in vivo CRISPR/Cas9 resources for gene overexpression and knockout

Jonathan Zirin, Harvard Medical School, Boston, MA

Why do we need a CRISPR library?

The "Phenotype Gap" – In FlyBase only 39% of D. melanogaster genes have molecular function information based on experimental evidence or inference from sequence or structural similarities.

Classical and insertion-based mutant alleles are available in stock centers for a similar fraction of sequence-located genes (6,146/14,898) and many of these have not been shown to alter gene function.

RNAi lines cover most of Drosophila protein-coding genes, but….

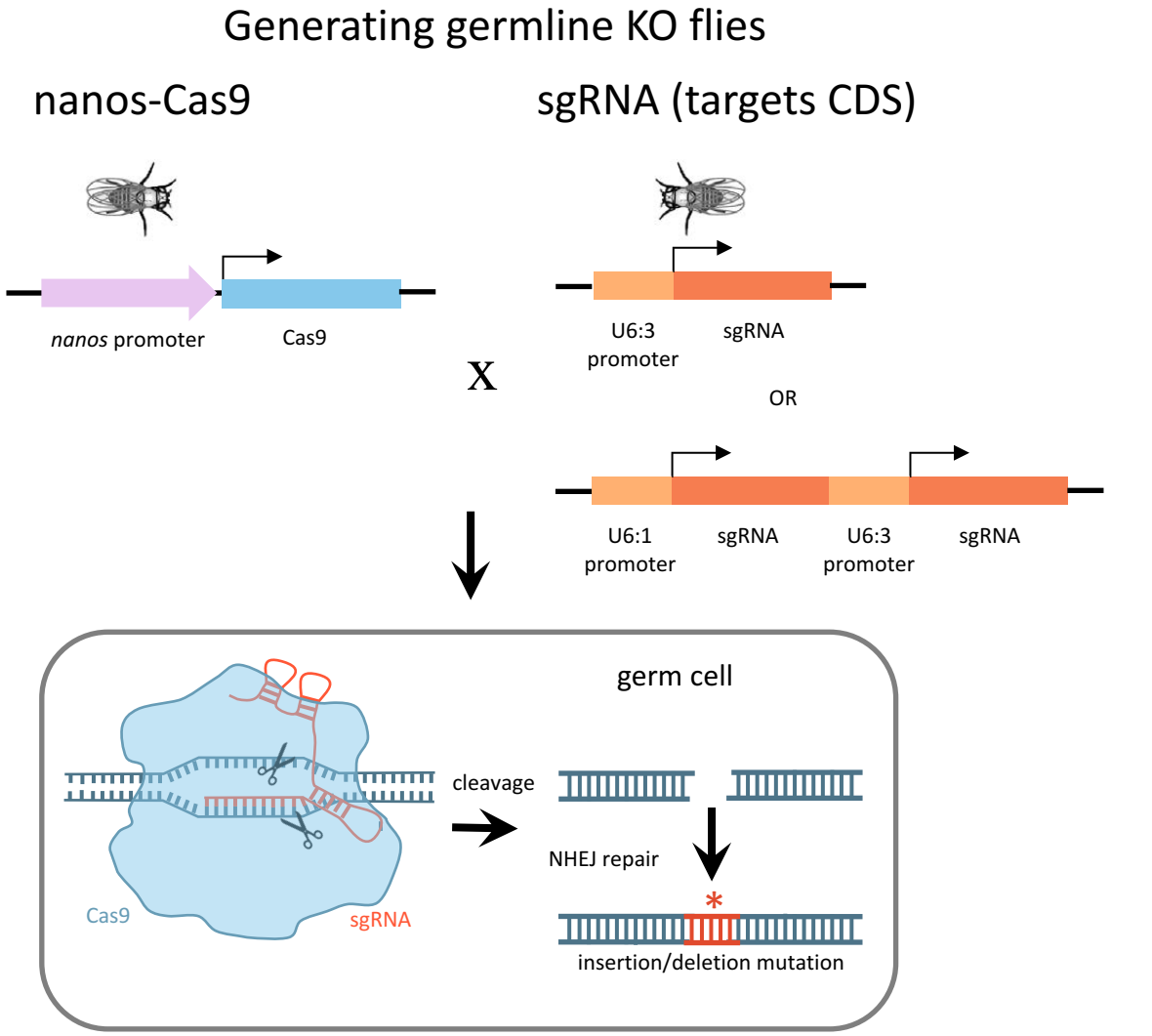
- Significant risks of false positive and false negative results
- Need 2+ RNAi lines per gene to confirm results

CRISPR/Cas9 can help address this Phenotype Gap

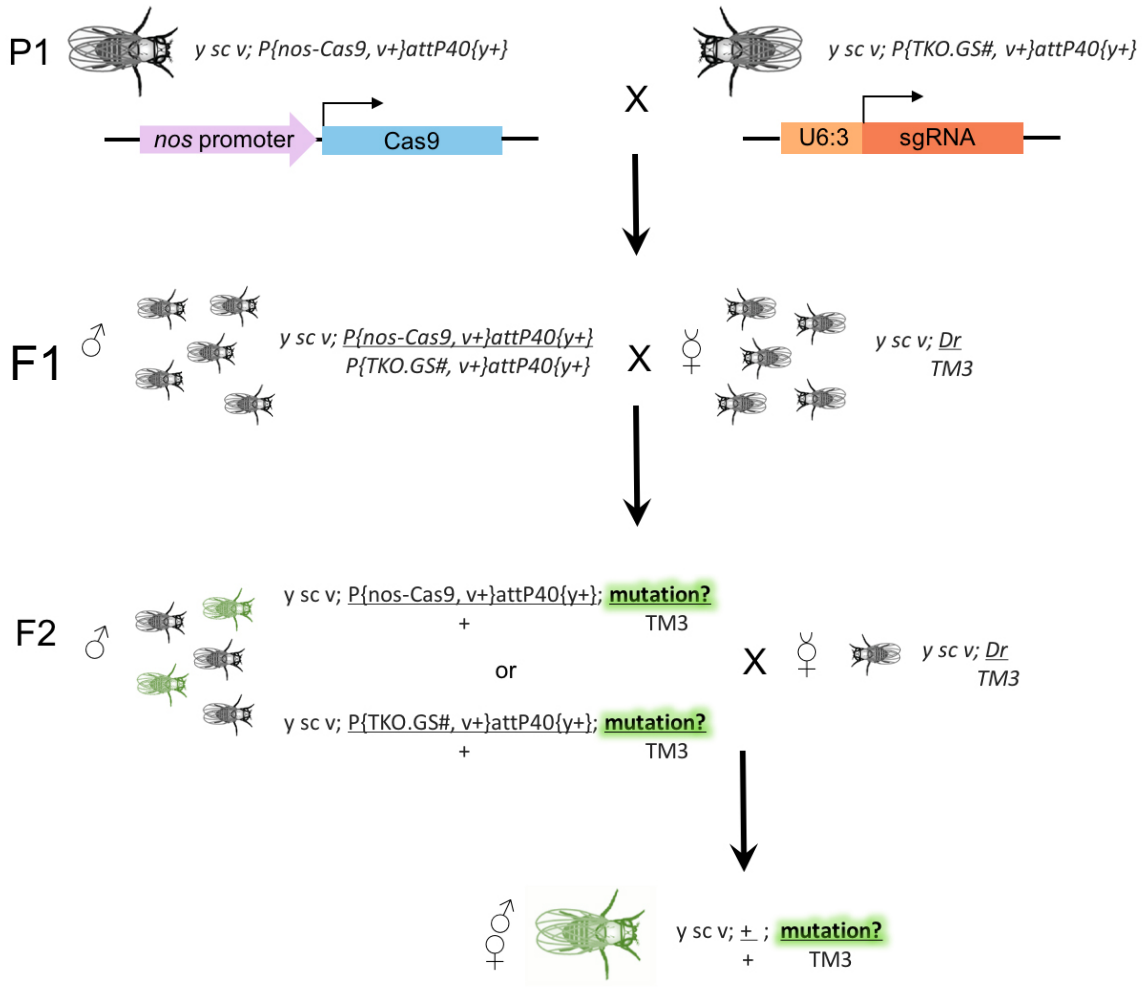
- Used in the germline to generate mutant stocks
- Used in the soma to generate mutant mosaics.
- dCas9 fusions for gene activation
- Genome engineering by homologous repair

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.

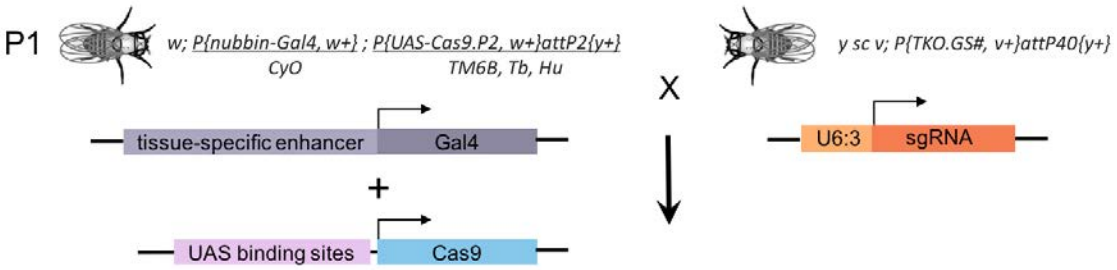
Step 3: cross male F1 progeny en masse to appropriate balancer strain for your target gene

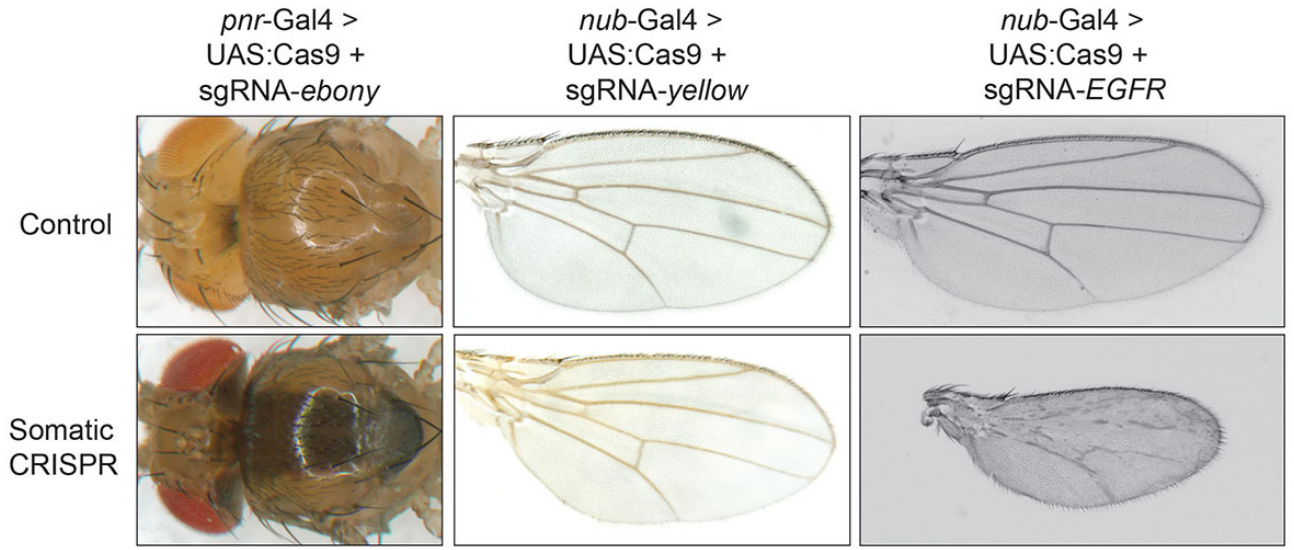
Step 4: collect male or female F2 progeny (some will be heterozygous mutants) and cross each individually to balancer stock.

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

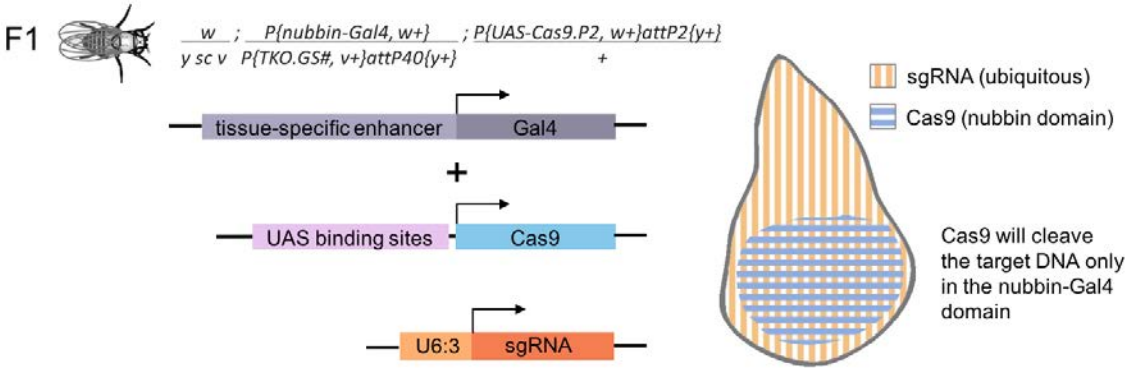
Using TRiP-KO stocks for mosaic KO

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

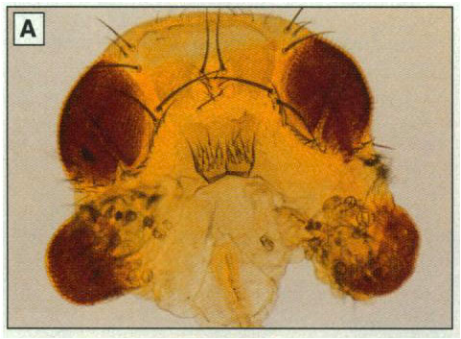
```
CCATTGGCTCAGATTGACGAGGCGCCGGCAACTAAAAGA   WT

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

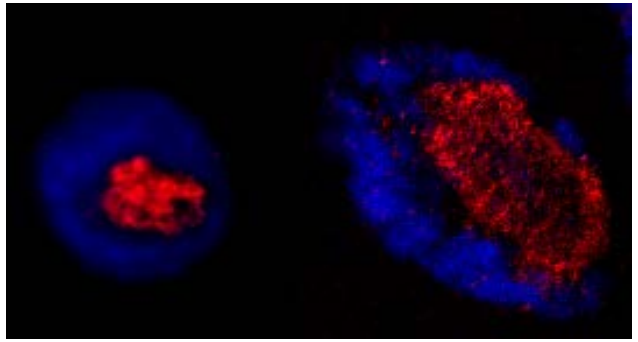
TRiP-CRISPR Overexpression (TRiP-OE)

Why overexpress a gene?

Overexpression of eyeless triggers a regulatory cascade that generates extra eyes



Eyeless overexpression
(Halder et al., 1995)



Myc overexpression
(Zirin and Perrimon, unpublished)

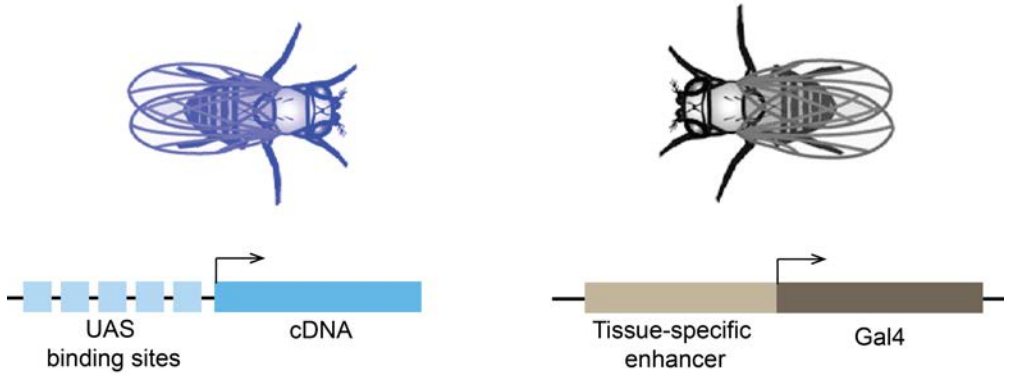
Overexpression of oncogenes the cause of many cancers. Here Myc overexpression causes the nucleolus to overgrow

Overexpression studies are useful for

- Determining the function of redundant genes (paralogs)
- Modifier screens
- Drug target screens

Current tools for over-expression in *Drosophila*

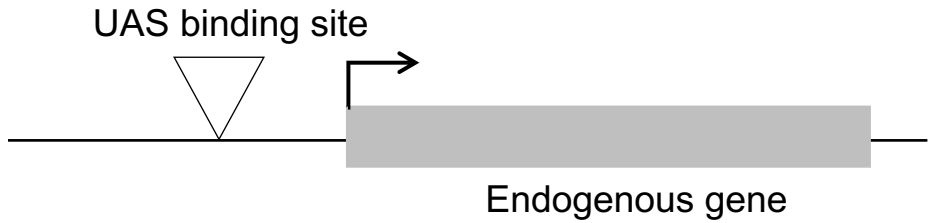
Gal4>UAS-cDNA



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)

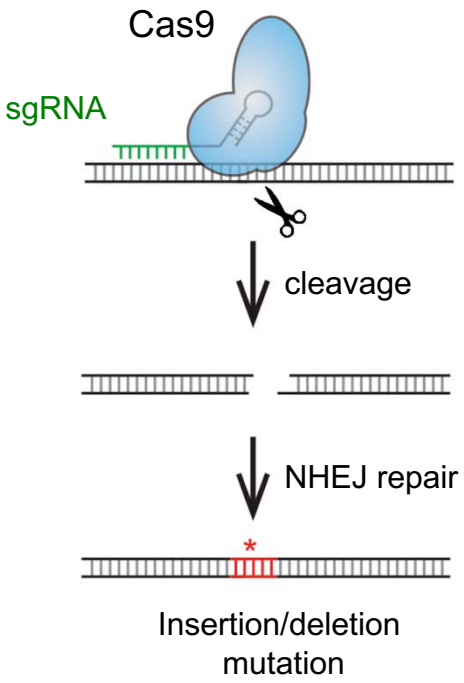


Limitations:

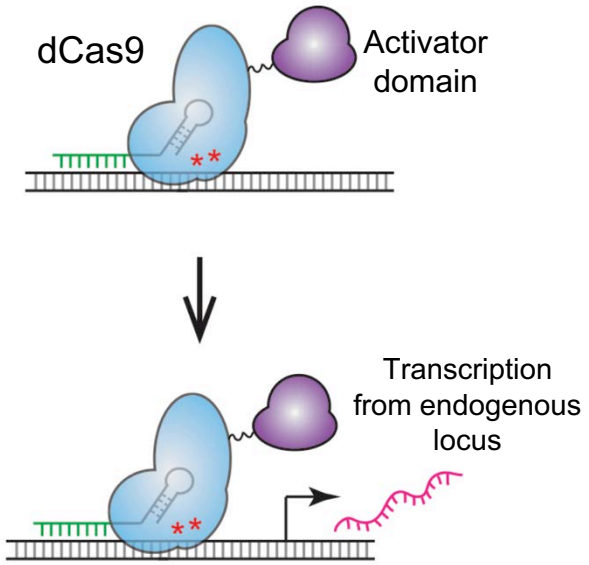
1) Expression is often very weak
2) Random integration
3) Disrupt gene

dCas9-fusions - a new tool for transcriptional activation

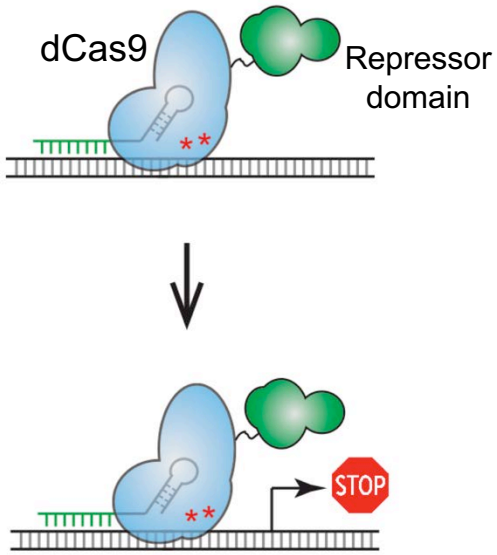
CRISPR



CRISPRa
Transcriptional activation



CRISPRi
Transcriptional repression



Point mutations D10A and H840A eliminate Cas9 endonuclease activity

VPR is a powerful Cas9 activator

Comparison of Cas9 activators in multiple species

Alejandro Chavez, Marcelle Tuttle, Benjamin W Pruitt, Ben Ewen-Campen, Raj Chari, Dmitry Ter-Ovanesyan, Sabina J Haque, Ryan J Cecchi, Emma J K Kowal, Joanna Buchthal, Benjamin E Housden, Norbert Perrimon, James J Collins & George Church

Nature Methods **13**, 563–567 (2016)
doi:10.1038/nmeth.3871
Download Citation
Genetic engineering | Transcription

Received: 29 January 2016
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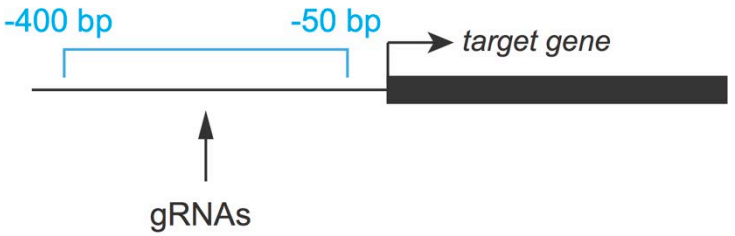
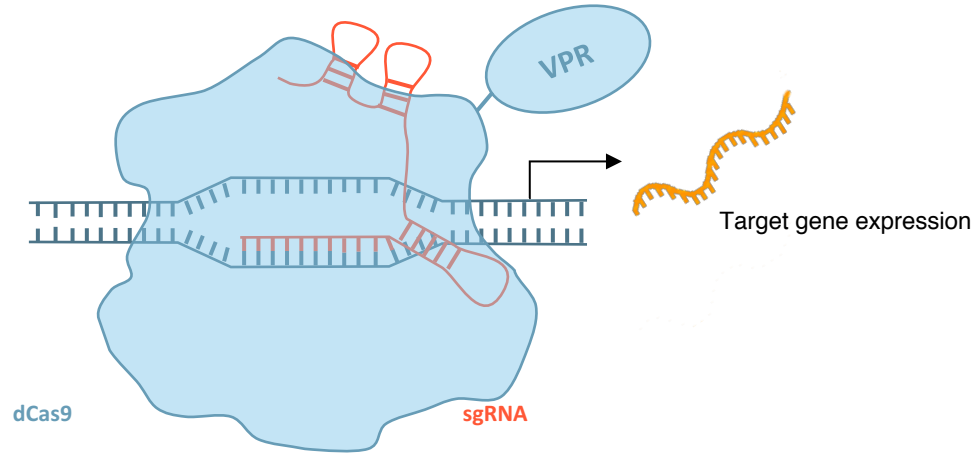
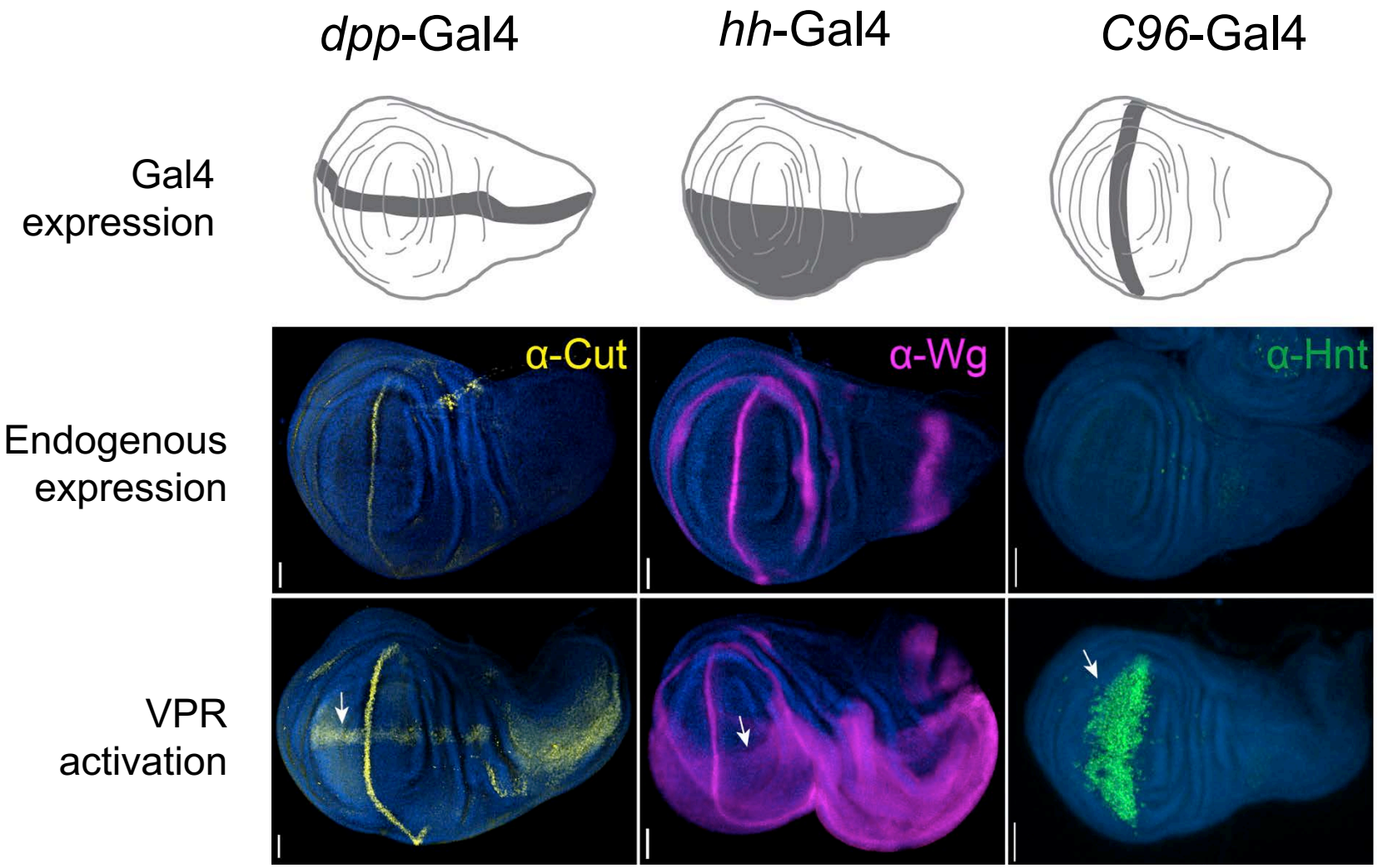
Published: 23 May 2016





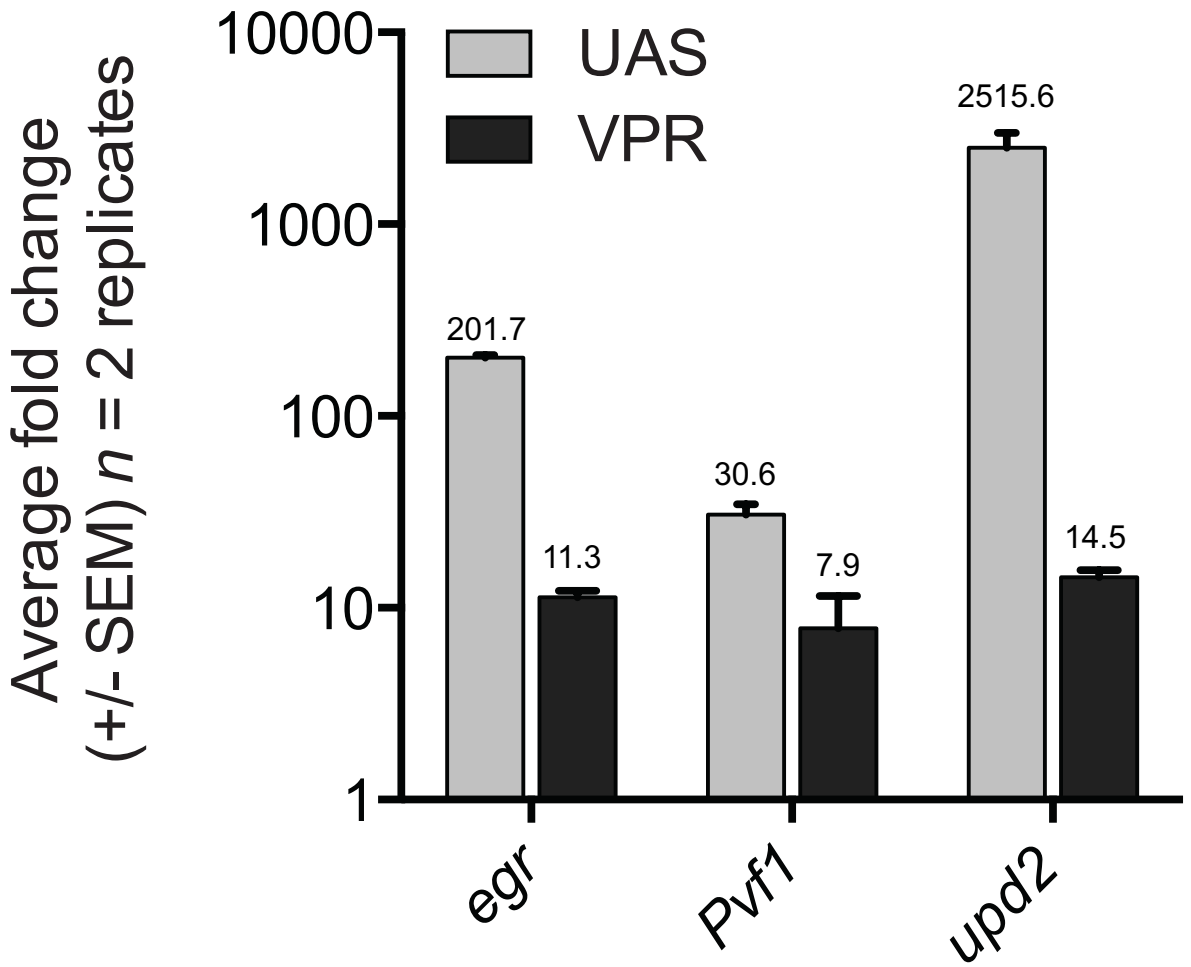
VP64-**p**65-**R**ta (VPR) – a tripartite activator, fused to nuclease-null Cas9

VPR activates a range of target genes *in vivo*



Images from
Ben Ewen-Campen

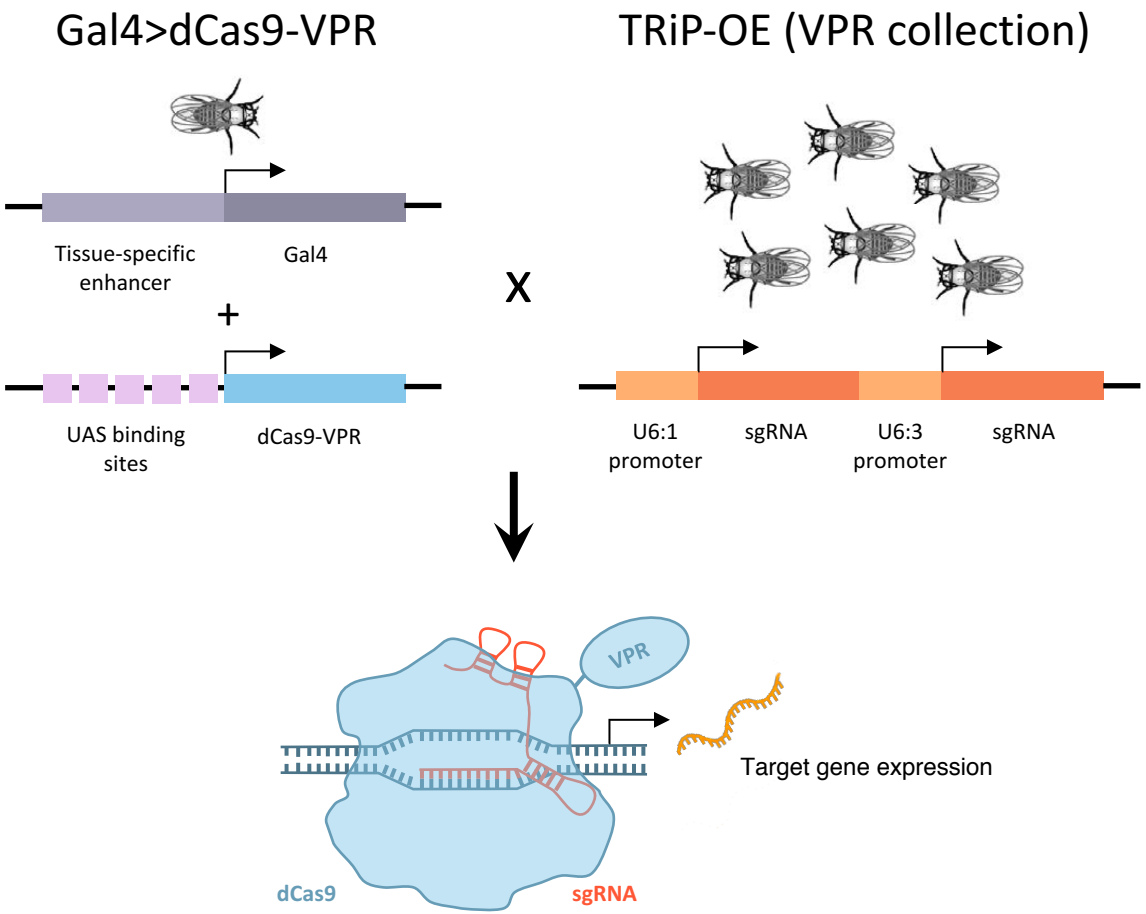
VPR vs UAS



- VPR is weaker than UAS
- Tested activation of 30 genes by VPR in vivo by qPCR
- from this group, almost 80% raised expression over 3-fold, and nearly 70% raised over 8-fold.

Data from Ben Ewen-Campen

The TRiP-OE VPR stock collection

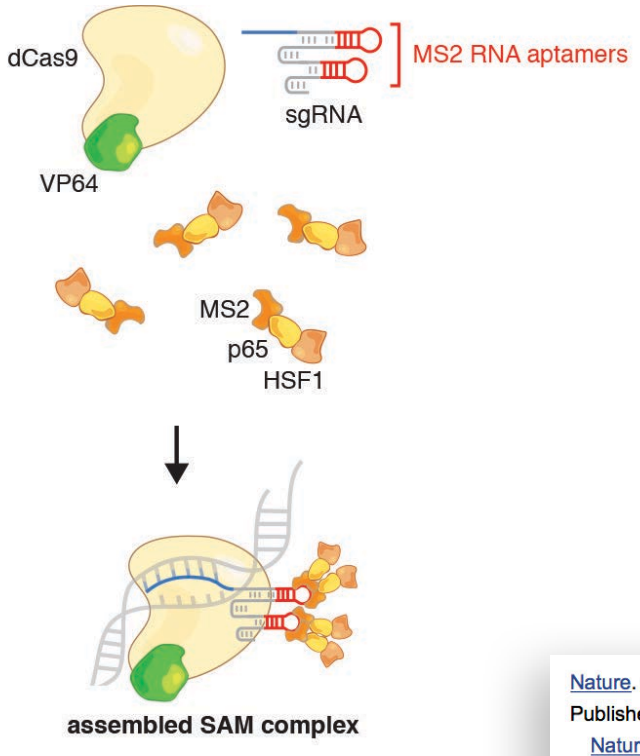


- A single gene is targeted by tandem expression of two sgRNAs from independent U6 promoters
- Stocks are made in the pCFD4 vector, developed by Fillip Port and colleagues
- Crossing TRiP-OE stocks to a Gal4 line expressing dCas9 fused to the chimeric activator domain VPR induces expression of the target 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.

Limitations of the VPR system

1) VPR typically requires two sgRNAs per target gene to reliably achieve consistent transcriptional activation, which greatly increases the cost and complexity of creating a large-scale resource for in vivo CRISPRa
2) Second, because CRISPRa requires three independent transgenes in a single fly (Gal4, UAS:dCas9-VPR, and sgRNA), the usage of this system is not as straightforward as standard Gal4-UAS based tools which only require a single genetic cross.
3) Experiments in Drosophila cell culture suggest that an alternative CRISPRa technique, synergistic activation mediator (SAM), outperforms VPR in direct comparisons. However, previous attempts to express SAM components in vivo have failed due to toxicity.

New OE library production based on SAM



CRISPR/Cas9 Synergistic Activation Mediator (SAM) is an engineered protein complex for the transcriptional activation of endogenous genes.

The SAM complex consists of three components:

- A nucleolytically inactive Cas9-VP64 fusion
- An sgRNA incorporating two MS2 RNA aptamers at the tetraloop and stem-loop
- The MCP-P65-HSF1 activation helper protein

The incorporation of three distinct activation domains - VP64, P65 and HSF1 – into the complex aids robust transcriptional activation through synergy.

Nature. Author manuscript; available in PMC 2015 May 5.
Published in final edited form as:
Nature. 2015 Jan 29; 517(7536): 583–588.
Published online 2014 Dec 10. doi: 10.1038/nature14136

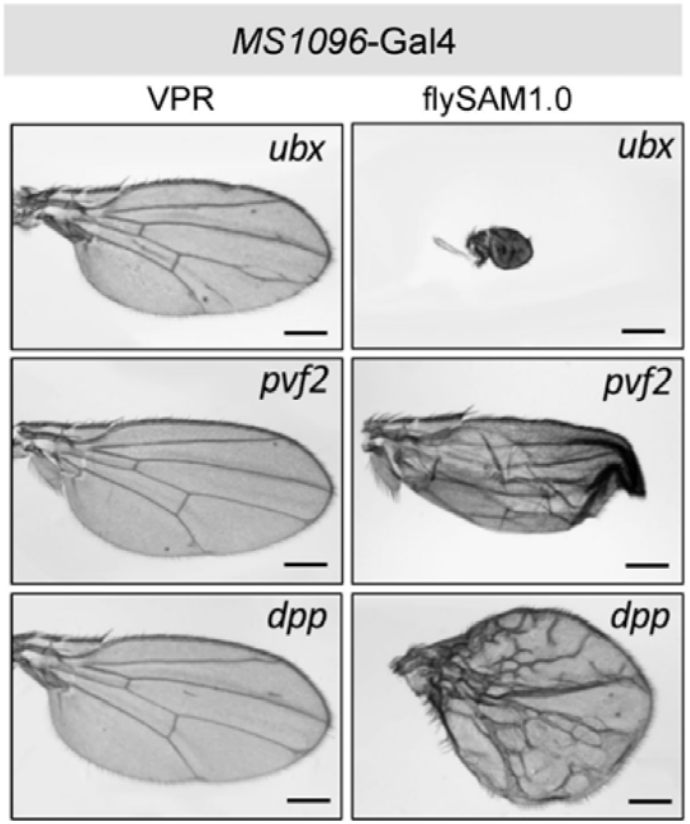
PMCID: PMC4420636
NIHMSID: NIHMS681876
PMID: 25494202

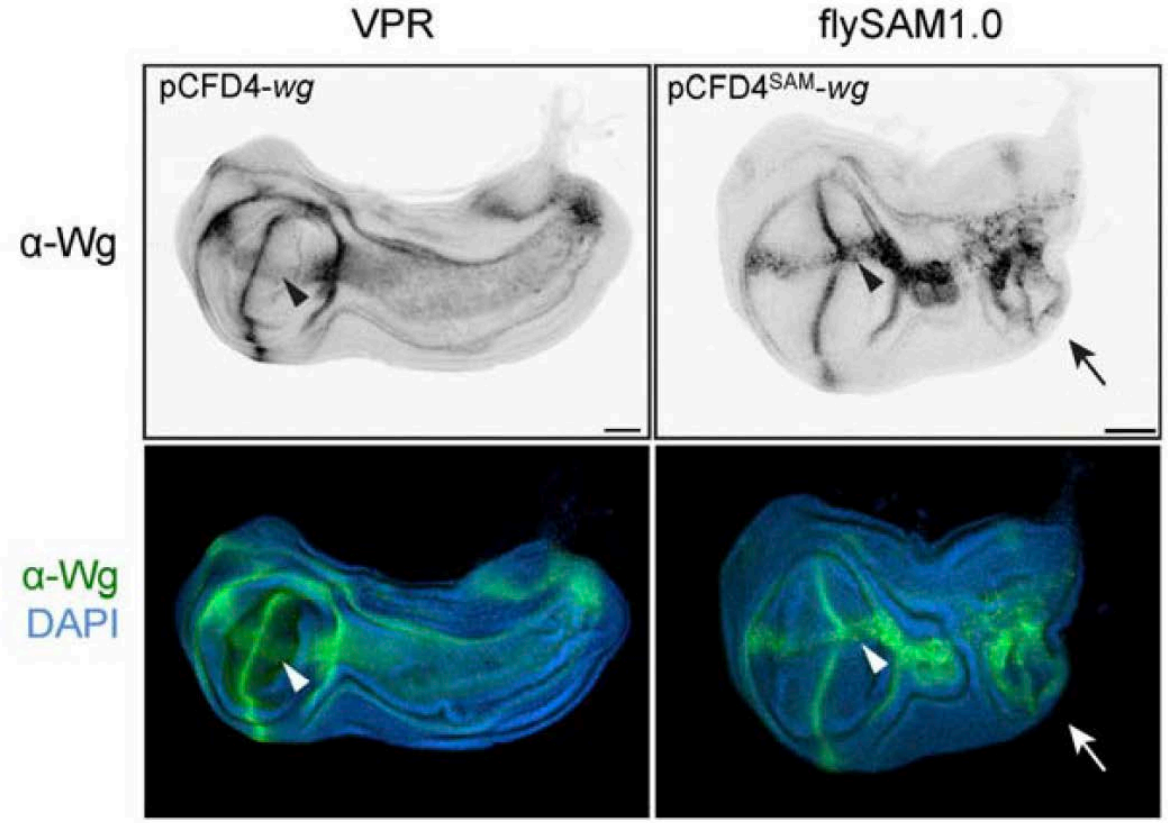
Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex

Silvana Konermann,[1,2,3,4,*] Mark D. Brigham,[1,2,3,4,*] Alexandro E. Trevino,[1,2,3,4] Julia Joung,[1,4] Omar O. Abudayyeh,[1,2,3,4] Clea Barcena,[1,2,3,4] Patrick D. Hsu,[1,2,3,4] Naomi Habib,[1] Jonathan S. Gootenberg,[1,2,3,4,5] Hiroshi Nishimasu,[6,7] Osamu Nureki,[6] and Feng Zhang[1,2,3,4,†]

flySAM1.0

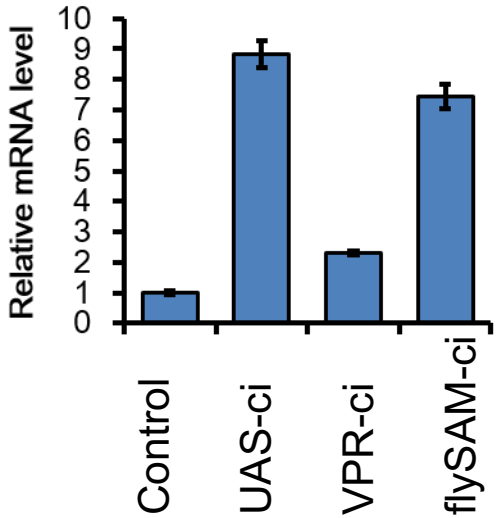
expresses the SAM components dCas9-VP64 and MCP-p65-HSF1 separated by a T2A self-cleaving peptide, under 10XUAS control

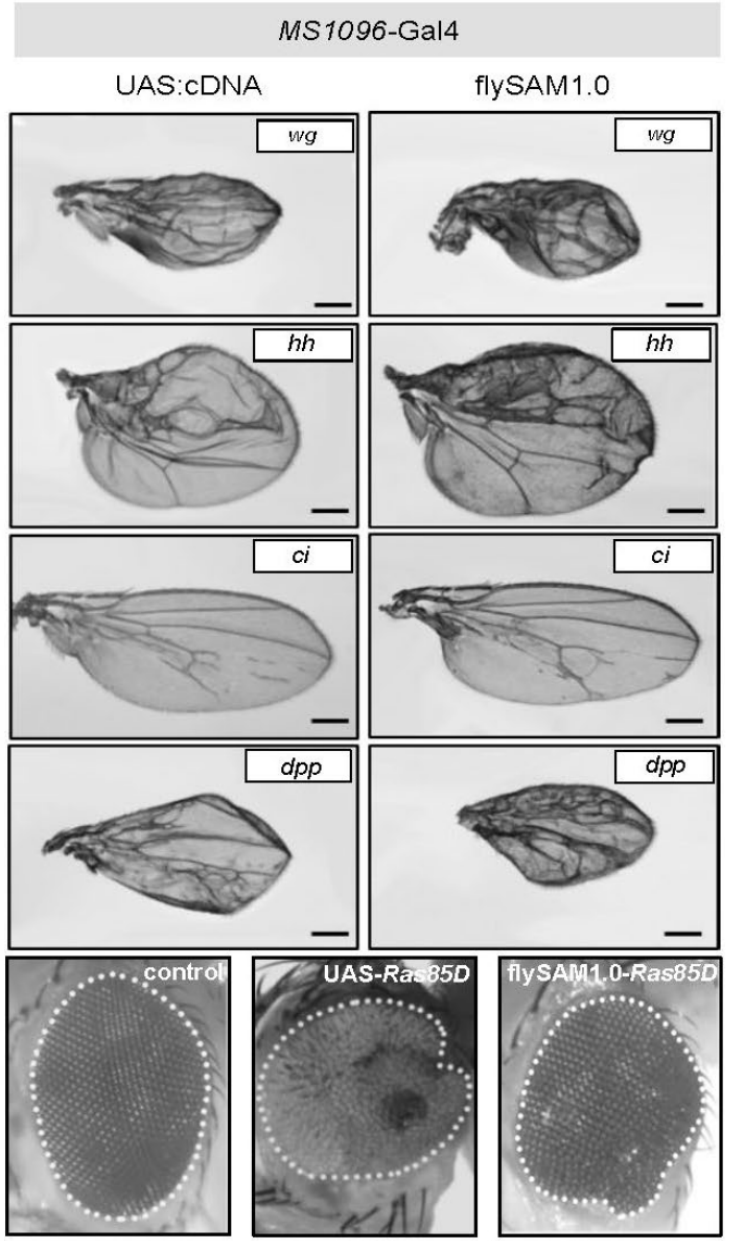




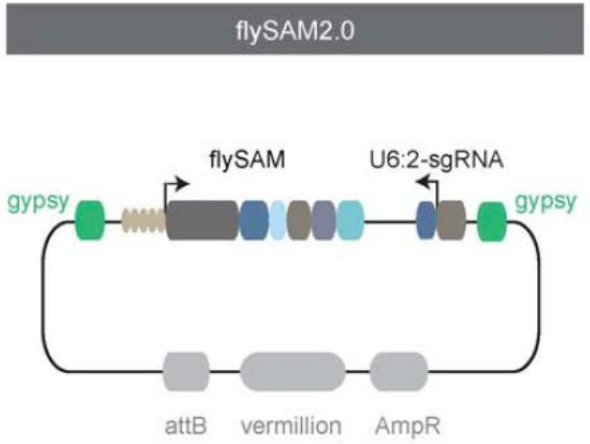
flySAM vs UAS

flySAM phenotypes recapitulate Gal4-UAS over-expression phenotypes.

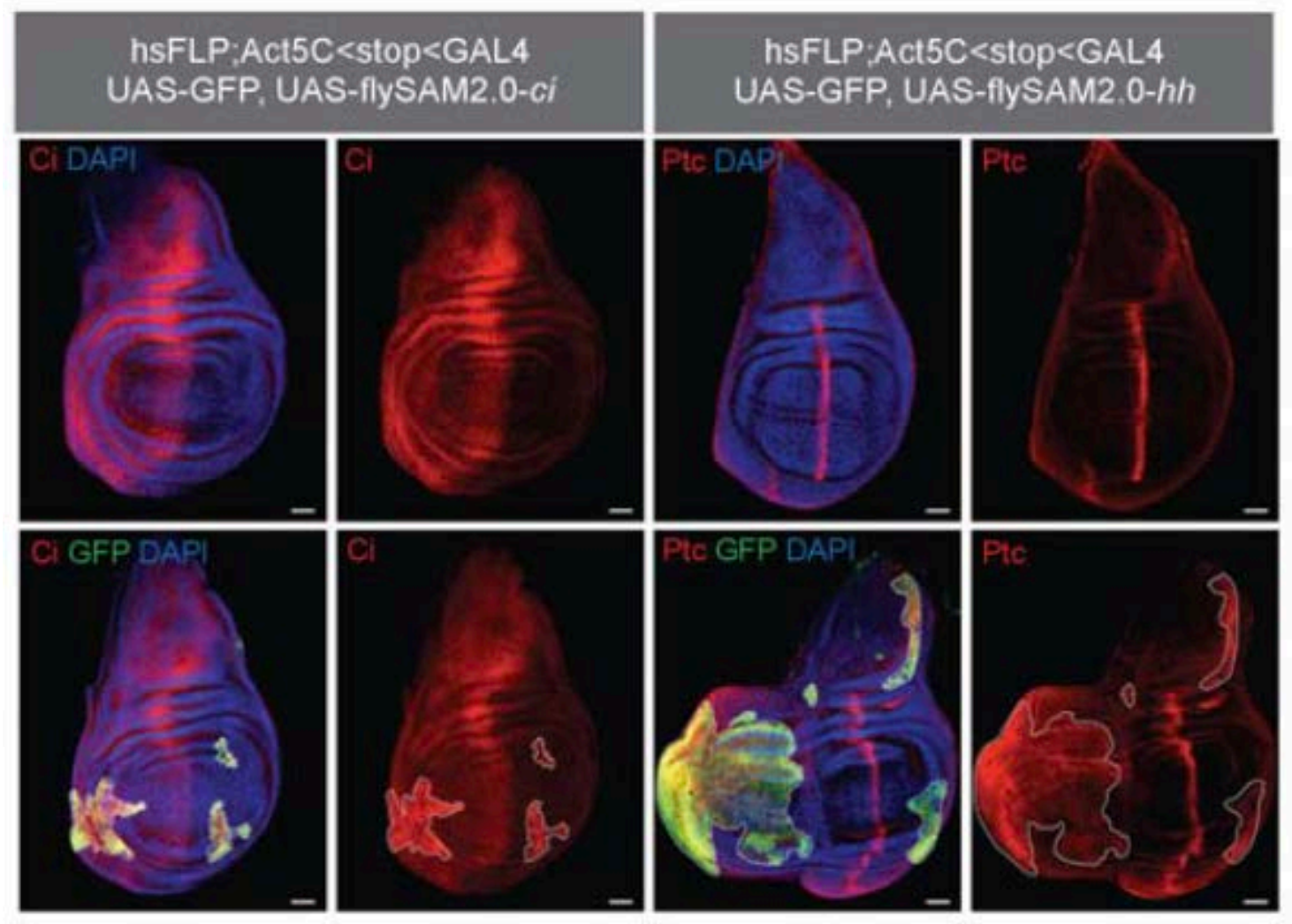




flySAM2.0 contains both the UAS:flySAM and the sgRNA in a single plasmid



Clonal CRISPRa using FLPout Gal4 > flySAM2.0 for ci and hh in L3 larval wing discs



For more information on CRISPRa in flies check out these publications

Cold Spring Harbor Laboratory

bioRxiv

THE PREPRINT SERVER FOR BIOLOGY

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New Results

Next generation CRISPR/Cas9 transcriptional activation in *Drosophila* using flySAM

Yu Jia, Rong-Gang Xu, Xingjie Ren, Ben Ewen-Campen, Rajendhran Rajakumar, Jonathan Zirin, Donghui Yang-Zhou, Ruibao Zhao, Fang Wang, Decai Mao, Ping Peng, Huan-Huan Qiao, Xia Wang, Lu-Ping Liu, Bowen Xu, Jun-Yuan Ji, Qingfei Lu, Jin Sun, Norbert Perrimon, Jian-Quan Ni

doi: https://doi.org/10.1101/252031

This article is a preprint and has not been peer-reviewed [what does this mean?].

Abstract | Info/History | Metrics | Supplementary material | Preview PDF

Optimized strategy for in vivo Cas9-activation in *Drosophila*

Ben Ewen-Campen, Donghui Yang-Zhou, Vitória R. Fernandes, Delfina P. González, Lu-Ping Liu, Rong Tao, Xingjie Ren, Jin Sun, Yanhui Hu, Jonathan Zirin, Stephanie E. Mohr, Jian-Quan Ni and Norbert Perrimon

PNAS August 29, 2017. 114 (35) 9409-9414; published ahead of print August 14, 2017.
https://doi.org/10.1073/pnas.1707635114

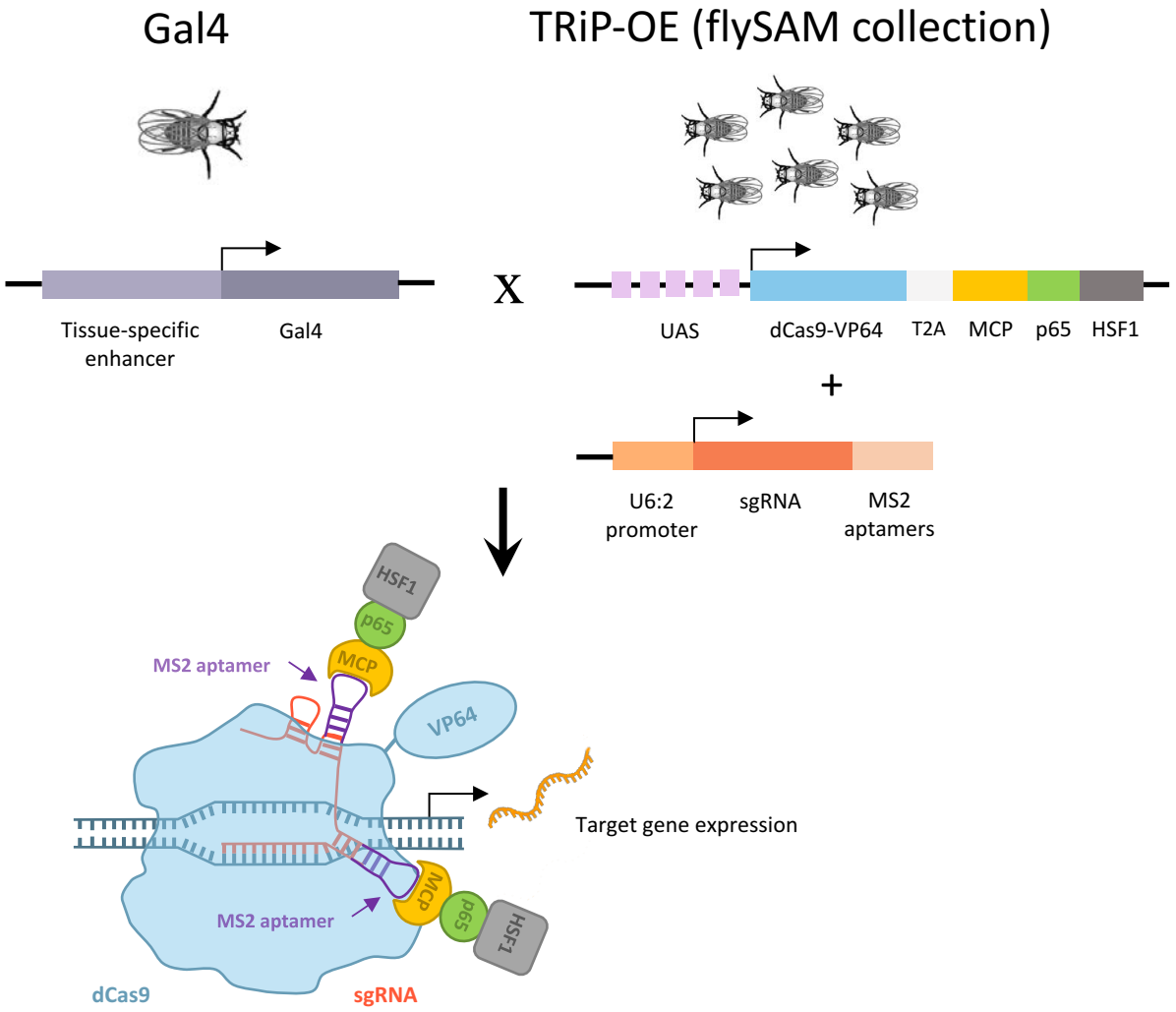
In Vivo* Transcriptional Activation Using CRISPR/Cas9 in *Drosophila

Shuailiang Lin, Ben Ewen-Campen, Xiaochun Ni, Benjamin E. Housden and Norbert Perrimon

GENETICS *October 1, 2015 vol. 201 no. 2 433-442;*
https://doi.org/10.1534/genetics.115.181065

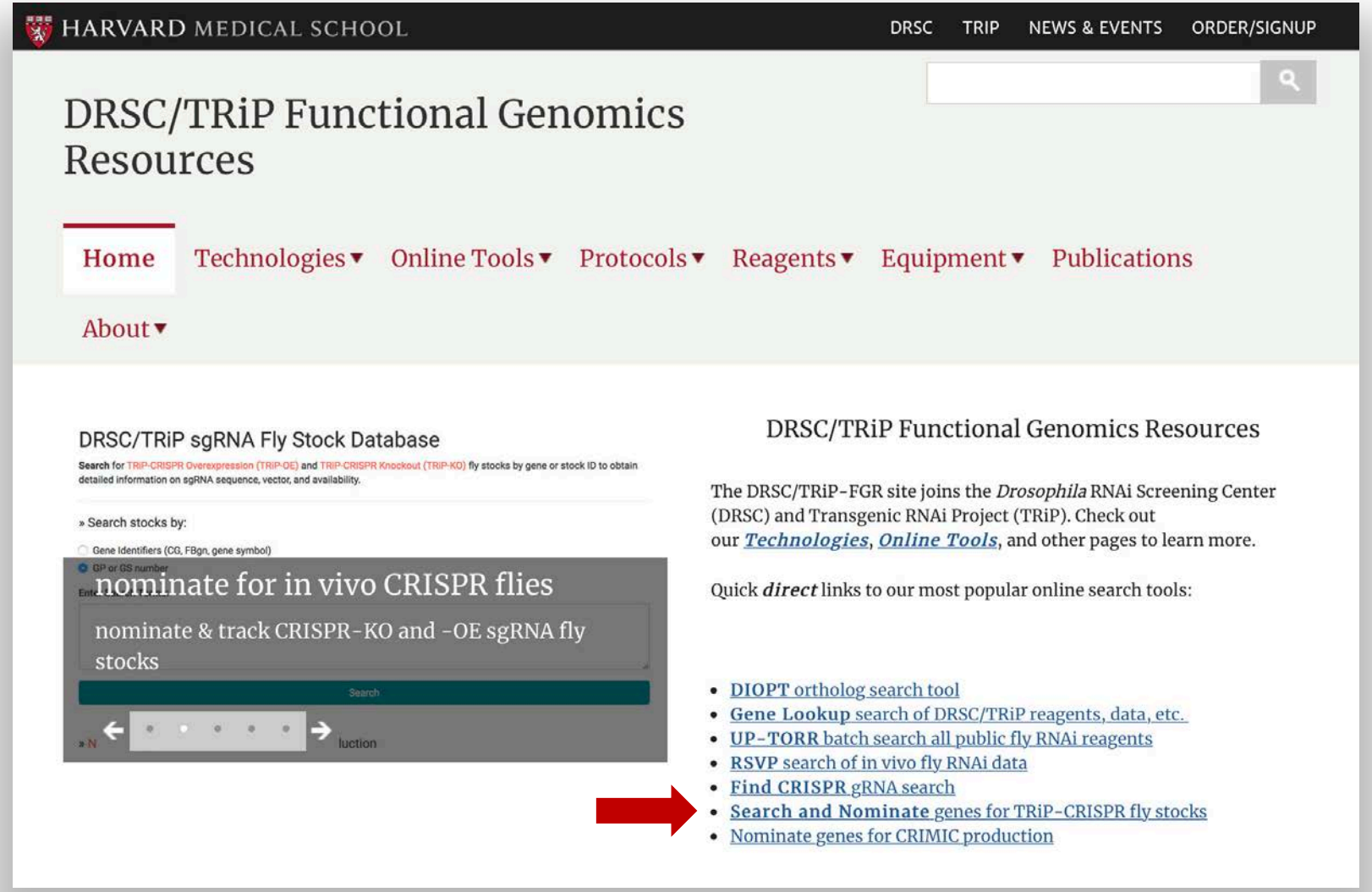
Article | Figures & Data | Supplemental | Info & Metrics | PDF

The TRiP-OE flySAM stock collection

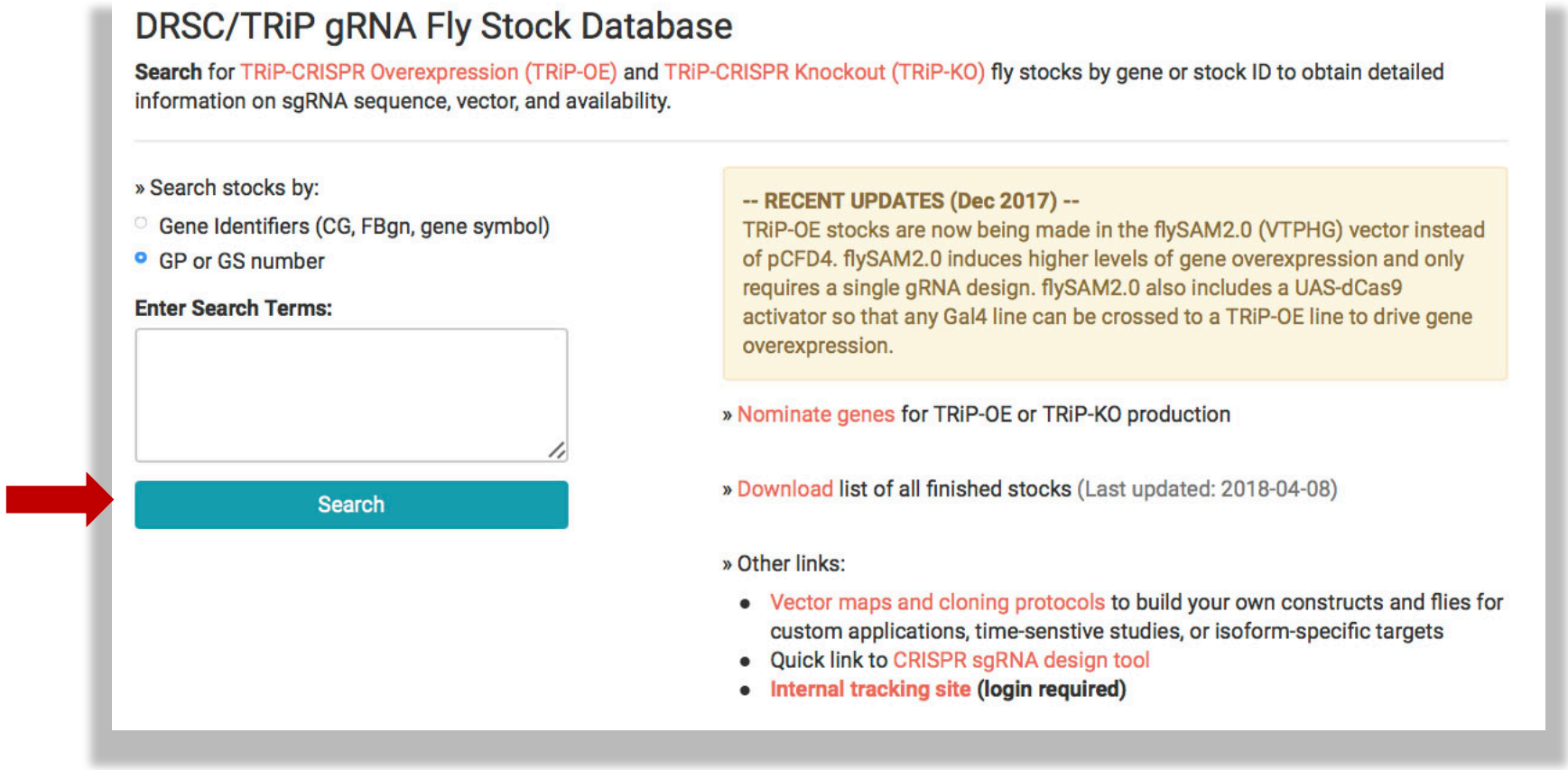


- New simplified strategy for *in vivo* CRISPR activation
- flySAM generates stronger overexpression phenotypes, comparable to Gal4/UAS
- A single gene is targeted by expression of one sgRNA from the U6:2 promoter
- Stocks are made in the flySAM2.0 vector, developed by Jian-quan Ni and colleagues
- TRiP-OE/flySAM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

Search and nominate sgRNA lines



sgRNA Fly Stock Database (http://www.flyrnai.org/tools/grna_tracker/)



Researchers can search TRiP-CRISPR stocks by gene identifier or by stock number and nominate genes for TRiP-OE or TRiP-KO production.

DRSC/TRiP gRNA Fly Stock Database

Search for TRiP-CRISPR Overexpression (TRiP-OE) and TRiP-CRISPR Knockout (TRiP-KO) fly stocks by gene or stock ID to obtain detailed information on sgRNA sequence, vector, and availability.

» Search stocks by:

- (•) Gene Identifiers (CG, FBgn, gene symbol)
- ( ) GP or GS number

Enter Search Terms:

Hh

Search

-- RECENT UPDATES (Dec 2017) --
TRiP-OE stocks are now being made in the flySAM2.0 (VTPHG) vector instead of pCFD4. flySAM2.0 induces higher levels of gene overexpression and only requires a single gRNA design. flySAM2.0 also includes a UAS-dCas9 activator so that any Gal4 line can be crossed to a TRiP-OE line to drive gene overexpression.

» Nominate genes for TRiP-OE or TRiP-KO production

» Download list of all finished stocks (Last updated: 2018-04-08)

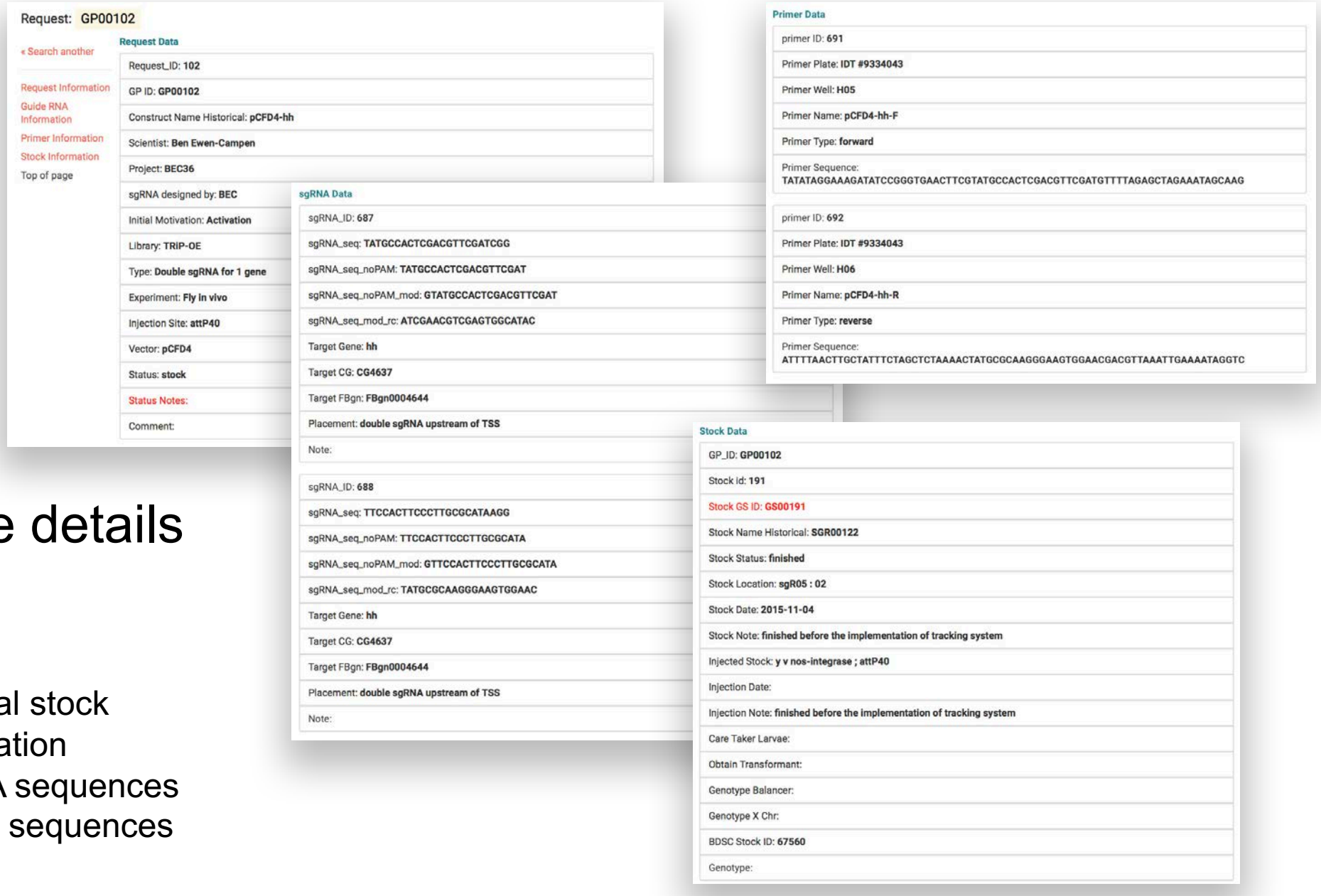
» Other links:

- Vector maps and cloning protocols to build your own constructs and flies for custom applications, time-senstive studies, or isoform-specific targets
- Quick link to CRISPR sgRNA design tool
- **Internal tracking site (login required)**

Export table

Construct (GP ID)	Status	TRiP Stock (GS ID)	BDSC Stock ID	Function	Library	Type	Gene	Placement	Vector	Injection Site
GP00102	stock	GS00191	67560	Activation	TRiP-OE	Double sgRNA for 1 gene	hh	double sgRNA upstream of TSS	pCFD4	attP40
GP01881	stock	GS00602	76431	Cut (KO)	TRiP-KO	Single sgRNA	hh	CDS	pCFD3	attP40

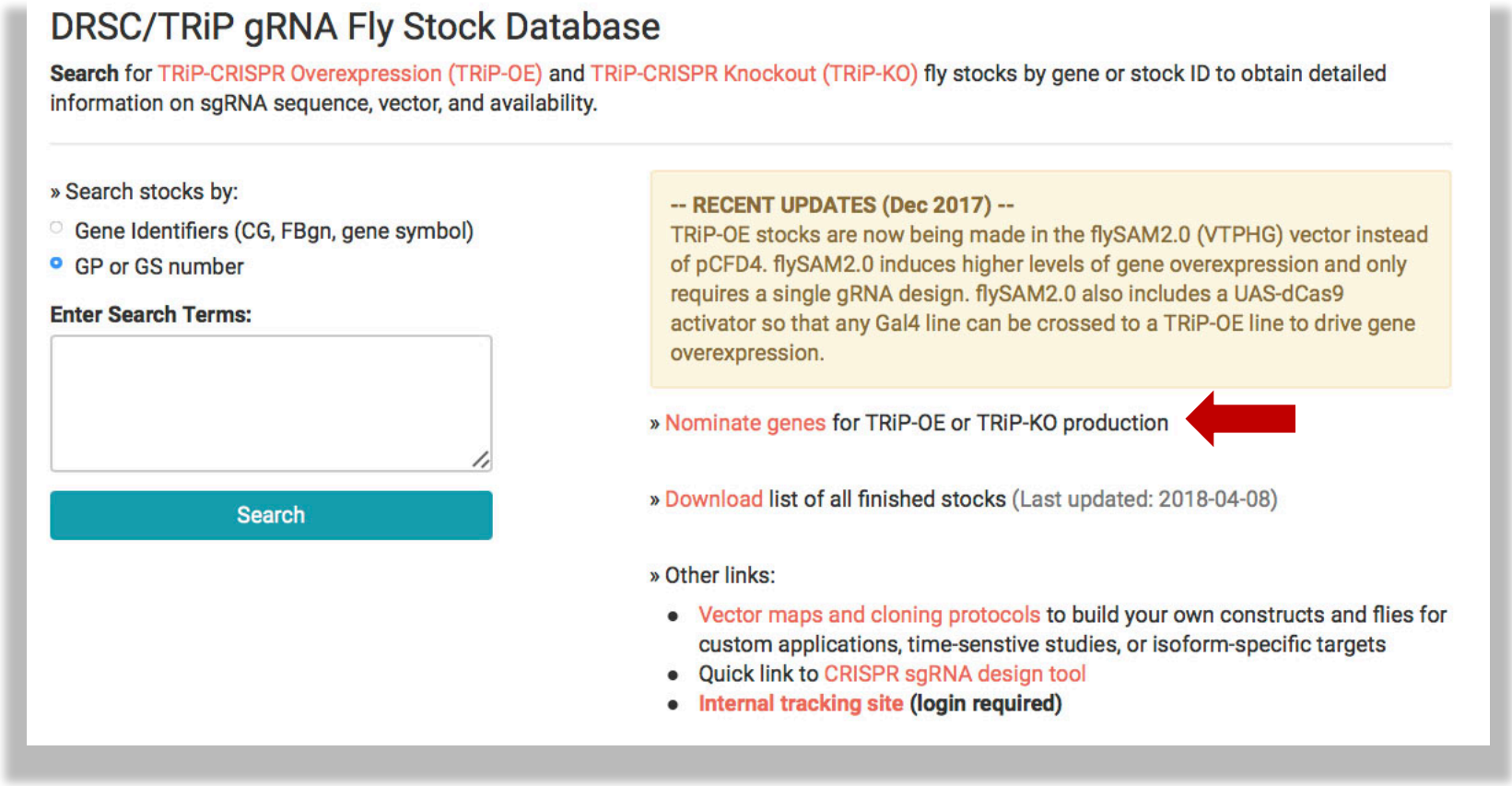
Showing 1 to 2 of 2 entries



On the details page:

- General stock information
- sgRNA sequences
- Primer sequences

We welcome community nominations



Nominate Genes

» Step 1: Download the appropriate template

- If you only have the **gene information**[?], download this template:
- If you have both **gene and gRNA information**, download this template:
- If you have **gene, gRNA, and primer information**, download this template:

» Step 2: Enter the project information

TRiP-OE stocks are now being made in the flySAM2.0 (VTPHG) vector instead of pCFD4. flySAM2.0 induces higher levels of gene overexpression and only requires a single gRNA design. flySAM2.0 also includes a UAS-dCas9 activator so that any Gal4 line can be crossed to a TRiP-OE line to drive gene overexpression.

Scientist*
Seth Brundle

Project*
cell transport

Email*
brundlefly@bsi.edu

gRNA Designed By*
Claire Hu

Enter "Claire Hu" if you only have gene information -- she will design the sgRNAs for you

Comment

Function*
Activation

Type*
Single gRNA

Vector*
flySAM2.0

Target*
Near TSS

Experiment Type*
Fly In vivo

Injection Site*
attP40 (chr2)

Clear Experiment Details

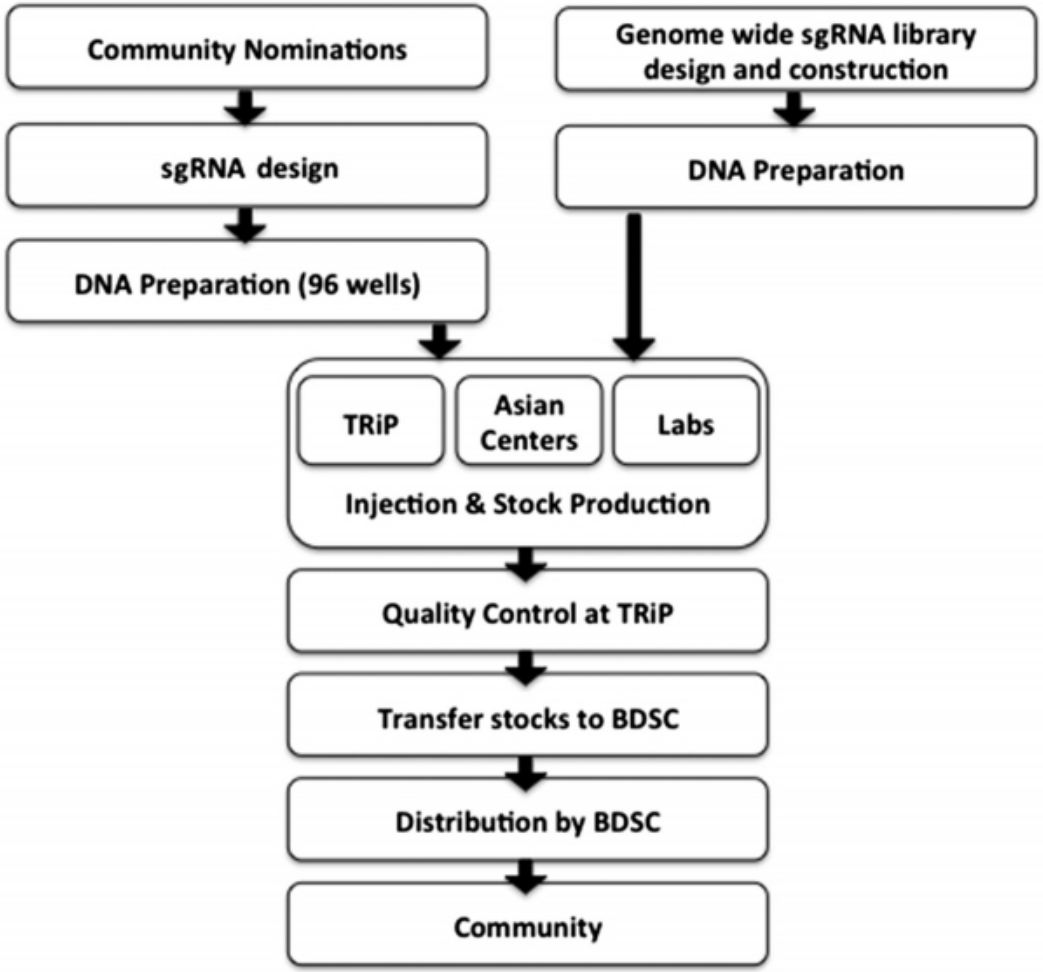
*required fields

» Step 3: Upload the template file

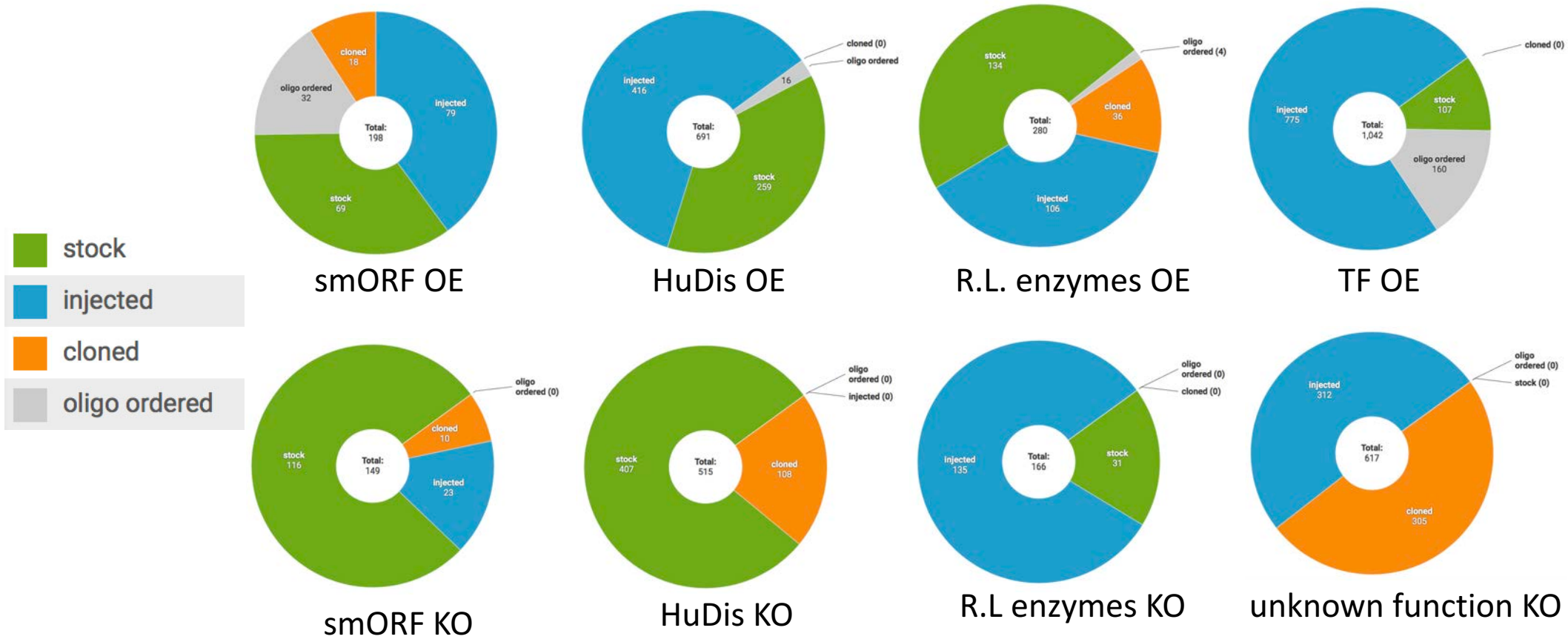
Choose File No file chosen

Submit

Your nomination will immediately enter the TRiP production pipeline



TRiP-CRISPR production is focused on community nominations and mini-libraries



To date the TRiP has produced **~2000** sgRNA fly stocks for either gene overexpression or gene cutting, with **~1500** more constructs in the transformation pipeline.

TRiP-CRISPR Toolbox stocks

dCas9-activator stocks

- GAL4/UAS expression of Cas9 proteins with dead nuclease activity (dCas9), fused to VPR transcriptional activator (dCas9-VPR)

- GAL4/UAS combined with temperature-sensitive Gal80 (tubGal80[ts])

allows greater control of spatial and temporal dCas9-VPR expression

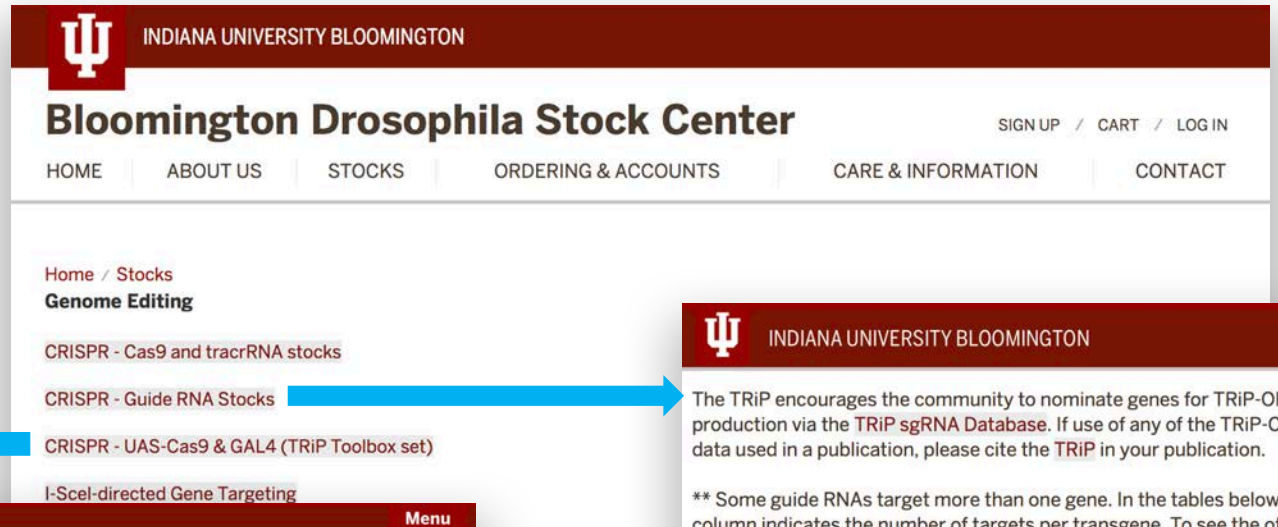
Stocks for mosaic knock-outs

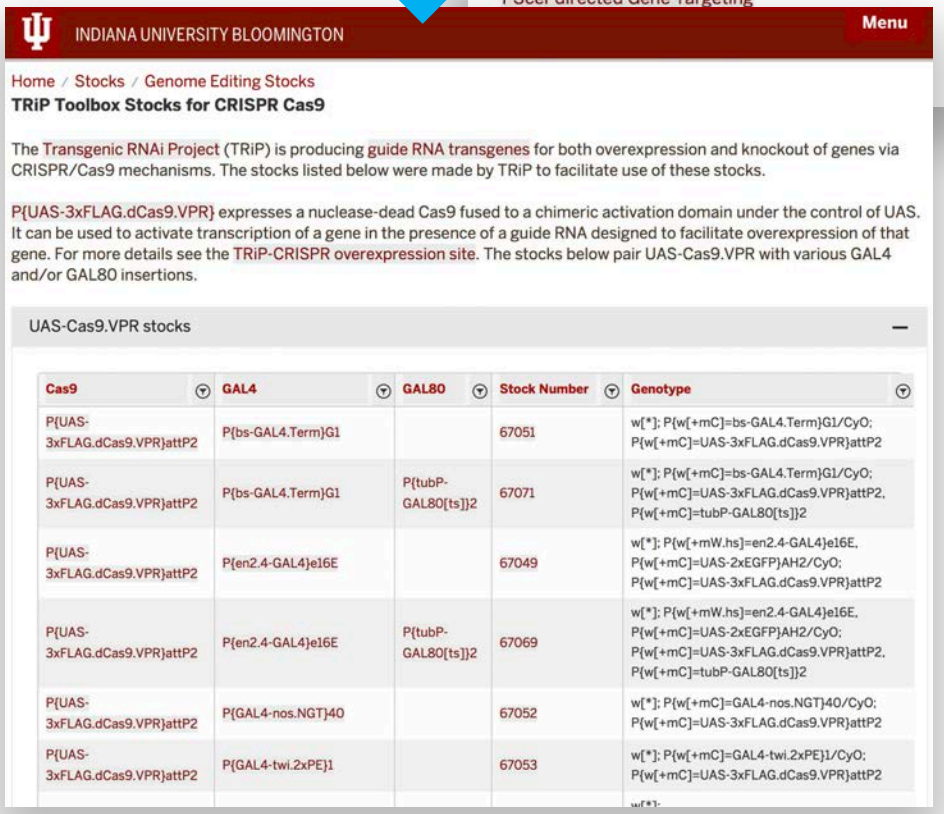
GAL4/UAS expression of wild type Cas9
used for generating mutant mosaics in the soma in cells expressing sgRNAs targeting the coding region (eg. TRiP-KO stocks)

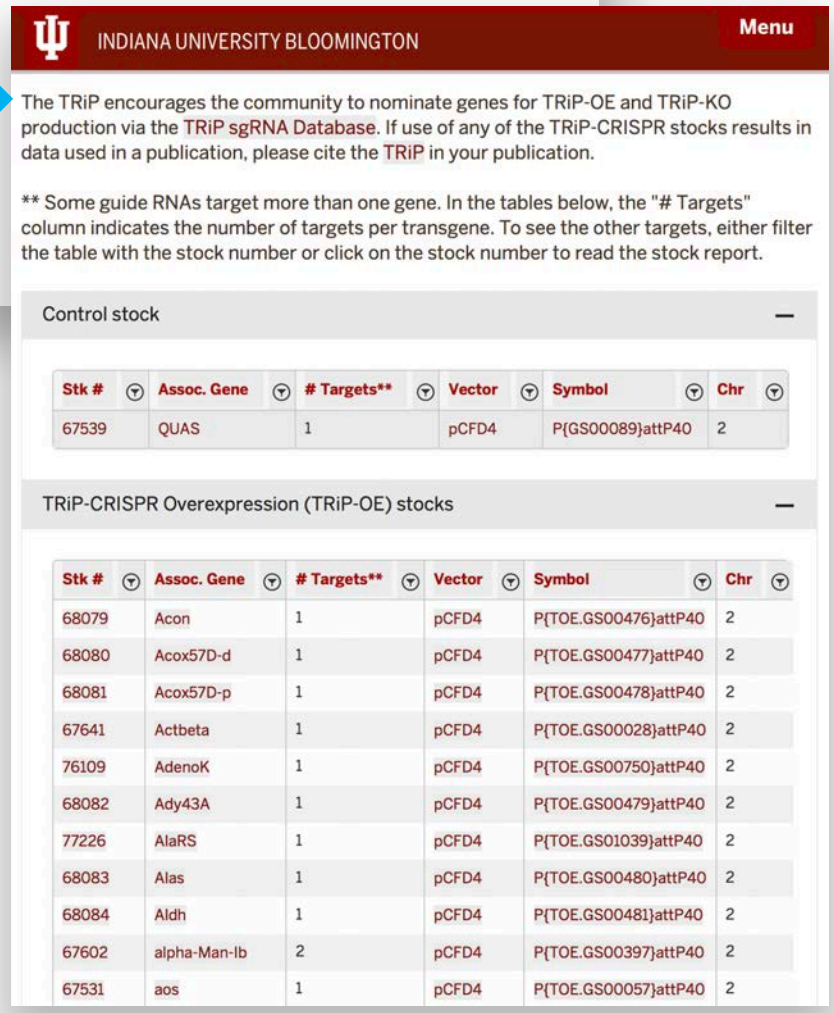
Stocks for germline mutants

germline-specific expression of wild type Cas9
used for generating small deletions and modifications in the germline in cells expressing sgRNAs targeting the coding region (eg. TRiP-KO stocks)

Search TRiP-CRISPR stocks at BDSC

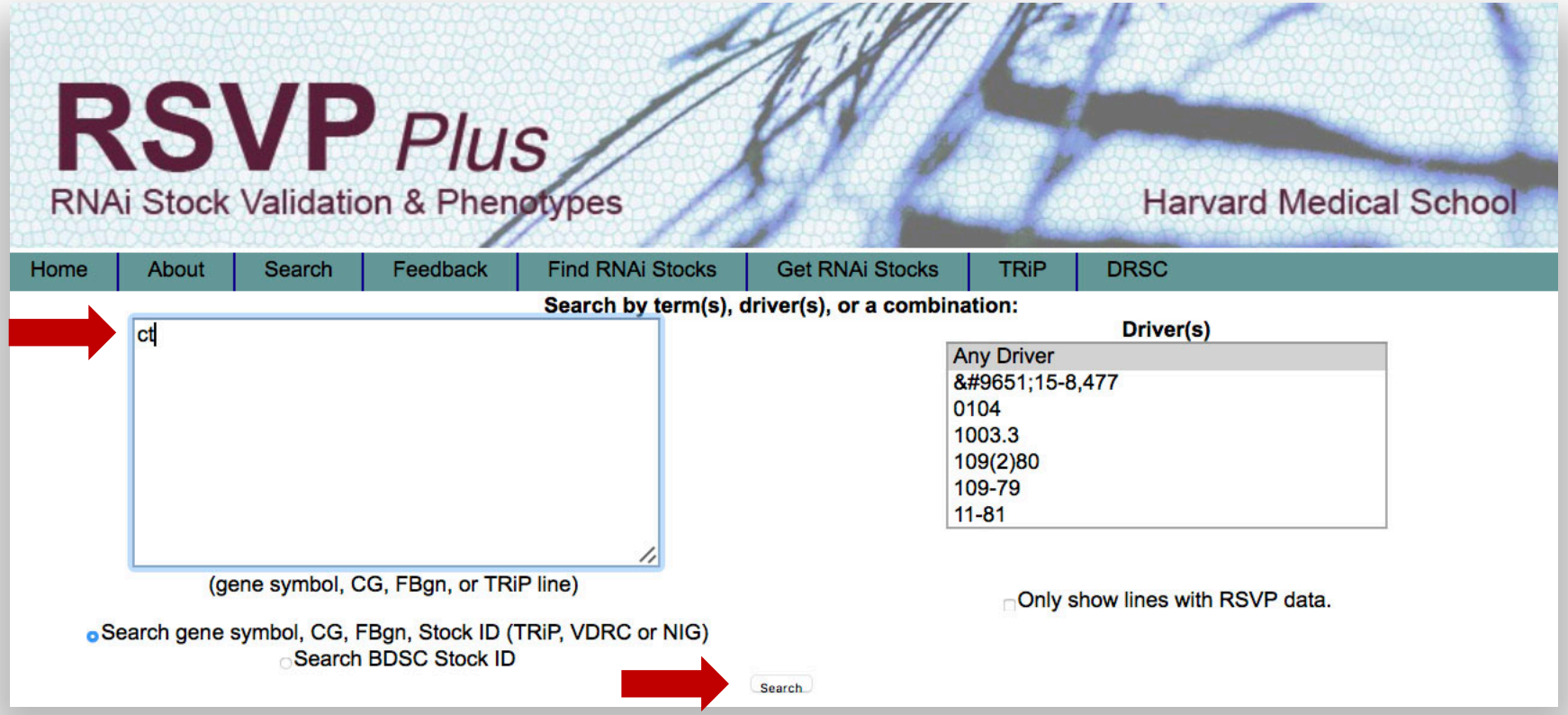






Search and add feedback for sgRNA lines at RSVP *Plus*

http://www.flyrnai.org/cgi-bin/RSVP_search.pl



RNAi Stock Validation and Phenotype *Plus* (RSVP *Plus*) allows for search and view of RNAi **and sgRNA** fly stocks from TRiP, VDRC, and NIG-Japan, along with any available data regarding knockdown or phenotypes observed for that fly stock and a given Gal4 driver (e.g. from publications).

RSVP Plus

RNAi Stock Validation & Phenotypes

Harvard Medical School

Home | About | Search | Feedback | Find RNAi Stocks | Get RNAi Stocks | TRiP | DRSC

Search by term(s), driver(s), or a combination:

ct

(gene symbol, CG, FBgn, or TRiP line)

Driver(s)

- Any Driver
- △15-8,477
- 0104
- 1003.3
- 109(2)80
- 109-79
- 11-81

Only show lines with RSVP data.

Search gene symbol, CG, FBgn, Stock ID (TRiP, VDRC or NIG)

Search BDSC Stock ID

Search

Download the Results (.xls format, with data summary).

	FBgn	CG	Gene Name	BDSC Stock ID	Detail Page	Line Source	Vector	Stock Type	Insert Site	Data
1	FBgn0004198	CG11387	cut	-	5687	VDRC-GD		RNAi		pnr-MD237
2	FBgn0004198	CG11387	cut	67524	GS00041	TRiP	pCFD4	sgRNA-OE	attP40	P{w[+mW.hs]=GAL4-dpp.blk1}
3	FBgn0004198	CG11387	cut	33967	HMS00924	TRiP	VALIUM20	RNAi	attP2	Act5C-Gal4 ppk.1.9 MTD-Gal4
4	FBgn0004198	CG11387	cut	29625	JF03304	TRiP	VALIUM10	RNAi	attP2	Act5C-Gal4 ppk.1.9 y w; UAS-dcr2; nanos-Gal4

4 results (1 genes).

Download the Results (.xls format, with data summary).

RSVP Plus

RNAi Stock Validation & Phenotypes

Harvard Medical School

P{TRiP.GS00041}attP40

Gene Information

Symbol: ct
CG: CG11387
FBgn: FBgn0004198

TRiP Stock Information

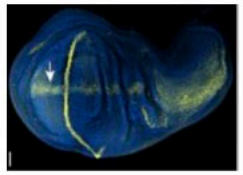
Collection: TRiP **Line ID:** GS00041 **Type:** sgRNA-OE
BDSC ID: 67524
Vector: pCFD4 **Insertion Site:** attP40

Validation Test Results

Driver: P{w[+mW.hs]=GAL4-dpp.blk1}
Driver BDSC: 67045
Contributor(s): Jonathan Zirin
Experiment Type : Activation
Reference: Optimized strategy for in vivo Cas9-activation in Drosophila. PUBMED PDF

Phenotype Results:

- **Phenotype:** ectopic cut expression: a stripe of ectopic Cut expression along the A/P boundary of the wing disc
- **Phenotype Anitbody Info:** mouse anti-Cut (2B10, DSHB, 1:10)
- **Phenotype Category:** wing imaginal disc
- **Temperature (°C):** 27.0



Community Input (*asterisk indicates required field*)

Contributor*:		Describe the phenotype.
Driver*:		
Driver BDSC:		
Temperature*:	°C	
qPCR data:	% Remaining after knockdown	
Cas9 variant*:	Cas9.P2 (cutting)	Other:
experiment:	knock-out	Other:
Phenotype?	○As expected ○None detected ○Unclear (difficult to determine phenotype) ○Novel (unexpected phenotype)	
Contributor Email*:	For follow-up only.	

Submit Input

RSVP Plus

RNAi Stock Validation & Phenotypes

Harvard Medical School

P{TRiP.GS00041}attP40

Gene Information

Symbol: ct
CG: CG11387
FBgn: FBgn0004198

TRiP Stock Information

Collection: TRiP **Line ID:** GS00041 **Type:** sgRNA-OE
BDSC ID: 67524
Vector: pCFD4 **Insertion Site:** attP40

Validation Test Results

Driver: P{w[+mW.hs]=GAL4-dpp.blk1}
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Experiment Type : Activation
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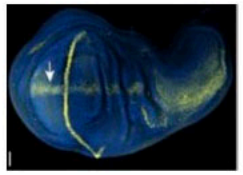


Community Input (*asterisk indicates required field*)

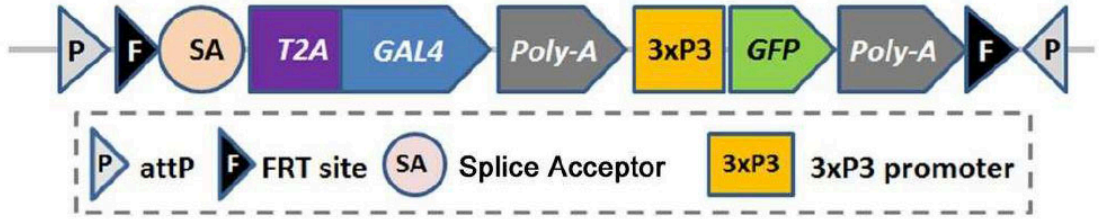
Contributor*:
Driver*:
Driver BDSC:
Temperature*: °C
qPCR data: % Remaining after knockdown
Cas9 variant*: ✓ Cas9.P2 (cutting) | Cas9.P (cutting) | dCas9.VPR (activation) | dCas9.VP64 (activation, flySAM) | other (please specify) — Other:
experiment: Other:
Phenotype? r (difficult to determine phenotype) Novel (unexpected phenotype)
Contributor Email*:

Describe the phenotype.

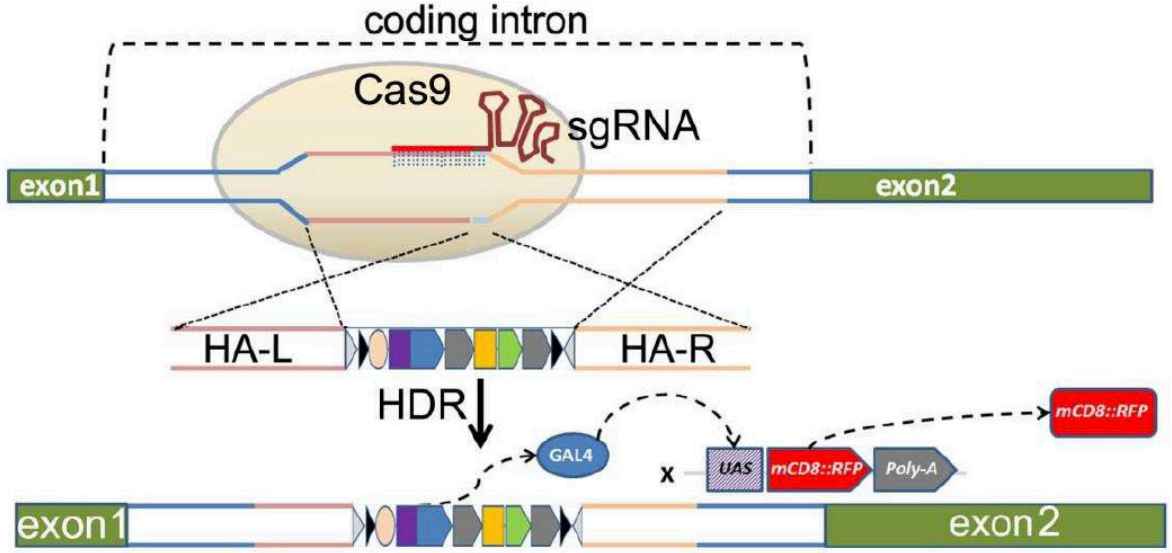
Submit Input

The CRIMIC Project

Structure of CRIMIC (CRISPR Mediated Integration Cassette) cassette (pM37)



Schematic of pM37 integration by CRISPR/*Cas9*



Collaboration with Hugo Bellen's group

Insert gene traps into the 5'-most intron that can be used to tag all or most of the predicted splice Isoforms.

A gene-specific *T2A-GAL4* library for *Drosophila*

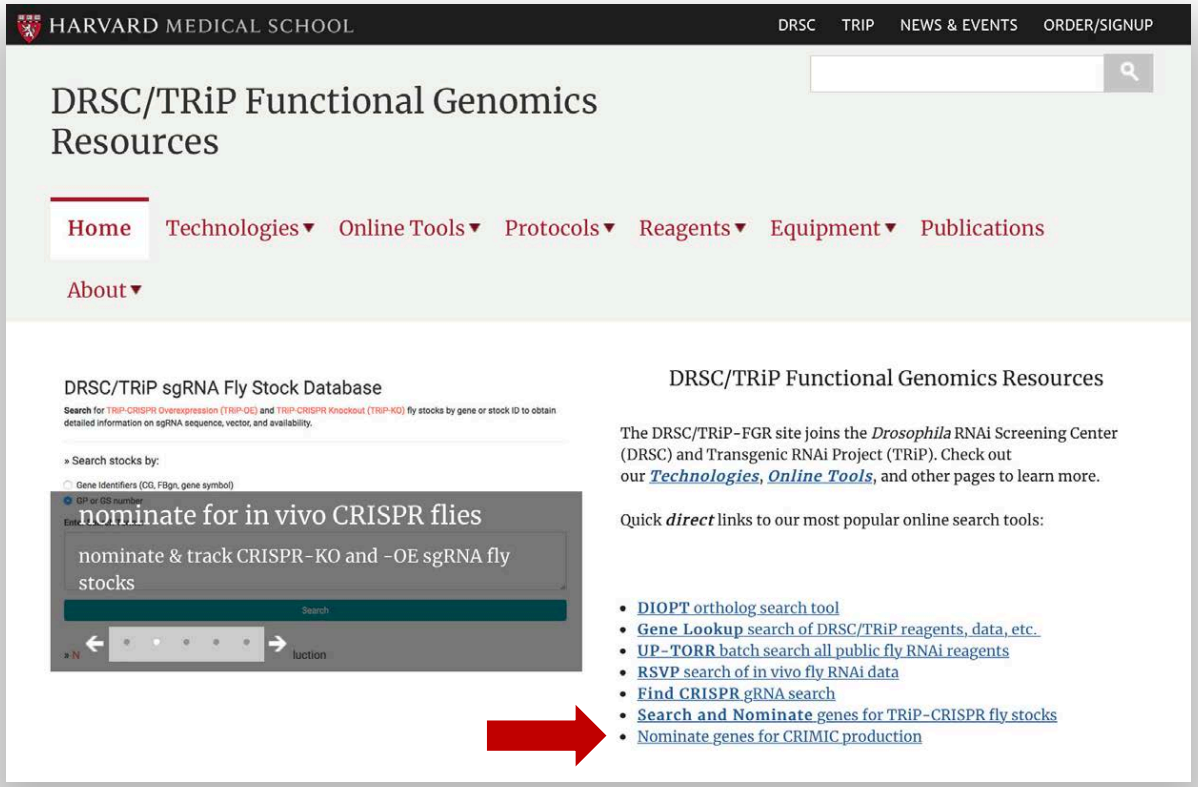
eLIFE

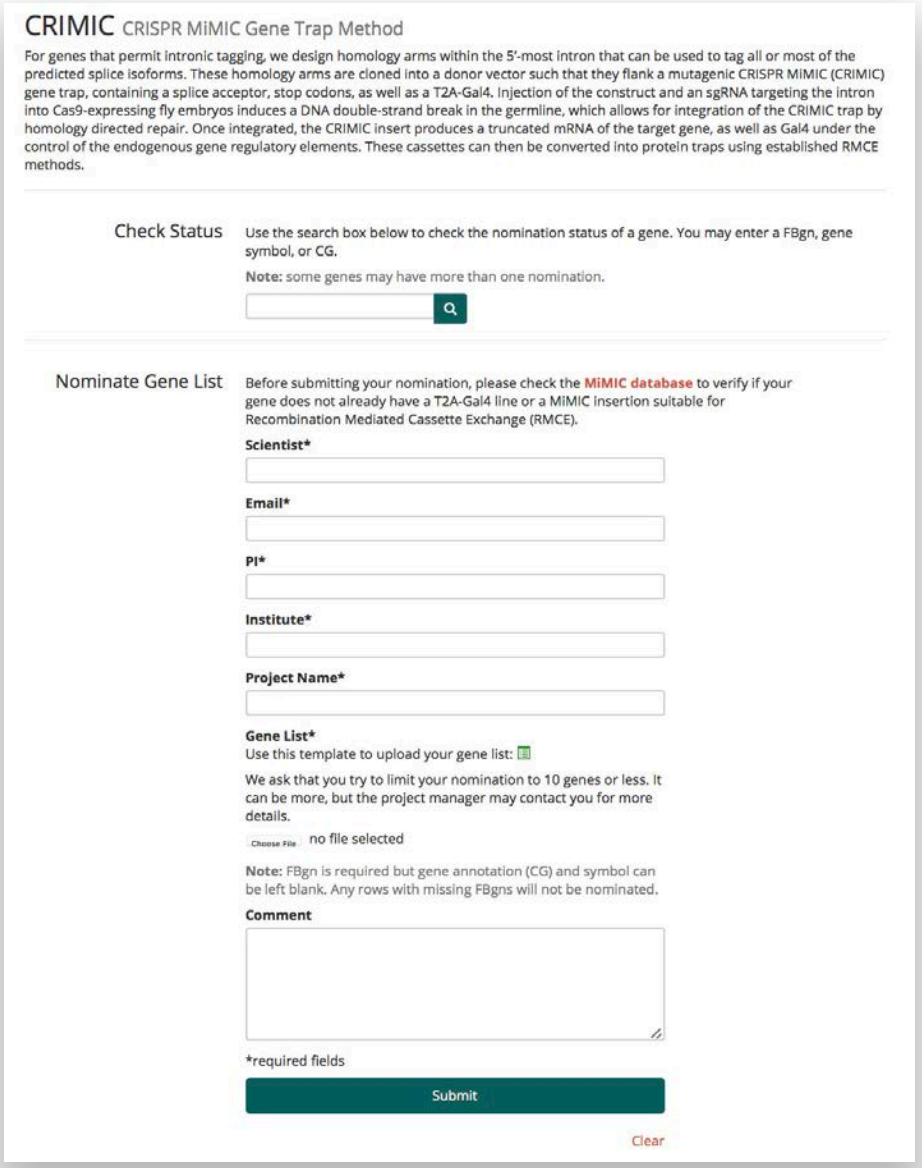
Pei-Tseng Lee, Jonathan Zirin, Oguz Kanca, Wen-Wen Lin, Karen L Schulze, David Li-Kroeger, Rong Tao, Colby Devereaux, Yanhui Hu see all »

Baylor College of Medicine, United States; Harvard Medical School, United States; Howard Hughes Medical Institute, Carnegie Institution for Science, United States

Nominate your gene for CRIMIC

http://www.flyrnai.org/tools/crimic/web/





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Howard Hughes Medical Institute

PgmNr 867: Cell-based screen technologies at the Drosophila RNAi Screening Center.

PgmNr 869: A new resource from the TRiP: sgRNA stocks for gene overexpression and knockout by CRISPR-Cas9.

PgmNr 876: DRSC Informatics Tools for Functional Genomics Studies – 2018 update.