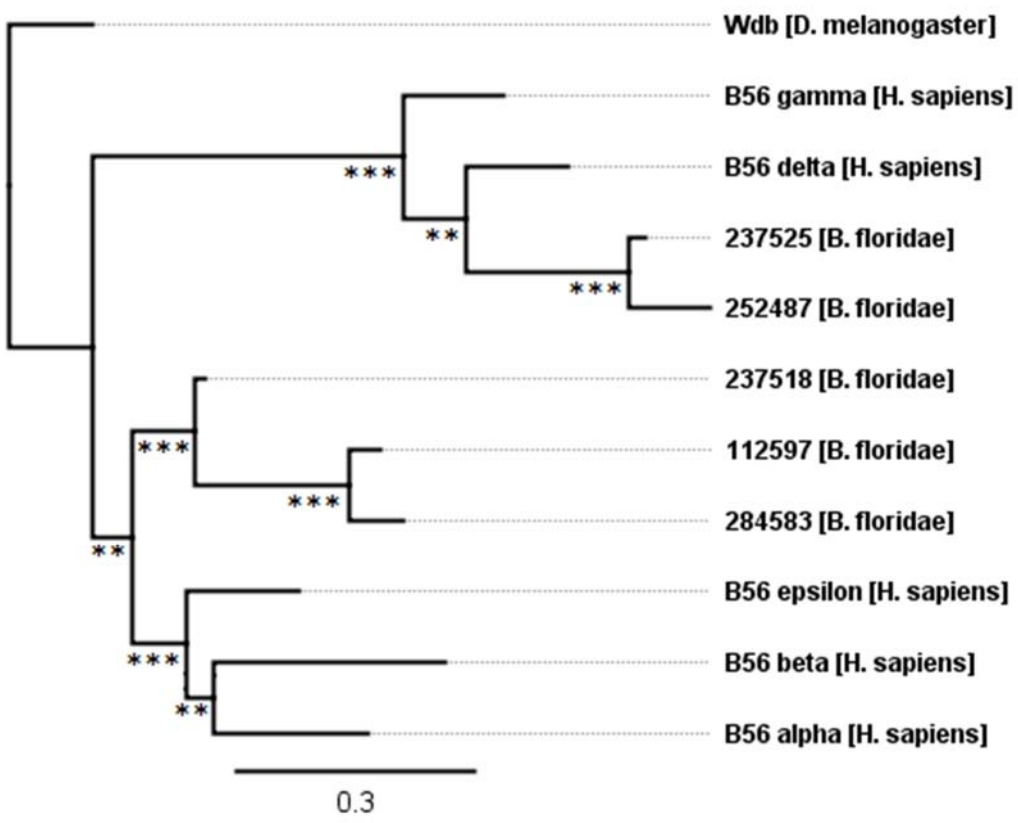


Figure 4. The Evolution of B56 Genes in the Simple Chordate *B. floridae. B. floridae* B56 isoforms have diverged into B56αβε and B56γδ subfamilies. The tree was rooted with *D. melanogaster* wdb. Horizontal lines are proportional to the substitution rate. The bar represents 0.3 changes per amino acid. Local support values are marked with *** and ** for 0.9–1.0 and 0.7–0.89.

The genome sequence of *P. marinus* (sea lamprey, a primitive vertebrate) was first reported in 2013, and is available at 5.0X whole genome coverage (Smith, et al., 2013,and e!Ensembl, 2014). We identified three *P. marinus* B56 genes: two B56αβε subfamily members and one B56γδ subfamily member (Table 2). Similar to *B. floridae*, one B56αβε subfamily member diverged from the B56αβε clade prior to isoform specialization (S4RGA7, Figure 6). However, S4RHV1 forms a clade with B56β, while S4RN43 forms a clade with B56δ. This suggests that *P. marinus* branched off from vertebrates after isoform specialization had started, but before it had been completed. *P.*

marinus' phylogenetic position suggests that it will possess a full complement of B56 genes; these genes will likely be revealed once a more complete coverage of the *P. marinus* genome is obtained. The five chordate B56 genes present in *B. floridae* are maintained in all chordates examined, with two exceptions, as described below.

The B56γδ clade has a two-fold lower substitution rate than the B56αβε clade before they first branch (leading to 12% and 25% divergence, respectively) (Appendix A). This finding correlates with the heat map (Figure 1), suggesting that the B56γδ clade is either newer than the B56αβε clade, or that it is under stronger selection to maintain its sequence. Our data suggest that the B56γδ clade has fewer substitutions because it resulted from the second genome-wide duplication, with the paralogs from the first genome-wide duplication being lost. However, our data does not rule out the possibility that the B56γδ clade may be more constrained. Future studies of synonymous/non-synonymous changes may determine the mechanism behind the conservation of the B56γδ clade, as well as the mechanism behind the limited B56 subfamily divergence in *B. floridae* and *P. marinus*.

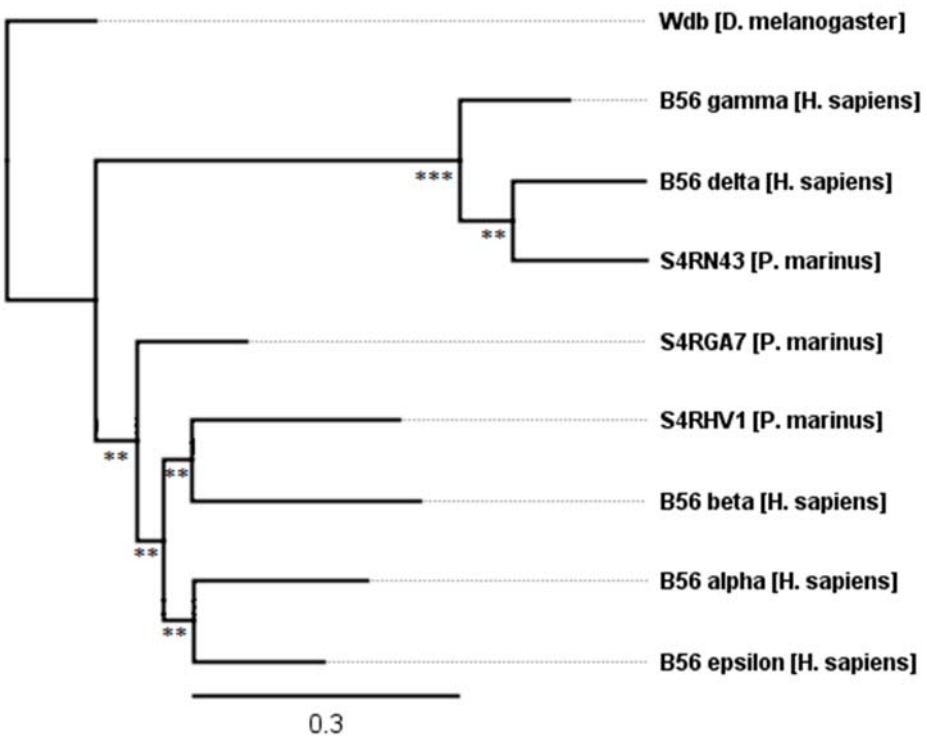


Figure 5. The Evolution of B56 Genes in the Simple Chordate *P. marinus*. One *P. marinus* B56 family member remains undifferentiated in the B56αβε subfamily while two correspond to the vertebrate isoforms B56α and B56δ. The tree was rooted with *D. melanogaster* wdb. Horizontal lines are proportional to the substitution rate. The bar represents 0.3 changes per amino acid. Local support values are marked with *** and ** for 0.9–1.0 and 0.7–0.89.

The B56αβε Subfamily

Within the B56αβε subfamily, individual B56 isoforms exhibited distinct levels of evolutionary change. *D. rerio* B56 αβε genes were most divergent from the rest of the species examined (Appendix A and Figure 7). This was not unexpected, as *D. rerio* (zebrafish) is the outlier of the vertebrate species examined. B56ε displayed the most stringent conservation, as it underwent 4% amino acid changes excluding *D. rerio*, and 13% amino acid changes including *D. rerio*. B56α displayed an intermediate level of conservation, as it underwent 8% amino acid changes excluding *D. rerio*, and 18% amino acid changes including *D. rerio*. B56β was the least conserved, as it underwent 23%

amino acid changes excluding *D. rerio*, and 29% amino acid changes including *D. rerio* (each also excluding *M. mulatta* (rhesus macaque)). *M. mulatta*'s B56β gene displayed an exceptionally high amino acid substitution rate, 25% since its divergence from other mammals. This was due in large part to a 63 amino acid region in the amino half of its core that lacks significant conservation with other B56 sequences. In addition, unlike B56α and B56ε, reptilian and amphibian B56β displayed a relatively high amino acid substitution rate, 14% versus 8% and 4% in B56α and B56ε, respectively, again suggesting reduced constraint on B56β sequence in these species (Appendix A). In summary, B56ε was under the strongest selective pressure to maintain its sequence, whereas B56α was under moderate selective pressure. B56β's selective pressure was similar to B56α in mammalian genes (excluding *M. mulatta*), but much looser in reptiles and amphibians. Alternatively, B56α and B56β may have been under positive selection.

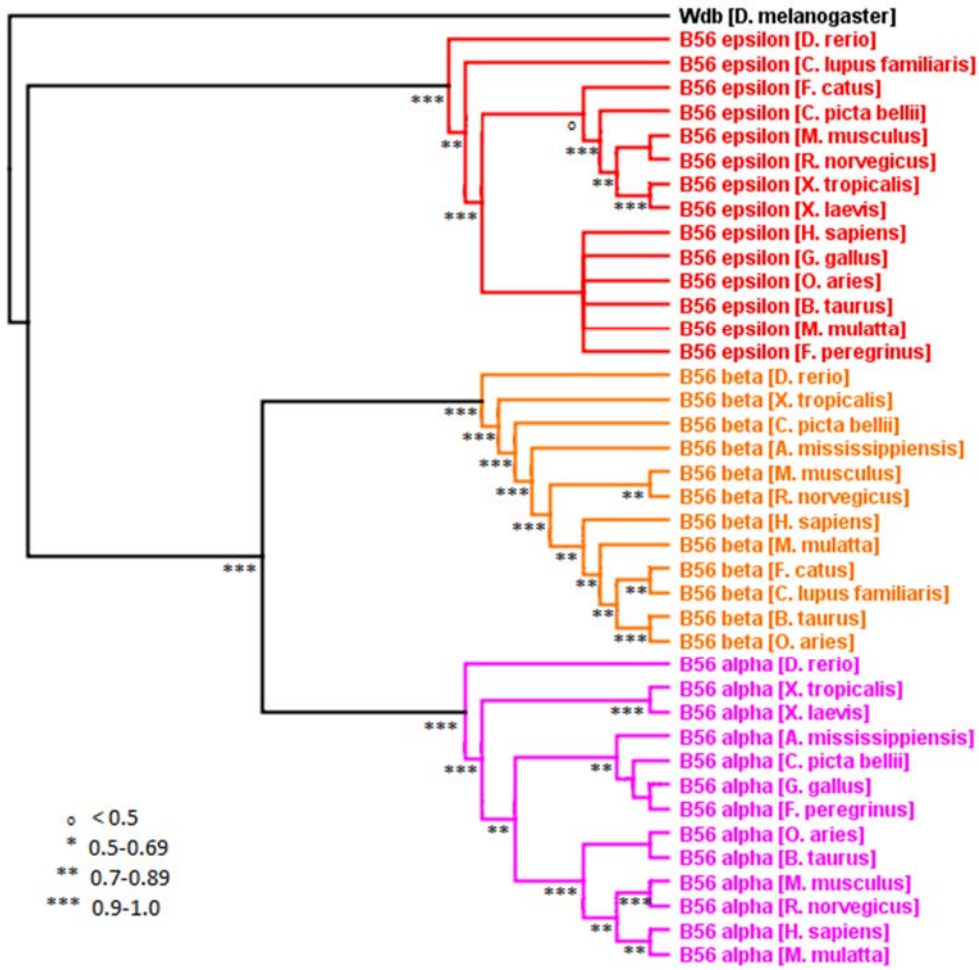


Figure 6. Vertebrate B56αβε Phylogenetic Tree. A B56αβε phylogenetic tree was built using FastTree 2. The tree was rooted with *D. melanogaster* wdb. Horizontal lines are not proportional to the substitution rate in order to display the clade topology. Local support values are marked with ***, **, and ° for 0.9–1.0, 0.7–0.89, and <0.5, respectively.

The evolution of the B56αβε subfamily is of particular interest, as isoforms within this subfamily have antagonistic effects on the canonical Wnt signaling pathway (Seeling, et al., 1999, and Yang, Wu, Tan, & Klein, 2003). B56ε is required for canonical Wnt signaling, whereas B56α inhibits Wnt signaling. There is also evidence suggesting that B56β has an inhibitory role (Seeling, et al., 1999). An earlier report used UPGMA to suggest that B56α and B56ε are more highly related to one another than to B56β (McCright, Rivers, Audlin, & Virshup, 1996). We carried out several analyses to sort out

the relationships within the B56αβε subfamily, using FastTree 2, Bayesian, and neighbor joining programs (Figure 7 and data not shown). The majority of our analyses showed that B56ε diverged prior to B56α and B56β. However, there were also instances where B56α and B56ε appeared more closely related. Therefore, our data is suggestive of B56ε being more distantly related to B56α and B56β, correlating with the functional data, but this conclusion is not robust. This ambiguity was likely due to the fact that there were few informational differences within the B56αβε clade.

The B56γδ Subfamily

A distinct analysis of the B56γδ subfamily was carried out to construct a B56γδ phylogenetic tree based on sequences specific for the B56γδ subfamily to gain insight that was not obtained from the global B56 analysis, which was based on the core domain. Both B56γ and B56δ vertebrate isoforms differed by approximately 12% when the B56δ/γ splice variants were not included in the analysis (Appendix A and Figure 8, and data not shown). With the inclusion of B56δ/γ, B56γ differed by 29%. This is due to the fact that B56δ/γ has an 82 amino acid amino-terminal region that is not related to the 19 amino acid amino-terminal region of B56γ/γ.

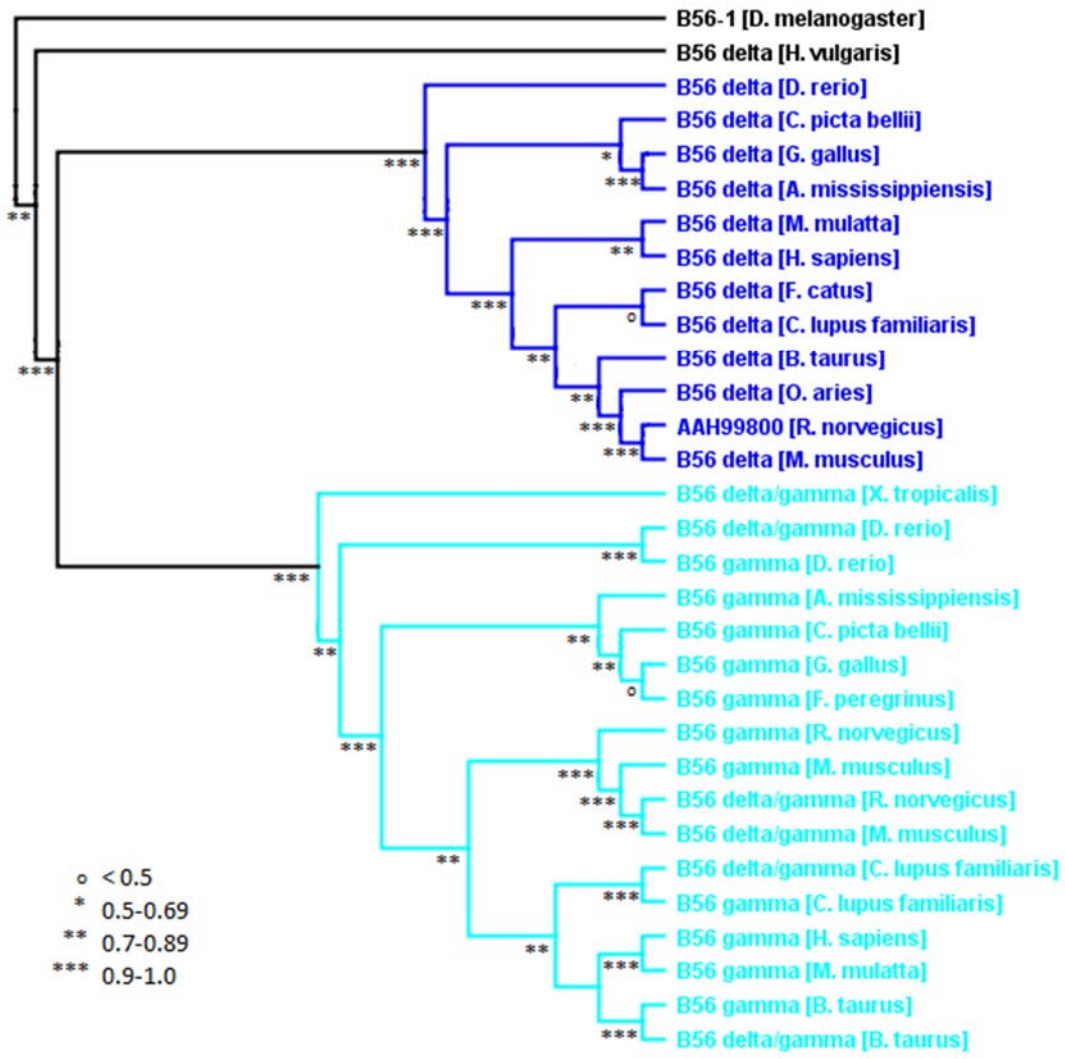


Figure 7. Vertebrate B56γδ Phylogenetic Tree. A B56γδ phylogenetic tree was built using FastTree 2. The tree was rooted with *D. melanogaster* B56-1. Horizontal lines are not proportional to the substitution rate in order to display the clade topology. Local support values are marked with ***, **, * and ° for 0.9–1.0, 0.7–0.89, 0.5–0.69, and <0.5, respectively.

H. vulgaris contains one B56 gene from each subfamily. The B56γδ family member of *H. vulgaris* segregated within the B56δ clade in the larger phylogenetic analysis of B56 (Appendix A). However, all other B56 proteins that were examined from nonchordate animal species did not segregate into distinct isoforms within the B56 subfamily clades. We therefore included the *H. vulgaris* B56γδ protein in our analysis of

the vertebrate B56γδ subfamily to more accurately place *H. vulgaris* B56γδ within the B56 tree. This B56γδ-specific analysis placed *H. vulgaris* B56γδ within the B56γδ subfamily but outside of the B56γ and B56δ isoform clades. Therefore, the *H. vulgaris* B56γδ protein now falls in line with other diploblasts (*A. queenslandica*), protostomes (*D. melanogaster* and *C. elegans*), and primitive deuterostomes (*S. purpuratus*) in which the B56 genes have not evolved into distinct isoforms.

The Loss of Vertebrate B56 Genes

B56δ was not found in *X. laevis* or *X. tropicalis* but was present in *A. mexicanum*, a closely related amphibian (Figure 9). As *X. tropicalis*'s genome has been completely sequenced, this strongly suggests that the B56δ gene was lost in these two *Xenopus* species. Within archosaurs, B56β was not found in *G. gallus* and *F. peregrinus* but was present in *Alligator mississippiensis*. As *G. gallus'*, *F. peregrinus'*, and *A. mississippiensis*'s genomes have all been completely sequenced, this strongly suggests that the B56β gene was lost in the Aves lineage. These two separate B56 gene losses suggest that B56 isoforms may share some overlapping functions. Since the amino- and carboxy-terminal variable domains of the protein are likely to be key in carrying out isoform-specific functions, similarities in these regions may be important in understanding the potential for functional overlap between B56 isoforms.

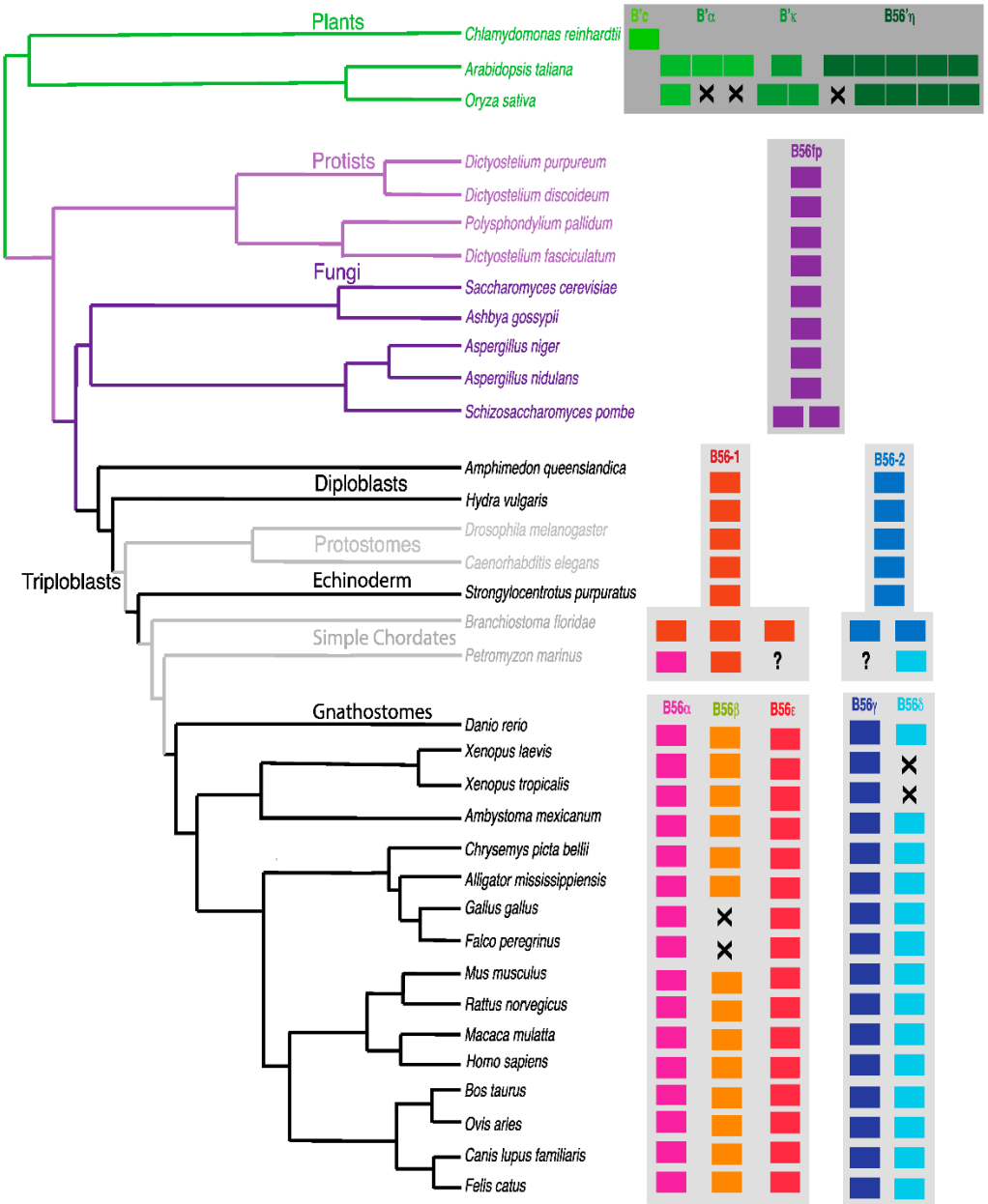


Figure 8. Distribution of B56 Genes in Plants, Protists, Fungi, and Animals. A species tree was constructed based on the Tree of Life (Maddison & Schulz, 2007). B56 genes are represented by rectangles; the absence of a B56 gene is signified with an X; uncertainty in the presence of a B56 isoform is signified by a question mark.

Overlapping functions would be more likely to occur within a B56 subfamily. For example, the function of B56δ in *Xenopus* is more likely to have been maintained by B56γ rather than by a B56 αβε family member, whereas the function of B56β in Aves would more likely be carried by B56α or B56ε. Indeed, the amino-terminal variable regions of human B56α, B56β, and B56ε are approximately 50% identical and 60% similar, while their carboxy-termini lack significant similarity. Therefore, the similarity of the amino-terminal domains in the B56αβε subfamily may provide sufficient functional overlap to allow the loss of one family member. The amino-terminal variable region of human B56γ and B56δ lack significant similarity, but their carboxy-termini possess approximately 50% identity and 56% similarity, therefore their carboxy-termini, but not their amino-termini, may provide some overlapping functions. As little is known about the substrate of each B56 isoform, it is hypothesized that since the core domain is highly conserved, it is the termini that determines the substrate of each isoform. A nonsynonymous/synonymous substitution analysis of the B56 genes showed that the B56αβε subfamily displayed higher purifying selection at the amino-termini than at their carboxy-termini (Qureshi, Cho, Choudhary, & Seeling, 2015). In the B56γδ subfamily, the carboxy-termini showed stronger purifying selection than its amino-termini. These findings indicated that in the B56αβε subfamily, the amino-termini interacts with the subfamily-specific protein partner, while the carboxy-termini encodes for the specificity within the subfamily. In the B56γδ subfamily the amino-termini encodes for the specificity, while the carboxy-termini interacts with the subfamily-specific protein partner.

Alternatively, we previously identified an evolutionarily conserved alternative splice form of B56γ that contains a B56δ-like amino-terminal variable region (Baek & Seeling, 2007). This B56δ/γ isoform may be sufficient to carry out B56δ-specific functions in *Xenopus*. Qureshi et al.'s findings support this idea that the amino-termini determined specific isoform can still maintaining the carboxy-termini subfamily identity thus carrying out binding to specific substrates (Qureshi, Cho, Choudhary, & Seeling, 2015). Indeed, as B56δ/γ and B56γ share their B56γ core and carboxy-termini, they are somewhat intermingled on the phylogenetic tree, with B56δ/γ and B56γ from the same species, such as *B. taurus, C. lupus familiaris*, and *D. rerio*, often segregating together (Figure 8).

Conclusions

The B56 gene family is highly conserved. B56 was present as a single gene in simple eukaryotes, but was duplicated prior to the divergence of protostomes and deuterostomes. Further duplications occurred in chordates, resulting in three B56αβε and two B56γδ genes. These genes remained similar to one another in simple chordates, but diverged into five distinct isoforms in vertebrates. B56ε was most highly conserved, followed by B56α, B56γ, and B56δ, which displayed an intermediate level of conservation; B56β was the least conserved. This divergence in vertebrates likely led to the ability of B56 family members to regulate numerous signal transduction pathways.

The deletion of B56δ in *Xenopus* species and B56β in Aves suggests that some B56 isoforms may have overlapping functions. However, in the case of B56δ, there exists an evolutionarily conserved mixed-isoform alternative splice form that contains a B56δ-like amino-terminal variable domain upstream of the B56γ core region (Baek & Seeling,

2007). This strengthens the argument that the variable regions largely determine isoform specificity, as the presence of a B56δ amino-terminal variable domain appears to compensate for loss of the B56δ.

CHAPTER III

Future Work

The upregulation of Wnt should result in patterning of cells that signal the formation of the body axis. This upregulation of Wnt signaling is analogous to that which causes tumor formation. Identifying the cause of tumor formation will help in the design of therapeutics that could restore normal PP2A activity. The expression of B56 in human colon cancer cell lines downregulates β-catenin and acts as a tumor suppressor, while the deletion of Aβ isoform in human colon tumors supports the idea that PP2A is indeed a suppressor (Polaki, 2000). There are two genes that code for the A subunit, Aα and Aß. These two genes share 87% identity in humans. The Aα is found in 90% of the PP2A holoenzymes, while Aß is only found in 10% of the PP2A holoenzymes in humans (Yang & Phiel, 2010). For wild type PP2A function, the A subunit must properly bind to the B and C subunits. In many types of cancer, it has been found that mutations in the A subunit inhibit this binding resulting in upregulation of the Wnt signaling pathway. P53S, V436A, E64G, and R418W are all point mutations of the A subunit of PP2A. P53S and V436A, were identified in the Aβ subunit. In a study by Wang, et al. on cancer cell lines, it was determined that the P53S mutation, found in lung cancer, occurs when a cytosine is mutated to a thymine coding for serine instead of the wild type proline (Wang, et al., 1998). Ruediger et al. found that this mutation reduces B72 binding (Ruediger, Pham, & Walter, 2001). Wang et al. looked at another point mutation found in a colon adenocarcinoma. A thymine is mutated to cytosine this causes the codon to code for Alanine instead of the wild type valine (V436A). These alteration affect the 11-15 repeats that are necessary for the binding of the Aβ subunit to the C subunit (Wang, et al., 1998).

This mutation had a reduction in the B72 subunit and C subunit binding (Ruediger, Pham, & Walter, 2001).

E64G and R418W mutations in the Aα subunit found in human lung and colon cancers, respectively (Ruediger, Pham, & Walter, 2001). Ruediger's in vitro study showed the Aα subunit mutations HEAT repeats are defective at interacting with the B and C subunits of PP2A (Ruediger, Pham, & Walter, 2001). This finding suggests that the Aα subunit plays a role in cancer and tumor development. These mutations were also defective at interacting with the B and C subunits. Not all mutations were equally defective, R418w and Δ171-589 mutations of the A subunit cannot bind any of the B and C subunits. The E64G mutation, however, bound all but B56. This suggests that in the embryos with the R418W and Δ171-589 mutations, the PP2A is not functional at all, while E64G loses only the B56 function. Ruediger's findings support the previous proposal that PP2A operates as a tumor suppressor. Mutations in Aα and Aß destroy PP2A's tumor suppressing function by inhibiting or altering the binding of the B or C subunits (Ruediger, Pham, & Walter, 2001). Ruediger et al. found the cancer-associated Aα mutation E64G increased the incidence of lung cancer in 50% to 60% of the transgenic mice that had been treated with benzopyrene, and they found that the tumor suppressor function of PP2A was dependent on the activation of p53 (Ruediger, Ruiz, & Walter, 2011). Multiple gene mutations that deregulate multiple signaling pathways are required for a tumor to form (Chial, 2007). PP2A A subunit mutations may occur frequently in cancers because multiple pathways can be deregulated such as p53, Wnt, etc. with a single point mutation. Figueroa-Aldariz et al. found that normal or cancer cells with regular Wnt signaling function express mRNAs coding for Aα and Aβ isoforms, but

the cancer cells with altered Wnt signaling do not express the Aβ isoform (Figueroa-Aldariz, Castaneda-Patlan, Santoyo-Ramos, Zentella, & Robles-Flores, 2014). This indicates that Aβ isoform functions as a tumor suppressor and when that function is lost the cell becomes cancerous.

B56 subunits are involved in many different signaling pathways. The A subunit mutations that are cancerous and affects B56 binding may alter the Wnt pathway. The proposed hypothesis is that mutations that inhibit the A subunit from binding to the B56 subunit will increase Wnt signaling. The question behind this research is how do the P53S, V436A, E64G, and R418W mutations of the A subunit affect Wnt signaling?

Xenopus laevis embryos will be comicroinjected with Wnt and the A subunit mutations in order to determine their effects on Wnt signaling. The night before the planned fertilization, the female *Xenopus* will be induced to lay eggs by injecting human chorionic gonadotropin hormones and kept overnight in 15^0C incubator. The next day the female will be "squeezed" to simulate the male trying to mate. The eggs will be fertilized using testes acquired from a survival surgery. Fertilized eggs will then be incubated in cysteine to remove the jelly coat to allow for microinjections and then rinsed in an R/3 solution to prevent the cysteine from degrading the embryos. The embryos will then go through cell division for approximately two hours until they reach the four cell stage.

A ficoll solution will be added to stabilize the embryonic membrane. At the four cell stage, the embryos will be comicroinjected with Wnt and the wild type or mutated PP2A A subunits. P53S, V436A, E64G, and R418W RNA will be comicroinjected into the embryos on the ventral side independently. The wild type A subunit, and β-galactosidase will be comicroinjected with Wnt as the positive and negative controls. β-

galactosidase will be used to equalize the RNA concentration being injected. The embryos will be left to develop for a period of approximately 72 hours, after which time the phenotype will be analyzed. When Wnt alone is injected into the embryos, Wnt signaling is upregulated and a secondary axis is produced. The negative control β-galactosidase does not play a role in the Wnt pathway, so when comicroinjected with Wnt it will produce embryos with a secondary body axis (Li, Yost, Virshup, & Seeling, 2001). Preliminary data shows that when wild-type A subunit is comicroinjected with Wnt it rescues the phenotype resulting in a normal embryo. Using this information, the phenotypes of the comicroinjected A mutations will be evaluated to determine their effects on the Wnt pathway. There are four possible outcomes for the microinjections: 1) the mutation will rescue the phenotype as it does with the wild-type A subunit, thus the mutation does not affect B56 binding nor Wnt signaling; 2) there will be a reduced level of rescue, due to reduced binding between the A subunit and B56; 3) the mutation will not rescue the phenotype, which indicates that the A subunit has lost its ability to downregulate Wnt signaling, or 4) the embryos will be more highly dorsalized, because Wnt signaling is activated due to a dominant-negative A mutation.

P53S has been shown to reduce the binding of the B72 subunit while C subunit binding is normal Therefore Wnt signaling may be upregulated due to the inhibition of the B72 subunit interaction with Naked Cuticle, which interacts with Disheveled and acts as a switch to downregulate Wnt signaling (Creyghton, et al., 2005). V436A has reduced B72and C subunit binding therefore the interaction with Naked Cuticle cannot occur and Wnt signaling cannot be regulated and the C subunit will not act as a Wnt pathway

repressor, thus Wnt will be upregulated (Creyghton, et al., 2005, and Ruediger, Pham, & Walter, 2001).

E64G inhibits the A subunit from binding to the B56 subunit thus we expect the mutation will not rescue the phenotype due to loss of binding and Wnt signaling will not be rescued. R418W inhibits the A subunit from binding to all B subunits as well as the C subunit. This is expected to inhibit all PP2A activity, which will upregulate Wnt signaling and produce secondary body axes when injected in the ventral side of the embryo. The injected embryos should not be rescued.

Preliminary results have been obtained. The β-galactosidase was an effective negative control resulting in dorsalization in one third of the embryos after injection. Wild type A subunit was found to rescue the phenotype in all of the injected embryos. E64G and Wnt have been comicroinjected and resulted in majority dorsalization. These results indicate that the E64G mutation affects the Wnt pathway as a dominant-negative mutation with more dorsalization than with the β-galactosidase control and therefore likely sequesters proteins from binding to the endogenous wild-type A subunit. The V436A and P53S mutations both exhibit low levels of dorsalization, indicating that it does not rescue the phenotype. The R418W mutation appears to exhibit a high percentage of dorsalization, however, less than E64G.

Alternatives Preliminary data suggests that the E64G mutation will act as expected but if it does not show the expected results of the A mutations upon replication of microinjection, it could be due to an expression from endogenous A subunit from the other allele that is strong enough to overcome the negative effects of the mutations and results in a normal phenotype. Although this is not expected, if this happens, the wild

type A subunit could be knocked down with MOs. Another reason could be the mutated RNA used is from the Aα subunit.

Normal or cancer cells with regular Wnt signaling function express mRNAs coding for Aα and Aβ isoforms, but the cancer cells with altered Wnt signaling do not express the Aβ isoform mRNA. They also found that Aβ protein levels are lost in all colon cancer cells which further indicates its role as a tumor suppressor (Figueroa-Aldariz, Castaneda-Patlan, Santoyo-Ramos, Zentella, & Robles-Flores, 2014). The Aβ subunit mutations may be more effective than the Aα mutations. If this is found true the Aβ DNA can be mutagenized and new RNA will be made. Another reason is the injected mutations are human genes. If they are found to be ineffective *Xenopus* genes could be mutated.

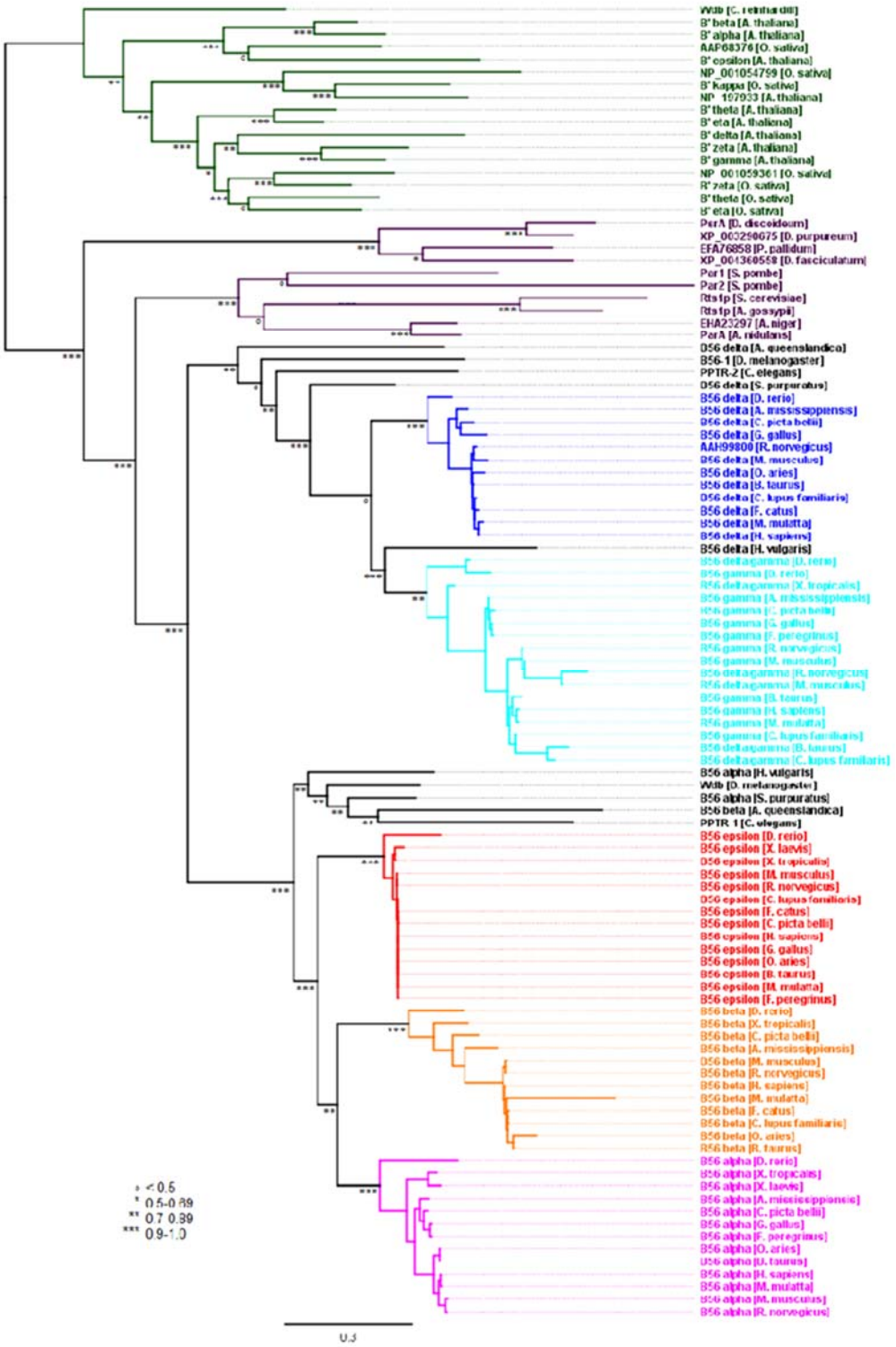
In vertebrates, the increase in Wnt signaling results in a secondary body axis, but in the colon it causes increased cell proliferation and tumor formation. Normal colonic cells proliferate at a rate that matches colonic cell death (Peifer & Polakis, 2000). When the numbers are matched the cells are sent a signal to stop proliferation. In most colon cancers, APC, a regulator of the Wnt pathway, is inactivated and the colonic cells are continuously increasing in number due to the signal being locked in an ON position (Peifer & Polakis, 2000).

APPENDIX A

A B56 phylogenetic tree

A B56 phylogenetic tree was built using FastTree 2. A B56 sequence was used in this analysis only if it contained 90% or more of the conserved core domain. Horizontal lines are proportional to the substitution rate. The bar represents 0.3 changes per amino acid. Local support values are marked with ***, **, *, and º for 0.9–1.0, 0.7–0.89, 0.5–0.69, and <0.5, respectively.

See next page for figure.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B" beta [A. thaliana]
B" alpha [A. thaliana]
AAP68376 [O. sativa]
B" epsilon [A. thaliana]
NP_001054799 [O. sativa]
B" kappa [O. sativa]
NP_197933 [A. thaliana]
B" theta [A. thaliana]
B" eta [A. thaliana]
B" delta [A. thaliana]
B" zeta [A. thaliana]
B" gamma [A. thaliana]
NP_001059361 [O. sativa]
B" zeta [O. sativa]
B" theta [O. sativa]
B" eta [O. sativa]
PerA [D. discoideum]
XP_002250675 [D. purpureum]
EFA76858 [P. pallidum]
XP_004360558 [D. fasciculatum]
Par1 [S. pombe]
Par2 [S. pombe]
Rts1p [S. cerevisiae]
Rts1p [A. gossypii]
EHA23297 [A. niger]
PerA [A. nidulans]
B56 delta [A. queenslandica]
B56-1 [D. melanogaster]
PPTR-2 [C. elegans]
B56 delta [S. purpuratus]
B56 delta [D. rerio]
B56 delta [A. mississippiensis]
B56 delta [C. picta bellii]
B56 delta [G. gallus]
AAH99806 [R. norvegicus]
B56 delta [M. musculus]
B56 delta [O. aries]
B56 delta [B. taurus]
B56 delta [C. lupus familiaris]
B56 delta [F. catus]
B56 delta [M. mulatta]
B56 delta [H. sapiens]
B56 delta [H. vulgaris]
B56 deltagamma [D. rerio]
B56 gamma [D. rerio]
B56 deltagamma [X. tropicalis]
B56 gamma [A. mississippiensis]
B56 gamma [C. picta bellii]
B56 gamma [G. gallus]
B56 gamma [F. peregrinus]
B56 gamma [R. norvegicus]
B56 gamma [M. musculus]
B56 deltagamma [R. norvegicus]
B56 deltagamma [M. musculus]
B56 gamma [B. taurus]
B56 gamma [H. sapiens]
B56 gamma [M. mulatta]
B56 gamma [C. lupus familiaris]
B56 deltagamma [B. taurus]
B56 deltagamma [C. lupus familiaris]
B56 alpha [H. vulgaris]
WWb [D. melanogaster]
B56 alpha [S. purpuratus]
B56 beta [A. queenslandica]
PPTR-1 [C. elegans]
B56 epsilon [D. rerio]
B56 epsilon [X. laevis]
B56 epsilon [X. tropicalis]
B56 epsilon [M. musculus]
B56 epsilon [R. norvegicus]
B56 epsilon [C. lupus familiaris]
B56 epsilon [F. catus]
B56 epsilon [C. picta bellii]
B56 epsilon [H. sapiens]
B56 epsilon [G. gallus]
B56 epsilon [O. aries]
B56 epsilon [B. taurus]
B56 epsilon [M. mulatta]
B56 epsilon [F. peregrinus]
B56 beta [D. rerio]
B56 beta [X. tropicalis]
B56 beta [C. picta bellii]
B56 beta [A. mississippiensis]
B56 beta [M. musculus]
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B56 alpha [G. gallus]
B56 alpha [F. peregrinus]
B56 alpha [O. aries]
B56 alpha [B. taurus]
B56 alpha [H. sapiens]
B56 alpha [M. mulatta]
B56 alpha [M. musculus]
B56 alpha [R. norvegicus]
* < 0.5
* 0.5-0.69
** 0.7-0.89
*** 0.9-1.0
0.5

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    - B56 regulates cell formation by inhibiting Wnt signaling. Mutations in the A subunit of the PP2A complex inhibit the binding to the B subunit which is hypothesized to cause an increase of Wnt signaling. This increase in Wnt causes tumor formation. *Xenopus laevis* embryos are microinjected with the mutated RNA to produce an overexpression of Wnt and excessive cell formation. The expected results are a dorsalized phenotype with a secondary body axis
    - Awarded $1,250 from Texas Academy of Science Grant, March 2015

- A Field Guide to the Amphibians and Reptiles of the Houston Museum of Natural Science
    - Published by HMNS, Spring 2016

- Evolutionary Analysis of the B56 Gene Family of PP2A Regulatory Subunits

    - Using bioinformatic phylogenetic analysis, the relationships of the evolutionarily conserved B56 isoforms were determined. The five isoforms α, β, δ, γ, and ε were compared across 33 species commonly used as model organisms.
    - Published in the International Journal of Molecular Sciences, May 2015
- Contributed to the Dissection Instructions in the BIOL 1413 General Zoology Manual by Rose (Fall, 2014)

- Herpetology Symposium, Oral Presentation, April 2016
- Texas Academy of Science Meeting, Oral Presentation, March 2016
- Society of Developmental Biology Conference, Poster Presentation, October 2015
- SHSU Undergraduate Research Symposium-Moderator, April 2015
- Texas Academy of Science Meeting, Poster Presentation, March 2015
- Graduate Student Research Exchange, Oral Presentation, February 2015
- Biological Sciences Graduate Research Symposium, Poster Presentation, December 2014
- SHSU Undergraduate Research Symposium, Poster Presentation, April 2014

Certification and Skills

- IACUC certifications
    - Working with Mice in Research- 01/14/2016
    - SHSU Research Administrators- 1/03/2014
    - RCR for Social, Behavioral, and Education (SBE) Sciences-11/03/2014
    - Working with Amphibians in Research Settings-10/06/2013
    - Working with the IACUC- 09/27/2013

- Maintained a *Xenopus laevis* research colony
- Trained undergraduates in colony maintenance and lab techniques
- Minimal work with mouse colony (three months)
- Assisted in teste removal-survival surgeries
- Animal dissections
- In vitro fertilization
- Microinjections of *Xenopus* embryos
- RNA purification and isolation
- Basic PCR and Western blotting techniques
- Bioinformatic analysis using MUSCLE and Geneious software

Organizations

- Society of Developmental Biology member, Fall 2015-present
- Texas Academy of Science member, Spring 2015-present

- Biological Sciences Graduate Student Organization (BSGSO), 2014-present
- Elliott T. Bowers Honors College, 2010-2014
- Golden Key National Honors Society, 2011-2014

Work Experience

- Brookshire Brothers Customer Service Clerk/Cashier-Spring 2014-Present
- Research Assistant- Summer 2016
- Teaching Assistant
  - Human Anatomy, Fall 2014 - Spring 2016
  - Vertebrate Embryology, Fall 2014, 2015
  - Cellular Biology, Spring 2014, 2015, 2016(Lead TA)
  - Zoology, Fall 2014
- Student Note-taker, Services for Students with Disabilities Center, Spring 2012-Spring 2014