Pathways Project: Annotation Walkthrough

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Prerequisites

- Understanding Eukaryotic Genes (UEG) Modules 1, 4, 5, & 6
- RNA-Seq Primer
- An Introduction to NCBI BLAST

Resources & Tools

- All links for this walkthrough, including the recommended prerequisites listed above, can be found on the Pathways Project page of the GEP website (thegep.org/pathways).
- Annotation Form
- Annotation Form Exemplar
- Annotation Workflow
- Glossary

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Introduction

The Pathways Project is focused on annotating genes found in well characterized signaling and metabolic pathways across the *Drosophila* genus. The current focus is on the insulin signaling pathway which is well conserved across animals and critical to growth and metabolic homeostasis. The long-term goal of the Pathways Project is to analyze how the **regulatory** regions of genes evolve in the context of their positions within a network. For a general project overview, see the 6-minute video.

This walkthrough illustrates how to apply the Genomics Education Partnership's (**GEP**) annotation strategy, for the Pathways Project, to construct a gene model for the *Ras homolog enriched in brain* (*Rheb*) gene (**target gene**) in *Drosophila yakuba* (**target species**). This walkthrough focuses on **annotation** of the coding regions only, so we won't annotate the **untranslated regions (UTRs)**, nor the transcription start site (TSS).

It is important to note that the *Rheb* gene in *D. yakuba* is a relatively straightforward annotation project in comparison to what your own project might be. Some of the steps in this walkthrough might appear to be excessive, but keep in mind that you will potentially have a more complex project, so it is important to follow this protocol as it will equip you with most of what you will need should you encounter complexities.

Throughout this walkthrough, text boxes with icons are used to assist you.

We recommend you follow the parts in the order they are presented, and then refer to parts when needed for your own project. Note that the figures have been configured to fit this document while still maintaining readability; therefore, your screen may differ slightly. Commas have been included with most **coordinates** to improve readability, but you do not have to enter them in the Genome Browser (navigation will work the same with or without commas). **Terms located in the Glossary are red and bolded typically the first time they are mentioned in the walkthrough and not necessarily every time they're mentioned.**

Part 1: Examine genomic neighborhood surrounding target gene in *D. melanogaster*

1. Navigate to the GEP UCSC Genome Browser Gateway Page.
2. Click on "*D. melanogaster*" in the "UCSC Species Tree and Connected **Assembly** Hubs" table (Figure 1).
3. Ensure "Aug. 2014 (BDGP Release 6 + ISO1 MT/dm6)" under the "*D. melanogaster* Assembly" field is selected.
4. Enter "**Rheb**" in the "Position/Search Term" field.
5. Click on the "Go" button (Figure 2).

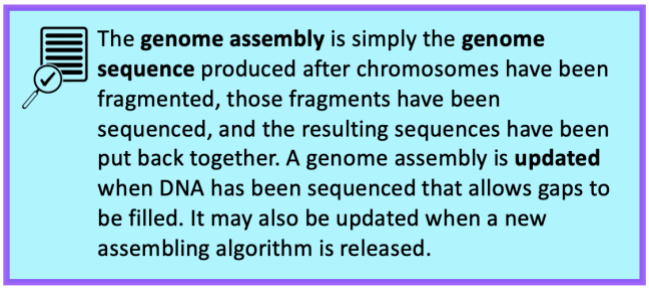


Figure 1 Review of content from UEG Module 1

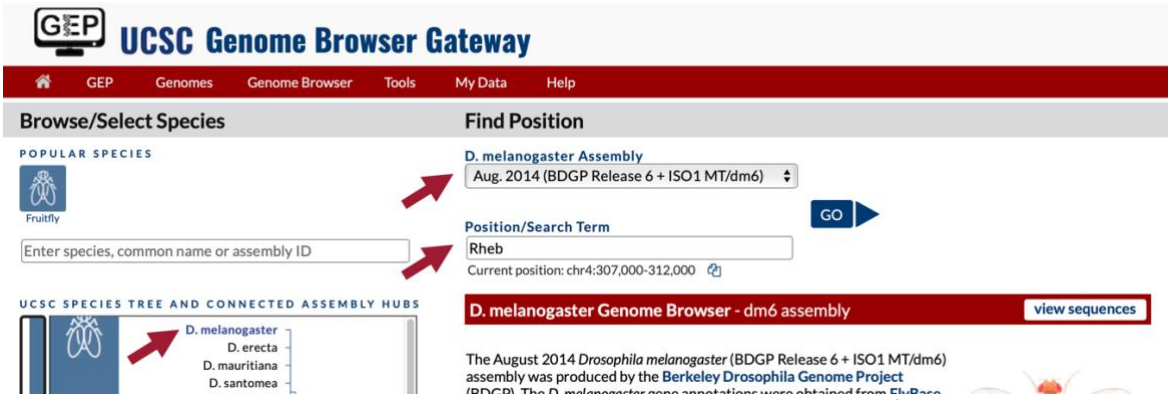


Figure 2 Navigate to the *Rheb* gene in the *D. melanogaster* Aug. 2014 (BDGP Release 6 + ISO1 MT/dm6) assembly.

6. On the following "**FlyBase** Protein-Coding Genes" page, click on "Rheb-RA at chr3R:5568921-5570491" (Figure 3).

Figure 3 Since both Rheb-RA and Rheb-RB span the same coordinates, it doesn't matter which of the two we select (Figure 4). Navigate to the *Rheb* gene in *D. melanogaster* by clicking on Rheb-RA (arrow).

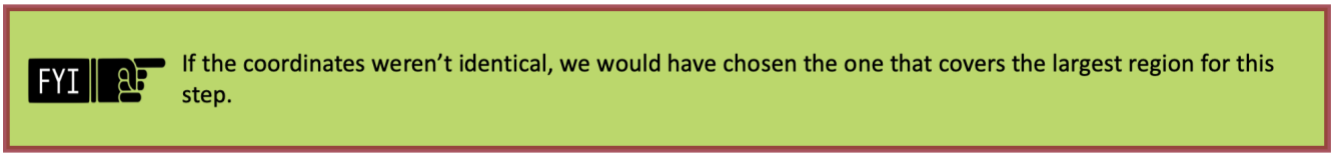


Figure 4 For Your Information — Isoforms with differing coordinates

7. Because the Genome Browser remembers our previous track display settings, click on “default tracks” in the display controls below the Genome Browser image (Figure 5).

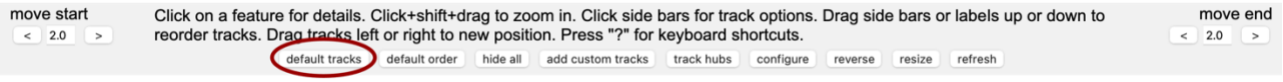


Figure 5 The “default tracks” button quickly removes any display changes you previously made and returns to the defaults.

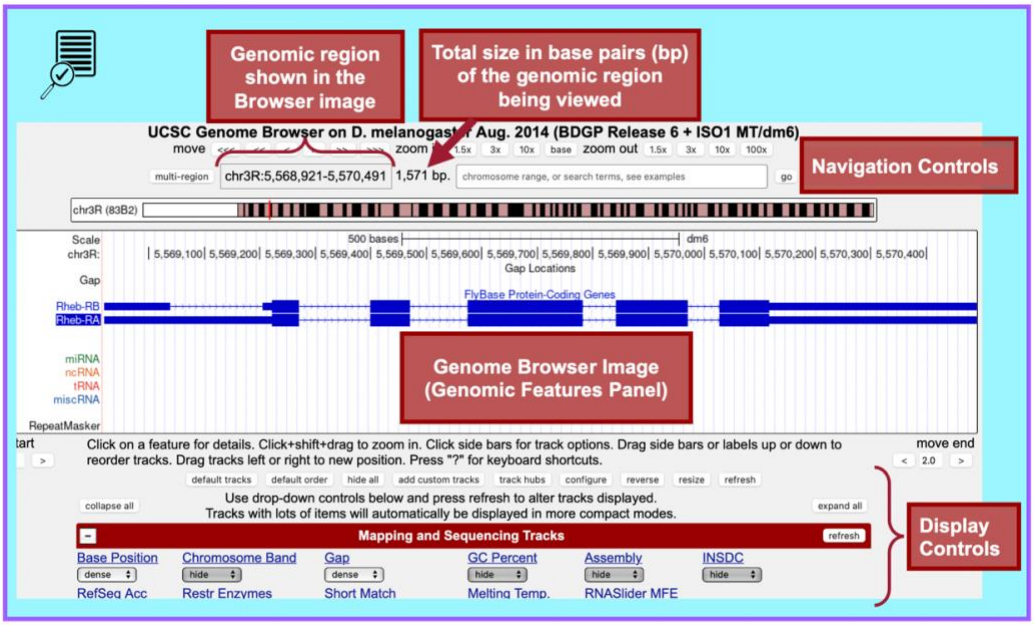


Figure 6 Review of content from UEG Module 1. There are three major sections of the Genome Browser—a set of navigation controls, the Genome Browser image, and a set of track display controls.

In the “FlyBase Protein-Coding Genes” track, we should now see the gene structure for the two **isoforms** (Figure 6) of the *Rheb* gene (i.e., Rheb-RA and Rheb-RB). Notice that *Rheb* is on the “chr3R” **scaffold** (Figure 7).

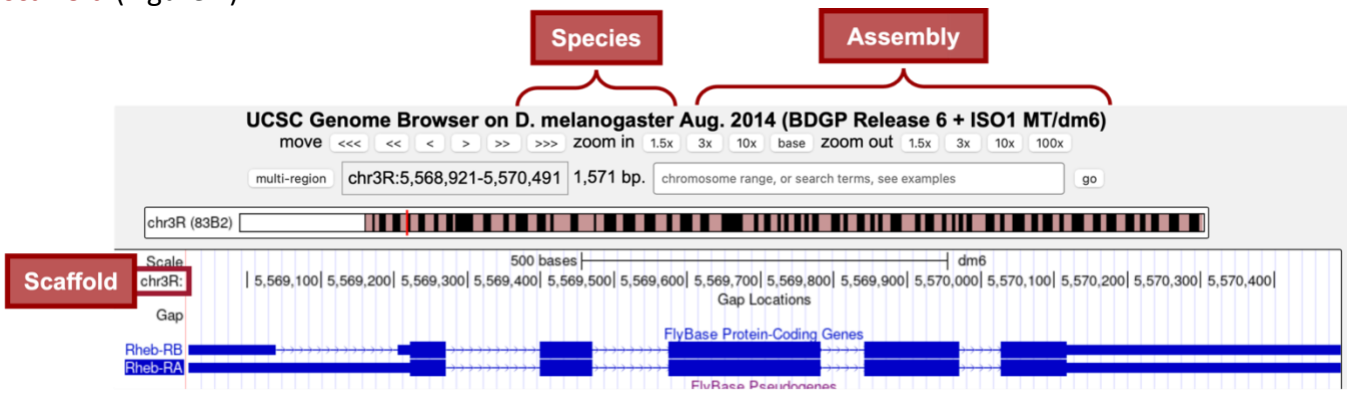


Figure 7 There are two isoforms of *Rheb* in *D. melanogaster* (Rheb-RA and Rheb-RB). *Rheb* is located on the “chr3R” scaffold of the *D. melanogaster* Aug. 2014 (BDGP Release 6 + ISO1 MT/dm6) genome assembly.

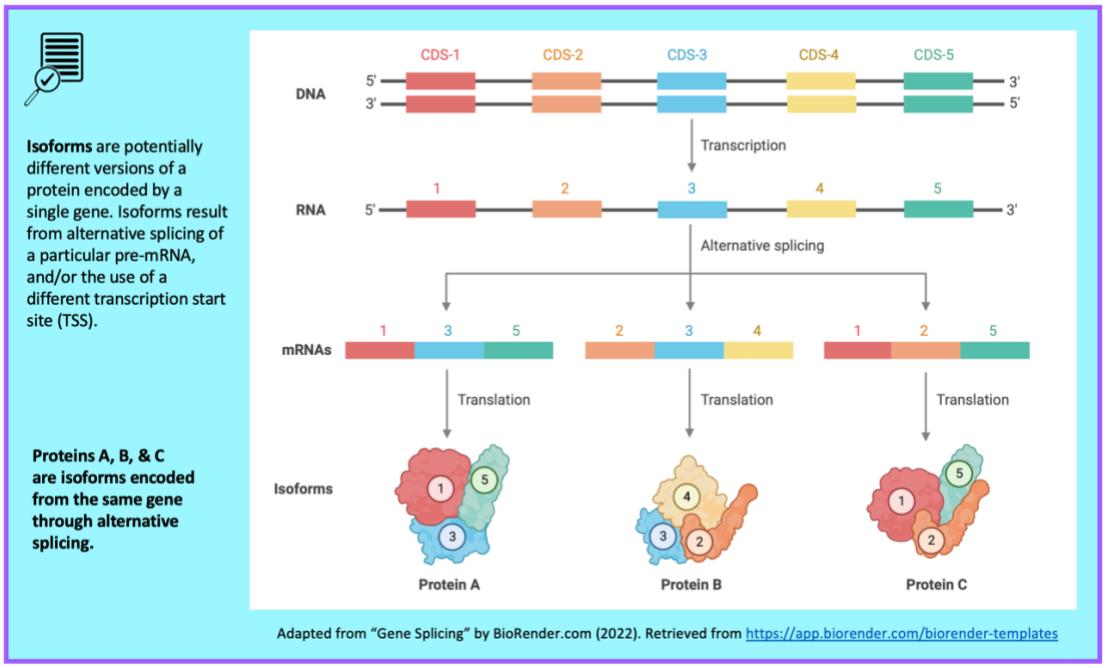


Figure 8 Review of content from UEG Module 6. For a more in-depth review of alternative splicing see Park et al. (2018).

8. Zoom out until you can see the nearest two genes on each side of *Rheb*.

We are now viewing the **genomic neighborhood** of the *Rheb* gene in *D. melanogaster* (i.e., region of the scaffold containing *Rheb* (target gene) and its neighboring two closest **upstream** genes and two closest **downstream** genes; Figure 9).

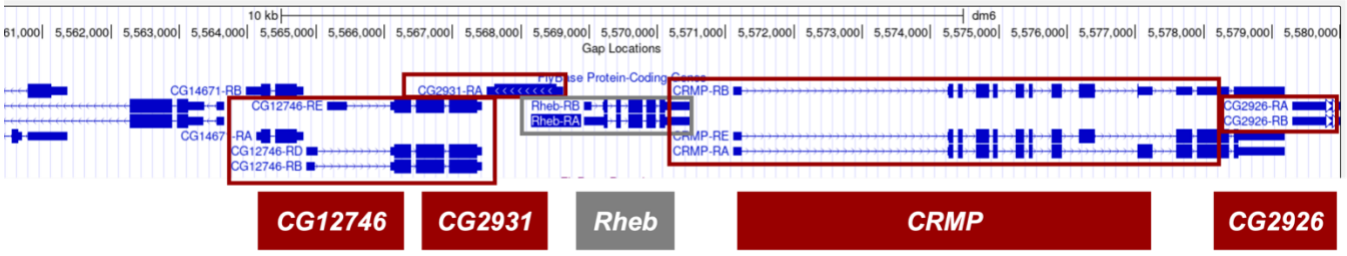


Figure 9 The genomic neighborhood of *Rheb* (grey) includes *CG12746, CG2931, CRMP*, and *CG2926* (Figure 10).

FYI

Each protein-coding gene annotated by FlyBase in *D. melanogaster* has an annotation symbol that begins with the prefix "**CG**" for **C**omputed **G**ene. Unless genes are characterized experimentally and formally named, they are referred to by this symbol. For example, in the figure above, three of the five genes—*CG2931, CG12746,* and *CG2926*—have not been named since they haven't yet been characterized experimentally. However, the Ras homolog enriched in brain and Collapsin Response Mediator Protein genes have been characterized by past scientific studies, and thus are referred to by their gene symbols *Rheb* and *CRMP*, respectively.

Figure 10 For Your Information — 'CG' prefix

Now we need to draw a sketch of the genomic neighborhood of *Rheb* in *D. melanogaster* (Figure 11). To do so, we must identify the direction of **transcription** for each gene by zooming in on an **intron** (or **exon**) of each gene.

9. Start by zooming in on an intron (or exon) of *Rheb*.
10. Since the arrows within the introns of *Rheb* point to the right, in our sketch we need to draw an arrow pointing to the right and label it *Rheb*.
11. Repeat Steps 9-10 for the two closest genes on both sides of *Rheb* (i.e., *CG12746, CG2931, CRMP*, and *CG2926*).

The arrows within the introns of *Rheb* point to the right; therefore, we know *Rheb* is transcribed from left to right and located on the positive strand of DNA in *D. melanogaster*. Since *Rheb* is our target gene (i.e., gene we are annotating), the two closest genes on its 5' side are considered **upstream** and the two closest genes on its 3' side are considered **downstream** (Figure 12).

- The two closest upstream genes to *Rheb* in *D. melanogaster* are *CG2931* and *CG12746*.
- The two closest downstream genes to *Rheb* in *D. melanogaster* are *CRMP* and *CG2926*.

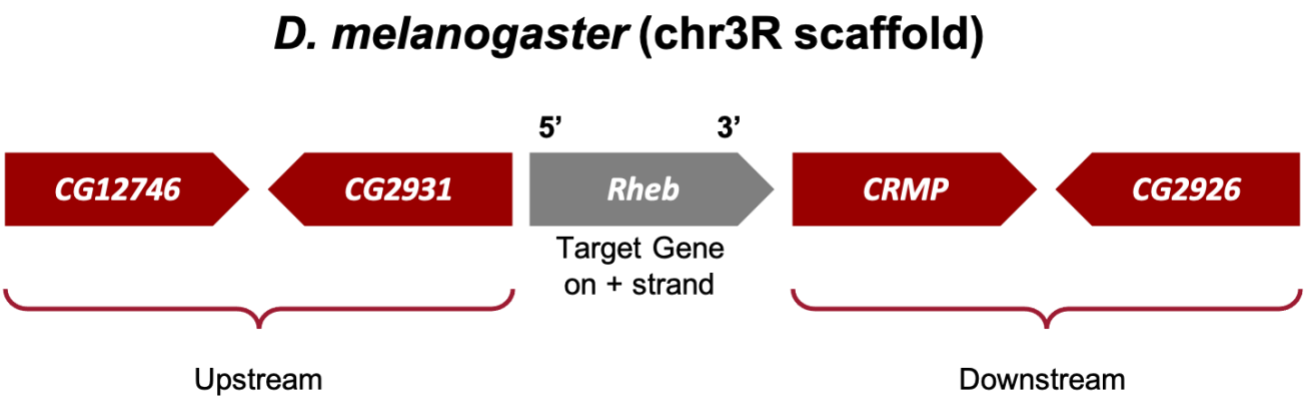
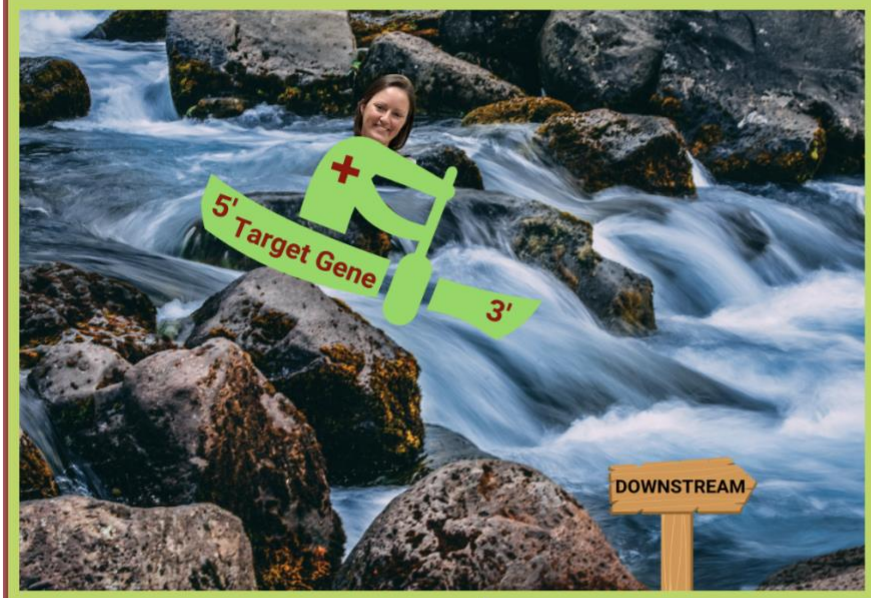


Figure 11 Sketch of the genomic neighborhood of *Rheb* in *D. melanogaster*. Since the direction of the arrows within their introns point to the right, *Rheb*, *CG12746*, and *CRMP* are on the positive strand. In contrast, *CG2931* and *CG2926* are on the negative strand since the direction of the arrows within their introns point to the left.

FYI

- **Upstream**: located on the 5' side of the target gene
- **Downstream**: located on the 3' side of the target gene

Genomic Neighborhood of Target Gene on Positive Strand of DNA



As we learned in Understanding Eukaryotic Genes: Module 1, genes on the same DNA molecule may be transcribed in opposite directions (i.e., genes on the positive strand of DNA are transcribed from left to right and genes on the negative strand of DNA are transcribed from right to left). Since genes have directionality, the areas of DNA we call upstream and downstream change depending on the orientation of the target gene (i.e., **upstream and downstream is determined in relation to the directionality of your target gene**).

Since river streams have directionality (i.e., flow in only one direction), to help you orient yourself, think of your target gene as a raft floating down a stream. Wherever the raft just floated *from* is "upstream" of its current location and wherever the raft is floating *to* is "downstream" of its current location. Notice how the areas of DNA we call upstream and downstream to the target gene change depending upon whether the target gene is on the positive or negative strand of DNA.

Genomic Neighborhood of Target Gene on Negative Strand of DNA

Figure 12 For Your Information — Upstream vs. downstream

Part 2: Identify genomic location of ortholog in target species

Now that we've examined the genomic neighborhood of our target gene, *Rheb*, in *D. melanogaster*, we need to identify the location of *Rheb* in our target species *D. yakuba*.

Part 2.1: Retrieve protein sequence of target gene in *D. melanogaster*

1. In the "FlyBase Protein-Coding Genes" track, click on "Rheb-RA" (Figure 13).

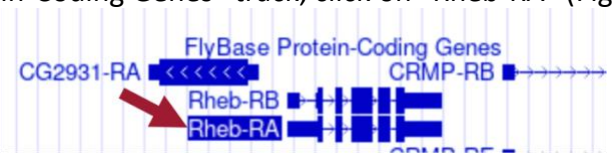


Figure 13 Click on "Rheb-RA" to view details regarding this protein-coding gene annotated by FlyBase.

2. Under the "Links to sequence" heading, click on the "Translated Protein" link (Figure 14, left).

We are now viewing the sequence for the 182 amino acids in the translated **protein** of Rheb-RA in *D. melanogaster* (Figure 14, right).

3. Copy the entire sequence (including the header) so we can use it in our ***tblastn*** search.

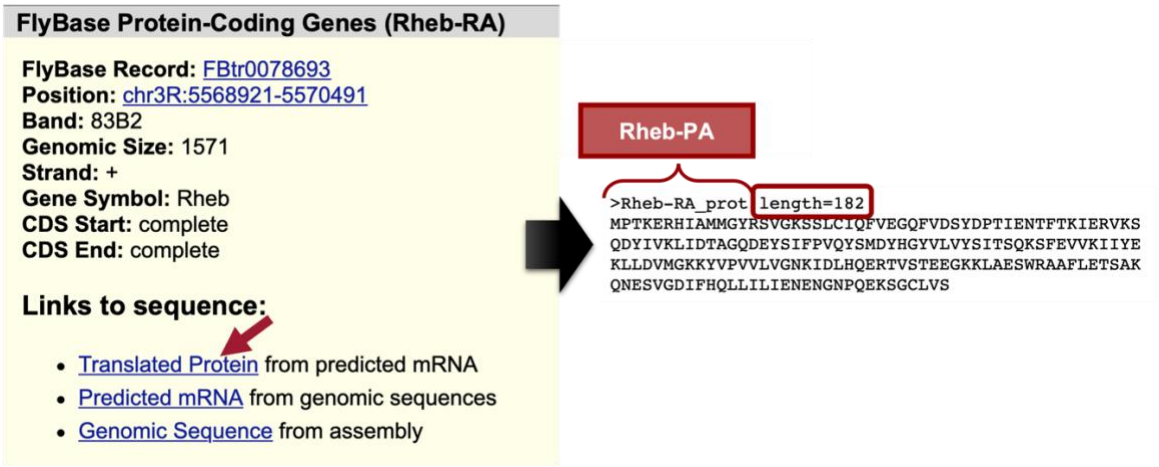


Figure 14 Click on the "Translated Protein" link for the Rheb-RA feature (Figure 15) to obtain the sequence in *D. melanogaster*. Notice the length of the protein is 182 amino acids (rectangle).

FYI What is the difference between Rheb-**RA** and Rheb-**PA**?

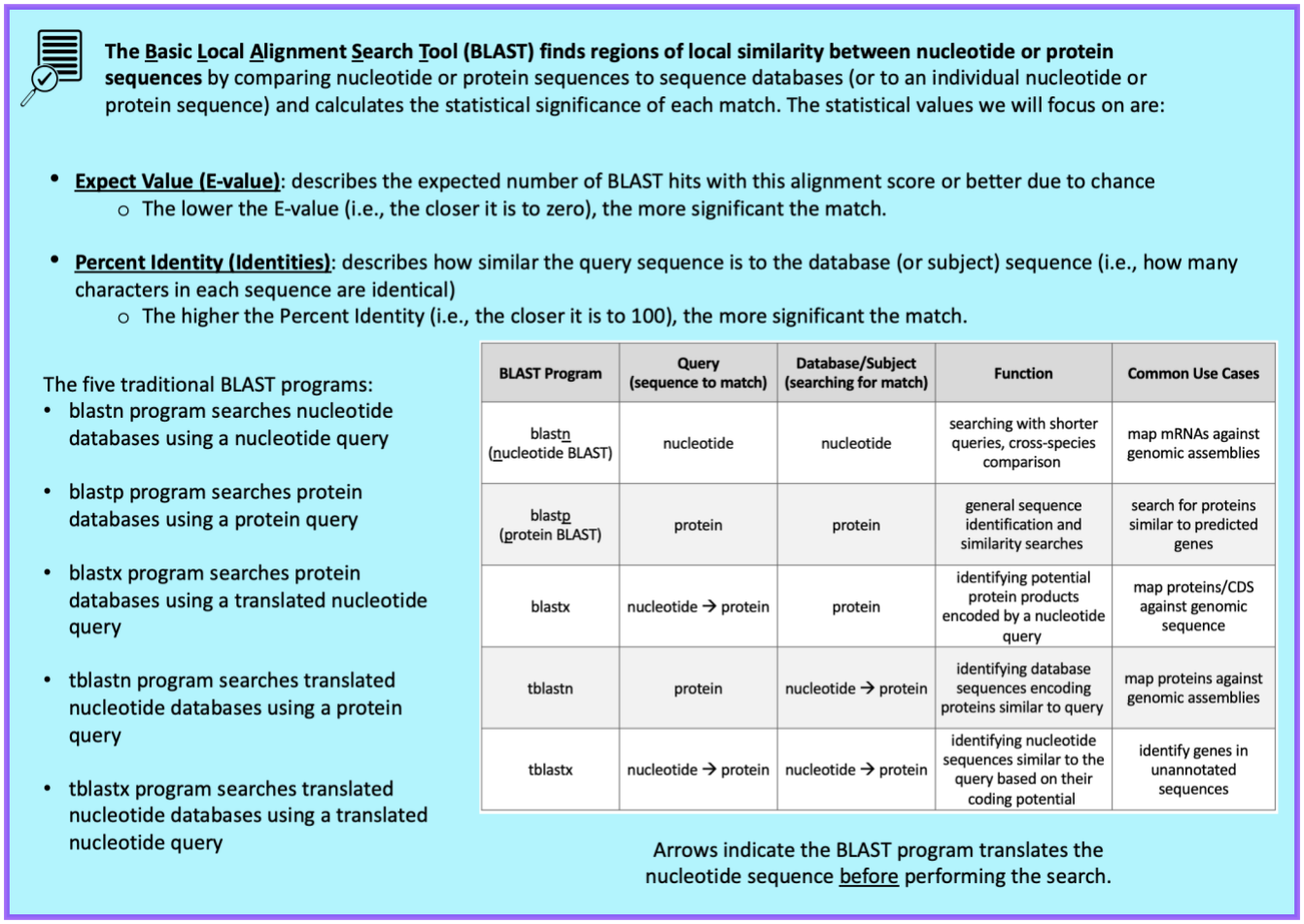
The prefix "Rheb" corresponds to the gene symbol. The 'R' or 'P' in the suffix designates the associated transcript (mRNA) or protein-product of the gene, respectively. The 'A' in the suffix refers to the A isoform of the gene.

Gene symbols (e.g., *Rheb*) are italicized while their mRNA and protein products are not (e.g., Rheb-RA and Rheb-PA).

Figure 15 For Your Information – Gene symbol, mRNA, and protein nomenclature

Part 2.2: Perform a BLAST search of *D. melanogaster* protein against the target species' genome

In Part 2.1, we retrieved the protein sequence for Rheb-PA in *D. melanogaster*. Now we need to perform a **BLAST** search (Figure 16) of Rheb-PA against the entire *D. yakuba* genome assembly.



The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between nucleotide or protein sequences by comparing nucleotide or protein sequences to sequence databases (or to an individual nucleotide or protein sequence) and calculates the statistical significance of each match. The statistical values we will focus on are:

- **Expect Value (E-value)**: describes the expected number of BLAST hits with this alignment score or better due to chance
  - The lower the E-value (i.e., the closer it is to zero), the more significant the match.
- **Percent Identity (Identities)**: describes how similar the query sequence is to the database (or subject) sequence (i.e., how many characters in each sequence are identical)
  - The higher the Percent Identity (i.e., the closer it is to 100), the more significant the match.

The five traditional BLAST programs:

- blastn program searches nucleotide databases using a nucleotide query
- blastp program searches protein databases using a protein query
- blastx program searches protein databases using a translated nucleotide query
- tblastn program searches translated nucleotide databases using a protein query
- tblastx program searches translated nucleotide databases using a translated nucleotide query

BLAST Program	Query (sequence to match)	Database/Subject (searching for match)	Function	Common Use Cases
blastn (nucleotide BLAST)	nucleotide	nucleotide	searching with shorter queries, cross-species comparison	map mRNAs against genomic assemblies
blastp (protein BLAST)	protein	protein	general sequence identification and similarity searches	search for proteins similar to predicted genes
blastx	nucleotide → protein	protein	identifying potential protein products encoded by a nucleotide query	map proteins/CDS against genomic sequence
tblastn	protein	nucleotide → protein	identifying database sequences encoding proteins similar to query	map proteins against genomic assemblies
tblastx	nucleotide → protein	nucleotide → protein	identifying nucleotide sequences similar to the query based on their coding potential	identify genes in unannotated sequences

Arrows indicate the BLAST program translates the nucleotide sequence before performing the search.

Figure 16 Review of An Introduction to NCBI BLAST — The Pathways Project annotation protocol uses *blastp* and *tblastn* searches.

Since we are looking for the **orthologous** region of *Rheb* in *D. yakuba*, we want BLAST to search the entire **genome** of *D. yakuba* to identify regions of **local similarity** with the protein sequence of *Rheb* in *D. melanogaster* we obtained in Part 2.1. In other words, we need to BLAST our *Rheb* protein sequence from *D. melanogaster* against the entire genome of *D. yakuba* to narrow down the possible regions where the *Rheb* ortholog could be in *D. yakuba*.

For this BLAST search, we will use the *tblastn* program to search the translated **nucleotide** database of our target species, *D. yakuba*, using the protein sequence of *Rheb* in *D. melanogaster* as our query.

- **Query**: sequence we are looking to match (i.e., protein sequence of *Rheb* in *D. melanogaster*)

- **Database (Subject)**: collection of sequences we are searching for matches (BLAST will translate the entire genome of *D. yakuba* before searching for a match to the Rheb-PA sequence from *D. melanogaster*)

1. Navigate to the Pathways Project Genome Assemblies page.
2. Click on the "Genome BLAST" link for *D. yakuba* (Figure 17).
   - Note: Here we are selecting the **entire genome** of *D. yakuba* **(~148 million bases)** as the database for our *tblastn* search (Figure 18).

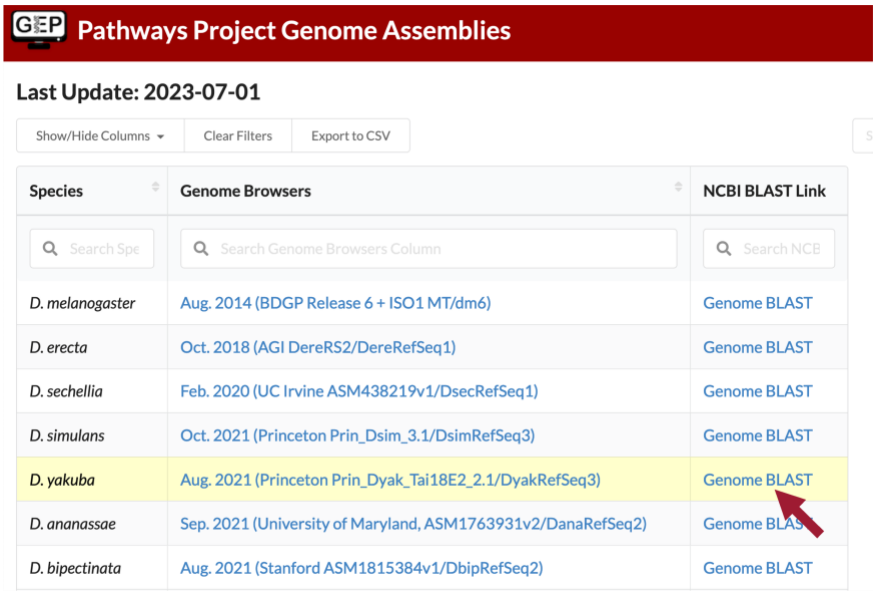


Figure 17 Click on the "Genome BLAST" link for *D. yakuba* in the "NCBI BLAST Link" column.

3. Paste the Rheb-PA sequence we copied from Part 2.1 (Figure 14) into the "Enter Query Sequence" text box (Figure 19).
4. Click on the "BLAST" button.

FYI The National Center for Biotechnology Information (NCBI) periodically updates the genome assemblies used for BLAST searches that can cause bookkeeping issues when they occur in the middle of a semester. Using the "Genome BLAST" links on the "Pathways Project Genome Assemblies" page ensures student annotators are navigating to the correct genome assembly database when performing their BLAST search against the entire genome of their target species. However, **this circumvention will only be needed in Part 2 of the Pathways Project protocol**. In Parts 3 and 5, we will navigate directly to the NCBI BLAST home page.

Figure 18 For Your Information – Pathways Project Genome Assemblies page

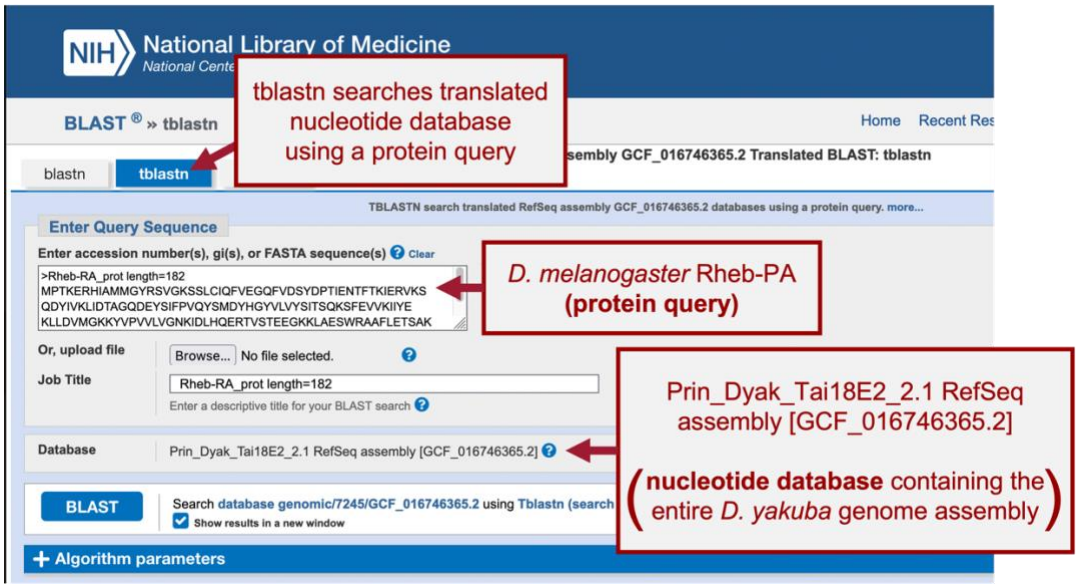


Figure 19 Configure *tblastn* to compare the *D. melanogaster* protein Rheb-PA (query) against the entire *D. yakuba* genome assembly (database). BLAST will translate the genome assembly before performing the search; thus, we are searching a translated nucleotide database using a protein query (i.e., *tblastn*).

When performing a search, BLAST may return any number of matches (often referred to as "hits") for regions of local similarity between our query sequence and database; however, each hit is not necessarily statistically significant. BLAST provides statistical scores to help us determine which **alignments** between the two sequences are statistically significant and which are **spurious** (i.e., likely occurred by chance alone and, therefore, are not evidence of real biological **conservation**). If BLAST returns multiple good hits (i.e., more than one match with a low **E-value** and a high sequence **identity**), we will need to investigate them all further to determine the most likely ortholog.

Our *tblastn* search found five regions within the translated *D. yakuba* genome that show similarities with the protein sequence of *Rheb* in *D. melanogaster* (Figure 21); however, only one of these is a good hit (*Drosophila yakuba* strain Tai18E2 chromosome 3R, Prin_Dyak_Tai18E2_2.1, whole genome shotgun sequence; sequence identity: 97.14% and E-value: 2e-78[1]). The second hit has a much higher E-value (7e-37) and much lower **percent identity** (43.75%), and this pattern of increasingly lower quality matches continues through the other three matches. Therefore, we will continue our analysis based on the **hypothesis** that the **putative ortholog** of Rheb-PA in *D. yakuba* is in the scaffold of chromosome 3R.

Notice that each of the genome regions of our five hits has a unique **accession number** (Figure 20). The accession number for the chromosome 3R scaffold in *D. yakuba* is NC_052530 (Figure 21, red arrow).

FYI Similar to how humans have unique fingerprints that can be used to identify them at crime scenes, each nucleotide and amino acid sequence is assigned a unique **accession number** to allow scientists to identify it from the millions of other sequences stored in a database.

Figure 20 For Your Information – Accession Numbers

[1] 2e-78 = 2 x 10^{-78}

Figure 21 The *tblastn* search of the *D. melanogaster* protein Rheb-PA (query) against the translated *D. yakuba* genome assembly (database) found five regions of similarity. The best match (black rectangle) is located on the "chromosome 3R" scaffold (pink arrow) (accession number: NC_052530; red arrow) of *D. yakuba* (Figure 22).

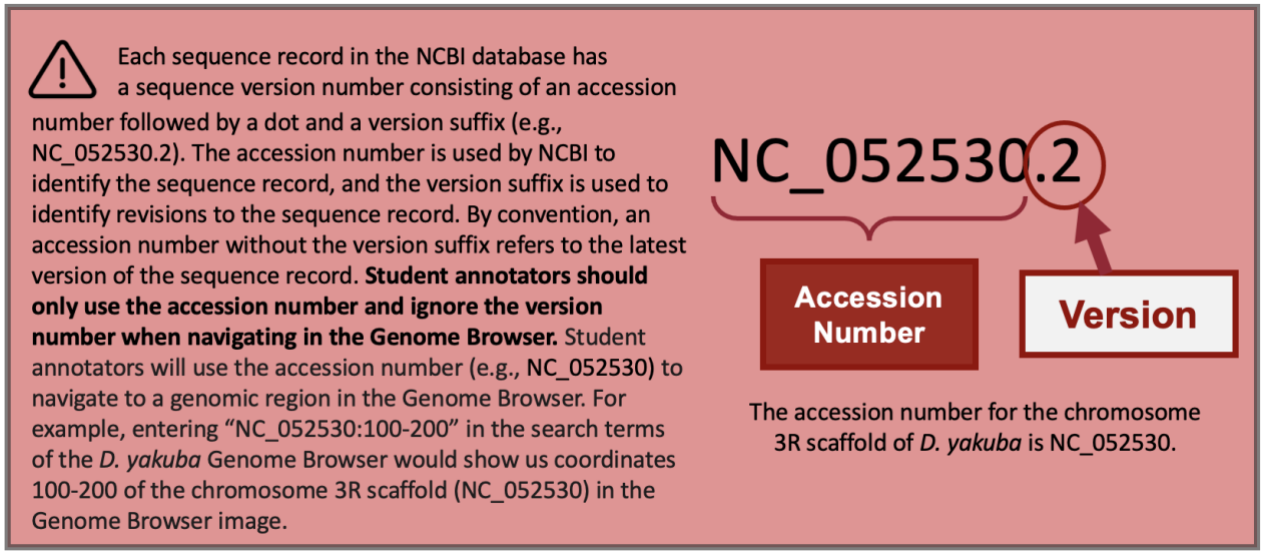


Figure 22 Caution – Accession Number

Part 2.3: Summarize *tblastn* results for protein on target species' scaffold

1. Click on the "*Drosophila yakuba* strain Tai18E2 chromosome 3R, whole genome shotgun sequence" link in the "Description" column to navigate to the alignment (Figure 21, left arrow).
2. In the blue toolbar for the BLAST hit, select the "**Subject** start position" option from the drop-down menu of the "Sort by" field to order the matches based on the start coordinates on *D. yakuba* NC_052530 scaffold in ascending order (Figure 23).

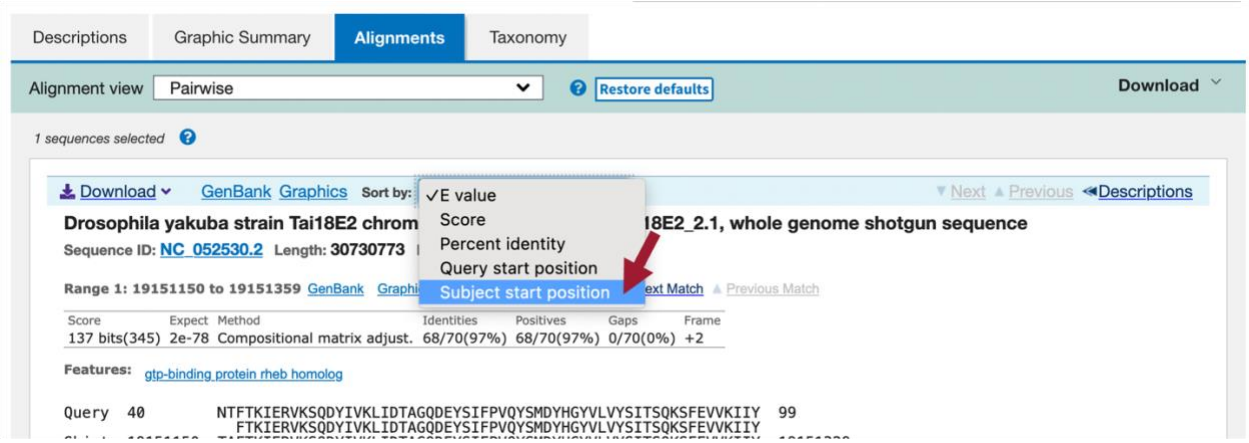


Figure 23 In the blue toolbar for the BLAST hit, select the "Subject start position" option from the drop-down menu of the "Sort by" field to order the matches based on the start coordinates of the *D. yakuba* NC_052530 scaffold in ascending order.

We should now see 9 ranges listed in ascending order of the subject start position (coordinate). Each **range** corresponds to a contiguous portion of the subject sequence (*D. yakuba* genome) that shows significant sequence similarity (i.e., matches) with a portion of the query sequence (*D. melanogaster* Rheb-PA). Here, the 9 ranges show matches between Rheb-PA in *D. melanogaster* (Query) and a range of coordinates from the NC_052530 scaffold in *D. yakuba* (Sbjct). For example, Range 1 shows a match between Rheb-PA in *D. melanogaster* (Query: 54 – 130) and coordinates 11,148,063 – 11,147,992 of NC_052530 scaffold in *D. yakuba* when translated in Frame -1. Range 1 has an E-value (Expect) of 3e-11 and a sequence identity of 40% (Figure 24, green).

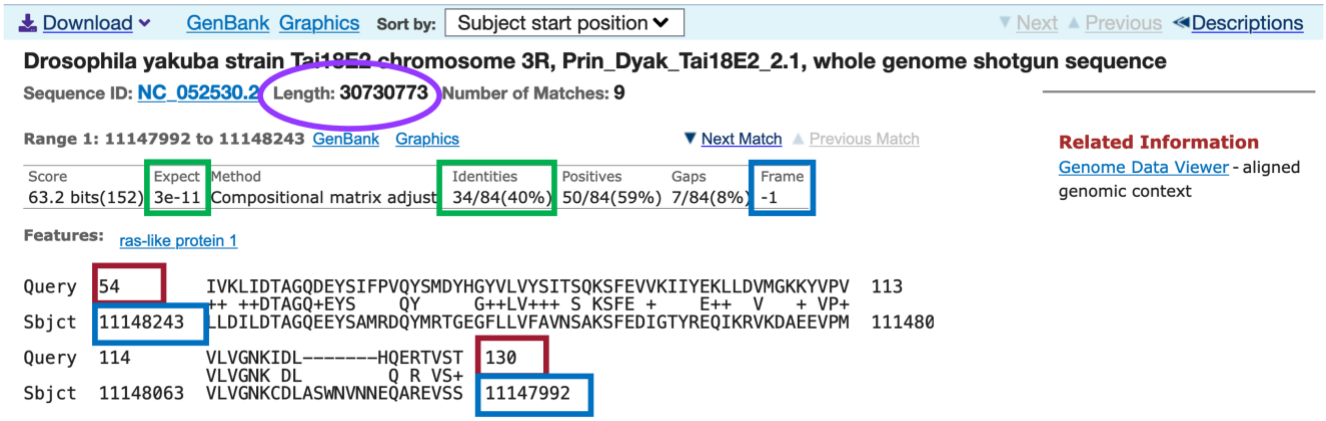


Figure 24 The Query coordinates (55 – 163) and Subject coordinates (7,623,630 – 7,623,953) are shown in red and blue, respectively.

Since the NC_052530 scaffold in *D. yakuba* is 30,730,773 **base pairs (bp)** long (Figure 24, purple circle), it is possible we will have some ranges (i.e., alignment matches or hits) that don't correspond to our ortholog; therefore, we need to examine each match more closely.

Remember that we are looking for matches with low E-values and high sequence identities, and there are five matches (ranges 5 – 9) that fit these criteria (E-value of 2e-78 and sequence identities that range from 83% to 97%). These five alignment matches are also **collinear** and appear on the same strand of DNA (+ Frame) (Figure 25).

The best collinear set of alignments to Rheb-PA is located at 19,150,809 – 19,151,699 on the NC_052530 scaffold of the *D. yakuba* genome assembly and the five alignment matches cover all 182 **amino acids** of Rheb-PA (Figure 25, arrow). Therefore, we will continue our analysis based on the hypothesis that the putative ortholog of Rheb-PA is located at *approximately* **19,150,809 – 19,151,699** on the NC_052530 scaffold of the *D. yakuba* genome assembly.

Range	*D. melanogaster*		Target Species		E-Value	Identities (%)	Subject Frame
	Query Start	Query End	Subject Start	Subject End			
1	54	130	11,148,243	11,147,992	3e-11	40	-1
2	6	44	11,148,568	11,148,431	0.002	46	-3
3	18	108	11,418,759	11,419,094	1e-06	28	+3
4	111	121	11,419,160	11,419,192	1e-06	73	+2
5	1	20	19,150,809	19,150,868	2e-78	90	+3
6	16	45	19,150,981	19,151,070	2e-78	83	+1
7	40	109	19,151,150	19,151,359	2e-78	97	+2
8	111	153	19,151,422	19,151,550	2e-78	93	+1
9	153	182	19,151,610	19,151,699	2e-78	93	+3

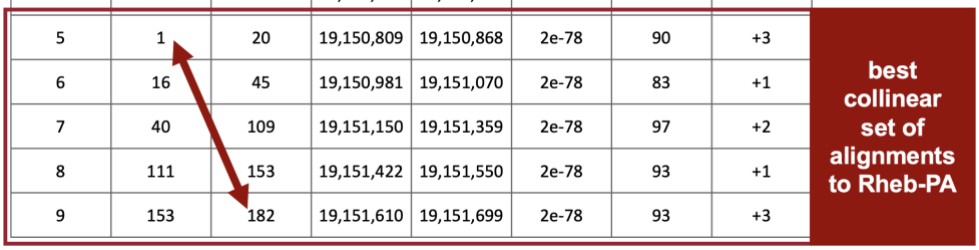


Figure 25 Summary of the *tblastn* search results for the 9 matches to Rheb-PA within the NC_052530 scaffold of *D. yakuba*. The best collinear set of alignments to Rheb-PA is located at 19,150,809-19,151,699. As we saw in Figure 14 Rheb-PA in *D. melanogaster* is 182 amino acids long and the above collinear set of alignments covers all 182 amino acids (arrow).

Part 3: Examine genomic neighborhood of putative ortholog in target species

In Part 1, we sketched the genomic neighborhood of *Rheb* in *D. melanogaster*. Here we will examine the genomic neighborhood of *Rheb* in *D. yakuba* (Figure 26) and then compare the order and orientation of these genes to what we found in *D. melanogaster*.

Based on **parsimony**, the genes surrounding *Rheb* in *D. yakuba* should be identical or very similar in sequence to the genes in the genomic neighborhood of *Rheb* in *D. melanogaster*. Additionally, the neighboring genes should also match in orientation (look at the direction of transcription of the neighboring genes; this is called local **synteny**). Since *Rheb* and *CG2926* are on the positive and **negative strand**, respectively, in *D. melanogaster*, these two genes should also be on different strands in *D. yakuba*.

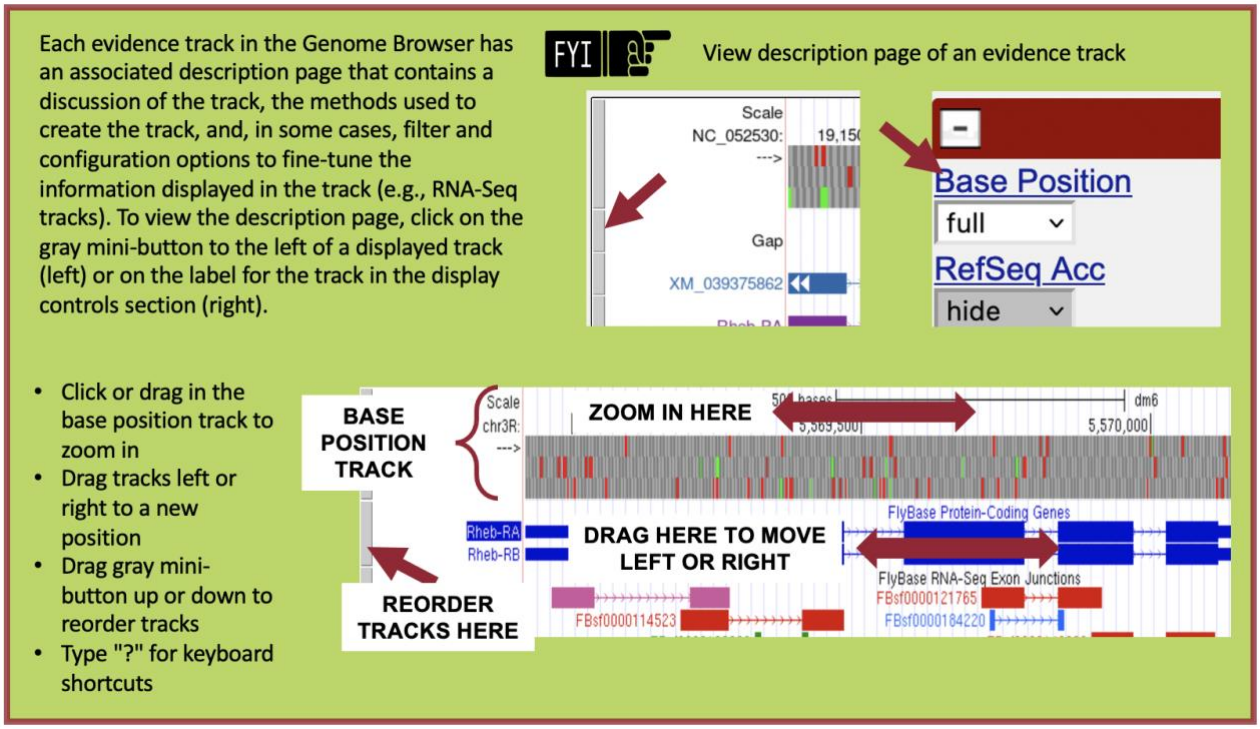


Figure 26 For Your Information — Navigating the GEP UCSC Genome Browser

Part 3.1: Examine evidence for a protein-coding gene in region surrounding the *tblastn* alignment in the target species

1. Navigate to the Genome Browser Gateway page.
2. Click on "*D. yakuba*" in the "UCSC Species Tree and Connected Assembly Hubs" table.
3. Under the "*D. yakuba* Assembly" field, confirm that "Aug. 2021 (Princeton Prin_Dyak_Tai18E2_2.1/DyakRefSeq3)" is selected (Figure 27).

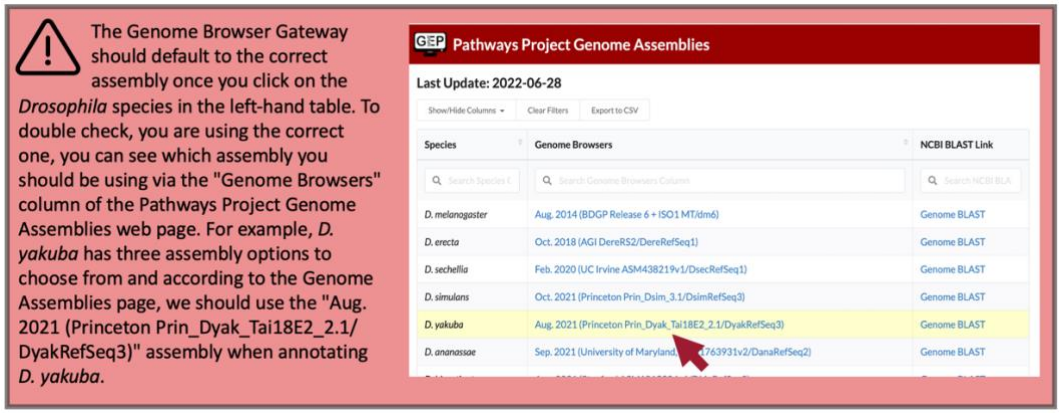


Figure 27 Caution – The "Genome Browsers" column of the Pathways Project Genome Assemblies page shows which assembly to use while annotating.

4. In Part 2, we determined the putative ortholog of Rheb-PA is located at approximately 19,150,809 – 19,151,699 on the NC_052530 scaffold of the *D. yakuba* genome assembly. Enter "**NC_052530:19,150,809-19,151,699**" under the "Position/Search Term" field to examine this region.
5. Click on the "Go" button (Figure 28).

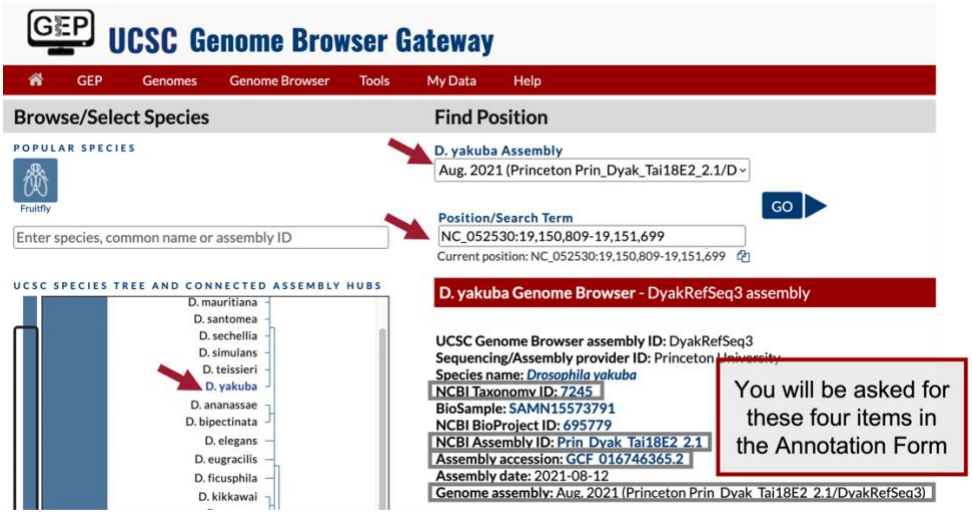


Figure 28 Navigate to the region surrounding the best collinear set of alignments to the *D. melanogaster* Rheb-PA protein in the *D. yakuba* Aug. 2021 (Princeton Prin_Dyak_Tai18E2_2.1/DyakRefSeq3) assembly (i.e., NC_052530:19,150,809-19,151,699).

6. In the list of buttons below the Genome Browser image, click on "default tracks" (Figure 5).
7. Zoom out 3x.

In the Genome Browser image, we should now see the following tracks (Figure 29):

- NCBI RefSeq Genes
- Spaln Alignment of *D. melanogaster* Proteins
- Gene Prediction Tracks (Figure 30):
  - GeMoMa Gene Predictions with RNA-Seq
  - N-SCAN PASA-EST Gene Predictions
  - Augustus Gene Predictions
- RNA-Seq for Adult Female
- RNA-Seq for Adult Male

Looking at the **NCBI** RefSeq Genes track, we notice that the alignment for the *coding regions* of the *D. yakuba* RefSeq **transcript** XM_039375862 against the NC_052530 scaffold of *D. yakuba* line up with the Spaln alignment to the *D. melanogaster* proteins Rheb-PA and Rheb-PB. Furthermore, the coding portions of the five alignment blocks are in congruence with the placements of the five **coding exons (CDS's)** predicted by GeMoMa, N-SCAN, and Augustus.

According to the RefSeq transcript XM_039375862 (Figure 29, red arrow), the putative (probable) ortholog of Rheb-PA is located at NC_052530:19,150,510-19,152,080 in *D. yakuba* and the region

spanning NC_052530:19,150,809-19,151,702, within the putative ortholog, corresponds to the alignment to the *coding* region of the RefSeq transcript.

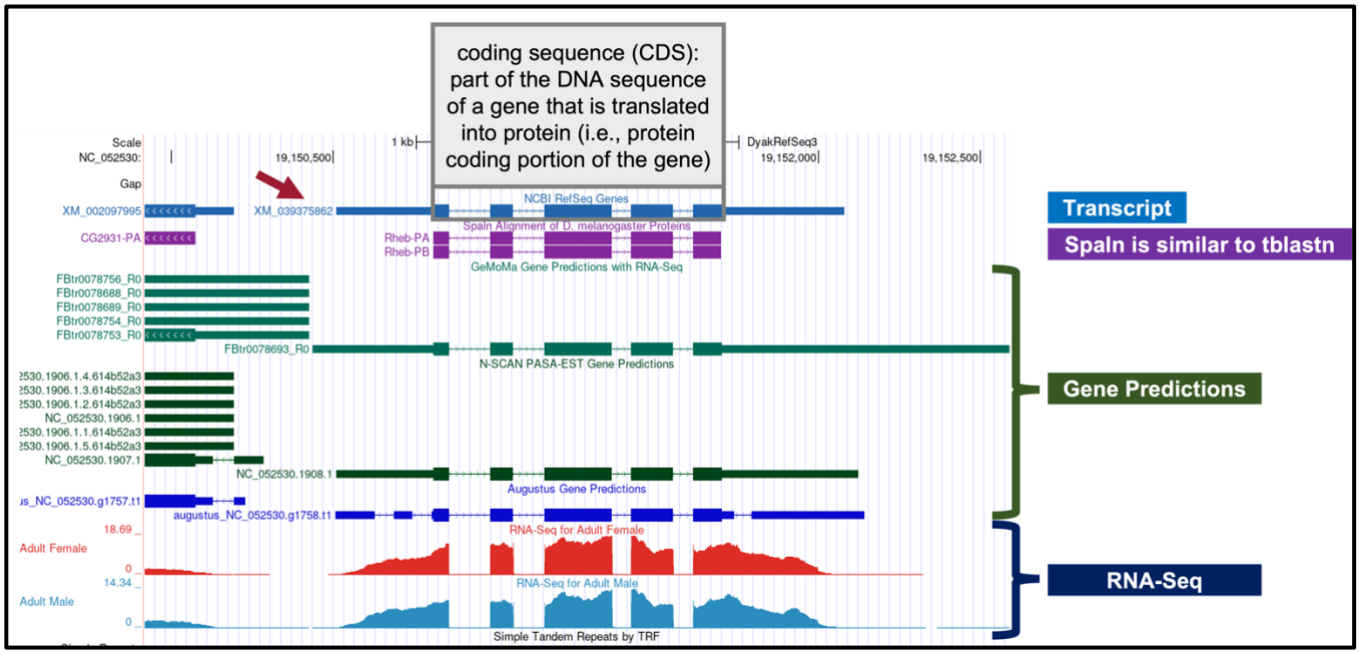


Figure 29 Genome Browser image of NC_052530:19,149,918-19,152,590 in *D. yakuba* (default tracks).

We cannot always trust that what we see in the Genome Browser is accurate, particularly for *Drosophila* species that are *more distantly related* to *D. melanogaster*. The gene prediction tracks, like their name implies, are predictions; thus, *your role, as a researcher in the Pathways Project,* is to help scientists studying these genes be confident in the specific model for the gene. *Your brain is far superior to a computer algorithm* in weighing conflicting evidence, thus your model will be more reliable than what a computer can produce alone. For example, in your own project, there might be a situation where a gene predictor(s) doesn't show a gene in an area that has an alignment to *D. melanogaster* proteins (or vice versa); therefore, you'd need to investigate that further.

Figure 30 Caution – To trust or not to trust the Genome Browser?

Part 3.2: Use synteny to gather additional evidence for the ortholog assignment

1. In the "NCBI RefSeq Genes" track shown in the Genome Browser image, click on "XM_039375862" (Figure 29, red arrow).
   - Note: XM_039375862 is the accession number for the *D. yakuba* RefSeq **mRNA** transcript that is aligned to this region of the *D. yakuba* scaffold.

Notice the position of XM_039375862 is "NC_052530:19,150,510-19,152,080" (Figure 31, black arrow).

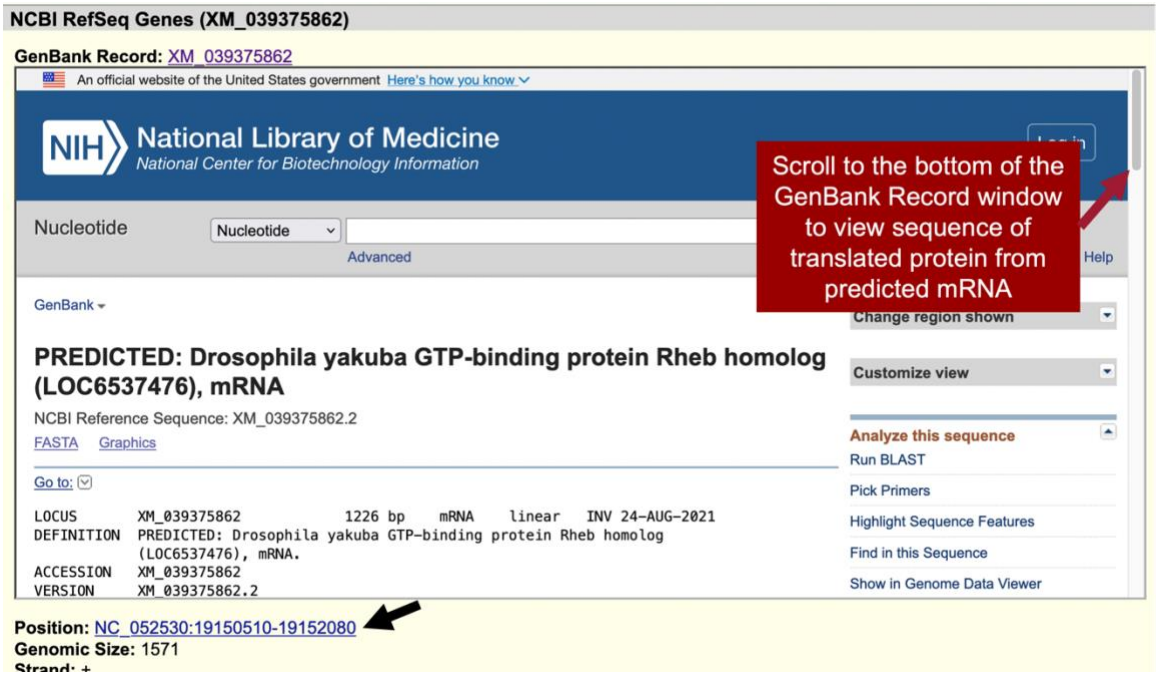


Figure 31 RefSeq Genes feature XM_039375862 is located at NC_052530:19,150,510-19,152,080 (black arrow).

2. Scroll to the bottom of the GenBank Record window to the "**translation**" sequence within the "CDS" section (Figure 32, rectangle).

We are now viewing the *computationally predicted* protein sequence of the putative ortholog to Rheb-PA in *D. yakuba*.

3. Copy the accession number for the translated protein sequence (Figure 32, arrow).

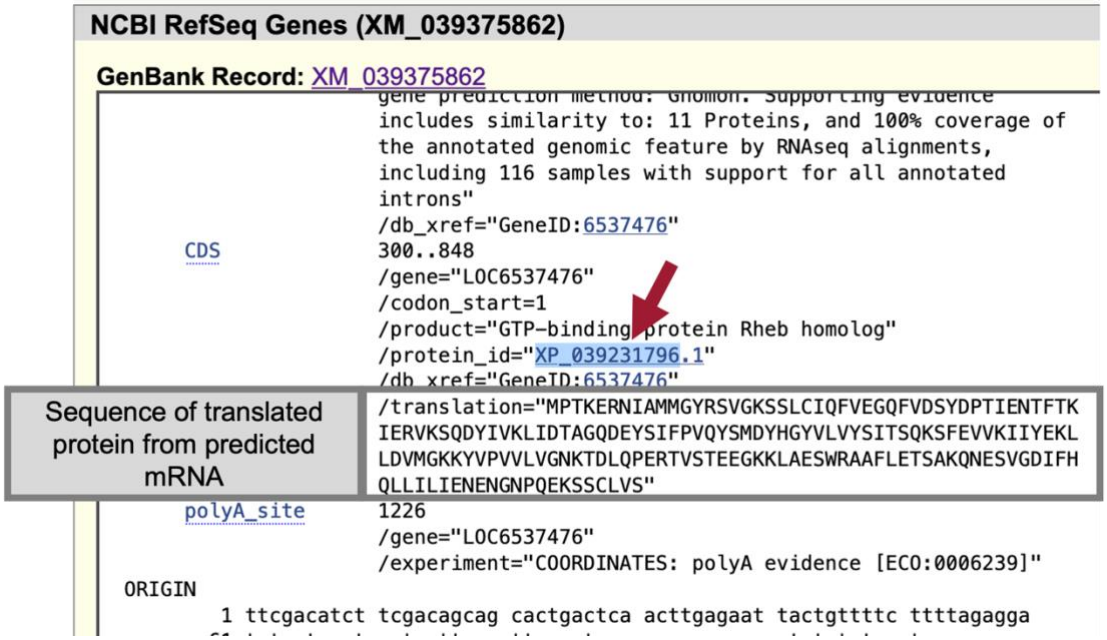


Figure 32 Scroll to the bottom of the GenBank Record window for the RefSeq Genes feature (XM_039375862) to view the sequence of the translated protein. Copy the accession number for the translated protein sequence labeled "protein_id" (see arrow) (Figure 33).

When we run BLAST for this protein, we can enter the accession number "XP_039231796" and the program will use the translated protein sequence shown in the grey box in Figure 32. Note: We recommend using the accession number instead of the actual sequence (i.e., MPTKERNIAMM…) to avoid formatting errors.

FYI The NCBI database of reference sequences (RefSeq) is a curated, non-redundant collection of naturally occurring DNA, RNA, and protein sequences. The RefSeq database includes both known (manually reviewed by NCBI staff or collaborators) and computationally predicted sequences.

Accession Prefix	Description
XM_	mRNA (predicted model)
XP_	protein (predicted model)
NP_	protein (known)

The two-letter prefix (followed by an underscore) of RefSeq accession numbers has an implied meaning with respect to the type of molecule it represents (e.g., known or predicted model, genomic scaffold, mRNA).

Figure 33 For Your Information – A complete list of RefSeq accession number prefixes is available in the NCBI Handbook.

4. Navigate to NCBI BLAST.
5. Click on the "Protein BLAST" button (Figure 34).
   - Note: This is a ***blastp*** search (Figure 16).

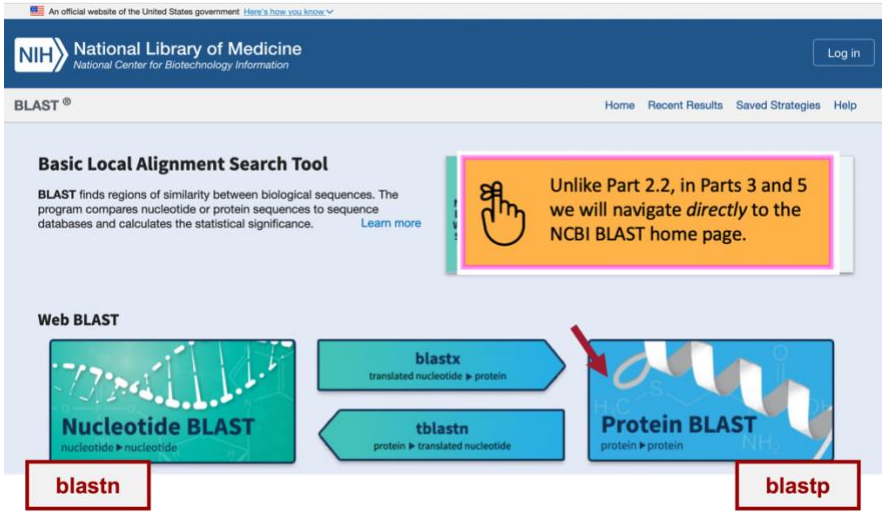


Figure 34 Navigate to the NCBI BLAST website and then click on the "Protein BLAST" button.

6. Paste the accession number for the translated protein sequence we copied from the GenBank Record for XM_039375862 (i.e., "**XP_039231796**") into the "Enter Query Sequence" text box (Figure 35).
7. Under "Choose Search Set," select "Reference proteins (**refseq_protein**)" as the "Database" to search.
8. In the "Organism" text box, enter "**Drosophila melanogaster (taxid:7227)**."
9. Select the check box next to "Show results in a new window" then click on the "BLAST" button.

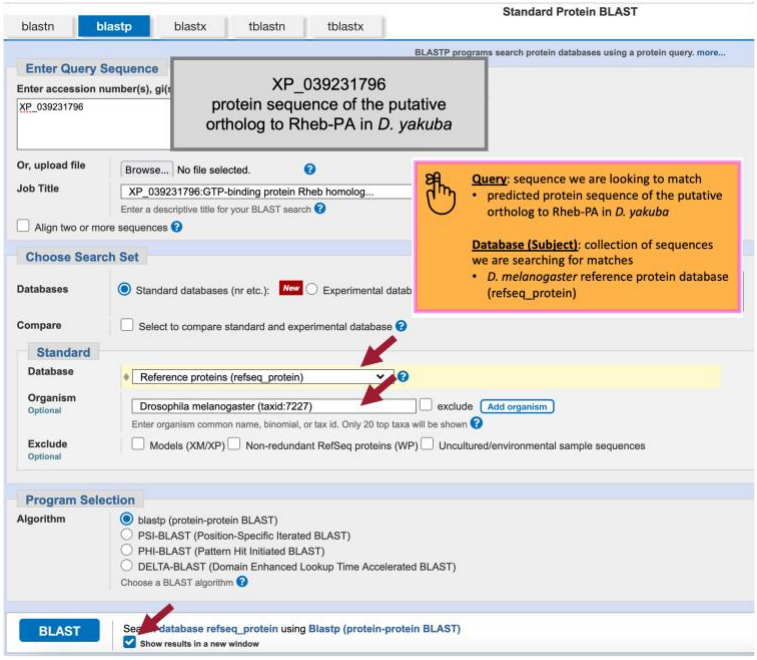


Figure 35 Configure the Protein BLAST (*blastp*) to compare the predicted protein sequence of the putative ortholog to Rheb-PA in *D. yakuba* (query; XP_039231796) against the *D. melanogaster* reference proteins (refseq_protein) database.

Our *blastp* search found 47 proteins within the *D. melanogaster* reference proteins (refseq_protein) database that show similarities with the protein sequence of the putative ortholog of Rheb-PA in *D. yakuba* (XP_039231796); however, only one of these is a good hit (Ras homolog enriched in brain, isoform A [*Drosophila melanogaster*]; accession: NP_730950, sequence identity of 97.25%, and an E-value of 6e-131). The remaining hits had much higher E-values (5e-41 to 0.002) and much lower percent identities (43.75% to 23.48%) (Figure 36). Therefore, the *blastp* search result shows that the *D. yakuba* RefSeq prediction XP_039231796 is most similar to the A isoform of *Rheb* among all the annotated proteins in *D. melanogaster*. Hence, based on **parsimony**, the *blastp* search result supports the **hypothesis** that XP_039231796 is the putative ortholog of *Rheb* in *D. yakuba*.

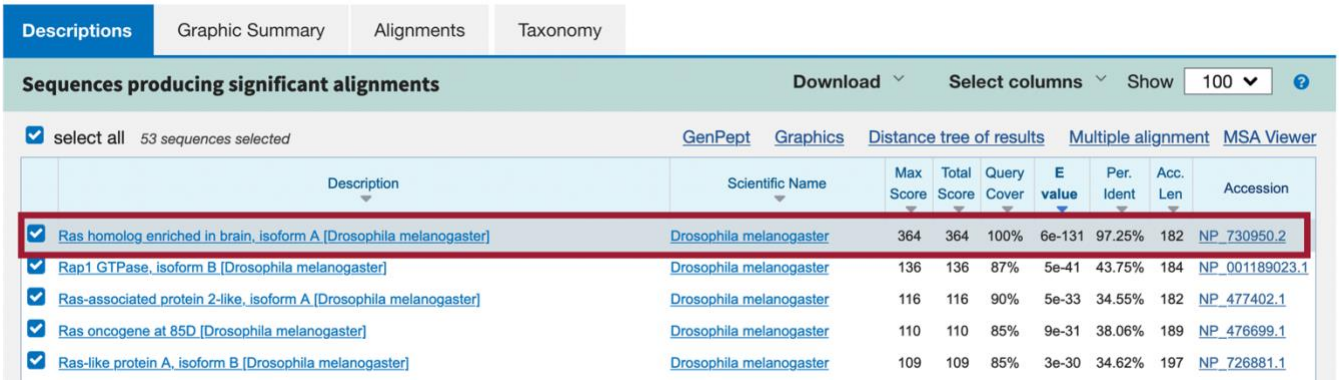


Figure 36 The best *blastp* match to the putative ortholog of Rheb-PA in *D. yakuba* is "Ras homolog enriched in brain, isoform A [*Drosophila melanogaster*]" (Accession: NP_730950) with an E-value of 6e-131 and a sequence identity of 97.25%.

FYI To help us stay oriented to the location of our target gene while looking at the neighboring genes, we can right-click on the the RefSeq transcript XM_039375862 and then select "Highlight XM_039375862." We should now see this region highlighted in blue. When we zoom out to examine the neighboring genes, we can easily visualize where our target gene is because it will remain highlighted. You can also highlight additional regions by repeating this process. To clear a highlight, right-click on the highlighted area and then select "Remove highlight." When right-clicking on the highlighted area, there's also a "Zoom to highlight" option.

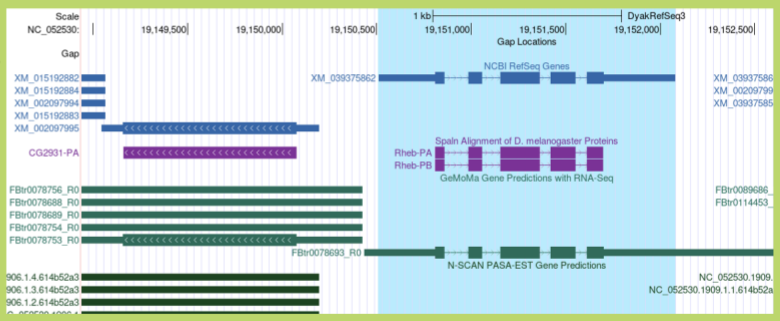


Figure 37 For Your Information – Highlighting regions in the Genome Browser

10. Now we need to repeat the *blastp* search (Steps 1-9 above) for the transcripts of the two neighboring genes on both sides of XM_039375862 (i.e., XM_002097995, XM_015192882, XM_002097997, and XM_002097998). See Figure 37 to help you stay oriented in the Genome Browser. See Appendix B for instructions on how to combine (or batch) multiple sequences.

FYI If the coding isoforms of your gene differ, it's recommended to use the longest one, that is best supported by the other lines of evidence, as the query for the *blastp* search. However, choosing a different isoform than the longest will likely still give the results you need.

Figure 38 For Your Information – Coding isoform lengths

Now that we've examined the genomic neighborhood of the putative ortholog (Figure 39), we need to identify the direction of transcription for the putative ortholog of Rheb-PA *and* the neighboring genes in *D. yakuba* and then use this information to draw a sketch of the genomic neighborhood. To do so, we need to repeat the process we followed in Part 1 (i.e., zoom into an intron (or exon) of each gene and draw arrows in the correct directions on our sketch).

11. Use the strategy described in Steps 9 – 11 of Part 1 to determine the orientations of the putative *Rheb* ortholog (XM_039375862) and the transcripts of the two neighboring genes on both sides (i.e., XM_002097995, XM_015192882, XM_002097997 and XM_002097998) (Figure 40).

blastp search results for the protein sequences of the genomic neighborhood of the target gene in the target species against the *D. melanogaster* reference proteins database (refseq_protein)						
		2nd Closest Upstream	Closest Upstream	Target Gene	Closest Downstream	2nd Closest Downstream
D. melanogaster	Gene Symbol	*CG12746*	*CG2931*	*Rheb*	*CRMP*	*CG2926*
	Strand (+/-)	+	-	+	+	-
Target Species	NCBI RefSeq Gene (mRNA) Accession	XM_015192882	XM_002097995	XM_039375862	XM_002097997	XM_002097998
	NCBI RefSeq Protein Accession	XP_015048368	XP_002098031	XP_039231796	XP_002098033	XP_002098034
	Strand (+/-)	+	-	+	+	-
Best blastp Result	Accession	NP_649551	NP_649552	NP_730950	NP_730954	NP_649554
	D. melanogaster Gene Symbol	*CG12746*	*CG2931*	*Rheb*	*CRMP*	*CG2926*
	E-Value	0.0	0.0	6e-131	0.0	0.0
	Percent Identity	84.30%	96.72%	97.25%	99.66%	86.18%

Figure 39 Summary of the *blastp* search results for the protein sequences of the two nearest predicted upstream and downstream neighbors to Rheb-PA in *D. yakuba* against the *D. melanogaster* reference proteins (refseq_proteins) database.

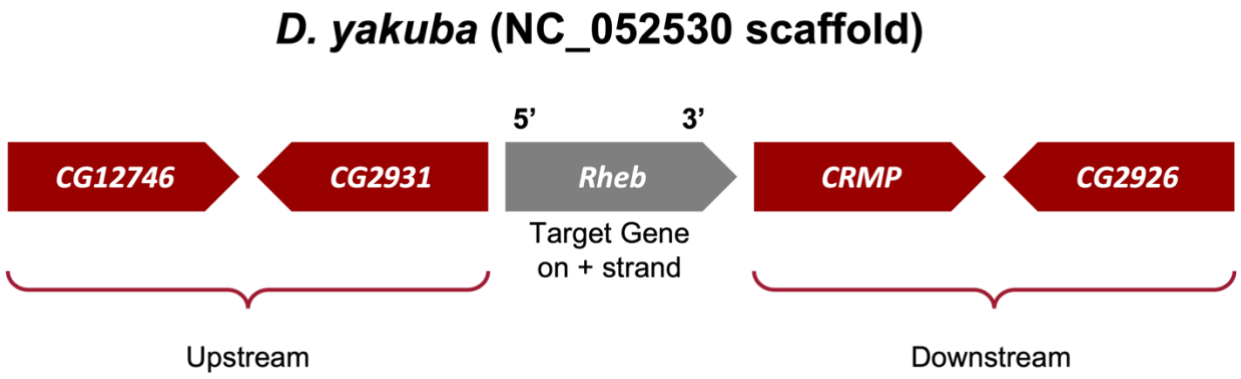


Figure 40 Sketch of the genomic neighborhood of the putative *Rheb* ortholog (on positive strand) in *D. yakuba*. The putative orthologs of *CG12746* and *CG2931* are located upstream of *Rheb*, and the putative orthologs of *CRMP* and *CG2926* are located downstream of *Rheb* in the *D. yakuba* scaffold, and each gene is on the +, -, +, and - strands, respectively.

If the **genomic neighborhood** looks similar between *Rheb* in *D. melanogaster* (Figure 11) and the putative ortholog in *D. yakuba* (Figure 40), we can be confident we have found the best candidate for the ortholog. However, if any of the information is inconsistent with this being a **locally syntenic region**

(**local synteny**: conservation of genomic neighborhood), we should inspect our other hits in the *tblastn* search (Part 2) to see if a different genomic region is a better match overall.

Examination of the genomic regions surrounding the *Rheb* gene in *D. melanogaster* (Figure 41; top) and the putative *Rheb* ortholog in *D. yakuba* (Figure 41; bottom) shows that the relative gene order (i.e., *CG12746*, *CG2931*, *Rheb*, *CRMP*, and *CG2926*) and orientations (+, -, +, + , -) are the same in the two species. Hence the synteny analysis supports the assignment of the *D. yakuba* **feature** at NC_052530:19,150,510-19,152,080 as an ortholog of *Rheb*.

- Note: If your target species' assembly happened to have been numbered from the opposite end of the relevant scaffold, the orientation (+ or – strand) of the orthologs could be the opposite (i.e., -, +, -, -, +) of what you see in *D. melanogaster* but still be syntenic.

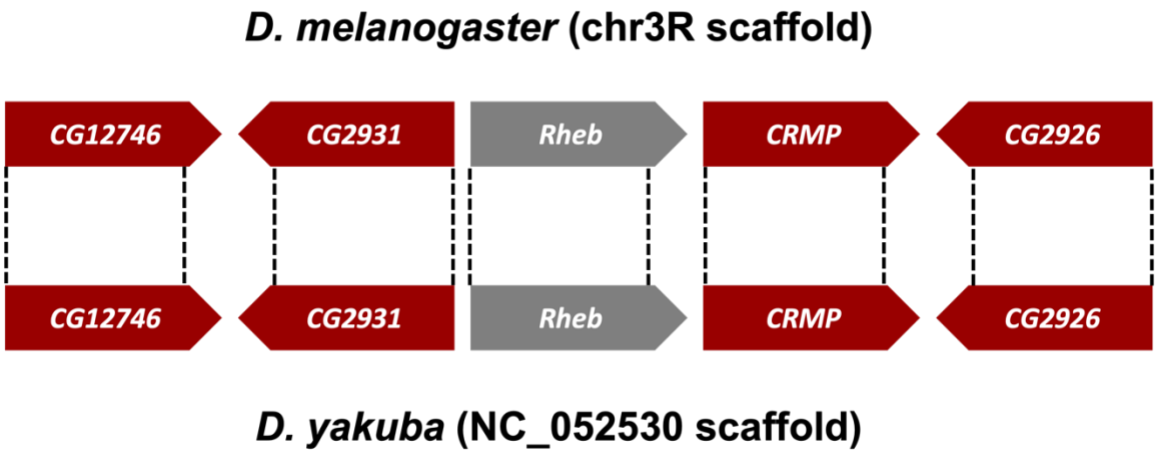


Figure 41 Comparison of the relative order and orientation of the genomic neighborhoods of *Rheb* in *D. melanogaster* (top) and *D. yakuba* (bottom).

Part 4: Determine target gene's structure in *D. melanogaster*

In Part 4 we will use the **Gene Record Finder**, which is a web tool that enables us to quickly identify the set of exons for a given gene and to retrieve their **Coding DNA Sequences (CDS's)**, also referred to as **coding exons**.

The Gene Record Finder will also provide details, such as number of isoforms, exon-intron structure and their coordinates, and transcript and protein information of the gene in question (in this case *Rheb*). **It is important to remember that the details provided by the Gene Record Finder are for the gene in the reference species, *D. melanogaster*.** We will use the details from *Rheb* in *D. melanogaster* to assist us with creating a gene model for *Rheb* in *D. yakuba*.

Before we can construct the orthologous gene model, we need to ascertain the gene structure (e.g., number of isoforms and CDS's) of the *D. melanogaster Rheb* gene using the Gene Record Finder.

1. Open a new web browser tab and navigate to the Gene Record Finder.
2. Enter "**Rheb**" into the text box (Figure 42).
3. Click on the "Find Record" button (Figure 43).

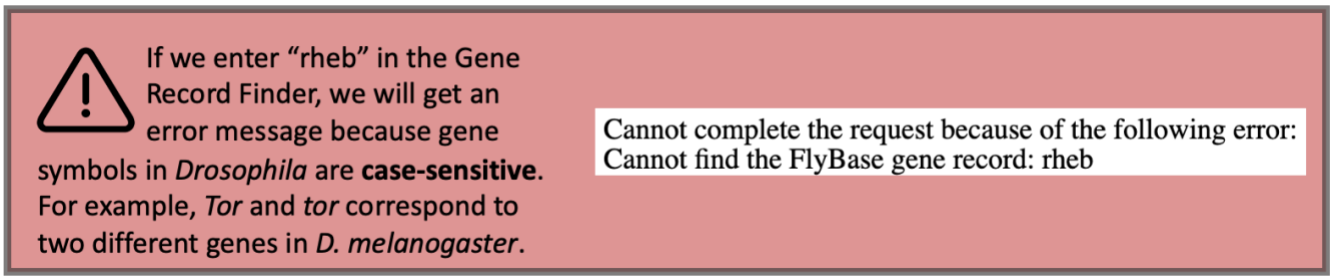


Figure 42 Caution – Gene symbols are case-sensitive.

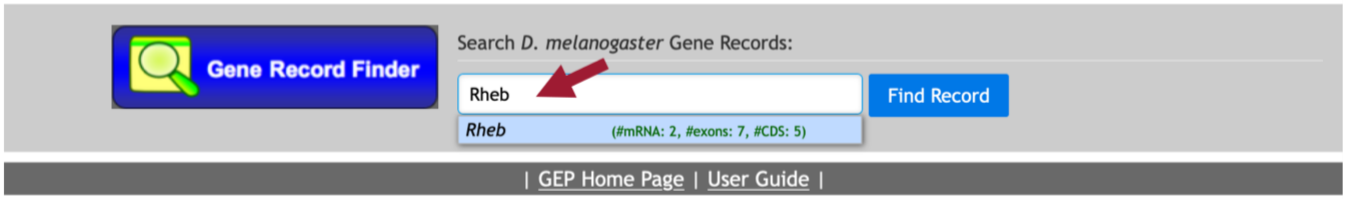


Figure 43 Use the Gene Record Finder to retrieve the gene record for the *Rheb* gene in *D. melanogaster*.

The Gene Record Finder shows that *Rheb* has two isoforms (A and B) in *D. melanogaster*. A graphical overview of the two isoforms is shown in the "mRNA Details" panel. The "CDS usage map" (under the "**Polypeptide** Details" tab) shows that both isoforms have the same set of coding exons (CDS's) (i.e., 1_9829_0, 2_9829_2, 3_9829_2, 4_9829_1, and 5_9829_0). (The coding exons are ordered from 5' to 3' (from left to right) in the CDS usage map.) Hence the differences between these two isoforms are limited to the untranslated regions (UTRs) (Figure 45).

Based on **parsimony** (i.e., minimizing the number of changes compared to *D. melanogaster*), we expect to find both the A and B isoforms of *Rheb* in our *D. yakuba* genome sequence. For this walkthrough, we will only focus on annotating the CDS's, which do not include the UTRs. Consequently, we only need to determine the coordinates of the five CDS's for *one* of the isoforms (e.g., isoform A) because the set of coding exons for both the A and B isoforms are identical (Figure 44).

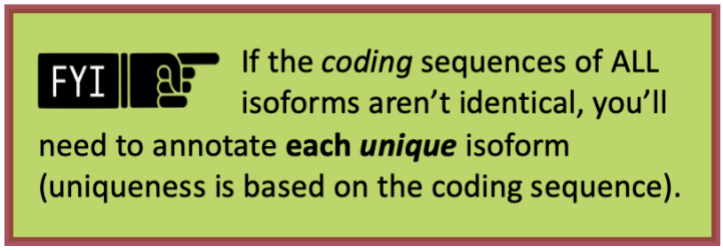


Figure 44 For Your Information – Unique isoforms

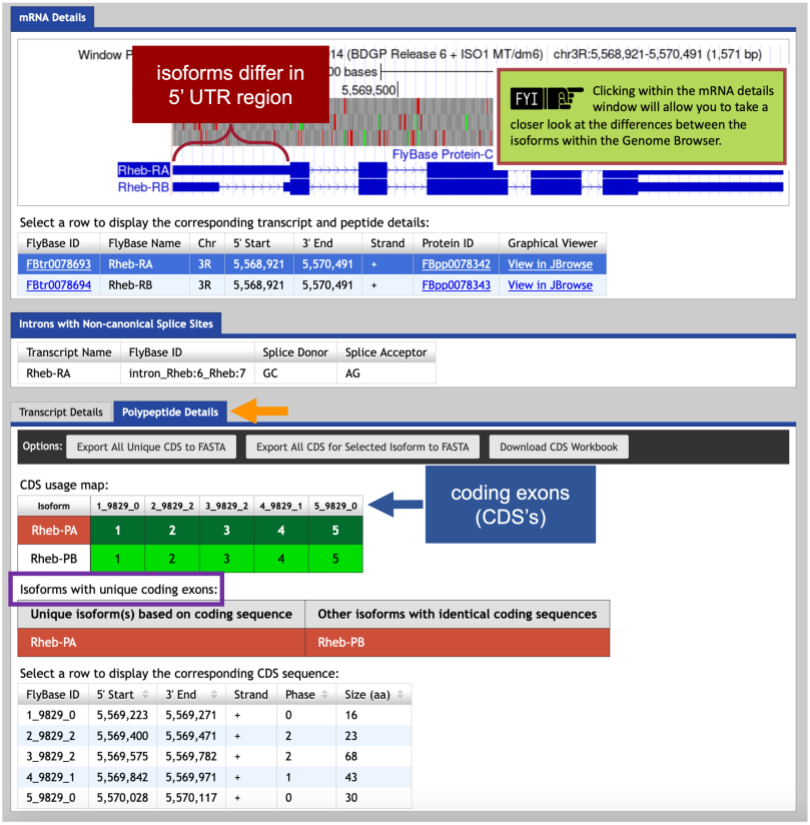


Figure 45 The "mRNA Details" panel of the Gene Record Finder shows that the *Rheb* gene has two isoforms in *D. melanogaster* (i.e., Rheb-RA and Rheb-RB). Under the "Polypeptide Details" tab, the "CDS usage map" indicates that both isoforms have five coding exons (CDS's), and the "Isoforms with unique coding exons" section shows that both isoforms have identical coding sequences.

Part 5: Determine approximate location of coding exons (CDS's) in target species

The initial *tblastn* search we performed in Part 2 helped define the search *region* for the putative ortholog within the genomic scaffold of the target genome, *D. yakuba*. The next step in our analysis is to determine the *approximate* coordinates of each coding exon (CDS) of Rheb-PA in *D. yakuba*. The *approximate* coordinates of each CDS can be determined by aligning *each* CDS of the gene in *D. melanogaster* against the search region (Figure 25) of the target genome (Figure 46).

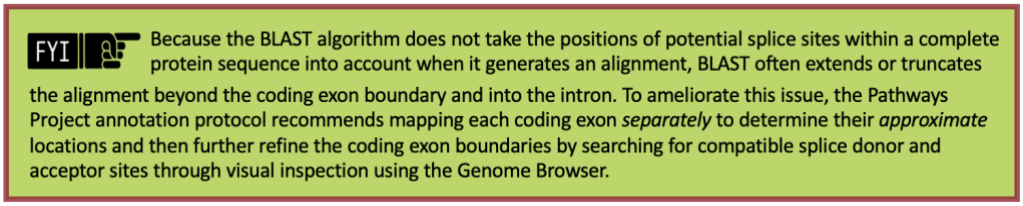


Figure 46 For Your Information – Visual inspection required

In addition to comparing a query sequence against a collection of subject sequences in a database (e.g., Part 2 of this walkthrough), the NCBI BLAST web service also allows us to compare two or more sequences against each other (using the program **bl2seq** (BLAST 2 sequences)).

To map the amino acid sequences of *each D. melanogaster* CDS against the *D. yakuba* scaffold, BLAST must translate the entire *D. yakuba* scaffold sequence in all six **reading frames** (i.e., three reading frames of the positive strand and three reading frames of the negative strand) and then compare each *conceptual translation* against each CDS sequence from *D. melanogaster*. Thus, we will conduct a *tblastn* search using the CDS as the query and the nucleotide sequence of the scaffold as the subject.

Here, we will perform five *tblastn* searches— using each *D. melanogaster Rheb* CDS sequence as the query and the accession number (NC_052530) we identified for the *D. yakuba* scaffold in Part 2 (Figure 21) as the database (subject) sequence.

1. Scroll down to the CDS usage map (under the "Polypeptide Details" tab). Since *Rheb* has 5 CDS's, we will need to run five different *tblastn* searches, one for each CDS. Let's start with CDS-1 (Figure 47).
2. To view the protein sequence for CDS-1, select row 1 (FlyBase ID: 1_9829_0).
3. Copy the protein sequence (including the header) shown in the pop-up window.

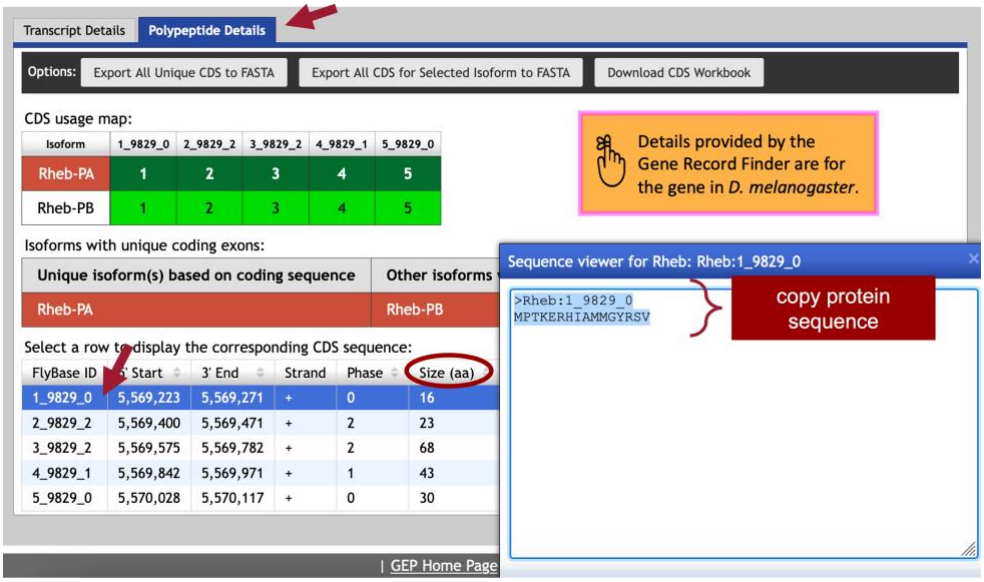


Figure 47 Use the Gene Record Finder to retrieve the amino acid sequence for CDS-1 (FlyBase ID: 1_9829_0). To obtain the sequence for CDS-1, select row 1 (left arrow) and then copy the protein sequence shown in the pop-up window (right). The "Size (aa)" column (circle) indicates how many amino acids (aa) are in each CDS (e.g., CDS-1 is 16 amino acids long).

4. To setup the *tblastn* search, navigate to the NCBI BLAST website.
5. Click on the "*tblastn*" image under the "Web BLAST" section (Figure 48).

Figure 48 Navigate to the NCBI BLAST website, and then click on the "*tblastn*" image.

6. Paste the sequence for CDS-1 (i.e., 1_9829_0) into the "Enter Query Sequence" text box (Figure 49).
7. Select the "Align two or more sequences" checkbox.
    - Note: NCBI BLAST is using the bl2seq (BLAST 2 sequences) program.
8. In the "Enter Subject Sequence" text box, enter the Accession Number for the *D. yakuba* scaffold (i.e., "**NC_052530**") we identified in Part 2.2.

In Part 2 we found the best collinear set of alignments to Rheb-PA in *D. yakuba* was at 19,150,809-19,151,699 on the NC_052530 scaffold. We can now use those coordinates to narrow down the search region of the *D. yakuba* scaffold by limiting the subrange of the NC_052530 scaffold to roughly 100,000 bp on each side of the collinear set of alignments.

The Subject subrange—19,051,000-19,252,000—is determined by subtracting 100,000 from the smaller coordinate (19,150,809), adding 100,000 to the larger coordinate (19,151,699), and then rounding each to the nearest thousandth.

9. Based on our analysis in Part 2.3 (Figure 25), limit the "Subject subrange" by entering from "**19051000**" to "**19252000**" (Figure 49).

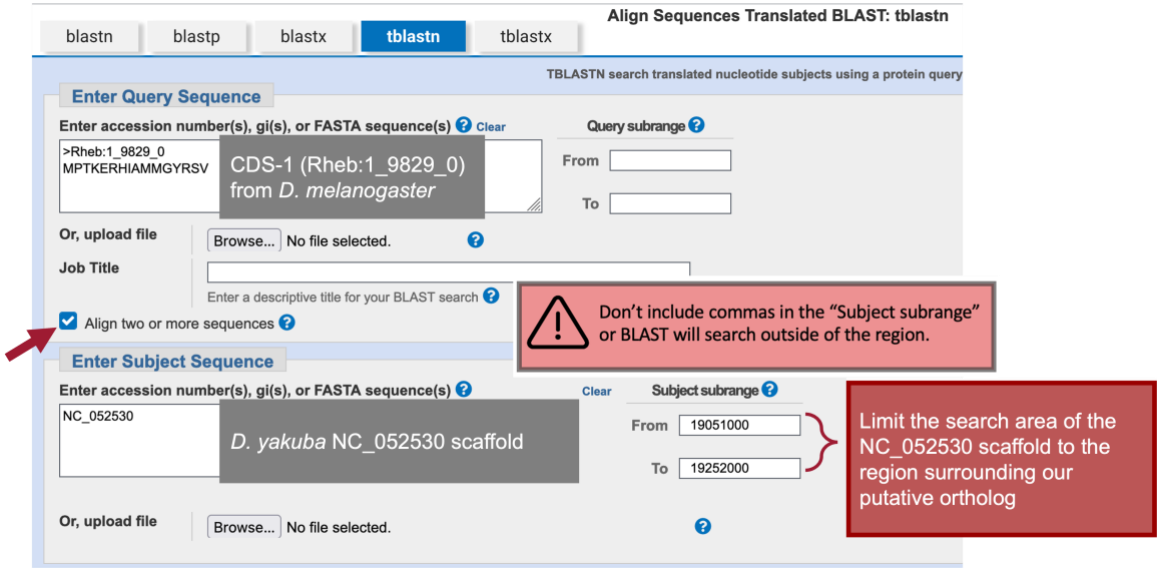


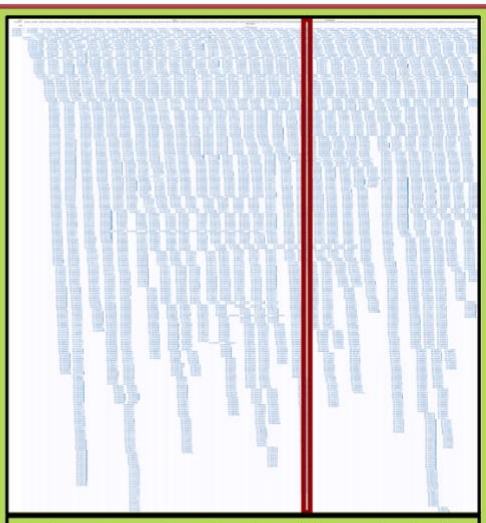
Figure 49 Configure *tblastn* to compare the *D. melanogaster* CDS-1 (query) against the *D. yakuba* NC_052530 scaffold (subject). In Part 2.3 we determined the putative ortholog of Rheb-PA is located at *approximately* 19,150,809 – 19,151,699 on the NC_052530 scaffold. The "Subject subrange" was used to limit the search region to 19,051,000-19,252,000, which was determined by subtracting 100,000 from the smaller coordinate (19,150,809), adding 100,000 to the larger coordinate (19,151,699), and then rounding each to the nearest thousandth (Figure 50).

FYI

The average novel contains roughly 400,000 characters (not including spaces). The NC_052530 scaffold in *D. yakuba* is 30,730,773 bp (or characters) long, which is roughly equivalent to 77 novels worth of information. Furthermore, since BLAST must translate the entire *D. yakuba* scaffold sequence in all six reading frames, that would be more like searching 462 novels to find a few pages of important information. While the English alphabet consists of 26 letters, the genomic alphabet consists of four (A, T, G, and C). With 462 novels worth of only A's, T's, C's, and G's BLAST must search, we're likely to get several spurious alignments; however, limiting the search region will increase the search's statistical power and reduce the number of spurious matches.

In Part 2 we found the best collinear set of alignments to Rheb-PA in *D. yakuba* was at 19,150,809-19,151,699 on the NC_052530 scaffold. We can now use those coordinates to narrow down the 462 novels worth of information to less than one novel by limiting the subrange of the NC_052530 scaffold to roughly 100,000 bp on each side of the collinear set of alignments.

The Subject subrange—19,051,000-19,252,000—was determined by subtracting 100,000 from the smaller coordinate (19,150,809), adding 100,000 to the larger coordinate (19,151,699), and then rounding each to the nearest thousandth.



The Genome Browser image above shows all the transcripts found within the NC_052530 scaffold of *D. yakuba*. Limiting the scaffold's search area to 19,051,000-19,252,000 (red rectangle), increases the statistical power of our search and helps filter out spurious matches.

Note: the Subrange can be larger or smaller than 100,000 bp on each side. Whatever you choose, be sure to generously round (like you wish your instructor would your grade ☺) in both directions.

Figure 50 For Your Information – Limiting search region

The default NCBI BLAST parameters are optimized for searching the query sequence against a large collection of sequences in a database. When we are using BLAST to compare only two sequences against each other, we need to change some of the algorithm parameters because the default parameters could potentially mask the conserved regions of the coding exon.

10. Click on the "+" icon next to "Algorithm parameters" to expand the section (Figure 51).
11. In the "Scoring Parameters" section, change the "Compositional adjustments" field to "No adjustment."
12. In the "Filters and Masking" section, uncheck the "Low complexity regions" checkbox in the "Filter" field.
13. Select the check box next to "Show results in a new window" then click on the "BLAST" button.

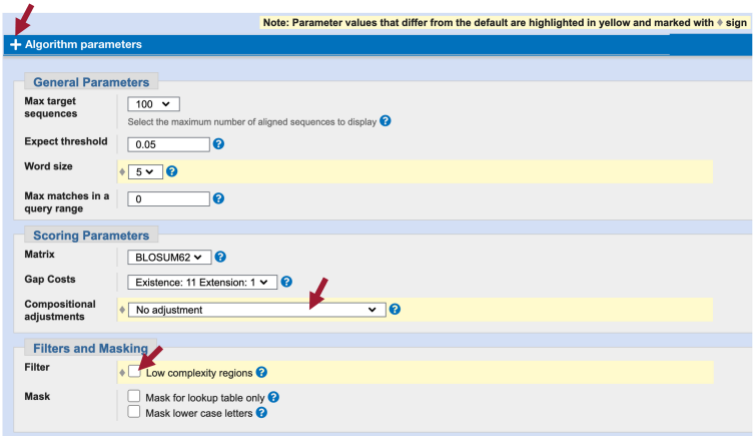


Figure 51 Adjust "Algorithm parameters" to decrease the likelihood that conserved regions of the coding exon will be masked.

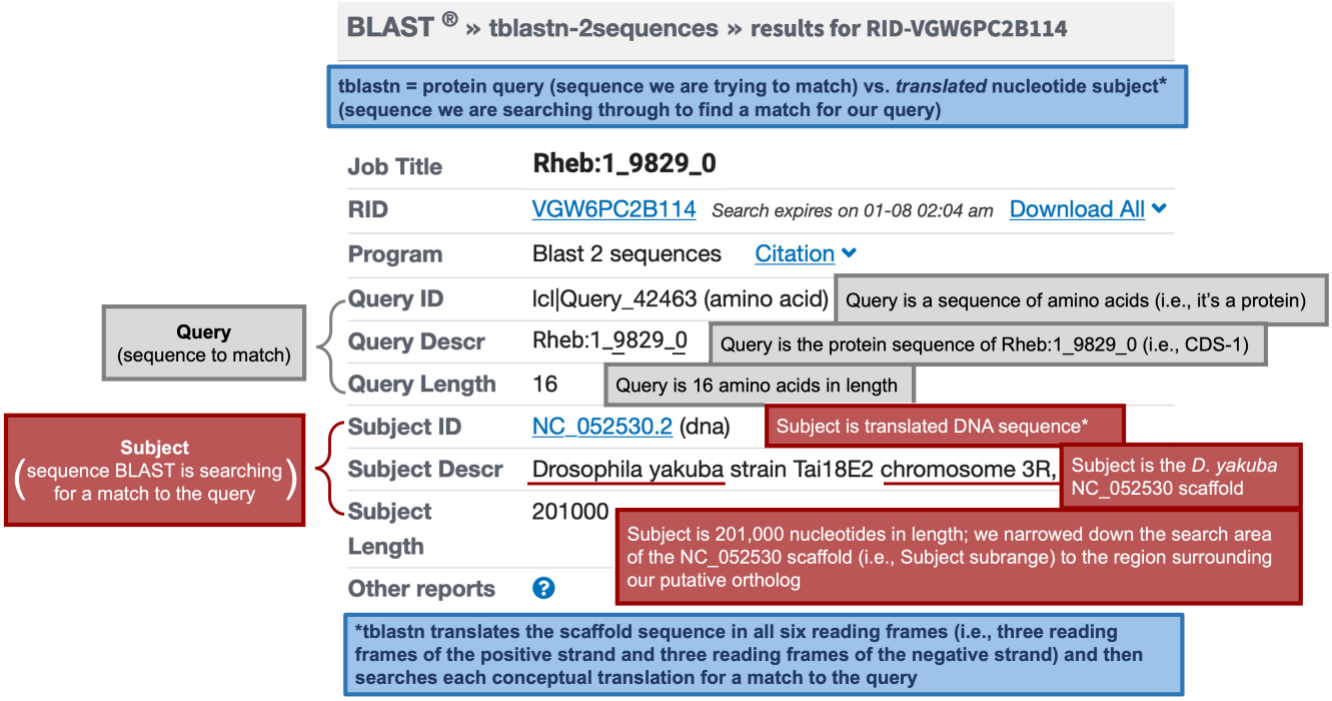


Figure 52 A summary of the *tblastn* search is shown at the top of the results page. The *D. melanogaster* CDS-1 query (Rheb:1_9829_0) is 16 amino acids in length and the program searched 201,000 translated nucleotides of the NC_052530 scaffold in *D. yakuba* to find a match for the 16 amino acids in CDS-1 of *D. melanogaster*.

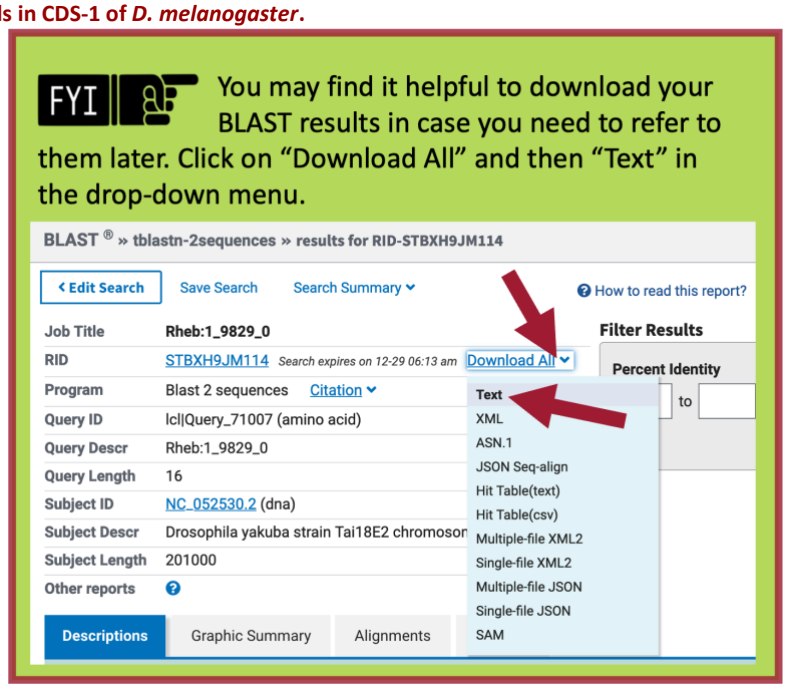


Figure 53 For Your Information— Download BLAST results

The *tblastn* results (Figure 52, Figure 53) show a single match (E-value: 1e-06; sequence identity: 93.75%) to CDS-1 (Rheb:1_9829_0).

14. Click on the "Alignments" tab to view the corresponding *tblastn* alignment (Figure 54).

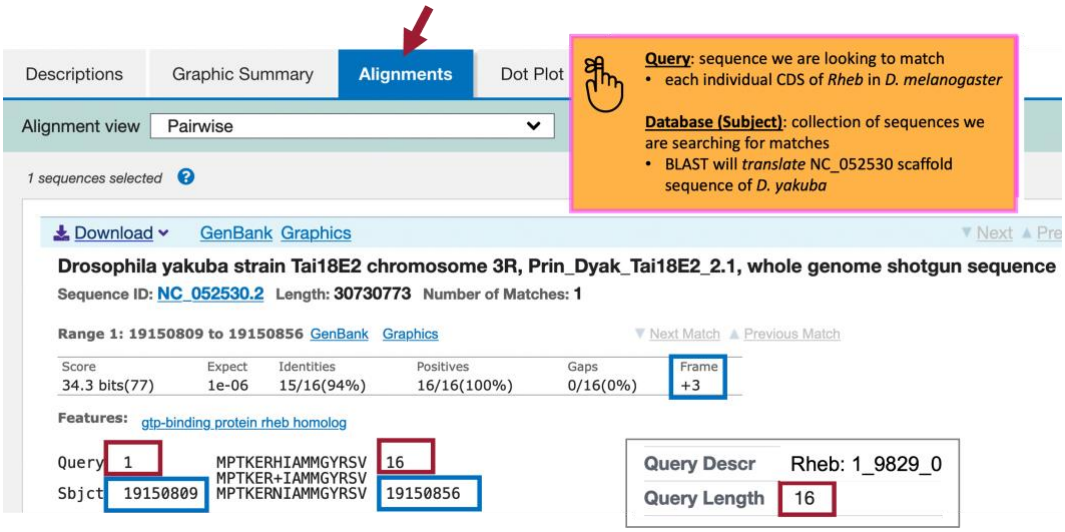


Figure 54 The *tblastn* alignment between the *D. melanogaster* CDS-1 (Query) against the *D. yakuba* NC_052530 scaffold (Sbjct) is located at 19,150,809-19,150,856 in the NC_052530 scaffold of *D. yakuba* (blue boxes) when the sequence is translated in Frame +3. This alignment covers all 16 amino acids of CDS-1 (red boxes).

The "Query" coordinates show that the alignment covers all 16 amino acids (aa) of CDS-1 (Figure 54, red boxes).

- NOTE: We can find the length (in aa) of CDS-1 in *D. melanogaster* using the Gene Record Finder ("Size (aa)" column under the "Polypeptide Details" tab; Figure 47, circle) or at the top of the *tblastn* search results (Figure 52).

The "Subject" coordinates correspond to the region within the NC_052530 scaffold of *D. yakuba* (i.e., 19,150,809 – 19,150,856) that shows sequence similarity to CDS-1 (Rheb:1_9829_0) from *D. melanogaster* when it is translated in the third reading frame of the positive strand (i.e., Frame +3). Hence, we can place CDS-1 of *Rheb* at *approximately* "**NC_052530:19,150,809-19,150,856**" in *D. yakuba*.

We can apply this same procedure to place the other four CDS's on the NC_052530 scaffold of *D. yakuba*. See Appendix A for instructions on how to combine (or batch) multiple sequences.

15. Copy the CDS-2 (Rheb:2_9829_2) sequence (along with the header) from the Gene Record Finder.
16. Return to the *tblastn* web browser and delete the CDS-1 sequence from the "Enter Query Sequence" textbox.
17. Paste the CDS-2 sequence in the textbox (leave everything else the same as we had for CDS-1).
18. Click on the "BLAST" button to run the *tblastn* search.
19. Click on the "Alignments" tab to view the corresponding *tblastn* alignment.
20. Repeat Steps 15-19 to BLAST the remaining CDS's (Figure 55).

CDS	FlyBase ID	Query Length Size (aa)	*D. melanogaster*		Target Species		Subject Frame
			Query Start	Query End	Subject Start	Subject End	
1	1_9829_0	16	1	16	19,150,809	19,150,856	+3
2	2_9829_2	23	1	23	19,150,987	19,151,055	+1
3	3_9829_2	68	1	68	19,151,156	19,151,359	+2
4	4_9829_1	43	1	43	19,151,422	19,151,550	+1
5	5_9829_0	30	1	30	19,151,613	19,151,702	+3

Figure 55 Summary of the *tblastn* CDS-by-CDS search results.

Examination of the subject ranges for the *tblastn* alignments of the five CDS's of *Rheb* in *D. yakuba* shows that they are collinear—CDS's 1-5 are placed on the positive strand and the subject ranges for the CDS's are in ascending order. Consequently, the CDS-by-CDS search results support the hypothesis that the putative (probable) ortholog of Rheb-PA is located at *approximately* 19,150,809 – 19,151,702 on the NC_052530 scaffold of the *D. yakuba* genome assembly (i.e., **NC_052530:19,150,809-19,151,702**).

Part 6: Refine coordinates of coding exons (CDS's)

Now that we've mapped each CDS separately to determine their *approximate* locations (Figure 55), we will further refine the CDS boundaries by searching for compatible **splice donor** and **splice acceptor** sites by visual inspection using the Genome Browser (Figure 56).

As part of the modENCODE project, RNA-Seq data for *D. yakuba* was generated using samples from adult females and males. These RNA-Seq **reads** (100–125 bp in length) are derived primarily from processed mRNA (i.e., after the introns have been removed). Hence, genomic regions with RNA-Seq read coverage usually correspond to transcribed exons, which include both the translated and untranslated regions.

The RNA-Seq tracks correspond to the samples from adult females (red) and males (blue) where RNA-Seq data is available (Figure 57). The height of the histograms within each track corresponds to the number of RNA-Seq reads that have been mapped to each position of the *D. yakuba* scaffold. By default, the scale of the "RNA-Seq Coverage" track will change automatically based on the minimum and maximum read depth within the genomic region being viewed.

Part 6.1: Verify start codon coordinates

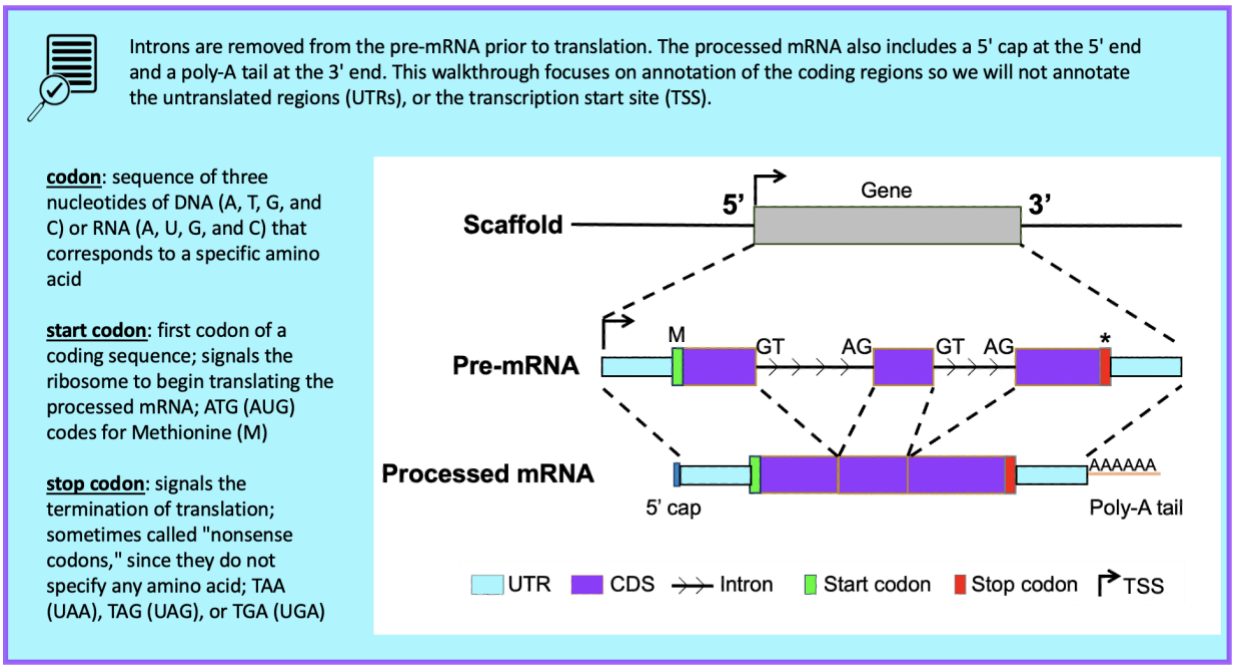


Figure 56 Review of RNA-Seq Primer

We need to ascertain whether the *tblastn* alignment for CDS-1 at 19,150,809-19,150,856 (Figure 57) is supported by RNA-Seq data and, if so, determine the location of the **start codon**.

1. Return to the Genome Browser for *D. yakuba*.
2. To examine the region of the *tblastn* alignment for CDS-1, enter "**NC_052530:19,150,809-19,150,856**" into the "enter position or search terms" text box.
3. Under the "Mapping and Sequencing Tracks," change the "Base Position" track to "full."
4. Click on any "refresh" button.

The RNA-Seq tracks for both samples show high RNA-Seq read depth within the *tblastn* alignment block (19,150,809-19,150,856), consistent with the hypothesis that this region is being transcribed in *D. yakuba*.

Notice that Frame +1 of this region has two **stop codons** (denoted in red with an '*') while Frames +2 and +3 have an **Open Reading Frame** (i.e., no stop codons are shown within Frames +2 or +3 of CDS-1).

5. To examine the region surrounding the start of the *tblastn* alignment to CDS-1, enter "**NC_052530:19,150,809**" into the "chromosome range or search terms" text box.
6. Click on the "go" button.
7. Zoom out 3x and another 10x.

In Part 5 (Figure 54), we found our *tblastn* alignment for CDS-1 begins at *approximately* 19,150,809 (Figure 57, highlighted blue). Examination of this region using the Genome Browser shows us that Frame +3 has a start codon in this location and is supported by multiple evidence tracks—the NCBI

RefSeq Genes and Spaln alignment of *D. melanogaster* proteins, and gene predictors GeMoMa, N-SCAN, and Augustus (Figure 57).

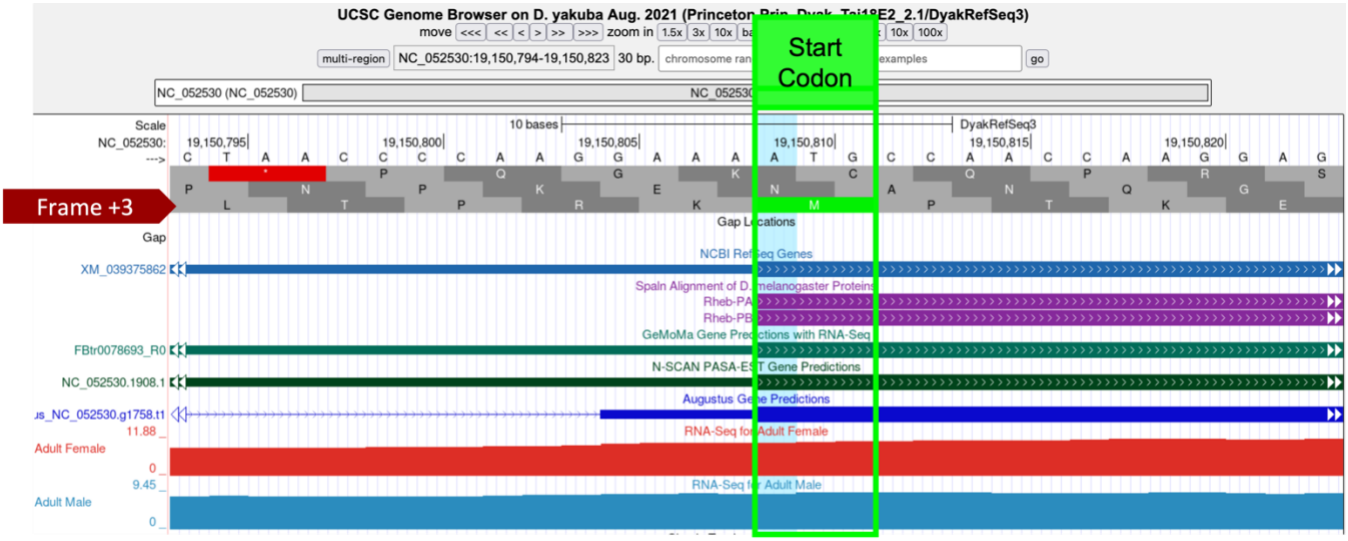


Figure 57 Our *tblastn* search placed the start of CDS-1 at *approximately* 19,150,809 (highlighted blue within green box). The start codon in Frame +3 at NC_052530:19,150,809-19,150,811 (green box) is supported by the NCBI RefSeq Genes and Spaln alignment of *D. melanogaster* proteins, and gene predictors GeMoMa, N-SCAN, and Augustus. Thus, the most likely translation initiation site for the Rheb-PA ortholog in *D. yakuba* is assigned to the position 19,150,809-19,150,811 on the NC_052530 scaffold.

The *tblastn* alignment for CDS-1 (CDS 1_9829_0) of *Rheb* in *D. melanogaster* against the *D. yakuba* scaffold encompasses all 16 amino acids of the CDS (Figure 54), and the alignment begins with a start codon at 19,150,809-19,150,811. Hence the most **parsimonious** gene model for Rheb-PA in *D. yakuba* would use the start codon at 19,150,809-19,150,811 in scaffold NC_052530.

Part 6.2: Verify stop codon coordinates

In Part 5 (Figure 55), we found our *tblastn* alignment for CDS-5 ends at *approximately* 19,151,702 when translated in Frame +3. The alignment covers all 30 amino acids of CDS-5 and ends with a stop codon (Figure 58).

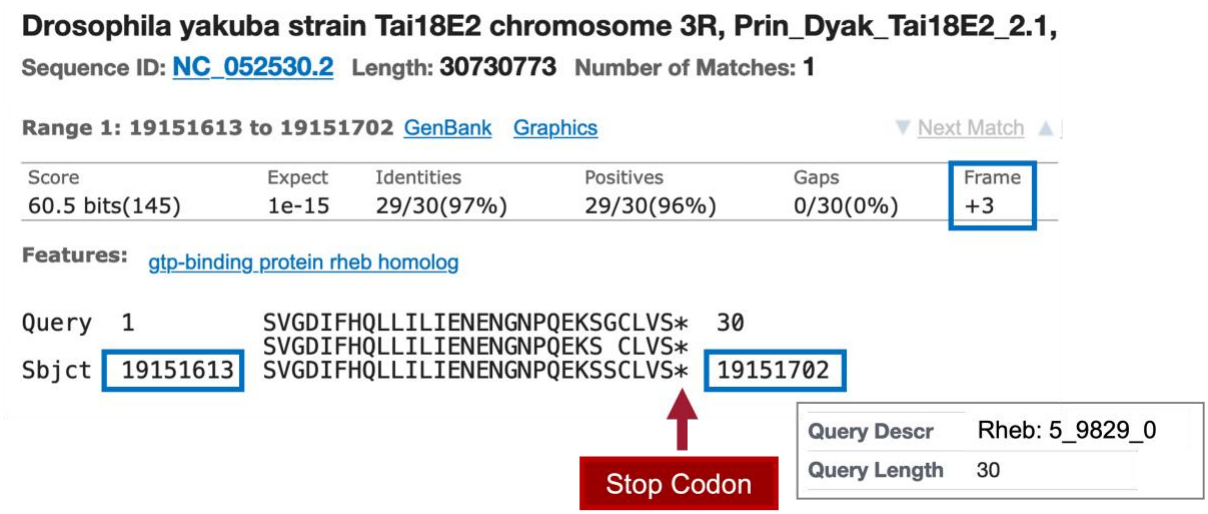


Figure 58 The *tblastn* alignment for the CDS-5 of Rheb-PA (5_9829_0) Query against the *D. yakuba* NC_052530 scaffold (Sbjct) placed this CDS at 19,151,613-19,151,702 (blue box) when the sequence is translated in Frame +3. The *tblastn* alignment covers all 30 amino acids of CDS-5, and it ends with a stop codon ('*'; red arrow).

1. To examine the genomic region surrounding the end of the *tblastn* alignment to CDS-5, enter "**NC_052530:19,151,702**" into the "chromosome range or search terms" text box.
2. Click on the "go" button.
3. Zoom out 3x and another 10x (Figure 59).

The stop codon at 19,151,700-19,151,702 is consistent with the NCBI RefSeq Genes and Spaln alignment of *D. melanogaster* proteins (stop codons don't become part of the protein rather they signal when translation should terminate and the newly made protein should be released), and gene predictors GeMoMa, N-SCAN, and Augustus.

Based on the *tblastn* alignment for CDS-5 and the available evidence on the Genome Browser, the stop codon for the Rheb-PA ortholog is placed at 19,151,700-19,151,702, and the last codon (S; Serine), before the stop codon, ends at 19,151,699 (Figure 59).

Note that the RNA-Seq read coverage tracks for both samples indicate that transcription extends beyond the stop codon. Based on the gene structure of Rheb-RA in *D. melanogaster*, the region with RNA-Seq read coverage that extends beyond the stop codon likely corresponds to the 3' untranslated region (UTR) of the last exon in *Rheb*.

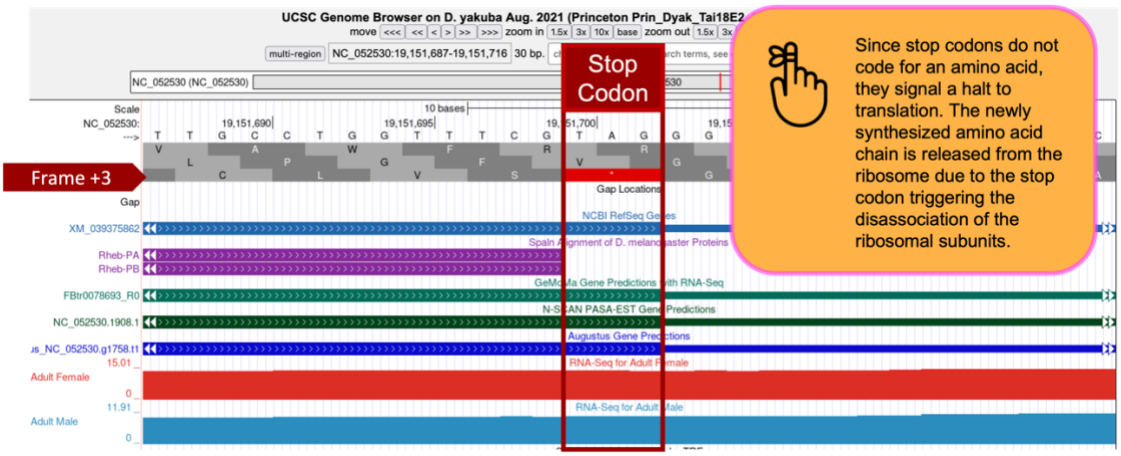


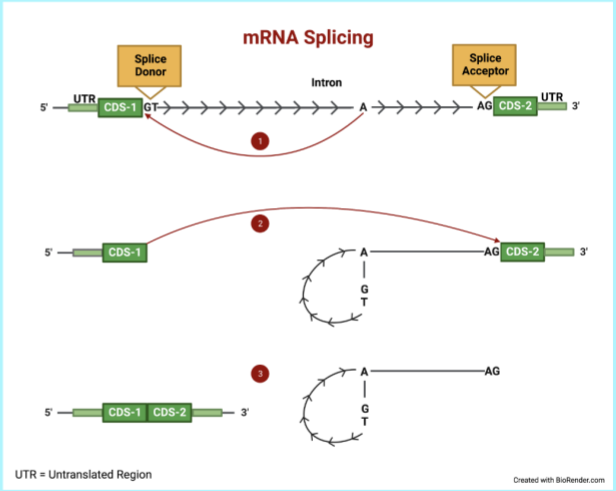
Figure 59 Based on the *tblastn* alignment for CDS-5 and the available evidence on the Genome Browser, the stop codon for the Rheb-PA ortholog is placed at 19,151,700-19,151,702, and the last codon (S; Serine), before the stop codon, ends at 19,151,699.

Part 6.3: Determine phases of donor and acceptor splice sites

During splicing, introns are spliced out (removed) from the pre-mRNA so that adjacent exons are placed next to each other. This means that the ends of an exon do not necessarily correspond to the ends of the complete codon. The number of nucleotides between the last complete codon and the splice donor site (e.g., CDS-1 in the image below) is known as the phase of the splice donor site. Similarly, the number of nucleotides between the splice acceptor site and the first complete codon (e.g., CDS-2 in the image below) is known as the phase of the splice acceptor site. Because the phases of the splice sites depend on the placement of the complete codon, the phases of the donor and acceptor sites are based on the reading frame of each CDS.

In addition, in order to maintain the open reading frame (ORF) across adjacent CDS's, the phases of the donor and acceptor sites of adjacent CDS's must be **compatible** with each other. Specifically, the **sum of the donor and acceptor phases of adjacent CDS's must either be 0 (i.e., no additional codon) or 3 (i.e., a complete codon)**. The use of incompatible splice donor and acceptor sites will introduce a frame shift into the translation of the CDS following the splice acceptor site.

In *D. melanogaster*, approximately 99% of introns have a **GT** splice donor site and 1% have a **GC** non-canonical splice donor site. Almost all introns have an **AG** splice acceptor site. The Pathways Project's comparative annotation protocol posits that all introns have a GT splice donor site and an AG splice acceptor site unless the *D. melanogaster* gene model uses a non-canonical splice site, or the non-canonical splice site is supported by RNA-Seq data.



Phase: number of bases between the complete codon and the splice site

Donor phase: number of bases between the **end of the last complete codon** and the splice donor site (GT/rarely GC)

Acceptor phase: number of bases between the splice acceptor site (AG) and the **start of first complete codon**

Phase **depends on the reading frame** of the CDS

Figure 60 Review of Understanding Eukaryotic Genes Module 4

Because the *tblastn* alignment for CDS-1 of *Rheb* terminates at 19,150,856 (Figure 54), we expect to find the splice donor site for CDS-1 at around position 19,150,856.

1. To examine the genomic region surrounding the splice donor site of CDS-1, enter "**NC_052530:19,150,856**" into the "chromosome range or search terms" text box.
2. Click on the "go" button.
3. Zoom out 3x and another 10x to examine the 30 bp surrounding this position.

The GT splice donor site closest to 19,150,856 (Figure 61, green box) is located at 19,150,858-19,150,859 (Figure 61, red box) which is supported by multiple lines of evidence—NCBI RefSeq Genes, Spaln alignment of *D. melanogaster* proteins, and the GeMoMa, N-SCAN, and Augustus gene predictions, and the RNA-Seq read coverage from samples of adult females and adult males.

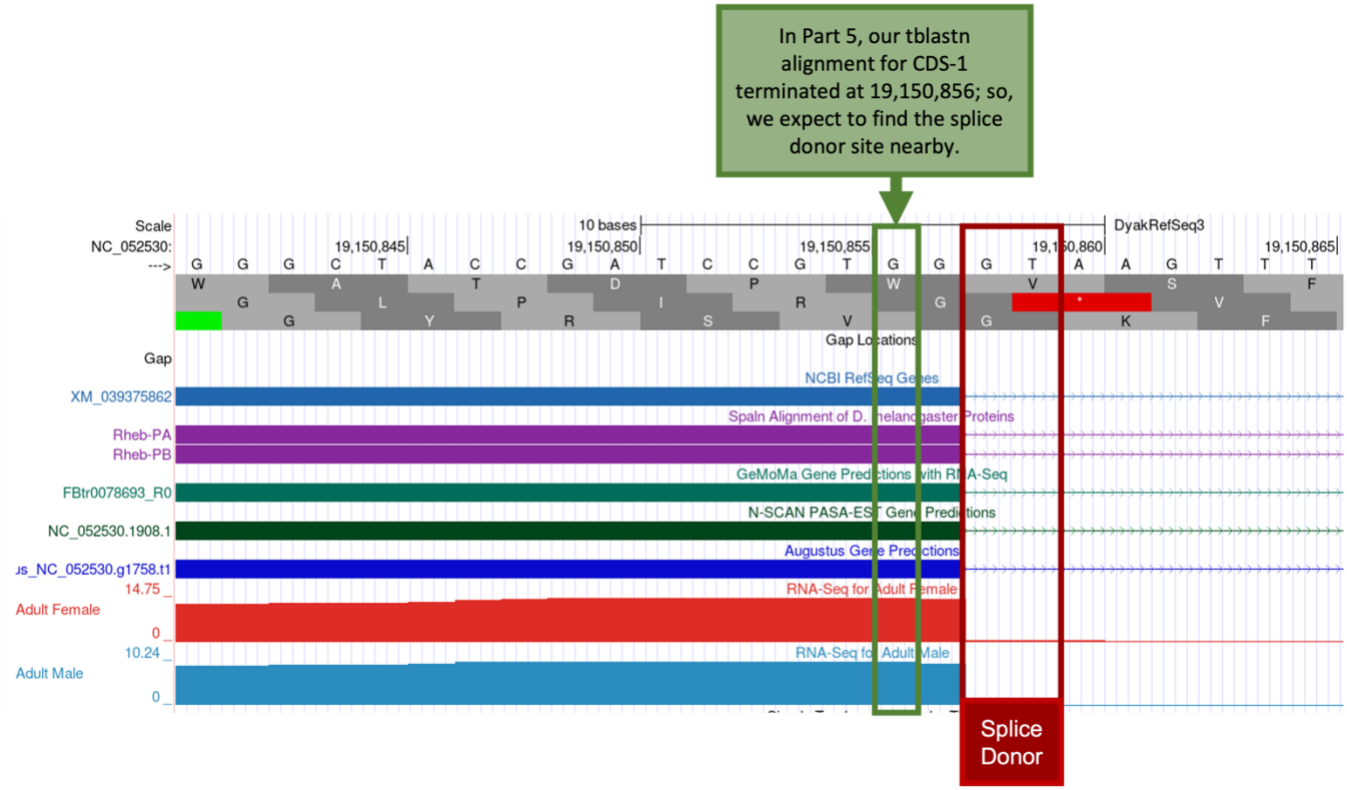


Figure 61 The splice donor site (GT) at 19,150,858-19,150,859 (red box) is supported by multiple lines of evidence and is near the *approximate* end coordinate of CDS-1—at 19,150,856—as determined by *tblastn*.

4. Zoom out far enough to see the entire length of CDS-1 and confirm that Frame +3 has an **Open Reading Frame (ORF)** (i.e., no stop codons are shown within Frame +3 of CDS-1).

Using *tblastn* in Part 5, we determined the *approximate* location of CDS-1 was 19,150,809-19,150,856. Using the Genome Browser, in Part 6.1 we verified the start codon of CDS-1 was in Frame +3 at 19,150,809-19,150,811 (Figure 57). We visually inspected the region surrounding the approximate end of CDS-1 and placed the splice donor site at 19,150,858-19,150,859, after which we confirmed Frame +3 of CDS-1 has an ORF. Now we need to determine the **phase** of the splice donor site for CDS-1.

5. Enter "**NC_052530:19,150,849-19,150,859**" into the "chromosome range or search terms" text box and click on "go."

In Figure 62, we see the last complete codon (i.e., containing three nucleotides) of CDS-1 before the splice donor site codes for the amino acid Tryptophan in Frame +1, Arginine in Frame +2, and Valine in Frame +3.

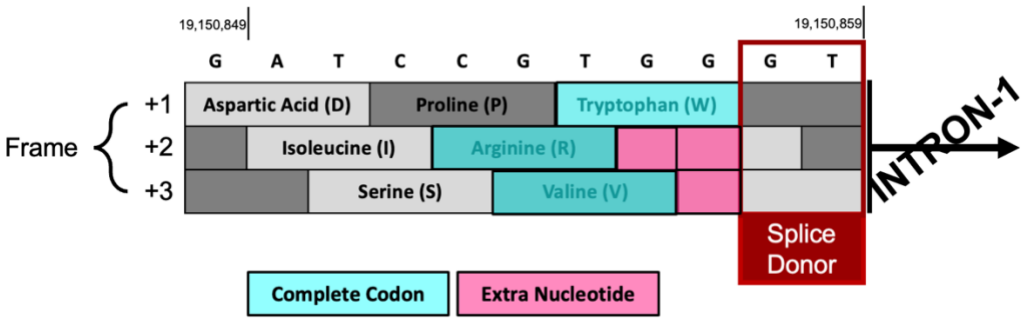


Figure 62 NC_052530:19,150,849-19,150,859. Since the splice donor site (GT) is at 19,150,858-19,150,859 (red box), the last coordinate of CDS-1 is, therefore, 19,150,857. The CDS-1 splice donor site has three possible phases (0, 1, or 2), which depend on the reading frame. Frame +1 ends in a complete codon, Frame +2 ends with two extra nucleotides, and Frame +3 ends with one extra nucleotide.

The splice donor site at 19,150,858-19,150,859 is in phase 0 relative to Frame +1 because CDS-1 ends in a complete codon and thus has no extra nucleotides. In contrast, Frame +2 and Frame +3 don't end in complete codons so they each have extra nucleotides. The splice donor site is in phase 2 relative to Frame +2 because there are 2 extra nucleotides (GG) after the last complete codon. The splice donor site is in phase 1 relative to Frame +3 since there is 1 extra nucleotide (G) after the last complete codon (Figure 62).

We previously determined CDS-1 is translated in Frame +3 (Figure 57); therefore, the last complete codon of CDS-1 (GTG which codes for Valine (V)) is located at 19,150,854-19,150,856 and there is one extra nucleotide (G at 19,150,857) between the last complete codon and the splice donor site. Hence, the CDS-1 splice donor site is in phase 1.

Our *tblastn* alignment in Part 5 placed CDS-2 at *approximately* 19,150,987 – 19,151,055 (Figure 55); therefore, we expect to find the splice acceptor site for CDS-2 around position 19,150,987.

6. To examine the genomic region surrounding the splice acceptor site of CDS-2, enter "**NC_052530:19,150,987**" into the "chromosome range or search terms" text box and click on "go."
7. Zoom out 3x and another 10x to examine the 30 bp surrounding this position (Figure 63).

There is only one potential **canonical** **("standard rule")** splice acceptor site (AG) in the 30 bp region surrounding the start of the *tblastn* alignment to CDS-2 (Figure 63, green box). The splice acceptor site is located at 19,150,983-19,150,984 (Figure 63, red box) and is supported by the NCBI RefSeq Genes and Spaln alignment of *D. melanogaster* proteins, the GeMoMa, N-SCAN, and Augustus gene predictions, and the RNA-Seq read coverage. Thus, CDS-2 starts at 19,150,985.

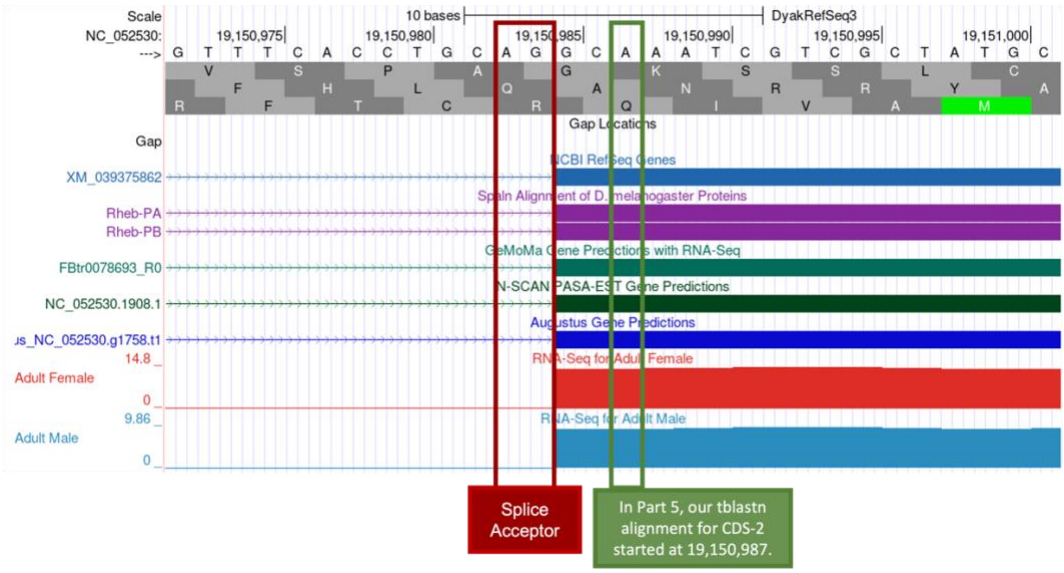


Figure 63 Our *tblastn* search placed the start of CDS-2 at *approximately* 19,150,987. The splice acceptor site (AG) is at 19,150,983-19,150,984 and CDS-2 starts at 19,150,985.

Now we need to determine the frame in which CDS-2 is translated.

8. Enter "**NC_052530:19,150,980-19,150,990**" into the "search terms" text box and click on "go" to zoom in on the region surrounding the splice acceptor site.

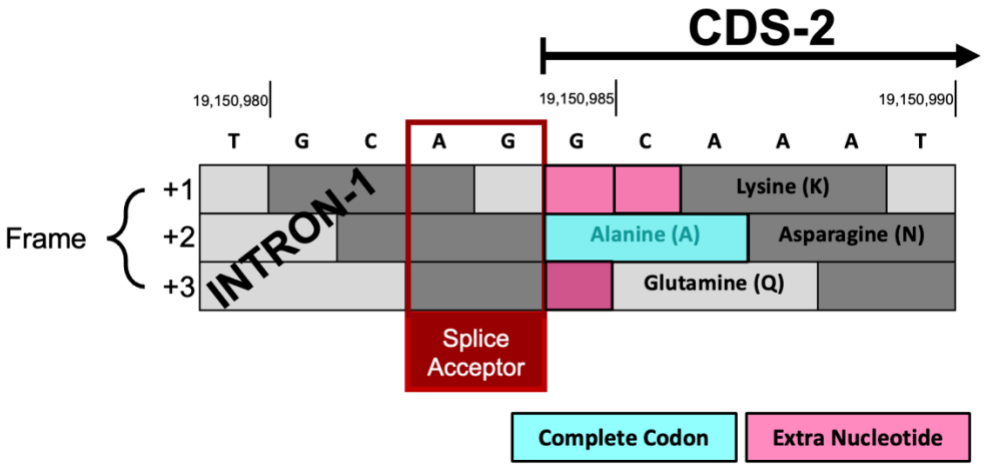


Figure 64 The CDS-2 splice acceptor site at 19,150,983-19,150,984 has three possible phases (0, 1, or 2), which depend on the reading frame.

Each frame can have extra nucleotides (0, 1, or 2) between the beginning of CDS-2 at 19,150,985 and the first complete codon of each frame (Figure 64).

- Frame +1 has two extra nucleotides (GC) before the first complete codon (Lysine; K).
    - Splice acceptor site is in phase 2 relative to Frame +1.

- Frame +2 begins with a complete codon (Alanine; A) so it has zero extra nucleotides.
    - Splice acceptor site is in phase 0 relative to Frame +2.

- Frame +3 has one extra nucleotide (G) before the first complete codon (Glutamine; Q).
    - Splice acceptor site is in phase 1 relative to Frame +3.

Since the CDS-1 splice donor site at 19,150,858 – 19,150,859 is in phase 1 relative to Frame +3 (i.e., CDS-1 ends with one extra nucleotide), the CDS-2 splice acceptor site must be in phase 2 (i.e., CDS-2 must begin with two extra nucleotides) to maintain the ORF after Intron-1 has been removed. Since **CDS-1** had **one extra nucleotide (G)** between the last complete codon (GTG = Valine; V) and the splice donor site, **CDS-2** must start with **two** extra nucleotides to join the extra one from CDS-1 because we **need 3 nucleotides to make a complete codon**.

Since CDS-2 is in Frame +1 and the first complete codon (AAA codes for K) is located at 19,150,987-19,150,989, there are **two nucleotides** (GC at 19,150,985- 19,150,986) between the potential splice acceptor site and the first complete codon. Hence, the CDS-2 splice acceptor site is in **phase 2**.

The extra nucleotides near the splice sites (i.e., G + GC) will form an additional amino acid (Glycine/G) after **splicing** (Figure 65). Collectively, our analysis suggests that CDS-1 ends at 19,150,857 with a phase 1 splice donor site and CDS-2 begins at 19,150,984 with a phase 2 splice acceptor site.

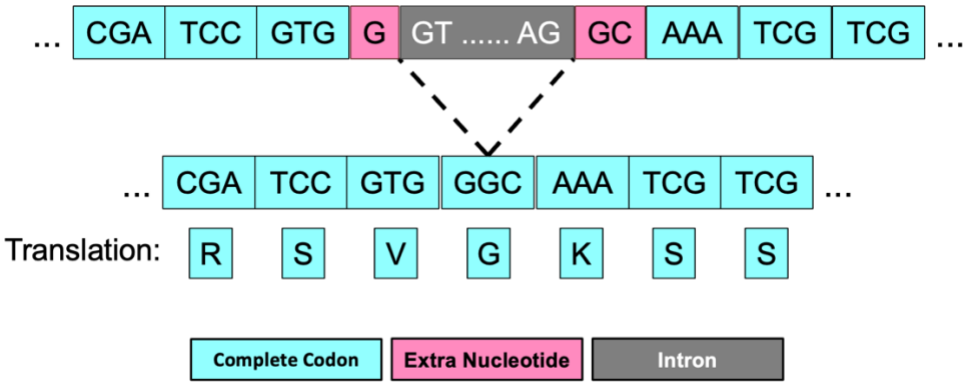


Figure 65 The phase 1 donor site (G) of CDS-1 combines with the phase 2 acceptor site (GC) of CDS-2 to form the codon GGC, which codes for a Glycine (G).

The same annotation strategy can be used to determine the phases for the remaining splice donor and splice acceptor sites between CDS-2 and CDS-3, CDS-3 and CDS-4, and CDS-4 and CDS-5 (Figure 66).

CDS	Frame	Splice Acceptor Phase	Splice Donor Phase
1	+3		1
2	+1	2	1
3	+2	2	2
4	+1	1	0
5	+3	0	

Figure 66 Summary of the phases of each splice donor and acceptor site in Rheb-RA.

We reviewed the phases of splice donor and acceptor sites in Figure 60. Recall that to maintain the open reading frame (ORF) across adjacent CDS's, the phases of the donor and acceptor sites of adjacent CDS's must be compatible with each other (i.e., the sum of the donor and acceptor phases of adjacent CDS's must either be 0 or 3.

Looking at the splice sites between CDS-1 and CDS-2, we see the splice donor phase of CDS-1 is one and the splice acceptor phase of CDS-2 is two. Thus, the sum of the donor and acceptor phases for Intron-1 is three (Figure 67).

CDS	Frame	Splice Acceptor Phase	Splice Donor Phase	Sum of Splice Donor and Splice Acceptor Phase	Does the sum of the donor and acceptor equal 0 or 3?
1	+3		1	1 + 2 = 3	✓
2	+1	2	1	1 + 2 = 3	✓
3	+2	2	2	2 + 1 = 3	✓
4	+1	1	0	0 + 0 = 0	✓
5	+3	0			

Figure 67 The sum of the adjacent splice sites is either zero or three; thus, the phases of our donor and acceptor sites of adjacent CDS's are compatible with each other.

Part 6.4: Use spliced RNA-Seq reads to verify coordinates for Intron-1

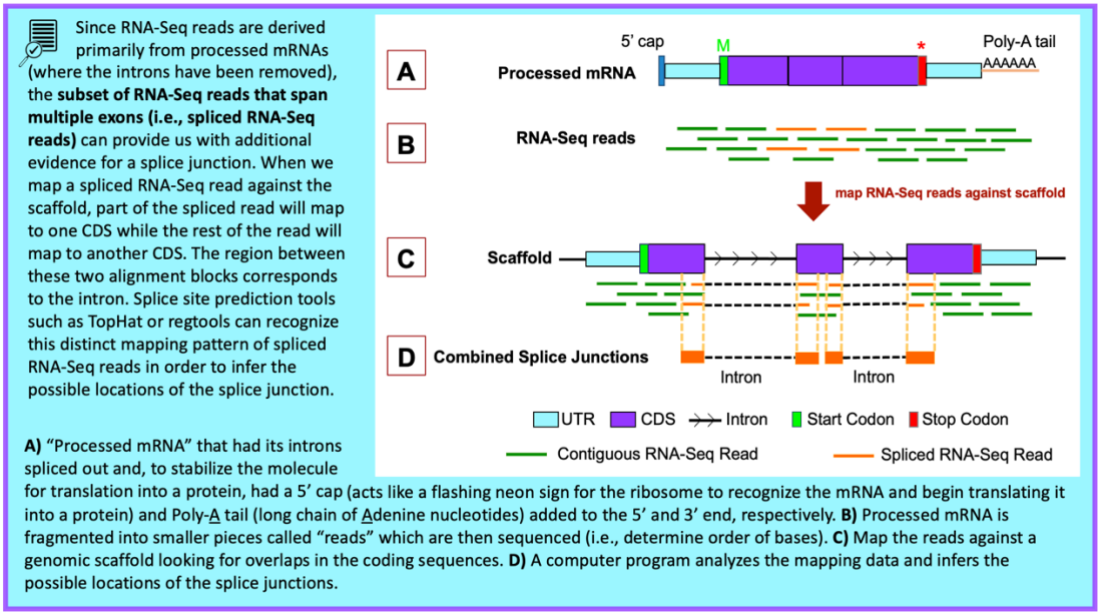


Figure 68 Review of splice junction predictions in Understanding Eukaryotic Genes Module 4; see RNA-Seq: Basics, Applications, and Protocol for a basic overview of RNA-Seq

1. Under the "RNA Seq Tracks," change the "Combined Splice Junctions" track to "pack."
    - Note: The "Combined Splice Junctions" track shows **splice junctions** extracted from spliced RNA-Seq reads that have been aligned to the genome.
2. Click on the "refresh" button.
3. To examine the region surrounding Intron-1 (i.e., intron between CDS-1 and CDS-2), enter "**NC_052530:19,150,858-19,150,984**" into the "search terms" text box.
    - Note: We found these coordinates in Part 6.3. Since our analysis in Part 6.3 suggested that CDS-1 ends at 19,150,857, Intron-1 should begin at 19,150,858; furthermore, we found that CDS-2 begins at 19,150,985 thus Intron-1 should end at 19,150,984.
4. Click on the "go" button then zoom out 3x to examine the 381 bp surrounding this position (Figure 69).

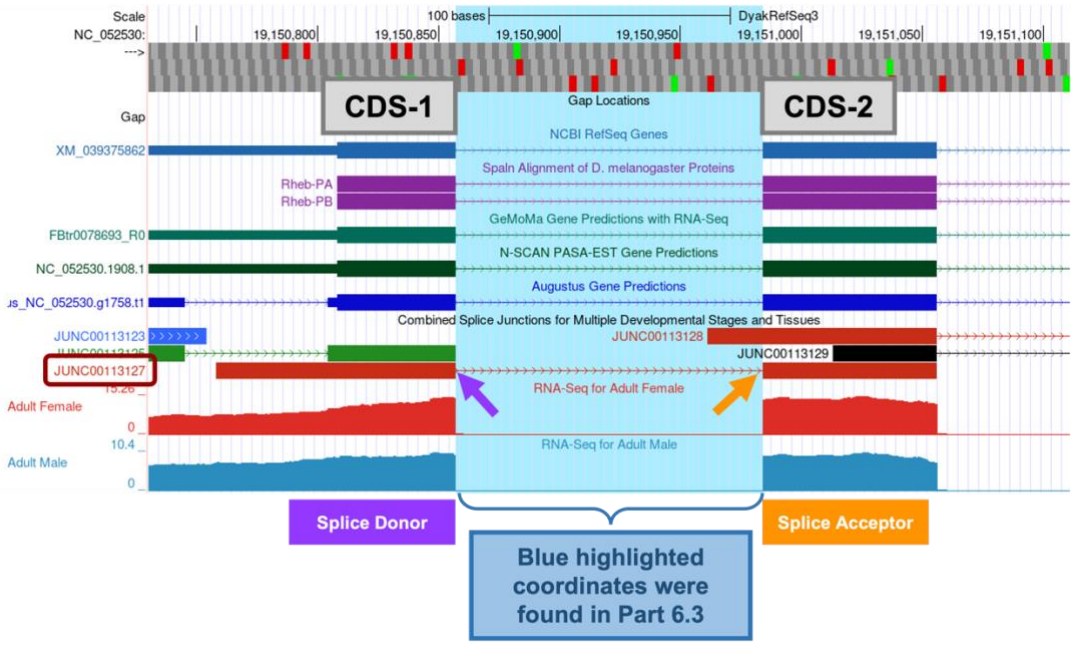


Figure 69 The splice junction prediction JUNC00113127 (red rectangle) connects CDS-1 with CDS-2. The predicted splice donor and acceptor sites are indicated by the purple and orange arrows, respectively.

There is only <u>one</u> splice junction predicted in this region.

5. To examine the splice donor site predicted by the splice junction JUNC00113127, zoom into the region surrounding the beginning of the intron predicted by this junction (Figure 69, purple arrow).
6. To examine the splice acceptor site predicted by the splice junction JUNC00113127, zoom into the region surrounding the end of the intron predicted by this junction (Figure 69, orange arrow).

The splice junction between the phase 1 donor site of CDS-1 and the phase 2 acceptor site of CDS-2 is supported by the NCBI RefSeq Genes and Spaln alignment of *D. melanogaster* proteins, gene predictors GeMoMa, N-SCAN, and Augustus, and the RNA-Seq data.

7. We can gather additional evidence to support this splice junction by clicking on "JUNC00113127" (Figure 69, red rectangle) and then examining the "Score" field (Figure 70, top).

The score tells us how many spliced RNA-Seq reads there are that support a predicted splice junction. Since JUNC00113127 has a score of 6257, that splice junction prediction is supported by 6,257 spliced RNA-Seq reads. Splice junction predictions are color-coded based on the number of spliced RNA-Seq reads that support the junction (i.e., their scores). Based on the color-coded table in the "Description" section, JUNC00113127 will be red in the Genome Browser image since greater than 1,000 spliced RNA-Seq reads support the feature (Figure 70, bottom).

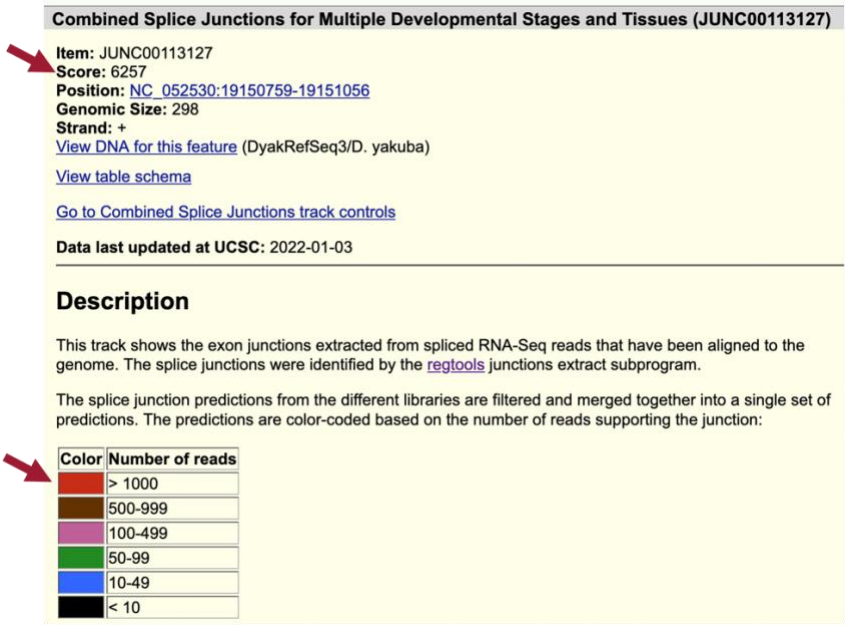


Figure 70 The splice junction JUNC00113127 has a score of 6257 indicating that it is supported by 6,257 spliced RNA-Seq reads; therefore, this feature will be red in the Genome Browser image.

Based on multiple lines of evidence, we can conclude the splice junction prediction JUNC00113127 is consistent with our splice donor site for CDS-1 at 19,150,858-19,150,859 and our splice acceptor site for CDS-2 at 19,150,983-19,150,984 that we annotated in Part 6.3.

Part 6.5: Use splice junction predictions to verify coordinates for second intron

The same annotation strategy can be used to determine the coordinates for Intron-2 between CDS-2 and CDS-3.

1. To examine the region surrounding Intron-2, enter "**NC_052530:19,151,055-19,151,156**" into the "search terms" text box.
    - Note: These coordinates can be found in the table in Figure 55.
2. Click on the "go" button.
3. Zoom out 3x to examine the 306 bp surrounding this position (Figure 71).

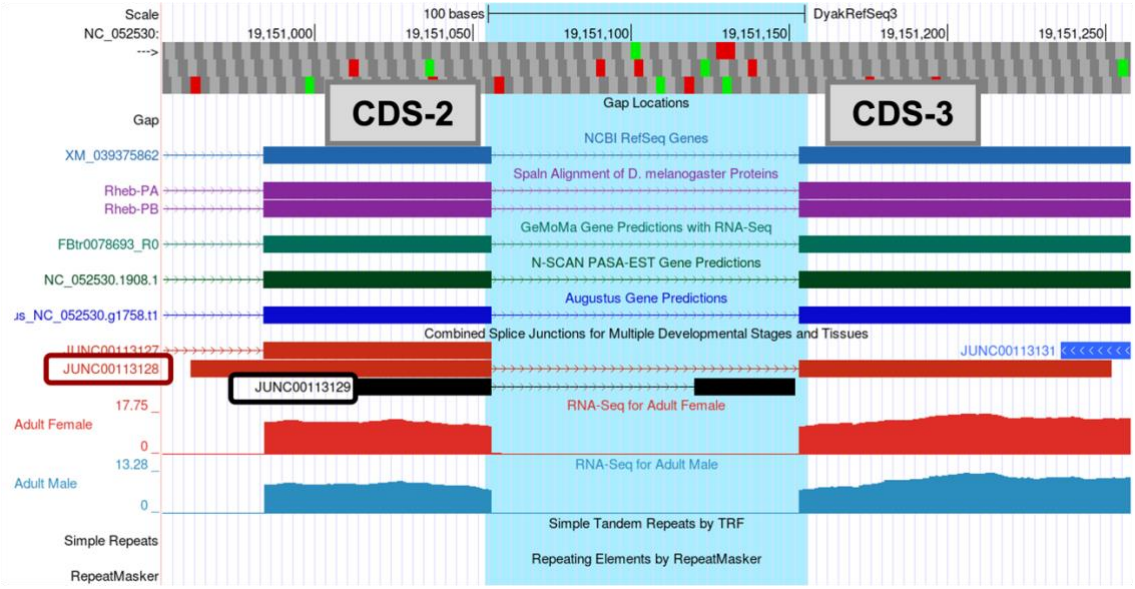


Figure 71 Predicted splice junctions JUNC00113128 (red rectangle) and JUNC00113129 (black rectangle) connect CDS-2 with CDS-3.

There are two splice junctions predicted in this region, JUNC00113128 and JUNC00113129.

The *tblastn* alignment for CDS-2 ends at 19,151,055 (Figure 55). The potential splice donor site at 19,151,057-19,151,058 for CDS-2 is supported by the splice junction predictions JUNC00113128 and JUNC00113129, the NCBI RefSeq Genes and Spaln alignment of *D. melanogaster* proteins, gene predictors GeMoMa, N-SCAN, and Augustus, and the RNA-Seq data. There is one nucleotide (A at 19,151,056) between the last complete codon (AAC) and the potential splice donor site. Hence, this splice donor site is in phase 1 relative to Frame +1.

The *tblastn* alignment for CDS-3 spans from 19,151,156-19,151,359 in Frame +2 (Figure 55). The potential splice acceptor site at 19,151,152-19,151,353 for CDS-3 is supported by the splice junction prediction JUNC00113128, the NCBI RefSeq Genes and Spaln alignment of *D. melanogaster* proteins, gene predictors GeMoMa, N-SCAN, and Augustus, and the RNA-Seq data. There are two nucleotides (CC at 19,151,154-19,151,355) between the first complete codon (TTC) and the potential splice acceptor site. Hence, this splice acceptor site is in phase 2 relative to Frame +2. This phase 2 splice acceptor site is compatible with the phase 1 splice donor site for CDS-2.

4. Click on the "JUNC00113128" feature to determine the number of spliced RNA-Seq reads that support this splice junction prediction (Figure 72).
5. Click on the back button of the web browser to return to the Genome Browser image.

Figure 72 The score for JUNC00113128 shows that this junction is supported by 5,875 spliced RNA-Seq reads.

In addition to the splice junction JUNC00113128, which supports the proposed splice acceptor site for CDS-3 at 19,151,152-19,151,353, there is another splice junction which suggests a different splice acceptor site (JUNC00113129) (Figure 71).

Thus, we need to investigate whether predicted junction JUNC00113129 is supported by other lines of evidence. CDS-3 in *D. yakuba* includes two methionine in Frame +2 (at 19,151,255- 19,151,257 and 19,151,348-19,151,350). Hence, the splice junction JUNC00113129 could indicate the presence of a novel isoform of *Rheb* in *D. yakuba.* However, when we assess the number of spliced RNA-Seq reads that support the splice junction JUNC00113129, we find that this junction is weakly supported by only 8 spliced RNA-Seq reads; thus, there is little evidence to postulate a **novel isoform** of *Rheb* in *D. yakuba* based on this splice junction prediction.

Note: In addition to the scores, when analyzing multiple splice junction predictions for an intron, be sure to confirm the predictions are on the same strand as the gene you're annotating. For example, if a splice junction is predicted in the negative strand and the *Rheb* gene is on the positive strand relative to the *D. yakuba* NC_052530 scaffold, you could eliminate that prediction.

Taking into account the splice site phases we found in Part 6.3 (Figure 66) and using Parts 6.4 and 6.5 as a guide, you will be able to find the start and end coordinates of the remaining CDSs for the gene model of Rheb-PA in *D. yakuba* (Figure 73).

Gene Model for Rheb-PA in *D. yakuba*						
CDS	FlyBase ID	Frame	Splice Acceptor Phase	Coordinates		Splice Donor Phase
				Start	End	
1	1_9829_0	+3		19,150,809	19,150,857	1
2	2_9829_2	+1	2	19,150,985	19,151,056	1
3	3_9829_2	+2	2	19,151,154	19,151,361	2
4	4_9829_1	+1	1	19,151,421	19,151,550	0
5	5_9829_0	+3	0	19,151,613	19,151,699	

Figure 73 Summary of the five CDS's in Rheb-PA. Stop codon is located at NC_052530:19,151,700-19,151,702.

Part 7: Verify and submit gene model(s)

Now that we've completed the annotation of Rheb-PA in *D. yakuba*, we need to verify our proposed gene model and prepare the corresponding sequence files for submission.

Part 7.1: Verify gene model of protein

Our analysis of the CDS-by-CDS *tblastn* alignments and the evidence tracks on the Genome Browser allowed us to precisely define the start and end positions of each of the five coding exons (CDS's) of

Rheb-PA. To verify that our proposed gene model satisfies the basic biological constraints (e.g., begins with a start codon, has compatible splice sites, and ends with a stop codon), we will check our gene model coordinates using the **Gene Model Checker**.

1. Open a new web browser tab and navigate to the Gene Model Checker (Figure 74).
2. In the "Project Details" section:
   - Species Name: select "*D. yakuba*"
   - Genome Assembly: select "Aug. 2021 (Princeton Prin_Dyak_Tai18E2_2.1/DyakRefSeq3)"
   - Scaffold Name: enter "**NC_052530**"
3. In the "Ortholog Details" section:
   - Ortholog in D. melanogaster: enter "**Rheb-PA**"
4. In the "Model Details" section:
   - Errors in **Consensus Sequence**? select "No"
   - Coding Exon Coordinates: enter a comma-delimited list of coordinates for the five CDS's as shown below **(commas separate adjacent CDS's; NO commas are included within each set of coordinates)**:
     19150809-19150857, 19150985-19151056, 19151154-19151361, 19151421-19151550, 19151613-19151699
   - Annotated Untranslated Regions? select "No"
   - Orientation of Gene Relative to Query Sequence: select "Plus" since Rheb-PA is on the positive strand relative to the scaffold
   - Completeness of Gene Model Translation: select "Complete"
   - Stop Codon Coordinates: click within the textbox and the coordinates will automatically populate
5. Click on the "Verify Gene Model" button to run the Gene Model Checker.

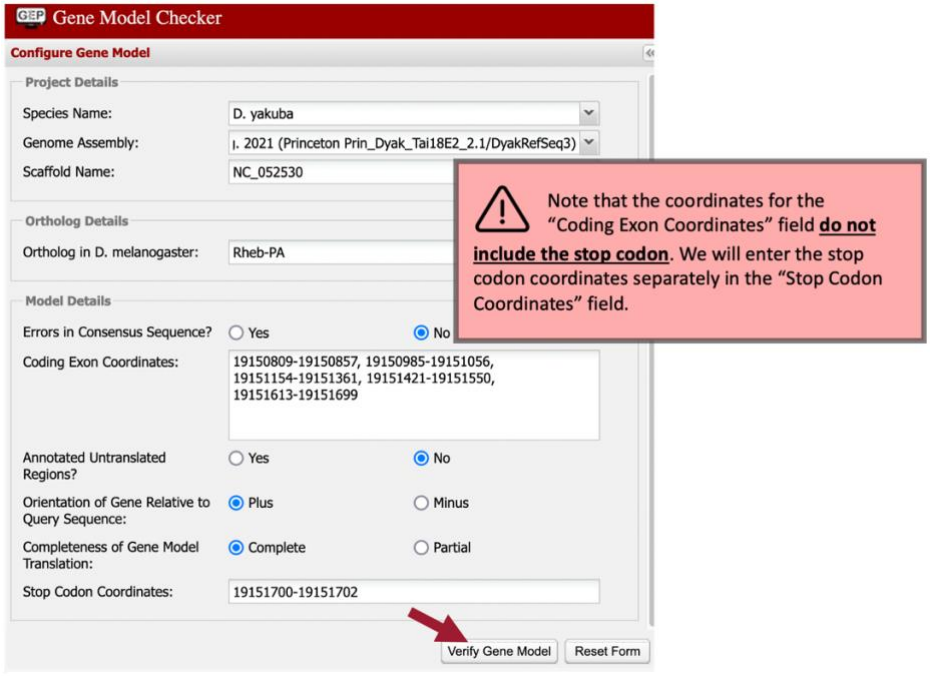


Figure 74 Verify the D. *yakuba* gene model for Rheb-PA using the Gene Model Checker.

Once the analysis is complete, the right panel of the Gene Model Checker contains the results. The "Checklist" tab enumerates the list of criteria that have been checked by the Gene Model Checker (Figure 75). For example, the Gene Model Checker verifies that our proposed gene model begins with a start codon and ends with a stop codon. It also verifies that the splice junctions contain the canonical ("standard") splice donor (GT) and acceptor (AG) sites. Some of the items on the checklist have been skipped because they do not apply to a complete gene (e.g., CDS-1 doesn't have a splice acceptor site and CDS-5 doesn't have a splice donor site).

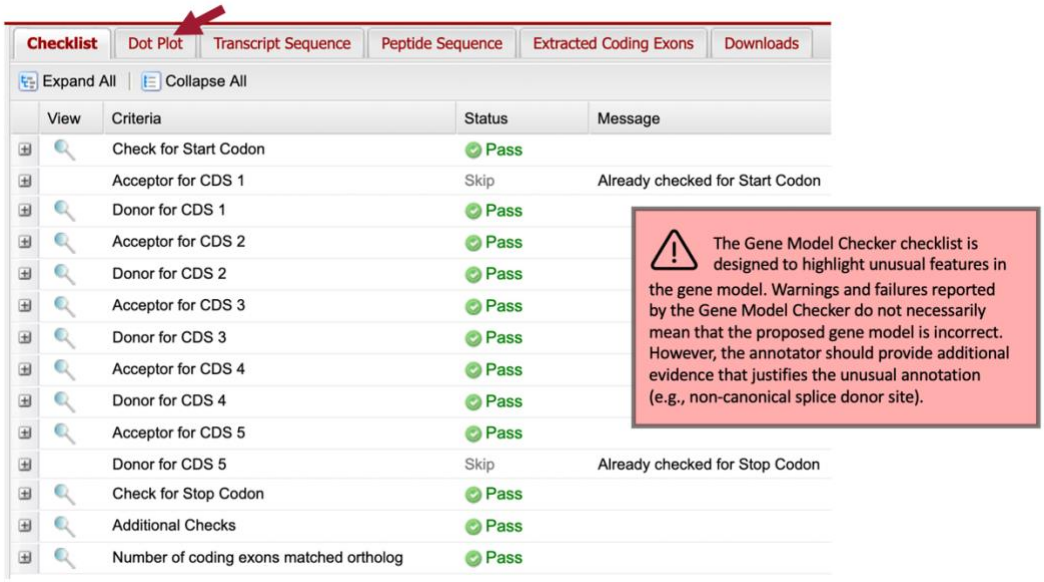


Figure 75 The Gene Model Checker checklist shows that the proposed gene model for Rheb-PA satisfies the biological constraints of most protein-coding genes (e.g., canonical start codon, stop codon, splice sites).

In addition to verifying the basic gene structure, the Gene Model Checker also compares the proposed gene model against the putative *D. melanogaster* ortholog using a protein alignment and a **dot plot** (Figure 77).

6. Click on the "**Dot Plot**" tab (Figure 75, arrow) to examine the dot plot between the *D. melanogaster* protein (x-axis) and the protein sequence for the submitted model in *D. yakuba* (y-axis) (Figure 76).

The alternating color boxes in the dot plot correspond to the different CDS's in the two sequences. Dots in the dot plot correspond to regions of similarity between the *D. melanogaster* protein and the submitted *D. yakuba* gene model. If the submitted sequence is identical to the *D. melanogaster* ortholog, then the dot plot will show a straight diagonal line with a slope of 1. Changes in the size of the submitted model compared to the *D. melanogaster* ortholog will alter the slope of this line.
In this case, the dot plot shows that the five CDS's of Rheb-PA in *D. melanogaster* and *D. yakuba* have similar lengths (compare the length shown on the x-axis to the length shown on the y-axis for each CDS). However, within a small region of CDS-4, the dot plot did not detect sequence similarity between the submitted model for *D. yakuba* and the *D. melanogaster* ortholog (Figure 76).

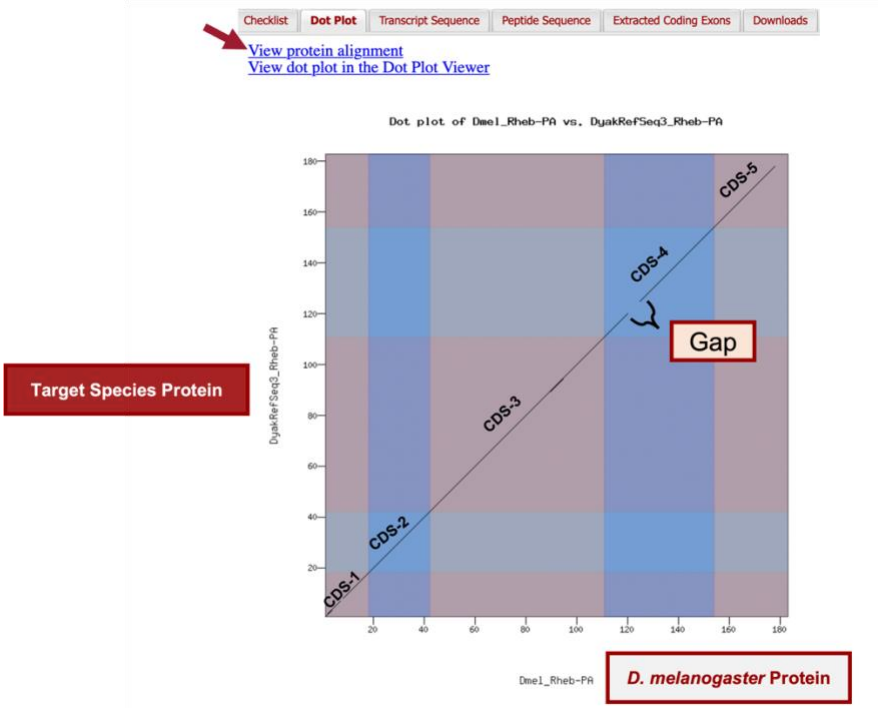


Figure 76 The dot plot comparing the *D. melanogaster* Rheb-PA (x-axis) with the submitted gene model in *D. yakuba* (y-axis) shows that the main differences between the two protein sequences are in CDS-4 and CDS-5.

To further investigate the dot plot, we will examine the protein alignment between the two sequences.

7. Click on the "View protein alignment" link above the dot plot (Figure 76, arrow).

FYI

The dot plot is a visual/graphical representation of the protein alignment showing the position of the amino acids for the *D. melanogaster* protein on the x-axis and the position of the amino acids for the target species protein on the y-axis; therefore, large gaps, regions with no sequence similarity, and any other anomalies seen in the dot plot can be located within the protein alignment.

Figure 77 For Your Information — Dot Plot

The protein alignment shows the comparison of the *D. melanogaster* protein against the conceptual translation for the submitted *D. yakuba* gene model. Like the dot plot, alternating colors correspond to the different CDS's (Figure 78).

The protein alignment between the *D. melanogaster* ortholog and the *D. yakuba* gene model shows that the five CDS's are 97.3% identical. The symbols in the match line denote the level of similarity ("*****" indicates conserved amino acids, "**:**" denotes amino acids with highly similar chemical properties). Hence, the *tiny* **gap** in the dot plot within CDS-1 can be attributed to similar, but not identical, amino acids near its center.

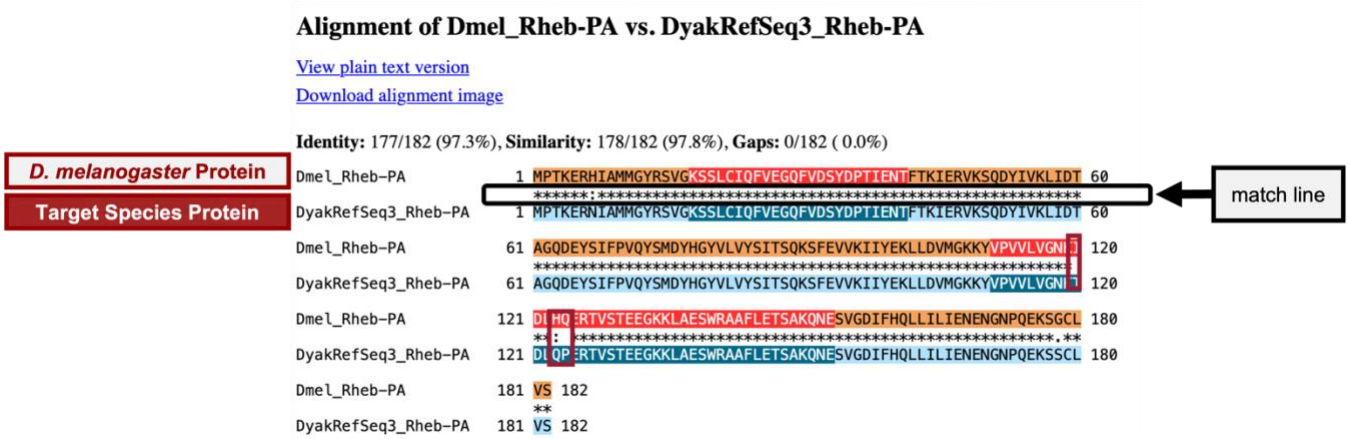


Figure 78 The protein alignment between *D. melanogaster* Rheb-PA versus the submitted gene model in *D. yakuba* shows that the tiny gap within CDS-4 in the dot plot can be attributed to three amino acid residues that differ between these two species (red boxes).

The protein alignment between the *D. melanogaster* Rheb-PA and the submitted gene model in *D. yakuba* shows that that the gap within CDS-4 in the dot plot can be attributed to differences in three amino acid residues.

We can view the submitted gene model within the context of the other evidence tracks in the Genome Browser.

8. Click on the “Checklist” tab (Figure 79, red arrow).
9. Click on the magnifying glass icon next to “Number of coding exons matched ortholog” (Figure 79, black arrow).
    - Note: A new window containing the Genome Browser will appear with our submitted gene model shown in the red “Custom Gene Model” track.

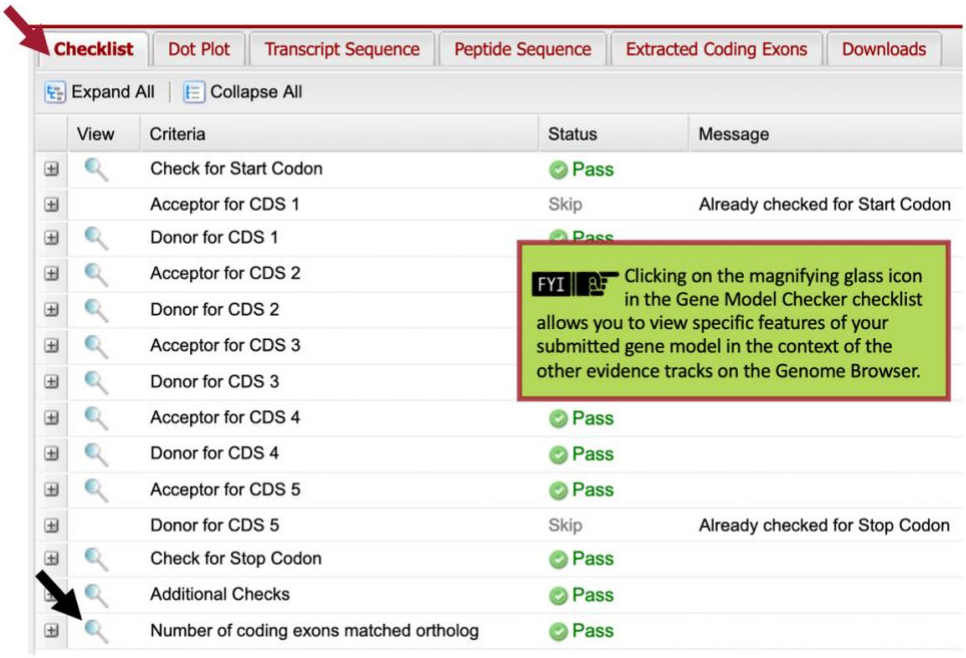


Figure 79 To view our gene model within the Genome Browser, click on the magnifying glass icon (black arrow).

Now we see our entire submitted gene model for Rheb-PA in *D. yakuba* (Figure 80).

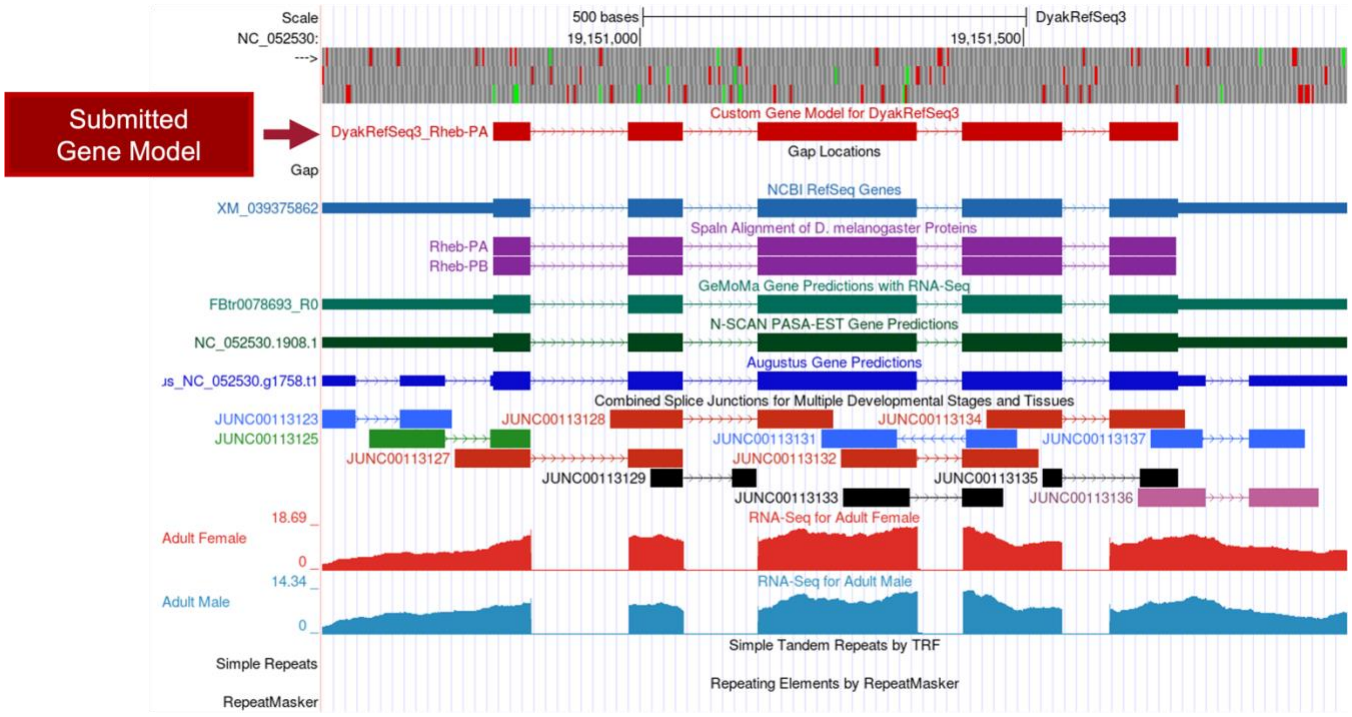


Figure 80 Our submitted gene model for Rheb-PA in *D. yakuba* is shown under the track title "Custom Gene Model for DyakRefSeq3."

Part 7.2: Download files required for project submission

In addition to the Pathways Project: Annotation Form, you must prepare three additional data files to submit a project to the GEP – a General Feature Format File (**GFF**), a Transcript Sequence File (**fasta**), and a Peptide Sequence File (**pep**). The Gene Model Checker automatically creates these three files for a specific isoform (e.g., Rheb-PA) when you verify a gene model.

1. You can download these files by clicking on the "Downloads" tab and then clicking on each of the links to save the files to your computer (Figure 81).

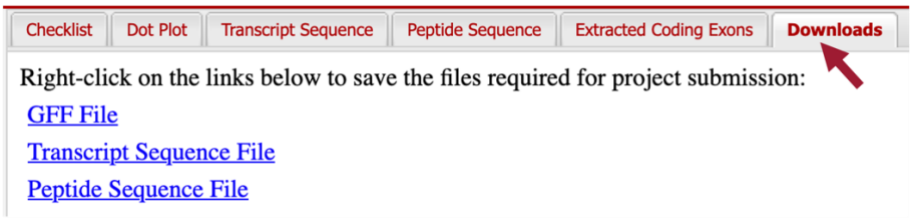


Figure 81 In preparation for project submission, click on the "Downloads" tab and save the GFF, transcript sequence, and peptide sequence files for the gene model to your computer.

Recall that *Rheb* in *D. yakuba* has two isoforms; the files we just downloaded were for the Rheb-PA isoform. Since the coding exons (CDS's) for both of our isoforms are identical, we don't need to verify any additional models. However, **if your project has multiple unique isoforms (i.e., don't all have identical coding regions) then you should repeat Parts 7.1 and 7.2 for each unique isoform.**

Part 7.3: Merge project files

Now we need to combine the GFF, transcript sequence, and peptide sequence files for all **unique** isoforms into a single file prior to project submission (we'll submit one merged file for each file type). We do NOT need to do this for *Rheb* in *D. yakuba*; however, we are including the steps for merging two separate files, so you'll know how to do so. Again, **if your project had only one unique isoform, you'd be done at this point. The following is merely to show you how to merge your files IF your project had multiple unique isoforms.**

1. Open a new web browser tab and navigate to the Annotation Files Merger.

Let's merge our two isoform GFF files first.

2. Change the "File Type:" to "GFF Files (.gff)."
3. Drag the two GFF files we downloaded for Rheb-PA and Rheb-PB to the "Drag and drop the files you want to merge here" section.
4. Click on the "Merge Files" button (Figure 82).

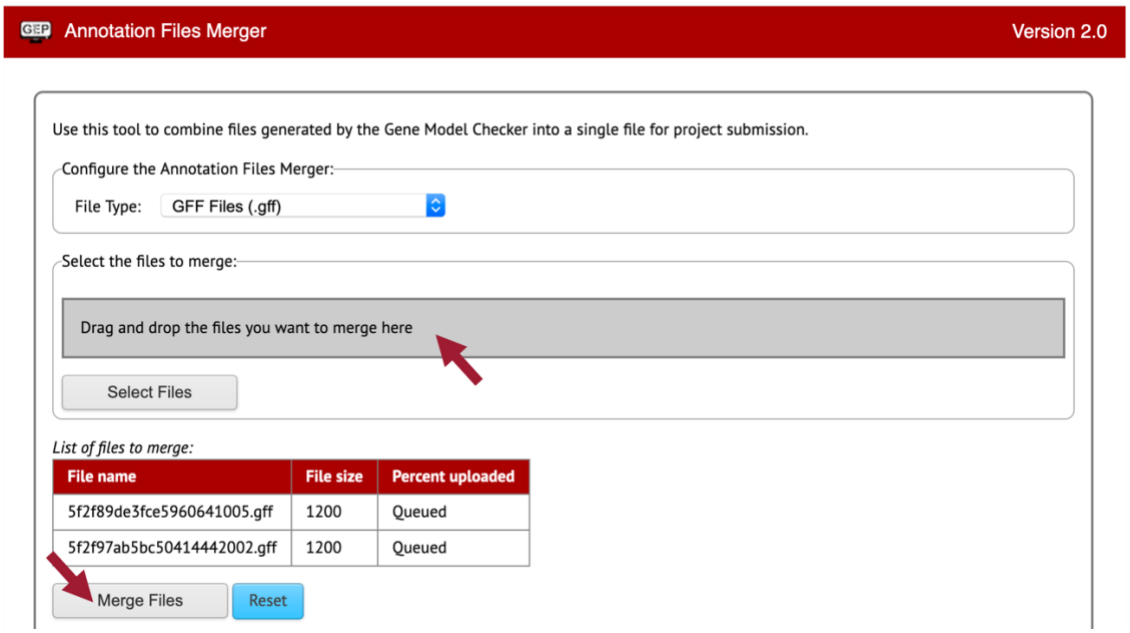


Figure 82 Merge the GFF files for Rheb-PA and Rheb-PB.

5. Download the merged GFF file by right-clicking (control click on macOS) on the "Merged File Link" (Figure 83).
6. Click on "Save Link As…"
7. Enter "**dyak_Rheb.gff**" as the file name. (If you are working on a different gene, use your assigned species ("d" followed by the first three letters of your species) + "_gene.filetype" as the format.)
    - For example, if you're annotating *Ilp8* in *D. grimshawi*, the merged GFF would be titled "dgrim_Ilp8.gff"

8. Once you click on the "Save" button, the merged GFF file should download onto your computer.

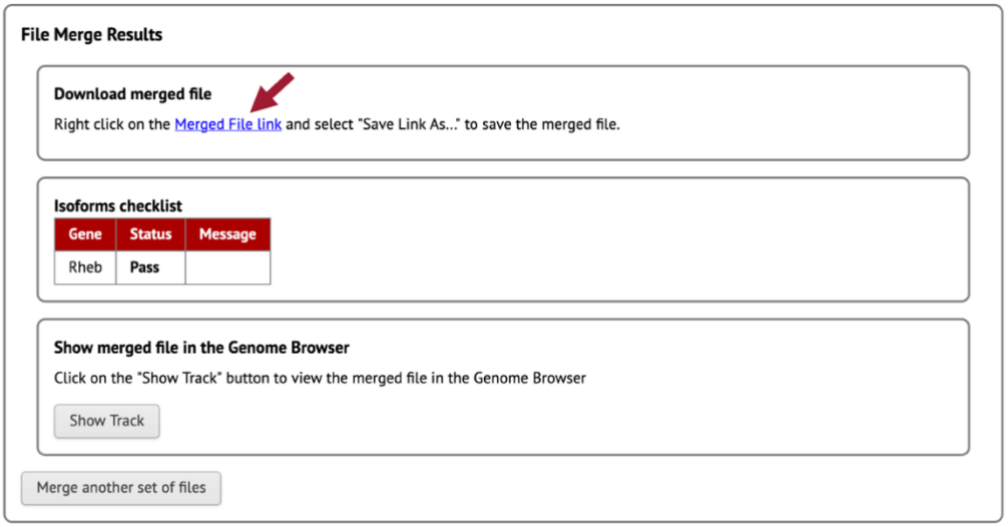


Figure 83 Download the merged GFF files for Rheb-PA and Rheb-PB.

Now we need to merge our two isoform Transcript Sequence Files (.fasta).

9. Click on the “Merge another set of files” button.
10. Change the “File Type:” to “Transcript Sequence Files (.fasta).”
11. Drag the two fasta files we downloaded for Rheb-PA and Rheb-PB to the “Drag and drop the files you want to merge here” section.
12. Repeat steps 4 – 6.
13. Enter “**dyak_Rheb.fasta**” as the file name.
14. Once you click on the “Save” button, the merged fasta file should download onto your computer.

Lastly, we need to merge our two isoform Peptide Sequence Files (.pep).

15. Click on the “Merge another set of files” button.
16. Change the “File Type:” to “Peptide Sequence Files (.pep).”
17. Drag the two pep files we downloaded for Rheb-PA and Rheb-PB to the “Drag and drop the files you want to merge here” section.
18. Repeat steps 4 – 6.
19. Enter “**dyak_Rheb.pep**” as the file name.
20. Once you click on the “Save” button, the merged pep file should download onto your computer.

Appendix A. Combining (or Batching) BLAST Searches

In Part 3.2, instead of running four individual *blastp* searches of the protein IDs of the nearest two neighbors upstream and downstream of the target gene, we could have combined all these searches (i.e., batched them) into a single search. Please note that this time saving method may be confusing for novice annotators.

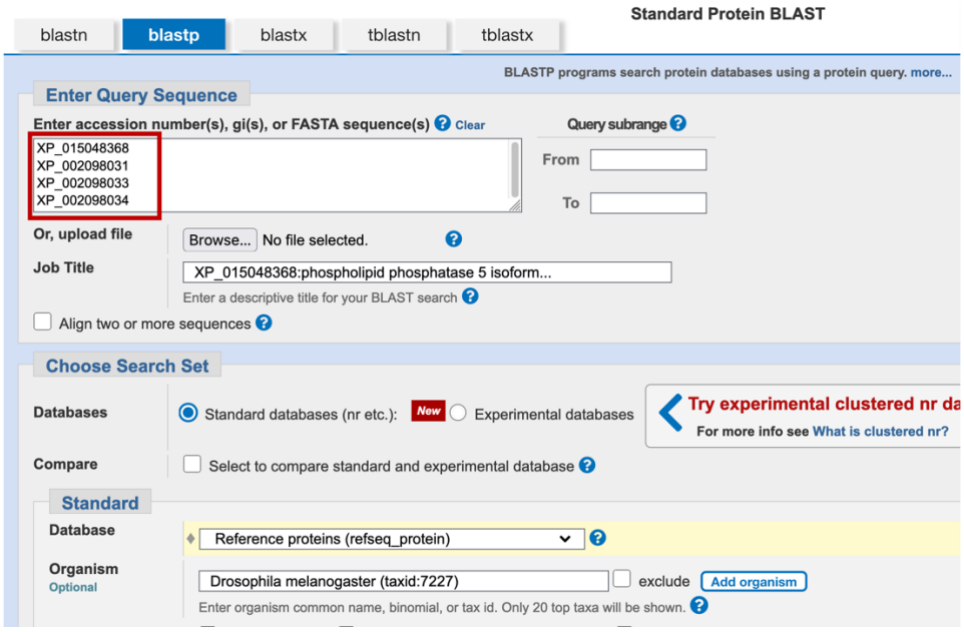


Figure 84 Enter Accession Numbers (one per line) in the "Enter Query Sequence" text box to run multiple query sequence searches at once.

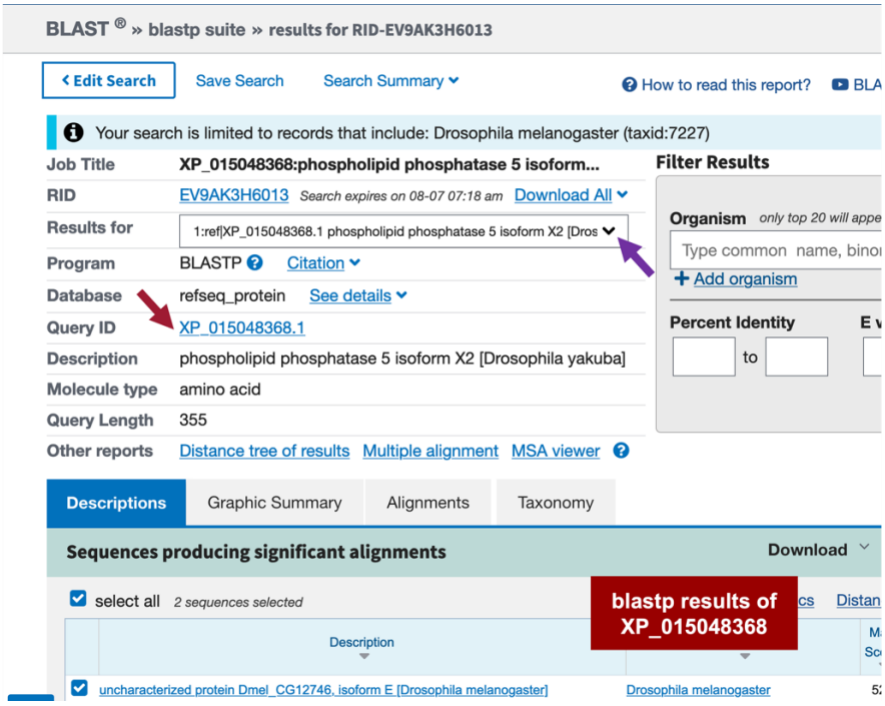


Figure 85 View of the *blastp* results for XP_015048368 (see the "Query ID" (red arrow) to determine which alignment you are viewing). Click on the "Results for" box (purple arrow) to see a drop-down menu that will allow you to view the other search results.

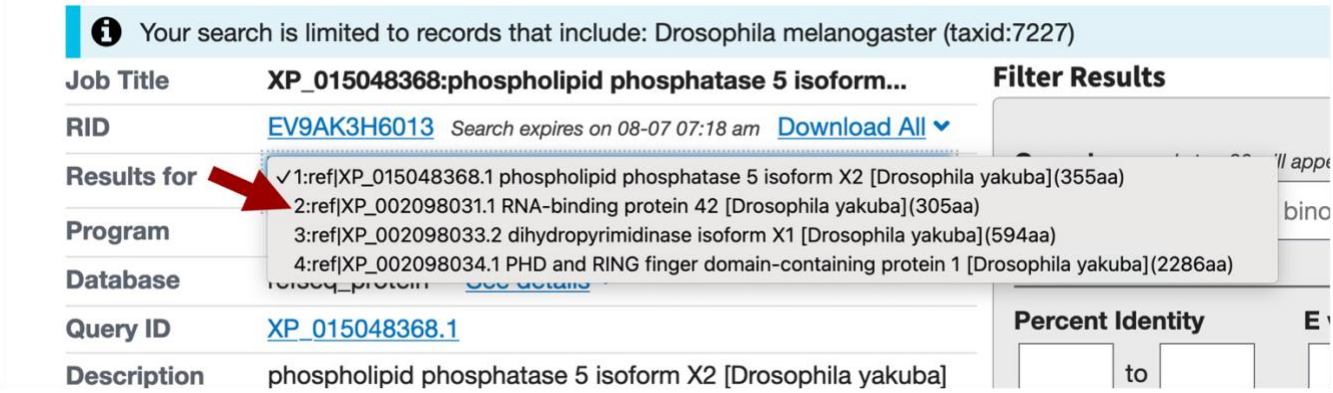


Figure 86 Click on the results for "XP_002098031" (arrow) to view the results of the closest upstream neighbor to our target gene.

In Part 5, instead of running five individual *tblastn* searches (one for each CDS), we could have entered all five CDS sequences into the "Enter Query Sequence" text box and performed all five searches at once.

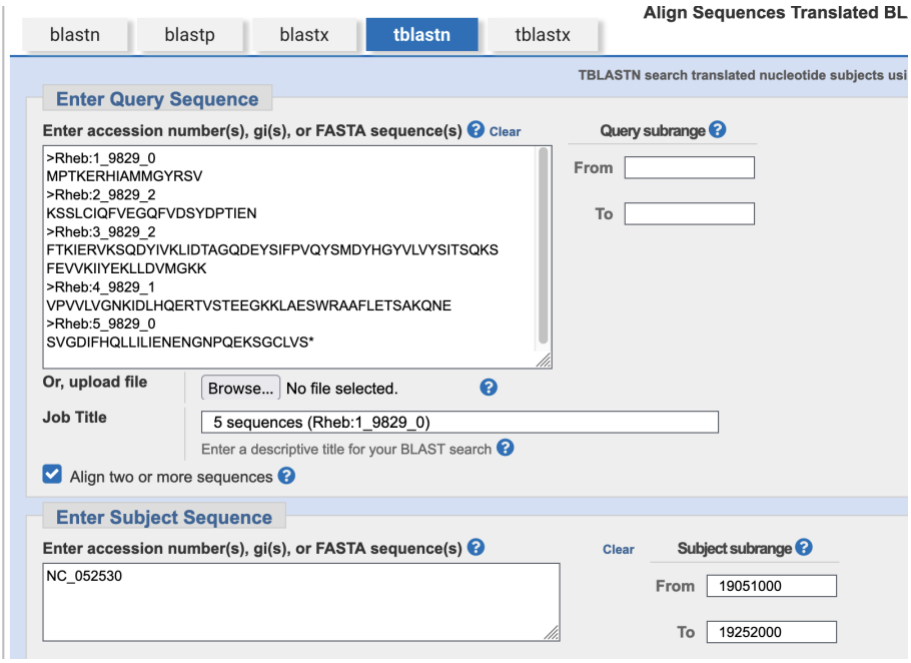


Figure 87 Copy and paste the header and sequence for each CDS into the "Enter Query Sequence" text box.