Cearley, Jamie A.

Huntingtin's role in HD and function in adult mouse.

Specific aim 1. We will generate an adult knock-out of the huntingtin gene using Cre/loxP technology. A membrane permeable cre recombinase will be delivered either stereotaxically (striatum specific) or intraperitoneal (IP) into adult JC1 mice by the method of Jo et al. (Jo, et al. 2001). JC1 mice contain loxP sites that flank the promoter and exon 1 of the wildtype huntingtin allele while the other *Hdh* allele is null. JC1 mice have been generated and tested by the author of this grant proposal in the laboratory of Peter Detloff.
Specific aim 2. We will assess the phenotype of JC1 mice that have lost huntingtin as an adult. Mice with systemic or exclusively striatal huntingtin loss will be analyzed for abnormalities including decreases in survival age, behavioral and neuroanatomic abnormalities.
Specific aim 3. We will assess the effects of huntingtin loss in JC1 mice on gene expression. Affymetrix chip analysis will be conducted on RNA samples from the striatum of JC1 knock-out mice versus controls. Particular interest will be paid to the expression levels of those genes implicated in HD (neurotransmitter receptors, calcium and retinoid signaling pathway components). Expression differences in the striatum will be confirmed by northern blot or real-time PCR. Results will be compared to the knock-in $Hdh^{(CAG)150}$ mouse model (Lin, et al. 2001). Differences in expression profiles between knock-in and adult knock-out would suggest long repeats cause cellular disturbances by a mechanism other than one involving a late onset loss of huntingtin function.
These specific aims will be used to address the following:
Hypothesis 1: Huntingtin serves a critical function in the fully developed mouse.
Hypothesis 2: A late onset loss of function of wildtype huntingtin contributes to the symptoms of HD.

Background and significance:

Huntington's disease (HD) is one member of the trinucleotide repeat disease family. This neurological disease is inherited in an autosomal dominant fashion and is caused by the expansion of a CAG repeat in the first exon of the huntingtin gene to a length greater than 35 units. HD usually manifests itself in the third or fourth decade of life with chorea, dementia, and intellectual deterioration followed by a slow 10 to 15 year decline to death (Harper, et al. 1991). A pathological hallmark of HD is the selective loss of medium sized spiny neurons in the striatum. More recently, neuronal intranuclear inclusions (NII's) have been found in affected regions of HD brains (DiFiglia, et al. 1997). No treatment is available to slow the progression of HD. The Huntington's Collaborative Research Group first discovered the gene in which the HD mutation resides in 1993. The gene codes for a protein of approximately 350Kd referred to as huntingtin.

The role of huntingtin in development: HD knock-out mice. Although the function of huntingtin remains a mystery, several mutant lines of mice show the mouse huntington's disease homolog (*Hdh*) to be essential for development. Three classical knock-outs of *Hdh* display a lethal phenotype at embryonic day 7.5 (Nasir, et al. 1995; Zeitlin, et al. 1995; MacDonald, et al. 1996). In all three knock-out lines development was arrested at the egg cylinder stage. Heterozygous knock-outs show few or no abnormalities, suggesting that one dose of wildtype *Hdh* is compatible with normal development and normal adult life. Others have gone beyond the analysis of classical knock-out mice to determine huntingtin's developmental role. Dragatsis et al. have conducted clever experiments to determine the embryonic function of huntingtin. It was shown that by injecting wildtype blastocysts with huntingtin null embryonic stem (ES) cells extensively chimeric embryos could develop well beyond the lethal point for classical knock-outs of embryonic day 7.5. Many highly chimeric embryos were still indistinguishable from their wildtype littermates as late as embryonic day 12.5. However, no live births of highly chimeric animals were observed, indicating that loss of huntingtin after this stage in development leads to death. There were live births of less chimeric animals but in every case they had neurologic abnormalities. These researchers next conducted the converse experiment by injecting huntingtin -/- blastocysts with wildtype ES cells. When these mice were highly chimeric they displayed the same lethal phenotype at embryonic day 7.5 as classical knock-outs. From these experiments it was concluded that huntingtin plays some role in trafficking nutrients to the developing embryo through the extraembryonic tissue (Dragatsis, et al. 1998).

A mouse containing a hypomorphic allele was used to show that mice with 25% of the normal expression of *Hdh* have severe problems with neurogenesis (White, et al. 1997). These mice survived well beyond embryonic day 7.5, the age at which the classical huntingtin knock-out mice died. One half of the mice expressing only 25% of the

endogenous huntingtin level were stillborn while those that were born alive only survived for a day or two. Both the stillborn and live-born pups were found to be grossly abnormal morphologically with regard to the nervous system while the remaining part of their bodies were normal. These data indicate a role for huntingtin in neurogenesis and a lack of function in the development of the remainder of the body. Comparing our studies of a systemic versus a striatum specific huntingtin knock-out will extend these studies by assessing huntingtin's function in organs other than the brain and in the adult as well. The early death of the hypomorphic mice does not allow assessment of the necessity of huntingtin in the fully developed adult.

To investigate further the effects of the loss of huntingtin in the mouse the Cre-loxP system has been used to make a knock-out of the huntingtin gene at embryonic day 15 or postnatal day 5 (Dragatsis, et al. 2000). In these mice recombination of the loxP sites and hence the knock-out occurred specifically in the forebrain and testis of mice resulting in neurodegeneration. Expression of Cre recombinase was driven by the Cam kinase 2a (CamK2a) promoter, which provided the tissue specificity of the knock-out. Expression of Cre and hence deletion of the huntingtin gene began at two different time points, embryonic day 15 or postnatal day 5, depending upon which Cre expressing founder line was used. Huntingtin was decreased to levels as low as 10% of endogenous in the forebrains of some of these mice correlating with neurodegeneration. Although the knock-out of the huntingtin gene occurs later in the development of these mice the possibility of developmental effects on the phenotype still cannot be ruled out as deletion began on or before postnatal day 5, a time when the nervous system of the mouse is still developing. The mice used in my experiments will be fully developed adults prior to deletion of *Hdh*.

The role of huntingtin in the adult and in HD. The embryonic lethality of the classic huntingtin knock-out has greatly hampered the investigation into huntingtin's normal function in the adult. There have been several roles for huntingtin proposed in recent years. The subcellular location of huntingtin suggests it may function in vesicle trafficking. Immunoelectron microscopy and subcellular fractionation of brain tissues reveals that huntingtin associates with clathrin coated vesicles, is enriched in synaptosomal membrane fractions, and associates with vesicle membranes and microtubules (DiFiglia, et al. 1995). All of this data suggests a role in cellular transport and neurotransmission. Huntingtin has also been proposed to be a negative regulator of apoptosis. Mice transgenic for a Huntington's disease homolog (*Hdh*)$^{(CAG)72}$ yeast artificial chromosome (YAC) construct show increased apoptosis in the testes when wildtype *Hdh* is not present. When wildtype *Hdh* is returned to this system protection from apoptosis in the testes is conferred (Zuccato, et al. 2001). One way to interpret these data is that the expression levels of huntingtin from the *Hdh*$^{(CAG)72}$ YAC construct are not high enough to prevent apoptosis in the testis and the addition of a wildtype gene is raising the level of huntingtin to a tolerable degree. This interpretation is plausible as the expression levels of the *Hdh*$^{(CAG)72}$ YAC construct have never been convincingly shown to be as high as endogenous levels. Huntingtin may play a role as a regulator of brain derived neurotrophic factor (BDNF) expression. BDNF levels are decreased in a tissue culture system when long repeat versions of huntingtin are expressed (Leavitt, et al. 2001). This finding is interesting in light of the role BDNF plays as a protector of striatal cells which are most vulnerable in HD (Nakao, et al. 1995). In support of huntingtin's ability to alter the expression of genes Kegel et al. have shown that wildtype huntingtin is present in the nucleus. Additionally, Kegel and colleagues provide evidence for huntingtin's function in assembling nuclear matrix bound protein complexes involved in transcriptional repression, RNA processing, and ribosomal biogenesis (Kegel, et al. 2002).

There are several experiments that might be interpreted as support for a late onset loss of function mechanism for HD. Kegel et al. suggest that mutant huntingtin could disrupt the function of the wildtype protein leading to neuronal dysfunction (Kegel, et al. 2002). Moreover, wildtype huntingtin protein is present in NII's (Narain, et al. 1999). It is possible that excessive sequestration of wildtype huntingtin protein into NII's hinders its ability to function leading to neuronal dysfunction. Also, Dragatsis and colleagues found symptoms similar to HD in their knock-out of the huntingtin allele at day e15 or p5 specifically in the forebrain and testis of mice (Dragatsis, et al. 2000). Finally, Auerbach and co-workers have recently shown that a reduced wildtype huntingtin level sensitizes the CNS to the toxic effects of a mutant huntingtin allele (Auerbach, et al. 2001). Although one can infer from these experiments that a late onset loss of wildtype function is contributing to HD, the developmental process confuses the interpretation of these experiments.

Experiments have been conducted that contest a late onset loss of function mechanism. First, a knock-out of the androgen receptor gene, responsible for spinobulbar muscular atrophy (SBMA) when the CAG repeat is expanded, does not lead to SBMA but rather testicular atrophy. Second, Ordway and colleagues have shown that expression of a long CAG repeat located in the Hprt gene of the mouse leads to a late onset neurological phenotype with symp-

toms similar to that of the trinucleotide repeat diseases. Importantly, Hprt knock-out mice display no phenotype, and Hprt is not functional in the animals made by Ordway. While this experiment does not eliminate the possibility of a role for wildtype trinucleotide repeat alleles in the disease mechanism, it does indicate that this role would not be exclusive in nature. Clearly, the CAG repeat was acting as the driving force apart from a loss of function of the wildtype allele in this case (Ordway, et al. 1997). Wheeler et al. have conducted an experiment aimed at testing the effects of the wildtype allele on the disease process (Wheeler, et al. 2002). In this experiment, *Hdh*^{Q111} knock-in mice carrying either a wildtype or knock-out allele on the other chromosome were examined for differences in early disease characteristics. They reasoned if the wildtype allele was having some type of protective influence, that ability would be lost in the mouse heterozygous for both the knock-in and the knock-out allele. Wheeler et al. concluded that there was no effect due to the loss of the wildtype allele and hence any affects that wildtype huntingtin has on the disease process are further downstream, perhaps in neurons that are already sensitized by the actions of the mutant allele. These results must be considered carefully as the phenotype of *Hdh*^{Q111} mice is quite subtle, making differences in severity difficult to detect. An adult knock-out would provide an essential reagent for determining whether a late onset loss of function mechanism is at work, and make it possible to definitively distinguish between the consequences of huntingtin loss as an adult and consequences resulting from loss during development.

Gene expression analysis to examine the effects of huntingtin loss. Gene expression arrays are a tool that can be used to screen for genes whose expression levels may be affected by a particular gene of interest, and to identify potential candidates for drug targeting. Multiple gene expression arrays, using Affymetrix chips, including those on HD patient material as well as mouse models of HD indicate that changes in the expression levels of many genes occurs early in the disease process (Cha, et al. 1998; Luthi-Carter, et al. 2000). The affected genes fall into a few categories including neurotransmitter receptors, and calcium and retinoid signaling pathway components. In each expression study to date, mutant huntingtin was expressed either as a full-length protein, from the endogenous locus, or as a truncated version from a transgene. With the mutant allele expressed in all of these types of studies it is impossible to determine what genes are directly or indirectly affected as a result of the presence of the mutant allele and what genes may be effected through the action or loss of the wildtype allele. It is not unlikely that some of these results are due to the loss of wildtype function as huntingtin is proposed to play a role in neurotransmission, those types of genes who's regulation is altered. It is also interesting to note that results from expression studies conducted on the R6/2 mouse line, which expresses human exon 1 sequence with a CAG repeat of approximately 150 units, and results from our lab conducted on *Hdh*$^{(CAG)150}$ mice, which express full length huntingtin with a 150 unit CAG repeat from the endogenous mouse locus, differ in some respects (Mangiarini, et al. 1996; Lin, et al. 2001). This is intriguing because these mice also differ in that the R6/2 mouse retains a wildtype copy of the huntingtin allele at the endogenous locus while *Hdh*$^{(CAG)150}$ mice were homozygous for the mutant allele and hence have no wildtype allele present. One possible explanation for these differences is the presence or absence of the wildtype allele. The experiments proposed here will provide useful information with regard to what genes are specifically affected by the loss of wildtype huntingtin in the absence of a mutant allele. By examining samples over a time course after injection of Cre we can begin to determine which genes are initially affected by loss and those that are further downstream, helping to place huntingtin in a pathway. These results will provide information that could potentially narrow the field of targets for drug therapy. Since the Affymetrix chip is a new technology whose results are often questioned we will use the chip study only as a preliminary screen. We will select approximately 10 genes showing differences from the chip results to confirm using Real-time PCR.

Cre/loxP mediated deletion. The Cre/loxP system has been used in many different ways to create a deletion or knock-out of a gene. Cre is a site-specific recombinase that specifically recognizes 34 base pair sequences called loxP sites. When Cre binds two loxP sites, they are brought together and a recombination event occurs leading to the deletion of any sequence lying between them. Hence, the presence of Cre is the key to controlling both the location and time of the deletion. Cre has been controlled in many different ways. For example, consider the work of Dragatsis et al. using the CamKII promoter to drive expression of Cre and create a knock-out of the huntingtin gene (Dragatsis, et al. 2000). The drawbacks of this technique are clear. For one, the time of expression is not completely at the discretion of the researcher. In this case expression occurred before development was complete, at e15 or p5. The CamKII promoter that is driving Cre expression determines the time of loss. It follows that the hurdle of getting past development cannot be overcome. Secondly, the location at which the expression occurs is also limited, in the case of Dragatsis et al., to the forebrain and testis. Without doubt, it would be more desirable in the case of huntingtin to create the deletion in a location more relevant to HD, such as the striatum. We plan to circumvent these prob-

lems, as Jo et al. have done, by the creation of a membrane permeable Cre (Jo, et al. 2001). By placing a membrane translocation sequence on the 3' end of Cre, and inserting this sequence into an expression vector, Jo et al. were then able to express and purify a membrane permeable Cre protein from bacteria (CreMTS). Jo and co-workers then delivered the CreMTS protein via IP injection into mice containing loxP sites flanking a reporter construct. They found that deletion occurred in all tissues examined including the brain at high levels. The advantages of this system are evident: First, the use of either IP or stereotaxic injection can easily control the location of Cre. Therefore, it can be used to knock-out huntingtin either systemically or specifically in the striatum allowing us to determine the necessity of huntingtin in different tissues. Second, Cre can be delivered at any time during the life of the mouse. By using CreMTS in conjunction with JC1 mice we will be able to create an adult knock-out of the huntingtin gene. The results will enable us to determine the necessity of huntingtin in the adult mouse, the adult brain, and whether symptoms resulting from adult loss of huntingtin mimic HD, suggesting a late onset loss of function mechanism for HD. In addition, we can use JC1 knock-out mice to test for changes in the expression levels of genes of interest, giving clues into the role of wildtype huntingtin in the adult mouse.

Preliminary Results: There are two main components needed in order to complete the specific aims laid out in this grant proposal: JC1 mice and CreMTS protein. JC1 mice, the first component, have already been made and tested. To make JC1 mice I utilized the optimized tag and exchange protocol described in Cearley et al. (Cearley, et al. 2001). The tag and exchange system, first described by Askew et al., makes it possible to create a modified allele without leaving a drug selectable marker behind (Askew, et al. 1993). The exchange construct (pJC4) consisted of a wildtype *Hdh* allele with the addition of two loxP sites, one just ahead of the promoter and a second in intron 1 (*Hdh*flox) (Figure 1c).

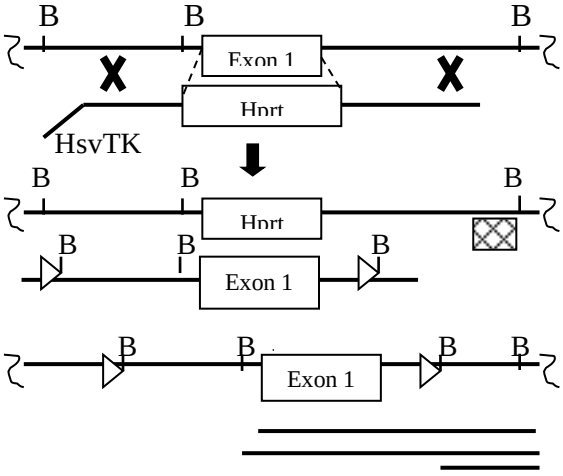

Figure 1. Tag and exchange of the *Hdh* locus. (a) Replacement of *Hdh* Exon1 with an Hprt minigene by conventional gene targeting. Upper line with break-markings represents the small portion of mouse chromosome 5 that contains exon 1 of the mouse *Hdh* locus. Desired crossover points are represented by 'X's. Boxes represent Exon1 of *Hdh* or the Hprt minigene. *Bsu*36I recognition sites are designated with the symbol 'B'. (b) Tagged chromosome resulting from recombination shown in panel (a). Hatched box represents homology to a probe used to confirm gene targeting. (c) pJC4 exchange gene targeting construct linearized with *Sac*II. Each open triangle represents an insertion containing a loxP site and a *Bsu*36I recognition site. These insertions flank a wildtype version of exon1. (d) The chromosome resulting from a correct exchange reaction using the construct shown in panel c and an ES cell containing the chromosome in panel (b). Below panel (d) bars represent the *Bsu*36I fragments expected for wildtype, tagged and exchanged chromosomes.

This positioning is critical in ensuring that the loxP sites do not affect normal transcription of the allele when in their non-recombined state. Also, by positioning the loxP sites so that they flanked the promoter region and exon 1 we were assured that deletion of the sequence between the loxP sites would render the allele null (a smaller deletion that created a null allele was previously published (Zeitlin, et al. 1995)). Four subcloned colonies of the tagged cell line (eJC23) were electroporated independently of each other with the exchange construct (pJC4). These

four electroporations resulted in 45 6-thioguanine (6-TG) resistant colonies, 6 of which were confirmed to be gene targeted by Southern blot for an average targeting frequency of approximately 13% (Figure 2).

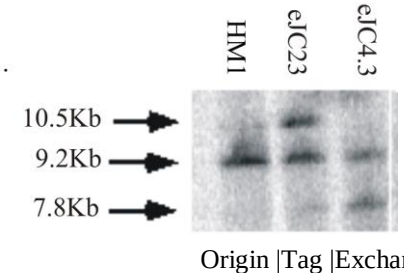

Figure 2. Southern blot of *Bsu*36I DNA fragments from tagged and exchanged cell lines. HM1 is the starting cell line. eJC23 has exon 1 of the *Hdh* locus replaced by an Hprt minigene. eJC4.3 was produced using the exchange construct pJC4 (*Hdh*flox). Map and probe are shown in Figure 1.

Mice were then generated from gene targeted embryonic stem (ES) cells containing the *Hdh*flox allele via blastocyst injection. Foster mothers gave birth to 62 pups, of which no less than 15 were chimeras. At least 4 of these chimeras transmitted the mutation to their offspring. These mice were then mated to *Hdh* knock-out mice that were also generated by the author of this application using the construct in figure 1b. The resulting offspring were heterozygous for both the knock-out allele and the *Hdh*flox allele. I will refer to these doubly heterozygous mice as JC1 mice. The presence of the null allele insures the removal of *Hdh* in each cell by a single recombination event.

Characterization of the *Hdh*flox allele. In order to insure that the *Hdh*flox allele was suitable for my purposes I undertook an extensive characterization of it. First, I investigated the possibility that the loxP sites would interfere with the expression of the *Hdh* allele. To test for loxP site interference we mated heterozygous *Hdh*flox mice to one another and searched for homozygous *Hdh*flox progeny, the rationale being that any gross disturbance of gene expression or dysfunction of the gene would likely produce a lethal phenotype (White, et al. 1997). Mice homozygous for the *Hdh*flox allele are produced in the expected numbers, appear normal, and live a typical life span as assessed by casual observation (Figure 3).

Figure 3. PCR across the loxP site in the *Hdh* flox allele. Wildtype band is 97 bp and *Hdh*flox band is 131 bp. A homozygote for the *Hdh*flox allele is indicated by a *.

I have shown that the *Hdh*flox allele in mouse brain expresses huntingtin protein in amounts comparable to wildtype *Hdh* (Figure 4).

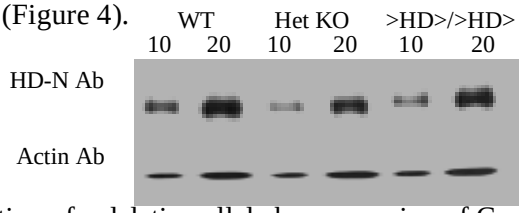

Figure 4. Western blot to detect huntingtin expression from the *Hdh*flox allele. Brain protein samples in 10 µg and 20 µg pairs from wildtype, heterozygous knock-out and homozygous *Hdh*flox animals.

Creation of a deletion allele by expression of Cre recombinase. Expressing Cre recombinase early in the development of the mouse tested LoxP functionality. We mated homozygous *Hdh*flox mice to mice carrying Cre as a transgene driven by the Cmv promoter (A gift from Dr. A. Nagy). These mice express Cre protein at the four-cell stage of the embryo. This mating resulted in mice heterozygous for the recombined *Hdh*flox (*Hdh*del) allele. The recombination of the loxP sites and the expected deletion was confirmed by Southern blot analysis (Figure 5).

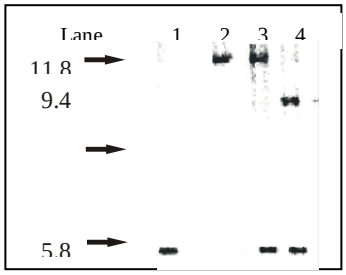

Figure 5. Southern blot to demonstrate loxP functionality. *Spe*I digests of mouse tail DNA showing the 2.4 Kb deletion of the sequence between loxP sites created by Cre recombinase. Wildtype band = 5.8 Kb, non-recombined *Hdh*flox band = 11.8 Kb, recombined *Hdh*flox (*Hdh*del) band = 9.4 Kb. lane 1 = wildtype, lane 2 = homozygous *Hdh*flox, lane 3 = heterozygous *Hdh*flox, lane 4 = heterozygous *Hdh*del.

A PCR assay to detect recombination at the *Hdh*flox locus has also been developed. This assay will allow us to detect recombination in very small amounts from DNA samples. By using primers that lie just outside the loxP sites, a unique band is amplified only when the loxP sites are recombined bringing the primers close together (Figure 6).

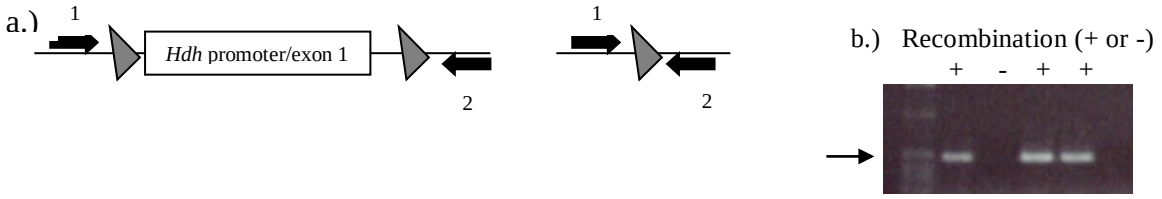

Figure 6. PCR to detect recombination at the *Hdh* locus. a.) Grey triangles represent loxP sites. Black arrows represent PCR primers 1 and 2. b.) PCR results from DNA isolated from mouse tail. The product from the non-recombined allele is too large to amplify while the product from the recombined allele is 465 base pairs. The arrow points to the 1Kb marker band of 516/506 base pairs (Gibco/BRL).

To ensure that the recombination of loxP sites in the *Hdh*flox allele would create a null allele, we mated the heterozygous knock-out mice (*Hdh*del) generated by cmvCre as described above to each other and observed for homozygous animals. Of 54 pups that were weaned 0 mice homozygous for the *Hdh*del allele were found showing that indeed a null allele was created. We next wanted to examine embryos at different ages to see if the phenotype of our homozygous knock-outs were similar to those previously published. Heterozygous knock-out pairs were allowed to mate and females were checked for plugs the following morning. On the specified day, mothers were sacrificed and embryos were dissected from the uterus. Embryos were dissected at ages ranging from embryonic day 7.5 to embryonic day 11.5. A total of 187 embryos were dissected and genotyped. The phenotype of the homozygous knock-outs was found to be much like that of previously published knock-outs further validating our knock-out allele. Based on these results, we are confident that this allele will behave as expected in JC1 mice, in the presence of CreMTS, to create a knock-out of the huntingtin allele.

Generating the Cre/MTS expression vector. A Cre gene with a membrane translocation signal was constructed as in Jo et. al. (Jo, et al. 2001). Briefly, Cre recombinase from bacterial phage P1 was isolated by PCR and a nuclear localization signal (NLS) from simian virus 40 (SV40) large-T antigen added to the 5' end of the Cre sequence by incorporation into the PCR primers. The NLS improves the performance of the Cre recombinase as reported by Jo et al. (Jo, et al. 2001). The entire PCR product was then cloned into the pET28a expression vector (Novagen) and the MTS from the Kaposi fibroblast growth factor (FGF-4) and a stop codon were added. The final clone (Figure 7) was sequenced from the start site through the stop codon to confirm that it is correct and in frame.

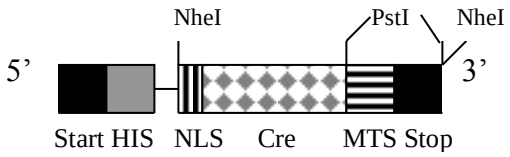

Figure 7. Map of pET28a/CreMTS. pET28a (Novagen) forms the backbone. Sequence containing an NLS Cre was generated by PCR and ligated into the NheI site of pET28a. The MTS was made as a pair of ligated synthetic oligos that were annealed and inserted into an engineered PstI site at the 3' end of Cre.

CreMTS protein expression from bacteria was confirmed by SDS-page and coomassie stain (Figure 8).

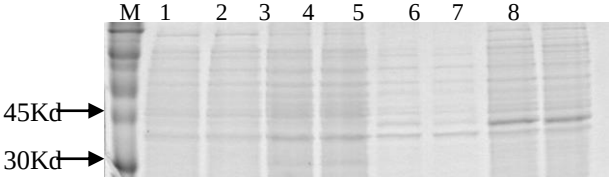

Figure 8. Coomassie stain of induced CreMTS protein in BL21 cells. Lanes are as follows: M = marker, 1=T0 of colony #1, 2=T0 of colony #2, 3=5hr uninduced colony #1, 4=5hr uninduced colony #2, 5=5hr, 1mM induction supernatant colony #1, 6=5hr 1mM induction supernatant colony #2, 7=pellet of lane 5, 8=pellet of lane 6. Expected size for CreMTS is 36.8Kd.

Purification of Cre/MTS and Assaying Cre enzyme activity. The HIS tag on the Cre/MTS protein was utilized to purify the protein. Following purification the protein was concentrated by ultrafiltration. To assay the activity of the Cre enzyme, the release of a circular plasmid inserted into a λ-phage (Novagen) by transformation of *E. coli* was measured as in Jo et al. A unit of Cre enzyme is defined, as by Jo et al., as that which produces 10^4 colonies (equivalent to $2x10^6$ circular molecules) in a 30-minute reaction.

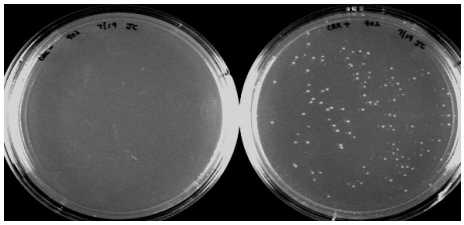

Figure 9. Representative plates from a functional assay using purified CreMTS proteinto release a circular plasmid containing an ampicillin resistance gene from lambda phage. The plate on the left served as a negative control where no creMTS protein added to the reaction. The plate on the right shows colonies resulting from the recombination of loxP sites by CreMTS protein to release the ampicillin resistant plasmid. When the activity was calculated it was found to be comparable to that of Jo et. al. (2001).

To begin, all experiments will be conducted using the same number of enzyme units as Jo et al. but will be modified if necessary (Jo, et al. 2001).

Research Design and Methods:

Specific Aim 1: We will generate an adult knock-out of the huntingtin gene using Cre/loxP technology.

Creating a systemic knock-out of the huntingtin gene in adult JC1 mice. As a pilot experiment 5 JC1 mice will be injected IP with 1 ml PBS alone and 5 will be injected with 2,250 U/g (CreMTS) in 1ml PBS on three consecutive days. Specific activity will be determined by the enzyme activity assay described above. Three days after the last injection, organs will be removed and assayed by PCR for recombination as described in the preliminary results section. Real-time PCR using the same primer concept as used in the PCR for recombination will also be conducted in order to quantitate, on the DNA level, the percentage of cells that have undergone recombination in any particular tissue. Because each cell has only one floxed allele (the other allele being null) an accurate assessment of the number of cells that have undergone recombination can be determined. The use of real-time PCR for genotype determination is a well-established procedure. A primer/probe set has been commercially designed and ordered from Applied Biosystems. The PCR primers flank the loxP sites and the target (a probe carrying a florescent tag) lies just outside of one of the loxP sites. Samples prepared and run in triplicate of the same tissue type from wildtype mice, un-injected, and injected JC1 mice will be compared. For comparison purposes a complementary PCR reaction will also be done in which the primers lie within the loxP sites. In this reaction only non-recombined alleles will produce a product. Our lab has access to an Applied Biosystems 7900HT Sequence Detection System. While there is no clear reason why we would not achieve the same high levels of deletion as Jo and co-workers, if we do not see desirable levels of recombination we will increase the dosage as well as the time course for Cre delivery. Once conditions for CreMTS delivery are optimized 20 JC1 mice will be injected with CreMTS protein, and 20 JC1 mice will be injected with PBS alone to serve as controls. These mice will be analyzed as described below.

Creating a striatum specific knock-out of the huntingtin gene in adult JC1 mice. To create a huntingtin knock-out exclusively in the striatum of JC1 mice we will use stereotaxic injection of CreMTS protein. The author of this grant has experience with stereotaxic injection into the mouse striatum. However, since CreMTS has not previously been delivered via stereotaxic injection we will conduct a pilot study to optimize the volume and concentration of CreMTS to be used. This pilot study will be based on results published for the injection of an adenoviral Cre into the mouse striatum (Kaspar, et al. 2002). For the pilot study only one side of the brain will be injected leaving the remaining side to serve as an internal control. Three days post injection the striatum from both sides of each animal will be removed. Additionally, the cortex, cerebellum, and hippocampus will be removed from the injected side of the brain. DNA will be isolated from these samples and tested by PCR for recombination. Any samples that are positive for recombination will then undergo quantitation of recombination using real-time PCR. Although this protocol originally delivered adenovirus exclusively to the striatum, if recombination is found in areas other than the striatum due to diffusion of the CreMTS protein a smaller volume of injection will be tested (Kaspar, et al. 2002). The mice injected with PBS alone will serve as controls for any dramatic phenotype that may result as a consequence of the injection procedure itself. Once conditions for injection are optimized, 20 ten-week-old JC1 mice will

be injected with CreMTS in PBS into the striatum on both sides of the brain. As controls, 15 ten-week-old JC1 mice will be injected with PBS alone. The mice will then be analyzed as described below.

Specific aim 2. We will assess the phenotype of JC1 mice that have lost huntingtin either systemically, or exclusively in the striatum, as an adult. To determine whether or not huntingtin is necessary for the survival of the adult mouse we will inject IP, 20 ten-week-old JC1 mice with 1ml of PBS alone as controls, and 20 ten-week-old JC1 mice with 1ml of PBS containing 2,250 U/g of Cre/MTS protein. Injections will be repeated on three consecutive days. The survival of these mice will then be monitored and their date of death plotted on a graph of time post injection versus percent of mice remaining alive in their category. The mice will be monitored until all the Cre/MTS injected mice are dead or until 1 year post injection, whichever comes first. At the death of each mouse or the end of one year, DNA will be isolated from the striatum, cortex, hippocampus, cerebellum, kidney, liver, muscle, and heart of these mice and the percentage of recombination determined by real-time PCR as described above. Therefore, we will be able to plot survival time relative to the percent of cells that have lost huntingtin. In order to compare the survival time of mice that have lost huntingtin systemically to those who have lost huntingtin exclusively in the striatum, this experiment will be repeated on mice that receive CreMTS protein via stereotaxic injection into the striatum as described above. In this case DNA will be isolated specifically from the striatum and percentage of recombination determined by real-time PCR. In combination these experiments will indicate the relative importance of huntingtin in other areas of the brain and body in comparison to the striatum.

Behavioral Analysis. In order to assess any behavioral phenotype that may occur in these animals a series of behavioral analyses will be conducted. The same mice as are included in survival determination will be used for these experiments. We will conduct tests once each week post injection up to 6 weeks post injection, then test frequency will be reduced to once every 3 weeks. Each mouse will be housed in an individual cage during the behavioral trials. The trials will consist of measurement of body weight, a simple open cage activity test, a tail suspension test, and a rotarod test. We will also record the frequency and duration of any convulsive spells that occur during behavioral trials. An infrared beam activity monitor will be used to assess the undisturbed activity of mice (A.B. Sieber, Rocky Mount, North Carolina) as previously described (Ordway, et al. 1997). JC1 knock-out mice may not survive long enough to conduct these behavioral trials. If this is the case our conclusion will be that huntingtin serves a critical function in the adult mouse. Assays such as the immunohistochemical analysis described below can still be conducted, and give meaningful information, if this is the case. The gene expression analysis can also be done even if survival time is short. Both the immunohistochemistry and gene expression studies will provide useful information regarding the function of huntingtin in the adult, and are likely to give information regarding it's role in HD in spite of a short survival time. Another possibility is that no behavioral phenotype will be seen in the JC1 knock-out animals. This does not eliminate the possibility of a pathological phenotype, nor does it mean that gene expression studies will not give useful information. There are two possible reasons that a behavioral phenotype may not be seen. One, huntingtin may not be necessary for survival and normal behavior as we are measuring it in the adult mouse. If this is the case, our conclusion will be that huntingtin does not serve a critical function in the adult mouse at least to the extent that huntingtin is deleted in our mice. Secondly, the lack of behavioral phenotype could be the result of a low percentage of cells that acquired the deletion of *Hdh*. This is not expected since Jo et al. found that IP injection of CreMTS resulted in recombination levels of 50% typically and often higher (Jo, et al. 2001). We would expect stereotaxic injection to produce a much higher percentage of recombination positive cells due to the direct delivery of CreMTS. However, if this is not the case we can conclude that huntingtin is not absolutely necessary in all cells for survival of the entire organism. Additionally, Jo and colleagues did not find 100% recombination in the tissues examined; there was a large concentration of recombination positive cells in areas located near blood vessels (Jo, et al. 2001). Therefore, our immunohistochemistry results can focus on areas near the blood vessels in our IP injected mice to yield useful information on the effects of huntingtin loss on the individual cell level.

Immunohistochemical Analysis. Immunohistochemistry will be used to determine if there are any pathological abnormalities in the adult JC1 knock-out mice. Since we do not yet know the effects of huntingtin loss in the adult mouse, the number and age of mice for these studies will be determined after the preliminary results are known for the survival and behavioral studies. Mice will be assessed at time points early as well as late in the course of any phenotype seen. If no phenotype is seen the mice will be assessed at one-year post injection. Mice will be fixed by transcardial perfusion with paraformaldehyde or formalin, cryoprotected, and shipped to Dr. Marian Difiglia's lab for neuroanatomical analysis. Cresyl violet will be used to assess any gross neuronal loss throughout the brain. AB-1 or HD-N antibody will be used to determine areas of huntingtin loss. The possibility of gliosis will be investigated

using a GFAP antibody. Additionally, we will look for evidence of markers related to HD such as enkephalin (lost early in HD) and somatostatin/ neuropeptide Y/ NADPH diaphorase (in neurons spared in HD), as well as BDNF and D1 receptors.

Specific aim 3. We will assess the effects of huntingtin loss in JC1 mice on expression of other genes implicated in HD. To detect changes in gene expression resulting from the loss of huntingtin, RNA from the striatum of mice stereotaxically injected with CreMTS protein or PBS only will be isolated and screened using Affymetrix chip analysis performed at UAB's gene expression core facility. The number and age of mice used for this study will be determined by the age of onset of any phenotype seen, the rate of phenotype progression, and the degree of deletion variability from mouse to mouse. All of these variables will be determined in specific aims 1 and 2. To conduct the study, both RNA and DNA will be isolated from the striatum of each mouse in the experiment using the Trizol reagent (Gibco/ Life Technologies). The DNA will be used to determine the percentage of recombination in that specific sample using the real-time PCR protocol described earlier. The RNA will be used for Affymetrix chip analysis. By determining the percentage of recombination in each sample we can compare changes in gene expression relative to the overall percentage of cells carrying the deletion. I will use the U74A version 2 gene chip. This chip contains probes for ~6000 known genes and ~6000 additional EST's. Those genes whose expression levels are changed significantly will be tested further by real-time PCR.

References:

Askew, G. R., (1993). Mol. Cell. Biol. 13(7): 4115-4124.
Auerbach, W., (2001). Hum Mol Genet 10(22): 2515-2523.
Cearley, J. A., (2001). Transgenic Research: 1-10.
Cha, J. H., (1998). Proc. of the Nat. Acad. of Sci.of the USA 95(11):6480-5.
DiFiglia, M., (1995). Neuron 14(5): 1075-81.
DiFiglia, M., (1997) Science 277(5334): 1990-1993.
Dragatsis, I., (1998). Development 125(8): 1529-39.
Dragatsis, I., (2000). Nat Genet 26(3): 300-6.
Harper, P. S., (1991). Huntington's Disease. London, Saunders.
Jo, D., (2001). Nature Biotechnology 19(October): 929-933.
Kaspar, B. K., (2002). Proc Natl Acad Sci U S A 99(4): 2320-2325.
Kegel, K. B., (2002). Journal of Biological Chemistry 277(March 1): 7466-7476.
Leavitt, B. R., (2001). Am J Hum Genet 68(2): 313-24.
Lin, C.-H., (2001). Human Mol. Gen. (In Press).
Luthi-Carter, R., (2000). Hum. Mol. Genet. 9: in press.
MacDonald, M. E., (1996). Cold Spring Harbor Symp. 61: 627-638.
Mangiarini, L., (1996). Cell 87(3): 493-506.
Nakao, N., (1995). Experimental Neurology 131(1): 1-10.
Narain, Y., (1999). Journal of Medical Genetics 36: 739-746.
Nasir, J., (1995). Cell 81(5): 811-23.
Ordway, J. M., (1997). Cell 91(6): 753-763.
Wheeler, V. C., (2002). Human Molecular Genetics 11(6): 633-640.
White, J.K., (1997). Nature Genetics 17(4): 404-410
Zeitlin, S., (1995). Nature Genetics 11(2): 155-63
Zuccato, C.C., (2001). Science Express: 1-6

Training goals during period of reward:

While performing the work proposed in this grant I will be trained in new techniques as well as increase my experience and familiarity with techniques still new to me. For example, I will be learning the latest techniques in Real-time PCR. Due to the nature of both Real-time PCR data as well as behavioral data I will have the opportunity to gain experience in statistical analysis. Also, I will become more proficient at stereotaxic injection into the mouse striatum as well as protein expression and purification from bacteria; two areas where my current experience is limited. The strong points of my experimental design include:

1. The use of a membrane permeable Cre recombinase to create an *Hdh* deletion in the adult mouse.
2. The possibility of obtaining and observing an adult striatum that has experienced the loss of *Hdh* for an extended period of time.
3. The ability to accurately quantitate the percentage of cells that have lost *Hdh* using Real-time PCR.

28a. Summary.

1992-1994 Southern Illinois University at Edwardsville

My Master's thesis project was aimed at producing recombinant human fibrinogen in potato tubers. Fibrinogen is a macromolecule consisting of three chains. During my time at SIUE I initiated this complex project by cloning the expression vector to produce the gamma chain of human fibrinogen. Another key component of this project was to have the ability to regenerate potato plants from leaf explants and produce tubers from them. The potato cultivar Katahdin was chosen for its extensive production of tubers. I formulated a protocol that would allow Katahdin to be regenerated from leaf explants for the first time and published these results in the American Potato Journal.

1994-2002 University of Alabama at Birmingham

During my time spent at UAB I have worked on Huntington's disease. In the course of this work I have produced multiple gene targeted mouse lines via blastocyst injection. I have also broadened my range of molecular biology techniques. It was a main focus in our lab to generate a mouse embryonic stem cell line that could be efficiently and repeatedly targeted at the huntingtin locus. I have independently developed such a cell line, optimized a targeting protocol and have published the results in Transgenic Research as a part of my dissertation work. A second goal of my own has been to create an adult knockout of the mouse huntingtin gene. I have made extensive progress toward this goal as can be seen in this grant proposal and am anxious to see the forthcoming results.

28b. Doctoral Dissertation

Cearley, Jamie A.

My dissertation work focused on overcoming limitations in making mouse models of trinucleotide repeat diseases. The trinucleotide repeat disease family is generally characterized by the expansion of a normally present CAG repeat (translated to polyglutamine) to a length of greater than 40 units. There is an inverse correlation between repeat length and onset of disease such that the longer the repeat the earlier the age of onset. Therefore the first question I sought to address was whether or not the mouse would respond to repeat length in the same fashion as humans. Another member of our lab had already conducted studies showing a late onset, progressive, neurological phenotype in mice with 146 CAG repeats located in the Hprt locus of the mouse. I conducted duplicate experiments on mice with 70 CAG repeats in the Hprt locus to see if the phenotype would be delayed as would be expected if the repeat behaved as it does in humans. I found that not only is the phenotype not delayed but it totally non-existent. These results indicated that it was going to take a much longer repeat to give a phenotype in the mouse than is needed in a human. Thus, in making mouse models of trinucleotide repeat diseases researchers are going to need to use repeats of much greater lengths than those seen in humans.

A second limitation that was overcome was the ability to efficiently and repeatedly target the *Hdh* locus in a mouse embryonic stem cell line. This repeated targeting is extremely useful in generating multiple mouse models of the disease. For example, mice with many different repeat lengths can be made. I made a mouse embryonic stem cell line and optimized a protocol that would allow for the efficient repeated targeting of the *Hdh* locus. This cell line and protocol has been used extensively in our lab as well as by others in the Huntington's research field. In addition, the optimized protocol for gene targeting that was published in Transgenic Research will be useful for others to do repeated gene targeting at other loci.

Lastly, due to the embryonic lethality of a classical huntingtin knockout mouse the function of the wildtype huntingtin protein in the adult mouse is not known. We also do not know whether or not the huntingtin protein is necessary for the survival of the adult mouse. To address this problem I have created a gene targeted mouse line (described in this grant) that will allow for the huntingtin gene to be knocked out after development is complete. These are the three main limitations that were overcome at least in part by my dissertation work.

28c. Publications.

Cearley JA and Detloff PJ (2001) Efficient repetitive alteration of the mouse Huntington's disease gene by management of background in the tag and exchange gene targeting strategy Transgenic Research 10(6): 489-499.

Lin C-H, Tallaksen-Greene S, Chein W-M, Cearley JA, Jackson WS et al. (2001) Neurological abnormalities in a knock-in mouse model of Huntington's disease Human Molecular Genetics 10(2): 137-144.

Ordway, J M, Cearley JA, and Detloff PJ (2001) Insights from mice carrying X-linked CAG-polyglutamine repeat mutations. In Glutamine Repeats and Neurodegenerative Diseases: Molecular Aspects. P. S. Harper and M.F. Perutz eds. Oxford University Press.

Ordway JM, Cearley JA and Detloff PJ (1999) CAG-polyglutamine-repeat mutations: independence from gene context The Royal Society 354:1083-1088.

Ordway JM, Tallaksen-Greene S, Gutekunst CA, Bernstein EM, Cearley JA et al. (1997) Ectopically expressed cag repeats cause intranuclear inclusions and a progressive late onset neurological phenotype in the mouse Cell 91:753-763.

Cearley JA and Bolyard MG (1997) Regeneration of solanum tuberosum cv. Katahdin from leaf explants in vitro American Potato Journal 74:125-129.

30b 5. Human Subjects and Vertebrate Animals.

Mice were chosen to conduct these experiments because they are currently the only animals amenable to gene targeting.

Cearley, Jamie A.

30c. Respective contributions.

Primarily a fellow co-worker and myself developed the idea and experimental design of the project proposed. The first draft of this grant was written independent of my sponsor Peter J. Detloff. The draft was then read and critiqued by Dr. Detloff for scientific soundness and grammatical correctness. His criticism focused mainly on the use of the Affymetrix chip to analyze differences in gene expression. He suggested I de-emphasize the use of the chip and increase the emphasis on Real-time PCR as a tool for assessing expression differences. I then included his suggestions and completed the final draft on my own and allowed my co-workers to review it before submission.

30d. Selection of Sponsor and Institution.

The University of Alabama at Birmingham is a growing, thriving research institution. I have been with this university and sponsor for the duration of my dissertation work. Peter Detloff and I have established an excellent working relationship that continues to grow and thrive. The project I have proposed in this grant is one that I have been intricately involved in the origination and experimental design of and have been working on for some time. Having graduated I have the option of moving to a new university and beginning a new work. However, having looked into the various opportunities it does not make economical sense for my family to move from our current location at this time to take another postdoctoral position elsewhere. The financial sacrifice is simply not worth any added benefit that may or may not come to my career by doing such. The project I have proposed is one that will and already has required me to learn entirely new techniques and will for the first time allow me to see a project of my own initiative to completion. These are both extremely valuable points and ones that I believe would be better accomplished in my current position than elsewhere. After completing the proposed project the prospects of moving to further my career will certainly be reconsidered.