ZNF423 patient variants, truncations, and in-frame deletions in mice define an allele-dependent range of midline brain abnormalities.

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ABSTRACT

Interpreting rare variants remains a challenge in personal genomics, especially for disorders with several causal genes and for genes that cause multiple disorders. *ZNF423* encodes a transcriptional regulatory protein that intersects several developmental pathways. *ZNF423* has been implicated in rare neurodevelopmental disorders, consistent with midline brain defects in *Zfp423*-mutant mice, but pathogenic potential of most patient variants remains uncertain. We engineered ~50 patient-derived and small deletion variants into the highly-conserved mouse ortholog and examined neuroanatomical measures for 791 littermate pairs. Three substitutions previously asserted pathogenic appeared benign, while a fourth was effectively null. Heterozygous premature termination codon (PTC) variants showed mild haploabnormality, consistent with loss-of-function intolerance inferred from human population data. In-frame deletions of select zinc fingers showed mild to moderate abnormalities, as did low-expression variants. These results affirm the need for functional validation of rare variants in biological context and demonstrate cost-effective modeling of neuroanatomical abnormalities in mice.

(149 words)

INTRODUCTION

Variant effect prediction remains a challenge in medical genomics [1, 2]. Progress from large reference databases such as ExAC [3], gnomAD [4], and UK Biobank [5] allow powerful statistical evidence against pathogenicity, based on allele frequency [6] for rare variants that had appeared unique to patients in smaller samples. Recessive phenotypes, low or context-dependent penetrance, effects on pre-term viability, and demography, however, may create exceptions often enough to be relevant to patients with rare disorders. Population frequencies also provide limited guidance for singleton and de novo variants. Predictive algorithms based on evolutionary constraint and structural motif requirements continue to improve, but evolutionary constraint is neither necessary nor sufficient for disease association as loss of human-specific traits may present as disease while evolution selects on subtler variation than disease presentation. Attempts to model prediction accuracy can suffer where ground truth is not available and clinical variant databases in current use include assertions often based on limited evidence. The problem can be particularly acute for disorders where a substantial number of genes are mutable to overlapping phenotypes, including ciliopathies such as Joubert syndrome and related disorders (JSRD). For example, *ZNF423* mutations have been reported as pathogenic in JSRD patients [7] and other neurodevelopmental disorders [8], but most patient variants have uncertain significance and even those asserted pathogenic in public databases rely on very limited data. This is true for many rare disorders.

Mice can be a useful model for *ZNF423* function. The ZNF423 orthology group is highly constrained across vertebrates [9]; after accounting for annotation differences in alternative 5' exons, mouse Zfp423 and human ZNF423 share >98% amino acid identity (99% in zinc finger domains). Most human variants will therefore be in sequence contexts that are similarly constrained in mice. Null mutations in mouse *Zfp423* have

defects in midline brain development similar to human JSRD, including anterior rotation of the cerebellar hemispheres and hypoplasia or agenesis of the cerebellar vermis with more modest effects on forebrain structures [10-12]. Roof plate defects in Zfp423 mutant mice also impact development of hindbrain choroid plexus [10, 13]. Other work in mice showed notable effects of Zfp423 on olfactory neurogenesis [14], neocortex development [15], adipogenesis [16-18], and wound healing [19]. Gene-trap alleles that reduced expression of an otherwise normal Zfp423 protein showed hypomorphic phenotypes, indicating a graded response to genetic function [10]. In cerebellum, loss of Zfp423 prevents or limits response to SHH by granule precursor cells ex vivo, consistent with a functional abnormality in the cilium [20]. ZNF423 homologs interact with a diverse set of lineage-determining or signal-dependent transcription factors in alternate and potentially competing complexes [7, 21-26] and mutational effect will likely depend on which if any of these contacts is altered [27]. Cell-based models may afford screening of all potential variants in a protein [28-30], but the limited context of cells ex vivo could miss key features of ZNF423 function as its expression is dynamic across communicating cell types during development, including both germinal zones in the cerebellum. Whether (or to what extent) all ZNF423 interactions and functions are required in any one cell type is not clear. With the ability to multiplex germline editing at high efficiency and specificity, mouse brain development might therefore be the simplest robust assay for impact of ZNF423 variants on human brain development, allowing quantitative assessment of variant effects on brain development at a useful scale while being agnostic to developmental stage or cell types in which specific functions are compromised.

Here we developed simple, quantitative measures with good statistical power to assess structural brain abnormalities in ~50 mouse strains with *Zfp423* mutations created by

genome editing. This extensive set of comparisons allowed us to show differences in sensitivity among phenotypes, test pathogenic potential of patient-derived and other variants, and identify previously unreported haploabnormality in null allele heterozygotes. Among patient-derived substitution alleles, H1277Y, at a zinc-coordinating histidine in the last of 30 C2H2 zinc fingers, was effectively null. By contrast, three other patient variants asserted pathogenic or likely pathogenic based on single patients and algorithmic predictions (R89H, P913L, and E1124K) appeared benign. Premature truncation variants, including humanized alleles that encode protein tails to model patient frame-shift variants, were predominantly null with no evidence for dominant negative activity. An early frame-shift variant in exon 3 was an exception, evading nonsense-mediated decay to produce a partial protein at reduced abundance and a partial loss-of-function phenotype. Null allele heterozygotes showed slightly lower weight, smaller cerebellar vermis, and shorter stride length than control littermates, providing functional evidence for loss-of-function intolerance observed in human population data. In-frame deletions had a range of domain-specific effects. Deletions that remove zinc finger 1, zinc fingers that bind BMP-dependent SMAD proteins, or a non-motif region containing two CXXC sites, and deletions that reduced overall protein expression showed measureable effects, while deletion of zinc finger 12 did not.

RESULTS

Induction of patient-derived variants and collateral mutations.

We developed an editing pipeline using standard CRISPR/Cas9 tools. We prioritized 13 *ZNF423* amino acid substitution variants and two frame-shifting alleles from patients (Supplementary Table S1). Four substitution alleles and one frameshift were published [7, 8] and reported in ClinVar [31] as pathogenic or likely pathogenic; a second

frameshift allele was reported in MyGene2 [32]. Other substitution alleles were patient variants of uncertain significance communicated by Drs. Joseph Gleeson and Friedhelm Hildebrandt. Targets were selected to include a range of predicted effects in commonly used variant effect algorithms and to include a range of allele frequencies in databases depleted for close relatives and patients with Mendelian disorders [3, 4]. We edited FVB/NJ embryos because this strain background improved both postnatal viability of the *Zfp423*^{nur12} null mutation and heterozygote breeding performance relative to C57BL/6J, without compromising penetrance of severe ataxia among surviving homozygotes. For most variants, we co-injected two or more alternative repair template oligonucleotides (Supplementary Table S2) to create silent substitution controls or edit adjacent sites in a single injection series.

We recovered 12 out of 15 designed mutations–10 of 13 intended substitution alleles and each of two patient frame-shift variants with humanized codons–as well as designed silent control edits and a large number of collateral mutations (Figure 1A, Supplementary Tables S3 and S4). Substitution alleles included sites that are highly conserved among vertebrates, including seven that were invariant among 165 curated orthologs [9]. Most lie in or adjacent to zinc finger (ZF) domains (Figure 1B) or a non-motif region containing a quartet of conserved cysteine residues with potential to form a C4-class zinc finger and for which common annotation tools showed wide disagreement on functional predictions (Table 1). Collateral variants included predicted loss of function (pLOF) alleles at several positions in the coding sequence and in-frame deletions that allowed additional probes of protein stability and domain-specific function in vivo. We included several variants that remove specific zinc fingers or conserved regions between zinc fingers in subsequent analyses. A simple measure of cerebellar vermis width from surface views of the brain was sensitive to heterozygosity for presumed null alleles and specific to variants that

removed critical residues or decreased protein abundance, while comparison among non-mutant littermate pairs suggested high sensitivity and power to detect modest differences in from 10-15 sample pairs (Figure 1C and results below).

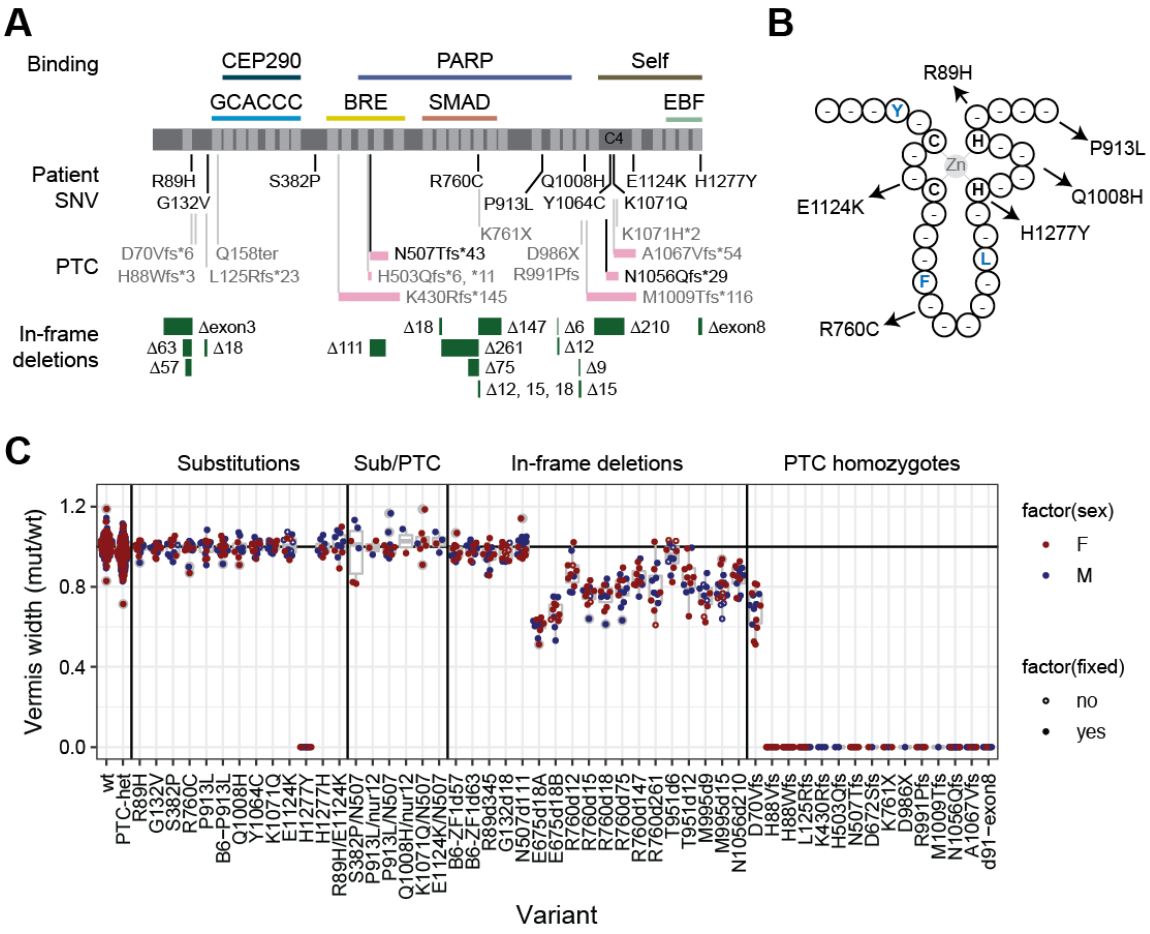


Figure 1. *Zfp423* mutations induced by genome editing affect vermis size.

(A) Locations of specific variants are indicated relative to ZNF423 RefSeq protein NP_055884 (dark grey), C2H2 zinc fingers (light grey), and reported binding activities. Frame-shift PTC variants show relative length of altered reading frame (pink bars). N507Tfs*43 and N1056Qfs*28 were humanized to encode the same frame-shifted peptide as reported patient variants. For in-frame deletions (green bars), the number of deleted base pairs is shown. (B) Schematic of a C2H2 zinc finger shows relative

positions of patient substitution alleles relative to consensus hydrophobic (blue) residues and required zinc-coordinating residues (black). (C) Ratio of mutant to control vermis width measured from surface views of same-sex littermate pairs. Each dot represents one littermate pair. Female pairs, red, male pairs, blue. A few pairs were measured as freshly dissected, unfixed material (open circles), which did not noticeably affect the relative measure. PTC heterozygous, H1277Y, each in-frame deletion except N507d111, and all PTC homozygous variants were significantly different from both the expected null model and empirical wild-type:wild-type comparisons.

Table 1. Effect predictions for *ZNF423* substitution variants modeled in this study.

Variant (domain)	hg38	gnomAD (MAF)	ClinVar	PolyPhen 2 (HVAR)	SIFT (Score)	PROVEAN	Mutation Taster (rankscore)	Mutation Assessor (rankscore)	VEST3 Rank score	CADD PHRED
R89H (ZF1)	16:49730782 C/T	18 (6.4 e-5)	Likely pathogenic	Benign (0.001)	Tolerated (0.282)	Neutral (0.19)	Polymorphism (0.261)	Neutral (0.016)	0.126	19.16
G132V (ZF1-2 linker)	16:49638757 C/A	0	-	Damaging (1)	Damaging (0.002)	Deleterious (-2.67)	Disease causing (0.81)	Low (0.225)	0.883	25.6
S382P (ZF8-9 linker)	16:49638008 A/G	215 (7.6 e-4)	Uncertain significance	Possibly damaging (0.72)	Tolerated (0.254)	Neutral (-1.65)	Disease causing (0.345)	Low (0.498)	0.682	24.4
R760C (ZF18)	16:49636874 G/A	11 (3.8 e-5)	-	Probably damaging (0.82)	Damaging (0.001)	Deleterious (-4.83)	Disease causing (0.588)	Low (0.304)	0.904	28.9
P913L (ZF21)	16:49636414 G/A	47 (1.7e-4)	Pathogenic	Probably damaging (0.92)	Damaging (0.010)	Deleterious (-3.87)	Disease causing (0.81)	Medium (0.833)	0.842	28.5
Q1008H (ZF24)	16:49636128 C/G	7 (2.8e-5)	-	Probably damaging (0.88)	Damaging (0.000)	Deleterious (-3.61)	Disease causing (0.457)	Medium (0.53)	0.848	25.3
Y1064C (C4)	16:49635961 T/C	1 (4.2e-6)	-	Probably damaging (0.75)	Damaging (0.031)	Deleterious (-5.02)	Disease causing (0.548)	Low (0.246)	0.815	28.1
K1071Q (C4)	16:49635941 T/G	0	-	Probably damaging (0.65)	Damaging (0.036)	Neutral (-1.52)	Disease causing (0.465)	Low (0.246)	0.577	28.5
E1124K (ZF26)	16:49635782 C/T	8 (3.2e-5)	Likely pathogenic	Possibly damaging (0.54)	Tolerated (0.092)	Neutral (-1.64)	Disease causing (0.548)	Medium (0.562)	0.883	23.6
H1277Y (ZF30)	16:49491301 G/A	0	Pathogenic	Benign (0.04)	Damaging (0.002)	Deleterious (-3.93)	Disease causing (0.53)	Medium (0.924)	0.951	24.5

Supplementary Table S1. Embryo injections and recovered mutations.

Supplementary Table S2. Oligonucleotide donor sequences.

Supplementary Table S3. Screening primers.

Supplementary Table S4. Genotyping assays and strains deposited to MMRRC.

Simple measures are highly sensitive to *Zfp423* variants.

To test variants for pathogenic potential at moderate to large scale, we looked for phenotypes that were easily obtained and robust across trials. We observed home cage behavior and videographed mice walking across an open stage to assess gross locomotor activity for each variant subjectively. We quantified viability, weight, and anatomical parameters of brains in both surface view and block face photographs as quantitative phenotypes. A subset of null-allele heterozygotes was tested in more detail for locomotor behaviors. To avoid potential confounding of age, sex, maternal care, or other factors that might vary across a large colony, all tests were conducted on co-housed, same-sex littermates and scored as mutant/control ratio (anatomy) or differences (behavioral latencies). Variants were studied concurrently by investigators blinded to genotypes.

Each measure showed large effects of *Zfp423* premature termination codon (PTC) alleles, with gross ataxia and cerebellar vermis hypoplasia showing complete penetrance. Predicted null variants had no detectable Zfp423 protein on Western blots, with a detection limit ≤5% of control littermate levels, except D70Vfs*6, which showed a reduced level of a lower molecular weight protein unique to that allele (Figure 2A). For protein-null alleles (PTC variants except D70Vfs*6), homozygotes were recovered at

reduced frequency and several null animals identified at P10-P15 died prior to assessment, showing a relatively broad window for lethal events (Figure 2B). Among survivors, locomotor disability was severe and never overlapped controls (Supporting videos). None of 72 *Zfp423*-null brains had measureable vermis in surface photographs (Figure 1D). Nearly half of those with mid-sagittal block face views (30/66) showed a small amount of cerebellar tissue, largely due to compression of lateral hemispheres toward the midline (Figure 2C-D). Midline fiber tract (corpus callosum and anterior commissure) and cortical thickness measures distinguished mutant from control groups, but with smaller relative magnitude and incomplete penetrance (Figure 2E-J). Among forebrain phenotypes, corpus callosum had the largest effect and highest penetrance, while anterior commissure effects were only evident across a larger population of animals. PTC homozygotes were smaller than littermates, with lower weight at sacrifice (Figure 2K-L). These results generalized findings from earlier studies and put quantitative parameters on magnitude and penetrance for hindbrain, forebrain, and weight measures.

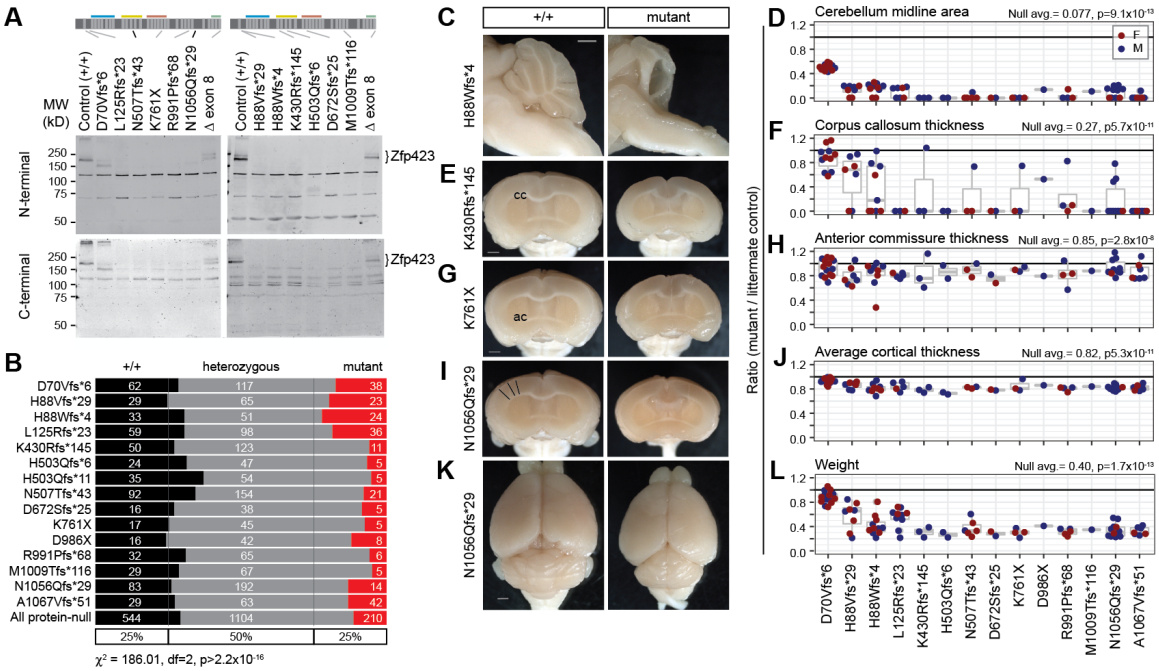


Figure 2. *Zfp423* frameshift and nonsense mutations are effectively null, except D70Vfs*6. (A) Western blots detected full length Zfp423 in perinatal cerebellum of control samples. D70Vfs*6 and a deletion of terminal exon 8 showed altered-size proteins at reduced levels. No consistent evidence for residual protein was seen for any other PTC variants, detection threshold ≤5% wild-type level. N-terminal, Bethyl A304. C-terminal, Millipore ABN410. Cross-reacting background bands were independent of PTC position. (B) Reduced frequency of homozygotes for each PTC at biopsy (P10-P20) from breeding records. Summary chi-square is for protein-null alleles (excluding D70Vfs*6). (C) Mid-sagittal images showed variable amount of midline cerebellar tissue in mutants, with residual tissue attributable to hemispheres. (D) Ratio of midline cerebellum area (mutant/control) from block face images. Coronal block face images showed abnormal forebrains, including (E, F) disrupted or reduced corpus callosum (cc), (G, H) reduced anterior commissure (ac), and (I, J) reduced cortical thickness, measured as the average radial distance at 15°, 30° and 45° from midline (black lines). (K) Representative surface view as measured in Figure 1. (L) PTC mutants had reduced body weight at sacrifice. (D,

F, H, J, L) Averages and Wilcoxon signed-rank test p-values for ratio = 1 for combined data from all PTC alleles excluding D70Vfs*6 are shown. Female pairs red, male blue. Scale bars, 1 mm.

Loss of function heterozygotes showed reduced vermis, weight, and stride without evidence for dominant negative activity.

Having a large set of protein-negative PTC alleles across the full of the coding sequence allowed us to compare presumptive null alleles and distinguish potential haploabnormalities from dominant negative effects. PTC variants that escape nonsense-mediated decay often enough to produce a variant protein can have dominant negative properties by decoupling functional domains [33]. Early PTCs can result in protein translation from an alternative initiation codon [34, 35] or exon skipping, while late PTCs could produce truncated proteins if they escape nonsense-mediated decay (NMD). *Zfp423* has a highly unusual gene structure, including 4-kb internal coding exon (Figure 1) and variant effects in situ might differ from assays performed in cell culture on compact gene structures according to the proposed “long exon rule” [35, 36]. In Chaki et al. [7], one of us (B.A.H.) speculated that JBTS19 patients carrying one PTC variant heterozygous to an apparently normal allele might have dominant negative activity, supported by transfection assays with a corresponding cDNA in a human cell line. To test this in vivo and to learn more about how PTC variants behave in the context of a very large exon structure, we examined 16 mouse lines carrying different PTC variants at distinct positions relative to exon boundaries and encoded protein domains.

Heterozygotes for PTC variants that do not produce detectable protein should also be a good test of sensitivity, since multiple labs previously reported only recessive phenotypes from several alleles [10-12, 27]. Physical measures were slightly decreased

on average in heterozygotes compared to sex-matched littermates across PTC variants for which homozygotes were protein-negative. Vermis width (Figure 3A) and weight at sacrifice (Figure 3B) each had a ~3-4% decrease with strong statistical support ($p<10^{-6}$), while anterior commissure measure had a 1.4% decrease with nominal support (p=0.046 after Benjamini-Hochberg correction for false discovery). In contrast, average thickness of cortex (p=0.11) and corpus callosum (p=0.38) showed little evidence for heterozygote effects, perhaps due to the modest effect size on cortex even in null animals and the comparatively high variance on corpus callosum measures.

Infrared fluorescence Western Blots from neonatal cerebellum (Figure 3C) supported average expression ratios between 0.56-0.73 with two different antibodies (N=29 comparisons for A304, 26 for ABN410), albeit with substantial variation across experiments (Figure 3D). Expression ratio greater than 0.5 is consistent with a proposed negative autoregulatory activity of Zfp423 [37]; it may also reflect changes in tissue composition of cell types and states, although the strongest source perinatally should be granule cell precursors, which decrease in mutant animals [10, 20]. With these caveats, identification of mild phenotypes in null heterozygotes places limits on the degree of protein functional deficit required to produce phenotypes relevant to disease modeling for structural abnormalities.

Quantified measures of locomotor function, performed by a core facility blind to genotype and experimental goals, supported a modest difference only in stride length for PTC heterozygotes. Joint analysis of two heterozygous variants, H503Tfs*11 (N=18 littermate pairs) and N1056Qfs*29 (N=24 littermate pairs), did not identify significant differences by genotype on a hanging wire task for grip strength and coordination (Figure 3E), elevated beam escape (Figure 3F), nor accelerating rotating rod (Figure 3G) tasks for locomotor

coordination. Footprint analysis for gait parameters showed ~5% reduced stride length that was independently significant for each variant, although potentially confounded by animal size as assessed by weight, with no detected difference in front or back stride width (Figure 3H). These data supported better sensitivity and cost effectiveness of anatomical measures than simple behavioral measures to perturbations in *Zfp423* function in mice.

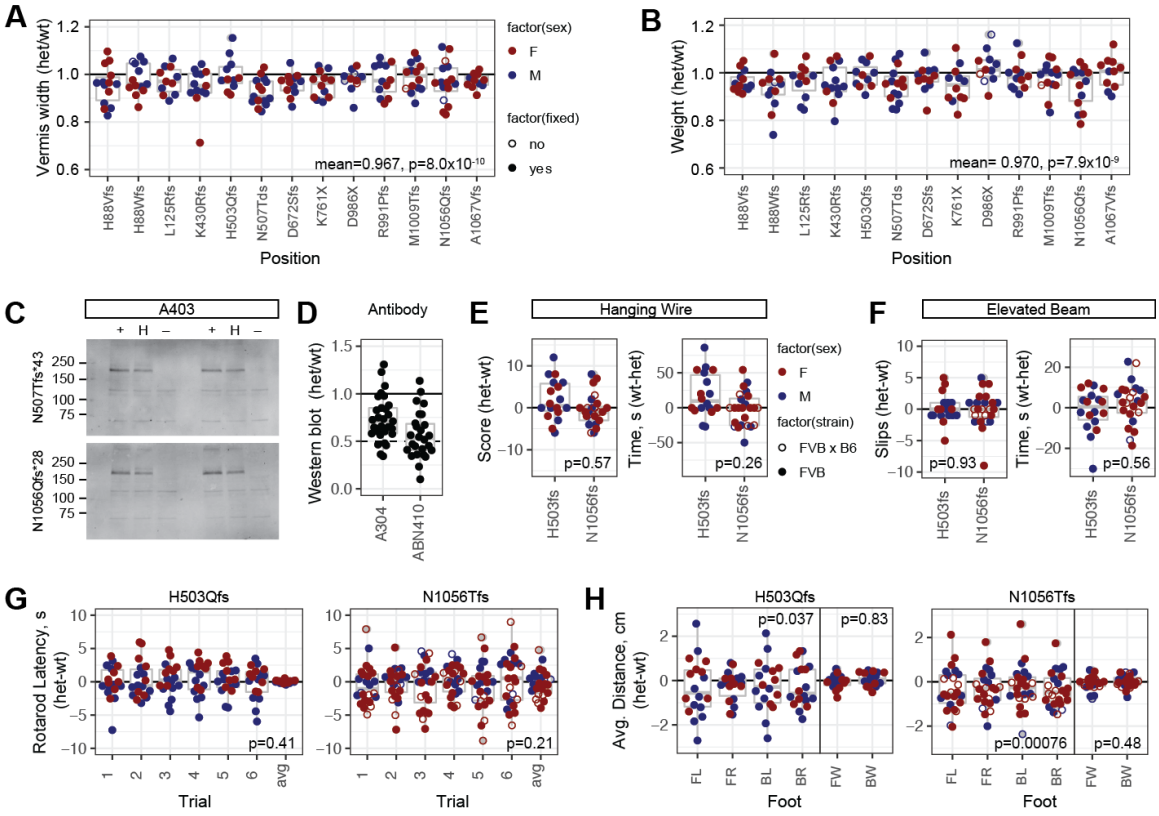


Figure 3. PTC heterozygotes have mild haploabnormalities. (A) Vermis width in heterozygotes for individual protein-negative PTC variants relative to control littermates. Y-axis was shifted relative to Figures 1-2 to emphasize distribution within the range of observed values. Bottom right corner, mean and p-value for all PTC heterozygotes, combined N=165, one-sample t-test for true ratio=1. (B) Weight at sacrifice, combined N=163. (C) Western blots showed reduced steady-state level of Zfp423 protein in neonatal cerebellum of PTC heterozygotes relative to reference littermate. (D)

Normalized values among PTC heterozygotes with independent antibodies showed typical values between 0.5 and 1 relative to control littermates in neonatal cerebellum. (E-H) Behavioral tests for locomotor coordination on littermate pairs for two PTC heterozygotes, H503Qfs*11 (N=18) and N1056Qfs*29 (N=24), either coisogenic on FVB (filled circles) or as B6xFVB F1 hybrids (open circles). (E) Hanging wire task performance scores and fall latencies. Wilcoxon signed rank test, N=43. (F) Elevated beam escape task. (G) Rotating rod fall latencies across six successive trials per pair and average ratio across all six trials. P-value is one-sample t-test for average difference being 0. (H) Footprint analysis for stride length in each paw and width between left and right paws. FL front left, FR front right, BL back left, BR back right, FW front paw stride width, BW back paw stride width. P-values for one-sample t-tests for average differences equal to 0.

Differential effects near either end of the open reading frame.

Despite being created on the more sensitive B6 background, D70Vfs*6 showed milder phenotypes than all other PTC variants, while a 91-bp deletion that removed just the nine terminal residues encoded by exon 8 on the FVB background appeared similar to null variants. Western blots showed that D70Vfs*6 produced a detectable pool of lower-molecular weight Zfp423 protein (Figure 4A), presumably by translational initiation after the introduced stop codon at position 72 in its first open reading frame. The next available in-frame AUG is at position 118, between ZF1 and ZF2, and would include each of the previously annotated binding domains. The exon 8 deletion produced a nearly full length protein, but one that must lack both histidine residues from ZF30, which is required for binding EBF proteins [26, 38] and at substantially reduced steady-state level (Figure 4B). D70Vfs*6 Homozygotes have mild to moderate ataxia and hypomorphic anatomical features, most notably vermis hypoplasia (Figure 4C, E). In

contrast loss of exon 8 had severe anatomical abnormalities, including nearly complete loss of vermis and corpus callosum in all six animals assessed (Figure 4D, E). Each of these mutations had significantly reduced frequency of homozygote offspring from heterozygote crosses (Figure 4F). These two mutations showed somewhat unexpected features of *Zfp423* functional organization and reinforced the need for empirical testing of predicted variant effects.

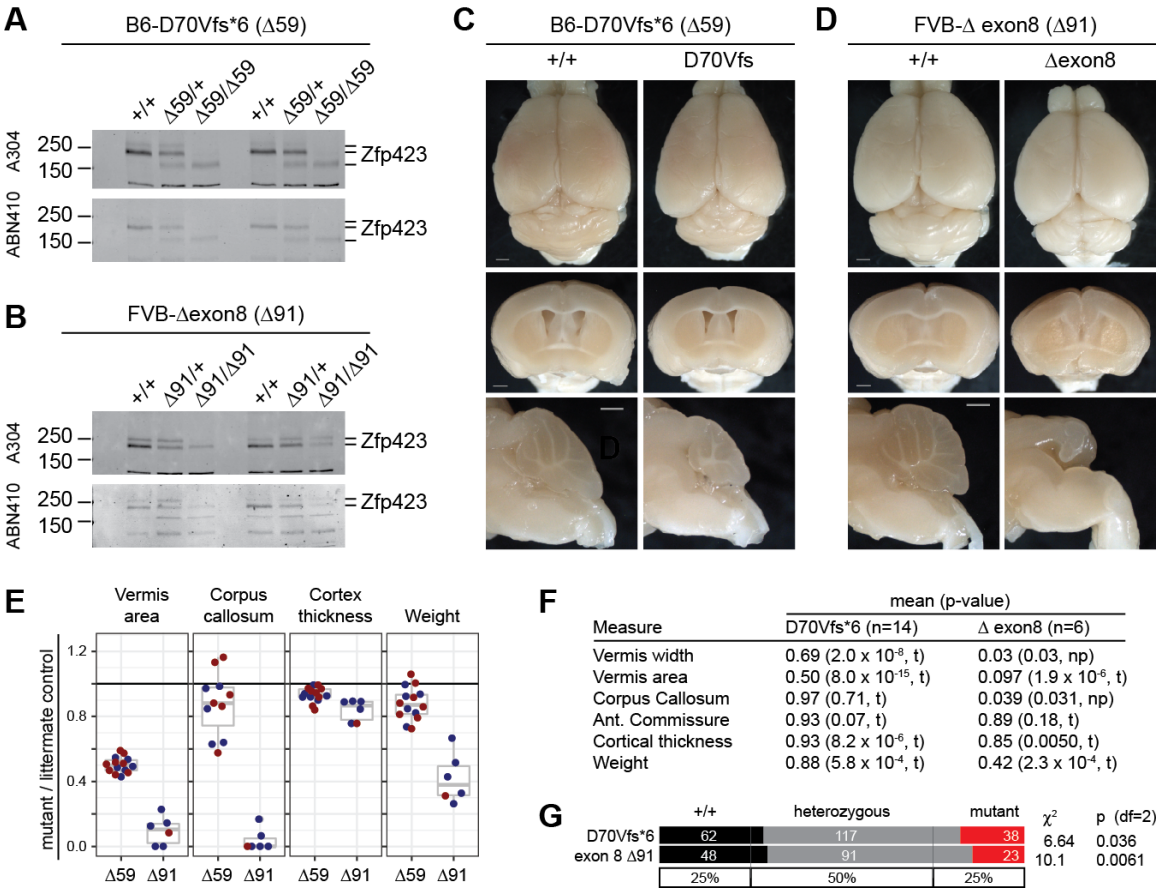


Figure 4. D70Vfs*6 is hypomorphic while deleting exon 8 is approximately null. (A) Westerns blots from neonatal cerebellum from independent trios with two different antibodies against Zfp423 showed a reduced amount of lower molecular weight protein derived from the D70Vfs*6 mutation. (B) Similar blots from two trios for deletion of exon 8, encoding the final nine amino acids of ZF30, showed reduced level of near-full length protein. (C) D70Vfs*6 anatomical phenotypes included reduced cerebellum size. Scale

bars, 1 mm. (D) Exon 8 deletion phenotypes approximate those of null alleles despite persistent protein. Scale bars, 1 mm. (E) Quantification of anatomical measures from D70Vfs*6 (Δ59) and exon 8 deletion (Δ91) homozygote and control littermate pairs. (F) P-values from one-sample t-test (t) or non-parametric Wilcoxon Signed Rank test (np) for anatomical measures. (G) Both D70Vfs and exon 8 deletion showed reduced frequencies of homozygotes in crosses.

H1277Y is pathogenic in mice, three other asserted mutations and six VUS are not. We assessed 10 patient-derived substitution alleles: four asserted pathogenic in ClinVar and six rare variants of uncertain significance spanning a range of allele frequencies in public databases from zero to 7.6 x 10^{-4} (Table 1 and Figure 1). Each position is highly conserved across vertebrates, except R89H. Eight lie in or adjacent to C2H2 zinc fingers (Figure 1B) or a putative C4 zinc finger [9]. Among these ten, only H1277Y showed a severe disease-related abnormality while none of the others was distinguishable from control littermates in our assays.

H1277Y was identified in a patient with cerebellar vermis hypoplasia, nephronophthisis, and perinatal breathing abnormalities [7]. By replacing a zinc-coordinating histidine with tyrosine, H1277Y should disrupt the structure of ZF30, which is required for interaction with EBF family proteins [26, 38]. Mice homozygous for the H1277Y variant showed many features of null alleles, including gross ataxia (Supplementary videos), vermis agenesis, reduced cortical thickness, and incomplete corpus callosum (Figure 5A) while a silent control edit was indistinguishable from control littermates (Figure 5B). Zfp423 H1277Y protein has substantially reduced abundance in neonatal cerebellum, similar to the exon 8 deletion and consistent with structural destabilization of the terminal zinc finger (Figure 5C). Quantitative measures from multiple same-sex littermate pairs show

full penetrance of severe defects in surviving mutants of both sexes for cerebellar vermis hypoplasia, loss of corpus callosum at the midline, cortical thickness, and body weight (Figure 5D). Retrospective analysis of breeding records showed reduced frequency of homozygotes for H1277Y, but not the silent substitution control allele (Figure 5E). These results confirm the pathogenic nature of H1277Y for structural brain abnormalities.

The other three asserted pathogenic variants were not sufficient to induce JSRD-like or other obvious phenotypes, nor were any of the tested VUS alleles. P913L was identified by homozygosity in a consanguineous patient with cerebellar vermis hypoplasia, nephronophthisis, and situs inversus [7]. R89H and E1124K were found together in a patient with macrocephaly, extended subarachnoid spaces, and thin corpus callosum [8]. Each of these variants was absent in contemporaneous control subjects. Each was later found in new and larger public databases, but only at low allele frequencies (1.8×10^{-4} to 3.2×10^{-5}, Table 1) and only as heterozygotes. Using a minimum 10 replicate sample pairs in mice, we did not identify any defect in vermis nor in midline forebrain phenotypes for P913L (Supporting Figure S5-1), nor for R89H or E1124K homozygous mice, nor for R89H/E1124K compound heterozygous mice (Supporting Figure S5-2). Substitution variants heterozygous to a null allele did not show any effect in smaller sample sizes. For P913L, we created the same mutation on the more sensitive B6 background, again with no evidence for an effect on brain structures typically affected in *Zfp423* mutants (Supporting Figure S5-1). Pooling all substitution variants except H1277Y and its silent control H1277H to test for a generalized substitution effect at high power (N=179 littermate pairs) did not produce statistical support for abnormality in any measure before correction for multiple tests ($p>0.15$ all tests). These data confirmed the pathogenic nature of H1277Y, but supported a more benign interpretation of all other non-synonymous substitutions tested.

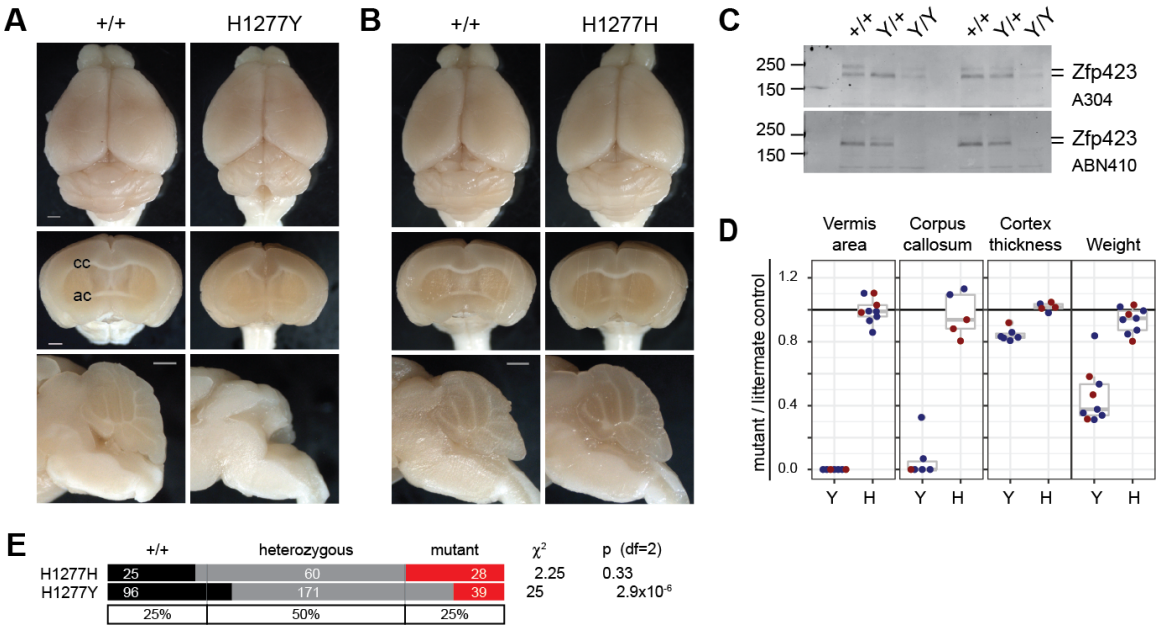
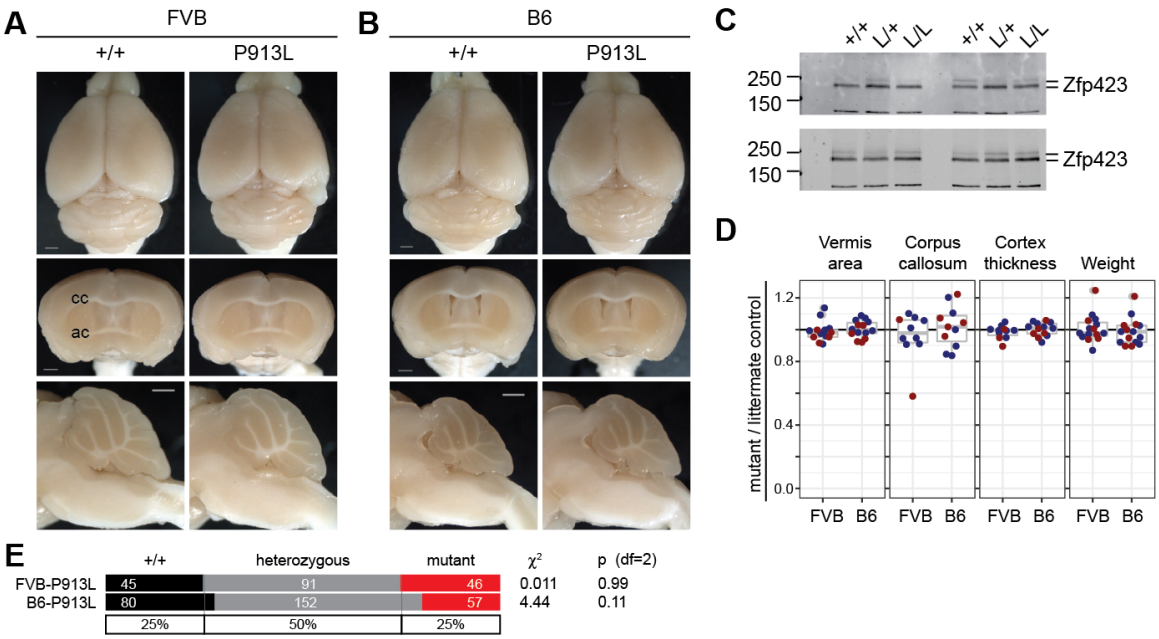
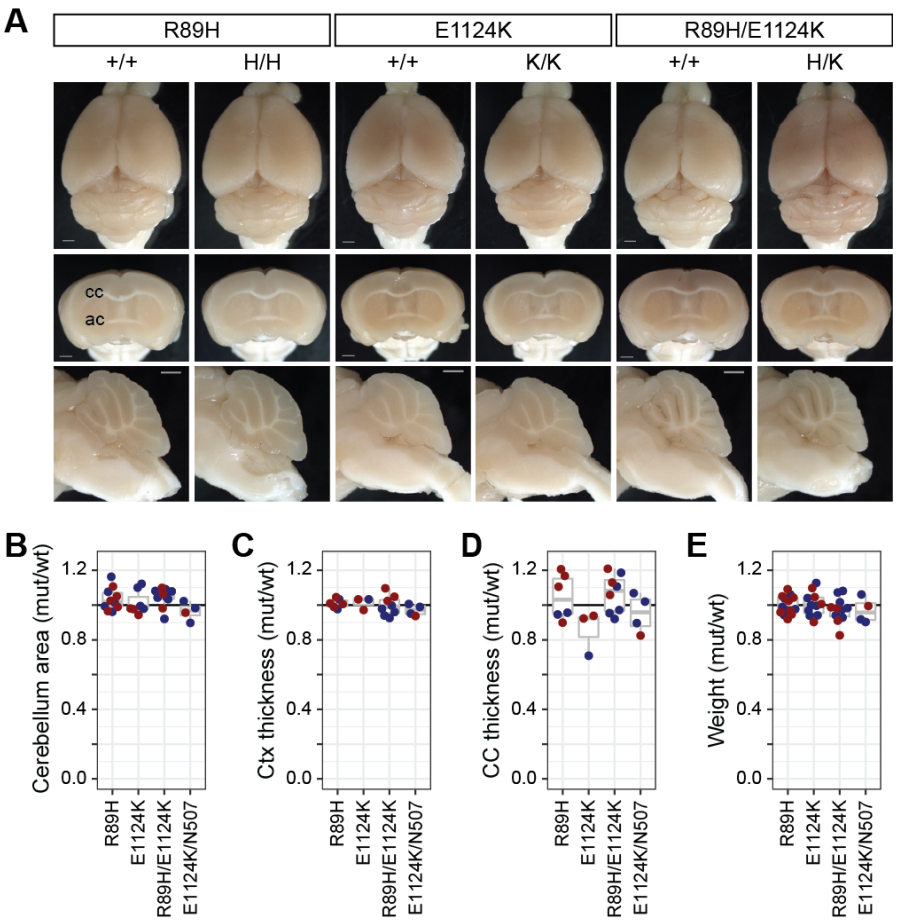


Figure 5. H1277Y is pathogenic and has reduced protein abundance. (A) Surface and block face views of same-sex littermates show H1277Y midline defects similar to null alleles, including complete loss of vermis, loss of corpus callosum crossing, and reduced cortical thickness. Scale bars, 1 mm. (B) Silent substitution H1277H is indistinguishable from control littermates. (C) Western blots with antibodies to amino-terminal (top) or carboxy-terminal (bottom) domains show dramatic loss of Zfp423 protein abundance. Results from two distinct trios shown. (D) Ratios relative to control same-sex littermates quantify loss of vermis, midline corpus callosum, cortex thickness, and body weight for H1277Y, but not H1277H. (E) Breeding records show reduced frequency of H1277Y, but not H1277H, homozygotes from heterozygote crosses.



Supporting Figure 5-S1. P913L is not pathogenic in mice. (A) Surface views of brains from control and mutant same-sex littermate pairs shows grossly normal brains for P913L substitution allele edited independently on FVB/NJ and C57BL/6 (B6) strain backgrounds. (B) Forebrain images show apparently normal structure for P913L mutant on both backgrounds, while highlighting different extent of lateral ventricles between strains at this place of section. (C) Western blots with antibodies to amino-terminal (top) or carboxy-terminal (bottom) domains show normal Zfp423 protein abundance. Results from two distinct trios shown. Ratios between same-sex littermates for (D) vermis area at midline, corpus callosum thickness at midline, average cortical thickness at 15°, 30° and 45° from midline, and body weight fail to identify defects in P913L homozygotes. Scale bars, 1 mm.



Supporting Figure 5-S2. R89H and E1124K are not pathogenic in mice. (A) Dorsal surface, coronal forebrain, and sagittal hindbrain views from control and mutant same-sex littermate pairs showed grossly normal brains for R89H homozygous, E1124K homozygous, or R89H/E1124K compound (trans) heterozygous animals on FVB/NJ background. Ratios between same-sex littermates for (B) vermis midline area, (C) average cortical thickness, (D) midline corpus callosum thickness, and (E) weight at sacrifice fail to identify significant deviations for any of these genotypes. Scale bars, 1 mm.

Deletions that remove SMAD-binding fingers or a potential C4-ZF domain produce intermediate alleles.

We examined several in-frame deletions (Figure 1A) for Zfp423 protein abundance and brain phenotypes. Recovered examples included distinct Zfp423 functional domains (Figure 6A), several of which had notable reductions in cerebellar vermis (Figure 6B). Several variants with strong phenotypes also significantly affected protein level (Figure 6C) and we could therefore not distinguish between functional requirement for a domain and destabilization of the protein due to awkward breakpoints, although comparison to null heterozygotes suggested that strong phenotypes associated with >75% of control expression levels probably indicates a sequence-specific function.

Cerebellar vermis was the most sensitive anatomic measure to in-frame deletions as a class (Figures 1C, 6D). Impact of intermediate alleles on corpus callosum measures had high variance that limited power after correction for multiple tests (Figure 6E) and lacked effect magnitude for simple measures of cortical thickness (Figure 6F). As a group, in-frame deletions slightly decreased body weight (Figure 6G).

Mutations that substantially reduced protein level also had the strongest effects on vermis measures. Overlapping deletions in ZF15 (E675Δ18A, E675Δ18B) and ZF24 (M995Δ9, M995Δ15), and a single small deletion in ZF18 (R760Δ18), each of which is predicted to destabilize the C2H2 structure by removing critical residues, all reduced protein expression level and reduced cerebellar vermis midline area by approximately half.

Four mutations removed significant protein-coding sequences without reducing measured protein level: G132Δ18 p.del(Leu125-Glu130), which removes 6 amino acids in the sequence between ZF1 and ZF2; N507Δ111 p.del(Arg500-Ile536), which removes ZF12 in the BRE-binding region; R760Δ261 p.del(E675-K761), which fuses ZF15 to

ZF18 while deleting ZF16-ZF17 in the SMAD-binding region (R760Δ147 and Δ75 also delete fingers within the SMAD region); and N1056Δ210 p.del(Thr1032-Gly1102)>Arg, which removes part of ZF25 and all of the C4 ZF-like sequence. Surprisingly, deletion of ZF12 (N507Δ111) in the annotated BRE-binding region had no measurable effect. The small deletion between ZF1 and ZF2 (G132Δ18) had a nominal effect (mean = 0.97, p = 0.035, one-sample t-test, N = 12). However, deletions in the SMAD-binding region that did not disrupt C2H2 structural elements and retained near-normal protein levels (R760Δ261, R760Δ147, and R760Δ75) each showed a strong intermediate phenotype, consistent with independent SMAD-domain deletions reported by Casoni et al. [27]. Deletion of ZF25 and putative C4 ZF (N1056Δ210) showed a similar degree of vermis hypoplasia, providing the first evidence for organic function of these domains. These results showed that *Zfp423* brain structural phenotypes were sensitive to most in-frame deletions, often including reduced protein levels, and that different ZF domains or clusters had different degrees of sensitivity. That in-frame deletions were hypomorphic rather than effectively null reinforced the idea that Zfp423 coordinates activities among its interaction partners rather than being an essential component of one pathway.

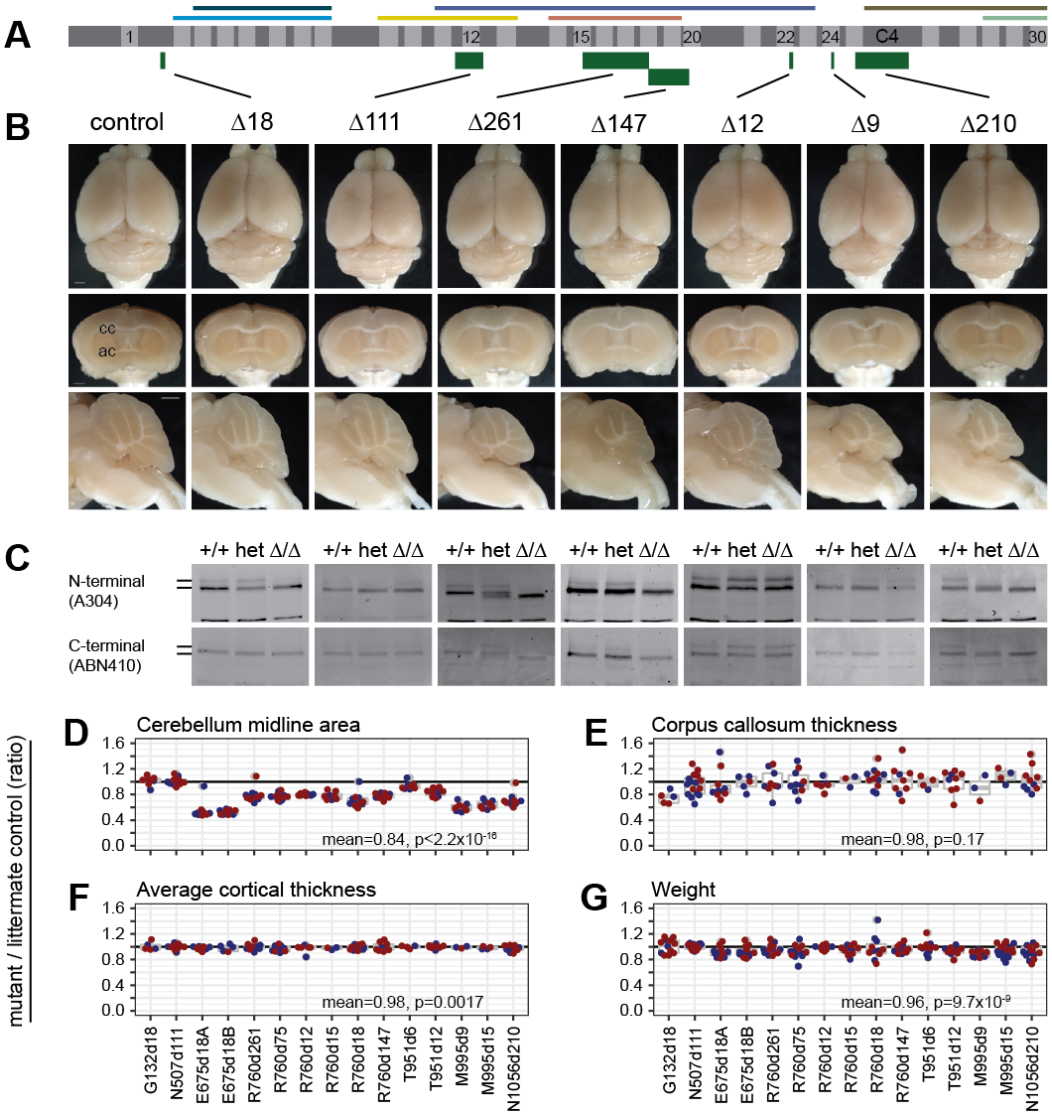


Figure 6. In-frame deletion that remove critical regions or reduce protein abundance are hypomorphic. (A) Schematic as in Figure 1 shows the locations relative to 30 C2H2 zinc fingers and known binding regions of in-frame deletions with example data below. (B) Surface views, coronal, and sagittal block face preparations of brains from typical control and in-frame deletions G132Δ18, N507Δ111, R760Δ261, R760Δ147, T951Δ12, M995Δ9, and N1056Δ210. Scale bars, 1 mm. (C) Western blots show Zfp423 proteins in newborn cerebellum for control, heterozygote, and mutant littermates for each variant. (D) Cerebellum midline area, (E) Corpus callosum midline thickness, (F) Cortex thickness as the average of three points at 15°, 30°, and 45° from

midline, and (G) Weight at sacrifice each expressed as the ratio of mutant to wild-type same-sex littermate controls for 15 in-frame deletion mutations.

ZF1 deletion has a reproducibly mild phenotype.

The D70Vfs*6 long open reading frame lacks N-terminal residues including the first zinc finger. To determine how much of this phenotype is attributable to loss of ZF1 rather than other N-terminal residues or reduced Zfp423 protein level, we examined three distinct deletions that remove ZF1. One in-frame deletion recovered in the course of modeling patient substitution variants on the FVB background removed all of exon 3 (R89 d345, Figure 1A, C), including ZF1 and ~40 other residues. To test the requirement for ZF1 specifically we constructed two smaller deletions (Δ57, Δ63) targeting just the ZF domain in the more sensitive B6 background (Figure 7A). Both targeted alleles expressed Zfp423 protein at levels similar to control littermates (Figure 7B, C) and differed only slightly in deletion breakpoints relative to the coding sequence (Figure 7D). All three mutations resulted in slightly smaller cerebellar vermis (Figure 7A, E), but less severe than D70Vfs*6 and comparable to reductions seen in null allele heterozygotes. This showed that while ZF1 contributed to Zfp423 function in hindbrain development, it had a smaller effect size than features required for protein production and stability or SMAD binding.

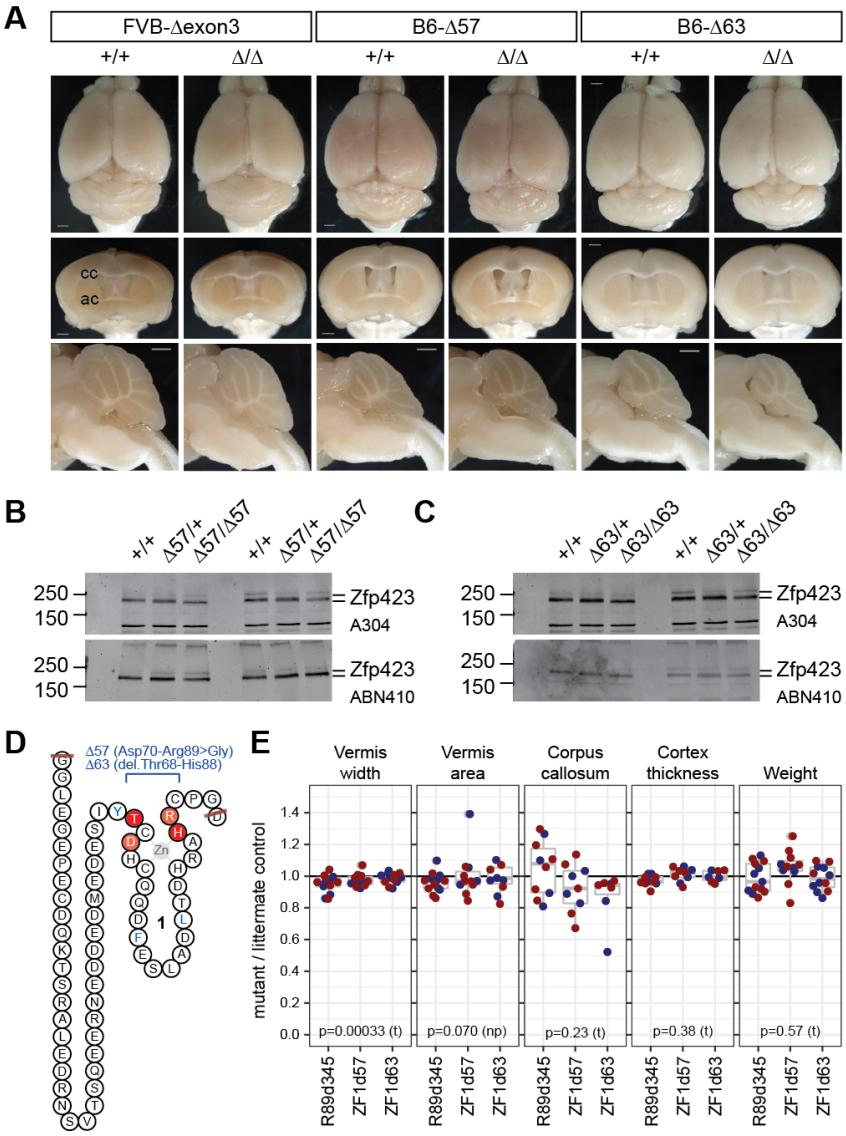


Figure 7. ZF1 deletions had modest, but measureable impact. (A) Surface views and block face views from control and mutant same-sex littermate pairs for in-frame deletions removing 345 bp (Δexon3) or smaller fragments within exon 3 (Δ57, Δ63) on FVB or B6 coisogenic backgrounds. Scale bars, 1 mm. (B, C) Western blots show similar levels of Zfp423 protein in control, heterozygote, and mutant newborn cerebellum for Δ57 and Δ63 mutations. (D) Peptide sequence encoded by exon 3, with zinc finger 1 schematized and deletion boundaries indicated in pink (Δ57) or red (Δ63). Structural hydrophobic residues are blue as in Figure 1B. (E) Ratios between mutant and same-sex littermate

controls for vermis width (redrawn from Figure C), vermis midline area, corpus callosum midline thickness, average cortical thickness, and weight at sacrifice are shown. P-values are shown for one-sample t-test (t) or non-parametric Wilcoxon signed rank test (np).

DISCUSSION

Genomic medicine for rare disorders is often limited by the ability to interpret rare variants. Databases such as ClinVar that house variants from multiple sources, but report pathogenic assertions based on varied, evolving, and sometimes unclear standards of evidence. Algorithmic predictions and cell models have many benefits, but are prone to errors if the input-output relationships of the assessment do not scale with the impact protein function on the relevant organ system. Here we showed that, for *ZNF423*, simple quantitative phenotypes and a small lab team can rapidly assess a large array of variants in whole-animal models in a cost-effective manner. We confirmed H1277Y as a pathogenic variant, while providing evidence against three other variants previously asserted pathogenic and six rare/singleton VUSs. The quantification of heterozygote phenotypes, as well as inclusion of collateral variants that remove domains or reduce protein abundance, demonstrated sensitivity of the simple assays to relatively modest genetic perturbation. Simple measures with low variance, such as vermis width, allowed high statistical power to detect structural changes less than 5% of mean values in the brains of even heterozygous animals. This strengthens the interpretation that substitution alleles with no abnormality are benign with respect to major ciliopathy phenotypes. By examining in-frame deletion variants, we confirmed the importance of the SMAD-binding ZF cluster, showed that deletion of ZF25 plus a conserved non-motif segment with potential to form a C4-class zinc finger is sufficient to cause intermediate

decrease in vermis size, and that ZF1 had detectable, but limited, impact on studied phenotypes despite strong conservation across species. Surprisingly, deletion of ZF12 in the BRE-interacting zinc finger cluster had no detectable effect in any of our outcome measures.

Limitations

Human and mouse brains, while homologous in structure, develop on different physical and temporal scales. Although ZNF423-homologous proteins are 98.5% identical in amino acid sequence and previous studies from several laboratories established strong homology between mouse *Zfp423* and human JSRD phenotypes, the degree of sensitivity to mutation could be non-linear. Amino acid substitutions that alter a binding surface could also have differential effects depending on conservation of specific binding partners. In order to test many variants rapidly at high power, we focused on simple and less time-consuming measures. It is possible that more intensive studies on any specific variant might identify a responsive phenotype, but these are unlikely to be severe in the context of laboratory mice. We also have not examined impacts on other systems, such as olfactory epithelium and adipose tissue, where Zfp423 null mutants have strong phenotypes. While acknowledging these caveats in principle, we nonetheless found high sensitivity of the mouse models to even modest perturbation, including heterozygosity for simple loss-of-function alleles.

Prospects

Well-powered results from experimental models should inform and modify clinical interpretation of rare alleles. Previous studies identified several variants studied here as

pathogenic or likely pathogenic in subjects with JSRD or other neurodevelopmental abnormalities based on being rare variants in a gene with known phenotypic overlap and other properties typically associated with causal variants. However, new evidence should update our expectations and interpretations. For example, P913L was proposed as causal based on its being homozygous in an affected child from a consanguineous pairing, not detected in a control population, and conserved across available vertebrate sequences. Subsequent studies, however, found this allele at modest frequency in the general population, which should reduce confidence in a pathogenic role. In our models, we see no evidence, even on the most sensitive genetic background, for pathogenic consequences of this allele, which should further reduce confidence in a pathogenic interpretation. This variant should now be regarded as likely benign. Similarly, R89H (which is not highly conserved) and E1124K (which is) were reported as likely pathogenic based on being rare or novel in humans and found together in a rare patient. Neither allele when homozygous, nor the two together in trans, nor E1124K in trans to a null allele showed any significant effect on anatomical measures. Interpretation of these variants should also now be modified to likely benign. In contrast, H1277Y, which was strongly predicted to destroy a critical structural residue in the final C2H2 domain, both reduced protein abundance and showed essentially null phenotypes. Interpretation of this variant should now be updated to experimentally supported pathogenic.

More generally, the ability of a single research team to validate the range of genetic perturbations that produce disease relevant phenotypes in a model should be considered as part of the evidence for model-based interrogation of clinical variants. Our results also reinforce the idea that evolutionary constraint is sensitive to much smaller effect sizes than Mendelian disorders. Variant effect predictions that account for this might improve the specificity of useful algorithms.

MATERIALS AND METHODS

Genome editing. All editing experiments used CRISPR/Cas9 ribonucleoproteins (RNPs) based on *S. pyogenes* Cas9. Guide sequences were selected for limited off-target potential using public, web-based tools [39, 40]. Modified (AltR) crRNA guides and tracrRNA were purchased from IDT. Standard and Hi-Fi variant Cas9 proteins were purchased from IDT and New England Biolabs. Single-stranded oligonucleotide donors for homology-dependent repair (Ultramers and Megamers) were purchased from IDT. Injections of FVB/NJ and C57BL/6N one-cell stage embryos were performed in the Rebecca and John Moores UCSD Cancer Center Transgenic Mouse Shared Resource. Guide sequences, predicted scores, RNP assembly conditions, and editing results are given in Supplementary Tables S1 and S2.

Mutation discovery and validation. Pups derived from injected embryos were screened for developmentally early mutations by PCR-based Sanger sequencing of 500-bp to 700-bp PCR products from crude tail tip lysis DNA preparations. Screening primer sequences are given in Supplementary Table S3. Transmission to F1 offspring was confirmed by allele-specific PCR and/or additional DNA sequencing (Supplementary Table S4). For mutations with large effects, predicted off-target sites were sequenced to reduce potential for false-positive effects. All variants were also studied across multiple lines and/or backcross generations to further guard against undetected collateral variants by segregation.

Variant effect prediction. PolyPhen2 [41], SIFT [42], PROVEAN [43], MutationTaster [44], MutationAssessor [45], VEST3 [46], CADD [47], and others were run on VCF files

for studied patient variants using wANNOVAR [48]. A subset of predictions were re-run through their stand-alone web pages for validation.

Stock maintenance. Mice were maintained by backcross to FVB/NJ or C57BL/6J and by intercrosses to obtain desired genotypes. Mice were maintained in a specific pathogen free (SPF) facility on 12 h light, 12 h dark cycle in high-density racks with HEPA-filtered air and ad libitum access to water and food (LabDiet 5P06).

***Western blots*.** Cerebellums were manually dissected from young litters (P0-P4) and individually frozen prior to genotyping. Samples from littermate pairs and trios with desired genotypes were homogenized in RIPA buffer supplemented with protease inhibitors (Millipore Sigma P8340) using a small glass dounce (20 strokes). Protein extracts were quantified with BCA assays. 52 µg samples run through Laemmli SDS-PAGE gels before transfer to nitrocellulose membranes (Bio-Rad 1620112). A subset of blots were incubated with Ponceau-S to visualize protein transfer and subjected to image analysis to quantify bulk protein per lane as a reference for subsequent measures. Zfp423 protein was detected using an antibody to residues 250-300 (A304, Bethyl Labs) or residues near the carboxylterminal end of the protein (ABN410, Millipore) with IR-700 conjugated goat anti-rabbit secondary antibody (Rockland 611130122) and detection on a LiCor Odyssey fluorescence imaging station. Processed blots were reprocessed with a cocktail of anti-phosphoprotein antibodies (Millipore Sigma, P3430, P3300, and P3555; with an IR-800 conjugated secondary antibody) as a proxy for total protein. Gel images were quantified in ImageJ and measurements were recorded as background-corrected Zfp423 signal normalized to either Ponceau-S or phosphoproteins.

Anatomical measures. Samples were prepared, photographed, and measured by an investigator blinded to genotypes. Brains were imaged through a dissecting microscope (Zeiss Stemi 2000-C) with a digital camera (Nikon DS-Fi1) using standardized zoom and distance settings and a standard ruler in frame to verify scale. Paired samples were processed together and imaged consecutively. Anatomical features were measured in ImageJ (v1.52a). For surface images, brains were aligned on a swivel-mount platform and photographed dorsal side up. Vermis width was measured at the middle of the folium-tuber lobule (VII). Cerebellar hemisphere height was measured as a vertical line drawn from the dorsal-most point of the simple lobule. Coronal and sagittal block face preparations were made using a standard mouse brain matrix (Zinc Instrument) with the sample aligned anteriorly. Coronal cuts were made at the rostral end of the optic chiasm, through the striatum. Sagittal cuts were made at the midline. Cut brains were mounted on a rotating platform to hold each surface perpendicular to the lens. For cortical thickness, three lines were drawn using the ImageJ ROI Manager, one each at 15 degrees, 30 degrees, and 45 degrees counter-clockwise from vertical, starting at a point where the line would be perpendicular to the angle of the brain surface, and ending at the dorsal side of the corpus callosum. The thickness of the corpus callosum and anterior commissure were measured with vertical lines at the midline and the width of the brain was measured with a horizontal line across the coronal surface at its widest point. Vermis area was measured from midline sagittal block face image using the polygon selections tool in ImageJ to manually define the region of interest.

Locomotor assays. Gross locomotor function was assessed in home cages and by allowing each test animal and same-sex littermate control to walk freely across a small stage with video recordings for a minimum of three crossings. Rotating rod, footprint pattern, hanging wire, and beam walking tests were performed on same-sex littermate

pairs in the Scripps Research Institute Animal Models Core Facility by staff blinded to genotype and hypothesis and following standard protocols. Animals included roughly equal numbers of male pairs and female pairs for each genotype tested.

Rotating rod test. Latency to fall from an accelerating rotating rod assessed a combination of proprioceptive, vestibular, fine motor, and motor learning capabilities required to avoid falling [49]. Animals were placed on the apparatus (Roto-rod Series 8, IITC Life Sciences, Woodland Hills, CA) prior to acceleration. Latency to fall was recorded by sensing platforms below the rotating rod. Mice were tested in two sets of 3 trials separated by 2 hours.

Footprint pattern test. Footprint pattern analysis assessed basic gait parameters [49-51]. Non-toxic paint was applied to each paw, with front and back paws distinguished by color. Each mouse was placed at one end of a runway covered in paper and allowed to walk until their paws no longer left marks. Forelimb and hindlimb stride lengths (left and right) and front and back leg stride widths were measured the average of three full strides was used for each mouse's values. Mice that did not make 3 measurable strides were excluded.

Hanging wire test. The hanging wire test assessed grip strength and coordination [52, 53]. Mice were held so that only their forelimbs contact an elevated metal bar (2 mm diameter, 45 cm long, 37 cm above the floor) parallel to the ground and released to hang. Each mouse had three trials separated by 30 seconds. Each trial was scored 0 (mouse fell off), 1 (hung onto the wire by two forepaws), 2 (hung onto the wire by two forepaws and attempted to climb onto the wire), 3 (hung onto the wire by two forepaws plus one or both hindpaws), 4 (hung onto the wire by all four paws plus tail wrapped), or 5 (escaped

to the ring stand holding the bar or climbed down the stand to the table). Latency to falling off was measured up to a maximum of 30 s.

Elevated beam test. Escape latency and observed foot slips during escape on a narrow beam further assessed locomotor coordination [49]. Three successive trials were recorded. Average escape time and total number of slips were compared between genotypes.

Statistical analyses. Target samples sizes were estimated from literature and refined according to power calculations based on the observed standard deviations for wild-type littermate pairs as an empirical null model. Retrospective analysis in the R package pwr (v1.2-2, https://github.com/heliosdrm/pwr) or stand-alone software G*power (v3.1.9.2, [54]). Hypothesis tests were performed in R (v3.5.1 [55]). A one-sample t-test was performed for same-sex littermate ratios = 1, or the non-parametric Wilcoxon test if the data distribution showed significant departure from normality by the Shapiro-Wilk test. Graphical output was in R base graphics or ggplot2 (v3.1.0 [56]) with ggbeeswarm (v0.6.0 https://github.com/eclarke/ggbeeswarm).

To estimate statistical power, we first analyzed non-mutant same-sex littermate pairs, for which data accumulated more quickly than for any specific variant. Vermis width and cortical thickness measures both showed paired sample ratios with low variance and approximately normal distribution. Power calculation for a one-sample t-test estimated 90% power to detect a 10% difference with 5-6 paired samples for a nominal alpha=0.05 or 10-12 samples after Bonferroni correction for ~50 variants tested for each measure. Other measured values had larger variance (and in some cases incomplete penetrance) and required transformation to meet normality. Vermis width was also the most sensitive

measure for variants with any effect (see below). Based on these observations, we set vermis as the primary outcome and 10 sex-matched littermate pairs as a target minimum sample size for testing quantitative effects of *Zfp423* variants.

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AUTHOR CONTRIBUTIONS

BAH conceived, designed, performed experiments, analyzed data, and wrote the manuscript. OD, RZ, RL, and DC refined methods, performed experiments, and analyzed data. All authors reviewed and agreed to the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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