Western Blotting NSD3: Further Testing of Commercial Antibodies

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Objective. To test commercial antibodies to NSD3 by western blotting whole cell lysates from cancer cell lines. Validated antibodies will be used as tools to study NSD3's function on chromatin.

Experimental Details:

1. Culture of Cell Lines

Cells were cultured at 37^{o}C - 5% CO2 following standard protocols. The cells were maintained at 0.5-2 x 10^6 cells / ml. All cell lines tested are of various leukemic backgrounds (described below), with the exception of MYC-driven H1299 non-small lung cancer (NSCLC) cells. NSD3 has been shown to bridge an interaction between BRD4 and Myc in this line (Li et al.(2017) - PMID 28205554) and could be useful to study NSD3's role at enhancers in cancer.

Cell Lines:

Name	Growth Media	Notes
H1299	RPMI +10% FBS & Pen/Strep	NSCLC P53 Null (ATCC CRL-5803)
UCSD-AML1(cherry)	IMDM +GMCSF +10% FBS & Pen/Strep	AML - EVI1 Overexpression (DSMZ no: ACC 691)
MOLM-13	IMDM +10% FBS & Pen/Strep	AML - MLL-AF9 Fusion (DSMZ no: ACC554)
MV4-11	IMDM +10% FBS & Pen/Strep	AML - MML-AF4 (ATCC CRL-9591)
K562	IMDM +10% FBS & Pen/Strep	CML - JQ1 resistant (ATCC® CCL-243)

2. SOP - Western Blot Analysis of Whole Cell Lysates

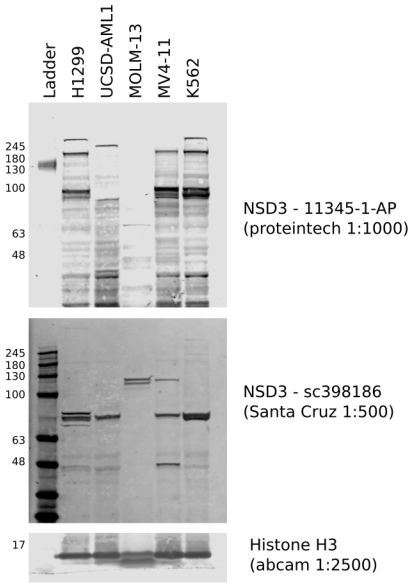
Lysis Buffer:
* 20 mM Tris-HCl pH8
* 150 mM NaCl
* 10 mM MgCl2
* 1mM EDTA
* 0.5 % Triton X-100

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

Protocol

1. ~2 ml of cells (~2 x 10^6) were pelleted by centrifugation at 300 x G for 5 min. With the exception of H1299, which are adherent. H1299 cells were trypsinized at ~80% confluence from a 10 cm dish, washed in PBS and spun to pellet.
2. The supernatant was removed and cells were washed 1x in 500 μL 1 x buffer, then resuspended in ~100 μL of lysis buffer, some adjustment to lysis volume made based on pellet size.
3. Lysates were incubated on ice for 10 min at RT , then SDS added to 1% (final concentration).

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.
6. Proteins transferred overnight at 30 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 1 hour at RT. Antibodies used and dilutions are indicated in Figure 1.
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)



Ladder = BLUeye Prestained (FroggaBio Cat# PM007)

3. Observations

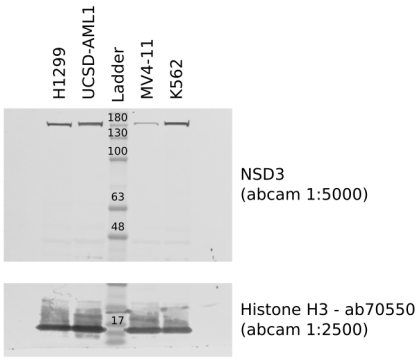
Both SantaCruz & proteintech NSD3 antibodies showed high-levels of background, unclear if any of these bands correspond to NSD3 long or short isoforms. To date, the NSD3 antibody from AbCam gives the cleanest results, a single band at correct MW for NSD3 long.This is shown in Figure 2, where several of these samples were re-run on a separate gel, using the protocol described above, and probed for NSD3 using ab137430. Strange, again in MOLM-13 cell lysates, prepared by myself and others, I do not detect a band for NSD3 (see Figure 3 - Ran cancer cell lysates provided by MS). This is likely due to protein degradation, as NSD3 does seem to be expressed at mRNA according to data from Broad's Cancer Cell Line Encyclopedia (analysis shown below).

Next Steps:

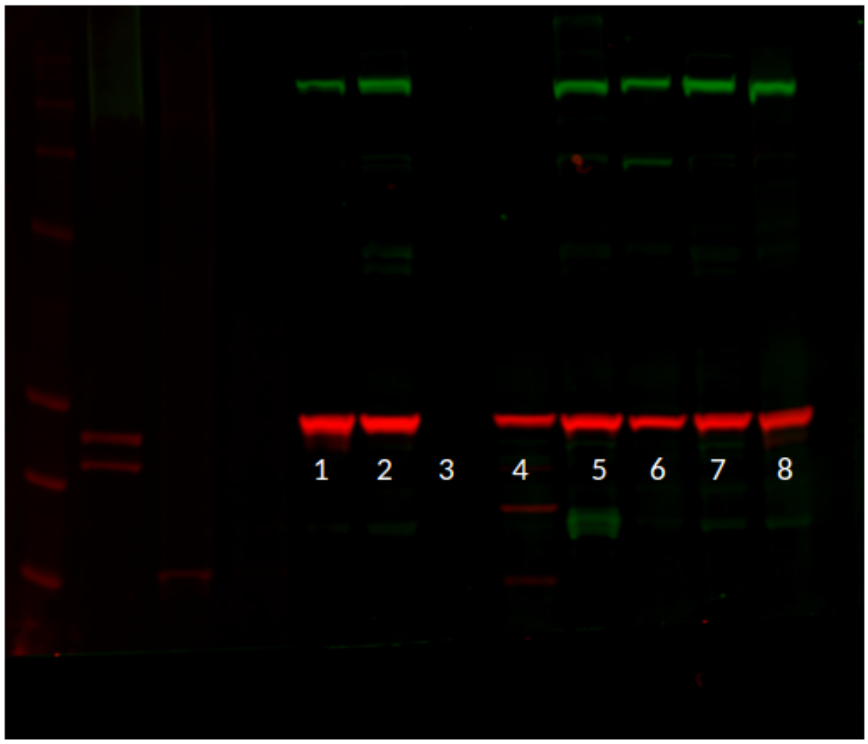
Next, I will be testing dox-inducible shRNA lentiviral expression constructs targeting NSD3 from a collection of in-house plasmids. With NSD3 knockdown cells, I will be able to further validate antibodies for subsequent experiments and screen for a phenotype.

4. Additional Data

Several cell samples described above were re-run on a gradient gel (Figure 2). Additionally, I was given a protein normalized lysates prepared by Magda in our lab to run (Figure 3). Finally, I obtained CCLE data and looked at NSD3 gene expression across AML cell lines in the data set.



Ladder = BLUeye Prestained (FroggaBio Cat# PM007)



1. LnCap
2. ZK251
3. Molm-13
4. TUP
5. SKMM2
6. Th70
7. HCT116
8. Mun75

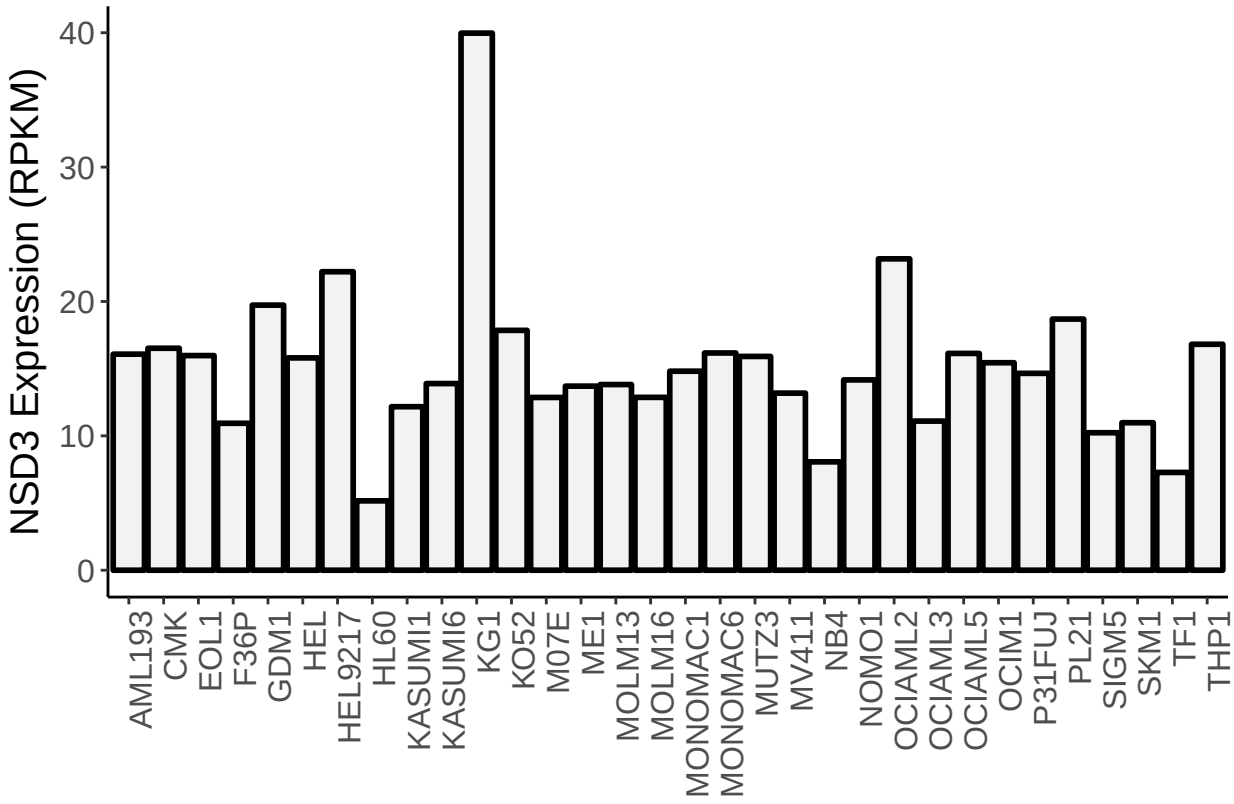
Green – NSD3 (AbCam ab137430)

Red – Tubulin

Ladder = BLUeye Prestained (FroggaBio Cat# PM007)

Plotting NSD3 Expression From CCLE Data in R

```
library(tidyverse)

# Gene expression data was downloaded from the Broad Institute's
# Cancer Cell Line Encyclopedia - https://portals.broadinstitute.org/ccle.
# Gene expression values are reported as RPKM.

ccle_meta <- read.csv(file = "./data/exp012/CCLE_sample_info_file_2012-10-18.txt",
                      sep = "\t")

ccle <- read_tsv(file="./data/exp012/CCLE_RNAseq_rpkm.txt", skip = 2)

#Identify AML Cell Lines in data set
ccle_meta_aml  <-  filter(ccle_meta,
                          Hist.Subtype1 == "acute_myeloid_leukaemia")
cells <- ccle_meta_aml$CCLE.name
cells <- as.character(cells)

# Filter gene expression data NSD3/WHSC1L1(ENSG00000147548) & AML Cell Lines
ccle_NSD3 <- filter(ccle, Name == "ENSG00000147548")
ccle_NSD3_AML <- select(ccle_NSD3, one_of(cells))
ccle_NSD3_AML <-  gather(ccle_NSD3_AML, Cell.Line, NSD3_Expression)
cell.lines <- gsub(pattern = "_HAEMATOPOIETIC_AND_LYMPHOID_TISSUE",
                   replacement = "", ccle_NSD3_AML$Cell.Line)
ccle_NSD3_AML$Cell.Line <- cell.lines

#Plot of NSD3 Expression in CCLE AML Cell Line Data
ggplot(ccle_NSD3_AML, aes(x=Cell.Line, y=NSD3_Expression)) +
  geom_bar(stat = "identity", fill = "gray95", color="black", size = 1) +
  theme_classic() +
  theme(text = element_text(size=15),
        axis.text.x = element_text(angle = 90, hjust = 1)) +
  xlab("") +
  ylab("NSD3 Expression (RPKM)")
```


NSD3 Expression (RPKM)
40
30
20
10
0
AML193
CMK
EOL1
F36P
GDM1
HEL
HEL9217
HL60
KASUMI1
KASUMI6
KG1
KO52
M07E
ME1
MOLM13
MOLM16
MONOMAC1
MONOMAC6
MUTZ3
MV411
NB4
NOMO1
OCIAML2
OCIAML3
OCIAML5
OCIM1
P31FUJ
PL21
SIGM5
SKM1
TF1
THP1

ExpID-012