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Review

Characterizing pathogenic processes in Batten disease: Use of small eukaryotic model systems

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Abstract

The neuronal ceroid lipofuscinoses (NCLs) are neurodegenerative disorders. Nevertheless, small model organisms, including those lacking a nervous system, have proven invaluable in the study of mechanisms that underlie the disease and in studying the functions of the conserved proteins associated to each disease. From the single-celled yeast, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, to the worm, *Caenorhabditis elegans* and the fruitfly, *Drosophila melanogaster*, biochemical and, in particular, genetic studies on these organisms have provided insight into the NCLs.
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1. Introduction

Several genes that cause different NCLs have been identified. Congenital NCL (CNCL), infantile NCL (INCL), late-infantile NCL, juvenile NCL (JNCL), adult NCL (Kuf's disease), Finnish variant late-infantile NCL, variant late-infantile Gypsy/Indian NCL, Turkish variant late-infantile NCL/Northern epilepsy harbor mutations in Cathepsin D, *PPT1* (formerly *CLN1*), *CLN2*, *3*, *4*, *5*, *6*, and *8*, respectively (see http://www.ucl.ac.uk/ncl/; [1] reviewed in [2,3]). However, for most of these proteins, the biological function and mechanism of disease development remains unclear. Evolutionary conservation of each NCL gene allows for their functional analysis in different model organisms. The lesser complexity of model organisms enables researchers to obtain information about a certain protein, the processes it is involved in, and to extrapolate that knowledge with the aim of unraveling the more complex systems in higher organisms. As such, combining the data from the different models will lead to an increased understanding of the pathways affected in NCL.

Known homologs to NCL proteins in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Caenorhabditis elegans* and *Drosophila melanogaster* and their high amino acid sequence similarity are shown by Multalin sequence alignment (Figs. 1 2 and 3) [4,5]. The homolog to CLN3 in the budding yeast *Saccharomyces cerevisiae* is Btn1p. Homologs of CLN3 and PPT1 in fission yeast *Schizosaccharomyces pombe* are

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Btn1p and Pdf1p, respectively. The ease of using yeast for genetic and biochemical studies has made it a powerful model organism for characterizing these disorders. Similarly, the nematode *Caenorhabditis elegans* has proven useful in the study of both INCL and JNCL, which are studied using the worm homologs PPT-1 and CLN-3, respectively. Along with its many other advantages, *C. elegans* has a well-studied nervous system making it useful in characterizing the disease mechanism and protein function in the nervous system. Like *C. elegans*, neuronal development and function has been well characterized in the fly. This, along with its sophisticated genetics, makes *Drosophila melanogaster* a valuable model system in the study of human disease, including CNCL, JNCL and INCL where Cathepsin D, CLN3 and PPT1 are studied. In this review, the advantages of each model will be outlined and current research using genetic and biochemical techniques, as well as advances in the characterization of lysosomal storage material, will be discussed.

2. Genetic and biochemical studies

2.1. Saccharomyces cerevisiae

The budding yeast *S. cerevisiae* has been used in scientific research for over half a century, resulting in a highly annotated genome and many optimized approaches and tools, coined "the awesome power of yeast genetics". *S. cerevisiae* has approximately 6000 Open Reading Frames (ORFs) spread across 16 fully-sequenced chromosomes. It is a single-celled eukaryote that stably exists in both the haploid and diploid state with a fast generation time. Two haploid strains of opposite mating type can fuse to form a diploid strain, allowing for complementation assays, haplo-insufficiency analysis, and essential gene deletion. Likewise, a diploid strain can sporulate into four haploid spores, allowing for genotypic analysis. Yeast readily takes up, recombines, and expresses foreign DNA, further providing relative ease in genetic manipulation. For example, a gene of interest can be intra-chromosomally tagged, allowing for endogenous expression of the tagged protein, which eliminates the need for specific antibodies and some of the artifacts resulting from overexpression. Entire ORFs can be replaced by reporter genes for promoter and expression analysis, or conversely, an inducible promoter can be inserted before a gene of interest to facilitate the study of essential genes. Making a deletion strain by introduction of a disruption cassette with a selectable marker by homologous recombination is straightforward and rapid. In fact, a deletion library of all nonessential yeast genes was recently made commercially available [6,7]. Likewise, libraries of almost all genes with green fluorescent protein (GFP), glutathione-S-transferase (GST), or tandem affinity purification (TAP) tags are widely used [8–11]. There are techniques accessible in yeast that are not yet optimized, or even available, for higher eukaryotes, such as suppressor screening and synthetic genetic arrays for identification of genetic interactions, demonstrating the power of yeast as a model for NCL [12].

S. cerevisiae is used to study the juvenile form of NCL in which *CLN3,* encoding a yet uncharacterized protein, is mutated [13]. Btn1p, also designated Yhc3p, is 39% identical and 59% similar in amino acid sequence to human CLN3 (Fig. 1) [14]. Btn1p is a 46 kDa integral membrane protein, and like CLN3, has several predicted phosphorylation, myristoylation, and glycosylation sites and is predicted to be farnesylated [4,15–20]. Furthermore, Btn1p and CLN3 are functional homologs, in that plasmid-derived CLN3 can complement for the absence of Btn1p in the *BTN1* deletion strain, *btn1*-Δ, suggesting that the primordial function of CLN3 is conserved in yeast [21,22]. This property of the yeast model has the potential as a powerful tool to test the functionality of CLN3 and Btn1p constructs. For example, it is unclear if GFP fusions of CLN3 are functional [23], and a simple complementation assay in the yeast model could test this. Importantly, the residues that are mutated in Batten disease patients are conserved in Btn1p, suggesting that the primary activity of the protein is conserved. Moreover, the yeast model has been used to test the CLN3 point mutants' ability to complement *BTN1* and it was found that disease severity correlated with the degree of complementation [22,24]. Human CLN3 has been localized to the lysosome in various studies and cell types [15,25] (reviewed in [26,27]). Similarly, Btn1p has been localized to the vacuole, the analogous structure to the higher eukaryotic lysosome, further emphasizing the relevance of the yeast model [28,29].

To date, Btn1p has been implicated in three main cellular processes, two of which have been validated in mammalian models. Although not completely independent, Btn1p has been linked to regulation of cellular pH, basic amino acid homeostasis, and nitric oxide production [29–32]. Using pH sensitive dyes, Pearce and colleagues first reported that the vacuolar pH in *btn1*-Δ cells was decreased as compared to *BTN1*$^+$ vacuoles at early growth and continued to increase throughout log and stationary phases [29]. More recently, it has been shown that as *btn1*-Δ cells grow, vacuolar pH will rise above that of normal [33]. Moreover, vacuolar pH in both normal and *btn1*-Δ strains was shown to be altered by extracellular pH. In *btn1*-Δ cells, activity of the plasma membrane H^+-ATPase is increased, likely acting to buffer altered vacuolar pH. Importantly, this led to the discovery of a plate phenotype for the *btn1*-Δ strain, which due to the increased plasma membrane H^+-ATPase activity has an elevated rate of media acidification. This elevated media acidification allows *btn1*-Δ cells to grow in the presence of D-(−)-threo-2-amino-1-[*p*-nitrophenyl]-1,3-propanediol (ANP), as the increased acidity of the medium results in ANP being non toxic to *btn1*-Δ cells whereas ANP is toxic to *BTN1*$^+$ cells [22,32] . In addition, when the vacuolar pH of *btn1*-Δ cells was artificially increased using chloroquine, the plasma membrane ATPase activity decreased strengthening the link between external and vacuolar pH [29,31]. Importantly, these studies demonstrated that human CLN3 could complement for the ANP phenotype in *btn1*-Δ yeast. The pH and ANP results are in contrast to the *Schizosaccharomyces pombe* model, where an increased vacuolar pH and sensitivity to ANP are observed in

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HsCLN3 MGGCAGSRRR FSDSEGEETV PEPRLPLLDH QGAHWKNAVG FWLLGLCNNF SYVVMLSAAH DILSHKRTSG NQSHVDPGPT
DmCLN3 MTATKQQEAE AEHDPQVVVT PGDASRSSRQ DRGLWRDLTS YWILGLCNNY GYVVMLSAAH DIIKQFN--- --------PN

HsCLN3 PIPHNSSSRF DCNSVSTAAV LLADILPTLV IKLLAPLGLH LLPYSPRVLV SGICAAGSFV LVAFSHSVGT SLCGVVFASI
DmCLN3 DESEESSSGR NCHLVSTGAI LLADVLPSLF VKILMPFFPF WVNF--RIAL AVAFSAAGFL LVGFANAEWM ALLGVIITSA

HsCLN3 SSGLGEVTFL SLTAFYPRAV ISWWSSGTGG AGLLGALSYL GLTQAGLSPQ QTLLSMLGIP ALLLASYFLL LTSPEAQDPG
DmCLN3 SSGIGETTFL AYSSRYNKNV ISTWSSGTGG AGVIGSLSYA SLRSLDFSPR DTMLVMLIFP AIEAFAFWLL LRRPQV-DIL

HsCLN3 GEEEAESAAR QPLIRTEAPE SKPGSSSSLS LRERWTVFKG LLWYIVPLVV VYFAEYFINQ GLFELLFFWN TSLSHAQQYR
DmCLN3 PVTTVEST-- EVLISDEKP- -------LVG FKEKLFYIKH LFKYMLPLCL VYFFEYFINQ GLFELVYFED IFLDKDSQYR

HsCLN3 WYQMLYQAGV FASRSSLRCC RIRFTWALAL LQCLNLVFLL ADVWFGFLPS IYLVFLIILY EGLLGGAAYV NTFHNIALET
DmCLN3 WLNVDYQIGV FISRSSVNLF QLDKIWLMSI FQFVNVVYFL TEVIWWYTPS IWIVFAIVLW EGLLGGGAYV NTFYRMSKEI

HsCLN3 SDEHREFAMA ATCISDTLGI SLSGLLALPL HDFLCQLS
DmCLN3 SPERQQFAMA MVVQSDSYGI ALAGFLAIPV HNAICGLPAA ARSLVW
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HsCLN3   MGGCAGSRRR FSDSEGEETV PEPRLPLLDH QG-AHWKNAV GFWLLGLCNN FSYVVMLSAA HDILSHKRTS GNQSHVDPGP
CeCLN3.1                               M VNWTNFRDIL AFWLLGLCNN YGYVIMLSAA EDILDDQKGT NSS----STN
CeCLN3.3                            MMDR VNWPTVRIAV AFFLIGLCNN YGYVIMLSAA EDILSEQHGK VKS----NA-
CeCLN3.2                          MDVASG GGRRPPRNSV AFWLLGLCNN FAYVVMLSAA KDILEKDAKH IEK----P--

HsCLN3   TPIPHNSSSR FDCNSVSTAA VLLADILPTL VIKLLAPLGL HLLPYSPRVL VSGICAAGSF VLVAFSHSVG TSLCGVVFAS
CeCLN3.1 SSTCETHLDS RHCQSISTGA VLLADNLPAL VVQTTFPFFM HRFPFGFRAF LVFLLQASSY FVVAYSKNIA MSLAGVCMAS
CeCLN3.3 TDLCLPAITK RECKP-PVAE VLLSDNLPSL IVKLTFPFFM DRFPFGFRVA IVCLLQATSY FVVAFSVSIP MSLAGVVFVS
CeCLN3.2 ---CREHVTT RECQMMSTGS VLLADIIPAL LIKITAPMFI HRVPFGIRHS IVVLLQASSF LIVGISDSTA LALFGVVLAS

HsCLN3   ISSGLGEVTF LSLTAFYPRA VISWWSSGTG GAGLLGALSY LGLTQ---AG LSPQQTLLSM LGIPALLLAS YFLLLTSPEA
CeCLN3.1 LGQGIGEITF LAMAAHYIPE TIASWSSGTG GAGLIGSFSY AFITQ---AG LSPSNTLLVQ LFIPVVFAGA YFFLLTIPPT
CeCLN3.3 LGGGLGEITF LGLSAHYQRI AIAGWSSGTG MAGLLGSFSY AFLTEPHMAN LTPKVALLIQ LFIPVLFAFA YFILLKKPES
CeCLN3.2 FGSGLGEISY LALSSNYPST VVASWSSGTG GAGLIGASAY ALLTDSKLLA ISPKHTMFIM LTLPALFSVS YWSILKIPHS

HsCLN3   QDPGGEEEAE SAAR------ -QPLIRTEAP ---------- ---ESKPGSS SSLSLRERWT VFKGLLWYIV PLVVVYFAEY
CeCLN3.1 VY-SPTIHPS TWIIPKNY-- -DKDVFEAAE ARGDVL---- ---NTKKVPQ RELGPLERIK LIGPLLYLMV PLATVYTAEY
CeCLN3.3 VY-SPTLDPK SWIVPKGY-- -DDFI----- -----V---- ---SEHRVPQ RELGPSDRLK LILPMLHLMI PLAIVYVGEY
CeCLN3.2 VQRAHFLQPS TWLVTGDFIP ADDMRREEVE EEEGLLGIRE DRENSVDVTS RRRQTSTTLE RVLPLLKFMI PLISVYLAEY

HsCLN3   FINQGLFELL FF---WNTSL SHAQQYRWYQ MLYQAGVFAS RSSLRCCRI- -RFTWALALL QCLNLVFLLA DVWFGFLPSI
CeCLN3.1 MINQGLTELI IFNCSQGLSL PLSSQYRWYQ VLYQLGVFIS RSSVKFFEMP LWLIWCLPIL QCVNMIFFFF EAVYWFTPTI
CeCLN3.3 MINQGMTQQI VFDCAHGFNL SLHSQYRWYQ VLYQFGVFVS RSSIRLVELP MWMLYLLPFL QLTNMLFFFF DALYWFVPQI
CeCLN3.2 YINQGLLELL EFDCSHGFSM SSESQYRWFQ VTYQLGVFIS RSSSNYVTIP TQYLKSLAVL QIFNAGFFTI TAIYSFLPHI

HsCLN3   YLVFLIILYE GLLGGAAYVN TFHNIALETS DEHREFAMAA TCISDTLGIS LSGLLALPLH DFLCQLS
CeCLN3.1 IIIFVLIVFE GLLGGSAYVN TYNKIHKKVN PDVREYSLSA ASMGNSLGTN IAAFLSIPLY NWVCTMKAPG R
CeCLN3.3 AIIFALIIFE GLFGGSSYVN TFHKIHNKVE PDVREYCLSA ASMGDSIGVN FAAGVSIPLH YWMCDQPARL
CeCLN3.2 LIAFLVILFE GLLGGASYVN TFRAVHKEIP ADSREFSMGV VSISDTIGIV FAGFLAMPVH NRICSMPM
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C

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HsCLN3  MGGCAGSRRR FSDSEGEETV PEPRLPLLDH QGAHWKNAVG FWLLGLCNNF SYVVMLSAAH DILSHKRTSG NQSHVDPGPT
ScBtn1p                             MSD KSH--QIYCY FWLFGLINNV LYVVILSAAV DIV------- -------GPT
SpBtn1p                         MIKLRLT KDA--KVGCC FLIFGLLNNL LYVIILSAAL DLV------- -------GAN

HsCLN3  PIPHNSSSRF DCNSVSTAAV LLADILPTLV IKL-LAPLGL HLLPYSPRVL VSGICAAGSF VLVAFSHSVG TSLCGVVFAS
ScBtn1p ---------- ----LPKSLV LLADIFPSLA IKL-CSPFFI DRIKYSYRIW SLITMSCLGM FLVSFKNLFV C-LLGISFAS
SpBtn1p ---------- ----VSKGVV LLSNIVPSLA CKLSASILHV HKFKFAKRIG FCVFMSILGM QWIAWSSSVP SKMLGVSLAA

HsCLN3  ISSGLGEVTF LSLTAFYPRA VISWWSSGTG GAGLLGALSY LGLTQA-GLS PQQTLLSMLG IPALLLASYF LLLTSPEAQD
ScBtn1p ISSGFGEVTF LQLTHYYKQI SLNGWSSGTG GAGIIGGASY MFLTSIFKVP VKLTLLVFS- LPPFAFLFYF KLESNDTNLT
SpBtn1p ISSSFGEISF LHLSSRYHSV SLPCWSSGTG LAGLFGASSY LVMTTWFNFS VRSTLIISSF LPLFLLIMYF FVLPESESTS

HsCLN3  PGGEEEAESA ARQPLIRTEA PESKPGSSSS L-----SLRE RWTVFKGLLW -YIVPLVVVY FAEYFINQGL FELLFFWNT-
ScBtn1p YQSLQQIDEA EDDQLVPFPV AFTHTNASQS LYSTRQHILQ TVKRLRRLVF PYMVPLTTVY LFEYLINQAV APTLLFPING
SpBtn1p PSINNNYTPI ESIDLRAGHV SFNFVNS--- -------LKQ TFIFMQPYLL SHMFPQFLVY FSEYTINIGV APTLLFP---

HsCLN3  ---------- SLSHAQQYRW YQMLYQAGVF ASRSSLRCCR IRFTWALALL QCLNLVFLLA DVWFGFLPSI YLVFLIILYE
ScBtn1p DERSKSMPFF FHKYRDIYVT YGTLYQLGVF ISRSFGHLMR MRSLYILAFL QGVNLCITVL QSWFYVTHSP WAVMILIFYE
SpBtn1p PEKAG----- FSSFRDFYPT YQTVYQIGVF LSRSSISFFT VPYLRTLAIT QFIILLFTIL QSALYLTSSY HFVLFLIFVE

HsCLN3  GLLGGAAYVN TFHNIALETS DEHREFAMAA TCISDTLGIS LSGLLALPLH DFLCQLS
ScBtn1p GFLGGASYVN TFLNILEQED PDETEFAMGA VSIADSFGVF LAALLGLGLE PKLCRHQIAD DRPWCRME
SpBtn1p GLIGGTVYVN VY-HSLQTTE SSQRELAIST VGSSDSSGIF LASLVSLFLE PSLCHFQADR GRDWCALT
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HsPPT1  MASPGCLWLL AVALLPWTCA SRALQHL--- -----DPPAP LPLVIWHGMG DSCCNPLSMG AIKKMVEKKI PGIYVLSLEI
DmPPT1  MISICCSRFS CILFLLFLIF SLVLSYIWWS PTKGGTNPEV LPVVLWHGMG DTCCVPFSLG AIMNLIVEQT KGGYVRSLQI

HsPPT1  GKTLMEDVEN SFFLNVNSQV TTVCQALAKD PKLQQGYNAM GFSQGGQFLR AVAQRCPSPP MINLISVGGQ HQGVFGLPRC
DmPPT1  GGNVLIDWQS GFFIHPNEQV DYVCKQLLQD EHLAKGYHAI GFSQGGQFLR AVAERCPNPP MRNLITLGGQ HQGIFGLPMC

HsPPT1  PGESSHICDF IRKTLNAGAY SKVVQERLVQ AEYWHDPIKE DVYRNHSIFL ADINQERGIN ESYKKNLMAL KKFVMVKFLN
DmPPT1  PTLTEKPCDY ITRLLDNAAY APEVQKALVQ ATYWHDPIME NKYRLGSTFL ADINNELFIN KFYIENLQKL KKFVMVQFLN

HsPPT1  DSIVDPVDSE WFGFYRSGQA KETIPLQETS LYTQDRLGLK EMDNAGQLVF LATEGDHLQL SEEWFYAHII PFLG
DmPPT1  DTIVQPKESQ WFQYYTTGQN KVIQPFTESK VYQD--LGLD KMHRQGQLVF LGVEGDHLAI SKAWFIQNIV PLLLEK
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HsPPT1   MASPGCLWLL AVALLPWTCA SRALQHLDPP APLPLVIWHG MGDSCCNPLS MGAIKKMVEK KIPGIYVLSL EIGKTLMEDV
Ceppt-1                                        MWHG MGDCCCNPLS MGSVKKLFEE QIPGVYVHSL QLGSSITKDI

HsPPT1   ENSFFLNVNS QVTTVCQALA KDPKLQQGYN AMGFSQGGQF LRAVAQRCPS PPMINLISVG GQHQGVFGLP RCPGESSHIC
Ceppt-1  EHGFYANTNE LVYMACIKIK NDPELKNGYN AIGFSQGAQF LRAVAQRCPN PPMKNLVSVG GQHQGVFGAP YCIGDNI-MC

HsPPT1   DFIRKTLNAG AYSKVVQERL VQAEYWHDPI KEDVYRNHSI FLADINQERG INESYKKNLM ALKKFVMVKF LNDSIVDPVD
Ceppt-1  NGVRRLIDLG AYLPFVQKRV VQAQYWHDPN QVEEYKKRSI FLADINNENN NNPTYKRNLL SLKNLVLVKF NQDHMVVPKD

HsPPT1   SEWFGFYRSG QAKETIPLQE TSLYTQDRLG LKEMDNAGQL VFLATEGDHL QLSEEWFYAH IIPFLG
Ceppt-1  SSWFGFYKDG DIDTILPMNE TDLYKEDRIG LKKLHESGRI HFMDVDGDHL QIPRSVLVND IIKKYFM
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C

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HsPPT1   MASPGCLWLL AVALLPWTCA SRALQHLDPP APLPLVIWHG MGDSCCNPLS MGAIKKMVEK KIPGIYVLSL EIGKTLMEDV
SpPdf1p        MKSF AIPIISLDKV RLAIND-GAS EQLPVVIWHG LGDTPTS-FT LTEVSQRVQK LTKGA-VYAI RVGDNEFEDI

HsPPT1   ENSFFLNVNS QVTTVCQALA KDPKLQQGYN AMGFSQGGQF LRAVAQRCPS PPMINLISVG GQHQGVFGLP RCPGESSHIC
SpPdf1p  KAGYLGKLED QLDEVCDLIG NEDSLSNGFY ALGLSQGGLF LRALAQTCDA AKIRSLITLG SPHSGINTIP GCSPTNLICK

HsPPT1   DFIRKTLNAG AYSKVVQERL VQAEYWHDPI KEDVYRNHSI FLADINQERG INESYKKNLM ALKKF---VM VKFLNDSIVD
SpPdf1p  AVVHSILGLG IWHSWIQNHV VQAQYYRTEK QYDKYLENNK FLTHLNNEV- LHDNYTRNIE KLKELDNLVA VSFERDDIVE

HsPPT1   PVDSEWFGFY RSGQAKETIP LQETSLYTQD RLGLKEMDNA GQLVFLATEG DHLQLSEEWF YAHIIPFLG
SpPdf1p  PPYSTGFGWI NETTG-ENIE MEDFVLY--E SLGLKDLVNQ GKLETISFPG RHLQMRWGDF DALVLKYFKD EKEEKTELEE

HsPPT1
SpPdf1p  STRPSNFLST YFVSPLVSAI DGTVDYLHGK SLFPEKRNFK ELTMRKRSIV TPEDSEEVYP YISEFVAASN VSEEKGPKSF

HsPPT1
SpPdf1p  ANLAFITIFS HFFYHIDDMW RSTLGLFSLI PQIIGIIYLT VMFTGRELDT FMQFGGQVVN EFINYVVKVS LKYPRPADIE

HsPPT1
SpPdf1p  YGVGYGMPSS HSQFMGFFSA YMIAWDYKYR RSQCFSMLSF AKYAIYLTLS TFVCSSRYLL DFHYLTQVVY GYMIGFGVGL

HsPPT1
SpPdf1p  FWVYLVGKLR SLGVTKWLLS LPPLQFFYIK DTIPHSKDNH KRQWLESKQF KNQKSN
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Fig. 2. Human PPT1 aligned with corresponding model organism homologs. Sequences were aligned using the multalin program. (A) Human PPT1 with fly PPT1; (B) Human PPT1 with worm PPT1; (C) Human PPT1 with yeast Ppt1p.

the deletion [34] (see below). Subsequent follow up studies on the pH of lysosomes from human fibroblasts indicated that pH was slightly elevated in JNCL, suggesting that indeed defects in CLN3 result in a disruption in the regulation of this organelle's pH [22,35]. However, limitations in what can be explored in cell culture means that it is not feasible to explore the possibility of a correlation between the dynamic change from lower to higher pH of the lysosome and the disease. Nevertheless, it is clear that the processes that govern vacuolar/lysosomal pH are disrupted in the absence of a functional Btn1p/CLN3 ultimately leading to an elevated pH of this organelle.

Besides pH, alterations in arginine levels in *btn1*-Δ cells have also been observed [21]. Both cytoplasmic and vacuolar arginine and lysine levels and ATP-dependent vacuolar arginine uptake are significantly decreased in *btn1*-Δ [21]. This is interesting since the vacuole acts as a storage organelle for sequestration of basic amino acids [36,37]. Like the pH defects, vacuolar arginine transport returns to normal when human CLN3 is expressed in *btn1*-Δ. Moreover, lysosomes isolated from JNCL patient human lymphoblasts cell lines demonstrate decreased arginine transport [21,38]. The role of Btn1p in both regulating vacuolar pH and arginine transport has recently been clarified. In a recent

Fig. 1. Human CLN3 aligned with corresponding model organism homologs. Sequences were aligned using the multalin program. (A) Human CLN3 with fly CLN3; (B) Human CLN3 with the three worm CLN3 sequences; (C) Human CLN3 with the yeast Btn1p sequences.

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HsCTSD   MQPSSLLPLA L--CLLAAPA S----ALVRI PLHKFTSIRR TMSEVGGSVE DLIAKGPVSK YSQAVPAVTE GPIPEVLKNY
DmCATHD  MQKVALLLVA FLAAAVAHPN SQEKPGLLRV PLHKFQSARR HFADVGTELQ QLRIR----- -------YGG GDVPEPLSNY

HsCTSD   MDAQYYGEIG IGTPPQCFTV VFDTGSSNLW VPSIHCKLLD IACWIHHKYN SDKSSTYVKN GTSFDIHYGS GSLSGYLSQD
DmCATHD  MDAQYYGPIA IGSPPQNFRV VFDTGSSNLW VPSKKCHLTN IACLMHNKYD ASKSKTYTKN GTEFAIQYGS GSLSGYLSTD

HsCTSD   TVSVPCQSAS SASALGGVKV ERQVFGEATK QPGITFIAAK FDGILGMAYP RISVNNVLPV FDNLMQQKLV DQNIFSFYLS
DmCATHD  TVSI------ -----AGLDI KDQTFAEALS EPGLVFVAAK FDGILGLGYN SISVDKVKPP FYAMYEQGLI SAPVFSFYLN

HsCTSD   RDPDAQPGGE LMLGGTDSKY YKGSLSYLNV TRKAYWQVHL DQVEVASGLT LCKEGCEAIV DTGTSLMVGP VDEVRELQKA
DmCATHD  RDPASPEGGE IIFGGSDPNH YTGEFTYLPV TRKAYWQIKM DAASIGD-LQ LCKGGCQVIA DTGTSLIAAP LEEATSINQK

HsCTSD   IGAVPLIQGE YMIPCEKVST LPAITLKLGG KGYKLSPEDY TLKVSQAGKT LCLSGFMGMD IPPPSGPLWI LGDVFIGRYY
DmCATHD  IGGTPIIGGQ YVVSCDLIPQ LPVIKFVLGG KTFELEGKDY ILRVAQMGKT ICLSGFMGLD IPPPNGPLWI LGDVFIGKYY

HsCTSD   TVFDRDNNRV GFAEAARL
DmCATHD  TEFDMGNDRV GFADAK

B

HsCTSD         MQPS SLLPLALCLL AAPASALVRI PLHKFTSIRR TMSEVGGSVE DLIAKGPVSK YSQA------ ---VPAVTEG
Ceasp-4  MNRCILLLLG ALLLVQGLHV HKRQQKLRTV SLKKQPTLRE TLLQAGSFET FAKHRHGYKK YLKTNGNHHF DKYQALNVEG

HsCTSD   PIPEVLKNYM DAQYYGEIGI GTPPQCFTVV FDTGSSNLWV PSIHCKLLDI ACWIHHKYNS DKSSTYVKNG TSFDIHYGSG
Ceasp-4  EIDELLRNYM DAQYFGTISI GTPAQNFTVI FDTGSSNLWI PSKKCPFYDI ACMLHHRYDS KSSSTYKEDG RKMAIQYGTG

HsCTSD   SLSGYLSQDT VSVPCQSASS ASALGGVKVE RQVFGEATKQ PGITFIAAKF DGILGMAYPR ISVNNVLPVF DNLMQQKLVD
Ceasp-4  SMKGFISKDS VCV------- ----AGVCAE DQPFAEATSE PGITFVAAKF DGILGMAYPE IAVLGVQPVF NTLFEQKKVP

HsCTSD   QNIFSFYLSR DPDAQPGGEL MLGGTDSKYY KGSLSYLNVT RKAYWQVHLD QVEVASGLTL CKEGCEAIVD TGTSLMVGPV
Ceasp-4  SNLFSFWLNR NPDSEIGGEI TFGGIDSRRY VEPITYVPVT RKGYWQFKMD KV-VGSGVLG CSNGCQAIAD TGTSLIAGPK

HsCTSD   DEVRELQKAI GAVPLIQGEY MIPCEKVSTL PAITLKLGGK GYKLSPEDYT LKVSQAGKTL CLSGFMGMDI PPPSGPLWIL
Ceasp-4  AQIEAIQNFI GAEPLIKGEY MISCDKVPTL PPVSFVIGGQ EFSLKGEDYV LKVSQGGKTI CLSGFMGIDL PERVGELWIL

HsCTSD   GDVFIGRYYT VFDRDNNRVG FAEAARL
Ceasp-4  GDVFIGRYYS VFDFDQNRVG FAQAKTADGR PVDPAPRPFR SVFDNESEES MEQDDE

C

HsCTSD          MQP SSLLPLALCL LAAPASAL-- --VRIPLHKF T------SIR RTMSEVGGSV EDLIAKG-PV SKYSQAVPAV
ScPep4p        MFSL KALLPLALLL VSANQVAAKV HKAKIYKHEL SDEMKEVTFE QHLAHLGQKY LTQFEKANPE VVFSREHPFF
SpSxa1p  MKASFFVFAI SALQALQASV ASAYSEVPGK RSVVLNLQHS QYDHVARKLE RTKVLNKRDS SGYPVLDLEY TDAGGYFANL

HsCTSD   TEGPIPEVLK NYMDA-QYYG EIGIGTPPQC FTVVFDTGSS NLWVPSIHCK LLDIACWIHH KYNSDKSSTY VKNGTSFDIH
ScPep4p  TEGGHDVPLT NYLNA-QYYT DITLGTPPQN FKVILDTGSS NLWVPSNECG SL--ACFLHS KYDHEASSSY KANGTEFAIQ
SpSxa1p  TLGSNERVYS LTLDTGSPYI WVTAKNITAL SASEIWSDTD GVDAGRSTSD IRTNACTNYT CFDYSSTTAR RTNSSTIGFL

HsCTSD   YGSGSLSGYL SQDTVSVPCQ SASSASALG- GVKVERQVFG EATKQPGITF IAAKFDGILG MAYPRISVNN VLPVFDNLMQ
ScPep4p  YGTGSLEGYI SQDTLSI--- ---------G- DLTIPKQDFA EATSEPGLTF AFGKFDGILG LGYDTISVDK VVPPFYNAIQ
SpSxa1p  ASYGDNTTVL GYNMVDNAYF AGLTLPGFEF GLATREYDSS QISVTPGIIG LSVAMTITGI SSDDKVVAFT PPTIVDQLVS

HsCTSD   QKLVDQNIFS FYLS-RDPDA QPGGELMLGG TDSKYYKGSL SYLNVTRK-- -AYWQVHLDQ VEVASGLTL- ----------
ScPep4p  QDLLDEKRFA FYLGDTSKDT ENGGEATFGG IDESKFKGDI TWLPVRRK-- -AYWEVKFEG IGLGDEYAE- ----------
SpSxa1p  ANVIDTPAFG IYLN------ EDVGELIFGG YDKAKINGSV HWVNISSSDD STFYSVNLES ITVTNSTSSN NVQSSKRSSK

HsCTSD   -CKEGCEAIV DTGTSLMVGP VDEVRELQKA IGAVPLIQGE YMIPCEKVST LPAITLKLGG -KGYKLSPED YTLKVSQAGK
ScPep4p  -L-ESHGAAI DTGTSLITLP SGLAEMINAE IGAKKGWTGQ YTLDCNTRDN LPDLIFNFNG -YNFTIGPYD YTLEVSGS--
SpSxa1p  DIEVNTTVTL DTGTVYIYLP EDTVESIADQ YQGIVSEYGY VVIYCDSFSD SDYISFNFGS DADFHVSVND LVIYRQES--

HsCTSD   TLCLSGFMGM DIPPPSGPLW ILGDVFIGRY YTVFDRDNNR VGFAEAARL
ScPep4p  --CISAITPM DFPEPVGPLA IVGDAFLRKY YSIYDLGNNA VGLAKAI
SpSxa1p  --TSGDICYL ALFEGDTSSY LLGQYFLQYV YSIYDWDAQK IGLAALNSNA TSTANHQILN INSALRSVTS GQSVSATPTV

HsCTSD
ScPep4p
SpSxa1p  SMSIAATSFG SSLVLTASAS PSSTSVDGSS SSDSSEASGA ASVGVSISAI VLCASTLISL LFA
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Fig. 3. Human CTSD aligned with corresponding model organism homologs. Sequences were aligned using the multalin program. (A) Human CTSD with fly CATHD; (B) Human CTSD with asp-4; (C) Human CTSD with the yeast Pep4p and Sxa1p sequences.

publication, the coupling of proton pumping and the activity of the vATPase was found to be dependent on extracellular pH [33]. Importantly, *btn1*-Δ, results in an alteration in the coupling of proton pumping and the activity of the vATPase [33]. Thus, defective arginine transport in *btn1*-Δ could result from an alteration in the regulation of the electrochemical gradient driving this transport [33]. Interestingly, subsequent studies aimed at dissecting out whether alterations in intracellular arginine impact cells lacking Btn1p (*btn1*-Δ) have revealed that overexpression of Can1p, the plasma membrane basic

amino acid transporter, is lethal [39,40]. It is tempting to speculate that the decrease in intracellular arginine and lysine levels may result from a buffering mechanism against arginine and lysine being toxic to cells lacking Btn1p [39,40]. Alternatively, it should be noted that *BTN1*+ cells with either endogenous or overexpressed levels of Can1p have similar rates of plasma membrane arginine uptake, suggesting that *btn1*-Δ cells may lack the ability to regulate the amount or activity of Can1p at the plasma membrane, resulting in arginine and lysine toxicity [40]. It is pertinent to point out that as amino acid levels, and possibly metabolism, are clearly affected by *btn1*-Δ, the studies described have avoided the typical use of auxotrophic markers for gene deletion and genetic studies [41]. In other words, *btn1*-Δ strains have identical amino acid growth requirements to that of wild type to avoid studying artifacts of altered amino acid metabolism.

A recent study has highlighted involvement of a third pathway in the absence of Btn1p, namely nitric oxide (NO) synthesis [42]. As arginine serves as the substrate for NO synthesis this is also likely linked to the aforementioned alterations in arginine levels. Specifically, a new publication by Osorio and colleagues demonstrated that *btn1*-Δ cells are more resistant to menadione due to defective synthesis of NO and consequent decreased levels of reactive oxygen and nitrogen species. If *btn1*-Δ cells are preincubated with arginine before menadione exposure, the phenotype is lost, suggesting that the decrease in nitric oxide results from the decrease in cellular arginine [42]. Although this phenotype may result as a consequence of the primary defect associated to lacking Btn1p, it could underlie pathophysiological aspects of the disease. It will be important to recapitulate these observations in human cell lines.

Microarray analysis of *btn1*-Δ strains revealed that *BTN2* mRNA levels were increased in a *btn1*-Δ background [29]. Btn2p interacts with Yif1p, Rhb1p, and Ist2p, with deletion of *BTN2* resulting in an altered localization of these proteins [30,43,44]. Yif1p is involved in ER to Golgi transport, Rhb1p is a small GTPase that has been implicated in plasma membrane arginine transport regulation, and Ist2p is a putative ion channel at the plasma membrane [45–48]. Taken together, these observations would suggest that Btn2p is involved in trafficking. Btn2p does not have a true mammalian homolog, however, as a cytosolic coiled-coil protein, it shows very specific domain similarity to the higher eukaryotic protein Hook1. Hook1 is a microtubule binding protein involved in trafficking to the late endosome, multivesicular body formation, and endosomal fusion [49–53]. Up-regulation of Btn2p may be explained by the pH alterations as it has been shown that decreases in vATPase activity can have an effect on protein trafficking and degradation [54–60]. Therefore, up-regulation of Btn2p may result in a compensatory response to minimize disruptions in protein trafficking in *btn1*-Δ cells due to altered intracellular pH.

In summary, it appears that the primary defect in *btn1*-Δ involves a disruption in regulation of vacuolar pH, suggesting that Btn1p functions in a pH regulatory pathway. However, at this point a direct role for Btn1p in arginine homeostasis, nitric oxide production or protein trafficking cannot be ruled out. It is important to note that studies thus far have focused on determining the role for Btn1p through the use of *btn1*-Δcells that lack Btn1p and are therefore based on a loss-of-function model, where the cell may be altering these pathways to correct for the loss of Btn1p. It is possible that pH alterations are a secondary consequence of the loss of Btn1p, and the primary defect is yet to be elucidated. Future studies are focused on looking on the direct function of Btn1p, especially its role in coupling transport mechanisms with vATPase activity. *S. cerevisiae* has played and will continue to play a strong part in the efforts to understand JNCL.

Recently, Cathepsin D mutations have been implicated in CNCL [1]. Cathepsin D, a lysosomal aspartic protease, is highly conserved throughout lower and higher eukaryotes. *S. cerevisiae* Pep4p shares 43% identity and 58% similarity with human Cathepsin D (Fig. 3) [61]. Pep4p is a nonessential enzyme required for activation of other vacuolar proteases and glucose-induced vacuolar degradation of peroxisomes, with deletion of *PEP4*, *pep4*-Δ, resulting in accumulation of vacuolar protease precursors and aberrant vacuolar morphology [62–69]. Because Pep4p and *pep4*-Δ are well characterized, *S. cerevisiae* could become a useful CNCL model.

2.2. *Schizosaccharomyces pombe*

Fission yeast, *Schizosaccharomyces pombe,* has recently been developed as a model system to study Batten disease. It is a popular model organism that over the past 50 years has greatly influenced the understanding of cell cycle control and cell division. It is a rod-shaped eukaryote that grows in a highly polarized manner and divides by medial fission. Fission yeast have 4912 genes organized onto three chromosomes, which is the smallest number of protein-coding genes yet recorded for a eukaryote. Like *S. cerevisiae,* fission yeast has a fast cell cycle and exists in both the haploid and diploid states. Genes can be readily deleted, mutated and tagged allowing for deletion/mutation characterization and localization studies. Inhibition of gene expression by RNAi is also under development. A deletion library of all nonessential genes is near completion, as is a library containing almost all the genes tagged with green fluorescent protein (GFP). cDNA and genomic libraries exist that allow for powerful suppressor screen studies. Importantly for the study of lysosomal storage disorders, fission yeast cells have large numbers of small vacuoles (50–80 per cell), the yeast equivalent of the lysosome [70]. Two NCL genes are conserved in *S. pombe*, namely *CLN1* and *CLN3*. The fission yeast homolog of human CLN3, termed Btn1p, is a predicted transmembrane protein of 396 amino acids that is 30% identical and 48% similar to its human counterpart (Fig. 1). Importantly, the same residues that are mutated in Batten disease patients are conserved in Btn1p, suggesting that they are important for the function of both *CLN3* and Btn1p.

Recent work has focused on cell morphology and vacuole function in a strain deleted for the *btn1* gene, *btn1*Δ. This strain is viable, but shows subtle and reproducible defects in cell cycle progression, with an increased cell size and an increase in the

number of mitotic and dividing cells [34]. Significantly, alterations in vacuole dynamics are evident. First, vacuoles from *btn1*Δ cells are larger than those of wild type cells with a mean size of 1.3 μm compared to 0.9 μm, and show a broader vacuole size distribution, suggesting an inherent defect in vacuole size regulation. Second, *btn1*Δ cells have an elevation in vacuole pH of 1 pH unit, (pH 5.1 compared to pH 4.1 for wild type cells) [34]. A correlation is known to exist between increased vacuole size and increased pH in fission yeast [71], and growth of the *btn1*Δ strain in acidic media (pH 4) was found to restore the vacuolar size defect to wild type levels [34]. Thus, vacuole size is a reflection of the increased pH of the vacuole in *btn1*Δ cells. *btn1*Δ cells were also found to be sensitive for growth in media containing 1 mM ANP. Although the mechanism for this sensitivity in fission yeast remains to be determined, growth of *btn1*Δ on plates containing 1 mM ANP was restored when this media was at pH 4 (S. Codlin, unpublished). Thus, sensitivity of *btn1*Δ cell appears to be related to pH homeostatic mechanisms. The increased vacuolar pH and ANP sensitivity in *S. pombe* are in contrast to *S. cerevisiae btn1-*Δ cells which have alterations in vacuolar pH that change through growth, and is resistant to ANP. Although intracellular pH in many yeast species including *S. cerevisiae* and *S. pombe* has reported to be similar [72,73], these studies indicate a clear difference in wild type vacuolar pH between other yeast and *S. pombe,* with *S. pombe* having a vacuolar pH near 4 and *S. cerevisiae* a pH around 6.2. Although, vacuolar pH for *S. pombe* was previously reported to be in the range of pH 6–6.2 [73], similar to that of *S. cerevisiae,* this study was performed in cells that were starved for nitrogen, and hence is not a measure of vacuolar pH in growing cells. In the *btn1*Δ *S. pombe* studies the fluorophore lysosensor green D189 which vividly fluoresces at the vacuole in wild type *S. pombe* was used. This compound fails to fluoresce above pH 6, supporting the results that the vacuole pH of wildtype *S. pombe* is less than pH 6 [34]. This, in turn, may be the basis for the difference in sensitivity to ANP and vacuole pH of *BTN1* deletions of the two different yeast models.

The vacuolar pH of *S. pombe* is similar to that of mammalian cells which are also acidic [74]. Expression of btn1 and CLN3 in fission yeast deleted for btn1 causes a decrease in pH of the vacuoles [34]. These results are in contrast to overexpression studies of CLN3 in HEK293 cells [35], where lysosomal pH is increased but in agreement with studies on JNCL fibroblasts that had a slightly elevated lysosomal pH [74]. Importantly, a clear genetic interaction has been established between *btn1* and the vATPase, the major complex associated with vacuolar acidification. Cells deleted for both *vma1* and *btn1* exhibited slow growth and synthetic lethality at 30 °C [34]. While the molecular basis of this interaction has not been determined, previously described studies in *S. cerevisiae* implicating Btn1p in the coupling of proton pumping and the activity of the vATPase [33], may provide a clue.

Overexpression of N-terminally fused GFP-Btn1p and GFP-CLN3 constructs in *btn1*Δ cells complemented the vacuolar size and pH defects as well as the subtle cell growth defects, proving that Btn1p and CLN3 are functional homologs. In fission yeast, both Btn1p and CLN3 traffic to the vacuolar membrane via FM4–64 stained pre-vacuolar compartments, suggesting an endocytic trafficking route for Btn1p. Localization of Btn1p using a functional GFP-tagged Btn1p to the vacuole membrane was Ras GTPase Ypt7p dependent, with Btn1p being excluded from the vacuole and held in prevacuolar compartments in *ypt7*Δ cells [34]. Cells deleted for both *ypt7* and *btn1* showing synthetic lethality at 36 °C and vacuoles in these cells were larger than those of cells deleted for *ypt7* alone and again showed reduced pH. Btn1p must therefore have a functional role prior to reaching the vacuole, and this function impacts on vacuolar function.

In addition to the subtle growth defects in cell cycle progression of *btn1*Δ cells at 25 °C, *btn1*Δ cells are also temperature sensitive for growth at 37 °C (S. Codlin, unpublished). *btn1*Δ cells passed through no more than three cell cycles at this temperature and subsequently lost rod-shaped morphology, resulting in swollen and rounded cells. Cell lysis was found to be the subsequent cause of cell death. Electron microscopy reveals grossly thickened cell walls and septum regions, suggesting defects in the development of the main cell wall components, the ά- and β-glucans. Indeed, *btn1*Δ cells were found to be highly sensitive to zymolase, a β-glucanase, but not to novozyme, an ά-glucanase. Also, the swelling phenotype and cell lysis were completely rescued by the addition of 1 M sorbitol, an osmolyte, to the media, suggesting that the swelling and lysis may be caused by aberrant osmoregulation in *btn1*Δ cells [34].

S. pombe also has a homolog *PPT1*, which encodes a lysosomal palmitoyl protein thioesterase 1 (Fig. 2) [75]. The *PPT1* gene product is mutated in INCL patients [75]. The *S. pombe* ORF SPBC530.12c encodes the homolog to human *PPT1*, denoted Ppt1p, fused to dolichol pyrophosphate phosphatase 1, Dolpp1p. The entire coding region is denoted *pdf1* and the resulting propeptide is cleaved into its two distinct proteins most likely by a kex-related protease [76]. Through deletion and complementation of *pdf1*, Cho and Hofmann observed that Ppt1p is not required for viability, but Dolpp1p is. However, Ppt1p is required for growth in sodium orthovanadate and basic pH, a phenotype that indicates lysosomal dysfunction in *S. pombe*, and, importantly, expression of human *PPT1* could complement for this phenotype suggesting that the primordial function of the protein is conserved [76]. This study again links the NCLs to pH alteration phenotypes and supports the use of fission yeast to study NCLs.

Fission yeast is also a potential model for CNCL as it has a homolog to Cathepsin D, which is implicated in CNCL [1]. The *S. pombe* protein Sxa1p is 22% similar and 40% identical in amino acid sequence to human Cathepsin D (Fig. 3). Sxa1p is an aspartyl protease involved in the mating pathway [77]. Thus, fission yeast has proved to be a valid model for several NCLs and will be valuable tool in the understanding of the molecular basis and progression of CNCL.

2.3. *Caenorhabditis elegans*

The nematode *Caenorhabditis elegans* is a relatively simple multicellular eukaryotic organism of approximately one mm in

length, which normally lives in soil [78]. These worms have a reproduction cycle of approximately 3 days and a life span of about two to 3 weeks. The *C. elegans* genome, divided over six chromosomes, has been completely sequenced, and is predicted to contain about 19,000 genes [79]. The existence of the hermaphrodite sex, which by self fertilization after a cross of two different mutant strains easily yields homozygote offspring and the possibility to grow large quantities for large genetic screens, has made this animal a popular model for genetic research [80]. Moreover, extensive research into the nematode anatomy and development has lead to a complete map of all 959 somatic cells and a full understanding of their cell lineage. The nervous system of the worm comprises of 302 neuronal cells with approximately 7600 synaptic junctions and is invariably wired [81]. The broad knowledge of the worm's neuronal anatomy and wiring combined with its genetic power make this organism very suitable to investigate the molecular mechanisms underlying neuronal disorders.

Homologs of the *CLN1* and *CLN3* genes involved in the infantile and the juvenile forms of NCL, respectively, have been identified in *C. elegans* (Figs. 1 and 2). Models for both diseases have been generated using worms with deletion mutations identified by the *C. elegans* knock-out consortium and other laboratories. The *ppt-1* mutant, PPT-1 being the homolog to the protein mutated in INCL patients, was isolated out of a nematode mutant library that was generated by chemical mutagenesis [82]. Two different *ppt-1* mutant strains were isolated, both carrying a deletion of at least two exons predicted to lead to truncation of the protein [83]. Although *ppt-1* mutants displayed no morphological, locomotion or neuronal defects, the onset of egg-laying was delayed for approximately 4 h. The *ppt-1* mutants appeared to carry more embryos than wild type, and 17.8% of them displayed "bagging", a reproductive phenotype found in only 1.6% of the wild type worms, where eggs hatch inside the parent. Since not all of the mutant worms that failed to lay them on time displayed the "bagging" phenotype, the effect of the mutation varies in severity. Life span and brood size of the *ppt-1* mutants were similar to wild type, when worms with the bagging phenotype were not taken into account. Furthermore, *ppt-1* mutants were suggested to have a decreased "health span", i.e. they appeared to age faster and were less motile earlier than wild type. In electron micrographs of *ppt-1* mutant nematodes, neuronal cells presented many enlarged mitochondria containing less cristae and whorling inner membranes. The *ppt-1* mutants contained 26–29% more mitochondria than wild type, and in six-day old adult worms the average size of *ppt-1* mutant mitochondria was severely decreased. Currently, it is unclear what caused this phenotype, but it is too mild to be used in genetic screens for genes that may enhance or suppress the mitochondrial effect. The *ppt-1* mutants, however, did not display two of the main characteristics of INCL, neuronal degeneration or accumulation of storage material. Nonetheless, the mitochondrial abnormalities of the *ppt-1* mutants suggest that these organelles are involved in NCL pathogenesis, as previously suggested [84–97] and have prompted further investigations into the integrity of mitochondria in INCL patients as well as other INCL model organisms.

The generation of worm models for juvenile NCL turned out to be more complicated: *C. elegans* is the only model organism with three *CLN3* homologs, designated *cln-3.1*, *cln-3.2*, and *cln-3.3*. The presence of three *CLN3* homologs in *Caenorhabditis briggsae*, a nematode species closely related to *C. elegans* suggests that the three genes have evolved before the separation of the two species, some 100 million years ago [97]. Assuming that the genes result from ancient duplications of a common ancestor, their genomic sequences have diverged beyond recognition, but the encoded protein sequences show considerable homology [98]. The degree of conservation across their complete protein sequences suggests that none of the genes is a pseudogene, which is expressed but has lost most of its original function, as was shown for the *elt-4* gene [99]. The biological reason for the existence of multiple CLN3 genes in the worm is unknown, but expression analysis of the *cln-3* genes using promoter–GFP fusion constructs indicated that these genes differ in their temporal and spatial expression patterns. Expression of *cln-3.1* was restricted to cells of the intestine and was observed in transgenic hermaphrodite and male worm embryos, larvae and adults [100]. The *cln-3.2* gene is expressed in cells of the hypoderm only in adult worms of both sexes. Expression of *cln-3.3* was detected in the intestinal muscle cells and hypoderm of adult hermaphrodite and male worms and also in posterior diagonal muscle cells of males. Except for co-expression of the *cln-3.2* and *cln-3.3* genes in hypodermal cells of adult worms, each of the *cln-3* genes is not expressed at detectable levels in all cells. It should be noted that this does not indicate that the cln3-1 genes are not expressed, but merely that expression levels in other cell types (neurons) are likely to be below the detection threshold of GFP by fluorescence microscopy.

Apart from the number of *CLN3* homologs, the nematode also differs from other model organisms in the organization and regulation of some of its genes. *C. elegans* is one of the few multi-cellular eukaryotic organisms in which several genes are organized in operons [101]. In *C. elegans* the question, whether the gene products of operons are functionally related or just linked to ensure coordinated temporal expression, cannot be answered unequivocally in all cases and remains a topic of investigation and discussion. The *cln-3.2* and *cln-3.3* genes have closely located upstream genes and may therefore be in an operon. In accordance with this, *cln-3.2* was found to be *trans*-spliced to a SL2 spliced leader, but *cln-3.3* is associated to an SL1 spliced leader. However, we cannot completely exclude that *cln-3.3* and its upstream gene, ZC190.2, are members of an operon, as the putative *cln-3.3* promoter–GFP fusion construct failed to cause GFP fluorescence in transgenic nematodes (De Voer *et al.*, unpublished results), whereas transgenic worms containing a larger upstream sequence including the ZC190.2 promoter and gene in front of the *cln-3.3* promoter–GFP fusion did show GFP fluorescence [100]. Since both reporter constructs contain an in-frame fusion of GFP to the first three exons of *cln-3.3* and thus are partial translational reporter constructs [102], the GFP fluorescence from the longer one can only be caused by the presence of additional cis-acting elements. Currently, it remains unclear whether the ZC190.2

promotor drives also the cln3.3 expression or whether cis-acting elements overlapping the ZC190.2 coding region are responsible for the observed expression pattern.

The *cln-3.2* gene is the fourth in an operon also containing *erm-1*, *dnj-4*, and *dhs-1*. The first gene, *erm-1*, encodes a protein with homology to ezrin, radixin, and moesin proteins of the ERM family of cytoskeletal linkers, and is involved in organism development and positioning of cell–cell contacts [103]. ERM proteins have diverse roles in cell architecture, cell signaling and membrane trafficking [104], and have recently been shown to be important for actin assembly by phagosomes, which may facilitate their fusion with lysosomes [105]. This gene is expressed from the two-cell stage onward throughout the entire life of the worm in epithelial cells lining the luminal surfaces of intestine, excretory canal, and gonad, whereas the *cln-3.2* gene was expressed in the hypoderm of adult worms [100]. This difference in expression between genes in the same operon could be caused by common errors in operon transcription, of which the probability decreases with increasing distance between the operon genes, or their mRNAs may be subject to differential mRNA destabilization [106]. Expression patterns of the other operonic genes, *dnj-4*, *dhs-1*, have not been reported, and RNAi knockdown of these genes did not result in obvious phenotypes (Wormbase website). Therefore, the function of these genes can only be derived from protein sequence homology. The DHS-1 protein has both chaperone and heat shock protein domains, which could indicate a role in protein folding. The dhs-1 protein has dehydrogenase and reductase domains and may have a function in metabolism of short chain alcohols. Although a role of ERM proteins in lysosome–phagosome fusion potentially connects it functionally with *cln-3.2*, it is unclear whether a functional relationship exists between *cln-3.2* and the other genes in this operon.

JNCL worm models with single *cln-3.1*, *cln-3.2*, or *cln-3.3* deletions were generated from the original mutants isolated from the deletion mutant libraries by out-crossing six times into wild type background to remove additional mutations [100]. Since the *cln-3* single mutant models had a wild type appearance, which might be caused by redundancy, they were crossed to generate three double and one triple *cln-3* mutant models. The *cln-3* triple mutant model was viable and superficially displayed wild type behavior and normal morphology, indicating that the *cln-3* genes are not essential. Comparison of the life span of the different models to wild type worms suggested the *cln-3.1* mutant has a shorter life span than wild type worms, while *cln-3.2* and *cln-3.3* single mutants have a normal life span. This effect becomes more prominent in the *cln-3* triple mutant when *cln-3.2* and *cln-3.3* are also deleted. The *cln-3.2* single mutant has a decreased brood size compared to wild type. The brood size of the *cln-3* triple mutant is decreased more prominently than that of the *cln-3.2* single mutant, even though the other single mutants do not have a significantly decreased brood size. To detect functional aberrations, the *cln-3* triple mutants have been investigated extensively, using assays for correct neuronal function and response to a diversity of external cues, such as temperature, touch, presence of other worms, mating behavior (De Voer et al., unpublished results). The integrity of the *cln-3* triple mutant nervous system was investigated using GFP which was expressed from the *unc-119* promoter in neuronal cells and was similar to wild type in all of the tests. Electron micrographs of *cln-3* triple mutant neurons did not reveal altered morphology or the presence of lysosomal storage material. The *cln-3* triple mutants could not be distinguished from wild type worms after staining with organelle or compound specific fluorescent dyes, Lysotracker Red, Acridine Orange, and Nile Red to assess whether lysosomes, acidic organelles and lipid content, respectively, were altered.

Human Cathepsin D, the protein mutated in CNCL patients, and the worm homolog asp-4 share 58% identity and 73% similarity (Fig. 3) [1]. Worm asp-4 is an aspartyl protease that mediates necrotic cell death and is required for neurodegeneration [107–109]. Like the other model organisms, CNCL research could benefit from work on the worm homolog of Cathepsin D.

2.4. Drosophila melanogaster

The evolutionary conservation of gene function between humans and *Drosophila* make it an ideal model system for the study of common eukaryotic cell biological mechanisms. Furthermore, the sophisticated cell biological and genetic reagents available in the fly provide the experimental tools for elucidating the biological function of any gene that may play a role in human disease processes. In regards to neurological disorders, a recent study has shown that of the molecularly defined human genetic disorders that produce mental retardation, which includes the NCLs, approximately 87% have a homolog in the *Drosophila* genome [110]. The success of using *Drosophila* as an important model of neurological disease is obvious in the contributions the fly has made to the understanding of several kinds of neuronal degeneration including Huntington's, Parkinson's, Alzheimer's, and several Spinocerebellar Ataxias (reviewed in [111,112]).

As with the other small eukaryotic model systems, *Drosophila* has clear homologs of the CNCL, JNCL, and INCL disease genes (Figs. 1–3). The analysis of several genes, including the INCL homolog *Drosophila ppt1*, suggests that the fruit fly will also be an extremely valuable model system to study lysosomal storage disorders and the NCLs in particular. For example, while the human homolog has yet to be implicated in an NCL disorder, mutations in a predicted lysosomal sugar carrier *benchwarmer* (*bnch*) produce both autofluorescent lipopigments and neural degeneration characteristic of NCLs [113,114]. Analysis of *bnch* mutations demonstrated endo-lysosomal trafficking defects, defective larval synapse development, impaired synaptic vesicle recycling, and age-dependent synaptic dysfunction [113,114]. Mutations in the fly homolog of the lysosomal aspartyl protease *cathepsinD* (Fig. 3), known to produce an ovine NCL and the recently identified congenital NCL, also produce autofluorescent storage material and a low level of age-dependent neurodegeneration in the adult *Drosophila* brain [115]. These fly mutants, along with the phenotypes of *ppt1*$^{-}$ flies described

below, suggests an evolutionary conservation of cell biological mechanisms in the fly that will be useful in our understanding of human NCL phenotypes [115,116].

The *Drosophila* PPT1 homolog is ~55% identical and ~72% similar to the human protein at the amino acid level (Fig. 2) [117]. The *ppt1* transcript appears to be expressed ubiquitously, although at different levels during all stages of fly development [117]. Consistent with the levels of mRNA, PPT1 enzymatic activity is present at varying levels in all tissues that have been tested [117]. The development of a *Drosophila* human disease model can be approached in two distinct, yet complementary ways by producing a loss-of-function mutation in the fly homolog or by using a gene-overexpression approach to dissect gene function [112]. In the first approach a disease model can be developed to observe whether aspects of the INCL phenotype are recapitulated in $ppt1^{-}$ flies. The second approach makes use of a modular misexpression system to overexpress *ppt1* in order to produce an *in vivo* assay of the protein's function. The results of both approaches for INCL in *Drosophila* have proven to be fruitful.

Loss of function analysis of the *ppt1* gene has been performed through the use of a small deficiency, RNA interference and several point mutations in the gene [116,117]. $Ppt1^{-}$ flies are viable, although with a reduced life span, and have storage material but no visible signs of neurodegeneration [116]. Targeted over-expression of *ppt1* in the developing *Drosophila* visual system using GMR-Gal4 leads to the loss of cells, including neurons, through apoptotic cell death both early in eye development and also after ommatidial differentiation has finished, yielding black ommatidial spots [118]. To determine whether the abnormal eye phenotypes were the result of increased levels of *ppt1* enzymatic activity or are due to an ectopic, non-wildtype function of the protein, an enzyme dead form of *ppt1* was also misexpressed. Expression of a *ppt1* serine 123 to alanine (S123A) catalytic mutant with GMR-Gal4 yielded no observable abnormal phenotypes when analyzed with SEM [118]. Further quantification of semi-thin retinal sections demonstrated a significant reduction in loss of rhabdomeres in *ppt1-S123A* over-expressing eyes when compare to overexpression of the wild type enzyme indicating that a majority of the *ppt1* over-expression phenotypes observed are due to increased levels of the wildtype protein activity [118]. These findings demonstrate that, while recessive mutations that severely decrease PPT1 activity cause neuronal cell death in INCL patients, increased levels of PPT1 activity can also lead to neurodegeneration, revealing that the precise level of PPT1 activity is important for neuronal cell survival [118]. The development of both loss- of-function and gain-of-function models for *ppt1* in *Drosophila* provides a firm base for the future examination of the cellular basis of neuronal dysfunction and degeneration in INCL patients.

The next step in NCL research is the elucidation of what cellular processes are being disrupted in the patient's neuronal cells that ultimately lead to dysfunction and degeneration. Towards this end, work in cultured $ppt1^{-/-}$ mouse neuronal cells demonstrated a reduction in synaptic vesicle pool size in the absence of large amounts of storage material suggesting that synaptic abnormalities may contribute to the early progression of INCL [119]. The *Drosophila* model system will also play an important role in this endeavor. The power of the *Drosophila* system lies in the ability to perform large-scale second site genetic modifier screens once a fly disease model has been produced [112]. These genetic screens allow the unbiased identification of the cellular pathways that are relevant to protein function and disease progression. For other neurodegenerative disorders, such as Huntington's disease, the results of *Drosophila* modifier screens have led to the identification of genes that may be therapeutic targets for the treatment of human patients [120]. Modifier screens focused on *ppt1* are presently underway in *Drosophila* the results of which will facilitate the identification of *in vivo* substrates and signaling pathways that are regulated by *ppt1* activity. While only *ppt1* has been extensively studied in *Drosophila*, the success of this work bodes well for future genetic analysis and functional characterization of the *Drosophila* JNCL gene, *cln3*.

Human cathepsin D (CTSD) and fly cathepsin D (CATHD) are 53% identical and 68% similar in amino acid sequence (Fig. 3). A Cathepsin D-deficient fly contains storage material that at the ultrastructural level is similar to the storage material in CNCL and INCL patients, indicating that the fly will be an appropriate organism to study the storage material and NCL-disease [1,115].

3. Autofluorescent storage material

The NCLs belong to a large group of disorders commonly termed lysosomal storage disorders. For a majority of these disorders, the substrates that accumulate and the mutated gene products have been identified. What remains unclear is why the build-up of these particular substrates produces the observed disease pathophysiology [121]. Is the substrate accumulation the primary cause of the disorders or rather a secondary symptom of the disease? In some cases it is becoming clear that the storage phenotype underlies more significant changes in cell physiology produced by the loss of a particular protein. The loss of the protein may have a primary effect on cellular processes such as cellular trafficking or signal transduction, which on their own, or through secondary effects, leads to the disease pathology [121]. Recent work in mice, sheep and humans has thoroughly described the cellular storage materials and progressive cellular pathology in affected NCL brains (reviewed in [122]). This work suggests that there is no clear connection between the amount of storage material and the observed neurodegeneration in these systems [122,123]. The analysis of the *cathepsinD* and *ppt1* fly mutants further supports this hypothesis [115,116].

The *ppt1* mutant flies show CNS-specific accumulation of autofluorescent storage material characteristic of the NCLs and abnormal cytoplasmic inclusions although they are different in morphology to the granular osmiophilic deposits typical of INCL patients [116]. Analysis of the adult nervous systems of the $ppt1^{-}$ flies by transmission electron microscopy reveals the presence of cytoplasmic deposits with some similarities to the

multi-laminate deposits present in Tay–Sachs patients [116]. Expression of a *ppt1* cDNA in the nervous systems of *ppt1*$^-$ flies using the Gal4/UAS system rescues the laminar deposit phenotype and the autoflouresence in *ppt1*$^-$ males was rescued by a genetic duplication of the *ppt1* chromosomal region located on the Y chromosome [116]. Taken together, these data suggest that the NCL-like phenotypes observed are due to the loss of *ppt1*.

In *C. elegans ppt-1* and *cln-3* triple mutant worms, storage of autofluorescent lipopigments could not be detected. Therefore, an attempt was made to increase the amount of lipopigments in the *cln-3* triple mutant worm model by overexpressing the main component of the storage material found in Batten disease patients, the hydrophobic subunit c of the mitochondrial ATP synthase [124–127]. The only homolog to the human *ATP5G1* gene encoding subunit c in *C. elegans* is *atp-9*. Overexpression of this worm homolog was deleterious to wild type animals, causing overall structural impairment, increased transparency, and near paralysis (De Voer et al., manuscript in preparation). On electron micrographs of worms overexpressing subunit c, damaged mitochondria could be observed, which is in accordance to the loss of mitochondrial staining with Mitotracker Red seen in these animals. A mild subunit c overexpression in a *cln-3* triple mutant background allowed the worms to survive, but did not result in an obviously different phenotype compared to mild subunit c overexpression in a wild type background.

It has been assumed that the use of single-celled organisms like yeast in the study of lipopigment was futile due to their short life-spans. Not only did they not live long enough to accumulate the storage material to a detectable level, but normal budding yeast have increased total cellular fluorescence as they age, making it difficult to detect the vacuolar storage material fluorescence above normal background fluorescence. However, with new, more sensitive techniques, yeast model organisms are likely to become increasingly an asset in the assessment of the processes that lead to storage material. For example, both yeast models do have detectable storage material. Autofluorescence in the *Schizosaccharomyces pombe* JNCL model has been observed upon growth at 37 °C in that cell swelling was accompanied by an increase in vacuolar autofluorescence and the accumulation of neutral lipids, both characteristic hallmarks of Batten disease (S. Codlin and S.E. Mole, unpublished). Furthermore, the *Saccharomyces cerevisiae* JNCL model shows a small amount of fluorescent material co-localizing with the vacuolar marker FM4-64 after entering the stationary phase of growth (N. Muzaffar and D. A. Pearce, unpublished). However, caution in interpreting subtle differences in the accumulation of storage material in yeast will be necessary as the time-frame for this accumulation is beyond the growth phases that are characteristically studied.

4. Perspectives

The model organisms *S. cerevisiae*, *S. pombe*, *C. elegans*, and *D. melanogaster* have pushed NCL research toward areas that, without them, probably never would have been uncovered. Most of what has been shown in the model organisms can be recapitulated in mice or humans making these small eukaryotes invaluable. In JNCL, they are responsible for uncovering the pH maintenance pathway for future studies. Research into INCL using the model organisms has brought us further in understanding the activity of PPT1 and what can occur in its absence. Model organisms will continue to be a cornerstone in NCL research.

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