# Labmeeting

06 / 16 / 2009

• Functional characterization of ataxin-2

What is the effect of an expanded polyQ repeat on the normal atxn2-fct.

What is the function of the wildtype protein >> pathways or network



 A2BP1 is recruited by ataxin-2 Recruitment is dosage dependent Deficiency of nuclear A2BP1 might lead to missplicing No differences between wt and expanded polyQ-forms



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 Artificial minigene construct with A2BP1 recognition site shows missplicing Missplicing is dosage dependent

Alteration of A2BP1 splicing by increasing Atxn2 levels



# **Endogenous Splicing**

### The search for the right gene(s)

- 1. Gene shows different splice forms in untransfected HEK293 and SH-SY5Y cells
- Expression in both cell lines is high enough for a good amplification out of cDNA
- 3. Exogenous expression of A2BP1 leads to an increase of A2BP1 spliced transcripts
- Addition of exogenous ataxin-2 leads to the absence of 'neuronal' transcripts in SH-SY5Y cells

### Sources:

- 1. Conboy paper with published mouse target primers (more specific primers needed)
- Target list from Zhang et al.
  Only computer predicition of A2BP1 spliced exons for most genes

# **Endogenous Splicing**

### Conboy genes:

HEK293

HIBIO

SHESTER

### Bin1: bridging integrator 1

Exon inclusion

### NF1: neurofibromin 1

stistst

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Exon inclusion(s)

# **Endogenous Splicing**

### Zhang et al. paper:

### Defining the regulatory network of the tissue-specific splicing factors Fox-1 and Fox-2

Chaolin Zhang,<sup>1,2,5</sup> Zuo Zhang,<sup>1,5</sup> John Castle,<sup>3</sup> Shuying Sun,<sup>1,4</sup> Jason Johnson,<sup>3</sup> Adrian R. Krainer,<sup>1,6</sup> and Michael Q. Zhang<sup>1,7</sup>

### SV2A: synaptic vesicle glycoprotein 2A



### Pumilo1 and ADAM15



# Interaction with A2BP1

- First identified interaction partner: A2BP1 (fox-1)
- Nuclear as well as cytoplasmic localization
- RNA binding motifs
- tissue-specific splicing
- mRNA splicing triggered by a specific recognition sequence: UGCAUGU
- Disease releated links:
  - A2BP1 gene maps to an locus for autism
  - Chromosome 16 translocation in two cases of epilepsy and mental retardation disrupt A2BP1 gene

# A2BP1 protein domains



RRM: Eucaryotic RNA Recognition Motif

# Interaction with A2BP1

- Another protein RBM9 has the identical recognition sequence as A2BP1
- Nuclear as well as cytoplasmic localization (predicted)
- RNA binding motifs
- two isoforms in human
- mRNA splicing triggered by a specific recognition sequence: UGCAUGU

RBM9	(fox-2)	

Ala-rich region

**RRM** 

expression pattern like a house-keeping gene

Does RBM9 rescue for A2BP1 malfunction ?

## Geneclip hairpin RNA vector to suppress RBM9



**Conclusions of A2BP1 Recruitment by Atxn2** 

Elevated levels of ataxin-2 alter the nuclear function of A2BP1 in a dosage dependent manner

Which reasons can cause elevated ataxin-2 levels in patients with expanded polyQ repeats?

## **Atxn2 Mediated Splicing Alterations of A2BP1**

### Which reason could lead to higher ataxin-2 levels in the PK cells of SCA2 patients

- Expanded polyQ-forms are more stable than wt form and degrade slower
  - → accumulation of ataxin-2 over time
- Expanded polyQ-forms decrease proteasomal degradation

→ all cellular proteins have longer half-life but control of expression might regulate toxic effects

Expanded polyQ lead to formation of protofibrils

→ Increase in misfolded proteins in the cell over time as protofibrils are hard to degrade in the UPS

protein accumulation >> increase in cellular stress like in PD
 has almost nothing to do with splicing differences

## Other ideas

### Inhibition of proteasome by expanded polyQ forms:

• Proteasomal degradation assay points out if proteasomal function is decreased due to inhibition of ataxin-2 polyQ forms

Increase of ubiquitinated proteins in the cell

→ Stable expressing ataxin-2 cells are needed

Formation of protofibrils by expanded polyQ's:

• Fluorescence correlation spectroscopy between wt and expanded polyQ repeats in stable expressing cell lines

→ Has to be done in cooperation with an experienced lab

## Ataxin-2 degradation: Kikume vector

# Degradation of different polyQ forms Photoconversion of Kikume-green to Kikume-red



## Ataxin-2 degradation: Kikume vector

### Kikume Experiment over 60 hours



### **Problems:**

- Movement of cells
- cell divisions
- cell death

 Whole field of view measurement is necessary and reduces accuracy
 → make it *hot*

## Ataxin-2 proteasomal inhibition

#### **Proteasome-GLO-Assay:**



Media without cells

Untreated, untransfected cells

Epoxomicin (10 uM), untransfected cells

Untransfected cells with treatment

Samples



Figure 1. Luminogenic substrates containing either Suc-LLVY, Z-LRR or Z-nLPnLD peptide sequences are recognized by the proteasome. Following cleavage of the substrate by the proteasome, aminoluciferin is released and serves as the substrate for firefly luciferase. Light is produced as a result of the luciferase reaction.

### Ataxin-2 proteasomal inhibition

### 48 hours transient expression



## Ataxin-2 proteasomal inhibition



Kikume expression seems higher than ataxin-2 expression levels >> higher degradation rate?

### Ataxin-2 expression vs protein level



### Ataxin-2 compared to neomycin resistance gene

Ataxin-2 expressing cells

Ataxin-2 expression compared to protein level

Protein extraction –

Ataxin-2 compared to neomycin resistance gene

















Kikume-green-atxn2-Q58

Kikume-green-atxn2-Q108

## **Generation of stable FLAG-atxn2 expressing cells**

### Control: untransfected





### Atxn2-Q22



2 ug/ml G418 48 h post transfection until control 1 shows no living cells

Switch to 1 ug/ ml to maintain line

## Stress response atxn2-overexpressing cells (transient transf.)

HEK293 cells after 0.5 mM sodium arsenite treatment

30 min

pEGFP-ataxin-2-Q22







# Stress response atxn2-overexpressing cells (transient transf.)

HEK293 cells after 0.5 mM sodium arsenite treatment

pEGFP-ataxin-2-Q58











# Stress response atxn2-overexpressing cells (transient transf.)

HEK293 cells after 0.5 mM sodium arsenite treatment

pEGFP-ataxin-2-Q108





## Endogenous Expression Levels of A2BP1, Atxn2 and RBM9

### CT values in different cell lines

	RBM9	A2BP1	Atxn2	
HEK293	23.27	0	27.37	
H1299	23.17	0	26.62	
A2058	22.78	0	26.7	
HTB10	21.16	35.3	25.67	
MG-63	23.51	0	28.11	
SH-SY5Y	23.30	31.42	27.63	
IMR-90	22.2	0	27.53	
MB-231	21.83	0	25.25	
HEK- <b>A2BP1</b>	23.85	18.79	27.84	
HEK-Atxn2	23.64	0	20.73	

→ RBM 9 and Ataxin-2 are expressed in each cell line > housekeeping genes?

- → A2BP1 is only expressed in neuronal cell lines at a very low level
- → A2BP1 has a much higher exogenous expression level than Atxn2 Atxn2 is less potent for transfection than A2BP1