I. INTRODUCTION
The increase in life expectancy, now into the eighth decade, has lead to a dramatic increase in the prevalence of many neurodegenerative diseases. For Alzheimer’s Disease, the most prevalent of all neurodegenerative disorders affecting 15 million people worldwide, it is expected that the number of affected individuals will be 13.2 million in the United States and 16.2 million in Europe by 2050 (Forman et al., 2004; WHO, 2002). Therefore, the development of effective treatments or preventive diagnostic methods for Alzheimer’s Disease or other neurodegenerative diseases is urgent, before the financial and social implications of this burden start to emerge. The research in this area becomes, consequently, of major interest.

The heterogeneous group of neurodegenerative disorders includes, among others, Alzheimer’s Disease (AD), Parkinson’s Disease (PD), transmissible spongiform encephalopathies (including animal and Human diseases), Amyotrophic Lateral Sclerosis (ALS), Huntington’s Disease (HD) and other related polyglutamine disorders including several forms of Spinocerebellar Ataxias (SCAs) (Soto, 2003). Most of these disorders are autosomal dominantly inherited, usually strike around mid-life and are characterized by neuronal damage and accumulation of toxic aggregating-prone proteins (Table 1). This neuronal damage is, intriguingly, restricted to specific neuronal population that are different in each disease. The brain region and type of neurons affected determines the characteristic clinical symptoms associated with each disorder. For example, cerebellar damage in AD patients occurs mainly in the hippocampus, entorhinal cortex and neocortex leading to dementia. In PD patients, cells of the substantia nigra degenerate, resulting in rigidity and tremor. HD patients show neurodegeneration in the striatum, which results in uncontrolled movements; whereas in SCA cell loss occurs mainly in the cerebellum, inducing ataxia. Finally, in ALS, the progressive paralysis observed in the patients results from the cellular damage in the spinal cord, brain stem and areas of the motor cortex. Note, however, that the association between
cellular populations affected and clinical symptoms is more evident in early stages of the disease, when damage is less extensive.

One of the most important effects of the mutations in the disease-causing genes is altered processing and accumulation of misfolded protein in inclusions and plaques (Figure 1). This is one of the major hallmarks, but also a highly controversial subject in what respects to the role of the protein aggregates, as the cause or consequence, of neurodegenerative disorders.

Table 1. Examples of neurodegenerative diseases characterized by aggregation and deposition of abnormal proteins

<table>
<thead>
<tr>
<th>Toxic Protein</th>
<th>Protein Deposit</th>
<th>Familial Disease</th>
<th>Gene mutated</th>
<th>Sporadic Disease</th>
<th>Risk Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>b-amyloid</td>
<td>Senile plaques</td>
<td>FAD</td>
<td>APP/PS1</td>
<td>Alzheimer Disease (AD)</td>
<td>Apoe4</td>
</tr>
<tr>
<td>Tau</td>
<td>Neuronal and glial inclusions</td>
<td>FTDP-17 inclusions</td>
<td>MAPT</td>
<td>AD and tauopathies</td>
<td>MAPT haplotype</td>
</tr>
<tr>
<td>a-synuclein</td>
<td>Lewy bodies</td>
<td>Familial Parkinson Disease (PD)</td>
<td>SNCA (a-synuclein)</td>
<td>Lewy body disease</td>
<td>SNCA haplotype</td>
</tr>
<tr>
<td>Polyglutamine repeat expansion</td>
<td>Nuclear and cytoplasmic inclusions</td>
<td>HD (huntingtin)</td>
<td>Not identified</td>
<td>Not applicable</td>
<td>Not identified</td>
</tr>
<tr>
<td>PrPsc</td>
<td>Protease-resistant PrP</td>
<td>Familial prion protein disease</td>
<td>PRNP</td>
<td>Sporadic prion protein disease</td>
<td>PRNP polymorphism</td>
</tr>
<tr>
<td>SOD</td>
<td>Hyaline inclusion</td>
<td>Autosomal dominant familial ALS</td>
<td>SOD1 (Cu/Zn SOD)</td>
<td>Sporadic ALS</td>
<td>Not identified</td>
</tr>
<tr>
<td>Abri/ADan</td>
<td>Amyloid plaques and angiopathy</td>
<td>Familial British/Danish dementia</td>
<td>BRI</td>
<td>Not identified</td>
<td>Not identified</td>
</tr>
<tr>
<td>Neuroserpin</td>
<td>Collins bodies</td>
<td>FENEB</td>
<td>SERPIN1 (neuroserpin)</td>
<td>Not identified</td>
<td>Not identified</td>
</tr>
</tbody>
</table>

* Tauopathies: Pick Disease (PiD), corticobasal degeneration and progressive supranuclear palsy.  
  * Four additional genes are implicated in familial PD including PARK2, UCHL1, DJ1 and PINK1. However, it is unclear if these genes are associated with Lewy Body (LB) pathology.  
  * Lewy body diseases: PD and Dementia with Lewy Bodies (DLB).  
  * CACNA1A encodes the α(1A) subunit of voltage-gated calcium channel, type P/Q.  
  * Detected by immunohistochemistry or biochemically after digestion with proteinase K.  
  * Familial prion protein disease: familial Creutzfeldt-Jakob Disease (CJD), Gerstmann-Scheinker disease and fatal familial insomnia.  
  * Sporadic prion protein disease: CJD, variant CJD, iatrogenic CJD and kuru.  
  * Familial encephalopathy with neuroserpin inclusion bodies (Forman et al., 2004).
Figure 1 – Protein aggregates and neurodegenerative diseases. Examples of protein aggregates present in a variety of neurodegenerative diseases are shown. Extra cellular amyloid plaques (white arrows) and intra-cytoplasmic neurofibrillary tangles (yellow arrows) are characteristic of AD, while in PD and ALS patients usually present intra-cytoplasmic aggregates. In individuals affected by Huntington's disease intranuclear inclusions of Huntingtin are normally detected. For some cases of transmissible spongiform encephalopathy, extra cellular prion amyloid plaques that are located in distinct brain regions can be observed. Even though these protein accumulations have a different composition, its ultra-structure seems to be similar and composed of an arrangement of fibrillar polymers (see image in the centre) (From Soto, 2003).

Over the past years a growing number of Human neurodegenerative diseases have been identified. Despite the effort of many towards the understanding of genes and mechanisms that lead to neuronal degeneration, it is still unclear what are the pathogenesis events that ultimately lead to neuronal cell death in each disorder. Due to the relevance of neurodegenerative diseases, we believe that it is of great importance to further characterize genes and pathways that are involved in the process of neurodegeneration.
Although tetra- and pentanucleotides repeats have also been associated with the development of neurological disorders (for example SCA10), trinucleotide repeats are the most common type of repeats that cause human disease (Orr and Zoghbi, 2007). Therefore, the focus of this thesis will be on this particular group of neurodegenerative disorders, the trinucleotide repeat disorders, specifically on polyglutamine diseases. A brief overview on this group of diseases will be presented.
1. **Trinucleotide Repeat Disorders**

Trinucleotide repeat disorders is a group of diseases that includes at least 16 disorders with a wide variety of clinical symptoms, from developmental childhood disorders (in the case of X-linked mental retardation syndromes) to late onset neurodegenerative diseases (such as HD or inherited Ataxias) and distinct molecular features (Orr and Zoghbi, 2007). This type of disorders can be categorized into two groups based on the location of the repeat expansion in the gene (Table 2). The first subclass includes diseases caused by expansion of exonic repeat sequences, normally CAG, which result in altered protein function. Because CAG encodes for glutamine, these disorders are referred as polyglutamine diseases. The second subclass includes diseases that are caused by expansions of intronic repeats or caused by expansions of regions located in the 3’ or 5’ Untranslated Regions (UTRs), in non-coding regions of the gene. These result in either loss of protein function or altered RNA function (Gatchel and Zoghbi, 2005; Orr and Zoghbi, 2007).

1.1. **Polyglutamine Diseases**

As mentioned earlier, several Human neurodegenerative diseases result from the expansion of a translated tract of consecutive CAG trinucleotide repeats. To date, this group includes nine genetically distinct disorders: Kennedy’s disease or Spinal-Bulbar Muscular Atrophy (SBMA), HD, Dentatorubral-Pallidoluysian Atrophy (DRPLA) and several distinct Spinocerebellar Ataxias (SCA1, SCA2, SCA3/Machado-Joseph Disease, SCA6, SCA7 and SCA17 (Nakamura et al., 2001; Zoghbi and Orr, 2000). All of these are dominantly inherited in an autosomal manner with the exception of SBMA, which is X-linked recessive. The normal function of many of the genes causing polyglutamine disorders remains unclear and the corresponding proteins have no structural similarity with each other, except for the polyglutamine tract itself. As discussed above, although the disease-causing proteins are ubiquitously expressed in the Central Nervous System (CNS), polyglutamine disorders are characterized by loss of specific neuronal...
populations, resulting in characteristic patterns of neurodegeneration and clinical manifestations. In all cases, the disease only occurs once the glutamine expansion surpasses a certain threshold (Ross, 1997). The clinical symptoms usually manifest around mid-life, and become progressively worse within the next two decades of life. Furthermore, longer repeats lead to earlier age of onset with very long expansions causing juvenile cases.

Table 2. Molecular and clinical features of trinucleotide repeat expansion disorders

<table>
<thead>
<tr>
<th>Disease</th>
<th>Mutation/repeat unit</th>
<th>Gene name (protein product)</th>
<th>Putative function</th>
<th>Normal repeat length</th>
<th>Pathogenic repeat length</th>
<th>Main clinical features</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SCA1</strong></td>
<td>(CAG)n</td>
<td>SCA1 (ataxin 1)</td>
<td>Transcription</td>
<td>6-39</td>
<td>40-82</td>
<td>Ataxia, slurred speech, spasticity, cognitive impairment</td>
</tr>
<tr>
<td><strong>SCA2</strong></td>
<td>(CAG)n</td>
<td>SCA2 (ataxin 2)</td>
<td>RNA metabolism</td>
<td>15-24</td>
<td>32-200</td>
<td>Ataxia, slow saccades, decreased reflexes, polyneuropathy, motor neuropathy, infantile variant</td>
</tr>
<tr>
<td><strong>SCA3 (MJD)</strong></td>
<td>(CAG)n</td>
<td>SCA3 (ataxin 3)</td>
<td>De-ubiquitylating activity</td>
<td>13-36</td>
<td>61-84</td>
<td>Ataxia, parkinsonism, severe spasticity</td>
</tr>
<tr>
<td><strong>SCA6</strong></td>
<td>(CAG)n</td>
<td>CACNA1A (CACNA1A)</td>
<td>P/Q-type 1A calcium channel subunit</td>
<td>4-20</td>
<td>20-29</td>
<td>Ataxia, dysarthria, nystagmus, tremor</td>
</tr>
<tr>
<td><strong>SCA7</strong></td>
<td>(CAG)n</td>
<td>SCA7 (ataxin 7)</td>
<td>Transcription</td>
<td>4-35</td>
<td>37-106</td>
<td>Ataxia, retinal degeneration, cardiac involvement in infantile variant</td>
</tr>
<tr>
<td><strong>SCA17</strong></td>
<td>(CAG)n</td>
<td>SCA17 (TBP)</td>
<td>Transcription</td>
<td>25-42</td>
<td>47-63</td>
<td>Ataxia, behavioural changes or psychosis, intellectual deterioration, seizures</td>
</tr>
<tr>
<td><strong>DRPLA</strong></td>
<td>(CAG)n</td>
<td>DRPLA (atrophin 1)</td>
<td>Transcription</td>
<td>7-34</td>
<td>49-88</td>
<td>Ataxia, epilepsy, choreoathetosis, dementia</td>
</tr>
<tr>
<td><strong>SBMA</strong></td>
<td>(CAG)n</td>
<td>AR (androgen receptor)</td>
<td>Steroid-hormone receptor</td>
<td>9-36</td>
<td>38-62</td>
<td>Motor weakness, swallowing difficulty, gynecomastia, hypogonadism</td>
</tr>
<tr>
<td><strong>HD</strong></td>
<td>(CAG)n</td>
<td>HD (huntingtin)</td>
<td>Signalling, transport, transcription</td>
<td>11-34</td>
<td>40-121</td>
<td>Severe movement abnormalities, chorea, dystonia, cognitive decline, psychiatric features</td>
</tr>
<tr>
<td><strong>FRDA</strong></td>
<td>(GAA)n</td>
<td>FRDA (frataxin)</td>
<td>Mitochondrial iron metabolism</td>
<td>6-32</td>
<td>200-1700</td>
<td>Sensory ataxia, cardiomyopathy, diabetes</td>
</tr>
<tr>
<td><strong>FRAXA</strong></td>
<td>(CGG)n</td>
<td>FMR1 (FMRP)</td>
<td>Translational regulation</td>
<td>6-60</td>
<td>&gt;200 (full mutation)</td>
<td>Mental retardation, macroorchidism, connective tissue dysplasia, attentional and behavioural abnormalities</td>
</tr>
<tr>
<td><strong>FRAXE</strong></td>
<td>(CCG)n</td>
<td>FMR2 (FMR2)</td>
<td>Transcription?</td>
<td>4-39</td>
<td>200-900</td>
<td>Mild mental retardation or learning impairment</td>
</tr>
<tr>
<td><strong>DM1</strong></td>
<td>(CTG)n</td>
<td>DMPK (DMPK)</td>
<td>RNA-mediated</td>
<td>5-37</td>
<td>50-1,000</td>
<td>Myotonia, weakness, wasting, cardiac conduction abnormalities, testicular atrophy, insulin resistance, cataracts, congenital form, potentially severe CNS involvement with mental retardation</td>
</tr>
<tr>
<td><strong>DM2</strong></td>
<td>(CTG)n</td>
<td>ZNF9 (ZNF9)</td>
<td>RNA-mediated</td>
<td>10-26</td>
<td>75-1,000</td>
<td>Similar to DM1, no congenital form</td>
</tr>
<tr>
<td><strong>FXTAS</strong></td>
<td>(CGG)n</td>
<td>FMR1 (FMRP)</td>
<td>RNA-mediated</td>
<td>6-60</td>
<td>60-200 (premutation)</td>
<td>Tremor/ataxia, parkinsonism, cognitive defects</td>
</tr>
<tr>
<td><strong>SCA8</strong></td>
<td>(CTG)n</td>
<td>SCA8 (transcribed/untranslated)</td>
<td>Unknown</td>
<td>16-34</td>
<td>&gt;74</td>
<td>Ataxia, slurred speech, nystagmus</td>
</tr>
<tr>
<td><strong>SCA10</strong></td>
<td>(ATTCT)n</td>
<td>Unknown</td>
<td>Unknown</td>
<td>10-20</td>
<td>500-4,500</td>
<td>Tremor, ataxia, dementia</td>
</tr>
<tr>
<td><strong>SCA12</strong></td>
<td>(CAG)n</td>
<td>PPP2R2B</td>
<td>Phosphatase regulation</td>
<td>7-45</td>
<td>55-78</td>
<td>Ataxia and seizures</td>
</tr>
<tr>
<td><strong>HD12</strong></td>
<td>(CTG)n</td>
<td>JPH3 (junctophilin 3)</td>
<td>PM/ER junction protein</td>
<td>7-28</td>
<td>66-78</td>
<td>Similar to HD</td>
</tr>
</tbody>
</table>
CACNA1A- calcium channel, voltage-dependent, P/Q type, 1A subunit; DMPK- dystrophia myotonica protein kinase; DRPLA- dentatorubral-pallidoluysian atrophy; DM- dystrophia myotonica; ER- endoplasmic reticulum; FRDA- Friedreich ataxia; FMRP- fragile X mental retardation protein; FMR1- fragile X mental retardation 1; FMR2- fragile X mental retardation 2; FXTAS- fragile X tremor/ataxia syndrome; HD- Huntington disease; HDL2- Huntington disease-like 2; MJD- Machado-Joseph disease; PM- plasma membrane; PPP2R2B- protein phosphatase 2 (formerly 2A) regulatory subunit B; SBMA- spinal and bulbar muscular atrophy; SCA- spinocerebellar ataxia; TBP- TATA box binding protein; ZNF9- zinc-finger protein 9. *Dystrophia myotonica 1 (DM1) differs from DM2 in that it also has a congenital form, and potentially severe CNS involvement with mental retardation (Gatchel and Zoghbi, 2005).

In spite of the effort to understand the genes and mechanisms that contribute to neuronal degeneration, it is still unclear which pathogenic events are relevant to each of these disorders. Several studies support the idea that protein modifications outside of the polyglutamine expansion, such as post-transcriptional modifications, are critical in determining the clinical and pathological effects of polyglutamine tracts and gives to the protein context an important role in pathogenesis (Chen et al., 2003; Emamian et al., 2003; reviewed in Gatchel and Zoghbi, 2005; Humbert et al., 2002; Humbert and Saudou, 2002; Orr, 2001; Riley et al., 2005; Steffan et al., 2004; Tsuda et al., 2005; Zoghbi, 1996). Also contributing to the idea that the polyglutamine tract is not the major contributor to pathogenesis is the observation that other repeats (CGG or CTG, among others) also induce disease phenotypes and that in several cases the “pathogenic” repeat is not translated (reviewed in Gatchel and Zoghbi, 2005; McLeod et al., 2005). However, the specific effects of each post-translational modification may vary from disease to disease and mutant protein. Despite the existence of common mechanism among polyglutamine diseases, that raise the possibility of conserved pathogenic mechanisms, the striking differences in clinical and pathological features also suggest disease specific pathogenesis.
1.1.1. Genetic Anticipation and Repeat Instability

Trinucleotide Repeat Diseases typically present unusual clinical and genetic patterns that cannot easily be explained based on the laws of classical Mendelian inheritance. One common feature to these diseases is Genetic Anticipation, which means, an earlier onset of the first symptoms, as well as increased severity of clinical symptoms throughout the generations. Anticipation can be explained by the increase in size of the expansion over generations, which usually occurs with paternal transmission, as a result of instability in the trinucleotide repeats. Note that longer tracts are more prone to experience an expansion mutation than shorter tracts. Unlike static mutations, that are stably transmitted to the offspring and retained in somatic tissues, repeat mutations are a dynamic process, where products continue to mutate within the tissues and along generations (Mirkin, 2007; Pearson et al., 2005). Moreover, the discovery of these dynamic mutations has provided a molecular explanation to the variety of the disease phenotypes and expressivity observed: larger expansions result in an earlier onset of the disease and in increased severity of the symptoms. Note also that DNA repeats are particularly prone to these type of expansions due to its unusual structural features that tend to disrupt cellular replication, repair and recombination machineries (Mirkin, 2007).

The phenomenon of Genetic Anticipation was first described in 1918 for Myotonic Dystrophy, a hereditary disorder. Anticipation was then observed in other hereditary disorders such as Huntington’s Disease, Friedrich’s Ataxia and several Spinocerebellar Ataxias, all caused by DNA repeat expansions within the disease-causing gene. More recently, it was described for the case of Fragile X Syndrome a particular form of anticipation, in which an increasing repeat’s length and penetrance (which can be explained as the probability of a specific mutation to result in the development of a disease) in succeeding generations is observed. This is known as the Sherman Paradox (Mirkin, 2006).

Although normal alleles are relatively stable on germ line transmission, mutant expanded alleles change in size in the majority of parent-descendent transmission, know as repeat instability. Somatic
instability can also occur, but is less common. Trinucleotide repeats that exceed a certain threshold (for example, ≈30 CAG repeats in the case of Huntington's Disease) show repeat instability during the replication process, which tends to be more significative with the increase in the length of the expansions. In most cases, ≈73%, repeat instability leads to expansion, but in some cases, contraction takes place (≈23%) (Trottier et al., 1994; Walker, 2007). Repeat instability appears to be more frequent and stronger when transmission is made from a male than from a female, and expansions, rather than contractions, tend to occur frequently. The simplest explanation to what properties of the repeats contribute to the propensity to expand is that the repetitive nature of this expandable elements leads to an occasional strand slippage during DNA replication (Mirkin, 2006). However this theory does not explain many of the features observed, including why not every repeat expands. It is, therefore, more likely that the mechanisms leading to repeat instability include not only DNA replication, but also DNA recombination, DNA repair or other additional mechanisms (Pearson et al., 2005).

1.1.2. “Gain of function” mechanisms and current models of disease

One major effort from those that work in the field is to learn what are the mechanisms that lead to neuronal degeneration and cell death.

The idea that polyglutamine disorders are caused by “gain of toxic function” mechanisms has been proposed. Evidence supporting this notion comes from different observations: 1) Mice models that lack the disease protein, for example, Ataxin-1 or Huntingtin (in the case of SCA1 and HD, respectively), are viable and fertile but do not display the characteristic SCA1 and HD clinical or neuropathological phenotypes. On the contrary, overexpression of the mutant Human proteins leads to cell loss and characteristic neurodegenerative phenotypes (Burright et al., 1995; Mangiarini et al., 1996; Matilla et al., 1998; Sisodia, 1998). 2) Deletions in the Huntingtin and androgen receptor genes (genes expanded in HD and SBMA, respectively) do not display the characteristic HD and SBMA clinical or
neuropathological phenotypes. 3) Levels of normal and mutant Htt are equivalent in HD patients (Sisodia, 1998). 4) Individuals homozygous for the HD-causing mutation have a more severe clinical phenotype than individuals that carry only one mutated allele (Wexler et al., 1987).

Therefore, even though in some cases the loss of function of the normal gene may contribute to disease, an important component of the pathology comes from a gain of function of the disease-causing protein.

There is also abundant evidence that protein context is important for polyglutamine toxicity. For example, post-transcriptional modifications outside of the polyglutamine tract are critical in determining the clinical and pathological effects of the corresponding proteins (Chen et al., 2003; de Chiara et al., 2005; Emamian et al., 2003; Gatchel and Zoghbi, 2005; Humbert et al., 2002; Humbert and Saudou, 2002; Orr, 2001; Riley et al., 2005; Steffan et al., 2004; Tsuda et al., 2005; Zoghbi, 1996).

There are disease mechanisms that are likely to be common to all polyglutamine diseases (Figure 2). For example, protein misfolding and protein turnover have been implicated in all of these disorders (Everett and Wood, 2004; Ghosh and Feany, 2004; Humbert and Saudou, 2002; Orr, 2001; Riley and Orr, 2006; Sisodia, 1998; Soto, 2003). Other mechanisms, such as altered gene expression, RNA processing, synaptic transmission, calcium homeostasis and dysfunction or endoplasmic reticulum impairment have been implicated with pathogenesis in one or more of these disorders (reviewed in Everett and Wood, 2004; Hoshino et al., 2004).

Besides these, other theories also propose that accumulation of mutant polyglutamine protein in insoluble inclusions recruits components of the ubiquitin–proteasome system and other protein quality-control pathways, although the implications of this for pathogenesis are unclear. Furthermore, the full-length polyglutamine protein can also be cleaved by proteases to form fragments, which might also mediate pathogenic effects. However, the relevance of each of these events to each disease has not been clarified yet.
Figure 2 – Possible mechanisms of pathogenesis in polyglutamine diseases. a - Alterations in protein conformation lead to aberrant interactions and accumulation in the cells. These aberrant interactions can result from enhanced or lost interactions. Note also that some interactions might remain unchanged. b - Expanded polyglutamine proteins might induce toxicity through a variety of mechanisms that range from altered transcription and RNA metabolism, mitochondrial dysfunction, proteolytic cleavage and protein accumulation in the cells. Q, glutamine; Ub, ubiquitin (From Gatchel and Zoghbi, 2005).

Despite all the work done in this area, we still have a poor understanding of the relevance of these mechanisms to each particular disease, and it
remains to be determined what mechanisms, if any, besides protein folding and turnover may be common to several disorders.

1.1.3. The role of neuronal aggregates in polyglutamine diseases

Polyglutamine diseases share a number of common features including progressive neuronal degeneration and formation of protein aggregates. These aggregates contain the mutant polyglutamine protein and other proteins such as ubiquitin, chaperones and proteasome subunits (Zoghbi and Botas, 2002). The neuronal aggregates can be mainly nuclear, Nuclear Inclusions (NIs), which are present in SCA1, SCA7 and SCA17. Neuronal aggregates can also be cytoplasmic (such as in the case of SCA6) or present in both cytoplasm and nucleus as in HD, DRPLA, SBMA, SCA2 and SCA3 (Al-Ramahi et al., 2007; Gatchel and Zoghbi, 2005).

The role of NIs in pathogenesis has been, however, a matter of much debate. At first, the predominant idea was that NIs are pathogenic as they may sequester essential proteins and interfere with their normal function (Davies et al., 1997; DiFiglia et al., 1997; Kazantsev et al., 2002; Perutz, 1999; Ross, 1997; Yamamoto et al., 2000). Supportive of the idea that aggregates are the trigger of the pathogenesis in polyglutamine disease is the fact that these neuronal aggregates containing expanded polyglutamine proteins were found in neurons affected in several of these disorders: SCA1, HD, SCA3, DRPLA and SBMA. However, a report by Orr and colleagues showed that even though aggregates could be relevant for the progression of the disease, they were not the pathological basis (Klement et al., 1998). An opposite view argues that NIs may play a defensive role by sequestering the soluble mutant protein, which is the toxic form, and preventing its deleterious interactions with other cellular proteins (Arrasate et al., 2004; Cummings et al., 1999b; Kayed et al., 2003; Kummerle et al., 1999; Orr, 2001; Saudou et al., 1998; Zoghbi and Botas, 2002). Therefore, aggregates would not be the main drivers of toxicity (Cummings et al., 2001; Everett and Wood, 2004; Kazemi-Esfarjani and Benzer, 2000; Klement et al., 1998; Orr, 2001). There is also a third theory, only with scattered supported, that assumes that NIs are neutral to the toxicity. In support of this are some
reports showing that polyglutamine toxicity might be modulated without affecting aggregation of the mutant protein (Cummings et al., 2001; Kazemi-Esfarjani and Benzer, 2000; Klement et al., 1998), while other reports show that aggregates are also present in unaffected areas of the brain (Everett and Wood, 2004; Orr, 2001) or that aggregates are not present, temporally and spatially, in the described patterns of neurodegeneration (Gutekunst et al., 1999).

Despite the extensive work on this matter, no definitive explanation regarding the role of aggregates in the development and/or progression of neurodegenerative diseases has been obtained.

1.1.4. Spinocerebellar Ataxia Type One (SCA1) and Huntington’s Disease (HD)

SCA1 and HD are autosomal dominant diseases caused by expansion of a polyglutamine tract on the N-terminal region of the Ataxin-1 and Huntingtin (Htt) proteins, respectively (Table 3). The gene products of the two diseases have no structural similarity to each other with the exception of the polyglutamine tract. SCA1 is characterized by loss of coordination, impaired balance, motor and cognitive decline. These clinical features are associated with degeneration of cerebellar Purkinje cells, spinocerebellar tracts and, to a lesser extent, brain stem nuclei (Zoghbi, 1995). In HD, clinical manifestations include involuntary movements (chorea), cognitive decline and psychiatric disturbances that lead to progressive dementia. These characteristic symptoms result mainly from degeneration of striatal neurons but also degeneration of cortex neurons (Cattaneo et al., 2005; Landles and Bates, 2004). For both disorders, there is an inverse correlation between the number of repeats and the age of onset of the first clinical symptoms.

A more detailed description of the two disorders is shown in the next two sections.
1.1.4.1. SCA1

SCA1 is a progressive neurodegenerative disorder affecting approximately 1 to 2 individuals in every 100,000, depending on geographical location and ethnic background (data from NAF, www.ataxia.org). SCA1 is characterized by loss of coordination, motor impairment and degeneration of cerebellar Purkinje cells, spinocerebellar tracts and brainstem nuclei. Symptoms usually develop in the third or fourth decade but anticipation has been noted in juvenile onset cases (Figure 3; Zoghbi, 1995).

The disease results from the expansion of a CAG trinucleotide repeat within the coding region of the disease gene, SCA1. In normal individuals, repeat length ranges from 6 to 39 interrupted glutamines, whereas in patients, disease alleles can be found with 40 to 82 uninterrupted glutamine repeats (Zoghbi and Orr, 2000). The repeat has a perfect CAG configuration in the expanded alleles whereas in normal alleles containing over 20 repeats, it is interrupted with 1 to 4 CAT repeat units. These CAT repeat units, encoding histidine, most likely maintain CAG repeat tract stability (Table 3). Both wild type and expanded alleles are transcribed, ruling out impairment of transcriptional efficiency in SCA1.

The SCA1 gene product, Ataxin-1 (locus 6p23), is a novel protein of 792-868 amino acids, depending on the glutamine track length, with a molecular mass of ≈90 kDa that is widely expressed in the central nervous system and peripheral tissues. Ataxin-1 localizes mainly in the nucleus of neuronal cells but it is cytoplasmic in other cell types. The C-terminal portion of the protein contains the nuclear localization signal (NLS), the self-association region (between amino acids 495 and 605), a 14-3-3 binding motif and also a region, spanning 130 amino acids, with homology to the transcription factor

<table>
<thead>
<tr>
<th>Disease</th>
<th>Gene Locus</th>
<th>Gene Product</th>
<th>Normal CAG(n)</th>
<th>Expanded CAG(n)</th>
<th>Protein Localization</th>
<th>Special Features</th>
<th>Brain regions most affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCA1</td>
<td>6p22-23</td>
<td>Ataxin-1</td>
<td>6-39</td>
<td>40-82</td>
<td>Nuclear in neurons</td>
<td>Normal alleles &gt;21 repeats interrupted with 1-4 CAT units</td>
<td>Cerebellar Purkinje cells, dentate nucleus, brainstem</td>
</tr>
<tr>
<td>HD</td>
<td>4p16.3</td>
<td>Huntington</td>
<td>11-34</td>
<td>40-121</td>
<td>Cytoplasmic and Nuclear</td>
<td>Intermediate alleles: 29-35</td>
<td>Striatum, cerebral cortex</td>
</tr>
</tbody>
</table>

(Zoghbi and Orr, 2000)
HBP1 (High Mobility Group (HMG) box containing protein-1) (Burright et al., 1997; Chen et al., 2003; Klement et al., 1998). This region is named AXH, from domain in Ataxin-1 and HMG box protein-1 (Chen et al., 2004; de Chiara et al., 2003).

Figure 3 – Correlation between severity of the mutation (or size of the trinucleotide repeat expansion; arrow on the top), time of onset of the symptoms of the disease (middle panel) and regions of the brain that are affected (bottom panel). Usually neuronal damage is restricted to specific neuronal sub-populations in each disease. However, severe mutations (such as very long CAG repeat expansions) will cause juvenile-onset cases. As a result, neuronal damage is less localized than in adult-onset patients, affecting brain regions that are unaffected in older affected individuals. This loss of specificity might be a consequence of the mutation being so severe that protein clearance is impaired even in those neurons that normally express low levels of the disease protein. Another alternative is that the mutant protein is so abundant that many more aberrant interactions with other proteins are taking place (Adapted from Zoghbi and Botas, 2002).

The murine Ataxin-1 protein shares 89% identity with the Human Ataxin-1 but contains only two glutamine repeats instead of the long track present in the Human protein. In Drosophila and C. elegans the proteins encoded by CG4547 and K04F10.1, respectively, share homology only with the AXH domain and lack the glutamine track, suggesting that this domain may act as an independent functional unit (Figure 4).
Ataxin-1 seems to play a role in synaptic plasticity and neuronal functions underlying some learning tasks function: SCA1 null mice display impaired spatial and motor learning and decreased paired-pulse facilitation in the CA1 area of the hippocampus (Matilla et al., 1998). Moreover, mice lacking Ataxin-1 display not only learning deficits but also altered hippocampal synaptic plasticity, but none of the abnormalities seen in Human SCA1 patients. On the other hand, mice expressing Ataxin-1 with an expanded CAG tract (82 glutamine residues) develop Purkinje cell pathology and ataxia. These results suggest that mutant Ataxin-1 gains a novel function that leads to neuronal degeneration. Again, this novel function might involve aberrant interaction(s) with cell-specific protein(s), which in turn might explain the selective neuronal pathology. Immunolocalization studies in affected neurons of patients and SCA1 transgenic mice showed that mutant Ataxin-1 localizes in ubiquitin-positive nuclear inclusions (NIs) that alter the distribution of the proteasome and certain chaperones. Further analysis of NIs in transfected HeLa cells established that the proteasome and chaperone proteins co-localize with Ataxin-1 aggregates. However, more recently it has been shown that neuronal dysfunction occurs without
impairment of the ubiquitin-proteasome system (Bowman et al., 2005). It was also found that overexpression of the chaperone HDJ-2/HSDJ in HeLa cells decreased Ataxin-1 aggregation, suggesting that protein misfolding might underlie NI formation. Studies in transgenic mice expressing expanded Human Ataxin-1 have demonstrated that the nuclear localization of mutant Ataxin-1, rather than aggregation, is crucial for cell death, and so for the development of the disease (Klement et al., 1998). There is increasing evidence that, due to Ataxin-1 nuclear localization, altered gene expression is an early effect (Humbert and Saudou, 2002; Yue et al., 2001).

Despite these evidences, a recent report by Zoghbi and colleagues shows that SCA1 pathological events might result from wild-type protein interactions with other proteins and not from novel interactions (Lam et al., 2006).

1.1.4.2. HD

HD is a genetic disease affecting around 8 in 100 000 people, varying according to the geographic location and ethnic background (www.neurologychannel.com/huntingtons; www.wrongdiagnosis.com/h/huntingtons_disease/stats-country.htm; http://genome.wellcome.ac.uk/doc_wtd020863.html). The disease is caused by a polyglutamine repeat expansion in the N-terminal of Huntingtin (Htt), a 348kDa cytosolic protein (locus 4p16.3). This is the largest of the polyglutamine disease proteins known to date (with 3144 amino acids) and its function is, for the most part, still unknown (Figures 5 and 6). It is expressed as 2 alternatively polyadenylated forms displaying different relative abundance in various fetal and adult tissues. The larger transcript is approximately 13.7 Kb and is expressed predominantly in adult and fetal brain whereas the smaller transcript of approximately 10.3 Kb is more widely expressed. Htt is a soluble protein extremely conserved across species, expressed ubiquitously in and outside of the nervous system and whose normal function appears to be critical for embryogenesis and mammalian development (Cattaneo et al., 2005; Lee et al., 2002; Rigamonti et al., 2001). It has also been proposed that Htt exerts a function in the control of neuronal survival and stability.
(Cattaneo et al., 2001). In HD patients, the striatum is most affected (in particular caudate nucleus and putamen), but degeneration is also observed in cerebellar cortex. However, in advanced stages of the disease, patients also show degeneration in other brain regions, such as the thalamus, cerebellum, substantia nigra, and in the subthalamic nucleus. Cerebellar atrophy can also occur in juvenile cases of the disease.

Figure 5 - Primary sites of neuronal loss in HD patients (Rubinsztein, 2002).

The Exon1 of the wild-type Htt gene (also called IT15 (Interesting Transcript 15) gene) contains the stretch of trinucleotide repeats, which is translated into consecutive glutamine repeats. Normal individuals have between 11 and 34 stable CAG repeat units. More than 36 glutamine repeats result in an unstable, expandable, disease-causing allele with incomplete penetrance. Disease alleles containing 40 to 50 glutamine repeats are found in 90-95% of the cases and correspond to cases where onset of the symptoms occurs around mid-life. Juvenile forms of HD are associated with alleles containing more than 70 repeats, and the longest allele described to date contains 121 CAG repeats (Zoghbi and Orr, 2000). The disease typically strikes individuals with 35 to 50 years of age and inevitably ends in death in 10 to 20 years after its onset. There is no treatment or cure for this disorder.

Htt contains multiple HEAT repeats, hydrophobic α-helices that mediates protein-protein interactions, which indicates that Htt might be a multifunctional scaffold protein. A number of proteins have been found to bind N-terminal Htt (some examples are shown in Table 4), and their binding is altered by expansion of the polyglutamine tract (Cattaneo et al., 2001; Goehler et al., 2004; Li et al., 2003). Due to its large size, it is not surprising
Molecular Mechanisms Involved in Polyglutamine-Induced Neurodegeneration

Joana Branco

that Htt interacts with many different proteins involved in distinct pathways. However, there is a limited knowledge regarding the pathological relevance of the interactions between Htt and its interacting proteins.

Figure 6 - Schematic representation of the Huntingtin amino acid sequence. (Q)n indicates the polyglutamine tract, and (P)n the polyproline region. The red squares highlight the three main clusters of HEAT repeats. The blue arrowheads indicate the calpain cleavage sites and their amino acid position, and the green arrows point at the caspase cleavage sites and their amino acid positions. B identifies the regions cleaved preferentially in the cerebral cortex, C indicates those cleaved mainly in the striatum, and A indicates the regions cleaved in both. NES is the nuclear export signal. Green and orange arrowheads indicate amino acid regions where protease cleavage occurs. The red and blue circles indicate post-translational modifications: ubiquitination (UBI) and/or SUMOylation (SUMO) (red), and phosphorylation at serine 421 and serine 434 (blue). The glutamic acid (Glu)-, serine (Ser)- and proline (Pro)-rich regions are indicated (serine-rich regions encircled in green) (Cattaneo et al., 2005).

Although the expanded CAG repeat plays a major role in the disease, the possibility of subtle dysfunctions of the Htt normal allele cannot be ruled out. Suggestions that loss of normal Htt function(s) might contribute to the disease come from work in cells and in mice, in which the absence of normal Htt was shown to decrease the survival and stability of neuronal cells (Cattaneo et al., 2001). In addition, mutant Htt can recruit normal Htt into insoluble aggregates both in vitro and in vivo, suggesting that the toxic effect of mutant Htt may include sequestration of the wild-type Htt protein or of its functions. Moreover, it was reported a correlation between the inhibition of the cleavage of endogenous wild-type Htt and prolonged survival (Cattaneo et al., 2001).
Table 4 - Partial list of proteins (and respective cellular pathways) interacting with the N-Terminal region of the Human Htt protein

<table>
<thead>
<tr>
<th>Cellular Pathway</th>
<th>Interacting Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcription</td>
<td>Sp1, RAP30, CBP, PS3, PQBP-1</td>
</tr>
<tr>
<td>Subcellular Localization</td>
<td>SD-95, tubulin, HIP1, HAP1</td>
</tr>
<tr>
<td>RNA processing</td>
<td>FBP-11, HYCP, symplekin</td>
</tr>
<tr>
<td>Receptors</td>
<td>EGFR, IP3R1</td>
</tr>
<tr>
<td>Degradation</td>
<td>E2-25 kd</td>
</tr>
</tbody>
</table>

Adapted from (Orr and Zoghbi, 2007)

2. Animal Models of Neurodegenerative Diseases

The study of the brain of normal and sick individuals provides a valuable insight of the mechanisms of pathogenesis of neurodegenerative diseases. However, the information obtained is limited to highly advance stages of the disease. Therefore, animal models are of great importance since they allow close monitorization of mechanisms leading to the characteristic neuropathology as well as the study of pre- and post-pathogenesis events. For that reason, many disease models have been established over the past years. Animal models also allow the analysis of modifier genes and performing drug screens. Although cell-based models provide valuable information, cell culture assays do not take into account physiological conditions and interactions with different cell types and tissues. Nevertheless, the use of cell culture and in vitro models can be of great relevance if combined with other animal models.

In the recent years many invertebrate systems, specifically Drosophila melanogaster (D. melanogaster). For simplicity, from this point on,
**Drosophila melanogaster** will be only referred as *Drosophila* and *Caenorhabditis elegans* (*C. elegans*), have been used to model neurodegenerative disorders, making use of the many advantages of those systems. A variety of strategies have also been used to successfully establish vertebrate models (especially mouse models) for these disorders. The use of these models is supported by the existence of highly conserved molecular pathways between the two species and by the powerful genetic and molecular tools available. A summary of the most significant animal models for both SCA1 and HD will be described next.

2.1. **ANIMAL MODELS FOR SCA1 AND SIGNIFICANT ADVANCES**

Some animal models have been generated in order to gain insight into the mechanisms and features associated with the SCA1 pathology. A brief description of those models will follow.

Mice lacking Ataxin-1 were first generated to provide information on the normal function of Ataxin-1 (Matilla et al., 1998). Ataxin-1 null mice are viable, fertile and display normal life span and somatic development with no major coordination problems. However, they do not show any evidence of the characteristic pathological features of SCA1. These observations clearly support the idea that SCA1 is not caused by the loss of function of Ataxin-1, but do not rule out a possible contribution of wild-type Ataxin-1 in SCA1 pathogenesis.

On the other hand, mice over expressing full-length expanded Human Ataxin-1 (hAtaxin-1^{82Q}) cause progressive ataxia, Purkinje cell degeneration and many other features associated with SCA1 pathogenesis, in a similar manner to what is seen in patients (Burright et al., 1995). This was actually the first transgenic mouse model. Since then it has been extensively used in many studies and has provided valuable insights into the mechanisms linked to SCA1 (Clark et al., 1997; Cummings et al., 1998; Cummings et al., 1999a; Fernandez-Funez et al., 2000; Lin et al., 2000; Serra et al., 2004; Skinner et al., 1997). However, because these mice express the mutant protein at high levels in specific cells, some pathological discrepancies are observed between Human
patients and this model. Extremely valuable information has also been generated by the work of Orr and collaborators (Klement et al., 1998). In this study, and making use of a transgenic mouse that express expanded Ataxin-1 (82 glutamines), the authors proved that the nuclear localization of the mutant protein, rather than its aggregation is crucial for pathogenesis to occur.

Ataxin-1 knock-in mice have also been created. Mice with a 78 CAG repeat insertion in the mouse SCA1 locus do not show the characteristic SCA1 phenotypes (Lorenzetti et al., 2000), but mice with a long CAG repeat expansion (154Q) reproduce many of the phenotypes observed in SCA1 patients (Watase et al., 2002). These features include cognitive defects, muscle wasting, premature death, as well as selective neurodegeneration. This model allows the possibility of studying the effects of mutant Ataxin-1 in its natural context, without the need of a cell specific promoter, therefore with no restrains regarding spatial and temporal expression and at its endogenous levels. Making use of these knock in mice, it was also possible to further explore the role of NIs in pathogenesis. Together, studies using knock in and transgenic mice have shown that the SCA1 pathogenic events are dependent on the glutamine tract length, the levels of expression of the protein and the duration of exposure of the cells to the mutant protein.

Taking advantage of the power of the Drosophila genetics, and using the GAL4/UAS system, SCA1 transgenic flies have also been generated (Fernandez-Funez et al., 2000). The GAL4/UAS system (Brand and Perrimon, 1993) allows the control of the level of transgene expression and to direct its expression to different cell types or even to specific neurons. Target expression of mutant Ataxin-1 leads to cell degeneration and several SCA1 cellular features, such as formation of NIs positive for ubiquitin, Hsp70 and proteasome components. Making use of this fly model, genetic screens were conducted. This allowed the identification of suppressors and enhancers of the SCA182Q-induced phenotypes. The modifiers identified are involved in a variety of cellular processes such as protein folding and clearance, RNA processing, transcriptional regulation and cellular detoxification. This model and the relevance of using
*Drosophila* as a model system will be further discussed in section 3 of this Introduction.

Transgenic mice and flies that express the Human Ataxin-1 protein bearing a non-pathogenic glutamine tract (containing 30 glutamines) have also been generated (Burright et al., 1995; Fernandez-Funez et al., 2000). These animals do not show the obvious phenotypes associated with SCA1 pathogenesis (and that are present in transgenic Ataxin-1 mice and flies bearing a pathogenic glutamine expansion, 82Q). However, when the Ataxin-110Q transgenes are expressed at very high levels, the animals show mild cell loss. The work done with these two transgenic organisms has revealed that wild-type Ataxin-1 can promote neurodegenerative phenotypes similar to the ones induced by expression of its mutant counterpart, as long as it is expressed at sufficiently high levels.

### 2.2. Animal Models for Huntington’s Disease and Significant Advances

Transgenic animal models of HD were first created in mice and afterwards in *Drosophila* and *C. elegans* (Faber et al., 1999; Jackson et al., 1998; Marsh et al., 2003; Rubinsztein, 2002). As for the SCA1 models, HD fly and mouse models show that pathology is dependent on the size of the polyglutamine tract and present many of the features of the disease, including formation of NIs, progressive degeneration and cell death.

Mice bearing only one functional copy of the *Htt* gene do not present the typical HD features. In contrast, transgenic mice carrying the mutant *Htt* gene (or a fragment of the *Htt* gene) or knock-in animals show some of the characteristic phenotypical and pathological hallmarks of HD (Table 5; reviewed in (Rubinsztein, 2002)). These observations support, once again, the current idea that polyglutamine disorders are caused, for most cases, by gain of function mechanisms, instead of mechanisms resultant of the loss of the normal function of the disease-causing protein. A transgenic HD mouse model expressing the N-terminal of the Htt protein with approximately 150 glutamines has provided the first evidence that the truncated form of the protein is sufficient for the development of HD-like phenotypes (Mangiarini...
et al., 1996). It also contributed to the identification of NIs that contains the mutant protein, a major hallmark of the disease. Note also that the use of or *knock-in* models has highlighted the relevance of the protein context, since the same glutamine repeat size is more toxic and induces disease phenotypes more severe in Ataxin-1 than in Huntingtin, a larger protein. Therefore, it may be possible that not only the context but also the size of the disease-causing protein contributes to the severity of the disease phenotypes.

Fly models of HD were also generated, expressing either different portions of the N-terminal of the Htt protein (Agrawal et al., 2005; Jackson et al., 1998; Kaltenbach et al., 2007; Kazemi-Esfarjani and Benzer, 2000; Lee et al., 2004; Steffan et al., 2001) or the full length Htt protein (Romero and Botas, unpublished data). As mentioned earlier, the models show mutant Huntingtin-induced degeneration, in a similar fashion to what is observed in Human patients and mice models. Also features of these models are the nuclear localization and age-dependent aggregation of the mutant protein as well as age of onset and the severity of the disease correlated with the length of the polyglutamine track. HD *Drosophila* models have also been used to conduct pharmacological studies, that hopefully will allow the development of treatments for this disease (Agrawal et al., 2005; Apostol et al., 2003; reviewed in Marsh and Thompson, 2006).
### Table 5 - Mouse models of HD

<table>
<thead>
<tr>
<th>Model</th>
<th>Onset</th>
<th>Symptoms</th>
<th>Neuropathological features</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Transgenic models</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 1 R6/2 line</td>
<td>144Q (expanded to 170-190Q) R6/1 line 155Q</td>
<td>86/2: 2 months R6/1 line 5 months</td>
<td>Intranuclear and neuropil aggregates throughout brain; Fewer dendritic spines</td>
<td>I</td>
</tr>
<tr>
<td>Full-length gene</td>
<td>72Q, 46Q or 18Q</td>
<td>72Q: 3 months</td>
<td>Inclusions in striatum</td>
<td>II</td>
</tr>
<tr>
<td>First 171 amino acids</td>
<td>82Q, 44Q or 8Q</td>
<td>82Q: 5 months</td>
<td>Neuronal degeneration in striatum</td>
<td>III</td>
</tr>
<tr>
<td>Full-length gene</td>
<td>89Q, 48Q or 16Q</td>
<td>89Q and 48Q: 4 months</td>
<td>Fewer inclusions throughout the brain</td>
<td>IV</td>
</tr>
<tr>
<td>Exons 1-3</td>
<td>CMV promoter</td>
<td>89Q</td>
<td>4 months</td>
<td>Not reported</td>
</tr>
<tr>
<td>3-Kb N-Terminal fragment</td>
<td>Rat neuron specific enolase promoter</td>
<td>100Q: 3-4 months</td>
<td>Inclusions in 100Q (few in 46Q); dystrophic neurites observed</td>
<td>V</td>
</tr>
<tr>
<td>Exon 1</td>
<td>Tet-off (CamKII-tTA) promoter</td>
<td>94Q</td>
<td>94Q: 2.5 months in 50% abnormal gait</td>
<td>Not observed</td>
</tr>
<tr>
<td><strong>Targeted models</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Knock-in</td>
<td>Endogenous mouse HD gene promoter (Hdh prom)</td>
<td>111Q, 92Q or 50Q</td>
<td>Cellular Phenotype nQ20/nQ111 show phenotype similar to Exon 1 144Q mice</td>
<td>VII</td>
</tr>
<tr>
<td>Knock-in neo and Hdh promoter</td>
<td>111Q or 20Q</td>
<td>HdhQ111Q111: +2 months</td>
<td>Age-dependent Htt nuclear re-localization at 1.5 months, inclusion &gt;6 months</td>
<td>VII</td>
</tr>
<tr>
<td>Knock-in Hdh promoter</td>
<td>80Q or 72Q</td>
<td>No movement disorder</td>
<td>None observed</td>
<td>VIII</td>
</tr>
<tr>
<td>Knock-in Hdh promoter</td>
<td>94Q or 71Q</td>
<td>No movement disorder</td>
<td>None observed</td>
<td>IX</td>
</tr>
<tr>
<td>Knock-in Hdh promoter</td>
<td>77Q</td>
<td>No symptoms</td>
<td>None observed</td>
<td>X</td>
</tr>
<tr>
<td>Knock-in Hdh promoter</td>
<td>150Q</td>
<td>60 weeks</td>
<td>Clasping gait deficit 25 weeks Inclusions in striatum</td>
<td>XI</td>
</tr>
</tbody>
</table>
3. **Drosophila as a Model System and its Potential**

Although mice and other mammalian model systems present extensive similarity to Humans and offer several genetic and genomic tools, the length in time and cost necessary to conduct experiments with those models, along with the extensive ethical requirements needed, highly disfavors its use when compared to fly models.

Several arguments are in favor of using *Drosophila* as a model system. First of all, *Drosophila*’s genome is already sequenced and most of the genetic pathways involved in normal development and disease conditions are conserved between *Drosophila* and mammals. Additionally, there are many powerful genetic tools available that allow, among other things, to screen for genetic modifiers. The identification of modifier genes of some of the characteristic features of certain disorders also provides potential pharmacologic targets. These types of screens are also possible because flies are relatively inexpensive, easy to manipulate and allow studies in a relatively short time frame. This is due to a relatively short life cycle and to a large generation of progeny (Figure 7). Therefore, the number of individuals that can be tested simultaneously is greater than the ones possible in experiments with other animal models such as mice. Furthermore, as mentioned before, there are many genetic tools available to manipulate gene expression and there is a great knowledge of the anatomy and the distinct phenotypes in the flies. Therefore, *Drosophila* is emerging as a model system with broad applications and numerous tools available to address both mechanistic questions and also to test therapeutic options.
Together, these aspects make flies a powerful and flexible model to study the function of a particular gene and, more importantly, to study Human disease genes and mechanisms of disease.

The first draft of the *Drosophila melanogaster* genome was released in 2000 (Adams et al., 2000; see also Rubin et al., 2000), and one of the most surprising discoveries that were found was the similarities to the Human genome. Since then, several groups have tried to fully complete the sequence and to annotate the information that it contains, as well as to extrapolate about function of many genes. Other groups are also devoting their effort to develop tools that explore the information on the fly’s transcriptome and other genomic tools (De Gregorio et al., 2001; Furlong et al., 2001; Giot et al., 2003; McDonald and Rosbash, 2001).

Historically, *Drosophila* is mainly known for its potential as a model for classical genetics studies (Rubin and Lewis, 2000), but the emergence of a vast array of genetic tools has broaden this concept. One of those tools is the possibility to generate transgenic animals. As mentioned earlier in section 2.1 of this Introduction, transgenic animals can be generated making use of the GAL4/UAS system described by Brand and Perrimon (Brand and Perrimon, 1993; Figure 8). This system allows targeted, spatially and temporally, expression of the gene of interest. Depending on position at which a specific transgene is inserted on the fly’s genome, different phenotypes can be observed that usually result from different expression levels of the transgene. Different transgene insertion sites are expressed at higher or lower expression levels due to surrounding chromatin sequences.
Furthermore, the expression levels can also be influenced by temperature.

**Figure 8** - The Gal4/UAS system. Using this system, a transgenic line containing a tissue-specific promoter that drives the yeast Gal4 transcription activator (driver) can be crossed to another fly line that contains the gene of interest, here represented by Gene X, under the control of the Gal4 responsive Upstream Activating Sequence (UAS) promoter. Interestingly, this system allows generating and maintaining over the generations flies that carry a gene that is lethal when over-expressed.

In the past years, several methods have been developed to conduct forward and/or reverse genetic screens based on gain of function and loss of function approaches (Brand and Perrimon, 1993; Rorth, 1996; Rorth et al., 1998; Rubin and Spradling, 1982; Spradling and Rubin, 1982; Spradling et al., 1999).

Forward Genetic Screens are more molecularly oriented than Reverse Genetic Screens. Forward genetic screens are designed to elucidate gene functions and interactions on the basis of their mutant phenotypes. These are usually based on traditional phenotypic or functional basis for identifying interesting mutants. Such mutants might be used to analyze a particular biological process or particular disease-induced phenotypes. However, forward genetic screens present some limitations as they only allow the evaluation of gene functions that are associated with easily measurable or visible phenotypes.

Reverse Genetics, on the other hand, are designed to unravel the function of a particular gene. In order to achieve that, mutations within the gene are induced and only then the mutant phenotype is analyzed. Reverse
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Genetics may use undirected (random mutagenesis) or directed (that allow specific inactivation of a gene) techniques. Once the gene is disrupted it is functionally characterized, and normal and mutant forms of the proteins or the mammalian counterparts are overexpressed in transgenic animals to assess its effects (Fortini and Bonini, 2000; Nairz et al., 2004).

The most powerful technique developed to perform the described genetic screens is transgenesis with mobile elements (disruption, overexpression or mis-expression of specific genes to create distinct expression patterns). The main advantage of this method compared to classical chemical methods of mutagenesis is the possibility to identify the targeted gene because molecular tags, like the white gene, can also be inserted. P-elements were the first mobile elements for germ-line transformation, and are still the most used system in Drosophila.

The forward and/or reverse genetic screens mentioned above are usually achieved by making use of these P-elements and EP vectors. P-elements are genetically engineered Drosophila transposable elements and are currently used for insertional mutagenesis, enhancer trap studies, targeted overexpression, RNA interference (RNAi) and homologous recombination, since they can be easily inserted into the fly’s genome (Rubin and Spradling, 1982; Spradling and Rubin, 1982; Spradling et al., 1999). The EP vectors derive from P-elements. EPs contain multiple binding sites for the yeast transcription activator Gal4 and, like P-elements, are introduced randomly in the genome (Rorth, 1996; Rorth et al., 1998). When the insertion of an EP vector is near a gene (preferably at the beginning of the gene), the expression of Gal4 induces overexpression of that gene (Figure 9). On the contrary, if the insertion of a P-element is within the gene sequence, it will induce gene disruption. However, this last possibility is very rare.

Even though P-elements are extremely powerful and flexible, they do show some limitations, such as the insertion site specificity. P-elements are preferentially inserted in the 5'-UTR region of the Drosophila genes, have a tendency for particular sequence motifs (AT-rich) and regions where other P-elements are inserted are preferential targets as well. Therefore, there are regions in the genome denominated as “hot spots”, were P-elements seem to
have a clear preference for insertion in particular genes, as well as “cold spots” in which P-elements have a strong bias against insertion in those particular genes, resulting in genes that do not have a single P-element inserted.

Figure 9 – Schematic view of the genetic screening process (top panel) and the loss of function and gain of function types of screen (bottom panels). The loss of function and gain of function screens make use of P- and EP elements, respectively (Modified from Rorth, 1996).

This limitation makes it difficult to generate insertions in some regions of the genome, and thus new alternative transposable elements with different site specificity or even no specificity at all are needed (Bonin and Mann, 2004; Ryder and Russell, 2003). In order to overcome part of these problems, Minos and piggyBac, among others, elements were generated (Bonin and Mann, 2004; Horn et al., 2003; Metaxakis et al., 2005; Thibault et
al., 2004). Of these, *piggyBac* elements are particularly important and a Gene Disruption Project allowed the creation of many new mutant lines (Figure 10; Thibault et al., 2004). These *piggyBac*’s are distinct from traditional P-elements as they do not show the same bias for insertion in 5’-UTR regions as P-elements and, therefore, are more effective. Furthermore, germ line *piggyBac* excisions are almost always precise, a characteristic not observed with P-elements.

The development of other techniques that allow gene silencing in *Drosophila* have also revealed to be an import tool (see for example Dietzl et al., 2007; Giordano et al., 2002; Kennerdell and Carthew, 1998; Kennerdell and Carthew, 2000; Lam and Thummel, 2000; Rong and Golic, 2000; Rong and Golic, 2001). Platform screening processes based on the combination of different techniques, like RNAi and small-molecule screens, are also being developed providing additional resources (Perrimon et al., 2007).

### 3.1. Models of Human Neurodegenerative Diseases in *Drosophila*

In the recent years, *Drosophila* has been widely used as a model system...
to study many Human diseases (see for example Bier, 2005; Bilen and Bonini, 2005; Celotto and Palladino, 2005; Fortini and Bonini, 2000; Marsh and Thompson, 2006; Michno et al., 2005; Sang and Jackson, 2005). As mentioned before, the comparison between Human and fly genomes has revealed strong evolutionary conservation both at structural and functional levels. Fly homologues of Human disease-associated genes have been identified for many disorders, including cancer and neurological disorders (Chien et al., 2002; Reiter et al., 2001). In fact, approximately 75% of the Human disease genes have a *Drosophila* homologue. This emphasizes the potential of using *Drosophila* as a model system to study mechanisms of disease and also for drug discovery (Mackay and Anholt, 2006; Manev et al., 2003; Marsh and Thompson, 2004; Tickoo and Russell, 2002). Also, the main aspects of cell biology, including gene expression regulation, membrane trafficking, cytoskeleton, neuronal connectivity, synaptogenesis, cell signaling and cell death, among others, are analogous in flies and Humans. Furthermore, *Drosophila* has a complex nervous system and brain with specialized functions such as vision, olfaction, learning and memory that can be used to study neurological disorders (Marsh et al., 2003). Together with the increasing number of unique genetic, proteomic and genomic tools, this makes *Drosophila* an excellent model to study Human diseases.

Modeling diseases in *Drosophila* is usually achieved by making use of the GAL4/UAS system described in the previous section (Figure 8), which allows the generation of transgenic flies that expressed the Human disease-causing protein. These transgenic flies also contain a tissue-specific promoter that is fused to the yeast GAL transcription factor. When these flies are crossed to flies that contain the gene of interest fused with to the yeast UAS, the offspring will express the disease-causing gene in specific tissues and in a controlled manner. Usually the expression of a specific gene of interest is driven to neurons (for example, using the *elav* or *nervana* drivers) or to all cells in the eye (using, for example the *GMR* driver). By making use of these type of drivers, toxicity can be evaluated, for example, by measuring loss of photoreceptor neurons in the eye, lethality of organism or behavioral
phenotypes (Marsh and Thompson, 2006). Even though these are the most common assays used, other methods can be applied.

**Figure 11 – How can flies help to study Human diseases?** After the disease-causing gene has been identified, two strategies might be adopted. After the fly orthologue of the Human disease-causing gene has been identified, its normal function can be studied, either by knocking it out or by overexpressing it in the flies. An alternative way is to generate transgenic flies that express the Human gene responsible for the disease. The phenotypes observed, as a result of the expression/knock-out of the proteins, can be used to extensively study its function and possible mechanisms underlying the development of the disease phenotypes. In addition, the models can be used to perform genetic screens and identify modifiers (enhancers and suppressors) of the disease phenotypes. The models can ultimately be used to screen for drug candidates that modulate the disease phenotypes that can, in the future, be tested in higher vertebrate models (Adapted from Michno et al., 2005).
In this context, *Drosophila* models of many neurodegenerative diseases have been developed, including polyglutamine diseases (example SCA1, HD and SCA3), tauopathies, Alzheimer’s disease, Parkinson’s disease or even non-coding triplet-repeat diseases (example Fragile X, DM1 and SCA8). Of these, many have been used to conduct genetic modifier screens that allow the identification of genetically interacting proteins (and thus pathways) that are able to modify the disease phenotypes. These types of screens have revealed to be a very useful strategy to identify pathogenic molecular mechanisms. For example, the relevance of heat-shock proteins/chaperones, protein degradation machinery and transcription regulation for polyglutamine diseases and the importance of kinases and phosphatases for the case of tauopathies were revealed (or confirmed) by genetic screens (Fernandez-Funez et al., 2000; Kazemi-Esfarjani and Benzer, 2000; Shulman and Feany, 2003). Typically this type of genetic screen is achieved by inducing the expression of the transgene of interest using an eye specific driver. The eye has revealed to be an excellent system to express these genes for several reasons:

1) The eye is a very sensitive organ and as a consequence, expression of a toxic protein generates a rough appearance that can be easily observed and, therefore phenotypes can be rapidly identified and studied.

2) The eye is not an essential tissue and, therefore, even though the expressed protein might be toxic, the flies are still viable (for most of the cases).

3) The photoreceptor cells in the eye are highly organized into ommatidia and so, quantitative measurements of cell loss can be achieved by counting the number of photoreceptor cells per ommatidia.

After an initial screening process using the eye assay, additional and more specific assays are usually done in order to further confirm the results obtained.

Besides the significant contribution to identify novel interactions and pathways, *Drosophila* is also extremely important tool to test specific hypothesis that result from preliminary genetic or pharmacologic screens or hypothesis that arise from studies done with other model
systems/organisms. Hence, the use of *Drosophila* in pharmacologic screens is becoming a valuable strategy (reviewed in Celotto and Palladino, 2005; Marsh and Thompson, 2006).

**Table 6 – Examples of Some Neuroprotective Genes and Compounds Identified Using *Drosophila* Models of Disease**

<table>
<thead>
<tr>
<th>Gene or Treatment</th>
<th>Effect/Mode of Therapy</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ataxin-3</td>
<td>Ubiquitin protease activity</td>
<td>I</td>
</tr>
<tr>
<td>C2-8</td>
<td>Reduce PolyQ aggregation</td>
<td>II</td>
</tr>
<tr>
<td>C4 sFv intrabodies</td>
<td>Reduce PolyQ aggregation</td>
<td>III</td>
</tr>
<tr>
<td>CREB-binding protein</td>
<td>Recovery of histone acetylation</td>
<td>IV</td>
</tr>
<tr>
<td>dHdj (HSP40)</td>
<td>Chaperone activity</td>
<td>V</td>
</tr>
<tr>
<td>dTPR2</td>
<td>Chaperone activity</td>
<td>V</td>
</tr>
<tr>
<td>Drobl-1</td>
<td>Mitochondrial homeostasis</td>
<td>VI</td>
</tr>
<tr>
<td>Geldanamycin</td>
<td>Hsp90 inhibitor</td>
<td>VII, VIII</td>
</tr>
<tr>
<td>Glutathione S-transferase</td>
<td>Suppressor of parkin pathology</td>
<td>IX</td>
</tr>
<tr>
<td>Hsp70</td>
<td>Chaperone activity</td>
<td>X, XI</td>
</tr>
<tr>
<td>SAHA</td>
<td>Histone deacetylase inhibitor</td>
<td>VIII</td>
</tr>
<tr>
<td>SUMO-1</td>
<td>Reduce PolyQ aggregation</td>
<td>XII</td>
</tr>
<tr>
<td>Suppressor peptides (3-14 + 3-17)</td>
<td>Supress ND in PolyQ</td>
<td>XIII</td>
</tr>
<tr>
<td>Y-27632</td>
<td>Inhibitits Rho-associated kinase</td>
<td>XIV</td>
</tr>
</tbody>
</table>

Adapted from (Celotto and Palladino, 2005). I (Warrick et al., 2005); II (Zhang et al., 2005); III (Wolfgang et al., 2005); IV (Steffan et al., 2001); V (Kazemi-Esfarjani and Benzer, 2000); VI (Senoo-Matsuda et al., 2005); VII (Auluck and Bonini, 2002); VIII (Agrawal et al., 2005); IX (Whitworth et al., 2005); X (Auluck et al., 2002); XI (Warrick et al., 1999); XII (Steffan et al., 2004); XIII (Ravikumar et al., 2004); XIV (Kazantsev et al., 2002); XV (Pollitt et al., 2003). HD, Huntington’s Disease; PD, Parkinson’s Disease; SCA3, Spinocerebellar Ataxia Type 3

### 3.1.1. Description of the models generated in the Botas laboratory

In order to gain insight into polyglutamine-induced neurodegeneration, *Drosophila* models of SCA1 and HD were generated in the Botas laboratory by expressing either normal or expanded Human *SCA1* or *Htt* transgenes (Figure 12). SCA1 transgenic flies express Human full-length Ataxin-1 encoding a polyglutamine tract of either 82 or 30 glutamines (Fernandez-Funez et al., 2000), whereas HD flies express the N-terminal portion of the Huntingtin protein (amino acids 1-336) including an expanded tract of 128...
glutamine repeats (Fernandez-Funez, Rincón-Limas and Botas, unpublished work; Branco et al., 2007; Kaltenbach et al., 2007). Transgenic flies that express the same N-Terminal fraction of the Huntingtin protein containing either 44 or 16 glutamines were also generated.

Figure 12 – Schematic representation of the SCA1 82Q and Htt128Q constructs. Top panel: A human SCA1 transgene encoding the full-length Ataxin-1 protein containing 82 glutamine repeats was used to generate the SCA1 Drosophila model. A similar transgene was generated encoding Ataxin-1 with 30 glutamines (data not shown). Bottom panel: An N-terminal fragment of the human Huntingtin cDNA encoding the first 336 amino acids of the protein (including 128 glutamine repeats) was used to generate the HD Drosophila model. Similar transgenes were also generated with 16 and 44 glutamines, but these do not show toxicity (data not shown). In all cases the transgenes were cloned downstream of UAS binding sites to confer regulation by the GAL4 transcriptional activator (Adapted from Branco et al., 2007).

In both SCA1 and HD models, overexpression of the expanded proteins in the eye leads to a phenotype characterized by degeneration and increased disorganization of the ommatidia and inter-ommatidial bristles that can be used to screen for interactors/genetic modifiers (Figure 13). Unlike flies expressing SCA1 82Q, that display a severe rough external eye phenotype (Figures 13. B, E and H; respective controls are shown in Figures 13.A, D and G; (Fernandez-Funez et al., 2000)), the N-terminal HD model flies show mild ommatidial disorganization and bristle loss phenotypes (Figures 13.C and F). However, longitudinal sections show severe disorganization, tissue loss and vacuolization in the retina of these flies (Figure 13.I). This effect is even more dramatic in aged flies, demonstrating that the degenerative phenotype is progressive (Figure 14).
Figure 13 - Expression of either Ataxin-1\textsuperscript{82Q} or Htt\textsuperscript{128Q} in *Drosophila* eye cells causes a degenerative phenotype. Shown are Light Microscope eye pictures (A-C), Scanning Electron Microscopy (SEM) eye pictures (D-F) and paraffin retinal sections (G-I). Wild type (control) flies show a highly organized pattern of the ommatidia and inter-ommatidial bristles, evident both in Light Microscope and SEM eye images (A and D, respectively). At the retinal level, these flies also display normal organization of cells in the eye (G). Overexpression of the SCA1\textsuperscript{82Q} transgene (B, E and H) induces a severe disruption of the external lattice of the eye accompanied by a dramatic reduction of the length of the retina (compare to the controls in A, D and G, respectively). In the Light Microscope eye pictures necrosis is also observed (black spots), indicating that cells are dieing (B). In the SEM image (E) it is evident the increased disorganization in the arrangement of ommatidia and inter-ommatidial bristles, along with cell loss. At the retinal level, note the drastic decrease in the length of the retina of these flies (H). Expression of the Htt\textsuperscript{128Q} transgene induces only a very mild external eye phenotype (C and F). In Light Microscope eye pictures we can observed a mild de-pigmentation of the cells (C), while in SEM images, abnormal organization of the inter-ommatidial bristles is detected (F). However, at the retinal level (I), expression of the Htt\textsuperscript{128Q} transgene causes shortening and disorganization of the retina, as well as a dramatic tissue loss. Genotypes: A, D, G) w;GMR-GAL4/UAS-GFP. B, E, H) yw, UAS-SCA1\textsuperscript{82Q}[F7]/+;GMR-GAL4/UAS-GFP. C, F, I) w; GMR-GAL4/ UAS-GFP; Htt\textsuperscript{128Q}[M64]/+. All flies shown are 6 days old except for (B, E and H) that are 1 day old. Scale bar in SEM pictures is equivalent to 100\textmu M (10\textmu M in the magnification insets) and equivalent to 10\textmu M in the retinal paraffin sections (Adapted from Branco et al., 2007).
Figure 14 – Expression of either Htt$^{128Q}$ in *Drosophila* eye cells causes shortening of the retina as well as tissue loss. These phenotypes become more evident and severe as the flies age (compare B and C), reproducing the progressiveness of the disease in this fly model. Genotype: w; GMR-GAL4/ UAS-GFP; Htt$^{128Q}$[M64]/+. Flies shown in panel A and C are six days old and one day old in (B). Scale bar is equivalent to 10µM (Adapted from Branco et al., 2007).

Furthermore, the models developed reproduce many of the cellular phenotypes observed in SCA1 and HD patients including the formation of nuclear inclusions and the progressive neuronal degeneration (Figures 14, 15 and 16). These data suggests that the mechanisms of pathogenesis in polyglutamine-repeat diseases are conserved between *Drosophila* and Humans.

Figure 15 – Expression of Ataxin-1$^{82Q}$ in *Drosophila* neurons causes progressive degeneration. Apterous-GAL4 (*apVNC-GAL4*) was used to express the expanded protein (red) and the τ-GFP reporter gene (green) in the adult CNS. a, Confocal image of cells in the CNS of a 45-day-old control fly shows normal axonal projections of interneurons. b, Magnification of image a showing ventral interneurons and their axonal projections. c, d, Magnification images of 1-day-old (c) and 45-day-old (d) SCA1$^{82Q}$ flies. In red, Ataxin-1$^{82Q}$ NIs are shown. While most cell bodies are
present at Day 1, by Day 45, much fewer cells are detected. Note that for SCA182Q-expressing flies the signal for axonal projections is weak since Day 1 (From Fernandez-Funez et al., 2000).

Figure 16 – Expression of Htt128Q in Drosophila promotes its aggregation into NIs. Confocal images of Drosophila neurons stained with anti-Huntingtin antibody. Images show high magnification views of adult ventral ganglion neurons in which Htt128Q and UAS-CD8:GFP transgenes are expressed with the OK107-GAL4 driver. UAS-CD8:GFP labels the neuronal cytoplasm (green, panel A) and the Htt signal, shown in red, was detected using anti-Htt antibody (B). Confocal images reveal accumulation of Htt in the nucleus and low levels of Htt in the cytoplasm (B). Large aggregates are only found in the nucleus. (C) Merged image of panels (A) and (B). Genotype: w; FRTG13, UAS-CD8:GFP/UAS-LacZ; Htt128Q[M64]/+; OK107-GAL4/+ Flies are 3 days old. Scale bar is equivalent to 10µM (Adapted from Branco et al., 2007).

The study by Botas and colleagues also revealed that the NIs observed in the SCA1 model are also positive for the Hsp70 chaperone, ubiquitin and proteasome components, in a similar manner to what is observed in patients (Figure 17; Fernandez-Funez et al., 2000).

Another interesting observation by Botas and colleagues was that when wild type Ataxin-1 protein (with 30 glutamine repeats, Ataxin-130Q) is expressed at sufficiently high levels, a degenerative phenotype is observed. This phenotype is similar to what it is observed when expanded Ataxin-1 (Ataxin-182Q) is expressed (Fernandez-Funez et al., 2000). The finding that even the wild type protein, when expressed at high levels, is sufficient to induce a degenerative phenotype was unexpected and suggested that Ataxin-1 toxicity might be a result of an excess of the protein.
As mentioned before, the fly eye is an excellent system to study numerous cellular processes. Therefore, using an eye assay as the main assay, where the integrity of the ommatidia is analyzed, two large genetic screens were designed to identify genes that modify the SCA1 neurodegenerative phenotype in the eye.

The first screen yielded modifiers of the SCA1 phenotype when gene activity was decreased and the second screen yielded SCA1 modifiers when gene activity was increased. Both suppressors and enhancers of the neurodegenerative phenotype were obtained from each screen.

Some of the modifiers identified in the screen highlighted the role of protein folding and protein clearance in the development of SCA1 pathogenesis, mechanisms previously known to be involved in polyglutamine-induced neurodegeneration. However, new mechanisms of polyglutamine pathogenesis were also revealed by the discovery of modifiers that are involved in RNA processing, transcriptional regulation and cellular detoxification (Fernandez-Funez et al., 2000). Interestingly, suppressors
were also identified either with no homology to any known protein and others whose identity was unclear.

The HD model generated by Botas land collaborators has also been used, more recently, to identify potential Htt interactors (Kaltenbach et al., 2007). The study used two complementary approaches, a High-Throughput Yeast Two-Hybrid Screening Assay and Affinity Pull-Down Assays followed by Mass Spectrometry, in order to identify Htt interactors. From the 234 Htt-associated proteins found using both methods, a group of 60 genes that encoded some of the interacting proteins found were tested in the HD model developed in the Botas laboratory in order to evaluate if they behaved as genetic modifiers of the degenerative phenotypes characteristic of our *Drosophila* model. The study highlighted the importance of proteins implicated in synaptic transmission, cytoskeletal organization, signal transduction and transcription for the pathogenesis of HD. It also emphasizes that some of the loss of function suppressors of toxicity identified can be putative pharmacological targets.

4. **AIM OF THE STUDY**

Towards differentiating between common and specific mechanisms among polyglutamine diseases, we performed a comparative genetic study using known SCA1 genetic modifiers as well as additional modifiers identified meanwhile, and analyzing its effects in HD. In order to achieve this aim, *Drosophila* was chosen as our model system. The eye assay, where the integrity of the photoreceptor cells of the eye is evaluated, was the primary method chosen to perform the comparative analysis. We have further analyzed the effect of some of the genetic modifiers found using additional assays to evaluate their effect at the neuronal level. Those assays were the Climbing Assay, in which the motor performance of the flies as a function of age is measured in a simple climbing test of negative geotaxis, and an Aggregation/Cell Viability Assay where the percentage of cells that contain nuclear aggregates is scored.
5. **Rationale and Significance**

Comparative analysis of genetic modifiers of disease using animal models is a powerful approach to identify common and distinct mechanisms of pathogenesis exerted by different polyglutamine proteins. *Drosophila* is well suited for this type of analysis because of the surprising similarity of its genome and the Human genome (Chien et al., 2002; Reiter et al., 2001), the ability to manipulate up or down the activity of most of its genes and the fast pace of experimentation. To date, only a small number of genes have been compared in fly models of neurodegenerative diseases (Ghosh and Feany, 2004). Together, these observations support the need of a broad study to identify mechanisms that mediate cell degeneration in different polyglutamine diseases. The knowledge of the specific mechanisms involved in each disorder and their relevance to the disease pathology will possibly allow the development of effective therapies.

We have taken advantage of *Drosophila* models of HD and SCA1 to compare genetic modifiers and mechanisms of pathogenesis of expanded Ataxin-1 and Huntingtin toxicity. In order to achieve that aim we have tested previously known *SCA1*82Q genetic modifiers (Fernandez-Funez et al., 2000) and some additional genes in the HD model generated in our laboratory.

As a result of our comparative genetic screen we found modifier genes that are common to the SCA1 and HD models and do not belong to the protein folding/ degradation machineries. Other modifier genes have disease model specific effects. Of these, a group modulates disease progression in both SCA1 and HD *Drosophila* models, but in opposite ways. This group of disease-specific modifier genes is particularly interesting as it may account for the exclusive mechanisms of pathogenesis in each disease. We have also found that the phenotypical modifications observed are not cell-type specific, since the results were comparable using both eye and behavioral assays.

Finally we have studied the formation of NIs. Our results suggest that polyglutamine toxicity is modulated with no direct correlation on the formation of aggregates.
Our results point to potential common therapeutic targets in novel pathways, and also to genes and pathways responsible for differences between Ataxin-1 and Huntingtin-induced neurodegeneration. Even though more studies will be needed to further exploit the exact mechanisms that are mediating the modulation of the disease phenotypes, the work developed during this PhD thesis represents the largest comparative screen performed to date and identifies some cellular pathways not previously implicated in, at least, one of the two disorders.
II. MATERIALS AND METHODS
Fly strains and constructs

SCA1^{82Q} and SCA1^{10Q} lines were previously described (Fernandez-Funez et al., 2000). Htt^{128Q} flies were generated by sub-cloning a portion of N-terminal of the Human Huntingtin gene (amino acids 1-336, including 128 glutamine repeats) into the Drosophila transformation vector pUAST (Brand and Perrimon, 1993). Transgenic flies were obtained following standard procedures for embryonic germ line injection and transformation. Similar transgenes were also generated with 16 and 44 glutamines.

Flies were maintained on standard cornmeal medium. Crosses were performed at 25°C or 27°C in order to induce medium or high expression levels of the transgenes. In this study, the GMR-Gal4, nervana-Gal4 (both obtained from Bloomington Stock Center; Indiana University, IN, USA) and OK107-Gal4 drivers (w; FRTG13, UAS-CD8: GFP;OK107-Gal4 – a gift from Dr. Hugo Bellen) were used.

All stocks used were obtained from the Bloomington Stock Center (Indiana University, IN, U.S.A.) with the exception of the following stocks: obtained from Exelixis (EP(2)2417, EP(3)3623, EP(3)651, EP(3)531, EP(X)1438, EP(3)411, EP(3)674, EP(X)1303, EP(2)866, EP(3)3375, EP(2)2039, EP(2)2300, EP(3)3463, EP(3)3378, EP(3)3461, EP(X)1412 and EP(X)1357) or from private collections: UAS-dAkt1, UAS-DPi3K^{92E} wt, UAS-14.3.3^{ex4} and 14-3-3-{e}^{ex4} (Chen et al., 2003); ps^{3} and ps^{449} were a gift from Dr. Deborah Andrew (Seshaiah et al., 2001; ). dAtx2 was kindly provided by Dr. L. Pallanck (Satterfield et al., 2002) and dIAP1 by Dr. Bruce Hay. For the studies with the Imd Pathway, the following stocks were kindly provided: UAS-PGRP-LCa, UAS-PGRP-LCx were a gift from Dr. Anderson (Choe et al., 2002) and DD1;PGRP-LC^{7454} and PGRP-LC^{612}, were provided by Dr. Royet (Gottar et al., 2002). The RNAi constructs αFADD-2, αFADD-3, αFADD-4, dTAK1-5, dTAK1-7 and dTAK1-9 were a gift from Dr. Ryu Ueda and Dr. Bruno Lemaitre (Leulier et al., 2002; Vidal et al., 2001). Additional mouse and Drosophila TAK1 alleles were kindly provided by Dr. Ueno (Takatsu et al., 2000). The UAS-DREDD constructs (UAS-DREDD-FL10, UAS-DREDD-FL12 and UAS-DREDD-FL11) were a gift from Dr. Bruno Lemaitre (Vidal et al., 2001); and the Relish UAS construct and imprecise excisions (Rel^{E20}, Rel^{E21}, Rel^{E23}, Rel^{E18}) were a gift from Dr. Hultmark (Hedengren et al., 1999).
Scanning Electron Microscopy (SEM)

Flies collected on the day of eclosion were serially dehydrated in ethanol, critical point dried, metal coated and analyzed in a Jeol JSM 6100 microscope.

Retinal paraffin sections

Adult fly heads were dissected, fixed in 4% formaldehyde overnight, dehydrated in serial dilutions of ethanol and embedded in Paraplast Plus paraffin. 10µm sections were obtained using an Olympus Cut4055 microtome and afterwards re-hydrated. Sections were stained with hematoxilin (Sigma), dehydrated and mounted using Permount mounting media (Fischer Chemicals). Samples were visualized in a Nikon Microphot-FXA microscope (Nikon) and the tissue morphology was analyzed. Images were obtained using the AxionCam MRc camera from Zeiss.

Climbing Assay

SCA1^{82Q} and Htt^{128Q} flies were crossed with nervana-GAL4 driver and adult females were collected for periods no longer than 24h. Their climbing ability was analyzed throughout their lifespan. Flies were transferred to vials containing new food everyday. The climbing performance is determined by counting the number of flies that climb above a 5cm line in 18 seconds, after being tapped to the bottom of an empty culture vial. This procedure was repeated ten times for each day in which the experiment was performed, and an average of the percentage of flies climbing per day was calculated and plotted using Microsoft Excel. The groups of thirty virgin female flies were kept in individual vials and their climbing performance was recorded, at least, every two days. A minimum of two replicas per genotype was tested. The experiments were performed approximately at the same time in the day to avoid the interference of circadian rhythm effects with the results.
Immunofluorescence

Adult ventral ganglions of aged flies were dissected in Phosphate-buffered Saline (PBS), fixed in 4% formaldehyde for thirty minutes and stained overnight with anti-Ataxin-1 (ata1 11NQ V, 1:750; a gift from Dr. Huda Zoghbi) or anti-Huntingtin (MAB5374, 1:100; Chemicon) antibodies. The secondary antibodies (anti-rabbit IgG conjugated with Cy3 and anti-mouse Texas Red, Jackson Laboratories and Molecular Probes) were used at a 1:200 dilution. Antibodies were diluted in PBS, 2% BSA. Samples were mounted using Vectashield mounting media (Vector Laboratories), visualized in a LSM510 Zeiss confocal microscope and analyzed using Image J software. Results from the quantifications done were calculated and plotted using Excel software and statistically analyzed by Student’s t-test.

Larval brain and ventral ganglion were dissected in the same conditions as described above and stained with anti-mub (Mub-pep1 (A1008a); 1:50). The secondary antibody (anti-mouse Texas Red, Molecular Probes) was used at a 1:200 dilution. Antibodies were diluted in PBS, 2% BSA. Samples were mounted using Vectashield mounting media (Vector Laboratories), visualized in a Nikon Microphot-FXA microscope (Nikon) and images obtained using an AxionCam MRc camera from Zeiss.

X-GAL Staining

Third instar larval ventral ganglions were dissected in PBS, fixed in 4% formaldehyde for fifteen minutes and then washed with PBS and stained overnight at 37°C with X-GAL solution (10 mM Na2HPO4/NaH2PO4 [pH 7.2], 150 mM NaCl, 1 mM MgCl2, 3 mM K4[FeII(CN)6], 3 mM K3[FeIII(CN)6] containing a 1/50 dilution of X-Gal (25 mg/ml in dimethyl formamide)). Samples were mounted using Vectashield mounting media (Vector Laboratories) and visualized in a phase contrast microscope (Nikon Microphot-FXA). Images were obtained using the AxionCam MRc camera from Zeiss.
Protein extraction and Western Blot assay

Western Blot (WB) assays were performed using 4 adult heads from each genotype tested. The tissue was homogenized in 30µl of Laemnli buffer (BioRad) using a motor pestle (Kontes). Samples were then heated at 95°C for 10 minutes, incubated on ice for 5 minutes and loaded in a pre-cast 7.5% (For Ataxin-1 WB assays) or 10% (for GFP WB experiments) Tris-HCl gel (Ready Gel, BioRad). Proteins were transferred to a nitrocellulose membrane (0.45µm, BioRad) at 200mA for 2 hours using a wet Trans-Blot Transfer Cell (BioRad). Membranes were blocked (PBS-0.05%Tween, 5%milk) overnight at 4°C. Antibodies were diluted in PBS, 1% BSA at the following final concentrations: anti-ata1 11NQ V (a gift from Dr. Huda Zoghbi), 1:1500; anti-tubulin (Hybridoma Bank), 1:2000; anti-GFP (Molecular Probes), 1:5000. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:5000, BioRad) antibodies were used as secondary antibodies. Signal was detected using chemiluminescence (ECL kit, Amersham). Protein expression levels were quantified using ImageQuant5.2 (Personal Densitometer SI, Molecular Dynamics). Values were normalized to the levels of an internal control (Tubulin or GFP). Both Western Blot and RT-PCR (see bellow) experiments were performed at a moderate temperature (25°C), and thus medium expression levels, to avoid reduction of Ataxin-1 levels due to an increase in degeneration and cell death.

RNA extraction and semi-quantitative RT-PCR

Trizol reagent (Invitrogen) was used to isolate total RNA (following the manufacture’s instructions) from adult fly heads. The RNA was then treated with RNase-free DNAsel I (15 minutes at room temperature, Invitrogen) and reverse transcribed using oligo(dT)s and SuperScript III First Strand cDNA Synthesis (Invitrogen). The PCR reaction was performed in MasterCycler Gradient equipment (Eppendorf) using Platinum Taq Polimerase (Invitrogen). PCR products were resolved in a 2% agarose gel and visualized in BioRad equipment. The SCA1 and rp49 (internal control) transcripts were amplified using the following primers: SCA1 Forward: 5'-GAG CTA AAG AGT GTG GAA GAC TTA AA-3'; SCA1 Reverse: 5'-CTC CAG TTA AA-3';
rp49 Forward: 5’-GAC GCT TCA AGG GAC AGT ATC TG-3’; rp49 Reverse: 5’-AAA CGC GGT TCT GCA TGA G-3’.
III. RESULTS AND DISCUSSION
The work developed during this PhD work was preceded by the publication of a list of genetic modifiers of expanded SCA1-induced toxicity found in the Botas laboratory (Fernandez-Funez et al., 2000).

Thus, the first years of this thesis were devoted to explore and further characterize some of the genetic modifications observed in (Fernandez-Funez et al., 2000), as well as dedicated to the study of other new genetic interactors. Note, however, that throughout the whole work many genes/alleles of genes were tested, but because the alleles being tested had an eye or neuronal phenotype on their own or because no conclusive modification of the induced toxicity was observed, those results are not reported here.

This thesis will be, therefore, divided in three separate sections, Part 1, Part 2 and Part 3.

In Part 1 we will present the results obtained with the exploratory work done on a previously known SCA1 genetic modifier and on an additional modifier identified meanwhile. This includes a complete characterization of the eye phenotypes and modifications observed, and in some cases, it also includes some additional experiments performed in order to try to prove genetic interactions with possible interactors. An example of this is the work developed with ariadne-2 and the Imd pathway.

In Part 2, the results presented correspond to the main focus of this thesis and it includes the results published in

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Comparative Analysis of Genetic Modifiers in Drosophila Points to Common and Distinct Mechanisms of Pathogenesis among Polyglutamine Diseases


doi:10.1093/hmg/ddm315
This comparative work includes some of the genes identified in the previous screen done in the Botas laboratory (Fernandez-Funez et al., 2000), as well as other new genes tested meanwhile.

Finally, in Part 3, we present additional results regarding some of the genes tested in (Branco et al., 2007). In this section, we present complementary assays and/or approaches performed in order to explore the mechanisms underlying the modification of polyglutamine toxicity in flies by Nup44A and mub.
Part 1

Genetic analysis of modifiers of the SCA1\textsuperscript{82Q}-induced toxicity
As mentioned earlier, the work leading to this PhD thesis started with the analysis of genes previously published as SCA1 genetic modifiers of a Drosophila model of the disease (Fernandez-Funez et al., 2000). Meanwhile, during this thesis work other genes were identified as genetic modifiers of the expanded SCA1-induced eye phenotype (for example pasilla). In this section, a general introduction about these genes, the results obtained and a brief discussion regarding the study of those genes will be presented. Note that other genes were studied during this thesis work, but because only very preliminary data was obtained (e.g., the chorein Drosophila homologue, CG2093) or because the study of that particular gene was then followed by other members of the Botas laboratory (e.g. EP3139/CG14959, later identified as a micro-RNA mir282), those will not be included in this thesis.

**A. ariadne-2 (ari-2)**

A loss of function mutation in ari-2 (ari-2⁰⁰⁷⁶⁸ or ari-2⁰⁰⁰⁰⁸⁶⁶, resultant of a P element insertion in the ari-2 genomic sequence) was identified as a suppressor of Ataxin-1⁰⁸²Q-induced toxicity in the genetic screen performed by Botas and collaborators (unpublished work, Figure 18).

![Figure 18 - ari-2 genomic region and localization of the P-element insertion.](http://www.fruitfly.org/cgi-bin/annot/gbrowse?name=gene:CG5709)

Sequence homology analysis reveals that ari-2 might act as an E3 ubiquitin-protein ligase, or as part of the E3 complex. E3 ligases accept ubiquitin from specific E2 ubiquitin-conjugating enzymes and then transfers it to its target substrates. The substrate, usually an abnormal protein, once it
has been ubiquitinylated, is directed to degradation, via the 26S proteasome subunit, and thus cleared from the cell. This avoids its accumulation and/or aggregation, which ultimately could lead to pathogenesis and/or cell death (Figure 19).

**Figure 19 – The Ubiquitin Proteasome System (UPS).** When activated by ubiquitin-activating enzyme E1, Ubiquitin is then transferred to an ubiquitin-carrier protein, the E2 protein (also known as a ubiquitin-conjugating enzyme). From E2, it can be transferred directly to the substrate, which in turn is bound specifically to an ubiquitin ligase, the E3 protein (A). This occurs when the E3 belongs to the RING finger family of ligases. In the case of a homologous to the E6-AP C Terminus (HECT) domain-containing ligase (B), the activated ubiquitin is transferred first to the E3, and then transferred to the E3-bound target substrate. The ubiquitinated substrates are next directed to the 26S proteasome complex, where they are degraded into short peptides and free and reusable ubiquitin (From Ciechanover and Brundin, 2003).

The E3 ligases are the most diverse group of the three kinds of enzymes involved in the cascade of events leading to ubiquitination. Therefore, E3’s are also the main determinants of the specificity and regulation of the ubiquitination process.

Both *Drosophila ariadne-2* (*ari-2*) and *ariadne-1* (*ari-1*), a structurally related protein, have been shown to interact with ubiquitin-conjugating
enzymes. Note that even though these two proteins are structurally similar and, therefore, belong to the same family of proteins, they are functionally different (Aguilera et al., 2000).

A more detailed analysis of the structure of ariadne-2 reveals the presence of RING domains separated by an IBR (“in between RINGs”) domain. The group of proteins containing these domains is, therefore, designated as RBR (RING-IBR-RING or RING-Between rings-RING) family of proteins. Comparative genomics indicate that RBR proteins have multiple roles, from quality control mechanisms to indirect transcription regulators (reviewed in Marin and Ferrus, 2002). Interestingly, a mutation in parkin, also an E3 ligase containing a RBR domain, is responsible for the Familial Autosomal-Recessive Juvenile Form of Parkinson’s Disease. Since this discovery, a significant effort has been devoted to the study of the RBR group of proteins and it is now suggested that RBR proteins are ubiquitin-ligases that function not only as key elements in the machinery that controls protein quality in the cell but also as regulators of transcription factors such as p53 or the Androgen Receptor (Marin et al., 2004).

The Ubiquitin Proteasome System is responsible for the regulation of a vast array of cellular processes and as a consequence, has been implicated, either as the primary caused or as a secondary effect, in human disease states (reviewed in Ciechanover and Brundin, 2003). These include several neurodegenerative disorders such as PD, AD and HD, but also immune and inflammatory diseases. Typically, the disorders associated with UPS can be of two types: 1) The ones resulting from a loss of function/mutation in an ubiquitin-system enzyme or target substrate; 2) Those resulting from a gain of function of an enzyme/protein involved in the UPS. While in the first case, the result is stabilization of the specific protein/target substrate, in the second case, the alteration induces an increased rate of degradation of the target protein (Ciechanover and Brundin, 2003).

Therefore, in this context, the observation that the loss of function of a putative ubiquitin ligase was a suppressor of the SCA1^{82Q}-induced toxicity was surprising, and in order to further confirm this result, transgenic flies...
overexpressing *ari-2* were generated (*ari-2*OE). As shown in Figure 20, while *ari-2*of is an excellent suppressor of the SCA1 model eye phenotype (Figures 20.B and 20.G), *ari-2*OE is responsible for an enhancement the phenotype (Figures 20.D and 20.I). Note, however, that overexpression of *ari-2* does not induce formation of an eye phenotype when expressed alone (Figures 20.E and 20.J).

![Figure 20](image)

**Figure 20 – Loss of function of *ari-2* suppresses SCA182Q-induced eye phenotype.** Light Microscope eye images (A-E) and SEM images of the external eye (F-J) of flies expressing SCA182Q and different levels of *ari-2* (A-D, F-I) and of flies overexpressing *ari-2* alone (E, J). Loss of function of *ari-2* improves the SCA182Q eye phenotype that shows less necrosis signs (B, compare with A), but also better organization and less fusion of the ommatidia (G, compare with F). Overexpression of *ari-2*, on the other hand, aggravates the eye phenotype of SCA182Q flies that show increased cell death (evident by the necrosis dots in the external eye (D, compare with C), degenerated ommatidia and abnormal distribution of inter-ommatidial bristles (compare I to the control shown in H). Note that *ari-2*OE does not display an eye phenotype when expressed alone (E and J, control is shown in Figure 12 A and D). Insets in SEM images show a higher magnification view of the ommatidia.

Genotypes: A,F) *yw, UAS-SCA182Q[F7]/+; GMR-GAL4/UAS-GFP*. B,G) *yw, UAS-SCA182Q[F7]/+; GMR-GAL4/ ari-2*of. C,H) *yw, UAS-SCA182Q[F7]/+; GMR-GAL4/UAS-GFP*. D,I) *yw, UAS-SCA182Q[F7]/+; GMR-GAL4/UAS-Flag-ari-2*4. E,J) *w; GMR-GAL4/ UAS-Flag-ari-2*4. All flies are 1 day old, with the exception of flies in panels (E) and (J) that are 6 days old. Flies were raised at 27°C (A, B, E, F, G and J) or at 25°C (C-D and H-I). Scale bar is equivalent to 100µM (10µM in the magnification insets of SEM images).
Even though surprising, because the modifications observed with both loss of function and overexpression of \textit{ari}-2 were so clear, we decided to further investigate this modification in order to try to understand the mechanism behind this genetic interaction.

Interestingly, a protein interaction map for \textit{Drosophila melanogaster}, based on yeast two-hybrid assays followed by a statistical analysis (Giot et al., 2003) confirmed a link between \textit{ari}-2 and the Ubiquitin-Proteasome Pathway (through the Ubiquitin Conjugating Enzymes UbcD10 and Ubc84D). Moreover, the data from Rothberg and co-workers also indicates that \textit{ari}-2 is linked with BG4 (a.k.a. as FADD), a protein involved in the Immune Defense (Imd) Pathway (Figures 21 and 22). The Imd signaling cascade is equivalent to the mammalian Tumor Necrosis Factor (TNF) signaling pathway (Hoffmann and Reichhart, 2002), and FADD/BG4 is known to be one key element in promoting activation of the Imd pathway (Naitza et al., 2002). According to this report, decreased levels of \textit{dFADD} renders flies more susceptible to Gram-negative bacterial infections.

This new interaction with the BG4, lead us to hypothesize that the modification of the SCA1-induced eye phenotype observed with different levels of \textit{ari}-2 could be mediated through the Imd Pathway and its interacting proteins.

![Figure 21- Possible interaction map linking \textit{ari}-2 and the Ubiquitin-Proteasome Pathway and Imd pathways (Adapted from Giot et al., 2003).]
Figure 22: Schematic representation of the Imd signaling pathway in *Drosophila* (a) and the comparison to the analogous mammalian TNF Pathway (b). Activation of the Imd pathway leads to the nuclear import of Relish (Rel)-type transcription factors, which promotes up-regulation of expression of specific anti-microbial genes that mediate the immune response against Gram-Negative bacteria. The red box denotes the possible link between Ari-2 and the Imd pathway, through the interaction with FADD/BG4.

Proteolitic Pathway and Imd Pathway was the discovery of another report in the literature that supported an involvement between these 2 pathways in flies (Khush et al., 2002). According to Lemaitre and collaborators the Ubiquitin-Proteasome Pathway represses the Imd Pathway in *Drosophila*. Also indicating a role of the Imd pathway in neurodegenerative diseases, typically characterized by a late onset, was the observation that genes involved in the immune response in *Drosophila* also played a role in the aging process (Brummel et al., 2004; Libert et al., 2006; Pletcher et al., 2005; Pletcher et al., 2002). Interestingly, it was recently reported by Crack and Bray a link between innate immunity (specifically the Toll pathway) and the development and regulation of CNS inflammation, neurodegeneration and brain trauma (Crack and Bray, 2007).

All together, this led us to hypothesize that decreased levels of ari-2 would promote an increased activity of the Imd pathway, that in turn would mediate the suppression of the SCA1-induced phenotype we observed. In order to further investigate this hypothesis we analyzed whether SCA1\(^{82Q}\)-induced phenotypes can be modulated by altered levels of the proteins involved in the Imd signaling cascade. If so, increased levels of the proteins downstream of FADD, and ultimately Relish, would mimic the effect of Ari-2\(^{LOF}\), and, therefore, would suppress Ataxin-1 toxicity in the eye. Conversely, overexpression of Ari-2, and enhancer of Ataxin-1-induced toxicity in the eye, would repress the Imd pathway. Therefore, lower levels of the proteins in the Imd pathway should also be enhancers of the SCA1\(^{82Q}\)-induced eye phenotype.

To test this hypothesis we have collected the available stocks (both from Bloomington and Szeged stock centers, but also from private collections) for some of the genes implicated in the Imd Pathway. In this way we have tested the following alleles (Table 7):
### Table 7 – Alleles of the Imd Pathway Tested

<table>
<thead>
<tr>
<th>GENES</th>
<th>ALLELES TESTED</th>
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| PGRP-LC | - One deficiency line (Df(3L)29A6), where a portion of the genomic region that contains PGRP-LC is removed)  
- P-element insertion (PGRP-LC^BG00650)  
- The following UAS constructs:  
  - PGRP-LCa, PGRP-LCx (a gift from Dr. Anderson (Choe et al., 2002))  
  - DD1;PGRP-LC^7454 and PGRP-LC^AE12, a gift from Dr. Royet (Gottar et al., 2002). |
| FADD/BG4 | - 3 RNAi constructs (αFADD-2, αFADD-3 and αFADD-4, a gift from Dr. Ryu Ueda and Dr. Bruno Lemaitre (Leulier et al., 2002)) |
| DREDD | - EP line (DREDD^Dd013412)  
- Three UAS constructs (UAS-DREDD-FL10, UAS-DREDD-FL12 and UAS-DREDD-FL11,) a gift from Dr. Bruno Lemaitre (Vidal et al., 2001) |
| TAK1 | - Three RNAi constructs (dTAK1-5, dTAK1-7 and dTAK1-9), a gift from Dr. Ryu Ueda and Dr. Bruno Lemaitre (Leulier et al., 2002; Vidal et al., 2001).  
  Note that other TAK1 alleles of both mouse and Drosophila were tested, but because they were either lethal when combined with overexpression of expanded Ataxin-1 or displayed an eye phenotype on their own; those results will not be referred here. Those additional alleles tested were kindly provided by Dr. Ueno (Takatsu et al., 2000). |
| Ird5 | - One P-element insertion (ird5^KG08072) |
| Relish | - Two P-elements (Rel^exo16, Rel^XG07752)  
- UAS construct and the corresponding imprecise excisions (Rel^E20, Rel^E21, Rel^E23, Rel^E38). The UAS construct and imprecise excisions were a gift from Dr. Hultmark (Hedengren et al., 1999) |
Some of the results obtained are shown below.

![Figure 23: Overexpression and loss of function of Relish do not modify SCA182Q-induced eye phenotype. Shown are SEM images of the external eye of flies expressing SCA182Q and different levels of Relish. As shown in panels B and D, neither overexpression nor loss of function of Relish significantly modify the Ataxin-1 eye phenotype (compare to controls in panels A and C, respectively). Insets show a higher magnification view of the ommatidia.

Genotypes: A,C) yw, UAS-SCA182Q[F7]/+; GMR-GAL4/UAS-GFP. B) yw, UAS-SCA182Q[F7]/+; GMR-GAL4/+; UAS-Relish/+. D) yw, UAS-SCA182Q[F7]/+; GMR-GAL4/+; Relneo36/+. All flies are 1 day old. Flies were raised at 27°C (A, B) or at 25°C (C-D). Scale bar is equivalent to 100µM (10µM in the magnification insets of SEM images).

Surprisingly, we found that overexpression of Relish did not had an effect on SCA182Q-induced toxicity in the eye (Figure 23). In agreement, the loss of function alleles of this gene (excision lines; data not shown) or any of the P-element insertion lines of Relish also did not had any effect (Figure 23). To confirm this observation we decided to also test some of the alleles of the genes that are upstream of Relish in the Imd pathway and also involved in other pathways. For example, FADD and DREDD are also implicated in apoptotic/cell survival pathways (Hu and Yang, 2000; Strasser and Newton, 1999; Tibbetts et al., 2003) and TAK1 is also involved in development, and in particular in the c-Jun N-Terminal Kinase (JNK) Signaling Pathway (Mihaly et al., 2001; Takatsu et al., 2000; Vidal et al., 2001).

Some of the results obtained are shown in Figure 24.
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Figure 24. DREDD\textsuperscript{OE}, FADD\textsuperscript{LOF} or dTAK1\textsuperscript{LOF} do not modify SCA1\textsuperscript{82Q}-induced eye phenotype. Shown are Light microscope eye images of the external eye of flies expressing SCA1\textsuperscript{82Q} and either DREDD\textsuperscript{OE}, FADD\textsuperscript{LOF} or dTAK1\textsuperscript{LOF}. As shown in panels B, C and D, neither overexpression of DREDD nor loss of function of FADD or dTAK1 significantly modify the Ataxin-1 eye phenotype (compare to control in panel A). Note that even though slightly more necrosis (black spots in the eye) is observed, the alteration is not significant.

Genotypes: A) yw, UAS-SCA1\textsuperscript{82Q}[F7]/+; GMR-GAL4/UAS-GFP. B) yw, UAS-SCA1\textsuperscript{82Q}[F7]/+; GMR-GAL4/+; UAS-DREDD\textsuperscript{FL12}+/+. C) yw, UAS-SCA1\textsuperscript{82Q}[F7]/+; GMR-GAL4/\alpha FADD-3. D) yw, UAS-SCA1\textsuperscript{82Q}[F7]/dTAK1-7; GMR-GAL4/+. Note that \alpha FADD-3 and dTAK1-7 are RNAi lines, and because of that the alleles tested might not be null alleles. All flies are 1 day old. Flies were raised at 27°C. Scale bar is equivalent to 100\mu M

The results shown above suggest that modulation of the protein levels of components of the Imd Pathway does not modify the SCA1\textsuperscript{82Q}-induced degeneration in the eye (Figure 24).

Therefore, our current results do not genetically support the evidences in the literature, and therefore, at this time, we cannot establish a relation between the Imd pathway and the Ubiquitin Proteasome Pathways through ari-2.

**DISCUSSION**

The Ubiquitin-Proteasome Pathway (UPS), and Ubiquitin Ligases in specific have been extensively implicated in Neurodegenerative Diseases (Ardley and Robinson, 2004; Ciechanover and Brundin, 2003) and even in cancer (Zhang et al., 2007). Interestingly, Ubiquitin Ligases have even been suggested as therapeutic targets (reviewed in Robinson and Ardley, 2004). The current idea is that in the occurrence of UPS impairment/inhibition the characteristic phenotypes associated to neurodegenerative diseases in

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general are aggravated/worsen. Therefore, the discovery that loss of function of a specific Ubiquitin Ligase ameliorated significantly the expanded Ataxin-induced toxicity in the eye was surprising.

Our initial hypothesis was that this modification could be mediated through the activation of the Imd pathway (Giot et al., 2003; Khush et al., 2002). As described above, this hypothesis had a strong support from other independent reports in the literature (Brummel et al., 2004; Giot et al., 2003; Libert et al., 2006; Pletcher et al., 2005; Pletcher et al., 2002). However, as shown, we did not find a genetic interaction between mutant Ataxin-1 toxicity and the activation of Imd Pathway. We have tested several alleles of genes implicated in the Imd pathway, but we were not able to prove significant differences in the toxicity in the eye as a result of its expression/loss of function. Even though the possibility of a link between Ataxin-1-induced pathogenesis and the Imd Pathway cannot be totally discarded (more alleles would have to be tested and maybe other assays would need to be conducted), there is little support to pursue this option.

In order to evaluate the relevance of ari-2 in SCA1 through the UPS, it would be interesting to test the effect of Ubc84D or UbcD10, the two other proteins also identified as ari-2 interactors in the study by Giot and collaborators (Giot et al., 2003; Figure 21) in SCA1^82Q-expressing flies.

Although it is possible that the specific alleles tested do not have an effect on the model, because different alleles of the same gene and also several genes of the pathway were tested, it suggests that the Imd Pathway is not implicated in pathogenesis of this SCA1 Drosophila model. Nevertheless, because TAK1 is also implicated in the JNK Pathway (Takatsu et al., 2000; Wang et al., 2003), we have decided to further test this hypothesis. Yet, this hypothesis needs careful examination as the JNK pathway has a strong correlation with the apoptotic pathway (but also with longevity as reported by Jasper and collaborators (Wang et al., 2003)) and, therefore, its effects can be masked. Preliminary work with some alleles of genes involved in the JNK pathway (Puc, Bsk and Hep) indicates that they are able to modulate the toxicity of Ataxin-1 in the eye. However, these alleles might display a phenotype on their own, due to their involvement in apoptosis and cell survival (data not shown). More studies will need to be conducted in the
future in order to explore this hypothesis.

Nevertheless, other options still need to be considered and tested. Some components of the UPS have also been implicated in SUMOylation. Even though related to ubiquitination, SUMOylation leads to modulation and/or stabilization of the protein’s activity rather than proteolysis. Therefore, ari-2 might be involved in a mechanism similar to this, instead of the obvious proteolitic pathway that leads to the degradation of the protein.

Also need to be taken into consideration is the hypothesis of ari-2 modulating the levels of another protein important for either the clearance of mutant Ataxin-1 or, on the other hand, a protein implicated in the survival of the cell. UPS is typically associated with protein degradation, but it is also crucial to numerous cellular functions not related to degradation, such as ribosome function, membrane protein transport and nuclear transport, or even in RNA metabolism (reviewed in Lucas et al., 2006). An influence of the UPS in gene transcription has also been suggested (Ciechanover and Brundin, 2003; Sugars and Rubinsztein, 2003). Additionally, there are proteins that are stable when ubiquitinated, as for example histones, and it is possible that this can be the case for, at least, one of the targets of ari-2.

All these options should be considered in future works aimed at identifying the exact mechanisms by which ari-2 modulates the toxicity of Ataxin-1 in a Drosophila model.

**B. pasilla (ps)**

*Drosophila* SCA1 genetic modifiers previously identified in a screen performed in the lab, highlighted the role of protein folding and protein clearance in the development of SCA1 characteristic phenotypes in the fly (Fernandez-Funez et al., 2000). These were mechanisms previous known to be implicated in polyglutamine-induced neurodegeneration.
However, new mechanisms of polyglutamine pathogenesis were also revealed by the discovery of modifiers that are involved in RNA processing, transcriptional regulation and cellular detoxification (Fernandez-Funez et al., 2000). At first, we were particularly interested to understand how did RNA processing affected SCA1 pathology in our fly model.

To address this question we decided to further study *mushroom-body expressed (mub)*, one of the modifiers identified in the screen. mub protein isoforms are related to a family of mouse and Human poly(rC) binding proteins (PCBP), namely PCBP-1, and PCBP-2 and PCBP-3. mub is also similar to the mouse and Human Nova proteins (Nova-1 and Nova-2), which are also implicated in a neuronal disease, Paraneoplastic Opsoclonus Myoclonus Ataxia - POMA (Figure 25; Grams and Korge, 1998). Nova is a nuclear protein normally expressed in the CNS where it regulates splicing, but in POMA patients the Nova proteins are present as auto-antigen throughout the body.

mub is an RNA-binding protein containing 3 KH-type domains, likely involved in regulation of alternative nuclear mRNA splicing, via spliceosome or another RNA-mediated processes (Adapted from www.flybase.net/reports/FBgn0014362.html; (Grams and Korge, 1998; Park et al., 2004)).

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**Figure 25** – Sequence homology within the KH domains between different RNA-binding proteins. Shaded residues match the KH domain consensus sequence. # indicates the number of KH domain used to produce this alignment and • marks the hydrophobic residues. In the first line: E and H indicate folding in beta-sheet and helical structures, respectively. The proteins used for this alignment are MUB, PCBP-1, PCBP-2, hnRNP K, hnRNP K, RPS15, TDP-43, PS1, TDP-43, Bic-C, Bic-C, PSI, Bic-C, SCP160, SCP160, Mer1, Mer1, etc.
Interestingly, mub protein is not the only “Nova-like” protein in *Drosophila*. Andrew and collaborators have identified another “Nova-like” protein in flies, pasilla (ps) (Seshaih et al., 2001). Therefore, we decided to test the effect of this protein in our SCA1 model. Interestingly, in a Yeast Two-Hybrid Assay, mub and ps were found as interactors (Giot et al., 2003). Even though this interaction was reported as “Low confidence”, it could mean that the two proteins could be part of a complex or act together transiently.

As mub, ps is described as an RNA-binding protein involved in regulation of alternative mRNA splicing or another RNA-mediated processes (Adapted from [www.flybase.net/reports/FBgn0026188.html](http://www.flybase.net/reports/FBgn0026188.html); (Park et al., 2004)). ps itself is alternatively spliced, generating 4 different splice forms: ps-A, ps-B, ps-C and ps-D (Figure 26).

**Figure 26** - Pasilla alternatively spliced predicted isoforms, ps-A, ps-B, ps-C, and ps-D. The splice forms were predicted from the analysis of seven different ps cDNAs. ORFs are indicated as filled boxes and open boxes indicate the Untranslated Regions. The ORFs span from exons 1a, 1b, or 1c through exon 5b for splice form ps-A and ps-C and through exon 6 for splice forms ps-B and ps-D. The Celera genomics predicts additional four genes in this interval: CG16765, CG16776, CG16777, and CG8144. CG16765 overlaps the first two exons of the ps-A splice form. CG16776 corresponds to the first exon of the ps-D splice form and CG8144 corresponds to exons 3-6 of splice forms ps-B and ps-C. The original ETPs P-element insertion maps between the first two exons of ps-A at position 181,167 and does not affect the accumulation of ps protein or ps function (From Seshaih et al., 2001).

Andrew and collaborators have created several alleles of ps by excisional mutagenesis (Figure 27; Seshaih et al., 2001).
Figure 27 – ps alleles map (by complementation analysis). ps maps to a genomic interval for which several deficiencies are available. Chromosomal regions removed by the deficiencies are indicated by dotted lines. pasilla is located in the cytological interval 85D16–85D17 and is deleted in Df(3R)GB104 and Df(3R)by416, but not in Df(3R)by62 (From Seshaiah et al., 2001).

Of those alleles, ps¹, ps², ps³ and ps⁴⁴⁹ were kindly provided by Dr. Andrew and were tested in our SCA1 Drosophila model.

Figure 28 – Decreased levels of ps improve the SCA¹⁸²Q-induced eye phenotype. Light Microscopic eye images (A–E) and SEM images of the external eye (F–J) of flies expressing SCA¹⁸²Q and different loss of function alleles of ps. With the exception of allele ps¹ (panels C and H), all other alleles of ps are suppressors of mutant Ataxin-1 toxicity in the eye. While ps² (panels D and I) is only a mild suppressor of the eye (note less ommatidia degeneration as well as its improved organization but still
some inter-ommatidial bristle loss and/or abnormal distribution), \( ps^{149} \) (panels B and G) and \( ps^t \) (panels E and J) are both good suppressors of the toxicity in the eye; compare to controls in panels A and F. Both \( ps^{149} \) and \( ps^t \) alleles induce an increased organization of the ommatidia and bristles, along with a clear decrease in cell death and in fused ommatidia. \( ps^t \) (panels C and H), however, does not restore the organization of ommatidia and bristles neither it improves cell degeneration. Insets in SEM images show a higher magnification view of the ommatidia.

Genotypes: A,F) yw, UAS-SCA1^{82Q}[F7]/+; GMR-GAL4/UAS-GFP. B,G) yw, UAS-SCA1^{82Q}[F7]/+; GMR-GAL4/+; \( ps^{149} / + \). C,H) yw, UAS-SCA1^{82Q}[F7]/+; GMR-GAL4/+; \( ps^t / + \). D,I) yw, UAS-SCA1^{82Q}[F7]/+; GMR-GAL4/+; \( ps^2 / + \). E,J) yw, UAS-SCA1^{82Q}[F7]/+; GMR-GAL4/+; \( ps^3 / + \). All flies were raised at 27°C and are 1 day old. Scale bar is equivalent to 100\( \mu \)M (10\( \mu \)M in the magnification insets of SEM images).

As shown in Figure 28, apart from the allele \( ps^t \), that can be responsible for the expression of one particular protein isoform, all other pasilla alleles are suppressors of the \( SCA1^{82Q} \)-induced eye phenotype. We observe that \( ps^{149} \), \( ps^2 \) and \( ps^t \) promote a decrease in necrosis and cell degeneration, evident both in the Light Microscope eye images (Figure 28.B and D-E, control is shown in panel A) and also in SEM images (Figure 28.G and I-J, control is shown in panel F). Furthermore, we also observe an increased organization of ommatidia and inter-ommatidial bristles. This suppression is particularly more effective with alleles \( ps^{149} \) and \( ps^3 \).

Regarding mub, the other RNA-binding protein that we were first interested in, the results obtained will be discussed in the Part 2 and 3 of this thesis.

As mentioned earlier in the Introduction (Section 3.1.1) we have also generated transgenic flies over expressing the N-terminal portion of the Huntingtin protein (Fernadez-Funez, Rincón-Limas and Botas, unpublished work; Branco et al., 2007; Kaltenbach et al., 2007); see also Part 2 of this thesis). The relevance of testing the same alleles in different disease models in \( Drosophila \) relays on the fact that it able us to identify common and distinct mechanisms between the two polyglutamine disorders.

Because we had already identified some genetic modifiers that were also able to modify the mutant \( Htt \)-induced eye phenotype (Branco et al., 2007 and this thesis), we decided to test \( ps \) alleles in our HD model.
As shown in Figure 29, and in contrast to mub, loss of function of pasilla does not modify the toxicity in the eye induced by overexpression of truncated Htt\textsuperscript{128Q} in the flies.

**Figure 29** – Loss of function of ps does not affect the Htt\textsuperscript{128Q}-induced toxicity in the eye. Shown are Retinal Paraffin sections of flies that over express the SCA1\textsuperscript{82Q} (A-D) or the Htt\textsuperscript{128Q} transgenes (E-H) and the different loss of function alleles of ps tested. Arrows indicate retina’s length. SCA1 and HD model controls are shown in (A) and (E), respectively. All the loss of function alleles of ps tested suppress the phenotype of SCA1\textsuperscript{82Q}-expressing flies (B-D), in accordance to the data presented in Figure 28. Note the increase in the length and organization of the retina. This suppression is particularly more evident for ps\textsuperscript{3} (panel D). In Htt\textsuperscript{128Q} flies (F-H), partial loss of function of ps does not induce a clear modification of the eye phenotype. Even though there is a slight decrease in the length of the retina, we can still observe a similar amount of tissue loss when compared to the control in panel E. Genotypes: A) yw, UAS-SCA1\textsuperscript{82Q}[F7]/+; GMR-GAL4/UAS-GFP. B) yw, UAS-SCA1\textsuperscript{82Q}[F7]/+; GMR-GAL4/+; ps\textsuperscript{449}/+. C) yw, UAS-SCA1\textsuperscript{82Q}[F7]/+; GMR-GAL4/+; ps\textsuperscript{2}/+. D) yw, UAS-SCA1\textsuperscript{82Q}[F7]/+; GMR-GAL4/+; ps\textsuperscript{449}/+. E) w; GMR-GAL4/ UAS-GFP; Htt\textsuperscript{128Q}[M64]/+. F) w; GMR-GAL4/+; Htt\textsuperscript{128Q}[M64]/ps\textsuperscript{449}. G) w; GMR-GAL4/+; Htt\textsuperscript{128Q}[M64]/ps\textsuperscript{2}. H) w; GMR-GAL4/+; Htt\textsuperscript{128Q}[M64]/ ps\textsuperscript{3}. Flies were raised at 25°C. All SCA1\textsuperscript{82Q} transgenic flies shown are one day old and all HD model flies are six days old. Scale bar is equivalent to 10µM.

Reinforcing the results in Figure 28, in Figure 29 we show that ps\textsuperscript{LOF} is a suppressor of the SCA1\textsuperscript{82Q}-induced toxicity in the eye. However, in the HD model (Figures 29. F-H), the phenotypes observed are not significantly
different from the control shown in Figure 29.E. Even though a mild decrease in the length of the retina is observed, there is still considerable tissue loss. Therefore, the results indicate that \( ps^{\text{LOF}} \) is a suppressor of the \( SCA1^{82Q} \) but not \( Htt^{128Q} \)-induced toxicity in the eye. This result re-enforces the existence of specific mechanisms leading to the pathology in each of the disorders.

In order to try to better understand the modification observed in the SCA1 model, we then decided to test if decreased levels of \( ps \) altered the amount of RNA (Figure 30) and/or soluble protein levels of Ataxin-1\(^{82Q} \) (Figure 31).

**Figure 30 – Loss of function of \( ps \) does not affect the \( SCA1^{82Q} \) mRNA levels.** Shown is a semi-quantitative RT-PCR with RNA extracted from adult heads (6 per genotype) of \( SCA1^{82Q} \) flies that carried loss of function alleles of \( ps \) and control flies (expressing only \( SCA1^{82Q} \)). No significant difference in the levels of \( SCA1 \) mRNA was detected. Rp49 was used as an internal control. A total of 3 independent experiments were performed.

Genotypes: \( SCA1^{82Q} \) \( yw, \) UAS-\( SCA1^{82Q}[F7]/+; \) GMR-GAL4/UAS-GFP, \( SCA1^{82Q}/ps^{j449}/yw, UAS-SCA1^{82Q}[F7]/+; \) GMR-GAL4/+; \( ps^{j449}/+. \) \( SCA1^{82Q}/ps^{j449}/yw, UAS-SCA1^{82Q}[F7]/+; \) GMR-GAL4/+; \( ps^{j449}/+. \) \( SCA1^{82Q}/ps^{j449}/yw, UAS-SCA1^{82Q}[F7]/+; \) GMR-GAL4/+; \( ps^{j449}/+. \)

As shown in the semi-quantitative RT-PCR (Figure 30), we observed that none of the \( ps^{\text{LOF}} \) alleles tested modify \( SCA1^{82Q} \) mRNA levels. Moreover, the modification of the eye phenotype observed cannot result of alterations at the level of soluble protein amounts in the cells, as revealed by the Western Blot assay performed (Figure 31).
Figure 31 – Soluble levels of the Ataxin-1\textsuperscript{82Q} protein are not altered by loss of function of \textit{ps}. Shown is a representative Western Blot comparing soluble protein levels of Ataxin-1\textsuperscript{82Q} (Ata1\textsuperscript{82Q}) in SCA1\textsuperscript{82Q}-expressing adult flies with decreased levels of \textit{ps}, or SCA1\textsuperscript{82Q} alone. Different \textit{ps} alleles were tested. Western Blot was performed using 4 adult heads per genotype. Control (lane 1) shows no signal for Ataxin-1\textsuperscript{82Q}. Ataxin-1\textsuperscript{82Q} was detected using anti-Ataxin-1 antibody (11NQ) and anti-tubulin antibody was used as loading control. As shown by the quantifications in the box at the bottom of the figure, no significant difference was observed between SCA1\textsuperscript{82Q}-expressing flies (lane 2) and any of the SCA1\textsuperscript{82Q} transgenic flies carrying decreased levels of \textit{ps} (lanes 3-6). Genotypes: 1) \textit{w};GM\textsuperscript{R}\textsuperscript{-}GAL4/\textit{UAS}-\textit{GFP}. 2) \textit{yw}, \textit{UAS}-SCA1\textsuperscript{82Q}[F7]/+; GM\textsuperscript{R}-GAL4/+; \textit{ps}\textsuperscript{1}/+. 4) \textit{yw}, \textit{UAS}-SCA1\textsuperscript{82Q}[F7]/+; GM\textsuperscript{R}-GAL4/+; \textit{ps}\textsuperscript{1}/+. 5) \textit{yw}, \textit{UAS}-SCA1\textsuperscript{82Q}[F7]/+; GM\textsuperscript{R}-GAL4/+; \textit{ps}\textsuperscript{j449}/+. 6) \textit{yw}, \textit{UAS}-SCA1\textsuperscript{82Q}[F7]/+; GM\textsuperscript{R}-GAL4/+; \textit{ps}\textsuperscript{j449}/+.

Our results show that flies co-expressing mutant Ataxin-1 and loss of function of \textit{ps} do not affect neither Ataxin-1\textsuperscript{82Q} RNA or protein levels, and suggests that the modification observed might be mediated through the interaction with other key proteins that affect toxicity and not by affecting the expression or protein synthesis of Ataxin-1 itself. \textit{ps} might also affect post-translational modifications of Ataxin-1\textsuperscript{82Q} but not its endogenous soluble levels.

\textbf{DISCUSSION}

RNA-mediated mechanisms and RNA binding proteins have been implicated in many neurodegenerative diseases and muscular dystrophies, promoting either gain of function mechanisms, altered splicing events or alterations in mRNA localization (Gallo et al., 2005; Gatchel and Zoghbi, 2005; Jin et al., 2007; Sofola et al., 2007a; Sofola et al., 2007b; Swanson and Orr, 2007). In fact, even in the genetic screen previously performed in our
laboratory, RNA-binding proteins were one of the representative groups of proteins identified as modifiers of the $SCA1^{82Q}$-induced toxicity in the eye (Fernandez-Funez et al., 2000).

According to our data, apart from one specific allele, all other pasilla alleles are suppressors of the $SCA1^{82Q}$-induced eye phenotype, both under Light microscope, in Scanning Electron Microscopy (SEM) images and in retinal paraffin sections. But in contrast to mub, which will be further discussed next (see also Branco et al., 2007), pasilla did not altered the soluble levels of Ataxin-1$^{82Q}$ mRNA or protein. ps also did not modify the characteristic eye phenotype of our HD model in the assay used and under our specific experimental conditions. Even though admitting that a negative result is hard to interpretate, we were tempted to suggest that ps, along with other genes that only modify the $SCA1^{82Q}$-induced eye phenotype or that modify SCA1 and HD models eye phenotypes differently (see also Branco et al., 2007), may contribute to some of the differences between SCA1 and HD.

Given the similarities between mub and ps, the results obtained with pasilla were surprising. Not only the same modification of the $SCA1^{82Q}$ induced eye phenotype (suppression) was caused by opposite amounts of the two proteins protein (overexpression for the case of mub and loss of function in the case of pasilla); but also they seemed to mediate different events leading to the toxicity characteristic the model (mub alters both mRNA and soluble protein levels of Ataxin-1$^{82Q}$, as it will be further discussed in Part 3 of the Results section of this thesis).

One possible explanation for such differences came after analyzing the observations made by Musunuru and Darnell (Musunuru and Darnell, 2004). According to the authors, all three KH domains in the Nova protein are responsible for the interaction and /or coordination of its RNA targets. In fact, the work presented by Musunuru and Darnell and previous reports suggests that all three KH domains in the Nova proteins have similar specificity to the core tetranucleotide UCAY (Jensen et al., 2000; Lewis et al., 2000; Musunuru and Darnell, 2004). Despite the high degree of sequence and structure homology between the Nova proteins and heterogeneous nuclear Ribonucleoprotein (hnRNP) E and hnRNP K, these groups of proteins
recognize different target sequences and have different functions. While the Nova proteins have been implicated in the regulation of pre-mRNA splicing, the hnRNP E and hnRNP K proteins are implicated in mRNA stabilization and translational control. In order to better understand the difference in sequence preferences between these two groups of proteins, Musunuru and Darnell made a sequence alignment of the KH domains of mammalian Nova-1 and Nova-2, the four variants of hnRNP E, hnRNP K and its Drosophila orthologues (pasilla, mub and Bancal, respectively). For this sequence alignment the authors have also included the yeast protein Pbp2p, which presumably represents the ancestor of the proteins mentioned above. The sequence alignment generated showed that an Arginine is invariably present at position 32 in the KH domains of all mammalian and Drosophila hnRNP E and hnRNP K variants, which was not present in neither two of the Nova proteins nor in its Drosophila orthologue pasilla (Figure 31; Musunuru and Darnell, 2004). This observation suggests that the Arginine residue at this particular position has a crucial role in determining the protein’s function, maybe dictating RNA specificity for some target sequences.

Unexpectedly, we show that mub and pasilla, although very similar in terms of structure and function (see also Park et al., 2004), they have different effects and specificities in our two fly models of SCA1 and HD. The relevance of the different single residue in the primary sequence of the two proteins, pointed by Musunuru and Darnell (Figure 32), in their cellular role needs to be confirmed. More experiments have to be conducted in order to further explore and understand the role and relevance of pasilla in SCA1 pathogenesis in the flies as well as to understand the molecular reasons that justify the fact that pasilla does not modify the eye phenotype of our HD model.
One other alternative hypothesis that could be further explored in order to understand the effect of pasilla in SCA1^82Q-induced toxicity in the flies comes from the observations made by Darnell and collaborators (Ule et al., 2005). According to this report, by using genome-wide alternative splicing Microarrays, the authors were able to identify the exons that are regulated by Nova, the human orthologue of pasilla. The aim was to gain insight into possible functions of Nova in the brain. Interestingly they found that a group of genes with defined functions in the brain, of which the alternative splicing was regulated by Nova, belong to a group of interacting proteins abundant at the synapses (the majority) or involved in axon guidance. Nova’s function can be, therefore, to coordinate and regulate specific neuronal processes. With their results, the authors also show that the levels of the RNAs regulated by Nova are constant in knockout and wild type brains, emphasizing the role of Nova as a regulator of the quality of the interactions occurring at the synapses (Ule et al., 2005).
One fruitful approach to undertake in the future could be to determine whether altered endogenous levels of pasilla modify the morphology or activity of specific proteins in the synaptic buttons of flies that over express the expanded Ataxin-1 protein.

Overall, the work with the pasilla gene here presented is, to our knowledge, the first report of the involvement of this gene in SCA182Q-mediated toxicity. More experiments will be needed to further understand the suppression observed and to explore its possible role as a therapeutic target for SCA1. However, the work here presented, sets up a number of observations regarding its effect as a modifier of the SCA1 model in flies.
Part 2

Comparative Analysis of Genetic Modifiers in *Drosophila* Points to Common and Distinct Mechanisms of Pathogenesis among Polyglutamine Diseases
In this part of the Thesis, the main focus is the comparative analysis of some genetic modifiers of our SCA1 model in the HD model. The results presented next are the major focus of the work in (Branco et al., 2007). A Supplemental Figure is shown in Section V, Appendix I. Some additional results obtained throughout this PhD work and that are not published elsewhere are present in Part 3 (included in III- Results and Discussion) of this thesis.

As mentioned before, the aim of this work was to identify common and distinct modifiers in SCA1 and HD Drosophila models generated in the Botas laboratory, making use, mainly, of a collection of known SCA1\textsuperscript{82Q}-induced toxicity in the eye.

**A. Degenerative phenotypes and NIs caused by targeted expression of either SCA1\textsuperscript{82Q} or Htt\textsuperscript{28Q} transgenes in Drosophila can be used for comparative analysis of modifier genes**

The directed overexpression (OE) of the transgene of interest was achieved by using the GMR-GAL4 driver, which directs expression to photoreceptor neurons and surrounding support eye cells. As mentioned earlier in the Introduction, overexpression of the human Ataxin-1 protein containing a polyglutamine tract of 82 glutamines (Ataxin-1\textsuperscript{82Q}, Figure 12 top panel) in the eye causes an external eye phenotype consisting of ommatidial disorganization and inter-ommatidial bristle loss that can be used to screen for genetic modifiers (Figures 13.B, and 13.E and control in Figures 13.A and 13.D, see also (Fernandez-Funez et al., 2000)). The flies expressing the SCA1\textsuperscript{82Q} transgene also display a retinal phenotype, characterized by a decrease in the length of the retina, tissue loss and shortened and abnormal rhabdomeres (Figure 13.H and control in Figure 13.G).

We generated transgenic flies over expressing the N-terminal portion of the Huntingtin protein (Figure 12 bottom panel). Unlike flies expressing Ataxin-1\textsuperscript{82Q} that display a severe rough external eye phenotype, the N-
terminal HD model flies show a slight disorganization of ommatidia and bristle loss (Figures 13.C and 13.F). However, when longitudinal sections of the retina are performed, we can observe a severe disorganization of the retina, along with dramatic tissue loss and vacuolization (Figure 13.I). Note that this phenotype is even more pronounced in aged flies (Figure 14).

Like in SCA1^{82Q}-expressing flies, in the HD model we observe formation of neuronal NIs, one of the hallmarks of both diseases (Figures 15; see (Fernandez-Funez et al., 2000) for expanded Ataxin-1 NIs). Using the OK107-GAL4 driver we direct the expression of the Htt^{128Q} transgene to specific neurons of the CNS. Immunofluorescence staining experiments reveal that the Htt protein is present both in large NIs and in a diffuse form that may represent soluble protein or small aggregates that cannot be resolved with the immunofluorescence method used (Figure 16). The percentage of cells that contain large macro-aggregates increases during the aging process of the fly (data not shown) in agreement with the progressiveness of the model.

Using the eye phenotypes described above as a primary assay, we investigated the effects of a collection of candidate modifier genes of the SCA1 in the HD model. This collection mainly includes modifier genes previously identified in a forward genetic screen using the SCA1 model (Fernandez-Funez et al., 2000). We found three different categories of modifiers: genes that modify SCA1^{82Q} and Htt^{128Q}-induced phenotypes similarly, genes that appear to modify only the SCA1 model, and genes that modify the two disease models in opposite ways.

### B. Genes that modify SCA1^{82Q} and Htt^{128Q}-induced phenotypes similarly

The genes in this group are listed in Table 8 and representative examples are shown in Figure 33. They include genes encoding signal transduction or RNA binding proteins that were not previously known to modulate the toxicity of different polyglutamine proteins. Interestingly, this group also includes specific transcription factors that modulate the toxicity of Ataxin-1^{82Q} and Htt^{128Q} in a similar manner.
**Table 8 – Genes that modify the toxicity of Ataxin-1^{82Q} and Htt^{128Q} similarly**

<table>
<thead>
<tr>
<th>Gene / Accession Number</th>
<th>Modification of the Retinal Phenotype</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
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<td><strong>Chaperones/Heat-Shock response</strong></td>
<td>Ataxin-1^{82Q}</td>
<td>Htt^{128Q}</td>
</tr>
<tr>
<td>DnaJ-1 / FBgn0015657</td>
<td>Su by OE</td>
<td>Su by OE</td>
</tr>
<tr>
<td>Hsp70</td>
<td>Su by OE</td>
<td>Su by OE</td>
</tr>
<tr>
<td></td>
<td>En by DN</td>
<td>En by DN</td>
</tr>
<tr>
<td><strong>Ubiquitin Proteasome Pathway</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eff / FBgn0011217</td>
<td>En by LOF</td>
<td>En by LOF</td>
</tr>
<tr>
<td>Ubc-E2H / FBgn0029996</td>
<td>En by LOF</td>
<td>En by LOF</td>
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<tr>
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<td>En by LOF</td>
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<td>En by LOF</td>
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<tr>
<td>skd / FBgn0003415</td>
<td>En by LOF</td>
<td>En by LOF</td>
</tr>
<tr>
<td>elB / FBgn0004858</td>
<td>En by OE</td>
<td>En by OE</td>
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<tr>
<td>Sir2 / FBgn002429</td>
<td>En by OE</td>
<td>En by OE</td>
</tr>
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<td>tara / FBgn0040071</td>
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<td>En by OE</td>
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<tr>
<td><strong>RNA Binding</strong></td>
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<tr>
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<td>pum / FBgn0003165</td>
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<tr>
<td><strong>Signal Transduction</strong></td>
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<td>d14-3-3ε / FBgn0020238</td>
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<td>Su by LOF</td>
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<td>FBgn0003691</td>
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Su - Suppressor; En – Enhancer; LOF – Loss of Function; OE – Overexpression; DN – Dominant Negative
Figure 33 - Genes that modify \textit{SCA1}^{82Q} and \textit{Htt}^{128Q}-induced phenotypes similarly

Paraffin retinal sections of flies expressing either \textit{SCA1}^{82Q} (A-H) or \textit{Htt}^{128Q} (I-P) transgenes are shown. Arrows indicate retina’s length. \textit{SCA1} and HD model controls are shown in (A) and (I), respectively. \textit{SCA1}^{82Q} flies carrying one functional copy of \textit{skd} show an enhanced phenotype characterized by disorganization and decrease in the length of the retina as well as tissue loss (B). In \textit{Htt}^{128Q} flies, partial loss of function of \textit{skd} also induces an enhancement of the \textit{Htt}^{128Q}-induced eye phenotype, most evident by a reduction of the length of the retina (J). \textit{SCA1}^{82Q} transgenic flies over expressing \textit{pum} show a more severe eye phenotype (C). These flies have a more disorganized retina with some tissue loss (compare to control in A). In \textit{Htt}^{128Q} flies, \textit{pum} overexpression clearly increases the amount of tissue loss (K). Decreased levels
of *pum*, on the other hand, are responsible for an increase in the length and organization of the retina both in SCA1 and HD models (D and L, respectively). In the HD model (L) note also that *pum-af* induces a decrease in tissue loss compared to control in (I). Overexpression of 14-3-3ε is a strong enhancer of both SCA1128Q and Htt128Q eye phenotypes (E and M). Note the dramatic decrease in the length of the retina that 14-3-3ε causes in both models, as well as an increase in tissue loss in the SCA1 model (E). Decreased levels of 14-3-3ε, on the other side, suppress both SCA1128Q and Htt128Q eye phenotypes (F and N). Note improved organization of the retina as well as decrease in tissue loss. SCA1128Q flies that also over express DIAP1 display a retinal phenotype very similar to wild-type flies (G and compare to Figure 1, panel E). Note the improvement of the retinal organization as well as length. The improvement is also evident in Htt128Q flies over expressing DIAP1 (O). Decreased levels of DIAP1 also induce a modification of the SCA1128Q and Htt128Q eye phenotypes (H and P, respectively). Note the decrease in the length of the retina, along with increased tissue loss. skdaf, *pum* (OE and LOF), 14-3-3ε (OE and LOF) and DIAP1 (OE and LOF) do not display an eye phenotype by themselves (see Suplemental Figure 1, Section V, Appendix I).


All SCA1128Q transgenic flies shown are 1 day old and all HD model flies are 6 days old. All flies were raised at 25°C. Scale bar is equivalent to 10µM.

**skuld** (*skd*) is an example of a transcription factor whose loss of function (LOF) allele enhances the toxicity of both Ataxin-1128Q and Htt128Q. As shown in Figures 33.B and 33.J, skdaf induces a decrease in the length of the retina and promotes increased disorganization and tissue loss in the eyes of both the SCA1128Q and Htt128Q-expressing flies, but does not have a phenotype on its own (Supplemental Figure 1.I in Section V, Appendix I).

*pumilio* (*pum*), encodes an RNA binding protein and also modifies SCA1 and HD model phenotypes similarly. Overexpression of *pum* enhances both SCA1128Q (Figure 33.C; (Fernandez-Funez et al., 2000)) and Htt128Q-induced eye phenotypes (Figure 33.K), but does not show a phenotype by itself (Supplemental Figure 1.F; Section V, Appendix I). Furthermore, loss of function of *pum* increases the thickness of the retina both in the SCA1 and HD *Drosophila* models, decreases cell loss (Figures 33.D and 33.L, respectively), and does not display an eye phenotype alone (Supplemental...
14-3-3e, encodes a protein involved in signal transduction and its overexpression enhances both SCA182Q and Htt128Q-induced eye phenotypes (Figures 33.E and 33.M, respectively). Note the drastic reduction in the length of the retina, accompanied with cell loss. In contrast, 14-3-3e loss of function suppresses both SCA1 and HD model phenotypes as it promotes an increase in length of the retina and improves its integrity (Figures 33.F and 33.N, respectively; (Chen et al., 2003)). Modulating the expression levels of 14-3-3e does not cause a phenotype in control eyes (Supplemental Figures 1.G and 1.O; Section V, Appendix I).

We have also tested the effect of an anti-apoptotic protein, Drosophila Inhibitor of Apoptosis (DIAP1) or thread (th), in the two models. Confirming previous results (Ghosh and Feany, 2004), we find that DIAP1 is a strong suppressor of SCA182Q-induced eye phenotype (Figure 32.G). We also found that DIAP1OE strongly suppresses the Htt128Q eye phenotypes and does not have a phenotype on its own (Figure 33.O and Supplemental Figure 1.H - Section V, Appendix I). Overexpression of DIAP1 in SCA182Q-expressing flies restores the organization and thickness of the retina, which becomes similar to wild-type retinas (compare Figures 13.G and 33.G). In the HD Drosophila model, overexpression of DIAP1 also improves the organization of the retina and reduces tissue loss (Figures 33.O and control in 33.I). Additionally, SCA182Q or Htt128Q-expressing flies that also carry a loss of function allele of DIAP1 (DIAP1LOF) show a clear enhancement of the eye phenotypes when compared to flies that express the SCA182Q or Htt128Q transgenes alone (Figures 33.H and 33.P, respectively; controls are shown in Figures 33.A and 33.I). Note the decrease in the length of the retina as well as increase in tissue loss. DIAP1LOF also does not display an eye phenotype on its own (Supplemental Figure 1.P in Section V, Appendix I).
C- Genes that modify Ataxin-1^{82Q} toxicity but not Htt^{128Q}-induced toxicity

This group includes modifiers of SCA1^{82Q}-induced toxicity previously reported (Fernandez-Funez et al., 2000), as well as new Ataxin^{82Q} modifiers reported here. They include both enhancers; i.e., Heat shock RNAω (Hsrω), Sc2, Rpd3, C-terminal Binding Protein (dCtBP), and Glutathion-S Transferase 1 (GstS1) as well as suppressors; i.e., pasilla (ps), Ataxin-2 (dAtx2) and Nucleoporin-44A (Nu44A) of Ataxin^{82Q} toxicity. In the context of the HD model, however, we were unable to detect significant modification of the eye phenotype by these genes (Table 9 and data not shown).

Table 9 – Genes that modify Ataxin-1^{82Q} toxicity only

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<th>Gene / Accession Number</th>
<th>Modification of retinal phenotype</th>
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<td>Ataxin-1^{82Q}</td>
<td>Htt^{128Q}</td>
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<td>None by LOF</td>
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<td>Sc^{2ds16}</td>
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</tbody>
</table>

Su - Suppressor; En – Enhancer; LOF – Loss of Function; OE – Overexpression

These are Ataxin-1^{82Q} modifier genes that do not alter Htt^{128Q}-induced toxicity in our assays and specific experimental conditions. They include genes in some of the functional categories already discussed above (Table 8), which might indicate that the genes presented here as modifiers of SCA1 but not HD models, may be responsible for some of the differences between the two diseases.

Because of the possible relevance of, at least some, of the genes presented in Table 8 to the toxicity in SCA1, we have performed some more experiments using our SCA1 Drosophila model with one of the genes included in this category, Nup44A. The additional results obtained...
(Aggregation/OK107 Assay) are presented in Part 3, Results Section of this thesis.

**D- Genes that modify SCA1^{82Q}- and Htt^{128Q}-induced phenotypes in opposite ways**

While for the group of genes above mentioned we can argue that we are not able to detect a modification in the HD model because of the assays and specific conditions used, for the group that will now be introduced, in which we observe opposite effects in the two disease models, the results clearly demonstrate differences between the two disease models.

Therefore, of all the genes tested, this group is perhaps the most intriguing. In Table 10 we list the genes that induce a clear modification of both SCA1^{82Q} and Htt^{128Q}-induced phenotypes, but in opposite directions. Therefore, these modifiers are good candidates to uncover specific mechanisms of Ataxin-1^{82Q} and Htt^{128Q} toxicity. Representative examples of this interesting class of modifiers are described below in more detail.

**Table 10 – Genes that modify the toxicity of Ataxin-1^{82Q} and Htt^{128Q} differently**

<table>
<thead>
<tr>
<th>Gene / Accession Number</th>
<th>Modification of retinal phenotype</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ataxin-1^{82Q}</td>
<td>Htt^{128Q}</td>
</tr>
<tr>
<td>RNA Binding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mub / FBgn0014362</td>
<td>Su by OE</td>
<td>En by OE</td>
</tr>
<tr>
<td></td>
<td>En by LOF</td>
<td>None by LOF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP(3)3623 mub^D496</td>
</tr>
<tr>
<td>Signaling transduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vib / FBgn0026158</td>
<td>Su by OE</td>
<td>En by OE</td>
</tr>
<tr>
<td>Akt / FBgn0010379</td>
<td>En by OE</td>
<td>Su by OE</td>
</tr>
<tr>
<td></td>
<td>Su by LOF</td>
<td>En by LOF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP(3)651, EP(3)513 UAS-Akt1 Akt1^{9426}</td>
</tr>
<tr>
<td>PI3K92E / FBgn0015279</td>
<td>En by OE</td>
<td>Su by OE</td>
</tr>
<tr>
<td>Unknown Function</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG14438 / FBgn0029899</td>
<td>Su by OE</td>
<td>En by OE</td>
</tr>
<tr>
<td></td>
<td>En by LOF</td>
<td>Su by LOF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP(X)1438 CG14438^{109226}</td>
</tr>
</tbody>
</table>

Su - Suppressor; En - Enhancer; LOF – Loss of Function; OE – Overexpression

**Akt1**

Ataxin-1 and Huntingtin have been reported as substrates of Akt1, a serine/threonine kinase involved in numerous cell survival pathways (Chen et al., 2003; Colin et al., 2005; Gines et al., 2003; Humbert et al., 2002; Rangone et al., 2005).
Overexpression of Akt1 enhances SCA1^{82Q}-induced eye and retinal degeneration but suppresses degeneration caused by overexpression of Htt^{128Q}, and does not cause an eye phenotype by itself (Figure 35 and Supplemental Figure 1.B in Section V, Appendix I). Externally, overexpression of Akt1 is responsible for an increased disorganization and degeneration of the ommatidia of Ataxin-1^{82Q} flies (Figures 35.A-B). Internally, Akt1^{OE} increases the disorganization of photoreceptors in the retina of Ataxin-1^{82Q} animals and also causes tissue loss (Figures 35.E-F). Conversely, in the HD model, overexpression of Akt1 is able to reduce tissue loss and to increase the thickness of the retina when compared to flies expressing Htt^{128Q} alone (Figure 35.H-I). Furthermore, reducing the endogenous levels of Akt1 by having one copy of a loss of function allele ameliorates the SCA1^{82Q}-induced eye phenotype. These flies have improved organization of the ommatidia (Figures 35.C, control in Figure 35.D) and increased length of the retina (Figures 35.E and 35.G). However, lowering Akt1 levels enhances the Htt^{128Q}-induced phenotype, as shown by a reduction in the length of the retina (Figure 35.J, control in Figure 35.H). Loss of function of Akt1 also does not
show eye degeneration on its own (See Supplemental Figure 1.J in Section V, Appendix I).

Figure 35 - Akt1 modifies expanded SCA182Q and Htt128Q-induced eye phenotypes differently. SEM images of the external eye (A-D) and paraffin retinal sections (E-J) of flies expressing SCA182Q (A-G) or Htt128Q transgenes (H-J) and different levels of Akt1. Insets show a higher magnification view of the ommatidia and arrows indicate retina’s length. Akt1 overexpression (Akt1OE) aggravates SCA182Q-induced eye degeneration (B and F). Externally, the ommatidia are more disorganized and in some cases fused (B and compare with control in A). The retina is shorter, less organized and shows more tissue loss compared to controls (F, control is shown in E). The opposite effect is observed in Htt128Q flies over expressing Akt1, whose retina is more organized, thicker and with less tissue loss (compare I to the control in H).

On the other hand, reduced levels of Akt (Akt1LOF) improve the eye phenotype of SCA182Q flies that show less fusion and partial recovery the shape of ommatidia (C, compare with control in D) as well as increase in the thickness of the retina (G, control in E). The opposite effect is observed in Htt128Q flies with reduced levels of Akt1 (Akt1LOF) that display a shorter retina when compared to the control (J, control is shown in H). Arrows indicate retina thickness. Note that Akt1OE and Akt1LOF do not display an eye phenotype by themselves (see supplemental data in Section V, Appendix I).
Genotypes: A,D,E) yw, UAS-SCA1^{82Q}[F7]/+; GMR-GAL4/UAS-GFP. B,F) yw, UAS-SCA1^{82Q}[F7]/+; GMR-GAL4/+. C,G) yw, UAS-SCA1^{82Q}[F7]/+; GMR-GAL4/+; Akt^{F7}+/+. H) w; GMR-GAL4/UAS-GFP; Htt^{128Q}[M64]/+. I) w; GMR-GAL4/+; Htt^{128Q}[M64]/UAS-Akt. J) w; GMR-GAL4/+; Htt^{128Q}[M64]/Akt^{F7}. All SCA1^{82Q} transgenic flies are 1 day old and all flies carrying the Htt^{128Q} transgene are 6 days old. Flies used for SEM analysis were raised either at 25°C (A,B) or 27°C (C,D). All flies for paraffin vertical sections were raised at 25°C (E-K). Scale bar in SEM pictures is equivalent to 100µM (10µM in the magnification insets) and equivalent to 10µM in the retinal paraffin sections.

**mushroom-body expressed (mub)**

Overexpression of mub, a RNA binding protein, suppresses (Figures 36.A-B and Figures 36.E-F), and an heterozygous loss of function allele of mub enhances the SCA1^{82Q}-induced eye phenotype (Figures 36.C-D and Figures 36.E and 36.G; (Fernandez-Funez et al., 2000)). Note that neither mub^{OE} nor mub^{OF} have an eye phenotype on their own (See Supplemental Figures 1.C and 1.K, respectively, in Section V, Appendix I).

While mub^{OE} induces an increase in the length of the retina of flies expressing SCA1^{82Q}, mub^{OF} allele causes a reduction of the retinal thickness and promotes tissue loss in the retinas of SCA1^{82Q}/mub^{OF} flies. Interestingly, overexpression of mub dramatically enhances the Htt^{128Q}-induced eye phenotype, reducing the length of the retina by more than 50% when compared to Htt^{128Q}-expressing flies (Figures 36.H and 36.I). On the other hand, mub^{OF} is a very weak suppressor of the Htt^{128Q}-induced eye phenotype, promoting a mild increase in the length of the retina (Figure 36.J and control in Figure 36.H). Because this modification was so mild we decided to consider it as "No Modification/None" in Table 10.

Since I was particularly interested in this modifier, additional experiments were conducted in order to try to understand the mechanisms by which mub modifies polyglutamine-induced toxicity. Those additional results are presented in Part 3 of the Results Section of this thesis.
Figure 36 - *mub* overexpression suppresses *SCA1*Q and enhances *Htt*Q eye phenotypes. SEM images of the external eye (A-D) and paraffin retinal sections (E-J) of flies expressing *SCA1*Q (A-G) or *Htt*Q transgenes (H-J) and different levels of *mub*. Overexpression of *mub* improves the *SCA1*Q eye phenotype that shows better organization and less fusion of the ommatidia (B, compare with A) and improved retinal morphology (F, compare with E). Loss of function of *mub* aggravates the eye phenotype of *SCA1*Q flies that show deteriorated ommatidia and abnormal inter-ommatidial bristles (compare C to the control shown in D). *mub*LOF also increases tissue loss and disorganization and decreases the length of the retina.

In contrast, in *Htt*Q flies, *mub*OE is a strong enhancer of the retinal phenotype drastically reducing the length of the retina (compare I to the control in H). The heterozygous loss of function allele of *mub* (*mub*LOF) is a very mild suppressor modestly improving the organization and retinal length of *Htt*Q flies (compare J to the control in H). Insets in SEM images show a higher magnification view of the ommatidia. Arrows in paraffin sections show retina’s length. Note that *mub*OE and *mub*LOF do not display an eye phenotype by themselves (see Supplemental Data in Section V, Appendix I).

Genotypes: A, D, E) *yw, UAS-SCA1*Q(F7)/+; GMR-GAL4/UAS-GFP. B, F) *yw, UAS-SCA1*Q(F7)/+; GMR-GAL4/+; *mub*P31263/+ C, G) *yw, UAS-SCA1*Q(F7)/+; GMR-GAL4/+; *mub*04093/+ H) *w, GMR-GAL4/UAS-GFP; HttQ[M64]/+. I) *w, GMR-GAL4/+; HttQ[M64]/mubP31263. J) *w, GMR-GAL4/+; HttQ[M64]/mub04093. All *SCA1*Q transgenic flies shown are 1 day old and all flies expressing mutant *Htt* are 6 days old. Flies used for SEM analysis were raised at 27°C (A,B) or 25°C (C,D). All flies for
paraffin vertical sections were raised at 25°C (E-J). Scale bar in SEM pictures is equivalent to 100µM (10µM in the magnification insets) and equivalent to 10µM in the retinal paraffin sections.

**vibrator (vib)**

*vib* encodes a *Drosophila* orthologue of mammalian proteins implicated in lipid transport (Allen-Baume et al., 2002). *vib* is involved in phosphatidylinositol transfer with a role in actin-based processes and signal transduction (Spana and Perrimon, 1999), and a recent report relates its function, more specifically, to cytokinesis (Gatt and Glover, 2006).

In the SCA1 *Drosophila* model, increased levels of *vib* suppress the Ataxin-182Q eye phenotype (Figure 37). At the external level, *vib*OE improves the organization of the ommatidia and eye bristles of flies expressing SCA182Q (Figure 37.B, control in Figure 37.A). Internally, it is responsible for an increase in the length of the retina in SCA182Q/vibOE flies in comparison to flies expressing SCA182Q alone (Figures 37.C-D).

In the HD *Drosophila* model, *vib*OE is an enhancer of the eye phenotype, severely reducing to almost 50% the length of the retina of Htt128Q flies, but does not display an eye phenotype on its own (Figures 37.F and control in Figure 37.E; See Supplemental Figure 1.D in Section V, Appendix I).

Because no loss of function alleles of *vib* were available at the time of this study, only overexpression alleles were tested. However, even though only one allele is shown, two distinct *vib*OE alleles were analyzed, and the results obtained for both alleles in the two fly models are identical (data not shown).
Figure 37 - Overexpression of vib suppresses SCA1^{82Q} and enhances Htt^{128Q} eye phenotypes. SEM images of the external eye (A-B) and paraffin retinal sections (C-F) of SCA1^{82Q} (A-D) or Htt^{128Q} (E-F) flies over expressing vib. vib^{OE} improves the shape and organization of ommatidia characteristic of SCA1^{82Q} flies (compare B with control in A). Internally, overexpression of vib increases the length of the retina and improves its morphology (compare D with control C). In contrast, in Htt^{128Q} flies, vib^{OE} is an enhancer of the eye phenotype. Overexpression of vib in Htt^{128Q} flies promotes a decrease in the length of the retina compared to the phenotype of control flies (compare F with control E). Two independent overexpression alleles of vib were tested and the results obtain for both are equal. For simplicity only one allele is shown here. Also, the vib^{OE} alleles do not display an eye phenotype by themselves (Supplemental Data (Section VI, Appendix I); data not shown). Insets show a higher magnification view of the ommatidia and arrows indicate the length of the retina.

Genotypes: A,C) yw, UAS-SCA1^{82Q}[F7]/+; GMR-GAL4/UAS-GFP. B,D) yw, UAS-SCA1^{82Q}[F7]/+; GMR-GAL4/+; vib^{EP(3)651}/+. E) w; GMR-GAL4/UAS-GFP; Htt^{128Q}[M64]/+. F) w; GMR-GAL4/+; Htt^{128Q}[M64]/vib^{EP(3)651}. All SCA1^{82Q} transgenic flies shown are one day old and all HD model flies are six days old. Flies used for SEM analysis were raised at 27°C. All flies for paraffin vertical sections were raised at 25°C (C-F). Scale bar in SEM pictures is equivalent to 100µM (10µM in the magnification insets) and equivalent to 10µM in the retinal paraffin sections.
**CG14438**

CG14438 encodes a protein with 21 zinc finger domains whose function is still unknown. Overexpression of CG14438 is one of the strongest suppressors of the Ataxin-182Q eye phenotype we have identified (Figure 38).

**Figure 38** - Overexpression of CG14438 is responsible for the suppression of the SCA182Q-induced eye phenotype, but also for the enhancement observed in flies expressing Htt128Q. SEM images of the external eye (A-D) and paraffin sections of the retina (E-J) of SCA182Q (A-G) or Htt128Q (H-J) flies expressing different levels of CG14438. Overexpression of CG14438 in SCA182Q flies dramatically improves the organization and shape of ommatidia (B, control in A). Internally, CG14438OE strongly suppresses the retinal SCA182Q phenotype. Note the increase in the length and the improved organization of the retina (F, compare to control in E). The loss of function CG14438 allele, on the other hand, aggravates the shape and organization phenotypes of ommatidia, as well as the inter-ommatidial bristle phenotype of SCA182Q eyes (C, control in D). At the retinal level, CG14438OE reduces the length of the retina (G, control is shown in E). In contrast, overexpression of CG14438 in Htt128Q flies severly reduces the length of the retina and aggravates its organization (I, control in H). Conversely, CG14438 loss of function in the context of expanded Htt promotes an increase in the length of the retina along with a decrease in tissue loss (J, control in H). Both CG14438OE and CG14438OF do not display an eye phenotype by themselves (see Supplemental Data in Section V, Appendix I). Insets in
SEM images show a higher magnification view of the ommatidia. Arrows in paraffin sections show retina’s length.

Genotypes: A,D,E) yw, UAS-SCA1\textsuperscript{82Q}[F7]/+; GMR-GAL4/UAS-GFP. B,F) yw, UAS-SCA1\textsuperscript{82Q}[F7]/CG14438\textsuperscript{P00143}; GMR-GAL4/+; C,G) yw, UAS-SCA1\textsuperscript{82Q}[F7]/ CG14438\textsuperscript{CG00226}; GMR-GAL4/+; H) w; GMR-GAL4/UAS-GFP; Htt\textsuperscript{128Q}[M64]/+. I) w, CG14438\textsuperscript{P00143}/+; GMR-GAL4/+; Htt\textsuperscript{128Q}[M64]/+. All SC1\textsuperscript{82Q} transgenic flies shown are one day old and all HD model flies are six days old. Flies used for SEM analysis were raised at 27°C (A,B) or 25°C (C,D). All flies for paraffin vertical sections were raised at 25°C (E-J). Scale bar in SEM pictures is equivalent to 100µM (10µM in the magnification insets) and equivalent to 10µM in the retinal paraffin sections.

At the external level, CG14438\textsuperscript{0} improves the organization of the ommatidia (Figure 38.B, control is shown in Figure 38.A). Internally, CG14438\textsuperscript{OE} strongly suppresses the phenotype of the retina whose length and organization are improved in SCA1\textsuperscript{82Q}/CG14438\textsuperscript{OE} flies compared to Ataxin-1\textsuperscript{82Q} alone. (Figure 38.F, control in Figure 38.E). On the other hand, loss of function of CG14438 enhances the SCA1\textsuperscript{82Q}-induced eye phenotype, but does not display an eye phenotype on its own (Figures 38.C-D, 38.E, 38.G and Supplemental Figure 1.M in Section V, Appendix I). Externally the ommatidia and inter-ommatidial bristles are more disorganized (Figure 38.C, control in Figure 38.D), and internally the retina is thinner and also less organized (Figure 37.G, control in Figure 38.E). In contrast, in the HD model, overexpression of CG14438 enhances the Htt\textsuperscript{128Q} eye phenotype and does not have an eye phenotype when expressed alone (Figures 38.H-I and Supplemental Figure 1.E shown in Section V, Appendix I). The length of the retina of Htt\textsuperscript{128Q}/CG14438\textsuperscript{OE} flies is dramatically reduced and there is also an increase in its disorganization in comparison to flies expressing Htt\textsuperscript{128Q} alone (Figure 38.I; compare with Figure 38.H). Conversely, the loss of function allele causes an increase in length of the retina, as well as a decrease in retinal tissue loss when compared to control flies (Figure 38.J and Figure 38.H).

**E- Validation of the Ataxin-1\textsuperscript{82Q} and Htt\textsuperscript{128Q} eye modifier genes in a motor performance assay**

To investigate whether the genetic interactions described above in the eye are also valid in the CNS, we carried out a motor performance test that
measures climbing ability of flies as function of age. We used the nervana-GAL4 driver, which directs expression of the transgenes of interest to the CNS, and measured the number of flies that are able to climb above a 5cm line marked in the vials in 18s. Using this assay we tested specific genes from Table 9 that do not display a phenotype by themselves when the nervana-GAL4 driver is used.

Wild type or other control strains perform well in the climbing assay until late in life (Figures 39.A and 39.B, blue line). In contrast, SCA182Q and Htt128Q-expressing flies perform well only as young adults, becoming progressively more impaired as they age (red line in Figures 39.A and 39.B, respectively). In SCA182Q-expressing flies, climbing performance is impaired by day 20-21 (when only 50% of the flies are still able to climb) in comparison to control flies that only start to lose their ability to climb by day 32 (Figure 39.A- red line, control in blue). Similarly, in the HD model, by day 24, the climbing performance is impaired (only 50% of the flies are still able to climb) while in the control, at this age, 70% of the flies are still able to climb (Figure 39.B- red line, control in blue).

As shown in Figure 39.A, overexpression of the Ataxin-182Q suppressors mub and vib improves the climbing performance of the SCA182Q-expressing flies (lines dark green and purple, respectively). Flies co-expressing mutant Ataxin-1 and either mub or vib are able to climb normally until a later age. Note that while ≈50% of the flies expressing SCA182Q fail to climb by day 21, in flies that over express either mub or vib in addition to SCA182Q it takes between 28 and 25 days, respectively, to observe a similar motor impairment.

The results obtained with the climbing assay for the HD model also mimic the ones previously obtained using the eye system: overexpression of mub and vib worsens the Htt128Q-induced motor performance (Figure 39.B, lines dark green and purple, respectively; flies expressing Htt128Q alone are represented by a red line). The climbing performance of Htt128Q/mubOE or Htt128Q/vibOE flies is impaired by day 19 and 21, respectively, where 50% of the flies fail to climb. In comparison, by this age, ≈80% of flies that express Htt128Q alone are still able to climb, and only by day 24 there is a decrease to
50%, in the percentage of flies able to climb. Note that mubOE and vibOE do not have a climbing phenotype on their own (Figures 39.A and 39.B, lines light green and orange, respectively).

Figure 39 - Modification of SCA1^{82Q} and Htt^{128Q} neuronal phenotypes in a motor performance assay. Quantification of the climbing performance of SCA1^{82Q} (A) and Htt^{128Q} (B) flies as a function of age. The nervana-GAL4 driver was used to express the mutant proteins in the CNS. (A) SCA1^{82Q} flies (red line) behave normally in this assay early in life but their motor performance is impaired prematurely when compared to controls (blue line). Note that 50% of controls still climb normally at day 30-32, whereas only 50% of SCA1^{82Q} flies climb normally at day 20-21. Overexpression of either mub (dark green) or vib (purple) partially restores climbing ability with 50% climbing at days 25-28. Additional controls that over express mub or vib in the absence of the SCA1^{82Q} transgene behave normally (light green and orange respectively). (B) Htt^{128Q} flies (red line) shown normal climbing performance early in
life but their motor performance also declines prematurely when compared to controls (blue line). Only 50% of Htt\textsuperscript{128Q} flies climb normally at day 23-24. Overexpression of \(mub\) (dark green) or \(vib\) (purple) aggravates the Htt\textsuperscript{128Q}-induced climbing phenotype. Note that 50% of the flies are impaired at days 19-21. Neither \(mub\)\textsuperscript{OE} nor \(vib\)\textsuperscript{OE} have a climbing phenotype on their own (light green and orange respectively). Error bars represent standard deviation.

Genotypes:  

Nerv\textgreater LacZ) w;\textit{nervana}\textendash GAL4\textasciitilde \textit{UAS}\textendash LacZ  
Nerv\textgreater LacZ; SCA1\textasciitilde 82Q) w;\textit{nervana}\textendash GAL4\textasciitilde \textit{UAS}\textendash SCA1\textasciitilde 82Q\textasciitilde [M6]/+  
Nerv\textgreater SCA1\textasciitilde 82Q\textasciitilde /\textit{mub}\textasciitilde 82Q) w;\textit{nervana}\textendash GAL4+/; \textit{UAS}\textendash SCA1\textasciitilde 82Q\textasciitilde /\textit{mub}\textasciitilde 82Q\textasciitilde [M6]/+  
Nerv\textgreater SCA1\textasciitilde 82Q\textasciitilde /\textit{vib}\textasciitilde 82Q) w;\textit{nervana}\textendash GAL4+/; \textit{UAS}\textendash SCA1\textasciitilde 82Q\textasciitilde /\textit{vib}\textasciitilde 82Q\textasciitilde [M6]/+  
Nerv\textgreater LacZ; Htt\textasciitilde 128Q) w;\textit{nervana}\textendash GAL4\textasciitilde \textit{UAS}\textendash Htt\textasciitilde 128Q\textasciitilde [M6]/+  
Nerv\textgreater Htt\textasciitilde 128Q\textasciitilde /\textit{mub}\textasciitilde 128Q) w;\textit{nervana}\textendash GAL4+/; \textit{UAS}\textendash Htt\textasciitilde 128Q\textasciitilde [M6]/\textit{mub}\textasciitilde 128Q\textasciitilde  
Nerv\textgreater Htt\textasciitilde 128Q\textasciitilde /\textit{vib}\textasciitilde 128Q) w;\textit{nervana}\textendash GAL4+/; \textit{UAS}\textendash Htt\textasciitilde 128Q\textasciitilde [M6]/\textit{vib}\textasciitilde 128Q\textasciitilde  

Therefore, the observation that certain modifier genes have opposite effects on Htt\textasciitilde 128Q and SCA1\textasciitilde 82Q-induced toxicity is not only valid in the eye, but it is also true when using a motor behavior test based on expression of these proteins in the CNS.

\textbf{F- Alteration of Ataxin-1\textasciitilde 82Q and Htt\textasciitilde 128Q protein aggregation in NIs does not correlate with suppression or enhancement of neurotoxicity}

We next investigated whether genes modifying the toxicity of Ataxin-1\textasciitilde 82Q and Htt\textasciitilde 128Q also modify their aggregation into NIs. Therefore, we generated flies that over express the SCA1\textasciitilde 82Q or Htt\textasciitilde 128Q transgenes under the control of \textit{OK107-GAL4}, a driver directing expression to well-defined subset of CNS neurons (Figure 40 and Figure 41).

\textbf{Figure 40 - Schematic representation of the adult brain and ventral ganglion (left) and confocal image of the CD8-GFP pattern of expression using \textit{OK107-GAL4} driver in the adult Ventral Ganglion}
In the brain, OK107-GAL4 directs expression of the transgene of interest to the mushroom bodies (green arrows) and to some parts of the optical lobes (black arrows). At the level of the Ventral Ganglion, OK107-GAL4 directs expression to very precise subset of cells and neuronal projections as shown in the image on the right, using CD8:GFP. (Adapted from Agrawal et al., 2005; Branco et al., 2007). Genotype of the image on the right: w; FRTG13, UAS:CD8-GFP/+; OK107-GAL4/+; Flies shown are two days old and were raised at 25°C.

Figure 41 - Expression pattern of the OK107-GAL4 driver in the Ventral Ganglion of adult. Confocal images of Drosophila neurons located in the ventral ganglion of adult flies that express UAS:CD8-GFP under the control of the OK107-GAL4 driver (A). This driver leads to expression in a cluster of neurons located at the posterior end of the ventral ganglion (red box). Note that CD8-GFP is also detected in the corresponding neuronal projections (arrowheads) that are particularly abundant in the posterior end of the ventral ganglion (B). Higher magnification view of the region highlighted in (A). Genotypes: A,B) w; FRTG13, UAS:CD8-GFP/+; OK107-GAL4/+. Flies shown are 2 days old and were raised at 25°C.

Figure 42 shows the results obtained for both SCA1 and HD Drosophila models (panels A-F and H-M, respectively) and the quantification of the percentage of cells that contain Ataxin-1 or Huntingtin aggregates (panels (G) and (N), respectively). Note that, with this driver and in the specific experimental conditions used, we observe cells with single or multiple NIs in the SCA1 model whereas in the HD model the majority of the cells have single NIs. Of these, we observe that ~43% of the Ataxin-1^82Q–expressing neurons have NIs, whereas ~13% of the neurons that express Htt^128Q have NIs. Also, while in the SCA1 model we can detect NIs of different size, in the HD model only cells with relatively small NIs are detected.
Overexpression of the Ataxin-1\(^{82Q}\) suppressor *CG14438* leads to an increase in the percentage of NIs containing Ataxin-1\(^{82Q}\) aggregates, when compared with appropriate *SCA1\(^{82Q}\)* controls that over express *LacZ* (Figures 42.A-B and Figure 42.G). Conversely, flies that express *SCA1\(^{82Q}\)* and carry the *CG14438\(^{LOF}\)* allele have fewer cells with Ataxin-1\(^{82Q}\) NIs than the corresponding controls (Figure 42.E and 42.F, quantification in Figure 41.G). In contrast to *CG14438\(^{OE}\)*, overexpression of *vib* or *CHIP*, also suppressors of Ataxin-1\(^{82Q}\) toxicity (Al-Ramahi et al., 2006; Branco et al., 2007), causes a decrease in the percentage of cells that contain NIs (Figures 42.C and 42.D, control in Figure 42.A; quantification in Figure 42.G). This observation is valid for both *vib* alleles tested (Figures 42.C and data not shown). In the HD model, overexpression of *CG14438*, enhancer of Htt\(^{128Q}\)-induced toxicity, also promotes an increase in the percentage of cells with NIs (Figures 42.I and 42.N, control is shown in Figure 42.H). However, we could not detect alterations in NIs formation with the *CG14438* loss of function allele (Figure 42.M, control is shown in Figure 42.L and quantification in Figure 42.N). Overexpression of *vib*, enhancer of Htt\(^{128Q}\) eye phenotype, for the two alleles tested, also increases the percentage of cells where Htt\(^{128Q}\) NIs can be detected (Figures 42.J and data not shown; quantification is shown in Figure 42.N). In contrast to what was seen for the SCA1 model, in the HD model overexpression of *CHIP* is responsible for a dramatic increase in the percentage of cells containing Htt NIs (Figure 42.K, control in Figure 42.H and quantification in panel N).

In summary, we identified genes modifying toxicity that also alter NI formation. However, there is no correlation between modification (suppression or enhancement) of the toxicity and modification (increase or decrease) in the formation of NIs.
Figure 42 – Modifications of the eye phenotype do not correlate with increase or decrease in the formation of nuclear inclusions. Confocal images of an anti-Ataxin-1 (A-F) or anti-Huntingtin (H-M) immunofluorescence staining in specific neurons of the CNS (adult ventral ganglion). The OK107-GAL4 driver was used to drive expression of FRTG13, UAS:CD8-GFP, SCA1\(^{82Q}\) or FRTG13, UAS:CD8-GFP; Htt\(^{128Q}\) transgenes and the genes to be tested. Arrowheads indicate cells with NIs. (G and N) show quantification of the percentage of cells that contain Ataxin-1 or Htt NIs, respectively.
Overexpression of CG14438 (a suppressor of SCA1<sup>82Q</sup>) leads to an increase in the percentage of cells with Ataxin-1<sup>82Q</sup> NIs (B, compare with control in A and quantification in the corresponding bar in panel G). Conversely, reduced levels of CG14438 (an enhancer of SCA1<sup>82Q</sup>) promote a decrease in the number of cells that contain NIs (F; control in E and quantification in G). On the other hand, overexpression of vib or CHIP, both suppressors of SCA1<sup>82Q</sup>, lead to a decrease in the number of cells with Ataxin-1 NIs (panels C and D, respectively; quantification in G). Note the dramatic decrease in NIs formation for the case of CHIP (D). Images in the right top corner of the chart in panel (G) illustrate a cell with diffused Ataxin-1 signal, a cell with a single Ataxin-1 NI and a cell with multiple NIs of the Ataxin-1 protein (from left to right).

As in the SCA1 model, in Htt<sup>128Q</sup> flies, overexpression of CG14438 induces an increase in the number of cells that contain NIs (I; control in H and quantification in N). Note however that CG14438 is an enhancer of the Htt<sup>128Q</sup>-induced eye phenotype. Loss of function of CG14438 failed to alter the percentage of cells with Htt NIs (M, control is shown in L; quantification in N). Similarly, vib is also an enhancer of the Htt<sup>128Q</sup>-induced eye phenotype, induces an increase in the number of cells that contain NIs (J, control is shown in H and quantification in N). On the other hand, CHIP, a suppressor of the HD model eye phenotype, increases dramatically the percentage of cells containing NIs (panel K, the corresponding control is shown in H and the quantification in N). In panel (N), the figure on the top right corner shows the difference between Htt NIs and diffuse Htt signal (from left to right). Error bars represent standard deviation. All the alterations observed in terms of percentage of cells containing NIs marked with an asterisk are statistically significant (Results were analyzed by Student’s t-test; *p<0.001 and **p<0.0001). For each experiment at least 8 independent animals (with an average of 32 cells each) were analyzed. Scale bar is equivalent to 10µM.

Genotypes: A) w; UAS-LacZ/+; FRTG13, UAS:CD8-GFP, UAS-SCA1<sup>82Q</sup>[M6]-R4/+; OK107-GAL4/+; B) w, CG14438<sup>PPO1438</sup>/+; FRTG13, UAS:CD8-GFP, UAS-SCA1<sup>82Q</sup>[M6]-R4/+; OK107-GAL4/+; C) w; FRTG13, UAS:CD8-GFP, UAS-SCA1<sup>82Q</sup>[M6]-R4/vib<sup>UAS:CD8-GFP</sup>, OK107-GAL4/+; D) w; UAS-CHIPs/+; FRTG13, UAS:CD8-GFP, UAS-SCA1<sup>82Q</sup>[M6]-R4/+; OK107-GAL4/+; E) w; FRTG13, UAS:CD8-GFP, UAS-SCA1<sup>82Q</sup>[M6]-R4/+; OK107-GAL4/+; F) w, CG14438<sup>B00228</sup>, FRTG13, UAS:CD8-GFP, UAS-SCA1<sup>82Q</sup>[M6]-R4/+; OK107-GAL4/+; H) w; FRTG13, UAS:CD8-GFP/UAS-LacZ; Htt<sup>128Q</sup>[M64]/+; OK107-GAL4/+; I) w, CG14438<sup>PPO1438</sup>/+; FRTG13, UAS:CD8-GFP/+; Htt<sup>128Q</sup>[M64]/+; OK107-GAL4/+; J) w; FRTG13, UAS:CD8-GFP/+; Htt<sup>128Q</sup>[M64]/+; vib<sup>UAS:CD8-GFP</sup>, OK107-GAL4/+; K) w; FRTG13, UAS:CD8-GFP/UAS-CHIPs; Htt<sup>128Q</sup>[M64]/+; OK107-GAL4/+; L) w; FRTG13, UAS:CD8-GFP/+; Htt<sup>128Q</sup>[M64]/+; OK107-GAL4/+; M) w, CG14438<sup>B00228</sup>/+; FRTG13, UAS:CD8-GFP/+; Htt<sup>128Q</sup>[M64]/+; OK107-GAL4/+.

All flies used are two days old and were raised at 25°C.

**DISCUSSION**

We have used Drosophila models of SCA1 and HD for a comparative analysis of genetic factors involved in Ataxin-1 and Huntingtin-induced neurotoxicity. For that, we have tested in an HD Drosophila model generated in the Botas Laboratory previously know genetic modifiers of a SCA1 Drosophila model also generated in the Botas Laboratory.
We found genetic modifiers involved in a variety of cellular functions that affect similarly the SCA1 and HD *Drosophila* models. For other genes we did not detect modification of Huntingtin toxicity. Surprisingly, we also identified modifier genes that have antagonistic effects on Ataxin-1 and Huntingtin toxicity. We found no consistent correlation between modifier gene activity and nuclear inclusion formation.

*Genes that modify the phenotypes of SCA1 and HD Drosophila models similarly*

Among this group of modifiers there are genes involved in protein folding, protein turnover, apoptosis and transcriptional regulation (Table 8). These are cellular processes that have been implicated in polyglutamine toxicity in general, and also in SCA1\(^{82Q}\) and Htt\(^{128Q}\)-induced neurodegeneration (Ghosh and Feany, 2004; Paulson et al., 2000; Riley and Orr, 2006; Soto, 2003; Sugars and Rubinsztein, 2003; Wellington and Hayden, 2000). The observation that they modulate similarly the Ataxin-1\(^{82Q}\) and Htt\(^{128Q}\) phenotypes in *Drosophila* provides further validation for these fly models, and suggests that novel modifiers identified in *Drosophila* may also be relevant to SCA1 and HD pathogenesis. Interestingly, we found specific transcription factors among the common modifiers suggesting that at least some of the genetic pathways responsible for altered transcription are common in HD and SCA1.

Other modifiers in this group are genes for which there was previously little evidence of their involvement in both Ataxin-1\(^{82Q}\) and Htt\(^{128Q}\) toxicity. These include *cpo* and *pum*, two genes encoding RNA binding proteins. RNA-mediated mechanisms and RNA binding proteins have been implicated in a number of neurodegenerative diseases and muscular dystrophies (Gallo et al., 2005; Gatchel and Zoghbi, 2005). These include disorders caused by expansion of non-translated repeats that lead (FXTAS, DM1 and DM2), or are suspected to lead (SCA8, SCA10, SCA12), to gain of RNA toxicity (Jin et al., 2003; Ranum and Day, 2004). Other neurodegenerative diseases may be caused by abnormal alternative splicing (FTDP-17, (Hutton et al., 1998) and ALS, (Robertson et al., 2003)), or by mutations in factors involved in splicing
or mRNA localization (SMA, (Frugier et al., 2002)). We reported that RNA binding proteins form a functional class among the Ataxin-1 genetic modifiers (Fernandez-Funez et al., 2000). However, Ataxin-1 itself was later shown to have RNA-binding activity in vitro (Yue et al., 2001), and whether other polyglutamine diseases besides SCA1 implicate RNA-mediated mechanisms remains unclear (Faber et al., 1998; Gallo et al., 2005; Kegel et al., 2002; Kita et al., 2002; McLeod et al., 2005; Peel et al., 2001). Our data provides strong support for the hypothesis that RNA binding proteins also have a role in Htt\textsuperscript{128Q}-induced neurodegeneration, and suggests that abnormal RNA-processing may be a more general pathogenic mechanism in neurodegenerative disorders than previously anticipated.

Two other genes not previously known to modify Ataxin-1\textsuperscript{82Q} and Htt\textsuperscript{128Q} toxicity in a similar manner are 14-3-3\(\epsilon\) and RhoGAPp190, both of which participate in a variety of signal transduction pathways. RhoGAP proteins mediate growth factor dependent regulation of GTPases (Bernards and Settleman, 2005), and have been implicated in cytoskeletal reorganization and axonal growth (Ng and Luo, 2004). 14-3-3 proteins are involved in many cellular processes, and they are binding partners of a large number of phosphoproteins (Kjarland et al., 2006). We found that loss-of-function of 14-3-3\(\epsilon\) suppresses, and overexpression enhances, both Ataxin-1\textsuperscript{82Q} and Htt\textsuperscript{128Q} toxicity. In the case of Ataxin-1\textsuperscript{82Q} we understand the mechanism behind these modifications. Upon phosphorylation of Ataxin-1\textsuperscript{82Q} by Akt, 14-3-3\(\epsilon\) binds and stabilizes the phosphorylated protein, which leads to its accumulation and neurotoxic effects (Chen et al., 2003). In the case of Htt\textsuperscript{128Q} the mechanism of modification is less clear. We expect, however, that it is different from the Ataxin-1\textsuperscript{82Q} mechanism because Akt has a protective role in Htt-induced neurodegeneration (Branco et al., 2007; Colin et al., 2005; Gines et al., 2003; Humbert et al., 2002; Rangone et al., 2005). 14-3-3 proteins co-localize with Huntingtin perinuclear inclusions in cell culture (Waelter et al., 2001), and interact with HAP-1, a protein involved in intracellular trafficking (Rong et al., 2007a). Thus, 14-3-3\(\epsilon\) may modify Htt\textsuperscript{128Q} toxicity by modulating the trafficking impairments caused by this expanded protein.
Genes that modify the phenotypes of SCA1 but not HD Drosophila models

These are Ataxin-1^{82Q} modifier genes that do not alter Htt^{128Q}-induced toxicity in our assays and experimental conditions. They include genes in some of the functional categories discussed above (Table 9). Although negative results are difficult to interpret, some of these genes may account for differences between SCA1^{82Q} and Htt^{128Q}-induced toxicity.

Modifier genes that have antagonistic effects on the phenotypes of SCA1 and HD models.

Surprisingly, we found modifier genes that are common to both disease models but have antagonistic effects on SCA1^{82Q} and Htt^{128Q}-induced toxicity (Table 10).

Of all the genes in this group, the best understood is Akt. As described above, Akt phosphorylates Ataxin-1 allowing binding of 14-3-3 proteins. This leads to increased Ataxin-1 stability and thus promotes neuronal degeneration (Chen et al., 2003). Huntingtin is also a substrate of Akt, and Akt phosphorylation of Htt at Serine 421 is crucial for neuroprotection mediated by the Insulin Growth Factor 1 (IGF-1) signaling pathway (Humbert et al., 2002; Rangone et al., 2004). In fact, Akt has been extensively implicated in the pathogenesis of HD (Colin et al., 2005; Gines et al., 2003; Humbert et al., 2002; Rangone et al., 2004).

The work by MacDonald and co-workers (Gines et al., 2003), suggests that the protective effect exerted by Akt in early stages of the disease is lost at end stages due to cleave of Akt by Caspase 3 into its inactive form (Colin et al., 2005). Furthermore, MacDonald and collaborators propose that stimulation of the Akt pathway in the late stages of the disease might be beneficial and an interesting therapeutic approach to further test, emphasizing once more the importance of the Akt pathway in the pathogenesis of HD (Gines et al., 2003). In addition, the work by Saudou and collaborators (Humbert et al., 2002) shows that the phosphorylation of Htt by Akt is essential in order to induce the neuroprotective effects of IGF-1, as mentioned earlier, but also that additional mechanisms are mediating this protective effect. Finally, also a work developed by Saudou and co-workers...
(Rangone et al., 2005), shows that Akt also protects from Htt toxicity by phosphorylating Arfaptin 2 and promoting proteasome function.

The results obtained with the HD *Drosophila* model generated in the Botas Laboratory here presented, need a careful interpretation. While in (Gines et al., 2003; Humbert et al., 2002), knock-in models or expression of the full length protein, respectively, are used, in (Colin et al., 2005 and Rangone et al., 2005), as in our study, N-terminal constructs were used. Therefore, the results by (Humbert et al., 2002) are relative to a phosphorylation in a Serine residue that is not present in the construct used to generate our fly model (Ser^{421}). However, and as referred in (Rangone et al., 2005), other Akt substrates can contribute to the pathogenesis observed in HD patients. Akt has been pointed as a regulator of ubiquitination, degradation and stabilization of other proteins, therefore, other key proteins yet to be identified as targets of Akt can interfere with toxicity in HD. Furthermore, as indicated in (Rangone et al., 2005), the pro-survival effect exerted by Akt can also be caused by interference with the endogenous levels of regulators of cell growth and/or cell survival.

Therefore, and because the N-terminal Htt fragment in *Htt^{128Q}* flies lacks Ser^{421}, Akt-mediated protection against *Htt^{128Q}* toxicity is likely indirect and mediated by CG17184, the *Drosophila* orthologue of Arfaptin 2, or another substrate of Akt. More studies will need to be conducted in order test this hypothesis or identify other substrates of Akt that interfere with *Htt^{128Q}*- induced toxicity.

Note, however, that our results with the *Drosophila* HD model are in agreement with the observations from the literature as overexpression of Akt improves, and reduced levels of Akt aggravate, the phenotype of *Htt^{128Q}*- expressing flies. Thus, these findings further validate the HD *Drosophila* model, and support the relevance of other modifiers of *Htt^{128Q}*-induced toxicity identified in this study.

*mub* encodes the *Drosophila* orthologue of human poly(rC) binding protein 3, and both are members of a conserved family of proteins that contain three RNA-binding KH domains, and have been implicated in mRNA
stabilization, translational activation, and translational silencing (Makeyev and Liebhaber, 2002). Overexpression of \textit{mub} ameliorates Ataxin-1\textsuperscript{82Q} but enhances Htt\textsuperscript{128Q} phenotypes in the eye; conversely, \textit{mub} loss of function aggravates Ataxin-1\textsuperscript{82Q} and mildly suppresses Htt\textsuperscript{128Q} phenotypes. These results were reproduced independently in a motor performance assay indicating that these interactions are not specific to the eye, and confirming their relevance to neurons. These observations further suggest that RNA-mediated mechanisms are relevant to Huntingtin neurotoxicity.

Over-expression of \textit{vib} suppresses SCA1\textsuperscript{82Q} and enhances Htt\textsuperscript{128Q}-induced toxicity, both in the eye and motor performance assays. \textit{vib} encodes a conserved phospholipid transporter, which are proteins involved in lipid metabolism and membrane trafficking, and implicated in numerous cell-signaling pathways (Routt and Bankaitis, 2004). Accumulating evidence suggests that the metabolism of phospholipids in neural membranes is abnormal in neurodegenerative diseases, such as Friedreich ataxia (Ross et al., 2000) and Alzheimer's Disease (Farooqui et al., 2004). Less evidence is available for the involvement of phospholipid metabolism and signaling in HD, and particularly in SCA1. However, Huntingtin has been implicated in membrane trafficking (Rong et al., 2007b; Truant et al., 2006). Also, it has been suggested that electrostatic interactions with acidic phospholipids mediate binding of Huntingtin to membranes (Kegel et al., 2005). The genetic interactions between \textit{vib} and Ataxin-1\textsuperscript{82Q} and Htt\textsuperscript{128Q} suggest that abnormal phospholipid metabolism may be a mechanism of pathogenesis also in polyglutamine diseases.

Over-expression of \textit{CG14438} suppresses Ataxin-1\textsuperscript{82Q} and enhances Htt\textsuperscript{128Q} eye phenotypes; conversely its loss of function enhances the Ataxin-1\textsuperscript{82Q} and suppresses the Htt\textsuperscript{128Q} phenotypes. \textit{CG14438} is an uncharacterized gene encoding a long protein (3321 amino acids). It contains 21 C2H2-type zinc-fingers, which are domains that can mediate interactions to DNA, RNA or other proteins. The unknown function of this \textit{Drosophila} gene provides little insight into possible mechanisms by which it may modulate SCA1\textsuperscript{82Q} and Htt\textsuperscript{128Q}–induced toxicity.
Alterations of Ataxin-1^{82Q} and Htt^{128Q} protein aggregation in NIs do not correlate with suppression or enhancement of neurotoxicity

For some modifier genes we investigated whether modulation of the eye and motor performance phenotypes correlates with changes in the formation of NIs. We found no direct correlation between NI formation and toxicity: for two Ataxin-1^{82Q} suppressors (vib^{OE} and CHIP^{OE}) the percentage of neurons containing NIs was reduced, whereas for a third suppressor the percentage of neurons containing NIs was increased (CG14438^{OE}). In the case of Htt^{128Q}, we found increased percentage of neurons containing NIs for both enhancers (CG14438^{OE} and vib^{OE}) and a suppressor (CHIP^{OE}) of the Htt^{128Q}-induced toxicity. These results are consistent with the idea that some modifiers may function by reducing the levels of mutant protein, while others function downstream of protein synthesis/clearance. Thus, our results argue that NI formation is not a good read-out of polyglutamine-induced neuronal toxicity, and argue for caution when interpreting results from chemical screens in which the screening assay is based on aggregation of the mutant protein.

In summary, this comparative study of SCA1 and HD models in Drosophila has lead to the identification of novel modifier genes that fall in two important categories. Those modifying SCA1^{82Q} and Htt^{128Q}-induced neurotoxicity in a similar manner point to new potential therapeutic targets that may be common to both diseases. A second class of modifiers genes has antagonistic effects on SCA1^{82Q} and Htt^{128Q}-induced toxicity and point to disease-specific mechanisms of pathogenesis. This last group will contribute to the understanding of the distinct clinical and pathological features among polyglutamine diseases.
Part 3

Other Relevant Data Obtained for Nup44A and mub
During the course of the past years, other assays and/or approaches were performed with some of the genes listed in Tables 8, 9 and 10. In the next pages we will present data obtained with Nup44A, a modifier of SCA1<sup>82Q</sup>-induced toxicity in the eye but not Htt<sup>128Q</sup>-induced degeneration, and also additional data for <i>mub</i>, one of the interesting modifiers that we have found that has an opposite effect in our SCA1 and HD <i>Drosophila</i> models. None of this data is included in what was published in (Branco et al., 2007). However, we have considered that the results obtained can be useful for future studies with any of these two modifiers, and decided to include it in this thesis. For <i>mub</i> we have significant more data since one of the first aims of this thesis was to understand the mechanisms underlying the modification induced by <i>mub</i> in the two fly models.

### A- Genes that modify SCA1 but not HD <i>Drosophila</i> models: the case of Nup44A

During the course of this genetic comparison we have identified genes that modify SCA1<sup>82Q</sup>-induced eye toxicity but not Htt<sup>128Q</sup>-induced eye phenotype (Table 9). Although negative results are difficult to interpret, some of these genes may be relevant for at least SCA1 pathogenesis, and may account for some of the clinical and pathological differences between SCA1<sup>82Q</sup> and Htt<sup>128Q</sup>-induced toxicity. Because of its possible relevance, we decided to investigate the role of nucleoporin-44A (Nup44A) for the pathogenesis of expanded Ataxin-1 in our SCA1 <i>Drosophila</i> model.

Overexpression of Nup44A (<i>Nup44A<sup>EP2417</sup></i>) was previously published as a suppressor of our SCA1 model (Fernandez-Funez et al., 2000). Nup44A encodes a nucleoporin, likely a constituent of a nuclear pore. Its function is estimated to be relevant for intracellular protein transport, exocytosis or RNA localization (inferred from sequence similarity; http://www.flybase.net/reports/FBgn0033247.html; Figure 43).
**Figure 43 – Predicted structure of the Nup44A protein.** Protein domains predicted by the SMART program provided by EMBL (http://smart.embl-heidelberg.de). Nup44A is composed of 5 WD-40 domains, also known as WD or beta-transducin repeats, which are short motifs (approximately 40 amino acid long) often terminating in a Trp-Asp (W-D) dipeptide. WD-repeat proteins are a large family found in all eukaryotes and are implicated in a variety of functions that can go from signal transduction and transcription regulation to cell cycle control and apoptosis. A common function of all WD-repeat proteins is coordination of multi-protein complex assemblies, where the repeating units function as a rigid scaffold for protein interactions. The specificity of the proteins is determined by the sequences outside the repeats themselves. Adapted from http://smart.embl-heidelberg.de/smart/show_motifs.pl.

BLAST analysis reveals homology to the human protein sec13-like or seh1L (E value: $1e^{-10^5}$), a protein part of a nuclear pore complex, Nup107-160. This protein shares 34% amino acid identity with yeast Seh1 and 30% identity with yeast Sec13 (Cronshaw et al., 2002; Enninga et al., 2003; Loiodice et al., 2004).

Our results show that overexpression of *Nup44A* restores ommatidial arrangement of the external eye in SCA1^{82Q} flies and increases the length of the retina (Figure 44). However, Htt^{128Q} flies do not seem to be affected by the overexpression of *Nup44A* (data not shown).
Figure 44 - Overexpression of Nup44A suppresses SCA1^{82Q}-induced eye phenotype. Light Microscope eye images (A-B) and SEM images of the external eye (C-D) of flies expressing SCA1^{82Q} and an overexpression of Nup44A (B and D) and of flies overexpressing SCA1^{82Q} alone (Control, A and C). Overexpression of Nup44A improves the SCA1^{82Q} eye phenotype that shows less necrosis signs (B, compare with A), but also better organization and less fusion of the ommatidia (D, compare with C). Insets in SEM images show a higher magnification view of the ommatidia.

Genotypes: A,C) yw, UAS-SCA1^{82Q}[F7]/+; GMR-GAL4/UAS-GFP. B,D) yw, UAS-SCA1^{82Q}[F7]/+; GMR-GAL4/ Nup44A^EP2417. All flies are one day old, and were raised at 27°C. Scale bar is equivalent to 100µM (10µM in the magnification insets of SEM images).

We know from previous results by Zoghbi and colleagues that the nuclear localization is crucial for the toxicity of Ataxin-1 (Klement et al., 1998) and, therefore, since Nup44A could be part of a membrane porous, we decided to investigate whether Nup44A affected the translocation of Ataxin-1 to the nucleus.

In order to answer this question we used the OK107 Gal4 Assay developed in the Botas laboratory, described in this thesis and in (Branco et al., 2007). The assay allows us over express the SCA1^{82Q} transgene under the control of OK107-GAL4, a driver directing expression to a defined subset of CNS neurons and to analyze the formation of NIs in the cells. We analyzed
alterations in the formation of NIs and counted the percentage of cells containing aggregates versus the number of cells expressing diffused Ataxin-1 signal. Note, once again, that what we have considered as “diffused” Ataxin-1 signal might be in fact micro-aggregates that because of the techniques used cannot be resolved as aggregates and are therefore considered as soluble Ataxin-1 protein.

Figure 45 – Modifications of the SCA182Q-induced eye phenotype by Nup44AOE does not interfere with the formation of nuclear inclusions. Confocal images of an anti-Ataxin-1 immunofluorescence staining of neurons located in the ventral ganglion of adult flies. The OK107-GAL4 driver was used to drive expression of the FRTG13, UAS:CD8-GFP, SCA182Q transgene and Nup44AOE. Top panels are representative images of the results obtained for the genotypes indicated and quantification of the percentage of cells that contain Ataxin-1 NIs is shown in the bottom panel. Overexpression of Nup44AOE (a suppressor of SCA182Q) does not modify the percentage of cells with Ataxin-182Q NIs (top right panel, compare with control in top left panel and quantification in the corresponding bars in the bottom panel). As shown both with the images and the quantification, the alteration in the percentage of cells with NIs is not statistically significant. Results were analyzed by Student’s t-test and the p value, marked with an asterisk is above 0.05 (*p>0.05). Images in the right top corner of the chart in the bottom panel illustrate a cell with diffused Ataxin-1 signal, a cell with a single Ataxin-1 NI and a cell with multiple NIs of the Ataxin-1 protein (from left to right). Error bars represent standard deviation. For each experiment at least 8 independent animals (with an average of 32 cells each) were analyzed. Scale bar is equivalent to 10µM.

Genotypes:
Top right panel) w; UAS-LacZ/Nup44AEP2417; FRTG13, UAS:CD8-
GFP, UAS-SCA1^{82Q}[M6]-R4/++; OK107-GAL4/+. All flies used were 2 days old and raised at 25°C.

As shown in Figure 45, overexpression of the Ataxin-1^{82Q} eye suppressor Nup44A does not alter the percentage of cells containing Ataxin-1^{82Q} aggregates, when compared with appropriate SCA1^{82Q} controls that over express LacZ in the same experimental conditions.

**DISCUSSION**

Here we have shown that Nup44A is a good suppressor of the induced toxicity of SCA1^{82Q} in the eye (Figure 44). However, in contrast to one of the hypothesis we have considered, in which Nup44A could be mediating the translocation of the mutant Ataxin-1 protein into the nucleus, we find that suppression of SCA1^{82Q}-induced eye by over expression of Nup44A is not due to alterations in the formation of NIs (Figure 45). We have shown previously in (Branco et al., 2007) and Part 2 of this thesis, that we find no direct correlation between NI formation and toxicity, since different Ataxin-1^{82Q} suppressors can increase or decrease the percentage of neurons that present NI formation (see (Branco et al., 2007); Figure 42). The result with Nup44A further reinforces the notion that screens mainly based on the evaluating the aggregation of the mutant protein per se need to be interpreted with caution, since formation of NIs might not be the best read-out of the toxicity in the cell.

Even thought the data presented here is inconclusive, the fact that Nup44A is such a potent suppressor of the SCA1^{82Q}-induced toxicity in the eye makes it a good candidate for future studies.

In order to further explore the hypothesis that Nup44A is mediating the translocation of Ataxin-1 between cytoplasm and nucleus more experiments need to be performed, such as analyze if the distribution of the Ataxin-1 protein is altered by overexpression of Nup44A by making cytoplasmic and nuclear extracts and measuring Ataxin-1 protein levels in the two extracts. It would also be interesting to test if, in the presence of drugs that are able to
block nuclear import, Nup44A is able to revert Ataxin-1 transport or if, in contrast, it does not have any effect. Also interesting would be to test other alleles of this gene that in the mean time have become available, but also to test some of the possible interactors of Nup44A indicated by Rothberg and colleagues in (Giot et al., 2003) and at the Curagen Drosophila Interaction Database (http://portal.curagen.com/cgi-bin/interaction/flyHome.pl) or in the bioGRID (www.thebiogrid.org) online portal.

In order to understand the mechanisms behind this modification, more experiments would need to be conducted, such as the ones suggested here.

B- Genes that modify SCA1^{82Q} and Htt^{128Q}-induced phenotypes in opposite ways: other data obtained for the study of mub

As mentioned earlier, overexpression of mub suppresses and a heterozygous loss of function allele of mub enhances the SCA1^{82Q}-induced eye phenotype. In contrast, overexpression of mub dramatically enhances the Htt^{128Q}-induced eye phenotype, but mub^{LOF} only mildly suppresses Htt^{128Q}-induced eye phenotype (this thesis and (Branco et al., 2007; Fernandez-Funez et al., 2000)). Note that a brief introduction to mub is also mentioned in Part 1, in the context of pasilla, another RNA-binding protein, as a modifier of SCA1^{82Q}-induced toxicity.

As mentioned earlier, at the beginning of this PhD thesis, one of our major interests was to understand how RNA processing affected SCA1 pathology in our fly model, and in specific how did mub had a role in polyglutamine-induced neurodegeneration. mub is an RNA-binding protein containing 3 KH-type domains, strongly expressed in the mushroom bodies thought the development (Figures 46 and 47). mub is likely involved in regulation of alternative mRNA splicing or another RNA-mediated processes (Adapted from www.flybase.net/reports/FBgn0026188.html; (Grams and Korge, 1998; Park et al., 2004)).
Figure 46 – **Predicted structure of the mub protein.** Protein domains predicted by the SMART program provided by EMBL (http://smart.embl-heidelberg.de). mub is composed of 3 K homology (KH) domains. The KH domain is an evolutionarily conserved sequence of around 70 amino acids present in a wide variety of diverse nucleic acid-binding proteins. However, it is not strictly proven the KH domains bind RNA. Like many other RNA-binding motifs, KH motifs are found in one or multiple copies and each motif is necessary for in vitro RNA binding activity. This suggests that they may function cooperatively or, in the case of single KH motif proteins, independently. The domain has been found in a number of proteins including eukaryotic and prokaryotic RS3 ribosomal proteins; vertebrate fragile X mental retardation protein 1 (FMR1); yeast Pab1-binding protein 2 PBP2; human high-density lipoprotein binding protein; and human onconeural ventral antigen-1 (NOVA-1). Adapted from http://smart.embl-heidelberg.de/smart/show_motifs.pl.

Figure 47 – **mub expression time course.** mub is expressed through the development of the flies, reaching its peak during the pupal stage (A and left panel in B). Note also that through their life span, for the majority of the time, mub is expressed at higher levels in adult females than it is in the males. Adapted from http://genome.med.yale.edu/Lifecycle/query_gen.php?input1=cg7437.

In order to confirm the suppression observed with the EP line (recovered from the original SCA1 genetic screen) and to understand the mechanisms by which *mub* modulates SCA1/HD pathogenesis in flies, two new and undescribed *mub* isoforms were generated (Laura Nino-Rosales and Juan
Botas, unpublished work). One isoform lacks amino acids 246 to 268, a region between the 2nd and 3rd KH domain (mub-1), and a second new isoform that lacks just four amino acids (from residues 92 to 95) between the 1st and 2nd KH domain (mub-2; Figure 48). mub-1 sequence corresponds to the sequence of mushroom-body expressed protein isoform C, CG7437-PC, while mub-2 sequence corresponds to the protein sequence of mushroom-body expressed isoform A, CG7437-PA.

These constructs were used to generate transgenic flies. Twenty-two mub-1 lines and thirty-one mub-2 lines were generated. However, the majority of the lines expressing any of the two isoforms failed to rescue the phenotype observed originally with the EP line. Only 4 lines mildly...
suppressed the characteristic SCA1 phenotype (data not shown). Because the mub gene undergoes alternative splicing (four alternatively spliced isoforms are generated) we hypothesized that maybe we could not be expressing the correct isoforms. Alternatively, several isoforms of mub may need to work together to suppress SCA1\textsuperscript{82Q}-induced eye phenotype in the flies. In order to answer this question we created transgenic flies expressing two of the mub isoforms generated that showed the best suppression of the eye phenotype and that expressed higher levels of the mub protein in a Western Blot assay (data not shown). However, we could still not observe significant modification of the SCA1\textsuperscript{82Q}-induced eye toxicity (data not shown). Because of these negative results we have decided to generate transgenic flies that express the full-length mub gene. Unfortunately, because the genomic region of mub extends for 18kb, and even though several techniques and approaches were used, we were unable to succeed with the cloning process.

Although we could not generate transgenic flies that express the full-length protein, we were able to prove that the modification observed with the EP line was in fact due to overexpression of mub. In a similar manner, we have observed that the P-element insertion (mub\textsuperscript{04093}) that caused an aggravation of the eye phenotype was the result of the insertion in the mub gene. To prove this, two approaches have been followed. While a peptidic antibody for mub was being generated in collaboration with UT Southwestern University in Dallas we decided to perform an X-Gal Staining with the P-element line we had been using as a Loss of Function allele and also to determine the precise genomic location of the insertion of this P-element.

**X-Gal Staining**

In order to perform this assay we took advantage of the fact that the P-element line mub\textsuperscript{04093} was generated with a P-LacZ construct in its structure, which can function as a reporter gene (Figure 49).

In this construct, the *Esherichia coli* LacZ gene is fused in frame with the P-element transposase gene, and, therefore, functions as a reporter of P promoter activity. The P-element promoter-LacZ fusion can be used as a
tissue specific expression method to visualize \textit{LacZ} expression by performing a X-gal Staining for \(\beta\)-galactosidase activity (Brand and Perrimon, 1993; Brook et al., 1993 and references therein; Rincon-Limas et al., 1999). \(\beta\)-galactosidase detection can also be used to identify patterns of gene regulation or as an enhancer trap approach, used as a screening method.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure49.png}
\caption{Molecular map of \textit{mub}\textsuperscript{04093} and its associated alleles. \textit{mub}\textsuperscript{04093} (P\{PZ\}mub\textsuperscript{04093}) is a transposable element with approximately 14.55 Kb inserted in the genomic region of \textit{mub}. The PZ element is an enhancer-sensitive P-element incorporating a P-transposase. The structure of \textit{mub}\textsuperscript{04093} also comprises the alleles \textit{ry}\textsuperscript{17.2}, \textit{EcoI}\textbackslash lacZ\textsuperscript{P\textbackslash T} (\(\beta\)-galactosidase foreign engineered gene, which expression is driven by the P\textbackslash T promoter) the and \textit{l(3)87DF}\textsuperscript{2}. Adapted from www.flybase.net/reports/FBti0005504.html and www.flybase.net/reports/FBal0043886.html.}
\end{figure}

Therefore, the X-Gal Staining protocol was used to detect the pattern of expression of \textit{mub}\textsuperscript{04093}.

As shown in Figure 50 (right panel), \textit{mub}\textsuperscript{04093} signal is present in the cells were \textit{mub} is normally expressed, namely the mushroom bodies. Some expression is also visible in the eye imaginal discs and along the ventral ganglion (Figure 50, right panel). Control larva stained in the same conditions as \textit{mub}\textsuperscript{04093} show no signal for X-Gal (Figure 50, left panel).
Figure 50 - X-Gal staining reveals that mub\textsuperscript{04093} signal match with the predicted mub expression pattern. Shown are images of a X-Gal Staining obtained following the protocol described in Materials and Methods. Control shows no X-Gal signal (left panel). Results obtained show that mub\textsuperscript{04093} signal (in blue) is present in the mushroom bodies (right panel- yellow circles), in the eye imaginal discs (right panel-red arrows) and also along the ventral ganglion.

Genotypes:
- Control \( \text{yw} \)
- mub\textsuperscript{04093} P(PZ)mub\textsuperscript{04093} ry\textsuperscript{506}/TM3, ry\textsuperscript{Rk} Sb\textsuperscript{i} Ser\textsuperscript{i}

**mub Antibody**

Using the antibody raised against a specific peptide of the mub protein, Mub-pep1 (A1008a), it was possible to stain the endogenous mub protein in the larval brain, ventral ganglion and imaginal discs (Figure 51, panels C-E). Confirming the results previously obtained with the X-Gal staining (Figure 50), mub is expressed mainly in the mushroom bodies and eye imaginal discs (Figure 51, panels C-E). Using the dpp-Gal4 driver, which specifically directs expression to the wing imaginal discs, we were able to further confirm the specificity of the antibody that was generated (Figure 51, panel A).
Figure 51 – mub is expressed in the mushroom bodies and in the eye discs. Shown are immunostainings performed with the peptidic antibody generated in collaboration with UT Southwestern Antibody Production Core revealing dpp-GAL4-induced mub expression (A) and endogenous mub signal (C-E). B shows a confocal image of larval brain and ventral ganglion. (A) Overexpression of mub under the control of the dpp-GAL4 driver shows signal in the normal pattern of expression in the wing imaginal disc (vertical row; arrowheads, right panel). No signal was detected in the control shown on the left panel, which expresses the neutral transgene LacZ also using the dpp-GAL4 driver. Wing discs are ventral side up. (B) Confocal image of the larval brain, eye imaginal discs and ventral ganglion highlighting the location of the eye imaginal discs and the mushroom bodies. (C, D) Immuno-stainings using Mub-pep1 antibody detect endogenous mub expression in the mushroom bodies (C) and in the eye imaginal discs (top view, D). (E) High magnification view of an imaginal eye disc showing mub staining in the tissue. Genotype: (A) Dpp-GAL4>LacZ; w;dpp-GAL4/UAS-LacZ; Dpp-GAL4>mub<sup>OE</sup> w;dpp-GAL4/++; mub<sup>EP(3)3623</sup>/+. (B) w; FRTG13, UAS:CD8-GFP/++; OK107-GAL4/+. (C-E) yw Larva used in (A) was raised at 27°C.

The results presented above show that the modification observed with the EP line results from the overexpression of the mub protein. Conversely, we also show that the P-element insertion (mub<sup>04093</sup>) used is inserted in the mub gene, as its pattern of expression coincides with the pattern of expression predicted for the mub protein. Furthermore, we have also
determined the genomic location of the \( mub^{04093} \) P-element insertion, by performing an Inverse PCR, and found that it is located within the genomic region that corresponds to the mub gene (data not shown).

Therefore, the EP and P-element lines that have been used are suitable to use in assays to test the effect of altered levels of mub, either increased levels, EP line (overexpression), or decreased amounts, P-element insertion line (loss of function).

\( mub^{OE} \) decreases mutant Ataxin-1 soluble protein levels in adult flies but not its mRNA levels

Next we wanted to evaluate the effect of \( mub \) in Ataxin-1/Huntingtin protein levels in order to gain insight into the mechanism by which \( mub \) modifies SCA1\(^{82Q} \) and Htt\(^{128Q} \)-induced eye degeneration. Because \( mub \) is a RNA binding protein we also wanted to know whether the suppression of the mutant Ataxin-1- and Htt-induced eye phenotypes was due to alterations at the level of the mRNA. Note that most of the experiments conducted, both Western Blot and RT-PCR were performed using animals raised at a lower temperature (25°C), and thus with lower expression levels, in order to avoid reduction of mutant Ataxin-1/Huntingtin levels due to an increase in degeneration and cell death.

Due to technical problems we were not able to perform the Western Blot experiments using \( Htt^{128Q} \)-expressing flies. Very likely, mutant Huntingtin protein adopts a tight secondary conformation that unable us to solubilize it and to run it in an acrylamide gel, even in highly stringent conditions. In our experiments, most of the Huntingtin protein remained in the stacking gel and only a small portion of it was separated in a gradient gel (data not shown). Because we do not believe that this small portion of mutant Huntingtin is representative of the total sample, the Western Blot results for the HD model are not shown and not taken into consideration. Furthermore, RT-PCR results obtained using \( Htt^{128Q} \) adult flies were not reproducible and could not be taken into consideration. Therefore, for now, and as a result of
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the a high variability in the Western Blot and RT-PCR results, in the specific conditions tested at this moment, for the HD model, these assays can not be used.

**mub and the variation of SCA1\(^{82Q}\) protein levels in the larval stage**

First, we tested the effect of altered levels of *mub*, both overexpression (OE) and loss of function (LOF) in soluble Ataxin-1 protein levels at the larval stage (third instar eye and brain imaginal discs) by Western Blot. The results obtained (Figure 52, panel A) show a slight increase of Ataxin-1 protein levels in SCA1\(^{82Q}\) flies co-expressing *mub*\(^{OE}\). In the same way, co-expression of the SCA1\(^{82Q}\) transgene and *mub*\(^{OF}\) allele showed a decrease in those same levels. Due to a tight secondary structure, it has been shown before that Ataxin-1\(^{82Q}\) is very prone to aggregate and its solubilization is very hard to achieve (Burright et al., 1995; Klement et al., 1998). Bearing this in mind, and assuming that the protein levels measured by Western Blot analysis reflect only the soluble portion of Ataxin-1 present in the cell, we decided to conduct an Immunohistochemistry Assay to approach this issue (Figure 52, panels B and C). With this assay it would be possible to quantify both soluble and aggregated protein in the cell. Surprisingly, when we stained third instar eye imaginal discs of flies expressing either the SCA1\(^{82Q}\) transgene alone or flies co-expressing SCA1\(^{82Q}\) and *mub*\(^{OE}\) or *mub*\(^{OF}\) we were not able to reproduce the results obtained by Western Blot. Furthermore, the results obtained seem to indicate that both *mub*\(^{OE}\) and *mub*\(^{OF}\), when co-expressed with SCA1\(^{82Q}\), decrease the Ataxin-1 protein levels in the cells (Figure 52, panel C). These results might indicate that variation in the endogenous *mub* protein levels does not affect Ataxin-1 protein levels, and that the results obtained could be an artifact or due to some other variations that occur as a result of the conditions and assay tested.

One obvious justification for this discrepancy of the results is related to the different sensitivity of the two assays used: by Western Blot we only measure the soluble fraction of the protein and with the immunofluorescence assay we detect both aggregated and soluble protein levels. Because of its tight secondary structure, it is possible that in the
presence of *mub* Ataxin-1 is more prone to aggregation and thus less likely to be solubilized and so can not be resolved in the acrylamide gel. Furthermore, because of the results obtained with the Immunofluorescence Assay, we decided that maybe we were not pursuing the best approach to answer the question of whether *mub* affects Ataxin-1 protein levels.

**Figure 52 - Altered levels of *mub* and the variation of the Ataxin-1^{82Q} protein levels in cells at the larval stage.** Western Blot (A) and Immunofluorescence (B and C) in third instar larval eye discs from flies expressing *SCA1^{82Q}* alone and flies co-expressing *SCA1^{82Q}* with *mub*^{OE}, *mub*^{LOF} or the control transgene *UAS-GFP*. Shown is a representative Western Blot (panel A) comparing soluble protein levels of Ataxin-1^{82Q} (*Ata1^{82Q}* in *SCA1^{82Q}* expressing adult flies with altered levels of *mub*, or *SCA1^{82Q}* alone; and a representative immuno staining of anti-Ataxin-1 in the larval eye disc (B) and the respective quantification of the levels of the protein in the different genotypes tested (C).

(A) The results obtained by Western Blot are shown by the quantifications in the box at the bottom of the figure in panel A. The assay was performed using 4 third instar larval eye discs per genotype. Control lane (1) shows no signal for Ataxin-1. Flies
over expressing \textit{SCA1}^{82Q} and \textit{mub} induce an increase in the soluble endogenous levels of the Ataxin-1 protein (lane 3), when compared with the appropriate \textit{SCA1}^{82Q} expressing control (lane 2) that also co-expresses the neutral transgene UAS-GFP. In a similar way, flies that over express the \textit{SCA1}^{82Q} transgene and have decreased levels of \textit{mub} show a decrease in the Ataxin-1 protein levels (lane 5 and compare with the control in lane 4).

\textbf{(B)} Confocal image showing the area in the late third instar imaginal eye disc that expresses the \textit{SCA1} transgene with the \textit{GMR-GAL4} driver. Arrowhead indicates morphogenetic furrow. The expression of Ataxin-1 extends from the morphogenetic furrow (arrowhead) to the most posterior part of the disc. \textbf{(C)} Quantification of the amount of anti-Ataxin-1 immunofluorescence in eye imaginal discs from third instar larvae expressing \textit{SCA1}^{82Q} (shown in arbitrary units). Quantification was carried out using four imaginal eye discs per genotype. Error bars indicate standard deviation. The alterations observed in terms of amount of fluorescence/levels of Ataxin-1 protein are statistically significant (Results were analyzed by Student’s t-test; *p<0.01 and **p<0.001). The results obtained show a decrease in the Ataxin-1\textsuperscript{82Q} protein levels when larvae co-express \textit{SCA1}^{82Q} and \textit{mub}\textsuperscript{OE} and \textit{mub}\textsuperscript{LOF} [(\textit{SCA1}^{82Q}>\textit{mub}\textsuperscript{OE}) and (\textit{SCA1}^{82Q}>\textit{mub}\textsuperscript{LOF})] when compared with the control (\textit{SCA1}^{82Q}/\textit{LacZ}).

Both immunofluorescence and Western Blot assays were probed with anti-Ataxin-1 antibody (11NQ). Anti-tubulin was used as a loading control for Western Blot Assays.

\textbf{Genotypes:}

\textbf{Western Blot Assay (Panel A)}

1. Control) \textit{w};\textit{GMR-GAL4}/+. 2. \textit{SCA1}^{82Q}>\textit{UAS-GFP}) \textit{yw},\textit{UAS-SCA1}\textsuperscript{82Q}[F7]/+;\textit{GMR-GAL4}/+. 3. \textit{SCA1}^{82Q}>\textit{mub}\textsuperscript{OE}) \textit{yw},\textit{UAS-SCA1}\textsuperscript{82Q}[F7]/+;\textit{GMR-GAL4}/+;\textit{mub}\textsuperscript{EP(3)3623}/+. 4. \textit{SCA1}^{82Q}) \textit{yw},\textit{UAS-SCA1}\textsuperscript{82Q}[F7]/+;\textit{GMR-GAL4}/+. 5. \textit{SCA1}^{82Q}>\textit{mub}\textsuperscript{LOF}) \textit{yw},\textit{UAS-SCA1}\textsuperscript{82Q}[F7]/+;\textit{GMR-GAL4}/+;\textit{mub}\textsuperscript{04093}/+. 6. \textit{SCA1}^{82Q}>\textit{mub}\textsuperscript{LOF}) \textit{yw},\textit{UAS-SCA1}\textsuperscript{82Q}[F7]/+;\textit{GMR-GAL4}/+;\textit{mub}\textsuperscript{04093}/+. 7. \textit{SCA1}^{82Q}>\textit{mub}\textsuperscript{LOF}) \textit{yw},\textit{UAS-SCA1}\textsuperscript{82Q}[F7]/+;\textit{GMR-GAL4}/+;\textit{mub}\textsuperscript{04093}/+. 8. \textit{SCA1}^{82Q}>\textit{mub}\textsuperscript{LOF}) \textit{yw},\textit{UAS-SCA1}\textsuperscript{82Q}[F7]/+;\textit{GMR-GAL4}/+;\textit{mub}\textsuperscript{04093}/+.

\textbf{Immunofluorescence Assay (Panels B and C)}

\textit{SCA1}^{82Q}/\textit{LacZ}) \textit{w};\textit{GMR-GAL4}/\textit{LacZ}.

\textit{SCA1}^{82Q}>\textit{mub}\textsuperscript{LOF}) \textit{yw},\textit{UAS-SCA1}\textsuperscript{82Q}[F7]/+;\textit{GMR-GAL4}/+;\textit{mub}\textsuperscript{04093}/+. \textit{SCA1}^{82Q}>\textit{mub}\textsuperscript{LOF}) \textit{yw},\textit{UAS-SCA1}\textsuperscript{82Q}[F7]/+;\textit{GMR-GAL4}/+;\textit{mub}\textsuperscript{04093}/+.

Since \textit{SCA1} is a late onset disease, we also hypothesized that maybe the larval stage was not the best developmental period to evaluate alterations in protein levels. We have shown before that our \textit{SCA1} and \textit{HD} \textit{Drosophila} models show many features also present in patients, including the progressiveness of the characteristic phenotypes ((\textit{Branco et al., 2007; Fernandez-Funez et al., 2000}) and this thesis). Furthermore, and using the \textit{OK107-Gal4} assay described earlier, we have observed that in larval stages we detect formation of a smaller percentage of cells with \textit{NIs} (data not shown). Therefore, we have decided to try to address the question of whether \textit{mub} affects Ataxin-1 protein levels in the cell by performing Western Blots using adult flies.

Carrying out these experiments in adult tissues has two problems. We might obtain results with artifacts due to availability of tissue, since the enhancers cause loss of cells (therefore less protein), while the suppressors
typically cause increased cell survival (therefore more protein). A second problem is related to the insoluble aggregates that both Htt\(^{128Q}\) and Ataxin-1\(^{82Q}\) form. These insoluble aggregates may difficult the quantification of the protein levels by Western Blot assays. In fact, we have already mentioned that for the case of Htt\(^{128Q}\)-expressing flies we cannot perform this type of assays. Bearing in mind these disadvantages, we still decided to perform the assay in adult flies and evaluate if mub affects Ataxin-1 protein and mRNA levels.

**mub\(^{OE}\) decreases Ataxin-1\(^{82Q}\) soluble protein levels in adults**

We then tested the effect of altered levels of mub, both overexpression and loss-of-function, on soluble mutant Ataxin-1 protein levels in adult flies (Figure 53). Our results show that in flies co-expressing mutant Ataxin-1 and overexpressing mub, the levels of the Ataxin-1 soluble protein are reduced to approximately 50% of its normal levels (Figure 53, lanes 2 and 3; *p value* < 0.0005). However, flies that carry mutant Ataxin-1 and mub loss-of-function do not show a significantly difference in the Ataxin-1 protein levels (Figure 53, lanes 4 and 5; *p value* < 0.06). It is possible that this results from the massive degeneration (and probably cell death) that occurs due decreased levels of mub, which is responsible for the enhancement of the SCA1\(^{82Q}\)-induced eye phenotype previously reported.
Figure 53 – *mub*<sup>OE</sup> modulates Ataxin-1<sup>82Q</sup> soluble protein in adult flies. Shown is a representative Western Blot of flies expressing SCA1<sup>82Q</sup> alone and flies co-expressing SCA1<sup>82Q</sup> and *mub*<sup>OE</sup>, *mub*<sup>LOF</sup> or the control transgene UAS-GFP. The quantification of the results obtained is shown in the box at the bottom of the figure. Control (lane 1) shows no signal for Ataxin-1. Overexpression of *mub* induces a significant decrease in the soluble levels of Ataxin-1 protein (lane 3, reduction to ~50% of the levels detected by densitometry in flies expressing SCA1<sup>82Q</sup>/UAS-GFP - lane 2, *p value*= 1.53x10<sup>-8</sup>). However, the increase in Ataxin-1 levels induced by *mub*<sup>LOF</sup> is not significant (compare lanes 4 and 5, *p*=0.057). Four adult heads lysates were used and the membrane was probed with anti-Ataxin-1 antibody (11NQ). Anti-tubulin antibody was used as loading control. A total of 11 independent experiments were performed. Genotypes:
1. Control) *w; GMR-GAL4/+.*
2. SCA1<sup>82Q</sup>/UAS-GFP) *yw, UAS-SCA1<sup>82Q</sup>[F7]/+; GMR-GAL4/UAS-GFP.*
3. SCA1<sup>82Q</sup>/ *mub*<sup>OE</sup> *yw, UAS-SCA1<sup>82Q</sup>[F7]/+; GMR-GAL4/+; *mub*<sup>P[NB3623]/+</sup>.
4. SCA1<sup>82Q</sup>) *yw, UAS-SCA1<sup>82Q</sup>[F7]/+; GMR-GAL4/+.
5. SCA1<sup>82Q</sup>/ *mub*<sup>LOF</sup>) *yw, UAS-SCA1<sup>82Q</sup>[F7]/+; GMR-GAL4/+; *mub*<sup>04093</sup>/+
Flies were raised at 25°C.

Because the decrease in Ataxin-1 protein levels observed was dramatic and consistent in all the experiments performed, we next wanted to know if this reduction was specific for Ataxin-1 or, in the other hand, common to all transgenes under the control of the Gal4/UAS system. To answer this question, we crossed flies expressing UAS-CD8:GFP transgene under the control of the *GMR-GAL4* driver with flies carrying *mub* overexpression and loss-of-function alleles. As shown in Figure 54, no significant difference was found between flies expressing SCA1<sup>82Q</sup>/ *mub*<sup>OE</sup> and SCA1<sup>82Q</sup>/UAS-GFP (lanes 1 and 2, *p value*= 0.18) or between flies expressing SCA1<sup>82Q</sup>/ *mub*<sup>LOF</sup> and SCA1<sup>82Q</sup> (lanes 3 and 4, *p*= 0.12). This means that both overexpression and loss-of-
function of *mub* do not affect the levels of the UAS-CD8:GFP transcript. Therefore, the results regarding Ataxin-1 are specific and not due to an effect of *mub* in the translational machinery/Gal4-UAS system.

**Figure 54** - *mub* does not alter CD8-GFP protein levels in adult flies. Shown is a representative Western Blot of flies co-expressing CD8-GFP with *mub*<sup>OE</sup>, *mub*<sup>LOF</sup> and the control transgene UAS-LacZ. Quantification of the signal is shown in the box on the bottom of the image in arbitrary units. The expression was driven by *GMR-GAL4* and the adult eye was used to perform this experiment. *mub*<sup>OE</sup> (lane 4, the respective control is shown in lane 3) decreases CD8-GFP protein levels while *mub* overexpression increases those levels (lane 1, control is shown in lane 1). However, this variation is not statistically significant (*p*<0.2 and *p*<0.12, respectively. Results were analyzed by Student’s T Test). Five adult heads lysates were used and the membrane was probed with anti-GFP antibody. Anti-tubulin antibody was used as loading control. Two independent experiments were performed.

Genotypes

1. CD8-GFP/UAS-LacZ) w; CD8-GFP, *GMR-GAL4/++;UAS-LacZ/+.
2. CD8-GFP/mub<sup>OE</sup>) w; CD8-GFP, *GMR-GAL4/++; *mub<sup>EP(3)3623</sup>/+.
3. CD8-GFP) w; CD8-GFP, *GMR-GAL4/+.
4. CD8-GFP/mub<sup>LOF</sup>) w; CD8-GFP, *GMR-GAL4/++; *mub<sup>04093</sup>/+.

Flies were raised at 25°C.

**Suppression of SCA1<sup>82Q</sup>-induced eye phenotype by mub<sup>OE</sup> is due to a decrease in Ataxin-1<sup>82Q</sup> soluble protein but not the SCA1<sup>82Q</sup> transcript levels**

Finally, we investigated whether this genetic interaction and decrease in Ataxin-1 protein levels takes place at the RNA level or if it is a post-transcriptional event.

We first tried to investigate the possibility that *mub* influences the
stability of the SCA1 mRNA by using Real Time Quantitative PCR. We have performed the assays ourselves and also in collaboration with the Micro-Array Core Facility at Baylor College of Medicine (data not shown). However, we could never obtained reproducible data and have, therefore, decided to perform semi-quantitative RT-PCR. The results obtained are shown in Figure 55.

**Figure 55** – mub modulates SCA182Q mRNA levels in adult flies in opposite ways. Shown is a representative image of a semi-quantitative RT-PCR performed with SCA182Q flies co-expressing altered levels of mub or the control transgene UAS-GFP. The RT-PCR was done with RNA extracted from adult heads (6 per genotype). mub overexpression (mubOE) promotes an increase in the levels of the transcript Ataxin-1 mRNA (lane 2 and the respective control is shown in lane 1), while mubLOF reduces those levels (lane 4 and compare with the control in lane 3). The ubiquitously expressed RP49 mRNA was used as an internal control for the reactions. Three independent RT-PCR reactions were performed. Quantification of the signal is shown in arbitrary units in the box at the bottom of the image.

Genotypes:
1. SCA182Q/UAS-GFP) yw, UAS-SCA182Q[F7]/+; GMR-GAL4/UAS-GFP.
4. SCA182Q/mubLOF) yw, UAS-SCA182Q[F7]/+; GMR-GAL4/+; mubLOF[320]/+.

Flies were raised at 25°C.

RT-PCR experiments show that the reduction in Ataxin-1 protein is not a consequence of reduced Ataxin-1 mRNA. In fact, we observed that overexpression of mub leads to an increased SCA1 mRNA levels and that mub loss-of-function decreases those same levels (Figure 55). These results are opposite to the ones observed at the protein level. This increase in the levels of transcript is likely a consequence of the suppression of the eye
phenotype mediated by overexpression of mub. The suppression is correlated with an increase in the number of cells, which probably justifies the increase in the mRNA levels and the decrease in the SCA1\(^{82Q}\) flies that carry a mub loss-of-function allele. Taken together, these results suggest that mub affects Ataxin-1 induced degeneration at the level of protein synthesis and/or protein stability.

**mub modifies Ataxin-1-induced neurodegeneration regardless of the length of the polyglutamine tract**

In order to evaluate whether the suppression of SCA1\(^{82Q}\)-induced eye phenotype by mub overexpression was dependent on its polyglutamine tract length, flies overexpressing mub were crossed with flies overexpressing wild type SCA1 (Human SCA1 gene containing 30 glutamine repeats, SCA1\(^{30Q}\)) in the eye. We have previously shown both in flies and mice that high expression levels of wild type SCA1 (SCA1\(^{30Q}\)) are also able to trigger neurodegeneration, but to a lesser extent when compared to SCA1\(^{82Q}\) induced neurodegeneration (Fernandez-Funez et al., 2000). SCA1\(^{30Q}\)-expressing flies have a mild eye phenotype at 27\(^\circ\)C, but at 29\(^\circ\)C they display a severe phenotype, showing degeneration of the ommatidia and increased disorganization.

Our results show, from SEM analysis, that co-expression of SCA1\(^{30Q}\) and overexpression of mub promotes a mild suppression of the eye phenotype, suggesting that the genetic interaction of Ataxin-1 and mub is not dependent on the glutamine repeats number per se (Figure 56). It also suggests that this interaction might be mediated through the backbone of the Ataxin-1 protein. This underlies the relevance of the protein structure independently of the repeats and might also explain why mub affects SCA1 and HD models characteristic phenotypes differently.
Figure 56 - mubOE suppresses SCA130Q eye phenotype. SEM photographs of the external eye of flies expressing SCA130Q alone (left panel) and flies co-expressing SCA130Q and mubOE (right panel). mubOE promotes a general improvement of the SCA130Q-induced eye phenotype showing more organized ommatidia and bristles. Insets show a higher magnification view of the ommatidia. Flies were raised at 29°C. Scale bar in SEM pictures is equivalent to 100µM (10µM in the magnification insets).

Genotypes:

SCA130Q>UAS-GFP) yw, UAS-SCA130Q[F1]/+; GMR-GAL4/UAS-GFP.
SCA130Q> mubOE yw, UAS-SCA130Q[F1]/+; GMR-GAL4/+; mubEP(3)3623/+.

DISCUSSION

With the additional data presented here, we have shown that mub, a RNA-binding protein composed of 3 KH domains, dramatically decreases the levels of the Ataxin-182Q protein in the adult flies. We have also shown that this decreased is not a consequence of decreased levels of the Ataxin-1 transcript, suggesting that mub affects SCA182Q-induced toxicity at the post-transcriptional level or that it interferes with the stability of the mutant protein. Furthermore, we have also shown that the modification that mub induces in the SCA182Q-induced eye phenotype is not dependent on the polyglutamine tract. The fact that mub also modifies the phenotype of wild-type Ataxin-1 (SCA130Q) suggests that mub interacts with the backbone of the Ataxin-1 protein. This observation might explain why mub affects SCA1 and HD Drosophila models in opposite ways. It also reinforces the relevance of the protein context to understand the mechanisms behind the toxicity observed in polyglutamine and other neurodegenerative disorders.
We have also tried to address the question of whether altered levels of mub affected the aggregation of Ataxin-1 and Htt mutant proteins into NIs, using the OK107 assay developed in the lab. However, we were not able to answer this question using this assay. OK107-Gal4 directs expression into specific subsets of cells in the CNS of the fly, in particular to the mushroom bodies, where mub is normally most expressed (Figure 57).

Preliminary experiments were performed using the OK107 assay, but our results showed that the expression of UAS:CD8-GFP, just by itself, was highly increased in flies that also over expressed mub, both using Immunofluorescence and Western Blot Assays (data not shown). Therefore we hypothesized that some type of parallel interaction could be occurring at the level of the mushroom bodies and interfering with the results. Thus, we decided that the OK107 assay was not suited to analyze whether mub affects aggregation of mutant Ataxin-1/Huntingtin into NIs.

The additional results presented in this Part of the thesis show that mub is an interesting modifier of SCA1\textsuperscript{82Q}-induced toxicity. Although a specific
mechanism of interaction with the toxicity was not identified, the data here presented contributes to the ongoing work of defining the specific mechanisms that involve RNA-based mechanisms (and the involvement of RNA-binding proteins) and the toxicity in the SCA1 *Drosophila* model.

In the future, an interesting approach would be to verify if the modification of the SCA1√260/Htt√280-induced phenotypes occurs via direct interaction of the mub protein with Ataxin-1/Huntingtin proteins or through interaction with an intermediate protein. With the antibody that we have also generated, it would also be interesting to see if mub and Ataxin-1/Huntingtin co-localize in the cell. Ultimately, another assay/approach will need to be developed in order to evaluate if altered levels of mub affect the aggregation of mutant Ataxin-1/Huntingtin proteins into NIs.
IV. CONCLUSIONS AND FUTURE PERSPECTIVES
We have used *Drosophila* models of SCA1 and HD for a comparative analysis of genetic factors involved in Ataxin-1 and Huntingtin-induced neurotoxicity. For that we have used, mainly, previously identified genetic modifiers of the SCA1 model.

We found genetic modifiers involved in a variety of cellular functions that affect similarly the SCA1 and HD *Drosophila* models. For other genes we did not detect modification of Huntingtin toxicity. Surprisingly, we also identified modifier genes that have antagonistic effects on Ataxin-1 and Huntingtin toxicity. Some of the opposing interesting modifiers were validated in the CNS using a neuronal assay, such as the climbing assay. We have also found no consistent correlation between modifier gene activity and nuclear inclusion formation.

To our knowledge, the study here presented represents the largest study to date comparing genetic modifiers between two polyglutamine disorders using fly models. However, and as most of the genetic screens performed, it is hard to identify a clear link between the observation of a genetic modifier that modulates specific phenotypes characteristic of the model being studied and a mechanism that explains the toxicity observed. Because of that, many have considered that genetic screens are merely a large collection of observations. However, we believe that the fact that two disorders have been compared in this study overcomes this negative aspect of the approach we have taken, in the sense that it may contribute to understanding why, at least for some specific cases, some of the characteristic clinical and pathological phenotypes polyglutamine diseases are so distinctive.

Many of the genes highlighted in this study can be the starting point for us or other groups to start a line of research on a specific gene, interaction or effect. However, our results support a model in which each disease should be analyzed separately and not in the context of other diseases. As we have shown, a genetic modifier for one of the diseases might have the opposite
effect in another one. Therefore, therapeutic approaches need to be carefully directed and taken into consideration the specific protein causing the disease (and its protein context) and not what is common among that specific group of diseases.

The results here present will also contribute to the creation of a network of proteins that interact with the disease-causing proteins. This will allow a better assessment of all the mechanisms and pathways leading to the toxicity observed not only in the animal models, but also, ultimately, in the patients. The work here presented does not intend to establish specific mechanisms or pathways leading to toxicity in polyglutamine disorders, but yet aims to contribute to the knowledge of the network of interactions behind the mechanisms that cause the disease clinical, phenotypical and molecular phenotypes observed in patients.

Furthermore, this work reinforces the importance of using genetic approaches in order to fully understand mechanisms underlying disease pathology, and also in this case, the relevance of the protein context for the development or progression of polyglutamine diseases and for the occurrence of distinct characteristic diseases phenotypes among this group of disorders.

Our results also support the recent evidences that focus on the function of the mutant protein itself and how protein context (and not just the polyglutamine tract on its own) can regulate the pathogenic events in each of these diseases.

As mentioned, for this study we have used genes previously identified as genetic modifiers of the SCA1 model developed in the Botas laboratory (Fernandez-Funez et al., 2000). As a result of this approach, many modifiers might have not been identified, and, therefore, an interesting approach would be to test some know HD model genetic modifiers (for example some of the genes identified in the study by Hughes and colleagues (Kaltenbach et al., 2007)) in the SCA1 fly model developed in the Botas Laboratory. Also interesting would be to test some of the known- SCA1 genetic modifiers in the newly developed full length HD model (Eliana Romero and Juan Botas,
He now knows that a collection of various proteins and pathways are involved in polyglutamine-induced neurodegeneration and that, most likely, some will be more relevant for one disorder than to another. The combination of the studies mentioned here and our study will likely open new and interesting avenues of research.

Figure 58 - Model for Cellular Pathogenesis. The figure here presented was specifically designed to illustrate some of the possible mechanisms involved in cellular pathogenesis in HD. With the obvious exception that Huntingtin is predominantly a cytoplasmic protein, many of the mechanisms here schematically represented can be adjusted to other polyglutamine disorders. For the specific case of HD, the normal function of Htt is yet to be clarified, but it is possible that it is involved in cytoskeletal functions or vesicle recycling. It may cycle to the nucleus and have a normal role in the regulation of gene transcription, but more studies will be needed in order to confirm this observation. The conformational changes resulting from the expansion of the trinucleotide repeats lead to unfolding/abnormal folding of the protein, and thus molecular chaperones can play an important role in this process. Proteolytic cleavage of the mutant protein can also occur, both in the cytoplasm and in the nucleus, depending on the protein. The mutant protein, once in the nucleus, forms NIs (note that in some cases NIs in the cytoplasm can also be observed). As discussed throughout this thesis, the role for toxicity of these aggregates of mutant protein and additional proteins (trapped in these aggregates) is still matter of much debate. However, nuclear toxicity is believed to be caused by disturbance of gene transcription, one of the main common molecular features of polyglutamine diseases. (Adapted from Ross, 2002)
One other line of investigation not mentioned in this thesis that is gaining relevance in the field is miRNAs. miRNAs have been implicated in many human diseases (reviewed in Hebert and De Strooper, 2007), and in particular in neurodegenerative diseases (Bilen et al., 2006a; Bilen et al., 2006b; Kim et al., 2007). Therefore, their role in the pathogenic mechanism is anticipated to be of greater importance than previously thought. However, more work will be needed in order to understand the clinical relevance of these molecules and, specially, to clearly define molecularly how they influence pathogenesis. Not only the miRNAs need to be the focus of more detailed studies, but also their interacting proteins and enzymes need to be further investigated.

Finding the mechanism by which the polyglutamine tract, and more interestingly, how the protein context of one specific protein, affects the activity of the disease protein as a whole might help research aimed at developing a therapeutic solution of this type of disorders. Either by interfering with the specific interactors that mediate the selective neuronal toxicity or by modulating the downstream events of those interactions, it will be possible to develop a therapeutic approach in the future. The findings obtained as a result of this PhD thesis reinforce that, in order to achieve effective treatments, each disease needs to be carefully studied in its own context, and not as part of a group of disorders.
V. APPENDIX I

SUPPLEMENTAL DATA
Supplemental Figure 1 - The modifier genes tested do not alter the eye phenotype of control flies. Paraffin sections through the retina of flies expressing Akt (OE and LOF, panels B and J, respectively), mub (OE and LOF, panels C and K, respectively), vib (panel D), skd (panel L), CG14438 (OE and LOF, panels E and M, respectively), pum (OE and LOF, panels F and N, respectively), and 14-3-3ε (OE and LOF, panels G and O, respectively) and DIAP1 (OE and LOF, panels H and P, respectively) under the control of the GMR-GAL4 driver. Control flies are shown in panels A and I. Note that the eyes shown do not display an eye phenotype on their own when compared to control eyes, the length of the retina is very similar to control eyes and there is no apparent tissue loss.
Genotypes: A) w; GMGAL4/UAS-GFP. B) w; GMGAL4/+; UAS-Akt/+. C) w; GMGAL4/+; mub\textsubscript{1346}+/+. D) w; GMGAL4/+; vib\textsubscript{3451}/+. E) w, CG14438\textsubscript{1431}+/; GMGAL4/+. F) w; GMGAL4/+; pum\textsubscript{1346}+/+. G) w; GMGAL4/+; UAS-14-3-3\epsilon\textsubscript{9or}/+. H) w, UAS-myc-DIAP1/+;GMGAL4/+. I) w; GMGAL4/+. J) w; GMGAL4/+; Akt\textsubscript{04226}+/+. K) w; GMGAL4/+; mub\textsubscript{04093}+/+. L) w; GMGAL4/+; skd\textsubscript{7062}/+. M) w, CG14438\textsubscript{G00226}/; GMGAL4/+. N) w; GMGAL4/+; pem\textsubscript{281}/+. O) w; GMGAL4/+;14-3-3\epsilon\textsubscript{281}/+. P) w; GMGAL4/+; th\textsubscript{/+}.

All eyes shown are from six days-old flies that were raised at 25°C. Scale bar is equivalent to 10\mu M.
VI. APPENDIX II

Comparative Analysis of Genetic Modifiers in *Drosophila* Points to Common and Distinct Mechanisms of Pathogenesis among Polyglutamine Diseases
Joana Branco, Ismael Al-Ramahi, Lubna Ukani, Alma M. Pérez, Pedro Fernandez-Funez, Diego Rincón-Limas, and and Juan Botas

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VII. APPENDIX III

PARTICIPATION IN OTHER PROJECTS/REPORTS
Al-Ramahi I, Lam YC, Chen HK, de Gouyon B, Zhang M, Perez AM, Branco J, de Haro M, Patterson C, Zoghbi HY, Botas J.

**CHIP protects from the neurotoxicity of expanded and wild-type Ataxin-1 and promotes their ubiquitination and degradation.**

J Biol Chem. 2006 Sep 8;281(36):26714-24

dAtaxin-2 mediates expanded Ataxin-1-induced neurodegeneration in a Drosophila model of SCA1.

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