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Review

# The relevance of the storage of subunit c of ATP synthase in different forms and models of Batten disease (NCLs)

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ABSTRACT

The discoveries of specific protein storage in the NCLs, particularly of subunit c of ATP synthase in most, and the sphingolipid activator proteins, SAPs or saposins A and D in CLN1, CLN10 and an unassigned form are reviewed. The subunit c stored in the relevant NCLs is the complete mature molecule including an unusual modification found only in animal species, trimethylation of its lysine-43. Because of its strongly hydrophobic and lipid-like properties subunit c is easily overlooked or incorrectly described. This is becoming more of a problem as subunit c is not detected in standard proteomic investigations. Methods are reviewed that allow its unequivocal characterisation. Subunit c storage and cellular storage body accumulation do not cause the neuropathology characteristic of these diseases. The function of the trimethyl group on lysine-43 of subunit c is considered, along with some indications of where its normal turnover may be disrupted in the NCLs.





## 1. Introduction

Mutations in 13 different genes have been described that lead to different forms of Batten disease (the neuronal ceroid lipofuscinoses, NCLs), and there may be more, [NCL mutation database; http://www.ucl.ac.uk/ncl, [1]]. These inherited, mainly childhood, fatal neurodegenerative diseases are grouped by the similarity of symptoms and pathologies. There are animal equivalents of many of these human diseases, both naturally occurring and constructed by genetic manipulations of mice. A defining feature shared in the NCLs is the accumulation of fluorescent, electron dense, lysosome derived storage bodies in cells in the brain, and in many other cells throughout the body. Historically differences in the ultrastructure of these lysosome derived organelles were used as the basis of classification. Despite these ultrastructural differences, all NCLs share the common feature of storage of specific proteins. Direct sequencing of storage body proteins established specific storage of subunit c of mitochondrial $F_1F_0$ ATP synthase, first in South Hampshire sheep with a CLN6 form [2–4], and extended to CLN2, CLN3, CLN5, CLN6, CLN7 and CLN8 [8]. The identification of these proteins and implications drawn from their storage are the subject of this review.

## 2. The identification of the specific proteins stored and other storage body components

The initial work characterising subunit c as the stored molecule relied on the use of automated N terminal Edman degradation of the stored protein. The dominance of subunit c storage was established by identification of the N-terminal 40 amino acids in the first sequencing runs where total storage body proteins were loaded on the sequencer [3]. This identified "the lipid binding subunit of ATP synthase", the name given in the database entry at the time. This is in fact the c subunit of ATP synthase, or the dicyclohexylcarbodiimide reactive proteolipid, routinely referred to as the DCCD-reactive proteolipid [9], and known earlier as the mitochondrial proteolipid, and sometimes subunit 9. Proteolipids were a new class of compounds discovered by Jordi Folch and Marjorie Lees, being entirely polypeptides or sometimes with covalently attached lipids, that are soluble in common lipid extraction solvents, particularly chloroform/methanol mixtures [11]. Further sequencing studies showed that the complete and normal 75 amino acid subunit c is stored, first in the CLN6 ovine and the human late infantile and juvenile forms, and revealed no truncation of the complete protein [4,6]. Western blotting and preliminary protein sequencing data also confirmed subunit c storage in affected English Setter (CLN8), Border Collie (CLN5) and Tibetan Terrier (ATP13A2, CLN12) dogs [12]. A comparison of the yield of the protein sequenced with the amount of total storage body protein loaded onto the sequencer allowed an estimate of the proportion that is subunit c. Over three quarters of the

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protein or half the storage body mass was determined to be subunit c this way, initially in the CLN6 affected sheep and CLN5 affected cattle [13–15]. These results agreed with the proportion of protein that was ether precipitated as proteolipid after chloroform/methanol extraction. The readability of the sequences extended up to 43 amino acids, the sequence being relatively free of interfering signals indicating a high level of purity. This was complemented by the dominance of the subunit c in proteins on polyacrylamide gel electrophoresis (PAGE) of total storage body proteins, visualised by a silver stain developed to detect this Coomassie blue insensitive protein, and compared with Western blots.

Lipids comprised approximately one third of the storage body mass and were determined as normal lysosomal components, augmented by the storage of a number of isoprenoids normally stored in lysosomes, there was no indication of any loss of polyunsaturated fatty acids to lipid peroxidation and the metals stored reflected normal metal metabolism [16–18]. No intrinsic fluorophor was found and it has been shown that the fluorescence (also referred to as autofluorescence) is an aggregate property of non-fluorescent protein and lipids. When these non-fluorescent protein and lipid molecules were combined, remarkably storage body like structures were created, that fluoresced strongly when irradiated at the characteristic storage body wavelengths [17,19,20].

These techniques were used to establish that lysosomal proteins, the sphingolipid activator proteins (SAPs or saposins) A and D, not $F_1F_0$ ATP synthase subunit c, were the major storage body components in the infantile CLN1 form [21] a form in Miniature Schnauzer dogs [22], and in a *CLN4*/*DNAJC5* Parry disease form [23,24]. Subunit c was also not stored in ovine and human NCLs caused by cathepsin D deficiency [25,26]. These forms differ from other NCLs by sharing a granular ultrastructure of the storage material, often referred to as granular osmiophilic deposits or GRODs, whereas the subunit c storing forms are characterised by the accumulation of curvilinear and/or fingerprint membranous profiles.

Storage of subunit c has also been inferred from immunohistochemical staining in a large number of cases, including CLNs 2,3,5,6,7, 8 and 11 [28]. In general results agree with protein sequencing studies but this technique provides results that are not as unequivocal, immunohistochemistry being a qualitative technique where even a minor amount of stored subunit c may give a strong signal. Furthermore the storage of subunit c is generalised in the NCLs, occurring in many cell types throughout the nervous system as well as many visceral tissues. Because of this generalised storage a case has been made to drop "neuronal" from the name, preferring instead "generalised ceroid-lipofuscinoses" or just "ceroid-lipofuscinoses," particularly in a veterinary pathology context. This avoids the confusion caused by the storage of some subunit c containing organelles in neurons in specific brain regions in some other lysosomal storage diseases that are clearly not NCLs, for instance in a mouse model of mucopolysaccharidosis IIIB [28]. This confusion has led to the mistaken conclusion that subunit c storage is not specific to the NCLs [29].

## 3. Additional Edman sequencing

Table 1 summarises unpublished results of sequencing studies of storage bodies isolated from animal models, performed while automated Edman degradation was still widely used. Storage bodies were isolated from various tissues by centrifugation following homogenisation of the tissues as described [3,6,16,30], total storage body proteins dissolved in 100% formic acid and loaded onto ABI automated Edman degradation protein sequencers and the major N-terminal sequences determined.

These results confirm $F_0$ subunit c storage in CLN3 affected mice [31], *nclf* CLN6 affected mice [32], CLN5 affected Border Collies [33], CLN8 affected English Setters [34] and CLN12 (ATP13A2) affected Tibetan Terriers [35]. The Tibetan Terrier result contradicts a claim that glial fibrillary protein (GFAP) and histone H4 accumulate in this disease [36]. However, as has been pointed out, this apparent accumulation is more likely to have arisen from contamination in the storage body preparations, the GFAP coming from the astrocytosis associated with the disease rather than being intrinsic to storage bodies [37]. Furthermore the gel from which the protein bands were cut for sequencing [36] does not include the low molecular weight region where subunit c would migrate.

**Table 1**
Edman degradation sequencing of storage body proteins from different forms of NCL.

| | |
|---|---|
| Dominant $F_0$ subunit c sequence[a] | DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPS. |
| CLN3 mouse brain | DIDTAAKFIG[b] |
| CLN6 mouse brain | DIDTAAKFIG[b] |
| Cathepsin D mouse brain | No dominant readable sequence[b] |
| Border Collie brain | DIDTAAKFIGAGAATV |
| Border Collie liver | DIDTAAKFIGAGAATVGVAGSGA– – – TVFG– L |
| English Setter brain | DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIG – ARN |
| English Setter liver | DIDTAAKFIGAGAATVGVAGS – A – IG – VFGSL-I – – ARNPS |
| Tibetan Terrier brain | DIDT – AKFIGAGAA – V |
| Second minor sequences found | |
| CLN6 mouse brain | APEY(A)IF |
| CLN3 mouse brain | APE |
| Dog $V_0$c sequence[c,d] | GPEY(A)SF(F)AVM(G)(A)SAAMVF.. |
| Border Collie brain[d] | GPEY(A)S – (F) |
| Border Collie liver[d] | GPEY(A)SF(F)AVM |
| English Setter brain[d] | GPEY(A)SF(F) – VM |
| English Setter liver[d] | GPEY(A)SF(F)AVM |
| Tibetan Terrier brain[d] | GPEY(A)SF(F)AVM |

[a] Identical for all mammals, including trimethylation of lysine-43 [35].
[b] Sequence only determined for the first 10 cycles.
[c] Inferred from the genomic sequence for the dog ATP6VO gene.
[d] Amino acids in brackets are those where the ATP synthase c subunit and the vacuolar ATPase c subunit have the same residue.

ATP synthase is a complex of 16 different polypeptides, a number of them in multiple copies, including eight c subunits in animals [38]. No other components of the ATP synthase complex were found in these experiments and the subunit c sequence was not determinable in attempted mixed sequencing of inner mitochondrial membrane proteins, so these results cannot be an artefact of inner mitochondrial membrane contamination of the storage body isolates. The amount of the subunit sequenced and the clarity of the sequence, in line with sequence determinations of purified proteins, indicate little or no storage of other mitochondrial ATPase subunits, or of any other inner mitochondrial proteins, also indicated by a comparison of PAGE of storage bodies and purified ATP synthase [4].

A second readable minor sequence was often found, being the N terminus of the $V_1V_0$ vacuolar ATPase subunit C (Table 1). This protein is homologous with a double copy of the mature mitochondrial $F_0$c subunit, and resides in the endosome–lysosome membrane as part of the proton pumping $V_1V_0$ vacuolar ATPase. Others have reported it to be a major storage body component in so-called "*mnd*" CLN8 affected mice [7,39]. Varying amounts have also been found in some human, canine and ovine storage body isolates [36]. These results suggest that the NCLs involve lesions in some common turnover pathway of both mitochondrial ATP synthase c and $V_0$ vacuolar ATPase C subunits [36]. However it is not certain that this vacuolar C subunit is an intrinsic storage body component, and it is entirely possible that it arises from lysosomal–endosomal membrane fragments co-sedimenting with the storage bodies during the isolation procedure. Vacuolar membranes are rich in this molecule, acidifying capacity being regulated by the binding of the $V_1$ segment of the complex to it [40].

SAPs A and D have also been detected in subunit c containing storage body isolates, but not to the same extent as the accumulation in the CLN1, CLN10 and unassigned Miniature Schnauzer forms [19,22,30].



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Again it is likely that this minor amount results from the presence of normal lysosomal components in the storage bodies, reflective of their lysosomal origin.

## 4. Subunit c storage, lipofuscin, "universal" proteomics and the biochemical lesions in the NCLs

Despite the robustness of these results they have not been universally accepted and numbers of papers and authoritative texts still refer to the storage material as the fluorescent product of lipid peroxidation cross-linked to proteins, or claim that it is not known. These claims are driven by dogma, not experimental investigations. The dogma arises from experiments in which various proteins and lipid peroxidation products were heated together to form lipofuscin like fluorescent aggregates [41,42], but are not supported by good structural studies of lipofuscin itself. As indicated above the storage bodies contain no such fluorescent compounds, their fluorescence being an aggregate property of non-fluorescent compounds [17,19,20].

There is another difficulty. Unfortunately subunit c will not be detected in many modern "universal" proteomic methods. Automated Edman degradation is now unusual, having been replaced by mass spectral methods of protein sequencing and analysis. Detecting subunit c by these methods is not straight forward. Special techniques are required for chromatographic isolation and mass spectral detection [43,44]. Trypsin does not cleave $F_0$ subunit c separated by polyacrylamide gel electrophoresis and chymotryptic digestion is required to provide fragments for LC-MS verification of lysine-43 trimethylation [38]. Subunit c also has a high propensity to irreversibly aggregate prior to gel electrophoresis, is insoluble in many solvents routinely used in protein analyses and is insensitive to Coomassie blue staining. For all these reasons the presence of subunit c can be overlooked in proteomic investigations and thus its storage in NCL samples can, and has been, overlooked. Unfortunately storage material in some of the more recently described NCLs is also ascribed to heterogeneous peroxidative linking of sugars, proteins and lipids with no rationale other than dogma, as in CLN11 associated with a mutation in the progranulin locus [45], this is in spite of positive subunit c immunohistochemistry [46].

## 5. Storage body accumulation does not cause the neuropathology

There is a longstanding paradigm in the lysosomal storage diseases that the storage material itself is the cause of the pathology, either because of its toxic nature or because it somehow blocks normal cell function. This is often used in the failed "rubbish disposal" portrayal of pathogenesis. There is no evidence of this in the subunit c storing NCLs. Subunit c containing storage bodies accumulate in most cells in most tissues, without any suggestion of tissue or organ failure or disruptions of cellular functions [47]. Even within the brain the pattern of storage body accumulation is independent on the progressive regional atrophy. For instance careful longitudinal studies of neuropathological changes in brains from presymptomatic sheep affected with a CLN6 ovine form showed that astrocytic activation and progressive transformation of microglia to brain macrophages started regionally, preceded neurodegeneration and spread to different cortical areas, most prominently regions associated with clinical symptoms [48]. In contrast, storage body accumulation was much more evenly spread across regions, indicating that neurodegeneration and storage body accumulation are independent manifestations of CLN6 mutation. Whereas storage body accumulation in the cerebellum of these sheep is similar to cortical accumulation the cerebellum remains virtually unchanged even at end-stage disease. There was no correlation between disease-related changes and the presence of storage bodies in thalamus and hypothalamus of these sheep, where storage bodies were abundant but there was no neurodegeneration or signs of activated astrocytes or microglia [49]. Unfortunately this failed rubbish disposal analogy is often used in lay explanations in an allusion that it will aid understanding when in fact it has the opposite effect.

## 6. The biochemical lesion

A longstanding paradigm in lysosomal diseases, arising from Garrod's insights on the nature of in-born errors of metabolism [50] is that the nature of the storage material should directly reflect the underlying enzyme, interpreted to mean that it should be a substrate of the missing enzyme activity. Traditionally this has been a partially degraded macromolecule but careful mass spectral characterisation of subunit c has shown that the complete and normal subunit is stored [44], including trimethylation of lysine-43 and cleavage of the lead sequence, strongly indicating that the stored protein has been processed through mitochondria prior to accumulation in lysosomes, but has been subjected to no degradation. One scenario that would fit with Garrod's proposal is that subunit c storing NCL genes function in a subunit c turnover pathway, an essential step of which is de-methylation of lysine-43. Further studies of the role of methylation of lysine-43 of the c-subunit of F-ATPases would be greatly aided by the identification of the modifying enzyme.

The biology and structure of the $F_0$c subunits supplies some clues as to why it may be stored in these NCLs. Recent studies have confirmed that the sequence of subunit c is very highly conserved in all metazoans (Animalia) both vertebrate and non-vertebrate, including absolute conservation of trimethylation of lysine-43 and three alanines, in the N-terminal α-helix at positions 13, 19 and 23 [38]. This highly conserved mature protein segment is in contrast to other areas of the gene product which are much less conserved, including the lead sequences in nuclear encoded subunit c, which guide the gene product to the mitochondria for import and are cleaved off in the process. Precursors of the human and bovine c-subunits, for example, are each encoded by three nuclear genes [51,52]. In each case, the products differ in the sequences of the N-terminal extensions that direct the proteins to the matrix of the mitochondria, but removal of the import sequences during the import process produces identical mature c-proteins.

Studies have shown that conservation of these residues in mature metazoan subunit c is critical to the way the subunit c interacts with cardiolipin in the inner mitochondrial membrane and allows an insulated c-rotor driving ATP synthesis made up of only 8 c subunits [53]. Replacement of the critical alanines by amino acids with larger side chains would destabilize the ring, and such residues can only be accommodated in the larger c-rings, such as those found in fungi and eubacteria. Replacement of these alanines by glycines would abolish hydrophobic packing interactions that contribute to the ring's stability.

Each complete rotation of the rotor produces three ATP molecules, one from each of the three catalytic sites in the $F_1$-domain [54], and requires the translocation through the membrane of one proton per c-subunit [53]. Thus, the number of translocated protons required to make each ATP is the number of c-subunits comprising the ring divided by three, a parameter referred to as the "energy cost" for making each ATP molecule [53]. The identity, or near identity, of the sequences of vertebrate c-subunits makes it highly likely that the $c_8$-rings observed in the bovine enzyme will persist throughout vertebrate F-ATPases, and hence the energy cost in their F-ATPases will be 2.7 translocated protons per ATP, the lowest value so far observed [38].

Trimethylation of the conserved lysine is restricted to Animalia and does not occur in species from the other kingdoms. The sequences of c-subunits from representatives of other opisthokont kingdoms (choanoflagellates, filasterea, icthyosporea and fungi) show that lysine-43 is conserved except in the fungus, *Pichia angusta*, where an arginine residue is substituted. However, in the two cases where the methylation status of the conserved lysine has been investigated, *Saccharomyces cervisiae* and *Yarrowia lipolytica*, it is not methylated [38]. Also, the three alanines in the N-terminal α-helix that are conserved in metazoans are frequently mutated to amino acids with large



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side chains in non-metazoans. It is of interest that no accumulation of subunit c has been reported in yeast models of NCLs, where causative gene homologs have been mutated. This fits with the idea that demethylation of lysine-43 is a critical step in subunit c turnover, and that this process is somehow perturbed in the subunit c storing forms of NCL. It also indicates limits as to what should be deduced from yeast or other non-metazoan models of NCLs.

Further studies of the role of methylation of lysine-43 of the c-subunit of F-ATPases and the NCLs would be greatly aided by the identification of the modifying enzyme(s). Until the recent reports of the first arginine and lysine methyltransferases found in the matrix of human mitochondria [55,56], it was not known whether such enzymes are associated with the mitochondrial matrix. In the case of the c-subunits in porifera, there can be little if any doubt that the methylation of subunit c is an event that takes place in the mitochondrial matrix as the sponge c-subunits are the products of the mitochondrial genomes [57], in contrast to other metazoans where the c-subunit is encoded by nuclear genes. Critical demethylation could be a mitochondrial, autophagic, endosomal or lysosomal event and the family of subunit c storing NCLs may be linked by lesions along this pathway.

## Transparency document

The Transparency document associated with this article can be found, in online version.

## Uncited references

[5,10,27]

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