© 2020. Published by The Company of Biologists Ltd | Development (2020) 147, dev191411. doi:10.1242/dev.191411

SPOTLIGHT

Using *Drosophila* to drive the diagnosis and understand the mechanisms of rare human diseases

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ABSTRACT

Next-generation sequencing has greatly accelerated the discovery of rare human genetic diseases. Nearly 45% of patients have variants associated with known diseases but the unsolved cases remain a conundrum. Moreover, causative mutations can be difficult to pinpoint because variants frequently map to genes with no previous disease associations and, often, only one or a few patients with variants in the same gene are identified. Model organisms, such as *Drosophila*, can help to identify and characterize these new disease-causing genes. Importantly, *Drosophila* allow quick and sophisticated genetic manipulations, permit functional testing of human variants, enable the characterization of pathogenic mechanisms and are amenable to drug tests. In this Spotlight, focusing on microcephaly as a case study, we highlight how studies of human genes in *Drosophila* have aided our understanding of human genetic disorders, allowing the identification of new genes in well-established signaling pathways.

KEY WORDS: Ankle2, *Drosophila*, Asymmetric division, Microcephaly, Model of human disease

Introduction

Every *in vivo* approach used to study genetic variants associated with human disease has benefits and caveats. Whether the system is based on human cells in culture, primates, rodents, fish, flies, worms or yeast, no model can recapitulate every aspect of human development and disease. In addition, the speed and costs associated with approaches in various model organisms can vary by orders of magnitude. Yet, effectively addressing how genetic variation might lead to human disease is important, both for diagnosis and for developing suitable therapies.

A number of established genetic model systems, such as worms, flies, fish and mice, offer the ability to test specific variants associated with human disorders *in vivo* (Wangler et al., 2017a). The fruit fly *Drosophila melanogaster* is particularly well suited for these studies because of the ease and sophistication of its genetic manipulation, its short generation time and the fact that about 75% of human genes have *Drosophila* homologs (Chow and Reiter, 2017; Reiter et al., 2001). Indeed, research on *Drosophila* in the Model Organism Screening Center (MOSC) of the Undiagnosed Diseases Network (see below) and in collaboration with other human geneticists has provided diagnoses for more than 25 rare human genetic diseases in less than 4 years (Bellen et al., 2019; Wangler et al., 2017a; 2017b). These include diseases caused by proteins associated with different organelles, such as mitochondria (Chao et al., 2017; Harel et al., 2016; Liu et al., 2017; Oláhová et al., 2018; Yoon et al., 2017), peroxisomes (Chao et al., 2016; Chung et al., 2020; Luo et al., 2017; Wangler et al., 2017b), lysosomes (Şentürk et al., 2019) and endosomes (Lin et al., 2018), as well as numerous developmental disorders (Ansar et al., 2018a; Ansar et al., 2018b; Link et al., 2019; Marcogliese et al., 2018).

Here, we provide an overview of how studies of *Drosophila* have contributed to the identification and characterization of human diseases. We then discuss the recent discovery of a number of genes associated with microcephaly in humans. These discoveries provide us with a better understanding of the molecular mechanisms underlying asymmetric cell division in neuroblasts in *Drosophila*, which in turn has led to the discovery of many homologs of *Drosophila* genes that are associated with microcephaly in humans.

Advances in human genetics, technologies and rare disease diagnoses

In the past, human geneticists mostly relied on large family pedigrees to identify genes associated with disease (Wijsman, 2012). However, with the advent of next-generation sequencing technology, it is now often possible to identify one or a few variants that are associated with disease in a single individual (Bamshad et al., 2019; Posey et al., 2019). Typically, 40-50% of pediatric diseases can be diagnosed by identifying variants in previously described disease genes using whole-exome sequencing (WES) combined with assessment of copy number variations (CNVs) (Liu et al., 2019; Ngo et al., 2020), or by performing whole-genome sequencing (WGS). However, the remaining cases (~50%) go undiagnosed (Adams and Eng, 2018). If additional tests or experiments are performed, a significant number of cases can eventually be diagnosed (Ramoni et al., 2017; Splinter et al., 2018). These often include sequencing the DNA of the patient's parents and other family members, the identification of other independent affected individuals through gene-matching algorithms (Buske et al., 2015; Sobreira et al., 2015) and RNA sequencing of blood or fibroblasts (Frésard et al., 2019).

Despite these efforts, there are still millions of individuals with undiagnosed diseases, and it is estimated that 6000-13,000 human genes remain to be associated with diseases (Bamshad et al., 2019). The National Institutes of Health has established and supported consortia such as the Centers for Mendelian Genomics (CMG) to uncover the genetics of Mendelian disease (Bamshad et al., 2012; Posey et al., 2019) and the Undiagnosed Diseases Network (UDN) (Ramoni et al., 2017; Splinter et al., 2018) to diagnose patients with rare diseases who typically undergo a long diagnostic odyssey. Other consortia include Finding of Rare Disease Genes (FORGE) (Beaulieu et al., 2014), Care4Rare Canada Consortium (Sawyer et al., 2016), Canadian Rare Diseases Models and Mechanisms Network (RDMM) (Boycott et al., 2020), Deciphering Developmental Disorders (DDD) (Firth and Wright, 2011), Undiagnosed Diseases

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Network International (UDNI) (Taruscio et al., 2015) and the International Rare Diseases Research Consortium (IRDiRC) (Gasser et al., 2012).

When a diagnosis cannot be reached for an individual after analyzing clinical and genetic information, genetic model organisms can be used to model human disease variants and assess their function in a developing or adult animal. *Drosophila* has been very successful in this respect, as 50-70% of human genes can rescue phenotypes associated with loss of the homologous gene in *Drosophila* (Bellen et al., 2019). Importantly, 75% of human genes have *Drosophila* orthologs (Reiter et al., 2001). Finally, 65% of the applicants to the UDN are children, and 65-70% have neurological symptoms (Splinter et al., 2018; Wangler et al., 2017a), highlighting that many undiagnosed and rare diseases are developmental in nature, often affecting neuronal development or function.

Using *Drosophila* to understand the mechanisms underlying human diseases

Pathways that regulate brain development are largely conserved between *Drosophila* and humans (Hirth and Reichert, 1999; Pires-daSilva and Sommer, 2003), and the *Drosophila* brain undergoes many of the same developmental processes as the human brain. Neuronal stem cell division and neurogenesis have been extensively investigated using *Drosophila* neuroblasts and their distinct lineages (Gallaud et al., 2017; Homem and Knoblich, 2012). Furthermore, the *Drosophila* nervous system has been crucial to the study of processes that regulate axonal guidance (Sáchez-Soriano et al., 2007) and the formation of neural circuits (Davis et al., 2020; Olsen and Wilson, 2008; Zheng et al., 2018). Hence, the developing brain (larval and pupal stages) or the adult brain in *Drosophila* can be used as models of human brain development and neurodegeneration and are useful to decipher human neurological disease; there are, of course, some aspects of human brain development and morphological features that cannot be effectively modeled in *Drosophila.* In addition, although ∼75% human genes do have a *Drosophila* ortholog, there are some (the remaining ∼25%) that do not, so investigations focused on these would be better suited in other model systems. In most cases, genes that cannot be modeled in flies due to lack of homologs can effectively be modeled in fish or mice.

To test the function of a human variant, and whether it might cause disease, *Drosophila* can be 'humanized'. This involves expressing the human protein in a *Drosophila* mutant background to assess whether it can rescue phenotypes associated with gene loss. This is most often achieved by using GAL4 drivers (Brand and Perrimon, 1993; Caygill and Brand, 2016; Duffy, 2002) or by inserting a disruptive GAL4 in the *Drosophila* gene (Bellen and Yamamoto, 2015; Diao et al., 2015) to drive a UAS-human reference cDNA (Kanca et al., 2017; Şentürk et al., 2019). If the human protein encoded by the cDNA can rescue phenotypes associated with loss-of-function mutations in *Drosophila*, then the function of the protein is conserved. Human variants can then be tested, and the following questions can be addressed: (1) Does the putative disease variant rescue the mutant phenotype or not; if so, how do the phenotypes associated with variant expression differ from those associated with the reference protein? (2) Does the reference or disease variant protein cause phenotypes in a wild-type animal, suggesting that they are dominant-negative or gain-of-function variants? (3) Can the function of the protein, and hence the mechanisms of disease pathogenesis, be tackled? (4) Do the identified proteins/pathways provide opportunities for development of therapies and drug testing? Answering these questions can provide a diagnosis, link many genes to known pathways and processes, and identify drugs that can be tested in patients (Bellen et al., 2019; Chung et al., 2020; Yoon et al., 2017).

An alternative approach to identifying genes associated with human disease is to carry out a forward genetic screen that probes disease-associated phenotypes in *Drosophila*. One such screen has been successful at identifying novel genes associated with human disease (Yamamoto et al., 2014), identifying 165 loci associated with developmental or neurodegenerative phenotypes. Of these, 93% had human homologs, and 61% are currently associated with human diseases based on MARRVEL (model organism aggregated resources for rare variant exploration, Wangler et al., 2017a), an online database integrating genetic information from humans and across species to provide a unified interface for gene, protein or disease investigation. However, 30% of the genes that are submitted by the UDN consortium have not been isolated in this or other genetic screens and have almost no annotation in any model organism. Moreover, existing annotations can be restricted to one species and are frequently cursory in nature (Wangler et al., 2017b). Hence, defining the function of human genes and their homologs associated with developmental diseases can provide crucial biological information about conserved developmental processes.

Drosophila as a model for diagnosing and investigating causes of microcephaly

In recent years, several developmental disorders have been identified, and the function of the corresponding genes have been characterized using *Drosophila*. These include genes associated with autism spectrum disorder (ASD) (reviewed by Bellosta and Soldano, 2019) and neurological syndromes that encompass developmental delay, intellectual disability, seizures or psychiatric problems (Halperin et al., 2019; Hubert et al., 2020; Kummeling et al., 2020; Muir et al., 2020; Nixon et al., 2019; Owings et al., 2018; Straub et al., 2018). Another example is congenital microcephaly (Jayaraman et al., 2018; Schoborg et al., 2019): a developmental disorder that can be caused by genetic mutation, virus exposure or environmental toxins (Devakumar et al., 2018; Pirozzi et al., 2018). Primary microcephaly is defined as a disorder in which individuals exhibit a smaller than normal head size, as measured by an occipital frontal circumference (OFC) that is 2-3 standard deviations (s.d.) below the average for the age and gender of the individual. Currently, 25 genetic loci are associated with primary autosomal recessive microcephaly (MCPH) (Jayaraman et al., 2018; Naveed et al., 2018) in the Online Mendelian Inheritance in Man (OMIM) database (Amberger et al., 2015). In recent years, the molecular function of some of these MCPH genes has been elucidated using *Drosophila* (Gambarotto et al., 2019; Lucas and Raff, 2007; Poulton et al., 2017; Ramdas Nair et al., 2016; Rujano et al., 2013; Schoborg et al., 2015; Schoborg et al., 2019; Singh et al., 2014). Below, we outline how studies of *Drosophila* have played an important role in elucidating some of the pathogenic mechanisms associated with microcephaly and in aiding microcephaly disease diagnosis.

Heterozygous missense mutations in the gene *ALFY* (also termed *WDFY3*) were recently identified in a large family with autosomal dominant primary microcephaly (Kadir et al., 2016). *ALFY* encodes a protein that functions in the removal of aggregated proteins by serving as a scaffold for autophagy-mediated removal (Filimonenko et al., 2010; Simonsen et al., 2004). To determine whether heterozygous variants in *ALFY* might lead to microcephaly, a C-terminal fragment consisting of 1226 amino acids of both wild-type and variant human *ALFY* was expressed in developing *Drosophila* brains (Kadir et al., 2016). This approach showed

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that, while pupal brains expressing wild type human *ALFY* appeared normal, animals expressing mutant *ALFY* failed to eclose (enter adulthood) and showed brain sizes 40-60% smaller than brains expressing wild-type *ALFY*. Additionally, expression of mutant *ALFY* in the eye resulted in a severe rough eye phenotype, while eyes expressing wild-type *ALFY* appeared normal. Based on further *in vivo* and *in vitro* analyses, the authors proposed a mechanism in which the expression of mutant *ALFY* causes sustained Wnt signaling in radial glial cells, which thereby promotes symmetric division of apical progenitor cells at the expense of asymmetric division and the generation of basal progenitor cells. This disruption leads to fewer neurons and eventually microcephaly (Kadir et al., 2016). Taken together, these results indicate that heterozygous variants in *ALFY* lead to microcephaly, likely in a dominant-negative fashion, and provide a diagnosis for MCPH as well as revealing the mechanism by which *ALFY* mutations contribute to disease pathogenesis.

Drosophila have also been valuable for studying mechanisms of microcephaly without having to use 'humanization'. For example, variants in *WDR81* – a gene that encodes WD repeat-containing protein 81 – were recently identified in five families with associated progressive microcephaly (Cavallin et al., 2017). Individuals often initially presented with mild microcephaly but then progressed to more severe phenotypes as they aged (OFC z=−5 to −10 s.d.). Neurological development was severely disrupted in these individuals, and MRIs showed significant defects. Studies assessing the function of *WDR81* in mutant human fibroblasts showed mitotic progression delay, and analogous studies in *Drosophila* neuronal stem cells (termed neuroblasts) with knockdown of *Drosophila WDR81* revealed a similar cell cycle phenotype (Cavallin et al., 2017). Together, these results indicate that WDR81 is an essential component of the cell cycle machinery and support the diagnosis of microcephaly induced by variants of *WDR81*.

Variants in *KATNB1,* which encodes a regulatory subunit of the microtubule-severing enzyme katanin, were found in five individuals with complex brain malformations, including lissencephaly (defects in the development of brain folds) and microcephaly (Mishra-Gorur et al., 2014). Mutations in *KATNB1* were shown to affect the mitotic spindle in dividing patient-derived fibroblasts, and corresponding studies in *Drosophila* showed reduced brain size after knockdown of the *Drosophila* ortholog of *KATNB1*, *kat80*. In addition, developing larval brains, which harbor neuroblasts that continuously divide to produce neurons for the entire adult brain, showed reduced numbers of neuroblasts with a significant delay in cell cycle progression and centrosome defects, correlating with the brain size reduction observed in third instar larvae. As mutations in both human and *Drosophila KATNB1* resulted in reduced brain size, it was concluded that *KATNB1* is likely associated with microcephaly in humans. Finally, it was noted that animals lacking *kat80* demonstrated neuronal aborization defects in *Drosophila*, indicating that variants in *KATNB1* could also affect the peripheral nervous system. Moving forward, it will be interesting to assess rescue by human *WDR81* or *KATNB1* and variants associated with human microcephaly in *Drosophila* models of asymmetric division and brain development.

Experiments using *Drosophila* have also helped to solve a case of a single individual affected by severe microcephaly; follow-up studies then led to the unraveling of the pathogenic mechanisms and to the discovery of many new genes associated with microcephaly in humans (Link et al., 2019; Yamamoto et al., 2014). A mutation in *Ankle2* was recovered from a large mutagenesis screen in *Drosophila* designed to identify novel mutations associated with neurodevelopmental or neurodegenerative phenotypes (Yamamoto et al., 2014). To link *Ankle2* to human disease, the human homolog (*ANKLE2*) was used to search the Baylor-Hopkins Center for Mendelian Genomics (BHCMG) exome database (Bamshad et al., 2012; Posey et al., 2019). A single individual was identified presenting with severe microcephaly (OFC z=−9 s.d.) and harboring compound heterozygous variants in *ANKLE2*. Both parents were carriers of the variants, fitting an autosomal recessive inheritance pattern. Mutations in *Drosophila Ankle2* also gave rise to a small brain phenotype in larvae due to a reduction in cell division, fewer neuroblasts and an increase in cell death (Yamamoto et al., 2014). To verify that *ANKLE2* was associated with human microcephaly, human wild-type and variant *ANKLE2* cDNAs were expressed in *Drosophila* mutant for *Ankle2*. Wild-type human *ANKLE2* rescued both lethality and brain size but the variants associated with human microcephaly did not, indicating that *ANKLE2* mutations in both *Drosophila* and humans cause reduced brain size (Link et al., 2019). These results confirmed that ANKLE2 function is conserved, and that variants in *ANKLE2* lead to primary microcephaly (MCPH16).

Additional studies showed that Ankle2 interacts with a kinase called Ballchen (VRK1 in humans) to regulate the asymmetric division of *Drosophila* neuroblasts (Link et al., 2019). It was also noted that the localization of key polarity proteins, including the PAR complex proteins aPKC, Par3 and Par6, was disrupted in dividing *Ankle2* mutant neuroblasts. PAR proteins have long been established as essential proteins for regulating polarity in many cell types, including neuroblasts, and are known to affect cell fate decisions when disrupted (Betschinger et al., 2003; Rolls et al., 2003; Suzuki and Ohno, 2006). Defects in PAR complex localization in *Ankle2* mutant animals resulted in defective asymmetric division and neuronal development, which contributed to their reduced brain volumes (Fig. 1). Using the human orthologs corresponding to the PAR complex and members of the *ANKLE2* pathway, the BHCMG and Baylor Genetics databases were searched, and additional individuals with microcephaly and neuronal phenotypes were identified (Table 1). Although VRK1 has previously been associated with pontocerebellar hypoplasia (Gonzaga-Jauregui et al., 2013), PAR complex members had not previously been associated with microcephaly phenotypes. This approach revealed that the ability to pinpoint where Ankle2 and Ballchen/VRK1 fit into the neuroblast division pathway using *Drosophila* allowed the identification of essential genes associated with cell polarity and human neurological disease (Link et al., 2019). In the future, studies using *Drosophila* and mice to investigate how human variants in PAR complex proteins lead to neuronal phenotypes could provide crucial insight into disease pathogenesis.

In related studies, ANKLE2 has been shown to interact with a Zika virus protein, NS4A (Shah et al., 2018). Expression of NS4A in *Drosophila* causes microcephaly phenotypes, while co-expression of human *ANKLE2* rescues NS4A-induced phenotypes. These investigations showed that NS4A expression causes microcephaly, and that this protein inhibits the function of ANKLE2, providing a compelling mechanism for Zika virus-induced microcephaly. Furthermore, NS4A-induced phenotypes are more severe in animals heterozygous for *Ankle2* mutations, suggesting that human variants in microcephaly loci such as *ANKLE2* may enhance Zika virus-associated microcephaly.

In summary, studies using *Drosophila* to assess *ANKLE2* function provided a diagnosis for the first patient, offered mechanisms of disease pathogenesis and allowed identification of additional patients with variants in the *ANKLE2* pathway. Furthermore, these

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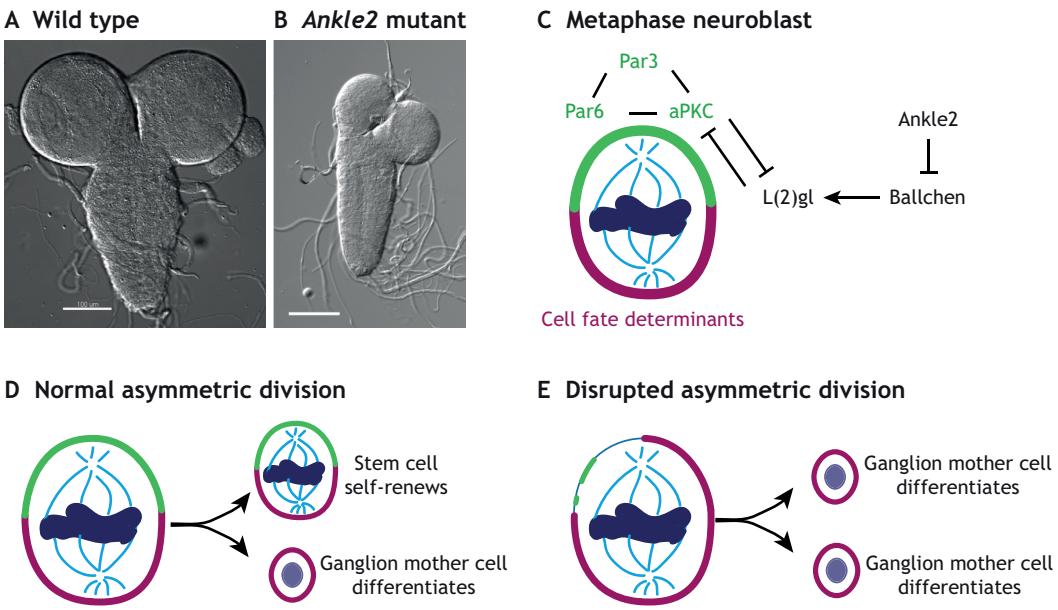

Fig. 1. Using *Drosophila* to identify an essential pathway that is required for brain development and is associated with human disease. (A,B) *Drosophila* third instar larval brains from (A) wild type and (B) severe *Ankle2* mutants with associated microcephaly. Scale bars: 100 µm. (C) Ankle2 regulates asymmetric division by interacting with a kinase, Ballchen. Together, these proteins affect L(2)gl and aPKC activity, causing defects during division, including perturbing the localization of key asymmetric proteins that regulate cell fate. These include PAR complex proteins (aPKC, Par3 and Par6), which are localized apically (green) and control the localization of cell fate determinants on the basal side of the cell (purple). (D) Normal asymmetric division of a neuroblast (when PAR proteins and cell fate determinants are segregated properly) produces a stem cell that can self-renew and a ganglion mother cell that will eventually differentiate. (E) In the case of disrupted asymmetric division (when PAR proteins are not properly localized to the apical membrane), cell fate determinant localization is expanded and two ganglion mother cells, which go on to differentiate, are produced at the expense of a neuroblast. Alternatively, cell death can occur (not shown).

experiments demonstrate that both genetic and viral-induced microcephaly result from inhibition of the same evolutionarily conserved pathway and provide evidence that genetic variation in humans may affect phenotypes associated with environmental pathogens. Obviously, not all processes that lead to microcephaly in human will be conserved. Indeed, some genes that have been shown to cause primary microcephaly in human do not have an obvious *Drosophila* homolog, and it is not yet clear whether a functional equivalent exists in *Drosophila*.

Conclusions and perspectives

When combined with human genetics, studies in *Drosophila* offer an efficient and effective approach that can aid both the diagnosis of disease and the elucidation of disease mechanisms. Although we have placed an emphasis on microcephaly here, *Drosophila* can be used for the study of many human developmental disorders. Mechanistic insights using *Drosophila* enable diagnoses for patients and often provide clues for therapeutic targets. As technologies advance with human genetics, model systems will be essential for assessing the function of the numerous variants associated with disease. *Drosophila* provides an opportunity to help diagnose cases with few affected individuals, as shown for *ANKLE2*, or in the case of *de novo* and dominant-negative mutations, such as those associated with *ALFY/WDFY3*. Furthermore, with established lines from the *Drosophila* Genetic Reference Panel (MacKay et al., 2012), the role of genetic variation

Table 1. Human orthologs corresponding to the ANKLE2 pathway and their associated phenotypes in humans

Drosophila	Human	DIOPT (18)	Human disease phenotype
Ankle2	*ANKLE2*	9	Severe microcephaly
ballchen	*VRK1*	14	Spinal muscular atrophy, microcephaly, short stature, progressive weakness, hypotonia and intrauterine growth retardation
	VRK2	11	Nanophthalmos
	VRK3	6	Severe microcephaly, brain malformations, seizures and intellectual disability
l(2)gl	*LLGL1*	15	In utero dilation of lateral ventricles, developmental delay, dolichocephaly and Arnold-Chiari Type A malformation
	LLGL2	11	Not known
aPKC	*PRKCI*	12	Not known
	PRKCZ	9	Not known
par3	*PARD3*	13	Not known
	PARD3B	11	Microcephaly and distal arthrogryposis
par6	*PARD6A*	12	Not known
	PARD6B	13	Not known
	PARD6C	14	Not known

DIOPT represents an ortholog prediction value. Confidence increases as value increases (highest=18).

and how it might affect phenotypes associated with rare human disease variants can be explored (Chow et al., 2016; Lavoy et al., 2018). These studies also highlight the fact that collaborations between patient families, clinicians, human geneticists and model system geneticists can and will continue to solve rare disease-related questions. In summary, a cooperative, multidisciplinary approach promotes a positive outcome for patients and advances our biological knowledge. Given that many thousands of diseases likely remain to be discovered, we anticipate that these discoveries are just the beginning of an exciting biological and therapeutic adventure.

Acknowledgements
We thank Angad Jolly for critical reading of the manuscript.

Competing interests
The authors declare no competing or financial interests.

Funding
This work was supported by the National Institutes of Health/National Institute of Neurological Disorders and Stroke (F32NS092270 to N.L.) and jointly funded by a National Human Genome Research Institute and National Heart, Lung, and Blood Institute grant to the Baylor-Hopkins Center for Mendelian Genomics (UM1 HG006542 to J.R.L, and NIH R01GM067858 and NIH R24OD022005 to H.J.B.). N.L. is supported by the Howard Hughes Medical Institute and H.J.B. is an Investigator of the Howard Hughes Medical Institute. Deposited in PMC for release after 12 months.

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