

The flower code and cancer development

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Abstract It has been postulated that the preliminary steps of cancer known as “cancerization field” could be mediated by a competitive mechanism among mutated and wild-type cells. Cell competition is a process of selection among populations of cells with different fitness: the best adapted cells (winners) survive and proliferate in the tissue at the expense of the less well adapted cells (losers), and these loser cells are eliminated from the tissue by apoptosis. However, the molecular mechanisms mediating this process and the genes involved are still unknown. A mechanism of cell-to-cell communication during cell competition known as the “flower code” has been recently proposed to distinguish loser from winner cells: *fwe*^{ubi} isoform is expressed ubiquitously in the imaginal disc while *fwe*^{lose} isoforms are expressed specifically during cell competition in the cells to be eliminated. Cell competition has been postulated to have implications in development, tissue homeostasis, regeneration and tumour development; the process of cell competition does not affect the total cell number and organ morphology is maintained because winner cells compensate for the loss. A role of cell competition

as the mechanism occurring during initial stages of tumour formation is currently under study.

Keywords Cell competition · *fwe* code · Cancer · *Drosophila*

Cell competition [1] is a process of selection among populations of cells with different proliferation rates or fitness [2]; during cell competition, the best adapted cells are favoured to continue and proliferate in the tissue while the less well adapted cells are eliminated from the tissue by apoptosis [2]. This process is phenotypically silent, as the total cell number and organ morphology is maintained because winner cells compensate for the loss [3]. Cell competition has been postulated to have implications in development, tissue homeostasis, regeneration and tumour development [4, 5]. However, the molecular mechanisms mediating this process and the genes involved are still unknown. Recent studies suggest that cell competition has a role as the mechanism occurring during initial stages of tumour formation [6]. It has been postulated that the preliminary steps of cancer known as “cancerization field” could be mediated by a competitive mechanism among mutated and wild-type (WT) cells [2]. The “flower code” has been recently proposed as a system to distinguish loser from winner cells [7]: *fwe*^{ubi} isoform is expressed ubiquitously in the imaginal disc while both *fwe*^{lose} isoforms are expressed specifically during cell competition in the cells to be eliminated. In this review we will assess the latest advances in cell competition and try to answer the question: does cell competition have a relevant role during cancer development?

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What is cell competition?

Cell competition was described in 1975 in *Drosophila* as a process that occurs among cells with different mitotic rates [1]. Heterozygous mutant cells for a ribosomal protein, so called “Minutes” ($M/+$), although viable, have a slower mitotic rate than WT cells. $M/+$ animals develop normally and do not have any morphological defects, however, when $M/+$ cells are confronted with WT cells in the imaginal disc of *Drosophila*, $M/+$ cells are eliminated by apoptosis [2]. Minutes were the original system to model cell competition; however, several other mutations have been related to competitive interaction processes: also mutations in other genes such as *lgl*, *dlg* or *scrib* behave similar to Minutes and induce cell competition [5, 8–14]. In those cases, the loser cells are the ones carrying the mutations. As a result of competition, *lgl*, *dlg* or *scrib* mutant cells are eliminated by apoptosis induced by surrounding WT cells.

In all the cases described above, WT cells are the winners and mutant cells are the losers. However, in 2004 a novel system of cell competition was described: Cell competition could also be induced by mutations in genes that provide an advantage to the cells over the WT tissue, thereby generating winners, i.e., increasing the activity of the protooncogene *Myc* [3, 15]. Cells that gain *Myc* activity can induce apoptosis in the neighbouring WT cells. Such *Myc* overexpressing cells grow at the expense of the neighbouring WT counterparts that in this situation are the “losers”. This phenomenon where the mutated cell acquires an advantage is known as supercompetition [3] and resembles a possible scenario where pretumoral cells will expand in a WT organism.

It is known that several mutations in different genes are necessary to generate a tumour [16], a minimum of 3–12 mutations depending on the cell type and tumour. Taking these observations together, it seems obvious to consider supercompetition as a possible mechanism during the initial steps of tumour formation, facilitating a process of field cancerization and increasing the possibility of further mutations that generate a tumour.

Cancer development is a challenging field that stimulates research in laboratories around the world; much effort is dedicated to the study of tumoral growth, but still little is known about the initial stages of tumour formation. A proposed mechanism for initial tumour progression is based on the “cancerization field” concept [6].

If a WT tissue is exposed to a mutagen that induces changes in an oncogene or tumour suppressor gene, one of these mutated cells could grow and colonize a region of the tissue by supercompetition. However, these cells do not induce any morphological defects and are therefore undetectable to the pathologist’s eye. If there is a novel exposure to the mutagen or the exposure is maintained, one of the cells in this patch could suffer further mutations and become malignant (Fig. 1). The larger this precancerous clone of cells is, the more probable a second and third

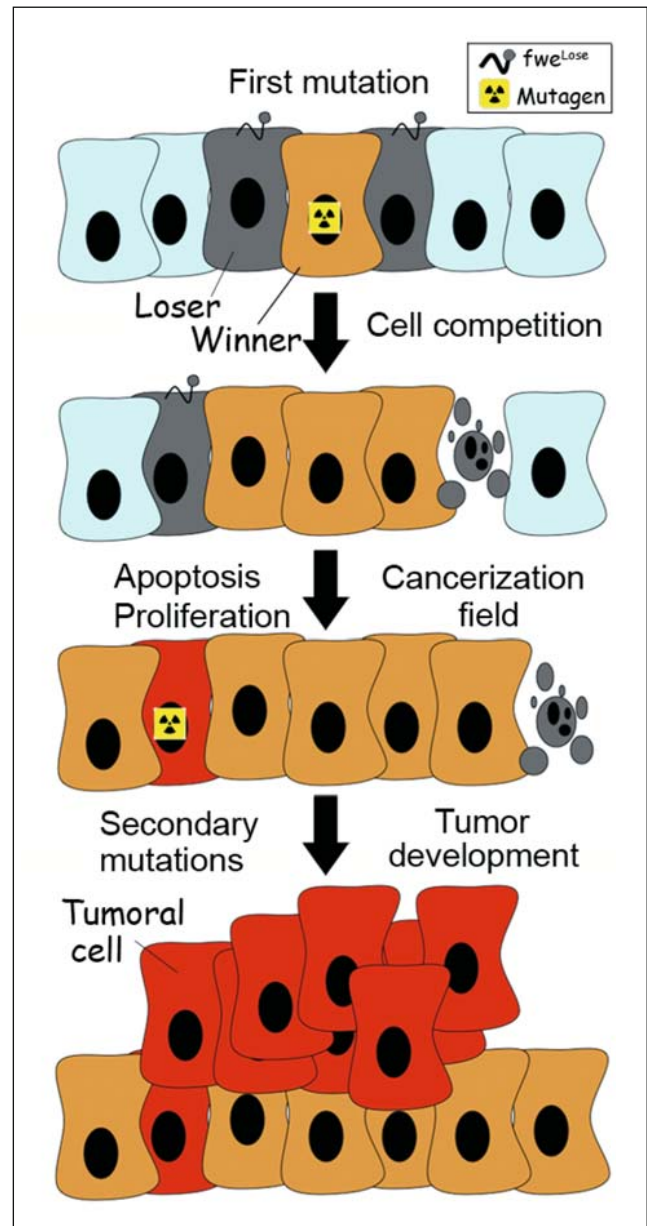


Fig. 1 Schematic representation of the early stem in tumorigenesis. Wild type epithelial cells express fwe^{ubi} normally in the tissue, after exposition to a mutagen, genetic changes occur in tumor suppressors or oncogenes that convert the WT cell in a supercompetitor. As a result, cell competition process is initiated and fwe isoforms differentiate winners (orange) from losers that express fwe^{Lose} isoforms (grey). Loser cells are eliminated from the tissue by apoptosis and winner cells can expand their territory generating a cancerization field. If further mutations occur affecting oncogenes and/or tumor suppressor genes in cells from this cancerization field a tumor could be generated

mutation could be, and therefore, the generation of tumoral cells. In this situation, it has been proposed that supercompetition could be a key event to regulate the expansion of the cancerization field and by this means, increasing the probability of acquiring further tumorigenic mutations [6]. Taking all this into account, supercompetition would be an

important process to be blocked in order to prevent tumour appearance and progression.

What pathologists identify as a “small tumour” already compromises one billion cells (10^9), which is the minimum detectable size. These tumours have already accumulated several mutations in either tumour suppressors or oncogenes and their potential malignancy is higher compared to the cells from the cancerization field with a single mutation. This “small tumour” would be only 10 cycling steps away from the maximal tumour size compatible with life (10^{12} cells) and would be already a fully tumoral mass [6].

So far, the mechanisms of these early events that take place in the process of field cancerization are unknown and the lack of specific markers makes difficult the detection of such pre-tumoral changes. It has been proposed that the expansion of pre-tumoral cells may not rely just on their intrinsic proliferative potential but could instead require a cell competition process to expand at the expense of surrounding WT cells [3, 5]; the challenge is how to explore this intriguing hypothesis.

Drosophila has emerged as an optimal system to model cell competition: the imaginal disc of the larvae is an epithelium where cell competition can be studied by generating clones of cells. The collection of tools available in *Drosophila* allows the induction of clonal expression systems [17], i.e., the FRT/Flp system [18], which makes possible the generation of cells with specific characteristics that grow and develop as clones in the imaginal disc. This system in combination with the expression system Gal4-UAS [19] permits the specific expression of RNAi constructs [20] or overexpression of genes in the cells of these clones. Both techniques together can be used to study the growth of genetically modified cells in contact with WT cells, thereby mimicking a cell competition scenario. The well known genetics of the fly and its fully sequenced genome facilitate the identification of candidate genes and their study. Moreover, the relevance of *Drosophila* to humans is perhaps best illustrated by the fact that more than 75% of the genes identified in human diseases have counterparts in *Drosophila* [21]. During the last decade, many fly models of tumoral growth have contributed to the identification of novel pathways mediating pathogenesis like Hippo-Warts-Salvador, Notch, Lgl-Scrib-dlg and EGFR-Pi3K among others [14, 22–27]. However, cell competition signalling pathways are still poorly understood [28–30].

Does cell competition occur in adult tissues?

So far all the results were referred to imaginal tissue in the *Drosophila* larva, however, experiments performed in the adult ovary of *Drosophila* demonstrate that cell competition can occur among stem cells competing in the same niche [31]. If a stem cell gains Myc, it is transformed into a winner compared to the neighbouring WT stem cells. This winner cell will then induce the elimination of the WT

stem cells from the niche and occupy the niche of the loser cells. In the case of the ovary, cell competition does not depend on apoptosis but forces the exclusion of the less competitive cell from the niche, favouring the expansion of the Myc stem cell. These experiments suggest that cell competition is an inter-cellular process with a role in development, stem cell niche maintenance and potentially in the initial steps of tumour formation.

Can we use this knowledge to design a therapy?

Early tumour detection is crucial for a better prognosis of cancer. If pre-tumoral fields could be identified early on, radio- and chemotherapy treatments might be more effective and patients' prognoses would be better. So far, preventive strategies such as healthy lifestyle, reduction of tobacco use, sun protection and hormonal treatment among others are used to reduce the probability of tumour appearance. However specific early identification of tumoral fields would improve the success of the treatments. On the other hand, if the key players of cell competition were identified, these early events could be detected and modulated, thereby delaying tumour progression. Moreover, metastatic cells also have to compete to colonize novel tissues in the organism and it has been postulated that the genes implicated in metastasis could be the same as the ones that are initially mutated in primary tumours [32], so cell competition could also be relevant for metastasis. These proteins would be ideal targets for specific treatments such as immunochemotherapy in order to delay the elimination of WT cells during tumour progression or target malignant mutated cells for elimination.

Specific markers in cell competition

It has been described that supercompetition can be modulated by two factors: (1) Compensating for the deficit of extracellular factors of loser cells by increasing endocytosis. This prevents the apoptosis and facilitates the survival of WT cells surrounded by Myc cells. As a consequence, winner cells cannot expand and the size of these Myc-patches is smaller [3]. (2) Inhibiting the apoptosis on the WT loser cells by p35 [2], Hid mutations [15] or dIAP1 [3], which equally allows the survival of WT cells and prevents the expansion of Myc winner cells. Since it was shown that apoptosis of the loser cells is necessary for the proliferation of Myc cells [3], this growth of the winner cells was defined as “apoptosis-dependent proliferation” [5].

Until now no cell competition specific gene was known, so it was not possible to design a strategy to modulate early tumoral steps. Whole genome screen analysis and functional validation by *in situ* hybridisation has emerged as a possibility to identify novel genes involved in cell competi-

tion [33]. Microarray techniques allowed the identification of candidate genes up- or down-regulated in cell competition in a whole genome search that could be crucial for this process [7]. RNAi techniques allowed the validation of these candidates *in vivo* and identified cell competition markers and/or regulators. The *Drosophila* S2 cell system has also contributed to come across the mechanisms implicated in cell competition [34]. A secreted molecule is thought to participate as a killing signal from the winners to induce apoptosis in the loser cells, however the role of this secreted signal during cell competition in flies or mammals has not been proven yet. The identity of this molecule is still unknown, however the TNF superfamily ligand in *Drosophila eiger* [35] can trigger JNK signalling and apoptosis [36] and has been proposed to play a role during tumour development in *Drosophila* [37–39]. Besides, the identification of this molecule and the signalling pathways implicated are still under study.

The flower code: extracellular tags that reveal the fitness of a cell to its neighbours

Recent research in cell competition has provided an early specific marker for loser cells and regulator of cell competition and supercompetition in the fly. The gene CG6151 is upregulated early during cell competition, before any other known event happens, suggesting that it could be an important player in Lose/Win decisions. Three different isoforms of CG6151 protein can be generated by an unknown mechanism, but all of them are associated to the membrane. In 2009, this gene was named Flower (*fwe*) due to its mutant “flowery” phenotype in the neuromuscular junction of *Drosophila larvae* [40]. Its product was proposed to be a calcium channel with a role in the nervous system synaptic endocytosis of *Drosophila*. In a different context, during cell competition, *fwe* isoforms are differentially expressed: a code composed by three different *fwe* isoforms can tag cells for their elimination or durability in the tissue [7]. The “*fwe* code” is composed by *fwe*^{ubi}, *fwe*^{LoseA} and *fwe*^{LoseB} isoforms generated by alternative splicing; *fwe*^{ubi} is ubiquitously expressed throughout the imaginal disc in all cells at similar levels during the entire development. *fwe*^{LoseA} and *fwe*^{LoseB} isoforms are upregulated specifically in “loser” cells during cell competition. Here the presence of the Lose isoforms mark cells as “losers” and force cells’ elimination from the tissue by apoptosis. This code implicates a cell-to-cell communication where a Lose-expressing cell is eliminated only if in contact with a WT (ubi-expressing) neighbouring cell. Expression of *fwe*^{LoseA} and *fwe*^{LoseB} isoforms throughout the entire animal does not increase apoptosis nor the elimination of cells in the organism. In addition, if a specific RNAi is expressed in all the cells to knock down *fwe* expression, WT loser cells do not undergo apoptosis and they are no longer eliminated from the imaginal disc; as a consequence, the expansion of the winner cells in this

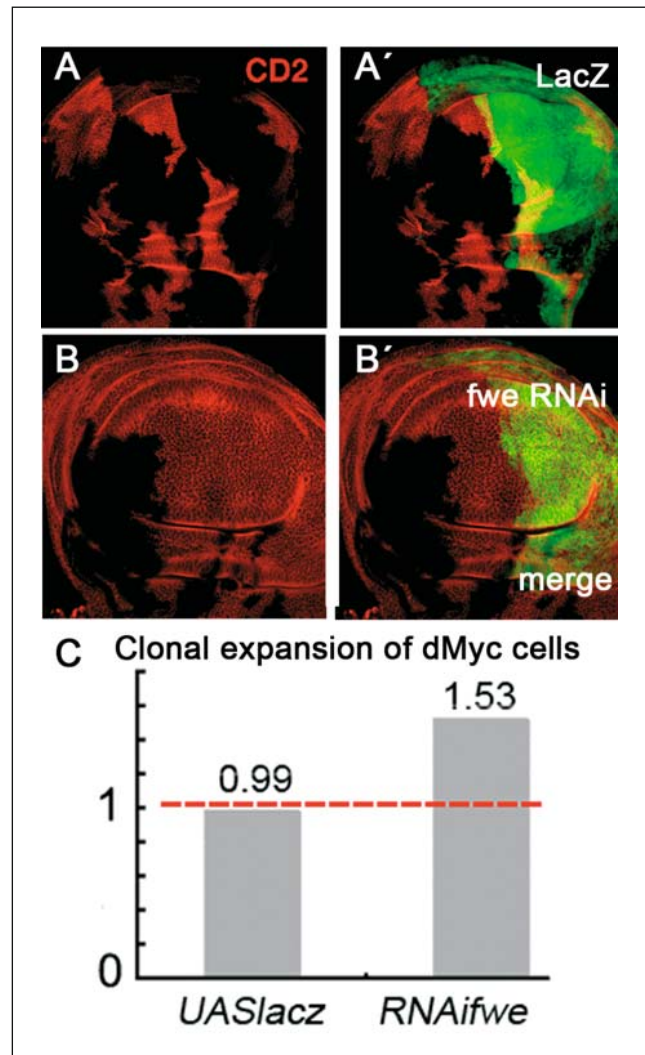


Fig. 2 Expansion of supercompetitor cells can be prevented knocking down *fwe*

Supercompetition assay performed in imaginal discs generating clones of Tub>Myc cells (black) in a Tub>CD2 background (red). Specific sequences under UAS enhancer were co-expressed with GFP (green) in the posterior compartment using an engrailed_Ga14 transgene. (A-A') Control experiment expressing UAS_LacZ. (B-B') knocking down total *fwe* levels can prevent the growth of Myc cells by cell competition. (C) Quantification of Myc cells area in the anterior compartment vs Myc cells area in the posterior compartment expressing LacZ or *fwe* RNAi.

tissue is reduced (Fig. 2). This code may rely on a threshold compared among cells to define the losers and the winners. In this way, the lack of *fwe* in a clone of cells surrounded by WT *fwe*^{ubi} expressing cells also promotes the elimination of the mutant cells identified as losers. Due to this role, the gene was also called *flower* after the so-called “Flower war” between the Aztecs and their neighbours [41]. This was a peculiar type of ancient war where, likewise, losers were not killed immediately, but rather captured, marked as “losers” with blue paint and eventually sacrificed later during an independent ritual [7, 42].

Flower is the first specific early marker and regulator of cell competition. Different isoforms can precisely mark loser and winner cells early in cell competition and its modulation can prevent the elimination of loser and Myc+ cell progression. The mouse orthologues are currently under study, but several isoforms have also been found and their expression is regulated during cell competition on mouse embryo, similarly to *Drosophila*. However, the eventual death of the loser cells depends on other signals, mainly the balance in the levels of two opposing secreted signals: (1) a protective signal encoded by *dSparc* and (2) an unknown killing signal [34, 43].

In the future, it will be important to explore the potential of treatments targeted to *fwe^{Loss}* isoforms to block the elimination of WT loser cells and thereby to stop the progression of pretumoral cells in mammals. If this was possible, early detection and treatments would open new avenues for cancer treatment, preventing the growth of pretumoral fields at a preclinical stage. To achieve these goals, further studies will be necessary to characterise the role of *fwe* in mammals and in tumour progression but also during development, regeneration and stem cell turnover.

Conflict of interest The authors declare that they have no conflicts of interest relating to the publication of this manuscript.

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