Abstract

Genetic analyses in Drosophila epithelia have suggested that the phenomenon of “cell competition” could participate in organ homeostasis. It has been speculated that competition between different cell populations within a growing organ might play a role as either tumor promoter or tumor suppressor, depending on the cellular context. The evolutionarily conserved Hippo (Hpo) signaling pathway regulates organ size and prevents hyperplastic disease from flies to humans by restricting the activity of the transcriptional cofactor Yorkie (yki). Recent data indicate also that mutations in several Hpo pathway members provide cells with a competitive advantage by unknown mechanisms. Here we provide insight into the mechanism by which the Hpo pathway is linked to cell competition, by identifying dMyc as a target gene of the Hpo pathway, transcriptionally upregulated by the activity of Yki with different binding partners. We show that the cell-autonomous upregulation of dMyc is required for the supercompetitive behavior of Yki-expressing cells and Hpo pathway mutant cells, whereas the relative levels of dMyc between Hpo pathway mutant cells and wild-type neighboring cells are critical for determining whether cell competition promotes a tumor-suppressing or tumor-inducing behavior. All together, these data provide a paradigmatic example of cooperation between tumor suppressor genes and oncogenes in tumorigenesis and suggest a dual role for cell competition during tumor progression depending on the output of the genetic interactions occurring between confronted cells.

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Introduction

Growth regulation requires the fine tuning between the rate of cell death and cell proliferation in developing organs. Studies in Drosophila have revealed that somatic cells within a growing epithelium compete with one another for contribution to the adult organ and this phenomenon, known as “cell competition” [1], is possibly conserved among organisms, for a review [2]. Cell competition was discovered several decades ago comparing the clonal growth parameters of Drosophila wild type cells (+/+ ) and slow-dividing Minute/+ cells [1]. From those analyses and recent data [3], it has been concluded that the contact between wild type and slow-growing cells, in genetic mosaics, favors the positive selection and clonal expansion of faster cells (winners) at the expense of slow-dividing ones (losers), although eventually the final number of cells in the organs is unaffected [3]. The biological function of cell competition remains unclear but it is thought to contribute to tissue homeostasis by coordinating the rate of cell proliferation and cell death [4,5]. One of the best examples illustrating cell competition was obtained from the analysis of Drosophila myc [4,5], opening to the speculation that this phenomenon might play a role in tumorigenesis [2,6], however the basis of cell competition in tumorous situations has just begun to be investigated [7]. myc is an evolutionarily conserved proto-oncogene associated with different cellular processes, including cell cycle progression, cell growth and apoptosis [8–11]. The function of dMyc protein is both necessary and sufficient to control rRNA synthesis and ribosome biogenesis [12]. In Drosophila, cells carrying hypomorphic alleles of dmyc are viable in a homotypic context, but they are outcompeted and excluded from the epithelium when
surrounded by wild type cells [5]. By contrast, dmyc overexpressing cells become “supercompetitors” able to kill wild type surrounding cells [4,5]. Remarkably, DMYC upregulation is related with many types of human cancers [13] and it favors the clonal expansion of cells carrying additional oncogenic mutations [14,15].

During the last years, the Hippo (Hpo) tumor suppressor pathway has emerged as a safeguard system restricting organ growth and preventing hyperplastic disease in metazoans [16,17]. Mutations in several members of this pathway have been associated with tumor formation both in Drosophila and in humans [18]. It has also been reported that mutations in many members of the Hpo pathway can rescue the viability of heterozygous M/+ cells in genetic mosaics [19], suggesting that these mutant cells behave as “supercompetitors”. Therefore the detailed analysis of Hpo pathway members appears to be an attractive model in which to evaluate the relationship between cell competition and tumor growth, as well as the molecular mechanisms required for this crosstalk. Hpo, Salvador (Sav) and Warts (Wts) constitute the core of the Hpo pathway that regulates by phosphorylation the downstream transcriptional co-activator Yorkie (Yki) [18,20]. The hyperphosphorylated form of Yki is retained in the cytoplasm [21,22], thereby preventing the expression of several target genes involved in cell proliferation control ([Cyclin E, E2F1, bantam miRNA] [16,23–25], cell death ([dIAP1]) [16] and cell signaling regulation ([dally and dally-like]) [26]. It has been demonstrated that Yki regulates its target genes by binding to Scalloped (Sd), a TEAD/TEF family transcription factor [27–30]. In addition, recent data indicate that Yki is also able to bind to the homeoprotein Homothorax (Hth) forming a complex which regulates the transcription of bantam in the eye disc [31]. The atypical cadherins Fat (Ft) [26,32–37] and Dachsous (Ds) [20,26,33,38], as well as the FERM-domain proteins Expanded (Ex) and Merlin (Mer) [39], have also been implicated in the pathway as upstream components. Although their biochemical functions are still uncertain, it is assumed that they converge on constraints compensating for excessive proliferation of the entire organ or toxicity caused by high and constant levels of Yki. Altogether, these results confirm the previously suggested supercompetitive properties of the Hpo pathway mutant clones [19] by revealing their ability to overgrow and eliminate surrounding wild type cells.

dmyc is a Hpo pathway target gene regulated by the activity of Yki

It is well documented that the confrontion of different levels of dMYC protein between two populations of cells either in vivo [4,5] or in cell culture [44] can trigger cell competition, however the molecular mechanism by which this occurs is unknown. In addition, MYC family oncogenes are frequently overexpressed in human cancers and it contributes to tumor progression of LAP-expressing cells (mammalian orthologue of yki) [17]. We have previously shown that a transcriptional activation of dmyc occurs in ft mutant tissues and that ft clones fail to grow in a dmyc hypomorphic background [45], indicating a possible regulation of this oncogene by the Hpo pathway. Moreover, the expression pattern of DMYC is complementary to that of Ds in the wing imaginal disc (Figure 2A), suggesting a possible functional interaction. To validate this hypothesis, we analyzed DMyc from being eliminated in a competitive background. Finally, we show that the relative levels of DMyc protein between neighboring cells are critical in order to define the role of cell competition during tumor progression.
expression in mutant clones for several members of the Hpo pathway and in yki over cells by immunofluorescence. Noticeably, we found that dMyc was upregulated in a cell-autonomous manner in yki over clones throughout the wing disc (Figure 2B and Figure S3), with the weakest activation in the lateral regions, and in a subset of clones mutant for several Hpo pathway members (Figure 2C–2F). These differences in dMyc activation between yki over clones and clones mutant for other members of the Hpo signaling pathway might be due to additional levels of regulation of the Hpo cascade operating on upstream members. According to our previous observations, we would predict a repression of dMyc upon Hpo pathway hyperactivation. To investigate this hypothesis, we expressed Hpo in the spalt expression domain of the developing wing disc. Since Hpo overexpressing cells die massively by apoptosis during development [25], we coexpressed the anti-apoptotic factor p35. As expected, cells coexpressing Hpo and p35 show reduced levels of dMyc with respect to the control (Figure S4A) in both late (Figure S4B) and early (Figure S4C) wing discs. Thus dMyc levels can be regulated by the Hpo pathway activity.

dmyc is transcriptionally regulated by Yki

dmyc was observed upregulated in RT-PCRs performed on ft mutant imaginal discs [45], suggesting that it could be a transcriptional target of the Hpo pathway. In order to investigate this, we first performed an in situ hybridization in Drosophila wing discs expressing yki under the control of the decapentaplegic (dpp) promoter. As expected, dmyc transcript is detectable in the dpp
domain both in yki and control dmyc-expressing discs (Figure 3A). No signal within the dpp domain was detected in dpp>Gal4 control discs (not shown). We were able to reproduce these data using a dmyc>lacZ line [46] which recapitulates accurately the dmyc pattern throughout the wing disc during development [7, 47]. As can be seen in Figure 3B, the βGal expression is increased in the dpp domain upon yki expression, indicating that Yki acts upon dmyc transcription. This result was supported using clonal analysis, both in yki<sup>trans</sup> cells, as shown in Figure 3C, and in cells mutant for ft (Figure S5). Altogether, these data demonstrate the ability of the Hpo pathway to regulate dmyc transcription in the imaginal wing disc.

Yki transcriptional activity depends on the formation of tissue-specific complexes with different partners such as Scalloped and Homothorax [27–31]. In order to study the contribution of Sd to dmyc upregulation by Yki in the wing disc, we generated yki<sup>trans</sup> clones coexpressing either a UAS-sd or a UAS-sd-RNAi construct (see Figure S6A for validation). As can be seen in Figure 3D, sd<sup>trans</sup>; yki<sup>trans</sup> clones overgrew relative to yki<sup>trans</sup> clones (compare with Figure 2B, 68% increase on average, n = 27, P < 0.005) confirming previous data [29], but we were not able to detect significant differences in dMyc protein levels compared to yki<sup>trans</sup> clones (n = 22, P = 0.43). As expected, control sd<sup>trans</sup> clones did not overgrow and did not deregulate dMyc (Figure 3E), demonstrating that Yki is required for dMyc upregulation. We were not able to recover sd-RNAi; yki<sup>trans</sup> clones in the wing pouch region, but clones generated in other territories of the wing disc, although large, did not upregulate dMyc (Figure 3F), nor showed the same degree of hyperplasia as Yki expression alone (Figure 1B–1E). sd-RNAi control clones were very small and did not deregulate dMyc (not shown). These data indicate a key role for Sd in vivo in upregulating dMyc in yki<sup>trans</sup> clones, and in contributing to the yki<sup>trans</sup> tumorous phenotype.

Interestingly, examination of dmyc locus revealed the existence of several CATTCCA repeats in non-coding regions of the gene, which perfectly match the mammalian [48, 49] and Drosophila [28, 29] TEAD/TEF family transcription factor consensus binding motifs (mammalian orthologues of Scalloped). In addition, these putative binding motifs for Yki/Sd complexes are evolutionarily conserved in D. simulans (Figure 3G) and relatively close to the insertion point of P elements that recapitulate the endogenous expression of the gene (dm<sup>PL35</sup> LacZ [50, 51] and dm<sup>BG02383</sup> Gal4 insertions - http://flybase.org/reports/FBti0018138.html).

To test the significance of these sequences in dmyc regulation, we generated a dmyc-firefly reporter containing the putative responsive elements for Yki/Sd complexes (Figure 3H) and performed a transient dual luciferase assay in S2 cells. As can be seen in Figure 3I, the reporter was specifically activated upon Sd and Yki cotransfection but, unexpectedly, the transfection of Yki alone was able to activate the reporter as efficiently as the cotransfection Yki/Sd (Figure 3I). This result suggests that in presence of high levels of Yki alone, additional partners such as Hth [31] could bind it and co-regulate dmyc expression.

Indeed, complementarily to Yki/Sd complexes, Yki/Hth complexes seemed to play the same role in the presumptive thoracic region of the wing disc. Supporting this conclusion, hth-RNAi; yki<sup>trans</sup> clones down-regulated dMyc in the notum (30% reduction on average, n = 15, P < 0.05, Figure S6B, yellow arrows) and did not grow as tumors in that region. By contrast, they were

Figure 2. dmyc oncogene is regulated by the Hpo pathway. (A) Double staining with anti-dMyc (red) and anti-Ds (green) reveals that dMyc and Ds proteins show a complementary pattern of expression in the wing disc; dMyc is highly expressed in the wing pouch and less in the notum, while Ds localizes mainly in the hinge and pleura, where dMyc expression is the lowest. (B) dMyc staining of yw, hs-Flp; actFRTy FRTGal4, UAS-GFP/ UAS-yki Flp-Out clones (GFP<sup>+</sup>) show increased dMyc protein level. (C–F) dMyc protein levels are increased in d<sup>trans</sup>, ft<sup>trans</sup>, ex<sup>trans</sup> and wts<sup>trans</sup> LOF clones (arrows). Clones were induced by the Flp/FRT system and are marked by the lack of GFP (0xGFP). Clones were induced at 42–54 h AEL (C–F) or at 54–66 h AEL (B).

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undistinguishable from yki over clones in the wing pouch region (Figure S6B, white arrowhead), where Hth expression is almost undetectable (Figure S6C). Altogether, these latter results indicate that Sd and Hth play a role in Yki-induced tumorigenesis by regulating dmyc expression in the wing disc, with Sd playing a more critical role in the pouch and Hth acting in the presumptive thorax.

dMyc upregulation enhances cell proliferation of the Hpo pathway mutant cells in an autonomous manner

With the aim to investigate the cell-autonomous contribution of dMyc overexpression to yki over phenotypes, we first compared the size of yki over clones with that of yki over; dmyc-RNAi clones (Figure 4, see also Figure S7A, S7A’, and [7] for RNAi construct validation). As expected, dmyc-RNAi clones showed a reduced number of cells with respect to that observed in wild type clones (21% reduction on average, compare Figure 4B and 4B’ with Figure 4A and 4A’, P<0.01). The reduction in cell number displayed by the yki over; dmyc-RNAi clones with respect to the yki over clones was even more evident (43% reduction on average, compare Figure 4D and 4D’ with Figure 4C and 4C’, P<0.01), and this percentage raised up to 65% (n = 87, P<0.001) when these clones were induced earlier in development (42–54h AEL), indicating a strong cell-autonomous requirement of dMyc protein for the expansion of yki over clones. We also observed that the non-autonomous apoptosis induced by yki overexpression was reduced upon dmyc deprivation (32% on...
average, n = 28, P<0.01, Figure S7B). These data suggest that dMyc upregulation promotes cell proliferation of yki\textsuperscript{over} clones in an autonomous manner, and also promotes their competitive behavior.

To further characterize this proliferation-promoting effect of dMyc, we compared the clonal behavior of various mutations in members of the Hpo pathway grown in two different genetic backgrounds: a wild type context and a genetic background overexpressing dMyc under the control of a hedgehog promoter in the posterior (P) compartment of the wing disc. We found that ft, ex and ds mutant clones were consistently larger in those territories expressing uniform levels of dMyc than in the wild-type background (Figure 5 and Figure S8). It is however described that the overexpression of dMyc is able to autonomously increase apoptosis [8–11]. In fact, the wild type tissue expressing high amounts of dMyc tends to die and does not overgrow (see active Caspase 3 stainings in Figure 5A and 5D). Noticeably, the apoptosis mediated by dMyc overexpression seems to be extremely reduced inside ft and ex clones (Figure 5A and 5D) with respect to the wild type surrounding territories, likely due to the upregulation of antiapoptotic genes such as bcl2, a target of the Hpo pathway [53]. This extra copy of dmyc gene plus an extra copy of the gene under the control of a tub promoter ensures two-to-threefold increase of dmyc transcript [5]. This extra copy of dmyc is located in a removable cassette between the tub promoter and a Gal4 cDNA. Upon tub promoter excision, the Gal4 promoter drives Gal4 expression in the clones and, as a result, those cells express lower levels of dmyc relative to the background and are rapidly eliminated from the tissue by cell competition. Only few genes have so far been found whose overexpression rescues cell viability in this context [5]. The relative difference in dMyc levels between yki-expressing cells and the surrounding tub>dmyc cells was minimized in a competitive background compared to a wild type context (compare Figure S7C and S7C′ with Figure 2B). In this competitive background, yki\textsuperscript{over} clones showed a diminished ability to overgrow compared to a wild type background (44% reduction on average, compare Figure 6C and 6C′ and Figure 6B and 6B′, P<0.01). Besides the reduction in size, yki\textsuperscript{over} clones showed an important reduction in clone number both in discs (Figure 6C) and adult wings (compare Figure S7E to Figure S7D). Moreover, yki\textsuperscript{over} clones induced earlier in development (42–54h AEL) were never recovered at the end of larval development (not shown). These data indicate that the competitive properties of yki\textsuperscript{over} cells are extremely reduced when they are surrounded by cells expressing very high amounts of dMyc.

We then performed the same competition assay as before while reducing dmyc activity inside the clones. We used the pupal lethal dmyc\textsuperscript{PL35} allele [49] and, taking advantage of dmyc locus association to chromosome X, we were able to analyze both female (heterozygous condition, the expression of dmyc is halved) and male (hemizygous condition, the expression of dmyc is completely removed) larvae. In dmyc\textsuperscript{PL35}+/+, tub>dmyc females, yki\textsuperscript{over} clones were smaller than those described in the previous assay (28% reduction on average, compare Figure 6D and 6D′ to Figure 6C and 6C′, P<0.05), whereas they were completely outcompeted by 48h after the heat shock in males (not shown). Since it has been observed that a dmyc\textsuperscript{PL35} heterozygous condition does not impair cell growth or proliferation rate [49], our results reveal an
important role for dmyc-induced cell competition in controlling the clonal expansion of yki

expression alone is not sufficient to prevent the elimination of yki mutants

yki LOF clones generated in a wild type background are not able to grow [16,25] and the ectopic expression of the antiapoptotic proteins dIAP1 [25] or p35 (Figure S10A) poorly rescues their viability, whereas a Minute background [33] or bantam overexpression within yki clones has been shown to partially rescue their growth [25]. Since our results have indicated that dmyc participates in tumor growth of the Hpo pathway mutant cells, we therefore analyzed if the expression of dMyc was sufficient to prevent the death of yki mutant cells. The overexpression of dMyc failed to rescue the viability of yki/

What is the contribution of dMyc to the Hpo pathway mutant phenotypes?

We found that dMyc upregulation is a common feature of Hpo pathway mutant cells. Since dmyc has been repeatedly associated with tumor progression and cell competition, we analyzed its role in the clonal expansion of Hpo pathway mutant cells. We observed that the reduction of dMyc expression restricts the ability of Hpo pathway mutant cells to proliferate (Figure 4), whereas its uniform overexpression strongly promotes their proliferation (Figure 5).
Figure 6. yki\textsuperscript{pro} clonal expansion is restrained by dmyc-induced cell competition. (A–D') Cell competition assay shows that, while wild type clones are outcompeted in this genetic background [5], the clonal expansion of yki\textsuperscript{pro} cells is partially restrained. Clones were induced at 60–84 h AEL and allowed to grow until 120 h AEL. Clones are GFP\textsuperscript{+}, nuclei are counterstained with DAPI. (A–A') wild type control clones generated through a tub\textsuperscript{+} tub–GFP \textit{UAS-GFP} \textit{Gal4} system. (B–B') yki\textsuperscript{pro} clones generated with the same system as the wild type control. (C–C') yki\textsuperscript{pro} clones in a tub\textsuperscript{+}dmyc background are smaller than in a wild type background. P<0.01. (D–D') dmyc\textsuperscript{E12} / +; tub–yki\textsuperscript{pro} clones in a tub–dmyc background are smaller than in C (P<0.01), confirming that the relative dMyc levels outside vs inside the clones affect the competitive ability of yki\textsuperscript{pro} cells. (A’–D’) Histograms showing the number of cells/clone of the genotypes indicated in A–D. In C and D discs are larger due to the overall increase in body size of tub\textsuperscript{>dmyc} individuals [59] SEM = Standard Error of the Mean.

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Furthermore, while dMyc-expressing wild type cells surrounding mutant clones are rapidly eliminated by autonomous apoptosis, Hpo pathway mutant cells are able to take advantage of dMyc role in protein biosynthesis and cellular growth to divide rapidly. This is a clear example of functional cooperation between different genes in order to favor tumor progression, but it also indicates a specific role of dMyc in promoting the clonal expansion of Hpo pathway mutant cells. According to these data, we conclude that dMyc behaves as a growth-promoting factor which sustains the hyperplastic phenotype of Hpo pathway mutant cells. Importantly, this specific cooperation might be evolutionarily conserved, since \textit{c-myc} appears to be upregulated in a murine model of YAP-induced carcinoma [17].

Relative levels of dMyc in neighboring cells restrict/ promote clonal expansion of hyperplastic cells, likely through cell competition

It has been suggested that cell competition may be a mechanism potentially restricting the clonal expansion of tumor cells [7], but it might also help faster proliferation of transformed cells. Our data indicate that Hpo pathway mutant cells are able to use high levels of dMyc to proliferate rapidly (Figure 5), but in a competitive context, neighboring cells express high levels of dMyc, clonal expansion of yki\textsuperscript{pro} cells is restrained (Figure 6), therefore suggesting a tumor suppressor role for cell competition. Conversely, dMyc upregulation in yki\textsuperscript{pro} clones grown in a wild type background favors their clonal expansion promoting cell autonomous proliferation and also conferring the ability to outcompete surrounding cells in a non-autonomous manner. These findings suggest that the phenomenon of cell competition may play a dual role in tumor progression depending on the output of the genetic interactions occurring between adjacent cells.

In summary, we have shown a tumor-braking gene network in \textit{Drosophila} epithelia which tightly controls cell proliferation, apoptosis and cell competition \textit{via} the Hpo pathway and dMyc expression. Importantly, YAP deregulation has been reported in several types of human cancers [54–56], therefore the mechanism of clonal expansion of Hpo pathway mutant cells in \textit{Drosophila} might be relevant to understand tumor progression in mammals.

Materials and Methods

Genotypes and clonal analysis

The fly strains used in the present work were obtained by the Bloomington Stock Center and are described at http://flybase.bio.indiana.edu. The following strains were instead obtained by: \textit{ykiB5} (B Edgar); \textit{hs-Gal4/UAS-yki\textsuperscript{B5}}, \textit{actFRTy}\textit{FRTGal4} (P Gallant); \textit{yki\textsuperscript{B5} UAS-GFP} [B Edgar]; \textit{yki\textsuperscript{B5} dmyc\textsuperscript{B5} wts\textsuperscript{B5} X1} (I Rodriguez). The UAS-RNAi constructs for \textit{dmyc}, \textit{sd} and \textit{hh} were obtained from the VDRC. All experiments were carried out at 25°C unless otherwise indicated.

MARCM UAS-yki twin-spot clones were induced at different stages of development by a 35-minutes heat shock at 37°C and larvae of the following genotype were dissected at either 84–100 h AEL or 120 h AEL: \textit{yw}, \textit{hs-Flp}, \textit{ub\textsuperscript{+}}, \textit{UAS-GFP}; \textit{FRT42D}, \textit{ub\textsuperscript{+}}\textit{UAS-yki\textsuperscript{B5}+/FRT40A, UAS-GFP} [B Edgar]; \textit{yw}, \textit{FRT40A, dmyc\textsuperscript{B5} (+ Rodriguez)}. The UAS-RNAi constructs for \textit{dmyc, sd} and \textit{hh} were obtained from the VDRC.

All experiments were carried out at 25°C unless otherwise indicated.
The size of non-confluent clones was measured drawing each Z-stack of the confocal images using ImageJ software [http://rswebw.nih.gov/ij]. Afterwards the area of the clones was normalized dividing by the area of the wing pouch, considered as the territory encircled by the first outer folding of the wing. In Figure S1, the narrower window of clonal induction allowed us to compare clone size without size normalization respect to the wing pouch. Statistical analysis was performed with Microsoft Excel and R (www.r-project.org). Statistical significance was determined by two tailed Student’s t test and reported as the associated probability value (P).

Flp-Out clones were induced at 60h AEL by a 8-minutes heat shock at 37°C; imaginal discs of the following genotype were dissected at 120h AEL:

yw, hs-Flp, act5FRTy3; FRTGal4, UAS-GFP
yw, hs-Flp, UAS-dmycRNAi/+; act5FRTy3; FRTGal4, UAS-GFP/+ yw, hs-Flp, act5FRTy3; FRTGal4, UAS-GFP/UAS-yki
yw, hs-Flp, UAS-dmycRNAi/+; act5FRTy3; FRTGal4, UAS-GFP/UAS-yki.

yw, hs-Flp/w, dmyc>lacZ535; act5FRTy3; FRTGal4, UAS-GFP/UAS-yki.

Cell competition assays were performed at 72h AEL inducing a 40-minutes heat shock at 36°C. Larvae of the following genotype were dissected at 120h AEL:

yw, tubFRTy3; FRTGal4/hs-Flp, UAS-GFP/+ yw, tubFRTy3; FRTGal4/hs-Flp, UAS-GFP/+; UAS-yki/+ yw, tubFRTy3; FRTGal4/hs-Flp, UAS-GFP/+; UAS-yki/+ yw, dmyc; tubFRTy3; FRTGal4/hs-Flp, UAS-GFP/+; UAS-yki/++

MARCM yki clones overexpressing p35, dMyc or both were generated at 48–72h AEL by a 45-minutes heat shock at 37°C and larvae were dissected 48h later.

Immunofluorescence

Immunostainings were performed using standard protocols. The following primary antibodies were used: mouse anti-dMyc (1:5, P Gallant), mouse anti-En (1:50, DSHB), rabbit anti-active Caspase 3 (1:100, Cell Signaling Technology), rabbit anti-p53 (1:1000, Stratagene), rabbit anti-Ds (1:100, D Strutt), rabbit anti-Hth (1:100, A Salzberg,[57]), mouse anti-dIAP1 (1:100, B Hay) and rabbit anti-βGal (1:400, F Graziani). Anti-mouse and anti-rabbit Alexa Fluor 555 (1:200) (Molecular Probes) and anti-mouse and rabbit anti-βGal (1:400, F Graziani). Anti-mouse and anti-Hth (1:400, A Salzberg,[57]), mouse anti-dIAP1 (1:100, B Hay)

Luciferase transient expression assays

Drosophila S2 cells were grown at 25°C in Schneider medium (GIBCO) supplemented with 10% heat-inactivated FCS and 100 units of penicillin.

1189 base pairs located in the second intron of the dmyc sequence (Figure 3H) were subcloned into a pGL3-basic vector (Promega) and co-transfected with Sd and/or Yki-expressing pAc5.1/V5-HisB plasmids [20] using Effectene Qiagen Transfection Kit. The primers used for that purpose were:

5’ CACCGGTTAACAGTTTGCCTGCTTGC 3’
5’ GCACCTCTAGACCATGGAAATTGTGGC 3’.

The PCR product was first cloned in pCR 2.1 TOPO-TA (Sigma) and then subcloned in KpnI/XhoI sites of pGL3 Promoter vector. For luciferase transient expression assays, 2×10^5 cells were plated in 96-well dishes. Cells were harvested at 48 hours after transfection and luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega). Dual-Luciferase measurements were performed using a FLUOstar Optima luminoimeter (BMG Labtech) and normalized to the Renilla luciferase activity using pAct5C-seapancy as an internal control. All transient expression data reported in this paper represent the means from three parallel experiments, each performed in triplicate. Average relative luciferase activity was graphed and statistically analyzed by the Student’s t-test.

Supporting Information

Figure S1 yki over cells supercompetitive behavior is indeed visible at 48h after induction. (A,B) yw, hs-Flp, tub-Gal4, UAS-GFP, FRT42D, tub-Gal80/FRT42D, Ubi-Gal4 (A) and yw, hs-Flp, tub-Gal4, UAS-GFP, FRT42D, tub-Gal80/FRT42D, Ubi-Gal4, UAS-yki/+ (B) clones induced at 54–66h AEL and dissected 48h after the heat-shock. Wild type and yki over clones are GFP+ and twin clones are marked by the lack of GFP. Cell death is assayed by active Caspase 3 immunoreactivity in red. Note that cell death is almost absent in the wild type experiment (A) and marks wild type cells in the yki over expression (B). (C-F) Histograms showing the surface area of wild type and yki over clones and respective twins. (C,F) Wild type clones (C) and their twins (D) display the same size profile. (E) The size profile indicates that yki over clones are larger than wild type controls (C) as than their wild type twins (F) after only 48h of growth in the wing. SEM = Standard Error of the Mean. P<0.0001.

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Figure S2 Hpo pathway LOFs induce cell competition. (A,B) Activated Caspase 3 staining of yw, hs-Flp/+; fl; act5FRT40A/ Ubi-GFP, FRT40A discs in which mutant clones (0xGFP) were grown for 48 hours (48–96 in A and 72–120 in B); apoptotic cell death occurs mainly in wild type cells surrounding the mutant clones (arrowheads). (C,D) Activated Caspase 3 staining of yw, hs-Flp/+; act5FRT40A/ Ubi-GFP, FRT40A (C) and hs-Flp/+; actx1, FRT82B/ Ubi-GFP, FRT82B (D) discs in which mutant clones (0xGFP) were grown for a longer period (48–108 and 48–120 hours respectively); apoptotic death is visible in both wild type (D, arrowheads) and mutant (D, arrows) cells.

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Figure S3 dMyc upregulation in yki over clones is cell-autonomous. dMyc staining in yw, hs-Flp/+; act5FRT40A/ UAS-GFP/UAS-yki imaginal wing discs, yki over clones (GFP+, in green) express high levels of dMyc (in red) compared to the endogenous background. Z-section indicates that dMyc (in red) up-regulation is confined to yki-expressing cells (in green). Found at: doi:10.1371/journal.pgen.1001140.s003 (0.51 MB TIF)
Figure S4  Hpo overexpression reduces dMyc protein levels. (A) dMyc staining in w; sal>Gal4/+; UAS-p35/+ imaginal wing discs. (B–C) dMyc staining of late (B) and early (C) w; sal>Gal4/+; UAS-Hpo/+; UAS-p35/+ imaginal wing discs. p35 is shown in the green channel and dMyc in red. As can be observed in the Z-sections dMyc abundance is lower inside the sal domain. The position of Z-section is indicated by white bars in the surface view of the wing discs.

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Figure S5  dmyc is transcriptionally upregulated in ft mutant clones. BGal staining (red) of dmyc>–lacZoff/+; hs-Flp; J[+]Zoff, FRT40/UbaGFP/hs, imaginal wing discs. As can be observed, a robust activation of dmyc regulatory sequences is visible within the mutant clones (arrows). Larvae were dissected at 120h AEL.

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Figure S6  Hh is necessary for Yki-induced dMyc overexpression in the presumptive thoracic region of the wing disc. (A) Wing from a w; UAS-sd-RNAi/en>Gal4 individual. For UAS-sd-RNAi line validation, we induced the expression of the sd-RNAi construct in the posterior compartment of the wing by means of the engailed (en) promoter. As can be observed, the wing lacks the posterior compartment (green-colored in the insert). (B) dMyc staining in w; hs-Flp/+; UAS-hh-RNAi; UAS-yki/actFT; hthGal4, UAS-GFP imaginal wing discs. Note that mutant clones (GFP+) overgrow and overexpress dMyc in the wing pouch region (white arrowhead) and in the notum region (yellow arrows). (C) For UAS-hh-RNAi line validation, we stained for Hh [57] w; hs-Flp/+; UAS-hh-RNAi; actFT>yHthGal4, UAS-GFP/+ wing discs. Hh expression is lacking in the clone originated in the pleural region (arrow).

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Figure S7  dmyc is involved in the competitive ability of yki. (A) dMyc levels are strongly affected inside dmyc-RNAi; yki[+]over clones. (A’) A projection along the Z axis of the clone presented in Figure A is shown. The percentage of dMyc abundance reduction inside the mutant clones calculated as the abatement of fluorescence intensity (see Methods - Immunofluorescence) with respect to the mutant clones calculated as the abatement of fluorescence intensity (see Methods - Immunofluorescence) with respect to the wild type background. (B–E) dMyc overexpression strongly enhances the proliferative activity of ft mutant cells; mutant clones are larger in dMyc-expressing territories ( posterior compartment in C) than in a wild type background ( anterior compartment in B). SEM = Standard Error of the Mean. P<0.001.

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Figure S8  dMyc overexpression boosts proliferation in ds mutant cells. (A) ds LOF clones (0xGFP) generated in a background where posterior cells ectopically express dmyc under the control of the hh promoter (on the right). dMyc overexpression strongly enhances the proliferative activity of ds mutant cells; mutant clones are larger in dMyc-expressing territories ( posterior compartment in C) than in a wild type background ( anterior compartment in B). SEM = Standard Error of the Mean. P<0.001.

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Figure S9  dMyc overexpression boosts proliferation of Hpo pathway mutant cells also when wild type cells are protected from cell death. ft (A–C) and ex (D–F) LOF clones (0xGFP) generated in a background where posterior (P) cells ectopically coexpress dmyc and dAPI upon inhibition of cell

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Figure S10  dmyc fails to rescue yki LOF upon inhibition of cell death. Three types of yki LOF clones were induced through the MARCM system. In (A), yki mutant clones were generated while overexpressing the antiapoptotic protein p35 (in red). (B) Overexpression of dmyc fails to rescue yki mutant cells viability and Caspase 3 activation (red arrows). (C) The overexpression of p35 and dmyc together also fails to rescue yki mutant cells viability.

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