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ALS SİNEK MODELİNDE NÖRODEJENERASYON MEKANİZMASININ PROTEOMIC VE GENETİK ANALİZİ

PROTEOMİC AND GENETİC ANALYSIS OF THE MECHANISM OF NEURODEGENERATION IN A FLY MODEL OF ALS

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ÖZET

Amaç : TDP-43; RNA bağlanma motifleri içeren ve pre-mRNA ekleme, transkripsiyon, mRNA stabilitesi ve mRNA transferinde yer aldığı rapor edilen oldukça korunmuş ve her yerde eksprese edilen nükleer proteindir. Son zamanlarda TDP-43 yapılan çalışmalar ile Amyotrofik Lateral Skleroz (ALS) hastalarının etkilenmiş beyin bölgelerinde gözlenen hücre içi inklüzyonların ana protein bileşeni olarak tanımlandı. Bu çalışmada Drosophila melanogaster'de TDP-43'ün analoğunun (TBPH'in) ALS modelindeki sinekler üzerinde proteomik ve genetik mekanizmaları içerisindeki rolünün araştırılması amaçlanmıştır.

Materyal-Metod : WIII8,UAS GFP, GMR,GMR TBPH genotiplerinde bakire dişi sinekler ve erkekler uygun besiyeri ortamında çiftleştirilerek farklı genotip ve fenotiplerde ALS için etki gösterebilen transgenik model sinekler üretildi. Transgenik sineklere TBPH entegre edildi. PCR , Jel Elektroforezi ve 2D kütle spektrofotometri yöntemleri kullanılarak TBPH mutantlarının analizi yapıldı.

Bulgular : TDP-43'ün analoğu olan TBPH'i eksprese eden farklı transgenik sinekler kullanılarak , TBPH'in ekspresyonunun kontrol gruplarına kıyasla nörodejenerasyona neden olup olmadığı kontrol edildi. TPBH'nin ekspresyonunun sineklerin gözlerinde siyah lekelerin (nekrozun) gelişmesine neden olduğu ve gözlerde nörodejenerasyon oluşturduğu görüldü. Dahası, mutasyon geçirmiş sinekler üzerinde 2D jel analizi ile protein agregasyonunda TBPH'in rolünü düşündüren sinek türlerine kıyasla diğer sineklerin gözlerinde azalmış yağ vücut proteinleri 1 (Fbp1) seviyeleri gösterildi.

Sonuçlar : Sonuç olarak TBPH'in Fbp1'nın üretimini artırarak nörodejeneratif sürece karıştığı görüldü.

Anahtar Kelimeler : ALS – Drosophila – TDP-43 –TBPH –Fbp1

ABSTRACT

Objective : *TDP-43 is a highly conserved and ubiquitously expressed nuclear protein containing RNA binding motives and reported to be involved in pre-mRNA splicing, transcription, mRNA stability and mRNA transport. Recently, TDP-43 was identified as the main protein component of the intracellular inclusions observed in affected brain areas of patients suffering from Amyotrophic Lateral Sclerosis (ALS).*

Material and Methods : *In WIII8, UAS GFP, GMR, GMR TBPH genotypes, virgin and male transgenic model flies were produced which were able to act for ALS in different genotypes and phenotypes by mating them in the appropriate medium. TBPH was integrated into the transgenic flies. TBPH mutants were analyzed using PCR, Gel Electrophoresis and 2D mass spectrophotometry methods.*

Results : *We have used different transgenic flies expressing TDP-43 analogue TBPH and checked whether the expression of TBPH is causing the neurodegeneration as compared to the control groups. Expression of TPBH caused development of black spot (necrosis) in the eyes of the flies clearly suggested neurodegeneration in the eye. Moreover, 2D gel analysis on TBPH mutated flies showed reduced fat body proteins 1 (Fbp1) levels in the eyes of flies as compared to wild type, suggesting the role of TBPH in the protein aggregation.*

Conclusion : *Our date showed TBPH is involved in the neurodegenerative process by enhancing the production of Fbp11.*

Key words : *ALS – Drosophila – TDP-43 –TBPH –Fbp1*

INTRODUCTION

1.NEURODEGENERATIVE DISEASES

The term neurodegenerative diseases indicates a group of pathologies characterized by the progressive loss of structure or functions of neurons. The loss of neuronal function can be caused either by the degeneration of a particular neuronal population or by the alterations of the glial cells, cell of support, with particular pathogenic manifestation. (Gama Sosa et al.,2012.) [1]

Even if neurodegenerative diseases have different clinical manifestations, they often share a common feature , the presence of insoluble protein aggregates, localized both at the intra- and extracellular level. The mechanisms by which a protein turns to be toxic causing a disease is still unclear, but mainly two possibilities can occur , either a loss of function or the acquisition of toxic properties.

1.1.AMYOTROPHİC LATERAL SCLEROSİS

Amyotrophic Lateral Sclerosis (ALS) is a rapidly progressive and fatal neurodegenerative disorder, for which there is no available cure, affecting 1 in 200.000 people (Cleveland et al.,2001) [2]. The founder of modern neurology, Jean Martin Charcot, first described and Lateral Sclerosis relates to the hardening of the anterior and lateral corticospinal tracts that occurs during motor neuron degeneration.

ALS is one of the most common neuromuscular diseases worldwide, and people of all races and ethnic backgrounds are affected. It generally strikes people between 40 and 60 years of age, but also younger and older people can develop the disease. Even if initial studies emphasized that ALS affects men more often than women , the additional more recent findings suggest an equal ratio between the genders (Worms,2001) [3].

Both sporadic and familial cases of the disorder have been reported , where sporadic ALS (SALS) amounts to 90-95% of cases and usually occurs in midlife, with a mean age of onset at 55-65 years, while just 5% of sALS cases have a juvenile onset, below the age 30. Familial ALS (FALS) accounts for 5-10% of the total ALS cases and in this inherited form of the disease the onset of symptoms typically appears a decide earlier than in SALS with a shorter survival period. (Veltema et al.,1990) [4]. Additionally, the inheritance in fALS is usually Mendelian, autosomal dominant with a has different Gaussian distribution fort he age of onset compared to sALS.

Motoneuron degeneration in ALS is characterized by the presence of intra-citoplasmic inclusions, Bunina bodies, ubiquitin-positive and TDP-43 positive (Piao et al.,2003)[5], and recently, it is also found that some patients with autosomal-dominant familial ALS (FALS) have point mutations in the gene that encodes Cu/Zn superoxide dismutase (SOD1) (Bowling et al.,1993)[6]. The SOD1 gene encodes the cytoplasmatic isoform of the superoxide dismutase, acting as antioxidant capable to convert oxygen free radicals in hydrogen peroxide, which can be eliminated from the cell protecting them from free radical accumulation , but it could in turn cause DNA and protein damages.Nevertheless the toxicity does not concern the reduction of the antioxidant activity, but it is the protein that gains a toxic function, since the mutated SOD1, missing a correct folding in the endoplasmic reticulum, accumulates in the motoneurons causing their death (Chia et al.,2010)[7]. Moreover the increased stress, induced by the presence of misfolded proteins, causes neurotoxic signaling from motoneurons that leads to a microglia activation. Microglia switches from an anti-inflammatory and neuroprotective state to a pro-inflammatory cytokines, that further enhances motor neuron stress and cell injury and initiates a self-propagating cycle of motor neuron injury and cell death (Appel et al.,2011)[8]. Although these mechanisms play a crucial role in developing neurodegeneration, they all are considered as secondary events in the causes behind ALS onset (Vucic S, Kiernan MC)[9]. Abnormalities in cortical and peripheral excitability in flail arm variant amyotrophic lateral sclerosis. (J Neurol Neurosurg Psychiatry. 2007;78:84952) [10].

As already mentioned, mitochondrial dysfunction plays a crucial role in the motor neuron degeneration and accumulating evidences suggests that

abnormalities in mitochondrial morphology and biochemistry contribute to the development of ALS. Functional defects and altered mitochondrial morphology such as fragmented network, swelling, and augmented cristae were found in soma and proximal axons of skeletal muscle and spinal motor neurons of ALS patients (Chung MJ, Suh YL)[11]. Ultrastructural changes of mitochondria in the skeletal muscle of patients with amyotrophic lateral sclerosis. (Ultrastruct Pathol. 2002;26:37)[12]. In addition, axonal transport of mitochondria along microtubules and mitochondrial dynamics may also be disrupted in the ALS pathology (Shi et al., 2010)[13].

Other genes could represent a cause or a risk factor for the pathology (Armon, 2005)[14], these genes are involved in metabolism processes, such as transcription, pre-mRNA splicing, ribonucleoprotein complex formation, transport , RNA translation and degradation (Strong, 2010) [16], and at the moment the genetic mutations observed in familial ALS involves mutation in: TAR DNA-binding protein-43 (TDP-43), fused in sarcoma/traslocated in liposarcoma (FUS/TLS), angiogenin (ANG), senataxin (SETX), survival of motor neuron (SMN) and elongator protein 3 (ELP3) (Strong, 2010.)[15]

Another pathogenic mechanism, involved in motoneuron degeneration, seems to be induced by glutamate excitotoxicity, since the over-stimullation leads to excessive calcium influx into the motoneuron, resulting in cell-death. Data obtained from ALS patients, animal and cellular models confirm the role of the excitotoxicity; in physiology, excitotoxicity is prevented by rapid binding and clearance of synaptic released glutamate by Na(+)-dependent glutamate transporters and amplified by defects of the glutamate transporter and of its receptor system (Foran and Trotti, 2009)[16]. In agreement with this theory of glutamate excitotoxicity, the antiglutamate agent, riluzole, appears to slow the progression of amyotrophic lateral sclerosis, since it reduces motoneuron damage by decreasing the release of glutamate so improving survival in patients with disease of bulbar onset, but it does not reserve the damage of motoneurons that has already occurred (Bensimon et al., 1994)[17].

1.2.TDP-43

1.2.1.TARDBP gene

TARDBP (TAR DNA Binding Protein) gene codes for a protein of 43kDa, called TDP-43, which was first isolated during a search for novel transcriptional inactivators able to bind the TAR DNA element of the HIV-1 virus (Ou et al.,1995)[18]. It is located at p36.21 of human chromosome 1, the gene is comprised of six exons, exon 1 is non-coding and of unknown function, while exons 2-6 are protein coding (Warraich et al., 2010)[19]. It has been

demonstrated that the transcript of mammalian TDP gene undergoes alternative splicing to generate a variety of different mRNAs, which supports the idea that the eukaryotic TDP gene have a functional complexity.

1.2.2.TDP-43 PROTEINOPATHIES

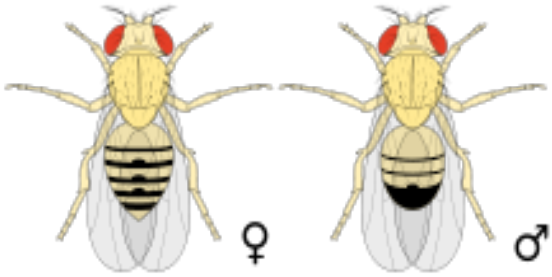
In 2006, Neumann et al. reported that TDP-43 was the main protein component of the ubiquitinated protein aggregates found in most cases of FTLD-U and in ALS. These aggregates appear unique to previously described aggregates, in that they don't form amyloid deposits and don't stain for tau, α-synuclein, ß-amyloid and expanded polyglutamines. Nowadays TDP-43 is considered the hallmark of ALS and FTLD-TDP.

In many other disorders, including AD and Parkinson disease (PD), TDP-43 pathology is an important but secondary histopathological feature of disease.

1.2.3. TDP-43 HISTOPATHOLOGY

TDP-43 histopathology in ALS is characterized by cytoplasmic inclusions of a skein-like or dense granular appearance and by clearance of TDP-43 from the nucleus, highlighted by absence of immunoreactivity (Geser et al., 2009)[20,].

3.*DROSOPHİLA* MODEL INTRODUCTION



Drosophila melanogaster belongs to the taxonomic order Diptera, in the family Drosophilidae. The species is known as the common fruit fly or vinegar fly.

Drosophila melanogaster is a model organism used for genetic research and its first application in a research lab.

3.1.WHY *DROSOPHİLA*?

* *Drosophila melanogaster* is one of the most well understood model organisms.

* *Drosophila* flies measure approximately 3 mm in length.

* *Drosophila* larvae are small, white and glossy with a similar appearance to worms. Within 5-6 days they increase around 1000-fold in weight.

* Adults in the wild are tan with black stripes on the back of the abdomen and vivid red eyes.

* Females live for about one month at room temperature but this can increase to over two months at lower temperatures.

* A female may lay 30-50 eggs per day throughout her lifetime at room temperature. Daily egg production is reduced at lower temperatures.

* *Drosophila* are ideal for the study of genetics and development.

* The complete genome sequence of the *Drosophila* was published in 2000.

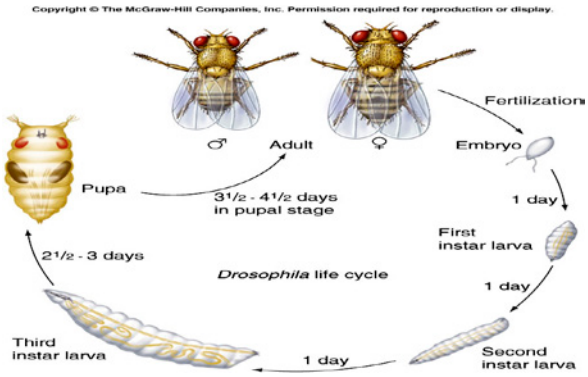
* 75 percent of the genes that cause diseases in humans have an homolog in the fruit fly.

* They are inexpensive to maintain in the laboratory.

Part of the reason people work on it is historical, so much is already known about it that it is easy to handle and well-understood - and part of it is practical: it's a small animal, with a short life cycle of just ten days, and it is an animal model very cheap and easy to keep in large numbers. Mutant flies, with mutations in any of the several thousand genes are available, and the entire genome has recently been sequenced. There ıs an hıgh level of homology between fruit fly and human genes, including many genes ınvolved ın human diseases and 77% of these present an homolog ın the fly genome. Neurological diseases are at the present tıme one of the most common topic of discussion during meeting presentations.

Drosophila model can recapitulate the key neuropathological features observed in affected individuals, both those triggered by gain-of function mechanisms (for example;polyglutamine disease) that can be generated using transgenes expressing the human protein and also those triggered by loss of function (for example; spinal muscular atrophy) generatıng knock out of the fly orthologs. (Therese A.Markow,Patrick M. O'Grady) [23]

3.1.2.Life Cycle



Drosophila life cycle

Image Source : Carolina Biological Supply Company.

Drosophila cultures are grown under laboratory conditions at 25° C in a ventilated oven set to a constant temperature with a fixed 60% level of humidity. Colorless glass or plastic culture tube of different sizes are preferred for the development of the flies since they allow a constant monitoring of the cultures.

Eggs and Embryos

Eggs are oval shaped, about 0.5 mm in diameter and 0.2 mm length. The dorsal side is flat compared to the ventral side. Two filaments located at the anterior dorsal side. It prevents the penetration into the egg soft nutrient medium and provide oxygen uptake. Eggs are coated with a protective chorionic and vitelline membranes. During kopulasyon, sperm is transferred to the female uterus, where can last for days. The eggs are fertilized in the uterus of the female. A single sperm enters the egg during fertilization by micropyle (monosperma a). The female lays her eggs after fertilization, either immediately or in the uterus through the first stages of embryonic development.

Larva

The larvae were hatched after about one day of embryonic development, and can be found on the surface of the medium where yeast powder, added to the fly food, implement the larval development with essential nutrients. The larvae of the first instar (L1), after one day development, evolved in second instar (L2) larvae, and a further day of development allow them to reach the third instar (L3) state. L3 larvae eat for 2 consecutive days during this transition period, and grow very rapidly, up to the moment in which they develop the pupal stage.

Pupa

At the end of the third larval stage, larvae climbing in a suitable place around the bottle to complete their development. Pupa are initially white, turning in a yellow color within two hours. During pupal stage there is no feeding and the metamorphosis occurs. This development at 25 ° C is completed in 10 days.

Adult flies

The newly born flies hatching the pupal cage are light-bodied but they turn dark within few hours. Flaps and other parts of the body is initially soft and air harden them in a few hours with oxygen exposure. The initially crinkled wings open rapidly with and they reach a normal adult individual look. (Therese A.Markow,Patrick M. O'Grady) [23]

MATERİAL AND METHODS

The content of the medium :

104 g of corn flour

94 g glucose

9 g yeast

6 g agar

1020 ml of distilled water

6 ml of propionic acid as preservative.

CULTURING DROSOPHILA STOCKS

Collecting Virgins

Virgin females of *Drosophilia melanogaster* are fondamental to set up crosses.

Females and males can be distinguished easily under a stereoscope becouse of their sexual dimorphism: females are larger than males with a striata abdomen, while males present a darker terminal part in the abdomen due to the exterior genitalia and the presence of the sex combs on the front legs, necessary to engage females during mating.

Flies to be collected are anesthetized with carbon dioxide, and poured on a fly pad where they are kept under a flux of gas to allow the selection of vergins females, the newly born flies that are not receptive to courting for about 8-12 hours after hatching. They can be easily recognized by the very light colour of their cuticule and a greenish spot that can be seen on the abdomen: the meconium. Ensuring that, after the first step of collection of the morning, there are no males in the culture tube, allow the colletion of all the females present within a window of time of 6-7 hours. In order to settle efficient crosses females flies must not be older that 10 days.

2. Collection of Males

Male flies can be harvested at any time.Usually 1-2 days old male flies are collected and used for cross setting.

3. Cross setting

Day 0: 10 females and 3-4 males are added to a

culture tube with *Drosophila* medium.

Day 5-6: Progenitors male and female flies are transferred into on a new tube with medium to start a new culture and a piece of filter paper is generally inserted in the tube with larvae inside to increase the surface for pupae development..

Day 10: New flies start to born after 10 days from the setting of the cross, at this point selection of the progeny occured or collection of virgins in the case of the need to set up new crosses.

DROSOPHİLA STOCKS GENOTYPE

1.WIII8 : Wild type fly, White eyes, straight wings

2.UAS GFPmCD8/CyO :UAS type fly, expresses GFP in combination with a driver fly

a) Homozygous condition: UAS GFPmCD8/UAS GFPmCD8 ; Red eyes,straight wings

b) Heterozygous condition: UAS GFPmCD8/CyO : Red eyes,curly wings

3.GMR Gal4/CyO : Driver fly,Red eyes,

a) Homozygous condition: GMR/GMR : Red eyes,straight wings

b) Heterozygous condition: GMR/CyO : Red eyes,curly wings

4.GMR Gal4,UAS TBPH line#1/CyO-GFP-gal80 : Recombinant fly, with curly wings, expresses TBPH, in the eyes when CyO-GFP-gal80 is removed

5.UAS TBPH/CyO : Red eyes, curly wings, expresses TBPH in combination with a driver fly

6.UASFbp1/UASFbp1 : Homozygous condition, Red eyes, long hair (Thoracic part hair) (like wild type), expresses Fbp1 in combination with a driver fly

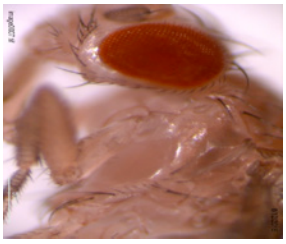
7.UASFbp1/Tm3Sb : Heterozygous condition, orange eyes, short hair (Thoracic part hair)

RESULTS and DİSCUSSİON

First, I analyzed the phenotypes derived from the crossing of the following *Drosophila* strains:

1.GMR/CyO × +/+;si RNA Fbp1/siRNAFbp1

With this control cross I verified if the direct silencing of the Fbp1 protein, in the eye of the fly, can cause an alteration of the eye phenotype .

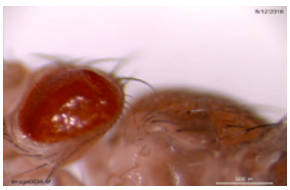
2.GMR,TBPH/CyOgal80 × W1118

This cross I used to demonstrate the neurodegeneration induced by TBPH when expressed alone in the eye.


3.GMR,TBPH/CyOgal80 × +/+;UAS Fbp1/Tm3Sb

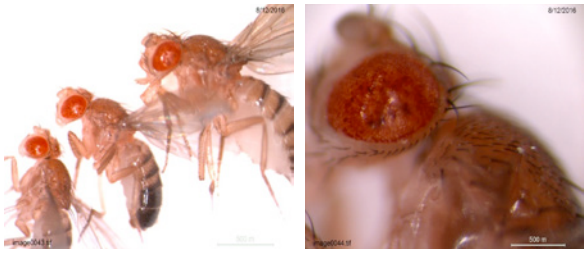
In this experiment we analysed whether the overexpression of Fbp1 was able to modify the neurodegeneration induced by TBPH in GMR,TBPH flies.

With this cross I managed to have the co-expression in the eye of the fly of the TBPH protein and of the Fbp1 protein. Thanks to Fbp1 expression I saw a mild recovery of the eye neurodegeneration induced by TBPH, none of the flies that I collected presented a black spots of neurodegeneration. The below pictures show the eye phenotype.

4.GMR,TBPH/CyOgal80 × UAS si RNAFbp1/ UAS si RNAFbp1

With this cross I managed to have the expression in the eye of the fly of the TBPH protein and also the siRNAagainst the Fbp1 mRNA that allow the reduction of Fbp1 protein level. The below pictures show the eye phenotype.



5.GMR/CyO × UASGFP/UASGFP

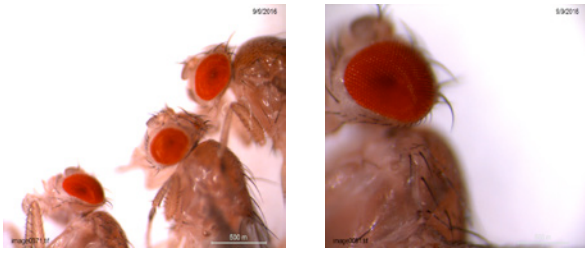
With this cross I had the negative control, the expression of an unrelated protein under the control of UAS, UAS GFP, in the eye of the fly. The below pictures show the eye phenotype.

6.GMR,TBPH/CyOgal80 × UASGFP/UASGFP

This cross I used as control of the coexpression of TBPH and an unrelated protein under UAS control. When GFP is co-expressed with TBPH the neurodegenerative damage in the eye of the progeny flies is still present and the phenotype is worst compare to the co-expression of UAS Fbp1. The below pictures are the experimental evidences.

7.GMR/CyO × +/+;UAS Fbp1/UAS Fbp1

This cross I used for direct check of the effect of Fbp1 expression in the eye .GMR is the driver fly and UAS Fbp1 is the transgenic one. With this cross it is possible to appreciate that the Fbp1 expression didn't cause any neurodegenerative phenotype in the eyes of the progeny.

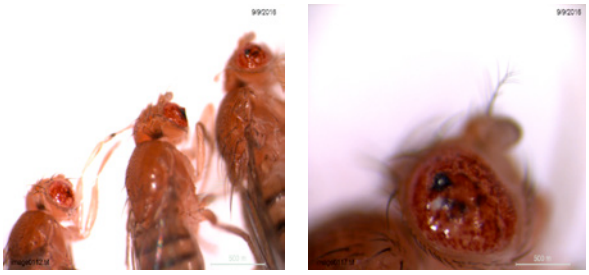


8.GMR,TBPH/CyOgal80 × UAS Fbp1/UAS Fbp1

Below the pictures of the eyes phenotypes.

9. UASGFP/UASGFP × GMR,TBPH/CyOgal80

This cross I used as control of the coexpression of TBPH and an unrelated protein under UAS control. When GFP is co-expressed with TBPH the neurodegenerative damage in the eye of the progeny flies is still present and the phenotype is worst compare to the co-expression of UAS Fbp1. The below pictures are the experimental evidences.



Also in this second set of crosses I confirm a mild recovery of the neurodegenerative phenotype of the eye when TBPH is co-expressed with the Fbp1 protein but not with an unrelated GFP protein.

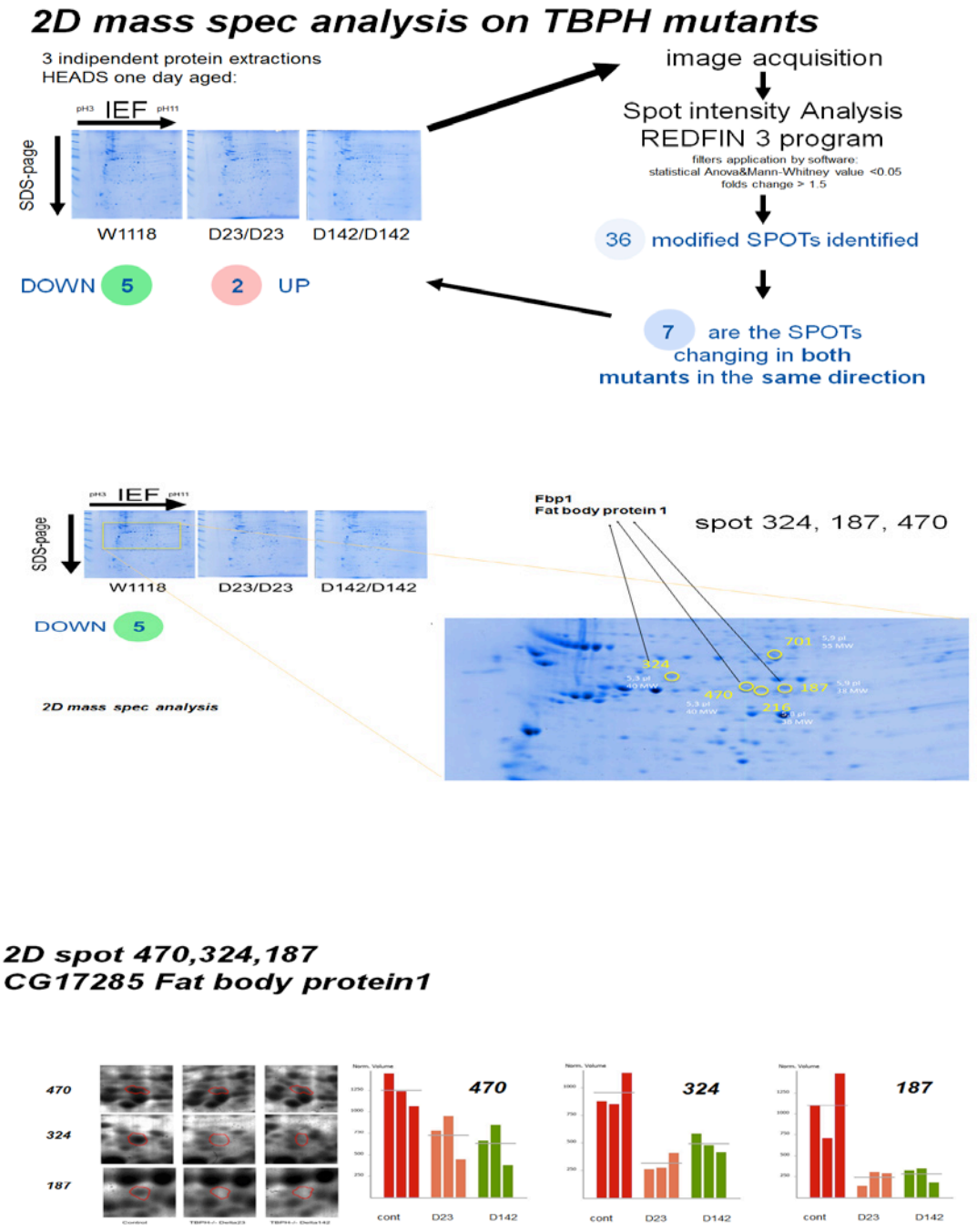


Fig . 2-D mass spec analysis of TPBH mutants. Mutations in the TBPH protien reduced the level of expression of FBP1 protein in both the mutants.

In conclusion, finally, we observed that in our recombinant fly model of neurodegeneration, the overexpression of TBPH through the UAS Gal4 system induced a strong damaged Drosophila eye due to tissue degeneration.with the appearance of spot of necrosis. This phenotype become suppressed with the co-overexpression of the Fbp1 gene.

My results indicate that the Fbp1gene interacts with the neurodegenerative mechanisms induced by TBPH and suggest that the Fbp1 protein may have an important role in the degenerative process observed during the disease.

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