

## THE RISE OF CANCER CELL POPULATIONS IN FLIES

What are some of the different cancer cell subpopulations arising in Chromosomal Instability induced tumors, and what are their molecular signatures, what insights do we get from the fly model?

FlyFucci2005

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*“Down to their innate molecular core, cancer cells are hyperactive, survival-endowed, scrappy, fecund, inventive copies of ourselves”*

Siddhartha Mukherjee, *The Emperor of All Maladies*

To our uncle, who is fighting against his metastatic cells.

## **Acknowledgments**

We would like to thank Crazy About Biomedicine for giving us the opportunity to work in IRB and letting us work in Marco Milan's lab. We would also like to use this opportunity to thank IRB for letting us use their installations for our project, as well as Dr. Marco Milan (Group Leader in Development and Growth Control Laboratory at IRB), who gave us the opportunity to do research on *Drosophila* in his lab. Also, this project wouldn't have been possible without the supervision of Kaustuv Ghosh (Ph.D. Student in the Development and Growth Control Laboratory at IRB) who guided us through all the stages of the experiments, taught us how to do all the processes and introduce us in the daily life in a research lab. Moreover, we extend our thanks to our tutor Mikel Martín, who helped us in this project, giving us instructions on what we had to do and supporting us on our way.

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## Abstract

This work aims to study different subpopulations of cancerous cells in tumors induced in *Drosophila melanogaster*, by chromosomal instability, making a group of cancer cells appear, specifically on the wing imaginal disc of the fly. To do this, we crossed flies with genetic modifications, so we got the flies with the genes included we wanted to study. We raised the flies and dissected the tissue we were studying, where the tumor was induced. Using immunofluorescence and advanced microscopy, we see how chromosomal instability, a hallmark of most cancers, is able to give rise to different heterogeneous populations of cancer cells. We also learned about the different states of the cell cycle of cancer cells, using Fly FUCCI. The results of the first experiment were that metastatic cells, which had the two reporters for JNK and JACK/STAT pathways, were the largest group, still, there was diversity, meaning that there is heterogeneity of cells from the same tumor. From the second experiment, we concluded that most migratory tumor cells are in the G2/M phase of the cell cycle. In doing so, we conclude that cancer cells that arise from the same tumor can be truly different from each other and that JNK and JAK are two genes that are induced upon tumorigenesis. On the other hand, most metastatic cells are in the final phase of the cell cycle. These results can open up new research lines, such as, the discovery of drugs or small molecules that can inhibit or eliminate, JAK or JNK, as they represent a large group of the total metastatic cells, the same is applied to the information about the phase of cancer cell's cycle.

**Keywords:** Chromosomal Instability, *Drosophila*, cancer cell heterogeneity, JAK, JNK, metastatic cells

## Resum

Aquest treball té com a objectiu estudiar diferents subpoblacions de cèl·lules canceroses en tumors induïts en *Drosophila melanogaster*, mitjançant la inestabilitat cromosòmica, fent que aparegui un grup tumoral, específicament en el disc imaginal de l'ala . Per fer-ho, vam crear mosques modificades genèticament amb els gens que volíem estudiar. Vam criar les mosques i dissecionar el teixit on es va induir el tumor. Utilitzant la immunofluorescència i microscòpia avançada, veiem com la inestabilitat cromosòmica, un segell distintiu de la majoria de càncers, és capaç de donar lloc a diferents poblacions heterogènies diferents de cèl·lules canceroses. A més, vam identificar l'etapa del cicle cel·lular on es trobaven les cèl·lules canceroses, emprant Fly FUCCI. Els resultats aconseguits del primer experiment van ser que les cèl·lules metastàtiques amb els dos marcadors per JNK i JACK/STAT representaven el grup més nombrós, tot i haver diversitat. Del segon experiment, vam concloure que la majoria de les cèl·lules tumorals migratòries es troben en la fase G2/M del cicle cel·lular. Amb això, arribem a la conclusió que les cèl·lules canceroses que sorgeixen del mateix tumor poden ser realment diferents les unes de les altres i que el JNK i el JAK són dos factors amb un paper important en la dispersió de metastasis. Aquests resultats poden obrir noves investigacions, que busquin fàrmacs o substàncies que inhibeixin o regulin, JAK o JNK, ja que representen un gran grup de les cèl·lules metastàtiques. La informació del cicle cel·lular es podria utilitzar de la mateixa manera.

**Paraules clau:** Cèl·lules metastàtiques, *Drosòfila*, heterogeneïtat de cèl·lules canceroses, JAK, JNK, inestabilitat cromosòmica.

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## INTRODUCTION

We all know that cancer is a very difficult disease to cure. We chose it because we are interested in the field of research and cancer, as it is a disease that affects many people, and is the second highest cause of death by disease. We wanted to be able to learn and study some features that make cancer so difficult to cure.

Furthermore, we reached out to Marco Milan's lab at IRB in Barcelona, there we had the opportunity to work on *Drosophila melanogaster*. Ph.D. student, Kaustuv Ghosh helped us during all the process of the experiment as he tried to make sure we understood all the processes and why we were doing those processes.

We started reading about *Drosophila*, how to induce cancer in them and some gene modification for the study of cancer and this was our **question**:

*What are some of the different cancer cell subpopulations arising in Chromosomal Instability induced tumors, and what are their molecular signatures, what insights do we get from the fly model?*

We also formulated a **hypothesis** before going into the project:

*"There must be some identifiable cellular factor other than time that influences the diversification of cancer cells."*

The **objectives** were as follows:

- *To know how cancer acts in a primary tumor.*
- *To understand how to work in a scientific research laboratory.*
- *To find out what are the factors that influence the emergence of the different populations of cancer cells.*
- *To learn how to do research on *Drosophila* as a model.*
- *To recognize the stages of cancer.*

The experiment consists of induction of chromosomal instability in different specimens of *Drosophila* which contains different genes and with that and fluorescence microscopy, we wanted to study the tumor heterogeneity and see how different cells from the same place are.

The aim of our study is to know how many cells migrate in a primary tumor placed amongst epithelial cells of *Drosophila* imaginal wing disc and study the differences between them.

We decided to focus on cancer cell's heterogeneity for the fact that we know it plays a role in cancerous cell diversification and so it helps us to answer our main question.

In order to achieve our objectives and to verify our hypothesis, we separated the project into two parts: a theoretical part, which includes information about features involved in the experiment, and a practical part, which includes two experiments we did in IRB Barcelona.

For the theoretical part, we have done a lot of research and read papers, books and articles in order to get into the subject and learn as much as possible. With all the knowledge we had, then we could start practicing and going to the laboratory to do the experiments.

The firsts days going to IRB, we got used to working with flies, learned about them, making them reproduce, selecting the ones of our interest and changing them from the tubes where the females had laid their eggs and transfer the tubes to hot water so that they grow faster. After that, we had to learn how to dissect the larvae. Then, we prepared the samples to observe at the microscope and image them. We got the images and the only thing left was to make stats and extract the results.

In the following pages, you will learn and see how an induced tumor in the epithelial tissue of a fly develops into metastatic cells and what are the different characteristics of the metastatic cell population that has grown in the tissue.



# THEORETICAL FRAME

## 1. Introduction to cancer biology

Cancer is a disease that is caused by the uncontrolled growth of cells from any part of the body. Cells in the human body have a life cycle, so when they grow old, are damaged, or become non-functional, they are programmed to die; this process is called programmed cell death or apoptosis. Apoptosis, as mentioned before, is the programmed death of a cell, which, when disrupted, can lead to the abrupt multiplication of cells, causing tumorigenesis. These tumors can be cancerous or non-cancerous.

Proto-oncogenes are genes that help cells to multiply. When proto-oncogenes are mutated, they can be activated when they shouldn't; these genes could turn into an oncogene due to these mutations. This can produce cancer caused by uncontrolled cell growth.

Tumor suppressor genes are those genes that slow down cell division or 'tell' when to die, also known as apoptosis, as mentioned before. When these genes do not work properly, cells start to grow faster and do not die, producing cancer. Tumor suppressors prevent a cell from transforming into a cancerous cell.

Overactivation of Proto-oncogene (by transformation into Oncogene) or hypo-activation of a tumor suppressor alone is insufficient to cause carcinogenesis. In addition, the malfunction of DNA repairing genes has a role in the evolution of this process.

Both of these mutations must be present simultaneously to initiate carcinogenesis.

### 1a. History of Cancer biology

Historically, cancer was thought to be something different from what it is; here are some of the old theories about what cancer was:

The first historical pieces of evidence of cancer are as ancient as the Edwin Smith Papyrus (1300 BC), where breast cancer was described and Ebers Papyrus (1300 BC) which described uterus, skin and other tumors. Ancient Egyptians believed cancer to be a cure. Hippocrates, the father of medicine, thought that the human body contained four humors (blood, yellow bile, black bile, and phlegm), and if there were an imbalance from black bile, there would be a development of cancer. This is a thought that extended over the Middle Ages.

By the 17th century, a theory about lymph fluid was thought to produce tumoral cells. They felt that tumors grew from lymph and were thrown out by the blood.

Johannes Müller published monographs about the nature and characteristics of cancer; he disproved the lymph theory and stated that cancer was composed of cells, not lymph, as his student, Virchow, affirmed that cancer cells were derived from other cells.

Rudolf Virchow proposed that the cause of cancer was chronic irritation, but Tiersch showed that metastasized cancer cells spread malignant cells, not any fluid.

From the 1800s to the 1920s, trauma was thought to be the cause of cancer, and also, until the 18th-century doctors and scientists believed that cancer was contagious and was spread because of parasites.<sup>1</sup>

In 1975, Harold Varmus and J. Michael Bishop, in their laboratory in San Francisco, observed and demonstrated that cell genomes carry genes called 'proto-oncogenes and tumor suppressors, which have the potential to produce cancer if they are mutated.

About three decades before, three theories explained why cancers originate; the first one was that cancer cells are so different because the cell's phenotype changed without the genome being modified. Virologists around the 1970s made the second hypothesis. They believed that some human tumor viruses were capable of transforming normal cells into cells that have uncontrolled growth. And the last of these three theories, which was the most convincing among scientists, was that some carcinogenic agents acted like mutagens. This hypothesis was later experimented in Bruce Ames's lab. Bruce Ames said that carcinogenic agents were able to cause damage to genes by damaging DNA. In the mid-1970s, studies showed that the human papillomavirus (HPV) strongly correlated to cervical carcinomas, Epstein-Barr virus (EBV) with Burkitt's lymphomas in Africa, and with nasopharyngeal carcinomas in Asia, or Hepatitis B and C with hepatocellular carcinomas in Eastern Asia.

An early 1974 work described that a gene called *src*, from the Rous sarcoma virus, carried the information of cancer being produced, this discovery, led Varmus and Bishop to launch research to investigate the origin of this virus-related gene, in this research, it was shown that *src* had a different behavior than other oncogenes. The *src* was found in chicken cells and thus to all the vertebrates. Varmus and Bishop called it *c-src* (Short form for cellular-*src*) to differentiate it from the *v-src*, the oncogene of the virus.

This whole discovery of this proto-oncogene made a change in cancer research because it concluded that normal cellular genes had the ability to make cancer. They were wondering if proto-oncogenes could be activated by a retrovirus. The answer was

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<sup>1</sup> Sudhakar, A. (2009). History of Cancer, Ancient and Modern Treatment Methods. Journal of Cancer Science & Therapy, 01(02), i-iv. <https://doi.org/10.4172/1948-5956.100000e2>

affirmative, and they determined that proto-oncogenes might be altered in the chromosome by affecting its coding sequences.

This result made another question pop up, whether the same proto-oncogene was the result of the activation by retroviruses. The answer to this question was that it happened through transfection too; transfection is the gene transference. Scientists extract DNA from chemically induced transformed mouse fibroblasts into normal cultured cells to know if DNA transferred from tumor cells induced certain types of malignant transformations into tumor cells. The transferred genes operated as donors in the tumor cells to induce those transformations.

As they were doing this experiment, they noticed that none of the retroviruses were active. In fact, they concluded that those oncogenes were related to the RAS gene, which is a family of oncogenes. Although they knew that tumor cells induced transformation to other cells, they were still missing some other questions without any answer. They still wanted to know how a normal proto-oncogene turned into a virulent oncogene. Within a few months of research, they discovered something new, they discovered that *myc* oncogene was found in some human hematopoietic cancer, this oncogene was activated by a chromosomal change. They concluded that a normal proto-oncogene could be activated by a retrovirus or by somatic mutations. This activation involved an amplification of genes due to a chromosomal translocation.

All these discoveries led to a model of cancer formation. This model said that mutagenic carcinogens entered a cell and changed a proto-oncogene, this changed proto-oncogene, induced the mutant cell, and it initiates a malignant growth, that -after years- it will turn into a bigger tumor containing new additional and cumulative mutations. Eventually they found out that during this process of growth, these cells go through different stages that, as the malignant cell grows, the next cells will be more malignant rather than the previous ones and so on.

By 1983, once discovered, another solution to this problem appeared. After numerous experiments, they figured out that more than one oncogene was needed to transform all the other cells into tumorigenic. Moreover, if they introduce only a RAS oncogene in embryonic cells, they could not transform those cells into tumors, but if they put a RAS oncogene with a *myc* oncogene both could transform those cells which are in the embryonic stage into tumors. After this discovery, they asked themselves what would happen if, instead of introducing a RAS oncogene and a *myc* oncogene, they introduced a RAS oncogene with adenovirus E1A oncogene, this oncogene is essential for encoding the proteins necessary for the replication of the adenovirus, the results were exactly the same as the other pair of oncogenes. This has a simple reason behind, that is because it seems these two oncogenes “work” together to transform normal cells into malignant. Later that year, they cloned copies of mutant oncogenes as RAS and *myc*, they introduced it into the mammary epithelium of pregnant mice

resulting in breast cancer at an extremely high rates, this demonstrated that mutant oncogenes played an important role in tumor's pathogenicity. But the tumor did not appear until a few months later, which showed that it required more than one alteration for the tumor to appear.

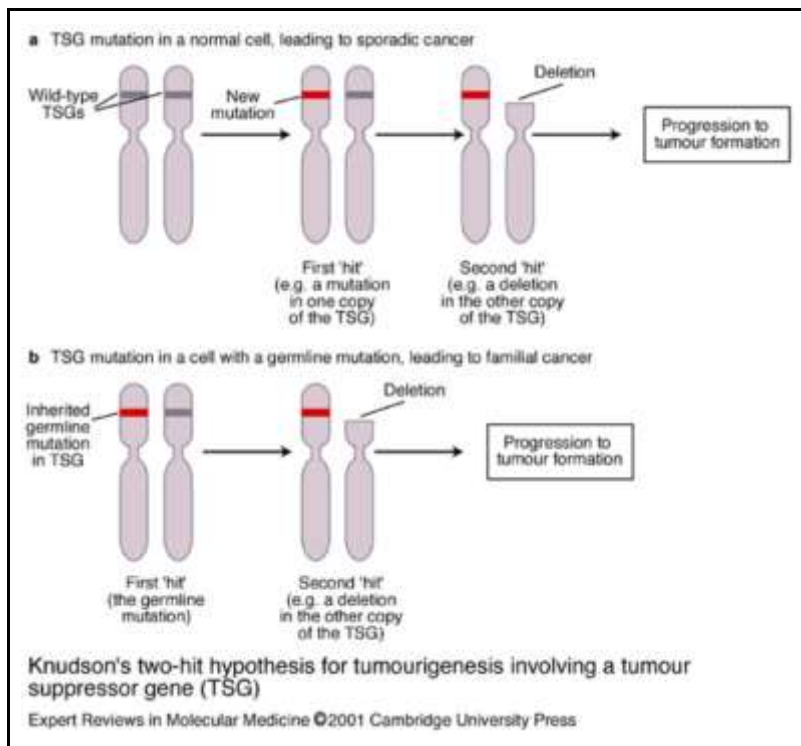
Not always the nature of these other alterations was clear, but they supposed that were somatic mutations, which created mutant growth control genes that with the cloned copies of mutant oncogenes start a malignant outgrowth. In the following years, they would keep working on with human tumors on mice, until they found out that those cells contained multiple mutant genes that help the tumor formation. This model is also known as the Multistep model of cancer.

Later that decade, experiments for the validation of the model that establishes that tumorigenesis implicates a cell which carries two or more mutant oncogenes, which are activated in each step of development of the tumor, proved certain difficulty for the validation. They found out that rarely two mutant oncogenes such as RAS or *myc* were found in the genome of tumor cells, so what were the possibilities that remained. One of the two different possibilities scientists determined was that the genome of a normal human tumor cell did not have mutated genes associated with, or that it had mutated genes, but they were not the ones that were studied in previous years, such as *myc* and RAS.

They discovered a group of genes that worked preventing cancer rather than making it grow bigger and bigger, they are called tumor suppressors. Henry Harris, a scientist from Oxford, was the first one to give indications of its existence. He experimented with different types of cells and tumor suppressors, which had different genomes, the aim of these experiments was to see which cell had the genome that would dominate over the other one. They concluded that most of the hybrid cells were non-tumorigenic, meaning that normal cells dominated over the other cells. In other words:

The alleles from the cancerous cells made a phenotype that was recessive to the normal cell phenotype, which also means that the normal cell phenotype was dominant. They assumed that normal cells had a gene that controls growth, which they were needed for normal proliferation. When this was first found out, they observed that, apparently, cancerous cells had lost this gene, so they experimented with cancerous cells and normal cells, and what happened was that the allele that was dominant in the normal cell took over the cancerous cell in order to restore the behavior of the hybrid cell. This is the reason why they are called *tumor suppressor genes* (TSG). They studied how they worked on retinoblastoma and eye tumors, this work was made by Alfred Knudson, who in the 1970s studied the genetics of these rare tumors. By doing research on retinoblastoma and familial cancer, he discovered that the retinoblastoma seemed to appear due to accidental somatic mutations, whereas the familial cancer appeared due to a mutation in the germline. Another difference Alfred Knudson observed, was that retinoblastoma needed two somatic mutations which affected the

lineage of cells from the retina, whereas the familial cancer only required a single mutation; but Knudson thought that, in order to trigger tumorigenesis, a second mutated gene was required, although, that defective allele was inherited from one of their parents. Knudson also remarked on the fact that for a retina cell to turn into a cancer cell it had to lose two mutated alleles, after some research Knudson concluded that both of the mutant genes were arisen by somatic mutations (Two-hit hypothesis). In 1979, analyzing karyotypes, they discovered that the q14 band of chromosome 13 was deleted and this was the locus for a gene called Rb.



**Fig. 1** Illustration of Knudson's two-hit hypothesis of tumor suppressor inactivation in familial (heritable) cancer.<sup>2</sup>

negatively, due to the loss of this tumor suppressor gene, making an uncontrolled cell growth that ends forming a cancerous tumor.

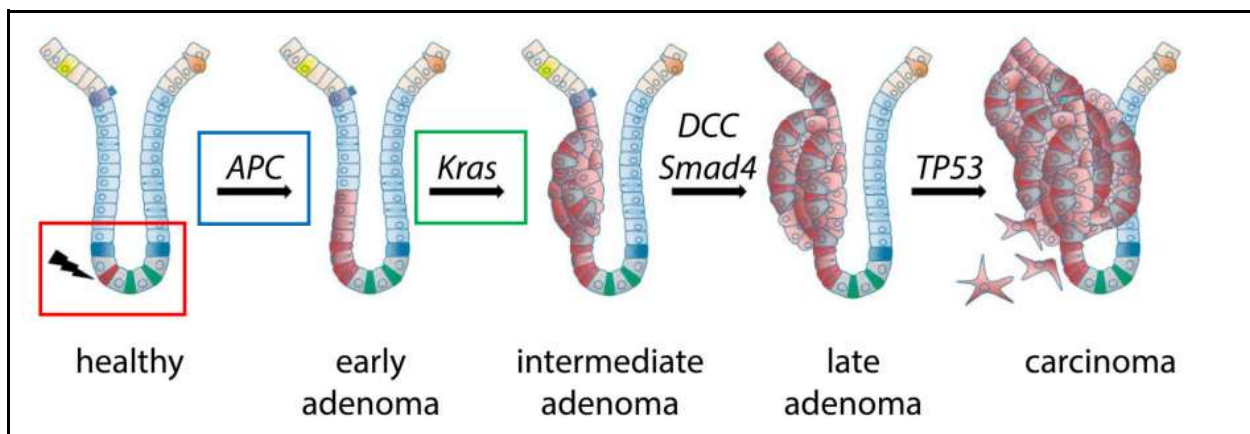
Harris and Knudson made it possible to understand the variety of familial cancer syndromes. Harris made his postulation about hybrid cells which permitted the discovery of TSG, as Knudson made the discovery of the RB gene. The discovery of the RB gene, made us able to know that by inheriting a non-correct functional allele the chances of having a retinoblastoma increases to a 90%, also inheriting a defective APC TSG increases the chances of having colon cancer. Today, we know that there are more than two dozen cancer syndromes associated with a non-correct functional TSG. Research on TSG is very difficult, because we can only observe them when they

<sup>2</sup> Knudson, A.G. (1971) Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A 68, 820-823, PubMed



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are defective from a cell genome, involving the inactivation of the two copies of this TSG. The probabilities of this happening are very low, around  $10^{-12}$  per cell cycle. The premalignant heterozygous cells carrying an intact allele of the TSG through a genetic mechanism cuts the arm with the wild functional TSG allele and replaces it with the non-functional mutated allele, transforming this cell into homozygous carrying the defective alleles of the TSG. This discovery made geneticists look for which chromosomal regions suffer what is called loss of heterozygosity (LOH) which affects very specific regions of the chromosomes of various human tumor cells. The fact that researchers identified and cloned such a group of TSGs associated with these LOH rich regions, made us come across a great discovery of a new group of growth-controlling genes. As mentioned before, researchers thought that most of the human tumor cells carried multiple mutated genes, but this discovery made us change this hypothesis, and now we know that a great part of the human tumor cells only carry one active oncogene with different inactivated TSGs, as Vogelstein and his team discovered in tumors containing the mutation of *K-ras* oncogene that were accompanied by mutations of three TSGs (APC, TP53 and DCC).



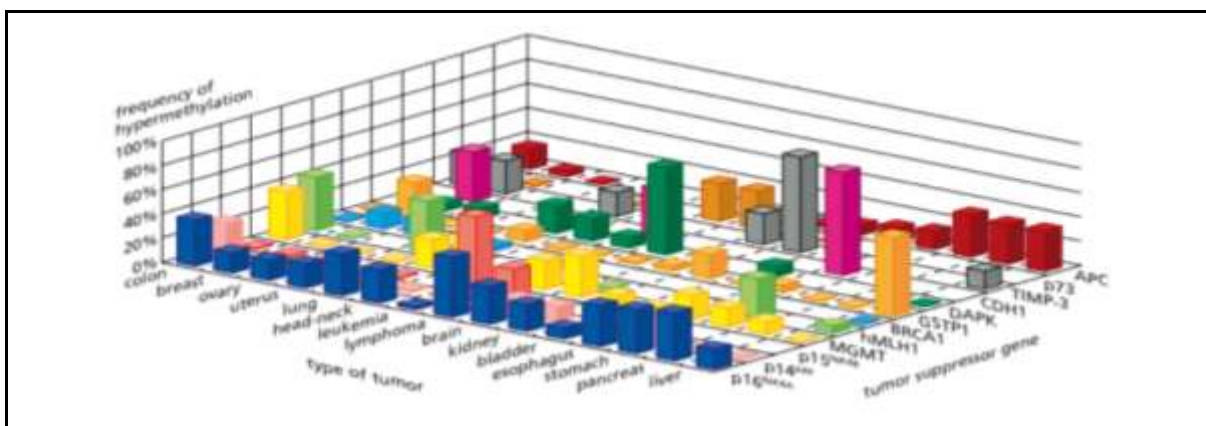
**Fig. 2** Genetic changes related with the colorectal carcinogenesis as the multistep model of Fearon and Vogelstein. In this model, Tumorigenesis progresses through a succession of genetic alterations. These alterations include oncogenes (*Kras*) and tumor suppressor genes like APC, DCC or TP53.<sup>3</sup>

We know that this event is always present, for the fact that the genetic studies held by Vogelstein showed us that TSGs are even more inactivated when oncogenes around are activated during the process of tumor generation. In the late 1980s, they discovered that numerous DNA tumor viruses aimed at two important TSGs: p53 and *pRB*. As a consequence, the cells deprived of these two regulators by non-related genetic mechanisms will lead to cancer and metastasis.

However, there is a way in which tumor formation can be accelerated: if there is an increased rate of gene mutation in cells, this means that the time required for cells to

<sup>3</sup> Michaela Krausova Laboratory of Cell and Developmental Biology Institute of Molecular Genetics AS CR, vvi. (n.d.). Czech Center for Phenogenomics. Retrieved July 8, 2022, from [https://www.phenogenomics.cz/files/2015/04/2015-03-24-Presentation\\_05\\_Krausova.pdf](https://www.phenogenomics.cz/files/2015/04/2015-03-24-Presentation_05_Krausova.pdf)

develop cancer will be reduced, thus the probability of someone having cancer will increase considerably. For instance, people that suffer from Xeroderma pigmentosum (XP), are very sensitive to UV rays, this exposure to UV rays create melanomas and carcinomas. In most of the humans, the thymine dimers created by UV radiation are quickly eliminated by the DNA, this elimination of dimers is accomplished by a cohort of DNA proteins which repairs very specific mutations in the DNA, although people suffering from Xeroderma pigmentosum have one of these proteins' repairers absent or defective; as a consequence, XP patients concentrate many mutations. Following this example, the same happens with hereditary non-polyposis colon cancer (HNPCC), a type of colon cancer, where the DNA mismatch repair genes affected are MSH2, MLH1, MSH6 or PMS2.



**Fig. 3** Methylation of multiple genes within tumor analyzed genomes, summarizing measurements of the methylation state of the promoters of different tumor suppressor genes. The height of each bar indicates the proportion of tumors of a given type in which a specific promoter has undergone methylation<sup>4</sup>

Moreover, recent research has shown that many breast cancers are associated with mutated versions of BRCA1 and BRCA2 genes, which were first thought they were TSGs but its behavior shown otherwise. Biochemical studies proved that BRCA1 and BRCA2 specified proteins that take part in repairing double-strands of DNA. There are lots of reasons that made scientists believe that bad-functional DNA repairers can produce human tumors, this loss can be somatic, although, occurs more frequently through epigenetic mechanisms. As mentioned earlier, there is the loss of two cellular genes during the course of tumor progression, those are TSGs and DNA repair genes, which are the cause of tumor formation, but if we think that cancer is a genetic disorder we are considering that these two genes are lost during a somatic mutation, so the phenotypic changes must be heritable, but there is a mechanism that does not depend on those genetic alterations: it depends on 5'-C-phosphate-G-3' (CpG) which is a sequence of dinucleotide, CpG that can be found nearby promoters of genes. When a DNA segment contains CpG which is methylated (MeCpG), the genes are inactivated by promoter repression without any mutation; for real, we can affirm that the function

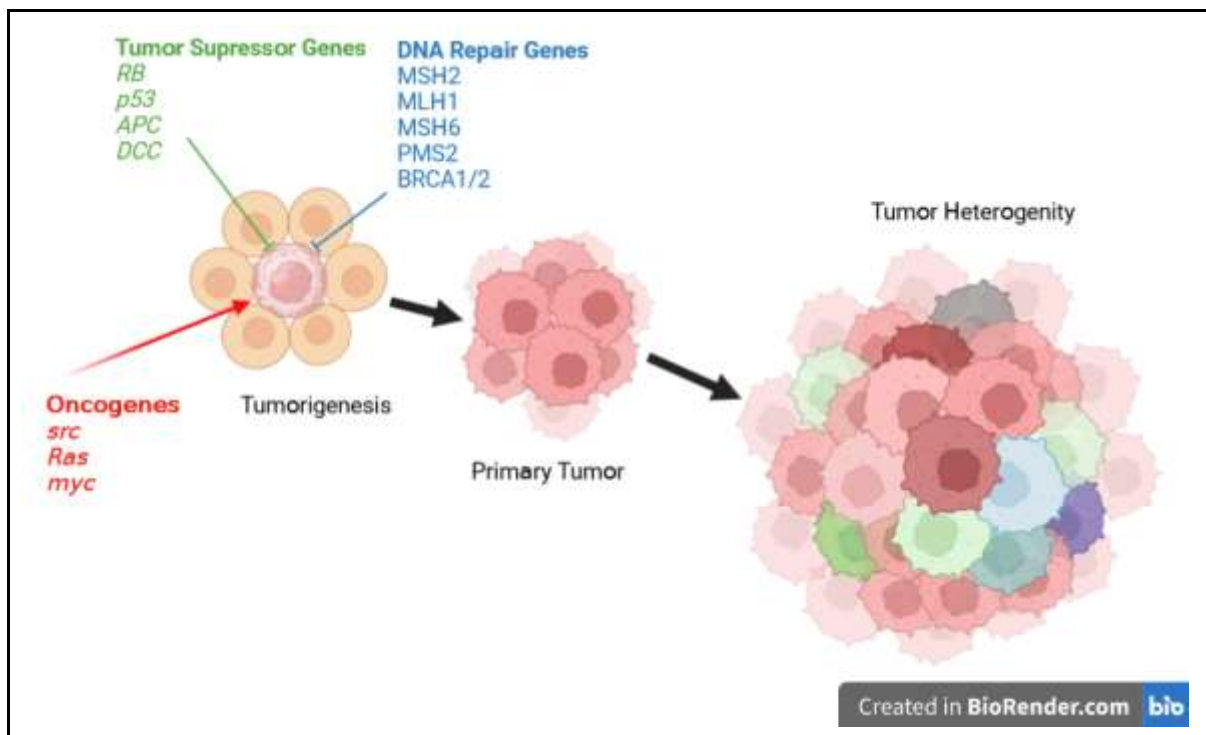
<sup>4</sup> Esteller M., Paul G. Corn, Stephen B. Baylin, James G. Herman; A Gene Hypermethylation Profile of Human Cancer. *Cancer Research* 15 April 2001; 61 (8): 3225–3229

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of TSGs and DNA repair genes are more likely to be lost by methylation rather than by any mutation.

But most of the carcinogens responsible for producing cancer in humans do not act as mutagens, they act as tumor promoters as they trigger the tumorigenesis by mutating very specific targets.

Cancerous cells also have the ability to transform and create blood vessels surrounding tumor masses, this process is called angiogenesis, and it involves complex interaction between cancer cells and its microenvironment.<sup>5</sup>



**Fig. 4** This is an image of the progress of formation of a tumor. Firstly, the oncogenes, such as *src*, *Ras* and *myc*, are activated and when they are activated, tumor suppressor genes (TSGs) and DNA repair genes deactivate, this is also known as tumorigenesis. After tumorigenesis has happened, a primary tumor is formed because the TSGs and the DNA repair genes are deactivated, and the cells start dividing uncontrollably. Finally, after these tumor cells have divided so many times, there are mutations that make them different among others, causing diversity of cancer cells and forming a heterogeneous tumor. Own production

Once the cancer appears, the formation of metastasis is the result of a large process that implicates cells capable of invading adjacent tissues or traveling to different parts of the body via blood and lymphatic systems. Cancer cell from a primary tumor must have been genetically altered multiple times, although, scientists and researchers thought that there was an alternative mechanism, which implicates epithelial cells

<sup>5</sup> Howley, P. M., Gray, J. W., Israel, M. A., Thompson, C. B., & Mendelsohn, J. (Eds.). (2015). *The Molecular Basis of Cancer*. Saunders/Elsevier.



undergo a big change that makes those cells to acquire motility and invasiveness, rather than needing to acquire lots of changes in order to execute the multistep metastasis cascade, some genotypes of certain primary tumor cells allows them to activate a biological embryonic cell program, for instance the Mesenchymal-Epithelial Transition (EMT), once activated, this EMT enables the carcinoma cells to complete most of the steps of the metastasis, leading to malignant behavior.

### **1b. What is a tumor, how does it originate, and how does heterogeneity originate?**

A tumor is a group of cancerous cells that arise in a place and there are a lot of them in a specific area. A tumor is made from carcinogenic cells that produce cancer stem cells and those make more cells, but the first ones are produced from a single cell; all the following ones arise from this one who suffered mutations and transformed into the first cancer cell. Heterogeneity can be seen because of the appearance of additional mutations from the original tumor cells. Different subtypes of cancer cells will arise from this primary tumor with new capabilities, making possible the evolution of the tumor.

There are two main types of tumors, the ones that grow locally without invading adjacent tissue and that are not causing major damages, they only grow in a place where they should not be, but those do not spread metastasis and cancerous cells. The other type, the ones that we know as malignant tumors, those which invade nearby tissues and spawn metastasis.

Tumors have the tendency to extend all over the body, indeed this tumor not only extends on a specific part of the body, but also grows in places quite distant from where the primary tumor was found.

Metastasis consists of a random spread of cancer cells from a primary tumor into another part of the body, from which those cells migrate and produce a colony, that will be the tumor and will start growing and growing more cells.

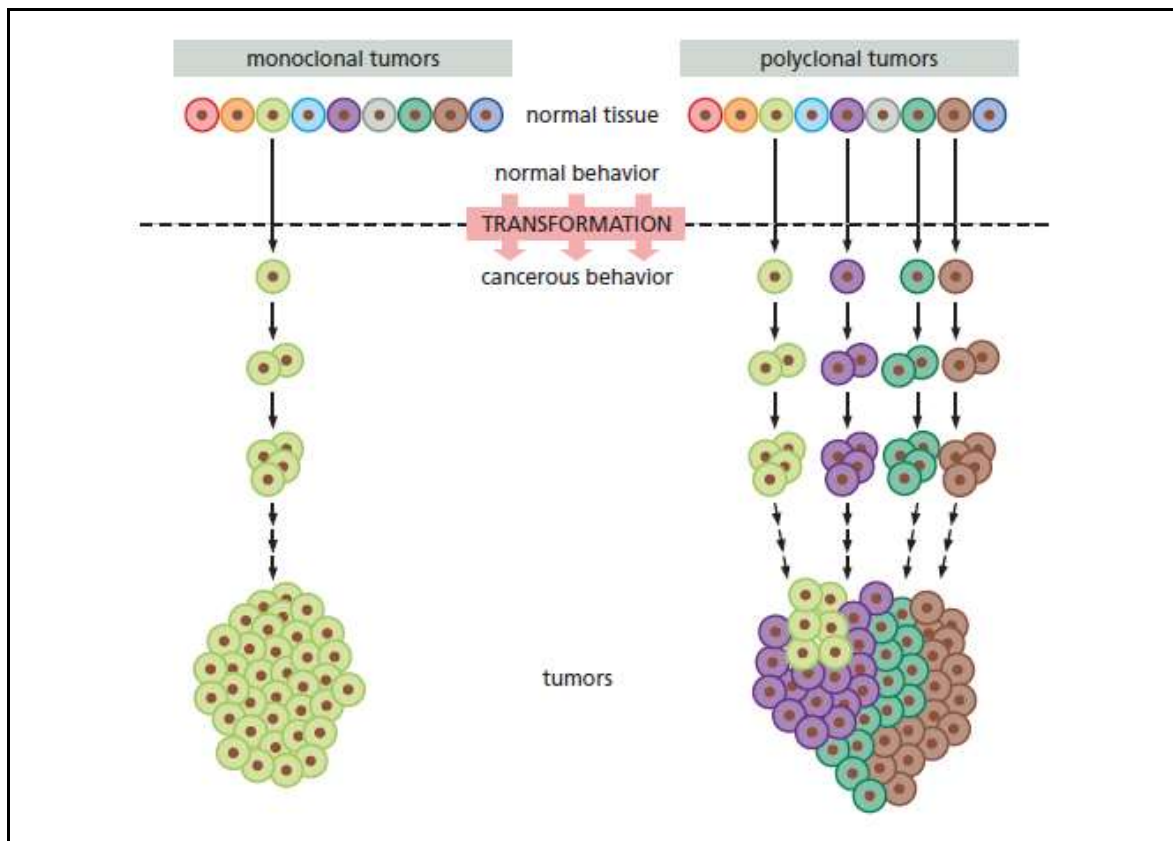
There are two principal types of malignant tumors, depending on its origin: Monoclonal and polyclonal.

Monoclonal tumors are those whose cells share a common one, from one cell arises two, and then four until there is a tumor, so all the cells are from the same type. All derive from a common ancestor cell.

Polyclonal are those whose origin is not a single cell but different types of cells from which more and more cells derive and form a tumor with different types of markers as there are different types of cancer cells. However, it can also happen that the tumor mass is composed by genetically distinct subpopulations of cells and do not give indication of common origin.

## 1. Introduction to cancer biology

In reality, when a tumor is formed it arises from a single cell (monoclonal), and by natural selection and the pressure from the surrounding cells, the tumor starts generating mutations, which makes the tumor more fit to survive. That is the main reason why there is such heterogeneity in tumors and also the fact that makes some drugs work for some cells, but without eliminating them, for the fact that not all the cells are as sensitive as each others.



**Fig. 5** There are two different types of tumors depending on its origin. The one in the left is a monoclonal tumor and consists of a tumor that is arisen by one specific type of cell, so the tumor will have only one type of cell. On the contrary, the one on the right is a polyclonal tumor and this one is a tumor that is formed by different types of cells and those clones forming a tumor with different cells. <sup>6</sup>

The heterogeneity from the tumors is caused by epigenetics variations and the addition of mutations. The first one consists of a variation on the expression of the signatures in a region of a tumor or genetic problems. Tumor heterogeneity basically means that tumor cells that proceed from a single primary tumor, after being reproduced and developed, show distinct phenotypic and morphological profiles. So, that they have differences between them. This is caused by an imperfection on the replication of the DNA that has acquired a mutation, that the cells from the primary tumor from which these cells came from did not have.

<sup>6</sup> Weinberg, R. A. (2014). *The Biology of Cancer*. Garland Science.

There are some diverse epigenetic variations that can affect the heterogeneity of the tumors and although those are not the same, all epigenetic variations that occur cause the heterogeneity.

Tumor microenvironment can influence through chemical signaling, and some regional differences can cause the heterogeneity between a primary tumor and the metastatic tumor formed from this first one.

### 1c. What are some of the hallmarks of cancer and how each of the hallmarks is exploited for treatment

The hallmarks of cancer as acquired functional capabilities that allow cancer cells to survive, differentiate, proliferate, and disseminate are acquired in different tumor types via distinct mechanisms with the course of multistep tumorigenesis. We can define summarize seven hallmarks according to Hanahan and Weinberg (Douglas Hanahan & Robert A. Weinberg, 2000) and what are the targets for treatments:

- *Selective growth and proliferation advantage:* Growth promoting is expected to be produced in high levels to promote tumor progression giving an advantage for the tumor survival through receptors overexpression, constitutive receptor activation, aberrant signaling and checkpoint disruption. Perhaps the most common alterations could be found in the regulation pathways, for example the *RAS* protein, which can be found in nearly 30% of cancers and about 90% of pancreatic carcinomas<sup>7</sup> or p53, mutated in the 50% of sequenced tumors<sup>8</sup>.

The therapies arresting cell cycle at the G1 phase will stop the proliferation and this could be possible through selectively blocking cell signaling, activation of epigenetically silenced tumor suppressor genes or microRNAs for inhibiting the proliferation farther than nonspecific mitostatic drugs or radiation.

- *Altered stress response for survival:* On the evolution of cancerous cells towards malignity, the altered stress response provides survival advantage. Under these stress conditions including excess signaling, DNA damage, hypoxia, nutrient scarcity and also cancer treatments, tumor cells produce reactive oxygen species (ROS) and become dependent on ROS scavenging systems.

One of the therapies to specifically kill these cancer cells is to inhibit NADPH-producing enzymes to increase the concentration of ROS and induce the ROS-mediated cancer cell death. Another way for the treatment are miRNAs which can regulate apoptosis

<sup>7</sup> Csermely, P., Korcsmáros, T., & Nussinov, R. (2016, July 6). Intracellular and intercellular signaling networks in cancer initiation, development and precision anti-cancer therapy. *RAS acts as contextual signaling hub*. *Semin Cell Dev Biol*, 58(55-59), 10.1016/j.semcdb.2016.07.005.

<sup>8</sup> Kim, M. P., Zhang, Y., & Lozano, G. (2015, Nov 11). Mutant p53: Multiple Mechanisms Define Biologic Activity in Cancer. *Front Oncol.*, 2015(5), 249. 10.3389/fonc.2015.00249

through promotion of the apoptosis pathways or inhibition of the cancer products (miRNAs, proteins ...) which arrest the process.

Some tumors have DNA repair pathways or proteins overexpressed, and it is common in radio or chemoresistant ones, in these cases, some DNA repair inhibitors could be a potential treatment without affecting the normal cells.

- *Vascularization:* Tumors do not grow more than 2 mm<sup>3</sup> without vascularization, the growth of tumor directed capillary networks creates a favorable microenvironment, promoting cancer growth, progression and metastasis.

Angiogenesis is the process of cell division of endothelial cells from another existing vessel, it happens because of the expansion of the vessels. Hypoxia is triggered when there is low oxygen in the tumor microenvironment, thus Hypoxia triggers hypoxia-inducible factor-1 alpha (HIF1- $\alpha$ ). Vascular endothelial growth factor (VEGF) is an HIF1- $\alpha$  induction-dependent factor and an inducer of vascularization. It was found that anthracycline treatment in prostate cancer-xenografted mice, which blocked the HIF1- $\alpha$  binding potential via a downregulation of the VEGF. The results of the treatment also showed that cellular proteases, for instance, matrix metalloproteinase (MMP) contributed to vascularization.

The MMP, which is activated by the HIF1- $\alpha$  incites angiogenesis. These changes affect vascularization by changing the tumor microenvironment.

The use of monoclonal antibodies against VEGF has shown effect at first stages, and the use of nanoparticles to deliver specific anti-angiogenic agents like endostatin has shown, in vitro, suppressions of proliferation of blood vessels (Saman et al., 2020).

- *Invasion and metastasis:* This is a multi-step process which involves the invasion of the cellular matrix and the surrounding stroma, intravasation, survival in the blood or lymph vessels, extravasation to distant places and adaptation to the new cell environment to develop a secondary tumor (colonization). Exosomes carry on different molecules like proteins, mRNAs, DNA and miRNAs for the preparation of the target of colonization.

It is known that the application of nanomedicine as a treatment for cancer has the potential to let clinicians solve some problems with different treatments. With nanomedicine, we can create nanoparticles (NP), that, eventually will be more effective than other types of treatments, matter of fact with NPs we will be able to target more specific cells, deliver the active drug inside the cell and reduce side effects.

Targeted therapies using radiation and immunotherapy as well as hormonal treatments are used against the metastatic cells.

- *Metabolic rewiring:* Is a hallmark which provides 'advantages' during the process of cancer formation in front of the high rate of proliferation. Pavlova and Thompson identified 6 hallmarks related to this metabolic rewiring. The first one they identified was that tumors had a non-regulated consumption of glucose and amino acids, the second one is that tumors take nutrients differently and also take them

opportunistically, which makes the tumor take more nutrients than required, using glycolysis and TCA cycles. Many cancer cells can oxidize great amounts of glucose to obtain ATP and lactate whereas some other cells can use this lactate in its metabolism. Other hallmarks that they observed were that tumors demanded more nitrogen, metabolic interactions with the microenvironment, among others. The three main objectives of the metabolic rewiring are the maintenance of the oxidative balance (to equilibrate the increase of ROS), the ATP generation and the synthesis of biomolecules to sustain the cell growth.

With the development of new rationale drugs combinations, which will implicate metabolic inhibitors for glycolysis and membrane transporters with traditional chemotherapy and radiotherapy will be a great way to manage the metabolic rewiring of cancerous cells.

- *The microenvironment:* There is a strong communication between stromal cells and cancerous cells, which creates a microenvironment during carcinogenesis, recruiting and reprogramming other cells in order to maintain the tumor growth. To put in contrast to this, components of the Tumor MicroEnvironment (TME) integrate very well with fibroblasts, which can provide selective growth and proliferation during the tumor formation. In this TME is very important the role of the communication between cells (receptor-ligand binding, gap junctions, nanotubes, mediators, extracellular vesicles) and it results in intratumoral heterogeneity where different cells play diverse functions allowing the tumor survival and expansion.

One of the therapeutic approaches is microenvironment-targeted therapy, where antibodies can inhibit extracellular ligand-receptor interactions. Another therapeutic way is the inhibition of the nanotubes and the exosomes. Finally, the other way is to use the cell-cell communication as a vector to deliver specific drugs<sup>9</sup>.

- *Immune modulation:* The immune system eliminates the malignant disease and proceeds to initiate three steps: elimination, equilibrium and escape. The first step consists of 4 subphases by which the immune system recognizes the tumor cells through innate immunity, secondly takes place the antigen presentation and activation of specific tumor-antigen T-lymphocytes, which is also made at the same time the activation of the cytotoxic mechanism and the last step is the recruiting of these T-lymphocytes to eradicate the tumor cells. After all of this process, the equilibrium stage initiates, in this stage there is a production of various tumor cells resistant variants and, by natural selection, the cells with low immunogenicity are selected. This is called the escape phase, and has 3 principles. The first one is that the tumor cells might be a lack of antigen recognition, the second one is resistance to cell death and the last one

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<sup>9</sup> Dominiak, A., Chelstowska, B., Olejarz, W., & Nowicka, G. (2020). Communication in the cancer microenvironment as a target for therapeutic interventions. *Cancers*, 12(5), 1232.

is the increasing of tolerance towards some immunosuppressive cells. Metabolic rewiring and microbiome are two relevant factors.<sup>10</sup>

It was discovered that ipilimumab which is a therapeutic drug that modulates T-cell pathways and have the potential to reinvigorate an antitumor immune response, meaning that, blocks a checkpoint molecule called cytotoxic T-lymphocyte antigen 4 (CTLA-4).

#### 1d. What is the most advanced treatment available for cancer

There are a lot of treatments available for cancer, for example surgery, chemotherapy, immunotherapy, radiotherapy, target drug therapy among others. This list of treatments are very common and some of them have quite good medical results, such as the reduction of the length of a tumor by chemotherapy.

Historically, the first treatments for cancer were some homemade remedies that the apothecaries recommended, such as fox lungs, boar's teeth, white coral and other unlikely remedies of questionable reliability. Sometimes, surgeons undertook some surgeries, such as mastectomies, under unsanitary conditions and without anesthesia. Later on, they started making operations with anesthesia to remove tumors, although sometimes it didn't work because they didn't know that the tumor had spread, and they would die because of its metastasis.

In the 19th century with the modern surgery advances and the discovery of anesthesia, most of the malignant tumors that could be operated, were removed through surgery, even though some of those, which spread beyond its primary tumor, would posteriorly cause the death of the patient.

By the end of the 19th century, the x-rays were discovered by the physicist Wilhelm Röntgen, which later, Becquerel, through the phosphorescence produced by potassium-uranium sulfate with light, deduced that a spontaneous emission of radiation was generated and was called Becquerel rays.

It was known that the radiation worked breaking down the DNA of every cell, which controls the process of cell division. However, the radiation kills all the cells, nonetheless, the cancer cells are easier to kill because they divide faster, and some healthy cells may survive.

Within this knowledge and other discoveries, the Nobel Prize, Marie Curie, discovered the radioactivity emitted by Polonium and Radium. It was accidentally observed that the people who manipulated the radium presented hand injuries due to their continuous

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<sup>10</sup> *Revisiting the hallmarks of cancer - PMC.* (2017, May 1). NCBI. Retrieved July 6, 2022, from <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5446472/>



manipulation. In 1901 Curie suggested that a small radium tube in contact with a superficial tumor could be inserted. This was the discovery of brachytherapy, and the first time radiotherapy was used for therapeutic purposes.

As has happened with many scientific discoveries, the chance has caused advances in science. This is the case of the chemotherapy that was discovered because of the use of mustard gas in the 1st World War. The effect this has on our body is at DNA level, as it damages all the DNA of the cells, making it impossible for them to reproduce and to carry out the cell division. It also reduces the formation of blood cells and there is an abnormal decrease in erythrocytes, leukocytes and thrombocytes. This gas, besides, produces the typical symptom chart of chemotherapy which consists of nausea, vomiting, alopecia, and increased vulnerability to infection, related to its affection to the immune system.

In gas mustard exposed patients, the initial increase in the total number of leukocytes was accompanied by an increase in the total number of leukocytes, those individuals who survived for several days developed a profound decrease in blood cells.<sup>11</sup>

Since leukocytes can divide rapidly, it was deduced that this chemical agent could be useful to destroy cancer cells that also divide rapidly.

The selective action of mustard gas, that affected only the white blood cells, suggested that it could be used to treat lymphoma.

A study was carried out, in which nitrogen mustard was used to treat a lymphoma and the tumor was greatly reduced. Because of those good results, agents extracted from nitrogen mustard were used as the first chemotherapy treatment.

Since then, more and more chemotherapy agents were discovered and put into practice to prevent cancer from spreading or in order to reduce its size, to make those tumors easier to strip.

After several years of research, scientists found that the best way to cure cancer and to not damage healthy cells from the body, such as chemotherapy or radiotherapy does, was to make target drug therapy or personalized medicine, attending each subtype of cancer cell with the knowledge over its genetic fingertip.

*Biomarker Testing* techniques are a way to look for genes, proteins, and other substances that can give us information about the characteristics of the cancer cells in each patient and, as a result, easier to find an effective way to wash them off it.

Nowadays, there is no 100% effective treatment that cures cancer, but there are some that in some percentage does.

It depends on the type of cancer and its location, but the most useful and advanced treatments are the following:

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<sup>11</sup> History of Cancer Treatments: Chemotherapy. (n.d.). American Cancer Society. Retrieved July 10, 2022, from <https://www.cancer.org/treatment/understanding-your-diagnosis/history-of-cancer/cancer-treatment-chemo.html>

1. Introduction to cancer biology

- *Targeted therapy*: This is a type of drug therapy that consists of a specific feature attack against cancer cells, and the objective is to stop the cancer from spreading and growing or from preventing metastasis through two ways: using small molecules which can enter inside the cell to act over specific cancer proteins or using monoclonal antibodies which recognize specifically cancer cells and cause the self-destruction, attach radioisotopes or active drugs, or enhancing the immune response against these labeled cancer cells.

- *Immunotherapy*: It is a cancer treatment that works by helping the immune system to recognize and kill cancer cells. This type of treatment is done by drugs that boost the immune system. This could be via immune checkpoint inhibitors to respond more strongly to cancer, monoclonal antibodies, vaccines or modified T-cells transfer (Chimeric Antigen Receptor T-Cells, otherwise CAR-T cells).

- *Hormone therapy*: Some cancers grow in response to particular hormones, so this therapy uses synthetic hormones to block the effect of the body's natural hormones.

- *Stem cell transplants*: Restores stem cells that grow into blood cells in people who have had theirs destroyed by high doses of chemotherapy or radiation therapy, so it works as an additive treatment for radiation or chemotherapy in order that our body doesn't get too weak from having those treatments done.

- *Photodynamic therapy*: It uses a drug activated by light to kill cancer and other abnormal cells. The photosensitizer produces oxygen radicals that kill cancer cells under exposure to laser or LED light.

- *Hyperthermia*: Is a type of treatment in which body cancerous tissue is heated to 45 °C through radiofrequency, laser ultrasounds or microwaves are used to help damage or even kill cancer cells with little injury to healthy tissue. It is used in a small group of very specific cancer tumors.<sup>12</sup>

Not as advanced as the others ones, but still really effective, are:

- *Surgery*: Even though it has been used for many years, it is still one of the most advanced and effective treatments for many cancers, such as small or primal ones. It is used for those which are located in accessible areas of the body and are easy to strip.

- *Radiotherapy and chemotherapy* (specially combined chemotherapy), although they have been used for many years and are not so new, work very well in many cases,

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<sup>12</sup> Types of Cancer Treatment - NCI. (n.d.). National Cancer Institute. Retrieved July 10, 2022, from <https://www.cancer.gov/about-cancer/treatment/types>



and despite the fact that they are not even close to 100% effective, they save many lives and are still the most used treatments. A new milestone for these therapies are radioimmunotherapy, where the radionuclides are attached to specific cancer antigen cells immunoglobulins, and epigenetic therapy, where the drugs act by reverting cancer cells into normal cells changing methylation state in the genome.

## 2. Introduction to *Drosophila*

*Drosophila melanogaster*, also known as the fruit fly or the vinegar fly, is a small and really common fly that can be found near rotted or unripe fruit almost everywhere.



**Fig. 6** This is an image of a *Drosophila melanogaster* specimen. André Karwath [CC BY-SA 2.5](https://creativecommons.org/licenses/by-sa/2.5/)<sup>13</sup>

It is used as a model organism to study with, and a wide variety of studies have been made with specimens of this type of fly. These studies vary between neurodegenerative diseases, immune system deficiencies, pathologies of the visual system, genetic studies and cancer.

There are many reasons why *Drosophila* is used as a model organism, some of those are that there are few ethical problems for using them in research.

They are not considered as mice, rats or other mammalian model organisms such as guinea pig, so the ethical problems are not as many, they also have a very short life cycle which makes them better to work with and there is no need to wait many days or even months to let them grow, as their life cycle finishes between two and three weeks. They are easy to find and really cheap to culture in a laboratory, the food they use is not expensive and the conditions that are needed for them to live are very normal (the adequate temperature for them is about 22-25 Celsius degrees).

Furthermore, they produce large numbers of externally laid embryos and their offspring are numerous.

Another reason is that they can be genetically modified in many ways, and they are not as difficult to modify genetically as other models. They have eight chromosomes, which makes it much easier to make modifications and see how these are and behave than if there were more. For example, humans have 46 chromosomes or mice have 40 chromosomes.

Despite being apparently very different from humans, in reality they are not, for the fact that *Drosophila* and the human being embryonic development and their genes, are quite similar.

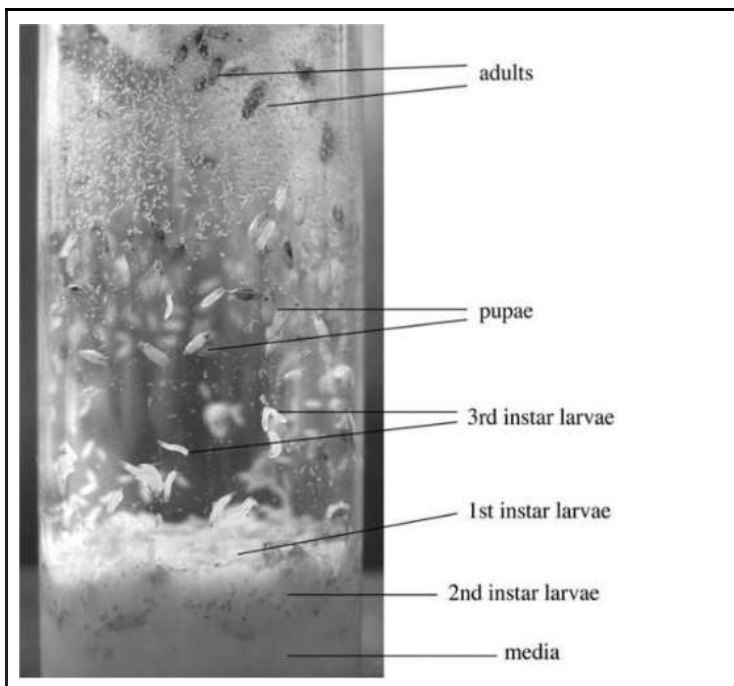
<sup>13</sup> Karwath, A. (2022, February 21). *Drosophila melanogaster*. Wikipedia. Retrieved March 19, 2022, from [https://es.wikipedia.org/wiki/Drosophila\\_melanogaster#/media/Archivo:Drosophila\\_melanogaster\\_-\\_side\\_\(aka\).jpg](https://es.wikipedia.org/wiki/Drosophila_melanogaster#/media/Archivo:Drosophila_melanogaster_-_side_(aka).jpg)

*Drosophila melanogaster* is really important for our experiment because we will carry out our experiment on these specimens, and we will use those to study cancer. It is one of the best model organisms to study on, and we will use few of those thanks to IRB Barcelona, where we will be performing the experiment, using their laboratories and material, under the supervision of an experimented researcher.

## 2a. *Drosophila* life cycle.

The *Drosophila* life cycle is divided into 4 major stages, embryo, larva, pupa and adult, all the cycle has an approximate duration of 10 days, and when those are adults, they can live between 2-3 months.

The first step of its life cycle is the embryo, a virgin female and a male meet, and the oocyte is fertilized by the male and the egg forms inside the abdomen of the female, after approximately 24 hours the egg is released, the embryo continues developing.



**Fig. 7** The different stages of the fly in a plastic flask where it grows and develops. On the top part there are adult flies, the pupa is the step before the adult, they are attached to the wall, and it is bringing to completion the metamorphosis. Before the pupa there are the three larval stages, the first one is when the larva is on the top of the media obtaining nutrients, in the second stage the larva has enough strength to thicken into the media and continue obtaining nutrients and before the pupa, in the third stage the larva comes out the food and starts climbing the wall.<sup>14</sup>

After another 24 hours, the larva starts to form. There are 3 stages of larva, the first one is the 1st instar larva, which has a duration around 1 day, and it begins to feed in on the surface of the medium where they are cultivated.

The 2nd instar larva burrows into the media and makes a hole in it, in order to get into the food, so it can have an easier way to get the necessary nutrients. In the next 24 hours, the 2nd instar larva will convert into the 3rd instar larva.

The 3rd instar larva stage, when the larva leaves the medium and starts climbing the walls to find a place where they can convert into pupa, the process from 3rd instar larva to pupa has an approximate duration of 3 days.

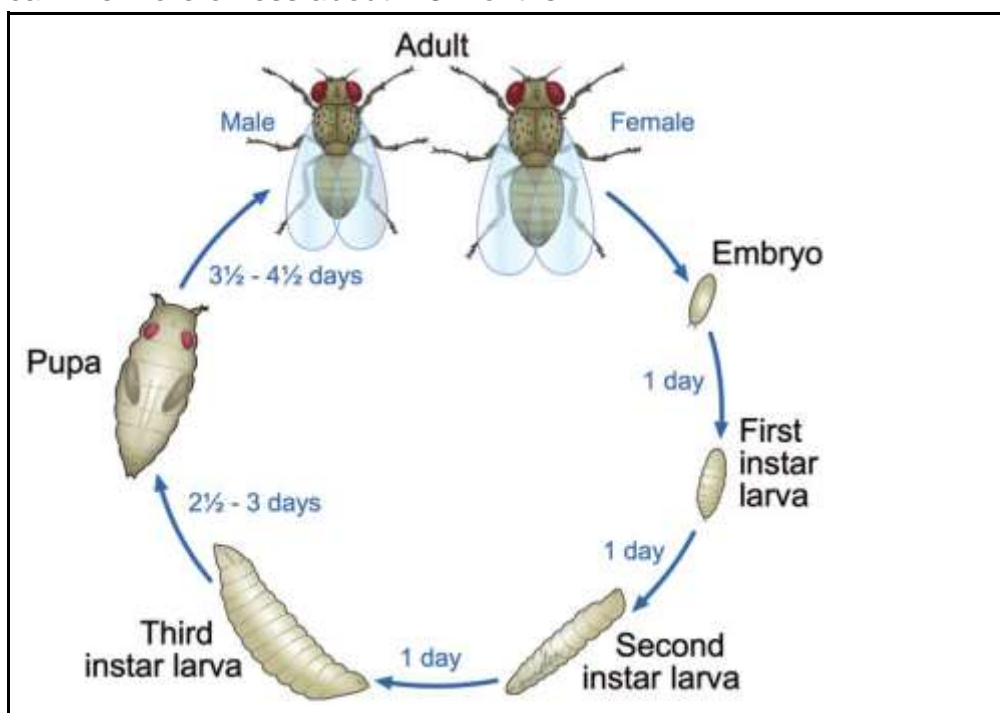
<sup>14</sup> Fernández-Moreno, M. A., Farr, C. L., Kaguni, L. S., & Garesse, R. (2007). *Drosophila melanogaster* as a model system to study mitochondrial biology. *Methods in molecular biology (Clifton, N.J.)*, 372, 33–49. [https://doi.org/10.1007/978-1-59745-365-3\\_3](https://doi.org/10.1007/978-1-59745-365-3_3)

## 2. Introduction to *Drosophila*

The next step is the pupal stage, that is when the fly is hooked in the wall of the flask and starts the metamorphosis. What happens is that the complete body of the larva converts into the adult and this happens because with all the nutrients that the larva acquired, the pupa can start to develop all the cells and organs from the adult fly and after 4 days, the pupal stage is finished and the pupa has converted into an adult, having all the organs developed in order to be an adult fly.

Next there is an image where the fly is observed in its different stages of growth, and it is seen how it changes its shape and appearance.

All the cycle has an estimated duration of 10 days and when it finishes, the adult fly can live more or less about 2-3 months.



**Fig. 8** The cycle of *Drosophila melanogaster* life, which is divided into 4 major stages (Embryo, Larva, Pupa and Adult) and has nearly 10 days of duration.<sup>15</sup>

### 2b. Rearing and mating *Drosophila* in laboratory

*Drosophila melanogaster*, apart from not having a lot of ethical problems when working with it or being very expensive, it is a great genetic model to study because of its

<sup>15</sup> Ong C., Lin-Yue Lanry Yung, Yu Cai, Boon-Huat Bay & Gyeong- Hun Baeg. (2014, 07 22). "Drosophila melanogaster as a model organism to study nanotoxicity". *Nanotoxicology*, 9(3), 396-403. [https://www.researchgate.net/publication/264127592\\_Drosophila\\_melanogaster\\_as\\_a\\_model\\_organism\\_to\\_study\\_nanotoxicity](https://www.researchgate.net/publication/264127592_Drosophila_melanogaster_as_a_model_organism_to_study_nanotoxicity) .

genetics and all the things that can be done with it, but it is also very important and useful because to breed them you do not need a very expensive habitat or extreme conditions. Some animals that have been used as model to study, cannot be stored in a laboratory, but with *Drosophila* only a plastic flask is needed, a medium so that it has all the necessary nutrients, cotton so that it does not escape and a stable temperature, all these elements can be easily found and are not unaffordable.

The utensils and ingredients that are needed consist of a plastic flask, some cotton, agar-agar, yeast, sugar or some type of molasses, propionic acid, corn flour and water.

To prepare the media it would have to be boiled and then mixed with the agar for around 4 minutes, all the sugar or molasses would need to be included in the mixture and return water into a boil.

Meanwhile, mix cold water and corn flour in a different container until there are no lumps left.

Add the two mixtures together and mix these two, then bring this mixture to a boil for 5 minutes, stirring continually. Let it sit until it reaches 60° C and add the propionic acid (it improves larval survival and supports growth).

Now the mixture is ready to be poured in a sterile flask, the amount that is needed to be poured inside is around 1 inch deep. We need to put fresh yeast inside before introducing the flies in. <sup>16</sup>



**Fig. 9** This is a *Drosophila melanogaster* laboratory culture-vial  
Autorstwa Masur - Praca własna, CC BY-SA 4.0 <sup>17</sup>

#### Culture media for flies

water .....	420 ml
agar .....	4,5 g
sugar/molasses .....	60 ml
corn flour .....	49 g
yeast .....	6,5 g
cold water .....	145 ml
propionic acid .....	3,4 ml

<sup>16</sup> Tyler, M. S. (2000). *Developmental Biology: A Guide for Experimental Study*. Sinauer Associates.

<sup>17</sup> Masur. (2011, 1 22). *Drosophila melanogaster* laboratory culture-vial. Retrieved 6 26, 2022, from [https://es.m.wikipedia.org/wiki/Archivo:Drosophila\\_melanogaster\\_laboratory\\_culture-vial.jpg](https://es.m.wikipedia.org/wiki/Archivo:Drosophila_melanogaster_laboratory_culture-vial.jpg)

## 2. Introduction to *Drosophila*

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This type of flask needs to be in rooms with a temperature of approximately 25°C, so flies have good condition to survive. As they don't need too much space, there can be lots of flasks in a tiny room, and it wastes much less than if it couldn't be stored this way.

To mate these flies, it is necessary to put some virgin female flies with male flies in a flask and then, after some days, if everything is good the female flies will have laid some eggs.

The eggs will be laid in the media and after some time, the larva will hatch from the egg, they will eat some media, convert into a big larva, then climb up the wall of the flask, convert into a chrysalid and after some days, they will be adult flies.

### 2c. Use of *Drosophila* as a genetic model system

*Drosophila melanogaster* is used as a model system for many reasons, one reason is that it doesn't need any expensive place to live or to be reared.

As explained before, everything that it needs is really accessible and economic.

Another reason is that there are not as many ethical problems with *Drosophila*, as it is an insect and its nervous system is not as complex as others, such as rats or mice, they are not mammals and, as insects, they aren't considered to be suffering while experimenting with them. There is no need to do paperwork for the procedures for the experiments, and it benefits a lot the experimental part as it is not necessary to wait for the response of the ethical group and the permission for doing the experiment. With flies, you just select some of those, propose the experiment to your superior and go on with the experiment.

So that they do not move and there is no possibility of something happening to them while they are being experimented on, they are put under anesthesia, normally they are exposed to a small amount of CO<sub>2</sub>.

In addition, *Drosophila* has a really short life cycle, which makes them reproduce certainly fast, and they can have many offspring as they produce numerous laid eggs.

Also, they can be genetically modified in many ways, which makes them an excellent study organism. Furthermore, the flies have its genome 60% homologous to human genome, and around 75% of the genes involved in human diseases have homologs in *Drosophila*, which makes them a super adequate organism to study with.

*Drosophila* only has four pairs of chromosomes, compared to 23 pairs in humans. This simplicity is one of the reasons why they are used in genetic studies; *Drosophila* genes can be mapped easily to investigate genetic transmission and a wide variety of diseases, where genes are involved.

*Drosophila* has a large number of anatomical features similar to humans, such as eyes, skin, brain, with which human diseases can be studied in these flies. As well as that, genetic markers can easily be identified in the microscope.

Another thing that makes *Drosophila* a good system, additionally to all said before, is that it is relatively small, so there can be a lot of them stored in a small space but are big enough to manipulate them.

Regarding cancer, this species of fly is used in research because it is very easy to manipulate them genetically and because they conserve most of the signaling pathways of cancer.

A great amount of signaling pathways that are responsible for controlling cell growth have a human equivalent in *Drosophila*.

There is a wide variety of recombination techniques that can be done in these flies and allows scientists to study the human combination genetic screens in flies.

Furthermore, *Drosophila* has some epithelial tissues, such as the larval imaginal disc, that is a perfect model to study epithelial cancer. Indeed, most of the cancers in humans happen in epithelial tissues and these larval organs are biological, biochemical and morphological comparable to humans, making these flies a great model to study cancer hallmarks.

In addition, through gene insertion methods, it is very easy to induce cancer in them.

## 2d. GAL4 UAS system

The GAL4/UAS system is a biochemical technique that is used to study gene expression and functions of some genes in determinate organisms.

GAL4 is a yeast transcription factor that recognizes and activates specific genes that are regulated by a UAS (Upstream Activation Sequence).

It has GAL4 that is a regulator of gene expression of galactose-induced genes and the UAS an enhancer to which GAL4 binds to activate a chosen gene that is wanted to study.

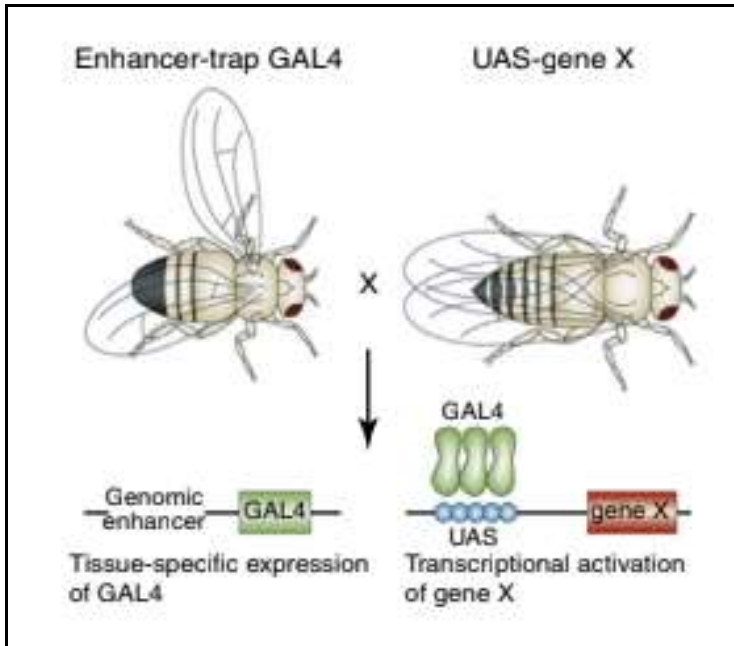
The UAS sequence with the gene of interest is only expressed when GAL4 is also expressed, this allows precise control and studies of its expression.

It works in the following way:

GAL4 is a transcriptional activator which binds to UAS enhancer sequence in the DNA. In this case, the fly induces a particular gene expression that is chosen that binds to UAS, and it is passed to the offspring to study whatever it is wanted.



2. Introduction to Drosophila



**Fig. 10** This is a graphic image that shows how GAL4 UAS system works. A male fly with an enhancer trap that is the GAL4 mates a female with the UAS and a gene, it produces an offspring that has the gene that is wanted.<sup>18</sup>

By doing this method, you can study specific genes because it enables you to choose the specific gene you planned to study mating to flies with only doing this technique. Although it can be a bit complex, with an enhancer or promoter and the GAL4 protein, plus another fly with the UAS and the gene of your choice, produce an offspring of a fly that has that gene because the GAL4 and the UAS allow us to do this.

Furthermore, there is Gal80, a GAL4 repressor that does not allow GAL4 to bind to UAS so the genes that were going to be expressed cannot.

Due to its easy use and manipulation, in addition to its approachability, we will use GAL4 UAS system to insert genomic enhancers and genes to the chromosomes of the flies we will study on, in our practical part.

We will use apterous (a gene that makes dorsal cells from the wing disc distinct from the ones from the ventral part) with Gal4 as a genomic enhancer and then will bind myrTom to UAS so where there is apterous, it will be present myrTom, that will give red color in that zone.

Also, we will bind bub3<sup>RNAi</sup> to UAS and p35, to create the tumor, as these are tumor agents provocateurs, so there will be a tumor wherever apterous is active.

We will, additionally, bind to UAS GC3Ai and in other flies we will bind Fly FUCCI to UAS.

**2e. Balancer chromosomes**

Balancer chromosomes are a type of genetically modified chromosome used for the maintenance of some genetic combinations or some deleterious mutations that are

<sup>18</sup> Gray, J. W., Mendelsohn, J., Israel, M. A., Thompson, C. B., & Howley, P. M. (Eds.). (2015). *The Molecular Basis of Cancer*. Saunders/Elsevier.



wanted to be sustained over generations in a group of flies. Making that the offspring of the flies also have that allele.

Permitting longer studies or analysis with this type of genes.

In *Drosophila*, there are matings that combine different genes and produce specific gene offspring. These can maintain combinations, but they can also make this mutation to be sustained for many offspring generations.

Fruit flies are diploid which means that their genomes are made up of two copies of each chromosome each one inherited from both their parents, these two chromosomes are homologous because they carry the same genes, but they came from different progenitors. The *Drosophila* genome has four pairs of homologous chromosomes, there are 2 sex chromosomes and a pair of chromosomes for chromosomes 2,3 and 4. If a desired mutation has been generated, and you want to be able to maintain that mutation over generations, you have to have two flies that are heterozygous for recessively inherited mutation, and they are left alone to multiply overtime, but they could potentially be producing more heterozygotes or flies that are homozygous not carrying the mutation.

If the mutation is recessive, you would not be able to tell, so what is done to make sure there is not a loss of this mutation is to mate the fly with the gene wanted with a fly that has a balancer chromosome. That creates a generation of flies, some of which carry the desired mutation.

Once this is done, the properties of the balancer chromosome will allow you to maintain this mutation stored over the balancer chromosome.

Then, there is no need to worry about making more crosses, since these will already have the desired mutation that you can work with it over time.

Meaning that mated offspring flies with a desired gene and a balancer chromosome will allow you to have lots of generations with the same genetic condition.

We know that if you were meeting two flies that are heterozygotes carrying both the balancer chromosome and the mutated chromosome of interest you have three potential offspring genotypes: a heterozygous that carries the mutated chromosome in the balancer chromosome, a homozygote carrying two mutated chromosomes and a homozygous carrying two balancer chromosomes and no mutated chromosome. Balancer chromosomes allow you to easily pick out the heterozygote without having to do any genetic analysis, so it is really easy for the scientists to pick the one that has both, the balancer and the gene because of physical features.<sup>19</sup>

Balancer chromosomes have three characteristics that allow scientists to maintain this mutation over offspring. First, balancer chromosomes contain multiple nested inversions which means that they prevent recombination between the balancer and the

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<sup>19</sup> Miller, D. E., Cook, K. R., & Hawley, R. S. (2019, Nov 7). The joy of balancers. *PLoS Genet.*, 15(11). 10.1371/journal.pgen.1008421

## 2. Introduction to Drosophila

mutated chromosome with the gene, keeping the mutation on the original chromosome, overtime crossing-over will not occur, and the mutated chromosome will not be altered in meiosis. Therefore, it makes sure that the mutation stays on the chromosome throughout generations.

The second important feature of balancer chromosomes is that they contain dominant markers that show up in the flies that carry those. This means that you can easily pick out flies that carry the balancer chromosomes, because these can be seen because of distinct physical features.

The third characteristic of balancer chromosomes is that they contain alleles that are lethal when carried homozygously meaning that a fly that carries two balancer chromosomes will not survive, and it will not be able to hinder the work that is done, with those that do contain both wanted characteristics.<sup>20</sup>

Balancer chromosomes contain phenotypic markers to allow the identification of these just by watching the individuals, letting know that it contains the balancer chromosome.

Furthermore, in the experiment, we will be using some balancer chromosomes. For example, Tm6B, a balancer chromosome that produces small body, and is linked to “upd3 lacZ :: UAS GC3Ai” in the same chromosome, and as it has a phenotypic marker, we can identify by sight. We will see that the larva has a smaller body and it is not normal. Since we want it to not have a small body, because we want it to express “upd3 lacZ :: UAS GC3Ai”, we will choose the ones that are normal, so we make sure they have what we want, if we did not use this marker we would not be able to know it. If it has small body, it will not have “upd3 lacZ :: UAS GC3Ai”.

Another one that we will use is CyO, a balancer chromosome, that it is characterized by giving wavy wings to adult flies. We will be using this one together with another one, as it will be joined with GFP because, we will be using larvae, so they have not developed the wings and GFP will help us to identify those just by seeing if there is green when seen in the fluorescence microscope. It will be in the chromosome with “ap Gal4 :: Fly FUCCI” and we will not choose CyO GFP as we want “ap Gal4 :: Fly FUCCI” to express in the offspring.

### 2f. RNA interference

It is a type of RNA molecule that eliminates the expression of some genes.

This process is done by double-stranded RNA, by means of transcriptional or translational repression.

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<sup>20</sup> How balancers work - Bloomington Drosophila Stock Center”: Indiana University Bloomington. (n.d.). Bloomington Drosophila Stock Center: Indiana University Bloomington. Retrieved August 8, 2022, from [https://bdsc.indiana.edu/stocks/balancers/balancer\\_intro.html](https://bdsc.indiana.edu/stocks/balancers/balancer_intro.html)

It is a natural mechanism for sequence-specific gene silencing that has possible applications in genomics, therapeutic treatments, cancer...

Due to its specific approach in silencing specific genes and the possibility of inhibiting tumor growth in addition to its precise targeting, it has been used and studied for cancer treatment.

Contrary to chemotherapy, RNAi has greater precision and only affects a specific selected group of cells, that is why it can be very useful and in the future help cancer treatment. Also, RNAi can enhance to some specific type of cancer cells or other, adding its possibility to carry therapeutic agents, can be a great tool for cancer treatment.

RNAi drug treatments work by silencing the cancer promoter genes, making the tumor unable to reproduce thanks to its drugs and its precision.

There are two types of RNA interference, that are microRNAi and small interference RNA (SiRNA).

The SiRNA are derived from longer double-stranded RNAi that are produced in the cell itself or delivered into cells to it experimentally.

Micro RNAi are another type of RNAi, most of those come from RNA that are transcribed in the nucleus, then fold and processed before being exported into the cytoplasm as double-stranded precursor microRNA.

These bind to Dicer, which is an endonuclease protein that cuts the RNA into short segments, then, the double-stranded RNA binds an Argonaute protein.

One selected RNAi remains bound to Argonaute, this is called the guide strand.

The combination of Argonaute and RNAi along with other proteins is named RNA induced silencing complex.

SiRNA directs the complex to bind specific messenger RNA, the targeting is precise because it's determined by base pairing between SiRNA and the target messenger RNA. Which makes the RNAi complex very precise. And after a process, it degrades the specific gene that has been targeted. Micro RNAi also guides the complex to mRNA.

It degrades the mRNA during translation, so they can inhibit the specific gene.<sup>21</sup>

Moreover, RNAi will help us in the practical part because it will prevent some determinate gene expression in the DNA of our flies that is not of our interest, and also it will not modify the already altered DNA of the flies we will study with.

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<sup>21</sup> "RNA Interference (RNAi) - NCBI." 9 nov. 2017, <https://www.ncbi.nlm.nih.gov/probe/docs/technai/>

## 2g. Protein Traps

It is a method that allows tagging endogenous proteins and the identification of those proteins that are of interest. Likewise, it allows the access to mutations trapped in genes using protein screens.

Additionally, it is a complementary method of gene screening, detection of changes in the genome. Without using any specific antibodies for each protein.

GFP (Green Fluorescent Protein) is a protein that allows the scientists to identify some specific genes using it as it emits bioluminescence.

A modification of the gene trap to identify subcellular located or transmembrane proteins using the specific antibodies for each protein is a good idea to identify those, but in practice it is a problem as it requires a lot of time and technique. Therefore, to solve this problem, protein-trap is an option.

The main idea of protein-trap was to tag proteins by localizing the epitope-tagged proteins using specific antibodies. But, using the GFP as the main tag, specific antibodies were not necessary.

Then, using a GFP protein-trap allowed epitope-tagging of proteins. So finally, the protein-trap could tell the location of the proteins wanted.<sup>22</sup>

In the experimental part, we will be using this tool to identify what are the larvae that have the cells of our interest and which don't. So if we mark a genotype that we don't want with GFP, and we put the larva in the microscope to observe, and we see green color, we will not choose that larva. This is the case of our experiment, in which we will be using a balancer chromosome called CyO and will be marked with GFP, so if we see green color, it will mean that it has CyO.

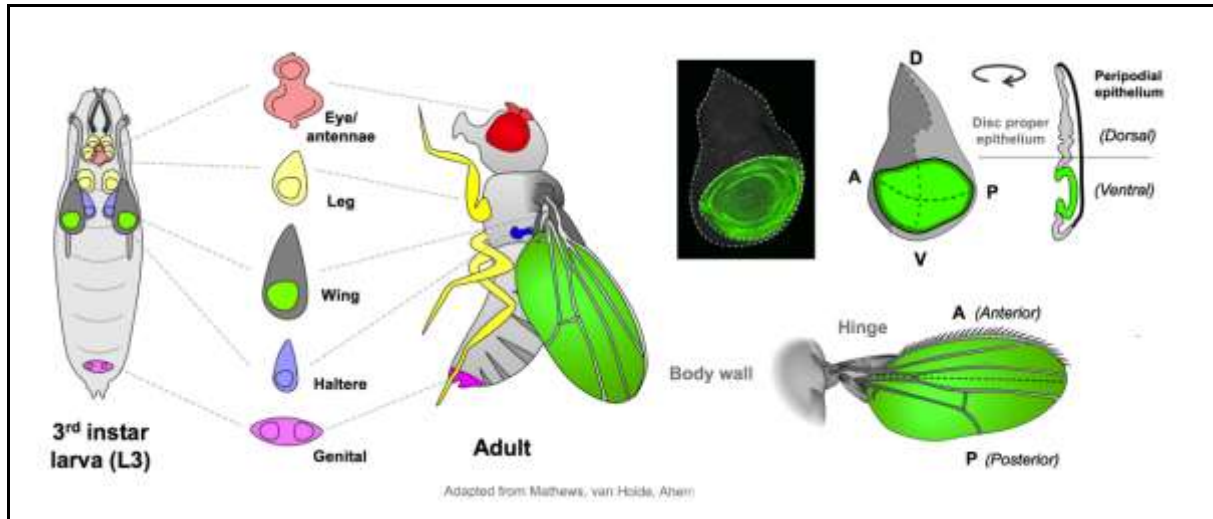
## 2h. Use of *Drosophila* imaginal disc as a model tissue

The *Drosophila* imaginal disc is an epithelial tissue that is located in the middle superior part of the larva.

The imaginal discs of *Drosophila* are used to study many things, for example the mechanisms of cell regeneration because of its high regenerative capacity, that is due to the big amount of mother cells that are in the larva, because a large, complex organ or part of the fly must emerge, so its cells must have great reproductive capacity to produce it.

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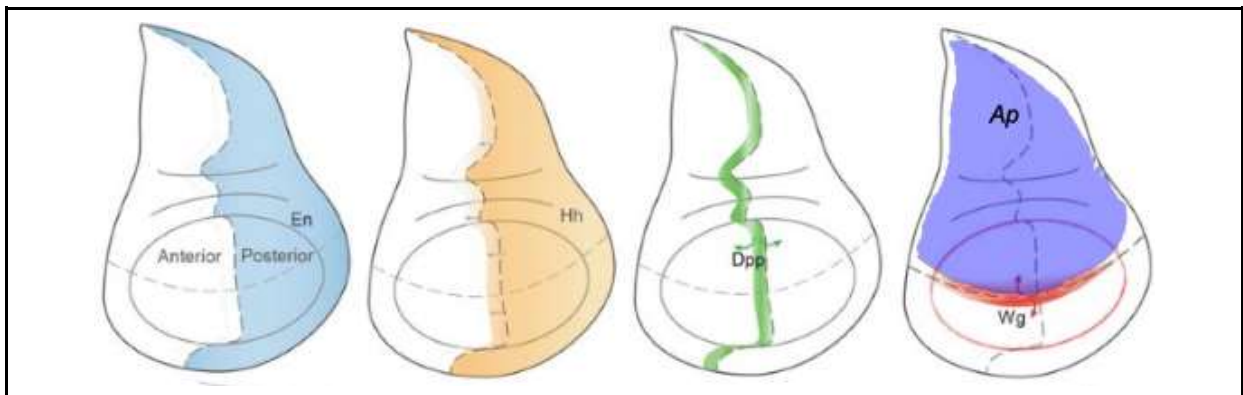
<sup>22</sup> Sineshchekova, O. O., Kawate, T., Vdovychenko, O. V., & Sato, N. (2004, February 2). Protein-trap version 2.1: screening for expressed proteins in mammalian cells based on their localizations. - BMC Molecular and Cell Biology. BMC Molecular and Cell Biology. Retrieved July 5, 2022, from <https://bmcmolcellbiol.biomedcentral.com/articles/10.1186/1471-2121-5-8#citeas>



**Fig. 11** As shown in the image, the *Drosophila* larva has many imaginal discs and the one that we are focused on is the wing disc (right), located in the middle top part of the larva, that produces the whole wing of the adult fly and it is divided in different segments (Dorsal/Ventral, Anterior/Posterior). Adapted<sup>23</sup>

Indeed, imaginal discs are a good model to study cancer, as it is a tissue where cells divide rapidly, like cancer would do and as many of these cells are mother cells, when cancer is studied there, many factors that are good for studying cancer, its spreading and division, imaginal disc is a great model tissue. In addition, it is relatively easy to get it and many things can be done in it.

These are used to study the response to some specific gene functions and when studying cancer, if it is applied to a specific gene to study it, this simple columnar epithelial tissue is a good model to study cancer stages and observe how it does develop. Furthermore, another reason why it is widely used is because of its ease in genetic manipulation.



**Fig. 12** A *Drosophila* wing imaginal disc. These images are showing some of the active genes: *Engrailed* (*En*) expressed in the posterior compartment which define the A/P boundary and inducing *Hedgehog* (*Hh*). *Hh* drives the expression of *Decapentaplegic* (*Dpp*). On the other hand, *Apterous* (*Ap*) is expressed in the dorsal compartment, define the D/V boundary and promotes the expression of *Wingless* (*Wg*) which as *Dpp* drives the development of the fly wing. Adapted<sup>24</sup>

<sup>23</sup> Research | The Harris Lab. Retrieved July 3, 2022, from <https://web.asu.edu/harris/research>

<sup>24</sup> Beira, J.V., Paro, R. *The legacy of Drosophila imaginal discs*. *Chromosoma* 125, 573–592 (2016). <https://doi.org/10.1007/s00412-016-0595-4>

In the practical part, we will be using some of these. What we will do is induce cancer in it, using the gene *apterous* (*Ap*). The tumor will be induced in the dorsal region, the top one, shown in the right-wing disc of **Fig. 12**. To perform the experiment, we will choose the larvae that contain the genes we want, we will dissect them, and we will keep the wing disc that we will dissect. After some process of preparation, we will be able to observe the wing discs under the microscope and extract the results.

### 3. Introduction to cell Biology

Cell biology is the field of biology that is concerned with the study of the development of the cells and its structure, behavior and function.

This field is of great importance in terms of the study of cancer, as cancer consists of an unusual behavior of a specific group of cells, it also studies the mechanisms that make the development and behavior of tumors.

Understanding the cell cycle is vital for understanding how cancer happens and what are the factors that produce it.

The cell cycle has some checkpoints and stages, to check if the development that has been done until that point is correct and if not, there are some mechanisms that help us to get rid of that cell.

This is the case of tumor suppressors, whose function is to stop the cell cycle and prevent the formation of malignant tumors.

One essential tumor suppressor is the tumor protein p53, that acts in the G1 checkpoint and blocks the cell cycle if the DNA of the cell is damaged, in order to save some possible problems and that cell doesn't continue.

If it happens to be DNA damaged, one thing that can be done is to repair this DNA by activating DNA repair enzymes. On condition that the damage is repaired, the p53 releases the cell, and it can continue the cell cycle, but if the damage is unfixable, p53 will activate the programmed cell death (apoptosis) thus the damage doesn't pass to others.

In cancer cells, the p53 is missing or defective, the cell might continue its cycle and produce cancer as it will start dividing uncontrollably and passing the damaged DNA to all of those cells.

This is the reason why understanding cell cycle and cell behavior is so important for cancer knowledge and study.

#### 3a. Cell cycle (Mitosis and meiosis)

The cell cycle is the period of time that goes from the birth of a cell until it divides and creates new cells. In this cell cycle, we can identify two phases, one called interphase and the other one called division.



In the interphase, we can differentiate three phases, the  $G_1$ , S and the  $G_2$ , although cells might enter another phase, which is called  $G_0$ , if inside those cells it manifests very specific genes and the cells can stay in  $G_0$  for long time. Also at the end of the  $G_1$  phase, mammal cells might enter a point called R, at this point cells cannot move forward phase S,  $G_2$  and the M phase.

During the  $G_1$  phase, the cell increases the volume of proteins and organelles.

In the S phase, the cell synthesizes completely a copy of the DNA and also duplicates the microtubule-organizing structure, which is the centrosome.

During the  $G_2$  phase, the cell continues to grow more, starts to make proteins and organelles and also recognizes the content for the mitosis.

The M phase comprehends two steps, the first one is the division of the nucleus, also known as mitosis, and the second step is the division of the cytoplasm.

The finality of the mitosis is that at the end of this process, there will be two cells with the exact same number of chromosomes as the cell where they came from.

Mitosis and cancer are very related for the fact that cancer cells tend to 'ignore' the checkpoints of mitosis, matter of fact, cancer can be also known as a disease from the mitosis.

Cancer cells never die, so they can add themselves as many telomeres as they want and won't stop duplicating.

The mitosis is regulated by several mitotic kinases that have been related with tumorigenesis. These kinases play a role during all the mitosis, regulating the DNA damage and the spindle assembly checkpoint before entering the mitosis, also matures and separates the kinetochore and the centrosome and even regulates the start and the end of mitosis.<sup>25</sup>

In the gonads, we have another type of cell division: the meiosis. Meiosis is the cell division exclusively for germ cells and instead of having the exact same number of chromosomes as in the mitosis, in meiosis the cells have the half of the cells they have come from. The meiosis comprehends two sequential divisions. In the first meiotic division, the cells will have half of the chromosomes of the mother cell and in the second meiotic division, the cells will have the same number of chromosomes as the mother. Before the first meiotic division, as in the mitotic cycle, there is a short period called interphase in which DNA duplicates, but in the second meiotic division there is not an interphase.

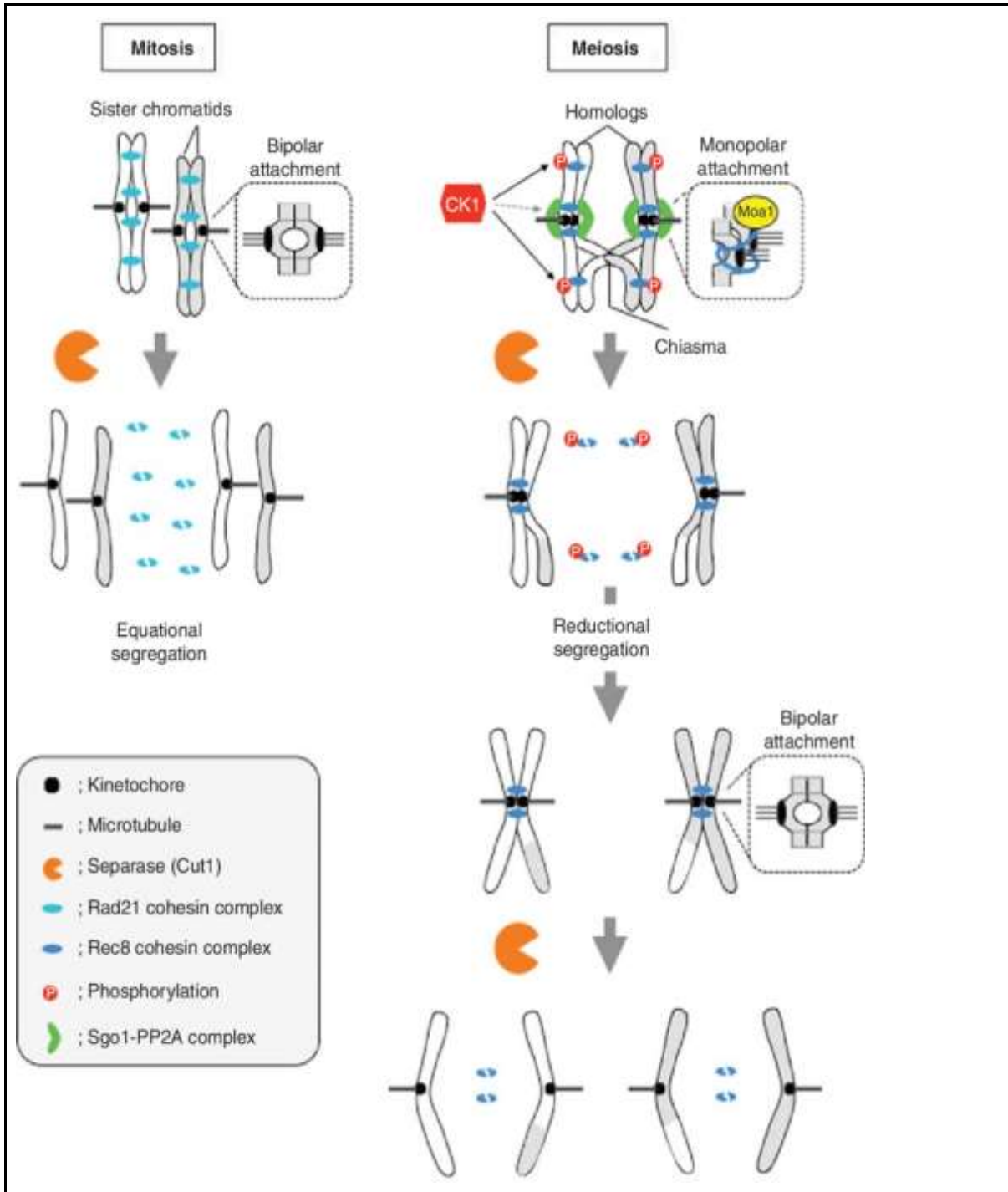
The meiosis is regulated by cyclin-dependent kinase (CDK) and its subunits. The CDK regulates meiosis-specific events.<sup>26</sup>

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<sup>25</sup>Ahmad, N. (n.d.). Regulation of mitosis via mitotic kinases: new opportunities for cancer management. PubMed. Retrieved July 7, 2022, from <https://pubmed.ncbi.nlm.nih.gov/17620424/>

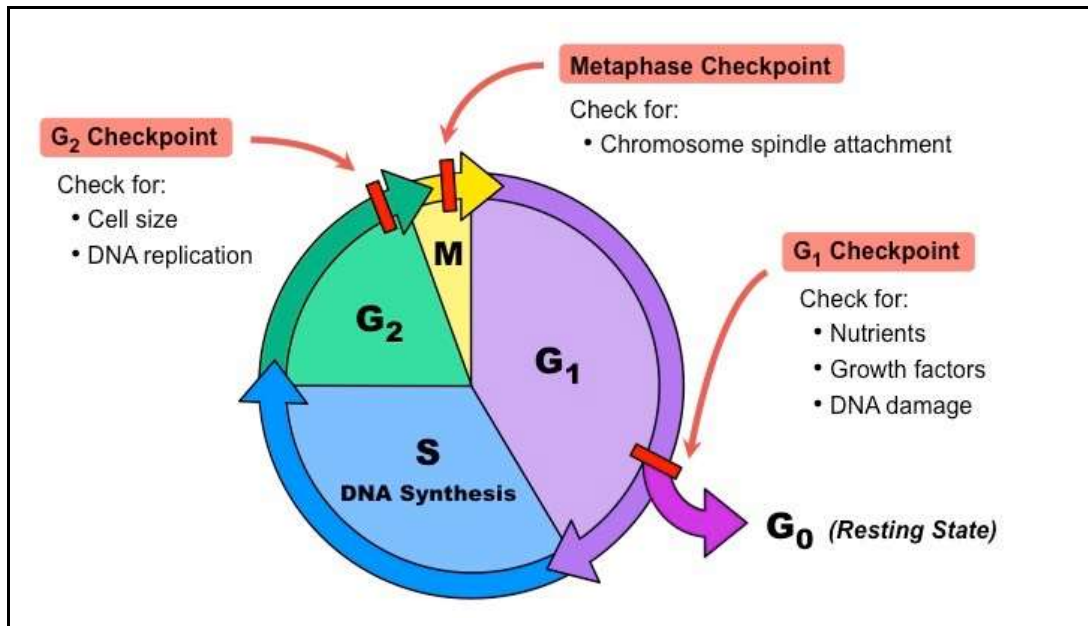
<sup>26</sup> MacKenzie, A. M., & Lacefield, S. (n.d.). CDK Regulation of Meiosis: Lessons from *S. cerevisiae* and *S. pombe*. MDPI. Retrieved July 7, 2022, from <https://www.mdpi.com/2073-4425/11/7/723/htm>





**Fig. 13** In this image, we can observe the chromosome segregation during mitosis and meiosis. On the left, what happens during mitosis is that, sister kinetochores are attached to the microtubules emanating from opposite poles. On the right, what happens in the meiosis I, is that the homologous chromosomes are recombined, and sister kinetochores attach to the microtubules emanating from the same spindle pole. During meiosis II, sister chromatids separate as in the mitosis.<sup>27</sup>

<sup>27</sup> Watanabe, Y. (n.d.). Chromosome segregation during mitosis and meiosis. (Left) During... | Download Scientific Diagram. ResearchGate. Retrieved July 7, 2022, from [https://www.researchgate.net/figure/Chromosome-segregation-during-mitosis-and-meiosis-Left-During-mitosis-sister\\_fig2\\_318652883](https://www.researchgate.net/figure/Chromosome-segregation-during-mitosis-and-meiosis-Left-During-mitosis-sister_fig2_318652883)



**Fig. 14** This is a graphic that shows the cell cycle, with its phases and the checkpoints that are in there to regulate that everything is correct, and the cell cycle can continue.<sup>28</sup>

The checkpoints are mechanisms that monitor the order of the events in the cell cycle. Checkpoints are important because they make sure that the daughter cells don't have any genetic error. As seen in **Fig. 14** there are 3 main checkpoints, the G<sub>1</sub> checkpoint checks for growth factors, nutrients and DNA damage and if there is damage the cell goes to the G<sub>0</sub> where a stop of the cycle occurs, and if there is no damage the cell goes to the next checkpoint. The second checkpoint is the G<sub>2</sub> in this checkpoint, it makes sure that the cell size is the correct one and that the DNA replication is alright. Finally, if everything is right, it goes to the final stage, the M, where the checkpoint looks for the chromosome spindle attachment. If there are no problems, the cell can initiate the division.

### 3b. Fly Fucci, cancerous cells difference in cell cycle stages and its contribution to microenvironment heterogeneity

Fucci (fluorescent ubiquitination-based cell cycle indicator) is a system that enables the scientist to see the cells in the different phases of the cell cycle progression *in vivo* (cells alive). It allows distinguishing and identifying when a cell is in each stage of the cell cycle.

In addition, the cells that are in the G<sub>1</sub> stage are marked in green fluorescence, whereas cells in stage S are red and G<sub>2</sub> or M are seen in yellow.

<sup>28</sup> "Cell Checkpoints. (n.d.). BioNinja. Retrieved July 6, 2022, from <https://ib.bioninja.com.au/standard-level/topic-1-cell-biology/16-cell-division/cell-checkpoints.html> .

While working with FUCCI, GAL4/UAS systems or QF/QUAS systems are needed, in order to make it easier to identify or follow the progression of some specific cells by marking them thanks to these systems.<sup>29</sup>

It is really useful as it can show how cells are in the different stages of the cell cycle and how cancer cells distinguish from each other depending on which point of the cycle they are. Which could help to understand the behavior and how the cancer cells are.

The cancerous cells do pass the cell cycle because of mistakes, furthermore, they do the cell cycle indefinitely, as the cancer cells do mitosis. In cancer cells, mitosis happens infinitely, this is why they spread so rapidly and, as the cell cycle has made mistakes, they divide uncontrollably and their DNA might be really different from each other's because of those mistakes.

Cancer cells utilize the telomerase (an enzyme that is involved in DNA replication) to add telomeric sections to the DNA during replication, causing the division of the cells instead of its death.

Tumor microenvironment is the surrounding tissue, space, and cells. These are continually changing due to the conversion and replacement of its cells and extracellular matrix.

Heterogeneity is the consequence of all these changes and distinguishes between all the surrounding cells and hallmarks, as a consequence, the cancer cells are different from each other. This is the reason why cancer is so difficult to treat.

Epigenetic changes that occur in the cells, the release of extracellular signals and tumor angiogenesis, beyond others, is how cancer cells affect the tumor microenvironment and boost its heterogeneity.

In the practical part, we will be using Fly FUCCI to study the stages of the cell cycle on cancer cells.

### 3c. Chromosomal Instability

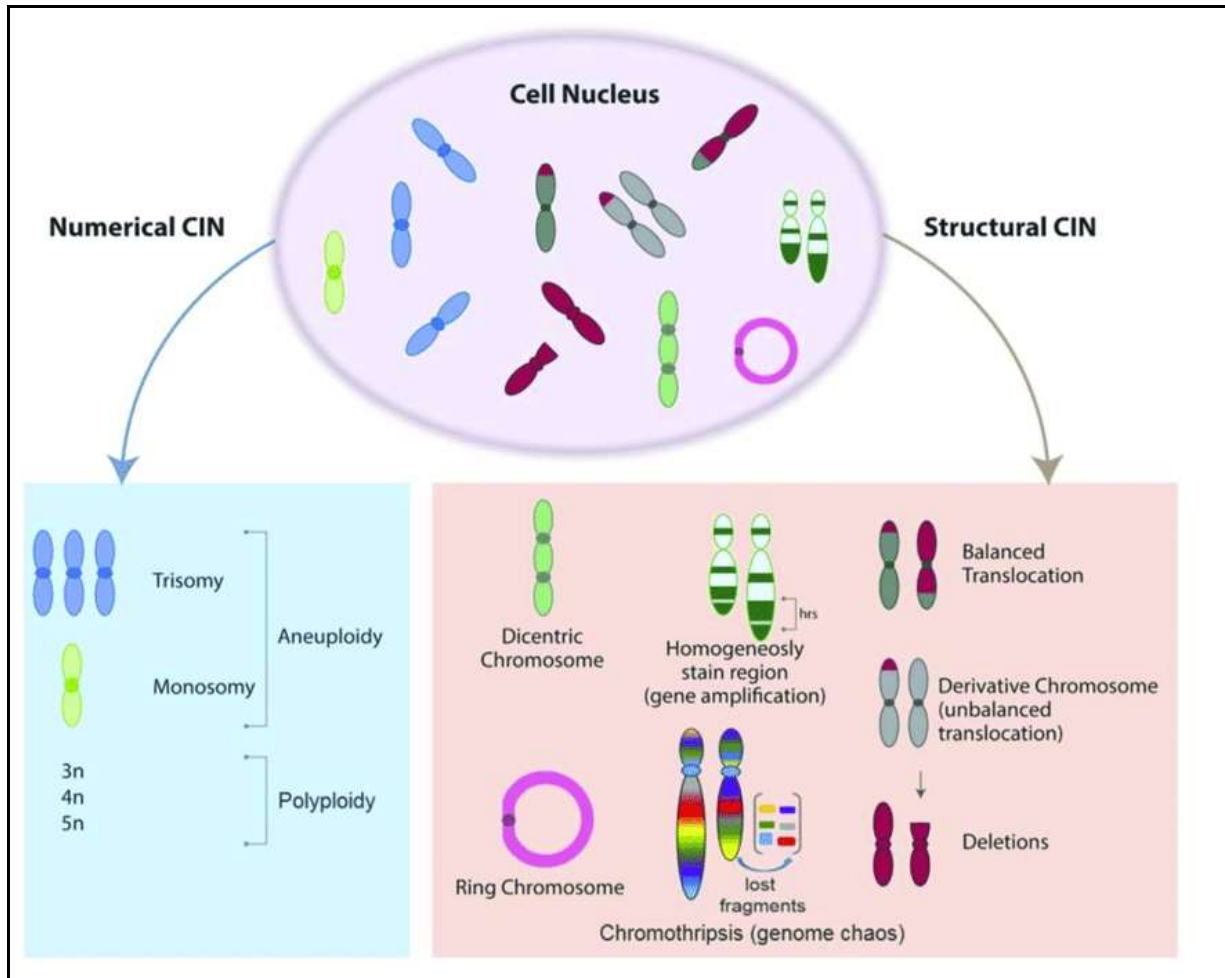
Chromosomal instability (CIN) is a hallmark of human neoplasms, and it has the ability to make tumors adapt and evolve. It is known that CIN has a very important role in cancer initiation, growth, metastasis and, at the end, tumor immunity and resistance to therapies. CIN causes aneuploidy, this is an altered number of chromosomes in the cell nucleus (numeric aneuploidy), or chromosomes with a defective or altered

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<sup>29</sup> Zielke N, Korzelius J, van Straaten M, Bender K, Schuhknecht GFP, Dutta D, Xiang J, Edgar BA. Fly-FUCCI: A versatile tool for studying cell proliferation in complex tissues. *Cell Rep.* 2014 Apr 24;7(2):588-598. <https://doi.org/10.1016/j.celrep.2014.03.020>

## 3. Introduction to cell Biology

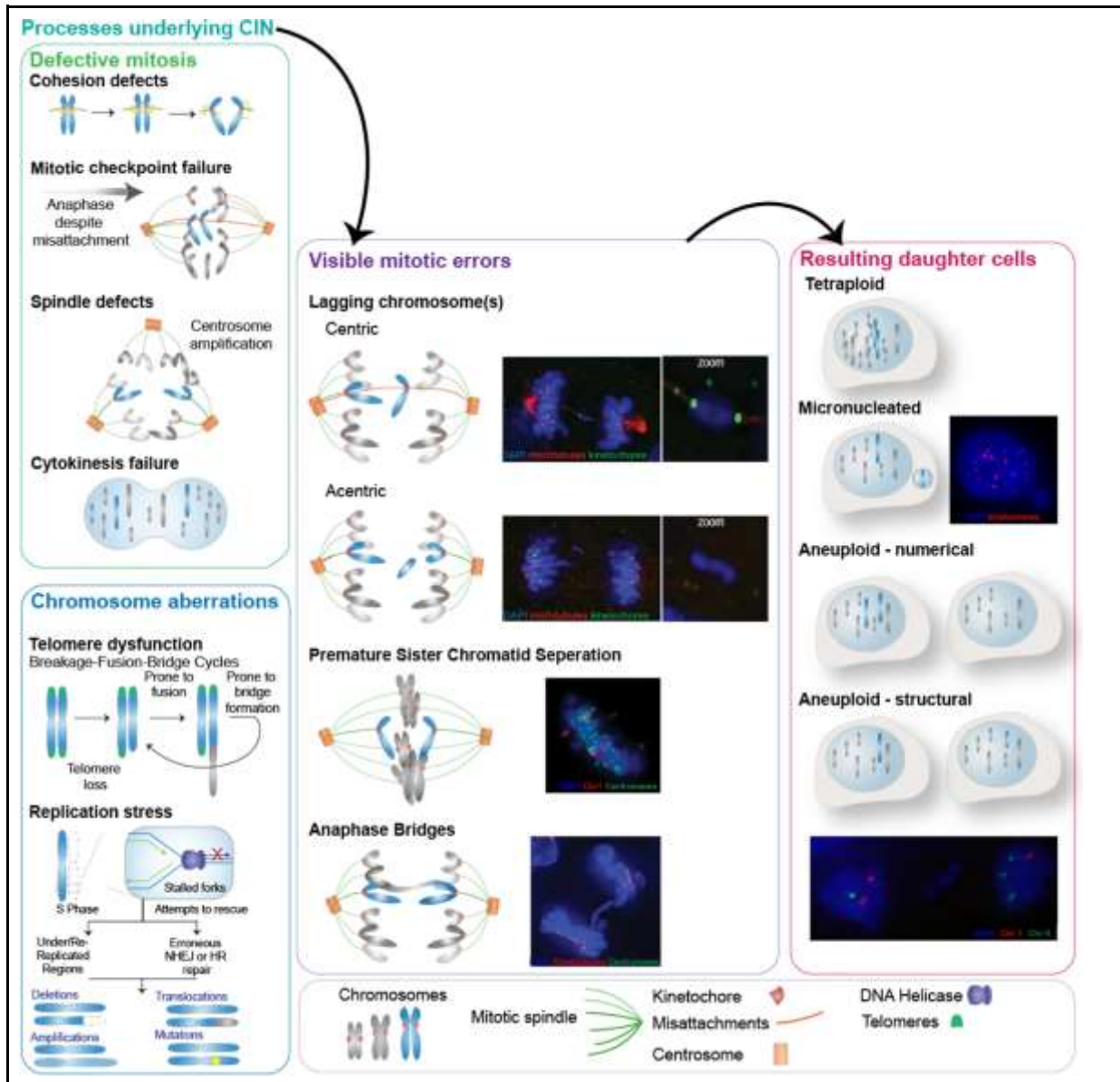
structure (structural aneuploidy). Chromosomal instability is the increased rate of acquisition of chromosomal abnormalities through mis-segregation of chromosomes and structural rearrangements such as translocation, deletions and inversions.



**Fig. 15** Types of chromosomal instability. There are two types of chromosomal instability, the first one is the structural, in this type of CIN, what happens is that the chromosomes suffer changes in their structure, for instance, instead of having one centromere they have two centromeres. And the other one is the numerical, in the numerical CIN what happens is that there are changes in the number of chromosomes, for example, instead of having two chromosomes, the cell has three or one.<sup>30</sup>

There has also been found that mitosis and CIN have a stretch relationship, for the fact that errors in the mitosis are the most common cause of chromosomal instability, matter of fact 90% of human tumors appear by errors in the mitosis, some of these errors can be: malfunctioning of SAC (spindle assembly checkpoint), bad cohesion between sister chromatids, incorrect timing for the chromosomes to separate, supernumerary centrosomes, defects in kinetochore-microtubule attachments. All of them due to dysregulation of proteins such as JNK and JAK.

<sup>30</sup> Vargas-Rondón, N., Villegas, V. E., & Rondón-Lagos, M. (2010). The role of chromosomal instability in cancer and therapeutic responses. *Cancers*, 10.(1), 4.



**Fig. 16** These are the processes that underlay CIN, for example, defects in the mitosis that encompasses cohesion defects, failure in the cytokinesis, this produces that during the stages of mitosis happen visible errors, such as bridges in the anaphase, separation of the chromatids before is the time, and this produces that the daughter cells suffer from numerical CIN. Also, CIN can underlie chromosomal aberrations which produce structural mutation, for instance, amplifications, deletions, translocation or different mutations.<sup>31</sup>

*Malfunctioning of the spindle assembly checkpoint (SAC):* Impairment of the SAC enables that the anaphase starts earlier and increases the possibilities of causing chromosome mis-segregation.

<sup>31</sup> Tijhuis, A.E., Johnson, S.C. & McClelland, S.E. The emerging links between chromosomal instability (CIN), metastasis, inflammation and tumour immunity. *Mol Cytogenet* 12, 17 (2019).



*Bad cohesion:* High level of the protein separases can induce sister chromatids disjunction, which could lead to a chromosome mis-segregation.

*Supernumerary centrosomes:* Extra centrosomes are produced by a deregulation of the centrosome duplication cycle or by cells that have become tetraploid.

*Defects in kinetochore-microtubule attachments:* These defects appear when the microtubules are released from its kinetochores, the attachment of the microtubules to their respective kinetochore is essential for the microtubules to remain to the spindle microtubules.<sup>32</sup>

In *Drosophila*, the malfunctioning of the SAC can cause segregation and aneuploidy. The wing imaginal disc in *Drosophila* is a structure that throughout the years has been used as a model for research on tumorigenesis, RNAi retrenchment of genes involved in the SAC produces CIN and aneuploidy in the imaginal disc. Too many aneuploidies cause cell deamination and apoptosis. In flies like *Drosophila*, the response to CIN is p53 independent and relies on the activation of JNK cascade, but in mammals the response is p53 dependent.<sup>33</sup>

### 3d. Cellular signaling

Cells typically communicate between them by means of proteins and other molecules which both are secreted to the extracellular space or through exosomes, which transport DNA, miRNAs, lipids, headstock proteins, transcription factors... which can induce distant cell's transformation. Another possible way of communication is by gap junctions, cell adhesion and tunnel nanotubes. Therefore, the substances can travel to other close cells.

All these types of cell signalings makes the cells respond to the microenvironment molecules and led the tumor cells to communicate between them and adapt through switching genes.

These proteins might be c-Jun N-terminal kinase (JNK) and cytokine-activated Janus kinase (JAK), JNK has been related to the mitogen activated protein kinase (MAPK). JNK regulates important cellular activities including cell proliferation, differentiation and apoptosis. JNK promotes tumor development in many human cancers, it is activated by environmental stress, for instance, heat or DNA damage. JNK involve receptors-independent stress-induced membrane changes that will be transmitted to the adaptor

<sup>32</sup> Memoli, V. (n.d.). *Mechanisms of Chromosomal Instability - PMC*. NCBI. Retrieved July 7, 2022, from <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3781365/>.

<sup>33</sup> Gerlach, S. U., & Herranz, H. (2020, June 3). *Genomic instability and cancer: lessons from Drosophila*. <https://royalsocietypublishing.org/doi/10.1098/rsob.200060>

proteins, which can activate the MAP4K and rarely the MAP3K for the JNK cascade, the next thing that happens is that the MAP3K transmit signals to the mitogen activated protein kinase (MAPKK), the most important are the MKK4 and the MKK7, as the main MAPKK the main JNK kinases are activated and then transmit the signals to another JNK level.<sup>34</sup>

The MMP1 is a readout of the JNK, that means that when the cell receives a signal from JNK that enters inside the cell, the MMP1 goes outside as a response. If we want to see how it is expressed, we can see it with an antibody attached to it. It happens the same with the TRE that is also a readout of JNK and to see it expressed we need to use RFP (red fluorescence protein).

JNK, reacts against hypoxia, lack or low amount of nutrients and ROS (Reactive Oxygen Species) are stressful conditions that, in JNK, can cause tumorigenesis or treatment resistance. In addition, JNK can promote components of the tumor microenvironment (TME), such as angiogenesis, inflammation, cancer stem cells (CSC), tumor stroma, extracellular matrix (ECM) and immune response. JNK can be activated in many ways, because of receptors such as hormones, inflammatory cytokines, oxidative stress, DNA damage or UV radiations. These, activate JNK via individual specific MAP3Ks, which, in turn, through MKKs (MKK7, MKK4) regulate JNK 1/2/3 allowing different targets for tumorigenesis occurrence like inflammation, migration, apoptosis, beyond others.<sup>35</sup>

For instance, in *Drosophila*, there is only a JNK protein, which is expressed by a gene basket that leads to the phosphorylation of the Jun and Fos factors. Furthermore, in the imaginal disc, JNK is not expressed until the tissue has been damaged or irradiated, it is also known that under the factors mentioned above, JNK triggers apoptosis, but it also has a very important role in tumorous cell proliferation.

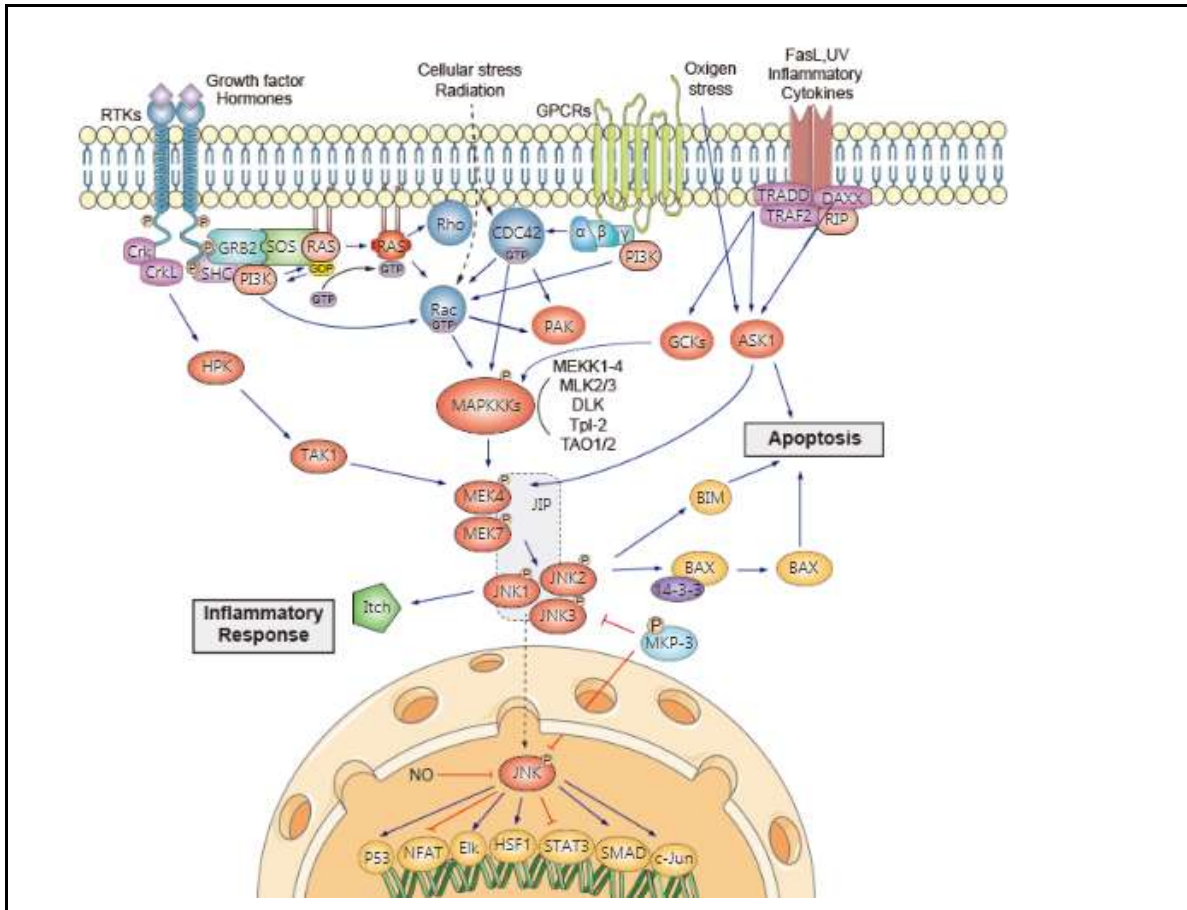
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<sup>34</sup> JNK Signaling Pathway. (n.d.). Creative Diagnostics. Retrieved July 7, 2022, from <https://www.creative-diagnostics.com/JNK-Signaling-Pathway.htm>

<sup>35</sup> Tam SY, Law HK. JNK in Tumor Microenvironment: Present Findings and Challenges in Clinical Translation. *Cancers (Basel)*. 2021 May 3;13(9):2196. doi: 10.3390/cancers13092196. PMID: 34063627; PMCID: PMC8124407.



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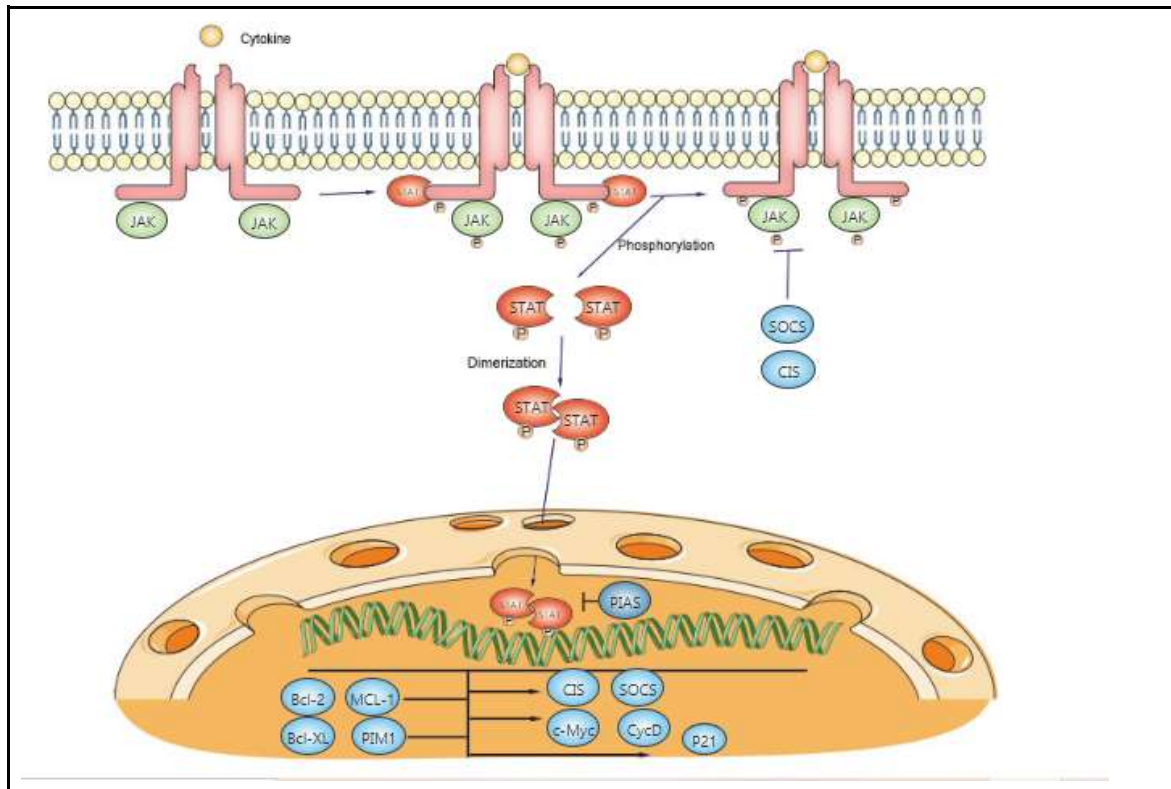
**Fig. 17** In this image we can see the JNK cascade and all of his components, we can also see how it progresses and all the steps it takes in order to complete it.<sup>36</sup>

The JAK-STAT, that is a way of cell signalling, has an important role in the control of immune response, dysregulation of the JAK-STAT process is related to immune disorders. This kinase transmits information from extracellular chemical signs to the nucleus. Most cytokines and growth factors transmit their signals through the JAK-STAT pathway, in most cases ligand bindings appear to promote the membrane receptors, bringing closely their related JAK, then the JAKs phosphorylate and activate each other, meaning that the STATs will be activated by the JAKs. Then the STATs move to the nucleus and regulate the expression of cytokine-sensitive genes. In conclusion, the JAK-STAT cascade provides mechanisms to translate the extracellular signs into a transcriptional response.<sup>37</sup>

<sup>36</sup> *JNK Signaling Pathway*. (n.d.). Creative Diagnostics. Retrieved July 7, 2022, from <https://www.creative-diagnostics.com/JNK-Signaling-Pathway.htm>

<sup>37</sup> *JAK-STAT Signaling Pathway*. (n.d.). Creative Diagnostics. Retrieved July 7, 2022, from <https://www.creative-diagnostics.com/jak-stat-signaling-pathway.htm>

As in the JNK signaling, the JAK-STAT signaling also has a readout, in this case it is called upd3 and in order to see it through the microscope it is used lacZ, which is a protein used in fluorescence microscopy to give color where the cells express the gene.



**Fig. 18** In this picture we can see the JAK-STAT cascade and all the components it takes to do it and complete it.<sup>38</sup>

P53 is a tumor suppressor gene that plays a role in the development and cellular growth, acting as a protector of the germline, as a tumor suppressor, p53 protects the cell from transformations and tumorigenesis by activating the transcriptional expression of certain genes that have the ability to induce cell growth arrests, apoptosis. It is also known that p53 can act as a transcriptional repressor and surviving gene which can regulate proliferation and angiogenesis pathways. Moreover, p53 also acts as a general regulator of cellular signaling pathways, and the MDM2, COP1, and ARF-BP1 proteins can join to the p53 and act as ubiquitin ligases resulting in its degradation, but the MDM2 protein is the worst regulator when is joined to the p53, for the fact that inhibits its biochemical activities.<sup>39</sup>

<sup>38</sup> JAK-STAT Signaling Pathway. (n.d.). Creative Diagnostics. Retrieved July 7, 2022, from <https://www.creative-diagnostics.com/jak-stat-signaling-pathway.htm>

<sup>39</sup> Zhang, R. (n.d.). The MDM2-p53 pathway revisited - PMC. NCBI. Retrieved July 7, 2022, from <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3721034/>

### 3e. Immunofluorescence: a method for visualizing proteins in cells

Immunofluorescence is a technique that uses fluorophores and is used to visualize antigens and proteins in cells, it can also be used to visualize the exact place where various components of the cell are. Fluorophore compounds emit light when they are exposed to certain wavelengths of light, this is essential for immunofluorescence. In order to detect expressions from proteins, the sample must be incubated with a specific antibody. There are two different ways of immunofluorescence, the direct immunofluorescence, that consists of a fluorophore attached to the antibody, which allows its detection. The other way is the indirect immunofluorescence, which involves the detection of the first antibody with a secondary antibody covalently coupled with the fluorophore, this method is more precise. The proteins or antigens can be visualized by a fluorescence microscope or a confocal microscope, depending on the biological question and the purpose of the observation.

Direct immunofluorescence involves the use of antibodies that have been related to the fluorophores. The sample is incubated with an antibody that has been marked, and then there is another antibody that is not marked, this last antibody must be removed in order to examine the sample.

In the indirect immunofluorescence, the sample is incubated with a non-marked antibody, which will be washed and will be incubated with a fluorophore-marked anti-immunoglobulin antibody, in that way the second antibody will reveal the presence of the first one. One advantage of using indirect immunofluorescence is that one fluorescent anti-immunoglobulin will be usable for many first antibodies.

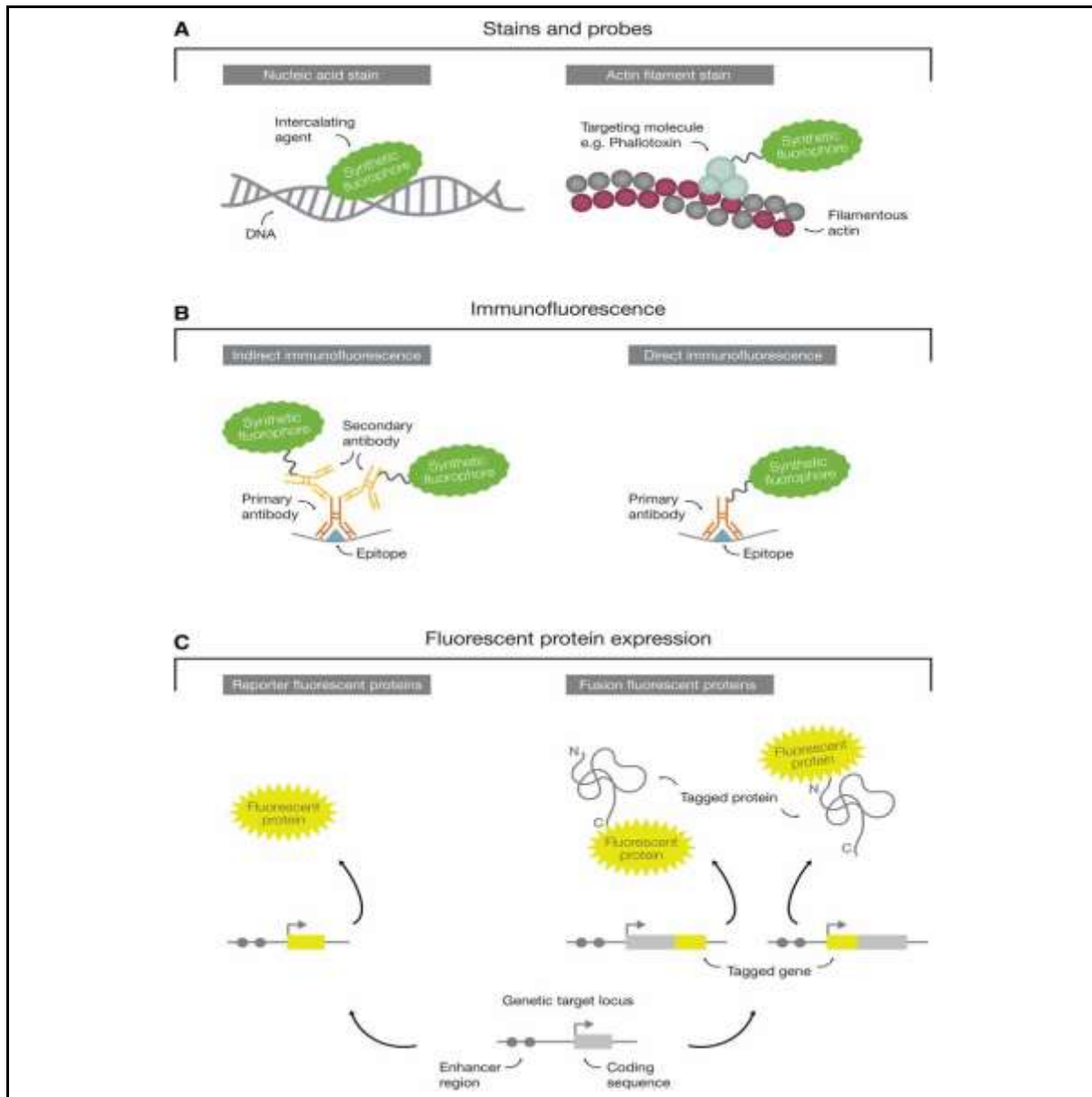
Also, the indirect way gives brighter fluorescence rather than the direct way, for the fact that the secondary antibodies may tie the first antibody, but one important disadvantage is that the anti-immunoglobulin, cannot differentiate between endogenous and exogenous immunoglobulin. For instance, the secondary antibody of a mouse anti-rabbit immunoglobulin would detect membrane immunoglobulin rabbit on its cells even if there are any other antibodies. The suppression of the first antibody is a very useful control to check if there are non-specific binding secondary antibodies via Fc receptors. Fc receptors are proteins located in the membrane of some cells which help a more precise observation of it.<sup>40</sup>

For our experiment, we will use fluorescent markers to identify different type of cells in the larva, more specifically the wing disc, for instance we will use a fluorescent marker called DAPI which is shown as blue to identify the nucleus of the cells, another fluorescence tool we will be using is GFP (green fluorescence protein) in this case we

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<sup>40</sup> F. Y. L., & Jalali, M. (Eds.). (2017). *Basic Science Methods for Clinical Researchers*. Elsevier ScienceJalali, M., Saldanha.

will use it to identify the GC3Ai that is a sensor of the caspases. Moreover, we will also use myrTom or RFP (red fluorescence protein) as an identifier of the apterous. To identify the upd3, we will make use of an antibody called lacZ that is expressed as white, that means that if any cell had a white fluorescent tag it would mean that it has the upd3 gene and this means that that cell would have JAK.



**Fig. 19** The first type of fluorescence labeling technique is the fluorescence stain and probes that interact directly with the components of the cell and also can be fused to target a molecule. The second method is called immunofluorescence, this method consists of synthetic fluorophores that attaches to specific antigens that target a protein. And the last method is the fluorescent protein expression that consists of endogenous expressions that provide a genetically encoded avenue in order to visualize the components of the cells.<sup>41</sup>

<sup>41</sup> Dunst, S., & Tomancak, P. (2019). Imaging flies by fluorescence microscopy: Principles, technologies, and applications. *Genetics*, 211(1), 15-34.

## 4. Advanced microscopy and analysis

Microscopy is a key element in the laboratory and experimental work, as it is sometimes very necessary to observe the results of an experiment and allows obtaining results that the naked eye of the human could not obtain itself.

As it is so necessary, for a long time scientists have been working on improving microscopes and having a better resolution of what is being observed, and as a result, developing many innovative techniques that allow them to observe with good quality what they want.

Fluorescence microscopy, used in combination with labeling methods like fluorescent antibodies or fluorescent proteins, let us recognize selected components of living matter. This is thanks to the discovery of the green fluorescent protein that was found in a jellyfish by Osamu Shimomura. Later, sequencing and cloning of it led to the visualization of some specific cellular components.

In addition, this technique not only allows to visualize and mark specific cellular features, but this one also can be used in another type of advanced microscopy, that is the confocal microscopy, that not only allows to detect the fluorescence but also has better resolution and one of its characteristics is that it allows to see the different layers of the sample giving a 3D image.

### 4a. Fluorescence Microscope

The fluorescence microscope is an optical microscope that uses fluorescence as a replacement for reflection and absorption of light. It is used for the fact that most of the components of a cell are colorless, and we cannot distinguish it with a normal microscope, but with this microscope we must dye the sample in order to observe it. The dye that is used with the fluorescence microscope is called fluorophores, fluorophores are molecules that can absorb the excitation light at a certain wavelength (mostly UV), and then emit the light at a major wavelength.

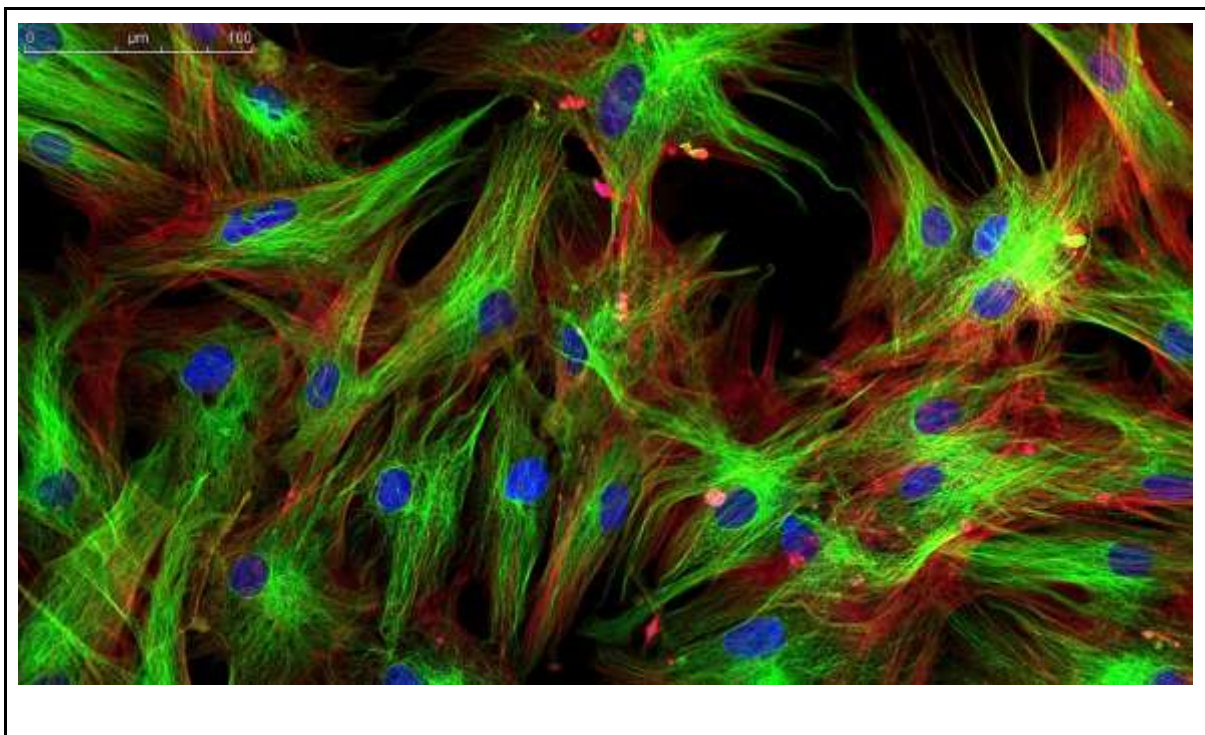
Its working is quite simple, as mentioned above the sample is dyed with fluorophore, and it is illuminated through the lens with a major energy source. The light is absorbed by the fluorophores and makes them to reproduce a longer or lower energy wavelength, also the fluorescence light can be separated from the surroundings, enabling us to only see the part that is fluorescing.



The basic function of it is to let light radiate to the sample, so later the microscope can classify the weakest emitted light from the image.

The microscope has a filter which can only pass-through radiation with a certain wavelength that tone with the fluorescing material, then the radiation impacts against the atoms in the sample and the electrons are exposed to a higher energy level, after this happens the electrons relax, and they emit light. In order to be observable through the human eye, the fluorescence emitted from the samples is separated from the brighter light, which goes to a filter.

Most of the fluorescence microscopes used in research are epi-fluorescent, that means that the excitation and the observation of the fluorescence happens above the sample, and for the brighter light it is usually used Xenon or Mercury arc-discharge.<sup>42</sup>



**Fig. 20** This is a picture of skin cells made with a fluorescence microscope.<sup>43</sup>

#### 4b. Confocal microscope

While most of the traditional fluorescence microscopes provide us the localization of compounds, the images might be blurry, for the fact that the emitted light which is

<sup>42</sup> "Rice, G. (2021, January 15). *Fluorescent Microscopy*. SERC - Carleton. Retrieved July 7, 2022, from [https://serc.carleton.edu/microbelife/research\\_methods/microscopy/fluomic.html](https://serc.carleton.edu/microbelife/research_methods/microscopy/fluomic.html).

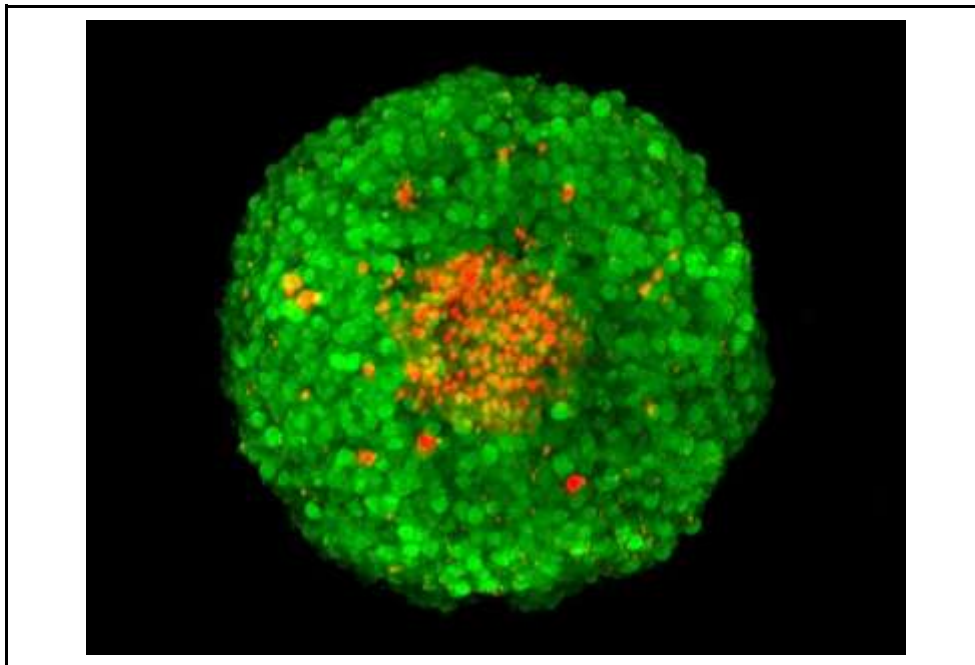
<sup>43</sup> Setting up and Adjusting a Fluorescent Microscope for Reliable Examination Results. (n.d.). Physik Instrumente. Retrieved July 7, 2022, from <https://www.physikinstrumente.com/en/knowledge-center/blog/setting-up-and-adjusting-a-fluorescent-microscope-for-reliable-examination-results/>

## 4. Advanced microscopy and analysis

collected to originate an image, comes from an optical plane within the sample. But the confocal microscope uses light from the laser through the objective, and any emission that is out-of-focus plane will be rejected by the confocal aperture.

This type of microscope is usually used to observe very detailed structures of cells, like the fluorescence microscope, cell structures can be ordered using immunofluorescence. But unlike the fluorescence microscope, this type of microscope enables us to take an image of sharp and clear images without any fluorescent light from the background, only using the fluorophores that must be in the sample, in order to see and distinguish a specific part of the sample. Moreover, we can analyze 3D structures by stacking up images from different angles.

The principle of the confocal microscope is similar to the widefield microscope, but the confocal one uses fluorescence that is included in the sample. Laser lights are focused on a defined spot at a determined depth of the sample, this will lead to the emission of fluorescent light to the point. As mentioned earlier, the confocal aperture rejects all the signals that are out of focus, making it possible to allow only the fluorescence light to enter the light detector.<sup>44</sup>



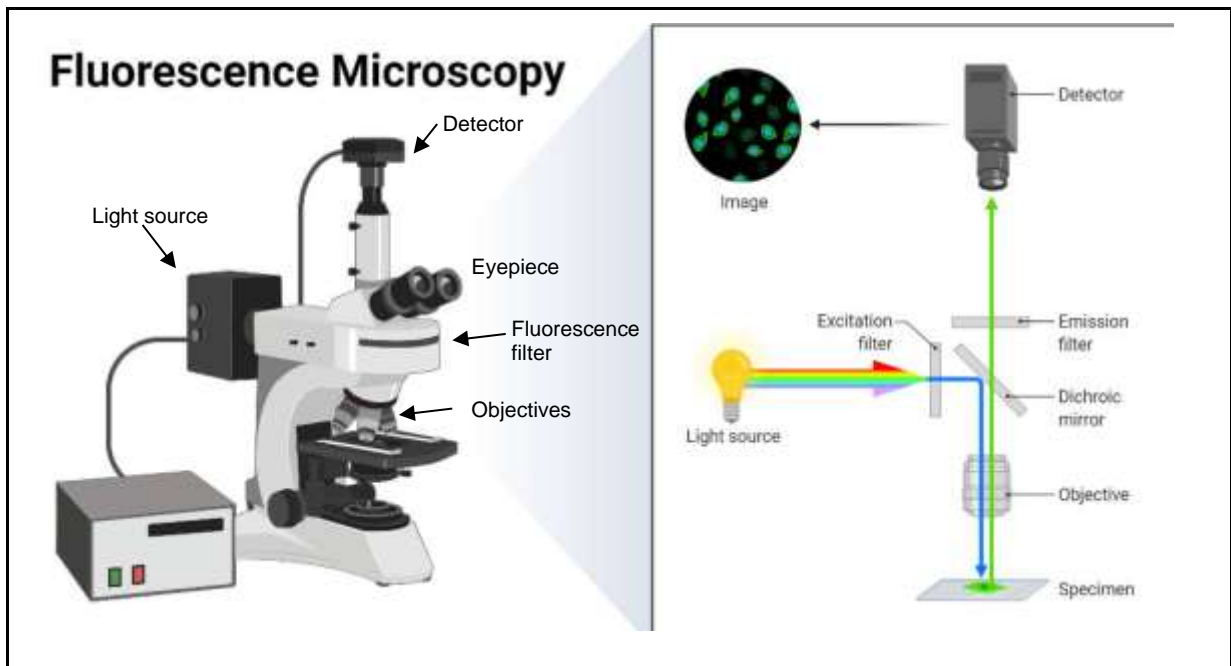
**Fig. 21** This is an image of an FDA/PI stained MCF-7 steroid, taken with a confocal microscope, unlike the fluorescence microscope, with the confocal microscope we can have a 3D model of the sample by stacking images.<sup>28</sup>

<sup>44</sup> Confocal Microscopy | Principle & Applications | ibidi. (n.d.). IbiDi. Retrieved July 7, 2022, from <https://ibidi.com/content/216-confocal-microscopy>



#### 4c. Image analysis and processing

For the analysis of the images, the fluorescence microscope has the components shown in the **Fig. 22**.



**Fig. 22** This type of microscope produces fluorescence. The main exterior pieces are: light source, detector, eyepiece, fluorescence filter and the objectives. And in the interior, there are different filters, such as the excitation filter and the emission filter in addition to the dichroic mirror that makes it produce those fluorescence images. <sup>45</sup>

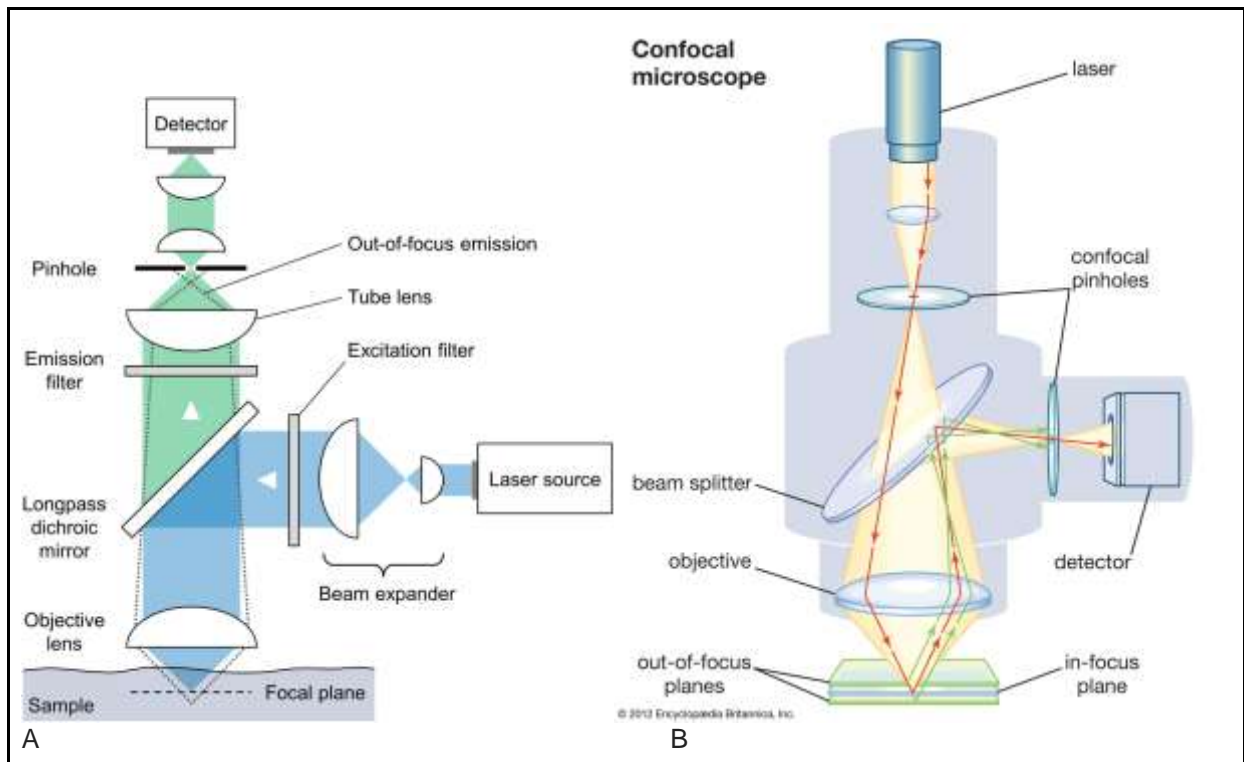
The principle of this microscope is that, in addition to the dye that is put to the sample, is to absorb the light that is reflected to its mirrors, the excitation of the light reveals the location of the fluorophores that are in the sample emitting longer light wavelengths and permitting to see the sample.

The light hits the different mirrors and passes through the filters, it is focused on the sample through the objectives, and the fluorescence emitted by the specimen is detected, to then be able to generate an image, such as the **Fig. 21**.

Unlike the fluorescence microscope, the confocal microscope only exposes a certain point of the sample to light, therefore only a piece is seen, but in the fluorescence microscope the entire specimen is affected evenly for light, resulting in a broader but not as accurate sample image. Also, the confocal microscope can scan different layers of the specimen at once and give the image of all the layers, but, as said before, it only focuses the light in a very specific point.

<sup>45</sup>Aryal, S. (2022, April 12). *Fluorescence Microscopy- Definition, Principle, Parts, Uses. Microbe Notes*. Retrieved July 7, 2022, from <https://microbenotes.com/fluorescence-microscope-principle-instrumentation-applications-advantages-limitations/>.

The confocal microscope structure is the following one:



**Fig. 23** These two images show two different types of confocal microscopes, on both there is a laser, detector, pinholes, beam splitter or mirrors, objective lens and focus/focal plane which is the piece, that helps the microscope take an image of the different layers, even though the laser and the whole set in general makes it possible. These two are a bit different in practice, there is almost no difference in the images taken, the only thing that makes it different is its internal construction. A<sup>46</sup>, B<sup>47</sup>

The confocal microscope uses the light from a laser that goes through the objective in order to excite the sample, there is a pinhole that rejects the emission of light that is out of focus, allowing only the light that is focused to go through that same pinhole. The principle of the confocal microscope is that instead of illuminating the whole sample, the laser light only illuminates a defined spot at a specific depth of the sample, due to this, we can see the different layers of the sample at once. A pinhole inside the optical pathways eliminates the parts of the sample that are unfocused, this allows only the fluorescence light that is from the focused part of the sample, to go through the light detector. Another feature is that, unlike the fluorescence microscope, with the

<sup>46</sup> Soliman, D. (2016, October). Augmented microscopy: Development and application of high-resolution optoacoustic and multimodal imaging techniques for label-free biological observation. DOI: 10.13140/RG.2.2.24410.03525

<sup>47</sup> Encyclopædia Britannica, Inc. Confocal microscope. Retrieved July 7, 2022, from <https://www.britannica.com/biography/Eric-Betzig#media/1/380582/136152> .

confocal microscope, if we scan the sample multiple times, using a software, we will actually be able to see the image in 3D.

In our case, we will use a software called FIJI, that will let us count all the migrating cells and classify them.

Thanks to the software, we will be able to observe all the layers from the wing disc image and eventually, identify cells that in a layer will not be shown but in another layer will be shown. We will also be able to modify the brightness, color saturation, so we can see the cells better. The different fluorescence colors will be shown in the specific cells that contain the markers.

## PRACTICAL FRAME

### Experiment

The aim of our experiment is to know how different cancer cells that arise from the same primary tumor diversify, also in which phase of the cell cycle are cancer cells most likely to be in, by means of a genetic tool named Fly FUCCI. We will be studying this by producing Chromosomal instability induced tumors in *Drosophila melanogaster* specimens.

*-Experiment 1:* JNK and JAK (JNK is one of the major signaling cassettes and has a really important role in cancer, controlling and regulating apoptosis and cell proliferation, JAK is involved in processes such as cell division, immunity, tumor formation and cell death. These two are often found in the early onset of tumors) will be marked, as these are present in all tumors and are some of the factors that make the tumors develop, migrate and endure. With the help of immunofluorescence, we will be able to identify which cells carry JAK, which will be marked with white color, so the cells that are white will be JAK-STAT positive. The ones that had JNK, will be expressed as green. Last but not least, there are also cells that may contain green and white at the same time. Which means they will be JAK and JNK positive. JAK-STAT and JNK will be monitored with two different fluorescent markers, namely, upd3-LacZ for JAK-STAT (receptor level activation) and GC3Ai (marker of JNK at the level of apoptosis).

This will show us the heterogeneity of the tumors, that is one of the reasons why cancer is so difficult to cure, as it might be easy to eliminate one type of cells but not all of them because they can be really different from each other.

We will quantify our results in order to understand in what proportions these different cells may be present in a heterogeneous tumor in which cells have diversified.

*-Experiment 2:* We will be studying the different states of the cell cycle stages in cancer cells through a tool called Fly FUCCI that will show us different colors for each state, and we will know in which cell stage are tumor cells more likely to be when proliferating and migrating from the primary tumor, which will give us additional information about tumor and how its cells behave.

## Procedure and materials

In order to carry out the experiment, we were let to do it in a scientific laboratory at the Institute for Research in Biomedicine (IRB) in Barcelona, specifically in Marco Milán's laboratory, under the supervision of Kaustuv Ghosh, a PhD student who works in Milán's laboratory. IRB is a Biomedical Research Institute, an investigation center dedicated to fundamental research in biomedicine. We got the opportunity to go there because of a program called "Crazy About Biomedicine". Marco Milán is the Group Leader of the Development and Growth Control Laboratory research group, he obtained his degree in Biology from the Universidad Complutense de Madrid, he got his doctorate in 1995 and since then, he has worked in different laboratories and has wide experience in the field of developmental biology and tissue biology of Chromosomal Instability. Kaustuv Ghosh, helped us throughout the whole process, and he studied at the Indian Institute of Science Education & Research Mohali. Kaustuv, although he is younger, has a lot of experience and has been working with *Drosophila* for many years.

To do our experiment, we will work with *Drosophila melanogaster*'s imaginal wing disc, to develop a malignant tumor and study its growth and development through induced chromosomal instability. GAL4/UAS system, RNAi of *bub3*, and overexpression of *p35* will be used to do so.

And afterwards, when the disc has the tumor, fluorescence microscopy will be used in order to distinguish each type of cell and tell which are migrating and in what group are they.

Crossing, dissection, Immunostaining, imaginal discs extraction and mounting, microscopy and analysis will be done to get the final conclusions.

The flies with the genetic changes and tools like markers or balancers will be mostly given to us from IRB from different international *Drosophila* Stock Centers, and we will be allowed to work with them with the supervision and help of Kaustuv Ghosh.

### 1. Crossing

To make the crosses we used tubes which contained the media, male flies and female flies and cotton to prevent the flies from flying away. To make the crosses correctly, we needed to separate the virgin female flies from the non-virgin flies, because if they were not virgin the genes of the offspring won't be the ones we wanted. We used a microscope to look for different visible markers the virgin females had in their abdomen and also its body was slightly bigger. Once this process was made, we did the same with males and when it was done, we introduced the virgin males with the virgin females that had the genotype we wanted, in order to obtain the desired offspring in a few days.

Later on, the flies that were on the tubes were put in a room at 25° C, the next day the tubes needed to be flipped in order to see if the females laid eggs and transfer the flies to another tube so that there could be space to lay new eggs and for them to develop. If they did so, the tubes that had eggs were put in a bath at 29° C to accelerate the cycle.

To swap the flies from their previous tube, we had to shake the tube while taking out the cotton so that the flies don't fly away when the cotton is taken. The new tube was put above the tube with the flies and then to make the change of tube we had to flip the tube, so the new tube now is below the old tube, and finally the cotton was put in the new tube when all the flies were in the new one. The eggs did not fall from the media because they were stuck in it.

In the experiment done, we used genetically modified flies that had manipulation in the chromosomes 2 and 3. To do so, we used different balancers, markers and genetic tools.

We were able to use flies that already had some of the genes and markers we needed because different stock centers already put those in the flies with different gene insertion methods and IRB bought those and let us use them.

Firstly, we had 5 different types of flies that we crossed to get the final result we wanted for experiment 1.

These are the 5 different types:

1.  $\frac{x}{x} / \frac{x}{y} ; \frac{+}{+} ; \frac{upd3 lacZ:: UAS GC3Ai}{Tm6B Gal80}$
2.  $\frac{x}{x} / \frac{x}{y} ; \frac{ap Gal4:: UAS myrtom}{CyO GFP} ; \frac{mkrs}{Tm6B Gal80}$
3.  $\frac{x}{y} ; \frac{ap Gal4:: UAS myrtom}{+} ; \frac{upd3 lacZ:: UAS GC3Ai}{Tm6B Gal80}$
4.  $\frac{x}{x} / \frac{x}{y} ; \frac{UAS bub3 RNAi:: UAS p35}{CyO GFP} ; \frac{+}{+}$
5.  $\frac{x^-}{x^-} ; \frac{+}{+} ; \frac{+}{+}$  (wild type for control)

To get the type of fly we wanted, we crossed  $\frac{x}{x} / \frac{x}{y} ; \frac{+}{+} ; \frac{upd3 lacZ:: UAS GC3Ai}{Tm6B Gal80}$  with  $\frac{x}{x} / \frac{x}{y} ; \frac{ap Gal4:: UAS myrtom}{CyO GFP} ; \frac{mkrs}{Tm6B Gal80}$

We obtained  $\frac{x}{y}$  ;  $\frac{ap\ Gal4::\ UAS\ myrtom}{+}$  ;  $\frac{upd3\ lacZ::\ UAS\ GC3Ai}{Tm6B\ Gal80}$  and crossed with  $\frac{x}{x}/\frac{x}{y}$  ;  $\frac{UAS\ bub3\ RNAi\ ::\ UAS\ p35}{CyO\ GFP}$  ;  $\frac{+}{+}$

To obtain the type of fly, we wanted.

-Experiment 1:

$$\frac{x}{x}/\frac{x}{y} ; \frac{ap\ Gal4\ ::\ UAS\ myrtom}{UAS\ Bub3\ RNAi\ ::\ UAS\ p35} ; \frac{upd3\ lacZ\ ::\ UAS\ GC3Ai}{+}$$

We could know that these flies had the genotype that we wanted because of physical features. These flies had no green fluorescent color when observed in the fluorescence microscope, neither in the head nor the tail, but we could see red color. In addition, we chose the ones that were not small body and as well as a delay in its development, which showed that it contained the tumor. All these features, indicated us that had the tumor (UAS Bub3 RNAi :: UAS p35), had upd3 lacZ :: UAS GC3Ai and everything that these flies contained in its genotype, was of our will, there was nothing that we did not want, so the experiment was successfully arranged.

-Control 1:

We crossed  $\frac{x^-}{x^-}$  ;  $\frac{+}{+}$  ;  $\frac{+}{+}$  with  $\frac{x}{y}$  ;  $\frac{ap\ Gal4::\ UAS\ myrtom}{+}$  ;  $\frac{upd3\ lacZ::\ UAS\ GC3Ai}{Tm6B\ Gal80}$

Then we selected the ones with RFP because of myrtom, so the flies had “ap Gal4 :: UAS myrtom” and were the ones with long body to give us this final result, that did not have cancer:

$$\frac{x}{x}/\frac{x}{y} ; \frac{ap\ Gal4\ ::\ UAS\ myrtom}{+} ; \frac{upd3\ lacZ\ ::\ UAS\ GC3Ai}{+}$$

To select the flies for the control, we did not mate the parent flies which contained UAS Bub3 RNAi :: UAS p35, as we do not want cancer to be in the control, so we matched the flies type 3 and 5 (normal/wild type), and we did the same as before (select the ones with large body, not Tm6B, select the ones with red color), but we did choose the ones that had developed on day number 5, so we knew, that the ones that did develop early, did not have the tumor. We had the control parents and experiment parents separated to make sure, there was not a tumor developed in the control, but to make sure that the control was as it should be, we chose the ones that were the fastest to reach the 3rd instar larva. So we knew that the tumor was not there.



For the experiment 2, we started with the following type of flies:

$$\frac{x}{y} ; \frac{ap\ Gal4}{CyO\ GFP} ; + \quad \text{crossed with} \quad \frac{x}{x} ; \frac{UAS\ Fly\ FUCCI}{UAS\ Fly\ FUCCI} ; +$$

$$\text{And we obtained: } \frac{x}{x} ; \frac{ap\ Gal4}{UAS\ Fly\ FUCCI} ; + \quad \text{crossed with: } \frac{x}{y} ; \frac{if}{CyO\ GFP} ; +$$

$$\text{We selected the ones that had green color. } \frac{x}{x} ; \frac{ap\ Gal4::\ UAS\ Fly\ FUCCI}{CyO\ GFP} ; +$$

$$\text{And finally, this } \frac{x}{x} / \frac{x}{y} ; \frac{ap\ Gal4::\ UAS\ Fly\ FUCCI}{CyO\ GFP} ; + \quad \text{was crossed with}$$

$$\frac{x}{x} / \frac{x}{y} ; \frac{UAS\ Bub3\ RNAi::\ UAS\ p35}{CyO\ GFP} ; +$$

And we obtained the flies for the *experiment 2*:

$$\frac{x}{x} / \frac{x}{y} ; \frac{ap\ Gal4 : : UAS\ Fly\ FUCCI}{UAS\ Bub3\ RNAi : : UAS\ p35} ; +$$

We knew that we selected this properly, because there was no green color in head and not in tail, it contained red color in the wing discs and were a bit delayed in its cycle. It all indicated that it had the tumor and the Fly FUCCI gene. The things that did not interest us, were not present, so we knew we were on the right track.

$$\text{For the control, we mate } \frac{x}{x} / \frac{x}{y} ; \frac{ap\ Gal4::\ UAS\ Fly\ FUCCI}{CyO\ GFP} ; + \quad \text{with } \frac{x^-}{x^-} ; + ; +$$

And obtained the final result for *control 2*:

$$\frac{x}{x} / \frac{x}{y} ; \frac{ap\ Gal4 : : UAS\ Fly\ FUCCI}{+} ; +$$

We knew that it was the control because it developed in 5 days and had no green color but red.

We had all the different types of flies separated and named, to know which were which. The physical features helped us to identify every type and also to make sure, like insurance, that the flies contained what we wanted.

The chromosomes are separated by “;” so we put the first chromosome, then “;” and the following chromosomes

+ : Means no changes in that chromosome, it is like a normal wild fly.

-*ap* (apterous): It is a gene which is activated in the dorsal part of the wing disc. Apterous is united with Gal4, so those cells that are in the dorsal part of the wing disc express Gal4 protein. As it is bound to Gal4, whatever gene that contains UAS, in those cells it will be expressed the gene product.

-*Upd3*: It is a cytokine that binds and marks JAK-STAT signalling pathway.

-*lacZ*: Is an antigen derived from bacteria that is tagged to *upd3*, so when a fly expresses *upd3*, it produces *lacZ*, against which we can use an antibody with fluorophore in order to distinguish the cells that expresses *upd3*.

-*GC3Ai*: Is a caspase activity sensor which makes green fluorescence in the cell where it detected the activity. And apoptosis inhibitor when it detects it. It is attached to UAS, so it is expressed in apterous. It will mark the cells that contain JNK.

-*Tm6B*: Is a balancer that produces a small body in larvae due to its phenotypic marker, so it can be identified by sight as the abdomen is much smaller than a normal one. It helps us identify the larvae that have the genes of our interest.

-*Gal80*: Is a protein which is an inhibitor of Gal4. We use it so that it does not let Gal4 do its job and produce a tumor because of UAS *Bub3* RNAi :: UAS *p35*. As Gal80 binds to UAS and does not let Gal4 do it.

-*myrTom*: It is a gene that encodes a protein called tomato, it is a variant of Green Fluorescent Protein, because it produces fluorescence red color, when observed in the fluorescence microscope. It will only be activated in *ap* domain, because *myrTom* is attached to UAS.

-*CyO GFP (CyO + GFP)*: *CyO* is a *Drosophila* balancer, which is recognizable because it gives a curvature to adult fly wings. It was also expressed as GFP tag making it easier to recognize it in the larval stage where the wings have not developed completely.

-*Bub3<sup>RNAi</sup>*: *Bub3* controls the SAC (spindle assembly checkpoint) complex and it does not permit the cell to correctly distribute the chromosomes, causing CIN (chromosomal

instability), producing RNAi and inducing the formation of tumors. It will be only activated in apterous as ap is attached to Gal4 and bub3 RNAi to UAS.

-*p35*: It is a protein with an inhibitory function on the apoptosis process used for tumor cell production. It is a baculoviral protein, it is normally not present in the system

-*Bub3 RNAi + p35*: This set produces tumor cells, as it has migratory and survival capacity, and as it goes all with UAS, it will produce cancer where Gal4 is, in this case in apterous, as it is bound to Gal4.

-Fly FUCCI: Is a tool developed by Zeilke et. al, and it is used for studying the cell cycle stages in the cells that are marked with it. The G1 stage cells are seen in green, S cells are red and M, G2 are seen as yellow.

## 2. Dissection

To accomplish the dissection correctly we had to use 2 forceps, PBS, a fluorescence microscope and a normal microscope (Nikon SMZ645).

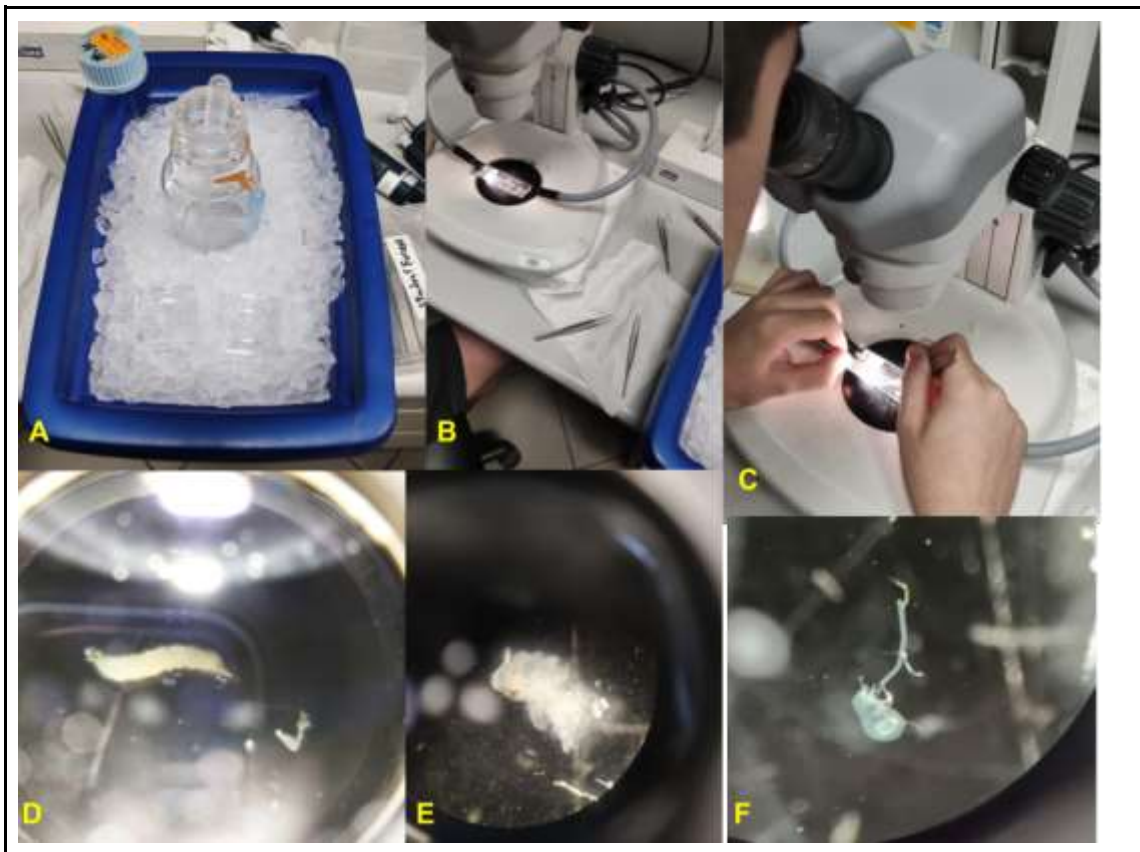
Initially we had to prepare the set, first, we had to take ice and put in a box where we would also add PBS in a container cube, 1 Eppendorf tube which contained 900  $\mu$ l of PBS and the larvae that had 6 days of age, for the fact that were in the 3rd instar larvae stage because were the ones of our interest. Moreover, we also selected the larvae that had a large body and RFP (red color/myrTom) that we could observe in the middle top part of its body through the fluorescence microscope for the experiment because those had the tumor. And for the control, we also used other ones with RFP and large body, and we knew they had no tumors because we did not put the factors that promote them and were the ones of 5 days all to make sure they did not have any tumor.

To start doing the dissection, we had to take both of our forceps, adjust the microscope and fill the container cube with PBS and put the selected larvae inside it. Next, we needed to put every larva in a slide with PBS to preserve the larva after putting it in the small container that is with ice and PBS, so it does not move, and we can do the dissection correctly. To do so, we had to place both forceps in the middle part of the larva and pull the posterior part with one of the forceps to get rid of the abdomen part and all the organs that we do not need. Then we will have the top and middle top part of the larva which contain the mouth, brain and the wing discs, beyond other organs and tissues.

After that, we will need to invert the larva to expose all the organs including the wing disc, in order to do this process, we need to open one forceps and introduce the tip in

the mouth while with the other one we push slightly the anterior part of the body making an inversion to expose the discs. When all this process is done, we placed the tissue in the Eppendorf tube.

When we had between 7-8 larvae dissected without more than 20 minutes from the first dissection to the last one and were all placed inside the Eppendorf in ice, the dissection process was done. These were needed to be placed in ice and PBS, so they do not degrade.



**Fig. 24** This is a group of images that show step by step all the process of dissection. As shown, in picture A, there is a bowl filled with ice and two containers with cold PBS. In the middle top part, B image shows the microscope with the lights on the sides and the sample in the middle that is seen in the microscope, on the right of the microscope there are two forceps that were used to do the dissection. On image C, we were in the process of dissecting. The image D shows what we saw in the microscope while dissecting and in the image in the middle, there is the top part of the larva that is the one we needed. And finally, in image F, we can see the wing imaginal disc dissected from that larva. Own file

### 3. Immunostaining

Immunostaining is done with antibodies that detect the protein so that we can see the fluorescence after all the processes, and it does not lose the different types of fluorescence that the tissue has. And its function is to preserve the cells and proteins as well as the fluorescence and markers.

After we dissected and put the tissues in cold PBS for not more than 20 minutes, we could start the immunostaining following the next steps.

- 1- Fix the samples at 900  $\mu$ l of PBS plus 100  $\mu$ l (about 37% of Formaldehyde) and put it on the nutating mixer for about 20' at room temperature.
- 2- Wash the Eppendorf 3 times and put again in the nutating mixer for 15' at room temperature, adding PBS 1x plus Triton 0.2%.
- 3- Block 30' with 300  $\mu$ l of BBT (PBT + 0.3% BSA + 250mM NaCl)
- 4- Add the primary antibody (mouse anti  $\beta$ Gal) with 50  $\mu$ l of BBT and leave it overnight at 4 °C.
- 5- Wash the samples 4 times with PBS and leave it in the nutating machine for 20' at room temperature.
- 6- Add the second antibody (Donkey anti-mouse antibody, which will be expressed as Far red) diluted in 1:150 - 200  $\mu$ l in BBT + DAPI 1:100 in a final volume of 100  $\mu$ l and incubate the sample in darkness at room temperature for 90'.
- 7- Wash the sample 4 times with PBT and put it moving at room temperature in darkness for 15',
- 8- Remove the PBT and add 200 ml of mounting medium and store at 4°C in darkness.

Now we were ready to mount the wing discs.

The PBT used was composed by 1 ml Triton 20% and 99 ml PBS for 100 ml.

BBT composition for every 100 ml was: 5ml NaCl (5M), 1ml BSA 30 %, 1 ml Triton 20% and 93 ml PBS.

This process was done with the experimental samples of the experiment 1 (upd3,GC3Ai), but with the experimental samples of the experiment 2 (Fly FUCCI) we did all the steps except 4,5 and 6, because the antibodies were not needed for Fly FUCCI.

#### 4. Mounting and imaginal discs extraction

Mounting is the process in which the samples are cleaned from fat and excess tissue is removed, just leaving the wing disc, and all the samples are put on the slides and prepared to observe.

First, we prepare all the sets like if we were going to dissect but instead of using two forceps, we used 1 forceps and 1 needle or two needles.

Little by little, with the maximum magnification of the microscope to observe correctly the wing disc, we separated the two wing discs from each tissue pulling the tissue that was stuck with the disc and carefully separating it, we obtained 13 wing discs from the experiment 1 experimental samples, we got 14 wing discs samples as control of this experiment. We did the same with the flies from experiment 2, and we obtained 38 wing discs that were for the study of the experiment and 2 control samples.

Every time we separated and got the wing disc, we put it in PBS and, when we had all, we placed them in a new and cleaned slide all in a row, so it is easier afterwards to observe in the microscope, and covered it with a coverslip. We put glue in the walls of the coverslip because we do not want it to fall and lose all the samples, we let it dry up and place it in a dark box in the fridge overnight.

Now the samples were ready to be observed in the confocal microscope.

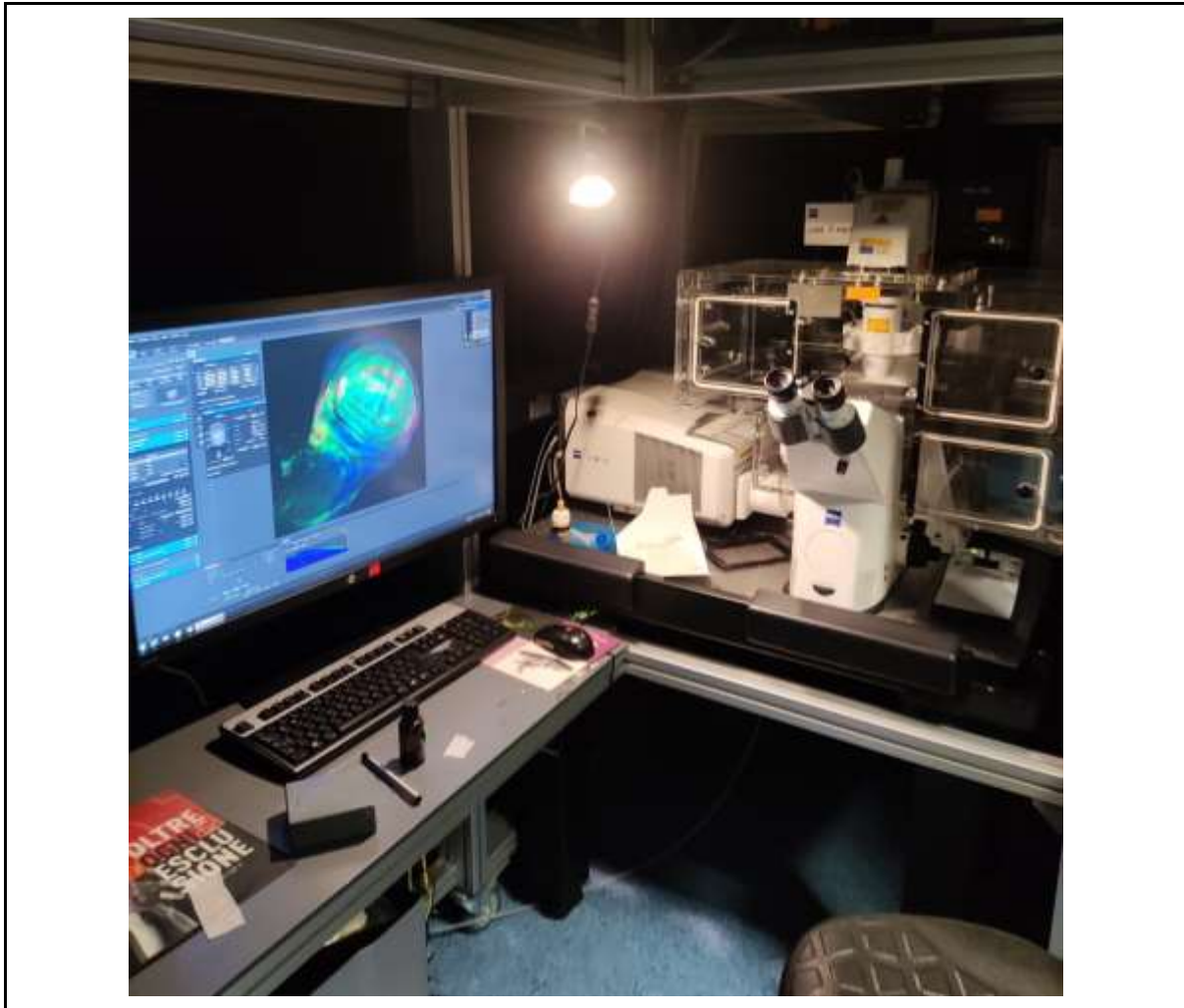
#### 5. Microscopy

For observing the wing disc, we used a ZEISS LSM780 confocal microscope, the eyepiece magnification was x10, and we used the objective of x25, so the total magnification was x250. Also, we took some images at higher magnification (x400), to see the tissue better.

One aspect of the confocal microscope was that as we were observing the samples, the images taken by the microscope were shown on a computer screen next to the microscope.

At the end of the process, we saw all the layers stuck in order, like a 3D image.





**Fig. 25** In this image, we can observe the set of a confocal microscope. On the upper shelf we can see that there is a machine attached with cables that go to the microscope, that is the fluorescence light. Right below is the microscope with a computer. We can observe that near the microscope there is a button to move and select the sample you want to observe through the lens of the microscope, there are also the windows on the sides, those are to move around the sample to make sure they are on sight. And on the other side of the confocal microscope there is a computer, on the computer we can see the images that the confocal microscope has taken, so then we can analyze it. (IRB BARCELONA, ZEISS LSM 780)

For identifying the cells, we used DAPI (blue color) for the identification of the nucleus of the cell, so we can identify the number of cells, then we used red color to identify the apterous, moreover, in the control the apterous is only expressed in the dorsal part of the larva body and in the experiment, as it promotes the migration and proliferation, we can identify that those red dorsal cells, have moved into other parts of the wing imaginal disc, this is why these are migrating cells, we used lacZ to create white color cells when it has upd3-lacZ, and we know that these cells have JAK and, finally, green color to identify GC3Ai, the detector of caspase action, so if a cell is green, it is positive in caspase and so, positive in JNK.

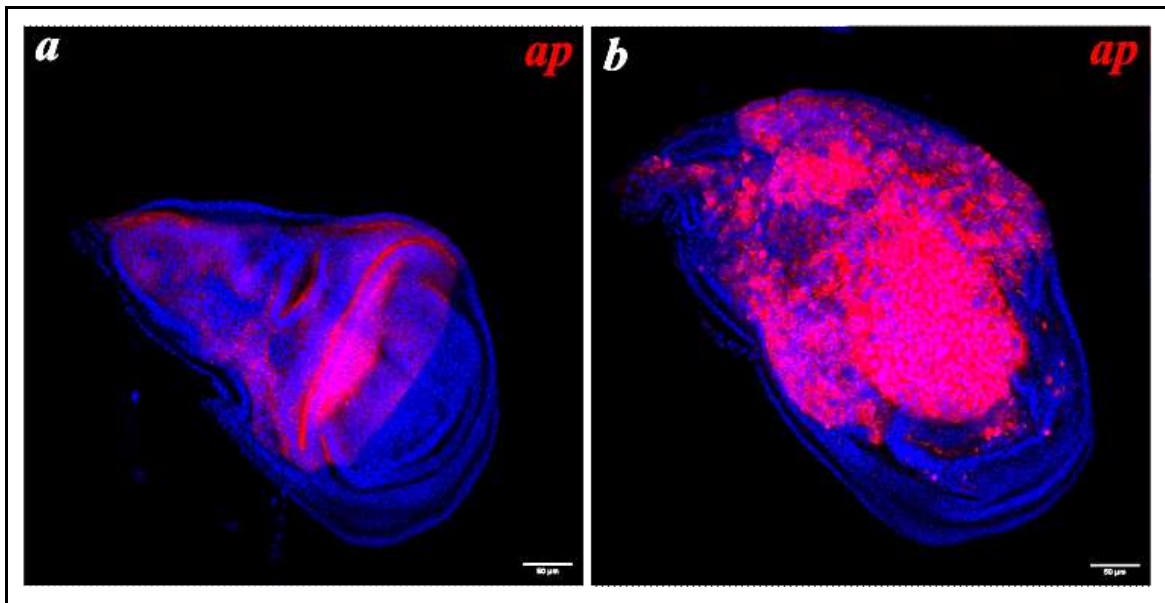
In order to process and analyze the images, we used a software called FIJI, that help us identify the migrating cells and identify whether they were ap positive, upd3-lacZ



positive, GC3Ai positive or if some cells were positive in all or negative in upd3-lacZ and GC3Ai. Also, we used Excel to make a table of the results.

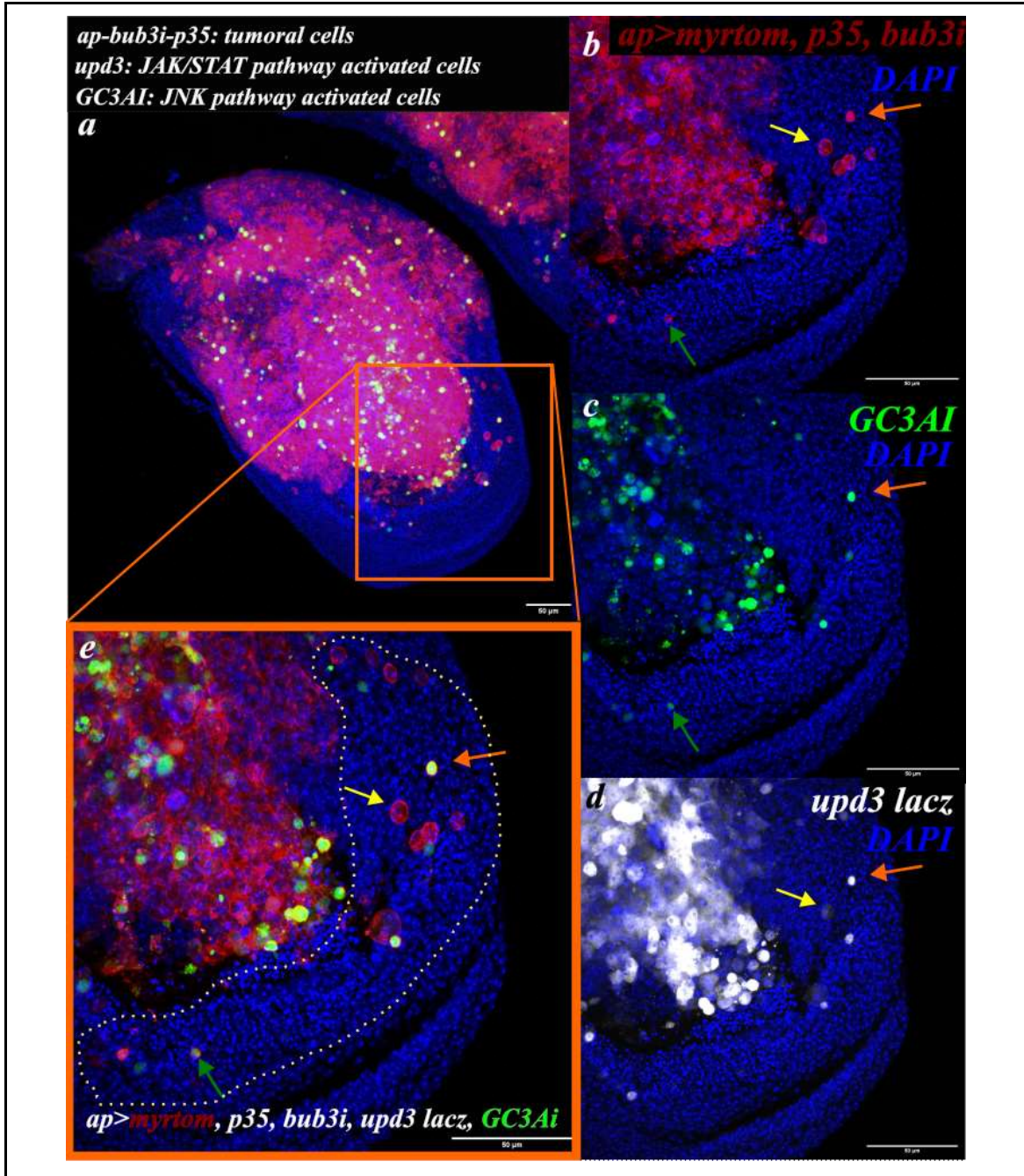
## Results experiment 1:

Through the confocal microscope, we observed and took microphotographies from the wing imaginal discs we dissected and used to obtain the results.



**Fig. 26** In this image, there is a comparative of the control and the experiment wing discs. We can see in our left, the image a, it has a "line" where from that point, there are no red marked cell and all the bottom part of it has no migratory cell, as this one has no cancer. On the other side, we can see more cells and there are some cells that are outside that line and are in the ventral (bottom) part of the wing disc. This one is positive for cancer and there are migrating cells that are marked with red that are migrating to other parts of the wing disc, and in a normal situation, these will go to other parts of the body. Own file

two images show the differences between a tissue with cancer and one without cancer. We can clearly see the difference of cell, as in the left picture, there is a section where no marked cell passes and all the cells are organized, whereas in b, we can see a clear disorder of cells and in the bottom part of the wing disc we can see some red spots, which are metastatic cells moving from the tumor. Here we can see what a tumor looks like and the clear difference between a wing disc with a tumor and the one without these



**Fig. 27** In this figure, we can see a collage that shows different parts of a wing disc that shows migrating cells. All the wing discs have a induced tumor and there are cells with the following markers, GC3Ai, upd3 lacZ and all the cells have DAPI, which shows the nuclei of the cell, so we know where are the cells and ap>myrtom-bub3i-p35. Image A is the image of the whole wing disc where we can see inside the square the place where there are the most migrant cells, that are the ones that are more separated and are mostly surrounded by blue. Image B, is the square, but it is only seen ap>myrtom-bub3i-p35, that means that they are migrant, and we do not know if they have the markers or not. In C, we see green cells, which means that is positive for GC3Ai. D, shows cells that are white, meaning that are positive for containing upd3 lacZ. And finally, in E, we see the square magnified but with all the fluorescence colors, so we can identify which cells have markers, the orange row, shows a cell that is metastatic, as we see in b, meaning that it has ap>myrtom-bub3i-p35, in c we see it contained GC3Ai and in d, it was also positive for upd3 lacZ, so this cell is positive for all. The yellow row, shows a cell that is metastatic and positive in upd3 lacZ because in d, we saw that it was white, not a lot but quite, so it is positive, and it contained ap>myrtom-bub3i-p35. And the green row shows a cell that is metastatic and GC3Ai positive, as we see in images b and c. Own file

In the collage, we have seen a positive tumoral wing disc, which has markers for cancer promoters. Through fluorescence, we can see different types of marked cells. In white, we see *upd3 lacZ* positive cells, which are the ones that contain JAK, in green, we have *GC3Ai*, which tells us that the cell marked contains JNK. In blue are marked all the cells, because DAPI shows the nucleus of the cell, so we can identify what is a cell and finally *ap>myrtom-bub3i-p35* which is in red are the cells where chromosomal instability was induced so are the cells that constitute the tumor, some of which migrate. The cells that are inside the dot line are the ones migrating, and these can be only red, which means that have no marker, only white, which means that have JAK, only green, so these have JNK and both white and green, which means that are double positive and contain JAK and JNK at the same time.

After we got the images, and we had analyzed it, the results we got from the control were the following:

In all the control imaginal wing discs we had dissected presented no sign of migrating cells. That means that the control was done correctly. We knew that the genes that we put were there as we could see the phenotypic, physical features, but the ones that produced cancer were not there and everything was normal.

After analyzing the wing discs of the first experiment, we did a table to express the obtained results of the analysis. Apart from that, we did some conclusions, for instance:

In all the wing discs of the first experiment, they all had migrating cells, which means that there was a tumor. There was not a common number of migrating cells in all the discs, as some might develop faster, and some tumors may migrate more than others.

Wing discs Ap BP Upd3 GC3Ai sample id	Total number of migrating cells (Red+Blue)	Total number and percentage of migrating cells Double positive GC3Ai + upd3 (Red Green blue white)	Total number and percentage of exclusively GC3Ai cells (Red, Green, blue)	Total number and percentage of exclusively upd3 cells (Red, white, blue)	Total number and percentage of migrating cells with no marker (neither white nor green)
1	81	33 (40.740 %)	17 (20.987%)	10 (12.345 %)	21 (25.926 %)
2	51	23 (45.098 %)	6 (11.764%)	7 (13.725 %)	15 (29.412 %)
3	47	22 (46.808 %)	7 (14.893%)	8 (17.021 %)	10 (21.276 %)
4	49	18 (36.735 %)	11 (22.448%)	6 (12.244 %)	14 (28.571 %)
5	43	16 (37.209 %)	6 (13.953%)	6 (13.953 %)	15 (34.883 %)
6	46	13 (28.261 %)	6 (13.043%)	10 (21.739 %)	17 (36.956 %)
7	37	11 (29.729 %)	6 (16.216%)	10 (27.027 %)	10 (27,027 %)

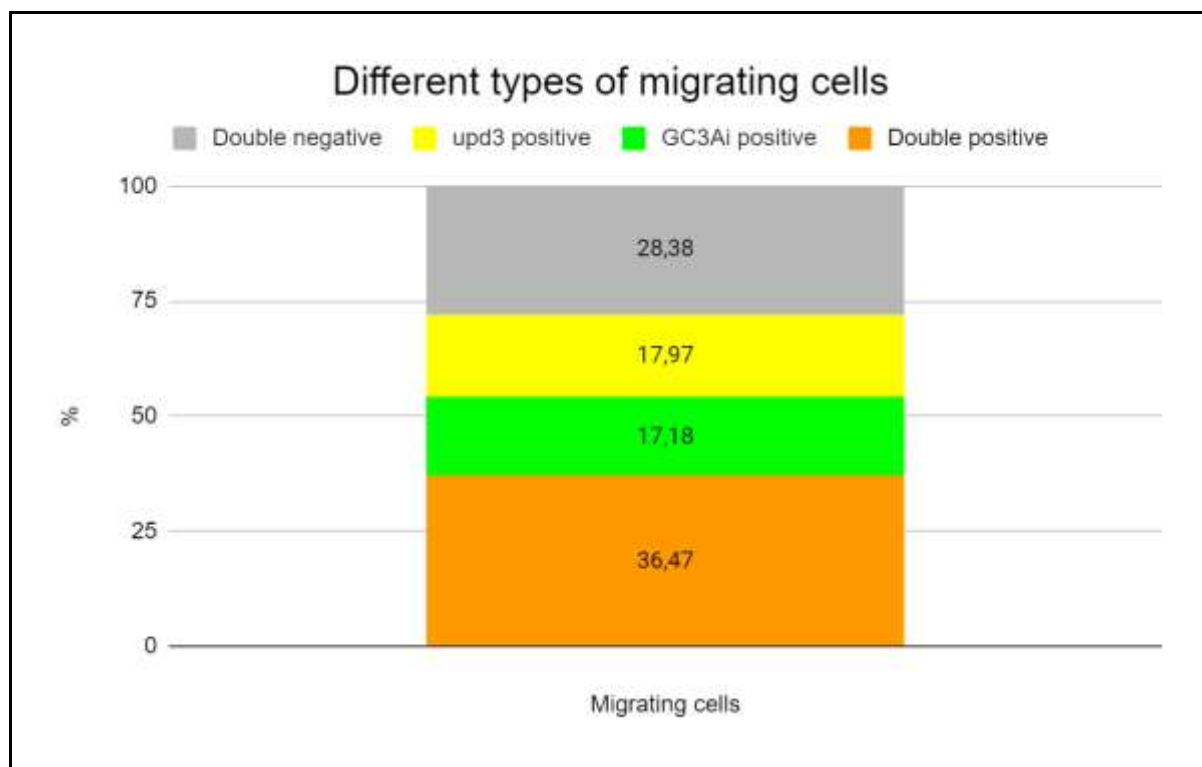
## PRACTICAL FRAME

8	45	18 (40 %)	14 (31.111%)	5 (11.111 %)	8 (17.777 %)
9	34	12 (35.294 %)	9 (26.470%)	6 (17.647 %)	7 (20.588%)
10	51	17 (33.333 %)	11 (21.568%)	8 (15.686 %)	15 (29.411%)
11	32	10 (31.25 %)	2 (6.25%)	7 (21.875 %)	13 (40.625%)
12	48	16 (33.333 %)	2 (4.1666%)	16 (33.333 %)	14 (29.166%)
13	44	16 (36.364 %)	9 (20.454%)	7 (15.909 %)	12 (27.272%)

**Table 1.** Counts of migrating cells positive for different markers in each wing discs (n=13)

In **table 1**, we have put all the percentages and number of cells that are of our interest, from all the wing discs from the experimental part of the experiment 1, we have counted all the migrating cells and from those, we counted which one were positive for each marker, as we could see the different fluorescence colors in the images that we did with the confocal microscope, and with the help of the software called FIJI, we were able to see and count the number of cells. There were cells that were migrating but had no positive color for upd3 or GC3Ai, so we knew that they were migrating cells, but these had a different type of promoter that we do not know.

With these results, we can confirm that there is heterogeneity in all tumors. From a primary tumor, many cells can arise and these can be really different from each other, we have seen that with the cells that had upd3 positive (means that these cells had JAK-STAT), some cells were GC3Ai positive (had presence of JNK) and there were some that had both of them. This is the proof of the heterogeneity of cancer. Besides that, there are cells that are negative, which means that they have other markers that we are not studying but are different from the ones we know, so there is even more heterogeneity as these are different types of cells deriving from the same tumor.



**Fig. 28** This is a graphic of all the percentages we have taken from the cell counting we did. As it is shown, the orange range corresponds to the double positive of migrating cells. The green range, is the one whose cells were positive for GC3Ai corresponds to all the migrating cells that are JNK positive. The yellow part, which is the upd3 positive, are 17,97% of the total of migrating cells that are positive for JAK-STAT, and an 28.38% that are double negative, meaning that they have neither JNK nor JAK. The whole percentage represents all the migrating cells. Own file

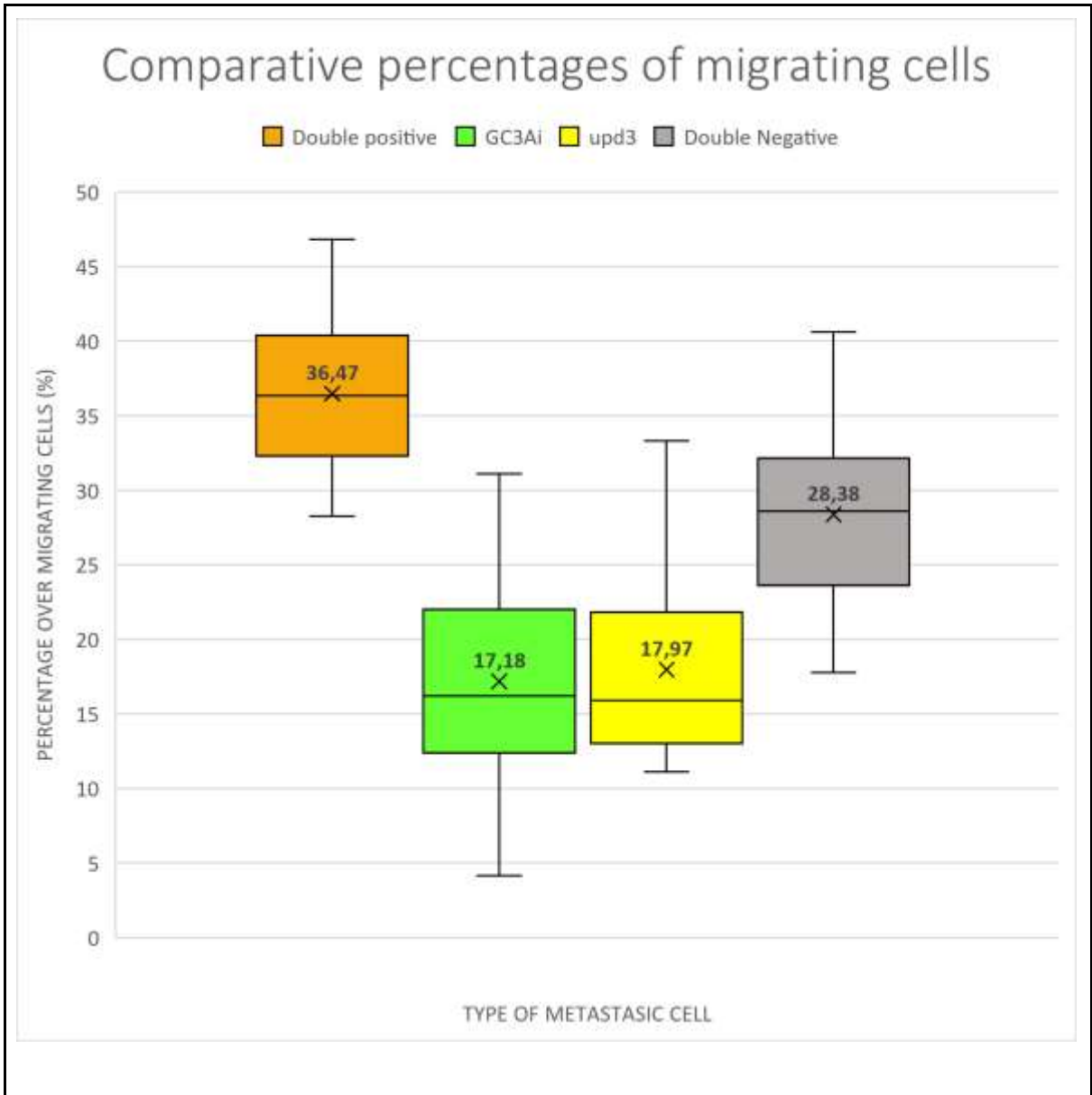
The results are the following: the great majority of positives were from the group of double positives, with a 36,47% of the total number of migrating cells and those had both, JAK and JNK.

The 17,97 % of the migrating cells were upd3 positive, meaning that it contained only JAK, and the percentage is very similar to the GC3Ai positive, which contained JNK and represents the 17,18 % of the total migrating cells.

And the remaining 28,38 % are those cells that had not JAK, nor JNK, but were migrant, these means that it didn't have the markers we put in the metastatic cells but as these were also metastatic and migrating, might have some cancer promoters that we are not studying, so we don't know what they had. There is a possibility that there are more variants of migrating cells, which we don't know yet.



PRACTICAL FRAME



**Fig. 29** This statistical graph shows the distribution of percentages of different types of metastatic cells, out of 13 wing discs and 608 migrating cells in total. We have 4 different groups, the double positive group, represented in orange, the GC3Ai group, represented in green, the upd3 positive group, that is yellow and finally the double negative group, represented in gray. In each one, the bottom segment of the box shows the lowest percentage of cells that were present in one wing disc, for example, the lowest percentage of GC3Ai positive cells found in a wing disc was 4,1 % of the total metastatic cells. The highest segment shows the highest percentage found in one sample, for example, in the double positive group, the highest percentage of cells that were present in a single wing disc was 46,8 % of the total metastatic cells.

The square represents the values from the first to third quartile, for example, the group of double negative square, include all the percentages of this group, that is calculation form all the samples between the first and third quartile, the line inside the box represents where is the 2nd quartile, that is the median of all the percentages (if we rank all the percentages from highest to lowest, and we have 13 samples, that line represents the value of the position 7). In the double negative group, this value is 28,58 % of the total metastatic cells of that wing disc. The X represents the arithmetic average, and the value is inside the box, for example, upd3 positive, the x (arithmetic average) is 17,97. Own file

The results we extract from this graphic are that double positive cells are the ones more present, because JAK and JNK together give superiority to cells which contain

them. Then we have a 28,38% of cells that are negative for both markers, which means that those have no presence of JAK nor JNK and that might contain other markers but that in this case we are not studying.

The ones with JAK constitute the 17,97% of the total percentage of migrating cells which is really close to the percentage of the ones with JNK, which is 17,18%. This tells us that containing JAK or JNK is very similar, and the advantage of having one of these two is very similar to having the other.

The ANOVA test checks if the difference between the averages of each type of metastatic cell is statistically significant in the studied samples. When performing a one-way ANOVA test, we try to determine if the difference between the averages reflects a real difference between the groups, or is due to the random noise inside each group, so we check if the results are statistically significant.

Pair	Difference	p-value
Double positive - JNK positive	19,2944	0,00000000846
Double positive - JAK positive	18,5029	0,0000000248
Double positive - Double negative	8,097	0,01508
JNK positive - JAK positive	0,7915	0,9899
JNK positive - Double negative	11,1973	0,0004222
JAK positive - Double negative	10,4059	0,001113

**Table 2.** Statistic table of ANOVA test of experiment 1 results

The **table 2** show us the comparison of the average of positives of each group promoters and tells us if it is significant and also a value, the lower the value the stronger the statistical significance.

So, it validates if there are statistically significant differences between the different cell populations.

The double positive has a really big significant difference between JNK and JAK groups, which is not as big but still significant with the double negative group.

JAK and JNK are not significantly different, as the difference between its percentages is from 0,79%

JAK and JNK versus double negative are both significantly different as the value is very low.

The lowest values, which mean that the difference is statistically significant, are the ones between double positive and JNK / JAK.



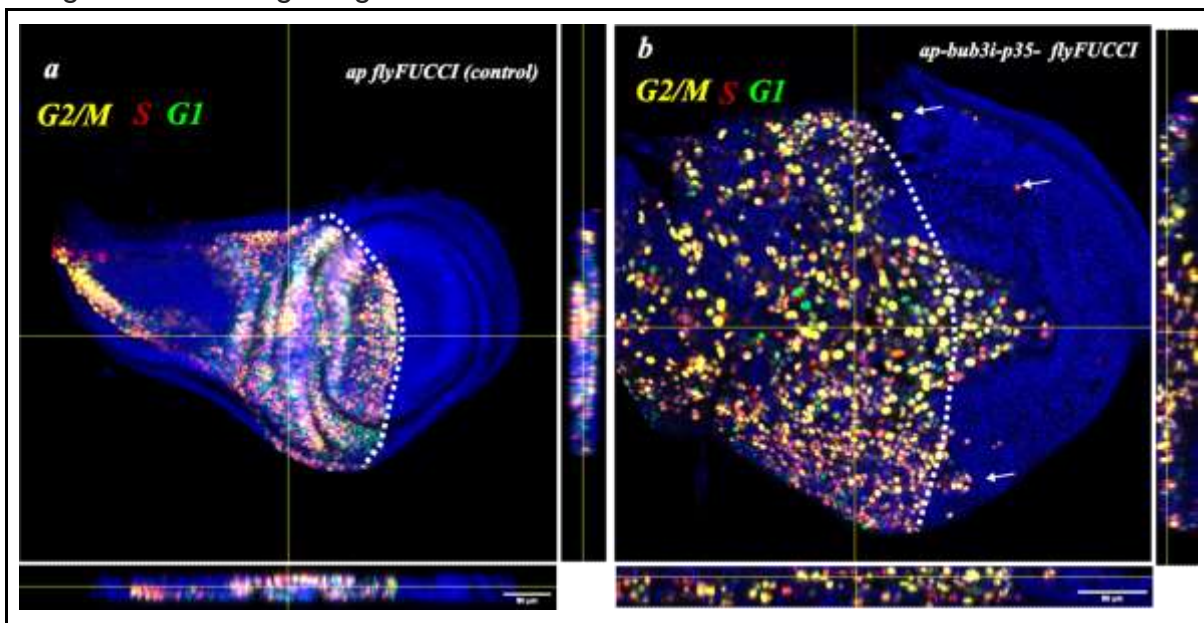
## Results experiment 2

After we did all the process, and we processed the images in the confocal microscope, both experimental and control, we obtained the images of the control from the Fly Fucci experiment, we did the quantification of the cells, counting all the cells that were migrating, and we classified them in 3 groups, that are the stages of the cell cycle (G1, S, G2/M).

The 38 imaginal discs of the experiment were done correctly, as we could see the 3 different types of cells and there was presence of metastatic tumor cells in all the samples.

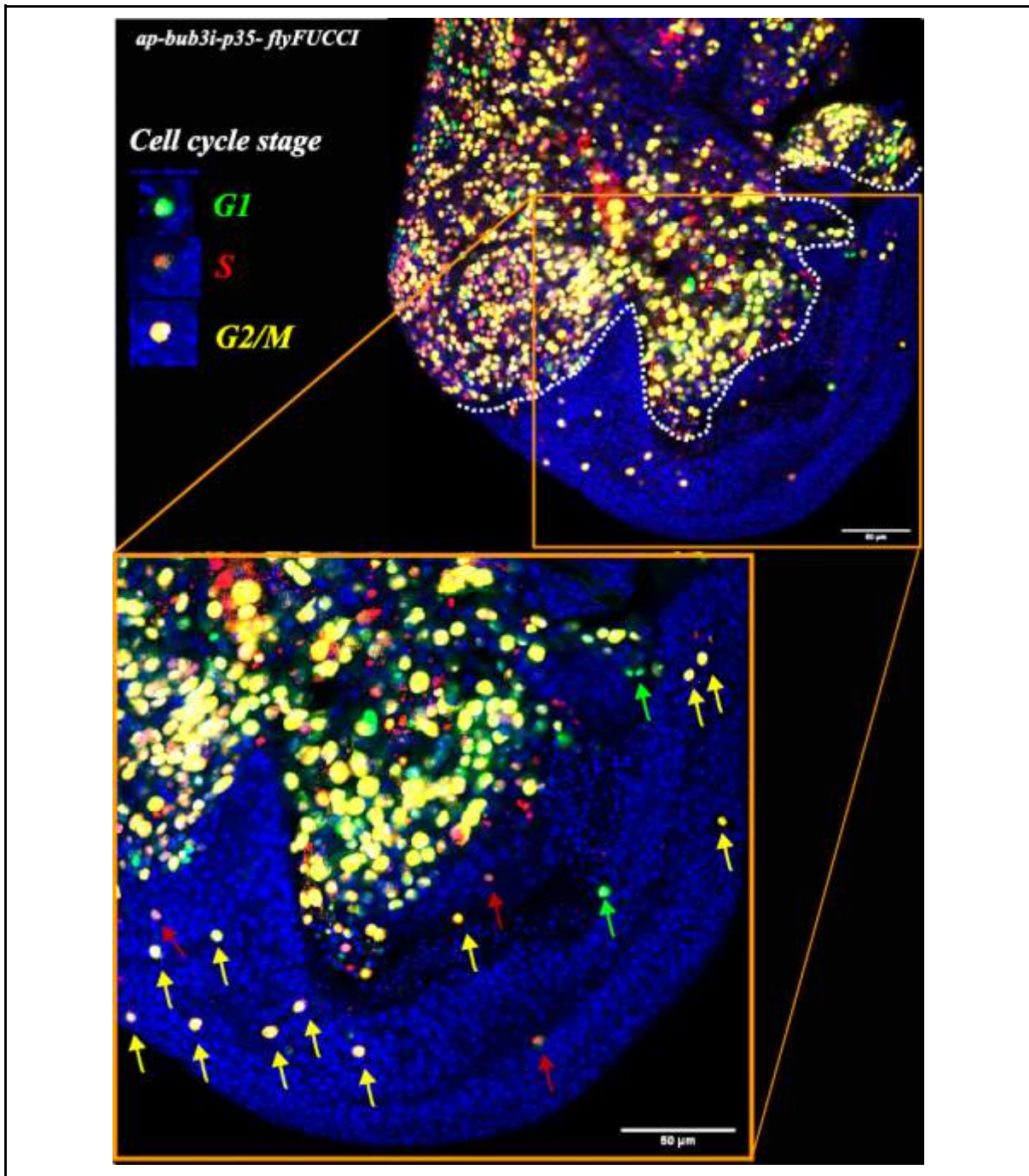
We concluded that the control was done correctly, for the fact that there was no sign of migrating cells, matter of fact all the cells were in the dorsal part of the wing disc which was the part they were meant to be.

We got the following images and made these:



**Fig. 30** Here, we can see two layers of two different wing discs, the superior one (a) is the control and the b is the one that has the tumor. In the control, the wing disc has Fly Fucci in apterous, that shows with different colors the cells with different cell cycle stages. G1, in green, S in red and G2/M in yellow. We cut the y axis, and the x, which are in the right and bottom of the image, we observe that it in the bottom image (x axis), there is a moment in which only blue color is seen, that is that already there are no cells with the Fucci fly and it indicates that the control is correct, since there are no cells outside the margin, the y axis, a vertical cut is seen, that there are a set of cells in different phases of the cycle but that they are cells more or less all the same although of different color. In the control we can clearly see that the cells are structured correctly and no cell is outside the white edge. Contrary to what happens in b, where there is a tumor and there are cells that are migrating, as the arrows indicate. There is a tumor in it and the cells are totally disordered and without control, in the y axis, there is a nonsense accumulation of cancer cells, and in the x axis, we see the group of cells and in the middle of blue, we see in the middle top part a few cells, these are the cells that have migrated from the set of cells. Own file

The results we get from these images are that we can clearly see a disorder of cells in the tumor wing disc and there is an accumulation of cells, a thing that does not happen in the control, where there is a correct order and disposition of the cells.



**Fig. 31** In this image we can observe a wing disc in which we can “separate” it in two parts, one part in which there are all the non-migrating cells, because it is the place where the tumor was induced and another part in which there are the migrating cells. As we can see, there are different types of colored cells. There are some cells that are in the G1 cells which are shown as green, other cells are in the S phase, which are expressed as red, and finally we can find cells in the G2/ M cells, which are expressed in yellow. Moreover, we can observe that the most abundant stage of cells is the G2/M phase. Own file

## PRACTICAL FRAME

This image shows us that there is a line from which the cells that supply from this are migratory tumor cells, and we see that there are a few of them. We also see that the cancerous body of this wing disc has a very uneven line, and unlike what we have seen before in the control, this was much more continuous. As we see, there is a great majority of G2/M (yellow) cells. There are 3 red cells, which are in phase S of the cell cycle and two which are in phase G1, which are seen in green.

When the quantification was done and all the wing discs were analyzed, we did the following table and some graphics based on the results of the data table.

Ap bub31 p35 fly Fucci sample	Total number of migrating cells	G1 (Green)	S (Red)	G2-M(Yellow)
1	46	3 (6,52%)	2 (4,35%)	41 (89,13%)
2	34	2 (5,88%)	3 (8,82%)	29 (85,29%)
3	23	2 (8,69%)	0 (0%)	21(91,31%)
4	31	1 (3,23%)	2 (6,45%)	28 (90,32%)
5	22	3 (13,64%)	3 (13,64%)	16 (72,73%)
6	31	2 (6,45%)	2 (6,45%)	27 (87,1%)
7	23	1 (4,35%)	2 (8,69%)	20 (86,96%)
8	27	1 (3,7%)	4 (14,81%)	22 (81,49%)
9	29	1 (3,45%)	3 (10,34%)	25 (86,21%)
10	22	0 (0%)	3 (13,64%)	19 (86,36%)
11	37	2 (5,41%)	3 (8,11%)	32 (86,48%)
12	25	2 (8%)	3 (12%)	20 (80%)
13	31	5 (16,13%)	5 (16,13%)	21 (67,74%)
14	22	3 (13,64%)	2 (9,1%)	17 (77,27%)
15	22	2 (9,1%)	5 (22,73%)	15 (68,18%)
16	29	4 (13,79%)	2 (6,9%)	23 (79,31%)
17	18	1 (5,56%)	2 (11,11%)	15 (83,33%)
18	26	0 (0%)	2 (7,69%)	24 (92,31%)
19	36	2 (5,56%)	3 (8,33%)	31 (86,11%)
20	24	2 (8,33%)	1 (4,17%)	21 (87,5%)
21	34	1 (2,94%)	6 (17,65%)	27 (79,41%)
22	33	5 (15,15%)	3 (9,1%)	25 (75,75%)

23	30	3 (10%)	4 (13,33%)	23 (76,67%)
24	36	5 (13,89%)	3 (8,33%)	28 (77,78%)
25	31	2 (6,45%)	1 (3,22%)	28 (90,33%)
26	39	2 (5,13%)	5 (12,82%)	32 (82,05%)
27	37	2 (5,41%)	4 (10,81%)	31 (83,78%)
28	32	3 (9,37%)	0 (0%)	29 (90,63%)
29	29	2 (6,9%)	3 (10,34%)	24 (82,76%)
30	22	1 (4,55%)	3 (13,64%)	18 (81,82%)
31	35	4 (11,43%)	2 (5,71%)	29 (82,86%)
32	26	3 (11,54%)	3 (11,54%)	20 (76,92%)
33	28	2 (7,14%)	3 (10,71%)	23 (82,15%)
34	34	2 (5,89%)	3 (8,82%)	29 (85,29%)
35	26	0 (0%)	0 (0%)	26 (100%)
36	27	2 (7,41%)	5 (18,52%)	20 (74,07%)
37	24	3 (12,5%)	2 (8,33%)	19 (79,17%)
38	33	2 (6,06%)	3 (9,09%)	27 (81,82%)
	<b>Average cells per disc</b>	<b>Average G1 cells per disc</b>	<b>Average S cells per disc</b>	<b>Average G2/M cells per disc</b>
	29,31	2,19	2,77	24,35
		<b>Percentage of G1 cells of the total</b>	<b>Percentage of S cells of the total</b>	<b>Percentage of G2/M cells of the total</b>
		7,58%	9,44%	82,98%

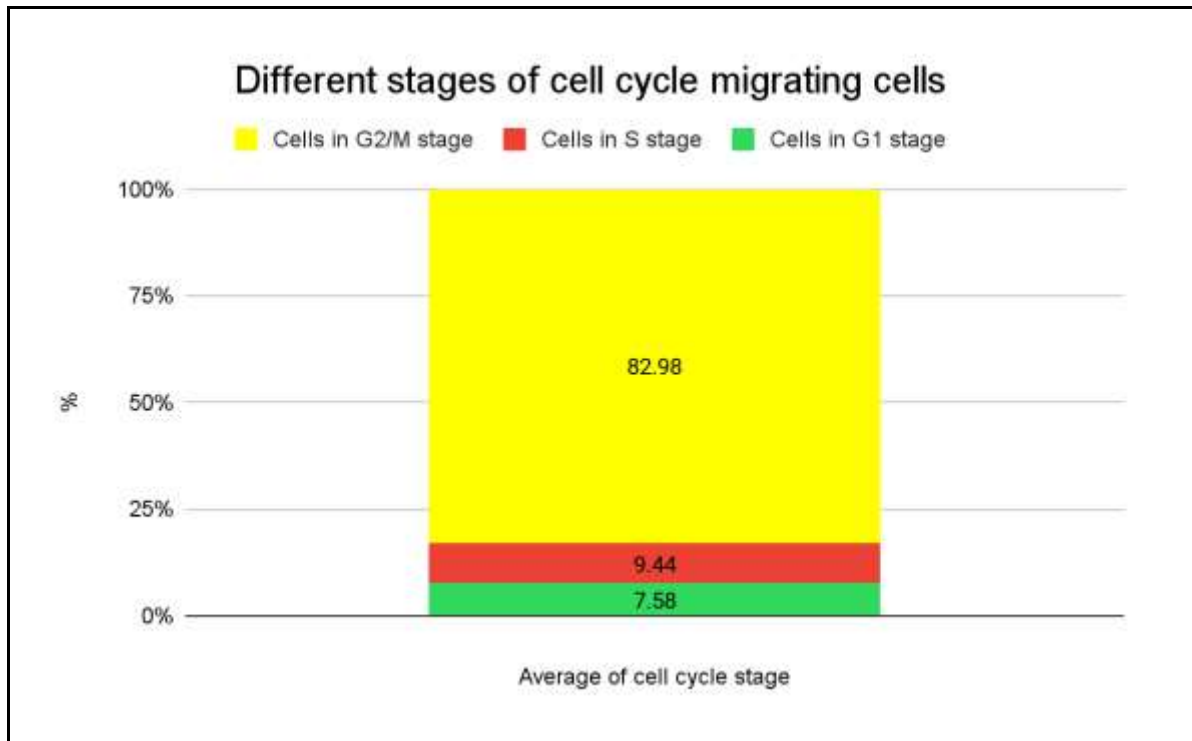
**Table 3.** Number of cells in a specific cell cycle stage found in each disc (n=38), their percentages and averages.

**Table 3** shows the total number of cells per disc and the % of cells in each wing disc. We have also put the average cells per wing disc in each cell cycle stage and the total % of cells per cell cycle stage. As in the first experiment, firstly, we had to count all the cells in each sample of wing discs, in this case 38 samples. After we did the counting, we needed to distinguish, with the help of FIJI, in which cell cycle stage each migrating cell was. After all the counting process was done, we had to make this data table in order to know the average G1, S and G2/M cells per wing disc so that later we could know the % of G1, S and G2/M cells of the total, as it is shown in the data table.

From the results we obtained, we can confirm that most of the migrating cells are in the G2/M phase rather than the G1 or the S phase. This phenomenon is caused because when we induced the tumor the cells had already duplicated its DNA, that is why there are fewer cells in G1 and S phase and more in the G2/ M phase. Another

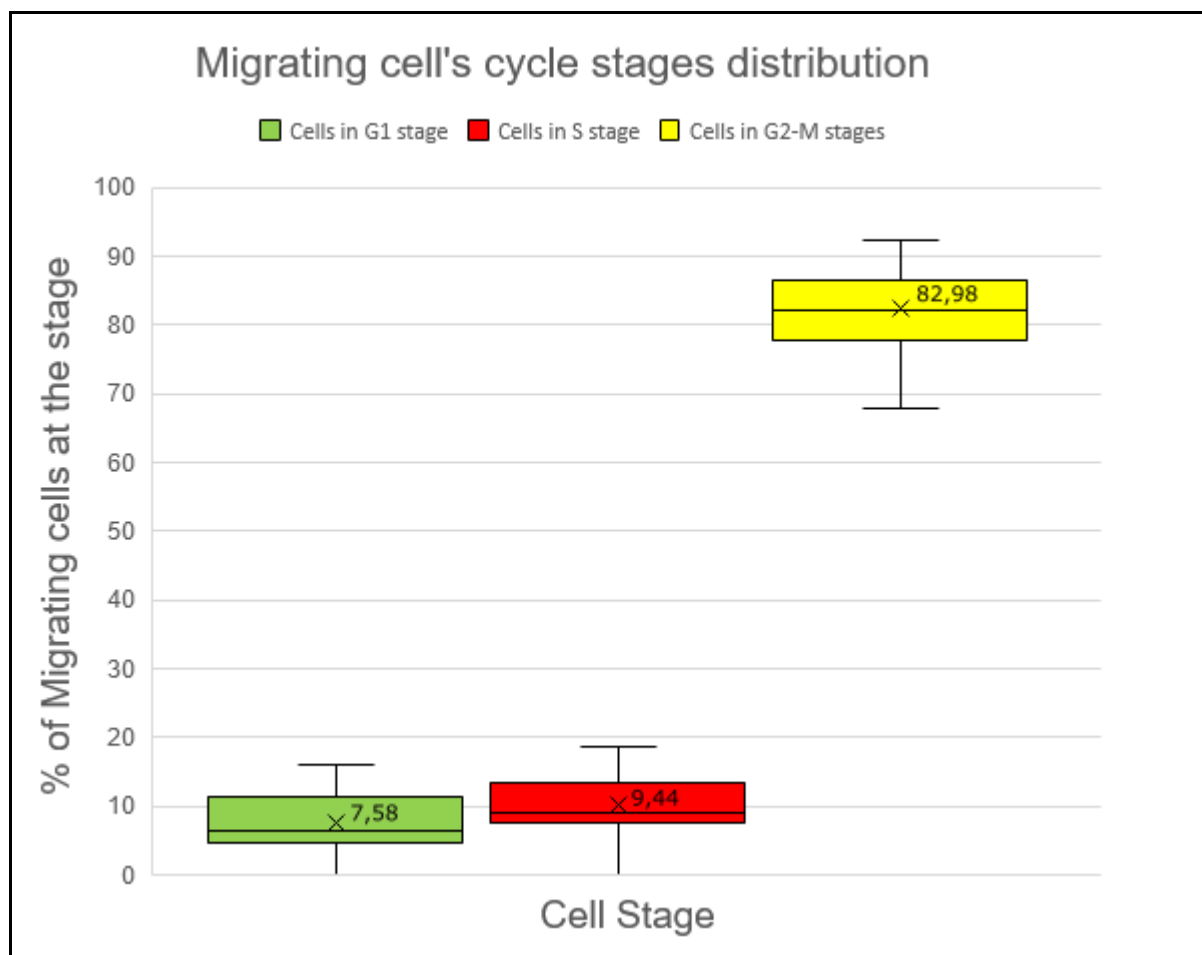
PRACTICAL FRAME

cause, that is related to the first one, is because when a tumor is induced the G1 and S phase shortener making it impossible to divide, for the fact that they will lose a lot of energy, this energy is needed for these cells to migrate so instead of wasting the energy in completing the cell cycle, the cells waste the energy in migrating, so the tumor still remains and survives.



**Fig. 32** In this graphic, we see the average percentage of cells that are in a phase of the cell cycle. We can see that in yellow represents the % of average cells that are in the G2/M stage, in red there are the cells that are in the S stage and in green are the cells that are in the G1 stage, which is the stage with the lowest percentage of cells. The stage G2/M is the stage of the cell cycle with the most percentage of cells, indeed, with a lot of difference. The stage S, that is the stage before the phase G2/M is superior to the G1 (green) stage, not with much difference, but we can see that G2/M stage has a really big difference between the other stages. Own file





**Fig. 33** In this graph, there is the distribution of migratory cells in the stages of the cell cycle, as in Fig.29, we have information of the percentages, such as, the lowest and highest percentage of cells in the same cell cycle stage and in the same wing disc, for example, in G1 stage (green), the lowest percentage is 0 % and the highest 16,13% of G1 cells in the same wing disc. The box, takes into account the percentages of cells from the first to third quartile. The line inside the box is the median value, that is the middle percentage of ranking of all the values, from higher percentage to lower. In case of S stage, this will be 10,34% from all the percentages. The X represents the arithmetic average, for example, in G2/M (yellow) stages, is the 82,98%. We can see that the yellow box has the higher percentage of migrating cells in that stage by a wide difference. The difference between the stage G1 and S is not that big. Own file

What we have seen in the two graphs above, is that, by far, the most abundant state in metastatic cells is the G2/M phase, while the s and G1 phases are much smaller, with little difference between them. This is because, as most cancer cells have already replicated their DNA and then, normally they have passed the S phase, they are in the G2/M phase, in which they must expend a lot of energy to reach the end of the cycle, something that they don't do because they must migrate to grow and proliferate the tumor and if they spend that energy migrating, they can't finish the cell cycle, that's why they mostly stay in the G2/M phase

In the next page, there is a table that tells us about the statistical differences between the percentages of the groups:

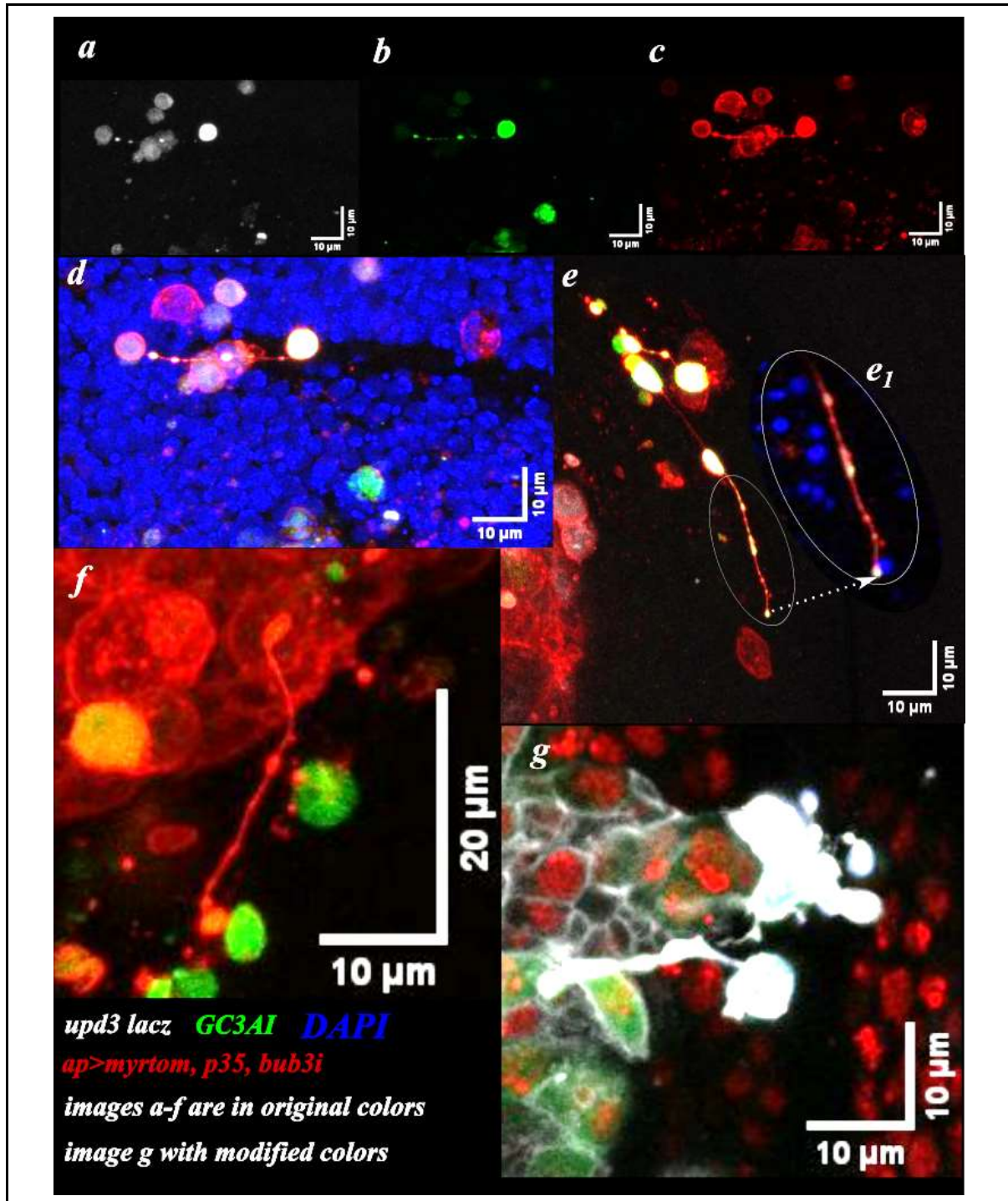
Pair	Difference	p-value
G1-S	2.1639	0.1885
G1-G2/M	75.4	$6.485 \cdot 10^{-11}$
S-G2/M	73.2361	$6.485 \cdot 10^{-11}$

**Table 4.** ANOVA statistical analysis comparing different cell cycle stages.

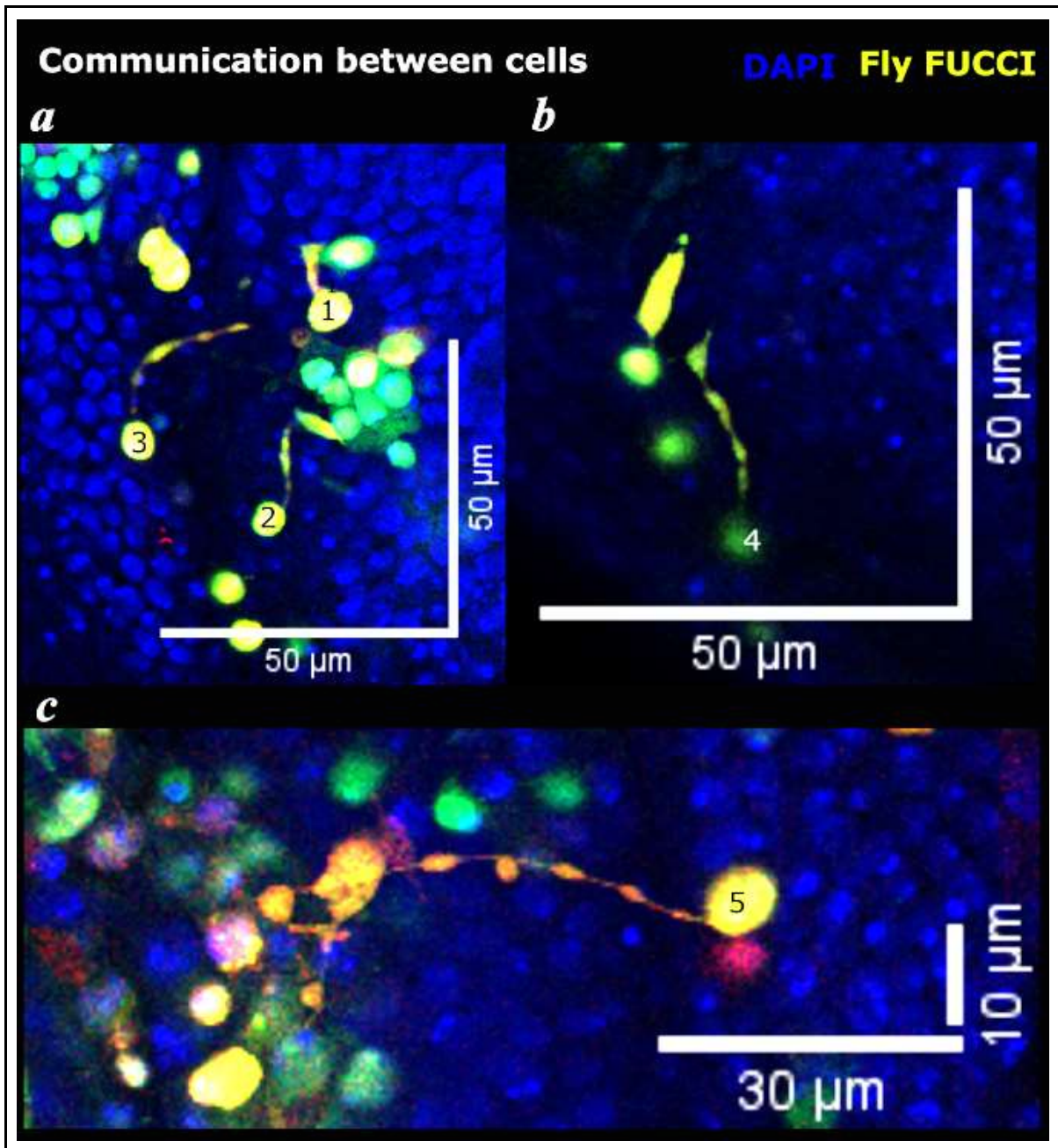
The information we get from **table 4** is that G2/M is statistically very different to G1 and S, as it's p-value is a minuscule number. And their numeric difference is 75,4 and 73,24 respectively.

Not as big but still contrasting is the value between G1 and S, that is about 0,18. ANOVA test, tells us that a smaller difference than 0,5 is statistically significant.





**Fig. 34** This is a collage of pictures taken from the confocal microscope from wing discs coming from experiment 1. All these pictures have filopodia, a structure that makes the cell communicate with other cells. The cells that are green contained GC3AI, the ones that were white *upd3 lacZ*, the blue was due to DAPI and the red was because of *ap>myrtom.p35, bub3i* and made that those cells were carcinogenic. In a, b, c and d we see the same cells but with different color, which just means that contained all the markers, and from those cells, the one in the middle was the one with filopodia and was communicating with the cell on the left. In e, we see a few filopodia, *e<sub>1</sub>* is the largest one, that communicates with one cell below itself. Also, one cell on its top has a filopodia to it and in the top of the image, we can see small filopodia. The image f, shows one red cell which filopodia is communicating with three different cells, which are also different to itself, as the cells to whom it is communicating are green whilst it is red. In g, we can see a filopodia directed towards the group of cells in its left, and the color was modified in order to distinguish better the cell that contains the filopodia. Own file



**Fig. 35** This is a composite image from experiment 2, that shows filopodia. These cells are from experiment 2 and were metastatic cells with Fly FUCCI, the blue color is for DAPI, that allows us to detect all types of cells. In a, b and c we can observe different sized filopodia, in a, we have filopodia 1, that is the shortest one and it is communicating with the cell on its top. 2 is a bit larger and it is communicating with the cell in its top, which it is at the same time in a group of cells. Filopodia 3 is larger but it is not directly touching a cell, so we can speculate that it might be sending signals or trying to reach a cell. In b, we can observe filopodia 4, that is communicating with the top left cell, and we can see that the last part of the structure is touching the other cell through a thin extension. Finally, in c we observe the largest filopodia, that has a large extension that is communicating with a few cells at the same time. Own file

Here we have some images of filopodia in migrating cells, that are moving through the surrounding tissues, communicating with neighbor cells and sending signals in order

to establish them in new areas. We see that this happens in both experiments and, what's more, it happens several times and on different wing discs. This might be for different reasons but what we think that happens is that the metastatic cells want to emit some kind of signal so that the other cancerous cells can also migrate.

## CONCLUSIONS

Taking into account the results obtained from experiment 1, we can firmly conclude that, in the primary tumors that we induce with different techniques in the wing discs of different *Drosophila melanogaster* specimens, in this case 13, there is heterogeneity since we have seen that there are at least 4 different types of cells that are arisen from the first primary tumor. By counting all the migrating cells, we concluded that the considerable majority of all the migrating cells were double positive (36.47%), meaning that they were positive for both JAK and JNK, JAK-STAT signalling pathway involved in immunity processes, cell division, cell death and tumor formation and JNK regulates cellular processes and activities involved in cancer such as migration and invasion, so we can speculate, that these two together provides advantage on these cells for proliferation and migration, so the tumor can spread faster and greater effectiveness. The percentage of the migrating positive cells in only one of the factors is practically the same on both (18%), and the percentage of the ones that are negative is around 28%.

The negative means that these may be positive for some markers we do not know because we are not looking or studying others, so we say it is negative for GC3Ai and upd3 markers.

We can assume that JNK and JAK together give a great advantage if they are both together, over the others. JAK and JNK for themselves are not so abundant and therefore not so advantageous. On the other hand, those cells that are negative, which means, that may have other markers, do not give them so much advantage to the migrating cells, as a consequence, they are not as abundant as double positive.

As not all flies develop at the same speed, and therefore neither their wing discs nor their cells do so at the same time as the other flies, there are wing discs that have more migratory cells than others. Despite this, all the samples have properly developed the tumor and all have at least 32 migratory cells.

The control went as it should, there was nothing strange and everything was normal and as it should be, there was not a single cell from apterous, from where we put the genes that had the markers present, we know because no marked cell was separated from the others. So the control was perfect.



In this second experiment, the control went as it should, the cells in the wing disc of the control because the apterous Fly FUCCI was in the gens, but we didn't put any tumor. All the cells are in the dorsal part, meaning that there were no migrating cells, we can also observe that there are cells from all the cell cycle stages, which are expressed in different colors. The G1 phase is expressed in green, the S phase is expressed in red and the G2 and M phase are expressed in yellow, and they were all equally distributed, there was not a majority of cells that were in the same stage.

The experiment without the control had 38 wing discs which showed migratory cells. The total number of migrating cells from experiment 2 was 1114 and the average of migrating cells per wing disc was 29,31. All the experimental wing discs had developed a tumor on it and all had metastatic cells, from those cells that were migrating, there was variety, but all the stages of the cell cycle were present. The control had 2 wing discs and everything was normal, there was presence of Fly FUCCI and no cell passed from the dorsal part of the wing disc. As we did in the control of the experiment 1, everything was normal and the only difference is that 1 had different markers and 2 contained Fly FUCCI, nothing strange was found in any of the control wing discs.

The average of cells per wing disc in stage G1 was 2,19 and the average of cells in the phase S was from 2,77 , while the average of migrating cells in the stage G2/M per wing disc was from 24,35 cells. And the percentages of each group were 7,58 % of cells in stage G1, 9,44 % of cells in stage S and 82,98 % of cells in stage G2/M.

We see that the most abundant stage where metastatic cells stayed in was G2, and moreover, statistically significantly different from the others. And there was not as much difference between G1 and S.

We know that a great majority of cancer cells have done replication, so they passed G1 and S phase, so these are in G2 stage, from which these cannot, pass from this stage, as they spend a lot of energy migrating, they no longer have energy to finish the cell cycle, that is why many remain in the G2/M phase, those that have not replicated their genetic information, are in the G1 or S phase, and they are pending to replicate their DNA. When cancerous cells migrate, they do not replicate their DNA, which means that, putting drugs that stop DNA replication will only make the size of the primary tumor smaller, and it will not have any effect on the migrating cells. This is why studying the features of G2/M stalled cells, we can derive new knowledge about how to kill metastasis and eventually, open a new research line.

## CONCLUSIONS

We also conclude that filopodia is a very common structure found in cells, in this case, cancer cell as we detected some of them in both experiments and in different wing discs, so we think that it might be a way that cells use to communicate and transmit signals between them, either for a function, an order, some exchange of matter, etc. With this, we have seen that filopodia are common in cancer cells and they might also use it to make cells migrate to other territories, so it might be a reason why cancer cells migrate.

Our objectives were the following:

- *To know how cancer acts in a primary tumor.*
- *To understand how to work in a scientific research laboratory.*
- *To find out what are the factors that influence the emergence of the different populations of cancer cells.*
- *To learn how to do research on *Drosophila* as a model.*
- *To recognize the stages of cancer.*

We have completed all of them, because we have learned the behavior of primary tumors and cancer and its stages, we went to a professional scientific research laboratory and worked there for some weeks and as well, we worked with *Drosophila*.

Our initial question was: *What are some of the different cancer cell subpopulations arising in Chromosomal Instability induced tumors, and what are their molecular signatures, what insights do we get from the fly model?*

The hypothesis we made was that: *There must be some identifiable cellular factor other than time that influences the diversification of cancer cells.*

We found out that the factors that influence the emersion of the different populations of cancer are the chromosomal instability, the cancer factors that are present in cancer cell in nature and the heterogeneity that these cause. The molecular signatures that are present is the change of DNA that happen in cancer cells, and that these changes may be different from one cell to another.

In cancer cells, tumorigenic proliferation and replication undergoes with a lot of stress and makes more mistakes in the DNA. So, their molecular signatures are the different DNAs of the cells.

The stages of the cell cycle are one of the factors that affect cancer, being G2/M stage the most abundant one.

The subpopulations of cancer cells, are the groups of cells which contain JNK and JAK, only JAK, only JNK or neither of those, we also saw that there are other subpopulations which do not contain these. Therefore, there were some subpopulations of cancer we identified but we do not know what they contain.

In short, our hypothesis is verified, as we have explained, there are some factors rather than time that have great influence in the diversification of cancer cells, their subpopulations and that are part of their molecular signatures.

What these results mean for cancer field and research is that, by knowing that JNK and JAK are present in 71,62 % of metastatic cell, according to our results, by themselves or both together, if some way is found to make these cells not develop or some way to inhibit them, it would mean a great advance in the treatment of cancer, since a large part of the migratory cells would be eliminated.

The same is applied to the results of the second experiment, because, knowing that around 83 % of the metastatic cells are in the stage G2/M of the cell cycle, can be useful information for research. With the knowledge that the 83% of metastatic cells are in the last two stages of the cell cycle, we can aim for the cells that are in the G2/M phase and attack them with some types of drugs making the cells die and therefore, reducing the extension of the tumor.

In addition, knowing that the malignant tumor cells we induced were done by bub3i and p35, this gives us information about how a cancer might be arisen, so new research lines about how to avoid or prevent bub3i and p35 or a way to eliminate those, so definitely eliminate the cancer, would be an interesting cancer research line.

Lastly, we have observed that double positive cells, have a more resistant membrane enabling them to migrate because of their resistance of osmotic and media changes, making those cells reach other places. So, with this information, we thought that it might be some relation between the double positive cells and their abundance in the metastatic cells, in addition to their emission of filopodia, which make them transmit messages to other cells to also migrate. The elasticity of the membrane is the factor that makes the cell more resistant to the osmotic changes and to the membrane deformation, which makes it more advantageous for the cell to migrate. Opening a new research field to know how to disable the signals that make the membrane of those double positive metastatic cells more resistant, will make the migrating process more difficult and it could be an interesting advantage in cancer research.



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