

参赛学生姓名： 蓝玖月

中学： 北京市第八中学

省份： 北京市

国家/地区： 中华人民共和国

指导老师姓名： 倪建泉，侯越

指导老师单位： 清华大学，北京市第八中学

论文题目： The preliminary construction of a novel DSRCT

model in *Drosophila* by integrating the *EWSR1::WT1* fusion

gene and *yki*^{3SA} into one strain

The preliminary construction of a novel DSRCT model in *Drosophila* by integrating the *EWSR1::WT1* fusion gene and *yki*^{3SA} into one strain

Lan jiuyue Beijing No.8 High School

Supervisor: Professor Ni jianquan Tsinghua University

Hou yue Beijing No.8 High School

Abstract

Desmoplastic small round cell tumor (DSRCT) is a highly aggressive sarcoma with a poor prognosis. It arises from a chromosomal translocation that produces the *EWSR1::WT1* (*E::W*) fusion gene, which encodes an aberrant transcription factor. Effective therapies remain unavailable, largely owing to the scarcity of disease models. A recent study of our lab aiming to establish a DSRCT model in *Drosophila* found that inserting the *E::W* fusion gene alone into the fly genome caused tissue shrinkage and limited polarity disruption in wing imaginal discs, rather than the proliferation and marked polarity disruption that had been predicted. To reproduce the predicted phenotype and thereby construct a more comprehensive DSRCT model, a genetic crossing experiment was designed using the UAS/*Gal4* binary system together with balancers and genetic markers, yielding a novel line carrying both the *E::W* fusion gene and *yki*^{3SA}, a transcriptional co-activator of cell growth. As a result, the desired line was obtained and epithelial polarity disruption was enhanced; however, the shrunk tissues were not rescued, indicating that the highly toxic *E::W* fusion protein may require lower expression levels to manifest its potential proliferative or tumorigenic effects. This study provides new evidence for the high toxicity of the *E::W* fusion gene and the cellular reprogramming effect of *yki*^{3SA} and offers deeper insights into the construction of *Drosophila* models of DSRCT.

Keywords: DSRCT; *yki*^{3SA}; *Drosophila* model; UAS/*Gal4* system; genetic crossing

Outline

1. Introduction	1
1.1 Overview of DSRCT	1
1.2 Current understanding of the pathogenesis of DSRCT	1
1.3 Existing DSRCT disease models	2
1.4 Drosophila as a model organism	2
1.5 RNA interference techniques and UAS/Gal4 System	3
1.6 Markers and Balancers	4
1.7 Preliminary research in our lab	5
1.7.1 Transgenic construct	5
1.7.2 E::W alone causes aberrant loss or shrinkage of wings in Drosophila	5
1.8 The yki3SA Tumor Model	6
1.9 Significance	6
2. Materials and methods	7
2.1 Materials	7
2.1.1 Culture of Drosophila	7
2.1.2 PCR-Based Genotyping	8
2.1.3 Dissection of Wing Imaginal Discs and Slide Preparation	8
2.2 Methods	8
2.2.1 Routine Maintenance of Drosophila	8
2.2.1.1 Husbandry and stock maintenance.	8
2.2.1.2 Complete and partial transfer of cultures.	9
2.2.2 Collection of Virgin Females	9
2.2.3 Genetic Cross Scheme	10
2.2.4 PCR-Based Genotyping of Co-Inserted Lines	11
2.2.5 Dissection of Wing Imaginal Discs and Slide Preparation	12
2.2.5.1 Dissection of Wing Imaginal Discs	12
2.2.5.2 Slide preparation and nuclear staining.	12
3. Results and analysis	13
3.1 phenotypic changes in the genetic crossing experiment	13

3.2 The morphological changes in wing imaginal discs.....	13
3.2.1 Despite gene integration, wing discs shrink	13
3.2.2 Co-integrated wing discs present more intense epithelial polarity disruption	14
4. Discussion.....	15
4.1 Why the shrinkage? — Toxicity of E::W	15
4.2 Why the remarkable pattern disruption? — yki3SA in the Fat-Hippo-Warts signaling pathway	16
4.3 Why the combination?	16
5. Future perspectives.....	16
5.1 To optimize expression levels of E::W	16
5.2 To assess potential cooperating genetic alterations	17
References.....	18

1. Introduction

1.1 Overview of DSRCT

Desmoplastic Small Round Cell Tumor (DSRCT) is a rare and highly aggressive soft tissue malignancy with a poor prognosis and limited standardized treatment options.^[1] It is driven by the fusion oncogene *EWSR1::WT1* (*E::W*), which is a product of chromosomal translocation t(11; 22)(p13; q12). The cellular origin remains undetermined, though it is believed to derive from highly undifferentiated mesenchymal cells. It mostly occurs in young males, in primary sites of the abdomen and pelvis. Current mainstream therapies include surgery, chemotherapy, targeted therapy and immunotherapy. Due to its strong invasiveness and general insensitivity to immunotherapy, targeted therapy has become the main focus of recent studies. While multiple downstream targets have been found, the molecular mechanisms triggered by gene fusion have remained elusive.

1.2 Current understanding of the pathogenesis of DSRCT

The defining molecular characteristic of DSRCT is the chromosomal translocation resulting in fusion between the *EWSR1* and *WT1* genes, forming the *EWSR1::WT1* fusion oncogene. *E::W* combines *EWSR1*'s transcriptional activation domain with *WT1*'s DNA-binding domain, creating an aberrant transcription factor. The chromosomal breakpoints in DSRCT patients vary, with the type 7/8 fusion (exons 1-7 of *EWSR1* fused to exons 8-10 of *WT1*) being most common, present in over 50% of cases.

Alternative splicing generates two isoforms (with/without KTS). Early studies show that the -KTS variant induced transformation in NIH3T3 cells². However, more recent studies reported that neither isoform transforms normal human mesothelial cell line LP93 or wild-type MEFs⁴, but both enhance colony formation in *p53*-mutant MEFs⁴. Yet, *p53* mutations are rare in DSRCT cases⁴.

E::W is essential for tumor cell survival, as its silencing inhibits proliferation and induces apoptosis^{5,6}. Mechanistically, *E::W* drives oncogenesis by: (1) activating proliferative pathways through targets like MYC⁵ and CCND1⁷, (2) modulating DNA damage response, (3) affecting growth factor signaling (*IGF2*, *FGFR4*), (4)

potentially activating *Wnt* signaling.

Despite these advances, current understanding of *E::W* mechanisms remains limited. The precise transcriptional networks regulated by *E::W*, tissue-specific cofactors enabling oncogenesis, and requirements for cooperating mutations are poorly understood.

1.3 Existing DSRCT disease models

Disease models such as cell lines, organoids, and patient-derived xenografts (PDXs) are key for investigating tumor molecular mechanisms and drug screening. However, DSRCT models remain severely limited. Only two in vitro models of DSRCT have been published: one primary patient-derived^[2] and one PDX-derived cell line^[3]. As for in vivo models, several PDX models have been established by transplanting DSRCT specimens into the flank of mice^[3]. Despite great contribution made, the in vitro models lack physiological context and the PDXs have unclear genetic backgrounds, lack of standardization and low success rate.

Therefore, the field notably lacks transgenic animal models, which would enable systematic investigation of disease mechanisms in physiologically relevant contexts and facilitate higher-throughput therapeutic screening.

1.4 *Drosophila* as a model organism

Drosophila serves as an ideal model organism in genetics and cancer research because its genome is highly homologous with humans (sharing around 80% of genes, covering about 75% of human disease genes). Therefore, many cancer-related pathways are conserved in *Drosophila*. In addition, the short life cycle, the ease of culture, the small genome size and extensive genetic toolkit make it ideal for studying disease mechanisms and therapeutic strategies.

A recent study demonstrates *Drosophila*'s value in fusion gene-driven sarcoma research by establishing a transgenic Ewing's Sarcoma model^[4]. Flies expressing the *EWS::FLI* fusion gene showed transcriptomic changes paralleling human EwS signatures and interacted with conserved protein complexes. This confirms that *Drosophila* can effectively recapitulate key molecular features of fusion oncogene-driven malignancies.

1.5 RNA interference techniques and UAS/Gal4 System

RNA interference (RNAi) refers to the silencing of gene expression in which single- or double-stranded RNA within the cell degrades the endogenous mRNA to which it is complementary. Building on this pathway, a technique has been developed that permits exogenous DNA integrated into the genome to be transcribed conditionally; the resulting exogenous RNA pairs with endogenous mRNA by base complementarity and thereby suppresses translation of the mRNA or targets it for degradation. This approach is known as conditional transgenic RNA interference (RNAi) technology.

Since its inception, this technology has progressed from the first generation through the second and third generations to the next-generation transgenic RNA interference system^[5-7]. The present study employs the new-generation transgenic interference technology developed by the *Drosophila* laboratory of Tsinghua University. In this system, a sh plasmid is injected into *Drosophila* embryos that express the integrase phiC31 and carry an integration site such as attP40, allowing the plasmid to be integrated into the genome at a defined locus^[8]. The integrated plasmid transcribes a pri-miRNA bearing a stem-loop structure, which is cleaved by the nuclear ribonuclease Drosha to remove its terminal sequences. The resulting pre-miRNA is exported from the nucleus and processed by Dicer1 into mature miRNAs of 22–24 nt. One strand of the mature miRNA can be incorporated into the Ago1-containing silencing complex; when its sequence is complementary to the 3' untranslated region (UTR) of an mRNA, translation of the target gene is repressed, whereas when it is highly complementary to the coding sequence, the mRNA is degraded. The new-generation transgenic interference technology offers several advantages, including high transcription levels, low basal expression, a low off-target rate, low toxicity and the capacity to clone multiple shRNAs into a single vector.

Although conditional transgenic RNAi was originally designed to silence gene expression conditionally at the post-transcriptional level, conditional gene expression can also be achieved by modifying the design of the plasmid. If the shRNA fragment is replaced with the gene of interest and the construct is integrated at the same site-specific locus through the procedure described above, the target gene can be transcribed in a conditional manner. Because the resulting transcript lacks a stem-loop structure, it is not cleaved by Drosha or processed by Dicer1; instead, it is exported from the nucleus

and translated directly into protein. Conditional expression of the target gene is thereby accomplished.

The conditional nature of conditional transgenic RNAi is realized through the UAS/*Gal4* binary system. Only when the *Gal4* protein and the UAS sequence are present simultaneously is the sequence downstream of UAS transcribed, while *Gal4* is expressed organ-, tissue- or cell-specifically, due to its specific enhancer.

As a transcriptional activator, *Gal4* can drive the transcription of DNA sequences downstream of the UAS element. As a result, after generating transgenic lines containing UAS-preceded genes of interest, conditional knockdown or expression of genes can be achieved by crossing these lines with *Drosophila* strains that express *Gal4* specifically in designated cells, tissues or organs.

In conclusion, the RNA interference techniques and the UAS/*Gal4* system serve as effective tools in silencing and activating designated genes conditionally.

1.6 Markers and Balancers

Accurately distinguishing among different genotypes is the central problem in *Drosophila* crossing experiments. Balancers and markers are effective tools that prevent recombination and allow flies of different genotypes to be told apart^[9]. With their help, the genotype of a fly can be read directly from its phenotype, which in turn makes it possible to design rational crossing schemes.

A salient feature of *Drosophila* genetics is that recombination is entirely absent in males. Although recombination is often reduced in the heterogametic sex of other species, in *Drosophila* it is exactly zero. While chromosomal recombination proceeds normally in females, however, the fly experimentalist's most distinctive tool — the balancer chromosome — can be exploited to select for the genotypes of interest. In a balancer, the order of an entire normal chromosome is scrambled by the numerous breaks and rejoins induced by radiation, so that during meiotic prophase it can no longer pair or recombine with its normal homolog. The concept of the balancer was first put forward by H. J. Muller, and the principle that multiply inverted chromosomes undergo little or no exchange with their homologs has held ever since.

Because balancers carry dominant and recessive mutations as markers, flies bearing

a balancer can be recognized by phenotype. Their transmission from parent to offspring can therefore be traced unambiguously, and, since they never recombine with their homologous chromosomes, the fate of the homolog can be inferred accordingly. This is the core principle underlying the design of *Drosophila* crossing schemes.

Balancers are available for the X chromosome and for chromosomes 2 and 3. On chromosome 2, for example, there is In(2LR)O, whose dominant marker is Curly wings (CyO); on chromosome 3, there are TM3, *Sb*, marked by Stubble (*Sb*, short bristles), and TM6B, marked by the dominant phenotypes of extra humeral bristles and a short body. Chromosome 4, by contrast, undergoes no recombination and therefore requires no balancer.

In addition, dominant and recessive markers can be used independently of balancers. *Sco*, for instance, is a dominant marker corresponding to the reduced-bristle phenotype and can be placed on the chromosome homologous to a balancer to report the fate of that homolog.

1.7 Preliminary research in our lab

1.7.1 Transgenic construct

To establish a *Drosophila* model of *E::W*-driven oncogenesis, our lab designed and constructed several transgenic constructs to express the *E::W* fusion protein in the wings of *Drosophila*. Given the prevalence of type 7/8 fusion variant in clinical samples, we selected this variant as the focus for our model. Similarly, the -KTS isoform has been chosen as the focus of this study due to its strong oncogenic effect compared with the +KTS isoform. Therefore, unless otherwise specified, “*E::W*” refers to the type 7/8 fusion variant -KTS isoform throughout this report.

1.7.2 *E::W* alone causes aberrant loss or shrinkage of wings in *Drosophila*

Various *Drosophila* tissues can be utilized to establish tumor models, with the most commonly used including the eyes, wings, intestine, etc. We crossed different tissue-specific *Gal4* driver lines with our constructed transgenic lines to overexpress *E::W*, then observed the resulting phenotypes. Previously, our lab found that when expressed under strong developmental drivers (*ptc-Gal4*, *en-Gal4*, *elav-Gal4*, *esg-Gal4*, *cg-Gal4*, *1824-Gal4*), the fusion protein induced embryonic lethality, even at reduced

temperatures (18°C), which normally decreases *Gal4* activity. Fortunately, the wing is one of the few tissues in which *E::W* can be expressed without causing embryonic or pupal lethality, although it leads to aberrant wing loss in adult *Drosophila* and shrinkage of wing imaginal discs in larvae.

Based on these findings, we suggest that the aberrant reduction of cells may relate to the overexpression of *E::W*, which surpasses the survival threshold of cells. In addition, other mutants may also be required to create the phenotype of the overproliferation of the tissue.

1.8 The *yki*^{3SA} Tumor Model

The Fat-Hippo-Warts signaling pathway regulates cell growth and comprises a series of genes; mutations or dysregulation of these genes cause tumorigenesis, an effect that has been verified in both *Drosophila* and mammals. In *Drosophila*, Yorkie (*Yki*) is a key gene of this pathway. When the three phosphorylation sites of *Yki* are mutated, the resulting *yki*^{3SA} allele greatly enhances the growth- and proliferation-promoting activity of the protein, which in turn drives cancer development^[10].

In recent studies, by cooperating with other factors causing neoplastic effect, *yki*^{3SA} displays a remarkable promoting effect on tumorigenesis. For example, the cooperation of Yorkie and *Ras* drives tumor progression, even though *Ras* paradoxically induces cellular senescence, which prevents tumorigenesis^[18]. Similar to *Ras*, *E::W* is also the major event of oncogenesis, yet negatively regulates the proliferation of cells due to its high toxicity.

Since *yki*^{3SA} promotes the growth and proliferation of cells, would the additional expression of *yki*^{3SA} in the *E::W* line rescue the phenotype of shrunk wing discs in *Drosophila* larvae?

Therefore, we conducted this study to introduce *yki*^{3SA} into the constructed *E::W*-inserted line based on genetic crossing techniques, wondering whether the co-insertion of *yki*^{3SA} and *E::W* would present the phenotype of overproliferation of wings, suggesting tumorigenesis in *Drosophila*.

1.9 Significance

Our proposed study would carry significant implications for DSRCT research and

potential therapeutic advancements:

First, establishing a *Drosophila* model of E::W-driven oncogenesis would address a critical gap in the field: the lack of accessible, genetically tractable in vivo models. Unlike existing cell lines and PDX models which are limited in availability and experimental flexibility, a *Drosophila* model would enable systematic investigation of E::W function in a whole-organism context with well-established genetic tools.

Second, such a model would provide a platform to rapidly identify and characterize signaling pathways dysregulated by E::W, potentially revealing novel therapeutic targets. The ability to perform large-scale genetic screens in *Drosophila* could accelerate discovery of interactions and pathway dependencies specific to E::W-driven oncogenesis that may be translatable to human disease.

Collectively, this work aims to establish a foundation for mechanistic understanding of E::W-driven oncogenesis and create a tractable platform for evaluating targeted therapeutic strategies, potentially benefiting patients with this rare but aggressive malignancy.

2. Materials and methods

2.1 Materials

2.1.1 Culture of *Drosophila*

- *Drosophila* culture vessels: polystyrene vials and bottles containing solid cornmeal-based medium, sealed with mite-proof latex foam plugs. (Provided by the laboratory; medium was confirmed free of fungal or bacterial contamination and showed no desiccation.)
- CO₂ delivery system: comprising a CO₂ cylinder (Beijing Huanyu Jinghui Jingcheng Gas), a bifurcated plastic tube connecting the cylinder to both a CO₂ gun and a porous CO₂ pad.
- Stereomicroscope (Olympus, model: SZX10)
- Feather (for gentle manipulation of anesthetized flies)
- Laboratory marker pen
- High-activity instant dry yeast powder (Angel, 500 g)
- Discarded *Drosophila* disposal solution (provided by the laboratory)

2.1.2 PCR-Based Genotyping

- 1.5 mL microcentrifuge tubes (Eppendorf tubes)
- Polypropylene pestles (for homogenizing individual flies)
- Sodium hydroxide (NaOH)
- Thermomixer (Eppendorf, model: Thermomixer compact)
- Tris-HCl buffer (pH 8.0)
- PCR primers (provided by the laboratory)
- 2× Taq PCR MasterMix (Taq mix)
- DNase-free distilled water (ddH₂O)
- Gradient PCR thermocycler (Eppendorf, model: Mastercycler nexus GSX1)
- DL5000 DNA Marker
- Gel documentation system with UV transilluminator

2.1.3 Dissection of Wing Imaginal Discs and Slide Preparation

- Coarse and fine Dumont forceps
- Glass 9-well plate
- Paraformaldehyde (PFA), phosphate-buffered saline (PBS), PBS containing 0.1% Tween-20 (PBST)
- Spiral mixer (Kylin-Bell, model: KB-3-D, 6 rpm)
- Fluorescence mounting medium
- Glass microscope slides and coverslips
- Inverted fluorescence microscope (Nikon, model: ECLIPSE Ti)

2.2 Methods

2.2.1 Routine Maintenance of *Drosophila*

2.2.1.1 Husbandry and stock maintenance.

All fly stocks were maintained at 25 °C and 56.5% relative humidity in a temperature-controlled incubator. Polystyrene vials were used for routine maintenance of most lines, each holding 50–100 adult flies; bottles, each accommodating 300–600 adults, were used exclusively for the genetic tool line (U7) among the parental stocks. For each cross, ten virgin females and five males were combined in a single vial at a 2:1 female-to-male ratio to reduce inter-male aggression.

2.2.1.2 Complete and partial transfer of cultures.

When the superficial layer of the medium began to loosen—an indicator of dense larval feeding—parental adults were either transferred wholesale to fresh vials or bottles, or sacrificed in the disposal solution. Progeny larvae subsequently pupated on the vessel walls. Pupal maturity was assessed by holding the vessel against a light source: pupae that had darkened and become translucent signaled imminent eclosion. Once approximately half of the pupae had eclosed, the surviving progeny were moved to fresh medium using the standard “fly flipping” technique. Briefly, the vessel was tapped firmly against the bench to dislodge adults from the walls, the foam plug was removed rapidly, and the open mouth of the old vessel was inverted over a fresh one so that living adults fell into the new vessel. Because dead flies adhered to the spent medium, they were not carried over. The entire transfer was completed within seconds to minimize escape, crushing by the plug, or contamination by wild flies.

When flies required phenotypic sorting, they were anesthetized with CO₂. The vessel was held inverted at an angle, and the nozzle of the CO₂ gun was inserted through the foam plug to deliver a brief pulse of CO₂. After the flies had fallen to the bottom, the vessel was tapped gently to prevent adhesion to the walls, the plug was withdrawn, and the flies were tipped onto the porous CO₂ pad for observation under a stereomicroscope. A feather was used to reposition individual flies for phenotypic scoring and cross setup^[11]. Following sorting, flies to be retained were guided into fresh vials with the feather, whereas unwanted flies were discarded into a killing jar. For either complete or partial transfers, the fresh medium could be scored crosswise with forceps and lightly dusted with yeast powder before introduction of flies to augment nutrition. The genotype and date of transfer were invariably recorded on the vessel wall.

2.2.2 Collection of Virgin Females

Female *Drosophila* are sexually unreceptive for the first 8 h following eclosion; females isolated within this window are referred to as virgins. Because an adequate supply of virgins is indispensable for controlled genetic crosses, virgin collection was performed as a daily routine. Given that eclosion predominates in the early morning, collections were initiated at approximately 08:00. When partial eclosion was noted, all adults were emptied from the vessel, and females exhibiting pale cuticle and a

characteristic dark abdominal spot (reflecting residual meconium) were selected as putative virgins and transferred to fresh, labeled vials. Collected virgins were aged for 2–3 days before being used in crosses.

2.2.3 Genetic Cross Scheme

Lines carrying the *E::W* fusion transgene inserted on the second chromosome and the *yki*^{3SA} transgene inserted on the third chromosome had been established previously in our laboratory. The genetic tool line U7, used for transgene integration, and the MS1096-*Gal4* driver line, which directs *Gal4* expression specifically to the wing imaginal disc, were obtained from the laboratory stock collection.

The crossing scheme proceeded as follows. U7 virgin females were crossed to *E::W* insertion males, and F₁ progeny exhibiting curly wings (*Cy*) and extra humeral bristles (*Hu*) were retained. In parallel, U7 virgin females were crossed to *yki*^{3SA} insertion males, and F₁ progeny exhibiting curly wings, extra humeral bristles, and wild-type (non-stubble) bristles were selected. The two F₁ populations were then intercrossed, and F₂ progeny carrying both curly wings and extra humeral bristles were retained as putative co-integrated lines. Individual F₂ males were crossed to MS1096-*Gal4* virgin females to generate F₃ progeny. After mating, each F₂ parent was sacrificed and its genotype confirmed by PCR amplification followed by agarose gel electrophoresis. F₃ progeny descended from a genotypically verified F₂ parent and exhibiting neither curly wings nor extra humeral bristles—that is, carrying both the *E::W* and *yki*^{3SA} transgenes in the absence of balancer chromosomes—were designated as tumor-bearing experimental flies. Third-instar F₃ larvae were harvested for dissection.

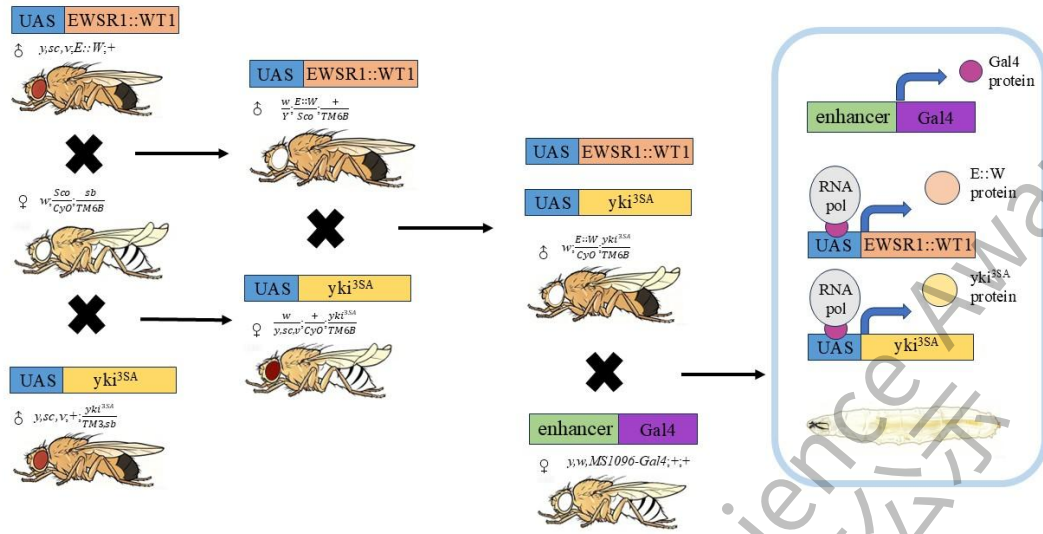


Figure 1. Genetic crossing scheme

2.2.4 PCR-Based Genotyping of Co-Inserted Lines

The appearance of white spots on the medium confirmed that the parental cross had been successful. Following the one-to-one crosses between co-integrated F₂ flies and MS1096-*Gal4* drivers, the F₂ parents were anesthetized and individually transferred to 1.5 mL microcentrifuge tubes. Each fly was frozen at -20 °C for 1 min and then homogenized thoroughly against the lower inner wall of the tube with a polypropylene pestle. Fifty microliters of 100 mM NaOH were added, and the homogenate was triturated several times before incubation at 95 °C for 10 min on the Thermomixer. Ten microliters of 1 M Tris-HCl (pH 8.0) were then added to neutralize the lysate.

Each PCR was assembled in a total volume of 50 µL containing 25 µL of 2× Taq MasterMix, 10 µL of ddH₂O, 2 µL each of forward and reverse primers (10 µM), and 1 µL of the cleared fly lysate as template. Thermal cycling was performed on an Eppendorf Mastercycler nexus GSX1 with the following program: an initial denaturation at 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; a final extension at 72 °C for 5 min; and a hold at 12 °C. Five microliters of DL5000 DNA Marker were loaded into the first well of an agarose gel, and 5 µL of each PCR product were loaded into adjacent wells. Electrophoresis was run for 10 min, after which the gel was visualized on a UV transilluminator and documented with a gel imaging system.

2.2.5 Dissection of Wing Imaginal Discs and Slide Preparation

2.2.5.1 Dissection of Wing Imaginal Discs

Four days after the parental crosses were established, third-instar larvae began wandering up the vessel walls, at which point they were collected with forceps for dissection.

A glass 9-well plate was positioned on the stage of a stereomicroscope, and the bilateral incident illumination was adjusted to evenly light the dissection field. Three adjacent wells were filled with sufficient PBS. A single larva was placed in the central well, where all dissection was performed; the well on one side served as a holding reservoir for dissected wing discs destined for imaging, and the well on the other side received discarded larval debris. Coarse forceps were held in the left hand and fine forceps in the right. The larva was grasped at approximately one-third of the body length from the posterior terminus and torn transversely into two fragments; the posterior portion was discarded. The coarse forceps were then used to immobilize the anterior fragment by its mouth hooks, while the fine forceps were drawn from the cut edge toward the head to gently peel back the transparent cuticle, everting the internal tissues and organs inside-out. The wing imaginal disc—a bilaterally symmetric, white, disc-shaped structure with superficial tracheal ramifications, typically situated near the larval head and often accompanied posteriorly by a smaller white disc, the leg imaginal disc—was identified among the everted viscera, detached with fine forceps, and transferred to the holding well.

2.2.5.2 Slide preparation and nuclear staining.

Dissected wing imaginal discs were gently transferred to microcentrifuge tubes containing sufficient paraformaldehyde (PFA) and fixed for 20 min at room temperature in the dark under a foil-covered lid. Following fixation, the discs were washed with ample 0.1% PBST and incubated for 10 min in the dark on a Spiral mixer. The discs were then immersed in a freshly prepared DAPI working solution (DAPI stock diluted 1:1001 in PBST, v/v) and incubated for 10 min in the dark on the Spiral mixer, followed by a final 10-min wash with 0.1% PBST under the same conditions.

For mounting, a drop of fluorescence mounting medium was dispensed onto the frosted end of a clean glass slide. Stained wing discs were aspirated from the

microcentrifuge tube and transferred to a PBS-filled well of the 9-well plate, where their number and position were confirmed under the microscope. Individual discs were then picked up with fine forceps and arranged within the mounting medium drop. After the position and orientation of each disc had been adjusted, a clean coverslip was lowered at an angle from one edge of the drop to minimize bubble formation. Slides were subsequently examined and imaged on an inverted research microscope.

3. Results and analysis

3.1 phenotypic changes in the genetic crossing experiment

The genetic crossing experiment in this study included four generations of *Drosophila*. As the figure shows, the phenotypes of *Drosophila* vary among generations, tracing the transmission of chromosomes. Thanks to the balancers and markers, with careful observation and categorization, acquisition of the desired line could therefore be ensured.

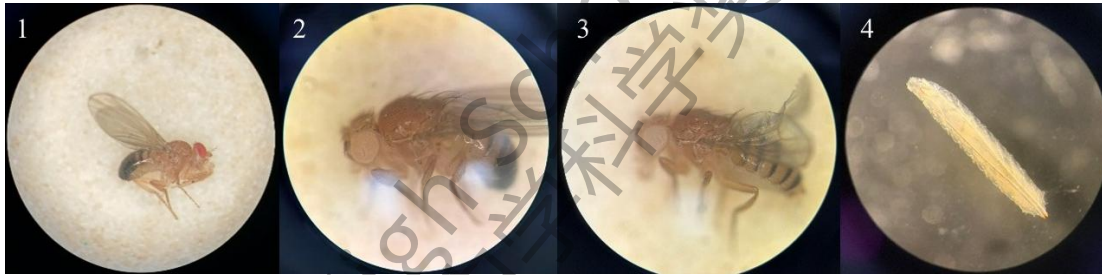


Figure 2. Phenotypic changes

1: $y, sc, v; E::W; +$. 2: $\frac{w}{Y}; \frac{E::W}{Sco}, \frac{+}{TM6B}$. 3: $w; \frac{E::W, yki3SA}{CyO}, \frac{+}{TM6B}$. 4: MS1096-Gal4> $w; \frac{E::W, yki3SA}{CyO}, \frac{+}{TM6B}$.

3.2 The morphological changes in wing imaginal discs

After dissecting wing imaginal discs and observing them under an inverted research microscope, we found the following results.

3.2.1 Despite gene integration, wing discs shrink

Compared with the control wing discs of the w^{1118} line, the yki^{3SA} -inserted wing discs showed a notable increase in size, implying remarkable proliferation and therefore serious carcinogenesis. The proliferation in the yki^{3SA} -inserted wing discs speaks volumes for the tumorigenic effect of yki^{3SA} alone.

In contrast, the $E::W$ -inserted wing discs shrank in size, indicating aberrant cell death,

which is consistent with previous results suggesting that $E::W$ alone is not capable of triggering DSRCT.

Surprisingly, the co-inserted wing imaginal discs shrank to approximately the same size as the $E::W$ -inserted wing discs. This result suggests that the addition of yki^{3SA} to the $E::W$ -inserted line failed to rescue the abnormal phenotype of shrunken wing discs driven by $E::W$.

Regarding cell number, although yki^{3SA} itself serves as an effective factor in causing proliferation, the addition of yki^{3SA} to the $E::W$ -inserted line had no remarkable effect on relieving aberrant cell death, not to mention generating DSRCT.

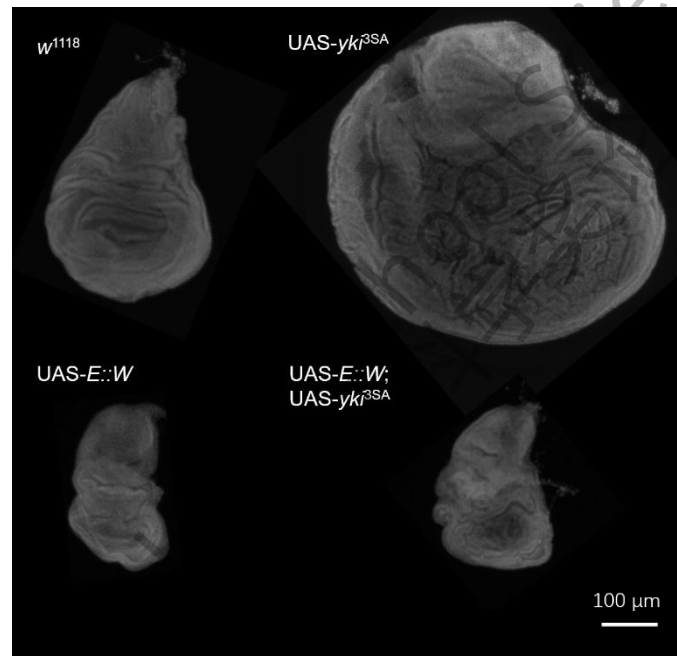


Figure 3. The dissected wing discs

3.2.2 Co-integrated wing discs present more intense epithelial polarity disruption

The addition of yki^{3SA} , although it failed to rescue the phenotype of aberrant cell death, did perturb the polarity of the wing disc epithelium.

In a normal *Drosophila* wing imaginal disc, the monolayer of epithelial cells is aligned within the plane according to a vector field of polarity, and characteristic patterns emerge as a result^[12]. The morphology of these patterns therefore offers a direct readout of epithelial cell polarity. As shown in the figure, in the wing disc as well, the patterns in the group carrying only the $E::W$ insertion, although shrunken on account of the reduced cell number, remain recognizable relative to the control, with the core

frame mostly unaltered. In every group that carried *yki3SA*, by contrast, dense and curved aberrant patterns appeared, and the original orientation of the pattern became almost impossible to discern. These results indicate that insertion of *yki3SA* substantially disturbs the polarity of the epithelial cells.

In terms of cell arrangement, then, the presence of *yki3SA* in the *E::W* strain promotes the malignant transformation of cells.

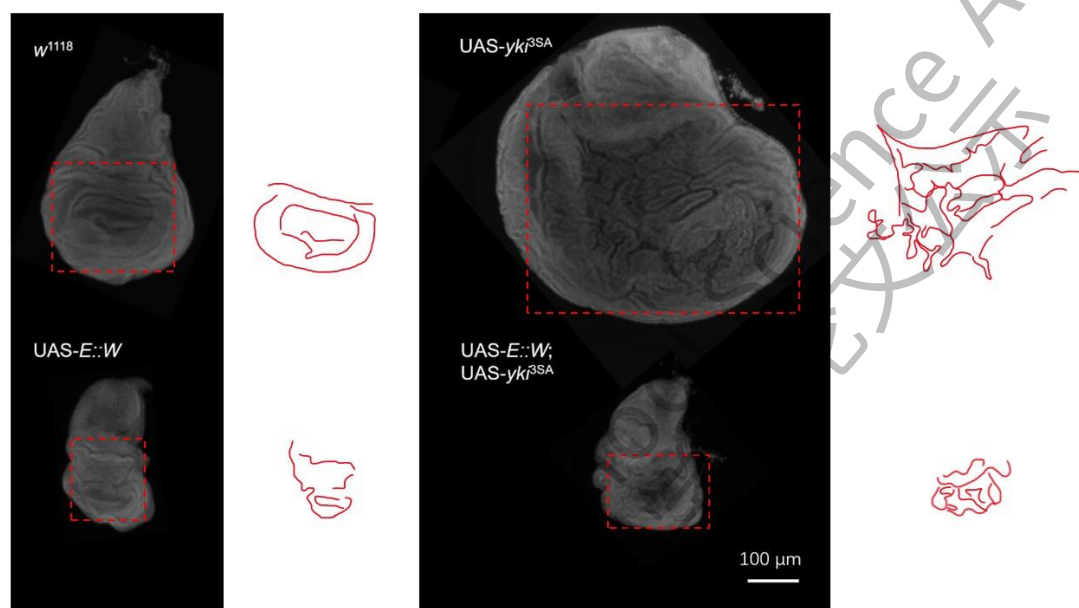


Figure 4. The epithelial polarity of wing discs

4. Discussion

4.1 Why the shrinkage? — Toxicity of *E::W*

E::W expression causes severe developmental defects.

In preceding studies in our lab, *E::W* caused embryonic lethality with most tested developmental drivers. Even with tissue-specific drivers that allow survival (MS1096-*Gal4*, *ey-Gal4*), severe tissue ablation occurred (wing ablation). Moreover, *E::W* expression inhibited normal cell proliferation in both normal human mesothelial cell lines^[13] and wild-type MEFs^[14]. The above evidence suggests that *E::W* has potent developmental toxicity.

In conclusion, the phenotype of shrunken wing imaginal discs is consistent with expectations, indicating that the toxicity of *E::W* may lead to cell death and therefore

overshadow its tumorigenic effect.

4.2 Why the remarkable pattern disruption? — *yki*^{3SA} in the Fat-Hippo-Warts signaling pathway

Yorkie in *Drosophila* (YAP in mammals) is a co-activator mediating transcriptional regulation in the *Drosophila* Fat-Warts-Hippo pathways. Yorkie is inhibited by Warts-mediated phosphorylation at the sites of S111, S168, and S250^[10].

The gene *yki*^{3SA} is an activated version of Yorkie. With the above three sites mutated, *yki*^{3SA} cannot be negatively regulated by Warts and therefore exerts a much stronger effect on promoting cell growth and preventing cell death^[15].

Previously, researchers found that the low-level or intermediate-level expression of *yki*^{3SA} has only a hyperplastic effect and no neoplastic effect on wing imaginal discs of *Drosophila*. For example, overexpression of *yki*^{3SA} alone produced overgrown discs but did not induce spindle alignment defects^[16], indicating that *yki*^{3SA} is not the decisive factor in cell polarity. However, the high activity of Yorkie causes not only the cell proliferation but also the disruption of cell fate by altering the expression of the selector genes in wing imaginal discs^[17].

Consistent with the above, in this study, the phenotype of the cell proliferation and the disruption of patterns in wing imaginal discs were observed, offering further evidence of the hyperplastic and cellular reprogramming effect of *yki*^{3SA}.

4.3 Why the combination?

As the wing imaginal discs with co-integration of the *E::W* fusion gene and *yki*^{3SA}, presented in the figure above, show no relief of aberrant cell death but remarkable pattern disruption, we can conclude that a modified preliminary DSRCT model in *Drosophila* has been built. Although the high toxicity of the *E::W* fusion gene blocks the way to cell proliferation, the phenotype of cell-fate reprogramming has been shown, thanks to the insertion of *yki*^{3SA}.

5. Future perspectives

5.1 To optimize expression levels of *E::W*

Since the severe ablation phenotypes observed might be due to the dose-dependent

E::W expression toxicity, masking potential proliferative or tumorigenic effects, we will generate new transgenic lines with reduced *E::W* expression levels using constructs containing fewer UAS repeats (e.g., 5xUAS-*E::W*, 3xUAS-*E::W*, 1xUAS-*E::W*). These lines will be crossed with the same tissue-specific drivers used previously. Phenotypes will be compared across different expression levels, focusing on tissue morphology, cell proliferation and tumor formation.

5.2 To assess potential cooperating genetic alterations

E::W may require additional genetic alterations, such as activation of oncogenic pathways or loss of tumor suppressors, to drive robust tumorigenesis, consistent with multi-step cancer models. The multi-step tumorigenesis theory states that tumor formation typically requires the cooperative action of multiple genes. Previous in vitro studies have demonstrated that *E::W* expression alone is insufficient to transform human mesothelial cells or wild-type MEFs into tumor cells, yet it can enhance proliferation in *p53*-mutant MEFs. This suggests that *E::W*-driven tumorigenesis likely requires additional alterations. Interestingly, *p53* mutations are rare in DSRCT4, indicating that other oncogenes or tumor suppressor mutations may be necessary cooperating factors in pathogenesis.

As a result of the failed rescue by *yki*³⁸⁴, we will use other genetic integration approaches to generate flies co-expressing *E::W* with known oncogenic drivers or in tumor suppressor mutant backgrounds relevant to cancer, such as *p53* RNAi.

References

- [1] Wei T, Zhao Q, Li Y. Current Status and Progress of Targeted and Immunotherapy for DSRCT[J/OL]. *Cancers*, 2026, 18 (11) : 1711. DOI:10.3390/cancers18111711.
- [2] Nishio J, Iwasaki H, Ishiguro M, 等. Establishment and Characterization of a Novel Human Desmoplastic Small Round Cell Tumor Cell Line, JN-DSRCT-1[J/OL]. *Laboratory Investigation* , 2002 , 82 (9) : 1175-1182. DOI:10.1097/01.LAB.0000028059.92642.03.
- [3] MARKIDES C S A, COIL D R, LUONG L H, 等. Desmoplastic small round cell tumor (DSRCT) xenografts and tissue culture lines: Establishment and initial characterization[J/OL]. *Oncology Letters* , 2013 , 5 (5) : 1453-1456. DOI:10.3892/ol.2013.1265.
- [4] Molnar C, Reina J, Herrero A, 等. Human EWS-FLI protein recapitulates in *Drosophila* the neomorphic functions that induce Ewing sarcoma tumorigenesis[J/OL]. *PNAS Nexus* , 2022 , 1 (4) : pgac222. DOI:10.1093/pnasnexus/pgac222.
- [5] 韩玉婷, 许博文, 李羽童, 等. 模式动物果蝇的基因调控前沿技术[J/OL]. *遗传*, 2022, 44 (1) : 3-15. DOI:10.16288/j.ycz.21-347.
- [6] Xu R-G, Wang X, Shen D, 等. Perspectives on gene expression regulation techniques in *Drosophila*[J/OL]. *Journal of Genetics and Genomics*, 2019, 46 (4) : 213-220. DOI:10.1016/j.jgg.2019.03.006.
- [7] Qiao H-H, Wang F, Xu R-G, 等. An efficient and multiple target transgenic RNAi technique with low toxicity in *Drosophila*[J/OL]. *Nature Communications*, 2018, 9 (1) : 4160. DOI:10.1038/s41467-018-06537-y.
- [8] Wang F, Qiao H-H, Xu R-G, 等. pNP Transgenic RNAi System Manual in *Drosophila*[J/OL]. *BIO-PROTOCOL*, 2019, 9 (3) . <https://bio-protocol.org/e3158>. DOI:10.21769/BioProtoc.3158.
- [9] Fly Pushing: The Theory and Practice of *Drosophila* Genetics[M].
- [10] Oh H, Irvine K D. In vivo analysis of Yorkie phosphorylation sites[J/OL]. *Oncogene*, 2009, 28 (17) : 1916-1927. DOI:10.1038/onc.2009.43.
- [11] 王霞, 沈达, 乔欢欢, 等. 实验用果蝇的饲养及管理[J/OL]. *BIO-PROTOCOL*, 2019.

<https://bio-protocol.org/e1010250>. DOI:10.21769/BioProtoc.1010250.

- [12] Aigouy B, Farhadifar R, Staple D B, 等. Cell Flow Reorients the Axis of Planar Polarity in the Wing Epithelium of *Drosophila*[J/OL]. *Cell*, 2010, 142 (5) : 773-786. DOI:10.1016/j.cell.2010.07.042.
- [13] Magrath J W, Espinosa-Cotton M, Flinchum D A, 等. Desmoplastic small round cell tumor: from genomics to targets, potential paths to future therapeutics[J/OL]. *Frontiers in Cell and Developmental Biology*, 2024, 12 : 1442488. DOI:10.3389/fcell.2024.1442488.
- [14] Bandopadhyay P, Jabbour A M, Riffkin C, 等. The oncogenic properties of EWS/WT1 of desmoplastic small round cell tumors are unmasked by loss of p53 in murine embryonic fibroblasts[J/OL]. *BMC cancer*, 2013, 13 : 585. DOI:10.1186/1471-2407-13-585.
- [15] Oh H, Irvine K D. in vivo regulation of Yorkie phosphorylation and localization[J/OL]. *Development (Cambridge, England)*, 2008, 135 (6) : 1081-1088. DOI:10.1242/dev.015255.
- [16] Dewey E B, Taylor D T, Johnston C A. Rolling in the mud: Hippo controls oriented cell division[J/OL]. *Cell Cycle*, 2016, 15 (5) : 607-608. DOI:10.1080/15384101.2015.1130578.
- [17] Bairzin J C D, Emmons-Bell M, Hariharan I K. The Hippo pathway coactivator Yorkie can reprogram cell fates and create compartment-boundary-like interactions at clone margins[J/OL]. *Science Advances*, 2020, 6 (50) : eabe8159. DOI:10.1126/sciadv.abe8159.
- [18] Ito T, Igaki T. Yorkie drives Ras-induced tumor progression by microRNA-mediated inhibition of cellular senescence[J/OL]. *Science Signaling*, 2021, 14 (685) : eaaz3578. DOI:10.1126/scisignal.aaz3578.