A homozygous KAT2B variant modulates the clinical phenotype of ADD3 deficiency in humans and flies

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Abstract

Recent evidence suggests that the presence of more than one pathogenic mutation in a single patient is more common than previously anticipated. One of the challenges hereby is to dissect the contribution of each gene mutation, for which animal models such as Drosophila can provide a valuable aid. Here, we identified three families with mutations in ADD3, encoding for adducin-γ, with intellectual disability, microcephaly, cataracts and skeletal defects. In one of the families with additional cardiomyopathy and steroid-resistant nephrotic syndrome (SRNS), we found a homozygous variant in KAT2B, encoding the lysine acetyltransferase 2B, with impact on KAT2B protein levels in patient fibroblasts, suggesting that this second mutation might contribute to the increased disease spectrum. In order to define the contribution of ADD3 and KAT2B mutations for the patient phenotype, we performed functional experiments in the Drosophila model. We found that both mutations were fully rescued the viability of the respective null mutants of the Drosophila homologs, hts and Gcn5,
suggesting that they are indeed pathogenic in flies. While the KAT2B/Gcn5 mutation additionally showed a significantly reduced ability to rescue morphological and functional defects of cardiomyocytes and nephrocytes (podocyte-like cells), this was not the case for the ADD3 mutant rescue. Yet, the simultaneous knockdown of KAT2B and ADD3 synergistically impaired kidney and heart function in flies as well as the adhesion and migration capacity of cultured human podocytes, indicating that mutations in both genes may be required for the full clinical manifestation. Altogether, our studies describe the expansion of the phenotypic spectrum in ADD3 deficiency associated with a homozygous likely pathogenic KAT2B variant and thereby identify KAT2B as a susceptibility gene for kidney and heart disease in ADD3-associated disorders.

Author summary

Genetic diseases with complex syndromic constellations may be caused by mutations in more than one gene. Most examples studied so far describe genetic interactions of known disease genes, suggesting that a large number of multilocus diseases remain unexplored. Assessment of mutation pathogenicity can be achieved using animal models. One main advantage of using Drosophila is that it allows easy in vivo gene manipulation in cell types that are relevant for the disease. Here, we report the pathogenicity of ADD3 mutations in three families with intellectual disability, microcephaly, cataracts and skeletal defects. Moreover, we provide evidence that the renal and cardiac phenotypes in one of the families could be unmasked by a homozygous variant in the lysine acetyltransferase encoding KAT2B gene. In Drosophila, this variant resulted not only in decreased viability, but also in functional defects in cardiomyocytes and nephrocytes, the latter being similar to mammalian podocytes. Our study implicates KAT2B as a susceptibility gene for steroid-resistant nephrotic syndrome (SRNS) and cardiomyopathy and emphasizes the importance of protein acetylation in kidney and heart function.

Introduction

The interrogation of the entire genome via next generation sequencing (NGS) technology has revolutionized clinical diagnostics. For medical genetics that traditionally focuses on finding monogenetic causes for Mendelian diseases, NGS has not only introduced much higher mutation detection rates but also unprecedented complexities. A recent retrospective analysis of more than 7000 exomes revealed multiple molecular diagnoses in around five percent of cases with suspected monogenic disease [1], suggesting that patients with multilocus diseases are underrecognized.

The phenotypic complexity of multilocus diseases, of which digenic disease represents the simplest and most common form, can be challenging for the physician, both when it comes to finding a diagnosis and to genetic counseling and risk assessment. Two distinct disease phenotypes in a single patient may present with a completely new clinical phenotype. On the other hand, two overlapping disease phenotypes may be misinterpreted as a single disease with increased severity. The underlying genetic defects are equally difficult to predict. Both compound phenotypes caused by mutations in two completely unrelated genes [1] and overlapping disease phenotypes caused by mutations in genes within the same pathway are possible [2–4].
But as genes can be pleiotropic, there are most likely many exceptions to this. Also, while two loci may be equal in importance [2], a second variant may simply enhance the general or organ-specific penetrance of a given mutation [4].

One important challenge is therefore to decompose the contributions of each gene mutation or variant to the clinical phenotypes in question. So far, most reports on digenic inheritance in Mendelian disease have focused on known disease genes [1, 5, 6]. However, the diagnosis is even more difficult when dealing with genes that have previously not been associated with any genetic diseases.

In this study, we identify three families with mutations in ADD3, encoding for adducin-γ, with intellectual disability, microcephaly, cataracts and skeletal defects, further supporting that ADD3 is a disease gene as previously reported for a single family [7]. We further use mutation validation in Drosophila and mammalian cell culture to demonstrate that in one of the families additional phenotypes in kidney and heart are associated with a homozygous missense variant in the lysine acetyltransferase KAT2B.

**Results**

**Clinical features of three families with intellectual disability and microcephaly**

Six individuals in three families (families A-C) with intellectual disability and varying degrees of microcephaly (Table 1) were identified for this study. Individuals from family A and B also shared bilateral cataracts, corpus callosum defects as well as specific skeletal defects such as shortening of the third and fourth metatarsals (Fig 1A–1D and Table 1), while the affected boy from family C suffered from epilepsy, severe speech delay and suspected cerebral palsy (Table 1).

The affected sibs in the consanguineous family A additionally presented with steroid-resistant nephrotic syndrome (SRNS), a progressive renal disease characterized by podocyte lesions and massive proteinuria [8], and cardiomyopathy (Table 1). For individual II-1 and II-3, proteinuria was first detected at 7 and 12 years of age, respectively, and end-stage renal disease was diagnosed a decade later. Individual II-6 was diagnosed with SRNS and end-stage renal disease at the age of 13 years. In kidney biopsies, individuals II-3 and II-6 (Fig 1E) both showed focal segmental glomerulosclerosis (FSGS). In the biopsy of individual II-6, whose renal disease was at a more advanced stage, hypertrophic and vacuolated podocytes (Fig 1E) as well as tubular atrophy, interstitial fibrosis and inflammatory cell infiltrates could also be found. In addition, all affected individuals from family A developed dilated cardiomyopathy with progressive heart failure and arrhythmia (Table 1). Cardiac failure was the cause of death for both individuals II-1 and II-3.

**Whole-exome sequencing identifies missense mutations in ADD3 in all families**

For the affected individuals in family A and C, the presence of mitochondrial disease was excluded by muscle biopsy. Moreover, high-resolution karyotypes were normal for all patients, and CGH arrays (performed for family B and C) did not show significant abnormalities. Consequently, whole exome sequencing (WES) was performed on two affected members of family A as well as on the affected individuals and the parents of family B and C, after obtaining written informed consent and study approval. WES led to the identification of recessive potentially damaging mutations in ADD3, all segregating with the disease as confirmed by Sanger sequencing (NM_016824.4: family A: homozygous c.1975G>C, p.E659Q; family B: compound
Table 1. Clinical phenotype of affected individuals.

<table>
<thead>
<tr>
<th></th>
<th>Family A</th>
<th>Family B</th>
<th>Family C</th>
<th>Kruer et al (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>II-1</td>
<td>II-3</td>
<td>II-6</td>
<td>II-3</td>
</tr>
<tr>
<td>Sex</td>
<td>F</td>
<td>F</td>
<td>M</td>
<td>NK</td>
</tr>
<tr>
<td>SRNS</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>NK</td>
</tr>
<tr>
<td>Age of onset</td>
<td>7</td>
<td>12</td>
<td>&lt;13</td>
<td>NA</td>
</tr>
<tr>
<td>of proteinuria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Renal histology</td>
<td>FSGS</td>
<td>FSGS</td>
<td>FSGS</td>
<td>NA</td>
</tr>
<tr>
<td>Age of ESRD (yrs)</td>
<td>17</td>
<td>27</td>
<td>13</td>
<td>NA</td>
</tr>
<tr>
<td>Heart disease</td>
<td>Dilated cardiomyopathy (dx 16 yrs), supraventricular arrhythmia (frequent auricular extra-systoles), heart failure</td>
<td>Dilated cardiomyopathy, arrhythmia</td>
<td>Dilated cardiomyopathy (dx 8 yrs), arrhythmia (ventricular hyperexcitation), heart failure</td>
<td>NK</td>
</tr>
<tr>
<td>Cataract</td>
<td>Congenital bilateral cataract</td>
<td>Congenital bilateral cataract</td>
<td>Bilateral cataract (6 yrs)</td>
<td>NK</td>
</tr>
<tr>
<td>Age at last</td>
<td>† 19</td>
<td>† 28</td>
<td>19.</td>
<td>TOP</td>
</tr>
<tr>
<td>examination vs † age (yrs)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations are as follows: CP, cephalic perimeter; ESRD, end-stage renal disease; F, female; FSGS, focal segmental glomerulosclerosis; yrs, years; M, male; MRI, magnetic resonance imaging NA, not applicable; NK, not known; SRNS, steroidresistant nephrotic syndrome; SD, standard deviation; TOP, termination of pregnancy; yrs, years †, deceased

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heterozygous c.86A>G, p.N29S; c.1588G>A, p.V530I (both on the same allele in the mother), c.995A>G, p.N332S (heterozygous in the father); family C: homozygous c.995A>G, p.N332S) (Fig 2A and 2B and Table 2). In 148,632 reference individuals from the gnomAD browser
The identified $ADD3$ mutations result in the substitution of amino acids located in the head and tail region of the protein product adducin-$\gamma$ (Fig 2B). In humans, adducins form heterotrimers that are composed of either adducin-$\alpha$ and $\gamma$ (the most widely expressed) or adducin-$\alpha$ and $\beta$ (restricted mainly to erythrocytes and specific brain regions) [9]. These heterotrimers regulate the actin cytoskeleton by capping the barbed ends of F-actin and by promoting the interaction between actin and spectrin [9, 10]. Recently, a homozygous mutation in $ADD3$ was shown to cause cerebral palsy, epilepsy, borderline microcephaly, thin corpus callosum...
KAT2B is a susceptibility gene for SRNS and cardiomyopathy.
and intellectual disability in one family [7]. As the phenotype of this family shows overlap with all our families, particularly family C, our study supports the pathogenicity of the previously identified ADD3 mutation.

A homozygous variant in KAT2B associates with the extended phenotypes in family A

Family A, which was characterized by additional cardiomyopathy and SRNS, exhibited another potentially damaging homozygous mutation in lysine acetyltransferase 2B (KAT2B). No other pathogenic variant was identified after applying a set of filters excluding common variants in the general population (dbSNP >1%) or in our in-house database as well as variants predicted not to be deleterious. The KAT2B variant (NM_003884.4; c.920T>C, p.F307S) segregated with the disease and was not present in the reference individuals from the gnomAD browser (Fig 2A and Table 2). KAT2B is known to acetylate a variety of substrates, including histones (preferentially H3), and to function as a transcription coactivator together with CBP/p300 [11–13]. The identified KAT2B missense variant affects a highly conserved amino acid within the PCAF homology domain (Fig 2C), which is required for the interaction with CBP/p300 [14].

By studying mRNA and protein expression in patient fibroblasts from affected members of family A using qPCR, western blotting and immunostainings, we found no significant decrease for adducin-γ at the mRNA or protein level (Fig 2D and S1A and S1C Fig). However, KAT2B protein (but not mRNA) levels were significantly reduced (Fig 2 and S1B and S1D Fig). Thus, we reasoned that the KAT2B variant could contribute to the extended phenotype observed in family A. To test this hypothesis, we decided to perform functional validation of both mutations in Drosophila melanogaster.

Table 2. Pathogenic genetic variants identified in affected individuals with overlapping syndromes.

<table>
<thead>
<tr>
<th>Family/ Individual</th>
<th>Gene</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Zygosity, Segregation</th>
<th>MT</th>
<th>SIFT</th>
<th>PolyPhen-2</th>
<th>gnomAD allele frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/II-3, II-6</td>
<td>ADD3</td>
<td>c.1975G&gt;C</td>
<td>p.E659Q</td>
<td>HOM, Segregation</td>
<td>DC</td>
<td>0.13 (T)</td>
<td>0.980 (PD)</td>
<td>4/246110 (no HOM)</td>
</tr>
<tr>
<td></td>
<td>KAT2B</td>
<td>c.920T&gt;C</td>
<td>p.F307S</td>
<td>HOM</td>
<td>DC</td>
<td>0 (D)</td>
<td>0.990 (PD)</td>
<td>Not reported</td>
</tr>
<tr>
<td>B/II-3, II-4</td>
<td>ADD3</td>
<td>c.86A&gt;G</td>
<td>p.N29S</td>
<td>het m</td>
<td>DC</td>
<td>0.25 (T)</td>
<td>0.653 (PoD)</td>
<td>17/276960 (no HOM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c.995A&gt;G</td>
<td>p.N332S</td>
<td>het p</td>
<td>DC</td>
<td>0.03 (D)</td>
<td>0.995 (PD)</td>
<td>176/276966 (no HOM)</td>
</tr>
<tr>
<td>C/II-1</td>
<td>ADD3</td>
<td>c.1388G&gt;A</td>
<td>p.V530I</td>
<td>het m</td>
<td>DC</td>
<td>0 (D)</td>
<td>1 (PD)</td>
<td>9/276822 (no HOM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c.995A&gt;G</td>
<td>p.N332S</td>
<td>HOM</td>
<td>DC</td>
<td>0 (D)</td>
<td>0.995 (PD)</td>
<td>17/276966 (no HOM)</td>
</tr>
</tbody>
</table>

Abbreviations are as follows: D, deleterious; DC, disease causing; het, heterozygous; HOM, homozygous; m, maternal; MT, mutationtaster; p, paternal; PD, probably damaging; PoD, possibly damaging; T, tolerated

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Adducin-γ E659Q is a hypomorphic mutation in Drosophila

Drosophila hu li tai shao (hts) corresponds to the sole homolog of all three adducin genes in humans. As previously described [15], htsnull hemizygous animals died at the late larval stage, with only a few escapers progressing into adult stage. The escapers showed rough eyes, uncoordinated movements and inability to fly leading to death within 24h after eclosion (S2A Fig). For mutation validation, we re-expressed in this htsnull background the human wild-type (WT) and mutant constructs using the ubiquitous driver tubulin (tub)-GAL4 (see S1 Table for precise genotypes). As E659, the amino acid mutated in adducin-γ, is located in a very poorly conserved region (Fig 2B), we performed rescue experiments with WT and mutated human adducin-γ. While re-expressing each of the adducins alone failed to rescue the viability, the co-expression of adducin-α and -γ (hereafter referred to as adducin-αγ WT) led to around sixty percent of viable mutant adults (Fig 3A). Importantly, when co-expressing adducin-γ E659Q together with adducin-α (adducin-αγ E659Q), we observed a significantly reduced partial rescue of fly viability (Fig 3A). The surviving animals did not present with any defects in eye and wing morphology (S2A Fig) but showed climbing impairment in a geotaxis assay (Fig 3B) [7]. To express the transgenes with endogenous expression levels, we also used an available GAL4 insertion in the hts locus. This insertion leads to a partial lethality over htsnull, which could be fully restored by adducin-αγ WT but not by E659Q (S3A Fig). Altogether, these results suggest that adducin-γ E659Q is a hypomorphic mutation.

KAT2B F307S is a loss-of-function mutation in Drosophila

Drosophila Gcn5 is homologous with KAT2B and its paralog KAT2A. Gcn5E333st hemizygous animals died at late larval stage/early pupal stage as previously reported for this null mutation [16]. The expression of Drosophila Gcn5 (hereafter referred to as Gcn5 WT) with tub-GAL4 or another ubiquitous driver (daughterless (da)-GAL4) led to a full rescue (S3B Fig and Fig 3C). By contrast, the expression of human KAT2A and KAT2B, either alone or in combination, did not restore the viability of the mutant (Fig 3C), suggesting that the human orthologs have evolved in structure and function in comparison to Gcn5. As the mutated amino acid in KAT2B, F307, is conserved in Drosophila Gcn5 (corresponding to Gcn5 F304), we re-expressed Gcn5 F304S in the Gcn5E333st hemizygous background (Gcn5 F304S). As a negative control, we re-expressed a predicted potentially damaging KAT2B variant (S502F corresponding to Gcn5 S478F) found in a homozygous state in a healthy individual from our in-house database. While Gcn5 S478F rescue animals were normal (Fig 3C and S3B Fig), Gcn5 F304S had a dramatically decreased viability with death occurring either in pupal stages or a few days after eclosion (Fig 3C). All adult escapers showed blistered wings, inability to fly and rough eyes and around 40 percent of the animals had defects in leg morphology (Fig 3D and 3E). Interestingly, this phenotype corresponds to what has previously been described for the deletion of the entire PCAF homology domain, where the mutation is localized [16]. In agreement with the proposed function of Gcn5 in histone acetylation [16], we further detected histone (H3K9) acetylation defects for Gcn5 F304S but not for Gcn5 WT and control animals, as assessed by immunoblotting of larval nuclear extracts (Fig 3F), suggesting that the mutation impairs the enzymatic activity of Gcn5. Altogether, the results suggest that KAT2B F307S is a loss-of-function mutation in Drosophila.

KAT2B F307S but not ADD3 E659Q causes cardiac defects in Drosophila

Since the presence of SRNS and heart defects in family A was the main phenotypic difference from the other families, we looked more specifically into the cardiac and renal system of the fly. The Drosophila heart is a tubular organ formed by contractile cardiomyocytes that pump
KAT2B is a susceptibility gene for SRNS and cardiomyopathy.
Fig 3. Effect of ADD3 and KAT2B mutations on viability and morphology in Drosophila. (A) Viability for hs<sup>md</sup> hemizygous flies and respective rescues with adducin (Add) construct(s) using tubulin-GAL4 (tub>). After 48h of egg laying on standard cornmeal/yeast food, viability was calculated as the percentage of hatching adults of the indicated genotype and normalized to the control. The control corresponds to the viable F1 trans-heterozygous flies obtained from the cross between Df(2R)BS C26 (harbouring the hts gene) and a non-overlapping deficiency on the same chromosome (Df(2R)247). Quantification is for >100 F1 eclosing flies/genotype/experiment in 5 independent experiments. Statistical analysis was performed using one-way ANOVA with Bonferroni post-test. (B) Negative geotaxis assay for one-day-old adult flies. Flies were transferred to a graduated tube, and after tapping, the length climbed in 8 sec was recorded [45]. Quantification was performed on 6 independent experiments with >38 flies/genotype using one-way ANOVA with Kruskal-Wallis post-test. ns, non significant (see S1 Table for details on transgenic flies). For all panels: ns, non significant, *p<0.05 **p<0.01, ***p<0.001 (see S1 Table for details on transgenic flies). (C) Viability for Gcn5<sup>md</sup> hemizygous flies and respective rescues with Gcn5 and KAT2A/B construct(s) using daughterless-GAL4 (da>). Viability was assessed as described in (A). Human KAT2B F307S and S502F mutations correspond to Gcn5 F304S and S478F mutations, respectively. Gcn5 S502F variant predicted to be deleterious (PolyPhen-2 score of 0.98) was found on a healthy individual at the homozygous state in our in-house exome database. The control corresponds to the viable F1 trans-heterozygous flies obtained from the cross between Df(3L)sex 204 (harbouring the Gcn5 gene) and a non-overlapping lethal mutant on the same chromosome (CG3104<sup>md(10)198</sup>). Quantification is for >100 F1 eclosing flies/genotype/experiment in 5 independent experiments. Statistical analysis was performed using one-way ANOVA with Bonferroni post-test. (D) Phenotype of Gcn5 WT, Gcn5 F304S and Gcn5 S478F rescue animals. Pictures correspond to adult flies one day post-eclosion and are representative of the defects found in wings (separated wing blades), legs (femur kinking, arrow) and eye (small and mild rough eye). Scale bars: wings: 500 μm, legs: 500 μm, eye: 200μm. (E) Quantification of the defects in wings, legs and eyes found in Gcn5 F304S rescue flies is for >100 F1 eclosing flies/genotype. (F) H3K9 acetylation levels of Gcn5<sup>md</sup> and Gcn5 WT and F304S rescue animals. Extracted nuclear proteins from 3rd instar (= late) larvae were analysed by western blotting normalized to non-acetylated Histone 3 (H3). Quantification is shown in the lower panel (n = 3 independent experiments; one-way ANOVA with Bonferroni post-test). For all panels: ns, non significant, *p<0.05 **p<0.01, ***p<0.001 (see S1 Table for details on transgenic flies).

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KAT2B is a susceptibility gene for SRNS and cardiomyopathy

The hemolymph (analogous to the blood in vertebrates) to the rest of the body. This organ system has proven to be an important tool for studying the genetics and pathophysiology of cardiac disease [17, 18]. Therefore, we studied heart function in adult adducin and Gcn5 rescue flies. As illustrated in the M-mode traces obtained from high-speed movies, adducin-αγ E659Q did not show any significant differences in heart period, cardiac output, fractional shortening and arrhythmia index when compared to adducin-αγ WT (Fig 4). By contrast, Gcn5 F304S flies showed prolonged heart period and reduced cardiac output compared to Gcn5 WT and control flies (Fig 5A–5E). Both Gcn5 WT and F304S rescue flies showed a reduction in the normal diastolic diameter compared to control flies (Fig 5F), but only for Gcn5 F304S there was a reduction in contractility, measured as fractional shortening (Fig 4G).

Moreover, the Gcn5 F304S mutant showed a more irregular heartbeat compared to Gcn5 WT, reflected by an increase in the arrhythmia index (Fig 5H). In further support of Gcn5’s requirement for normal heart function, the silencing of Gcn5 with a heart-specific driver (tin>GAL4) led to a decreased cardiac output, an increased arrhythmia index and shortened diastolic diameter (S4B–S4D Fig). Interestingly, while the knockdown of hts did not cause any significant heart phenotypes (S4A–S4F Fig), the co-expression of hts<sup>RNAi</sup> and Gcn5<sup>RNAi</sup> significantly aggravated the heart period length and the arrhythmia index observed upon single knockdown of Gcn5 (S4A and S4C Fig). The silencing efficiency for both RNAi lines were confirmed by qPCR and immunocytochemistry (S5A–S5D Fig). Altogether, the results suggest that Gcn5 is important for heart function in Drosophila and that Hts deficiency can increase the phenotypic consequences of Gcn5 knockdown.

KAT2B F307S but not adducin-γ E659Q causes renal defects in Drosophila

The fly kidney is composed of garland and pericardial nephrocytes (Fig 6A) that perform the filtration of the hemolymph and Malpighian tubules that function as excretory tubes. The surface of nephrocytes is decorated with actin-anchored slit diaphragms showing high molecular similarity with those of mammalian podocytes [19–21]. Therefore, nephrocytes have successfully been used to functionally validate candidate genes for SRNS [22, 23].

By immunostaining, we observed that endogenous Hts localizes below the slit diaphragms at the cell cortex of larval garland nephrocytes (Fig 6B). A similar localization pattern was
found when adducin-γ was overexpressed with its binding partner adducin-α. In this case, adducin-γ protein levels were significantly increased compared to expressing adducin-γ alone, suggesting that the stabilization by adducin-α is a prerequisite for proper function (S6 Fig).

For Gcn5, we found a prominent expression in nephrocyte and podocyte nuclei (Fig 6C). The endogenous localization patterns were specific as they were lost upon hts and Gcn5 knockdown, respectively (S5A and S5B Fig).

To study the requirements of Hts for the integrity of the slit diaphragm, we performed immunostainings for Kirre, the ortholog of the mammalian slit diaphragm protein Neph1 [24]. In line with the proposed role for adducin-γ in cortical actin regulation [25], we found that hts null larval garland nephrocytes showed a decrease of Kirre between adjacent nephrocytes (Fig 7A and 7B). When rescued with adducin-αγ WT and E659Q transgenes, however, no major differences with respect to Kirre localization were seen in larval garland nephrocytes.
Similarly, the morphology and number of adult pericardial nephrocytes were normal in both rescue animals compared to the control (Fig 7C and 7D). Gcn5E333st hemizygous larvae presented with morphologically normal nephrocytes. Yet, we did detect a decreased H3K9 acetylation at this stage in the nephrocyte nuclei of the mutant, which could be rescued by Gcn5 WT but not by Gcn5 F304S (Fig 8A). Moreover, the majority of adult Gcn5 F304S escapers showed mislocalized and/or abnormally shaped pericardial nephrocytes in the adult stage that were often reduced in number (Fig 8B–8D), consistent with previously reported characterizations of important podocyte genes [26–28].

Together, the results demonstrate that, while Hts is important for nephrocyte function, the ADD3 missense mutation identified in family A is alone insufficient to cause a renal phenotype in flies. By contrast, Gcn5 F304S seems to impair Gcn5 function in nephrocytes.

Synergistic effects of hts and Gcn5 in Drosophila nephrocytes

To address any functional synergism between Gcn5 and Hts in nephrocytes, we performed nephrocyte-specific silencing of Gcn5 and hts alone or in combination. In larval nephrocytes,
the double knockdown of Gcn5 and hts caused an increase in Kirre mislocalization, compared to the single knockdowns (S7A and S7B Fig). Moreover, while in 3-day-old adults hts knockdown did not affect pericardial nephrocyte number (Fig 9A–9C), a significant decline of differentiated nephrocytes could be observed in 15-day-old adults (Fig 9D). Similarly, the nephrocyte-specific expression of Gcn5 RNAi caused a progressive decline of differentiated pericardial nephrocytes at 15 days, but not at 3 days post-eclosion (Fig 9A–9D). By contrast, the double knockdown of Gcn5 and hts caused a significant loss of differentiated nephrocytes already at 3 days post-eclosion (Fig 9A–9C).

Making use of the double knockdown phenotype, we also addressed the synergism between ADD3 and KAT2B mutations from family A. Considering that the KAT2B variant corresponds to an almost complete loss of function mutation, we performed a double knockdown of Gcn5 and hts rescued only with the adducin-αγ transgenes, thereby avoiding the complexity of bringing all the Gcn5 and hts alleles as well as the GAL4 driver and respective rescue constructs together in one fly. In this setting, the adducin-αγ WT combination partially rescued the loss of pericardial nephrocytes in 3-day-old adult flies (Fig 10A and 10B). By contrast the expression of the adducin-αγ E659Q combination showed the same degree of nephrocyte loss as the double knockdown at this stage. Together, these results suggest a functional interaction between KAT2B and ADD3 mutations in the nephrocyte, which may be of relevance for the renal phenotypes in family A.

Synergistic effects of adducin-γ and KAT2B in human podocytes
To validate our findings in human cells, we studied adducin-γ and KAT2B in cultured human podocytes. While KAT2B was as expected found in nuclei of podocytes, adducin-γ localized to
Fig 7. Garland nephrocyte phenotype of hts<sup>null</sup> and adducin-αγ rescue mutants. (A) Kirre and Pyd localization in hts<sup>null</sup> and rescue mutant garland nephrocytes. Dissected nephrocytes of the indicated genotypes were stained for Kirre (red) and Pyd, corresponding to Neph1 and ZO-1 in vertebrates, (blue). Arrowheads show areas of cell fusion. Scale bar: 10 μm. (B) Quantification of nephrocytes showing a continuous Kirre staining using >9 samples/genotype from 3 independent experiments. Statistical analysis was performed with Kruskal-Wallis with Dunn’s post-test. ns, non significant, *p<0.05, ***p<0.001 (see S1 Table for details on transgenic flies). (C) Pericardial nephrocytes in adducin-αγ WT and E559Q rescue and control adult flies at 15 days post-eclosion were stained for the differentiation markers Kirre (red) and Pyd (blue). Note that hts<sup>null</sup> is lethal at this stage. Scale bar: 30 μm. (D) Quantification of the number of pericardial nephrocytes from n>8 samples/genotype in 3 independent experiments. Statistical analysis was performed using one-way ANOVA with Bonferroni’s post-test. ns, non significant (See S1 Table for details on transgenic flies).

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Fig 8. Effect of Gcn5/KAT2B variant on histone acetylation and survival of Drosophila nephrocytes. (A) Acetylated H3K9 in larval garland nephrocytes of Gcn5<sup>null</sup> and Gcn5 WT and mutant rescue animals. Dorothy (Dot)-GAL4 (a nephrocyte specific driver) is used in combination with da-GAL4 as the latter shows only minor expression in nephrocytes. Garland nephrocytes of the indicated genotypes were stained for acetylated H3K9 (red) and Hoechst (blue). Scale bar: 5 μm. (B) Pericardial nephrocytes in adult Gcn5 rescue mutant flies (7–15 days after eclosion) that express transgenic GFP (green) driven under the Hand promoter (Hand-GFP), specific for nephrocytes and cardiomyoblasts. Dissected pericardial nephrocytes were fixed with PFA and observed directly for GFP signal. Scale bar: 30 μm. (C) Pericardial nephrocytes in adult Gcn5 rescue mutants (7–15 days after eclosion). Dissected pericardial nephrocytes of the indicated genotypes were stained for the differentiation markers Kirre (red) and Pyd (blue). Scale bar: 30 μm. (D) Quantification of the pericardial nephrocyte defects found in Gcn5<sup>null</sup> rescue mutants (n>13/genotype; 3 independent experiments; Chi-square test). Nephrocytes with abnormal phenotypes included nephrocytes with abnormal distribution, abnormal shape, multinucleated or fragmented nuclei and reduced number of nephrocytes (<20). Phenotype severity was scored as normal (0), medium (1), intermediate (2) and severe (>2). For all panels: ns, non significant, ** p<0.01, *** p<0.001 (see S1 Table for details on transgenic flies).

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the cell periphery similar as in nephrocytes (S8A–S8D Fig). Both localization patterns were specific, as they were reduced upon lentiviral transduction of respective shRNAs and could be restored by re-expression of both wild-type and even mutant adducin-γ and KAT2B (S8A–S8D Fig).

Fig 9. Number of pericardial nephrocytes in single and double knockdown of hts and Gcn5. (A–D) Dot-GAL4-mediated knockdown of hts or/and Gcn5 in pericardial nephrocytes. Pericardial nephrocytes of adult flies with the Hand-GFP background were observed directly for GFP signal after fixation (A). Immunostaining was performed for the differentiation markers Kirre (red) and Pyd (blue; B). Images are representative of pericardial nephrocytes dissected from adult flies at 3 days post-eclosion. Scale bars: 30 μm. Graphs represent quantification of the number of pericardial nephrocytes at 3 days (C) and 15 days post-eclosion (D) using >15 samples/genotype from 3 independent experiments. Statistical analysis was performed with Kruskal Wallis with Dunn’s post-test. For all panels: ns, non significant, **p<0.001 (See SI Table for details on transgenic flies).

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To address the phenotypic effects of the ADD3 and KAT2B knockdowns, we analyzed adhesion and migration, which are processes typically affected in kidney diseases such as SRNS [29, 30]. While podocyte adhesion was reduced in the single ADD3 and KAT2B knockdowns, the double knockdown showed additive effects (Fig 10C). With regard to migration, the single
knockdown showed a mildly increased migration. By contrast, the double knockdown led to a strongly impaired migration (Fig 10D), providing further evidence for potential synergistic effects of mutations in both genes in the kidney.

Discussion

Here, we identify ADD3 mutations in three different families with similar neurological, skeletal and ophthalmological phenotypes, thereby consolidating and expanding the mutational and phenotypic spectrum of ADD3 deficiency reported initially. Moreover, we use functional validation in Drosophila and human cells to characterize the contribution of an additional variant in KAT2B to the extended phenotype featuring one of the ADD3 families. While such additional variants are commonly excluded from WES datasets without even performing functional validation, particularly when the respective gene functions seem to be unrelated to the disease(s) in question, our results demonstrate that both the ADD3 and the KAT2B mutation could be pathogenic. Moreover, we show that ADD3-associated phenotypes can be unmasked by additional Gcn5/KAT2B deficiency in nephrocytes and human podocytes. Our study thus provides an example of a genetic disease where the tissue manifestation could be influenced by a second homozygous mutation on another chromosome.

KAT2B has previously not been associated with any genetic disease. The severity of the KAT2B variation in the fly model compared with the relatively late-onset cardiac and renal defects in the patients indeed suggests a partial functional redundancy due to gene duplication in vertebrates. Accordingly, it has been shown that in mouse development loss of KAT2B can be compensated for by KAT2A [31]. Nevertheless, KAT2B is strongly expressed in mouse heart and kidney, particularly podocytes [32, 33], while KAT2A has a more widespread expression pattern [14]. Moreover, mouse and zebrafish studies have shown that KAT2B can perform functions that are non-redundant with KAT2A, even in the heart [34–39], indicating that KAT2B deficiency alone could be sufficient for any clinical manifestations. What remains to be seen is whether the clinical impact of KAT2B deficiency needs to be uncovered by a sensitized genetic background, such as the ADD3 mutation, or whether it is the other way around. While our data do not exclude either possibility, it is interesting that rare but otherwise uncharacterized variants of KAT2B have been found to be enriched in a patient cohort with sporadic FSGS [32], suggesting that KAT2B could be a susceptibility factor for FSGS forms with different primary causes.

At the level of protein function and disease mechanism, the precise mode of interaction between adducin-γ and KAT2B is equally unclear. Apart from histones, KAT2B has recently been shown to acetylate a variety of proteins [40]. Among them are also cytoskeletal regulators, that when mutated cause FSGS or cardiomyopathy (e.g. actinin-4, TTC21B and myosin-7). Thus, it is possible that adducin-γ could also be a target of KAT2B-dependent acetylation. Vice versa, any influence of adducin-γ deficiency on the activity of KAT2B cannot be excluded. Apart from more mechanistic functional studies, the identification of more patients with the same or other variants in only ADD3 or KAT2B combined with careful characterization of their phenotypes will be crucial to define the precise role of each gene and their potential functional interaction in humans.

Materials and methods

Ethical statement

Following informed written consent, we obtained clinical data, blood samples and skin biopsies from the affected individuals. This study was conducted with the approval of the Comité
Whole exome sequencing

Whole-exome sequencing (WES) was performed for affected individuals II-3 and II-6 from family A and for the two parents and the affected sib from family B and family C. Whole-exome capture was performed with the Agilent SureSelect Human All Exon Kit, 51Mb, V4 (family A), the Roche MedExome kit (family B) or a proprietary system from GeneDx (family C). The enriched library was then sequenced on either Life Technologies SOLID (paired end with 75+35 base pair (bp) reads; family A) or Illumina systems (family B: 2x150 bp reads; family C: 2x100bp read). Images were analyzed and the bases were determined according to Lifescope or bcl2fastq Conversion Software v2.17.Variants were called as described [41].

Fly strains and generation of transgenic flies

Crosses were maintained on standard cornmeal-yeast food at 25˚C except for RNAi crosses (29˚C). The fly stocks were used in this study can be found in S1 Table. For Hts null rescue constructs we used an N-terminal V5 tagged human ADD3 (clone IMAGE: 6649991), WT or carrying the E659Q mutation, and the N-terminal HA tagged human ADD1 (gift from Vann Bennett, Duke University). For Gen5 null rescue constructs we used the C-terminal HA tagged human KAT2B (clone IMAGE: 30333414), the N-terminal Flag tagged human KAT2A (gift from Laszlo Tora, Institut de Gene bique et de Biologie Moleculaire et Cellulaire, Strasbourg) and the C-terminal Flag tagged Drosophila Gcn5, WT (gift from Clement Carrè, University Pierre et Marie Curie) or carrying the mutations F304S or S478F (corresponding to human mutation F307S and S502F). All mutations were inserted using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol. Subsequently, the rescue constructs were subcloned into a pUASTattB vector (gift from Konrad Basler, University of Zurich) and injected into flies at attP landing sites by Bestgene, USA.

Cell culture

A conditionally immortalized human podocyte cell line developed by transfection with the temperature-sensitive mutant (tsA58) of the SV40-T-antigen-encoding gene, was kindly provided by Dr. Saleem (University of Bristol). In brief, the cells proliferated at the permissive temperature of 33˚C, whereas growth arrest and differentiation were induced by incubation at the nonpermissive temperature of 37˚C for 14 days. Cells were grown with 7% CO2 in RPMI 1640 medium supplemented with 10% fetal bovine serum, insulin-transferrin-selenium, glutamine, penicillin and streptomycin (all from Life Technologies).

Primary skin fibroblasts were obtained from individual II-3 and II-6 from family A and two different age matched controls. These cells were grown in OPTIMEM medium supplemented with 20% fetal bovine serum, glutamine, penicillin and streptomycin (all from Life Technologies) at 37˚C with 7% CO2.

Establishment of lentiviral cell lines

Small hairpin RNAs (shRNAs) Scramble (Scb) or targeting the 3'UTR of human ADD3 and KAT2B mRNA in the lentiviral vector pLKO.1 were purchased from Sigma (ADD3 clone: NM_019903.3-2280s1c1 TRCN0000123024; KAT2B clone: NM_003884.4-3192s2c1, TRCN0000364135). Lentiviral particles containing these constructs were produced in human embryonic kidney 293T cells as previously described [42]. ShScb, ADD3 or KAT2B depleted
podocytes were obtained by transduction with the respective shRNAs lentiviral particles and subsequent puromycin selection.

Human ADD3 and KAT2B, were subcloned from human full-length cDNA (ADD3: clone IMAGE: 6649991; KAT2B clone IMAGE: 3033341) into the expression vectors pLentiGIII and PLEX-MCS, respectively. An HA tag was added in frame, before the stop codon, to the C terminus of ADD3 and KAT2B. The ADD3 E659Q and KAT2B F307S mutations found in affected individuals were introduced with the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. All constructs were verified by sequencing. ADD3 or KAT2B depleted podocytes were transduced with WT or mutant ADD3 or KAT2B lentiviral particles, respectively.

RNA extraction, RT-PCR and real time quantification

Total RNA was isolated using a Qiagen RNA extraction kit (Qiagen), following the manufacturer's instructions. cDNA was prepared using reverse transcriptase Superscript II (Invitrogen). PCR was performed using ReadyMix Taq PCR (Sigma). After RNA extraction and cDNA preparation by RT-PCR, relative expression levels of genes of interest were determined by real-time PCR using the Absolute SYBR Green ROX Mix (ABgene) and specific primers as follows: ADD3 forward 5'-CTTCTGGAATTGTTGTGATAAG-3' and reverse 5'-CTGGTGCGGCATGATCTC3'; KAT2B forward 5'-ATCACACGGCTGCTTTGAC3' and reverse 5'-CACCAATAACACCCCATCTT-3'; hts forward 5'-ATCACACGGCTGCTTTGAC3' and reverse 5'-CAGGGTGGCCATGATCTC3'; Gcn5 forward 5'-CGATCGTCCAAGCAGTGAG-3' and reverse 5'-TCCGCCTTGACTCTTC3'. Experiments were repeated at least three times and gene expression levels were normalized to human HPRT or Drosophila melanogaster actin.

Immunoblotting

Total cell or third instar larvae total protein extractions were performed and the resolved proteins were probed using the primary antibodies: anti-PCAF rabbit monoclonal (3378, Cell Signaling, 1:1000), anti-adducin-γ mouse monoclonal (sc-74474, Santa Cruz, 1:1000) and anti-α tubulin mouse monoclonal (T5168, Sigma Aldrich, 1:5000). For immunoblotting of nuclear extracts [43], the primary antibodies anti-acH3K9 rabbit polyclonal (39918, Active motif, 1:1000) and anti-H3 mouse monoclonal (61475, Active motif, 1:1000) were used as well as the corresponding HRP-conjugated secondary antibodies (Amersham ECL, GE healthcare and Invitrogen). Bands were visualized using Amersham ECL Western Blotting Detection Reagent (GE Healthcare) and quantified by densitometry using Image J software.

Immunofluorescence

Fibroblasts or podocytes were plated on noncoated coverslips or coverslips coated with rat-tail collagen type I (Corning), respectively. After 48h of culture cells were fixed with 100% ice-cold ethanol. Cells were incubated with a blocking solution (PBS, 1% BSA, and 0.1% tween 20) and further permeabilized for ten minutes with PBS 0.1% Triton. Incubation with the following primary antibodies was done ON at 4˚C: anti-PCAF mouse monoclonal (sc-13124, Santa Cruz, 1:100), anti-adducinγ rabbit polyclonal (sc-25733, Santa Cruz, 1:100) and anti-HA (11867 423 001, Roche, 1:200).

For immunofluorescence in Drosophila, garland and pericardial nephrocytes were dissected from third instar larvae and adults, respectively, and fixed for 20 minutes in 4% paraformaldehyde at room temperature and stained according to standard procedures. For Kirre stainings an alternative fixation method ("heat fixation") was used: nephrocytes were heat-fixed for 5 seconds at 90 ˚C in 0.7% NaCl/0.05% TX-100 solution. The following primary antibodies were

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used: anti-Hts mouse monoclonal (#1B1 deposited to the Developmental Studies Hybridoma Bank (DSHB) by Lipshitz, H.D.), anti-Gcn5 rabbit polyclonal (gift from Jerry Workman, Stowers Institute for Medical Research, Kansas, 1:200) anti-Kirre rabbit polyclonal (gift from Karl Fischbach, Institute for Biology, Freiburg, Germany, 1:200), anti-Pyd2 mouse monoclonal (deposited to the DSHB by Fanning, A.S, 1:100), anti-acH3K9 rabbit polyclonal (#06-942, Upstate, 1:100), AlexaFluor488-conjugated anti-horseradish peroxidase (Jackson Immunoresearch, 1:400), anti-HA (11 867 423 001, Roche, 1:200) and anti-V5 rabbit polyclonal (v8137 Sigma, 1:200). The corresponding anti-isotype AlexaFluor antibodies (ThermoFisher Scientific, 1:200) were used at room temperature for 2 hours. Nuclei were stained with Hoechst. Confocal images were obtained with a Leica TCS-SP8 confocal microscope, and post-treatment analysis was performed with Image J software.

### Statistical analyses

Results are presented as means ± standard error or standard deviation for the indicated number of experiments. Statistical analysis of continuous data was performed with two-tailed Student t test for pairwise comparisons or one-way analysis of variance for comparisons involving three or more groups, with Dunnet’s, Bonferroni or Dunn post hoc test, as appropriate. Pearson’s chi-squared test was used for analysis of categorical data. Linear relations between variables were analysed using linear regression analysis. P<0.05 was considered statistically significant. Analysis was carried out with GraphPad Prism software. (’p<0.05; **p<0.01, ***p<0.001, ****p<0.0001). All experiments were performed at least three times.

### Supporting information

#### S1 Fig. mRNA levels and subcellular localization of ADD3 and KAT2B in control and patient fibroblasts.

(A, B) ADD3 (A) and KAT2B (B) mRNA levels in patient fibroblasts were assessed by quantitative PCR. Experiments were repeated at least three times and gene expression levels were normalized to the housekeeping gene HPRT. Statistical analysis was performed using student’s t-test; ns, non-significant. (C, D) Immunostaining was performed for adducin-γ (green; C) and KAT2B (green; D) in control and patient fibroblasts. Nuclei were stained with Hoechst (blue). Note the loss of nuclear staining for KAT2B in patient fibroblasts. Scale bars: 10 μm.

(TIF)

#### S2 Fig. Morphology of htsnull flies and adducin-αγ WT or E659Q rescue mutants.

Representative pictures of htsnull and respective adducin-αγ WT and E659Q rescue mutants one day post-eclosion. htsnull flies have rough eye and motor coordination defects and are unable to fly. The ubiquitous co-expression of adducin-α and -γ using tub-GAL4 rescues these defects regardless of the presence of the E659Q mutation. Scale bars: upper panel: 1mm, wings: 500μm, eye: 200μm.

(TIF)

#### S3 Fig. Rescue experiments with alternative drivers.

(A) Rescue of hts mutant viability defects with adducin-αγ transgenes driven by a GAL4 insertion in the endogenous hts locus. After 48h of egg laying on standard cornmeal/yeast food, viability was calculated as the percentage of eclosing adults of the indicated genotype and normalized to the control. The control corresponds to the viable F1 trans-heterozygous flies obtained from the cross between Df(2R) BSC26 (harbouring the hts gene) and a non-overlapping deficiency on the same chromosome (Df(2R)247). The insertion of GAL4 in hts locus leads to partial lethality which is completely rescued by adducin-αγ WT but not by E659Q. Quantification is for >100 F1 eclosing flies/
genotype/experiment in >5 independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett’s post-test. (B) Viability for Gcn5null hemizygous flies and respective rescues using tubulin-GAL4 (tub>). Viability was assessed as described in (A). The control corresponds to the viable F1 trans-heterozygous flies obtained from the cross between Df(3L)sex204 (harbouring the Gcn5 gene) and a non-overlapping lethal mutant on the same chromosome (CG310gRM0010). Quantification is for >100 F1 eclosing flies/genotype/experiment in >5 independent experiments. Statistical analysis was performed using one-way ANOVA with Bonferroni post-test. For all panels: ns, non significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

S4 Fig. Effect of cardiac-specific depletion of hts and Gcn5 on Drosophila heart function. (A-F) Tin-GAL4 driver was used to knockdown hts or/and Gcn5 in cardiomyocytes, and different heart parameters were analyzed in 3 week-old adult flies. Two separate control RNAi lines (TRIP and KK) were used to match Gcn5RNAi (TRIP) and htsRNAi (KK), respectively. For quantification, 19–30 flies were analyzed. Statistical analysis was performed using one-way ANOVA and Tukey’s multiple comparison for all parameters except arrhythmia index, which was analysed using Mann-Whitney-Wilcoxon. For all panels: ns, non significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

S5 Fig. Hts and Gcn5 RNAi validation in Drosophila. (A, B) Nephrocyte-specific knockdown of hts (A) and Gcn5 (C) in nephrocytes was performed using using prospero (pros)-GAL4. Dissected garland nephrocytes of the indicated genotypes (see also S1 Table) were stained for Hts (green; A) and Gcn5 (green; C). Nuclei were stained with Hoechst (blue). Scale bars: 10 μm. (B, D) Hts (B) and Gcn5 (D) RNAi knockdown validation was performed with tub-GAL4 and the fat body-specific lpp-GAL4, respectively. Note that the knockdown of Gcn5 with tub-GAL4 and da-GAL4 was lethal in the embryonic stage and thus could not be used for knockdown validation.

S6 Fig. Adducin-α and -γ co-expression in garland nephrocytes. The knockdown of hts and simultaneous re-expression of human HA-tagged adducin-α and V5-tagged adducin-γ was performed in garland nephrocytes with prospero (pros)-GAL4 (see S1 Table for details on transgenic flies). Dissected garland nephrocytes of the indicated genotypes were stained for HA (green) and V5 (red). Nuclei were stained with Hoechst (blue). Scale bar: 10 μm.

S7 Fig. Kirre localization in nephrocytes in single and double knockdown of hts and Gcn5. (A) Pros-GAL4-mediated knockdown of hts and/or Gcn5 in garland nephrocytes. Dissected garland nephrocytes of the indicated genotypes were stained for Kirre (red) and Pyd (blue). Scale bar: 10μm. (B) Quantification of nephrocytes showing a continuous Kirre staining using >12 samples/genotype in 3 independent experiments. Statistical analysis was performed with Kruskal Wallis with Dunn’s post-test.

S8 Fig. Expression and subcellular localization of adducin-γ and KAT2B in podocytes. (A) Cell lysates from undifferentiated podocytes were analysed by western blotting using anti-adducin-γ. Anti-α-tubulin was used as a loading control. (B) Differentiated podocytes were stained for adducin-γ (green), HA (magenta) and DNA (blue). (C) Undifferentiated podocyte cell lysates were analysed by western blotting using the anti-KAT2B. Anti-α-tubulin was used
as a loading control. (D) Differentiated podocytes were stained for KAT2B (green) and HA (red). Scale bars: 20 μm.

**S1 Table. Fly strains used in this study.**

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KAT2B is a susceptibility gene for SRNS and cardiomyopathy


