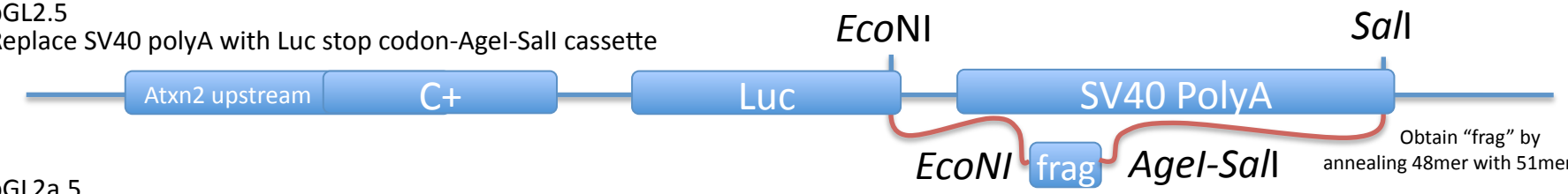
Lab Meeting 12/12/08

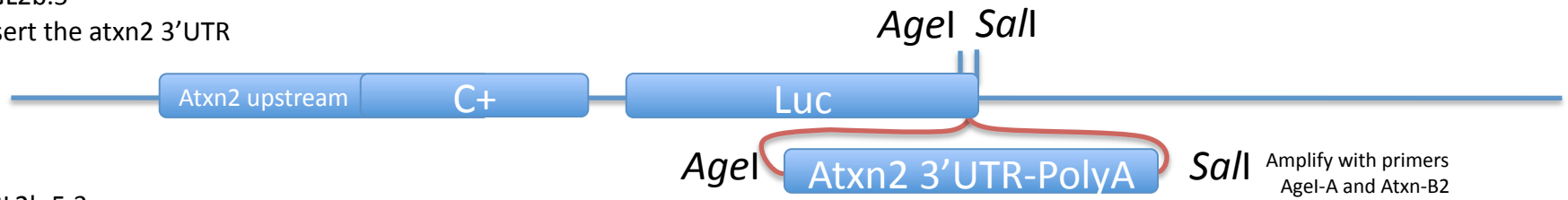
Cloning pGL2-5.A.3 and pDsRed-5.A.3 (or pLacZ-5.A.3)

pGL2.5
Replace SV40 polyA with Luc stop codon-AgeI-SalI cassette

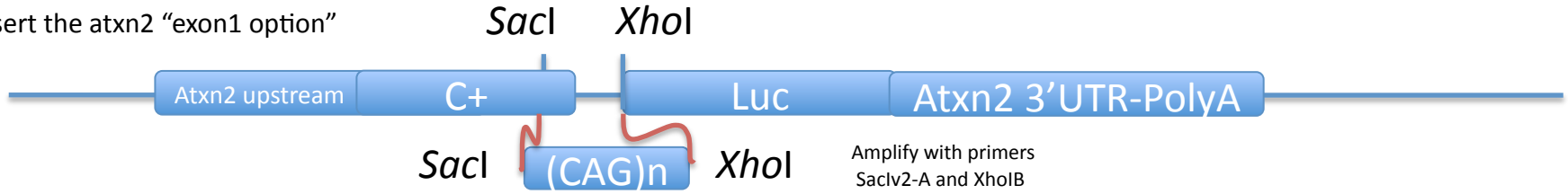


pGL2a.5
Remove junk upstream of the luciferase gene

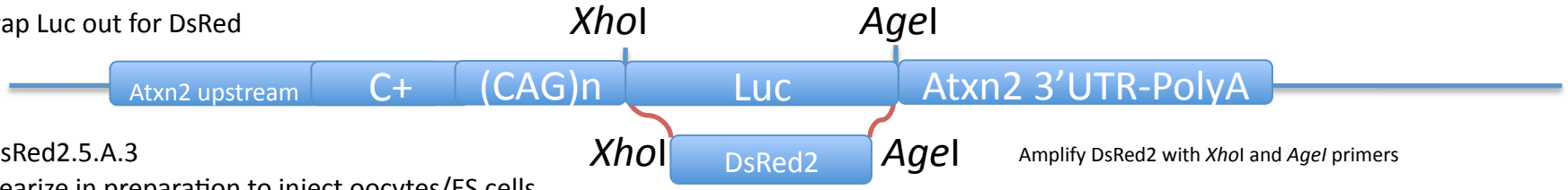
pGL2b.5
Insert the atxn2 3'UTR



pGL2b.5.3
Insert the atxn2 "exon1 option"



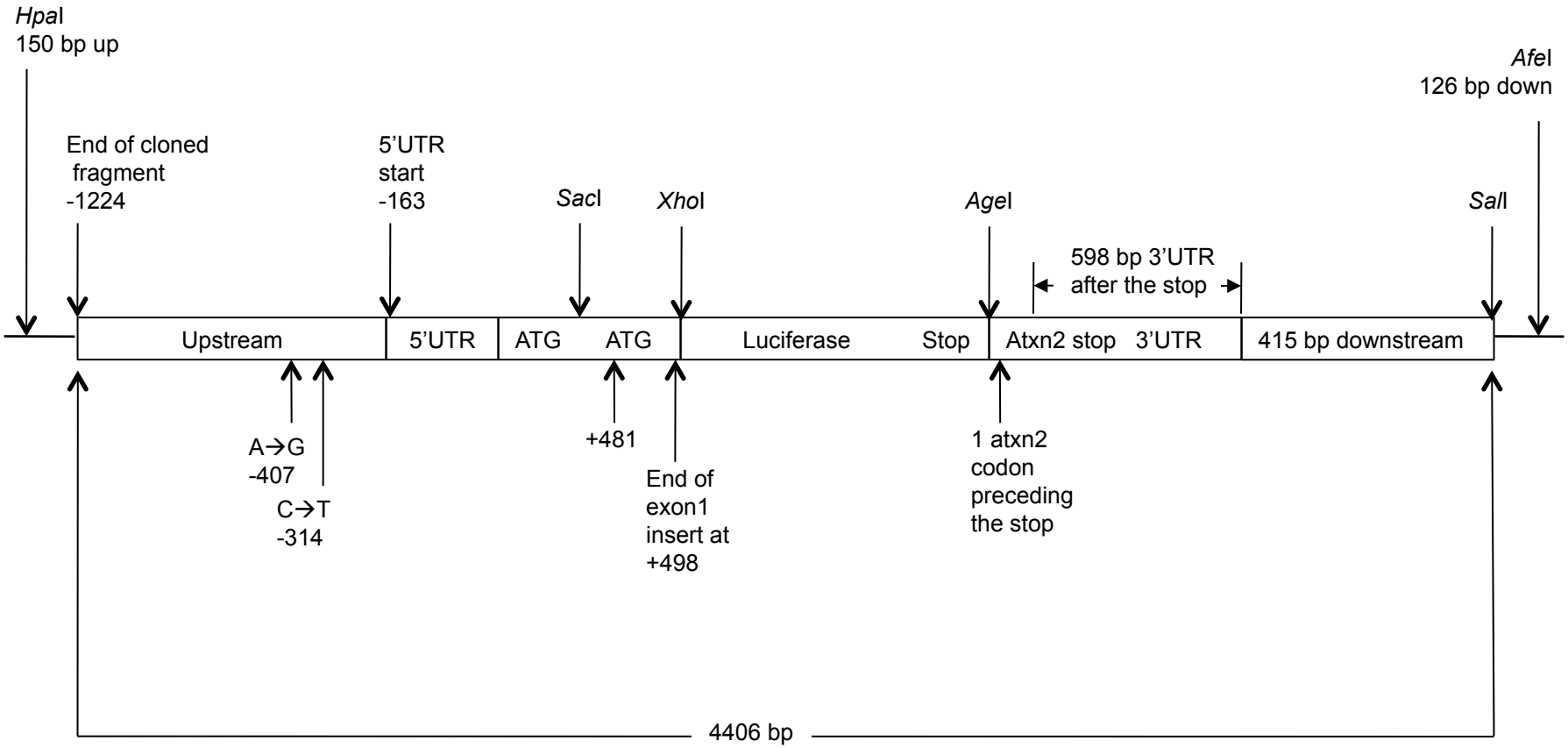
pGL2b.5.A.3
Swap Luc out for DsRed



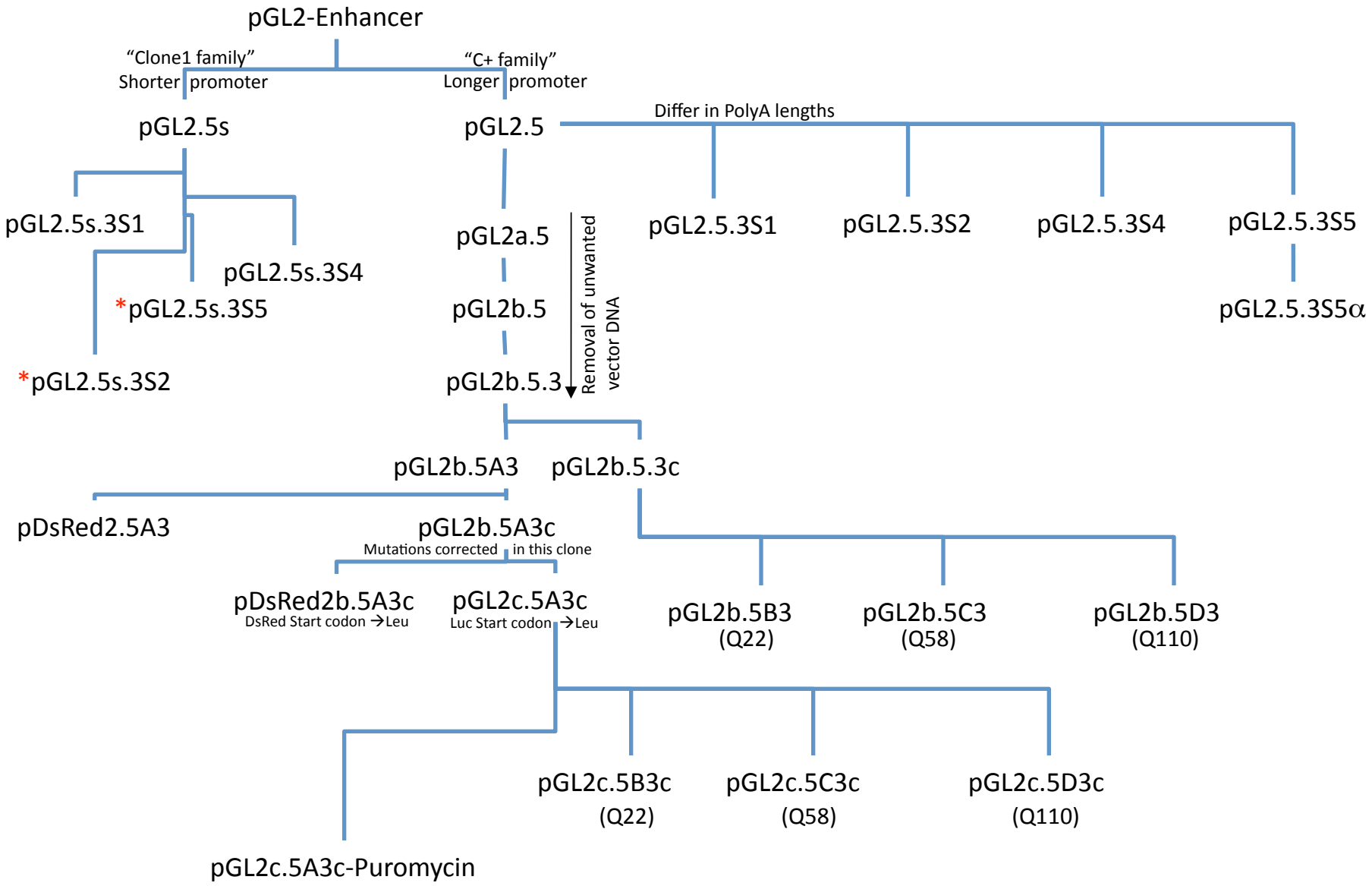
pDsRed2.5.A.3
Linearize in preparation to inject oocytes/ES cells

pGL2c.5A3c

Revised map



Summary of all clones



Updated Feb 2, 2009

Key:

pGL2.5 5 denotes "C+" and 5' denotes "clone1"

pGL2a lowercase letters designate vector modifications, changes to up or downstream sequence of the luc gene

pGL2b.5.3 3 denotes atxn2 3'-UTR

pGL2.5.3S1 S number (S1..S5) denotes the primer set used to obtain different atxn2 3'-UTRs

pGL2b.5.A.3 uppercase letters designate the form of the completion of the atxn2 Exon1 (with or without CAGn)
A=includes 2nd ATG before the polyglutamine; B=(CAG)22; C=(CAG)58, D=(CAG)110.

pGL2.5.3S5α symbols indicate subsequent modification to atxn2 3'UTRs

pDsRed2.5.A.3 pDsRed2 indicates that luciferase was swapped out of the pGL2 construct and replaced with DsRed2 from pDsRed2

pDsRed2b.5.A.3 The b in pDsRedb indicates that the start codon in DsRed2 was changed to CTG to encode leucine.

pLacZ.5.A.3 pLacZ indicates that luciferase was swapped out of the pGL2 construct and replaced with LacZ from pLacZ

pGL2b.5.A.3.c Two accidental mutations in the 5'UTR corrected

* A red asetricks indicates that the construct and everything below it in the family has not yet been made

Start Codon Choices

pGL2b.5.A.3	pDsRed2.5.A.3	pDsRed2b.5.A.3
First Start Codon: 2154 bp 718 aa 78.9 kDa	First Start Codon: 1179 bp 393 aa 43.2 kDa	First Start Codon: 1179 bp 393 aa 43.2 kDa
Second Start Codon: 1674 bp 588 aa 61.4 kDa	Second Start Codon: 699 bp 233 aa 25.6 kDa	Second Start Codon: 699 bp 233 aa 25.6 kDa
Luciferase Start Codon: 1650 bp 550 aa 60.5 kDa	DsRed2 Start Codon: 678 bp 226 aa 24.8 kDa	DsRed2 Start Codon: None

Transfection of SHSY5Y or HEK293 with pGL2b.5.A.3 and pRL.SV40

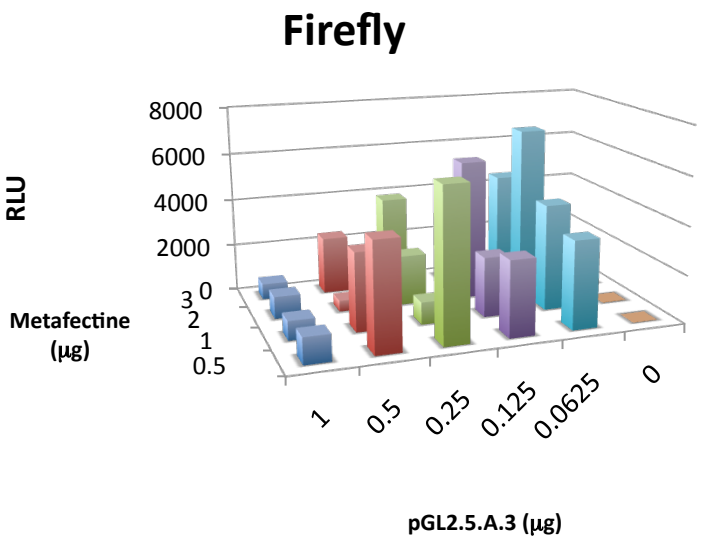
24-well dish

		pGL2b.5.A.3 (ug) + 0.04 ug pSV40-RL					
		1	0.5	0.25	0.125	0.0625	0
Metafectine (ul)	3	1:3	1:6	1:12	1:24	1:48	-
	2	1:2	1:4	1:8	1:16	1:32	-
	1	1:1	1:2	1:4	1:8	1:16	-
	0.5	1:0.5	1:1	1:2	1:3	1:4	-

Mix DNA and 15 ul serum free media. Add Metafectine and incubate 20 minutes. Add 300 ul cells in growth media. Assay for luciferase after 48 hours.

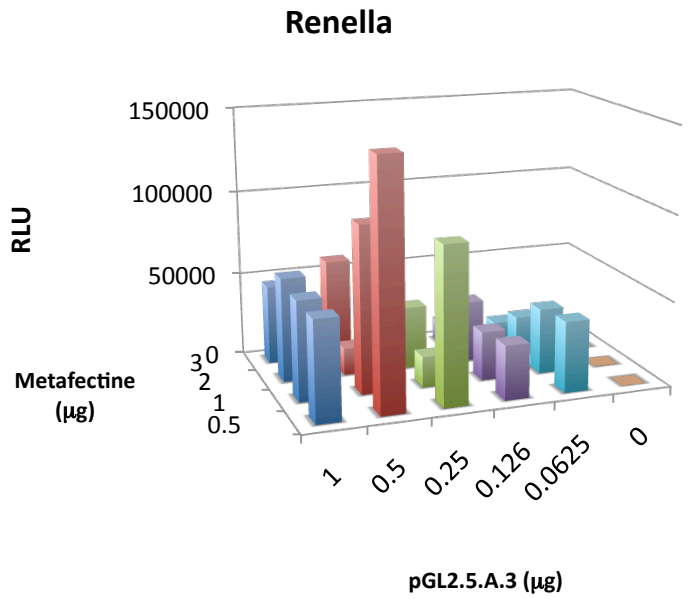
Results in next slides

HEK293



Ave RLU from 2 reads		pGL2b.5.A.3 (ug) + 40 ng pSV40-Luc					
		1	0.5	0.25	0.125	0.0625	0
Metafectine (ul)	3	622	2394.5	3877	4345	4475	17
	2	923	456.5	2084	5847.5	7003	22
	1	798.5	3251	924	2489.5	4408	17.5
	0.5	1078	4444.5	6270.5	3139.5	3653.5	11

Standard Deviations (single transfections read in duplicate)					
50.24542344	3.535533906	226.27417	39.59797975	28.28427125	2.828427125
11.3137085	44.54772721	114.5512986	208.5965005	240.4163056	1.414213562
33.23401872	43.84062043	31.11269837	10.60660172	91.92388155	4.949747468
155.5634919	28.99137803	300.520382	296.2777413	395.2726907	1.414213562



Ave RLU from 2 reads		pGL2b.5.A.3 (ug) + 40 ng pSV40-Luc					
		1	0.5	0.25	0.125	0.0625	0
Metafectine (ul)	3	46566.5	58545	19912	13238	9541.5	148.5
	2	61226	15915	36592	36192	23228.5	80
	1	58556.5	97741	18082	28683.5	38629.5	182.5
	0.5	58498.5	142960	92245.5	31869.5	41620	73.5

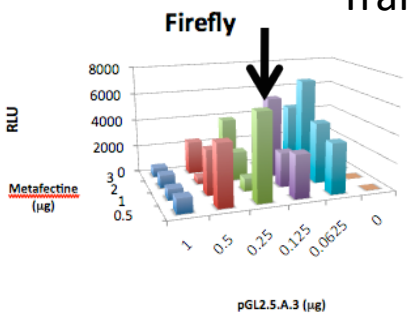
Standard Deviations (single transfections read in duplicate)					
959.5439021	562.8569978	309.7127702	12.72792206	180.3122292	30.40559159
305.4701295	21.21320344	519.0163774	502.0458146	159.0990258	14.14213562
272.2361108	330.9259736	164.0487732	348.6036431	47.37615434	37.4766594
45.96194078	1764.938526	193.0401513	24.74873734	627.9108217	7.778174593

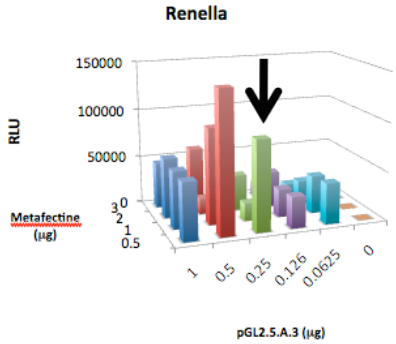
11/23/08

Transfection of SHSY5Y with pGL2 plasmids and pRL.SV40

24-well plate

11/26/08

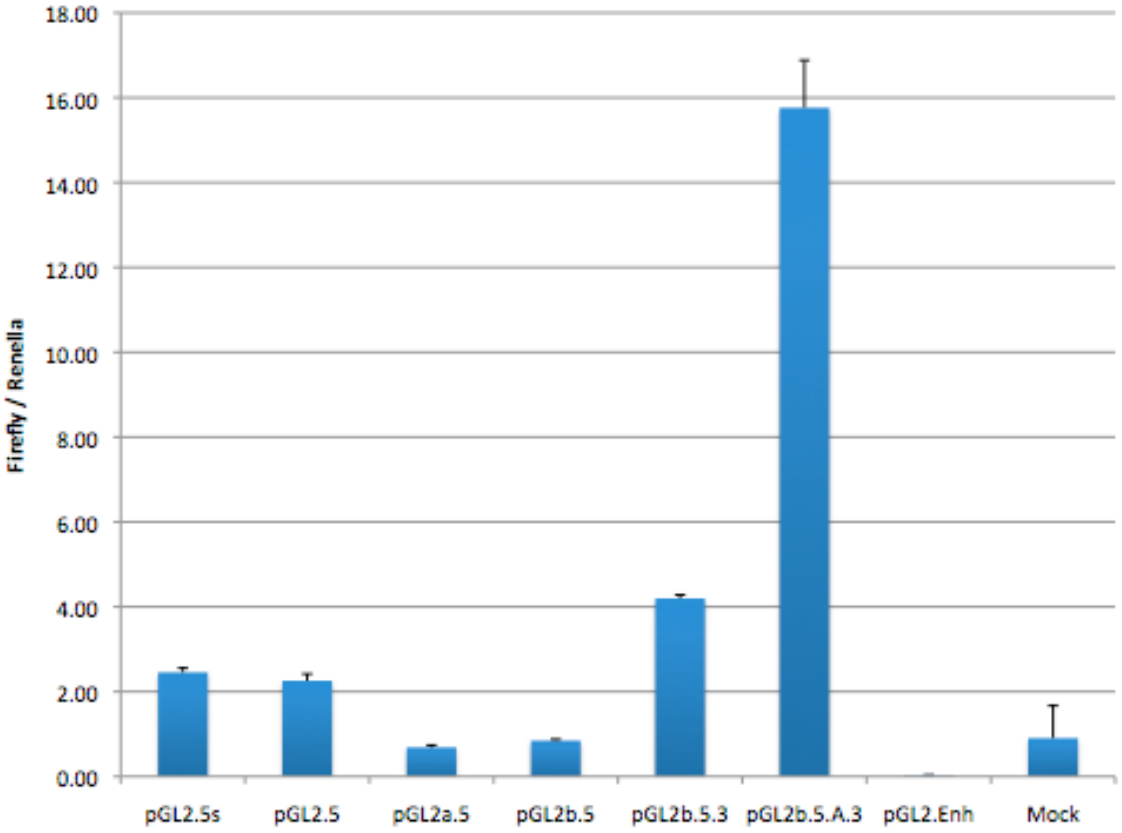




← Used conditions of the 11/23/08 experiment

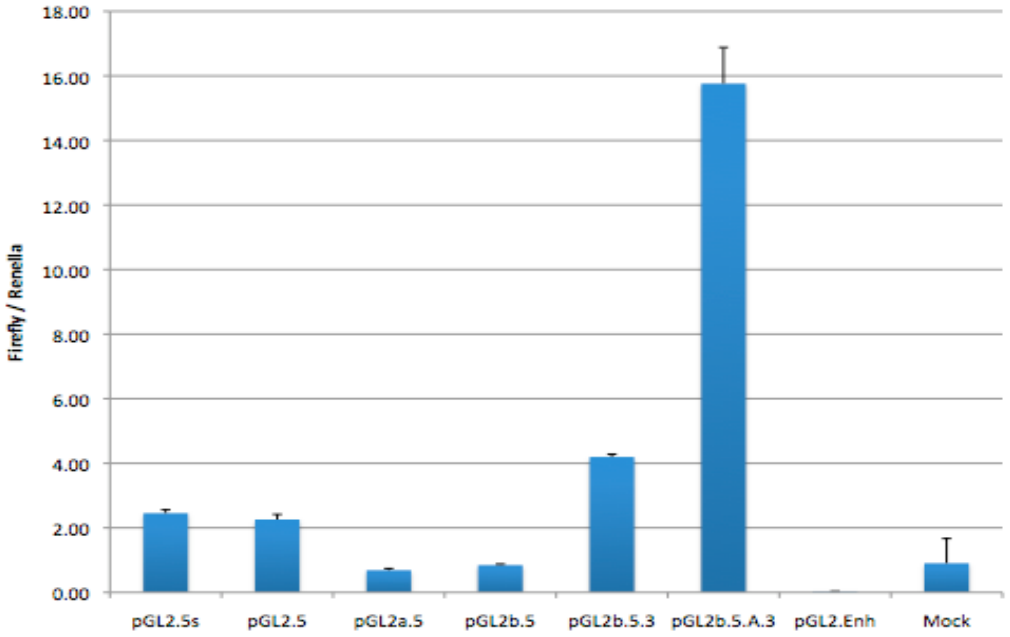
Mixed 250 ng DNAs and 15 ul serum free media. Added 0.5 ul Metafectine and incubated 20 minutes. Added 300 ul cells in growth media. Transfected each in duplicate in 24 well plate. Assayed each transfection for luciferase in triplicate after 48 hours.

Construct	Firefly (ave)	Renella (ave)	Ratio (Mean ± SD)
pGL2-5s (C1)	424	173.5	2.46 ± 0.10
pGL2-5 (C+)	441	196	2.26 ± 0.16
pGL2a.5	270	394.75	0.69 ± 0.05
pGL2b.5	377	446.5	0.85 ± 0.03
pGL2b.5.3	2274	542.25	4.20 ± 0.09
pGL2b.5.A.3	6185	388	15.76 ± 1.12
pGL2.Enh	103	3506.25	0.03 ± 0.001
Mock	7	10.75	0.90 ± 0.77
No Cells	4	5	0.6 ± 0.38



Preparation of pGL2b.5.A.3 for drug screening (and promoter analysis ongoing)

These are a series of plasmids prepared with the purpose of making pGL2b.5.A.3 as an end product with all vector junk sequence eliminated. Only 6bp restriction sites lie between exon1/ luciferase/and 3'utr. The final addition of exon1 puts the luciferase in frame (its out of frame in all others). The luciferase ATG is present in all clones. We are in the process of removing the luciferase ATG from 5A3. We hope to use pGL2b.5A3 for compound screening.



Transient transfections of HEK293 cells were performed in 24 well plates with constant amount of Renella plasmid. After 48 hours cells were collected in 160 ul of luciferase reagent, and 75 ul was placed in each of 2 wells of a 96 well plate. Firefly luc was read, 37 ul Stop N Glow reagent was added per well, and Renella luc was read. Transfections were in duplicate, luciferase reads were in duplicate, means and standard deviations resulted from four values.

Correction of 2 point mutations found in Molly's C+ and Clone1 clones

We used Gene Editor to simultaneously correct two point mutations.

The mutations are

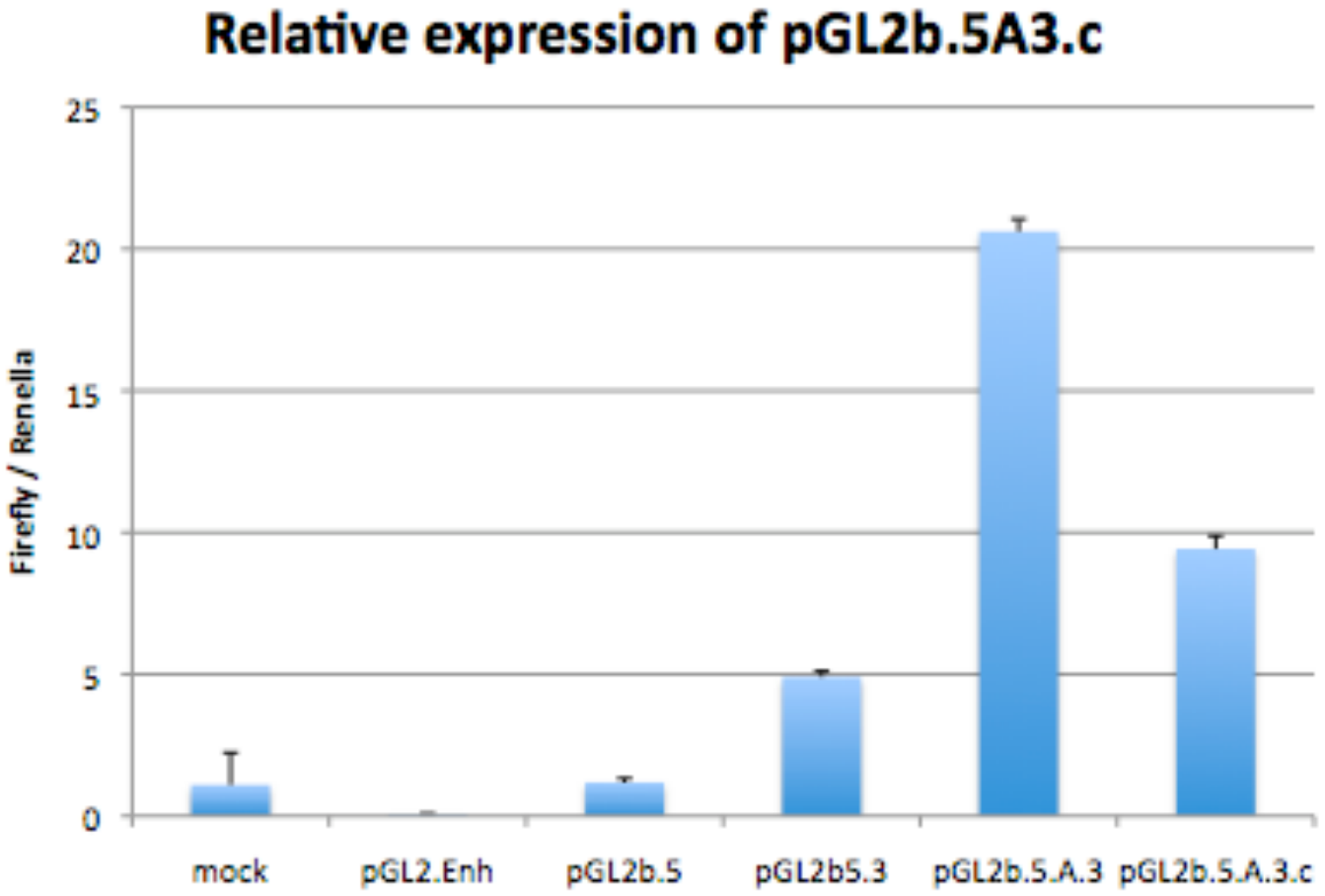
gtagcaAgacac…….gaagatgCtga correct sequence
gtagcaGgacac…….gaagatgTtga mutated sequence

Located at -406 and -313 in the 5' UTR of the *atxn2* gene relative to the 1st ATG
(-244 and -151 relative to the transcription start site)

pGL2b.5.3 Both mutations fixed…. named pGL2b.5.3.c
pGL2b.5.A.3 Both mutations fixed….. named pGL2b.5.A.3.c

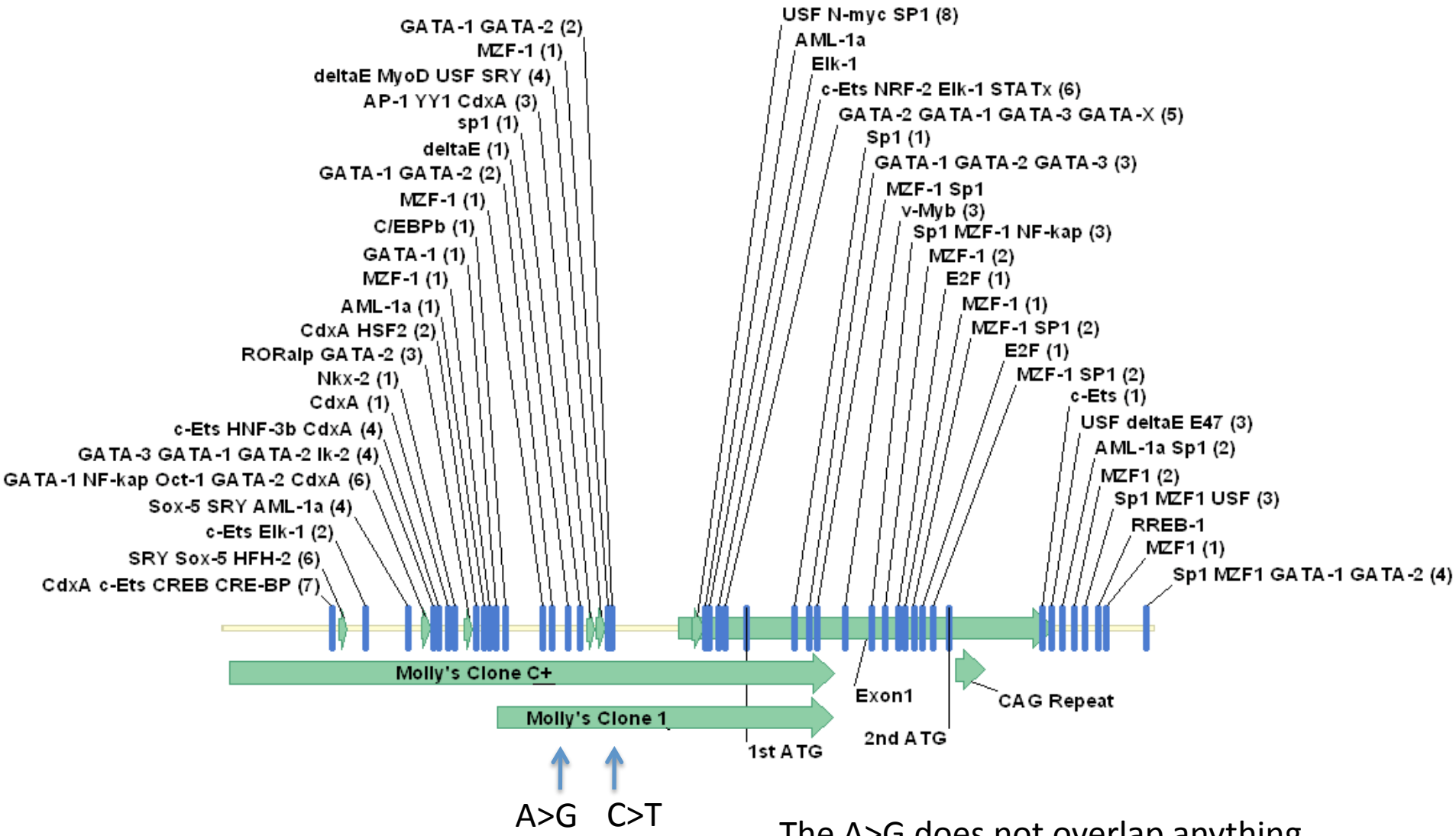
Sequences returned 12/5/08

The presence of the 2 point mutations appears to enhance expression



12/8/08

Putative transcription factor binding sites in *Atxn2*



Lyf1 and p300 further downstream

The A>G does not overlap anything

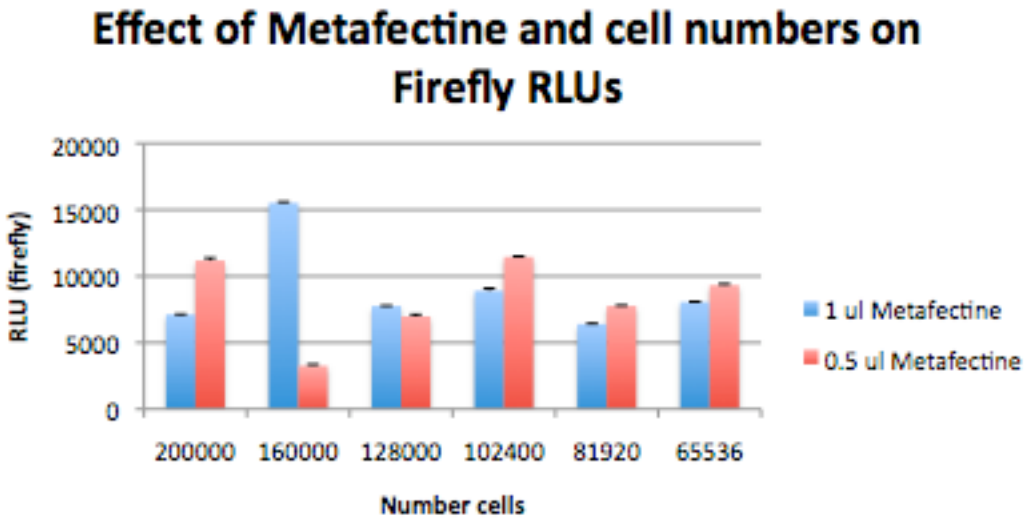
The C>T is in the center of a GATA-1 site

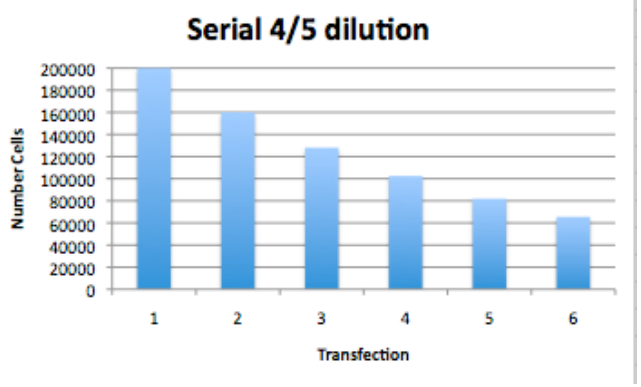
Some things about these transcription factors…

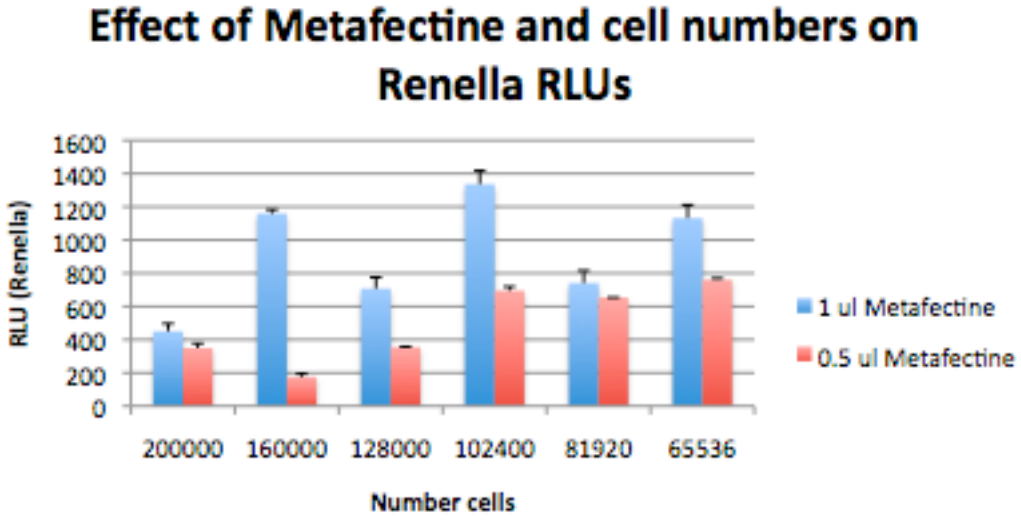
CDXA	caudal type homeobox 1 (CDX1)
SRY	Sex determining region Y (HMG box family)
SOX-5	SRY related HMG box
Nkx-2	Homeodomain transcription factor
C-Ets	encoded by protooncogene
CREB	in HD, binds atxn3, inhibits neuronal death, expanded polyQs bind TAFII130 which binds CREB
CRE-BP	= CREB
Elk1	mapk pathway
AML-1a	RUNT family of transcription factors, antagonizes AML-1b activated genes
GATA	cell growth, cancer, erythroid development
IK2	also in alpha-synuclein
HNF-3b	hepatocyte nuclear factor, also called FoxA2
RORalpha	controls melatonin signaling, circadian rhythms
MZF1	N-cadherin expression, proliferation, tumorigenesis
USF	upstream transcription factor, implicated with SP1 in amyloid expression
N-Myc	regulates parkin expression, reactive astrocyte related neurodegenerative disorders
E2F	helix turn helix, retinoblastoma, ovarian cancer
SP1	upregulated in AD
v-Myb	derived from c-Myb (truncation), transforming, cancer
RREB	Ras responsive transcription factor, binds RRE, cancer, ras raf signaling
Lyf1	shown to activate polyQ proteins (Okazawa 2003 Cellular and moleculr life sciences 60)
P300	binds atxn3

Effect of cell numbers on Metafectine transfection

Mixed 250 ng of pGL2b.5A3 + 80 ng pRLSV40 and 15 ul serum free media. Added Metafectine and incubated 20 minutes. Added 300 ul cells in growth media and plated into a single well of a 24 well plate. Assayed each transfection for luciferase in duplicate after 48 hours.



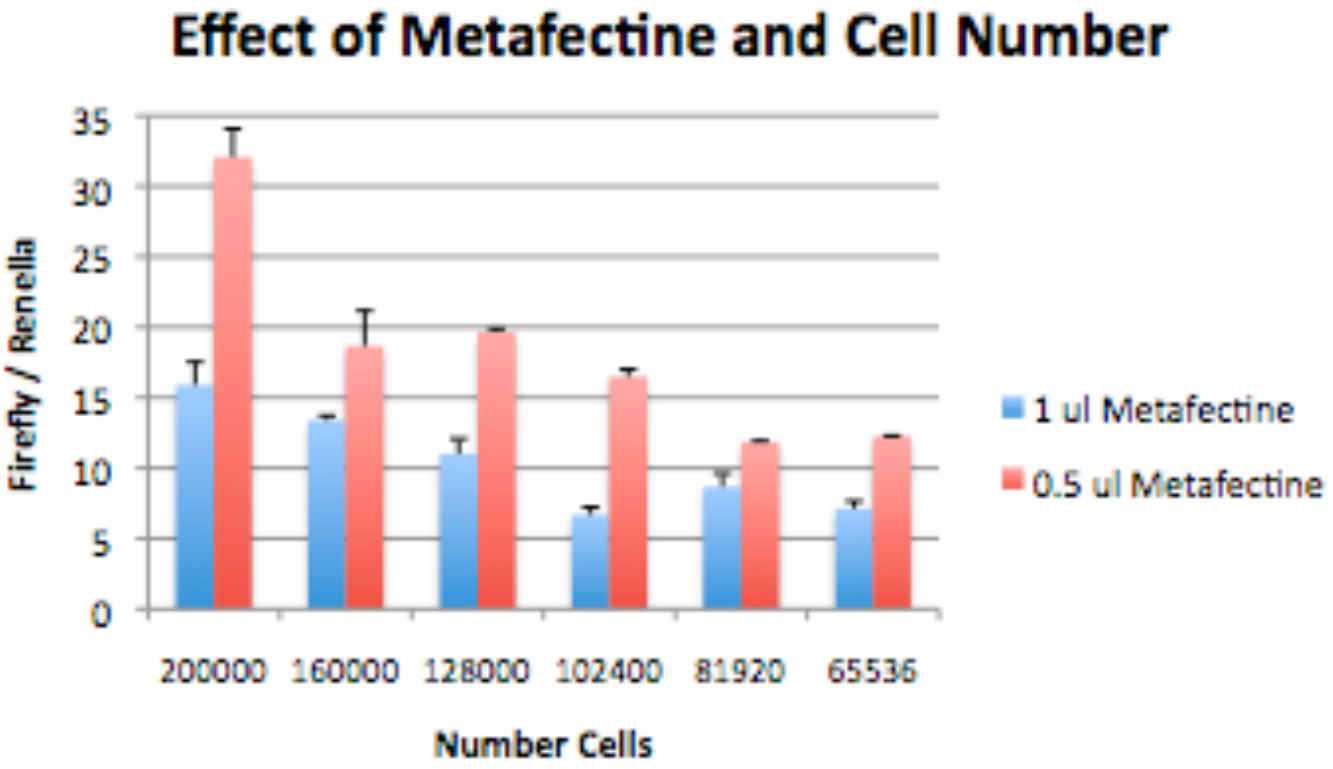




HEK293 cells

12/08/08

Effect of cell numbers on Metafectine transfection… cont.



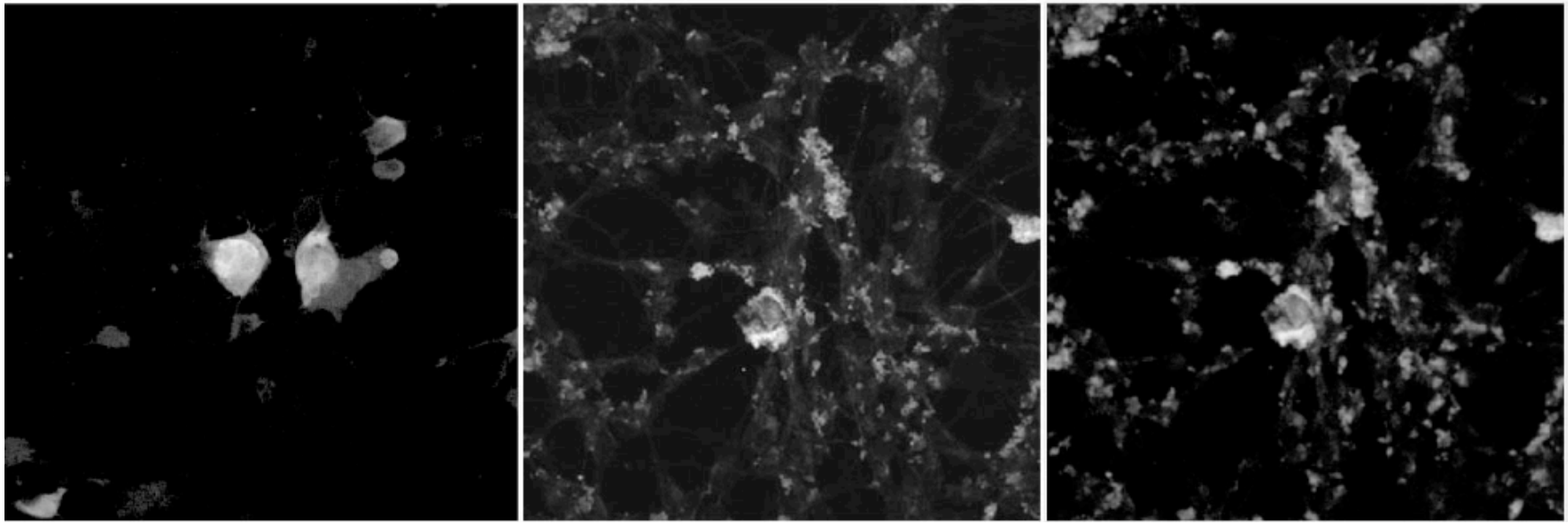
Transfection of SHSY5Y with pDsRed2-5.A.3

Constructs Used:
pDsRed2
pDsRed2-2.5.A.3
Mock

Mixed DNAs and 15 ul serum free media. Added 2 ul Metafectine and incubated 20 minutes. Added 300 ul cells in growth media. Transfected each in duplicate in 24 well plate. Wells had HCl treated coverslips. Coverslips were paraformaldehyde treated 15 min at RT, PBS washed and mounted 48 hours post transfection. Note that I meant to add 1 ul Metafectine to match conditions of 11/19/08 but instead I added 2.

11/23/08

Transfection of DsRed2 plasmids in SHSY5Y cells

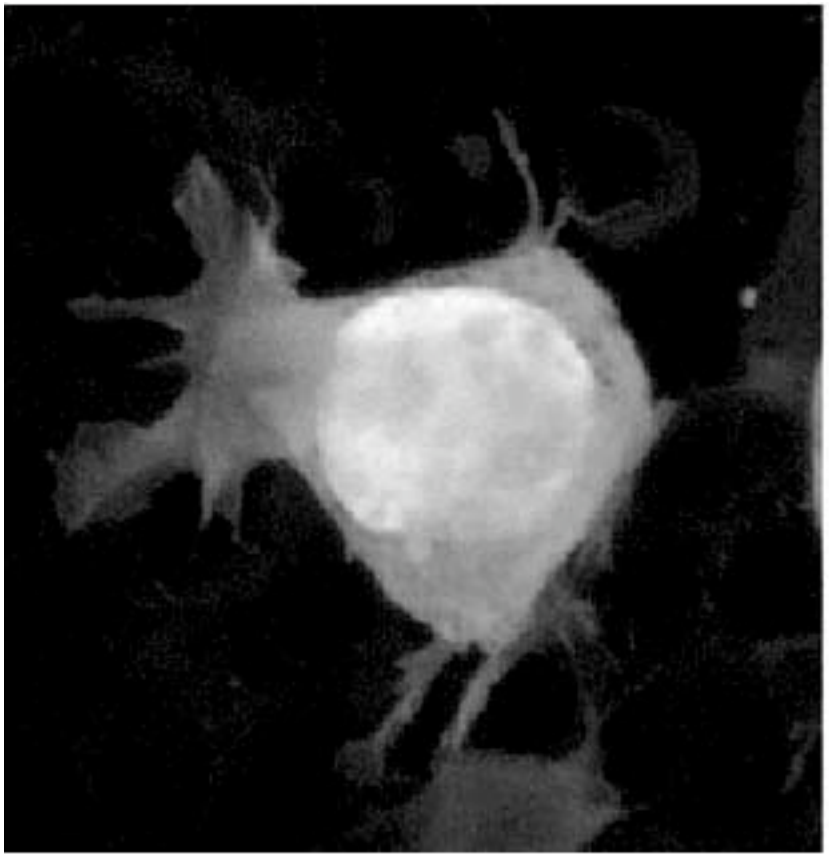


pDsRed2

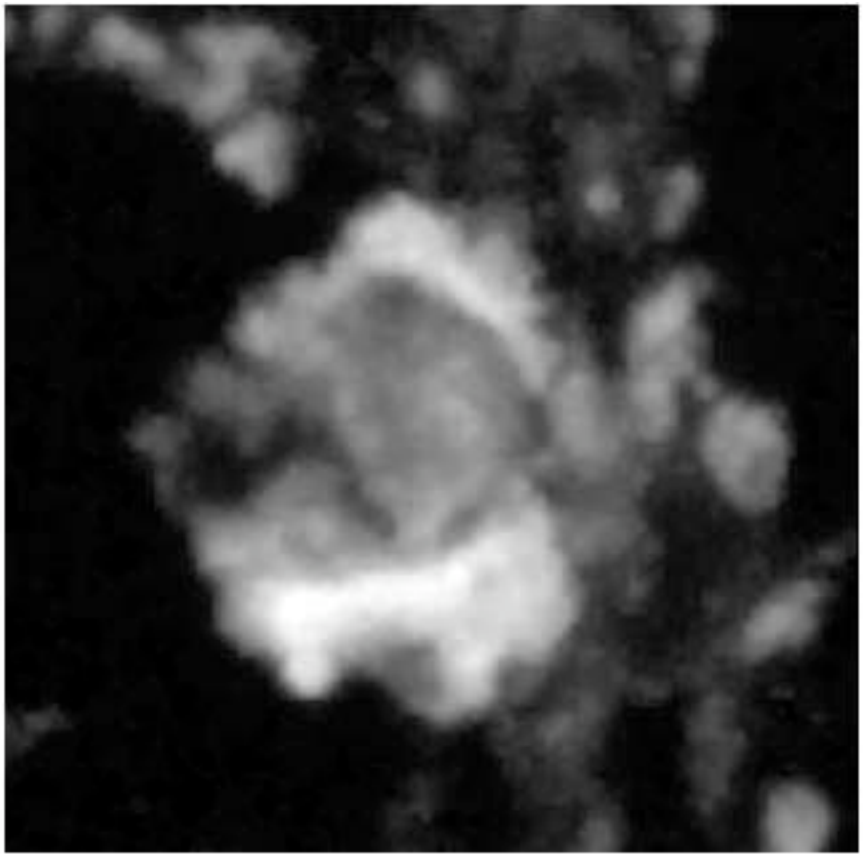
pDsRed2-5.A.3

pDsRed2-5.A.3
"Unmixed"

DsRed2 plasmids in SHSY5Y cells…closer up



pDsRed2



pDsRed2-5.A.3

CpG Island Prediction

Human sequence searched

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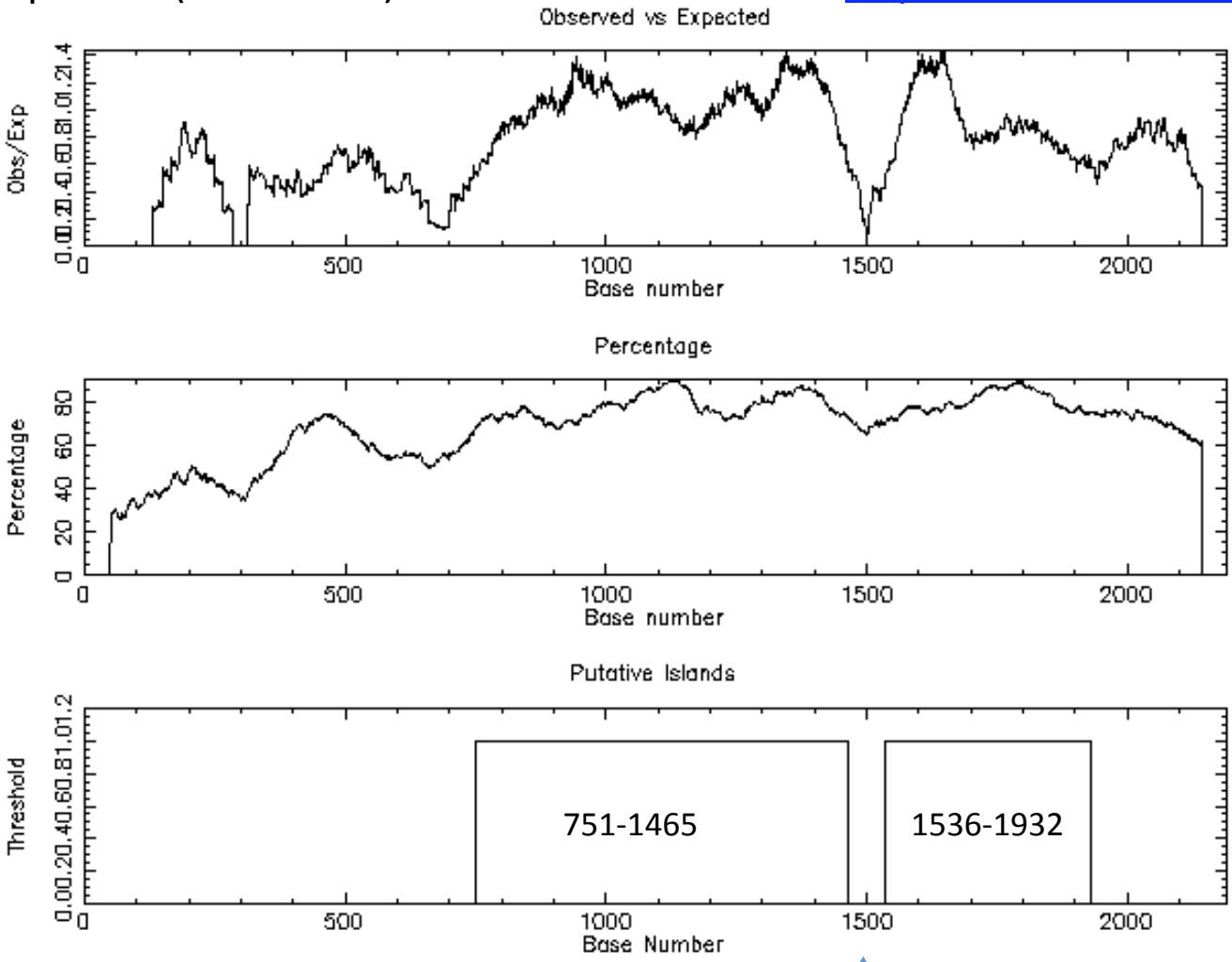
CpG Plot (online tool) http://www.ebi.ac.uk/emboss/cpgplot/



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

Hypothetical Atxn2 Function in Protein Translation

In C. elegans Atxn2 represses translation via MEX-3 and GLD-1 (Ciosk et al., 2004)

MEX-3 is a cytoplasmic RNA binding protein that associates in large RNP nuclear foci in arrested C-elegans oocytes. Select RNAs are enriched in these RNP foci, not including all RNA types. MEX-3 mediates translational repression in C. elegans (Schisa et al 2001). Post-transcriptional RNP-containing bodies include P bodies (processing bodies characterized by RNA degradation) and stress granules (stalled translation complexes requiring eIF2α phosphorylation) and include such things as RNAs, decapping proteins, and eIF4E (Anderson and Kedersha 2006). There are 4 human homologs of MEX-3 (*hMex-3A*, *hMex-3B*, *hMex-3C*, & *hMex-3D*); *hMex-3A* and *hMex-3B* co-localize with Argonaute (AGO1 & AGO2, RNA silencing proteins) in P bodies of MCF7, and *hMex-3C* directly bound AGOs outside P bodies (Buchet-Poyau et al., 2007). *hMex3* proteins bind select RNAs via their KH domains.

The lecture on Tueday by Joan Steitz described how AGO-bound miR369-3 bound ARE in TNFa to activate protein translation in growth arrested cells in a FXR1-dependent manner or to inhibit proteins translation in proliferating cells in a FRX1-independent manner.

A hard to find published abstract showed that Tino was hMex3 and that it bound an ARE in bcl-2 mRNA. A microarray approach to finding binding sites revealed sequences that more than 50% contained consensus sequence homology for binding by Quaking, the human homolog of GLD-1 (Donnini et al., American Journal of Pathology Sept 08 Vol 173 Supplement).

GLD-1 binds the TGE element located in the 3'UTR of the C. elegans tra gene to repress tra expression allowing hermaphrodite spermatogenesis.

Atxn2 interacts with DEAD/H-box RNA helicase DDX6 in P-bodies and stress granules. Overexpression of atxn2 reduced the number of P-bodies but not stress granules. Underexpression of atxn2 reduced the number of stress granules.

→ But what is the function of atxn2 in stress granules and P-bodies?
→ Can we pull down atxn2 and determine specific messages it bound?