Recent studies have identified both recessive and dominant forms of mitochondrial disease that result from ATAD3A variants. The recessive form includes subjects with biallelic deletions mediated by non-allelic homologous recombination. We report five unrelated neonates with a lethal metabolic disorder characterized by cardiomyopathy, corneal opacities, encephalopathy, hypotonia, and seizures in whom a monoallelic reciprocal duplication at the ATAD3 locus was identified. Analysis of the breakpoint junction fragment indicated that these 67 kb duplications were likely mediated by non-allelic homologous recombination at regions of high sequence identity in ATAD3A exon 11 and ATAD3C exon 7. At the recombinant junction, the duplication allele produces a fusion gene derived from ATAD3A and ATAD3C, the protein product of which lacks key functional residues. Analysis of fibroblasts derived from two affected individuals shows that the fusion gene product is expressed and stable. These cells display perturbed cholesterol and mitochondrial DNA organization similar to that observed for individuals with severe ATAD3 deficiency. We hypothesize that the fusion protein acts through a dominant-negative mechanism to cause this fatal mitochondrial disorder. Our data delineate a molecular diagnosis for this disorder, extend the clinical spectrum associated with structural variation at the ATAD3 locus, and identify a third mutational mechanism for ATAD3 gene cluster variants. These results further affirm structural variant mutagenesis mechanisms in sporadic disease traits, emphasize the importance of copy number analysis in molecular genomic diagnosis, and highlight some of the challenges of detecting and interpreting clinically relevant rare gene rearrangements from next-generation sequencing data.

Since its initial association with a neurological disorder, it has become apparent that disruption of the ATAD3 cluster, and more specifically ATAD3A (MIM: 612316), is a significant cause of pediatric disease. Variants at this locus are associated with a wide phenotypic spectrum, including pontocerebellar hypoplasia, hereditary spastic paraplegia, and a syndromic neurological disorder characterized by peripheral neuropathy, hypotonia, cardiomyopathy, optic atrophy, cerebellar atrophy, and seizures: Harel-Yoon syndrome (HAYOS [MIM: 617183]). The different phenotypes can be attributed to a spectrum of disease-causing variants that includes bi-allelic hypomorphic variants, bi-allelic deletions, and monoallelic dominant-negative missense variants. Here, we report two de novo intergenic duplications in the ATAD3 cluster identified in five unrelated neonates with shared phenotypes including corneal clouding, cardiomyopathy, hypotonia, and white matter changes, thus expanding the genotype spectrum of ATAD3-related disorders.

The ATAD3 cluster is composed of three paralogs with extensive sequence homology, formed through tandem segmental duplication: ATAD3A, ATAD3B (MIM: 612317), and ATAD3C (MIM: 617227). ATAD3A and ATAD3B are protein-coding genes of near identical sequence, differing primarily due to a stop-loss mutation in ATAD3B that extends the protein by 62 amino acids; ATAD3C is not known to be expressed. ATAD3A is a transmembrane ATPase, which is predicted to form hexamers,
a fraction of which is found at contact sites between the inner and outer mitochondrial membranes\textsuperscript{5} in complex with TSPO, CYP11A1, and OPA1.\textsuperscript{6} ATAD3 has also been shown to interact with mitochondrial nucleoprotein complexes and to play roles in mtDNA organization and replication.\textsuperscript{2,7,8} More recently it has been shown to interact with Drp1/DNM1L to support Drp1-induced mitochondrial division,\textsuperscript{9} a process that drives mtDNA segregation.\textsuperscript{10,11} Concordantly, ATAD3 dysfunction and deficiency have a wide range of effects on mitochondrial structure and function, characterized by disturbed mitochondrial morphology and fission dynamics,\textsuperscript{3,6} loss of cristae,\textsuperscript{12} perturbed mtDNA and cholesterol metabolism, impaired mitochondrial steroidogenesis,\textsuperscript{2,13} and decreased levels of some mitochondrial oxidative phosphorylation (OXPHOS) components.\textsuperscript{12} It is not clear whether the disruption to the inner mitochondrial membrane, mtDNA, and OXPHOS complexes are due directly to the absence of ATAD3\textsuperscript{4,12} or whether they are consequences of changes to membrane architecture resulting from an altered cholesterol content\textsuperscript{4,13} or a combination of the two.

We report de novo ATAD3 duplications identified in five unrelated neonates through exome sequencing. Clinical exome sequencing failed to identify any alternative molecular diagnosis potentially causative of the phenotype, which is characterized by seizures (four of the five neonates) and fetal akinesia and contractures (in three case subjects). A clinical summary is shown in Table 1 and clinical case reports are detailed in the Supplemental Note. Informed consent was obtained and all processes adhered to local and national ethical standards. The duplication in the ATAD3 cluster was also detected by arrayCGH for those subjects studied (subjects four and five). The duplication is predicted to be the product of

\begin{table}
\centering
\caption{Clinical Features of Individuals with Duplication in ATAD3 Gene Cluster}
\begin{tabular}{|c|c|c|c|c|c|}
\hline
& Subject 1 & Subject 2 & Subject 3 & Subject 4 & Subject 5 \\
\hline
Sex & male & female & male & female & male \\
\hline
Gestation & term & 38 weeks & term & 33+3 weeks & term \\
\hline
Apgars at birth & 3 & poor & 1 & 5,8,9 & 1,0 \\
\hline
Chronological age at death & 3 days & 6 weeks & 5 days & 6 weeks & 4 weeks \\
\hline
Cardiomyopathy & HCM & DCM & DCM; cardiomegaly & HCM; cardiomegaly & HCM; cardiomegaly \\
\hline
Congenital cataracts & ✓ & ND & ND & ND & ND \\
\hline
Corneal opacity & ✓ & ✓ & ✓ & ✓ & ✓ \\
\hline
Postnatal hypotonia & ✓ & ✓ & ✓ & ✓ & ✓ \\
\hline
Abnormality of the external genitalia & cryptorchidism and micropenis & ND & ND & ND & hypospadias \\
\hline
Seizures & ✓ & diffuse abnormalities on EEG & ND & diffuse abnormalities on EEG & ✓ \\
\hline
Encephalopathy & ✓ & ND & ✓ & ND & ND \\
\hline
Brain findings & ND & white matter changes; simplified gyral patterning; cerebellar atrophy (MRI) & widespread hypoxic brain damage (post-mortem) & diffuse bilateral abnormal subcortical, periventricular, and deep white matter; abnormal MR spectroscopy & white matter changes, generalized reduction of brain volume (MRI); abnormal MR spectroscopy (lactate peak) on day 9 \\
\hline
Contractures/fetal akinesia & fetal akinesia & ND & contractures & ND & contractures \\
\hline
Edema/fetal hydrops & ND & fetal hydrops; edema & fetal hydrops & ND & ND \\
\hline
Metabolic investigations & increased excretion of fumarate, malate, 2-ketoglutarate, 3-methylglutaconate, and 3-methylglutarate & lactic acidosis & ND & lactic acidosis; increased excretion of 2OH butyrate, fumarate, and 3OH isobutyrate & lactic acidosis, increased excretion of fumarate, malate on day 22 \\
\hline
Prior genetic investigations & ArrayCGH; Prader-Willi; SMA & prenatal aneuploidy & ND & arrayCGH; 202 gene mitochondrial panel & arrayCGH, 27 gene glycogen storage disease panel \\
\hline
\end{tabular}
\end{table}

HCM, hypertrophic cardiomyopathy; DCM, dilated cardiomyopathy; SMA, spinal muscular atrophy; ND, not detected.
non-allelic homologous recombination (NAHR) between regions of high sequence homology in \( \text{ATAD3C} \) and \( \text{ATAD3A} \) (Figure 1A) and encompasses \( \text{ATAD3C} \) exons 8–12, \( \text{ATAD3B} \), and \( \text{ATAD3A} \) exons 1–11 (Figures 1B, S1, and S2).

PCR and Sanger sequencing confirmed the presence of the duplications, which showed a 1.2 kb proband-specific amplicon (1.6 kb for subject four due to alternative primer design; data not shown). No PCR product was amplified in DNA derived from unaffected parents, consistent with a de novo event, and proband-parent relationships were confirmed for all case subjects during exome analysis. The 5’ end of the PCR amplicon was derived from \( \text{ATAD3A} \) exon 10, while the 3’ was derived from \( \text{ATAD3C} \) intron 7. The breakpoints of the duplication identified in subject four were found to differ from those identified in the other case subjects. The duplications are considered functionally equivalent as their protein products are predicted to be identical, differing at a single intronic nucleotide. These results are consistent with tandem duplication without inversion, as described for \( \text{NC}_{000001.11} \) (GRCh38: 1456616_1524663dup (subjects 1–3 and 5) and \( \text{NC}_{000001.11} \) (GRCh38: 1456890_1524937dup (subject 4). The duplications are predicted to maintain the copy-number of \( \text{ATAD3A} \) and \( \text{ATAD3C} \), create a fusion gene, \( \text{ATAD3A-C} \), composed of \( \text{ATAD3A} \) (UniProt: Q9NVI7-2, residues 1–405) and \( \text{ATAD3C} \) (UniProt: QST2N8-1, residues 231–411) (Figures 1B and 1C).

We performed multiple complementary in silico analyses to characterize the effect of the duplication. Multiple sequence alignment of \( \text{ATAD3A} \) (NC_000001.11 (GRCh38):1512151–1534687) and \( \text{ATAD3C} \) (NC_000001.11 (GRCh38):1449689–1470158) showed the genes have an overall sequence identity of approximately 56%. The duplications occur at a 673 bp region with near-complete sequence identity between \( \text{ATAD3A} \) and \( \text{ATAD3C} \) (Figure 1A). In silico splicing analysis of \( \text{ATAD3A-C} \) showed that the splice sites are maintained (Figure S3). Pairwise alignment of \( \text{ATAD3A} \) (GenBank: NM_001170535.2; Q9NVI7-2) and \( \text{ATAD3A-C} \) (UniProt: Q9NVI7-2, residues 1–405, and UniProt: QST2N8-1, residues 231–411) primary amino acid sequences showed that they are of identical length and differ at 29 residues (Figure S4). Seven of the variants (p.Val450Ile, p.Asn454Cys, p.Gln455His, p.Asp465Asa, p.Arg466Cys, p.Asn469Asp, and p.Glu469Val) lie within the ATPase domain (residues 348–474; Pfam: PF00004) (Figure 1D, underline; Figure 1E), while the remaining 22 are present outside of a known functional domain (p.Glu482Ala, p.Phe489Leu, p.Asp490Asn, p.Lys491Glu, p.Gln502Arg, p.Ser516Leu, p.Val518Ile, p.Gly527Cys, p.Glu529Lys, and p.Glu545Lys) or within a region of predicted intrinsic disorder (p.Thr556Ala, p.Glu482Ala, p.Asp490Asn, p.Lys491Glu, p.Gln502Arg, p.Ser516Leu, p.Val518Ile, p.Gly527Cys, p.Glu529Lys, and p.Glu545Lys) or within a region of predicted intrinsic disorder (p.Thr556Ala, p.Arg557Cys, p.Ala561Phe, p.Lys568Met, p.Cys570Arg, p.Ala574Gly, p.Gly576Arg, p.Arg579Pro, p.Gly580Glu, p.Pro583Gln, p.Ser584Pro, and p.Pro585Ser). DeepLoc (v1.0) was used to predict the subcellular localization of
ATAD3A and ATAD3A-C. The tool was able to correctly predict that ATAD3A is transported into the mitochondrial membrane. There was no change in this prediction for ATAD3A-C. Together, these analyses indicate that the fusion transcript is likely to be correctly transcribed and translated and maintain the signals necessary for native subcellular localization. We next modeled the composition of ATAD3 hexamers using a binomial distribution based on two copies of ATAD3A and one copy of ATAD3A-C. It is predicted that 8.8% of ATAD3 hexamers would be comprised solely of wild-type ATAD3A monomers, while 91.2% would contain at least one copy of the ATAD3A-C fusion protein (Figure S5).

To experimentally assess the predictions of the *in silico* analyses we amplified a ~1.8 kb product by reverse transcription PCR on RNA extracted from fibroblasts (subject 4), using a primer pair specific to ATAD3A and ATAD3C. Sanger sequencing of this product showed a sequence identical to the predicted ATAD3A-C transcript (Figure S6). We found that the 5’ region of the ATAD3A-C fusion transcript corresponds to that of ATAD3A, splicing isoform two. Western blotting showed that fibroblasts (subject 1) harboring the duplication had higher expression of ATAD3, compared to controls (Figures 2A and 2B). The upper of the two bands is where ATAD3B migrates and so the increased signal is attributed to the additional copy of ATAD3B. As ATAD3A is not fully duplicated, the increased signal of the lower band suggests that the ATAD3A-C protein product is expressed and stable. ATAD3 is an established mitochondrial protein,7 and antibody labeling of ATAD3 in fibroblasts of subject 1 revealed a distribution similar to control cells and to the mitochondrial outer membrane protein TOMM20 (Figure S7). Therefore, both the duplicated ATAD3B and the ATAD3A-C fusion gene product appear to be targeted to the mitochondria.

Variants in *bor*, an ATAD3A homolog in *Drosophila melanogaster*, are associated with a reduction in the number of mitochondria and mitochondrial structural abnormalities1 and bi-allelic ATAD3 cluster deletions have been shown to cause mitochondrial structural abnormalities and impaired cholesterol metabolism in human fibroblasts.2 Therefore, we assessed mitochondrial morphology and cholesterol levels in our cellular models. In subject 1-derived fibroblasts, free-unesterified cholesterol assessed by filipin staining was significantly higher than control subjects and was similar to cells with pronounced ATAD3 deficiency caused by bi-allelic ATAD3 cluster deletions2 (Figures 2C and 2D). Many fibroblasts (subject 1) showed aggregations of mitochondria, and swollen and rounded organelles (Figure 3A; circled). Nevertheless, cells with an extensive and interconnected mitochondrial network were also apparent (Figure 3A). Immunostaining for DNA indicated that the swollen mitochondria contained accumulations of mtDNA (Figure 3B; arrows). These features are similar to those associated with ATAD3 cluster
ATAD3A. Duplications were carried either by unaffected parents or probands whose clinical features were inconsistent with a probable metabolic disorder, were above the age of 1 year at their last clinical assessment, and were alive at the point of recruitment. Confirmation testing was not undertaken for the apparently benign duplications, and the precise breakpoints have not been determined. Nevertheless, the presence of multiple ATAD3B duplications in this study cohort suggests that the duplication of ATAD3B and increased ATAD3B gene dosage alone is not causative of this severe phenotype, but rather the NAHR-derived recombinant ATAD3A-C gene and novel protein product generated by the de novo mutational event.

We have identified two de novo duplications within the ATAD3 cluster in five unrelated individuals whose clinical presentation suggested a metabolic disorder. ATAD3 gene defects were recently recognized as a cause of human disease, accounting for a growing number of phenotypes and cases. Dominant-negative ATAD3A missense variants have been reported in individuals affected with hypotonia, optic atrophy, axonal neuropathy, hypertrophic cardiomyopathy, and hereditary spastic paraplegia. Bi-allelic ATAD3 cluster deletions result in a more severe phenotype with pontocerebellar hypoplasia and death in the majority of case subjects within the first week of life similar to bi-allelic ATAD3A deletions. These case subjects with a monoallelic ATAD3 gene cluster duplication extend the genotype spectrum of ATAD3-related disorders.

The phenotype of the neonates with ATAD3 duplications shows overlap with the previously reported cases associated with pathogenic variation at this locus noting corneal clouding, cardiomyopathy, hypotonia, white matter changes, seizures, fetal akinesia, and contractures. All subjects with duplication died within 6 weeks of life. Although four neonates had low Apgar scores and required intensive clinical management from birth, subject 4 was born prematurely (33+3 weeks), achieved high Apgar scores, had a less severe perinatal course, and presented 3 weeks later with severe lactic acidosis (Table 1 and Supplemental Note). The subjects did not present with obvious signs of mitochondrial distress, and this study highlights the importance of considering mitochondrial genes even in atypical cases, such as these.

The ATAD3A-C fusion protein is uniquely associated with the severe neonatal phenotype and therefore is likely causal. It is expressed and stable (Figures 2A, 2B, and S6) and has the correct subcellular localization (Figure S7). The fusion protein differs from ATAD3A at 29 amino acid residues within the C-terminal region, including a highly conserved residue within the ATPase domain, p.Arg466Cys (Figure 1D; arrow and Figure 1E). The equivalent residue is conserved in all multimeric AAA-domain containing ATPases and functions as an arginine finger, a trans-acting residue that binds to the γ-phosphate of ATP in the neighboring monomer. Multiple recurrent missense variants have been reported at the equivalent arginine finger residue, Arg499, in SPAST (Figure 4) and cause...
autosomal-dominant hereditary spastic paraplegia (SPG4 [MIM: 182601]). These variants have been shown to result in the complete loss of SPAST ATPase activity, leading to disease through a dominant-negative mechanism.

We suggest that the ATAD3 duplications described here act through the same mechanism: through incorporation of a non-functional monomer derived from the novel fusion protein into more than 90% of ATAD3 hexamers (Figure S5).

Our data suggest that the generation of the fusion protein causes this lethal neurological disorder through disruption of mitochondrial and cholesterol metabolism (Figures 2C, 2D, and 3). This reinforces the links between ATAD3, cholesterol, and mtDNA metabolism. Considering the majority of the cholesterol in mitochondrial membranes co-purifies with mtDNA and increasing or decreasing cholesterol availability markedly alters mtDNA organization, then cholesterol dyshomeostasis evidently disrupts mtDNA metabolism. ATAD3 has links to cholesterol metabolism through partner proteins, TSPO, CYP11A1, and SPTLC. ATAD3 also co-purifies with the mitochondrial protein synthesis machinery, mtDNA, and mitochondrial cholesterol, and there is evidence that the mitochondrial nucleoprotein complexes are interlinked. Hence perturbed cholesterol-containing micro-domains could be the common factor linking all the features associated with ATAD3 deficiency.

Copy number variants (CNVs) pose a practical challenge in genomic analysis, in both their detection and interpretation. Determining how to analyze and interpret rare CNVs which intersect common benign CNVs is not trivial. The high frequency of benign duplications seen in the ATAD3 region coupled with high sequence homology of the three genes means that pathogenic duplications could potentially be missed. This study highlights the importance of systematic CNV analysis, particularly of genomic intervals prone to instability, where a clinical presentation is consistent with a monogenic disorder.

The high frequency at which this specific ATAD3 duplication was identified within this cohort suggests that for all clinical suspicions of severe neonatal disorder of unknown origin, negative for known mitochondrial variants and mitochondrial nuclear genome panels, the ATAD3 locus should be carefully evaluated for single nucleotide, copy-number, and structural variants.

Figure 4. Protein Modeling of ATAD3 Hexamer and 3D Alignment against SPAST ATPase Domain
(A) Hexameric structure of ATAD3A ATPase domain (amino acids 348–474), modeled in SwissModel using PDB: 6f0x (H. sapiens, TRIP13) as a template. A single monomer is highlighted in violet. (B) Single ATAD3A monomer (violet) aligned to H. sapiens SPAST ATPase domain (blue). (C) The ATAD3A arginine finger, Arg466 (yellow) which is changed to a cysteine in the ATAD3A-C fusion gene, is overlaid with the SPAST arginine finger (Arg499; orange).

Supplemental Data
Supplemental Data can be found online at https://doi.org/10.1016/j.ajhg.2020.01.007.

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Declaration of Interests

Baylor College of Medicine (BCM) and Miraca Holdings have formed a joint venture with shared ownership and governance of Baylor Genetics (BG), which performs clinical microarray analysis and clinical exome sequencing. J.R.L. serves on the Scientific Advisory Board of BG. J.R.L. has stock ownership in 23andMe, is a paid consultant for Regeneron Pharmaceuticals, has stock options in Lasergen, and is a co-inventor on multiple United States and European patents related to molecular diagnostics for inherited neuropathies, eye diseases, and bacterial genomic fingerprinting. The other authors declare no competing interests.

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Web Resources

CBS DeepLoc, http://www.cbs.dtu.dk/services/DeepLoc/
DECIPHER, https://decipher.sanger.ac.uk/
EBI KAlign, https://www.ebi.ac.uk/Tools/msa/kalign/
EBI MUSCLE, https://www.ebi.ac.uk/Tools/msa/muscle/
Ensembl Genome Browser, http://www.ensembl.org/index.html
OMIM, https://www.omim.org/
Swiss-Model, https://swissmodel.expasy.org/
Uniprot, https://www.uniprot.org/

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