Environmental toxins vs genes associated with PD

PARKIN!

hypotheses

1) Altering the level of specific PD-associated genes reduces the ability of DA-like cells to protect themselves from exposure to specific environmental toxin.
2) Exposure to sustained sublethal doses of environmental toxins overtime exacerbates the development of PD phenotypes in transgenic/or gene ko of animal models of PD associated genes.

specific Aims

Aim 1: Generation of cell lines expressing shRNAs or siRNA against PD associated genes (PARK2, PINK1, and DJ1) and controls.

Aim 2: Characterization of the effectiveness of shRNAmi and shRNAi knock-down by Western blots and quantitative RNA PCR and RNA microarrays.

Aim 3: Investigating the ability of PD-associated genes shRNA and siRNA transfected cell lines and mouse ko model to protect themselves against paraquat cytotoxicity and other environmental toxins.

background

Basic facts about Parkinson Disease

Incidence: About 1 million patients with PD in the US.

Age range: 40-70 for PD; younger than 20 for juvenile parkinsonism.

Neuropathology: Loss of dopamine-producing nigral neurons in the substantia nigra subcompacta, presence of Lewy bodies in the surviving neurons.

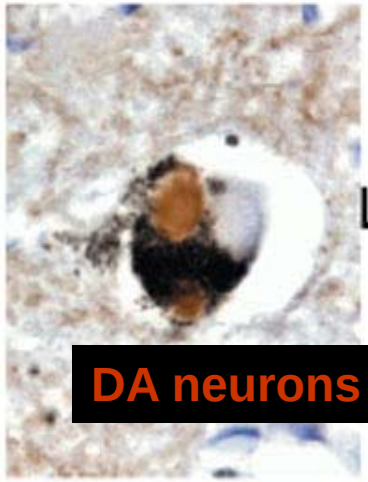
Causative factors: Environmental or Genetic Factor(s), or interaction of both.

Phenotypes: TRAP

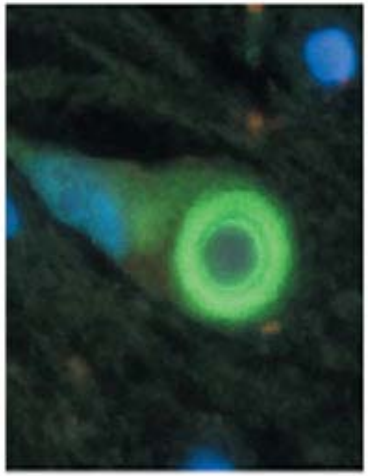
- Resting **T**remor: movement at rest-most suggestive of PD.
- Cogwheel **R**igidity;
- Bradykinesia/ **A**kinesia: Unable to move.
- **P**ostural reflex impairment: Can't control posture=push then fall

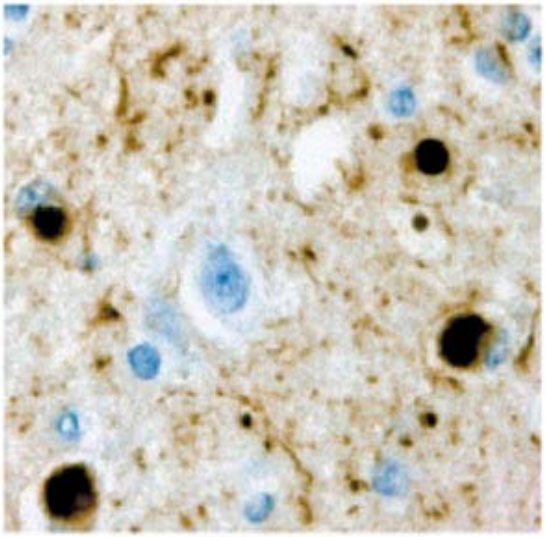
Types of Lewy bodies and LB neurites

1) **LBs in DA neurons are used to define classical PD.**
2) **However, LBs or Lewy neurites are also found in other areas of the brains such as the neocortex, amygdala, and hippocampus in later stages of PD or AD.**
3) **LB is a spherical shape body composing of several proteins. Most of these proteins such as alpha synuclein, parkin, and tau are later found to be involved in causing PD or other degenerating diseases.**

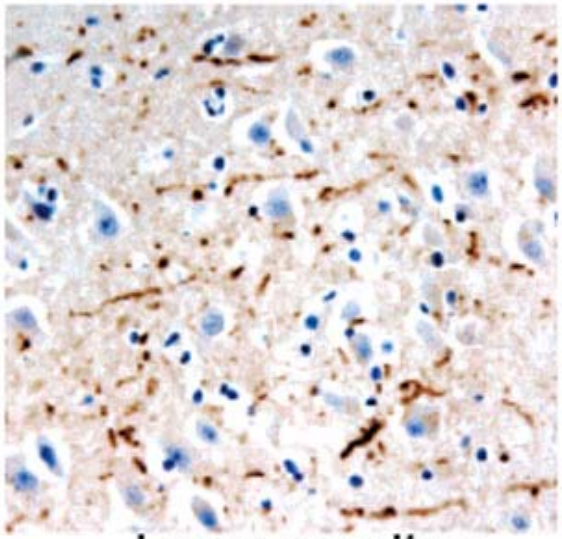


Classical LBs in pigmented neurons of the brainstem.





Cortical LBs in the neocortex &amygdala

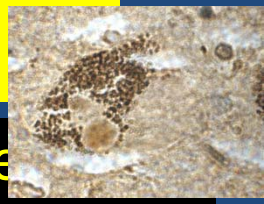


Lewy neurites in the hippocampus

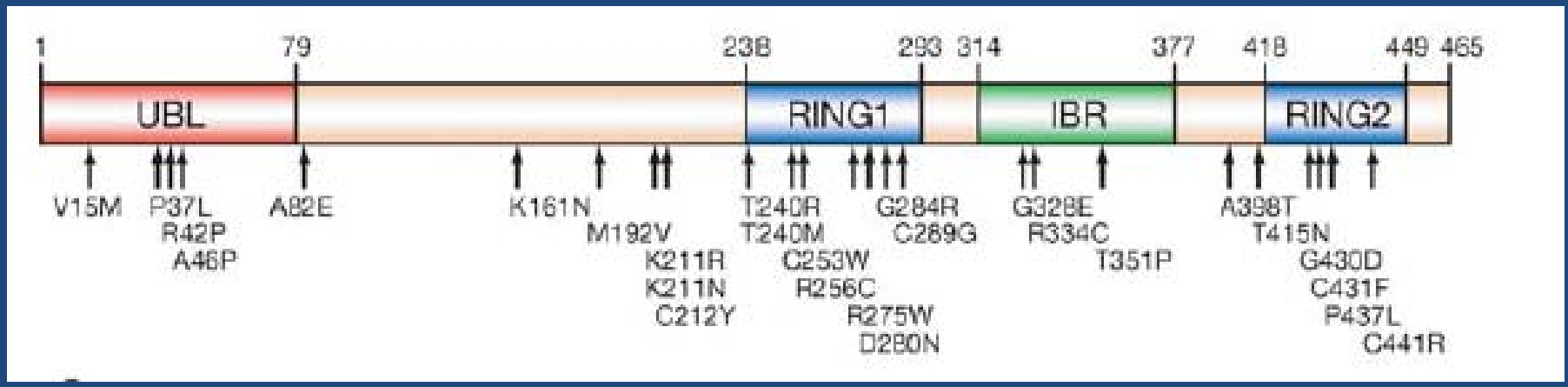
Name	Gene locus	Gene	Inheritance
PARK1/4	4q21	α-Synuclein	Autosomal dominant/synaptic vesicle
PARK2	6q	Parkin	Autosomal recessive/synaptic-everywhere
PARK3	2p	?	Autosomal dominant-
PARK5	4p	UCH-L1	Dominant (rare-one family)
PARK6	1p	PINK1	Autosomal recessive/mitochondria
PARK7	1p	DJ-1	Autosomal recessive/mito-
PARK8	12p	LRRK2	Autosomal dominant
PARK9	1p	ATP13A2/ lysosomal ATPase	Autosomal recessive/lysosomal
PARK10	1p	?	?
PARK11	2q	?	?
PARK12	Xq21-q25	?	?
PARK13	2p12	HTRA2, serine protease	Identified previously by yeast-two hybrid interaction with presenilin 1-an AD gene

JUST PARKIN

PARK2 autosomal recessive juvenile parkinsonism (AR-JP) and parkin

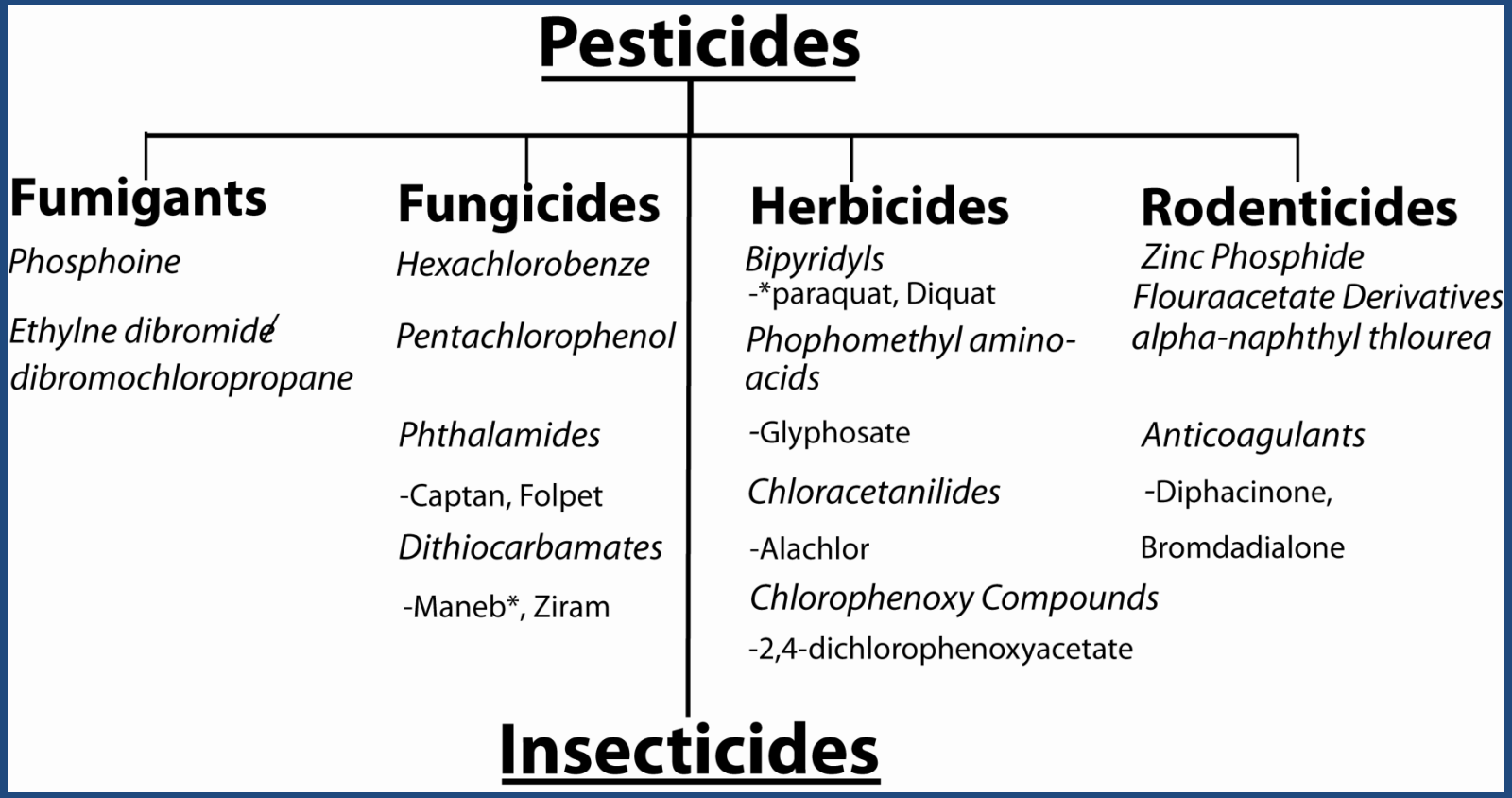


- Parkin is an E3 ubiquitin ligase, most mutations are localize the functional motifs-ubiquitin like domain, the RING1, RING2, In-Between Rings region.
- Parkin KO showed moderate (E3 KO) or no (exon-2 KO) defect on dopamine transmission and no dopaminergic neuron degeneration. Some substrates have been shown to be increased in Parkin KO mice.
- Overexpression of parkin protected cells against environmental toxins.



Brief introduction on pesticides

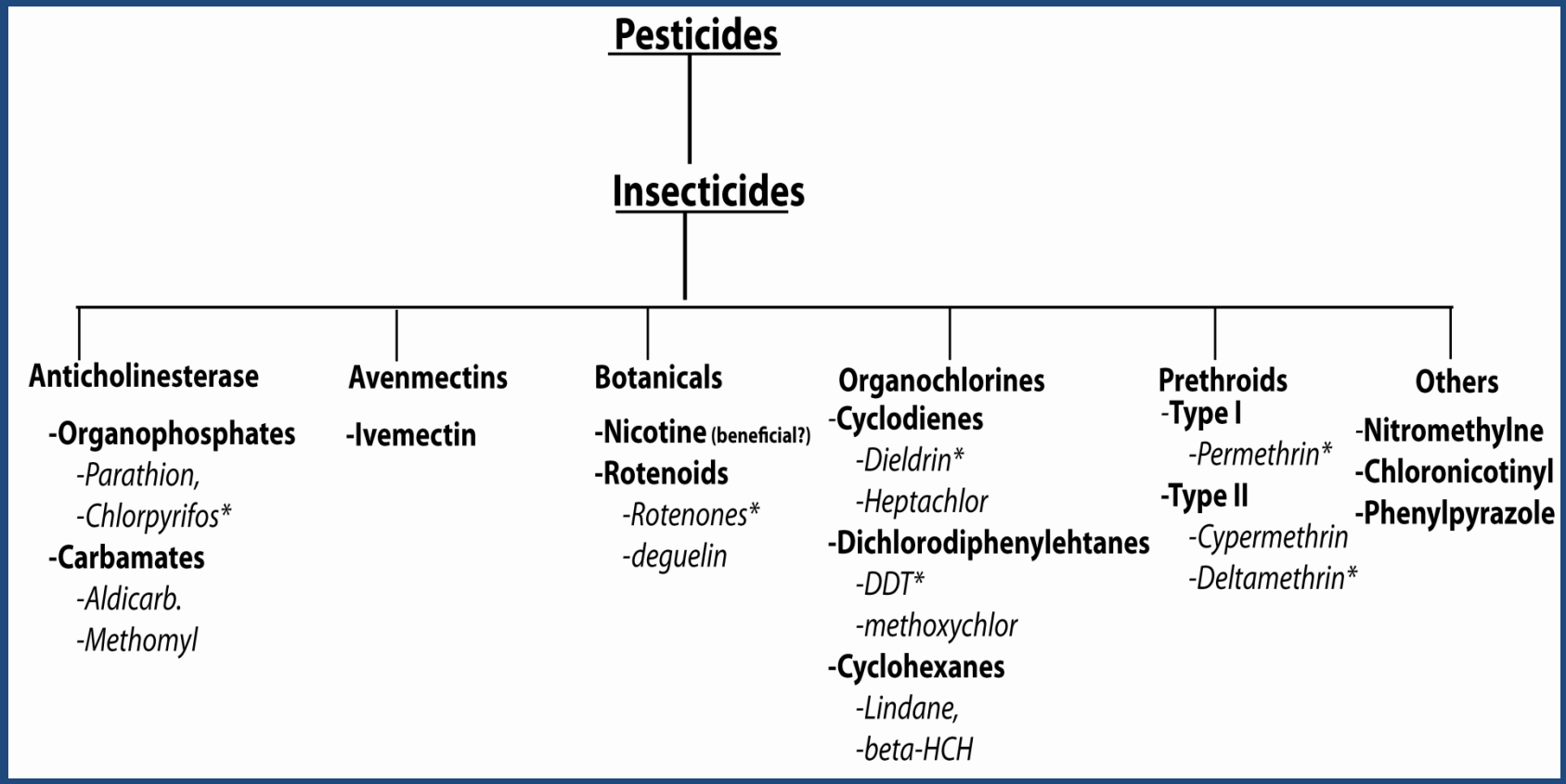
Classes of Pesticides



- *Three classes of Pesticides:* ***Herbicides, Fumicides, and Insecticides***

Hatcher JM et al, 2008_Review: Trends in Pharm. Sci. vol 29:322-329

SubClasses of Insecticides



- *There are several subclasses of **Insecticides, many compounds are neurotoxic.***

Hatcher JM et al, 2008_Review: Trends in Pharm. Sci. vol 29:322-329

Paraquat and DJ1 and models of PD associated genes

Paraquat caused Selective Degeneration of DA Neurons

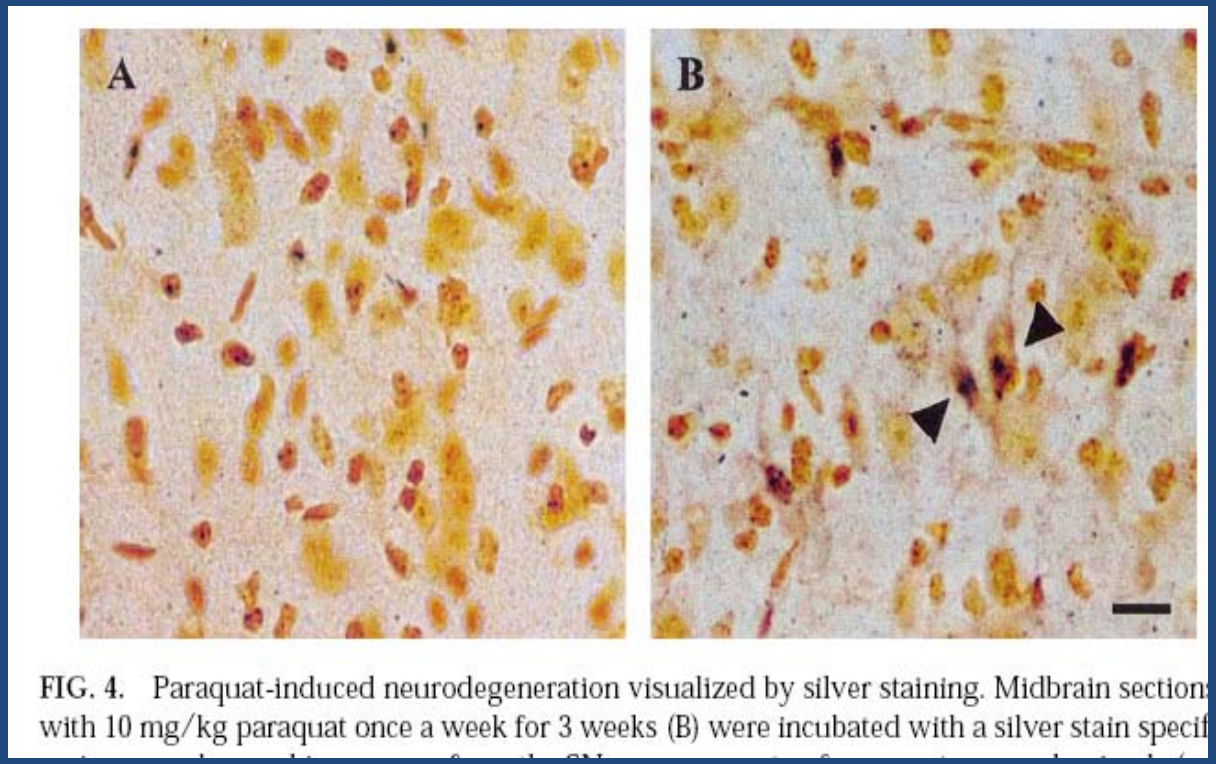


FIG. 4. Paraquat-induced neurodegeneration visualized by silver staining. Midbrain section
with 10 mg/kg paraquat once a week for 3 weeks (B) were incubated with a silver stain specif

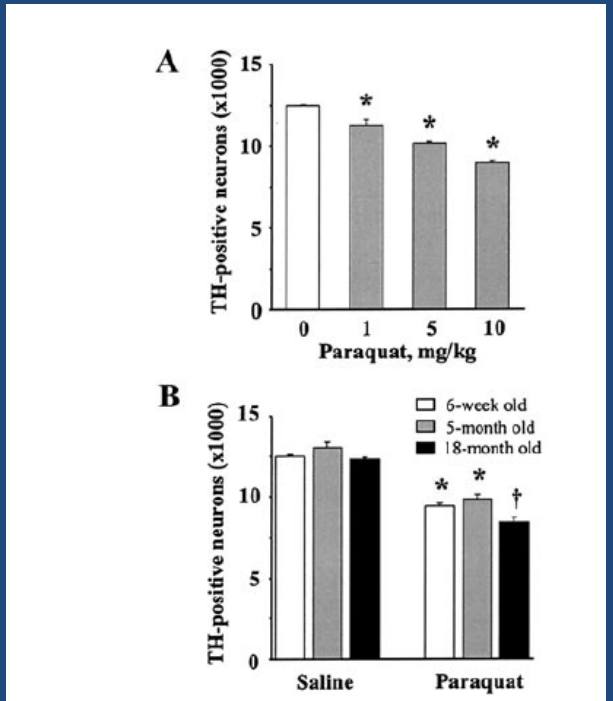
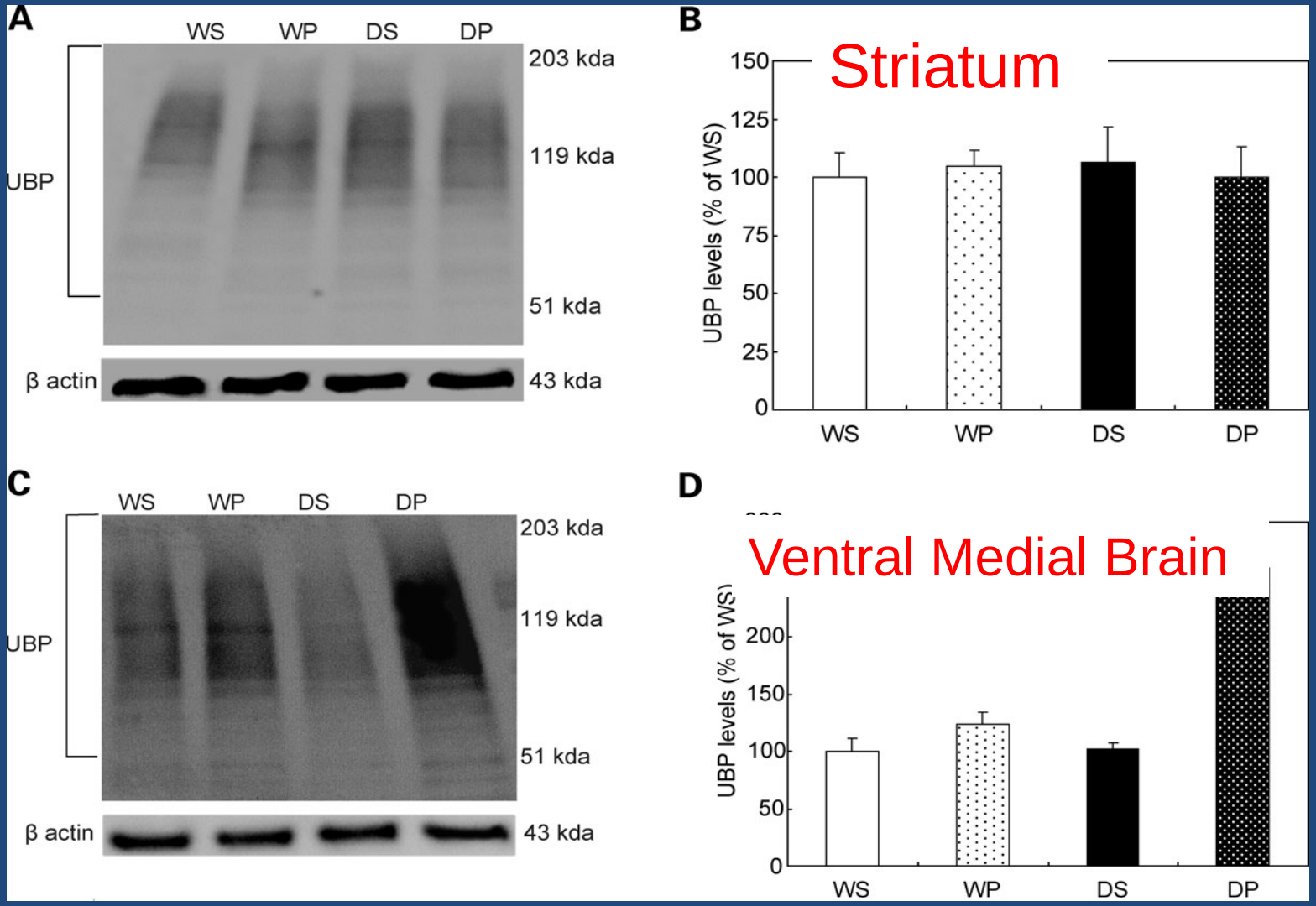


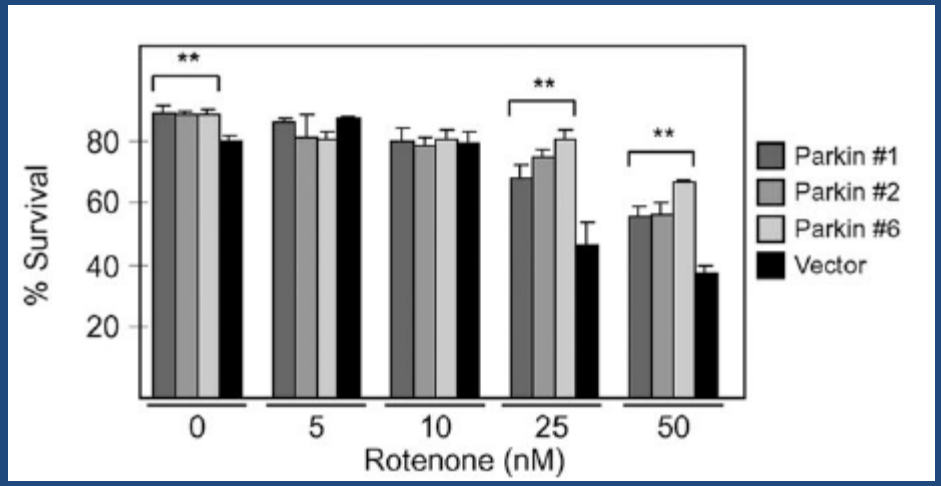
FIG. 3. Paraquat-induced nigral cell loss. TH-positive
were counted using a stereological technique in the SN pa

- McCormack AL et al, Neurobio. 10:119-127 (2002)

Effects of paraquat on the levels of ubiquitinated proteins (UBP) in the brain of DJ-1-deficient and wild-type mice.

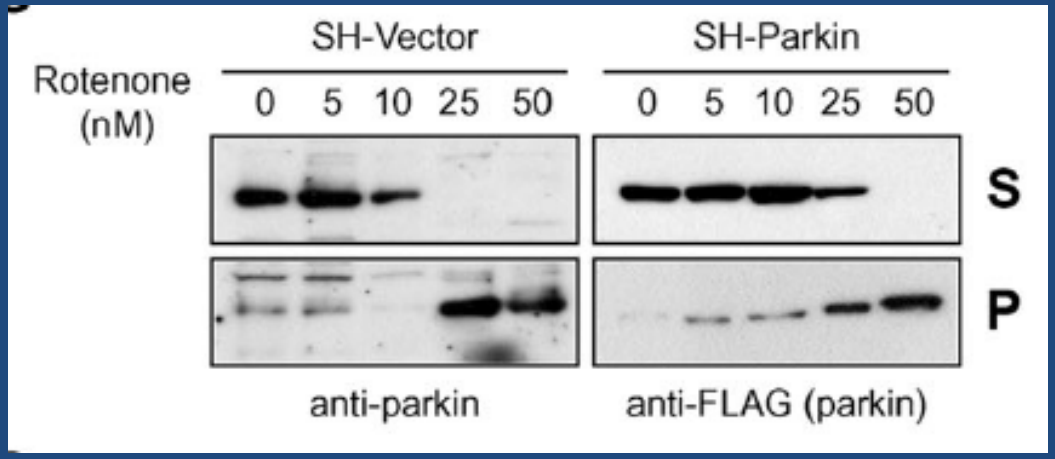


Overexpression of parkin protects cells against rotenone exposure (SH-SY5Y cells)



Overexpression of Parkin protected SH-SY5Y cells against rotenone

Rotenone treatment promoted parkin aggregation.



Wang C. et al.
Hum Mol Genet,
2005, Vol. 14, No. 24

Aim 1: Generation of PARK2 shRNA/siRNA cell lines

HEK293 and Neuro2a cell line

shRNA and siRNA plasmid

1) Origene mouse siRNAs Plasmids (5,430 bp)_G50, G51, G52, and G53, and two controls: TR3007 (vector) and TR3008.
2) pGENEClip (4,758 bp)-062M, 118M (mouse) and 116H and 062H plasmids.
3) pGIPz-shRNAmir (11,774 bp) from OPEN BIOSYSTEM: human shRNAmir lentiviral based plasmid : pGIPz-empty plasmid, pGIPz-84517, pGIPz-84518, and pGIPz-84520.

SIMILARITY OF ORIGEN MOUSE PARK2 OLIGOS WITH HUMAN PARK2 SEQUENCE

- G50 Query 1 CTTGAC**A**CG**A**GTGGACCT**G**AGCAGC**CATA** 29
- hu 404 CTTGAC**T**CG**G**GTGGACCT**C**AGCAGC 428

- G51 Query 1 GATAGTGTTTGTCAGGTTCAACTCCAGC**T** 29
- hu 104 GATAGTGTTTGTCAGGTTCAACTCCAGC 131

- G52 Query 3 **CT**GTCCTGGT**C**TTCCAGTG**TAACCACCGT** 29
- hu 843 GTCCTGGT**T**TTCCAGTG 859

- G53 Query 5 ACCACCAAGCC**T**TGTCC**T**CGCTGC**A** 29
- hu 1341 ACCACCAAGCC**C**TGTCC**C**CGCTGC 1364

SIMILARITY OF pGENECLIP PARK2 OLIGOs (Dan) WITH Mouse PARK2 SEQUENCE

- 1B: 116H hu 5 GGAACATCACTTGCATTACGT 16
- mouse 784 CATCCCTTGCAT 795

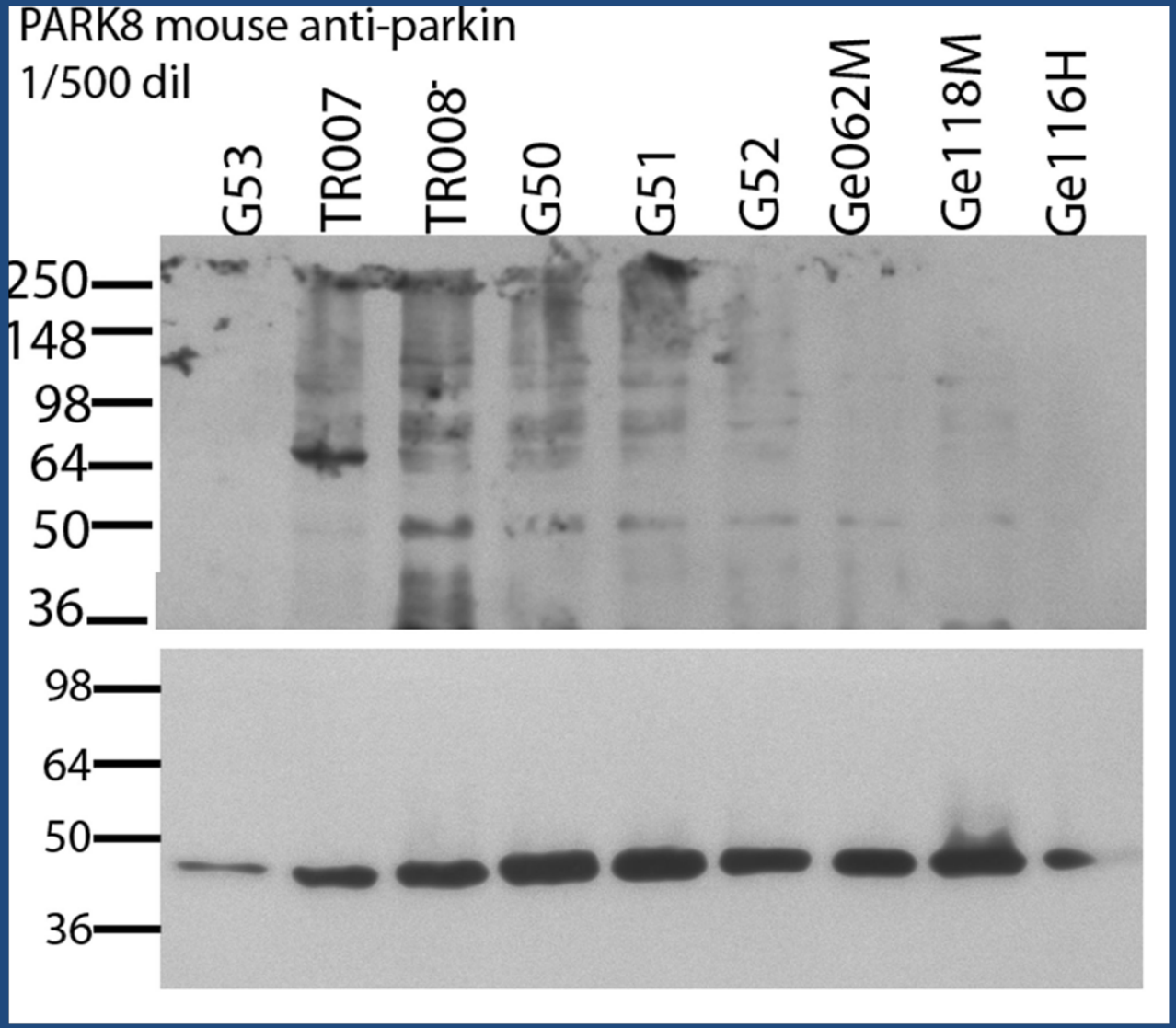
- 2B:62H Query 1 GCTGTCCCAACTCCTTGATTA 21
- mouse 954 GCTGTCCCAACTCCCTGATTA 974

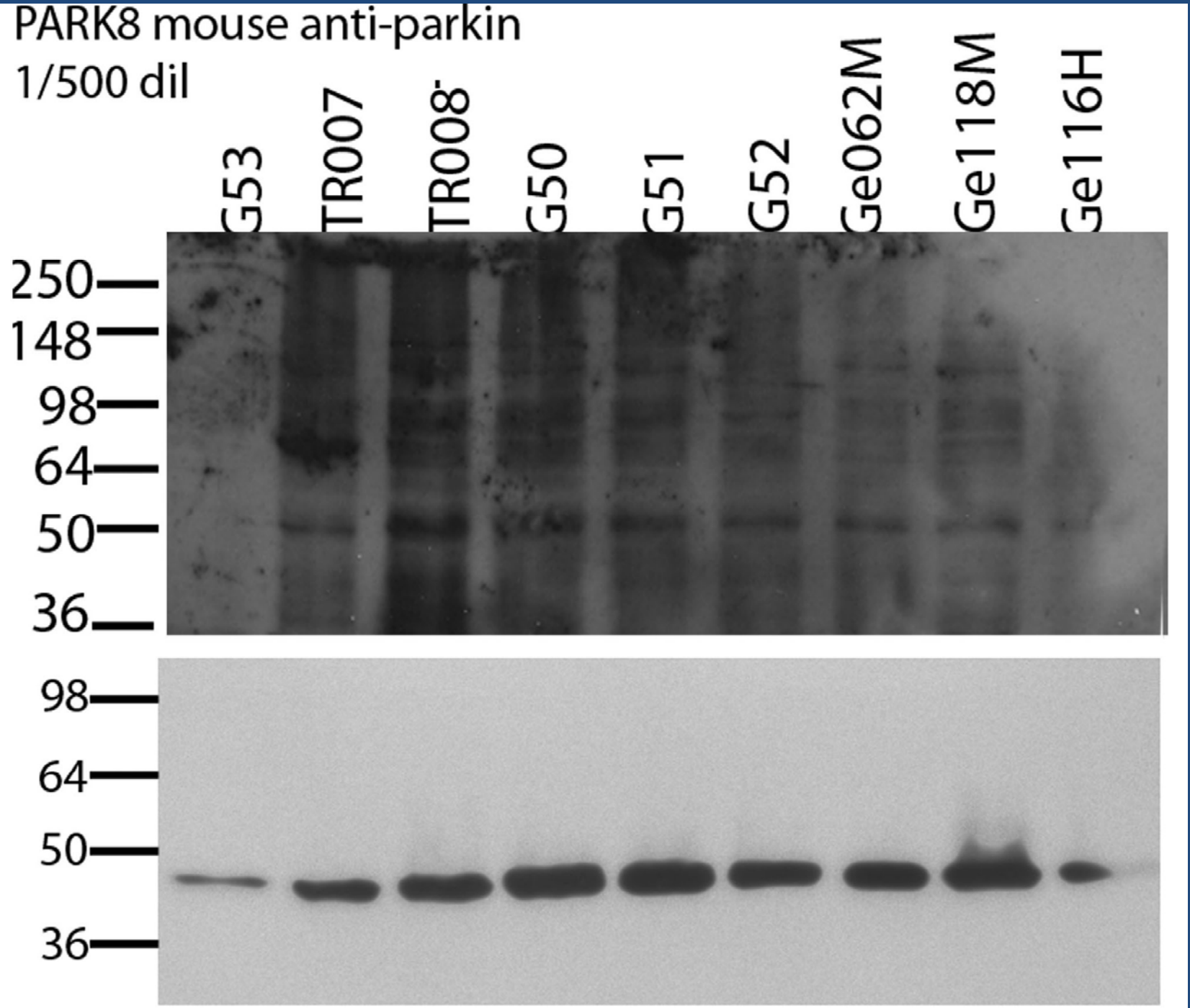
- 3B: 62M Query 1 GCTGTCCCAACTCCCTGATTA 21
  mouse 954 GCTGTCCCAACTCCCTGATTA 974

4B: 118M: Query 1 GCTGCAACGTGCCAATTGAAA 21
mouse 1338 GCTGCAACGTGCCAATTGAAA 1358

Transient transfection of mouse PARK2 siRNA plasmid into neuro2a

- Transfection-1 x 10^6 cells per well in 6-well plate.
- Grow for 48 hrs post-transfection, extract protein, determine protein concentration using Pierce BCA kit.
- Western blot: 50 ug per well.
- Detect with mouse anti-parkin MAB5512 and PARK8 antibodies.


PARK8 mouse anti-parkin
1/500 dil
G53
TR007
TR008-
G50
G51
G52
Ge062M
Ge118M
Ge116H
250
148
98
64
50
36
98
64
50
36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250
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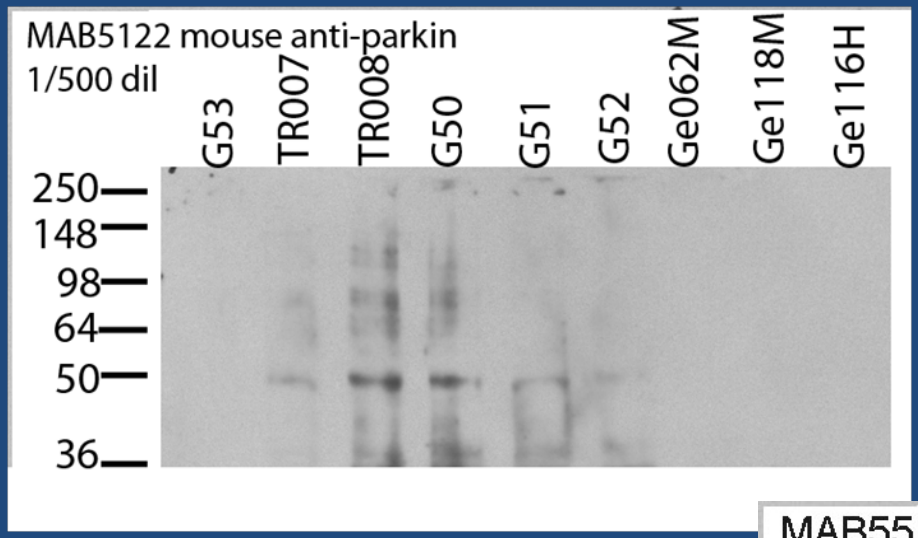
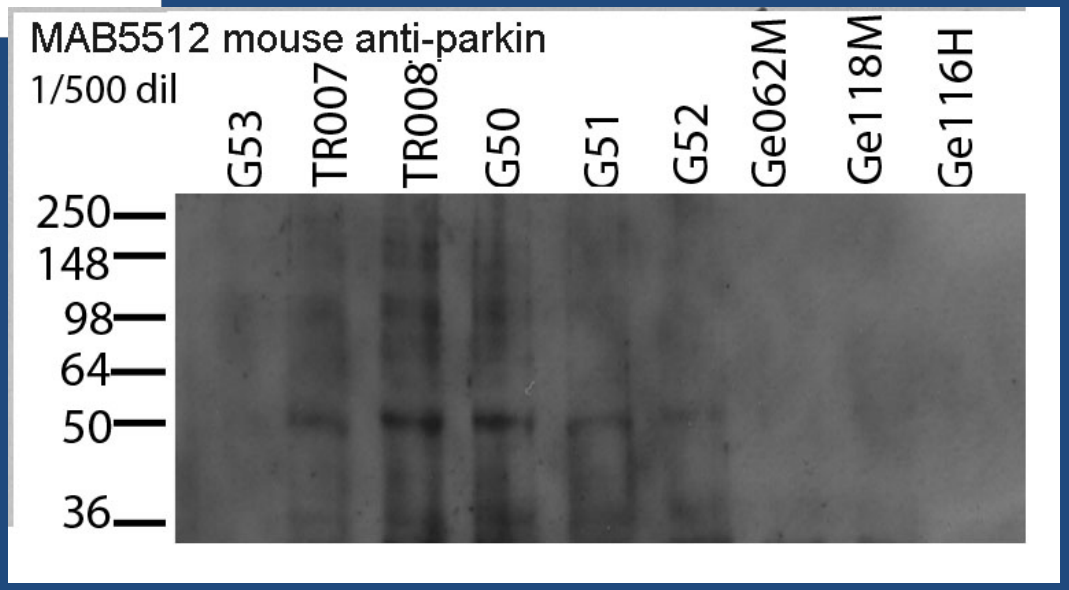
With MAB5512





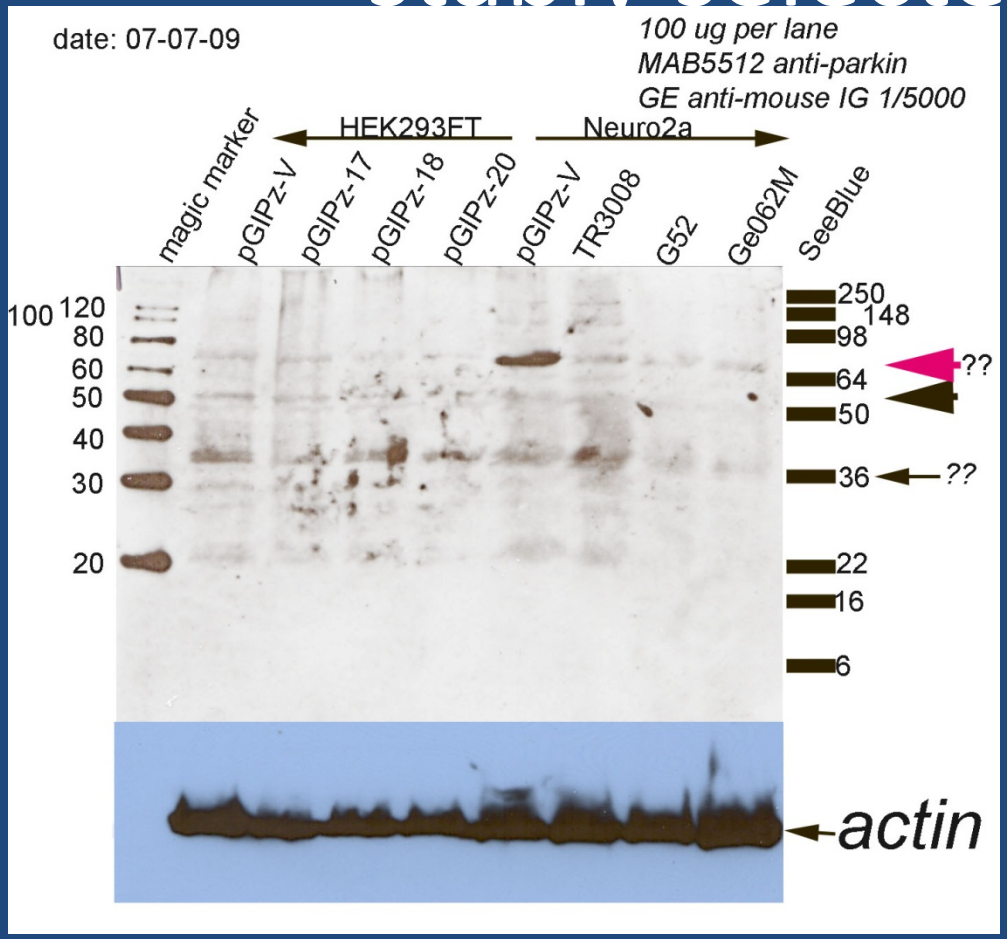
Stable selection

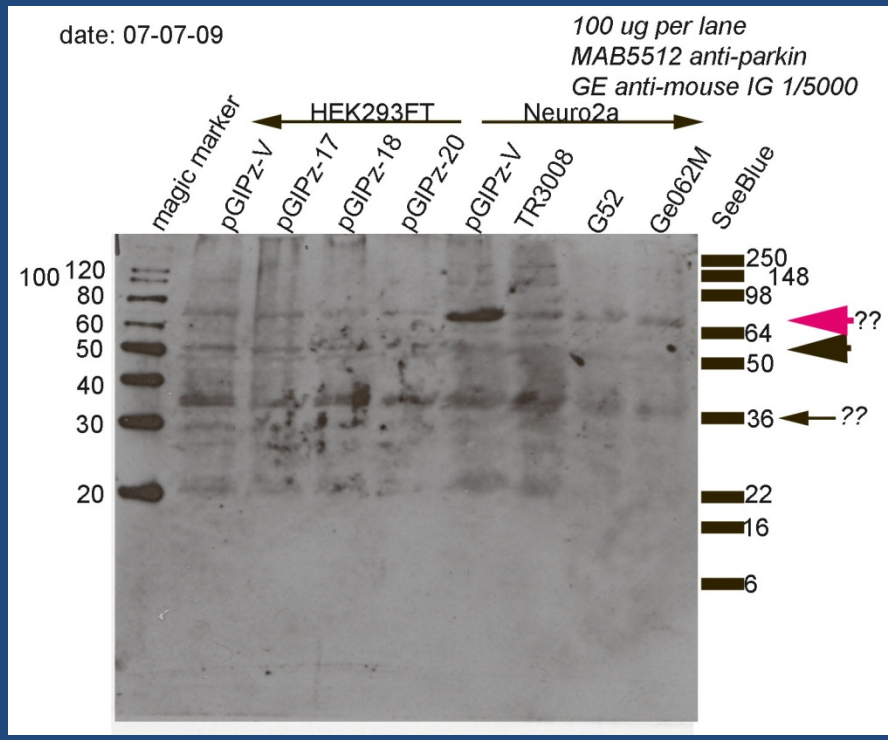
- Number of cells per well of 6-well plate:
  - HEK293 cells: plate at 500,000 cells
  - Neuro2a: plate at 1 x 10^6 cells
  - SH-SY5Y: plate at 1 x 10^6 cells
- Transfection reagent: FuGene:
  - FuGENE:DNA ratio: 8:2 for all cell line
  - Transfection efficiency:
    - HEK293 CELLS: 60-70%, STRONGLY FLUORESCENT FOR pGIPz
    - SH-SY5Y-ATTC: <1% TRANSFECTED FOR pGIPz PLASMIDS.
    - NEURO2A: 60-70%, STRONGLY FLUORESCENT- ORIGENE PLASMID (GFP MARKER),
- Selection Reagent: Puromycin
  - HEK293: SELECT AT 6 ug/ml FOR 1 WEEKS, GO DOWN TO 4 ug/ml THEREAFTER.
    - MANY WEAKLY FLUORESCENT CELLS AND ABOUT 10% WERE STRONGLY FLUORESCED. VERY FEW NONFLUROESCENT CELLS.
  - Neuro-2a: SELECT AT 4 ug/ml FOR 2 WEEKS, THEN DOWN TO 2 ug/ml THEREAFTER.
    - BOTH WEAK AND STRONG FLUORESCENT CELLS WERE OBSERVED.
  - SH-SY5Y CELLS: SELECT AT 0.01 ug/ml FOR 2 WEEKS, GO DOWN TO 0.008 ug/ml PUROMYCIN
    - AFTER 2 WEEKS, A VERY FEW FLUROSCENT CELLS SURVIVED, MOST OF THESE ALSO DIE AFTER A WHILE.
    - NOT SUCCESSFUL YET!

Cell lines (mixed) on hand

1) 2 separate lines of HEK293FT pGIPz-84517
2) 2 separate lines of HEK293FT pGIPz-84518
3) 2 separate lines of HEK293FT pGIPz-84520
4) 2 separate lines of HEK293FT pGIPz-vector
5) 1 line of Neuro2a G50 (in 2-well of 6-well dish)
6) 1 line of Neuro2a G51 (1 dish ~20% confluence-recently from 2 wells of 6-well dish)
7) 1 line of Neuro2a G52 (1 flask)
8) 1 line of Neuro2a G53 (1 dish ~90% confluence).
9) 1 line of Neuro2a TR3007 (1 dish ~90% confluence).
10) 1 line of Neuro2a TR3008 (noneffective human siRNA, flask-confluent)
11) 1 line of Neuro2a Geneclip-062M (flask-confluent)
12) 1 line of neuro2a Geneclip-118M – (1 dish- 80%)
13) 1 line of neuro2a Geneclip-062H (1 dish, 80%)

Western blot of HEK and Neuro2a stably selected cell lines





Upcoming Analyses

- Western blots: are being conducted on available cell lines.
- Quantitative RT-PCR: Preparing cells for RNA isolation.
- Cell death assays: Best cell lines will be selected for paraquat studies-MTS assay, sytox-Red assay
- Mitochondrial stability and conductance assays:

siRNA vs. miRNA

Conservation of small-RNA silencing pathways in eukaryotes

Small RNA	Size (bases)	Mechanism of action	Eukaryotes conserved in
siRNA	~21-25	PGTS (RNA degradation or translaltional arrest) CDGS	Plants, animals, fungi, ciliates
miRNA	~21-25	PTGS (RNA degradation or translational arrest). CDGS (to a lesser extent)	Plants, animals
piRNA	~24-31	PTGS (RNA degradation) CDGS (to a lesser extent)	Animals

1) PGTS-posttranslational genes silencing
2) CDGS-chormatin-dependent gene silencing pathways = assembly of small RNA complexes on nascent transcripts and includes both transcriptional gene silencing (TGS) and co-transcriptional gene silencing (CTGS) events.
3) CTGS_ chromatin-dependent processing and degradation of the nascent transcript.

Danesh Moazed (2009), Nature 452: 413-420

Mechanism of miRNA/siRNA translation suppression