Interplay between Notch Signaling and Epigenetic Silencers in Cancer

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Abstract

Given its role in the development and self-renewal of many tissues, it is not surprising that a prominent role has recently been proposed for the Notch signal transduction pathway in tumor development. However, exactly how Notch hyper-activation promotes oncogenesis is poorly understood. Recent findings in Drosophila melanogaster have linked the Notch pathway to epigenetic silencing and the tumor suppressor gene Rb during tumorigenesis. Because aberrant epigenetic gene silencing contributes to the pathogenesis of most human cancers, these findings may provide a new focal point to understand how Notch is associated with cancers, and to help develop better selective cancer therapies. (Cancer Res 2006; 66(18): 8931-4)

Background

Cancer is a highly varied and complex disease considered until recently to be underpinned by genetic abnormalities. However, epigenetic alterations of gene expression now also seem to contribute to the pathogenesis of cancer. Thus, epigenetic processes are responsible for the inactivation of tumor suppressors and tumor-related genes in many cancer cells, particularly through abnormal histone modifications and aberrant DNA methylation. These epigenetic modifications permit heritable gene silencing that could, in turn, contribute to the initiation and progression of cancer, and to metastasis (1, 2). In contrast to genetic changes (i.e., mutations), the reversibility of epigenetic changes has led to an expansion of epigenetic therapies targeting histone deacetylases (HDAC) and DNA-demethylating agents (for reviews, see refs. 3, 4).

In normal cells, the epigenetic inheritance of transcription patterns (i.e., cellular memory) helps to maintain cell identity, as well as regulating senescence and stem cell renewal. For example, proteins of the PcG form a part of the memory machinery and they maintain transcriptional repression of genes and have been implicated in the maintenance of stem cell pluripotency and plasticity (see for example, ref. 5). In humans, the up-regulation of PcG genes has been associated with many types of invasive carcinomas, lymphomas, and leukemia (6, 7). To understand how cancer initiates, and to develop specific therapies, we must determine how these damaging epigenetic alterations arise in the precursor cells of cancer, and why specific genes are targeted for epigenetic silencing in such cells. To address these questions directly, animal models are required in which the role of different epigenetic silencing elements and transforming networks can be defined in vivo.

Despite the tradition of studying cancer in mouse models, the fruit fly, Drosophila melanogaster has recently emerged as a powerful model system for these purposes (8). Indeed, a genetic screen in the Drosophila eye revealed an unexpected collaboration between epigenetic silencing pathways and the Notch signaling pathway during tumorigenesis (9). The Notch signaling pathway is important in many biological processes as it governs binary cell fate decisions, patterning, cell proliferation and growth, apoptosis, differentiation, and migration. This developmental pathway was first identified in Drosophila before mammalian Notch receptors (NOTCH1-4) and ligands of the Delta (DLL-1, DLL-3, and DLL-4) and Serrate (JAGGED-1, and JAGGED-2) family were shown to act in mammalian development, organ self-renewal, and diseases such as cancer (10).

Aberrant activation of the NOTCH1 receptor is estimated to participate in 50% of all pediatric and in almost all of the most common subtypes of T cell acute lymphoblastic leukemias (11). Moreover, inappropriate activation of the NOTCH transduction pathways has also been associated with a wide range of solid tumors (12) and, in some contexts, Notch has been shown to function as a tumor suppressor gene; but how the deregulation of Notch pathways contributes to the pathogenesis of cancer in vivo remains unclear. Accordingly, it is imperative to identify the oncogenes and tumor suppressors that cooperate with Notch pathways to promote tumorigenesis. The recent demonstration that Notch and epigenetic silencing pathways cooperate to promote tumorigenesis is a first step in this direction (9). If this holds true for human cancers, these discoveries open the possibility to treat Notch-associated cancers with epigenetic therapies.

Key Findings

The early growth and patterning of the Drosophila eye depends on the autonomous and nonautonomous activity of the Notch signal transduction pathway (13). The Delta and Serrate ligands locally activate the Notch receptor along a dorsal/ventral organizer. Inappropriate inactivation of the Notch pathway or the expression of dominant-negative Notch receptors impairs eye growth, whereas constitutive activation of this pathway or ectopic ligand expression induces eye overgrowth. Given the increasing relevance of the Notch pathway in human cancers, we established a high-throughput forward genetic screen in the fly eye to search for “gain-of-expression” mutations that interact with the Notch pathway and that convert tissue overgrowths into tumors. For this, we exploited the “large eye” phenotype caused by overexpression of the Notch ligand Delta (14) coupled to the Gene Search (GS) transposon system to systematically generate gain-of-expression mutations (15). This P element–based GS transposon contains five tandem Gal4
Figure 1. Top, the proposed mechanism by which cancer is initiated by the Notch signaling pathway and the PcG-mediated cellular memory machinery. The hyperactivation of the Notch pathway initiates the repression of target genes, such as the Rbf gene. Subsequently, Pipsqueak (Psq) and/or Lola could bind to the repressed genes and enforce gene silencing via the recruitment of HDAC and histone lysine methyltransferases. Thereafter, binding of bromodomain and chromodomain proteins, such as Pc, to the methylated H3 lysine residues initiates chromatin condensation and “permanently” silences the Notch target genes. This would foment tumor growth and metastasis. Bottom panels, overexpression of the Notch ligand Delta (ey-Gal4 > Dl) or deregulation of Psq and Lola (data not shown) alone is insufficient to promote tumor formation. However, the coexpression of psq, lola, and Delta in the developing eye tissue induces the development of highly invasive tumors. Macroscopically visible metastases (red) can be found throughout the body of the fly (9).
binding sites (upstream activating sequences, UAS) capable of overexpression or misexpressing gene(s) laying on either side of the GS insertion. The GS lines were crossed with ey-Gal4 flies carrying a Delta transgene (UAS-Delta), which drives eye-specific expression of the UAS-linked genes throughout the proliferative phase of the imaginal eye disc (14).

This screen identified a GS line, that when coupled to Delta overexpression, produced massive overgrowths and tumors (9). Some of these mutant flies display macroscopically visible secondary eye tumors within the head, thorax, and abdomen, hence the line was named “eyeful” (Fig. 1). These secondary eye growths (metastases) have ragged borders and a disrupted basal membrane, and they invade their surrounding tissue (9). The eyeful GS-element causes the deregulation of two neighboring genes, longitudinal lacking (lola) and pipsqueak (psq; ref. 9). The lola gene encodes 25 alternatively spliced mRNAs that generate 19 different transcription factors. All of these transcription factors share a BTB domain (Broad complex, Tramtrack, Bric-a-brac, also known as POZ for Poxviruses zinc-finger), and all but one is spliced to unique exons encoding zinc finger motifs (16). The psq gene encodes four different proteins, three of which contain a BTB domain in the NH2 terminus. Furthermore, two of the BTB-containing isoforms and the isoform that lacks the BTB domain have four tandem copies of a conserved DNA-binding motif known as the Psq helix-turn-helix (HTH) motif (17, 18).

To determine whether the lola and psq genes contribute to the tumor phenotype, we introduced point mutations into the lola or psq genes. All psq knockouts induced on the eyeful chromosome prevented the eyeful GS line from producing eye tumors and metastases (9). In contrast, whereas all lola mutations reduced the size of the eye tumors, including a null allele of lola, metastases still developed (9). Thus, transcription of both genes is required for tumorigenesis, although Psq seems to be more important for tumor growth and metastasis.

Six of the psq mutations (missense, nonsense, small deletion, and splice) disrupt or completely delete the conserved BTB or the sequence-specific DNA-binding domains. Thus, protein-protein interactions via the BTB domain as well as DNA binding are likely to be involved in oncogenesis. The human oncogenic proteins, PLZF and BCL-6, associate with HDACs, corepressors, and PcG repressors through their BTB domain (ref. 19, and citations therein). Although a direct interaction of the BTB domain of Psq and/or Lola with HDACs and Polycomb repressors remains to be shown, Psq is present in a multiprotein complex that displays PcG repressor and HDAC activity (reviewed in ref. 20). The Psq-type HTH motif binds to the GAGAG sequence, which is present in many Polycomb-responsive elements of homeotic box (hox) genes, as well as at hundreds of other chromosomal sites. Thus, Psq and Lola could be required for sequence-specific targeting of Polycomb complexes to particular genes. Indeed, genetic studies have identified both Psq and Lola as members of the PcG family (9, 20). We therefore speculated that the deregulation of psq and lola might induce tumorigenesis through aberrant epigenetic silencing of genes that contribute to the uncontrolled growth of tumor cells.

Epigenetic processes alter the accessibility of transcriptional regulators to chromatin via the local or global modification of histones and DNA (20, 21). Covalent histone modifications include: lysine acetylation, lysine and arginine methylation, serine/threonine phosphorylation, ADP-ribosylation of glutamic acid, and sumoylation and ubiquitination of lysines. Because lysine (K) methylation of histone H3 is important in both fly and mammalian epigenetic control (21), we analyzed this modification with specific antibodies in mutant eye discs. We observed the loss or strong reduction of histone H3K4 trimethylation (H3K4me3) in the developing eye tissue from which the tumor arises. Interestingly, this epigenetic modification identifies open chromatin and transcriptional activation, and thus, the strong reduction in histone H3K4me3 signifies that the chromatin in the mutant tissue has been condensed or silenced. Strikingly, we found a small but significant reduction of this epigenetic marker in the overgrown eye tissues when only the Delta gene is overexpressed (9). Together, these results suggest that the Notch pathway controls normal tissue growth and tumorigenesis at least in part through epigenetic silencing.

The two H3 methyl tags associated with epigenetic gene silencing and chromatin condensation in flies and mammals involve H3 methylation at K9 and K27 (20). These events are coordinated by the Suppressor of variegation 3-9 [Su(var)3-9] (H3K9me) and Enhancer of zeste [E(z)] (H3K27me) genes (19, 20). Human homologues of these two fly genes are often up-regulated in cancer cell lines (6, 7, 20), and thus, inhibitors of HDACs are promising drugs for patients with cancer (1–4). It was therefore critical for us to determine whether endogenous Rpd3/HDAC and the histone lysine methyltransferases Su(var)3-9 and E(z) were required for tumorigenesis in vivo. Extra Sex Comb (Esc) and its human counterpart, EED, are essential for the E(z)/EZH2-mediated methyltransferase activity (20, 21). Furthermore, binding of the chromodomain protein Pc/HPC to methylated H3K27 is required to stably silence gene transcription. Thus, we tested the need for these genes in a heterozygous assay in vivo. Complete loss of function of any of these epigenetic genes confers a pleiotropic phenotype or early lethality (20), whereas the heterozygous state (i.e., a 50% reduction in gene dosage) enables us to develop a reliable and sensitive assay. If the Rpd3/HDAC, E(z), Su(var)3-9, Pc, and Esc genes are required for Psq and Lola-mediated aberrant epigenetic repression, giving half the dosage should significantly impede tumor development. Indeed, the eye phenotype in flies with a mutant copy of the Rpd3, Su(var)3-9, E(z), Pc, or Esc suggested a clear involvement of these epigenetic repressors in the development of tumors.

In searching for tumor suppressor and tumor-related genes whose silencing might be aberrant in these tumors, we found that the retinoblastoma family protein (Rbf) gene expression was down-regulated in tumors, whereas the expression of a second Rbf2 gene was unaffected (9). Interestingly, Rbf gene expression was also down-regulated in the eye tissue upon overexpression of the growth-promoting Delta protein alone (9), showing that the Rbf gene is a target of the Notch pathway. More importantly, reestablishing Rbf gene expression in the mutants prevented the tumor phenotype. In contrast, further reducing the activity of endogenous Rbf by introducing a mutant copy of this gene notably enhanced the tumor phenotype (9). These results clearly link the Notch signaling pathway to cell cycle control. RBF1, together with p53, participates in one of the most important tumor suppressor pathways in humans. RBF1, like its fly counterpart, Rbf, acts as a constraint on the G1-S transition and therefore its inactivation is often considered an important step towards malignancy (22). In humans, the loss of RBF1 expression can occur through epigenetic mechanisms, specifically due to the aberrant DNA hypermethylation of its promoter (1). In insects, such as D. melanogaster, there is little DNA methylation in the genome. Nevertheless, we found that when compared with the wild-type, the promoter and transcription start regions of the Rbf gene are methylated in tumors (9).
Significance and Future Directions

These recent findings reveal the unexpected cooperation between the Notch pathway and PcG-mediated cellular memory to promote tumor initiation and metastasis in *D. melanogaster* (see model, Fig. 1, top). They also imply that the growth control normally exerted by the Notch pathway involves epigenetic changes, such as depleting histone H3K4 methylation and down-regulating Rbf gene expression, thereby linking histone modification and cell cycle control with the Notch developmental pathway. Given that both Notch hyper-activation and aberrant gene silencing have been identified in human cancers, these discoveries in the fruit fly may provide a useful new focus for research into Notch-associated cancers.

Finally, the striking *eyaful* mutant may help us to understand the initial events that trigger tumor metastasis. In most types of malignant tumors, it takes years for a tumor to accumulate all the genetic and epigenetic alterations required to transform it from a premalignant lesion into an invasive cancer. In flies, which have a relatively short life cycle, the time for malignant tumor development seems to be very compressed, as this study (9), and earlier studies (8), have affirmed. One possible way to accelerate the metastatic development of tumors is if the same combination of genes which initiated the tumor are also responsible for its metastasis. Hence, we expect further studies of the *eyaful* and other fly mutants to provide more insights into the genetic and epigenetic changes that activate (or inactivate) metastasis-initiating genes, driving stationary epithelial cells to become invasive (i.e., to become motile and capable of degrading the basal lamina), and to metastasize. Although there are clearly vast differences in the physiology of flies and mammals, the basic processes and rules that govern the transformation of a healthy cell into a cancerous cell are likely to be similar.

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