

# **Integrin-mediated cell migration is blocked by inhibitors of human neuraminidase<sup>1</sup>**

Feng Jia, Md. Amran Howlader, and Christopher W. Cairo\*

Alberta Glycomics Centre, Department of Chemistry, University of

Alberta, Edmonton, Alberta, Canada T6G 2G2

\*Corresponding author. Tel.: 780 492 0377; fax: 780 492 8231; e-mail:

[ccairo@ualberta.ca](mailto:ccairo@ualberta.ca)

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<sup>1</sup> Abbreviations used: CER, ceramide; C9-4HMT-DANA, 9-[4-hydroxymethyl-[1,2,3]triazol-1-yl]-2,3-didehydro-N-acetyl-neuraminic acid; DANA, 2,3-didehydro-N-acetyl-neuraminic acid; ECM, extracellular matrix; FN, fibronectin; hNeu, human neuraminidase enzyme; HPTLC, high performance thin layer chromatography; LN, laminin; 4MU-NANA, 4-methylumbelliferyl-N-acetyl-neuraminic acid; pfNeu, *C. perfringens* neuraminidase; SPT, single particle tracking; TIRF, total internal reflection fluorescence

## **Abstract**

Integrins are critical receptors in cell migration and adhesion. A number of mechanisms are known to regulate the function of integrins, including phosphorylation, conformational change, and cytoskeletal anchoring. We investigated whether native neuraminidase (Neu, or sialidase) enzymes which modify glycolipids could play a role in regulating integrin-mediated cell migration. Using a scratch assay, we found that exogenously added Neu3 and Neu4 activity altered rates of cell migration. We observed that Neu4 increased the rate of migration in two cell lines (HeLa, A549); while Neu3 only increased migration in HeLa cells. A bacterial neuraminidase was able to increase the rate of migration in HeLa, but not in A549 cells. Treatment of cells with complex gangliosides (GM1, GD1a, GD1b, and GT1b) resulted in decreased cell migration rates, while LacCer was able to increase rates of migration in both lines. Importantly, our results show that treatment of cells with inhibitors of native Neu enzymes had a dramatic effect on the rates of cell migration. The most potent compound tested targeted the human Neu4 isoenzyme, and was able to substantially reduce the rate of cell migration. We found that the lateral mobility of integrins was reduced by treatment of cells with Neu3, suggesting that Neu3 enzyme activity resulted in changes to integrin–co-receptor or integrin–cytoskeleton interactions. Finally, our results support the hypothesis that inhibitors of human Neu can be used to investigate mechanisms of cell migration and for the development of anti-adhesive therapies.

## **Keywords**

glycolipid; ganglioside; neuraminidase; sialidase; integrin; cell migration

## 1. Introduction

Cell migration is critical for normal development and plays an important role in many disease states. The seeding of tumors by cell metastasis is a target for cancer therapies; therefore, a more detailed understanding of mechanisms which contribute to cell migration are needed.[1] The extracellular matrix (ECM) surrounds tissues and organs, providing a substrate which adhesion receptors bind to. Cells then use this matrix as an anchor through association of receptors to the cytoskeleton. Integrins are a major class of adhesion receptors that interact with the ECM, and which participate in transmembrane signaling.[2, 3] The integrins are heterodimeric proteins which vary by cell type, with  $\alpha4\beta1$  and  $\alpha5\beta1$  being the most prevalent on epithelial cells,[4, 5] making them potential targets for cancer therapies.[6]

The  $\beta1$  integrins are regulated through a variety of biochemical mechanisms, including phosphorylation, clustering, conformational change, and lateral association with proteins and glycolipids.[7, 8] Glycolipids are known to participate in integrin regulation. For example, GT1b negatively regulates cell motility, spreading, and adhesion on fibronectin (FN) through direct molecular interactions with the  $\alpha5$  subunit of the  $\alpha5\beta1$  integrin.[9-12] GM3 has been proposed to associate with the  $\beta1$  integrin.[13] GM3 also affects cell migration and invasion by altering the expression of matrix metalloproteinase-9 (MMP-9), a co-receptor which

directly interacts with the  $\alpha 5\beta 1$  integrin.[14] Other integrin-associated proteins, including EGFR and the urokinase-type plasminogen activator receptor (uPAR) interact with specific glycolipids which regulate their activity.[15, 16] Thus, glycolipid composition of the membrane may directly or indirectly modulate integrin function.

Glycolipid composition of the plasma membrane is regulated through biosynthesis, catabolism, exocytosis, and endocytosis.[17, 18] Sialic acid residues are typically at terminal positions within glycolipids, and their content is regulated by sialidase (also called neuraminidase, Neu) and sialyltransferase enzymes (SiaT). Most SiaT are found in intracellular compartments,[19] although, some SiaT activity has been found in the extracellular space.[20-22] The GM3 synthase (GM3S), a SiaT, has been shown to modulate the migration of cells in diabetes.[23] Human Neu enzymes (hNeu) have been linked to the regulation of cancer cell proliferation and motility.[24] Two isoenzymes of hNeu, Neu3 and Neu4, are known to modify the glycolipid composition of cells.[25, 26]

Glycolipid processing enzymes are known to have a variety of roles in the regulation of cell motility, adhesion, and signaling through integrins. Neu3 expression alters the proliferation of cancer cells,[27] and the enzyme has been shown to be a positive regulator of cell migration in renal and prostate cancers.[28, 29] Lactosyl ceramide (LacCer), a product of Neu activity on GM3 substrates, is

known to mediate neutrophil adhesion, phagocytosis, and superoxide generation.[30-32] Importantly, studies in animals have found that prostate cancer cells with suppressed Neu3 activity have reduced metastasis and growth.[33, 34] Neu4 has been shown to negatively regulate cell motility in matrigel.[35] Therefore, evidence suggests that Neu3 and Neu4 can play an important regulatory role in integrin-mediated cell migration. Specific inhibitors of hNeu isoenzymes would then be useful tools for the development of therapeutics. However, few selective and potent inhibitors of hNeu have been reported or tested for this purpose.

Our group has been working to develop specific inhibitors of hNeu in order to investigate their role in adhesion and cancer.[36] The general sialidase inhibitor, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (DANA), has activity against human, bacterial, and viral neuraminidase enzymes.[37, 38] Previous studies have shown that DANA has a low micromolar IC<sub>50</sub> against all isoenzymes of hNeu.[39] We have reported inhibitors with specificity for Neu2, Neu3,[37, 39] and Neu4.[40] A selective inhibitor of Neu4 has been shown to alter stem-cell marker expression and suppress proliferation of glioblastoma cells.[41] However, neither DANA nor these newly identified compounds have been used to investigate the role of native sialidase enzymes in the regulation of integrin adhesion. Evidence of the ability of Neu inhibitors to alter integrin-mediated cell migration would validate this family of enzymes as potential targets for anti-adhesive therapeutics.[6, 42]

Herein we report our investigation of the role of human neuraminidase enzymes in the regulation of  $\beta$ 1 integrin-mediated cell migration of transformed cells on a FN substrate. We implemented a scratch assay to measure changes in the rate of cell migration.[43] Additionally, we employed immunofluorescence and single-particle tracking (SPT) to observe changes in the subcellular location and diffusion of integrin receptors within the membrane. To gain insight into the role of specific glycolipids in this process, we tested the effect of recombinant glycolipid processing enzymes (Neu3 and Neu4), a general neuraminidase inhibitor (DANA), and exogenous gangliosides (LacCer, GM1, GM3, GD1a, GD1b and GT1b) on cell migration. Critically, we tested the only known nanomolar-active inhibitor of a single Neu isoenzyme (Neu4) and found that the compound was an excellent inhibitor of cell migration.

## **2. Results**

### *2.1. Glycolipid composition of cells is altered by Neu treatment and inhibition.*

Cellular gangliosides can be qualitatively analyzed by high-performance thin layer chromatography (HPTLC).[44] Treated cell extracts were analyzed to determine changes in sialo- and asialo-glycolipids. Individual glycolipid bands attributed to LacCer or GM3 were integrated by densitometry, and a ratio was determined by comparison with that of GM3 (LacCer:GM3; asialo:sialo) (**Figure S1**). Examination of lipids extracted from cells treated with the NanI sialidase from

*C. perfringens* (pfNeu) showed an increase in asialo glycolipids. Treatment with the sialidase inhibitor, DANA, showed an opposite effect with a decrease in the asialo:sialo ratio of glycolipids, confirming inhibition of native Neu activity. A small increase in asialo glycolipids was observed when cells were treated with recombinant human Neu3 enzyme.[39] These results confirmed that exogenous sialidase enzymes can alter membrane glycolipid composition, and that inhibition of native sialidase enzymes resulted in an increase of sialic acid-containing glycolipids.

## *2.2. Human Neu3 and Neu4 have $\alpha(2,3)$ - and $\alpha(2,8)$ -sialidase activity for glycolipids*

To provide insight into the specificity of these enzymes for complex ganglioside substrates, we tested the activity of recombinant Neu3 and Neu4 *in vitro*. [39, 45, 46] Lipid substrates were incubated with the enzyme and the products were detected using HPTLC. Substrates were used at identical concentrations and were incubated with enzyme samples calibrated for similar specific activity using a 4MU-NANA assay.[46] Gangliosides GM1, GM3, GD1a, GD1b, and GT1b were tested with three neuraminidase enzymes: Neu3, Neu4, and pfNeu (**Figure S2**). The data confirmed that Neu3, Neu4, and pfNeu were able to cleave the  $\alpha(2,3)$  linked sialic acid of GM3; however, none of the enzymes were able to cleave the same linkage in GM1 at an appreciable rate – not even the promiscuous pfNeu

enzyme.[47] We suspect that the resistance of the internal  $\alpha(2,3)$  linkage of GM1 may indicate that the recognition of the substrate requires a free C4-hydroxyl of the internal galactose residue.[46] We observed that GT1b, which contains both  $\alpha(2,3)$  and  $\alpha(2,8)$  linkages was a substrate for all three enzymes. In this experiment, a new band was observed for all three enzymes at the  $R_f$  of GM1, as well as an intermediate band which was ascribed to GD1b. Further testing of both GD1a and GD1b confirmed that GD1a was a substrate for all three enzymes, while GD1b showed only trace cleavage by Neu3 (**Table S1**). These data are consistent with an  $\alpha(2,8)$  activity for both Neu3 and Neu4; however, the absence of a GD1a intermediate in the GT1b cleavage experiments implies that the  $\alpha(2,3)$  activity is much greater than that of the  $\alpha(2,8)$  of both enzymes. The observed specificity of all three enzymes is summarized in **Figure 1**.

### *2.3. Cell migration on fibronectin is modulated by Neu3 and Neu4*

We proceeded to test the role of hNeu in cell-matrix interactions using a cellular migration assay. A variety of assays can be used to evaluate changes in cell adhesion and migration.[48] We selected a scratch assay as it is easy to implement, and could be used to measure small changes in cell migration rates using microscopy.[49] We used two human epithelial cell lines: HeLa and A549. Lung epithelia has been shown to express Neu3,[50] where they play a role in cellular migration.[51] In order to provide a controlled surface with known integrin-matrix

interactions, substrates were coated with fibronectin (FN), which primarily interacts with the  $\alpha 4\beta 1$  and  $\alpha 5\beta 1$  integrins.[52] Control experiments confirmed that treatment of the substrate with FN was required for cell attachment, and blocking with an anti- $\alpha 5$  integrin antibody resulted in cell detachment. Thus, while these cells express other integrins, the matrix used in the experiment restricts interactions with the substrate to those between FN and  $\alpha 5\beta 1$  integrin.

Cells were grown to a monolayer on FN-coated plates, and a wound was generated using a pipette tip.[43] Representative raw data are shown for the control (buffer treated) condition in **Figure S3**. We found that the data for both cell lines were well fit as a line ( $r^2$  values 0.92-0.99). To avoid enzyme degradation treatment with Neu3 and pfNeu were conducted over a 3 hr period; Neu4 experiments were conducted over 1 hr period, (all with four time points including  $t = 0$ ). In control experiments, FN-coated plates were pre-treated with Neu3 and pfNeu enzymes. No effect on cell migration was observed for Neu-treated FN (**Figure S4**), allowing us to conclude that effects from the enzyme are the result of changes in cellular targets rather than the ECM substrate. With our assay established, we examined conditions that could contribute to changes in cell migration.

Among the neuraminidase enzymes tested with HeLa cells, Neu3, Neu4, and pfNeu were all found to increase the rate of cell migration (**Figure 2a**). The effect of Neu3 was confirmed to be a result of enzyme activity, as an inactive

Neu3(Y370F) mutant showed no effect on migration rates.[46] Importantly, increased amounts of pfNeu enzyme activity (3.4-fold in relative units, RU) were required to achieve a similar enhancement as that seen for 1 RU of Neu3. This finding is consistent with Neu3 having greater specificity for the target substrate(s) which result in altered migration. Corresponding experiments in A549 cells found that Neu3 and pfNeu had the opposite effect as that seen in HeLa cells, with significant reduction in the rate of cell migration (**Figure 2b**). The effect of Neu3 was again dependent upon enzyme activity, as the Neu3(Y370F) protein did not alter the rate of migration from control. Experiments with Neu4 in both cell lines found that the enzyme increased the rate of cell migration, with a larger effect seen in A549 cells than in HeLa.

To explore the role of integrin glycosylation in our observations, we performed immunoblots of integrin  $\beta 1$  glycoprotein from HeLa cells after treatment with Neu (**Figure S5**). We observed that the  $\beta 1$  subunit showed a less diffuse, and lower molecular weight band after pfNeu treatment consistent with loss of sialic acids. Furthermore, treatment with Neu3 showed a similar, but less substantial change in migration. We conclude that the integrin, or other glycoproteins, may be desialylated by Neu3. Previous work has noted that EGFR is desialylated due to Neu3 activity.[53]

#### *2.4. Cell migration on fibronectin is modulated by Neu inhibitors*

We next tested compounds which could affect native Neu activity. Compounds tested included DANA, a non-selective Neu inhibitor; TQ a purported Neu4-activator [54]; and cytoD, a cytoskeleton disruptor known to affect integrin function [55]. The non-selective Neu inhibitor, DANA (100  $\mu$ M), substantially suppressed cell migration to almost half of the rate of control in both cell lines tested (**Figure 2b & 2d**). Sialic acid (Neu5Ac) was tested as an analog of DANA, and showed only a minor suppressive effect on cell migration. Vehicle controls (buffer with 0.1% DMSO) were used to compare the activity of thymoquinone (TQ) and cytoD. TQ induced a small increase in the rate of cell migration in both cell lines; consistent with the effect of Neu4 treatment on both cell lines. Although this observation may be the result of Neu4 activation, further experiments are required to confirm the operative mechanism and selectivity of this effect.[56] Experiments with low concentrations of cytoD resulted in unstable monolayers of cells which were much more sensitive to damage when scratched at the start of the assay. As a result, we were only able to measure the effect of cytoD by using very low concentrations of the drug in HeLa. As expected, we found that cytoD inhibited cell migration consistent with integrin-cytoskeletal interactions being required for efficient cell migration.[57]

Our observations with DANA-treated cells suggested that hNeu inhibitors

could act as potent anti-adhesives. DANA has previously been shown to partially inhibit the adhesion of stimulated neutrophils to endothelial cells.[58] Since DANA has such broad specificity, we decided to test an inhibitor, C9-4HMT-DANA, previously identified to specifically target Neu4.[40] We tested C9-4HMT-DANA in the A549 cell line as it showed the most significant response to Neu4 activity. We found that C9-4HMT-DANA was a substantially more potent inhibitor of A549 migration as compared to DANA, resulting in activity at 200-fold lower concentration (0.5  $\mu$ M, **Figure 2d**).

#### *2.5. Cell migration on fibronectin is modulated by glycolipids*

We next examined the effect of specific glycolipids on cell migration when added exogenously to cells. Glycolipids were selected based on our substrate studies (*vide supra*) to include possible native products (LacCer, GM1, GD1a) and substrates (GM3, GD1a, GD1b, GT1b) of Neu3 or Neu4 enzymes. Glycolipids are known to insert into the plasma membrane when added exogenously to cells,[59, 60] and may also undergo endocytosis.[61, 62] We chose conditions that were expected to favor membrane insertion of glycolipids, including the use of serum in the media and a low concentration of the exogenous lipid.[63] We found similar effects on cell migration rates between both cell types (**Figure 3**). The addition of LacCer resulted in enhanced cell migration in both cell lines. However, GM3 addition resulted in enhanced migration of HeLa cells, but did not have any

significant effect in A549 cells. All other complex gangliosides tested (GM1, GD1a, GD1b, and GT1b) resulted in suppression of cell migration in both cell lines.

#### *2.6. Migration inhibitors are not toxic to cells*

It is possible that the reduced migration of cells treated with DANA, sialic acid, or C9-4HMT-DANA could be the result of toxic effects of these reagents. To test this hypothesis, we treated cells under the same conditions and measured their viability using an MTS assay (**Figure S6**). We observed little or no effect of any of the conditions tested on cell viability. Therefore, we concluded that the effects on cell migration were a result of direct or indirect biochemical effects on the integrin-FN system rather than a result of cellular toxicity.

#### *2.7. Localization of integrins by immuno-fluorescent labeling*

In order to obtain additional data on the interaction between gangliosides and integrin receptors on the cell membrane, we employed immuno-fluorescence microscopy. In human epithelial cell adhesion, the primary integrins that bind with ECM are the  $\alpha 5\beta 1$  and  $\alpha 4\beta 1$  integrins.[64] Initial attempts to visualize the  $\alpha 4\beta 1$  integrin using a Cy5-anti- $\alpha 4$  antibody conjugate on HeLa cells were not successful. We attribute this finding to the low expression levels of  $\alpha 4\beta 1$  integrin found on adherent cells.[65] Labeling of cells with a Cy5-anti- $\alpha 5$  antibody conjugate allowed visualization of the integrin receptors using total internal reflection fluorescence (TIRF) microscopy (**Figure 4**). Integrin staining revealed a large number of small

puncta on the cell membrane, with most of the integrin receptors at the edge of the cell-substrate contact area. These puncta are likely microclusters of receptors, and appear to be relatively diffuse on the cell surface. In order to detect membrane ganglioside localization on cells, we used CTB conjugated with a fluorescent dye (FITC), which may co-localize with ganglioside components of lipid rafts. Two-color images of HeLa cells stained with the Cy5-antibody conjugate and CTB-FITC suggested only partial co-localization of gangliosides with integrin receptors. These findings do not rule out the formation of domains below optical resolution, but support that no gross changes in integrin organization occur upon treatment with Neu (e.g. capping.)

#### *2.8. Lateral mobility of the $\alpha 5$ integrin is altered by Neu3 treatment*

The lateral mobility of adhesion receptors can provide critical insights into mechanisms of regulation. For example, the formation or release of cytoskeletal contacts would manifest as a respective decrease or increase in lateral mobility.[66] We set out to study the lateral mobility of the  $\alpha 5\beta 1$  integrin under conditions which we found could alter cell migration rates (*vide supra*). We tested three conditions which we expected could alter  $\alpha 5$ -integrin lateral mobility, including treatment with DANA, GM1, and recombinant Neu3. HeLa cells were first sparsely stained with Cy5-anti- $\alpha 5$  antibody, and cells were imaged by TIRF over 10 sec to observe the movement of receptors. Data for each condition was obtained from 10 videos

collected over two separate experiments. The video data were converted to trajectories,[67] and analyzed according to a standard MSD analysis.[68, 69] The MSD curve was fit to determine the diffusion coefficient using a standard microdiffusion analysis. To identify if our data were consistent with a single or multiple populations, we analyzed the cumulative probability distribution (CPD) of the diffusion coefficients. We found that these data could be described by a single log-normal distribution, and fit values were determined (**Table S2, Figure S7**). The diffusion data show that  $\alpha 5$ -integrin does not show significant changes relative to control when cells are treated with DANA or GM1 (**Table 1**). However, treatment with the Neu3 enzyme resulted in a significant decrease in the mean of the log-normal distribution. A visualization of the data as a CPD is shown in **Figure 5** showing the reduced diffusion rates of the  $\alpha 5$  integrin on Neu3-treated cells. We interpret these data as an indication of increased cytoskeletal contact or ligand engagement by the  $\alpha 5\beta 1$  integrin due to Neu3 activity.

### 3. Discussion

Human neuraminidase enzymes have been shown to alter the migration of transformed cells in vitro and in vivo. [34, 70] However, the precise mechanism of this effect remains unclear. In this study, we have investigated the role of Neu enzymes and glycolipid composition on integrin function. We employed a scratch assay to measure cell migration rates under treatment. In the two cell lines tested, we found that treatment with exogenous Neu generally increased cell migration. One exception to this was the treatment of A549 cells with Neu3, which resulted in a decrease in migration rates. To probe the role of native glycosylhydrolase activity, we treated cells with Neu inhibitors. We found that the non-selective inhibitor, DANA, was able to inhibit cell migration at high concentrations. More importantly, we confirmed that a Neu4-selective inhibitor, C9-4HMT-DANA, was a potent inhibitor of cell migration. Our results implicate a role for changes in glycolipid content as treatment of both cell lines with LacCer resulted in an increase in cell migration rates, while complex gangliosides (GM1, GD1a, GD1b, and GT1b) reduced migration. Finally, experiments which observed the lateral mobility of  $\alpha 5 \beta 1$  integrin showed a clear decrease in the diffusion coefficient of Neu-treated cells; suggesting that cytoskeletal contacts for the integrin are reduced. Together, these results provide a window into the complex interplay of glycoconjugates and cell-surface receptors which regulate cell motility.

Membrane-associated and lysosomal glycosylhydrolases can exert control over the composition of membrane glycoconjugates. In this study we employed enzymes with substrate selectivity that should favor changes to glycolipid content: Neu3 is thought to target only glycolipids, while Neu4 cleaves both glycolipids and glycoproteins.[71, 72] It is important to note that we cannot rule out a role for changes to the glycosylation of glycoproteins which could result from Neu3 or Neu4 treatment.[53] Alteration of glycoprotein sialic acid content is known to play a role in processes including insulin signaling, adhesion, and metastasis.[73-75] These effects may be due to exposure of cryptic galectin binding sites or loss of Siglec binding sites.[76, 77] Alternatively, the enzyme activities studied here may also exert their effects through changes to glycolipid composition of the membrane.

The effects of Neu isoenzymes with a preference for glycolipids and the addition of specific glycolipids lend support to the glycolipid hypothesis. Other evidence is consistent with the hypothesis that changes in glycolipid-receptor interactions may account for our observations. Disruption of ganglioside biosynthesis has previously been found to affect cell migration.[78] Gangliosides which are substrates for Neu3 and Neu4 include GT1b, GD1a, GD1b, and GM3. Increased expression of Neu3 altered the level of gangliosides in human lymphoma cells.[79] Studies have suggested that Neu3 could cleave  $\alpha(2,3)$ ,  $\alpha(2,6)$ [80, 81], and  $\alpha(2,8)$ [82] sialic acid linkages in gangliosides. Ganglioside substrates of Neu3 and

Neu4 are known to have specific interactions with integrins [9, 13] or their co-receptors (i.e. EGFR, MMP-9, uPAR).[14-16] EGFR signaling has been linked to Neu3 activity, and the enzyme has been found to co-precipitate with EGFR.[83] Furthermore, Neu3 may also change the glycosylation state of EGFR itself.[53] Previous reports have found GM3-associated  $\beta$ 1-integrins have reduced adhesion to FN, and GM1 clusters modulate integrin adhesion.[13, 84] Thus, changes to ganglioside composition may alter integrin-co-receptor interactions.[85] Despite these potential interactions, our data can also be interpreted by focusing on the role of LacCer in cell migration.

The asialo-glycolipid, LacCer, is well known as a regulator of cell migration.[86, 87] Our results are in agreement with previous findings that the addition of LacCer to cells increased rates of migration.[28] It is worth noting that GM3 is a substrate for both Neu3 and Neu4, and processing by these enzymes would generate LacCer as a product. Increased amounts of LacCer in the membrane would be expected to induce clustering of  $\beta$ 1 integrins.[87] Furthermore, LacCer induces  $\beta$ 1 integrin conformational change to an activated form, priming it for ligand engagement. LacCer increases Caveolin-1 (Cav-1) mediated endocytosis of integrins, and the glycolipid is internalized along with the receptor.[88] The interaction of LacCer with integrins is further supported by the observation that Neu3 associates with Cav1 and integrins.[33, 89]

We propose that the regulation of integrin-mediated cell migration by glycolipids can be understood with a model composed of a series of coupled equilibria as follows (**Figure 6**). Glycosphingolipids act as negative or positive regulators through binding interactions or the formation of membrane microdomains. Binding interactions can negatively regulate integrins by blocking adhesion (Figure 6a), or by shunting co-receptors via complex formation (Figure 6b). Remodeling of membrane glycosphingolipids by Neu may disrupt receptor-ganglioside complexes and allow for the formation of microdomains enriched in asialoglycolipids (Figure 6c). These domains may encourage the formation of complexes that enhance integrin adhesion, and trigger integrin conformational change. The activated integrin within the microdomain will have one of two fates: it will be endocytosed (Figure 6d), or it will engage and bind with its extracellular ligand (Figure 6e). Endocytosis will provide for recycling of the un-engaged integrin to another location on the cell, or else degradation of the integrin after trafficking to the endosome (Figure 6f). Alternatively, engaged integrin-ligand complexes will become anchored to the cytoskeleton in order to strengthen the adhesion.[90] In effect, this model increases the ability of the integrin to sample the cell surface and localizes important co-receptors within a domain. This model is also consistent with the localization of Neu3 to membrane ruffles.[91]

Our finding that Neu3 has opposite effects on migration depending upon

cell type likely indicates that Neu can play different roles depending on the tissue it is expressed in. Previous studies have found that the same change in glycosylation of integrin co-receptors can have opposing effects depending on the integrin substrate (FN vs LN).[92] Additionally, Neu3 overexpression has been found to have no effect on cell migration in lung endothelia,[51] and to have differential effects on adhesion depending on the substrate.[33] Thus, it is not unexpected that the same Neu may have different effects on cell migration based on cell type, substrate or enzyme concentration, or other factors.

#### **4. Conclusion**

The remarkable influence of sialic-acid containing glycoconjugates on the function of adhesion receptors like the integrins may suggest that strategies to interfere with the distribution of native sialic acid content in the host could have unexpected impacts.[93] Indeed, treatment of endothelial cells with pfNeu is known to degrade the integrity of microvasculature, and disruption of adhesion receptor-ECM interactions is likely involved.[94] Thus, a more complete understanding of the specific substrates and activities of Neu will be necessary to advance potential therapeutics into clinical use.

Our findings strongly suggest that the targeting of human Neu isoenzymes with small molecule inhibitors should be more fully investigated for therapeutic applications. Although few isoenzyme-specific inhibitors of Neu are currently

known, work from several groups has begun to identify more potent and selective inhibitors.[36] We were able to show that a potent Neu4 inhibitor was able to significantly suppress cell migration. Unfortunately, Neu3-specific inhibitors of similar potency are not currently available. It should be noted that Neu3 and Neu4 have similar substrate activities, and may therefore show compensation in the presence of specific inhibitors.[81] Additional experiments will likely be needed to resolve the respective roles in glycoprotein or glycolipid targets of Neu3 and Neu4 enzymes. Future work should examine the role of native Neu activity in different cell types and their influence on different adhesion receptors or integrin-substrate pairs.

## **5. Materials and Methods**

### *5.1. Reagents*

Purified gangliosides LacCer, GM1 and GM3 were purchased from Avanti Polar lipids, Inc. (USA). Gangliosides GD1a, GD1b, and GT1b were obtained from Santa Cruz Biotechnology (USA). The Cy5-NHS ester was obtained from GE Healthcare (USA). An anti-CD49e ( $\alpha 5$  integrin, clone SAM-1) antibody was purchased from Santa Cruz Biotechnology (USA). The anti-CD49d ( $\alpha 4$  integrin, clone BU49) antibody was purchased from ThermoScientific. Fluorescent conjugates of anti-CD49e or anti-CD49d were generated by reaction with the Cy5-NHS ester, and purified by gel filtration chromatography using the manufacturer's protocol. Cholera toxin B (CTB) from *Vibrio cholera* was used as either a FITC-labeled conjugate obtained from Sigma Aldrich (Milwaukee, WI, USA), or a TRITC-labeled conjugate obtained from List Biological Laboratories, Inc. (USA).

Thymoquinone (TQ) was obtained from Sigma Aldrich. C9-4HMT-DANA, 9-[4-hydroxymethyl-[1,2,3]triazol-1-yl]-2,3-didehydro-N-acetyl-neuraminic acid, was prepared as previously described.[40] The *Clostridium perfringens* neuraminidase, was obtained from SigmaAldrich and had a molecular weight most consistent with the NanI isoform (Figure S8).[95] The enzyme is referred to as pfNeu throughout. The human Neu3, Neu3(Y370F), and Neu4 enzymes were produced recombinantly and purified as previously described.[45, 46] Toxicity assays were performed using CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, USA).

### 5.2. Cell lines

The HeLa cell line (cervical epithelial) used was a generous gift of Dr. R.E. Campbell (University of Alberta). The A549 cell line (lung epithelial) was obtained from ATCC (Manassus, VA, USA). All cells were cultured in DMEM (Gibco, Invitrogen, USA) containing 10% Fetal Bovine Serum (Hyclone, Thermo, USA) and penicillin/streptomycin (Gibco, Invitrogen, USA).

### 5.3. Cell migration assays and data analysis.

Plates (12-well) were pre-treated with 10  $\mu\text{g mL}^{-1}$  of FN for 2 hours at 37 °C or 4 °C overnight, and then blocked with 0.2 mg  $\text{mL}^{-1}$  of BSA in PBS buffer for 1 hour at 37 °C. Cells were counted and seeded into wells (HeLa, 4 x 10<sup>5</sup> cells  $\text{mL}^{-1}$ ; A549, 5 x 10<sup>5</sup> cells  $\text{mL}^{-1}$ , 1 mL for each well) with fresh medium (1 mL). Plates were incubated overnight at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. Cells were treated with the indicated condition, and the cell monolayer was carefully scratched with a gel-loading pipette tip (200  $\mu\text{L}$ ). Exogenous GSL samples were prepared by dissolution in PBS at 100X concentration. The stock solution of GSL was then diluted into fresh cell media (1 mL, DMEM, 10% FBS) already in the plate wells to give a final concentration of GSL at 10 ng  $\text{mL}^{-1}$ . Images of the wound were collected once every hour over 3 hours (6 fields along the scratch were acquired at each time point per well), and each condition was run in four separate wells. Image

analysis software (NIS-Elements v3.5, Nikon USA) was used to measure the distance between the cell front at each side of the scratch, and fit using linear regression according to  $y = ax + b$ , where  $a$  is the rate of migration, and  $b$  is the width of the wound at  $t = 0$ . To assure that all enzyme samples used were of the same activity, we calibrated the activity of individual samples using a fluorogenic assay based on the 4MU-NANA substrate [46]. Since our experiments were to be conducted in cell culture at physiological pH, we conducted the assay at pH 7. All Neu samples were used at an activity of 500  $\mu$ U per sample at pH = 7. In the case of Neu3(Y370F), the protein concentration was matched to the Neu3 sample at 1 RU. Cells treated with Neu4 were observed over 1 hr, due to instability of the enzyme and compared to the 1 hr control for statistical analysis.

#### *5.4. Cell toxicity*

Toxicity of migration conditions to cells were tested using an MTS assay using the manufacturer's protocol (Promega). Briefly, a 96-well plate was loaded with  $5 \times 10^3$  cells per well, and buffer containing the appropriate condition to be tested. Cells were incubated for 48 hours and then 20  $\mu$ L of the MTS solution was added to each well and incubated for another 2 hours. The absorbance in each well was measured using a plate reader at 490 nm.

#### *5.5. Lipid extraction and thin layer chromatography*

Cells were treated for 3 hours using the indicated conditions following the migration assay protocol. At the end of the incubation, cells were harvested with a cell scraper. Cells were suspended in PBS and washed 2 times. After the addition of 5 volumes of  $\text{CHCl}_3/\text{MeOH}$  (2:1), the solution was sonicated for 5 min and left to stand overnight at room temperature. The sample was centrifuged to remove cellular debris, dried under a stream of  $\text{N}_2$  and then dissolved in  $\text{CHCl}_3/\text{MeOH}$  (2:1). The resulting solution was desalted using a  $\text{C}_{18}$  column, and analyzed by HPTLC with  $\text{AcOH}/n\text{-BuOH}/0.5\%$  aqueous  $\text{CaCl}_2$  (1:2:1) or  $\text{CHCl}_3/\text{MeOH}/0.25\%$  aqueous

KCl (60:40:8) as eluent. Lipids were then stained by an orcinol-H<sub>2</sub>SO<sub>4</sub>-EtOH solution and heated for 30 min at 100 °C. The bands were analyzed by densitometry using ImageJ [96] with four separate plates and the ratio of LacCer/GM3 was calculated and then normalized to control conditions. For substrate studies, gangliosides GM1, GM3, GD1a, GD1b, and GT1b were dissolved in sodium bicarbonate buffer (200 µL, pH 4.7, at 0.25 mg mL<sup>-1</sup> final concentration). The indicated neuraminidase enzyme was then added to the solution (0.1 mg mL<sup>-1</sup> final concentration) and incubated at 37 °C for 1 hour. The mixture was then analyzed by HPTLC as described above.

### *5.7. Immunofluorescence imaging*

Cells were cultured in a FN-treated confocal dish overnight at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. Cells were washed with PBS before labeling. HeLa cells were labeled with Cy5-anti-CD49e (50 ng) and FITC-CTB (5 µg) for 10 min, and then washed again with PBS. Live cells were imaged by epifluorescence (CTB) and total-internal reflection fluorescence (TIRF) microscopy (integrin) on a Nikon ECLIPSE *Ti* microscope system. Images were processed with NIS-Elements v3.5 (Nikon, USA).

### *5.8. Single-Particle Tracking*

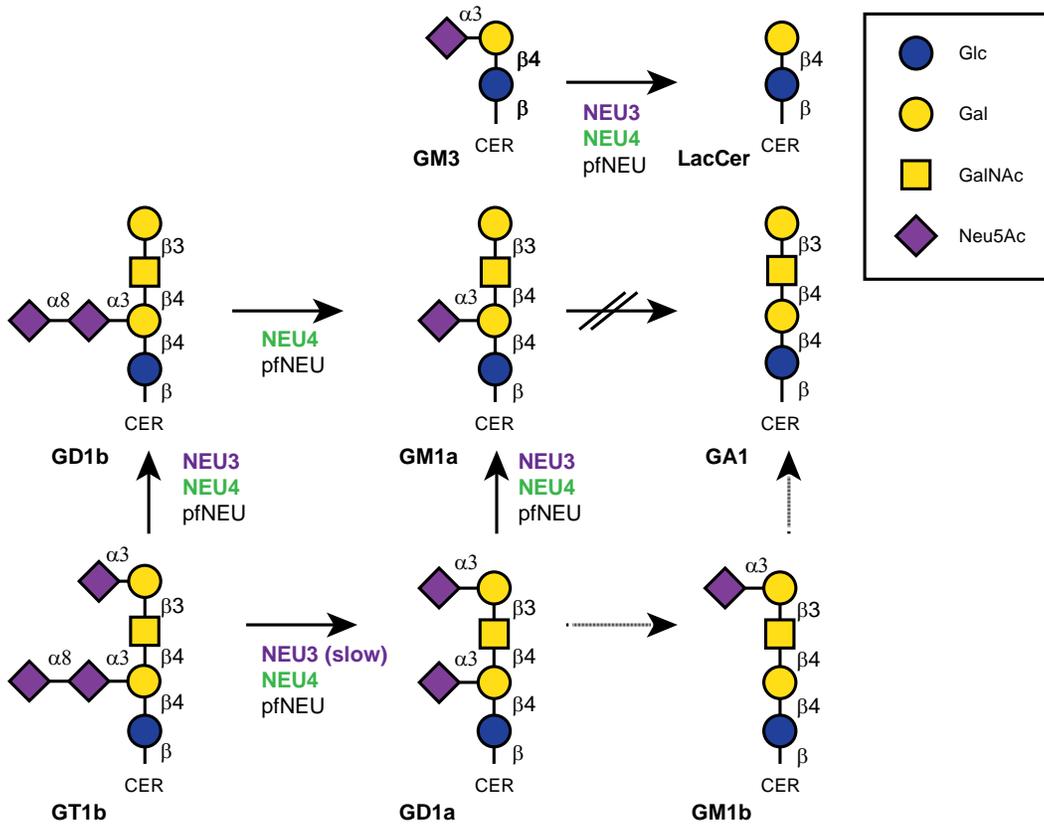
HeLa cells were cultured in a FN-treated confocal dish overnight at 37 °C in a humidified incubator with 5% CO<sub>2</sub>, and then washed with PBS before incubation with the indicated condition for 3 hrs. Cells were washed with PBS twice, followed by addition of Cy5-anti-CD49e (50 ng) for 10 min at room temperature. Cells were washed twice with PBS to remove excess antibody. Video images of labeled cells were taken in TIRF with a 60x objective (NA = 1.4) and 1.5x amplification on a Nikon ECLIPSE *Ti* microscope system. Data were processed using and NIS-Elements v3.5 (Nikon, USA). Single-particle trajectories were analyzed using U-Track [67], and the resulting data was processed using a custom software package

developed in MATLAB (Mathworks, Framingham MA, USA) [69] to calculate the microdiffusion coefficient of each trajectory [68].

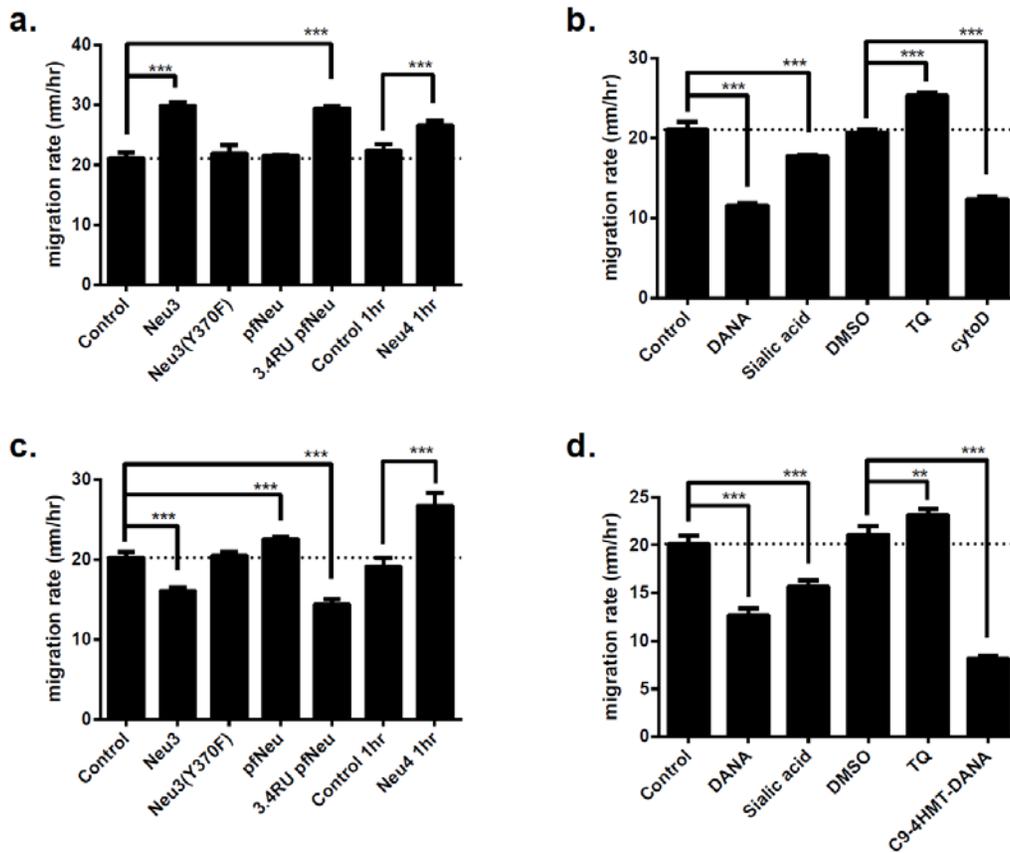
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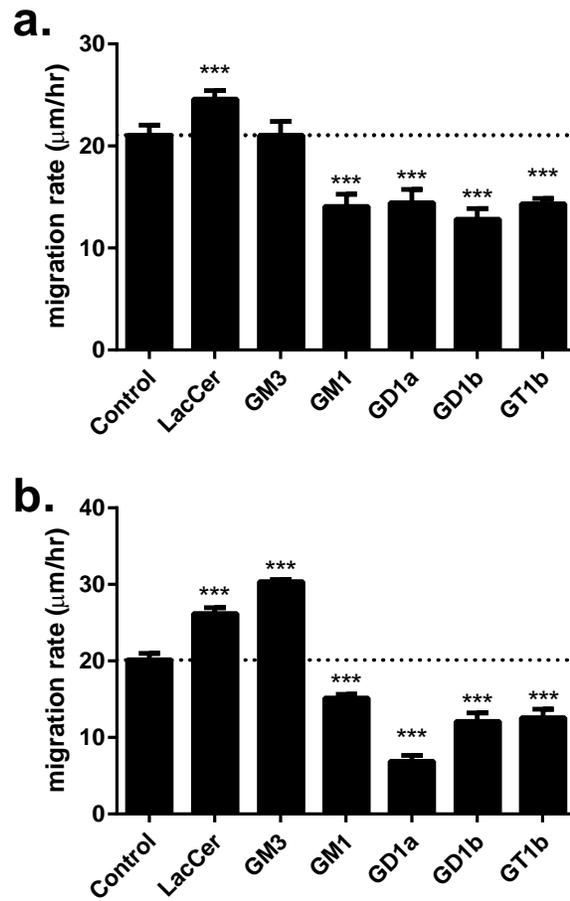
## Figures and Tables



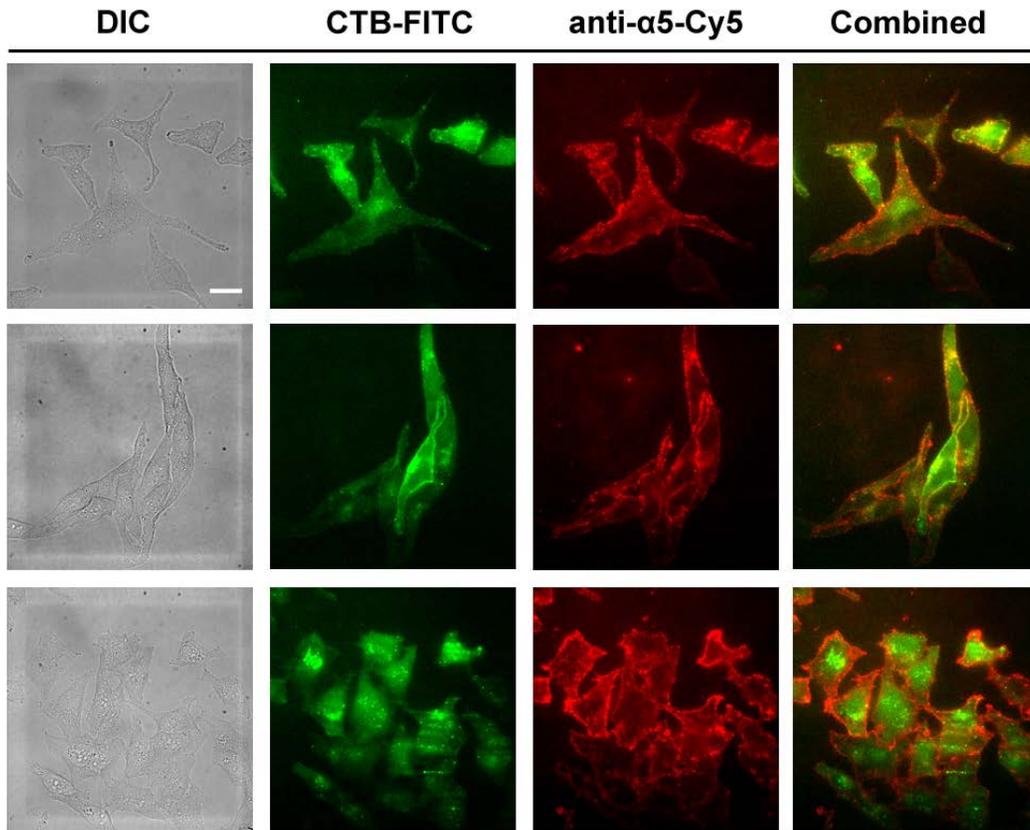
**Figure 1: Summary of the observed substrate activity of Neu3, Neu4 and pfNeu for ganglioside substrates.** Glycosphingolipid structures are shown using a symbolic representation, with the stereochemistry of linkages indicated. Solid arrows indicate conversion by the enzymes listed, dashed arrows indicate that the conversion was not tested, if the reaction was tested but no product was observed the arrow has a double hash. See Supporting Information for TLC data supporting the conclusions shown.



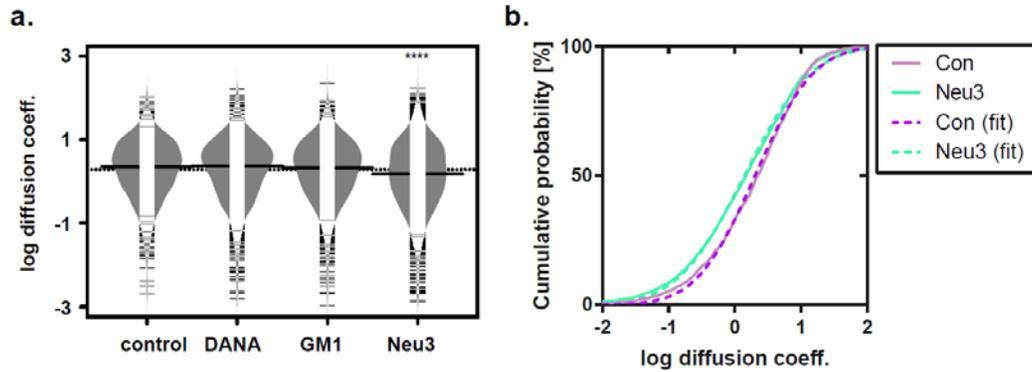
**Figure 2: Cell migration of HeLa and A549.** The rates of migration of **a.** HeLa and **c.** A549 monolayers to treatment with neuraminidase enzymes or drugs (**b.** and **d.**, respectively) was determined. Cells were prepared as described in Materials & Methods, conditions are as indicated in PBS. The concentration of compounds used were: DANA, 100  $\mu\text{M}$  and sialic acid, 100  $\mu\text{M}$ . Compounds with reduced solubility were compared to a vehicle control of PBS containing 0.1% DMSO with concentrations of cytochalasin D (cytoD), 25  $\text{ng mL}^{-1}$ ; thymoquinone (TQ), 10  $\mu\text{M}$ ; and C9-4HMT-DANA, 0.5  $\mu\text{M}$ [40]. Each condition was repeated 3 times. Data were compared to the indicated controls using a t test; \*\*,  $p < 0.005$ ; \*\*\*,  $p < 0.001$ . Error is shown as the standard deviation.



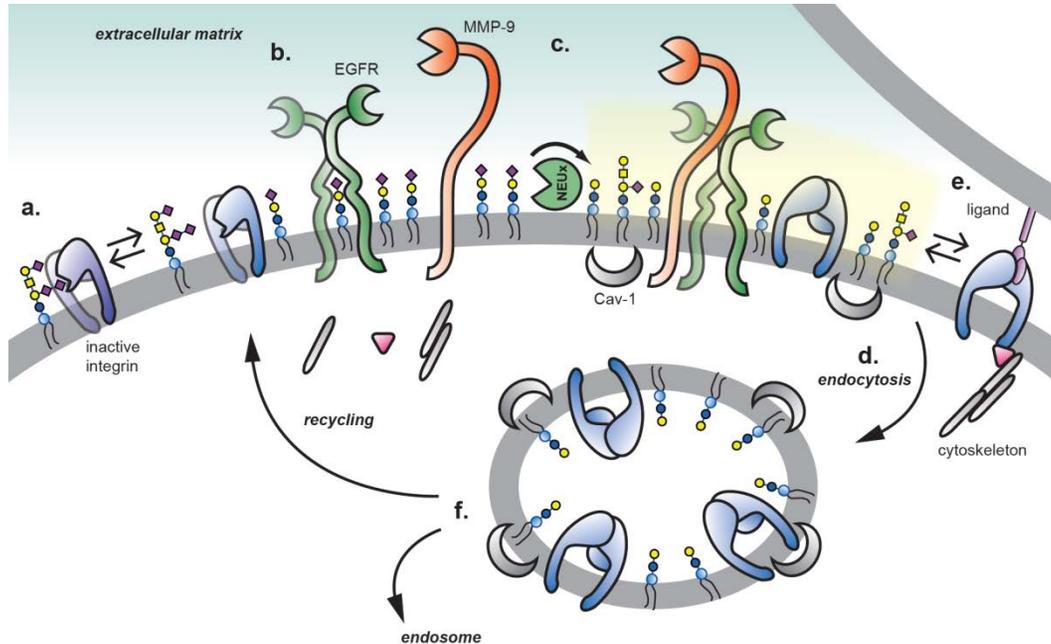
**Figure 3: Cell migration of HeLa and A549 treated with glycolipids.** Monolayers of **a.** HeLa or **b.** A549 cells were prepared as described in Materials & Methods, and treated with PBS containing the indicated glycolipid (final concentration  $10 \text{ ng mL}^{-1}$ ). Each condition was repeated 3 times. Treatments were compared to control (PBS only) using a t test; \*\*\* =  $p < 0.001$ . Error is shown as the standard deviation.



**Figure 4: HeLa cells labeled with CTB-FITC and Cy5-anti- $\alpha 5$  antibody.** HeLa cells were labeled with anti- $\alpha 5$  integrin (red) and FITC-conjugated cholera toxin B (CTB-FITC, green). Images are shown for transmitted (DIC) and TIRF (FITC, TRITC). Scale bar = 25  $\mu\text{m}$ .



**Figure 5: Diffusion of integrins is altered by Neu3 treatment.** Diffusion of integrin receptors was determined using SDT. **a.** Diffusion coefficients of individual trajectories are shown for each condition (see Materials & Methods). Data are the same as those summarized in Table 1. **b.** Comparison of integrin diffusion coefficients using a cumulative probability distribution (CPD). Data are the same as those summarized in Table 1. Only control and Neu3 conditions are shown for clarity. Log transformed data were used to generate a cumulative frequency distribution with GraphPad Prism, and then fit to a cumulative Gaussian distribution. Diffusion coefficients are in log(10) transformed units of  $\times 10^{-10}$  [ $\text{cm}^2\text{s}^{-1}$ ] or  $\times 10^{-2}$  [ $\mu\text{m}^2\text{s}^{-1}$ ].



**Figure 6: Model of glycolipid interactions with membrane components.**

Glycolipids engage in a variety of interactions in the membrane which modulate receptor function. **a.** Sialylated glycolipids, such as GT1b, may bind to the  $\alpha 5 \beta 1$  integrin and block it from binding to ligand. **b.** Co-receptors, such as EGFR, can also engage with sialylated lipids (GM3), which prevent interaction with other proteins which regulate adhesion (MMP-9). **c.** Ganglioside-modifying Neu enzymes (Neu3 and Neu4) can remodel the ganglioside composition. Desialylated lipids (LacCer, GM1) can become enriched in membrane microdomains. Domains allow for the adoption of an active conformation of the integrin receptor and recruitment of co-receptors. **d.** Activated integrin may be endocytosed through a Cav-1 mechanism. **e.** However, if the integrin becomes engaged with an appropriate ligand, it can recruit cytoskeletal-binding proteins which will stabilize the adhesion. **f.** Endocytosed integrin may be recycled to the cell surface, or else be directed to the endosome.

**Table 1. Diffusion of  $\alpha 5\beta 1$  on HeLa cells**

		$D_{\text{micro}}^{\dagger}$			
Condition	N	mean (linear)	median (linear)	mean (log transformed)	median $\ddagger$ (log normal)
Control	1027	$5.2 \pm 0.3$	2.2	$1.9 \pm 1.1$	$1.9 \pm 0.2$
DANA	1249	$5.6 \pm 0.3$	2.3	$1.8 \pm 1.1$	$1.8 \pm 0.2$
GM1	1586	$5.1 \pm 0.3$	2.2	$1.8 \pm 1.0$	$1.8 \pm 0.2$
Neu3	2493	$5.1 \pm 0.2$	1.5	$1.3 \pm 1.0^{****}$	$1.3 \pm 0.1$

Values listed are either the arithmetic mean, arithmetic median, mean of log transformed data, or the median determined for a log normal distribution (see Supporting information.) Error is given as the standard error of the mean.  $\dagger$ , Diffusion coefficients are in units of  $\times 10^{-10}$  [ $\text{cm}^2\text{s}^{-1}$ ] or  $\times 10^{-2}$  [ $\mu\text{m}^2\text{s}^{-1}$ ]; Data were compared to control to determine p values; \*\*\*\*,  $p < 0.0001$ .  $\ddagger$  Median calculated based on a lognormal fit as described in supporting information.

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