



# Combined sialic acid and histone deacetylase (HDAC) inhibitor treatment up-regulates the neuroblastoma antigen GD2

Received for publication, March 6, 2018, and in revised form, January 14, 2019. Published, Papers in Press, January 22, 2019, DOI 10.1074/jbc.RA118.002763

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Edited by Luke O'Neill

Neuroblastoma cells highly express the disialoganglioside GD2, a tumor-associated carbohydrate antigen, which is only sparsely expressed on healthy tissue. GD2 is a primary target for the development of immunotherapy for neuroblastoma. Immunotherapy with monoclonal anti-GD2 antibodies has proven safety and efficacy in clinical trials and is included in the standard treatment for children with high-risk neuroblastoma. Strategies to modulate GD2 expression in neuroblastoma could further improve anti-GD2-targeted immunotherapy. Here, we report that the cellular sialylation pathway, as well as epigenetic reprogramming, strongly modulates GD2 expression in human and mouse neuroblastoma cell lines. Recognition of GD2 by the 14G2a antibody is sialic acid-dependent and was blocked with the fluorinated sialic acid mimetic Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac. Interestingly, sialic acid supplementation using a cell-permeable sialic acid analogue (Ac<sub>5</sub>Neu5Ac) boosted GD2 expression without or with minor alterations in overall cell surface sialylation. Furthermore, sialic acid supplementation with Ac<sub>5</sub>Neu5Ac combined with various histone deacetylase (HDAC) inhibitors, including vorinostat, enhanced GD2 expression in neuroblastoma cells beyond their individual effects. Mechanistic studies revealed that Ac<sub>5</sub>Neu5Ac supplementation increased intracellular CMP-Neu5Ac concentrations, thereby providing higher substrate levels for sialyltransferases. Furthermore, HDAC inhibitor treatment increased mRNA expression of the sialyltransferases GM3 synthase (ST3GAL5) and GD3 synthase (ST8SIA1), both of which are involved in GD2 biosynthesis. Our findings reveal that sialic acid analogues and HDAC inhibitors enhance GD2 expression and could potentially be employed to boost anti-GD2 targeted immunotherapy in neuroblastoma patients.

Neuroblastoma is a childhood malignancy that originates in the sympathetic nervous system and is responsible for 12% of cancer-related deaths in children younger than 15 years (1). Although, the prognosis of high-risk neuroblastoma patients has improved over the last decades, their long-term survival still remains poor despite the intensive multimodal treatment (2). All neuroblastomas express high levels of GD2, a ganglioside composed of a ceramide anchored in the outer leaflet of the plasma membrane and a glycan moiety extending into the extracellular milieu. The glycan consists of glucose, galactose, GalNAc, and two sialic acid residues (Neu5Ac) that branch from the galactose residue (Fig. 1A). GD2 is produced from its precursor gangliosides GM3 and GD3 by sequential activity of the glycosyltransferases GM3 synthase (ST3GAL5), GD3 synthase (ST8SIA1) and GD2 synthase (B4GALNT1), respectively (Fig. 1A) (3). GD2 is homogeneously and highly expressed by neuroblastoma cells, whereas in healthy tissues it is expressed only by neurons, skin melanocytes, and peripheral pain fibers (4–6). This preferential expression on neuroblastoma cells makes GD2 a key target for the development of immunotherapy for neuroblastoma, ranked 12th of 75 of all important tumor antigens identified by the National Cancer Institute (7).

Over the past 3 decades, GD2 has been used as the primary target for the development of immunotherapeutic monoclonal antibodies. Monoclonal anti-GD2 antibodies effectively mediate the lysis of neuroblastoma cells via antibody-dependent cell-mediated cytotoxicity (ADCC)<sup>2</sup> involving natural killer cells and granulocytes as well as complement-dependent cytotoxicity (6, 8–12). Anti-GD2 antibodies, e.g. dinutuximab, proved safe and efficacious in clinical trials and are therefore included in the routine treatment of high-risk neuroblastoma (5, 13–18). More recently, GD2 has also been explored as a

This work was supported by Grant KUN2015-7604 (to G. J. A., T. J. B., and C. B.) from the KWF Kankerbestrijding (Dutch Cancer Society) and by continuous financial support from the Villa Joep Foundation (to P. M. H. and G. J. A.) and Stichting Vrienden KOC Nijmegen (to P. M. H. and G. J. A.). The authors declare no potential conflicts of interest.

This article contains Figs. S1–S4.

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<sup>2</sup> The abbreviations used are: ADCC, antibody-dependent cell-mediated cytotoxicity; CAR, chimeric antigen receptor; HDAC, histone deacetylase; PNA, peanut agglutinin; ECL, *Erythrina crista-galli* lectin; SNA-I, *Sambucus nigra* I lectin; MALII, *Maackia amurensis* lectin II; GSL-I, *Griffonia simplicifolia* I; PHA-L, *Phaseolus vulgaris* L; LCA, *Lens culinaris*; PE, phycoerythrin; FBS, fetal bovine serum; MEM, minimum essential medium.

## Boosting GD2 expression in neuroblastoma

target for T-cell immunotherapy by incorporating the antibody specificity into chimeric antigen receptor (CAR) T cells. In a small patient cohort, GD2-specific CAR-T cell administration was well-tolerated and was associated with tumor regression and necrosis in half of the patients (19). The long-term follow-up showed low-level persistence of CAR T cells, which was associated with clinical benefit, including three complete responses (20). Based on these encouraging results, several clinical phase I trials are currently testing third- and fourth-generation GD2-specific CAR T cells, including combinations with immune checkpoint-blocking antibodies (21, 22). Next to monoclonal antibodies and CAR T cells, GD2 is also a potential target for carbohydrate-based neuroblastoma vaccines (23, 24).

Despite these advances in neuroblastoma immunotherapy, still around half of the patients eventually show progressive disease (25). Combining immunotherapy with other tumor-targeting therapies could further improve the treatment of neuroblastoma. We have recently reported that histone deacetylase (HDAC) inhibitors could be successfully applied together with anti-GD2 antibody as immune-combination therapy in a pre-clinical model (26, 27). The HDAC family controls gene expression at the epigenetic level by removing acetyl groups from histones and from nonhistone proteins (28). HDAC inhibitors are emerging as potent anticancer drugs that induce cell cycle arrest and differentiation in neuroblastoma and other cancer types (29, 30). Using a murine neuroblastoma model resembling the immunobiology of human neuroblastoma, our group recently reported that the pan-HDAC inhibitor vorinostat synergized with anti-GD2 mAb therapy in reducing neuroblastoma tumor growth (27). Vorinostat created a more immunopermissive tumor microenvironment, but it also enhanced GD2 expression on neuroblastoma cells by increasing GD2 synthase (B4GALNT1) protein but not mRNA levels.

Here, we report that the fluorinated sialic acid analogue  $Ac_53F_{ax}Neu5Ac$  potentially blocked GD2 expression, whereas the cell-permeable, acetylated sialic acid  $Ac_5Neu5Ac$  boosted GD2 expression on neuroblastoma cells. In view of the total cellular sialylation pathway, the GD2 biosynthesis pathway in neuroblastoma cells appeared highly sensitive to the effects of the sialic acid analogues. Moreover, we found that sialic acid supplementation combined with various HDAC inhibitors strongly increased GD2 expression. As a result of  $Ac_5Neu5Ac$  addition, intracellular CMP-Neu5Ac levels, the substrate for sialyltransferases, increased strongly. In addition, HDAC inhibitor treatment increased the expression of sialyltransferases involved in GD2 biosynthesis, thereby providing mechanistic insights into the strong combination effect of  $Ac_5Neu5Ac$  and HDAC inhibitors. In conclusion, this study provides a rationale for boosting GD2-targeted neuroblastoma immunotherapy through sialic acid engineering and/or HDAC inhibition.

## Results

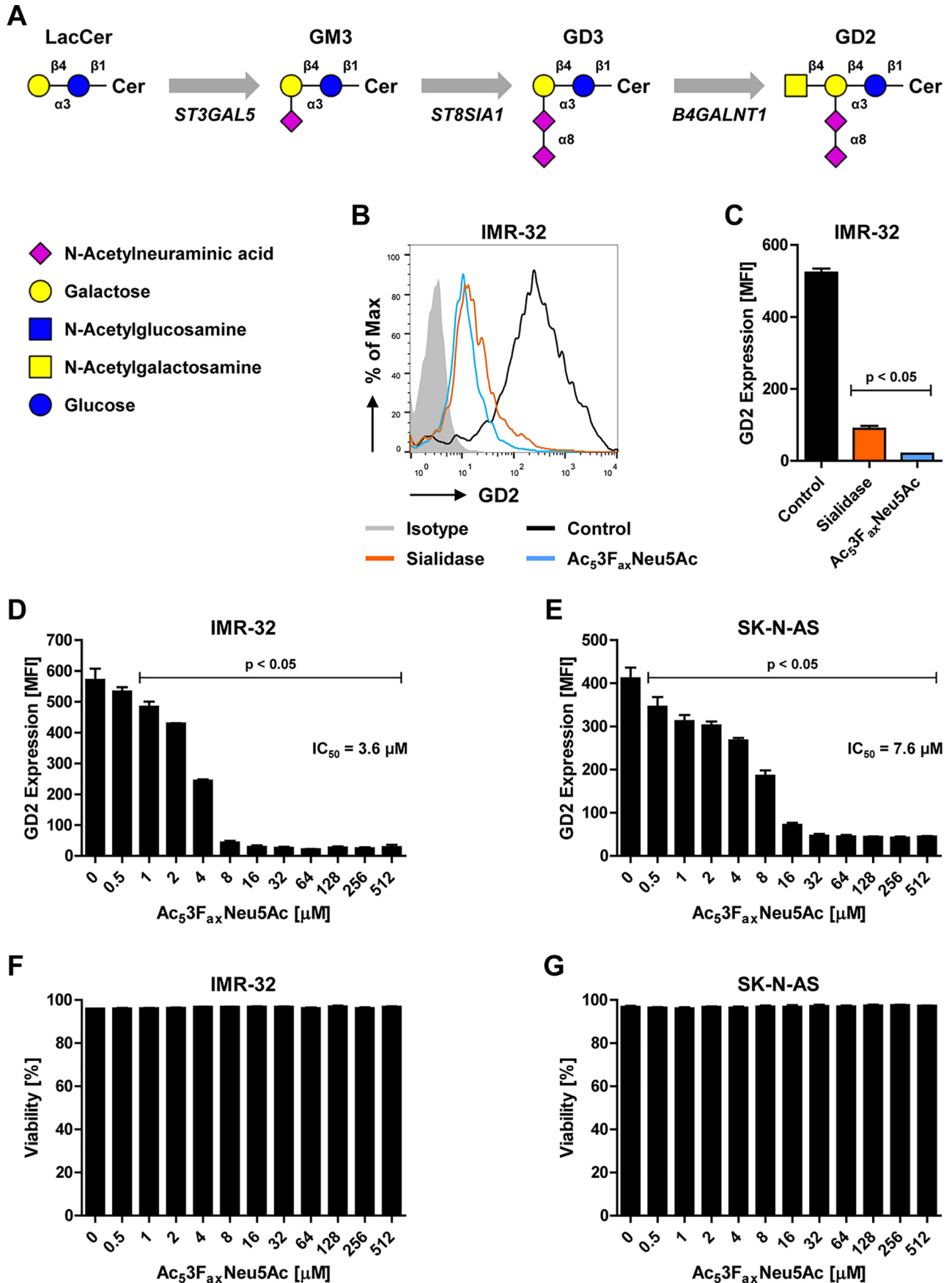
### *Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac blocks GD2 expression in neuroblastoma cell lines*

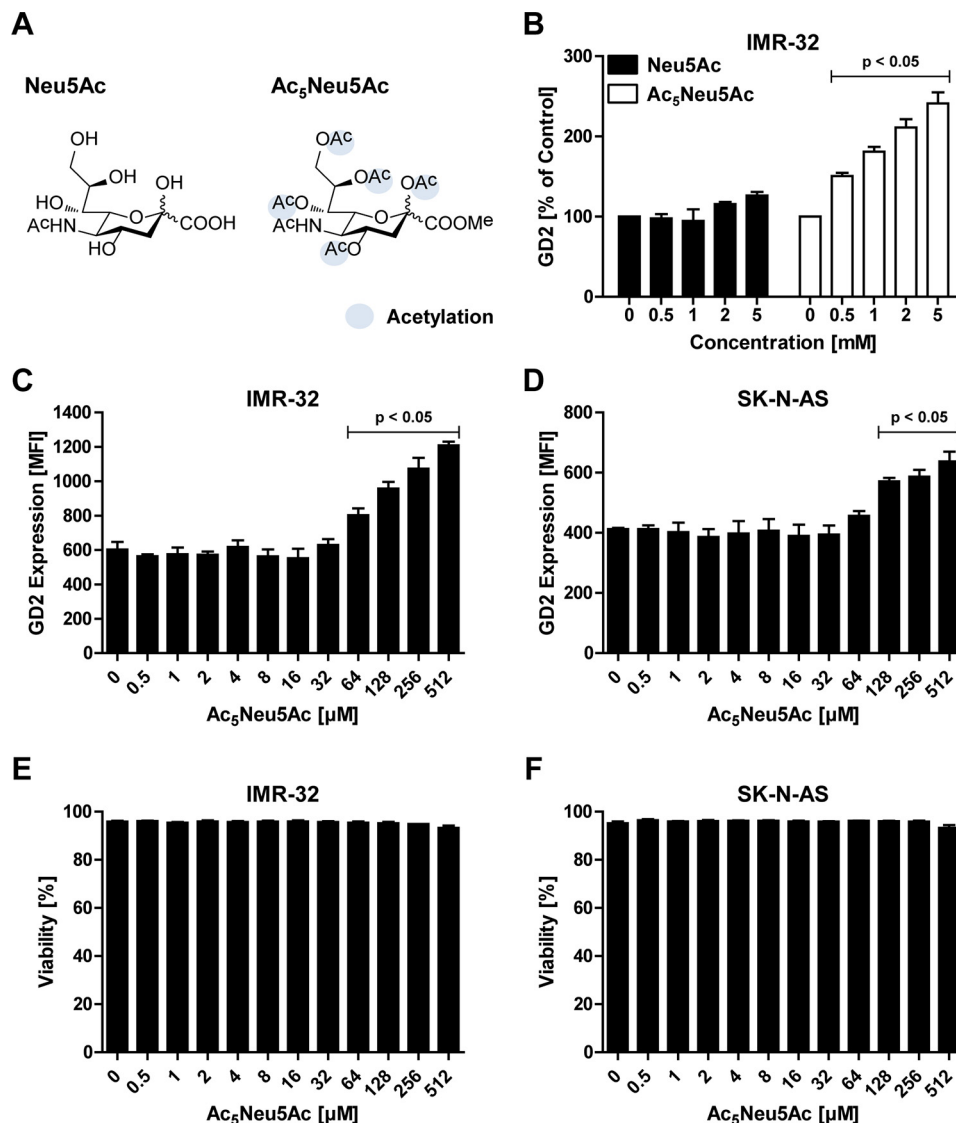
The tumor antigen GD2 carries two sialic acid residues, one that is linked via an  $\alpha 2,3$  linkage to galactose and a second one that is linked to the sialic acid via an  $\alpha 2,8$  linkage (Fig. 1A).

These linkages are produced by GM3 synthase (ST3GAL5) and GD3 synthase (ST8SIA1), two sialyltransferases that transfer sialic acids to the GD2 precursors GM3 and GD3, respectively (Fig. 1A). The availability of GM3 and GD3 is therefore rate-limiting for GD2 synthase activity and the production of GD2 (3, 31). We and others have shown previously that a cell-permeable fluorinated sialic acid analogue,  $Ac_53F_{ax}Neu5Ac$ , inhibits sialyltransferases and blocks  $\alpha 2,3$  and  $\alpha 2,6$  sialylation in human cells (32–35). Here we investigated whether  $Ac_53F_{ax}Neu5Ac$  is capable of blocking the production of the disialoganglioside GD2 in human neuroblastoma cell lines. Human IMR-32 cells were treated with the sialic acid inhibitor  $Ac_53F_{ax}Neu5Ac$  (100  $\mu M$ ) for 3 days or with *Clostridium perfringens* sialidase for 1 h after which membrane GD2 expression was measured by flow cytometry using the anti-GD2 antibody 14G2a. Both enzymatic removal of sialic acid and metabolic sialic acid blockade with  $Ac_53F_{ax}Neu5Ac$  almost completely abolished GD2 staining on IMR-32 cells (Fig. 1, B and C). To determine the effective dose of the sialic acid inhibitor, increasing concentrations of  $Ac_53F_{ax}Neu5Ac$  were added to IMR-32 and SK-N-AS cells, and the expression of GD2 was analyzed. GD2 expression in both cell lines was reduced in a dose-dependent manner with an  $IC_{50}$  of 3.6  $\mu M$  for IMR-32 cells and 7.6  $\mu M$  for SK-N-AS cells (Fig. 1, D and E). Higher concentrations, around 8  $\mu M$  for IMR-32 and 32  $\mu M$  for SK-N-AS, reduced GD2 expression by more than 90%. Importantly, at all concentrations used, the fluorinated sialic acid showed no effect on cell viability in either of the cell lines (Fig. 1, F and G). These results confirm that GD2 recognition by the anti-GD2 antibody 14G2a depends on sialic acid residues and identify  $Ac_53F_{ax}Neu5Ac$  as a potent metabolic inhibitor of GD2 expression.

### *Cell-permeable sialic acid enhances GD2 expression*

Having shown that GD2 expression can be blocked with the metabolic inhibitor of sialylation  $Ac_53F_{ax}Neu5Ac$ , we next investigated whether GD2 expression can be influenced by supplementing the sialylation pathway with exogenous sialic acid. Physiologically, sialic acids are poorly cell-permeable because of their negative charge and hydrophilic properties. In addition, mammalian cells lack an active uptake system for sialic acids (36). To overcome this obstacle, ester-linked acetyl groups were added to the sialic acid sugar, yielding a peracetylated sialic acid,  $Ac_5Neu5Ac$ , which is cell-permeable (Fig. 2A). Inside the cell, the acetyl groups are removed by cytoplasmic esterases and the sialic acid enters the sialylation pathway (37, 38). To test the effect of control Neu5Ac and  $Ac_5Neu5Ac$  on GD2 expression, IMR-32 cells were incubated for 3 days with increasing concentrations of the sialic acids and analyzed by flow cytometry. Although the addition of up to 5 mM Neu5Ac showed no significant effect on GD2 expression, peracetylated sialic acid  $Ac_5Neu5Ac$  significantly enhanced GD2 expression at concentrations of 0.5 mM and higher (Fig. 2B). Subsequently, the titration of  $Ac_5Neu5Ac$  into IMR-32 and SK-N-AS cell cultures confirmed that GD2 expression was increased in a dose-dependent manner without affecting cell viability (Fig. 2, C–F). In IMR-32 cells, the GD2 expression was doubled by adding  $Ac_5Neu5Ac$  concentrations above 128  $\mu M$ , and in SK-N-AS expression levels could be increased about 1.5 times. Similar results were obtained using *N*-acetylmannosamine (Man-





**Figure 2. Peracetylated sialic acid enhances GD2 expression.** *A*, structural representation of Neu5Ac (*left*) and the peracetylated form Ac<sub>5</sub>Neu5Ac (*right*). *B*, effect of sialic acid supplementation on GD2 expression of IMR-32 cells. The cells were cultured for 3 days with 0–5 mM Neu5Ac or Ac<sub>5</sub>Neu5Ac, and GD2 expression was assessed by flow cytometry. The *bar diagram* shows the mean percentage of GD2 expression ± S.E. of IMR-32 cells treated with Neu5Ac or Ac<sub>5</sub>Neu5Ac normalized to control (*n* = 3). *C* and *D*, IMR-32 and SK-N-AS cells were cultured for 3 days with 0–512 μM Ac<sub>5</sub>Neu5Ac and stained with anti-GD2 antibody. *Bar diagrams* show GD2 expression of IMR-32 cells (*C*) and SK-N-AS cells (*D*) as mean fluorescence intensity ± S.E. of three independent experiments. *E* and *F*, effect of Ac<sub>5</sub>Neu5Ac on cell viability. *Bar diagrams* show mean percentage of viable IMR-32 cells (*E*) and SK-N-AS cells (*F*) ± S.E. in culture after treatment with 0–512 μM Ac<sub>5</sub>Neu5Ac for 3 days (*n* = 3).

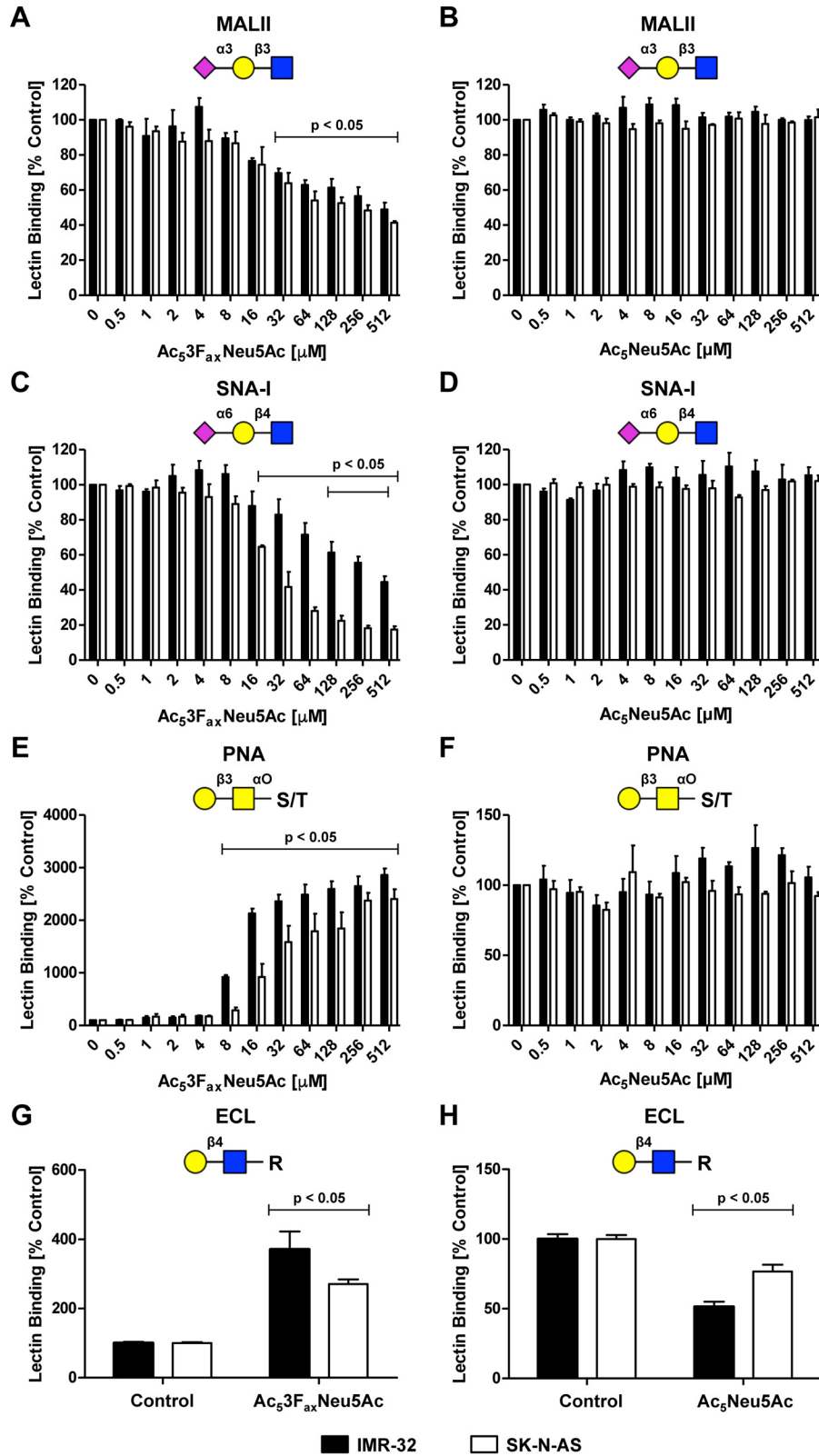
NAc) supplementation, the biological precursor of sialic acid (Fig. S1). Altogether, these results indicate that increased intracellular sialic acid availability leads to higher GD2 expression in neuroblastoma cells.

**GD2 expression is sensitive to sialic acid modulation relative to overall sialylation**

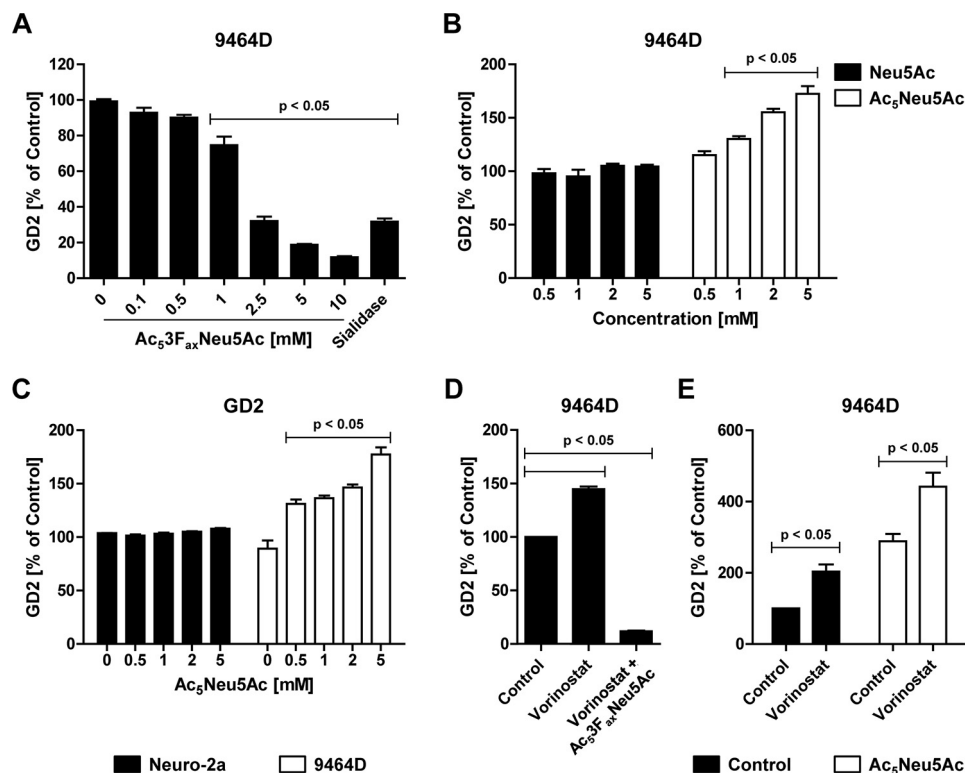
To determine the effect of sialic acid analogues on total cell surface sialylation, neuroblastoma cells were incubated for 3 days with increasing concentrations of the sialic acid inhibitor

Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac or supplemented with cell-permeable Ac<sub>5</sub>Neu5Ac. Cell surface sialylation was analyzed by flow cytometry using carbohydrate-binding lectins. IMR-32 and SK-N-AS cells expressed high levels of α2,3-linked and α2,6-linked sialic acids as detected with the lectins MALII and SNA-I, respectively, and uncapped glycans were barely detected using PNA lectin (Fig. S2). The sialic acid inhibitor Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac reduced α2,3 and α2,6 sialylation in a dose-dependent manner in both cell lines (Fig. 3, *A* and *C*). Consistent with decreased sialic acid expression, exposure of terminal β1–3– and β1–4–

**Figure 1. Recognition of GD2 by the 14G2a antibody is sialic acid dependent.** *A*, schematic representation of the GD2 biosynthesis pathway. *B* and *C*, binding of the anti-GD2 antibody 14G2a to control or sialidase- or Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac-treated IMR-32 cells. *A* representative *histogram* shows GD2 expression as detected by flow cytometry (*B*), and the *bar diagram* shows GD2 expression as mean fluorescence intensity ± S.E. of three independent experiments (*C*). *D* and *E*, effect of metabolic sialic acid inhibition on GD2 expression. *Bar diagrams* show GD2 expression as mean fluorescence intensity ± S.E. on IMR-32 cells (*D*) and SK-N-AS cells (*E*) treated with 0–512 μM Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac for 3 days (*n* = 3). *F* and *G*, effect of Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac on cell viability. *Bar diagrams* show mean percentage of viable cells ± S.E. in the IMR-32 (*F*) and SK-N-AS (*G*) cultures after treatment with 0–512 μM Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac for 3 days (*n* = 3).



**Figure 3. Effect of sialic acid analogues on overall cell surface sialylation.** A–F, IMR-32 cells and SK-N-AS cells were cultured for 3 days with 0–512 μM Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac or Ac<sub>5</sub>Neu5Ac, and cell surface sialylation was assessed by flow cytometry using fluorescent lectins. The lectins recognize α2,3-linked sialic acids (MALII), α2,6-linked sialic acids (SNA-I), or terminal β-galactose (PNA), respectively. Bar diagrams show average binding ± S.E. normalized to control of MALII (A and B), SNA-I (C and D), or PNA (E and F) on IMR-32 cells and SK-N-AS cells treated with Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac or Ac<sub>5</sub>Neu5Ac (n = 3). G–H, IMR-32 and SK-N-AS cells were cultured for 3 days with Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac (32 μM) or Ac<sub>5</sub>Neu5Ac (2 mM). Cells were stained with the ECL lectin, which recognizes uncapped terminal Galβ1–4GlcNAc residues. Bar diagrams show average binding ± S.E. normalized to control of ECL on IMR-32 cells and SK-N-AS cells treated with Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac (G) or Ac<sub>5</sub>Neu5Ac (H) (n = 2).



**Figure 4. Sialic acid analogues and vorinostat modulate GD2 expression in murine 9464D cells.** *A*, effect of  $Ac_53F_{ax}Neu5Ac$  on GD2 expression in 9464D cells. 9464D cells were cultured for 3 days with 0–10 mM  $Ac_53F_{ax}Neu5Ac$  or treated for 1 h with *C. perfringens* sialidase, and GD2 expression was determined by flow cytometry. The bar diagram shows the mean percentage of GD2 expression  $\pm$  S.E. normalized to control ( $n = 3$ ). *B*, effect of sialic acid supplementation on GD2 expression of 9464D cells. The cells were cultured for 3 days with 0–5 mM Neu5Ac or  $Ac_5Neu5Ac$ , and GD2 expression was assessed by flow cytometry. The bar diagram shows the mean percentage of GD2 expression  $\pm$  S.E. of 9464D cells treated with Neu5Ac or  $Ac_5Neu5Ac$  normalized to control ( $n = 3$ ). *C*, Neuro-2a or 9464D cells were cultured for 3 days with 0–5 mM  $Ac_5Neu5Ac$  and stained with anti-GD2 antibody. Bar diagrams show the mean percentage of GD2 expression  $\pm$  S.E. ( $n = 3$ ). *D*,  $Ac_53F_{ax}Neu5Ac$  blocks vorinostat-mediated up-regulation of GD2. 9464D cells treated with 5 mM  $Ac_53F_{ax}Neu5Ac$ , and during the last 24 h of the culture 256 nM vorinostat was added. The bar diagram shows the mean percentage of GD2 expression  $\pm$  S.E. normalized to control ( $n = 3$ ). *E*,  $Ac_5Neu5Ac$  and vorinostat boost GD2 expression. 9464D cells were treated for 3 days with 2 mM  $Ac_5Neu5Ac$ . On the last 2 days of the culture, 256 nM vorinostat was added, and GD2 expression was assessed. The bar diagram shows the mean percentage of GD2 expression  $\pm$  S.E. normalized to control ( $n = 2$ ).

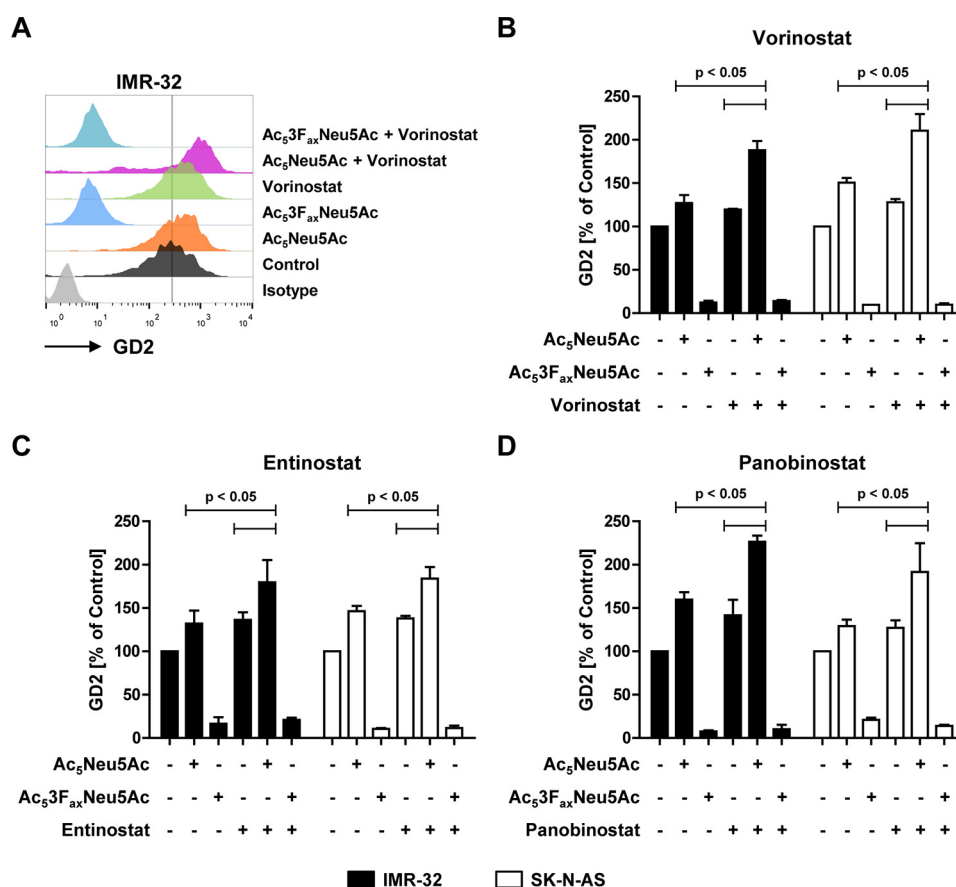
linked galactose residues increased as reflected by enhanced binding of the lectins PNA and ECL, respectively (Fig. 3, E and G). The maximum level of inhibition observed with 512  $\mu$ M  $Ac_53F_{ax}Neu5Ac$  was 50 and 60% for  $\alpha$ 2,3-linked sialic acids and 50 and 80% for  $\alpha$ 2,6-linked sialic in IMR-32 and SK-N-AS cells, respectively. In contrast, GD2 expression was almost completely lost at concentrations as low as 16  $\mu$ M, a concentration at which the overall cell surface sialylation was only slightly reduced.

$Ac_5Neu5Ac$  supplementation did not result in significant changes in total cell surface sialylation as detected by MALII and SNA-I, even at a concentration of 512  $\mu$ M (Fig. 3, B, D, and F, and Fig. S2). Only ECL staining revealed a significantly lower exposure of terminal  $\beta$ 1–4-linked galactose residues upon treatment with a high concentration (2 mM) of  $Ac_5Neu5Ac$  (Fig. 3H). Overall glycosylation was not altered upon  $Ac_5Neu5Ac$  supplementation (Fig. S3, A–C).

In contrast,  $Ac_5Neu5Ac$  concentrations from 128  $\mu$ M and higher significantly increased GD2 expression (Fig. 2). Collectively, these findings suggest that the GD2 biosynthesis pathway is highly sensitive to both sialic acid inhibition and supplementation as compared with overall cell surface sialylation of neuroblastoma cells.

#### Combined effect of sialic acid analogues and HDAC inhibitors on GD2 expression

Previously, we reported that the pan-HDAC inhibitor vorinostat enhances GD2 expression in the 9464D neuroblastoma mouse tumor model *in vitro* and *in vivo*. The enhanced GD2 expression induced by vorinostat resulted in improved anti-GD2 antibody-mediated ADCC and reduced tumor growth (27); the vorinostat-increased GD2 expression in 9464D neuroblastoma cells was caused by increased GD2 synthase protein but not mRNA levels. These findings prompted us to assess the combined effect of sialic acid analogues and HDAC inhibitors on GD2 expression in murine 9464D and the human IMR-32 and SK-N-AS cell lines. First, we confirmed the effects of the sialic acid analogues in the murine neuroblastoma cells. 9464D cells were cultured for 3 days with 0–10 mM  $Ac_53F_{ax}Neu5Ac$  or were treated for 1 h with *C. perfringens* sialidase.  $Ac_53F_{ax}Neu5Ac$  inhibited GD2 expression in this murine neuroblastoma cell line, although higher concentrations were needed compared with the human cells (Fig. 4A). Similar to the human neuroblastoma cells, the addition of peracetylated sialic acid  $Ac_5Neu5Ac$ , but not the nonpermeable sialic acid Neu5Ac, significantly enhanced GD2 expression at concentrations of 1 mM and higher (Fig. 4B). Importantly,  $Ac_5Neu5Ac$  supplementation did not induce GD2 expression in



**Figure 5. Combined effect of Ac<sub>5</sub>Neu5Ac and HDAC inhibitors on GD2 expression in human neuroblastoma cells.** A–D, IMR-32 and SK-N-AS cells were cultured for 2 days with control, Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac (32 μM), or Ac<sub>5</sub>Neu5Ac (2 mM) and for another day with or without HDAC inhibitors; GD2 expression was determined by flow cytometry. A representative histogram shows GD2 expression of IMR-32 cells treated with sialic acid analogues alone or in combination with 32 nM vorinostat (A). Bar diagrams show the mean GD2 expression ± S.E. normalized to control of IMR-32 and SK-N-AS cells incubated with Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac or AcNeu5Ac in combination with 32 nM vorinostat (B), 32 nM entinostat (C), or 1 nM panobinostat (D). Data from three independent experiments are shown.

GD2 synthase negative Neuro-2a neuroblastoma cells, in various nonneuroblastoma tumor cell lines, or in noncancerous human or mouse cells (Figs. 4C and S4). These data confirm that the increased availability of sialic acids enhances GD2 biosynthesis also in murine neuroblastoma cells.

Next, we assessed the combined effect of HDAC inhibitors and the sialic acid analogues on GD2 expression. Murine 9464D and human IMR-32 and SK-N-AS neuroblastoma cells were treated for 48 h with control or Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac and for another 24 h with vorinostat, after which GD2 expression was analyzed. In line with our previous study, vorinostat treatment enhanced GD2 expression in all three neuroblastoma cell lines. Cells treated with Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac lost expression of GD2 even in the presence of vorinostat, confirming that the HDAC inhibitor-induced up-regulation of GD2 is also sialic acid-dependent (Figs. 4D and 5, A and B). Subsequently, neuroblastoma cells were cultured with the cell-permeable sialic acid Ac<sub>5</sub>Neu5Ac followed by vorinostat. Strikingly, this combination significantly increased GD2 expression in both murine and human neuroblastoma cell lines beyond the effect of the individual compounds (Figs. 4E and 5, A and B). Whereas Ac<sub>5</sub>Neu5Ac and vorinostat alone each enhanced GD2 expression about 1.5-fold, the combined treatment resulted in more than 2-fold higher GD2 expression. Notably, vorinostat treatment showed no significant effect on overall cell surface sialylation or overall gly-

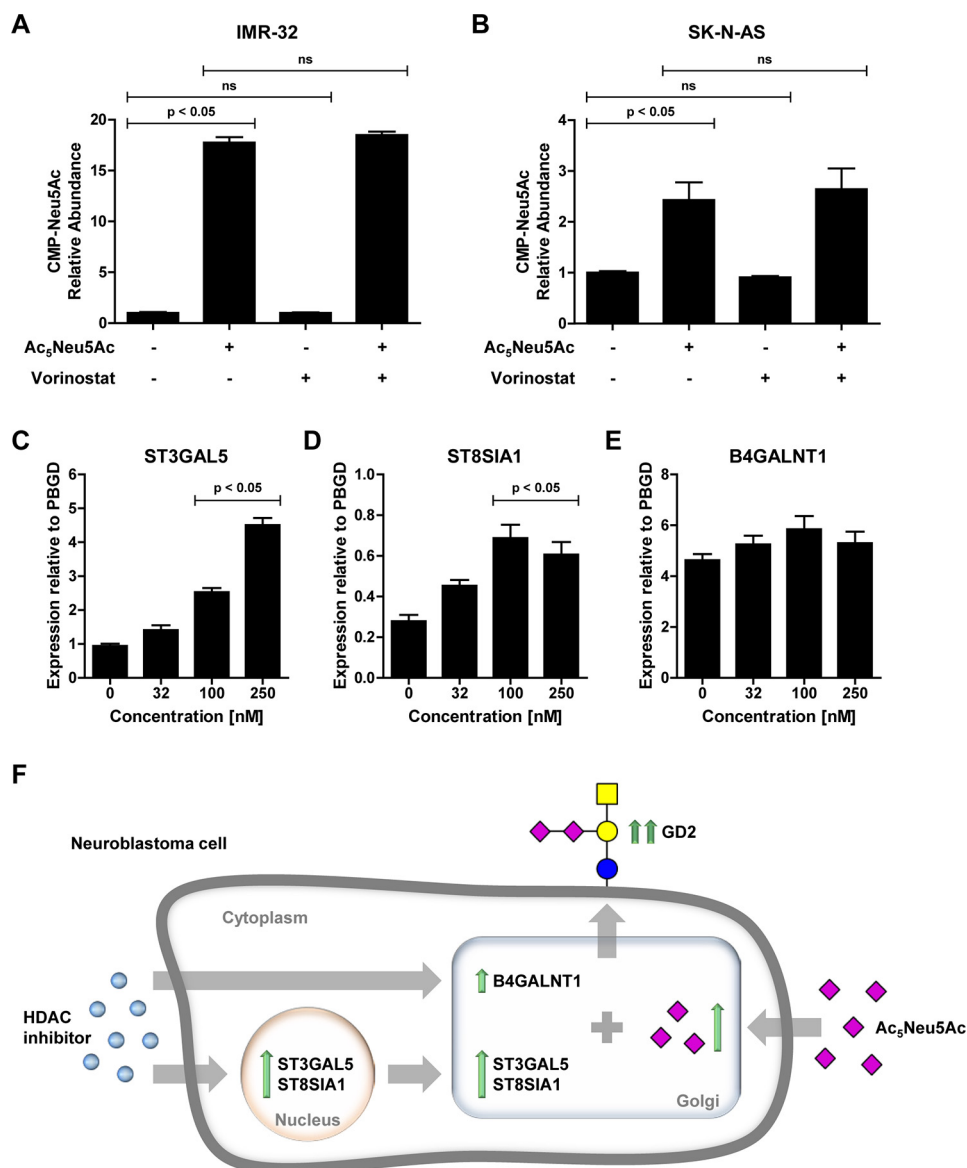
cosylation (Fig. S3). Furthermore, the class I HDAC inhibitor entinostat and the pan-HDAC inhibitor panobinostat also increased GD2 expression to a similar extent as vorinostat (Fig. 5, C and D). In conclusion, these data show that the combination of sialic acid supplementation and HDAC inhibition results in an even stronger up-regulation of GD2 expression in neuroblastoma cells.

#### Ac<sub>5</sub>Neu5Ac increases intracellular CMP–Neu5Ac levels, and vorinostat induces expression of sialyltransferases involved in GD2 biosynthesis

To investigate the effect of sialic acid supplementation on substrate availability for sialyltransferases, we assessed intracellular CMP–Neu5Ac levels in IMR-32 and SK-N-AS cells treated with Ac<sub>5</sub>Neu5Ac, vorinostat, or a combination of the two. We found that supplementation with Ac<sub>5</sub>Neu5Ac increased CMP–Neu5Ac levels around 20-fold in IMR-32 cells and around 3-fold in SK-N-AS cells. Vorinostat alone did not affect CMP–Neu5Ac abundance and had no additional effect on the Ac<sub>5</sub>Neu5Ac-induced increase (Fig. 6, A and B). These results show that Ac<sub>5</sub>Neu5Ac supplementation increases intracellular CMP–Neu5Ac levels, thereby enhancing substrate availability for sialyltransferases.

We reported earlier that vorinostat increases GD2 synthase protein levels without affecting mRNA expression. Others have reported that GD2 expression also depends strongly on GM3

## Boosting GD2 expression in neuroblastoma



**Figure 6.** Ac<sub>5</sub>Neu5Ac increases intracellular CMP-Neu5Ac levels, and vorinostat induces sialyltransferase expression. *A* and *B*, Ac<sub>5</sub>Neu5Ac increases intracellular CMP-Neu5Ac levels in IMR-32 cells (*A*) and SK-N-AS cells (*B*). Cells were cultured for 2 days with control or Ac<sub>5</sub>Neu5Ac (2 mM) and for another day with or without vorinostat (32 nM); intracellular CMP-Neu5Ac levels were determined by MS. Relative abundance  $\pm$  S.E. relative to control is presented. *C–E*, vorinostat induces sialyltransferase expression in IMR-32 cells. Cells were treated with 0–250 nM vorinostat for 5 h, and expression of glycosyltransferase genes involved in GD2 biosynthesis was determined by quantitative PCR. Mean expression values  $\pm$  S.E. relative to PBGD of ST3GAL5 (*C*), ST8SIA1 (*D*), and B4GALNT1 (*E*) are presented from three independent experiments. *F*, schematic model showing the proposed mechanism of enhanced GD2 expression induced by HDAC inhibitors and peracetylated sialic acid.

and GD3 synthase expression (27, 39). Therefore, we investigated whether vorinostat induces changes in the expression of genes involved in GD2 biosynthesis. IMR-32 cells were treated with 0–250 nM vorinostat for 5 h, and expression of glycosyltransferase was determined by quantitative PCR. In line with our previous findings, vorinostat had no significant effect on B4GALNT1 expression but up-regulated ST3GAL5 and ST8SIA1 expression in a dose-dependent manner (Fig. 6, *C–E*). These results show that vorinostat mainly affects ST3GAL5 and ST8SIA1 expression, sialyltransferases that generate GM3 and GD3 gangliosides, the precursors for GD2 synthase. Notably, expression of GD1b synthase (B3GALT4), which converts GD2 to GD1b, was not detected in IMR-32 cells (data not shown). Altogether, these findings show that the combination of

increased intracellular CMP-sialic acid levels and increased ST3GAL5 and ST8SIA1 expression induced by Ac<sub>5</sub>Neu5Ac and HDAC inhibition, respectively, results in increased expression of GD2 on neuroblastoma cells.

### Discussion

In this study, we investigated the possibility of using sialic acid analogues alone or in combination with HDAC inhibitors to increase GD2 expression in human and mouse neuroblastoma cells. First, we found that the fluorinated sialic acid analogue Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac potently inhibited GD2 expression in human neuroblastoma cells in the low micromolar range. Second, we demonstrated that increasing intracellular CMP-sialic acid levels enhanced GD2 expression on cells, whereas overall



cell surface sialylation remained largely unaltered. Third, a combination treatment of Ac<sub>5</sub>Neu5Ac with different HDAC inhibitors showed an additive effect and strongly boosted GD2 expression. This effect was strongly associated with enhanced expression of the sialyltransferases ST3GAL5 and ST8SIA1 upon HDAC inhibitor treatment.

GD2 expression depends on the availability of GM3 and GD3, two sialic acid-carrying gangliosides that are produced by the sialyltransferases GM3 synthase and GD3 synthase, respectively (3, 31). Previous studies by other groups and our own group have shown that the fluorinated sialic acid analogue Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac blocks sialic acid expression with high efficiency and selectivity (32–35). In the present study we found that this fluorinated sialic acid analogue is also a potent inhibitor of GD2 expression, consistent with previous data using sialidase (40). The fluorinated sialic acid analogue almost completely blocked GD2 expression in both human neuroblastoma cell lines at concentrations in the low micromolar range. This effect of Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac is most likely attributable to inhibition of the two sialyltransferases ST3GAL5 and ST8SIA1 that generate GM3 and GD3, respectively, thereby depleting the substrate for GD2 synthase. Murine 9464D cells were less sensitive to inhibition of GD2 expression with Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac, which may be caused by the differences in esterase expression required to remove the acetyl moieties. Neu5Ac carries a natural acetyl group on C-5, and four acetyl groups were added to C-1, C-7, C-8, and C-9 to increase cell permeability. These acetyl groups need to be removed, at least partially, by esterases before the supplemented sialic acid can act as a substrate or inhibitor. Interestingly, next to the natural C-5 acetyl modification, sialic acids can be 9-O-acetylated inside the cell, and this form of sialic acid is incorporated into GD2 (41, 42). The 14G2a antibody used recognizes both GD2 forms, and it has been suggested that O-acetyl-GD2 is expressed on neuroblastoma cells but not on peripheral nerves. Therefore, anti-O-acetyl-GD2 antibodies might show higher tumor specificity and less adverse effects in future (43). In addition, unlike human cells, mouse cells can produce N-glycolylneuraminic acid (Neu5Gc), a hydroxylated form of Neu5Ac (36, 44, 45). Possibly, this difference could account for the lower sensitivity of the murine cells to manipulation with Neu5Ac analogues. Overall the influence of the diverse sialic acid modifications in GD2 biology needs to be investigated further. Interestingly, the low micromolar concentrations used in the human cell lines caused only a mild reduction of total α2,3-/α2,6-sialylation. These findings indicate that the ganglioside synthesis pathway resulting in GD2 expression is more sensitive to metabolic sialic acid blockade compared with the synthesis pathway of other sialoglycans as detected using MALII and SNA-I lectin.

Although sialic acid blockade abrogates GD2 expression, the addition of Neu5Ac analogues could enhance GD2 expression in neuroblastoma cells by increasing intracellular CMP-sialic acid levels. Importantly, at the effective concentrations no change in overall sialylation was detected, indicating that the GD2 biosynthesis pathway in neuroblastoma cells is relatively more sensitive to changes in intracellular sialic acid levels compared with overall cell surface sialylation. This finding is particularly important, as sialic acids have been shown to promote

tumor growth and suppress the immune system (46–49). The IMR-32 and SK-N-AS cells we used are naturally highly sialylated as evidenced by strong binding of MALII and SNA-I lectin and almost no binding of PNA lectin. Possibly, overall sialylation could not be enhanced by Ac<sub>5</sub>Neu5Ac treatment because of the high endogenous sialylation of the neuroblastoma cell lines and the absence of uncapped glycans, which could serve as a substrate for sialyltransferases. Only exposure of terminal β1–4-linked galactose residues showed a significant decrease upon treatment with high Ac<sub>5</sub>Neu5Ac concentrations as detected by the lectin ECL. The decrease in ECL binding could be explained by sialylation of lipids, like GD2, and/or glycoproteins. GD2 expression, however, could be readily enhanced by sialic acid supplementation suggesting that this pathway is not saturated in the tested neuroblastoma cell lines. Presumably, the increased bioavailability of CMP-Neu5Ac for sialyltransferases facilitates GM3 and GD3 production and thus raises substrate levels for GD2 synthase. Further research is needed to investigate the precise relationship between intracellular sialic acid levels and disialoganglioside synthesis.

Previously, we showed that the HDAC inhibitor vorinostat enhanced GD2 expression in neuroblastoma cells *in vitro* as well as *in vivo*. Treatment with vorinostat and anti-GD2 antibody synergistically reduced neuroblastoma growth in mice (27). We now showed here that the combination of Ac<sub>5</sub>Neu5Ac with three different HDAC inhibitors, vorinostat, entinostat, and panobinostat, had an additive effect on GD2 expression. No induction of GD2 by this combination treatment was observed in neuroblastoma cells, nonneuroblastoma tumor cell lines, or normal cells negative for GD2. These findings suggest that sialic acid supplementation and HDAC inhibition act only on cells with endogenous GD2 synthesis capacity. Furthermore, HDAC inhibition showed no significant effect on CMP-sialic acid levels or overall cell surface glycosylation suggesting that they increase GD2 expression by a different mechanism. Suzuki *et al.* (50) have provided evidence that the expression of GD3 synthase, and especially GD2 synthase, is regulated at the epigenetic level. We have observed previously that the treatment of neuroblastoma cells with vorinostat enhances GD2 synthase protein levels but not mRNA expression in 9464D cells (27). In line with these findings, vorinostat had no significant effect on GD2 synthase (B4GALNT1) mRNA expression in human neuroblastoma cells or on CMP-Neu5Ac levels, but it strongly up-regulated GM3 synthase (ST3GAL5) and GD3 synthase (ST8SIA1) gene expression. The additive effect of peracetylated sialic acid and HDAC inhibition on GD2 expression is therefore the overall result of (i) increased intracellular CMP-sialic acid levels, (ii) up-regulated sialyltransferase expression, and (iii) higher GD2 synthase protein expression levels (as shown schematically in Fig. 6F). Further research is needed to investigate how HDAC inhibition regulates GD2 synthase stability as well as sialyltransferase expression. Moreover, this combination treatment could also influence the expression levels of other glycosphingolipids that are synthesized by GD2 synthase and sialyltransferases or that are derived from GD2 (*e.g.* GD1b) (3). Mass spectrometry analysis would be useful in further detailing the effects of the combination treatment presented here on glycosylation, including glycosphingolipid expression.

## Boosting GD2 expression in neuroblastoma

Current immunotherapy efforts in neuroblastoma, like mAb and CAR-T cell therapy and carbohydrate vaccines, rely on the recognition of GD2 on the neuroblastoma cells. Increasing GD2 expression in neuroblastoma cells by using engineered sialic acid supplementation and HDAC inhibition may further increase the efficacy of current and future GD2-targeted immunotherapy in neuroblastoma patients.

### Experimental procedures

#### Reagents and antibodies

*C. perfringens* sialidase, Neu5Ac, ManNAc, SYBR Green, and primers were purchased from Sigma-Aldrich. Ac<sub>5</sub>Neu5Ac and Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac were synthesized as described previously and dissolved in PBS (51). Mouse anti-GD2 antibody (clone 14G2a) was purified from a hybridoma cell line (obtained from Dr. Reisfeld, Scripps, La Jolla, CA) (52). Purified mouse IgG2a isotype antibody, PE-conjugated goat anti-mouse Ig, and streptavidin-PE were purchased from BD Biosciences (BD Pharmingen™) and eFluor 780 viability dye from eBioscience, Inc. (San Diego, CA). The HDAC inhibitors vorinostat (suberoylanilide hydroxamic acid, MK0683), panobinostat (LBH589), and entinostat (MS-275) were purchased from Selleckchem (Houston, TX) and dissolved in DMSO. Carbo-Free blocking solution and biotin-MALII (*Maaackia amurensis* lectin II), biotin-SNA-I (*Sambucus nigra* I agglutinin), biotin-PNA (peanut agglutinin), biotin-GSL-I (*Griffonia simplicifolia* I), biotin-LCA (*Lens culinaris*), biotin-PHA-L (*Phaseolus vulgaris*), and biotin-ECL (*Erythrina crista-galli*) were obtained from Vector Laboratories Inc. (Burlingame, CA).

#### Cell culture

Human IMR-32 (ATCC, CCL-127) neuroblastoma cells and the murine neuroblastoma cell lines 9464D (obtained from Dr. Orentas, National Institutes of Health, Bethesda, MD) and Neuro-2a (ATCC, CCL-131) were cultured in Dulbecco's modified Eagle's medium—GlutaMAX (Gibco/Invitrogen) with 10% FBS (Greiner Bio-One), 1% nonessential amino acids (Gibco), 50 μM 2-mercaptoethanol (Sigma-Aldrich), and 1% antibiotic-antimycotic solution (Gibco). Human SK-N-AS neuroblastoma (ATCC, CRL-2137) cells were cultured in Dulbecco's modified Eagle's medium—GlutaMAX (Gibco) with 10% FBS (Gibco), 1% nonessential amino acids (Gibco), 2 mM glutamine (Lonza, Walkersville, MD), and 1% antibiotic-antimycotic solution. B16-F10 (ATCC, CRL-6475) melanoma cells were cultured in minimum essential medium (MEM; Gibco) containing 5% FBS, 1% MEM nonessential amino acids, 0.15% sodium bicarbonate (Gibco), 1 mmol/liter sodium pyruvate (Gibco), 1.5% MEM vitamins (Gibco), and 1% antibiotic-antimycotic solution. 3T3 fibrosarcoma cells (ATCC, CRL-1658) were cultured in Dulbecco's modified Eagle's medium—GlutaMAX with 10% FBS, 1 mmol/liter sodium pyruvate, and 1% antibiotic-antimycotic solution. HeLa cells (ATCC, CCL-2) were cultured in Dulbecco's modified Eagle's medium—GlutaMAX with 10% FBS, 1% nonessential amino acids, 1 mmol/liter sodium pyruvate, and 1% antibiotic-antimycotic solution. Mouse mammary gland (NMuMG) epithelial cells (ATCC, CRL-1636) were cultured in Dulbecco's modified Eagle's medium—GlutaMAX with 10% FBS, 1 mmol/liter

sodium pyruvate, 2 mM glutamine, 10 μg/ml insulin (Sigma-Aldrich), and 1% antibiotic-antimycotic solution. The murine fibroblast cell line B6MEC was cultured in Iscove's modified Dulbecco's medium (Gibco) with 5% FBS (Greiner Bio-One), 50 μM 2-mercaptoethanol (Sigma-Aldrich), and 1% antibiotic-antimycotic solution. Human primary dermal fibroblasts were kindly provided by the Department of Dermatology, Radboud University Medical Center, Nijmegen, the Netherlands. Primary dermal fibroblasts were isolated from skin tissue biopsies taken from patients undergoing elective surgery after written consent and were maintained in Dulbecco's modified Eagle's medium—GlutaMAX with 10% FBS, 1 mmol/liter sodium pyruvate, and 1% antibiotic-antimycotic solution. Cryopreserved cells were resuscitated and used within 3 month after thawing. All cells were incubated in a humidified CO<sub>2</sub> incubator at 37 °C in 5% CO<sub>2</sub> and tested regularly for mycoplasma contamination using a mycoplasma detection test (Lonza, Basel, Switzerland).

#### Sialic acid analogue and HDAC inhibitor treatment

To inhibit sialic acid expression, IMR-32, SK-N-AS, or 9464D cells were cultured for 3 days with increasing concentrations of Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac. For enzymatic removal of sialic acid expression, the cells were incubated for 1 h with 200 milliunits/ml *C. perfringens* sialidase in serum-free medium at 37 °C. To assess the effect of Neu5Ac, Ac<sub>5</sub>Neu5Ac, and ManNAc on GD2 expression and total sialylation, the cells were cultured for 3 days in the presence of different concentrations of these sialic acid analogues. To study the effect of HDAC inhibitors on GD2 expression, cells were treated with different concentrations of vorinostat, entinostat, panobinostat, or DMSO control for 24 h. In addition, the cells were cultured for 48 h with 32 μM (IMR-32 or SK-N-AS), 5 mM (9464D) Ac<sub>5</sub>3F<sub>ax</sub>Neu5Ac, or 2 mM Ac<sub>5</sub>Neu5Ac before the HDAC inhibitors were added to the culture medium for 24 h. For IMR-32 and SK-N-AS cells, 32 nM vorinostat, 32 nM entinostat, or 1 nM panobinostat were used, and for 9464D cells 256 nM vorinostat was used. B16-F10, 3T3, NMuMG, B6MEC, and HeLa cells and primary dermal fibroblasts were cultured for 48 h with 2 mM Ac<sub>5</sub>Neu5Ac and for another 24 h in combination with 32 or 256 nM vorinostat.

#### Antibody staining, lectin staining, and flow cytometry

To detect GD2 expression, IMR-32 and SK-N-AS cells treated with sialic acid analogues, sialidase, or HDAC inhibitors were stained for 20 min at 4 °C with mouse anti-GD2 antibody or isotype antibody in PBA (1 × PBS, 1% BSA, and 0.02% sodium azide). The cells were washed with PBA and incubated for 20 min at 4 °C with PE-conjugated goat anti-mouse IgG antibody. After extensive washing, the cells were resuspended in PBA for flow cytometry analysis. Additionally, cell viability was determined by staining the cells with eFluor 780 viability dye following the manufacturer's instructions. Cell surface sialylation was assessed by staining the cells with the biotinylated lectins MALII (α2,3-linked sialic acids), SNA-I (α2,6-linked sialic acids), PNA (β-galactose), GSL-I (GalNAc and galactose), LCA (branched (N-)glycans and mannose), PHA-L (complex branched glycans), and ECL (uncapped terminal Galβ1-4GlcNAc residues). The cells were washed in 1 × Carbo-Free blocking solution containing 1 mM CaCl and 1 mM MgCl and incubated for 45 min at

4 °C with biotinylated MALII (5 µg/ml), SNA-I (1 µg/ml), PNA (5 µg/ml), GSL-I (5 µg/ml), PHA-L (5 µg/ml) and LCA (5 µg/ml) or for 30 min at 4 °C with biotinylated ECL (0.5 µg/ml). The cells were washed with PBA and incubated for 20 min at 4 °C with streptavidin-PE and then washed and resuspended in PBA for analysis. Cell fluorescence was measured using a CyAn ADP flow cytometer (Beckman Coulter), and the data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR). IC<sub>50</sub> values for the inhibition of GD2 expression with Ac<sub>5</sub>F<sub>ax</sub>Neu5Ac were calculated using Prism 5 software (GraphPad, Inc., La Jolla, CA).

### CMP-Neu5Ac quantification

IMR-32 and SK-N-AS cells were cultured on 6-well plates for 72 h with or without 2 mM Ac<sub>5</sub>Neu5Ac, and for the last 24 h of the culture 32 nM vorinostat or DMSO control was added. Cells were washed twice with ammonium carbonate buffer (pH 7.4) and snap-frozen in liquid nitrogen. Cell extracts were prepared by incubating the wells twice for 3 min with cold extraction buffer (acetonitrile:methanol:water, 2:2:1 (v/v/v)). The samples were centrifuged for 3 min at 13,000 rpm and vacuum-dried. The extracts were reconstituted in 100 µl of MilliQ and analyzed by reversed-phase ion pair chromatography (Agilent Technologies 1290 Infinity LC) coupled to a triple quadrupole mass spectrometer operating in negative ion mode (Agilent Technologies 6490 Triple Quad MS). CMP-sialic acid levels were expressed as the relative ratio of all nucleotide sugars.

### RNA isolation and quantitative PCR

IMR-32 cells were treated for 5 h with 0, 32, 100, or 250 nM vorinostat, and total RNA was isolated from neuroblastoma cells using an RNA isolation kit (Zymo Research, Irvine, CA) following the manufacturer's instructions. RNA samples were in-column DNase I-treated, and concentrations were quantified by spectrophotometry. cDNA was synthesized using random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). mRNA levels for the genes of interest were determined with a Bio-Rad CFX96 sequence detection system (Bio-Rad) with SYBR Green. The reaction mixtures and program conditions used were as recommended by the manufacturer; data were analyzed with the CFX Manager V1.6 (Bio-Rad) and checked for correct amplification and dissociation of the products. mRNA expression was determined relative to the housekeeping gene porphobilinogen deaminase (*PBGD*). Primer sequences as follows were derived from the Harvard University PrimerBank: ST3GAL5\_FW\_TCCCTGCAATGGTACACCC; ST3GAL5\_RV\_ACTTGGGACGACATTCC-TTCT; ST8SIA1\_FW\_GTCCTCTGTTGGCTCTACATCT; ST8SIA1\_RV\_CCCCGTCATACCACATGCTC; B4GALNT1\_FW\_CAGAAACAAGTCCGAGCTATTGA; B4GALNT1\_RV\_GAGGGGCTGAACTTCCACAC.

### Statistical analysis

Significance between two groups was calculated using a Student's *t* test, and comparisons between multiple groups were made using one-way analysis of variance followed by Bonferroni's correction using Prism 5 software (GraphPad Inc.). *p* values < 0.05 were considered significant.

*Author contributions*—R. J. E. v. d. B., M. K., T. J. B., M. H. d. B., C. B., and G. J. A. conceptualization; R. J. E. v. d. B., M. W., I. C. B., E. D. K.-R., and C. B. data curation; R. J. E. v. d. B., M. K., M. W., I. C. B., E. D. K.-R., M. v. S., D. J. L., and C. B. investigation; R. J. E. v. d. B., M. K., T. H., M. v. S., D. J. L., T. J. B., M. H. d. B., and C. B. methodology; R. J. E. v. d. B. and C. B. writing-original draft; M. K., L. B., T. H., T. J. B., M. H. d. B., P. M. H., and G. J. A. writing-review and editing; L. B., T. H., and T. J. B. resources; M. H. d. B., P. M. H., and G. J. A. project administration; P. M. H. and G. J. A. supervision.

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