Identification of drugs targeting Ataxin 2

Constructs for compound screening

Preliminary luciferase assays

Functional model for testing drugs in context of ataxin 2 function

Brief note on progress of construct for our transgenic reporter mouse

Lab Meeting of Thursday 3/26/09

Study Rationale

There are no drugs for the treatment of SCA2. None to treat SCA2 or to modify the phenotype / reduce symptoms. This study is intended to identify the first SCA2 drugs and test them in cellular and animal models.

The objectives of this study are

1) To identify drugs for the treatment of SCA2.

2) To identify regions of the *ATXN2* promoter targeted by such drugs.

3) To identify regions of the *ATXN2* 3'-UTR targeted by such drugs.

4) To establish a functional model of *ATXN2* for testing drugs efficacy.

5) To test efficacy of *ATXN2* drugs in existing SCA2 mice or in *ATXN2* reporter mouse.

Hypothesis:

SCA2 phenotype modifying drugs can be identified by high-throughput compound screening using a luciferase reporter plasmid of the following structure:

[*ATXN2* promoter]-[luciferase]-[*ATXN2* 3'UTR/PolyA]

Secondary hypotheses:

The CAG repeat alters the expression of *ATXN2*.

Specific regions of the *ATXN2* gene targeted by these drugs can be identified by screening reporter plasmids with deletions through the promoter region:

…regions of deletions in constructs whose luciferase expression is not affected by various drugs may indicate sites where drugs directly interact with the *ATXN2* gene to modify expression, or binding sites for transcription factors that are targeted by these drugs.

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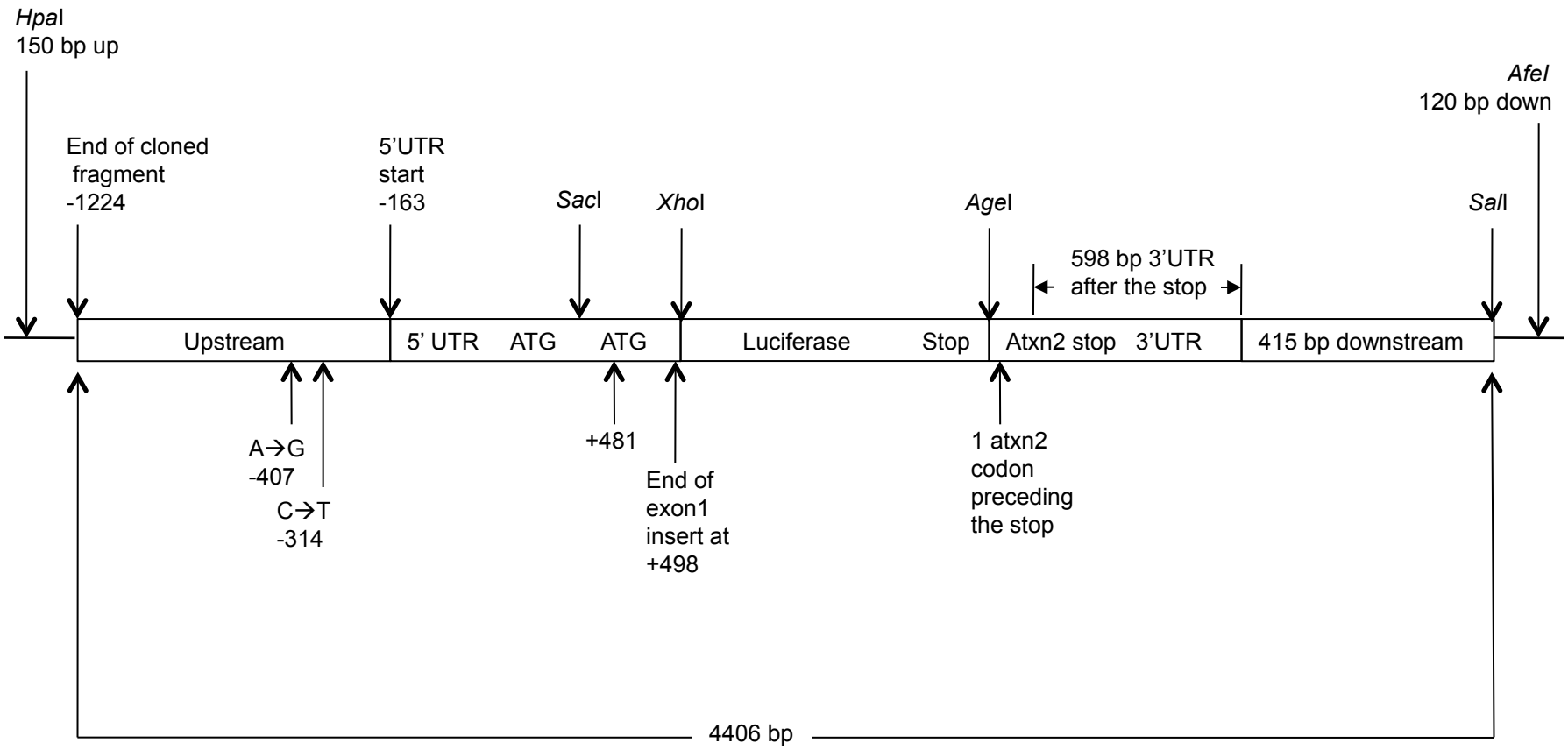
pGL2c.5A3c

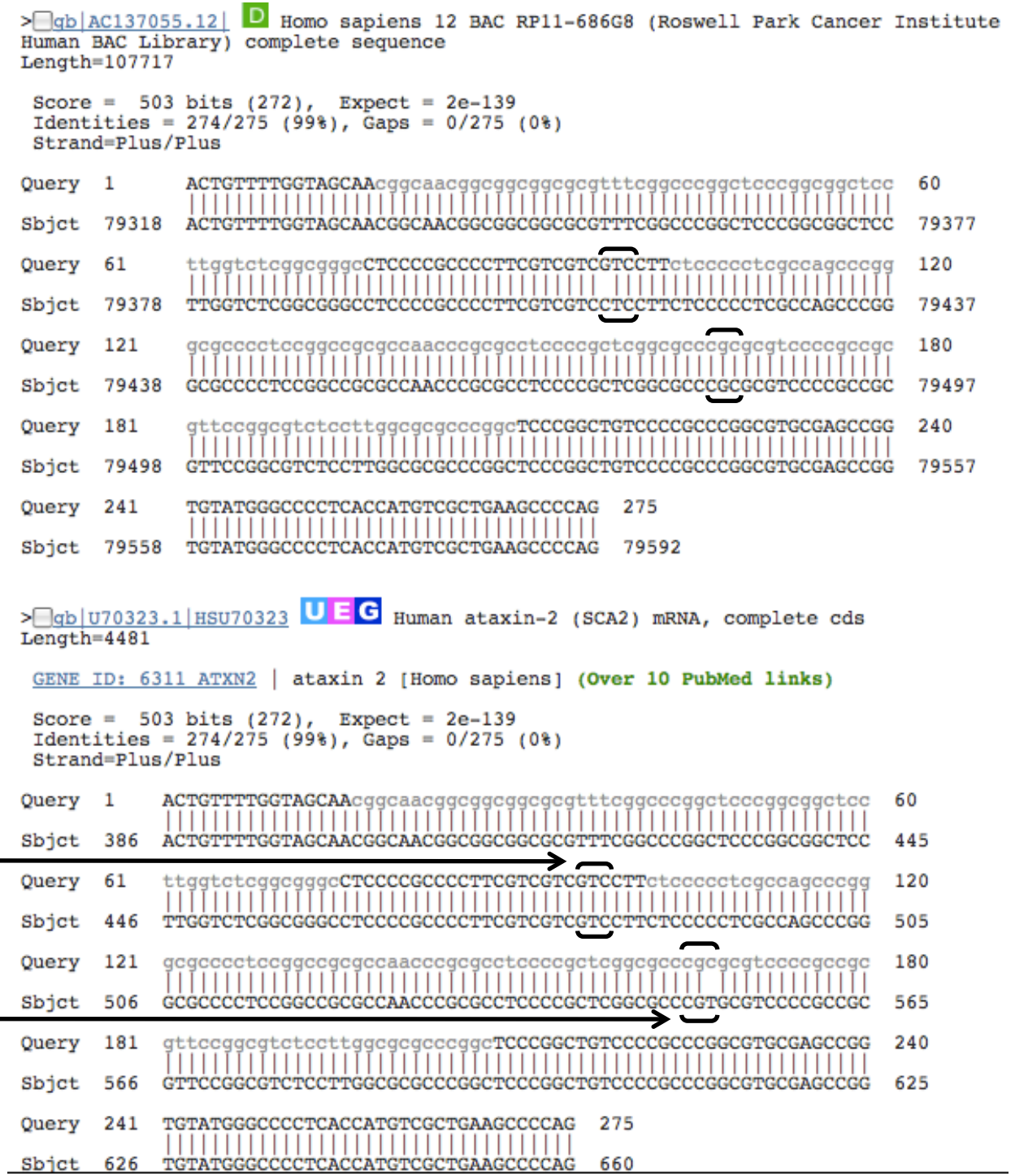

*Hpa*I
150 bp up
End of cloned fragment
-1224
5'UTR
start
-163
*Sac*I
*Xho*I
*Age*I
*Sal*I
*Afe*I
120 bp down
598 bp 3'UTR
after the stop
Upstream
5' UTR
ATG
ATG
Luciferase
Stop
Atxn2 stop
3'UTR
415 bp downstream
A→G
-407
C→T
-314
+481
End of
exon1
insert at
+498
1 atxn2
codon
preceding
the stop
4406 bp

We overlooked a sequence variation in our clone.

Leu → Val. It is located between the 1st and 2nd start codons so if the 1st start codon isn't utilized the protein sequence will be conserved.

Our variant was found in an Atxn2 sequence by Blast

Arg (conserved)



“common type” ??

```
/translation="MRSAAAAPRSPAVATESRRFAAARWPGWRSLQRPARRSGRGGGG
AAPGPYPSAAPPPPGPGPPPSRQSSPPSASDCFGSNGNGGGAFRPGSRRLLGLGGPPR
PFVVLLLPLASPGAPPAAPTRASPLGARASPPRSGVSLARPAPGCPRPACEPVYGPLT
MSLKPQQQQQQQQQQQQQQQQQQQQQPPPAAANVRKPGGSGLLASPAAAPSPSSSS
```

Leu → Val

```
/translation="MRSAAAAPRSPAVATESRRFAAARWPGWRSLQRPARRSGRGGGG
AAPGPYPSAAPPPPGPGPPPSRQSSPPSASDCFGSNGNGGGAFRPGSRRLLGLGGPPR
PFVVVLLPLASPGAPPAAPTRASPLGARASPPRSGVSLARPAPGCPRPACEPVYGPLT
MSLKPQQQQQQQQQQQQQQQQQQQQPPPAAANVRKPGGSGLLASPAAAPSPSSSSV
```

In fact, there are two Blast hits for this variation:

Accession # U70323

Submitted by Pulst et al., 1996

Has both the L105 → V and the conserved Arg “wobble”

Accession # Y08262

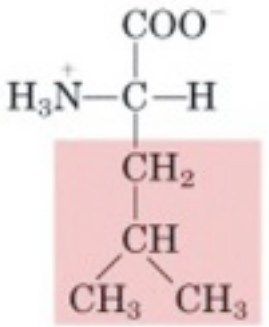
Submitted by Imbert et al., 1996 (the French group)

Has the L105 → V but not the c→t change in the Arg codon

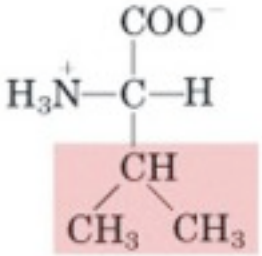
Could not find this variant in Ensembl

Chimps, macacks and Auburger and others have L105.

It may not matter, Leucine and Valine are very similar nonpolar residues:



Leucine



Valine

Luciferase assays

Luciferase assays of some of our constructs follows here…

…including a review of some old data and some new data too.

Luciferase assay protocol

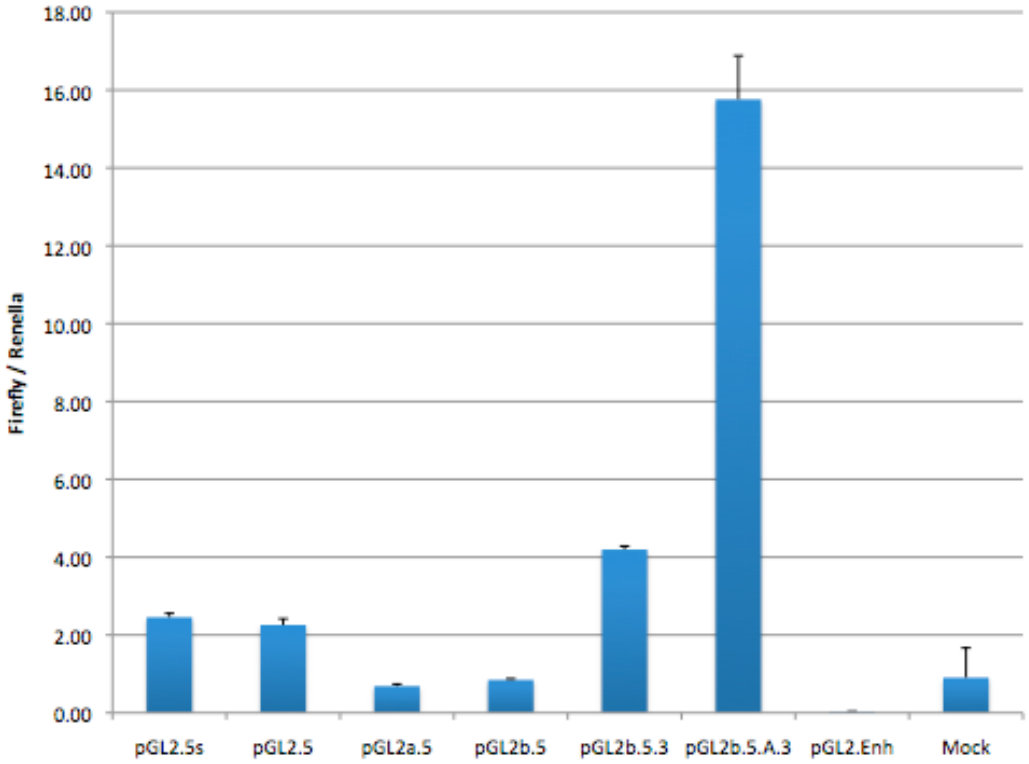
The assays are all conducted by transfecting 250 ng of reporter plasmid with 40 ng pRL-SV40 into 300 ul HEK293 cells plated in 24 well plates for 48 hours.

After 48 hours growth media was removed and 250 ul luciferase assay reagent was added, cells were suspended and 70 ul was distributed among wells of a solid white 96 well plate. Luminescence from firefly luciferase was read from the top. Next an a half volume (35 ul) of Stop-n-Glow reagent was added and Luminescence from Renella luciferase was read.

Values are reported as firefly/Renella. Background values were taken as well but not subtracted because they are usually so low relative to the true signal that subtraction wasn't necessary.

Each mean and standard results from three individual transfections (3 wells) each read in triplicate.

Luciferase assays using the first set of clones we made



pGL2.5s [shorter upstream]-[vector junk]-/-[M-Luc]-[SV40 PolyA]
pGL2.5 [longer upstream]-[vector junk]-/-[M-Luc]-[SV40 PolyA]
pGL2a.5 [longer upstream]-[vector junk]-/-[M-Luc]-[No PolyA]
pGL2b.5 [longer upstream]-[less vector junk]-/-[M-Luc]-[No PolyA]
pGL2b.5.3 [longer upstream]-[less vector junk]-/-[M-Luc]-[*ATXN2* PolyA]
pGL2b.5A3 [longer upstream]-[*ATXN2* Exon1]-[M-Luc]-[vector PolyA]
pGL2.Enh [No promoter]-[M-Luc]-[SV40 PolyA]
Mock No DNA, just water

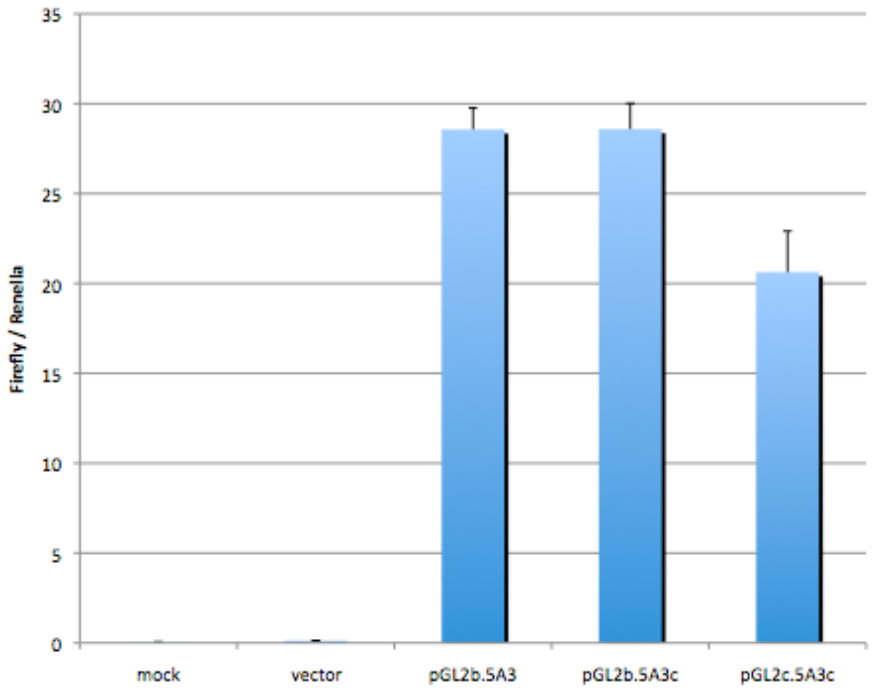
Notable Findings

Loss of polyA ↓ luc

Atxn2 poly ↑ luc

Frame correction ↑ luc

Comparison of before and after mutation correction and luc ATG removal



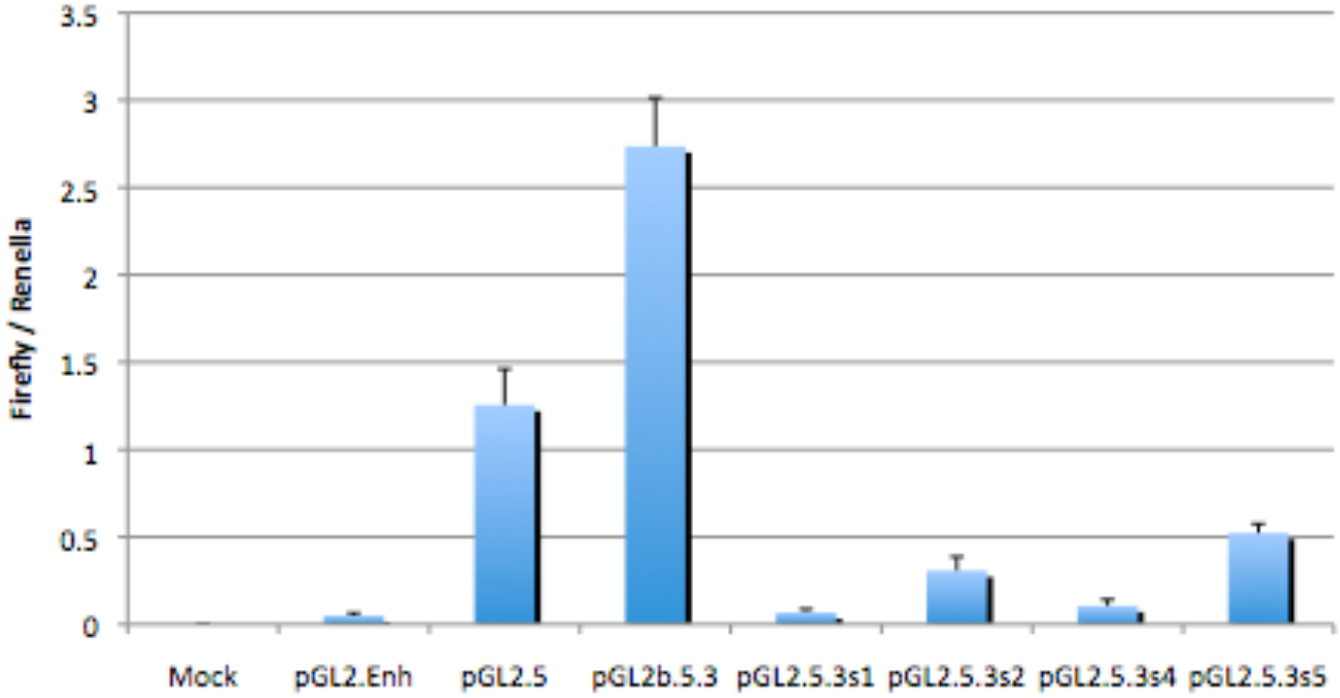
Notable Findings

Fixing the mutations didn't change luc expression.

Removing the luciferase ATG slightly reduced expression.

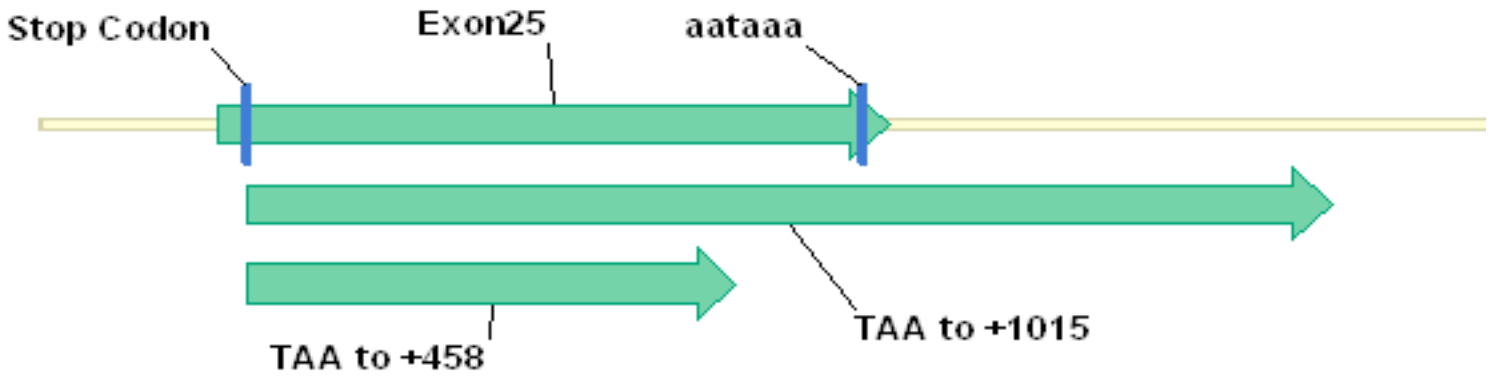
Mock	No DNA, just water	
pGL2.Enh (vector)		[No promoter]-[M-Luc]-[SV40 PolyA]
pGL2b.5A3		[upstream]-[*ATXN2* Exon1]-[M-Luc]-[vector PolyA]
pGL2b.5A3c	(mutations corrected)	[upstream]-[*ATXN2* Exon1]-[M-Luc]-[vector PolyA]
pGL2b.5A3c	(Luc ATG removed)	[upstream]-[*ATXN2* Exon1]-[Luc]-[vector PolyA]

Comparison of deletions in the 3’ UTR / PolyA region



Description of how the plasmid types differ				
	promoter	SV40 PolyA	Atxn2 PolyA	
Mock	n/a	n/a	n/a	
pGL2.Enh	no promoter	all	None	
pGL2.5	atxn2 promoter	all	None	Atxn2 Poly A portion drawn schematically
pGL2b.5.3	atxn2 promoter	None	-4 to +1015	.TAA.....
pGL2.5.3s1	atxn2 promoter	1-551	-104 to +458	.....TAA.....
pGL2.5.3s2	atxn2 promoter	1-551	-104 to +1015	.....TAA.....
pGL2.5.3s4	atxn2 promoter	1-551	-4 to +458	.TAA.....
pGL2.5.3s5	atxn2 promoter	1-551	-4 to +1015	.TAA.....
			^bp numbers are relative to the T in the Atxn2 TAA stop codom	
		^bp numbers are relative to the T in the Luciferase stop codon preceding the vector SV40 PolyA		

Loss of a bunch of adenines and a polyadenylation signal appears to associate with reduced luciferase expression



(fragment shown in blue below)

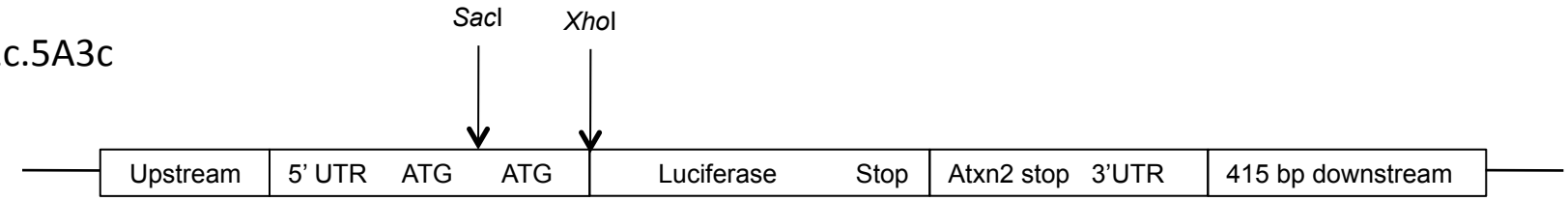
```
   1  CCCAACCCTC CCCACTTTGG TGCAGATGGG AGGGGGAAAA GCGAATTCAA TTTTGAGTTT TGTTCAGCTA GCACGAGGAT AGTTTACAAT CATGTGCTGC
      GGGTTGGGAG GGGTGAAACC ACGTCTACCC TCCCCCTTTT CGCTTAAGTT AAAACTCAAA ACAAGTCGAT CGTGCTCCTA TCAAATGTTA GTACACGACG

 101  AGAGACACTA GGCTGATGTG TGGTGTTGCC AGTTTTCTGT TTCAATGTTC GCTTTTCTTT TTACAGTACA AGCCCACCAC CAACAGCAGT TGTAAGGCTG
      TCTCTGTGAT CCGACTACAC ACCACAACGG TCAAAAGACA AAGTTACAAG CGAAAAGAAA AATGTCATGT TCGGGTGGTG GTTGTCGTCA ACATTCCGAC

 201  CCCTGGAGGA ACCGAAAGGC CAAATTCCCT CCTCCCTTCT ACTGCTTCTA CCAACTGGAA GCACAGAAAA CTAGAATTTC ATTTATTTTG TTTTTAAAAT
      GGGACCTCCT TGGCTTTCCG GTTTAAGGGA GGAGGGAAGA TGACGAAGAT GGTTGACCTT CGTGTCTTTT GATCTTAAAG TAAATAAAAC AAAAATTTTA

 301  ATATATGTTG ATTTCTTGTA ACATCCAATA GGAATGCTAA CAGTTCACTT GCAGTGGAAG ATACTTGGAC CGAGTAGAGG CATTTAGGAA CTTGGGGGCT
      TATATACAAC TAAAGAACAT TGTAGGTTAT CCTTACGATT GTCAAGTGAA CGTCACCTTC TATGAACCTG GCTCATCTCC GTAAATCCTT GAACCCCCGA

 401  ATTCCATAAT TCCATATGCT GTTTCAGAGT CCCGCAGGTA CCCCAGCTCT GCTTGCCGAA ACTGGAAGTT ATTTATTTTT TAATAACCCT TGAAAGTCAT
      TAAGGTATTA AGGTATACGA CAAAGTCTCA GGGCGTCCAT GGGGTCGAGA CGAACGGCTT TGACCTTCAA TAAATAAAAA ATTATTGGGA ACTTTCAGTA

 501  GAACACATCA GCTAGCAAAA GAAGTAACAA GAGTGATTCT TGCTGCTATT ACTGCTAAAA AAAAAAAAAA AAAAAAATCA AGACTTGGAA CGCCCTTTTA
      CTTGTGTAGT CGATCGTTTT CTTCATTGTT CTCACTAAGA ACGACGATAA TGACGATTTT TTTTTTTTTT TTTTTTTAGT TCTGAACCTT GCGGGAAAAT

 601  CTAAACTTGA CAAAGTTTCA GTAAATTCTT ACCGTCAAAC TGACGGATTA TTATTTATAA ATCAAGTTTG ATGAGGTGAT CACTGTCTAC AGTGGTTCAA
      GATTTGAACT GTTTCAAAGT CATTTAAGAA TGGCAGTTTG ACTGCCTAAT AATAAATATT TAGTTCAAAC TACTCCACTA GTGACAGATG TCACCAAGTT

 701  CTTTTAAGTT AAGGGAAAAA CTTTTACTTT GTAGATAATA TAAAATAAAA ACTTAAAAAA AATTTAAAAA ATAAAAAAAG TTTTAAAAAC TGATCAAGTT
      GAAAATTCAA TTCCCTTTTT GAAAATGAAA CATCTATTAT ATTTTATTTT TGAATTTTTT TTAAATTTTT TATTTTTTTC AAAATTTTTG ACTAGTTCAA

 801  AGTGTGTGTC TGTATAAGCT ACTTCTTTGT AGGATACTTA ATATCAAAGC AGGTGTGCTA AGGGTGCATT TTGAATATCC CGGAAGGTAG CTTTGAAATG
      TCACACACAG ACATATTCGA TGAAGAAACA TCCTATGAAT TATAGTTTCG TCCACACGAT TCCCACGTAA AACTTATAGG GCCTTCCATC GAACTTTAC
```

AATAAA

Exon end

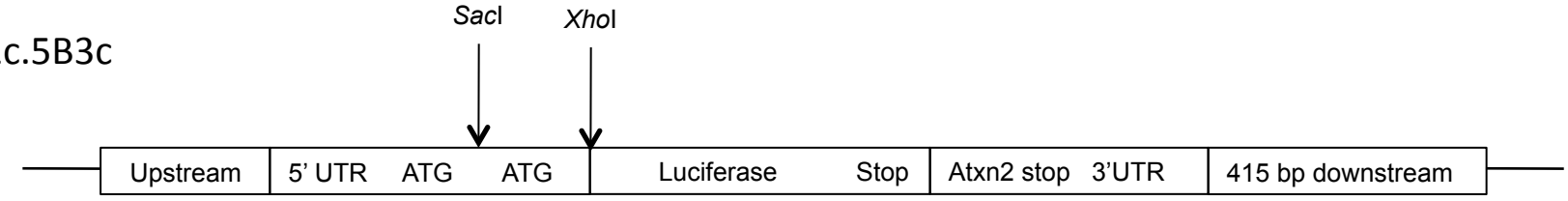
Constructs with different CAG repeats

pGL2c.5A3c



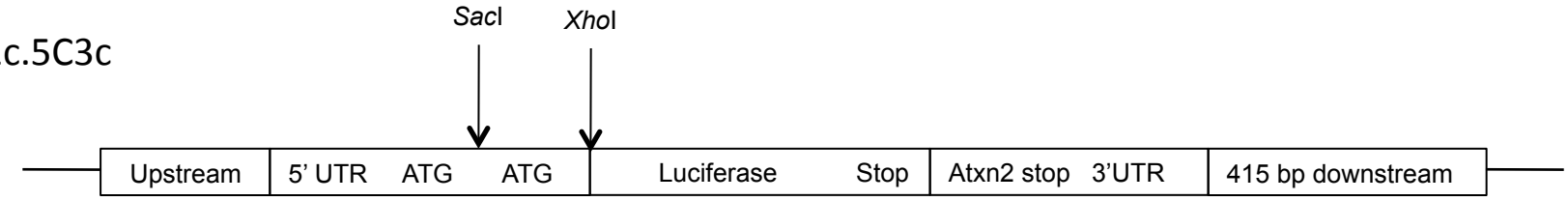
MSLKJPQ

pGL2c.5B3c



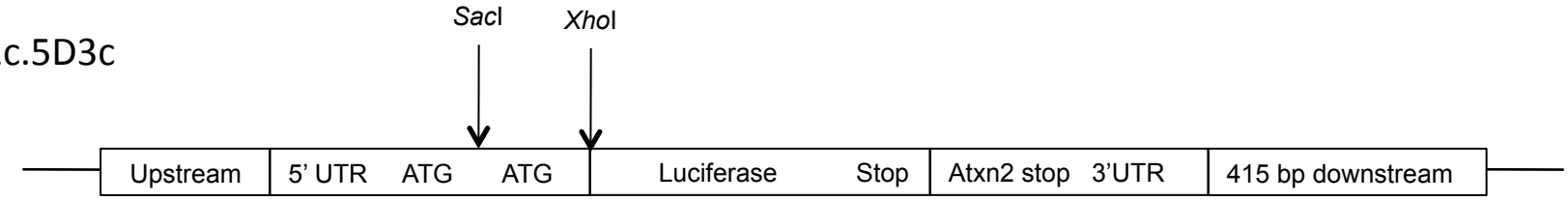
MSLKJPQ$_{22}$PPPAAADVRKPGGSGLLASPPAAAPSPSSSSVSSSSA

pGL2c.5C3c



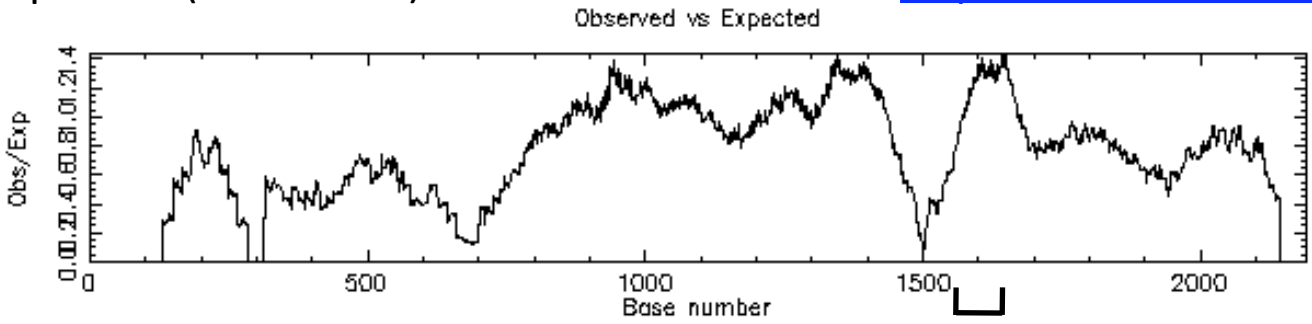
MSLKJPQ$_{58}$PPPAAADVRKPGGSGLLASPPAAAPSPSSSSVSSSSA

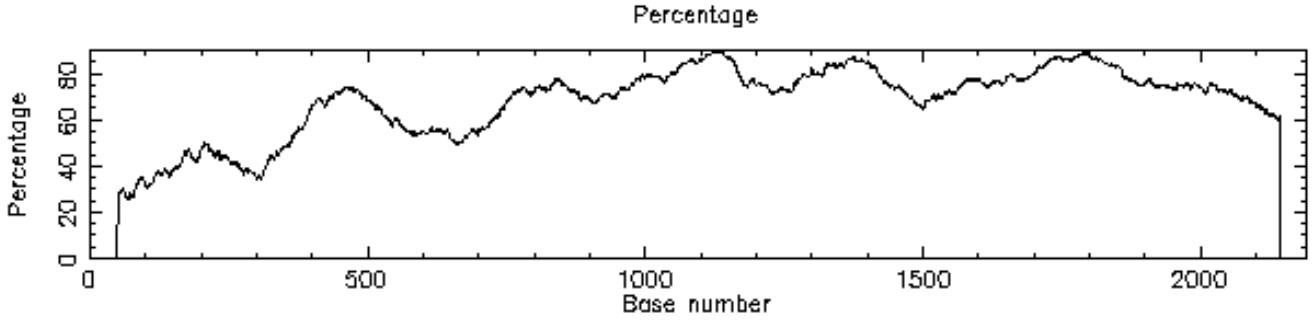
pGL2c.5D3c

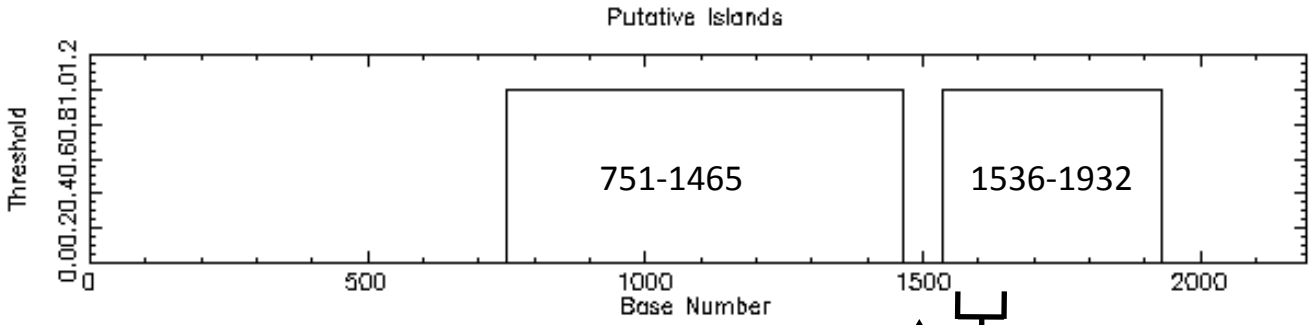


MSLKJPQ$_{110}$PPPAAADVRKPGGSGLLASPPAAAPSPSSSSVSSSSA

CpG Plot (online tool) http://www.ebi.ac.uk/emboss/cpgplot/








```
CPGPLOT islands of unusual CG composition
EMBOSS_001 from 1 to 2192

     Observed/Expected ratio > 0.60
     Percent C + Percent G > 50.00
     Length > 200

Length 715 (751..1465)

Length 397 (1536..1932)
```

CAG: 1484-1552

The cloned region ds of the CAG repeat is 108 bp, or 1553-1630 on this map.

How CpG plot predicts CpG islands

CpGs islands are estimated based on a sliding window that moves over a test sequence. The window size is default at 100 bp.

The Observed number of CpG patterns in a window is simply the count of the number of times a 'C' is found followed immediately by a 'G'. The Expected number of CpG patterns is calculated for each window as the number of CpG dinucleotides you would expect to see in that window based on the frequency of C's and G's in that window. Thus, the Expected frequency of CpG's in a window is calculated as the number of 'C's in the window multiplied by the number of 'G's in the window, divided by the window length.

Expected = (number of C's * number of G's) / window length

Percentage = Percentage of CpGs over a window

Threshold = 17 CpGs within a window, a somewhat arbitrary value

disclaimer, this doesn't mean I understand it all…

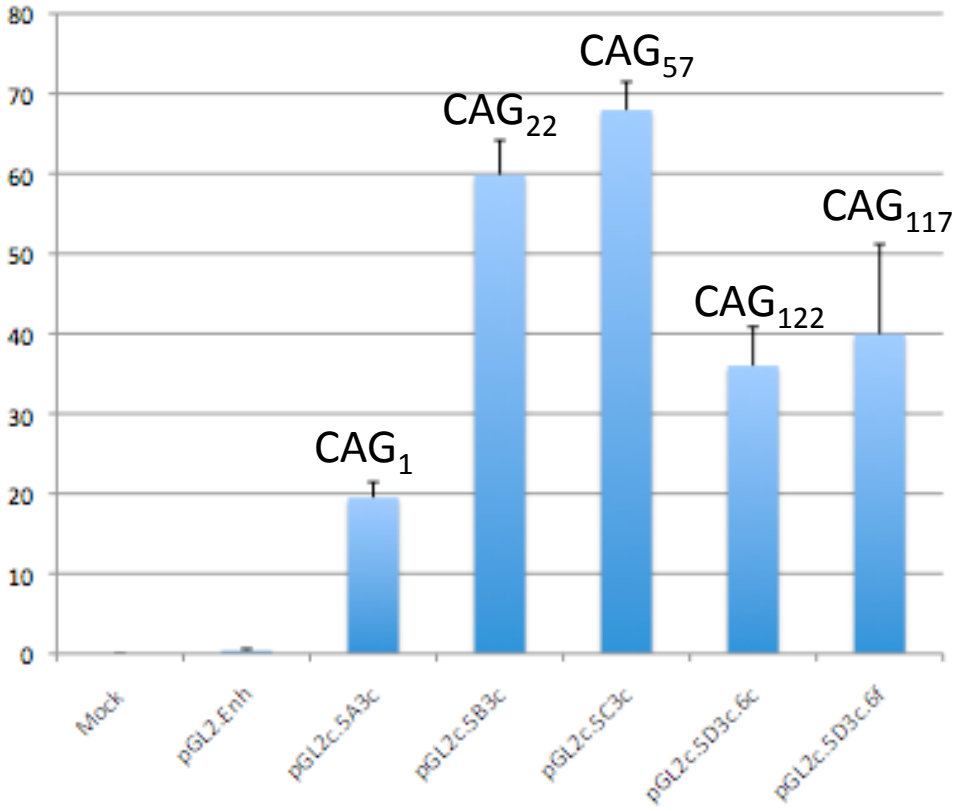
Numbers and structures of the CAGs we cloned

A = CAG1	CAG
B = CAG22	$(CAG)_8CAA(CAG)_4CAA(CAG)_8$
C = CAG57	$(CAG)_{57}$
D(6c) = CAG122	$(CAG)_{81}CGG(CAG)_{40}$
D(6f) = CAG117	$(CAG)_{88}CGG(CAG)_{28}$

Note that CGG encodes Arginine

Furtado et al. (2004) showed $(CAG)_{38}(CGG)_1(CAG)_7$ in a Chinese-American family with early onset parkinsonism-predominant SCA2 and proposed the interruption serves to stabilize the long repeat.

Comparison of constructs with different CAG repeats.



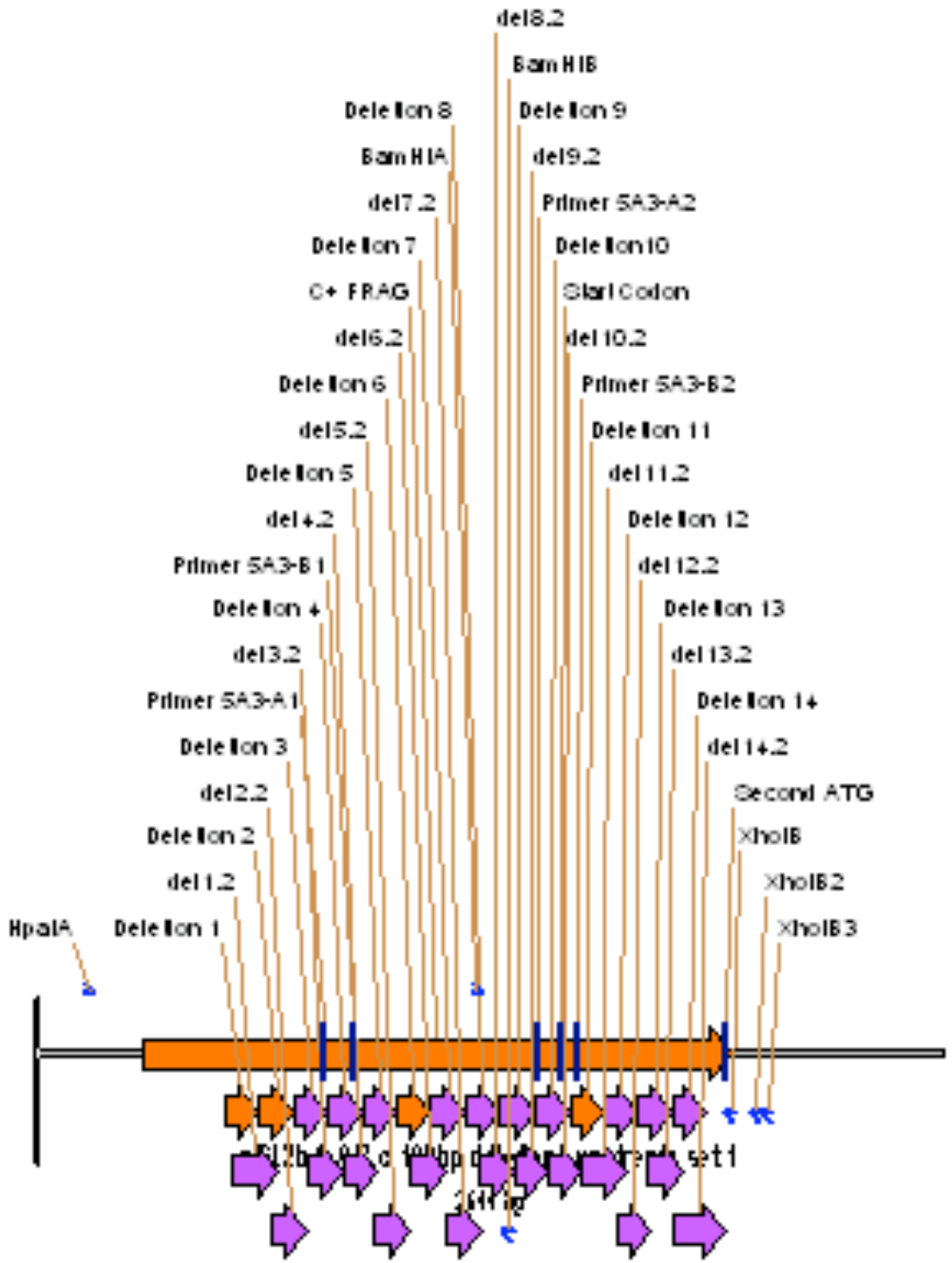
3/4/09

Progress on cloning deletion constructs

Purpose of these deletions:

Should we find experimental compounds that reduce luciferase expression but fail to reduce luciferase expression from particular deletion constructs then the regions of deletion in those constructs may define sites where compounds directly interact or sites of interaction by transcription factors that are targeted by these drugs.

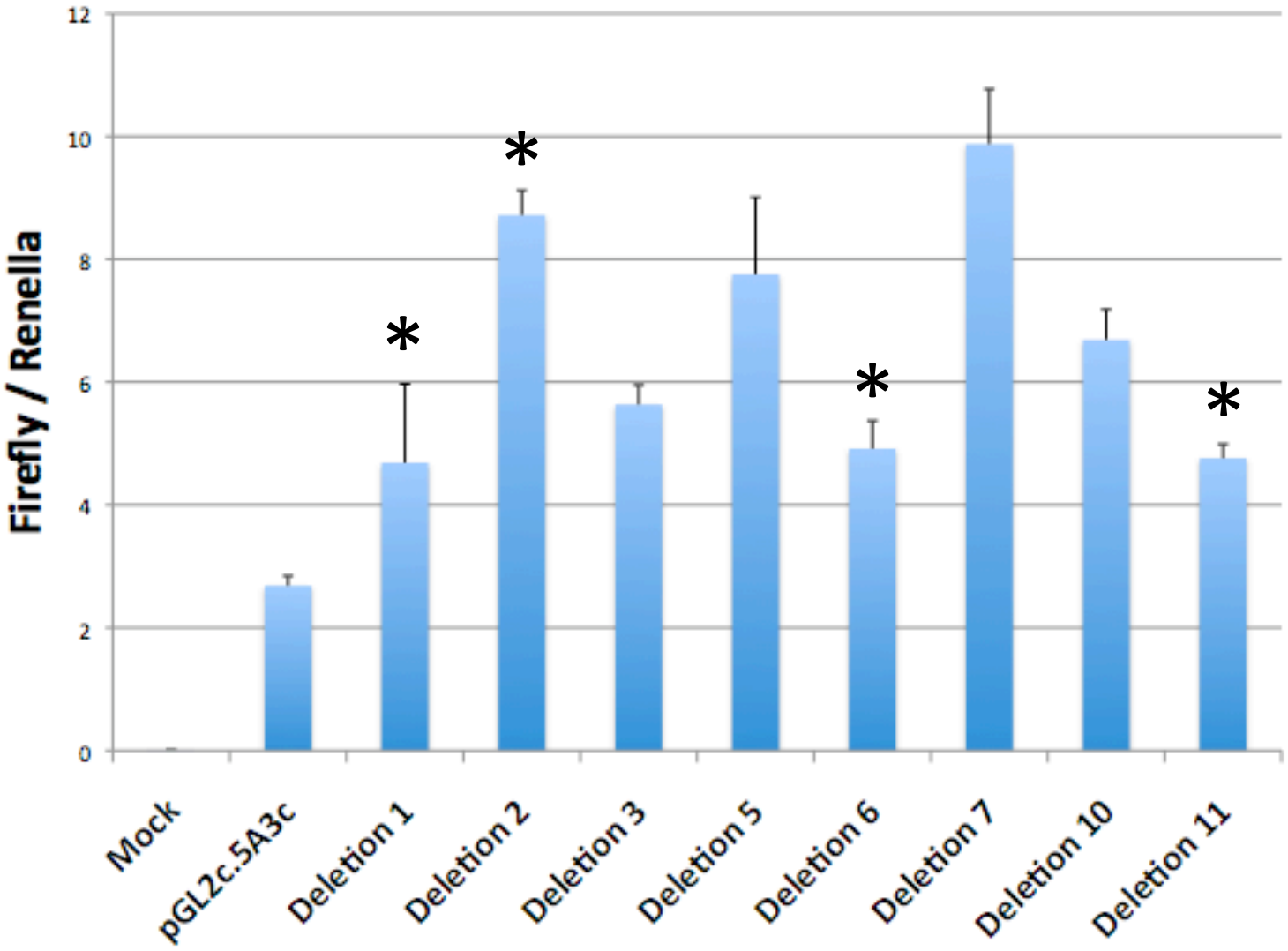
5’-UTR Deletions



Deletion cloning progress (Upstream half only, Deletion #s only, Del1.#s still to do)

	Sequence Verified	Still screening	Still cloning	Preliminary Luc Assay done
Deletion1	x			x
Deletion2	x			x
Deletion3		x		x
Deletion4		x		
Deletion5		x		x
Deletion6	x			x
Deletion7		x	x	x
Deletion8		x		
Deletion9		x		
Deletion10		x		x
Deletion11	x			x
Deletion12			Replating ligaton	
Deletion13		x		
Deletion14			Difficult to clone	

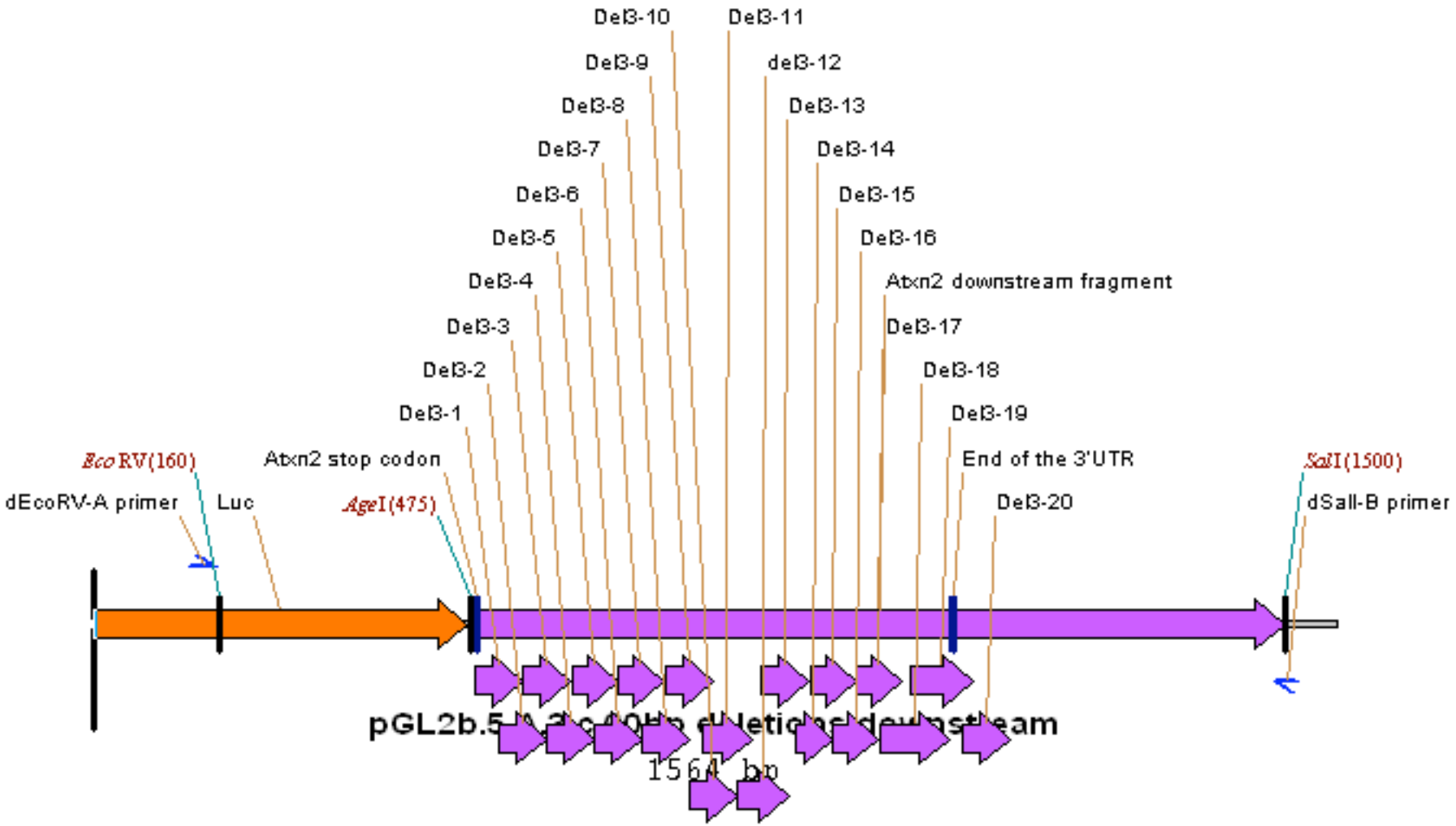
Results of preliminary luciferase assays of deletions

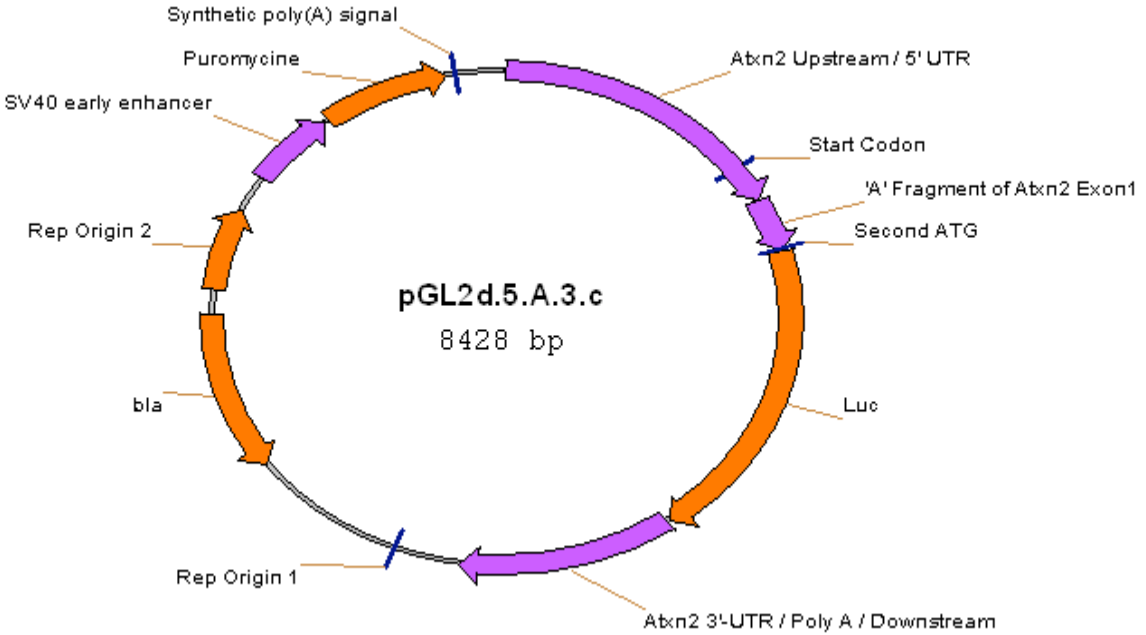


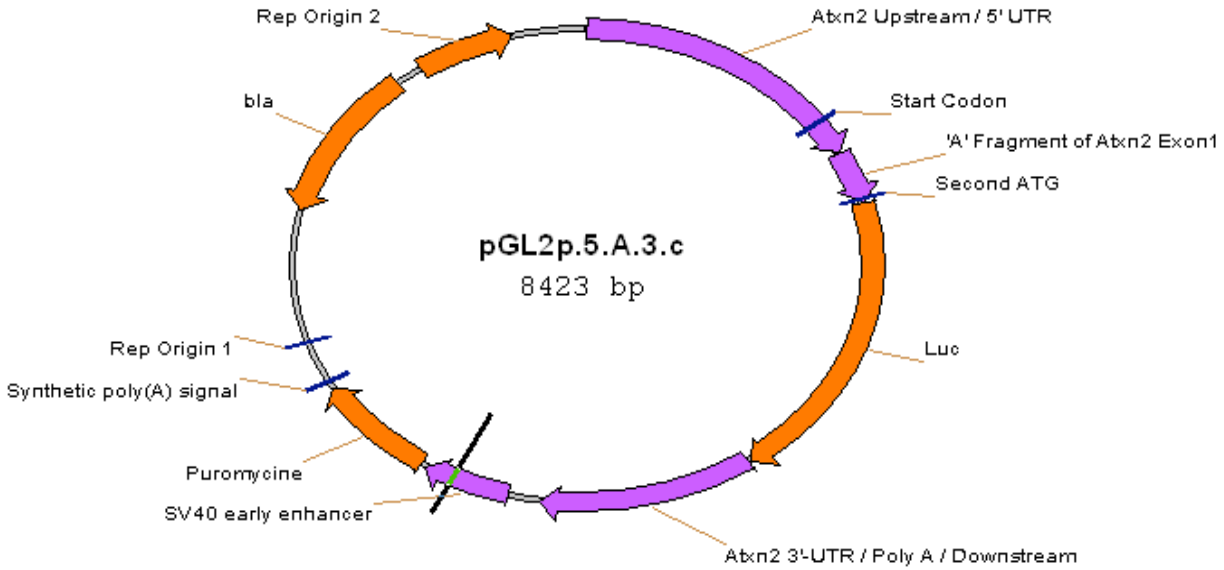
* = Deletion Verified

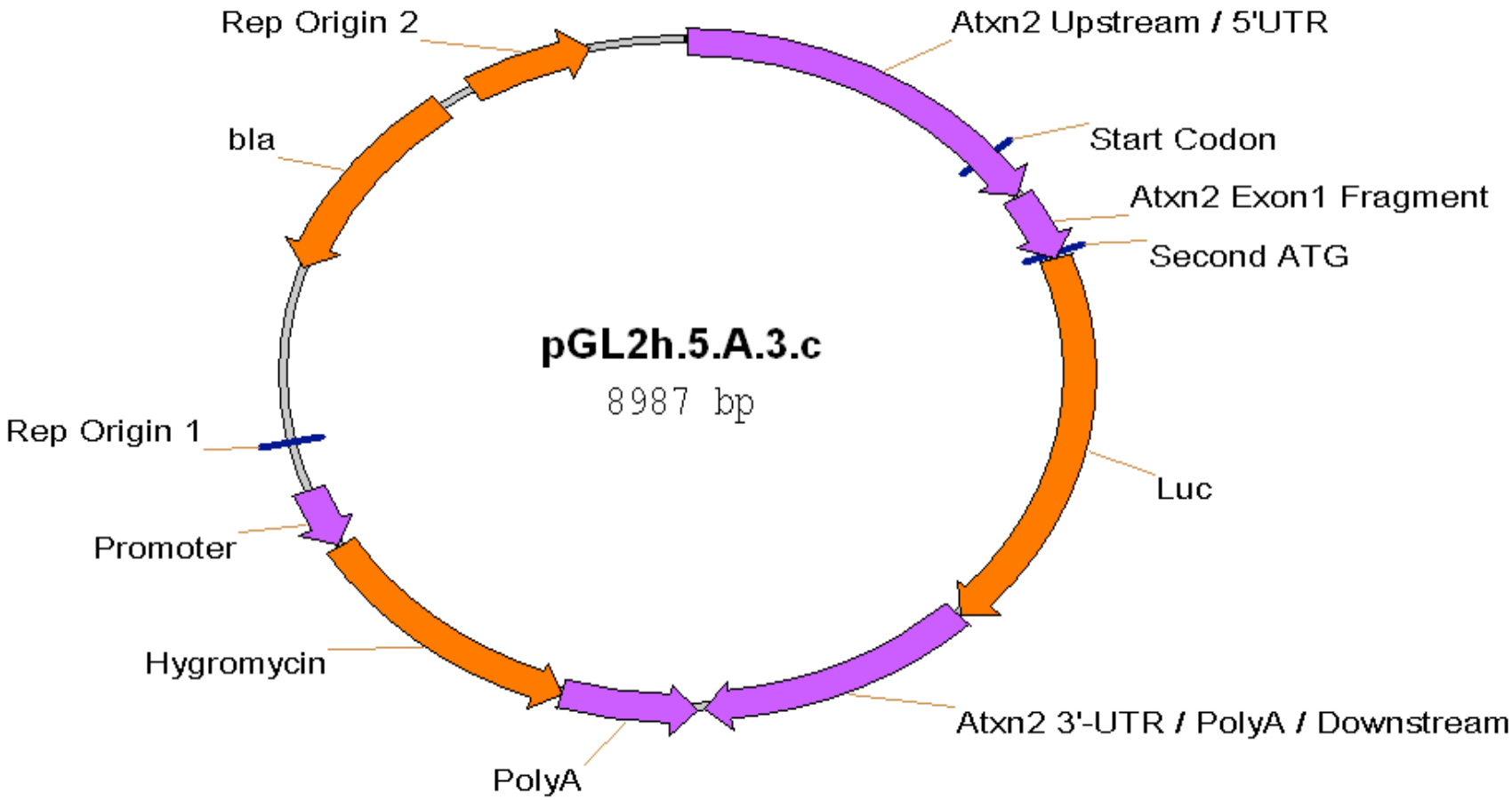
Note: this experiment appeared to have failed…reasons why include probably the use of our replacement HEK293 cells without optimization of transfection conditions, indicated by low value for pGL2c.5A3c which normally is ~ 20, and poor growth and detached cells after transfection in many of the wells.

3’-UTR deletions planned




Synthetic poly(A) signal
Puromycine
SV40 early enhancer
Rep Origin 2
bla
Rep Origin 1
Atxn2 Upstream / 5' UTR
Start Codon
'A' Fragment of Atxn2 Exon1
Second ATG
Luc
Atxn2 3'-UTR / Poly A / Downstream
pGL2d.5.A.3.c
8428 bp


Rep Origin 2
bla
Rep Origin 1
Synthetic poly(A) signal
Puromycine
SV40 early enhancer
Atxn2 Upstream / 5' UTR
Start Codon
'A' Fragment of Atxn2 Exon1
Second ATG
Luc
Atxn2 3'-UTR / Poly A / Downstream
pGL2p.5.A.3.c
8423 bp


Rep Origin 2
Atxn2 Upstream / 5'UTR
bla
Start Codon
Atxn2 Exon1 Fragment
Second ATG
pGL2h.5.A.3.c
8987 bp
Rep Origin 1
Luc
Promoter
Hygromycin
Atxn2 3'-UTR / PolyA / Downstream
PolyA

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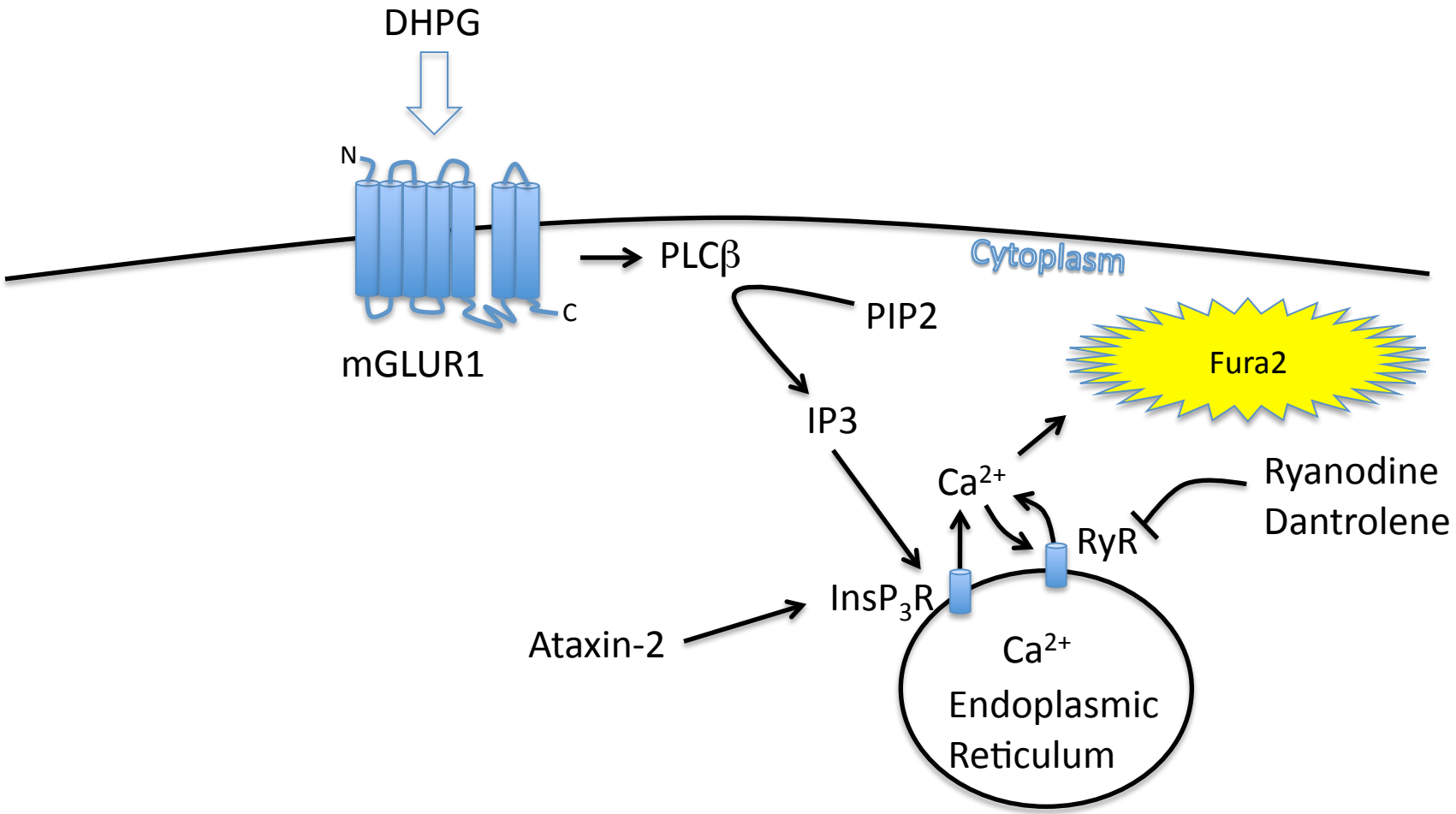
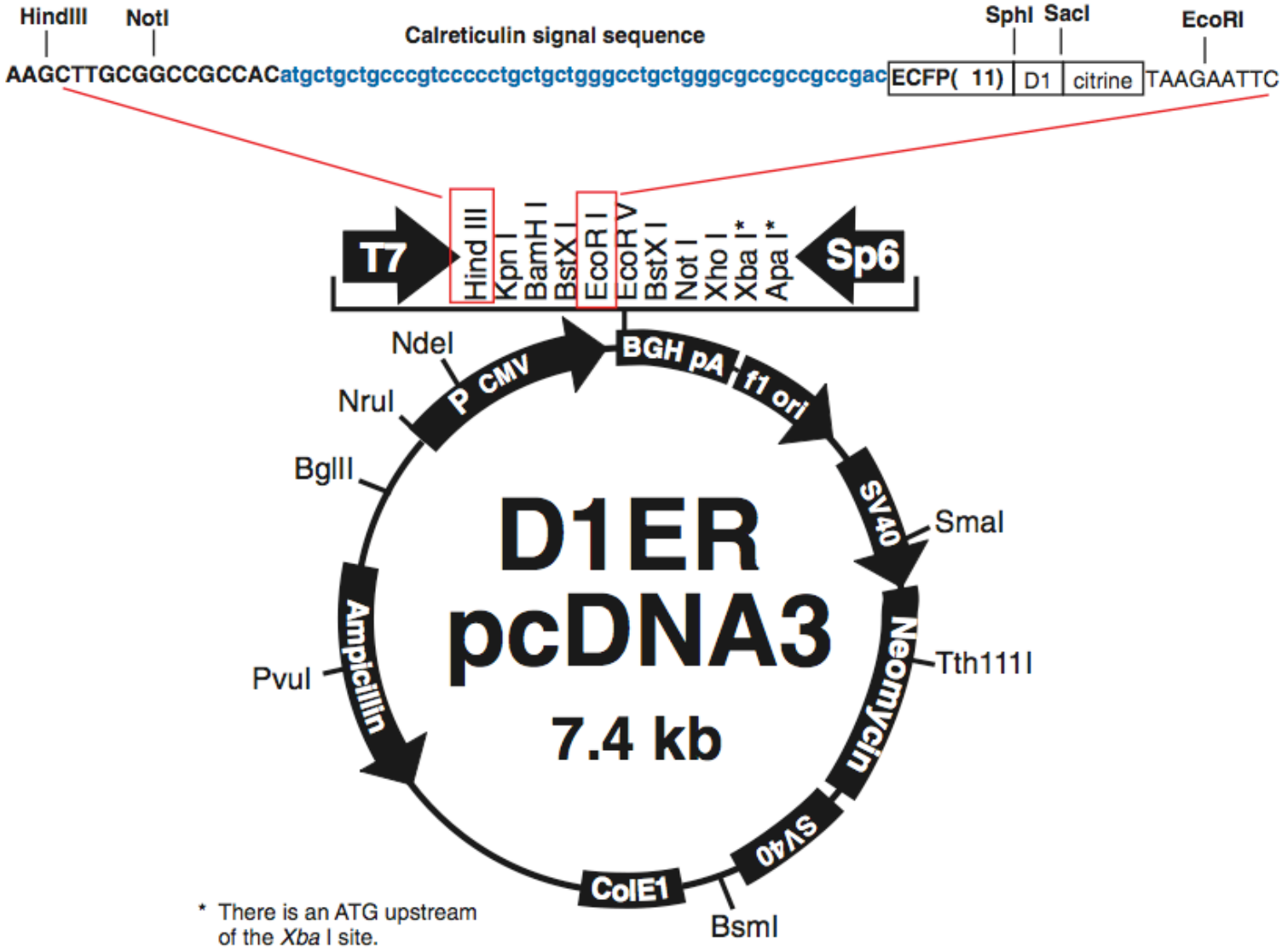
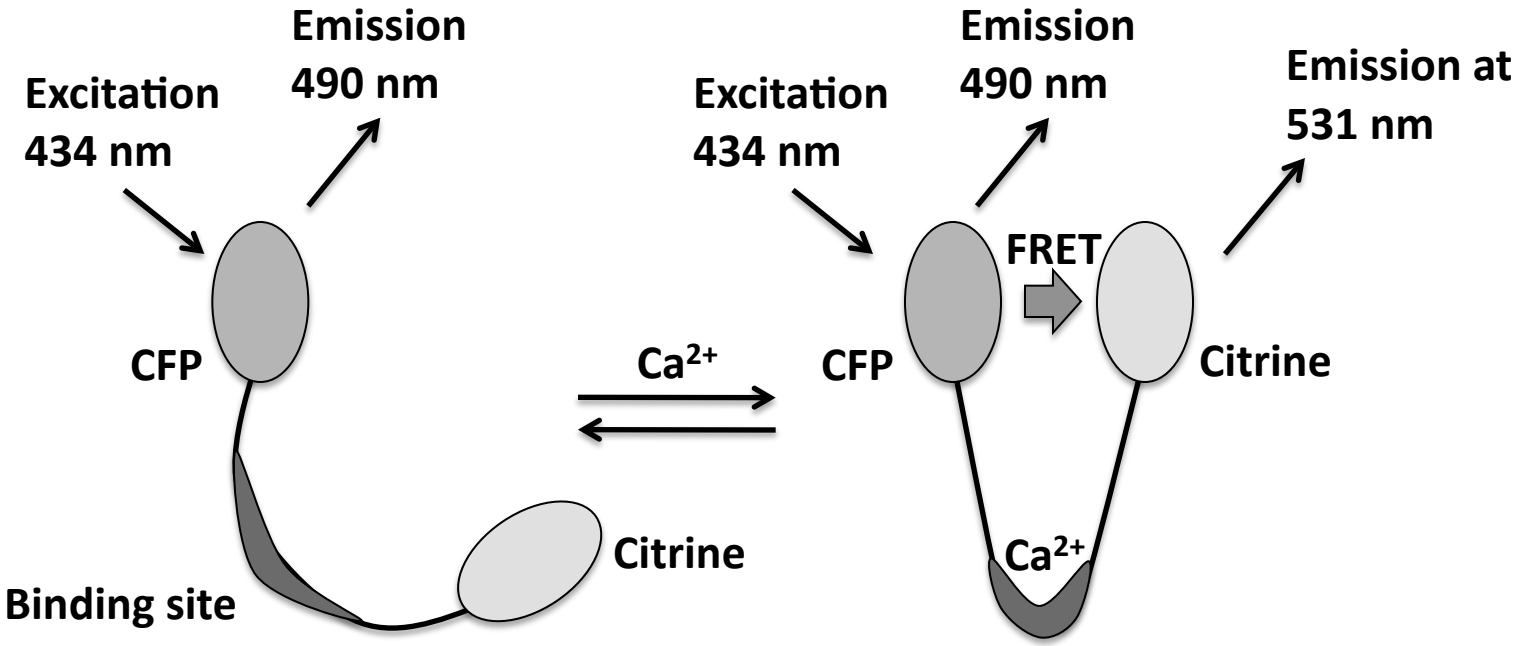
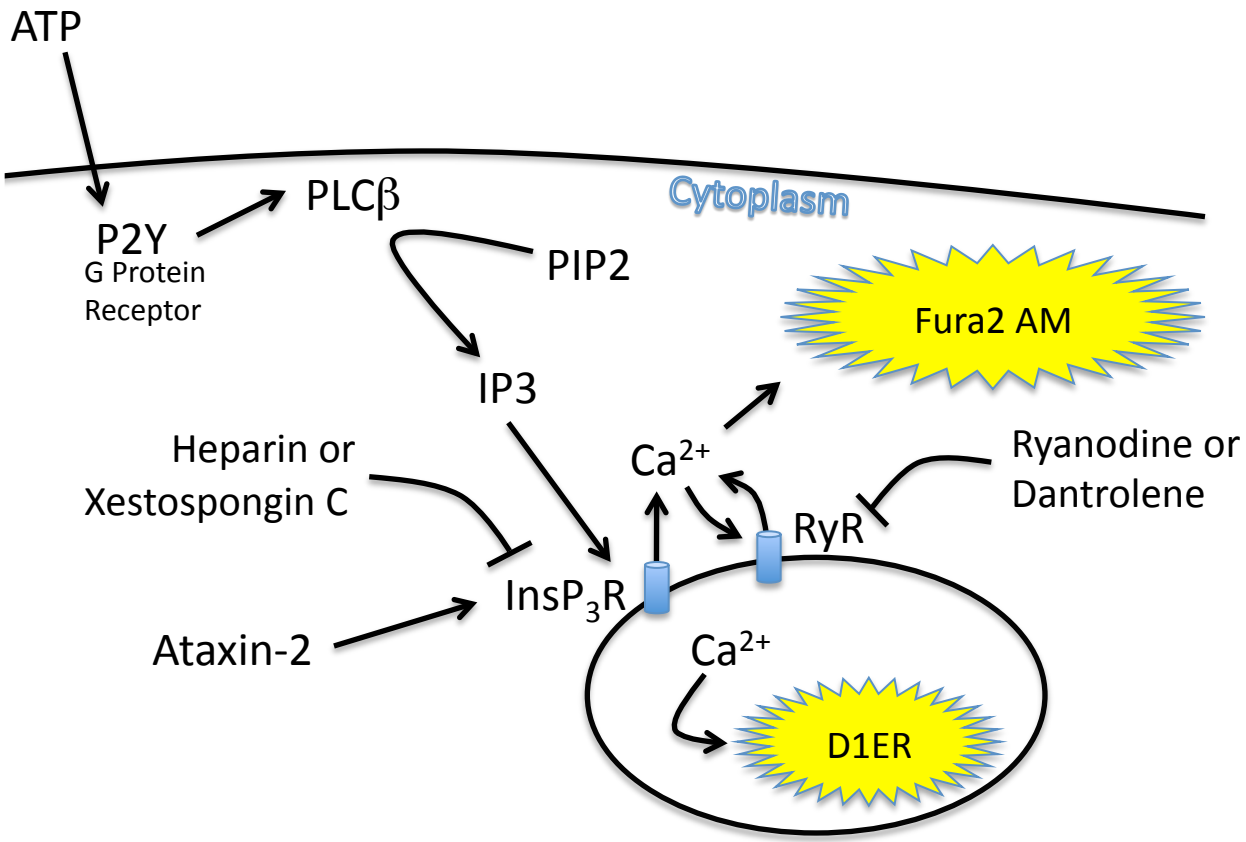

DHPG
N
C
mGLUR1
PLCβ
Cytoplasm
PIP2
Fura2
IP3
Ca2+
Ryanodine
Dantrolene
RyR
InsP3R
Ataxin-2
Ca2+
Endoplasmic
Reticulum

D1ER, a genetically encoded calcium sensor targeted to ER, from Roger Tsien's lab



D1ER


Excitation
434 nm
Emission
490 nm
CFP
Citrine
Binding site
Ca2+
Excitation
434 nm
Emission
490 nm
FRET
Emission at
531 nm
CFP
Citrine
Ca2+


ATP
P2Y
G Protein
Receptor
PLCβ
Cytoplasm
PIP2
Fura2 AM
IP3
Heparin or
Xestospongin C
Ca2+
Ryanodine or
Dantrolene
RyR
InsP3R
Ataxin-2
Ca2+
D1ER

Can Zn or Cd activate *ATXN2* expression?

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– Letter –

GENE EXPRESSION PROFILE IN HUMAN CELLS EXPOSED TO ZINC

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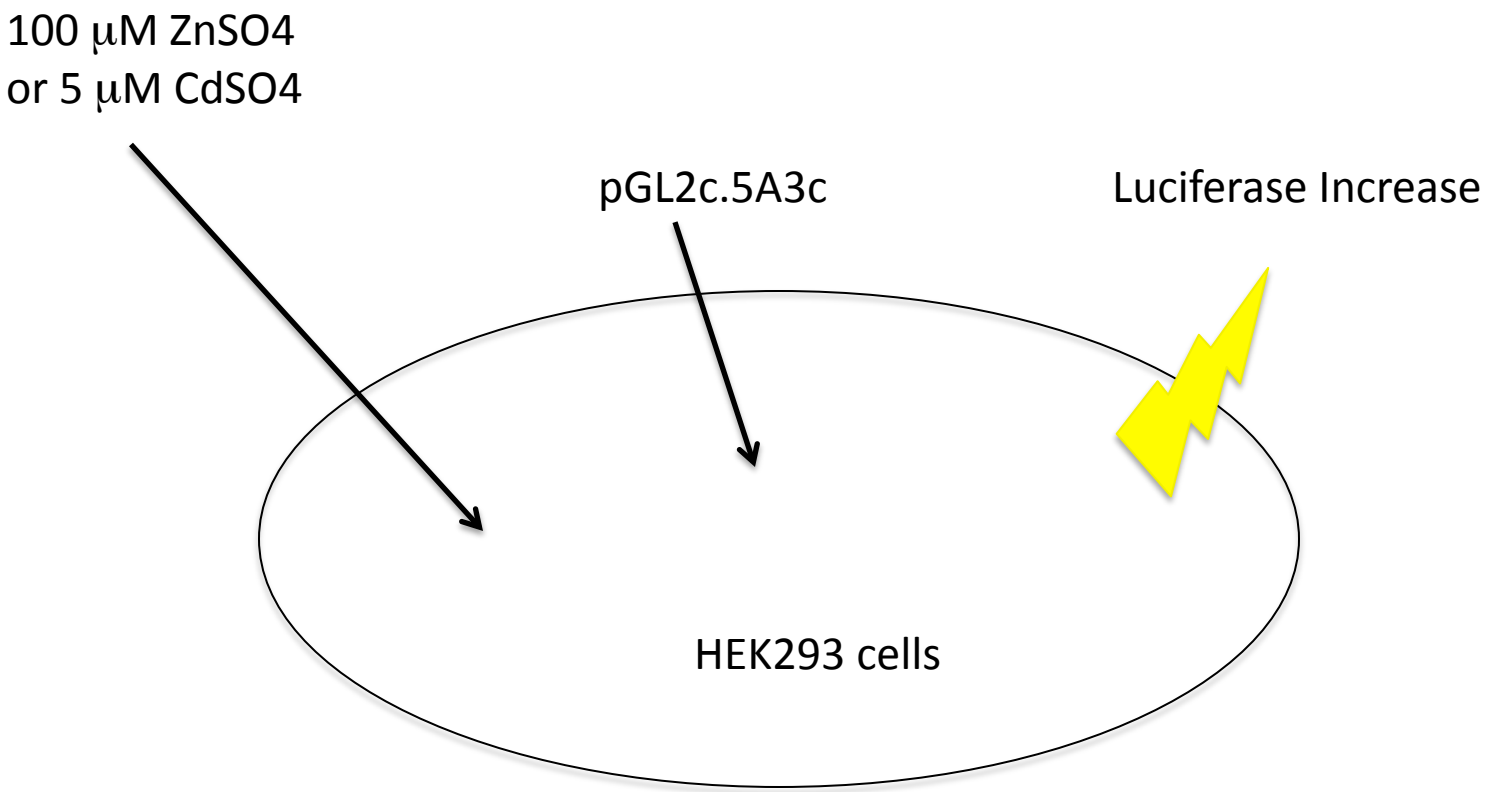
ABSTRACT — Although Zn is an essential trace metal for humans, a comprehensive view of its effects on cellular functions has not been obtained. We used a DNA microarray to assess transcriptional alterations in human HeLa cells after exposure to a moderate concentration of Zn (100 μM $ZnSO_4$). Out of 9,182 human genes, expression was increased in 7 genes and decreased in 4 genes twofold or greater. Four of the 7 upregulated genes were those coding for metallothionein isoforms or related proteins. An unexpectedly small extent of changes in gene expression might reflect rapid sequestration of Zn ions by metallothioneins, and the absence of most of the other protective responses indicated the non-toxic nature of Zn at this concentration. Comparison with our previous DNA microarray results for 5 μM $CdSO_4$-exposed HeLa cells revealed several genes that are regulated by both metals in parallel, and a gene reciprocally regulated by them.

Table 1. Gene expression induced by 100 μM Zn.

	100 μM Zn	5 μM Cd	Gene	
1.	23.7	58.8	MT-1L	(metallothionein-1L)
2.	8.6	*	EST similar to MT-1F	(metallothionein-1F)
3.	5.5	5.3	EST similar to MT-1B	(metallothionein-1B)
4.	4.5	6.5	MT-1E	(metallothionein-1E)
5.	3.5	–	Dsg2	(desmoglein 2)
6.	3.4	3.3	human hbc647 mRNA sequence	
7.	2.6	2.9	ataxin 2	(spinocerebellar ataxia 2)

The 2nd and 3rd leftmost columns show induction ratios by 100 μM $ZnSO_4$ and 5 μM $CdSO_4$ (data from Yamada and Koizumi, 2002 are indicated for comparison), respectively. Genes are arranged by an order of induction by Zn. EST, expressed sequence tag. *, no corresponding probe was present on the DNA microarray used; –, a corresponding probe was present on the DNA microarray but the change in expression was less than twofold.



What use is this? Could also test deletion constructs…
Maybe it could indicate that Zn in the diet of pts should be avoided.

Identification of drugs targeting Ataxin 2

Constructs for compound screening

Preliminary luciferase assays

Functional model for testing drugs in context of ataxin 2 function

Brief note on progress of construct for our transgenic reporter mouse

Progress on a transgenic mouse

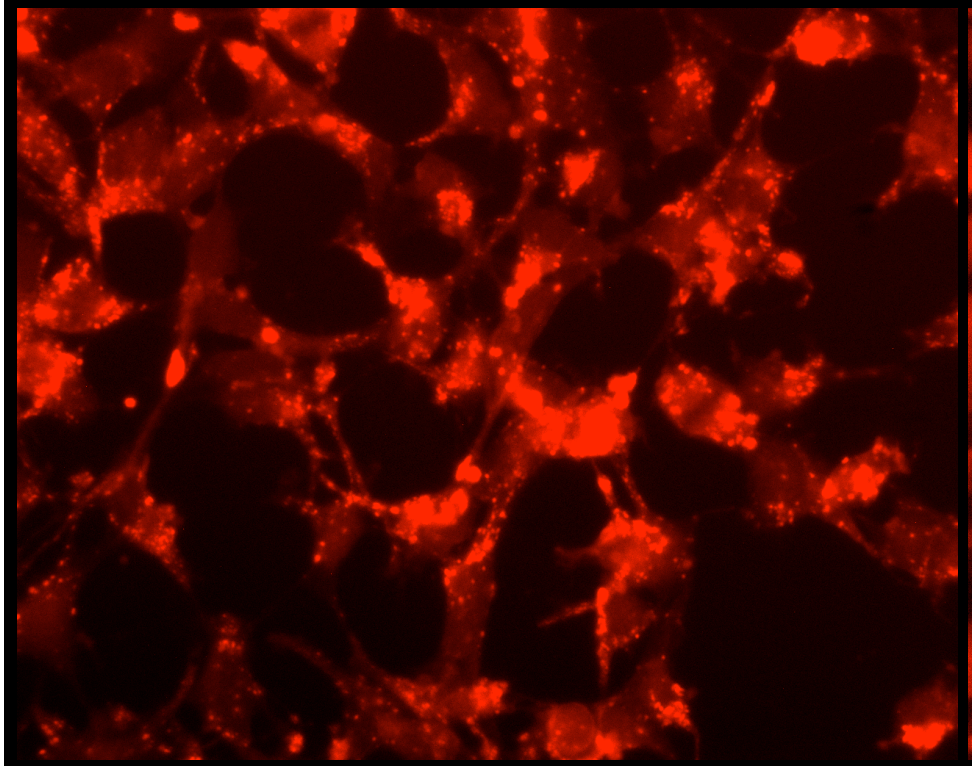
The construct is cut and purified

We want to run some verifications first, including

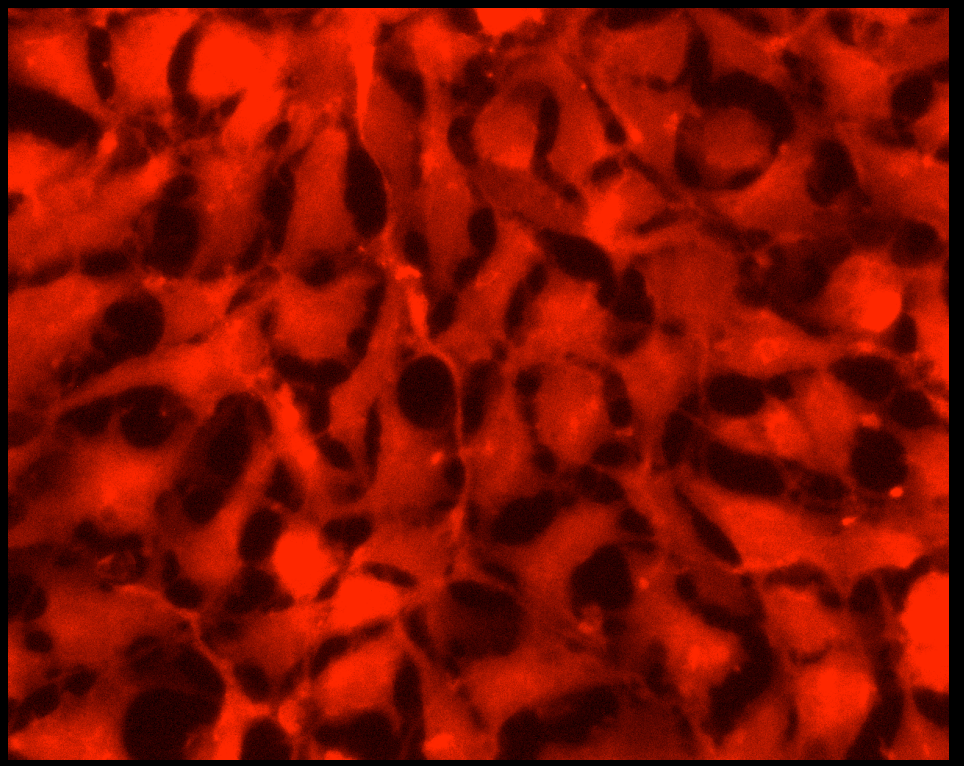
Sequencing

Transfection of linearized construct, is it red

DsRed construct transfected in HEK293 cells



pDsRed2b-5A3c



pDsRed2

The fusion is targeted to some structure…

Assistance with screening here on campus…

Gretchen King (585 Building E. of Eccles)

- She is an aquarist, does no research
- She works for David Grunwald who can help with zebrafish ideas

David Jones in HCI & USTAR (585-6107)

- Bought a NPS library of compounds
  - (Natural Products ? Or library from NPS Pharmaceuticals?)
- Are trying to establish a core for screening
- Might be able to help with our planning