NEURAL TUBE DEFECTS

Risk of meningomyelocele mediated by the common 22q11.2 deletion

Keng Ioi Vong[1,2]†, Sangmoon Lee[1,2]†, Kit Sing Au[3], T. Blaine Crowley[4], Valeria Capra[5], Jeremiah Martino[6], Meade Haller[7], Camila Araújo[8], Hélio R. Machado[8], Renee George[1,2], Bryn Gerding[1,2], Kiely N. James[1,2], Valentina Stanley[1,2], Nan Jiang[1,2], Kameron Alu[1,2], Naomi Meave[1,2], Anna S. Nidhiry[2], Fiza Jiwani[1,2], Isaac Tang[1,2], Ashna Nisal[1,2], Ishani Jhamb[1,2], Arzoo Patel[1,2], Aakash Patel[1,2], Jennifer McEvoy-Venneri[1,2], Chelsea Barrows[1,2], Celina Shen[1,2], Yoo-Jin Ha[1,2], Robyn Howarth[1,2], Madison Strain[9], Allison Elizabeth Ashley-Koch[9], Matloob Azam[10], Sara Mumtaz[11], Gyang Markus Bot[12], Richard H. Finnell[13], Zoha Kibar[14], Ahmed I. Marwan[15], Gia Melikishvili[16], Hal S. Meltzer[17], Osvaldo M. Mutchinick[18], David A. Stevenson[19], Henry J. Mroczkowski[20], Betsy Ostrander[21], Erica Schindewolf[22], Julie Moldenhauer[22], Elaine H. Zackai[4], Beverly S. Emanuel[4], Sixto Garcia-Minaur[23], Beata A. Nowakowska[24], Roger E. Stevenson[25], Maha S. Zaki[26], Hope Northrup[3], Hanna K. McNamara[27], Kimberly A. Aldinger[27,28,29], Ian G. Phelps[28], Mei Deng[28], Ian A. Glass[28], Spina Bifida Sequencing Consortium‡, Bernice Morrow[30], Donna M. McDonald-McGinn[4,31], Simone Sanna-Cherchi[6], Dolores J. Lamb[7,32], Joseph G. Gleeson[1,2]*

Meningomyelocele is one of the most severe forms of neural tube defects (NTDs) and the most frequent structural birth defect of the central nervous system. We assembled the Spina Bifida Sequencing Consortium to identify causes. Exome and genome sequencing of 715 parent-offspring trios identified six patients with chromosomal 22q11.2 deletions, suggesting a 23-fold increased risk compared with the general population. Furthermore, analysis of a separate 22q11.2 deletion cohort suggested a 12- to 15-fold increased NTD risk of meningomyelocele. The loss of *Crkl*, one of several neural tube–expressed genes within the minimal deletion interval, was sufficient to replicate NTDs in mice, where both penetrance and expressivity were exacerbated by maternal folate deficiency. Thus, the common 22q11.2 deletion confers substantial meningomyelocele risk, which is partially alleviated by folate supplementation.

Meningomyelocele (MM), a subtype of spina bifida, is the most common developmental anomaly of the central nervous system and is a severe type of neural tube defect (NTD). MM often requires either pre- or postnatal surgical repair, resulting in major long-term complications such as paraparesis, bowel and bladder incontinence, and learning difficulties. Many patients require assistance with ambulation, leading to psychological and socioeconomic sequelae. Most patients with MM also have Chiari type II malformation and resultant hydrocephalus.

Prevalence of MM was historically greater than 1 per 2500 live births before folic acid (FA) fortification, though this varied by country and ancestry (*1*). The introduction of FA into grains in many parts of the world beginning in 1998 reduced prevalence (*2*), but MM remains a particular challenge in some populations, including geographies without FA fortification or with FA-depleted soil. Causes of MM remain mostly unknown, although heritability is estimated at 70% (*3*) and environmental factors in addition to FA—such as maternal diabetes; drugs, including valproate; and mycotoxins like fumonisin—increase risk (*4*, *5*). Rare and ultrarare V[illegible] pathway variants, found in 1 to 3% of patients, also contribute (*6*, *7*), but risk attributed to more common genetic variants remains unexplored.

To better understand the genetic architecture of MM, we established the Spina Bifida Sequencing Consortium (SBSC) in 2015 to aggregate MM trios from diverse ethnic groups and FA-exposure histories. The entry criteria were restricted to the most severe form of MM that was compatible with long-term survival, with the presence of open neural tube tissue and hydrocephalus due to Chiari type II malformation, which required shunt placement surgery, where both the parents and proband were available for DNA sampling. This trio approach was selected because of success in other complex or multigenic conditions such as congenital heart disease and autism spectrum disorder (*8*, *9*), where high-impact de novo mutations under strong purifying selection might increase MM risk, possibly modified by FA and other environmental factors.

From the 715 SBSC trios that underwent either whole-genome or whole-exome sequencing, we identified six patients who harbored previously unrecognized de novo or inherited 22q11.2 deletions (22q11.2del); this mutation was the most common recurrent genetic finding. Our results suggested that patients with MM are 22.98 times more likely to harbor 22q11.2del compared with the general population. Moreover, in a cohort of 1522 subjects ascertained for 22q11.2del, the most common cause of DiGeorge syndrome (*10*), we identified eight patients with MM. Thus, risk of MM for those with 22q11.2del was approximately 12.28- to 15.54-fold greater than expected. Among the 10 protein-coding genes within the minimal consensus LCR22C-D interval, *CRKL*, which encodes the CRK-like proto-oncogene adaptor protein, emerged as the strongest candidate, where

[1]Department of Neurosciences, University of California, San Diego, La Jolla, CA 92093, USA. [2]Rady Children's Institute for Genomic Medicine, San Diego, CA 92123, USA. [3]Department of Pediatrics, McGovern Medical School at the University of Texas Health Science Center at Houston (UTHealth) and Children's Memorial Hermann Hospital, Houston, TX 77030, USA. [4]22q and You Center, Division of Human Genetics, Children's Hospital of Philadelphia and Department of Pediatrics, Perelman School of Medicine of the University of Pennsylvania, Philadelphia, PA 19104, USA. [5]Genomics and Clinical Genetics Unit, IRCCS Istituto Giannina Gaslini, 16147 Genova, Italy. [6]Division of Nephrology, Department of Medicine, Columbia University, NY 10027, USA. [7]Center for Reproductive Medicine, Department of Molecular and Cellular Biology and Scott Department of Urology, Baylor College of Medicine, TX 77030, USA. [8]Department of Surgery and Anatomy, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto 14040-900, Brazil. [9]Duke Molecular Physiology Institute, Duke University Medical Center, Durham, NC 27710, USA. [10]Pediatrics and Child Neurology, Wah Medical College, Wah Cantt, Punjab 47000, Pakistan. [11]Department of Biological Sciences, National University of Medical Sciences (NUMS), Punjab 46000, Pakistan. [12]Neurosurgery Division, Department of Surgery, Jos University Teaching Hospital, Jos 930105, Nigeria. [13]Center for Precision Environmental Health, Departments of Molecular and Human Genetics, Molecular and Cellular Biology and Medicine, Baylor College of Medicine, Houston, TX 77030, USA. [14]Department of Neurosciences, University of Montreal and CHU Sainte Justine Research Center, Montreal, QC H3T 1C5, Canada. [15]Division of Pediatric Surgery, University of Colorado School of Medicine, Children's Hospital of Colorado, Colorado Fetal Care Center, Aurora, CO 80045, USA. [16]Department of Pediatrics, MediClubGeorgia Medical Center, Tbilisi 0160, Georgia. [17]Department of Neurosurgery, University of California San Diego, Rady Children's Hospital, San Diego, CA 92123, USA. [18]Department of Genetics, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, 14080 Mexico City, Mexico. [19]Division of Medical Genetics, Stanford University, Palo Alto, CA 94305, USA. [20]Division of Medical Genetics, University of Tennessee Health Science Campus, Memphis, TN 38163, USA. [21]Division of Pediatric Neurology, Primary Children's Hospital, University of Utah, Salt Lake City, UT 84113, USA. [22]Center for Fetal Diagnosis and Treatment, Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA. [23]Clinical Genetics Section, Institute of Medical and Molecular Genetics, University Hospital La Paz, 28046 Madrid, Spain. [24]Department of Medical Genetics, Institute of Mother and Child, Kasprzaka, 01-211 Warsaw, Poland. [25]JC Self Research Institute of Human Genetics, Greenwood Genetic Center, Greenwood, SC 29646, USA. [26]Clinical Genetics Department, Human Genetics and Genome Research Division, National Research Centre, Cairo 12311, Egypt. [27]Center for Integrative Brain Research, Seattle Children's Research Institute, Seattle, WA 98101, USA. [28]Departments of Pediatrics, University of Washington, Seattle, WA 98105, USA. [29]Department of Neurology, University of Washington, Seattle, WA 98105, USA. [30]Division of Translational Genetics, Department of Genetics, Albert Einstein College of Medicine, Bronx, NY 10461, USA. [31]Department of Human Biology and Medical Genetics, Sapienza University, 00185-Rome RM, Italy. [32]Department of Urology, Center for Reproductive Genomics, Englander Institute for Precision Medicine, Weill Cornell Medicine, New York, NY 10021, USA.
***Corresponding author. Email: jogleeson@health.ucsd.edu**
†These authors contributed equally to this work.
‡Spina Bifida Sequencing Consortium collaborators and affiliations are listed at the end of this paper.

Table 1. Clinical manifestation of MM patients in the SBSC with 22q11.2del. A total of 715 probands from the SBSC were analyzed. Six patients with MM were identified. CPC, choroid plexus cauterization; ETV, endoscopic third ventriculostomy; F, female; GA, gestational age; IVF, in vitro fertilization; M, male; VPS, ventriculoperitoneal shunt.

Patient number	P1 (6338-2-1)	P2 (6803-2-1)	P3 (6903-2-1)	P4 (6353-2-1)	P5 (7431-2-1)	P6 (7036-2-1)
General characteristics						
Birth year	2016	2017	1994	2013	2002	1988
Sex	M	M	M	M	F	F
Gestational age (weeks)	38.5	33.5	38	36	39	37.5
Birth weight (g)	2576	1474	3373	3170	2600	2818
Length at birth (cm)	53	39	58.3	51	46	50.5
Head circumference at birth (cm)	37.5	27	38.5	36	35.5 (at 1 month)	34.5
Ethnicity	Hispanic	European American	European American	European American	Brazilian	Hispanic
Country of birth	United States	United States	United States	United States	Brazil	Mexico
22q11.2 deletion syndrome features						
22q11.2del type	A-D (de novo)	A-D (de novo)	B-D (inherited)	B-D (de novo)	C-D (de novo)	C-D (inherited)
Dysmorphic craniofacial features	Yes	Yes	None	Yes	None	None
Congenital heart disease	None	None	None	None	None	None
Genitourinary anomaly	None	None	Unilateral renal aplasia	None	None	None
NTD						
NTD subtype	MM + Chiari type II	MM + Chiari type II	MM + Chiari type II	MM + Chiari type II	MM + Chiari type II	MM + Chiari type II
MM level	L4-L5	L5-S1	L3-L4	L3-L5	L3-L4	L4-L5
Fetal surgery	None	None	None	25 weeks GA	None	None
Hydrocephalus surgery	VPS at 3 days old	ETV/CPC at 1 month old	VPS at 2 days old	VPS at 11 months old	VPS at 20 days old	ETV/CPC at 1 month old
Ambulate independently	No	Yes (delayed)	No	Yes (delayed)	No	No
Assisted ambulation	Walker	Leg braces	Wheelchair	Leg braces	Wheelchair	Wheelchair
Family history of NTD	None	None	None	Yes, maternal great aunt	None	None
Periconceptional FA fortification	Yes	Yes	No	Yes	No	No
Psychomotor or neurological phenotype						
Language	Normal	Delayed	Delayed	Delayed	Delayed	Normal
Gross motor upper	Normal	Normal	Normal	Delayed	Normal	Normal
Fine motor upper	Normal	Delayed	Delayed	Delayed	Delayed	Normal
Seizure	None	Febrile seizure	None	None	None	None
Brain imaging result	Chiari type II, stable hydrocephalus	Chiari type II, stable hydrocephalus	Chiari type II, stable hydrocephalus	Chiari type II, stable hydrocephalus	Chiari type II, stable hydrocephalus	Chiari type II, stable hydrocephalus
Other notable health conditions	None	IVF pregnancy (healthy dizygotic twin sister), reflux, hearing aids	Autism spectrum disorder	Neurogenic bladder	Early puberty, polycystic ovarian syndrome	None

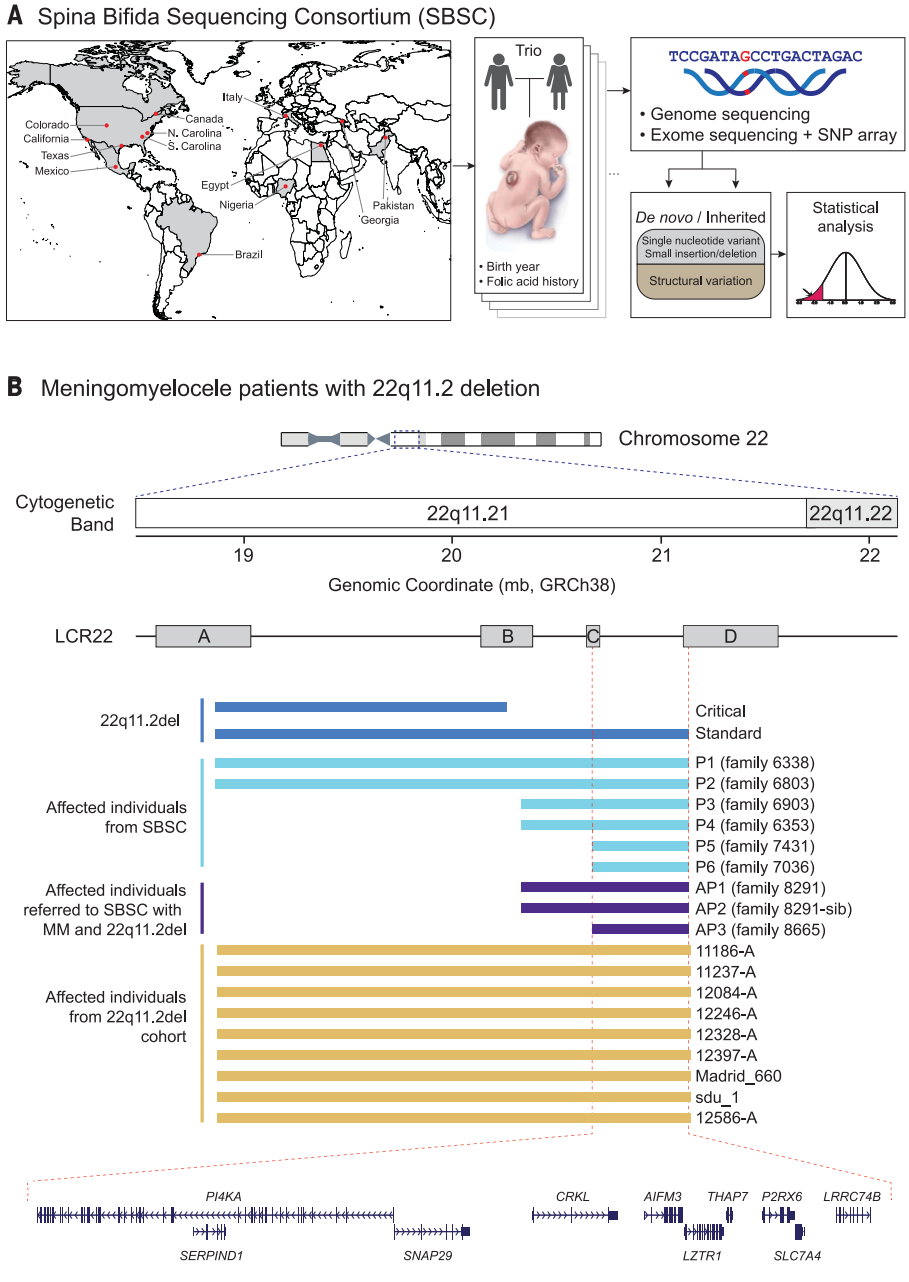

Fig. 1. Deletions in chromosome 22q11.2 associated with MM. (**A**) Trios of MM were recruited to the SBSC from multiple countries, including the United States, Mexico, Brazil, Canada, Italy, Georgia, Egypt, Nigeria, and Pakistan. Red dots indicate city locations of recruitment centers. DNA samples from trios were evaluated by whole-exome sequencing and/or whole-genome sequencing to identify de novo or inherited genetic variants. SNP, single-nucleotide polymorphism. (**B**) The standard and critical deletions of 22q11.2 LCRs (LCR22) for DiGeorge risk span 2.5 or 1.5 Mb, respectively (blue bars). Six patients with MM (P1 to P6, cohort size n = 715) showed chromosomal 22q11.2 deletion (22q11.2del, light blue bars): four de novo (P1, P2, P4, and P5) and two inherited from a healthy parent (P3 and P6). Whereas approximately 85% of 22q11.2del spanned the classic LCR22A-D region (*35*), two-thirds of MM cases showed partial deletions, either LCR22B-D or LCR22C-D (P3 to P6). Another three MM patients with 22q11.2del were referred to our study (purple bars, AP1 to AP3): two siblings with an inherited LCR22B-D deletion [AP1 and AP2, as previously described (*14*)] and a patient with an LCR22C-D deletion (AP3). The consensus LCR22C-D deletion interval in all MM patients includes 10 protein-coding genes. In addition, nine MM patients (yellow bars) were identified, eight from the Children's Hospital of Philadelphia (CHOP) and one from Spain (madrid_660), which is part of the International 22q11.2 Brain and Behavior Consortium, all of whom had LCR22A-D deletions. Only patients from CHOP were included for odds ratio calculation.

we found that loss contributes to NTD risk in a FA-dependent fashion in mouse.

MM is associated with 22q11.2del

As an effort to investigate the genetic risk factors for MM, we established the SBSC to aggregate trios for gene discovery (Fig. 1). Most previous MM genetic studies focused on single-nucleotide variants or small insertion-deletions for only affected individuals, and few interrogated for structural variation (SV) (*11*). In this study, we focused on de novo SVs that can affect multiple genes by sequencing 555 and 160 MM family trios from the SBSC cohort with whole-exome and whole-genome methods, respectively.

We identified six patients with 22q11.2del (P1 to P6); the mutation was inherited in two patients and de novo in four (Fig. 1, Table 1, and figs. S1 to S3). In addition, three patients were identified who had other chromosomal de novo SVs that were not recurrent (tables S1 and S2). Therefore, 22q11.2del was the most recurrent single genetic variation from the SBSC cohort, and other de novo SVs appeared only rarely in MM. Two of six patients had the standard 3-Mb deletion flanked by low-copy repeats (LCRs) LCR22A and LCR22D (LCR22A-D), whereas two had the atypical LCR22B-D deletion and two had the atypical LCR22C-D deletion. As evaluated by whole-genome sequencing in all subjects, LCR22C-D was deleted across all the MM patients, suggesting a minimal consensus interval.

All deletions were de novo, except those in P3 (LCR22B-D) and P6 (LCR22C-D), for whom parents were asymptomatic and unaware that they were carriers (Fig. 1 and Table 1). Individuals with 22q11.2del other than LCR22C-D displayed various degrees of facial dysmorphism, and P3 had unilateral renal agenesis associated with 22q11.2 deletion syndrome (*12*). The 22q11.2del frequency in the SBSC cohort was 0.839% (6/715), which was significantly higher than frequencies in the gnomAD database of more than 10,000 healthy individuals (0.0368%, 4/10,847; odds ratio for patients versus controls of 22.98, $P = 9.16 \times 10^{-6}$, 95% confidence interval (CI) = 6.47 to 81.61) and a population control database (0.0051%, 1/19,584; odds ratio for patients versus controls of 165.64, $P = 1.27 \times 10^{-8}$, 95% CI = 19.91 to 1377.71) (fig. S4 and table S3) (*13*). Half of the patients were born after FA supplementation was implemented in their home country (Table 1 and table S4). These results suggest an association between the presence of 22q11.2del and MM risk.

We identified three additional individuals with MM and 22q11.2del who were referred to the SBSC for analysis (AP1 to AP3; Fig. 1 and table S5), two of whom were described before (*14*). These individuals were excluded from odds ratio calculation but confirmed the link

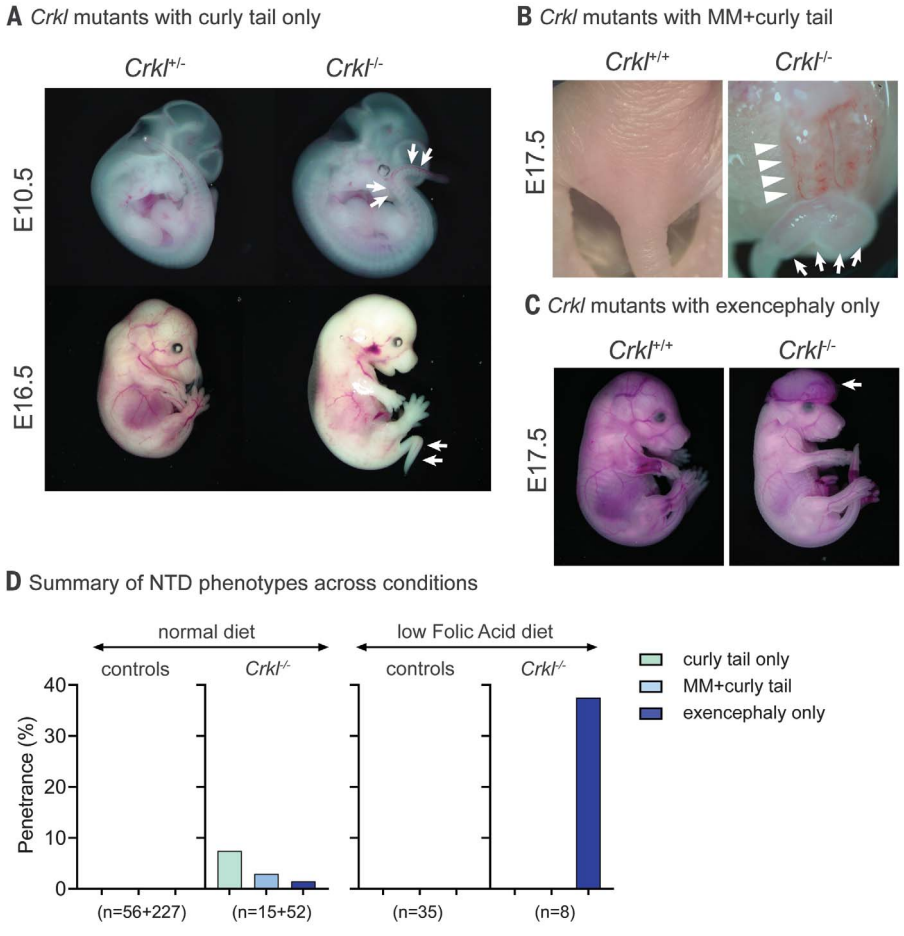

Fig. 2. NTDs in *Crkl* mutants are exacerbated by a FA-restricted diet. (**A**) Brightfield images showing gross morphology of control and *Crkl* null mutants that displayed a curly tail. White arrows identify the caudal neural tube and tail in control and $Crkl^{-/-}$ embryos. (**B**) Brightfield images of *Crkl* mutant embryos with curly tail (white arrows) and open neural tissue (white arrowheads) showing MM. (**C**) Brightfield image of *Crkl* mutant with exencephaly, a severe form of NTD. The white arrow identifies exposed brain tissue. (**D**) Penetrance of NTD phenotypes across genotypes and FA-diet treatments. No controls ($Crkl^{+/+}$ and $Crkl^{+/-}$) displayed NTD phenotypes on either a normal or a low-FA diet. With control diet (FA at 3 ppm), $Crkl^{-/-}$ mutants displayed curly tail at 7.46% penetrance (5/67) or, rarely, curly tail together with MM (2.99%, 2/67) or exencephaly (1.49%, 1/67). The penetrance for exencephaly increased significantly in $Crkl^{-/-}$ mutants after a FA-restricted diet (37.5%, 3/8, odds ratio of 34.96, P = 0.0031, 95% CI = 3.05 to 400.71), whereas curly tail or MM was not observed (see also fig. S8).

between the two conditions. The same SV analysis pipeline was performed on 704 control trios from the Simons Simplex Collection (SSC), which is composed of healthy siblings of children with autism and their parents (*15*). None had 22q11.2del (P = 0.01323).

To further verify the risk of MM conferred by 22q11.2del, we analyzed the frequency of MM in an independent cohort ascertained for the presence of 22q11.2del (n = 1522) and identified eight patients with MM who had the common LCR22A-D deletion (Fig. 1 and table S6). Approximately 59.6% of these patients were born in the United States after the mandate of FA fortification. The frequency of MM (0.526%, 8/1522) was significantly higher in these individuals than in the general population (odds ratio for patients versus controls of 12.28, $P = 9.01 \times 10^{-7}$, and 95% CI = 5.77 to 26.17, when comparing with the prevalence of MM before FA fortification; and odds ratio for patients versus controls of 15.54, $P = 1.88 \times 10^{-7}$, and 95% CI = 7.18-33.62, when comparing with the prevalence of MM in the general population after FA fortification) (*2*). Through the International Brain and Behavior Consortium, we also identified an additional 22q11.2del patient who displayed MM and who was also excluded from odds ratio calculation (Fig. 1 and table S6).

All individuals with 22q11.2del and MM identified in our study shared the LCR22C-D as a consensus interval, within which there are 10 protein-coding genes (table S7). Given that only one copy of the interval was deleted, any variants on the intact allele would be hemizygous, and thus we looked for rare (<2% allele frequency from gnomAD) coding variants on the intact LCR22C-D haplotype. We identified one hemizygous synonymous variant in *LZTR1*. Variants in *SNAP29* (*16*) and *AIFM3* (*14*) were previously reported, which were predicted to be benign (supplementary text) (*17*). This argues against loss of a second copy of a 22q11.2 gene as the mechanism. We identified likely nonpathogenic missense variants of *MYO1E*, *SHROOM3*, *ANKRD6*, *CELSR1*, and *GLI3*, which were all inherited and nonrecurrent—either not reported in ClinVar or predicted to be benign—and have relatively high allele frequency in gnomAD (table S8).

CRKL as the major driver of MM risk

Next, we sought to identify genes within LCR22C-D that are responsible for MM risk. To understand how gene expression was affected, RNA sequencing (RNA-seq) of five fibroblast lines from 22q11.2del patients with MM was performed. As expected, expression of only the genes within the deletion showed substantial decrease, suggesting that phenotypes did not arise from chromatin shuffling–related changes in the expression of genes flanking the deletions (fig. S5 and table S9). Among the 10 protein-coding genes, *CRKL* is the least tolerant to loss [loss intolerance probability (pLI) score of 0.45, whereas none of the others have a pLI score greater than 0.01; table S10]. Spatial transcriptomics of murine embryonic day 10.5 (E10.5) sections suggested that none of the 10 candidates were exclusively expressed in the neural tube (fig. S6), but RNA-seq suggested that *Crkl* had the strongest expression in the murine E9.5 and E10.5 developing neural tube, followed by *Aifm3* and *Pi4ka* (fig. S7).

To correlate these findings with human embryos, we next performed single-nucleus RNA-seq at Carnegie stage 16 (CS16) and confirmed robust expression of *CRKL*, *PI4KA*, and *LZTR1* in neural tube progenitors, neurons, and neural crest (fig. S7). However, mouse

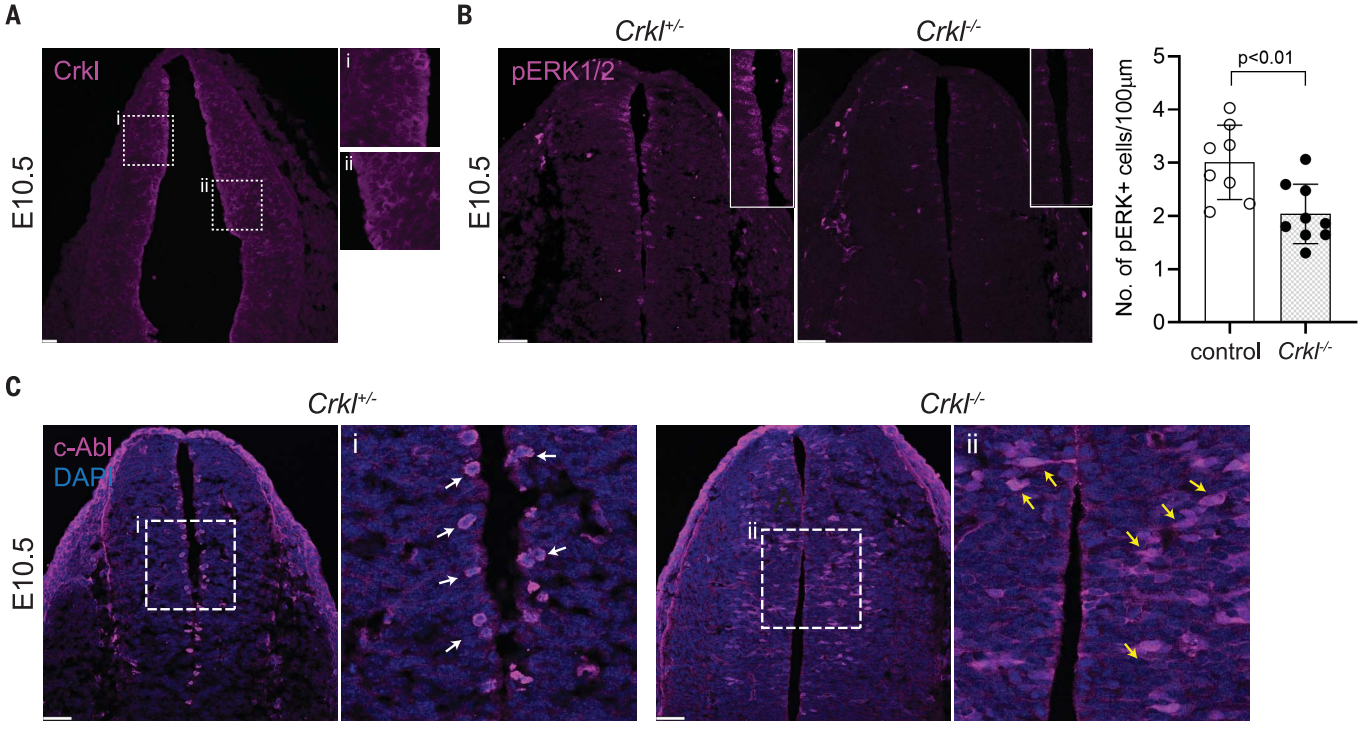

Fig. 3. Signaling defects in *Crkl* mutant neural tube. (**A**) CRKL immunostaining in E9.5 mouse showing broad expression in neural tube. Insets i and ii show corresponding magnified areas. (**B**) Phosphorylated ERK1/2 immunostaining of E9.5 mouse neural tube of control and *Crkl*$^{-/-}$ embryos. Insets show high magnification of the ventricular zone. The bar graph shows the quantification of ERK1/2 phosphorylation cells within the ventricular zone, from at least eight images of two animals per genotype (P = 0.0062). Error bars indicate standard deviation. (**C**) c-Abl localization in control predominantly excluded from the nucleus (white arrows in inset i) versus greater accumulation in the nucleus (yellow arrows in inset ii) in *Crkl*$^{-/-}$. Insets i and ii show corresponding magnified areas. DAPI, 4′,6-diamidino-2-phenylindole. Scale bars are 20 μm in (A) and 50 μm in (B) and (C).

loss-of-function alleles for some of these genes, for example, *Snap29* and *Lztr1*, were previously reported without observable NTDs in large numbers of mutant animals (*18*, *19*) and were thus deprioritized (*17*). We prioritized three candidate genes from LCR22C-D interval for further analysis: *AIFM3*, *CRKL*, and *PI4KA*.

First, we knocked out *Crkl* with a previously reported exon 2 deletion allele (*20*). Because the environment or FA level present in rodent chow could affect NTD penetrance (*21*, *22*), we analyzed the *Crkl* phenotypes at two independent vivaria [University of California, San Diego (UCSD) and Baylor College of Medicine] using chows with different FA content on a nearly isogenic B6/SvJ background. We observed curly tail, a morphological deformity previously associated with murine NTDs (*23*), in 26.67% of null embryos and no controls in the first cohort (4/15) [3 parts per million (ppm) FA in diet]. In the other cohort of 52 *Crkl* null embryos, 1.9% showed exencephaly (1/52), 3.8% (2/52) had curly tail with MM, and 1.9% showed curly tail alone (1/52) (4 ppm FA in diet) (Fig. 2 and fig. S8). These results suggest that *Crkl* loss can increase risk of NTD and that additional factors such as environment or diet may modify risk.

We thus repeated the *Crkl* null breeding on the same B6/SvJ background in a single vivarium at UCSD using FA-replete (2 ppm) versus FA-deficient diets (0.2 ppm) ad libitum for 6 weeks before mating. *Crkl* mutant embryos on a low–maternal FA diet displayed a notably increased prevalence of exencephaly (37.5%, 3/8), a severe form of murine NTD (Fig. 2 and fig. S8; odds ratio for low FA diet versus control diet of 34.96, P = 0.0031, 95% CI = 3.05 to 400.71), whereas no control littermates had NTDs. None of the embryos showed curly tail. Moreover, the exencephaly phenotype was replicated in 1 out of 10 homozygous floxed conditional *Crkl* allele mice carrying *Hoxb7-Cre*, which expresses Cre recombinase in the neural tissue but not in the epidermis or mesoderm (*24*). Although exencephaly was rarely observed in this line, these results suggest that *Crkl* mediates its effect in a spatially restricted fashion in neural tissues (fig. S9).

To exclude other genes in the LCR22C-D interval, we also used CRISPR-Cas9 to knock out *Aifm3* and *Pi4ka*, which are the only other genes that share strong expression in the neural tube (table S11). We found that *Pi4ka*$^{-/-}$ was uniformly lethal before neurulation, whereas all 11 *Aifm3*$^{-/-}$ mice were viable without NTDs. To assess possible gene-gene interaction, compound mutations for *Aifm3* and *Pi4ka* were created. None of the 17 *Aifm3*$^{+/-}$*Pi4ka*$^{+/-}$ or 22 *Aifm3*$^{-/-}$*Pi4ka*$^{+/-}$ mice showed NTD phenotypes (table S12). Together, although not exhaustively excluding other genes in the interval, these results suggest that *Crkl* can influence NTD risk in a spatially specific and FA-dependent manner.

CRKL enhances ERK phosphorylation to promote cell shape changes during lens development (*25*). Also, studies in *Xenopus* demonstrated that ERK activity peaks during neural plate folding and promotes neural tube closure (*26*). In addition, knockout of the Gβ1 subunit of the heterotrimeric G protein, a downstream effector of G protein–coupled receptor signaling, results in NTD with 40% penetrance, presumably owing to reduced ERK phosphorylation and neural progenitor proliferation (*27*). CRKL protein was most abundant along the apically located cells in the neural tube ventricular zone, as visualized both in mouse and human embryos (Fig. 3 and fig. S7). ERK phosphorylation was significantly depleted in the neural tube of all *Crkl* mutants, especially in the progenitors at the ventricular zone (Fig. 3; P = 0.0062). However, *Crkl* mutants

showed no impairment of cell proliferation in the neural tube (fig. S10), suggesting that ERK function during neural tube closure was independent of cell proliferation and instead regulated other cellular process like cytoskeletal remodeling, as described previously in other contexts (*28*, *29*).

CRKL functions as a critical effector of c-Abl, a non–receptor tyrosine kinase that mediates cytoskeleton remodeling, where cytoplasmic translocation of c-Abl from the nucleus to focal adhesions contributes to adhesion-dependent actin reorganization. Although c-Abl was predominantly localized to the cytoplasm in neural tube cells in control embryos during neural tube closure, *Crkl* mutants showed aberrant nuclear accumulation of c-Abl, suggesting altered signaling (Fig. 3). These findings suggest that *Crkl* loss may contribute to NTD risk in 22q11.2del patients.

Discussion

In this work, we demonstrate that 22q11.2del in the minimal consensus LCR22C-D region increases the risk for MM through analysis of two separate cohorts: one ascertained for MM and the other ascertained for 22q11.2del. We found that the risk of identifying 22q11.2del in MM is approximately 22.98 times greater than expected by chance, and the risk of identifying MM in association with 22q11.2del is approximately 12.28 to 15.54 times greater than expected by chance. 22q11.2del is the most recurrent genetic variant in the SBSC cohort identified to date. We identified six patients with 22q11.2del from our SBSC cohort and nine additional patients from the 22q11.2del cohorts. Thus, in addition to the rare and ultrarare genetic variants previously linked to MM, we now report a substantially increased risk from the recurrent 22q11.2del.

22q11.2 deletion syndrome is the most common clinically relevant microdeletion in humans, occurring in approximately 1 in 992 pregnancies and 1 in 2148 live births (*30*). Although the standard 22q11.2del encompasses the LCR22A-D region, analysis of our MM patients suggests a minimal LCR22C-D risk interval. The LCR22C-D deletion is much less common than the standard LCR22A-D deletion, occurring just once in gnomAD and not at all in Decipher databases, which have collectively more than 46,000 subjects. The 10 genes within the interval were each considered as candidates, prioritized by gene expression within the murine embryo. Knockout of the five genes with the highest level of expression, either previously published or generated in this work, revealed that only *Crkl* was sufficient to replicate NTDs in mice, although with expressivity and penetrance of 7 to 37.5%, depending on dietary FA.

Crkl mutant mice displayed three different NTD-related phenotypes, which varied dependent on FA intake, including curly tail, curly tail with MM, or exencephaly. These traits were observed in a nearly pure C57BL/6 genetic background, arguing against genetic modifiers and instead suggesting that they are part of the same NTD spectrum. Recently, *CRKL* emerged as a driver for cardiac and renal anomalies associated with 22q11.2 deletion syndrome (*12*, *20*, *31*), and, correspondingly, our subject P3 was noted to have unilateral renal agenesis. These data suggest that *CRKL* may be required for multiple aspects of embryogenesis.

Our results demonstrate that dietary FA insufficiency markedly increases both the penetrance and expressivity of NTDs in *Crkl* mutant mice, suggesting that FA replete and deficient states were associated with different frequencies and types of NTDs. The exact mechanism by which *Crkl* loss imposes NTD risk remains to be explored, but we observed altered ERK phosphorylation and nuclear localization of the *Crkl* binding partner c-Abl before neural tube closure in *Crkl* mutants, suggesting that these cytological defects establish risk, which is then further modified by FA. However, whether FA modulates NTD risk through this pathway is unclear. Because *CRKL* exhibits a somewhat ubiquitous expression pattern, it remains unclear which cell type or types are responsible for neural tube closure. We note that three of six SBSC patients were born after FA supplementation, suggesting that 22q11.2del-associated MM is at least partially resistant to maternal FA supplementation. No rare inherited or de novo intragenic variants of *CRKL* were identified, suggesting that additional cis or trans genetic or environmental modifying factors, particularly FA, may contribute to the risk of NTDs in humans. Although *CRKL* is the most likely candidate driving MM risk, the potential involvement of other LCR22C-D genes cannot be completely excluded.

22q11.2 deletion syndrome is associated with birth defects, medical conditions, cognitive deficits, and psychiatric phenotypes (*20*, *30*, *32*). Most 22q11.2del occur de novo, owing to the presence of LCRs that result in nonallelic homologous recombination. Several case reports of patients with MM and 22q11.2del—some de novo and some inherited—are consistent with our findings (*14*, *33*, *34*). In conclusion, our findings identify the common 22q11.2del associated with risk of MM and suggest mechanisms by which FA could mitigate MM risk in humans.

REFERENCES AND NOTES

1. S. Lee, J. G. Gleeson, *Trends Neurosci.* **43**, 519–532 (2020).
2. J. Williams *et al.*, *MMWR Morb. Mortal. Wkly. Rep.* **64**, 1–5 (2015).
3. H. J. Blom, G. M. Shaw, M. den Heijer, R. H. Finnell, *Nat. Rev. Neurosci.* **7**, 724–731 (2006).
4. R. H. Finnell *et al.*, *Front. Genet.* **12**, 659612 (2021).
5. J. Gelineau-van Waes, K. A. Voss, V. L. Stevens, M. C. Speer, R. T. Riley, *Adv. Food Nutr. Res.* **56**, 145–181 (2009).
6. K. S. Au *et al.*, *Sci. Rep.* **11**, 3639 (2021).
7. Z. Kibar *et al.*, *N. Engl. J. Med.* **356**, 1432–1437 (2007).
8. J. Homsy *et al.*, *Science* **350**, 1262–1266 (2015).
9. F. K. Satterstrom *et al.*, *Cell* **180**, 568–584.e23 (2020).
10. D. M. McDonald-McGinn, H. S. Hain, B. S. Emanuel, E. H. Zackai, in *GeneReviews*, M. P. Adam *et al.*, Eds. (University of Washington, 1993).
11. P. Wolujewicz *et al.*, *Genet. Med.* **23**, 1211–1218 (2021).
12. E. Lopez-Rivera *et al.*, *N. Engl. J. Med.* **376**, 742–754 (2017).
13. R. L. Collins *et al.*, *Nature* **581**, 444–451 (2020).
14. C. Leoni *et al.*, *Am. J. Med. Genet. A.* **164A**, 2701–2706 (2014).
15. G. D. Fischbach, C. Lord, *Neuron* **68**, 192–195 (2010).
16. D. M. McDonald-McGinn *et al.*, *J. Med. Genet.* **50**, 80–90 (2013).
17. Supplementary text is available in the supplementary materials.
18. A. Cuevas-Navarro *et al.*, *eLife* **11**, e76495 (2022).
19. V. Keser *et al.*, *Commun. Biol.* **2**, 375 (2019).
20. M. Haller, Q. Mo, A. Imamoto, D. J. Lamb, *Proc. Natl. Acad. Sci. U.S.A.* **114**, 4981–4986 (2017).
21. J. D. Gray *et al.*, *Hum. Mol. Genet.* **19**, 4560–4572 (2010).
22. K. Y. Leung, S. C. De Castro, D. Savery, A. J. Copp, N. D. Greene, *Brain* **136**, 2836–2841 (2013).
23. H. W. van Straaten, A. J. Copp, *Anat. Embryol.* **203**, 225–238 (2001).
24. Y. Ahn, H. E. Mullan, R. Krumlauf, *Dev. Biol.* **388**, 134–144 (2014).
25. T. N. Collins *et al.*, *eLife* **7**, e32586 (2018).
26. E. B. Sequerra, R. Goyal, P. A. Castro, J. B. Levin, L. N. Borodinsky, *J. Neurosci.* **38**, 4762–4773 (2018).
27. H. Okae, Y. Iwakura, *Dev. Dyn.* **239**, 1089–1101 (2010).
28. M. C. Mendoza, M. Vilela, J. E. Juarez, J. Blenis, G. Danuser, *Sci. Signal.* **8**, ra47 (2015).
29. M. C. Mendoza *et al.*, *Mol. Cell* **41**, 661–671 (2011).
30. D. M. McDonald-McGinn *et al.*, *Nat. Rev. Dis. Primers* **1**, 15071 (2015).
31. D. L. Guris, J. Fantes, D. Tara, B. J. Druker, A. Imamoto, *Nat. Genet.* **27**, 293–298 (2001).
32. D. M. McDonald-McGinn, *Am. J. Med. Genet. A.* **176**, 2055–2057 (2018).
33. R. E. Nickel, R. E. Magenis, *Am. J. Med. Genet.* **66**, 25–27 (1996).
34. R. E. Nickel *et al.*, *Am. J. Med. Genet.* **52**, 445–449 (1994).
35. I. M. Campbell *et al.*, *Am. J. Med. Genet. A.* **176**, 2058–2069 (2018).

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Spina Bifida Sequencing Consortium
Allison Elizabeth Ashley Koch[1], Hal S. Meltzer[2], Joan Le[3], Kit Sing Au[4], Hope Northrup[4], Gyang Markus Bot[5], Valeria Capra[6], Richard H. Finnell[7], Zoha Kibar[8], Philip J. Lupo[9], Helio R. Machado[10], Camila Araújo[10], Tony Magana[11], Ahmed I. Marwan[12], Gia Melikishvili[13], Osvaldo M. Mutchinick[14], Roger E. Stevenson[15], Anna Yurrita[16], Maha S. Zaki[17], Sara Mumtaz[18], José Ramón Medina-Bereciartu[19], Caroline M. Kolvenbach[20], Shirlee Shril[20], Friedhelm Hildebrandt[20], Mahmoud M. Noureldeen[21], Aida MS. Salem[21], Yukitoshi Takahashi[22], Hormos Salimi-Dafsari[23], H. Westley Phillips[24], Brian Hanak[25], Bülent Kara[26], Ayfer Sakarya Güneş[26], David D. Gonda[3], Salman Kirmani[27], Tinatin Tkemaladze[28], Joseph G. Gleeson[29,30]*

[1]Duke Molecular Physiology Institute, Duke University Medical Center, Durham, NC 27710, USA. [2]WVU School of Medicine, Rockefeller Neuroscience Institute, Morgantown, WV 26506, USA. [3]Rady Children's Hospital, San Diego, CA 92123, USA. [4]Department of Pediatrics, McGovern Medical School at the University of Texas Health Science Center at Houston (UTHealth) and Children's Memorial Hermann Hospital, Houston, TX 77030, USA. [5]Neurosurgery Division, Department of Surgery, Jos University Teaching Hospital, Jos 930105, Nigeria. [6]Genomics and Clinical Genetics Unit, IRCCS Istituto Giannina Gaslini, 16147 Genova, Italy. [7]Center for Precision Environmental Health, Departments of Molecular and Human Genetics, Molecular and Cellular Biology and Medicine, Baylor College of Medicine, Houston, TX 77030, USA. [8]Department of Neurosciences, University of Montreal and CHU Sainte Justine Research Center, Montreal, QC H3T 1C5, Canada. [9]Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030, USA. [10]Department of Surgery and Anatomy, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto 14040-900, Brazil. [11]Mekelle University, Mekelle, Ethiopia. [12]Division of Pediatric General, Thoracic and Fetal Surgery, Children's Hospital, University of Missouri School of Medicine, Columbia, MO 65211, USA. [13]Department of Pediatrics, MediClubGeorgia Medical Center, Tbilisi 0160, Georgia. [14]Department of Genetics, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, 14080 Mexico City, Mexico. [15]J.C. Self Research Institute of Human Genetics, Greenwood Genetic Center, Greenwood, SC 29646, USA. [16]Francisco Marroquín University, Guatemala City, Guatemala. [17]Clinical Genetics Department, Human Genetics and Genome Research Division, National Research Centre, Cairo 12311, Egypt. [18]Department of Biological Sciences, National University of Medical Sciences (NUMS), Punjab 46000, Pakistan. [19]Venezuela Association of Spina Bifida, Caracas, Venezuela. [20]Boston Children's Hospital, Harvard Medical School, Boston, MA 02115, USA. [21]Beni-Suef University, Beni-Suef, Egypt. [22]NHO Shizuoka Institute of Epilepsy and Neurological Disorders, Shizuoka, Japan. [23]Department of Pediatrics, University Hospital Cologne, University of Cologne, 50937 Cologne, Germany. [24]Department of Pediatric Neurosurgery, Stanford University, Palo Alto, CA 94304, USA. [25]Children's Hospital of Orange County, Orange, CA 92868, USA. [26]Department of Child Health and Diseases, Department of Child Neurology, Umuttepe Campus, Kocaeli University, Kocaeli, Turkey. [27]Division of Women and Child Health, Human Development Program, The Aga Khan University, Karachi 74800, Pakistan. [28]Department of Molecular and Medical Genetics, Tbilisi State Medical University, Tbilisi, Georgia. [29]Department of Neurosciences, University of California, San Diego, La Jolla, CA 92093, USA. [30]Rady Children's Institute for Genomic Medicine, San Diego, CA 92123, USA.
*Point of contact for the Spina Bifida Sequencing Consortium.
Email: jogleeson@health.ucsd.edu

SUPPLEMENTARY MATERIALS

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Materials and Methods
Supplementary Text
Figs. S1 to S10
Tables S1 to S12
References (*36*–*51*)
MDAR Reproducibility Checklist

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