

1 ***Acat1* gene KO restores TGN cholesterol deficiency in**
2 **mutant NPC1 cells and expands mutant *Npc1* mouse lifespan**
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22 **Abstract**

23

24 Niemann-Pick type C (NPC) is a neurological disorder with no cure. NPC proteins deliver
25 cholesterol from endosomes to other compartments including trans-Golgi network (TGN) and
26 endoplasmic reticulum (ER). Acyl-coenzyme A:cholesterol acyltransferase 1 (ACAT1) is a
27 resident ER enzyme that converts cholesterol to cholesteryl esters for storage. Here, we report
28 the surprising finding that in a mutant *Npc1* mice, *Acat1*-deficiency delayed the onset of weight
29 loss and declining motor skill, prolonged lifespan, delayed Purkinje neuron death, and improved
30 hepatosplenic pathology. Furthermore, syntaxin 6, a cholesterol-binding t-SNARE normally
31 localized to TGN, is mislocalized in mutant NPC cells. However, upon ACAT1 inhibition this
32 mislocalization is corrected, and increase the level of a few proteins further downstream. Our
33 results imply that ACAT1 inhibition diverts a cholesterol storage pool in a way that replenished
34 the low cholesterol level in NPC-deficient TGN. Taking together, we identify ACAT1 inhibition as
35 a potential therapeutic target for NPC treatment.

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42 Introduction

43
44 Niemann-Pick disease type C (NPC) is a genetically recessive neurodegenerative disease
45 caused by mutations in *Npc1* (1), (2) or in *Npc2* (3). Loss of in NPC1 or NPC2 function results in
46 the accumulation of cholesterol (4) as well as various sphingolipid species (5), mainly within late
47 endosomes/lysosomes (LE/LYS). NPC disease shares many similar attributes with Alzheimer's
48 disease, and is colloquially referred to as juvenile Alzheimer's disease. As such any mechanistic
49 and therapeutic findings in NPC disease may have broad application to other
50 neurodegenerative diseases. The lipid accumulation seen in NPC occurs in all tissues, and
51 results in neurodegeneration as well as malfunctions in liver and lung. In brain, the most
52 extensive cell death occurs in cerebellum, with preferential loss of Purkinje neurons. In terms of
53 potential therapies, miglustat, a glycosphingolipid synthesis inhibitor, has demonstrated
54 significant efficacy (6). However, miglustat is not FDA-approved as an NPC therapy.
55 Intrathecal delivery of 2-hydroxypropyl- β -cyclodextrin, a water-soluble molecule that binds
56 cholesterol, reduces neurological disease progression (7), (8), but the clinical benefit of
57 cyclodextrin has yet to be clearly demonstrated in ameliorating the disease pathology in NPC1
58 patients. Given the current lack of approved NPC treatments, there remains a critical need to
59 develop therapeutic approaches for the treatment of NPC disease.

60
61 Cholesterol is an essential lipid molecule needed for cell growth and function. Cells acquire
62 cholesterol through exogenous sources as well as through *de novo* biosynthesis. Exogenous
63 cholesterol enters cells mainly through receptor-mediated endocytosis. Subsequently, it is
64 distributed to various membrane compartments for utilization, feed-back regulation of
65 cholesterol metabolism, and storage as cytoplasmic cholesteryl ester lipid droplets [Reviewed in
66 (9)]. Distribution of cholesterol from the LE/LYS to other membrane compartments requires
67 NPC1 and NPC2. Both NPC1 and NPC2 bind to cholesterol (10), (11), (12), (13). These two
68 proteins work in concert to export cholesterol from LE to other membrane organelles [reviewed
69 in (14)]. In cells with NPC mutations, buildup of cholesterol and other lipids occurs within
70 LE/LYS, while leaving other membrane compartments, including plasma membrane (PM) (15),
71 (16), endoplasmic reticulum (ER) (17), (18), (19), (20), peroxisomes (21), and trans-Golgi
72 network (TGN) (22), (23) relatively deficient in cholesterol. In mutant NPC cells, abnormal
73 membrane cholesterol distribution cause malfunctions in LE/LYS (24), and in other membrane
74 organelles (23), (25). Cholesterol overload in LE/LYS also causes cholesterol accumulation in
75 the inner membranes of mitochondria (26), (27), (28), (29).

76 In addition to receiving cholesterol from exogenous uptake, cells produce cholesterol from *de*
77 *novo* biosynthesis. Upon synthesis at the ER, sterols quickly move to the PM within a few
78 minutes, via mechanisms independent of NPC1 (30). Cholesterol from both endogenous and
79 exogenous sources traverse among various membrane compartments [reviewed in (31), (32)].
80 To prevent overaccumulation of free cholesterol in cells, which would result in cellular toxicity
81 [reviewed in (33), acyl-coenzyme A:cholesterol acyltransferase 1 (ACAT1) (also called sterol O-
82 acyltransferase 1 [SOAT1]) (34), converts a certain portion of cellular cholesterol to cholesterol
83 esters. Furthermore, ATP binding cassette transporter A1 (ABCA1) [reviewed in (35)].
84 removes excess cholesterol through a lipid efflux process.

85
86 ACAT1 is a membrane protein residing at the ER (36); in addition, a certain portion of ACAT1 is
87 found close to other cellular organelles including PM (37), recycling endosomes, (38), and TGN
88 (39). In mutant NPC cells, the absence of functional NPC1 or NPC2 considerably slows delivery
89 of cholesterol from LE/LYS to ER; however, a significant amount of cholesterol can translocate
90 from the PM to the ER for esterification in an NPC-independent manner (16, 40-43), (44, 45).
91 Here, we hypothesize that in mutant NPC cells, ACAT1 inhibition causes the NPC-independent
92 cholesterol pool arriving at ER to build up. Once built-up, this cholesterol pool moves away from
93 ER to other subcellular membrane compartments, including TGN, to fulfill their needs for
94 cholesterol. To test this hypothesis, we adopted a transgenic mouse model-based approach.

95
96 A mutant mouse model for NPC disease (*Npc1^{nmf}* mouse) was discovered and characterized by
97 Maue *et al.* (46). This *Npc1^{nmf}* mouse model has a C57BL/6J genetic background, and a single
98 D1005G-*Npc1* mutation located within the cysteine-rich luminal loop of the NPC1 protein; which
99 is comparable to mutations that commonly occur in human *Npc1* patients. Homozygous
100 *Npc1^{nmf/nmf}* mice begin to die by 90 days (almost 13 weeks) after birth; and exhibit a phenotype
101 that mimics the late onset, slowly progressing form of NPC disease. In contrast, heterozygous
102 *Npc1^{nmf/+}* mice seem to exhibit a generally normal mouse phenotype and are fertile. In the
103 studies presented here, we bred heterozygous *Npc1^{nmf/+}* mice with global *Acat1^{-/-}* (*A1^{-/-}*)-deficient
104 mice (47), which also have a C57BL/6J genetic background, to produce *Npc1^{nmf/nmf}.A1^{+/+}* and
105 *Npc1^{nmf/nmf}.A1^{-/-}* mice. We then performed paired studies *in vivo* by using sex and age matched
106 *Npc1^{nmf/nmf}.A1^{+/+}* (*Npc1^{nmf}*) and *Npc1^{nmf/nmf}.A1^{-/-}* mice (*Npc1^{nmf}.A1^{-/-}*). We used littermates from
107 *Npc1^{+/+}.A1^{+/+}* mice (WT) and *Npc1^{+/+}.A1^{-/-}* mice (*A1^{-/-}*) produced from the same breeding
108 experiments as non-diseased controls. In addition, we isolated embryonic fibroblast cells from
109 *Npc1^{nmf}*, *Npc1^{nmf}.A1^{-/-}*, WT, and *A1^{-/-}* mice to perform paired studies *in vitro* in primary cell

110 culture. Furthermore, to evaluate the relevance of our findings in the context of human disease,
111 we monitored the effect of a small molecule ACAT1-specific inhibitor K604 on human fibroblast
112 (Hfs) cells isolated from several patients with NPC disease as well as on a human fibroblast
113 cells isolated from a patient with a related lysosomal storage disease Niemann-Pick type A
114 (NPA). The results of both these *in vivo* and *in vitro* approaches are reported here.

115

116

117 Results

118

119 ***Acat1* gene deficiency (*A1*^{-/-}) increased life span and reduced weight loss in *Npc1*^{*nmf*} mice.**

120 To evaluate effects of *A1*^{-/-} on lifespan of homozygous *Npc1*^{*nmf*} mice, mice with four genotypes
121 (WT, *A1*^{-/-}, *Npc1*^{*nmf*}, and *Npc1*^{*nmf*}:*A1*^{-/-}) were fed a regular chow diet and their lifespans were
122 assessed. The age of death for *Npc1*^{*nmf*} mice with or without *A1* were determined as the point
123 where mice could no longer take in food or water, as described previously (46). Results (**Fig.**
124 **1A**) show that the median survival for *Npc1*^{*nmf*} and *Npc1*^{*nmf*}:*A1*^{-/-} mice is 113 days and 138 days
125 respectively, with the mean survival being 102 days and 137 days, respectively.

126

127 Overall, *A1*^{-/-} increased *Npc1*^{*nmf*} mutant mouse lifespan by 34%. In control experiments, no
128 spontaneous deaths occurred in either WT mice or *A1*^{-/-} mice. We next evaluated the effect of
129 *A1*-deficiency on the weights of *Npc1*^{*nmf*} mice, starting at 6 weeks of age. Results (**Fig. 1B; right**
130 **hand panel**) show that *Npc1*^{*nmf*} mice start to lose weight at around 9 weeks of age. In contrast,
131 mice lacking *A1* gene did not begin to lose weight until 13 weeks of age. Thus, the lack of *A1*
132 delayed the onset of weight loss observed in *Npc1*^{*nmf*} mice. In control experiments (**Fig. 1B; left**
133 **hand panel**) at 6 weeks of age, *A1*^{-/-} mice weighed slightly less than WT mice, but at 8 weeks of
134 age or older, the difference in weights between these 2 genotypes disappeared.

135

136 ***A1*^{-/-} reduced foam cell pathology in liver/spleen and Purkinje neuron loss in *Npc1*^{*nmf*}**
137 **mice.** Niemann-Pick disease exhibits accumulation of large foamy macrophages in various
138 tissues. To determine if *Acat1* inhibition had any effect on the foam cell pathology in *Npc1*^{*nmf*}
139 mouse, we isolated tissues from WT, *A1*^{-/-}, *Npc1*^{*nmf*}, and *Npc1*^{*nmf*}:*A1*^{-/-} mice at 80 days (P80) of
140 age, and performed histological staining analyses. When compared to *Npc1*^{*nmf*} mice,
141 *Npc1*^{*nmf*}:*A1*^{-/-} mice have significantly reduced foam cell pathology in liver (**Fig. 2A; right panels;**
142 **top vs. bottom**) and spleen (**Fig. 2B; right panels; top vs. bottom**). In lung, however, foam
143 cell pathology in these two genotypes are comparable (**Fig. 2C; right panels; top vs. bottom**).

144 The result of the control experiments confirmed that neither WT mice nor global *A1*^{-/-} mice
145 exhibit significant foam cell pathology in any of these three tissues (**Fig. 2A-C; left panel; top**
146 **vs. bottom**). Previous studies in mutant NPC animals have demonstrated that extensive
147 Purkinje neuron cell death occurs prior to the death of the animals. To evaluate the effect of *A1*^{-/-}
148 on *Npc1*^{*nmf*} Purkinje neurons, cerebellum was isolated from P80 mice brain, and the number of
149 Purkinje neurons were counted after histochemical staining of thin slices. As shown in **Fig. 2D**,
150 when compared to values that found in WT mice and in *A1*^{-/-} mice, *Npc1*^{*nmf*} mice had less than

151 20% residual Purkinje cells remaining, while in the *Npc1^{nmf}:A1^{-/-}* mice, the numbers of residual
152 Purkinje neurons remaining was more than 30% of WT values (**Fig. 2D; right panels**). To
153 validate the results shown in Fig. 2D, we isolated cerebellar tissue from the brains of P50 and
154 P90 mice, and compared the levels of calbindin mRNA, as calbindin expression is specific to
155 Purkinje neurons in the cerebellum. The results of P50 cerebellum (**Fig. 2E; A**), calbindin mRNA
156 levels in WT and *A1^{-/-}* mice were comparable (first 2 bars on left). In *Npc1^{nmf}* mice (the 3rd bar
157 from left), the expression was significantly reduced by 55% of the values found in WT and *A1^{-/-}*
158 mice, but the expression was almost completely restored in *Npc1^{nmf}:A1^{-/-}* mice (4th bar from left)
159 to about 90% of values found in WT and *A1^{-/-}* mice. The results obtained from the P90
160 cerebellum (**Fig. 2E; B**) show that calbindin expression in *Npc1^{nmf}* mice was drastically reduced
161 to about 10% of values found in WT and *A1^{-/-}* mice, but was partially restored in *Npc1^{nmf}:A1^{-/-}*
162 mice, to about 30% of values found in WT and *A1^{-/-}* mice. Overall, these results demonstrate
163 that in the *Npc1^{nmf}* mouse model, *A1* gene ablation significantly decreases the loss of Purkinje
164 neurons in brain.

165

166 ***A1^{-/-}* ameliorated the motor deficits and behavior of *Npc1^{nmf}* mice.** Mutant *Npc1^{nmf}* mice
167 exhibit a decline in their motor performance, beginning at 11 weeks of age (46). We monitored
168 the motor performance of WT, *A1^{-/-}*, *Npc1^{nmf}*, and *Npc1^{nmf}:A1^{-/-}* mice beginning at 9 weeks of age
169 by using a rotarod test. The results show that for *Npc1^{nmf}* mice, the mean age of failing off or not
170 running on the rotarod test occurred at around 12 weeks of age, whereas for *Npc1^{nmf}:A1^{-/-}* mice,
171 the mean age of failing occurred at 18 weeks of age (**Fig. 2F**). Results from control experiments
172 show that at the same age, neither WT mice nor *A1^{-/-}* mice fail the rotarod test. Since the rotarod
173 test does not assess muscle strength per se, these results do demonstrate that *A1* gene
174 ablation improves sensorimotor coordination and behavior in the *Npc1^{nmf}* mice.

175

176 **Cholesterol content, cholesterol esterification, and cholesterol distribution in mouse**
177 **embryonic fibroblasts (MEF) from WT, *A1^{-/-}*, *Npc1^{nmf}*, and *Npc1^{nmf}:A1^{-/-}* mice.** To determine
178 whether *A1^{-/-}* alters the total free cholesterol content in *Npc1^{nmf}* mouse brain, we first isolated
179 whole brains from WT, *A1^{-/-}*, *Npc1^{nmf}*, and *Npc1^{nmf}:A1^{-/-}* mice at postnatal day 90 (P90) and
180 measured their total free (unesterified) cholesterol content. The results show that the cholesterol
181 content in these samples are comparable to one another (**Fig. 3A**).

182

183 In the central nervous system, however, the bulk of cholesterol exists in the membranes of a
184 variety of cell types, including the glial-derived myelin sheath, making measurements of bulk

185 cholesterol less informative when considering the effects of NPC1 deficiency, as well as the
186 effects of ACAT1 inhibition on membrane cholesterol distribution at the cellular level. Parallel
187 cultures of primary fibroblasts isolated from normal mice and from mice with single mutations
188 have been used extensively as a model system to investigate the effects of single-gene
189 mutations at the biochemical level. We therefore used mouse embryonic fibroblasts (MEFs) to
190 address this question. We isolated MEFs from WT, $A1^{-/-}$, $Npc1^{nmf}$, and $Npc1^{nmf};A1^{-/-}$ mice, grew
191 them in DMEM with 10% serum in monolayers until confluent, and then harvested the cells for
192 the analysis of total free cholesterol content. Results (**Fig. 3B**) show that the free cholesterol
193 content in these four cell types are very similar, demonstrating that under the condition
194 employed, neither NPC1 mutation nor ACAT1 blockage significantly affects the total cellular free
195 cholesterol content.

196
197 We next measured relative cholesterol ester biosynthesis rates in these cells by feeding labelled
198 ^3H -oleate to the intact cells for 20-min and then measuring the ^3H -cholesteryl oleate that was
199 produced. The results (**Fig. 3C**) show that when grown in lipoprotein-containing medium (10%
200 serum), WT cells exhibit an ample cholesterol ester biosynthesis rate. As expected, in cells
201 without ACAT1 (i.e., $A1^{-/-}$ cells and $Npc1^{nmf};A1^{-/-}$ cells), the cholesterol ester biosynthesis rate
202 was only 2% that of WT cells. The residual amount of cholesteryl oleate in ACAT-deficient cells
203 formed are perhaps due to the presence of ACAT2 (48). In $Npc1^{nmf}$ cells, the rate was around
204 34% that of WT cells (**Fig. 3C**). When these four cell types were grown in 10% delipidated
205 serum medium (i.e., medium devoid of exogenous cholesterol), the cholesteryl ester
206 biosynthesis rate in WT cells decreased drastically; these decreases also occurred in the other
207 three cell types (**Fig. 3D; lanes 5-8**); whereas, in contrast, when low-density lipoproteins (LDL)
208 were added to the delipidated serum (DLS) medium for 3 h, 6 h, or for 24 h (indicated as LDL (3
209 h), LDL (6 h) or LDL (24 h) below the X-axis in **Fig. 3D**), cholesteryl ester biosynthesis rate was
210 restored in WT cells in a time-dependent manner. Adding LDL also increased cholesteryl ester
211 biosynthesis rate in mutant $Npc1^{nmf}$ cells, but this increase was not nearly as what was observed
212 in WT cells (**Fig. 3D; comparing 3rd bars vs. 1st bars**). These results show that within the 3-6 h
213 period, mutant $Npc1^{nmf}$ cells fail to efficiently utilize LDL-derived cholesterol for esterification,
214 indicating the importance of NPC1 in delivering cholesterol from endosomes to the ER. As
215 expected, in $A1^{-/-}$ cells or in $Npc1^{nmf};A1^{-/-}$ cells, within the 3-6 h time frame, the LDL-dependent
216 increase in cholesterol ester biosynthesis rate was abolished (**Fig. 3D; comparing 2nd bars
217 and 4th bars vs. the 1st bars**). When cells were exposed to LDL for 24 h, a large increase in
218 cholesterol ester biosynthesis rate occurred in $Npc1^{nmf}$ cells but not in cells without A1 (**Fig. 3D**;

219 comparing last 4 bars at the LDL (24 h) time point). Our earlier results (**Fig. 3C**) show that,
220 when maintained in the lipoprotein- containing medium at a steady state, *Npc1^{nmf}* cells exhibit a
221 residual cholesterol ester biosynthesis rate of about 34% compared to WT cells. These results
222 support the notion (described in the Introduction) that, in addition to using cholesterol derived
223 from the NPC-containing late endosomes for esterification, cells can also use other cholesterol
224 sources for esterification at the ER, in an NPC-independent manner.

225
226 ACAT1 plays a key role in cholesterol storage via its conversion of cholesterol to cholesterol
227 esters. In both WT and *Npc1^{nmf}* MEFs, preventing cholesterol storage by inhibiting ACAT1 may
228 cause significant alteration(s) in cholesterol distribution among various membrane organelles.
229 To test this possibility, we performed intact cell staining with filipin, a naturally fluorescent small
230 molecule that binds to cholesterol, and then viewed the labeled cells with spinning disc confocal
231 fluorescence microscopy. The results show that in WT and *A1^{-/-}* cells (**Fig. 4A; 1st and 2nd**
232 **columns**), the cholesterol-rich domains, as represented by areas with strong filipin staining (**1st**
233 **row**), were mostly located in peripheral regions of the cytosol, whereas in mutant *Npc1^{nmf}* cells
234 (**Fig. 4A; 3rd column**), the cholesterol-rich domains were primarily peri-nuclear in their
235 localization. It was previously shown that LE/LYS exhibit non-random, bidirectional movements
236 between the nucleus region and the cell surface; when mutant NPC1 cells were laden with
237 cholesterol and other lipids, the movements of LE/LYS towards the cell periphery became
238 sluggish, presenting cholesterol-rich particles primarily to the perinuclear location (49). The
239 results shown in Fig. 4A; 3rd row confirms these findings. Importantly, in mutant *Npc1^{nmf}* cells,
240 *A1^{-/-}* caused most of the cholesterol-rich particles to become primarily peripherally located (**Fig.**
241 **4A; the 4th column**). We also calculated total cell fluorescence intensity of filipin per cell and
242 found that the values among these four cell types were comparable (**Fig. 4B**). Overall, these
243 results imply that *A1^{-/-}* corrected the late endo/lysosomal cholesterol sequestration defects seen
244 in the mutant NPC1 cells without significantly altering the total cholesterol content in these cells.
245 We also noted that when compared to WT cells, *A1^{-/-}* cells contained more cholesterol-rich
246 domains that are scattered throughout the cytoplasm (**Fig. 4A; the 2nd column**), suggesting that
247 in WT cells, *A1^{-/-}* causes significant cholesterol accumulation in certain internal membrane
248 organelle(s). The nature of these cholesterol-rich internal organelles is unknown at present.

249
250 To examine the membrane cholesterol distribution in these four cell types with another method,
251 we took a biochemical approach, and performed subcellular fractionation of post-nuclear cell
252 homogenates prepared from the MEFs of these four different genotypes, using OptiPrep density

253 gradient ultracentrifugation. This method produces partial separation of various membrane
254 organelles, based primarily on their buoyant densities (50), (51), (22). After OptiPrep
255 fractionation we analyzed the distribution of various membrane organelles in the fractions by
256 performing Western blot analyses of protein markers for LE/LYS, TGN, ER, caveolae, and
257 mitochondria (**Fig. 4C**). We also analyzed cholesterol content in each fraction (**Fig. 4D**).
258 Western analyses (**Fig. 4C**) show that in all four cell types, LE/LYS (LAMP1 positive) were
259 tightly enriched in fractions #1 to #3 (with light density) and the mitochondria (cytochrome C
260 oxidase positive) were tightly enriched in fractions #9 to #11 (with heavy density). Mutant
261 *Npc1^{nmf}* cells, but not *Npc1^{nmf}:A1^{-/-}* cells, exhibited additional LAMP1 positive signals in fractions
262 #5 to #9. The ER (calnexin positive) exhibited a broader range of densities but was similarly
263 enriched in #6 to #10 in all cell types. The PM (caveolin 1 positive) was enriched in fractions #5
264 to #8 in WT cells (with medium density), but in *A1^{-/-}* cells was enriched in later fractions (#7 to
265 #9). This result suggests that *A1^{-/-}* may cause an increase in buoyant density of the PM. Similar
266 to the WT cells, in mutant *Npc1^{nmf}* cells and in *Npc1^{nmf}:A1^{-/-}* cells, the PM were enriched in
267 fractions #5 to #8. However, the PM in mutant *Npc1^{nmf}:A1^{-/-}* cells exhibited a broader range in
268 buoyant densities. These results show that except for the PM in *A1^{-/-}* cells and the abnormal
269 LAMP1 positive signal in mutant *Npc1^{nmf}* cells, the buoyant densities of LE/LYS, mitochondria,
270 ER, and PMs in these four cell types are comparable. In contrast, the syntaxin 6 rich-fractions
271 (**Fig. 4C; 2nd row**) exhibited large variation in densities: in WT cells, they are enriched in
272 fractions #5 to #6; in *A1^{-/-}* cells, in fractions #8 to #10; in *Npc1^{nmf}* cells, in fractions #8 to #11, in
273 *Npc1^{nmf}:A1^{-/-}* cells, in fractions #5 to #9. The results of cholesterol content analyses in the
274 OptiPrep fractions (**Fig. 4D**) show that as expected, in WT cells, cholesterol was highly enriched
275 in the PM fraction (#5 to #8), and membranes with slightly heavier densities, including the ER
276 membranes (#7 to #9), whereas the mitochondrial membranes had much less cholesterol
277 content. In *A1^{-/-}* cells, membranes with heavier densities, including the ER (#7 to #9) and
278 mitochondria (#9 to #11), may be enriched in cholesterol. However, this interpretation is not
279 definitive, because in the *A1^{-/-}* cells, the PM fractions increased in buoyant densities, such that
280 they overlapped significantly with the ER fractions (#7 to #9) (**Fig. 4C; 4th row**). In mutant
281 *Npc1^{nmf}* cells, as expected, cholesterol was highly enriched in the light density late
282 endo/lysosomes (LE/LYS) fraction (fractions #1 to #3). In the mutant *Npc1^{nmf}:A1^{-/-}* cells, the
283 Golgi-like membranes (#6 to #9), the PM-like membranes (#5 to #8), and the ER-like
284 membranes (#7 to #9), all become relatively enriched in cholesterol. These results corroborate
285 with the cholesterol distribution in intact cells (shown in Fig. 4A), and support the interpretation
286 that while in *Npc1^{nmf}* cells cholesterol is mostly sequestered within LE/LYS, with the deletion of

287 the *A1* gene in *Npc1^{nmf}:A1^{-/-}* cells most of the sequestered cholesterol appears redistributed to
288 various other membrane organelles, including Golgi, ER, PM, and mitochondria.

289

290 **Effects of *A1^{-/-}* on syntaxin 6 and golgin 97 localization in intact mutant *Npc1^{nmf}* MEFs.**

291 The result presented in **Fig. 4C** (2nd row) showed that the syntaxin 6-rich membranes isolated
292 from the four different MEFs exhibited large variation in buoyant densities. Syntaxin 6 binds to
293 cholesterol (52), and is one of the t-SNARE proteins present in vesicles that participate in
294 various membrane fusion events. The syntaxin 6-rich vesicles move dynamically between
295 various membrane organelles. In normal cells, most of the syntaxin 6 signal is found at the
296 TGN (53). The TGN is rich in cholesterol content (54), and plays key roles in transporting
297 proteins and lipids to various other membrane compartments, including the PMs and
298 endosomes. In mutant NPC cells, the TGN fails to receive LDL-derived cholesterol from the late
299 endosomes (22). This deficiency causes syntaxin 6 to be mislocalized from the TGN, and
300 instead it exhibits an abnormal, scattered cytoplasmic pattern (25). Importantly, treating mutant
301 NPC1 cells with cholesterol/cyclodextrin complex, or with high concentration of LDL for 24 h
302 restores the syntaxin 6 signal to the typically one-sided, perinuclear Golgi localization pattern
303 observed in normal cells (25). In mutant NPC cells, other SNARE proteins such as syntaxin 16,
304 VAMP3, VAMP4, do not show cholesterol-sensitive localization patterns (25). Based on these
305 previous findings, as well as results presented in **Fig. 4B,C,D**, we postulated that *A1^{-/-}* may
306 correct the mis-localization pattern of syntaxin 6 observed in mutant *Npc1^{nmf}* MEFs. To test this
307 possibility, we performed immunofluorescence confocal microscopy in fixed, intact cells using
308 antibodies specific for syntaxin 6. The results (**Fig. 5A**) show that, in WT cells and in *A1^{-/-}* cells
309 most of the syntaxin 6 signal was highly polarized to only one side of the space adjacent to the
310 nucleus. This pattern is a typical Golgi distribution pattern found in mammalian cells (55). In
311 contrast, in mutant *Npc1^{nmf}* cells (**Fig 5A**), a significant portion of the syntaxin 6 signal was
312 distributed in scattered cytoplasmic vesicular structures; these structures were located at the
313 space around both sides of the nucleus. This result confirms the findings by Reverter *et al.* (25).
314 Importantly, *A1* deletion (*A1^{-/-}*) in mutant NPC cells (**Fig 5A**) largely restored the syntaxin 6
315 distribution pattern back to the one-sided, peri-nuclear pattern observed in WT and *A1^{-/-}* cells. To
316 quantitate the difference of syntaxin 6 positive signals observed in the MEFs of these four
317 different genotypes, we adopted the procedure developed by Mitchel *et al.* (55), by measuring
318 the “Reflex angle”, defined as the angle subtended by the edges of the syntaxin 6-positive
319 signals in the confocal images, using the center of the nucleus (DAPI positive signal) as the
320 vertex. The results (**Fig. 5B**) show that for WT and *A1^{-/-}* cells, the reflex angle was very similar :

321 119° or 126° respectively; for mutant *Npc1^{nmf}* cells, the reflex angle was much larger (346°). For
322 mutant *Npc1^{nmf}:A1^{-/-}* cells, the reflex angle was largely restored, to 160°.

323

324 The results of control experiments (**Fig. 5C**) show that the level of syntaxin 6 protein in all four
325 cell types examined were comparable. Together, these results show that *A1^{-/-}* largely restores
326 the mis-localization of syntaxin 6 observed in mutant *Npc1^{nmf}* cells, without affecting the syntaxin
327 6 protein content.

328 To strengthen these results, we sought to examine the localization pattern of a second protein
329 marker of the TGN. Golgins are long coiled-coil peripheral membrane proteins located mainly at
330 the membrane surface of the TGN (56). In humans there are four golgins, each playing a
331 distinct role in membrane protein transport events. Using specific antibodies against golgin 97
332 (GCC97), we performed double immunofluorescence experiments to compare the localization
333 patterns of syntaxin 6 and golgin 97 in parallel cultures of four MEF cell types. The results show
334 that, in WT cells (**Fig. 5D, 1st row**), golgin 97 exhibited a typical polarized Golgi distribution
335 pattern. In *A1^{-/-}* cells (**Fig. 5D, 2nd row**), most of the golgin 97 exhibited a similar pattern to what
336 was found in WT cells; with a small portion also appearing as part of small, punctate structures,
337 perhaps as parts of the internal membrane organelles. In *Npc1^{nmf}* cells (**Fig. 5D; 3rd row**), golgin
338 97 became dispersed, and scattered within the space around the nucleus. This distribution
339 pattern is clearly distinct from that observed in WT cells and in *A1^{-/-}* cells. Importantly, in
340 *Npc1^{nmf}:A1^{-/-}* cells (**Fig. 5D; 4th row**), the abnormal golgin 97 localization pattern in *Npc1^{nmf}* cells
341 was largely corrected. For clarity, enlarged (6-fold) versions of the merged images are provided
342 (**Fig. 5D**). We next performed double immunofluorescence experiments and monitored the
343 degree of apparent colocalization between syntaxin 6 and golgin 97 in these four cell types (**Fig.**
344 **5 D, E**). The results show that in WT and *A1^{-/-}* cells, the apparent colocalization between
345 syntaxin 6 and golgin 97 was relatively low (between 21% to 32%). Mutation in NPC1 caused
346 the colocalization index to increase to 51%; while *A1 deletion (A1^{-/-})* in the mutant *Npc1^{nmf}* cells
347 reduced the % colocalization index back to 24%, a value comparable to those observed in WT
348 and *A1^{-/-}* cells. Together, these results shown in **Fig. 5A-E** suggest that mutation of NPC1
349 causes a certain portion of the TGN membrane to become deficient in cholesterol; this
350 deficiency causes both syntaxin 6 and golgin 97 to become mislocalized from the rest of the
351 TGN. Importantly, ACAT1 blockage in mutant NPC1 cells largely corrects the abnormal
352 localization patterns of both syntaxin 6 and golgin 97.

353

354 **Effects of $A1^{-/-}$ on the levels of cation-dependent manose-6-phosphate receptor (CD-**
355 **M6PR), and cathepsin D protein contents in mutant $Npc1^{nmf}$ MEFs.** Syntaxin 6 is involved in
356 the anterograde vesicular trafficking of M6PRs/lysosomal hydrolase complexes (57). The cation-
357 dependent (CD) and cation-independent (CI) mannose-6-phosphate receptors (CD-M6PR and
358 CI-M6PR) deliver newly synthesized lysosomal enzymes, which carry the mannose-6-
359 phosphate signal, from the TGN to the late endosomes. The M6PRs then recycle back to the
360 TGN for re-utilization, as reviewed in (58). Previously, Kobayashi *et al.* (59) showed that in
361 mutant NPC cells, the CD-M6PR localization pattern was altered, from mainly residing at the
362 TGN to mostly residing in the cholesterol-laden late endosomes. Ganley and Pfeffer (60)
363 showed that in mutant NPC cells, cholesterol accumulation caused CD-M6PR to be missorted
364 and rapidly degraded in late endo/lysosomes. Since we found that in mutant $Npc1^{nmf}$ cells, $A1^{-/-}$
365 rescued syntaxin 6 from mislocalization (**Fig. 5A,B**), we suspected that $A1^{-/-}$ may also affect the
366 M6PR protein expression in mutant $Npc1^{nmf}$ cells. To test this possibility, we performed Western
367 blot analyses to examine the levels of CD-M6PR protein in parallel cultures of MEFs of the four
368 genotypes. The results (**Fig. 6A**) show that when compared to WT cells, $Npc1^{nmf}$ cells express
369 CD-M6PR at levels less than 20% of values found in WT cells. In contrast, the $Npc1^{nmf};A1^{-/-}$ cells
370 expressed CD-M6PR at levels more than 2-fold that of WT cells. This result suggests that
371 deleting $A1$ was indeed more than sufficient to correct the low level of expression of the CD-
372 M6PR protein observed in $Npc1^{nmf}$ cells. Interestingly, we also found that $A1^{-/-}$ cells expressed
373 CD-M6PR level at 50% of values found in WT cells. At present, the significance of this finding is
374 unclear at present.

375
376 Cathepsin D is one of the major lysosomal enzymes that require M6PRs for processing at the
377 TGN. From the TGN, the cathepsin D/M6PR complexes move to LE/LYS for maturation by
378 proteolysis. Cathepsin D exists in various forms, including the precursor, intermediate, and
379 proteolytically cleaved mature heavy chain and light chain. These forms exhibit different
380 molecular weights on SDS-PAGE (61). Since we found that the expression level of CD-M6PR is
381 very low in mutant $Npc1^{nmf}$ cells and $A1^{-/-}$ corrected this deficiency (**Fig. 6A**), we postulated that
382 mutant $Npc1^{nmf}$ MEFs may express lower level of cathepsin D protein (the mature heavy chain),
383 and that $A1^{-/-}$ in mutant $Npc1^{nmf}$ MEFs may correct this abnormality. To test this possibility, we
384 performed Western blot analysis with a highly specific, monoclonal antibody against the
385 cathepsin D mature form heavy chain. The results (**Fig. 6 B**) show that in both WT cells and $A1^{-/-}$
386 MEF cells, this antibody recognized the mature form of cathepsin D heavy chain, which has an
387 apparent size of 30-32 kDa. Furthermore, WT and $A1^{-/-}$ cells expressed the mature form at

388 comparable levels. In contrast, the mutant *Npc1^{nmf}* cells expressed cathepsin D at a level 64%
389 less than that of WT cells and *A1^{-/-}* cells. In addition, the size of the mature form of cathepsin D
390 present in the *NPC1^{nmf}* cells is slightly smaller (by 2-3 kDa) than that found in WT and *A1^{-/-}* cells,
391 suggesting that within the LE/LYS of *Npc1^{nmf}* cells, abnormal proteolytic cleavage of cathepsin D
392 might occur. The *Npc1^{nmf}:A1^{-/-}* cells expressed cathepsin D with the same size as found in WT
393 cells and *A1^{-/-}* cells, with protein levels higher than those found in WT and *A1^{-/-}* cells by 40%.
394 Together, these results show that in *Npc1^{nmf}* cells, *A1^{-/-}* reversed the diminished levels of CD-
395 M6PR and cathepsin D protein expression, consistent with both of these proteins being
396 downstream targets of syntaxin 6 mediated vesicular trafficking. We were curious as to whether
397 the diminished cathepsin D protein content observed in *Npc^{nmf}* cells may affect their ability to
398 degrade proteins. To address this issue, we monitored the degradation of long-lived proteins by
399 using the procedure described by Auteri *et al.* (62). The result (**Fig. 6C**) shows that, instead, the
400 proteolysis of long-lived proteins in WT, *A1^{-/-}*, *Npc1^{nmf}*, and *Npc1^{nmf}:A1^{-/-}* MEF cells was
401 comparable. This finding is consistent with the work of Pacheco *et al.* (63), who showed that
402 normal and NPC1-deficient human fibroblast (Hf) cells, the degradation of long-lived proteins
403 was comparable.

404

405 **Effects of ACAT1 inhibition on ABCA1 and NPC1 protein levels in MEFs, in mouse**

406 **cerebellum, and in human fibroblasts (Hfs).** The results described above show that in
407 *Npc1^{nmf}* cells, the cathepsin D protein content is significantly decreased, and that the decrease
408 can be corrected by *A1^{-/-}*. To substantiate this finding, we sought to identify the relevant
409 downstream targets of cathepsin D mediated signaling and decided to focus on the ATP-binding
410 cassette transporter A1 (ABCA1). ABCA1 functions as a key cellular cholesterol efflux protein
411 [reviewed in (64)]. It is transcriptionally regulated by liver X receptors (LXRs) (65), (66) and post-
412 translationally regulated by various degradation mechanisms (67). In mutant *Npc1* Hfs, Choi *et*
413 *al.* showed that the levels of both ACAT1 mRNA and protein are down regulated (68). In
414 addition, in macrophages and other cells, Haidar *et al.* showed that the ABCA1 protein content
415 is up-regulated by cathepsin D through a post-translational mechanism yet to be defined (69). In
416 various mammalian cell lines examined, blocking ACAT1 either by genetic inactivation or by
417 using a small molecule ACAT inhibitor increased the ABCA1 protein content; with the degree of
418 the ACAT inhibition affecting ABCA1 levels in a cell type dependent manner (70). On the other
419 hand, whether ACAT1 inhibition can increase ABCA1 in mutant NPC cells had not been
420 reported previously. Since we found that mutant *Npc1^{nmf}* MEF cells expressed the mature form
421 of cathepsin D at a level significantly lower than that in WT cells (**Fig. 6B**), we postulated that

422 this abnormality may cause mutant *Npc1^{nmf}* MEF cells to express the ABCA1 protein at a lower
423 level, and that *A1^{-/-}* may be able to correct this deficiency. To test this possibility, we performed
424 Western blot analyses in parallel cultures of WT, *A1^{-/-}*, *Npc1^{nmf}*, and *Npc1^{nmf}:A1^{-/-}* MEFs. The
425 results show that the ABCA1 protein content in mutant *Npc1* cells was lower than in WT cells or
426 in *A1^{-/-}* cells, and that *A1^{-/-}* in mutant *Npc1^{nmf}* cells restored ABCA1 protein content to similar
427 level found in the WT cells (**Fig. 7A**). We prepared MEFs from WT and mutant mice that
428 completely lacking NPC1 (the *Npc1^{nih}* mouse model with a BALB/c genetic background), and
429 found that the ABCA1 protein content in the *Npc1^{nih}* MEFs was also significantly lower than that
430 in the control WT MEF cells (**Fig. 7A; top row**; comparing the last 2 lanes on the right). These
431 results show that in MEFs, *A1^{-/-}* restore the diminished protein content of ABCA1 in *Npc1^{nmf}*
432 cells. We also performed Western blot analyses to monitor the NPC1 protein content (**Fig. 7A;**
433 **second row**) that in mutant *Npc1^{nmf}* cells, NPC1 protein content was significantly lower than
434 that in WT or *A1^{-/-}* cells, confirming our previous report (46). *A1^{-/-}* in mutant *Npc1* cells had a
435 tendency to increase mutant NPC1 protein content, but the effect did not reach statistical
436 significance (**Fig. 7A**). To serve as a control, additional results show that unlike WT MEFs,
437 NPC1 protein was completely absent from *Npc1^{nih}* MEFs (**Fig. 7A**). We next determined the
438 level of ABCA1 mRNA in parallel cultures of WT, *A1^{-/-}*, *Npc1^{nmf}*, and *Npc1^{nmf}:A1^{-/-}* MEFs by RT-
439 PCR. The results (**Fig. 7B**) show that, similar to the finding by Choi *et al.* in Hfs (68), the ABCA1
440 mRNA level in *Npc1^{nmf}* cells is lower than that in WT cells. Furthermore, in both WT and *Npc1^{nmf}*
441 cells, *A1^{-/-}* caused increase in the level of ABCA1 mRNA. This result supports the interpretation
442 that in mutant NPC1 cells, *A1^{-/-}* restored the level of ABCA1 protein, at least in part, by restoring
443 cathepsin D function in late endo/lysosomes.

444

445 To test the *in vivo* significance of these findings, we next performed similar Western blot
446 analyses of homogenates prepared from P80 mouse cerebellum. The results show that in
447 mutant *Npc1* cerebellum, *A1^{-/-}* significantly increased ABCA1 protein abundance (**Fig. 7C; left**
448 **panel at the bottom**; comparing the 3rd bar versus the 4th bar). *A1^{-/-}* had a tendency to also
449 increase the mutant NPC1 protein content, but the effect did not reach statistical significance
450 (**Fig. 7C**; right panel at the bottom; comparing the 3rd bar versus the 4th bar). These results
451 demonstrate that the restorative effect of *A1^{-/-}* on the low level of ABCA1 protein found in mutant
452 NPC1 MEF can be replicated in P80 mouse cerebellum.

453

454

455 To determine if the beneficial effect of $A1^{-/-}$ on increasing the ABCA1 protein level in MEFs with
456 an NPC mutation can also be observed in Hfs isolated from patients, we treated Hfs isolated
457 from one normal individual, four NPC1 patients, and one Niemann-pick disease type A (NPA)
458 patient (as indicated in **Fig. 7D**) with the small molecule ACAT1 specific inhibitor K604 at 0.5 μ M
459 for 24 h. At this concentration, K604 is expected to inhibit the ACAT1 enzyme activity in intact
460 cells by approximately 70-80% (71). The results (**Fig. 7D, E**) show that K604 modestly
461 increased the ABCA1 protein in normal Hf, and in one of four different mutant NPC1 Hfs (line
462 GM03123). In the second Hf with mutant NPC1 (GM18453), in one Hf with mutant NPA
463 (GM00112), and in one Hf with mutant NPC2 (GM17910), K604 tended to increase the ABCA1
464 protein, but the difference did not reach statistical significance. These results show that the
465 enhancing effects of ACAT1 inhibition on ABCA1 protein levels observed in mutant $Npc1^{nmf}$
466 MEFs can be demonstrated in at least some Hfs with NPC1 mutations.

467 Discussion

468

469 We genetically inhibited ACAT1 in a mouse model of NPC1 disease (*Npc1^{nmf}*) and show that *A1^{-/-}*
470 delays the onset of weight loss and declining sensorimotor skill, ameliorates certain systemic and
471 neuropathological NPC disease hallmarks, and prolongs the life span by 34%. This “rescue” of
472 *Npc1^{nmf}* mice by *A1^{-/-}* is rather surprising, and while similar attempts to extend lifespan have been
473 made by genetic crossing of mutant NPC1 mice with mice lacking genes that encode one of the
474 following proteins: LDL receptor (72), ApoE (73), SRBI (74), GM2 synthetase (75), GM3
475 synthetase (76), glucocerebrosidase 2 (77), Tau (78), and RIPK1 (a key protein that mediates
476 necroptosis) (79), to our knowledge the current study is the first to demonstrate that knockout of
477 a single gene can extend the life span of a mutant *Npc1* mouse model by more than 30%.

478

479 To provide a mechanistic basis for the beneficial effects of ACAT1 inhibition at the cellular level,
480 we studied MEFs isolated from WT, *A1^{-/-}*, *Npc1^{nmf}*, and *Npc1^{nmf}:A1^{-/-}* mice, and show that in
481 mutant NPC1 MEFs, ACAT1 inhibition alters membrane cholesterol distribution, and restores
482 the mislocalization of syntaxin 6, a cholesterol-binding t-SNARE that normally localizes to the
483 TGN and mediates the anterograde vesicular trafficking of the M6PRs/lysosomal hydrolase
484 complexes. In addition to syntaxin 6, we show in mutant NPC1 cells that golgin 97, a different
485 TGN marker, also mis-localized, and *A1^{-/-}* can restore normal golgin 97 localization at the TGN .
486 These results suggest that mutation of NPC1 causes a certain portion of the TGN membrane to
487 become deficient in cholesterol and that ACAT1 inhibition in mutant NPC1 cells corrects the
488 abnormal localization patterns of both syntaxin 6 and golgin 97 by replenishing cholesterol in the
489 TGN membrane. We also show that mutant NPC cells express diminished level of protein
490 further downstream, such as CD-M6PR and cathepsin D (one of the key lysosomal hydrolases),
491 and that *A1^{-/-}* restores the levels of these proteins as well. These results imply that *A1^{-/-}* restores
492 the localization and functionality of syntaxin 6 in mutant NPC1 cells. To link the changes in
493 cathepsin D with its downstream target(s), we also show that *A1^{-/-}* restores the diminished
494 ABCA1 protein content observed in mutant NPC1 cells and in mutant NPC1 cerebellum. Based
495 on these results, we propose a model to explain the actions of ACAT1 blockage: In mutant NPC
496 cells, the inability of NPC to export cholesterol from the LE/LYS causes several membrane
497 organelles downstream of NPC mediated cholesterol trafficking pathway to become deficient in
498 cholesterol which leads to malfunctions in these organelles. ACAT1 resides at a certain
499 subdomain (designated as the A1 domain) of the ER. Despite the mutation in NPC, certain
500 cholesterol continues to arrive at the A1 domain, in an NPC independent manner, to be

501 esterified by A1. ACAT1 inhibition causes the cholesterol pool associated with the A1 domain to
502 translocate to other subcellular membrane compartments. The diversion of the cholesterol
503 storage pool fulfills the needs of these membrane compartments for cholesterol and helps them
504 regain their proper function. In the current work, we demonstrate that the TGN is one of the
505 recipient organelles that benefits from ACAT1 inhibition. Results presented in Fig. 4A-D provide
506 indirect evidence suggesting that in mutant NPC cells, $A1^{-/-}$ may also increase the cholesterol
507 content in other membrane compartments including the PM and perhaps the limiting membrane
508 of the LE/LYS. Further investigations will be required to test these possibilities. It is possible that
509 cholesterol translocation processes exist between the A1 microdomain and the microdomains
510 present in other subcellular organelles, and that inhibition of A1 facilitates the cholesterol
511 transfer from the A1 domain to these other membrane organelles. Multiple contact sites have
512 been shown to exist between the ER membranes and other membrane organelles including PM,
513 Golgi, mitochondria, and endosomes, as reviewed in (80), (81), (82), (83). The sterol transfer
514 process between the A1 microdomain and other membrane microdomain(s) may occur through
515 these membrane contact sites. Future work will be needed to reveal the molecular nature of the
516 hypothetical cholesterol translocation pathways between the A1 domain and other membrane
517 organelles.

518
519 The current study identifies ACAT1 as a new potential target for treating patients with NPC
520 disease. It is important to evaluate the pros and cons of using small molecule ACAT inhibitors
521 to treat NPC disease and other related diseases. At the cell culture level, studies have shown
522 that when the cholesterol efflux process is absent (i.e., by placing cells in growth medium
523 without any cholesterol acceptors present, or by studying cells that lack ABCA1), ACAT
524 inhibition produced cytotoxicity (84), (33). When the cellular cholesterol efflux process is active
525 (i.e., by including cholesterol acceptors such as apoA1 or serum lipoproteins in the growth
526 medium), however inhibiting A1 does not cause detectable cellular toxicity (85), (86), (87). It is
527 possible that the buildup of free (unesterified) cholesterol in cell membranes may need to reach
528 certain threshold level before it becomes toxic to cells. At the *in vivo* level, mouse gene KO (KO)
529 studies show that *Acat1* KO ($A1^{-/-}$) mice exhibit dry skin, dry eye syndrome (88), (89), and
530 leukocytosis (90), but their adrenal functions are normal (47) and their ability to learn and
531 memorize are also normal (91). At the human level, at least two adult humans with presumed
532 homozygous knockout mutations for *SOAT1* or *SOAT2* have been identified, and neither has
533 obviously noticeable issues (92). Interestingly, recent studies have shown that, in mouse
534 models, total $A1^{-/-}$ reduces pathologies associated with Alzheimer's disease (91). Myeloid $A1^{-/-}$

535 suppresses atherosclerosis development and progression (93), (94), and suppresses diet
536 induced obesity (95). Additional studies show that inhibiting A1 suppresses the development
537 and progression of pancreatic cancer (96), suppresses the development of hepatocellular
538 carcinoma (97), and potentiates the antitumor activities of cytotoxic T cells (98). Collectively,
539 these studies suggest that if employed properly, ACAT1 may be a promising target for treating
540 multiple human diseases. ACAT inhibitors of various structural types are available. In many
541 cases they were developed with the original intention to treat cardiovascular diseases, and
542 several of these inhibitors have passed a phase-I safety test for anti-atherosclerosis treatment.
543 For safety reasons, it will be important to begin testing the ACAT inhibitors in NPC patients (and
544 patients with other diseases) who do not also have partial deficiencies in other genes involved in
545 the cellular cholesterol efflux process.

546

547 **Materials and Methods**

548

549 **Materials**

550

551 **Chemical reagents:**

552

553 Fetal bovine serum was purchased from Sigma. Iron-supplemented calf serum was purchased
554 from Atlanta Biologicals. OptiPrep was obtained from Axis-Shield. ³H-labeled acetate and ³H-
555 labeled oleate were acquired from PerkinElmer. All chemicals (analytical grade) were purchased
556 from Sigma-Aldrich or Fisher. Low-density lipoproteins (LDL) from fresh human blood and
557 delipidated serum from fetal bovine serum stock were prepared as previously described (99).

558

559 **Histological reagents:**

560

561 Reagent type	Designation	Source or	Identifiers	Additional
562 or resource		reference		information
563 2° Antibody	Alexa fluor 568	Molecular Probe		1:500
564 2 °Antibody	Alexa fluor 488	Molecular Probe		1:500
565 Antibody	ABCA1	Novus	NB-400-105	1:500
566 Antibody	B-Tubulin	GenScript	A01717	0.16 µg/ml
567 Antibody	Calnexin	GenScript	A01234	1 µg/ml
568 Antibody	Cathepsin D	Santa Cruz	SC-6486	1:200

574

575	Antibody	Caviolin 1	Santa Cruz	SC-894	1:500
576					
577	Antibody	CD-M6PR	Novus	NBP-1-20167	1:500
578					
579	Antibody	Cyto C Oxidase I	Santa Cruz	SC-58347	1:200
580					
581	Antibody	Golgin 97	Invitrogen	A-21270	1:500
582					
583	Antibody	LAMP1	Cell Signaling	D401S	1:1000
584					
585	Antibody	NCP1	William S Garver's lab		
586					
587	Antibody	Syntaxin 6	Andrew A Peden's lab		
588					
589	Antibody	Vinculin	Millipore	05-386	1:2500
590					
591	Fluorescent Probe	DAPI	CalBiochem	26898	0.2 µg/ml
592					
593					
594	Fluorescent Probe	Filipin	Sigma	F-9765	0.05 mg/ml
595					
596					
597					

Primers used for ACAT1 mouse genotyping:

600	WT:	Forward primer:	5'-GGTGTTCACATGGTGCACAGATAC-3'
601		Reverse primer:	5'-GACTTTTCAATGAGGTTGGTCACA-3'
602		Amplicon size:	445 bp
603			
604	Mutant:	Forward primer:	5'-GGTGTTCACATGGTGCACAGATAC-3'
605		Reverse primer:	5'-AGGATCTCCTGTCAT CTC ACC TTG CTC CTG
606		Amplicon size:	1052 bp
607			
608			

Primer sequences used for real time PCR (RT-PCR) analysis:

Gene	Sense/Antisense	Amplicon size (bp)	
613	Calbindin	5'-GAGCTATCACCGGAAATGAAA-3'	152
614		5'-AATTCCTCGCAGGACTTCAG-3'	
615			
616	ABCA1	5'-GGTTTGGAGATGGTTATACAATAGTTGT-3'	96
617		5'-TTCCCGGAAACGCAAGTC-3'	
618			
619	GAPDH	5''-ATGGTGAAGGTCGGTGTG-3'	186
620		5'-CATTCTCGGCCTTGACTION-3'	

621 **Methods**

622

623 **Animal maintenance:** Mice were fed *ad libitum* with standard chow diet, maintained in a
624 pathogen-free environment in single-ventilated cages, and kept on a 12 h light/dark schedule,
625 using Dartmouth Animal Research Center Institutional Animal Care and Use Committee–
626 approved protocol number 00002020. Animals were checked daily for their entire lifespan.
627 When *Npc1^{nmf}* mice began to have trouble reaching food, wet food pellets were placed on the
628 bottom of their cage for the remainder of their life. Death was marked as the point where the
629 mice could no longer ingest food or water.

630

631 **Mouse breeding:** The heterozygous mutant NPC1 mouse in C57BL/6J background
632 (*Npc1^{nmf/wt}.Acat1^{+/+}* mouse; from Jackson laboratories) was crossed with *Acat1^{-/-}* mouse (global
633 ACAT1 KO mouse) [(47); received from Dr. Sergio Fazio in C57BL/6J background], to produce
634 *Npc1^{nmf/wt}.Acat1^{-/-}* mice and *Npc1^{nmf/wt}.Acat1^{+/+}* mice. After two rounds of breeding, the resultant
635 *Npc1^{nmf/wt}.Acat1^{-/-}* mice were set up as breeding pairs to generate the *Npc1^{nmf/nmf}.Acat1^{-/-}* mice
636 (Designated as *Npc1^{nmf}.A1^{-/-}* mice) and *Npc1^{wt/wt}.Acat1^{-/-}* mice (Designated as *A1^{-/-}* mice). The
637 *Npc1^{nmf/wt}.A1^{+/+}* mice were set up as breeding pairs to generate *Npc1^{nmf/nmf}.A1^{+/+}* mice
638 (Designated as *Npc1^{nmf}* mice) and *Npc1^{wt/wt}.A1^{+/+}* mice (Designated as WT mice).

639

640 **NPC1^{nmf} mouse genotyping:** The protocol described by (46) was followed with minor
641 modification: 10 µl of reaction buffer containing 10 ng of mouse-tail genomic DNA, 1x TaqMan
642 genotyping master mix, and 1x SNP Custom TaqMan SNP assay mixture. The PCR reaction
643 was carried out by amplifying at 95°C for 5 min, followed by 45 cycles of: 92°C for 15 s and
644 60°C for 1 min.

645

646 **ACAT1 mouse genotyping:** PCR conditions were: 94°C for 1.5 min, followed by 35 rounds
647 of: 94°C for 30 s, 62°C for 60 s, 72°C for 60 s. Lastly, 72°C for 2 min. The primers used are
648 described above in the Materials.

649

650 **Mouse motor skills:** Mouse motor skills were assessed by RotaRod test using a
651 commercially available instrument (purchased from Med Associates Inc, Fairfax, VT) in a
652 manner similar to what was previously reported (100), with slight modifications. Briefly, after a
653 brief initial training period, mouse motor skills were monitored from six weeks postnatal age until
654 failure. Each week mice were given three consecutive trials on a constant speed rotarod at 24
655 rotations per min for up to 90 s for each of the three trials. WT and *A1^{-/-}* mice passed all trials

656 running for at least 10 s on any of the three consecutive trials during every week assessed. Age
657 of rotarod test failure in *Npc1^{nmf}* and *Npc1^{nmf}:A1^{-/-}* mice was measured as the age at which mice
658 failed to run on the rotarod for at least 10 s during at least one of the three consecutive trials.
659 Rotarod trial failure included falling off the rod before 10 s or freezing and claspings to the rotarod
660 and not running or moving.

661
662 **Histological analyses:** Hematoxylin and Eosin staining of mouse liver, spleen and lung
663 tissues, and Purkinje neurons in cerebellum at postnatal day 80 were performed by the
664 Histology Service at the Jackson Laboratory, using standard protocols in a Leica Autostainer XL
665 automated processor.

666
667 **Cell culture:** MEFs were isolated according the procedure described (101). MEF were grown
668 as monolayers at 37°C with 5% CO₂ in DMEM supplemented with 10% serum and MEM non-
669 essential amino acids (Gibco), or with 5% delipidated fetal bovine serum and 35 μM oleic acid,
670 and with penicillin/streptomycin. Each experiment was performed with cells grown in triplicate
671 dishes.

672
673 **Degradation of long-lived proteins in MEFs:** The procedure described in (62), (63) was
674 adopted with minor modification. MEFs were seeded on 12-well plates at a density of 0.012x10⁶
675 cells in triplicate. Media were replaced the night before the experiment. Cells were rinsed with 2
676 ml of MEM and labeled with 1 ml of 2 μCi/ml of ³H leucine in MEM+10% serum. At time zero,
677 cells were washed twice with HBSS, then chased by 1 ml MEM+2.8 mM leucine without serum.
678 At each time point indicated, media were transferred to a microcentrifuge tube, trichloroacetic
679 acid added to a final concentration of 20% and BSA to a final concentration of 3 mg/ml.
680 Samples were incubated at 4°C for 1 h, centrifuged at 15,000g at 4°C for 5 min, supernatants
681 and pellets were collected. Ecoscint H was added for scintillation counting. Cells were washed
682 with PBS and incubated in 0.1 M NaOH in 0.1% deoxycholate for 1 h. 40 μl in duplicates were
683 aliquoted for protein determination.

684
685 **Western blot analyses:**
686 From either freshly isolated mouse cerebellum tissues or tissue culture cells: it was prepared in
687 either 10% SDS (for syntaxin 6, CD-M6PR, NPC1, or cathepsin D), or RIPA buffer (for ABCA1),
688 plus protease inhibitor (Sigma), and homogenized in a stainless-bed Bullet Blender twice for 3
689 min each at 4°C. Homogenized lysates were run on a 6% gel (for ABCA1), 10% gel (NPC1,
690 LAMP1) or 12% gel (for Syntaxin 6, CD-M6PR, Cathepsin D, Caveolin 1, and Cytochrome C

691 Oxidase), and transferred to PVDF membrane in Towbin buffer. Signal intensities were
692 normalized to vinculin (117-kDa) or B tubulin (50-kDa) expression by NIH Imaging software.

693
694 **RNA isolation and real-time PCR (RT-PCR) experiments:** Total RNA was isolated from
695 TRIzol reagent (Invitrogen) following the manufacturer's instruction. The RNA was dissolved in
696 sterile water treated with DNaseI (Ambion). 1 µg of RNA was used to synthesize cDNA,
697 according to the instructions in the BioRad iScript cDNA Synthesis Kit. Real-time PCR was
698 carried out using the iTaq Universal SYBR Green Supermix (from Bio-Rad) with the Applied
699 Biosystems Step One RT-PCR system. Relative quantification was determined by using the
700 delta CT method. The primer sequences used are listed above in the Materials. The PCR
701 reaction conditions were as described previously (91).

702
703 **Subcellular fractionation:** Procedures were carried out as described previously (22). Cells
704 grown in one 150-mm dish to near confluence were washed twice with phosphate-buffered
705 saline, once with homogenization buffer (HB, 250 mM sucrose, 20 mM Tris-HCl, pH 7.4, 1 mM
706 EDTA), harvested to 1 ml HB with protease inhibitors, and homogenized by using a stainless-
707 steel homogenizer with 40 strokes. The post-nuclear supernatants were placed onto the top of
708 an 11 ml 5-25% OptiPrep discontinuous gradient in HB. The gradient was centrifuged at
709 200,000 x g (40,000 rpm) for 3 h in a Beckman SW41 rotor; 15 fractions (800 µl each) were
710 collected from the top.

711
712 **Lipid syntheses in intact cells:** The analysis of cholesterol biosynthesis in intact cells was
713 carried out as previously described (102), exposing cells to ³H-labeled acetate for 1 h followed
714 by lipid extraction. The measurement of cholesterol esterification in intact cells was performed
715 by exposing cells to ³H-labeled oleate/BSA for 1 h followed by lipid extraction and analysis, as
716 previously described (103).

717
718 **Fluorescence microscopy:** MEF cells were cultured on poly-D-Lysine (70-150kDa) glass
719 coverslips (MatTek) in 12-well plates for 24 h, and fixed in 4% paraformaldehyde (EMS) at RT
720 for 10 min. After washing with PBS, the cells were permeabilized with 0.3% Triton X-100 for 20
721 min. Cells were then washed with PBS prior to blocking with 5% goat serum in PBS for 1 h at
722 RT, followed by staining with primary antibodies for at least 1 h at RT. Cells were washed with
723 PBS before and after incubation with Alexa Fluor 568 or Alexa Fluor 488 as the secondary
724 antibodies at 1:500 dilution for 1 h at RT, DNA was counter stained with DAPI (0.2 µg/ml) at RT
725 for 10 min to visualize the nucleus.

726 To identify cellular cholesterol, cells were fixed in 4% paraformaldehyde (without the use of
727 detergent), after several washes, cells were pre-incubated with 1.5 mg/ml glycine in PBS for 10
728 min at RT, then incubated with Filipin (50 µg/ml) at RT for 1 h as previously described (104).
729
730 Images were acquired by using the Andor W1 Spinning Disk Confocal system (Nikon Eclipse Ti
731 inverted microscope, and Andor Zyla camera), with a 60x oil-immersion lens, using three laser
732 lines (403-nm laser for DAPI, 488-nm and 561-nm filters for FITC and Texas Red respectively).
733 Z-stacked fluorescent images were taken by 11 optical slices at 0.2 µm intervals to enhance the
734 spatial signal allocation. Images were visualized by using Fiji-Image J Software, and processed
735 using Nikon Elements to create the “Maximum Intensity Projection”, and calculate the Pearson’s
736 correlation coefficient. The Reflex angle was determined according to Mitchel *et al.* (55), as the
737 angle subtended by the edges of the positive fluorescence signals, using the center of the
738 nucleus (based on the DAPI positive signal) as the vertex.

739
740 **Statistical analysis:** Statistical comparisons were made by using a two-tailed, unpaired
741 student *t* test according to GraphPad Prism 8. Difference were considered significant when the
742 *P* value was less than 0.05. ($p < 0.0001$ ****, $p < 0.001$ ***, $p < 0.01$ **, $p < 0.05$ *). n.s. indicates
743 the differences were not significant.
744

745 **Author contributions and Conflict of interest statement**

746 M.A.R., C.C.Y.C., and T.-Y.C. designed research; C.C.Y.C., M.A.R., E.M.M., and M.H., P.S
747 performed research; R.A.M., A.P., and W.G. contributed new reagents/analytic tools; C.C.Y.C.,
748 M.A.R., R.A.M. and T.-Y.C. analyzed data; T.-Y.C., M.A.R., and C.C.Y.C., wrote the paper. All
749 authors approved the final version of the manuscript.

750 The authors declare no conflict of interest.

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1086 Abbreviations

1087

1088 **ABCA1**: ATP- binding cassette protein A1; **ACAT/SOAT**: Acyl-coenzyme A:cholesterol
1089 acyltransferase/sterol O-acyltransferase; **KO**: gene knockout; **A1^{-/-}**, **Acat1^{-/-}**: ACAT1 gene
1090 ablation; **CD-M6PR**: cation-dependent mannose-6-phosphate receptors; **ER**: endoplasmic
1091 reticulum; **PM**: plasma membrane; **TGN**: trans-Golgi network; **LE**: late endosomes; **LXRs**: liver
1092 X receptors; **NPC**: Niemann-Pick type C; **Npc1^{nmf/nmf}**, **Npc1^{nmf}**, or **Npc1^{nmf164}**: An *Npc1* disease
1093 mouse model with D1005G mutation; **NPA**: Niemann-Pick type A.

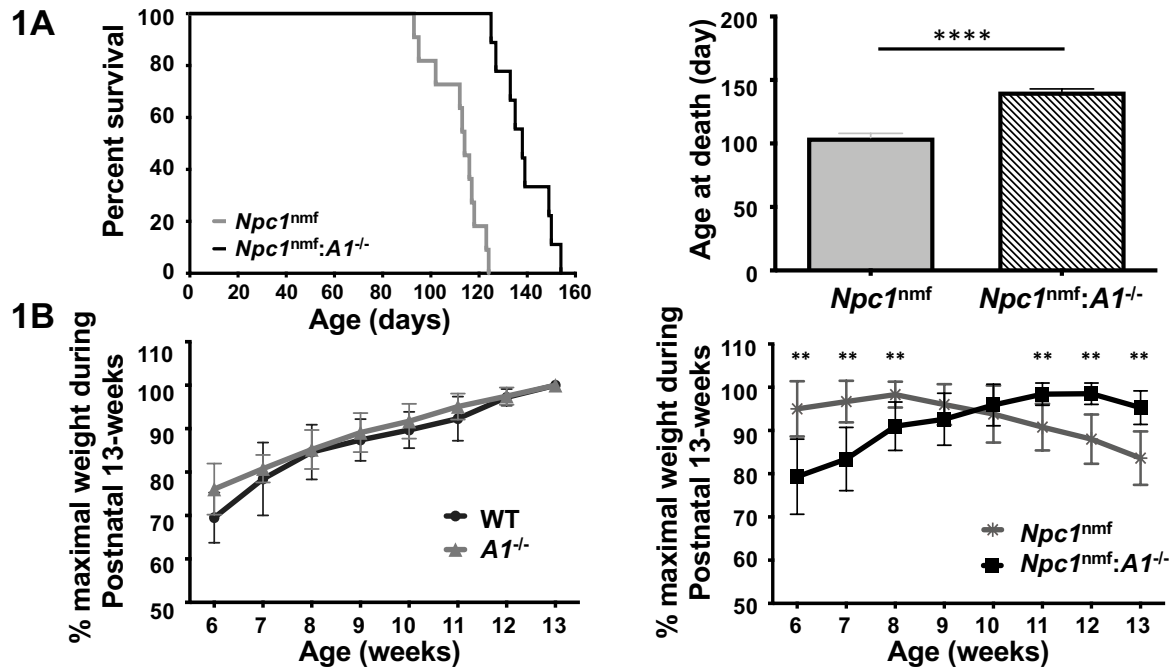


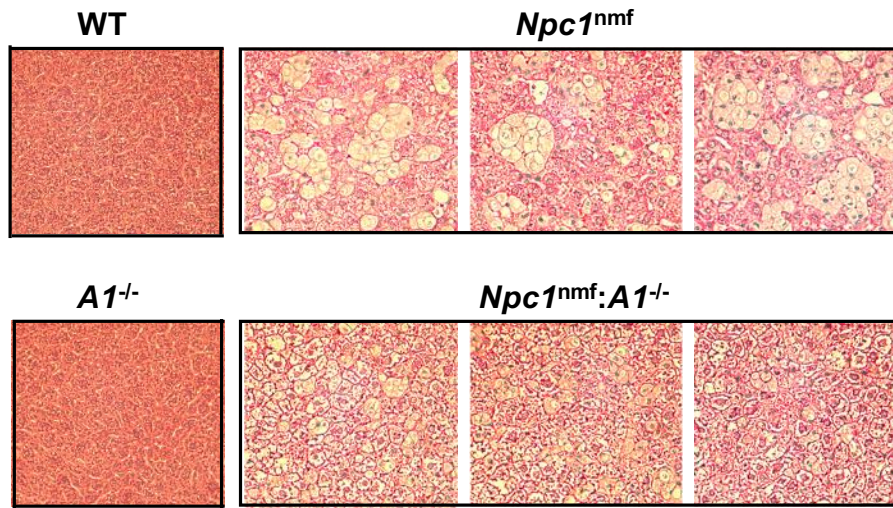
Fig. 1. Effect of *A1^{-/-}* on the life span and weight loss of *Npc1^{nmf}* mice.

A. *A1^{-/-}* increases life span of *Npc1^{nmf}* mice by 34%. Median survival (left panel) for *Npc1^{nmf}* mice and for *Npc1^{nmf}:A1^{-/-}* mice is 113 days and 138 days respectively. Mean survival (right panel) is 102 days and 137 days, respectively. N=18 mice for *Npc1^{nmf}* and N=16 for *Npc1^{nmf}:A1^{-/-}* mice. Equal numbers of male and female mice were used, and the procedure described in (46) was adopted to define death of the *Npc1^{nmf}* mouse. The *p*-value for survival curves is <0.0001.

B. *A1^{-/-}* delays weight loss of *Npc1^{nmf}* mice (right panel) but not WT mice (left panel). Weight measurement began at 6 weeks of age. Data are expressed as percent maximum weight during the first 13 weeks. N=10 mice per group with equal numbers of males and females evaluated. Error bars indicate 1 SEM. In the right panel, except for weeks 9 (*p* = 0.2) and 10 (*p* = 0.4), the *p*-value for each week is <0.003. In the left panels, there are no significant differences.

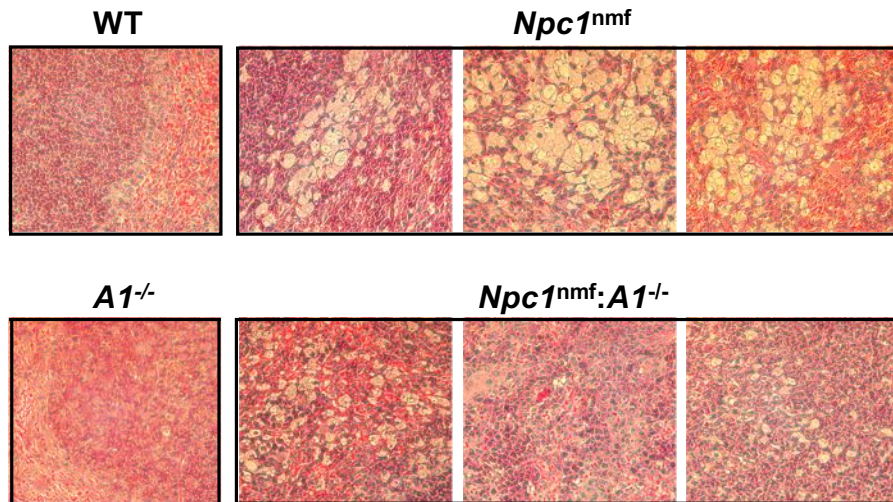
2A

P80 Liver Histology



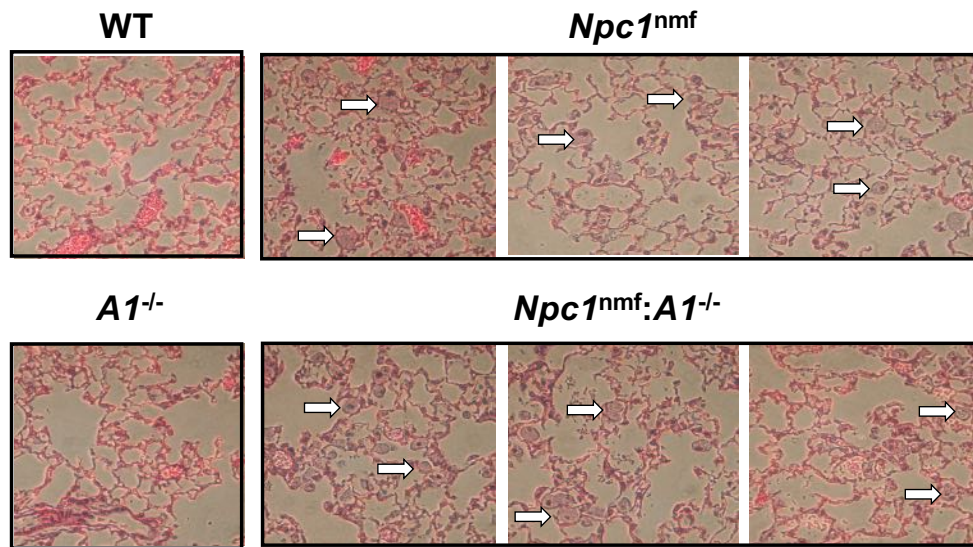
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P80 Spleen Histology



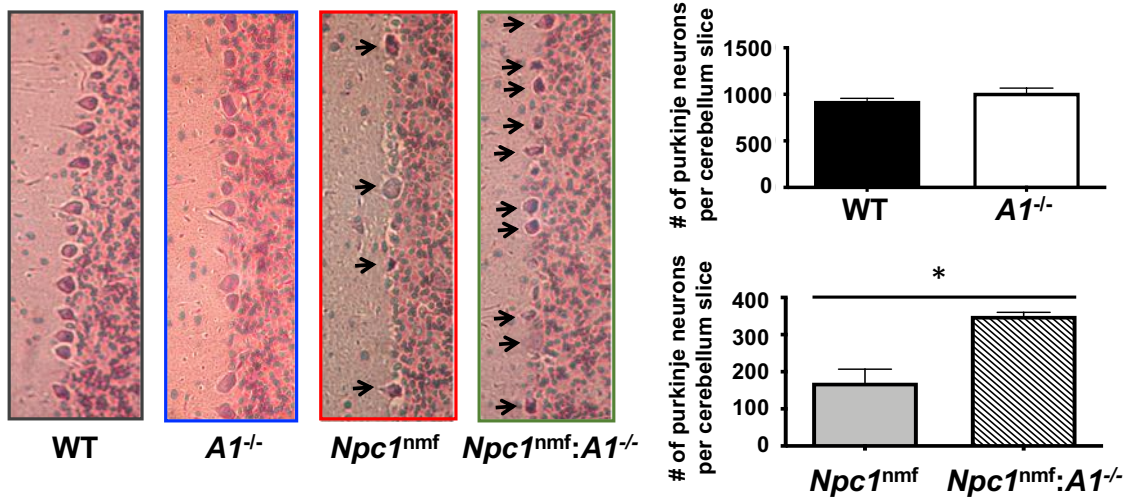
2C

P80 Lung Histology

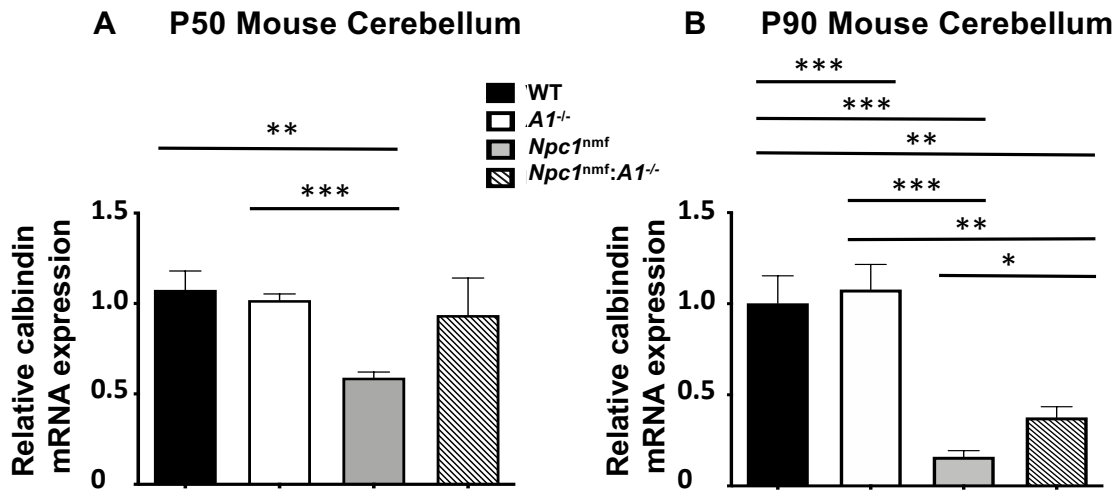


2D

P80 Cerebellum Histology



2E



2F

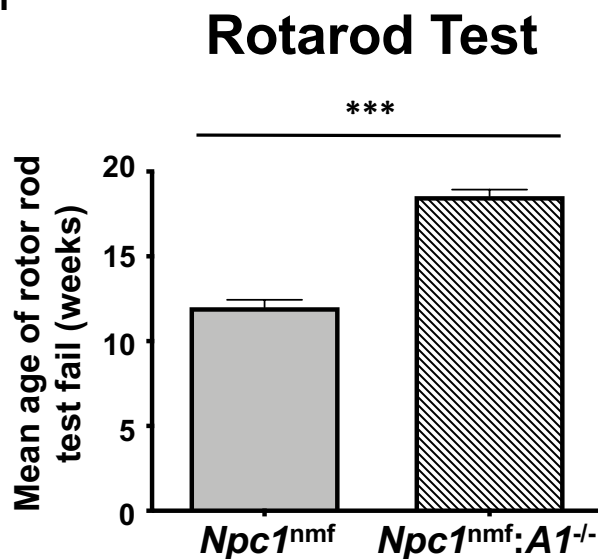


Fig. 2. Effect of *A1*^{-/-} on cellular pathology, neuronal loss, and motor deficits in *Npc1*^{nmf} mice.

A-C. *A1*^{-/-} improves macrophage foam cell pathology in liver (**A**), in spleen (**B**), but not in lung (**C**) of the *Npc1*^{nmf} mice. Tissues shown were isolated from mice at P80, fixed, sectioned, and processed for H&E staining. Lungs were perfused through the trachea. All mice were collected at the same magnification (40x). Results are representative of the 3 mice per group. In **2C**, arrows point at foam cells.

D. *A1*^{-/-} reduced Purkinje neuron death in *Npc1*^{nmf} mice, without affecting Purkinje neuron numbers in WT mice. Representative images of the cerebellum in the WT, *A1*^{-/-}, *Npc1*^{nmf}, and *Npc1*^{nmf}:*A1*^{-/-} mice were collected at the same magnification (10x) and highlight the relative

number of Purkinje neurons in each case. The relative number of Purkinje neurons (indicated by arrows in the images) was quantitated by counting in two separate cerebellar lobes. N=2 to 4 animals per group. Error bars indicate 1 SEM. For the right bottom panel, $p < 0.05$.

E. *A1*^{-/-} reduces the loss of calbindin expression in *Npc1*^{nmf} mouse cerebellum without affecting calbindin expression in WT mouse cerebellum. Cerebellar tissue was isolated from the brains of P50 (left panel) and P90 mice (right panel) and calbindin mRNA levels were measured by RT-PCR, with GAPDH also measured for normalization. Tissues were collected from 3 mice of each genotype.

F. *A1*^{-/-} prevents the decline in motor skill in the *Npc1*^{nmf} mouse. Mice were tested each week in 3 consecutive trials on a rod rotating at a constant speed (24 rpm) for up to 90 s per trial. The fail time was defined as the age at which the mouse failed to stay on the rod at least 10 s during one of the 3 trials or froze on the rotarod and did not move. N=13 mice for *Npc1*^{nmf} and N=11 mice for *Npc1*^{nmf}:*A1*^{-/-}. Comparable numbers of male and female mice were evaluated. The p -value is < 0.001 .

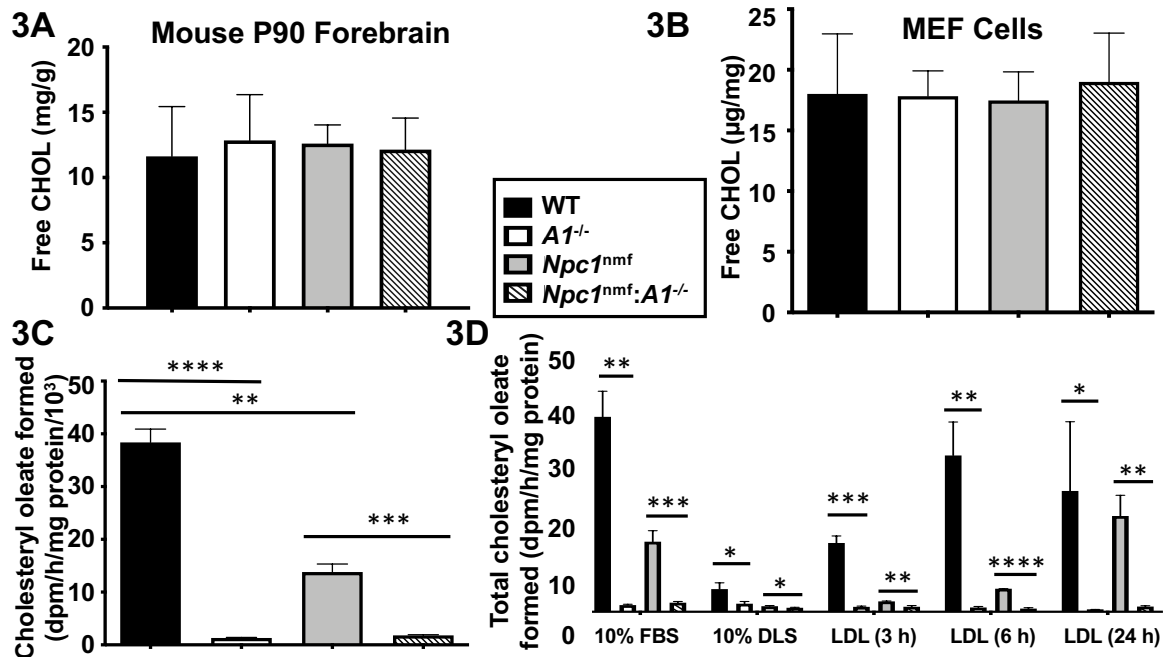
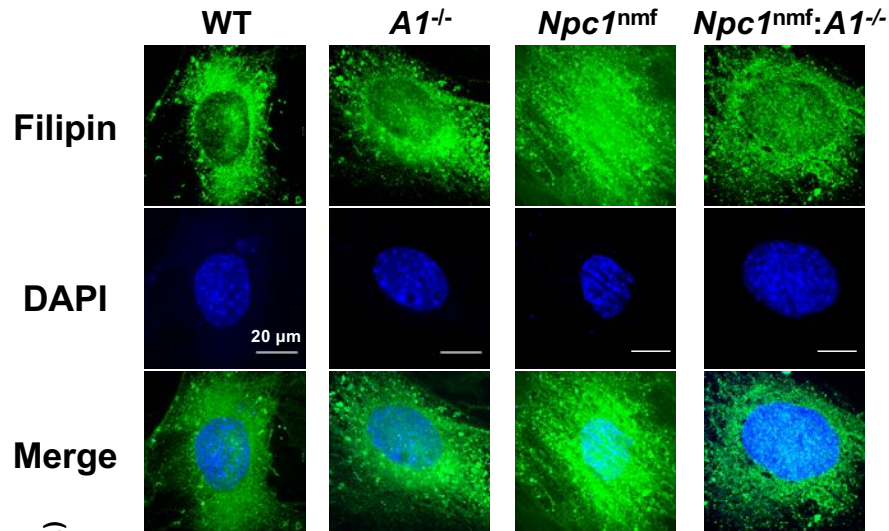


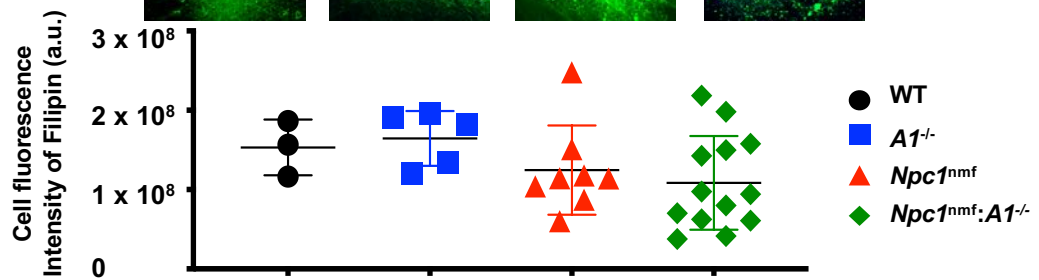
Fig. 3. Cholesterol content and cholesteryl ester biosynthesis.

- A. Free cholesterol content in P90 WT, *A1*^{-/-}, *Npc1*^{nmf}, and *Npc1*^{nmf}:*A1*^{-/-} mouse forebrain.** Tissues were homogenized in chloroform: methanol 2:1, filtered by Whatman filter paper, dried under nitrogen then resuspended in methanol. Free cholesterol content was determined in triplicate by using Wako's Free Cholesterol E kit. N= 5 mice (3 male and 2 female) per group. Error bars indicate 1 SEM.
- B. Free cholesterol content in mouse embryonic fibroblasts (MEFs).** MEF were seeded in 6-well dishes at 200,000 cells/well and grown in DMEM plus 10% serum to near confluence. After three washes with PBS, cells were scraped off the dish to form suspensions in PBS and were used for protein measurement and lipid extraction by chloroform/methanol. Cholesterol content was determined as described in part A above. Data shown is from 3 platings per genotype.
- C. Cholesteryl ester biosynthesis in MEFs continuously grown in lipoprotein containing medium.** Cells were grown as described in part B above. Cholesteryl ester biosynthesis in intact cells was described in (103). Data shown is from 3 platings per genotype.
- D. Cholesterol ester biosynthesis in MEFs grown in cholesterol containing medium (10% FBS), cholesterol free medium (10% DLS), or 10% DLS in response to LDL feeding for 3 h, 6 h, or 24 h.** Human LDL and delipidated fetal bovine serum were prepared as described (103).

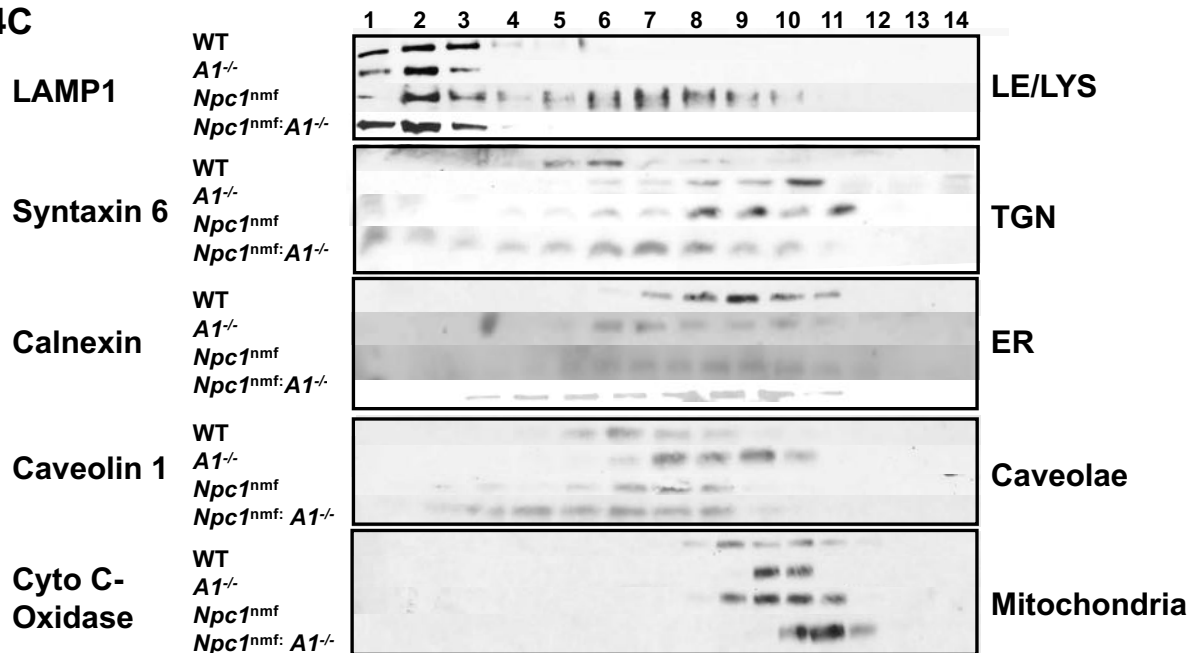
4A



4B



4C



4D

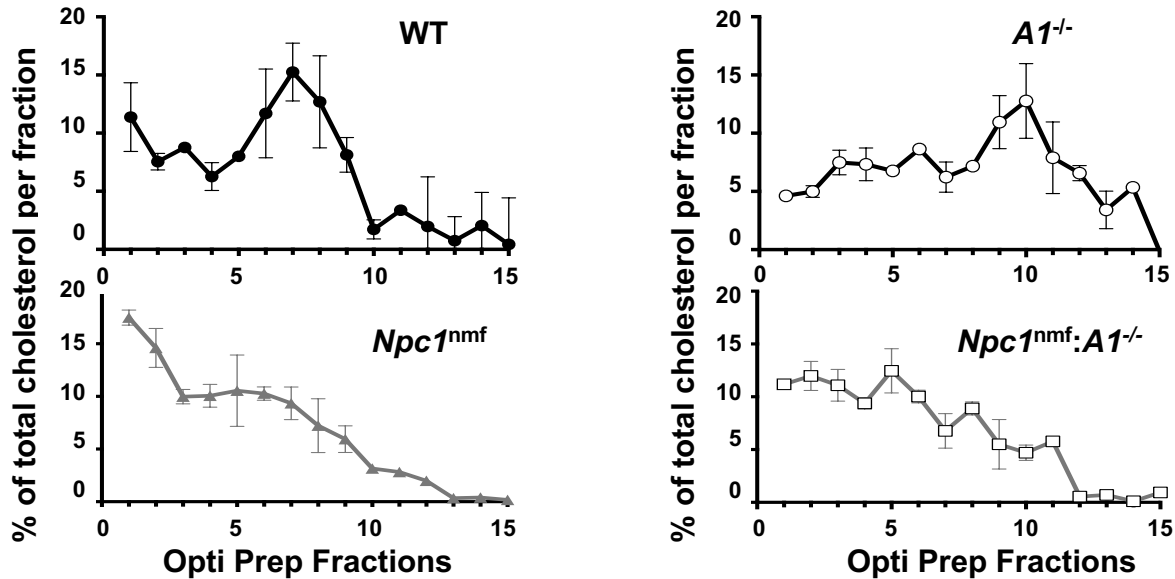


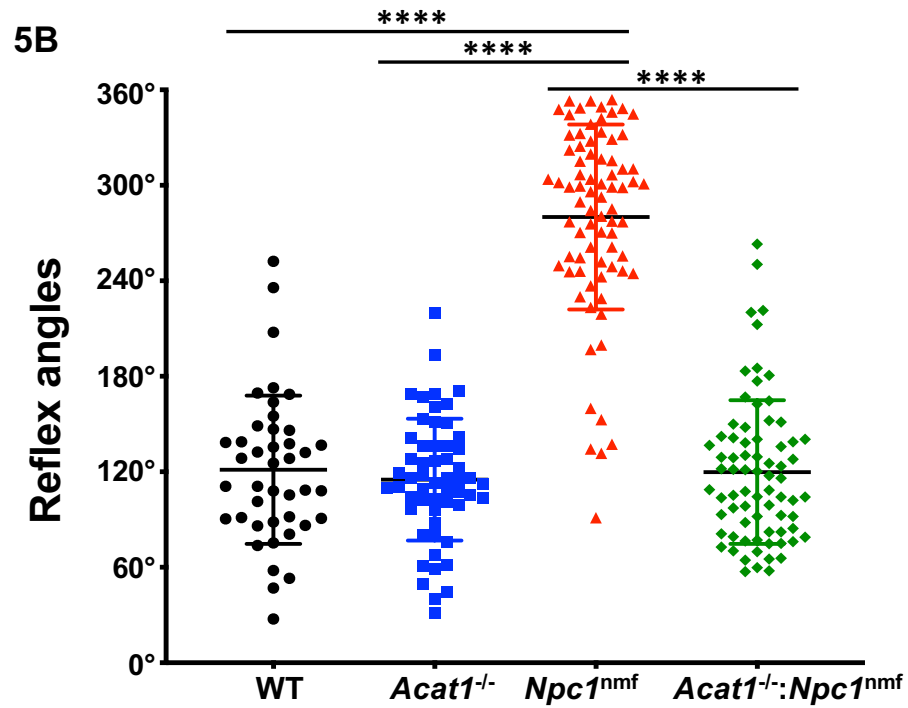
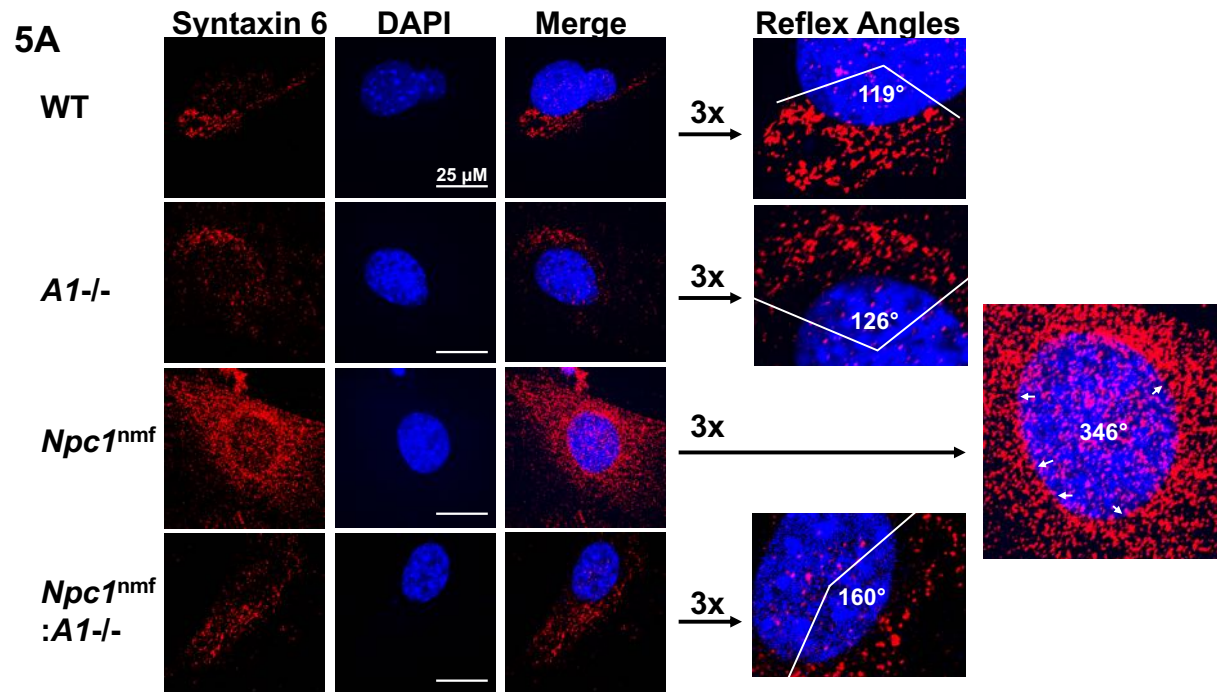
Fig. 4. Cholesterol distribution in mouse embryonic fibroblasts (MEFs).

A. MEFs from WT, $A1^{-/-}$, $Npc1^{nmf}$, and $Npc1^{nmf};A1^{-/-}$ mice were seeded on poly-d-lysine treated glass coverslips in 12-well plates. Filipin staining was carried out according to (104).

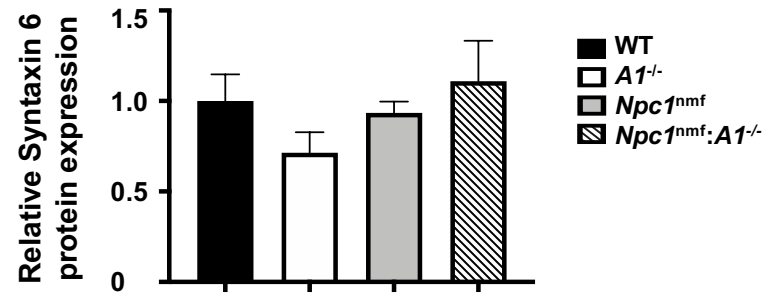
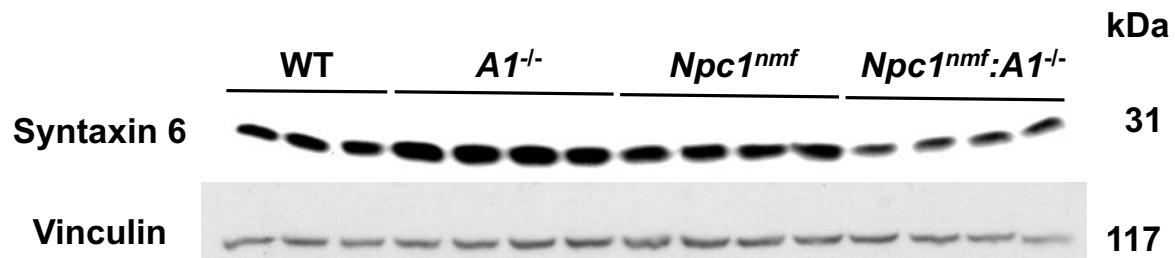
B. Total fluorescence intensity per cell. Fluorescence was quantified using NIH Image J. Each point represents the value obtained for an individual cell.

C. Localization of subcellular organelles after cell fractionation. Antibodies for specific protein markers (listed on far left) were used to localize specific organelles (listed on right).

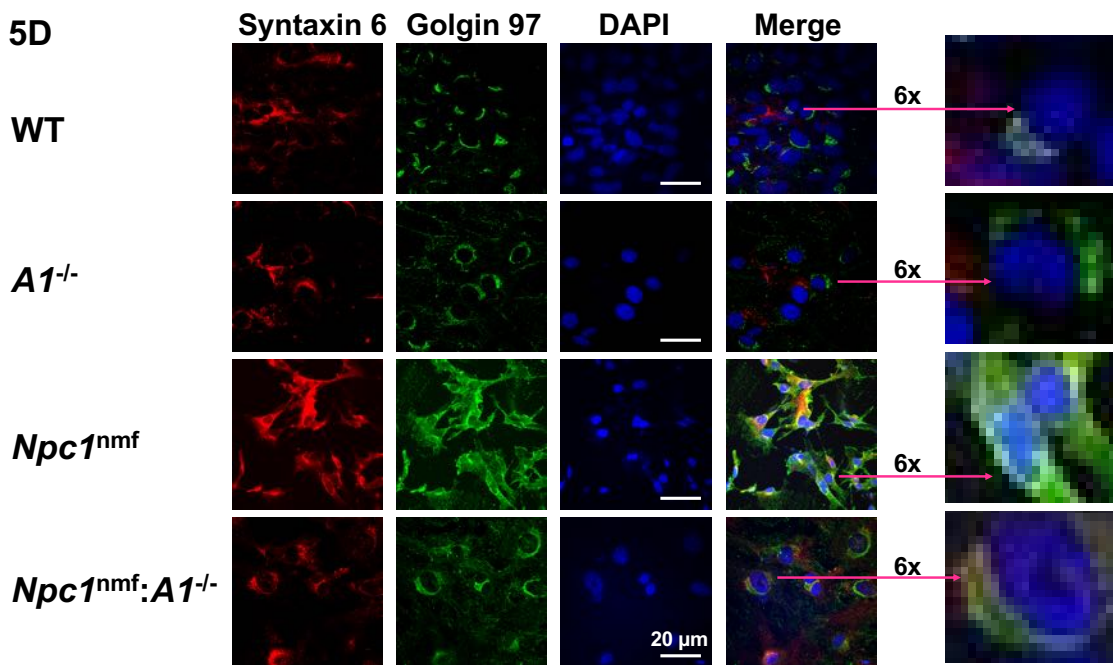
D. Cholesterol distribution in various subcellular organelles. For each cell type, cells from a single 15-cm dish were grown in medium plus 10% serum until confluent. Cell homogenization and OptiPrep density gradient ultracentrifugation, were carried out as described (22). Fourteen fractions were collected from each sample, with each fraction analyzed in triplicate for cholesterol content using a Wako kit. The values reported are normalized to the total cholesterol content present in all fractions. Data are means \pm 1 SD from 2 separate experiments.



5C



5D



5E

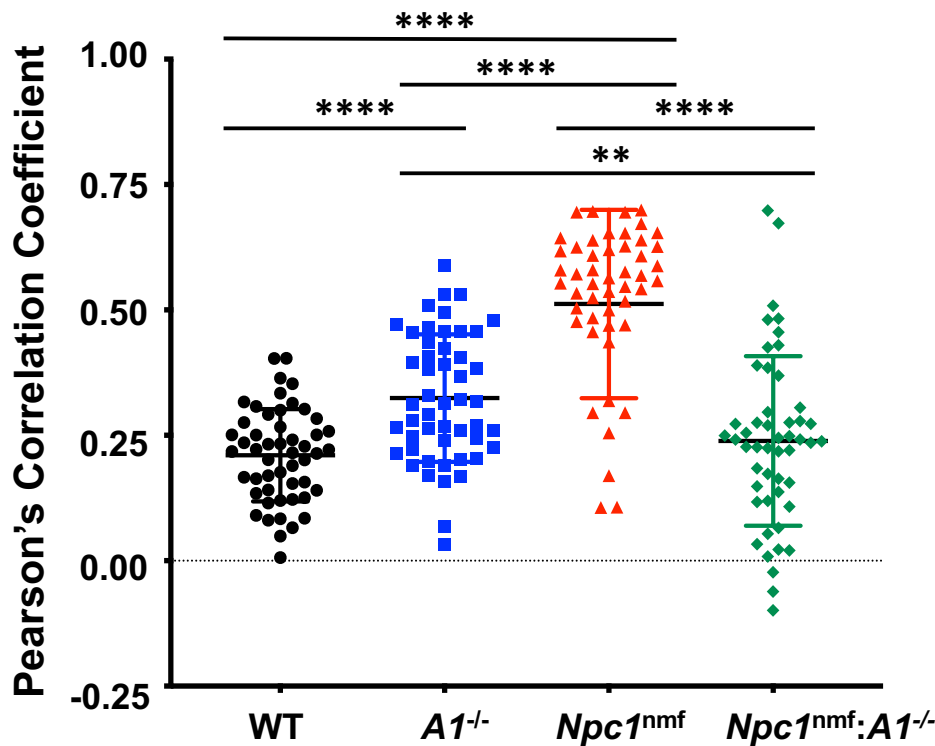


Fig. 5. Localization of syntaxin 6 and golgin 97 in intact mouse embryonic fibroblasts (MEFs).

A. The methods used for the detection of fluorescence-labeled **syntaxin 6** in MEFs are described in the Methods. Fiji-ImageJ software was used to calculate the "Reflex angle", which is indicated in the images on the right.

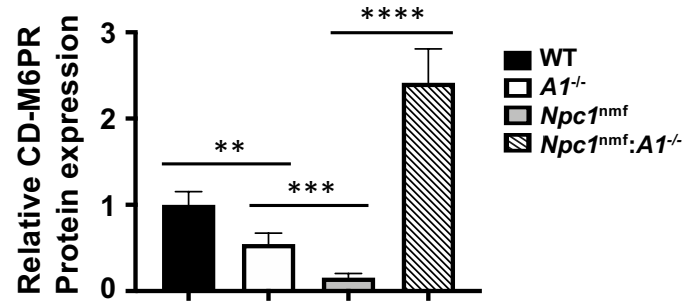
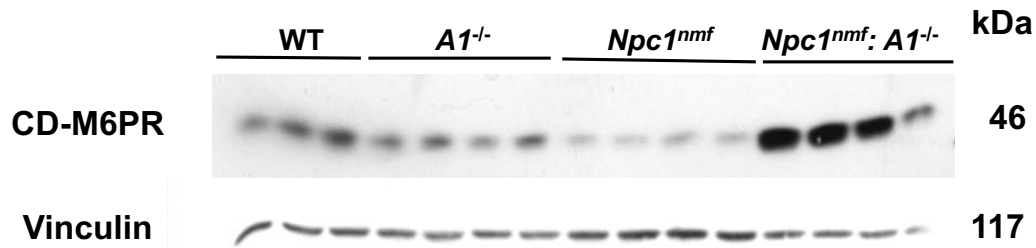
B. The Reflex Angle of individual cells from WT, *A1*^{-/-}, *Npc1*^{nmf}, and *Npc1*^{nmf}:*A1*^{-/-} mice. Between 50 to 70 cells were measured for each genotype.

C. Relative syntaxin 6 protein content in lysates of MEFs. Cells were seeded in 60 mm culture dishes and grown in DMEM plus 10% serum until confluent. Then 200 μ l of 10% SDS were added per dish, and 150 μ g of the solubilized protein was loaded per lane for Western blot analyses. The vinculin signal was used as the loading control. Error bars indicate 1 SEM.

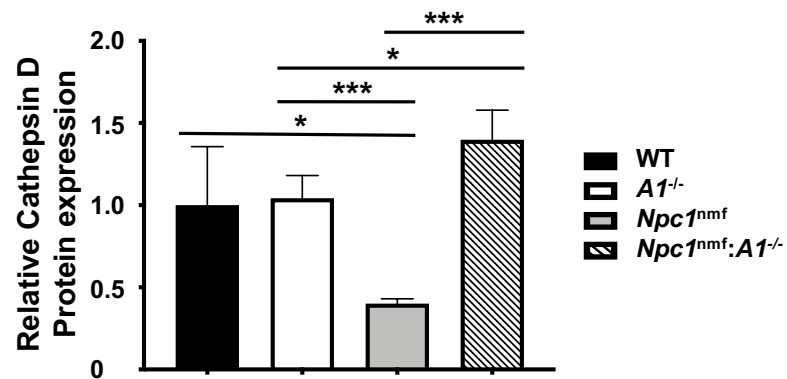
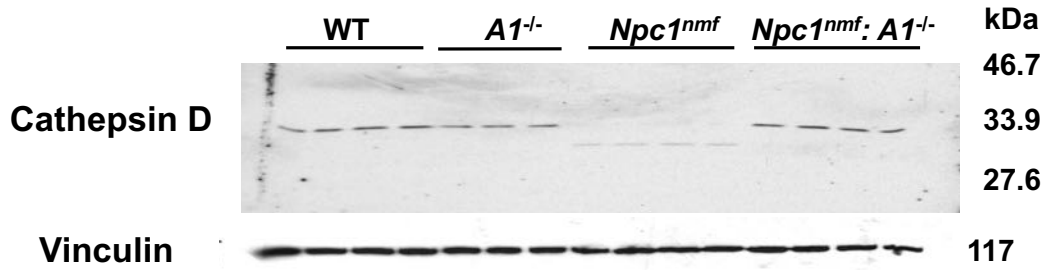
D. Double immunofluorescence of syntaxin 6 and golgin 97 in fixed, intact MEFs. The conditions used were the same as described in part A above.

E. Degree of apparent colocalization between syntaxin 6 and golgin 97 in MEFs. Nikon NIS-Element AR Imaging Software was used to create the Maximum Intensity Projection used to calculate the degree of colocalization, which is reported as Pearson's correlation coefficient. Each data point represents the result from an individual cell, and images from more than 50 cells were collected for each genotype. GraphPad Prism's One-way ANOVA multiple comparisons method was used for statistical analysis.

6A



6B



6C

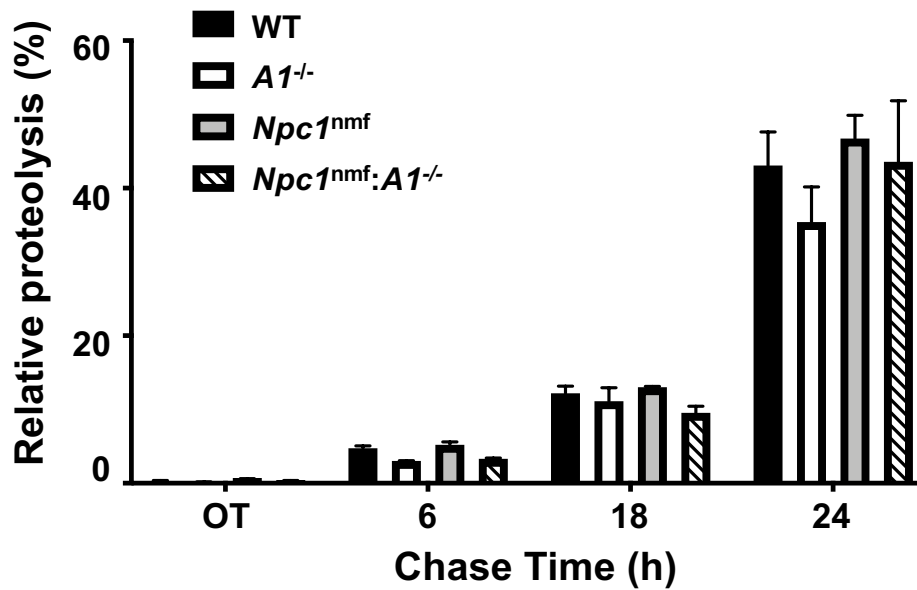


Fig 6. Analyses of various protein content and degradation of long-lived proteins in mouse embryonic fibroblasts (MEFs) from WT, A1^{-/-}, Npc1^{nmf}, and Npc1^{nmf}:A1^{-/-} mice.

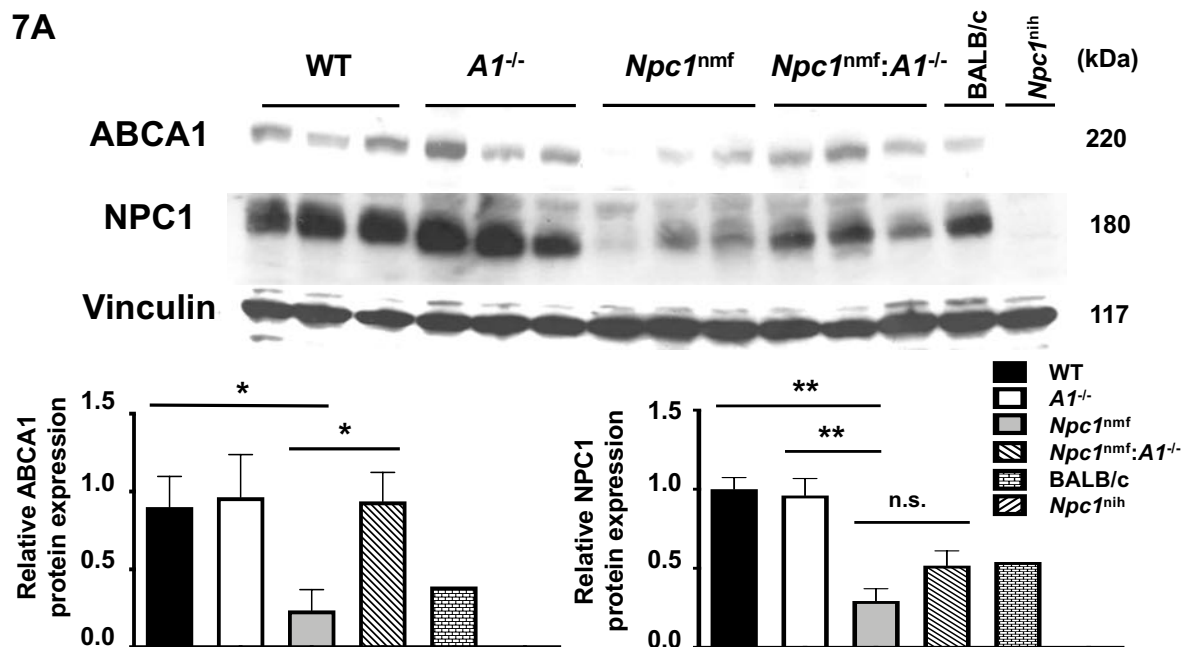
For parts A and B, the cell growth conditions were the same as described in Fig. 5C. Vinculin signal was used as the loading control.

A. The relative contents of CD-M6PR protein (46-kDa) in MEFs.

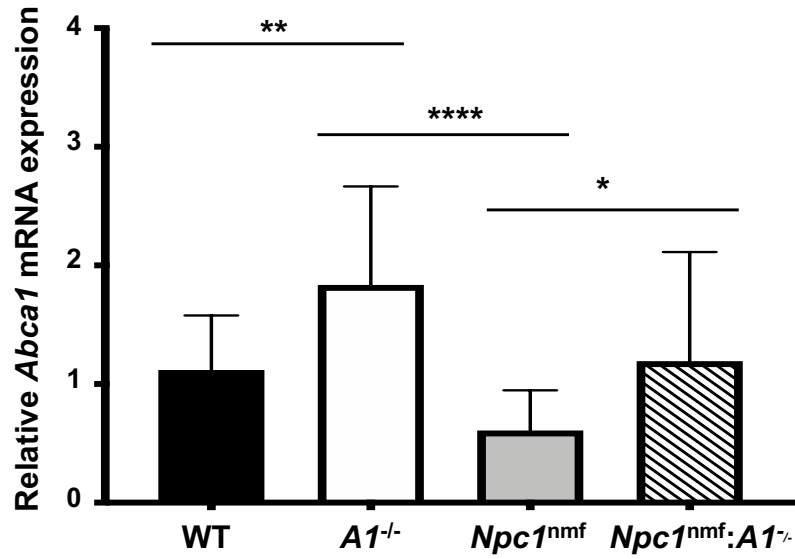
B. The relative contents of cathepsin D protein (heavy chain of the mature enzyme) (30 to 32-kDa in WT, A1^{-/-}, and Npc1^{nmf}:A1^{-/-} cells. And 28-kDa in Npc1^{nmf} cells).

C. The degradation of long-lived proteins in MEFs from WT, A1^{-/-}, Npc1^{nmf}, and Npc1^{nmf}:A1^{-/-}. The analysis of protein degradation was conducted as described in the Method. Results are reported as relative proteolysis (%). Error bars indicate 1 SEM.

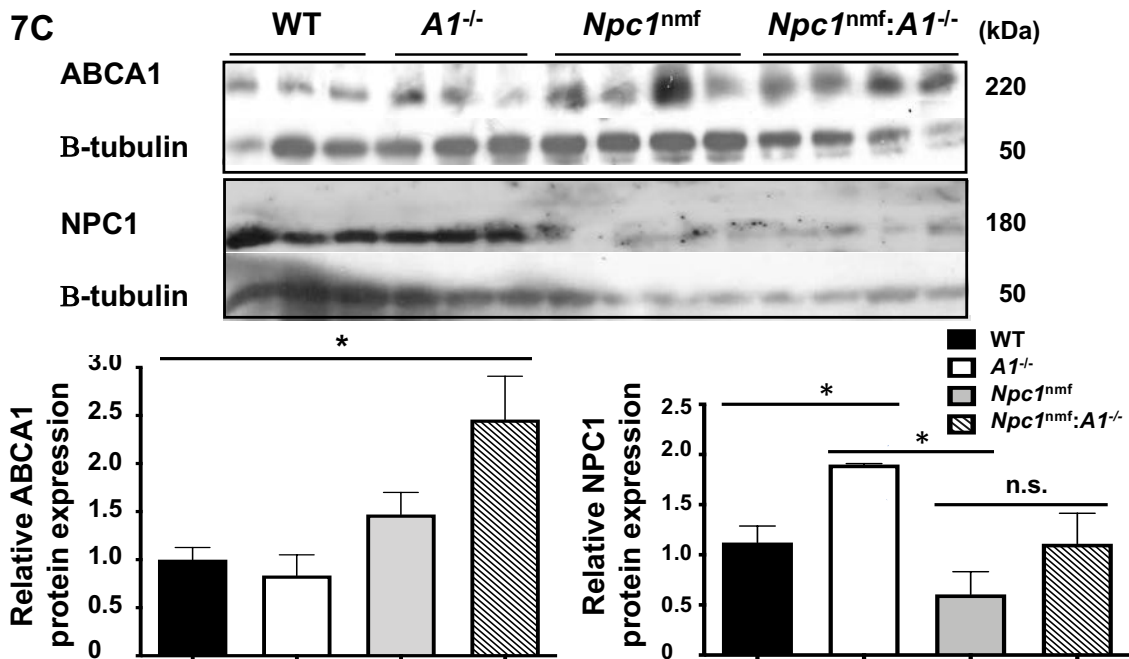
7A



7B



7C



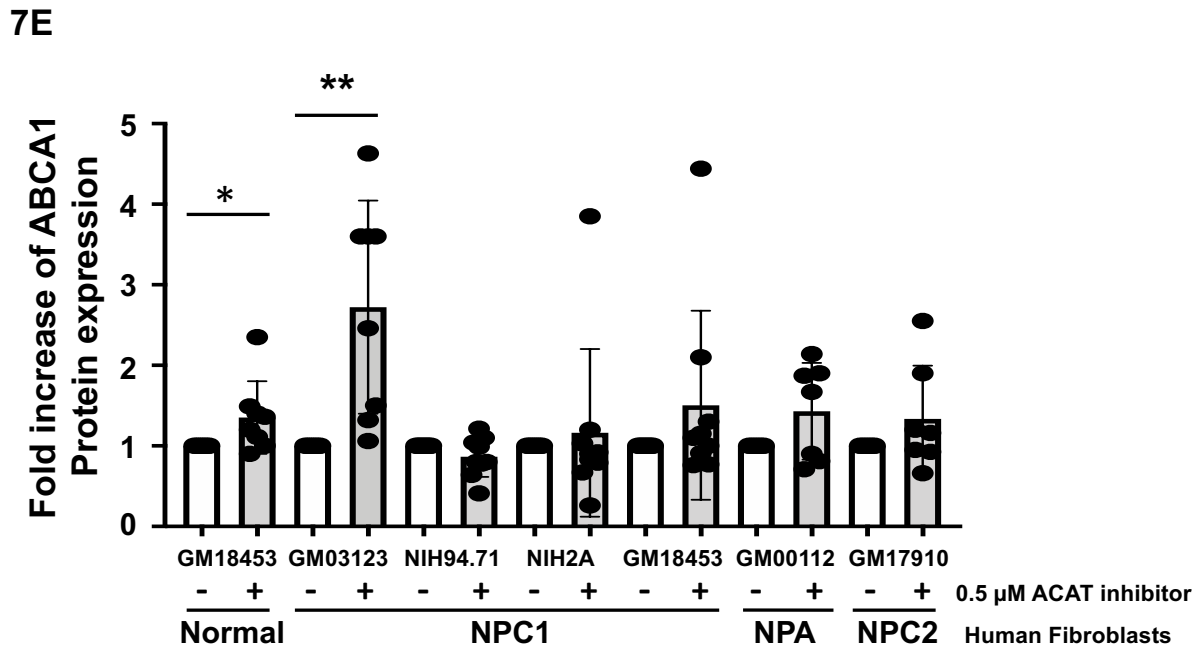
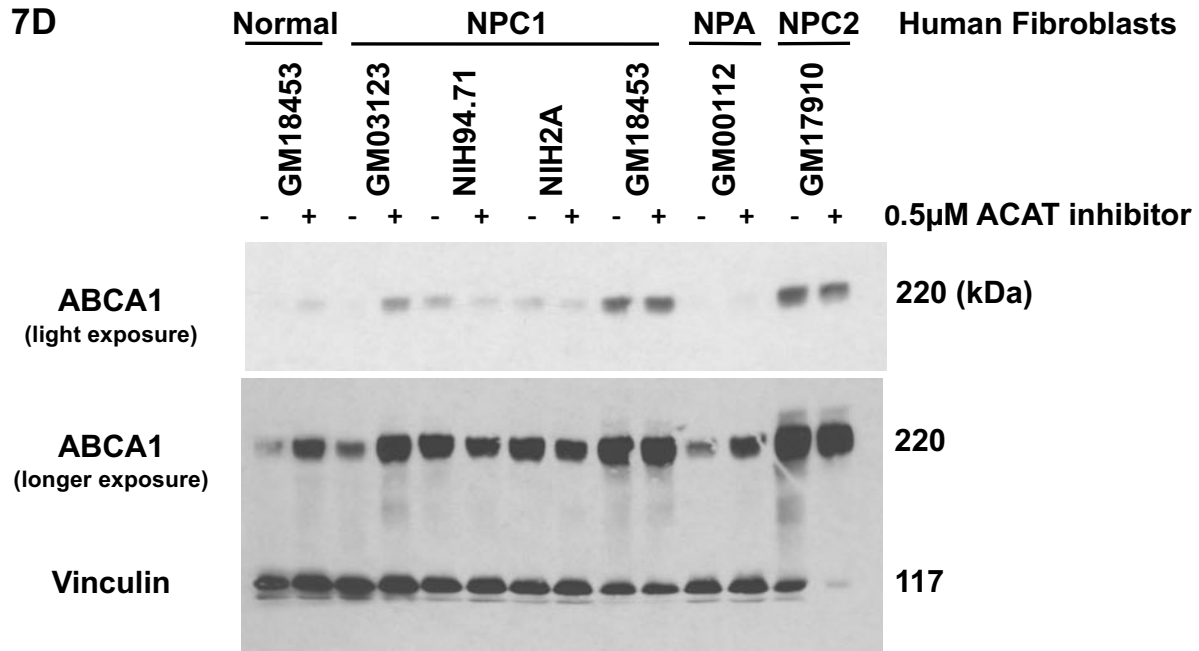


Fig. 7. ABCA1 and NPC1 expression in WT, $A1^{-/-}$, $Npc1^{nmf}$, and $Npc1^{nmf};A1^{-/-}$ cells and tissues.

For parts A, B, and D, the cell growth conditions were the same as described in Fig. 5C.

For parts A, B, and C, cells and tissues from all four genotypes of mice (WT, $A1^{-/-}$, $Npc1^{nmf}$, and $Npc1^{nmf};A1^{-/-}$) were analyzed.

A. Expression of ABCA1 (220 kDa) and NPC1 (180 kDa) proteins in MEFs.

B. Expression of *Abca1* mRNA in MEFs.

- C.** Expression of ABCA1 and NPC1 proteins in P80 mouse cerebellum. Tissues were prepared as described in Methods. $p < 0.05^*$, n.s., not significant.
- D.** Expression of ABCA1 protein in human fibroblasts (Hfs). The vinculin signal was used as the loading control.
- E.** Relative expression of ABCA1 protein in human fibroblasts (Hfs) cultured in the presence or absence of 0.5 μM of ACAT inhibitor K604.