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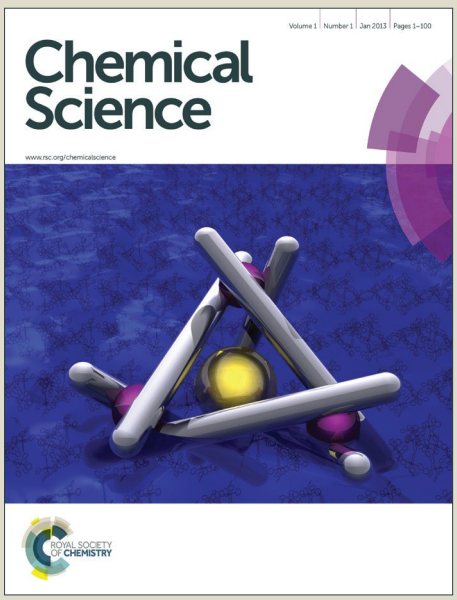





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Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

# Redirecting immunity via covalently incorporated immunogenic sialic acid on the tumor cell surface

**Xuanjun Wu,[a,‡] Bijuan Lin,[a,‡] Hu Zhao,[a] Yunpeng Tian,[a] Jiahuai Han,[b] Jian Liu,[a] and Shoufa Han[a,*]**

***Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX***

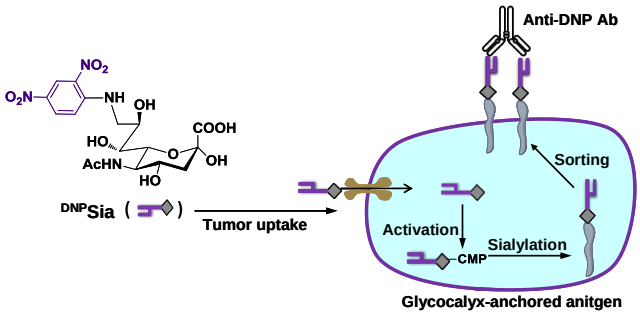
**DOI: 10.1039/b000000x**

Techniques eliciting anti-tumor immunity are of interest for immunotherapy. We herein report covalent incorporation of non-self immunogen into tumor glycocalyx by metabolic oligosaccharide engineering with 2,4-dinitrophenylated sialic acid ($^{DNP}$Sia), which enables marked suppression of pulmonary metastasis and subcutaneous tumor growth of B16F10 melanoma cells in mice preimmunized to produce anti-DNP antibodies. Located at exterior glycocalyx, $^{DNP}$Sia is well positioned to recruit antibodies. Given the high levels of natural anti-DNP antibodies in humans and ubiquitous sialylation across cancers, $^{DNP}$Sia offers a simplified route to redirect immunity against diverse tumors without recourse to preimmunization.

## Introduction

Immune systems eradicate deleterious "non-self" cells while saving "self" cells by sensing cell surface biomarkers. As cancers evade immune surveillance, extensive effort has been devoted to redirect immunity against tumors by targeting biomarkers that are often nonimmunogenic. For instance, cytotoxic T cells genetically engineered with chimeric antigen receptors are actively explored for targeting tumor surface antigens.[1] However, challenges remain with these self-antigen targeted therapies owing to the presence of self-antigens on normal tissues and inability to turn off persistent T cell activity.[2] Alternatively, ligand−antigen adaptor molecules that bind avidly to tumor surface receptors (e.g. folate receptor) enable exogenous antigens displayed on tumors to trigger immune responses.[3] Albeit powerful, these approaches rely on tumor-specific high affinity receptors that are often not defined in many cancers.

Mammalian cells are covered with a dense layer of glycans which, known as glycocalyx, mediates diverse cellular events such as immunological recognition and cancer metastasis.[4] Sialic acids (Sia) are a family of 9-carbon monosaccharides commonly located at cell surface glycan termini.[5] Hypersialylation contributes to metastatic potentials of many cancers,[6] and facilitates tumor evasion of immune surveillance.[7] Cell surface sialosides has been engineered with exogenous *N*-acyl mannosamines, metabolic precursors of Sia.[8] However, the oligosaccharide engineering approach using peracetylated sugars is of low cell type- or tissue- specificity, leading to expression of *N*-acyl Sia in diverse tissues in animals.[9] We recently observed marked propensity of tumors to take up Sia derivatives with selected substitutions at C-9 in mice[10] We herein report covalent incorporation of a non-self immunogen into tumor glycocalyx with DNP-conjugated Sia ($^{DNP}$Sia) to redirect immunity against tumors. $^{DNP}$Sia effectively accumulates in tumors and is covalently installed into cell surface glycocalyx by endogenous sialylation pathway. Mice preimmunized with DNP-labelled keyhole limpet hemocyanin ($^{DNP}$KLH) display marked antitumor effects against $^{DNP}$Sia-displaying B16F10 murine melanoma cells. Complementing receptor-ligand affinity based tumor coating,[3] this work suggests an alternative approach for tumor therapy via metabolically incorporated non-self antigen.




**Scheme 1.** Schematic for incorporation of non-self antigen into glycocalyx. $^{DNP}$Sia uptaken by tumors is metabolically transferred to glycoconjugates. The neosialoconjugates sorted to cell surface enable $^{DNP}$Sia well positioned to trigger immunity.

## Results and discussion

### Metabolic incorporation of $^{DNP}$Sia on cell surface glycocalyx

Sia chemically modified with C-9 substitutions are often compatible with cellular sialylation, leading to incorporation of abiotic Sia into glycoconjugates. For instance, FITC-labelled Sia sialylates proteins in permeabilized CHO cells.[11] Sia bearing aromatic azide is effectively incorporated into CD22 on B cell surface.[12] We recently observed that Sia with hydrophobic groups at C-9 preferentially accumulates in tumors in mice.[10] Encouraged by these observations, we explored the efficacy of $^{DNP}$Sia incorporated on glycocalyx for tumor suppression.

We first probed the impact of the spacers of DNP-Sia diads on cell surface sialoside expression. $^{DNP}$Sia featuring an amino spacer was synthesized from nucleophilic aromatic substitution of 2,4-dinitrofluorobenzene with 9-amino-Sia whereas $^{DNP\text{-}Tz}$Sia bearing a 1,2,3-triazole (Tz) linker was synthesized by copper (I)-catalyzed azide−alkyne cycloaddition of 9-azido-Sia with 2,4-dinitro-1-propargylamino-benzene (Fig. 1A, Scheme S1-2, ESI†). B16F10 cells, poised to hypersialylation,[13] were cultivated in Dulbecco's modified Eagle medium (DMEM) spiked with methyl esters of $^{DNP\text{-}Tz}$Sia or $^{DNP}$Sia (Scheme S1 and S2, ESI†), and then stained with biotin-labelled anti-DNP antibodies (Ab) and phycoerythrin (PE)-labelled streptavidin to probe the degrees of cell surface DNP. Confocal microscopic images reveal bright PE fluorescence confined on plasma membrane of cells treated with

methyl ester of $^{DNP}$Sia or $^{DNP\text{-}Tz}$Sia whereas no signal is observed in control cells (Fig. 1B). Flow cytometry analysis revealed 4-fold enhancement of cell surface $^{DNP}$Sia (MF = 7221) on relative to $^{DNP\text{-}Tz}$Sia (MF = 1686) (Fig. 1C). Western blotting confirms high abundance of $^{DNP}$Sia-bearing proteins over $^{DNP\text{-}Tz}$Sia-bearing proteins (Fig. 1D). These results validate covalent incorporation of $^{DNP}$Sia into glycocalyx with superior efficacy relative to $^{DNP\text{-}Tz}$Sia. $^{DNP}$Sia was also effectively installed on cell surface of Raw 264.7 macrophages, HeLa cells, L929, SMMC-7721 and U87-MG cells (Fig. S1, ESI†), demonstrating compatibility of $^{DNP}$Sia with sialylation pathway of diverse cancer cell lines. The immunostaining of cell surface DNP shows that glycocalyx-anchored $^{DNP}$Sia is well positioned to recruit anti-DNP Ab. We then monitored temporal changes of cell surface $^{DNP}$Sia on B16F10 cells cultivated in fresh DMEM. Albeit decaying over time, the levels of glycocalyx-anchored $^{DNP}$Sia remained high after 24 h incubation (Fig. S2, ESI†). In addition, cells surface $^{DNP}$Sia was shown to be more resistant to sialidase-mediated hydrolysis relative to Sia (Fig. S3, ESI†), which is beneficial for in vivo immunotherapy.

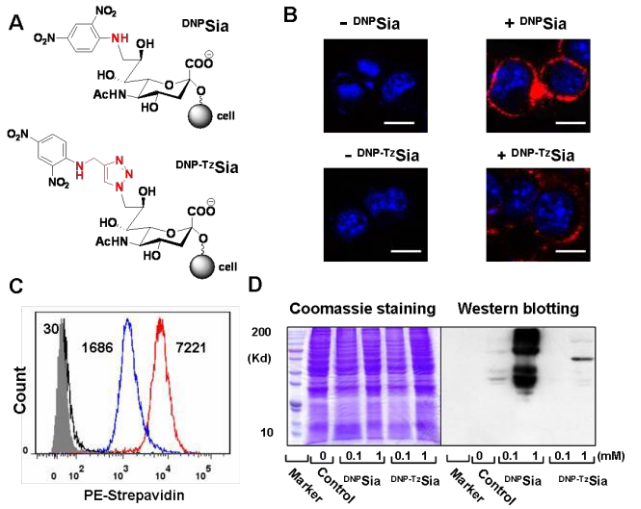



**Fig. 1** Incorporation of DNP-Sia diads into cell glycocalyx. Chemical structures of sialoaides of $^{DNP}$Sia and $^{DNP\text{-}Tz}$Sia displayed on cell surface (A). B16F10 cells treated with methyl esters of $^{DNP\text{-}Tz}$Sia or $^{DNP}$Sia (0, 1 mM) were stained with biotin-labelled anti-DNP Ab, PE-labelled streptavidin, DAPI specific for nucleus, and then analysed by confocal fluorescence microscopy (B) or flow cytometry (C) with mean channel fluorescence (MF) indicated. Bars: 10 μm. (D) Western blot of lysate of cells treated with DNP-Sia diads. Protein loading was verified by Coomassie blue staining.

### Anti-tumor effects by glycocalyx-anchored $^{DNP}$Sia in mice

Next, $^{DNP}$Sia was assessed for its influence on tumor cell proliferation in vitro. B16F10 cells pretreated without or with $^{DNP}$Sia were maintained in fresh DMEM for 24-72 h. No detrimental effects of $^{DNP}$Sia were observed on cell growth (Fig. 2A; Fig. S4, ESI†). With the negligible effects on cell growth in vitro, $^{DNP}$Sia was then explored for its capability to redirect immunity *in vivo*. C57BL/6 mice were immunized with $^{DNP}$KLH and subsequently boosted with another injection. Enzyme-linked immunosorbent assay (ELISA) shows anti-DNP Ab in serum from treated mice are 5-fold higher than untreated mice (Fig. 2B), proving induction of DNP-specific Ab by injected $^{DNP}$KLH. $^{DNP}$Sia-displaying B16F10 cells ($^{DNP}$Sia$^{+}$) and cells devoid of $^{DNP}$Sia ($^{DNP}$Sia$^{-}$) were subcutaneously inoculated into left or right flank of $^{DNP}$KLH-treated mice, respectively. The tumor from $^{DNP}$Sia$^{+}$ cells exhibits about 75% volume reduction compared to from $^{DNP}$Sia$^{-}$ tumor 7 days after inoculation whereas the tumor formation of $^{DNP}$Sia$^{-}$ cells with natural Sia was largely unaffected in DNP-immunized mice relative to unimmunized mice (Fig. 2C). In unimmunized mice the tumor from $^{DNP}$Sia$^{+}$ cells was about 50% smaller relative to $^{DNP}$Sia$^{-}$ cells, whereby retarded growth of $^{DNP}$Sia$^{+}$ tumor is likely due to endogenous low levels of anti-DNP Ab (Fig. 2B). In addition, time course monitoring reveals consistent and obvious suppression of tumor formation from implanted $^{DNP}$Sia$^{+}$ cells over $^{DNP}$Sia$^{-}$ cells in $^{DNP}$KLH-immunized mice (Fig. 2D) in the early stage, demonstrating synergistic effects of anti-DNP Ab and $^{DNP}$Sia on anti-tumor response.

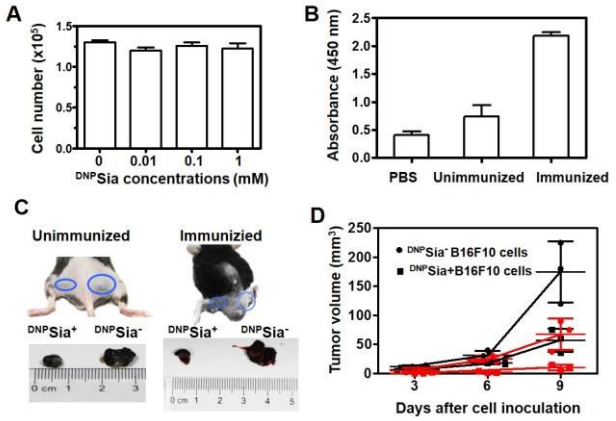



**Fig. 2** $^{DNP}$Sia mediated anti-tumor responses in mice. (A) Effects of $^{DNP}$Sia on cell proliferation. B16F10 cells pretreated with $^{DNP}$Sia (0-1 mM) were cultured in fresh DMEM for 24 h prior to cell number determination. (B) ELISA of anti-DNP Ab in serum from C57BL/6 mice untreated or treated with injection of $^{DNP}$KLH. (C) Suppressed growth of inoculated $^{DNP}$Sia$^{+}$ B16F10 cells over $^{DNP}$Sia-free cells that are injected in opposite flanks of mice. Tumors were excised 7 days post inoculation. (D) Anti-DNP Ab mediated inhibition of $^{DNP}$Sia$^{+}$ B16F10 cells and $^{DNP}$Sia$^{-}$ cells subcutaneously inoculated in mice unimmunized (in dark) or immunized with $^{DNP}$KLH (in red). Assays were performed in triplicate each using 3 mice. Error bars represent ±SD of experimental data on a representative assay.

To further evaluate therapeutic scope of this approach, $^{DNP}$KLH-immunized mice subcutaneously inoculated with B16F10 cells were treated by tail-veil injection of PBS or $^{DNP}$Sia after tumor transplantation. $^{DNP}$Sia treatment resulted in 50-90% reduction in tumor volume in the early stage (6-9 days after cell inoculation) as compared to control mice treated with PBS (Fig. 3), proving the effectiveness of $^{DNP}$Sia for systemic tumor suppression.

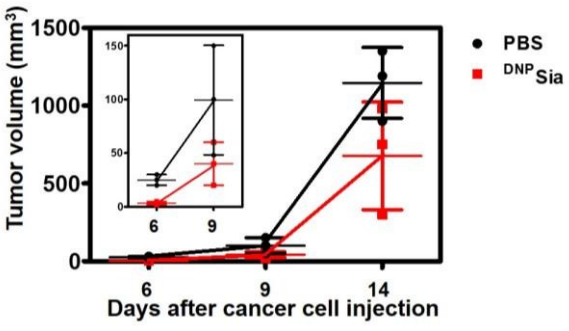



**Fig. 3** Tumor suppress with intravenously injected $^{DNP}$Sia in mice. $^{DNP}$KLH-immunized C57BL/6 mice were subcutaneously inoculated with B16F10 cells, and then treated with tail-veil injected PBS (100 μL) or $^{DNP}$Sia (30 mg kg$^{-1}$) at 3$^{rd}$, 6$^{th}$, and 9$^{th}$

day post cell inoculation. Tumor volumes were monitored over time. The enlarged insert shows the correlation of early stage tumor volume vs time. Assays were performed in triplicate each using 3 mice. Error bars represent ±SD of experimental data on a representative assay.

Metastasis is a major cause of cancer-associated mortality. Cell surface sialosides are critical for cancer metastasis, promoting the use of sialyltransferase inhibitors to decrease cancer sialylation.[14] We therefore evaluated the effects of $^{DNP}$Sia on B16F10 metastasis with an experimental pulmonary metastasis model. $^{DNP}$Sia$^{+}$ B16F10 cells were injected into $^{DNP}$KLH-immunized mice by tail vein. Lungs and representative organs were isolated 7 days after cell administration. Metastase in the lung from mice treated with $^{DNP}$Sia$^{+}$ cells is significantly smaller than that from mice treated with $^{DNP}$Sia$^{-}$ cells (Fig. 4, Fig. S5, ESI†), revealing the capability of $^{DNP}$Sia to inhibit metastasis. Our results suggest an alternative approach against metastasis with chemically modified Sia on cell surface.

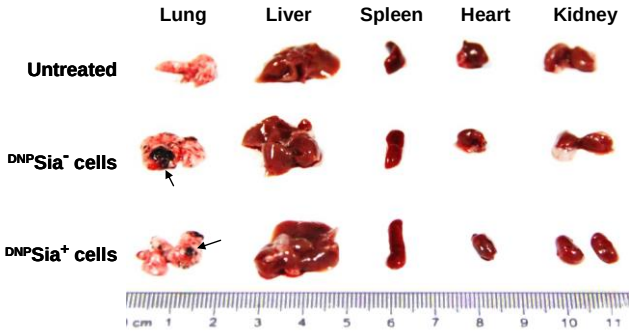



**Fig. 4** Glycocalyx-anchored $^{DNP}$Sia decreases pulmonary metastasis of B16F10 cells in mice. $^{DNP}$KLH-immunized C57BL/6 mice were untreated or treated with $^{DNP}$Sia$^{-}$, or $^{DNP}$Sia$^{+}$ B16F10 cells by tail-veil injection, respectively. The organs were excised 7 days after injection. The arrows denote metastases.

Compared with affinity based tumor decoration,[3b, 3d, 15] we employ antigens covalently installed on glycocalyx via metabolic sialylation pathway to elicit antitumor responses. Historically, sialosides with selected N-acyl groups at C-5 have been reported by Guo's group and Bertozzi's group to be more immunogenic than natural sialoside.[16] The feasibility of this approach has been examined in both cell and mice models with exogenous N-acyl mannosamines, metabolic precursor of C-5-N-acyl Sia.[16e, 17] This abiotic Sia-mediated cancer therapy entails tandem preimmunization to elicit Ab specific for the abiotic sialosides, and subsquent tumor expression of the abiotic Sia. These studies lay the foundation for unnatural Sia mediated immunotherapy. One percent of circulating antibodies in humans binds DNP.[18] Of note, our approach directly uses DNP-bearing Sia for metabolic tumor engineering, and offers a simplified immunotherapy by recruiting high levels natural anti-DNP Ab in humans without recourse to preimmunization.

### Biodistribution of $^{DNP}$Sia in tumor-bearing mice

To probe $^{DNP}$Sia biodistribution, C57BL/6 mice bearing subcutaneous B16F10 tumors were treated with $^{DNP}$Sia by tail-veil injection. Tumor and selected organs were excised 1 h post-injection and examined for $^{DNP}$Sia expression. Immunostaining reveals intense fluorescence in tumors whereas negligible to moderate fluorescence is present in kidney, heart, spleen, lung and liver (Fig. 5), showing that $^{DNP}$Sia is preferentially taken up and incorporated into glycoconjugates by tumors in mice. $^{DNP}$Sia displayed compromised tumor accumulation as the tumor volume increases (data not shown). Time course study shows that tumor-associated $^{DNP}$Sia decreased over time and yet remained substantial at 24 h post-injection (Fig. 6). By contrast, $^{DNP}$Sia incorporated on heart and liver decreased to baseline levels by 4 h of sugar administration (Fig. S6, ESI†), suggesting in vivo clearance of non-self antigen which offers a means to temporarily turn off immunity and thus is beneficial for decreased systemic toxicity after treatment. Although B16F10 is a cell line featuring oversialylation,[13] the elevated expression of tumor surface $^{DNP}$Sia sialoside might also benefit from rapid cell division and glycoprotein biosynthesis in tumor cells relative to normal tissue and organs. The lower levels of $^{DNP}$Sia on heart tissues could trigger off-target immune responses. In future, selective expression of $^{DNP}$Sia on tumors could be potentiated with the aid of emerging vectors for tumor-specific delivery of Sia.[14b, 19]

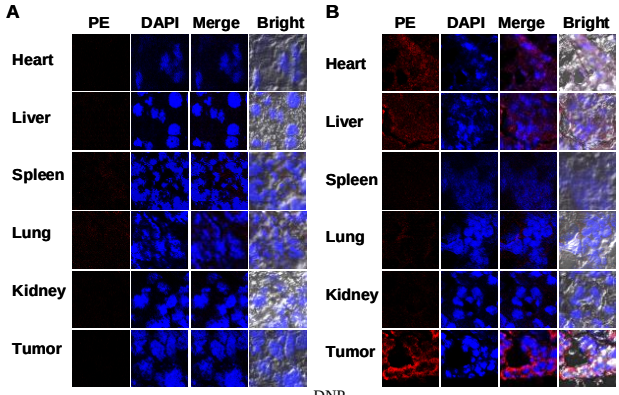



**Fig. 5** In vivo distribution of $^{DNP}$Sia. C57BL/6 mice bearing subcutaneous B16F10 tumors were injected by tail veil with PBS (A), or $^{DNP}$Sia (60 mg kg$^{-1}$) (B). The tumor (10 mm$^3$) and organs were excised 1 h post-injection, sectioned, and stained with biotin-labeled anti-DNP Ab, PE-labeled streptavidin and DAPI prior to fluorescence analysis.

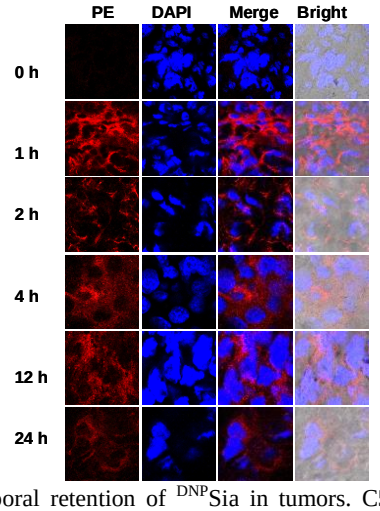



**Fig. 6** Temporal retention of $^{DNP}$Sia in tumors. C57BL/6 mice with B16F10 tumors (10 mm$^3$) were injected with $^{DNP}$Sia (60 mg kg$^{-1}$) by tail veil. At 0-24 h after probe injection, the tumors were excised, sectioned, and stained with biotin-labeled anti-DNP Ab, PE-labeled streptavidin, and DAPI prior to fluorescence detection.

### Cytotoxicity of $^{DNP}$Sia

Low toxicity is critical for agents to be administered *in vivo*. To probe the systemic toxicity, $^{DNP}$Sia was injected into healthy mice by tail vein at dose of 300 mg kg$^{-1}$, which is 10-fold higher than the dose used for systemic tumor suppression mentioned in Fig. 3. No signs of abnormal behaviors and death were observed on mice up to 14 days after injection. Histological analysis reveals that

morphologies of organs from mice untreated or treated with $^{DNP}$Sia were virtually identical (Fig. S7, ESI†), indicating that $^{DNP}$Sia is of low systemic toxicity.

## Conclusions

We have demonstrated the use of $^{DNP}$Sia, a non-self immunogen tagged monosaccharide, to trigger immunity against tumors in mice. $^{DNP}$Sia is preferentially taken up by tumors and then metabolically incorporated into cell surface, enabling marked suppression of pulmonary metastasis of $^{DNP}$Sia-bearing B16F10 melanoma cells and suppression of subcutaneous tumor formation by intravenously injected $^{DNP}$Sia in $^{DNP}$KLH-immunized mice. Given the high levels of natural anti-DNP antibodies in humans,[18a] $^{DNP}$Sia on outmost glycocalyx is well positioned to recruit pre-existing antibodies and might offer a simplified immunotherapy in humans without recourse to preimmunization. Compared to ligand-receptor affinity mediated tumor targeting,[3] our approach takes advantages of widespread cellular sialylation pathway to covalently install non-self antigen conjugated Sia on tumor glycocalyx, which in principle could increase the immunogenicity of diverse tumors with a broad range of immunogens.

## Notes and references

[a] *State Key Laboratory for Physical Chemistry of Solid Surfaces, Department of Chemical Biology, College of Chemistry and Chemical Engineering, the Key Laboratory for Chemical Biology of Fujian Province, The MOE Key Laboratory of Spectrochemical Analysis & Instrumentation, and Innovation Center for Cell Signaling Network, Xiamen University;* [b]*State key Laboratory of Cellular Stress Biology, Innovation Center for Cell Signaling Network, School of Life Sciences, Xiamen University, Xiamen, 361005, China; Tel: 86-0592-2181728; E-mail: shoufa@xmu.edu.cn;*

‡*Both authors contributed equally to this work.*

*Acknowledgments: This work was supported by grants from NSF China (21572189, 21272196), the Fundamental Research Funds for the Central Universities (20720160052, 20720150047); 973 program 2013CB933901, PCSIRT, and open project grants from State Key Laboratory of Cellular Stress Biology, Xiamen University; Dr. J. Han was supported by grants from NSF China (91429301, 31420103910, 31330047, 31221065),the National Scientific and Technological Major Project (2013ZX10002-002), the Hi-Tech Research and Development Program of China (863program; 2012AA02A201), the 111 Project (B12001), the Science and Technology Foundation of Xiamen (3502Z20130027), the National Science Foundation of China for Fostering Talents in Basic Research (J1310027) and The Open Research Fund of State Key Laboratory of Cellular Stress Biology, Xiamen University.*

† Electronic Supplementary Information (ESI) available on synthesis of DNP conjugated Sialic acids, Western blot analysis, treatment and analysis of sugar-treated cells and mice; See DOI: 10.1039/b000000x/

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