PDF Distribution Version

May 2019

Functional Genomics Resources for Drosophila Research from the DRSC/TRiP

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Director of DRSC/TRiP Functional Genomics Resources

Look out for helpful hyperlinks throughout the presentation

@smohrfly
@DRSC_TRiP

HARVARD MEDICAL SCHOOL

PART 1:
in vivo fly stock production

Transgenic RNAi Project (TRiP)-KO and –OE sgRNA fly stocks

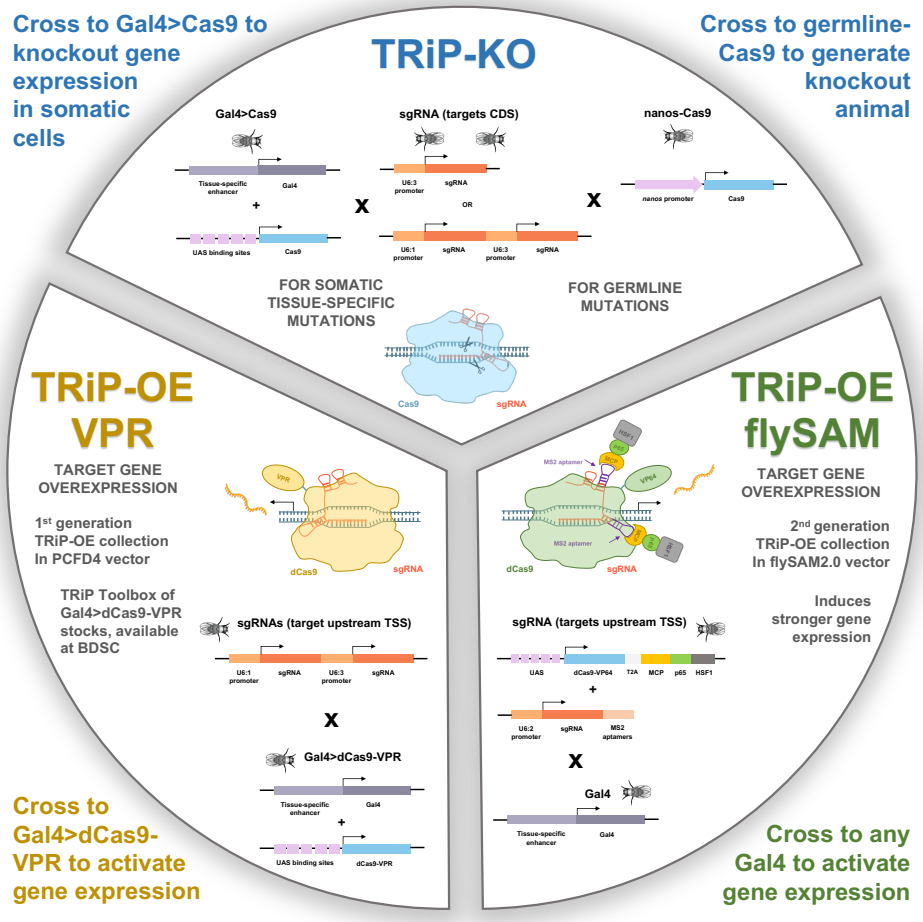
For tissue-specific CRISPR knockout or activation

https://fgr.hms.harvard.edu/using-trip-crispr-lines

https://fgr.hms.harvard.edu/sgrna-vectors

https://www.flyrnai.org/tools/grna_tracker/web/

TRiP-CRISPR overview



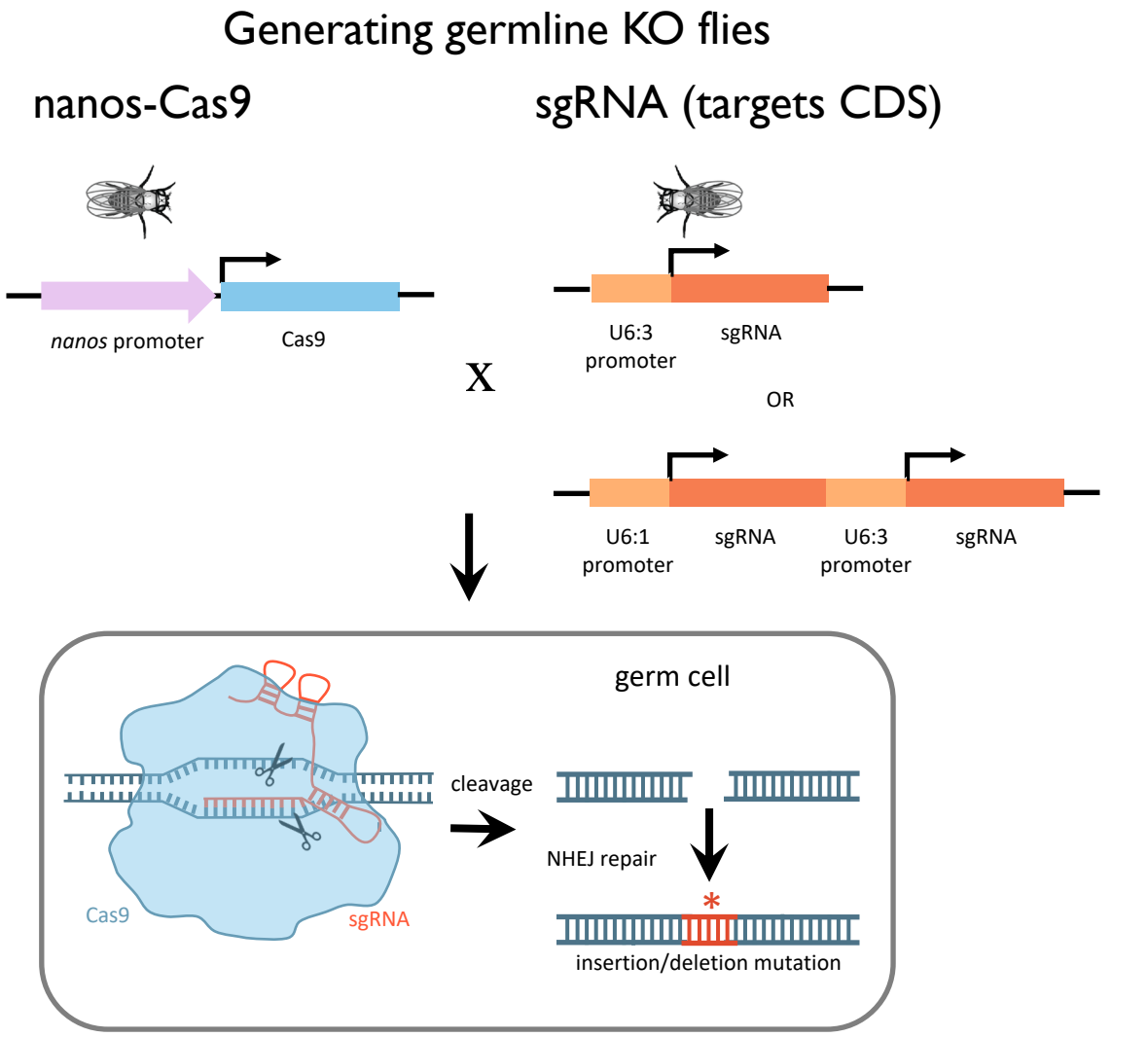
	TRiP-OE			TRiP-KO
Collection	VPR	flySAM	flySAM.dCas9	
Function	Gene Activation	Gene Activation	Gene Activation	Gene Cutting
Vectors	pCFD4	U6B-sgRNA2.0	flySAM2.0	pCFD3, pCFD4, pCFD6
Cross to	Gal4+ dCas9-VPR	Gal4+ flySAM	Gal4	Gal4+ Cas9
Notes	Use with TRiP-CRISPR Toolbox	Use with TRiP-CRISPR Toolbox	flySAM.dCas9 contain both sgRNA and activator complex	Use with TRiP-CRISPR Toolbox
Stocks	2,128	216	262	2,145

https://fgr.hms.harvard.edu/using-trip-crispr-lines

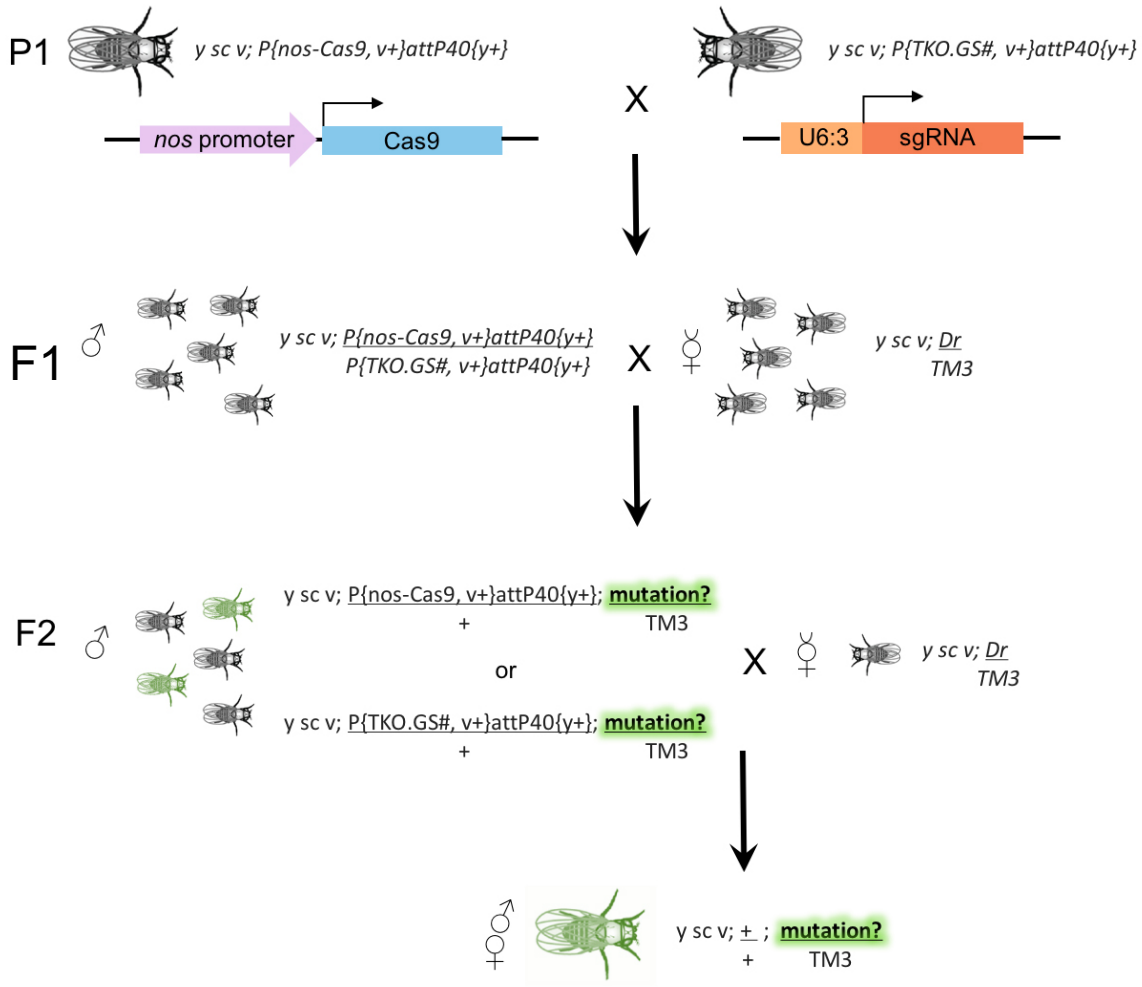
https://fgr.hms.harvard.edu/sgrna-vectors

TRiP-CRISPR Knockout (TRiP-KO)

- TRiP-KO flies ubiquitously express sgRNAs targeting gene coding sequence.
- Target most 5' sequence that will mutate all/most isoforms
- Mutant animals can be produced by simply crossing TRiP-KO flies to germline-specific-Cas9



Using TRiP-KO stocks to make mutants



Step 1: cross nanos-Cas9 (nos-Cas9) stock to individual TRiP-KO stock.

Step 2: collect at least 15 male F1 progeny containing both nos-Cas9 and sgRNA transgenes.

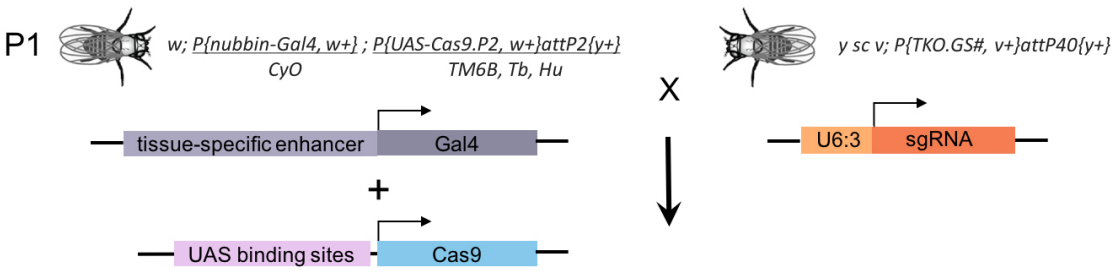
Step 3: cross male F1 progeny en masse to appropriate balancer strain for your target gene

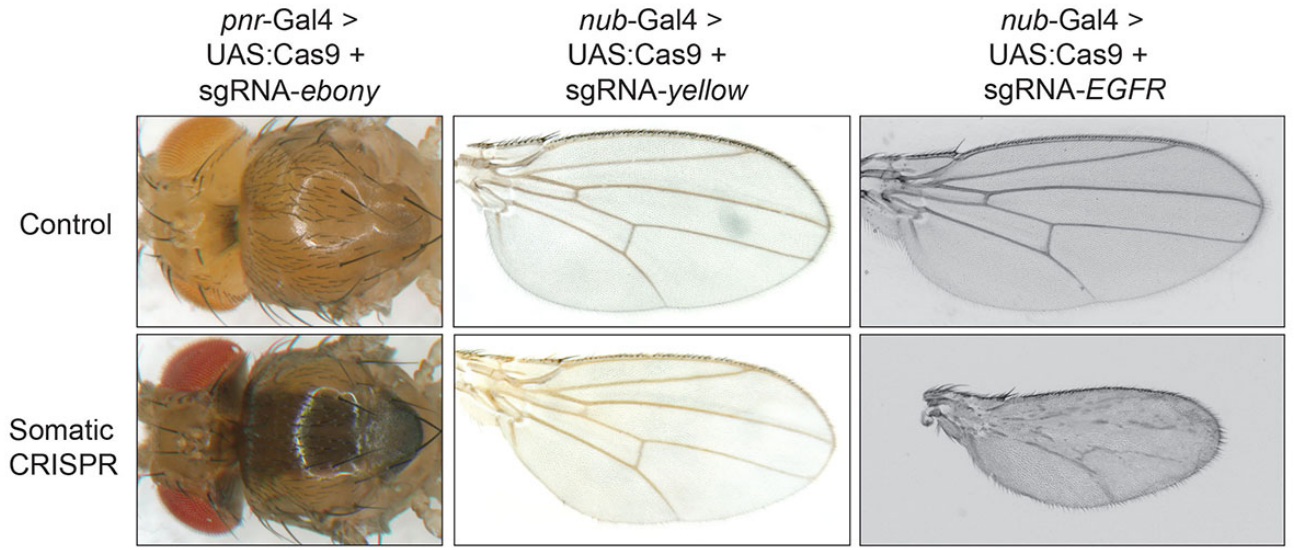
Step 4: collect male or female F2 progeny (some will be heterozygous mutants) and cross each individually to balancer stock.

Step 5: Screen mutations by restriction profiling, endonuclease assays or high-resolution melt assays (HRMAs) and confirm by PCR and sequencing

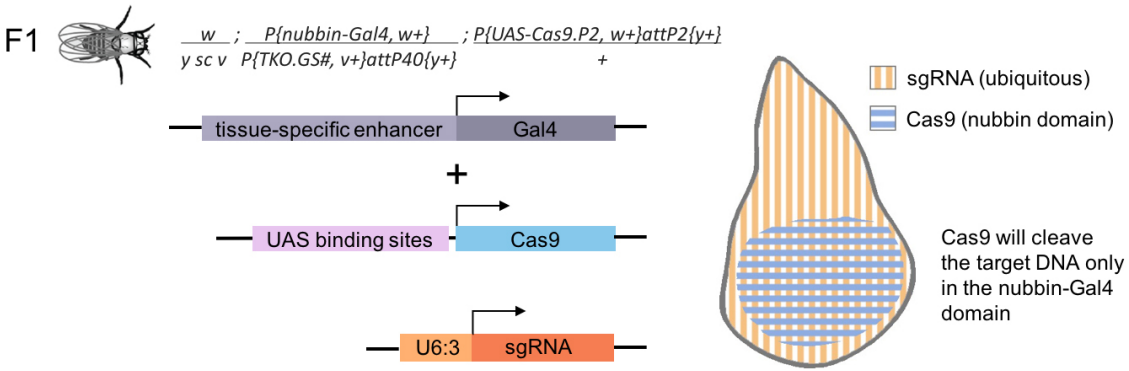
Using TRiP-KO stocks for mosaic KO

Step 1: Step 1: cross tissue specific-Gal4 + UAS-Cas9 stock to individual TRiP-KO stock.





Step 2: collect male or female F1 progeny containing tissue-specific-Gal4, UAS-Cas9 and sgRNA transgenes and analyze phenotype.



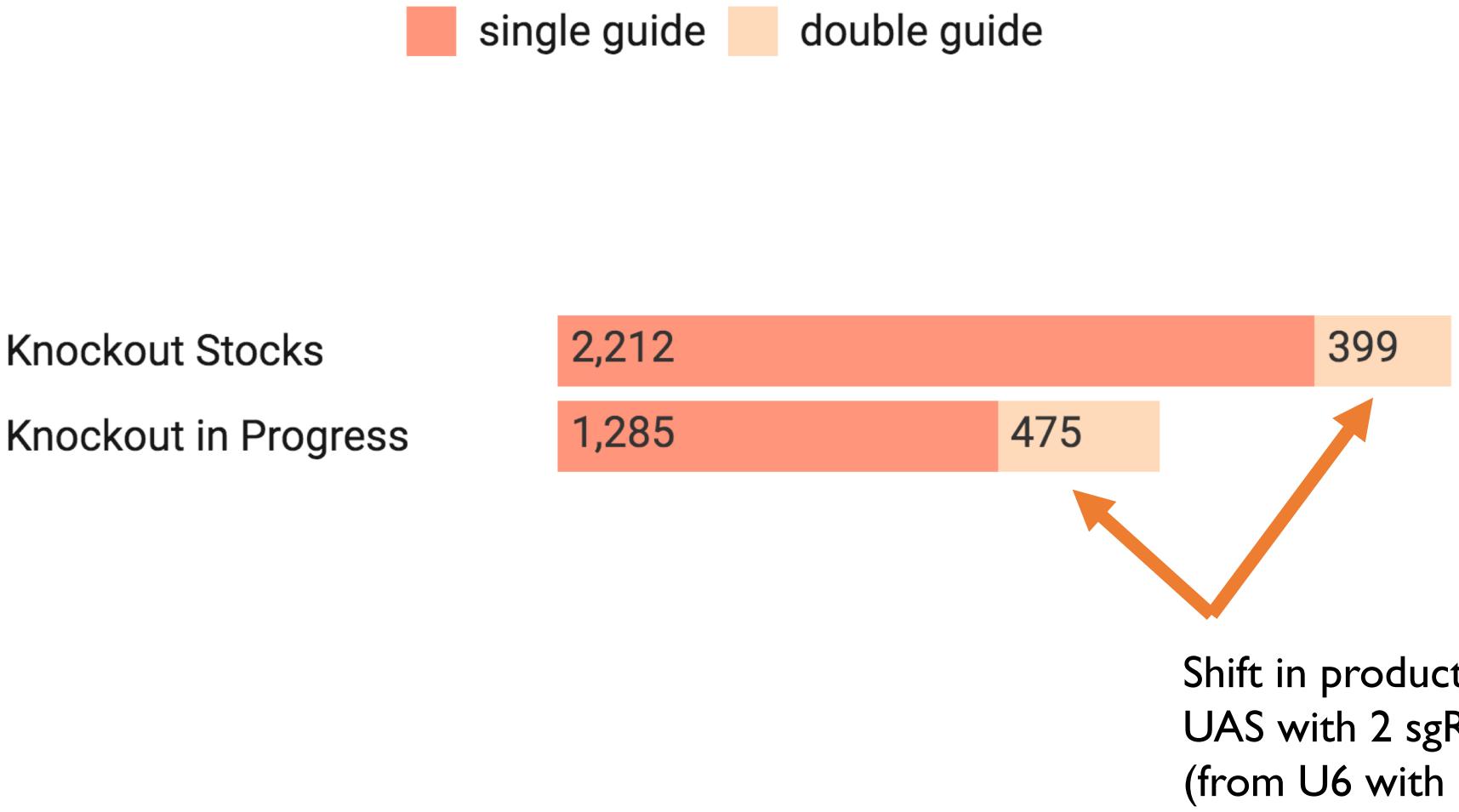
Two guides are better than one: the problem of in-frame deletions

```
CCATTGGCTCAGATTGACGAGGCGCCGGCAACTAAAAGA   WT

CCATTGGCTCAGATTGACG-----CCGGCAACTAAAAGA   *
CCATTGGCTCAGATTGA-----CGCCGGCAACTAAAAGA   *
CCATTGGCTCAGATTG-------GCCGGCAACTAAAAGA   *
CCATTGGCTCAGATTGACGAG-------CAACTAAAAGA   *
CCATTGGCTCAGATTGACGAa--------AACTAAAAGA   *
CCATTGGCTCAGA-------------------------    *
```

Numbers as of May 2019

TRiP-CRISPR sgRNA Production

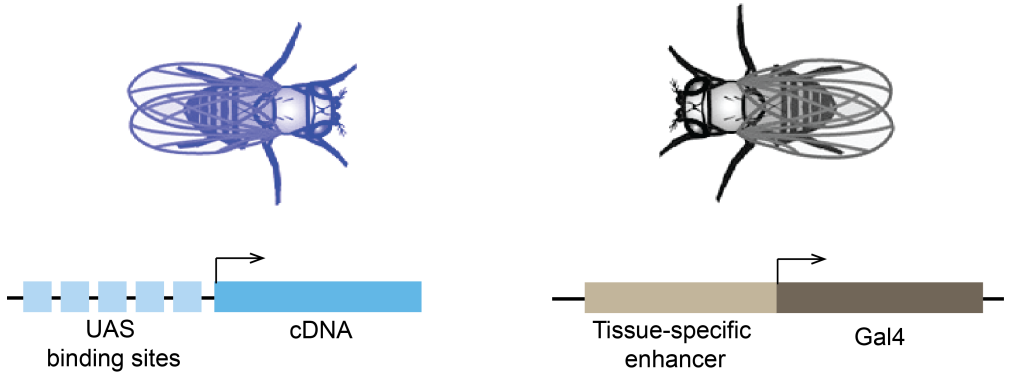


https://fgr.hms.harvard.edu/using-trip-crispr-lines

https://fgr.hms.harvard.edu/sgrna-vectors

Previous tools for over-expression in *Drosophila*

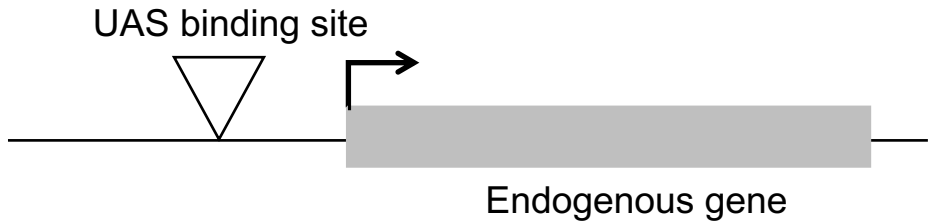
Gal4>UAS-cDNA



Limitations:

1) Expression is often at extremely high levels – possible toxicity
2) Cloning challenges – multiple isoforms, very large genes, etc.
3) Very hard to scale to a genome-wide resource

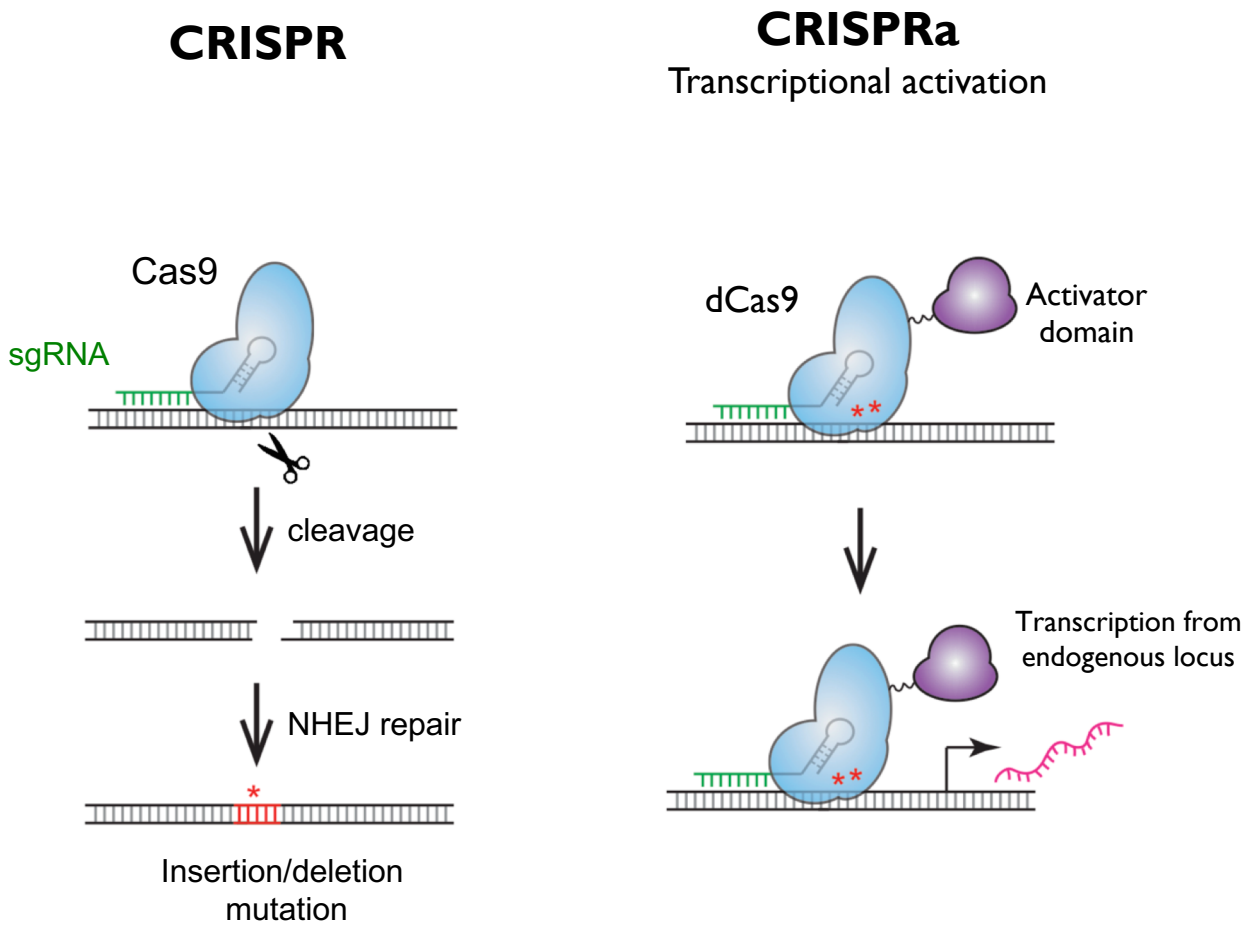
"EP Collection" (Rørth 1996)



Limitations:

1) Expression is often very weak
2) Random integration
3) Disrupt gene

dCas9-fusions for transcriptional activation

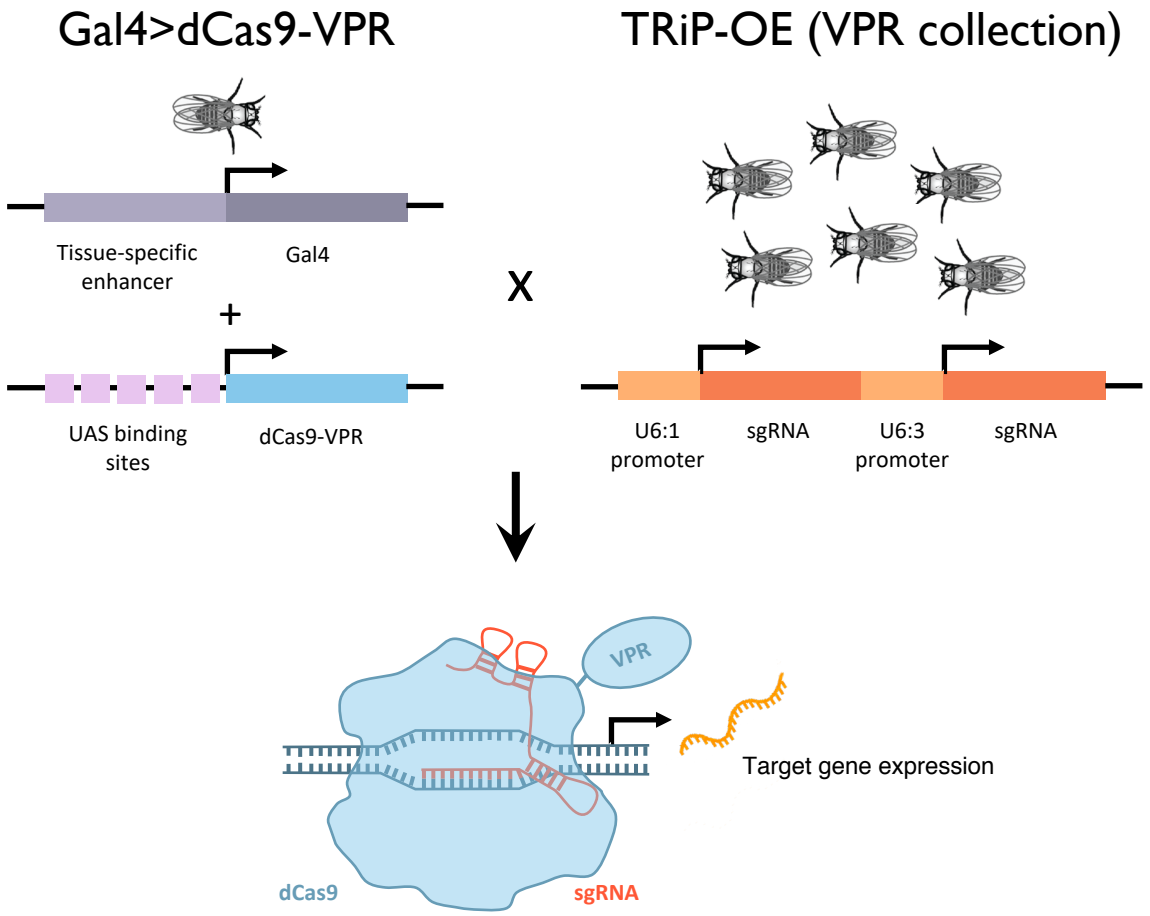


Point mutations D10A and H840A eliminate endonuclease activity (dCas9)

CRISPRa with VPR: fusion of dCas9 with VPR hybrid activaction domain leads to up-regulation

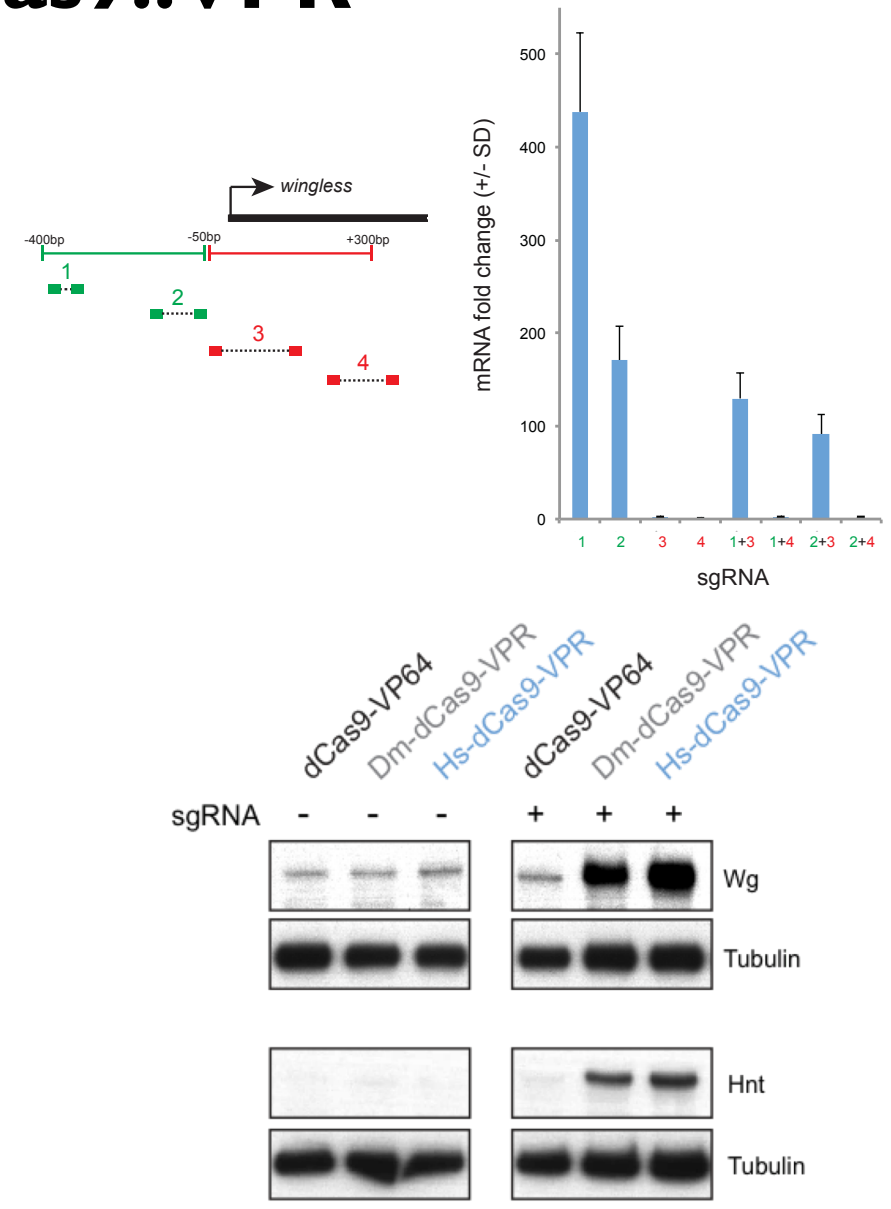
CRISPRa with FlySAM: fusion of dCas9 and modification of sgRNA backbone with MS2 loops + MS2 binding protein fused to additional activation domain

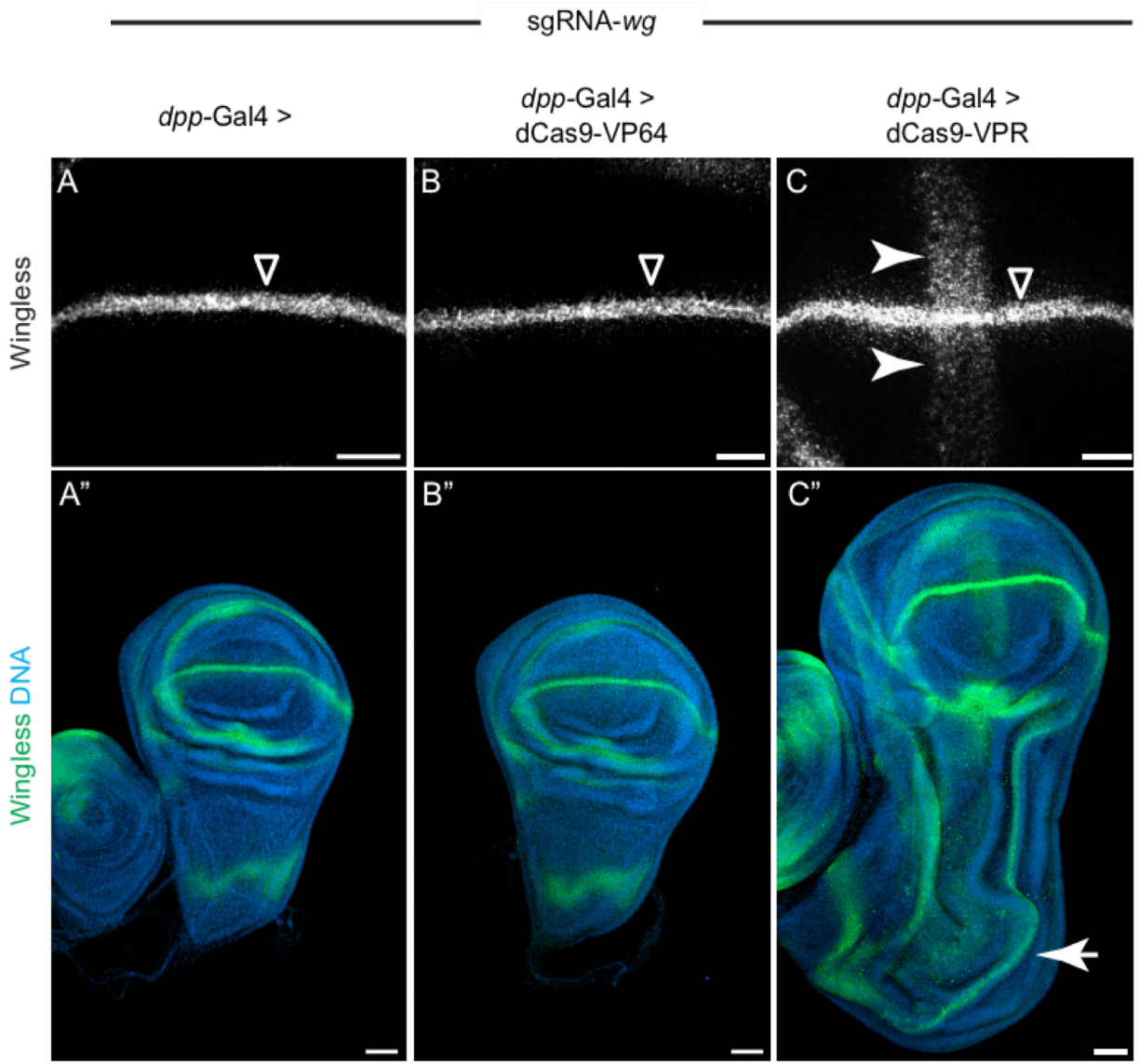
TRiP-Over-Expression (OE) stock collection—VPR



- A single gene is targeted by tandem expression of two sgRNAs from independent U6 promoters
- Stocks are made in the pCFD4 vector, developed by Fillip Port and colleagues
- Crossing TRiP-OE stocks to a Gal4 line expressing dCas9 fused to the chimeric activator domain VPR induces expression of the target gene
- Can also generate Indels and larger genomic deletions by crossing to Gal4>Cas9 flies
- Can only express wildtype proteins – no tags, no dominant negatives, etc..
- Can have off-targets if two genes are nearby.

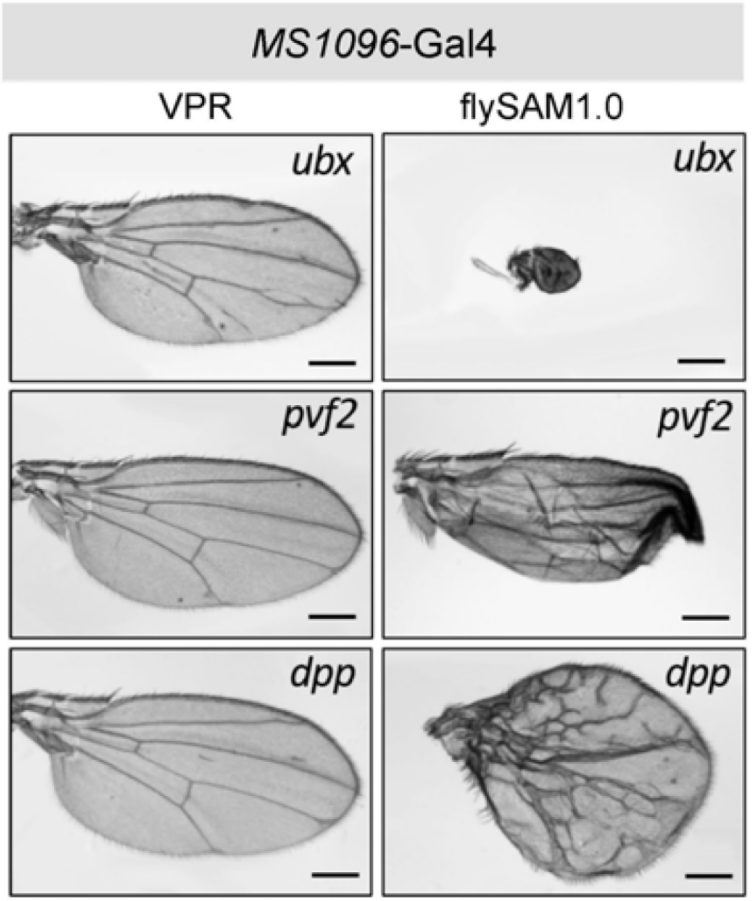
Cas9::VPR

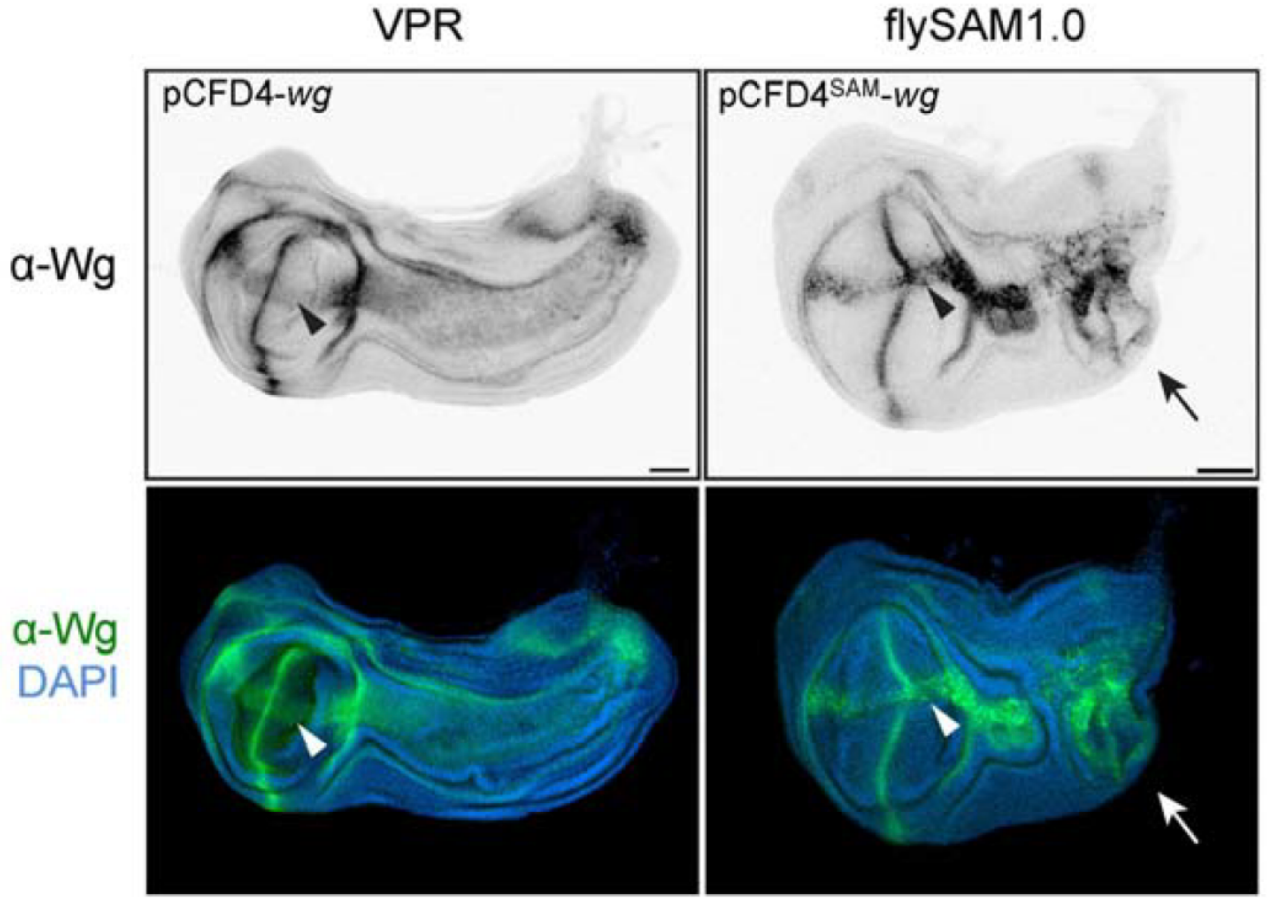




Ewen-Campen et al. 2017 *PNAS*

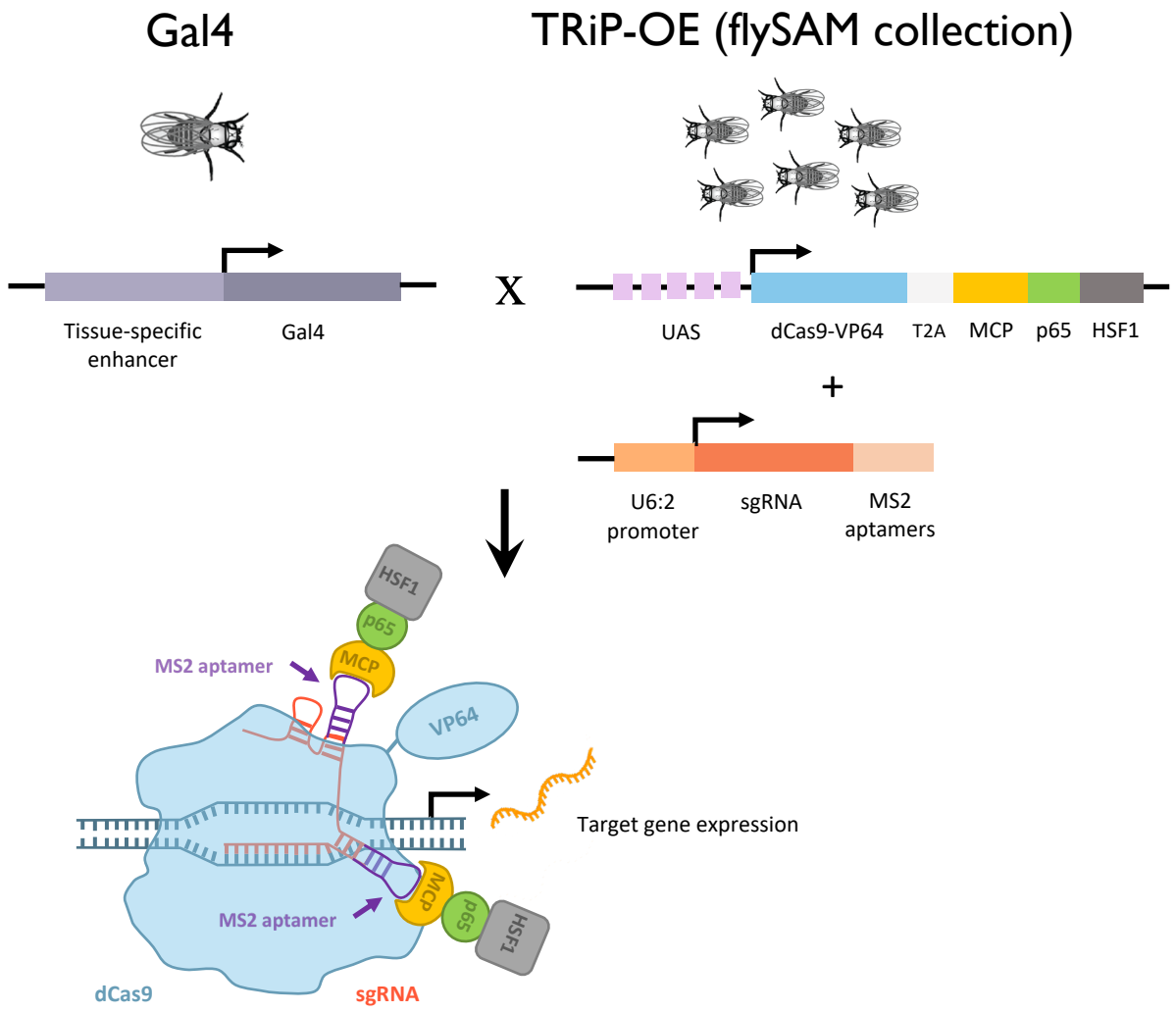
VPR vs. FlySAM





Jia et al. 2018 *PNAS*

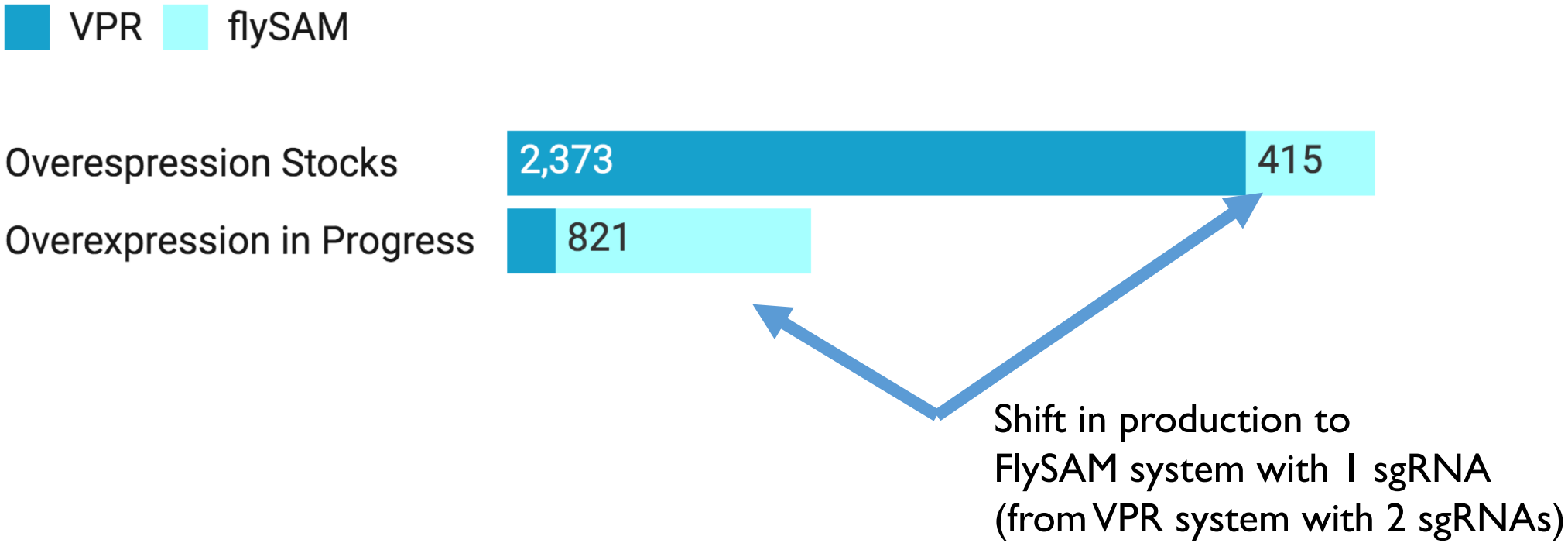
TRiP-Over-Expression (OE) stock collection—flySAM



- Simplified strategy for *in vivo* CRISPR activation
- Stronger overexpression phenotypes
- A single gene is targeted by expression of one sgRNA from U6:2 promoter
- Stocks are made in the flySAM2.0 vector, developed by Jian-quan Ni and colleagues
- TRiP-OE/flySAM stocks contain UAS-Cas9, so simply crossing to a Gal4 induces expression of the target gene

Numbers as of May 2019

TRiP-CRISPR sgRNA Production

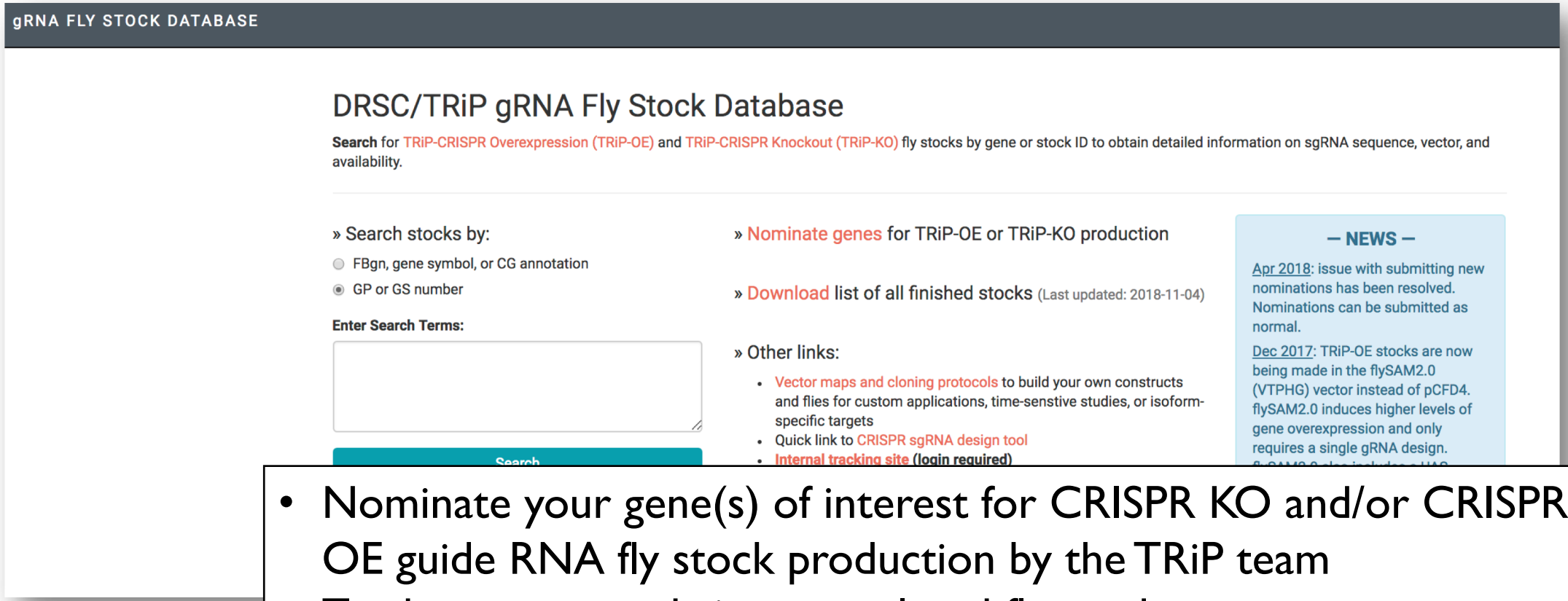


https://fgr.hms.harvard.edu/using-trip-crispr-lines

https://fgr.hms.harvard.edu/sgrna-vectors

gRNA Fly Stock Database

Online portal for community nomination, production tracking, information



- Nominate your gene(s) of interest for CRISPR KO and/or CRISPR OE guide RNA fly stock production by the TRiP team
- Track progress and view completed fly stocks

https://www.flyrnai.org/tools/grna_tracker/web/

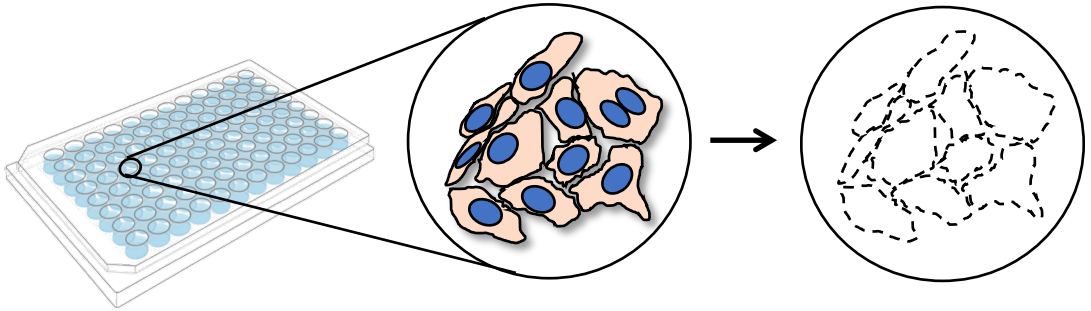
PART 2:
cell-based screens and cell lines

Drosophila RNAi Screening Center (DRSC) RNAi and CRISPR screening

And knock-in of GFP for live-cell visualization of organelles and compartments

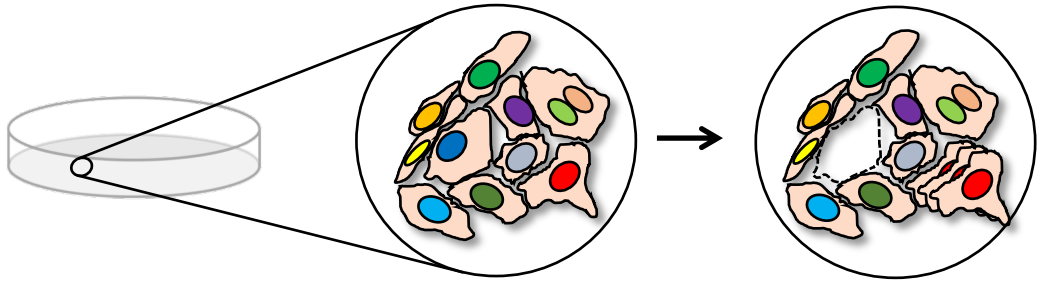
Arrayed Screens

- Micro-well plates
- Liquid handling automation
- Assay period of days
- Automated high-content assays
- Look up ‘hits’ in a database

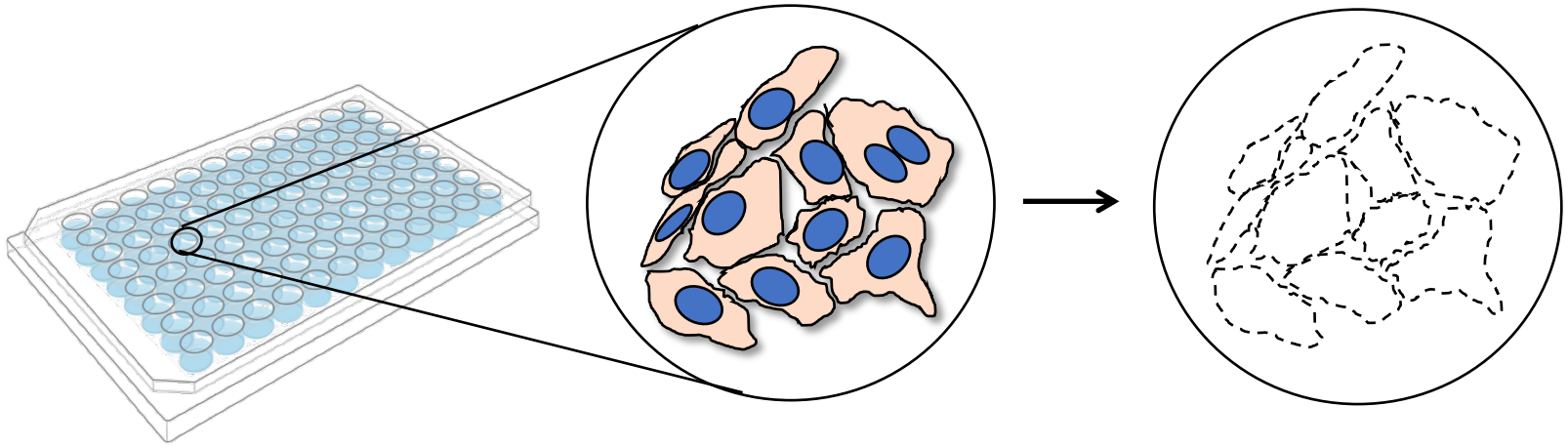


Pooled Screens

- In bulk culture
- No automation needed
- Assay period of weeks
- Drop-out or selection assays
- NGS readout to identify ‘hits’



Since 2003:
Arrayed Format Screening
Double-stranded RNAs (dsRNAs)

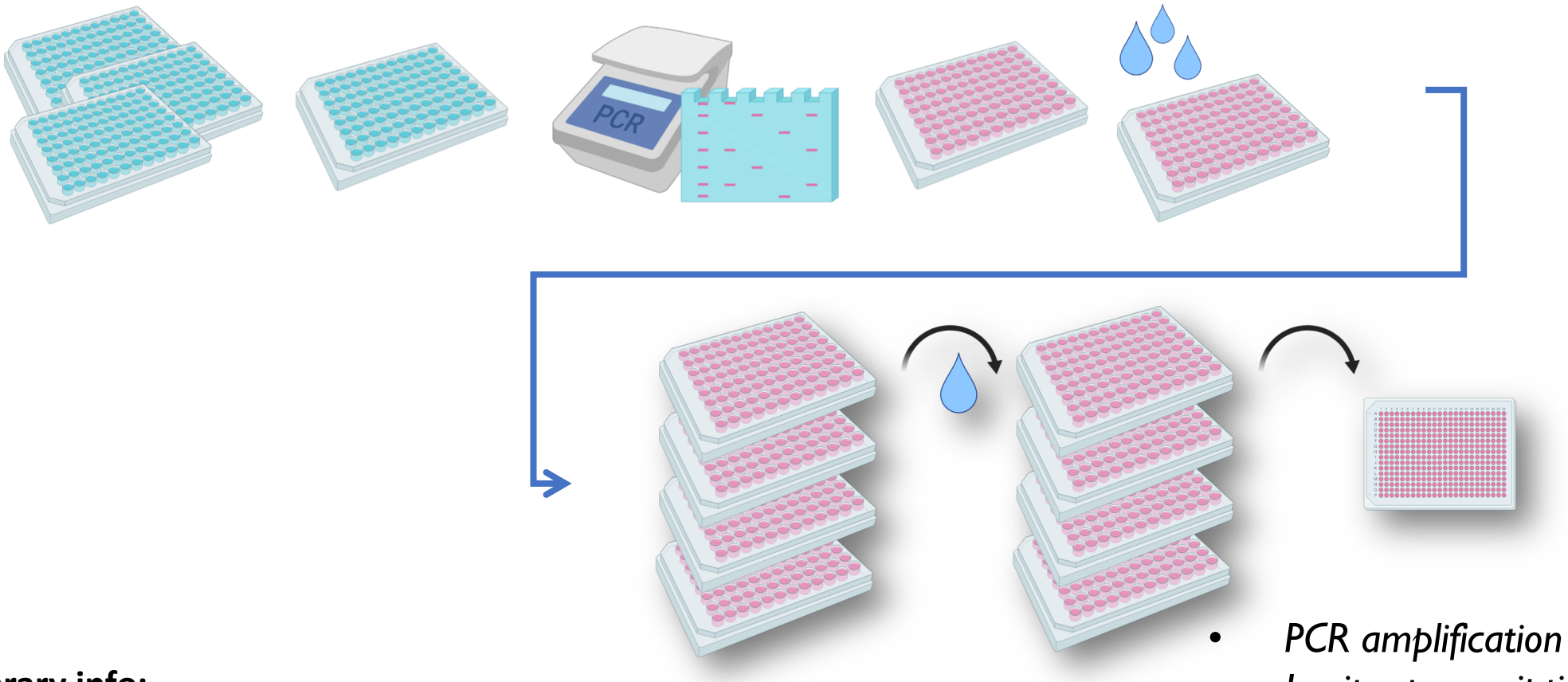


DRSC dsRNA library production

- Automated, fully in vitro process (no bacterial transformation steps)
- T7 primer used to re-amplify PCR amplicons (2-3 designs/gene, all genes)
- PCR amplicons used as template for in vitro transcription
- dsRNAs are normalized and re-arrayed
- Concentrated dsRNAs are diluted to assay-ready concentration
- dsRNAs are aliquoted to 384-well plates for cell-based assays

Library info:
https://fgr.hms.harvard.edu/fly-cell-rnai-libraries
https://fgr.hms.harvard.edu/drsc-20-genome-wide-screening-library
https://fgr.hms.harvard.edu/drsc-focused-sub-libraries
Protocols:
https://fgr.hms.harvard.edu/drsc-cell-rnai

dsRNA production workflow



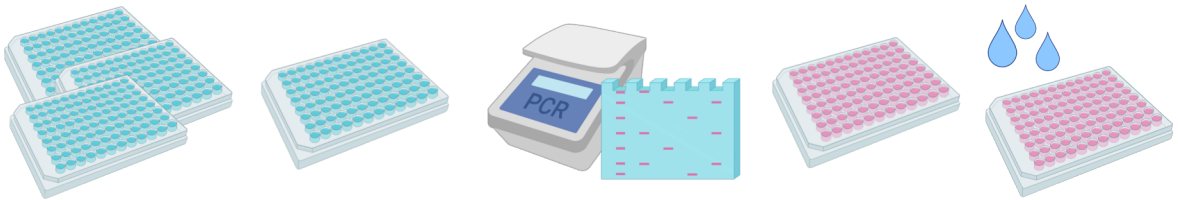
- *PCR amplification of templates*
- *In vitro transcription to make dsRNA*
- *Normalization of concentrations*
- *Dilution to 1x concentration*
- *Assay-ready 384-well plate production*
- *Quality control & analysis throughout*

Library info:
https://fgr.hms.harvard.edu/fly-cell-rnai-libraries
https://fgr.hms.harvard.edu/drsc-20-genome-wide-screening-library
https://fgr.hms.harvard.edu/drsc-focused-sub-libraries

Protocols:
https://fgr.hms.harvard.edu/drsc-cell-rnai

Cell-based RNAi libraries and services

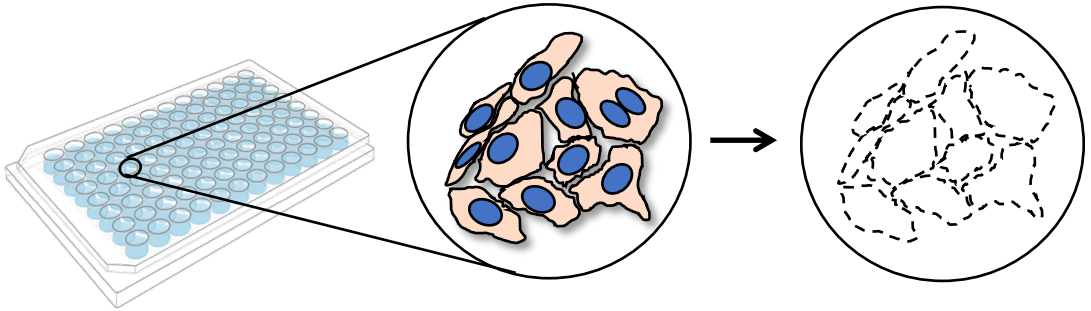
- **>200 RNAi screen projects since 2003**
  - View public datasets:
    - https://www.flyrnai.org/screensummary



- **Full-genome dsRNA library**
- **Focused sub-libraries** (kinases, transcription factors, etc.)
  - Recently developed: "FDA" library targeting fly orthologs of human genes that encode proteins that are known drug targets
  - Recently updated: Ubiquitin-related library
  - View library list and info:
    - https://fgr.hms.harvard.edu/drsc-focused-sub-libraries
- **Custom 'cherry-pick' of templates**
  - one, a few, or many—for small-scale studies, establishing controls, etc.
- **Custom dsRNA synthesis based on our existing templates**
  - billed by the 96-well plate—e.g. for a cell-based functional assay of candidates identified using another 'omics approach, enough dsRNA for many assays
- **Screen on-site at DRSC, e.g. with GE IN Cell automated confocal microscope**
- **-or- Screen at a screening center local to your area (libraries shipped to you)**

Arrayed Screens

- Micro-well plates
- Liquid handling automation
- Assay period of days
- Automated high-content assays
- Look up ‘hits’ in a database



Pooled Screens

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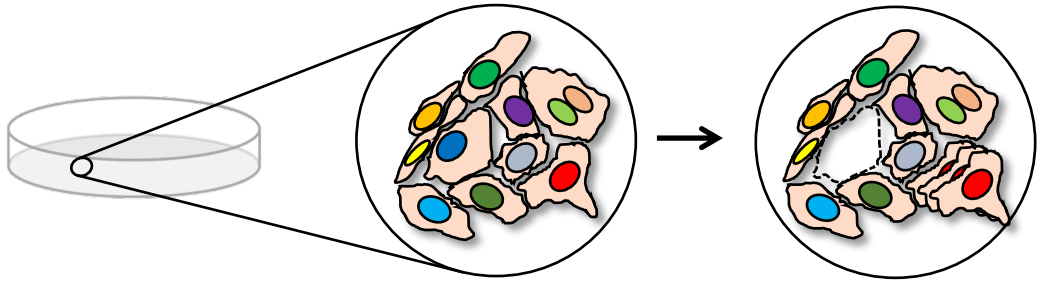


Pooled screens in Drosophila culture cells

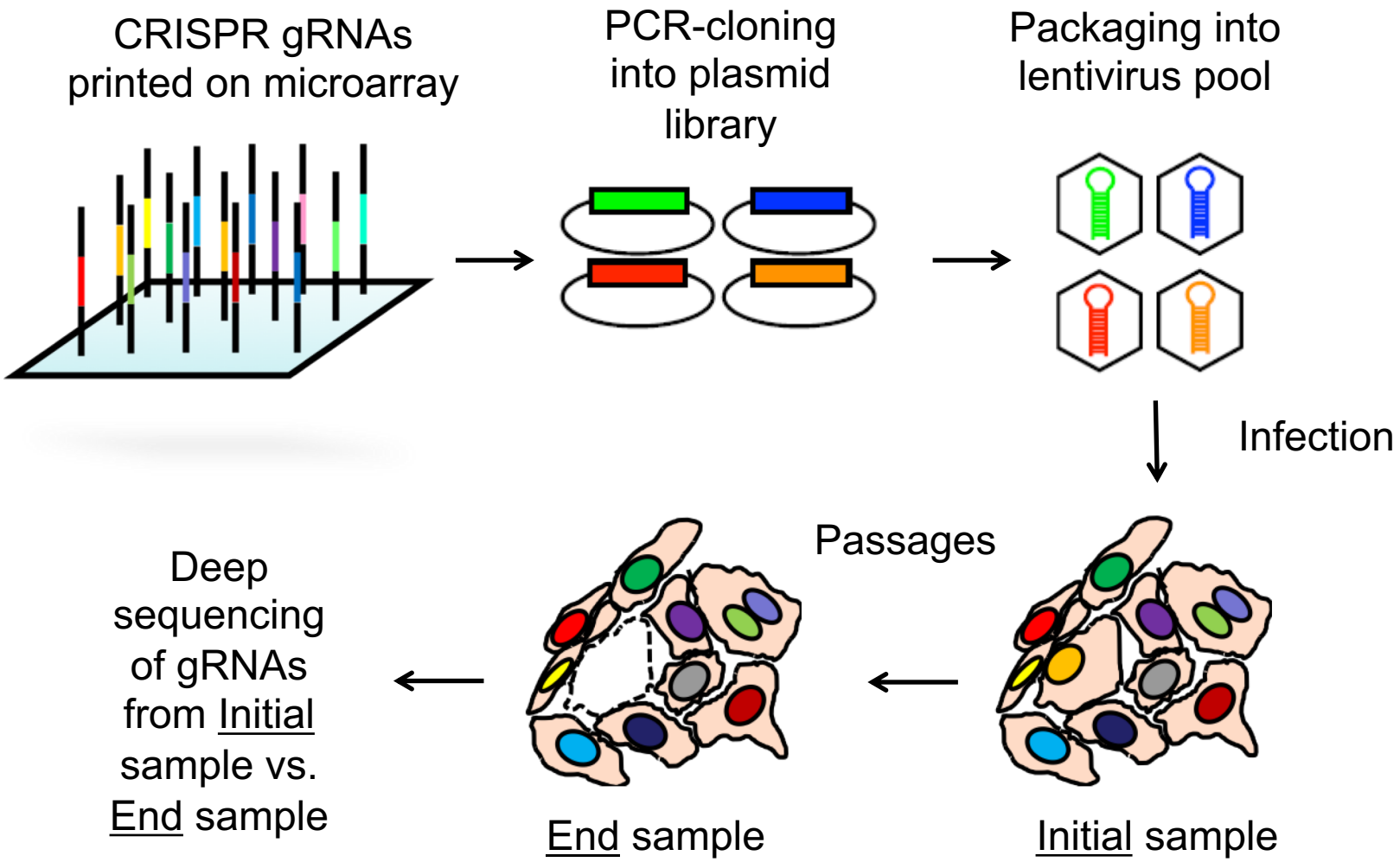
Why?

- Low gene redundancy vs. humans
- Relevance to humans, insect disease vectors, insect pests
- Different types of assays vs. arrayed-format RNAi assays

How?

- Reagent delivery method—transfection
- Reagent library design—DRSC bioinformatics
- Reagent library production—on-chip, 6 sgRNAs per gene
- Reagent integration into genome? Had been a barrier—no longer!

Mammalian genome-scale CRISPR knockout screen workflow



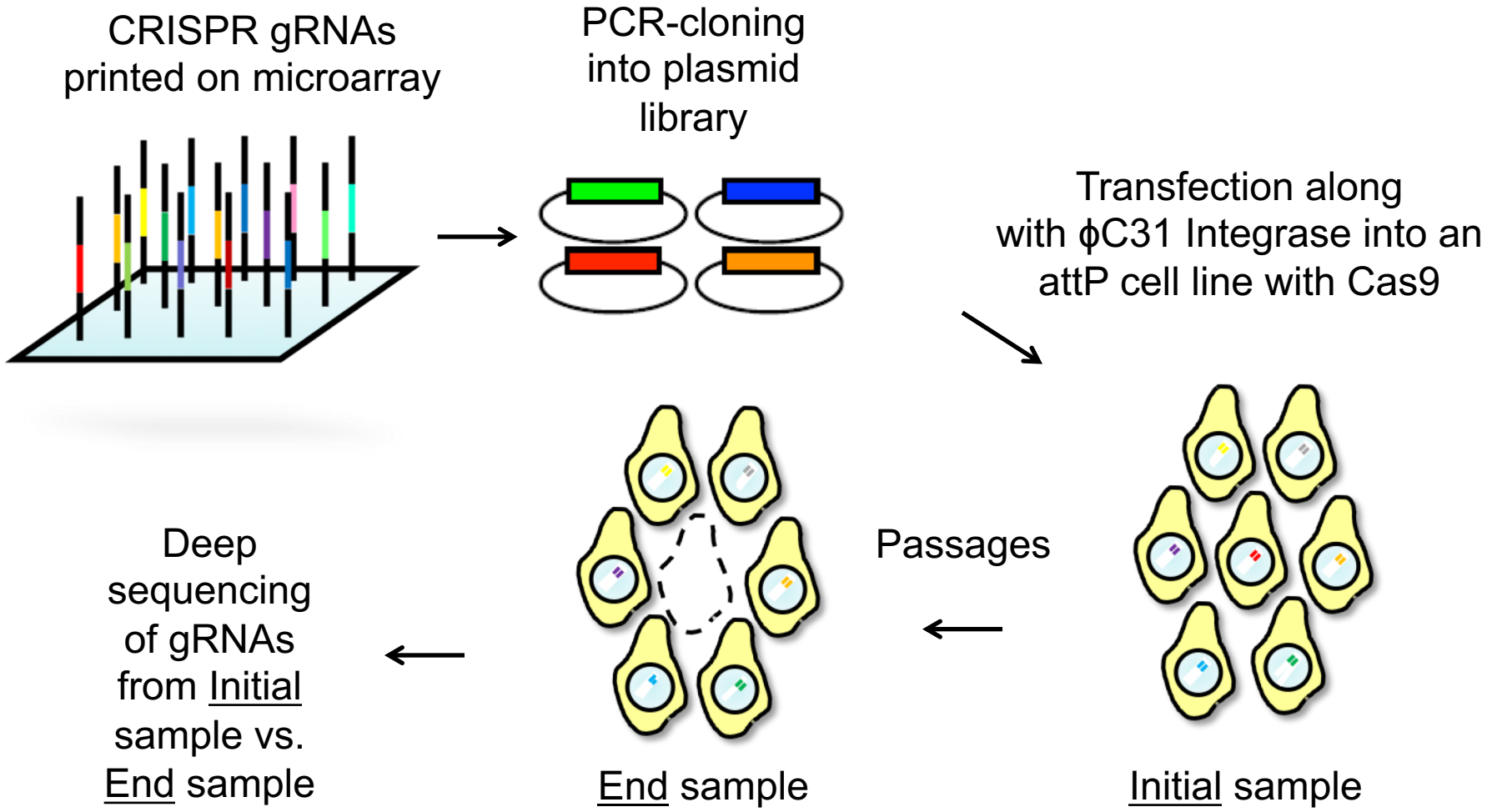
- **Lentiviral delivery results in integration into genome**
- **Amplification of integrated sgRNAs allows for identification of hits**

But:
lenti system doesn't work in fly cells—so,

How integrate sgRNAs into fly cells?

Answer: attP landing sites

Drosophila genome-scale CRISPR knockout screen workflow



Viswanatha et al. (2018) *eLife*

CRISPR pooled-format screens in fly cells:
Relevant publications and data sets

Proof-of-principle screens:

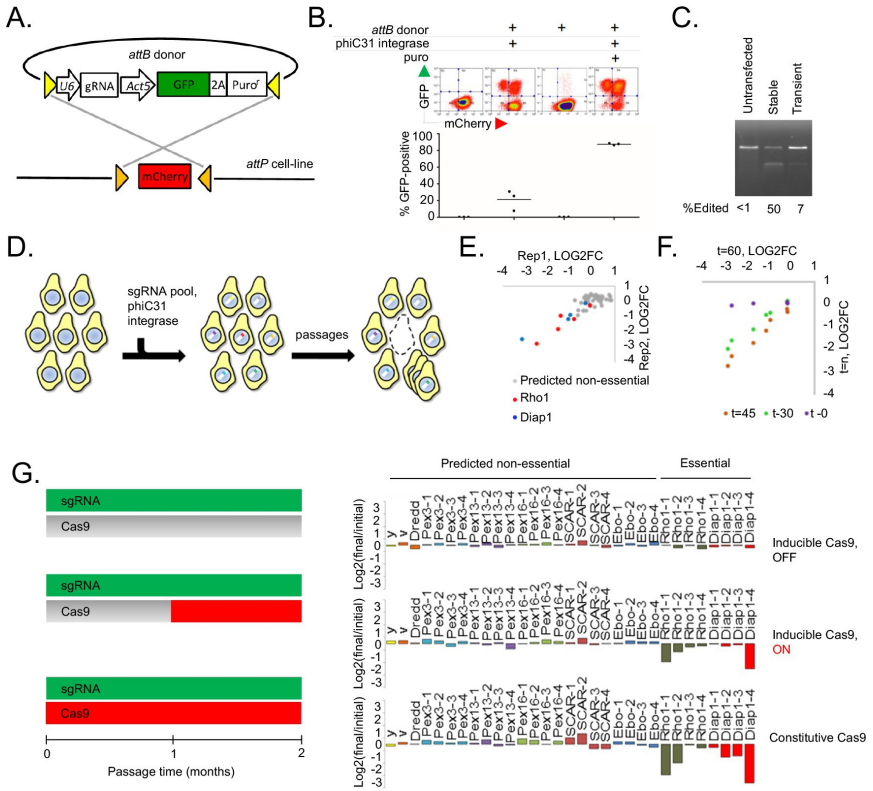
Viswanatha R, Li Z, Hu Y, Perrimon N. **Pooled genome-wide CRISPR screening for basal and context-specific fitness gene essentiality in Drosophila cells**. Elife. 2018 Jul 27;7. pii: e36333. PMID: 30051818

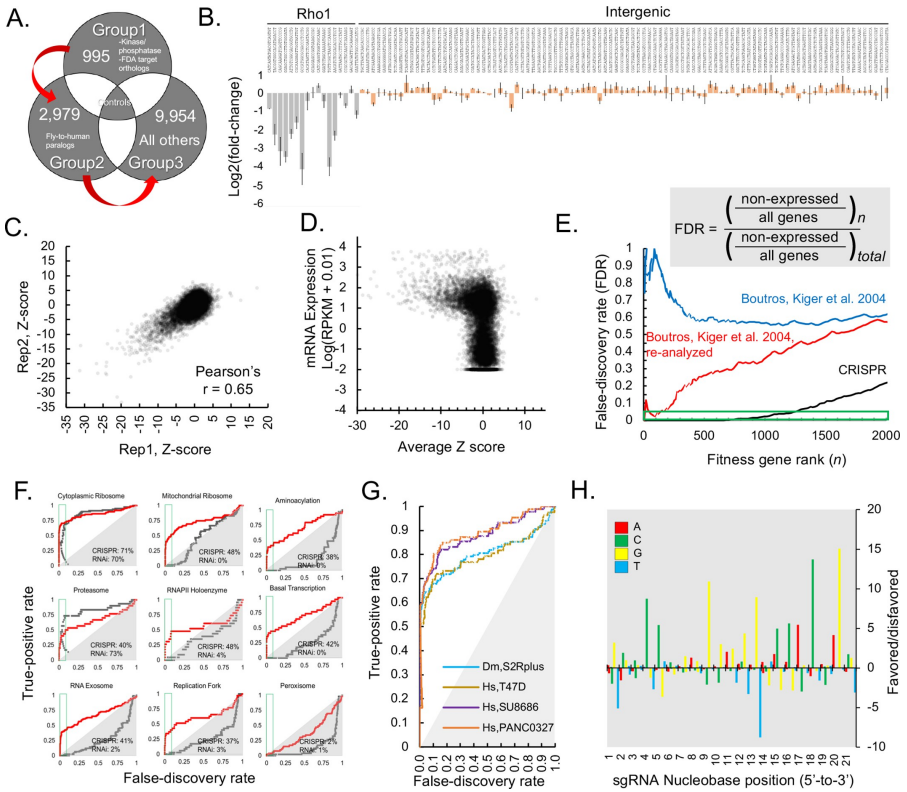
Example use to identify new gene functions:

Okamoto N, Viswanatha R, Bittar R, Li Z, Haga-Yamanaka S, Perrimon N, Yamanaka N. **A Membrane Transporter Is Required for Steroid Hormone Uptake in Drosophila**. *Dev Cell*. 2018 Nov 5;47(3):294-305.e7. PMID: 30293839

Note: Raw data download is available for Viswanatha et al. (2018)

CRISPR pooled-format screens in fly cells:
proof-of-concept screens





Optimization

- Delivery
- Timing

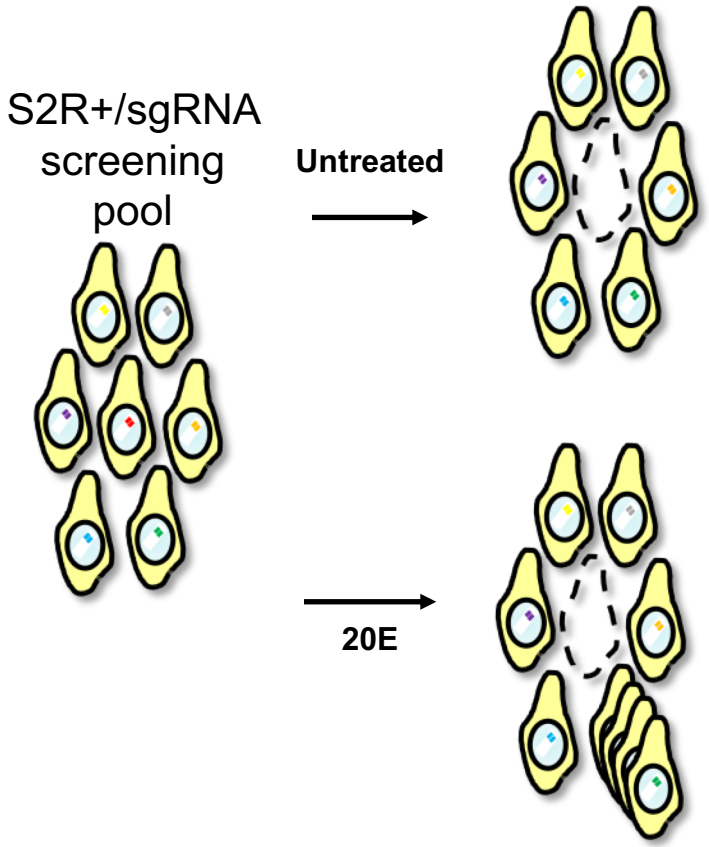
Application

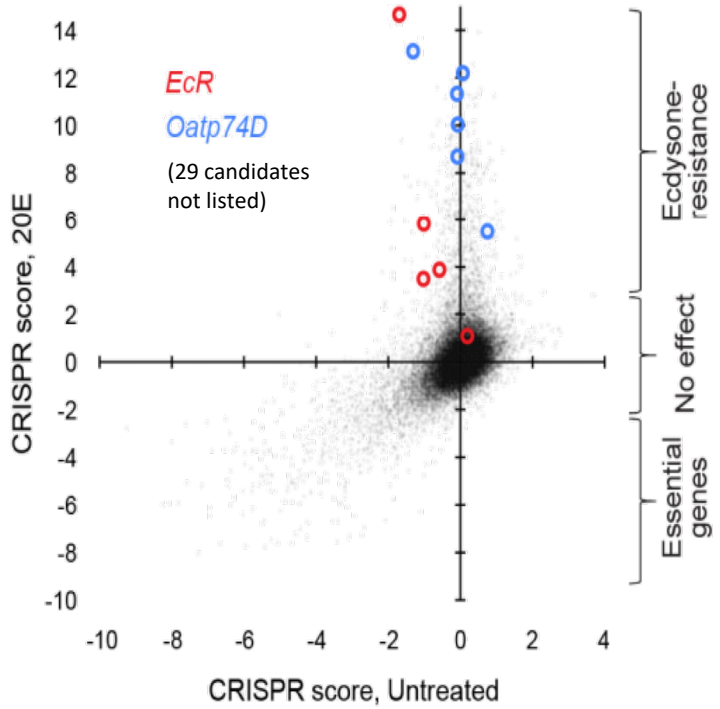
- Large-scale library
- Identify essential genes

[Viswanatha et al. (2018) *eLife*]

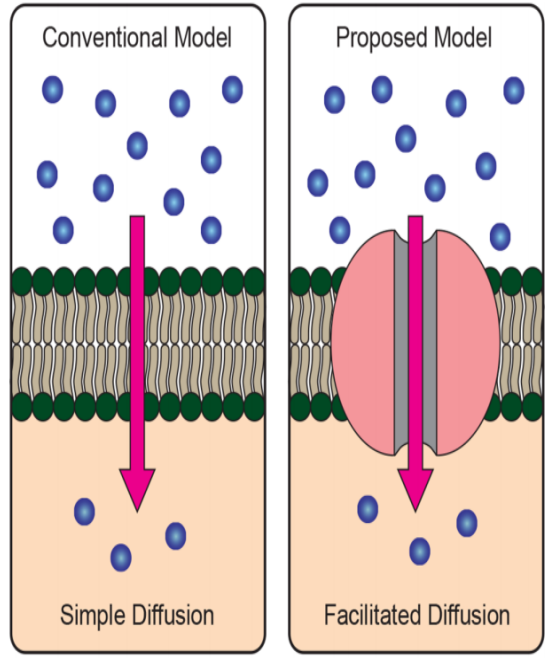
CRISPR pooled-format screen—example selection assay

Screen assay approach: Identify resistance to proliferative block caused by 20E treatment





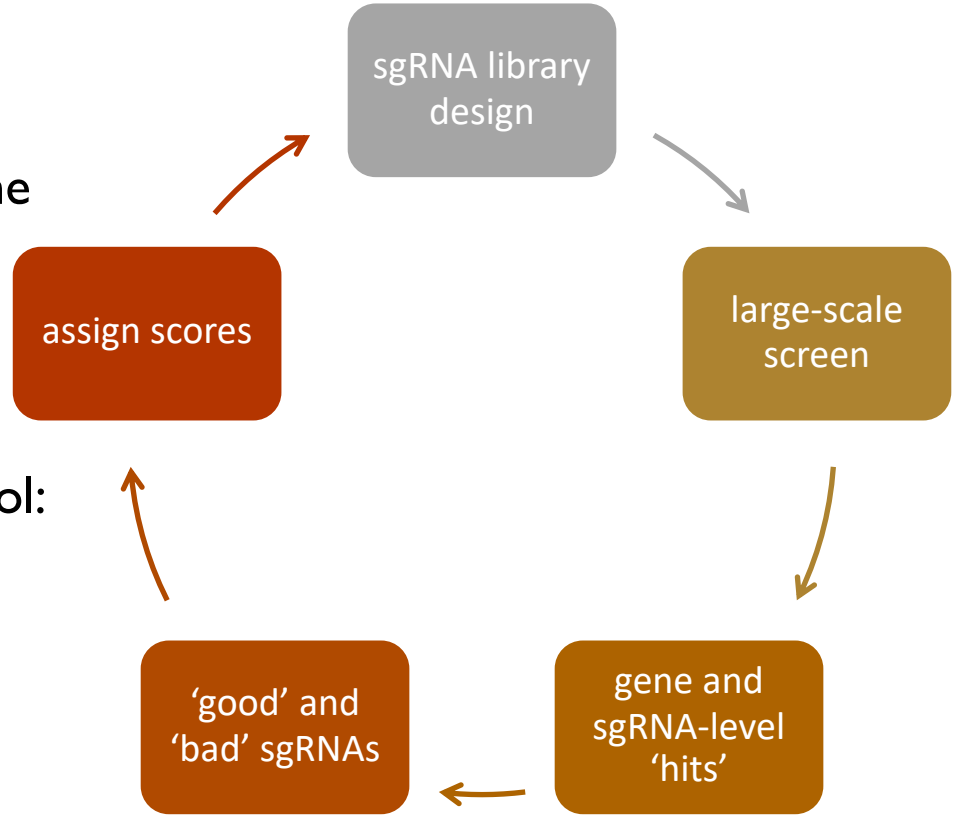
Results led to revised model for entry of ecdysone into cells



Okaoto, Viswanatha et al. (2018) *Dev Cell*

Machine Learning approach to sgRNA design

- Genome-wide library in the Viswanatha et al. pooled CRISPR studies includes ~6 sgRNAs per gene
- Data sets generated in the study provides opportunity to use machine learning to identify new rules, assign scores
- Define hits at gene level, then identify 'good' and 'bad' designs
- ML approach applied using 'good' and 'bad' as training set
- ML-based scores to be added to our Find CRISPRs sgRNA design tool:
  - https://www.flyrnai.org/crispr/
- Follow-up on ML results currently in progress
  - *Testing if ML-assigned scores are predictive of performance*
  - *Expanding the screen library to ~8 sgRNAs per gene coverage*



Virtuous cycle of ML-informed design, experimental testing, and new library development

Merckaert, Hu, Viswanatha, Rodiger, Mohr, Perrimon, unpublished

CRISPR pooled-format screens

How might they be useful to your studies?

Example assays

- Drop-out screen for essential genes (e.g. in new cell lines)
- Drop-out screens in a knockout cell vs. parental cell to identify synthetic lethal genes
  - To uncover genetic networks (e.g. for poorly understood genes)
  - To find potential new drug targets (e.g. starting with tumor suppressor KO cells)
- Select for drug or other cytotoxin resistance
  - To identify transporters or entry factors
  - To study relevant biological process(es)
- Select by transwell migration assay or other physical separation
- Select by flow cytometry (e.g. loss or gain of a fluorescence marker)

Additional applications of the approach

- In other *Drosophila* cell lines (add attP, add Cas9)
- With other Cas9 derivatives/types (e.g. CRISPR activation—pilot screen in progress)
- In other insect cell lines (add attP, add Cas9, design library)
  - e.g. mosquito vectors of disease—assay development in progress

… also in cell lines …

New GFP-tagged cell line resource for:

- Small-scale live or fixed-cell studies
- High-throughput screens, e.g. arrayed-format image-based screens
- Proteomics, e.g. using reagents directed against GFP

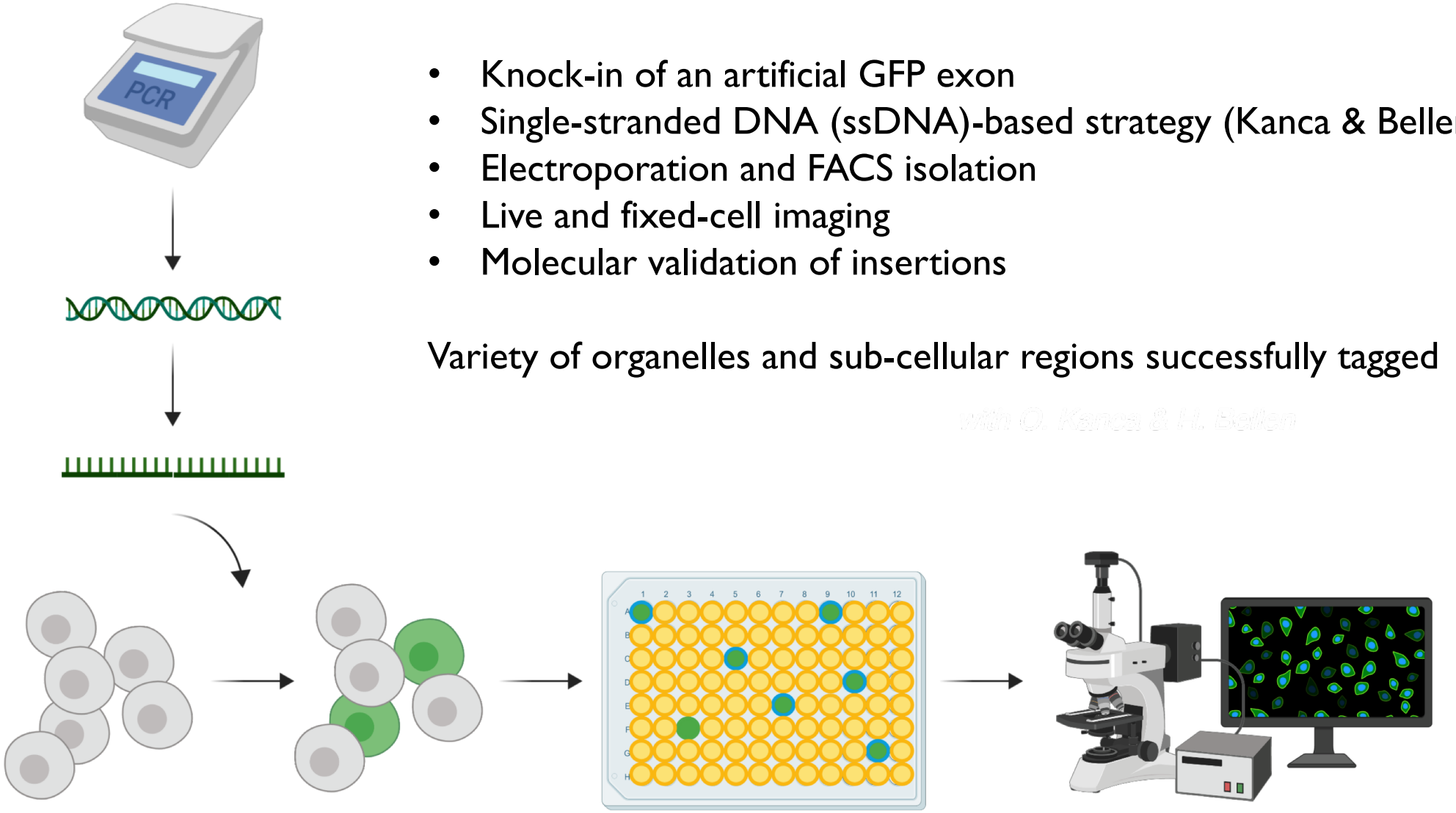
New Resource of GFP-tagged cell lines

- GFP tagging of organelles and sub-cellular compartments
  - Targets based on yeast, mammalian literature
  - ~two dozen candidate proteins identified to 'paint' various cell regions
- Overall workflow improved by switching from plasmid-based donors with long homology arms to single-stranded DNA donors with shorter homology arms (collaboration with Kanca & Bellen)
- GFP-tagged cell line workflow:
  - Parental cell lines is a DRSC S2R+ cell line (mCherry+ Cas9+) (available at DGRC)
  - Electroporated, FACS single-cell isolated, then validated by live imaging, fixed imaging (Ab co-stain), molecular characterization of insertion site
  - Validated cell lines being deposited at DGRC (Bloomington) for distribution, see "S2R+" section on cell lines catalog

Summary info & links to distribution:

https://fgr.hms.harvard.edu/crispr-modified-cell-lines

GFP-tagged cell line resource—workflow summary



- Knock-in of an artificial GFP exon
- Single-stranded DNA (ssDNA)-based strategy (Kanca & Bellen)
- Electroporation and FACS isolation
- Live and fixed-cell imaging
- Molecular validation of insertions

Variety of organelles and sub-cellular regions successfully tagged

with O. Kanca & H. Bellen

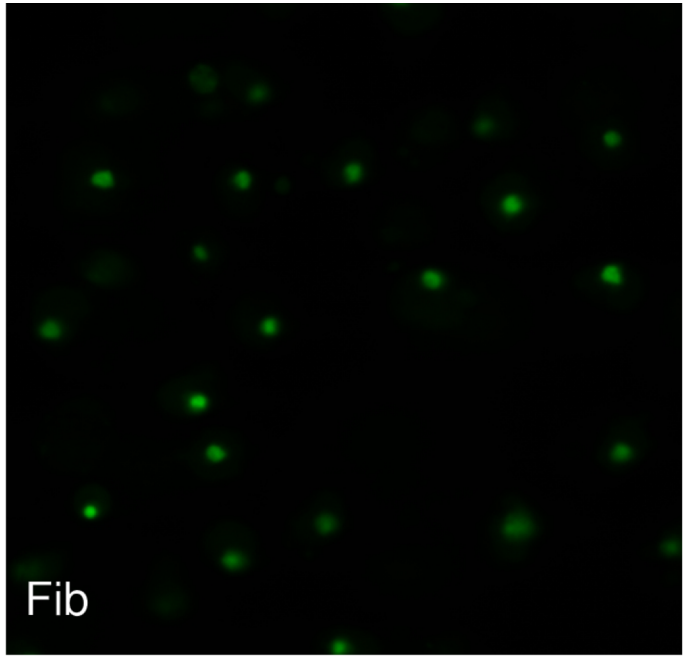
https://fgr.hms.harvard.edu/crispr-modified-cell-lines

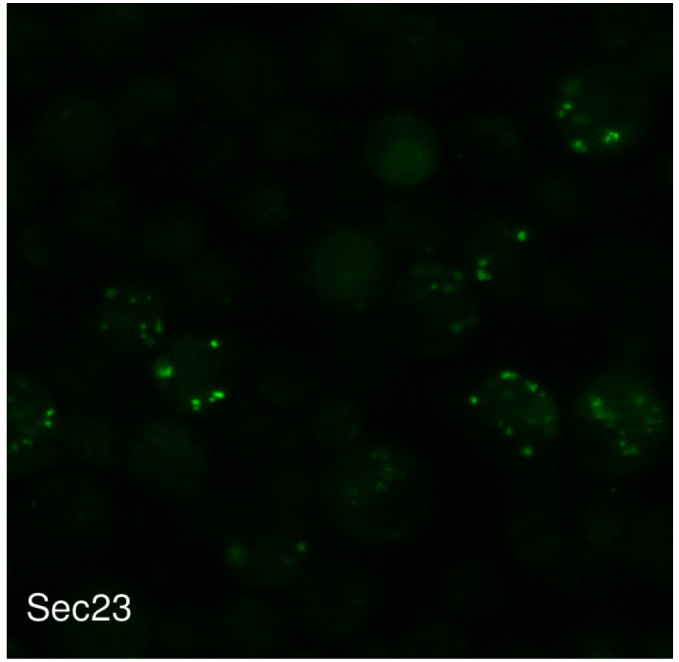
GFP-tagged cell lines—summary

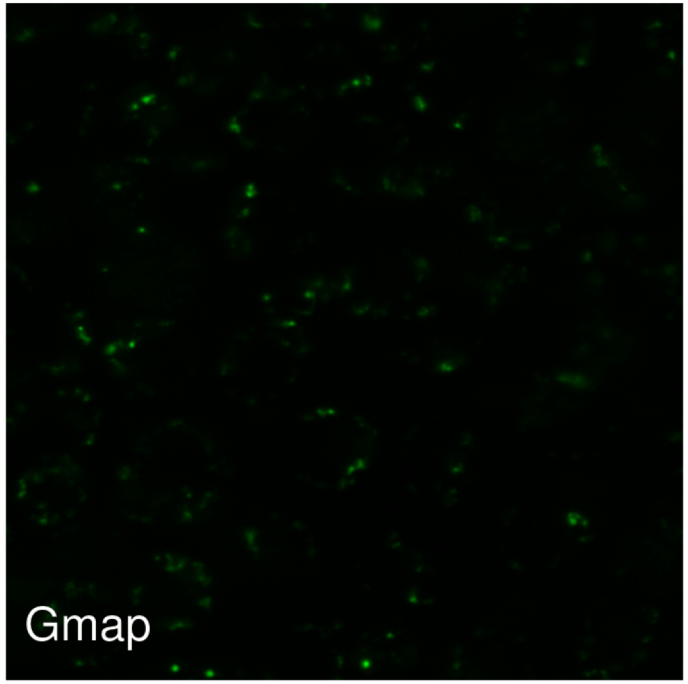
Links to DGRC's cell line catalog (more tagged cell lines are on the way from DRSC to DGRC)

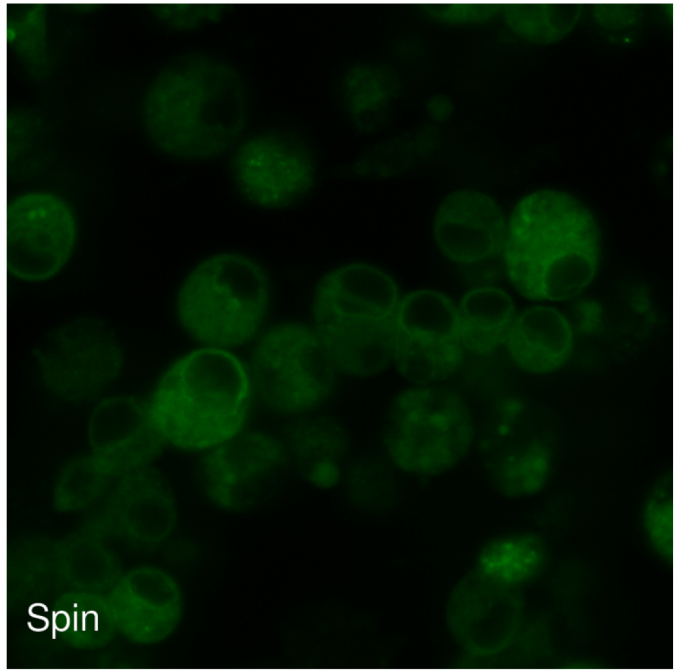
Organelle	GFP Fusion With	clones obtained	population imaged	clones imaged	insertion sequence verified	Immuno-staining	Correct GFP localization	DGRC product ID
Endoplasmic reticulum (ER)	Calnexin99A	16			Y	a-Cnx99a	Y	273
Endoplasmic reticulum (ER), transitional	Sec23	30			Y	--	?	sent
Endosomes, recycling	Rab11	23			Y	--	?	274
Golgi (cis-Golgi)	Gmap	10			Y	a-GMAP	Y	276, 277
Golgi (trans-Golgi)	Golgin245	1			Y	a-Golgin245	Y	280
Kinetochore	Polo	2			Y	a-aTub	Y	275
Lysosomes	spin	2			Y	--	?	sent
Lysosomes	Arl8	9			Y	a-Arl8	Y	sent
Nuclear membrane, inner	Lamin	53			Y	a-Lamin	Y	sent
Nucleolus	Fibrillarin	14			Y	a-Fib	Y	278 & 279

https://fgr.hms.harvard.edu/crispr-modified-cell-lines





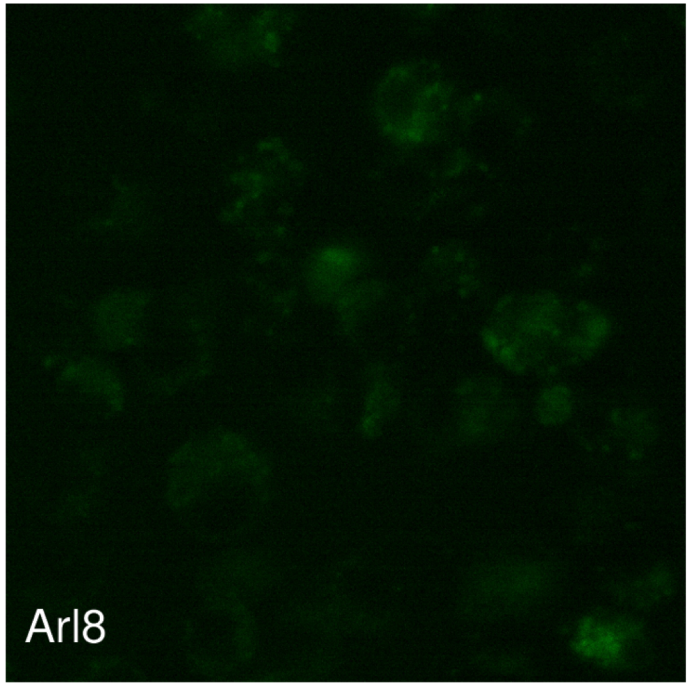


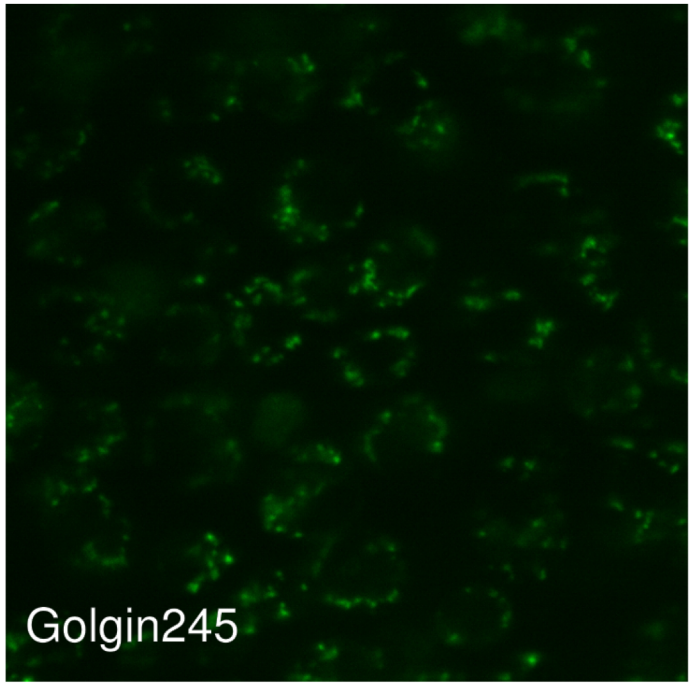


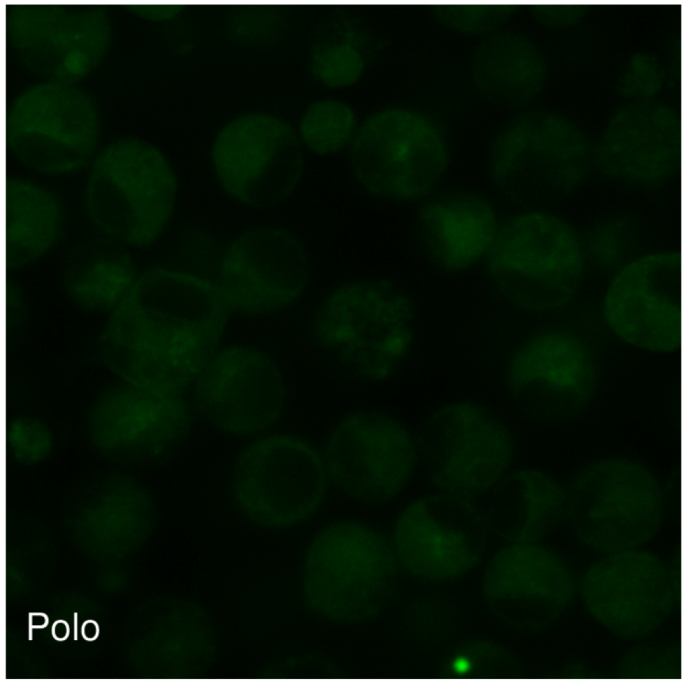
This slide and next—live imaging of GFP-tagged cell lines

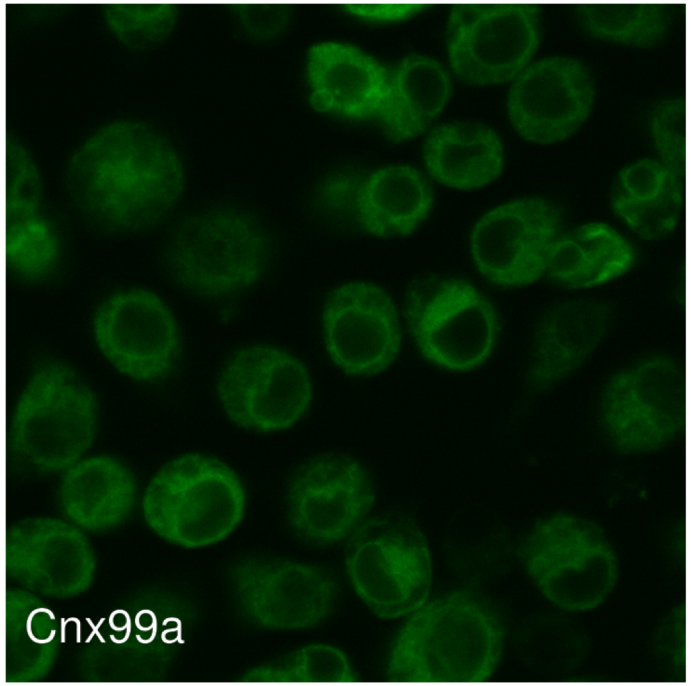
To view a time-lapse movie of Polo::GFP

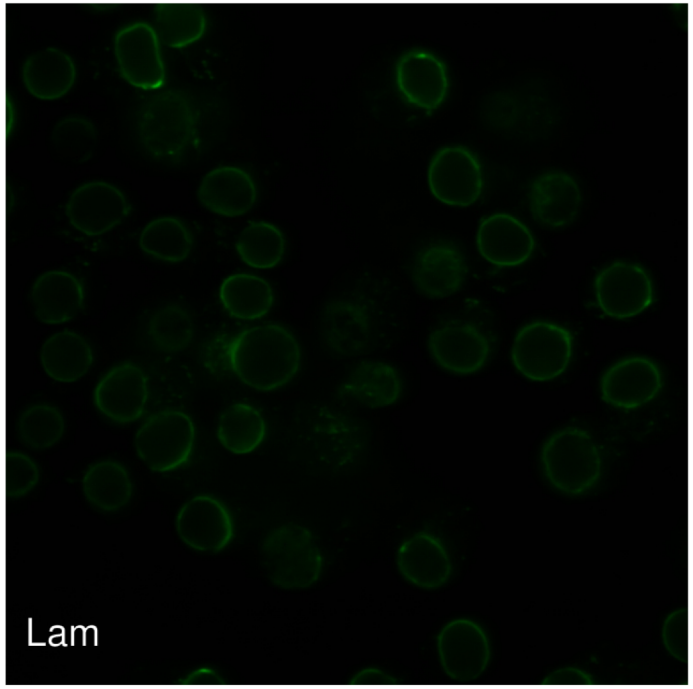
CLICK HERE

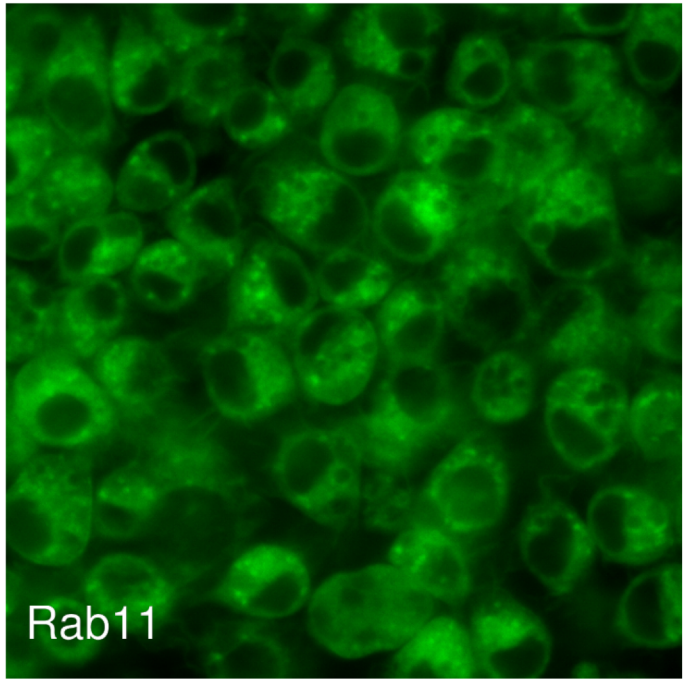

Arl8


Golgin245


Polo


Cnx99a

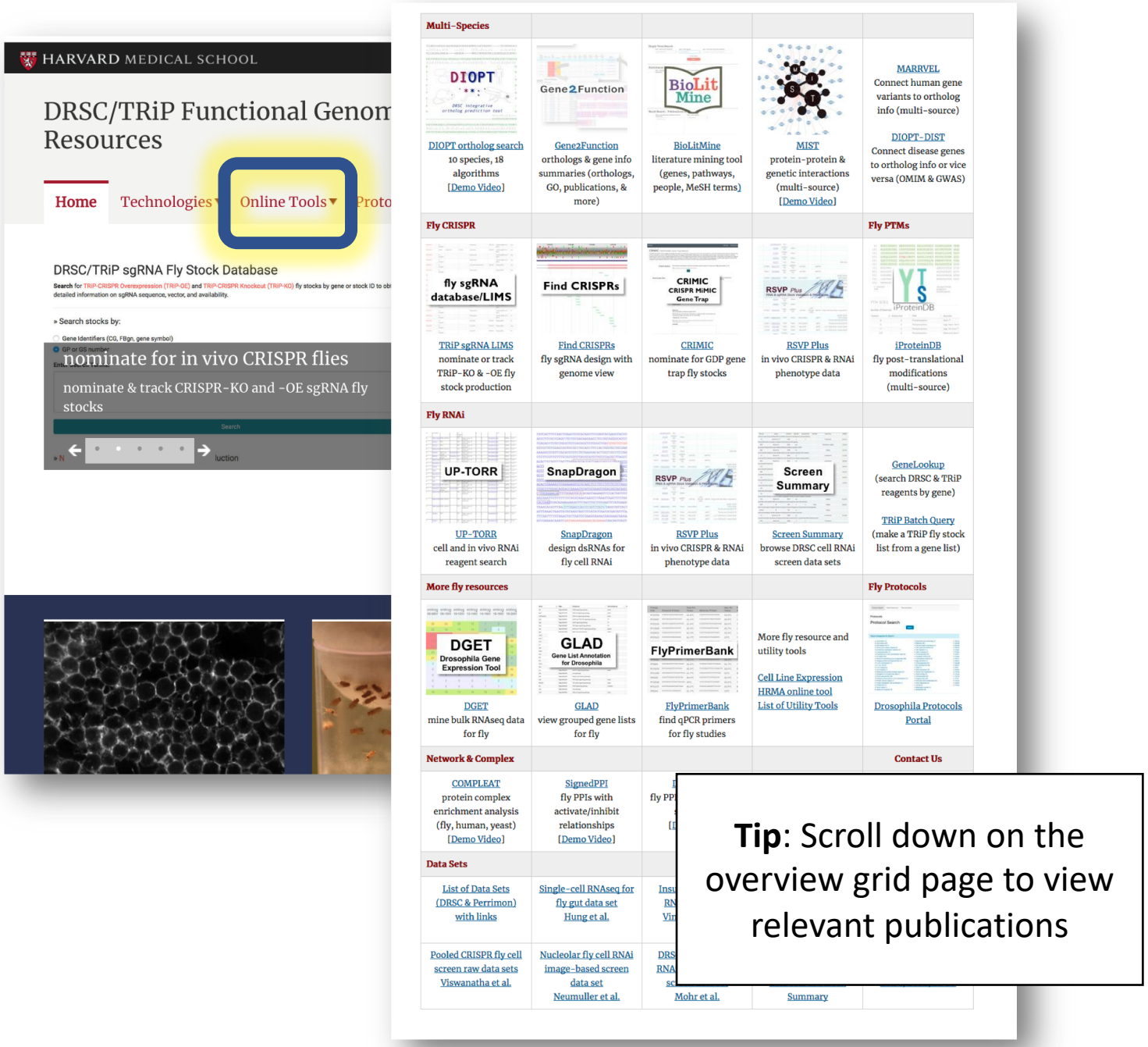

Lam


Rab11

PART 3: bioinformatics resources

New online tool overview page—view our suite of online resources

Brief introduction to newest online resources iProteinDB and BioLitMine



Tip: Scroll down on the overview grid page to view relevant publications

Shown to the left:
New online grid view of DRSC online resources

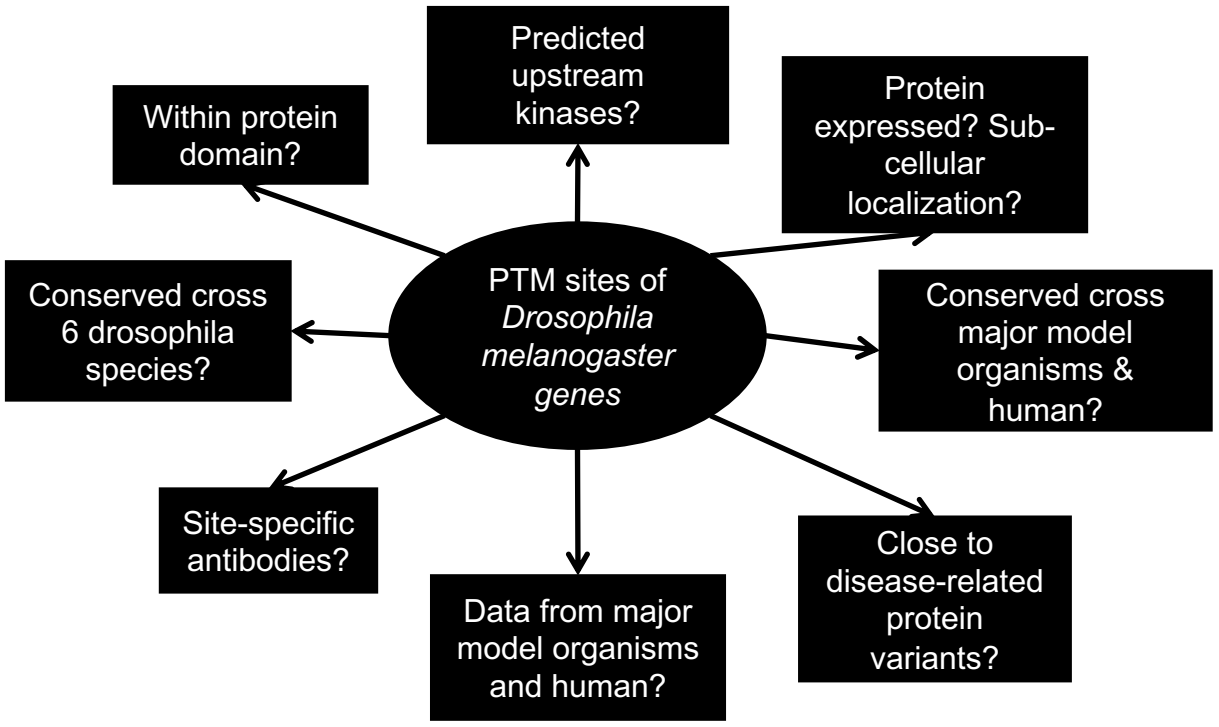
Resources include:
Multi-species resources
Fly-specific resources
Reagent design and info
Data mining and navigation
Large-scale data sets

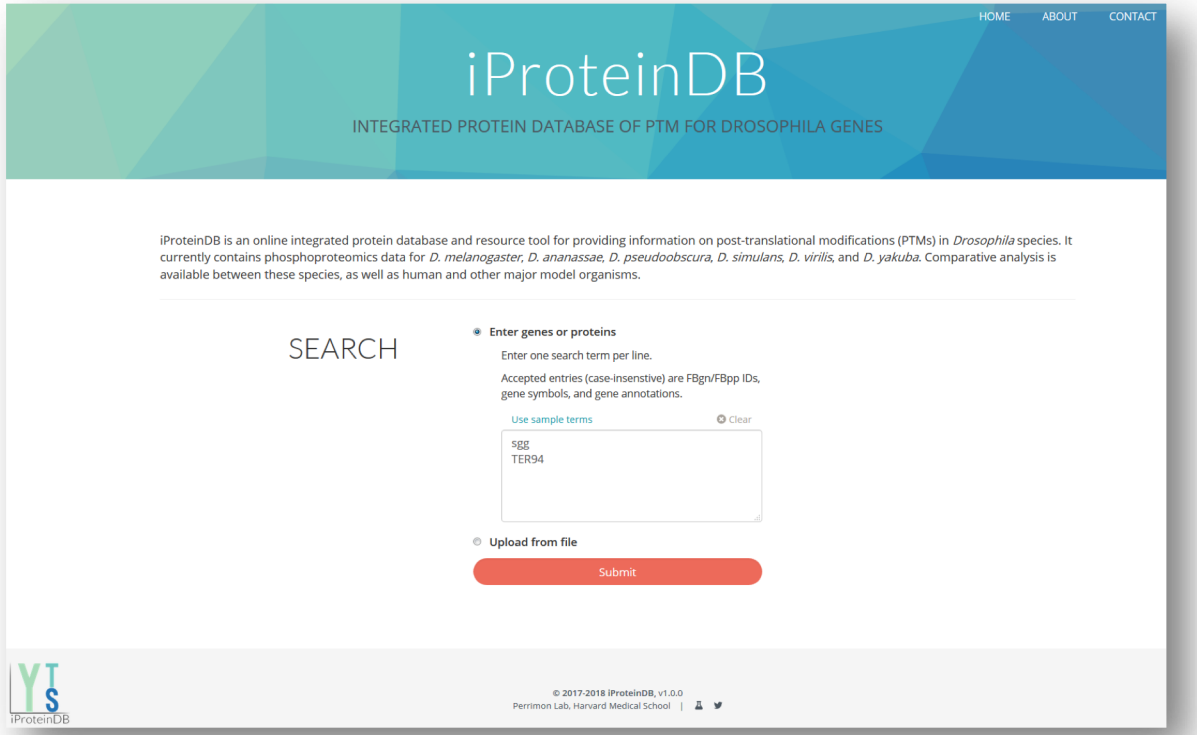
Most popular resource:
DIOPT integrated ortholog search tool

Newest resources:
iProteinDB
BioLitMine

Have you seen?
FlyPrimerBank-–qPCR primer designs
DGET RNAseq search & visualize
Fly Protocols Portal
Gene2Function

iProteinDB: Database of fly post-translational modifications





iProteinDB

Hu et al. *G3*

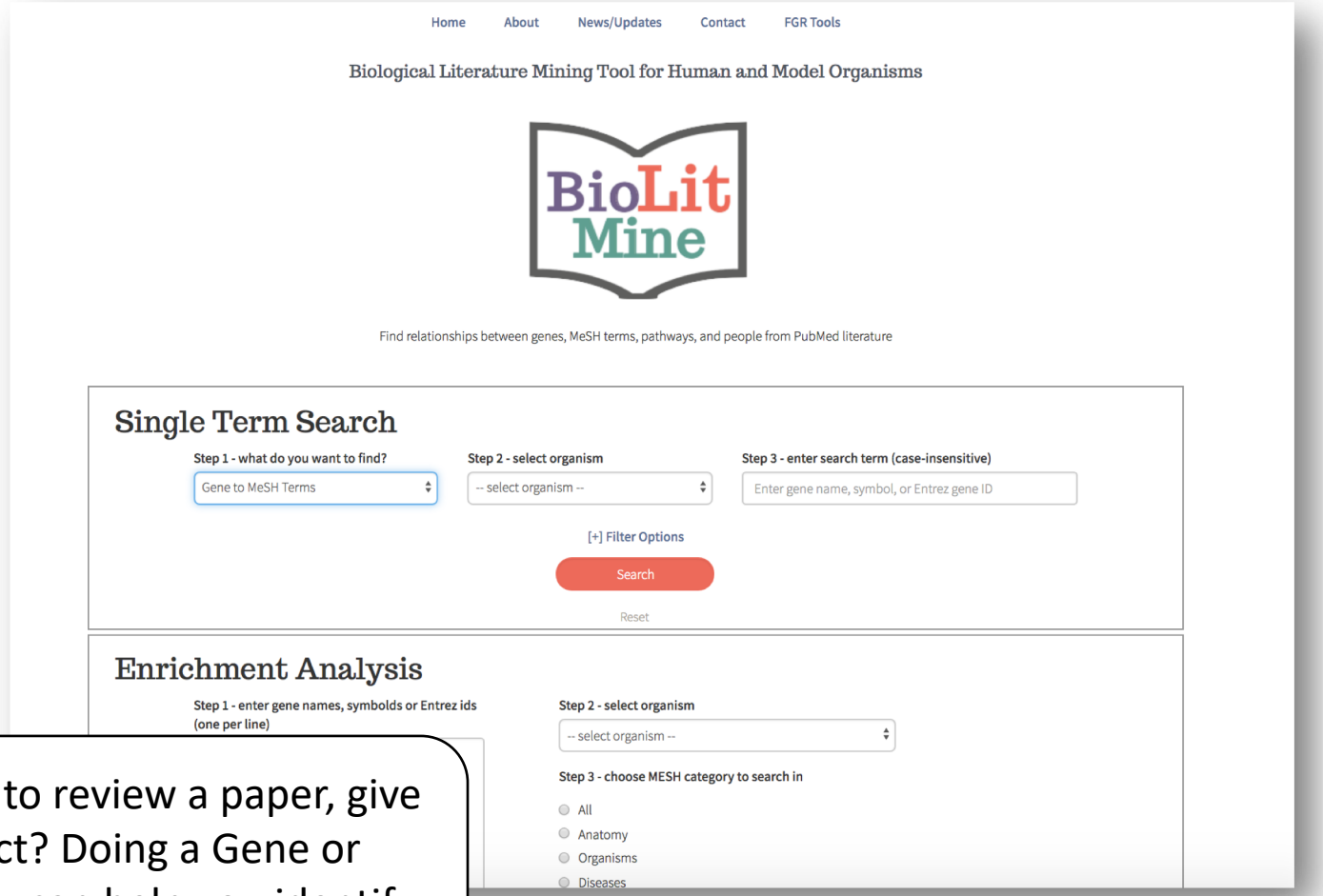
BioLitMine: text mining of PubMed literature

Connect:

Medical Subject Heading term → genes

pathway → genes

etc.



Tip: Looking for experts to review a paper, give a talk, advise on a project? Doing a Gene or Pathway to People query can help you identify experts who've published relevant work.

BioLitMine

Hu et al. unpublished

Keep up-to-date with new resources, technologies, etc.

- Visit the News section of our website
- Follow us on Twitter @DRSC_TRiP

Future directions:

- New emphasis on technology development in partnership with specific projects
- CRISPR activation pooled screening—proof-of-concept studies in process
- Application of CRISPR screen technologies to mosquito cell lines
- New technologies related to proteomics and protein detection
- Updates to the website on these and other topics
- *Your ideas and input are always welcome*

Interested in outreach within and beyond our community?

So are we. Check out for example these resources:

drosophilaresearch.org

flydiseasemodels.blogspot.com

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dsRNA and GFP cell line workflow illustrations drawn using BioRender

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perrimon lab website

We are here to help!
For screen consultation,
new collaborations,
& new projects,
contact:
stephanie_mohr@hms.harvard.edu

NIH NIGMS
NIH ORIP OFFICE OF RESEARCH INFRASTRUCTURE PROGRAMS
hhmi Howard Hughes Medical Institute
DF/HCC Dana-Farber/Harvard Cancer Center
NCI Comprehensive Cancer Center
A Cancer Center Designated by the National Cancer Institute