CRM1-dependent export. Indeed, leptomycin B (LMB), an exquisitely specific inhibitor of CRM1, induces the accumulation of nuclear APC. Importantly, β-catenin localization is also subject to CRM1, and this seems to be mediated through APC — in APC-mutant colorectal cancer cells that accumulate β-catenin in the nucleus, re-expression of APC induces nuclear export and subsequent cytoplasmic degradation of β-catenin. Mutation of the NES in APC abrogates both effects.

In the second paper, Bienz et al.[5] reach essentially the same conclusions, in that APC utilizes the CRM1 pathway to shuttle nuclear β-catenin to the cytoplasmic destruction complex. The studies, however, differ in critical details. Whereas Bienz and colleagues notice the N-terminal NES, they home in on 2 potent NESs that coincide with 2 of the 20-amino-acid repeats found in β-catenin. These repeats, along with the more N-terminally located 15-amino-acid repeats, have previously been shown to bind to β-catenin and to regulate its activity. These NES sequences are conserved between all known APC proteins in vertebrates and flies. Removal of the NESs from the central region of APC by mutation impairs the ability of the protein to export and degrade nuclear β-catenin. As a consequence, NES-mutant APC could not efficiently repress the constitutive activity of β-catenin–TCF in the TOPFLASH reporter assay.

Significantly, Bienz and colleagues scrutinized the accumulated mutational data of APC in colorectal cancer. It had previously been noted that the vast majority of the — almost invariably truncating — mutations map to a small region termed the mutation-cluster region (MCR). The authors found a steep drop in the incidence of mutations directly at the first of the two NESs (Fig. 1). In other words, there seems to be a strong selective pressure in the tumours to remove both NESs, implying that the NESs participate in the tumour-suppressor activities of APC. Also important is the earlier observation that truncated APC molecules usually retain their β-catenin-binding 15-amino-acid repeats, as well as at least 1 of the 20-amino-acid repeats[6]. The resulting truncated APC molecule can bind to β-catenin but fails to export it to the cytoplasm, thus explaining the accumulation of nuclear β-catenin in colon-cancer cells.

It is evident from both studies that APC shuttles between nucleus and cytoplasm by means of the CRM1 pathway. It is also likely that this process has a function in the nuclear export and cytoplasmic degradation of β-catenin. The relative importance of the different NESs identified in the two studies remains open. It is possible that the APC molecule requires a minimum of two functional NES sequences in order to deliver nuclear β-catenin for cytoplasmic degradation. A critical experiment to address the importance of the NES sequences for cancer would be their removal from the wild-type APC protein by gene disruption in the mouse germline.

The proposed mechanism poses several other questions. Is the shuttling-and-delivery mechanism the only role of APC in the functioning of the destruction complex? If, as proposed by Bienz and colleagues, mutant APC binds to β-catenin and retains it in the nucleus, how is it then released to bind to TCF? Does APC exploit its ability to track along the cytoskeleton to deliver β-catenin to the destruction complex, or even to adherens junctions? The ever-growing crowd of researchers studying the Wnt pathway will undoubtedly tackle these issues in the near future. □

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The ins and outs of p53

Karen H. Vousden and George F. Vande Woude

Moving proteins between cellular locations is proving to be a tightly regulated process, and provides an important mechanism for controlling protein function. The tumour-suppressor protein p53 has been shown to use microtubules to aid nuclear localization, a finding that raises interesting questions about the action of microtubule-disrupting chemotherapeutic drugs.

Like the parents of adolescent children, cell biologists routinely ask their favourite protein questions like "who are you hanging around with?" and "where are you going?" After a decade of intensive study of the former question, leading to the identification of seemingly endless combinations of protein–protein interactions, emphasis is now shifting, and interest in the regulation of protein subcellular localization is increasing. On page 709 of this issue, Giannakakou et al.[1] report that the localization of p53 is regulated through interactions with the microtubule network, and furthermore, that the nuclear localization of p53 requires the activity of a microtubule-associated molecular motor. This study builds on an emerging theme that even relatively small proteins use microtubules and molecular motors to move through the cytoplasm, in this case to successfully find and enter the nucleus.

Regulation of protein activity is a complex and multifaceted process. Many proteins only function in certain cellular compartments, and so one efficient way to control their activity is to govern where they are within the cell. In particular, the shuttling of proteins such as transcription factors in and out of the nucleus provides an extremely fast and sensitive mechanism for the regulation of activity. We now have a fairly detailed understanding of how proteins enter and leave the nucleus, with the help of import and export receptors that recognize nuclear-localization or nuclear-export signals within the proteins that need to move from one place to the other[2]. But how do proteins get close enough to the nucleus to engage the import machinery? Although it is possible that the whole process depends on diffusion, a picture is emerging in which navigation of proteins through the cytoplasm is a highly organized process that uses microtubule highways and specific motor proteins. The microtubule network shows an inherent polarity from the dynamic 'plus' end (usually at the cell periphery) to the relatively stable 'minus' end (at the centrosome, usually near, or even linked to, the nucleus). Numerous motor proteins can move along microtubules in one direction or the other, transporting their cargo towards the centre or the edge of the cell. Although the ability of these microtubule highways to transport large structures such as organelles or viruses has been known for some time, it has only recently become evident that microtubules and molecular
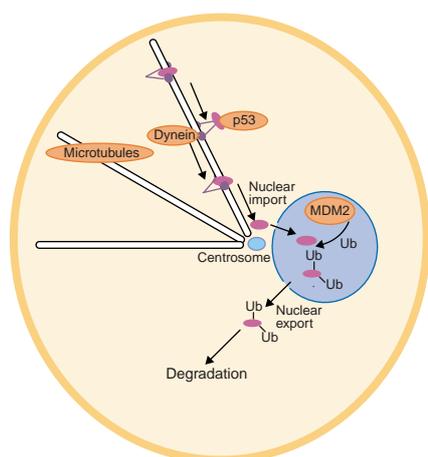
Figure 1 Model for regulation of the subcellular localization of p53. Association with microtubules directs p53 towards the nucleus, using the minus-end-directed molecular motor dynein, where import receptors recognize localization signals in the C terminus of p53. Export from the nucleus requires nuclear-export sequences in p53 and MDM2 activity, possibly through ubiquitination of p53. Defects in the import pathways, or failure to inhibit the export pathways, could compromise the ability to activate p53 and contribute to tumour development. Ub, ubiquitin.

motor proteins can also participate in the navigation of proteins through the cytoplasm of an intact cell.

p53 is a transcription factor that functions to prevent the growth of abnormal or damaged cells through several mechanisms, the best understood being the activation of cell-cycle arrest and apoptosis[3]. The p53-driven elimination of aberrant cells represents an important component of our defence against tumour development, and loss of the p53 response is virtually an obligatory step during carcinogenesis. As p53 has such a potent ability to stop cell growth, normal dividing cells take pains to restrain p53 activity through regulation of the expression, stability, activity and localization of proteins. Previous studies have shown that p53 functions in the nucleus, and the report from Giannakakou and colleagues indicates that this localization of p53 may require an interaction with the microtubule network and the activity of dynein, a minus-end-directed microtubule motor[4], that would move p53 along the microtubules towards the nucleus. The study shows that transport along the microtubules occurs only in response to stress, and that disruption of the microtubule network or interference with dynein function impedes localization of p53 to the nucleus, and so inhibits activation of a p53 response. Interestingly, the amino-terminal

region of p53, which is either directly or indirectly responsible for the interaction with microtubules, is distinct from the carboxy-terminal nuclear-localization signals. It therefore seems possible that these mechanisms are independent, with p53 hitching a ride on the microtubules to get close to the nucleus and then being handed off to the transport receptors for the final ride through the nuclear pore into the nucleus. It is worth remembering, however, that an interaction with microtubules does not necessarily help translocation of all proteins to the nucleus. Indeed, a recent study of Smads has shown that binding to the microtubules may prevent, rather than assist, nuclear translocation[5]. Moreover, movement along microtubules can occur in both directions, and another tumour-suppressor protein, APC, has recently been shown to travel along microtubules in the opposite direction to p53, accumulating at the plasma membrane and possibly contributing to cell migration[6].

Although getting p53 into the nucleus is obviously important in allowing p53 to function, dampening p53 activity in normally dividing cells seems to require transport of p53 out of the nucleus. Critical to the regulation of p53 is the interaction with MDM2, the expression of which is activated by p53 and participates in a negative feedback loop[7]. MDM2 is a RING-finger protein that can target both itself and p53 for ubiquitination, thereby driving degradation of p53 through the proteasome. Interestingly, several lines of evidence indicate that this degradation of p53 occurs in the cytoplasm, although both MDM2 and p53 are nuclear proteins, and a model has emerged in which p53 must be exported from the nucleus to be degraded. This is nicely demonstrated by the use of inhibitors of the nuclear-export machinery, which results in stabilization and activation of p53. Several models of how p53 is exported from the nucleus have been put forward. MDM2 contains nuclear-export signals and in one model, p53 hitches a ride with MDM2 out of the nucleus[8]. Subsequent identification of nuclear-export signals in p53 has complicated this model, and there is some evidence that the export of p53 continues unabated even in the absence of MDM2 (ref. 9). Now a further wrinkle on the system has been uncovered by two studies that have shown that the ubiquitin-ligase activity of MDM2 is necessary for p53 export, but export of MDM2 itself is not required[10,11]. Although not shown directly, these results indicate that ubiquitination of p53 may be necessary for its export, perhaps by revealing the nuclear-export sequence in its C terminus.

Together, the studies indicate that, in the absence of stress signals, p53 activity may be regulated both by suppressing localization to the nucleus and by enhancing transport out of the nucleus. In response to the appropriate signals, nuclear import of p53

is activated, whereas export is inhibited, so maximizing the accumulation of p53 in the nucleus. In tumours that retain wild-type p53 (roughly 50%), defects in either of these pathways would impinge on the ability to activate the p53 response. Several tumour-associated defects have been described that lead to a failure to block MDM2 function in response to stress signals, and so result in an inability to stop the nuclear export and degradation of p53. Examples include mutations of kinases that phosphorylate p53 and MDM2 to prevent their interaction, and loss of expression of inhibitors of the ubiquitin-ligase activity of MDM2, such as ARF[12]. However, a significant number of tumours with wild-type p53, particularly breast cancers and neuroblastomas, seem to be insensitive to the tumour-suppressor protein because p53 is held in the cytoplasm. Although previous studies have shown that cytoplasmic localization of p53 in tumour cells may be the result of a hyperactive nuclear-export system[13], the study by Giannakakou and colleagues raises the possibility that cytoplasmic retention may also result from defects in the ability of p53 to associate with microtubules in response to stress. How this is regulated remains a mystery, but it is always tempting to speculate that phosphorylation, or other stress-induced modifications of p53, may be important.

One of the principal interests in p53 is its potential as a target for tumour therapy. Reactivation of p53 in tumours that retain the wild-type protein seems a reasonable goal, and direct inhibition of MDM2 function has been a popular strategy. This approach is unlikely to be effective, however, in tumours in which p53 never gets into the nucleus because of defects in the microtubule transport system described here. Furthermore, the importance of microtubules in p53 function may have important repercussions in tumour therapy, as microtubule-disrupting drugs like taxol are commonly used for chemotherapy. Somewhat paradoxically, several previous studies have shown that disruption of microtubules results in activation of p53 (ref. 14), and there are opposing views over whether or not p53 influences sensitivity to taxol[15,16].

… This study builds on an emerging theme that even relatively small proteins use microtubules to move through the cytoplasm, in this case to successfully find and enter the nucleus…

Although the exact contribution of p53 to the response to microtubule-damaging drugs clearly needs further clarification, the potential impact of the findings of Giannakakou and colleagues on understanding how anti-microtubule drugs influence p53 function *in vivo* in tumours and normal cells is striking. In cancers that retain wild-type p53, and in which chemosensitivity may be determined, in part, through p53, treatment with anti-microtubule drugs may reduce the p53 response and so be counterproductive. The study also raises important questions concerning the response of normal cells to these types of drugs. For example, can taxol, when used in combination with p53-activating drugs like adriamycin or 5-fluorouracil, interfere with damage repair in normal cells? More importantly, if taxol prevents normal p53 function in a manner similar to the effects observed with the p53 inhibitor PFT-α (ref. 17), then this anti-microtubule drug could also reduce the serious side effects of chemotherapeutic drugs and radiation therapy. This would certainly be an exciting and unexpected new twist on the use of this class of compounds in combined chemotherapeutic regimens. □

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Chemical genetics: new tools for understanding signalling networks

Doug Kellogg

Protein kinases play an integral role in the regulation of virtually every major cellular and developmental process, and an understanding of how protein kinases function in vivo is therefore of central importance to cell biology. Newly developed protein-kinase inhibitors provide powerful tools for understanding the functions of kinases in vivo.

Thus far, our understanding of how protein kinases function *in vivo* has come largely from the analysis of phenotypes caused by conditional mutations, gene deletions or regulated expression. Although these approaches have been highly informative, they suffer from several limitations. Inactivation of protein-kinase function using conditional mutations or regulated expression can take many minutes or hours, whereas signalling events often occur on a time scale of seconds or minutes. Furthermore, cells carrying mutations or gene deletions often have to be grown for many generations before they can be studied, which can make it difficult to determine which phenotypes are due to primary defects. In addition, growth of mutant cells for many generations can result in the accumulation of suppressor mutations that allow the cell to adapt to the loss of a protein kinase. A final problem is that many kinases carry out functions that are independent of their kinase activity, and these can be difficult to discern using standard techniques.

These problems could be circumvented if one had cell-permeable inhibitors that allowed rapid inhibition of specific kinases *in vivo*. So far it has been difficult to find such inhibitors because all kinases share a highly conserved ATP-binding site, and compounds that inhibit one kinase therefore usually inhibit other kinases as well. However, two recent papers by Bishop *et al.*[1,2] describe an elegant solution to this problem and thereby provide a new means with which to understand signalling networks.

As a starting point for this work, Bishop and colleagues decided that the best way to inhibit a specific kinase would be to use site-directed mutagenesis to engineer a kinase with unique properties. In all kinases, the adenosine ring of ATP is closely surrounded by amino-acid side chains that form the ATP-binding site (Fig. 1). A conserved, bulky amino-acid side chain forms part of this pocket, and it was found that this residue can be mutated to glycine in many kinases without destroying kinase activity. Most importantly, this amino-acid change generates a unique ATP-binding site with an extra pocket. A rational molecular design approach was then used to search for molecules that fit uniquely into the engineered ATP-binding site, but not into the ATP-binding site of the wild-type kinase or of any other kinase. This led to the synthesis of a group of compounds that have bulky hydrophobic groups substituted on an adenosine-like molecule called PP1. These PP1 analogues fit into the active site of the engineered kinase and function as highly potent and specific competitive inhibitors of kinase activity without affecting wild-type kinases. To test the general usefulness of this approach, the authors introduced space-creating mutations into the ATP-binding sites of five different kinases, including members of both tyrosine and serine/threonine kinase families. All of these 'analogue-sensitive' kinases were strongly inhibited at low nanomolar concentrations of PP1 analogues, whereas the wild-type versions of the same kinases were unaffected. It seems likely, therefore, that this approach will work for most protein kinases.

The *in vivo* usefulness of analogue-sensitive kinases has been dramatically demonstrated by experiments carried out using four different protein kinases in both budding yeast and mammalian cells[1–3]. Initial experiments were carried out with the V-src tyrosine kinase, an oncogene product that induces transformation of cultured mammalian cells. An analogue-sensitive version of V-src induces a transformed phenotype that can be reversed by addition of inhibitor, demonstrating *in vivo* inhibition of kinase activity. Similar results have more recently been obtained for the serine/threonine kinases Fus3, Cdc28 and Cla4 in budding yeast.

Fus3 is a member of the mitogen-activated