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Dictyostelium discoideum: Novel Insights into the Cellular Biology of Neurological Disorders

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1. Introduction

Here we will review the somewhat surprising, and novel emerging use of the classic model organism *Dictyostelium discoideum*, a species of soil-living amoeba, as a valuable biomedical tool to study the function of mutant genes implicated in human neurological disorders. Many neurodegenerative disorders, although related by their destruction of brain function, display remarkable cellular and/or regional pathogenic specificity most likely initiated through some deregulated aspect of the activity of the mutant protein. However, the expression of many neurodegenerative disease genes, including but not limited to *huntingtin* (*HTT*), the *ataxins*, the *presenilins* (*PSEN1/PSEN2*) are not simply localized to neurons but are found ubiquitously expressed throughout peripheral tissues. It is safe to say that when considering neurodegeneration, a term used to describe the progressive loss of function or death of neurons; it is paramount to properly understand the earliest precipitating events that lead to neuronal pathogenesis in order to ultimately develop long-term effective therapies. This means, in no unequivocal terms, that it is critical to understand the normal function of the disease-causing gene. Unfortunately, the normal function(s) of a large majority of mutant genes remain largely unknown which may in fact delay, and often precludes the development of assays for rapid and accurate assessment as to how the mutation initiates disease. As a result, a variety of lower and higher metazoan model systems have been established that range from worms to flies to mice, all designed to better understand neurological disease in humans. So the question arises: how reasonable is the use of simple organisms to study neurological disorders especially when the model of choice does not contain neurons? Historically, I believe the evidence suggesting the usefulness of using simple organisms to understand the etiology of cellular pathology cannot be denied. But using an organism without a central nervous system to understand diseases of the brain? The intrinsic complexity of the brain, without question, greatly complicates the accessibility to study the biochemical processes that contribute to pathology. Furthermore, functional genomic studies through the targeted deletion of genes may preclude their study due to essential cellular roles. As a consequence, determining the direct functional role and subsequent alteration of function by the mutation through phenotypic analysis is once again complicated further. Specifically, this has been true for huntingtin, the causative agent of Huntington's disease, and the beta-amyloid precursor protein (APP) and presenilins, responsible for the onset of Alzheimer's disease (AD) as well as a host of other

neurological disorders where candidate mutant genes have been discovered but cannot readily be assigned a function. Reverse genetics in mice, the "gold standard" in human disease models, have shown that a number of disease genes including those mentioned previously are critical components involved in embryonic developmental. So again, the question might arise why *Dictyostelium*? The haploid life cycle of *Dictyostelium* is fascinating from a biological perspective, and provides unique opportunities to assess disease-related questions because it can be studied naturally as both single, dynamic independent cells and as a *bona fide* multicellular organism in a highly meaningful way with exceptional cellular clarity. Second, and importantly, unlike metazoans, growth precedes development in this eukaryotic organism. As such, targeted deletions of homologous disease genes that do not affect growth can be readily studied in great biochemical detail when the cells are induced to proceed through development with unparalleled synchronous timing and a robustly consistent pattern of gene expression even across evolutionarily divergent species (Parikh et al. 2010). Finally, since the completion of the *Dictyostelium* genome project a number of reviews on the use of this model system to elucidate mechanisms of disease including actin pathologies, mitochondrial disease, human lysosomal and trafficking disorders, host-pathogen interactions and anti-cancer drug action have been described (Carnell & Insall, 2011; Clark, 2010; Francione et al. 2011; Maniak, 2010; R.S. Williams et al. 2006). As will be reviewed below, the evolutionary conservation between *Dictyostelium* and human genes implicated in neurodegeneration, combined with the organisms genetic tractability and easy cellular accessibility, it can be argued that this model system, along side many other human diseases, also provides a fertile environment for discovering normal gene function(s) that cause neurodegeneration with the significant potential for translational and validation studies in higher eukaryotes including both mouse and human systems.

2. Contributions of *Dictyostelium* to neurodegenerative disease-gene function

When readers of this chapter pause to think about *Dictyostelium*, if at all, at least not since their Introductory Biology course back in University, I have a hunch that they might recollect faint memories of laboratory demonstrations involving cAMP-mediated chemotaxis or the observation of various developmental structures including fruiting bodies made up of spores and stalks that form from starved individual amoebae. Whereas bacteria, yeasts and several other invertebrate or vertebrate model systems are well known for their additions to our understanding of basic biology and human disease, *Dictyostelium*, unfortunately is rarely included among this list. However, quietly, over the last few years, primarily following the publication of the genome, public availability of RNA seq data and DNA microarray data, research conducted on the social amoeba has revealed some very common cellular features shared across various phyla, and almost uncannily, that many of its proteins are more similar to human orthologues than are those of *S. cerevisiae* (Eichinger et al. 2005). Moreover, unlike yeasts, the 34 Mb, *Dictyostelium* genome is densely populated with 12,646 genes and is surprisingly close in size to the 13,676 genes found in the *Drosophila* genome (Adams et al. 2000). I wish to suggest that, despite the wide evolutionary distance that separates humans and the social amoeba, mutations in *Dictyostelium* can provide direct insight into human neurological disease processes. In this chapter I will discuss in detail research using this organism on genes known to cause neurodegenerative disorders including Alzheimer's disease, Huntington's disease and Hirano bodies. However,

Dictyostelium also contain many other genes related to neurological disease including, but not limited to Adrenoleukodystrophy (*ABCD1*), Amyotrophic lateral sclerosis (*SOD1*), Miller-Dieker Lissencephaly (*LIS1*), Parkinson's disease (*UCHL1*) and Neuronal ceroid lipofuscinosis (*PPT1, CLN2, CLN3, CLN5*) (Eichinger et al. 2005). Later in this chapter I will introduce an emerging role for *Dictyostelium* in pharmacogenetics. More specifically, discoveries in *Dictyostelium* on the effects of valproic acid have uncovered common modes of action in mammalian neurons (R.S. Williams, 2005). Lastly, I will describe in detail *in vitro* studies using the unique small molecule DIF-1 (differentiation-inducing factor-1; 1-(3, 5-dichloro-2, 6-dihydroxy-4-methoxyphenyl) hexan-1-one), a morphogen produced by *Dictyostelium*, that has been shown to have anti-amyloid properties in a wide variety of mammalian cell types (Myre et al. 2009). DIF-1 selectively reduces the amyloidogenic processing of wild type and mutant APP (e.g., early onset mutations) which dramatically prevents production of Abeta40 and Abeta42, believed to be the causative agent of AD. Alzheimer's disease, the most common form of dementia among older adults, is nonreversible, and with the number of AD cases on the rise in the absence of effective treatments, it becomes even more urgent to explore and identify molecules with potential therapeutic potential. In essence, as a research community, what I hope to convince you of is that it is important we "leave no stone unturned".

2.1 *Dictyostelium* – Emergence of a classic model organism for the study of human disease

Dictyostelium discoideum is a species of soil-living amoeba belonging to the Kingdom Amoebozoa and phylum Mycetozoa. *Dictyostelium*, now commonly referred to as social amoeba, is a eukaryote that transitions from a collection of individual, self-sustainable phagocytic amoebae into a multicellular slug and then into a fruiting body within its life cycle (Figure 1a).

Upon starvation conditions, *Dictyostelium* transitions from a unicellular to a *bona fide* multicellular organism. During this period of multicellular development, the cells execute a series of morphological changes that proceed in defined stages over a 24 hour period. Within the first six hours of development, cells secrete, and undergo chemotaxis toward cyclic adenosine monophosphate (cAMP) to form aggregation territories. The secretion of cAMP promotes a G protein-coupled receptor signaling cascade that results in the formation of discrete mounds that may contain as many as 100,000 cells (Parent & Devreotes, 1996; Soderbom & Loomis, 1998). As development continues, cells within the mound remain motile and are directed to differentiate into either prestalk or prespore cells, leading to morphogenetic changes yielding a multicellular stalk, supporting a ball of encapsulated dormant spores (J.G. Williams et al. 1989). The entire process showing the various developmental structures is beautifully depicted in Figure 1b by scanning electron microscopy (SEM) and although the description of the life cycle appears simplistic, the process in actuality is quite complex with still many unresolved questions including the transition from growth to development, on how cells secrete cAMP, regulate organism size, and initiate cell-fate choices to name but a few. A PubMed search of the term *Dictyostelium* retrieves well over 7,000 articles with more than 600 reviews available to the reader that explore the life cycle in much greater detail than is presented here. It is now quite clear that *Dictyostelium* possesses signal-transduction pathways that are closely related to those of

metazoa. Yet, with at least one major and exploitable fundamental difference: animals undergo embryogenesis, that is to say they develop from a single cell by a combination of cell division, morphogenetic movements and differentiation followed by growth of the organism. Whereas with *Dictyostelium*, growth precedes development, and in contrast, development requires no growth, and multicellularity is achieved by the concerted aggregation of many unicellular amoebae. This greatly simplifies the study of development and provides an exceptionally easier route to examine numerous cellular functions including cytokinesis, endocytosis, secretory pathways, protein trafficking, intracellular signaling, gene transcription and regulation, cell-cell communication and adhesion, differentiation and many molecular and biochemical aspects of random and directed cell motility. In addition, *Dictyostelium* cells undergo a relatively simple program of multicellular development, which in many ways resembles animal development. With the sequencing of the genome we are now entering a renaissance in the use of this model organism as a biomedical research tool.
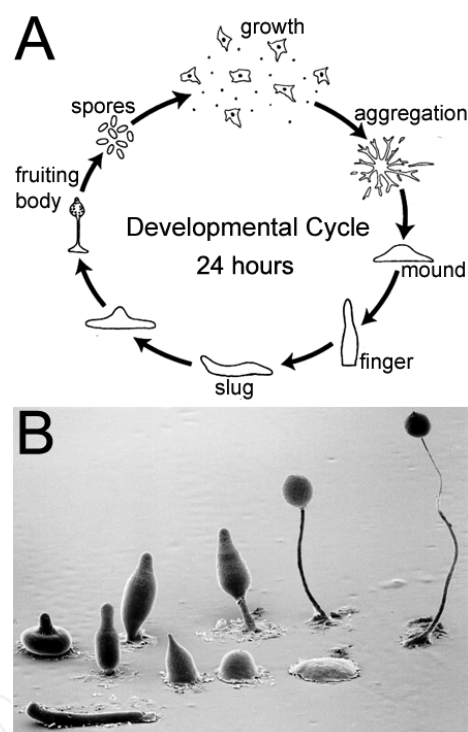

Fig. 1. The life cycle of *Dictyostelium discoideum*. (A) Most of its life exists in its growth phase, as a haploid social amoeba preying upon bacteria in the soil and dividing by mitosis. Once the food source is depleted cells enter into a 24 hour multicellular developmental program. During this transition, amoebae aggregate towards secreted cAMP by chemotaxis in the thousands to form a tight mound and then enter a stage where cells begin to differentiate. Cells within the mound remain motile and are directed to differentiate, by secreted morphogens, into either prestalk or prespore cells, culminating to form a fruiting body comprised of a multicellular stalk that supports a ball of encapsulated dormant spores. (B) Scanning electron microscopy showing the various structures formed during development. Permissions: CC Creative Commons Attribution - Share Alike 3.0, David Brown & Joan E. Strassmann. SEM courtesy of MJ Grimson & RL Blanton, Biological Sciences Electron Microscopy, Texas Tech University.

As will be discussed in more detail later, *Dictyostelium* offers numerous advantages as an experimental organism. Its haploid genome is gene dense, and where higher eukaryotes may express a large number of similar genes with redundant functions, *Dictyostelium* often carries only a single homologous or orthologous gene. In addition, GFP-fusions, expressed sequence tags, RNAi and just about all molecular biochemical experimentation can be performed using the organism with exceptional cellular clarity between differentiated cell types. Most importantly, genetic manipulation of *Dictyostelium* by targeted gene deletion using homologous recombination can be performed relatively quick and for a fraction of the cost of higher eukaryotic systems. In order to unveil the functions of homologous proteins by studying them in simple model systems, it is important that these functions retain a respectable level of conservation through the course of evolution. In many cases, it is possible to demonstrate directly, the conservation of function(s) of homologous proteins among species by expressing a recombinant version of the protein in a null genetic background and showing the restoration of phenotypic deficiencies. Importantly, to suggest the use of *Dictyostelium* as a model system to understand the normal function of human neurological disease genes, when the difficulty of using more complex eukaryotic models have either failed or provided only limited indirect information, there must, at the very least be a significant level of homology present. Sequencing of the *Dictyostelium* genome revealed that many genes encode proteins that meet this criterion of homology to their mammalian counterparts, and some have been shown to be extremely conserved throughout evolution at the amino acid level (>70%) (e.g., calmodulin has 88% identity, actin has 94% identity; see www. http://dictybase.org) and, are also functionally equivalent in an experimental setting. In *Dictyostelium*, some proteins that retain functional equivalence include but are not limited to Vmp1, a vacuolar membrane protein, involved in vesicle trafficking, autophagy, growth and morphogenesis in mammals but not yeast; Rbl1, retinoblastoma-like protein, which causes loss of retina structure/architecture, and ABP34, an actin-bundling protein that causes the formation of Hirano bodies in mammalian neurons (reviewed in Annesley & Fisher, 2009). Moreover, the sequencing of the *Dictyostelium* genome revealed, at a highly conservative and stringent threshold value of $e \leq 10^{-40}$, the presence of at least 64 proteins involved in a variety of human diseases that were similar in size and amino acid identity extending over 70% of the protein (Eichinger, et al. 2005). However, at this level of stringency, the number of orthologous *Dictyostelium* proteins involved in human diseases (e.g., PSEN1, PSEN2, HTT, Neurofibromatosis 1, LRRK2) are not as readily recognized, and so the actual number of predicted human disease proteins present in the genome is very likely to be much higher (Bosgraaf & Van Haastert, 2003; McMains et al. 2010; Myre et al. 2011; S. Zhang et al. 2008). Future biomedical research using this organism will undoubtedly continue to illuminate many more human disease genes with conserved function. Finally another strength that supports the use of this model are the multiple facets of the life cycle, where it can be observed at the organismal, cellular, biochemical and molecular level, all in the same system and with an extremely limited degree of artifact that is far more common among mammalian cell culture. This includes the issues of biological contamination of mammalian cell culture; between 11 percent and 15 percent of cultures in U.S laboratories have been or are infected with species of *Mycoplasma* (Lincoln & Gabridge, 1998), which affect the host cells' metabolism, morphology, cause chromosomal damage, and can provoke cytopathic responses, rendering any data from contaminated cultures unreliable. There are no reports that I am aware of detailing infection of *Dictyostelium* cultures with

Mycoplasma, or, surprisingly, no large DNA virus that infects *Dictyostelium* have been detected thus far. Taken together, the simplicity of its life cycle makes *Dictyostelium* a valuable model organism to study conserved genetic, cellular, and biochemical processes with a very high degree of translation in other model organisms from flies to zebrafish.

2.2 Alzheimer's disease

Dementia is an acquired condition typified by a severe decline in memory and other cognitive abilities. It represents a recession from an individuals established level of intellectual capacity that sufficiently interferes with their everyday ability to function. Alzheimer's disease (AD) is the most common cause of dementia, estimated to contribute to about 60 to 70% of cases (Barker et al. 2002). Estimates suggest that ~ 5.3 million Americans are afflicted with AD (http://www.alz.org), or ~1 in 8 people older than 65, with an estimated worldwide prevalence of AD approaching 30 million people and a quadrupling of numbers expected to occur over the next 40 years. The time course of AD averages 7 to 10 years, characterized by loss of specific neuronal populations and synapses in the cerebral cortex and certain subcortical regions that culminates in extreme atrophy of the diseased regions, including degeneration in the temporal lobe, parietal lobe, and parts of the frontal cortex and cingulate gyrus (Wenk, 2003). AD is a terminal disease that culminates in death. Impaired recent memory usually is an initial symptom of AD, but other cognitive deficits (e.g., changes in attention, problem-solving abilities) may also be present. Amyloid plaques and neurofibrillary tangles (NFTs), the characteristic pathological hallmarks of AD, are believed to be causative and accrue in the years preceding clinical symptoms.

2.2.1 The amyloid precursor protein and discovery of presenilin-dependent gamma-secretase activity

Amyloid plaques are accumulations of insoluble protein aggregates in the extracellular space of the brain. The principal proteinaceous component of plaques is the Abeta peptide, a 38– to 43–amino acid peptide derived from a much larger pre-cursor protein, the amyloid precursor protein (APP) (Golde et al. 2000; Hardy & Selkoe, 2002). APP is a type I integral membrane protein with a single-pass C-terminal transmembrane domain. Several isoforms of APP have been identified, the most common being comprised of 695, 751, or 770 amino acids, are highly expressed in the central nervous system and particularly enriched in neurons. Abeta peptides are derived from APP through the sequential enzymatic activity of two proteases, beta-secretase (BACE) and gamma-secretase, respectively. In addition to Abeta, these cleavage events also liberate an APP intracellular fragment (AICD) that has been suggested to have a transcriptional function (Cao & Sudhof, 2001). APP was identified as the first gene in which mutations in the coding sequence cause early-onset familial Alzheimer's disease (FAD) (Goate et al. 1991; Levy et al. 1990). The identification and location of mutations within the APP gene have provided important clues regarding the potential pathological mechanisms that lead to increased Abeta production in the brain in FAD, but have not been as insightful in the pathology of late-onset AD in which individuals do not contain mutant APP. The normal function of APP is not well understood. However, a review on the function and proteolytic processing of APP is beyond the scope of this chapter and can be thoroughly reviewed elsewhere (Y.W. Zhang et al. 2011).

In addition to APP, mutations in two other genes have been identified that when mutated result in the rare, autosomal dominant forms of early-onset FAD: presenilin-1 (*PSEN1*) (Levy-Lahad, 1995) and presenilin-2 (*PSEN2*) (Sherrington et al. 1995). These genes encode highly homologous, ubiquitously expressed multiple transmembrane domain proteins in which a vast number of mutations have been identified in FAD families. Several years passed before the AD field determined that PSEN1 and PSEN2 were integral parts of the elusive gamma-secretase (De Strooper et al. 1998). Finally, a series of seminal reports provided independent evidence supporting the hypothesis that presenilins provided the catalytic core of gamma-secretase (De Strooper et al. 1999, Struhl & Greenwald 1999, Wolfe et al. 1999). This was ultimately proven by the finding that transition-state, gamma-secretase inhibitors, targeted the presenilins (Esler et al. 2000, Li et al. 2000). The protein composition of the gamma-secretase complexes is now largely resolved (De Strooper 2003). The different gamma-secretases each consist of at least four proteins that are all required for proteolytic activity (Edbauer et al. 2003, Kimberly et al. 2003): APH-1, PEN2, and nicastrin (Edbauer et al. 2003; Yu et al. 2000). In contrast with this basic knowledge, tantalizing questions regarding the physiology, the cell biology, and the structure-function relation of these intriguing gamma-secretases will keep research groups around the world busy for several years largely because the normal function(s) of the presenilins, and other protein components that make up the gamma-secretase complex, remains unclear. *PSEN1* knockout mice die *in utero* and have a phenotype similar to that of Notch (another single-pass transmembrane domain protein) knockout mice (Thinakaran, 2002). However, what is clear is that the gamma-secretase complex directly cleaves the C-terminal transmembrane protein stubs of Notch, APP, APLPs and many other substrates. The mechanism by which gamma–secretase cleaves its substrates is not the focus of this chapter and the process has been described in great detail elsewhere (De Strooper & Annaert, 2010). Here we focus on the normal biological function of the presenilins which has been difficult to determine largely due to the fact that in mammals, it is an essential gene and knockout mice die *in utero*.

2.2.2 The role of presenilins in higher organisms

Notch is a protein involved in cell fate decisions during development and consequently the Notch field of research continues to provide novel insights into various cellular physiological processes, including the biology of the presenilins (Kopan & Ilagan 2009). Furthermore, the central role of presenilin-dependent gamma-secretase activity in Notch signaling has been unequivocally demonstrated in a long series of genetic experiments in various organisms including flies and worms (Levitan & Greenwald, 1995). In mammalian species, the situation is much more complicated because of gene duplication (*PSEN* and *APH-1*) and embryonic lethality of knocking out the *PSEN* and *Aph-1* genes (Ma et al. 2005). Moreover, in adult animals, partial or tissue-specific knockouts of PSEN-1 lead to gastrointestinal bleeding, hair-color changes, and immune problems (Tournoy et al. 2004). In addition, more than 60 other proteins have been proposed or identified as substrates for the gamma-secretases (McCarthy et al. 2009, Wakabayashi & De Strooper 2008). Almost all candidates are type I integral membrane proteins, and they obey the same consecutive-cleavage process as was outlined for APP and has suggested that in many cases, the whole PSEN1 process is aimed at clearance of transmembrane domain stubs

from the cell membrane. Protein transport and turnover has also been suggested as a function of presenilin based upon work indicating that several members and/or effectors of the Rab family of small GTPases interact with PSEN (Dumanchin et al. 1999). Most recently it was suggested that PSEN1 is involved in the maturation of the V0a1 subunit and subsequent assembly of the V-ATPase proton pump, providing an explanation for the defects in acidification of cellular degradative compartments in *Psen* knockout cells (Lee et al. 2010). Despite a number of reports suggesting that PSEN mutations also affect Ca^{2+} signaling by disturbing the Ca^{2+} pool in the ER (Bezprozvanny & Mattson 2008), the origin of this disturbance is highly debated, contradictory and the literature remains confusing. What is true is that more is known about the function of presenilin substrates and their intracellular cleavage domains (ICDs) than presenilin itself (reviewed in Krishnaswamy et al. 2009). Of particular importance regarding this chapter, the function of PSEN-dependent gamma-secretase in more distantly related and unicellular species has not been demonstrated or explored to any comparable extent. PS-like components have been identified in moss (Khandelwal et al. 2007), but gamma-secretase activity could not be detected. Overall, a lot of difficult work is still needed in order to decipher the function of presenilin in different gamma-secretase complexes in mammals, but to also uncover the normal *in vivo* role of the protein in other readily available model systems including lower organisms.

2.2.3 The identification of presenilins and gamma-secretase activity in *Dictyostelium*

A recent development in *Dictyostelium* biomedical research was the identification of homologous genes to presenilin 1, presenilin 2, nicastrin, aph-1 and pen-2, the core components that constitute the gamma-secretase complex (McMains et al. 2010). As discussed in humans, mutations in the presenilins, which are integral for protease activity are intimately involved in the aberrant proteolytic processing of APP in a pathway that leads to the condition of early onset familial Alzheimer disease. How the defects cause disease, or more importantly the time course or precipitating events that lead to the pathogenesis of AD are not known, and as such, highlight the importance of uncovering the normal function of the gamma-secretase complex responsible for production of the toxic Abeta peptide. In this section, I discuss the importance of having a viable, organismal model, to aid in further understanding the normal physiological role of the presenilins and gamma-secretase from a conserved and evolutionary point of view in a system that has no Notch or APP equivalent (Eichinger et al. 2005). To study the gamma–secretase in *Dictyostelium* in great detail, strains were created containing single and/or double gene disruptions for *ps1-*, *ps2-*, *aph1-*, *ncst-* and *ps1/ps2-*, *aph1/ps2-*, *ncst/ps2-*, and *aph/ncst-* nulls. It is important to note that mutants of these genes in *Dictyostelium* are viable, unlike the embryonic lethality seen in mammalian systems, and that mutant amoebae show a variety of phenotypic defects during specific periods of development. Moreover, extensive sequence analysis reveals that the *Dictyostelium* presenilins are structurally similar to proteases in this family of proteins rather than the related signal peptidyl proteases (SPP) and that phylogenetic analysis firmly places them among this group (Figure 2A-D), and a majority of the mutations in the human PS1 gene associated with early-onset familial Alzheimer's disease alter residues (Kim & Kim, 2008) that are conserved in both *Dictyostelium* PS proteins (McMains et al. 2010).
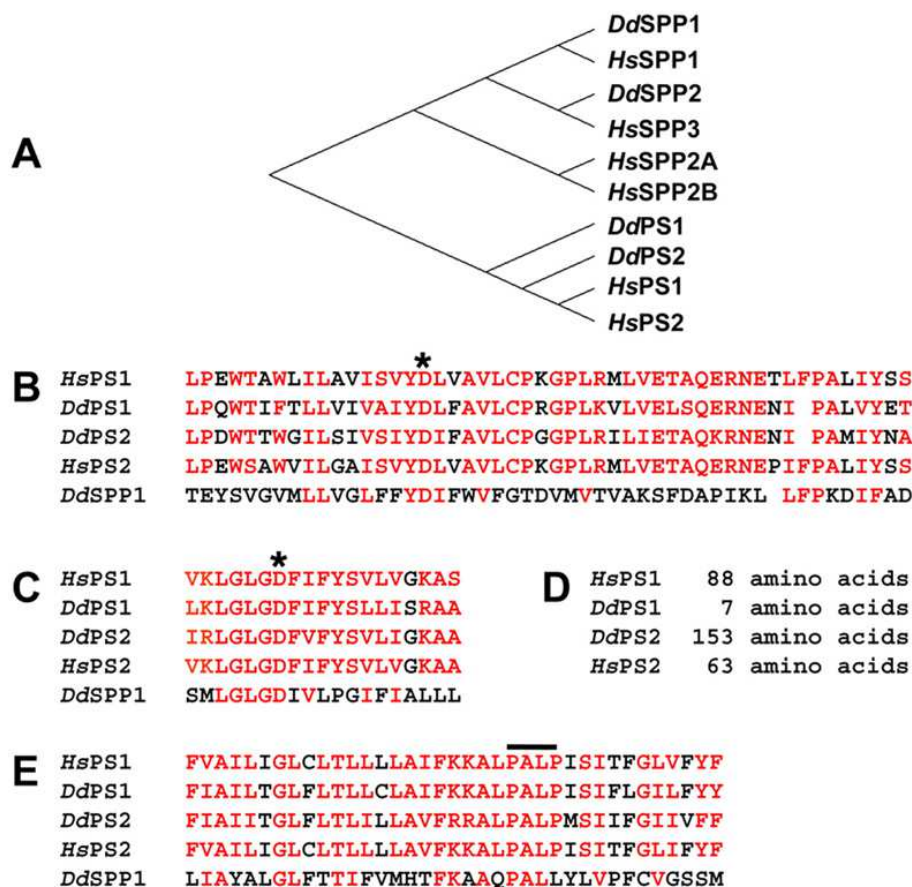
Fig. 2. *Dictyostelium* PS proteins align with human presenilins. (A) Phylogenic comparison of amino acid sequences of *Dictyostelium* and the human PS and signal peptidyl proteases (SPP) proteins. The *Dictyostelium* PS proteins cluster closely with human PS. (B-D) Sequence alignments of functional domains of PS and SPP proteins. Similar amino acid residues are indicated in red. Residue alignment surrounding the N-terminal enzymatic aspartate (*) is shown. Residue alignment surrounding the C-terminal enzymatic aspartate (*) is shown. Residue alignment within the conserved, *overlined* PALP domain region. Permissions: McMains, V.C. et al. (2010). *Dictyostelium* possesses highly diverged presenilin/gamma-secretase that regulates growth and cell-fate specification and can accurately process human APP: a system for functional studies of the presenilin/gamma-secretase complex. *Dis. Model. Mech.* 3, pp. 581-594.

2.2.4 The presenilin gamma–secretase complexes regulate cell differentiation and phagocytosis in *Dictyostelium*

Cells deficient for *ps1* did not show post-aggregative developmental defects compared to cells deficient for *ps2* suggesting that in *Dictyostelium*, PS1 gamma–secretase complexes may carry out different functional activities than PS2 gamma–secretase complexes during specific phases of the life cycle. This observation fits well with the suggestion that that there may be several distinct presenilin complexes that have different biological activities (Gu et al. 2004). Wild type and *ps1*- cells are able to complete development within 24 hours, whereas *ps2*-, *aph*1- and *ncst*- mutants not only form fewer fruiting bodies, but predominantly arrest as abnormal intermediate structures within the same time frame (McMains et al. 2010). It

should be noted that the observed development defects were more pronounced in the double mutants and so PS1 might also contribute some functional activity during development. The authors also show that in *Dictyostelium* gamma-secretase complexes function to regulate cell differentiation as measured by cell-type specific gene expression and firmly establish that PS gamma–secretase activity is required in a cell-autonomous pathway that determines cell fate during development (McMains et al. 2010). Once again, these *in vivo* observations support a large body of suggestive evidence for presenilin gamma–secretase playing an essential role in cellular and neuronal differentiation during neural development and adult neurogenesis in mouse and zebrafish models (Baumeister, 1999; van Tijn, 2011). The authors also found that cells deficient for gamma-secretase display a reduced ability for phagocytosis. Quantification of phagocytic rates for the various mutant strains in comparison to wild type cells revealed that cells lacking PS1 had highly reduced rates of phagocytosis, but this was not the case for *ps2-* cells. The described phagocytosis defect supports observations that mammalian presenilins are present in lysosomal membranes, but also strengthens the argument that distinct presenilin complexes exist and have different biological activities. Further to this, when wild type amoeba were made to express a variant of human APP, the cells were clearly able to process APP in a manner identical to mammalian cells resulting in the differential production of C-terminal fragments and Abeta40/Abeta42, the toxic fragment believed to be the causative agent in AD (Figure3).

This work demonstrates the evolutionary conservation of this key regulatory enzyme and establishes the ability to now use the powerful molecular genetics of *Dictyostelium* to test a variety of biochemical predictions using an *in vivo* system. Therefore the most obvious value stemming from this work for the study of presenilin cellular functions and neurological disease falls into three categories. The first being the development of a viable organismal system to study both the ancient and conserved function(s) of the presenilin-dependent gamma-secretase complex, and the possibilities for elucidating presenilin-independent function(s) (Neely et al. 2011); secondly, the exciting prospects surrounding the finding that proteolytic processing of APP occurs in *Dictyostelium* exactly the same way as has been shown animal cells. This finding is important not only because it provides an excellent system in which to test therapeutic agents that target Abeta production *in vivo*, but it is remarkable that this enzyme, given the evolutionary distance between *Dictyostelium* and humans, is capable of cleaving a non-endogenous substrate to produce C-terminal fragments and ratios of secreted Abeta40/Abeta42 identical to mammalian systems. It should be mentioned that a survey of the *Dictyostelium* genome does not readily identify or predict the presence of either the known alpha–secretase or beta–secretase (BACE) (Eichinger et al. 2005) and suggests the presence of yet to be identified first-cleavage sheddases either unique to *Dictyostelium* or potentially conserved across metazoan species. Moreover, this model offers a biochemical clarity not offered by any other system to date to study gamma–secretase function directly, free from the multiple secondary effects that can arise from perturbation of proteolysis of more than 60 identified substrates (McCarthy et al. 2009). Experiments will need to be conducted to elucidate the mechanism by which *Dictyostelium* produces gamma–secretase targets from human APP in the absence of these characterized mammalian enzymes. Unfortunately, from the point of view of therapeutically targeting gamma-secretase it may also suggest that this enzyme is indeed a "membrane proteasome" with less stringent substrate requirements for target specificity than previously thought (Hemming et al. 2008). Clearly, this work further demonstrates the importance in

understanding how gamma-secretase complexes recognize and differentiate between their many substrates which undoubtedly are essential for understanding the role of presenilins in biological processes. Lastly, this study provides strong *in vivo* evidence for presenilin function in regulating phagocytosis, only previously suspected in animal cell culture (Jutras et al. 2005). Further studies will be required to determine whether the mammalian *PSEN* gene can rescue *Dictyostelium ps1*-null phenotypes and proteolytic processing of human APP.
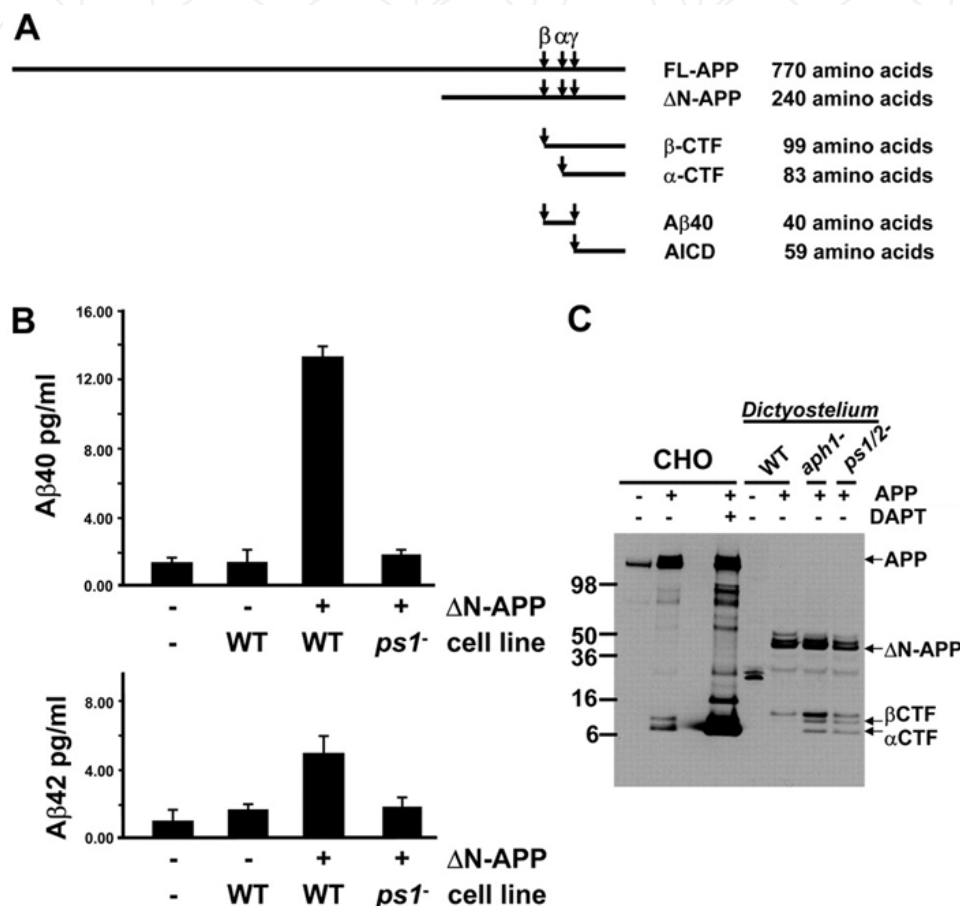

Fig. 3. *Dictyostelium* have PS-dependent gamma-secretase activity that processes human APP to release Abeta peptides. (A) Schematic showing various APP derived products and their respective sizes. (B) ΔN-APP, a truncated human APP expressed in transformed wild type (WT) and ps1-null cells were analyzed for secreted levels of Abeta40 and Abeta42 peptides by quantitative ELISA. Fresh media and media conditioned by native WT cells were used a negative controls. Bars indicate standard errors derived from two independent experiments, each with two replicates. (C) Protein samples were collected from native and APP-expressing CHO cells untreated or treated with DAPT, from growing WT *Dictyostelium* or WT, *aph1*-null and *ps1/2*-null *Dictyostelium* that express ΔN-APP. APP expression and processing was determined by immunoblot assay using anti-APP C-terminus antibodies. Permissions: McMains, V.C. et al. (2010). *Dictyostelium* possesses highly diverged presenilin/gamma-secretase that regulates growth and cell-fate specification and can accurately process human APP: a system for functional studies of the presenilin/gamma-secretase complex. *Dis. Model. Mech.* 3, pp. 581-594.

2.3 Huntington's disease

Huntington's disease (HD) is a fatal neurodegenerative disease (HD Collaborative Research Group, 1993) also known as Huntington's chorea, that affects muscle coordination and leads to cognitive decline, dementia and eventually death. The molecular mechanisms underlying the disease have been widely studied yet the normal function of the protein responsible for the disease remains largely unknown. HD is a monogenic neurological disorder that occurs because of a mutation in the huntingtin protein (HTT). The disease is caused by an unstable, but normally polymorphic, CAG trinucleotide repeat within the coding region of the *htt* gene that leads to the expansion (>35) of a homopolymer stretch of glutamine (Q) residues near the amino-terminus of the protein (HD Collaborative Research Group, 1993). Mutant HTT confers a gain-of-function property to some aspect of the protein, but recent evidence also suggests that mutant HTT impairs the ability of normal huntingtin protein to exert molecular activities that are fundamental for the survival and functioning of the neurons that predominantly degenerate in the disease (Cattaneo et al. 2005). Huntingtin is a large (350 kDa) alpha-solenoid HEAT (*h*untingtin, *e*longation factor 3, the *A* subunit of protein phosphatase 2A, and *T*OR1) repeat protein (Andrade & Bork, 1995) that bears little resemblance to any known protein. After almost 20 years of research and more than 13,000 research articles, the normal function of HTT, which is expressed in all cells, both neuronal and non-neuronal, still remains unclear for a number of reasons (reviewed in Zuccato et al. 2010).

The large size of HTT makes it challenging to perform biochemical experiments and develop robust structure/function assays. Manipulating such a large DNA fragment by conventional molecular cloning techniques is fraught with problems including sequence stability in bacterial hosts, expansion/contraction of the polyQ and deletions of the polyproline region. Consequently, few studies have been able to address the cellular function(s) of full-length HTT and its dysfunction(s) associated with the disease compared to the vast amount of literature on the amino-terminal fragment, which in essence, removes the mutation from the context of the full-length protein. One might be inclined to argue that studying N-terminal polyQ fragments is tantamount to research on polyQ disease rather than HD. However, the point of this chapter is not to create controversy as one cannot deny that much has been learned about HTT fragment toxicity in studies that utilize the N-terminal fragment. The few genetically precise mouse models for HD (knock-in mice that harbor the full-length mutant protein) have contributed significantly towards understanding HTT normal function, but these mice are laborious to create, expensive to maintain and the results expensive and difficult to validate. Moreover, sub-cellular localization, which often provides clues to protein function, has also been an arduous task due to conflicting reports between various antibodies and the effects that different immunocytological methods can have upon the apparent location of HTT in the cell (reviewed in Hughes & Jones, 2011). Lastly, a number of methods are available to map protein-protein interactions as a means to elucidate protein function. With each approach come strengths and weaknesses regarding specificity and/or sensitivity of the interaction, and again in the case with HTT, its large size and differential affinity for a variety of antibodies has made uncovering its normal function difficult to interpret. As such, a single large scale screening experiment identified at least 234 proteins that interact with mutant huntingtin (Kaltenbach et al. 2007). In this study, the authors assessed five different recombinant wild type and mutant HTT fragments spanning the length of the protein using yeast two-hybrid (Y2H) and affinity pull-down/mass

spectrometry (MS) protein interaction screens. How these interacting partners ultimately contribute to understanding HTT normal function remains to be seen, and again, the interpretation of the results must be closely scrutinized as the screen used fragments and was not performed with full-length HTT, which when mutated, is the actual causal agent of HD.

2.3.1 Higher organism and cellular models of Huntington's disease

Genetic evidence in humans and mouse models of Huntington's disease suggests that the disease mutation acts through the deregulation of some aspect of the protein's normal function(s). Over time, the consequence of carrying the HD mutation is an extensive amount of neurodegeneration characterized by the preferential loss of efferent medium spiny neurons in the striatum of the basal ganglia. This massive amount of neuronal loss is primarily responsible for the typical HD symptoms (Reiner et al. 1988). However, it is now well known that as the disease progresses, a more widespread degeneration of the brain ensues and also involves cortical structures (Rosas et al. 2003, 2005). Thus, defining the disease-producing 'gain-of-function' - either a polyglutamine-length dependent increase or deregulation of a normal huntingtin activity or the introduction of a novel polyglutamine-length dependent activity, will without a doubt require an understanding of the protein's normal function(s) beyond what is currently feasible methodologically when using post mortem human brain samples.

I will now offer a brief look at the wide range of HD animal models currently available to the HD community. These models have been used to investigate pathological pathways, molecular targets, and potential therapeutics. A wide variety of species, including invertebrates such as *Drosophila melanogaster*, non-mammalian species (e.g., *Danio rerio*) and mammals, including mice, rats, sheep and non-human primates, have also been genetically engineered to model the HD mutation. Unfortunately, many of these models, that express either truncated or full-length human or mouse mutant huntingtin, display significant phenotypic discrepancies within species that are often attributed to differences in strains or protein context (HTT fragments vs full-length). However, with their size, organ capacity, and close resemblance to human physiology, these models are particularly well-suited for preclinical trials and long-term safety studies. The limitations associated with these models are often ethical concerns, the fact they take a considerable amount of time (e.g., years) to assess and are incredibly costly to maintain. Moreover, modeling the HD mutation, a human disease and without knowing the normal function of the protein, relies primarily on re-creating human symptoms in non-human systems. Although these systems are extremely important tools to understand HD, a large majority of studies focus upon the late end-stage disease symptoms and thus offer minimal insight into early manifestation including HTT normal function. An attempt to describe the phenotypic differences between these entirely different model systems is well outside the scope of this chapter and can be reviewed elsewhere (Zuccato et al. 2010). The existence of HD cell lines, which allow for the stable or inducible expression of wild-type or mutant huntingtin, have been, to a finite degree, useful for the dissection of huntingtin function and assessment of potential therapeutic compounds (Rigamonti et al. 2007; Varma et al. 2007). Further, *in vitro* cell lines often do not show overt defects suggesting they are unable to reproduce the pathophysiological mechanisms induced by the mutant gene.

I have established that the dominant nature of Huntington's disease (HD) and its tissue specificity relative to the fact that it is ubiquitously expressed indicate that the HD mutation may initiate pathogenesis through some unique characteristic activity of the huntingtin protein, making it critical that we understand the normal biochemical function(s) of huntingtin. This is true with respect to developing safe effective treatments. However, the truth is, we don't currently know the details of huntingtin's function nor do we have a ready assay for its rapid and accurate assessment. As mentioned earlier, many established model systems are based upon an amino-terminal fragment that results in perturbation of many cellular processes but these remove the polyglutamine tract from its normal protein context. Therefore, one cannot understate that strategies are badly needed to reveal full length huntingtin-dependent biochemical processes that contribute to HD pathology. Because of the evolutionary conservation of huntingtin, lower organism systems offer an attractive and affordable route that has yet to be exploited to understand huntingtin function. Consequently, we have used the model organism *Dictyostelium* to generate a viable huntingtin-null organism and have defined the various phenotypes.

2.3.2 The identification of *Dictyostelium* huntingtin

Use of the social amoeba *Dictyostelium* to uncover normal function(s) for HTT with a particular focus on the importance of how these functions might illuminate our total understanding of the early vulnerability of specific neuronal populations to mutant HTT is the long-term goal that requires validation in cross-species HTT-null mutants and *in vitro* cell lines. The *Dictyostelium* genome was screened and found to contain a single gene with sequence homology to human huntingtin. Bioinformatic and phylogenetic analysis of the primary amino acid sequence placed the protein firmly within the huntingtin family, including size, and the presence of numerous HEAT and HEAT-like repeats. In developing mutants' deficient for HTT we have determined that in *Dictyostelium*, cells are viable, unlike the embryonic lethality seen in higher eukaryotes. Moreover, *htt*- cells are fragile in that they display a number of subtle phenotypes which suggests that HTT is involved in a number of cellular processes (Myre et al. 2011). We show that huntingtin deficiency impacts upon growth, osmoregulation, cation homeostasis, cell motility, cell shape, chemotactic cAMP relay, homotypic cell-cell adhesion and cell fate determination in *Dictyostelium*. Importantly, these phenotypic deficits are greatly dependent upon the environment in which the cells are placed which suggests that HTT may serve as a multifunctional protein that aids in translating extracellular signals into cellular processes. These findings support the large body of evidence that suggest in metazoa, huntingtin is a multifunctional protein with suggested roles in embryogenesis, cell fate, cytoskeleton, apoptosis, BDNF production and signaling, vesicular and mitochondrial transport, iron homeostasis, autophagy, energy metabolism and transcriptional regulation (Cattaneo et al. 2005; Lumsden et al. 2007; reviewed in Imisario et al. 2008). Major issues of concern regarding HTT function is the inability to discern if these hypotheses correlate with each other, are mutually exclusive to one another, and which, if any, directly involve HTT or are secondary effects to HTT deficiency or mutant HTT activity. The power behind the *Dictyostelium* system is that it allows for the identification of different *in vivo* HTT functions at the level of both single cells and a multi-cellular environment all in the same organism and with a biochemical and cellular clarity that will identify functions that directly depend on HTT.

2.3.3 *Dictyostelium* cells deficient for huntingtin display cell shape and osmoregulatory defects

When *htt*- cells are induced to develop under non-nutrient, low ionic strength phosphate buffer (KK$_2$; ~40 mOsmol/L), the cells immediately elicit a round phenotype suggesting the presence of an actin-cytoskeleton defect, and a role for HTT in modulating the ability of cells to adapt to stress or changes in their ionic environment (Myre et al. 2011). Under these conditions and in large contrast to wild type cells, HTT-null cells show a disorganized cytoskeleton with a reduction of F-actin at the cortex of the cell (Figure 4A-D). This fits well with reports that mutant HTT in cells from HD patients or when normal huntingtin levels are reduced cells display defective actin-remodeling under conditions of stress (Munsie et al. 2011). *In vitro* binding of the HTT N-terminus to F-actin has been reported (Angeli et al. 2010). Together with our *in vivo* observations indicates a normal, perhaps transient, function for HTT in the regulation of the actin cytoskeleton. Considering the importance of extracellular tonicity to neuronal function, this model provides a valuable tool to uncover mechanisms by which HTT regulates intracellular ionic stores. The extreme cell rounding observed in low osmotic buffer also hinted at a potential defect in osmoregulation. In *Dictyostelium*, osmoregulation is maintained by a bladder-like intracellular membranous organelle called the contractile vacuole (CV) that also functions as a highly efficient acidic Ca^{2+}-store (Zhu & Clarke, 1992). When wild-type cells are placed in water, several small contractile vacuoles form within 30 minutes and, over time, the cells compensate for the sudden change in their osmotic environment, whereas *htt*- cells fail to form vacuoles, rapidly swell and undergo complete lysis within 5-6 hours (Myre et al. 2011) (Figure 4E). This finding is conducive with the role of the cortical cytoskeleton and F-actin crosslinking proteins that protect cells against osmotic stress ensuring both cell size and shape, and suggests a scaffolding role for HTT between the cytoskeleton, actin-remodeling proteins and the function of the CV system in *Dictyostelium*. These results are also consistent with mammalian studies that huntingtin regulates neurological processes including actin-rich dendritic spine formation and membrane branching (Dent et al. 2011; Ferrante et al. 1991). We suggest that the defective CV system in *htt*- cells renders cells not only sensitive to extreme hypoosmotic shock, but secondarily affects intracellular ion homeostasis. As a consequence, unless specific cations (Ca^{2+} or Mg^{2+}) are provided exogenously *htt*- cells appear unable to initiate cAMP-induced Ca^{2+}-transients that may act in a feedback loop to positively reinforce cAMP relay which impairs chemotaxis during *Dictyostelium* development. Although a role for HTT in chemotaxis has not been established in mammalian systems, a consensus is gradually emerging that the dyshomeostasis of Ca^{2+} is an important factor in the linkage of the HTT mutation to the onset and progression of the disease (reviewed in Bezprozvanny, 2007) and together suggest abnormal Ca^{2+} signaling and other Ca^{2+} signaling proteins should be explored further as an evolutionary conserved role for HTT.

2.3.4 Huntingtin regulates cell fate and differentiation during development in *Dictyostelium*

Furthermore, this study also provides compelling evidence to suggest HTT modulates cell fate and differentiation in *Dictyostelium* (Myre et al. 2011) (Figure 5). Using chimeras, we have shown that when *htt*- cells are challenged to differentiate in the presence of wild type cells, they fail to populate the prespore region of the slug, and as a consequence do not become spores. Differentiation defects in huntingtin deficient cells during development has

also been observed in vertebrates. In zebrafish, reduced expression of huntingtin differentially targets development of telencephalic neurons compared to mid- and hind-brain (Henshall et al. 2009). In mouse chimeras, *Hdh-/-* cells also preferentially colonize the hypothalamus, midbrain, and hindbrain relative to the telencephalon and the thalamus during early development (Reiner et al. 2001). Thus, like these latter neuronal
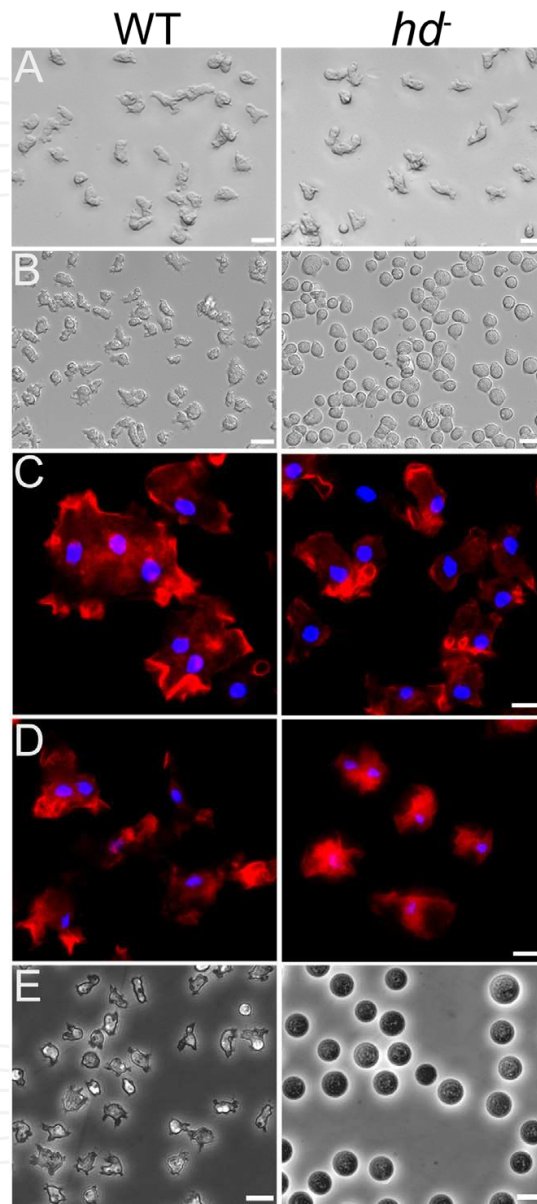

Fig. 4. *Htt-* cells round up under nutrient stress affecting F-actin localization and fail to osmoregulate. (A) The morphology of *htt-* cells as an adherent culture was similar to wild-type cells (left panel). (B) Removal of nutrients and replacement with starvation buffer caused rounding of *htt-* cells. (C) F-actin staining (*red*) in wild type and *htt-* cells is similar. (D) Aberrant localization of F-actin occurs in starved *htt-* cells. Nuclei are counterstained with Hoechst 33342 (*blue*) (E) Unlike WT cells (*left*) *htt-* cells are extremely sensitive to hypoosmotic shock, and begin to lyse after ~3 hours. Permission: Myre M.A. et al. (2009). Deficiency of huntingtin has pleiotropic effects in the social amoeba *Dictyostelium discoideum*. *PLoS Genetics*, 7(4), pp. e1002052.

populations, *Dictyostelium* cells require huntingtin for the proper development of viable spores in the presence of wild-type cells. Not surprisingly, the data thus far do not provide a simple definition for a single normal function for huntingtin, but, as huntingtin deficiency in *Dictyostelium* produces pleiotropic effects throughout the life cycle, the findings are consistent with the consensus from mammalian studies that huntingtin is a multifunctional protein that can impact upon many biochemical processes. Importantly, the existence of a *Dictyostelium* ortholog of human huntingtin, the viability of the null *htt⁻* mutant, and its discrete, readily assayed deficiency phenotypes indicate that this haploid organism provides an effective genetic model system to identify molecular and cellular processes affected by the loss of huntingtin function. Exciting new avenues of research have emerged in delineating which of these functions are conserved in mammals. Ultimately, determining to what degree these functions are altered by expansion of the polyglutamine tract in human huntingtin will also provide much needed insights into the mechanism by which mutant huntingtin triggers HD pathogenesis.
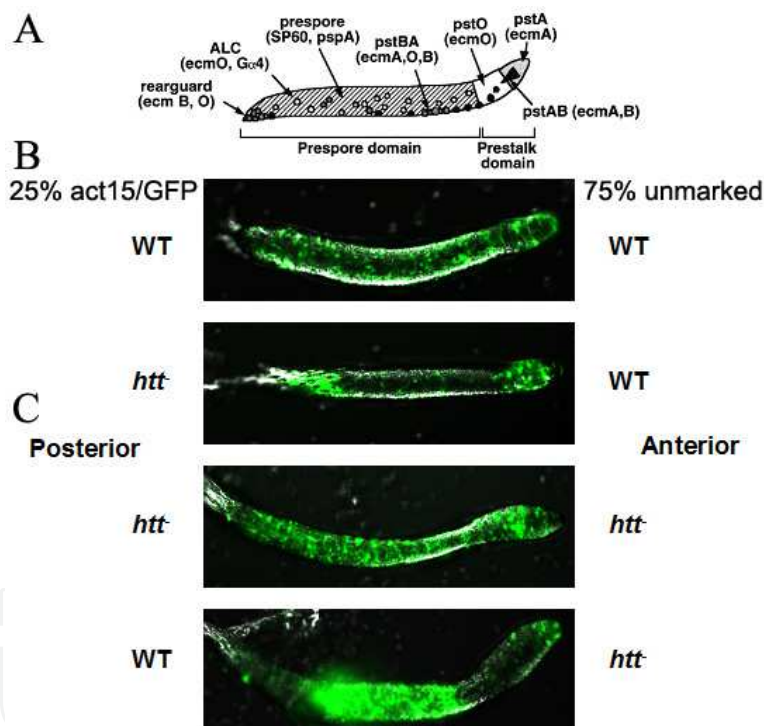

Fig. 5. Huntingtin regulates prespore/spore differentiation cell-autonomously. (A) Schematic representing the position and distribution of cell types in the slug. The prestalk (front) and prespore (rear) domains are *underlined*. Cell types within each region are shown. (B) GFP was expressed in htt- cells and their position in chimeras with unlabelled wild type cells was monitored. GFP:*htt⁻* cells fail to populate the prespore domain. (C) Reverse experiment in which GFP: WT cells in chimeras with unlabelled *htt⁻* cells occupy the prespore domain. Permission: Myre M.A. et al. (2009). Deficiency of huntingtin has pleiotropic effects in the social amoeba *Dictyostelium discoideum*. *PLoS Genetics*, 7(4), pp. e1002052.

2.4 Hirano bodies – *Dictyostelium* as the first available cultured cell model of Hirano bodies

Hirano bodies, first described more than 40 years ago, are bright eosinophilic intracytoplasmic inclusions/protein aggregates which have a highly characteristic crystalloid fine rod structures that occur preferentially in the neuronal processes of individuals with a number of neurodegenerative diseases including Alzheimer's disease, Creutzfeldt-Jacob disease, amyotrophic lateral sclerosis, parkinsonism-dementia and Pick's disease (Cartier et al. 1985; Hirano, 1995). Therefore, Hirano bodies are largely considered as a pathological hallmark of postmortem brain samples from patients suffering from neurodegenerative disorders and thus represent "tombstones" that mark neuronal cell death. Although Hirano bodies are most often encountered in neurons of the central nervous system, they have been reported in glial cells, in peripheral nerve axons, and in extraocular muscles of eyes (Tomonaga, 1983) suggesting that they may be more than products of cell death. It should be noted that Hirano bodies are also increasingly observed as a function of age in individuals without any obvious underlying neurodegeneration (Gibson & Tomlinson, 1977). The importance of these findings remains to be addressed. Hirano bodies are complex neuronal inclusions comprised of a variety of proteins including actin and actin-associated proteins (cofilin and alpha–actinin), tau, a C-terminal fragment of APP, microtubule associated bundling proteins (MAPs), and neurofilament proteins (Hirano, 1994). Moreover, they are not specific to humans and have been described in various experimental animals. Although their existence has been reported for decades, understanding the underlying pathological molecular mechanism responsible for their formation has been precluded by the lack of either an *in vitro* or *in vivo* experimental model system. In this section I highlight the development of *Dictyostelium* as the first available cultured cell model of Hirano bodies (Maselli et al. 2002). Of critical importance here is that research using *Dictyostelium* has shown that the formation of Hirano bodies is a pathological event and not simply related to cell death.

I have already established that abnormal protein aggregation results in formation of distinct types of protein assemblies frequently associated with neurodegenerative disease. Maselli et al. 2002 managed to show that expression in *Dictyostelium* of the CT fragment (amino acids 124-295) of the 34 kDa protein, a protein that exhibits activated actin binding and calcium-insensitive actin filament cross-linking activity induced cells to form ellipsoidal paracrystalline inclusions that contain ordered assemblies of F-actin, CT-myc, myosin II, cofilin and alpha-actinin that remarkably resembled the structure of Hirano bodies. They extended their findings to mammalian murine cells and discovered that F-actin rearrangements caused by a reduction in stress fibers accumulate as numerous punctate foci and large aggregates: Hirano bodies (Maselli et al. 2002). This suggested that the failure to regulate either the activity and/or affinity of an actin cross-linking protein can provide a signal for formation of Hirano bodies. Whereas wild type cells complete development in 24 hours, they found that CT-myc cells contained significantly higher amounts of F-actin, were able to form fruiting bodies, but displayed delayed developmental timing by ~6 hours. Further to this, an accumulation of F-actin was also shown to occur in murine cells expressing CT-myc. More importantly, this first report established for the first time that formation of Hirano bodies is not restricted to mammalian cells or nerve cells, occurs as a consequence of aberrant function of the actin cytoskeleton and demonstrated the physiological effects that Hirano bodies can exert on normal cell function. Because of the

development of this system using *Dictyostelium*, the formation of Hirano bodies and induction of F-actin rearrangements in mammalian cell cultures by expression of the CT 34-kDa actin-bundling protein has now been shown to occur in HEK 293, HeLa, Cos7 cells, neuroblastoma and astrocytic cells, and in primary neurons (Davis et al. 2008).

The abundant detection of Hirano bodies in numerous neurodegenerative diseases suggest the complex aggregate might be involved in normal and neuronal disease processes. This prompted the study to determine how Hirano bodies are turned over in the cell. Hirano bodies in cultured mammalian cells are frequently observed within membrane-bound vesicles and therefore suggested they might be degraded by autophagosomes. Strains of *Dictyostelium* mutants with defects in autophagy and the use of proteasome inhibitors revealed that these aggregates are turned over by autophagic processes as well as the ubiquitin-proteasome pathway (Kim et al. 2009). Importantly, the authors were able to also show that Hirano bodies are also turned over in human H4 cells by both the proteasome and autophagy. These studies support the concept that the ability of clearance mechanisms to keep pace with accumulation of aggregates may have a major impact on disease progression and further showcase how the lower eukaryote *Dictyostelium* can be used to gain novel insight into mechanisms that potentially contribute to human neurodegenerative disease.

3. Pharmacogenetics

Pharmacogenetics is generally regarded as the study or clinical testing of genetic variation that gives rise to differing response to drugs, while pharmacogenomics is the broader application of genomic technologies to new drug discovery and further characterization of older drugs. Pharmacogenetics refers to genetic differences in metabolic pathways which can affect individual responses to drugs, both in terms of therapeutic effect as well as adverse effects. This section will discuss in detail, the discovery that *Dictyostelium* naturally produces small-molecules with anti-amyloidogenic properties and therefore serves as a valuable resource of novel compounds with the potential to become lead molecules in clinical trials with the long-term goal of treating neurodegenerative diseases such as AD. Importantly, *Dictyostelium* can serve as a high-throughput *in vivo* tool with the potential to identify common modes of action, toxic and therapeutic dosing and adverse effects with greater biochemical clarity than *in vitro* screening methods. One may even argue that in some cases, the use of this model system has the potential to rapidly and cost-effectively act as proof-of-principle for future validation studies to improve the safety of certain drugs. Very early during the development and clinical analysis of new pharmaceuticals, it is imperative to determine the teratogenic potential of promising drugs. *Dictyostelium* has successfully been used as a simple biological system to study the teratogenic effects of valproic acid (VPA) and valproic acid-analogues with results that are consistent with data obtained in mammalian teratogenicity assays (Dannat et al. 2003; Tillner et al. 1998). In keeping with this, *Dictyostelium* has also provided much needed detailed insight into the common mechanistic action of specific neuropsychiatric therapies for bipolar disorder including lithium and VPA, whose mode of action remains poorly understood (R.S. Williams et al. 2002). Early work was able to show that in *Dictyostelium*, drugs that provide a neuroprotective effect in individuals with bipolar disorder work through a common mechanism involving the activation of MAPK signaling and increased ERK2 phosphorylation by reducing protein kinase A (PKA) signaling (Eickholt et al. 2005). More

recently, data from *Dictyostelium* models strongly suggest that VPA rapidly immobilizes phospholipid signaling through the modulation of phosphoinositide levels independent of inositol regulation which coincides with a reduction in endocytic trafficking and that VPA when tested in mammalian cells also abrogates this process as measured through depolarization-dependent neurotransmitter release in rat nerve terminals (Chang et al. 2011; Xu et al. 2007). The use of *Dictyostelium* as a model system to understand the common mechanism of action of drugs to treat bipolar disorders will not be discussed any further in this chapter as it has been recently and extensively reviewed (Ludtmann et al. 2011) but confirms the existence of conserved drug-sensitive signaling pathways between *Dictyostelium* and mammalian model systems.

3.1 Differentiation-Inducing Factor-1 from *Dictyostelium* has anti-amyloid properties

As discussed earlier in this chapter, many theories regarding the pathogenesis of AD resulting in abnormal Abeta production or clearance that include inflammation, oxidative stress, metal ion dysregulation and Ca^{2+}-dyshomeostasis, but the exact mechanisms leading to Abeta accumulation remain unclear (reviewed in Walsh & Selkoe, 2004). However, more and more evidence has suggested that irregular cell cycling in selective neuronal populations may contribute to the initiation of AD pathogenesis (reviewed in Herrup & Arendt, 2002; Nagy et al. 1998). Although a controversial area of AD research given the post-mitotic nature of neurons, a variety of cell cycle regulatory proteins, including proliferating cell nuclear antigen, cyclin
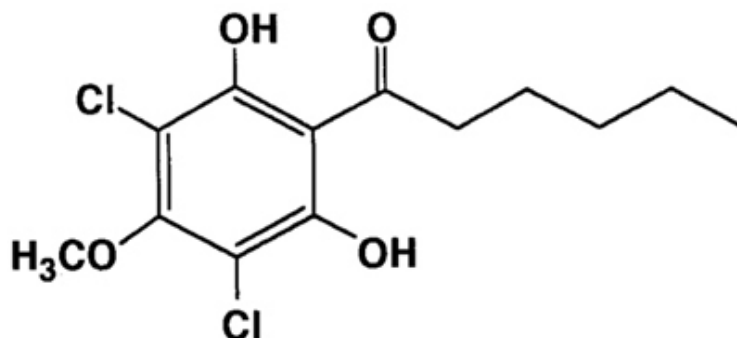
Fig. 6. Chemical structure of DIF-1. Differentiation-Inducing Factor-1 (DIF-1) (l-[3, 5-dichloro-2, 6-dihydroxy-4-methoxyphenyl]-l-hexanone) is a chlorinated alkyl phenone, isolated from the social amoeba *Dictyostelium discoideum*. Permission: Myre M.A. et al. (2009). Reduced amyloidogenic processing of the amyloid beta-protein precursor by the small-molecule Differentiation Inducing Factor-1. *Cellular Signalling*, 21(4), pp. 567-76.

D1, Cdk4, and cyclin B1, are often detected in animal models of AD well before the presence of plaques, and in human brain regions that display AD pathology (Khurana et al. 2006; Yang et al. 2006). Even though APP processing is clearly aberrant in neuronal signaling pathways, the relevance of neuronal cell cycle re-entry to this process remains unclear. Interestingly, APP processing is elevated in pancreatic cancer, oral squamous cell carcinomas and colon carcinomas, which strongly suggest that APP proteolysis is linked to physiological processes involving cellular proliferation and that treatment of cells with antisense oligonucleotides against APP reduced both secreted APP and proliferation

(Hanzel et al. 2003; Ko et al. 2004). Collectively, it would appear that genetic and/or environmental factors (e.g., oxidative and metabolic insults on brain aging) that impede upon the tight regulation of the cell cycle could indeed impact upon Abeta formation. I spoke earlier in this chapter on the various uses of *Dictyostelium* to understand eukaryotic cellular processes and the role that secretion of small chemical morphogens have on the coordinated cellular differentiation of amoeba into prestalk or prespore cells (J.G. Williams et al. 1989). One particular morphogen is a unique chlorinated alkyl phenone, (l-[3, 5-dichloro-2, 6-dihydroxy-4- methoxyphenyl]-l-hexanone) which has been termed Differentiation-Inducing Factor-1 (DIF-1) (Figure 6) (Kay & Jermyn, 1988). Even though the intracellular DIF-1 receptor(s) have yet to be identified in *Dictyostelium*, and no structural analogs of DIF-1 have been reported in mammalian systems, novel functions for DIF-1 in inhibiting the proliferation of mammalian tumors concomitant with decreased expression of specific cell cycle markers (e.g., cyclin D1 and beta-catenin) have been reported (Yasmin et al. 2005). Of particular interest and in support of the role of APP in cellular proliferation was the finding that mutant presenilin variants that bind beta-catenin significantly modify cell growth *in vivo* (Chevallier et al. 2005). Based on all of these lines of independent evidence, we hypothesized that DIF-1 might function as a novel small molecule with the ability to modulate APP catabolism in mammalian cells. Importantly, while there is no cure for Alzheimer's disease, the most common form of dementia, and no treatment to reverse or halt its progression, a small number of medicines are available that help treat symptoms in some people with the disease. These generally fall within the class of drugs known as cholinesterase inhibitors, but unfortunately are limited in their efficacy. At least 80 drugs, from vitamin E to Liptor™ to Abeta immunotherapies have been or are currently being tested in clinical trials. Unfortunately, the large majority of drugs fail or are discontinued from clinical trials, and to discuss the many drugs being tested is well beyond the scope of this chapter. However, it should be noted that *Dictyostelium* might also serve as a source of natural molecules with biomedical potential in the treatment of neurodegenerative disease.

3.1.1 DIF-1 reduction of APP secretion Abeta production in cultured mammalian cells is cell cycle-dependent

I will now discuss DIF-1, a unique, small-molecule from *Dictyostelium* and its ability to significantly reduce Abeta production in a large variety of mammalian cells from mouse embryonic fibroblasts to neuronally derived mouse N2a and human SH-SY5Y cells (Myre et al. 2009). In all cell types tested, DIF-1 inhibited cellular proliferation without inducing any detectable cytotoxic effects and simply washing the cells with phosphate buffered saline restored their rate of proliferation. Moreover, the cytostatic effect of DIF-1 was coupled with an average decrease of 82.41±0.74% in cyclin D1 levels and an increased number of cells in G0/G1 (Myre et al. 2009). Since healthy post-mitotic neurons are relatively locked in a G0/G1 state to prevent proliferation or migration (Arendt, 2003) and aberrant cyclin D1/cdk4 expression has been reported in AD-diseased brain regions suggesting entrance into S-phase (Khurana et al. 2006; Yang et al. 2006) one might speculate that as a consequence of aberrant cycling, associated with age-dependent, environmentally acquired insults, creates a biochemical state that results in increased APP processing. To see if cyclin D1 levels were correlated with either an increase or decrease in APP processing we treated cells with DIF-1 and monitored the levels of secreted APP and Abeta, and the proteolysis of APP into alpha- and beta-C-terminal fragments, respectively (Figure 7a-e).
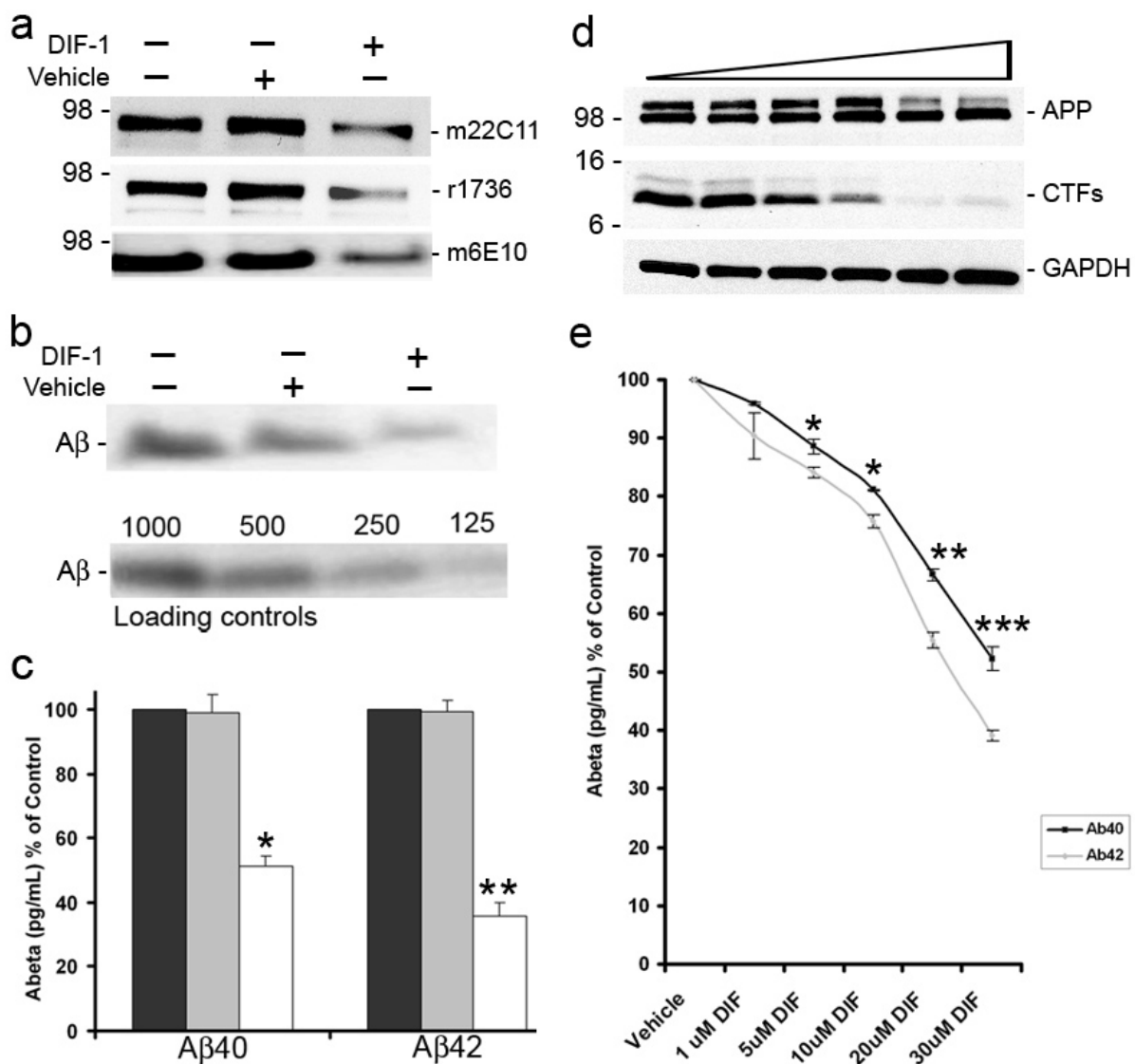
Fig. 7. DIF-1 reduces secretion of APP and inhibits Abeta production. (a) Western blot analysis shows CHO cells treated with DIF-1 show reduced levels of secreted APP into the media as analyzed using three different anti-APP N-terminus antibodies (*shown on right*). (b) Western blot analysis shows DIF-1 also reduced total secreted Abeta in conditioned media. (c) Levels of Abeta40 and Abeta42 were measured by sandwich ELISA. The graph represents the percent average reduction of Abeta (±S.D.) (n=3) compared to controls. Black bars = non-treated cells, grey bars = vehicle and white bars = DIF-1 treated CHO cells. One asterisk represents a P value < 0.003; two asterisks represent a P value < 0.001. (d) Extracts from CHO cells that stably overexpress BACE1 were treated with increasing concentrations of DIF-1 (0, 1, 5, 10, 20 and 30 µM) (*triangle*) show reduced proteolysis and CTF levels (e) The levels of secreted Abeta40 and Abeta42 is significantly reduced in conditioned media from CHO cells that stably overexpress BACE1 as measured by sandwich ELISA. One asterisk represents a P value < 0.003, two asterisks represent a P value < 0.001, and three asterisks represent a P value < 0.0005. Permission: Myre M.A. et al. (2009). Reduced amyloidogenic processing of the amyloid beta-protein precursor by the small-molecule Differentiation Inducing Factor-1. *Cellular Signalling*, 21(4), pp. 567-76.

As shown in figure 7, DIF-1, in a dose-dependent manner, decreased the level of secreted APP compared to controls, reduced APP maturation and beta-CTF levels and is associated with a differential reduction in the levels of Abeta40 (44.2±3.4%) and Abeta42 (66.9±2.6%). Taken together, the data suggests that in the presence of DIF-1, APP processing into Abeta either occurs less during the G0/G1 phase of the cell cycle or is directly affected by DIF-1. Importantly, these results were obtained from CHO cells with increased BACE1 expression and suggest that BACE1 activity is most likely not inhibited or targeted by DIF-1. The effect of DIF-1 on APP processing is also not a result of a general disruption in metabolic processing of type I transmembrane proteins, as when we investigated its effect on a highly related protein, APP-like protein 1 (APLP1), DIF-1 did not affect APLP1 maturation or CTF production.

3.1.2 The mechanism by which DIF-1 reduces of Abeta production involves APP phosphorylation at Thr668

Notably, APP that is phosphorylated at residue Thr668 appears to be a preferred substrate for amyloidogenic cleavage (Vingtdeux et al. 2005). We next assessed whether DIF-1 alters the level of APPT668 phosphorylation and found that in the presence of DIF-1, APP phosphorylation at Thr668 is drastically reduced (88.7± 3.16%) (Myre et al. 2009) and implies that the mechanism by which DIF-1 reduces amyloidogenic processing of APP is dependent upon the phosphor-state of this residue. In order to determine the importance of the Thr668 residue with respect to the effect of DIF-1 on APP metabolism, we generated constructs that result in changes of the Thr688 residue to either Thr688A or Thr688E which would result in the production of APP molecules that cannot be phosphorylated at Thr668. Unexpectedly, DIF-1 had no affect on the metabolic processing of either APPT668A or APPT668E as compared to wild type APP751. The APPT668E and APPT668A mutations abolished the effect of DIF-1 on APP maturation, CTF levels and total Abeta production. This result was surprising since the T668E mutation was expected to mimic a permanently phosphorylated form of APP. We then performed a bioinformatic analysis of the APP C-terminal sequence which predicts that mutating residue Thr668 to any other amino acid (with the exception of serine) in this region no longer meets the criteria as a potential group IV WW-binding motif (Ser/Thr-Pro). This form of WW domain is a small phosphoserine- or phosphothreonine-binding protein module found in various proteins that participate in cell signaling or binding regulation, including physiological substrates of enzymes, in a phosphorylation-dependent manner (Lu et al. 1999). We suggest that a glutamic acid residue cannot compensate for the actual phosphorylation of Thr668 with respect to the mechanism of DIF-1 action on APP processing. Future work on the mechanism of DIF-1 regarding APP processing and phosphorylation of Thr668 will hopefully elucidate how this residue functions in a modulatory manner in the production of Abeta.

The normal function of APP has remained elusive since its' initial discovery in 1987 and consequently, knowledge of the biology behind AD pathogenesis remains limited. This research is important because it has confirmed the importance of deregulated phosphorylation of APP and identification of cell-cycle markers in postmortem AD brains as important pathogenic mechanisms. Moreover, DIF-1 represents a novel small-molecule with the potential for therapeutic investigation into the pathological mechanisms that result in AD with the long-term possibility to serve as a lead molecule in the development of structural analogs in the pursuit of AD therapies. Further to this, DIF-1 serves as yet another

compound the will help to realize the promise in the area of "chemical genomics." The goal of this application of genomics is to expand the universe of small molecules that can be used as probes for understanding biological pathways. While the human genome appears to contain more than 20,000 genes, the entire current pharmacopoeia targets only about 500 genes and their products (Drews, 2000). The use of chemical biological tools provides another resource that will compliment existing models and may allow for the discovery of alternative pathways that regulate Abeta production. We believe that our findings set the stage for the use of DIF-1 as a molecular probe to further elucidate cell cycle-dependent signaling mechanisms that regulate amyloidogenic processing of APP in mammalian systems and further suggest that *Dictyostelium* is a potential resource for unique molecules with biomedical relevance.

4. Future value and limitations of *Dictyostelium* as a biomedical model

All eukaryotic organisms share a cellular organization made up of functionally distinct, membrane-enclosed compartments such as the nucleus, endosome, mitochondria and endoplasmic reticulum/Golgi. In addition, similar, if not identical, mechanisms across diverse species control the cell cycle and division, the establishment of cell polarity (e.g., polarity and motility of chemotaxing *Dictyostelium*) in unicellular as well as multicellular eukaryotes. It is therefore important to not overlook the many basic molecular biological processes which are shared by all eukaryotes, of varying complexities, and in doing so exploit the strengths of each model system. It is my hope that this review exemplifies how the lower organism *Dictyostelium* fulfills the necessary scientific requirements that qualify its future use as an invaluable tool to understand the conserved cellular roles of genes implicated in neurodegenerative disorders. I believe most would agree that it is of fundamental importance to gain as much understanding about the earliest underlying pathogenic processes of any devastating human disease in order to increase drug efficacy and/or appropriate therapeutic targeting, but to also work towards a cure. This section will outline the cost-effectiveness of *Dictyostelium*, the rapid nature in which reverse genetics and genetic modifier screens can be employed to mine even the most subtle of phenotypes, and the recent discoveries of cell-cell signaling molecules that were previously thought to only exist in higher metazoan systems. Taken together, the genetic tractability of this model organism has the magnificent capacity in certain circumstances to illuminate protein function(s) and mechanisms that are conserved in higher organisms by careful, cross-species phenotypic analysis and biochemical structure/function studies.

4.1 *Dictyostelium* – A valuable model organism for biomedical research

For any model system to be of practical use, not only should it allow for the study of numerous basic and specialized physiological processes, but it must also do so in a cost-effective fashion. *Dictyostelium* undeniably offers great economic advantages over other eukaryotic systems as cells can grow in simple medium at room temperature effectively limiting the need for costly growth incubators. Furthermore, *Dictyostelium* cells can grow to very high cell density (10^{11} clonal cells) in a few days without the need for highly sophisticated equipment. The multicellular development is much shorter compared to mammalian systems and can be induced to proceed in a highly synchronous manner allowing for the rapid detection of developmental phenotypes. The haploid nature of the

genome allows one to create gene deletions at a rate unparalleled in mammalian systems including the creation of strains that carry multiple gene deletions. Quite simply when all things are considered, the cell culture conditions are simple, inexpensive, less time consuming, and not as labor intensive as they are for mammalian cells, thereby making it a highly attractive, complementary biological system. As such, *Dictyostelium*, as a model system, has been chosen by the National Institutes of Health as part of its Model Organism Initiative.

Dictyostelium is a powerful molecular system as it offers most of the numerous molecular genetics techniques available for mammalian cells, including gene knock-out, gene knock-in, restriction enzyme-mediated mutagenesis (REMI), RNAi, and inducible gene expression, allowing a wide range of biological questions to be tested. The haploid nature of the genome clearly allows for the unbiased discovery of direct and indirect genetic modifiers of disease through comprehensive genetic screens. The successful use of cDNA complementation /multi-copy suppression strategies that correct phenotypic defects is well described and can be used in combination with the REMI technique to perform saturation screens aimed at recovering genetic modifiers (Kuspa & Loomis, 1994; Robinson & Spudich, 2000). REMI involves the transformation of cells with a mixture of plasmid DNA linearized with a restriction enzyme, along with a restriction enzyme capable of creating compatible cohesive ends in the genome (Kuspa, 2006). This powerful screening technique is not available in higher organisms, and in addition, makes it possible to carry out genetic selection schemes and screens in which greater than a billion progeny can be generated and tested. Genetic schemes of this kind are effective at isolating potential second-site intragenic suppressor loci as well as saturating for second-site mutations which modify the phenotype of a given mutant. Clearly this model system has no equal for establishing the networks of gene action involved in basic cell biological processes. However, one limitation of using unicellular organisms as models for assessing the function of genes involved in disease is that pathologies which affect specific organs cannot be as readily assessed as it might be in the relevant organism. This limitation may also apply to genes that are present in unicellular organisms but are required in a more stringent manner in specialized tissues, or expressed as a variety of different isoforms in specific cell types. Although *Dictyostelium* develops into a bona fide multicellular organism, it does present a limitation in the number of cell types that can be analyzed and therefore discoveries made using this organism must be rigorously assessed in the relevant mammalian cell type.

4.1.1 The surprising discovery of neurotransmitter homologues in *Dictyostelium*

Of the variety of mechanisms evolved that allow for cell-to-cell communication in animals, none maybe more complex than that within the mammalian brain. However, there is evidence to suggest that some of these mechanisms were already in use by pre-metazoan cells, prior to the development of nervous systems, and surprisingly, there exists a body of evidence for a functional role of neurotransmitter receptor homologues in the social amoeba *Dictyostelium*. Unlike the unicellular yeasts, *Dictyostelium* is not a confined immotile unicellular organism but a model system with a short life-cycle that comprises both cellular and multicellular developmental stages. Even in its amoeboid stage *Dictyostelium* can be considered a dynamic organism that is capable of carrying out many physiological processes once thought to only occur in higher metazoan systems. A prime example of this is the recent findings that the family of G-protein coupled receptors (GPCRs) linked to GABA$_B$ receptors, that are typically

found in the central and peripheral autonomic nervous system that respond to a wide range of signals (e.g., main neurotransmitters glutamate, gamma-aminobutyric acid and for Ca^{2+}) were considered animal-specific and were not found outside the metazoan branch until the *Dictyostelium* genome was sequenced (Eichinger et al. 2005; Prabhu & Eichinger, 2006). In total, 17 genes encoding GABA_B like receptors were identified based upon homology and are named GrlA through GrlR (GABA_B or metabotropic glutamate – receptors like proteins) (Prabhu et al. 2007). *Dictyostelium* produces GABA in both growing and developing cells (Anjard & Loomis, 2006). Two genes exist in the *Dictyostelium* genome that encodes the enzyme glutamate decarboxylase which converts glutamate to GABA. Signaling in the central nervous system is mediated to a large extent by glutamate and GABA. These intercellular signaling molecules and their receptors have been found in *C. elegans*, *Drosophila* and all vertebrates but are not found in protists or yeasts. Glutamate and GABA activate ionotrophic and metabotropic receptors on the surface of neurons to initiate and modulate neurotransmission and also play roles in peripheral tissues. The generation of a *grlE*–null strain by homologous recombination found that *Dictyostelium* cells of this strain do not produce SDF-2, a secreted peptide similar to the mammalian neuropeptide diazepam binding inhibitor (DBI), and as a consequence, impairs the timing of sporulation in response to GABA (Anjard & Loomis, 2006). This suggests that GrlE is the GABA receptor in *Dictyostelium* and the findings have revealed that GABA functions not only as a neurotransmitter but has an early intercellular signaling role setting the stage for future studies on the molecular evolution of nervous systems.

Neurotransmitter homologues in *Dictyostelium* are not limited to glutamate and GABA signaling. Five genes homologous to animal P2X receptors have also been identified in *Dictyostelium* (named p2XA through p2XE) (Fountain et al. 2007; Fountain & Burnstock, 2009) and allows for the evolutionary study of purinergic receptors. In *Dictyostelium*, P2XA is an exclusively intracellular receptor, which is in contrast to the predominant cell surface expression of animal receptors, and localizes to the membrane of the contractile vacuole (Fountain et al. 2007). Deletion of the p2XA gene results in an osmoregulatory phenotype, where the cell volume decrease in response to hypoosmotic stress is abolished (Fountain et al. 2007). Although it is clear that the activity of this receptor is not entirely similar to the function of neuronal P2X receptors, these findings do imply that P2XA is absolutely required for the function of an intracellular compartment and has lead to the analysis of potential roles for P2X receptors in mammalian vacuoles (Qureshi et al. 2007). Glutamatergic, GABAergic and purinergic receptors are all present in *Dictyostelium* which represents a fascinating opportunity to garner insight into how these signaling molecules have evolved to function in neuronal signaling in animals and additionally, suggests neurotransmitters were in operation well before the development of the synapse.

The fact that *Dictyostelium*, the ancestor of vertebrate and invertebrate model organisms, appears as a highly evolved organism that had already invented complex interacting systems to control development, cellular physiology, and cellular behavior has profound implications for biomedical genetic research. The central points that I have explored in this chapter can be broadly put into two categories: (1) the great advantages of model organisms, specifically *Dictyostelium*, for identifying and understanding genes that are altered in heritable human diseases and (2) the functions of many of those genes and the evidence that they were present long ago and have remained largely intact in both vertebrate and invertebrate lineages during the course of evolution. New perspectives on disease

pathologies are often a result of data collected from unbiased experimental approaches or saturation screens. Often, the identification of novel genetic modifiers then become prime targets of investigation in mammalian systems to explore their potential effects as players in the pathogenesis of disease and as possible alternative therapeutic targets. The logic behind using lower organisms to perform these screens is to partially cripple a process or pathway with a mutation which affects one component and then search for alterations in other genes encoding component functions in the same system. Importantly, one of the often overlooked but added values from studies using simple models is that they have the potential to open radically new perspectives in the study of a given pathology.

5. Conclusion

Several *Dictyostelium* genes are homologous or at the very least, retain a significant amount of structural conservation to human genes making the organism a useful biomedical model system. With the entire genome now sequenced and publicly available in a model organism database called dictyBase, these sequences will allow molecular and cellular biologists to examine with more clarity the complex multifunctional aspects of gene function. Coupled with translational experiments and validation in higher eukaryotes, research that utilizes *Dictyostelium* I believe, will expedite and contribute to our understanding of human neurological diseases. Individual cell behavior accounts for the many phases of health and pathogenic mechanisms that initiate disease. As described earlier, this has been elegantly portrayed in *Dictyostelium* with respect to the precise production of beta-amyloid from the heterologous expression of human APP. Chemotaxis is a critical cellular function with implications in immune health and embryogenesis and the discovery that *Dictyostelium* cells deficient for HTT show highly defective chemotactic behavior coupled to cytoskeletal/membrane deficits under conditions of low ionic conditions may reveal conserved mechanisms for this protein in axon guidance, neuritic extensions and embryogenesis. In this chapter I have provided a list of examples where studies in *Dictyostelium* have provided information that support, complement, or enhance the understanding of the defects that underlie human neurodegenerative diseases or trafficking disorders, or may do so in the future. Future endeavors with this organism that increase the amount of translational research conducted in higher organism or neuronal cell models will be beneficial for understanding both *Dictyostelium* cellular physiology and, more importantly, the cellular mechanisms of human disease. Indeed, bioinformatic analysis of the *Dictyostelium* genome reveals many homologies with animal genes associated with disease, drug targets, and the biosynthesis, storage and reception of neurotransmitters. I propose that multiple model systems can and should be employed in the cross-genomic analysis of human neurodegenerative disease genes to address multiple basic eukaryotic cellular functions (e.g., *Dictyostelium*), to their assembly into various types of more complex molecular pathways (e.g., flies and worms), and then validated and accurately assessed in models of human neurodegenerative disease (e.g., mice).

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7. References

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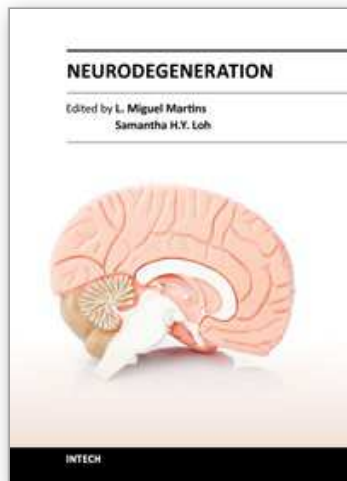
Neurodegeneration

Edited by Dr. L. Miguel Martins

Currently, the human population is on a collision course for a social and economic burden. As a consequence of changing demographics and an increase in human individuals over the age of 60, age-related neurodegenerative disorders are likely to become more prevalent. It is therefore essential to increase our understanding of such neurodegenerative disorders in order to be more pro-active in managing these diseases processes. The focus of this book is to provide a snapshot of recent advancements in the understanding of basic biological processes that modulate the onset and progression of neurodegenerative processes. This is tackled at the molecular, cellular and whole organism level. We hope that some of the recent discoveries outlined in this book will help to better define the basic biological mechanisms behind neurodegenerative processes and, in the long term, help in the development of novel therapeutic approaches.

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