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Neural rosettes formation as a newly emergent huntingtin function

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Valentina Lo Sardo
Matr. R07518

TUTOR:
Prof. Elena Cattaneo

COORDINATORE DEL DOTTORATO:
Prof. Guido Franceschini

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_____Introduction

Huntington's disease (HD) is a dominant inherited neurodegenerative disorder that is caused by an unstable expansion of a CAG repeat within the coding region of the IT-15 gene (HDCRG 1993). The gene encodes for a protein called huntingtin, and the mutation results in an elongated stretch of glutamine near the N-terminus of the protein (HDCRG 1993). Prevalence of the mutation is 4–10 cases per 100,000 in populations of Western European descent, with many more at risk of having inherited the mutant gene. Overtime, the consequence of carrying the HD mutation is a massive brain neurodegeneration characterized by the prevalent loss of efferent medium spiny neurons in the striatum (caudate nucleus and putamen) of the basal ganglia, which is primarily responsible for the typical HD symptoms (Reiner et al 1988). However, it is now well established that a more widespread degeneration occurs in the brain and also involves cortical structures (Rosas et al 2005; Rosas et al 2003; Rosas et al 2008). Since HD is caused by a single mutation, the introduction of the mutant gene into non-human primate, mouse, fly, fish, and worm has generated disease models. This single mutation in huntingtin is the triggering event that endows the protein with new toxic functions that are deleterious for brain cells. Although it is well established that the disease occurs as a consequence of an expanded polyQ above 35 and that the polyQ length accounts for the disease onset, some evidence now also points to the loss of physiological activities of the normal protein as contributing to disease pathogenesis and, in particular, to its selectivity. (Cattaneo et al 2005; Zuccato et al 2007). Although a number of molecular dysfunctions have been elucidated and contribute to explain the early deterioration of the spiny-projection GABAergic neurons of the striatum, the exact mechanisms whereby mutation in huntingtin causes the observed neuronal degeneration, despite a ubiquitous expression, are still unclear. Evidence shows that the pathophysiology of HD may arise both from cell autonomous processes within vulnerable neurons and dysfunction of interneuronal interactions, specifically at the level of the cortical-striatal afferents (Cepeda et al 2007; Fan et al 2007; Zuccato et al 2007).

Huntingtin protein

Huntingtin (htt) is a protein product of IT15 gene. It is completely soluble protein of 3,144 amino acids (aa) that carries a polyglutamine (polyQ) tract in its N-terminus. When this expands over 36 units, the protein becomes toxic and Huntington's disease (HD) develops. It has many potential domains, the boundaries and activities of which are not fully understood. Huntingtin is expressed ubiquitously in humans and rodents, with

highest levels in the neurons of the CNS (DiFiglia et al 1995; Ferrante et al 1997; Fusco et al 1999; Trottier et al 1995; Velier et al 1998). Particularly, huntingtin is eriched in cortical pyramidal neurons in layers III and V that project to the striatal neurons (Fusco et al 1999). Humans and rodents have two mRNAs that are generated by alternative cleavage and polyadenylation of the primary transcript, producing a long and a short 3-untranslated region (UTR) that differ by 3 kb (Lin et al 1993; Li net al 1994). In both organisms, the long-UTR transcript is predominantly expressed in the brain, whereas the short-UTR transcript is more widely expressed (Lin et al 1993). Intracellularly, mammalian huntingtin is associated with a variety of organelles, including the nucleus, endoplasmic reticulum, Golgi complex, and mitochondrion (Hilditch-Maguire et al 2000; Hoffner et al 2002; Kegel et al 2002; Panov et al 2002, Strehlow et al 2007). It is also found within neurites and at synapses, where it associates with various vesicular structures such as clathrin-coated vesicles, endosomal compartments or caveolae, as well as microtubules (DiFiglia et al 1995; Hilditch-Maguire et al 2000; Hoffner et al 2002; Velier et al 1998). This widespread subcellular localization does not facilitate the definition of its function

Huntingtin protein structure

The primary amino acid sequence of huntingtin reveals little, as there are only a few known sequence motifs and no structural domains with defined functions (Figure 1). One obviously important portion of the mammalian protein is the polyQ region itself, which is also present in many transcription factors and aberrantly expanded in at least eight other disease-causing proteins (Everett et al 2004). Because each of these diseases is characterized by the loss of a specific subset of neurons, it is previously proposed that sequences around the glutamine tract might have a crucial role in disease pathogenesis (Cattaneo et al 2001). The polyQ stretch in huntingtin begins at the eighteenth amino acid, and, in unaffected individuals, contains up to 34 glutamine residues (HDCRG 1993). In 1994, Perutz *et al.* showed that this portion forms a *polar zipper* structure, and suggested that its physiological function is to bind transcription factors that contain a polyQ region (Perutz et al 1994); it has now been shown that wild-type huntingtin interacts with several partners and that the polyQ tract is a key regulator of such binding (Harjes and Wanker 2003; Li and Li 2004; Goehler at al 2004). Clabough and collegues have generated mice with a precise deletion of the short CAG triplet repeat encoding 7Q in the mouse HD gene Hdh (DeltaQ/DeltaQ). Hdh (DeltaQ/DeltaQ) mice are

born with normal Mendelian frequency and exhibit only subtle phenotypes, i.e., defects in learning and memory test. The authors suggest that the polyQ tract is not required for essential function of huntingtin but instead may modulate a normal function of the protein. In higher vertebrates, the polyQ region is followed by a polyproline (polyP) stretch, which might help to keep the protein in solution (Clabough et al 2006). Downstream of these regions are the so-called HEAT repeats, which are~40-amino-acid-long sequences that occur multiple times within a given protein and are involved in protein–protein interactions (Andrade and Bork 1995; Neuwald and Hirano 2000). Bioinformatic analyses have found 36 putative HEAT repeats in huntingtin (Takano and Gusella 2002). Subsequently, three main clusters of HEATs have been identified. The repeats are well conserved in huntingtin from all vertebrates, which may indicate that it interacts with the same proteins across vertebrates, whereas 28 putative consensus repeats have been found in *Drosophila melanogaster* huntingtin, whose degree of conservation with respect to humans has yet to be fully defined. In 2001, Andrade et al. (Andrade et al 2001) reported that HEAT repeats could be organized in three-dimensional structures called ROD. More recently, a new neural network for the prediction of *rod repeats* has been applied to huntingtin, and three domains of ROD have been found that defined H1 covering from amino acids 114 to 413, H2 comprised between 672 and 969, and H3 between 2667and 2938 (Palidwor et al 2009). The study revealed also for the first time the presence of intra molecular interactions between single RODs of human huntingtin, suggesting the possibility of homodimerization of huntingtin through inter- and intramolecular association of the RODs domain. The presence of HEAT repeats suggests that huntingtin may exert its physiological function by using different protein partners.

Huntingtin contains a functionally active carboxy (C)-terminal nuclear export signal (NES) sequence and a less active nuclear localization signal (NLS), which might indicate that the protein (or a portion of it) is involved in transporting molecules from the nucleus to the cytoplasm (Xia et al 2003). This hypothesis is supported by huntingtin's perinuclear and nuclear distribution, and the recent demonstration that the 17 amino acids before the polyQ region interacts with the nuclear pore protein TPR (translocated promoter region), which exports proteins from the nucleus. Removal of these amino acids causes huntingtin to accumulate in the nucleus (Cornett et al 2005). Huntingtin also contains three well-characterized protease cleavage consensus sites (Goldberg et al 1996; Wellingtonet al 1998; Wellington et al 2000; Gafni and Ellerby 2002; Gafni et al

2004), where cleavage generally leads to fragments of both normal and mutant huntingtin, although the latter is more susceptible to proteolysis and generates fragments that are found in the cytoplasm and nucleus (Davies et al 1997; DiFiglia et al. 1997; Kim et al 2002; Lunkes et al 202; Wellington et al 2002). Caspase cleavage sites are typically conserved in vertebrate huntingtin, but not in that of *D. melanogaster* (Goldberg et al 1996; Wellingtonet al 1998; Wellington et al 2000; Wellington et al 2002; Li et al 1999). Functionally active calpain cleavage sites have been described for mouse and human huntingtin (Gafni and Ellerby 2002; Gafni et al 2004). Other sites, the exact amino acid positions of which are not well defined, are preferentially cleaved in some brain regions (Mendle-Mueller et al 2001). The contribution of huntingtin proteolysis to cell function is unclear. However, modifications in the activity of caspase and calpain reduce the proteolysis and toxicity of the mutant protein, and delay disease progression (Wellington et al 2000; Gafni et al 2004; Ona et al 1999). Huntingtin is subject to four types of post-translational modification: the amino (N)-terminal lysines K6, K9 and K15 compete for sumoylation and ubiquitination (Steffan et al 2004; Kalchman 1996) and phosphorylation at serines 421 and 434 influences cleavage and toxicity, and is reduced in HD (Humbert et al 2002; Warby et al 2005; Luo et al 2005; Hackman et al 2000). Huntingtin is also palmitoylated by its co-partner, huntingtin-interacting protein 14 (HIP14, a palmitoyl transferase), but the precise amino acid position involved is unknown. The palmitoylation of huntingtin is consistent with its proposed role in regulating vesicular trafficking, because palmitoylated proteins are often involved in the dynamic assembly of components that control vesicle trafficking and synaptic vesicle function (Huang et al 2004; DiFiglia et al 1995). Therefore, huntingtin might have flexible or multifunctional structures capable of assuming specific conformations and activities depending on its binding partners, subcellular location, and time of maturation in a given cell type and tissue. Recently Bezprozvanny and colleagues used X-ray crystallography at atomic resolution to show that polyglutamine in huntingtin adopts multiple flexible conformations (-helix, random coil, and extended loop) (Kim et al 2009).

Huntingtin Functions (Figure 2)

Huntingtin in embryonic development. Two years after the cloning of the gene, huntingtin was shown to be essential for embryonic development as its complete inactivation in huntingtin knock-out mice (*Hdh*-/-) causes embryonal death before day 8.5, i.e., before

gastrulation and the formation of the nervous system (Duyao et al 1995; Nasir et al1995; Zeitlin et al 1995). The basis of this effect appears to be increased apoptosis in the embryonic ectoderm shortly after the onset of gastrulation. It is known that the defect in development observed in the huntingtin knock-out mice embryos derives from a defect in the organization of extraembryonic tissue, possibly as a consequence of an alteration in the nutritive function of the visceral endoderm (Dragatsis et al 1998). Intriguingly, the inactivation of huntingtin gene does not reveal a phenotype in *Drosophila melanogaster* embryos, further reinforcing the evidence that the protein in the protostome branch may have different functions (Zhang et al 2009). Unexpectedly, *Drosophila* huntingtin is required for maintaining the mobility and long-term survival of adult animals, and for modulating axonal terminal complexity in the adult brain (Zhang et al 2009). With the progression of embryonic development, experimental reductions of huntingtin levels to below 50% cause defects in the epiblast, the structure that will give rise to the neural tube, and profound cortical and striatal architectural anomalies (Auerbach et al 2001; White et al 1997). Defects in the formation of most of the anterior regions of the neural plate, specifically in the formation of telencephalic progenitor cells and the preplacodal tissue, have been recently found by Henshall et al. (Henshall et al 2009) that used morpholinos to reduce huntingtin levels in the developing zebrafish. These data indicate that, in addition to its early extraembryonic function, at post gastrulation stages huntingtin participates in the formation of the CNS. To further investigate the role of huntingtin in development, Dragatsis and colleagues (Dietrich et al 2009) have inactivated the *Hdh* gene in Wnt1 cell lineages using the Cre loxP system of recombination, demonstrating that conditional inactivation of the *Hdh* gene in the midbrain and hindbrain results in congenital hydrocephalus. These results implicate huntingtin also in the regulation of cerebralspinal fluid (CSF) homeostasis (Dietrich et al 2009). Analyses of chimeras created by blastocyst injectionof *Hdh* -/-ES cells have provided further insights into the role of wild-type huntingtin in brain maturation by showing that it is critical for establishing and maintaining especially cortical and striatal neuronal identity (Reiner et al 2001). Although some brain regions were appropriately colonized by *Hdh* -/-neurons, few donor cells were found in cerebral cortex, striatum, basal ganglia, thalamus, and the Purkinje cell layer of the cerebellum, thus suggesting that huntingtin plays a specific role in neuronal survival in these brain regions. Preliminary analyses of blastocysts injected chimeras at E12.5 showed ongoing *Hdh* -/-cell degeneration specific to the striatum, cortex, and thalamus, thus supporting the

view that neuroblasts in these areas need to synthesize huntingtin if they are to progress in development and differentiation (Reiner et al 2003). These data indicate that huntingtin is required at different steps of embryonic development and that its total absence or 50% reduced presence generates a very early phenotype in mice. Moreover, the HD mutation does not seem to abrogate the developmental functions of huntingtin, as HD patients pass development and symptoms only start to manifest several years after birth. Human mutant huntingtin can compensate for the absence of endogenous huntingtin, by rescuing the embryonic lethality of mice homozygous for a targeted disruption of the endogenous *Hdh* gene (Leavitt et al 2001). Thus it is reasonable to suggest that huntingtin's function during embryonic development is independent of the length of the polyQ. Mice carrying 50% full-length wild-type huntingtin (i.e., one allelic dose) reach normal adult life (Duyao et al 1995; Nasir et al1995; Zeitlin et al 1995). However, one of three generated heterozygous knock-out mice, which still expresses a 20-kDa N-terminal portion of wild-type huntingtin, at adulthood shows behavioral abnormalities, cognitive deficits, and significant neuronal loss in the subthalamic nucleus (Nasir et al 1995; O'Kusky et al 1999). This phenotype may be ascribed to reduced huntingtin function(s) and/or to a dominant negative effect driven by the remaining N-terminal fragment over the full-length wild-type protein, regardless of the CAG tract. Although HD mice are born with no apparent defects, a recent study from Mehler's group suggests that developmental abnormalities occur in a knock-in mouse model of HD (HdhQ111) compared with a knock-in mouse model expressing only 18 CAG (HdhQ18) (Molero et al 2009). By analyzing the expression of markers of MSNs such as Islet1, dopamine and cAMP-regulated phosphoprotein, 32-kDa (DARPP32), and mGluR1 in the striatum of HdhQ111 embryos at E17.5, the authors report an impairment in the acquisition of the cytoarchitecture of striatal subcompartments, suggesting abnormalities in MSN specification and maturation. They also show that neural progenitor cells from embryonic striatum exhibit *in vitro* reduced proliferative potential, enhanced late-stage self-renewal, and impaired generation of MSN subtypes. Yet, HdhQ111 mice show a very mild disease phenotype and no striatal neurons degeneration. Although more work is required, the hypothesis that HD may be a developmental disease is of interest, especially given that recent preliminary functional MRI analyses (fMRI) studies indicate that subtle brain structure abnormalities may be present in children at risk for HD (age 7–18) who are estimated to be decades from diagnosis and may be indicative of developmental defects (Nopoulos et al 2009). More

recently, Godin and colleagues has found that inactivation of the mouse huntingtin gene in neural progenitor promotes neurogenesis, induced premature differentiation and alter cell fate of cortical progenitors by mechanisms involving mitotic spindle orientation (Godin et al., 2010).

Huntingtin in adult brain. In vitro and *in vivo* studies have shown that wild-type huntingtin has a pro-survival role. The first *in vitro* demonstration of an antiapoptotic role of wild-type huntingtin came from experiments performed by our group on immortalized cell lines. We found that overexpression of wild-type huntingtin in brain-derived cells protects them from toxic stimuli (Rigamonti et al 2000; 2001). One year later, similar results have been obtained by Rubinsztein and colleagues (Ho et al 2001) who showed that wild-type huntingtin can protect neuroblastoma and kidney cell lines from death triggered by the mutant protein. Additional confirmation of an antiapoptotic role of huntingtin came from studies by Hayden's group. They found that primary striatal neurons from YAC18 transgenic mice overexpressing full-length wild-type human huntingtin were protected from apoptosis compared with cultured striatal neurons from non transgenic littermates and YAC72 mice expressing mutant human huntingtin (Leavitt et al 2006). On the contrary, cells depleted of wild-type huntingtin were more sensitive to apoptotic cell death and showed increased level of caspase-3 activity, with respect to control cells (Zhang et al 2006). Some of the mechanisms by which wild-type huntingtin protects cells from apoptotic cell death have been elucidated. We found that wild-type huntingtin blocks the formation of a functional apoptosome complex and the consequent activation of caspase-3 and caspase-9 (Rigamonti et al 2000; 2001). In line with these findings, a study from Friedlander and colleagues (Zhang et al 2006) showed that wild-type huntingtin blocks apoptosis by physically interacting with active caspase-3, thereby inhibiting its proteolytic activity. Other authors found that huntingtin inhibits the formation of the proapoptotic HIPPI-HIP1 complex, thus interfering with procaspase-8 activation and apoptotic cell death (Gervais et al 2002; Hackam et al 2000). Further studies revealed that wild-type huntingtin is a substrate for Akt, a serine/threonine kinase that activates pro-survival pathways, and may participate in the phosphoinositide3-kinase (PI3K)-Akt pathway by stimulating the expression of prosurvival genes, whereas death genes such as BAX or Bcl-2 are repressed (Rangone et al 2004). In 2000 Zeitlin's group showed that neuronal inactivation of the huntingtin gene in adult mice by means of a Cre/LoxP site-specific recombination system driven by the neuronal-specific subunit of the calcium-

dependent calmodulin kinase-2 (Camk2a) promoter, causes apoptotic cells in the hippocampus, cortex, and striatum, and a lack of axon fibers (Dragatsis et al 2000, Rigamonti et al 2000). This animal model showed a progressively more severe limb-clasping upon-tail suspension phenotype, a feature also observed in transgenic HD mouse mutants. Further studies provided *in vivo* evidence that huntingtin regulates the balance between neuronal survival and death and that the levels of huntingtin modulate neuronal sensitivity to excitotoxic neurodegeneration (Leavitt et al 2006). In fact, the overexpression of wild type huntingtin in YAC18 transgenic mice was found to confer significant protection against apoptosis triggered by excitotoxicity (Leavitt et al 2006). Moreover, endogenous huntingtin is reduced following ischemic injury through a caspase mediated process, while overexpression of wild-type huntingtin two to three times with respect to levels in wild type mice protects against ischemic injury in an huntingtin dose-dependent manner (Zhang et al 2003). More recently, the antiapoptotic role of huntingtin has been highlighted also in non mammalian models. In fact, apoptotic cell death has been found in zebrafish embryos in which huntingtin has been knocked down by morpholino technology (Diekmann et al 2009). Huntingtin morpholino-injected zebrafish showed a massively increased cell death as indicated by caspase-3 activity especially in the midbrain/hindbrain region of the developing zebrafish embryo. This increased apoptosis was accompanied by a severe underdevelopment of the CNS (Diekmann et al 2009).

Huntingtin and gene transcription. Biological and molecular findings have linked wild-type huntingtin to BDNF, a neurotrophin that is particularly important for the survival of striatal neurons and for the activity of the cortico-striatal synapses (Zuccato et al 2007). BDNF colocalizes with huntingtin in cortical neurons that project to the striatum and, despite some reports of BDNF mRNA transcription in adult striatal neurons, it is well established that most of the striatal BDNF is produced in the cerebral cortex and delivered to the striatal neurons via the cortico-striatal afferents (Altar et al 1997; Baquet et al 2004; Fusco et al 1999). Several findings have led to the conclusion that wild-type huntingtin contributes to the pool of BDNF proteins produced in the cerebral cortex and that a loss or reduction in wild-type huntingtin activity diminishes BDNF production and delivery to striatal targets, thus likely contributing to selective degeneration of those neurons (Zuccato et al 2007; 2001; 2008; 2003; Gauthier et al 2004). Wild-type huntingtin stimulates cortical BDNF protein production by acting at the level of BDNF gene transcription as shown by *in vitro* and *in vivo* data. In fact,

cultured brain cells overexpressing wild-type huntingtin produce increased BDNF mRNA and protein levels. Studies on YAC mice expressing increased full-length wild type huntingtin levels (YAC18) also show high BDNF protein levels in the cerebral cortex, as a consequence of the positive regulation by wild-type huntingtin of the BDNF gene transcription (Zuccato et al 2001). Therefore, higher striatal BDNF levels are found in the mice (Zuccato et al 2001). Conversely, reduced BDNF mRNA levels are found in brain samples from wild-type huntingtin-depleted mice as well as in heterozygous huntingtin knock-out mice (Zuccato et al 2007; 2003). A similar 50% BDNF reduction is found in whole zebrafish embryos in which huntingtin has been knocked down (Diekmann et al 2009). A more thorough assessment of the molecular mechanism by which wild-type huntingtin affects BDNF gene transcription has shown that the normal protein specifically regulates the activity of one of the BDNF II promoters. The investigation of the mechanism by which wildtype huntingtin stimulates BDNF gene transcription has concentrated on BDNF promoter exon II, within which a conserved 21- to 23-bp DNA response element (RE1/NRSE) is recognized by REST/NRSF transcriptional regulator. REST/NRSF was identified in 1995 as a protein that binds the RE1/NRSE silencing sequences present in the rat *Scn2a2* and *Stmn2* (*SCG10*) genes. We have found that the RE1/NRSE silencing activity is inhibited in the presence of wild-type huntingtin, through recruitment and sequestration of REST/NRSF into the cytoplasm; REST/NRSF is therefore prevented from binding to, and activating, the nuclear RE1/NRSE regulatory elements. Huntingtin does not seem to interact with REST/NRSF directly, but rather seems to be part of a complex that contains HAP1 and RILP, a protein that directly binds REST/NRSF and promotes its nuclear translocation (Shimojo et al 2008). Huntingtin may therefore act in the nervous system as a general facilitator of neuronal gene transcription for a subclass of genes.

Huntingtin role in axonal and vesicle transport. Huntingtin is found predominantly in the cytoplasm of neurons and is enriched in compartments containing vesicle-associated proteins (Velier et al 1998). In line with this finding, it is retrogradely transported in the rat sciatic nerve where it associates with vesicles and microtubules (Block-Galarza et al 1997). Further evidence of a role of huntingtin in intracellular transport came from a study by Goldstein's group (Gunawardena et al 2003). They found that reduction in *Drosophila* huntingtin disrupts axonal transport (Gunawardena et al 2003). Wild-type huntingtin is also involved in fast axonal trafficking of mitochondria in mammalian neurons (Trushina et al 2004). In primary striatal neurons taken from mice expressing

only one copy of the wildtype allele or 50% of normal huntingtin levels upon CRE-mediated recombination (knock-out), mitochondria became progressively immobilized (Trushina et al 2004). This effect was significantly stronger in complete knock-out neurons than in those with a 50% loss of huntingtin, which points to a dose-dependent effect. Several reports indicate that wild type huntingtin regulates axonal transport by participating in the assembly of the motor complex on microtubules. It has been proposed that huntingtin associates with motor proteins via HAP1, a protein that has been shown to interact with both huntingtin and the p150 subunit of dynactin, thereby enabling retrograde transport and perhaps anterograde transport (Gunawardena et al 2005). Further evidence implicating huntingtin, HAP1, and p150(glued) comes from studies that have highlighted huntingtin's role in the control of BDNF vesicle transport (Gauthier et al 2004).

Huntingtin and synaptic activity. Normal communication between neurons is regulated by a number of proteins in the synapse. Normal huntingtin interacts with cytoskeletal and synaptic vesicles proteins essential for exo- and endocytosis at the synaptic terminals, thus participating in the control of synaptic activity in neurons (Smith et al 2005). One early finding shows that wild-type huntingtin directly binds the SH3domains of PSD95 (Sun et al 2001). PSD95 is a key molecule in synaptic transmission and a component of the membrane associated guanylate kinase (MAGUK) protein family that binds the NMDA and kainate receptors at the postsynaptic density (Maue et al 1990). A decreased interaction of mutant huntingtin with PSD95 has been described in HD, suggesting that more PSD95 is released in HD, thus affecting the activity of NMDA receptors, and possibly leading to their overactivation/sensitization and to excitotoxicity (Sun et al 2001). More recent data show that huntingtin may also take part in the presynaptic complex through its interaction with HIP1, which has been recently associated with the presynaptic terminal (Parker et al 2007). Furthermore, huntingtin can bind to PACSIN1/syndapin, syntaxin, and endophilin A, which collectively play a key role in synaptic transmission, as well as in synaptic vesicles and receptor recycling. These interactions depend on the length of the polyglutamine repeat and are enhanced by the presence of an expanded CAG, leading to impairment of synaptic transmission in HD (Smith et al 2005).

Huntingtin Through Evolution

Most of the known huntingtin protein homologues belong to vertebrates and show a high degree of conservation throughout their length, as murine huntingtin homologue with a 86% of similarity. All mammalian huntingtin homologues shared an high conserved sequences, while high differences are concentrated in polyQ tract. In 1996 Pecheux and co-authors studied huntingtin homologues in different mammalian species, among other gorilla, pig and dog. In particular the study is focused on the region of huntingtin that contained the polyQ. In all investigated species, the number of CAG found is equal or inferior to the normal range of human IT15 alleles. The longer uninterrupted CAG stretch was found in the pig and consists of 18 CAG. Interestingly in all these species the CAG tract is interrupted by a CAA triplet that encoded for glutamine. This interruption could lead a more stably polyQ tract (Pecheux et al 1996). The huntingtin sequence is well conserved also in vertebrate as zebrafish homologue of huntingtin protein has 79% of homology sequence with 4 glutamine (Karlovich et al. 1998). The most divergent vertebrate species (i.e., *Homo sapiens* and the *Fugu* fish) show 80% conservation. Interestingly, because of the presence of shorter introns, the *Fugu* gene contains 67 exons as the human gene, but it spans over a region of 22kb (Sathasivam et al 1997).

The only entirely known invertebrate amino acid sequence is from *Drosophila melanogaster*, in the protostome branch, which is characterized by five 20–50% conserved regions distributed throughout the length of the protein (Li et al 1999; Zhang et al 2009). We therefore speculated that *Drosophila* huntingtin represents a residue of the ancestral huntingtin molecule at the origin of the protostoma-deuterostoma branches, suggesting that huntingtin is, evolutionarily, an old gene (Cattaneo et al 2005). In line with this hypothesis, huntingtin is present in an old deuterostome, the tunicata *Halocynthia roretzi* (sea pineapple) and in the echinodermata *Heliocidaris herithrogramma (*sea urchin) (Kauffman et al 2003). More recently, work from Andrade's group has predicted the presence of the protein in ancient organisms such as the amoeba *Dyctostelium discoideum* and nematode *C. elegans*, but not in the *Saccharomyces cerevisiae* or in previously divergent plants, thus confirming the ancient origin of huntingtin (Palidwor et al 2009).

The analysis of huntingtin tissue distribution in species of the phylogenetic tree lead to interesting information from the expression of the huntingtin gene in two basal deuterostomes. Deuterostomes consist of two primary clades: one, the chordates, which

contains the ascidian, cephalochordates, and vertebrates; and a second clade, which contains the hemichordates and echinoderms. Preliminary analyses of huntingtin mRNA expression and distribution in the primitive chordate *Halocynthia roretzi* and in the echinoderm *Heliocidaris herithrogramma* (sea urchin) detected huntingtin mRNA at all stages of development. Interestingly, C-terminal huntingtin sequence seems confined to non neural tissues in the echinoderm *Heliocidaris herithrogramma*. On the contrary, huntingtin has a ubiquitous distribution in the primitive chordate *Halocynthia roretzi* with increased levels of expression in the nervous system. In ancestral deuterostomes, the huntingtin expression pattern is prevalently non neural and that neural expression of the HD gene may be a chordate evolutionary novelty (Kauffman et al 2003). More recently, we have investigated huntingtin expression during development of the cephalochordate lancelet (*Branchiostoma floridae*) (Candiani et al 2007). The development of the nervous system of lancelet is in fact particularly close to that of vertebrates as it includes vertebrate-like anatomical characteristics such as cephalization and a dorsal nerve cord. We found that in lancelet, huntingtin expression is detectable by in situ hybridization starting from the early neurula stage, where it is found in cells of the neural plate. At later stages, it is retained in the neural compartment but also it appears in limited and well defined groups of non neural cells. At subsequent larval stages, huntingtin expression is detected in the neural tube, with the strongest signal being present in the most anterior part (Candiani et al 2007). This study shows for the first time a subregionalization of huntingtin's expression in the nervous system (Candiani et al 2007). When huntingtin distribution has been analyzed in the protostome branch, in the divergent organism *Drosophila melanogaster*, an ubiquitous expression of huntingtin has been revealed (Li et al 1999; Zhang et al 2009). Furthermore, when huntingtin has been genetically inactivated in *Drosophila*, the larva developed without any defect in the gastrulation process, suggesting that, in contrast to what happens in the deuterostome branch, the protein is not involved in controlling embryo development (Cattaneo et al 2005).

Loss of Wild-Type Huntingtin Function in HD

The above data indicate that wild-type huntingtin has beneficial activities in the mature brain. It is therefore possible that its loss in human HD reduces the ability of neurons to survive and to counteract the toxic effects of the mutant protein. In some mouse models, homozygosity for the HD mutation leads to a more severe phenotype than

heterozygosity for an equivalent CAG expansion in the HD gene (Wheleer et al 1999; Reddy et al 1998). Similarly, a small cohort of HD patients homozygous for the CAG expansion seems to show a more severe disease progression than those who were heterozygous for the mutation (Squitieri et al 2003). Further studies in lymphoblastoid cell lines revealed that homozygotes have a more aggressive molecular phenotype than heterozygotes (Maglione et al 2006, Squitieri et al 2006).

Hayden's group demonstrated the *in vivo* contribution of the loss of wild-type huntingtin function in HD. YAC128 mice were initially crossed with mice heterozygous for the targeted inactivation of the mouse HD gene (*Hdh*+/-mice) to generate YAC128 mice heterozygous for the targeted inactivation of *Hdh* (YAC128 +/-). These mice were then crossed with *Hdh* +/- mice to generate YAC128 mice homozygous for the targeted inactivation of *Hdh* (YAC128 -/- mice) (Van Raamsdonk et al 2005). The phenotypic severity of YAC128 +/+ mice (carrying extra copies of mutant huntingtin with 128Q repeats in normal huntingtin background) was compared with that observed in double mutant YAC128-/- mice that do not express endogenous wild-type huntingtin but express the same amount of the mutant protein with 128Q. The complete loss of wild-type huntingtin in the YAC128 -/- mice led to a slight worsening of striatal atrophy and neuronal loss and a small but significant decrease in the neuronal cross-sectional area. YAC128 -/- mice also showed behavioral and motor abnormalities. In addition, testicular atrophy and degeneration were markedly worsened in the absence of wild-type huntingtin. These data suggest that the elimination of wild-type huntingtin expression in YAC128 mice results in the exacerbation of behavioral deficits and survival, with a mild worsening of neuropathology. The absence of severe striatal abnormalities led to the suggestion that the striatal phenotype is primarily dependent on mutant huntingtin toxicity. Considering a different experimental approach with non mammalian models of HD, Zhang et al. (Zhang et al 2009) have recently shown that the removal of endogenous *Drosophila* huntingtin significantly accelerates the neurodegenerative phenotype associated with a *Drosophila* model of polyglutamine huntingtin toxicity (HD-Q93), further suggesting that disrupting the normal function of huntingtin might contribute to HD pathogenesis.

Several studies have evaluated the impact of wild type huntingtin overexpression in HD. In 2001, Hayden's team showed that expression of mutant huntingtin in the absence of wild-type huntingtin results in massive apoptotic cell death in the testes of male mice, but that the observed cell death can be modulated by the expression of normal

huntingtin. In fact, no evidence of apoptosis is seen in the testes of mice expressing human mutant huntingtin when endogenous wild-type huntingtin is expressed from both *Hdh* alleles (Leavitt etal 2001). These data indicate that wild-type huntingtin reduces the cellular toxicity of mutant huntingtin *in vivo* in the testes of mice (Leavitt etal 2001). Similar results were obtained in *in vitro* experiments involving non neuronal cells, showing that overexpression of wild-type huntingtin reduces the polyQ toxicity induced by an exogenous mutant huntingtin construct (Ho et al 2001). Moreover, overexpression of wild-type huntingtin rescued the activity of a tk-RE1/NRSE-cat construct in109/7Q knock-in cells, indicating that the wild-type protein inhibits the silencing activity of the RE1/NRSE and promotes BDNF gene transcription in HD (Zuccato et al 2003). Similarly, the expression of wild-type huntingtin in 109/109Q knock-in cells seems to be able to rescue the decreased transport and release of BDNF, although the number of analyzed cells was a limiting factor in the experiment (Gauthier et al 2003). However, one should expect that higher levels of wild-type huntingtin may be required to overcome the dysfunctions caused by mutant huntingtin.

Mouse Embryonic Stem cells

Mouse embryonic stem (ES) cells are permanent cell lines derived from the inner cell mass of blastocyst (Evans and Kaufman, 1981; Martin, 1981). These cells have three main hallmarks: they undergo symmetrical self-renewing divisions, they are pluripotent with capacity to differentiate into all fetal and adult cell lineages, and they can incorporate into embryos and contribute to functional tissue generation (Smith 2001). ES cells are commonly cultured in in the presence of feeder layer (MEF-Murine Embryonic Fibroblast), with bovine serum (as BMP4 source) and LIF (Leukemia inhibitory factor). The feeder layer is used as a source of LIF and as a trophic support, but in the presence of serum and LIF ES cells are able to maintained self-renewal also plated on gelatin coating, without feeder. LIF and BMP4 source are able to sustain self-renewal through the inhibition of differentiation stimuli. LIF, is a cytokine that acts with its receptor LIFR activating Stat3 pathway, preventing ES cells endoderm differentiation. BMP4, a member of TGFβ family, bind its receptor and activates Smad signal. This causes the activation of Id (inhibition of differentiation) genes transcription and prevents trophoectoderm differentiation, promoting plurypotency. Recent studies have demonstrated that self-renewal conditions are well preserved in serum free media in the presence of ERK and GSK3β inhibitors (Ying 2008 et al, Wray et al 2010). Ying and

collegues demonstrated that these condition allow to promote self-renewal, by an upstream mechanism with respect LIF and BMP4. During the years ES cells become a useful tool to explore the mechanism directing embryonic development and cell fate and differentiation. ES cells are a valid instrument to genetic manipulation and to study development in in vitro system. Many researcher groups, during the last fifteen years developed some experimental protocol to drive ES cells in many differentiated cell lines. To date, the best-studied mode of ES cell differentiation is the formation in suspension culture of multicellular aggregates called Embryoid Bodies (EBs). Within these aggregates, complex interactions between heterologous cell types result in the induction of differentiation of stem cells to derivatives of all three embryonic germ layers. Plating of the embryoid bodies causes further differentiation and outgrowth. In particular neural differentiation of ES cells has been achieved by several different protocols, some of which are strikingly different. In the protocols that were published first, EBs were treated with retinoic acid at different time windows and then plated on to different substrates (Bain et al 1995; Strubing et al 1995; Fraichard et al 1995). Cells with overt neuronal morphology appeared after plating, and were found to express neuron specific genes such as neurofilament light chain (NEFL), microtubule- associated proteins 2 (MAP2), synaptophysin and others. These cells were found to respond to a range of neurotransmitters and depolarizing currents, confirming that they were indeed excitable neurons. Glial cell types also appeared in such differentiated cultures, as judged both by morphology and expression of specific glial markers. The majority of glial cells produced were astrocytes, but oligodendrocytes have also been generated and selectively expanded from EB cultures. In 2003 Ying and colleagues set up a new neural differentiation protocol in monolayer culture. On withdrawal of self-renewal stimuli, serum and LIF, ES cells will readily generate neural progenitors. Cells are plated at low density, and after 4 days neural precursor markers Sox-1 still appears. Neural commitment requires the absence of exogenous serum factors or bone morphogenetic proteins (BMPs), which act as potent antineural factors, and appears to be driven by autocrine signals, including fibroblast growth factors (FGF4). This protocol allow to produce neuron and glial cells with high efficiency of conversion after 10-15 days of monolayer culture (Ying et al 2003).

_____Aim of the project

The aim of my PhD project is to explore the normal function of huntingtin to understand how it may be involved in the pathogenesis of HD, a cascade of events that affects many brain neurons and kills medium spiny striatal and cortical neurons. Although the acquired toxicity of mutated protein, there are some clues to the individual activities of the normal huntingtin in mammalian brain. At the present time there is no clear understanding of its overall role in nervous system development or maintenance, or whether some portions of huntingtin have evolved specific functions for mammalian neurons. To attend this purpose we studied normal huntingin function during early neural development.

Using phylogenetic approach we purpose a study to understand how this function is evolved in higher species. One primary route for investigating the normal function of proteins is to make cross-evolutionary comparisons of huntingtin homologues in several species. Bioinformatic multiple alignment of huntingtin homologues is useful to obtain sequence informations about its evolution and potential functional domains. Our hypothesis is that huntingtin has neuronal function that have specifically evolved along the deuterostome branch leading to mammals, and are associated with protein domains arising during phylogenesis. In particular we focused our attention on N-terminal portion of huntingtin, containing the typical polyQ tract.

To check the activity of huntingtin domains (in particular N-terminal ones) we use a cell system depleted of endogenous huntingtin i.e. Embryonic Stem cells (ES). Thanks to ability of the ES cells to reproduce early embryonic development, we want to use this system as a tool to study neural development in *in vitro* paradigma. Through neural differentiation protocol we analyze the role of huntingtin during ES conversion in neural precursors and then in mature neurons. Using huntingtin null ES cells we check at the phenotype occurs in absence of huntingtin during neurulation. This phenotype will be characterized and analyzed under the control mechanism.

To test possible newly developed huntingtin neuronal functions we propose some rescue experiments in huntingtin null cells expressing the N-terminal portion of several huntingtin homologues. This study leads us to understand how the activity of the N-terminal domain evolved during phylogenesis revealing the reconstruction of huntingtin neuronal function in different evolved species.

On these bases we think it is important to reveal early biological cellular activities mediated by huntingtin and critical for neural development, the underlying molecular mechanisms, when they emerge along evolution, and if and how these activities may be

associated to specific portions of the protein.

Results

1. A bioinformatic study to reveal huntingtin putative domains

In the first part of my PhD project I contributed to a bioinformatic study about huntingtin primary sequence. The aim of this study was to reconstruct huntingtin evolution to better understand the structure and its particular subdivision in possible protein domains. We make a wide-ranging comparative analysis of huntingtin homologues in both deuterostome and protostome branches (includes 17 sequences from 11 vertebrates, 2 tunicates, and 4 insects) to compare the primary sequence of the protein homologues through multiple alignment. The alignment of huntingtin from vertebrates reveals a high percentage of homology in the primary sequence. This similarity excludes the possibility to obtain some informations about the evolution of the protein and the presence of potential functional domains.

To reconstruct the evolution of the sequence of huntingtin we choose to cloned huntingtin homologues from more ancient species. To add an important point in evolution, we cloned the most ancient deuterostome homologue (i.e., sea urchin *Strongylocentrotus purpuratus* huntingtin), which is present at the base of the deuterostome–protostome divergence and is one of the oldest still living deuterostome organism. Sea urchin is a not chordate and it is characterized by a relative poor organized nervous system. These features led us to analyze a huntingtin homologue in a specie more divergent from mammalians. Along the deuterostome branch we cloned also huntingtin from *Branchyostoma floridae* and *Ciona intestinalis*, chordate but not vertebrate, more evolved compared to sea urchin. Comparison of the gene structure of the entire group of homologues also showed that the gene has evolved along the deuterostome branch by allowing a progressive increase in the number of exons depending on phylogenetic distance, whereas the evolution of the gene (and protein) in the protostome branch is more heterogeneous. This suggests that the protostome branch has less stringent functional constraints and that the function of the protein in protostomes may be involved in different biological functions. The study of primary aminoacid sequences of different huntingtin homologues also highlighted a number of other important aspects:

1) huntingtin consists of 3 major conserved regions corresponding to blocks 1–386 (htt1), 683–1,586(htt2), and 2,437–3,078 (htt3) of human htt;

2) huntingtin primary sequence follows a more progressive and linear evolution along the deuterostome branch and is more heterogeneous in the protostomes;

3) the polyQ evolution is a characteristic typical of deuterostomes whose appearance dates back to sea urchin divergence and whose position is conserved, whereas its length increases; (Figure 3A)
4) the Ciona genus has lost the polyQ while accumulating more differences in its N-terminal fragment;
5) the drosophilids accumulate differences in the N-terminal portion of the protein due to a large aminoacid insertion without any polyQ;
6) when polyQ length increases along vertebrates and couples with the polyP tract, the conservation of the N-terminal domain becomes more stringent.
We speculate that the evolution of the primary huntingtin aminoacid sequence parallels the particular evolution of the nervous system. At a biological level:
1) the sea urchin nervous system is poorly organized in comparison with that of vertebrates;
2) although belonging to the chordates, Ciona has a totally differently organized nervous system from that of vertebrates;
3) vertebrates all share the same structural organization of the nervous system, whose complexity increases progressively with the development of the most anterior brain structures (telencephalon);
4) the structuring of the nervous system along the protostome branch has followed a different type of developmental program (metamerism).
At anatomical level, the evolution of the nervous system along the deuterostome branch has progressively increased its anterodorsal positioning. On these evidences we suggest that, along the deuterostome branch, huntingtin may have become progressively more important for nervous system (Tartari et al. 2008).

1a. The evolution of the polyQ

Through our bioinformatic studies we observed a particular evolution of the characteristic polyQ tract. This sequence, that is probably envolved in protein-protein interaction, is a feature of deuterostomes, since in protostomes huntingtin homologues (i.e. *Drosophila melanogaster*) there is no presence of any polyQ tract. This repeat appears at the base of deuterostome branch in sea urchin specie, as a NHQQ sequence seem to be a primitive polyQ. Along the phylogenetic tree the polyQ expands and maintained the same position. In *Branchyostoma floridae*, cephalochordate, huntingtin has QQ tract. In more evolved species as vertebrates the polyQ became composed by 4Q

in fish and other vertebrate. The poly Q increased in length through the evolution. It expands from vertebrates to mammals, as rodents had 7-8Q and it reached its maximum expansion in humans in which polymorphic stretch is present. In protostomes no polyQ has been found probably as a consequence of a different evolution of huntingtin sequence, and possible relative domains.

1b. Huntingtin has different protein domains

The bioinformatic study we performed gave us some interesting informations about the presence of different protein domains. The multialignment of different huntingtin homologues allow us to reconstruct three main region of huntingtin protein. Huntingtin seems to have three main block, corresponding to position 1–386 (htt1), 683–1,586(htt2), and 2,437–3,078 (htt3) of human htt. The C-terminal portion of the protein is well conserved during evolution, while the N-terminal tract exhibits more recent evolution of the sequence (Figure 3B). For this reasons we speculate that huntingtin critical domain resides in the N-terminal portion of the protein, in which the polyQ first arose 450 MYA and has been specifically maintained (except in Ciona) in the same position although gradually expanding. A particular function of huntingtin could be evolved in parallel during phylogenesis in more evolved species. As the appearance of the polyQ is shown only in deuterostomes it is also possible that, further in the evolution, huntingtin acquired in this branch a glutamine-dependent function, which is particularly important for neurons.

2. Producing expression vectors bearing N-terminal portion of huntingtin from different species

To reconstruct the huntingtin evolution and to understand better its function during phylogenesis, we decided to test the activity of several huntingtin homologues. We expressed these molecule in a cell system depleted of endogenous huntingtin, i.e. Embryonic Stem cells lacking huntingtin. Firstly we looked at the phenotype occured in absence of huntingtin in ES cells in self-renewal condition and applying neural differentiation protocol. In parallel we cloned different huntingtin homologues from key species in phylogenetic tree. Then we checked the activity of these homologues in the ability to rescue huntingtin depletion phenotypes.

Through multialignment analysis we firstly designed and produced several expression vectors containing the N-terminal portion of different huntingtin homologues,

approximately the first 500 aa of the primary sequence. The homologues we choosed are from deuterostomes: *Homo sapiens*, *Mus musculus*, *Branchyostoma floridae*, *Ciona intestinalis*, *Strongylocentrotus purpuratus*. We choose also a protostome homologue *Drosophila melanogaster* and *Dyctiostelium discoideum* as the first multicellular organism in which huntingtin protein is found (Figure 4). We cloned the fragments by PCR amplification, using primers containing tag-HA sequence to detect different huntingtin fragments. *Strongylocentrotus purpuratus, Ciona intestinalis, Branchiostoma floridae, Mus musculus* and *Homo sapiens* huntingtin was produced by specific cDNA amplification. The constructs containing *Dyctiostelium discoideum* and *Drosophila melanogaster* huntingtin were produced by a collaboration of Prof. Gusella (Massachusetts General Hospital Cambridge, MA). After amplification by PCR each fragment was transfered in a pCAG expression vector. We test the expression of constructs by transfection in mammalian cells. After transfection the presence of the huntingtin fragment was evaluated by immunocytochemistry 48hs post-transfection.

3. Developing a cellular tool to study huntingtin function

To study the involvement of huntingtin during the early phase of neural development we set up an *in vitro* paradigm of embryonic development. We choose Embryonic Stem cells (ES) and their ability to differentiate in a wide range of cell types. To study the role of huntingtin during embryogenesis we use an huntingtin depleted ES cell line, in which two alleles of the *Hdh* gene have been inactivated ($Hdh^{ex4/5}$) (Duyao et al., 1995), and their wild-type counterparts. ES $Hdh^{ex4/5}$ line was produced exciding the exons 4 and 5 of huntingtin gene. As a consequence of this deletion, in these cells huntingtin a shorter mRNA was produced but no protein is translated.

3a. Huntingtin depleted cells are more vulnerable to serum deprivation under self-renewal conditions

First of all we characterized ES lacking huntingtin (ES $Hdh^{ex4/5}$) under self-renewal conditions. Cells were assayed by immunoreactivity to typical embryonic stem cells markers as the transcription factor Oct4. Wild-type and $Hdh^{ex4/5}$ ES cells are both positive for Oct4 and shown the same morphology (Figure 5).

Then we check whether the absence of huntingtin can alter the typical ES cells features. We choose two different assay to test cell viability: MTT assay and Caspase-3 active assay.

Firstly MTT reduction to formazan was used. The growth rate of huntingtin depleted ES was compared to the one of wild-type cells over time under basal proliferation conditions. The resulting growth curve of undifferentiated ES cells expressing both (ES *Hdh*$^{+/+}$), and none (ES *Hdh*$^{ex4/5}$) huntingtin alleles reveals that the absence of huntingtin does not impair the growth rate within the first 48 hours of culture. Only after 48-96 hours, in proliferation medium, huntingtin-depleted ES cells start to show a lower capacity to reduce MTT, meaning that less viable ES *Hdh*$^{ex4/5}$ cells were present at those time points, when compared to both ES *Hdh*$^{+/+}$ cell line. The same assay was repeated with cells kept in proliferation media but deprived of serum for up to 96 hours. It is important to note that under this condition of serum deprivation, none of the cell lines undergo differentiation. Under this stress-induced condition, the growth curve of two cell lines was affected, and the number of viable huntingtin depleted cells was significantly lower than that of wild- type cells (Figure 6A).

Then we check huntingtin knock out cells in a well establish huntingtin activity, such as active Caspase-3 assay. Wild-type cells and *Hdh*$^{ex4/5}$ are plated in normal growth condition. After 24hs, complete medium was replaced with serum deprived medium and the active Caspese-3 is measured after 24 hours of serum deprivation. ES *Hdh*$^{ex4/5}$ cells show a greater increase of caspase-3 activity, when compared to wild-type cells. These findings clearly indicate that huntingtin-depleted mouse embryonic stem cells (ES *Hdh*$^{ex4/5}$) are particularly vulnerable to serum deprivation (Figure 6B).

3b. "Rosetteless phenotype" in absence of huntingtin during neural differentiation

To study huntingtin role during neural development we have subjected ES cells *Hdh*$^{ex4/5}$ and their wild-type counterparts to a neural differentiation protocol in monolayer culture, according to Ying et al., 2003 (Figure 7). This protocol provides a simple, rational system for conversion of ES cells into neural precursors and thence into neurons and glia, which likely results from a better mimicry of the events that occur in the embryo. Particularly, during the first stage of neural development (day 6-8) ES wild-type cells are converted into neural precursors forming neural tube-like rosettes, radially organized columnar epithelial cells. As neural rosettes continue to grow *in vitro*, they mimic the processes occurring during neurulation and neural tube growth. Rosette cells gradually give rise to differentiated cells such as neurons migrating radially away from the rosettes structure. Neural rosettes are positive for neuroectodermal markers such as Nestin, a protein of intermediate filament, N-cadherin, typical protein of neural

adherent junctions, ZO-1 typical protein of the tight junction. Nestin staining shows the presence of neural precursors radially arranged, along all rosettes structure. N-cadherin and ZO-1 staining have a particular luminal localization, indicating a strict apical-basal polarity. As neuroephitelium *in vivo* neuroephitelial cells inside rosettes are anchored to one another apically through junctional complex characterize by more apical tight junction expressing ZO-1 followed by N-cadherin expression in adherent junction. Under differentiation condition we find that in absence of huntingtin aberrant spatial organization of neural precursors has been observed at day (6-8) as judged by Nestin, N-cadherin and ZO-1 expression, which we named "*rosetteless phenotype*" (Figure 8). We show a severe reduction in neural rosettes number and size in spite of a comparable number of Nestin positive cells. We show N-cadherin and ZO-1 missing the typical luminal staining suggesting a particular disorganization of neural precursors. Then we tested this phenotype also in an other cell line lacking huntigntin. The model we chose, null huntingtin ES (*Hdh*$^{pr/ex1}$), is characterized by the deletion of the promoter and the exon 1 of huntingtin gene. In these cells no mRNA production is detected (Zeitlin et al., 1995). We test this cell line to the ability to form rosettes under neural differentiation protocol. Our data demonstrate that "*rosetteless phenotype*" is evident also in this cell line, confirming that this phenotype is due to the absence of huntingtin, and it is cell-line independent (Figure 8). We quantified this disorganization by using the imaging software ImageJ. We performed an immunocytochemistry for Nestin neural marker. 12 different fields were acquired at fluorescence microscope. We calculated the total surface occupied by Nestin positive cells in each field. After that, we design a mask for each rosette in the field, then we measure the surface occupied by Nestin positive cells IN and OUT of the rosettes (Figure 9A). Quantification by this imaging technique showed an approximately 70% reduction of Nestin positive cells inside rosettes in both the two different batches of cell lines depleted by endogenous huntingtin (Figure 9B).

3c. Validation of "Rosetteless phenotype"

RNA interference approach has been used to analyse the role of endogenously expressed huntingtin on rosettes formation. Huntingtin siRNA was delivered at day 0 of the monolayer protocol and decreased huntingtin up to 40% of the scrambled-transfected level after 24 hours. The reduction is maintained until day 5, a time point at which the fate of neural precursor cells has been already defined (Figure 10A). At day 8 huntingtin

knockdown in *Hdh*$^{+/+}$ causes defects of neural precursors spatial organization, with respect to cells treated with scramble RNAi (Figure 10B).

On the contrary, expression of full-length mouse huntingtin in a null huntingtin background led to a nearly complete rescue of the "*rosetteless phenotype*". We described the presence of neural rosettes as in Hdh$^{+/+}$ cells, as an approximately 65% increased of Nestin positive cells inside rosettes was found (Figure 10C-D).

We also established co-culture systems to understand whether the presence of wild-type cells can induce *Hdh*$^{ex4/5}$ to produce neural rosettes. Wild-type cells (*Hdh*$^{+/+}$) have been equally mixed with cells lacking huntingtin stably expressing eGFP (*Hdh*$^{ex4/5}$ *eGFP*) and wild-type cells stably expressing eGFP (*Hdh*$^{+/+}$*eGFP*) have been mixed with cells lacking huntingtin (*Hdh*$^{ex4/5}$). Cells were subjected at neural monolayer differentiation as above. Cells have been fixed at day 8 and immunodecorated for Nestin, N-cadherin and eGFP. We observed the formation of neural rosettes composed exclusively by *Hdh*$^{+/+}$ cells, whereas *Hdh*$^{ex4/5}$ cells still remained unable to form rosettes in both the experimental conditions (Figure 11). Therefore the ability to form rosettes is a cell-autonomous process. Together these results show that wild-type ES cells does not exert a positive effect on cells that are null for huntingtin gene, and no secreting factors support huntingtin depletion defect.

Altogether these data indicate that huntingtin is involved in the process underlying the formation of neural rosettes, suggesting a possible role of the protein in regulating early phases of neural development.

3d. Neural precursors defects leads to vulnerable mature neurons in absence of huntingtin

During the first stage of neural differentiation we shown that cells lacking huntingtin exhibit a defect in spatial organization in spite of immunoreactivity to neuroectodermal markers. We also testing the ability of huntingtin knock out cells to undergo terminal differentiation to product mature neurons and glial cells. We performed some experiments of monolayer differentiation until 21 days of differentiation. During the late stage of maturation, we check the presence of mature neurons, MAP2 (Microtubule Associated Protein) and astrocytes GFAP (Glial Fibrillary Associated Protein) reactive. At D14 of differentiation we found the same rate of mature neurons and glia cells, indicating that "*rosetteless phenotype*" didn't impair the abilty of huntingtin knock out cells to differentiate in mature neural cells (Figure 12). Some of mature neurons

observed are GABA (Gamma Amino Butyric Acid) positive, demonstrating the ability of cells lacking huntingtin to differentiate in GABAergic neurons, as allowing by Ying protocol. In spite of an equal rate of differentiation, we also observe a decrease in the number of mature neurons at D21 of neural differentiation protocol, suggesting that the absence of huntingtin causes the production of more vulnerable neurons. Therefore we check the immunoreactivity for GAP-43 (Growth Associated Protein) at D21. GAP-43 is a protein expressed at high levels in neuronal growth cones during development. We can observed a reduction of GAP43 positive cells, suggesting mature neurons from *Hdh*$^{ex4/5}$ cells are defective in protrusions growth and and have less viability *in vitro* until the end of differentiation (Figure 12).

4. Huntingtin drives rosettes formation through N-cadherin-mediated cell adhesion

To understand better the mechanism causing "*rosetteless phenotype*", firstly, we assay the migration ability of huntingtin knock out cells, to test if defects in migration events could led to impairment in rosettes formation. By computerized video time-lapse microscopy we found that wild-type cells and huntingtin-depleted cells show similar migration speed (*Hdh*$^{+/+}$:0,42um/min±0,27 and *Hdh*$^{ex4/5}$:0,33 um/min±0,22), then we excluded the possibility that defects in cell migration may be responsible for the *"rosetteless phenotype"* (Figure 13).

Then we want to check if the aberrant spatial organization of neural precursors cells in rosettes may be due to defects in cell anchoring. We focussed on adhesion molecules as potential intermediates of huntingtin function at the stage of rosettes formation. Among the several candidates, N-cadherin, is typical located into adherent junction and it is critically involved in the regulation of cell adhesion and cell migration in the CNS (Halbleib and Nelson, 2006). N-cadherin is a cell surface glycoprotein mediating Ca^{2+}-dependent homophilic cell adhesion and it is mainly expressed in neuronal cells, physically associated with NMDA receptor (Husi et al 2000). It is involved in neurite outgrowth, neuron pathfinding and in synaptic structure it ensures cell-cell comunication. Moreover N-cadherin has a role in the maintenance of epithelial integrity, heart tube formation, neurulation and somitogenesis (Radice et al 1997). Several studies demonstrate that N-cadherin has a foundamental role in neurulation. N-cadherin null embryos die at E9.5, because of a cell adhesion defect in the developing myocardium (Radice et al 1997), and neurulation defects also occur, the neural tube is still formed

but has an abnomal apparence (Radice et al 1997). N-cadherin morpholino in Zebrafish causes blockage of neural tube formation. The cells are not defective in their ability to form protrusions, but they are unable to mantain them stably (Hong and Brewster 2006).

For these reasons we choose N-cadherin as candidate to investigate whether N-cadherin-pathway could be affected in the absence of huntingtin, contributing to the "*rosetteless phenotype*".

4a. Loss of wild type huntingtin does not affect N-cadherin expression level

First of all we check the expression level of N-cadherin during neural differentiation, assaying mRNA and protein levels. N-cadherin mRNA levels are assayed in $Hdh^{+/+}$ and $Hdh^{ex4/5}$ from D0 to D7 of differentiation protocol by Real-time PCR. No changes in N-cadherin mRNA levels were observed in the absence of huntingtin, at the same stages (Figure 14A). We performed also western blotting analyses to detect N-Cadherin protein level on $Hdh^{+/+}$ and $Hdh^{ex4/5}$ cells during the initial stage of the monolayer protocol (day 0, 5, 6, 7 and 8). No differences in full-length N-cadherin levels have been found in $Hdh^{+/+}$ and $Hdh^{ex4/5}$ starting from day 5. These preliminary analysis demonstrate that there is no change in the total amount of N-cadherin in $Hdh^{ex4/5}$ cells with respect the control cells.

4b. Alterated N-cadherin cleavage occurs in huntingtin depleted cells

In physiological condition N-cadherin is subjected to a cleavage by metalloproteinase to regulate cell-cell interactions. N-cadherin full-length is a 135 kDa protein characterized by an N-terminal extracellular domain, directly involved in cell-cell interaction; and a C-terminal cytoplasmatic domain that interact with β-catenin, which in turn is linked with the cytoskeleton. N-cadherin is cleaved by two different metalloproteinase. The first cut is due to the activity of ADAM-10 metalloproteinase that generates a N-terminal 95 kDa fragments (NTF) and C-terminal 40 kDa fragments (CTF1), which can be further processed by PS/g-secretase complex into soluble 35 kDa fragments (CTF2) (Reiss et al. 2005). In this work we wanted to study N-cadherin cleavage mechanism to understand whether alteration in this process could have an effect in "*rosetteless phenotype*". We performed some western blotting analysis to evaluate N-cadherin fragments amount in our cell lines. We found that level of CTF1 fragment is increased in $Hdh^{ex4/5}$ compared to $Hdh^{+/+}$ from day 5 to day 8, reaching a peak at day 8 in which 8-fold increased has been

observed. Similar results have been obtained in *Hdh*$^{pr\text{-}ex1}$ compared to *Hdh*$^{+/+}$ at day 8 (Zeitlin et al. 1995) (Figure 14B-C).

We want to analyse also the CTF-1 production using a typical pharmacological treatment with Ionomycin (2.5 μM concentration for 30 min at day 8). This compound is commonly used to promote cadherin shedding through stimulation of calcium influx (Marambaud et al. 2002). We performed treatments with Ionomycin at D8 of differentiation and after 30 minutes cells were lysated. In these condition both *Hdh*$^{+/+}$ and *Hdh*$^{ex4/5}$ lines is responsive to the treatment, but *Hdh*$^{ex4/5}$ cells shown an higher amount of CTF-1 fragment (Figure 14D). These data suggest that huntingtin controls the proteolytic processing of N-cadherin, since in its absence an aberrant cleavage process occurs. Therefore this alteration could regulate the anchoring of neural precursors into the rosettes.

4c. Huntingtin controls ADAM-10 activity

Multiple line of evidence indicates ADAM10 is the major metalloproteinase responsible for N-cadherin cleavage in neuronal cells. Therefore, we investigated ADAM10 level and activity in control and *Hdh*$^{ex4/5}$ cells. Western blot for ADAM10 showed three immunoreactive bands at 100 kDa, 80 kDa and 60 kDa, respectively indicating ADAM10 precursor, the partially processed form and the catalitically active protein. We found that levels of precursor and inactive form of ADAM10 are similar between control and knock-out cells. On the contrary, the amount of the processed and active 60-kDa form shows a 2-fold increase in *Hdh*$^{ex4/5}$ compared to *Hdh*$^{+/+}$ (Figure 15A-B). We suggest increase N-cadherin cleavage in the absence of huntingtin is due to increased activity of ADAM10. Accordingly the production of CTF1 fragment was completely blocked with the ADAM10 inhibitor Tissue Inhibitor of MMPs-1 (TIMP1) both in wild-type and knock-out cells (Figure 14D). Altogether these data indicate that normal huntingtin controls ADAM10-mediated cleavage of N-cadherin.

4d. Validation of huntingtin role in ADAM-10 mediated N-cadherin cleavage

To confirm the possible link between ADAM-10, N-cadherin and huntingtin, we have performed some experiments modulating N-cadherin and ADAM-10 expression. We have expressed a mutant form of N-cadherin (HA tagged) resistant to ADAM-10 cleavage in *Hdh*$^{ex4/5}$ cells. Stable *Hdh*$^{ex4/5}$ cell line overexpressing mutated form of N-cadherin was produced and subjected to neural differentiation. We observed that the mutant N-

cadherin transgene rescued the "*rosetteless phenotype*", suggesting that abnormal cleavage of N-cadherin is responsible for the observed defect in rosette formation (Figure 16A). Moreover rosettes produced in the presence of mutated N-cadherin are immunoreactive for HA, demonstrating that full-length N-cadherin is responsible of rosettes maintenance. Moreover, the production of CTF1 fragment is blocked in *Hdh$^{ex4/5}$* cells (Figure 16B-C). Similar results have been obtained when ADAM-10 has been knock-down in *Hdh$^{ex4/5}$* cells by RNAi, leading to a significant decrease in the production of CTF1 (Figure 17A-B). These data confirm that huntingtin controls the formation of neural rosette by modulating ADAM-10-mediated N-cadherin cleavage.

5. Assaying the activity of huntingtin N-terminal domain by rescue experiments in huntingtin depleted cells

Our recent bioinformatics studies and cross-evolution comparison of huntingtin homologues suggest that huntingtin function(s) may be embedded into three possible protein domains, one of which, the N-terminal fragment, containing the polyQ and more recently evolved, may be responsible for the neuronal functions of the protein (Tartari et al. 2008). We have chosen to perform rescue experiments in huntingtin-null ES cells to evaluating the activity of the N-terminal huntingtin fragment and to test the hypothesis of a progression in neuronal function of the N-terminal portion during evolution. We selected a range of N-terminal fragments of huntingtin from ancient species and organisms of the deuterostome branch - with progressively more evolved nervous systems, one specie in the protostome branch and a more ancient specie before the divergence of protostome-deuterostome. The huntingtin homologues we used are from: *Dictyostelium discoideum* (*Dd*), *Strongylocentrotus purpuratus* (*Sp*), *Mus musculus* (*Mm*), *Homo sapiens* (*Hs*) with 15Q or mutated form with 128Q, *Drosophila melanogaster* (*Dm*) (Figure 18).

5a. Production and characterization of stable cell lines of Hdh$^{ex4/5}$ expressing N-terminal fragment from different species

ES *Hdh$^{ex4/5}$* are transfected with pCAG expression vectors bearing the N-terminal fragment of huntingtin from different homologues. We have cloned N-terminal huntingtin cDNA from *Dyctiostelium discoideum*, a motile soil amoeba at the point of transition from unicellular to multicellular organisms with no nervous system, at the base of the Metazoan phylogenetic tree. We have also cloned the N-terminal huntingtin

from *Strongylocentrotus purpuratus* (sea urchin) the oldest deuterostome still living with a primitive nervous system organized into radial nerves. Then, N-terminal huntingtin from organisms with a nervous system of increased complexity such as *Mus musculus* and *Homo sapiens* have been included. We also introduced a protostome specie in the study to confirm the specificity of this huntingtin function in deuterostomes derived species, *Drosophila melanogaster*. We also chose to clone the C-terminal portion of murine huntingin as a control to assay specific activity of N-terminal domain.

All constructs have been transfected in ES *Hdh*$^{ex4/5}$. For constructs bearing N-terminal from *Dd*, *Sp*, *Dm* and C-terminal fragment from *Mm*, we use expression vectors containing the transgene-HA tagged for the detection. 24 hours after transfection the cells are selected with puromycin antibiotic, to select stable cell lines. After 10 days of antibiotic selection resistant cells are pulled and maintained in normal ES culture condition. We selected the following cell line:

- ES *Hdh*$^{ex4/5}$ N-*Dd*
- ES *Hdh*$^{ex4/5}$ N-*Sp*
- ES *Hdh*$^{ex4/5}$ N-*Mm*
- ES *Hdh*$^{ex4/5}$ N-*Hs 15Q*
- ES *Hdh*$^{ex4/5}$ N-*Hs 128Q*
- ES *Hdh*$^{ex4/5}$ N-*Dm*
- ES *Hdh*$^{ex4/5}$ C-*Mm*

To test the expression of the transgene, we assayed cell lines by western blot and immunocytochemical analysis. All stable cell lines expressed the transgene as shown using huntingtin antibody or HA antibody (Figure 19A-B).

5b. Rescue assay during neural differentiation: the ability to form rosettes is a huntingtin function included in its N-terminal domain

First, we expressed the first 550 aa of *Mus musculus* huntingtin in *Hdh*$^{ex4/5}$ that have been subjectd to neuronal differentiation towards the monolayer protocol. A full-rescue of the "*rosetteless phenotype*" has been observed similarly to what observed after full-length wild-type huntingtin transfection. Importantly, no rescue in rosettes formation has been detected when C-terminal portion of huntingtin was transfected in *Hdh*$^{ex4/5}$ (Figure 20). Rescue of the "*rosetteless phenotype*" is independent from transgene level, since low or high amount expression of N-terminal mouse huntingtin both led to rosettes formation in *Hdh*$^{ex4/5}$. These data demonstrate that the N-terminal portion of

huntingtin is a protein domain that controls the process of rosettes formation during early neural development.

5c. The rosetteless phenotype is rescued by N-terminal domain from species with a more evolved nervous system

Then, we tested the possibility that the ability to regulate rosettes formation carried by the N-terminal domain is a property of huntingtin that has emerged during deuterostome evolution. All transgenic cell lines expressing the N-terminal portion of huntingtin were tested during neural differentiation and their ability to complement defects at the level of rosettes formation and N-cadherin cleavage, previously observed in *Hdh*$^{ex4/5}$ cells, has been evaluated. The cells have been subjected to neuronal differentiation in monoculture and immunodecorated at day 8 for Nestin, N-Cadherin and ZO-1. We quantified the "*rosetteless phenotype*" with imaging analysis we set up for this protocol. Our experiments indicate that overexpression of N-terminal huntingtin from *Dictyostelium discoideum* shows null ability to restore "*rosetteless phenotype*" in *Hdh*$^{ex4/5}$ cells. An approximately 5% rescue capability was found when *Strongylocentrotus purpuratus* N-terminal huntingtin has been expressed. On the contrary, N-terminal portions from species with a progressively more evolved nervous system (*Mus musculus* and *Homo sapiens*) show full rescue of the "*rosetteless phenotype*". Moreover, rosettes formation is strongly impaired and similar to what observed in *Hdh*$^{ex4/5}$ cells when N-terminal from *Drosophila melanogaster* was expressed, thus suggesting that huntingtin has not evolved cell-adhesion functions in the protostome branch (Figure 21A-B).

Accordingly, N-cadherin cleavage (determined by measuring CTF-1 production) still remains high in *Hdh*$^{ex4/5}$ cells expressing the N-terminal huntingtin from *Dyctiostelium dyscoideum* and *Strongylocentrotus purpuratus*. On the contrary, CTF-1 level is significantly decreased when huntingtin from more evolved organisms is expressed (i.e. *Mus musculus* and *Homo sapiens*) (Figure 22A-B). As a consequence of the rescue in "*rosetteless phenotype*" we observed a restoring of GAP43 staining at D21 of differentiation in cells expressing human and mouse N-terminal portion, in spite of a null rescue in transgenic line overexpressing C-terminal portion of huntingtin (Figure 22C).

5d. The impact of the polyQ tract in "rosetteless phenotype"

Therefore we asked whether an expanded polyQ could affect the process of rosette formation and cell adhesion function. We overexpressed N-terminal human mutant huntingtin with 128Q in *Hdh*$^{ex4/5}$ cells and found that the "*rosetteless phenotype*" and abnormal N-cadherin cleavage were partially rescued after N-terminal mutant huntingtin expression (approximately 20%) (Figure 23A-B-C). Moreover, after 21 days of neuronal differentiation the number of MAP2 positive cells is not reduced, and GAP43 staining showed no particularly differences in the mutant huntingtin expressed cells (Figure 23D). These data suggest that more slight impairments could occur in the presence of mutation, as a consequence of an aberrant neural precursors organization, in spite of a correct differentiation of neurons. Altogether these results suggest that the polyQ in the pathological range alters huntingtin functions during development, possibly causing subtle molecular and cellular abnormalities that compromise aspects of the specification and maturation of neurons and subsequently make them more vulnerable to late life stressors.

5e. The anti-apoptotic activity of huntingtin is an old born function conserved during evolution

We then screened the same *Hdh*$^{ex4/5}$ lines expressing the N-terminal fragments of huntingtin homologues for the ability to modulate a previously identified huntingtin non-brain specific function (i.e. the ability to protect by apoptotic cell death by blocking caspase-3 activity). We found that caspase-3 activity is significantly reduced in *Hdh*$^{ex4/5}$ cells expressing N-terminal domain from ancient and more evolved species, with respect to *Hdh*$^{ex4/5}$ cells (Figure 24). These data indicate that the anti-apoptotic activity is an ancestral function of huntingtin that has born in ancient organisms such as *Dictyostelium discoideum* and has been maintained during deuterostome evolution up to mammals.

Discussion

Huntingtin is a large, ubiquitous, partially unknown protein, in which mutation in its N-terminus is the cause of genetic, neurodegenerative disease called Huntington Disease. From the discovery in 1993 of the disease causing gene encoded for huntingtin, some research groups had focus their attention to the molecular mechanism of HD to reveal the basis of the pathology. Several studies during the years had demonstrated that mutation occurred in huntingtin gene cause the production of a mutated protein, that exert its toxic activity, lead to neuronal death. Although the presence of mutated huntingtin, from one decade it is known that the loss of wild-type protein can contribute to pathogenesis of HD (Cattaneo et al 2000). This hypothesis had a new impact in HD research since many scientific groups focused their efforts in study phenotypes caused by loss of normal huntingtin. This studies revealed that huntingtin is embryonic lethal during embryogenesis and it exerts a fundamental role in the adult brain as a protein sustaining viability and health of mature neurons. Huntingtin is shown to be involved in neuron viability and maintenance, but its really and univocal function is already unknown. Huntingtin is a protein *sui generis*, with no particular sequence homology with other known protein. Some studies turn to understand its structure not reveal definitive informations about its possible structure and domains.

This study, object of my PhD project is focused on understand normal huntingtin function. First of all, we performed a bioinformatic study in which multialignment of 17 different homologues of huntingtin, both in deuterostome and protostome branches (includes 17 sequences from 11 vertebrates, 2 tunicates, and 4 insects) were analyze. Through this analysis we dissect the primary aminoacid sequence of huntingtin to reveal the presence of possible protein domains.

Studying huntingtin protein structure

In the first part of my project we performed a bioinformatic analysis of the primary aminoacid sequence of several huntingtin homologues in order to reconstruct the evolution of huntigntin protein along the evolution. We choose some known homologues of huntingtin from deuterostomes and protostomes (i.e. *Mus musculus, Homo sapiens, Drosophila melanogaster*) and we cloned also huntingtin from new ancient species (i.e. *Strongylocentrotus purpuratus, Ciona intestinalis, Branchiostoma floridae*). Our comparison of huntingtin homologues in multialignment analysis first revealed that huntingtin has three major domains. During phylogenesis C-terminal domain is maintained conserved in metazoan, while N-terminal portion presents a more re-arranged sequence showing more recent evolution. In deuterostomes N-terminal

domain has a linear evolution from the echinoderms to mammals, with a particular expansion of the polyQ tract, while protostomes diverged with a different aminoacid sequence and the characteristic lost of polyQ. The aminoacid primary sequence trend suggest us that huntingtin could maintain a conserved function associated to its C-terminal domain during phylogenesis while N-terminal portion specified a different function in protostomes and deuterostomes. Moreover, look at the particular evolution and specification of the nervous system structures, and complexity along deuterostomes, we speculate that the evolution of the primary huntingtin aminoacid sequence parallels the particular evolution of the nervous system. We therefore speculated that the N-terminal portion of huntingtin could be endowed with functional activities that become progressively more specialized during deuterostome evolution and critical for adult neurons (Tartari et al. 2008).

Huntingtin function during neural development

To analyse the role of normal huntingtin since its early embryonic function we choose to study what is the role of the protein during the early phase of embryo development, in particular during the early phases of neural development. To dissect how this function is acquired during phylogenesis we choose to study neural differentiation process and the activity of the N-terminal domain during neurulation.

We selected Embryonic Stem cells and their ability to differentiate in all cell type of the body, as a tool to study early embryonic development. We performed some experiments to understand the role of huntingtin during the first stages of neural commitment and the followed neuronal differentiation.

Using huntingtin deficient embryonic stem (ES) cells and an in *vitro* neural differentiation protocol that allow the multistep processes occurring during neural development, we have found that huntingtin regulates biology of neural rosettes. Neural rosettes are an early transient neural structure composed of radially organized columnar epithelial cells resembling *in vitro* the processes occurring during neurulation. The differentiation potential of neural rosettes corresponds to that of the neural-plate stage cells, suggesting they can mimic *in vitro* the neural tube growth. We found that huntingtin loss causes a defect in spatial organization of the developing neural progenitors, which results in decrease of rosettes number and size, which we called "*the rosetteless phenotype*". In the absence of huntingtin we observed a normal commitment of ES cells into neural precursors, as demonstrates by the immunoreactivity to typical neural precursors markers Nestin and N-cadherin, however the neural precursors

produced is unable to formed neural rosettes. These data suggest that, by controlling rosettes formation, huntingtin may participate at the cytoarchitecture of the developing CNS. We test this activity of huntingtin modulating its expression by several experimental approaches.

i) We check the *rosetteless phenotype* in two different batches of huntingtin depleted cells (*Hdh*$^{4/5}$ and *Hdh*$^{pr\text{-}ex1}$), to be sure that the phenotype we observed is cell line independent. With this experiments we demonstrate that *rosetteless phenotype* is indipendent by the variability of different cell lines.

ii) Then we knock down endogenous huntingtin in wild-type cells demonstrating that *rosetteless phenotype* occurs downregulating the expression of huntingtin.

iii) Cell depleted of endogenous huntingtin are able to restore rosettes formation activity through a stably over-expression of full-length huntingtin protein.

iv) To test whether the presence of wild-type cells can rescue the defect in huntingtin depleted cells, we peformed some co-colture experiments to test if released factors by wild-type cells can contribute to restoring huntingtin depletion effects. Our data demonstrated that no exogenous factors can rescue *rosetteless phenotype* in huntingtin null neural progenitor cells.

All these experiments demonstrate that huntingtin has a key role in the formation of neural tube-like rosettes, since its absence lead to a severe disorganization of neural precursors cells. As a consequence of the *rosetteless phenotype* we observed that huntingtin knock out cells are able to differentiate in mature neurons and glia, as described in literature, but neurons formed are more vulnerable.

Huntingtin regulates cell adhesion N-cadherin mediated

In the second part of the project we try to understand better the underlying mechanism that occurs to cause *rosetteless phenotype*. We shown that huntingtin modulates rosettes formation by regulating cell-cell interaction mechanisms. We propose a novel partner of huntingtin that is crucial for cell adhesion at adherent junctions, N-cadherin. Several studies indicate N-cadherin undergoes ADAM-10 mediated cleavage in its extracellular domain yielding fragments of major importance for the regulation of cell adhesion, cell migration and neurites outgrowth (Reiss et al., 2005; Halbleib and Nelson, 2006). We performed some experiment to reveal the role of huntingtin in cell-adhesion mechanism. Although the same level of mRNA and total full length N-cadherin protein in wild-type and knock out huntingtin cells, we observed increased N-cadherin cleavage in the

absence of huntingtin during neural development. Higher level of CTF-1 has been found in neural precursors cells depleted of huntingtin, which is responsible for defects in neural cells spatial organization. We found that higher activity of ADAM10 is responsible for the increased N-cadherin shedding observed in the absence of huntingtin. Some genetic modulation experiments demonstrated that huntingtin acts in concert with N-cadherin and ADAM-10 in rosettes formation.

i) Silencing ADAM-10 in huntingtin null ES cells can rescue *rosetteless phenotype*

ii) Overexpression of mutant form of N-cadherin in ADAM-10 cleavage site is able to restore rosette formation ability in huntingtin null ES cells.

Therefore, we concluded that, by controlling ADAM-10 activity, huntingtin regulates N-cadherin mediated cell-cell adhesion mechanisms during neurulation, leads to a correct formation of neural-tube like rosettes.

Huntingtin cell adhesion function emerges in deuterostome evolution

Finally we want to detect whether N-terminal portion of huntingtin is responsible of this neural huntingtin function (i.e. neural rosettes formation), and how this function is evolved during phylogenesis. We observed that huntingtin N-terminal domain, but not C-terminal one is able to rescue *rosetteless phenotype*, when overexpressing in huntingtin knock out cells, demonstrating that N-terminal domain is a functional domain of the protein responsible of a neural developmental function of huntingtin. Previous bioinformatics data showed that the N-terminal fragment is the most recent evolved portion of huntingtin during deuterostome evolution. In this study we provide a reconstruction of the functional activity of the N-terminal domain of huntingtin during evolution. We provide evidence that the N-terminal huntingtin from lower species with no-nervous system, such as *Dyctiostelium discoideum* or with a poor organized nervous system as *Strongylocentrotus purpuratus* is not endowed with neuronal properties (i.e. the ability to regulate the neural precursor spatial organization). On the contrary, expression of N-terminal huntingtin from *Mus musculus* and *Homo sapiens*, show complete rescue of the "*rosetteless phenotype*". Our data show also that the expanded polyQ partially affects the neuronal activities associated with the N-terminal domain, since *rosetteless phenotype* is partially restored after transfection of mutated form of N-terminal human huntingtin (with 128Q). We also demonstrate that N-terminal from *Dyctostelium discoideum, Strongilocentrotus purpuratus, Mus musculus and Homo sapiens* rescue apoptotic cell death in a null huntingtin background, indicating that huntingtin ability to regulate apoptosis is an ancient acquisition of the protein that has been

maintained during evolution. We suggest that huntingtin was born to serve primordial non-neuronal functions and it is only at later stages that it acquired additional neuronal functions, in coincidence with the formation of the nervous system. These neuron-specific functions have been progressively imposed to the N-terminal domain in higher species, coinciding with the acquisition of an increasingly more complex CNS.

Conclusion

In the present study we propose a new function of huntingtin during neural differentiation and one possible underlying mechanism. We also show that this function has emerged during deuterostomes evolution and it is exerted by the huntingtin N-terminal domain. We have highlighted new molecules and pathways involved in huntingtin's neuronal activities. Two novel potential huntingtin targets have been highlighted, the cell adhesion molecule N-cadherin and metalloproteinase ADAM10.

Future studies will be direct on understanding better N-cadherin-ADAM10-Huntingtin regulation in the presence of mutated form of huntingtin and how this mechanism is affected during embryonic development and in the adult brain in a typical Huntington disease *scenario*.

Materials and Methods

Mouse ES cell colture

We used mouse Embryonic Stem cell lines either wild-type and homozygous for a knockout mutation in the gene encoding for huntingtin protein. R1 (*Hdh*$^{+/+}$), R1-Hdh4/5-1d1(*Hdh*$^{4/5}$) (Duayo et al., 1995), and 129/Sv (*Hdh*$^{+/+}$*), 129/SvHdh-/- (*Hdh*$^{pr\text{-}ex1}$) were used (Zeitlin et al., 1995).

ES cells were maintained in Glasgow Minimal Essenzial Medium supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS-EuroClone, Italy), 1mM β-mercaptoethanol (GIBCO), 100 μM non essential amino acids (GIBCO), 1 mM sodium piruvate (GIBCO), 2 mM L-glutamine (EuroClone), 100 u/ml penicillin, 100 μg/ml streptomiycin (EuroClone) and 1000 U/ml murine Leukemia Inhibitor Factor (LIF-ESGRO) (Chemicon) on gelatinised tissue culture flasks. Cells were passaged every 2 days using 0.05% trypsin-EDTA.

Mouse ES cell monolayer neural differentiation

Mouse embryonic stem cell lines used for monolayer neural differentiation (Ying, 2003) experiments. Before initiating differentiation, ES cells were plated at high density (2x106 cells/ T25 flask) and cultured for 24 hours in standard ES cell medium containing LIF (as described above). Undifferentiated ES cells were dissociated using 0.025% trypsin (Gibco, Invitrogen) solution at 37oC, and then plated onto 0.1% gelatin-coated tissue culture plastic at a density of 1.0x104/ cm2 in N2B27 serum-free medium. N2B27 consists of a 1:1 ratio of DMEM/F12 (Gibco) and Neurobasal (Gibco) media supplemented with 0.5% N2 (Gibco), 1% B27 (Gibco) and 0.2% 2-mercaptothanol (Gibco, Invitrogen). The medium was then changed every other day. From day 9 N2B27 the cell colture medium become a 1:4 mixture of the same supplemented media. For some experiments, cells were replated after 7 days onto poly-ornitin (Sigma) and laminin (Gibco, Invitrogen) coated plastic at a density of 0.5- 1.5x104/ cm2. A supplement of 10-20ng/ml of b-FGF (PeproTech, TebuBio) in the medium used for the replating step improved cell viability. Media was then changed every 3-4 days and monolayer cultures were kept under differentiation for up to 21 days.

DNA Transfections

ES *Hdh*$^{ex4\text{-}5}$ cells were plated in self renewal conditions at a density 1-1.5*10^4/cm^2 and transfected after 24 hours with different expression vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). To produce stable cell lines expressing N-terminal

fragment of huntingtin after 24 hours from lipofection cells were selected with puromycin (2 μg/ml) for 15 days. Puromycin-resistant clones were collected. RT-PCR was performed to analyze the presence of the transgene and the protein was assayed by Western blotting.

Constructs

The cDNA of the N-terminal part of huntingtin from different species were cloned in pCAG expression vector carrying an IRES element and a puromycin resistance cassett. The constructs product are: pCAG*Dd*N-500-HA, pCAG*sp*N-519-HA, pCAG*mm*N-548, pCAG*mm*N-548-HA, pCAG*hs*-N652-15Q, pCAG*hs*N-652-128Q, pCAG*mm*C-500 (respectively carryng the N-terminal portion of Dictyostelium discoideum, Strongylocentrotus purpuratus, Mus musculus, Homo sapiens). Constructs with huntingtin from Dictyostelium, Strongylocentrotus are tagged with HA (hemagglutinin) tag. The N-terminal portion of Dyctyostelium huntingtin was provided by Professor James Gusella (Massachusetts General Hospital, Boston). 3xFLAG 7Q full-lenght mouse huntingtin was kindly provided by Scott Zeitlin's laboratory, University of Virginia School of Medicine. Other constructs using for transfection: *pCAG-Ncadherin-wt, pCAG-Ncadherin-GDmutant,* carrying respectively cDNA of murine N-cadherin in wild-type form and mutated on ADAM-10 cleavage site (Uemura et al…)

siRNA transfection

For knock-down of huntingtin*,* and scrambled control 100 nM siRNA from Dharmacon were transfected in R1 ($Hdh^{+/+}$), using Amaxa mouse ES cell nucleofector kit (A-24 program). For knock-down of ADAM-10 pmol siRNA from Invitrogen (Carlsbad, CA, USA) were trasfected in ES $Hdh^{ex4/5}$ with lipofectamine2000. After 24 hours transfected cells were plated for differentiation toward the monolayer protocol.

Pharmacological treatments

$Hdh^{ex4\text{-}5}$ and wild-type cells at day 8 of neural differentiation were incubated for 30 min with Ionomycin 2.5 μM or for 1 hours with TIMP 34 μM.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were permeabilized in PBS 1X-0.5% Triton X-100 and blocked with 5% Fetal Calf Serum for

1 h. Primary antibodies were diluted in blocking buffer and applied overnight at 4°C. After 3 washes in PBS, appropriate secondary antibodies conjugated to Alexa fluorophores 488 or 568 (Molecular Probes, Invitrogen Carlsbad, CA, USA) 1:500 in blocking solution were applied for 1 h at room temperature. Cells were incubated for 10 minutes with Hoechst 33258 (5 μg/ml; Molecular Probes, Invitrogen). The cells were then washed twice in PBS buffer. Images were acquired with a Leica DMI 6000B microscope (with LAS-AF imaging software) and then processed with Adobe Photoshop and ImageJ software.

Rosettes quantification

The rosettes quantification was performed using cells cultures at day 8 of neural-differentiation stained for Nestin as described previously. Ten images of random fields for every cells line in each experiment were acquired. Each experiment was independently repeated at least three times. Image J analysis software was applied for every image to calculate the area occupied by Nestin positive cells as total normalizing value in each field. The shape of rosettes is marked out, Nestin-positive cells present in the rosettes were measured as area occupied by Nestin positive cells IN Rosettes and remaining Nestin signal as area occupated by Nestin positive cells OUT of rosettes.

Protein Lysates and Western blotting

Cells were washed with cold PBS 1X and harvested in RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% SDS, 1% Nonidet P40, 0.5% Sodium deoxycholate) added with PMSF 1mM, DTT 1mM and protease inhibitor (SIGMA). After 30 minutes of incubation at 4 °C, lysates were cleared by centrifugation for 30 min at 12.000×*g* and 4 °C and the resulting supernatant collected. Protein concentration was determined using BCA assay (Pierce) and amounts between 50-80 μg were loaded on 10% SDS-page gel and transferred onto nitrocellulose membrane. Membranes were saturated with 5% nonfat dry milk (Biorad) in TBS 1X-Tween20 0.1% and incubated with primary antibodies at room temperature for 2 hrs or overnight at 4 °C. Filters were washed three times and then incubated for 1 h at room temperature with secondary antibody (peroxidase conjugate, Biorad, 1:3000), finally the filters were washed three times with TBS1X-0.1% Tween-20. The SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used to visualize immunoreactive bands by exposure to Amersham Hyperfilm ECL (GE Healthcare).

Antibodies used for Western blotting and immunocytochemistry

Monoclonal anti-HA Covance (IF 1:500; WB 1:1000);

Monoclonal anti-Huntingtin Mab2166 Millipore (IF 1:800; WB 1:2000);

Monoclonal anti-MAP2 Becton Dickinson (IF 1:1000);

Monoclonal anti-N-cadherin Becton Dickinson (IF 1:1000; WB 1:4000);

Monoclonal anti-Nestin Millipore (IF 1:200; WB 1:1000);

Monoclonal anti-OCT4 Santa Cruz (IF 1:100);

Monoclonal anti-Tubulin Sigma (WB 1:3000);

Polyclonal anti-ADAM-10 (*kindly provided by Paul Saftig lab at the University of Kiel*) (WB 1:1000);

Polyclonal anti-GFAP Dako (IF 1:1000);

Polyclonal anti-ZO-1 Zymed (IF 1:50).

RNA extraction and reverse transcription

Total RNA was extracted from cells in self-renewal condition or during differentiation protocol with Trizol reagent (Invitrogen, Carlsbad, CA, USA). Pellets containing 2-5x10^6 cells were lysate in 1 mL of Trizol reagent. Clorophorm was added 200 µL/mL of Trizol. Samples were vortexed vigorously for 15 seconds and centrifuged at 9.000×*g* for 15 minutes at 4°C. Following centrifugation, the mixture separates into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The aqueous phase, containing RNA was transferred into fresh tube. One volume of 2-Propanolol (SIGMA) was added and RNA was precipitated at 12.000×*g* for 30-40 minutes at 4°C. Removed the supernatant completely, the pellets was washed once with 1 mL of 75% ethanol. Samples were centrifuged at 12.000×*g* for 10 minutes. Pellets were resuspended in an appropriate volume of ultra-pure water (SIGMA). Total RNA concentration was measured with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). To detect mRNA levels, 1000 ng of total RNA was reverse-transcribed to single-stranded cDNA with Superscript reverse transcriptase (Invitrogen, Carlsbad, CA, USA) 1U/reaction and random primers 250 ng.

Real-time PCR

We used an iCycler Thermal Cycler with a Multicolour Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). All reactions were performed in a total volume of 25 µL that contained 5 µl of cDNA, 50 mM KCl, 20 mM Tris-HCI, pH 8.4, 0.2 mM dNTPs, 25

units/mL iTaq DNA polymerase, 3 mM MgCl2, SYBR Green I with 10 nM fluorescein and stabilisers (iQTM SYBR Green Supermix-Biorad, Hercules, CA, USA), and 0.3 mM each of forward and reverse primers. The amplification cycles consisted of an initial denaturing cycle at 95°C for 3 min, followed by 45 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Fluorescence was quantified during the 60°C annealing step, and product formation was confirmed with a melting curve analysis (55°C–94°C). Primers sequence are:

ADAM-10 Fw GGAAGCTTTAGTCATGGGTCTG

ADAM-10 Rev	CTCCTTCCTCTACTCCAGTCAT
N-Cadherin Fw	AACACAGCCACAGCCGTCATC
N-cadherin Rev	CTTTGTCCGTGACAGTTAGGTTG
Huntingtin Fw	CGCTATGGAACTGTTTCTGCTGTG
Huntingtin Rev	CTGTAGCCTTGGAAGATTAGAATCCATC

MTT Assay

Cells were plated in triplicates into twenty-four well plates (IWAKI, Japan) at a density of 2x104/ well in supplemented GMEM as indicated above. After 12 hours incubation at 37oC, medium was replaced in half the wells with serum-deprived medium (composition: GMEM supplemented as described above but without serum), and in the remaining half of the wells with fresh complete GMEM. All cells were then incubated at 37oC for the following time points 6, 12, 24, 36, 48 and 96 hours. At each time point, cells were exposed to 3- [4.5-dimethylthiazol-2-phenyl]-2.5-diphenyl-tetrazolium bromide, and mitochondrial formazan release was quantified at 560nm using a plate reader (Bio-Rad).

Caspase-3 activity Assay

For each tested condition, cells were plated in triplicates in a 96 well plate with transparent bottom (PBI) at a density of 5 000 cells/ well. After 36 hours incubation at 37oC, half wells received 100µl of freshly prepared complete GMEM and the other half 100µl GMEM lacking serum. After 3 hours incubation, 20µl of Cell Titer-Blue ® Reagent (Cell Titer-Blue ® Assay- Promega) was added to each well and plates were incubated for another 3 hours at 37oC. Fluorescence was recorded at 550/595nm (plate reader-

Bio-Rad) to measure cell viability. At this point, an opaque sticker was added to the transparent bottom of the 96 well plate and an equal volume (120μl) of Caspase-Glo 3/7 Reagent was added to each well. After 1 hour of room temperature incubation, luciferase achieved the steady state and luminescence was recorded with a luminometer (Veritas-Microplate Luminometer, Turner Byosistems). Each experiment was independently repeated at least three times. Results are presented as the ratio of Fluorescence/ Luminescence recorded for each tested condition.

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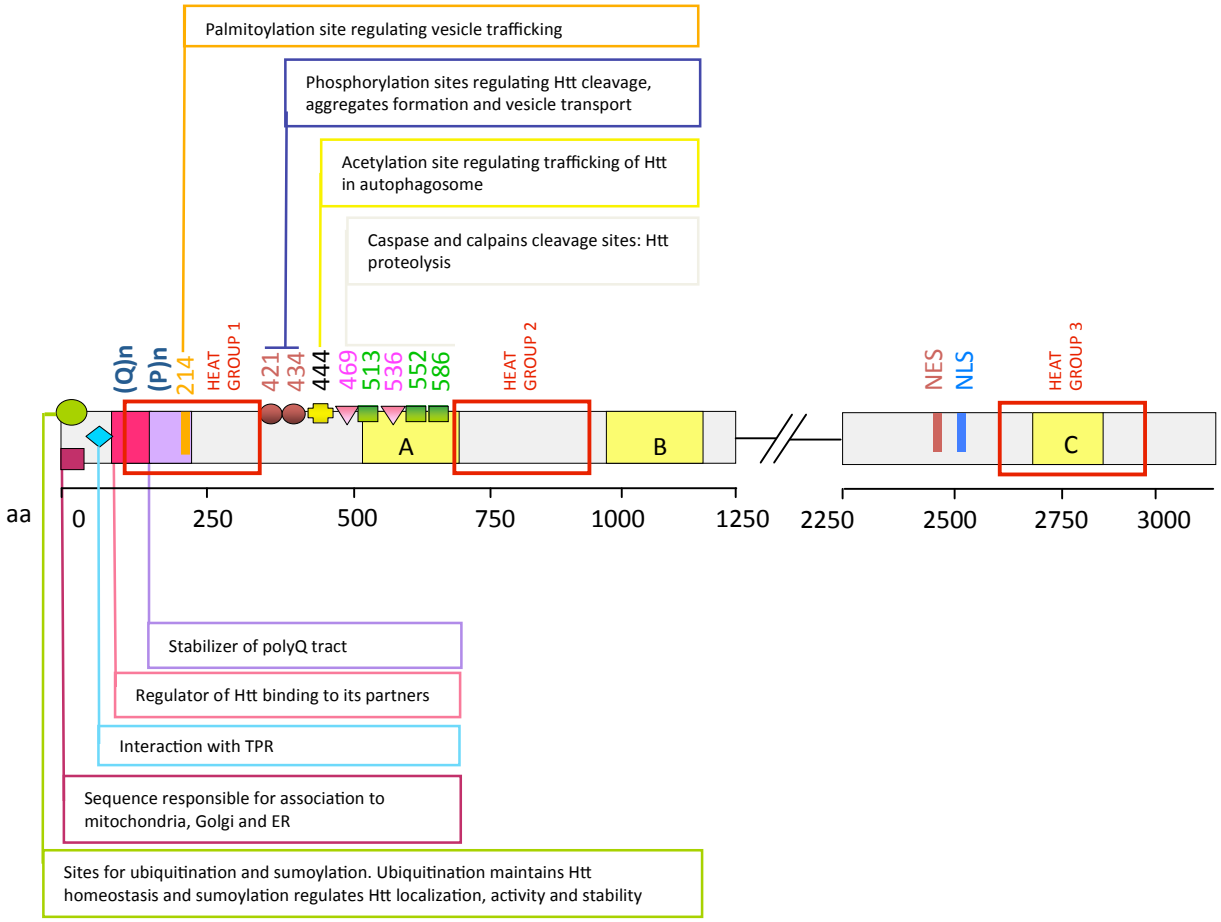


Figure 1. **Huntingtin aminoacid primary sequence:** schematic representation of huntingin primary aminoacid sequence and the relative aminoacid sites and consensa known of protein.

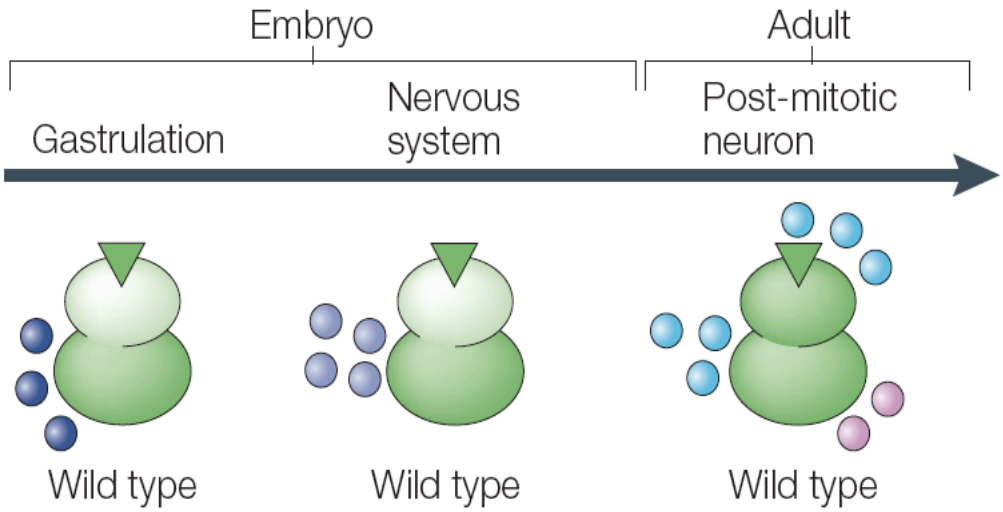


Figure 2. **Huntingtin function during the lifetime**

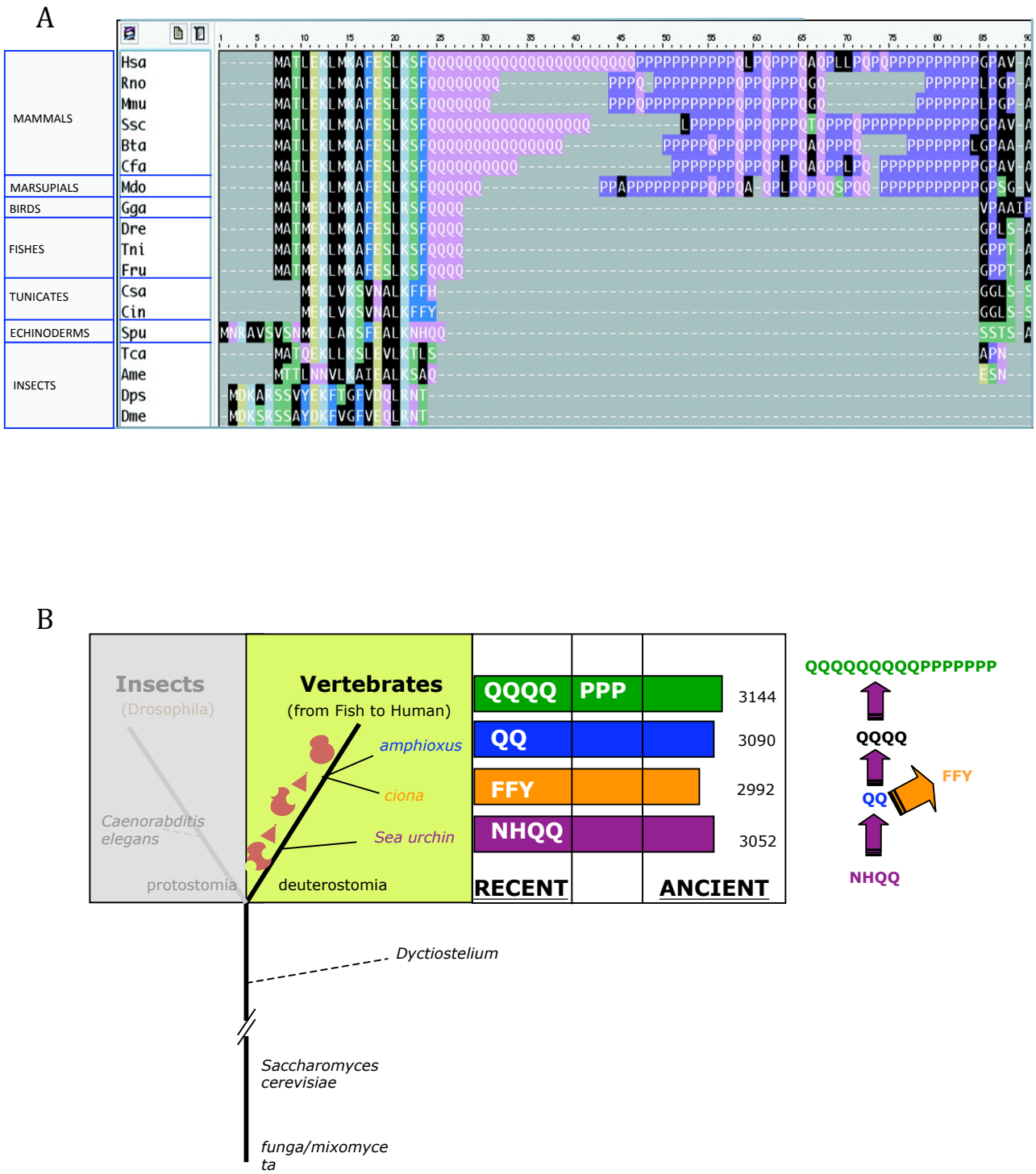


Figure 3. **The evolution of polyQ:** A) N-terminal portion of the wide-ranging multialignment of 18 huntingtin homologues. PolyQ appears in echinoderm *Strongylocentrotus purpuratus* and expands along deuterostomes. B) Schematic representation of the three main huntingtin domains.

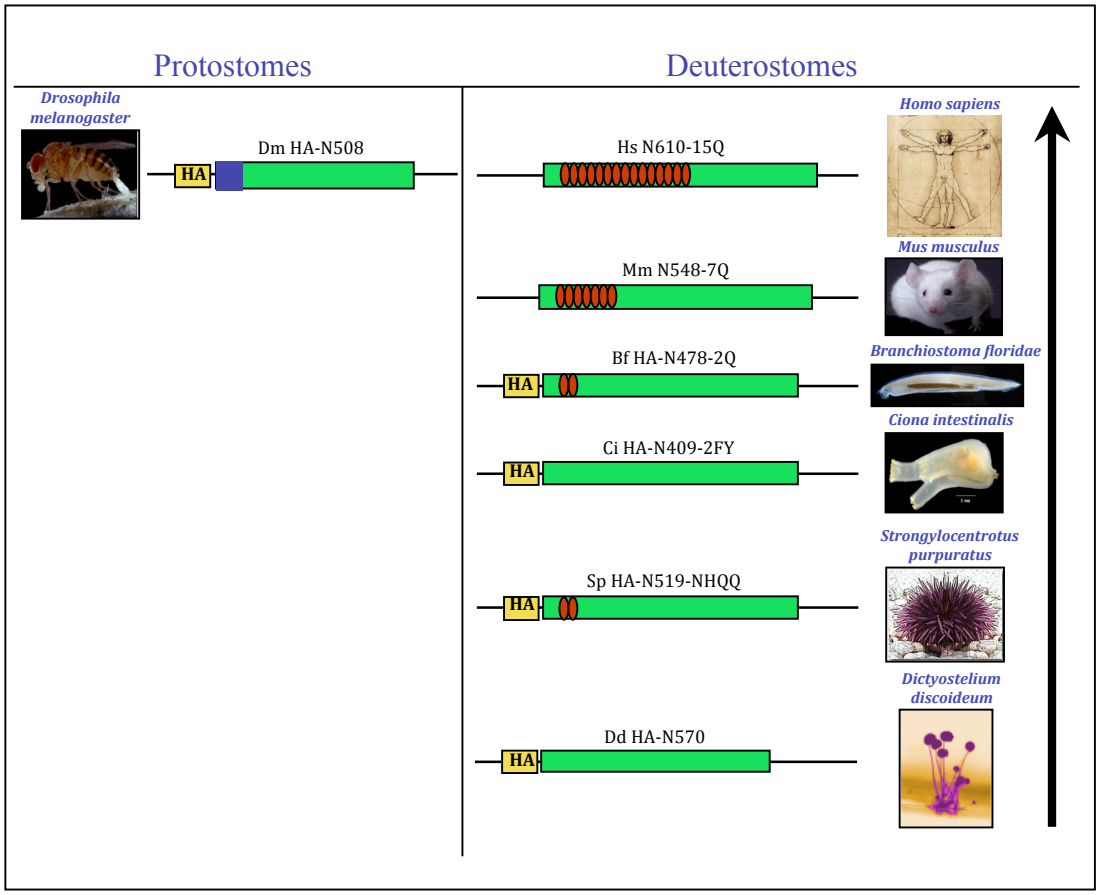


Figure 4. **Cloning huntingtin homologues :** Schematic representation of expression vectors produced, containing N-terminal portion of different huntingtin homologues.

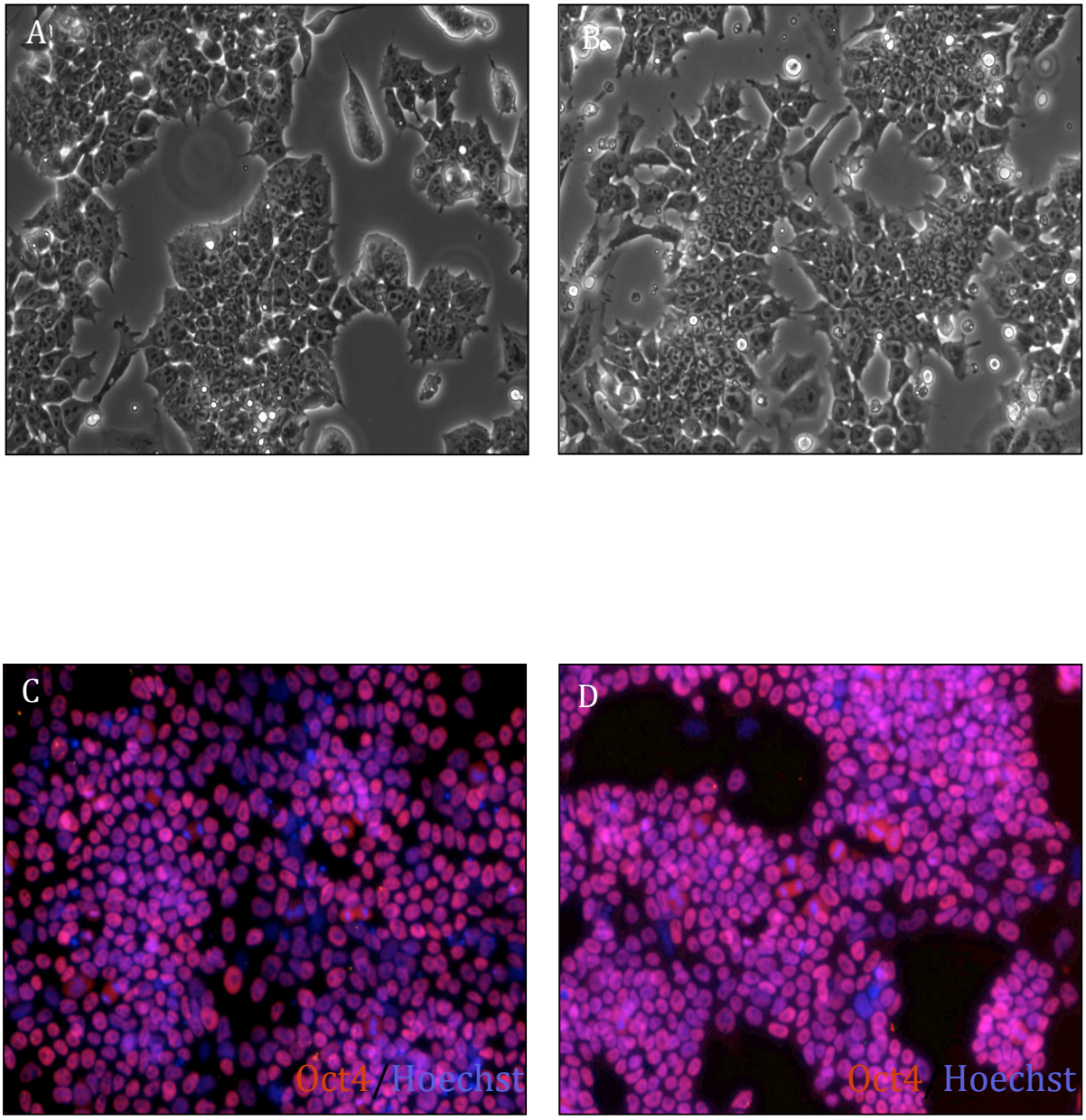
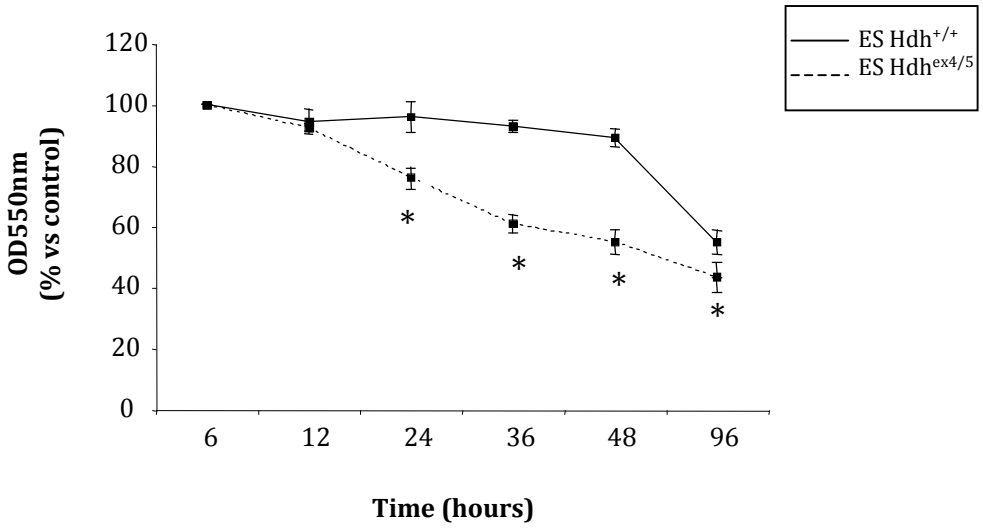


Figure 5. **Embryonic stem cells lacking huntingtin in self-renewal conditions:** Phase contrast images of wild-type cells (A) and huntingtin knockout cells (B) in self renewal conditions. Immunostaining for Oct4 plutipotency marker in wild-type cells (C) and huntingtin knockout cells (D).

A



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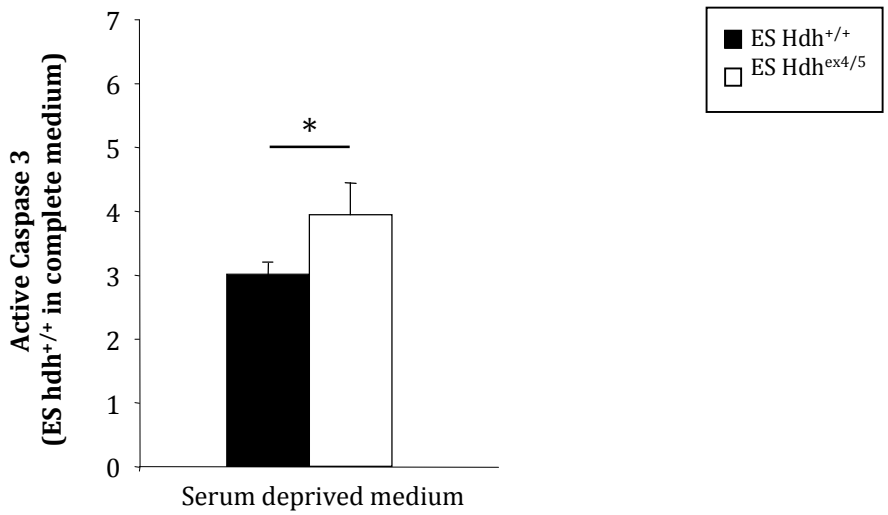


Figure 6 . **Huntingtin knock out cells in sel-renewal assays:** A) MTT assay 24 hs post serum deprivation (*p< 0,05 Anova Test). B) Caspase-3 activity assay 24 hs post serum deprivation (*p< 0,05 Anova Test).

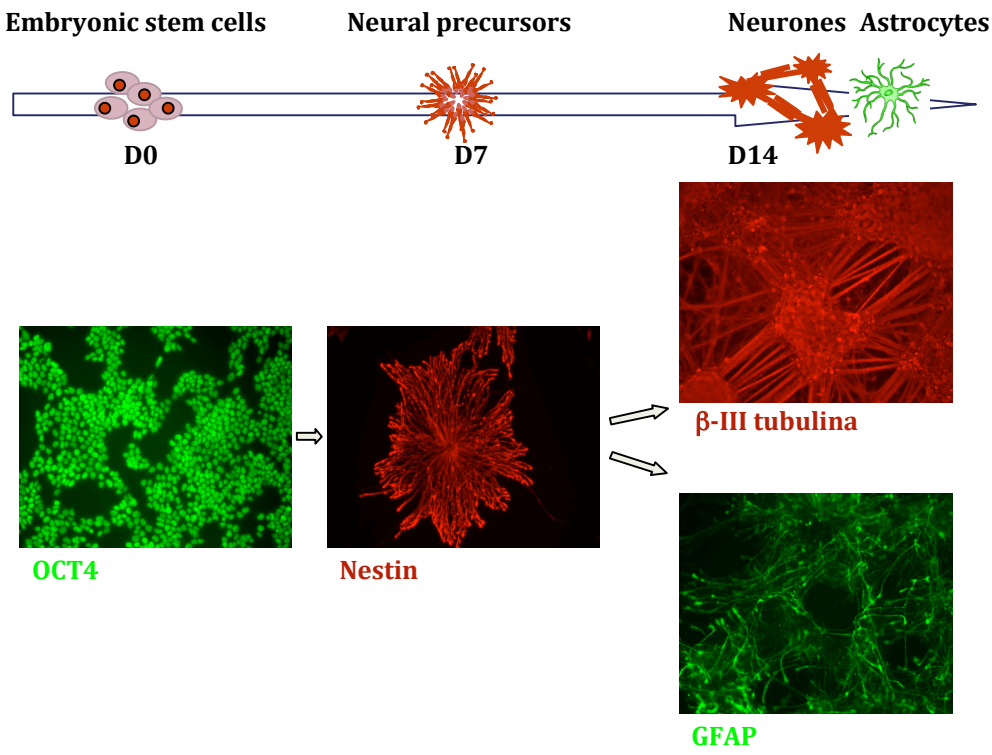


Figure 7. **Schematic representation of monolayer neural differentiation protocol by Ying et al.** Embryonic stem cells Oct4 positive in pluripotency conditions are firstly converted in neural precursors cells Nestin positive. After 14 days of neural differentiation neural precursors cells differentiate in neurons, b-III tubulin positive and GFAP positive astrocytes.

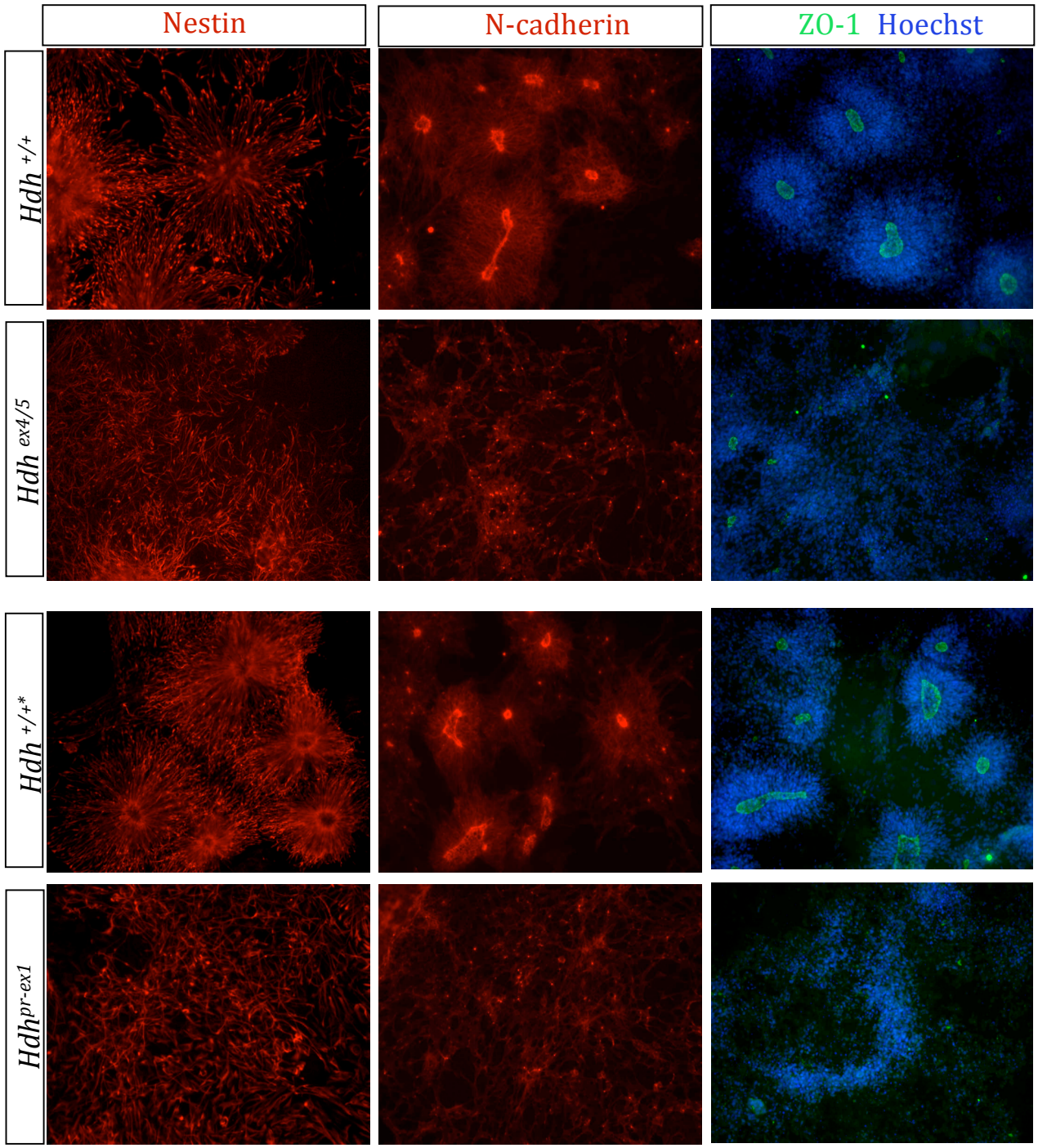
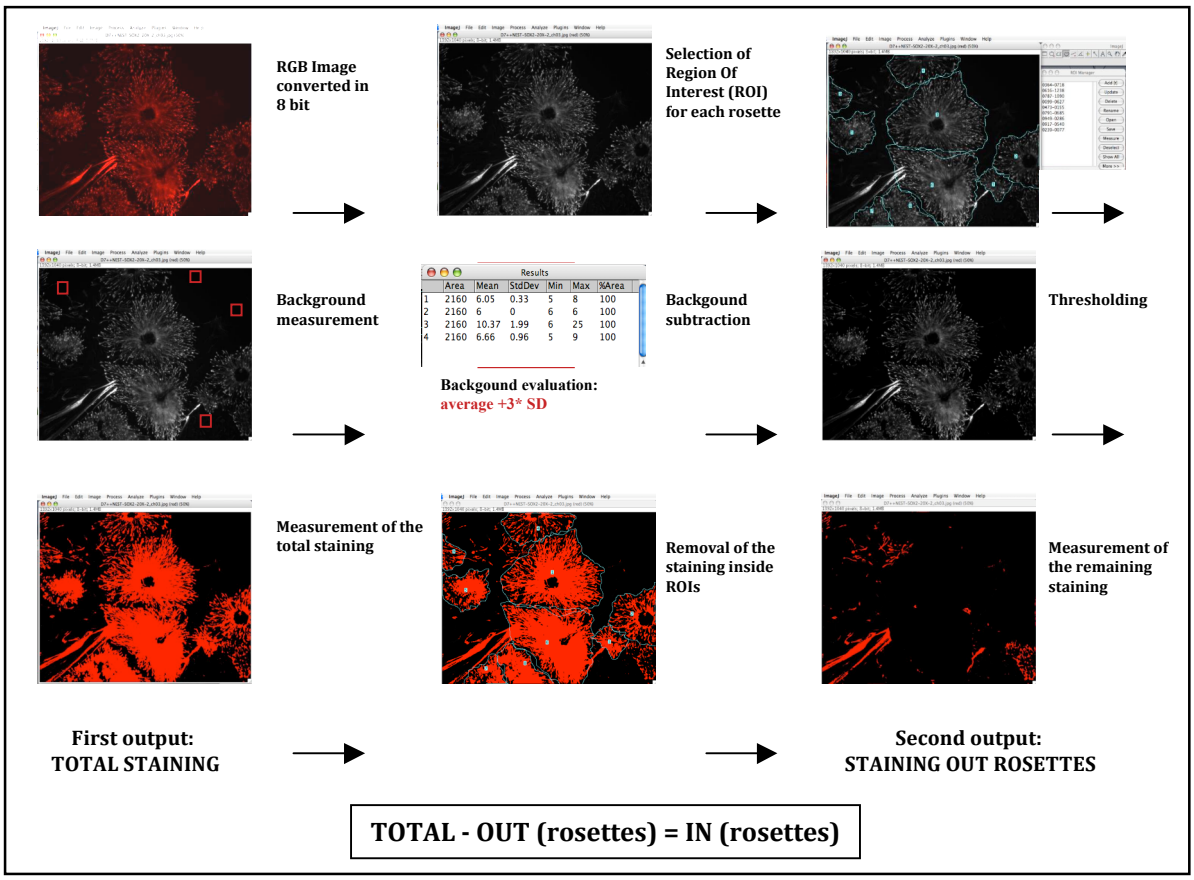


Figure 8 . **"*Rosetteless phenotype*" in absence of huntingtin:** Immunocytochemistry analysis at day 8 of neural differentiation protocol in two different batches of huntingtin depleted cells (*Hdh*$^{ex4/5}$ and *Hdh*$^{pr\text{-}ex1}$) and their wild-type counterpart (*Hdh*$^{+/+}$ and *Hdh*$^{+/+*}$). Cells are immunodecorated with Nestin, N-cadherin and ZO-1 markers.

A



B

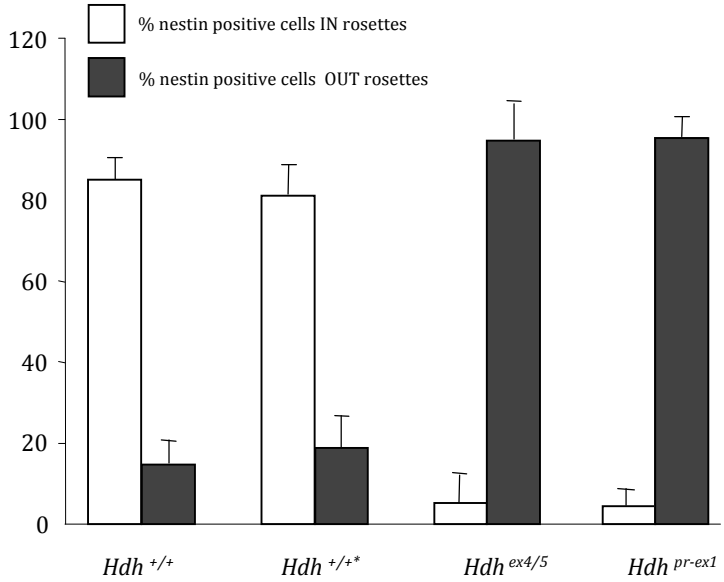


Figure 9. **Set up of a method to quantify "*rosetteless phenotype*":** A) Imaging method to quantify Nestin positive cells area IN and OUT of the rosettes. B) Quantification of "*rosetteless phenotype*" in the two batches of wild-type cells and huntingtin depleted cells at day 8 of differentiation protocol.

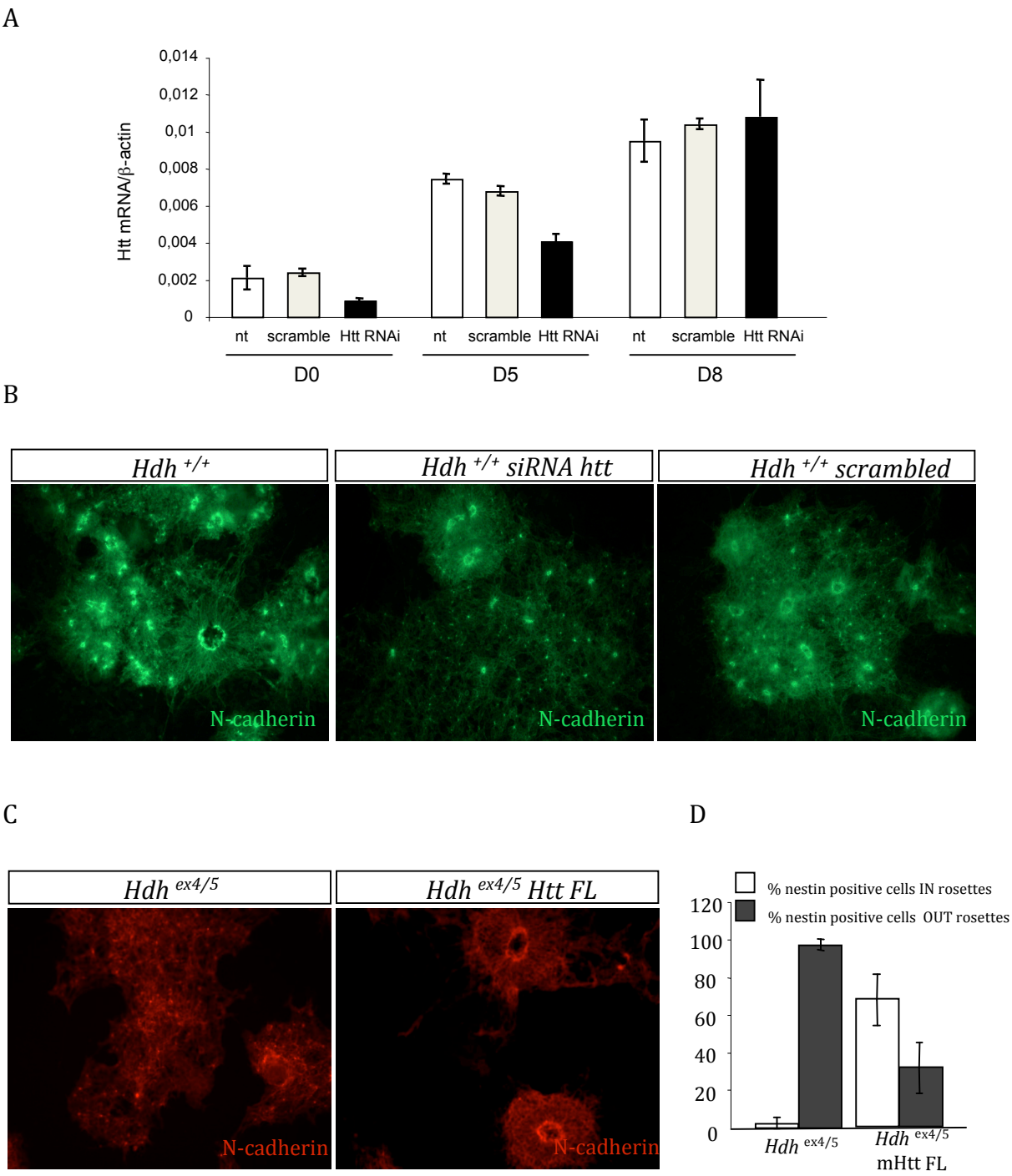


Figure 10. **Validation of "*rosetteless phenotype*" modulating huntingtin expression level:** A) qPCR for huntingtin mRNA expression level in *Hdh*$^{+/+}$ treated with huntingtin and scrambled RNA interference at D0, D5 and D8 of differentiation protocol. B)Immucytochemistry for N-cadherin marker at day 8 of neural differentiation protocol in wild-type cells no treated and with siRNA huntingtin and scrambled control. C) Immucytochemistry for N-cadherin marker at day 8 of neural differentiation protocol in huntingtin knockout cells no treated and stably transfected with full-length murine huntingtin. D) Rosettes quantification of stably transfected huntingtin depletd cells with full-length murine huntingtin and no treated counterpart.

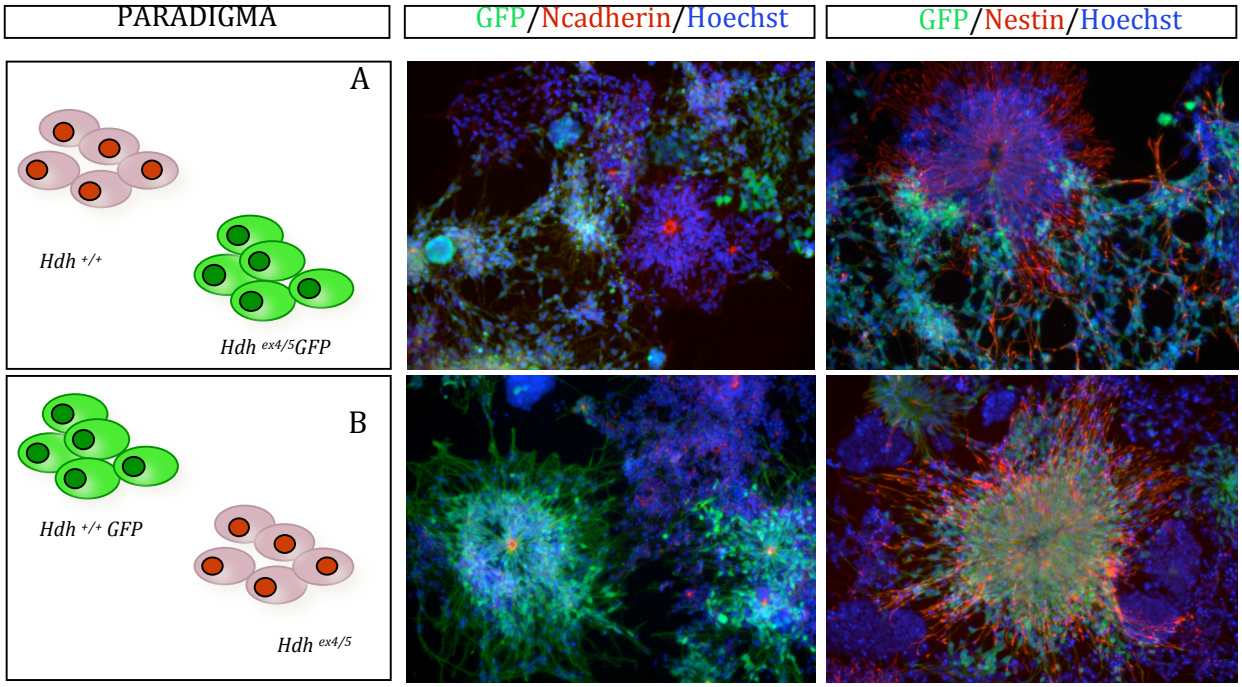


Figure 11. **Validation of "*rosetteless phenotype*" with co-colture experiments:** A)wild-type cells (*Hdh*$^{+/+}$) have been equally mixed with cells lacking huntingtin stably expressing eGFP (*Hdh*$^{ex4/5}$ eGFP); B) wild-type cells stably expressing eGFP (*Hdh*$^{+/+}$*eGFP*) have been mixed with cells lacking huntingtin (*Hdh*$^{ex4/5}$). Double immunostaining at D8 of differentaition protocol for Nestin/eGFP and N-cadherin/eGFP is shown.

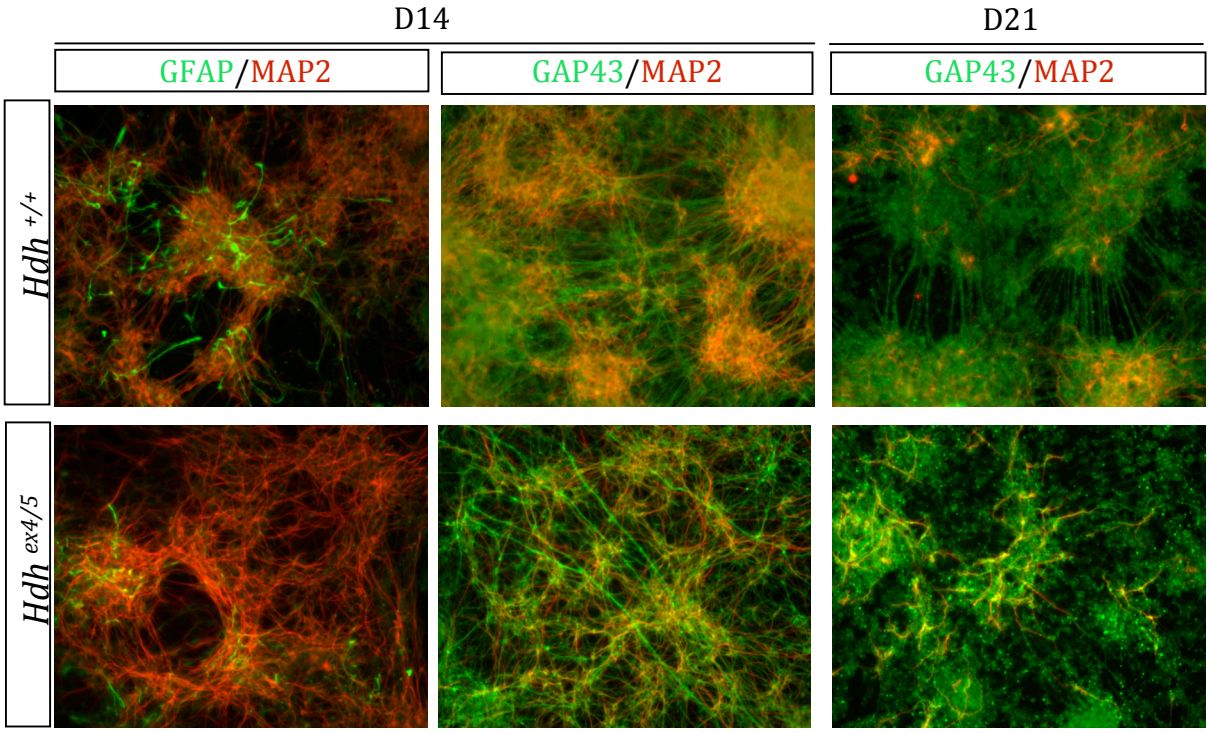


Figure 12. **Huntingtin knock out cells terminal differentiation:** Immunocytochemistry analysis at the late stage of neural differentiation protocol, D14 and D21. Staining for MAP2 and GAP43 mature neuronal markers and GFAP glial marker.

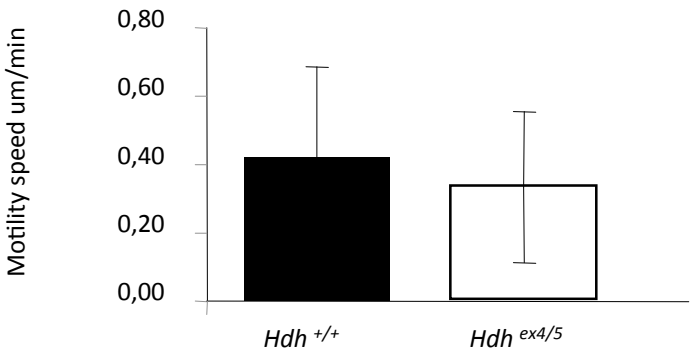


Figure 13. **Migration analysis of huntingtin knock out cells during neural differentiation:** Migration analysis by microscope time lapse. Quantification of motility speed of the cells (um/min) during differentiation from day 2 to day 8.

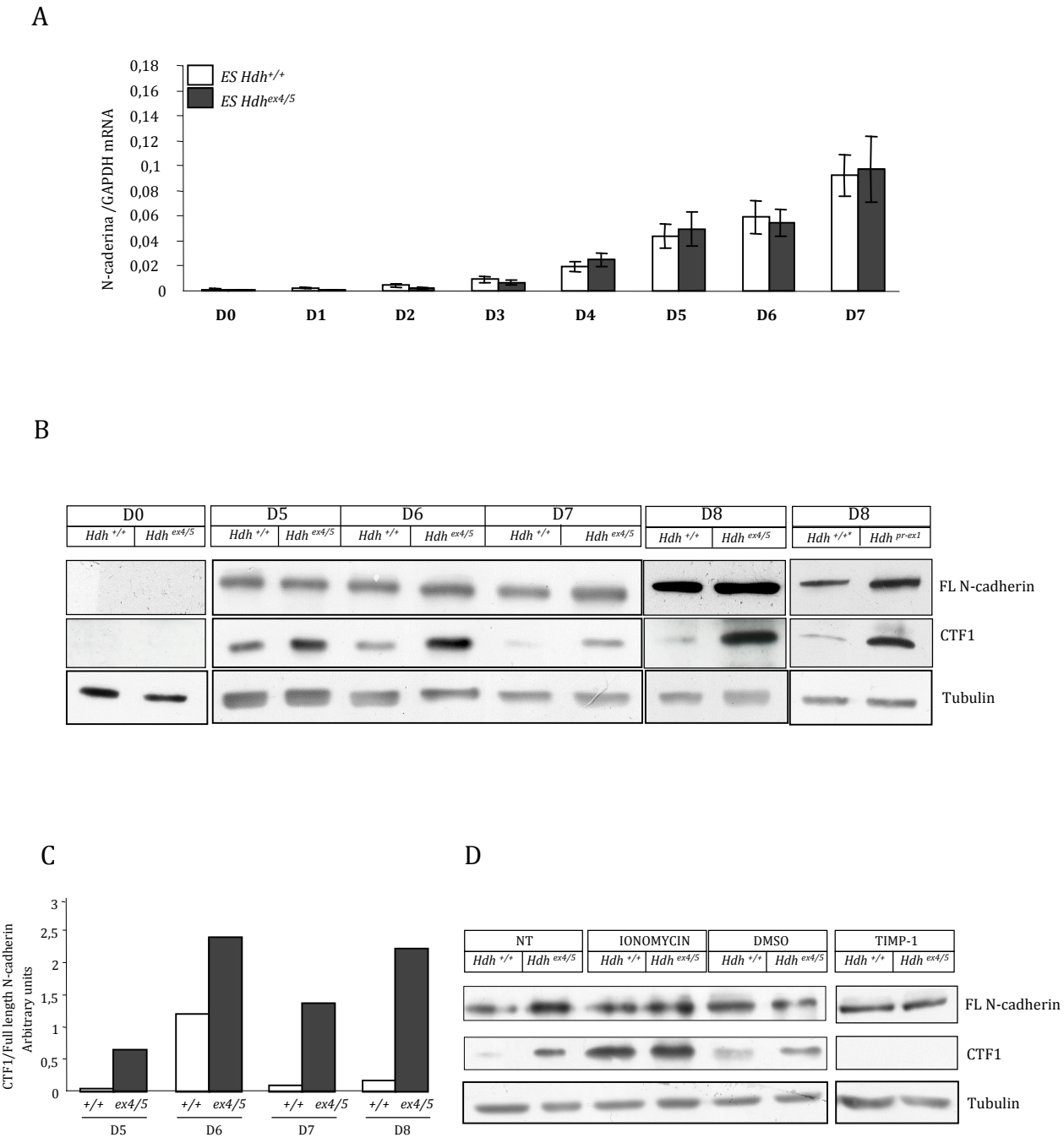
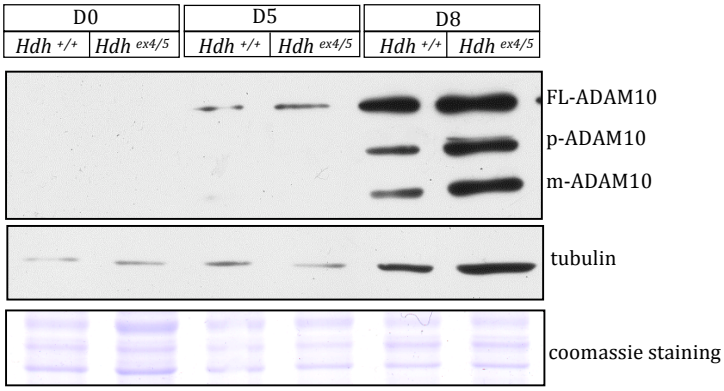


Figure 14. **N-cadherin analysis during differentiation:** A) mRNA level of N-cadherin by Real time PCR; B) Immunoblotting for full-length N-cadherin and CTF-1, during neural differentiation (D0, D5, D6, D7, D8) on total protein lysates from $Hdh^{+/+}$ and $Hdh^{ex4/5}$; $Hdh^{+/+*}$ and $Hdh^{pr\text{-}ex}$ at D8; C) Graphs show densitometric analysis of CTF1 over full-length N-cadherin. D) Full-length N-cadherin and CTF1 levels after pharmacological treatments $Hdh^{+/+}$ and $Hdh^{ex4/5}$ with Ionomycin and TIMP-1. NT, untreated cells. DMSO, vehicle

A



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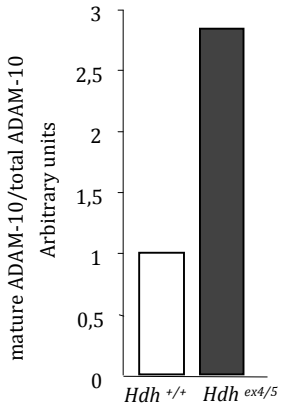


Figure 15. **ADAM-10 activity during neural differentiation:** A) Immunoblotting for ADAM-10 during monolayer differentiation: p, ADAM-10 precursor; m, mature active form of ADAM10. B) Graphs show densitometric analysis of ADAM10 mature form amounts compared to total amount of ADAM-10.

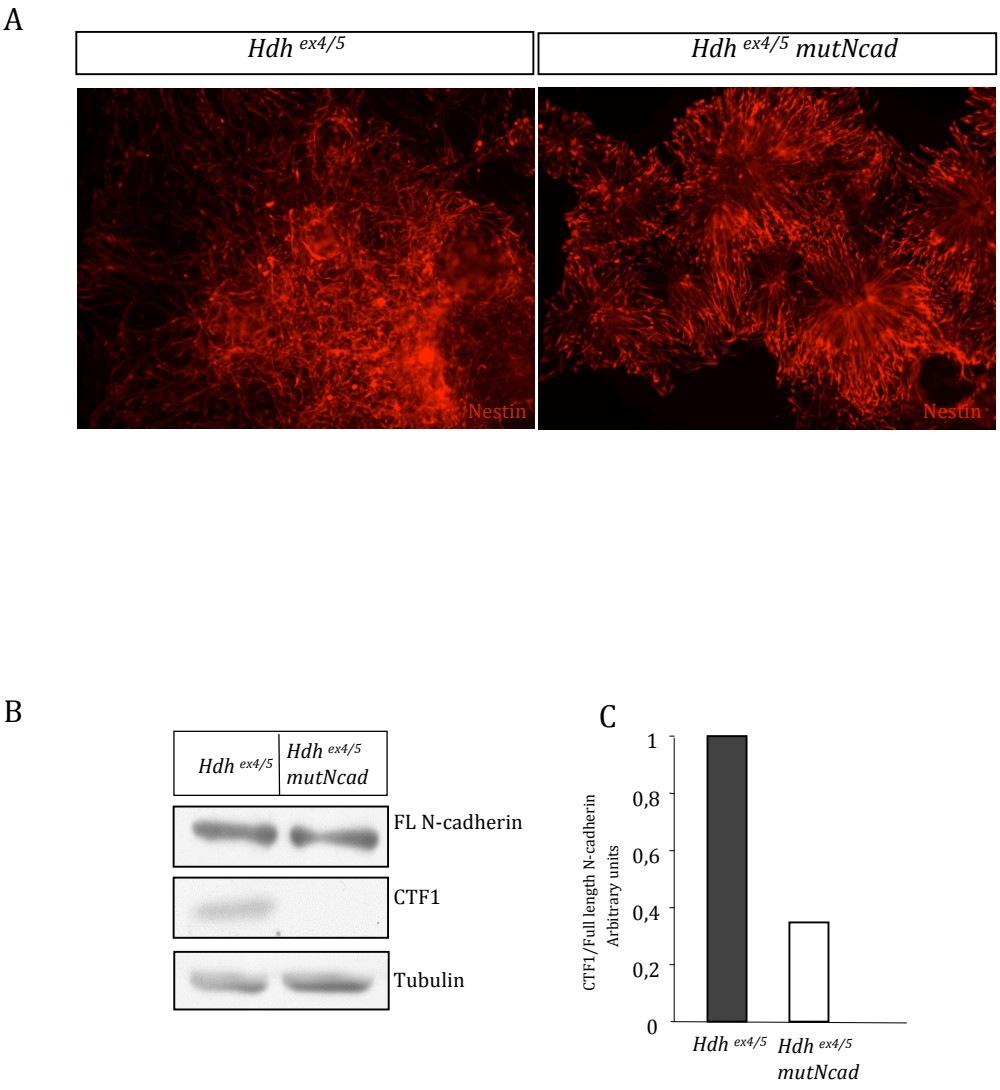
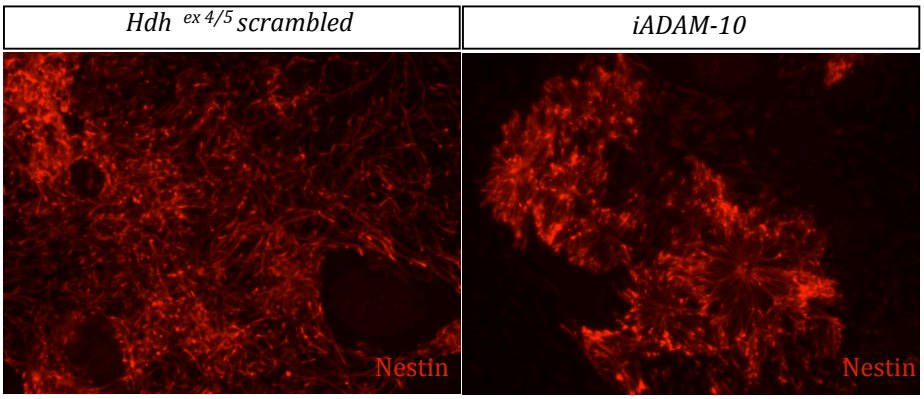


Figure 16. **Expression of mutated form of N-cadherin in *Hdh*$^{ex4/5}$cells :** A) Mutant N-cadherin expression in *Hdh*$^{ex4/5}$cells. Immunostaining for Nestin at D8 of the monolayer protocol is shown. B) Immunoblotting for N-cadherin and CTF1 at D8 of monolayer differentiation protocol in *Hdh*$^{ex4/5}$ expressing mutant N-cadherin and parental cells. C) Graphs show densitometric analysis of CTF1 over full-length N-cadherin.

A



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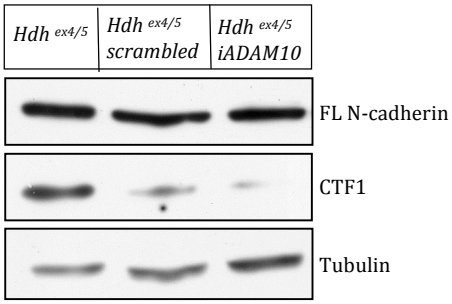


Figure 17. **Silencing of ADAM-10 in *Hdh*$^{ex4/5}$cells :** A) ADAM-10 knock-down in *Hdh*$^{ex4/5}$ cells. Immunostaining for Nestin at D8 of the monolayer protocol is shown. B) Immunoblotting for N-cadherin and CTF1 at D8 of monolayer differentiation protocol in *Hdh*$^{ex4/5}$ knock-down for ADAM-10 and parental cells.

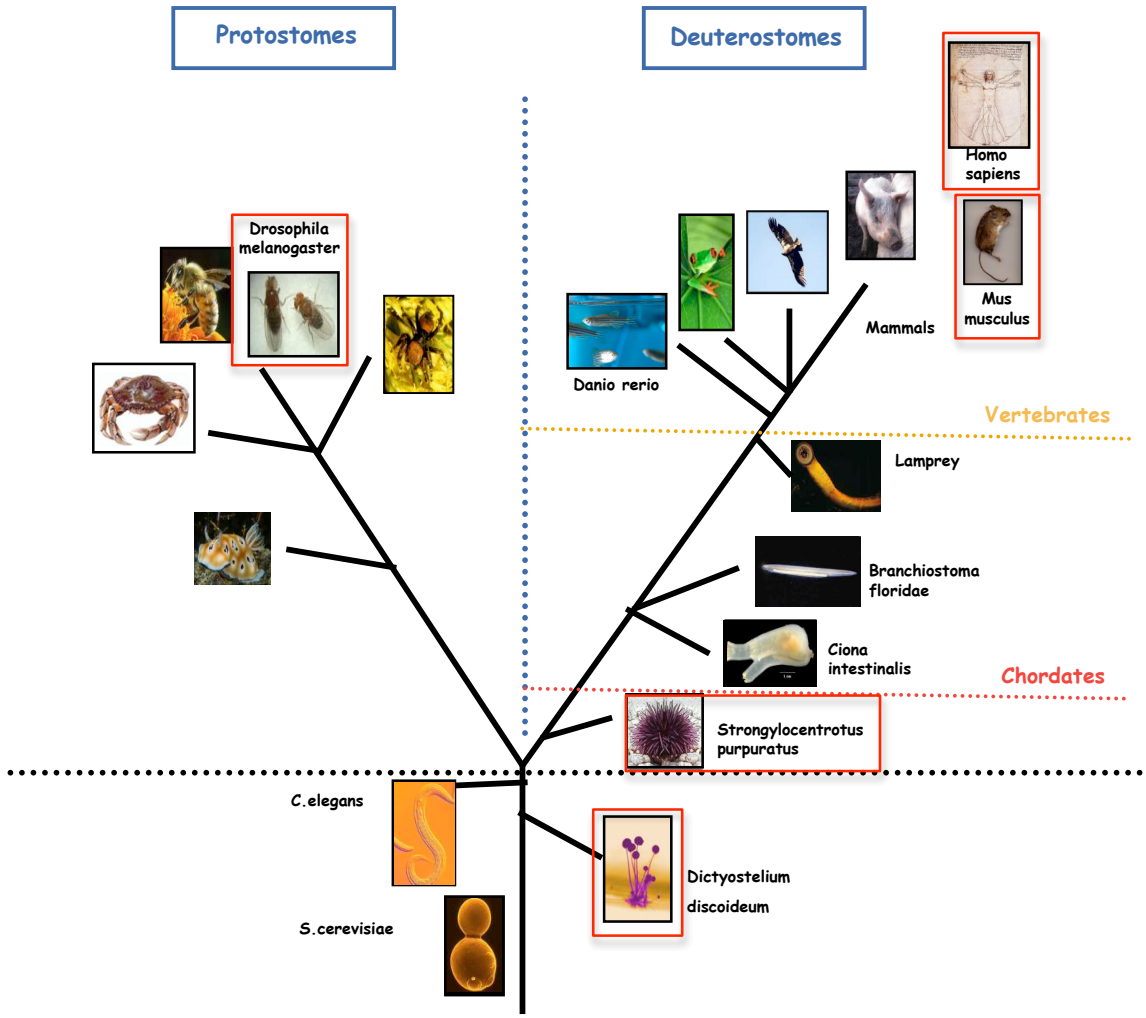


Figure 18. **Schematic representation of phylogenetic tree.** Homologues assayed for N-terminal activity are highlighted in red box *Homo sapiens*, *Mus musculus*, *Strongylocentrotus purpuratus, Drosophila melanogaster, and Dictyostelium discoideum.*

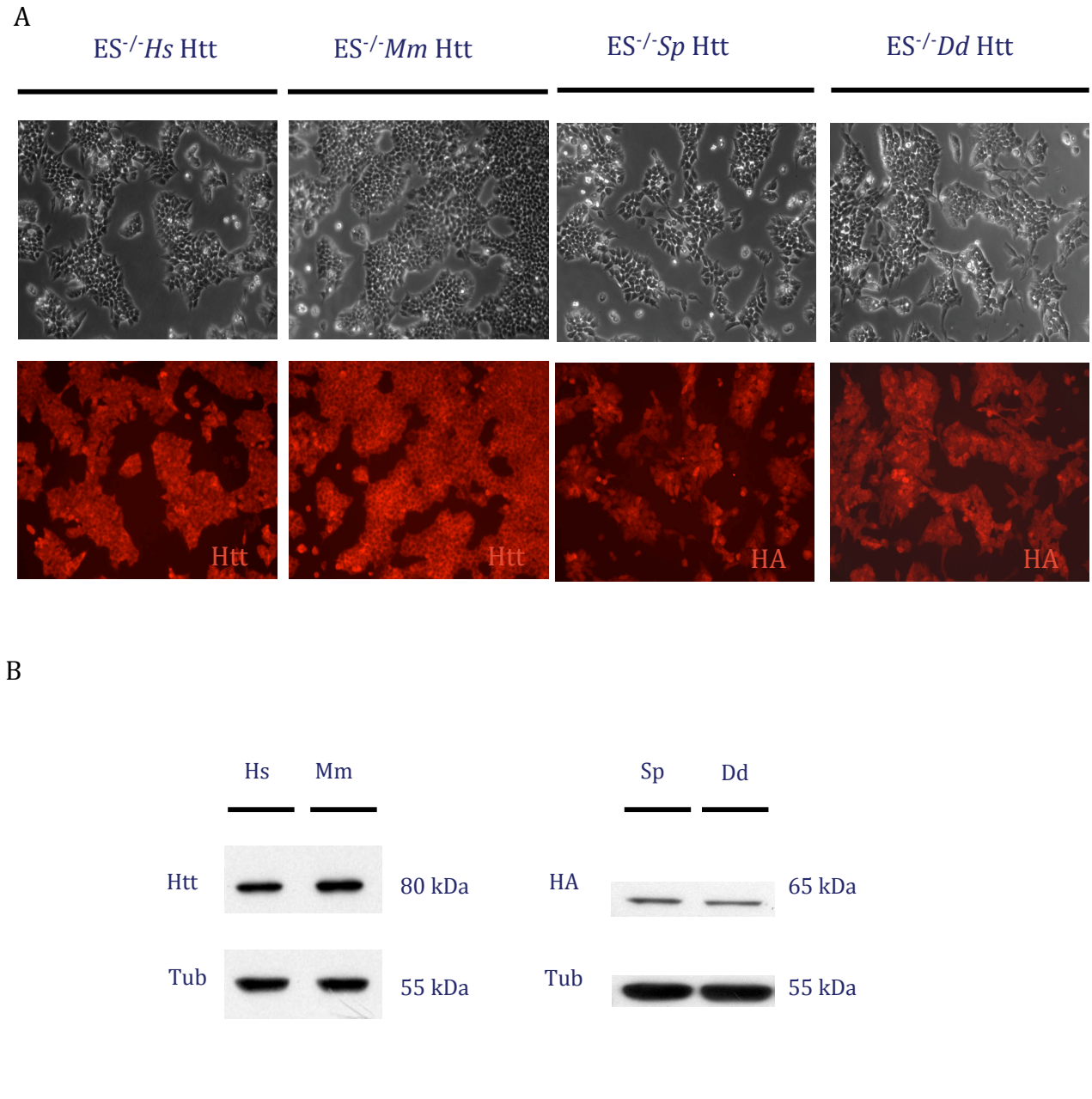


Figure 19. **Characterization of ES $^{Hdh4/5}$ stably expressing N-terminal huntingtin from different species:** Immunocytochemistry A) and immunoblotting B) to detect transgene expression in stable cell lines. Cell lines produced express the N-terminal portion from *Hs* Homo sapiens*; Mm* Mus musculus*; Sp* Strongylocentrotus purpuratus; *Dd* Dictyostelium discoideum.

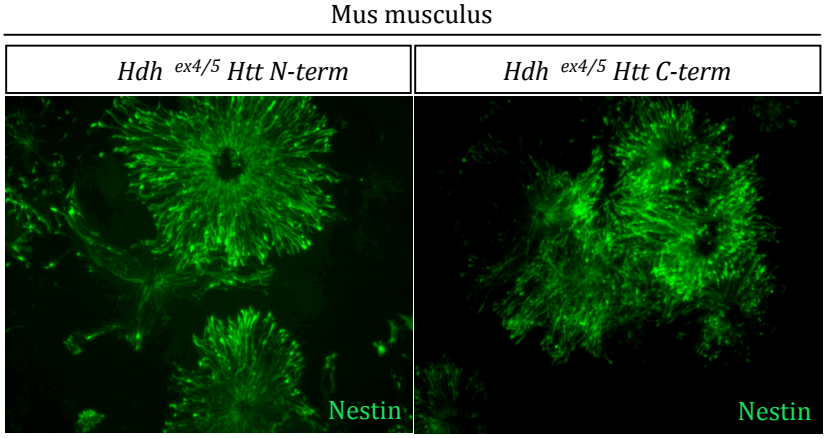
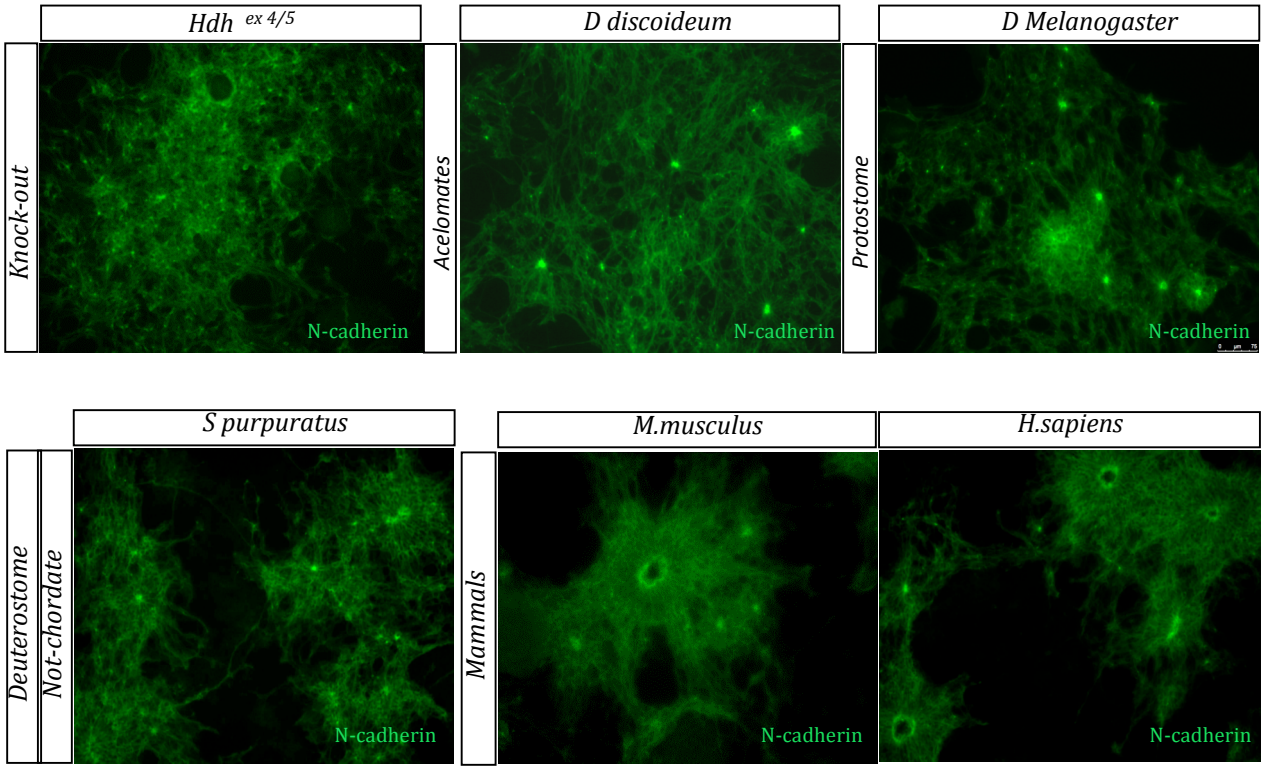


Figure 20. **The ability to form rosettes is N-terminal huntingtin function :** Immunocytochemistry for Nestin at D8 of neural differentiation in ES *Hdh*$^{ex4/5}$ stably expressing N-terminal and C-terminal portion of murine huntingtin.

A



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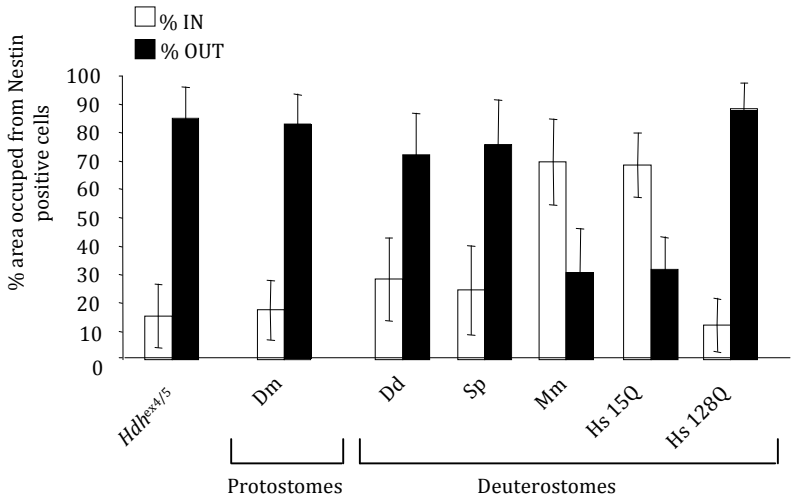

Figure 21. **The ability to form rosettes is a function evolved in deuterostomes:** A)Immunocytochemistry for N-cadherin at D8 of neural differentiation in ES *Hdh*$^{ex4/5}$ stably expressing N-terminal portion of huntingtin from *Dictyostelium discoideum, Strongylocentrotus purpuatus, Mus musculus and Homo sapiens and Drosophila melanogaster.* B) Rosettes qunatification at day 8 of neural differentation in all transgenic cell lines.

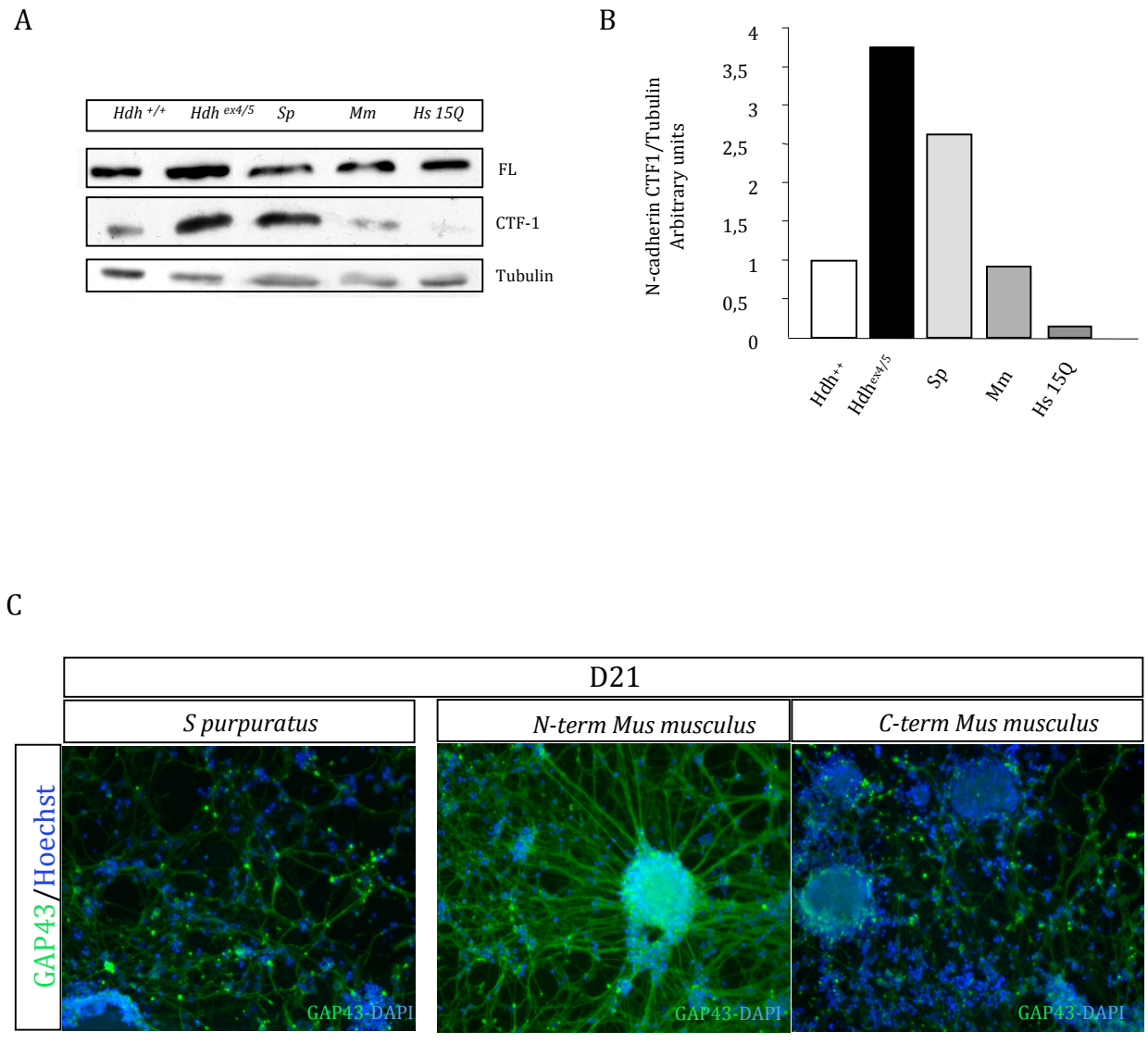

Figure 22. **N-cadherin cleavage during phylogenesis and rescue of "*rosetteless phenotype*":** A) N-cadherin immunoblotting with detection and quantification B) of the CTF-1. C) Immonostaining for neuronal markers GAP43 at D21 of neural differentiation in stable cell lines expressing respectively: *Strongylocentrotus purpuratus* huntingtin and murine N-terimal and C-terminal domain of huntingtin.

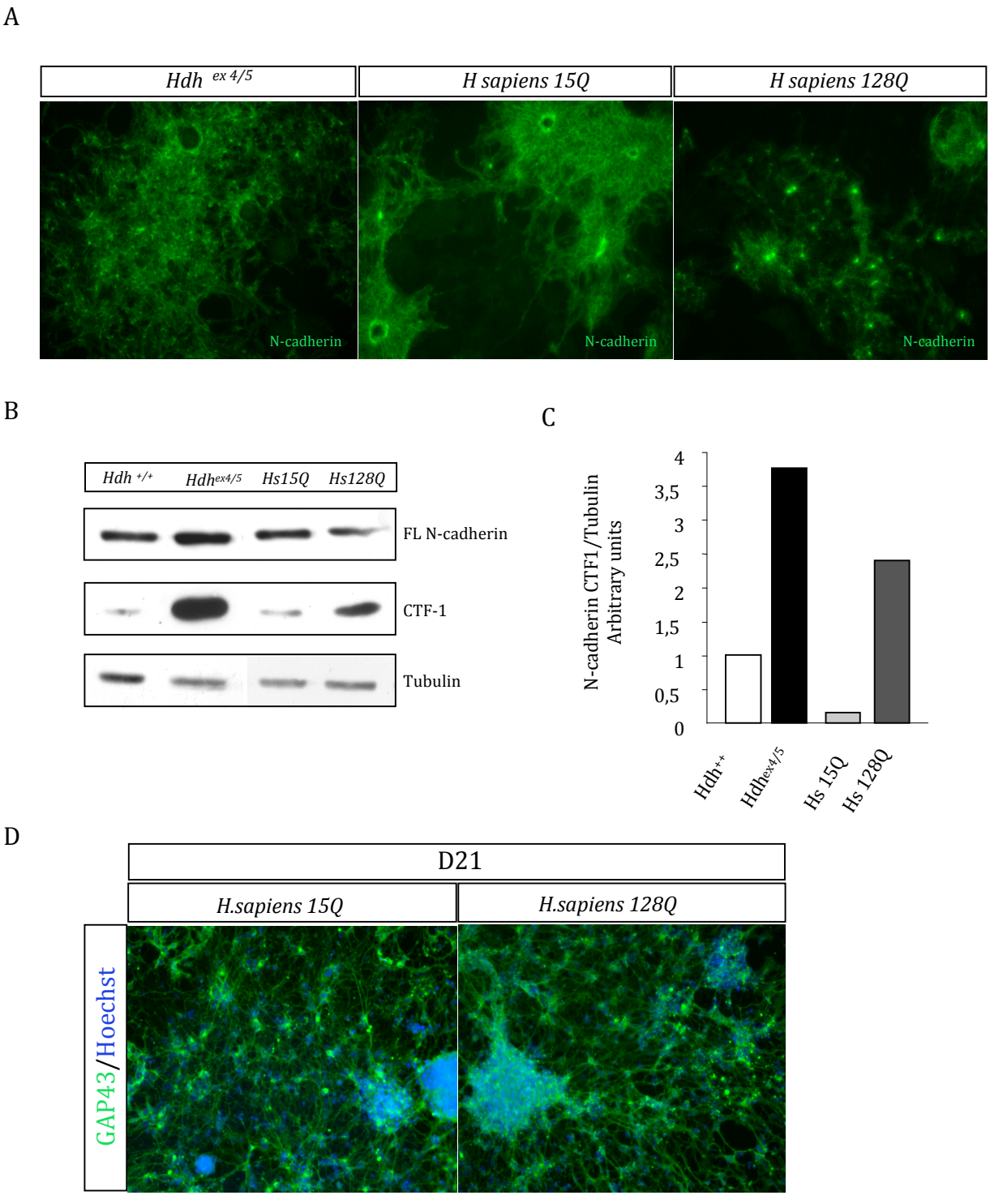

Figure 23. ***Rosetteless phenotype*** **in the presence of expanded polyQ:** A) Immunocytochemistry for N-cadherin at D8 of neural differentiation in ES *Hdh*$^{ex4/5}$ stably expressing N-terminal portion of human huntingtin with 15Q and 128Q. B) N-cadherin immunoblotting with detection and quantification C) of the CTF-1. D) Immonostaining for neuronal markers GAP43 at D21 of neural differentiation.

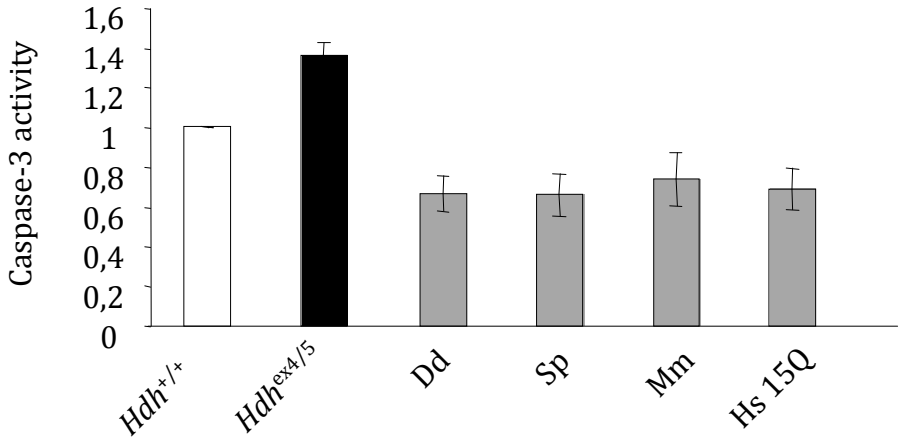

Figure 24 **Anti-apoptotic activity is an ancestral huntingtin function:** Caspase-3 activity assay in all transgenic cell lines in self-renewal condition.