
Figures and figure supplements

A *Drosophila* screen identifies NKCC1 as a modifier of NGLY1 deficiency

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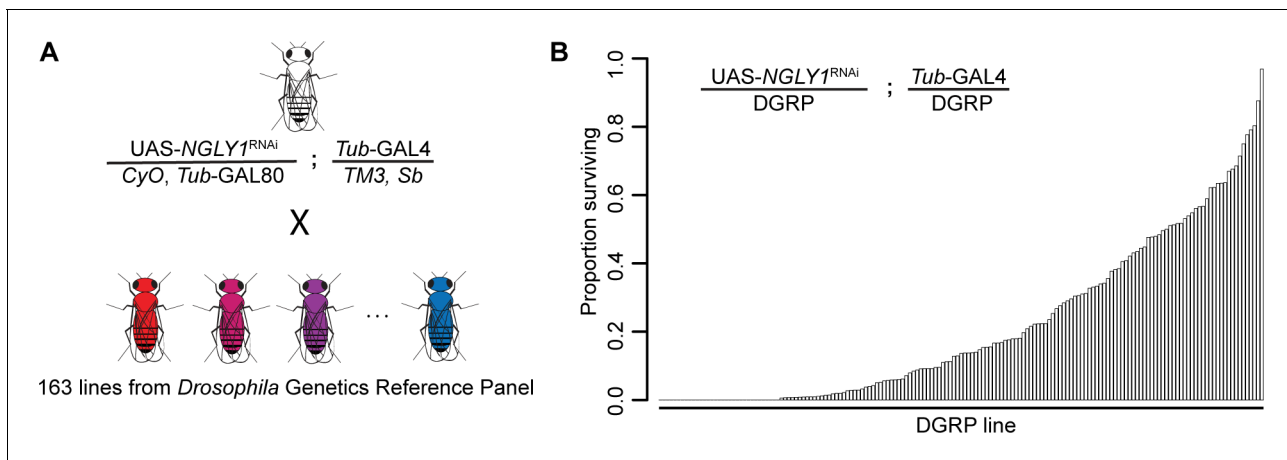


Figure 1. Lethality phenotype of *NGLY1* knockdown is highly modifiable by strain background. (A) *Drosophila* cross for *NGLY1* knockdown in each *Drosophila* genetic reference panel (DGRP) strain. (B) Proportion of *NGLY1* knockdown flies surviving for each cross was calculated based on the number eclosing compared to the expected number. Expected number was based on the largest control balancer class for each cross.

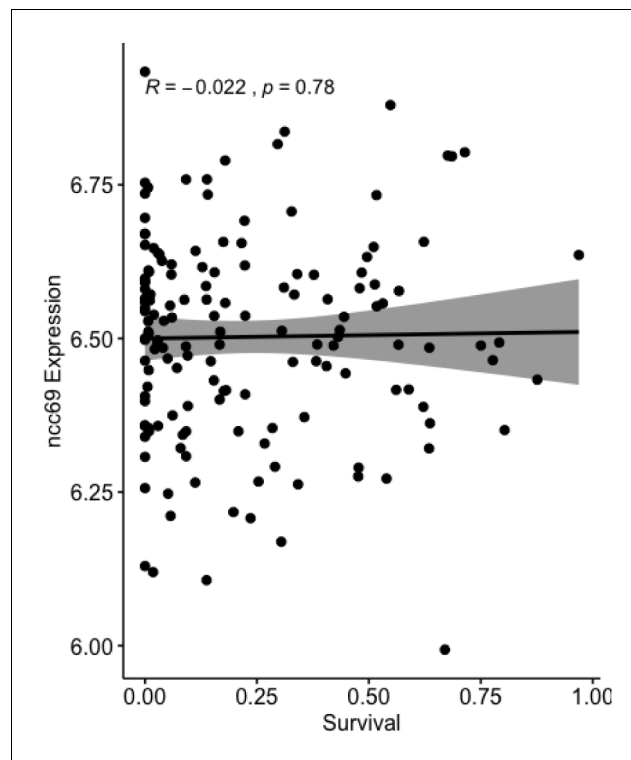


Figure 1—figure supplement 1. Ncc69 expression is not correlated with survival. Survival upon knockdown of *NGLY1* was plotted versus baseline expression levels of *Ncc69* in the DGRP ($r = -0.022$, $N = 154$, $p = 0.78$). DGRP gene expression data was taken from [Huang et al., 2014](#).

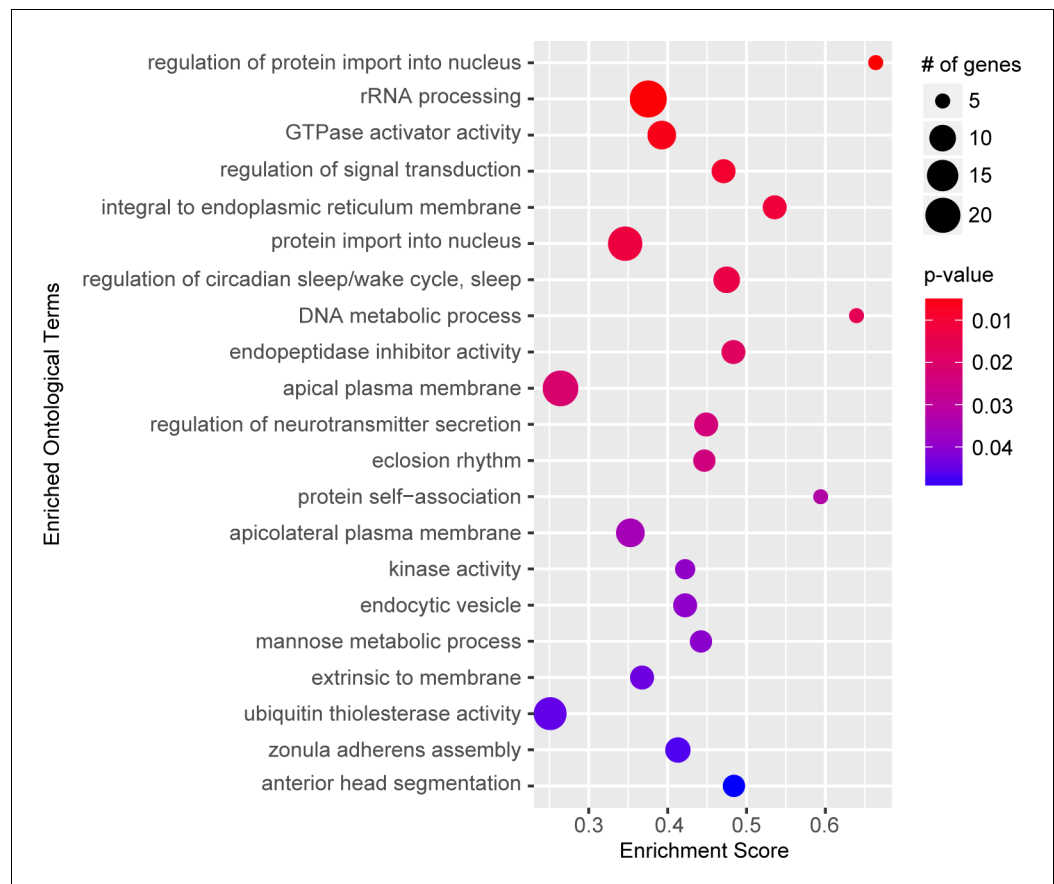


Figure 2. Gene set enrichment analysis. Top significant ontological categories identified by GSEA. p-values are indicated by red-to-blue gradient, with red the lowest p-values and blue the highest p-values. Gene number identified in each category is indicated by the size of the circle.

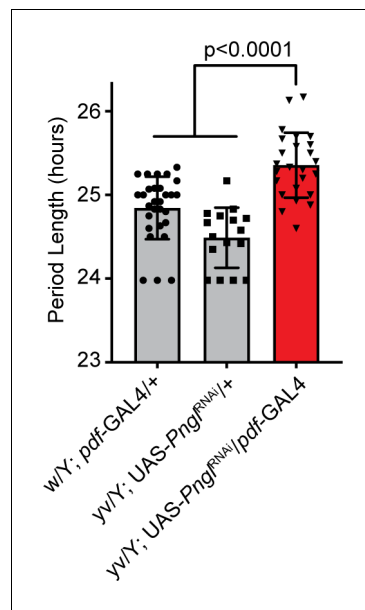


Figure 2—figure supplement 1. *NGLY1* knockdown causes circadian rhythm defect. Activity monitor was used to analyze 2- to 5-day-old flies for 1 week in complete darkness after 3 days of entrainment in a 12 hr light, dark cycle. At least 15 flies were analyzed for each genotype. Period length was calculated from activity using ClockLab. One-way ANOVA gave an overall $p < 0.0001$. Subsequently Tukey's test was used to calculate individual adjusted p -values between genotypes shown on the graph.

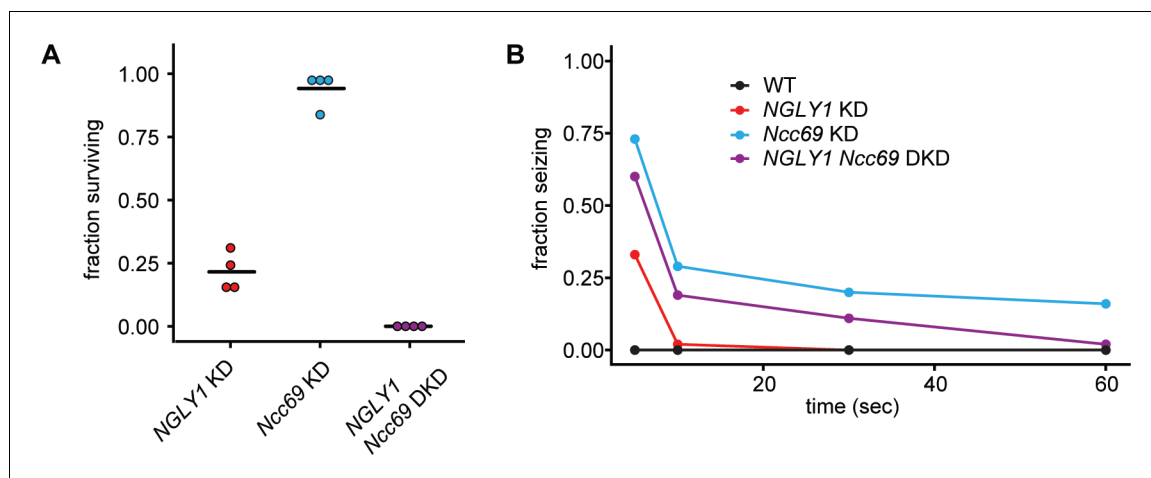


Figure 3. NGLY1 and Ncc69 interact genetically in *Drosophila*. (A) Proportion of flies surviving to eclosion in ubiquitous knockdowns. NGLY1 knockdown (KD) are UAS-*Pngl*^{RNAi}/+; *Tubulin-GAL4*/+. Ncc69 KD are UAS-*Ncc69*^{RNAi}/+; *Tubulin-GAL4*/+. NGLY1 Ncc69 double knockdown (DKD) are UAS-*Pngl*^{RNAi}/+ UAS-*Ncc69*^{RNAi}/*Tubulin-GAL4*/+. Four separate matings were performed for each cross with at least 40 offspring generated for the balancer control for each. Fraction surviving is calculated compared to balancer offspring. Chi-square analysis was performed for the total number of flies compared to expected Mendelian numbers. NGLY1 KD $\chi^2 = 109.7$, $p < 0.0001$; Ncc69 KD $\chi^2 = 1.002$, $p = 0.3168$, and NGLY1 Ncc69 DKD $\chi^2 = 186$, $p < 0.0001$. (B) Bang sensitivity assay to assess seizures in glial knockdown flies. WT flies are attP2 and attP40. NGLY1 KD are UAS-*Pngl*^{RNAi}/+; *repo-GAL4*/+. Ncc69 KD are UAS-*Ncc69*^{RNAi}/+; *repo-GAL4*/+. NGLY1 Ncc69 DKD are UAS-*Pngl*^{RNAi}/+; UAS-*Ncc69*^{RNAi}/*repo-GAL4*. For each genotype, at least 45 4- to 7-day-old females were used to calculate the percent seizing at a given time after vortexing. Repeated measures ANOVA p -value=0.000176.

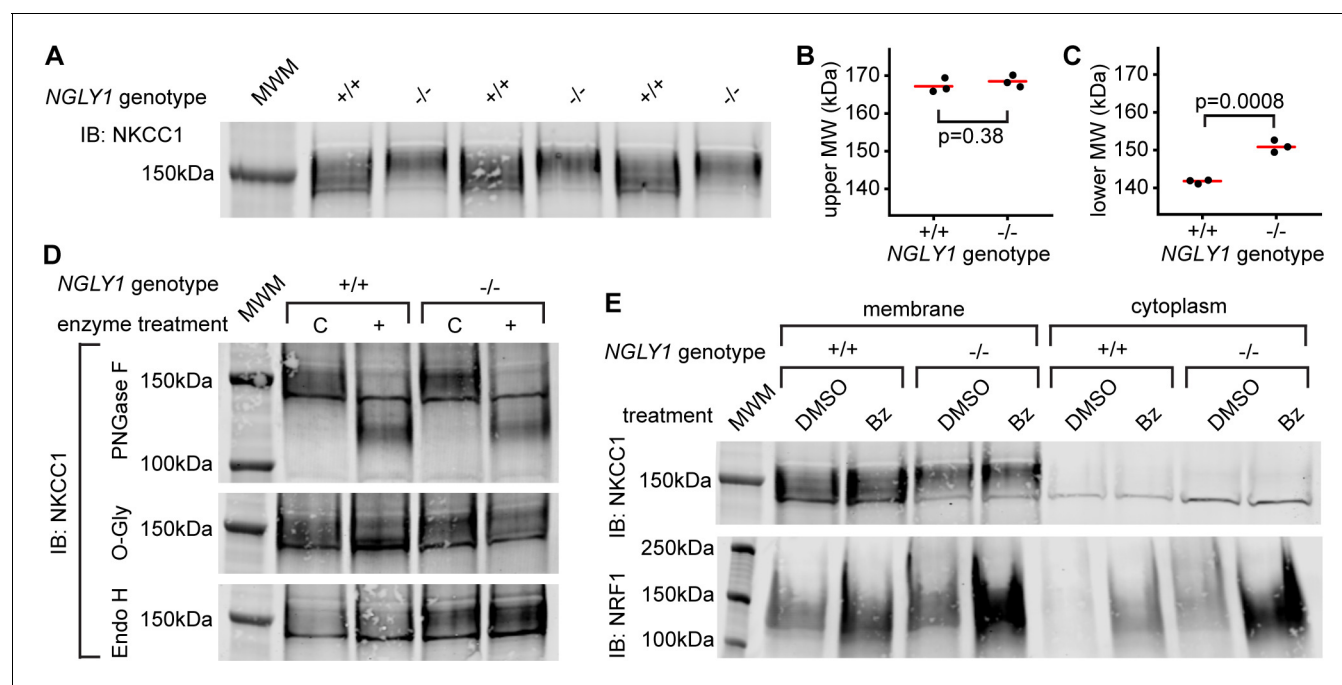


Figure 4. Endogenous NKCC1 is altered in NGLY1-deficient MEFs. (A) Control (+/+) and NGLY1 null (-/-) MEFs were grown to confluency and then lysed to isolate the membrane and cytoplasmic fractions. Three separate membrane lysates for both genotypes were analyzed by immunoblotting for NKCC1 compared to a molecular weight marker (MWM). Blot was used for molecular weight calculations of the upper-most limit (see un-cropped blot in **Figure 4—figure supplement 1**) (B) And the lower-most limit (C) of the protein band. Red bar represents the mean. Two-tailed t-test was used to calculate p-values. (D) Membrane lysates from MEFs were treated with N-Glycosidase F (PNGase F), O-Glycosidase (O-Gly), or Endoglycosidase H (Endo H) for 1 hr then analyzed by immunoblot. Control (C) Samples were treated in all the same conditions but without the added enzyme. (E) MEFs were treated with 500 nM bortezomib (Bz) or equal volume of vehicle control (DMSO) for 4 hr then lysed to collect membrane and cytoplasmic fractions. Lysates were analyzed by immunoblotting for NKCC1. NRF1 was analyzed as a positive control of proteasome inhibition. Dark band at about 140 kDa in both (A), (D), and (E) is believed to be non-specific.

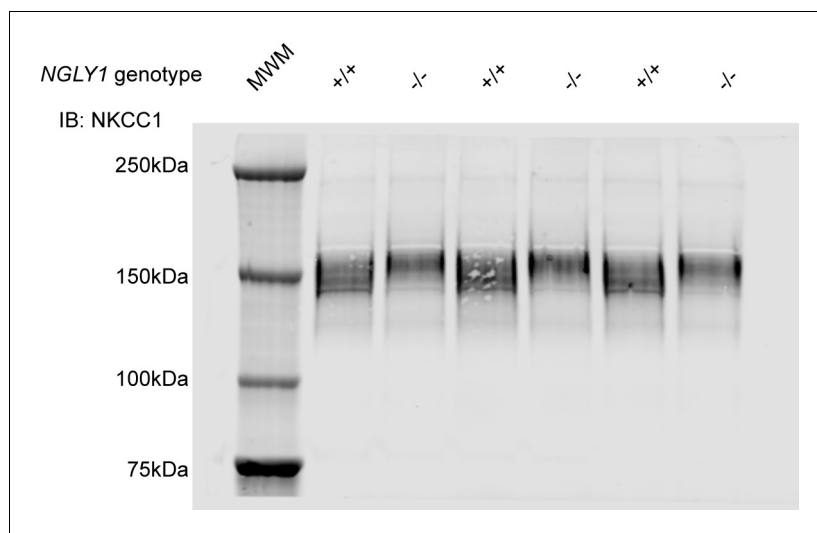


Figure 4—figure supplement 1. Full western blot of NKCC1 in NGLY1 +/+ and -/- MEFs. Tris-acetate gel was run at 150V for 2.5 hr to sufficiently distinguish between the different molecular weights of NKCC1, and therefore the 75 kDa band of the protein marker was the bottom-most band. The 250, 150, 100, and 75 kDa molecular weights were used for a standard curve in the Li-cor software Image Studio, so that the molecular weight of the NKCC1 bands could be calculated.

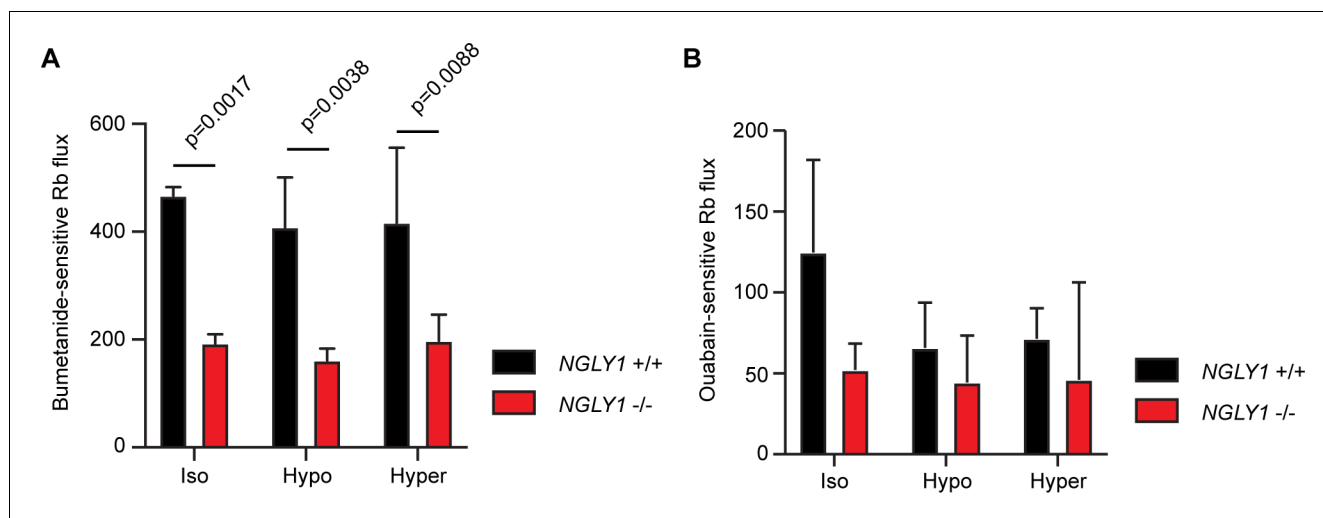


Figure 5. *NGLY1* $-/-$ MEFs show decreased NKCC1-specific ion flux. (A) Bumetanide-sensitive ^{86}Rb flux was measured in *NGLY1* $+/+$ and *NGLY1* $-/-$ MEFs to measure NKCC1 activity. Flux was examined in three bath conditions, isotonic (iso), hypotonic (hypo), and hypertonic (hyper). There was a significant effect of genotype ($p<0.0001$) in two-way ANOVA, with no significant effect of condition ($p=0.5756$) or interaction ($p=0.8075$). Adjusted p-values for Sidak's multiple comparisons test between *NGLY1* $+/+$ and *NGLY1* $-/-$ are shown in the figure. (B) Ouabain-sensitive ^{86}Rb flux was measured in *NGLY1* $+/+$ and *NGLY1* $-/-$ MEFs to measure Na^+/K^+ -ATPase activity in the same three conditions as in A. There were no significant effects of genotype ($p=0.0516$), condition ($p=0.3047$) or interaction ($p=0.4711$) by two-way ANOVA, indicating the *NGLY1* knockout has a specific effect on NKCC1 activity without affecting Na^+/K^+ -ATPase activity.

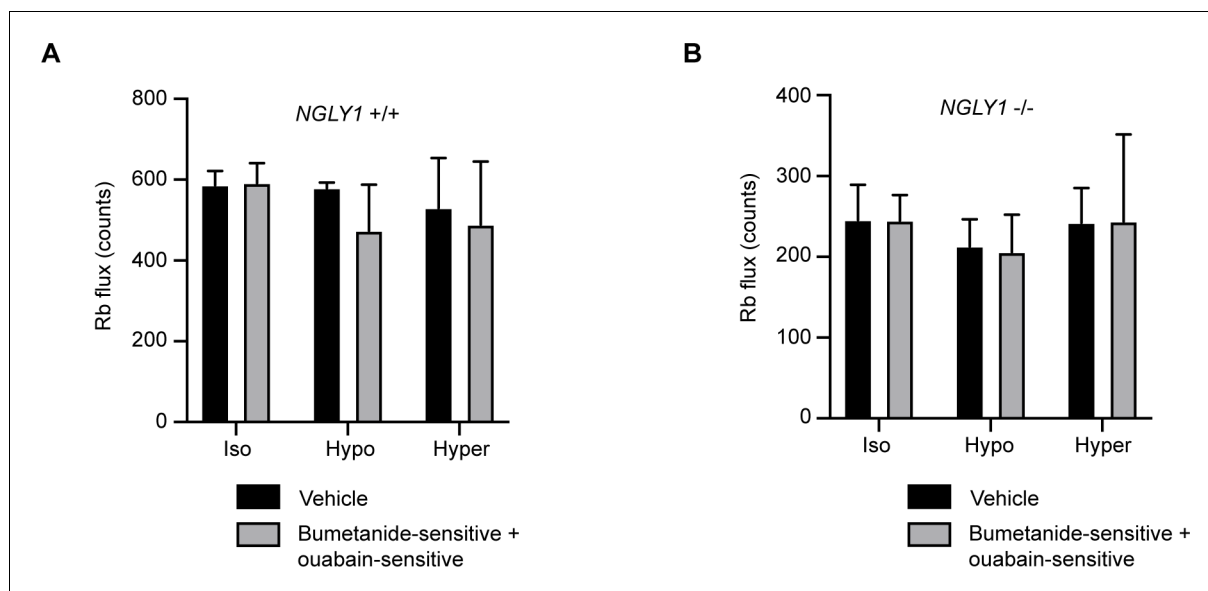


Figure 5—figure supplement 1. ^{86}Rb uptake in MEFs occurs through bumetanide-sensitive and ouabain-sensitive pathways. Either *NGLY1* $+/+$ (A) or *NGLY1* $-/-$ (B) MEFs were incubated with DMSO as a vehicle control or with the NKCC1 inhibitor, bumetanide, or the Na^+/K^+ -ATPase inhibitor, ouabain. ^{86}Rb flux was measured in three conditions: isotonic (iso), hypotonic (hypo), and hypertonic (hyper). The sum of bumetanide-sensitive and ouabain-sensitive flux was compared to vehicle control. There were no significant effects of genotype ($p=0.3267$), condition ($p=0.3602$) or an interaction ($p=0.6244$) in *NGLY1* $+/+$ cells, nor of genotype ($p=0.9422$), condition ($p=0.4987$) or an interaction ($p=0.9909$) in *NGLY1* $-/-$ cells by two-way ANOVA, indicating that in both cell types ^{86}Rb flux was comprised of the bumetanide-sensitive and ouabain-sensitive activities.