Step by Step, understanding the background of cancer:

Analysis of the protumoral role of the JNK pathway in *Drosophila melanogaster*

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Abstract

S'ha demostrat que la inestabilitat genòmica és una característica promotora del càncer, i la majoria dels carcinomes esporàdics presenten un augment en la taxa de canvis en el nombre de cromosomes, conegut com a inestabilitat cromosòmica (CIN). Malgrat això, el paper de la CIN en l'impuls del caràcter invasiu encara no està clar. En aquest treball, utilitzem cèl·lules epitelials de *Drosophila melanogaster* per abordar el paper de la CIN en el desenvolupament tumoral. Mostrem que el blocatge dels gens del punt de control de la mitosi (*bub3*) en *Drosophila melanogaster* condueix a una mort cel·lular programada, que depèn de l'activació de la via de senyalització quinases c-Jun N-terminal (JNK). Quan evitem que les cèl·lules entrin en el procés de l'apoptosi amb la proteïna p35, la CIN condueix a un creixement neoplàstic i impulsa la tumorigènesi. Les cèl·lules amb un nombre anormal de cromosomes es desprenen de l'epiteli principal i envaeixen els teixits veïns. En aquests tumors també es va observar un reporter de la via de JNK, la MMP1 (matrix metalloproteinase 1), implicada en la degradació de la membrana basal. JNK s'activa en les cèl·lules que es desprenen i impulsa el creixement excessiu dels teixits, la invasió i la degradació de la membrada basal. També vam demostrar que el bloqueig de la via de JNK rescata parcialment les cèl·lules amb CIN de formar un tumor. En conjunt, els nostres resultats sostenen la proposta que la CIN promou un comportament ràpid i invasiu a les cèl·lules epitelials i reforcen el paper proliferatiu de la via de JNK.

Paraules claus: *Drosophila melanogaster,* càncer, tumors, JNK, inestabilitat cromosòmica

Se ha demostrado que la inestabilidad genómica es una característica promotora del cáncer, y la mayoría de los carcinomas esporádicos presentan un aumento en la tasa de cambios en el número de cromosomas, conocido como inestabilidad cromosómica (CIN). Sin embargo, el papel de la CIN en el impulso del carácter invasivo todavía no está claro. En este trabajo, utilizamos células epiteliales de Drosophila melanogaster para abordar el papel de la CIN en el desarrollo tumoral. Mostramos que el bloqueo de los genes del punto de control de la mitosis (*bub3*) en Drosophila melanogaster conduce a una muerte celular programada, que depende de la activación de la vía de señalización quinasas c-Jun N-terminal (JNK). Cuando evitamos que las células entren en el proceso de la apoptosis con la proteína p35, la CIN conduce a un crecimiento neoplástico e impulsa la oncogénesis. Las células con un número anormal de cromosomas se desprenden del epitelio principal e invaden los tejidos vecinos. En estos tumores también se observó un reportero de de la vía de JNK, la MMP1 (matrix metalloproteinase 1), implicada en la degradación de la membrana basal. El programa transcripcional JNK se activa en las células que se desprenden e impulsa el crecimiento excesivo de los tejidos, la invasión y la degradación de la membrada basal. También demostramos que el bloqueo de la vía de JNK rescata parcialmente a las células con CIN de formar un tumor. En conjunto, nuestros resultados apoyan la propuesta de que la CIN promueve un comportamiento rápido e invasivo en las células epiteliales y refuerzan el papel proliferativo de la vía de JNK.

Palabras clave: *Drosophila melanogaster,* cáncer, tumores, JNK, inestabilidad cromosómica

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Introduction

I consider myself honored to have been selected to participate in the "Crazy About Biomedicine" program, promoted by the Catalunya La Pedrera Foundation. And it was through this program that I was given the opportunity to collaborate on research with the Barcelona Institute for Research in Biomedicine (IRB) in a laboratory with a tutor specialized on the subject of my choice. With her help and during the month of July 2021, I was able to carry out the practical part of my research in the Marco Milán laboratory.

When given the choice to select a research topic, I decided to dedicate myself to the molecular processes that affect and promote cancer. Cancer is a very complex disease and one of the major medical mortality causes worldwide, which is why I felt prompted to dive myself into it. According to the National Center for Health Statistics, in 2021, 1.898.160 new cancer cases and 608.570 cancer deaths will likely occur only in the United States. And despite cancer's mortality peak being left behind in the 20th century, due to improvements in early detection and treatment, and the cancer death rate steadily falling year after year since; the deeper and unknown causes of the disease remain a complex mystery even to the most thorough researchers.

Within the biological and chemical processes that take place during the development of cancer, I had the opportunity to focus on one of the mechanisms involved in tumor activation: the JNK pathway. This pathway plays a key role in regulating the immune response to tumors in their early stages and poor regulation of this mechanism is related to the development of cancer. The main objective of this work is to analyze the role of the JNK signaling pathway in the promotion of tumor development. At the same time, there are some other specific goals, such as generating a model of chromosomal instability in which cell death is blocked; using *Drosophila melanogaster*'s as a model.

The hypothesis to going into the investigation was the following:

- "The activation of the JNK pathway may have a pro-proliferation role and cells with chromosomal instability may have an invasive behavior"

In order to achieve the objectives and to verify the hypothesis, the work consists of two parts: a theoretical part, with a thorough description of the concepts involved in the experiment, and a practical part consisting of the experiments themselves, performed entirely on the IRB facilities.

Theoretical frame

Cancer

History of cancer

Cancer is an old disease, as of today, the oldest description of cancer (without using the word cancer) was discovered in Egypt and dates back to about 1600 BC. It is a copy of part of an ancient textbook on trauma surgery, that describes breast cancer. The writing says about the unknown disease: “there is no treatment”.

The word cancer is attributed to Hippocrates (460-370 BC), also known as the “Father of Medicine”. He differentiated between non-ulcer forming and ulcer-forming tumors, using the terms *carcinos* and *carcinoma* to describe them. In Greek, these words refer to crab, and probably he used them because the blood vessels that branched from his patients’ tumors reminded him of the claws of a crab. Later on, Celsus, a Roman physician, translated the Greek term into *cancer,* the word for crab in Latin. Another Greek physician, Galen, used the work *oncos*, Greek for swelling, to describe tumors. Although the word is not used for malignant tumors, Galen’s term is now used as a part of the name for the cancer specialty: oncology.

At the beginning of the 15th century, scientists developed a greater understanding of the human body, however, it was John Hunter (1728-1793) who first suggested that some cancers could be cured by surgery, and also described when cancer should be operated on. It could be removed if the tumor had not invaded nearby tissue and was, as he wrote, “moveable”. A century later, the development of anesthesia allowed surgery to flourish and cancer operations were developed, such as radical mastectomy to “cure” breast cancer, where the breast, the chest muscles and all of the lymph nodes were removed.

In the late 19th century, the development of better microscopes made the examination of cells and cellular activity possible. Study of cancer tissues and tumors revealed that cancer cells were different in appearance from normal cells. In the early 20th century improvement were made to the understanding of the structures, functions and chemistry of living organisms. Researches pursued different theories of the origin of cancer, as a viral cause of cancer in chickens was documented in 1911 while also chromosomal abnormalities were being investigated as a possible cause.

In 1896 Wilhelm Roentgen presented the “X-ray”, and three years later radiation was used to treat cancer, however, later on, it was discovered that radiation could cause cancer as well as cure it. In the 1940s the first drug treatment was induced, causing a brief reduction of tumors, opening the doors to the cure by pharmacological agents. Sidney Farber, known as “The Father of modern chemotherapy” did a rational drug design and administrated it to children with acute lymphoblastic leukemia, resulting in brief remissions. Later on, some other scientists demonstrated that the use of other drugs could also lead to remissions.

The 20th century had lots of other discoveries, as the consent of the TNM (Tumor Node Metastasis), the classification that helps to know whether it is a tumor, its length and the type. In the 1950s, Farber and his colleagues achieved the first remissions in Wilms' tumor, by employing chemotherapy with surgery and radiation therapy. That same decade, tobacco smoking was linked to lung cancer, leading to the first anti-smoking campaign in modern history.

Since then, fundamental and clinical biomedical research has advanced the understanding of cancer. Using molecular biology and molecular genetics, scientists are making great improvements in mapping the links between chromosomes, genes and cancer. At the same time, research is also focused on cancer prevention and reducing cancer risk, as well as detecting it early. (Faguet, 2015; Mukherjee, 2010)

As Siddhartha Mukherjee said in his book: The Emperor of All Maladies: A Biography of Cancer: *“Nineteenth-century doctors often linked cancer to civilization: cancer, they imagined, was caused by the rush and whirl of modern life, which somehow incited pathological growth in the body. The link was correct, but the causality was not: civilization did not cause cancer, but by extending human life spans—civilization unveiled it.”* Perhaps this is the weight of longevity, but is the duty of each generation to try to lighten the load.

What is cancer?

Cancer is a heterogeneous group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. Normally, human cells grow and multiply parodically and regularly, through the process called cell division, to form new cells as the body needs them to replace the old or the dead ones and thus maintain the proper functioning of our organs. At the same time, the body has a cell self-destruction process, apoptosis, that destroy the abnormal or damaged cells.

However, sometimes this process breaks down and damaged or abnormal cells grow and multiply. Cells and their descendants lose the ability to die and divide almost without limit. These cells may form tumors, that are lumps of tissue. It should be noticed that although the vast majority of cancers form solid tumors, there are exceptions such as leukemia, which is a cancer of the blood.

Tumors

We understand tumors as an abnormal growth of tissues with clusters of cells that are capable of growing and dividing uncontrollably. However, not all tumors are cancer, as there are two types of tumors (Cooper & Hausman, 2017):

- **Benign or noncancerous tumor**: it tends to grow slowly and is localized, so it doesn't spread to other parts of the body or the blood. Most benign tumors are not harmful, as they can be removed, however, they can cause pain if they press against nerves or blood vessels and can trigger the overproduction of hormones.

- **Malignant or cancerous tumor:** it tends to grow very fast and very aggressively, without regard to the stimuli it receives from the environment around them. Malignant tumors tend to invade tissues that are close to them, such as organs, and they can invade tissues that are far from the tumor, causing metastasis, when the cells break allay from the original tumor and travel through the blood or the lymph system, forming another tumor in other organs or tissues of the body.

Carcinogenesis

Is a multistep process that involves the cooperation of mutations in signaling, cell cycle, cell death and metabolic pathways, as well as interactions between the tumor and the microenvironment (Figure 1). This process leads to cancer and has three main steps (Scott et al., 1984; Weston & Harris, 2016):

1. **Initiation:** results from an irreversible genetic alteration in a cell's DNA, like a carcinogen agent (such as chemicals, exposure to the sun or tobacco) that causes a mutation in a cell. If the cell is able to divide, it begins to multiply quickly and transmits the mutation to its offspring[1].

2. **Promotion:** is the interval during which increased cellular proliferation occurs and initiated cells clonally expand. There are, therefore, more mutated cells that divide more and more rapidly. They also have the ability to influence normal cells that are surrounding them, thus obtaining the food and oxygen that they provide. They continue to grow and reproduce until they acquire the ability to move and invade other tissues.

3. **Progression:** the cancer cells separate from the original tumors and travel through the blood or the lymphatic system to reach a new organ or tissue to form a new tumor.

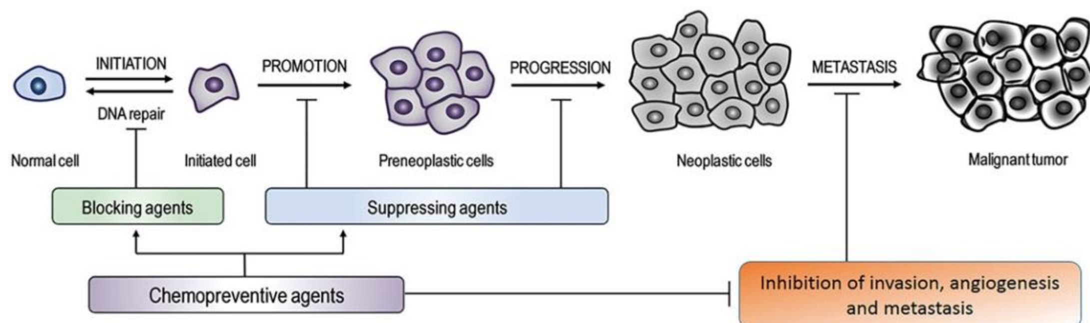

Figure 1: ***Representation of carcinogenesis phases: initiation, promotion, progression and metastasis.*** *Extracted from*: (Siddiqui et al., 2015)

[1] The product of reproduction, a new organism produced by one or more parents

Hallmarks of cancer

A mentioned before, cancer is a disease with a multistep development in which normal cells evolve progressively to a tumor state, acquiring a succession of characteristics and capabilities, called “Hallmarks of cancer” (Figure 2). These are the six “Hallmarks of cancer” (Hanahan & Weinberg, 2000):

1. **Self-sufficiency in Growth Signals:** normal cells require stimulatory signals before they can move into an active proliferative state, while tumor cells generate many of their own growth signals, thereby reducing their dependence on stimulation from their tissue microenvironment. Cancer cells can also switch the types of extracellular receptors, favoring ones that transmit pro-growth signals.

2. **Insensitivity to Antigrowth Signals:** in normal tissues, multiple antiproliferative signals operate to block the growth and the proliferation of the cells. However, cancer cells evade these antigrowth signals to prosper and continue growing. Usually, it is due to the mutations of the so-called “tumor suppressors genes”, that control the response the cells give to the signals.

3. **Tissue invasion and metastasis:** this enables cancer cells to escape the primary tumor mass and colonize new tissues and organs in the body where, initially, nutrients and space are not limited. Cancer cells are able to break the walls that separate the tissues and cross the walls of the blood vessels, incorporating into the blood and proliferating in another area of the body.

4. **Limitless replicative potential:** one of the factors that make the number of cells so high in a malignant tumor is the ability to divide almost infinitely. In almost 90% of cases, this characteristic is acquired when, due to mutations in the DNA, the enzyme telomerase is activated. In the division of a normal cell, a small portion at the end of each chromosome, called a telomere, is lost; this means that in each copy a part of the DNA disappears. The accumulation of these losses brings the cell to a critical point where it loses its ability to replicate and activates apoptosis. In this way, healthy cells have a certain number of programmed divisions, but in carcinogens, the enzyme telomerase prevents this loss of DNA in telomeres, which makes them "immortal” (Shay & Bacchetti, 1997).

5. **Sustained angiogenesis[2]:** oxygen and nutrients supplied by the vasculature are crucial for the cell to function and survive, obligating cells in a tissue to reside near a capillary blood vessel. Once the tissue is formed, angiogenesis is carefully regulated. The cells within proliferative lesions initially lack angiogenic ability, curtailing their capability to expand, so to progress, incipient tumors must develop angiogenic ability (Bouck et al., 1996).

6. **Evading apoptosis:** when normal cells age or are damaged, they activate the process of apoptosis, enabling the body to limit the growth and get rid of cells with mutations in the DNA. Cancer cells gain the ability to prevent the normal cycle of cell death so they can accumulate in the body and continue to increase.

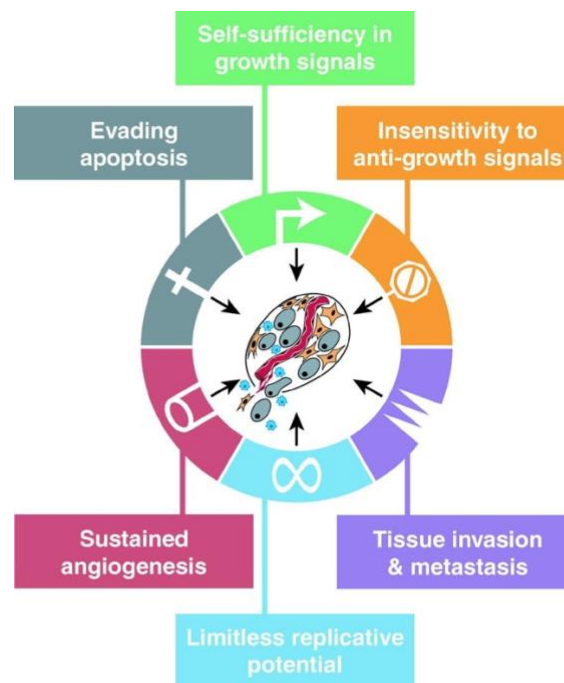

*Figure 2: **Hallmarks of cancer.** Extracted from: (Hanahan & Weinberg, 2000)*

[2] The development of new blood vessels

New Hallmarks

Both Douglas Hanahan and Robert A. Weinberg, the authors of the original Hallmarks of cancer, published a review in 2011 with the discovery of new Hallmarks (Hanahan & Weinberg, 2011). They added two acquired capabilities that all cancerous cell develops, as the other six they had described, and two necessary characteristics that must be given for a cell to acquire all the Hallmarks and be considered carcinogenic (Figure 3). The two new Hallmarks are:

7. **Reprogramming Energy Metabolism:** even with the increase in the volume of blood that tumors receive, there can often be a lack of oxygen or nutrients in it that prevents it from obtaining energy due to the agglomeration of cells that are formed in tumors. This lack of components would be fatal to any healthy cell, as it uses oxygen to transform glucose into energy through a process of aerobic metabolism. Cancer cells, however, acquire the ability to change the method of obtaining energy according to the number of nutrients and oxygen in their environment. In conditions where oxygen is scarce, these vary from an aerobic to an anaerobic (oxygen-free) method to metabolize glucose and obtain energy.

8. **Evading Immune Destruction:** cells and tissues are monitored by an ever-alert immune system, which is responsible for recognizing and eliminating the vast majority of incipient cancer cells. Although the process used by cancer cells is unknown, there is evidence that they are able to prevent them from being destroyed by the immune system to some degree, allowing them to proliferate and invade other tissues.

The enabling characteristics are:

1. **Genome Instability and Mutation:** the acquisition of the capabilities is most acquired, directly or indirectly, through changes in the genomes of cancer cells. Multistep tumor progression can be portrayed as a succession of clonal expansions, each triggered by the chance acquisition of an enabling mutant genotype. The genome maintenance system detects and resolves defects in the DNA to ensure that rates of spontaneous mutations are low, so a defect in this system is advantageous for tumor progression.

2. **Tumor-Promoting Inflammation:** inflammation can contribute to multiple hallmark capabilities by supplying molecules to the tumor microenvironment, including growth factors, survival factors, or enzymes that facilitate angiogenesis, invasion and metastasis. Additionally, inflammatory cells can release chemicals that are mutagenic for nearby cancer cells.

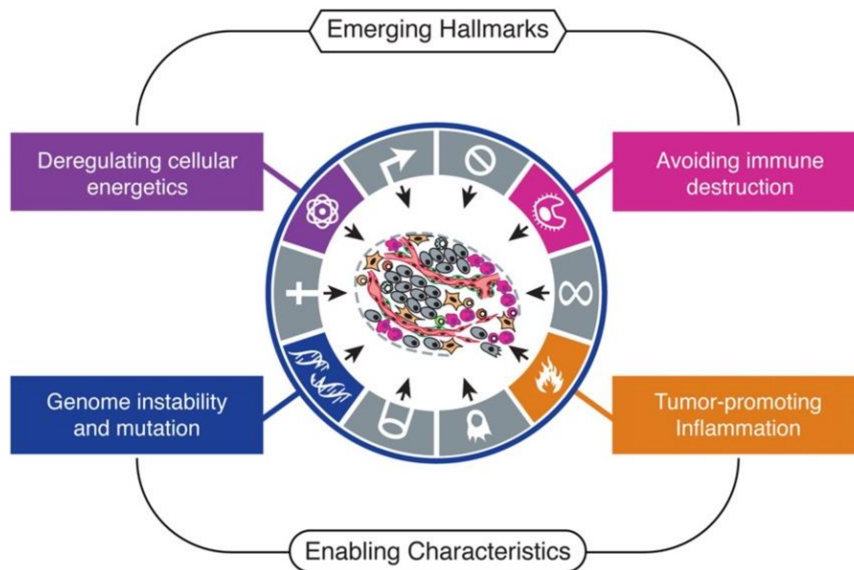

*Figure 3: **Emerging Hallmarks and Enabling Characteristics.** On top, the new two hallmarks, and at the bottom are the two enabling characteristics. In grey, the six previously described hallmarks. Extracted from: (Hanahan & Weinberg, 2011)*

Involved genes

Cancer is caused by the malfunction of genes that regulate cell proliferation. There are different kinds of regulatory genes (Yarbro, 1992):

- **Tumor suppressor genes:** encode proteins that limit cell growth by monitoring how quickly cells divide into new cells, repair mismatched DNA and control when a cell dies. When a tumor suppressor gene mutates, cells grow uncontrollably and eventually form a tumor. Some examples are BRCA1, p53 or TP53.

- **Protooncogenes:** encode proteins that stimulate cell growth and cell division. They are very important at certain stages of life, especially during embryogenesis. When they mutate, they are transformed into oncogenes.

- **Oncogenes:** encode proteins that activate cell multiplication with abnormal growth of the tissues on which they act, which lead to the formation of tumors

and often inhibit apoptosis. Two common oncogenes are HER2, a protein that controls cancer growth and spread and the RAS family of genes, which makes proteins involved in cell communication pathways, cell growth and cell death,

Causes

The alteration in the cell development and the mutations is usually an interaction between the genetic factors of the person and some extern agent. The most important ones are (Das et al., 2020):

- **Physical carcinogens:** ultraviolet and ionizing radiations (Michor et al., 2011).
- **Chemical carcinogens:** such as asbestos (used a few decades ago in the construction), tobacco smoke components, aflatoxins…
- **Biological carcinogens:** certain viruses, bacteria and parasites.

Aging is another of the key factors, as the accumulation of risk factors over time adds up to the loss of effectiveness of the cell repair mechanism. The habits of individuals are also key factors that can condition the development of cancer: incorrect eating habits, smoking, alcoholism, sedentary lifestyle or obesity (Ames et al., 1995).

Treatments

As mentioned before, one of the Hallmark of cancer is its ability to avoid the immune system, making it almost impossible for the own body to kill the tumor. For this reason, cancer is not a disease that can be easily cured, however, thought the year, a large variety of treatments have been developed (Falzone et al., 2018):

- **Chemotherapy:** uses drugs to prevent cancerous cells from growing and multiplying. It also affects some healthy cells so, between doses, recovery periods must be spent.
- **Radiation therapy:** uses high-powered energy beams, such as X-rays, to damage the DNA of the cancerous cell and cause them to die or grow much slower. As a contradiction, it can damage healthy cells close to the tumor.

- **Surgery:** the tumor is removed, usually followed by other treatments such as chemotherapy or radiation, as a large part of the diseased cells have been removed, but some may persist. It is a very effective method in certain types of cancer, such as breast cancer.
- **Immunotherapy:** stimulates the patient's immune system with pharmacological substances so that it is the one who attacks the cancer cells.
- **Hormone therapy:** it is used for cancers that are fueled by hormones, such as breast and prostate cancer. Hormones are removed or their effects are blocked so that the cancer cells stop growing.

Research on cell signaling pathways

The genetic alterations in cancer cells can be connected with signaling pathways that control processes associated with tumorigenesis (Sever & Brugge, 2015). Cell signaling governs basic cellular activities and coordinates cell actions through a complex coordination of responses to the cellular microenvironment. Cells communicate with each other, producing different intermediate signal molecules that target cells recognize, allowing them to develop a specific response. Signals are picked up by the cell through specific receptors that can be found both in the cell membrane and inside it (Aldridge et al., 2006).

Signaling process

The process is divided into three main parts (Figure 4). First, a specific molecule binds to a receptor during cell communication. The transmission of the signal inside the cell is possible thanks to the enzymes[3], which help the different chemical reactions to take place in the messenger molecules throughout the process. The signal can be amplified at any point in the pathway, meaning that a single signal molecule can produce responses involving the participation of thousands of molecules. Signaling pathways end up generating responses in the cell such as activating gene expression, altering metabolism, initiating the process of cell division or triggering apoptosis among many others (Cooper & Hausman, 2017).

[3] A substance that acts as a catalyst in living organism, regulating chemical reactions

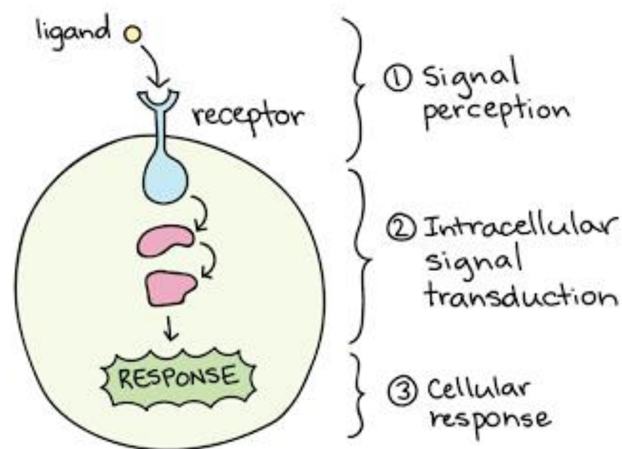

Figure 4: ***Signaling mechanism.*** *The three main steps in cell signaling and where they take place in the cell. Extracted from:* https://www.khanacademy.org/science/ap-biology/cell-communication-and-cell-cycle/cell-communication/a/introduction-to-cell-signaling

Activation and deactivation of the pathways

Proteins can be activated and inactivated in various ways, one of the most common ways to alter protein activity is the addition of phosphate. Kinases are molecules that transport phosphate groups to specific proteins. Cells contain different kinases that phosphorylate different targets. However, phosphorylation is not permanent, as cells have enzymes called phosphatases, which remove a phosphate group from their targets (Valdespino-Gómez et al., 2015).

Response to signals

Signaling pathways generate various cellular responses that can be divided into two groups:

- **Molecular-level:** changes in the expression of certain genes or the activity of particular proteins

- **Macroscopic level:** changes in the external behavior or the appearance of the cell, such as cell migration or cell death (both caused by molecular changes)

Drosophila melanogaster

Introduction

The species *Drosophila melanogaster*, also known as the fruit fly or the vinegar fly, is a small insect that pertains to the order of Diptera. The word *Drosophila* is derived from the Greek word “drósos”, which means dew loving. Like most other flies, this species is harmless, despite having gained a bad reputation due to its habit to fly near decomposing garbage or fruit. However, it has made itself noticeable in laboratories and research as a model organism[4] (Yamaguchi & Yoshida, 2018).

D. melanogaster has been extensively studied for more than one hundred years. It has mostly been used to study genetics, molecular and developmental biology and mutagenesis, from human disease modelling to behaviour and aging. *D. melanogaster* genome was sequenced in 2000 (Adams et al., 2000) one year before the human one (Venter et al., 2001). These genomes revealed homologies between *D. melanogaster* and humans. As remarkable as it can sound, D. *melanogaster* shares 60% of the DNA with humans, however, nearly 75% of human disease-causing genes seem to have a homolog in the fly (Pandey & Nichols, 2011a; Reiter et al., 2001; Yamamoto et al., 2014).

In nature, *D. melanogaster* has been found in all continents, except Antarctica. The fruit fly lives in a very large gamma of regions, being the two only aspects that limit its regions temperature and water availability. Adult flies can’t resist cold temperatures in high latitudes, having also in these locations less food available. *D. melanogaster* is fertile and vital in a range from 12 to 35 °C and 60% of humidity (Demerec, 1950).

[4] Non-human species that has been widely studied and is used by researchers in laboratories to understand biological processes. In researching human disease, model organism allows a better understanding of the disease process without harming humans.

Classification

As an organism, the fruit fly has its own taxonomy[5] (Table 1).

*Table 1: **Taxonomy of D. melanogaster.** The table shows the taxonomy of the species D. melanogaster, starting at the larger and more global group until the species, the smaller subgroup. Adapted from: https://www.uniprot.org/taxonomy/7227*

DOMAIN	Eukaryota
KINGDOM	Animalia
PHYLUM	Arthropoda
CLASS	Insecta
ORDER	Diptera
FAMILY	Drosophilae
GENUS	Drosophila
SPECIES	melanogaster

[5] The classification of organisms

Morphology

Larvae

External structure:

They have a small head distinguished by the mouth hooks, that give the segment a prominent dark colour. They also have eight abdominal segments and three thorax segments. At the border of every segment, some tiny hooks prevent the larvae from sliding backwards while moving forward. The body wall consists of an external cuticle and the cellular epidermis below, and it is very flexible. In some stages of the development (first instar larvae), they also have spiracles located on both sides of the first thoracic segment. These spiracles have papillae with terminal openings through which air can enter the body. (Graf et al., 1992)

Internal structure:

Larvae are transparent, and under transmitted light, some other anatomical details can be studied. Under the tracheae, the white band of the fat bodies can be seen. This is the main respiratory system in the larvae. They stand from the two spiracles (open to the exterior) to the telson the two posteriors' spiracles. The gonads are located in the fifth abdominal segment, they are translucent when compared to the fat bodies, which appear white.

The gut starts as a muscular pharynx and continues as an oesophagus, which runs through the middle of the brain in the thoracic region. Near the oesophagus, there are the salivary glands. The gastric area is where the oesophagus empties and continues into the midgut, which empties into the hindgut. The heart extends anteriorly as an aorta. The circulatory system is open. Near the brain are the imaginal disc, appearing as small teardrop-shaped packets, with a connection to the tracheal system. In Figure 5 we can see a representation of a larva and some of its parts. (Demerec & Kaufmann, 1961; Tyler, 2000)

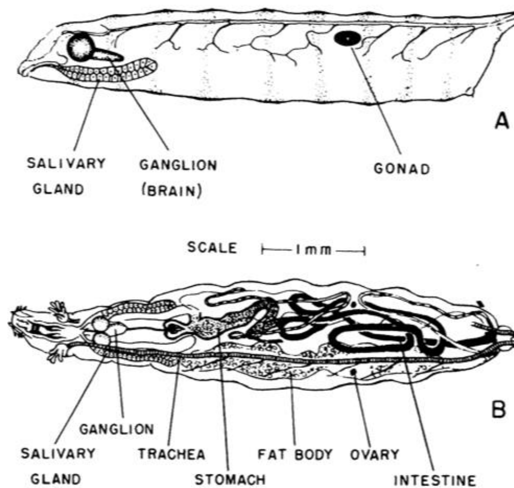

Figure 5: ***Diagrams of a third-instar larva of Drosophila melanogaster*****.** A: Lateral view, showing approximate locations of salivary glands, ganglion and gonads. B: Dorsal view of female larva, showing approximate locations of some of the larva parts. Extracted from: *(Demerec & Kaufmann, 1961)*

Imago or adult fly

Like most other insects, *D. melanogaster* has six legs. The wild type fly has big red eyes and a brown-yellow body, with black lines in the abdomen (Figure 6). They are approximately 3 mm long and 2 mm in diameter weigh 1mg. Their body is divided into three parts (Graf et al., 1992):

1. **Head:** there are two eyes (wild type eyes are red, but a common mutation is the one with white eyes), a mouth and two antennas. The external skeleton protects the soft tissues inside the head (Cagan, 2009).

2. **Thorax:** it is the region in the middle. In the inferior region, it has six legs, and in the superior part, the wings.

3. **Abdomen:** it contains the reproductive and intestinal systems.

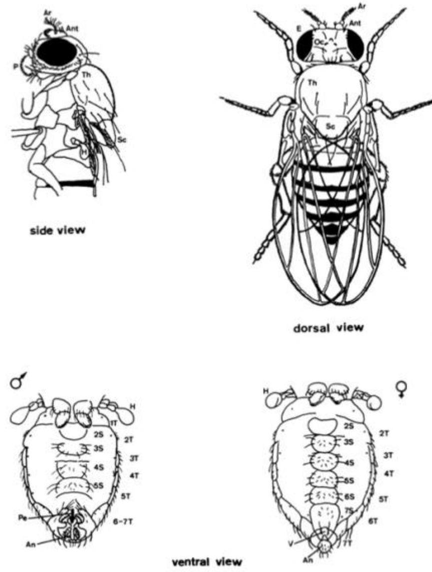

Figure 6: ***External morphology of adult flies.*** *An = anus, Ant = antenna, Ar = arista, E = compound eye, H = haltere, Oc = ocelli, P = proboscis, Pe. Extracted from:* (Graf et al., 1992)

General characteristics

Life's circle

Reproduction and embryonic development

D. melanogaster's reproduction is relatively fast, as when maintained at 25°C it takes 10 days to complete the life cycle (Figure 7), and a single fertile mating pair can produce hundreds of genetically identical offspring, contrasting with other model organisms, that can take over 3 months to produce little offspring (Pandey & Nichols, 2011b).

Fecundation is internal, and sperm is stored within the female's body. As in all insect species, *D. melanogaster* lays eggs, which are placed on fruit, so when they become fly larvae, they can consume the fruit on which they were laid (Patterson & Stone, 1952).

The life cycle starts with a single cell, the zygote[6], which contains all the information needed about the new fly. First of all, the blastoderm[7] is produced by the activation of specific genes with undifferentiated cells, which then will become the epidermis[8], the nervous system, the digestive tract, the muscles and the fat.

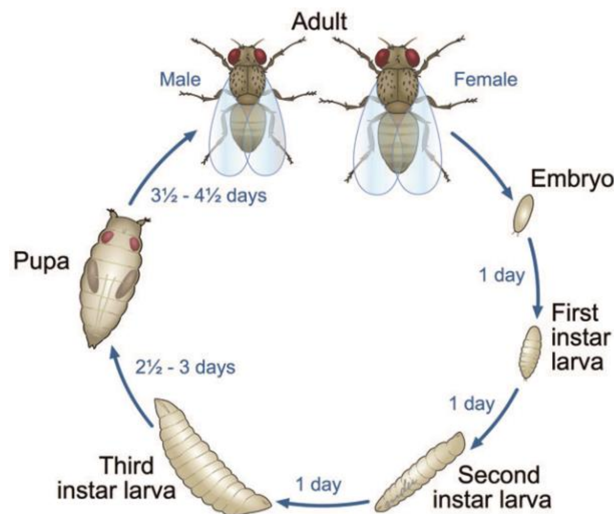

*Figure 7: **Life circle of Drosophila melanogaster at 25°C**. Extracted from:* (Ong et al., 2015)

[6] A eukaryotic cell formed by fertilization between a female gamete (egg, or ovum) and a male gamete (sperm)
[7] The layer of cells that later divides into three germ layers from which the embryo develops
[8] The layer of cells that make up the skin

Stages and duration

Like all flies, *D. melanogaster* has an indirect development through a complete metamorphosis: from the egg comes out a larva, that posterior to its three larvae stages, is transformed into a pupa, which develops into an imago, or adult. That is why *D. melanogaster* is a holometabolous[9] insect, and has, therefore, four stages: embryo, larva, pupal and adult. The duration of these stages varies with the temperature. At 25°C the life cycle is completed in about 10 days, while at 20°C it can take about 15 days or at 10°C that cycle may be prolonged up to 57 days. However, exposure to temperatures above 30°C may kill or sterilize the flies (Demerec & Kaufmann, 1961).

The Egg:

The spermatozoa penetrate the egg through a small opening as the egg passes through the uterus. Immediately, the meiotic divisions are completed and the zygote is produced. It is about 0.5 mm long, and the dorsal side is flatter than the ventral surface. As shown in Figure 8, it has a pair of filaments that help the egg to float and prevents it from sinking into the semiliquid food on which it may be laid. The eggs can be laid shortly after they are penetrated by the sperm or they can be retained in the uterus. (Demerec & Kaufmann, 1961)

Figure 8: Egg of Drosophila melanogaster. Extracted from: https://en.wikipedia.org/w/index.php?title=Drosophila_melanogaster&oldid=1037486498

The Larva:

The first instar larva emerges from the egg and it is like a small worm, which feeds on the substrate that the eggs were laid on (fruits in nature, small media culture in laboratories). 25 hours later, it becomes a larger wormlike form, the second instar larva,

[9] Insects that have a larval and a pupal stage prior to the adult stage

and finally, 24 hours later it turns into the third instar larva, which can reach a length of about 4.5 mm. The larva has small black pieces in the anterior region, the jaws, which are constantly moving (Figure 9). They are transparent and contain some complexes called imaginal discs, which are the primordia for later structures. The larva grows by molting, where at each molt the cuticle is shed and has to be rebuilt again, however, the growth of internal organs proceeds independently of the molting process. (Demerec, 1950; Parvathi et al., 2009). The larva is commonly used to study developmental and physiological processes. (Yamaguchi & Yoshida, 2018)

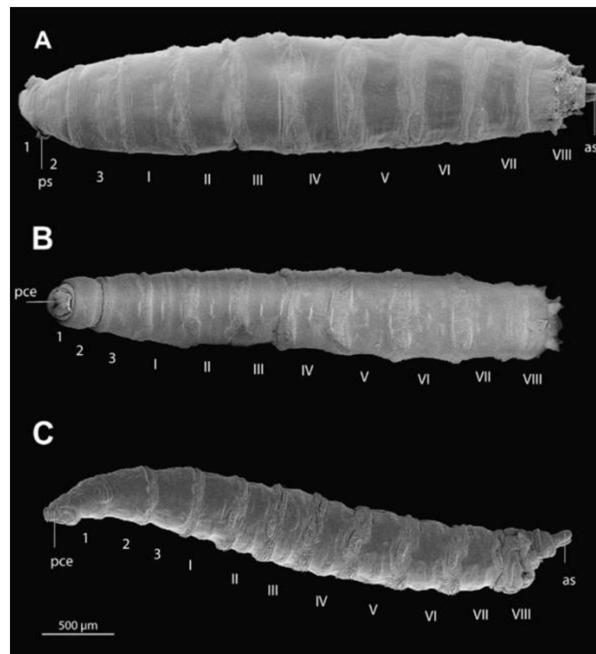

Figure 8: ***Drosophila melanogaster larva.*** *(A) Dorsal; (B) ventral; (C) lateral. Extracted from:* (Wipfler et al., 2013)

The Pupa:

When larvae are preparing to pupate, they creep from the culture medium and search for some dry surface. Soon the larva shortens and gradually acquire its pupal shape (Figure 10). Metamorphosis involves the destruction of certain larva tissues and the organization of adult structures. Most organs and structures come from the imaginal discs, which are primitive cell complexes (Parvathi et al., 2009)

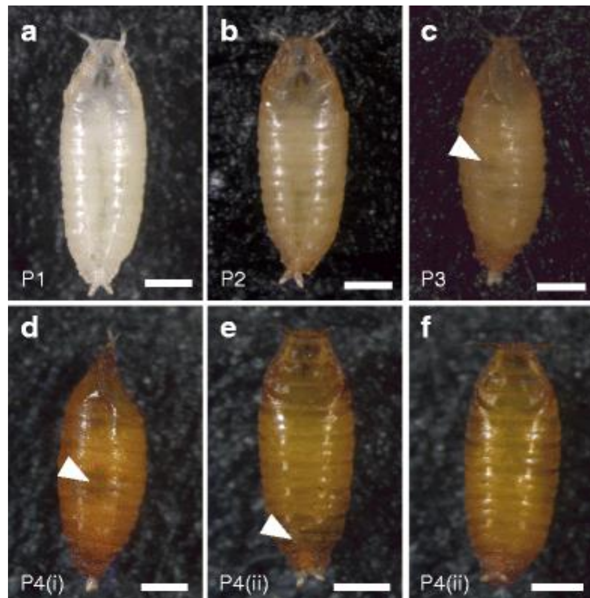

Figure 10: ***Pupal stages of Drosophila melanogaster.*** *Extracted from:* (Fukutomi et al., 2017)

The adult:

The adult fly emerges from the pupa. They are fragile and pallid, with not fully expanded wings. A few hours later, theses flies get the dark colour.

Sexual dimorphism

The female fly has XX chromosomes, while the male is XY. The X chromosome is approximately one-fifth of the fly genome, while the Y chromosome has little gens in it (Adams et al., 2000). Having just one chromosome X leads to the development of the male, and two XX leads to a female. To make crossings, it is important to determine the sex of the flies. Some of the easiest differences that can be seen are (Figure 11) (Parvathi et al., 2009):

- **The size**: the female is larger than the male
- **The shape of the abdomen**: the tip of the abdomen is more rounded in the male, and elongated in the female
- **The marking on the abdomen**: the abdomen of the female has alternating dark and light bands, while the last segments of the male are fused.

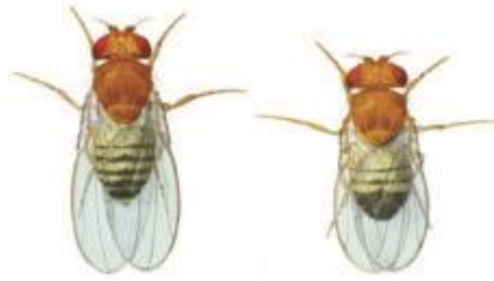

Figure 11: ***Female and male adult in Drosophila melanogaster****. On the right, the female representation of the fruit fly, and on the left is the male one. Extracted from:* (Parvathi et al., 2009)

Virgin females

Female flies have the ability to store sperm after a single mating, so if the female used for a cross is not a virgin, the genotype of the male will be unknown. Young flies are more elongated, slightly pigmented and with folded wings, and in the early hours of the fly as an adult organism, a dark greenish region, the meconium, will be shown on the lower abdomen (Markow, 2011).

The genome

As mentioned before, the *D. melanogaster* genome was sequenced in March 2000 (Adams et al., 2000), and it was the first complex organism to be studied genetically. The haploid genome size is estimated to be 175 Mb[10], containing approximately 15.000 genes (Bosco et al., 2007).

Chromosomes

D. melanogaster is a diploid organism, just as humans, which means that it has two copies of every chromosome, one inherited from the father and the other from the mother. This species has only four pairs of chromosomes, so when compared to the 23 pairs of chromosomes that the human species has, it is a simple organism.

The first chromosome is the X and it is the sex chromosome (X/X indicates female fly while X/Y indicates it is a male, however, X/O flies are males due to the decreased X dosage (Erickson & Quintero, 2007), this first chromosome only has approximately the 20% of the genetic information. Both chromosomes 2 and 3 contain 40% of the information each one, leaving the fourth chromosome with only 1% of the genes. That is why the fourth chromosome is not usually studied or mutated because it has few genes in it.

[10] Million base pairs

Use in research

History

The fruit fly was introduced to laboratory research more than a hundred years ago, specifically, in 1902 by William E. Castle, at Harvard University. To date, six Nobel's Prizes in "Physiology or Medicine" have been given to scientists for their work using *D. melanogaster* (Figure 12).

This began in the early 20th century when Tomas H. Morgan and his team used the fruit flies to prove that genes are located in chromosomes and that they are inherited together. Some years later, one of Morgan's disciples, Hermann J. Müller used the X-ray technique to induce mutations on a large scale, discovering the radiation effects on mutagenesis.

Approximately fifty years later, Eric Wieschaus, Cristiane Nüsslein-Volhard and Edward B. Lewis used *D. melanogaster* to understand the genetic control of the development of the embryo. Many of the genes they defined as important for fly development have been shown to be critical for other organisms, such as humans.

In 2004 and 2011, R. Axel unravelled the olfactory system and J. Hoffman studied innate immunity. The last Drosophila Nobel Prize was given to Jeffrey C Hall, Michael Rosbash and Michael W. Young for uncovering the molecular mechanisms that control circadian rhythms. Nowadays, *D. melanogaster* is being used to study Alzheimer's disease, Parkinson's, longevity, stems cells and cancer among other biological questions. (Stephenson & Metcalfe, 2013; Yamaguchi & Yoshida, 2018)

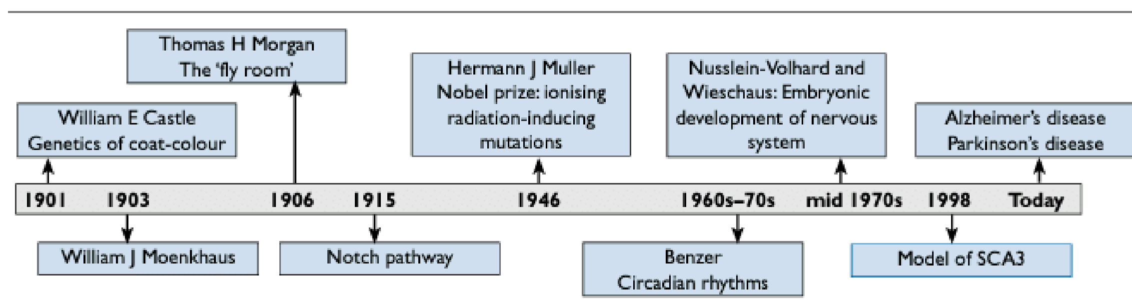

Figure 12: ***Timetable of the use of Drosophila melanogaster in the history of Medical and scientific research.*** *Extracted from: (Stephenson & Metcalfe, 2013)*

Why is Drosophila so useful for laboratory research?

D. melanogaster has some characteristics, both biological and genetic, that make the fruit fly an ideal organism to study (Table 2). The ones that stand up are (Parvathi et al., 2009; Yamaguchi & Yoshida, 2018):

- **It has a small genome**: when compared to the 23 pairs of chromosomes that a human cell has, having only four chromosomes makes the manipulation of the genome easier. Therefore, being able to manipulate some genes helps us understand their function. Moreover, the giant chromosomes in the salivary glands of the mature larvae show more structural detail than normal chromosomes do and they are present during interphase when usually chromosomes cannot be seen.

- **They are easy and inexpensive to culture, maintain and work within laboratories**: the only material necessary to maintain *D. melanogaster* is small glass tubs with food (usually cornmeal and agar-agar) and some cotton in the top to prevent the flies from leaving. Like most other live organisms, a room where the temperature is steady will be needed.

- **Their short life cycle**: in about 12 days, the flies go from embryonic to adult fly, allowing quick crosses and experiments with more than one generation.

- **They produce a large number of offspring**: this allows data collection and statistical analysis. One single female fly can lay down up to 120 eggs per day (Shapiro, 1932).

- **Its development is external**: it is very easy to follow it with only a microscope.

- **There are a large number of genetically defined mutants**: many mutants and transgenic[11] flies can be obtained from stock canters. In addition, a lot of information about other experiments and discoveries is available.

- **There are few ethical concerns**: because being an insect, it is outside animal laws in many countries, allowing the easiest manipulation and stock care.

[11] An organism with one or more DNA sequences from another unrelated species, that has been introduced artificially

Table 2: ***Comparison of model organisms commonly used in biomedical research.*** *Extracted from:* [***https://invivobiosystems.com/disease-modeling/worms-flies-fish-comparison-common-model-organisms/***](https://invivobiosystems.com/disease-modeling/worms-flies-fish-comparison-common-model-organisms/)

	mouse	mammalian cell culture	zebrafish	*Drosophila*	*C. elegans*
Life cycle (time to obtain fertile adults)	50-60 days	n/a	10-12 weeks	12 days	3.5 days
Brood size	6-12 pups per month	n/a	~200 eggs per week	~120 eggs per day	~140 eggs per day
Standard of sharing mutants and reagents		X	X	X	X
Behavior relatively easy to study			X	X	X
No safety and ethics constraints			X	X	X
Mutant strains can be frozen and revived easily		X			X
Easy single cell laser ablation					X
Distinct tissues and high cell diversity	X		X	X	X
Structure of nervous system	Brain Spinal cord		Brain Spinal cord	Brain Ganglia	Somatic neurons Pharyngeal neurons
Number of neurons in adult	> 70,000,000		~10,000,000	> 100,000	302
Connectome	Partial	Partial	Partial	Partial	Complete
Gene homology for human diseases	>90%	>90%	84%	77%	65%
Large library of mutants and transgenics readily available	X	X		X	X
Price to acquire mutant strain	~$200	~ $500	$200-$900	$14-$300	$0-$100
Fully annotated genome		-unknown mutations -variabilities in reagents -unknown cell origins -contaminations -lack of reproducicbility	multiple misalignments	X	X
Easy genetic manipulation		X	X	X	X
Easy and fast transgenic rescue		X	X	X	X
Amenable to drug testing	X	X	X	X	X
High throughput drug screening		X	X	X	X

Genetics toolkit

One of the characteristics of *D. melanogaster* that make it a good model organism is its genetic tools and having high genetic manipulation available to study gene function, or the activity and the regulation of some pathways. The most important tools to work with the fruit fly are:

- **Genetic marker:** a specific DNA sequence or a gene with a known physical location on a chromosome. It can be used to identify individuals or species or to link an inherited disease with the responsible gene (Boopathi, 2013). For example, the larval size, the wing size, the eye and body colour…

- **Reporter genes**: genes that enable the localisation of a segment of DNA or the monitoring of gene expression. Many genes' functions and products are complicated to assay or maybe even unknown. To avoid this, the reporter gene is attached to the regulatory sequence[12] of the gene, and if the gene is finally

[12] A segment of a nucleic acid molecule which is capable of increasing or decreasing the expression of specific genes

expressed, the protein of the reporter gene will too. They are often used as an indication of whether a gene has been expressed in a cell or an organism. Commonly reporter genes involve visually identifiable characteristics, like fluorescent and luminescent proteins (Clark et al., 2019).

- **Balancer chromosomes:** genetically engineered chromosomes used to stably maintain mutations in the stock without a selection process. *D. melanogaster* cannot be frozen or archived, so mutants have to be continuously propagated. Mutations are only viable in heterozygotes so they cannot be maintained through generations and continually lead to the production of wild-type organisms. To prevent that, the homologous wild-type chromosome is replaced with a balancer. They have different characteristics that allow them to balance and control the offspring (Hales et al., 2015):

    - **Dominant phenotypical (genetic) marker:** each balancer chromosome expresses a different character in the phenotype, enabling the distinction between the flies with the balancer and the ones without it using just a magnifying glass.

    - **Inversions, deletions translocations:** this avoids the recombination of the balancer chromosomes with its homologous chromosomes during the stage of meiosis.

    - **Recessive lethal marker:** when carried in homozygous, the fly with two balancer chromosomes will die or will be infertile, allowing scientists to only pick up the flies needed for the experiment without other genotypes in the middle.

- **Bank of mutants:** throughout the year of research, there has been generated mutants for almost every gene. To generate the first mutants, chemical compounds and X-rays were used, however, the most recent technique is CRISPR-Cas9.

- **The GAL4/UAS system:** a dual system that allows controlling gene expression in a temporal and spatially regulated fashion. GAL4 is a yeast transcription factor that recognizes and activates specific genes that are regulated by a UAS

(upstream activating sequence) that is placed before the gene or the RNAi of interest. One of the progenitors will carry the driver of the gene expression (GAL4) and the other progenitor will carry the UAS sequences with the gene of interest. In the progeny, both the GAL4 and the UAS sequence will come together and regulate gene expression (Busson & Pret, 2007).

- **Driver line**: the GAL4 gene is expressed in tissue-specific patterns

    - **Tissue promoter**: a DNA sequence that will mark the expression domain area of the GAL4 protein. At the same time, this will be the promoter sequence that will initiate gene transcription.

    - **GAL4**: the protein used as a transcription factor that adheres specifically to UAS sequences. This protein comes from yeast *Saccharomyces cerevisiae* and (Brand & Perrimon, 1993) cloned the transcription factor GAL4 into a vector that can be integrated into the genome.

    - **Responder line:** the gene of interest is under UAS control. UAS is the sequence where the GAL4 protein is adhered to give the signal to initiate the transcription of the gene or the RNAi, which is located near the UAS sequence (Hales et al., 2015; Mcguire, 2004).

    - **UAS:** it is found in a different gene from the GAL4, which makes it possible for a fly to contain a UAS with genes behind that express a tumour but that it does not for lack of the GAL 4 protein. Similarly, a fly can contain GAL4 and not express any character due to the lack of a UAS. That is why the system needs two parts to work.

    - **GEN:** the DNA sequence that is activated due to the binding of GAL4 with UAS.

    - **RNAi:** interference RNA is a sequence that aims to adhere specifically to an mRNA in order to block its functionality. They work as a zipper, and they are made up of nucleotides

complementary to the mRNA, being able to adhere permanently to the mRNA.

In this system another protein can be involved too, the GAL80, which has the ability to adhere specifically to the GAL4 protein, making a blockage. This allows the fly to have both the GAL4 protein and the UAS sequence but not the gene transcription, because the protein is blocked and cannot adhere to the UAS. GAL80 has a thermosensitive variant (GAL80ts) that allows us to express the gene in a particular moment, as when it is between 18 and 29 °C it is functional and allows the inhibition of GAL4, but when it is above this interval it is not functional and allows GAL4 to activate the UAS sequence. This is useful when we want to express the tumor in a particular stage, like the adult fly, as if the tumor is activated before, the fly would never reach that stage (Suster et al., 2004)

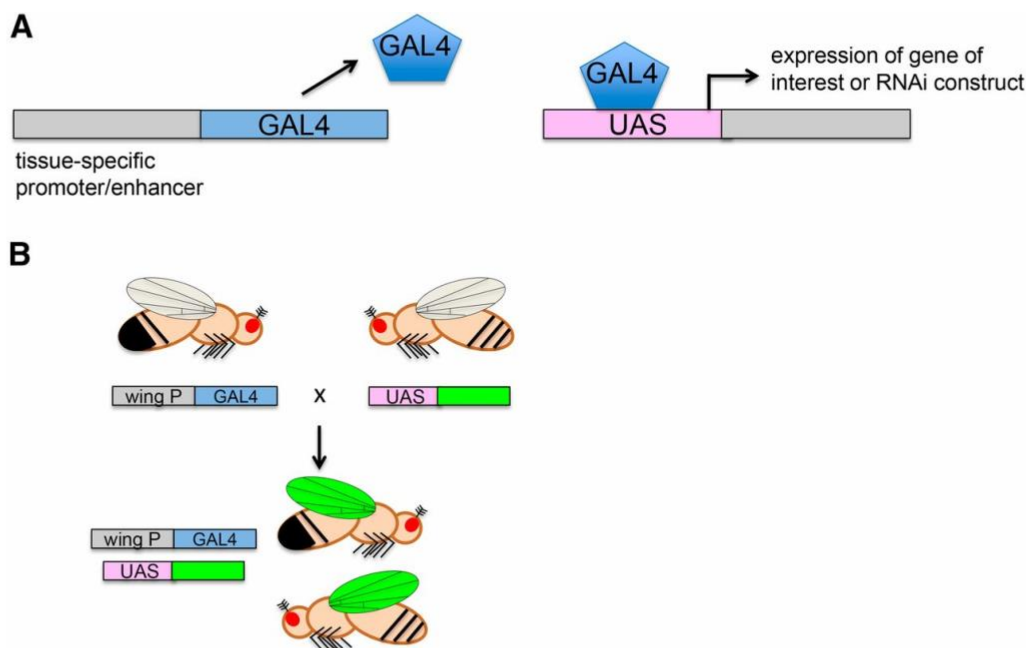

*Figure 13: **GAL4/UAS System for modular expression of genes in specific tissues.** A: flies carrying a driver with a tissue-specific promoter placed on the gene encoding the yeast GAL4 transcription factor (left) and the gene of interest placed on the UAS sequence (right). B: transgenic flies carrying either of the two parts alone (top) without expressing the gene of interest, but when crossed into the same fly, the tissue-specific promoter (a wing promoter) drives expression of GAL4, which turns on the gene of interest (indicated by green) in the specific tissue. Extracted from:* (Hales et al., 2015)

Drosophila in the oncological research

Chromosomal Instability (CIN)

As mentioned before, mutations and genomic instability are one of the two characteristics that allow the development of different Hallmarks of cancer. CIN refers to a higher-than-normal rate of missegregation of chromosomes or parts of chromosomes during mitosis, due to a defective cell cycle quality control mechanism (Thompson et al., 2010; Wei et al., 2016).

The most common direct consequence of CIN is aneuploidy. CIN induction in some tissues has been one of the most widely used techniques for decades in laboratories to cause the production of tumors in animals such as the fruit fly. Although the terms CIN and aneuploidy are closely related they are not synonymous and therefore cannot be used interchangeably. The differences between the two words are as follows (Schukken & Foijer, 2018):

- **There may be aneuploidy without CIN.** In many cases, the alteration of the usual number of chromosomes or aneuploidy may appear regardless of chromosomal instability as a result of a possible poor distribution of chromosomes with the consequent expansion of this initial cell with aneuploidy. In these cases, the tumors that may appear will have a homogeneous alteration of the normal number of chromosomes as all cells will come from it and have the same abnormal number of chromosomes.

- **CIN causes aneuploidy in many cases.** On the other hand, when an organism has CIN, it will most likely appear a tumor formed mostly by cells with different or heterogeneous aneuploidies because in all cell divisions will vary the number of chromosomes that will have the two descending cells by chromosomal instability.

In the last decade, most models for CIN have been developed in *D. melanogaster* due to the unique benefits it offers for understanding the functioning of CIN in live animals and also thanks to the multiple advantages it offers not only for maintaining but also for all the reasons mentioned that make it an excellent animal as a genetic model.

Despite the various existing mechanisms for the induction of CIN in the fruit fly, one of the most widely used systems is the mentioned GAL4/UAS. A mention before, it allows the expression of a gene or RNAi in the F1 generation without either parent express. This system presents different possibilities when choosing both the promoter area and the sequence of bases that UAS will activate, which enhances its use.

In this case, we chose to choose the *Apterous* domain zone and an RNAi that will block the production of the Bub3 protein to produce CIN. This protein is responsible for the formation of the mitotic spindle and the correct distribution of chromosomes. Later, when we talk about crosses between flies, we will get further into it.

Dominant-negative

A mutation of certain genes that code for proteins in such a way that they express an excess of a non-functional one. This mutation causes that without diminishing the production of the functional proteins the effectiveness decrease (fewer molecules will do their job). It is called a negative dominant because it only takes one mutation in one of the alleles of the gene for non-functional protein molecules to begin to occur. This achieves a dominant effect even though it has only one mutation in one of the two genes. These variations result in a loss of protein function if functional, making it less functional or not useful for its original role (Sheppard, 1994).

Imaginal Discs

They are sacs of epithelial cells that are found in the larva of insects with complete metamorphosis and represent the cuticular structures of the adult (wings, legs, antennae, mouth, genitals ...) which will form during pupal metamorphosis. In this work, we will use the imaginal wing disk as an epithelial study model as we have these multiple tools for genetic manipulation and simplicity in being extracted from the larva.

The epithelial cells that make up the disc acquire imaginary tissue identity through positional information, that is derived from the interpretation of signals they receive from the outside such as "Wingless (Wg)" and "Decapentaplegic (Dpp)". The identity of each cell type is determined by the location of the cells within the tissue. The signaling to which the cells are subjected varies in the different zones of the disc, a fact that gives rise to different identities. Compartments are units of lineage, so the acquisition of anterior or

posterior identity in a cell implies that all cells descending from it will have the same identity as the stem cell (Lawrence & Struhl, 1996).

Approximately in the second larval stage, the second territorial segregation occurs in the imaginal wing disc with the appearance of the dorsal (D) and ventral (V) compartments. The gene responsible for giving dorsal identity to cells is *Apterous* and their absence determines cells as ventral. Approximately at the same time a third division is established that differentiates cells of the thorax (notum) and cells of the wing.

Like all epithelia, the imaginary wing disk is always oriented and arranged in the same way with a polarity (Simons & Mlodzik, 2008). Specifically, this type of epithelial tissue has an apicobasal polarity, which means that they have a membrane that separates them from other tissues at the base, and are joined together by various proteins on the sides. The loss of this polarity is mostly caused by the appearance of a tumor and the uncontrolled growth of cells.

Imaginary discs have provided a very useful platform for studying fundamental aspects of biology, mainly due to their accessibility, and to the development of methods that allow the manipulation of the genetic content of cell populations within the discs (Beira & Paro, 2016). The use of imaging discs as experimental systems also overcomes various limitations such as lethal embryonic mutations, because portions of mutant tissue can be generated and analysed at later stages of development. This is possible because there are significant technical advances that have contributed to the development of this field, mainly the GAL4/UAS system which, as we have already mentioned, allows the detection or specific expression of a specific segment and its possible combination, with the generation of homozygous mutant clones within a tissue without mutations.

JNK signaling pathway

The Jun N-terminal kinase (JNK) is a member of the mitogen-activated protein kinase[13] family. It appears to be conserved in all animal species where it regulates important physiological functions involved in apoptosis, cell migration proliferation and regeneration (Bosch et al., 2005; Kanda, 2004). A principal feature of the JNK pathway

[13] an enzyme that catalyzes the transfer of a phosphate group from ATP to a specified molecule

is that it is activated in response to stress or pro-inflammatory cytokines (Noselli & Agnès, 1999).

In *D. melanogaster,* there is a single JNK protein, encoded by the gene *basket (bsk)*. The activation of Bsk leads to the phosphorylation of the transcription factors Jun and Fos that regulate the activity of genes responsible for the various cellular functions associated with JNK activity. In the case of the imaginal discs, JNK is not expressed during normal development, but it is activated after tissue damage or irradiation (Pérez-Garijo et al., 2009). The activity of JNK under those conditions triggers apoptosis and the subsequent elimination of the cells expressing the pathway. However, it has been shown that JNK may also have a pro-proliferation activity. Sustained JNK expression is linked with the formation of large tumor overgrowth in the imaginal discs (Pinal et al., 2018).

Practical frame

Materials and Methods

This practical study was conducted at the Institute for Research in Biomedicine (IRB) Barcelona, specifically in the laboratory of Marco Milán, under the supervision of Mariana Muzzopappa, during the summer of 2021. This research aimed to check whether the JNK pathway is activated or not in the process of tumorigenesis. *D. melanogaster*'s imaginal discs, immunofluorescence techniques, different transgenic lines and confocal microscopes were used to analyse the induced chromosomal instability. The GAL4/UAS system was used to express in a certain tissue and time an interference RNA that cancelled the function of *Bub3* (a gene that intervenes in the mechanism of control of cell division (Spindle assembly checkpoint gene)). When blocked, errors occurred in chromosome segregations, so that some cells inherited an altered number of chromosomes. This generates aneuploid cells, which induce the formation of a tumor.

Procedure

1. **Crossing**

   Before starting the experiment, it was necessary to obtain the necessary *D. melanogaster* specimens. To obtain the phenotypically and genotypically flies desired, we made crosses between existing samples in the laboratory[14].

   First of all, a differentiation between males and females was made, as they shared habitat in the stock tubes. Once the flies were separated by sex, it was necessary to identify the virgin samples.

   Once located the virgin sample, they were placed in different tubes. Next, we repeated the process of differentiation but to acquire the male specimens, and we introduce them in the tubes where the virgin flies were previously introduced.

   Flies remained in these tubes for twenty-four hours at 25°C. After that day, flies were transferred to a new tub. It is important to change the adult container flies every day, to avoid an accumulation of flies in different phases of the developmental cycle. In order to transfer the flies, the cotton that acts as a stopper is removed while shaking the bottom. As a result, the parental generation falls

[14] Flies used can be seen in the Key Resources Table section

due to gravity to the new tube while the eggs remain in the old tube, as they are attached to the food.

After forty-eight hours, the tubes were moved to water tanks that act as temperature regulators at 29°C, since at this temperature the proteins increase their efficiency.

In the different experiments performed, we used flies with genetic manipulations on chromosomes two and three, as they are the largest and most important ones. The scheme used when crossing flies is shown in Figure 14.

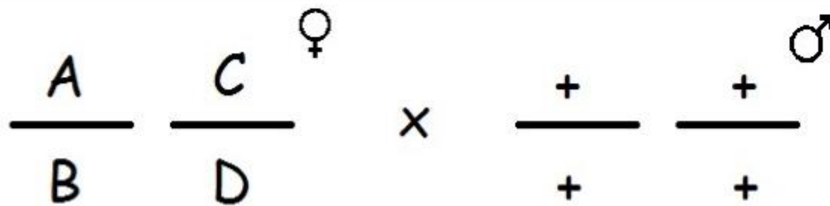

Figure 14: ***Schematic representation of chromosomes 2 and 3 at the crossing of D. melanogaster.*** Letters A and B represent chromosomes 2 and 2' of the fly while C and D are 3 and 3' (one inherited from the mother and one from the father). The "+" symbol means that the fly does not contain any transgenic and is therefore Wild Type (WT).

We made three parallel crosses to obtain the different genotypes:

Control: *Ap-GAL4, UAS-MyrT*

We selected male and female flies of two previous lines with the transgenic sequence that was of interest to us: Ap-GAL4, UAS-MyrT All offspring will maintain the mutation because the parents are homozygous for this information. The sequence has three parts:

- Ap (Apterous): Promoter zone that is located at the beginning of the sequence and that contains the information for the domain zone where the genes that follow it will be expressed, specifically the GAL4 gene. In this case, the genes that follow this promoter zone will be expressed in the imaginary wing discs of *D. melanogaste*r.
- GAL4: The gene that will transcribe for the GAL4 protein will be activated in the *Apterous* domain area, as it is located just behind it. GAL4 will always activate all the UAS sequences contained in the fly, by also expressing them only in the domain area of the Apterous promoter.

- UAS-MyrT: A gene that encodes a protein called Tomato that emits a wavelength corresponding to the color red under a fluorescence microscope. This gene will only be activated in the *Apterous* domain zone.

CIN with tumor: $\frac{Ap-GAL4,UAS-MyrT,UAS-bub3RNAi}{CyO}$; $\frac{UAS-p35}{Tm6,tb(G80)}$

- ***Bub3^{RNAI}:*** will be responsible for causing CIN as it will produce an interference RNA (RNAi) that will adhere to the mRNA responsible for the production of Bub3 protein forming an RNAm + RNAi complex that will be degraded without being able to produce Bub3. This protein has a very important function, as it controls points of cell mitosis. Specifically, it is one of the groups responsible for the formation of the mitotic spindle and therefore for the correct distribution of chromosomes in the process of cell replication. When this protein is non-existent, the cell is unable to properly distribute chromosomes in mitosis, which causes CIN and induces the formation of tumors throughout the imaginary wing of the fly. This RNAi will only be activated in the *Apterous* domain area because it is activated only where the GAL4 protein is produced.

- CyO: This is a *D. melanogaster* balancer, which is phenotypically easy to recognize in adults by the characteristic curvature they have on their wings.

- TM6, GAL80: (TM6 + GAL80) In this sequence we find two different genes. On one hand, there is the TM6 rocker that contains the phenotypic marker Tubby, due to the characteristic rounded shape and smaller than usual of the larva. On the other hand, it also has the gene that will produce the GAL80 protein, an inhibitor of GAL4.
  Containing two genes, TM6, GAL80 has two functions:
  - TM6: Help us to distinguish by phenotype which larva do not contain the genes that interest us and therefore will not cause CIN.
  - GAL80: It allows us to maintain the line, since if the offspring could not produce this protein, the CIN would have formed in F1, so that

the GAL4 of the sequence Ap-GAL4, UAS-bub3RNAi would not have been inhibited.

- p35: a protein with an inhibitory function on the apoptosis process, which is directly related to the JNK pathway in *D. melanogaster.*

  The 4 possible genotypes are:
  - Ap-GAL4, UAS-bub3RNAi; p35
  - Ap-GAL4, UAS-bub3RNAi; TM6GAL80
  - CyO; TM6, GAL80
  - CyO; p35

  Of these, we are only interested in Ap-GAL4, UAS-bub3RNAi; p35 as it is the only one that will have CIN and therefore a tumor in the imaginal wing disk. In the case of Ap-GAL4, UAS-bub3RNAi; TM6, GAL80 there will be no tumor due to the function that GAL80 performs by blocking GAL4 and consequently blocking all UAS and RNAi that it activates.

  Given that we work with larvae of *D. melanogaster*, to rule out the 3 possible genotypes that do not interest us we must look in a magnifying glass which larvae are smaller and rounded than usual, and therefore contain the TM6 balance, thus ruling out 2 of the possible genotypes. As for the other option, we don't want, we'll need a fluorescence microscope to look at which larvae emit red light, discarding those that don't (CyO) and therefore do not contain the MyrT gene.

Rescued CIN:

$$\frac{UAS-bsk-DN}{mmp1-GFP};\frac{Ap-GAL4,UAS-MyrT,UAS-bub3RNAi}{CyO};\frac{UAS-p35}{Tm6,tb(G80)}$$

- *Bsk^{DN}*: is a kinase that phosphorylates the transcription factor that goes to the nucleus and activates gene expression. By using a negative dominant, we block its function and therefore, the JNK pathway ceases to be active, the tumor does not grow because it depends on this signaling pathway.

- *Mmp1-GFP*: the matrix metalloprotease-1 is expressed when the JNK signaling pathway is activated, so it will let us see if the JNK has been active (Uhlirova & Bohmann, 2006). The Green Fluorescence Protein (GFP) will let us see the expression under the microscope.

2. Dissection ((Spratford & Kumar, 2014))

Five days after the cross, the imaginal discs of the larvae were dissected. An ordinary compound microscope (two eyepieces) was used. The slide on which the larvae are placed must contain a saline solution called Phosphate-buffered saline (PBS) (Table 3), which is a buffer solution commonly used in biological research. This buffer helps maintain a constant pH.

Table 3: composition of PBS 1X. Extracted from: ("Phosphate-Buffered Saline (PBS):," 2006)

Salt	Concentration (mmol/L)	Concentration (g/L)
NaCl	137	8.0
KCl	2.7	0.2
Na_2HPO_4	10	1.42
KH_2PO_4	1.8	0.24

Before dissecting the imaginary discs, it was necessary to choose the right larvae. In the tubes where the crosses have taken place and which consequently have the resulting eggs, the larvae were born. There are two types: tumor (its genotype indicates the formation of the tumor in the imaginary disc) and non-tumor (its genotype is not the one with the induced CIN). In our experiment, we only needed those that develop the tumor.

To separate the two kinds of larvae, it was needed to consider which chromosome balancer had been used:

- **CyO; GFP:** CyO causes adult specimens to have wavy wings, and GFP, a fluorescence-producing protein, can be observed with a confocal microscope from the first larval stage.
- **TM6Btb; Gal-80:** its generic marker is tb (tubby-tagged), which cause larvae to have a much smaller size than the wild type larva.

Considering the above, the larvae that we choose should be large in size and they should have GFP expressed. In addition, tumor larvae need more time to hatch because their genotypic errors cause delayed embryonic development. Therefore, the first larvae to appear will usually be non-tumorous.

Once the sample were prepared and the larvae were selected, using two tweezers (one in each hand) we prepared the dissection and followed the following steps:

1- Fill a Petri dish with PBS and place it on ice and prepare and Eppendorf with 900 μl of OBS
2- Place larvae in the Petri dish and identify where the head is, by looking at the mouth hooks
3- Clasp the larva with the forceps and cut it in half and remove the part of the abdomen opposite to the head, as it will not be necessary. One pair of forceps should be used to grab the larva gently while the other pair is used to hold the larva
4- Release the larva from the forceps and allow the guts and the fat bodies of the larva to spill out. This allows for the imaginal discs to remain in their normal conformation and prevents them from being deformed
5- With one pair of forceps grab the anterior part of the larvae and use the other pair to push the mouth hooks inside the larvae, trying not to tighten it too much. Make an inversion with the other tweezer in order to expose the imaginary discs
6- Insert the remaining of the larva into an Eppendorf tube, containing 900 μl of PBS 1X. Leave the tubes on ice so they don’t degrade

3. Immunostaining

Immunostaining is the most common technique for tissue analysis. It is a general term in biochemistry that refers to any use of an antibody-based method for the detection of a specific protein in a sample. Immunohistochemistry or IHC (staining of tissue sections) is perhaps the most commonly applied technique in the immunostaining technique. Two types can be differentiated (Figure 15) (Maity et al., 2013):

- Direct immunostaining: in which the specific antibody that binds to the substance to be detected is labeled with a chemical fluorescent.

- Indirect immunostaining: with this technique, the antibody signal expands when two layers of antibodies are produced. The first antibody binds to the antigen of the protein and the second antibody to the first antibody. The primary antibody can bind to more than one secondary antibody to amplify the signaling.

In this experiment, to test whether the JNK pathway is activated during tumor formation and development, we use indirect immunostaining to recognize proteins and transcription factors with antibodies.

Primary antibodies bind to antigens (proteins that we want to be labeled), but their fluorophore is too weak to be distinguished under a microscope, so a secondary antibody is used to recognize part of the primary antibody which is species-specific. The fluorophores of the secondary antibodies allow us to see where the primary antibody is, and therefore the protein we want to distinguish in the cellular tissue.

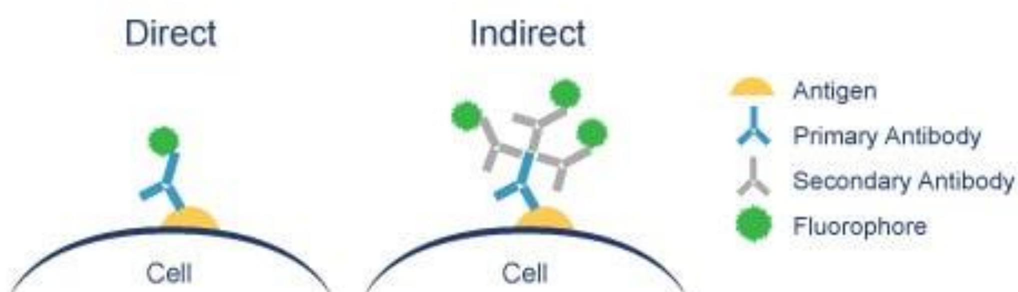

Figure 15: Direct and indirect immunofluorescence techniques. Extracted from: (Maity et al., 2013)

Products used during the process (Table 4.):

*Table 4: **Products used during the immunostaining process and its functions***

PBT	Solution composed of PBS (99%) and detergent (1%) used for washing
Formaldehid	Toxin organic molecule of Formula CH_2O. In preparations, it fixes the tissues by giving them rigidity so that they do not break so easily
BSA	Protein used as a blocker in solutions. By acting as a blocker, it binds nonspecifically to different tissues and molecules, which will allow for cleaner staining, as there are molecules that we don’t want that could adhere to the primary antibodies. These molecules will be removed from the solution once they join the BSA and perform the corresponding washes
BBT	Solution used to block
Mounting Media	Mixture used to preserve both fluorophores and larval tissues

Procedure:

1. Fix with PBS + 4% Formaldehyde for 20 minutes in motion (900 microliters of PBS 1X +100 microliters of Formaldehyde 37%). As the Eppendorf tubes with the imaginary discs contain already 900 µl of PBS, add only 100 µl more of Formaldehyde (FA). This fixing substance is carcinogenic and very toxic, so it Is necessary to use a camera and gloves. FA causes proteins to form a compact mass that, when bound, causes mild dehydration so that once the antibody is introduced, it can adhere effectively to the antigen.
2. Wash three times 10 minutes with PBT (PBS 1X + Triton 0.2%) to remove the remnants of Formaldehyde, as this would cause the decomposition of the antibodies. In addition, PBT permeabilizes the tissue and allows better

penetration. The Eppendorf tubes are placed in the mixer so that the PBT passes through and cleans all the tissues.

3. Block 20 minutes with BBT (PBT +0.1% BSA + 250 mM NaCl). A protein has both positive and negative charges, then the antibodies can bind by electrostatic instead of specificity. BBT surrounds the tissue and this causes the antibody to bind only by specificity (it blocks non-specific sites in the tissue).
4. Add the primary antibody (Mouse monoclonal anti-WG) diluted in BBT, in a final volume of 50 microliters overnight at 4°C.
5. Wash four times 20 minutes with PBT at room temperature using the mixer.
6. Add the secondary antibody (Cy2 AffiniPure Donkey Anti-Moues IgG (H+L)) diluted in BBT (1:200). Secondary antibodies, as previously described, recognize some of the primary antibodies and are specific, and also have fluorophores that allow us to see where the primary antibodies are. Also add DAPI (4',6-diamino-2-phenylindole), which is a fluorescent marker that binds strongly to regions rich in adenine and thymine in DNA sequences, thus marking nuclei preventing tissues from being invisible. It provides a blue color.
7. Wash 4 times 15 minutes with PBT stirring at room temperature and in complete darkness. The Eppendorf tubes can be covered in aluminum foil so that they don't get any light.
8. Remove PBT and add Mounting media (Glycerol 80% in PBS +nPG) and leave overnight at 4°C.

4. Extraction of imaginary discs

Once we had applied the two antibodies to the half larvae, we needed to extract the imaginary discs from the entire structure. Using a tweezer in one hand and a punch in the other, we set out to first identify the larval organs. The discs must be removed carefully by pushing with the punch and holding the larva with the tweezers. Once we had the desired structures extracted, they were placed on another slide that contained Mounting Medium. We marked in each of them with a permanent marker the genotype of the disk we were going to observe. The half larva that contained the remaining structures that weren't needed for this experiment were removed.

5. Microscopy

We used the ZEISS focal length microscope to observe the discs. The magnification of the eyepiece with which we looked at them is X10 and in the revolver of lenses, we chose the one of X25, achieving a total increase of X250. In order not to overly vary the refractive index between the different media of the preparation, we used 70% glycerol as an immersion liquid between the eyepiece and the discs.

A confocal microscope is a microscope capable of obtaining three-dimensional images of an object, such as a cell. It is based on a principle similar to that of a fluorescence microscope, but two confocal diaphragms are used - that is, it has the same focus - one located in front of the sample and the other behind; in this way the lighting can be directed to a single point in the sample.

A laser is used as a light source, and with its light, the sample is swept at all points of the volume of the object, plane by plane. Many two-dimensional images are created that a computer interprets and then ends up generating a complete three-dimensional image.

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-WG (4D4)	Developmental Studies Hybridoma bank (DSHB)	RRID: AB_528512
Cy2 AffiniPure Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch	Code: 715-225-151
Chemicals, Peptides and Recombinant Proteins		
DAPI	Sigma Aldrich	Code: 28718-90-3
Experimental Models: Organisms/Strains		
UAS-bub3^{RNAi}	VDRC Stock Center	RRID: VDRC_21037
UAS-bsk^{DN}	Bloomington Drosophila Stock Center	RRDI: BDSC_6409
UAS-p35	Bloomington Drosophila Stock Center	RRDI: BDSC_5073
UAS-myristoylated-Tomato (myrT)	Bloomington Drosophila Stock Center	RRDI: BDSC_32222
ap-GAL4	Bloomington Drosophila Stock Center	RRDI: BDSC_3041
Software and algorithms		
Fiji	Fiji	https://fiji.sc
GraphPad Prism 7 Project	GraphPad	RRDI: SCR_002798
Excel	Microsoft Excel 2016	N/A

Experimental model and subject details

Fly strains

Flies were maintained on standard medium (4% glucose, 55g/L yeast, 0,65% agar, 28 g/L wheat flour, 4 m/L propionic acid and 1,1 g/L nipagin)[15]. The following stocks were used: *UAS-bub3*^{RNAi} from the Vienna *Drosophila* RNAi Center, VDRC, Austria; *UAS-bsk*^{DN}, *UAS-p35, UAS-myristoylated-Tomato (myrT),* and *ap-GAL4* from Bloomington Drosophila Stock Center, IN, USA.

Genetic Analyses

To generate CIN, female flies carrying the *ap-GAL4* driver were crossed to male flies carrying the *UAS-bub3* and *UAS-p35* transgenes. Fertilized females were allowed to lay eggs at 25 °C and the resulting larvae were switched to 29 °C 48 h after egg laying. Wing imaginal discs were dissected after 5 days and both female and male larvae were used. Both control and tumorigenesis flies were grown at the same time.

Method Details

Immunohistochemistry and Confocal Imaging

Late third instar larvae (110 h after egg laying) were selected, and wing imaginal disc were dissected in phosphate buffered saline (PBS), fixed for 20 minutes in 4% formaldehyde in PBS and stained with antibodies in OBS with 0.3 BSA, 0.2% Triton X-100. The following primary antibodies at following dilutions were used: Donkey Anti-Mouse IgG (1:1o0; H+L; Jackson ImmunoResearch); mouse anti-Wg (1:20; 4D4, DSHB). Leica TCS SO5 MP confocal microscope was used to take high resolution images.

Flow Cytometry Analysis

Wing discs of larvae of the following genotypes (1) *ap-GAL4, UAS-MyrT/UAS-bub3-I,MMP1-GFP;UAS-p35* and (2) *ap-GAL4, UAS-MyrT/ MMP1-GFP;UAS-p35/UAS-bsk-DN* were dissected in cold PBS, dissociated with Trypsin-EDTA, fixed with formaldehyde 4% for 20 min and permeabilized in ethanol 70% for 2 hours. Fixed cells were stained with DAPI. Delaminated cells (MyrT-positive, MMP1-GFP-positive) activate JNK and drive *MMP1-GFP* expression. Excitation of the sample was carried out using a Coherent Enterprise II argon-ion laser. Excitation with the UV laser (440 nm) allows the acquisition of DNA content, the blue laser (530 nm) fluorescence from GFP and the green laser (610 nm) fluorescence from Tomato.

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[15] More about the flies maintenance in Appendix I

Results

Once the samples were observed with the confocal microscope, the images were exported to a computer using the FijiImageJ software. This is an image processing program that uses Java. It supports standard image processing functions, such as logical and arithmetic operations between images, contrast manipulation, edge detection, and medium filtering. It performs geometric transformations such as scaling, rotation, and flips. The program supports any number of images at a time, limited only by the available memory.

With FijiImageJ we selected the images from the samples where what we wanted to demonstrate was better visualized. This program also allowed us to retouch the images and make it easier to distinguish the compartments and regions under study.

In every cross we analysed:

1. DAPI expression in blue fluorescence
2. Mmp1 expression in green fluorescence
3. The *Apterous* domain morphology in red fluorescence

Control: Ap-GAL4, UAS-MyrT

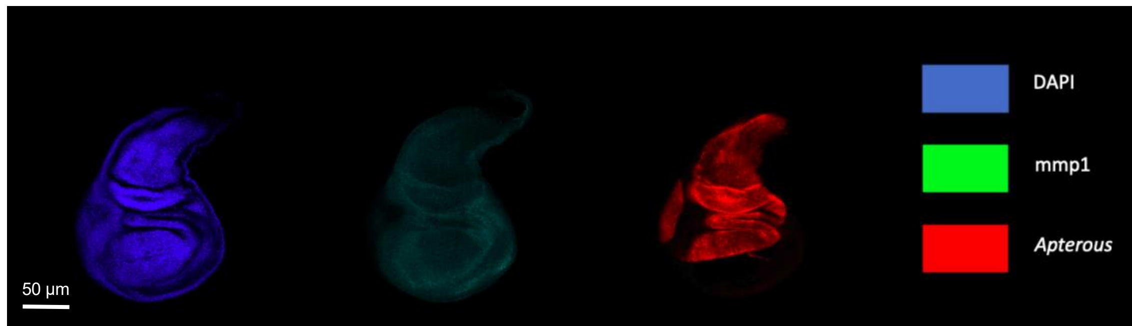

Figure 16: ***Fluorescence emitted by wing imaginal disc with the*** **Ap-GAL4, UAS-MyrT genotype**

- **DAPI and morphology:** cells nuclei are stained just as a Wild Type fly. All the compartments can be easily distinguished.

- **Mmp1:** the fluorescence levels are very low, as there is no mmp1 expression, which means that the JNK pathway has not been activated. All the disc has the same fluorescence, without difference between the *Apterous* domain and the others.

- ***Apterous*** **domain:** identic to a Wild Type fly, with the *Apterous* zone marked with the MyrTomato protein, as the genotype indicates that the MyrT protein should be expressed only in that domain.

This control cross is of great importance as it is on this where we will later contrast the results we will obtain in the other samples. If we compared the fluorescence obtained in the experiments with a WT fly, we would make a mistake, since it must be taken into account that the ApMyrT line has in itself a higher number of cell death than a wild-type fly, as they are transgenics in heterozygosity of an *Apterous* gene with several mutations.

CIN with tumor: $\frac{Ap-GAL4,UAS-MyrT,UAS-bub3RNAi}{CyO};\frac{UAS-p35}{Tm6,tb(G80)}$

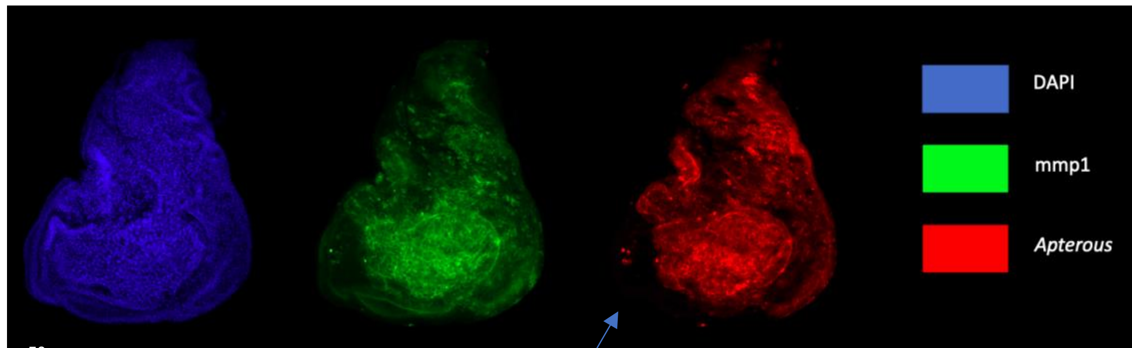

*Figure 17: **Fluorescence emitted by wing imaginal disc with the*** $\frac{Ap-GAL4,UAS-MyrT,UAS-bub3RNAi}{CyO};\frac{UAS-p35}{Tm6,tb(G80)}$ **genotype**

- **DAPI and morphology:** the disc is overloaded; it even comes out of the screen. It is visibly bigger than the control, so the tumor can already be seen.

- **Mmp1:** the protein is expressed in greater quantities than in the Control, meaning that the JNK pathway has been activated and has produced mmp1 proteins. We can see that the protein is overexpressed in the *Apterous* domain area, as it is where the CIN was induced and consequently where the JNK pathway initiated.

- ***Apterous* domain:** it has increased its size, allowing us to see the tumor. There are also cells that are migrating from the initial tumor to invade other tissues and metastasize. As the compartments and domain areas are affected by the tumor, there isn't a clear view of the *Apterous* domain area.

Rescued CIN: $\frac{UAS-bsk-DN}{mmp1-GFP};\frac{Ap-GAL4,UAS-MyrT,UAS-bub3RNAi}{CyO};\frac{UAS-p35}{Tm6,tb(G80)}$

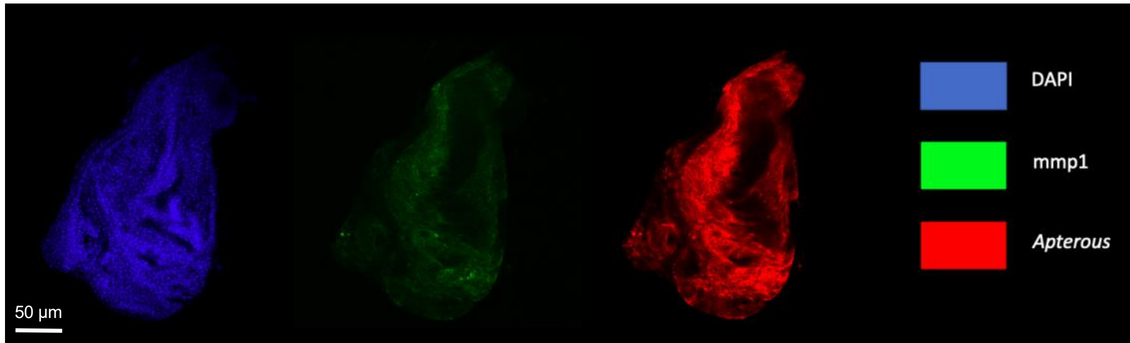

Figure 18: ***Fluorescence emitted by wing imaginal disc with*** $\frac{UAS-bsk-DN}{mmp1-GFP};\frac{Ap-GAL4,UAS-MyrT,UAS-bub3RNAi}{CyO};\frac{UAS-p35}{Tm6,tb(G80)}$ **genotype**

- **DAPI and morphology:** the disc is not much bigger than the Control one, however, the edges and the domains are not clear and it is difficult to distinguish them. As most cells in the *Apterous* domain have CIN, it has affected the morphology of the disc.

- **Mmp1:** the protein is expressed in greater quantities than in the Control but much less than the previous one, as the JNK has been blocked. Because we have used a dominant-negative mutation of the gene *basket,* in its majority the JNK has not been activated, but we can see some cells that have the pathway activated, as the dominant-negative mutation maintains some functional proteins.

- ***Apterous* domain:** the *Apterous* domain is not clear, because all cells in it have CIN. The disc has an irregular form and morphology, without a clear distribution or differentiation of the compartments.

Comparison

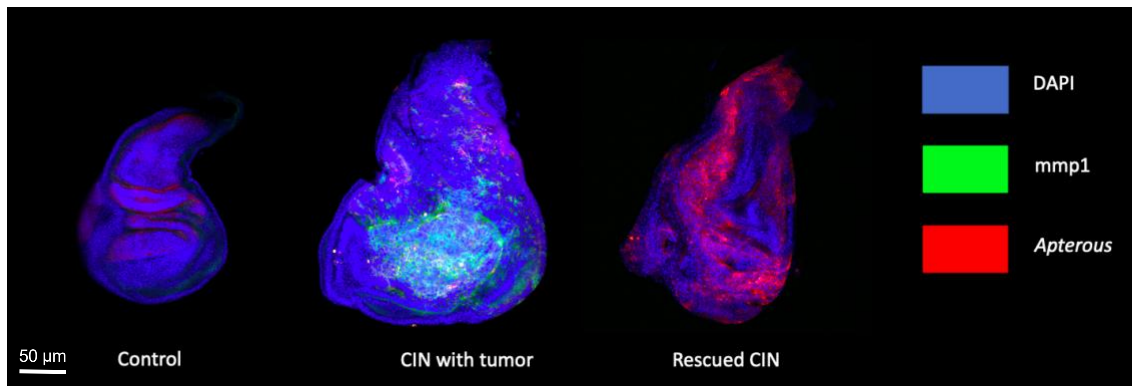

Figure 19: ***Fluorescence emitted by wing imaginal disc of the three crosses in this experiment, control CIN with the formation of a tumor and without the formation of one.*** *All three channels, blue, red and green fluorescence appear in the same image.*

The resulting wing primordia were massively overgrown, and the cell population where CIN was induced invaded the neighboring, wild-type territory. Invading cells were non-apoptotic, as shown by DAPI staining of their nuclear morphology. The mode of migration observed in all cases was as single cells.

So far, we have provided evidence that cell delamination and JNK activation is linked to high levels of aneuploidy. Our results indicate that CIN-induced JNK activation has an important role in organizing hyperplastic overgrowth of the tissues, as it was expressed in the tumor growth (Figure 19). When the JNK activity was blocked, the tissue overgrowth caused by CIN was rescued (Figure 20). Expression of MMP1 in delaminating cells degrades the underlying basement membrane thus facilitating tissue invasiveness. Remarkably, although JNK plays a critical role in driving tumorigenesis, JNK activation is not enough to drive tumorigenesis.

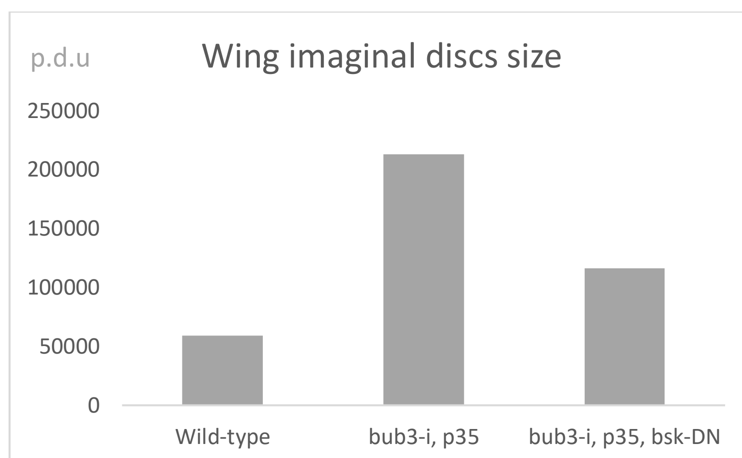

Figure 20: **Histogram plotting the wing imaginal discs size** measured in arbitrary unit of the indicated genotypes under the *ap-gal4* driver. The graphic indicates the average size measured after 5 days of induction.

Discussion

Perhaps the most accepted hypothesis on the role of CIN in tumorigenesis is the one that proposes that CIN is a source of mutability (like loss or gain of certain chromosomes carrying tumor suppressor genes or oncogenes) that helps the tumor cell population growth. CIN has been linked to metastatic progression in human tumors (Turajlic & Swanton, 2016) too.

Here, we used an epithelial model of *D. melanogaster* that has many features of CIN-induced tumorigenesis to address whether CIN is able to induce by itself and activate molecular mechanisms such as the JNK signaling pathway. We used RNAi forms of the *bub3* gene to present evidence that CIN is indeed able to induce an active invasive behavior. Cellular invasion is most probably not a direct consequence of gain or loss of specific chromosomes, as CIN-induced aneuploid cells did not have a signature of pro-invasiveness genes (Clemente-Ruiz et al., 2016).

In our study, aneuploidy-induced cells became mobile, expressed the secreted matrix-metalloproteinase-1 (MMP1) and degraded the membrane, thus supporting the role of CIN in driving tissue invasiveness, which promotes tumor growth. The expression of MMP1 depends on the activity of the JNK pathway. The activation of this pathway was required for CIN-induced tumorigenesis, as blocking the activity of this kinase rescued CIN-induced tumor-like overgrowth.

Work in flies has demonstrated that the cellular response to aneuploidy is tissue-dependent (Gogendeau et al., 2015), so further work is required to characterize the diversity of cellular responses to aneuploidy in different tissues and to address where the invasive behavior reported in this work is specific to epithelial cells.

The results reinforce the tumor-promoting role of the JNK signaling pathway (Pérez et al., 2017), since when it was blocked, the tumor growth was inferior. The JNK pathway possesses the ability to release proliferative signals that can stimulate the growth of the tissue nearby. In this study, we reinforce that the presence of the baculovirus protein p35 prevents the destruction of the cells (Wells et al., 2006). Undead cells remain alive for the rest of the development and keep secreting mitogenic factors, thus generating tumor growth.

Regarding *D. melanogaster,* there are several examples in which sustained JNK activity causes tumorigenesis in imaginal disc, due to persistent proliferative signaling from JNK-expressing cells (Brumby, 2003; Johnston et al., 1999). In our experiment, we believe that the size and shape of the *Apterous* domain ensure that many of the cells inside the group are beyond cell competition and therefore can continue proliferating. In this situation, tumor cells die at the border, but they are continuously replaced by neighbors.

However, it has also been shown that the activity of JNK after tissue damage triggers apoptosis and the subsequent elimination of the cell. JNK is primarily activated by stress factors and is critical for the apoptotic (Bosch et al., 2005; Pérez-Garijo et al., 2009). Stress treatments like X-radiation induce JNK activity, which in cells open to apoptosis leads to the demise of the cells. However, cells unable to enter apoptosis survive stress treatments and acquire persistent JNK activity for the rest of the development. This activity is translated into the formation of overgrowth.

It is clear from the above that the JNK pathway, through its pro-apoptotic and pro-proliferative functions, is involved in relevant physiological processes like tumorigenesis. However, the molecular and genetic mechanisms behind the various roles of JNK are not well known, especially in the pro-proliferation function (Pinal et al., 2018).

The diversity of cellular functions of the JNK pathway underscores the diversity of disease conditions in which JNK is implicated, including cancer. Activation of JNK is found in many cancer cell lines as well as in patients samples (Wagner & Nebreda, 2009). Our findings are consistent with the hypothesis that the JNK proteins may promote tumor development in a tissue-specific manner, and suggest that selective inhibition of these kinases may be effective in halting specific tumor formation.

The physiological and pathological functions of JNK have been very difficult to predict because of the contradictory role of JNK in promoting cell survival and proliferation and cell death too. Despite the latest efforts in the field, there is still no consensus on whether components of the JNK signaling pathway are suitable drug targets for cancer therapy (Tournier, 2013), however, it is important to highlight the importance of understanding fully both roles and the molecular basis in order to validate the therapeutic potential of JNK inhibition (Bubici & Papa, 2014).

The communication between tumor cells and their microenvironment is largely implicated in neoplastic growth. Cancer research needs *in vivo* investigation and the use of model organisms contribute to answering the request. In this study, we have used *Drosophila melanogaster*, and because of the anatomical differences between flies and humans, the use of *D. melanogaster* as a cancer and a CIN model has been fundamental to understanding the basic processes and pathways involved in the regulation and creation of cancers and tumors. Additional cancer hallmarks, cell metabolism reprogramming, inflammation and resistance to cell death have been studied and characterized in the fruit fly (Mirzoyan et al., 2019). This model, as described in the theoretical background, is greatly helping in dissecting the contribution of specific molecular pathways. In the last 30 tears, there has been a great development in genetic tools in *D. melanogaster*, and for this reason, we anticipate that the use of the fruit fly will move fast into the field of cancer.

Limitations

The empirical results of this study have to be seen in light of some limitations. The number of the samples in our study is significant small, as we only did three crosses, and may be difficult to find significantly relationships from the data. Normally a larger sample size is required to ensure a representative distribution, however, there is reliable data about the JNK signaling pathway so it is easier to find a trend and a meaningful relationship between the JNK pathway and the pro-proliferation role. In further studies, more crosses and more genotypes should be studied.

After completing the interpretation of the findings, it has been pointed out that we could have conducted a tougher analysis of the results, with quantification of loss of heterozygosity and cell death, as none of the data collected is quantitative. Future research should revise the specific method for gathering data.

This experiment should have also been repeated at least two times more, as the effect of a variable on an outcome or a random error could have affected our results. To maximize the accuracy of the results, a scientific experiment should be conducted at least three times.

Although the use of genetic screens of *D. melanogaster* provides an unbiased approach to gain insight into human disease, fly mutations may not be precise representations of human disease mutations. Furthermore, while the GAL4/UAS system is an extremely effective and versatile system, it is after all an overexpression system that may give rise to the difference from the clinical situation.

Conclusion

Throughout this work, the initial hypothesis has been tested by verifying the protumoral role of the JNK cell signaling pathway in *Drosophila melanogaster*. It has been confirmed with the experiments that this pathway has a function that favors the growth and malignancy of the tumor when in abnormal and aneuploidy cells apoptosis cannot be activated.

In the theoretical part of the work, different basic concepts of cancer have been studied, such as the changes that cells acquire to become cariogenic or the hallmarks of cancer. It has also been centered on the use of *Drosophila melanogaster* as a model organism to study cancer. In the experimental part, a genetic induction of chromosomal instability was made, in order to check whether the JNK was involved in the process of tumorigenesis.

It is clear that discovering how the cell carries out all the processes is key to better understanding the complex mechanism of cancer. The idea of this work is to see that from basic research we can find the cellular mechanisms involved in tumor activation, through an experimental design.

A better understanding of signaling pathways allows us to predict how the cell will act in different scenarios and can help us find an answer to problems that might arrive. In cancer, knowing the basic cell function and responses is a very important factor, which should be taken into account.

The use of *Drosophila melanogaster* as a model organism allows for rapid experimentation and advancement, although results obtained in genetic models, such as those in this work, must be analyzed in a higher organism, to see if the mechanisms are conserved. Assuming that in mammals there are three different JNKs pathways that participate in processes necessary for cell life, the possible future avenues of research based on the result of this work could be large.

Personally, this work has helped me to enter the world of scientific research and to work in a laboratory. In addition, I have learned to design an experimental method: pose a problem, formulate a hypothesis, do experiments, analyze the results obtained and draw a conclusion.

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Other resources

Additional papers

- Hales, Karen G et al. “Genetics on the Fly: A Primer on the Drosophila Model System.” *Genetics* vol. 201,3 (2015): 815-42. doi:10.1534/genetics.115.183392

  Great guide on *Drosophila melanogaster* as a model organism and the genome features and its Genetic Toolkit

- Tolwinski, Nicholas S. “Introduction: Drosophila-A Model System for Developmental Biology.” *Journal of developmental biology* vol. 5,3 9. 20 Sep. 2017, doi:10.3390/jdb5030009

  Introduction to *Drosophila melanogaster* as a model organism

- Fernández-Moreno, Miguel Angel et al. “Drosophila melanogaster as a model system to study mitochondrial biology.” *Methods in molecular biology (Clifton, N.J.)* vol. 372 (2007): 33-49. doi:10.1007/978-1-59745-365-3_3

  Provides great information for *Drosophila melanogaster* as a model organism, explaining its life cycle and genetics

- Serras, Florenci and Casanova, Jordi. “*Drosophila melanogaster* com un organismo model: una Font d’informació en recerca biomédica.” *Treb. Soc. Cat. Biol.,* vol. 62 (2011) 123-130. doi: 10.2436/20.1501.02.108

  Introduction to *Drosophila melanogaster* as a model organism and its connection to cancer research

- Vidal, Marcos, and Ross L Cagan. “Drosophila models for cancer research.” *Current opinion in genetics & development* vol. 16,1 (2006): 10-6. doi:10.1016/j.gde.2005.12.004

  Introduction to *Drosophila melanogaster* as a model organism for cancer research and discoveries made using it

- Richardson, Helena E, and Marta Portela. “Modelling Cooperative Tumorigenesis in *Drosophila*.” *BioMed research international* vol. 2018 4258387. 6 Mar. 2018, doi:10.1155/2018/4258387

Provides information about *Drosophila melanogaster* as a model organism for cancer research and some theoretical explications on oncogenes and tumorigenesis

- Liu Dawei et al. "*Drosophila* as a model for chromosomal instability." *AIMS Genetics* vol. 2 (2014) 1, 1-12. doi:10.3934/genet.2015.1.1

  Excellent study on chromosomal instability in *Drosophila melanogaster*

- Dekanty, Andrés, and Marco Milán. "Aneuploidy, cell delamination and tumorigenesis in Drosophila epithelia." *Cell cycle (Georgetown, Tex.)* vol. 12,5 (2013): 728-31. doi:10.4161/cc.23949

  Explains that induced aneuploidy cells develop tumor growth due to the activation of two involved gens in tumorigenesis

- Milán, Marco et al. "Aneuploidy and tumorigenesis in Drosophila." *Seminars in cell & developmental biology* vol. 28 (2014): 110-5. doi:10.1016/j.semcdb.2014.03.014

  Oriented to the study of aneuploidy and tumorigenesis induced in *Drosophila melanogaster*

- Benhra, Najate et al. "Chromosomal Instability Induces Cellular Invasion in Epithelial Tissues." *Developmental cell* vol. 47,2 (2018): 161-174.e4. doi:10.1016/j.devcel.2018.08.021

  Explains that chromosomal instability promotes and invasive behavior and introduces the JNK signaling pathway

Additional books

- Perveen FK, *Drosophila melanogaster- Model for Recent Advancers in Genetics and Therapeutics*, IntechOpen, February 28th 2018. doi: 10.5772/67731.

  Provides information about *Drosophila melanogaster* morphology, life cycle and genetics

Webography

- Institute for Research in Biomedicine: https://www.irbbarcelona.org/en

  Provides access to all publications of *Drosophila melanogaster* experiments in the IRB Barcelona

- National Centre for Biotechnology Information: https://www.ncbi.nlm.nih.gov

  Covers all the information related to biological or biomedical aspects. It also contains several links to other resources, like Pubmed

- FlyBase: http://flybase.org

  Database on *Drosophila melanogaster*, with genes present in it, its functions and a bank of mutants

- Fruitfly: https://www.fruitfly.org

  The “Berkeley Drosophila Genome Project” contains information about the *Drosophila* genome, like maps, sequences and mutagenesis projects

- Interactive fly: https://www.sdbonline.org/sites/fly/aimain/1aahome.htm

  Contains information about processes and genes involved in the development and it contains the *Drosophila* Development Atlas

- Bloomington: https://bdsc.indiana.edu

  One of the most important centers where *Drosophila* stocks can be bought

- UniProt: https://www.uniprot.org

  Provides information about proteins

- FlyView: http://flyview.uni-muenster.de

  Contains another Atlas where gene expression during *Drosophila*’s development can be seen

- Google Scholar: https://scholar.google.com

  Provides a simple way to search for scholarly literature and scientific articles

Annex I

Maintenance and breeding of *Drosophila melanogaster*

Bottles and vials

Morgan used glass milk bottles for his experiments, however, for ease of culturing and transferring flies, uniform bottles and vials are the best approach. Bottles are used for the maintenance of large populations of flies whereas culture vials are useful for maintaining smaller populations. Glass is effective, however, if dropped 2 weeks of data could be lost, so autoclaved sterile plastic vials are preferable.

Media

Composition of the media used at fly facilities (Tab. 5):

Media	Composition (for 42.5 liters to fill 4250 vials with 10 ml of food each)
Water	39 l
Yeast	675 g
Soy flour	390 g
Yellow cornmeal	2850 g
Agar	225 g
Light corn syrup	3 l
Propionic acid (> 99 % pure, 13.4 M)	188 ml

Environment

The optimum condition is a temperature of 25 °C and 60% humidity. In these conditions, generation time is shorter. Most large fly laboratories maintain stocks that are not in everyday use at 18 °C on a four- or five-week generation cycle. It is important to avoid too overcrowded cultures, and only 20 flies or so should be transferred, to ensure that both sexes are present. It is very good practice to keep the old cultures for two weeks at 18°C the transfer so that they can be used as a backup

Anesthetizing flies

Carbon dioxide (CO_2) keeps flies immobile for long periods of time with no side effects. In most laboratories, flies are placed on porous pads connected to a source of CO_2 and moved with a brush while viewed with a stereomicroscope.

Transferring flies

Flies should be transferred every 10 to 14 days. To transfer without anesthetizing gently tap the old vial with flies in it on a soft surface, so the flies go to the bottom of the vial. Then quickly remove the cotton plug, and invert the vial in top of the new vial. Then gently tamp the two vials to force flies down into the new vial.

Killing flies

An unfortunate necessity when using flies is killing them when they are no longer needs. A bottle with ethanol is generally used, so when flies are no longer needed, they are dumped into ethanol where they drown.

Annex II

Scientific paper

During the summer of 2021, I was selected to participate in the “Joves i Cièincia” program, also promoted by Catalunya La Pedrera Foundation. For there, I wrote a scientific paper of my research, following the structure of those kinds of literature.

Abstract

Genomic instability has been proved to be an enabling characteristic of cancer, and most sporadic carcinomas show an increased rate of changes in chromosome number, known as chromosomal instability (CIN). However, the role of CIN in driving invasiveness remains unclear. In this work, we use *Drosophila melanogaster* epithelial cells to address the role of CIN in tumor development. We show that depletion of spindle assembly checkpoint genes (e.g. *bub3*) in *Drosophila* leads to programmed cell death and apoptotic response, which depends on the activations of the c-Jun N-terminal kinase (JNK) signaling pathway. When we prevent cells from undergoing apoptosis with the p35 protein, CIN leads to a neoplastic overgrowth and drives tumorigenesis. Cells with an abnormal number of chromosomes delaminate from the main epithelium and invade neighboring tissues. A JNK transcriptional target MMP1 (matrix metalloproteinase 1), involved in basement membrane degradation, was also observed in these tumors. The JNK transcriptional program is activated in delaminating cells and drives tissue overgrowth, invasiveness and basement membrane degradation. We also showed that the blockage of the JNK pathways partially rescues CIN cells from forming a tumor. Altogether, our findings support the proposal that CIN promotes a rapid and invasive behavior in epithelial cells and reinforce the pro-tumorigenic role of the JNK pathway.

Introduction

Genomic instability was proposed as a cause of cancer over 100 years ago (Hardy & Zacharias, 2005). Since then, it has been observed in most solid tumors (Duijf & Benezra, 2013) and it was described as an enabling characteristic of cancer (Hanahan & Weinberg, 2011). There are various forms of genomic instability. Among the most common ones in cancer is chromosomal instability (CIN), which is defined as an elevated rate of gains and losses of whole chromosomes in cancer cells compared to normal cells (Hoevenaar et al., 2020a). Studies have suggested that CIN is associated with a poor clinical outcome in solid tumors (Walther et al., 2008). The consequence of CIN is aneuploidy, where the number of chromosomes in a cell deviate from the haploid number of chromosomes (Weaver & Cleveland, 2006). High levels of CIN in metastatic human tumors may contribute to the invasion of tissue by facilitating the gain of oncogenes and the loss tumor suppressor genes (Bakhoum et al., 2018; Bakhoum & Cantley, 2018; Vargas-Rondón et al., 2017).

Despite the relevance of CIN for tumorigenesis growth, its role is poorly understood. In fact, the use of CIN is frequently inconsistent and imprecise (Geigl et al., 2008). There is a growing number of genetic studies trying to understand the mechanisms behind CIN. However, it has been criticized whether those experiments mimic the genetics events that lead to CIN during tumor progression (Bakhoum & Swanton, 2014). Different studies show that almost every major oncogenic pathway can be implicated in CIN (Yates & Campbell, 2012). Due to chromosome missegregation can, in turn, activate oncogenic pathways, it has been proposed that this relationship can be also bidirectional (Yates & Campbell, 2012). So far, the most accepted hypothesis for cellular behavior underlying CIN proposes that it is a source of mutability that help tumor cells to pass through critical steps of tumorigenesis.

Drosophila melanogaster is a useful model organism to study genetics and tumorigenic action of CIN in the epithelial tissues (Müller, 2000). As an animal model system, *D. melanogaster* has many significant advantages because it is easy to manipulate gene expression at any stage of development in any tissue of interest (D. Liu et al., 2015). *D. melanogaster* provides an *in vivo* system to study genetic interactions, pathways dissections and modelling of most hallmarks of cancer (Brumby & Richardson, 2005a; Tipping & Perrimon, 2013). Additionally, it is important to mention that the simple organization of *D. melanogaster* epithelial tissues has contributed to describing aneuploid cells in driving tumor growth.

In this study, we used *D. melanogaster* to understand the role of CIN in tissue growth. CIN was generated with the knocked down of the spindle assembly checkpoint gene *bub3*, which leads to the production of highly aneuploid cells to activate the c-Jun N-terminal kinase (JNK)-dependent apoptosis. When highly aneuploid cells were

prevented from entering this process with the p35 protein, JNK activation induced overgrown tissues and invasiveness.

The aims of this work were to use *Drosophila melanogaster'*s wing epithelium to present evidence that CIN induces an invasive behavior in aneuploid cells. We also aimed to study the pro-proliferation role of the JNK pathway in *Drosophila.* We found that aneuploid cells delaminate from the epithelium and activate a JNK-dependent transcriptional response that drives tissue neoplastic growth through the expression of the mitogenic gene wingless/wnt and the matrix metalloproteinase 1 (MMP1). The observation that CIN induces a rapid response relaying on the activity of the JNK stress response pathway in highly aneuploid cells opens the possibility that aneuploidy plays a key role in tumorigenesis. Altogether, these results provide evidence to understand the correlation between aneuploid cells and tumor progression in a CIN context.

Materials and Methods

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-WG (4D4)	Developmental Studies Hybridoma Bank (DSHB)	RRID: AB_528512
Cy2 AffiniPure Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch	Code: 715-225-151
Chemicals, Peptides and Recombinant Proteins		
DAPI	Sigma Aldrich	Code: 28718-90-3
Experimental Models: Organisms/Strains		
UAS-bub3^{RNAi}	VDRC Stock Center	RRID: VDRC_21037
UAS-bsk^{DN}	Bloomington Drosophila Stock Center	RRDI: BDSC_6409
UAS-p35	Bloomington Drosophila Stock Center	RRDI: BDSC_5073
UAS-myristoylated-Tomato (myrT)	Bloomington Drosophila Stock Center	RRDI: BDSC_32222
ap-GAL4	Bloomington Drosophila Stock Center	RRDI: BDSC_3041
MMP1-GFP	(Uhlirova & Bohmann, 2006a)	N/A
Software and algorithms		
Fiji	Fiji	https://fiji.sc
GraphPad Prism 7 Project	GraphPad	RRDI: SCR_002798
Excel	Microsoft Excel 2016	N/A

Experimental model and subject details

Fly strains

Drosophila melanogaster used were grown on standard medium (4% glucose, 55g/L yeast, 0,65% agar, 28 g/L wheat flour, 4 m/L propionic acid and 1,1 g/L nipagin). The stocks of flies used were: *UAS-bub3*^{RNAi} from the Vienna *Drosophila* RNAi Center, VDRC,

Austria; *UAS-bsk*^{DN}, *UAS-p35, UAS-myristoylated-Tomato (myrT),* and *ap-GAL4* from Bloomington Drosophila Stock Center, IN, USA.

Genetic Analyses

Female flies with the *ap-GAL4* driver in their genotype were crossed to male flies with the *UAS-bub3* and *UAS-p35* transgenes, in order to induce chromosomal instability (CIN). Once the females had been fertilized, they laid eggs at 25 °C and the eggs were switched to 29 °C 48 h after egg laying. Five days later, larvae wing imaginal discs were dissected. Control flies were raised in parallel and subjected to the same experimental conditions (temperature and time of induction).

Method Details
Immunohistochemistry and Confocal Imaging

A period of 110 h after the eggs had been laid, late third instar larvae with the wanted genotype were selected and wing imaginal discs were dissected in phosphate-buffered saline (PBS), fixed for 20 minutes in 4% formaldehyde in PBS and stained with antibodies in OBS with 0.3 BSA, 0.2% Triton X-100. The primary antibodies and the dilutions used were: Donkey Anti-Mouse (1:100; H+L; Jackson ImmunoResearch); mouse anti-Wg (1:20; 4D4, DSHB). The Leica TCS SO5 MP confocal microscope was used to take images.

Flow Cytometry Analysis

Wing discs of larvae of the genotypes (1) *ap-GAL4, UAS-MyrT/UAS-bub3-i, MMP1-GFP; UAS-p35* and (2) *ap-GAL4, UAS-MyrT/ MMP1-GFP;UAS-p35/UAS-bsk-DN* were dissected in cold PBS, dissociated with Trypsin-EDTA, fixed with formaldehyde 4% for 20 min and permeabilized in ethanol 70% for 2 hours. Fixed cells were stained with DAPI. Delaminated cells (which were MyrT-positive and MMP1-GFP-positive) activated the JNK pathway. Excitation of the sample was carried out using a Coherent Enterprise II argon-ion laser. Excitation with the UV laser (440 nm) allowed fluorescence from DAPI, the blue laser (530 nm) fluorescence from GFP and the green laser (610 nm) fluorescence from Tomato.

These materials and methods have been described in previous works (Benhra et al., 2018; Dekanty et al., 2012).

Results

CIN leads to an invasive behavior

The *Drosophila melanogaster* primordia of adult wings (wing imaginal discs) is a high proliferation epithelial monolayer that proliferates during larval development (Beira & Paro, 2016). These structures have been useful as a model system to understand the molecular mechanisms underlying tumorigenesis growth (Beira & Paro, 2016; Brumby & Richardson, 2005b; Pagliarini, 2003).

Genome instability and mutation are enabling characteristics of cancer (Hanahan & Weinberg, 2011). In order to study tumorigenesis growth, we induced chromosomal instability (CIN) in the imaginal discs with the GAL4-UAS system (Brand & Perrimon, 1993). This system allowed us to knock down the expression of the *bub3* gene with an interference RNA (RNAi). The *bub3* gene is involved in the spindle assembly checkpoint and prevents early anaphase entry and mitotic exit, creating a mechanism for correct chromosomal segregation (Li et al., 2009). The mutation of this gene causes chromosomal segregation errors, induces a high level of CIN and is known to cause the most frequent defect associated with human cancer (Long et al., 2021; Morais da Silva et al., 2013).

CIN-induced aneuploid cells delaminate and activate the JNK-signaling pathway, which leads to the entrance to an apoptotic program. In order to prevent the aneuploidy cells from dying, we also expressed the baculovirus protein p35, a known inhibitor of the apoptosis process that leads to tissue overgrowth (Hay et al., 1994). The activation of the JNK signaling pathway was monitored by the expression of the matrix metalloproteinase 1 (MMP1), a direct transcriptional target of the JNK signaling pathway. This proteinase is known to contribute to basement membrane degradation (Srivastava et al., 2007; Uhlirova & Bohmann, 2006b). MMP1 expression was blocked with the expression of a dominant-negative version of *basket,* the main gene that controls JNK in *Drosophila* (Biteau et al., 2011).

We used the *apterous-Gal4* driver to express the transgenes in the dorsal compartment of the wing primordium, and cells were labeled with a targeted fluorescent protein Tomato (Myristoylated Tomato, MyrT). The transgenes were expressed in developmental compartments, where cell populations do not mix and invasive behavior can be easily seen.

CIN-induced cells were able to delaminate and invade the neighboring compartment (arrows in Figure 1A), which means that they were located outside the compartment of origin. Delaminating cells were aneuploid and the level of CIN was very high. The apterous domain area boundaries of wing imaginal discs with CIN were irregular and not organized, showing the invasive behavior of the CIN population (Figure 1B).

The resulting wing primordia were massively overgrown, and the cell population where CIN was induced invaded the neighboring, wild-type territory (Figure 1A). Invading cells were non-apoptotic, as shown by DAPI staining of their nuclear morphology (Figure 1A). Delaminating cells showed a high percentage of 4n DNA content compared with non-delaminated cells due to the blockage of the *bub3* gene. The mode of migration observed in all cases was as single cells (arrows in Figure 1A). Control wing discs were grown in parallel to avoid variability of the phenotype due to external factors. In control wing discs expressing only the Tomato protein zero migrating cells and no tumor growth were observed (Figure 1B). Taken all together, these results indicate that highly aneuploid cells have an active migratory behavior and proliferate from the epithelia.

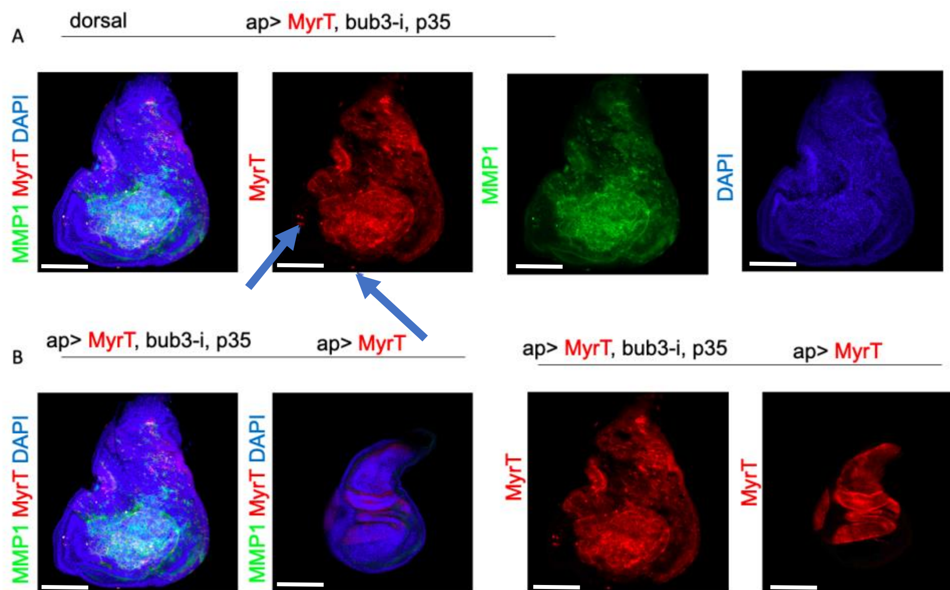

Figure 3: CIN leads to an Invasive Behavior
(A): Basal section of larval wing primordia expressing the indicated transgenes (*bub3-i, p35*), MyrT (red) under control of the *ap-gal4* driver and MMP1-GFP (green). Wing discs were stained with DAPI (blue). Blue arrows point MyrT-labeled cells invading the neighboring compartment.
(B): Comparison between the dorsal section of larval wing primordia expressing the indicated transgenes (*bub3-I, p35*) and MyrT (red) under control of the *ap-gal4* driver and the control group. CIN-induced cells have disorganized the dorsal segment when compared to the wild-type and have caused tissue overgrowth.
Scale bars, 50 µm

The JNK pathway promotes invasiveness and tumor growth

The c-Jun N-terminal kinase (JNK) pathway is involved in various stress responses and tissue damage in *Drosophila*. The signaling pathway plays an important role in the tumorigenic behavior of epithelial cells (Brumby, 2003; Pinal et al., 2018; Uhlirova & Bohmann, 2006b). CIN-induced tumorigenesis relies on aneuploid cells delaminating from the main compartment, activating JNK.

As aneuploid cells activate JNK-dependent transcriptional program that causes tissue overgrowth (Dekanty et al., 2012) we decided to address whether the JNK activation is

involved in the invasive behavior of CIN-induced cells. Interestingly, the invasive behavior of aneuploid cells was rescued when the JNK pathway was blocked. Not only did the blockage of the JNK pathway rescue tissues size but the number of invading cells from the dorsal area also decreased (Figure 3A, 3C, quantification in 2). In this cross, the number of apoptotic cells was largely, but not completely rescued (Figure 3C).

So far, we have provided evidence that cell delamination and JNK activation is linked to high levels of aneuploidy. Our results indicate that CIN-induced JNK activation has an important role in triggering neoplastic growth in the tissue, as it was expressed in the growing tumor (Figure 3B). Additionally, we observed a large number of cells located on the basal side of the epithelium that are non-apoptotic (Figure 3B). When the JNK activity was blocked, the tissue overgrowth caused by CIN was rescued (Figure 3A, quantification in Figure 4). Expression of MMP1 in delaminating cells degrades the underlying basement membrane thus facilitating tissue invasiveness (Figure 3B). Remarkably, although JNK plays a critical role in driving tumorigenesis, JNK activation is not enough to drive tumorigenesis (Igaki et al., 2006).

Altogether, we conclude that JNK induces the expression of mitogens like Wingless and MMP-1, which are responsible for tissue overgrowth and basal membrane degradation, a requisite for tissue invasiveness.

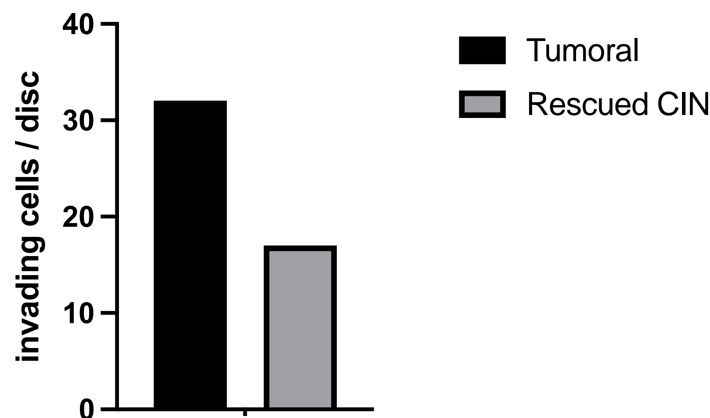

Figure 4: CIN-induced tissue overgrowth
Histogram showing the number of individual cells per disc invading the neighboring compartment after 5 days of induction in wing discs of the indicated genotypes (bub3-I, p35 / bub3-I, p35, bsk-DN)

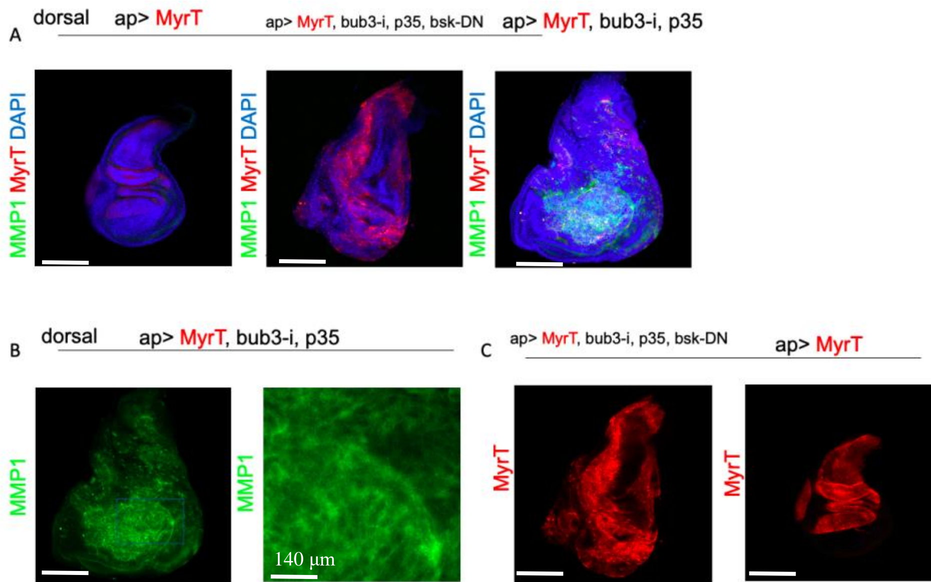

Figure 3: The JNK pathway promotes invasiveness and tumor growth

(A): Comparison between the dorsal section of larval wing primordia expressing the indicated transgenes (wild-type /*bub3-I, p35/ bub3-I, p35, bsk-DN*) MyrT (red) and MMP1 (green) under control of the *ap-gal4* driver. Wing discs were stained with DAPI (blue). The blockage of the JNK pathway through a dominant-negative form of the gene *basket* leads to significant tissue overgrowth and MMP1 expression.

(B): Dorsal section of larval wing primordia expressing the indicated transgenes (*bub3-I, p35*) under control of the *ap-gal4* driver. Wing discs were marked with MMP1 (green). Magnifications of squared regions are shown, pointing to the degradation of the membrane due to the JNK activation.

(C): Comparison between the dorsal section of larval wing primordia expressing the indicated transgenes (wild-type /*bub3-I, p35, bsk-DN*) and MyrT (red) under control of the *ap-gal4* driver. The blockage of the JNK pathway through a dominant-negative form of the gene *basket* leads to a partial rescue from tissue overgrowth.

Scale bars, 50 μm

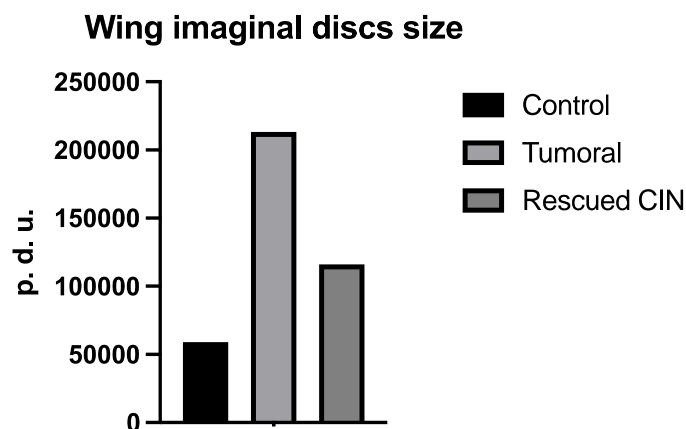

Figure 4: CIN and JNK activation leads to tissue overgrowth

Histogram plotting the wing imaginal discs size measured in arbitrary unit (p.d.u.) of the indicated genotypes (wild-type /*bub3-I, p35/ bub3-I, p35, bsk-DN*) under the *ap-gal4* driver. The graphic indicates the average size measured after 5 days of induction.

Discussion

One of the most accepted hypothesis about the role of CIN in tumorigenesis establishes that it is a source of mutability that helps the tumor cell population growth (Hoevenaar et al., 2020b). This means loss or gain of certain chromosomes carrying tumor suppressor genes or oncogenes (Thomas & Shi, 2017). Recently, CIN has been also linked to metastatic progression in human tumors (Turajlic & Swanton, 2016). However, the role of CIN in tumorigenesis growth has not been largely investigated.

Here, we used an epithelial model of *D. melanogaster* to address whether CIN can induce and activate molecular mechanisms such as the JNK signaling pathway by itself. We used RNAi forms of the *bub3* gene to present evidence that CIN is indeed able to induce an active invasive behavior. However, cellular invasion is most probably not a direct consequence of gain or loss of specific chromosomes because CIN-induced aneuploid cells did not have a signature of pro-invasiveness genes (Clemente-Ruiz et al., 2016).

In our study, aneuploid cells became mobile, expressed the secreted matrix-metalloproteinase-1 (MMP1) and degraded the membrane. Therefore, this supports the role of CIN in driving tissue invasiveness and promoting tumor growth. The activation of this pathway was required for CIN-induced tumorigenesis because blocking the activity of this kinase rescued CIN-induced tumor-like overgrowth.

Previous studies have shown that the JNK signaling pathway has a tumor-promoting role. When this cascade is blocked, the tumoral growth is inferior (La Marca & Richardson, 2020). The JNK pathway has the ability to release proliferative signals that can stimulate the growth of the tissue nearby (Pérez et al., 2017). In this study, we reinforce the role of the baculovirus protein p35 in preventing cell death (Wells et al., 2006). Undead cells remain alive for the rest of the development and keep secreting mitogenic factors, thus generating tumor growth (Pinal et al., 2019).

In *D. melanogaster,* there are several examples showing that JNK activity causes tumorigenesis in imaginal disc (Brumby, 2003; Johnston et al., 1999). In our experiment, we believe that the size and shape of the *Apterous* domain ensure that many of the cells inside the group are beyond cell competition and, therefore, can continue proliferating. In this situation, tumor cells die at the border, but they are continuously replaced by neighbors.

However, it has also been shown that the activity of JNK after tissue damage triggers apoptosis and the subsequent elimination of the cell (Bosch et al., 2005; Pérez-Garijo et al., 2009). However, cells unable to enter apoptosis survive stress treatments and

acquire persistent JNK activity for the rest of the development. This activity is translated into the formation of overgrowth.

The diversity of cellular functions of the JNK pathway reflects the wide range of diseases in which JNK is implicated, including cancer. Activation of JNK is found in many cancers' cell lines as well as in samples of patients (Wagner & Nebreda, 2009). Our findings are consistent with the hypothesis that the JNK-target proteins may promote tumor development in a tissue-specific manner. This suggests that selective inhibition of these kinases may be effective in halting specific tumor formation.

The physiological and pathological functions of JNK have been very difficult to predict. This is because of the contradictory role of JNK in promoting cell survival and proliferation and cell death too (J. Liu & Lin, 2005). Despite the latest efforts in the field, there is still no consensus on whether components of the JNK signaling pathway are suitable drug targets for cancer therapy (Tournier, 2013). However, it is important to highlight the importance of understanding fully both roles and the molecular basis to validate the therapeutic potential of JNK inhibition (Bubici & Papa, 2014).

The communication between tumor cells and their microenvironment is largely implicated in neoplastic growth. *In vivo* research is required to understand cancer and the use of model organisms will help to meet this need. In this study, the use of *D. melanogaster* as a cancer and a CIN model has been fundamental to understanding the basic processes and pathways involved in the regulation and progression of cancers and tumors. Additional cancer hallmarks, cell metabolism reprogramming, inflammation and resistance to cell death have been studied and characterized in the fruit fly (Mirzoyan et al., 2019). As described earlier, this model is greatly helping in dissecting the contribution of specific molecular pathways to tumorigenesis. In the last 30 years, there has been a great advance in genetic tools in *D. melanogaster*, and for this reason, we anticipate that the use of the fruit fly will move fast into the field of cancer.

Limitations

The empirical results of this study have to be seen in light of some limitations. The number of samples in our study is significantly small. We only did three crosses and it may be difficult to significantly find relationships from the data. Normally a larger sample size is required to ensure a representative distribution. However, there is reliable data about the JNK signaling pathway. It was easy to find a trend and a meaningful relationship between the JNK pathway and the pro-proliferation role. In further studies, more crosses and more genotypes should be studied.

After completing the interpretation of the findings, it has been pointed out that we could have conducted a tougher analysis of the results. It will include quantification of loss of heterozygosity and cell death, as none of the data collected is quantitative. Future research should revise the specific method for gathering data.

To make our analysis more robust, this experiment should have also been repeated at least two times more. This is because the effect of a variable or a random error could have affected our results. To maximize the accuracy of the results, a scientific experiment should be conducted at least three times.

Although the use of genetic screens of *Drosophila melanogaster* provides an unbiased approach to gain insight into human disease, fly mutations may not be precise representations of human disease mutations. Furthermore, while the GAL4/UAS system is extremely effective and versatile, it is after all an overexpression system that may differ from the clinical scenario.

Previous work in flies has demonstrated that the cellular response to aneuploidy is tissue-dependent (Gogendeau et al., 2015). Further research is required to characterize the diversity of cellular responses to aneuploidy in different tissues and to address where the invasive behavior reported in this work is specific to epithelial cells.

Conclusion

In summary, we have provided evidence that the JNK signaling pathway has a protumoral role and is activated in delaminating cells after CIN induction. We showed that CIN leads to a neoplastic overgrowth and the blockage of the JNK pathway partially rescues cells with chromosomal instability (CIN) from forming a tumor. All the evidence described here, using *Drosophila melanogaster* as a model organism, should unravel novel therapeutic targets and can help to decipher the multiple JNK activities. Altogether, this work will pave the road to open promising clinical perspectives to treat diverse pathological conditions, such as cancer.

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