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Linking model systems to cancer therapeutics: the case of Mastermind

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Genetics, and more recently genomics, reveal striking conservation in the fundamental signaling pathways that underlie normal and aberrant cell processes. Consequently, various genetic model organisms are now attracting the interest of biomedical scientists who are focused on therapeutic approaches to human disease. There are now several examples of studies in which Drosophila seems likely to facilitate advances in potential therapies, and a recent report has demonstrated the utility of the fly model for understanding and treating human disease. Basic developmental genetic information first obtained in Drosophila was used to design a therapeutic block to oncogenic Notch signaling that was associated with leukemia in mice. The story of Notch signaling in Drosophila demonstrates the potential for standard Drosophila molecular genetics in developing therapeutic strategies that are relevant to human disease.

Flies as models of disease

By all appearances flies and humans are quite different. Nevertheless, their genetics and underlying DNA sequences are remarkably similar. Many human disease genes have sequences common with those in Drosophila (Bier, 2005). This genetic similarity supports the notion that basic research in Drosophila will accelerate translational research for a wide range of diseases.

Disease-orientated Drosophila research generally follows one of three paradigms. The first uses flies to define the cellular and developmental roles of previously identified human disease genes or their fly equivalents. This is very common. The second uses the appearance of disease-like phenotypes in the fly to predict candidate human disease genes. This has been particularly successful in the areas of cell proliferation control and cancer. The Hippo and Archipelago tumor suppressor pathways were identified in Drosophila imaginal disc cells using this approach (Harvey and Tapon, 2007; Minella and Clurman, 2005). The third paradigm uses Drosophila as a screening platform to identify manipulations that reverse disease-like states. These manipulations commonly take the form of second-site modifier mutations that identify candidates for rational drug design in vertebrate cells. One example is the identification of mutations that selectively kill fly cells lacking *Rbf* (Edgar et al., 2005), whose human homolog, the retinoblastoma tumor suppressor gene *RB1*, is probably genetically or functionally inactivated in all human cancers. In a less common form of this third paradigm, flies are used as a preclinical 'staging ground' to identify and validate the in vivo efficacy of small molecules that revert disease-like phenotypes.

Despite the obvious promise of these three research paradigms for Drosophila, there are few instances in which information derived from Drosophila has been applied towards therapeutic or clinical approaches in humans. The wealth of data from Drosophila translates slowly into well-founded therapeutic strategies relevant to vertebrate cells. For example, the basic elements of Ras-MAPK (mitogen-activated protein kinase) signaling modules were first elucidated more than 15 years ago by studies in the fly eye (Wassarman et al., 1995), but the information from these studies has yet to profoundly influence clinical treatment for human cancers with elevated Ras activity.

Nevertheless, the utility of Drosophila to improve human therapies is now becoming evident. A recent report by Moellering et al. (Moellering et al., 2009) defines a new paradigm bridging classical Drosophila developmental genetics and potential clinical treatments. They describe a peptide-based anticancer strategy founded directly on genetic analysis of Notch signaling in Drosophila.

Preclinical studies in Drosophila

The history of treatment screening in flies is dominated by searches for small molecules with bioactivity in neurodegenerative and cancer models. One early study showed that flies lacking *bubblegum* gene function exhibit phenotypes similar to the human neurodegenerative disease adrenoleukodystrophy, and that feeding these flies a component of the dietary supplement 'Lorenzo's oil' could prevent the development of neuropathology (Min and Benzer, 1999). Perhaps because of the ease of achieving neuron-specific transgene expression in Drosophila, more recent work in neuropathology centers on gain-of-function models, with a special focus on pathology produced by neuronal expression of expanded polyglutamine (polyQ)-containing fragments of the Huntington's disease protein, Huntingtin (Htt). Steffan et al. (Steffan et al., 2001) used such a model to show that small-molecule inhibitors of the histone deacetylase class of enzymes (HDACs) protect against Htt-induced neurodegeneration. These data are now supported by work showing genetic interactions between fly HDAC genes and *htt* transgenes (Pallos et al., 2008). As a consequence of these studies, one FDA-approved HDAC inhibitor is in clinical trials to treat neurodegeneration. An Htt-polyQ fly model has also been used as a secondary screen to test

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the bioactivity of compounds that modulate autophagy in yeast. This approach identified three compounds that protected fly neurons in vivo against polyQ toxicity (Sarkar et al., 2007). Zhang et al. (Zhang et al., 2005) also found that a compound selected to inhibit polyQ aggregation in yeast inhibited neurodegeneration in flies overexpressing a neurotoxic fragment of Htt. A very similar approach by Desai and colleagues (Desai et al., 2006) identified five compounds (of which three are already FDA-approved drugs) that could rescue neurodegeneration in a fly Htt model. In addition to these examples of treatment screening in Htt models, Drosophila has also been used to identify small compounds that suppress neurological phenotypes associated with mutations in the fly version of the fragile X mental retardation gene (Chang et al., 2008). Thus, work in Drosophila models of neurodegeneration is beginning to make important contributions to the development of human treatments.

There are also examples of small-molecule anticancer treatments developed in flies that may soon affect human treatments. For example, Jaklevic et al. (Jaklevic et al., 2006) took advantage of the radiosensitive phenotype of Drosophila larvae with mutations in the Grapes (CHK1) checkpoint kinase to screen for small molecules that sensitize the organism to sublethal doses of radiation. This effort identified known and approved radiosensitizing drugs, suggesting that the fly has considerable potential to identify additional candidate anticancer compounds. Witte et al. (Witte et al., 2009) used a glioblastoma model in the Drosophila nervous system to validate the in vivo antitumor activity of the epidermal growth factor receptor tyrosine kinase inhibitor gefitinib, the phosphoinositide 3-kinase inhibitor wortmannin, and the Akt kinase inhibitor triciribine, suggesting that large-scale screens in this model might identify novel antiglioblastoma therapies. In a similar strategy, Vidal et al. (Vidal et al., 2005) used the Drosophila eye as an in vivo model to validate the ability of vandetanib, a compound that antagonizes activity of the receptor RET, to suppress a RET-driven model of multiple endocrine neoplasia type 2 (MEN2). Vandetanib has completed Phase II clinical trials (Wells et al., 2010) and might become the first approved chemotherapeutic for MEN2.

Genetics steps up

The classical strength of Drosophila as a model organism is its utility in identifying and characterizing essential genes. In the cancer field, Drosophila has been used to identify and characterize multiple, conserved tumor-regulatory pathways. An excellent example of this is the Hippo signaling pathway, which links nuclear proliferative and apoptotic transcriptional programs to planar cell polarity pathways, morphogen gradients, and adhesion molecules (Badouel et al., 2009). Yet, translating this basic knowledge of developmental mechanisms into potential pharmacological treatments is slow. This might be changing, given a recent report (Moellering et al., 2009) of using a peptide antagonist of Notch signaling to treat a mouse model for T-cell acute lymphoblastic leukemia (T-ALL). This inhibitory peptide was identified as a result of standard developmental studies in Drosophila, which highlights its value as a model. The utility of the fly is evident in the history of Notch signaling, which includes the development and characterization of a Notch antagonist, and a potential therapeutic for T-ALL.

Fundamentals of Notch signaling

The Notch pathway is an ancient, conserved signaling mechanism that influences numerous processes, ranging from cell proliferation and lateral inhibition to cell survival and stem-cell–niche interaction (Artavanis-Tsakonas et al., 1999; Fortini, 2009). Moreover, along with a small group of other pathways, including Wingless, Hedgehog, receptor tyrosine kinase, transforming growth factor β (TGFβ), nuclear receptor and JAK-STAT (Gerhart, 1999), it represents an example of how extracellular cues are integrated and translated into alternative patterns of gene expression and cell differentiation. Notch signaling includes the regulation of binary cell-fate decisions among groups of cells that are initially equivalent in developmental potential, as well as inductive interactions between different cell types.

The canonical Notch pathway (Artavanis-Tsakonas et al., 1999) involves a core set of proteins including cell-surface Notch receptors, ligands of the Delta and Serrate class, and nuclear proteins including CSL (CBF1/SuH/Lag-1) and Mastermind (Mam). Upon ligand binding, serial proteolysis of Notch releases an intracellular fragment, N^{IC}, that assembles with CSL and Mam in the nucleus. This ternary transcription complex transmits the signal, leading to activation of target genes. As the cell biology of Notch signaling was described, a more extensive array of proteins was found to contribute to the signaling cascade, including proteins that glycosylate, phosphorylate, ubiquitylate, regulate gene expression and endocytose core Notch components (Kopan and Ilagan, 2009; Fortini, 2009). Notch pathway genes were first associated with wing-margin formation and lateral inhibition in the Drosophila central nervous system (CNS), but the pathway has since been associated with myriad functions inside and outside the CNS in metazoans. In Drosophila, these include myogenesis, cardiogenesis, hematopoiesis, eye development and leg segmentation. In vertebrates, beyond its analogous and classic role in the nervous system, Notch functions include somitogenesis, lymphopoiesis, induction of left-right asymmetry, and intestinal, kidney, vascular and limb-bud development (Lai, 2004; Fortini, 2009).

Drosophila studies uncover the Notch signaling circuit

Drosophila embryos carrying lethal mutations in Notch pathway genes lose lateral inhibition and exhibit overgrowth of the nervous system, termed the ‘neurogenic’ phenotype. Insufficient Notch pathway activity at the wing margin leads to loss of margin bristles and formation of wing-blade notches, a phenotype that defined the *Notch* gene name.

The power of Drosophila as a genetic tool derives from its use in straightforward genetic screens and sensitive tests for genetic interactions among groups of extant mutations. Several structures, including wing, eye and bristle, require Notch function for proper development and are readily assayed for phenotypic changes in adult flies. This has allowed numerous genetic investigations to identify and characterize interacting components of Notch signaling. For example, the original *mam* alleles were isolated as zygotic lethal mutations in Drosophila (Nusslein-Volhard et al., 1984) that exhibited the classic neurogenic CNS phenotype described above. However, strong genetic interactions between *mam* and *Notch* mutations that affected the wing structure suggested an intimate functional relationship (Xu et al., 1990),

which is now known to reflect physical interaction of their gene products (Nam et al., 2006). Such formal genetic studies led to the elaboration of the canonical Notch pathway and the discovery of their cognate vertebrate genes and disease loci. Clues to potential therapeutic applications also derived from these original Drosophila studies.

Disease connections

Based on the wide spectrum of Notch function in vertebrates, it is not surprising that aberrant Notch activities in humans are associated with developmental abnormalities and numerous cancers. For example, the autosomal dominant Alagille syndrome is associated with liver, heart, eye and skeletal defects caused by loss of function of the Notch-ligand-encoding *JAG1* gene, an ortholog of Drosophila *Serrate*. Two additional disorders – spondylocostal dysostosis, affecting vertebral segmentation, and CADASIL, associated with degeneration of vascular smooth muscle cells – are caused by mutations in the *DLL3* and *NOTCH3* genes, respectively (Gridley, 2003).

Within the context of cancer alone, Notch exhibits diverse roles. It is described as an oncogene, a tumor suppressor gene, an inhibitor of apoptosis and a promoter of angiogenesis, among other descriptions (Zeng et al., 2005; Talora et al., 2008). Consequently, rational targeting of Notch signaling, designed to downregulate its activity during oncogenesis, is a very active area of study (Rizzo et al., 2008). One of the more promising therapeutic targets of Notch is the ternary activating transcription complex comprising N^{IC}, CSL and Mam, which associates with DNA regulatory elements of Notch-regulated loci (Kopan and Ilagan, 2009). Several strategies are possible for reducing the output from this complex. These include small-molecule disrupters of the functions of proteins, such as γ-secretase, which is a protease responsible for the generation of N^{IC} (Li et al., 2009), as well as expression of shortened versions of proteins, such as Mam, that can block assembly of the activation complex through a dominant-negative effect, as described below (Fig. 1).

Definition of Mam truncations as inhibitors of the Notch pathway

After Drosophila *mam* gene sequences were identified, the Mam protein was found to associate with chromosomes and to contain three conserved domains of charged amino acids: one basic and two acidic (Smoller et al., 1990; Bettler et al., 1996). One strategy to investigate a functional role of Mam used truncated polypeptides that lacked sequences carboxy to the basic-charge domain. It was predicted that truncations eliminating these segments, but retaining nuclear targeting via the basic domain, might produce dominant Notch-like phenotypes. In principle, such truncations could compete with wild-type Mam protein and act in a dominant-negative fashion (Fig. 1). Indeed, it was found that Mam truncations elicited strong Notch pathway phenotypes when expressed in various tissues (Helms et al., 1999). This genetic approach was thus the first demonstration that a Mam fragment containing the conserved basic domain can act as a potent inhibitor of Notch signaling in an otherwise normal genetic background.

It was later shown that Drosophila and mammalian Mam bind to N^{IC} through the basic domain and stabilize a complex that also contains CSL; this complex mediates activation of Notch target loci

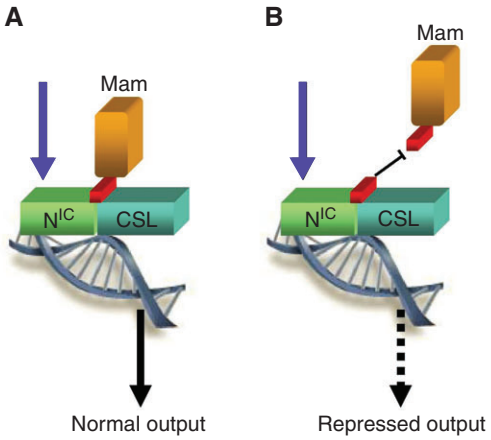

Fig. 1. Inhibition of the nuclear Notch activation complex. (A) Notch activation involves signals (purple arrow) that lead to production of an intracellular segment (NIC) that enters the nucleus. NIC associates with CSL, forming a binding groove for the basic domain (denoted in red) of Mam. This can lead to normal pathway output via gene activation, or excessive output in the case of oncogenic Notch mutations such as in T-ALL. (B) Studies in Drosophila have demonstrated that expression of truncated Mam proteins containing the basic domain can repress Notch pathway activation during normal development. Moellering et al. (Moellering et al., 2009) designed a peptide to a 16-residue portion of the basic domain and used it to repress pathway output (dotted arrow) associated with an oncogenic version of Notch. This repression derives from the peptide blocking wild-type Mam from interacting with the complex.

in cell culture (Petcherski and Kimble, 2000; Wu et al., 2000; Kitagawa et al., 2001). Moreover, truncations of the human version, MAML1, were also shown to exhibit Notch-inhibitory activity. Crystallography of the nuclear Notch ternary complex showed that the MAML1 basic domain binds tightly to a groove formed at the interface between the ankyrin repeats of N^{IC} and portions of CSL (Nam et al., 2006). This physical interaction presumably contributes to structural changes in the complex that mediate gene activation (Kopan and Ilagan, 2009). Thus, Mam is a coactivator in Notch signaling.

Linking truncated Mam to potential therapeutics

Moellering et al. (Moellering et al., 2009) show the application of earlier findings in a well-described case of oncogenesis resulting from hyperactivation of Notch signaling: T-ALL. They designed a 16-residue peptide from the basic region of MAML1. In wild-type MAML1 this segment binds at the interface of N^{IC} and CSL (Nam et al., 2006). The peptide was modified to stabilize its α-helical conformation by introduction of a hydrocarbon staple along one face of the helix; this method enhances protein-protein interactions and might be generally useful for future therapeutic applications. Cells endocytose the exogenous, low molecular weight peptide, which readily enters the nucleus. The stapled MAM peptide competes with full-length MAML1 to associate with N^{IC} and CSL. In cell culture, this peptide also represses Notch reporter expression in lines that express constitutively activated Notch, and it downregulates Notch target loci in human leukemic cells with activated Notch. The consistent effects of the MAML1 peptide on

Notch target expression indicated a potential therapeutic role and, indeed, the peptide elicited a dose-dependent regression of tumor in a mouse model of T-ALL when injected intraperitoneally.

A murine *Notch1* T-ALL retroviral construct was used to infect bone-marrow cells constitutively expressing firefly luciferase so that the authors could follow the tumor burden. Infected cells were transplanted into isogenic recipients, which developed T-ALL. The tumors in transplanted animals could be quantified with bioluminescence imaging, with or without peptide treatment. All mice receiving a twice-daily peptide regimen showed a significant decrease in tumor burden. The major implication of these data is that the MAM peptide reduced the tumors via a direct inhibition of the Notch ternary transcription complex. When viewed in historical context, this remarkable study almost completes a circuit from basic developmental genetics to successful treatment of a vertebrate cancer model, and in the process highlights the growing relevance of genetic models to translational research.

Conclusion

Drosophila is an established model for gene discovery and cell signaling, but recent studies demonstrate its potential for testing therapeutic strategies. As we learn more about human genomic structure at the DNA and chromatin (epigenetic) level, and their modifications associated with disease, it is highly likely that the relevance of the Drosophila model will continue to grow.

Flies and humans are similar in some key genetic pathways, but they are not equivalent. Human genetic complexity remains a major hurdle to drug development. Humans have multiple copies of Notch-pathway loci, including three homologs of *mam* (McElhinney et al., 2008), and the pathway has wide-ranging and crucial developmental effects. This means that even carefully designed drugs might not act as predicted for therapeutic uses. This complexity, along with roles for Mam outside of Notch signaling and potential off-target drug effects, indicates a continued steep climb towards successful clinical applications. However, the relative simplicity of the fly lends itself to early screening studies, which makes it a tractable research tool from the phases of initial discovery to drug screening.

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COMPETING INTERESTS

The authors declare no competing financial interests.

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