Genomics Education Partnership
thegep.org

P A T H W A Y S

Pathways Project Annotation Primer

Adapted from Wilson Leung
Katie M. Sandlin
Last Update: 08/20/2024

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Outline

- Overview of comparative genome annotation
- Pathways Project annotation strategy
  - Types of evidence
  - Analysis tools
  - Web databases
- Annotation of a single isoform of *Rheb* in *D. yakuba*

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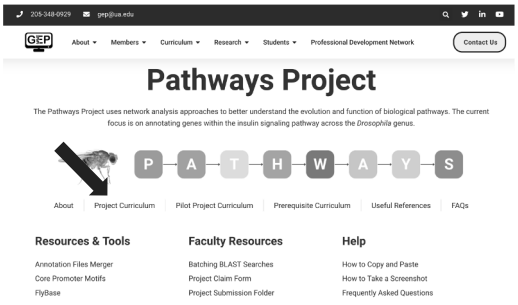
Pathways Project: Annotation Walkthrough



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Pathways Project: Annotation Walkthrough

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Pathways Project: Annotation Walkthrough

- https://thegep.org/lessons/ksandlin-walkthrough-drosophila_pathways/

Pathways Project: Annotation Walkthrough
Katie Sandlin, Wilson Leung, & Laura Reed

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

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
- Reference Glossary

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Annotation: Adding **labels** to a sequence

- **Genes**: Novel or known genes, pseudogenes
- **Regulatory Elements**: Promoters, enhancers, silencers
- **Non-coding RNA**: tRNAs, miRNAs, siRNAs, snoRNAs
- **Repeats**: Transposable elements, simple repeats
- **Structural**: Origins of replication
- **Experimental Results**:
  - DNase I Hypersensitive sites
  - ChIP-chip and ChIP-Seq datasets (e.g., modENCODE)

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```
AAACAACAATCATAAATAGAGGAAGTTTTCGGAATATACGATAAGTGAAATATCGTTCT
TAAAAAAGAGCAAGAACAGTTTAACCATTGAAAACAAGATTATTCCAATAGCCGTAAGA
GTTCATTTAATGACAATGACGATGGCGGCAAAGTCGATGAAGGACTAGTCGGAACTGGA
AATAGGAATGCGCCAAAAGCTAGTGCAGCTAAACATCAATTGAAACAAGTTTGTACATC
GATGCGCGGAGGCGCTTTTCTCTCACGATGGCTGGGGATGCCAGCACGTTAATCAGGAT
ACCAATTGAGGAGGTGCCCCAGCTCACCTAGAGCCGGCCAATAAGGACCCATCGGGGGG
GCCGCTTATGTGGAAGCCAAACATTAAACCATAGGCAACCGATTTGTGGGAATCGAATT
TAAGAAACGGCGGTCAGCCACCCGCTCAACAAGTGCCAAAGCCATCTTGGGGGCATACG
CCTTCATCAAATTTGGGCGGAACTTGGGGCGAGGACGATGATGGCGCCGATAGCACCAG
CGTTTGGACGGGTCAGTCATTCCACATATGCACAACGTCTGGTGTTGCAGTCGGTGCCA
TAGCGCCTGGCCGTTGGCGCCGCTGCTGGTCCCTAATGGGGACAGGCTGTTGCTGTTGG
TGTTGGAGTCGGAGTTGCCTTAAACTCGACTGGAAATAACAATGCGCCGGCAACAGGAG
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ATCGGCCGGAATGNTAANGAATTAATCAAATTTTGGCGGACATAATGNGCAGATTCAGA
ACGTATTAACAAAATGGTCGGCCCCGTTGTTAGTGCAACAGGGTCAAATATCGCAAGCT
CAAATATTGGCCCAAGCGGTGTTGGTTCCGTATCCGGTAATGTCGGGGCACAATGGGGA
GCCACACAGGCCGCGTTGGGGCCCCAAGGTATTTCCAAGCAAATCACTGGATGGGAGGA
ACCACAATCAGATTCAGAATATTAACAAAATGGTCGGCCCCGTTGTTATGGATAAAAAA
TTTGTGTCTTCGTACGGAGATTATGTTGTTAATCAATTTTATTAAGATATTTAAATAAA
TATGTGTACCTTTCACGAGAAATTTGCTTACCTTTTCGACACACACACTTATACAGACA
GGTAATAATTACCTTTTGAGCAATTCGATTTTCATAAAATATACCTAAATCGCATCGTC
```

Start codon	Coding region	Stop codon
Splice donor	Splice acceptor	UTR

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Evidence-based annotation

- Human-curated analysis
  - Higher accuracy than standard *ab initio* and evidence-based gene finders
- **Goal**: **collect**, **analyze**, and **synthesize** all the available evidence to create the best-supported gene model
  - Example: 4591-4688, 5157-5490, 5747-6001

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Collect, analyze, and synthesize

- **Collect:**
  - GEP UCSC Genome Browser
  - Conservation (BLAST searches)
- **Analyze:**
  - Interpreting Genome Browser evidence tracks
  - Interpreting BLAST results
- **Synthesize:**
  - Construct the best-supported gene model based on **potentially contradictory** evidence

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Evidence for gene models

(in general order of importance)

1. Conservation
   - Sequence similarity to genes in *D. melanogaster*
   - Sequence similarity to other *Drosophila* species (Multiz)
2. Expression data
   - RNA-Seq, EST, cDNA
3. Computational predictions
   - Open reading frames; gene and splice site predictions
4. Tie-breakers of last resort
   - See the "Annotation Instruction Sheet"

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GEP Annotation Strategy

- Use *D. melanogaster* as reference
  - *D. melanogaster* is very well annotated
  - Use sequence similarity to infer homology
- Minimize changes compared to the *D. melanogaster* gene model (**parsimony**)
  - Coding sequences evolve slowly
  - Exon structure changes **very** slowly

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Annotation Goals

- Identify the ortholog of a gene of interest from an **informant** genome (*D. melanogaster*) in the **target** genome (e.g., *D. yakuba*)
- For **ALL** unique isoforms, identify and map the locations of all coding exons (**CDS**) in the target genome

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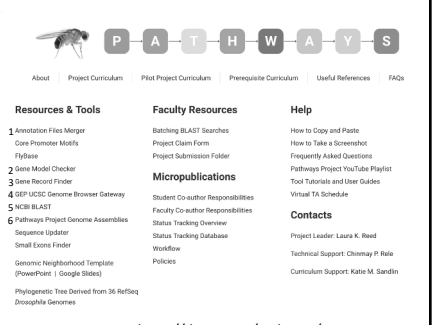
Resources

1. Merge files before submission
2. Check that proposed gene model meets basic biological constraints (e.g., has start and stop codon)
3. Look up information on *D. melanogaster*
4. Local mirror of UCSC Genome Browser
5. *blastn* and *tblastn**
   * Align two or more sequences
6. Whole genome BLAST



https://thegep.org/pathways/

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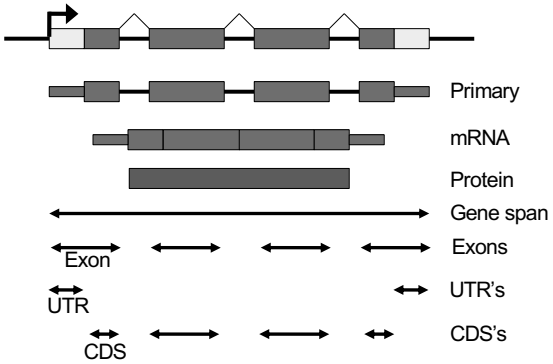
Gene Structure Terminology



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Nomenclature for *Drosophila* genes

- Every *D. melanogaster* gene has an annotation symbol
  - Begins with the prefix **CG** (**C**omputed **G**ene)
- Some genes have a different **gene symbol** (e.g., *Rheb*)
- Suffix after the gene symbol denotes different isoforms
  - mRNA = **-R**; protein = **-P**
  - Rheb-RA = Transcript for the A isoform of *Rheb*
  - Rheb-PA = Protein product for the A isoform of *Rheb*

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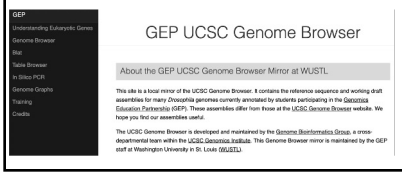
Two different versions of the UCSC Genome Browser

Official UCSC Version
https://genome.ucsc.edu/

GEP projects which use UCSC Assembly Hubs:
- Parasitoid Wasps
- Puerto Rican Parrot
- Detoxification Genes



GEP Version
https://gander.wustl.edu/

GEP projects which use the custom mirror of the UCSC Genome Browser:
- F Element
- Pathways

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UCSC Genome Browser

- Provide a graphical view of genomic regions
  - Sequence conservation
  - Gene and splice site predictions
  - RNA-Seq data and splice junction predictions
- BLAT – **B**LAST-**L**ike **A**lignment **T**ool
  - Map protein or nucleotide sequence against an assembly
  - Faster but less sensitive than BLAST
- Table Browser
  - Access raw data used to create the graphical browser

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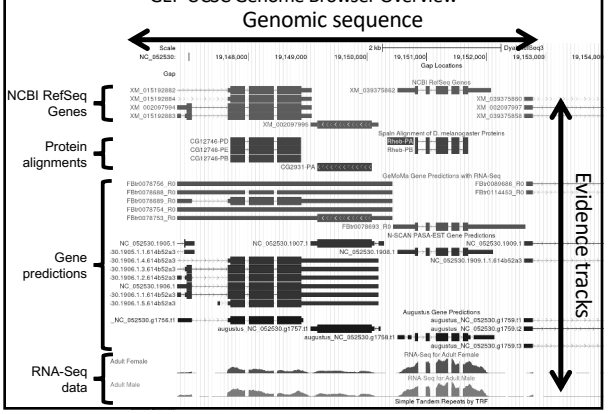
GEP UCSC Genome Browser Overview



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Control Display of Evidence Tracks

- Five different display modes:
  - Hide: track is **hidden**
  - Dense: all features appear on a **single line**
  - Squish: overlapping features appear on **separate lines**
    - Features are **half the height** compared to full mode
  - Pack: overlapping features appear on **separate lines**
    - Features are the **same height** as full mode
  - Full: each feature is displayed **on its own line**
    - Set **"Base Position" track to "Full"** to see the amino acid translations
- Some evidence tracks only have a subset of these display modes

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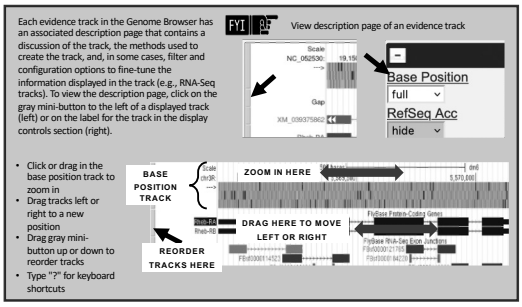
GEP UCSC Genome Browser



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Annotation Workflow

1. Examine genomic neighborhood surrounding target gene in *D. melanogaster*
2. Identify genomic location of ortholog in target species
3. Examine genomic neighborhood of putative ortholog in target species
4. Determine structure of target gene in *D. melanogaster*
5. Determine approximate location of coding exons in target species
6. Refine coordinates of coding exons
7. Verify and submit gene model

Repeat steps 5-7 for each unique isoform

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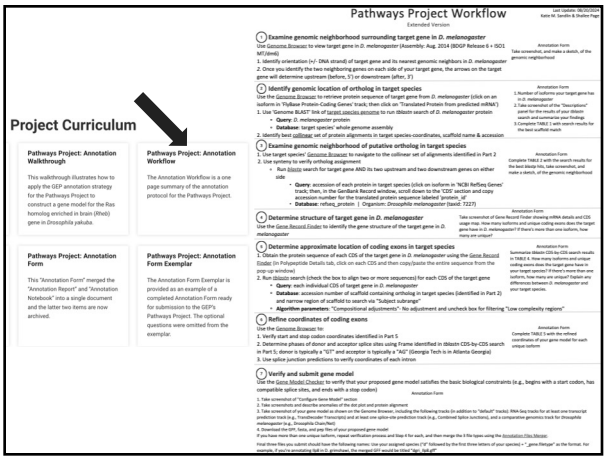

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Step 1

- Genomic neighborhood: A local region of the genome containing a small number of genes (5-15) or other features (e.g., repeats). The genomic neighborhood, as related to the Pathways Project, is the **region containing the target gene and its neighboring two closest upstream genes and two closest downstream genes**.
- Target gene: *Rheb*
- Targe species: *D. yakuba*

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Step 1: Examine genomic neighborhood surrounding target gene (*Rheb*) in *D. melanogaster*

- Use Genome Browser to view target gene in *D. melanogaster* (Assembly: Aug. 2014 (BDGP Release 6 + ISO1 MT/dm6)
  1. Identify orientation (+/- DNA strand) of target gene and its nearest genomic neighbors in *D. melanogaster*
  2. Once you identify the two neighboring genes on each side of your target gene, the arrows on the target gene will determine upstream (before, 5') or downstream (after, 3')

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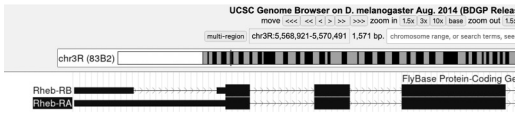
Determine the orientation of a feature in the Genome Browser

- Use the direction of the arrows within introns



- If the viewing region for the feature does not contain introns, use the direction of the arrows within exons

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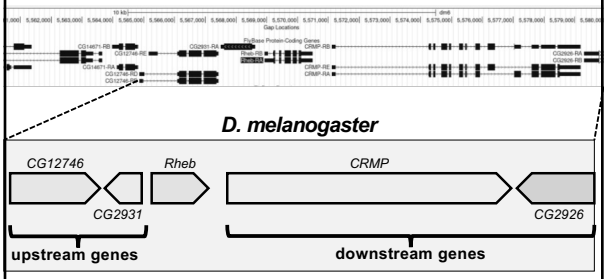
Region surrounding the *D. melanogaster Rheb* gene



Once you identify the two neighboring genes on each side of your target gene, the arrows on the target gene will determine upstream (before, 5') or downstream (after, 3')

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Annotation Workflow

1. Examine genomic neighborhood surrounding target gene in *D. melanogaster*
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Repeat steps 5-7 for each unique isoform

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Step 2: Identify genomic location of ortholog in target species (*D. yakuba)*

Use the Genome Browser to **retrieve protein sequence of target gene from *D. melanogaster*** (click on an isoform in 'FlyBase Protein-Coding Genes' track; then click on 'Translated Protein from predicted mRNA')

1. Use 'Genome BLAST' link of target species genome to run *tblastn* search of *D. melanogaster* protein
   - **Query**: *D. melanogaster* protein
   - **Database**: target species' whole genome assembly
2. Identify best **collinear** set of protein alignments in target species-coordinates, scaffold name & accession

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Detect sequence similarity with BLAST

- BLAST = **B**asic **L**ocal **A**lignment **S**earch **T**ool
- Why is BLAST popular?
  - Provide statistical significance for each match
  - Good balance between sensitivity and speed
- Identify **local** regions of similarity

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Common BLAST programs

- Except for *blastn*, all alignments are based on comparisons of protein sequences
  - Alignment coordinates are relative to the **original sequences**
- Decide which *BLAST* program to use based on the type of query and subject sequences:

Program	Query	Database (Subject)
blastn	Nucleotide	Nucleotide
blastp	Protein	Protein
blastx	Nucleotide → Protein	Protein
tblastn	Protein	Nucleotide → Protein
tblastx	Nucleotide → Protein	Nucleotide → Protein

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

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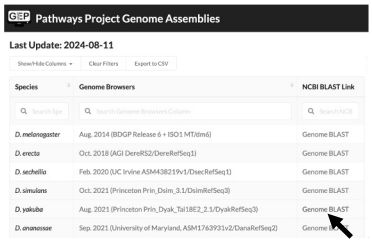
Genome BLAST links for the Pathways Project

- Limit BLAST searches to the **specific genome assembly** that is part of the Pathways Project



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Identify genomic location of ortholog in target species (*D. yakuba)*

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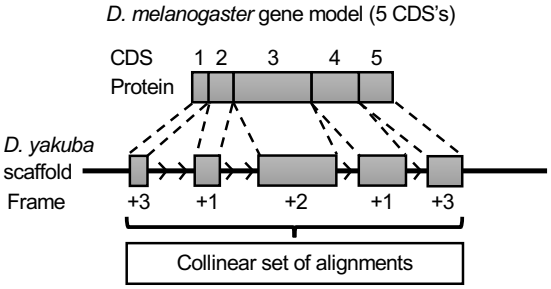
Expect **multiple alignment blocks** when aligning a protein with multiple CDS's against a scaffold



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Use the **Hit Table** to determine the best collinear set of *tblastn* alignments

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

best collinear set of alignments to Rheb-PA

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Genome databases use **different names** (sequence IDs) to refer to the **same sequence**

- INSDC (GenBank, EBI, DDBJ): CM028602.2
- NCBI RefSeq: NC_052530.2
- UCSC Genome Browser: NC_052530

NCBI RefSeq records are derived from, but might not be identical to, the source INSDC records

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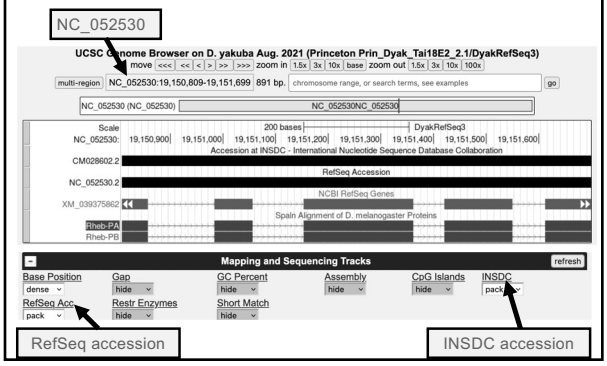
GEP UCSC Genome Browser supports INSDC and RefSeq accession numbers



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Repeat steps 5-7 for each unique isoform

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Step 3: Examine genomic neighborhood of putative ortholog in target species (*D. yakuba*)

1. Use target species' Genome Browser to navigate to the collinear set of alignments identified in Part 2
2. **Use synteny to verify ortholog assignment**
   - Run *blastp* search for target gene AND its two upstream and two downstream genes on either side
     - **Query**: accession of each protein in target species (click on isoform in 'NCBI RefSeq Genes' track; then, in the GenBank Record window, scroll down to the 'CDS' section and copy accession number for the translated protein sequence labeled 'protein_id'
     - **Database**: refseq_protein | Organism: Drosophila melanogaster (taxid: 7227)

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Use the NCBI RefSeq Genes predictions to gather **additional evidence** for the ortholog assignment

- Each bioinformatics tool has different strengths and weaknesses
- Trade-offs:
  - Sensitivity versus specificity
  - Required computational resources versus accuracy
- Program parameters often determined by heuristics
  - Optimized for the whole genome assembly

Need to evaluate and reconcile potentially contradictory results on the genome browser

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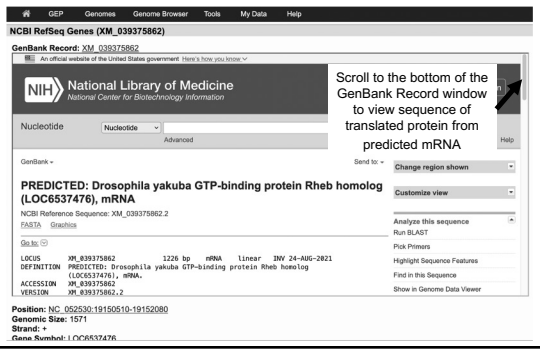
Retrieve the protein sequence for the NCBI RefSeq Gene prediction



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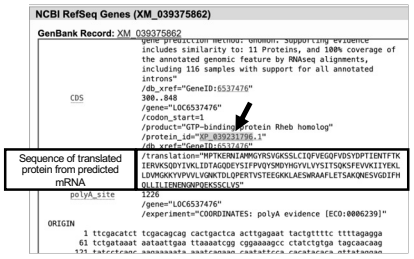

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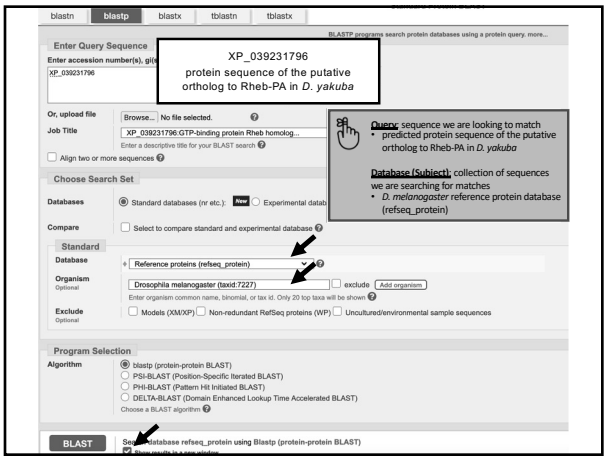

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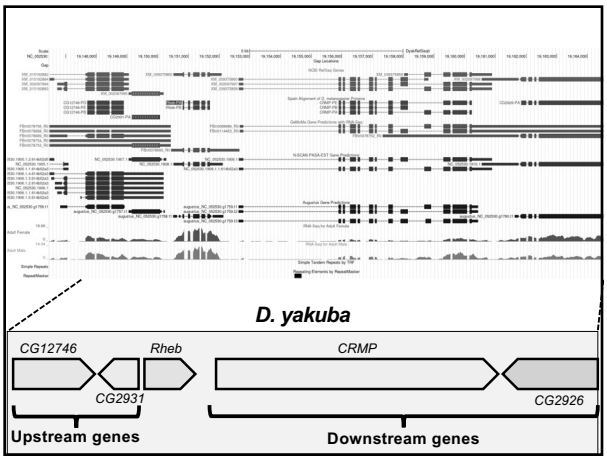

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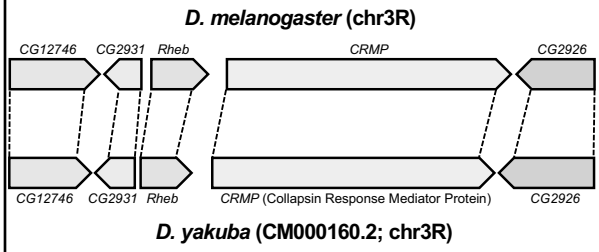
Synteny analysis provides additional evidence for the ortholog assignment



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Repeat steps 5-7 for each unique isoform

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Step 4: Determine structure of target gene (*Rheb*) in *D. melanogaster*

- Use the Gene Record Finder to identify the gene structure of the target gene (Rheb) in *D. melanogaster*

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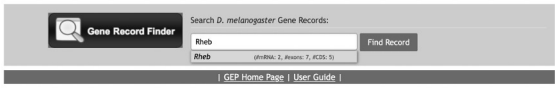
Retrieve the *Rheb* record for *D. melanogaster* using the Gene Record Finder

- Type ***Rheb*** into the search box, then press [Enter]



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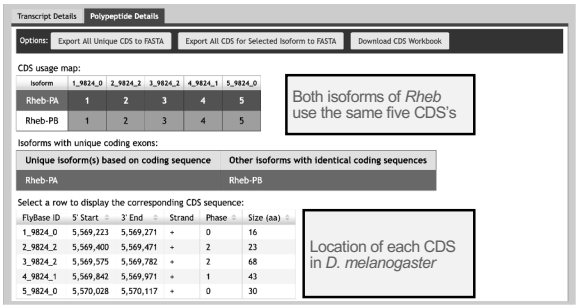
The A and B isoforms of *Rheb* use the **same set of CDS's** in *D. melanogaster*



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Step 5: Determine approximate location of coding exons in target species

1. Obtain the protein sequence of each CDS of the target gene in *D. melanogaster* using the Gene Record Finder (in Polypeptide Details tab, click on each CDS and then copy/paste the entire sequence from the pop-up window)

2. Run *tblastn* search (**check the box to align two or more sequences**) for each CDS of the target gene
   - **Query**: each individual CDS of target gene in D. melanogaster
   - **Database**: accession number of scaffold containing ortholog in target species (identified in Part 2) and narrow region of scaffold to search via "Subject subrange"
   - **Algorithm parameters**: "Compositional adjustments"- No adjustment and uncheck box for filtering "Low complexity regions"

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Detect conserved *D. melanogaster* CDSs in the target genome with *tblastn*

- Coding sequences evolve slowly
- Exon structure changes **very** slowly
- Initial hypothesis:
  - Gene structure is conserved between *D. melanogaster* and the ortholog in the target species
- Map each CDS separately to the target genome

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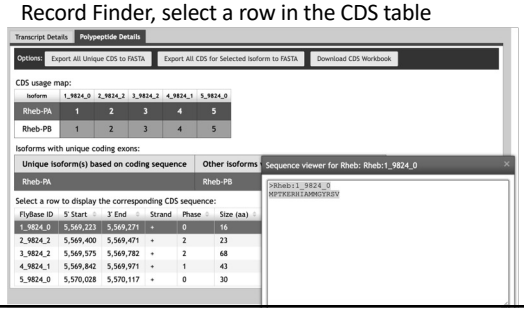
Retrieve the *D. melanogaster* CDS sequence from the Gene Record Finder

- In the **Polypeptide Details** section of the Gene Record Finder, select a row in the CDS table



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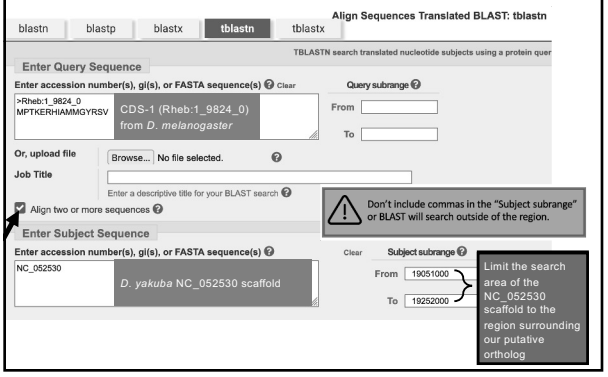
Compare the *D. melanogaster* CDS 1_9824_0 against the *D. yakuba* scaffold NC_052530



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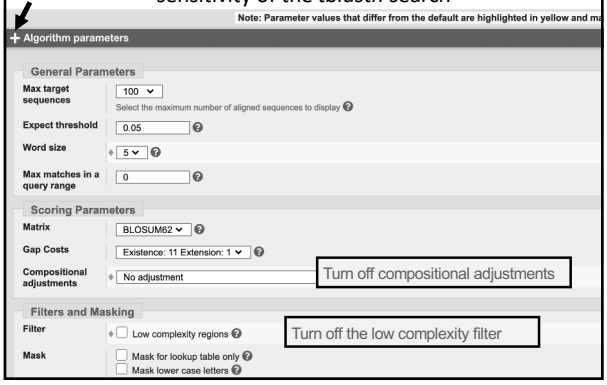
Customize **algorithm parameters** to increase the sensitivity of the *tblastn* search



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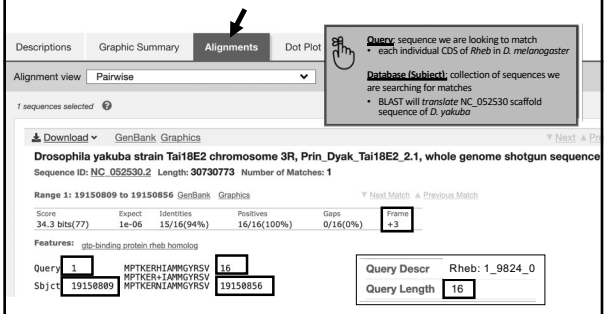
Record the results of the *TBLASTN* alignment to CDS 1_9824_0



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Summary of the *tblastn* results for *Rheb* CDS's against the *D. yakuba* scaffold NC_052530

CDS	FlyBase ID	Query Length Size (aa)	*D. melanogaster*		Target Species		Subject Frame
			Query Start	Query End	Subject Start	Subject End	
1	1_9824_0	16	1	16	19,150,809	19,150,856	+3
2	2_9824_2	23	1	23	19,150,987	19,151,055	+1
3	3_9824_2	68	1	68	19,151,156	19,151,359	+2
4	4_9824_1	43	1	43	19,151,422	19,151,550	+1
5	5_9824_0	30	1	30	19,151,613	19,151,702	+3

- Query: CDS's from the *D. melanogaster Rheb* gene
- Subject: *D. yakuba* scaffold NC_052530

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Annotation Workflow

1. Examine genomic neighborhood surrounding target gene in *D. melanogaster*
2. Identify genomic location of ortholog in target species
3. Examine genomic neighborhood of putative ortholog in target species
4. Determine structure of target gene in *D. melanogaster*
5. Determine approximate location of coding exons in target species
6. Refine coordinates of coding exons
7. Verify and submit gene model
8. Repeat steps 5-7 for each unique CDS found

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Step 6: Refine coordinates of coding exons

Use the Genome Browser to:

1. Verify start and stop codon coordinates identified in Part 5

2. Determine phases of donor and acceptor splice sites using Frame identified in *tblastn* CDS-by-CDS search in Part 5
   - donor is typically a "GT" and acceptor is typically an "AG" (Georgia Tech is in Atlanta Georgia)

3. Use splice junction predictions to verify coordinates of each intron

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The proposed gene model should satisfy **basic biological constraints***

- Coding regions start with a **methionine**
- Coding regions end with a **stop codon**
- Gene should be on only one strand of DNA
- Exons appear in order along the DNA (collinear)
- Intron sequences should be at least **40 bp**
- Intron starts with a **GT** (or rarely GC)
- Intron ends with an **AG**

*There are known exceptions to each rule

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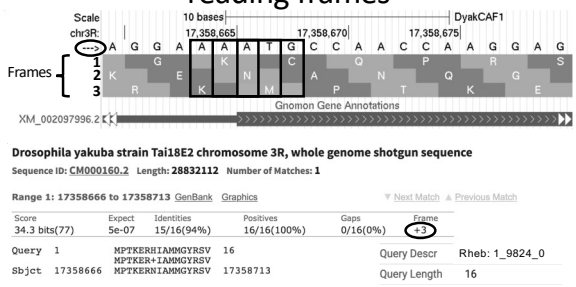
A genomic sequence has 6 different reading frames



- **Frame**: Base to begin translation relative to the first base of the sequence

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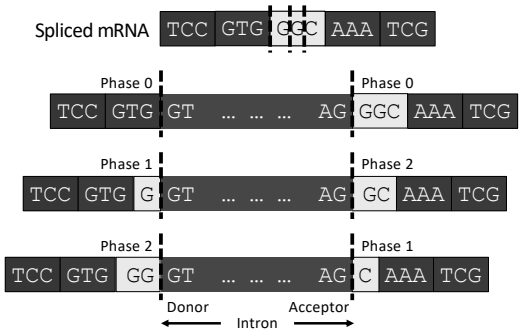
A codon could be derived from nucleotides in adjacent exons



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Splice donor and acceptor phases

- **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/GC)
  - Acceptor phase: Number of bases between the splice acceptor site (AG) and the **start of the first complete codon**
- Phase **depends on the reading frame of the CDS**

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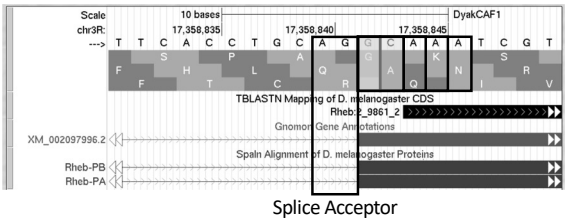
Phase depends on the reading frame



- Phase of acceptor site:
  - Phase **2** relative to frame **+1**
  - Phase **0** relative to frame **+2**
  - Phase **1** relative to frame **+3**

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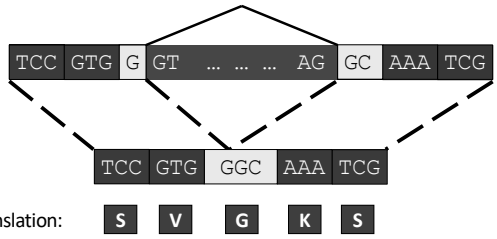
Phase of donor and acceptor sites must be compatible

- Extra nucleotides from donor and acceptor phases will form **an additional codon**
- Donor phase + acceptor phase = **0** or **3**



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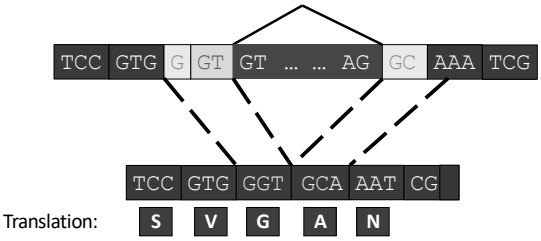
Incompatible donor and acceptor phases results in a frame shift



- Phase 0 donor is incompatible with phase 2 acceptor; use prior GT, which is a phase 1 donor

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Interpreting RNA-Seq data

- RNA-Seq evidence tracks:
  - RNA-Seq coverage (read depth)
  - Splice junction predictions (*TopHat*, *regtools*)
  - Assembled transcripts (*Cufflinks*, *Oases, StringTie*)
- Positive results very helpful
- Negative results less informative
  - **Lack of transcription ≠ no gene**
- GEP curriculum:
  - RNA-Seq Primer
  - Browser-Based Annotation and RNA-Seq Data

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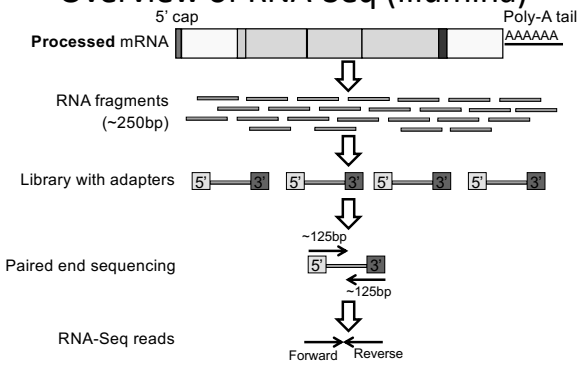
Overview of RNA-Seq (Illumina)



Wang Z et al. Nat Rev Genet. 2009 10(1):57-63.

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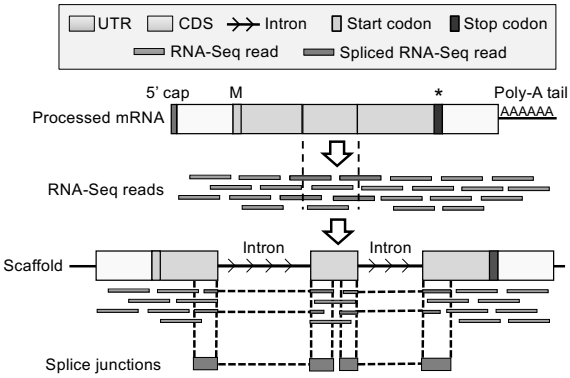
Use spliced RNA-Seq reads to identify splice sites



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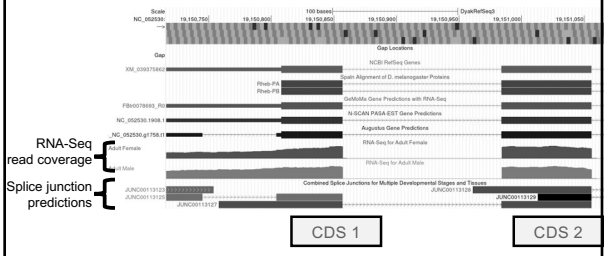
RNA-Seq evidence tracks on the GEP UCSC Genome Browser



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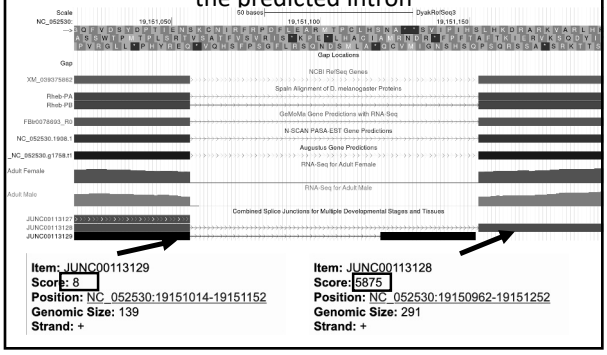
Splice junction score corresponds to the **number of spliced RNA-Seq reads** supporting the predicted intron



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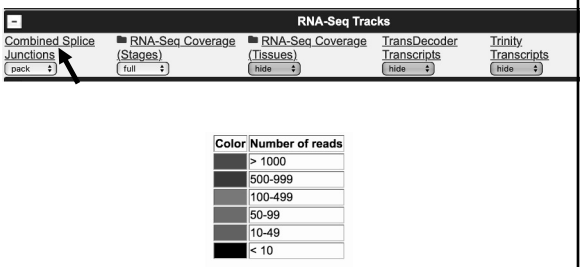
Color of the splice junctions correspond to the number of reads supporting the junction



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Gene model for Rheb-PA in *D. yakuba*

Gene Model for Rheb-PA in *D. yakuba*						
CDS	FlyBase ID	Frame	Splice Acceptor Phase	Coordinates		Splice Donor Phase
				Start	End	
1	1_9824_0	+3		19,150,809	19,150,857	1
2	2_9824_2	+1	2	19,150,985	19,151,056	1
3	3_9824_2	+2	2	19,151,154	19,151,361	2
4	4_9824_1	+1	1	19,151,421	19,151,550	0
5	5_9824_0	+3	0	19,151,613	19,151,699	

Stop codon: 19,151,700-19,151,702

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Annotation Workflow

1. Examine genomic neighborhood surrounding target gene in *D. melanogaster*
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6. Refine coordinates of coding exons
7. Verify and submit gene model

Repeat steps 5-7 for each unique isoform

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Step 7: Verify and submit gene model

- Use the Gene Model Checker to verify that your 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)

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Verify the final gene model using the Gene Model Checker

- Examine the checklist and explain any errors or warnings in the Pathways Project Annotation Report
- View your gene model in the context of the other evidence tracks on the Genome Browser
- Examine the dot plot and explain any discrepancies in the Annotation Report
  - Look for large vertical and horizontal gaps
  - See the "**Quick Check of Student Annotations**" document on the GEP web site

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Annotation Workflow

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