SIGNAL TRANSDUCTION

Emerging from hibernation

In keeping with its namesake, Hedgehog (Hh) — or, at least, components of the Hh pathway — has come out of hibernation. Thanks to work by Phil Beachy and colleagues, reported in *Science*, Dally-like protein (Dlp) and casein kinase 1α (CK1α) have newly assigned roles in Hh signalling.

Because classical-genetic approaches to studying Hh signalling have their limitations, the authors chose to use RNA interference (RNAi) to disrupt gene function, and they combined this approach with a cultured-cell assay to screen *Drosophila* genes. The assay involved transfecting cells derived from the wing imaginal disc (cl-8 cells) with a Hh-pathway-responsive luciferase reporter and double-stranded RNA (dsRNA) for RNAi. The assay used exogenous Hh ligand, so it rules out components involved in Hh synthesis or distribution, and is also quantitative. RNAi targeting of the positive regulatory elements of the pathway — Smoothened (Smo), Fused (Fu) and Cubitus interruptus (Ci) — inhibited the response to Hh, whereas targeting negative elements such as Costal 2 (Cos2) and Patched (Ptc) activated or enhanced the response. The assay can be used to study gene interactions and epistasis, as several dsRNA species can be combined.

To test the system, the authors used a library containing dsRNAs that corresponded to all of the kinases and phosphatases predicted from the *Drosophila* genome sequence. Several dsRNA pools that affected reporter activity were identified and, when re-screened, three dsRNAs emerged. Of these targets, CK1α had not previously been implicated in Hh signalling.

Extending the screen to a dsRNA library based on the *Drosophila* Gene Collection Release 1 — which contains cDNAs corresponding to ~43% of the genes predicted from the *Drosophila* genome sequence — identified four gene targets that had not previously been implicated in Hh signalling. CK1α was one; another was Dlp, a glycipan heparin sulphate proteoglycan. Both are thought to function in the Wingless (Wg) signalling pathway.

Immunostaining showed that Dlp localized to the surface of cl-8 cells. RNAi of Ptc suppressed the requirement for Dlp in the Hh response, which indicates that Dlp could function upstream or at the level of Ptc. As Ptc is a membrane protein, Dlp might help deliver Hh to Ptc by concentrating Hh on the cell surface and, indeed, preliminary evidence showed that Dlp associates with Hh. Consistent with such a role, when Hh was expressed in

ANTIBIOTIC RESISTANCE

A costly mistake

With up to one-third of the world's population estimated to be infected with *Mycobacterium tuberculosis* (MTB), resistance to antibiotic treatments is a huge potential problem. A report in *Cell* by Clifton Barry, Valerie Mizrahi and colleagues now takes us a step closer to understanding how this resistance might arise.

While in its host, MTB encounters various adverse conditions that can damage its DNA. To generate strains that can survive under these conditions, the bacterium undergoes genetic mutation. But this can lead to antibiotic resistance if these mutations arise in genes such as *rpoB*, the mutation of which confers resistance to Rifampicin (Rif).

How do these mutations arise? One idea is that, in response to the attack mounted by human macrophages, the so-called 'SOS response' is activated. This pathway, which is regulated by the RecA protein, repairs the damaged DNA. But it is thought to do so using 'error-prone' DNA polymerases, which have a low rate of fidelity, so they introduce mutations as they complete the repair.

Helena Boshoff, a postdoctoral fellow, first confirmed that MTB has a damage-inducible mutagenesis system. She observed a 20–50-fold increase in Rif-resistant mutants when the bacteria were treated with a DNA-damaging agent (ultraviolet (UV) light).

Next, microarray analysis was used to identify which genes were induced in response to UV damage. Of the 158 candidates, only one — *dnaE2* — encoded a known DNA polymerase. The authors therefore disrupted the *dnaE2* gene in *Mycobacterium*, and showed that the observed resistance to Rif (and streptomycin) after UV irradiation was completely lost in these mutants. This resistance could be restored, however, by complementation with an extra copy of *dnaE2*.

To see whether DnaE2 might function as an error-prone polymerase, Boshoff looked at the types of mutations that arose in wild-type strains after UV treatment. She found that a high proportion (35.6%) had a double CC to TT transition, which is typical of the error-prone repair of a DNA lesion. By contrast, this characteristic mutation was not seen in the *dnaE2*-deficient strains. This is a surprising result, as enzymes of the family to which DnaE2 belongs were not thought to introduce errors while copying DNA.

So might DnaE2 be involved in drug resistance *in vivo*? To test this, the authors infected mice with wild-type, *dnaE2*-knockout or *dnaE2*-complemented knockout strains of MTB, irradiated them, then treated them with Rif. The result was clear — drug resistance emerged more frequently in the wild-type and complemented strains, than with the *dnaE2* knockouts. And the implication is equally clear; DnaE2 is not only the main regulator of DNA-damage-induced mutagenesis in MTB, but it's also a new potential target for preventing the emergence of drug resistance in this pathogen.

Alison Mitchell

References and links

ORIGINAL RESEARCH PAPER Boshoff, H. I. M. *et al.* DnaE2 polymerase contributes to *in vivo* survival and the emergence of drug resistance in *Mycobacterium tuberculosis*. *Cell* **113**, 183–193 (2003)

a membrane-anchored form, RNAi of Dlp didn't block the signal response, which indicates that Dlp might usually concentrate the Hh signal.

Indications from the library screens were that CK1α might control basal pathway activity, as dsRNA increased basal reporter activity. Ci was required for this increase, whereas Smo and Fu were not, implying that CK1α is upstream or at the level of Ci, but downstream of Smo and Fu.

As a regulator of the basal activity of Hh and Wg signalling pathways, CK1α could function as a tumour suppressor in several cancers that are associated with overactivation of one or the other pathway. Similarly, deletion of a human chromosomal region that contains GPC6, a mammalian glycipan member that is closely related to Dlp, is associated with many human malformations. So, too, are mutations in one of the two remaining genes identified in the screen that had not previously been implicated in Hh signalling. Hopefully, discovering the functions of these genes in the Hh pathway won't be too prickly a problem!

Katrin Bussell

References and links

ORIGINAL RESEARCH PAPER Lum, L. *et al.* Identification of Hedgehog pathway components by RNAi in *Drosophila* cultured cells. *Science* **299**, 2039–2045 (2003)

CYTOKINESIS

Time to separate?

At the end of cell division, daughter cells separate from each other by a process known as cytokinesis. Now, in a report in *Science*, Timothy Mitchison and colleagues establish the usefulness of a fast-acting and reversible inhibitor in beginning to uncover the mechanisms involved in the spatial and temporal control of cytokinesis in mammalian cells.

Using a high-throughput screening assay to find small-molecule inhibitors of nonmuscle myosin II — a major component of the cytokinesis furrow that provides the force needed for furrow ingression — the authors identified blebbistatin. They found that blebbistatin disrupted cytokinesis in vertebrate cells by the rapid and reversible inhibition of ingression. But assembly of the furrow and the microtubules that position it was similar to controls. The timing of mitotic exit was also unaffected, with chromosomal decondensation and nuclear envelope re-formation taking place as normal.

Mitchison and colleagues then investigated the timing of C phase — the period during which cytokinesis can occur. At around one hour, the duration of C phase in blebbistatin-arrested cells was similar to that in cells treated with an actin-depolymerizing drug, and the authors concluded that "…an unidentified cell-cycle signal terminates C phase…". C phase is triggered by the start of anaphase and requires the anaphase-promoting complex and ubiquitin-mediated proteolysis. So, Mitchison and colleagues tested whether a proteasome inhibitor, MG132, had any effect. Indeed, in the presence of blebbistatin, MG132 significantly increased the percentage of binucleate cells retaining a myosin II ring, and the authors estimated that MG132 more than doubles the length of C phase. In addition, these cells showed abnormal organization and localization of myosin II and another major furrow component, anillin. So, ubiquitin-dependent proteolysis is important for C-phase exit in mammalian cells.

Next, the authors examined the spatial control of cytokinesis. They arrested cells in mitosis using monastrol (an inhibitor of the mitotic kinesin Eg5), released them into blebbistatin to initiate anaphase and furrow assembly, and then tested the effects of several drugs. By depolymerizing microtubules with nocodazole, Mitchison and colleagues showed that continuous microtubule-to-cortex communication is necessary for maintaining the localization of furrow components. In these cells, separated nuclei collapsed back together, indicating that the midzone is necessary to keep the nuclei apart until cytokinesis is completed.

The addition of staurosporine, a broad-spectrum kinase inhibitor, caused extensive disorganization of midzone microtubules. So, the authors looked at the effects of inhibiting the kinase aurora B, which has been implicated in midzone organization. ATP competitive inhibitors of aurora B produced similar results to staurosporine. In addition, staurosporine and a Rho-kinase inhibitor both showed that localization of myosin II to the cytokinesis furrow is controlled independently of anillin localization. Neither cyclin-dependent kinase (CDK) inhibitors nor monastrol affected the cytology of blebbistatin-arrested cells. This suggests that Eg5 might not be necessary for organizing spindle bipolarity once anaphase has been initiated, and that, although CDKs might be involved in C-phase timing, they do not have a central role in maintaining the contractile ring after anaphase.

So, the use of blebbistatin has allowed the further dissection of the proteins and processes involved in cytokinesis, and "New drugs that target guanosine triphospates, membrane dynamics, and mitotic motors will be useful in further dissecting the logic of cytokinesis".

Natalie Wilson

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