They found evidence that the Beringian wolves were morphologically different from modern North American wolves and from Pleistocene wolves from more southern regions. Moreover, the differences in morphology suggest that the Pleistocene Beringian wolves were adapted to hunting and scavenging members of the now extinct megafauna, a conclusion supported by isotope analysis. Finally, these wolves not only represented a different ecomorph, they were also genetically distinct. Not a single sequence of their mitochondrial DNA haplotypes exactly matched sequences found in modern and historical wolves identified to date. However, some of the sequences perfectly matched, albeit only for short stretches, sequences obtained from Eurasian Pleistocene wolves [15], from as far west as the Czech Republic. Thus, Pleistocene wolves across Northern Eurasia and America may actually have represented a continuous and almost panmictic population that was genetically and probably also ecologically distinct from the wolves living in this area today. Despite their high mobility, these wolves did not escape the megafaunal extinctions at the end of the Pleistocene, even though the causes of their extinction are unclear. The specialised Pleistocene wolves, thus, did not contribute to the genetic diversity of modern wolves. Rather, modern wolf populations across the Holarctic are likely be the descendants of wolves from populations that came from more southern refuges as suggested previously for the North American wolves [14]. If this is true for a highly mobile and ecologically adaptable species like the gray wolf [16], it is also likely to be true for other surviving species.

The results obtained by Leonard *et al.* [5] clearly call for an extension of interdisciplinary studies, both on Eurasian wolves and other surviving holarctic species. As shown for bisons, most of the genetic diversity of megafaunal animals may have been lost at the end of the Pleistocene, even in surviving species [4]. Moreover, most of this diversity seems to have accumulated during the 100,000 years between the last two glacial maxima 130,000 and 30,000 years ago, respectively. Both the genetic diversity and the ecological adaptations of populations may therefore be much more ephemeral than previously believed. While the results by Leonard *et al.* [5] do not immediately help in deciphering the causes of Late Pleistocene extinctions, they show that the ecological and population changes occurring at that time were rather complex and cannot simply be explained by the survival of some species and extinction of others.

References

1. Barnosky, A.D., Koch, P.L., Feranec, R.S., Wing, S.L., and Shabel, A.B. (2004). Assessing the causes of late Pleistocene extinctions on the continents. Science *306*, 70–75.
2. Barnes, I., Matheus, P., Shapiro, B., Jensen, D., and Cooper, A. (2002). Dynamics of Pleistocene population extinctions in Beringian brown bears. Science *295*, 2267–2270.
3. Guthrie, R.D. (2006). New carbon dates link climatic change with human colonization and Pleistocene extinctions. Nature *441*, 207–209.
4. Shapiro, B., Drummond, A.J., Rambaut, A., Wilson, M.C., Matheus, P.E., Sher, A.V., Pybus, O.G., Gilbert, M.T.P., Barnes, I., Binladen, J., *et al*. (2004). Rise and fall of the Beringian steppe bison. Science *306*, 1561–1565.
5. Leonard, J.A., Vilà, C., Fox-Dobbs, K., Koch, P.L., Wayne, R.K., and Van Valkenburgh, B. (2007). Megafaunal extinctions and the disappearance of a specialized wolf ecomorph. Curr. Biol. *17*, 1146–1150.
6. Reed, F.A., and Tishkoff, S.A. (2006). African human diversity, origins and migrations. Curr. Opin. Genet. Dev. *16*, 597–605.
7. Roberts, R.G., Flannery, T.F., Ayliffe, L.K., Yoshida, H., Olley, J.M., Prideaux, G.J., Laslett, G.M., Baynes, A., Smith, M.A., Jones, R., *et al*. (2001). New ages for the last Australian megafauna: continent-wide extinction about 46,000 years ago. Science *292*, 1888–1892.
8. Fiedel, S., and Haynes, G. (2004). A premature burial: Comments on Grayson and Meltzer's "Requiem for overkill". J. Archaeological Sci. *31*, 121–131.
9. Grayson, D.K., and Meltzer, D.J. (2003). A requiem for North American overkill. J. Archaeological Sci. *30*, 585–593.
10. Martin, P.S. (1984). Prehistoric overkill: the global model. In Quaternary Extinctions: A Prehistoric Revolution, P.S. Martin and R.G. Klein, eds. (Tucson: University of Arizona Press), pp. 354–403.
11. Steadman, D.W., Martin, P.S., MacPhee, R.D., Jull, A.J., McDonald, H.G., Woods, C.A., Iturralde-Vinent, M., and Hodgins, G.W. (2005). Asynchronous extinction of late Quaternary sloths on continents and islands. Proc. Natl. Acad. Sci. USA *102*, 11763–11768.
12. Turvey, S.T., Oliver, J.R., Narganes Storde, Y.M., and Rye, P. (2007). Late Holocene extinction of Puerto Rican native land mammals. Biol. Lett. *3*, 193–196.
13. Vila, C., Amorim, I., Leonard, J., Posada, D., Castroviejo, J., Petrucci-Fonseca, F., Crandall, K., Ellegren, H., and Wayne, R. (1999). Mitochondrial DNA phylogeography and population history of the grey wolf *Canis lupus*. Mol. Ecol. *8*, 2089–2103.
14. Leonard, J.A., Vila, C., and Wayne, R.K. (2005). Legacy lost: genetic variability and population size of extirpated US grey wolves (*Canis lupus*). Mol. Ecol. *14*, 9–17.
15. Stiller, M., Green, R.E., Ronan, M., Simons, J.F., Du, L., He, W., Egholm, M., Rothberg, J.M., Keates, S.G., Ovodov, N.D., *et al*. (2006). Patterns of nucleotide misincorporations during enzymatic amplification and direct large-scale sequencing of ancient DNA. Proc. Natl. Acad. Sci. USA *103*, 13578–13584.
16. Stahler, D.R., Smith, D.W., and Guernsey, D.S. (2006). Foraging and feeding ecology of the gray wolf (Canis lupus): lessons from Yellowstone National Park, Wyoming, USA. J. Nutr. *136*, 1923S–1926S.

Max Planck Institute for Evolutionary Anthropology, Deutscher Platz 6, D-04103 Leipzig, Germany.
E-mail: hofreite@eva.mpg.de

DOI: 10.1016/j.cub.2007.06.031

Neurite Outgrowth: A Flick of the Wrist

A new study has shown that, near the tip of a growing axon, dephosphorylation of the microtubule-associated protein Doublecortin is controlled by protein phosphatase 1 and its regulator spinophilin. This results in spatially regulated microtubule bundling within the axon and more efficient axon outgrowth.

Leif Dehmelt and Shelley Halpain

Wiring up the nervous system requires that neurons migrate, extend axons and make axonal-guidance decisions with relatively high spatial and temporal fidelity. When such 'geographic' events go awry — even within a subset of neurons — the development of

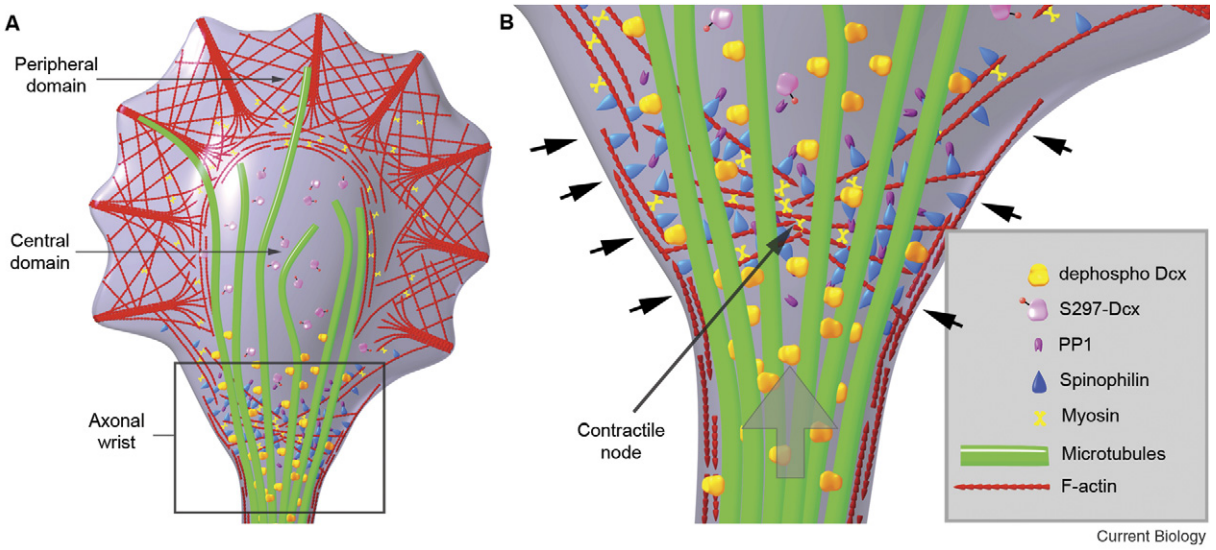

Figure 1. Growth cones.

(A) Two main subregions of a growth cone, the peripheral domain and the central domain, can be distinguished. The peripheral domain is characterized by F-actin-rich filopodia, separated by a looser meshwork of F-actin. The central domain contains organelles and splayed microtubules. The intersection between these domains is sometimes called the transition zone, which harbors contractile actomyosin structures called actin arcs [15]. Bielas *et al.* [1] introduce an additional region, the axonal wrist: an area in which the splayed microtubule array found in the central domain coalesces into a densely bundled microtubule array. (B) The adapter protein Spinophilin is enriched in the axonal wrist, where it is thought to recruit the protein phosphatase 1 (PP1), which in turn dephosphorylates position serine 297 on Doublecortin (Dcx). Dephosphorylation at this site enhances the interaction between doublecortin and microtubules, leading to enhanced microtubule stability. Microtubule bundle formation likely involves several additional key players. Contractile forces (small black arrows) originate from cortical actomyosin and point towards a contractile node near the axonal wrist [10]. Together with additional dynein-mediated forces acting on the microtubule array [11] (large transparent arrow), microtubules could be forced into a straight, bundled configuration. MAPs such as Doublecortin might either stabilize such linkages, or mediate the linkage of actomyosin contractility to the microtubule array.

neural maps and circuitry can become severely impaired. The coordinated regulation of the neuronal cytoskeleton is key to these morphological events. Many neuron-specific regulators of the cytoskeleton have been identified, and their activity is often controlled through protein phosphorylation. But the significance of such regulation for neuromorphogenesis remains poorly understood. A new paper by Bielas *et al.* [1] compellingly unites studies of knockout mice with cell biological and molecular experiments to reveal a regulatory pathway that is important in axon outgrowth.

The microtubule cytoskeleton plays a key role in the formation of both axons and dendrites (collectively termed 'neurites'). Early in the genesis of neuronal morphology, microtubules become reorganized from the radial arrays, which are characteristic of most non-neuronal cells, into parallel arrays that mediate the formation and maintenance of neurites. Such bundles of microtubules are closely packed within the neurite shaft, but they typically splay out into a looser, more disorganized pattern within the hand-shaped growth cone that tips the nascent neurite [2] (Figure 1). Microtubules within the neurite shaft are also fairly stable, whereas those within the growth cone are much more dynamic, polymerizing and depolymerizing with higher frequency. If the growth cone is likened to a hand, then the neurite shaft corresponds to the arm, and the region between them to the wrist. It is at this 'wrist' that the transition between bundled and non-bundled (and between stable and unstable) microtubules is found.

Neurons express specialized microtubule-associated proteins (MAPs) that promote microtubule reorganization and regulate microtubule stability. One such MAP is the X-linked gene product Doublecortin, so named because mutations in its microtubule binding domain cause neuronal migration defects in humans. In females carrying these mutations, some migrating cortical neurons come to rest anomalously between cell layers, resulting in a 'double cortex'; in males, the mutations result in lissencephaly ('smooth brain'). Neuronal migration defects are less severe in *doublecortin* knockout mice; however, new evidence indicates that axon outgrowth is impaired in knockout mice [1] as well as in humans with doublecortin mutations [3].

Doublecortin stabilizes microtubules by binding between protofilaments and counteracting their natural tendency to bend outward [4], thereby preventing microtubule 'catastrophes' — a term used to describe the conversion of a growing microtubule into a shrinking microtubule. The interaction between Doublecortin and microtubules is regulated by protein kinases, including JNK, PKA, MARK and Cdk5 [5–7]. Interestingly, knock-out animals lacking Cdk5 show similar lamination defects to mice lacking Doublecortin, suggesting that the two proteins might act in the same pathway *in vivo*. Studies also

suggest that Cdk5 is the dominant kinase for phosphorylating Doublecortin at residue serine 297 *in vivo*. Although this site is located outside the microtubule-binding domains of Doublecortin, phosphorylation reduces its ability to bind and stabilize microtubules and thus inhibits these main functions of doublecortin [7].

Bielas *et al.* [1] now propose a molecular mechanism for how the phosphorylation of Ser 297 is spatially controlled within subregions of the growth cone. The experimental data indicate that the adapter protein spinophilin [8] (also known as neurabin II [9]) targets protein phosphatase 1 (PP1) to the axonal wrist, where PP1 then dephosphorylates Doublecortin at the serine 297 site (Figure 2). The authors used a phosphoepitope-specific antibody to confirm that phosphorylation at serine 297 is low within this axonal domain. Neurons cultured from *doublecortin* knockout mice are impaired in neurite outgrowth, and show greater separation of microtubules within the axon shaft. Likewise, neurons from *spinophilin* knockout mice, or neurons depleted in PP1 catalytic subunit by *in utero* RNA interference, showed a similar impairment of microtubule bundle formation. Furthermore, exogenous spinophilin was able to rescue the bundling defect in *spinophilin* knockout neurons, but not when its doublecortin binding site was mutated.

Together these results demonstrate a requirement for Doublecortin–Spinophilin interaction in microtubule bundling in the axonal wrist. Such results provide a cellular substrate for Bielas *et al.*'s [1] finding that doublecortin and spinophilin cooperate *in vivo* to enable formation of the corpus callosum and anterior commissure, two brain tracts that require long-distance axonal growth. It remains to be discovered how spinophilin achieves its localized enrichment within the axonal wrist, but it is reasonable to speculate that this could involve its known actin binding activity [9].

How does doublecortin promote microtubule bundling? *In vitro* studies suggest that at high concentrations Doublecortin by itself is sufficient to bundle microtubules [4]. However, microtubule spacing within such bundles (~10 nm) is shorter than the microtubule spacing normally seen within the axon shaft (20–30 nm). Further work is needed to ascertain whether doublecortin itself mediates direct microtubule crosslinking *in vivo*.

Other mechanisms might work together with Doublecortin to bundle microtubules within the axonal wrist. Zhang *et al.* [10] have proposed that growth cone microtubules are compressed towards a contractile node located near the axonal wrist by virtue of actomyosin contractility (Figure 1B). Bielas *et al.* [1] suggest that the Doublecortin–Spinophilin complex can crosslink the microtubule and actin cytoskeletons *in vitro*. It would be interesting to investigate whether this interaction plays a role in linking actomyosin contractility to microtubule bundle formation within the axonal wrist, or if other microtubule–actin crosslinkers play a dominant role. Motor protein MAPs, such as dynein, can help microtubules generate an outward force during neurite extension [11]. The coordinated actions of outward forces and inward contraction on stabilized microtubules might be sufficient to squeeze microtubules into parallel arrays independent of physical crosslinks. Interestingly, Doublecortin and Lis1, another gene product linked to lissencephaly, form functional complexes with dynein to regulate nuclear migration [12]. Perhaps a similar complex functions in neurite outgrowth.

Although the timing of axon outgrowth *in vivo* is delayed in *doublecortin* single knockout animals, long-distance axon tracts do eventually reach their targets [1]. So other microtubule regulators must cooperate with Doublecortin during microtubule bundle formation. Prime candidates for such factors are MAPs of the MAP2/tau or MAP1B families, as well as other members of the doublecortin family, such as Doublecortin-like kinase [13]. Mice that singly lack either MAP2 or MAP1B show no gross developmental defects; however, double knock-out animals lacking both proteins display severe phenotypes, suggesting functional redundancy, even between these structurally unrelated MAPs [14]. Certain impairments in MAP1B/MAP2 double knockout mice, such

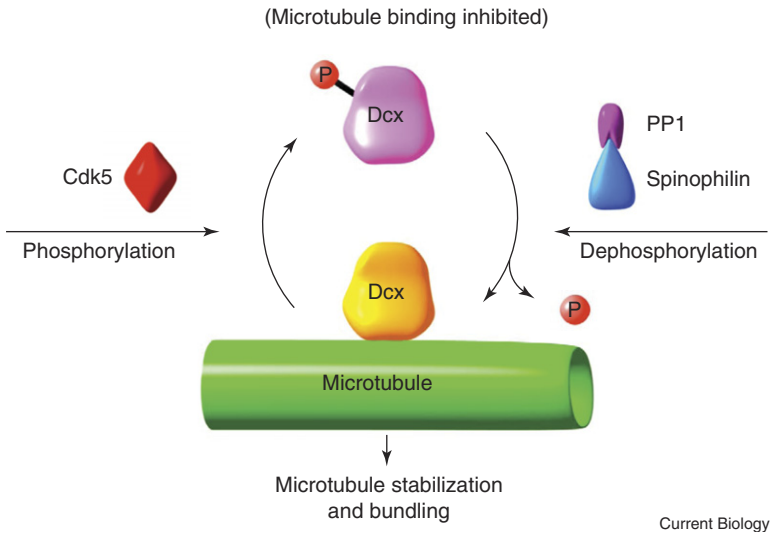

Figure 2. Doublecortin and microtubules.

The interaction between Doublecortin and microtubules is modulated by phosphorylation at residue serine 297. The dominant protein kinase that phosphorylates this site is Cdk5 [7]. Bielas *et al.* [1] have now shown that Spinophilin mediates protein phosphatase 1 (PP1) dependent dephosphorylation of Doublecortin. Thus, the Spinophilin–PP1 complex can reactivate doublecortin and thereby enhance microtubule stabilization and bundling.

as lamination defects, seem strikingly related to the *doublecortin* phenotype. Furthermore, knock-out animals lacking either MAP2 or MAP1B show slight impairments in microtubule bundling in growth cones — an effect that is exaggerated in MAP1B/MAP2 double knockout mice [14]. Thus, it would be of interest to investigate whether Doublecortin cooperates with MAP1B and MAP2 in microtubule bundle formation.

The study by Bielas *et al.* [1] draws attention to a little appreciated cytoskeletal mechanism in growth cones: microtubule bundling. It will be important and challenging to unravel the precise roles and redundancies of key players in this phenomenon.

References

1. Bielas, S.L., Serneo, F.F., Chechlacz, M., Deerinck, T.J., Perkins, G.A., Allen, P.B., Ellisman, M.H., and Gleeson, J.G. (2007). Spinophilin facilitates dephosphorylation of doublecortin by PP1 to mediate microtubule bundling at the axonal wrist. Cell *129*, 579–591.
2. Dehmelt, L., and Halpain, S. (2004). Actin and microtubules in neurite initiation: are MAPs the missing link? J. Neurobiol. *58*, 18–33.
3. Kappeler, C., Dhenain, M., Phan Dinh Tuy, F., Saillour, Y., Marty, S., Fallet-Bianco, C., Souville, I., Souil, E., Pinard, J.M., Meyer, G., *et al*. (2007). Magnetic resonance imaging and histological studies of corpus callosal and hippocampal abnormalities linked to doublecortin deficiency. J. Comp. Neurol. *500*, 239–254.
4. Moores, C.A., Perderiset, M., Kappeler, C., Kain, S., Drummond, D., Perkins, S.J., Chelly, J., Cross, R., Houdusse, A., and Francis, F. (2006). Distinct roles of doublecortin modulating the microtubule cytoskeleton. EMBO J. *25*, 4448–4457.
5. Gdalyahu, A., Ghosh, I., Levy, T., Sapir, T., Sapoznik, S., Fishler, Y., Azoulai, D., and Reiner, O. (2004). DCX, a new mediator of the JNK pathway. EMBO J. *23*, 823–832.
6. Schaar, B.T., Kinoshita, K., and McConnell, S.K. (2004). Doublecortin microtubule affinity is regulated by a balance of kinase and phosphatase activity at the leading edge of migrating neurons. Neuron *41*, 203–213.
7. Tanaka, T., Serneo, F.F., Tseng, H.C., Kulkarni, A.B., Tsai, L.H., and Gleeson, J.G. (2004). Cdk5 phosphorylation of doublecortin ser297 regulates its effect on neuronal migration. Neuron *41*, 215–227.
8. Allen, P.B., Ouimet, C.C., and Greengard, P. (1997). Spinophilin, a novel protein phosphatase 1 binding protein localized to dendritic spines. Proc. Natl. Acad. Sci. USA *94*, 9956–9961.
9. Satoh, A., Nakanishi, H., Obaishi, H., Wada, M., Takahashi, K., Satoh, K., Hirao, K., Nishioka, H., Hata, Y., Mizoguchi, A., *et al*. (1998). Neurabin-II/spinophilin. An actin filament-binding protein with one pdz domain localized at cadherin-based cell-cell adhesion sites. J. Biol. Chem. *273*, 3470–3475.
10. Zhang, X.F., Schaefer, A.W., Burnette, D.T., Schoonderwoert, V.T., and Forscher, P. (2003). Rho-dependent contractile responses in the neuronal growth cone are independent of classical peripheral retrograde actin flow. Neuron *40*, 931–944.
11. Dehmelt, L., Nalbant, P., Steffen, W., and Halpain, S. (2007). A microtubule-based, dynein-dependent force induces local cell protrusions: Implications for neurite initiation. Brain Cell Biol., [Epub 13 March 2007].
12. Tsai, L.H., and Gleeson, J.G. (2005). Nucleokinesis in neuronal migration. Neuron *46*, 383–388.
13. Weimer, J.M., and Anton, E.S. (2006). Doubling up on microtubule stabilizers: synergistic functions of doublecortin-like kinase and doublecortin in the developing cerebral cortex. Neuron *49*, 3–4.
14. Teng, J., Takei, Y., Harada, A., Nakata, T., Chen, J., and Hirokawa, N. (2001). Synergistic effects of MAP2 and MAP1B knockout in neuronal migration, dendritic outgrowth, and microtubule organization. J. Cell Biol. *155*, 65–76.
15. Schaefer, A.W., Kabir, N., and Forscher, P. (2002). Filopodia and actin arcs guide the assembly and transport of two populations of microtubules with unique dynamic parameters in neuronal growth cones. J. Cell Biol. *158*, 139–152.

Department of Cell Biology and Institute for Childhood and Neglected Diseases, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, USA.
E-mail: shelley@scripps.edu

DOI: 10.1016/j.cub.2007.05.059