



Actin, Spectrin, and Associated Proteins Form a Periodic Cytoskeletal Structure in Axons Ke Xu *et al. Science* **339**, 452 (2013); DOI: 10.1126/science.1232251

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passive 5mC depletion (fig. S23) (18), which may contribute to the partial demethylation observed in Tet1 and Tet2 knockdown PGCLCs. Thus, whereas in zygotes 5mC reprogramming is mechanistically compartmentalized into TET3-mediated 5hmC conversion of the paternal genome and direct passive 5mC depletion on the maternal genome (12, 19-21), both of these mechanisms operate together in PGCs (Fig. 4B). In addition, up-regulation of the base excision repair (BER) pathway in PGCs may both protect against cumulative genetic damage and act as an auxiliary active demethylation mechanism, perhaps for specific loci (22, 23). Reprogramming in PGCs therefore involves multiple redundant mechanisms to reset the epigenome for totipotency, which accounts for the apparent fertility (albeit subfertile) of mice lacking individual components, such as Tet1 (24). The existence of multiple mechanisms may also underpin the comprehensive nature of DNA demethylation in PGCs (3). Nonetheless, some rare single-copy sites of CpG methylation escape from 5mC erasure (25), which may provide mechanistic avenues for investigations into transgenerational epigenetic inheritance.

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### Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1229277/DC1 Materials and Methods Figs. S1 to S24 References (25, 26)

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# Actin, Spectrin, and Associated **Proteins Form a Periodic Cytoskeletal Structure in Axons**

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Actin and spectrin play important roles in neurons, but their organization in axons and dendrites remains unclear. We used stochastic optical reconstruction microscopy to study the organization of actin, spectrin, and associated proteins in neurons. Actin formed ringlike structures that wrapped around the circumference of axons and were evenly spaced along axonal shafts with a periodicity of ~180 to 190 nanometers. This periodic structure was not observed in dendrites, which instead contained long actin filaments running along dendritic shafts. Adducin, an actin-capping protein, colocalized with the actin rings. Spectrin exhibited periodic structures alternating with those of actin and adducin, and the distance between adjacent actin-adducin rings was comparable to the length of a spectrin tetramer. Sodium channels in axons were distributed in a periodic pattern coordinated with the underlying actin-spectrin-based cytoskeleton.

ctin plays critical roles in shaping and maintaining cell morphology, as well as Lin supporting various cellular functions, including cell motility, cell division, and intracellular transport (1). In neurons, actin is essential for the establishment of neuronal polarity, cargo transport, neurite growth, and stabilization of synaptic structures (2-4). Despite its importance, our understanding of actin structures in neurons remains incomplete. Electron microscopy has shown detailed actin ultrastructure in growth cones and dendritic spines (5, 6), in which actin is the dominant cytoskeletal protein, but little is known about the organization of actin in the axonal and dendritic shafts (4). These neurites contain a high density of different types of cytoskeletal filaments, such as microtubules and neurofilaments (6-8). Hence, the challenge of resolving the organization of actin in axons and dendrites requires imaging tools with both high spatial resolution and molecular specificity.

A prototypical actin-spectrin-based cytoskeleton structure is found in red blood cells (erythrocytes) (9, 10), where actin, spectrin, and associated proteins form a two-dimensional (2D) polygonal network (mostly composed of hexagons and pentagons) underneath the erythrocyte membrane (11, 12). Spectrin analogs have been found in many other animal cells (9, 10), including neurons (13, 14). These analogs play important roles, ranging from regulation of the heartbeat

to stabilization of axons, formation of axon initial segments and nodes of Ranvier, and stabilization of synapses in neurons (9, 10, 15). An erythrocyte-like, polygonal lattice structure has been observed for spectrin in the Drosophila neuromuscular junction (16), and models similar to the erythrocyte cytoskeleton have also been proposed for other systems (10). However, the ultrastructural organization of spectrin in nonerythrocyte cells is largely unknown due to similar challenges in imaging. Recent advances in superresolution fluores-

cence microscopy (17, 18) allow resolutions down to ~10 nm to be achieved with molecular specificity, providing a promising solution to the above challenges. In particular, superresolution studies of neurons have provided valuable structural and dynamic information of actin in dendritic spines (19-22). In this work, we used a superresolution fluorescence imaging method, stochastic optical reconstruction microscopy (STORM) (23-27), to study the 3D ultrastructural organization of actin and spectrin in neurons.

To image actin in neurons, we fixed cultured rat hippocampal neurons at various days in vitro (DIV) and labeled actin filaments with phalloidin conjugated to a photoswitchable dye, Alexa Fluor 647 (Invitrogen, Carlsbad, CA) (Fig. 1) (28). To identify axons and dendrites, we immunolabeled MAP2, a microtubule-associated protein enriched in dendrites, or NrCAM, a cell adhesion molecule found in the initial segments of axons (15), using a dye of a different color (Fig. 1) (28). In the conventional fluorescence images (Fig. 1, A, D, and F), MAP2 specifically stained dendrites, and NrCAM specifically labeled the initial segments of axons, whereas actin was found in both dendrites and axons.

Next, we used 3D STORM (27) with a dualobjective astigmatism-imaging scheme (28, 29)

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to image the ultrastructure of actin. Only a sparse subset of the labeled Alexa 647 molecules were activated with 405-nm light at any instant and imaged with 647-nm light using a continuous illumination and detection mode (30). The xy and z coordinates of the activated molecules were determined from the centroid positions and ellipticity values of their images, respectively (27, 29). Iterating this procedure allowed numerous molecules to be localized and a 3D superresolution image to be constructed from the coordinates of these molecules.

In contrast to the conventional images, individual actin filaments were resolved in the STORM images (Fig. 1, B, C, E, and G, and fig. S1B) (28). We observed long actin filaments in dendrites (Fig. 1, B and C, and fig. S1B). These filaments were primarily distributed within a cortical layer beneath the plasma membrane and largely ran along the long axes of dendrites, though crossing filaments were also observed (Fig. 1, B and C). Our findings here were focused on dendritic shafts and do not exclude the possibility of different actin organizations in dendritic spines (3, 6).

In contrast, we observed a drastically different organization of actin in axons. Actin filaments appeared to be arranged into isolated rings that wrapped around the circumference of the axons (Fig. 1, E and G). These rings were periodically distributed along the axonal shafts, forming a ladderlike, quasi-1D lattice with a longrange order (Fig. 1, E and G). Examination of neurons at different developmental stages showed that the periodic actin pattern started to appear at ~5 DIV, became clearly visible at ~7 DIV



**Fig. 1.** STORM imaging reveals distinct organization of actin filaments in the axons and dendrites of neurons. (**A**) Conventional fluorescence image of actin (green) and a dendritic marker, MAP2 (magenta), in a cultured hippocampal neuron fixed at 7 DIV. (**B**) Three-dimensional STORM image of actin in a dendritic region corresponding to the white box in (A). The *z* positions in the STORM image are color-coded according to the color scale, with violet and red indicating the positions closest to and farthest from the substratum, respectively. (**C**) Magnification of the region inside the red box in (B). The *yz* cross section corresponding to the white-boxed region is shown in the inset. (**D**) Conventional fluorescence image of actin (green) and MAP2 (magenta) in a neuron fixed at 12 DIV. (**E**) Three-dimensional STORM image of actin in a region containing axons (devoid of the dendritic marker MAP2), corresponding to the yellow box in (D). The *yz* cross sections corresponding to the white-boxed regions are shown in the insets. The 3D STORM image of a region containing a dendrite of this neuron is shown in fig. S1B (*28*). (**F**) Conventional fluorescence image of actin (green) and an axon initial segment marker, NrCAM (magenta), in a neuron fixed at 9 DIV. (**G**) Three-dimensional STORM image of actin in a region containing to the yellow box in (F).

(fig. S2) (28), and was maintained in older mature neurons (e.g., 28 DIV) (fig. S3) (28). In mature neurons, more than 80% of the neuronal processes that could be identified as axon shafts (that is, neurites positive for NrCAM or those devoid of MAP2 and wider than 50 nm) exhibited the periodic, ladderlike actin pattern, whereas some filopodia-like processes branching from axon shafts did not (fig. S3). Although long actin filaments were also observed along some axons, especially in thicker axons (Fig. 1, E and G, and figs. S2E and S3B), the periodic, ringlike pattern was the most pronounced actin feature in the axon shafts. We found similar periodic patterns in both the initial segments of axons (Fig. 1G) and distal axons that were not close to the cell body (figs. S1B and S3).

The periodicity of the actin pattern in axons appeared highly regular (Fig. 2). Projection of the 3D images of actin to the long axis of the axon led to well-separated peaks with nearly identical spacing (Fig. 2A). A Fourier transform of the 1D projection yielded fundamental frequencies and overtones corresponding to a spatial period of ~190 nm (Fig. 2B). Statistical analysis of the spacings showed a narrow distribution with a mean value of 182 nm and a SD of 16 nm (Fig. 2C).

The quasi-1D, periodic actin structure prompted us to search for a molecular mechanism underlying this organization. In particular, the isolated actin ringlike structures with ~180- to 190-nm spacing did not appear to form a cohesive network by themselves. Thus, we reasoned that a linker component may be present to connect the actin structures into a network, thereby giving mechanical support to the membrane, and to provide a mechanism for the regular spacing between the actin rings. We hypothesized that spectrin could be a good candidate for this linker component. The rod-shaped spectrin tetramers, typically 150 to 250 nm in length, cross-link short actin filaments in the erythrocyte membrane cytoskeleton to form a 2D, polygonal network (9-12). Spectrin analogs, in particular aII-BII spectrin, are present in the mammalian brain (10, 13, 14). Moreover, brain spectrin interacts with actin, and spectrin tetramers isolated from the brain exhibit a rodlike shape, with an average length of 195 nm (14), comparable to the spatial periodicity we observed for the actin rings.

To test this hypothesis, we performed 3D STORM imaging of  $\beta$ II-spectrin, which is enriched in distal axons (*31*). We immunolabeled  $\beta$ II-spectrin with the use of an antibody (*28*) that specifically targeted the C terminus of  $\beta$ II-spectrin, which should label the center of the rodlike  $\alpha$ II- $\beta$ II spectrin tetramer (*9*, *10*). If the adjacent actin rings are connected by spectrin tetramers, we expect the centers of the spectrin tetramers to also form a periodic pattern of ringlike structures with a quantitatively similar periodicity. We observed highly periodic, ringlike structures for the C terminus of  $\beta$ II-spectrin in axons (Fig. 3A and

figs. S4 and S5) but not in dendrites (fig. S5) (28). Statistics of the spacings between adjacent rings gave a mean value of 182 nm and a SD of 18 nm (Fig. 3B), which are nearly identical to the values measured for the actin rings (Fig. 2C), suggesting that spectrin and actin may form a coordinated periodic network.  $\beta$ IV-spectrin, a spectrin subtype specifically located in the axon initial segment (fig. S6) (15, 28, 32), also exhibited a quasi-1D, periodic pattern (Fig. 3, C and D) similar to those of actin and  $\beta$ II-spectrin, consistent with the observation that the periodic actin structure was detected in both axon initial segments (Fig. 1G) and distal axons (figs. S1B and S3).

In the erythrocyte cytoskeleton, the network formed by short actin filaments (12 to 16 monomers) and spectrin tetramers contains other proteins, such as adducin (a protein that caps the growing end of actin filaments and promotes the binding of spectrin to actin) and ankyrin (a protein that helps to anchor spectrin to the membrane) (9, 10). Thus, we also probed the distributions of these molecules in axons. Immunolabeled adducin formed periodic, ladderlike structures in axons (Fig. 3E), with a periodicity quantitatively similar to those of the actin and spectrin structures (Fig. 3F). Immunolabeled ankyrin-B exhibited a semiperiodic pattern with a similar periodicity but a less regular distribution compared with those observed for actin, spectrin, and adducin (fig. S7) (28). The less regular distribution is expected for ankyrin-B because each spectrin tetramer contains two separate ankyrinbinding sites that are both away from the center of the spectrin tetramer, neither of which is necessarily occupied by ankyrin (9, 10).

To confirm that the periodic actin-spectrin structure exists in the brain, we performed STORM imaging of hippocampal tissue slices of adult mice (fig. S8) (28). Because axons, dendrites, and cell bodies are densely packed in all three dimensions in brain tissues, we needed a positive marker to unambiguously identify axons in these experiments. We used BIV-spectrin, which is specifically localized to the initial segments of axons (fig. S6) (15, 28, 32), as such a marker. We performed STORM imaging on either immunolabeled BIV-spectrin or phalloidin-labeled actin. We observed quasi-1D, periodic structures for both BIV-spectrin (fig. S8, A and B) and actin (fig. S8, G and I) in axon segments stained by BIV-spectrin, with periodicities quantitatively similar to those observed in cultured neurons (fig. S8, C to E and J to L).

Next, we performed two-color STORM imaging of cultured neurons to determine the relative positions of the molecular components observed in the quasi-1D, periodic axonal cytoskeleton (Fig. 4). To this end, we labeled actin and spectrin (or adducin) with spectrally distinct photoswitchable dyes (28). We detected highly regular, alternating patterns of actin and spectrin in axons with the spectrin stripes falling midway between adjacent actin stripes (Fig. 4A and fig. S9A) (28).



**Fig. 2.** Actin filaments in axons form a quasi-1D, periodic structure with a uniform spacing of ~180 to 190 nm. (**A**) Three-dimensional STORM image of a segment of axon (top) and the distribution of localized molecules after the 3D image was projected to one dimension along the axon long axis (bottom). (**B**) Fourier transform of the 1D localization distribution shown in (A). The Fourier transform shows a fundamental frequency of (190 nm)<sup>-1</sup> and an overtone. (**C**) Histogram of the spacings between adjacent actin ringlike structures (N = 204 spacings). The red line is a Gaussian fit with a mean of 182 nm and a SD of 16 nm.



**Fig. 3.** Spectrin and adducin exhibit quasi-1D, periodic patterns in axons, quantitatively similar to that observed for actin. (**A**) Three-dimensional STORM image of  $\beta$ II-spectrin in axons.  $\beta$ II-spectrin is immunostained against its C-terminal region, which is situated at the center of the rodlike  $\alpha$ II- $\beta$ II spectrin tetramer. (Inset) The *yz* cross section of the boxed region showing the ringlike structure. The smaller white box denotes the position of the inset image. (**B**) Histogram of the spacings between adjacent spectrin rings (*V* = 340 spacings). The red line is a Gaussian fit with a mean of 182 nm and a SD of 18 nm. (**C** and **D**) Same as (A) and (B) but for  $\beta$ IV-spectrin, which is specifically located in the initial segments of axons.  $\beta$ IV-spectrin is immunostained against its N-terminal region, which corresponds to the ends of the spectrin tetramer. The red line superimposed on the histogram is a Gaussian fit with a mean of 194 nm and a SD of 15 nm (*V* = 88 spacings). (**E** and **F**) Same as (A) and (B) but for adducin, an actin-capping protein. The red line superimposed on the histogram is a Gaussian fit with a mean of 187 nm and a SD of 16 nm (*V* = 216 spacings).

Given that the antibody specifically labeled the C-terminal region of the  $\beta$ II-spectrin, which is at the center of the  $\alpha$ II- $\beta$ II spectrin tetramer, and that the spacing between adjacent actin stripes was comparable to the length of the spectrin tetramer, our observations suggest that spectrin tetramers are aligned longitudinally along the axonal shaft and connect the adjacent actin ringlike structures. Treatment with an actin-depolymerizing drug, latrunculin A, not only eliminated the periodic actin structures of spectrin (fig. S10) (28), suggesting that the actin and spectrin structures are interconnected.

The adducin stripes, on the other hand, appeared to colocalize with the actin stripes (Fig. 4B and fig. S9B) (28). Given that adducin caps one end of actin filaments, this result suggests that the ringlike actin structures probably do not represent long, continuous filaments spanning the entire ring, but rather are made of capped, short filaments aligned along the circumferential direction of the axon, probably facilitated by actinbinding or –cross-linking proteins (2, 3). As described earlier, long actin filaments running along axons were sometimes observed, but the filaments did not appear to have a well-defined spatial relation with either spectrin or adducin (fig. S9A and B). Consistent with the above

Fig. 4. Actin, spectrin, and adducin form a coordinated, guasi-1D lattice structure in axons, and sodium channels are distributed in a periodic pattern in coordination with the actin-spectrinbased submembrane cytoskeleton. (A) Two-color STORM image of actin (green) and βII-spectrin (magenta). βII-spectrin is immunostained against its C-terminal region, which is situated at the center of the spectrin tetramer. (B) Two-color STORM image of actin (green) and adducin (magenta). (C) Two-color STORM image of BII-spectrin (green) and adducin (magenta). (D) Two-color STORM image of sodium channels (Na<sub>w</sub> green) and BIV-spectrin (magenta), BIV-spectrin is immunostained against results,  $\beta$ II-spectrin (C terminus) and adducin stripes also alternated with each other along the axon (Fig. 4C and fig. S9C) (28).

To further analyze the data quantitatively, we calculated the cross-correlation between the two color channels for each of the three combinations: actin/spectrin, actin/adducin, and spectrin/adducin (Fig. 4E). The cross-correlation was determined for the 1D localization distributions, as shown in fig. S9 (28). The ~180- to 190-nm periodicities were apparent for all three correlation functions (Fig. 4E). The actin-adducin pair appeared to be correlated, exhibiting a maximum near zero shift, indicating that actin and adducin colocalize with each other. The actinspectrin (C terminus of BII-spectrin) and adducinspectrin (C terminus of BII-spectrin) pairs were both anticorrelated, exhibiting a minimum at zero shift and maxima at ~90- to 100-nm shifts, indicating that the centers of the spectrin tetramers lie halfway between the adjacent actin-adducin rings.

Finally, to assess the possible functional implication of this quasi-1D, periodic cytoskeletal structure, we performed two-color imaging of  $\beta$ IV-spectrin and sodium channels (Na<sub>v</sub>) in the axon initial segments (28). Sodium channels are enriched in the axon initial segments and are important for the generation of action potentials

(15, 33). We immunostained the N-terminal region of BIV-spectrin, which is at the two ends of the spectrin tetramer. Interestingly, Nav and the N terminus of BIV-spectrin exhibited alternating periodic patterns, with Nav being most abundant approximately halfway between the ends of the spectrin tetramers (Fig. 4, D and E, and fig. S9D) (28). Thus, the periodic distribution of  $Na_v$ is most likely coordinated by the underlying periodic cytoskeleton structure, presumably through ankyrin-G (34), which interacts with both sodium channels and BIV-spectrin (9, 15, 35, 36). The periodic pattern of Nav appeared less regular than that of  $\beta$ IV-spectrin, potentially because each spectrin tetramer contains two separate Nav binding sites (through ankyrin-G) that are not fully occupied, or because not every Nav molecule is anchored to the underlying cytoskeleton. It is also possible that the antibody against Na<sub>v</sub> was less specific.

The above results suggest that the cortical cytoskeleton of axons is composed of short actin filaments that are capped by adducin at one end and arranged into ringlike structures, which wrap around the circumference of the axon (Fig. 4F). Spectrin tetramers connect the neighboring actin/adducin rings along the long axis of the axon, tightly regulating the periodicity of the cytoskeleton structure to ~180 to 190 nm and



its N-terminal region, which is situated at the two ends of the spectrin tetramer. The distributions of the localized molecules along the axon shafts are shown in fig. S9 (28). (E) Spatial correlations between actin and the  $\beta$ II-spectrin C terminus [(A), black], between actin and adducin [(B), blue], between adducin and the  $\beta$ II-spectrin C terminus [(C), red], and between sodium channels and the  $\beta$ IV-spectrin N terminus [(D), green]. The correlation function is calculated for varying relative shifts between the two color channels along the axons. (F) A model for the cortical cytoskeleton in axons. Short

actin filaments (green), capped by adducin (blue) at one end, form ringlike structures wrapping around the circumference of the axon. Spectrin tetramers (magenta) connect the adjacent actin/adducin rings along the axon, creating a quasi-1D lattice structure with a periodicity of ~180 to 190 nm. The letters "C" and "N" denote the C terminus (magenta triangles) and N terminus (magenta squares) of  $\beta$ -spectrin, respectively. Ankyrin and sodium channels, not shown in the model, also form semiperiodic patterns in coordination with the periodic cytoskeletal structure.

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giving the axonal cytoskeleton a long-range order. Despite the molecular composition differences between the axon initial segments and distal axons [for example, ankyrin-G and BIVspectrin are confined in the axon initial segment by an exclusion effect of the distal axon proteins ankyrin-B and ßII-spectrin (31)], the cytoskeletal organization is similar between the initial and distal segments of the axons, both adopting a quasi-1D, periodic structure. Interestingly, we found this periodic cytoskeleton structure to be present only in axons, not in dendrites, which instead primarily contained long actin filaments running along the dendritic axis. Although the microscopic interactions between the molecular components of the axon cytoskeleton are probably similar to those between the erythrocyte analogs (9, 10), the overall structure of this quasi-1D, periodic cytoskeleton in axons is distinct from the 2D, pentagonal or hexagonal structure observed for the erythrocyte membrane cytoskeleton (11, 12). In Drosophila motoneuron axons near the neuromuscular junctions, spectrin and ankyrin appear to organize into an erythrocytelike, pentagonal or hexagonal lattice structure (16), which is distinct from the quasi-1D, periodic, ladderlike structure that we observed in the axons of vertebrate brains. Whether the difference is due to invertebrate versus vertebrate animals or peripheral versus central nervous systems is a topic for future investigations.

The periodic, actin-spectrin-based cytoskeleton observed here may not be involved in myosindependent axonal transport. If the analogy to the erythrocyte membrane cytoskeleton holds, the capped short actin filaments in the ringlike actin structures in axons are probably bound by tropomyosin (9, 10), which could potentially prevent the binding of myosins. Myosin-dependent axonal transport could, however, be mediated by the long actin filaments that run along the axon shaft. The quasi-1D, periodic, actin-spectrin cytoskeleton may instead provide elastic and stable mechanical support for the axon membrane, given the flexibility of spectrin. Elastic and stable support is particularly important for axons, because they can be extremely long and thin and have to withstand mechanical strains as animals move (37). Indeed, the loss of  $\beta$ -spectrin in Caenorhabditis elegans leads to spontaneous breaking of axons, which is caused by mechanical strains generated by animal movement and can be prevented by paralyzing the animal (37). The highly periodical submembrane cytoskeleton can also influence the molecular organization of the plasma membrane by organizing important membrane proteins along the axon. We found that sodium channels were distributed periodically along the axon initial segment in a coordinated manner with the underlying actin-spectrin cytoskeleton. An axonal plasma membrane with periodically varying biochemical and mechanical properties may not only influence how an action potential is generated and propagated, but might also affect how axons interact with other cells.

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### Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1232251/DC1 Materials and Methods Figs. S1 to S10 References (*38–42*)

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# **Comparative Analysis of Bat Genomes Provides Insight into the Evolution of Flight and Immunity**

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Bats are the only mammals capable of sustained flight and are notorious reservoir hosts for some of the world's most highly pathogenic viruses, including Nipah, Hendra, Ebola, and severe acute respiratory syndrome (SARS). To identify genetic changes associated with the development of bat-specific traits, we performed whole-genome sequencing and comparative analyses of two distantly related species, fruit bat *Pteropus alecto* and insectivorous bat *Myotis davidii*. We discovered an unexpected concentration of positively selected genes in the DNA damage checkpoint and nuclear factor  $\kappa$ B pathways that may be related to the origin of flight, as well as expansion and contraction of important gene families. Comparison of bat genomes with other mammalian species has provided new insights into bat biology and evolution.

B in the mammalian clade Laurasiatheria (1). Although consensus has not been reached on the exact arrangement of groups within Laurasiatheria, a recent study placed Chiroptera as a sister taxon to Cetartiodactyla (whales + eventoed ungulates such as cattle, sheep, and pigs) (2). The Black flying fox (*Pteropus alecto*) and David's Myotis (*Myotis davidii*) represent the Yinpterochiroptera and Yangochiroptera suborders, respectively, and display a diverse range of phenotypes (Fig. 1). Captive colonies, immortalized cell lines, and bat-specific reagents have been developed for these two species; however, genomic data are currently unavailable.

The most conspicuous feature of bats, distinguishing them from all other mammalian species, is the capacity for sustained flight. Positive selection in the oxidative phosphorylation (OXPHOS) pathway suggests that increased metabolic capac-