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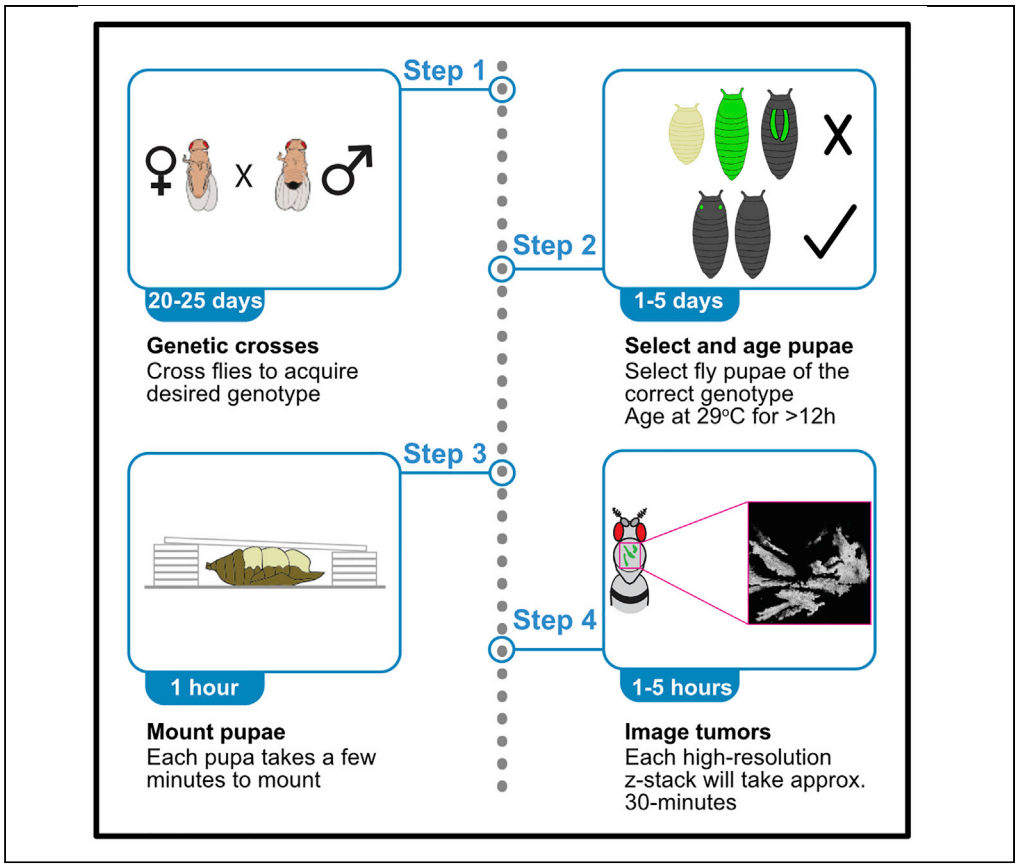
Generation and live imaging of tumors with specific genotypes in the living fly pupa



This protocol describes the step-by-step generation of tumors with specific genotypes on the dorsal thorax epithelium of the fly. This *in vivo* system allows the imaging of tumor cell morphology and behavior in high spatial and temporal resolution. Phenotypes such as cell invasion, cell division, and tumor size can be quantified and compared to specific controls or to the neighboring wild-type tissue. Thus, this model allows the study of conserved genes that enhance or suppress epithelial tumor progression.

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Highlights

Tumors with specific genotypes can be genetically generated on the back of the fly

Tumors can be imaged in real time in the living fly pupa

The system is flexible, allowing a combination of mutant alleles and UAS-transgenes

This system is ideal for both screening and gene characterization

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Generation and live imaging of tumors with specific genotypes in the living fly pupa

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SUMMARY

This protocol describes the step-by-step generation of tumors with specific genotypes on the dorsal thorax epithelium of the fly. This *in vivo* system allows the imaging of tumor cell morphology and behavior in high spatial and temporal resolution. Phenotypes such as cell invasion, cell division, and tumor size can be quantified and compared to specific controls or to the neighboring wild-type tissue. Thus, this model allows the study of conserved genes that enhance or suppress epithelial tumor progression.

For complete details on the use and execution of this protocol, please refer to Canales Coutiño et al. (2020).

BEFORE YOU BEGIN

Experimental design

This protocol describes the generation of positively marked tumors with specific genotypes on the dorsal thorax epithelium of the fly. To achieve this, we combined the Flp/FRT system (Xu and Rubin, 1993), the mosaic analysis with a repressible cell marker (MARCM) technique (Lee and Luo, 2001), and Pannier-Gal4 (Pnr-Gal4). The MARCM technique allows the positive labeling of homozygous mutant clones, which when combined with Ubx-Flp and Pnr-Gal4, allows us to generate positively marked homozygous mutant clones specifically within the epithelium of the fly pupal notum (the dorsal thorax). To model the multistep nature of tumorigenesis and tumor progression, multiple genetic lesions can be generated specifically within the labeled tumor tissue.

We carried out a large-scale genetic screen using this model, by generating *lethal (2) giant larvae*^{4} homozygous mutant clones (*lgl*^{4}). Tumors lacking *lgl* generate large, partially multi-layered tumors, and present a low-level invasive phenotype, representing an ideal scenario for screening for enhancers or suppressors of tumor progression. We characterized the cohesin complex subunits as tumor suppressor genes using this model (See expected outcomes and Canales Coutiño et al., 2020). For a detailed rationale of our experimental design and for the results of the 497 different genes we studied using this model, see Canales Coutiño et al. (2020).

The generation of homozygous mutant *lgl*^{4} tumors with the knockdown of an additional gene specifically within the mutant tissue will be described in detail in the main section of this protocol. However, we want to emphasize the versatility of this model and stress that the background mutation is not limited to a specific tumor suppressor gene. In this section we will provide the guidelines and indicate the critical aspects to consider before selecting candidate background genes.

1. Select the ideal background gene and identify (or generate) a suitable mutant fly stock.

a. Ideally the desired mutant allele should be together with an FRT (flippase recognition target) site on the same chromosome arm; this is to allow for Flp/FRT site-directed recombination (Xu and Rubin, 1993). Flybase.com has extensive information about mutant alleles that are commercially available. If the desired mutant allele does not reside together with an FRT site, the mutant allele and FRT site can be recombined together, see Greenspan (2004). The FRT site used will depend on the chromosome arm on which the mutant allele resides. Multiple FRT sites are available on all chromosome arms (other than chromosome 4). For example, if the desired mutation resides on the left arm of chromosome 2, an appropriate FRT site would be FRT40a.
b. Once a suitable line has been acquired or generated, positively marked homozygous mutant clones can be generated using the MARCM system (Lee and Luo, 2001). We recommend selecting a variety of candidate background genes and mutant alleles at this early stage. This will allow you to test for the ability of specific mutant alleles to generate large clones on the dorsal thorax of the fly. Cell lethality and/or cell competition could result in very small or no clones forming, so it would be advisable to screen through a number of candidates prior to proceeding.

2. Identify a fly stock with an identical FRT site and tubulin Gal80 (tubGal80) (e.g., FRT40a, tubGal80). Flippase induced recombination between the mutant gene and tub-Gal80 is necessary to (1) generate homozygous mutant clones and (2) remove suppression of Gal4 mediated transgene expression. Flippase requires two identical FRT sites in order to induce post-mitotic recombination.
3. Select a gene or group of genes that will be studied in addition to the background mutation; here the aim is to determine whether an additional gene alteration affects tumor behavior.

Identify any UAS lines to either downregulate or overexpress the specific genes of interest (from here onwards referred to as UAS-transgenes).

CRITICAL: It is important that the transgene is downstream of the UAS enhancer. This will allow transgene expression to be under the control of the GAL4 activator protein, and GAL80 repressor protein, thereby limiting transgene expression to the mutant cells.

Optional: When using RNA interference (RNAi) to inhibit target gene expression, we recommend utilizing at least two independent UAS-RNAi lines. This is to prevent potential errors due to off target effects, or inefficient gene knockdown.

4. Build the required stocks and design a cross scheme.

This protocol describes the fly cross scheme for a background gene located on the 2nd chromosome and a transgene on the 3rd chromosome (Figure 1). Background genes and transgenes located on different chromosomes will require the design of a slightly different cross scheme.

Obtaining flies of interest and fly pushing

Once the specific genes of interest have been identified, fly stocks carrying the required genotypes must be obtained before starting the protocol. There are four main international *Drosophila* libraries that offer genome-wide resources: the Vienna *Drosophila* Resource Center (VDRC), Bloomington *Drosophila* Stock Center (BDSC), National Institute of Genetics (NIG-Fly), and Kyoto Stock Center. Fly stocks can be purchased from any of these libraries.

5. Refer to the key resources table of this protocol for the fly lines that need to be ordered to generate *lgl* mutant tumors.
6. Upon arrival, verify that the flies express the correct genetic markers (Table 2, Figure 2).

CRITICAL: Markers are essential to track the genotypes of interest. Incorrect markers or the absence of them can indicate stock contamination.

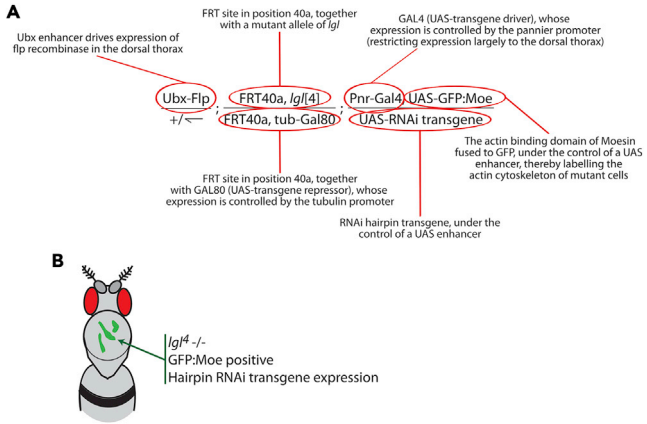

Figure 1. .Protocol overview

(A) Genotype of interest showing fly chromosomes I-III, each transgene and mutation used in this protocol is described in detail.

(B) Schematic illustrating how clones with distinct genotypes are generated on the back of the fly. The MARCM system is employed to generate mutant clones specifically within the fly dorsal thorax, through the use of Ubx-Flp. This generated GFP:Moe-labeled *lgl4* homozygous mutant clones. RNAi transgene expression, and therefore gene KD, is restricted to the labeled *lgl4* mutant tissue.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Flies required for this protocol		
D. melanogaster: w; FRT40a, tub-Gal80; MKRS / TM6b	This study	N/A
D. melanogaster: w; FRT40a, tub-Gal80	Bloomington *Drosophila* Stock Center	5192
D. melanogaster: w; ; UAS-transgene	Various (see experimental design)	N/A
D. melanogaster: Ubx-Flp; FRT40a, lgl^4 / CyO-GFP; Pnr-Gal4, UAS-GFP:moe / TM6b	This study	N/A
D. melanogaster: Ubx-Flp; FRT40a; Pnr-Gal4, UAS-GFP:moe / TM6b	This study	N/A
Flies recommended for the customization of this protocol		
D. melanogaster: y, w; ; Ubx-Flp	Bloomington *Drosophila* Stock Center	42719
D. melanogaster: w; ; UAS-GFP:moe	Bloomington *Drosophila* Stock Center	31776
D. melanogaster: UAS-GFP:moe, w	Bloomington *Drosophila* Stock Center	31774
D. melanogaster: w; UAS-GFP:moe / SM6a	Bloomington *Drosophila* Stock Center	31775
D. melanogaster: w; UAS-VC3Ai; Pnr-Gal4 / TM6b	Bloomington *Drosophila* Stock Center	84340
D. melanogaster: Ubx-FLP, tub-GAL80, FRT19a	Bloomington *Drosophila* Stock Center	42731
D. melanogaster: y, w; tub-Gal80, FRT40a / CyO	Bloomington *Drosophila* Stock Center	5192
D. melanogaster: y, w; FRT42d, tub-Gal80 / CyO, y[+]	Bloomington *Drosophila* Stock Center	9917
D. melanogaster: y, w; tub-Gal80, FRT80b	Bloomington *Drosophila* Stock Center	5191
D. melanogaster: y, w; FRT82b, tub-Gal80	Bloomington *Drosophila* Stock Center	5135
Chemicals, peptides, and recombinant proteins		
Yeast	Lesaffre UK and Ireland	Saf-Levure active dry yeast
Soy flour	Holland and Barrett UK	029185
Coarse yellow cornmeal	Spices of India UK	TRS RFG004-p
Agar	Fisher Scientific UK	Acros organics 10048991
Light corn syrup	brake.co.uk	A 26941
Propionic acid	Merck Life Sciences UK	W292400-1KG-K
10S VOLTALEF injection oil (or a different oil matching the refraction index of the microscope objective being used)	VWR UK	24627.188

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Microscope slides	Fisher Scientific UK	12383118
22 × 22 mm Coverslips	Fisher Scientific UK	12333128
22 × 50 mm Coverslips	Fisher Scientific UK	12383138
Clear nail polish	Poundland UK	121474
CO2 porous polyethylene gas diffuser, to anaesthetize flies for observation	flystuff.com	59-114
Dissecting stereomicroscope, to screen for genetic markers and identify males/females	Leica Microsystems	Leica M60
Fluorescence stereomicroscope, to screen for flies expressing GFP markers	Leica Microsystems	Leica MZ10F
Confocal microscope, to image the mutant clones	Zeiss	Zeiss LSM 880

MATERIALS AND EQUIPMENT

Fly food preparation

Standard fly food preparation is based on the BDSC recipe, adapted from (Lakovaara, 1969), refer to their recipe for detailed instructions.

The materials for 42.5 liters of food are as follows:

- 39L of water
- 675 g of yeast
- 390 g of soy flour
- 2,850 g of yellow cornmeal
- 225 g of agar
- 3L of light corn syrup
- 188 mL of propionic acid

Prepare fly food in advance and store at 4°C for maximum of one week

STEP-BY-STEP METHOD DETAILS

First cross

Timing: 30–45 min followed by 10–13 days incubation

The generation of positively marked tumors is achieved by selective breeding of transgenic and mutant flies. Male flies carrying the UAS-transgene line in the 3rd chromosome will be crossed to virgin female flies with the FRT site and tubGal80 on the 2nd chromosome (Figure 3). Genetic markers will be used to identify and track the progeny carrying the transgenes of interest.

1. Collect approximately 20 virgin female flies of the stock w; FRT40a, tub-Gal80; MKRS / TM6b
   a. Verify that the stock is expressing both MKRS and TM6b genetic markers.
      i. Flies must exhibit the phenotypes corresponding to these markers, namely: tubby (short, fat body), humeral (extra macrochaetes) and stubble hairs (Table 2, Figures 2B and 2C).

⚠ CRITICAL: Females must be virgin flies, otherwise the F1 progeny will be mixed with flies of the original stock.

⚠ CRITICAL: Verify that the genetic markers are correctly expressed, this is critical in all steps.

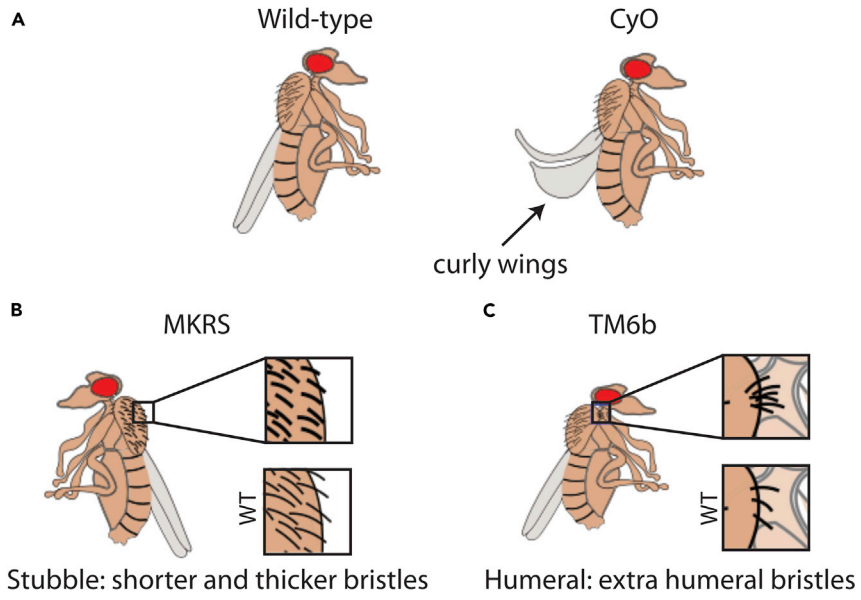

Figure 2. Dominant markers used in this protocol

(A) CyO dominant marker: Curly wings - adult flies have curly instead of straight wings.

(B) MKRS dominant marker: Stubble - flies have shorter and thicker bristles.

(C) TM6b dominant markers: Humeral and tubby. Humeral - flies have additional bristles in the humerus; tubby - flies are shorter than wild-type. Tubby is more easily identifiable at pupal stages (see Figure 4).

2. Collect males of the specific UAS-transgene of interest located on the 3rd chromosome.
   a. Select approximately 5 males of the stock w; ; UAS-transgene
3. Place the collected virgin flies from step 1 with the males from step 2 in a fresh vial of food.
4. Incubate at 25°C
   a. Flip the parent flies to a fresh vial of food twice a week. Keep both the old vials (containing the freshly laid eggs and larvae) and the new vials (containing the adult flies) at 25°C.
   b. Adult wild type flies emerge within approximately 10 days, although some mutant flies might take longer. To prevent progeny from mixing with the parents, do not use vials that are 17 days or older at 25°C.

⚠ CRITICAL: Do not let the F1 progeny mix with the parent flies.

Second cross

Timing: 30–45 min in 3–4 consecutive days followed by 5–8 days incubation

The aim of this step is to cross specific F1 progeny males carrying the FRT site, tubGal80 and UAS-transgene with virgin females carrying the *lgl* mutant allele, FRT site, Ubx-Flp, Pnr-Gal4 and UAS-GFP:moe (Figure 3).

5. Select approximately 5 TM6b positive males from the progeny of the first cross.
   a. Discard the females and screen the male F1 progeny for the tubby (short, fat body) and humeral (extra macrochaetes) genetic markers (Table 2, Figure 2B and 2C). Discard MKRS positive flies as they do not have an identifiable phenotype at the pupal stage; a pupal marker is required to confirm the presence of the UAS-transgene in the F2 progeny.

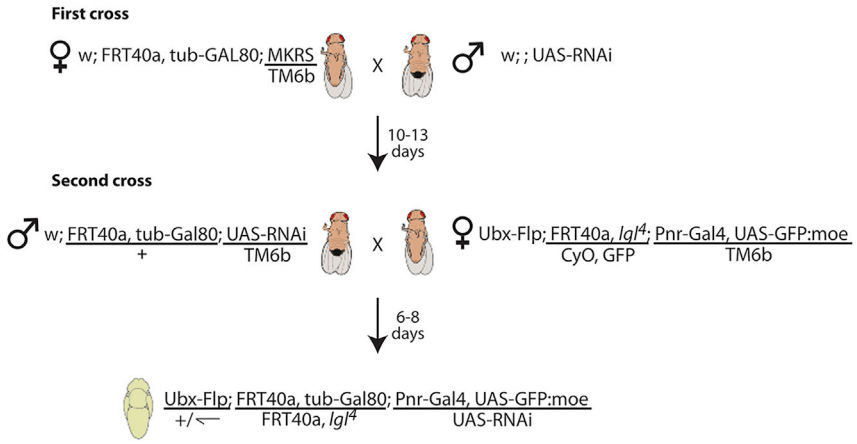

Figure 3. Cross scheme

Cross scheme required for the generation of positively marked lethal (2) giant larvae[4] (*lgl*^{4}) homozygous mutant tumors, with the knockdown of an additional gene specifically within the mutant tissue. First cross, females containing a specific FRT site (in this case, in position 40a) and tub-Gal80 are crossed with UAS-RNAi males. Second cross, male F1 progeny of the correct genotype are identified by the absence of short hairs (MKRS) and crossed with females of the genotype shown. Estimated waiting times are indicated for each step. For a description of each transgene and mutation refer to Figure 1 and Table 1. For the description of each genetic marker and balancer chromosome refer to Figure 2 and Table 2.

Optional: The first cross can be moved to 18°C once the males have been selected and the second cross has been carried out. Fly development is slowed at 18°C, and the first cross should be kept in case additional second crosses have to be set.

6. Collect approximately 20 virgin female flies of the stock Ubx-Flp; FRT40a, *lgl*^{4}/CyO-GFP; Pnr-Gal4, UAS-GFP:moe / TM6b
   a. Verify that the stock is expressing CyO-GFP.
      i. Flies must have curly wings and ubiquitous GFP expression (Table 2, Figure 2A).
   b. It is normal to have a mix of TM6b positive and negative flies in this stock, since Pnr-Gal4, UAS-GFP:moe is semi-lethal and some flies will be homozygous.
7. Place the collected males from step 5 with the virgin flies from step 6 in a fresh vial of food.
8. Set up the genetic crosses for the controls. A number of controls can be used in this experiment including (1) clones expressing the UAS-transgene without the background mutation, (2) clones homozygous mutant for the background mutation but without UAS-transgene expression, and (3) wild-type clones (no background mutation and no transgene expression). All controls will be labeled with GFP:moe
   a. To generate UAS-transgene clones without the *lgl*^{4} mutation, cross the males from step 5 to virgin females of the following genotype: Ubx-Flp; FRT40a; Pnr-Gal4,UAS-GFP:moe / TM6b.
   b. Only one cross is required to generate *lgl*^{4} mutant clones without the UAS-transgene. Cross the female virgins from step 6 with males of the following genotype: w; FRT40a, tub-Gal80.
   c. For wild type clones, only one cross is required. Cross Ubx-Flp; FRT40a; Pnr-Gal4,UAS-GFP:moe / TM6b female virgins to w; FRT40a, tub-Gal80 males.
   d. Follow the same subsequent steps for the controls. All controls are treated in the same conditions as experimental flies.
9. Incubate at 25°C
   a. Flip the parent flies to a fresh vial of food twice a week. Keep both the old vials (containing the freshly laid eggs and larvae) and the new vials (containing the adult flies) at 25°C.
   b. Flies will reach the pupal stage after approximately 5–8 days. Check the vials regularly, they will be ready for the next step (pupa collection) as soon as pupae start to form.

Table 1. Genetic constructs

Genotype	Abbreviation	Description
P{Ubx-FLP}	Ubx-Flp	Expresses Flp recombinase under the control of the Ultrabithorax (Ubx) enhancer
P{neoFRT}40A	FRT40a	Carries an FRT site at position: Chr 2, 40A3, 2L:21794705..21794705
P{UAS-GMA}	UAS-GFP:moe	Expresses the actin-binding domain of moesin tagged with GFP[S65T] under the control of a UAS enhancer
P{tubP-GAL80}	tub-Gal80	Expresses GAL80 'ubiquitously' under the control of the alphaTub84B promoter
P{GawB}pnr[MD237]	Pnr-Gal4	Expresses GAL4 in dorsal cells along the length of the fly under the control of the pannier (pnr) promoter.
P{UAS-IhRNAi}	UAS-RNAi	Expresses dsRNA for RNAi under UAS control

Table outlining the function of genetic constructs incorporated into the *Drosophila* genome.

Optional: We recommend setting at least two second crosses for each genotype to have sufficient F2 progeny.

Identify F2 pupae of interest

Timing: 5–10 min every hour for 4–5 h. Can be done daily until the target n number has been reached.

In this step, the F2 progeny will be collected at the pre-pupal stage and screened for the correct genetic markers.

10. Screen the F2 vials and collect all the TM6b negative pre-pupae at 0 h APF (after puparium formation) using a damp small brush. This step lasts approximately 10-min. Brown pupae can be discarded or left attached to the sides of the vials.
    a. The pre-pupal phase is a short transitional phase of approximately 30-min, between the larval and the pupal stages. Pre-pupae, unlike larvae, are immobile and are attached to the side of the vial, and unlike pupae, have a white/pale-yellow color (Figure 4A). White pre-pupae are designated to be at 0 h APF.
    b. Pupae carrying the TM6b balancer are identified by their short tubby phenotype (Table 2, Figure 4B). By selecting against TM6b, the collected pupae will carry the UAS-transgene, Pnr-Gal4 and UAS-GFP:moe.

⚠ CRITICAL: Flies must be collected at 0 h APF to be able to accurately determine the age of the pupa before imaging the next day.

11. Place the pupa on a petri dish covered with tissue paper soaked in water. Clearly label the exact time the pupa was collected and the genotype. This will allow you to correctly age the pupae prior to mounting.
12. Screen the collected pre-pupae for fluorescent markers.
    a. Discard CyO-GFP positive flies. These flies can be easily identified by their ubiquitous GFP expression (Table 2, Figure 4C). CyO-GFP negative flies will possess both the FRT site and *lgl* mutant allele.
    b. Discard GFP positive salivary glands. The salivary glands are two parallel tubes located on the ventral side of the pupa (Figure 4D). Pnr-Gal4 drives UAS-transgene expression (including UAS-GFP:moe) in the notum and salivary glands (Giagtzoglou et al., 2005). Flies that possess tubGal80 will repress Gal4 activity in the salivary glands, thus this tissue should be UAS-GFP:moe negative.
    c. Pupae of interest must be (1) non-tubby, and (2) GFP-positive only in small clones in the notum area. Entirely non-fluorescent pupae must also be collected, as the clones may be too small for detection when using a fluorescence dissecting stereomicroscope.

Table 2. Balancer chromosomes and genetic markers

Name	Chromosome	Type	Phenotype
CyO-GFP (Curley of Oster)	II	Balancer chromosome	Curly wings and ubiquitous GFP
MKRS	III	Marker	Stubble
TM6b (In(3LR)TM6)	III	Balancer chromosome	Tubby (short, fat body) and Humeral (extra macrochaetes)

13. Incubate the pupae carrying the correct markers at 29°C overnight.
    a. Pupae are ready to mount from 12 h APF. Prior to 12 h APF epithelia and puparium are attached together, therefore removal of the pupal case is not possible before this stage.
    b. Pupae are imaged between 20–24 h APF. We found these stages to be the most ideal for the study of tumor progression as the tissue is in a post-mitotic stage. At 29°C, pupae reach this age within approximately 18-h.
    c. Pupae that are older than 24 h APF can still be imaged. However, macrochaetae specification and eventually hair growth can potentially interfere with the observation of phenotypes.

Optional: We recommend collecting white pre-pupae every hour between 12–4 p.m. Pupae will then be ready for imaging the next morning.

Pupa mounting for live imaging

Timing: 1 h

This section describes the steps for mounting the pupa and the preparations needed for high resolution confocal imaging. It is recommended that you mount multiple pupae, ready for imaging. Image the pupae from oldest to youngest, since the pupae continue to age whilst mounted. Always image control pupae that are the same age as the experimental pupae.

14. Assemble the slides (Figure 5, Methods video S1)
    a. Grab a microscope slide and place double sided sticky tape lengthways along the center of the slide (Figure 5B).
    b. Remove the protective paper to uncover the upper sticky side.
    c. Assemble 2 stacks of approximately 3–5 square coverslips (22 × 22 mm). Use nail polish to glue the square coverslips together. The number of coverslips used will depend on the size of the pupa, which does vary. Usually, the posterior side of the animal is larger than the anterior and may require an additional coverslip.
    d. Use nail polish to glue the stack of coverslips to the anterior and posterior ends of the microscope slide. This will create a bridge to prevent squashing of the pupa once the 22 × 50 mm coverslip is placed, in step 18.
15. Place the pupa on the center of the slide, on top of the sticky tape, with the ventral side facing down. Wait approximately 10-min for the pupa to fully attach to the tape. This will allow the immobilization of the pupa during imaging.

16. Carefully remove a small region of the pupal case with tungsten forceps to expose the notum (see Figures 5A and 5B, Methods video S1).
17. Spread one side of a rectangular coverslip (22 × 50 mm) with a thin layer of 10S voltalef injection oil to create an interface between the coverslip and the exposed notum.
18. Place the coverslip on top of the pupa, the side covered with oil facing down.
    a. The rectangular coverslip should be resting on the square coverslip stacks from step 14.

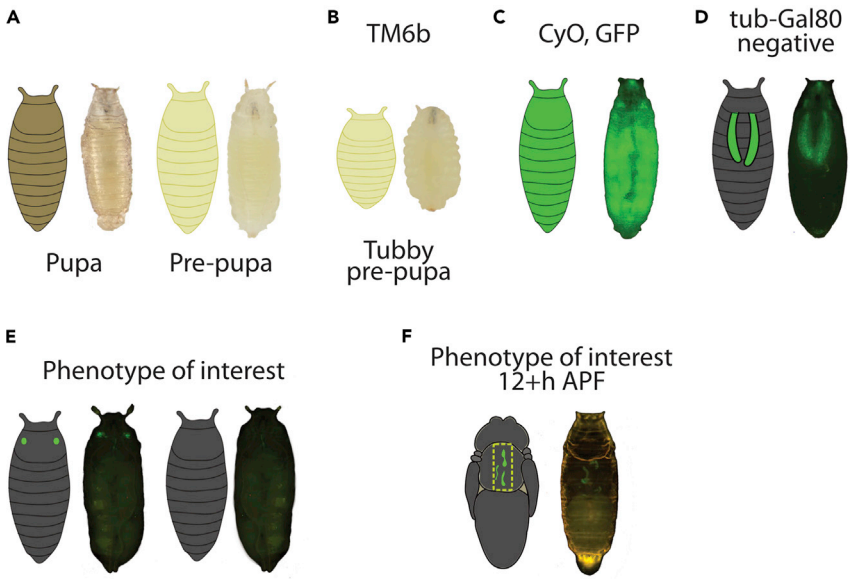

Figure 4. Pupa screening

Representative images of pupae expressing different markers, with emphasis on the selection of animals due to the presence/absence of genetic markers. An image is accompanied by a cartoon representation to emphasize the phenotypes to be identified.

(A) The pre-pupal stage is a short transitional phase between the larval and the pupal stages. Pre-pupae, unlike larvae, are immobile and are attached to the side of the vial, and unlike pupae, have a white/pale-yellow color. White pre-pupae are designated to be at 0 h APF (after puparium formation); flies should be collected at the pre-pupal stage.

(B–D) Phenotypes to discard. (B) TM6b positive pupae are tubby (shorter and fatter than wild-type); collect only TM6b negative pupae. (C) Discard Cyo-GFP pupae. These are easily identifiable as they express ubiquitous GFP. (D) Discard pupae with fluorescent salivary glands, as they did not segregate tub-Gal80.

(E and F) Phenotype of interest at pre-pupal stages (E) and at imaging stages (≥12 h APF) (F). Pupae of interest must be (1) non-tubby, and (2) GFP-positive only in small clones in the notum area. Entirely non-fluorescent pupae must also be collected, as the clones may be too small for detection when using a fluorescence dissecting stereomicroscope.

b. Verify that the rectangular coverslip is touching the notum (Figures 5C and 6). An interface on the notum (and occasionally also the head) should be clearly visible. Avoid forming air-bubbles between the notum and the coverslip.
c. Adjust the bridge if necessary. Add or remove square coverslips from either side of the stack if necessary. Troubleshooting
d. Once happy with the size of the interface, fix the long coverslip to the square coverslip stacks using nail polish.
e. Keep assembled slides in a humid atmosphere, to avoid animal desiccation, until ready for imaging.

Confocal live imaging

Timing: 3 h

This section describes the settings used for live imaging of tumors using a confocal microscope.

19. Equip the confocal microscope with a 40× oil immersion objective and a 488 nm laser.
20. Carefully identify and focus the GFP positive clones on the dorsal thorax of the pupa (Figure 4F).
    a. The animal should be GFP negative. Only clusters of cells in the dorsal thorax of the pupa should be GFP:moe positive. Troubleshooting

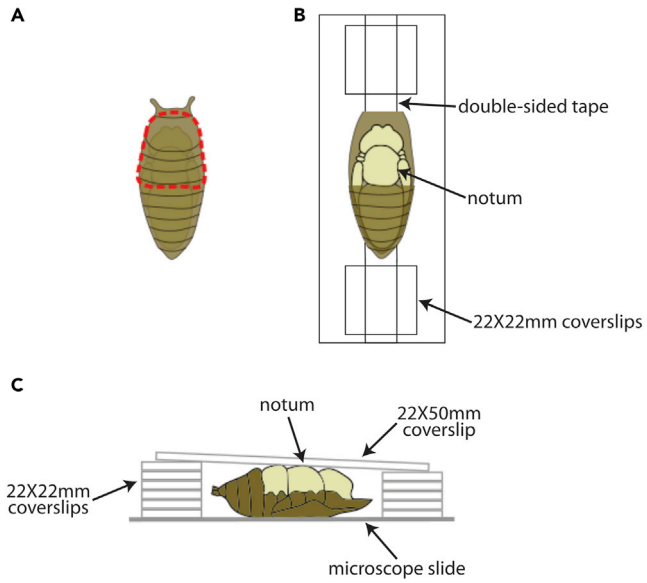

Figure 5. Pupa mounting for live imaging

(A and B) Pupal case removal. The brown pupal case interferes with the fluorescent signal and must be removed before imaging. Use forceps to uncover the head and dorsal thorax region of the animal. (A) Use tungsten forceps to pull the pupal case away from the pupa body, red dashed lines indicate the recommended cut lines. (B) Only uncover the head and dorsal thorax region of the animal. It is important to handle the pupa gently and pull the case without piercing the body of the pupa; the animal must be alive at the time of imaging.

(C) A pupa mounted and ready for imaging. Double sided sticky tape is used to glue the pupa to the microscope slide. Stacks of square coverslips on the edges of the slide form a bridge to prevent squashing. A rectangular coverslip with a thin layer of immersion oil is placed on top of the square coverslip stacks. Refer to steps 14–18 of this protocol.

21. For initial screening, the whole notum should be imaged with a single z-stack (use two z-stacks if the clones are very large and do not fit one field of view). Set the confocal microscope to take optical slices every 1 μm, from the cuticle to the basal lamina (until no GFP:moe signal is observed). You should image the entire dorsal thorax epithelial sheet at a resolution of 1024 × 1024 (375 × 375 μm).
22. Image at least 5 animals per genotype.
    a. This model is highly reproducible, we have found that 5 animals provide statistically relevant data.
    b. Tumors generated with different background genes may require more animals per genotype. Determine the most appropriate sample size by performing a power calculation.

EXPECTED OUTCOMES

Once the 3D confocal images have been generated, proceed to image analysis. We recommend creating a spreadsheet to keep record of the phenotypes analyzed. This protocol is well suited for large-scale genetic screens and in-depth characterization of individual genotypes. Depending on the type of study, qualitative, semi-quantitative and quantitative analysis of phenotypes can be performed. We have previously used this model to analyze 33 different phenotypic categories; see Figure 7 and the quantification and statistical analysis section of this protocol for more details. Additionally, refer to Figure 8 for an example of the characterization of cohesin complex subunits in tumor progression using this model.

Once interesting phenotypes have been identified, different confocal settings should be used (e.g., higher magnification, and/or higher resolution) to obtain high quality images of your specific phenotype. Time-lapses can be acquired in addition to the 3D confocal images. Time-lapses can be used

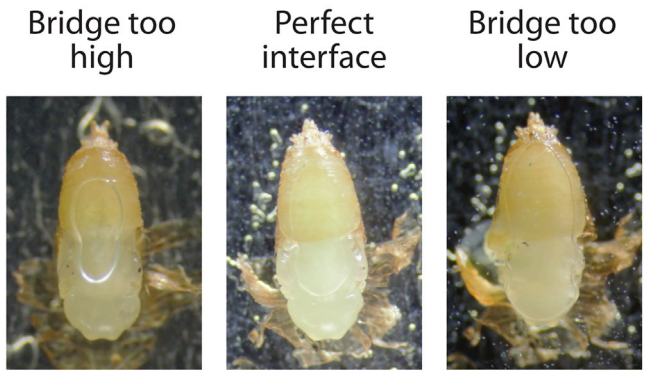

Figure 6. Oil interphase for confocal imaging

Examples of the most common mistakes during pupa mounting.

(A) The square cover slip bridge is too high. The oil interphase on top of the notum is very small and either only a very small area of the notum can be imaged, or the images will appear blurry. Remove one cover slip at a time until the interphase reaches the ideal size (see B).

(B) Correct mounting, the number of square cover slips is ideal, and the rectangular cover slip is in contact with the entire notum.

(C) The square cover slip bridge is too low. The rectangular cover slip will be too close to the pupa and it will be squashed and perhaps damaged. Animals that have been damaged due to squashing cannot be used.

for the study of dynamic processes such as cell division, cell invasion, protrusion extension and retraction, see (Canales Coutiño et al., 2020; Cohen et al., 2010; Couto et al., 2017; Georgiou et al., 2008; Georgiou and Baum, 2010). Additionally, the pupal notum can be removed from the animal and maintained in the presence or absence of drugs, fixed and stained, or processed for electron microscopy (Georgiou et al., 2008; Georgiou and Baum, 2010). Finally, additional reporters can be used, other than or as well as GFP:moe, to study different cellular structures, for example histone or tubulin markers to study cell division (Canales Coutiño et al., 2020).

QUANTIFICATION AND STATISTICAL ANALYSIS

1. Semi-quantitative analysis. Suited for large-scale screens. Refer to Table 3 and Figure 7 for example phenotypes to analyze and how to identify them.
   a. Create a database file to input all phenotypes and scores. See (Canales Coutiño et al., 2020) and also https://flycancerscreen.nottingham.ac.uk/ for examples of possible phenotypic categories and scoring approaches.
   b. Compare each phenotype against the background mutation. For example, for many phenotypic categories we used a scoring system that reflected the fact that the additional genetic lesion (in our case gene knockdown) could affect specific aspects of tumor behavior either positively or negatively. We gave lgl^4 phenotypes (the background mutation) a score of 0. We then scored animals with additional gene knockdown and used a scoring range of +2 to −2, where +2 strongly enhanced the lgl^4 phenotype, −2 strongly inhibited the lgl^4 phenotype, and 0 had no effect on the lgl^4 phenotype (Table 3 and Figure 7).
      i. We recommend that at least two different scientists perform a blind scoring of each animal.
      ii. Use averages of scores for data interpretation thereby reducing bias.
   c. Use R software to identify the statistically significant genes.
      i. Genes with a mean score above (positive) or below (negative) the interquartile range (IQR) from the median can be selected as target genes.
2. Quantitative analysis.
   a. Perform exact counts or measurements using imageJ/FIJI. You can download this software here: https://imagej.net/Fiji
      i. Phenotypes that are easily quantified include cell division, cell invasion and clonal area.
   b. Compare the genotype of interest against the control.

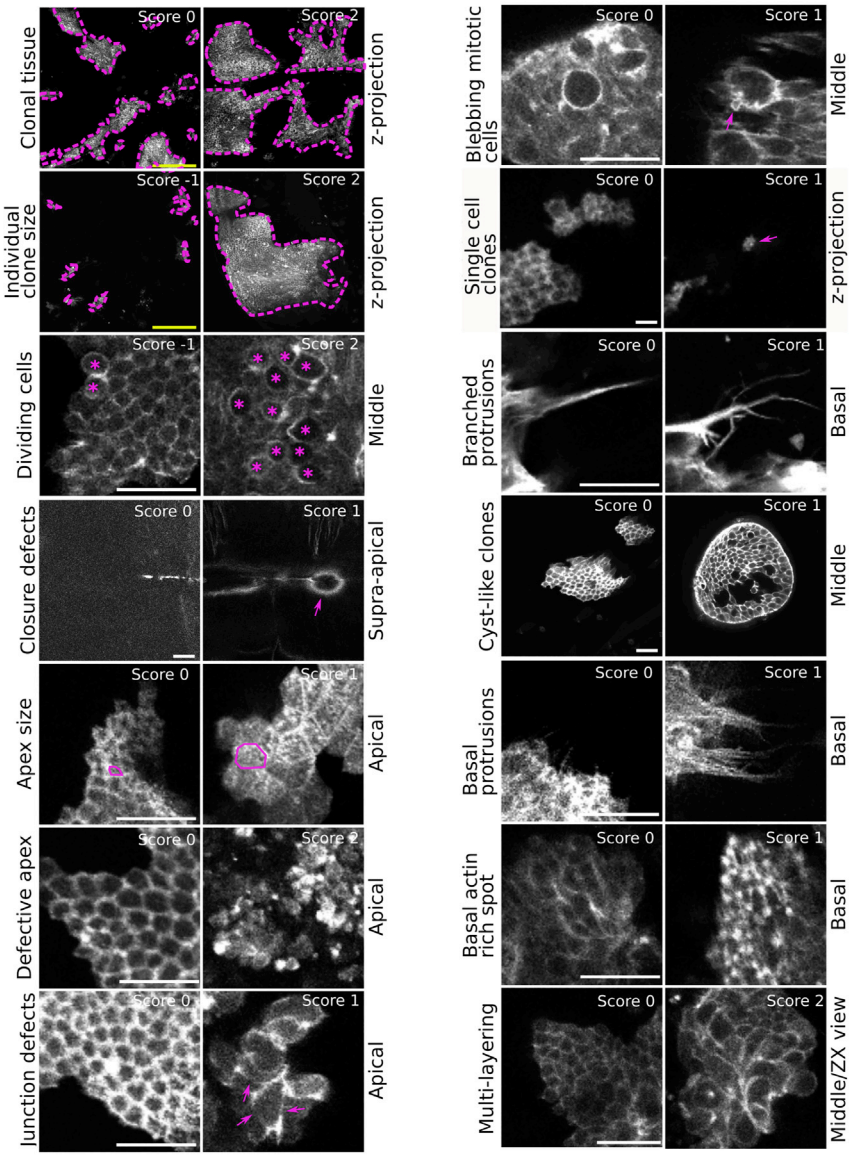

Figure 7. Expected phenotypes and scoring system

Representative examples of phenotypes that can be analyzed. The phenotype is specified at the left of each image. The z position within the cell is written at the right of each image. Refer to Table 3 for a detailed description of each phenotype and what the scoring system represents. Magenta dashed line, arrows, and asterisks point to the specific cells that represent the phenotype of interest. White scale bars: 20 μm, yellow scale bars: 100 μm.

c. Perform the most appropriate statistical analysis to determine statistical significance.

LIMITATIONS

This model provides high flexibility and can be adapted to the study of many different genotypes. However, the expression of transgenes that are on the same chromosome as the background gene is not practical for large-scale genetic screens (i.e., in this protocol the background gene is *lgl*^{4}, and transgene expression in chromosome II cannot be achieved by a simple cross scheme).

Additionally, the use of Pnr-Gal4, localized on the 3rd chromosome, restricts the use of background mutations to genes localized on the X or 2nd chromosome, to allow homozygous mutant clones to

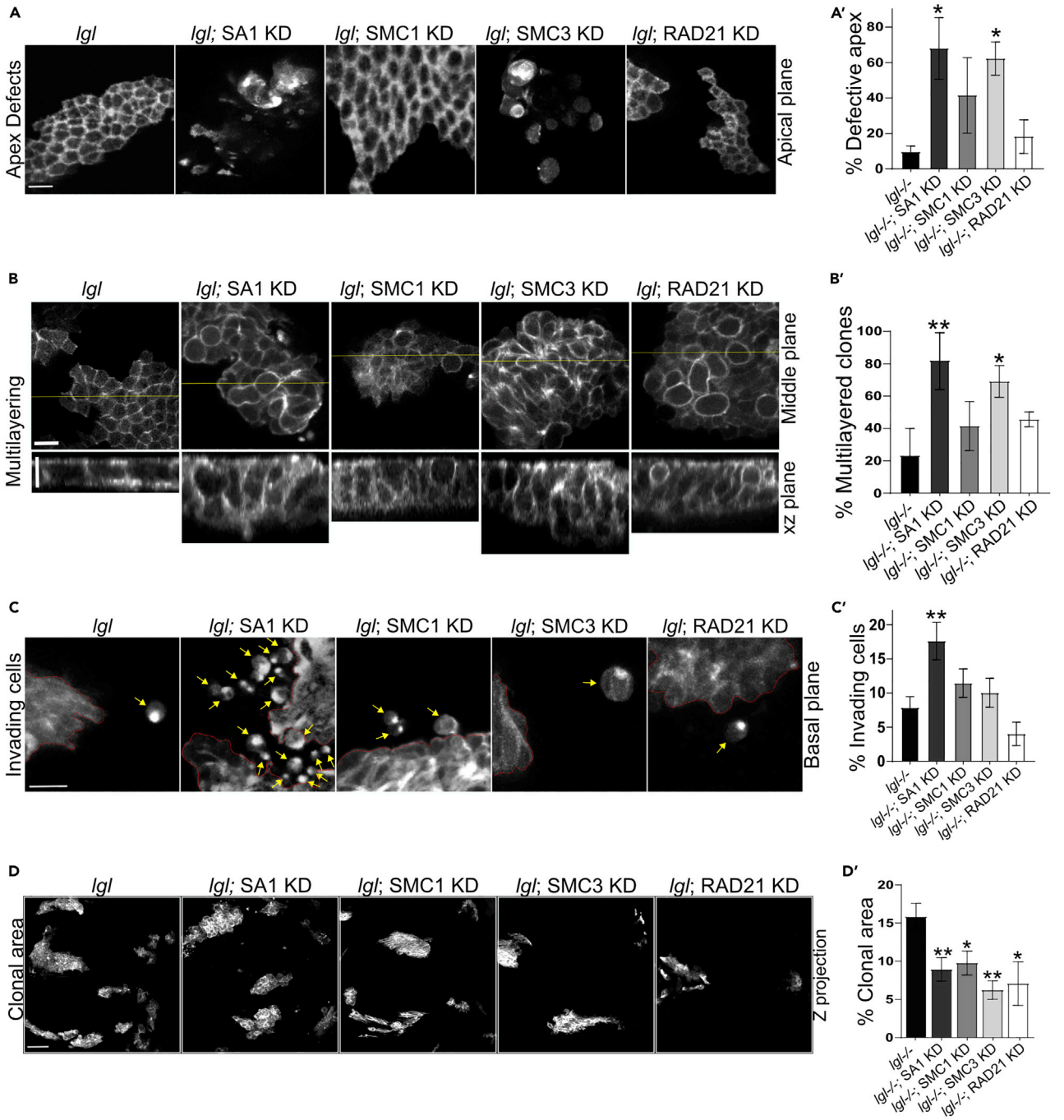

Figure 8. Cohesin complex subunit characterization

GFP:moe positively labeled *lgl* mutant clones with additional knock down of a specific cohesin complex subunit (SA1, SMC1, SMC3, or RAD21), as specified at the top of each image.

(A) Example images showing the apices of mutant cells. SA1 and SMC3 KD clones lost normal geometrical apical shape. (A′) Quantification of defective apex from (A).

(B) Example images at an intermediate confocal plane (top) and an orthogonal view of the z-stack (bottom). Multilayered clones were frequently observed with cohesin subunit KD. (B′) Quantification of multilayered clones from (B).

(C) Example images at a basal confocal plane. SA1 KD increased the number of invading cells. (C′) Quantification of invading cells from (C).

(D) Sample images of Z projection from the apex to the base of the clones, highlighting clonal area. (D′) Quantification of clonal area from (D). Scale bars: 10 μm. Statistical analysis: Student's t test. *p<0.05. **p<0.01. Error bars represent ± SEM. (B) and (C) reprinted with permission from Canales Coutiño et al. (2020).

Table 3. .Phenotypes and scoring system

Category	Data type	Scoring system	Description
No clones	Yes/No	No (0), Yes (1)	No labeled cells are observed
Not developed	Yes/No	No (0), Yes (1)	Animal did not reach the pupal stage (lethal)
Malformed	Yes/No	Normal (0), malformed (1)	Animal was too malformed to mount
Clonal tissue	Number	0–5% (−2), 5–15% (−1), 15–35% (0), 35–50% (1), 50% + (2)	Area covered by the labeled cells
Individual clone size	Number	mostly small (−1), mixture/mostly medium (0), mostly large (1), very large (2)	Size of the individual clones
Dividing cells	Number	none (−2), little (−1), medium (0), lots (1), extensive (2)	Number of cells in mitosis, identified by the mitotic cell rounding
Invading cells	Number	none (−2), very few (−1), medium (0), lots (1), extensive (2)	Number of cells that detached from the epithelium basally
Type of invasion	Number	mostly non-polarized (−1), mixture (0), mostly polarized (1)	Describes the actin localization within the invading cell. Polarized describes the accumulation of actin on one side of the cell.
Cuticle	Yes/No	normal (0), deep folding (1)	Folding in the supracellular cuticle
Closure defects	Yes/No	No (0), Yes (1)	Failure in the dorsal closure process.
Apex size	Number	very small (−2), small (−1), normal (0), large (1)	Size of the apical area of the cells
Defective Apex	Number	none (0), up to 50% (1), >50% (2)	Amount of apex that lost their geometrical shape
Junction defects	Yes/No	No (0), Yes (1)	Junctional breaks
Cell body (rounding)	Number	none (−1), 0–15% (0), 15–50% (1), 50% (2)	Number of rounded cells that lost their columnar shape
Polarized cytoskeleton	Number	none (−1), very few (0), medium (1), lots (2)	Non-symmetrical actin accumulation
Basal bundles	Yes/No	No (0), Yes (1)	Formation of protrusions bundles
Long apical/intermediate protrusions	Yes/No	No (0), Yes (1)	Length of apical/intermediate protrusions
Blebbing mitotic cells	Yes/No	No (0), Yes (1)	Presence of blebs in mitotic cells
Thick protrusions	Yes/No	No (0), Yes (1)	Protrusion width
Bright protrusions	Yes/No	No (0), Yes (1)	Protrusion brightness
Branched protrusions	Yes/No	No (0), Yes (1)	Presence of branches in the protrusions
Cyst-like clones	Yes/No	No (0), Yes (1)	Presence of rounded cyst-like clones
Apoptotic	Number	none(0), little (1), medium (2), lots (3)	Apoptotic estimation by identifying ruptured, fragmented or highly abnormal cells.
Basal protrusions	Number	absent (−3), small (−2), normal (−1), long (0), very long (1)	Length of basal protrusions
Basal actin rich spot	Number	No (0), Yes (1)	Actin rich spots present basally
Multi-layering	Number	none (−1), 0–15% (0), 15–50% (1), 50+% (2)	Loss of contact inhibition, presence of cells growing on top of each other
Cell length	Number	short <7 um (−2), normal 8–12 um (−1), long (0) 13–19 um, very long (1) 20 um+	Cell length in microns (from apex to basal)
Single cell clones	Yes/No	No (0), Yes (1)	Presence of single cell clones

be generated. This limitation can be overcome by using a different Gal4 driver (e.g., tub-Gal4), given that its pattern of expression will be restricted to the Ubx-flp region. However, this modification of the protocol should be optimized since we have not tested it.

TROUBLESHOOTING

Problem 1

Flies express incorrect or unexpected genetic markers. This could be due to an incorrect genotype in the original stock, cross contamination and/or accidental mixing of progeny with the parents.

Potential solution

Always verify the genetic markers of the fly stocks before use. Be very careful of cross contamination during fly flipping to new vials of food and when analyzing flies on the CO2 pad. Do not keep genetic crosses in the same vial for longer than 8 days and do not collect progeny from vials older than 18 days to prevent parents mixing with the progeny.

Problem 2

No clones are formed, instead the entire dorsal thorax is GFP:moe positive.

Potential solution

Carefully select pupae before imaging. Salivary glands must be GFP:moe negative (Figure 4), otherwise tub-Gal80 was not segregated to the fly and the transgenes will be expressed in the wild type cells.

Problem 3

No animals of the required genotype are observed

Potential solution

This would indicate lethality. It is possible that expression of the UAS-transgene, either alone, or in combination with the underlying mutation, causes lethality at developmental stages. One way around this is to rear the flies at lower temperatures. The Gal4/UAS system is known to be temperature sensitive, therefore lower expression of the UAS-transgene can be achieved by keeping flies at lower temperatures.

Problem 4

Blurry confocal images.

Potential solution

Correct pupal mounting is critical for high resolution imaging. Each pupa has a slightly different size and the coverslip bridge must be carefully adjusted for each fly. If the images are blurry, verify that the rectangular (24 × 50 mm) coverslip and the immersion oil is in contact with the dorsal thorax of the fly, creating an adequate interface (Figure 6).

Problem 5

Animal contents are released from the pupa.

Potential solution

Animal contents will seep from the animal if the animal is accidentally stabbed during mounting or if the square cover slip bridge is too low for the pupa, thereby squashing the animal. Always handle the pupa with care. Refer to Methods video S1 and Figure 6 of this protocol for a reference on how the pupae should be handled during mounting.

Problem 6

Pupa has shrunk (possibly leading to no, or a very small, interface) or looks highly wrinkled during confocal imaging.

Potential solution

This is likely due to animal desiccation. The pupa should be kept in a humid atmosphere during live imaging. Refer to steps 11 and 18 of this protocol for detailed information.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Marios Georgiou marios.georgiou@nottingham.ac.uk

Materials availability

Unique fly lines generated in the lab, but not deposited in stock centers, are available upon request.

Data and code availability

All the data generated in the original study is available at https://flycancerscreen.nottingham.ac.uk/

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100672.

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AUTHOR CONTRIBUTIONS

B.C.C. and M.G. wrote the paper. E.S. and Z.M. helped with animal imaging for the figures and movie.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Canales Coutiño, B., Cornhill, Z.E., Couto, A., Mack, N.A., Rusu, A.D., Nagarajan, U., Fan, Y.N., Hadjicharalambous, M.R., Castellanos Uribe, M., Burrows, A., et al. (2020). A genetic analysis of tumor progression in *Drosophila* identifies the cohesin complex as a suppressor of individual and collective cell invasion. IScience *23*. https://doi.org/10.1016/j.isci.2020.101237.

Cohen, M., Georgiou, M., Stevenson, N.L., Miodownik, M., and Baum, B. (2010). Dynamic filopodia transmit intermittent Delta-Notch signaling to drive pattern refinement during lateral inhibition. Dev. Cell *19*, 78–89. https://doi.org/10.1016/j.devcel.2010.06.006.

Couto, A., Mack, N.A., Favia, L., and Georgiou, M. (2017). An apicobasal gradient of Rac activity determines protrusion form and position. Nat. Commun. *8*, 15385. https://doi.org/10.1038/ncomms15385.

Georgiou, M., and Baum, B. (2010). Polarity proteins and Rho GTPases cooperate to spatially organise epithelial actin-based protrusions. J. Cell Sci. *123* (Pt 7), 1089–1098. https://doi.org/10.1242/jcs.060772.

Georgiou, M., Marinari, E., Burden, J., and Baum, B. (2008). Cdc42, Par6, and aPKC regulate Arp2/3-mediated endocytosis to control local adherens junction stability. Curr. Biol. *18*, 1631–1638. https://doi.org/10.1016/j.cub.2008.09.029.

Giagtzoglou, N., Koumbanakis, K.A., Fullard, J., Zarifi, I., and Delidakis, C. (2005). Role of the Sc C terminus in transcriptional activation and E(spl) repressor recruitment. J. Biol. Chem. *280*, 1299–1305. http://www.jbc.org/content/280/2/1299.abstract.

Greenspan, R.J. (2004). Fly pushing : the theory and practice of *Drosophila* genetics (Cold Spring Harbor Laboratory Press).

Lakovaara, S. (1969). Malt as culture medium for *Drosophila* species (Drosophila Information Service), p. 44.

Lee, T., and Luo, L. (2001). Mosaic analysis with a repressible cell marker (MARCM) for *Drosophila* neural development. Trend. Neurosci. *24*, 251–254. https://doi.org/10.1016/s0166-2236(00)01791-4.

Xu, T., and Rubin, G.M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. Development (Cambridge, England) *117*, 1223–1237.