A Novel Correlational Study of Myosin Heavy Chain 16 and Skull Shape Evolution

Abstract:

Myosin Heavy Chain 16 (MYH16) is a super fast-twitch masticatory muscle protein expressed in most organisms. In 2004, Dr. Hansel Stedman's lab discovered a mutation in MYH16 in the human lineage, and hypothesized that it led to the increased brain size in humans relative to non-human primates. Due to the lack of modern bioinformatics tools at their disposal at the time, Dr. Stedman could not fully prove or disprove this hypothesis, leading to intense debate. In an attempt to provide more information on this subject, this project seeks for a relationship between the presence/absence of MYH16 and brain size relative to the organism's body. This study found that for organisms with mutated MYH16, there was a correlation between mutation and brain size, but for organisms with deleted MYH16, there was no such correlation. Though not a definitive answer, the results of this study provide an intriguing modification to Dr. Stedman's original theory.

1. Introduction:

Evolution, at its most basic level, is the origination of a new species from an existing species. Organisms evolve as their genes change due to mutations of any kind, and within the broad scope of evolution, one can focus on the evolution of genes to understand how exactly one species evolved from another (Reece, 2014). Genes evolve through the process of molecular evolution, in which DNA is mutated in some manner and a new protein comes into existence (Reece, 2014). If the new protein is in no way detrimental to the survival of the organism, said organism will be considered fit, or likely to pass on its genetic material through reproduction (Reece, 2014). This would allow the gene, and thus its protein, to get passed on within the species indefinitely. Depending on the severity of the change the mutation brings about, organisms with this mutated protein may be considered a new species. For example, Charles Darwin claims that for any species of wolf in mountainous or lowland regions, “the wolves inhabiting a mountainous district, and those frequenting the lowlands, would naturally be forced to hunt different prey; and from the continued preservation of the individuals best fitted for the two sites, two varieties might slowly be formed” (Darwin, 1859). These varieties would be different solely through genetic changes that are beneficial to the survival of organisms in their respective areas - such as shorter legs for wolves in the mountains - brought about by changes in genes, which make proteins, that constitute the organisms.

Variations between organisms are primarily created through the mechanism of natural selection. Natural selection is the theory that organisms considered more fit than other organisms will pass on its genes (Reece, 2014). Fitness is the ability for an organism to survive in an area

and reproduce (Reece, 2014). For example a crocodile with a stronger bite will be more likely to capture prey and survive, so it will be more fit to reproduce. Thus, the crocodiles with stronger bites may be "selected for," and pass on their genes to their offsprings (Erickson et al. 2012). Over time, the stronger crocodiles will constantly be selected for, and may become their own species of crocodile, with a skull shape to account for increased jaw muscle size and strength (Erickson et al. 2012). Simultaneously, genes can be selected against if they are mutated in such a way as to harm the fitness of an organism. For example, weakened jaws in a mutated crocodile would be unlikely to get passed on because they would probably limit the ability for an organism to survive, and consequently reproduce (Erickson et al. 2012).

In 2004, researchers at the University of Pennsylvania noticed an unusual exception to this rule. Namely, they discovered that an unusually strong muscle protein found in the jaws of many animals called Myosin Heavy Chain 16 (MYH16) had a destructive 2 base pair deletion in humans. A deletion is where part of the gene's DNA sequence is omitted and the protein that comes from the sequence may change severely with a frameshift deletion (a deletion where one or two bases are omitted). After some analysis, these researchers concluded that this mutated myosin (a muscle protein), which was rendered nearly useless by its mutation, was selected for and therefore conserved in *Homo sapiens* (Stedman et al. 2004). They argued that the mutation that weakened MYH16 must have been beneficial to *Homo sapiens* in some way, as it was conserved for over 2 million years. Based on the location of MYH16 in chimpanzees and a few other organisms that have MYH16, the researchers drew the conclusion that the presence of mutated MYH16 in human face muscles led to the development of the large brain cap in our species (Stedman et al. 2004). Their reasoning revolved solely around the large strength of

MYH16 exerting force on the bones of skulls in other organisms, arguing that animals who carried the weakened form of MYH16 would not need crests in their skulls, and thus their braincaps could develop more (Stedman et al. 2004).

With growing technology in the field of bioinformatics, there is more information readily available on evolution as a whole (Stamatakis, A. 2014). Researchers in Rochester, New York recently used phylogenetic analysis, a subset of bioinformatics, in order to trace back the whole evolutionary tree of a protein called importin (Mason, D. A. et al. 2009). They took samples of the protein from a series of organisms and aligned the sequences using protein alignment software (Mason, D. A. et al. 2009). They then used analysis of introns and sequence mutations to order the proteins in order of evolution by group of importin and organism kingdom in a phylogenetic tree shown in figure 1 (Mason, D. A. et al. 2009).

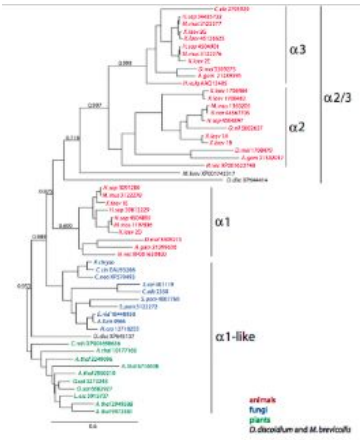

FIgure 1: The complete phylogenetic tree detailing the evolution of importin

With similar methods, one could analyze other proteins more in depth and possibly look at their effects on a specific phenotype. If needed, the same procedure above could be used in conjunction with other research methods to trace back any given protein. For MYH16, the use of the above methods could show the evolution of MYH16 and detect specific MYH16 samples.

Looking back at MYH16, one could use this new information to ask whether there is substantial evidence for or against the brain cap hypothesis proposed in the 2004 Nature paper. In order to answer this, one would need to find animals with full length MYH16 and separate animals with mutated MYH16 or no MYH16 at all. This brings up another question: How would

one find organisms with and without MYH16? From there, how could one fully determine the effects of MYH16 on skull shape? And most importantly, can the presence or lack of a single muscle protein really change something as concrete as skull shape at all? In order to look into these questions, one would have to search for information on bioinformatics and creating phylogenetic trees as well as information on general evolution. The parameters for these searches would likely consist of searching only academic papers that are peer edited. Phylogenetic trees are visual representations of molecular evolution, often used in molecular biology/evolutionary studies to show evolutionary trends and similarities. For the sake of this paper, phylogenetic trees will be used to form an expansive tree detailing the main types of myosin, and from there, finding potential MYH16 candidate myosins in organisms and linking them to their nearest related protein.

Figure 2: Skull crests are raised sections of bone on the skulls of organisms that are typically directly above or attached to the skull cap

1.1: Literature Review

Currently, there are two main competing theories to the evolution of skull shape in *Homo Sapiens*. As mentioned earlier, in 2004 Dr. Hansell Stedman hypothesized that the lack of an ultra fast-twitch myosin heavy chain (MYH16) allowed the skulls of early human ancestors to lose their crests and evolve to increase the size of the human braincap (Stedman et al. 2004). However, other experts in the field, like Dr. Alan Walker from Penn State find this theory too simplistic and describe it as “an extremely unlikely proposition” because of the lack of evidence

supporting Stedman's claim (Walker, 2004) . This lack of evidence for Stedman's hypothesis, however, is defensible since the paper was written in 2004, and most of the powerful bioinformatics programs one would use to justify his hypothesis were not developed until at least 2014 (Stamatakis A. 2014).

That being said, many researchers, like Dean Falk and Gabrielle Russo, oppose Stedman's hypothesis and instead promulgate the theory that the human skull increased in size after the shift to bipedalism in primates (Russo G. A. et al. 2017 ; Falk, D. 1990). Falk provided the bulk of her data for this claim in 1990, using what she called the "radiator theory". Falk states that the heat and position of blood vessels in the brain and neck shifted after the rise of bipedalism thereby increasing blood flow to the brain and allowing for more oxygen per unit time to enter the brain. A favorable environment was created for growth in human brain size (Falk D. 1990). Further evidence for this claim was provided by Russo in 2017 through the trend he discovered relating primate skull shape changes directly to bipedalism in the fossil record (Russo, 2017). In both of these studies, the changes had a direct correlation to the position of the face and jaw horizontally, which connects to a separate study by Daniel Lieberman in which it was noted that human jaw evolution may have actually come from the types of food that human ancestors ate (Lieberman, 2011). Lieberman published his study along with hundreds of other hypotheses and studies in his book titled *The Evolution of The Human Head* (Lieberman 2011). In this book, Lieberman claims that as humans shifted to eating softer, semi-processed food, the human jaw shifted too in response to less strain (Lieberman, 2011).

George Perry similarly argues against the theory proposed by Dr. Stedman in his claim that the protein's pseudogenization does not line up with the proper timing to have any effect on

human skull shape (Perry, 2004). However, unlike the aforementioned studies, Stedman previously refuted Perry's claims with direct letters to Nature. He outlined the binary model of evolution, in which he points out that the chimpanzee DNA has full MYH16, so it would be impossible for the event to occur before human divergence, and any skull shape changes would be forced to pop up around the same time.

1.2: The Gap

As mentioned in the literature review, a large controversy in the scientific community revolves around human skull evolution. The two main sides of this theory are the "radiator theory," which details the hypothesis that bipedalism led to changes in skull shape (Falk, D. 1990), and Stedman's 2004 hypothesis, which looks at MYH16 and its possible effects on skull shape. The main reason that these two theories have neither been proven nor disproven is that there has never been an in-depth study of the evolution of MYH16, so researchers have not yet examined the exact effect on skull shape for multiple animals in order to draw a conclusion about MYH16 and its possible effects in humans. The gap, therefore, would be the lack of knowledge about MYH16's presence in other animals and the effects that this protein may have on skull shape. This research would not provide a definite explanation for the evolution of skull shape in humans, but would rather provide information on a possible correlation between MYH16 presence and skull shape which, used in conjunction with other research in the future, may in some way influence the acceptance or dismissal of the theory hypothesized by Stedman in 2004. This gap prompts the overall question - "Is there a correlation between animals having mutated

or no MYH16 and having a significantly changed skull shape or brain size from their most related unmutated counterparts?"

2. Methods:

The general methods of this paper can be split into three main stages. The first stage of the project consisted of creating a phylogenetic tree, similar to the tree made by Mason et. al. in their study of importin (Mason, D. A. et al. 2009). The second stage consisted of an in-depth study of MYH16 in organisms, tracking potential for expression and mutation through syntenic genes and novel techniques. The final stage was a statistical analysis of the results, analyzing any potential correlation between the presence of MYH16 and its mutations and changed skull shape.

2.1 Phase One:

In phase one, I made a phylogenetic tree to provide easy identification of MYH16. Due to the lack of notation on MYH16 in current genome databases, it would be impossible to follow conventional search protocols, where you would just search for the protein on genome browsers. Thus, I needed to develop a method of discerning MYH16 from other similar proteins. In order to create the tree, I found all major Myosin proteins correctly expressed in humans on uniprot (uniprot.com), a protein database. I then took those protein sequences, and using the procedure outlined by Mason et. al. (Mason, D. A. et al. 2009), I aligned them in MacVector, an application for computational biology. Aligning the proteins meant that I used the program to match up similar sequences, pointing out differences among the proteins (Reece, 2014). After aligning them, I used the phylogenetic tree function of MacVector to create a phylogenetic tree, showing

the evolution of the myosin proteins. To ensure accuracy, I included MYH16 from major phyla in the protein alignment. As expected, the tree (see figure 3) showed its accuracy by correctly positioning the evolution of the myosins with the commonly accepted evolutionary order of the phyla.

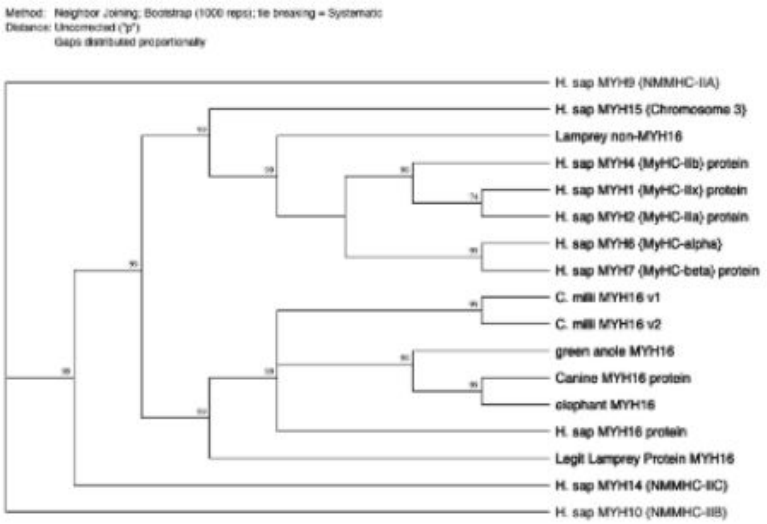

Figure 3 The final phylogenetic tree showing the evolution of MYH16 as compared to other myosins in humans. Included above are major organisms that show the accuracy of the tree

However, in order to add myosins from every major phyla, I had to trace back the protein through time on my own and find early forms of myosin. The easiest way to do this was to use gene synteny as described by Barbazuk *et. al.* in their study of the zebrafish genome (Barbazuk e. Al., 2000). Gene synteny, in a very broad sense, is the general order of genes on a chromosome, and due to the likelihood that chromosomes stay relatively consistent over time, genes next to each other tend to remain next to each other in subsequent species. In my study, MYH16 is syntenic (close to or next to) the genes arpc1a, smurf1, and importin, all of which are widespread proteins. Importin's evolution has been previously described by Mason et. al., so I

was able to easily identify MYH16 in most well annotated animal genomes with genome maps as I went to earlier diverging species for samples in the tree. If more genomes had genome maps, I would be able to skip the tree and use synteny as my sole identifier of MYH16, but the lack of widespread genome maps would limit my sample size. Thus, I was only able to trace the protein through well mapped genomes, and through this method I found that around the time of evolution of the elephant shark, synteny for MYH16 is broken. To search further organisms, like the lamprey, I had to use proteins from organisms after them, like the elephant shark, then scan the genome for its most similar sequence using the NIH software BLAST. Using this method, I found the main myosin 16 expressing organisms after the evolution of the lamprey (where I hypothesize MYH16 first evolved), and I added some of their sequences to my phylogenetic tree.

2.2 Phase Two:

Phase two included more generally finding MYH16 and mutated forms in as many organisms as possible. Through phase one I already had a large number of MYH16 samples, but I needed to find organisms without it as well. Stedman's original paper notes a deletion of the MYH16 gene in the mouse genome, so I began my second stage by analyzing the mouse genome. The genome map for the mouse genome (right) shows importin (labeled KPNA7) and arpc1a directly next to each other, showing a complete deletion of MYH16.

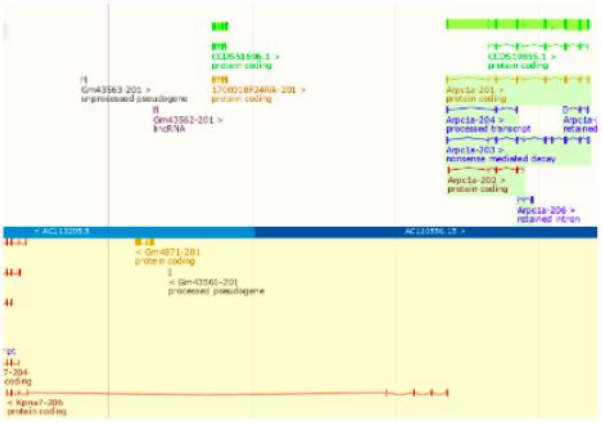

Figure 4 Notice that the KPNA7 gene (bottom left) goes directly to the edge of the arpc1a gene (top right).

After seeing this deletion, I realized that other organisms may have the same type of mutation, so my study would need to encompass those organisms. I therefore examined each organism with a genome map for MYH16 deletions like the one shown above. Stedman's hypothesis was peculiar in that it related specifically to humans because they have mutated MYH16 that isn't necessarily deleted. In order to address this difference, I needed to find other organisms with similar mutations. Other studies in this scenario would sequence the Myosin proteins present in the organisms to find potential mutations. However, that would have a significant cost, and would require muscle samples (specifically face muscles, where MYH16 is expressed) from every organism in the study. Given this dilemma, the only solution was to create a novel mutation detection technique.

Understanding that any given organism's most nearly related counterpart would have an almost identical protein, a direct comparison technique would show mutations. Point mutations between the two proteins would be impossible to show because we would not know if the protein evolved to have that different amino acid or if it was a genuine mutation. We could show, however, major frameshift mutations by comparing the protein sequences directly. In order to do this, I used the general sequence comparison techniques described in Stamatakis' 2004 paper. I took the genomic DNA sequences directly from ensembl (a website with genomes and their annotations), and then I ran them through softberry (another website that predicts genes) to get the protein sequence. The unique side of this technique comes once I had the sequence, when I used a dot plot matrix to compare the proteins directly, and for any major mutations, the matrix would show a lack of conserved sequences. To double check the accuracy of the program, I also ran DNA vs. protein matrices that would show the same frame-shift or other large mutation if

there was one. As shown below, the protein vs. DNA matrix shows the same frameshift mutation that is shown in protein vs. protein for the dolphin MYH16.

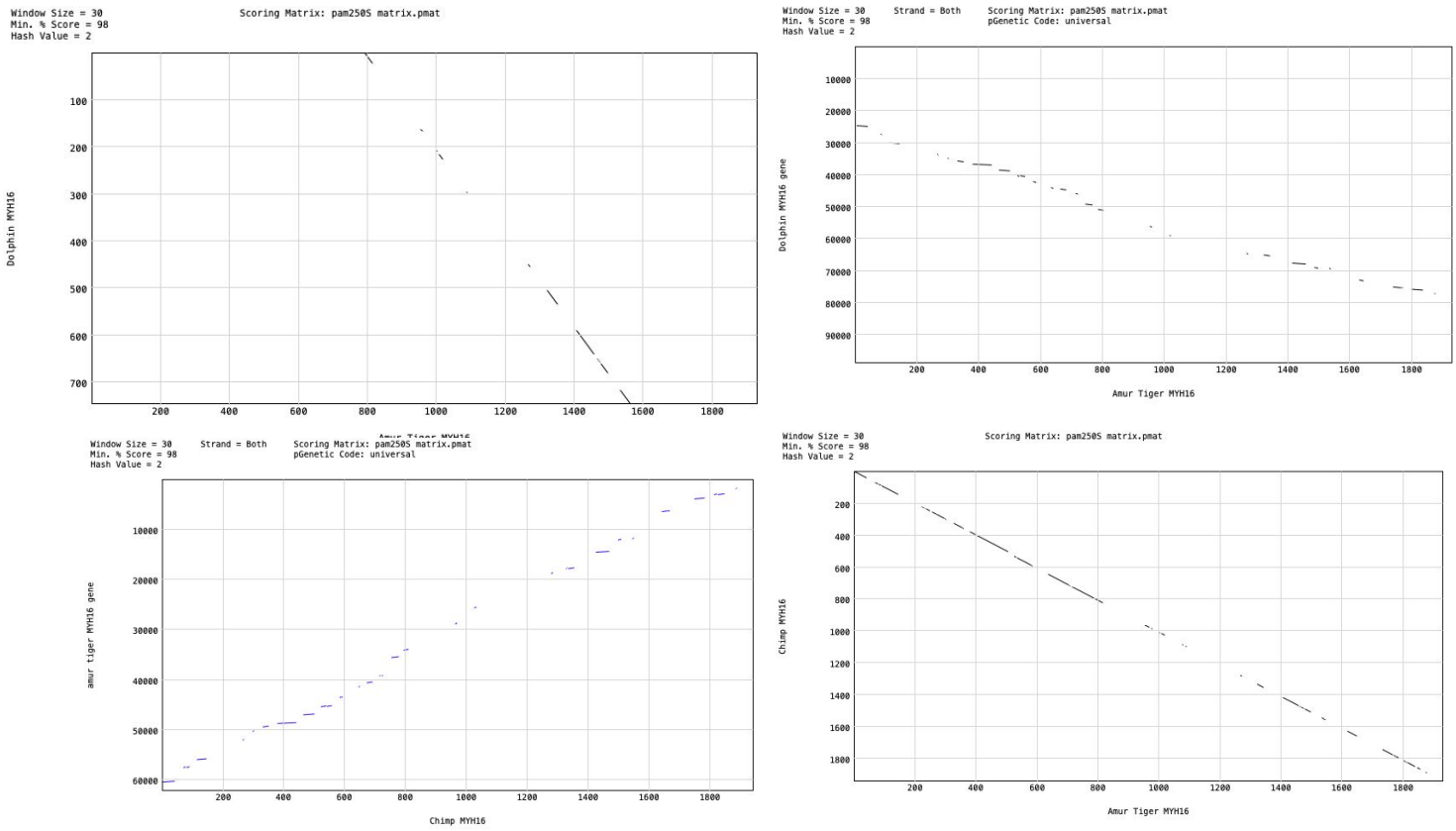

Figure 5: The top dot plots show dolphin matrices with clear mutations. As is visible, the dolphin matrices intersect the axes directly, rather than intersecting the corners like the lower control plots that also show more conserved lines in the tiger and chimpanzee which do not have mutations in MYH16.

For some organisms, it was imperative to first make sure that the protein examined was MYH16. In order to do this, I aligned the protein sequence found through the ensembl computer program with the phylogenetic tree and examined similarity as shown below. An example is shown

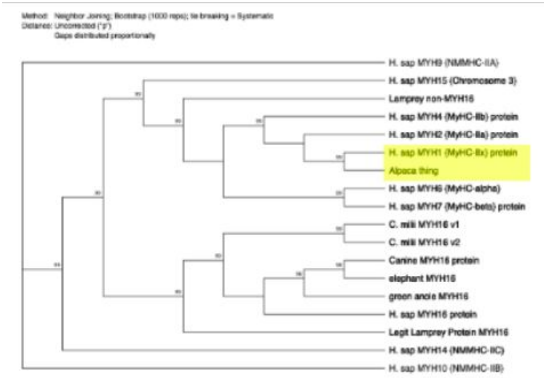

Figure 6 The unknown alpaca protein groups with MYH1 rather than MYH16, implying that it is a form of MYH1

in figure 6, in which the nearest related protein to MYH16 in the alpaca genome was actually just a form of myosin 1. This protein was therefore not included in the analysis, and the Alpaca was counted as an organism with a deleted MYH16.

In the case of proteins that showed potential frameshift deletions, to make sure that the frameshift was a pronounced mutation, I used PHYRE 2 protein modeling software to create a potential protein as shown below, and checked for clear mutations. This process added a significant amount of time in ascertaining every mutation. However it assured accuracy in predictions as some mutations may not alter the overall protein shape/functionality.

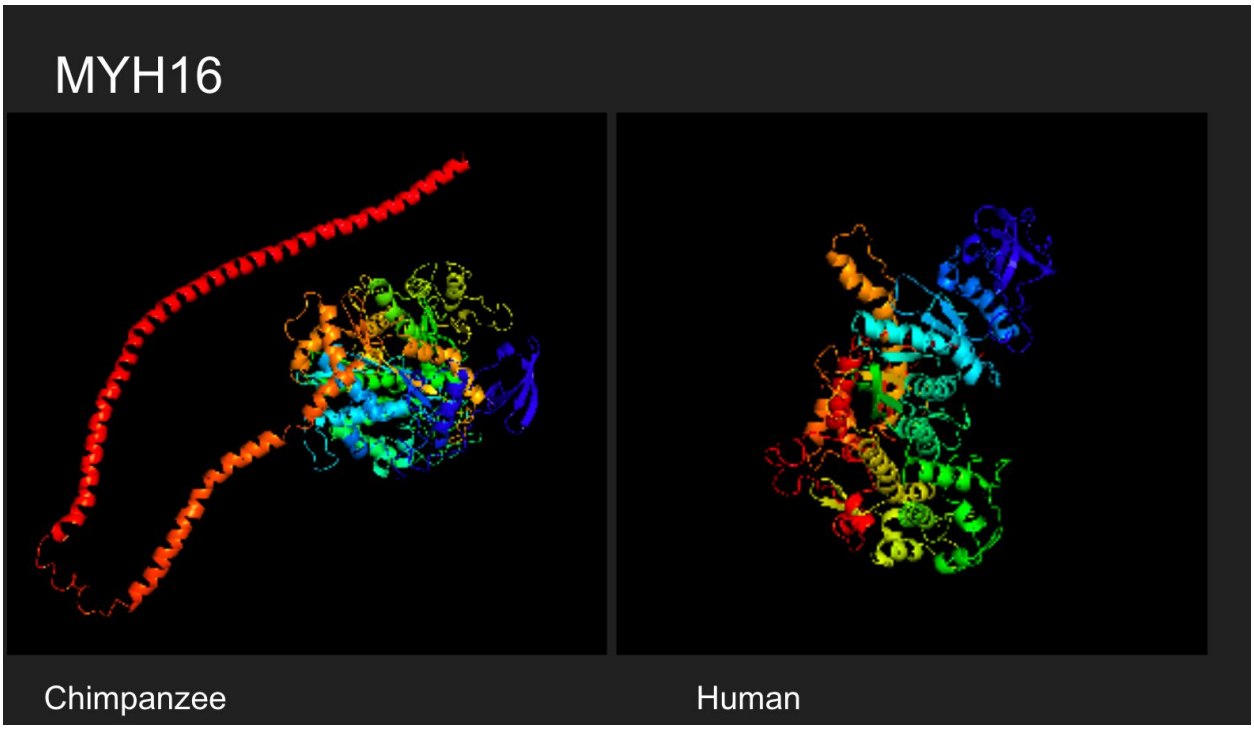

Figure 7: Models of human and chimpanzee MYH16 show the severity of the frameshift mutation in human MYH16

2.3 Phase Three:

After having found as many organisms with MYH16, without MYH16, and with mutated MYH16 as possible, I needed to compare their brain masses to see if we could demonstrate the effect that Dr. Stedman proposed. I therefore took each organism with full length MYH16 and compared it directly with an organism similar to it that either lacked or had mutated MYH16. I then found their brain masses and body weights online, and I examined pictures of the skull for crests and mandibles. After recording all that data, I calculated proportional brain mass to body weight using Dr. Osvaldo Cairó's encephalization quotient equation and analyzed potential trends using correlational equations and scatter plots with t-tests.

3. **Data and analysis**:

In this experiment, the data from phase one was used to complete a phylogenetic tree of Myosins, shown below:

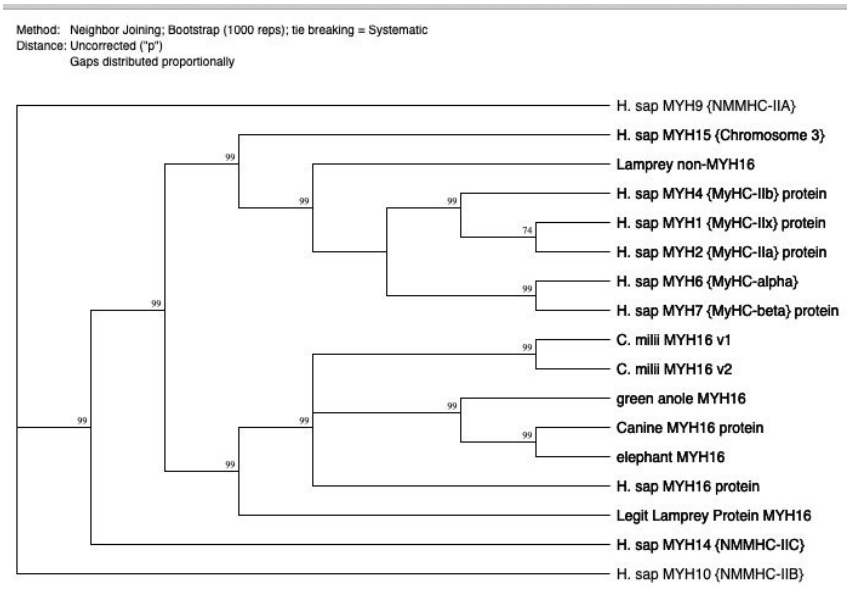

Figure 1 pt. 2: Final phylogenetic tree

The phylogenetic tree was then used to help determine the presence of MYH16 for each organism, while other values for organism mass, brain mass, etc. were found in studies like Hrdlička's book titled *Brain Weight in Vertebrates*. The values for brain weight and organism mass were used to compute the encephalization quotient of the organism. Encephalization quotients (EQ) are commonly accepted to show a more accurate representation of braincap volume and brain weight as a proportion of the organism's weight than a simple division of brain mass by body weight could represent since the brain to body mass proportion is skewed by larger organisms like elephants (Cairó, 2011).

Figure 8: Encephalization Quotient (EQ) equation

$$EQ = \text{brain} - \text{weight} \,/\, (0.12 \times \text{body} - \text{weight}((2/3))$$

Due to the size of the data table used, it will not be posted directly in this paper, but can be found in the appendix. More general values are shown in the chart below:

Figure 9: Average EQ and Correlational Values for Organism Groups

MYH16 Status	Unmutated MYH16	Mutated MYH16	Deleted MYH16	Mutated or Deleted MYH16
Average EQ	1.02	2.94	1.27	2.34
Correlation Coefficient Between EQ and MYH16 Status	N/A	0.46	0.19	0.12

From the data in this study, there is no clear correlation between the group of organisms with mutated or nonexistent MYH16 and an increased EQ (r^2=0.12, p= not significant). Though there is no general correlation for organisms without MYH16 (r^2=0.19, p= not significant), there is a strong correlation between increased EQ and mutated MYH16 (r^2=0.46, p<0.05). This would further support the above conclusions.

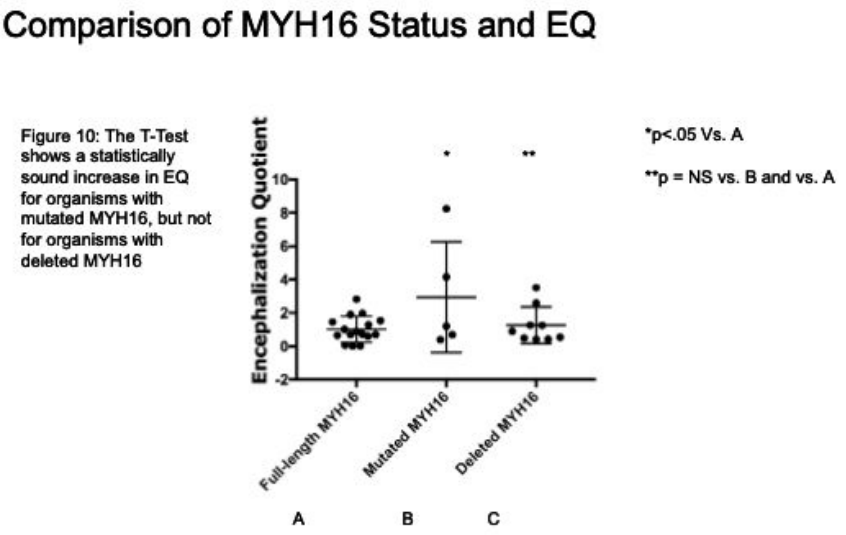

Figure 10: The T-Test shows a statistically sound increase in EQ for organisms with mutated MYH16, but not for organisms with deleted MYH16

Though this data shows a correlation between MYH16 being mutated and the organisms having an increased EQ, one can not exactly claim that the presence or lack of MYH16 is what causes this difference. Correlation is not causation, and so we can say that there is a correlation that partially supports Stedman's hypothesis, but without further research, one can not directly prove or disprove his hypothesis. Future studies may build upon the implications of this study by examining directly MYH16 and its mutations in these organisms as well as MYH16's potential effect on muscle fiber size in its mutated state.

For a more in depth look at the mechanisms behind the brain size increase, I examined the correlation between brain crests and the presence or lack of MYH16 and I compared brain crests to EQ. I found that there was no actual correlation between the presence of brain crests and a decreased EQ. However, I concluded that the lack of fully functioning MYH16 correlated directly to a lack of brain crests. This may mean that Dr. Stedman's hypothesis on the

mechanism behind the brain cap increase was wrong. However, as I was unable to physically obtain these animal skulls, I had to rely on photos to see if they had skull crests, so it is equally likely that this limitation skewed my data, and prevented accurate predictions in the correlation between brain crests and mutated or null MYH16 as well as the correlation between brain crests and EQ.

Conclusion:

In conclusion, this paper finds that Dr. Stedman's 2004 hypothesis relating mutations or deletions of MYH16 and larger brains in homosapiens is not supported by the data. There is, however, a clear correlation between larger brain size and the mutation of MYH16 vs. unmutated MYH16 in the organisms studied. This may imply that MYH16 in its mutated state somehow increases brain size. Possibly the mutated myosin binds to the actin, taking up binding sites for other myosins. The mutated MYH16 would then fail to provide force since it would lack a tail to wrap with other myosins, so it would travel without resistance to the end of the actin, possibly damaging the sarcomeric membrane and thus harming muscle fibers. Though this finding was interesting, there was no supported explanation for the increased brain size as the data showed no statistical correlation between a lack of skull crests and an increased EQ.

Though the findings of this paper will likely influence the scientific community in support of MYH16 mutations leading to increased brain size, the methods of this paper provide a separate implication. Most studies examining and comparing two proteins with potential mutations typically sequence the proteins directly from organisms and model them. In this paper, it was impossible to obtain the organisms and tissue samples for the above procedure, so I had to

use a novel method of mutation detection with three seperate assurances. This method showed consistency, and may be easily replicable as a low cost and time replacement for the typical sequencing method. In the future, scientists may be able to apply the methods of this paper to assist in their own studies.

In further studies, scientists could also further investigate the mechanisms of human brain size increase. Due to the relatively small sample size of this paper (n = 32), future researchers may want to continue correlational studies of this nature by obtaining more organisms. The sample size of this paper was limited by the number of sequenced genomes available online, but with lab access, future researchers could easily replicate the methods above on a larger scale using cDNA. In addition, studies examining the efficacy of the methods in this paper may help revolutionize computational biology mutation detection techniques.

In terms of the perspectives from my literature review, the conclusions alter Dr. Stedman's original hypothesis. They in no way impact Dean Falkner's claims, but they may influence her ideas on the human skull evolution. Also, these conclusions may support Dr. Lieberman's claim on soft foods leading to brain size evolution. It is possible that without selective pressure for strong bite forces, humans developed and could sustain the mutation discussed in this paper.

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Appendix

Raw Data Values for Organism Mutation Types and Corresponding Masses and EQ

Organism with Mutated/null MYH16	Type of mutation	brain mass (g)	presence of a lower mandible	presence of skull crests	Organism mass (g)	EQ
Human	Frame-shift	1400	yes	no	70000	8.242570
hedgehog	frame-shift	3.35	yes	no	771	0.398420
Dolphin	Frame-shift	1550	no?	no	227500	4.159209
Sheep	Frame-Shift	140	yes	no	90,718	0.693424
tree shrew	frame-shift	3.14	yes	no	133	1.205115
Duck	Pseudogene	6.61	no-ish	no	1,992	0.417518
Goat	pseudogene?	115	yes	no	27660	1.257369
Alpaca	deletion	1221	yes	no	68038	0.9
Zebrafish	Deletion	0.25	yes	no	0.6	3.514302
Mouse	Deletion	0.4	yes	no	23	0.494585
Hamster	deletion	1.4	yes	no	37	1.260840
Chicken	deletion	4	no-ish	no	635	0.541432
koala	deletion	19.2	yes	no	9,525	0.427291
Sloth	deletion	24.2	yes	kind of	907	2.582719
Painted Turtle	None	50	yes	yes	400	0.921007
Naked Mole Rat	None	2	yes	yes	34.019	0.190494
Chimpanzee	None	384	yes	yes	50000	0.282933
Coelecanth	None	3	yes	no	54431	0.002088
squirrel	None	7.6	yes	no	453	0.128849
Black Bear	None	450	yes	yes	108862	0.197376
Platypus	None	9	no	yes?	1814.37	0.060500
Dog	None	72	yes	yes	11000	0.145569
Great White Shark	None	35	yes	yes	430000	0.006143
Tiger	None	263.5	yes	yes	219992	0.072306
Elephant	None	5,000	yes	no	5896700	0.153190
Cow	None	423	yes	no	465,000	0.070475

Pig	None	180	yes	no	90718	0.089154
Ferret	None	8	yes	yes	1360	0.065172
Horse	None	655	yes	no	521,000	0.101161
Cow	None	423	yes	no	465,000	0.070475