PhD Thesis

Application of a *Drosophila* model to study Huntington's disease pathogenesis *in vivo*

László Bodai

Supervisor: Prof. J. Lawrence Marsh
Department of Developmental and Cell Biology
University of California, Irvine
Irvine, CA, USA

2004

1. CONTENTS

1. CONTENTS **2**

2. ABBREVIATIONS **3**

3. INTRODUCTION **5**

4. LITERATURE OVERVIEW **7**

4.1. HUNTINGTON'S DISEASE IS A NEURODEGENERATIVE DISORDER 7
4.2. GENETICS OF HUNTINGTON'S DISEASE 8
4.3. MOLECULAR BASIS OF HD PATHOGENESIS 11
4.3.1. Aggregation and protein turnover *12*
4.3.2. Transcriptional dysregulation *15*
4.3.3. Ca^{2+} homeostasis and mitochondrial dysfunction *19*
4.3.4. Loss of wild-type Huntingtin function *20*
4.4. THE ROLE OF *DROSOPHILA* MODEL SYSTEMS 22

5. AIMS **25**

6. MATERIALS AND METHODS **28**

6.1. *DROSOPHILA* MEDIA AND COMPOUNDS 28
6.2. *DROSOPHILA* STOCKS AND CROSSES 28
6.3. PSEUDOPUPIL ANALYSIS 29
6.4. QUANTIFICATION OF TRANSGENE EXPRESSION 30
6.5. SINGLE EMBRYO GENOMIC PCR 30
6.6. STATISTICAL ANALYSIS 31

7. RESULTS **33**

7.1. ESTABLISHMENT OF A *DROSOPHILA MELANOGASTER* MODEL OF HD FOR COMPOUND SCREENING AND GENETIC TESTS 33
7.1.1. STOCK CHARACTERIZATION 33
7.1.2. MEDIA AND SOLVENT OPTIMIZATION 36
7.1.3. TESTING THE SENSITIVITY OF THE TRANSGENIC SYSTEM 37
7.2. INVESTIGATION INTO THE ROLE OF DISTURBED PROTEIN ACETYLATION IN POLYGLUTAMINE DISORDERS 42
7.2.1. GENETIC RESTORATION OF THE ACETYLATION BALANCE SUPPRESSES POLYQ PATHOLOGY *IN VIVO* 43
7.2.2. HDAC INHIBITOR COMPOUNDS RESCUE POLYQ PATHOLOGY 45
7.2.3. DIVERSE HAT AND HDAC ENZYMES PLAY ROLE IN HD PATHOLOGY ... 51

8. DISCUSSION **57**

9. SUMMARY **64**

10. ACKNOWLEDGEMENTS **67**

11. REFERENCES **69**

2. ABBREVIATIONS

ANOVA	ANalysis Of VAriance
AR	Androgen Receptor
AT	AcetylTransferase
BDNF	Brain-Derived Neurotrophic Factor
CBP	CREB Binding Protein
CR	Congo Red
CtBP	C-terminal Binding Protein
CRE	cAMP Response Element
CREB	cAMP Response Element Binding protein
DMSO	DiMethyl SulfOxide
DRPLA	DentatoRubral-PallidoLuysian Atrophy
DTMCT	DunneTt's Multiple Comparison Test
DNMCT	DuNn's Multiple Comparison Test
GFP	Green Fluorescent Protein
GNAT	GCN5-related N-AcetylTransferase
HAT	Histone AcetylTransferase
HD	Huntington's Disease
HDAC	Histone DeACetylase
HPRT	Hypoxanthine PhosphoRibosylTransferase
Htt	Huntingtin
IT15	Important Transcript 15
kDa	kiloDalton
KWOWA	Kruskal-Wallis non-parametric One Way ANOVA
LOF	Loss Of Function
MW	Molecular Weight
MWRST	Mann-Whitney Rank Sum Test
MYST	MOZ, Ybf2, Sas2 and Tip60-related acetyltransferase
NII	neuronal intranuclear inclusion
OWA	One Way ANOVA
PCAF	p300/CBP Associated Factor
PCR	Polymerase Chain Reaction

polyQ	polyglutamine
Q48	48 repeats long polyQ transgene
Q108	108 repeats long polyQ transgene
SAHA	SuberoylAnilide Hydroxamic Acid
SBMA	Spinal and Bulbar Muscular Atrophy
SCA	SpinoCerebellar Ataxia
SNKMCT	Student-Newman-Keuls Multiple Comparison Test
TG	TransGlutaminase
tTG	tissue TransGlutaminase
TSA	TrichoStatin A
TWA	Two Way ANOVA
UAS	Upstream Activating Sequence
wt	wild-type

3. INTRODUCTION

Several hereditary, late-onset neurodegenerative disorders, including Huntington's disease, Kennedy's disease and several types of spinocerebellar ataxias are caused by the expansion of an endogenous CAG repeat in the corresponding disease gene. The elongated trinucleotide repeat translates to an expanded polyglutamine (polyQ) domain and causes dominant gain of function disease. The severity of these disorders is dependent on the length of the polyQ tract, and the number of glutamine residues necessary to cause a disease phenotype shows similar threshold levels among most of the polyglutamine repeat diseases. The aberrant polyQ domains are thought to cause structural changes in the affected proteins, leading to aberrant protein interactions such as those that allow formation of extra- and intranuclear aggregates, which are a hallmark feature of this class of disorders. Beside self-association, polyQ domains are also able to mediate direct interactions with other proteins and sequester them to aggregates. The depletion of some essential proteins may be the basis of pathogenesis.

The molecular mechanisms involved in polyglutamine pathogenesis are still not fully understood and are in the focus of interest. Animal model systems may serve as useful and essential tools to investigate the molecular details of the pathogenic process *in vivo.* Beside their research benefits, model systems can also be used for efficient screening of chemical compound libraries in order to identify drugs for therapeutic purposes. We have developed a transgenic *Drosophila melanogaster* model of polyglutamine diseases, which recapitulates the common characteristics of these disorders, such as aggregate formation, movement impairments, progressive neurodegeneration and early death. The modifiable phenotype of the transgenic animals allowed us to perform genetic and compound feeding tests. Thus, this model was extensively used in our experiments.

Histone acetyltransferase (HAT) enzymes play an important role in the establishment of the proper transcriptional activity and fine tuning of

several other cellular processes. To establish the correct acetylation pattern, the proper balance of HATs and histone deacetylases (HDACs), the opposing enzyme group, is necessary. It has been found that polyQ containing proteins physically interact with the catalytic domain of several HAT enzymes, and that this aberrant interaction results in the reduced enzymatic activity of the affected acetyltransferase proteins *in vitro*. These results suggested that unbalanced acetylation activity might be one of the components of polyglutamine pathogenesis. Using our *Drosophila* model system we were able to prove the *in vivo* relevance of this interaction. Our work demonstrated that distorted protein acetylation balance is one of the key factors of polyglutamine pathogenesis and pointed to HDAC inhibitor compounds as candidate drugs for effective treatment.

4. LITERATURE OVERVIEW

4.1. Huntington's disease is a neurodegenerative disorder

Huntington's disease (HD) is an inherited, late-onset neurodegenerative disorder. Symptoms typically begin between the ages of 35 and 50, although more severe cases exhibit juvenile onset. The disease is progressive and ultimately leads to death, usually 15-20 years after the first appearance of symptoms.

The symptoms of HD comprise motor, cognitive and psychiatric abnormalities. Motor disturbances include choreiform involuntary movements and impairment of voluntary movements. Chorea affects the limbs and trunk, and tends to plateau in the later stages of disease. In contrast, voluntary movement abnormalities, which, among others, include slow and uncoordinated fine motor movements, continue to progress until the final stage. Juvenile onset HD is characterized by somewhat different motor symptoms including bradykinesia, dystonia and rigidity. Cognitive deficits, such as impaired memory retrieval and slow cognitive functions, also progress as the disease develops. Psychiatric symptoms, comprising depression, irritability and apathy, are also commonly associated with HD and may lead to social withdrawal and often suicide.

Progressive and selective neuronal loss is characteristic of HD. In advanced cases, the atrophy of the central nervous system may lead to 25-30% loss of total brain weight. The most affected region is the corpus striatum with about 57% loss in its caudate nucleus and 65% loss in its putamen part in postmortem brain samples. The most affected cells in the striatum are the medium spiny neurons, while other cell types are relatively preserved. There is also a significant neuronal loss in the cerebral cortex, which affects predominantly the pyramidal neurons in layers III, V and VI. Most of these neurons seem to project to the thalamus. In advanced cases, neurodegeneration can also be observed in

other brain areas, such as the thalamus, hypothalamus and amygdala. Cerebellar loss is characteristic to juvenile onset cases.

The neuropathology of HD is in good accordance with its symptoms. Degeneration of the basal ganglia may lead to the early mild impairment of cognitive and mental functions, whilst neuronal loss in the cerebral cortex may cause the later developing, more severe dementia. Interruptions in the basal ganglia – cerebral cortex – thalamus neuronal circuit may be responsible for the motor symptoms of the disease. [Vonsattel and DiFiglia, 1998; Ross and Margolis, 2001]

4.2. Genetics of Huntington's disease

Huntington's disease is inherited in an autosomal dominant fashion. A major breakthrough towards the better understanding of HD occurred in 1993, when the gene responsible for the disorder, *IT15* (*Important Transcript 15*), was identified [Group T. H. s. D. C. R., 1993]. *IT15* is located at cytological position 4p16.3 and is composed of 67 exons, which encode a 348 kDa, widely expressed protein, called Huntingtin (Htt). It has two transcription variants that only differ in their 3' untranslated region. The first exon of the gene contains an instable, polymorphic CAG repeat that translates to an uninterrupted polyglutamine (polyQ) stretch. It is now widely accepted that the expansion of the CAG repeat is in causative association with HD, the resulting elongated polyQ track being responsible for the disease phenotype.

The length of the polyQ repeat is a key determinant of disease development and course. Healthy individuals have less than 35 glutamine residues. Polyglutamine tracks longer than 35 residues are considered to be expansions and cause HD with very high penetrance. Expansions over 40 are fully penetrant [Rubinsztein *et al.*, 1996; Duyao *et al.*, 1993]. Patients with the juvenile-onset form of the disease typically have elongations more than 60 residues long. There is a good inverse

correlation between polyQ repeat length in the expanded range and the age of disease onset – the longer the repeat is the earlier the first symptoms appear [Stine *et al.,* 1993]. The number of CAG triplets also influences the meiotic instability of HD alleles. Normal alleles are quite stable with a repeat number change in less than 0.5 % of meioses. On the other hand, expanded alleles (ranging from (CAG40) to (CAG70)) are highly unstable and 70% of meioses result in alleles with altered triplet repeat numbers [Zuhlke *et al.*, 1993].

Another feature of HD inheritance is anticipation - a shift towards earlier age of onset in successive generations of affected families. Anticipation is a characteristic of paternal transmission with a mean advancement of 8 years, whereas maternal transmission usually does not result in change of the age of onset [Ranen *et al.*, 1995]. The basis of this difference is the increased risk of expansion during spermatogenesis compared to oogenesis [Zuhlke *et al.*, 1993].

Following the identification of the mutation causing HD, it remained unclear whether the loss of normal Huntingtin function (and hence haploinsufficiency of *IT15*) or a new function gained by the elongation of the polyglutamine stretch was responsible for the disease. Although the fact that heterozygous loss of one of the *IT15* alleles (in Wolf-Hirschhorn syndrome) does not cause HD phenotype [Housman, 1995] suggests that a toxic gain of function is the basis of pathology, only the studies in animal models could elucidate this question. In mouse, similarly to humans, absence of one of the *Hdh* (the mouse homolog of *IT15*) alleles does not cause HD-like phenotypes [Duyao *et al.*, 1995; Nasir *et al.*, 1995]. Transgenic mouse models have been generated that carry a cDNA encoding an N-terminal truncated fragment [Mangiarini *et al.*, 1996], the full-length cDNA [Reddy *et al.*, 1998] or the full-length genomic region (including introns and regulatory sequences) of *IT15* [Hodgson *et al.*, 1999] along with two wild-type copies of the mouse *Hdh*. These transgenic strains show the characteristic features of HD, such as lack of coordination, involuntary movements, seizures [Carter *et al.*, 1999; Murphy *et al.*, 2000], intranuclear aggregates [Reddy *et al.*, 1998]

(discussed in the next section), striatal neuronal loss [Hodgson *et al.*, 1999] and electrophysiological abnormalities [Laforet *et al.*, 2001], providing evidence that expanded alleles are dominant even if two wild-type copies of the gene are present. Similar results were obtained from knock-in mouse models, in which the endogenous *Hdh* was engineered to encode an abnormally long polyglutamine track [Shelbourne *et al.*, 1999; Lin *et al.*, 2001]. The significance of the polyglutamine expansion (that is, the disease-gene independent toxic effect) was also inspected in model organisms. In a mouse model, an expanded CAG repeat was inserted into a gene unrelated to neurodegenerative disorders, the *hypoxanthine phosphoribosyltransferase (HPRT)*. The mutated *HPRT* gene caused late-onset, progressive neurological phenotypes [Ordway *et al.*, 1997]. In *Drosophila*, expression of an elongated polyglutamine polypeptide proved to be sufficient to result in neuronal loss and reduced viability [Marsh *et al.*, 2000]. These experiments strongly indicated that a new toxic function gained by the expansion of the CAG repeat is the major causative agent of HD pathogenesis. Even so, the contribution of loss of wild-type Huntingtin function to the disease cannot be ruled out.

Huntington's disease is not the only disorder caused by the detrimental effects of a polyglutamine track expansion. Several inherited neurodegenerative diseases are caused by the expansion of an unstable CAG repeat in the coding region of the disease-genes (Table 1). This class of disorders includes Huntington's disease, spinal and bulbar muscular atrophy (SBMA), dentatorubral-pallidoluysian atrophy (DRPLA) and several types of spinocerebellar ataxias (SCA1, SCA2, SCA3 (also known as Machado-Joseph disease), SCA6, SCA7 and SCA17). (For review, see [Zoghbi and Orr, 2000]) Although the genes affected (*Huntingtin*, *androgen receptor (AR)*, *atrophin 1*, *ataxin 1, 2, 3, 6, 7* and *TATA binding protein*, respectively) have divergent functions, and the targeted brain regions are distinct, polyglutamine diseases share several common features. They are all progressive, dominantly inherited (except for SBMA) and cause neuronal dysfunction or neuronal loss in the CNS. Manifestation of overt symptoms typically begins in middle adulthood and progression of the

disease takes several years or even decades. The well characterized genetic background and the large number of *in vitro* and *in vivo* model systems developed make HD suitable as a model for all polyglutamine diseases. Still, investigation of other polyglutamine induced disorders is essential, since their comparison may shed light on such important areas of research as the role of protein context in disease development or the molecular basis of selective neuronal loss in different diseases.

Disease	Position	Protein	Mw (kDa)	Normal repeat range	Pathogenic repeat range
HD	4p16.3	Huntingtin	348	6-35	36-121
SBMA	Xq11-q12	Androgen receptor	99	9-36	38-62
DRPLA	12p13.31	Atrophin-1	124	3-36	49-88
SCA 1	6p23	Ataxin-1	87	6-44	40-82
SCA 2	12q24	Ataxin-2	90	14-32	33-37
SCA 3	14q24.3-q31	Ataxin-3	42	12-40	55-86
SCA 6	19p13	P/Q Ca^{2+} channel	160-250	4-18	21-30
SCA 7	3p21.1-p12	Ataxin-7	95	7-17	38-200
SCA 17	6q27	TATA binding protein	41	29-42	47-55

Table 1.: Polyglutamine diseases. The main properties of the disease genes and encoded proteins are shown. [Jana and Nukina, 2003]

4.3. Molecular basis of HD pathogenesis

Although Huntington's disease is caused by a single gene mutation, its pathogenesis involves disturbances in several cellular functions. The efforts in HD research during the last decade identified protein turnover, transcriptional regulation, Ca^{2+} homeostasis, mitochondrial functions and apoptosis as affected processes. In this section I will present a summary of the current molecular models of HD pathogenesis.

4.3.1. Aggregation and protein turnover

The formation of large visible protein aggregates is a hallmark feature of Huntington's disease and the majority of other polyglutamine diseases. Expanded polyglutamine proteins in most cases form neuronal intranuclear inclusions (NII), as has been shown for DRPLA, HD, SBMA, SCA1, SCA3 and SCA7 [Jana and Nukina, 2003]. NIIs are mainly composed of truncated fragments, rather than the full-length, of the polyQ containing proteins, as shown by inclusions in DRPLA, HD, SBMA and SCA3 patient samples being reactive only to antibodies directed against the polypeptide region containing the polyQ domain [Zoghbi and Orr, 1999]. Huntingtin itself is a substrate of caspases [Wellington *et al.*, 2000] and calpains [Goffredo *et al.*, 2002]. The cleavage by caspase-3 releases an N-terminal truncated fragment of expanded Htt that is more susceptible to aggregation and more toxic than full-length Htt [Wellington *et al.*, 2000; Chen *et al.*, 2000].

The exact role of aggregate formation in polyglutamine induced neurodegeneration is still not clearly understood, and it is also not evident whether aggregates are detrimental, neutral or even beneficial in terms of pathology. Inclusion formation correlates with polyQ induced cell death in cell culture [Ho *et al.*, 2001], and aggregate formation precedes the manifestation of symptoms in some transgenic mouse models [Davies *et al.*, 1997]. The number of NIIs in the cerebral cortex of postmortem HD brain samples correlates with the length of the polyQ track [Becher *et al.*, 1998]. These observations support a view of the detrimental nature of inclusion formation. On the other hand, the tissue selectivities of neuronal cell death and aggregation do not correspond, as aggregation is most frequent in the cortex while the most extensive cell death occurs in the striatum [DiFiglia *et al.*, 1997; Gutekunst *et al.*, 1999]. Furthermore, striatal cell culture [Saudou *et al.*, 1998; Kim *et al.*, 1999] and transgenic mouse [Klement *et al.*, 1998; Cummings *et al.*, 1999] experiments dissociating neuronal dysfunction and visible aggregate formation suggest that aggregation is unlikely to be an exclusive cause of polyQ

pathogenesis. However, suppression of aggregation by inhibitor compounds [Smith *et al.*, 2001; Apostol *et al.*, 2003], recombinant proteins [Kazantsev *et al.*, 2002] or over-expressed molecular chaperones [Jana *et al.*, 2000] typically relieve pathology, suggesting that the aberrant protein interactions allowing aggregate formation are key determinants of pathology.

Two mechanisms have been proposed to explain how expanded polyglutamine repeats induce aggregate formation: covalent cross-binding by tissue transglutaminase (tTG) and "polar zipper" formation. In 1993 Green hypothesized that polyQ repeats beyond a threshold may turn the affected protein into a transglutaminase substrate (TGs catalyze a reaction between a polypeptide-bound glutamine and the ε-amino group of a polypeptide-bound lysine residue forming a covalent crosslink [Greenberg *et al.*, 1991]) and the crosslinked products may form insoluble aggregates [Green, 1993]. In support of this hypothesis are the findings that TG activity is elevated in HD brains compared to healthy control [Karpuj *et al.*, 1999; Lesort *et al.*, 1999]; that polyglutamine polypeptides [Kahlem *et al.*, 1996; Cooper *et al.*, 1997] and full-length Huntingtin [Kahlem *et al.*, 1998] are good tTG substrates *in vitro*, and that tTG associates with the truncated form of expanded Htt in cell culture [Chun *et al.*, 2001a]. On the other hand, cell culture experiments demonstrated that neither expanded nor wild-type Htt is modified by tTG *in situ* [Zhang *et al.*, 1998], and that tTG is excluded from Htt aggregates [Chun *et al.*, 2001b]. However, proteins associated with the N-terminal fragment of mutant Htt are polyaminated by tTG [Chun *et al.*, 2001a] and this interaction proposes an alternative role for tTG in HD.

The “polar zipper” model of aggregate formation hypothesizes that β-strands of poly-L-glutamine repeats may be linked together by hydrogen bonds between their main-chain and side-chain amides. This interaction leads to the creation of inter-molecular, cylindrical β-sheet structures and rapid aggregation of polyglutamine containing proteins [Perutz *et al.*, 1994; Perutz and Windle, 2001]. PolyQ polypeptides form large aggregates consisting β-sheets [Perutz *et al.*, 1994] and incorporation of

polyglutamine repeats into a monomeric protein induced its multimerization [Stott *et al.*, 1995], both supporting the idea of polar zipper formation. Based on the facts that the age of onset is an exponential function of polyglutamine repeat length and that the probability of neural cell death is constant with time in HD, Perutz also hypothesized aggregate nucleation as the rate limiting step in neurodegeneration, and hence the polyQ aggregates as the means of neurodegeneration [Perutz and Windle, 2001].

One of the most important consequences of aberrant protein folding and NII formation is the sequestration of important cellular proteins into aggregates and/or their binding by soluble forms of the polyglutamine containing proteins. The main groups of proteins co-localizing with NIIs are molecular chaperones, proteasome components and transcriptional factors (discussed in the next subsection). The presence of the chaperone proteins Hsp70 and Hsp40 [Jana *et al.*, 2000], ubiquitin and components of the 26S proteasome [Alves-Rodrigues *et al.*, 1998; Chai *et al.*, 1999] in NIIs indicates that polyQ proteins are recognized as misfolded proteins and attempts are made to refold or degrade them. This presumption is supported by the finding that in an inducible mouse model of HD, once the expression of mutant *Htt* is stopped the disappearance of aggregates and improvement of overt symptoms is observed [Yamamoto *et al.*, 2000]. Accordingly, chaperone overexpression suppresses cell death in cultured mammalian cells [Jana *et al.*, 2000] and also suppresses polyQ induced neurodegeneration in transgenic flies [Warrick *et al.*, 1999; Kazemi-Esfarjani and Benzer, 2000] and mice [Cummings *et al.*, 2001]. Conversely, compromising the ubiquitin - proteasome pathway enhances neurodegeneration in *Drosophila* [Chan *et al.*, 2002b]. Overwhelming amount of polyglutamine containing proteins may deplete chaperoning and proteasomal factors by trapping them into aggregates. In addition, polyQ domains seem to directly inhibit proteasomal functions [Goellner *et al.*, 2003]. Impairment of proteasomal activity may endanger important regulatory processes, such as cell cycle control, and induce endoplasmic reticulum stress via the accumulation of misfolded proteins [Nishitoh *et*

al., 2002] - and ultimately lead to cell death.

4.3.2. Transcriptional dysregulation

Transcriptional dysregulation is one of the best studied mechanisms of polyglutamine pathology. Gene expression changes may be the primary steps in these diseases, according to the fact that neuronal genes are down-regulated in SCA1 mice before the manifestation of symptoms [Lin *et al.*, 2000]. Several proteins involved in transcriptional regulation contain polyQ domains and the nuclear localization of polyQ inclusions also makes transcription factors potential targets of sequestration. Transcription factors associated with polyglutamine proteins include $TAF_{II}130$ [Shimohata *et al.* 2000], Sp1 [Li *et al.*, 2002a], p53 [Steffan *et al.*, 2000], CtBP [Kegel *et al.*, 2002], NCoR [Boutell *et al.*, 1999], NF-κB [Takano and Gusella, 2002], CA150 [Holbert *et al.*, 2001], TBP [Kim *et al.*, 2002] and several proteins (CBP, p300, P/CAF, Sin3A) involved in histone acetylation [Bodai *et al.*, 2003]. Amongs these, the contribution of histone acetyltransferases (HATs) and histone deacetylases (HDACs) to HD pathogenesis is the most extensively studied, and being in the focus of this thesis, will be discussed in most detail.

Core histones, the building blocks of nucleosomes, are targets of several covalent modifications. One of these modifications is acetylation, that is controlled by two classes of enzymes with opposing effects. Histone AcetylTransferases (HATs) acetylate free or nucleosomal histones, while Histone DeACetylases (HDACs) are responsible for the removal of acetyl groups. HAT proteins can be classified into several evolutionarily conserved but divergent protein families based on the homology between acetyltransferase (AT) domains and between other domains they bear. The majority of the non-AT domains mediate protein-protein interactions, thus allowing AT enzymes to participate in the formation of large protein complexes [Sterner and Berger, 2000, Marmorstein, 2001a]. Similarly to acetyltransferases, histone deacetylases can also be classified to protein

families. Class I and II (homologues of the yeast Rpd3 and Hda1 proteins, respectively) HDACs show close similarity to each other in their catalytic domain, while Class III proteins (sirtuins) are completely different from the former two families in both the structure of the catalytic domain and the mechanism of catalysis [Marmorstein, 2001b]. Similarly to HATs, HDACs also tend to form large protein complexes, which often associate with co-repressor proteins to direct the deacetylase activity to genes for silencing [Ng and Bird, 2000]. The balance of the acetyltransferase and deacetylase activities regulates the proper transcriptional competence of target genes. In particular, genes with highly acetylated nucleosomes at their promoter sequences generally have high transcriptional activity, while genes with deacetylated nucleosomes are silenced [Berger, 2002]. Recent results implicate a more sophisticated model, which proposes that the pattern of acetylation acts as a code allowing access of particular transcription factors to the promoter [Jenuwein and Allis, 2001; Agalioti *et al.*, 2002]. Thus, it is not the absolute level of acetylation, but the acetylation pattern of specific lysine residues of specific histones that influences transcriptional activity on the target sites. Apart from acetylation of core histones, several other proteins are targets of this posttranslational modification as well. These include non-histone chromatin components, transcription factors, nuclear receptor co-activators, and proteins not involved in gene expression [Sartorelli *et al.*, 1999; Barlev *et al.*, 2001; Caillaud *et al.*, 2002; Hubbert *et al.*, 2002]. Thus, sequestration of HAT proteins can disturb gene expression by altering the acetylation state of histones and transcription factors; and they may also influence cellular processes that are not related to transcription.

Multiple lines of evidence have demonstrated the physical interaction of polyglutamine proteins and histone acetyltransferases. *In vitro*, recombinant CREB binding protein (CBP) binds to a truncated, expanded Htt polypeptide through its acetyltransferase (AT) domain [Steffan *et al.*, 2000; Steffan *et al.*, 2001], implicating that other HAT enzymes may also be sequestered by polyQ. Accordingly, binding was shown to p300 (a close homolog of CBP) and p300/CBP Associated Factor

(P/CAF), a HAT enzyme without polyglutamine domain. These interactions reduced the acetyltransferase activity of the affected proteins *in vitro* [Steffan *et al.*, 2001]. Cell culture and *in vivo* observations also confirmed the sequestration of HATs. In cell culture, endogenous CBP has been found to co-localize with intranuclear aggregates composed of mutant androgen receptor, causing a remarkable decrease in soluble CBP levels [McCampbell *et al.*, 2000]. Similar redistribution of CBP to nuclear inclusions was observed using mutant *ataxin*-3 [McCampbell *et al.*, 2000; Chai *et al.*, 2001] and *HD* constructs [Nucifora *et al.*, 2001].

The interaction of CBP and polyglutamine bearing proteins was further confirmed in model organisms and patient tissues. Endogenous CBP co-localizes with aggregates of expanded AR proteins in transgenic mice and in tissue samples derived from SBMA patients [McCampbell *et al.*, 2000]. Similarly, it is sequestered into aggregates formed by an amino-terminal fragment of expanded Huntingtin protein in transgenic mice [Steffan *et al.*, 2000; Nucifora *et al.*, 2001], and is found in inclusions in HD, DRPLA [Nucifora *et al.*, 2001] and SCA7 [Takahashi *et al.*, 2002] postmortem brain samples. In addition to the aberrant redistribution of the protein, soluble CBP protein levels were found strikingly reduced in postmortem HD brain samples, further emphasizing the relevance of reduced CBP levels associated with sequestration of CBP into aggregates [Nucifora *et al.*, 2001]. The interaction of polyglutamine bearing proteins and HAT enzymes reduced the acetylation level of core histones [McCampbell *et al.*, 2001; Steffan *et al.*, 2001, Igarashi *et al.*, 2003] and silenced transcriptional activity in reporter gene assays [McCampbell *et al.*, 2000; Nucifora *et al.*, 2001; Li *et al.*, 2002b; Steffan *et al.*, 2000], suggesting that sequestration of HATs may disturb transcriptional regulation as part of the pathogenesis.

Gene expression profiling revealed that the expression of elongated CAG repeats results in acetylation related transcriptional changes in the disease state. Gene array experiments performed on HD transgenic mice [Luthi-Carter *et al.*, 2000] and cell culture samples [Wyttenbach *et al.*, 2001] revealed 110 genes with reduced transcriptional levels. 13 of these

(which is about one third of the genes with characterized regulatory sequences) were shown by database analysis to contain *cAMP response element* (*CRE*) binding sites in their regulatory regions. The expression of these genes is predicted to be influenced by CREB and its co-factor, CBP, supporting the view that sequestration of CBP affects transcription. Despite these results, reporter gene experiments performed to investigate the role of CRE-mediated transcription in HD provided conflicting results so far. In an inducible cell culture model of HD, significant down-regulation of CRE-dependent transcription was observed as early as 12 hours following mutant *Htt* transgene induction [Sugars et al., 2004]. In contrast, CRE-mediated transcription was found to be increased in a transgenic mouse model of HD [Obrietan and Hoyt, 2004]. However, overlapping (63% match) results derived from the comparison of the gene expression profiles of HD and DRPLA transgenic mice, and similar results obtained from northern analysis of transgenic SBMA and SCA7 mice samples [Luthi-Carter *et al.*, 2002] implicate the general role of transcriptional dysregulation in polyglutamine diseases. Further evidence linking HAT sequestration to transcriptional changes arose from a yeast model [Hughes *et al.*, 2001], where expression of an expanded polyQ protein was found to induce transcriptional changes similar to those observed in strains deficient for the components of the SAGA histone acetyltransferase complex.

Rescue experiments showed that increased HAT and reduced HDAC activity relieves polyQ pathology, presumably by the restoration of the correct acetylation balance. Over-expression of exogenous *CBP* rescued the toxic effects of expanded *AR* [McCampbell *et al.*, 2001; McCampbell *et al.*, 2000], *HD* and *atrophin-1* [Nucifora *et al.*, 2001] in cell culture assays. Similarly, up-regulation of endogenous *nejire*, the *Drosophila* homologue of *CBP*, rescued the polyglutamine induced neurodegenerative phenotype in *Drosophila* [Taylor *et al.*, 2003]. Conversely, a loss of function allele of the HDAC co-factor *Sin3A* rescued viability and progressive neurodegeneration in a *Drosophila* model of HD [Steffan *et al.*, 2001]. Furthermore, LOF alleles of *Sin3A* and the HDAC gene *Rpd3* also modulated the phenotype

of SCA1 flies [Fernandez-Funez *et al.*, 2000]. Restoration of the acetylation balance by the administration of HDAC inhibitor compounds also relieved pathology in cell culture [McCampbell *et al.*, 2001], yeast [Hughes *et al.*, 2001], *Drosophila* [Steffan *et al.*, 2001] and murine [Hockly *et al.*, 2003] model systems.

As several HAT enzymes can bind polyglutamine proteins and this interaction leads to decreased acetylation activity, processes HATs are involved in may be negatively affected, which, in turn, can lead to disturbed cellular functions. The exact mechanism whereby transcriptional dysregulation induces cellular dysfunction is still not clear. Thus, down-regulation of some dose sensitive factors and the cumulative action of moderate effects are also conceivable.

4.3.3. Ca^{2+} homeostasis and mitochondrial dysfunction

Mitochondrial defects have been suspected to contribute to HD pathogenesis as chronic exposure to low doses of the mitochondrial toxin 3-nitropropionic acid reproduces the symptoms of HD both in animals and humans [Ho *et al.*, 2001]. Recently, Panov and his colleagues have demonstrated the damage of mitochondrial Ca^{2+} handling in HD [Panov *et al.*, 2002]. In their experiments, mitochondria isolated from lymphoblasts of HD patients depolarize in response to Ca^{2+} challenge at much lower concentrations than control mitochondria. Similar results were found by challenging mitochondria derived from neurons of transgenic animals: mitochondria from brains of transgenic mice expressing an expanded *Htt* allele took up lower amounts of intracellular Ca^{2+}, and had substantially lower Ca^{2+} retention capacity than wild-type controls. These effects seem to precede overt symptoms by several months in transgenic mice, underscoring the potential pivotal role of mitochondrial dysfunction in HD. The mechanisms underlying these electrophysiological changes are not yet understood, but formation of polyQ composed ion channels able to span lipid bilayers [Hirakura *et al.*, 2000; Monoi *et al.*, 2000] may

contribute.

The observations described above fit well into the picture that is being formed based on knowledge accumulated from experiments studying Ca^{2+} dependent processes in HD. Excitotoxicity, neuronal death as a result of exposure to excitatory neurotransmitter analogs, has been proposed as one of the mechanisms responsible for neurodegeneration in HD. This hypothesis is based on the findings that striatal injection of glutamate agonists leads to symptoms mimicking those of HD in rodents and primates [Beal *et al.*, 1986; Ferrante *et al.*, 1993], and that vulnerability to excitotoxic cell death is increased in cells transformed with expanded *polyQ* transgenes and also in transgenic mouse models of HD [Zeron *et al.*, 2002]. Excitotoxic insults result in Ca^{2+} flux into the neuron, and hence may trigger the depolarization of the mitochondrial membrane and release of intra-mitochondrial Ca^{2+}. Release of intra-mitochondrial Ca^{2+} may induce release of cytochrome C to the cytosol and induce apoptotic effects. Activation of caspase-3 leads to the cleavage of mutant Huntingtin [Kim *et al.*, 2001], releasing the cytotoxic N-terminal fragment, the main component of intranuclear inclusions. Increased Ca^{2+} also induces calpains, calcium-dependent non-caspase cysteine proteases. Besides the mutant form, wild-type Htt is also a target of calpains [Goffredo *et al.*, 2002], and its cleavage may abolish its anti-apoptotic effects and the trophic support it provides to neurons by the enhancement of the expression of *brain-derived neurotrophic factor* (BDNF).

4.3.4. Loss of wild-type Huntingtin function

Though the theory of a toxic gain of function as the causative of Huntington's disease is widely accepted, current investigations indicate that wild-type Huntingtin plays important roles in neurons, and loss of wt Htt function may lead to striatal vulnerability and contribute to pathology.

Huntingtin is essential in embryonic development as homozygous knockout mice die during gastrulation [Duyao *et al.*, 1995; Zeitlin *et al.*,

1995]. A single copy of the gene is sufficient for normal development in mice, but further reduction of *Htt* activity results in abnormal brain development [White *et al.*, 1997]. Furthermore, conditional inactivation of wt *Htt* in the brain leads to motor deficits, striatal degeneration and early death in adult mice [Dragatsis *et al.*, 2000].

Among other proteins, Htt interacts with HAP1 and Hip-1, both of which are associated with membrane vesicles and cytoskeletal components, suggesting a role in intracellular vesicle trafficking and synaptic functions [Gusella and MacDonald, 1998]. Htt also associates with clathrin coated vesicles involved in endocytosis [Velier *et al.*, 1998; Sipione and Cattaneo 2001].

Wild-type Huntingtin also has anti-apoptotic functions. In cell culture, wt Htt inhibited the autoprocessing of procaspase-9 into its two active fragments, resulting in inhibition of the apoptotic cascade [Rigamonti *et al.*, 2001]. Moreover, wt Htt interacts with the pro-apoptotic protein Hip-1, participating in caspase-8 activation [Gervais *et al.*, 2002], and this interaction is reduced with increasing polyQ repeat length [Wanker *et al.*, 1997]. Sequestration of Hip-1 by wt Htt may lead to the suppression of apoptosis, while polyQ expansion abolishes this effect. In accordance with these results, wt *Huntingtin* has been shown to be protective against apoptotic stimuli, such as serum deprivation and 3-nitropropionic acid [Rigamonti *et al.*, 2000], and its over-expression also protects from the toxic effects of an expanded *Huntingtin* allele in transgenic mice [Leavitt *et al.*, 2001].

Understanding the normal function of unexpanded Huntingtin protein may also shed light on the tissue selectivity of neuronal death seen in HD patients. Recent searches for striatum specific Huntingtin functions led to the identification of a process which may contribute to the selective neuronal loss. Striatal neurons, the most affected cells in HD, require brain-derived neurotrophic factor (BDNF) for their differentiation and survival [Ivkovic and Ehrlich, 1999]. BDNF is synthesized by cortical neurons and transported to the striatum via cortico-striatal afferents [Altar *et al.*, 1997]. Wild-type Huntingtin has been found to up-regulate

the production of BDNF on the level of gene transcription while mutant Huntingtin suppressed its expression [Zuccato *et al.*, 2001]. Similarly, in transgenic mice, over-expression of wt *Htt* up-regulated the BDNF protein level in the cerebral cortex whilst mutant *Htt* caused a significant decrease in cortical BDNF production and cortically derived BDNF level in striatum [Zuccato *et al.*, 2001]. These results indicate that loss of wt Huntingtin function may lead to insufficient trophic support thus putting striatal neurons at risk of degenerating.

4.4. The role of *Drosophila* model systems

The significance of the contributions that cell culture and transgenic animal model systems make to the research of polyglutamine induced diseases is indisputable (Table 2). These disorders affect the central nervous system, limiting human molecular studies to the analysis of postmortem brain samples. The information collected this way applies only to the final stage of the diseases and little can be learned concerning the earlier aspects and mechanism of pathology. Moreover, the slow progression of the disease makes human clinical studies quite difficult and expensive. On the other hand, transgenic animal and cell culture models are cheap, relatively fast and allow the spatial and temporal manipulation of transgene expression. Thus, the course of the disease can be modified and different aspects of pathology can be investigated in a reasonable time frame. These features make animal models well suited for studying polyQ pathology, and their application led to several key findings in this field [Rubinsztein, 2002].

The first transgenic mouse model of HD expressing a truncated N-terminal fragment of Htt with an expanded polyQ domain was reported in 1996 [Mangiarini *et al.*, 1996]. Since then, animal models of several polyglutamine disorders have been generated in various model organisms (see Table 2.). The question is, how *Drosophila* models can contribute to

	Caenorhabditis	Drosophila	Mouse
Models in use	HD	HD, SBMA, SCA1, SCA3	HD, DRPLA, SBMA, SCA1, SCA2, SCA3, SCA7
Aggregation	✓	✓	✓
Cell death		✓	✓
Progressive degeneration		✓	✓
Late onset		✓	✓
Neuronal dysfunction	✓	✓	✓

Table 2.: Transgenic animal models recapitulate several characteristic features of polyglutamine diseases. (Bodai *et al.*, 2003)

polyglutamine research and what they can provide in comparison with other models. Flies are cheap, have a short life cycle and are easy to handle. As flies are multicellular organisms, *Drosophila* models are better suited to mimic the characteristics of human disorders than yeast or cell culture models. The majority of pathological features of HD can be recapitulated in transgenic fly models, including dominant gain of function neurotoxicity, nuclear inclusion formation, progressive neurodegeneration, behavioral abnormalities and early death [Chan, 2002a]. The main power of *Drosophila* lies in its well characterized genetics and genomics, and the wide range of powerful genetic tools developed during nearly a century of fruit fly research. Thanks to these features, *Drosophila* is exceptionally well suited for both forward and reverse genetic approaches. Several studies have proven their effectiveness in hypothesis-driven analysis of candidate processes of polyQ pathogenesis [Warrick *et al.*, 1999; Steffan *et al.*, 2001; Chan *et al*, 2002b], and the power of genetic screening was also demonstrated in the identification of pathological pathways [Kazemi-Esfarjani and Benzer, 2000, Fernandez-Funez *et al.*, 2000]. Besides this, *Drosophila* models are useful tools for chemical compound screening [Apostol *et al.*, 2003]. The screening of chemical compound libraries to search for potential drugs beneficial in HD primarily takes place in cell culture systems, often based on inhibition of aggregate formation. The number of false positives is rather large in these systems. Thus, validation in animal models is necessary and *Drosophila* may be a fast and cost effective screening step

prior to mouse tests.

Taken together, *Drosophila* models of polyglutamine disorders contribute to both the characterization of molecular aspects of pathology and identification of compounds affecting pathological pathways. Thus, they play important role in the development of evidence-based medicine for these diseases.

5. AIMS

5.1. Establishment of a *Drosophila* model system of HD that makes the testing of compounds and genetic factors possible

In order to analyze the molecular pathomechanisms of Huntington's disease *in vivo*, we decided to build up a transgenic *Drosophila melanogaster* model system that enables us to test the effects various factors exercise on polyQ induced phenotypes. To be able to use such a system for genetic tests and chemical compound screening, several tasks had to be done.

a. Characterization of transgenic *Drosophila* stocks expressing polyglutamine or *Htt* transgenes under the control of the bipartite UAS/GAL4 expression system. Determine which transgenic stock combinations have suitable phenotypes to reflect changes in polyQ pathology.

b. Optimize fly culturing conditions for feeding experiments.

c. Determine the highest applicable dose of the organic compound solvent dimethyl sulfoxide (DMSO) in feeding experiments.

d. Test the sensitivity of the *Drosophila* model system by challenging it with Congo red (CR), a chemical suppressor of aggregate formation, and with loss of *C-terminal Binding Protein*.

- Determine the applicable dose for Congo red.
- Examine the effect of CR on the semilethal phenotype of *polyQ* expressing flies.
- Determine whether CR exerts influence on *polyQ* induced degeneration of photoreceptor neurons.
- Investigate whether partial loss of *CtBP*, a genetic enhancer of SCA1 phenotypes, modifies *Q93Httexon1* induced phenotypes in a genetic

interaction test.

5.2. Investigation into the role of disturbed protein acetylation in Huntington's Disease

Previous experiments indicated that histone acetyltransferase enzymes bind to polyQ proteins, and thus might be involved in polyQ pathogenesis. To investigate the role altered protein acetylation plays in polyQ pathogenesis *in vivo*, we decided to undertake the following tasks.

a. Ascertain whether genetic restoration of the acetylation balance by mutants reducing deacetylase activity suppresses polyglutamine induced pathology *in vivo*. Analyze the influence that partial loss of the HDAC co-repressor *Sin3A* exerts on the semilethal and progressive neurodegenerative phenotypes of transgenic flies expressing mutant *Htt*.

b. Investigate the effects of HDAC inhibitor compounds on polyQ pathology *in vivo*.

- Determine the applicable doses of the HDAC inhibitors butyrate and suberoylanilide hydroxamic acid (SAHA).
- Test whether HDAC inhibitors affect the semilethal and neurodegenerative phenotypes of *polyQ* expressing transgenic flies.
- Examine whether HDAC inhibitors suppress the phenotypes of transgenic flies expressing an expanded polyQ domain in disease-gene context.

c. Determine whether the influence of HATs and HDACs on polyQ pathology is the result of additional effects of several proteins, or whether a few loci account for the majority of the effects.

- Validate deficiencies for HAT and HDAC genes.
- Examine the effects HAT genes belonging to different protein families have on polyglutamine induced phenotypes using genetic interaction

tests.

- Determine whether deacetylase enzymes belonging to different HDAC classes are able to suppress polyQ phenotypes in transgenic flies.

6. MATERIALS AND METHODS

6.1. *Drosophila* media and compounds

Drosophila stocks were maintained on Jazz food (Applied Scientific) or standard cornmeal – yeast – sucrose *Drosophila* medium. For chemical compound experiments freshly prepared Heidi-food (9.3 g agar, 61.2 g cornmeal, 129.4 g dextrose, 32.4 g dry yeast and 10 ml 10% Tegosept for 1 liter medium) was used. Compounds along with a few drops of food color were added directly to freshly prepared media cooled to 45 °C. Compound supplemented media were stored at 10 °C and used within a few days.

Congo red (Sigma) was dissolved in water at the concentration of 20 mM. Suberoylanilide hydroxamic acid (Calbiochem) stock solution was prepared by dissolving the compound in DMSO at the concentration of 20 mM. Butyrate (Sigma) was used as free acid.

6.2. *Drosophila* stocks and crosses

Expression of polyglutamine containing proteins were driven by the bipartite UAS/GAL4 expression system in transgenic flies. Flies expressing *polyQ* or *Htt* transgenes under the control of a yeast upstream activating sequence (UAS) were generated in our laboratory previously [Marsh *et al.*, 2000]. The transgenic stock used in our experiments were *w; P(w^{+mC} = UAS - Q48 + myc/flag)$^{\#36}$* ; *w; P(w^{+mC} = UAS - Q108)$^{\#16}$* and *w; P(w^{+mC} = UAS - Q93Httexon1)$^{\#4F1}$*. GAL4 drivers (*w; P(w^{+mc}; w+; elav - GAL4)/CyO, P(w^{+mc} = Act - GFP)JMR1CyO actin-GFP* and *w P(w^{+mW}.hs = GawB)elavC155*) and HAT/HDAC stocks (*Df(1)ovo44/FM6 ; Df(1)C149/FM6 ; Df(1)JA26/FM7c ; Df(1)N12, ras^{1} v^{1}/FM6 ; Df(1)JA27/FM7c ; Df(2L)XE-3801/CyO, P{ry$^{+t7.2}$=sevRas1.V12}FK1 ;*

Df(2L)spd, al^{1} *dp*^{ov1}*/CyO ; In(1)w*^{m4h} *y*^{1}*; Df(2L)TE29Aa-11/CyO ; P{ry*$^{+t7.2}$*=PZ}Sir2*^{05327} *cn*^{1}*/CyO; ry*^{506} *; P{ry*$^{+t7.2}$*=PZ}Sin3A*^{08269} *cn*^{1}*/CyO; ry*^{506} *; P{ry*$^{+t7.2}$*=PZ}Rpd3*^{04556} *ry*^{506}*/TM3 ry*^{RK} *Sb*^{1} *Ser*^{1} *; Df(3L)iro-2, Sb*$^{sbd-2}$*/TM3 Sb*^{1} *; y*^{1} *w***; P{w*$^{+mC}$*=lacW}Mi-2*^{j3D4}*/TM6B Tb*^{1} *; Taf250*^{1} *red*^{1} *e*^{1}*/TM3 Sb*^{1} *; Df(3R)p25, red*^{1} *e*^{1}*/TM3 Sb*^{1} *; Df(3R)ry*^{75}*, kar*^{2}*/TM3 Sb*^{1} and *Df(3R)96B/TM3, P{w*$^{+m*}$*=*}**, Sb*^{1}) were obtained from the Bloomington *Drosophila* Stock Center.

For compound feeding experiments transgenic flies were crossed to flies expressing the yeast GAL4 transcriptional activator driven by the neuron specific promoter *elav* that is expressed in all neurons from embryogenesis on, and their progeny were raised on media supplemented with the specific compounds. For testing the effects of *Sin3A* on polyQ phenotypes we crossed *w; P(w*$^{+mC}$*=UAS-Q93Httexon1)*$^{\#4F1}$ virgins to *w P(w*$^{+mW}$*.hs = GawB)elavC155/Y; Sin3A*^{08269}*/Bc Gla* males, and analyzed the phenotypes of the emerging female progeny. HAT / HDAC interaction crosses were performed by crossing *w P(w*$^{+mW}$*.hs = GawB)elavC155/Y; P(w*$^{+mC}$ *= UAS - Q93Httexon1)*$^{\#4F1}$*/TM6* males to females carrying the specific mutations or deficiencies over appropriate balancer chromosomes. Crosses with the *w; P(w*$^{+mC}$ *= UAS - Q48 + myc/flag)*$^{\#36}$ and *w; P(w*$^{+mC}$ *= UAS - Q108)*$^{\#16}$ stocks were done at 27 °C while *w; P(w*$^{+mC}$ *= UAS - Q93Httexon1)*$^{\#4F1}$flies were mated at 25 °C.

6.3. Pseudopupil analysis

Pseudopupil analysis allows the number of rhabdomeres in the ommatidia of the compound eye to be counted. Anesthetized adult flies were decapitated and their heads were mounted on a microscope slide by dipping one eye in a drop of nail polish. Eyes were covered with immersion oil and analyzed with a Nikon EFD-3/Optiphot-2 microscope using a X50 immersion objective, and photographed with a Spot camera.

6.4. Quantification of transgene expression

The expression levels of *Huntingtin* transgenes in the presence or absence of HDAC inhibitors were measured by western analysis. Synchronyzed eggs of the genotype of *w P(w^{+mW}.hs = GawB)elavC155/w ; P(w^{+mC} = UAS – Q93Httexon1)$^{\#4F1}$/+* were collected and transferred into vials containing media supplemented with 2 µM SAHA, 100 mM butyrate or solvent alone. Five days after egg laying, wandering L3 larvae were collected and their brains dissected in PBS. A western blot of extracts of larval brains expressing *Q93Httexon1*, and treated either with solvent alone or solvent with SAHA or butyrate was probed with anti-Htt antibody. Similar amounts of protein were loaded, as determined by Bradford assays and confirmed by Coomassie staining of the gel. (Western blotting was performed by Joan S. Steffan.)

6.5. Single embryo genomic PCR

For validating the deficiencies, stocks carrying the deletions over balancer chromosomes with a GFP transgene were established. Genomic DNA samples were prepared from embryos homozygous for the deletions. GFP negative embryos were collected 14-18 hours after egg laying. Single embryos were squashed in 20 µl squash buffer (10 mM Tris pH 8.0, 1 mM EDTA, 25 mM NaCl) containing 200 mg/ml proteinase K. After 30 minutes of incubation at 37 °C, the enzyme was inactivated for 2 minutes at 95 °C. 5 µl aliquots of the samples were used as template in conventional 20 µl PCR reactions. Each sample was analyzed by three PCR reactions: a reaction specific for the gene of interest, a reaction specific for p53 (positive control) and a reaction specific for GFP (genotype control). At least five homozygous embryos were analyzed for each tested deletion. Samples of GFP positive embryos were used as positive controls. The PCR primers used were the following: *GFP* forward: CAT GGC CAA

CAC TTG TCA CT, *GFP* reverse: ATC TTG GGA AAG GGC AGA TT; *p53* forward: GTC CGC TGT CAA AAT CAC CT, *p53* reverse: GGC GAC GTA GAT TCT CTT GG; *CG10899* forward: CGA GGA GGT TGT CTG GGT TA, *CG10899* reverse: CCA TAA TCC AAT GCC AGC TT; *CG14222* forward: AGG ATC GGA TTA GCC GAG AT, *CG14222* reverse: GGG CGC TAC TGG GTT GATA; *CtBP* forward: AGG CAG TGG GAG CTC TGAT, *CtBP* reverse: AGA TGA CGT TGA AGC CGA AC; *EG:EG0007.7* forward: TCA CAA TGC CTC CGT ATC AG, *EG:EG0007.7* reverse: CCA GTC TTT GGG TGT CCA GT; *HAT* forward: AAA GAC GTA ACG GAC GAG GA, *HAT* reverse: CAG GCA CAG ATG CTG ACA GT; *HDAC3* forward: TTG TCC TGT ATG CGA AGA CG, *HDAC3* reverse: TGG TTG TGG TTC AGC TTC TG; *HDAC4* forward: AGC GTA GGC AGC AAA AGA AC, *HDAC4* reverse: TTA TAG TAG CCC GGC TGT CC; *mof* forward: TGC AGT TTG GCA ACT ACG AG, *mof* reverse: ACT ACC GAT TAC GCC CTC CT; *P/CAF* forward: TCA AGG AAA ACC AAC CCA TC, *P/CAF* reverse: CCG GGT CTT ACT TTC TGC AC; *Smrter* forward: GAG TTG CAG CAC AGA AGC AA, *Smrter* reverse: TCG CTG AGT GCA TCG TAC TT; *Su(var)205* forward: CAA GCG AAA GTC CGA AGA AC, *Su(var)205* reverse: AGT TGG TTG GGT GGT TTT GA.

6.6. Statistical analysis

Experimental data sets were statistically analyzed using SigmaStat (SPSS, Inc). Data derived from groups subjected to two experimental conditions were analyzed by Two Way ANOVA (TWA). Multiple data sets collected from experiments testing one experimental condition were analyzed by One Way ANOVA (OWA) or Kruskal-Wallis non-parametric ANOVA (KWOWA), if the distribution of data was non-normal. ANOVA tests were followed by multiple comparison tests employing Student-Newman-Keuls (SNKMCT), Dunnett's (DTMCT) or Dunn's (DNMCT) methods. Two groups of data were compared by the non-parametric Mann-Whitney Rank Sum Test (MWRST). Category data were analyzed

using Yates' corrected X^2 probe. A $P<0.05$ was considered to be statistically significant.

7. RESULTS

7.1. Establishment of a *Drosophila melanogaster* model of HD for compound screening and genetic tests

7.1.1. Stock characterization

Our *Drosophila* model is based on the neuron specific expression of polyglutamine and *Htt* transgenes in transgenic flies. Targeted expression of the transgenes is controlled by the GAL4/UAS system [Brand and Perrimon, 1993]. In this bipartite system, expression of the gene of interest is controlled by the yeast *upstream activator sequence* (UAS). The transcription of the gene downstream of UAS depends on the presence of the yeast transcriptional activator, GAL4. In the absence of GAL4, the transgene is transcriptionally silent. To activate its expression, flies carrying the UAS construct are mated to flies expressing a GAL4 transgene. In their progeny, expression of the gene of interest will follow the spatial and temporal expression pattern of the GAL4 driver (Fig. 1).

Several transgenic *Drosophila* lines expressing *polyQ* or *Htt* transgenes under the control of UAS were established in our laboratory previously [Marsh *et al.*, 2000]. Polyglutamine transgenes encode proteins with 48 or 108 uninterrupted glutamine repeats alone, removed from their disease gene context. *Htt* transgenes encode the first exon of the *Huntingtin* gene with 50 or 93 uninterrupted CAG repeats. To build up an *in vivo* system that makes the testing of chemical compounds and genetic factors influencing *polyQ* induced phenotypes practical, the characterization of these transgenic lines was necessary. We used the semilethal phenotype of polyglutamine expressing flies as a marker to estimate the sensitivity and responsiveness of the different lines to factors affecting polyQ pathology. The viability of flies was measured at several

temperatures (25, 27 and 29 °C) as the expression of the transgenes is enhanced at higher temperatures. We wanted to choose such lines for further experiments that show severe semilethal phenotypes and are highly sensitive to the expression levels of the transgenes. These lines were expected to be appropriate to reflect changes in polyQ pathogenesis through suppression or enhancement of their phenotypes.

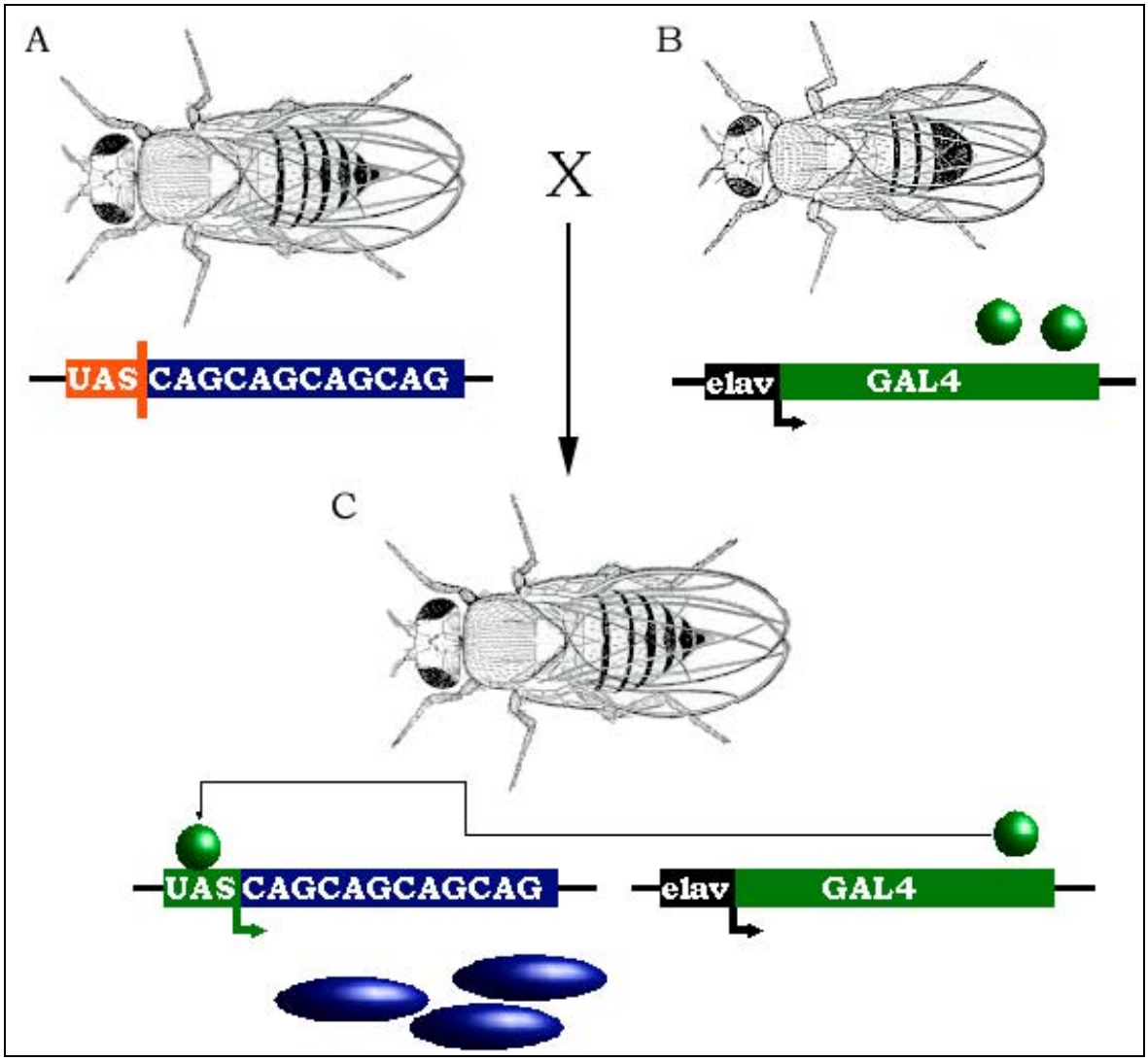

Figure 1.: Neuron specific expression of polyglutamine proteins by the UAS/GAL4 system. A transgenic line carrying a polyglutamine encoding transgene under the control of UAS (**A**) is crossed to a transgenic GAL4 driver line (**B**). The expression of the GAL4 transcriptional activator is directed by the *elav* neuron specific promoter. In neuronal cells of their progeny carrying both transgenes (**C**) the GAL4 protein binds to the UAS sequence and activates the expression of the polyglutamine encoding transgene.

Flies expressing a 108 repeats long *polyQ* transgene (*Q108*) were crossed to flies carrying an X chromosomal neuron specific GAL4 source,

P(w^{+mW}.hs = GawB)elavC155, and the percentages of progeny reaching pupariation were recorded [Table 3]. 48 repeats long *polyQ* transgenes (*Q48*) were driven by a second chromosomal neuron specific GAL4 source, *P(w^{+mc}; w+; elav - GAL4).* In this case, the eclosion rate of *Q48* expressing flies was compared to that of control animals [Table 4]. Flies carrying the first exon of the *Htt* gene with 50 or 93 residues long polyglutamine repeats were crossed to flies carrying the X chromosomal GAL4 source, and the percentages of eclosing progeny were recorded [Table 5]. After the evaluation of the results, we chose the transgenic lines *w; P(w^{+mC} = UAS - Q108)$^{\#16}$* (viability changed from 26% to 3% in the 25 °C - 29 °C temperature range), *w; P(w^{+mC} = UAS - Q48 + myc/flag)$^{\#36}$* (viability changed from 53% to 10.5% in the 25 °C - 29 °C temperature range) and *w; P(w^{+mC} = UAS - Q93Httexon1)$^{\#4F1}$* (viability changed from 23% to 6% in the 25 °C - 29 °C temperature range) for further work.

	Q108 #12	***Q108 #14***	***Q108 #16***
25 °C	49 %	12 %	26 %
27 °C	33 %	7 %	23 %
29 °C	28 %	1 %	3 %
Range	21 %	11 %	23 %

Table 3.: Pupariation rates of *Q108* expressing flies at different culturing temperatures.

	Q48#13	***Q48#25***	***Q48#30***	***Q48#31***	***Q48#32***	***Q48#36***	***Q48#42***
25 °C	57 %	78 %	81 %	41 %	94 %	53 %	76 %
29 °C	32 %	16 %	28 %	5.5 %	113 %	10.5 %	66 %
Range	25 %	62 %	53 %	35.5 %	19 %	42.5 %	10 %
Rhabdo-meres	5.41	4.39	4.98	3.07	7	5.68	6.45

Table 4.: Eclosion rates of *Q48* expressing flies at different culturing temperatures. The average number of rhabdomeres per ommatidia at 25 °C are also shown.

	Q50E1 #B2	***Q50E1 #Y4***	***Q93E1 #4F1***	***Q93E1 #16F1***	***Q93E1 #24***
25 °C	66 %	84 %	23 %	32 %	0 %
27 °C	55 %	63 %	13 %	34 %	0 %
29 °C	37 %	48 %	6 %	30 %	0 %
Range	29 %	36 %	17 %	2 %	0 %

Table 5.: Eclosion rates of *Httexon1* expressing flies at different culturing temperatures.

7.1.2. Media and solvent optimization

Dimethyl sulfoxide is a frequently used solvent for organic compounds, and it is also frequently utilized as a drug delivery vehicle. To be able to screen a wide array of chemical compounds in our *Drosophila* model system, the toxic effect of DMSO had to be determined. We tested the toxicity of DMSO on wild-type *Canton S* flies by culturing them on media supplemented with increasing doses of the compound. The test was performed at 27 °C on two types of fly media available in our lab: Heidi and Jazz food. Synchronized eggs were collected on grape juice plates and transferred into vials (100 eggs/vial) supplemented with 0%, 0.1%, 0.2%, 0.5%, 1% or 2% DMSO. The number of alive animals in the first larval, third larval, pupal and adult developmental stages was counted in three vials per concentration (Fig. 2).

The percentages of L1 larvae hatched were similar on the two media tested (80% and 84% in the case of Jazz and Heidi food, respectively) and did not depend on the concentration of DMSO administered. In the percentage of animals reaching pupariation and eclosion we found a marked difference between the two media. Flies cultured in Jazz food had significantly lower survival rates at these developmental stages at all DMSO concentrations tested than those cultured on Heidi food ($P<0.001$, TWA, Student-Newman-Keuls MCT), and also showed greater deviations between replicates. Thus, we concluded that Heidi food is more suitable for feeding tests and used this medium in later experiments.

We have found that low doses of DMSO do not exhibit toxic effects. Flies grown on 0%, 0.1%, 0.2% or 0.5% have similar pupariation (62%, 62%, 64% and 61%, respectively) and eclosion (59%, 54%, 57% and 56%, respectively) rates. At the concentration of 1% the toxic effect of DMSO became obvious ($P<0.05$, KWOWA, DTMCT). At 1% DMSO the pupariation rate drops to 29% and only 1.4% of the animals eclose. No animals grown on media complemented with 2% DMSO reach pupariation. Based on these results we determined the highest applicable dose of DMSO as 0.5%.

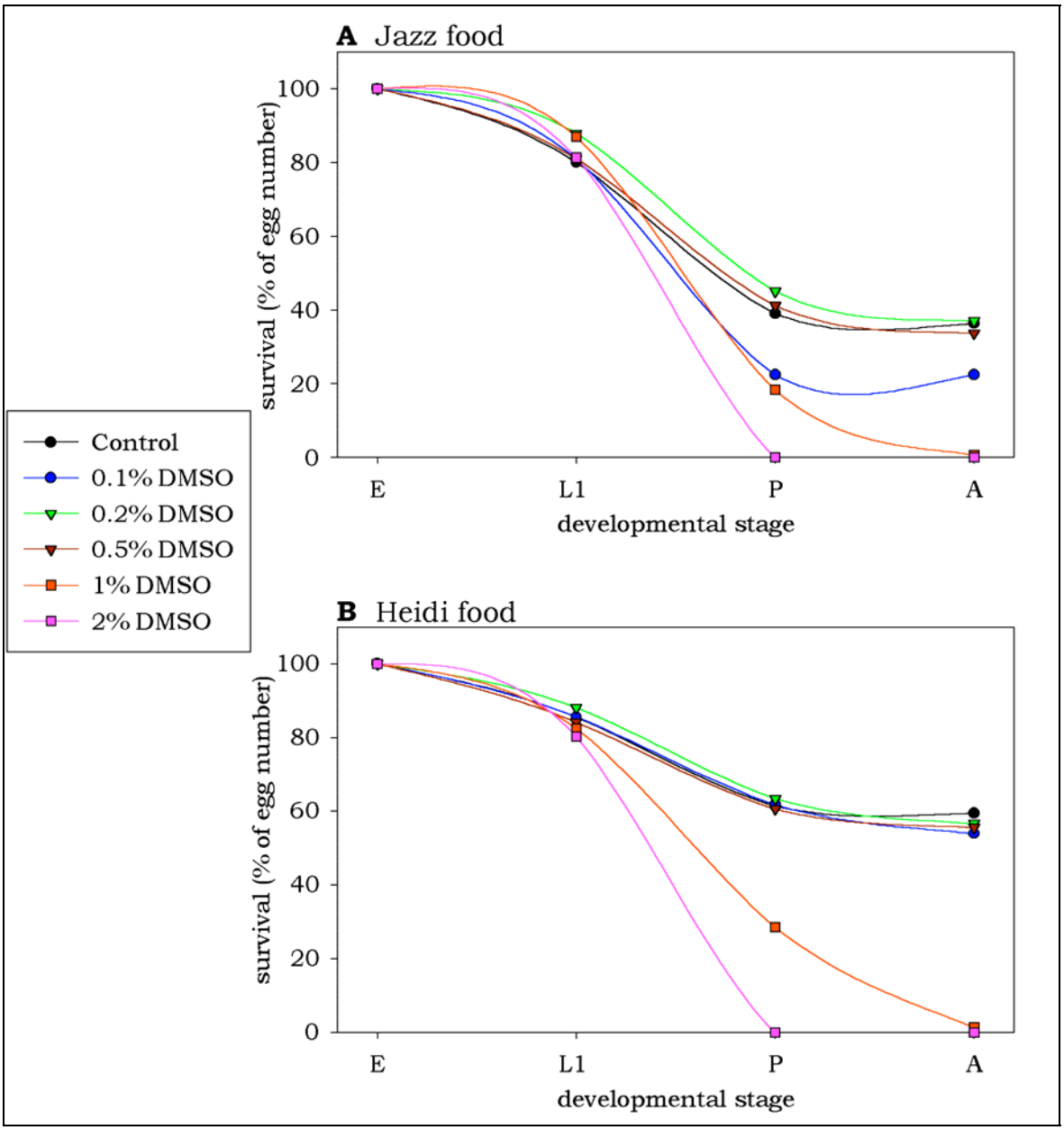

Figure 2.: Toxicity of DMSO for *Canton S* flies at different developmental stages. **A.** Flies cultured on Jazz food exhibit low viability at 27 °C. **B.** DMSO at concentrations up to 0.5 mM does not decrease the eclosion rate of flies grown on Heidi food. Higher doses result in lethality.

7.1.3. Testing the sensitivity of the transgenic system

One of the main goals in developing a transgenic *Drosophila* model for HD was to establish an assay that allows fast and reliable *in vivo* validation of chemical compounds proven to be effective inhibitors of polyQ pathogenesis in cell culture. Such an intervening step of drug

screening excludes numerous compounds giving false positive results in cell culture experiments, and in this way reduces the number of drugs to be tested in more time consuming and costly mammalian model systems. To validate the ability of our model to indicate effective inhibitors of polyglutamine pathogenesis by altered phenotype of *polyQ* expressing flies, we tested a previously characterized inhibitor of polyQ toxicity, Congo red. Congo red, a histological dye commonly used to detect amyloid deposits, inhibits aggregate formation of expanded Htt *in vitro* [Heiser *et al.*, 2000], in cell culture [Apostol *et al.*, 2003] and in HD transgenic mouse organotypic hippocampal slice cultures [Smith *et al.*, 2001 Neurobiol Dis].

Prior to testing the effects of Congo red on polyglutamine expressing flies, initial toxicity tests were performed in wild-type flies. Synchronized *Canton S* flies were grown in vials (100 eggs/vial) containing media supplemented with 0.5 mM, 1 mM or 2mM Congo red at 27 °C. After 14 days the number of eclosed flies were recorded and compared to that of flies grown on control food (Fig. 3A). Congo red did not have toxic effects at the concentrations of 0.5 and 1 mM but a slight decline of viability (88% of the control) was observed at 2 mM. Higher concentrations of CR up to 5 mM were also tested to determine a more detailed dose-response curve, but the detrimental effect of the compound on the structure of the fly medium made the interpretation of data impossible.

After the applicable dose of Congo red was determined, we performed feeding experiments to evaluate the potential suppressor effect of CR on polyQ induced phenotypes. As a primary assay, the viability of flies expressing a 48 (Q48) or 108 (Q108) residue long polyglutamine polypeptide was measured. Expression of the transgenes was driven by a second chromosomal *P(w^{+mc}; w+; elav - GAL4)* neuron specific GAL4 source. Both combinations resulted in intermediate phenotypes allowing the detection of both suppressive and enhancing effects. *w; P(w^{+mc}; w+; elav - GAL4)/CyO, P(w^{+mc} = Act - GFP)JMR1CyO actin-GFP* females were crossed to *w; $Q48^{\#36}$* or *w; $Q108^{\#16}$* males at 25 °C. Synchronized eggs were transferred to vials (50 eggs/vial) containing media supplemented

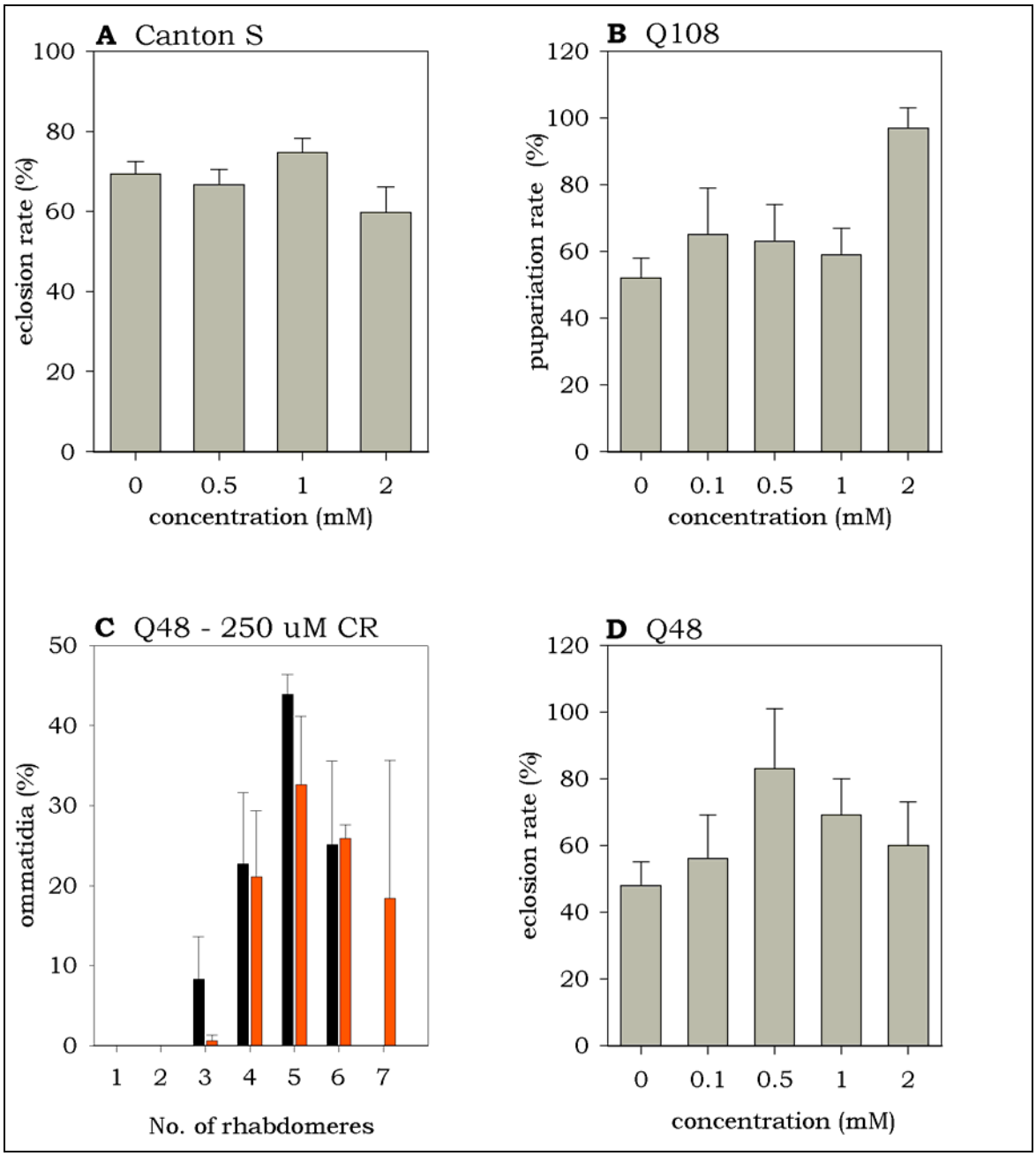

Figure 3.: Congo red suppresses polyglutamine induced phenotypes of *Q48* and *Q108* expressing transgenic flies. **A.** CR does not decrease the eclosion rate of wild-type flies at concentrations up to 1mM. **B.** Administration of CR suppresses larval lethality of *Q108* expressing flies. Bars represent the pupariation rate. **C.** Administration of 250 mM CR suppresses photoreceptor neuron degeneration in 6 days old *Q48* expressing flies. A shift towards ommatidia with higher rhabdomere numbers can be observed compared to untreated control (black). **D.** CR increases the survival rate of *Q48* expressing flies. Bars represent the eclosion rate.

with 0 mM, 0.1 mM, 0.5 mM, 1 mM or 2 mM Congo red, and incubated at 27 °C. For testing the viability of *Q48* expressing flies, the number of eclosed *w; Q48/elav>GAL4* animals were compared to that of *w; Q48/CyO, actin>GFP* control. The viability of *Q108* expressing flies was

determined as the ratio of GFP negative and positive pupae on the 8th day after egg laying. In the case of *Q48*, the viability of flies grown on control food was 48%. The eclosion rate increased to 56% in media supplemented with 0.1 mM Congo red and the maximal rescue (83% viability) was observed in flies fed with 0.5 mM CR. Higher doses of CR resulted in lower viability rates compared to this value (69% and 60% viability in the case of 1 mM and 2 mM CR, respectively), presumably because of toxic side effects of the compound (Fig. 3D).

Different dose response were observed in flies expressing *Q108*. In this case, lower doses of Congo red had moderate effect (65%, 63% and 59% viability in the case of 0.1 mM, 0.5 mM and 1 mM CR, respectively) compared to the baseline (52%), while 2 mM CR caused nearly complete rescue (97%, Fig. 3B). The difference in dose responses may be attributed to several independent effects. First, Congo red influences polyQ pathogenesis by inhibiting aggregate formation. Since the length of the polyglutamine repeat influences the kinetics of aggregation, the optimal concentration of CR to rescue polyQ pathogenesis might be different for polyQ proteins with different repeat numbers. Secondly, the dose response may be influenced by the developmental stage that is evaluated. Though flies do not feed while in the pupal stage, toxic effects of high doses of accumulated CR may negatively affect the eclosion rate. Conversely, the concentration of CR may diminish during the four day pupal period, thus releasing aggregate formation from suppression and leading to decreased viability.

To ascertain whether Congo red also suppresses polyglutamine induced neurodegeneration, we examined the number of rhabdomeres, subcellular light gathering structures, in the compound eyes of CR fed flies. In the *Drosophila* compound eye, arrays of photoreceptor neurons form a repeating trapezoidal arrangement of seven visible rhabdomeres. Rhabdomeres can be visualized by the pseudopupil technique (Fig. 4). The average number of rhabdomeres per ommatidium provides information about the state of photoreceptor neurons. *elav>GAL4* driven expression of *Q48* leads to a progressive loss of visible rhabdomeres

indicating neurodegeneration in the compound eye.

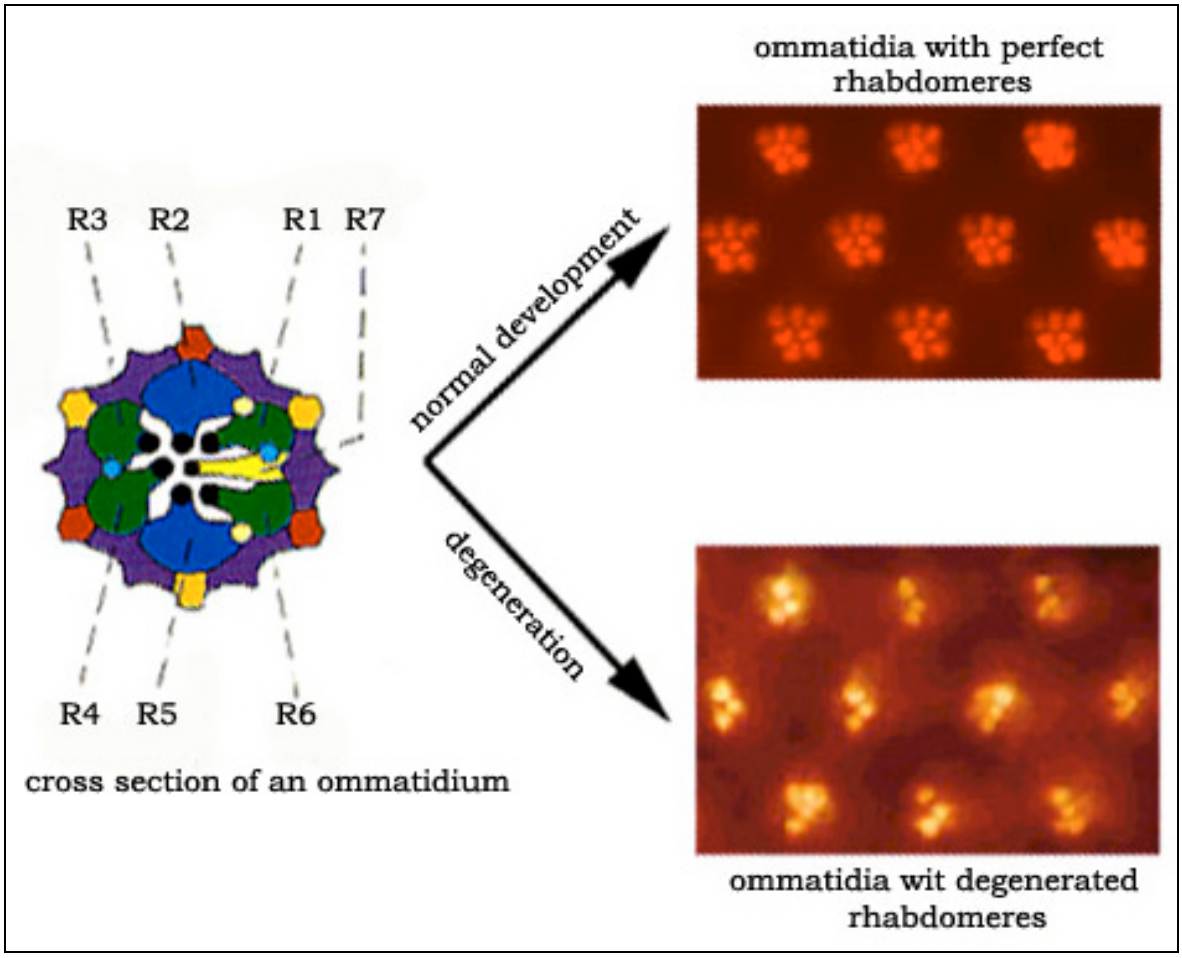

Figure 4.: Visualization of rhabdomeres by the pseudopupil technique. Each ommatidia of the compound eye contains eight photoreceptor neurons, seven of which (R1-R7) have visible rhabdomeres (black dots). If visualized by the pseudopupil technique, a normal eye shows a field of regularly arranged ommatidia with seven bright dots (rhabdomeres). Neurodegeneration leads to the reduction of rhabdomere numbers.

Congo red was tested and found to suppress this effect at the concentrations of 2.5-2500 μM. The most evident rescue was observed at 250 μM ($P<0.001$, MWRST). At this concentration, CR increased the average number of visible rhabdomeres of 10 day old flies from 4.4 to 4.7 (Fig. 3C). At higher doses, the suppression of neurodegeneration was reduced, presumably due to the toxic effects of CR.

Having seen that polyQ phenotypes are modifiable by chemical compounds, we were interested whether genetic factors may cause similar effects. To test this possibility, we crossed *w P(w^{+mW}.hs = GawB)elavC155/Y; P(w^{+mC} = UAS - Q93Httexon1)$^{\#4F1}$/TM6* males to females

carrying the Df(3R)ry75 deletion, that uncovers the *C-terminal Binding Protein* (*CtBP*) gene, and the progeny were raised at 25 ºC. CtBP interacts with Huntingtin [Kegel *et al.*, 2002], and enhanced the *polyQ* induced eye phenotype in a transgenic *Drosophila* model of SCA1 [Fernandez-Funez *et al.*, 2000]. We have found that hemizygosity of the *CtBP* gene reduced the viability of *Q93Httexon1* expressing animals to 66% (P=0.091, X^2 probe) compared to control animals wild-type for the *CtBP* gene. Thus, loss of *CtBP* caused similar effects in our model than in a SCA1 model.

Taken together, feeding experiments and genetic tests proved that our transgenic *Drosophila* model is a sensitive system capable of indicating conditions exerting influence on polyglutamine pathology. Thus, transgenic flies can be used to screen chemical compound libraries and mutant collections as well as to conduct hypothesis-driven *in vivo* studies.

7.2. Investigation into the role of disturbed protein acetylation in polyglutamine disorders

Several important cellular proteins have been found to interact with mutant Htt, establishing the sequestration model of polyQ pathogenesis. The sequestration model hypothesizes that depletion of certain cellular factors by binding to polyQ proteins may have a fundamental role in pathogenesis. Previous *in vivo* [*Fernandez-Funez* et al., 2000] and *in vitro* [Steffan *et al.*, 2000] experiments demonstrating the interaction of polyglutamine polypeptides with proteins participating in histone acetylation/deacetylation attracted our attention to this protein modification system.

In close collaboration with Leslie M. Thompson's laboratory at UCI, we decided to investigate the potential role of altered acetylation balance in HD. *In vitro* binding experiments performed in the Thompson lab showed that a polypeptide encoded by the first exon of *Htt* with 51

glutamines binds to the acetyltransferase domains of the HAT proteins CBP and P/CAF. Accordingly, they also found that expanded Htt polypeptides inhibited the acetyltransferase activity of CBP, p300 and P/CAF.

7.2.1. Genetic restoration of the acetylation balance suppresses polyQ pathology *in vivo*

If inhibition of acetyltransferase activity by polyglutamine containing proteins has a significant role in the pathogenic process, then one may expect that suppression of the opposing enzyme group, the histone deacetylases, may relieve pathology. Inhibition of HDAC activity either genetically or pharmacologically would correct the acetylation balance in the nucleus, and thus potentially restore the normal functioning of genes and proteins affected by low acetyltransferase activity. To test this possibility *in vivo*, we manipulated deacetylase activity by introducing a *Sin3A* loss of function allele, $Sin3A^{08269}$, to our *Drosophila* HD model system. *Sin3A* encodes an evolutionarily conserved co-repressor protein that is a component of HDAC complexes, thus its loss affects the activity of several HDAC proteins.

For the test, we crossed transgenic virgins carrying the first exon of *Htt* with an expanded CAG repeat (*w; Q93Httexon1*$^{\#4F1}$) to males carrying a GAL4 driver and the *Sin3A* mutation (*w P(w*$^{+mW}$*.hs = GawB)elavC155/Y; Sin3A*^{08269}*/BcGla*). Expression of the *Q93Httexon1* transgene results in ~70% lethality, early adult death and progressive degeneration of photoreceptor neurons. The females emerging from this cross express the *Q93Httexon1* transgene and provide information about the effects of *Sin3A,* while males do not express the transgene and serve as controls. We have found that heterozygous presence of the *Sin3A* LOF allele, $Sin3A^{08269}$, significantly increased the viability of *Q93Httexon1* expressing flies from 29% to 65% at 25 °C ($P<0.001$, X^2 probe). At 27 °C, where the

expression of the polyQ induced phenotype is more severe, *Sin3A*^{08269} still rescues viability from 22% to 37% [P<0.05, X^2 probe].

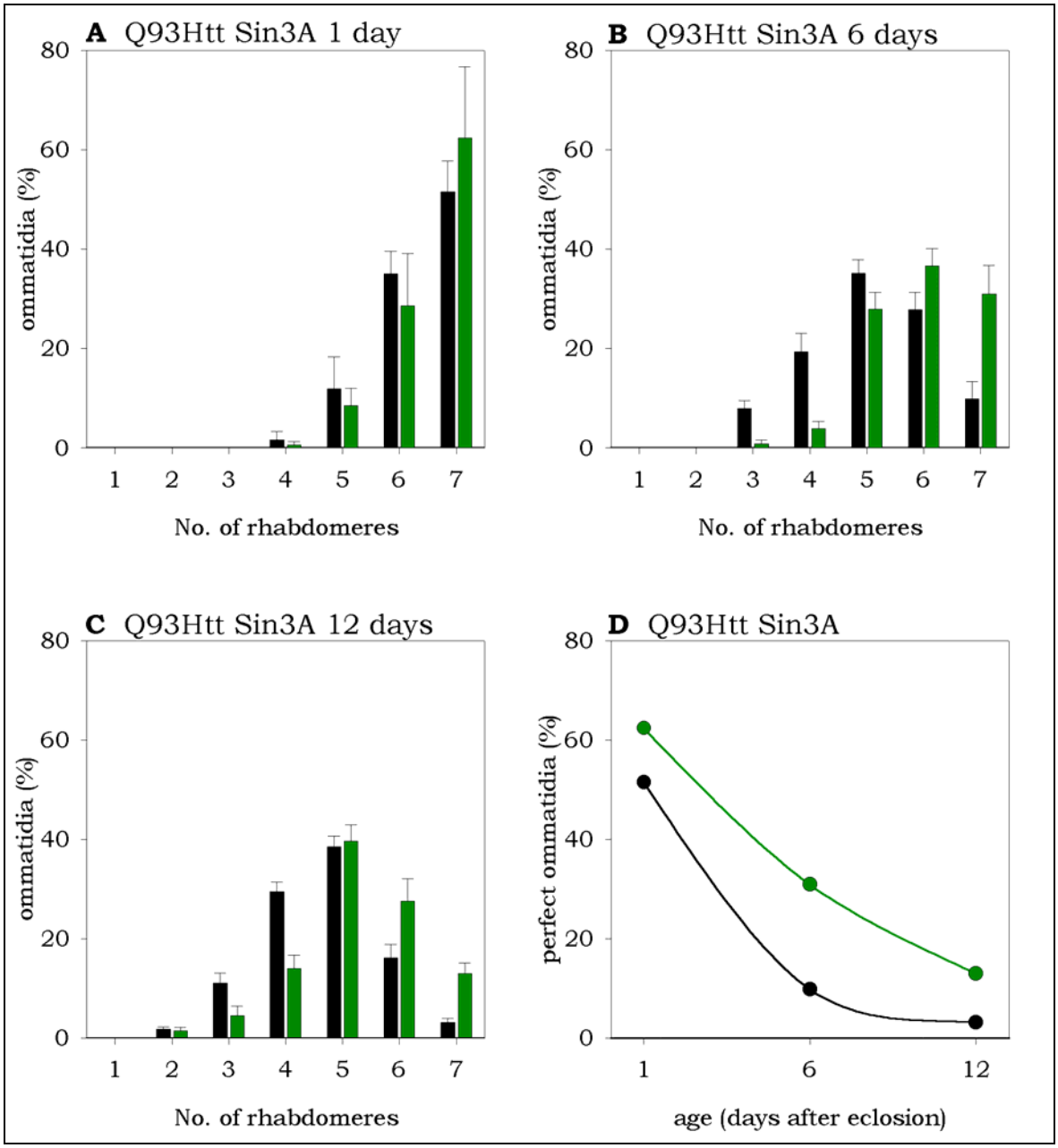

Figure 5.: Genetically reducing deacetylase activity in *Sin3A* heterozygotes slows progressive neurodegeneration of flies expressing *Q93Httexon1*. The number of rhabdomeres per ommatidium of 1 day old, 6 days old and 12 days old flies (**A**, **B** and **C**, respectively) expressing *Q93Httexon1* (black) shows progressive loss. Partial loss of *Sin3A* (green) suppresses neurodegeneration at any time point. **D.** The percentage of perfect ommatidia decreases rapidly in *Q93Httexon1* expressing flies (black). The speed of progressive neurodegeneration is reduced in flies heterozygous for *Sin3A* (green).

Loss of *Sin3A* function also suppresses the rate of neurodegeneration. Expression of the *Q93Httexon1* transgene leads to

progressive loss of rhabdomeres. The average number of rhabdomeres per ommatidia was 6.35 on the first day after eclosion. This value declined to 5.13 and 4.66 at days 6 and 12 after eclosion, respectively. *w P(w^{+mW}.hs = GawB)elavC155/w; Q93Httexon1/Sin3A^{08269}* flies exhibited similar average rhabdomere number (6.52) on the first day after eclosion as their control siblings. In contrast, the average number of rhabdomeres was 5.9 and 5.29 on the 6th and 12th day post-eclosion, respectively (Fig. 5A, B and C). Thus, the reduced activity of the Sin3A transcriptional co-repressor significantly (P<0.05, KWOWA, DNMCT) slowed the progression of *Htt* induced neuronal loss.

7.2.2. HDAC inhibitor compounds rescue polyQ pathology

Having seen that interfering with deacetylase activity genetically caused a marked rescue of polyglutamine induced phenotypes in *Drosophila,* we were interested whether administration of chemical compound inhibitors of HDAC proteins causes similar effects. Two HDAC inhibitor compounds with different action mechanism and enzyme specificity were used in these experiments (Fig. 6). Butyrate is a broad-spectrum, non-specific inhibitor of histone deacetylases with unknown mechanism of action. In contrast, suberoylanilide hydroxamic acid (SAHA) selectively inhibits Class I and II HDACs by plugging their catalytic pocket, while does not affect Class III HDAC proteins, homologs of the yeast Sir2 protein.

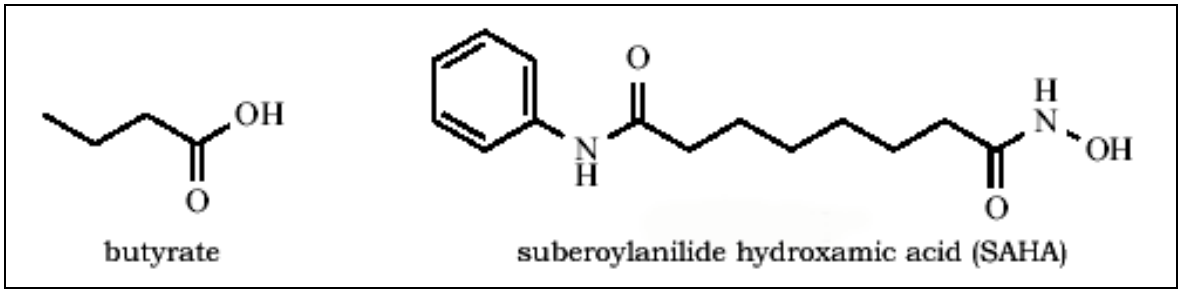

Figure 6.: Chemical structure of the histone deacetylase inhibitor compounds, butyrate and SAHA.

To determine the toxicity of HDAC inhibitors, synchronised *Canton S* animals were raised at 27 °C in vials (50 animals/vial) supplemented with 10 mM butyrate, 50 mM butyrate, 100 mM butyrate, 0.5 μM SAHA, 2 μM SAHA or 10 μM SAHA. After 14 days the number of eclosed flies were recorded and compared to that of flies grown on control food. Low doses of the inhibitors did not affect survival negatively but the highest concentrations of both compounds decreased viability slightly (Fig. 7A, $P<0.05$ in the case of 10 μM SAHA, TWA, DTMCT) marking the highest applicable doses.

We tested the effects of SAHA and butyrate on three genetic combinations expressing different transgenes: *Q108*, *Q48* or *Q93Httexon1*. To analyze the phenotypes of polyQ expressing animals, *w; P(w^{+mc}; w+; elav - GAL4)/CyO, P(w^{+mc} = Act - GFP)JMR1CyO actin-GFP* females were crossed to *w; Q48$^{\#36}$* or *w; Q108$^{\#16}$* males. To test the effect of the inhibitors on flies expressing a truncated Htt fragment, *w; Q93Httexon1$^{\#4F1}$* virgins were crossed to *w P(w^{+mW}.hs = GawB)elavC155/Y* males. Synchronized eggs were collected and transferred to vials containing media supplemented with 10 mM, 50 mM or 100 mM butyrate; 0.5 μM, 2 μM or 10 μM SAHA or control media (Heidi-food in the case of butyrate and Heidi-food supplemented with 0.5% DMSO in the case of SAHA). Flies expressing *Q48$^{\#36}$* or *Q108$^{\#16}$* were grown at 27 °C while flies expressing *Q93Httexon1$^{\#4F1}$* were raised at 25 °C.

As expression of *Q108* results in very poor adult survival, the effect of drugs on viability of *Q108* expressing animals were determined as the pupariation rate of GFP negative (polyQ expressing) larvae on the 8th day after egg laying. We have found that butyrate increased the pupariation rate at all concentrations tested (Fig. 7B, $P<0.05$ at 10 mM and 50 mM, OWA, DTMCT). The rescue peaked at 50 mM, reaching 71% increase in pupariation rate compared with untreated control. In contrast, SAHA did not have a pronounced effect on the viability of *Q108* expressing animals at the concentrations tested.

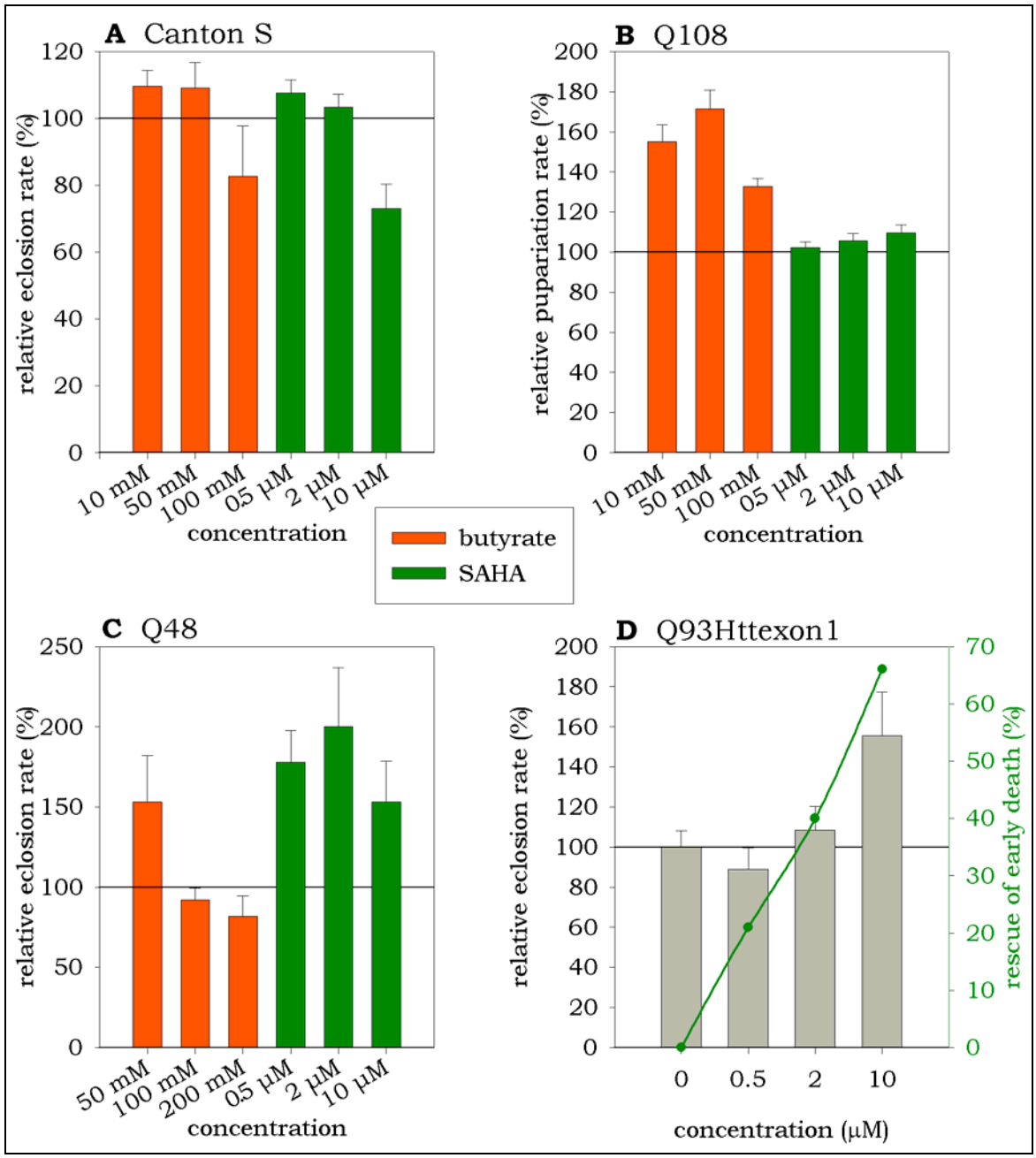

Figure 7.: Administration of histone deacetylase inhibitor compounds suppresses polyglutamine induced lethal phenotypes of *Q48*, *Q108* and *Q93Httexon1* expressing transgenic flies. **A.** Wild-type flies fed with 100 mM butyrate or 10 µM SAHA show lower viability compared to untreated control. Bars represent the relative eclosion rate expressed in the per cent of the eclosion rate of untreated control. **B.** Administration of butyrate suppresses larval lethality of *Q108* expressing flies. Bars represent the relative pupariation rate expressed in the per cent of the pupariation rate of untreated control. **C.** Administration of butyrate or SAHA increases the survival of *Q48* expressing flies. Bars represent the relative eclosion rate expressed in the per cent of the eclosion rate of untreated control. **D.** Administration of SAHA improves the viability of *Q93Httexon1* expressing flies. Bars represent the relative eclosion rate expressed in the per cent of the eclosion rate of untreated control. Line graph shows the rescue of early adult death. Survivors were counted on the sixth day post-eclosion and rescue was calculated as follows: (per cent surviving-per cent surviving on solvent alone)/(1-per cent surviving on solvent alone).

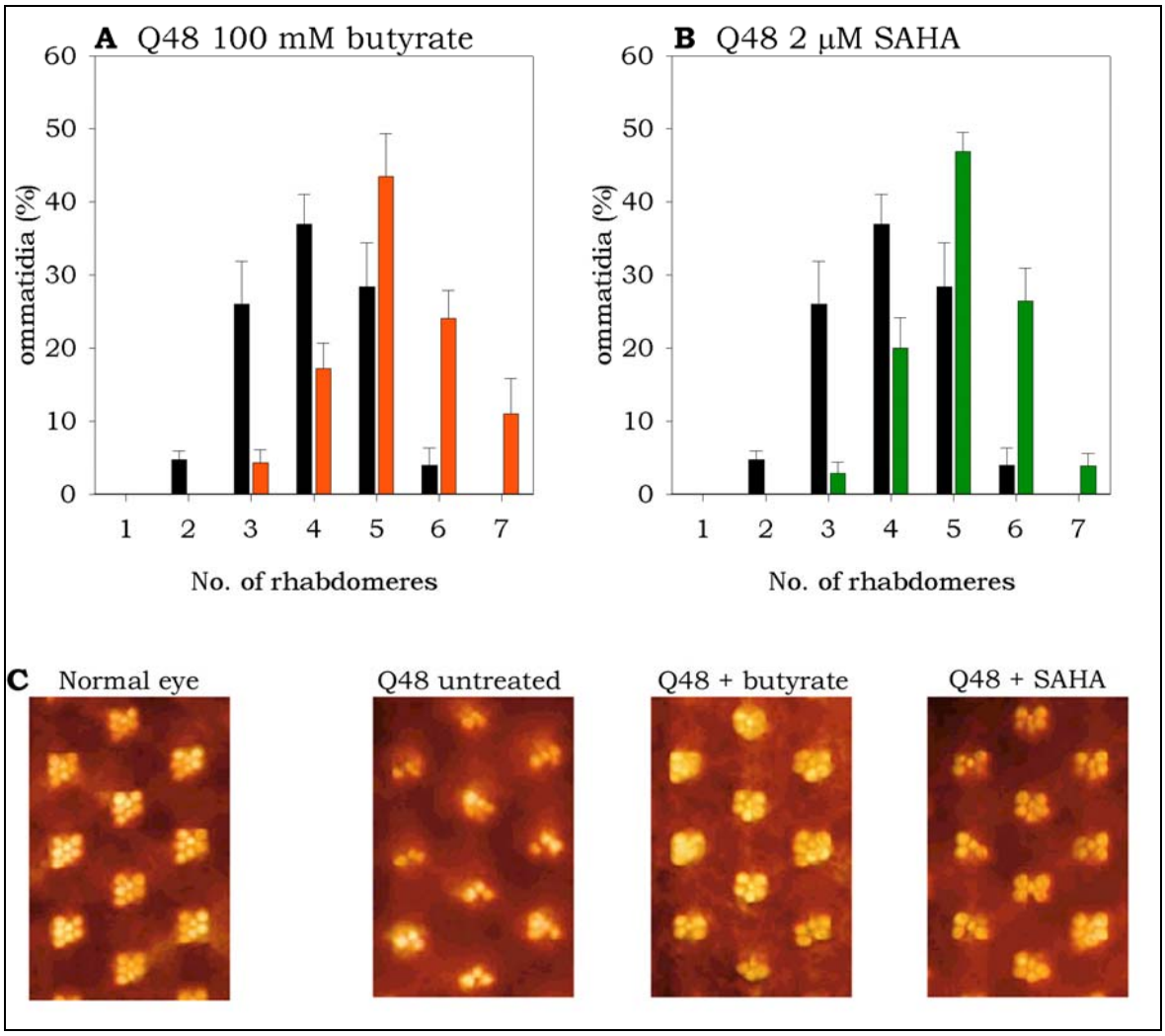

Figure 8: HDAC inhibitor compounds suppress the degeneration of photoreceptor neurons of *Q48* expressing flies. **A.** The number of rhabdomeres per ommatidium at 6 days after eclosion is markedly improved in *Q48* expressing flies fed 100 mM butyrate (red) compared to untreated control (black). **B.** SAHA suppresses neurodegeneration of *Q48* expressing flies. The number of rhabdomeres per ommatidia 6 days after eclosion is markedly improved in flies fed 2 µM SAHA (green) compared to untreated control (black). **C.** Photographs of ommatidia from *Q48* expressing flies with and without HDAC inhibitors.

Rearing larvae that express the Q48 polypeptide on butyrate or SAHA containing food reduced both polyQ induced lethality and the level of neurodegeneration. Flies fed 50 mM butyrate showed 53% higher eclosion rate than their control siblings (Fig. 7C). However, viability declined at higher doses of butyrate, presumably due to the toxic side effects of the compound. Similarly, lower doses of SAHA caused a robust increase in eclosion rate reaching 100% over the control value at the concentration of 2 µM, but the level of rescue declined at the highest concentration (Fig. 7C). Rescue of progressive degeneration of

photoreceptor neurons was monitored using the pseudopupil technique. Control flies that express the *Q48* transgene but did not receive HDAC inhibitors showed a significant decrease of rhabdomere number over time (average of 5.47 at day 1 versus 3.92 at day 6). HDAC inhibitor compounds suppressed the progressive degeneration of photoreceptor neurons (Fig. 8). The average number of visible rhabdomeres 6 days after eclosion was 5.12 in flies fed 100 mM butyrate and 5.00 in flies fed 2 μM SAHA ($P<0.05$, KWOWA, DNMCT).

HDAC inhibitor compounds also rescued the phenotypes of flies expressing a truncated Htt fragment with 93 glutamine residues. The viability of *Q93Httexon1* expressing flies increased at high doses of SAHA, reaching 55% higher eclosion rate than untreated control siblings at the concentration of 10 μM (Fig. 7D). SAHA also suppressed early adult death. 47% of *Q93Httexon1* expressing flies die during the first 6 days after eclosion. Administration of SAHA reduced this ratio in a concentration dependent manner, reaching the lowest death ratio (16%) at the concentration of 10 μM (Fig. 7D, $P<0.001$, KWOWA). The effects of HDAC inhibitors on *Q93Httexon1* expression induced progressive neurodegeneration were similar to those found in the case of *Q48* expressing flies, i.e. a shift was observed towards ommatidia with higher number of rhabdomeres. In the case of SAHA, the average number of rhabdomeres in 6 days old control flies was 4.59. All tested concentrations of SAHA significantly ($P<0.05$, KWOWA, DNMCT] suppressed neurodegeneration, resulting average rhabdomere numbers of 4.89, 5.39 and 5.19 in animals fed 0.5 μM, 2 μM and 10 μM SAHA, respectively (Fig. 9A, B and C). Butyrate also suppressed progressive neurodegeneration ($P<0.05$ in the cases of 30 mM and 100 mM, KWOWA, DNMCT). The average number of rhabdomeres per ommatidia was 5.33, 5.56 and 5.88 in 6 days old flies fed with 10 mM, 30 mM and 100 mM butyrate versus 5.22 in 6 days old control flies (Fig. 9D, E and F).

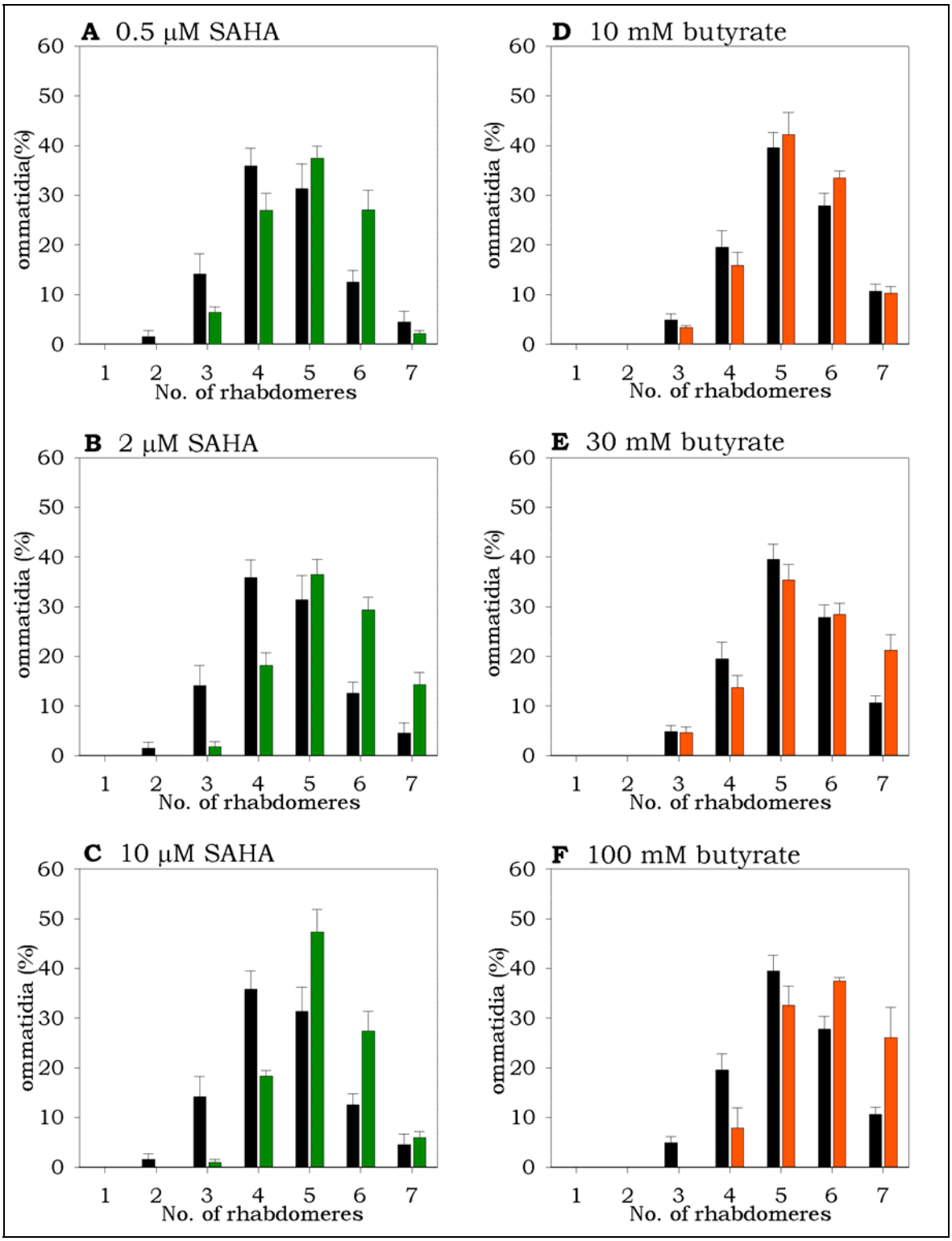

Figure 9.: Administration of HDAC inhibitor compounds suppresses the degeneration of photoreceptor neurons of *Q93Httexon1* expressing flies. The number of rhabdomeres per ommatidium at 6 days after eclosion is improved in *Q93Httexon1* expressing flies fed 0.5 µM, 2 µM or 10 µM SAHA (**A**, **B** and **C**, respectively) or 10 mM, 30 mM or 100 mM butyrate (**D**, **E** and **F**, respectively) compared to untreated control (black).

To rule out the possibility that the rescue of neurodegeneration, lethality and early adult death by HDAC inhibitors was due to altered

expression of the polyglutamine transgenes in the presence of HDAC inhibitors, we used western analysis to compare the level of transgene expression in larval brains expressing *Q93Httexon1* in the presence and absence of butyrate or SAHA (Fig. 10, western blotting was performed by Joan S. Steffan). We found transgene expression unaltered by the presence of HDAC inhibitors.

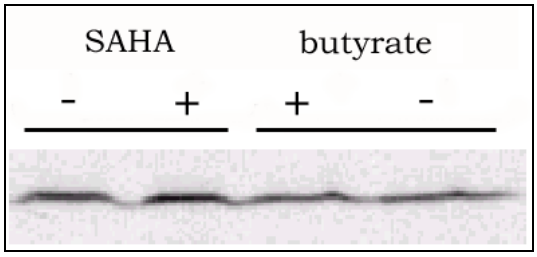

Figure 10.: *Htt* transgene expression in the presence of HDAC inhibitors. Western analysis of extracts from brains of *Q93Httexon1* expressing larvae fed 2 µM SAHA (+), 100 mM butyrate (+) or solvent alone (-) shows that HDAC inhibitor compounds does not alter *Q93Httexon1* transgene expression. Loading controls were performed by Bradford assay and Coomassie staining to ensure that equal amounts of protein are in each lane.

Taken together, the data provided above proves that HDAC inhibitor compounds suppress the semilethal, early death and neurodegenerative phenotypes induced by the expression of pure polyglutamine polypeptides as well as polyglutamine domains embedded in a disease gene context. These results not only support the hypothesis that reduced acetylation activity contributes to polyQ pathogenesis, but also point to HDAC inhibitor compounds as candidates to treat Huntington's Disease and related disorders.

7.2.3. Diverse HAT and HDAC enzymes play role in HD pathology

We have found that expanded polyQ proteins inhibit HAT activity *in vitro*, and that inhibition of the deacetylation process either genetically or pharmacologically ameliorates polyQ induced phenotypes *in vivo*. These data strongly implicate the state of acetylation in the pathogenic process. There are several structural classes of HATs and HDACs (Marmorstein,

2001), plus a range of co-factors that establish the correct acetylation pattern of histones and other proteins. The reagents and genetic factors we utilized in previous experiments primarily targeted Class I and II HDACs. Now we wanted to examine whether Class III HDACs exert similar influence on polyQ phenotypes, and wanted to analyze the contributions of HAT proteins belonging to different families to the pathogenic process. In other words, we wanted to know whether the effects of HATs and HDACs on pathology are the result of many small incremental effects, or whether a few loci account for the bulk of the effects observed.

To achieve this goal, we used genetic interaction tests, i.e. we examined the effects of genes involved in protein acetylation in flies expressing the *Q93Httexon1* transgene. To test the effects of HAT and HDAC genes, for which LOF alleles were not available, we used overlapping deletions. Deletions were chosen based on their published breakpoints and the inferred cytological positions of the genes of interest. The overlap of genes and corresponding deletions were validated by genomic PCR reactions using gene specific, *GFP* specific (homozygosity control) and *p53* specific (PCR positive control) primer pairs. Genomic DNA templates were prepared from single embryos derived from stocks carrying the deletions over a *GFP* positive balancer chromosome. A deletion was confirmed to uncover the gene of interest if the gene and *GFP* specific primers gave negative, while the *p53* specific primers gave positive results in PCR reactions. Using this method, we tested 14 deficiencies and validated deletions to uncover three HAT, two HDAC and three other genes (Table 6).

In viability assays, we tested the effects of *P/CAF* (a GNAT family HAT), *HAT* (a MYST family HAT), *Taf250* (a general transcription factor HAT), *Rpd3* (a Class I HDAC), *Sir2* (a Class III HDAC), *Mi-2* (a HDAC Class I co-repressor) and *Su(var)205* (encoding HP1, a heterochromatic protein). Genetic interaction tests were done by crossing *w P(w^{+mW}.hs = GawB)elavC155/Y; P(w^{+mC} = UAS - Q93Httexon1)$^{\#4F1}$/TM6* males to females carrying the specific mutations or deficiencies over appropriate balancer

chromosomes, and raise their progeny at 25 °C. Results were evaluated by comparing the phenotypes of progeny expressing the *Q93Httexon1* transgene and simultaneously heterozygous for the gene of interest with the phenotypes of *Q93Httexon1* expressing control animals. We have found that flies carrying deletions uncovering the *P/CAF* (*Df(3L)iro-2*) and *HAT* (*Df(2L)spd*) acetyltransferase genes had significantly lower viability than control animals, as expected. In contrast, $Taf250^{1}$, LOF allele of a TFIID subunit with HAT activity, did not have serious effect. Similarly to the previous results with *Sin3A*, LOF alleles of the genes involved in histone deacetylation, $Sir2^{05327}$ and $Mi\text{-}2^{j3D4}$, suppressed the *Q93Httexon1* induced semilethal phenotype. Loss of the non-acetylation-related heterochromatic protein, HP1, did not have significant effect on viability, showing that not every chromosomal functions are involved in HD pathogenesis. (Fig. 11).

Deficiency	**Cytology**	**Gene affected**	**Inferred cytology**	**Overlap**
Df(1)ovo44	4A4-5; 4F11-12	*EG:EG0007.7*	4B1-2	NO
Df(1)C149	5A8-9; 5C5-6	*mof*	5C2-4	YES
Df(1)JA26	11A1; 11D-E	*HDAC4*	11E1-3	YES
Df(1)JA26	11A1; 11D-E	*Smrter*	11B16-17	YES
Df(1)N12	11D1-2; 11F1-2	*HDAC4*	11E1-3	YES
Df(1)N12	11D1-2; 11F1-2	*Smrter*	11B16-17	NO
Df(1)JA27	18A5; 18D	*CG14222*	18D8-10	NO
Df(2L)XE-3801	27E2; 28D1	*HAT*	27F3	NO
Df(2L)spd	27D-E; 28C	*HAT*	27F3	YES
Df(2L)TE29Aa-11	28E4-7; 29B2-C1	*Su(var)205*	28F1-4	YES
Df(3L)iro-2	69B1-5; 69D1-6	*P/CAF*	69C2-4	YES
Df(3R)p25	85A3; 85B1	*HDAC3*		NO
Df(3R)ry75	87D2; 87D14	*CtBP*		YES
Df(3R)96B	96A21;96C2	*CG10899*	96B2-4	YES

Table 6.: PCR based molecular confirmation of deletions overlapping HAT or HDAC genes

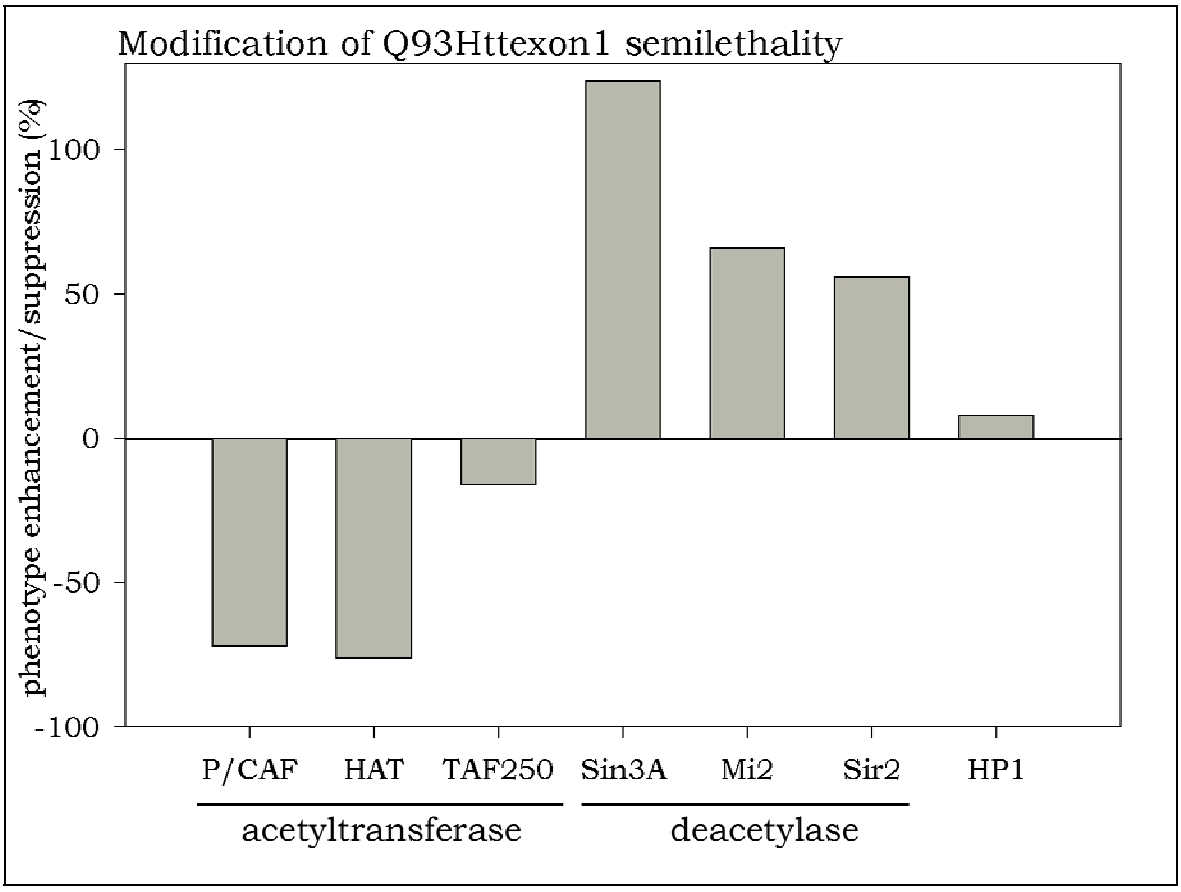

Figure 11.: Genetically altering the acetylation/deacetylation balance modifies the semilethal phenotype of *Q93Httexon1* flies. Loss of function of P/CAF and HAT acetyltransferases enhances the semilethal phenotype. On the other hand, flies heterozygous for the *Sir2* deacetylase or for the *Mi2* HDAC co-repressor show increased viability. Partial loss of *TAF250* and HP1 does not alter viability significantly. Bars represent the eclosion rate difference between experimental and control animals in the per cent of the eclosion rate of control.

The effects caused by partial loss of the activity of the *Rpd3* (a Class I HDAC gene), *Sir2* and *Mi-2* genes were further analyzed to evaluate the role HDAC proteins with different catalytic activities may play in the reversal of polyQ induced pathology. We tested the influence of the LOF alleles $Rpd3^{04556}$, $Sir2^{05327}$ and $Mi\text{-}2^{j3D4}$ on polyQ induced early adult death. We have found that heterozygosity of any of the three genes led to increased longevity (Table 7). The suppression of neurodegeneration was monitored using the pseudopupil technique (Fig. 12). The average number of rhabdomeres per ommatidium in six days old control flies was 4.83. This value increased significantly to 5.18 and 5.29 in flies heterozygous for *Rpd3* and *Mi-2*, respectively. Interestingly, though *Sir2* increased the viability and longevity of *Q93Httexon1* expressing flies, it did

not suppress the degeneration of photoreceptor neurons.

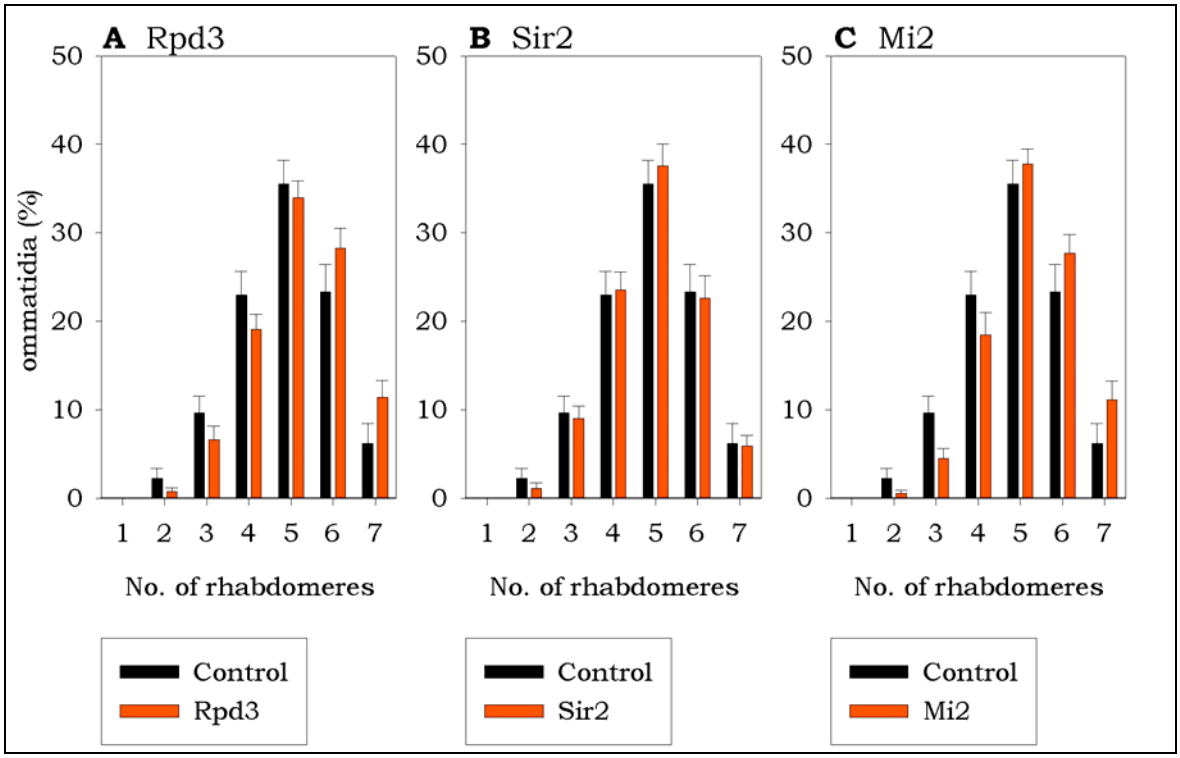

Figure 12.: Reduced HDAC Class I activity rescues neurodegeneration in flies expressing *Q93Httexon1*. **A.** The number of rhabdomeres per ommatidia is improved in *Q93Httexon1* expressing flies heterozygous for the *Rpd3* Class I histone deacetylase. **B.** Heterozygous loss of the HDAC Class III deacetylase *Sir2* does not rescue polyglutamine induced neurodegeneration. **C.** Partial loss of *Mi2*, a HDAC Class I co-repressor, suppresses photoreceptor neuron degeneration in flies expressing *Q93Httexon1*.

These results (summarized in Table 7) suggest that several HAT proteins belonging to different protein families are involved in the pathogenesis of HD. Though the genetic interactions examined here may reflect indirect effects, they indicate that the reduced activity of one key factor is not solely responsible for the phenotypes. Similarly, polyQ induced phenotypes can be suppressed by hindering the activity of HDAC proteins with different mechanisms of catalysis.

Genotype	***Targeted gene; Function***	***Viability***	***Longevity***	***Neuro-degeneration***
Df(3L)iro-2	*P/CAF*; HAT, GNAT family	26% P<0.001	N/D	N/D
Df(2L)spd	*HAT*; HAT, MYST family	22% P=0.009	N/D	N/D
$Taf250^{1}$	*Taf250*; HAT	84% P=0.377	N/D	N/D
$Rpd3^{04556}$	*Rpd3*; HDAC, Class I	N/A	222% P<0.001	Suppressor P<0.05
$Sir2^{05327}$	*Sir2*; HDAC, Class III	157% P=0.008	169% P<0.001	no effect
$Mi\text{-}2^{j3D4}$	*Mi-2*; DNA helicase, HDAC corepressor	167% P=0.005	172% P=0.001	Suppressor P<0.05
Df(2L)TE29Aa-11	*Su(var)205* (HP1); hetero-chromatic protein	108% P=0.766	N/D	N/D

Table 7.: HAT mutations enhance the phenotypes of *Q93Httexon1* expressing flies while HDAC LOF alleles suppress them. Viability shows the eclosion rate of *Htt* expressing flies heterozygous for the corresponding gene, expressed in the per cent of the eclosion rate of *Htt* expressing control animals. Longevity shows the ratio of flies surviving 6 days post-eclosion as the per cent of surviving control animals. Neurodegeneration evaluations are based on pseudopupil analysis.

8. DISCUSSION

Polyglutamine diseases are caused by the expansion of a stretch of uninterrupted glutamine residues in various, otherwise unrelated proteins. Although the first polyglutamine induced neurodegenerative disorder, Huntington's disease, was described in 1872, there is still no effective cure for HD or for any other polyQ disease. There are two main factors hindering the development of treatment strategies for HD. For one thing, HD pathology involves progressive degeneration and loss of neurons in the central nervous system. The tissue selectivity and course of the disease prevents the identification and analysis of molecular mechanisms underlying pathology in humans. Secondly, the dominant gain of function feature of the mutations responsible for the disease makes the causes of pathology hard to explore. While identification of a genetic loss of function as a cause of pathogenesis directly presents ways of treatment by reducing the need for the affected factor, in the case of a toxic gain of function all of the aberrant activities and interactions of the mutant protein have to be discovered and analyzed. These newly gained activities often have little to do with the wild-type function of the affected protein, making them complicate to deal with. However, with the development and application of *in vitro* and *in vivo* model systems, a large body of knowledge has accumulated about the molecular pathology of polyglutamine diseases in the last decade. Due to the research efforts made in this field, our understanding of HD pathogenetic mechanisms reached a level where rational drug discovery and design are rendered possible.

We have developed a transgenic *Drosophila* model system of Huntington's disease and polyglutamine induced disorders in general. One of our main goals with this model was to utilize it in *in vivo* drug screening. Drug discovery utilizes *in vitro*, cell culture and *in vivo* methods, each having special advantages. *In vitro* screens are high throughput, sensitive and allow the identification of compounds directly affecting the specific mechanism tested. Cell culture screens make the

monitoring of permeability and toxicological features of the drugs and their molecular interactions with endogenous factors possible. Animal (mouse) models reflect all of the expected and unexpected biochemical and physiological effects of the tested drug and its derivatives at the organism level. These steps form a narrowing pipeline of drug discovery. The throughput of this pipeline radically decreases as higher level tests consume more and more money and time. Our goal was to insert a new step into this process, which makes the prescreening of compounds identified in cell-free or cell culture screens possible, before testing them in mice. In our *Drosophila* model, expression of *UAS-Htt* and *UAS-polyQ* transgenes are driven by a GAL4 source that is under influence of the neuron specific *elav* promoter. This model recapitulates several characteristic features of HD, for example, late onset progressive pathology, aggregate formation, neurodegeneration, loss of motor function and early death. Quantifiable phenotypes, such as reduced viability and neurodegeneration present an opportunity to test the effects of various factors on polyglutamine induced pathology. To be able to utilize the model in practice, we tested and selected transgenic lines having sensitive enough phenotypes to reflect the changes in pathology, and optimized culturing conditions. To prove the ability of the system to detect factors affecting polyQ pathology, we tested the effects of Congo red, a previously characterized inhibitor of HD aggregate formation, in polyQ expressing flies. We have found that CR suppressed both the reduced viability and neurodegeneration induced by polyQ expression. Similarly, we tested our system with a deletion uncovering the *CtBP* gene, and found that partial loss of *CtBP* enhanced polyQ phenotype, as was described previously in a transgenic model of SCA1 [Fernandez-Funez *et al.*, 2000]. By these experiments, we learned that transgenic *polyQ* flies are suitable to reflect the effects of drugs and genetic factors by changes in their phenotypes.

Besides compound screening, we also utilized the transgenic model in hypothesis-driven analysis of transcriptional dysregulation, one of the processes underlying polyglutamine pathology. Former experiments indicated that the histone acetylation system may be involved in

polyglutamine pathogenesis. In 2000, a study from the Thompson lab demonstrated that CREB binding protein (CBP), a polyglutamine domain containing transcriptional co-activator with HAT activity, and *Sin3A*, a HDAC co-repressor, both bind to expanded polyglutamine polypeptides *in vivo* [Steffan *et al.*, 2000]. In the same year, Fernandez-Funez and colleagues showed that mutations in several genes involved in histone deacetylation (*Rpd3, Sin3A, Sir2*) modified the eye phenotype of SCA1 transgenic flies [Fernandez-Funez *et al.*, 2000]. These results urged us to investigate the effects of the interactions of HATs and HDACs with polyQ proteins. Our collaborators in the Thompson lab have found that a truncated Htt protein with 51 glutamines binds to the acetyltransferase domains of the HAT proteins CBP and P/CAF. This interaction inhibited the acetyltransferase activity of CBP and P/CAF *in vitro.* Moreover, the acetylation levels of histones H3 and H4 were reduced in PC12 cell lines expressing Httexon1 proteins. These findings suggested that the acetyltransferase activity of several HAT proteins may be compromised by the aberrant interaction with polyQ proteins. Having seen that polyQ proteins bind to and inhibit histone acetyltransferases *in vitro,* we wanted to ascertain whether this interaction has relevance *in vivo*. We hypothesized that if reduced activity of HAT proteins contributes to polyQ pathogenesis then inhibition of the opposing enzyme group, the HDACs, will relieve the phenotypes of *polyQ* and *Httexon1* expressing flies. To rule out the possibility that a potential rescue will be a result of a specific side effect of HDAC inhibition, we used both genetic and pharmacological approaches. To reduce HDAC activity in flies we introduced LOF alleles of the HDAC genes *Rpd3* and *Sir2*, or those of the HDAC co-repressors genes *Sin3A* or *Mi-2*. Partial loss of these genes rescued both semilethal and progressive neurodegenerative phenotype of *Httexon1* expressing flies. Similar results were observed by feeding the flies with the HDAC inhibitor compounds, butyrate and SAHA. HDAC inhibitors suppressed the semilethal, early death and neurodegenerative phenotypes of both *polyQ* and *Httexon1* expressing flies. These results validated the *in vivo* relevance of reduced HAT activity in polyQ diseases.

Taken together, our findings suggest a model of transcriptional dysregulation (Fig. 13) in polyglutamine disorders carried out by inhibited catalytic activity of HAT proteins. If these enzymes physically interact with either soluble or aggregated polyglutamine proteins, several processes they are involved in (e.g. transcription) may be negatively affected, which in turn can lead to disturbed cellular functions. This model is supported by several studies reporting reduced acetylation of core histones and perturbed transcriptional activity in polyglutamine model systems [Luthi-Carter *et al.*, 2000; McCampbell *et al.*, 2000; Steffan *et al.*, 2000; Hughes *et al*, 2001; McCampbell *et al.*, 2001; Nucifora *et al.*, 2001; Steffan *et al.*, 2001; Wyttenbach *et al.*, 2001; Luthi-Carter *et al.*, 2002]. However, it was still not clear whether reduced activity of some key factors (CBP, for example) or additive effects of several affected HAT proteins are responsible for the symptoms. Similarly, the types of HDACs relieving symptoms, if inhibited, were also not known. To answer these questions, we tested HAT and HDAC genes belonging to different families in transgenic flies expressing *Httexon1*. We have found that reduced activity of HAT proteins belonging to the GNAT and MYST families worsened polyQ phenotypes but Taf250 did not have a significant effect. This result supported our former view that a wide range of only partially related HAT proteins may interact with polyglutamines, but also showed that not every protein with AT activity is involved. Interaction crosses with HDACs provided similar results. Mutant alleles of the members of both major types of HDACs suppressed polyQ phenotypes indicating that inhibition of either type can have therapeutic benefits. These results together suggest that the majority of the effects HATs and HDACs exert on pathology are rather due to the modification of the general acetylation balance than to the dysfunction of few key factors.

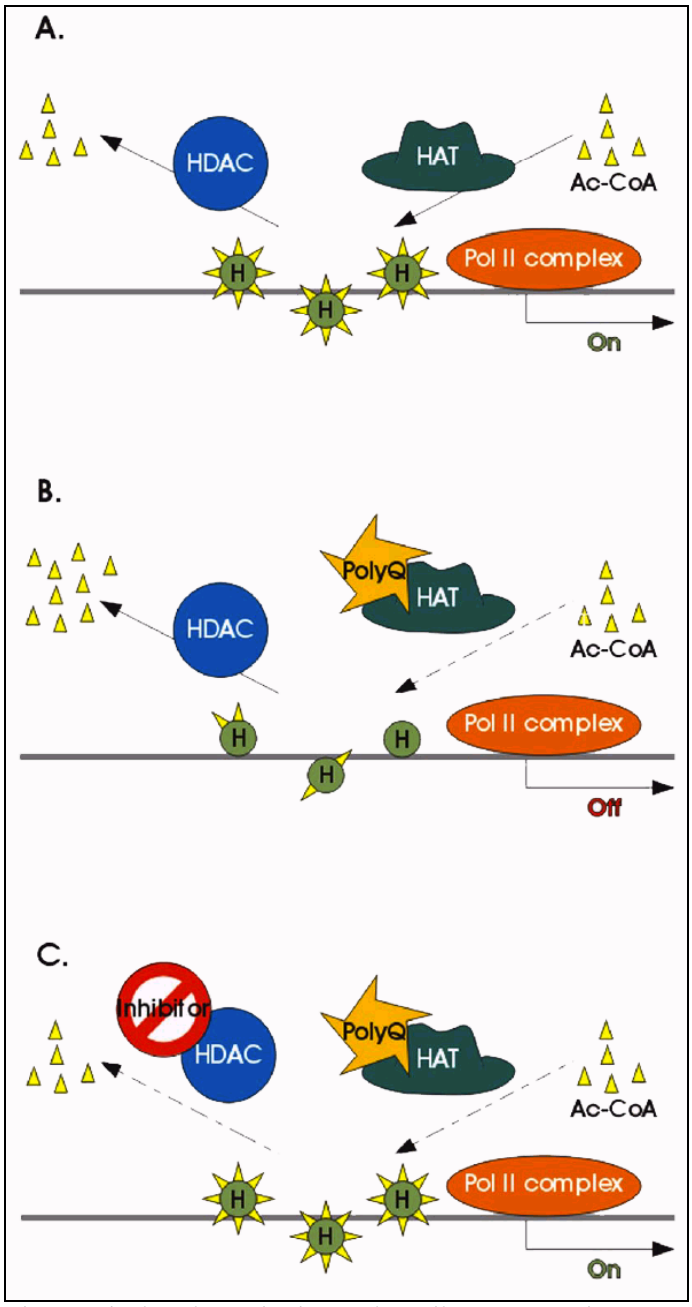

Figure 13.: Altered acetylation in polyglutamine diseases and restoration of acetylation state by histone deacetylase inhibitors. **A.** The acetylation state of histones (H) and proper gene expression levels are maintained by two opposing enzyme groups, histone acetyl transferases (HATs) and histone deacetylases (HDACs). **B.** In disease state however, polyQ proteins can interact with HATs distorting the fine balance established by HATs and HDACs, which then leads to decreased histone acetylation levels and repressed transcription. **C.** The effect of polyglutamine proteins can be counteracted by administering HDAC inhibitors. These compounds reduce the activity of deacetylase enzymes and restore the balance of HATs and HDACs. [Figure taken from Bodai *et al.*, 2003]

Our results established the deacetylation process as a potential candidate for medical intervention. HDAC inhibitor compounds can restore the dynamic equilibrium between the acetylation and deacetylation

processes, and in so doing, they can suppress the pathogenic effects of polyglutamines (Fig. 13C). The first compound found to be able to inhibit HDAC catalytic activity was the short chain fatty acid molecule butyrate and its derivatives [Riggs *et al.*, 1977; Candido *et al.*, 1978]. The major disadvantages of these drugs are that they are non-specific [Chung *et al.*, 2000] and the useful dose is rather high. Characterization of more specific natural inhibitors, such as trichostatin A (TSA) [Yoshida *et al.* 1990], made the development of highly specific synthetic HDAC inhibitors possible. Crystal structure analysis of an HDAC – TSA complex revealed that the hydroxamic acid group of TSA chelates the Zn^{2+} cation at the catalytic active site, while its aromatic group binds to the rim of the catalytic pocket [Finnin *et al.*, 1999]. Several synthetic analogs mimicking the molecular structure of TSA have been developed in the last few years [Furumai *et al.*, 2001; Sternson *et al.*, 2001]. These drugs contain a functional group (hydroxamate [Remiszewski *et al.*, 2002], epoxide [Kijima *et al.*, 1993] or phenilene diamine [Suzuki *et al.*, 1999] groups, for example) responsible for interacting with the catalytic active site connected to a cap group (cyclic tetrapeptide [Furumai *et al.*, 2001], aryl [Ueasato *et al.*, 2002], succinimide [Curtin *et al.*, 2002] or sulfonamide [Fournel *et al.*, 2002] groups, for example) that plugs the catalytic pocket. One of these drugs is SAHA, a highly specific and potent HDAC inhibitor, that is effective in suppressing polyglutamine pathology in transgenic flies. Class III HDACs (sirtuins) possess a NAD dependent catalytic domain [Landry *et al.*, 2000; Moazed, 2001] and are not sensitive to the compounds described above. However, non-hydrolysable NAD analogs are potent inhibitors of sirtuins [Landry *et al.*, 2000]. Nicotinamide, which is nutritionally equivalent to niacin or vitamin B3, also blocks deacetylation by product inhibition [Bitterman *et al.*, 2002]. Thus, niacin, as an easily available drug, may also have potentialities in therapy, if it proves to be effective in the suppression of polyglutamine symptoms.

First and last, we established a *Drosophila* model system of HD suitable to genetic and drug testing, proved the *in vivo* relevance of altered protein acetylation in polyglutamine disorders and pointed to HDAC

inhibitor compounds as candidate drugs for the treatment of these devastating diseases.

9. SUMMARY

At least nine hereditary neurodegenerative disorders, including Huntington's disese, are caused by the expansion of an unstable endogenous CAG repeat in the coding regions of otherwise unrelated disease genes. The expanded trinucleotide repeats translate to uninterrupted arrays of glutamine residues in the corresponding proteins. Expanded polyQ stretches are thought to alter the structure of the affected protein and mediate aberrant protein – protein interactions. In order to be able to analyze the molecular pathomechanisms of polyglutamine diseases *in vivo*, we developed a transgenic *Drosophila melanogaster* model system of Huntington's Disease. Our goal with this system was two kinds: to use it as an *in vivo* screening tool in genetical and chemical screens and to use it for hypothesis testing in the investigation of candidate processes underlying the molecular pathomechanisms of HD.

- We built a transgenic system in which the neuron specific expression of polyglutamine encoding transgenes is controlled by the bipartite UAS/GAL4 expression system.

- In order to choose such transgenic lines that are appropriate to reflect changes in polyQ pathogenesis we tested the viability of the UAS-polyQ lines in combination with neuron specific GAL4 drivers at several culturing temperatures. Based on their norms of reaction three lines (a *Q48*, a *Q108* and a *Q93Httexon1* line) were selected for further experiments.

- We optimized media and culturing conditions for compound feeding screens.

- We determined that the highest dose of DMSO, an organic solvent used in feeding screens, that do not exhibit toxic effects at any developmental stages is 0.05%.

- We validated the model systems ability to respond to factors influencing polyglutamine pathology. We challenged it with Congo red, a chemical inhibitor of aggregate formation, and found that CR rescued both viability and progressive neurodegeneration of polyglutamine expressing flies. We also tested the effects of loss of *CtBP*, a genetic enhancer of polyQ phenotypes, and got similar results than those that had been described previously in a transgenic SCA1 model.

- We hypothesized that inhibition of histone acetyltransferase activity has a significant role in polyQ pathology, thus reducing the histone deacetylase activity will relieve symptoms. We have found that a loss of function allele of the *Sin3A* gene, encoding a HDAC co-repressor protein, suppressed the phenotypes elicited by *Q93Httexon1* expression.

- We administered non-toxic doses of the HDAC inhibitor compounds butyrate and suberoylanilide hydroxamic acid to transgenic flies expressing a polyglutamine domain either alone or embedded in a disease gene context. We found that both compounds suppressed the polyQ induced phenotypes.

- By genomic PCR we tested 14 deficiencies and validated deletions to uncover three HAT, two HDAC and three other genes

- We set up genetic interaction crosses to investigate whether the effects of HATs and HDACs on pathology are the result of many small incremental effects or whether dysfunction of a critical HAT enzyme is responsible for the majority of effects. Interaction crosses with histone acetyltransferases showed that HAT enzymes belonging to distinct protein families are able to modify the inspected phenotype. Similarly, loss of HDAC enzymes with distinct mechanism of catalysis rescued polyQ phenotypes.

Taken all together, we established a transgenic *Drosophila* model of polyglutamine disorders, demonstrated that reduced acetylation activity contributes to polyQ pathogenesis *in vivo* and identified HDAC inhibitor compounds as candidates to treat Huntington's disease and related disorders.

10. ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my PIs, Larry Marsh and Leslie Michels Thompson, for creating the opportunity to join their groups and take a part in their projects. During the two years I spent at UCI I learned a lot from them about critical thinking, teamwork, project management and the importance of concepts such as 'spin', 'big picture' and 'cost-benefit ratio'.

I am most grateful to Judit Pallos whose contribution was essential to the successful execution of this project. She not only helped with the planning of experiments and evaluation of the results but also took her share of the benchwork. Judit is an exceptionally sharp girl who combines clear thought with precision (and high spirits, too).

I give my thanks to all members of the Marsh and Thompson labs, especially Barbara L. Apostol, Tamás Lukácsovich, Natalia Slepko, Joan S. Steffan, Péter Vilmos and Heli Walker for their collaboration, help and support.

I thank the crew of Zot n' Go South for fueling my neuronal networks with their excellent coffee.

I am also grateful for all of those members of the Drosophilist community of Szeged who helped me to develop my theoretical knowledge and manual skills during my student years. You know who you are.

I would like to express my thanks to my parents and family who were never really concerned about fruit flies or HD but raised me and supported through all these years.

And a final thanks goes to the following organizations for providing

financial support to our work: the Hereditary Disease Foundation, the Huntington's Disease Society of America and the National Institute of Health.

11. REFERENCES

Agalioti, T., Chen, G., and Thanos, D. (2002). Deciphering the transcriptional histone acetylation code for a human gene. Cell 111, 381-392.

Altar, C. A., Cai, N., Bliven, T., Juhasz, M., Conner, J. M., Acheson, A. L., Lindsay, R. M., and Wiegand, S. J. (1997). Anterograde transport of brain-derived neurotrophic factor and its role in the brain. Nature 389, 856-860.

Alves-Rodrigues, A., Gregori, L., and Figueiredo-Pereira, M. E. (1998). Ubiquitin, cellular inclusions and their role in neurodegeneration. Trends Neurosci 21, 516-520.

Apostol, B. L., Kazantsev, A., Raffioni, S., Illes, K., Pallos, J., Bodai, L., Slepko, N., Bear, J. E., Gertler, F. B., Hersch, S., et al. (2003). A cell-based assay for aggregation inhibitors as therapeutics of polyglutamine-repeat disease and validation in Drosophila. Proc Natl Acad Sci U S A 100, 5950-5955.

Barlev, N. A., Liu, L., Chehab, N. H., Mansfield, K., Harris, K. G., Halazonetis, T. D., and Berger, S. L. (2001). Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. Mol Cell 8, 1243-1254.

Beal, M. F., Kowall, N. W., Ellison, D. W., Mazurek, M. F., Swartz, K. J., and Martin, J. B. (1986). Replication of the neurochemical characteristics of Huntington's disease by quinolinic acid. Nature 321, 168-171.

Becher, M. W., Kotzuk, J. A., Sharp, A. H., Davies, S. W., Bates, G. P., Price, D. L., and Ross, C. A. (1998). Intranuclear neuronal inclusions in Huntington's disease and dentatorubral and pallidoluysian atrophy: correlation between the density of inclusions and IT15 CAG triplet repeat length. Neurobiol Dis 4, 387-397.

Berger, S. L. (2002). Histone modifications in transcriptional regulation. Curr Opin Genet Dev 12, 142-148.

Bitterman, K. J., Anderson, R. M., Cohen, H. Y., Latorre-Esteves, M., and Sinclair, D. A. (2002). Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. J Biol Chem 277, 45099-45107.

Bodai, L., Pallos, J., Thompson, L. M., and Marsh, J. L. (2003). Altered protein acetylation in polyglutamine diseases. Curr Med Chem 10, 2577-2587.

Boutell, J. M., Thomas, P., Neal, J. W., Weston, V. J., Duce, J., Harper, P. S., and Jones, A. L. (1999). Aberrant interactions of transcriptional repressor proteins with the Huntington's disease gene product, huntingtin. Hum Mol Genet 8, 1647-1655.

Brand, A. H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118,401-15.

Caillaud, A., Prakash, A., Smith, E., Masumi, A., Hovanessian, A. G., Levy, D. E., and Marie, I. (2002). Acetylation of interferon regulatory factor-7 by p300/CREB-binding protein (CBP)-associated factor (PCAF) impairs its DNA binding. J Biol Chem 277, 49417-49421.

Candido, E. P., Reeves, R., and Davie, J. R. (1978). Sodium butyrate inhibits histone deacetylation in cultured cells. Cell 14, 105-113.

Carter, R. J., Lione, L. A., Humby, T., Mangiarini, L., Mahal, A., Bates, G. P., Dunnett, S. B., and Morton, A. J. (1999). Characterization of progressive motor deficits in mice transgenic for the human Huntington's disease mutation. J Neurosci 19, 3248-3257.

Chai, Y., Koppenhafer, S. L., Shoesmith, S. J., Perez, M. K., and Paulson, H. L. (1999). Evidence for proteasome involvement in polyglutamine disease: localization to nuclear inclusions in SCA3/MJD and suppression of polyglutamine aggregation in vitro. Hum Mol Genet 8, 673-682.

Chai, Y., Wu, L., Griffin, J. D., and Paulson, H. L. (2001). The role of protein composition in specifying nuclear inclusion formation in polyglutamine disease. J Biol Chem 276, 44889-44897.

Chan, H. Y. (2002a). Fly-ing from genes to drugs. Trends Mol Med 8, 99-101.

Chan, H. Y., Warrick, J. M., Andriola, I., Merry, D., and Bonini, N. M. (2002b). Genetic modulation of polyglutamine toxicity by protein conjugation pathways in Drosophila. Hum Mol Genet 11, 2895-2904.

Chen, M., Ona, V. O., Li, M., Ferrante, R. J., Fink, K. B., Zhu, S., Bian, J., Guo, L., Farrell, L. A., Hersch, S. M., et al. (2000). Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. Nat Med 6, 797-801.

Chun, W., Lesort, M., Tucholski, J., Faber, P. W., MacDonald, M. E., Ross, C. A., and Johnson, G. V. (2001a). Tissue transglutaminase selectively modifies proteins associated with truncated mutant huntingtin in intact cells. Neurobiol Dis 8, 391-404.

Chun, W., Lesort, M., Tucholski, J., Ross, C. A., and Johnson, G. V.

(2001b). Tissue transglutaminase does not contribute to the formation of mutant huntingtin aggregates. J Cell Biol 153, 25-34.

Chung, Y. L., Lee, Y. H., Yen, S. H., and Chi, K. H. (2000). A novel approach for nasopharyngeal carcinoma treatment uses phenylbutyrate as a protein kinase C modulator: implications for radiosensitization and EBV-targeted therapy. Clin Cancer Res 6, 1452-1458.

Cooper, A. J., Sheu, K. F., Burke, J. R., Onodera, O., Strittmatter, W. J., Roses, A. D., and Blass, J. P. (1997). Polyglutamine domains are substrates of tissue transglutaminase: does transglutaminase play a role in expanded CAG/poly-Q neurodegenerative diseases? J Neurochem 69, 431-434.

Cummings, C. J., Reinstein, E., Sun, Y., Antalffy, B., Jiang, Y., Ciechanover, A., Orr, H. T., Beaudet, A. L., and Zoghbi, H. Y. (1999). Mutation of the E6-AP ubiquitin ligase reduces nuclear inclusion frequency while accelerating polyglutamine-induced pathology in SCA1 mice. Neuron 24, 879-892.

Cummings, C. J., Sun, Y., Opal, P., Antalffy, B., Mestril, R., Orr, H. T., Dillmann, W. H., and Zoghbi, H. Y. (2001). Over-expression of inducible HSP70 chaperone suppresses neuropathology and improves motor function in SCA1 mice. Hum Mol Genet 10, 1511-1518.

Curtin, M. L., Garland, R. B., Heyman, H. R., Frey, R. R., Michaelides, M. R., Li, J., Pease, L. J., Glaser, K. B., Marcotte, P. A., and Davidsen, S. K. (2002). Succinimide hydroxamic acids as potent inhibitors of histone deacetylase (HDAC). Bioorg Med Chem Lett 12, 2919-2923.

Davies, S. W., Turmaine, M., Cozens, B. A., DiFiglia, M., Sharp, A. H., Ross, C. A., Scherzinger, E., Wanker, E. E., Mangiarini, L., and Bates, G. P. (1997). Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. Cell 90, 537-548.

DiFiglia, M., Sapp, E., Chase, K. O., Davies, S. W., Bates, G. P., Vonsattel, J. P., and Aronin, N. (1997). Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science 277, 1990-1993.

Dragatsis, I., Levine, M. S., and Zeitlin, S. (2000). Inactivation of Hdh in the brain and testis results in progressive neurodegeneration and sterility in mice. Nat Genet 26, 300-306.

Duyao, M., Ambrose, C., Myers, R., Novelletto, A., Persichetti, F., Frontali, M., Folstein, S., Ross, C., Franz, M., Abbott, M., and et al. (1993). Trinucleotide repeat length instability and age of onset in Huntington's disease. Nat Genet 4, 387-392.

Duyao, M. P., Auerbach, A. B., Ryan, A., Persichetti, F., Barnes, G. T., McNeil, S. M., Ge, P., Vonsattel, J. P., Gusella, J. F., Joyner, A. L., and et al. (1995). Inactivation of the mouse Huntington's disease gene homolog Hdh. Science 269, 407-410.

Fernandez-Funez, P., Nino-Rosales, M. L., de Gouyon, B., She, W. C., Luchak, J. M., Martinez, P., Turiegano, E., Benito, J., Capovilla, M., Skinner, P. J., et al. (2000). Identification of genes that modify ataxin-1-induced neurodegeneration. Nature 408, 101-106.

Ferrante, R. J., Kowall, N. W., Cipolloni, P. B., Storey, E., and Beal, M. F. (1993). Excitotoxin lesions in primates as a model for Huntington's disease: histopathologic and neurochemical characterization. Exp Neurol 119, 46-71.

Finnin, M. S., Donigian, J. R., Cohen, A., Richon, V. M., Rifkind, R. A., Marks, P. A., Breslow, R., and Pavletich, N. P. (1999). Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature 401, 188-193.

Fournel, M., Trachy-Bourget, M. C., Yan, P. T., Kalita, A., Bonfils, C., Beaulieu, C., Frechette, S., Leit, S., Abou-Khalil, E., Woo, S. H., et al. (2002). Sulfonamide anilides, a novel class of histone deacetylase inhibitors, are antiproliferative against human tumors. Cancer Res 62, 4325-4330.

Furumai, R., Komatsu, Y., Nishino, N., Khochbin, S., Yoshida, M., and Horinouchi, S. (2001). Potent histone deacetylase inhibitors built from trichostatin A and cyclic tetrapeptide antibiotics including trapoxin. Proc Natl Acad Sci U S A 98, 87-92.

Gervais, F. G., Singaraja, R., Xanthoudakis, S., Gutekunst, C. A., Leavitt, B. R., Metzler, M., Hackam, A. S., Tam, J., Vaillancourt, J. P., Houtzager, V., et al. (2002). Recruitment and activation of caspase-8 by the Huntingtin-interacting protein Hip-1 and a novel partner Hippi. Nat Cell Biol 4, 95-105.

Goellner, G. M., and Rechsteiner, M. (2003). Are Huntington's and polyglutamine-based ataxias proteasome storage diseases? Int J Biochem Cell Biol 35, 562-571.

Goffredo, D., Rigamonti, D., Tartari, M., De Micheli, A., Verderio, C., Matteoli, M., Zuccato, C., and Cattaneo, E. (2002). Calcium-dependent cleavage of endogenous wild-type huntingtin in primary cortical neurons. J Biol Chem 277, 39594-39598.

Green, H. (1993). Human genetic diseases due to codon reiteration: relationship to an evolutionary mechanism. Cell 74, 955-956.

Greenberg, C. S., Birckbichler, P. J., and Rice, R. H. (1991). Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. Faseb J 5, 3071-3077.

Group, T. H. s. D. C. R. (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. Cell 72, 971-983.

Gusella, J. F., and MacDonald, M. E. (1998). Huntingtin: a single bait hooks many species. Curr Opin Neurobiol 8, 425-430.

Gutekunst, C. A., Li, S. H., Yi, H., Mulroy, J. S., Kuemmerle, S., Jones, R., Rye, D., Ferrante, R. J., Hersch, S. M., and Li, X. J. (1999). Nuclear and neuropil aggregates in Huntington's disease: relationship to neuropathology. J Neurosci 19, 2522-2534.

Heiser, V., Scherzinger, E., Boeddrich, A., Nordhoff, E., Lurz, R., Schugardt, N., Lehrach, H., and Wanker, E. E. (2000). Inhibition of huntingtin fibrillogenesis by specific antibodies and small molecules: implications for Huntington's disease therapy. Proc Natl Acad Sci U S A 97, 6739-44.

Hirakura, Y., Azimov, R., Azimova, R., and Kagan, B. L. (2000). Polyglutamine-induced ion channels: a possible mechanism for the neurotoxicity of Huntington and other CAG repeat diseases. J Neurosci Res 60, 490-494.

Ho, L. W., Carmichael, J., Swartz, J., Wyttenbach, A., Rankin, J., and Rubinsztein, D. C. (2001). The molecular biology of Huntington's disease. Psychol Med 31, 3-14.

Hockly, E., Richon, V. M., Woodman, B., Smith, D. L., Zhou, X., Rosa, E., Sathasivam, K., Ghazi-Noori, S., Mahal, A., Lowden, P. A., Steffan, J. S., Marsh, J. L., Thompson, L. M., Lewis, C. M., Marks, P. A., and Bates, G. P. (2003) Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, ameliorates motor deficits in a mouse model of Huntington's disease. Proc Natl Acad Sci U S A 100, 2041-6.

Hodgson, J. G., Agopyan, N., Gutekunst, C. A., Leavitt, B. R., LePiane, F., Singaraja, R., Smith, D. J., Bissada, N., McCutcheon, K., Nasir, J., et al. (1999). A YAC mouse model for Huntington's disease with full-length mutant huntingtin, cytoplasmic toxicity, and selective striatal neurodegeneration. Neuron 23, 181-192.

Holbert, S., Denghien, I., Kiechle, T., Rosenblatt, A., Wellington, C., Hayden, M. R., Margolis, R. L., Ross, C. A., Dausset, J., Ferrante, R. J., and Neri, C. (2001). The Gln-Ala repeat transcriptional activator CA150 interacts with huntingtin: neuropathologic and genetic evidence for a role

in Huntington's disease pathogenesis. Proc Natl Acad Sci U S A 98, 1811-1816.

Housman, D. (1995). Gain of glutamines, gain of function? Nat Genet 10, 3-4.

Hubbert, C., Guardiola, A., Shao, R., Kawaguchi, Y., Ito, A., Nixon, A., Yoshida, M., Wang, X. F., and Yao, T. P. (2002). HDAC6 is a microtubule-associated deacetylase. Nature 417, 455-458.

Hughes, R. E., Lo, R. S., Davis, C., Strand, A. D., Neal, C. L., Olson, J. M., and Fields, S. (2001). Altered transcription in yeast expressing expanded polyglutamine. Proc Natl Acad Sci U S A 98, 13201-13206.

Igarashi, S., Morita, H., Bennett, K., M., Tanaka, Y., Engelender, S., Peters, M. F., Cooper, J. K., Wood, J. D , Sawa, A., and Ross, C. A. (2003). Inducible PC12 cell model of Huntington's disease shows toxicity and decreased histone acetylation. Neuroreport 14, 565-8.

Ivkovic, S., and Ehrlich, M. E. (1999). Expression of the striatal DARPP-32/ARPP-21 phenotype in GABAergic neurons requires neurotrophins in vivo and in vitro. J Neurosci 19, 5409-5419.

Jana, N. R., and Nukina, N. (2003). Recent advances in understanding the pathogenesis of polyglutamine diseases: involvement of molecular chaperones and ubiquitin-proteasome pathway. J Chem Neuroanat 26, 95-101.

Jana, N. R., Tanaka, M., Wang, G., and Nukina, N. (2000). Polyglutamine length-dependent interaction of Hsp40 and Hsp70 family chaperones with truncated N-terminal huntingtin: their role in suppression of aggregation and cellular toxicity. Hum Mol Genet 9, 2009-2018.

Jenuwein, T., and Allis, C. D. (2001). Translating the histone code. Science 293, 1074-1080.

Kahlem, P., Green, H., and Djian, P. (1998). Transglutaminase action imitates Huntington's disease: selective polymerization of Huntingtin containing expanded polyglutamine. Mol Cell 1, 595-601.

Kahlem, P., Terre, C., Green, H., and Djian, P. (1996). Peptides containing glutamine repeats as substrates for transglutaminase-catalyzed cross-linking: relevance to diseases of the nervous system. Proc Natl Acad Sci U S A 93, 14580-14585.

Karpuj, M. V., Garren, H., Slunt, H., Price, D. L., Gusella, J., Becher, M. W., and Steinman, L. (1999). Transglutaminase aggregates huntingtin into nonamyloidogenic polymers, and its enzymatic activity increases in Huntington's disease brain nuclei. Proc Natl Acad Sci U S A 96, 7388-

7393.

Kazantsev, A., Walker, H. A., Slepko, N., Bear, J. E., Preisinger, E., Steffan, J. S., Zhu, Y. Z., Gertler, F. B., Housman, D. E., Marsh, J. L., and Thompson, L. M. (2002). A bivalent Huntingtin binding peptide suppresses polyglutamine aggregation and pathogenesis in Drosophila. Nat Genet 30, 367-376.

Kazemi-Esfarjani, P., and Benzer, S. (2000). Genetic suppression of polyglutamine toxicity in Drosophila. Science 287, 1837-1840.

Kegel, K. B., Meloni, A. R., Yi, Y., Kim, Y. J., Doyle, E., Cuiffo, B. G., Sapp, E., Wang, Y., Qin, Z. H., Chen, J. D., et al. (2002). Huntingtin is present in the nucleus, interacts with the transcriptional corepressor C-terminal binding protein, and represses transcription. J Biol Chem 277, 7466-7476.

Kijima, M., Yoshida, M., Sugita, K., Horinouchi, S., and Beppu, T. (1993). Trapoxin, an antitumor cyclic tetrapeptide, is an irreversible inhibitor of mammalian histone deacetylase. J Biol Chem 268, 22429-22435.

Kim, M., Lee, H. S., LaForet, G., McIntyre, C., Martin, E. J., Chang, P., Kim, T. W., Williams, M., Reddy, P. H., Tagle, D., et al. (1999). Mutant huntingtin expression in clonal striatal cells: dissociation of inclusion formation and neuronal survival by caspase inhibition. J Neurosci 19, 964-973.

Kim, S., Nollen, E. A., Kitagawa, K., Bindokas, V. P., and Morimoto, R. I. (2002). Polyglutamine protein aggregates are dynamic. Nat Cell Biol 4, 826-831.

Kim, Y. J., Yi, Y., Sapp, E., Wang, Y., Cuiffo, B., Kegel, K. B., Qin, Z. H., Aronin, N., and DiFiglia, M. (2001). Caspase 3-cleaved N-terminal fragments of wild-type and mutant huntingtin are present in normal and Huntington's disease brains, associate with membranes, and undergo calpain-dependent proteolysis. Proc Natl Acad Sci U S A 98, 12784-12789.

Klement, I. A., Skinner, P. J., Kaytor, M. D., Yi, H., Hersch, S. M., Clark, H. B., Zoghbi, H. Y., and Orr, H. T. (1998). Ataxin-1 nuclear localization and aggregation: role in polyglutamine-induced disease in SCA1 transgenic mice. Cell 95, 41-53.

Laforet, G. A., Sapp, E., Chase, K., McIntyre, C., Boyce, F. M., Campbell, M., Cadigan, B. A., Warzecki, L., Tagle, D. A., Reddy, P. H., et al. (2001). Changes in cortical and striatal neurons predict behavioral and electrophysiological abnormalities in a transgenic murine model of Huntington's disease. J Neurosci 21, 9112-9123.

Landry, J., Slama, J. T., and Sternglanz, R. (2000). Role of NAD(+) in the

deacetylase activity of the SIR2-like proteins. Biochem Biophys Res Commun 278, 685-690.

Leavitt, B. R., Guttman, J. A., Hodgson, J. G., Kimel, G. H., Singaraja, R., Vogl, A. W., and Hayden, M. R. (2001). Wild-type huntingtin reduces the cellular toxicity of mutant huntingtin in vivo. Am J Hum Genet 68, 313-324.

Lesort, M., Chun, W., Johnson, G. V., and Ferrante, R. J. (1999). Tissue transglutaminase is increased in Huntington's disease brain. J Neurochem 73, 2018-2027.

Li, F., Macfarlan, T., Pittman, R. N., and Chakravarti, D. (2002b). Ataxin-3 is a histone-binding protein with two independent transcriptional corepressor activities. J Biol Chem 277, 45004-45012.

Li, S. H., Cheng, A. L., Zhou, H., Lam, S., Rao, M., Li, H., and Li, X. J. (2002a). Interaction of Huntington disease protein with transcriptional activator Sp1. Mol Cell Biol 22, 1277-87.

Lin, C. H., Tallaksen-Greene, S., Chien, W. M., Cearley, J. A., Jackson, W. S., Crouse, A. B., Ren, S., Li, X. J., Albin, R. L., and Detloff, P. J. (2001). Neurological abnormalities in a knock-in mouse model of Huntington's disease. Hum Mol Genet 10, 137-144.

Lin, X., Antalffy, B., Kang, D., Orr, H. T., and Zoghbi, H. Y. (2000). Polyglutamine expansion down-regulates specific neuronal genes before pathologic changes in SCA1. Nat Neurosci 3, 157-163.

Luthi-Carter, R., Strand, A., Peters, N. L., Solano, S. M., Hollingsworth, Z. R., Menon, A. S., Frey, A. S., Spektor, B. S., Penney, E. B., Schilling, G., et al. (2000). Decreased expression of striatal signaling genes in a mouse model of Huntington's disease. Hum Mol Genet 9, 1259-1271.

Luthi-Carter, R., Strand, A. D., Hanson, S. A., Kooperberg, C., Schilling, G., La Spada, A. R., Merry, D. E., Young, A. B., Ross, C. A., Borchelt, D. R., and Olson, J. M. (2002). Polyglutamine and transcription: gene expression changes shared by DRPLA and Huntington's disease mouse models reveal context-independent effects. Hum Mol Genet 11, 1927-1937.

Mangiarini, L., Sathasivam, K., Seller, M., Cozens, B., Harper, A., Hetherington, C., Lawton, M., Trottier, Y., Lehrach, H., Davies, S. W., and Bates, G. P. (1996). Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. Cell 87, 493-506.

Marmorstein, R. (2001a). Structure of histone acetyltransferases. J Mol Biol 311, 433-444.

Marmorstein, R. (2001b). Structure of histone deacetylases: insights into substrate recognition and catalysis. Structure (Camb) 9, 1127-1133.

Marsh, J. L., Walker, H., Theisen, H., Zhu, Y. Z., Fielder, T., Purcell, J., and Thompson, L. M. (2000). Expanded polyglutamine peptides alone are intrinsically cytotoxic and cause neurodegeneration in Drosophila. Hum Mol Genet 9, 13-25.

McCampbell, A., Taye, A. A., Whitty, L., Penney, E., Steffan, J. S., and Fischbeck, K. H. (2001). Histone deacetylase inhibitors reduce polyglutamine toxicity. Proc Natl Acad Sci U S A 98, 15179-15184.

McCampbell, A., Taylor, J. P., Taye, A. A., Robitschek, J., Li, M., Walcott, J., Merry, D., Chai, Y., Paulson, H., Sobue, G., and Fischbeck, K. H. (2000). CREB-binding protein sequestration by expanded polyglutamine. Hum Mol Genet 9, 2197-2202.

Moazed, D. (2001). Enzymatic activities of Sir2 and chromatin silencing. Curr Opin Cell Biol 13, 232-238.

Monoi, H., Futaki, S., Kugimiya, S., Minakata, H., and Yoshihara, K. (2000). Poly-L-glutamine forms cation channels: relevance to the pathogenesis of the polyglutamine diseases. Biophys J 78, 2892-2899.

Murphy, K. P., Carter, R. J., Lione, L. A., Mangiarini, L., Mahal, A., Bates, G. P., Dunnett, S. B., and Morton, A. J. (2000). Abnormal synaptic plasticity and impaired spatial cognition in mice transgenic for exon 1 of the human Huntington's disease mutation. J Neurosci 20, 5115-5123.

Nasir, J., Floresco, S. B., O'Kusky, J. R., Diewert, V. M., Richman, J. M., Zeisler, J., Borowski, A., Marth, J. D., Phillips, A. G., and Hayden, M. R. (1995). Targeted disruption of the Huntington's disease gene results in embryonic lethality and behavioral and morphological changes in heterozygotes. Cell 81, 811-823.

Ng, H. H., and Bird, A. (2000). Histone deacetylases: silencers for hire. Trends Biochem Sci 25, 121-126.

Nishitoh, H., Matsuzawa, A., Tobiume, K., Saegusa, K., Takeda, K., Inoue, K., Hori, S., Kakizuka, A., and Ichijo, H. (2002). ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. Genes Dev 16, 1345-1355.

Nucifora, F. C., Jr., Sasaki, M., Peters, M. F., Huang, H., Cooper, J. K., Yamada, M., Takahashi, H., Tsuji, S., Troncoso, J., Dawson, V. L., et al. (2001). Interference by huntingtin and atrophin-1 with cbp-mediated transcription leading to cellular toxicity. Science 291, 2423-2428.

Obrietan, K., and Hoyt, K. R. (2004). CRE-mediated transcription is increased in Huntington's disease transgenic mice. J Neurosci 24, 791-6.

Ordway, J. M., Tallaksen-Greene, S., Gutekunst, C. A., Bernstein, E. M., Cearley, J. A., Wiener, H. W., Dure, L. S. t., Lindsey, R., Hersch, S. M., Jope, R. S., et al. (1997). Ectopically expressed CAG repeats cause intranuclear inclusions and a progressive late onset neurological phenotype in the mouse. Cell 91, 753-763.

Panov, A. V., Gutekunst, C. A., Leavitt, B. R., Hayden, M. R., Burke, J. R., Strittmatter, W. J., and Greenamyre, J. T. (2002). Early mitochondrial calcium defects in Huntington's disease are a direct effect of polyglutamines. Nat Neurosci 5, 731-736.

Perutz, M. F., Johnson, T., Suzuki, M., and Finch, J. T. (1994). Glutamine repeats as polar zippers: their possible role in inherited neurodegenerative diseases. Proc Natl Acad Sci U S A 91, 5355-5358.

Perutz, M. F., and Windle, A. H. (2001). Cause of neural death in neurodegenerative diseases attributable to expansion of glutamine repeats. Nature 412, 143-144.

Ranen, N. G., Stine, O. C., Abbott, M. H., Sherr, M., Codori, A. M., Franz, M. L., Chao, N. I., Chung, A. S., Pleasant, N., Callahan, C., and et al. (1995). Anticipation and instability of IT-15 (CAG)n repeats in parent-offspring pairs with Huntington disease. Am J Hum Genet 57, 593-602.

Reddy, P. H., Williams, M., Charles, V., Garrett, L., Pike-Buchanan, L., Whetsell, W. O., Jr., Miller, G., and Tagle, D. A. (1998). Behavioural abnormalities and selective neuronal loss in HD transgenic mice expressing mutated full-length HD cDNA. Nat Genet 20, 198-202.

Remiszewski, S. W., Sambucetti, L. C., Atadja, P., Bair, K. W., Cornell, W. D., Green, M. A., Howell, K. L., Jung, M., Kwon, P., Trogani, N., and Walker, H. (2002). Inhibitors of human histone deacetylase: synthesis and enzyme and cellular activity of straight chain hydroxamates. J Med Chem 45, 753-757.

Rigamonti, D., Bauer, J. H., De-Fraja, C., Conti, L., Sipione, S., Sciorati, C., Clementi, E., Hackam, A., Hayden, M. R., Li, Y., et al. (2000). Wild-type huntingtin protects from apoptosis upstream of caspase-3. J Neurosci 20, 3705-3713.

Rigamonti, D., Sipione, S., Goffredo, D., Zuccato, C., Fossale, E., and Cattaneo, E. (2001). Huntingtin's neuroprotective activity occurs via inhibition of procaspase-9 processing. J Biol Chem 276, 14545-14548.

Riggs, M. G., Whittaker, R. G., Neumann, J. R., and Ingram, V. M. (1977). n-Butyrate causes histone modification in HeLa and Friend

erythroleukaemia cells. Nature 268, 462-464.

Ross, C. A., and Margolis, R. L. (2001). Huntington's Disease. Clinical Neuroscience Research 1, 142-152.

Rubinsztein, D. C. (2002). Lessons from animal models of Huntington's disease. Trends Genet 18, 202-209.

Rubinsztein, D. C., Leggo, J., Coles, R., Almqvist, E., Biancalana, V., Cassiman, J. J., Chotai, K., Connarty, M., Crauford, D., Curtis, A., et al. (1996). Phenotypic characterization of individuals with 30-40 CAG repeats in the Huntington disease (HD) gene reveals HD cases with 36 repeats and apparently normal elderly individuals with 36-39 repeats. Am J Hum Genet 59, 16-22.

Sartorelli, V., Puri, P. L., Hamamori, Y., Ogryzko, V., Chung, G., Nakatani, Y., Wang, J. Y., and Kedes, L. (1999). Acetylation of MyoD directed by PCAF is necessary for the execution of the muscle program. Mol Cell 4, 725-734.

Saudou, F., Finkbeiner, S., Devys, D., and Greenberg, M. E. (1998). Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. Cell 95, 55-66.

Shelbourne, P. F., Killeen, N., Hevner, R. F., Johnston, H. M., Tecott, L., Lewandoski, M., Ennis, M., Ramirez, L., Li, Z., Iannicola, C., et al. (1999). A Huntington's disease CAG expansion at the murine Hdh locus is unstable and associated with behavioural abnormalities in mice. Hum Mol Genet 8, 763-774.

Shimohata, T., Onodera, O., and Tsuji, S. (2000). Interaction of expanded polyglutamine stretches with nuclear transcription factors leads to aberrant transcriptional regulation in polyglutamine diseases. Neuropathology 20, 326-333.

Sipione, S., and Cattaneo, E. (2001). Modeling Huntington's disease in cells, flies, and mice. Mol Neurobiol 23, 21-51.

Smith, D. L., Portier, R., Woodman, B., Hockly, E., Mahal, A., Klunk, W. E., Li, X. J., Wanker, E., Murray, K. D., and Bates, G. P. (2001). Inhibition of polyglutamine aggregation in R6/2 HD brain slices-complex dose-response profiles. Neurobiol Dis 8, 1017-1026.

Steffan, J. S., Bodai, L., Pallos, J., Poelman, M., McCampbell, A., Apostol, B. L., Kazantsev, A., Schmidt, E., Zhu, Y. Z., Greenwald, M., et al. (2001). Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in Drosophila. Nature 413, 739-743.

Steffan, J. S., Kazantsev, A., Spasic-Boskovic, O., Greenwald, M., Zhu, Y.

Z., Gohler, H., Wanker, E. E., Bates, G. P., Housman, D. E., and Thompson, L. M. (2000). The Huntington's disease protein interacts with p53 and CREB-binding protein and represses transcription. Proc Natl Acad Sci U S A 97, 6763-6768.

Sterner, D. E., and Berger, S. L. (2000). Acetylation of histones and transcription-related factors. Microbiol Mol Biol Rev 64, 435-459.

Sternson, S. M., Wong, J. C., Grozinger, C. M., and Schreiber, S. L. (2001). Synthesis of 7200 small molecules based on a substructural analysis of the histone deacetylase inhibitors trichostatin and trapoxin. Org Lett 3, 4239-4242.

Stine, O. C., Pleasant, N., Franz, M. L., Abbott, M. H., Folstein, S. E., and Ross, C. A. (1993). Correlation between the onset age of Huntington's disease and length of the trinucleotide repeat in IT-15. Hum Mol Genet 2, 1547-1549.

Stott, K., Blackburn, J. M., Butler, P. J., and Perutz, M. (1995). Incorporation of glutamine repeats makes protein oligomerize: implications for neurodegenerative diseases. Proc Natl Acad Sci U S A 92, 6509-13.

Sugars, K. L., Brown, R., J. Cook, L., Swartz, J., Rubinsztein, D. C. (2004) Decreased cAMP Response Element-mediated Transcription: an early event in exon 1 and full-length cell models of Huntington's disease that contributes to polyglutamine pathogenesis. J Biol Chem 279, 4988-99.

Suzuki, T., Ando, T., Tsuchiya, K., Fukazawa, N., Saito, A., Mariko, Y., Yamashita, T., and Nakanishi, O. (1999). Synthesis and histone deacetylase inhibitory activity of new benzamide derivatives. J Med Chem 42, 3001-3003.

Takahashi, J., Fujigasaki, H., Zander, C., El Hachimi, K. H., Stevanin, G., Durr, A., Lebre, A. S., Yvert, G., Trottier, Y., The, H., et al. (2002). Two populations of neuronal intranuclear inclusions in SCA7 differ in size and promyelocytic leukaemia protein content. Brain 125, 1534-1543.

Takano, H., and Gusella, J. F. (2002). The predominantly HEAT-like motif structure of huntingtin and its association and coincident nuclear entry with dorsal, an NF-kB/Rel/dorsal family transcription factor. BMC Neurosci 3, 15.

Taylor, J. P., Taye, A. A., Campbell, C., Kazemi-Esfarjani, P., Fischbeck, K. H., and Min, K. T. (2003) Aberrant histone acetylation, altered transcription, and retinal degeneration in a Drosophila model of polyglutamine disease are rescued by CREB-binding protein. Genes Dev 17, 1463-8.

Uesato, S., Kitagawa, M., Nagaoka, Y., Maeda, T., Kuwajima, H., and Yamori, T. (2002). Novel histone deacetylase inhibitors: N-hydroxycarboxamides possessing a terminal bicyclic aryl group. Bioorg Med Chem Lett 12, 1347-1349.

Velier, J., Kim, M., Schwarz, C., Kim, T. W., Sapp, E., Chase, K., Aronin, N., and DiFiglia, M. (1998). Wild-type and mutant huntingtins function in vesicle trafficking in the secretory and endocytic pathways. Exp Neurol 152, 34-40.

Vonsattel, J. P., and DiFiglia, M. (1998). Huntington disease. J Neuropathol Exp Neurol 57, 369-384.

Wanker, E. E., Rovira, C., Scherzinger, E., Hasenbank, R., Walter, S., Tait, D., Colicelli, J., and Lehrach, H. (1997). HIP-I: a huntingtin interacting protein isolated by the yeast two-hybrid system. Hum Mol Genet 6, 487-495.

Warrick, J. M., Chan, H. Y., Gray-Board, G. L., Chai, Y., Paulson, H. L., and Bonini, N. M. (1999). Suppression of polyglutamine-mediated neurodegeneration in Drosophila by the molecular chaperone HSP70. Nat Genet 23, 425-428.

Wellington, C. L., Singaraja, R., Ellerby, L., Savill, J., Roy, S., Leavitt, B., Cattaneo, E., Hackam, A., Sharp, A., Thornberry, N., et al. (2000). Inhibiting caspase cleavage of huntingtin reduces toxicity and aggregate formation in neuronal and nonneuronal cells. J Biol Chem 275, 19831-19838.

White, J. K., Auerbach, W., Duyao, M. P., Vonsattel, J. P., Gusella, J. F., Joyner, A. L., and MacDonald, M. E. (1997). Huntingtin is required for neurogenesis and is not impaired by the Huntington's disease CAG expansion. Nat Genet 17, 404-410.

Wyttenbach, A., Swartz, J., Kita, H., Thykjaer, T., Carmichael, J., Bradley, J., Brown, R., Maxwell, M., Schapira, A., Orntoft, T. F., et al. (2001). Polyglutamine expansions cause decreased CRE-mediated transcription and early gene expression changes prior to cell death in an inducible cell model of Huntington's disease. Hum Mol Genet 10, 1829-1845.

Yamamoto, A., Lucas, J. J., and Hen, R. (2000). Reversal of neuropathology and motor dysfunction in a conditional model of Huntington's disease. Cell 101, 57-66.

Yoshida, M., Kijima, M., Akita, M., and Beppu, T. (1990). Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J Biol Chem 265, 17174-17179.

Zeitlin, S., Liu, J. P., Chapman, D. L., Papaioannou, V. E., and

Efstratiadis, A. (1995). Increased apoptosis and early embryonic lethality in mice nullizygous for the Huntington's disease gene homologue. Nat Genet 11, 155-163.

Zeron, M. M., Hansson, O., Chen, N., Wellington, C. L., Leavitt, B. R., Brundin, P., Hayden, M. R., and Raymond, L. A. (2002). Increased sensitivity to N-methyl-D-aspartate receptor-mediated excitotoxicity in a mouse model of Huntington's disease. Neuron 33, 849-860.

Zhang, J., Lesort, M., Guttmann, R. P., and Johnson, G. V. (1998). Modulation of the in situ activity of tissue transglutaminase by calcium and GTP. J Biol Chem 273, 2288-2295.

Zoghbi, H. Y., and Orr, H. T. (1999). Polyglutamine diseases: protein cleavage and aggregation. Curr Opin Neurobiol 9, 566-570.

Zoghbi, H. Y., and Orr, H. T. (2000). Glutamine repeats and neurodegeneration. Annu Rev Neurosci 23, 217-247.

Zuccato, C., Ciammola, A., Rigamonti, D., Leavitt, B. R., Goffredo, D., Conti, L., MacDonald, M. E., Friedlander, R. M., Silani, V., Hayden, M. R., et al. (2001). Loss of huntingtin-mediated BDNF gene transcription in Huntington's disease. Science 293, 493-498.

Zuhlke, C., Riess, O., Bockel, B., Lange, H., and Thies, U. (1993). Mitotic stability and meiotic variability of the (CAG)n repeat in the Huntington disease gene. Hum Mol Genet 2, 2063-2067.