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REVIEW

Modeling Pathogenic Variants in the RNA Exosome

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Exosomopathies are a collection of rare diseases caused by mutations in genes that encode structural subunits of the RNA exosome complex (EXOSC). The RNA exosome is critical for both processing and degrading many RNA targets. Mutations in individual RNA exosome subunit genes (termed EXOSC genes) are linked to a variety of distinct diseases**.** These exosomopathies do not arise from homozygous loss-of-function or large deletions in the EXOSC genes likely because some level of RNA exosome activity is essential for viability. Thus, all patients described so far have at least one allele with a missense mutation encoding an RNA exosome subunit with a single pathogenic amino acid change linked to disease. Understanding how these changes lead to the disparate clinical presentations that have been reported for this class of diseases necessitates investigation of how individual pathogenic missense variants alter RNA exosome function. Such studies will require access to patient samples, a challenge for these very rare diseases, coupled with modeling the patient variants. Here, we highlight five recent studies that model pathogenic variants in EXOSC3, EXOSC2, and EXOSC5.

Keywords: RNA exosome; exosomopathies; EXOSC3; EXOSC2; EXOSC5; Pontocerebellar Hypoplasia

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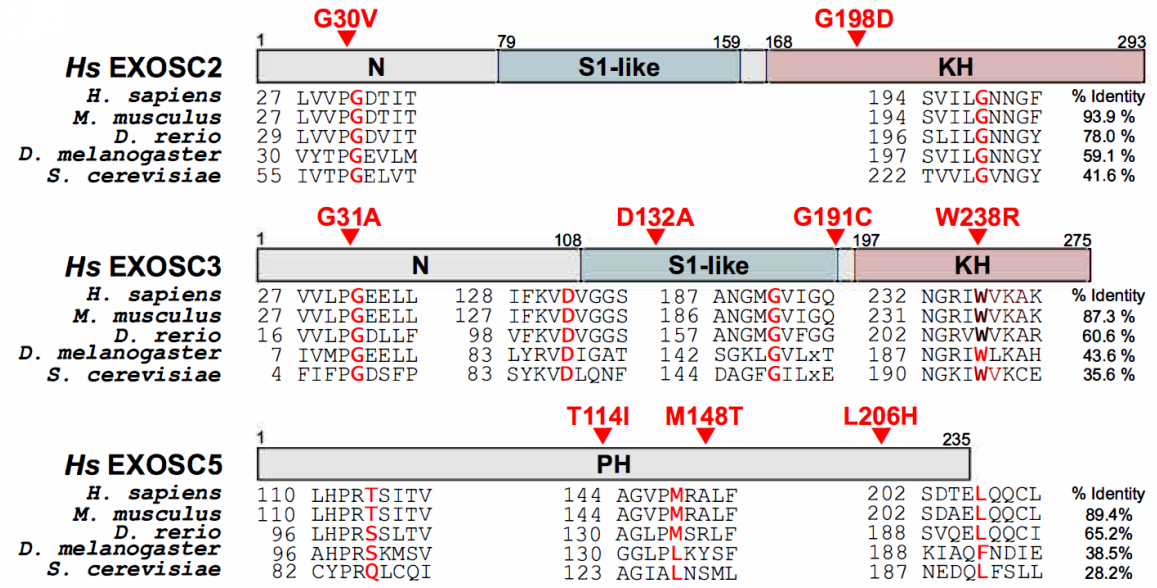
Abbreviations: RNA, ribonucleic acid; EXOSC, exosome component; PCH, pontocerebellar hypoplasia; SHRF, short stature, hair loss, retinitis pigmentosa, distinct facies; rRNA, ribosomal RNA; snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; tRNA, transfer RNA; CUTs, cryptic unstable transcripts; PROMPTs, promoter upstream transcripts; mRNA, messenger RNA; CRISPR, clustered regularly interspaced short palindromic repeats.

Introduction

The RNA exosome is a 10-subunit complex responsible for essential RNA processing and degradation in both the cytoplasm and the nucleus (Figure 1). The ribonuclease activity of the RNA exosome is critical for both RNA quality control and precise processing of key RNAs, including ribosomal RNA (rRNA) [1]. As shown in Figure 1, the 10 subunits of this complex are organized into a non-catalytic cap composed of three subunits (EXOSC1-3), a barrel-shaped non-catalytic core composed of six subunits (EXOSC4-9), and one catalytic 3′-5′ exo/endoribonuclease subunit that sits at the base of the core (DIS3) [2-6]. Most target RNAs are threaded through the cap and the central

A



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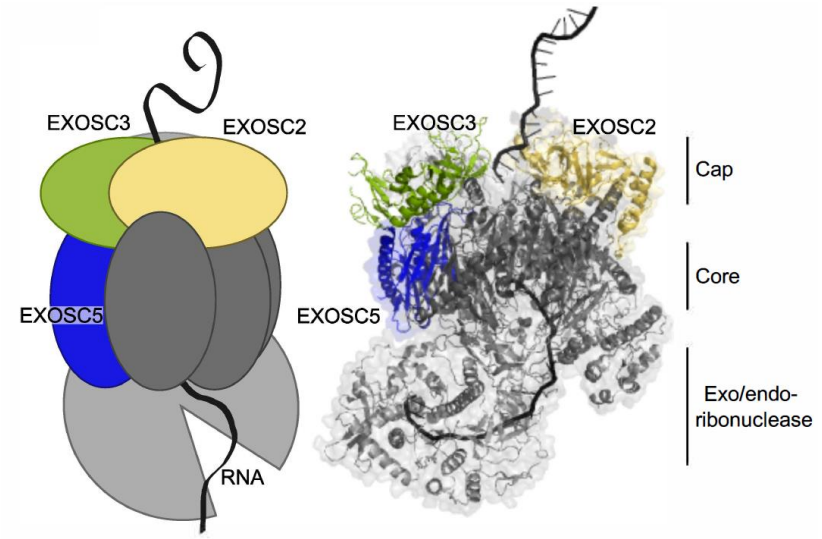

Figure 1. Pathogenic missense variants in structural subunits of the RNA exosome cause human disease with diverse clinical presentations. A) The domain structures of EXOSC2, EXOSC3, and EXOSC5 are shown. Sequence alignments of human (*H. sapiens*), mouse (*M. musculus*), zebrafish (*D. rerio*), fruit fly (*D. melanogaster*), and yeast (*S. cerevisiae*) orthologs are depicted below the structures to highlight the conserved residues altered in disease and the flanking conserved regions. Numbers preceding the sequences indicate the amino acid position and the red arrows above the domain structures indicate the approximate location of the respective amino acid substitution. The overall percent identity of the EXOSC orthologs compared to human EXOSC proteins is shown to the right of the sequence alignments. **B)** A cartoon rendering of the human RNA exosome is shown on the left and a structural model of the complex (PDB #6D6Q) is displayed on the right. The cap (EXOSC1-3), the core (EXOSC4-9), and the catalytic exo/endoribonuclease (DIS3) are labeled and the EXOSC2 (yellow), EXOSC3 (green), and EXOSC5 (blue) subunits altered in disease are highlighted.

channel of the barrel to reach DIS3 for processing and/or degradation [7, 8]. The RNA exosome is evolutionarily conserved, and all subunits analyzed are essential in any model organisms where studies have been performed [9-16].

Genes encoding subunits of the RNA exosome complex were initially discovered in a genetic screen for rRNA processing mutants in budding yeast [1, 17]. Studies in *S.*

cerevisiae demonstrate that 1) each subunit is essential for survival, 2) the RNA exosome processes and degrades target transcripts in a 3'-5' orientation, and 3) conditional mutations in genes encoding the RNA exosome subunits impair RNA metabolism [1, 13, 17, 18]. Early structures of the RNA exosome from a number of organisms provided key insight into how this complex could process and decay RNA [19-21]. Since then, a number of both structural and *in vitro* biochemical studies have been employed to understand the many functions of this critical complex [22, 23]. The RNA exosome regulates/processes several classes of RNAs in different cellular compartments [24-27]. In the nucleolus, RNA exosome-mediated processing is essential for the production of mature rRNA [24]. Within the nucleus, this complex also processes and/or degrades small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), tRNAs, cryptic unstable transcripts (CUTs) in yeast, and promoter-upstream transcripts (PROMPTs) in mammals [18, 25, 28-30]. In the cytoplasm, key targets include normal mRNAs in the turnover pathway and aberrant mRNA transcripts, such as those lacking a stop codon, in quality control pathways [31]. In addition, the RNA exosome regulates the levels of a variety of different transcripts [2, 24, 25]. To recognize and process/degrade distinct targets, the RNA exosome interacts with cofactors, proteins that associate with the complex [2]. Several RNA exosome cofactors that serve as RNA helicases, scaffolds, additional ribonucleases, and polyadenylases have been described [2, 5-7, 28, 32-35]. Cofactors have been primarily characterized in budding yeast, but more recent studies have identified mammalian cofactors [2, 27, 36, 37], providing fundamental insights into RNA exosome specificity for processing and decay of target RNAs.

Pathogenic missense variants in subunit genes of the RNA exosome are linked to disease.

Although the function of the RNA exosome is essential [17, 38], a number of studies have now identified mutations in genes encoding structural subunits of this complex linked to diverse clinical presentations (Table 1). These diseases are termed *exosomopathies* [10]. The initial report linking RNA exosome genes to disease described several pathogenic variants in *EXOSC3* that cause pontocerebellar hypoplasia type 1b (PCH1b) [16]. Subsequent studies linked *EXOSC2* [39], *EXOSC8* [9], *EXOSC9* [10], and *EXOSC5* [40] to a variety of clinical presentations [41].

Although the clinical presentations of exosomopathies are variable, impacts on the cerebellum are a common feature. In four of the five exosomopathies described to date, patients present with abnormal development of the cerebellum (cerebellar hypoplasia or pontocerebellar hypoplasia) or degeneration of the cerebellum. The cerebellar pathology is quite diverse and is typically associated with additional clinical manifestations [41]. Mutations in *EXOSC3* give rise to PCH1b, a disease characterized by atrophy of the cerebellum and the pons [16, 42]. *EXOSC8* mutations cause pontocerebellar hypoplasia type 1 c (PCH1c), characterized by hypomyelination with spinal muscular atrophy and cerebellar hypoplasia [9]. *EXOSC9* mutations give rise to pontocerebellar hypoplasia type 1 d (PCH1d), a spinal motor neuronopathy coupled with cerebellar atrophy [10, 43]. While only a few patients with mutations in *EXOSC5* have been described, these patients also show cerebellar abnormality as a common clinical feature [40]. In contrast to the other *EXOSC* mutations, pathogenic variants in *EXOSC2* only cause mild/borderline cerebellar atrophy and patients present with short stature, hearing loss, retinitis pigmentosa, and distinctive facies (denoted as SHRF) [39]. Whether the cerebellar hypoplasia and atrophy observed in the exosomopathies are part of a clinical spectrum that results from the same pathological process and molecular mechanism or distinct manifestations is still unknown.

Little is understood about why these pathogenic missense variants in genes encoding structural subunits of an essential complex that is ubiquitously expressed give rise to a broad range of clinical presentations. All patients with exosomopathies described thus far have at least one missense variant in an *EXOSC* gene (Table 1) [41]. Some patients are homozygous for the same missense variant, others are compound heterozygous for different missense variants, and some patients have a missense variant inherited *in trans* to a deletion or loss-of-function variant. The complete loss of the RNA exosome is lethal [9-16]; therefore, the missense variants likely provide residual RNA exosome function in all patients. These specific variants in different *EXOSC* genes may underlie the disparate clinical presentations of patients. The pathogenic amino acid changes could alter the function of individual subunits or the integrity of the RNA exosome complex, ultimately affecting downstream RNA targets.

Studies to define the molecular mechanisms underlying pathology in exosomopathies have used several approaches: 1) immortalized patient cells, 2) deletion or depletion of the affected *EXOSC* gene, and 3) modeling of the pathogenic missense variants in either model genetic systems or cultured cells. Ultimately, understanding how defects in RNA exosome function contribute to disease pathology will require studies investigating how the pathogenic amino acid substitutions impact the function of the complex. At this time, *in vivo* studies that model missense mutations to understand how disease-linked amino acid changes could alter RNA exosome function have employed budding yeast and *Drosophila*

melanogaster. Here, we highlight recent

Table 1. RNA Exosomopathy Pathogenic Missense Variant Models

Structural subunit gene	Genotype[i]	Pathogenic variants	Exosomopathy	Pathogenic Variant Models
EXOSC1				
EXOSC2	Homozygous	G30V	SHRF [39][ii]	
	Heterozygous	G30V/G198D	SHRF	[iii]
EXOSC3	Homozygous	D132A	PCH1b [16, 54]	
	Homozygous	G31A	PCH1b	
	Homozygous	G191C	PCH1b	
	Heterozygous	G31A/W238R	PCH1b	[iv] [v]
	Heterozygous	D132A/del[vi]	PCH1b	
EXOSC4				
EXOSC5	Homozygous	L206H	Novel exosomopathy [40]	
	Homozygous	M148T	Novel exosomopathy	
	Heterozygous	T114I/del[vi]	Novel exosomopathy	
EXOSC6				
EXOSC7				
EXOSC8	Homozygous	S272T	PCH1c [9]	
	Homozygous	A2V	PCH1c	
EXOSC9	Homozygous	L14P	PCH1d [46]	

D. melanogaster
S. cerevisiae
Cultured cell lines
Patient samples/cultured patient cells

[i]Homozygous or compound heterozygous
[ii]Short stature, hair loss, retinitis pigmentosa, distinct facies (SHRF)
[iii]HEK293T cells were transfected with either the EXOSC2 G30V or the EXOSC2 G198D variant.
[iv]Haploid budding yeast either expressed the yeast variant corresponding to EXOSC3 G31A or EXOSC3 W238R.
[v]Neuro2A cells were transfected with the variant corresponding to either EXOSC3 G31A or EXOSC3 W238R.
[vi]Genetic deletion

studies that model pathogenic missense variants in *EXOSC3* [11, 12, 14], *EXOSC2* [15] and *EXOSC5* [40].

EXOSC3 mutations impair RNA exosome function and organism viability.

Initial studies to explore the functional consequences of pathogenic variants in *EXOSC3* employed the budding yeast model system [11, 12]. The first observation from these studies is that pathogenic variants in *EXOSC3* modeled in yeast did not severely impact yeast cell growth or viability. This result is perhaps not surprising as each subunit of the RNA exosome is essential in the systems where this has been tested [1, 9-16, 44]. The presumption is that changes that significantly impair the function of this essential complex, which might impart a growth defect in yeast cells, may not be compatible with the human developmental program.

Although severe growth defects were not observed, both these studies showed that the yeast EXOSC3 variant corresponding to EXOSC3 W238R (W195R in yeast EXOSC3) conferred a temperature-sensitive growth defect when expressed as the sole copy of yeast *EXOSC3* [11, 12]. Moreover, these cells showed a significant impact on RNA processing and degradation mediated by the RNA exosome.

Yeast *EXOSC3* variants corresponding to EXOSC3 G31A (G8A in yeast EXOSC3), G191C (G148C in yeast EXOSC3), and W238R (W195R in yeast EXOSC3), showed impaired rRNA processing with the most profound effects evident for the yeast W195R variant [11]. Fasken *et al.* also observed misprocessing and accumulation of several RNA exosome targets, including CUTs and pre-snRNA, in cells expressing the W195R variant as the sole copy of yeast EXOSC3 [12]. In contrast, no effect on cytoplasmic RNA exosome function was detected [12]. The yeast model studies thus indicate that the W195R variant impairs cell function and significantly hinders RNA processing, suggesting that the EXOSC3 W238R variant is quite deleterious. This allele has only been identified in the compound heterozygous state in patients [16, 42], raising the possibility that EXOSC3 W238R may not confer sufficient RNA exosome activity to support life as a homozygous variant.

To begin to address how pathogenic amino acid substitutions could impair RNA exosome function, one study compared both the steady-state levels and stability of the yeast EXOSC3 variants to the wild-type protein [12]. Results of this analysis demonstrated that the yeast W195R variant protein is unstable when expressed as the sole copy of the yeast EXOSC3 protein and becomes further destabilized under conditions where a wild-type copy of the subunit is also present. These results suggest that perhaps the pathogenic subunits are not as efficiently incorporated into the complex as the wild-type subunits, or once pathogenic subunits are incorporated, the complex is not as stable. These yeast studies were complemented by analyzing mouse EXOSC3 variant protein levels in cultured mouse neuronal cells. This analysis showed that the steady-state level of the mouse EXOSC3 protein modeling the W238R variant is reduced in these cells compared to wild-type EXOSC3 [12]. Thus, a decrease in overall complex level could contribute to pathology in exosomopathies, as suggested from analyses of other *EXOSC* variants, such as EXOSC9 [10]; however, it is difficult to reconcile the very diverse clinical presentations of these diseases with a simple loss of or decrease in overall complex function.

Beyond affecting protein levels, pathogenic amino acid substitutions could also alter key interactions with other RNA exosome subunits or with the associated cofactors. Indeed, one study demonstrated that the yeast EXOSC3 W195R variant corresponding to EXOSC3 W238R shows decreased affinity for a cofactor, Mpp6 (MPHOSPH6 in humans), compared to wild-type yeast EXOSC3 [6]. These results support a model where altered interactions with RNA exosome cofactors could contribute to disease pathology.

Studies modeling pathogenic variants in *EXOSC3* have been extended to *Drosophila*. This system enables the effects of the EXOSC3 variants on the nervous system and brain to be studied within a genetically tractable system. A previous study in *Drosophila* demonstrated that RNA exosome subunits are essential in flies [45], consistent with the results obtained in budding yeast [1]. In the present study, CRISPR/Cas9 genome editing was used to engineer pathogenic variants of *EXOSC3* into the *Drosophila* genome [14]. This is the first study that analyzes RNA exosome mutations recapitulated at the genome level in a multi-tissue organism. The study modeled three patient genotypes [16, 42]: homozygous G31A, homozygous D132A, and D132A over a deficiency to model patients heterozygous for the D132A pathogenic variant inherited *in trans* to a deletion in *EXOSC3*. Results of this analysis show a striking genotype-phenotype correlation with respect to fly viability, lifespan, and locomotor function. The pathogenic variants that are most severe in patients [46] correlate with those that cause the most striking phenotypes in flies.

These mutant *EXOSC3* flies show morphological defects in the mushroom body, the area of the fly brain that controls learning and memory [47], that also correlate with the severity of the different *EXOSC3* alleles modeled. Finally, RNA sequencing of the heads of these mutant flies revealed an increase in the steady-state levels of a number of important neuronal transcripts, a result that is consistent with the role of this complex in RNA decay. This study developed a multi-cellular model to explore the consequence of pathogenic RNA exosome variants and provided insight into target RNAs affected in this model [14].

SHRF-causing pathogenic variants in *EXOSC2*.

Mutations in *EXOSC2* give rise to a novel syndrome characterized by short stature, hair loss, retinitis pigmentosa, and distinctive facies (SHRF) [39]. A recent study combined analysis of patient samples, biochemical approaches, and studies in *Drosophila* to explore the functional consequences of pathogenic missense variants in *EXOSC2* [15]. Biochemical analyses of both patient cells and transfected cell lines demonstrated that the G198D variant, but not the G30V variant, affects EXOSC2 protein stability and interactions with other RNA exosome components. While these authors did not create a fly model of the pathogenic variants in *EXOSC2*, they did test whether the EXOSC2 G30V variant could rescue defects observed in the eye in rare "escapers" where fly *EXOSC2* was deleted. The eye defect was partially rescued when the human wild-type *EXOSC2* gene was expressed, but not the pathogenic variant, providing evidence that EXOSC2

G30V does not retain the function of wild-type EXOSC2 [15]. RNA-sequencing on patient samples identified several dysregulated autophagy pathway genes [15]. In addition, in patient-derived B-lymphoblast cells with mutations in *EXOSC2*, overall RNA exosome subunit abundance is reduced and EXOSC2 proteins are unstable [15]. These findings are consistent with other studies that show a decrease in RNA exosome subunit levels in patient-derived samples [10], but do not readily explain why patients with mutations in different *EXOSC* genes display such a variety of clinical presentations.

Novel *EXOSC5* mutations impair RNA exosome activity.

A recent study reported five patients with biallelic variants in the *EXOSC5* gene [40]. Three of the four patients who learned to walk demonstrated ataxia, and four of the five patients' brain imaging showed hypoplasia of the cerebellum or cerebellar vermis [40]. This study employed three approaches to examine the link between *EXOSC5* and disease pathology; two of these approaches modeled the pathogenic variants that have been identified in *EXOSC5*. The initial approach employed zebrafish to assess the requirement for EXOSC5 in neurodevelopment has also been employed for the analysis of *EXOSC3* [16], *EXOSC8* [9], and *EXOSC9* [10]. The CRISPR-Cas9 system was employed to generate an allele with predicted loss of *EXOSC5* function. Consistent with previous studies of other RNA exosome subunit genes, zebrafish lacking EXOSC5 showed profound growth, developmental, and brain morphology defects [40]. To extend this analysis and explore the functional consequences of pathogenic variants in *EXOSC5*, the missense variants identified in EXOSC5 (I114T, M148T, and L206H) were all modeled in budding yeast. Only the yeast EXOSC5 variant corresponding to EXOSC5 L206H (L191H in yeast EXOSC5) showed growth defects, manifested as temperature-sensitive growth. Immunoblotting showed no statistically significant change in yeast EXOSC5 protein levels for any of these variants. Consistent with the growth defect observed, the yeast variant corresponding to EXOSC5 L206H showed defects in U4 snRNA and 7S pre-rRNA processing [40]. Biochemical studies in cultured mouse neuronal cells were performed to explore the interactions of mouse EXOSC5 variants with other subunits of the RNA exosome. Interestingly, the EXOSC5 L206H and I114T variants showed a decreased interaction with multiple other subunits of the complex. No defect in interaction was detected for the EXOSC5 M148T raising the question of how this amino acid substitution contributes to pathology.

Conclusions and Future Perspectives

The model organisms used to study RNA exosome biology have shed light on the complicated cellular roles of this multi-functional protein complex. Thus far, pathogenic missense variants in *EXOSC* genes that encode RNA exosome subunits have been linked to diverse clinical presentations with the majority causing some degree of cerebellar hypoplasia and/or atrophy. These *EXOSC* variants appear to have tissue-specific consequences. An ideal system to further explore the consequences of these pathogenic variants would be in an affected patient tissue or cultured primary cells. These samples are difficult to obtain because the disease is rare with only small numbers of patients identified to date. The cerebellum consists largely of Purkinje and granule cells [48]; therefore, an ideal *in vitro* system would be a genome-edited cerebellar cell-type organoid because the three-dimensional shape allows for multiple cell types. *In vivo* studies in model organisms thus far have allowed for simple recapitulation of pathogenic variants. Budding yeast are advantageous because they are simple to use and multiple functions of the conserved RNA exosome complex can be readily assayed. However, yeast lack relevant cell or tissue types. *Drosophilae* are multi-tissue organisms with a complex nervous system and a brain, which have been used extensively to model human disease [49]. However, fly brain structures lack elements of the human brain and some neurons are not well conserved [50].

A mammalian system would provide further insight into how specific pathogenic amino acid variants alter the function of the RNA exosome and cause diverse biological changes that underlie pathology. Thus far, no whole organism mouse models for mutations in any RNA exosome gene have been described. One study employed an *ex vivo* approach to swap exons 2 and 3 within *EXOSC3* in B cells [51]. The authors exploited this *ex vivo* system to explore the downstream effects of the loss of this RNA exosome subunit in B cells and identify key RNA exosome targets in this cell type [51]. A transgenic mouse that uses genome editing to incorporate pathogenic missense variants of *EXOSC* genes into the genome would greatly advance research in this field. Currently, the Jackson Laboratory site [52] lists CRISPR-generated knockout mouse strains for *EXOSC1* and *EXOSC2*, but there are no reports of researchers attempting to derive or analyze these strains.

A proposed model suggests that depletion of a subunit in model organisms recapitulates the patient disease because immunoblots of patient fibroblasts, myoblasts, and skeletal muscle samples show an overall steady-state decrease in protein levels of the affected subunits and other subunits in the complex [9, 10]. However, this model is difficult to reconcile with patients that show variable clinical presentation. Some of the *EXOSC* pedigrees identified to

date also call into question whether the primary driver of pathology is a decrease in RNA exosome subunit or complex levels. Several of the pedigrees for patients include seemingly unaffected parents with presumed loss of function of one *EXOSC* allele [16, 40, 41, 46]. Consistent

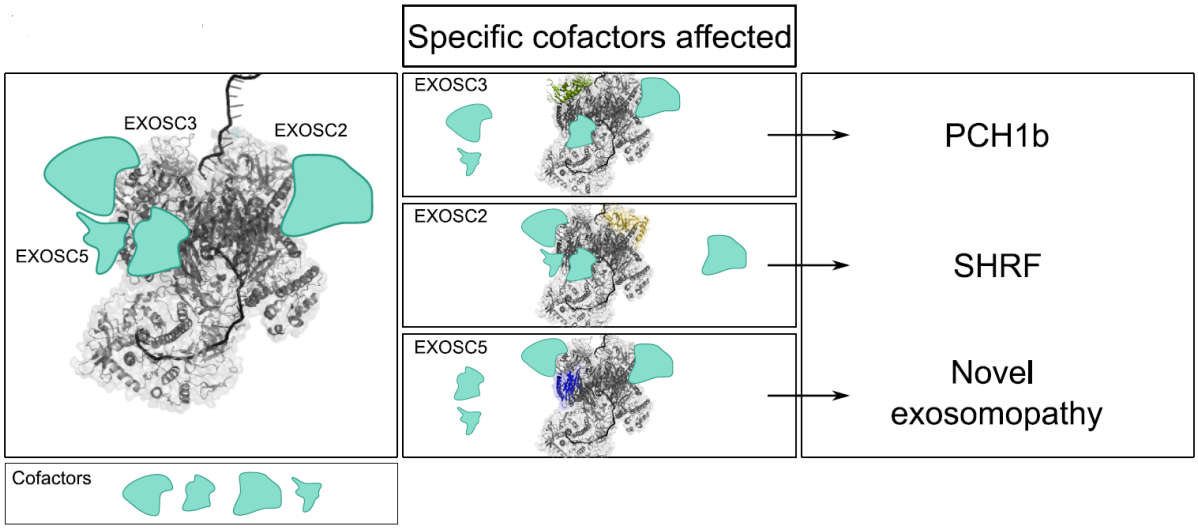

Figure 2. A proposed model of how pathogenic missense variants in *EXOSC* genes could contribute to variation in clinical presentation. The RNA exosome interacts with specific cofactors to confer specificity for target transcripts (left). Most cofactors have been identified and studied in budding yeast or cultured cells. Different pathogenic variants in structural subunits of the RNA exosome could disrupt interactions with a subset of cofactors. These cofactors could be critical in the cerebellum or other tissues susceptible to pathology. Interestingly, the four genes that encode RNA exosome subunits that include cerebellar atrophy and/or hypoplasia as a major clinical component, EXOSC3, EXOSC5, EXOSC8, and EXOSC9 are present on one face of the complex. In contrast, the cap subunit, EXOSC2 is located on the opposite side of the complex. Thus, loss of interactions with specific cofactors could contribute to the variable symptoms described in SHRF.

with this idea, flies heterozygous for a deficiency that removes the *Drosophila EXOSC3* gene do not show any of the phenotypes noted in the disease model flies [14]. These flies appear similar to wild-type flies in all assays performed, showing that even with presumably only 50% of expression of the *EXOSC3* gene, no phenotype is detected. Likely, pathology results from some combination of a decrease in overall RNA exosome levels and consequences of the specific pathogenic variants present. Further studies that examine the pathogenic missense variants of the *EXOSC* genes as well as the full RNA exosome complex are required to fully understand the molecular defects that contribute to pathology.

Experimental systems that deplete or delete an *EXOSC* gene provide critical insight but also simplify a more complex story. A major challenge remains understanding why the clinical presentation of patients with mutations in genes that encode a single complex show such variable pathology with some overlapping, but some distinct tissues affected. At the mechanistic level, pathogenic amino acid changes could, and certainly do [10], decrease the overall levels of the individual RNA exosome subunit and/or the complex. The changes might also alter the function of the complex at a molecular level, disrupting RNA binding or interactions with other subunits or cofactors. Moreover, certain cell types may express specific RNA exosome cofactors that interact and stabilize the RNA exosome, whereas other affected tissues may not express these cofactors (Figure 2). Loss of interactions with these cofactors could contribute to altered stability of the complex or changes in target specificity. These potential mechanistic consequences would all affect exosome-mediated RNA processing/decay.

New pathogenic *EXOSC* alleles are being reported by physicians worldwide. To date, pathogenic variants in *EXOSC1*, *EXOSC4*, *EXOSC6,* and *EXOSC7* have not been reported (Table 1); however, this is likely to change. GeneMatcher®, a freely available website designed to enable connections between clinicians and researchers [53], will likely continue to lead to identification of new alleles in these genes and in new genes as recently illustrated in the collaborative study of *EXOSC5* [40]. As additional pathogenic variants are identified, this could provide insight into whether there is a common mechanism

underlying pathology. A combination of approaches that includes functional studies in model organisms and analysis of patient samples will be critical to understand the mechanisms that underlie pathology. Further insight into the molecular consequences of single amino acid changes in *EXOSC* genes via genome-edited model systems will be required to paint the full canvas of RNA exosome biology and disease.

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Conflicting Interest

The authors have declared that no conflict of interest exists.

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