Transfection of NSD3-tareting siRNA in H1299 Cells

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Objective. I have identified a putative phenotype in H1299 cells upon overexpression of the NSD3 short isoform. I am also interested in any phenotype the results from decreasing the amount of NSD3 in cells. To do so, I am first testing conditions for RNAi-mediated knockdown of NSD3 in H1299 cells by treating cells with several concentrations of siRNA and evaluating knockdown by western blotting.

1. Experimental Details

1.1 Reverse Transfection of NSD3-targeting NSD3

1. H1299 cells were trypsinized, resuspended in media and counted.
2. Next, siRNA transfections were prepared with RNAiMAX (Invitrogen/ Thermo - 13778075) following reagent protocol.
3. Briefly, 7.5 ul RNAiMAX reagent was diluted in 150 ul Opti-MEM (Invitrogen - 31985062) per 6 well.
4. siRNA was then diluted in 150 ul Opti-MEM as described in the table below.

Well	siRNA Conc. (nM)	siRNA Volume
1	0	0
2	1	0.2
3	5	1
4	10	2
5	20	4
6	50	10

5. Diluted siRNA was then added to diluted transfection reagent & incubated for 5 min at RT.
6. H1299 cells were plated at 50 000 cells per well of a 6 well with siRNA-lipid complexes and media topped up to 2 ml per well.
7. Cells were then incubated for 72 hours and then harvested for western blotting.

1.2 Western Blot

Lysis Buffer:
* 20 mM Tris-HCl pH8
* 150 mM NaCl
* 10 mM MgCl2
* 1mM EDTA
* 0.5 % Triton X-100

Add fresh protease Inhibitors (100x) & benzonase (10 000x) prior to lysis.

1. 60 uL of lysis buffer with fresh protease inhibitors and benzonase was added directly to each well of the plate.
2. Lysates were incubated on ice for 5 min at RT , then SDS added to 1% (final concentration).
3. Lysates were collected into 1.5 ml eppendorf tubes.

4. BCA protein concentration estimation was performed using Pierce BCA Protein Assay Kit (Cat# 23225).
5. ~50 μg of total protein was run on a NuPAGE 4-12% Bis-Tris Protein Gel (NP0322BOX) in 1x MOPS Running Buffer at 100 volts. Gels were run in duplicate to probe using two different NSD3 antibodies.
6. Proteins transferred at 1.5 hrs at 80 volts in 1 x tris-glycine transfer buffer to a 0.2 μm PVDF membrane.
7. Membrane blocked in 5% milk in PBS-T (1x PBS - 0.1% Tween-20) for 30 min at RT.
8. Membrane cut and probed with antibodies diluted in 5% BSA in PBS-T for overnight at 4^oC. Dilutions shown below:

Target	Supplier - Cat#	Dilution
NSD3 (RabMono)	AbCam - ab180500	1:5000
NSD3 (RabPoly)	ProteinTech - 11345-1-AP	1:1000
Actin (MouseMono)	AbCam - ab3280	1:5000

9. Membranes washed 3x - ~10 min in PBS-T.
10. Incubated with secondary LiCor antibodies to mouse and rabbit (diluted - 1:5000) in Licor Odyssey Blocking buffer (927-40000) diluted 1 in 5.
11. Membranes washed 3x - ~10 min PBS-T and one additional wash in 1 x PBS for 5 min.
12. Blots were imaged on a Licor Odyssey CLx Imaging System.
13. Annotated results shown below (Figure 1)

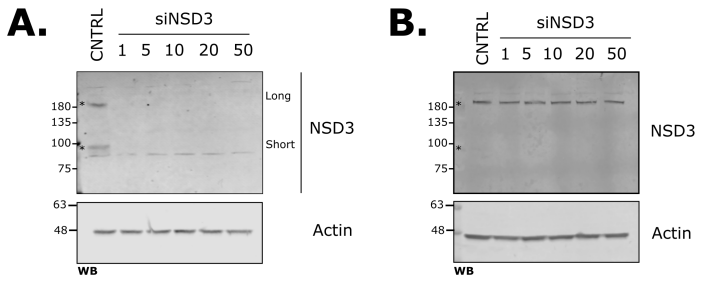

Figure 1: Western blot of H1299 Cells Treated with NSD3-targeting siRNA. (A) ProteinTech NSD3 antibody (B) AbCam antibody

2. Results & Obeservations

Observations. Suprisingly, with the ProteinTech antibody we observe robust knockdown of NSD3 long and short isoforms with as low as 1 nm siRNA. However, for the abcam antibody the upper band, which should correspond to the long isoform, is persistant across all concentrations of siRNA tested. While, this antibody is advertised as knockout validated and displayed recognition of the NSD3-3xFLAG transgene (exp-017), it does not seem recognize the form of the protein that is targeted by siRNA-mediated degradation or the endogenous short isoform. Moving forward, I will work with ProteinTech antibody to validate any gene knockdowns. Next, I plan on assaying proliferation in NSD3 knockdown cells.

ExpID-019