Grant Application to the Keith Hayes Foundation

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Title: Developing gene therapy for *RBCK1* deficiency.

Background

A group of clinical investigators from the US and three European countries (Sweden, Germany, and France) have identified mutations in a novel gene in ten patients from eight families. All had childhood-onset myopathy and eight also showed rapidly progressive cardiomyopathy requiring heart transplant in four. The patients (5 women and 5 men) had abundant accumulation of polyglucosan (an abnormal amylopectin-like polysaccharide with excessive number of poorly branched glucosyl chains) both in skeletal muscle and in the heart. [1].

The patients were homozygous or compound heterozygous for mutations in the heme-oxidized IRP2 ubiquitin ligase 1 (*HOIL-1*, HGNC Approved Gene Symbol: *RBCK1*).

Interestingly, a report by Boisson et al. (2012)[2] described two young siblings and one unrelated child with failure to thrive, chronic auto inflammation, and recurrent episodes of sepsis associated with loss-of-function mutations in *RBCK1.* Two of the children died from sepsis at ages 8 and 3.5 years and one child, who had allogeneic bone marrow transplantation at 13 months of age, died from sudden respiratory distress at 4 years of age. Similar to our patients, inclusions of polyglucosan were identified in skeletal muscle, heart and liver.

The finding of mutations in a ubiquitin ligase instead of a “canonical” glycogen metabolism enzyme came as a surprise, but – on second thought – there is a precedent for this situation. One of the two main mutated proteins in Lafora disease, devastating myoclonic epilepsy of young adults, is an ubiquitin ligase called malin, and the pathological hallmark of Lafora disease, the Lafora bodies, are composed of polyglucosan (PG).

The central question has been raised by malin deficiency in Lafora disease and by the new myopathy cardiomyopathy with mutations in *RBCK1* is how a dysfunction of the ubiquination system affects glycogen metabolism and results in PG accumulation.

To develop therapeutic strategies is of course the ultimate goal of translational research, especially for a monogenic disease that can be rescued by a gene therapy that helps patients affected by crippling myopathy and often fatal cardiomyopathy.

Abstract

Ubiquitin is a well-conserved eukaryotic protein, which, as its name implies, is expressed ubiquitously in all eukaryotic cells. Similar to phosphorylation, ubiquitination covalently binds ubiquitin via its c-terminal to a lysine residue on the target protein. This covalent modification may act in different ways. It may target a protein for proteasome-mediated degradation or for localization to membranes, or it may modify its activity as does phosphorylation. Therefore, deficiencies of ubiquitination or deubiquitination may result in a wide variety of diseases. RBCK1 an E3 ubiquitin ligase one of those proteins that control ubiquitination. Deficiency of RBCK1 causes either a childhood immune deficiency or a severe myopathy/cardiomyopathy. Although the pathogenic mechanism is not understood, deficiency of RBCK1 causes significant changes in the quality and quantity of glycogen. Therefore, disease has a pathological signature of accumulation of polyglucosan (PG) in tissues. PG is a poorly branched glucose polymer, which precipitates in the cell.

In this proposal, we aim to restore the defective RBCK1 function in patient cells by using a vector that can also be used to produce adeno associated virus serotype 9 (AAV9) for gene therapy. We will further test the safety of the AAV9 carrying *RBCK1* (AAV9-*RBCK1*) in mice. Our results will: determine if exogenous expression of RBCK1 can reverse PG deposition in (a) fibroblasts and (b) safety in normal mouse.

We will use the results of these experiments to prove that adenoviral vectors that can deliver RBCK1 resolves PGB aggregates and be a treatment for patients with RBCK1 deficiency.

Our Aim:

PG and its aggregates known as PG bodies (PGB) are formed in the absence of two known E3 ubiquitin ligases, malin and RBCK1 [2-4]. Although in both cases the cell accumulates PG, the two resulting diseases are very different. Malin deficiency is found in 30% of patients with Lafora disease (LD), characterized by severe myoclonus epilepsy, aphasia, dementia, and death within 10 years of disease onset. RBCK1 deficiency has been reported in a childhood form of immune deficiency [2] and in a severe form of myopathy/cardiopathy. Both malin and RBCK1 have E3 ubiquitin ligase activity and in their absence PG accumulates. PG also accumulates in glycogen branching enzyme (GBE) deficiency (glycogen storage diseases type IV, GSD IV). As the name implies, GBE adds new branches to the growing linear chains of glycogen. The clinical spectrum of GSD IV is different from the presentations of both malin and RBCK1 deficiencies. GSD IV has two main variants related to the degree of residual enzyme activity. Total loss of enzyme activity is fatal in infancy mainly due to liver and neuromuscular problems. However, the presence of 10 to 20% of GBE activity allows patients to survive to adulthood and the late-onset disease that follows affects upper and lower motor neurons, simulating amyotrophic lateral sclerosis (ALS). This disorder is also known as adult polyglucosan body disease (APBD).

In this proposal, we will try to cure PG accumulation in RBCK1 deficiency and prove that gene therapy will be a viable solution for patients affected by RBCK1 deficiency.

Our aim is to investigate if exogenous expression of RBCK1 reverses PG formation. We will design and test an adenovirus serotype 9 (AAV9) mediated gene therapy to deliver the normal RBCK1 gene to patient fibroblasts to show that regaining RBCK1 function will prevent the formation of PGB. Adenovirus we will produce will also be tested in mice for adverse biological interactions.

Our results will provide evidence for the benefits of gene therapy and reason to investigate further for clinical applications. We will generate a viral vector that can be used for gene therapy in patients with RBCK1 deficiency.

Innovation: We will develop a viral vector that can be used for gene therapy in patients with RBCK1 deficiency.

Background and Significance

Ubiquitin in eukaryotes is a highly conserved 76-amino-acid polypeptide that can be covalently attached to other proteins through an enzymatic cascade involving three enzymes: E1, E2, and E3[5]. In humans, there are two E1 enzymes, around forty E2 enzymes, and hundreds of E3 enzymes. Ubiquitination can be reversed by deubiquitinating enzymes (DUBs), which also form a large family, consisting of ~100 members in humans[6]. The ubiquitin 'signal' is recognized by specific ubiquitin 'receptors' that contain one or more ubiquitin-binding domains[7]. There are more than 20 different types of ubiquitin-binding domains embedded in a large variety of cellular proteins. Most ubiquitin-binding domains bind to ubiquitin with low affinity, which indicates that this binding is highly dynamic and specifically regulated. Thus, ubiquitin conjugation and de-conjugation systems, together with ubiquitin-binding proteins, are the basic molecular machineries for ubiquitin-mediated regulation of diverse cellular processes.

The addition of ubiquitin onto a protein has occasionally been called a "kiss of death" because it often commits the labeled protein to degradation in the proteasome, a barrel-shaped protein complex where proteins are disassembled by proteases[8]. This post-translational modification is carried out by a set of three enzymes, E1, E2 and E3. Ubiquitin has to be activated by the ubiquitin-activating enzyme E1 before being transferred to E1's active site, the amino acid cysteine. This transfer requires ATP, making the process energy-dependent. The ubiquitin molecule is then passed on to the second enzyme of the complex, E2 (ubiquitin-conjugating enzyme), before reaching the final enzyme, E3, the ubiquitin protein ligase, which recognizes and binds the target substrate, thus "tagging" it with the ubiquitin. The process can be repeated until a short chain is formed, with three or more ubiquitin molecules usually targeting the protein to the proteasome[8, 9].

The multiple E2 and E3 combinations define substrate specificity. E2 and E3 belong to large protein families, but - while E2 share many well-conserved catalytic domains - E3 ligases only share a few conserved motifs and are, therefore, very specific. As the three-steps process advances, specificity increases: E1 interacts with all E2s, but these interact with a more limited subset of E3s, which in turn target a limited array of protein substrates, based on shared recognition motifs within the proteins to be labeled. This enables the ubiquitination-proteasome pathway to be highly specific in the selection of the proteins to be labeled[8].

Ubiquitin labeling is not always fatal for the protein, and several non-proteolytic functions are associated with the addition of a single ubiquitin molecule (mono-ubiquitination) or with specific types of polyubiquitination. Mono-ubiquitination can alter the fate of the protein in a less drastic fashion, for example affecting its cellular location, its function, or its degradation through lysosomes. Also, the process is reversible, with enzymes (deubiquitinases) able to cleave ubiquitin from its target[9]. Ubiquitin is the "founding member" of a family of ubiquitin-like proteins (such as the SUMO protein) and - unlike ubiquitin - many of the other family members have non-degradative functions in the cell. For example, modification of proteins with small ubiquitin-related modifier, known as SUMOylation, often increases the protein lifespan and stability. This process is also linked to nuclear-cytosolic transport, regulation, and transcription[10].

Following addition of a single ubiquitin moiety to a protein substrate (mono ubiquitination), further ubiquitin molecules can be added to the first, yielding a poly-ubiquitin chain. In addition, some substrates are modified by addition of ubiquitin molecules to multiple lysine residues in a process termed multi-ubiquitination. As discussed, ubiquitin possesses a total of 7 lysine residues. Historically, the original type of ubiquitin chains identified were those linked via lysine 48. However, more recent work has uncovered a wide variety of linkages involving all possible lysine residues[11, 12], plus chains assembled on the N-terminus of a ubiquitin molecule ("linear chains")[13]. Work published in 2007 has demonstrated the formation of branched ubiquitin chains containing multiple linkage types[14]. "Atypical" (non-lysine 48-linked) ubiquitin chains have been discussed in a review by Ikeda & Dikic[15].

The ubiquitination system functions in a wide variety of cellular processes, including the following: antigen processing, apoptosis, organelle biogenesis, cell cycle and division, DNA transcription and repair, differentiation and development, immune response and inflammation, neural and muscular degeneration, morphogenesis of neural networks, modulation of cell surface receptors, ion channels and the secretory pathway, response to stress and extracellular modulators, ribosome biogenesis, and viral infections.

Chains linked through a specific lysine residue determine the faith of poly-ubiqutinylated proteins

The most studied polyubiquitin chains – lysine 48-linked - target proteins for destruction, a process known as proteolysis. At least four ubiquitin molecules must be attached to lysine residues on the condemned protein in order for it to be recognized by the 26S proteosomal complex. Lysine 63-linked chains direct the localization of proteins. Mono-ubiquitination also targets the localization of proteins to the proteasome[16]. The proteasome is a barrel-shaped structure with two chambers within which proteolysis occurs. Proteins are rapidly degraded into small peptides (usually 3-24 amino acids in length). Ubiquitin molecules are cleaved off the protein immediately prior to destruction and are recycled for further use. Although the majority of proteasomal substrates are ubiquitinated, there are examples of non-ubiquitinated proteins targeted to the proteasome[17, 18].

It has repeatedly been shown that ubiquitination affects the status of proteins. Notably, ubiquitin ligases have been found to be defective in two PG diseases, LD and a novel disorder manifesting either as infantile immunodeficiency or as myopathy/cardiopathy[1, 4]. How ubiquitin ligation affects the structure and amount of glycogen is still a mystery. It has been suggested that ubiquitination may modify enzymes that control the synthesis and degradation of other enzymes involved in glycogen synthesis. However, at least in Lafora disease, neither the amount nor the activity of two glycogenosynthetic enzymes, glycogen synthase and glycogen branching enzyme, were affected in ways that would explain abnormal glycogen synthesis[4, 19].

HOIL-1 deficiency has been shown to cause immune deficiency and PG myopathy/cardiopathy but the mechanism leading to PG accumulation has not been explained [1, 2].

In this proposal we aim at explaining the mechanism and nature of the PG-ubiquitin aggregates and how they differ from those that accumulate in GBE deficiency. We will study these mechanisms in human skin fibroblasts that do accumulate ubiquinated PG. We have in hand human skin fibroblasts that contain diastase-resistant PAS positive material (the histological “signature” of PG). One of the fibroblast lines contains a homozygous R515H mutation in *GBE1*, which is of APBD and causes relatively mild branching enzyme deficiency and PG accumulation. The second human skin fibroblast cell line contains a missense mutation that truncates the RBCK1/HOIL-1 and, like malin, E3 ubiquitin ligase deficiency, causes PG accumulation through an unknown mechanism.

Preliminary experiments:

Deficiency of RBCK1 causes polyglucosan accumulation in muscle and heart: Muscle biopsy and heart autopsy tissues obtained from patients were formalin fixed and sectioned according to standard procedures. Hematoxylin and eosin staining show inclusions in muscle tissue same inclusions are also present in sections stained with Gomeri trichrome. In order to determine the nature of these inclusion tissue sections were stained with ubiquitin to determine if they are protein or associated with proteins, or carbohydrate depositions, they are stained with Periodic Acid Schiff base (PAS) before and after diastase digestion. Diastase is an enzyme also known as alpha amylase that digests glycogen. If the glycogen is branched poorly, diastase works very slowly and PAS stained inclusion bodies remain in tissue sections. This is a standard method widely used to test the quality of glycogen in histochemistry. These staining clearly indicate that inclusion bodies are polysaccharide in nature but they are not normal glycogen because they survive diastase digestion. Same pattern was also present in skin fibroblast culture of a skin fibroblast with R515H mutation in *GBE1* (figure 2). Unlike muscle or liver, fibroblasts do not store detectable glycogen. In order to increase glycogen content fibroblasts are cultured to ~70% confluence in normal growth media with 10% fetal bovine serum and two more days in a serum free high glucose DMEM media supplemented with 100 micro molar cobalt chloride. Cobalt chloride stabilizes hypoxia inducible factor, which increases glucose import and glycogen synthesis and allows the accumulation of glycogen.

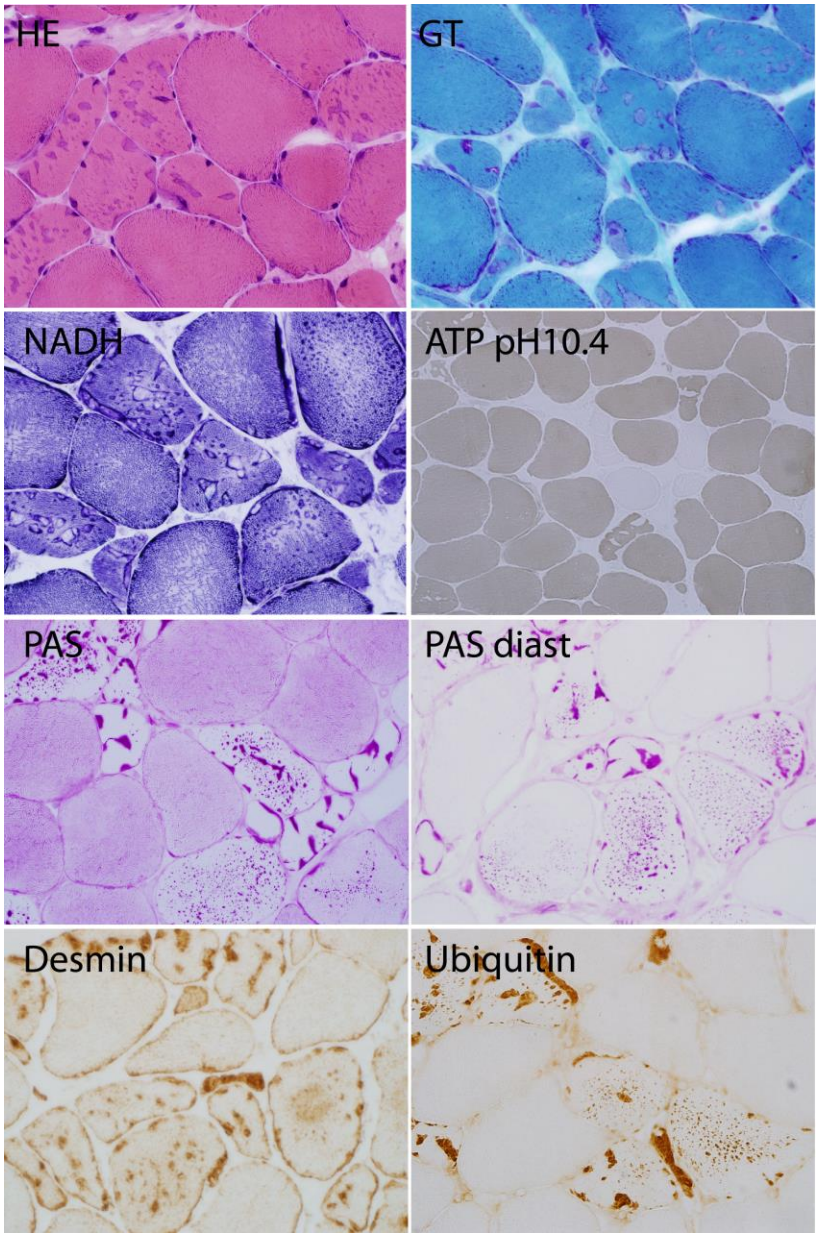

Figure 1. Characteristic morphological alterations of skeletal muscle and myocardium. Staining of skeletal muscle cryostat sections with periodic acid-Schiff (PAS) reagent demonstrates that numerous fibers (arrows) lack the normal inter-myofibrillar glycogen but show accumulation of PAS positive material (Panel A). Unlike normal glycogen, the storage material is not removed by amylase treatment (Panel B, arrows) and is ubiquitinated as shown by immunohistochemistry with an anti-ubiquitin antibody (NCL-UBIQ, Novocastra) (Panel C, arrows). Electron microscopy demonstrates that the accumulated polyglucosan is different from normal glycogen and consists of partly filamentous material (Panel D, arrows).

Muscle biopsies from patients HII:1 (Panel E), DII:1 (Panel F), EII:3 (Panel G) and CII:3 (Panel H) demonstrate similar abnormally accumulated PAS-positive material in fibers that are typically depleted of glycogen.

Paraffin section from the myocardium (patient BIII:1) stained with PAS demonstrates PAS positive material in all cardio myocytes (Panel I, arrows), with ultrastructural characteristics of polyglucosan (Panel J).

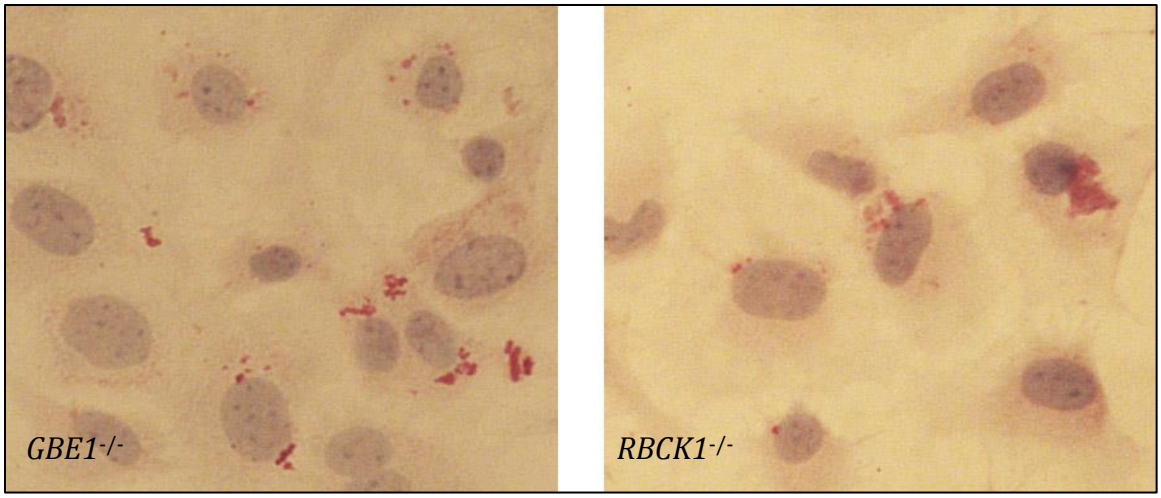

Figure 2. *GBE1* (left) and *RBCK1* (right) deficient skin fibroblasts showing PAS stained red polyglucosan accumulations.

Generation of RBCK1 expression vector:

Normal human RBCK1 cDNA (NM_031229) is purchased from Origene. We excised 1987 bp long RBCK1 coding sequence by *BsrBI* and *NspI* restriction digestion and cloned the cDNA in to AAV9 vector. Prior to cloning we linearized the AAV vector by *EagI* digestion (Fig 3). We will test this vector for protein expression in human cell lines (Fig3).

Research Plan:

Specific Aim: Does gene therapy reverse PG formation?

Working hypothesis: Polyglucosan body disease caused by RBCK1 deficiency is a condition should be reversed when a normal RBCK1 is provided to the tissue.

Our proposed work is summarized in figure 3. We will deliver normal RBCK1 to the cell and tissue via AAV9. We have cloned RBCK1 coding sequence in to a pAAV-RBCK1 vector which will allow us to pack *RBCK1* gene in to serotype 9 adeno associated virus. AAV9 has been shown to be the safest and most effective tool for gene therapy[20]. AAV9-RBCK1 will be injected in to normal mice and production of the RBCK1 protein will be assessed by human RBCK1 specific antibodies. Mice will be followed for two years to determine if AAV9-RBCK1 mediated gene expression and protein is safe. We will compare AAV9-RBCK1 treated mice to AAV9-GFP treated mice. In our laboratory we have used AAV9-GFP as a control to specific gene expression and transfection of the target tissues.

In order to determine if the vector we designed for gene therapy is successful we will measure gene expression and glycogen accumulation. In cell culture and normal mouse. In tissue culture, we will use patient fibroblasts that shows the accumulation of PGB. We will transfect the patient and control fibroblasts with the vector that we will use for AAV9-RBCK1 production. We will use vector only because AAV9 is not a good system for cell culture. In RBCK1 and GFP transfected cells we will measure production of RBCK1 protein, and accumulation of PGB. In normal mouse model we will test the transfection efficiency in tissues by monitoring human RBCK1 protein by western blotting and observing animals for adverse effects of AAV9 gene therapy system alone and exogenous expression of human RBCK1.

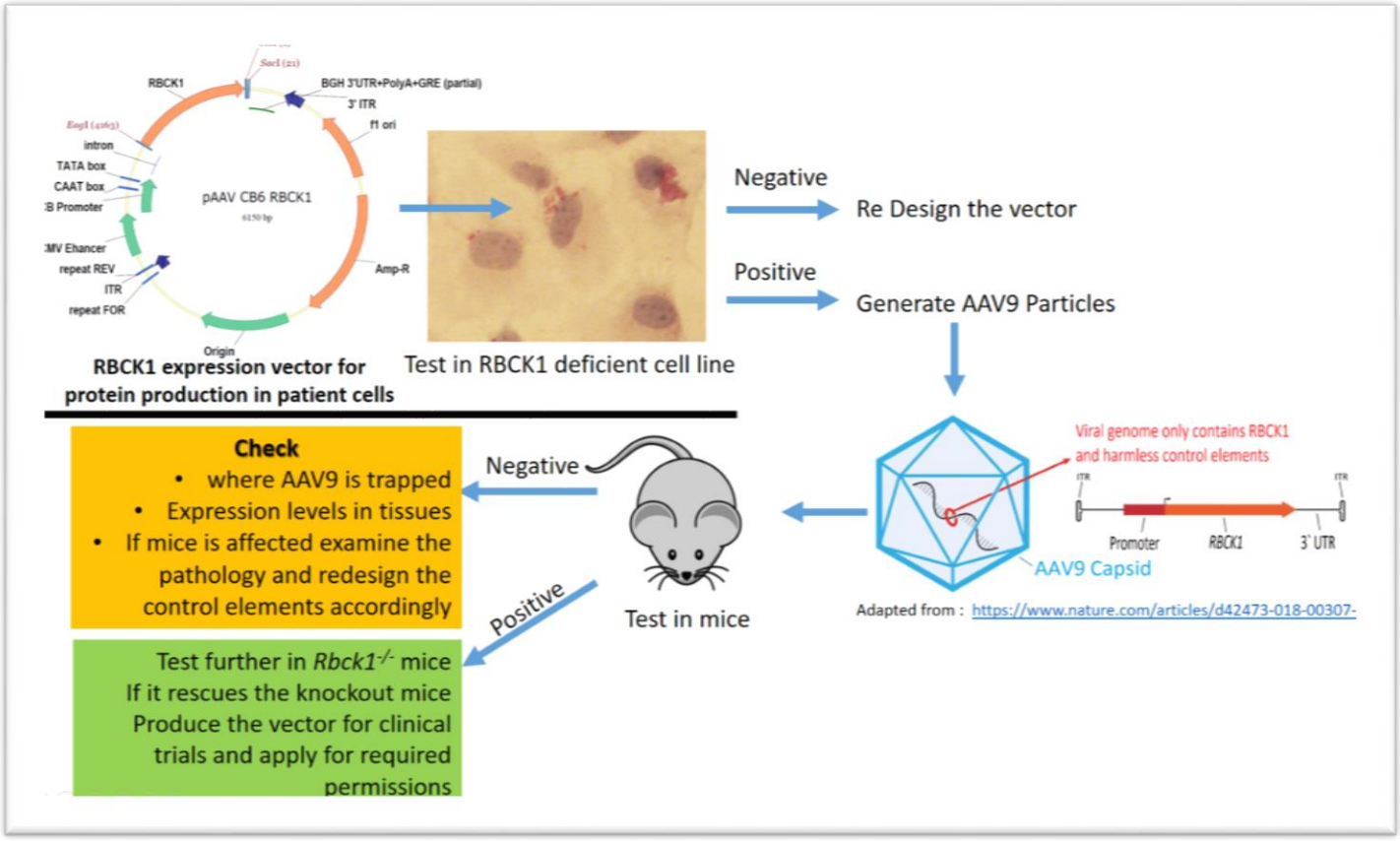

Figure 3. Flow chart outlines the milestones of generating AAV9-*RBCK1* for gene therapy.

Expected results and alternative strategies:

Transfection of patient cells: We have a good tissue culture model of PGB accumulation in RBCK1 deficient fibroblast cell line. Therefore, we will be able to show the absence of PGB in transfected cell line with successful production of the normal RBCK1. We will measure the successful transfection by co-transfection with GFP where all transfected cells will show green fluorescence. We expect all the patient cells with green fluorescence express normal RBCK1 and devoid of PGB. Untransfected cells will lack of green fluorescence and have PGB.

In mouse model we will monitor the expression of RBCK1 in tissues. Heart and skeletal muscles are the primary systems affected. Therefore, we will test them for successful expression of the gene. Unfortunately, we do not have *RBCK1* knockout mouse in which we would be able to show that we can prevent PGB accumulation. Therefore, we will only monitor the expression and tissue distribution. RBCK1 has been shown to play a role in NFkB pathway. Therefore, in the mice we will particularly observe the NFkB mediated gene expression. We will test this by western blot using the antibodies to proteins controlled by NFkB signaling [21].

In case if we see an adverse effect of RBCK1 expression in mouse model. Based on the tissue involved, we may use different promoters that restrict the production of RBCK1 to heart and muscle. We will use this strategy only to prove that our AAV9-RBCK1 works and eliminating excess production relieve adverse effects. Patients are lacking RBCK1 in all tissues therefore we do not expect any adverse effect in clinical application due to over expression of RBCK1. However, treatments in mice, will tell us which organ system must be monitored closely and if it involves tissues other than muscle and heart we may avoid it in clinical trials by changing the promoter that controls the RBCK1 production in a tissue specific manner [22].

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Proposed Budget:

Cell Culture:	$5150
AAV9-RBCK1 production and reagents	$6950
RBCK1 plasmid purification and antibodies	$3005
Animal Purchase and housing	$2700
Technician	$4750
Total cost is estimated	$20604

We expect to complete experiments in 6 months. Treatments in mice will take longer depending on the life span of the treated mice. Because we would like to obtain data on long term effects of the AAV9-*RBCK1* treatment, six mice will be observed for at least two years.