The N-end rule and regulation of apoptosis

Alexander Varshavsky

The ubiquitin-dependent N-end rule pathway targets proteins for degradation through their destabilizing N-terminal residues. This pathway is known to control the import of peptides, chromosome stability and cardiovascular development. A new report identifies yet another function of the N-end rule pathway: the regulation of apoptosis through degradation of *Drosophila melanogaster* DIAP1.

Apoptosis (programmed cell death) in a multicellular organism makes possible the selective elimination of supernumerary, damaged or otherwise abnormal cells. In most cases, the apoptotic death of a cell is caused by the activity of caspases, a set of sequence-specific intracellular proteases that are normally dormant, but which can be activated by signals that induce apoptosis[1,2]. Elaborate mechanisms have evolved to activate caspases through many and varied physiological inputs while maintaining a robust negative control over the activation process. The key caspases must be activated to a significant threshold level before committing a cell to apoptotic death. Inhibitors of apoptosis (IAPs) have a major role in the negative control of caspase activation[3]. The ubiquitin (Ub) system, a large array of proteolytic and non-proteolytic pathways that involve Ub-protein conjugation, is also essential for regulation of apoptosis: the major IAP proteins are specific Ub ligases whose ability to ubiquitylate either themselves or caspases (and other IAP-bound proteins) is important for IAP functions[3–7].

On page 467 of this issue, Ditzel, Meier and colleagues report a major new connection between the Ub system and apoptosis[8]. They demonstrate that *Drosophila* DIAP1, an inhibitor of apoptosis, is cleaved at position 20 by activated caspases, yielding a slightly shorter version of DIAP1 that is degraded by the Ub-dependent N-end rule pathway. This degradation, which does not involve the Ub ligase activity of DIAP1, is shown by the authors to be important for the function of DIAP1 as an inhibitor of apoptosis[8].

The N-end rule relates the *in vivo* half-life of a protein to the identity of its N-terminal residue[9–12]; the underlying proteolytic pathway is called the N-end rule pathway (Fig. 1). Its Ub ligases target protein substrates that bear specific (destabilizing) N-terminal residues. The corresponding degradation signal (degron), called the N-degron, consists of a substrate's destabilizing N-terminal residue and an internal Lys residue, the latter being the site of formation of a substrate-linked poly-Ub chain. A ubiquitylated substrate is processively degraded by the 26S proteasome[10]. Because an N-degron must be produced through a proteolytic cleavage that yields a destabilizing N-terminal residue, a nascent N-end rule substrate contains a cryptic N-degron, called a pro-N-degron.

In the yeast *Saccharomyces cerevisiae*, where a mechanistic understanding of the pathway is most detailed, the Ub ligase UBR1 recognizes (binds to) the primary destabilizing N-terminal residues Arg, Lys, His, Phe, Trp, Leu, Tyr and Ile (Fig. 1)[10,13]. Several other N-terminal residues function as tertiary (Asn and Gln) and secondary (Asp and Glu) destabilizing residues in that they are recognized by UBR1 after their enzymatic conjugation to Arg, a primary destabilizing residue. With N-terminal Asn and Gln, their conjugation to Arg is preceded by their enzymatic deamidation[14]. In metazoans, but not in fungi such as *S. cerevisiae*, N-terminal Cys is yet another tertiary destabilizing residue, in that the arginylation of Cys is preceded by its (apparently) enzymatic oxidation[12] (Fig. 1).

One function of the N-end rule pathway is regulation of peptide import. The UBR1 Ub ligase of the *S. cerevisiae* N-end rule pathway contains a distinct (third) substrate-binding site[13] that recognizes an internal (non-N-terminal) degron in the pathway's substrates, such as the transcriptional repressor CUP9. Dipeptides with destabilizing N-terminal residues bind to UBR1, thereby increasing the ability of UBR1 to target CUP9 for degradation[11]. The resulting decrease in CUP9 concentration causes derepression of the *PTR2* gene, which encodes a peptide transporter. Through this positive feedback in the UBR1–CUP9–PTR2 circuit, cells can sense the presence of extracellular peptides and react by accelerating their uptake[11].

Another function of the N-end rule pathway is the maintenance of chromosome stability. SCC1, a subunit of the *S. cerevisiae* cohesin complex, is cleaved by the ESP1 separase at the metaphase–anaphase transition, yielding a C-terminal fragment of SCC1 that bears N-terminal Arg and is degraded by the N-end rule pathway[15]. In *ubr1*Δ cells, failure to degrade the SCC1 fragment results in chromosome instability[15]. In mammalian cells, a fraction of the orthologous SCC1 (RAD21) cohesin subunit is also cleaved by separase at the metaphase–anaphase transition[16], and the resulting C-terminal fragment of SCC1, similarly to its yeast counterpart, is a short-lived substrate of the mammalian N-end rule pathway (J. Zhou, Y. T. Kwon and A.V., unpublished observations). The N-end rule pathway is also required for cardiovascular development in mammals[12].

We now learn about yet another function of this pathway: the regulation of apoptosis. Although caspases have been known to produce protein fragments bearing destabilizing N-terminal residues[10], the work by Ditzel *et al.*[8] demonstrates for the first time that at least one such fragment — of *Drosophila* DIAP1 — is, in fact, a physiological N-end rule substrate and that degradation of the fragment by the N-end rule pathway is important for regulation of apoptosis. *Drosophila* DIAP1 is the key inhibitor of apoptosis in *Drosophila*: a deletion of its gene results in apoptotic death of most cells in early *Drosophila* embryos[3]. Caspase inhibition is the major function of DIAP1 and other IAP proteins in all multicellular organisms, from animals to plants. A typical IAP protein is a complicated device: in addition to its ability to bind, and thereby to inactivate, specific caspases, it also contains a RING domain and can therefore function as a Ub ligase, mediating either self-ubiquitylation or ubiquitylation of IAP-bound proteins[3].

Reaper, Grim and Hid in *Drosophila*, and Smac/DIABLO in mammalian cells function as activators of apoptosis. These proteins counteract the protective effects of IAPs by competing with caspases for binding to IAPs, thus increasing the levels of active caspases. Activators of apoptosis can also increase the rate of self-ubiquitylation (and subsequent degradation) of IAP proteins. In addition, at least some of these activators can inhibit cellular protein synthesis. This

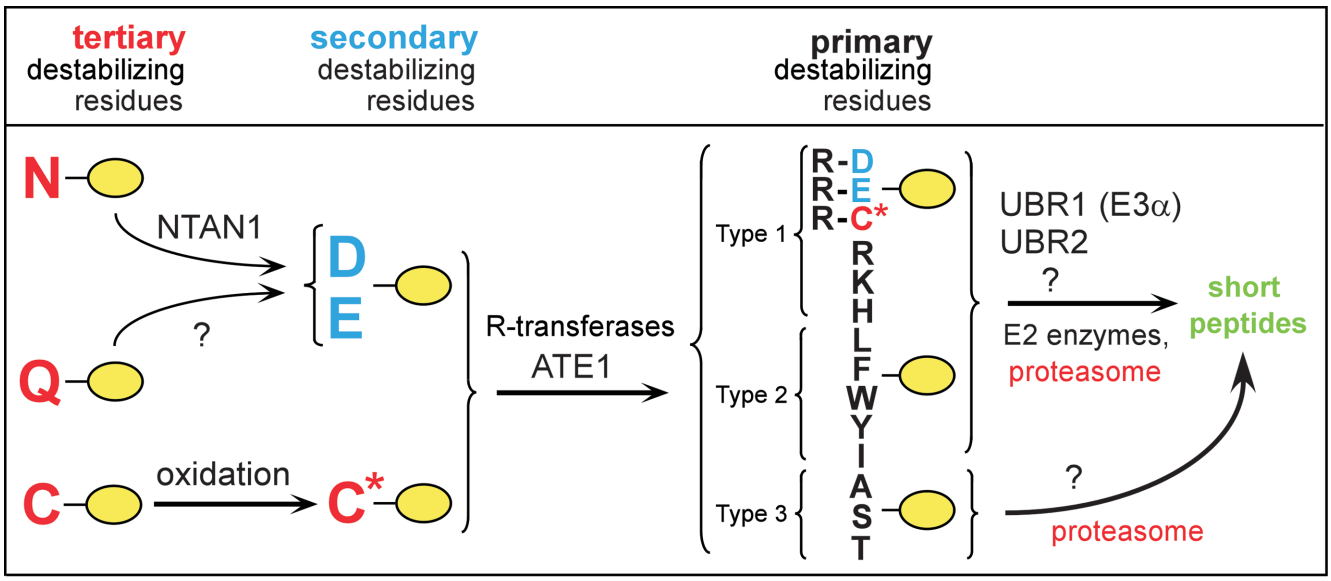


Figure 1 **The N-end rule pathway in mammals. N-terminal residues are indicated by single-letter abbreviations. Ovals denote the rest of a protein substrate. The Asn-specific N-terminal amidase NTAN1 converts N-terminal Asn into Asp[14]. N-terminal Gln is deamidated by another (unidentified) amidase. N-terminal Asp, Glu and Cys are conjugated to Arg by Arg-tRNA protein transferases (R transferases), encoded by the *ATE1* gene[12]. N-terminal Cys is arginylated after its (apparently) enzymatic oxidation to either sulphinic or cysteic acid residue (denoted as C*)[12]. In the yeast *S. cerevisiae*, the primary destabilizing N-terminal residues Arg, Lys, His (type-1 residues), and Phe, Leu, Trp, Tyr and Ile (type-2 residues) are recognized by the type-1 and type-2 substrate-binding sites of UBR1 in a complex with the RAD6 ubiquitin-conjugating enzyme (E2)[10,13]. In mammals, the same residues are recognized by either UBR1 or UBR2 (in complexes with their cognate E2 enzymes), and also by another, unidentified E3 (ref. 22). In mammals, but not in yeast, Ala, Ser and Thr are primary (type-3) destabilizing residues recognized by an uncharacterized Ub ligase. Both *S. cerevisiae* UBR1 and its mammalian counterparts contain yet another (third) substrate-binding site that targets proteins through their internal (non-N-terminal) degrons[11,13].**

preferentially decreases the concentrations of IAP proteins, whose *in vivo* half-lives tend to be shorter than the half-lives of active caspases[3]. The IAPs fight back, in part through their ability to be destroyed by the Ub system together with IAP-bound activators of apoptosis[7]. In a non-apoptotic cell, the IAPs keep an upper hand by being in stoichiometric excess over the activators of apoptosis and by physically sequestering the (relatively few) molecules of activated caspases, which are produced at significant rates even in the absence of pro-apoptotic signals.

The results by Ditzel *et al.*[8] have now added the N-end rule pathway to this complex picture. It was found that activated caspases cleave the 438-residue DIAP1 protein at position 20, resulting in an almost full-length DIAP1 bearing N-terminal Asn, a tertiary destabilizing residue (Fig. 1). The Asn-bearing DIAP1 fragment is degraded by the N-end rule pathway in a manner that does not require the Ub-ligase activity of DIAP1 itself[8]. One would expect a short-lived protein bearing N-terminal Asn to be targeted for degradation by the deamidation–arginylation branch of the N-end rule pathway (Fig. 1). Indeed, by using RNA interference to reduce the expression of the *Drosophila ATE1*-encoded R-transferase (Fig. 1), Ditzel *et al.*[8] demonstrated that degradation of the Asn-bearing DIAP1 fragment required arginylation. Remarkably, the caspase-mediated cleavage at position 20 that converts DIAP1 into an N-end rule substrate was found to be essential for the ability of DIAP1 to suppress the pro-apoptotic activity of Reaper[8]. In experiments with whole flies and cells in culture, Ditzel *et al.*[8] went on to show that degradation of the DIAP1 fragment by the N-end rule pathway was a major cause of the anti-Reaper activity of DIAP1 .

Thus, paradoxically, although the N-end rule-mediated degradation of DIAP1 decreases the level of this key apoptotic inhibitor and would therefore be expected to lower the apoptotic threshold, this degradation is important for DIAP1-mediated inhibition of apoptosis. A parsimonious interpretation of these findings[8], in conjunction with earlier evidence, is that the anti-apoptotic activity of DIAP1 is mediated by Ub-dependent codegradation of both DIAP1 and its associated pro-apoptotic ligands, such as caspases and Reaper. This codegradation could be achieved either through the N-end rule pathway or through the Ub-ligase activity of DIAP1 itself. Exactly how these alternative targeting routes are chosen for individual DIAP1 molecules and the exact stoichiometries of codegraded DIAP1-bound ligands remains to be determined. At least one mammalian counterpart of *Drosophila* DIAP1, called XIAP, is also cleaved by activated caspases[17]. The resulting C-terminal fragment of XIAP bears N-terminal Ala, a type-3 primary destabilizing residue in the mammalian N-end rule (Fig. 1; the type-3 branch of the N-end rule pathway is the least explored one; its Ub ligase remains to be characterized).

Fig. 2 provides a partial list of fly and mammalian proteins that are known to be cleaved by caspases to yield C-terminal fragments with destabilizing N-terminal residues. Thus far, the Asn-bearing C-terminal fragment of *Drosophila* DIAP1 is the only member of this list that has actually been shown to be a physiological N-end rule substrate[8]. The set of proteins whose cleavage by caspases produce putative N-end rule substrates encompasses a broad

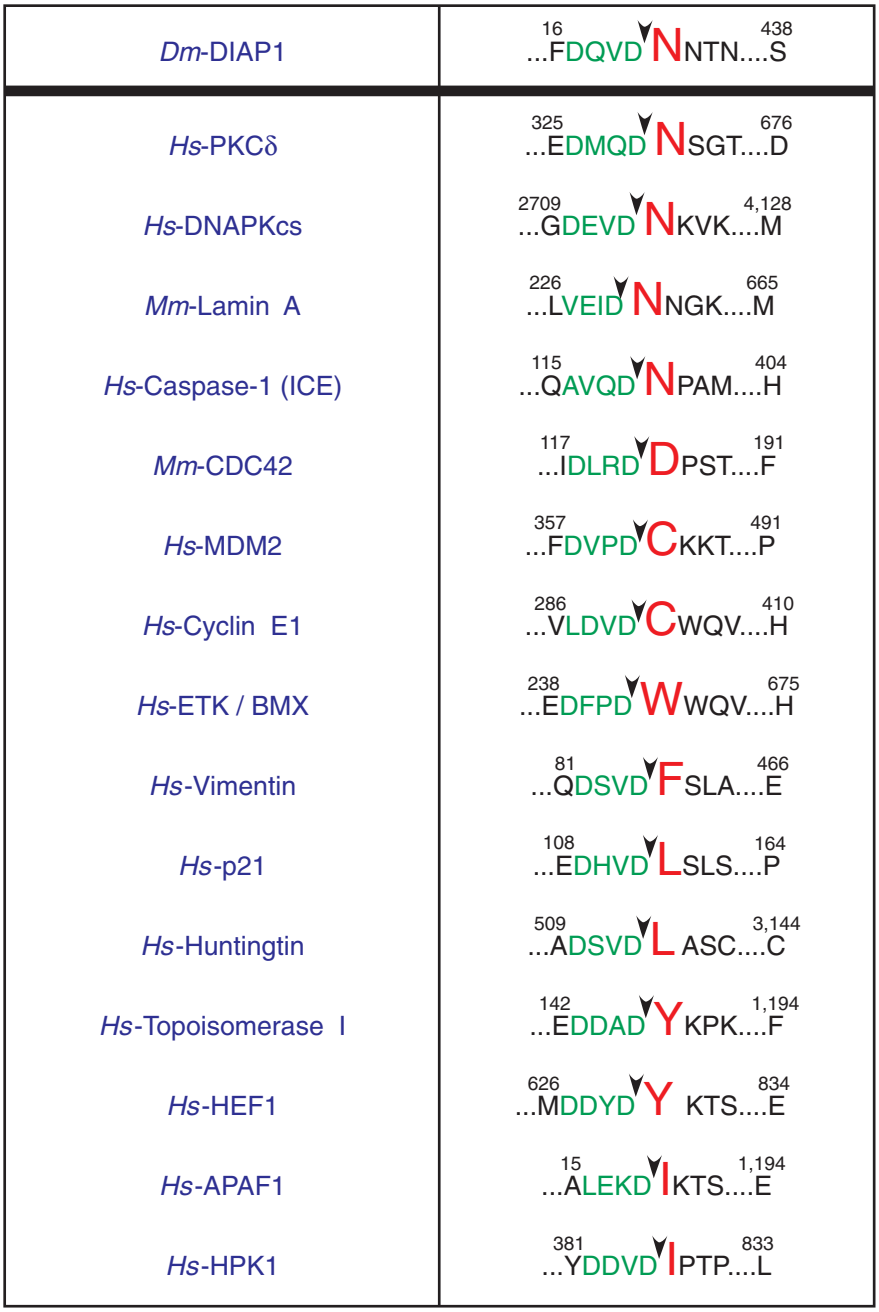


Figure 2 **Confirmed and putative N-end rule substrates produced by caspases. Amino-acid residues are indicated by single-letter abbreviations. The caspase-recognition motifs (green) precede an arrowhead indicating the site of cleavage and a destabilizing residue (red) that becomes N-terminal in a caspase-produced fragment. The numbers represent a residue preceding a caspase recognition motif and the last encoded residue of the protein, respectively. The prefixes *Dm*, *Hs* and *Mm* refer to proteins of *Drosophila melanogaster*, *Homo sapiens* and *Mus musculus*, respectively. All fragments in this list were actually observed (the corresponding papers will be cited in a more detailed review elsewhere). Thus far, the degradation by the N-end rule pathway and the physiological relevance of this degradation have only been demonstrated for *Drosophila* DIAP1 (ref. 8).**

range: phosphokinases, Ub ligases, transcription factors, DNA topoisomerases and regulators of the cell cycle (Fig. 2). This set includes both inducers and inhibitors of apoptosis; it also contains proteins, such as lamin and vimentin, that are normally cleaved by caspases at later (committed) stages of apoptosis. Caspases also have apoptosis-independent functions[18]. In addition, the suppression of caspase activation *in vivo* is not tight enough to preclude caspase activation entirely. Therefore even a normal, unstressed cell invariably contains significant levels of caspase-produced protein fragments, including the fragments described in Fig. 2. If a caspase-produced C-terminal fragment of a given protein has a pro-apoptotic activity or is otherwise toxic (such examples are known), one way to reduce accumulation of this fragment would be to make it short-lived. This teleology, which is additional to and distinct from the DIAP1-type function of the N-end rule pathway[8], may be a major source of evolutionary pressure that resulted in the frequent presence of destabilizing residues at the amino termini of protein fragments produced by caspases.

The phenotypes that result from the absence of the deamidation–arginylation branch of the N-end rule pathway vary greatly in their severity amongst different organisms. For example, arginylation-deficient *ate1Δ S. cerevisiae* cells are otherwise seemingly normal[19]. The arginylation- deficient *ATE1*$^{-/-}$ mutant of the plant *Arabidopsis thaliana* is viable but exhibits delayed leaf senescence[20]. Furthermore, arginylation-deficient *ATE1*$^{-/-}$ mice die as embryos, at least partly from cardiovascular defects[12]. In contrast, *NTAN1*$^{-/-}$ mice, which lack the Asn-specific N-terminal amidase but retain the arginylation step of the N-end rule pathway, exhibit abnormal behaviours but otherwise seem to be wild type[14]. Although the null *ate1* phenotype of *D. melanogaster* remains to be determined, it will probably be severe, because even heterozygous *ATE1*$^{+/-}$ flies in a genetic background that ectopically expresses Reaper in the developing retina fail to eclose and die in their pupae cases[8].

Processing proteases that convert pro-N-degrons into N-degrons have long been envisioned as regulated entry points to the N-end rule pathway[9,10]. Such proteases include both methionine aminopeptidases (Met-APs)[21] and cohesin-cleaving separases[15]. The study by Ditzel *et al.*[8] has now demonstrated that caspases, too, can function as specific entry points to the N-end rule pathway and that degradation of a caspase-produced N-end rule substrate such as DIAP1 by this pathway is important for the control of apoptosis. Given this insight and the broad range of caspase-produced protein fragments that are putative N-end rule substrates (Fig. 2), it would be particularly informative to identify among such fragments those that are actually destroyed by the N-end rule pathway and to determine the functions of this degradation. The set of proteases that produce physiological N-end rule substrates is unlikely to be confined to

Met-APs, caspases and separases. For example, the cleavage specificity of calcium-activated proteases called calpains and the properties of other, less well characterized cytosolic and/or nuclear proteases suggest that they, too, may function as upstream components of the N-end rule pathway. The DIAP1 findings by Ditzel *et al.*[8] are exciting not only because of what they tell us about the link between apoptosis and the N-end rule pathway. They also suggest the existence of other caspase-produced N-end rule substrates, of which the list in Fig. 2 is just the start. These substrates remain to be identified and understood in the context of N-end rule's physiology. □

Alexander Varshavsky is in the Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA.
e-mail: avarsh@caltech.edu

1. Metzstein, M. M., Stanfield, G. M. & Horvitz, H. R. *Trends Genet.* **14,** 410–416 (1998).
2. Guo, M. & Hay, B. A. *Curr. Opin. Cell Biol.* **11,** 745–752 (1999).
3. Salvesen, G. S. & Duckett, C. S. *Nature Rev. Mol. Cell Biol.* **3,** 401–410 (2002).
4. Yoo, S. J. Y. *et al. Nature Cell Biol.* **4,** 416–424 (2002).
5. Yang, Y., Fang, S., Jensen, J. P., Weissman, A. M. & Ashwell, J. D. *Science* **288,** 874–877 (2000).
6. Ryoo, H. D., Bergmann, A., Gonen, H., Ciechanover, A. & Steller, H. *Nature Cell Biol.* **4,** 432–438 (2002).
7. Olson, M. R. *et al. J. Biol. Chem.* **278,** 4028–4034 (2003).
8. Ditzel, M. *et al. Nature Cell Biol.* **5,** 467–473 (2003).
9. Bachmair, A., Finley, D. & Varshavsky, A. *Science* **234,** 179–186 (1986).
10. Varshavsky, A. *Proc. Natl Acad. Sci. USA* **93,** 12142–12149 (1996).
11. Turner, G. C., Du, F. & Varshavsky, A. *Nature* **405,** 579–583 (2000).
12. Kwon, Y. T. *et al. Science* **297,** 96–99 (2002).
13. Du, F., Navarro-Garcia, F., Xia, Z., Tasaki, T. & Varshavsky, A. *Proc. Natl Acad. Sci. USA* **99,** 14110–14115 (2002).
14. Kwon, Y. T. *et al. Mol. Cell. Biol.* **20,** 4135–4148 (2000).
15. Rao, H., Uhlmann, F., Nasmyth, K. & Varshavsky, A. *Nature* **410,** 955–960 (2001).
16. Hauf, S., Waizenegger, I. C. & Peters, J.-M. *Science* **293,** 1320–1323 (2001).
17. Deveraux, Q. L. *et al. EMBO J.* **18,** 5242–5251 (1999).
18. Algeciras-Schimnich, A., Barnhart, B. C. & Peter, M. E. *Curr. Opin. Cell Biol.* **14,** 721–726 (2002).
19. Balzi, E., Choder, M., Chen, W., Varshavsky, A. & Goffeau, A. *J. Biol. Chem.* **265,** 7464–7471 (1990).
20. Yoshida, S., Ito, M., Gallis, J., Nishida, I. & Watanabe, A. *Plant J.* **32,** 129–137 (2002).
21. Davydov, I. V. & Varshavsky, A. *J. Biol. Chem.* **275,** 22931–22941 (2000).
22. Kwon, Y. T., Xia, Z., Davydov, I. V., Lecker, S. H. & Varshavsky, A. *Mol. Cell. Biol.* **21,** 8007–8021 (2001).

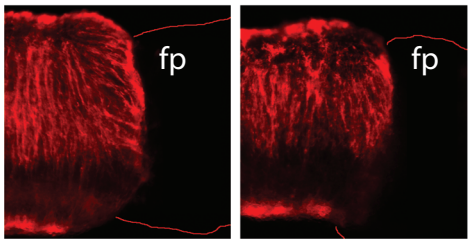
Morphogens recycled

Despite its eventual complexity, the developing nervous system seems to be directed by an elegant economy of cues. We are beginning to learn that factors can perform more than one job during its development, and in the April issue of *Cell* (**113,** 11–23 (2003)), Tessier-Lavigne and colleagues provide compelling evidence that Sonic Hedgehog (Shh), a morphogen known for its role in neural tissue patterning, also functions later to attract navigating axons.

During development of the nervous system, axons are guided by a combination of attractive and repulsive cues. In the embryonic spinal cord, a subset of axons are attracted from the dorsal neural tube towards the floor plate, where they form axon commissures. It is well established that outgrowth of these axons and their subsequent attraction towards the floor plate is directed by the long-range chemoattractant Netrin-1. However, the defects observed in *Netrin-1* mutants have hinted that residual chemoattractant activity may reside in the floor plate in its absence and raise the possibility that a second attractant is guiding the axons. Tessier-Lavigne and colleagues now show that this second signal is provided by Shh. Interestingly, unlike Netrin-1, Shh cannot function as a permissive factor to stimulate the outgrowth of these axons *per se*, but seems to be important both *in vitro* and *in vivo* for their subsequent routing.

The authors find that both COS cells and tissue explants expressing Shh, similarly to those expressing Netrin-1, can reorientate commissural axons. The chemoattractant effects of Shh seem to require Smoothened (Smo; see figure), a transmembrane protein known to function downstream of Shh in other contexts. To exclude the possibility that Shh might affect guidance indirectly, they also confirmed that it cannot induce repatterning of older spinal cord tissue in which the axons grow, as it can earlier in development. Moreover, they showed that applying a soluble source of Shh close to growing axons in culture was sufficient to trigger axon turning, an effect that also required Smo activity.

Finally, they asked whether Shh signalling is required *in vivo*. Using the Cre-LoxP recombinase system, they selectively



Smo activity in the floor plate. *Netrin-1*-mutant floor plate attracts commissural axons, revealing a second chemoattractant from the floor plate (left). Cyclopamine, an inhibitor of Smo, inhibits the Netrin-independent activity of the floor plate, indicating that Shh signalling is required for this activity (right). fp, floor plate. Reprinted from Charron *et al.* 113, 11–23 © (2003) with permission from Elsevier.

disrupted Smo function in subsets of dorsal spinal cord cells, including commissural neurons, and showed that this results in defects in axon guidance. Importantly, this suggests that the Shh signal is required autonomously in the commissural neurons themselves.

Together, these findings show that in addition to its earlier role in directing ventral tissue patterning, Shh functions later as a chemoattractant, guiding axons towards the floor plate. This adds to a rapidly growing list of factors that are re-used by the embryo for diverse functions during development. We know that another family of morphogens, the bone morphogenetic proteins (BMPs), which pattern the dorsal spinal cord, function later to repel commissural axons away from the dorsal midline. So a model begins to emerge where Shh and BMPs, which initially cooperate during patterning along the dorsoventral axis, pair up again to lend commissural axons a guiding hand.

ALISON SCHULDT