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**Elevated small GTPase activation influences the cell proliferation signaling control
in Niemann-Pick type C fibroblasts**

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Summary

Niemann-Pick type C (NPC) disease is characterized at the cellular level by the intracellular accumulation of free cholesterol. We have previously identified a similar phenotype in cystic fibrosis (CF) cell models that results in the activation of the small GTPase RhoA. The hypothesis of this study was that NPC cells would also exhibit an increase in small GTPase activation. An examination of the active, GTP-bound form of GTPases revealed a basal increase in the content of the active-form Ras and RhoA small GTPases in NPC fibroblasts compared to wt controls. To assess whether this increase in GTP-bound Ras and RhoA manifests a functional outcome, the expression of the proliferation control proteins p21/waf1 and cyclin D were examined. Consistent with increased GTPase signaling, p21/waf1 expression is reduced and cyclin D expression is elevated in NPC fibroblasts. Interestingly, cell growth rate is not altered in NPC fibroblasts compared to wt cells. However, NPC sensitivity to statin treatment is reversed by addition of the isoprenoid geranylgeranyl pyrophosphate (GGPP), a modifier of RhoA. It is concluded that Ras and RhoA basal activation is elevated in NPC fibroblasts and has an impact on cell survival pathways.

Introduction

At the cellular level, Niemann-Pick type C (NPC) disease is characterized by improper intracellular cholesterol accumulation in the late endosomal/lysosomal compartments [1]. Previously, we have demonstrated that NPC fibroblasts share similar cell signaling protein expression profiles with model cells of cystic fibrosis (CF) including expression alterations in the transforming growth factor- β 1 (TGF- β 1) signaling protein SMAD3, signal transducer and activator of transcription-1 (STAT1), and the small GTPase RhoA [2]. Included in the signaling alterations associated with NPC fibroblasts was an inability to induce the expression of the inducible nitric oxide synthase (NOS2) [2]. Reduced NOS2 expression is consistent with an increase in the activation of the small GTPase RhoA, a known negative regulator of NOS2 expression [3-5]. The hypothesis of this study is that small GTPase activation is increased in NPC cells and influences cellular functions.

Relatively little is currently known about the cell signaling consequences of losing NPC1 function, but there are suggestions of altered GTPase signaling. Garver et al have demonstrated that protein kinase C (PKC) isozymes and pp60-src exhibit increased expression in liver homogenates in *Npc1*^{-/-} mice [6]. This manuscript provides evidence of the involvement of caveolin-1 containing fractions in the NPC cell signaling regulation, and Gingras et al have shown that RhoA GTPase is localized in caveolin-1 containing membrane domains in endothelial cells [7]. In addition, Taggart et al report that the caveolin-1 scaffolding domain is essential in co-localizing PKC and RhoA in smooth muscle [8]. Taken together, these data suggest a possible role for RhoA in NPC-related signaling alterations. Similarly, indications of reduced peroxisome proliferator-

activated receptor- γ (PPAR γ) function in NPC cells have been reported [9]. RhoA has been demonstrated to be an effective inhibitor of PPAR γ signaling suggesting a possible source for altered PPAR regulation [10]. Although these cell-signaling alterations described in NPC do not conclusively implicate a role for increased GTPase activity, they are consistent with an alteration in the GTPase pathway and warrant a further examination of GTPase activation.

The goal of this manuscript is to examine small GTPase signaling and to determine if NPC cells exhibit alterations consistent with elevated GTPase activation. A prediction of elevated GTPase signaling, particularly RhoA and Ras, is a skewing of cell-signaling regulation to favor proliferation. For example, a study by Liberto et al demonstrated that elevated Ras and RhoA signaling in breast cancer cells resulted in reduced p21/waf1 and elevated cyclin D protein expression [11]. These signaling parameters were examined in NPC fibroblasts to test whether they would exhibit these changes consistent with elevated GTPase activation. The signaling relationship to be examined in this study is shown in figure 1.

Results from this study demonstrate that both RhoA and Ras GTPases exhibit increased basal activation in NPC fibroblasts compared to control cells. Consistent with the work by Liberto et al, the expression of p21/waf1 is reduced in NPC cells and the expression of cyclin D is elevated. It is concluded from this study that both RhoA and Ras GTPases are basally more active in NPC cells compared to controls and that this activation impacts the expression of proliferation control signaling proteins. Increased activation of small GTPases, particularly RhoA, is likely the source of the observed

sensitivity of NPC cells to apoptosis mediated by inhibition of HMG-CoA reductase [12, 13].

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Methods

Cell Culture - Niemann-Pick fibroblasts (NPC) (GM03123A) containing two missense mutations in the NPC1 gene were obtained from Coriell Cell Repository (Camden, NJ). Control human fibroblasts (CRL-2076) were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were grown at 37°C in 95% O₂ -5% CO₂ on Falcon 10 cm diameter tissue culture dishes in MEM Eagle with 2mM L-glutamine containing 15% fetal bovine serum (FBS) and 1u/ml penicillin/streptomycin.

GTPase activity assays – Rho and Ras activity were assayed using kits obtained from Upstate Biotechnology, Lake Placid, NY) according to manufacturer instructions. Details of the RhoA assay are as follows: Rho activity was determined using a Rho Activation Assay Kit (Upstate Biotechnology, Lake Placid, NY). This assay was used previously to determine RhoA activity in CF samples [14,15]. Cells were placed in 300ul 1x Mg²⁺ lysis buffer (25mM HEPES, pH 7.5, 150mM NaCl, 1% Igepal CA-630, 10mM MgCl₂, 1mM EDTA, and 2% glycerol) on ice and homogenized for 60 s. Homogenates were centrifuged at 14,000x g for 5 min at 4 ° C to clear cellular debris and protein concentration was determined. To assay for GTP- γ -S-dependent activation of RhoA, samples were either left untreated or loaded with GTP- γ -S. 5ul of 10mM GTP- γ -S was added to each respective aliquot and incubated for 30 minutes at 30 °C. The reaction was terminated by placing samples on ice and adding 32ul 1M MgCl₂. Active (GTP / GTP- γ -S-bound) Rho was isolated by pull-down assay: 50ul (30 μ g) of Rhotekin Rho Binding Domain (RBD) agarose bead slurry (50mM Tris, pH 7.5, 0.5% Triton X-100, 150mM NaCl, 5mM MgCl₂, 1mM dithiothreitol, 0.1mM PMSF 1ug/ml each of aprotinin and

leupeptin) was added to each 500ul aliquot and incubated for 45 minutes at 4 °C with gentle agitation. The Rhotekin-RBD agarose bead – Rho complex was pulled-down by brief centrifugation (10 s, 14,000x g, 4 °C). The supernatant was removed and the beads washed three times. The beads were resuspended in 40ul Laemmli buffer containing 5% 2-mercaptoethanol and western-blot analysis performed using Anti-RhoA antibody from Santa Cruz biotechnologies (Santa Cruz, CA). (1:1000 dilution) incubated overnight at 4 °C. The effect of U18666a (5 µg/mL for 24 h) from Biomol (Plymouth Meeting, PA) on RhoA activation was determined using the G-LISA RhoA activation assay from Cytoskeleton (Denver, CO). Manufacturer protocols were followed.

Western Immunoblotting. Antibodies against p21/waf1 (mouse) and cyclin D (mouse) were obtained from Santa Cruz biotechnologies (Santa Cruz, CA). Antibody against actin (rabbit) was obtained from Sigma-Aldrich (St Louis, MO). Protein samples were prepared by homogenizing 60mm dishes of cultured cells in ice cold lysis buffer (50mM Tris, pH 7.5, 1% Triton X-100, 100mM NaCl, 50mM NaF, 200uM Na₂VO₄, and 10ug/mL pepstatin and leupeptin) for 30 minutes at 4°C while shaking. Cell lysates were microcentrifuged at 4°C at 14,000 rpm for 10 mins. Proteins were separated using SDS-PAGE containing 20 to 40ug of protein of 6-12% acrylamide gel. The samples were transferred to an Immobilon-P membrane (Millipore, Bedford, MA) at 15V for 30 min. The blots were blocked overnight in phosphate buffered saline (PBS: 138mM NaCl, 15mM Na₂HPO₄, 1.5mM KCl, and 2.5mM KH₂PO₄) containing 10% nonfat dehydrated milk and 0.1% Tween-20 at 4°C. Blots were incubated overnight with primary antibodies (both at 1:1000 dilution) in PBS containing 10% nonfat dehydrated milk and 0.1%

Tween-20. Blots were washed three times in PBS and incubated in secondary antibody conjugated to horseradish peroxidase for one hour at room temperature (dilution 1: 4000, Sigma). Blots were washed again in PBS before visualizing using Super Signal chemiluminescent substrate (Pierce, Rockford, IL) and exposing the membrane to Kodak scientific imaging film (Kodak, Rochester, NY). Quantification of protein expression was accomplished by densitometry software on the VersaDoc (Quality One, BioRad, Hercules, CA).

Immunostaining - Briefly, cells were grown 55-60% confluency on Fisherbrand coverslips. Cells were rinsed three times with phosphate buffered saline (PBS) and then fixed with 2% paraformaldehyde for approximately 30 minutes. Cells were rinsed once more with PBS and then permeabilized in 0.1% NP-40 (Sigma-Aldrich, St Louis, MO) in PBS for 30 minutes at room temperature. Cells are washed 3x in PBS and blocked with 10% goat serum in PBS for 30 minutes at room temperature. Cells are then incubated with anti-cyclin D (1:100) from Santa Cruz biotechnologies (Santa Cruz, CA) in 10% goat serum in PBS for 1 h at room temperature. After washing slide 3x in PBS, cells are incubated with Alexa-fluor 488 goat anti-mouse IgG at 10ug/ml in 10% goat serum in PBS for 1 h at room temperature in the dark. Results were visualized in the ultraviolet range using wide field microscope on a Zeiss Axiovert 200 and Metamorph software. A 40X oil objective with a GFP filter was used for all images.

Proliferation Studies - Cells were treated with mevastatin (50 μ M) with or without geranylgeranylpyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) (Sigma-Alrich

(St Louis, MO)) at 10 μ M for 72 hours. Cell permeable C3 transferase (C3) was obtained from Cytoskeleton (Denver, CO). Cells were treated with C3 (0.5 ng/mL) for 24 h. Cells were plated in 96 well plates (3,000 cells/well) in 100 μ L 0.5 % serum and growth-factor free media for 24 hours prior to treatment. Proliferation was assayed by CellTiter 96 AQueous One Solution Cell Proliferation Assay purchased from Promega (Madison, WI) and used per manufacturer's instructions. After treatment, 20 μ L CellTiter 96 AQueous One Solution Reagent was added to each well in a sterile fashion and the plates were incubated for 2 hours at 37C/5% CO₂. Absorbance was read at 490 nm using a 96 well plate reader. The MTS tetrazolium salt in the reagent is bioreduced into a colored formazan product that is proportional to the number of living cells in culture. Results are expressed as percent absorbance at 490 nm of untreated controls. Groups are compared using ANOVA with the Duncan's multiple range test.

Results

Small GTPase activation – Previous work has demonstrated that NPC cells and CF cells exhibit similar alteration in signaling protein profiles [2]. It has also been established that the small GTPase RhoA exhibits elevated activation in CF cells and tissues [4]. The hypothesis being tested is that the cholesterol accumulation characteristic of NPC leads to elevated basal GTPase activation leading to altered cell signaling regulation. To test this hypothesis, the basal activation state of RhoA was examined in wt and NPC human fibroblasts. The GTP-bound form of RhoA GTPase was determined by rhotekin pull-down assay and the relative content of RhoA-GTP was normalized to total RhoA content. RhoA exhibits increased GTP binding in NPC fibroblasts compared to wt fibroblasts indicating a basal increase in RhoA GTPase signaling as a result of lost NPC1 function (figure 2A,B).

In order to determine if the elevated basal active state of the small GTPases is specific for RhoA in NPC fibroblasts, the GTP-bound form of Ras GTPase was also examined. Consistent with RhoA activation, Ras GTP-bound content is also elevated in NPC fibroblasts compared to wt controls (figure 2C,D). These data demonstrate that NPC-related alterations in small GTPase regulation not specific for simply RhoA and impact both farnesyl- (Ras) and geranylgeranyl-modified (RhoA) GTPase. These data also suggest that RhoA and Ras GTPases are strong candidates for effectors of NPC-related signaling alterations.

Regulation of GTPase regulated proteins – A basal increase in small GTPase signaling is predicted to result in alterations in GTPase-dependent protein regulation. The goal of these studies is to examine a signaling pathway established to be influenced

by small GTPases as a measure of functional influence of increased basal GTP-bound content of both Ras and RhoA in NPC fibroblasts. The pathway examined was the pathway identified by Liberto et al demonstrating that elevated Ras and RhoA signaling in breast cancer cells resulted in reduced p21/waf1 and elevated cyclin D protein expression. These signaling parameters were examined in NPC fibroblasts to test whether they would exhibit changes consistent with elevated GTPase activation. Consistent with the hypothesis, the cyclin kinase inhibitor p21/waf1 exhibits decreased expression in NPC fibroblasts compared to wt controls (figures 3A-B).

Correspondingly, cyclin D content is greater in NPC fibroblasts compared to wt controls (figure 4A-B). To further assess cyclin D expression, wt and NPC fibroblasts were immunostained for cyclin D content. As shown in figure 4C-E, NPC fibroblasts exhibit increased cyclin D content than wt controls. Both cell types are cultured, proliferating cells. It is expected that cyclin D, a cell cycle regulator, will be localized to the nucleus in both cell types. However, overall content of cyclin D in NPC cells is elevated. The above data demonstrate that cell signaling alterations associated with elevated Ras and RhoA GTPase activation are present in NPC fibroblasts.

The above data describe alterations in GTPase activation in NPC cells and changes in p21/waf1 and cyclin D that are consistent with increased GTPase signaling. In order to determine if cholesterol accumulation characteristic of NPC is the mechanistic source of these signaling changes, wt fibroblasts were treated with U18666a (5 $\mu\text{g/mL}$) to induce cholesterol accumulation and then examined for RhoA activation and cyclin D expression. U18666a (3-beta-[2-(diethylamino)ethoxy]androst-5-en-17-one) is a class 2 amphiphile known to inhibit intracellular cholesterol transport and lead to NPC-like free

cholesterol accumulation [16]. RhoA activation measured by immunodetection of GTP-bound RhoA content is elevated (figure 5A) and cyclin D expression is increased (figure 5B,C) by U18666a exposure. RhoA-GTP content increases approximately 10% after 24 h treatment ($p = 0.01$). The measurement of transient GTP binding by RhoA is the best current approach to measure RhoA activation. However, this increase likely does not represent a completely quantitative measure of RhoA activation, only a qualitative trend towards increased activation. An indirect, although more stable, measure of RhoA signaling is the expression of cyclin D. Cyclin D expression increases by approximately 80% ($p = 0.002$) after U18666a treatment. These data suggest that intracellular accumulation of cholesterol is the mechanistic trigger for these cell-signaling alterations.

Proliferation – The signaling profile of elevated active GTPase content, reduced p21/waf1 and elevated cyclin D content would be predicted to favor proliferation and cell survival pathways of NPC fibroblasts compared to wt controls. One potential mechanism for this result is that altered cell signaling skews the regulation of cell survival through the GTPase-dependent pathway. Previous work has demonstrated that NPC cells are sensitive to cell death caused by inhibitors of cholesterol synthesis [12,13]. Inhibitors of cholesterol synthesis also prevent the production of the isoprenoids farnesyl- (FPP) and geranylgeranyl pyrophosphate (GGPP). Ras and RhoA are covalently modified by the addition of FPP and GGPP, respectively, allowing the GTPases to be properly localized and activated. Thus, cholesterol synthesis inhibitors effectively reduce small GTPase function [17]. We hypothesized that NPC sensitivity to cholesterol synthesis inhibition is due to elevated Ras and RhoA signaling and should be reversible with the addition of either FPP or GGPP as a means to specifically modulate Ras and RhoA signaling.

Treatment of NPC fibroblasts with mevastatin, an inhibitor of HMG-CoA reductase, leads to cell death as previously described. However, the addition of GGPP completely rescues mevastatin sensitivity and FPP partially rescues NPC cells (figure 6A). These data support the suggestion that NPC survival control is skewed towards dependence on RhoA signaling.

To more directly confirm these results, the influence of the RhoA inhibitor C3 (0.5 ng/mL, 24 h) on wt and NPC fibroblast survival was examined. C3 exposure resulted in only $4.9 \pm 4.5\%$ (not significant) cell death in wt fibroblasts. However, C3 treatment of NPC fibroblasts resulted in $20.0 \pm 5.7\%$ cell death ($p = 0.003$) (figure 6B). These data demonstrate a NPC sensitivity towards RhoA inhibition and further support an intrinsic alteration of RhoA signaling in NPC.

Discussion

The above data demonstrate that NPC fibroblasts exhibit a basal increase in the GTP-bound active form of both Ras and RhoA small GTPases compared to wt fibroblasts. However, an elevation of GTP-bound Ras and RhoA does not necessarily lead to an increase in cellular responses dependent on these pathways. To test whether signaling responses influenced by small GTPase function are impacted in NPC fibroblasts, p21/waf1 and cyclin D expression were examined. Based on the work by Liberto et al, an increase in both Ras and RhoA signaling should lead to a decrease in p21/waf1 content and an increase in cyclin D protein expression [11]. NPC fibroblasts exhibit this same profile in p21/waf1 and cyclin D expression supporting the hypothesis that Ras and RhoA signaling is functionally elevated in the absence of NPC1 function. Previous work had demonstrated that NPC cells are sensitive to statin-induced cell death; and this manuscript demonstrates the reversibility of this sensitivity with the addition of GGPP, a RhoA modifying isoprenoid. More specifically, data demonstrate increased NPC sensitivity to RhoA inhibition compared to wt fibroblasts further supporting the hypothesis that RhoA signaling is basally increased in NPC.

Although these pathways need to be examined in neuronal cell models of NPC, alterations in the regulation of cell survival in relation to lost NPC1 function potentially impacts the poorly defined process of neurological decline in NPC disease. One model of neurodegeneration in Alzheimer's suggests that improper entry into a cell division cycle leads to neuronal cell death [18]. Several reports have now identified an increase in cellular proliferation markers, including cyclin D, as a precursor to neuronal degeneration in Alzheimer's [19-22]. The skewing of cell survival regulation towards dependence on

the RhoA signaling pathway in NPC cells may facilitate the improper entry into cell division leading to neurological decline.

Support for such a process being involved in NPC neurological pathology is demonstrated in the work by Zhang et al [23]. Zhang et al demonstrate the mice lacking NPC1 expression exhibit increased activation of cdk5, cdk2, and cdk4. Treatment of NPC mice with two cdk inhibitors, roscovitine and olomoucine, demonstrated efficacy in modulating neurological symptoms. Among the benefits of cdk treatment were reduced tau phosphorylation, modulation of Purkinje cell death, and improved motor function [23]. These findings are consistent with data from multiple Alzheimer's studies implicating cdk signaling, particularly cdk5, in the pathogenesis of neurological decline [24-30]. However, recent work by Hallows et al provides evidence against the role of cdk5 in the pathogenesis in NPC by ablating the expression of the cdk5 activator p35 [31]. The lack of cdk5 involvement and the efficacy of roscovitine and olomoucine in modulating NPC neurological phenotypes points to the involvement of cdk2 [31]. A manuscript by Guo et al demonstrates directly the ability of RhoA to regulate the activation of cdk2, supporting the suggestion that small GTPase signaling is potentially involved in NPC pathogenesis [32].

This manuscript demonstrates that NPC fibroblasts exhibit an inherent increase in small GTPase activation and that this signaling pathway impacts how cell survival is regulated. These data explain previous observations of NPC cell sensitivity to cholesterol synthesis inhibition and point to a potential signaling source of pathogenesis in NPC. In addition to the regulation of cell growth as described above, other NPC phenotypes that are influenced by either RhoA or Ras mediated signaling include PPAR γ activation [10]

and the control of neurite outgrowth [33,34]. Elevated signaling through small GTPases may represent a branch point that links several phenotypic manifestations of lost NPC1 function.

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Figure legends

Figure 1. Schematic diagram of proposed signaling relationships in NPC fibroblasts.

Figure 2. RhoA-GTP and Ras-GTP content in NPC and wt fibroblasts. A) Representative gel of RhoA-GTP pull-down assay. B) Densitometry analysis of RhoA-GTP content. Data are normalized to total RhoA protein (RhoA-GTP/total RhoA). Results are obtained from three replicate experiments. Error bars represent SEM and significance is determined by t-test; * $p = 0.006$. C) Representative gel of Ras-GTP pull-down assay. D) Densitometry analysis of Ras-GTP content. Data are normalized to total Ras protein (Ras-GTP/total Ras). Results are obtained from three replicate experiments. Error bars represent SEM and significance is determined by t-test; * $p = 0.05$.

Figure 3. Protein expression of p21/waf1 in NPC and wt fibroblasts. A) Representative gel showing reduced p21/waf1 expression in NPC cells. Actin is shown as a protein loading control. B) Densitometry analysis of p21/waf1 expression normalized to actin expression (p21/actin). Results are obtained from three replicate experiments. Error bars represent SEM and significance is determined by t-test; * $p < 0.001$.

Figure 4. Cyclin D expression and localization in wt and NPC fibroblasts. A) Representative gel showing elevated cyclin D expression in NPC cells. Actin is shown as a protein loading control. B) Densitometry analysis of cyclin D expression normalized to actin expression (cyclin D/actin). Results are obtained from four replicate experiments. Error bars represent SEM and significance is determined by t-test; * $p = 0.05$.

Immunostain data shown are representative images of cyclin D in C) NPC fibroblasts, D) wt fibroblasts, and E) NPC fibroblasts with secondary antibody only. Images are representative of three separate experiments.

Figure 5. Effect of U18666a treatment on RhoA activation and cyclin D expression. A) RhoA activation in wt fibroblasts treated with U18666a (U18; 5 $\mu\text{g}/\text{mL}$) for 24 h as measured by immunodetection of GTP-bound RhoA content. Error bars represent SEM and significance is determined by t-test; * $p = 0.03$; $n=8$ for untreated and $n=14$ for U18 treated cells over three experiments. B) Representative gel demonstrating increased cyclin D expression in response to U18 (5 $\mu\text{g}/\text{mL}$) treatment. C) Cyclin D protein expression in wt fibroblasts treated with U18 (5 $\mu\text{g}/\text{mL}$) for 24 h as measured by Western blot. Error bars represent SEM and significance is determined by t-test; * $p = 0.03$; $n=8$ for untreated and $n=10$ for U18 treated cells over three experiments.

Figure 6. RhoA-dependent cell survival in NPC fibroblasts. A) Data demonstrate that FPP (10 μM) partially reverses and GGPP (10 μM) completely reverses cytotoxicity in NPC fibroblasts induced by treatment with the HMG-CoA reductase inhibitor mevastatin (50 μM). Results are representative of three separate experiments with ten replicates per experiment. Error bars represent SEM and significance is determined by ANOVA with the Duncan's multiple range correction; ** $p < 0.001$ and * $p < 0.01$ compared to untreated samples. B) The influence of the RhoA specific inhibitor (C3; 0.5 ng/mL) on cell survival in wt (open bars) and NPC (black bars) fibroblasts. Results are representative of five separate experiments with three to five replicates per experiment. Error bars

represent SEM and significance is determined by t-test; * $p = 0.003$ compared to untreated samples.

ACCEPTED MANUSCRIPT

Figure 1.

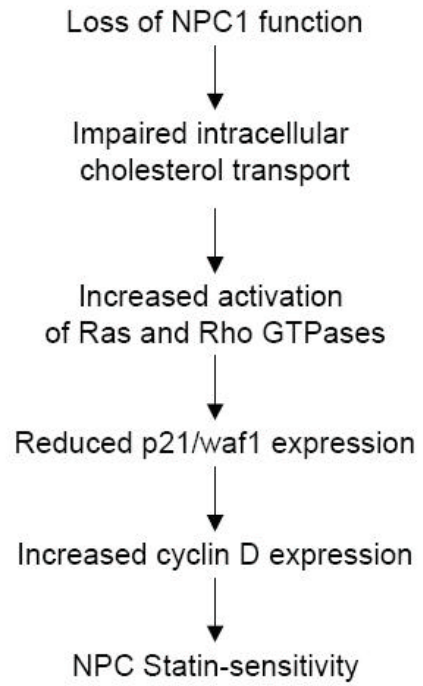


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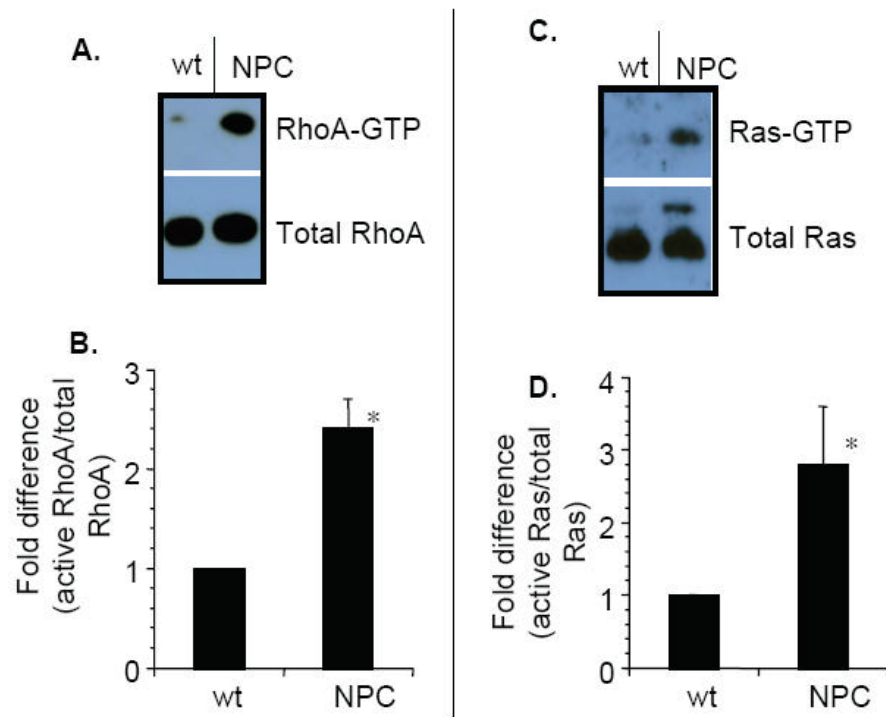


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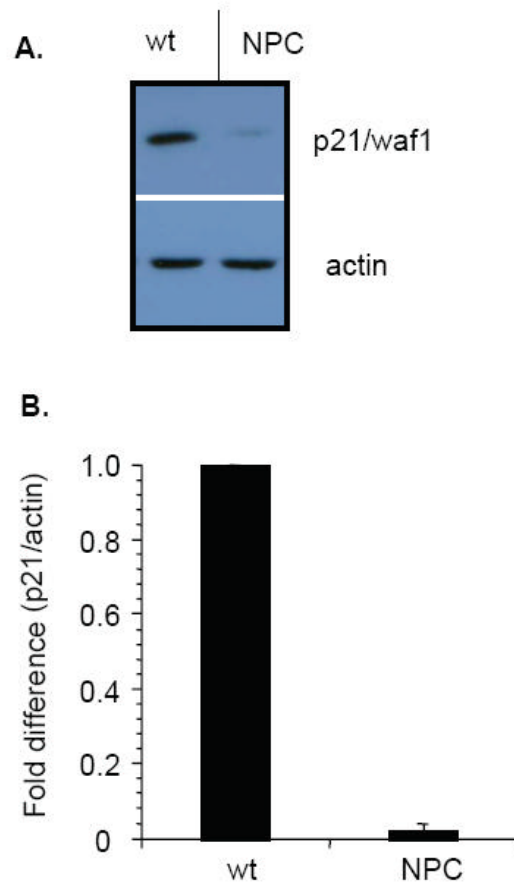


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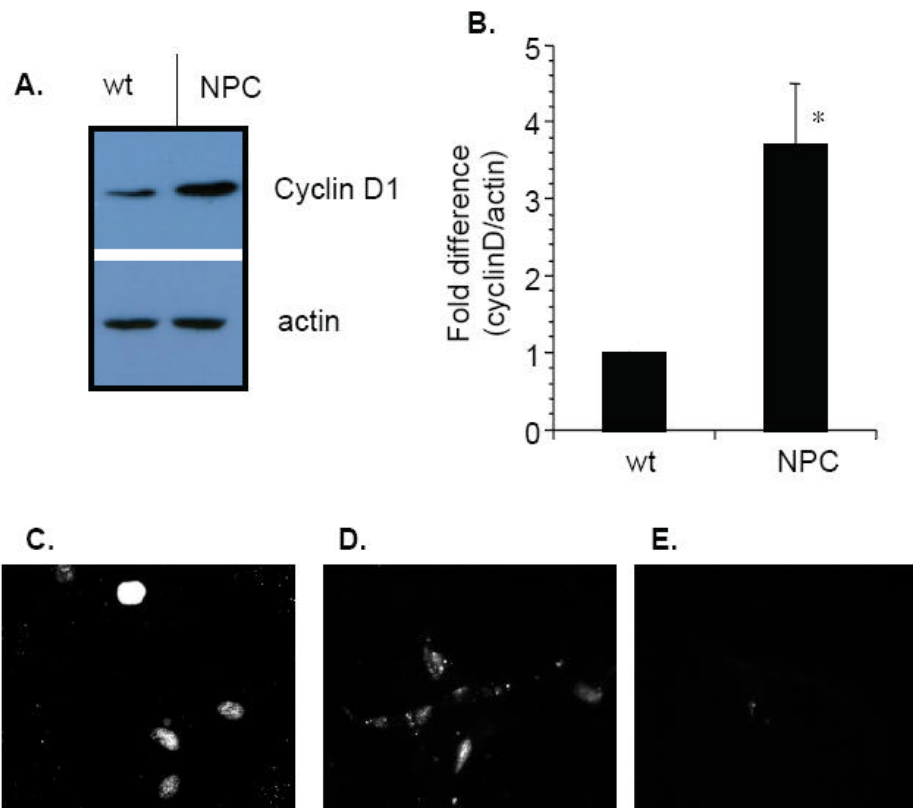


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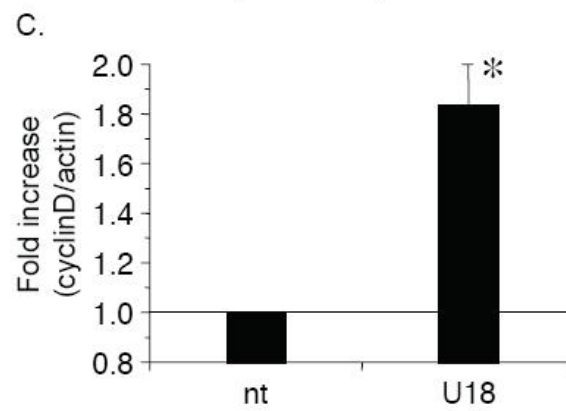
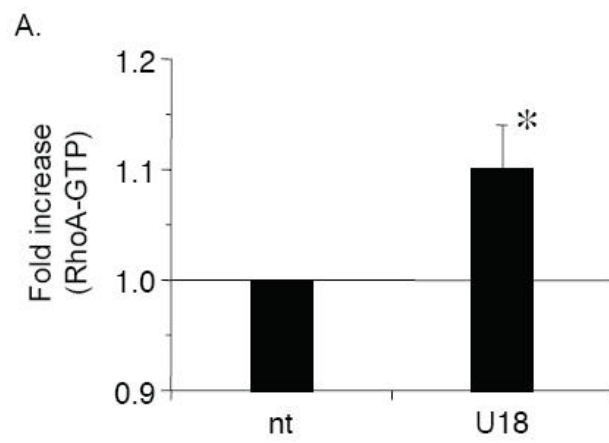


Figure 6.

