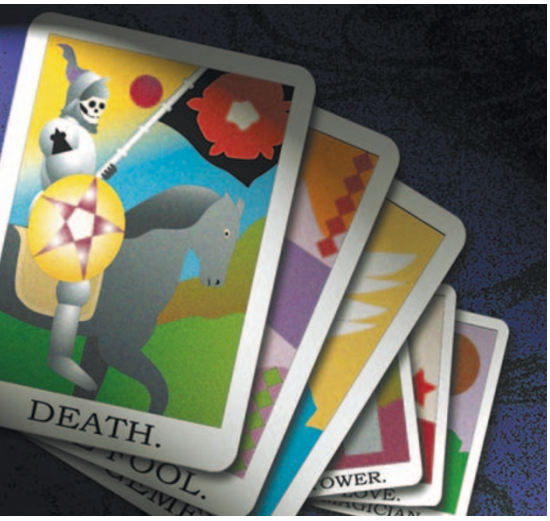
APOPTOSIS

Conveying a deadly message



Reactive oxygen species (ROS) are implicated in apoptosis, and the p66 isoform of the adaptor protein Shc regulates ROS metabolism and apoptosis. So could there be a direct connection between $p66^{Shc}$ and ROS release? Giorgio *et al.* reveal all in *Cell*.

$p66^{Shc}$ is known to be required for mitochondrial depolarization and for cytochrome *c* release in response to pro-apoptotic stimuli. And, because blocking the permeability transition pore of mitochondria inhibited this pro-apoptotic function, the group's first step was to see whether $p66^{Shc}$ could directly induce permeability transition. When allowed to enter the mitochondrial intermembrane space, recombinant $p66^{Shc}$ indeed increased the permeability of isolated mitochondria. $p66^{Shc}$ is known to be enriched in the inner membrane fraction of mitochondria and, consistent with this, the authors found it within the inner mitochondrial space.

With the localization of $p66^{Shc}$ pinpointed, Giorgio *et al.* then showed that $p66^{Shc}$ was essential for the increase in mitochondrial ROS that occurs after treatment with pro-apoptotic stimuli and that a functional electron transport chain (ETC) was a requisite. They ruled out the possibility that the increase in ROS was the result of decreased scavenging or occurred indirectly through mitochondrial swelling.

Unlike antimycin A, for example, which generates the ROS superoxide ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) by blocking the ETC, $p66^{Shc}$ didn't inhibit respiration. It required a functional ETC to stimulate the production of H_2O_2 ($O_2^{-\bullet}$ was not generated), which implies that $p66^{Shc}$ reduced oxygen using certain components of the ETC. Could $p66^{Shc}$ itself mediate electron transfer? The results from the group suggested that it could, and indicated a redox potential for $p66^{Shc}$ of –35 mV. As this value is closest — among the redox systems present in mitochondria — to that of cytochrome *c*, the authors investigated whether $p66^{Shc}$ could exchange electrons with cytochrome *c*, and found that it could. Specifically, $p66^{Shc}$ could interact with, and oxidize, cytochrome *c in vitro*. In doing so, oxygen was reduced and H_2O_2 was formed *in vitro* and *in vivo*.

SIGNAL TRANSDUCTION

What's in a name?

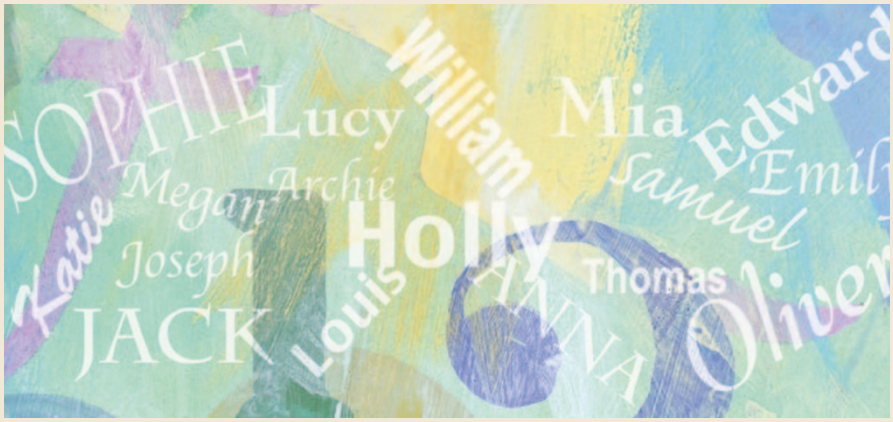
Cell migration and proliferation are just two of the processes regulated by Ras proteins, through Raf-mediated activation of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) cascade. Rajalingam and colleagues, reporting in *Nature Cell Biology*, now show that the evolutionarily conserved and ubiquitously expressed protein prohibitin certainly doesn't live up to its name, instead 'activating' the Ras-induced ERK/MAPK pathway and 'promoting' epithelial cell migration.

The authors had previously identified prohibitin in an RNA interference (RNAi)-based screen, and, in the present study, they showed that suppressing prohibitin expression by RNAi reduced cell spreading and increased cell–cell adhesion in treated HeLa cells and in other cancer cell lines. The RNAi-treated cells, when viewed by scanning and transmission electron microscopy, had almost no intercellular spaces and lacked any membrane protrusions. Confocal microscopy showed that this resulted from increased levels of the adherens junction proteins cadherin and β-catenin at regions of cell–cell contact, which stabilized the junctions.

When epidermal growth factor (EGF) was added to prohibitin-depleted HeLa cells — to stimulate the EGF receptor (EGFR) and its relative HER2 — these cells could not migrate efficiently on collagen as they normally do. The authors subsequently found a similar phenotype when cells were depleted of EGFR or HER2.

Members of the EGFR family signal through Ras proteins to ERK/MAPK (through Raf and MEK (MAPK and ERK kinase)) and to Akt (through phosphatidylinositol 3-kinase). Inhibiting prohibitin expression reduced basal and EGF-induced phosphorylation of ERK/MAPK, but had no effect on Akt, nor on the protein levels of Ras, C-Raf, MEK1/2 or ERK/MAPK1/2. However, prohibitin reduction did block the EGF-induced phosphorylation of C-Raf on Ser338. Phosphorylation on Ser338 is required for C-Raf activation after Ser259 is dephosphorylated, and high levels of Ser259-phosphorylated C-Raf were seen in the absence of prohibitin. So EGF-induced C-Raf activation requires prohibitin.

This being the case, Rajalingam and colleagues surmised that direct C-Raf activation should overcome the formation of increased intercellular adhesion and the reduced ERK/MAPK phosphorylation that occurred in the absence of prohibitin — which it did, when indirectly activated, constitutively expressed or artificially targeted to the membrane. At the membrane, Raf is usually recruited by Ras to caveolae, and the authors managed to detect prohibitin and C-Raf in the caveolin-1-rich fractions of HeLa cells and other cancer cell



The authors then used the fact that neither the p46 nor p52 isoforms of Shc influence ROS regulation or apoptosis to implicate the N-terminal region (which contains the collagen-homologous-2 (CH2) and phosphotyrosine binding (PTB) domains) of $p66^{Shc}$ in cytochrome *c* binding. Further probing uncovered a 44-residue region just N-terminal to the PTB domain which mediated cytochrome *c* binding, with the identification of E132, E133 and W134 as essential residues for redox activity and cytochrome *c* binding. Mutations that impair this redox activity not only impaired the ability of $p66^{Shc}$ to mediate ROS production, but also abrogated its ability to induce mitochondrial permeability transition and subsequent apoptosis. So $p66^{Shc}$ seems to be "an atypical signal transducer that converts proapoptotic into redox signals".

Katrin Bussell

References and links

ORIGINAL RESEARCH PAPER Giorgio, M. *et al.* Electron transfer between cytochrome *c* and $p66^{Shc}$ generates reactive oxygen species that trigger mitochondrial apoptosis. *Cell* **122**, 221–233 (2005)

lines. Despite EGF stimulation, C-Raf could not be detected in caveolae in prohibitin-depleted cells. Prohibitin and C-Raf interacted *in vitro* and endogenously in cells, and this direct interaction was necessary for EGF to activate C-Raf. Prohibitin was also needed for Ras to interact with, and to activate, C-Raf.

C-Raf activation by Ras requires, among many events, a 14-3-3 protein to be displaced from Ser259-phosphorylated C-Raf. After this Ras-mediated event, Ser259 is dephosphorylated and C-Raf can associate with the membrane, where it is phosphorylated on Ser338 and Tyr341 for full activation. Adding EGF resulted in the dephosphorylation of Ser259 on C-Raf and its subsequent membrane localization only when prohibitin was present. Even Ras activation by EGF couldn't promote 14-3-3 displacement from C-Raf if prohibitin was missing. Prohibitin is therefore needed for Ras to displace 14-3-3. As a Ser259Ala mutant of C-Raf, which cannot bind 14-3-3 and binds to Ras with a high affinity, could rescue C-Raf activation in prohibitin-depleted cells, Rajalingam and colleagues ended their report by suggesting that Ser259 is the site from which 14-3-3 is displaced by Ras and prohibitin.

Katrin Bussell

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CHROMATIN

Forming a silent structure

Heterochromatin is essential for correct centromere and telomere function, and has an important role in silencing genes at specific loci. In the current model for heterochromatin formation in fission yeast, the protein Rik1 recruits the histone methyltransferase Clr4 to a target locus. Clr4 then methylates histone 3 (H3) on K9, and this modification results in the recruitment of other proteins that nucleate the structural spreading of heterochromatin.

In *Genes & Development*, Peterson and colleagues now give new insights into heterochromatin formation through the identification of novel Rik1-associated proteins. Using tandem affinity purification and mass spectrometry, they showed that the following proteins associate with Rik1: Clr4, histone H2B, two novel proteins, which they named Rik1-associated factor-1 (Raf1) and Raf2, and Pcu4 and Pip1, which are components of cullin-dependent ubiquitin ligases.

The finding that Pcu4 and Pip1 copurified with Rik1 indicated that Rik1 might be associated with ubiquitylation processes, and the authors showed that, in the presence of recombinant ubiquitin-activating and ubiquitin-conjugating enzymes, the Rik1 complex polyubiquitylated the candidate substrate H2B *in vitro*. They also showed that Raf2 and Clr4 are components of this ubiquitin-ligase complex.

Next, Peterson and co-workers studied the role of the Rik1-associated proteins in heterochromatin-related functions. First, they studied the effect of the novel proteins Raf1 and Raf2 on the transcriptional silencing of transgenes that had been inserted into centromeric heterochromatic regions. They showed that deleting *rik1*, *clr4*, *raf1* or *raf2* resulted in the derepression of these transgenes, but that such deletions did not affect the expression of a transgene in a euchromatic region. These proteins therefore specifically function on heterochromatic loci.

Rik1 and Clr4 are required for the centromeric pattern of H3 methylation — an enrichment of H3-K9 methylation and a reduction in H3-K4 methylation. So, do Raf1 and Raf2 affect this methylation pattern? Deleting *raf1* or *raf2* had the same affect as deleting *rik1* or *clr4* — that is, it caused a decrease in H3-K9 methylation and an increase in H3-K4 methylation. Together with the transgene silencing assays, these data indicate that Raf1 and Raf2 are novel components of the silencing

machinery that affect the regulation of H3 methylation in heterochromatin.

Peterson and colleagues then studied the role of the ubiquitin-ligase activity of the Rik1 complex in heterochromatin formation. Using a dominant-negative allele of Pcu4, they showed that Pcu4 contributes to heterochromatic silencing. More specifically, a Pcu4-dependent ubiquitin ligase has a role in establishing heterochromatin domains by preventing inappropriate H3-K4 methylation.

So, these authors have identified two novel proteins — Raf1 and Raf2 — that are essential for transcriptional silencing in centromeric heterochromatin, as well as a "...novel Rik1-associated E3 ubiquitin ligase that is required for heterochromatin formation". Future studies will address the precise molecular roles of these proteins.

Rachel Smallridge

References and links

ORIGINAL RESEARCH PAPER Horn, P. J., Bastie, J.-N. & Peterson, C. L. A Rik1-associated, cullin-dependent E3 ubiquitin ligase is essential for heterochromatin formation. *Genes Dev.* **19**, 1705–1714 (2005)

FURTHER READING Maison, C. & Almouzni, G. HP1 and the dynamics of heterochromatin maintenance. *Nature Rev. Mol. Cell Biol.* **5**, 296–305 (2004)

WEB SITE

Craig Peterson's laboratory: http://www.umassmed.edu/pmm/faculty/peterson.cfm