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From Drosophila to humans: Myc-mediated clone competition as an evolutionary trait of tumour progression

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Abstract

Cell competition is a phenomenon first reported in *Drosophila melanogaster*, proven to exist also in mammals. It describes the result of a mechanism of fitness comparison undertaken by cells inhabiting the same tissue, that leads to the elimination of the weakest cells and, in the physiology, to the formation of a homogeneous organ. Over the years, many molecules have been identified that are involved in cell competition and among them MYC oncoprotein: from *Drosophila* to mammals, cell populations characterised by higher expression of MYC induce apoptotic death of the neighbours, allowing the fittest to acquire an advantage in space occupancy.

MYC-mediated cell competition has been found to be at work with a dual role in *Drosophila* epithelial tumours: it seems indeed to play tumour suppressive functions at cancer onset and to support cancer growth in frankly malignant masses. Clear evidence about its occurrence in human cancers is however still missing and, above all, nothing is known about a possible role for cell competition in cancer evolution.

My work defined the presence of markers of MYC-mediated cell competition in primary and secondary human carcinomas and demonstrated through experiments in human cancer cell lines that MYC modulation is *per se* sufficient to induce competitive behaviours in both genetically distant and identical cells. Noteworthy, MYC under-regulation in the fittest cell line is sufficient to undermine its competitive *status*, suggesting a role for MYC-mediated cell competition in the selective growth of tumour clones and, as a consequence, in cancer evolution. In addition, I was able to demonstrate a functional cooperation between MYC and p53 in this phenomenon.

The data obtained in the *Drosophila* model, where MYC over-expressing and MYC knock-down clones have been induced within a growing tumour, suggest that MYC-mediated cell competition is normally at work in these malignant cells, and it shapes cancer evolution through the elimination of the less fit cells (with lower levels of MYC) and the expansion of the most performant ones (with higher levels of MYC), demonstrating an evolutionary role played in defining the composition and the size of the final mass.

Altogether, my results show that MYC-mediated cell competition plays a role in the selection of the most performant cells within the tumour and represents an important step towards the understanding of the evolutionary mechanisms underlying tumourigenesis, defining for the first time the amazing contribution of cell competition to tumour progression.

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Chapter 1

Introduction

The evolutionary transition from the unicellular to a multicellular organisation led the organisms to acquire several benefits coming from increased complexity, functional specialisation of cells and increased size. Many requirements for multicellular organisation, such as cell adhesion, cell-cell communication and coordination and programmed cell death (PCD), have been developed in time and finely tuned through regulatory circuits. Multicellularity is characterised by cooperation among cells for the development, maintenance and reproduction of the organism. Complex multicellularity and cooperation underlying it have evolved independently multiple times [1].

Cells are carefully programmed to collaborate in the creation and maintenance of the diverse tissues, that make possible organism survival.

The maintenance of complexity depends on a multitude of strategies and involves wound repair and replacement of cells that have suffered attrition after extended periods of life. Breakdown of this equilibrium can lead cells to escape from developmental constraints, resulting in uncontrolled proliferation and altered behaviour [2].

Such changes make the cells incompatible with the assigned roles in structure and physiology, as cells no longer obey the rules that drive normal tissue construction and maintenance, and undergo various diseases, among which cancer.

Despite the extraordinary safeguard mechanisms adopted by the organism to prevent their appearance, cancer cells seem to have just one aim: making copies of themselves [3].

Cancer figures among the leading causes of morbidity and mortality worldwide (second in 2013), with approximately 14.1 million new cases and 8.1 million cancer-related deaths reported in the World Cancer Report 2014. The number of new cases is expected to rise over the next two decades.

Among men, the 5 most common sites of cancer incidence site reported in 2014 were lung, prostate, colorectum, stomach and liver; among women the 5 most common sites were breast, colorectum, cervix, lung, and stomach [4].

The heterogeneity and complexity of cancer led to the accepted notion that it represents a large group of different diseases involving dynamic changes in the genome.

It is now widely accepted that it results from the clonal accumulation and cooperation of various genetic and epigenetic lesions causing the progressive transformation of normal somatic cells into malignant derivatives which can grow, invade and migrate uncontrollably.

Two general classes of genes are typically affected in cancer cells: oncogenes and tumour suppressor genes (TSGs).
The first are aberrantly activated and represent dominant gain of function (GOF) mutations of genes (proto-oncogenes) generally involved in growth and division promotion and protection against PCD.
TSGs are inactivated in cancer cells, resulting in loss of normal cellular functions such as accurate DNA damage repair, control over the cell cycle, cell polarity and adhesion within tissues [3].
Tumour is a cell disease, and all tissues are composed of cells and cell products, so virtually cancer can arise in any tissue, but those of epithelial origin, called carcinomas, are the most frequent form, representing about 90% of all human malignant tumours. These tumours are responsible for more than 80% of the cancer-related deaths in the western world.

Moreover, tumours are capable to move within the human body: in many patients, multiple secondary tumours are discovered even distant from the primary site as a consequence of the tendency of cancer cells to spread throughout the body and establish new malignant colonies.

The first carcinogenic events generally lead to local hyperplasia with possible consequent formation of metaplasia and dysplasia. From this stage the lesion can evolve in an *in situ* carcinoma, which already exhibits strong alterations in cell physiology and behaviour, such as loss of differentiation, loss of apical-basal cell polarity and loss of tissue organisation, but the lesion is still confined within a specialised type of extracellular matrix (ECM), the basement membrane (or basal lamina), an acellular sheet that separates epithelial cells from the underlying layer of supporting connective tissue, the stroma.
At this stage, tumour-induced angiogenesis can also be observed, with neo-vascularisation of the neoplastic mass.
Finally, the tumour becomes invasive when basement membrane degradation occurs and cells disseminate to the neighbouring tissues. These cells can eventually intravasate into lymph or blood vessels, allowing their passive transport to distant organs.
At secondary sites, carcinoma cells can extravasate seeding micrometastases that in some cases will form secondary lesions, the final step of this sequence of events often termed invasion-metastasis cascade [3].

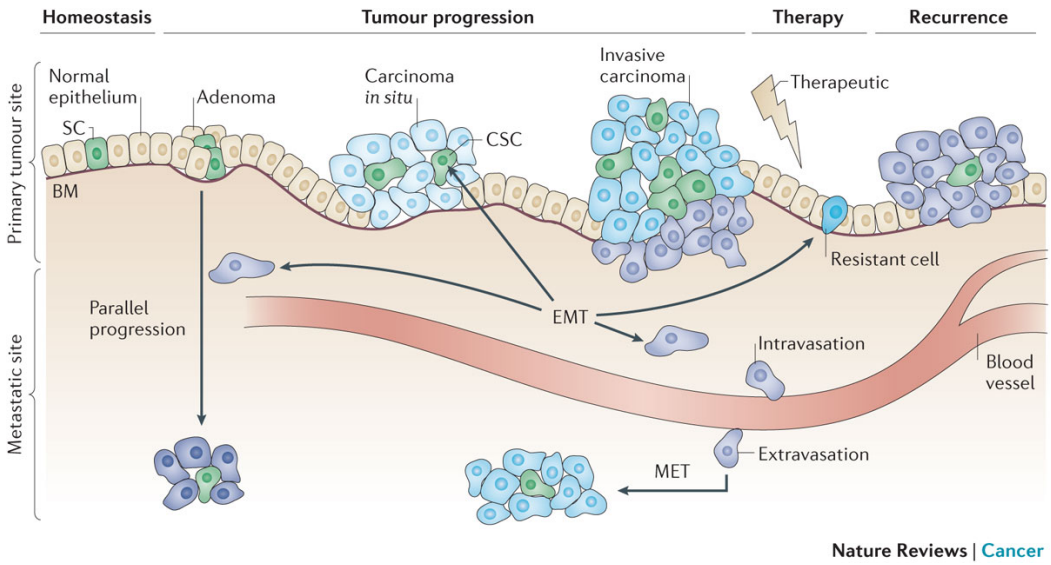


Figure 1.1: Epithelial tumour progression and evolution [5].

1.1. THE HALLMARKS OF CANCER

Cancer research has generated a rich body of evidence, revealing carcinogenesis as a multistep process, in which each step enables the cell to collect genetic alterations that, at the end, drive the transformation of normal human cells into malignant derivatives.

The number of cancer-associated genes identified has surpassed the original prediction of the gene mutation theory, according to which only a limited number of key cancer genes was thought to be responsible for cancer onset. In order to categorise all cancer-related mechanisms, to understand the common biological basis for the huge number of gene mutations involved and to reconcile the differences between theoretical prediction and clinical fact, various biological capabilities and enabling characteristics of cancer, shown to facilitate tumour growth and metastasis, have been summarised in eight hallmarks [6].

As tumour development proceeds *via* a process formally analogous to Darwinian evolution [7], the succession of genetic changes, eventually conferring some advantages, leads to the progressive conversion of normal human cells into cancer cells.

There are more than 100 distinct types of cancers and several subtypes of tumours can be found within specific organs. But, despite this remarkable diversity, it is possible to identify essential physiologic alterations acquired by cells in order to progress towards malignancy [8].

Hanahan and Weinberg proposed eight common traits of cancer, also known as the hallmarks of cancer: sustaining proliferative signals, evading growth suppressors, resisting

cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis [8].

The hallmarks of cancer, shared by most human tumours, constitute an organising principle that provides a logical framework for understanding the remarkable diversity of neoplastic diseases.

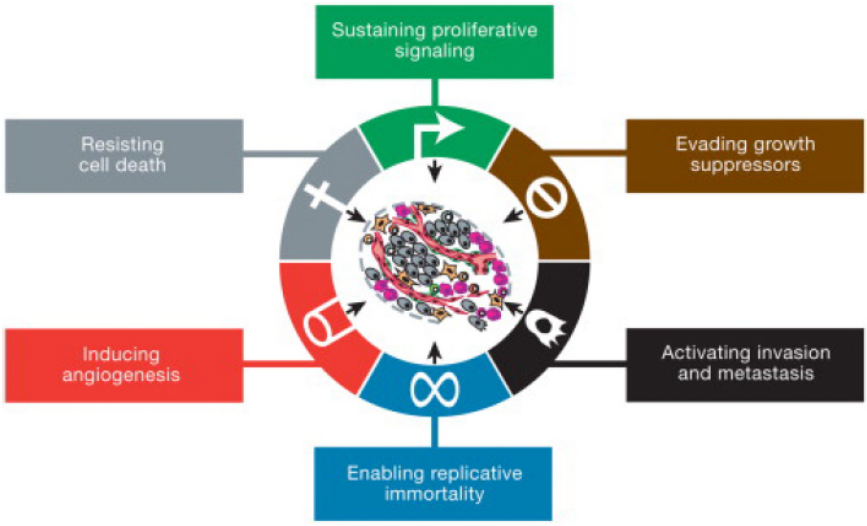


Figure 1.2: The six Hallmarks of cancer [8] proposed by Hanahan and Weinberg in 2000.

Self-Sufficiency in Growth Signals

The main feature of cancer is abnormal proliferation. No type of normal cells can proliferate without mitogenic growth signals (GS) transmitted by transmembrane receptors, allowing cells to move from a quiescent state into an active proliferation state characterised by stimulating growth, cell cycle entry and cell division.

Tumour cells generate many of their own growth signals, by reducing dependence from their normal tissue microenvironment. In fact, many cancer cells can acquire the ability to synthesise growth factors to which they are responsive, creating a positive feedback signalling loop often termed autocrine stimulation [9]. Cancer cells become independent through constitutive activation of components involved in proliferation, followed by oncogene deregulation. This is probably best exemplified by the Ras/MAPK (Mitogen Activated Protein Kinase) signalling cascade, as activating mutations of its members are found in a significant percentage of human cancers: as an example, RAS proteins are present in structurally altered forms, which in turn activate mitogenic proteins, such as MYC [10].

Of note, growth deregulation is carried out by both cancer cells and normal tissue surrounding the tumour mass [8] [9].

Insensitivity to Anti-Growth Signals

Tissue homeostasis is also maintained by powerful anti-proliferative programmes that cancer cells need to modify for proliferation to occur.

Anti-proliferative signals include either soluble growth inhibitors and immobilised inhibitors embedded in the extracellular matrix and on the surfaces of nearby cells.

Anti-growth signals block proliferation using two strategies: force cells to enter G_0 phase or a post-mitotic state, usually associated with the acquisition of specific differentiation-associated traits. Relief of proliferation inhibition is generally obtained through a LOF of TSGs; inactivating mutations of these genes are a common feature of all tumours. Inhibition of terminal differentiation, allowing the cells to turn to a fully proliferative phenotype, is rather achieved through oncogene activation [8] [9].

Evading Apoptosis

Further to the over-expressed pro-growth proteins and the insensitivity to the anti-growth signals, the ability of the tumour cell population to expand is strongly determined by its capacity to evade PCD.

The apoptotic programme is present in latent forms in all cell types. Once activated by several physiologic signals as chemical and mechanical stresses, hyper-proliferation, elevated levels of oncogene signalling and DNA damage, PCD, composed by a series of steps, is achieved in a span of 30-120 minutes: cellular membranes are disrupted, the cytoplasmic and nuclear structures are broken, the cytosol is extruded, the chromosomes are degraded and the nucleus is fragmented, while the remaining cell corpse is engulfed by nearby cells and disappears within 24 hours.

The apoptotic machinery can be broadly divided into two classes of components: sensors, which recognise pro-apoptotic signals and induce the activation of effectors, which respond by releasing in the cytosol the mitochondrial cytochrome C, a potent catalyst of apoptosis.

The way most tumour cells use to evade apoptosis is based on the increased expression of anti-apoptotic proteins as Bcl-2, and the inactivation of pro-apoptotic genes through which p53 is a well-known example. The p53 tumour suppressor protein can enable apoptosis by

upregulating pro-apoptotic Bax in response to DNA damage, so stimulating mitochondria to release Cytochrome C [8] [9].

Limitless Replicative Potential

Although tumour cells can acquire autonomy from the growth signals, insensitivity to anti-growth signals and resistance to apoptosis, these features do not ensure expansive tumour growth. Almost all mammalian cells are able to undergo a limited number of growth and division cycles before irreversibly entering a non-proliferative status named senescence, as they have an intrinsic cell-autonomous programme that limits their multiplication. Work on cell cultures demonstrated a finite replicative potential, after which cells stop growing and undergo senescence [12]. Senescence can be circumvented in cultured human fibroblasts by disabling pRB and p53 tumour suppressor proteins, following which these cells enter a crisis state. The crisis output is characterised by massive cell death, karyotypic disarray associated with end-to-end chromosome fusions and (occasional) cellular immortalisation.

Moreover, cell cycle seems to be linked to telomere ends. Each cell division, telomeres undergo progressive shortening until they reach a critical length at which proliferation stops.

Telomere maintenance is evident in all types of malignant cells [13]: in most of them the telomerase enzyme, a special DNA polymerase which adds hexa-nucleotide repeats onto the ends of telomeric DNA, is upregulated [8] [9].

Sustained Angiogenesis

Oxygen and nutrients are crucial for cell function and survival, obligating all cells to reside within a small distance from a capillary blood vessel. The development of new blood vessels - the process named angiogenesis - is transitory and carefully regulated.

Cells composing an aberrant proliferative lesion initially lack angiogenic abilities, curtailing their capability for expansion. To progress to a larger size, incipient neoplasia must develop angiogenic ability [8] [9].

There are positive and negative counterbalancing signals to regulate angiogenesis. The initiating signals are mediated by vascular endothelial growth factors (VEGF) and fibroblast growth factors (FGF1/2): first evidence about this was reached when anti-VEGF antibodies were used to block VEGF molecules during neovascularisation and growth of subcutaneous tumours in mice [8] [9].

Grifoni and colleagues have defined in *Drosophila,* where the oxygen is conveyed to the internal organs through an interconnected tubular network called "tracheal system", that the regulation is significantly analogue to that of mammalian angiogenesis: in an epithelial cancer model, *Drosophila* FGF/FGFR (FGF Receptor), encoded by the *branchless* (*bnl*) and *breathless* (*btl*) genes respectively, carry out the functions of VEGF/VEGFR in inducing angiogenesis [14].

Tissue Invasion and Metastasis

Invasion and metastasis are exceedingly complex processes, and their genetic and biochemical determinants remain not explained at all in their complexity.

During the development of most types of human cancer, primary tumour masses can spread invading adjacent tissues or distal sites, where they may form new colonies. Metastases are responsible for the 90% of cancer mortality. The invasive and metastatic process is a multistep mechanism. The process begins with local invasion of pioneer cells from the primary mass to the nearby surrounding tissues, followed by intravasation of cancer cells into blood or lymphatic vessels where they transit till they are stopped. Cancer cells can reach the parenchyma of a tissue (through a mechanism termed extravasation) followed by the formation of small micromasses, and if the microenvironment features are permissive, they may grow into a macroscopic tumour (colonisation).

To allow cell intravasation, it is important that the epithelial cells change their programmed information, acquiring the ability to invade, to resist apoptosis and to disseminate. This happens because cells develop a new regulatory programme, named the "epithelial-mesenchymal transition" (EMT).

The EMT programme can be transiently or stably activated by cancer cells during invasion and metastasis, orchestrated through a set of pleiotropically acting factors. Evidence from developmental genetics indicates that contextual signals received from neighbouring cells are involved in the activation of these factors. In the invasive and metastatic process, a crosstalk between cancer and stromal cells is involved. This observation indicates that the phenotype of high-grade malignancies do not arise in a strictly cell-autonomous manner, and their behaviour cannot be simply understood through tumour genome analysis.

Other two mechanisms of invasion have been identified: collective invasion, where cells migrate in clusters, and amoeboid invasion in which individual cells slide through existing interstices.

In some types of cancer, the primary tumour may release systemic suppressor factors that render such micro-metastases dormant, as revealed clinically by explosive metastatic growth. Other macroscopic metastases may erupt decades after a primary tumour has been removed.

Clinical evidence shows as individual types of carcinoma form metastases only in a limited subset of target organs, for a phenomenon called “tissue tropism” or “homing”. The reasons for this selectivity are still not completely understood. Substantial progress is currently being made to define a metastatic signature of gene expression changes which correlate with the establishment of macroscopic metastases in specific tissues. This would also help clarify how metastasis-promoting genetic alterations are selected within the primary tumour: whether these mutations are also beneficial to primary growth and thus are fixed before cells start to disseminate, or whether they are stochastically accumulated as “passenger" mutations that only subsequently give an advantage before to be fixed. Alternatively, cells may leave the tumour in a partially metastatic-competent condition and further evolve once they experience the new environment-associated pressure. Indeed, an increasing body of evidence seems to point towards a parallel progression model for certain types of carcinomas, according to which mutant cells disseminate relatively early, from pre-neoplastic lesions, and undergo genetic diversification and selection within the distant target organs, thus showing a rather different genetic profile with respect to the primary tumour [8] [9].

Enabling Characteristics

When we talk about cancer we have always to consider the acquired functional capabilities that allow cancer cells to survive, proliferate and disseminate; these functions are made possible by enabling characteristics that drive cells to switch into a cancer state. Most prominent is the development of genomic instability, which can generate random mutations which allow cancer cells to acquire multiple hallmarks. In fact, despite the extraordinary capacity of the genome control systems used to solve DNA defects, during the first stages of tumourigenesis cancer cells increase their rate of mutation by increasing cell cycle speed.

Another important feature of cancer cells is the acquisition of an inflammatory state of pre- and malignant lesions, driven by the immune defense system, used to promote tumour progression.

In fact, evidence shows as tumours are densely infiltrated by cells of either innate and adaptive immune system. Paradoxically, the tumour-associated inflammatory response enhances tumourigenesis by supplying bio-active molecules to the tumour microenvironment, including growth factors that sustain proliferative signalling, survival factors that limit cell death, pro-angiogenic factors, extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion and metastasis, inducing signals that lead to activation of EMT. The surrounding inflammation generates the conditions for the formation of a pre-cancerisation field, in which the future tumour cells switch to an active state [11].

Emerging Characteristics

A characteristic which plays an important role in cancer expansion and aggressivity is cancer cells' capacity to reprogramme metabolism. In fact, even in presence of oxygen, these cells switch to a less efficient glycolytic metabolism, the so-called Warburg effect. The glycolytic afflux can be regulated by oncogenes such as MYC and RAS (as well as a hypoxic condition). The significance of this is not fully understood, but an explanation can be found in the fact that these cells, following to fitness comparison with *wild-type* cells, may over-proliferate and colonise the great part of the tissue [15]. A second emerging hallmark in tumour formation involves mechanisms of immune evasion, as anti-tumour immunity creates a significant barrier to tumour formation and progression [11].

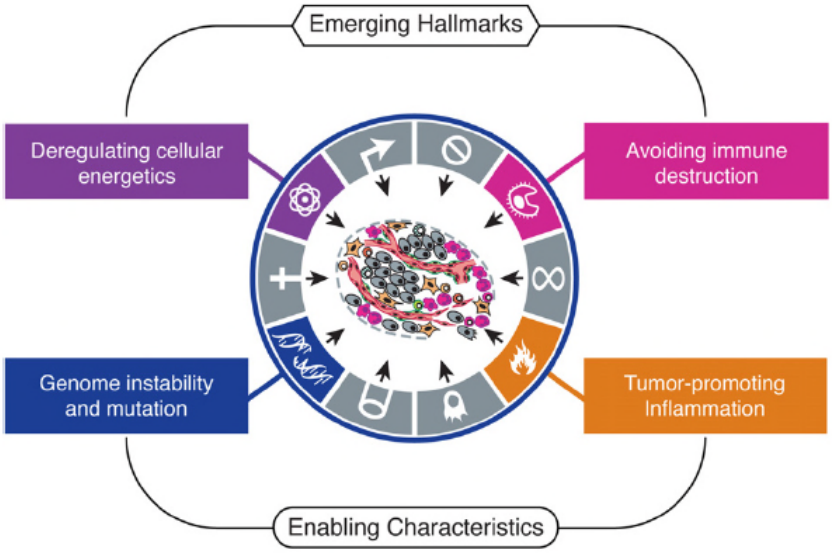


Figure 1.3: The Emerging Hallmarks and Enabling Characteristics [11] Hanahan and Weinberg added to their 2011 review: *Hallmarks of Cancer: The Next Generation.*

1.2. CANCER EVOLUTION

Cancer can be considered as a reiterative process of clonal expansion characterised by genetic diversification and clonal selection within the adaptive landscapes of tissue ecosystems. The current molecular cancer research is based on the "gene mutation theory" based on the evidence that gene aberrations and stepwise gene mutations drive cancer progression. While the hallmarks of cancer have been influential as they provided a rationale for clinical approach in cancer research, cancer dynamics cannot be lonely summarised by isolated and fixed molecular principles [6], as neoplasms are microcosms of evolution [7]; in the same way, the laws that rule cancer mutations are highly mutant.

Within a neoplasm, a mosaic of mutant cells compete for space and resources, evade predation by the immune system and cooperate to colonise new organs.

The idea of cancer as an evolutionary disease is not new. A tumorous mass is a heterogenic population in terms of genetics and epigenetics, and obey the Darwinian evolutionary laws. Organism-level and gene-level evolution led to achieve general tumour-suppression mechanisms and oncogenic vulnerabilities in our genomes through several levels of selection: neutral and non-neutral mutations, genetic drift, epigenetics, natural and artificial selections, colonisation and dispersion [7]. Mutations modify cell fitness and affect cellular fate through genetic drift and Darwinian selection by which the tumour mass can survive and expand itself. Cell fitness is shaped by its interactions with cells and other factors in its microenvironment, as the reproductive power of species depends on the environment [7].

Various forms of mutation have a role in the neoplastic progression. Studies of heterogeneity in tumours show that there is extensive cytogenetic, genetic and epigenetic variability in cancer cell populations, and the degree of variability can predict progression towards malignancy.

Genetic instability generates genetic heterogeneity, and this point may be recognised as a hallmark of cancer. Each clone in each cancer in each patient has a unique genome profile [16]. Researchers have shown that the type of environmental insults selects against the checkpoints that they trigger, as cells that lose checkpoints can reproduce more quickly than non-mutated populations [17].

Mutation frequency studies and measurements in cell culture calculated the sequence mutation rate at 10^{-6}-10^{-7} per *locus* per cell generation [18]. The estimation of number of mutations necessary to cause cancer is about 3-12 mutations for different forms of cancer [19]. But a genetic mutation might not always affect cells, as most of them can be neutral

mutations, so the spontaneous rate of somatic mutation is not high enough to generate the many mutations needed for neoplastic expansion. To solve this paradox, also known as the Loeb paradox, two hypotheses have been proposed: a genetically unstable phenotype increasing mutation rate might arise, or clone expansion generates target populations large enough to produce the necessary subsequent mutations [20], both resulting in a big heterogeneity of clones and cancers. To explain this great heterogeneity, over the decades, researchers have developed several theories based on the consideration of the tumour as a living organism in continuous interaction with its surrounding environment.

Two of the most quoted hypotheses are based on the **clonal evolution theory** and the cancer **cell plasticity theory**. Both theories, strongly connected to each other, explain how tumour clones achieve a high degree of cellular, phenotypic and functional heterogeneity.

Through mechanisms of cellular plasticity cancer cells can acquire functions and roles distant from their committed fate that, through typical Darwinian evolutionary mechanisms, result in the selection of aggressive clones characterised by high cell fitness and clonal expansion.

1.2.1. Clonal Evolution

As Peter Nowell showed in his work about tumour evolution, cancer is driven by stepwise somatic-cell mutations with sequential sub-clonal selection within tissue ecosystem [16].

Given the strong selective pressure imposed by the microenvironment surrounding cancer cells, just a little proportion of mutated cells can undergo neoplastic events that are not stalled and aborted.

In advanced malignancy, because of limited resources, cancer-suppressive mechanisms and potent artificial selection in form of drugs, the time used for cancer symptoms to emerge is quite long (depending also on the primary location).

The advanced malignancy growth respects the Gompertzian function [21], according to which cancer cells doubling time (1-2 days) is much faster than tumour doubling time (around 60-200 days). The Gompertzian growth explains the long time period usually required for cancer symptoms to emerge and the time needed to provide a positive selection of variant cells able to resist and overgrow. So, natural selection in tumours takes place, in the same way as selection in organisms, by competition for space and resources.

Cancer happens because of the exposure of the cells to genetic instability that could create mutations. But mutation rate varies substantially between different genomic regions [22] and the mutagenic processes are essentially blind or non-purposeful [23], and clones

evolve through the interaction of selectively advantageous driver lesions (gain and/or loss of functions), selectively neutral lesions (also known as *passenger*) and deleterious lesions. But mutated cells have an increased mutation rate, the meaning of this is that some of the phenotypic silent mutations or the *passenger* lesions drive other genetic changes allowing clonal expansion. The driver mutations give, as in the case of glioblastoma or pancreatic cancers, an average fitness advantage of only 0.4 % (using a non-spatial population genetics model to quantify the selective advantage provided by driver mutations) [24]. So the dynamics of somatic evolution depends on the interaction of mutation rate and clonal expansion.

The model of clonal evolution suggests that a series of clonal expansions grow to dominate the neoplasm (for the mechanism of clonal sweeps), until a next mutation is achieved. If or when the second mutation occurs, the expansion of both clones is restrained by mutual competition (clonal interference). Given the large population size and the high mutation rate typical of neoplasms, clonal competition is probably common [26] [27].

The scientific community is long debating about the way mutations have to occur to allow clonal evolution. The two main theories are based either on the *gradualism theory*, by which lesions accumulate over time in undetected sub-clones that finally appear following neoplasm expansion, or on the *punctuated equilibrium theory*, by which few big mutational events generate multiple lesions across the genome.

The latter theory is most accepted simply because the impossibility, in almost all cancers, to find out intermediate clones (in B-cell chronic lymphocytic leukaemia the frequency is <0.001%) to prove the gradualism hypothesis.

However it remains unclear whether sub-clone diversification reflects the impact of driver mutations and selective advantage, genetic drift of selectively neutral mutations or epigenetic alterations [27].

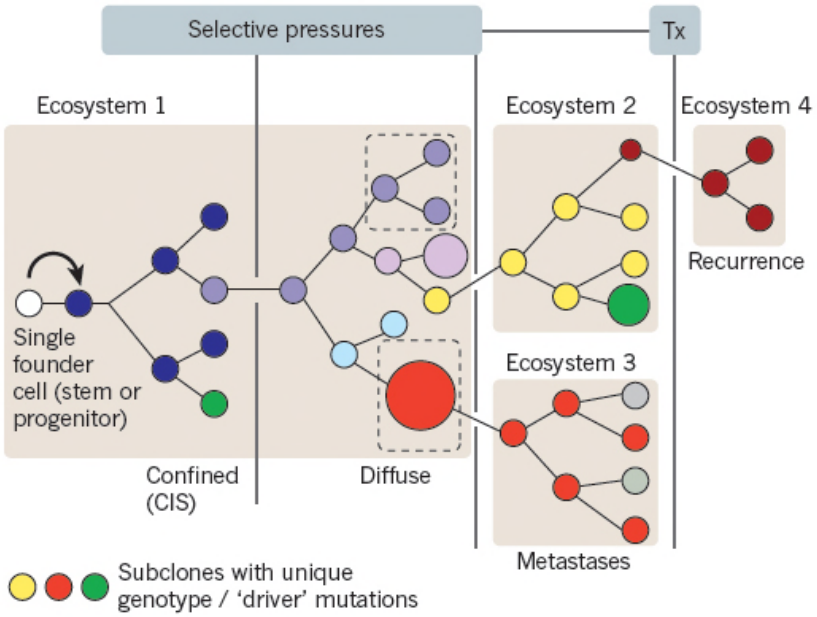


Figure 1.4: The branched architecture of evolution [28]. Representative pattern for common solid cancer evolution: selective pressure allows some mutant subclones to expand and other become extinct or remain dormant. Vertical lines represent the selective pressures in different habitats. TX represents therapy.

1.2.1.1. Sub-clonal segregation of mutations and clonal architecture

Most histopathological evidence supports the classical model of clonal evolution followed by sub-clonal dominance and/or selective sweeps.

The evidence of the complex pattern of sub-clonal segregation of mutations came from a large data of deep sequencing and single-cell analysis. By comparing the mutational genomes of the sub-clones, it is possible to trace their evolution.

Evidence of clonal evolution from a common ancestral cancer cell has been found in identical twins with a concordant acute leukaemia with metastatic lesions: divergent cancer clone genotypes and phenotypes correspond to allopatric (genetic isolation) speciation in separate natural habitats [29] (Fig 1.5).

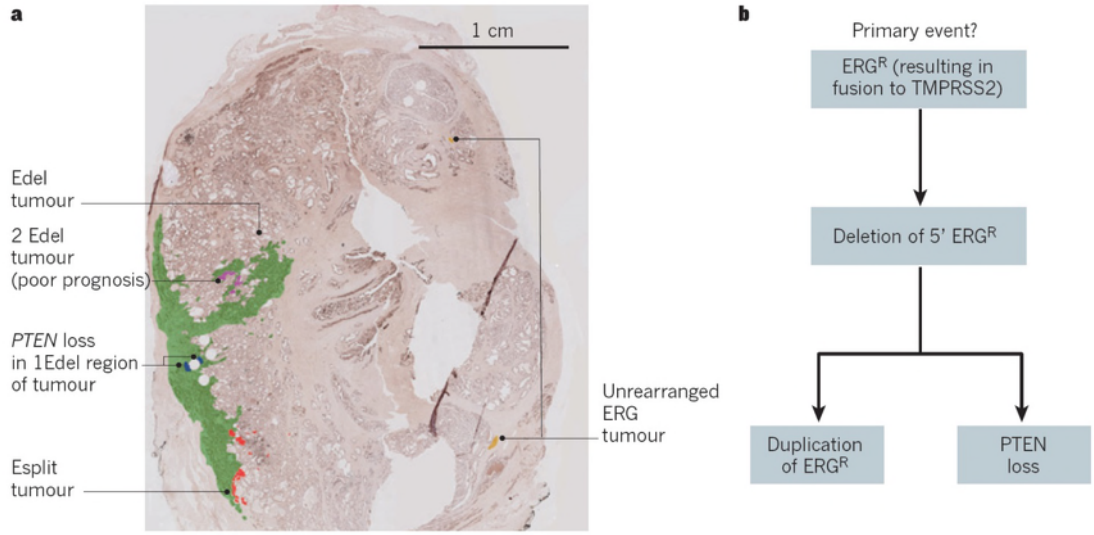


Figure 1.5: Prostate topography of cancer subclones [28]**. A**. Tissue section of prostate to detect genetic events: TMPRSS2- ERG fusion (ERG *via* rearrangement ERG^R) and *PTEN* loss. **B.** Presumed sequence of clonal events.

The level of biodiversity within the sub-clonal structure can be measured, and also, as the profile of sub-clones within a neoplasm can be used to determine the molecular clocks linked to time events in the history of the neoplasm, it has been shown to be a robust biomarker for predicting progression in some cancer diseases.

1.2.2. Cancer Cell Plasticity

Tissue and organ physiological developmental processes are based on the capacity of the stem/progenitor cell pools to activate different pathways allowing them to undergo a committed fate.

The commitment is controlled in a strict way by epigenetic regulators and transcription factors that organise the specific gene expression patterns of each lineage. This mechanism also occurs by de-differentiation phenomena by which differentiated cells can change their normal developmental programme and acquire a new fate, even distant from that of the lineage they come from.

Many studies during the years proved the de-differentiation of committed cells to a totipotent stage: after Brigs and Kings in 1952 [30] generated *frog* tadpoles by transplanting the nucleus from the blastula into *frog* enucleated oocytes, demonstrating the possibility for pluripotent cells to acquire a totipotent fate, in 1962 Gurdon and collaborators [31] generated *Xenopus* by transplanting nuclei from intestinal epithelial cells

into *Xenopus* oocytes, showing that committed nuclei may change their somatic fate into a germinal fate.

Strong evidence in mammals came from the cloning of Dolly the sheep [32] [33], where Wilmut and collaborators in 1997 proved that changes in cell fate did not involve irreversible nuclear changes: the fact that a lamb was derived among others from an adult mammary gland cell confirmed that differentiation of that cell did not involve irreversible modifications of the genetic material required for development and there were changes in gene expression but not in gene sequence, indicating that the developmental fate restrictions occurring during normal development can also result from epigenetic modifications [34].

Cellular plasticity based on epigenetic memory and involving chromatin regulators (*e.g.* Trithorax and Polycomb group Proteins [35]) is a fundamental trait of normal organ and tissue development.

The *de novo* progenitor state (acquired progenitor state) is not as stable as expected, and cell plasticity is indeed involved in several diseases mainly characterised by abnormal cellular reprogramming.

The plasticity of the very first cells harbouring oncogenic alterations plays an important role in cancer development, leading the cell to acquire stem cell proprieties and allowing it, in the worst case scenario, to develop a tumour mass. Different types of cells contribute to the tumour structures and while the great majority of them are differentiated, there is a small percentage of Cancer Stem Cells (CSCs) that are important for replenishing cancer mass. The CSC theory considers cancer as any other stem-cell-maintained tissue, as only CSCs are able to reform the tumour if injected in a responsive model [36]. So, this theory explains how tumour is not only a result of genetic mutations affecting oncogenes and TSGs, but it comes from altered cell-fate programmes, where committed cells are able to de-differentiate to ensure a cancer stem pool (fig 1.6a). Conventional therapy is known to be ineffective at eliminating CSCs, which allow tumour cells to eventually repopulate the organ (fig 1.6b); however, targeting CSCs will leave the bulk of the tumour intact. A remaining tumour cell could then convert into a CSC, allowing for tumour recurrence and metastasis. Combination therapies targeting both CSCs and non-CSCs are likely needed to better prevent tumour recurrence and metastasis [37].

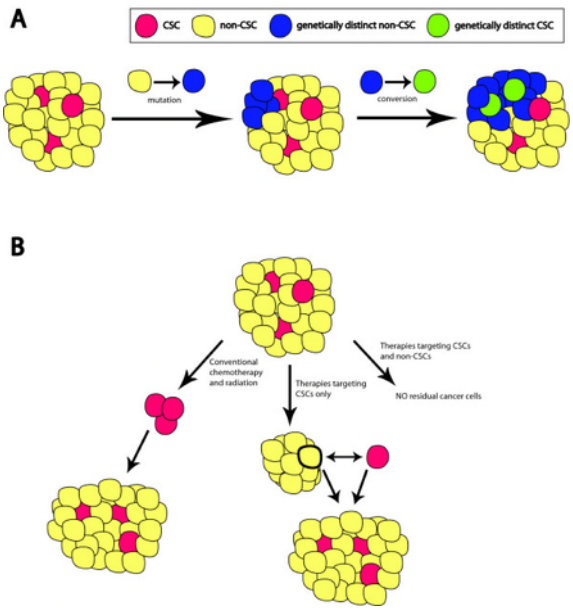


Figure 1.6: Plasticity of cancer stem cells [37]. **A**). The initial tumour is composed of non-cancer stem cells (CSCs; yellow) and rare CSCs (red). Non-CSCs within the tumour can mutate in non-CSC (blue). Spontaneous conversion of new non-CSC (blue) into a new CSC (green) provides tumour with genetically distinct CSCs (red and green) **B).** Conventional therapy and future therapy.

Evidence of this kind of cell plasticity can be found in haematopoietic tumours, where leukaemia stem cells are very similar to myeloid populations with the haematopoietic stem cell programme activated [38], and in some epithelial cancers where an embryonic stem cell-like (ESC-like) programme can be activated by oncogenes to allow, for intestinal cancers, to generate cancer cells from the crypt stem cells.

Plasticity during tumour development is comparable to normal development, where cell fate is programmed in early progenitors, but the final effects of these programming events are only evident in differentiated cells.

1.3. EPIGENETICS AND CANCER

In the study of cancer and its mutations it cannot be excluded an amount of non-genetic mechanisms that drive positive selection during growth, including signalling plasticity, quiescence and epigenetic changes [39]. Global changes in the epigenetic landscape are a hallmark of cancer: its disruption can lead to altered gene function and malignant transformation [39]. Epigenetics describes the study of heritable and non-heritable changes in gene expression that occur independently of changes in primary DNA sequence, involving the organisation of genome and chromatin structure and influencing the ability of genes to be activated or silenced. The sum of these modifications, collectively referred to

as epigenome, provides a mechanism for cellular diversity by regulating how genetic information can be accessed by the cellular machinery [40]. The majority of these modifications are maintained through cycles of cell division allowing cells that share the same genetic information to have the same epigenetic organisation. The rate of epigenetic changes, also known as epi-mutations, has been estimated to be higher than the genetic changes, and could thus be a major determinant of clonal evolution [41]. As epi-mutations affect cell phenotype, they can also underlie natural selection.

Failure of the epigenome machinery can result in activation or inhibition of various signalling pathways, leading to several diseases such as cancer [42] [43] where they play important roles from the early stages to progression in conjunction with genetic mutations or deletions. In addition to tumour suppressors' inactivation and oncogenes' activation, coming from a general genomic instability, they could serve as a second hit required for cancer initiation according to the Knudson's "two hits model". Among the types of epigenome aberrations found in cancers, the hypo-methylation of CpG islands in promoters, repetitive elements, introns and genes play a significant role. A low rate of methylation in transposons, e.g., increases genome instability induced by their translocation or rearrangements or leading to gene activation [44]. In contrast, hyper-methylation of TSGs can lead to gene inactivation, such as it happens to BRCA1 and p16 [45].

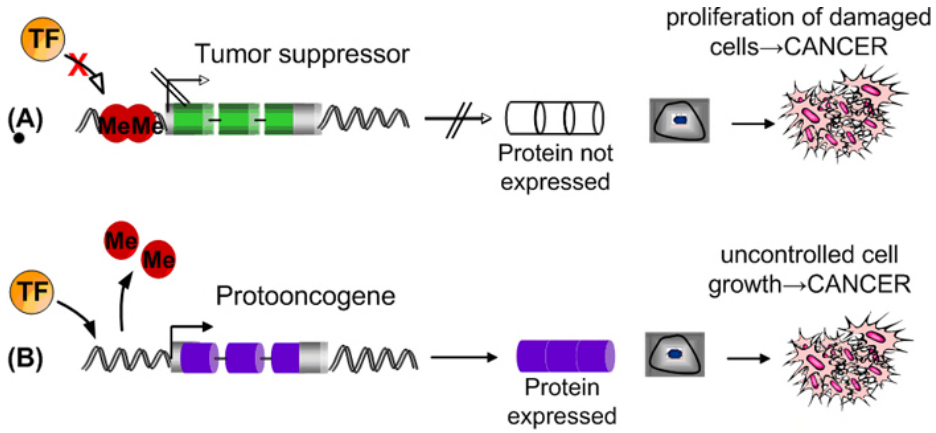


Figure 1.7: Epigenetic gene silencing (**B**) and activation (**A**) [46]. Two possible mechanisms by which epigenetic modifications can lead to cancer.

Other modifications include changes in histone epigenetics, deregulation of miRNA, the modified nucleosome positioning system and the stem cell fate epigenetics-induced modifications.

The CSCs model suggests that epigenetic changes, which occur in normal stem or progenitor cells, are the earliest events in cancer initiation [47]. Since epigenetic mechanisms are central to the maintenance of stem cell identity and epigenetic changes drive the early stages of cancer progression, these same changes may modify the fate of cells, inducing de-differentiation, one of the most common events in tumourigenesis.
For example, silencing of genes involved in the maintenance of stem/progenitor cell state, such p16 or APC, early markers of colon cancer, is induced by DNA hyper-methylation [48]; the Polycomb Complex Group proteins (PcG), involved in the silencing of developmental regulators in ES cells, are upregulated in various forms of cancer through hyper-methylation of the Polycomb repressive marks, suggesting another link between cancer and epigenetic regulations [49].

1.4. MODELLING CANCER IN DROSOPHILA

Cellular and animal models are contributing to the acquisition of knowledge on the genetic basis of cancer, and among all models *Drosophila melanogaster*, the fruit fly, has been largely used to investigate the mechanisms underlying the behaviour of cancer cells growing in heterotypic backgrounds: mutant cells growing in heterotypic contexts can mimic the typical clonal formation of a tumour and allow researchers to study the interactions with adjacent populations of cells bearing different genetic arrangements [50].
Drosophila and human epithelia are comparable in structure and composition and more than 50 *Drosophila* genes have been identified in the control of proliferation of cells in a variety of tissues including embryonic tissues, nervous system, imaginal discs, larval hematopoietic and adult gonadal tissues [51]. Genes and pathways controlling proliferation and growth in *Drosophila* are largely conserved in humans, therefore the study of *Drosophila* TSGs and proto-oncogenes has produced a strong contribution to a better understanding of cancer biology in humans. Nowadays *Drosophila* represents an invaluable model in cancer research, as the powerful and sophisticated genetic tools available allow to study the clonal effects of multiple genetic manipulations on populations of cells which are embedded in and interact with the surrounding normal tissue.
The first tumourous mutation was described in the 60's by Elizabeth Gateff: this mutation affected a *locus* named *lethal giant larvae, lgl,* that acted in a recessive manner, mimicking a TSG. In the following years, thanks to the development and improvement of genetic and molecular tools to allow wide genetic screens, several other TSGs were identified in

Drosophila that caused uncontrolled proliferation leading to tumour growth, and proved to be functionally conserved in mammals and also altered in human cancers.

1.4.1. lethal giant larvae is a TSG

Conventionally only the genes that cause over-proliferation in *Drosophila* can be defined as fly TSGs and they are classified into two main categories [52]:

- Hyperplastic TSGs are those genes that when mutated display extensive over-proliferation of the imaginal epithelia without affecting cell monolayer organisation, and mutant tissue is able to differentiate into rudimental adult organs.
- Neoplastic TSGs are classified as genes that, when mutated in the imaginal epithelia, cause over-proliferation accompanied by loss of apical-basal cell polarity; moreover mutant tissue never differentiate. When transplanted into adult w*ild-type* hosts, the mutant tissue from neoplastic TSGs is able to overgrowth, spread and kill the host [53]. Among the neoplastic tumour suppressors so far identified, *lethal giant larvae*, *discs large (dlg)* and *scribble (scrib)* are of particular interest as they encode conserved proteins involved in the establishment and maintenance of epithelial apical-basal polarity as well as in proliferation control. The regulation of epithelial cell polarity is a crucial issue in cancer biology as its loss is a key feature of carcinomas [54], [55].

1.4.2. lethal giant larvae and Cell Polarity

Most cell types are polarised in space with distinct structural orientations, following Planar Cell Polarity (PCP) and Apical-Basal Polarity (A/B) established by distinct protein localisation patterns that allow cells to perform specialised functions. Correct establishment and maintenance of cell polarity is required for the development and homeostasis of all metazoans and disruption of cell polarity is one of the early events in carcinogenesis and represents a hallmark of cancer [56].

While PCP describes the coordinated alignment of cells across the tissue plane [57], the A/B polarity is defined as the existence of cells which display an upper, apical pole and a lower, basal pole (fig 1.17), essential for adhesion, communication, morphogenetic properties of epithelia and to signal between the interior of the organism and the external environment. Loss of A/B cell polarity is a specific feature of the *lgl* LOF phenotype.

The *Lgl*/*Scrib*/*Dlg* complex establishes and maintains the basal-lateral cell domain, antagonising the activity of two other protein complexes: *Crumbs/Stardust/PATJ* (apical domain) and *Bazooka(Par3)/Par6*/*aPKC* (sub-apical domain).

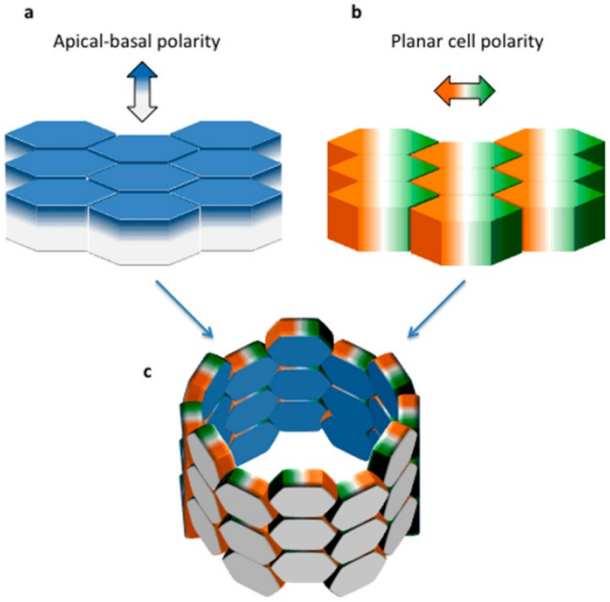


Figure 1.8: Schematic representation of an epithelium showing an **A**) apical-basal polarity and **B**) planar Polarity. **C**) their appearance in association is shown in a tubular structure [58].

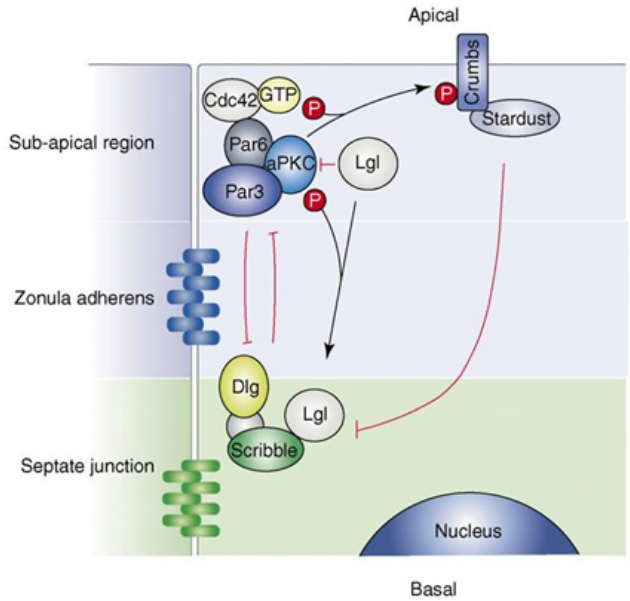


Figure 1.9: A model of localisation and collaboration of A/B polarity complexes [59]. Crumbs/Stardust complex identifies the apical domain; Bazooka(Par3)/Par6/aPKC localise at the sub-apical region and Scribble/Dlg/Lgl complex lies at the baso-lateral domain.

The *Crumbs/Stardust/PATJ* complex establishes the apical identity of polarised cells and is required for the formation of the adherens junctions (AJs) in *Drosophila* and in mammalian epithelial cells. It consists of three main components: a transmembrane protein, Crumbs (Crb), and two adaptor proteins, Stardust (Sdt) and PATJ [54]

The *Bazooka(Par3)/Par6*/*aPKC* complex is immediately below the *Crumbs/Stardust/PATJ* complex at the sub-apical region. It is composed of two scaffold proteins, Bazooka (Baz) or Par3, Par6 and an atypical Protein Kinase, aPKC. This complex defines the region in which the zonula adherens (ZA) is formed. In mammalian cells this complex localises at tight junctions and is required for AJs formation [54].

The *Scrib*/*Dlg/Lgl* complex is linked to cell polarity and cell proliferation [60] and LOF phenotypes of these three TSGs are very similar [50].

Scribble is a LAP (Leucine-rich repeats And PDZ domain) family protein and has four PDZ domains at the C-terminus and sixteen Leucine Rich Repeats (LRR) at the N-terminus; it is encoded by a single gene both in *Drosophila* and vertebrates.

Discs large is a MAGUK protein and contains three PDZ an SH3 and a GUK domain. Several mammalian orthologues of *Drosophila dlg* have been identified.

Lethal giant larvae encodes a protein, Lgl, rich in WD40 repeats, predicted to fold into two β-propeller domains at the N-terminus, as its mammalian orthologues, Lgl-1 and Lgl-2 [54]. Fly Lgl and human Lgl-1 show the significant sequence similarity of 62.5% (with conservative amino acids changes) associated with functional conservation: Lgl-1 expression is indeed able, to substitute for *Drosophila* Lgl [61]. Analogue rescuing ability has been demonstrated for rat Dlg [62] and human Scrib [63]. These three proteins are mutually dependent for correct localisation [60] in *Drosophila*, although direct interactions have never been proven.

Dlg and Scrib localise at the membrane cortex, at *Drosophila* septate junctions (SJs). Lgl co-localises with Dlg and Scrib at SJs but it is also found in the cytoplasm. It has been demonstrated that Lgl localisation at the membrane depends on its phosphorylation in conserved residues by aPKC of the Baz complex. When Lgl is phosphorylated its interaction domain is hidden, resulting in its exclusion from the cell cortex thus binding aPKC with Par6 excluding Bazooka (Par3) [64]. In fact, cortical spreading of aPKC in epithelial tissues causes Lgl inhibition at the baso-lateral domain with polarity defects, similar to what is observed in *lgl* mutants [65].

The Crb complex is recruited by the Baz complex at the apical domain and seems to further antagonise the activity of the Lgl complex by blocking its spreading along the lateral membrane domain. Crb is phosphorylated by aPKC and this event is required for its correct apical localisation. As expected from the high level of functional conservation, mammalian Lgl-1 and Lgl-2 are also excluded from the apical domain due to aPKC phosphorylation and are able to bind Par6 and aPKC preventing their association with Par3 and thus the baso-lateral spreading of apical complexes [54]. Moreover Lgl-1 subcellular localisation seems to play a crucial role in human cancer, as its cytoplasmic enrichment correlates to aPKC lateral spreading and cancer progression in ovarian carcinomas [65].

1.4.3. lethal giant larvae and Tumourigenesis

lgl LOF causes neoplastic over-proliferation in *Drosophila* imaginal epithelia and neuroblasts of the larval brain. These structures show loss of A/B polarity, sustained growth and disruption of tissue architecture which result in an over-extended larval period and pre-pupal lethality. Cell growth appears to be slow in *lgl* mutants; as $lgl^{-/-}$ cells are unable to exit cell cycle, discs reach an enormous cell number and eventually form large masses which fail to form proper cellular contacts and do not differentiate.

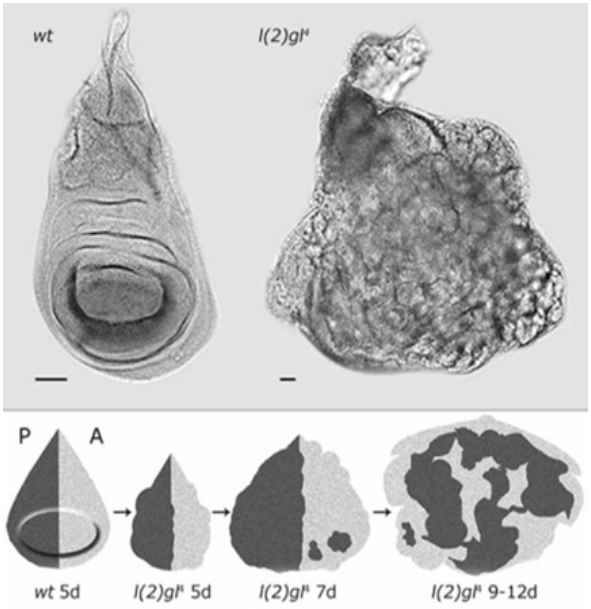


Figure 1.10: *Wild-type* (left) and *l(2)gl*^{4} (right) imaginal discs with evident over-growth and loss of layer organisation in *lgl* mutant epithelium [66].

lgl mutants also show loss of positional cues, in fact cells straddle the anterior-posterior compartment boundary [67], which separates two independent developmental units that never intermix. Moreover they are able to fuse with nearby tissues so displaying local invasiveness [68].

Overgrowth might be hypothesised to be a direct consequence of loss of cell polarity in *lgl* mutant tissues. Loss of membrane compartmentalisation could indeed alter distribution of diffusible signals that regulate proliferation and their receptors, and delocalisation of signalling molecules both on cell surface and within the cytoplasm could result in simultaneous deregulation of several different pathways so triggering changes in cell metabolism and proliferation rate. Evidence exists that demonstrate a specific signalling function of the *Dlg/Lgl/Scrib* complex during proliferative control. In fact, polarity alteration by other means does not induce over-proliferation in imaginal discs, mutation of E-cadherin is the best example [69].

It was demonstrated that the proliferative defects described above are due to the deregulation of the Hippo (Hpo) pathway, a very conserved signalling network that plays a central role in the control of epithelial organs, establishing for the first time a direct link between *lgl* and cell proliferation control [70].

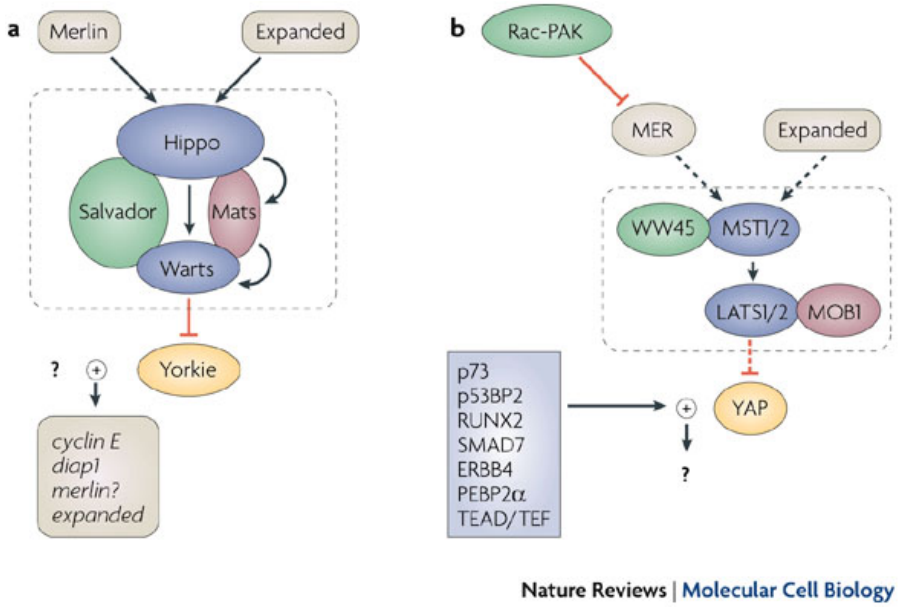


Figure 1.11: Hippo pathway components in **A**) *Drosophila melanogaster* and **B**) mammals [71].

The Hpo downstream effects are mediated by its effector: Yorkie (Yki) in *Drosophila* and YAP (Yes-Associated Protein) in mammals, a transcription factor regulated by two core proteins, Hpo and Warts (Wts), in *Drosophila*. Following physiological regulation, a large fraction of Yki is phosphorylated by Wts at multiple residues and sequestered in the cytoplasm by 14.3.3 proteins, but when a cell undergoes genetic or mechanical stress, core components are inactivated, unphosphorylated Yki enters nucleus and co-activates transcription of several target genes involved in cell growth and resistance to apoptosis, such *cyc E* (found over-expressed in $lgl^{-/-}$ clones [72], *dIAP1* (*Drosophila* Inhibitor of APoptosis 1) and the miRNA *bantam* [73].

Mutations in major components of the Hpo pathway, including *expanded (ex), fat (ft), Salvador (Sav)* or *wts*, lead to competitive interactions that trigger death of the *wild-type* surrounding cells, revealing a competitive behaviour. This is caused by the hyper-activation of Yki/YAP which is maintained in its unphosphorylated, nuclear form, increasing the expression of several targets involved in cell survival and proliferation [74].

A link between organ growth and nutrient availability has recently been found in the *Drosophila* wing with TOR (Target Of Rapamycine) regulating Yki downstream of the Insulin receptor by a separate and novel mechanism. TOR signalling controls nuclear Yki by allowing it to access to its target genes: when TOR activity is inhibited (low nutrients), Yki accumulates in the nucleus but is sequestered from its normal growth-promoting target genes, a phenomenon called "nuclear seclusion", thus scaling wing size downwards [75].

These were exciting findings because the Hpo pathway has only recently emerged as a major and highly conserved growth regulatory pathway that has also critical tumour suppressor functions [86] [89].

lgl was shown to take part in the regulation of this pathway: *lgl* mutant cells in the eye imaginal disc show upregulation of Hpo pathway target genes as cyclin E and dIAP1, as well as genes belonging to the negative feedback loops such as *ex*; consistently, in $lgl^{-/-}$ cells Yki was found to accumulate in the nucleus and the phosphorylated form levels were reduced. Removal of one copy of Yki partially reduced Hpo target genes, indicating that Yki activity is required for $lgl^{-/-}$ proliferative defects. *lgl*-dependent regulation of Yki has also been observed in the wing imaginal disc but it was proposed to occur through a different mechanism [78].

1.4.4. Mammalian Lgl-1 and Lgl-2

Mislocalisation, loss or decreased expression of *Lgl-1/2* (in man, *Hugl-1/2)*, suggest a role for these genes in tumourigenesis. It has been observed that *Hugl-1* transcript results decreased or completely absent in variety of human epithelial tumours such as breast, lung, prostate, ovarian cancers, melanomas [61] [79] and colorectal cancer, where *Hugl-1* loss was found to be associated with advanced stages and lymph node metastases [80]. Its loss has further been correlated with reduced survival in glioblastoma [81] and aberrant splicing variants with hepatocellular carcinoma progression [82]. Moreover, Hugl-1 seems to play a crucial role in ovarian carcinomas as its cytoplasmic localisation correlates to aPKC lateral spreading and cancer progression [65]. This clearly drives a strong parallel with the phenotypes observed in *Drosophila lgl* mutant epithelial tissues. Interestingly, pronounced similarities with the *Drosophila lgl* mutant were also found in *Lgl-1* knock-out (KO) mice. *Lgl-1*$^{-/-}$ individuals presented at birth severe brain dysplasia due to an abnormal expansion of progenitor cells, unable to exit cell cycle and to differentiate. These cells formed neuroepithelial rosette-like structures, similar to the meroblastic rosettes found in human primitive neuroectodermal tumours (PNETs) occurring at pediatric age [83].

Noticeably, human *Hugl-1* gene maps to 17p11.2, a region often shown to undergo chromosomal breakage in human PNETs [84]. *Hugl-2* has been found to be a negative target of ZEB1, a master regulator of EMT [85] suggesting that its loss in epithelial cells may favour the acquisition of mesenchymal, migration-prone behaviour.

1.5. CELL COMPETITION

"When the suspects give contrasting information, ask the neighbours:
some of them will tell you exactly what is happening next door. Get ready to listen".

Cell competition (CC) is a non cell-autonomous phenomenon occurring between adjacent cell populations showing different fitness/proliferation rates. It was described for the first time in *Drosophila melanogaster* by Morata and Ripoll [86] while they were analysing the effects on organ development of a group of dominant, recessive-lethal mutations *Minute (M*) affecting various ribosomal proteins (*Rp*). The researchers demonstrated in the developing wing imaginal disc (the *Drosophila* larval primordium of the adult wing and thorax, see Section Materials and Methods) that the emergence of *wild-type* cells in a *Minute* background triggered a battle for field occupancy between the two cell populations: the *wild-type* population overgrew (Winner cells) and the *Minute*/+ cells, characterised by

a lower proliferative rate, lost the competition undergoing Caspase-3 mediated apoptosis (Loser cells) and disappeared from the adult organ. Contrarywise, *Minute/+* clones generated in a homotypic context (in *M/+* individuals) are viable and fertile, although they show an impairment in proliferation due to a low efficiency in ribosome assembly [86].
CC thus appears to be a mechanism of "quality check" that maximises tissue robustness through destruction of sub-optimal cells during development.

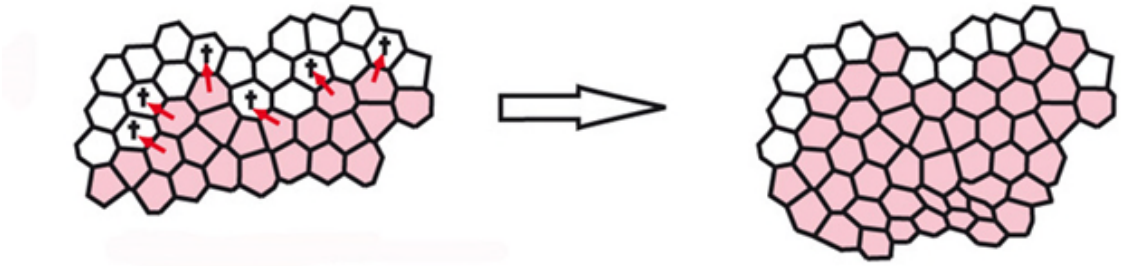


Figure 1.12: Cell competition: pink cells are characterised by a higher proliferation rate than white cells. Through mechanisms of cell competition, pink cells overgrow at the expense of the white cells. Adapted from [87]

Mutations in Rp genes also occur in mammals. In 2004, it was observed that cells mutant for the mouse RpL24 riboprotein gene (*Bst*) were out-competed by *wild-type* cells in developing chimeric blastocysts [88]. This was the first time that CC was demonstrated to be at work also in mammals.
Several molecules have emerged to be associated with the winner status by improving cell proliferation rate and cellular fitness, first in *Drosophila* then in mammals: one of them is the Decapentaplegic (Dpp)/TGFβ protein.
Researchers found a reduction in Dpp signalling in out-competed cells. This molecule is involved in cell survival and when its pathway is active, it inhibits the transcription repressor Brinker (Brk), which would otherwise lead to the activation of the JNK pathway, causing death of the cells under competitive stress, that are eventually eliminated from the epithelium [89].
Apoptosis is required for CC to occur; inhibition of cell death by the baculovirus IAP p35 decreases winners' proliferation rate and losers' apoptosis. In fact, CC seems also to require the elimination of apoptotic cells by means of engulfment (phagocytosis) by surrounding tissue, as loss of function of genes involved in this process prevents competition [90]; a basal extrusion of the dead cells has also been frequently observed as a process to eliminate apoptotic cells [91].

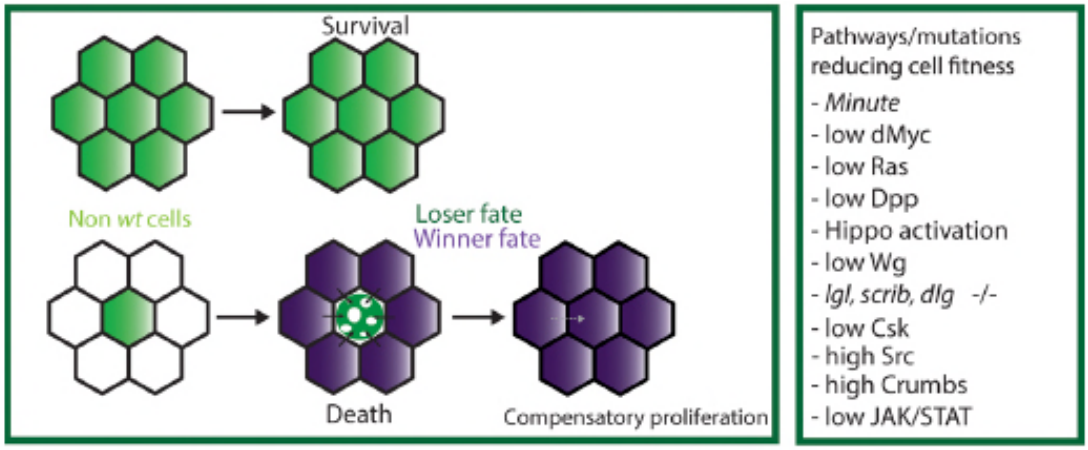


Figure 1.13: Genetic impairments in the loser cells [92]**:** in "classical" CC, mutant cells (green) survive in a homotypic environment (top), whereas they are eliminated when surrounded by *wild-type* cells (white, bottom). Subsequently, *wild-type* cells replenish the tissue by compensatory proliferation. Purple colour defines a winner fate.

1.5.1. Phases of Cell Competition

Based on the study of CC in the *Drosophila* imaginal wing disc, it has been possible to schematise the main phases of the mechanism.

- Mutational events in cells that generate a reduced fitness creating a difference in proliferation or growth rate [93], metabolic rate or protein synthesis [94].
- Deprivation of morphogens and survival signals in loser cells, as it has been suggested by the "ligand capture" hypothesis by which cells compete for extracellular survival and growth factors [93].
- Both winner and loser cells "communicate" each other and actively participate in the competitive process, as Senoo-Matsuda and colleagues demonstrated that soluble factors from both populations are necessary for death of the loser cells [95]. Both populations are modified during the early stage of the process. SPARC [94] and FWE^{lose} [96] are up regulated in loser cells and the winner cells are characterised by a metabolic change, known as Warburg effect [97].
- Activation of apoptosis in the loser cells and, after that apoptotic bodies are extruded from the epithelium, phagocytic mechanisms start to clear the apoptotic debris [98].
- Apoptosis-induced proliferation of the winner cells. CC is a process that assures an organ to reach a proper size with fit and functional cells composing it; unfit cells are promptly removed from the growing tissues and substituted by fitter cells, avoiding their expansion during development. Upon death and removal of unfit

cells, winner cells are stimulated to repopulate the developing organ undergoing additional proliferation, that has been named "apoptosis-dependent proliferation" because it does not occur if apoptosis is blocked in loser cells [56] [58].

1.5.2. Mechanics of Cell Competition

One hallmark of CC is the elimination of loser cells when they are close to winner cells, and this occurs through Caspase-3 mediated apoptosis [91] induced by signals emanated from both cell populations [95].

Caspase-3 induced apoptosis is a fundamental step for the progression of competitive interaction, and its inhibition has demonstrated to block CC [99].

Most of this apoptosis is observed at the boundary between two clones, winner cells and loser cells, as CC is based on direct cell-cell interaction between two different types of clones [100].

Successively, winners cells can overgrow through apoptosis-induced proliferation [101] at the expense of the losers, as the final tissue/organ size is not reduced despite the elimination of loser cells [50] [51]. This suggests that dying cells secrete mitogenic factors which increase the proliferation rate of the neighbouring cells (via Dpp and Wg secretion, and the JNK-dependent secretion of Unpaired (Upd), the ligand of the JAK-STAT cascade in *Drosophila*) [92]. On the contrary, loser cells are less sensitive to these mitogenic signals.

CC may require the function of the *brinker (brk)* gene, whose expression is normally repressed by Dpp signalling and is thus upregulated in slow-growing cells. Excess of Brk activates the JNK pathway, which in turn triggers apoptosis in these cells. The slow-proliferating cells upregulate Brk levels owing to a disadvantage in competing for, or in transducing, the Dpp survival signal. This evokes the ligand-capture hypothesis and may function either in a short- or in a long-range fashion. This sequence of events might represent a general mechanism by which weaker cells are eliminated from a growing population [91].

Recent work indicates that, in some cases, winner cells may not produce a specific pro-apoptotic ligand. Instead it has been suggested that winner cells could be engulfing loser cells [102].

Mechanical stress has also been proposed to contribute to CC [103] [104] [105]. Qiang Sun and colleagues demonstrated as human cells can directly compete by a mechanism of

engulfment called entosis. Through entosis, cells are engulfed or cannibalised while alive, and subsequently undergo cell death. The researchers found that the identity of engulfing and engulfed cells is driven by mechanical deformity controlled by RhoA and actomyosin, where tumour cells with high deformability preferentially engulf and outcompete neighbouring cells characterised by low deformability in a heterogeneous population, demonstrating that a mechanical differential between winner and loser cells is required for entosis to proceed [56] [103].

Entosis, in association with emperipolesis, cannibalism and phagocytosis, describes the "Cell-in-Cell" mechanisms by which cells are internalised to undergo different fates. Part of these structures are associated with cancer, and function as tumour accelerators or tumour suppressors (fig 1.15).

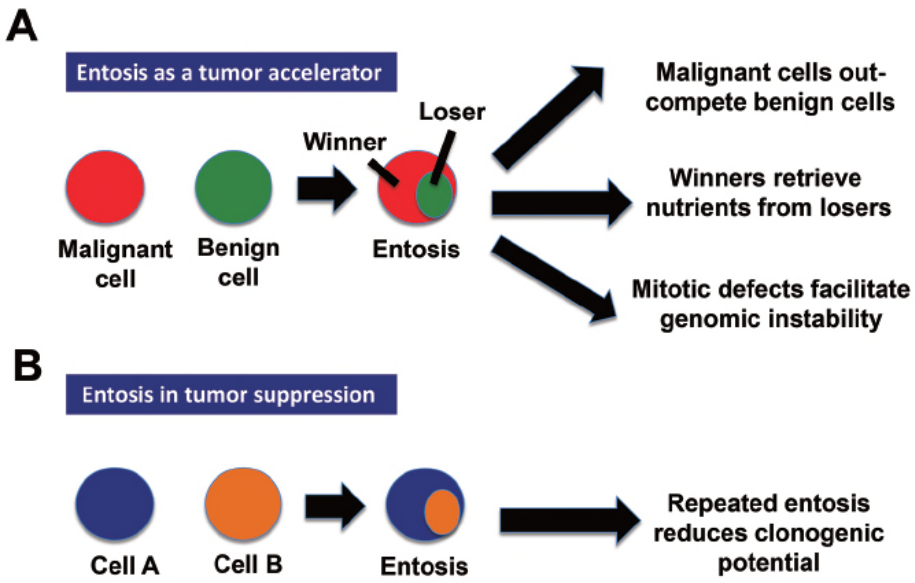


Figure 1.14: A dual role for entosis in cancer [106]**. A)** entosis as a pro-tumourigenic process. **B) e**ntosis as a tumour-suppressive mechanism.

Another process indirectly associated with apoptosis in the epithelia is the eviction of cells from the epithelial sheet. This can occur either towards the apical surface (a process known as extrusion) or towards the basal surface (a process known as delamination). Delamination only disrupts cell-cell contacts, whereas extrusion causes detachment from the extracellular matrix, as well as loss of cell-cell contacts. In both cases, unless it is part of a programmed process such as EMT, exit from the epithelium leads to the loss of essential survival signals and thus apoptosis and phagocytosis [102].

A recent study performed in MDCK cells provided evidence that crowding induces delamination. In fact, cell crowding may cause random buckling of weaker cells. In this

case, buckling would entail loss of the ability to withstand compression within the junctional area. Junctional buckling is expected to cause compensatory expansion of the cell surface elsewhere and hence activation of Piezo (a mechanically activated channel) [107]. Alternatively, cell compression could lead to other types of membrane deformation that could activate Piezo. This view suggests that compression and stochastic variations in the mechanical resilience of a cell are key to extrusion. As most compression probably occurs in fast-growing cells this view is unlikely to apply directly to CC, although a rigorous assessment will require three-dimensional modelling [102].

More recently, several studies identified MYC as a key regulator of CC. Clones of cells bearing hypomorphic *dm* mutations (the *locus* that in *Drosophila* encodes for the MYC protein, also known as dMyc), although viable in a homotypic environment, died when generated in a *wild-type* imaginal disc [108]. In 2004, two papers clearly demonstrated that this phenomenon was triggered by the difference in MYC expression levels between the two adjacent cell populations [93] [109].

1.5.3. MYC/dMyc Oncoprotein

MYC family proteins are evolutionarily conserved basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factors that control about the 30% of all the expressed genes. They are involved in several cellular functions as: cell cycle progression, growth, metabolism, apoptosis, transcriptional and post-transcriptional regulatory mechanisms, non-coding RNAs, stem cell biology and cancer development.

MYC oncoproteins can bind DNA at regulating regions, called E-Box sequences, following heterodimerisation with specific binding partners. Mammals possess three members of the MYC family: c-MYC, N-MYC and L-MYC (Figure 1.10).

The *c-MYC* gene is ubiquitously expressed in dividing cells and normally downregulated in quiescent cells; *N-MYC* and *L-MYC* are expressed during particular stages of the embryonic development and in some immature hematopoietic and neuronal cells [65] [66].

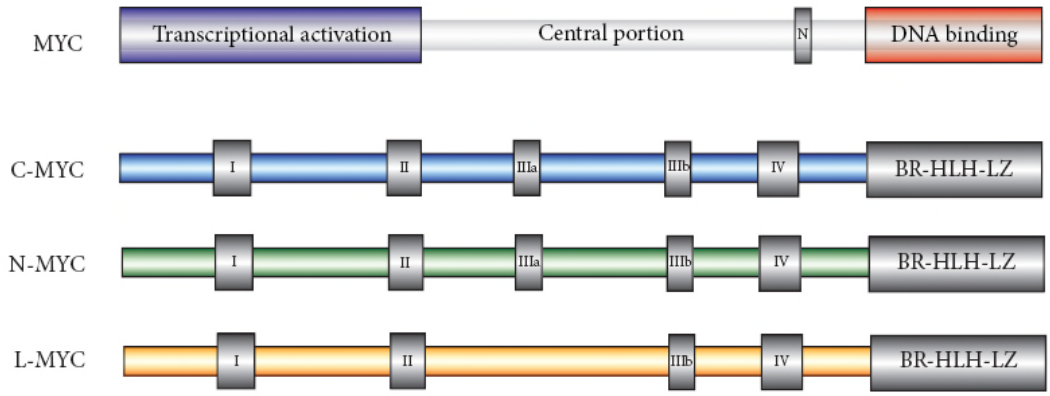


Figure 1.15: The MYC family members [112]. The image at the top shows a generic representation of a mammalian MYC protein, indicating the transcriptional activation domain, the central portion, the canonical nuclear localisation sequence (N) and the region involved in DNA binding via interaction with MAX. Below is a representation of conserved sequences present in C-, N-, and L-MYC family members. c-MYC is drawn to scale at 439 amino acids. N- and L-MYC proteins are different in length (464 and 364 amino acids, resp.) due to differences in the length of non-conserved sequences, but are drawn to highlight the conservation and relative location of *MYC* boxes.

MYC family proteins attained plain interest when their role in cancer development and progression was unveiled. Three mechanisms by which MYC oncoprotein can be activated and involved in cancer have been identified: **insertional mutagenesis**, **chromosomal translocation** and **gene amplification**.

Early evidence for insertional mutagenesis involved in MYC-associated cancerisation emerged in the study of leukaemogenesis induced by avian myelocytomatosis retrovirus (MC29), an acutely transforming virus. Even if the scientific community was reluctant to accept the idea of a viral integration in human genome, the analysis of DNA and RNA from avian leukosis virus (ALV) and the analysis of RNA chimeras showed the existence of a viral-cellular integration in specific sites in the genome, often nearby an oncogene control region. MYC was the first cellular oncogene shown to be activated by retroviral promoter insertion [66] [68].

A genetic analysis of the Burkitt Lymphoma, a cancer of the lymphatic system characterised by MYC over-expression, revealed that the production of MYC mRNA resulted from a recombination between the immunoglobulin (Ig) heavy chain *locus* and the MYC oncogene [114]. This recombination came from a gross translocation of chromosomes 14, 2 or 22, which harbour the Ig heavy and light chain genes, to chromosomes 8, nearby the MYC gene coding region. The result of this translocation was the MYC mRNA transcription under the control of the strong Ig promoter. This evidence

was consistent with the finding, during the same year, of the Philadelphia chromosome involved in chronic myeloid leukaemia, that through the 22-9 chromosomal translocation, generated a fusion protein BCR-ABL responsible for the disease [115].

Many different kinds of tumours are characterised by karyotypical abnormalities and the manner by which these aberrations can drive cancerous growth is also dependent on oncogene amplification. The role of MYC amplification involved in tumour emerged in the study of neuroblastoma. Amplification of N-MYC, normally expressed during neural development, was discovered in a panel of human neuroblastoma cell lines and tumour samples and was associated with poor prognosis [116].

In contrast to chromosomal translocations in haematopoietic cancers, activation of the MYC genes by amplification is commonly detected in human solid tumours [111].

In the early 1980s, the debate about MYC was very rich of new daily findings and it became important to define the basic knowledge about the protein role in normal development. It was shown that MYC is a nuclear protein that binds double stranded DNA [117], whose stability and activity depend on Thr58 and Ser62 phosphorylations [118], and whose expression pattern is invariant throughout the cell cycle [119].

A direct link between mitogenic stimulation of quiescent cultured cells and a rapid induction of MYC mRNA was established in 1983: the highest RNA levels were reached within 2 hours from the mitogenic stimulus in the presence of cycloheximide, an inhibitor of protein synthesis. MYC mRNA and protein [67] [68] showed a short half-life and were both expressed at constant levels once cells entered cell cycle [119]. Anti-proliferative signals were shown to trigger a rapid decrease of MYC levels. These data indicated that MYC expression and activity are both tightly regulated in non-transformed cells and respond quickly to stimuli from the extracellular milieu.

A multitude of signal transduction pathways have evolved to keep MYC expression under tight control.

Drosophila MYC (*dMyc*), transcribed from the *diminutive locus*, is an onco-protein that shares both structure and function with its human homologue [58] [75].

dMyc contains several functional domains including the conserved basic helix-loop-helix zipper domain (bHLH/LZ) present at the C-terminus and used for dimerisation with *Drosophila* Max protein, the most conserved member of the *Drosophila* MYC network.

At its N-terminus dMyc contains several conserved motifs including the conserved MYC Boxes I and II which are partially required for MYC transcriptional activities; the

conserved MYC Box III and IV containing the acidic region, whose mutation analysis revealed a novel conserved function in controlling MYC protein stability [120].

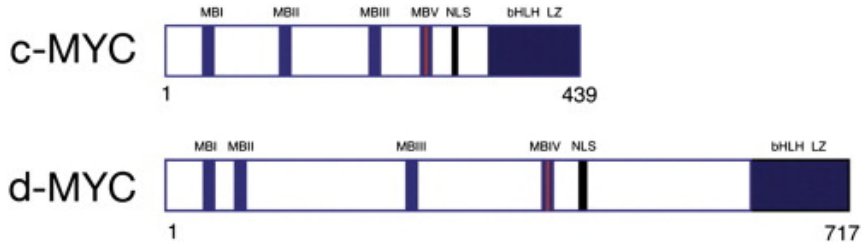


Figure 1.16: MYC proteins. Schematic representation of MYC proteins [121]. Comparison between human c-MYC and *Drosophila* d-MYC oncoproteins.

Experiments where *Drosophila* Myc cDNA was used to rescue proliferation defects of mouse embryonic fibroblasts from c-MYC mutant mice [122] and developmental defects of *Drosophila* dm^{PG45} hypomorphic mutants were rescued by expression of human c-MYC cDNA [123] demonstrated that *Drosophila* and vertebrate MYC can functionally substitute each other. Moreover these results showed that many MYC functions are conserved from insects to mammals.

MYC protein family exerts several activities, most of them through the MYC/MAX/MNT complex. MYC is largely responsible for growth control and organ size: overexpression of dMyc in large territories of the *Drosophila* imaginal disc induces growth by accelerating the G1/S transition of the cell cycle, while entry into the M-phase is limited by the availability of other molecules developmentally expressed (*e.g.* CDC25), thus limiting hyperproliferation [121].

MYC influences the expression of a large set of genes involved in several cellular processes and a preeminent group of MYC-activated targets encode for proteins involved in ribosome biogenesis and energetic metabolism [124]: MYC involvement is also demonstrated in the regulation of metabolic pathways such as glycolysis and glutaminolysis [125].

Moreover, high levels of Myc in *Drosophila* are demonstrated to cause cell-autonomous apoptosis through the expression of the pro-apoptotic genes *hid*, *grim*, *reaper* and *sickle* [126].

1.5.4. MYC-Mediated Cell Competition (MMCC)

CC collected the interest of cancer researchers when it was associated to cancer through the discovery of super-competitors. Super-competitor mutations increase cell fitness and lead to clonal overgrowth at the expense of the surrounding *wild-type* tissue, similarly to what happens in the early stages of tumour progression [127].

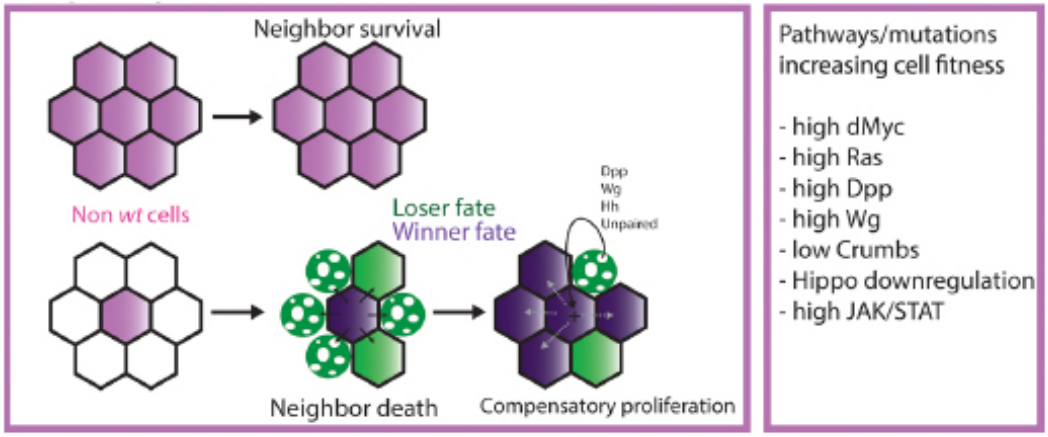


Figure 1.17: "Super-competitors" [92]**:** supercompetitor cells (light purple) do not induce apoptosis when surrounded by cells with the same genotype (top), whereas they can grow at the expenses of the surrounding *wild-type* cells (white) by inducing their death (bottom). Growth of the winner cells is induced by compensatory proliferation through the secretion of Dpp, Wg, Hh, and Unpaired from the dying cells (dark gray arrow), or a non-cell autonomous down-regulation of the Hippo pathway induced by dying cells (not depicted). Purple cells are the winner cells, green cells are the loser cells.

Clones expressing high dMyc levels within a *wild-type* tissue overgrow at the expense of the surrounding tissue to fill all the compartment [86] [101]. Clone expansion requires the elimination of the surrounding loser cells through *Caspase3*-mediated apoptosis; these *wild-type* loser cells appear to be eliminated within eight cell diameters away from the MYC-expressing cells [109] as CC seems to be mediated by soluble factors [95].

Similar evidence was found using a tandem duplication model, in which sibling clones resulted composed of cells expressing four copies (4x*dmyc*) and two copies (2x*dmyc*) of the *diminutive* (*dm*) gene. In these experiments the 4x*dmyc* clones were larger than the 2x*dmyc* clones and also larger than 4x*dmy*c clones growing in a homotypic background; similarly, the 2x*dmyc* clones were smaller then nearby 4x*dmyc* clones and also smaller than 2x*dmyc* clones growth in a homotypic background. The meaning of these studies is that the cellular behaviour is not fixed by genotypes but is adaptable to different contexts, and that the competition is based on relative and not absolute levels of *MYC* [93].

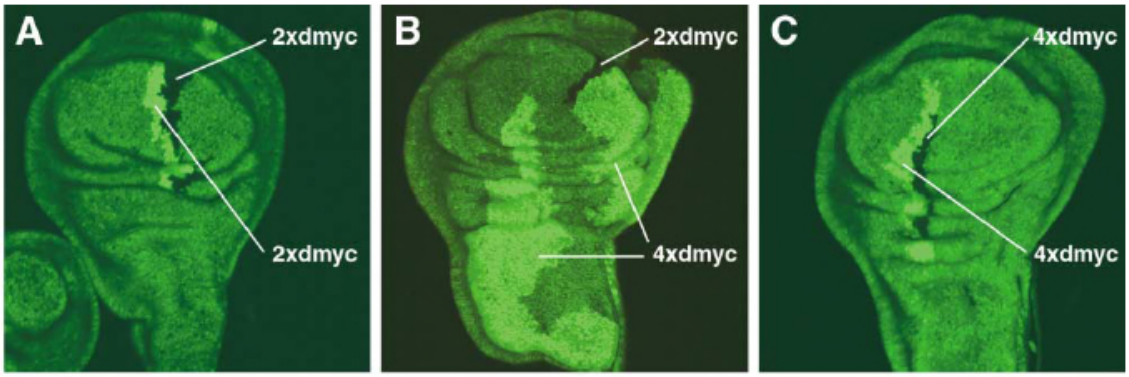


Figure 1.18: Cell Competition in tissues mosaic for cells with different copy numbers of the *dmyc* gene [93]: **A**) control twin clones which both express two copies of *dmyc*. **B**) experimental clones which either express four (GFP^{2+}) or two copies (GFP^{-}) of *dmyc*. The *4xdmyc* clones are larger than the *2xdmyc* clones. **C**) control twin clones which both express four copies of *dmyc*.

As *MYC* has a role in ribosome regulation, it has been hypothesised that MMCC may require its ability to influence ribosomal activity. Consistent with this idea, *MYC*-expressing clones bearing a ribosomal protein mutation were no longer able to out-compete surrounding cells [93]. Directly, the under-regulation of protein synthesis seems not to be sufficient to trigger MMCC, since the expression of *PI3K* (protein synthesis regulator) was demonstrated to be unable to induce CC [109].

The phenomenon of CC is mediated by different accessibility to growth signals: winner cells, due to their optimal growth status, would be able to capture more survival factors than loser cells, that instead would die by *JNK*-mediated apoptosis. To confirm this hypothesis and to test the "rescue" properties of some genes implicated in cell growth, researchers carried out a CC assay inducing mutant clones of the chosen genes in a MYC-overexpressing background: mutations that provided cells with an advantage in this competitive context were defined "super-competitors".

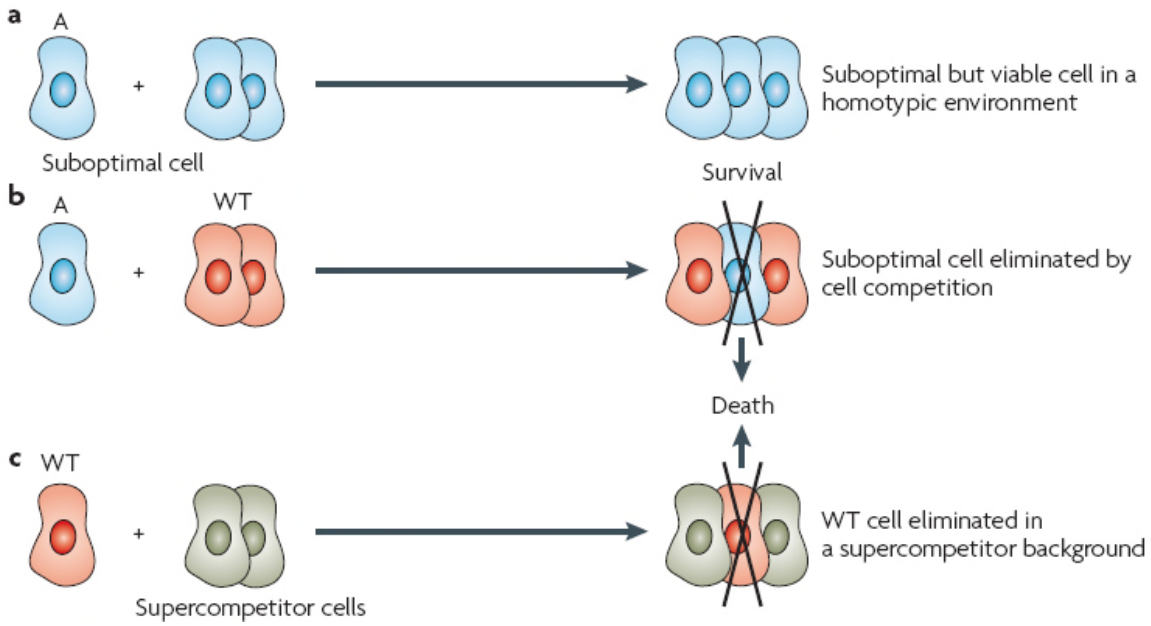


Figure 1.19: MYC-mediated CC [128]**: A)** a clone of cells with genotype A is viable in a homotypic context**. B)** cells with genotype A are culled by apoptosis when surrounded by *wild-type* cells. **C)** a case of super-competition.

1.5.5. Factors Involved in Cell Competition

CC is a process that requires communication between distinct cell populations. However, the molecular mechanisms underlying this dialogue are still unclear [102].

MMCC seems to be also mediated by soluble factors [95]. The study was carried out on *Drosophila S2* cells, where cells over-expressing *MYC* and control, GFP-positive cells, were co-cultured. The two populations were separated by porous filters that were permissive only to the transit of soluble molecules. Over a short period of time, the control GFP-positive cells were induced to die, while death of the MYC-expressing cells did not increase above background, demonstrating that CC did not require a cell-cell contact. In addition, medium from co-culture experiments was able to induce death of *wild-type* cells and not MYC-expressing cells when plated in single plates, and medium from single cultures was not sufficient to trigger CC, demonstrating that both populations are needed to trigger competitive behaviours.

Another important evidence about factors released into the competitive medium has been collected in a recent work. Patel and colleagues carried out a miRNAs multiple screening by which they identified miRNAs that target conserved CC-related genes in *Drosophila melanogaster*. This study led to the identification of nine potential CC-associated miRNAs in the *Drosophila* genome and, importantly, eighteen human homologs of these nine

potential *Drosophila* miRNAs are well reported for their involvement in different types of cancers [129].

CC can also be mediated by membrane proteins as Flower (Fwe), an evolutionary conserved membrane channel protein. Different Fwe isoforms were observed to tag "winner" and "loser" cells in *Drosophila* imaginal epithelia. Fwe is present in three isoforms with different C-terminal extracellular domains; the Ubi form is constitutively expressed by the epithelial cells of the discs, while the Lose-A and Lose-B forms are only present in the loser cells. The confrontation between the Ubi and the Lose forms was postulated to be the cause for loser cells to die, since knockdown of the *fwe* gene resulted in a reduced expansion of clones over-expressing MYC [96].

Another factor known to mark loser cells in *Drosophila* is SPARC (Secreted Protein Acidic and Rich in Cysteine). This molecule has a protective role in loser cells because its expression inhibits Caspase-3 activation. Like Fwe, SPARC seems to be actively expressed during CC as there is no rescue of apoptosis out of a competitive context [96].

1.5.6. The Hippo Pathway and Cell Competition

In both *Drosophila* and mammals, the Hpo signalling pathway regulates growth by preventing the transcriptional co-activator Yki/YAP (*Yes Associated Protein*) from entering nucleus and activating its downstream targets [77] .

Epithelial cells show strong polarisation along the apical-basal axis and epithelial cancer cells often exhibit absence or mislocalisation of polarity proteins and MYC upregulation [121].

Oncoprotein MYC is a transcriptional target of Yki/YAP and through a mechanism of negative feedback regulates MYC at both the transcriptional and post-transcriptional levels [130]. When MYC is downregulated in Yki-overexpressing clones these is no death of the surrounding cells, suggesting that MYC is the driving force of Yki-induced competition, and that relaxation of the Hpo pathway does not induce CC on its own, but it rather acts through direct regulation of MYC expression [77].

Drosophila lgl and its human homologue *HUGL-1* [61] are TSGs encoding scaffold proteins involved in the maintenance of A/B polarity in the epithelial cells. The mutant phenotype of these genes is dictated by the context in which the clones grow. In a homotypic context, cells mutant for *lgl* overgrow leading to the formation of a neoplastic mass (figure 1.19 A adapted: suboptimal cells are $lgl^{-/-}$ mutant clones) [131]; conversely, if the clones are surrounded by *wild-type* cells, they are eliminated from the tissue by the

activation of the JNK pathway (figure 1.19 B adapted). Overexpression of MYC in the *lgl*$^{-/-}$ clones not only rescues their viability but promotes malignant growth and induces death in the surrounding *wild-type* cells, turning *lgl*$^{-/-}$ cells from losers into super-competitors (figure 1.19 C adapted) [132].

Cells depleted of *lgl* show nuclear accumulation of the transcription factor Yki, where it can promote the expression of genes involved in cell survival and proliferation, among which MYC plays a role in transforming these cells into super-competitors [93]. Deregulation of the Hpo pathway also contributes to the overgrowth that characterises the oncogenic cooperation between activated Ras/Raf and polarity gene loss [133]. Yki targets are indeed upregulated in *Ras*^{V12}*; lgl*$^{-/-}$ tumours.

The constitutive activation of the oncoprotein Ras (e.g. Ras^{V12}) in cells mutant for *lgl* leads to the formation of growing neoplastic masses capable to invade and to create metastasis. The effect of Ras^{V12} on the neoplastic mutant cells appears to include super-competitive properties provided by a cell-autonomous increase in MYC expression. Mutations in the Hpo pathway give rise to hyperplastic growth, thus a cooperation between loss of apical-basal polarity and hyperplasia seems to be necessary and sufficient for pushing the cells towards a malignant phenotype.

MYC has been identified as a downstream effector of the Hpo pathway also in mammals; YAP promotes c-MYC transcription in a hepatocellular carcinoma mice model [121] and both are necessary for carcinogenesis in a nude mouse model [134]. YAP shows nuclear accumulation in several human tumours, partly associated with high MYC expression [135].

1.5.7. MMCC in Mammals

A decade later its discovery in *Drosophila*, MMCC was also found involved in mammalian development. Researchers induced functional genetic mosaics in the mouse and observed that imbalanced MYC levels in the epiblast (an early embryonic structure) were sufficient to induce the expansion of MYC over-expressing cells at the expenses of the *wild-type* cells, which were eliminated by apoptosis. Moreover, they noticed that *wild-type* cells composing the early embryo were heterogeneous in MYC content during development with a CC-like mechanism to refine the final epiblast selecting for cells with higher MYC levels [136].

Sancho and colleagues observed that defective embryonic stem cells underwent apoptosis during embryo development through a mechanism driven by MYC differences within the

embryo [137]. The first experimental evidence suggesting a role for MMCC in mammalian carcinogenesis was carried out in a familiar polyposis murine model, where APC cells deprived of c-Myc were out-competed by surrounding, c-Myc expressing *wild-type* cells, reverting the malignant phenotype [138]. An example of MMCC in mammals comes from a work conducted by Mamada and colleagues in 2015. The researchers established an *in vitro* model system that showed CC in mouse NIH3T3 embryo fibroblast cells. Co-culture of TEAD activity-manipulated cells with normal cells caused CC. Cells with reduced TEAD activity became losers, while cells with increased Tead activity became super-competitors. TEAD is a target that YAP binds to control cellular response and directly regulated Myc RNA expression, and cells with increased Myc expression became super-competitors [93] also in mouse [136]. The researchers demonstrated that NIH3T3 cells show CC mechanisms similar to those regulated by Yki and Myc in *Drosophila* [139] .

1.5.8. Cell Competition and Cancer

The origin of the vast majority of human tumours is monoclonal, descending from single progenitor cells through several rounds of mutation and selection, finally establishing a detectable mass. Even if an advanced mass is simple to find and to study, it is difficult to track early events. These initial events are vital for tumour progression and their understanding might help treat cancer successfully. CC may act during the early stages of cancer progression, where clones characterised by high fitness out-compete the unfit.

The initial expansion may increase the probability of accumulation of other mutations in subsets of cells, that would confer new oncogenic properties. As tumours are very heterogeneous, CC might provide a further selection for some kind of rapidly growing cells. In fact, hundreds of mutations are present and selected during cancer development. Selection mechanisms reflect interaction between tumour and normal cells during the progression of cancer. Some of these mutations may be selected through effects of CC, so they might not be required for tumourigenesis but might affect the chances of progression. Tumour cells may out-compete normal cells, such as super-competitors out-compete *wild-type* cells [140]. The first gene that demonstrated to induce CC in *Drosophila* when over-expressed was dMyc. Clones of *wild-type* cells are out-competed when nearby cells express high dMyc levels [50] [75]. Contemporary, researchers found that mutations inactivating the Hpo pathway can also cause super-competition in the presence of *wild-type* cells [141]. As Myc family genes and deregulation of Hpo pathway components are

involved in cancer, super-competitors have been hypothesised to be involved in the early stages of cancer progression.

Very recent evidence produced by two research groups defined the involvement of CC in different stages of tumour progression. Suijkerbuijk and colleagues demonstrated the involvement of clonal competition in driving the growth of tumour mass in a *Drosophila* model of $APC^{-/-}$ intestinal adenomas. Adenomatous $APC^{-/-}$ cells showed higher Yki/YAP activity via JNK pathway activation than *wild-type* surrounding tissue. Moreover they demonstrated that the prevention of CC through apoptosis inhibition restored host tissue growth and contained adenoma expansion, focusing on the importance of CC in adenoma progression in flies [142]. Eichenlaub and colleagues provided evidence that, in a *Drosophila e*pithelial cancer model, CC drives primary tumour formation and secondary tumour progression. Cells expressing EGFR together with the conserved microRNA miR-8 acquired super-competitive properties. Activating mutations in EGFR have long been recognised as driver mutations in human cancer. EGFR overexpression in wing imaginal disc cells leads to tissue hyperplasia, with accelerated cell proliferation accompanied by increased apoptosis. When combined with cooperating factors as miR-8 expression, a microRNA shown to downregulate the expression of the Septin family protein Peanut, involved in cytokinesis failure, EGFR overexpression can lead to neoplasia and metastasis through the induction of apoptosis and engulfment of the nearby *wild-type* cells [143].

In recent years, researchers postulated two different outcomes for CC during the early stages of tumour progression: "**field cancerisation**" [144] and elimination of pro-tumour cells, as an **intrinsic tumour suppressor mechanism**, to protect tissue from the very early mutated clones on the onset of tumour development [83] [102] [110].

The concept of field cancerisation was first introduced to explain the presence of histologically abnormal tissue surrounding oral squamous-cell carcinoma. According to one model, a cell sustains an initiating mutation. After massive proliferation, some of its clonal descendants may acquire a second mutant allele, and "initiated" cells may then proliferate and eventually occupy a large field of epithelium in which the chances of development of a malignant neoplasm are higher [100]. Field cancerisation has been observed in many epithelia, including the head and the neck region, lung, bladder, cervix, colon, breast, Barrett oesophagus and actinic keratosis, dysplastic precursors of squamous cell carcinoma [128].

1.6. p53 PROTEIN FAMILY

As p53 TSG has been found to be mutated in more than 50% of human cancers, it has attracted the interest of numerous researchers [146].

p53 protein was first identified in 1979 as a transformation-related protein characterised by a strong accumulation in cancer cell nuclei, able to bind simian virus 40 (SV40) large T antigen [147] and showing oncogenic activity. About 10 years later it was shown that those forms of the p53 protein were the result of missense mutations [148] and evidence from the first KO animal models, during the early 1990s, clarified the central role in tumour suppression of *wild-type* p53 [149]. During the years, several biological functions have emerged in which the p53 protein plays fundamental roles, such as apoptosis, development, differentiation, DNA recombination and cellular senescence [150].

p53 is part of a protein family including two other members: p63 and p73 [151] [152], considered the ancestors of p53 for the strong structural and functional relations: p53 seems to have evolved a tumour suppressive function in higher organisms [152]. The human gene encoding the p53 protein, TP53 (tumour protein 53), spans a 20 kb region on chromosome 17 [153]. Human p53 is a nuclear transcription factor of 53 KDa composed of 393 amino acids organised in several structural and functional domains [154]. The central region of p53, p63 and p73 is evolutionary conserved in *humans*, *Drosophila melanogaster* and *Caernorhabditis elegans* [155] and structural studies have revealed that majority of p53 missense mutations found in cancers are located in this region [156].

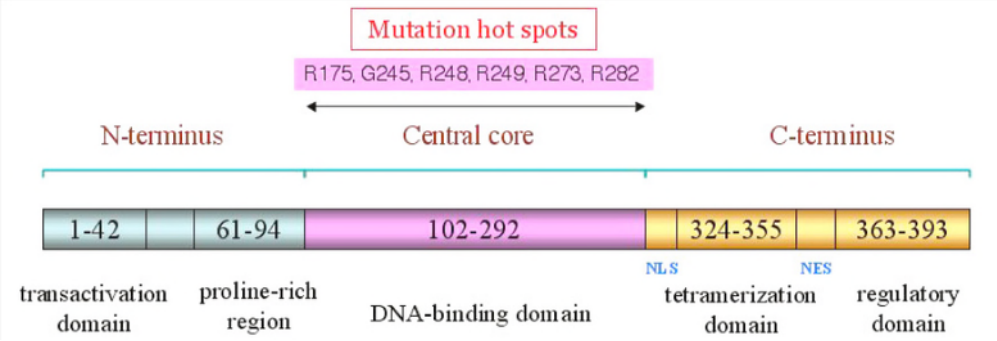


Figure 1.20: A schematic representation of the p53 structure with the hot spot mutation region and the principal missense mutations highlighted [146].

1.6.1. The Physiological Functions of p53

During the last 40 years, plenty of evidence demonstrated how p53 can be considered one of the main regulators of cell integrity.

Many genes have been found to be transcriptional targets of the protein, as *wild-type* p53 can be activated by a number of stresses within the cell, including hypoxia, DNA damage and oncogene activation [157].

During cell life, *wild-type* p53 protein is present and sequestered by the Mouse Double Minute 2 homologue (MDM2), so its transcriptional activity is inhibited. MDM2 is an important negative regulator of the p53 tumour suppressor protein and functions both as an E3 Ubiquitin ligase that recognises the N-terminal trans-activation domain (TAD) of the p53 protein, and as an inhibitor of p53 transcriptional activation [158]. Additionally, a homologue of MDM2, MDMX, also serves as a negative regulator of p53, as MDM family members are found overexpressed in a myriad of neoplasms showing nonfunctional *wild-type p53* [159].

In response to various types of stress, p53 enters nucleus, carries out its functions as a sequence-specific transcription factor, acting as a homotetramer and binding to p53 response elements on inhibiting cell cycle progression, promoting senescence, inducing apoptotic cell death or acting in metabolic processes [157] [160] [161]. Beside this, protein-protein interactions may be implicated in other functions of the protein [162].

The downstream targets are differentially activated depending on the cell type, on the damage and on various other not already identified parameters.

p53 exerts its role on **cell cycle progression and genome stability** by inducing a transient G1 cell cycle arrest in response to DNA damages [146].

One of the most important functions of p53 is to **induce apoptosis** in damaged cells. p53 transcriptionally activates the expression of several pro-apoptotic BCL-2 family proteins and, by interacting with pro-apoptotic and anti-apoptotic factors in the cytoplasm and at the mitochondrial membrane, it can lead to the activation of caspase-induced apoptosis. Additional experiments showed that disruption of the apoptotic pathway downstream of *wild-type* p53 by overexpressing BCL-2 or dominant-negative forms of the caspase 9 promotes lymphomagenesis [163].

p53 may also induce **senescence** in response to oncogene activation or telomere dysfunction and is dependent on the p53-mediated transcriptional activation of p21, which is a cyclin-dependent kinase (CDK) inhibitor that halts the cell cycle in the G_1 phase.

The maintenance of cellular homeostasis is a p53 role performed in case of metabolic stress when cells go through starvation. Upon glucose deprivation, MDH1 (Malate DeHydrogenase 1), the enzyme that catalyses the reversible oxidation of malate to oxaloacetate, recruits p53 and stabilises it inducing cell cycle arrest [164]. AMPK, a nutrient stress sensor, activates p53 by phosphorylation or acetylation [165]. This mechanism involves also ribosome proteins that sequester MDM2, favouring p53 over-expression [166].

In metabolism, p53 protein plays its role by limiting the glycolytic rate through the inhibition of glucose uptake by the glucose transporters type 1, 3 and 4 (GLUT1, 3, 4), This in turn slows glucose oxidation to pyruvate by phosphofructokinase 1 (PFK1) and phosphoglycerate mutase (PGM) through direct or indirect inhibition of these enzymes. This is followed by promotion of pyruvate conversion in acetyl-CoA and upregulation of the mitochondrial oxidative phosphorylation [167]. The balance of glucose metabolism in favour of energy production is physiological in some organs, as heart and brain. Until p53 is active, it promotes cell survival maintaining metabolic homeostasis, and when the *wild-type* p53 function is lost, cancer cells can however survive and divide by exploiting the glycolytic switch [168]. This metabolic switch that increases glycolytic flux, typical of cancer cells, is known as the Warburg effect [169].
Another important aspect of a tumour is the capacity to **interact with the immune system**: p53 participates also in this aspect by inducing the activation of cytokines and pro-inflammatory agents that act to decrease tumour size and angiogenic potential [170].

1.6.2. p53 as a Tumour Suppressor

First evidence about p53's role in cancer biology was found in late 1960s, when germline mutations in *TP53* were associated with the Li-Fraumeni Syndrome (LFS), an autosomal dominant disorder predisposing individuals to breast cancer, sarcomas and other neoplasms [171] [172] [173].
p53 is not involved in physiological development, but animals harbouring a p53 deletion are extremely cancer-prone [149] [174]. Loss of p53 in human tumours induces formation of more aggressive and vascularised masses [175].
Nowadays it is widely acknowledged that p53 compromised functions are the most common genetic events in human cancer [150]: while almost 50% of all tumours exhibit mutation of p53, in many other its normal function is attenuated by several mechanisms

acting in its modulation as the up-regulation or activation of negative regulators, as MDM2 or MDMX found over-expressed in a variety of neoplasms. [158].

In addition to the discussed roles of p53 in cell cycle regulation, senescence and apoptosis, further functions are associated with the inhibition of differentiated cell reprogramming to pluripotent stem cells (PSCs), [176] so it is plausible that p53 suppresses tumourigenesis by contrasting cell stemness.

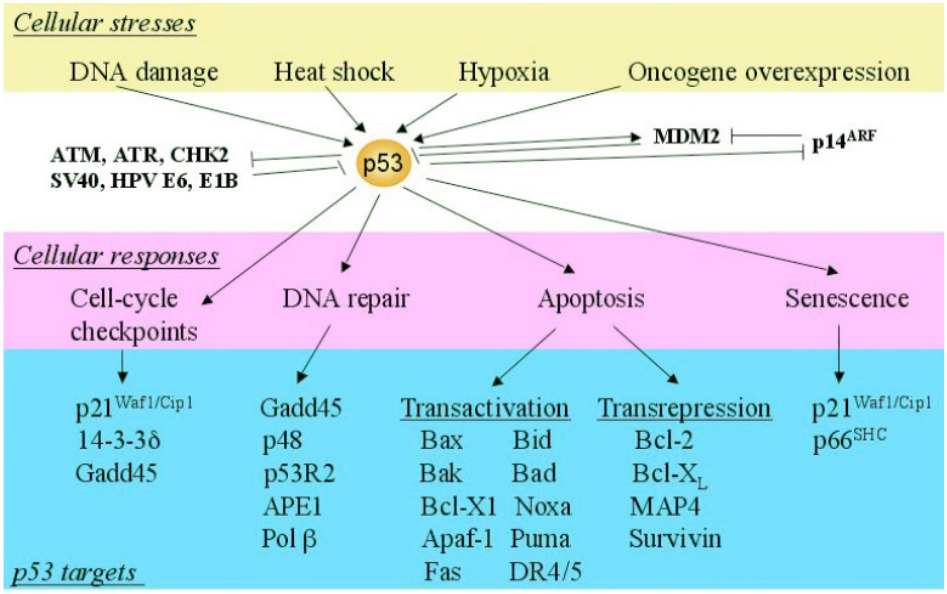


Figure 1.21: The Stress Response Pathway, in which p53 protein exerts several functions by activating different downstream targets [146].

1.6.3. p53 and the "Gain of Function" Hypothesis

A difference between *p53* and many other TSGs is the appearance of a huge number of missense mutations along its sequence: the vast majority of tumour suppressors, in fact, is inactivated by deletions [177], but in *p53*, a great number of cancer-associated mutations are single base substitutions resulting in a full-length active protein [178].

The mutational hot spots in the *p53* sequence are concentrated in the region encoding the DNA-binding domain (Fig 1.16). The mutant products fall into two broad categories: the *Contact mutations*, affecting residues involved directly in DNA contacts without altering p53 conformation, and the *Structural mutations*, that cause a conformational change in the core domain [179]. While *wild-type* p53 has a very short half-life when unreleased, some of these stable mutants have a prolonged half-life and can create precipitates inside cancer cells' nuclei [180] [181] [182].

Many of these stable mutants of p53 can exert a dominant-negative (DN) effect on the remaining *wild-type* protein [183] and such dominant activity may be affected by either formation of mutant/*wild- type* p53 co-tetramers or by the incorporation of the *wild-type* form into mutant tetrameric aggregates [184]. Interestingly, missense mutation of p53 are often followed by Loss of Heterozygosity (LOH) at the corresponding *locus* [185] [186].
The sum of all these observations led researchers to elaborate the "*gain of function*" hypothesis, either because many missense mutations of p53 are not equivalent to its loss of function, or because the strong selection of some of these forms upon the *wild-type* p53 protein suggests an important role in tumourigenesis.
There is also evidence that mutant p53 exerts its pro-oncogenic activities independent of its effects on the *wild-type* form [187] and many studies have also demonstrated a poor prognosis for many kinds of human tumours with missense mutations of p53 [178]. p53 pro-oncogenic activity may be carried out through tree different mechanisms: first, tumour cells could be selected for loss of *wild-type* p53; second, p53 mutants could lose their tumour-suppressive functions while retaining other aspects that can be involved in tumourigenesis: third, mutant p53 proteins could acquire neomorphic activities that improve cell performance and tumour growth [188].

1.6.4. p53 and Cell Competition

Myc supercompetitor behaviour in *Drosophila melanogaster* has been demonstrated to be dependent on the p53 *wild-type* function [168] by de la Cova and colleagues in 2014.
Researchers have analysed an increase in metabolic requirement of Myc-overexpressing cells during CC. This increased requirement is not observed in cells of the same genotype growing in a homotypic context.
Myc protein overexpression alters mitochondrial morphology and impairs COX activity of complex IV (a common trait in many cancers) which results in a reduced electron transport chain and in a reduced cellular ATP pool, followed by an increased glycolysis, a typical trait of cancer cells described as the Warburg effect [169]. The role of p53 is to balance these stressful metabolic changes occurring in Myc-overexpressing cells (where p53 *m*RNA is increased) by promoting OXPHOS and restraining glycolysis [168]. COX deficiency and low steady-state ATP levels are accompanied by a p53-dependent increase in *scox* mRNA, the cytochrome c oxidase 1 homologue in *Drosophila* [189], to maintain metabolic homeostasis and assure fitness protection to Myc-overexpressing cells.

In competitive co-culture assays, p53 inhibition by *p53 dsRNAs* downregulates glycolysis in Myc-overexpressing cells, and *glut1* and *glut3* expression is abolished. The result of this metabolic change is that Myc super-competitor cells lose their capability to out-compete loser cells and to expand and colonise the tissue [168].

The Aim of the Study

Tumour is a complex and heterogeneous disease in which cancer cells, in constant communication with their surrounding environment, undergo selective processes aimed at gaining strength and malignancy in order to protect their unlimited replicative power and ensure potentially endless descendants.

The aim of this study is to find an essential signature of MYC-mediated cell competition in human cancers and demonstrate a role for this phenomenon in shaping cancer evolution through continuous selection of the fittest cells within the expanding mass.

Chapter 2

Results and Discussion

Part 1

2.1. Definition of an Essential Signature of MYC-Mediated Cell Competition in Human Epithelial Cancers

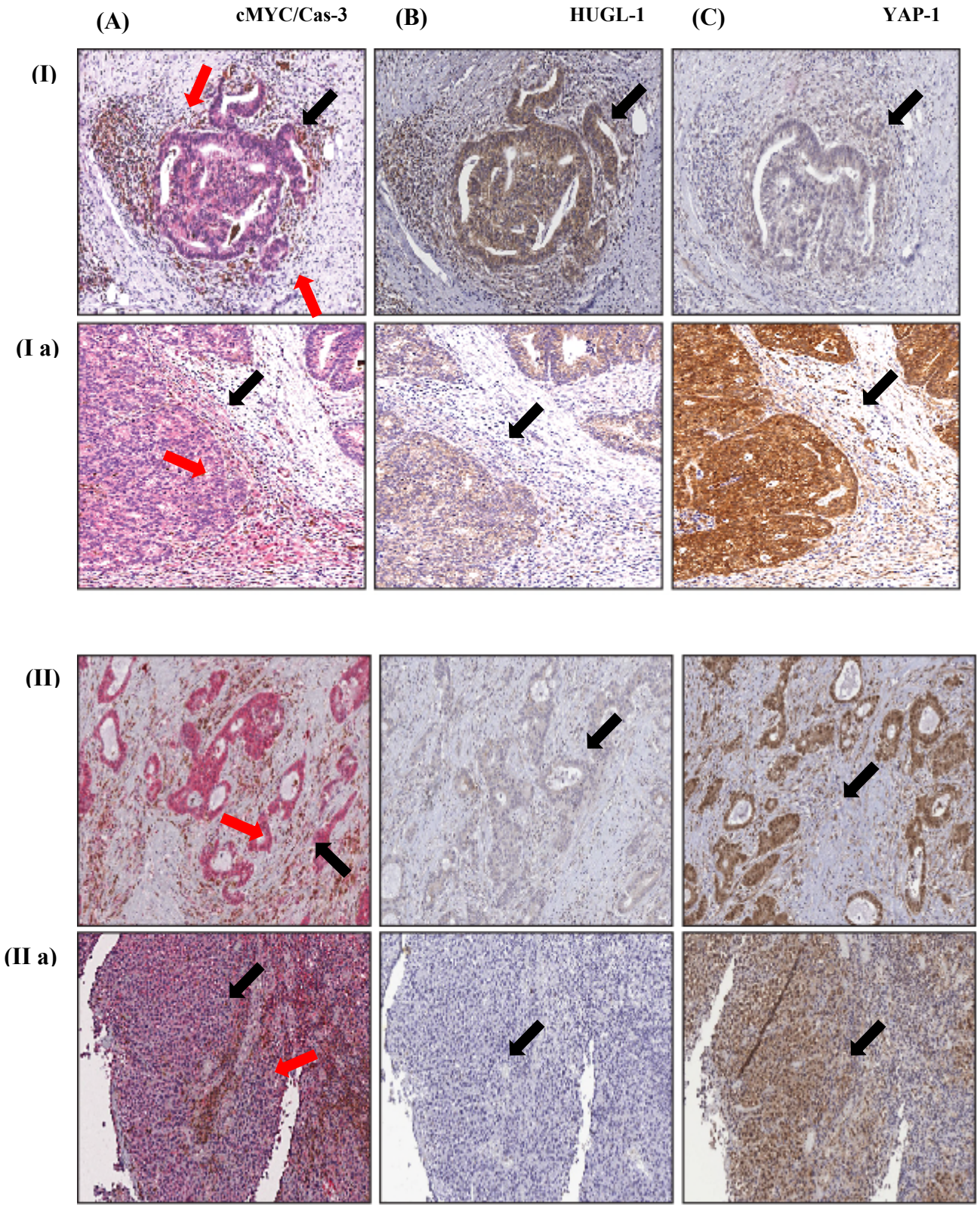
In *Drosophila*, dMyc oncoprotein was found to play a role in transforming cells into super-competitors able to overgrow at the expense of surrounding non over-expressing dMyc cells that are eliminated by *Caspase-3* mediated apoptosis [93].

cMYC over-expression is frequently observed in human cancers [112].

cMYC transcription is promoted by YAP (Yes-Associated Protein) in several carcinoma mice models [121] and both these proteins are necessary to induce carcinogenesis in nude mouse models [134]. YAP is found accumulated in the nucleus of several human tumours and it is frequently associated with a high cMYC expression profile [135].

YAP (Yki in *Drosophila*) nuclear translocation is induced by Hpo pathway alterations in both *Drosophila* and humans [190]. These alterations can be triggered by many physical and biochemical stimuli [191] among which loss of A/B cell polarity is prominent in epithelial carcinogenesis [50]. HUGL-1/Lgl delocalisation/absence [61] has been demonstrated to cause YAP/Yki nuclear translocation in human and *Drosophila* cancers. [65] [79] [80] [82].

To verify if signs of MMCC were present in human carcinomas, I hence defined an essential signature of CC in which cMYC overexpression was searched within tumour cells in association with HUGL-1 mislocalisation/absence and YAP nuclear enrichment, as both are involved in Hpo-dependent cMYC overexpression, and *Caspase-3* positive cells were searched out of the tumour mass.


(A)
cMYC/Cas-3
(B)
HUGL-1
(C)
YAP-1
(I)
(I a)
(II)
(II a)

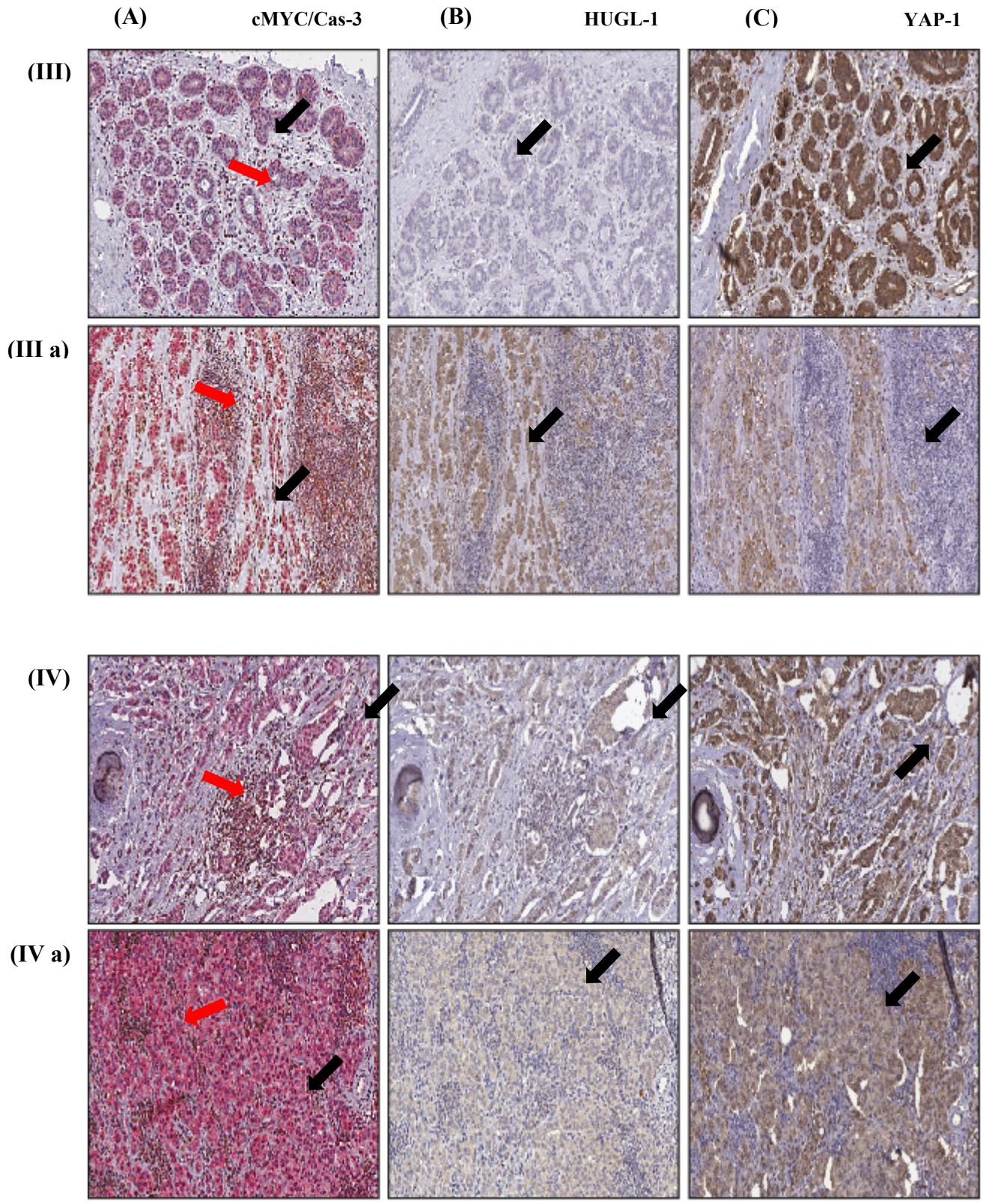


Figure 2.1: IHC on human carcinomas (I: colon, II: lung, III: breast lobular, IV: breast ductal) and respective secondary lesions (I a: liver , II a: brain, III a: lymph node, IV a: lymph node). Comparison of the same regions. **A.** Double staining for cMYC (pink) and Cleaved Cas-3 (brown) **B.** Staining for HUGL-1 (brown) **C.** Staining for YAP (brown). Black arrows indicate tumour clones, red arrows indicate lower cMYC levels and Cleaved Cas-3 positive clones (signs of CC). 100X magnification.

In the great part of the samples investigated it was possible to see as MMCC is engaged between tumour cells and the surrounding stromal cell populations.

As expected, tumour cells (red arrow) are characterised by HUGL-1 cytoplasmic localisation/absence, YAP nuclear persistence and cMYC upregulation, while surrounding stromal and immune cells, characterised by a lower cMYC expression level (black arrows), are positive to Cleaved Caspase-3, suggesting they are undergoing apoptosis.

This evidence indicates that CC driven by local differences in cMYC expression is a mechanism by which either primary epithelial tumours and respective metastases (with characteristic loss of A/B polarity and deregulation of the Hpo pathway) with high cMYC levels lead, while expanding, the surrounding non-tumour cells to apoptotic death.

Potentially, CC persists until a difference in cMYC levels is found between neighbouring cells.

2.2. Clone Competition - MMCC Within the Cancer Mass.

An interesting observation emerged while analysing the IHC slides in search of CC markers: MMCC seemed to exist even within different tumour cells. In Figure 2.2, adjacent tumour masses are characterised by different patterns of HUGL-1 and YAP (fig 2.2 B and C, black arrows) and different cMYC levels (fig 2.2 A, black arrows). The mass showing lower cMYC levels displays some cells positive to Cleaved Caspase 3 antibody (fig 2.2 A, red arrow), revealing an elimination in progress. This evidence suggests a role for cMYC in clone selection. This kind of MMCC will be referred to as Clone competition.

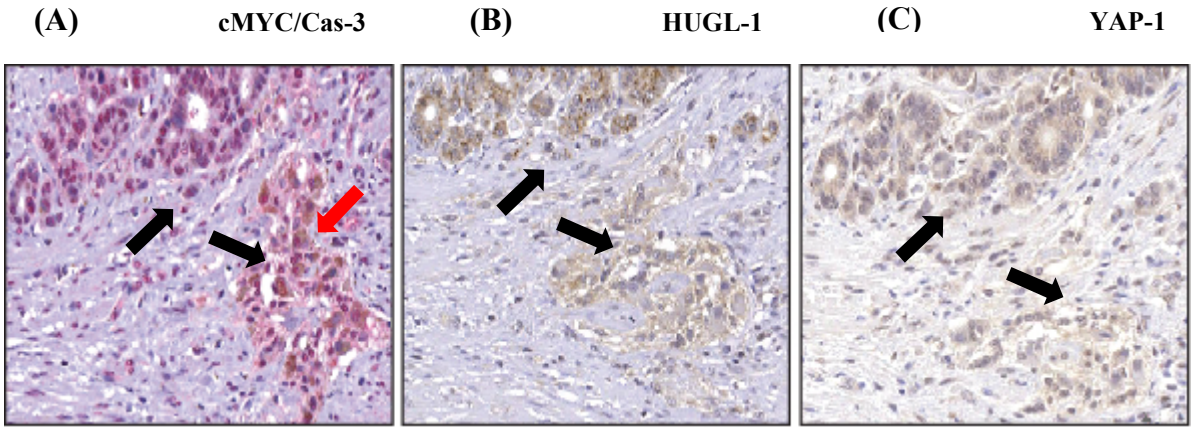


Figure 2.2: IHC on a colon carcinoma sample. Comparison of the same region in sequential slices. **A.** Double staining for cMYC (pink) and Cleaved Cas-3 (brown) **B.** Staining for HUGL-1 (brown) **C.** Staining for YAP (brown). Black arrows indicate tumour clones, the red arrow indicates lower cMYC and Cleaved Cas-3 positive clones. 200X magnification.

Signs of clone competition were found in a large panel of primary carcinomas (fig. 2.3 A) and correspondent secondary tumours (fig 2.3 B), where tumour clones surrounded by higher cMYC-expressing cells undergo apoptosis (fig. 2.3 A and B, black arrows).

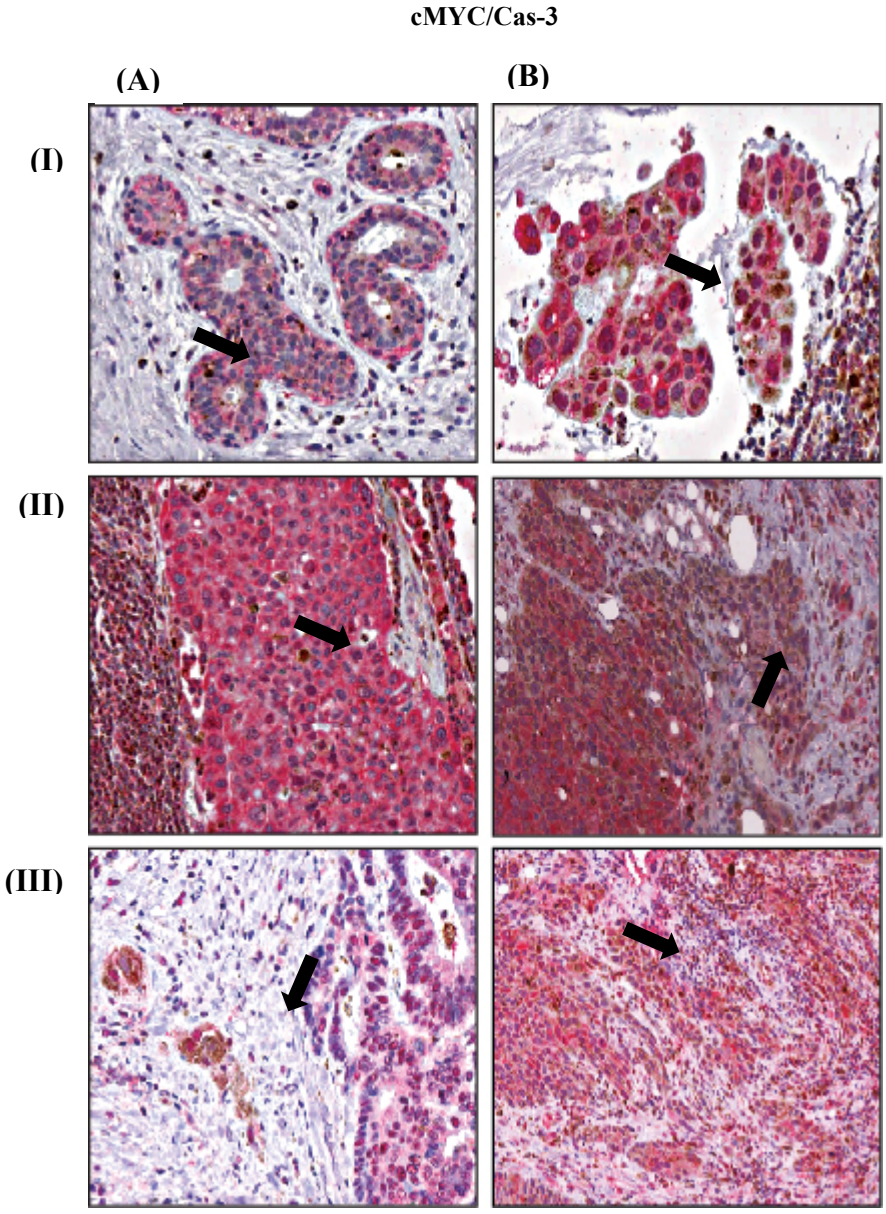


Figure 2.3: IHC on several primary (**A**) and corresponding secondary (**B**) tumours, MYC (pink) and Cleaved Cas-3 (brown). **I- A.** Ductal breast carcinoma **I- B.** Lymph node metastasis **II- A.** Lung poorly differentiated carcinoma **II- B** Brain metastasis **III-A**. Colon adenocarcinoma **III- B.** Liver metastasis. Black arrows indicate cancer clones characterised by low MYC expression and Cleaved Cas-3 positive staining. 200X magnification.

Taken together, these results obtained by IHC analysis let us hypothesise an important role played by MMCC during carcinogenesis: while CC is useful to shape the tumour mass, enhancing growth at the expense of the surrounding tissue, the mechanism of clone competition selects for clones with higher fitness within the tumour mass, characterised by

elevated cMYC oncoprotein levels. Cell competition and clone competition can be seen as two faces of the same coin, playing integrative roles in promoting malignancy and aggressiveness of solid tumours. This study on CC seems also to suggest that elimination of the weaker cells allows the stronger population to overgrow [93] [130].

2.3. An in Vitro Model to Study MMCC in Genetically Distant Cells

IHC analysis revealed that signs of MMCC are present in primary and secondary tumours, and this phenomenon seems to occur both between cancer/stromal cells and cancer/cancer cells characterised by a different expression of cMYC oncoprotein. MMCC has been demonstrated to be at work *in vitro* in *Drosophila* cell lines [95] [168] and in mouse fibroblasts [139] but its role has never been investigated between different cancer cells.

To evaluate whether cMYC protein differences are sufficient to drive CC between genetically distant cancer cells, I performed Cell Competition Assays (CCAs). CCA is an *in vitro* cell assay designed to assess competitive interactions occurring between co-plated cell populations compared to their behaviour in separate conditions.

I performed CCAs using two cell line pairs coming from different carcinomas: lung carcinoma H460 and H1975, colorectal carcinoma LS174T and LoVo. All the cell lines were previously characterised for cMYC levels (figure S.I. 1).

The first assay has been performed between lung carcinoma cell lines: H460 and H1975 (figure 2.4), with H460 harbouring higher cMYC levels (figure S.I. 1).

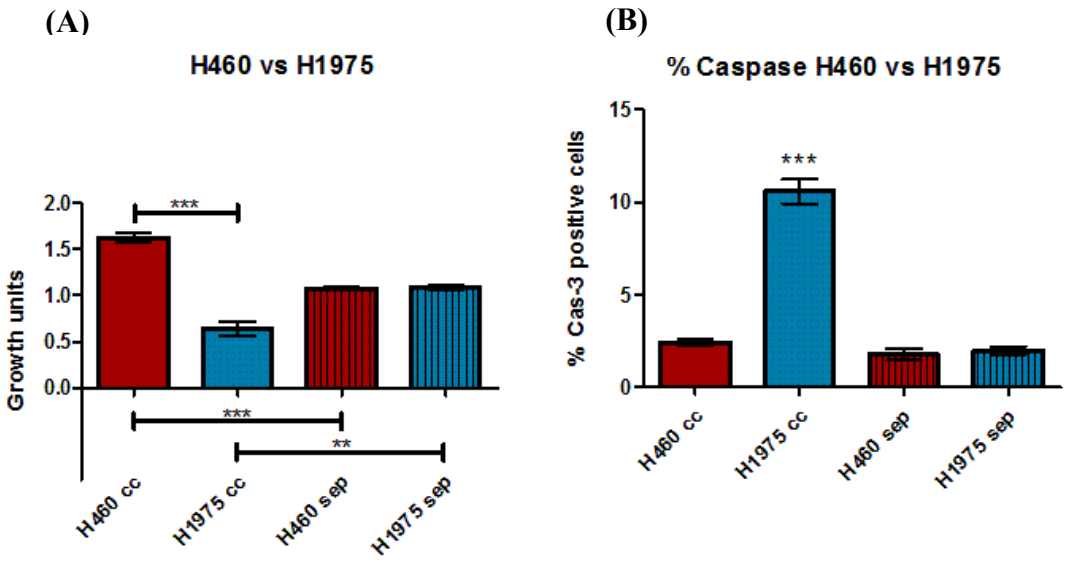


Figure 2.4: CCA on H460 and H1975 lung cancer cell lines. Co-cultured cell lines, indicated with **cc,** represent the first two bars of each graph; separated cell lines, indicated with **sep,** are the second two bars of each graph. **A)** Growth after 5h of culture; each assay was repeated 3 times and counted twice. **B)** Apoptotic cell percentage calculated by IF after 5h of culture. H1975 cell line shows the highest Caspase-3 percentage when co-cultured with H460. Each IF was repeated 4 times and 4 fields were counted in each slide. ± SEM is indicated.

As can be seen in Figure 2.4 A, H460 cell line in co-culture (red bar) showed a significantly higher growth rate than the H1975 in co-culture (blue bar) and than H460 in a separate condition (red striped bar), showing high competitive advantage; H1975 showed a significant decrease in growth units in co-culture (blue bar) compared to its growth in a separate condition (blue striped bar).

The level of Cleaved-Caspase 3 positive cells in the loser population was more than three times higher compared to the same cell population in separate conditions (Fig. 2.4 B, blue bar), defining a competitive interaction between the two cell lines in which winners induced the losers to undergo apoptosis. The increase of apoptosis in co-culture is a consequence of the activation of competitive interaction between the two populations and explains the low cell number found after the CCA. From the CCA and the Cleaved-Caspase 3 IF we can infer that cells carrying higher cMYC levels are able to out-compete genetically different cells with lower MYC levels.

The CCA was repeated using other two cell lines from different cancers (colorectal adenocarcinoma), where LS174T is characterised by a higher cMYC protein level (figure S.I. 1).

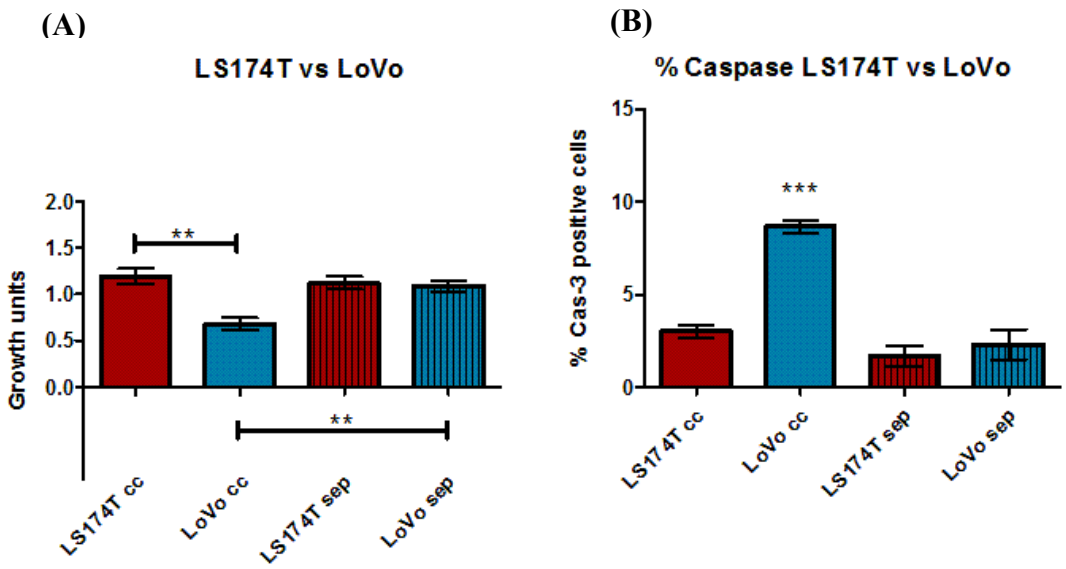


Figure 2.5: CCA on LS174T and LoVo colon cancer cell lines. Co-cultured cell lines, indicated with **cc,** represent the first two bars of each graph; separated cell lines, indicated with **sep,** are the second two bars of each graph. **A)** Growth after 5h of culture; each assay was repeated 4 times and counted twice. **B)** Apoptotic cell percentage calculated by IF after 5h of culture. LoVo cell line shows the highest Caspase-3 percentage when co-cultured with LS174T. Each IF was repeated 4 times and 4 fields were counted in each slide. ± SEM is indicated.

As showed in the CCA graph (Figure 2.5 A), the growth units of the line expressing less cMYC are lower in co-culture than those of the same cell line in separate conditions, and this is associated with a higher percentage of positive Cas-3 cells (figure 2.5 B).

These results obtained in cell lines coming from different tumours confirm that cMYC can play a role in driving competitive interaction between different cancer cells.

To assess if cMYC downregulation in the winner cell line was sufficient to lose the competitive drive, I performed a CCA on the LS174T and LoVo cell line pairs (figure 2.6) following cMYC chemical inhibition in the winner LS174T line, while the LoVo loser cell line was used in native conditions.

Following chemical inhibition, cMYC protein levels were assessed by western blot (WB) in treated LS174T and mocked LoVo. As can be seen in Figure S.I. 2, cMYC inhibition induced a drastic decrease in cMYC levels in the treated LS174T line (lane 2), that were not rescued at the native levels (lane 4) also 5 hours after inhibition (lane 3).

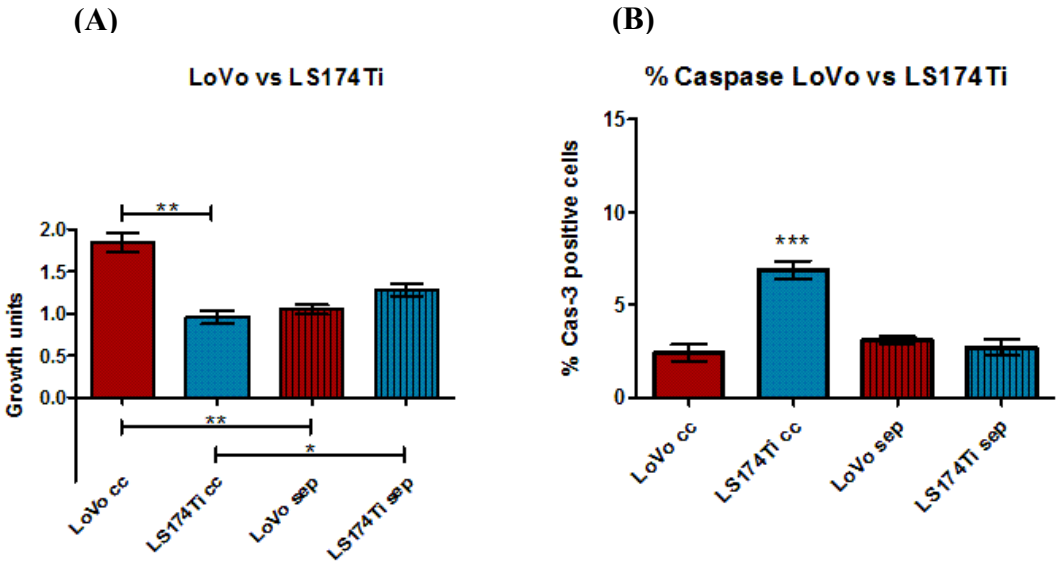


Figure 2.6: CCA on LoVo and LS174Ti colon cancer cell lines. Co-cultured cell lines, indicated with **cc,** represent the first two bars of each graph; separated cell lines, indicated with **sep,** are the second two bars of each graph. **A)** Growth after 5h of culture; each assay was repeated 4 times and counted twice. **B)** Apoptotic cell percentage calculated by IF after 5h of culture. LS174Ti (treated with the inhibitor 10058:F4) shows the highest Caspase-3 percentage when co-cultured with native LoVo Each IF was repeated 4 times and 4 fields were counted in each slide. ± SEM is indicated.

This CCA showed how cMYC inhibition in the LS174T cell line was sufficient to confer a competitive drive to the natively loser LoVo cell line. In fact, co-cultured LT174Ti displayed lower growth units than in separate conditions and the highest percentage of Cas-3 positive cells.

This was clear demonstration that, whatever the genetic distance of two cell populations inhabiting the same field, cMYC modulation is sufficient to change their competitive *status*, suggesting a role for MMCC in the selective growth of early diverged clones and, as a consequence, in cancer evolution.

2.4. An in Vitro Model to Study MMCC in Genetically Identical Cells

The IHC investigation revealed the presence of clones, within the carcinoma mass, characterised by different cMYC expression but sharing some molecular features (HUGL-1 delocalisation and YAP nuclear accumulation). This finding may be ascribable to a phenomenon of late genetic drift within the tumour. In a tumour mass composed of sibling cells, some local event may indeed trigger a sudden cMYC up-regulation. In this scenario, MMCC could play a role in the selection of the fittest clones.

In order to evaluate whether differences in MYC levels were sufficient to drive competitive interactions in siblings from the same cancer cell line, I performed CCAs co-culturing native cells and cells from the same line in which cMYC-MAX activity was previously inhibited by the same drug as in the previous assays. All the cell lines used in

the assays have been previously characterised for the expression of cMYC protein and cMYC target genes before and after the chemical treatment (figure S.I. 3 A and B).
CCAs were performed on the same carcinoma cell lines as in the previous assays: lung carcinoma H460, lung adenocarcinoma H1975 and colorectal adenocarcinoma LoVo and LS174T.

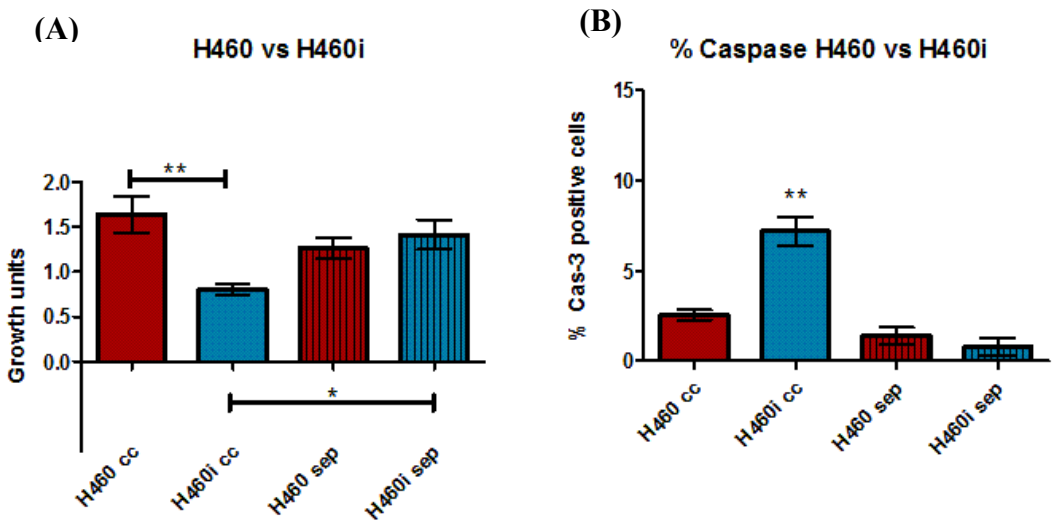


Figure 2.7: CCA on H460 lung cancer cell lines. Co-cultured cell lines, indicated with **cc,** represent the first two bars of each graph; separated cell lines, indicated with **sep,** are the second two bars of each graph. **A)** Growth after 5h of culture; each assay was repeated 4 times and counted twice. **B)** Apoptotic cell percentage calculated by IF after 5h of culture. H460i (treated with the inhibitor 10058:F4) shows the highest Caspase-3 percentage when co-cultured with mock-treated H460. Each IF was repeated 4 times and 4 fields were counted in each slide. ± SEM is indicated.

In this CCA, after 5h of co-culture (fig 2.7 A) the H460 cells treated with the 10058:F4 inhibitor (blue bar) show a significant decrease in growth compared to that of the same cell population plated in separate conditions (blue striped bar); its decrease is associated with a higher percentage of Cas-3 positive cells (fig 2.7 B, blue bar). Simultaneously, the native H460 cell population in co-culture (fig 2.7 A, red bar) exhibits a significative overgrowth compared to its competitor and shows an increased growth if compared to the H460 population plated in separate conditions (red striped bar). Moreover, no significant increase in Cas-3 positive cells was found. Notably, the two cell populations in separate conditions show a similar growth (fig. 2.7 A, red striped and blue striped bars) and a similar Cas-3 positive cell percentage (fig. 2.7 B, red striped and blue striped bars).
This evidence suggests that the inhibition of MYC-MAX activity in the H460 cell line lowered its fitness and made it undergo apoptotic death as a consequence of the co-presence of the H460 fitter cells.

Similar results have been obtained from the CCA performed on H1975 (figure 2.8 A- B), LS174T (figure 2.9 A- B) and LoVo (figure 2.10 A- B) cell lines.

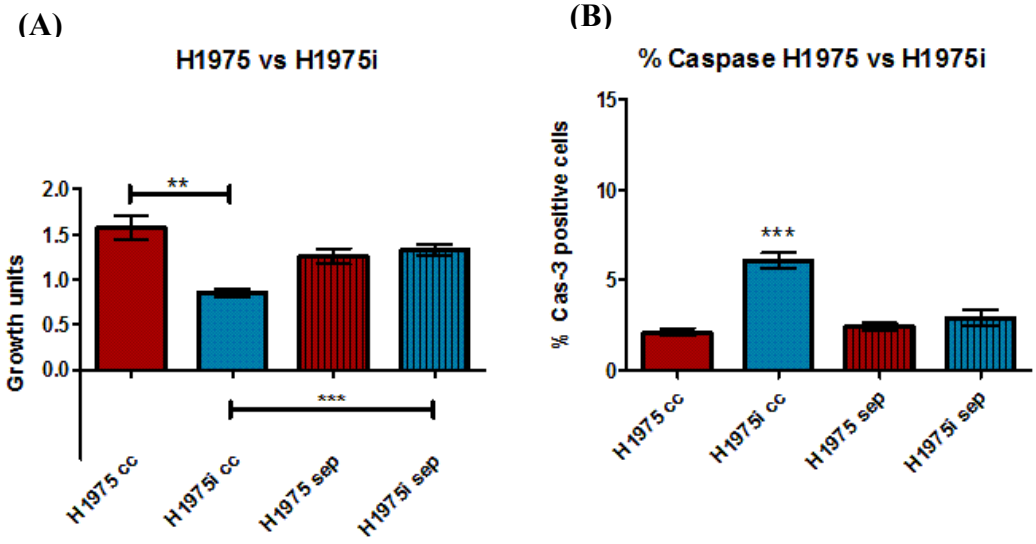


Figure 2.8: CCA on H1975 lung cancer cell lines. Co-cultured cell lines, indicated with **cc,** represent the first two bars of each graph; separated cell lines, indicated with **sep,** are the second two bars of each graph. **A)** Growth after 5h of culture; each assay was repeated 3 times and counted twice. **B)** Apoptotic cell percentage calculated by IF after 5h of culture. H1975i (treated with the inhibitor 10058:F4) shows the highest Caspase-3 percentage when co-cultured with mock-treated H1975. Each IF was repeated 4 times and 4 fields were counted in each slide. ± SEM is indicated

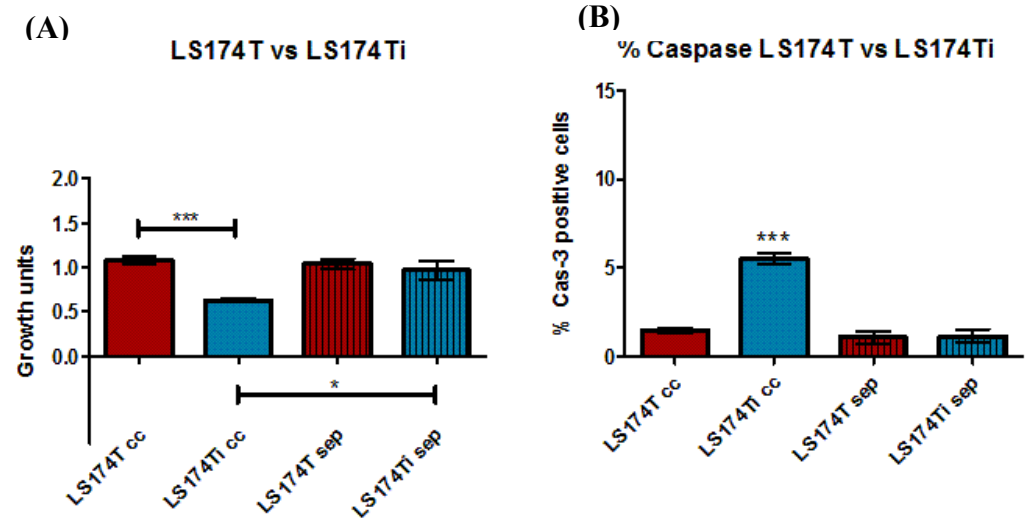


Figure 2.9: CCA on LS174T colon cancer cell lines. Co-cultured cell lines, indicated with **cc,** represent the first two bars of each graph; separated cell lines, indicated with **sep,** are the second two bars of each graph. **A)** Growth after 5h of culture; each assay was repeated 3 times and counted twice. **B)** Apoptotic cell percentage calculated by IF after 5h of culture. LS174Ti (treated with the inhibitor 10058:F4) shows the highest Caspase-3 percentage when co-cultured with mock-treated LS174T. Each IF was repeated 4 times and 4 fields were counted in each slide. ± SEM is indicated.

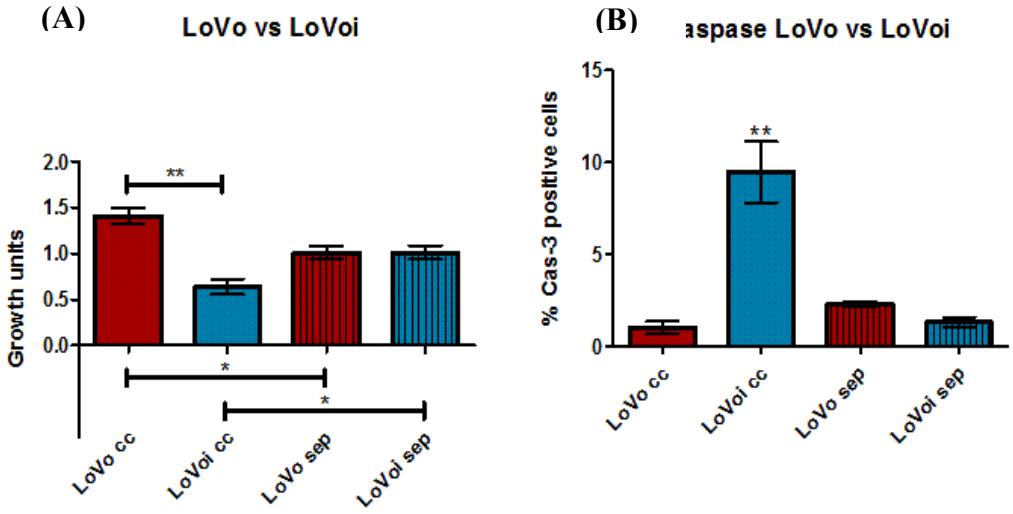


Figure 2.10: CCA on LoVo colon cancer cell lines. Co-cultured cell lines, indicated with **cc,** represent the first two bars of each graph; separated cell lines, indicated with **sep,** are the second two bars of each graph. **A)** Growth after 5h of culture; each assay was repeated 3 times and counted twice. **B)** Apoptotic cell percentage calculated by IF after 5h of culture. LoVoi (treated with the inhibitor 10058:F4) shows the highest Caspase-3 percentage when co-cultured with mock-treated LoVo. Each IF was repeated 4 times and 4 fields were counted in each slide. ± SEM is indicated.

During the early stages of cancer development, given the strong selective pressure imposed by the microenvironment, cancer clones can acquire different mutations and only a little proportion of mutant cells, that are not stalled or aborted, are able to initiate a malignant growth.

According to the model of clonal evolution, starting from a cell within a new lesion, the accumulation of mutations gives rise to several phenotypically and genotypically divergent clones until a local new pressure wave or additional mutations occur that regulate the expansion of different clones by mutual competition, called *clonal interference* [7] [16] [28].

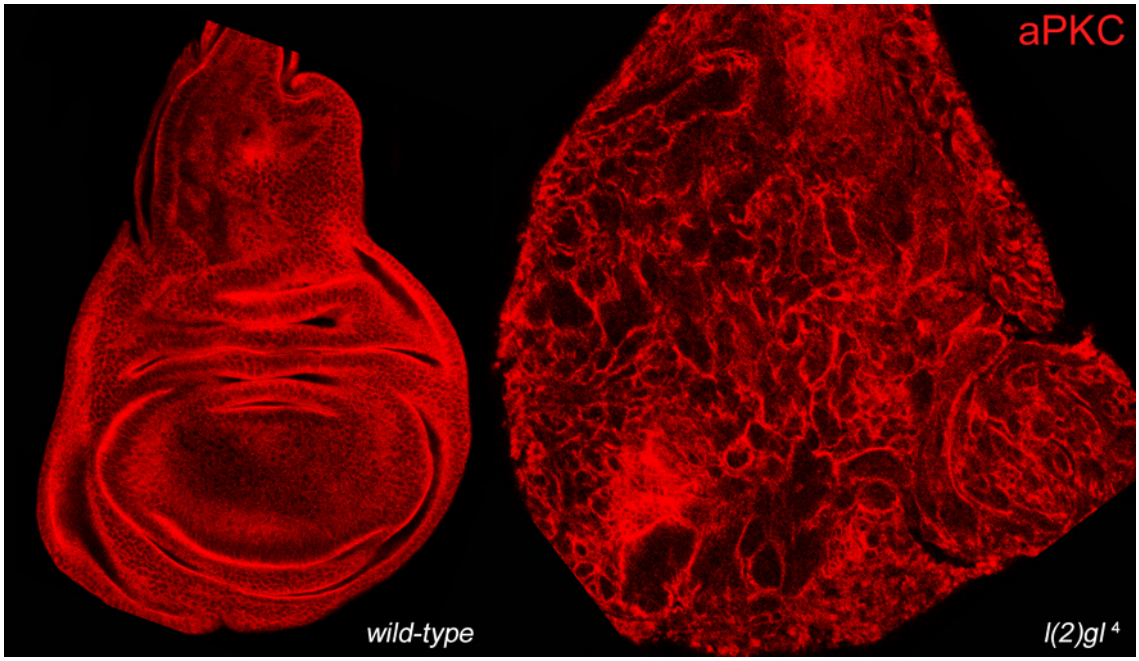
Our results obtained *in vitro* suggest that either genetically distant or identical cancer clones may compete each other following sudden changes in cMYC expression. This phenomenon may represent a *selective interference* within tumour bulks, able to drive clone evolution and eventually shaping cancer mass.

2.5. An in Vivo Model to study MMCC in Cancer

The data collected from the CCAs performed in cell culture let us hypothesise that, whatever the mutational burden of a cell population and whatever its fitness, Myc inhibition weakens its overall performance while sharing its living space with Myc-expressing neighbours.

That's all the functional information we can get from an *in vitro* assay, but many issues remain unaddressed: what happens to cancer cells if they start to up- or down-regulate Myc while embedded in an expanding tumour? And what happens to neighbours? Do local competitive interactions have an impact at a distance or is their effect just sensed in a restricted area? What is the impact of MMCC on the overall tumour size?

To answer these evolutive questions, I carried out an *in vivo* assay that allowed induction of Myc up- or down-regulating clones within a tumour mass growing in the animal and collection of a series of retrospective data.



The image on the right is a section of an epithelial tumour growing within the *l(2)gl*^{4} mutant larva. As described in the *Introduction*, in fact, the *lgl* LOF mutation in a homotypic background triggers loss of A/B cell polarity and uncontrolled proliferation (see the *wild-type* organ on the left for a comparison).

Epithelial tumours induced by the LOF of neoplastic TSGs *in Drosophila* are known to show altered metabolism, dedifferentiation and upregulation of cytokine-like ligands, which drive tumour overgrowth [192]. In addition, several oncoproteins as Myc, dpERK

and pAKT are found overexpressed in theses organs, together with JNK-dependent expression of MMPs, nuclear accumulation of the Hpo pathway downstream effector Yki with its target dIAP1, and stabilisation of the HIF1α *Drosophila* homologue Sima in response to oxygen shortage [14]. Overall tissue architecture is compromised and cancer cells/clones undergo massive molecular and morphological separation. Despite *lgl* mutation is ubiquitous in the animal, each cell seems indeed to develop different disregulation patterns within the growing masses [14] and possibly different genetic and epigenetic alterations emerge that, as it happens in mammalian cancers, shape cancer evolution through sub-clone competition and interference [16] [28]

lgl zygotes lacking maternal contribution die as embryos; maternal supply is hence sufficient to sustain the earliest stages of development [193] since Lgl is a highly stable protein[194]. Several cell cycles are indeed required before Lgl is completely depleted from the cell and its LOF phenotypes become evident; as a consequence, *lgl* mutant discs are not frankly malignant until 5 days from egg laying, despite they are composed at this stage of about one-third of the cells found in a *wild-type* disc [52]. Larval development lasts in the mutant larvae for additional 5-7 days, with late individuals showing huge masses that completely fill their anterior half.

In this context, I induced *l(2)gl*^{4} (*neutral*), *l(2)gl*^{4}, *UAS-HAdm* (*myc*^{OVER}) or *l(2)gl*^{4}, *UAS-dmRNAi* (*myc*^{KD}) clones at different stages of cancer progression and analysed, later in development, the phenotypic consequences of clone induction..

2.5.1. Induction of MMCC in Genetically Distant Cells: the 6+2 Scheme

With the aim to study how the emergence of MMCC in a late stage of cancer growth can shape both cell behaviour and overall tumour mass, I induced *neutral*, *myc*^{OVER} and *myc*^{KD} clones in an *lgl* LOF background at day 6, at a stage in which the wing imaginal discs show obvious neoplastic growth, and collect target tissues after two additional days of development (see *In Vivo Materials and Methods* for details). At day 8, GFP-positive larvae were selected under a fluorescence microscope, discarding those bearing large clones in the fat bodies, as it is known that Myc modulation in those tissues modifies organismal growth through the release of insulin-like peptides by the brain IPCs [195]. After having captured disc image with a dedicated camera to calculate disc volume, I dissociated them and counted GFP-positive and GFP-negative cells. As can be appreciated in Figure 2.11, GFP-positive cell percentage was much higher in the *myc*^{OVER} sample than

in the *neutral* control (35,44% compared to 20,31%) and, contrarywise, it was significatively decreased in the *myc*^{KD} sample (8,35% compared to 20,31%).

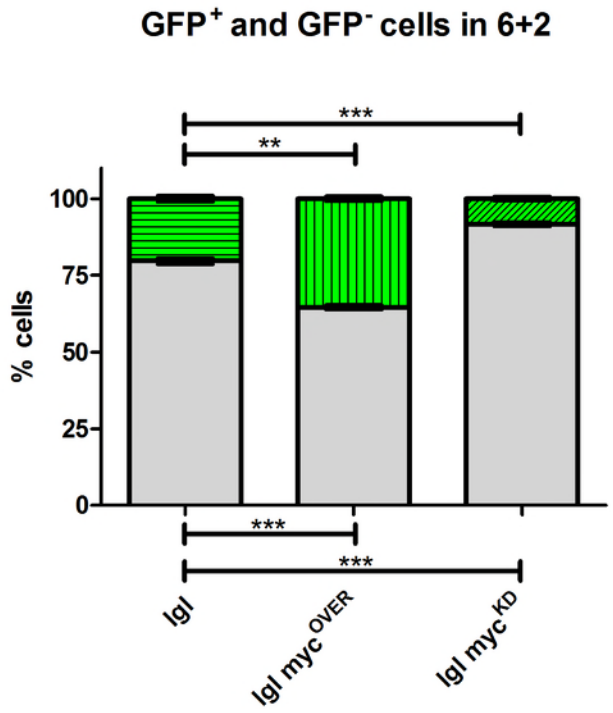


Figure 2.11: Percentage of GFP-positive (Green) and GFP-negative (Grey) cells from 8 days-old *lgl*, *lgl myc*^{OVER} and *lgl myc*^{KD} dissociated wing discs. Each assay was repeated 3 times and counted twice. ± SEM is indicated.

This result showed that Myc clonal upregulation in a tumour context is sufficient to drive expansion of the Myc-overexpressing population and that, on the contrary, Myc downregulation in cancer cells restrains their proliferation.

But what about the final tumour mass? I calculated the volume of each disc approximating its shape to a prolate spheroid, whose volume can be obtained by the formula: $4/3\pi a^2 b$.

As can be seen in Figure 2.12, while at day 6, before clone induction, the masses of the three samples were comparable in size, after two additional days of growth they appeared amazingly different: the average size of the *myc*^{OVER} sample displayed an about 2.5 fold increase with respect to that of the *neutral* sample and, contrarywise, tumour mass appeared reduced to one-half in the *myc*^{KD} sample.

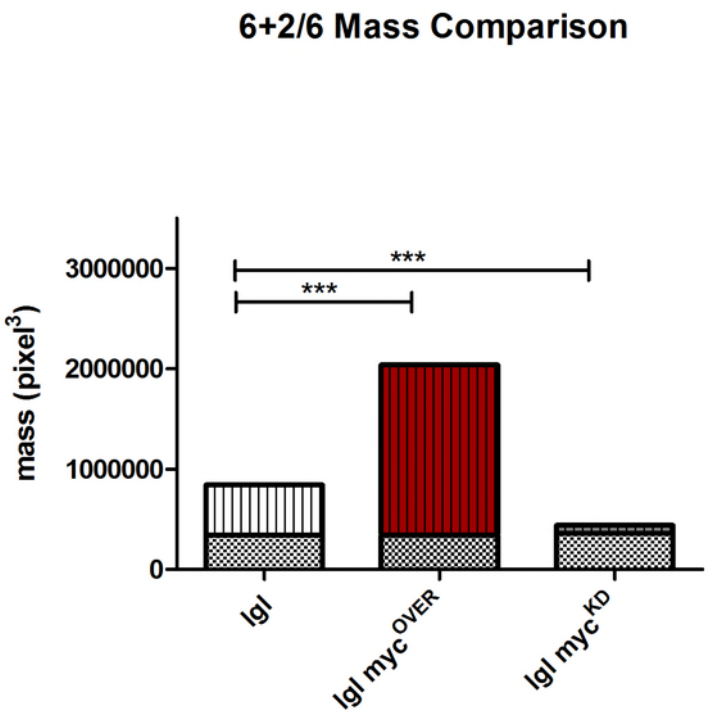


Figure 2.12: Masses of *lgl*, *lgl myc*^{OVER} and *lgl myc*^{KD} cancer tissues before (6 days, lower areas) and after (8 days, upper areas) clone induction. Each assay was repeated 3 times and counted twice. ± SEM is indicated.

This was convincing evidence that both up- and downregulation of Myc during cancer progression can help shape cancer evolution and, as a consequence, final size.

With the aim to analyse the contribution of the GFP-positive and the GFP-negative cells to the masses grown after clone induction (that is from day 6 to day 8), I calculated the average volumes of *lgl*, *lgl myc*^{OVER} and *lgl myc*^{KD} cells (see *In Vivo Materials and Methods* for details):

lgl cells: $GFP^+ = 0.752\ px^3$; $GFP^- = 0.752\ px^3$

lgl myc^{OVER} cells: $GFP^+ = 0.989\ px^3$; $GFP^- = 0.8\ px^3$

lgl myc^{KD} cells: $GFP^+ = 0.518\ px^3$; $GFP^- = 0.62\ px^3$

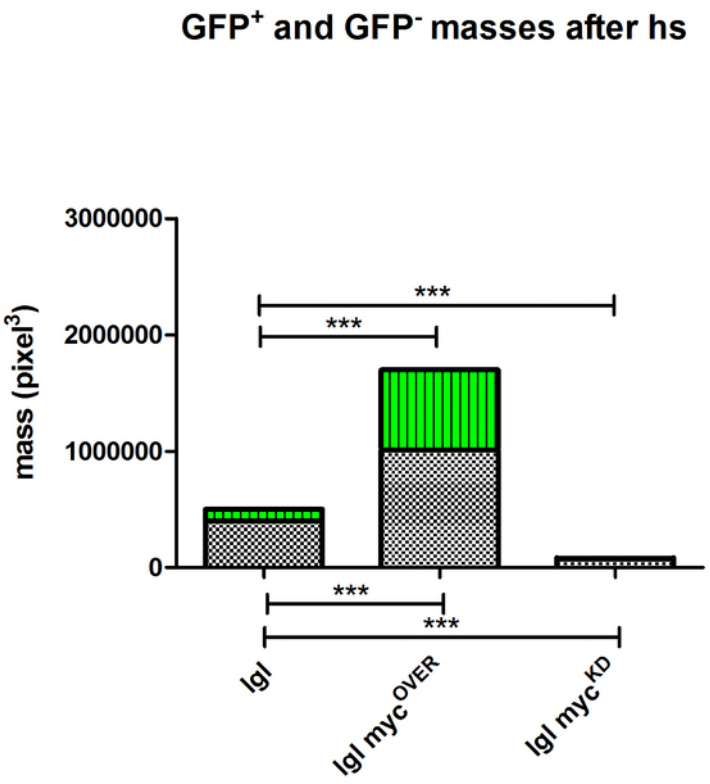


Figure 2.13: Relative masses occupied by the GFP$^+$ (Green) and the GFP$^-$ (Grey) cells in *lgl*, *lgl myc^{OVER}* and *lgl myc^{KD}* cancer tissues grown after clone induction. ± SEM is indicated.

Of note, as can be observed in Figure 2.13, both GFP$^+$ and GFP$^-$ populations expanded in *lgl myc*^{OVER} tumours with respect to the neutral *lgl* masses and, on the other hand, both GFP$^+$ and GFP$^-$ populations collapsed in *lgl myc*^{KD} samples. The GFP$^+$ *Myc*^{OVER}/*neutral* mass ratio was 6.75 and the GFP$^-$ *Myc*^{OVER}/*neutral* mass ratio was 2.53, while the GFP$^+$ *Myc*^{KD}/*neutral* mass ratio was 0.057 and the GFP$^-$ *Myc*^{KD}/*neutral* mass ratio was 0.19. Let's take a closer look to these numbers. In both *Myc*^{OVER} and *Myc*^{KD} samples, the major contribution to mass increase/decrease is due to the GFP$^+$ population, demonstrating a genuine autonomous role of Myc modulation on cell growth and proliferation. The evidence that the untouched populations follow the same trend of the manipulated populations let us hypothesise that *Myc*^{OVER} and *Myc*^{KD} cells prime some non-autonomous phenomena which in turn amplify/constrain overall growth.

I thus performed IF assays for Myc and Cas3 proteins, with the aim to investigate what happens at the interface between the GFP$^+$ and the GFP$^-$ cells during cancer expansion.

In Figure 2.14 a tumourous organ is represented in which neutral, *lgl* clones have been induced. Independently of the GFP signal, we can observe several cell clusters in which Myc staining is lower compared to the surrounding cells, that result positive to Cas3 signal (arrows). This means that MMCC may be regularly at work in these masses.

In the successive Figure (2.15) I show a closer view of a similar phenomenon: cells expressing very low levels of Myc (dotted line) surrounded by cells with high Myc expression are committed to die, as it is confirmed by a strong Cas3 staining.

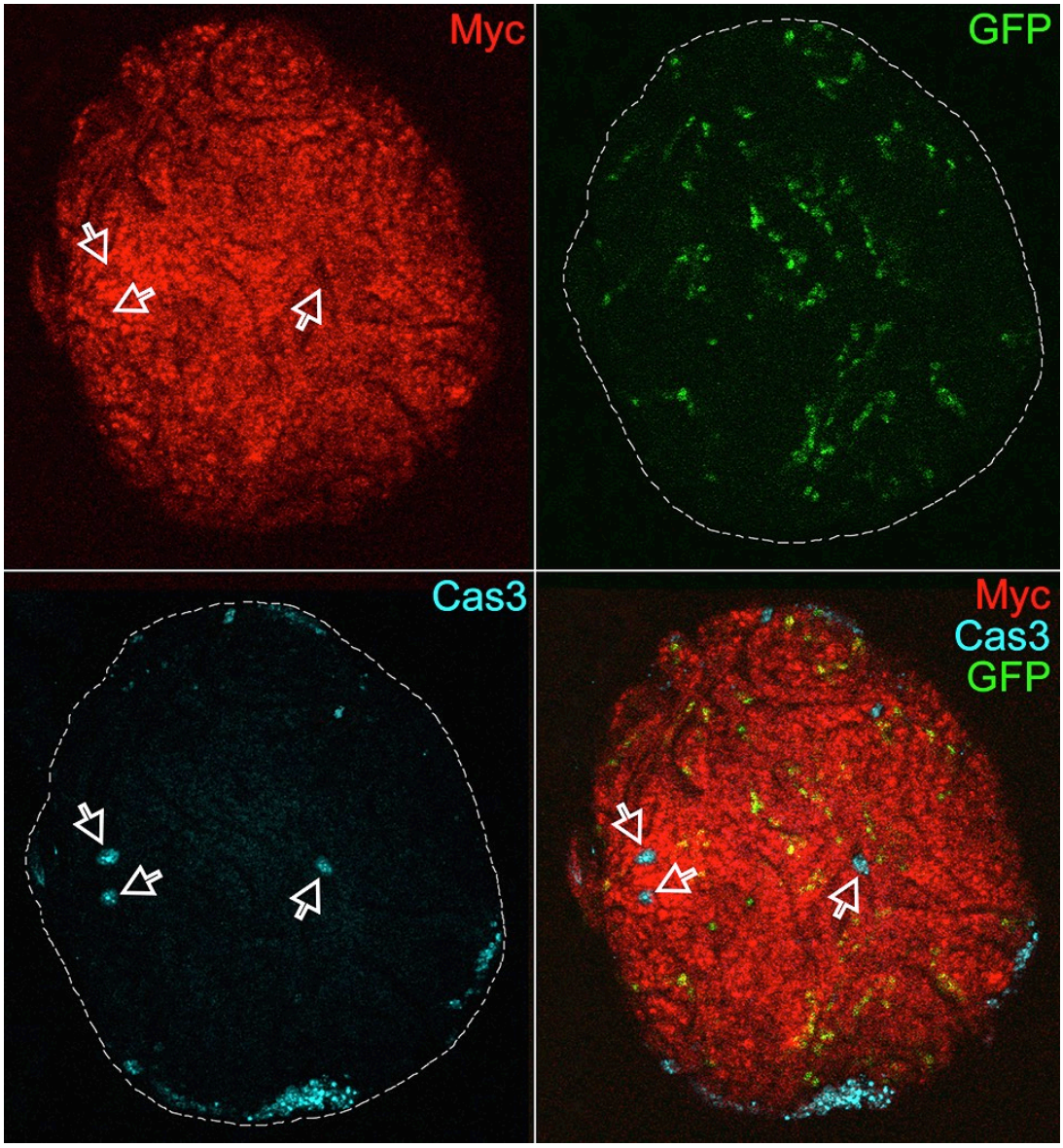


Figure 2.14: GFP^{+} neutral clones induced in an *l(2)gl*^{4} mutant background. Myc staining is in red and Cas3 staining is in cyan. The arrows indicate cells with low Myc protein positive to a-Cas3 antibody. Magnification is 400X.

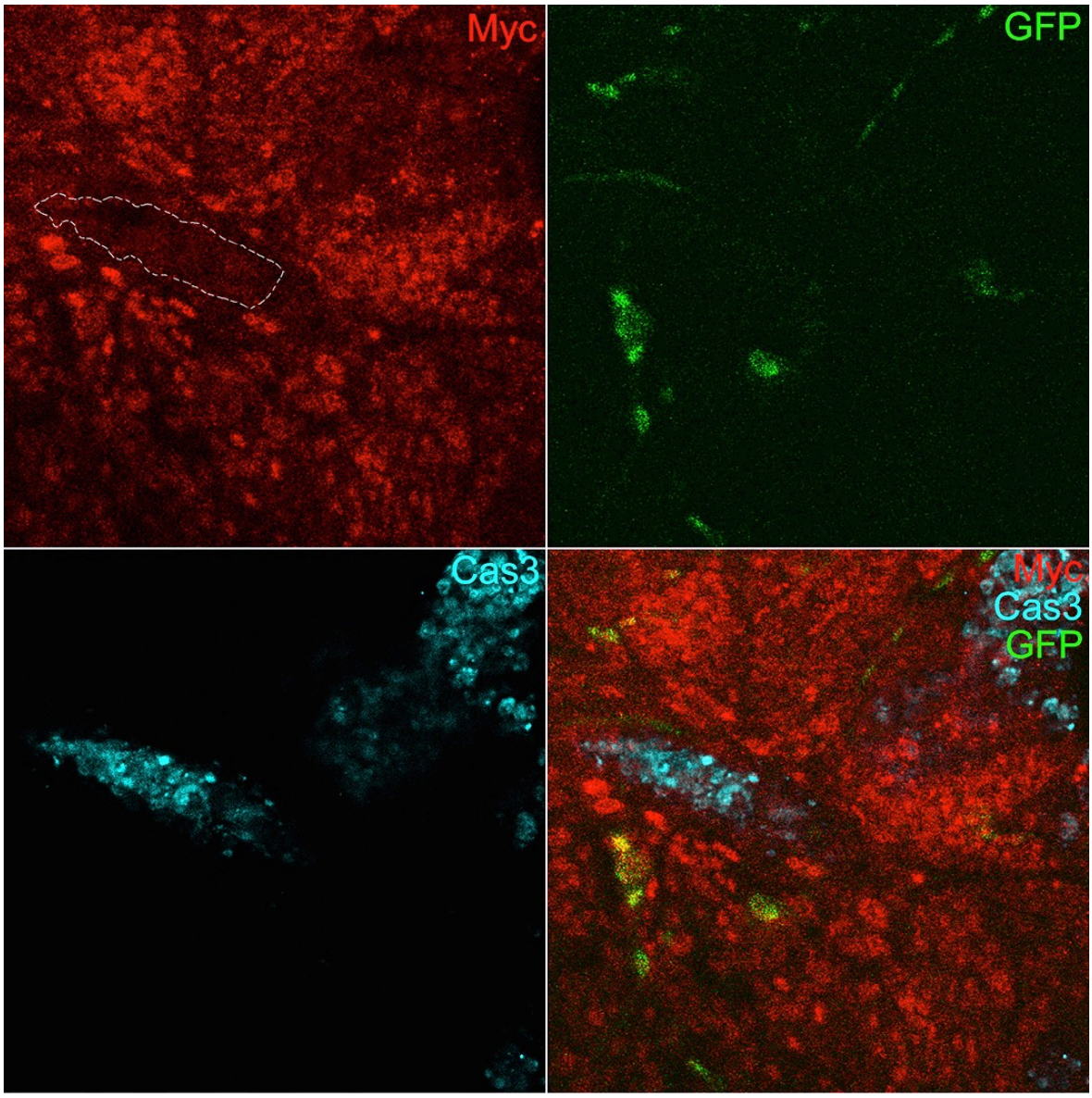


Figure 2.15: GFP^{+} neutral clones induced in an *l(2)gl*^{4} mutant background. Myc staining is in red and Cas3 staining is in cyan. The dotted line encircles a Cas3-positive area showing low Myc levels with respect to the neighbouring cells. Magnification is 800X.

These data suggest that MMCC is normally at work in these tumours, and it shapes cancer evolution through the elimination of the less fit cells (with lower levels of Myc protein) allowing the expansion of the most performant ones (with higher levels of Myc protein). Concerning MMCC, these cancers seem thus to recapitulate what happens in physiological conditions during *Drosophila* [93] [109] and mammalian [136] [137] development, but growth is not allometric in cancer and final size may be surprising.

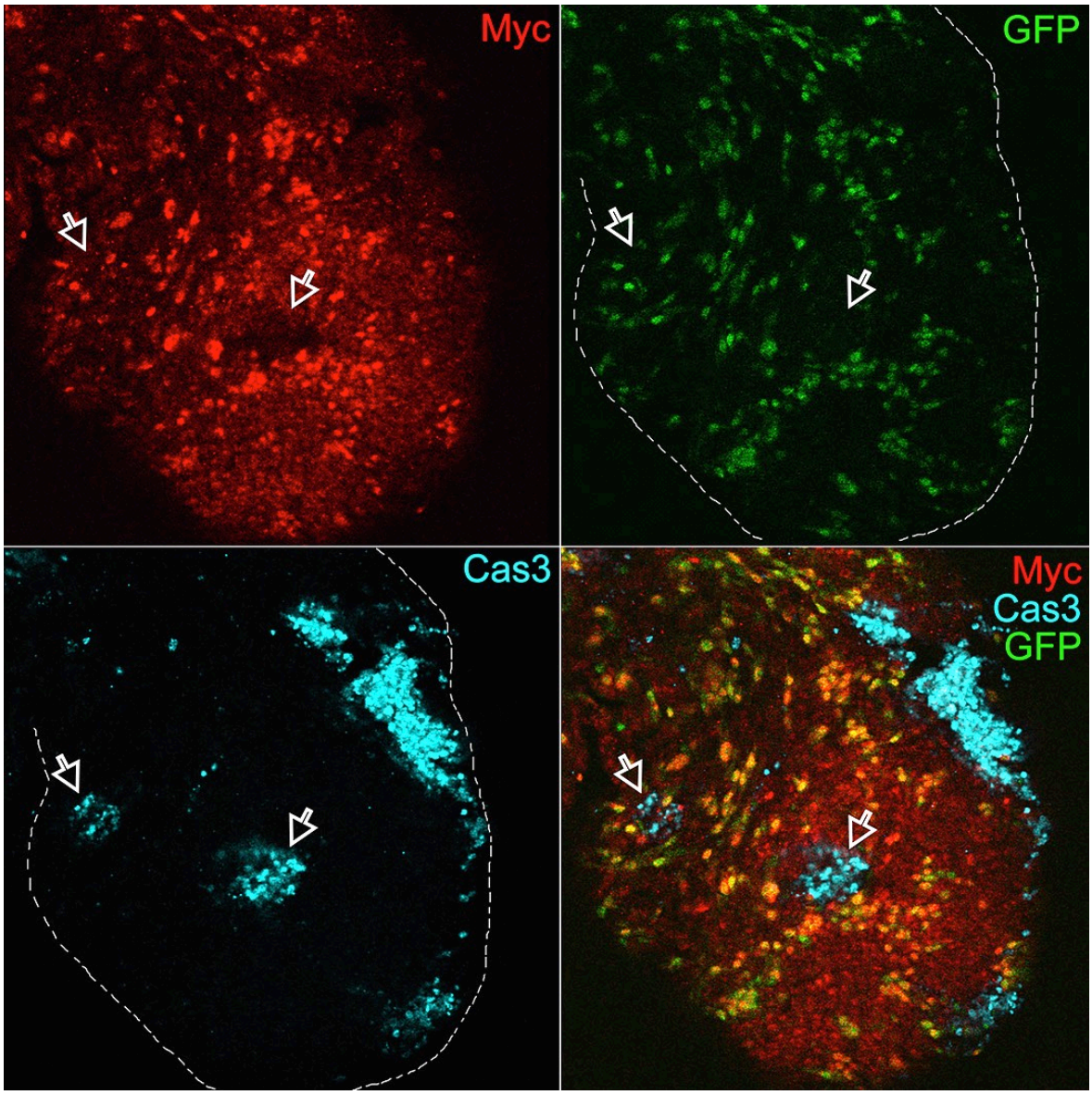


Figure 2.16: GFP^{+} *lgl* *myc*^{OVER} clones induced in an *l(2)gl*^{4} mutant background. Myc staining is in red and Cas3 staining is in cyan. The arrows indicate GFP^{-}, Cas3-positive areas showing lower Myc levels with respect to the neighbouring cells. Magnification is 400X.

A partial answer came from the IF carried out on *myc*^{OVER} samples. As can be seen in Figure 2.16, several cell groups with low Myc levels encircled by or adjacent to GFP-positive cells with high Myc expression show strong Cas3 signal (arrows indicate some). This demonstrates that, through clonal induction of Myc, we are enhancing the same mechanism as in the previous experiment: in this case, cells catching an "overdose" of Myc respond showing super-competitive behaviour in the tissue.

Figure 2.17 focuses closely on the same phenomenon: GFP-negative cells (marked by a dotted line) surrounded by high GFP-positive, high Myc-expressing neighbours succumb, losing the battle for space occupancy within the growing mass.

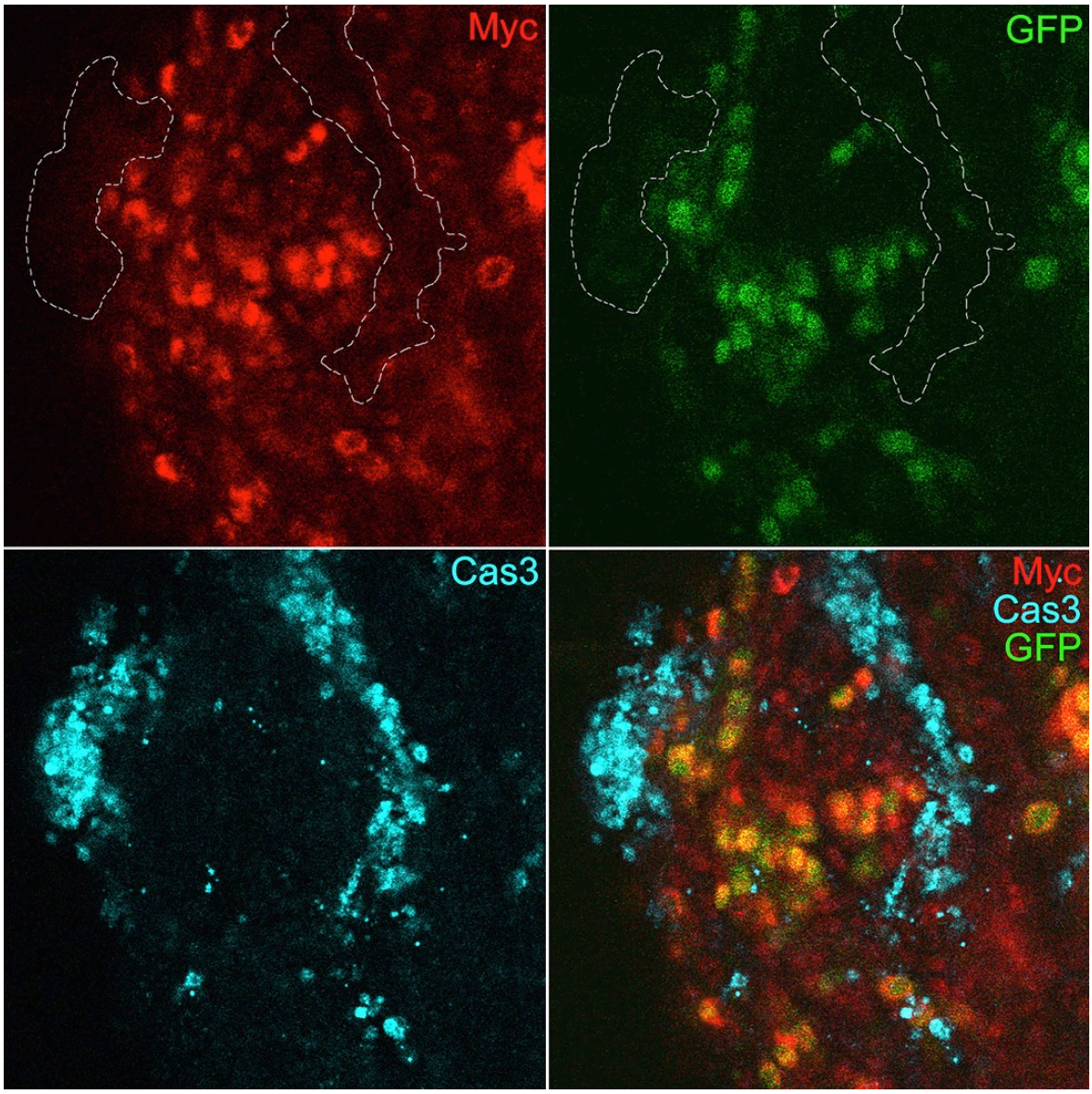


Figure 2.17: GFP^{+} *lgl* *myc*^{OVER} clones induced in an *l(2)gl*^{4} mutant background. Myc staining is in red and Cas3 staining is in cyan. The dotted lines indicate GFP^{-}, Cas3-positive areas showing lower Myc levels with respect to the neighbouring cells. Magnification is 800X.

Another interesting observation concerns the fact that I found very few *myc*^{OVER} cells dying within the sample I examined: this sounded unusual, as Myc ectopic expression is known to induce massive autonomous cell death in the fruit fly [126]; it is however known that, also in mammals, normal cells respond to high Myc levels by undergoing apoptosis, whereas tumour cells resist the apoptotic effects of Myc [196]. The cells in which I induced Myc upregulation have definitely passed multiple rounds of apoptosis, and survivors are somehow "addicted" to Myc: an overdose of this protein can thus be redirected to boost growth, proliferation and competitive drive.

In addition, those cells show deregulation of the Hpo pathway with nuclear accumulation of the anti-apoptotic protein dIAP1[14], which directly targets effector caspases in *Drosophila* [197].

Beside a huge expansion of the winner population, an increase of the GFP-negative mass was also noticed (Figure 2.13); a possible explanation for this observation is that some signalling molecules are secreted by both losers and winners, such as cytokine-like ligands, [192] that, catched by surrounding competent cells, may help amplify the original signals and contribute to the overall growth of the tumour.

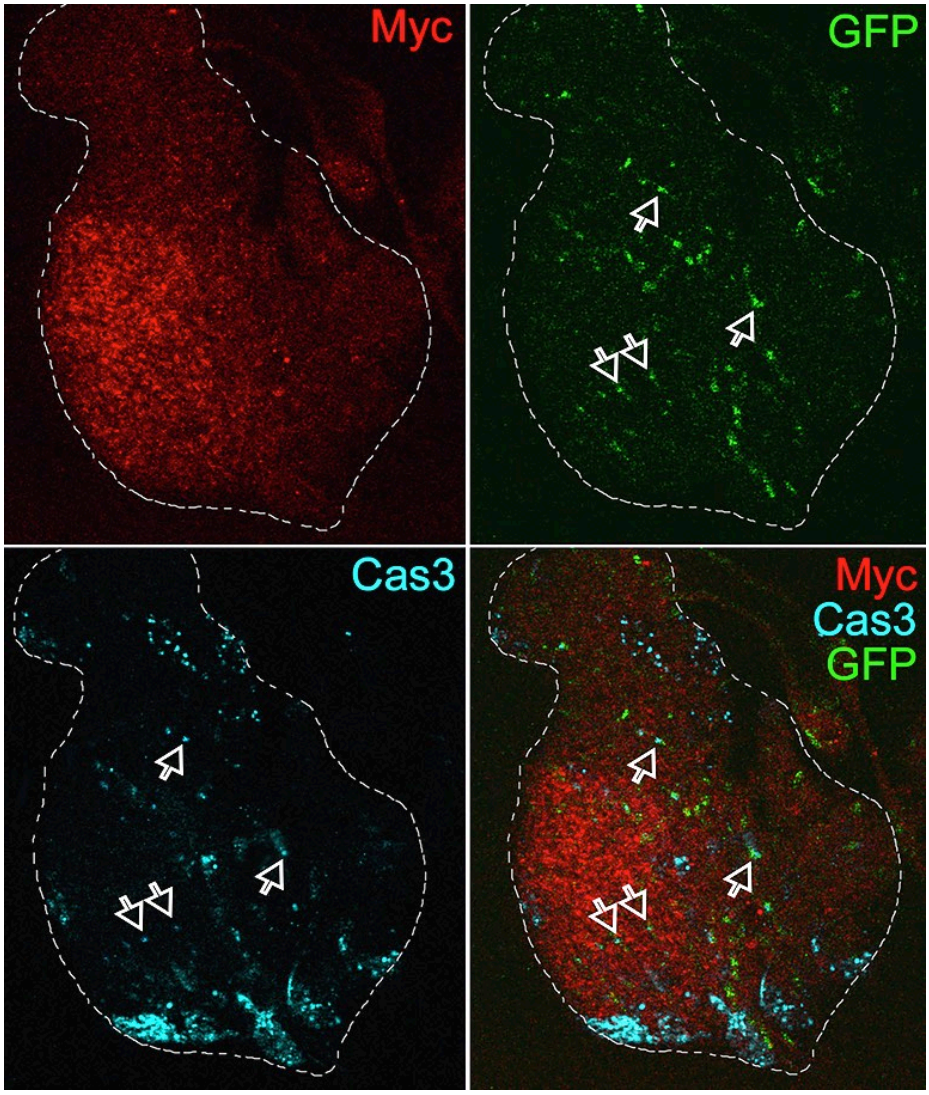


Figure 2.18: GFP^{+} *lgl myc*^{KD} clones induced in an *l(2)gl*^{4} mutant background. Myc staining is in red and Cas3 staining is in cyan. The arrows lines indicate GFP^{+}, Cas3-positive cells showing low Myc levels with respect to the neighbouring cells. Magnification is 400X.

Opposite results have been obtained following induction of *myc*^{KD} clones in the growing cancers. As can be observed in Figure 2.18, discs are smaller with respect to both *neutral*

and *myc*^{OVER} samples (Figg. 2.14 and 2.16) and express low levels of Myc protein. This restriction in organ size had already been observed by mass calculation (Figure 2.12) and, as for the previous sample, GFP-negative cells seem to follow the same trend of the GFP-positive population; in this case, native cells are under-represented with respect to the control sample (Figure 2.13).

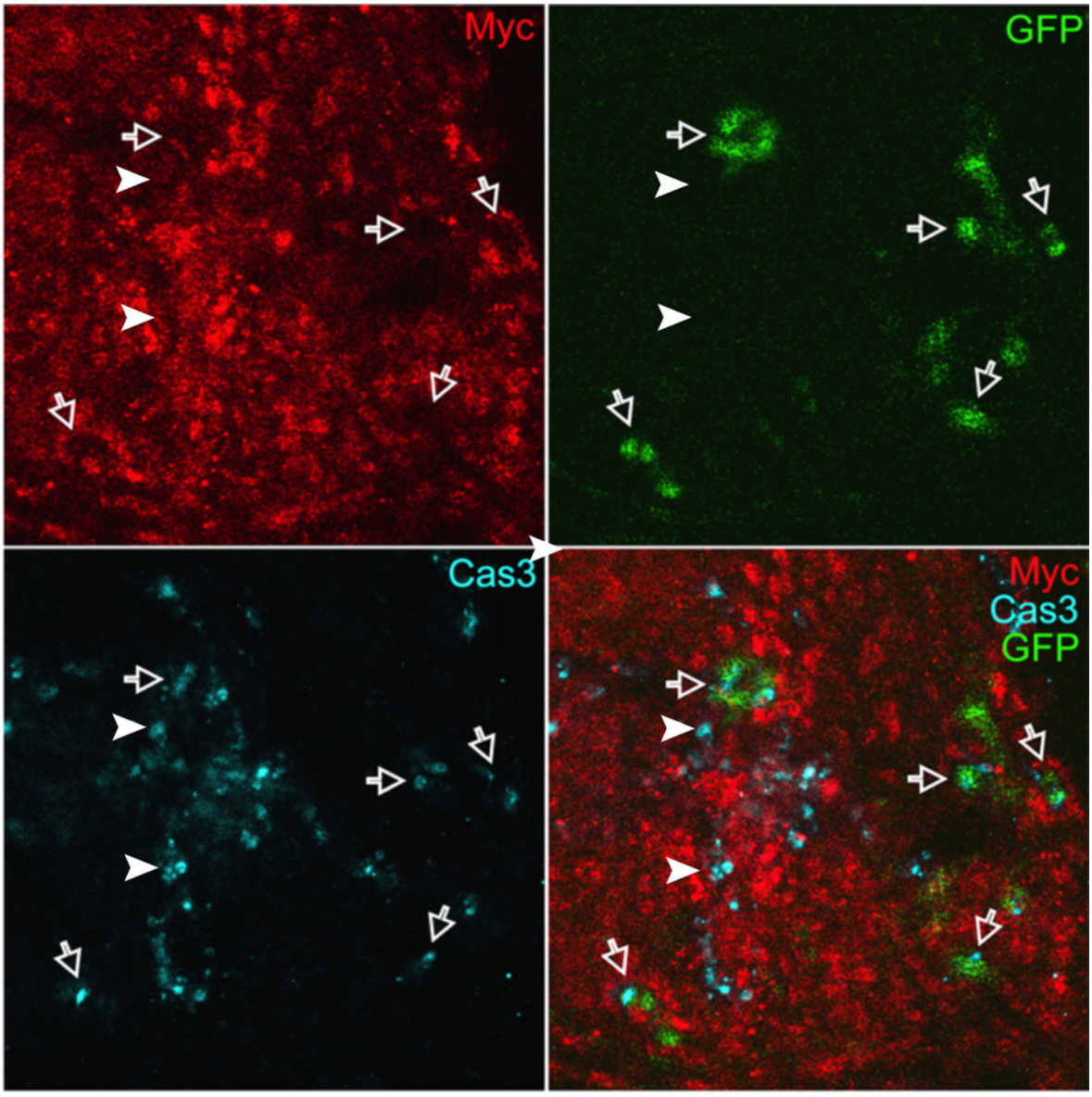


Figure 2.19: GFP^{+} *lgl myc*^{KD} clones induced in an *l(2)gl*^{4} mutant background. Myc staining is in red and Cas3 staining is in cyan. The arrows lines indicate GFP^{+}, Cas3-positive cells showing low Myc levels with respect to the neighbouring cells. The arrowheads point to native GFP^{-}, Cas3-positive cells showing low Myc levels with respect to the neighbouring cells. Magnification is 800X.

In Figure 2.19 a closer view is presented in which a myriad of GFP-positive, *myc*^{KD} cells undergo PCD. In addition, arrowheads indicate some native, Myc-downregulating cells that are out-competed by neighbouring Myc-positive cells.

Possibly, the introduction of a number of losers (*myc*^{KD}) in the system triggers a "relaxation" of the original MMCC: the native cells expressing low levels of Myc are now able to outcompete the newcomers as lower Myc levels are sufficient to win the competition in this context. The stressful metabolic cost of the winners can thus be limited, inducing cells to slow down their overall growth and proliferation rates.

2.5.2. Induction of MMCC in Genetically Related Cells: the 2+6 Scheme

After having observed what happens following induction of *neutral*, *myc*^{OVER} and *myc*^{KD} clones at a stage in which the wing imaginal disc is frankly malignant, we were interested in carrying out the same experiments as above in an epithelial organ well before the onset of cancer. As specified above, *lgl* mutant epithelia do not show any signs of neoplasia until the Lgl protein is completely depleted, that is 5 days from egg deposition. I thus induced the mutant clones at 2 days from egg laying and let larvae develop for additional 6 days before dissection and analysis. Cell count was performed as in the previous scheme and the results are illustrated in Figure 2.20.

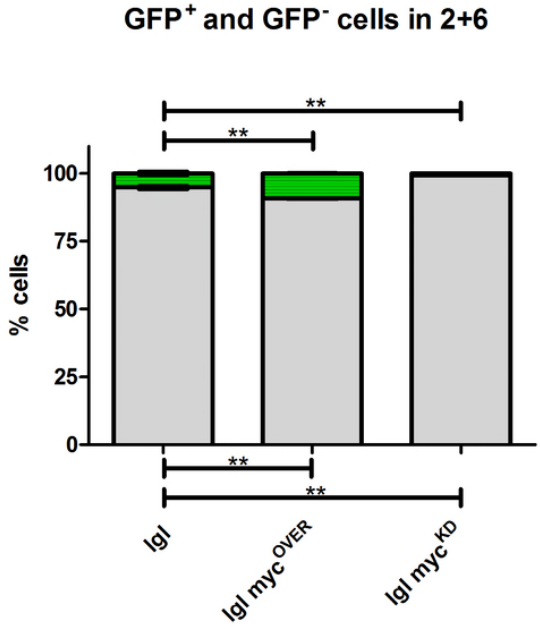


Figure 2.20: Percentage of GFP-positive (Green) and GFP-negative (Grey) cells from 8 (2+6) days-old *lgl*, *lgl myc*^{OVER} and *lgl myc*^{KD} dissociated wing discs. Each assay was repeated 3 times and counted twice. ± SEM is indicated.

As can be appreciated in the figure, I was able to find very few GFP-positive cells compared to the previous scheme, although the relative numbers gave similar information: the GFP-positive cells in the *myc*^{OVER} sample were about 2-fold those found in the *neutral* sample and the *myc*^{KD} masses contained about 1/10 GFP^{+} cells with respect to the *neutral* masses. This was convincing evidence that, whatever the stage at which Myc deregulation occurs in a developing mass, its increase supports autonomous growth and its reduction is detrimental for the cell.

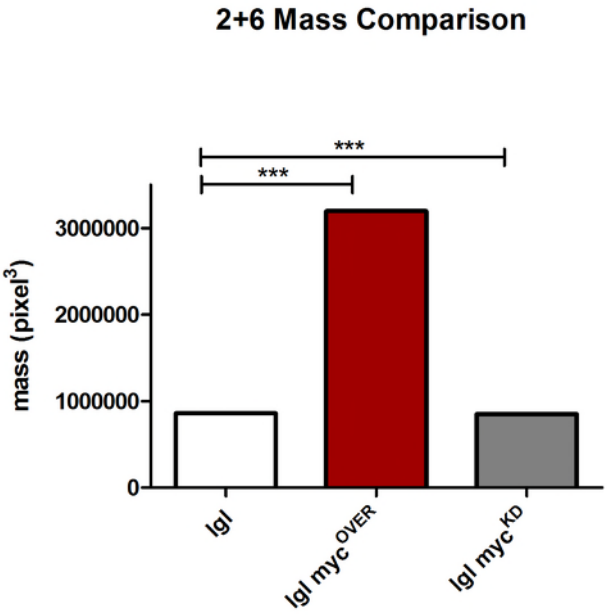


Figure 2.21: Masses of *lgl*, *lgl myc*^{OVER} and *lgl myc*^{KD} cancer tissues at 6 days from clone induction. Each assay was repeated 3 times and counted twice. ± SEM is indicated.

A surprising result came from mass calculation: while *myc*^{KD} samples were comparable in size to the *neutral* controls, *myc*^{OVER} tumours showed enormous growth, with the final mass more than threefold the control one. As this did not correlate with a GFP-positive huge number, some questions have to be addressed before drawing opportune conclusions. In principle, clone induction has similar efficiency when heat pulse duration, temperature and other experimental conditions are similar; this implies that a comparable percentage of cells undergoes Flippase-mediated excision in the target tissue. Being the 2-days *lgl* wing disc composed of about one-hundred cells, it it plausible that very few are modified by our manipulation, and this explains why the *myc*^{KD} sample was comparable in size to the neutral one: the few *myc*^{KD} cells induced at the very beginning of development are

immediately out-competed by the sorrounding epithelium and the tumour follows its native course.

It is more difficult to find a convincing reason for the behaviour of the *myc*^{OVER} tumours: the few mutant cells induced at the beginning of disc development may justify the scarce percentage of GFP-positive cells found in this sample, but this does not account for the huge non-autonomous mass overgrowth. It is possible that local MMCC provokes a wave of pro-growth signals across the expanding mass and that competent cells are able to amplify these signals, but it is also possible that the GFP construct got lost because of genomic instability happened in these masses. It is indeed demonstrated that some tumours show genomic instability in *Drosophila* [198], but it doesn't not seems to be associated with all the neoplastic mutations [199]. Current work is aimed at addressing this interesting issue.

Chapter 2

Results and Discussion

Part 2

The premise of the study

Lgl/HUGL-1 is a protein involved in the maintenance of the A/B cell polarity; cells mutant for *lgl/HUGL-1* loosen each other contacts, grow in 3D and the epithelium shows deep alterations in its overall architecture [52]. Loss of cell polarity induces, in turn, inactivation of the Hpo pathway, Yki/YAP nuclear accumulation and transcription of many proliferative and anti-apoptotic targets, included dMyc/MYC [130][134]. Cells overexpressing MYC can, in some contexts, trigger competitive interactions and expand at the expense of the normal surrounding tissue, eliminated by apoptotic death.

p53 is one of the most frequently mutated genes in human cancers and the function of its several mutant products and dominant negative forms is not clear.

A very recent work focused the attention on a fundamental role for p53 in MMCC in *Drosophila*, according to which the presence of the *wild-type* p53 protein in Myc over-expressing cells is required to gain a winner status and out-compete the neighbours [168].

Our IHC analysis showed that some essential traits of CC in *Drosophila* [52][130][66] are conserved in a large collection of samples from primary and secondary human carcinomas.

In addition to this, our *in vitro* experiments defined a role for cMYC in driving the winner fate during competitive interactions in human cancer cells lines, and its down-regulation was found to be sufficient to revert the phenomenon.

Finally, our *in vivo* investigations demonstrated how MMCC is involved in shaping the final tumour mass and in selecting the fittest cells in *Drosophila* epithelial tumourigenesis.

The Aim of the study

This part of the study aims at characterising the role of p53 in MMCC using cellular and animal cancer models in which p53 function will be opportunely modulated.

2.6. MMCC in Human Carcinomas Seems to be Associated with p53 Protein Status

Although the most part of the primary and secondary carcinomas analysed by IHC showed evident signs of MMCC (Fig 2.1), clear evidence was missing in some samples. In the view of the very recent work by the Johnston's group [168]. I decided to correlate the status of p53, evaluated through an antibody that recognises both the *wild-type* and the mutant forms of the protein, with MMCC in the same samples previously analysed (fig. 2.22).

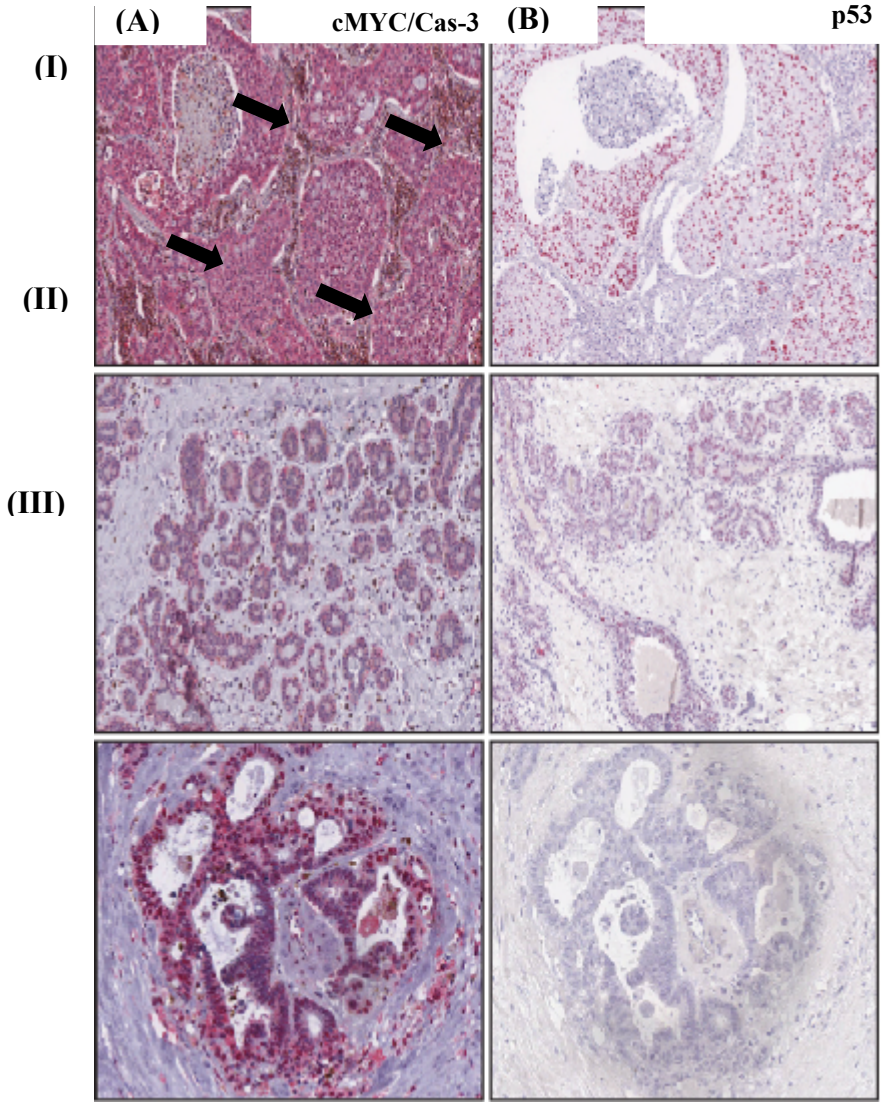


Figure 2.22: IHC on several human carcinomas: (**A**) MYC (pink) and Cleaved Cas-3 (brown). (**B**) p53 (pink) **I-.** lung adenocarcinoma (A) signs of CC: cancer cells show high levels of c-MYC protein surrounded by stromal cells with high staining for Cleaved Caspase-3; (B) p53 staining shows high accumulation of the protein in the nucleus of cancer cells (200X). **II-** ductal breast carcinoma**. (**A**)** signs of CC (B) presence of p53 with no massive nuclear accumulation (100X). **III-** colon adenocarcinoma **(**A**)** no evident signs of CC, **(**B**)** absence of p53 staining, **(**200X). The arrows point towards the dying cells.

The following expected scenario was found: in human samples where markers of MMCC were found (fig. 2.22 IA and IIB, black arrows indicate Cas-3 positive cells) p53 nuclear staining was evident (fig 2.22 IIA and IIB), while in samples where the Cas-3 signal resulted absent in the stroma surrounding the cMYC-expressing mass (fig. 2.22 III- A), p53 staining was negative.

This qualitative analysis led us to perform *in vitro* CCAs to study p53 implication in mammalian MMCC.

2.7. The Winner Status of Cancer Cells Requires p53 Function

To experimentally evaluate the involvement of the *wild-type* p53 function in MMCC in cancer, I performed the same CCAs as in Part 1 using two cell lines from the same human colorectal carcinoma: HCT116 (hereafter called HCT116^{wt}) and HCT116$^{p53-/-}$.

The HCT116$^{p53-/-}$ cell line was established by targeted homologous recombination by Bunz and colleagues from the parental colorectal carcinoma HCT116^{wt} line [200].

The two cell populations are genetically identical, except for the p53 mutation: HCT11$^{p53-/-}$ harbours the p53 exon 3 deletion, resulting in an inactive form of the protein unable to be revealed by the p53 antibody Do-1; cMYC protein levels were comparable in the two cell lines (fig. S.I. 5).

I performed the first CCA between the HCT116^{wt} and the HCT116^{wt} treated with the 10058:F4 inhibitor (fig. 2.23) to evaluate whether differences in cMYC may activate competitive behaviours also in this cell population. Preliminary characterisation of the cell lines used in this part of the work, before and after treatment with the drug 10058:F4, has been performed (fig. S.I. 6 A and B).

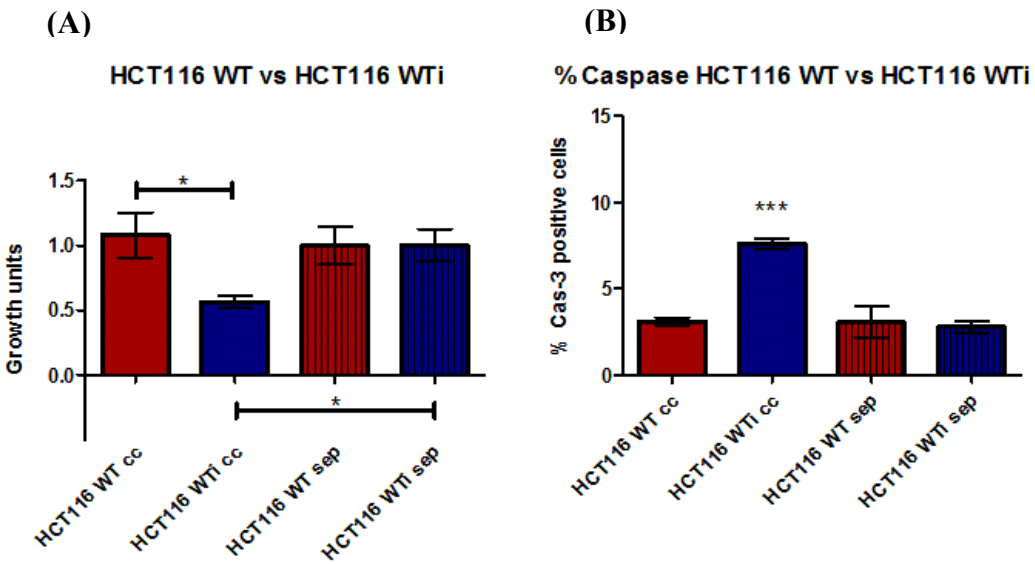


Figure 2.23: CCA on HCT116^{wt} and HCT116^{wt}i colon cancer cell lines. Co-cultured cell lines, indicated with **cc,** represent the first two bars of each graph; separated cell lines, indicated with **sep,** are the second two bars of each graph. **A)** Growth after 5h of culture; each assay was repeated 3 times and counted twice. **B)** Apoptotic cell percentage calculated by IF after 5h of culture. HCT116^{wt}i (treated with the inhibitor 10058:F4) shows the highest Caspase-3 percentage when co-cultured with mock-treated HCT116^{wt}. Each IF was repeated 4 times and 4 fields were counted in each slide. ± SEM is indicated.

As can be noted in Figure 2.23 A, after 5 hours of CCA the two cell lines show different growth rates in co-culture and in separate conditions: the HCT116^{wt} in a native condition (red bar) overgrow compared to the HCT116^{wt}i (blue bar), whereas the two cell populations show similar growth rates in separate conditions (red striped and blue striped bar). The growth disadvantage of the treated cell population in co-culture is associated with the highest Cas-3 positive signal (fig. 2.23 B, blue bar).

These data show that MMCC can be induced in this cell line.

With the aim to evaluate if p53 protein played a role in loser cells during MMCC, I performed a CCA between the HCT116^{wt} and the HCT116$^{p53-/-}$ treated with the inhibitor 10058:F4 (fig. 2.24) as above.

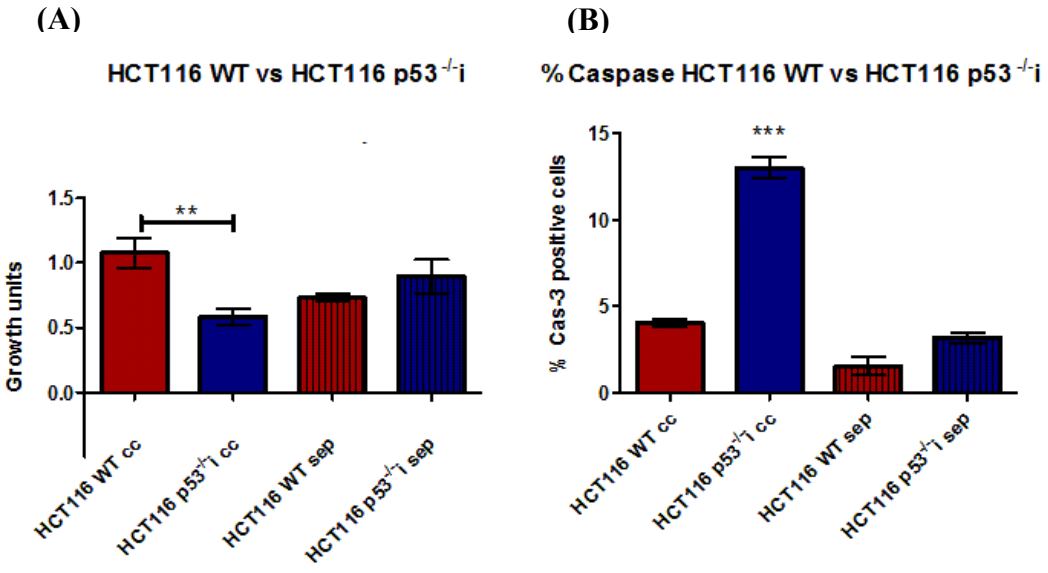


Figure 2.24: CCA on HCT116^{wt} and HCT116$^{p53-/-}$i colon cancer cell lines. Co-cultured cell lines, indicated with **cc,** represent the first two bars of each graph; separated cell lines, indicated with **sep,** are the second two bars of each graph. **A)** Growth after 5h of culture; each assay was repeated 4 times and counted twice. **B)** Apoptotic cell percentage calculated by IF after 5h of culture. HCT116$^{p53-/-}$i (treated with the inhibitor 10058:F4) shows the highest Caspase-3 percentage when co-cultured with mock-treated HCT116^{wt}. Each IF was repeated 4 times and 4 fields were counted in each slide. ± SEM is indicated.

The assay showed similar results as those from the previous one, as the HCT116^{wt} and HCT116$^{p53-/-}$i in co-culture exhibited the typical competitive behaviour according to the growth units (fig. 2.24 A) and the profile of Cas-3 staining (fig 2.24 B). The result of this experiment, associated with a CCA performed using two other cancer cell lines, H460 and H1299 (fig. S.I. 7), in which the first line has higher expression level of cMYC protein than the H1299, which harbor an homozygous partial deletion of the p53 protein (fig. S.I. 8), strongly indicated that p53 LOF in loser cells does not affect MMCC both in genetically distant and identical cancer cell lines.

Finally, to verify whether p53 *wild-type* protein is required in the winner cells to induce MMCC, I performed a CCA between HCT116$^{p53-/-}$ and HCT116^{wt}I cell lines, where the potential winner (based on cMYC levels) harbours a p53 deletion.

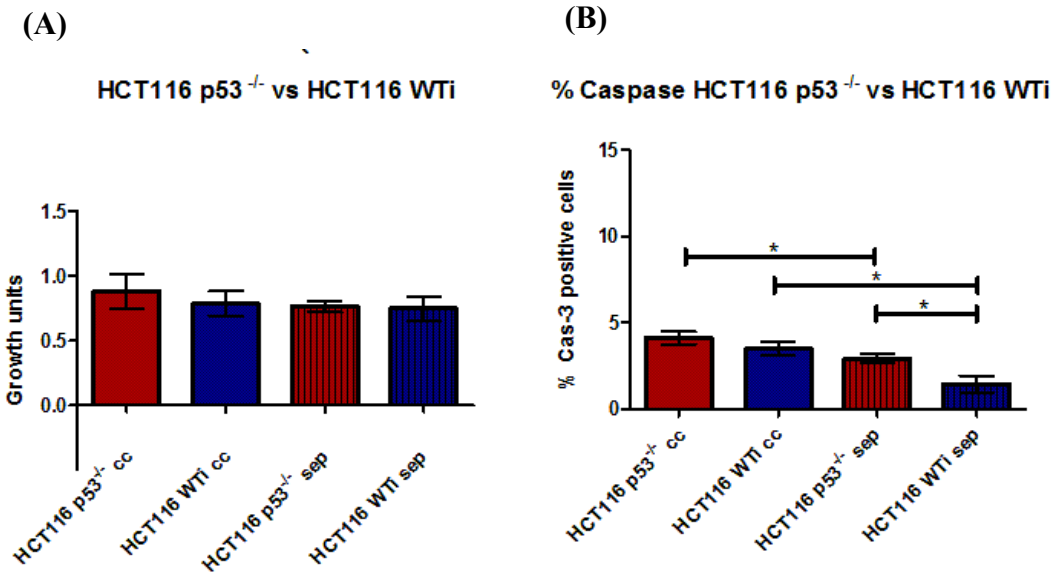


Figure 2.25: CCA on HCT116$^{p53-/-}$ and HCT116^{wt}i colon cancer cell lines. Co-cultured cell lines, indicated with **cc,** represent the first two bars of each graph; separated cell lines, indicated with **sep,** are the second two bars of each graph. **A)** Growth after 5h of culture; each assay was repeated 4 times and counted twice. **B)** Apoptotic cell percentage calculated by IF after 5h of culture. Each IF was repeated 4 times and 4 fields were counted in each slide. ± SEM is indicated.

The graph shown in Figure 2.25(A) illustrates how the potential winner cell line does not show differences in growth units compared to the potential loser population, defining the absence of competitive interactions, coherent with the hypothesis that p53 is required in MYC-overexpressing cells to drive CC. No significative variations emerged in proliferative units between co-culture and separate conditions.

Moreover, as can be seen in Figure 2.25 (B), the percentage of Cleaved-Caspase 3 positive cells is coherent with the CC assay count: none of the cell lines showed an increase in apoptosis in co-culture.

Further confirmation of this behaviour was achieved through a CCA assessed between the HCT116$^{p53-/-}$ and HCT116$^{p53-/-}$i lines (fig. 2.26 A and B): it can be seen that competition does not occur between the p53 KO lines even if a strong difference in cMYC protein levels is induced between the two.

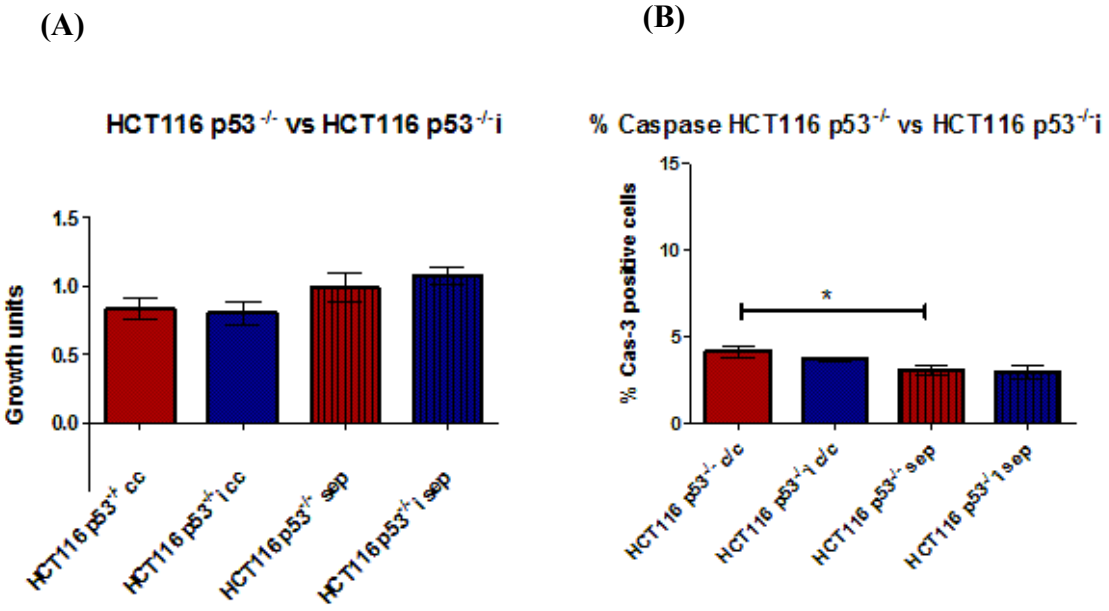


Figure 2.26: CCA on HCT116$^{p53-/-}$ and HCT116$^{p53-/-}$i colon cancer cell lines. Co-cultured cell lines, indicated with **cc,** represent the first two bars of each graph; separated cell lines, indicated with **sep,** are the second two bars of each graph. **A)** Growth after 5h of culture; each assay was repeated 3 times and counted twice. **B)** Apoptotic cell percentage calculated by IF after 5h of culture. Each IF was repeated 4 times and 4 fields were counted in each slide. ± SEM is indicated.

The two cell populations in co-culture showed a similar growth (fig. 2.26 A, red and blue bars) lower than that observed in separate conditions (fig. 2.26 A, striped bars), and their Cas-3 positive cells (fig. 2.26 B, red and blue bars) are coherently slightly higher than in separate conditions (fig. 2.26 B, striped bars).

The results of these experiments confirmed the hypothesis that a p53 *wild-type* function is required also in humans for MMCC to occur. The effect is not organ-specific as the same results have been obtained using the lung cancer line H1299 (fig. S.I. 9), whose its MYC-MAX activity has previously been inhibited (fig. S.I. 10)

2.8. Differences in p53 Status do not Drive CC in Cancer Cells

Noteworthy, a different *status* of p53 is not sufficient to induce MMCC in HCT116 cancer cell lines.

This evidence was obtained from a CCA where the HCT116^{wt} and the HCT116$^{p53-/-}$ cell lines, expressing similar levels of the cMYC protein (fig S.I. 5), were used for the assay.

(A) **(B)**

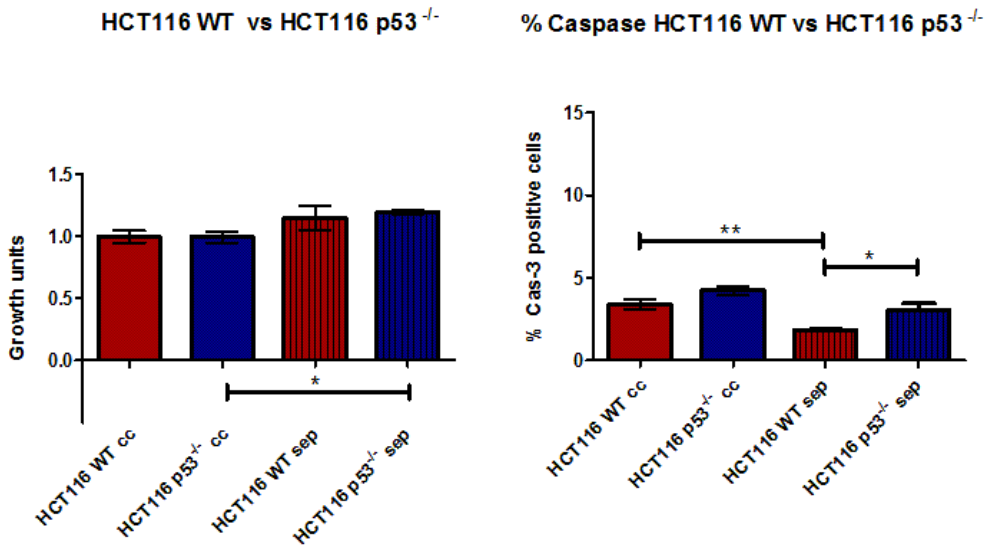


Figure 2.27: CCA on HCT116^{wt} and HCT116$^{p53-/-}$ colon cancer cell lines. Co-cultured cell lines, indicated with **cc,** represent the first two bars of each graph; separated cell lines, indicated with **sep,** are the second two bars of each graph. **A)** Growth after 5h of culture; each assay was repeated 4 times and counted twice. **B)** Apoptotic cell percentage calculated by IF after 5h of culture. Each IF was repeated 4 times and 4 fields were counted in each slide. ± SEM is indicated.

The growth units graph (fig. 2.27) does not show any competitive interactions at work as the two cell lines in co-culture (fig. 2.27 A, red and blue bars) exhibit similar proliferative rates. This seems to be a typical growth profile of populations where competitive interactions are relaxed (see fig 2.27).

Their apoptotic death (fig. 2.27 B, red and blue bars) appear to be higher than cells grown in separate wells, maybe as a result of the stress induced by the co-presence.

Altogether, these results demonstrate that p53 function is necessary downstream of MYC upregulation in the winner cells to assure their survival, growth and competitive capabilities.

2.9. Back to Drosophila: in Vivo CCA in a Cooperative Model of Carcinogenesis

de la Cova and colleagues demonstrated that p53 *wild-type* function is required in Myc-overexpressing cells to successfully out-compete the neighbours and overgrow during normal development [168], but evidence is still missing about a similar role of p53 in MMCC during cancer growth.

After having performed the CCA in human cancer cell lines and having collected plenty of data about a possible role of p53 in MMCC *in vitro*, we wanted to confirm this evidence *in vivo*.

I carried out a series of experiments with the aim to investigate the contribution of p53 to MMCC as a specific trait of carcinogenesis. I took advantage of a cooperative cancer model that uses the *l(2)gl*^{4} tumour suppressor mutation induced in a clonal fashion together with the over-expression of Myc (Myc^{over}); the cooperation between *lgl* LOF and Myc GOF is indeed known to trigger competitive overgrowth in the wing disc [141].

Through the use of the MARCM system (see *Materials and Methods*) I induced the following clones:

- *lgl* mutation (*l(2)gl*^{4}) together with Myc overexpression (Myc^{over});
- *lgl* mutation (*l(2)gl*^{4}) together with Myc overexpression (Myc^{over}) and p53^{KD} (an RNAi construct that decreased the p53 transcript to about 50% at 25°C, mimicking a heterozygous condition, (fig. S.I. 11);
- *lgl* mutation (*l(2)gl*^{4}) together with Myc overexpression (Myc^{over}) and p53^{DN} (used as a p53 LOF [201]);

As can be seen in Figure 2.28, *l(2)gl*^{4}*, Myc*^{over} clones are able to overgrow in any region of the disc. Of note, these mutant clones often appear to merge and many GFP-positive cells are found scattered throughout the wing pouch, a clear sign of migration (see Fig. 2.28 A) attributable to the malignant nature of these cells, as *Drosophila* normal or hyperplastic clones grow compact, with daughter cells remaining side by side (141). This feature seems to be rescued in both *l(2)gl*^{4}, Myc^{over}, *p53*^{KD} and *l(2)gl*^{4}, Myc^{over}, *p53*^{DN} clones (Fig. 2.28 B- C).

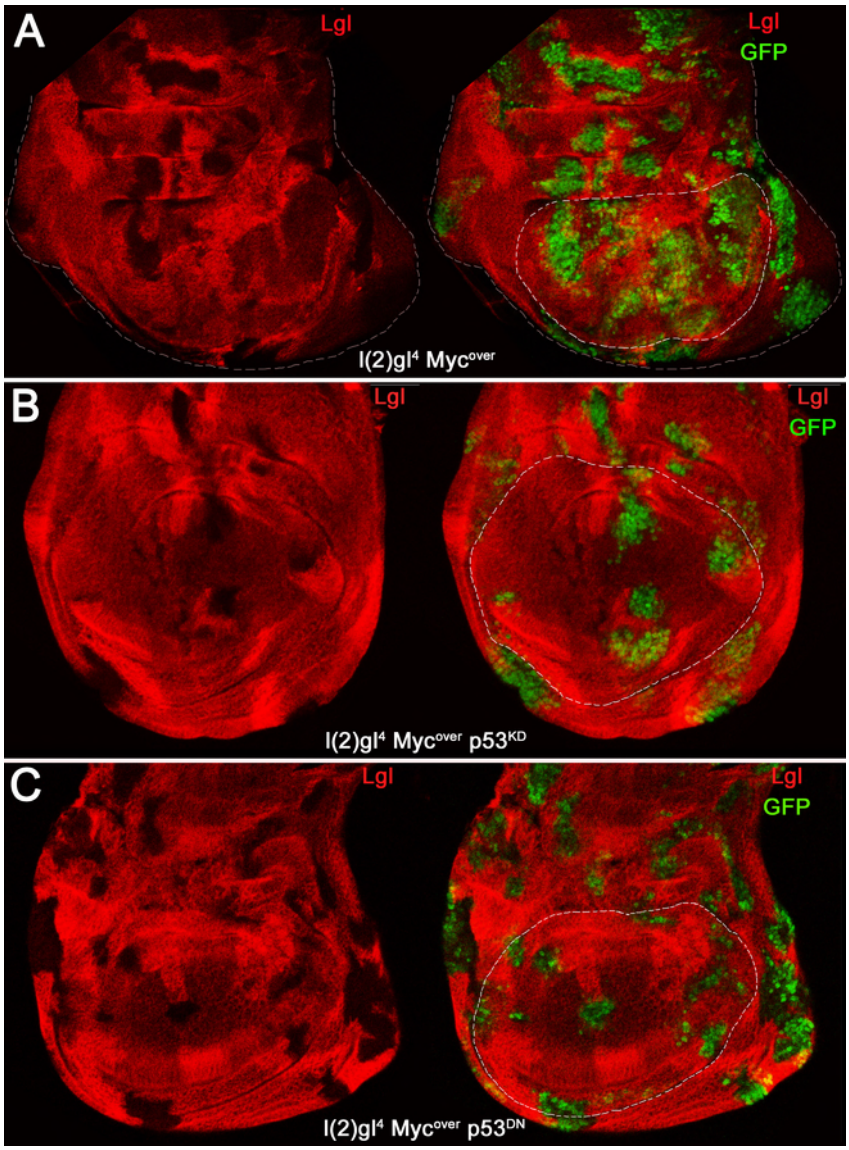


Figure 2.28: Imaginal wing discs from *l(2)gl*^{4}*, Myc*^{over} (**A**), *l(2)gl*^{4}*, Myc*^{over}*, p53*^{KD} (**B**) and *l(2)gl*^{4}*, Myc*^{over}*, p53*^{DN} (**C**) L3 larvae. GFP^{+} clones are black (0XLgl) and twin clones are intense red (2XLgl), while the background is red (1XLgl). The dotted line encircles the wing pouch region of the discs.

To support this morphological evidence, I carried out a measurement of the clonal mutant areas in the wing pouch region (encircled by dotted lines in Figure 2.17) whose results are illustrated in figures 2.18- 2.20.

The statistical analysis performed on *l(2)gl*^{4}, Myc^{over}, *p53*^{KD} clones showed that average clone area is reduced to about 50% (fig. 2.30) respect to that of the *l(2)gl*^{4}, Myc^{over} clones (Figure 2.29), and to about 25% in *l(2)gl*^{4}, Myc^{over}, *p53*^{DN} clones (Figure 2.31).

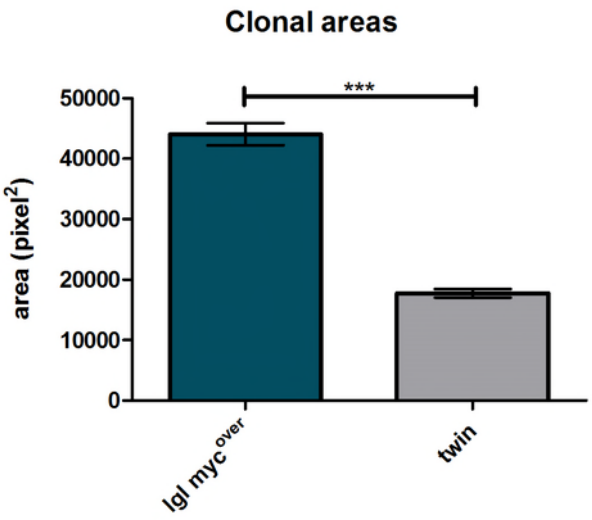


Figure 2.29: Average clonal area of *l(2)gl*^{4}*, Myc*^{over} clones (blue) and wild-type twins (grey). For each sample, 20 clones in different wing discs have been measured at 200X magnification. Error bars represent the SEM.

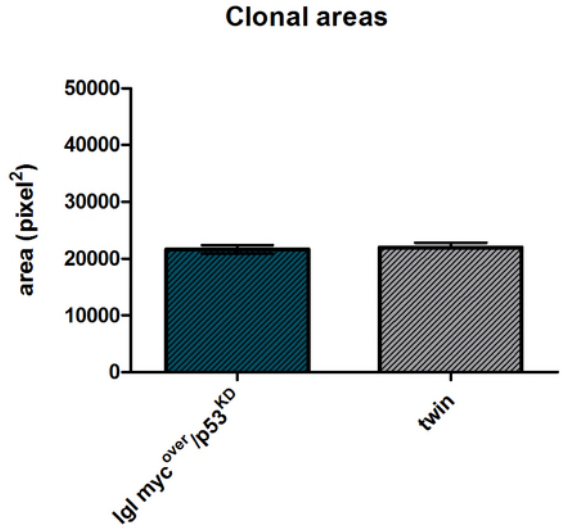


Figure 2.30: Average clonal area of *l(2)gl*^{4}*, Myc*^{over}*, p53*^{KD} clones (blue) and wild-type twins (grey). For each sample, 20 clones in different wing discs have been measured at 200X magnification. Error bars represent the SEM.

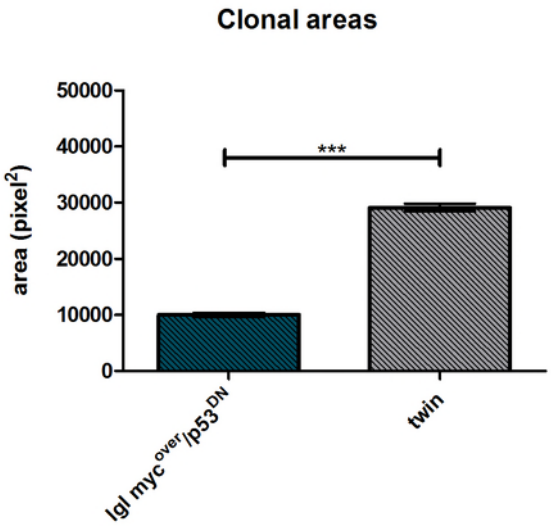


Figure 2.31: Average clonal area of *l(2)gl*^{4}*, Myc*^{over}, *p53*^{DN} clones (blue) and wild-type twins (grey). For each sample, 20 clones in different wing discs have been measured at 200X magnification. Error bars represent the SEM.

These results suggest that p53 partial/complete LOF is able to restrict both clonal expansion and malignancy of *l(2)gl*^{4}, Myc^{over} cells in the wing disc. This cell-autonomous growth restriction is a counterintuitive evidence, as heterozygous/homozygous p53 loss is known to support growth and malignancy in a variety of biological systems [202]. The oncogenic side of p53 has just begun to be investigated, but it is possible that in this particular trait of tumourigenesis, that is MMCC, p53 loss perturbs the metabolic *status* of the Myc^{over} cells thus decreasing their neoplastic potential.

Further to show the cell-autonomous role of p53 in tumourigenesis, my analysis on clonal areas highlighted an interesting non-autonomous trait of the phenomenon we are investigating.

As can be seen in Figure 2.32, the *wild-type* twins of the *l(2)gl*^{4}, Myc^{over}, *p53*^{KD} clones are not statistically different from the *wild-type* twins of the *l(2)gl*^{4}, Myc^{over} clones. This means that a partial loss of p53 is able to rescue growth and malignancy of the *l(2)gl*^{4}, Myc^{over} cells, but it does not seem to affect their competitive properties.

On the contrary, the *wild-type* twins of the *l(2)gl*^{4}, Myc^{over}, *p53*^{DN} clones are statistically greater with respect to both others. Being the twins of all the mutant clones composed of genetically identical cells (*wild-type*), this statistical difference is obviously due to the different genotypes of the neighbour mutant clones, showing that complete removal of p53 severely impairs *l(2)gl*^{4}, Myc^{over} cells' competitive drive.

Taken together, these data seem to indicate that clonal expansion and competitive ability are separable traits of cancer. Further analysis is however necessary to confirm and examine in depth this interesting evidence.

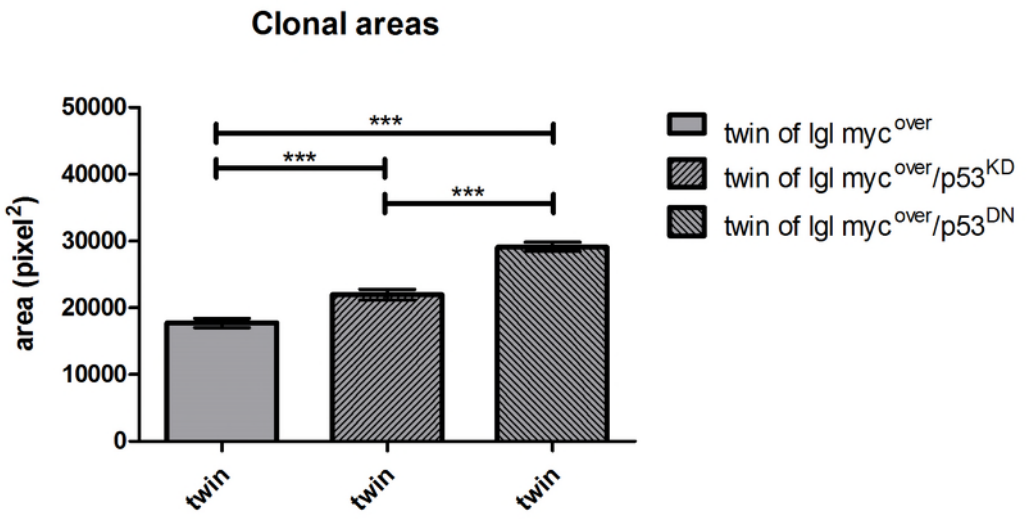


Figure 2.32: Average clonal area of the wild-type twins of *l(2)gl*^{4}*, Myc*^{over} (left bar); *l(2)gl*^{4}*, Myc*^{over}*, p53*^{KD} (central bar) *and l(2)gl*^{4}*, Myc*^{over}*, p53*^{DN} (right bar) clones. For each sample, 20 clones in different wing discs have been measured at 200X magnification. Error bars represent the SEM.

Chapter 3
Conclusions

Cancer is a heterogeneous and complex disease, with cells showing distant phenotypes as a result of dynamic changes in their genome.

Its extraordinary variety led researchers to arrange a taxonomic organisation where *quasi*-classes, genera and species characterised by divergent cells of origin make every cancer individually unique.
Cancer evolves by continuous processes of clonal expansion, genetic diversification and clonal selection within tissue ecosystems. Therapeutic intervention may decimate cancer clones and disrupt their habitats, but it provides unavoidably a potent selective pressure for the expansion of resistant variants.
To understand cancer genetic dynamics it is important to recognise it as a population in constant crosstalk with its surrounding environment. Thus cancer has to be viewed and analysed as an evolving ecosystem where cells are in constant communication and interaction with their environment. Populations, in order to use more and more resources to survive and proliferate, can elaborate strategies of ecological interactions listed as positive, as commensalism, synergism and mutualism, and negative as predation, parasitism, amensalism and competition, where one population is inhibited or eliminated by another one that gains advantages in space and resources.

The work I presented demonstrates that MYC-Mediated Cell Competition (MMCC) is at work during cancer progression, and this phenomenon seems to represent a central mechanism necessary for cancer clone selection and tumour mass expansion.

My investigation revealed typical signs of MMCC in human carcinomas, not only between cancer cells and the surrounding stromal tissue but, surprisingly, clear signs were also found within tumour cells, making us speculate that CC is used by the tumour mass to kill neighbours and gain a bigger amount of available resources and space, but also to select for the fittest clones amid cancer sub-populations. In this context, MMCC and its selective weapon, the MYC protein, represent a clear example of *clonal interference* aimed at gaining even more resistant behaviours.

I then developed an *in vitro* clone competition assay to demonstrate that MMCC is able to shape clone evolution in different contexts. I co-cultured pairs of genetically distant cell lines (to mimic MMCC between early diverged cancer clones) and genetically identical

cell lines (to mimic MMCC between late diverged cancer clones) and the results I obtained confirmed that MYC protein modulation is *per se* sufficient to subvert their competitive behaviours.

At this point, in order to find some answers to the evolutive questions related to MMCC, I chose to find a way to define the contribution of MMCC to tumour evolution *in vivo*, and I decided to develop a clone evolution assay in *Drosophila*.

I used *lgl* LOF mutant animals, known to undergo loss of apical-basal cell polarity and uncontrolled proliferation of the epithelial tissues, that show altered metabolism, dedifferentiation, upregulation of cytokine-like molecules and many other malignant features, described along the main text. In this *lgl* LOF background, I induced *neutral*, myc^{OVER} and myc^{KD} clones at different stages of cancer progression, and precisely at six days of development, a stage in which the wing imaginal disc shows obvious neoplastic growth, and at two days of development, when *lgl* mutant epithelia are still phenoypically normal. Target tissues were collected at eight days of development.

The first, interesting observation was that MMCC is normally at work in these tumours through the continuous elimination of the less fit cells (with lower levels of Myc protein), allowing the expansion of the strongest (with higher levels of Myc protein) (*paragraph 2.5*.).
The induction of Myc over-expressing clones conferred cells a super-competitive behaviour, enhancing the overall competitive interactions within the tumour mass and, as a result, tumours overgrew (fig 2.13). As the growth of the tissue was also contributed by the native population (fig 2.13), although to a lesser extent than the myc^{OVER} cells, this was clear demonstration that cancers behave like a cell community in which growth signals are systemically released and sensed, eventually generating an important expansion of the tumour bulk if compared to controls (fig 2.12).
On the other hand, the final size of the masses in which I induced myc^{KD} clones resulted significantly reduced, and also in this case both myc^{KD} and native cells contributed to the mass collapse, consistent with a signal of collective relaxation.

These were both important and amazing results, as they seem to suggest that while MMCC acts in a *leading role* in shaping the tumour mass as a consequence of mechanisms of

positive and negative pressure, the tumour cells at a distance from the *happening* are however able to detect that selective pressure and gain higher Myc expression to survive, thus acting in *supportive roles*: "*when the going gets tough the tough get going"*.

In addition, my study defined the presence of a functional cooperation between MYC and p53 in MMCC in cancer, as p53 LOF in the winner cells make them unable to grow and out-compete the neighbours.
p53 is one of the most frequently mutated genes in human cancers and the function of its several mutant products and dominant negative forms is not clear. As its tumour suppressor functions are well-known, many efforts are being made by the scientific community to reactivate its function in cancer.
But if p53 *wild-type* protein is also necessary to Myc-expressing cells to grow and out-compete neighbours during cancer progression, an oncogenic side of *wild-type* p53 should also be considered.
Given this dual role of p53, a better characterisation of all its functions and its mutated forms seems to urge in order to evaluate its actual role in cancer history.

Altogether, my results showed that MMCC plays an active role in the selective pressure we observed during cancer growth, favouring the most performant cells within the tumour and opposing to the expansion of the weakest derivatives. This evidence represents an important step towards the understanding of the evolutionary mechanisms underlying tumourigenesis.

Chapter 4

Materials and Methods

4.1. Ex Vivo and In Vitro Materials and Methods - Parts 1 and 2

Immunohistochemistry (IHC)

Immunohistochemical staining for c-MYC (mouse monoclonal 9E10; *DSHB*; 1:50), YAP-1 (rabbit polyclonal, *Cell Signaling*, 1:150)), HUGL-1 (by courtesy of Dennis Strand, rabbit polyclonal, 1:550) and Cleaved Caspase-3 (rabbit polyclonal, #9961 *Cell Signaling*, 1:250) was carried out on 5 μm thickness FFPE (formalin-fixed paraffin-embedded) histological sections of several types of human epithelial primary and secondary tumour samples (see tab. 1).

The whole set of sequential slides was first heat-deparaffinised (30 min at 65°C), rehydrated and antigen retrieval has been carried out with citric acid pH 8 at 95^{o}C for 30 minutes.

The stainings for YAP-1 and HUGL-1 were performed on separate slices using *Thermo Scientific, UltraVision kit* and each specific HRP-secondary antibody was revealed using DAB+ reaction.

The staining for c-MYC and Cleaved-Caspase-3 were performed on the same section using *Dako EnVision G/2 Doublestain System kit*, Rabbit/Mouse (DAB + / Permanent Red) which allows a double antigen hybridisation in the same slide through specific primary antibody recognition by HRP (Horseradish peroxidase enzyme), and A/P (Alkaline phosphatase enzyme)- conjugated polymer secondary systems.

All primary antibodies were incubated at 4°C overnight, and all secondary HRP/AP-conjugated polymers at room temperature for 10 minutes.

Slides were counterstained with Hematoxylin (nuclear dye), dehydrated, mounted and analysed. IHC for p53 (mouse monoclonal, DO-1, *abCAM*) has been carried out using the "Automatic Ventana BenchMark XT" Immunoistochemistry System.

For cancer and stroma identification, all the samples were analysed by an expert pathologist prior and after staining.

Primary Mass	Secondary Lesions
Ductal carcinoma in situ (DCIS)- Left breast	Lymph Node Metastasis
Lobular carcinoma in situ (LCIS)- Right breast	Lymph Node Metastasis
Mucinous Cystadenocarcinoma- Left breast	Lymph Node Metastasis
Ductal invasive G3 carcinomas- Left breast	Lymph Node Metastasis
Ductal invasive G2 carcinomas- Left breast	Lymph Node Metastasis
Infiltrating papillary thyroid carcinoma	Lymph Node Metastasis
Lung adenocarcinoma (NSCLC)- Left lobe	Brain Metastasis
Lung poorly differentiated carcinoma (NSCLC)- Left lobe	Brain Metastasis
Lung squamous cell carcinoma (SCC)- Left lobe	Brain Metastasis
Pleomorphic adenoma of the cheek	Brain Metastasis
Oesophageal carcinoma	Brain Metastasis
Colorectal Adenocarcinoma	Liver Metastasis
Colorectal Adenocarcinoma	Liver Metastasis
Colorectal Adenocarcinoma	Liver Metastasis
Endometrial carcinoma	Colorectal Metastasis

Table 4.1: Some of the cases analysed by IHC staining

Cell Cultures

Human Carcinoma cell lines (see tab. 2) from ATCC were cultured in DMEM-containing 10% heat-inactivated FBS (fetal bovine serum), 2mM of glutamine and 1% antibiotics (100 U/ml of Penicillin/ 100 µg/ml of Streptomycin) in 5% CO_2 humidified air atmosphere at 37°C.

Cell lines	Cancer Type:	Notes:
H460- ATCC® HTB-177™	Lung Carcinoma (LCLC)	
H1975- ATCC® CRL-5908™	Lung adenocarcinoma (NSCLC)	
H1299- ATCC® CRL-5803™	Lung Carcinoma (NSCLC)	Homozygous partial deletion of the p53 protein.
LS174T- ATCC® CL-188™	Colorectal adenocarcinoma	
LoVo- ATCC® CCL-229™	Colorectal adenocarcinoma	
HCT116- ATCC® CCL-247™	Colorectal carcinoma	
HCT116 $p53^{-/-}$	Colorectal carcinoma	p53 exon 3 deletion [203].

Table 4.2: Human carcinoma cell lines used in this study and their characteristics.

Cell Count

Cell were washed in PBS 1X, detached with trypsin 1X, collected in DMEM medium, pelleted by centrifugation and suspended in DMEM 10% FBS complete medium. Count and viability were quantified by cell counting with Trypan Blue exclusion using the Bürker's chamber applying the following equation:

[(Average n° of Trypan blue negative cells in 9 squares · 10^4) · Dil. Factor] · ml of cell suspension, obtaining the total number of viable cells suspended in the initial volume.

10058:F4 Treatment

The MYC-MAX small molecule inhibitor 10058:F4 (*Sigma-Aldrich*) was used to reduce MYC-MAX activity [204] through its binding to the MYC bHLHZip dimerisation domain. MYC-MAX small molecule inhibitor 10058:F4 was dissolved in dimethyl- sulphoxide (DMSO).

To assess the inhibitory effect of the drug, cells were seeded in a 6-well plate at a density of $5x10^5$ cells per well and allowed to settle for 24 hours at 5% CO_2 humidified air atmosphere at 37°C, before starting the treatment. 10058:F4 (and an equivalent amount of DMSO for controls) was applied at different concentrations with fresh medium.

After 24 hours of treatment, cells were washed in PBS 1X, detached with trypsin 1X, collected in DMEM and treated for RNA isolation for following qRT-PCR (to assess transcripts variations) or treated for total protein extraction for Western Blot analysis.

In CCAs, the 10058:F4 inhibitor was used at a concentration of 60µM as lower concentrations did not show significant effects on MYC-MAX activity and higher concentrations induced autonomous cell death.

Cells for CCAs (see further) were plated as before, where under treatment cells were plated with 60µM of 10058:F4 and non-treated cells were incubated with an equivalent amount of DMSO. After 24 hours at 5% CO_2 humidified air atmosphere at 37°C, cells were washed in PBS 1X, detached with trypsin 1X, collected in DMEM, counted and used for the assays.

PKH67 (Sigma-Aldrich) Cell Membrane Labelling

PKH67 is a fluorescent cell membrane dye characterised by a long aliphatic carbon tail than binds the phospholipid bilayers of the cell membrane, while its fluorogenic moiety is exposed near the outer surface of the cell. It is widely used to for *in vitro* and *in vivo* cell tracking. This tracker is characterised by no cytotoxic effects and reduced cell-cell transfer.

PKH67 was used to label one of the two populations used in the CCAs (see further) according to the manufacture's bulletin: cells were gently detached with trypsin 1X and washed twice with minimum DMEM medium, as serum interferes with labelling efficiency. Cells were counted with trypan blue exclusion method and $5/7x10^6$cells were used for the staining. Simultaneously, PKH67 was mixed with 2 ml of Diluent C solution to obtain a 10μM staining solution, then applied to the cell pellet and incubated for 5 minutes at room temperature. The reaction was then blocked with an equal volume of FBS for 1 minute at room temperature, as the serum competes for the aliphatic carbon tail binding to the cell membrane. Stained cells were centrifuged for 10 minutes at 400g, complete DMEM was added and cell viability and fluorescent intensity were assessed.
To exclude side effects of PKH67 on CCAs, a test on two different cell lines was performed in which the two cell populations were alternatively stained, and no differences in the final results were appreciable between the two conditions.

Cell Competition Assays (CCAs)

To experimentally evaluate the phenomenon of MMCC in human carcinoma cell lines, a cell-based assay was designed and performed.
Each assay was carried out by using two different human carcinoma cell populations characterised by different cMYC expression levels or two sub-populations from the same cell line in which one was previously treated with the MYC-MAX 10058:F4 inhibitor. The cell lines used in each CCA showed comparable proliferation rates.
One of the two populations forming the assay was previously labelled with the aim to distinguish the two cell lines during and after the assay.
The cell lines were counted with the Trypan blue exclusion method and, in a 6-wells plate, $2,5x10^5$ cells from each line were plated in co-culture; simultaneously, $5x10^5$ cells from each population were plated in separate conditions, both in standard growth and medium conditions. After 5 hours incubation, the wells were gently washed in PBS 1X, cells were detached with trypsin 1X, collected and counts were assessed in a Bürker's chamber using a wide-field fluorescence microscope. The number of cells obtained after 5 hours incubation has been normalised to the number of the cells plated at the beginning, obtaining the growth units for each cell line in the different conditions. Cell competition outcome was evaluated after 5 hours of co-culture according to literature's information [95]. As a control, we however performed some cell counts also after 12 and 24 hours, but we were not able to find significative changes in the final results. Each experiment has

been performed at least 3 times independently and each single count was repeated twice. Data were statistically analysed using GraphPad Prism® free software.

Immunofluorescence (IF) on CCAs

IF assays were performed to verify the apoptotic rate of the cells during the CCAs, by using a specific antibody directed against the active form of the Caspase 3 (Cleaved-Caspase 3), as loser cell elimination is Caspase 3 dependent in *Drosophila* [98] [93] [205] and in mammals [206].

A cover slip previously washed in EtOH 100% was settled on the bottom of every well of a 6-wells plate coated with collagen 50ng/µl in glacial acetic acid 0,02N and incubated for 1 hour at room temperature under a laminar flow hood. The excess collagen was aspirated, plates were washed twice in PBS 1X and cells were seeded as previously described.

After 5 hours growth, cells were gently washed in PBS 1X and fixed with Paraformaldehyde 2% (PFA) for 15 minutes at room temperature and rinsed 3 times with PBS-T (PBS 1X + Triton 0,1%) for 5 minutes. Cells were incubated for 30 minutes with Normal Donkey Serum 4% (NDS) diluted in PBS-T. Finally, Cleaved Caspase-3 antibody (rabbit polyclonal, 9961; *Cell Signaling*; 1:250) in PBT/NDS 4% was incubated overnight at 4°C. The secondary anti-rabbit antibody, (AlexaFluor™ 555, *Invitrogen*; 1:300) in PBT/NDS 4% was incubated for 1 hour at room temperature. A solution containing DAPI 2µg/ml PBS1X was used in the final wash to counterstain cell nuclei. The coverslips were mounted using Fluoromount™ glycerol-mounting medium (*Sigma- Aldrich*) and visualised under a wide-field fluorescence microscope. Four fields were counted in each slide; each experiment was repeated at least 3 times. All data were statistically analysed using GraphPad Prism® free software.

Total Protein Extraction, SDS PAGE and Western Blotting

Sub-confluent cells from a 100mm dish were washed in PBS 1X, detached with Trypsin 1X, collected in DMEM, centrifuged and lysed in RIPA buffer added with protease inhibitors (Complete, *Roche;* PMSF, *Sigma-Aldrich*) for 15 minutes at 4°C. Following chemical lysis, sonication was carried out to increase the detergent effect of RIPA buffer and break chromatin aggregates. After centrifugation, the supernatant containing the total protein extract was collected and quantification was carried out using the BCA protein method (*Pierce™ BCA Protein Assay Kit*).

50µg lysate were mixed with Laemmli Loading buffer 4X and denatured for 10 minutes at 100°C. Samples were resolved on 4/10% acrylamide/bisacrylamide gel for 90 minutes, 120Volt at room temperature. After migration, proteins were transferred to a nitrocellulose membrane in which immuonodetection is successively carried out. The transfer reaction was performed at constant amperage (300mA) for 1 hour and 45 minutes at 4°C in Tris-Glycine/Methanol based transfer buffer. Blocking in 4% Milk solution has been applied for 1 hour at room temperature to the membrane before incubation with the primary antibodies overnight at 4°C. Primary antibodies used were: anti-c-MYC (Rabbit, *Santa Cruz*, N-262; 1:1500), anti-p53 (Do-1, mouse, *AbCAM*; 1:1000) and anti-β-Actin (Rabbit, *Jackson ImmunoResearch*; 1:2000). The HRP-conjugated secondary antibodies used were goat anti-rabbit and anti-mouse (*Jackson ImmunoResearch* 1:2000) for 1 hour at room temperature. The HRP-ECL reaction has been revealed using Bio-Rad Chemidoc™.

Ripa buffer	**1X Running buffer**	**1X Transfer buffer**	**1X Blocking solution**
50mM TrisHCl pH7.5	10% Tris- Glycine	5% Tris- Glycine	20mM TrisHCl pH8
150mM NaCl	0.1% SDS	20% Methanol	20mM NaCl
0.5% Na Deoxycholato	mqH_2O	mqH_2O	25gr Powder milk
1% NP40			0.05% Tween20
0.1% SDS			mqH_2O
1mM PMSF			
1X Complete (*Roche*)			
mqH_2O			
4X Laemmli buffer	**10% Running gel**	**4% Stacking gel**	
250mM TrisHCl pH6.8	10% Acry/ bisacryl	4% Acry/ bisacryl	
40% Glycerol	300mM TrisHCl pH8.8	150mM TrisHCl pH6.6	
5% SDS	0.1% SDS	0.1% SDS	
0.005% Bromoph. Blue	0.1% APS	0.1% APS	
mqH_2O	0.05% TEMED	0.05% TEMED	
	mqH_2O	mqH_2O	

Table 4.3: Western blot Solutions

RNA Extraction and Purification, RT-PCR, Sybr Green qPCR

Cells from 100mm dishes were directly scraped in TRI-Reagent® (*Sigma-Aldrich*) and processed or stored at -80°C before being processed.

The larvae were obtained from a culture of 5 days after egg laying. About 15 larvae were homogenised in a vial with TRI Reagent® (*Sigma- Aldrich*), centrifuged for 5 minutes at 12000g at 4°C and the supernatant was collected to be processed.

300µl of chloroform were added to the samples containing 1ml of TRI Reagent® (*Sigma-Aldrich*) solution and vortexed for 10 seconds. The samples were incubated for 10 minutes

at room temperature and centrifuged for 12 minutes at 12000g at 4°C. The RNA-containing aqueous phase was transferred to a new tube, where 750µl of isopropyl alcohol were added. Samples were mixed gently, incubated 10 minutes at room temperature and centrifuged for 12 minutes at 12000g at 4°C. The supernatant was removed, the pellet was washed three times with 1 ml 75% EtOH and centrifuged at 7500g for 5 minutes at 4°C. The supernatant was removed and the dried pellet was eluted at 55°C for 10 minutes in 50µl of mqH_2O. A DNAse I-treatment followed to avoid genomic contamination.

cDNA synthesis was performed using total DNA free-RNA with oligo(dT) in a 0,2ml tube, and using the ThermoScript™ RT-PCR system. The ThermoScript is an engineered avian reverse transcriptase with reduced RNase H activity that shows high thermal stability and produces high amounts of full-length cDNAs. Each mix is prepared as follows: 1µg di RNA + 2X RT Reaction Mix (oligo dT 2,5µM, random examers 2,5 ng/µl, $MgCl_2$ 10 mM, dNTPs) + RT enzyme Mix (Retrotranscriptase e RNase OUT) + mqH_2O up to the final volume.

SYBR GreenER qPCR SuperMix (*Invitrogen*) is a ready-to-use cocktail containing all components, except primers and template, for real-time quantitative PCR (qPCR) on ICycler BioRad real time instruments that support normalisation with Fluorescein Reference Dye at a final concentration of 500nM. It combines a chemically modified "hotstart" version of TaqDNA polymerase. SYBR GreenER qPCR SuperMix is supplied at a 2X concentration and contains hot-start TaqDNA polymerase, SYBR GreenER fluorescent dye, 1 µM Fluorescein Reference Dye, $MgCl_2$, dNTPs and stabilisers. The SuperMix formulation is compatible with melting curve analysis.
The amplification is based on 40 cycles x 3 steps: after 3 minutes denaturation at 95°C, each amplification step includes: 30 second at 95°C (denaturation), 15 seconds at 60°C (annealing temperature for each primer pair), 30 seconds at 72°C (elongation step). The resulting graph is the relative quantity of the target gene transcript compared to the transcript quantity of the reference gene. For each sample, an amplification curve is shown in a Cartesian graph: the x axis represents the cycle number and the y axis represents the Relative Fluorescence unit which is dependent on the amplified cDNA molecules.

Gene	Primer sequences	Size (bp)	Melting (°C)	GenBank Accession
Homo sapiens				
c-MYC	Forward: 5'-GAGGAGGAACAAGAAGATGAGG-3' Reverse: 5'-TCCAGCAGAAGGTGATCCA-3'	100	60	NM_002467.4
BRCA1	Forward: 5'-GGTGGTACATGCACAGTTGC-3' Reverse: 5'-ACTCTGGGGCTCTGTCTTCA-3'	240	60	NM_007294.3
BRCA2	Forward: 5'-CCACAGCCAGGCAGTCTGTAT-3' Reverse: 5'-AGAACACGCAGAGGGAACTTG-3'	96	60	NM_000059.3
GUSB	Forward: 5'-AGCGTGGAGCAAGACAGTGG-3' Reverse: 5'-ATACAGATAGGCAGGGCGTTCG-3'	198	60	NM_000181.3
GAPDH	Forward: 5'-AAGGTGAAGGTCGGAGTCAAC-3' Reverse: 5'-GAGTTAAAAGCAGCCCTGGTG-3'	68	59	NM_002046.5
Drosophila melanogaster				
dMyc	Forward: 5'-CGGGAGTCAATAACAAAGTG-3' Reverse: 5'-GCTGCATACTAAGCTCCTTC-3'	423	58	NM_080323.4
dMax	Forward: 5'-CGACATAGACATCGAAAGTG-3' Reverse: 5'-TGCGTCTACTGAAGTCCTG-3'	434	56	NM_140840.4
DmP53	Forward: 5'-CCAAGCTAGAGAATCACAAC-3' Reverse: 5'-AGGCAGAAGACTAAGGAAG-3'	487	56	NM_206545.2
GFP	Forward: 5'-GGATGCTCTTGGCTCTTC-3' Reverse: 5'-GACAATCTTCTGGTGTCTGG-3'	352	55	
dACT5C	Forward: 5'-GAAGAAGTTGCTGCTCTG-3' Reverse: 5'-CATCAGGTAGTCGGTCAA-3'	564	54	NM_167053.2

Table 4.4: Real Time PCR primers used and main features. c-MYC: Homo sapiens v-myc avian myelocytomatosis viral oncogene homologue ; BRCA1: Homo sapiens Breast cancer 1; BRCA2: Homo sapiens Breast cancer 2; GUSB: Homo sapiens Glucuronidase, beta (reference gene); GAPDH: Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (reference gene); dMyc: *Drosophila melanogaster* Myc oncogene; dMax: *Drosophila melanogaster* Max; Dmp53: *Drosophila melanogaster* p53; GFP: Green Fluorescence Protein construct; dACT5C: *Drosophila melanogaster* Actin (reference gene).

Statistics

For CCAs, all values are the mean of at least 3 independent experiments where each single count was repeated twice; for IF, the percentage of Casp-3 positive cells are the mean of 4 fields counted for each experiment. Student's *t* test was performed to determine significance (two-tailed, unequal variance). *p* values are as follows: $p \leq 0.05$=*, $p \leq 0.01$=**, $p \leq 0.001$=***. All error bars are ± standard error of the mean (SEM), if not differently indicated. All data were statistically analysed using GraphPad Prism® version 5.01 for windows, GraphPad Software, San Diego California USA: www.graphpad.com

4.2. In Vivo Materials and Methods

Drosophila melanogaster has been used as animal model and the experiments were performed in a larval organ that is widely used for CC assays: the wing imaginal disc.

The original lines and the crosses were reared at 25°C on a medium composed of water, agar, glucose, baker yeast, cornmeal in the right proportions. A chemical is finally added to prevent moulds.

Mutant and transgenic lines described have been obtained, if not otherwise specified, from the *Bloomington Drosophila Stock Center*, Indiana. For information about mutations, transgenes and enhancers please see: http://flystocks.bio.indiana.edu/

Drosophila Model Tissue: The Imaginal Wing Disc

Imaginal discs are larval epithelial organs that give rise to adult structures and appendages. The imaginal wing disc, precursor of the adult wing, is composed of a pseudostratified columnar epithelium of undifferentiated and proliferating cells that represent the actual imaginal disc, and by a squamous epithelium that forms the peripodial membrane (Fig.4.1 A). The first will originate the integument and the wing, the second will originate the epithelial veil that welds the structures [207].

When it is formed during embryonic development, the wing imaginal disc comprises around 20 cells [207]. These cells intensely proliferate during the second and third larval instars to generate a disc of around 50,000 cells in the late third instar (96h after hatching). By this stage, the wing primordium is established and its major elements can be identified.

The centrifugal regions will originate the dorsal and ventral body wall thorax structures: notum and pleura. The middle region will give rise to the hinge, while the central region, the wing pouch, is the presumptive territory from which the wing lamina differentiates.

(Fig.4.1 B, [207]) For this reason, the wing pouch is defined as the distal region of the wing disc while hinge and pleura are considered as proximal.

The wing imaginal disc represents an excellent model widely used to study growth and proliferation control in epithelial tissues. It is morphologically and biochemically very similar to mammalian epithelia and it encounters a dramatic increase in cell number in a relatively short length of time with an average cycle time of 8.5h [208].

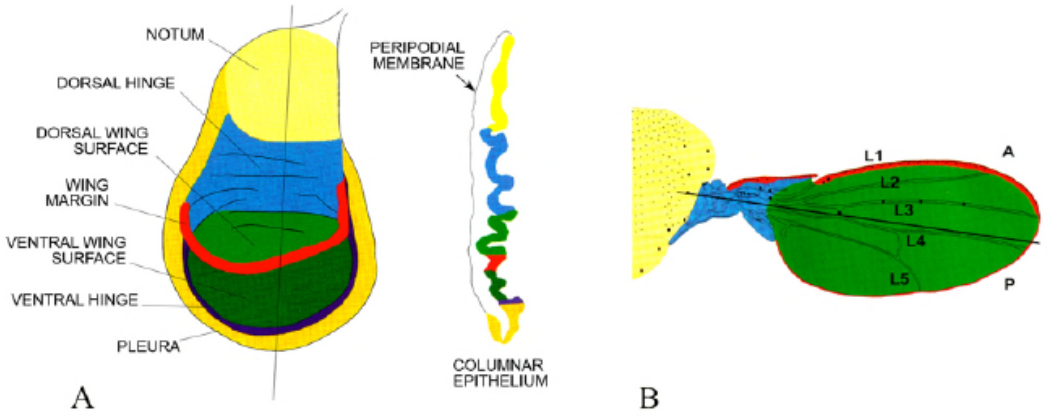


Figure 4.1 . **A)**: A third instar imaginal wing disc. The presumptive regions that correspond to the adult wing structures (shown in b) are labelled. Dorsal and ventral wing surfaces compose the wing pouch. A cross section of the epithelium and the peripodial membrane is also shown on the right. **B)** Adult wing. the different structures are coloured according to the presumptive territories they originate from (shown in A). Longitudinal veins (L 1-5), anterior (A) and posterior (P) compartments are also indicated. Adapted from [207].

4.2.1. Drosophila Methods Used in Part 1

Mutations

***lgl*^{4} (*lethal giant larvae*)** recessive lethal mutation of the *lgl* gene caused by a spontaneous loss of the subtelomeric 21A2 region;

y*^{67c23} *(yellow) X-associated recessive mutation: the body is yellow and the mouth apparatus of the larva is brown;

w*^{1118} *(white) X-associated recessive mutation: the eye is white because of a complete lack of pigmentation;

Bc (Black cells) II-associated dominant mutation: the larval crystal cells are substituted by melanotic masses;

***Cy* (*Curly*)** II-associated dominant mutation; wings are curled upward;

***Gla (Glazed*)** II-associated dominant mutation: the eye is reduced and misshaped and ommatidia appear fused.

***Hu* (*Humeral*)** III-associated dominant mutation; alteration of humeral bristles number;

***Tb* (*Tubby*)** III-associated dominant mutation; shorter and thicker larvae, pupae and adults.

Balancer Chromosomes

In(2LR)GlaBc II chromosome carrying multiple arrangements associated with *Gla* and *Bc* dominant markers, recessive lethal;

In(2LR)O (CyO - Curly of Oster) II chromosome carrying multiple arrangements associated with *Cy* dominant marker, recessive lethal;

In(2LR)SM5 II chromosome carrying multiple arrangements associated with *Cy* dominant marker, recessive lethal;

In(3LR)TM6b II chromosome carrying multiple arrangements associated with *Hu* and *Tb* dominant markers, recessive lethal.

Transgenes

hs-Flp X-associated construct encoding the yeast recombinase Flippase under the control of a heat-shock promoter;

act5c>CD2>Gal4 III-associated Flp-out construct driven by *actin5c* gene promoter, bearing CD2 cDNA between FRT sequences (>) (Paola Bellosta);

UAS-GFP III-associated construct encoding a nuclear GFP protein downstream of a UAS enhancer;

UAS-HAdmyc III-associated construct encoding the fly Myc protein with a HA epitope downstream of a UAS enhancer (Paola Bellosta);

UAS-dmRNAi II-associated construct encoding an interfering RNA specific for the *dm* transcript downstream of a UAS enhancer (VDRC 2847); this construct knocks down the *dm* transcript of about 50% (Figure S.I. 12), mimicking a heterozygous condition.

Flp-out system [209]

The Flp-Out technique allows clonal expression of UAS constructs combining the UAS-Gal4 system with Flp-FRT mediated recombination. A Flp-Out construct consists in a constitutive promoter (in this case, *actin5c*) followed by an FRT cassette, a sequence encoding a cell marker with a polyA terminator (in this case, *CDC2*), a second FRT cassette and a *Gal4* sequence. When activated, Flippase expression can induce excision of the DNA sequence comprised between the two FRT sites placed in the same orientation. Following this, the constitutive promoter can thus transcribe the Gal4 sequences that will, in turn, drive the expression of all the UAS-transgenes present in the line, including a visible cell marker (in this case, UAS-GFPnls) to allow clone identification.

Lines and Crosses:

Driver line:

yw, hs-Flp; *l(2)gl*^{4}*/CyO*; *act5c>CD2>Gal4, UAS-GFPnls/TM6b*

Responder lines:

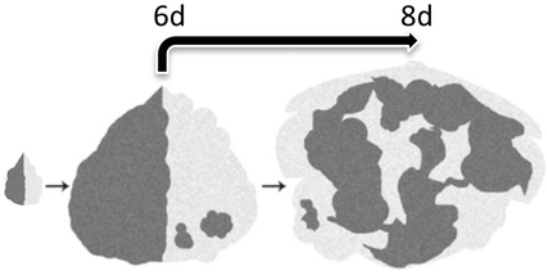
1. *w; l(2)gl*^{4}*/SM5*
2. *w; l(2)gl*^{4}*, UAS-dmRNAi/SM5*
3. *w; l(2)gl*^{4}*/In(2LR)GlaBc; UAS-HAdm*

Larvae of interest:

1. *yw, hsFlp/w; l(2)gl*^{4}*/l(2)gl*^{4}*; act5c>CD2>Gal4, UAS-GFPnls/+*
2. *yw, hsFlp/w; l(2)gl*^{4}*/l(2)gl*^{4}*, UAS-dmRNAi; act5c>CD2>Gal4, UAS-GFPnls/+*
3. *yw, hsFlp/w; l(2)gl*^{4}*/l(2)gl*^{4}*; UAS-HAdm,/act5c>CD2>Gal4, UAS-GFPnls*

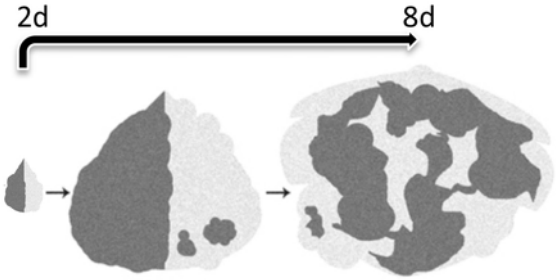
In those larvae, I induced *l(2)gl*^{4} neutral clones (1), *l(2)gl*^{4} *Myc*^{KD} clones (2) or *l(2)gl*^{4} *Myc*^{OVER} clones (3) at different stages of cancer development, as follows:

Myc Deregulation in Genetically Distant Cells: 6+2 Scheme



144±4h larvae grown at 25°C were heat-shocked for 2 minutes at 37°C in an eppendorf immersed in a water bath. After the heat-shock, larvae were transferred to fresh food and allowed to grow for additional 2 days at 25°C. Finally, wing discs were collected from GFP^{+} larvae for successive analyses.

Myc Deregulation in Genetically Related Cells: 2+6 Scheme



48±4h larvae grown at 25°C were heat-shocked for 8 minutes at 37°C in a food vial immersed in a water bath. After the heat-shock, vials were immersed in fresh water for 5 minutes, then transferred for additional 6 days at 25°C. Finally, wing discs were collected from GFP^{+} larvae for successive analyses.

Mass calculation

144±4h and 192±4h larvae were dissected at 4°C in PBS1X (Phosphate Buffer Saline, pH 7.5) under a stereoscope, imaginal wing discs were isolated from the carcasses and transferred in clean PBS1X. A picture of the discs collected each day was captured at 25X using a dedicated digital camera. Disc areas in $pixel^2$ were measured using ImageJ Software from NIH and areas were transformed in volumes following the same formula as for prolate spheroids: $4/3\ \pi\ a^2b$.

Cell volume calculation

Sequential Z stacks were taken under a confocal microscope for each 6+2 sample stained for the membrane marker aPKC (Santa Cruz, 1:200) and the cell area of all the GFP^+ and GFP^- cells included in 4 different fields were measured using ImageJ Software by NIH. Cell volume has been calculated by approximating cell shape to a sphere.

Dissociation and Cell count

Wing discs collected each day were washed twice in PBS1X and incubated with gentle agitation for 2.5 hr in 1 ml PBT (4.5 mg/ml porcine trypsin-EDTA [Sigma-Aldrich] in PBS1X). Cell count was carried out using the Bürker's chamber applying the following equation: [(Average n° cells in 9 squares · 10^4) · Dil. Factor] · ml of cell suspension, obtaining the total number of cells suspended in the initial volume.

Statistics

All values are the mean of at least 3 independent experiments where each single count was repeated twice. The number of wing discs analysed was 30÷90 for each sample. *p* values are as follows: $p \leq 0.05$=*, $p \leq 0.01$=**, $p \leq 0.001$=***. All error bars are ± standard error of the mean (SEM), if not differently indicated. All data were statistically analysed using GraphPad Prism® version 5.01 for windows, GraphPad Software, San Diego California USA: www.graphpad.com

Immunofluorescence

192±4h larvae from the 6+2 scheme were dissected in PBS1X (Phosphate Buffer Saline, pH 7.5), fixed in 3,7% formaldehyde (Sigma) diluted in PBS1X and permeabilised in PBT 0,3% for 90 minutes. They were then incubated with the primary antibody solution (PBT/BSA 2%) overnight at 4°C. The next day the carcasses were washed and incubated

for 2 hours at room temperature with a mix of the secondary antibodies. Finally, wing imaginal discs were isolated under a stereoscope and mounted in FluoromountG (Beckman Coulter). Samples were analysed with Leica TSC SP2 laser confocal microscope and entire images were processed with Adobe Photoshop software. All the images shown represent a single confocal stack. Primary antibodies: anti-cleaved Cas3 (rabbit polyclonal, #9961; *Cell Signaling*; 1:100) and anti-Myc (mouse, P. Bellosta, 1:5). Secondary antibodies: 555 Alexa Fluor anti-mouse 1:200 and Cy5 DyLight Jackson Laboratories anti-rabbit 1:500.

4.1.2. Drosophila Methods Used in Part 2

Mutations

***lgl*^{4} (*lethal giant larvae*)** recessive lethal mutation of the *lgl* gene caused by a spontaneous loss of the subtelomeric 21A2 region;

y*^{67c23}*(yellow) X-associated recessive mutation: the body is yellow and the mouth apparatus of the larva is brown;

w*^{1118} *(white) X-associated recessive mutation: the eye is white because of a complete lack of pigmentation;

Bc (Black cells) II-associated dominant mutation: the larval crystal cells are substituted by melanotic masses;

***Gla (Glazed*)** II-associated dominant mutation: the eye is reduced and misshaped and ommatidia appear fused.

Balancer Chromosomes

In(2LR) GlaBc, II chromosome carrying multiple arrangements associated with *Gla* and *Bc* dominant markers, recessive lethal.

Transgenes

hs-Flp X-associated construct encoding the yeast recombinase Flippase under the control of a heat-shock promoter;

tub-Gal4 X-associated construct encoding the yeast Gal4 protein under the control of the tubulin promoter;

tub-Gal80 II-associated construct encoding the yeast Gal80 protein under the control of the tubulin promoter;

UAS-lglRNAi III-associated construct encoding a double strand interfering RNA specific for the lgl transcript downstream of a UAS enhancer (VDRC 51249);

UAS-GFP X-associated construct encoding a nuclear GFP protein downstream of a UAS enhancer;

UAS-HAdmyc III-associated construct encoding the fly Myc protein with a HA epitope downstream of a UAS enhancer (Paola Bellosta);

UAS-p53RNAi III-associated construct encoding a double strand interfering RNA specific for the p53 transcript downstream of a UAS enhancer (#41720);

UAS-p53DN III-associated construct encoding a dominant negative form of the fly p53 protein downstream of a UAS enhancer (#8421).

MARCM (Mosaic Analysis with a Repressible Cell Marker) (Lee and Luo 1999)

In *l(2)gl*^{4} heterozygous individuals I induced the expression, by mitotic recombination, of homozygous *l(2)gl*^{4} clones bearing the UAS-HAdm and/or UAS-p53^{KD} or UAS-p53^{DN} constructs. The mosaic technique allowed to define the criteria of CC and the role of p53 in tumour growth. This has been reached by comparing the clonal areas of the GFP-positive mutant clones to those of the respective *wild-type* twin clones. The flippase enzyme, that gives rise to mitotic recombination, was activated by a heat-shock promoter, hence the larvae underwent 20 minutes heat-shock at 37^{o}C 48±4 hours after egg laying. After 72 hours, L3 larvae bearing GFP clones were dissected in PBS1X, fixed in formaldehyde 3,7% and frozen in 70% ethanol in PBS 1X.

The following crosses were carried out to perform this analysis:

♀♀ yw,hs-Flp,tub-Gal4,UAS-GFP; tub-GAL80, FRT40A (driver line)

X

1. ♂♂ w; l(2)gl^{4}, FRT40A/GlaBc; UAS-HAdm, UAS-lgl^{KD}

An irrelevant (RNAi)lgl^{KD} construct was added to this cross to make Gal4 titration comparable in all the progenies and as a control of the RNAi machinery activation.

2. ♂♂ w; l(2)gl^{4}, FRT40A/GlaBc; UAS-HAdm, UAS-p53^{KD}
3. ♂♂ w; l(2)gl^{4}, FRT40A/GlaBc; UAS-HAdm, UAS-p53^{DN}

Larvae of interest:

1. yw,hsFlp,tubGal4,UAS-GFP/w; l(2)gl^{4}, FRT 40A/ tubGal80,FRT40A;UAS-HAdm,UAS-lgl^{KD} /+
2. yw,hsFlp,tubGal4,UAS-GFP/w;l(2)gl^{4},FRT40A/tubGal80,FRT40A;UAS-HAdm,UAS-p53^{KD}/+
3. yw,hsFlp,tubGal4,UAS-GFP/w;l(2)gl^{4},FRT40A/tubGal80,FRT40A;UAS-HAdm,UAS-p53^{DN}/+

Upon the heat shock, recombination occurs and mutant cells face while growing with *wild-type* twin cells in which the recombination event has placed two copies of the tub-Gal80

repressor, so that they do not express GAL4 and the UAS-related products. By comparing the clonal areas of mutant and twin clones it was possible to evaluate both growth rate and competitive abilities of the mutant cells. The analysis was restricted to the wing pouch region so to make it more homogeneous and significant. The clonal areas were measured in $pixel^2$ by using the ImageJ Software from NIH and normalised to the wing pouch total area so to bypass the differences in wing disc overall dimensions. A statistic analysis was performed whose parameters are reported in the figure legends.

Immunofluorescence

Larvae collected at the end of the larval life were used for the IF analysis using the anti-Myc and anti-Lgl antibodies. Larvae were dissected in PBS1X (Phosphate Buffer Saline, pH 7.5) and fixed in 3,7% formaldehyde (Sigma) diluted in PBS1X. The larval carcasses collected each day, with the imaginal discs attached to the cuticle, were frozen after dehydration in a crescent alcoholic scale from 30% to 70% EtOH. Immediately before IF, the carcasses were rehydrated and permeabilised in PBT 0,3% for about 40 minutes. They were then incubated with the primary antibody solution (PBT/BSA 2%) overnight at 4^oC. The next day the carcasses were washed and incubated for 2 hours at room temperature with a mix of the secondary antibodies. Finally, wing imaginal discs were isolated under a stereoscope and mounted in FluoromountG (Beckman Coulter). The images for clonal area measurement were captured under a wide-field fluorescence Microscope (Nikon Eclipse 90i) and measured using the ImageJ software from NIH. Samples were analysed with Leica TSC SP2 laser confocal microscope and entire images were processed with Adobe Photoshop software. All the images shown represent a single confocal stack. Primary antibodies: anti-Lgl (rabbit, D. Strand, 1:400) and anti-Myc (mouse, P. Bellosta, 1:5). Secondary antibodies: 555 Alexa Fluor anti-rabbit 1:500 and Cy5 DyLight Jackson Laboratories anti-mouse 1:200.

Chapter 5

Supplementary Information

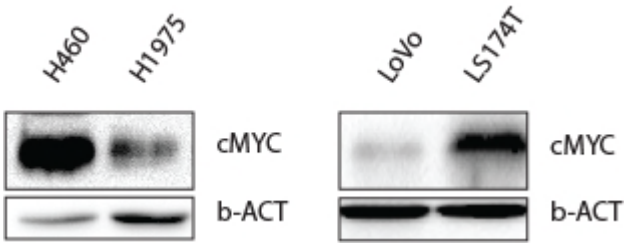


Figure S.I. 1: Comparison of cMYC protein expression in the cell lines used for CCAs. Western blot analysis of cMYC and β-Act as normaliser in H460, H1975, LoVo and LS174T.

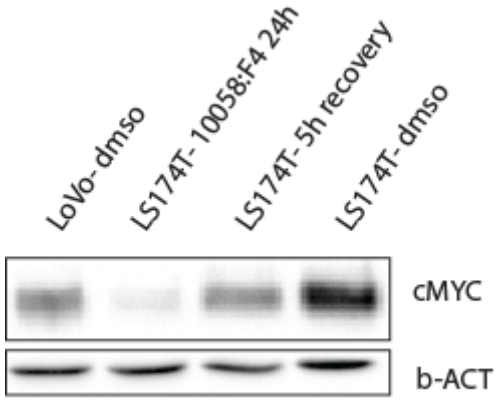
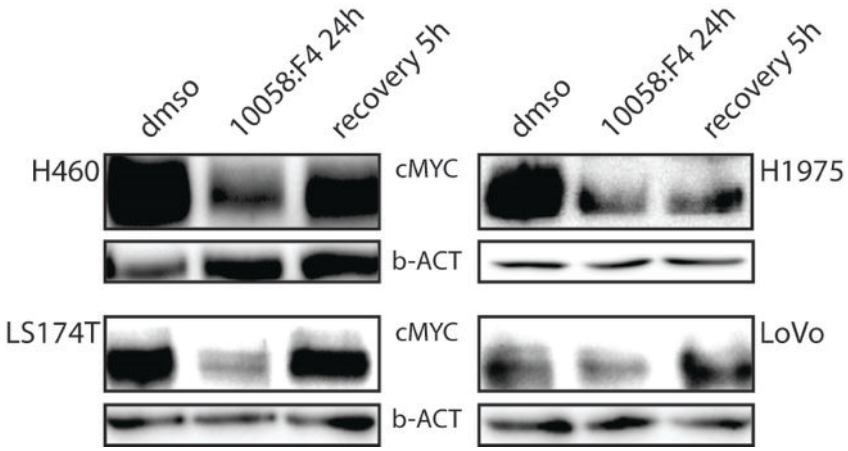


Figure S.I. 2: cMYC protein characterisation in LoVo and LS174T cell lines used in CCA before and after 10058:F4 treatment. Total proteins for Western blot analysis were extracted after the LoVo (lane 1) and LS174T (lane 4) were mock-treated with DMSO for 24h; LS174T was treated with 60μM 10058:F4 for 24h (lane 2); the same treated cell line was recovered in complete medium for 5h post 10058:F4 treatment (lane 3). β-ACT was used as normaliser.

(A)



(B)

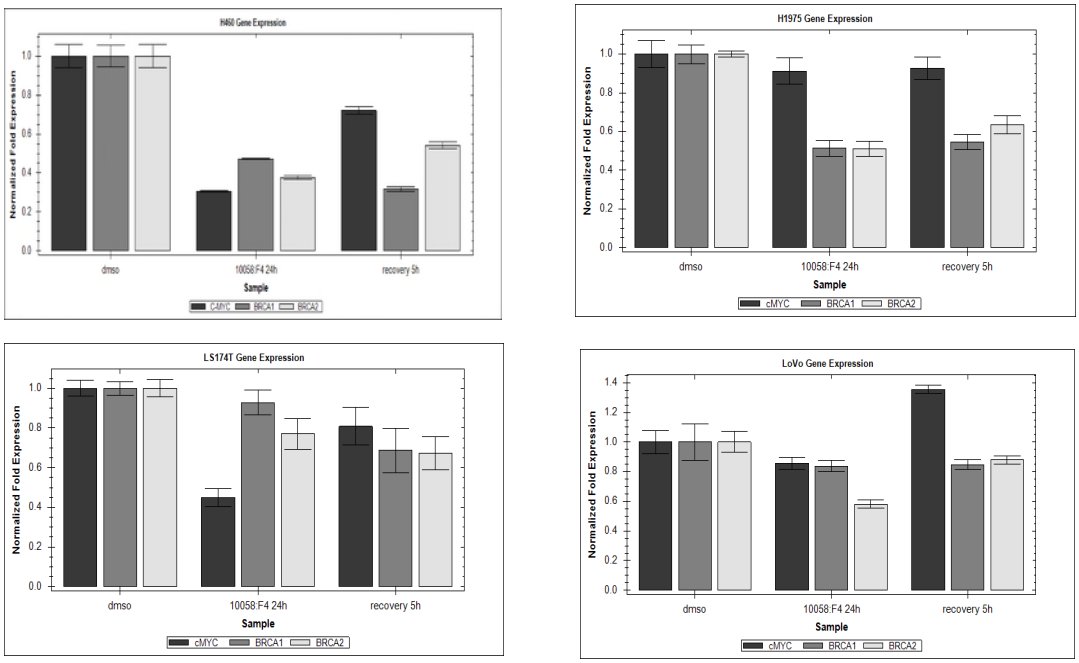


Figure S.I. 3: Characterisation of cell lines used in CCA before and after *10058:F4* treatment. Total Proteins for Western blot analysis and total RNAs for qReal Time PCR analysis were extracted after the cells were treated with DMSO for 24h (*dmso lane*); treated with 60µM 10058:F4 for 24h (*10058:F4 24h lane*) and were recovered in complete medium for 5h post 10058:F4 treatment (*recovery 5h lane*). (**A.**) Western blot analysis of cMYC and β-Act as normaliser in H460, H1975, LS174T and LoVo. (**B.**) qReal Time PCR in H460, H1975, LS174T and LoVo on *c-MYC* (dark grey bar), *BRCA1* (grey bar), *BRCA2* (light grey bar)*; GUSB* was used as reference gene.

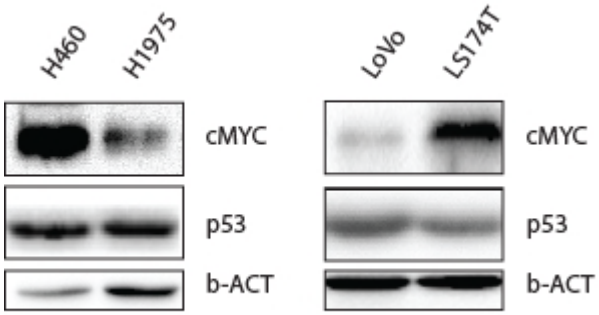


Figure S.I. 4: Western blot analysis on cMYC, p53 and β-Act as normaliser in H460, H1975, LoVo and LS174T.

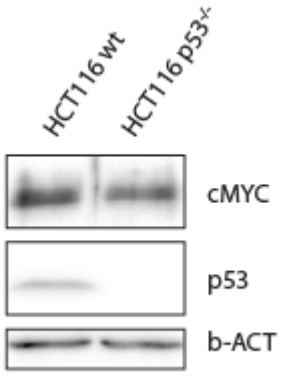


Figure S.I. 5: Western blot analysis on cMYC, p53 and β-Act as normaliser in HCT116^{wt} and HCT116$^{p53-/-}$.

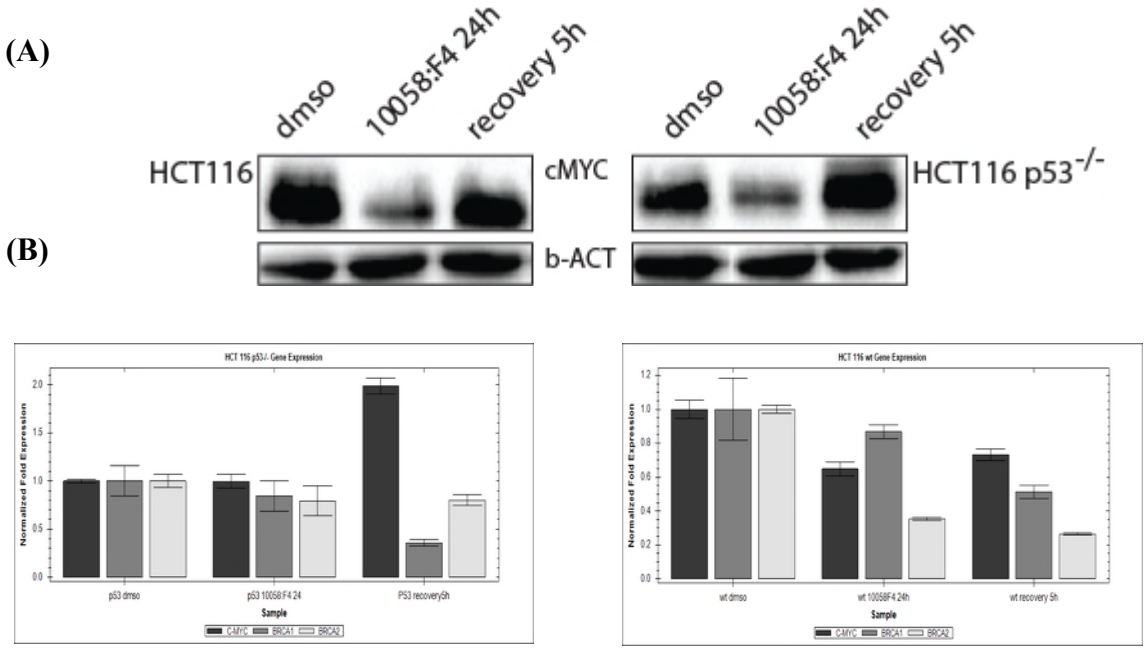


Figure S.I. 6: Characterisation of HCT116^{wt} and HCT116$^{p53-/-}$ after 10058:F4 treatment. Total proteins for Western blot analysis and total RNAs for qReal Time PCR analysis were extracted after the cells were treated with DMSO for 24h (*dmso lane*); treated with 60µM 10058:F4 for 24h (*10058:F4 24h lane*) and recovered in complete medium for 5h post 10058:F4 treatment (*recovery 5h lane*) (**A.**) Western blot analysis on cMYC, and b-ACT as normalizer. (**B.**) qReal Time PCR on *c-MYC (*dark grey bar), *BRCA1* (grey bar), *BRCA2* (light grey bar)*; GUSB* was used as reference gene.

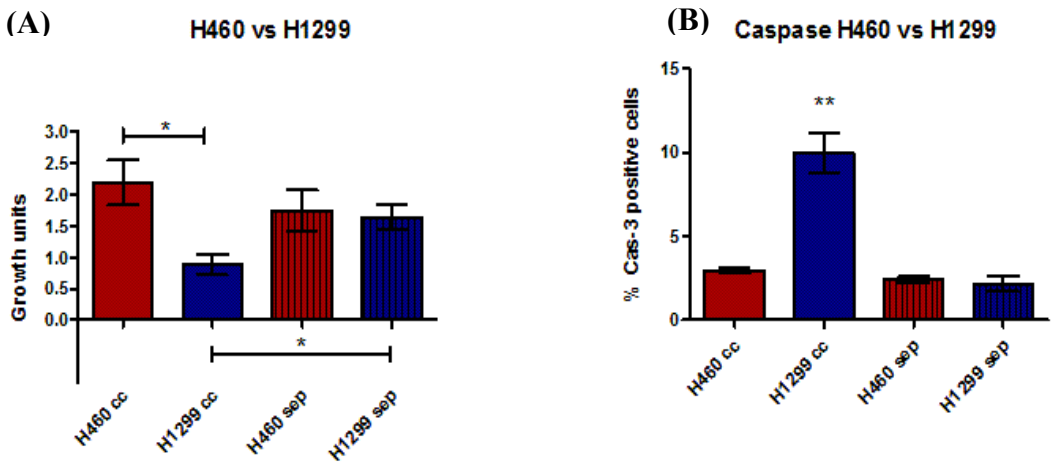


Figure S.I. 7: CCA between H1299 and H460. H460 wins the competition; H460 shows higher cMYC expression level and presence of p53 protein; H1299 exhibits a lower cMYC protein level than H460 and absence of p53. (**A**) H1299 shows the profile of a loser cell; growth after 5h of culture; each assay was repeated 3 times and counted twice. Its growth in co-culture is lower than H460 in the same condition and lower than the two populations in separate conditions. (**B**) Cas-3 profile confirms H1299 loser behaviour. Each IF was repeated 4 times and 4 fields of each slides were analysed, counted twice. ±SEM indicated. These data confirm that p53 LOF in loser cells does not interfere with CC.

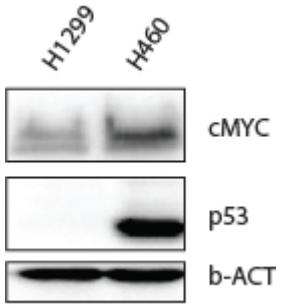


Figure S.I. 8: Western blot analysis on cMYC, p53 and β-Act as normaliser in H1299 and H460. H460 show higher cMYC expression and presence of p53 protein; H1299 exhibits lower cMYc protein level than H460 and absence of p53.

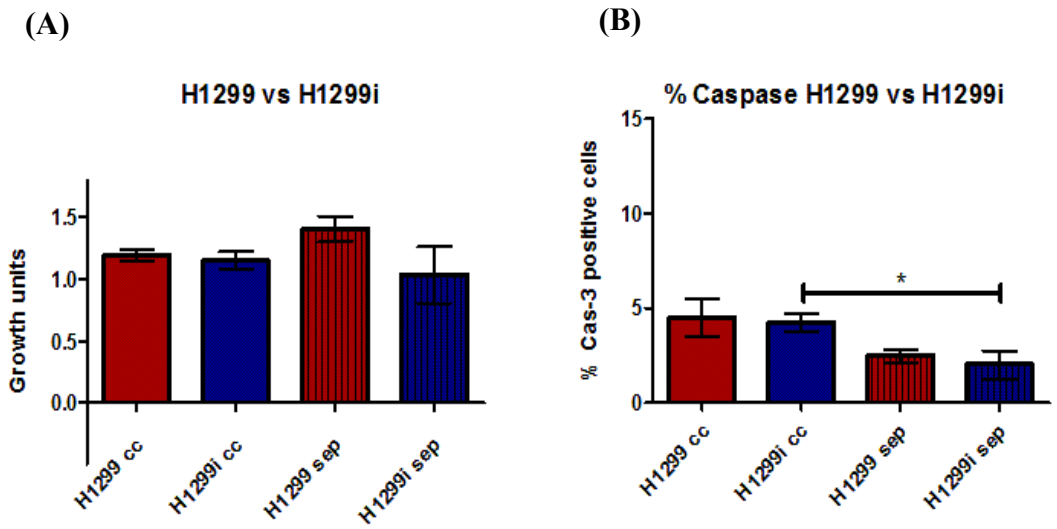


Figure S.I. 9: CCA between H1299 and H1299 treated with the 10058:F4 inhibitor. (**A**) H1299 and H1299i show similar growth when co-cultured (red and blue bar); their proliferation rate in co-culture is similar to that in separate conditions (striped red and striped blue bars). Growth after 5h of culture; each assay was repeated 4 times and counted twice. (**B**) Percentage of cells positive for cleaved caspase-3 is similar for co-cultured cell populations (red and blue bar) and cells in separate conditions (striped red and striped blue bars). No signs of CC are evident. Each IF was repeated 4 times and 4 fields of each slide were analysed, counted twice. ±SEM indicated. These data confirm that p53 *wild-type* function is needed in the potentially winner population for CC to occur.

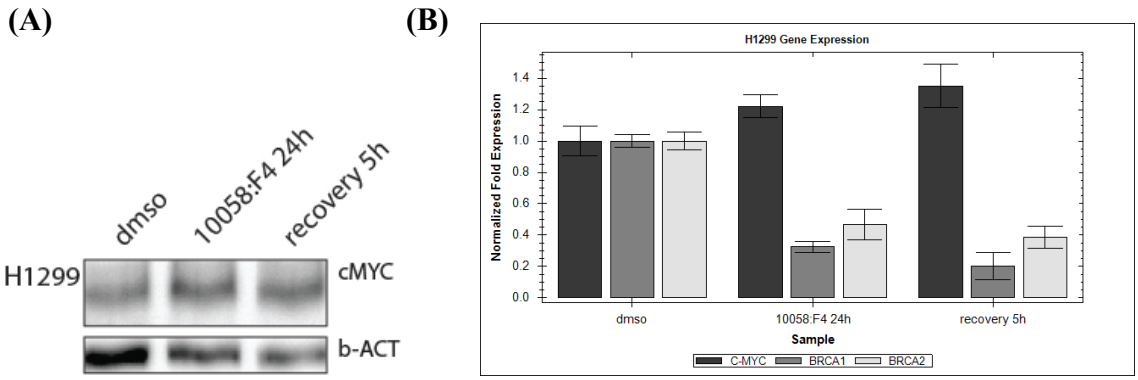


Figure S.I. 10: Characterisation of H1299 after 10058:F4 treatment. Total Proteins for Western blot analysis and total RNAs for qReal Time PCR analysis were extracted after the cells were treated with DMSO for 24h (*dmso lane*); treated with 60µM 10058:F4 for 24h (*10058:F4 24h lane*) and were recovered in complete medium for 5h post 10058:F4 treatment (*recovery 5h lane*). (**A.**) Western blot analysis on cMYC and β-Act as normaliser (**B.**) qReal Time PCR on *c-MYC* (dark grey bar), *BRCA1* (grey bar), *BRCA2* (light grey bar)*; GUSB* was used as reference gene.

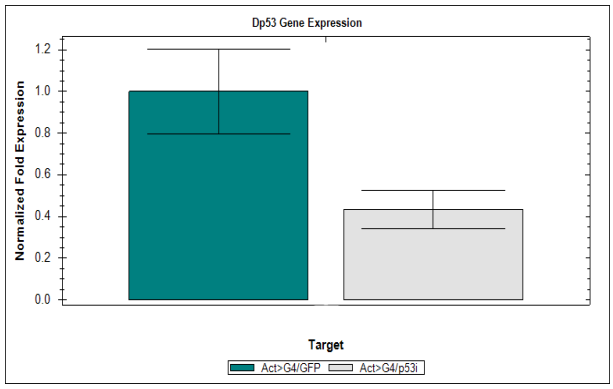


Figure S.I. 11: Characterisation of dp53 RNA interference on dp53 gene transcript. The relative quantity of p53 mRNA was assessed by qReal Time PCR in L3 larvae bearing a p53 RNAi construct under the control of the act5c-Gal4 driver. mRNA from L3 larvae with a UAS-GFP cassette under the control of the same driver was used as a control. *ACT5C* was used as reference gene.

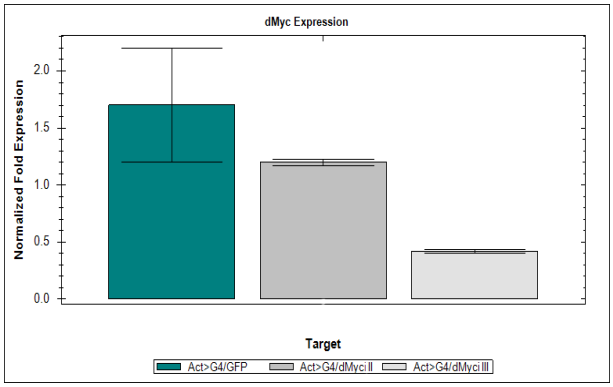


Figure S.I. 12: Characterisation of dmyc RNA interference constructs on chromosome II and III. The relative quantity of dmyc mRNA was assessed by qReal Time PCR in L2 (for the III chromosome construct) and L3 (for the II chromosome construct) larvae bearing different dmyc RNAi constructs under the control of the act5c-Gal4 driver. mRNA from L3 larvae with a UAS-GFP cassette under the control of the same driver was used as a control. *ACT5C* was used as reference gene.

Chapter 6

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