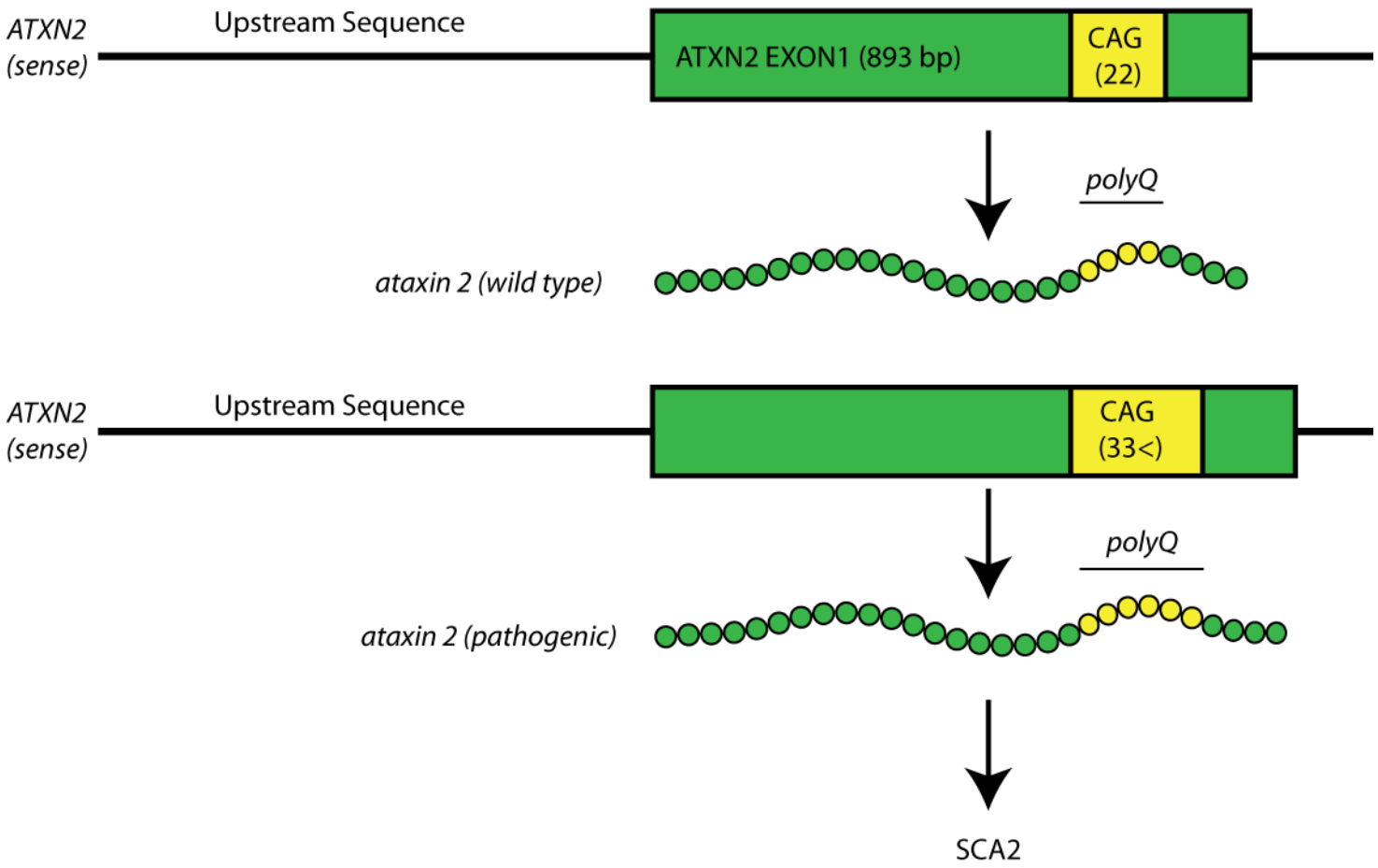
Lab Meeting November 2013

Brandon Henrie

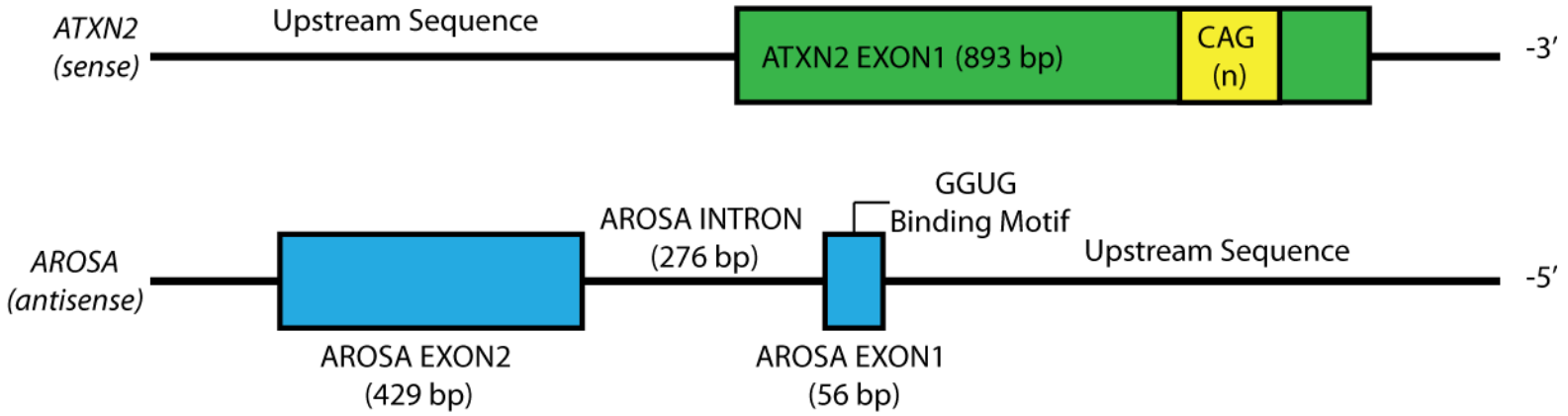
ATXN2-promoter associated antisense long non-coding RNA AROSA regulates ATXN2 expression.

Project 1

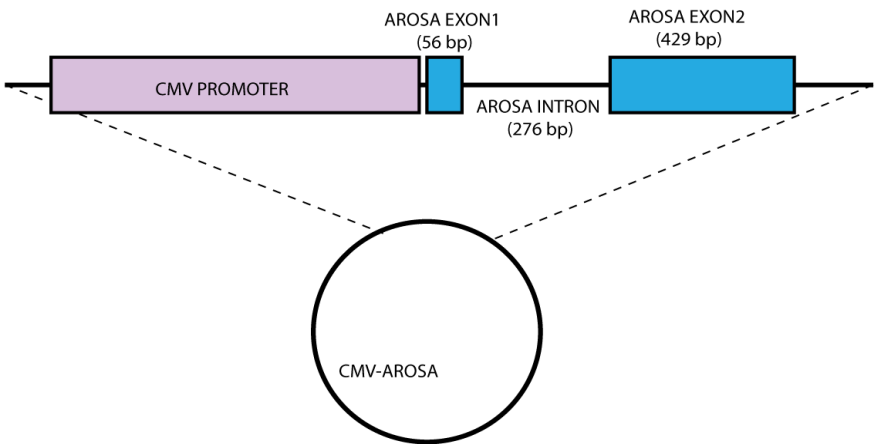
Repeat expansion in ATXN2 causes SCA2

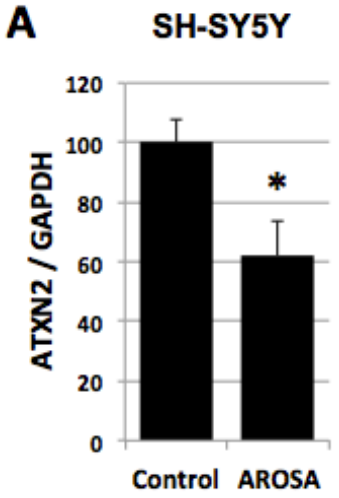


The ATXN2-promoter associated antisense long non-coding RNA AROSA regulates ATXN2 expression



Expression of AROSA lowered ATXN2 expression





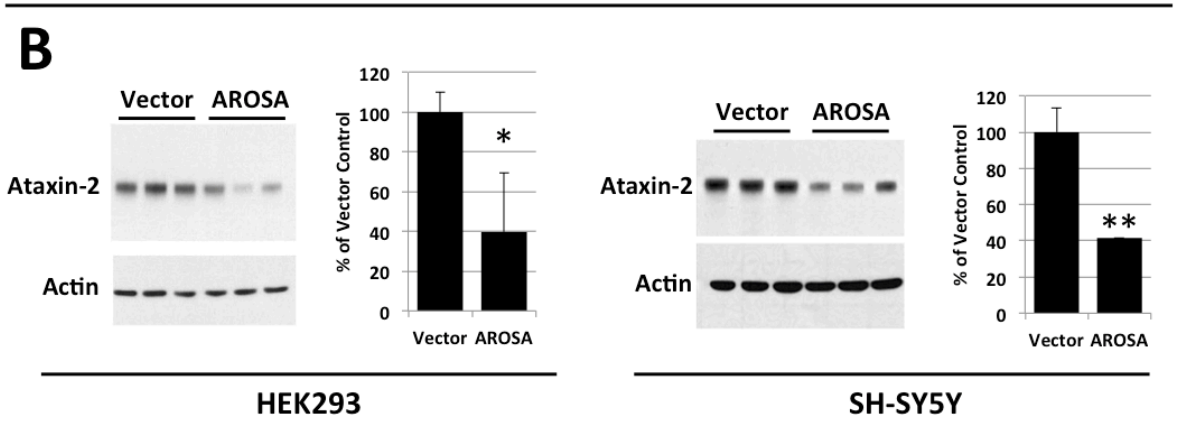


Figure 2

Daniel R. Scoles†, Lance T. Pflieger, Khanh K. Thai, Warunee Dansithong, Sharan Paul, Stefan M. Pulst.

Hypothetical models for the mechanism of AROSA function

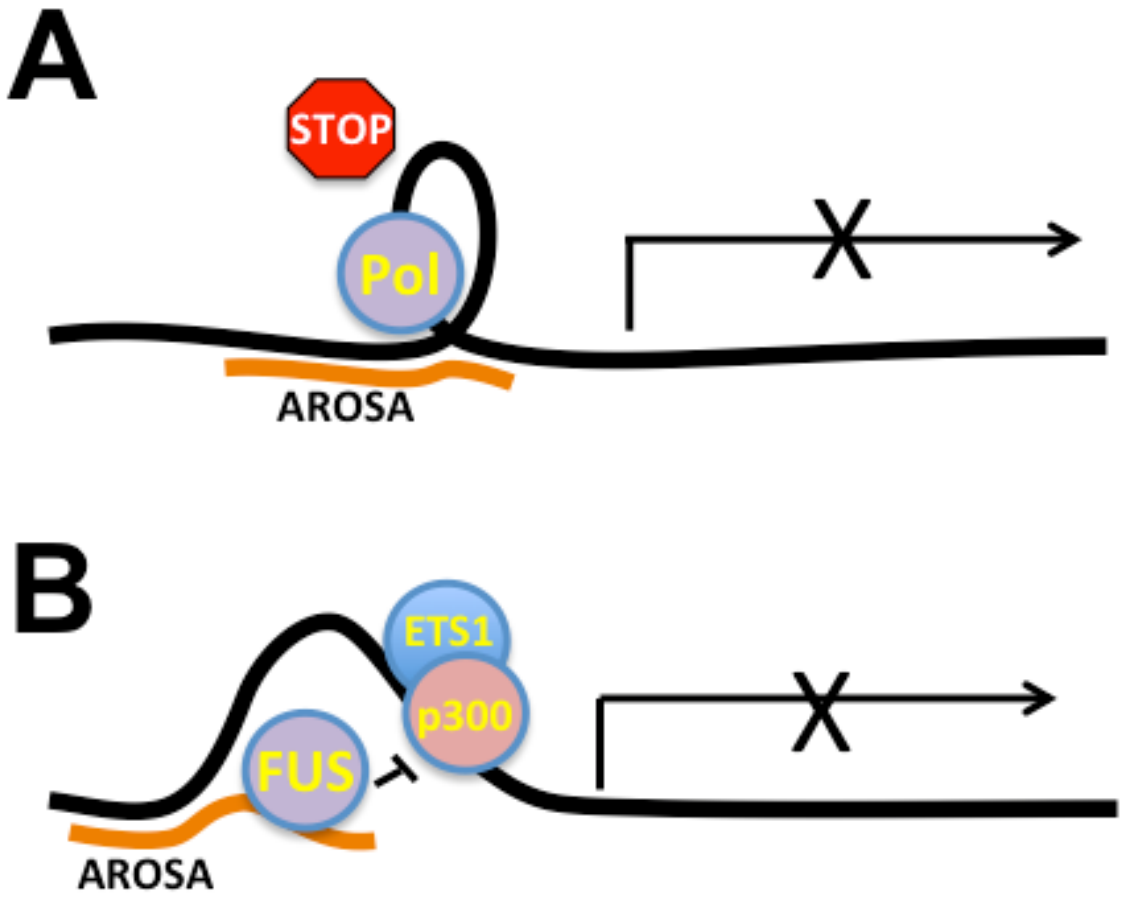


Figure 5

Daniel R. Scoles†, Lance T. Pflieger, Khanh K. Thai, Warunee Dansithong, Sharan Paul, Stefan M. Pulst.

Hypothetical models for the mechanism of AROSA function

Three testable predictions of our hypothesis:

1-FUS will bind AROSA.

2-Mutation of the GGUG motif in AROSA will abrogate AROSA-mediated regulation of ATXN2 expression.

3-Knockdown of FUS will abrogate AROSA-mediated regulation of ATXN2 expression.

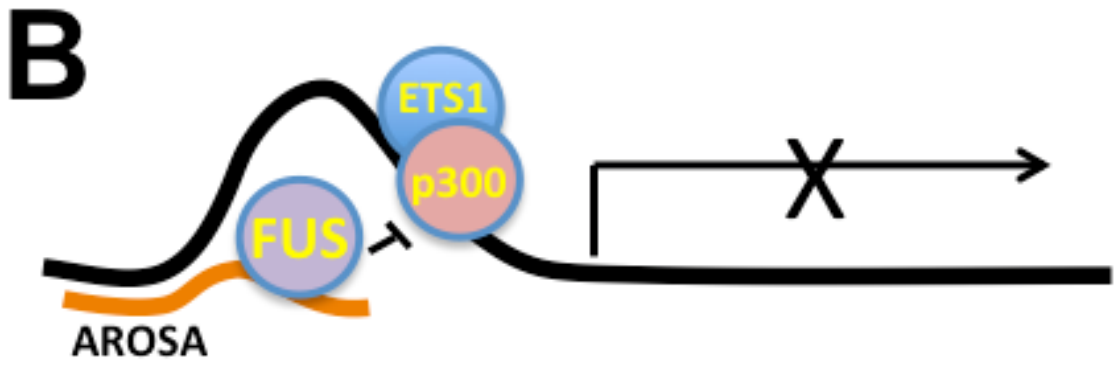
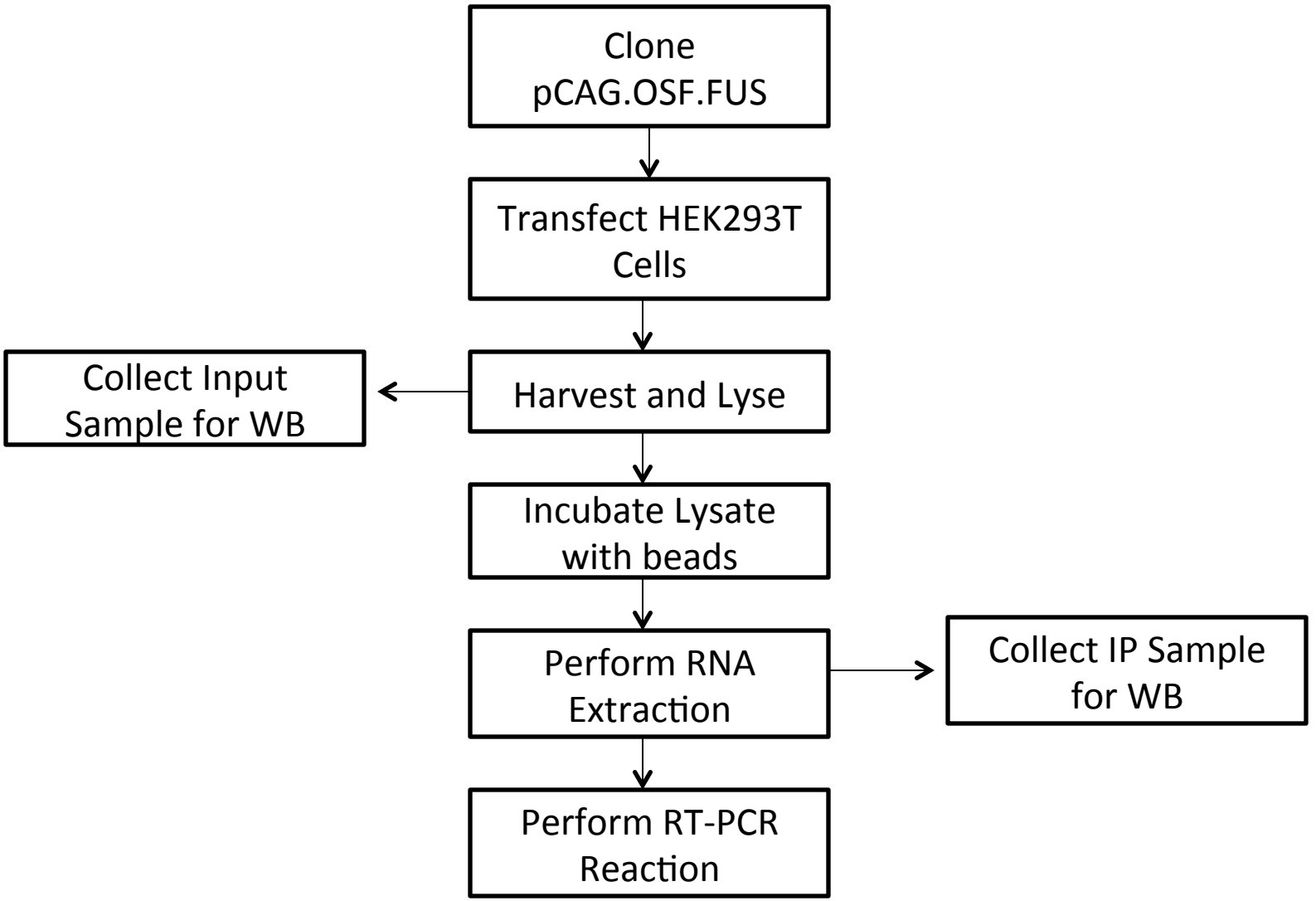


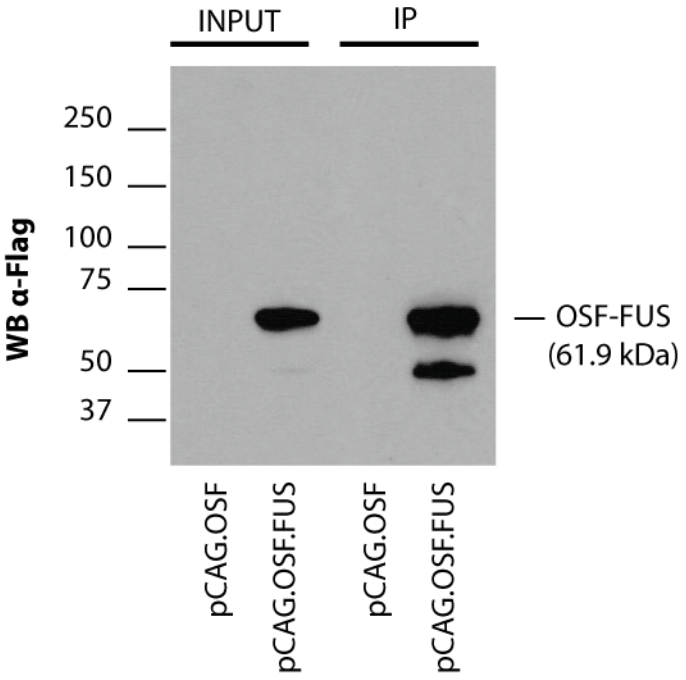
Figure 5

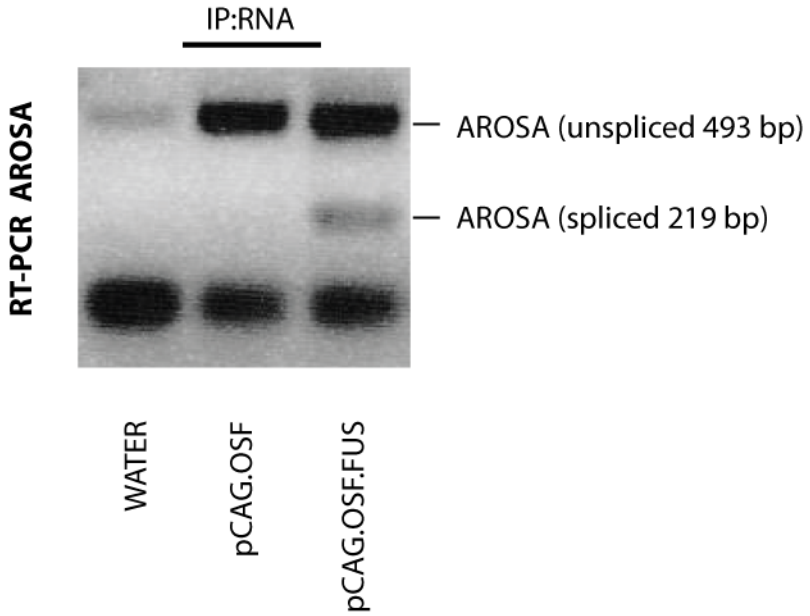
Daniel R. Scoles†, Lance T. Pflieger, Khanh K. Thai, Warunee Dansithong, Sharan Paul, Stefan M. Pulst.

Prediction 1: FUS will bind AROSA Strategy

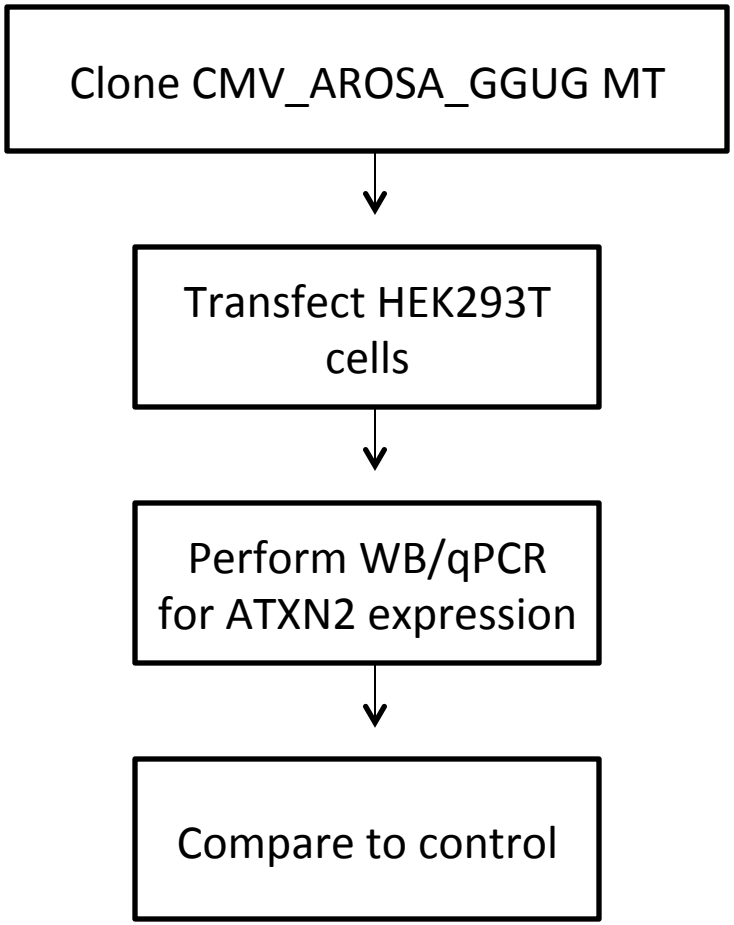


Prediction 1: FUS will bind AROSA Results

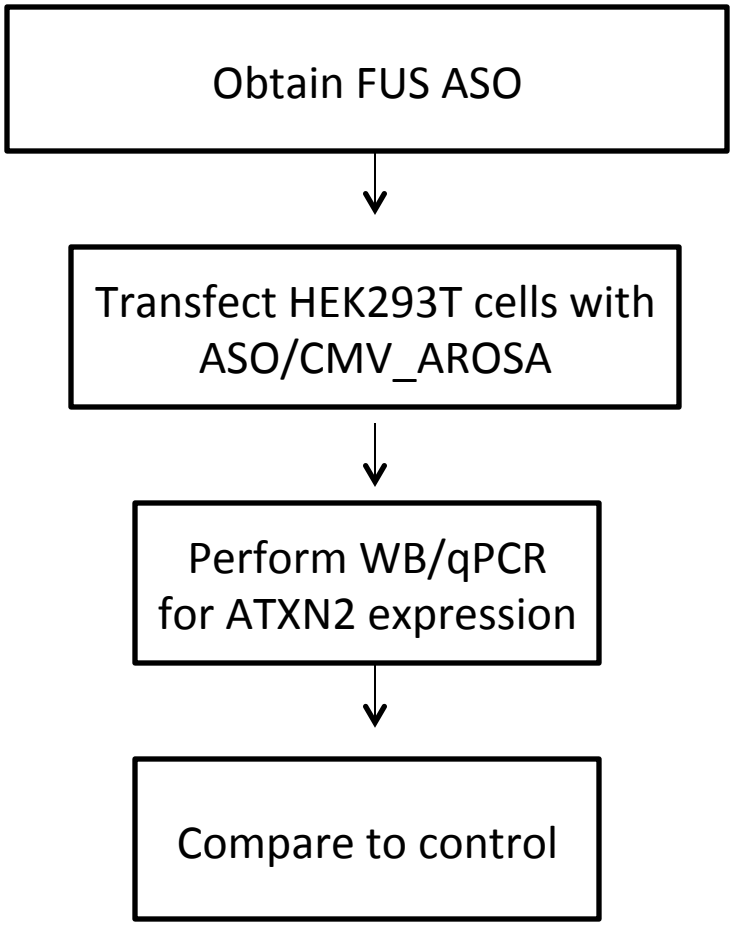




Prediction 2: Mutation of the GGUG motif in AROSA will abrogate AROSA-mediated regulation of ATXN2 expression Strategy



Prediction 3: Knockdown of FUS will abrogate AROSA-mediated regulation of ATXN2 expression Strategy



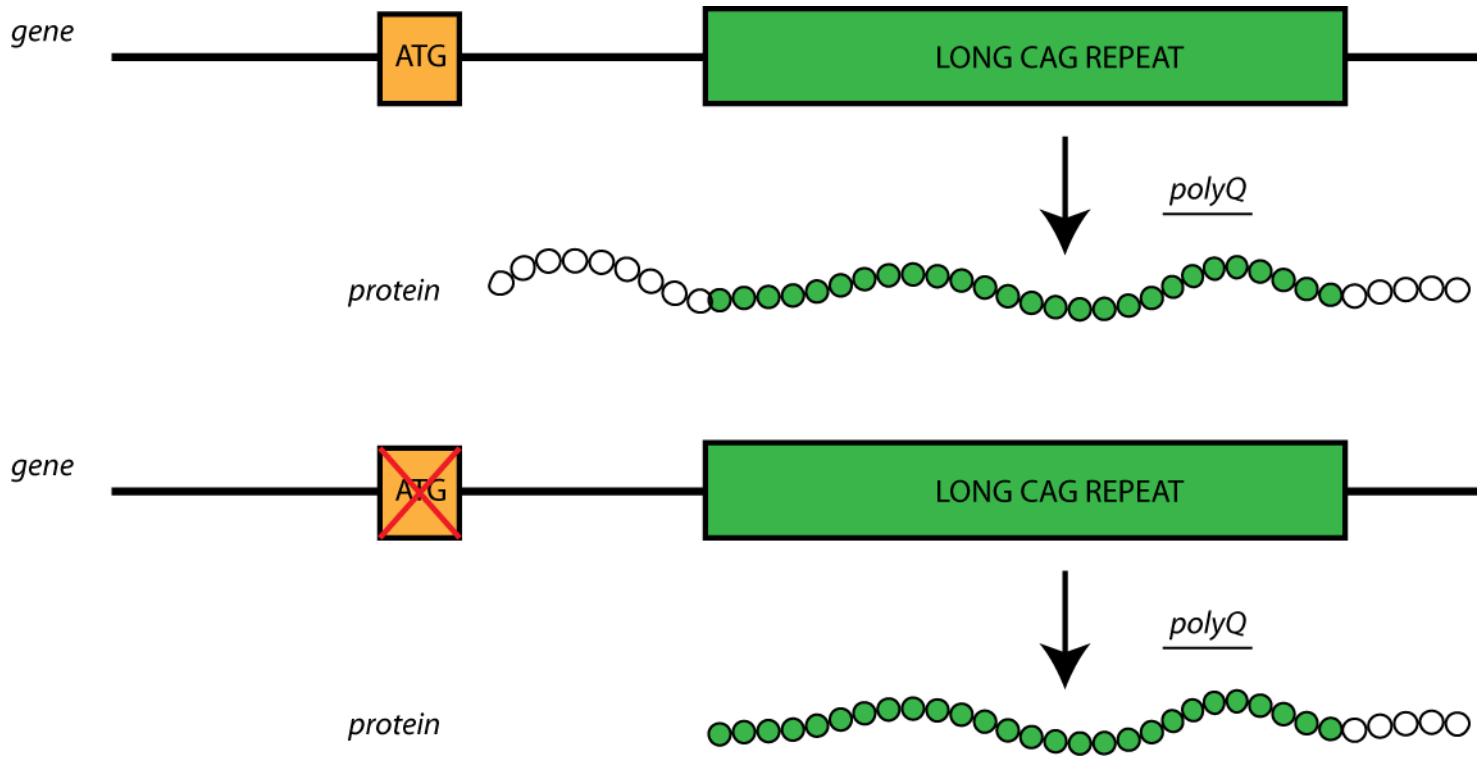
Project 1 Summary

- **Spliced AROSA appears to bind FUS.**
  - Attempt to remove unspliced AROSA from RT PCR.
    - Optimize PCR with RT-PCR primers spanning EXON1:EXON2 gap.
    - Elute FUS from beads to reduce non-specific binding.
- **Cloned CMV_AROSA wild type and CMV_AROSA (GGUG) Mutant.**
  - Determine if the AROSA GGUG mutant attenuates ATXN2 expression.
- **Requested FUS ASO.**
  - Determine if ASO-mediated knockdown of FUS abrogates AROSA regulation of ATXN2 expression.

Expanded CAG repeat in the spinocerebellar ataxia type 2 (SCA2) gene *ATXN2* does not induce repeat associated non-AUG translation (RAN translation) in cell models

Project 2

Non-AUG-Initiated Translation (RAN Translation)



Zu et al. 2010

Diagram of plasmid constructs

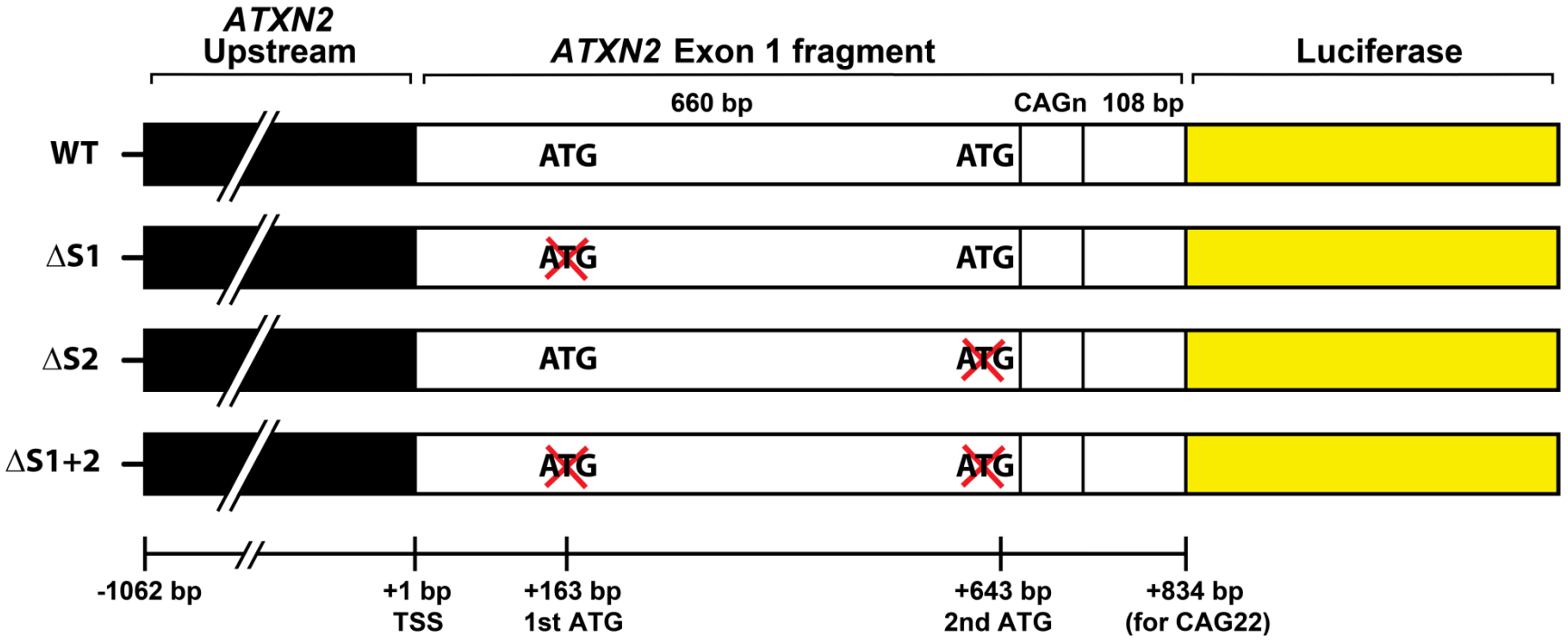


Figure 1

Lance W. Petersen, Khanh K. Thai, Lance T. Pflieger, Stefan M. Pulst, Daniel R. Scoles.

Luciferase assays showed that the expression of *ATXN2-luc* depends upon the second start codon

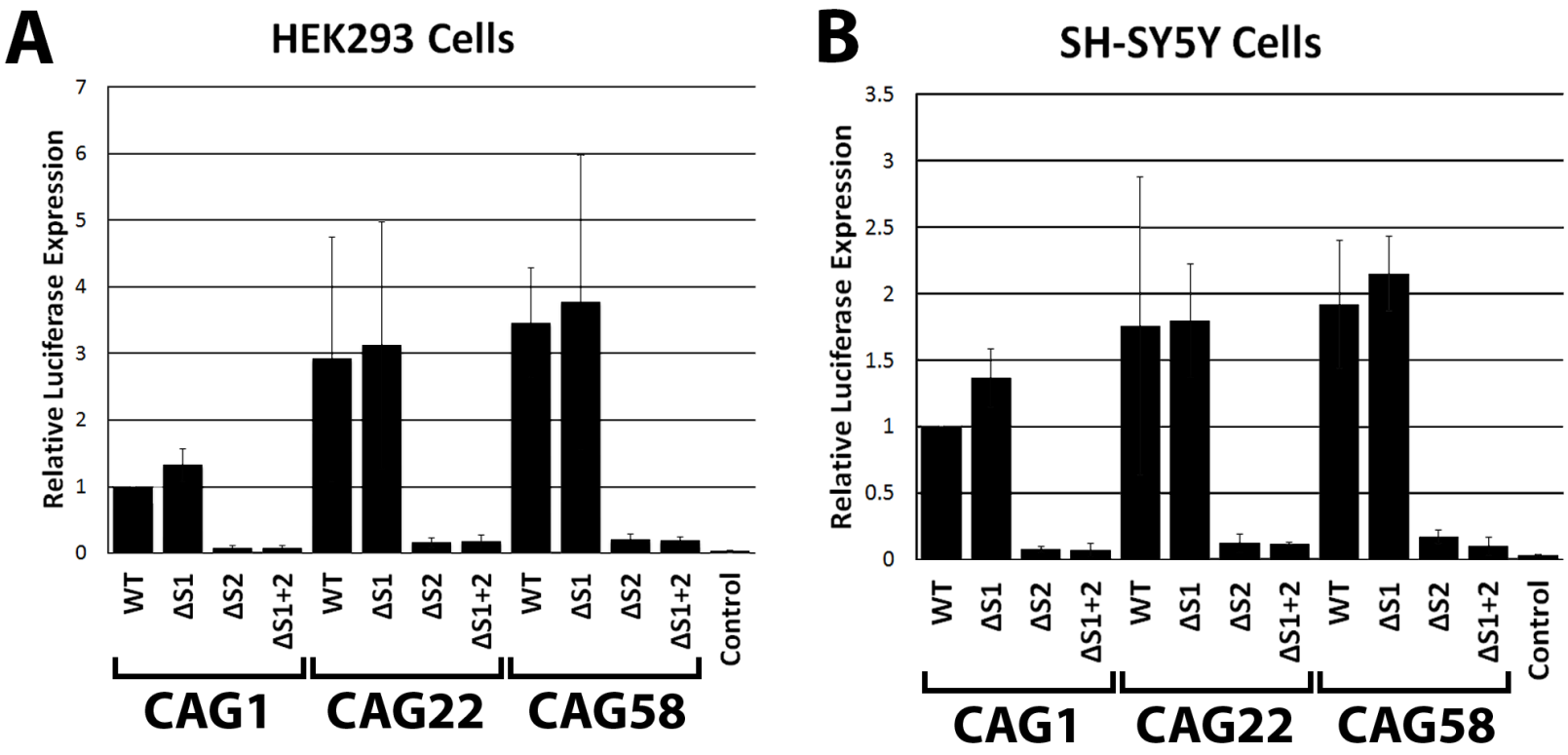


Figure 2

Lance W. Petersen, Khanh K. Thai, Lance T. Pflieger,
Stefan M. Pulst, Daniel R. Scoles.

Western blots showed no evidence of *ATXN2-luc* expression when the second start codon was deleted

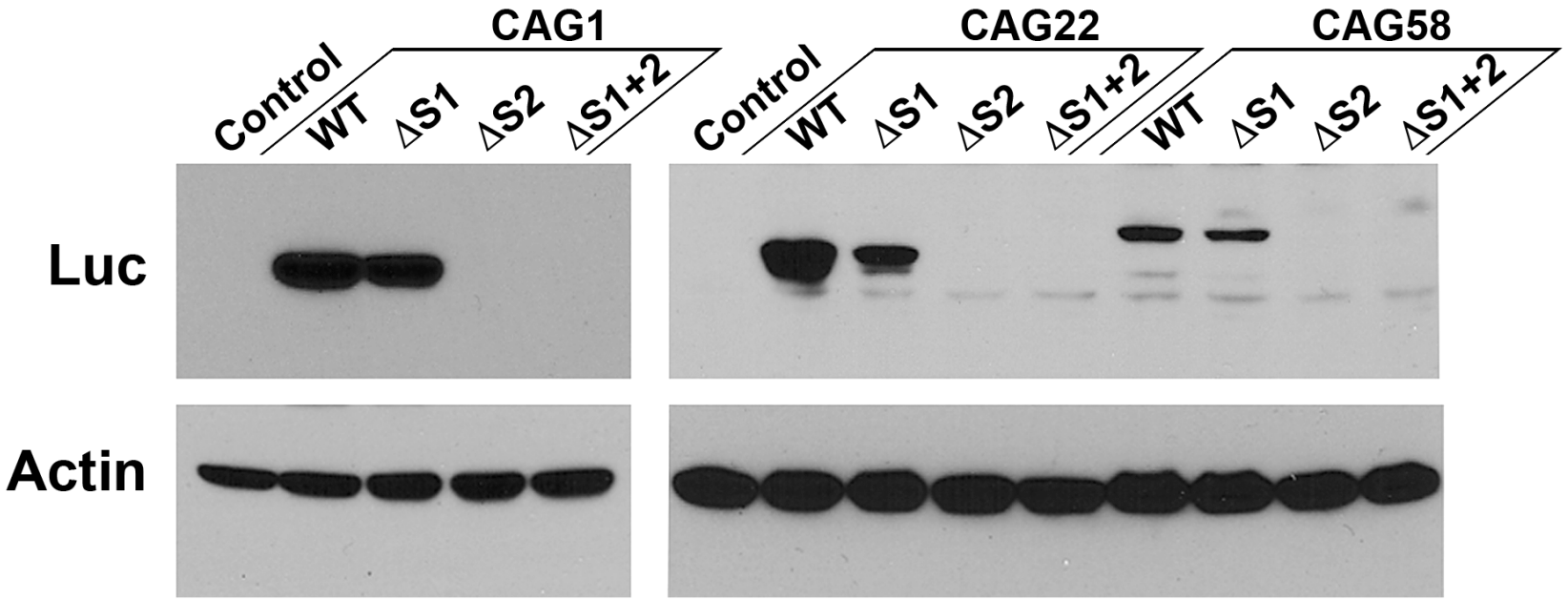


Figure 3

Lance W. Petersen, Khanh K. Thai, Lance T. Pflieger, Stefan M. Pulst, Daniel R. Scoles.

ATXN2-luc plasmids with luciferase placed in the polyalanine or polyserine frames generated no luciferase activity

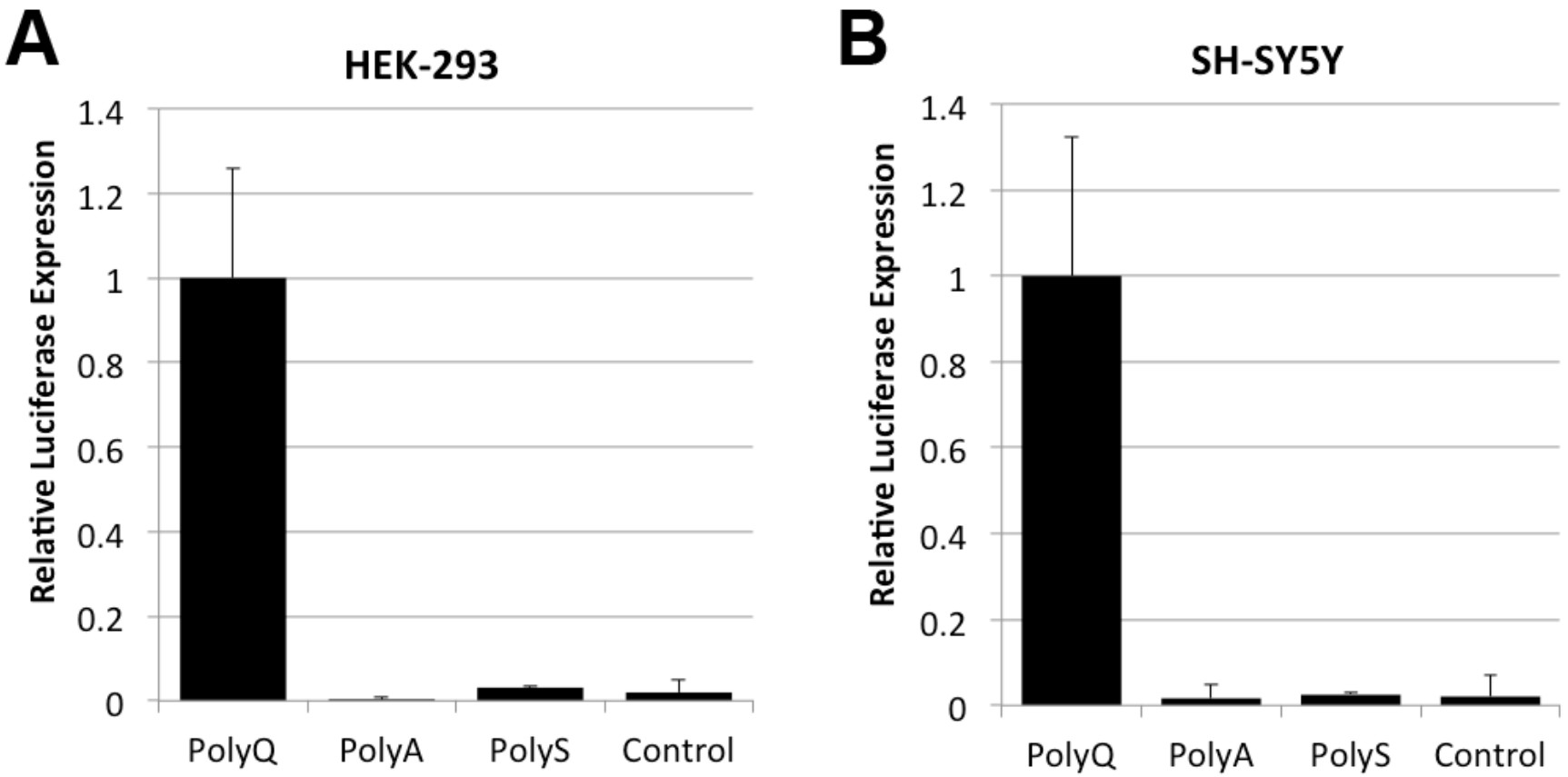


Figure 4

Lance W. Petersen, Khanh K. Thai, Lance T. Pflieger, Stefan M. Pulst, Daniel R. Scoles.

PLoS One Decision Letter

Reviewer 1

- Manuscript lacks positive control.
- Data are over-interpreted.
- Used the wrong cell type (HEK293 instead of HEK293T) and justify use of SH-SY5Y because RAN was not ever observed in HEK293 or SH-SY5Y
- How do we know lack of detecting RAN translation is due to ATXN2 flanking sequence and not the luciferase tag?
- Size markers needed on the western blots.
- Figure 3 should show the full blot because RAN proteins are smaller.
- Reprobe luciferase blots with 1C2 antibody.
- All luciferase assay data should be paired with independent westerns with luciferase and 1C2 antibody.
- Negative *in vitro* data with even with proper controls is still insufficient to claim there exists no RAN translation.

Reviewer 2

- This reviewer agrees previous studies with only 20 bp flank are "simple" vs. our study with native promoter.
- Include additional controls.
- Include more repeat lengths.
- Suggests testing that ATG→CTG does not block transcription by RT-PCR.

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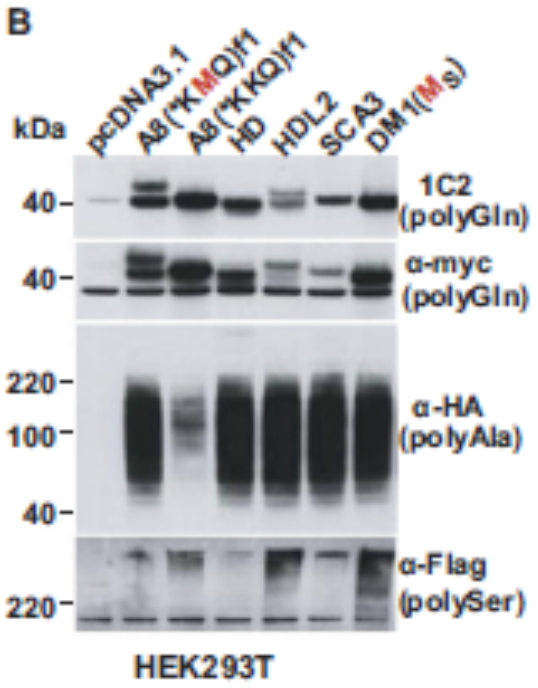
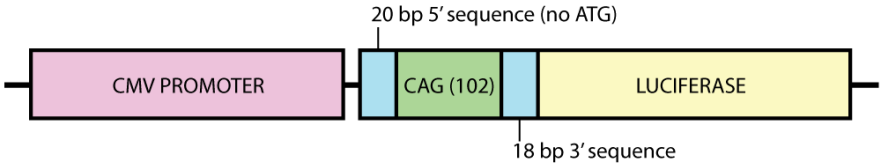
Creating a Control for RAN Translation



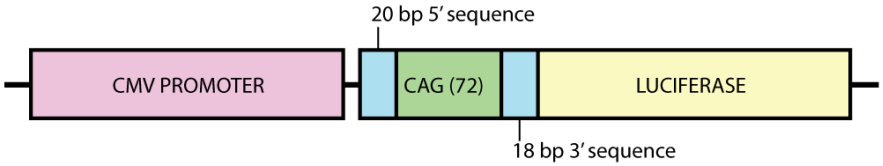
Zu et al. 2010

Clones for RAN Translation Control

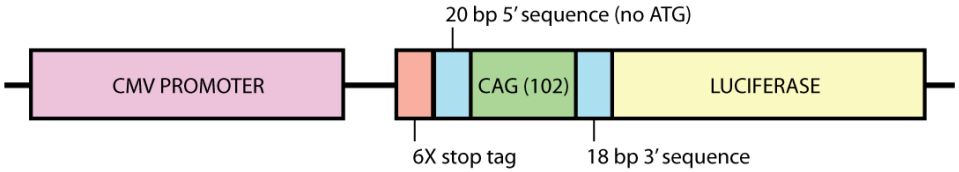
CMV_ATXN2_LUC



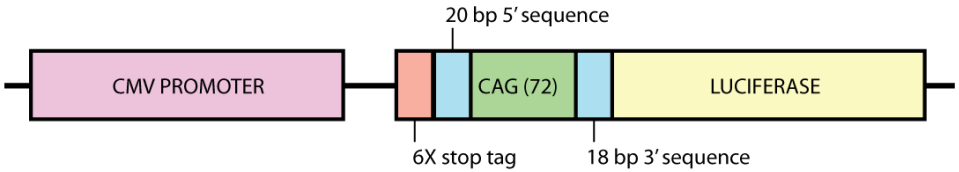
CMV_SCA3_LUC



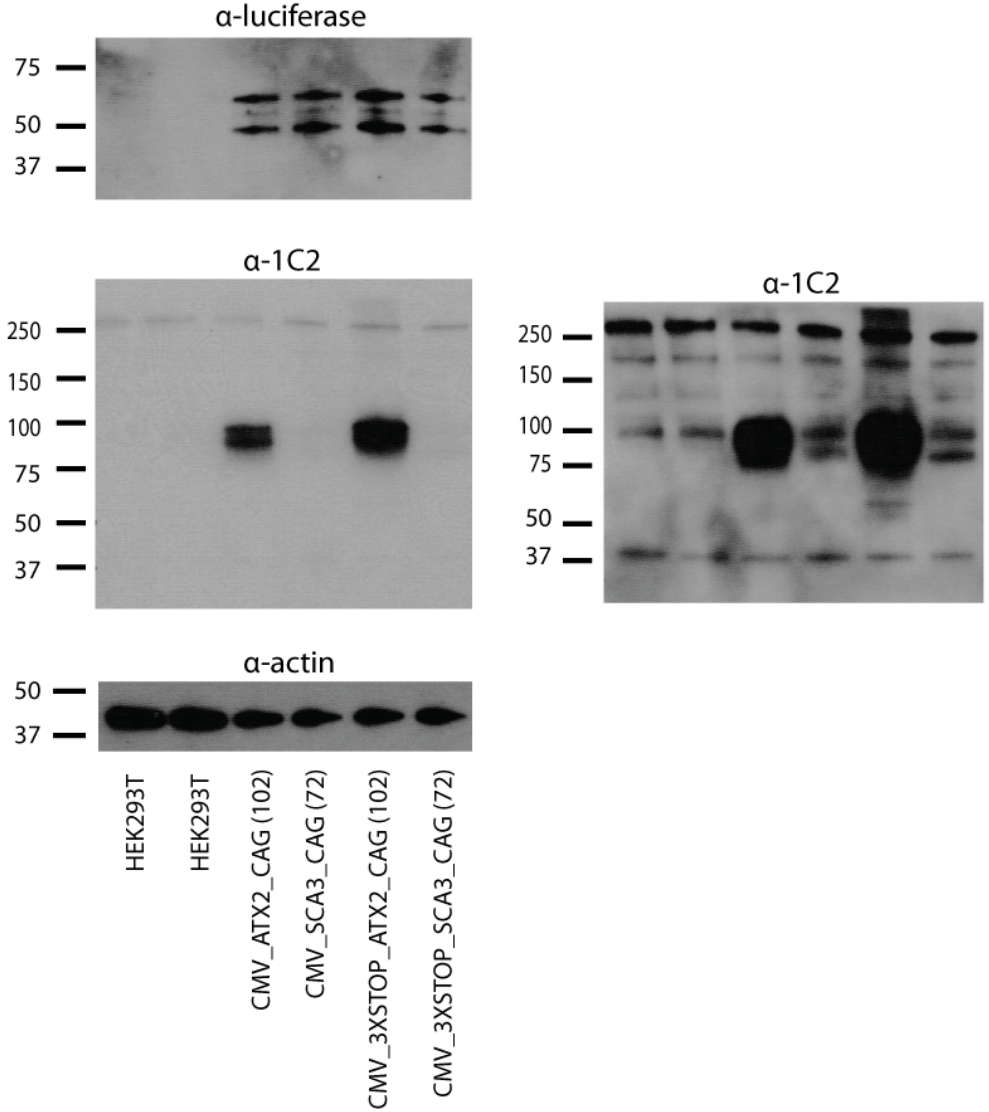
CMV_6XSTOP_ATXN2_LUC



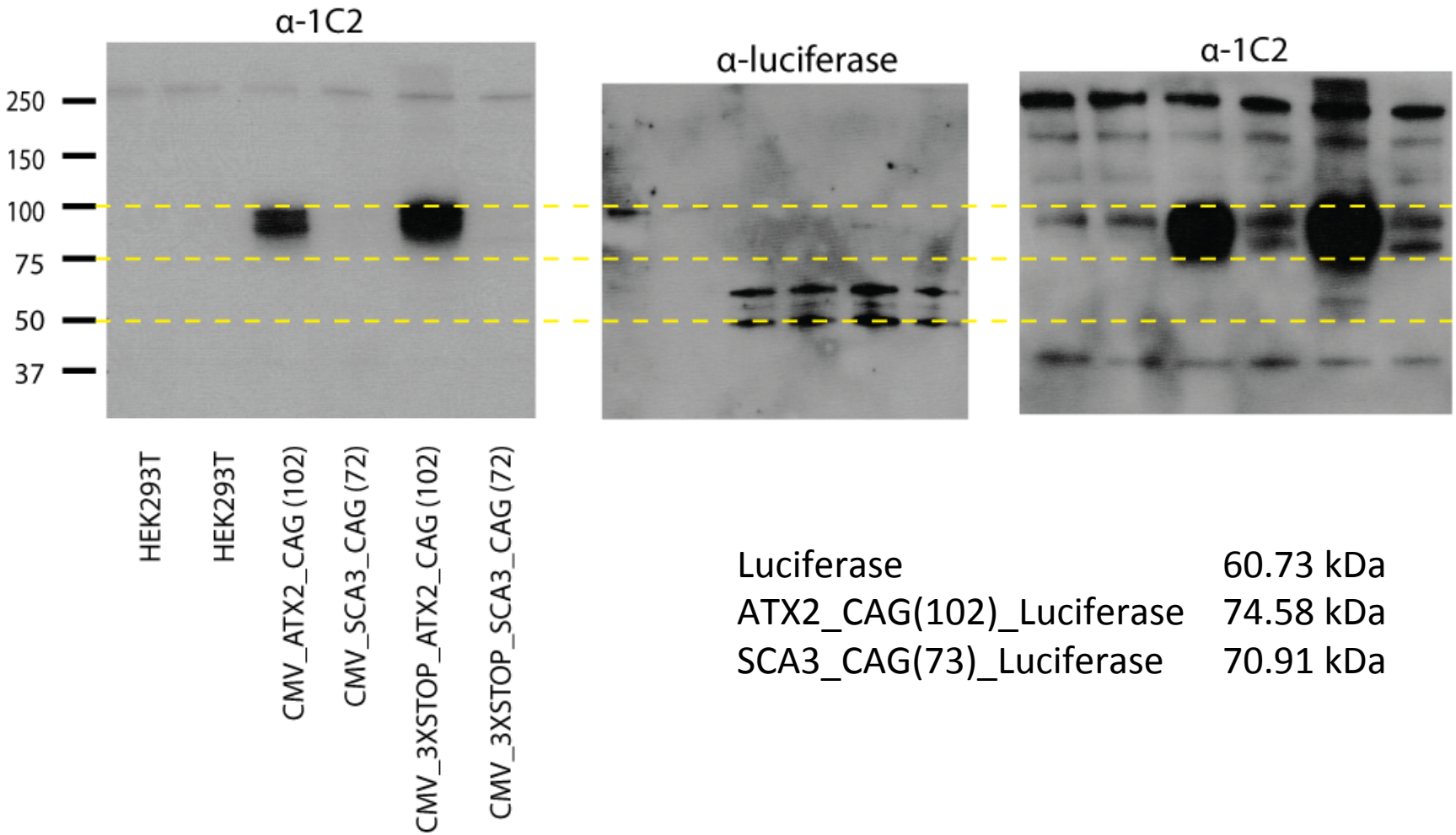
CMV_6XSTOP_SCA3_LUC



ATXN2 undergoes RAN translation in the presence of a CMV promoter

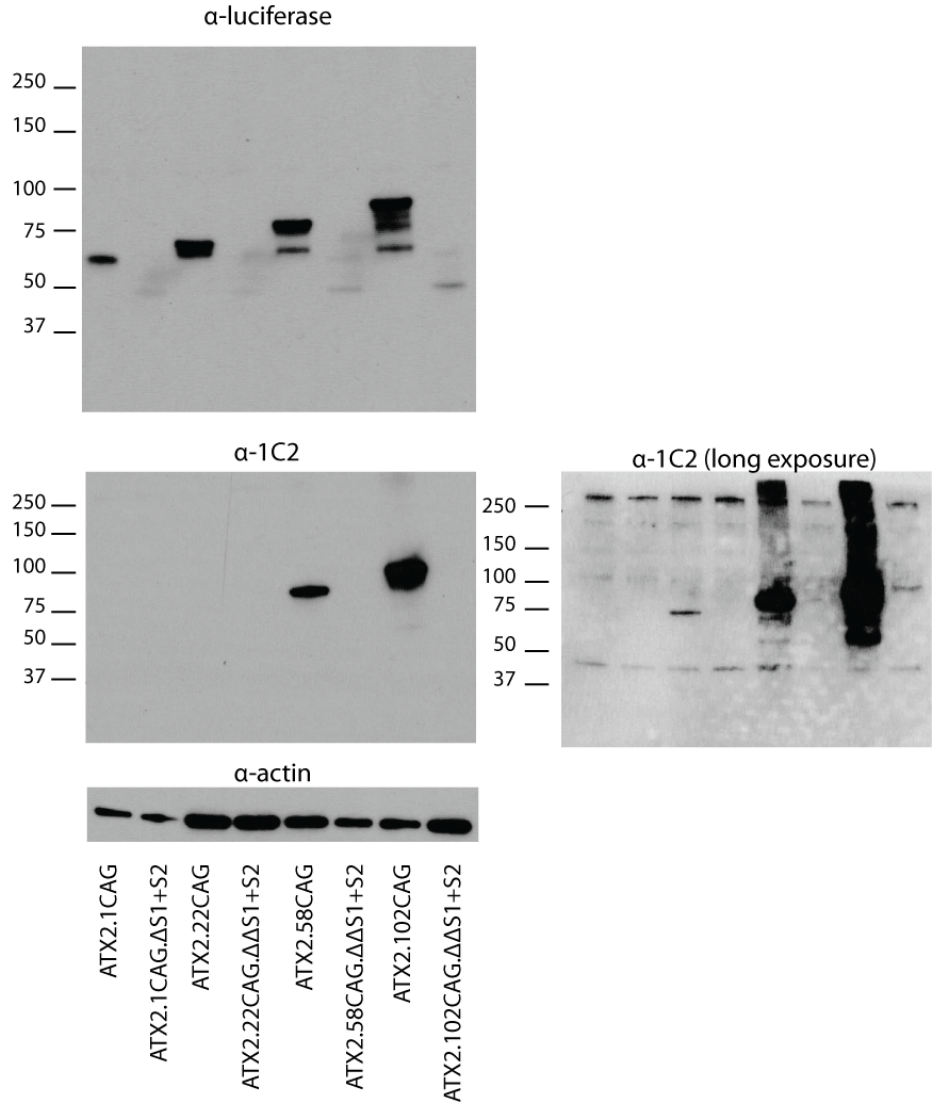


ATXN2 RAN translation products detected by luciferase and 1C2 are different sizes



Luciferase	60.73 kDa
ATX2_CAG(102)_Luciferase	74.58 kDa
SCA3_CAG(73)_Luciferase	70.91 kDa

ATXN2 undergoes RAN translation in the presence of the native promoter



Project 2 Summary

- **ATXN2 appears to undergo RAN translation in the presence of a CMV promoter**
  - Repeat and add appropriate controls.

- **ATXN2 appears to undergo weak RAN translation in the presence of the native promoter**
  - Repeat to confirm.

Additional things we should do:

1. Verify no difference for RAN translation in HEK293 vs HEK293T so Peterson's data will be accepted.

2. Transfect HEK293/T with Vector, ATG-ATXN2 and CTG-ATXN2, then perform RT-PCR using ATXN2 primers.

Wang et al discovered FUS bound *CCND1* promoter associated lncRNAs via a GGUG sequence and that the lncRNAs shifted FUS conformation to bind CBP/p300 to inhibit CBP/p300 HAT activity inhibiting transcription.

Wang et al. Nature 454:126-130; 2008
Induced ncRNAs Allosterically Modify RNA Binding Proteins *in cis* to Inhibit Transcription.

"Here, we report that an RNA-binding protein, TLS (same as FUS), serves as a key transcriptional regulatory sensor of DNA damage signals that, based on its allosteric modulation by RNA, specifically binds to and inhibits CBP/p300 HAT activities on a repressed gene target, cyclin D1 (CCND1). Recruitment of TLS to the CCND1 promoter to cause gene-specific repression is directed by single stranded, low copy number ncRNA transcripts tethered to the 5' regulatory regions of CCND1 that are induced in response to DNA damage signals. "

Multiple papers show ETS1 interacts CBP/p300. Since ETS1 binding site is near the *AROSA* sequence in *ATXN2*, this suggests relevance to SCA2

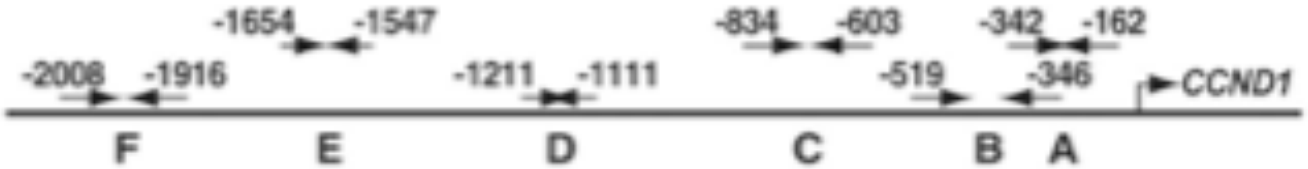
Ras/mitogen-activated protein kinase signaling activates Ets-1 and Ets-2 by CBP/p300 recruitment.
Foulds CE, Nelson ML, Blaszczak AG, Graves BJ.
Mol Cell Biol. 2004 Dec;24(24):10954-64.

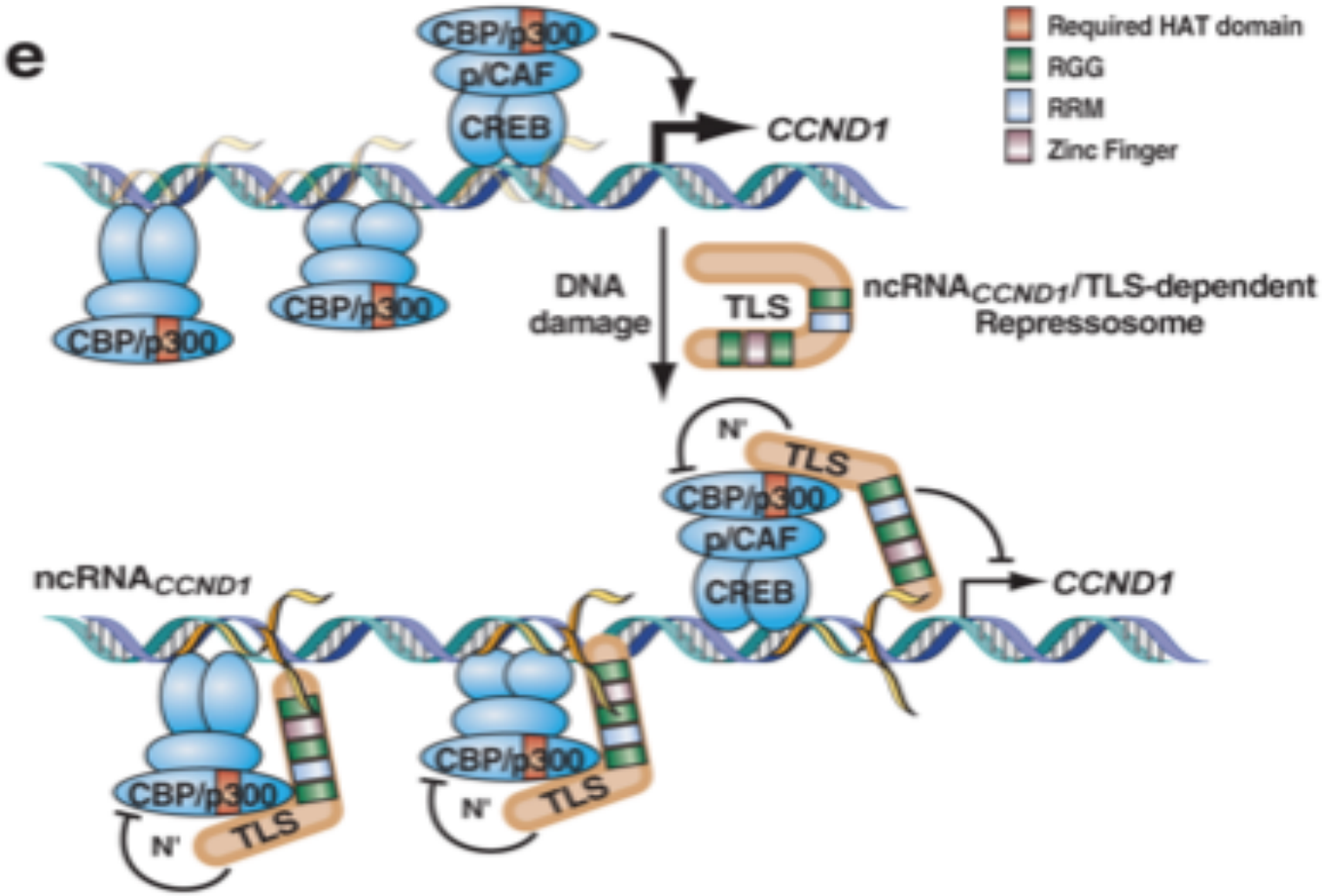
p300/cAMP-responsive element-binding protein interactions with ets-1 and ets-2 in the transcriptional activation of the human stromelysin promoter.
Jayaraman G, Srinivas R, Duggan C, Ferreira E, Swaminathan S, Somasundaram K, Williams J, Hauser C, Kurkinen M, Dhar R, Weitzman S, Buttice G, Thimmapaya B.
J Biol Chem. 1999 Jun 11;274(24):17342-52.

and another on the next slide…

The promoter associated cyclinD1-ncRNA was induced by ionizing radiation

There are multiple lncRNAs in *CCND1*





A role for CREB binding protein and p300 transcriptional coactivators in Ets-1 transactivation functions.

Yang C, Shapiro LH, Rivera M, Kumar A, Brindle PK.

Mol Cell Biol. 1998 Apr;18(4):2218-29.

The Ets-1 transcription factor plays a critical role in cell growth and development, but the means by which it activates transcription are still unclear. Here we show that Ets-1 binds the transcriptional coactivators CREB binding protein (CBP) and the related p300 protein (together referred to as CBP/p300) and that this interaction is required for specific Ets-1 transactivation functions. The Ets-1- and c-Myb-dependent aminopeptidase N (CD13/APN) promoter and an Ets-1-dependent artificial promoter were repressed by adenovirus E1A, a CBP/p300-specific inhibitor. Furthermore, Ets-1 activity was potentiated by CBP and p300 overexpression. The transactivation function of Ets-1 correlated with its ability to bind an N-terminal cysteine- and histidine-rich region spanning CBP residues 313 to 452. Ets-1 also bound a second cysteine- and histidine-rich region of CBP, between residues 1449 and 1892. Both Ets-1 and CBP/p300 formed a stable immunoprecipitable nuclear complex, independent of DNA binding. This Ets-1-CBP/p300 immunocomplex possessed histone acetyltransferase activity, consistent with previous findings that CBP/p300 is associated with such enzyme activity. Our results indicate that CBP/p300 may mediate antagonistic and synergistic interactions between Ets-1 and other transcription factors that use CBP/p300 as a coactivator, including c-Myb and AP-1.