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On the Biochemical Associations of FoxO3a and SirT1 with the Stress Resistance of Cells from the Slow Senescing Snell *dw*arf Mouse

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SUMMARY/ ABSTRACT

Tailskin fibroblasts from multiple genotypes of slow aging mice have been shown to be resistant to a broad spectrum of toxicants. The molecular determinants for this *in vitro* effect, as well as for the delayed/ decelerated senescence of these mice, are uncertain. Here, we have extended this phenomenon of *in vitro* cellular stress resistance to neurons derived from the cerebral cortex of the Snell Dwarf Mouse. We further investigated the role of the transcription factor FoxO3a and the protein deacetylase SirT1, proteins known to positively mediate cellular stress-resistance, in this paradigm. We found that Snell Dwarfs have a greater proportion of nuclear-localized FoxO3a within their cerebrums than their littermate controls and that the same is true for their unstressed fibroblasts *in vitro*; yet, Snell Dwarf fibroblasts did not differ in FoxO3a properties in response to the application of three different concentrations of two disparate stresses. Similar results were obtained for SirT1, although SirT1 content did increase under the mild cellular stress of serum deprivation. Taken together, these results depict stress resistance in non-fibroblast cell types of incontrovertible physiological import explanted from slow aging mice. Also, these results strongly suggest that neither FoxO3a nor SirT1 robustly regulate the stress-resistance of Snell Dwarf Mouse cells *in vitro*, and thus might not play a role in other slow aging mammalian *in vitro* models in which stress resistance has been documented. That cerebral neurons *ex vivo* and unstressed fibroblasts *in vitro* display FoxO3a concentrations suggestive of increased activity introduce the possibility that FoxO3a might partially mediate the *in vivo* retardation of senescence of these mice.

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82 **INTRODUCTION**
83
84 The process that converts healthy adults into frailer adults with progressively increased
85 risks of illness, injury, and death known as aging [miller (1999)] has been a topic of considerable
86 interest and study since time immemorial. Mammalian models for delayed/ decelerated
87 senescence include dietary regimens instituting “undernutrition without malnutrition”, such as
88 caloric restriction [McCay *et al.* (1935), Masoro (2001), Weindruch and Walford (1988)] or
89 Methionine restriction [miller *et al.* (2005)], and multiple mutants with somatotrophic defects
90 (dwarf mice) [Brown-Borg *et al.* (1996), Flurkey *et al.* (2001), Coschigano *et al.* (2003)]. The
91 most reliable single biomarker for identifying an intervention that genuinely retards senescence
92 is the documentation of longevity within a survivorship assay. Animals subjected to these
93 environmental or genetic manipulations have been shown not only to have increased mean and
94 maximal survivorship but to display multiple corroborative hallmarks of delayed or
95 decelerated/attenuated/ameliorated senescence [bartke (2006)]. Much work has been done to
96 establish the concomitants of their defects in growth hormone signaling, and include decreased
97 serum IGF1 concentrations, hypoinsulinemia & insulin sensitivity, hypoglycemia, and decreased
98 tumorigenesis & incidence of neoplastic diseases [miller/bartke review, Ikeno *et al*. (2003)]; yet
99 more work is required to investigate the proteins underpinning these results. One of these slow
100 aging, long-lived hyposomatotrophic mutants, the Snell Dwarf Mouse (Pit1$^{dw/dw}$) [Snell (1929)]
101 has a hypomorphic *dw* mutation at the Pit1 gene locus, affecting the development of its anterior
102 pituitary [Camper *et al.* (1990)]; it is the subject of this current work.
103
104 On a cellular level of experimental gerontology, fibroblasts derived from the skin of some
105 examples of these slow aging dwarf mice have been shown to be resistant to a panoply of stress
106 treatments, including hydrogen peroxide, paraquat, cadmium, heat, ultraviolet light, methyl
107 methanesulfonate, the metabolic inhibition induced by low glucose concentrations, and the
108 mitochondrial electron transport chain Complex 1 inhibitor rotenone [Murakami *et al.* (2003),
109 Salmon *et al.* (2005), Leiser *et al.* (2006)]. Concurrent results have also been seen for studies
110 correlating fibroblast stress resistance with species maximal lifespan [Harper *et al*. (2006)].
111 These results dovetail with findings in less complex organisms that stress resistance correlates
112 highly, albeit not perfectly, with increased lifespan [Johnson (2005), Tower review]. It has been
113 proposed that this multiplexed stress resistance could be causal, at least in part, for the observed
114 amelioration of aging [Martin *et al.* (1996), Kowald and Kirkwood (1994), Murakami *et al.*
115 (2003)].
116
117 The *Fo*rkhead-bo*x* Class *O* Alphanumeric Code Designation *3a* (FoxO3a) transcription
118 factor is a known regulator of mammalian stress resistance and is hypothesized to be important
119 for life expectancy [Greer and Brunet (2005), Furukawa-Hibi *et al*. (2005)]. The *Caenorhabditis*
120 *elegans* ortholog (based on sequence similarity) of FoxO3a, DAF-16, has been extensively
121 documented to be necessary for the longevity-conferring effects of many mutations that increase
122 lifespan in the worm [Kenyon (2005)] and to be sufficient for mild longevity [Henderson and
123 Johnson (2001)]. The *SIR T*wo-like Number *One* protein, SirT1, is the mammalian ortholog
124 (based on sequence similarity) of the *Saccharomyces cerevisiae* Silent Information Regulator 2
125 (SIR2). This nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylase has been
126 shown to engender greater cellular resistance to death of mammalian cells (2 germane refs.), its
127 worm and *Drosophila melanogaster* “homologs” (SIR-2.1 and dSIR2, respectively) have been

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128 shown to be sufficient for increased lifespan in those species [Tissenbaum & Guarente (2001),
129 Rogina & Helfand (2005)], and SirT1 has been promulgated as being relevant for mammalian
130 aging; possibly even being an anti-aging factor that mediates the benefits of caloric restriction
131 [Guarente & Picard, (2005), Sinclair & Guarente (2006), Chen & Guarente (2007)]. What is
132 more, SirT1 has been shown to be transcriptionally regulated by FoxO3a [Nemoto *et al.* (2004)].
133
134 Here, we expand the stress resistance-slow aging correlation by documenting the stress
135 resistance of neurons derived from the cerebral cortices of Snell Dwarf Mice to the
136 neuroexcitotoxin kainic acid (kainate). This establishes that the stress-resistance of these greatly
137 intriguing mice exists in multiple cell types, and in cell types of high physiological relevance that
138 could be more facilely related to cellular mediators of decelerated senescence. We further
139 determine the concentrations of proteins previously shown to be crucial for stress resistance in
140 mammalian cells and hypothesized to be necessary for longevity, namely FoxO3a and SirT1. We
141 find that FoxO3a levels are higher in unstressed tailskin-derived fibroblasts, and that SirT1 is
142 higher in serum-deprived cells, from Snell Dwarfs. Additionally, we find that FoxO3a is not
143 regulated, whether via translation or unphosphorylation, in a manner that would suggest that it
144 mediates the stress-resistance differences previously observed amongst Snell Dwarfs and their
145 littermate controls; similar data suggesting a lack of necessity for SirT1 in the stress-resistance of
146 these cells was also observed. Considered together, these findings suggest that proteins other
147 than FoxO3a and SirT1 mediate the *in vitro* (and putatively *in vivo*) superior stress resistance of
148 the Snell Dwarf Mouse, but that these two proteins could act *in vivo* to mediate the attenuation of
149 aging (to include the engendering of longevity) of the Snell Dwarf Mouse.
150
151 **RESULTS**
152
153 *Neuroexcitotoxicity Assay*
154
155 To expand the prior discoveries of stress resistance seen in tail-derived dermal fibroblasts
156 from the Snell Dwarf, we tested whether neurons derived from cerebral cortices of Snell Dwarf
157 Mice were more resistant to neuroexcitotoxicity incited via kainic acid than those of littermate
158 control mice. This assay assessed the stress resistance of neurons containing glutamate or N-
159 methyl-D-aspartate (NMDA) receptors and thus attempted to broaden the cell-type breadth of the
160 stress resistance effect of the Snell *dw* mutation. We found that Snell-derived neurons had over
161 one-fold enhanced mean survivorship (44.5% survival for the dwarf-derived neurons and 20.3%
162 for the control counterparts; Student's *t*-test *p*-value < 0.03) (Figure 1).
163
164 *Immunoblotting for FoxO3a in Cerebral Cortical Tissue*
165
166 To probe the role of FoxO3a in regulating the stress resistance of Snell Dwarf Mouse
167 cells, we assessed the presence of 1) the total protein and 2) the phosphorylated isoform of the
168 protein that is phosphorylated (at least) at the Serine residue at position 253; this phosphorylation
169 site is within the Nuclear Localization Sequence (NLS) of FoxO3a, and is preferentially
170 phosphorylated by protein kinases AKT serine/threonine kinase 1 (Akt) [Greer and Brunet
171 (2005)]. The Ser253-phosphorylated isoform should be sequestered in the cytoplasm and thus
172 not be transcriptionally potent; whereas the unphosphorylated isoform should be permitted
173 residence in the nucleus, and thus be able to induce the transcription of stress-resistance genes

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174 [Furukawa-Hibi *et al.* (2005)]. Therefore, phosphorylation status was used as a surrogate for the
175 sub-cellular localization, and thus potential activity, of FoxO3a.
176
177 To investigate the molecular underpinnings for the above-determined neuronal stress-
178 resistance, we investigated the concentration and phosphorylation status of FoxO3a in the
179 cerebral cortex of the dwarf mice and their littermate controls. We found that Snell Dwarf
180 cortices had a lesser proportion of phosphorylated FoxO3a polypeptides, with their mean
181 Ser253-FoxO3a:Total-FoxO3a ratio being 76% of that of the control mice (Student's t-test $p <$
182 0.02) (Figure 2); this would imply that Snell Dwarf neurons have more of their FoxO3a in their
183 nuclei than those of their littermates do. We also found that there was no difference in the total
184 concentration of FoxO3a in these cerebral cortices (mean dwarf value was 107% of mean control
185 value (Student's t-test $p = 0.75$).
186
187 *Immunoblotting for FoxO3a in Tailskin-Derived Fibroblasts*
188
189 Next, we examined the effect of cellular stress on FoxO3a concentrations and ratios in
190 Snell Dwarfs and their controls. We performed these experiments in the fibroblast stress system
191 previously used to establish that tailskin-derived primary fibroblasts from Snell Dwarfs have
192 multiplexed stress resistance [Murakami *et al.* (2003), Salmon *et al.* (2005)]. (These experiments
193 could not be conducted in the cerebral cortical neurons *in vitro* because the protein sample yield
194 from those cultures was too low for reliable immunodetection.)
195
196 For unstressed fibroblasts, we found that the ratio of the phosphorylated isoform of the
197 stress resistance-inducing transcription factor FoxO3a to the total concentration was 72% of that
198 in the control-derived fibroblasts (Student's t-test p-value < 0.0004) (Figure 3A). We
199 concurrently learned that the mean total concentration of FoxO3a was 30% higher in Snell Dwarf
200 fibroblasts than in those of their littermates (Student's t-test p-value < 0.04) (Figure 3B).
201
202 During the initial fibroblast stress analysis, it was discovered that a serum-deprivation
203 step in which the fibroblasts spend 20-24 hours in a serum-free Dulbecco's Modified Eagle
204 Medium (DMEM) formulation was necessary for the Snell Dwarf Mouse stress resistance to
205 manifest [Murakami *et al.* (2003)]. Under this intermediate situation, we observed no difference
206 in the Ser253-FoxO3a:Total-FoxO3a ratio (n = 19; Student's t-test p-value = 0.46).
207
208 Paraquat is an herbicide that causes oxidation-related toxicity by instigating the
209 generation of superoxide anions ($O_2^{-\cdot}$) (paraquat ref.). It was also consistently found to be one of
210 the most potent toxins for eliciting a differential stress response from our *in vitro* Snell Dwarf
211 fibroblast system. With regards to FoxO3a, the results for fibroblasts stressed with three different
212 concentrations (20, 100, and 200 μM) of the endogenous oxidant paraquat across a broad toxicity
213 range showed no difference in the ratio of Ser253-FoxO3a to total FoxO3a in dwarf mice
214 compared to their littermate controls (Student's t-test p-values equal to 0.63, 0.09, and 0.89 for
215 20, 100, and 200 μM paraquat, respectively) (Table 1B).
216
217 For fibroblasts stressed with three highly varying concentrations (1, 10, and 20 μM) of
218 the highly toxic heavy metal Cadmium, the results comparing Snell Dwarfs to their littermate

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219 controls were as was the case for paraquat (Student's *t*-test *p*-values equal to 0.34, 0.21, and 0.74
220 for 1, 10, and 20 μM Cadmium, resp.) (Table 1B).
221
222 For both 10 μM paraquat and 1 μM cadmium, we also stressed fibroblasts for 6 hours.
223 This was done in order to determine if a longer treatment period was required to elicit a
224 macromolecular response; and also because the original fibroblast stress survival assays were
225 designed for the cells to be incubated in the treatment-containing medium for 6 hours [Murakami
226 *et al*. (2003)]. Yet, even for this extended period of stress, there were no differences amongst
227 Snell Dwarfs and their littermate controls in stress response insofar as phosphorylation of
228 FoxO3a (6 hr. paraquat: n = 6, Student's *t*-test *p*-value = 0.13; 6 hr. Cadmium: n = 4, Student's *t*-
229 test *p*-value = 0.37).
230
231 *Immunoblotting for SirT1 in Tailskin-Derived Fibroblasts*
232
233 Due to the relation of SirT1 to the survival of cells *in vitro* (Sinclair ref.) and its
234 connection to FoxO3a [Nemoto *et al.* (2004)], we decided to assess the differences, if any, in
235 SirT1 concentration between the Snell Dwarf Mouse and its littermate control. Concerning
236 immunoblotting dwarf and control fibroblasts under the stress conditions described above (1, 10,
237 or 20 μM Cadmium; or 20, 100, or 200 μM paraquat), the only difference detected was for the
238 serum-free culture medium condition: a 53% increase in SirT1 concentration was observed in the
239 dwarfs relative to their littermate controls (Student's *t*-test *p*-value < 0.007) (Figure 4A). Upon
240 stress induction, no differences in SirT1 concentration were observed [(Student's *t*-test *p*-values
241 equal 0.31, 0.12, 0.99, 0.92, 0.26, 0.25, and 0.25 for Complete DMEM (Figure 4B-C), 20, 100,
242 and 200 μM paraquat (Figure 4B) and 1, 10, and 20 μM Cadmium (Figure 4C), resp.].
243
244 **DISCUSSION**
245

246 - recapitulate: *in vivo*-like condition (complete DMEM, explant) suggests *dw/dw*
247 mice hyper-vigilant against stresses via nuclear-loc. and conc. of FoxO3a; stress-
248 resistance poss. also mediated by increased [SirT1]
249
250 - emphasize breadth and stringency of analysis; address lack of signaling pathway
251 depth
252
253 - address variation in data-points by noting comparable variation in previous miller
254 lab. Snell Dwarf articles and WB images from Ames *dwarf* Mice (Sharp and
255 bartke, '05)
256
257 - discuss implications of SirT1 increase under serum-starvation in light of SirT1's
258 reliably documented role in response to nutrient deprivation
259
260 - mention Maynard and miller, '06's counterintuitive Hsp70 results
261
262 - mention miller, '91 and bartke papers on theme of "gerontology as oncology"
263 when discussing physiological effect of increase FoxO3a/SirT1 activity
264

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265 ○ discuss Harper et al., '06?!?!?
266
267 ○ coda, inc. reference to how Longevity Dividend Concept suggests multiple
268 indications might be ameliorated by basic gerontology
269
270 **EXPERIMENTAL PROCEDURES/ MATERIALS & METHODS**
271
272 Snell Dwarf Mice and littermate controls were maintained as previously [Harper *et al*.
273 (2006)]. Snell dwarf (*dw/dw*) mice, and heterozygote (*dw*/+) controls were bred as the progeny of
274 (DW/J × C3H/HeJ) *dw*/+ females and (DW/J × C3H/HeJ)F1 *dw/dw* males. Mice used were
275 sacrificed with carbon dioxide. All procedures involving animals were approved by the Unit for
276 Laboratory Animal Medicine (ULAM) of the university of michigan – ann arbor.
277
278 Neurons were cultured as previously described [Brewer (1997), Brewer and Torricelli
279 (2007)] using the consumables described; save that neither basic fibroblast growth factor (FGF2)
280 nor gentamycin were used, so as to avoid equivocal interpretation of the results. This cell
281 culturing methodology keeps the population of non-neuronal cells below 5% [Brewer (1997)]. 50
282 µM kainic acid (Tocris Bioscience, Ellisville, MO) was applied to 5 day-*in-vitro* (DIV) neuronal
283 cultures for 24 hours. 15 µg/mL fluorescein diacetate (FDA) (Sigma-Aldrich, St. Louis, MO) and
284 4.6 µg/mL propidium iodide (P.I.) (Sigma-Aldrich, St. Louis, MO) were added and fluorescence
285 microscopy was performed with a Leica DMIRB fluorescent inverted microscope (Leica
286 Microsystems GmbH, Wetzlar, Germany). Live cells fluoresced green under blue light excitation
287 due to active incorporation and lysis of FDA, and dead cells fluoresced red under green light
288 excitation due to the passive accumulation of P.I. Live cells/total cells were scored [Zachary S.
289 Pincus & Robert Sapolsky (MS thesis) (2002), Guo *et al.* (1999), Brewer (1997)]. Low-
290 abundance cells that clearly were not neurons, such as astroglia or microglia, were not scored.
291
292 Fibroblasts were cultured as previously [leiser *et al*. (2006)]. Third or fourth passage
293 fibroblasts were stressed with either 1, 10, or 20 µM Cadmium chloride (Sigma-Aldrich, St.
294 Louis, MO) or 20, 100, or 200 µM methyl viologen/ paraquat (Sigma-Aldrich, St. Louis, MO)
295 for 30 minutes or 6 hours in the 37°C/5% CO_2/9% O_2 incubator used for their culturing (?????,
296 ?????). Protein samples were then promptly collected with cold RIPA cell lysis buffer (ref.)
297 supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) in a 4°C cold
298 room.
299
300 The following antibodies were used in this study: FoxO3a (Catalog # 9467) (Cell
301 Signaling Technology, Danvers, MA), Phospho-FoxO3a (Ser253) Antibody (Cat. # 9466) (Cell
302 Signaling Technology, Danvers, MA), SirT1 (Cat. # 12193) (Abcam, Cambridge, MA), β-
303 Tubulin (Cat. # 6046) (Abcam, Cambridge, MA), alkaline phosphatase-conjugated goat anti-
304 rabbit secondary (Cat. # sc-2057) (Santa Cruz Biotechnology, Santa Cruz, CA).
305
306 Sodium Dodecyl Sulfate – PolyAcrylamide Gel Electrophoresis (SDS-PAGE)-based
307 (Western) Immuno-Blotting [Towbin *et al.* (1979)] was performed as previously described
308 [Maynard and miller (2006)]; save that molecular weight standards (Cat. # sc-2035) (Santa Cruz
309 Biotechnology, Santa Cruz, CA) and broad range markers (Cat. # sc-2361) (Santa Cruz
310 Biotechnology, Santa Cruz, CA) were used.

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311
312 Student's *t*-tests were performed on Microsoft Excel (Microsoft Corporation, Redmond,
313 WA).
314
315 **ACKNOWLEDGMENTS**
316
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318 Kyoko Yasumura for technical assistance.
319
320 **FUNDING DISCLOSURE**
321
322 This study was funded by a National Institute on Aging (N.I.A.) T32 National Research
323 Service Award (T32-AG000114) and N.I.A. grants AG024824 & AG023122).
324
325 **REFERENCES**
326
327 **FIGURE LEGENDS**
328
329 **FIGURES**
330

Figure 1. Adult *dw/dw* Neurons have Greater Kainate-resistance than *dw/+*

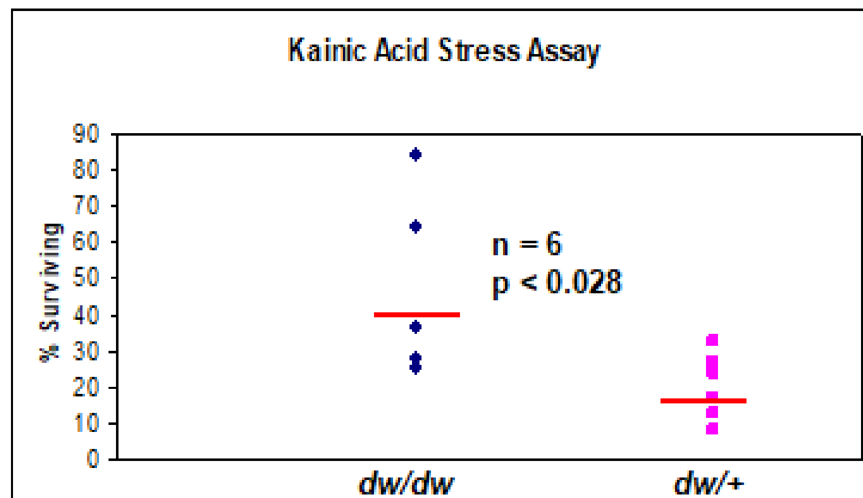

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Figure 2. *dw/dw* Cerebral Cortices Have Lower (Ser253:Total) for FoxO3a

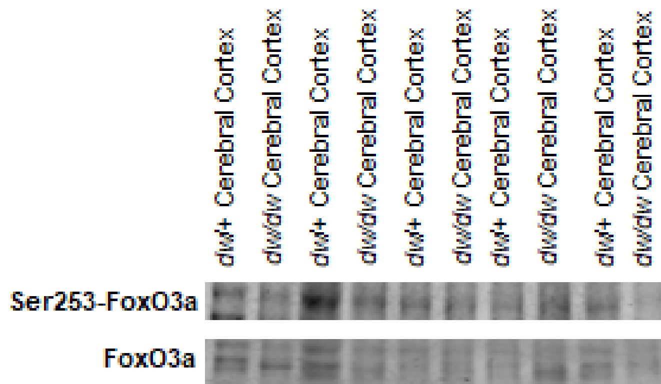

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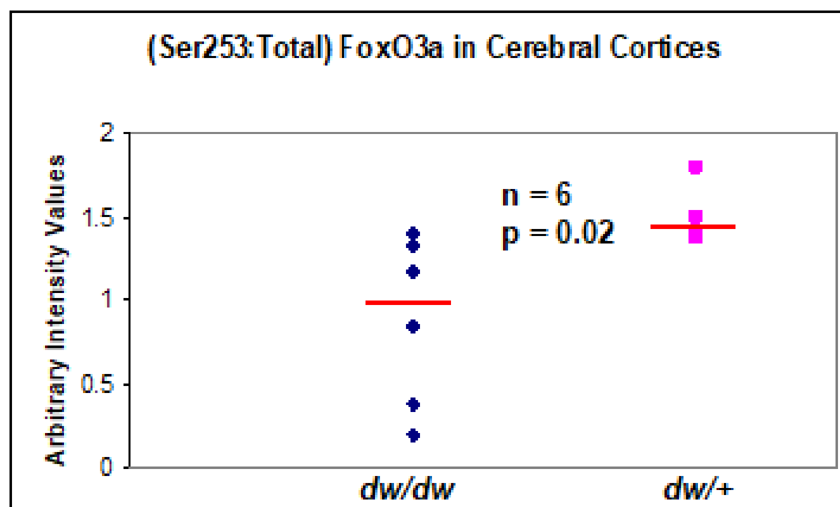
Figure 2. *dw/dw* Cerebral Cortices Have Lower (Ser253:Total) FoxO3a



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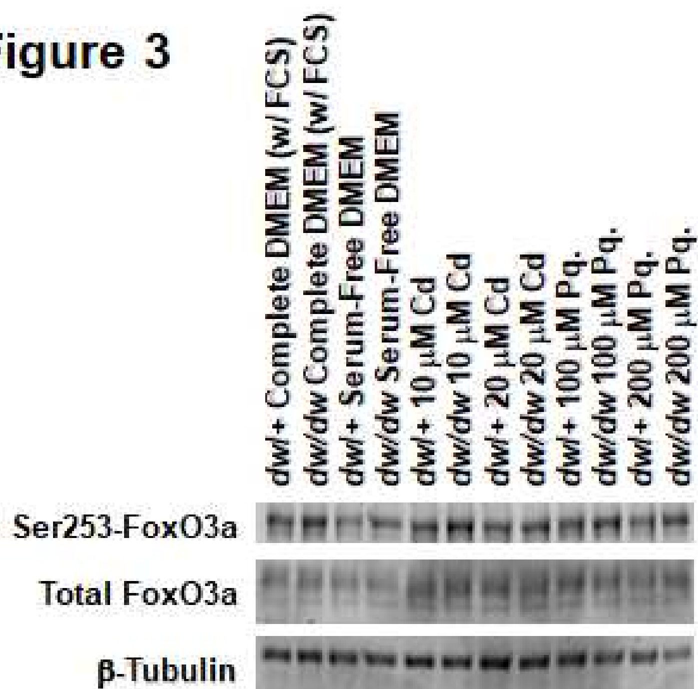

334

Fig. 3A. *dw/dw* Tailskin Fibroblasts have Greater Concentration of FoxO3a than *dw/+*

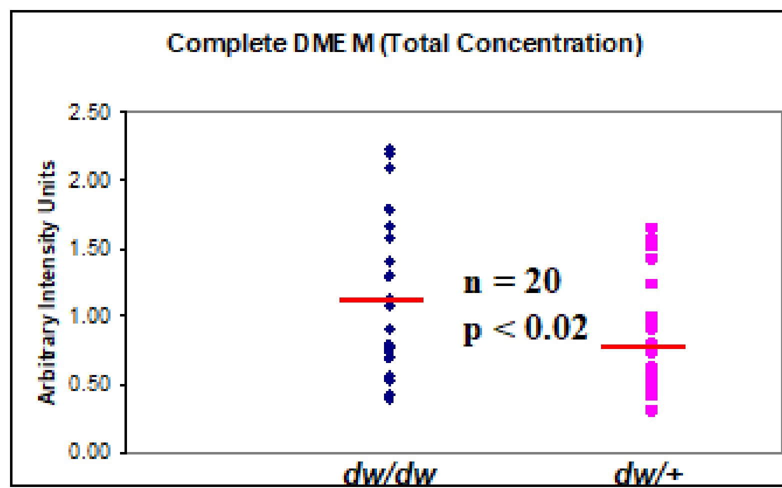

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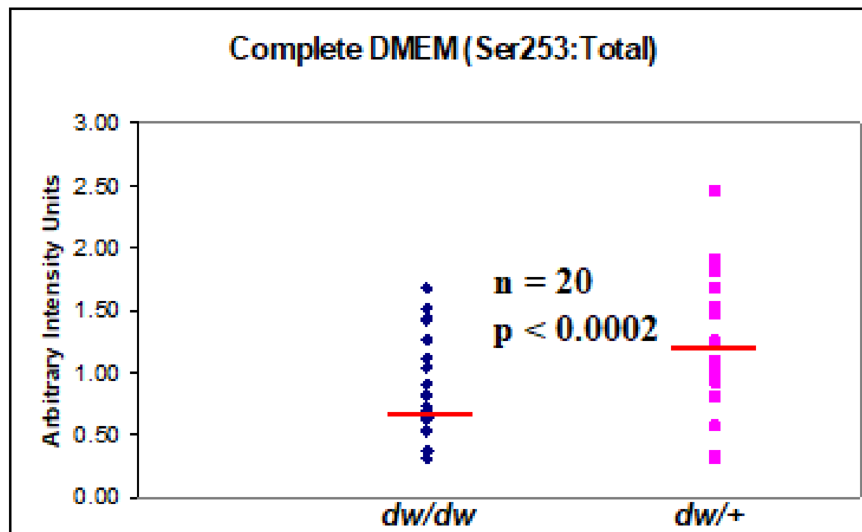
Fig. 3B. *dw*/+ Tailskin Fibroblasts have Greater Ser253-phosphorylated FoxO3a than *dw/dw*



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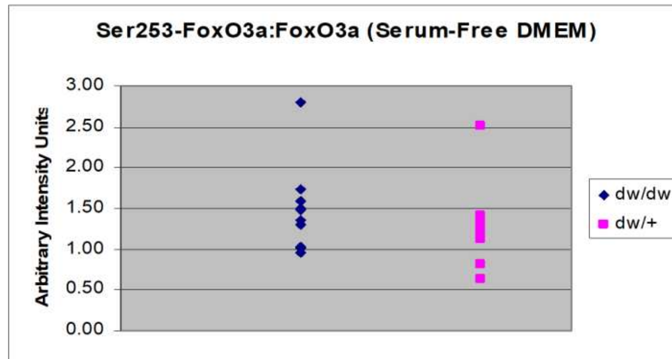
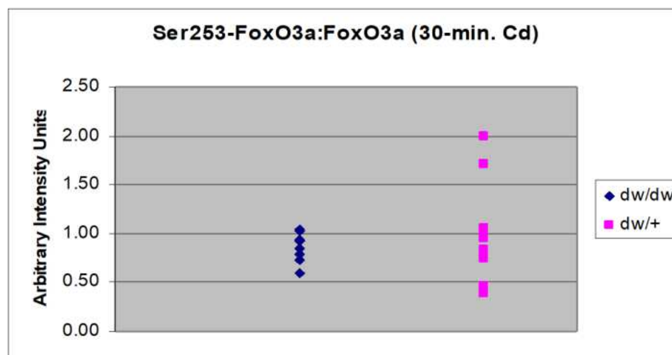


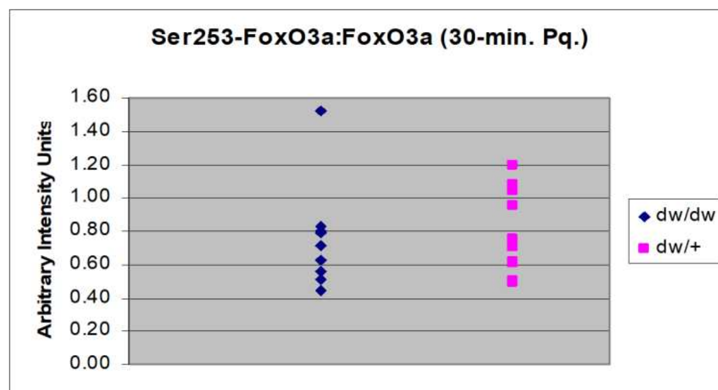
Genotype: Median +/- St. Dev.
dw/dw: 1.42 +/- 0.53
dw/+: 1.23 +/- 0.64

347 **Student's *t*-test p-value**: 0.4
348
349



Genotype: Mean +/- St. Dev.
dw/dw: 0.85 +/- 0.17
dw/+: 0.95 +/- 0.60

350 **Student's *t*-test p-value**: 0.37
351
352



Genotype: Median +/- St. Dev.
dw/dw: 0.67 +/- 0.31
dw/+: 0.73 +/- 0.26

353 **Student's *t*-test p-value**: 0.63

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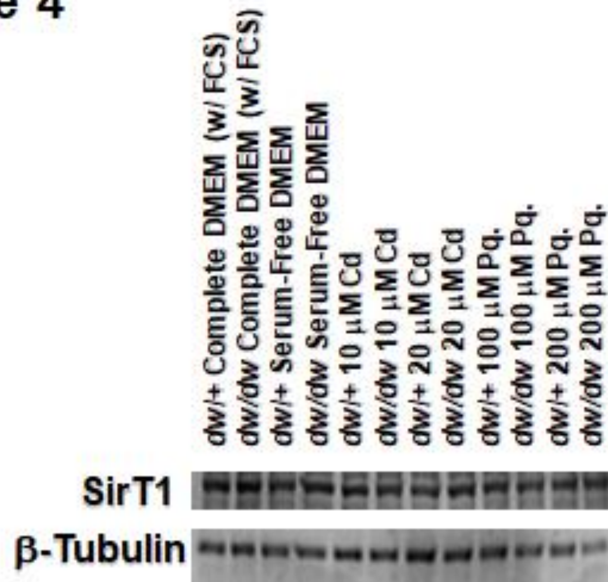

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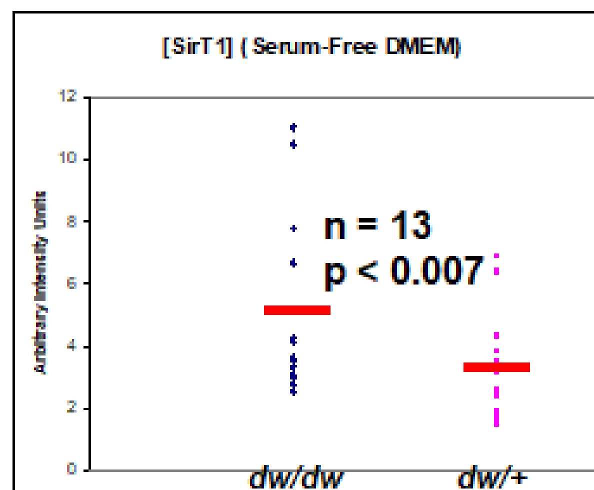
Figure 4A. More SirT1 in *dw/dw* Fibroblasts in Serum-Free DMEM



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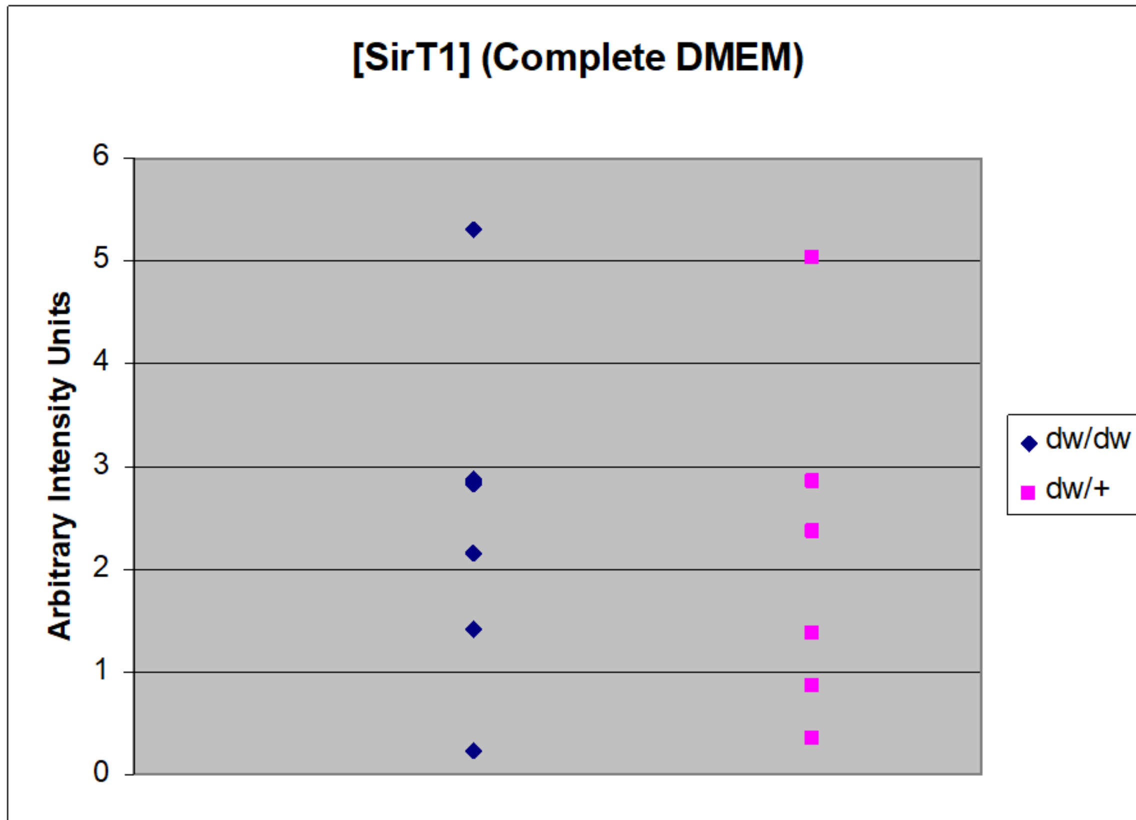

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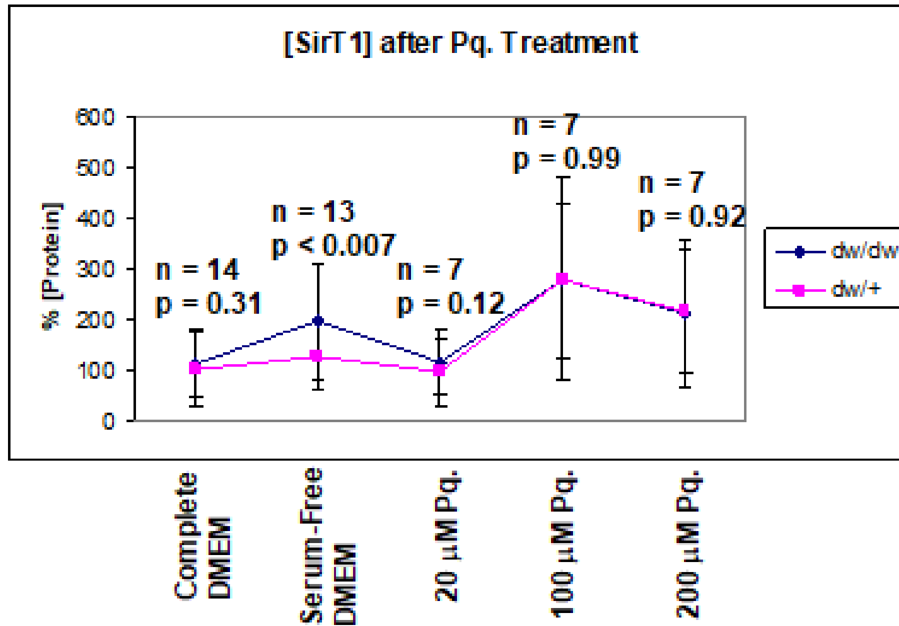
Figure 4B. [SirT1] after Treatment with Pq.



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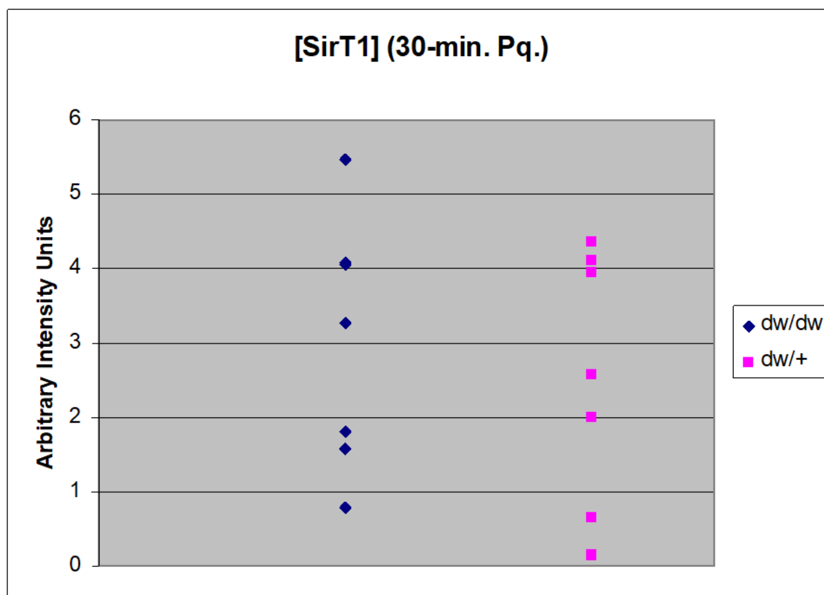

Genotype: Mean +/- St. Dev.
dw/dw: 3.01 +/- 1.67
dw/+: 2.53 +/- 1.70

Student's *t*-test p-value: 0.12

360

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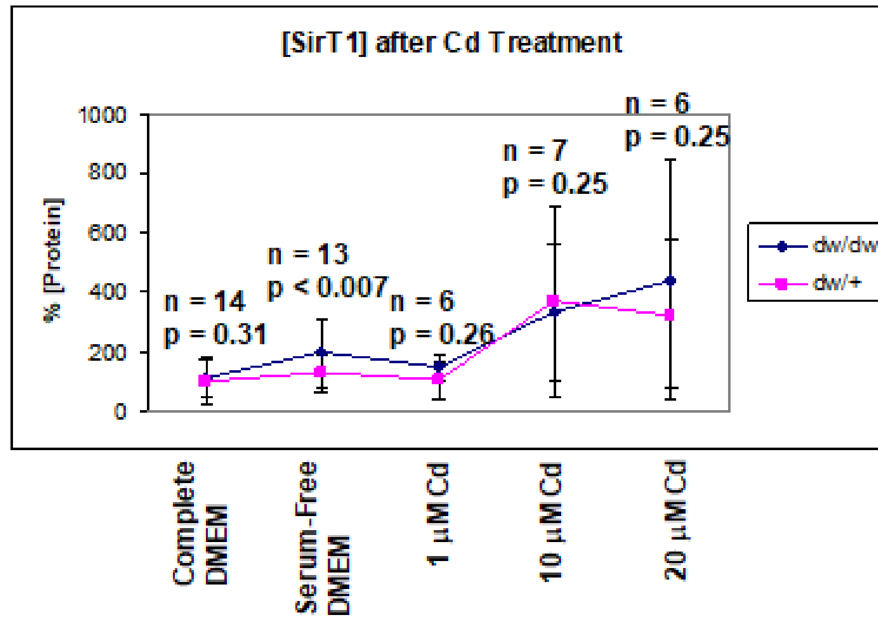
Figure 4C. [SirT1] after Treatment with Cd



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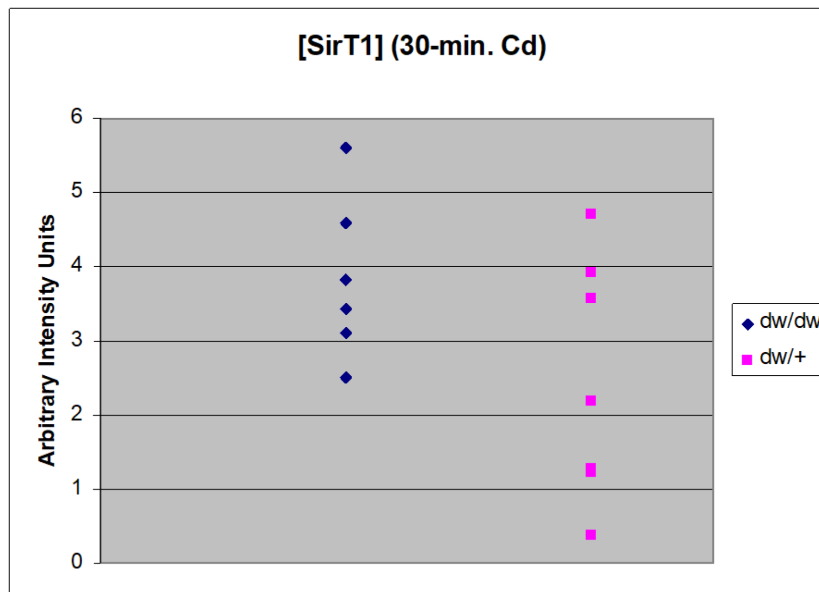

Genotype: Mean +/- St. Dev.

dw/dw: 3.84 +/- 1.77

dw/+: 2.46 +/- 1.62

Student's *t*-test p-value: 0.26

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364 **TABLES**

Table 1A. Conditions where *dw/dw* did not Differ from *dw*/+ in Total FoxO3a Concentration

In vitro Milieu	Sample Size	*dw*/+ Mean +/- S.D.	*dw/dw* Mean +/- S.D.	Paired Student's *t*-test
Serum-deprivation	17			p = 0.65

Table 1B. Conditions where *dw/dw* did not Differ from *dw*/+ in Ser253-phosphorylated:Total FoxO3a Ratio

In vitro Milieu	Sample Size	*dw*/+ Mean +/- S.D.	*dw/dw* Mean +/-S.D.	Paired Student's *t*-test
Serum-deprivation	17			p = 0.46
1 μM Cd	9			p = 0.34
10 μM Cd	7			p = 0.21
20 μM Cd	6			p = 0.74
20 μM Pq.	10			p = 0.63
100 μM Pq.	7			p = 0.09
200 μM Pq.	7			p = 0.89

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