CELL CYCLE

Simply effective

The mechanisms that underlie biological processes often turn out to be more complex than was initially thought. However, a recent study of the spindle checkpoint, which was reported by Hongtao Yu and colleagues in *Molecular Cell*, hints at a regulatory mechanism of striking simplicity.

The spindle checkpoint ensures the accurate separation of chromosomes by blocking the ubiquitin-ligase activity of the anaphase-promoting complex/cyclosome (APC/C) in response to improper chromosome alignments and inappropriate tension of the microtubular spindle. Many proteins are involved in controlling this checkpoint, so its regulation is complex. In particular, the Bub1 kinase interacts with and regulates several other checkpoint proteins and was expected to be an upstream component of the checkpoint. But, Yu and colleagues now show that, surprisingly, Bub1 inhibits the activity of APC/C directly.

The authors first showed that a significant fraction of HeLa cells that had been depleted for Bub1 using RNA interference (RNAi) failed to undergo mitotic arrest after treatment with nocodazole (a spindle-damaging agent). This indicates that Bub1 is indeed required for the spindle checkpoint.

Because Bub1 contains a protein-kinase domain, Yu and co-workers tested whether Bub1 phosphorylates Cdc20, which is a key regulatory protein of APC/C, and found that it did. By contrast, a kinase-deficient form of Bub1 failed to phosphorylate Cdc20. When the authors next tested the effects of Bub1-mediated phosphorylation of Cdc20 on the ubiquitylation activity of APC/C^{Cdc20}, they found that substoichiometric amounts of Bub1 were sufficient to inhibit APC/C^{Cdc20}. Also, the kinase-defective Bub1 mutant failed to inhibit APC/C^{Cdc20}. Together, these observations indicate that Bub1 catalytically inhibits APC/C^{Cdc20} by phosphorylating Cdc20.

Using mass spectrometry, the Yu team mapped the *in vivo* phosphorylation sites of endogenous Cdc20, which had been purified from nocodazole-treated cells. They identified six sites, all of which were also phosphorylated by Bub1 *in vitro*. That Bub1 phosphorylates Cdc20 *in vivo* was confirmed by checking the phosphorylation status of Cdc20 in Bub1-depleted cells — the slower-migrating, phosphorylated forms of Cdc20 that were present in wild-type, nocodazole-treated cells were absent in Bub1-depleted cells.

Next, the authors prepared a Cdc20 mutant ($Cdc20^{BPM}$) in which all six phosphorylation sites had been mutated. Bub1 failed to inhibit the ubiquitylation activity of APC/$C^{Cdc20BPM}$, which shows that Cdc20 — rather than APC/C

SIGNALLING

The stretch effect

If you tread on someone's toe, the response is usually quite vocal. But, beyond the yelps, what might be happening when force is applied at the cellular level? Tamada, Sheetz and Sawada investigated how forces on the extracellular matrix (ECM) are transduced into intracellular biochemical signals, using

cells that have been stripped of their cell membrane and soluble proteins. Stretching the resultant 'Triton cytoskeletons' initiated signalling to the small GTPase Rap1, as outlined in *Developmental Cell*.

When cells were extracted using Triton X-100, the remaining complex contained mainly cytoskeletal and adhesion proteins, and only a few membrane lipids or cytoplasmic proteins. Nevertheless, the authors found that, when these Triton cytoskeletons were stretched, they could activate Rap1 that was present in added cytoplasmic extracts. Such stretch-mediated Rap1 activation is known to occur in intact cells.

On investigating potential upstream candidates, the authors found that two components of an added soluble cytoplasmic extract, the Rap1 guanine nucleotide-exchange factor C3G and the adaptor protein CrkII — which are both involved in activating Rap1 — bound to the Triton cytoskeletons in a stretch-dependent manner. Activation of Rap1 was prevented if C3G was depleted from the extracts, so the Rap1 response to Triton-cytoskeleton stretching depended on C3G.

In response to various stimuli, CrkII–C3G binds to tyrosine-phosphorylated proteins through the Src-homology-2 (SH2) domain of CrkII. And, accordingly, Tamada, Sheetz and Sawada noticed an increase in phosphotyrosine levels in several protein components of Triton cytoskeletons in response to stretch. The prime suspects for this phosphorylation were Src-family kinases (SFKs), and a selective SFK inhibitor prevented this stretch-induced phosphorylation response and the ability of fluorescently tagged CrkII to bind to stretched Triton cytoskeletons. A candidate substrate for such phosphorylation was Cas (Crk-associated substrate), a significant amount of which remained on Triton cytoskeletons. The authors' assumptions proved correct — tyrosine phosphorylation of Cas in Triton cytoskeletons increased in response to stretch. And CrkII no longer bound to Triton cytoskeletons of $Cas^{-/-}$ cells, which indicates that CrkII binds directly to tyrosine-phosphorylated Cas.

Does this affect all parts of the cell in the same way? The greatest stresses are expected to occur at the regions of cell–ECM contact. At these points, Tamada, Sheetz and Sawada observed an increase in tyrosine phosphorylation in Triton cytoskeletons in response to externally applied force — no cytosolic molecules were required. A similar localized increase in tyrosine

itself — is required for the inhibition of APC/C^{Cdc20}.

Yu and colleagues isolated Bub1 from nocodazole-treated (metaphase-arrested) cells and from thymidine-treated (G1/S-arrested) cells. The kinase activity of Bub1 that was purified from the former cells was much higher than from the latter, which shows that the kinase activity is specifically activated after spindle-checkpoint activation. Overexpression of the non-phosphorylatable Cdc20^{BPM} mutant caused 40–50% of cells to arrest in mitosis, compared with >90% in the case of wild-type protein. So, elimination of the Bub1 phosphorylation of Cdc20 causes a substantial, yet partial, spindle-checkpoint defect. This is consistent with the existence of other APC/C-inhibitory mechanisms.

The spindle checkpoint is extremely sensitive, and Yu and colleagues propose that the catalytic mechanism for Bub1-mediated APC/C inhibition "…might be partially responsible for this remarkable sensitivity…"

Arianne Heinrichs

References and links

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FURTHER READING Margolis, R. L. Bub1, a gatekeeper for Cdc20-dependent mitotic exit. *Dev. Cell* **7**, 634–635 (2004)

phosphorylation was seen when intact, non-Triton-treated cells were stretched. And fluorescently tagged CrkII was seen to move to adhesion sites and to colocalize with Cas in response to stretching in intact cells.

So stretching Triton cytoskeletons and intact cells induces CrkII–C3G–Rap1 signalling. How the signalling is initiated is unknown, but it's likely that proteins are unfolded or distorted when force is applied, which could create new binding sites for other proteins. As Rap1 is known to induce integrin-mediated adhesion, its activation at cell–ECM sites could well stabilize such contacts. Recent insights into the activation of integrins by Rap1 come from the studies of Lafuente *et al.*, who cloned and characterized RIAM, a Rap1–GTP-interacting adaptor molecule. When overexpressed, RIAM induced β_1- and β_2-integrin-mediated adhesion and influenced actin dynamics.

Katrin Bussell

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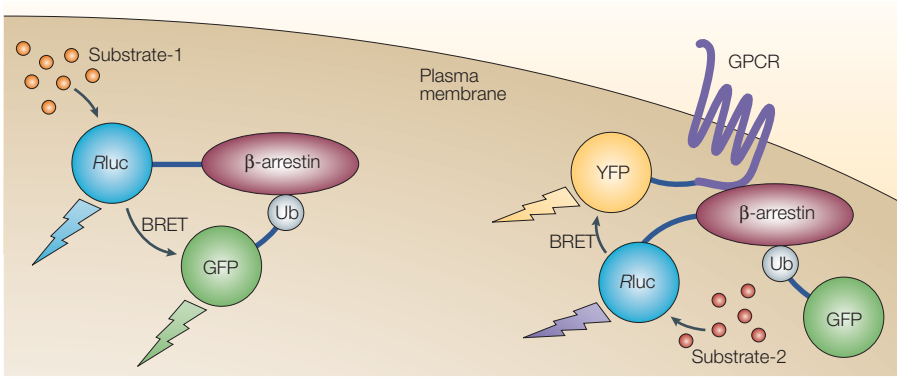

TECHNIQUE

Monitoring modification

Ubiquitylation is a reversible post-translational modification that has important roles in processes such as protein degradation and intracellular signalling. Yet, despite a growing interest in ubiquitylation, a tool for studying the dynamics of this process has been lacking. Now though, in *Nature Methods*, Bouvier and colleagues describe a technique that can be used to detect *in situ* changes in protein ubiquitylation.

Their work revolved around bioluminescence resonance energy transfer (BRET), which allows protein–protein interactions to be detected in real time *in vivo*, and β-arrestin — a protein that is ubiquitylated in response to G-protein-coupled receptor (GPCR) activation.

They made a *Renilla*-luciferase–β-arrestin construct (*R*luc–β-arrestin) and a green-fluorescent-protein–ubiquitin construct (GFP–Ub; mutant ubiquitin was used to prevent polyubiquitin-chain formation), and added a specific *R*luc substrate (substrate-1) to cells co-expressing these constructs. Substrate-1 hydrolysis by *R*luc emits light that overlaps with the excitation spectrum of GFP. So, if GFP–Ub is covalently conjugated to *R*luc–β-arrestin, BRET occurs and a fluorescent signal that originates from GFP should be detected.

Indeed, a BRET signal was detected, and the signal increased with increasing concentrations of GFP–Ub until it reached a plateau (weaker, linear signals were obtained using GFP alone or a GFP–Ub construct that could not be conjugated to *R*luc–β-arrestin). Furthermore, no signal was obtained when GFP–Ub and *R*luc were co-expressed. These data therefore confirm that the signal reflects the covalent attachment of GFP–Ub to β-arrestin in *R*luc–β-arrestin.

Next, the authors showed that this technique can be used to study receptor-regulated ubiquitylation by monitoring energy transfer between GFP–Ub and *R*luc–β-arrestin in the presence of GPCRs. When they activated the receptors using selective agonists, the BRET signal increased in a dose-dependent manner.

It has been proposed that GPCR activation might regulate β-arrestin ubiquitylation and its recruitment to the GPCR. By using two different substrates for *R*luc and a GPCR–yellow-fluorescent-protein construct (V_2R–YFP), Bouvier and colleagues were able to monitor these two events simultaneously. They split a cell culture expressing GFP–Ub, *R*luc–β-arrestin and V_2R–YFP into two samples, and added substrate-1 to one sample (substrate-1 hydrolysis produces light that excites GFP–Ub) and substate-2 to the other (substrate-2 hydrolysis emits light that excites V_2R–YFP). They found that the activation of V_2R–YFP produced BRET signals indicative of the concomitant ubiquitylation and recruitment of β-arrestin.

Finally, Bouvier and co-workers followed the real-time kinetics of agonist-promoted β-arrestin ubiquitylation in cells co-expressing GPCRs that interact with β-arrestin transiently (class A) or stably (class B). They found that class-B-GPCR activation resulted in a more stable ubiquitylation of β-arrestin than class-A-GPCR activation. The nature of the interaction between the receptor and β-arrestin therefore effects the dynamics of β-arrestin ubiquitylation.

So, using β-arrestin as a model, these authors have shown that BRET can specifically detect basal and regulated ubiquitylation processes in living cells. Their assay will therefore be useful "…for studying the dynamic ubiquitination of proteins and for understanding which cellular functions are regulated by this post-translational event."

Rachel Smallridge

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WEB SITE

Michel Bouvier's laboratory: http://www.mapageweb.umontreal.ca/bouvier/