Expression of NSD3short-3xFLAG in Multiple Cell Lines

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2018-02-26

Objective.To generate cell lines stably expressing NSD3-Short-3xFLAG in relevant cell backgrounds. These cell lines can be used to assay phenotypes associated with over-expression and to study NSD3-short protein-protein / protein-chromatin interactions. The lentiviral vectors used here have been modified by replacing an IRES bicistronic element with a T2A self-cleaving peptide sequence (cloning described in exp-016) for expression of NSD3short-3xFLAG and a puromycin selection marker.

1. Experimental Details

1.1. Producing Lentivirus in HEK293-T Cells

1. 24 hours prior to transfection 2 x 10^6 HEK293T cells were plated in 10 cm^2 dishes.
2. The following day cells were transfected with lentiviral construct and packaging vectors using jetPrime (Polyplus - Cat # 114-15), following manufacturer's instructions.
3. Cells were incubated overnight and the following morning HEPES (pH 7.5) was added to 10 mM to maintain pH.
4. 48 hours following transfection media was collected and the HEK293T cells bleached and discarded.
5. Media containing virus was passed through a 0.45 μm PES filter (Millipore) to remove cell debris.

Transfection Mix for 1 x 10 cm Dish

Lenti Plasmid	Concentration - Volume (5ug)	Lenti Packaging (5ug)	jetPrime Buffer	jetPrime
EmptyVector	458 ng/ul - 11 uL	5 uL	0.5 mL	20 uL
NSD3-3xFLAG(WT)	253.4 ng/ul - 19.8 uL	5 uL	0.5 mL	20 uL
NSD3-3xFLAG(W284A)	254 ng/ul - 19.7 uL	5 uL	0.5 mL	20 uL

Lenti Packaging Mix 1:1:1 - 1 μg/ul (Provided by DB)
pMDG - Envelope - Addgene #12259
REV pRSV - Rev - Addgene #12253
RRE pMDLg - Gag & Pol - Addgene #12251

1.2 PEG Concentration of Virus

5x PEG Solution
* 20% PEG8000
* 1.2 M NaCl
* 1 mM EDTA pH 8
-Dissolve in water, and filter 0.2 um.

1. PEG solution was added to clarified media to 1x (2.5 ml to 10 ml viral media)
2. Virus was incubated overnight at 4 ^{o}C.
3. Following day, samples were centrifuged at 1500 x G for 30 min to pellet PEG/viral particles.
4. The supernatant was discarded, then samples were spun again for 5 min and remaining supernatant pipetted off.
5. Viral pellet was than resuspended in 1 ml cold 1x PBS (10x concentrated viral stock), with a portion being used directly to transduce H1299 cells and the remainder stored at -80^{o}C for transduction of other cell types.

1.3 Transduction of H1299 Cells

To transduce cells for stable I selection, I followed a protocol outlined by AddGene (https://www.addgene.org/protocols/generating-stable-cell-lines/). The protocol outlined below describes the protocol for H1299 cells. However, the same process was used for multiple cell lines with only slight variations.

1. Prepare media (RPMI - 10% FBS + Pen/Strep) containing 2μg/mL polybrene.
2. The 10x virus prep was diluted into polybrene containing media at the following dilutions and added (500 μ/L per 6 well dish).

Dilution Setup:

Dilution	Volume 10x Virus	Volume Media (uL)
0	0	500
1:5	100	400
1:50	10	490

3. To reverse transduce H1299 cells, H1299 cells were trypsinized, counted and resuspended at 100 000 cells /ml in polybrene containing media (2 μg/mL).
4. 1 mL of cell suspension was added to each well containing diluted virus and incubated overnight.
5. The following morning cells were washed in 1x warmed PBS and fresh media added.
6. Approximately 48 hrs following transfection, media was changed and fresh media containing 2 μg/mL puromycin (Sigma) was added.
7. Cells were cultured for ~2 weeks in the presence of puromycin 2 μg/ml, at which point there remained no viable cells in the no virus control.

1.4 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. 2 ml of cells (~2 x 10^6) were pelleted by centrifugation at 300 x G for 5 min.
2. The supernatent was removed and cells were washed 1x in 500 μL 1 x buffer, then resuspended in 100 μL of lysis buffer.

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. Dilutions shown below:

Target	Supplier - Cat#	Dilution
NSD3 (RabMono)	AbCam - ab180500	1:5000
FLAG (MouseMono)	Sigma - F3165	1:20000
Histone-H3 (RabPoly)	AbCam - ab70550	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 & 2)

1.5 Imaging on Incucyte

1. H1299 cells (EV, NSD3short-3xFLAG[WT], and NSD3short-3xFLAG[W284A]) were plated at 2000 cells per well of a 96 well plate in quadruplicate.
2. Plates were incubated for 72 hours and for images per well taken using an IncuCyte live cell imaging platform.
3. IncuCyte software was used to estimate cellular eccentricity. (Results shown in figure 1C & figure code shown at the end of this document).

2. Result & Oberservations

Here, I successfully transduced multiple cell lines and selected for stable transgene expression. Importantly, replacing the IRES with a T2A sequence within the vector greatly increased transduction efficiency. This protocol was used to infect multiple cell types including H1299 (figure 1), as well as HeLa and MV4;11 cells (Figure 2). Interestingly, H1299 cells overexpressing either the wild-type or PWWP1 mutant displayed an altered morphology, appearing more elongated and in some instances with extended projections (Figure 1B). This phenotype was quantified by measuring morphological eccentricity using the IncuCyte imaging system (Figure 1C), with lower values indicated a more rounded shape. This observation indicates that H1299 lung cancer cells may be a useful backdrop to study NSD3's function in the context of cancer.

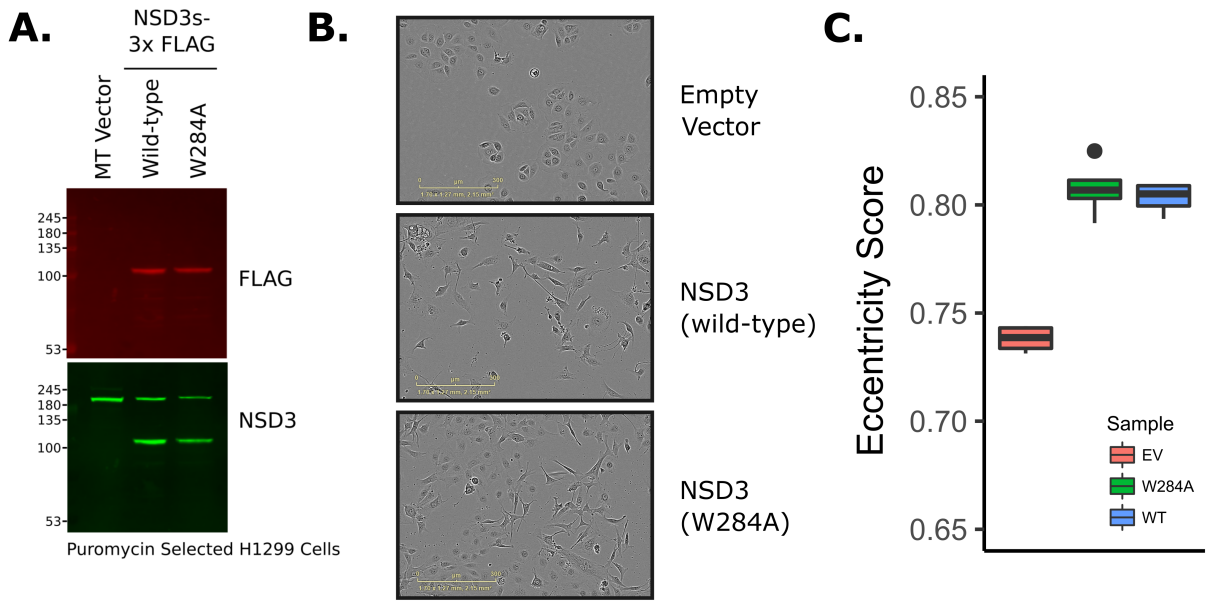

Figure 1: **Expression of NSD3short-3xFLAG in H1299 Lung Cancer Cells** (A) Western blot of NSD3 and FLAG in stable cell lines (B) Represesntaive phase constrast images (C) Boxplot of cellular eccentricity (mean of 4 images across 4 wells per sample)

R Code for Plotting Figure 1.C

```
require(tidyverse)

# Read TSV containing incucyte data
data <- read_tsv(file="./data/exp018/H1299_Eccentricity_13032018.tsv.txt")

#Order data for plotting
data$Sample <- factor(data$Sample,
                      levels = c("EV", "WT", "W284A"))

#Pull values for significance testing
EV <- data %>%
    filter(Sample == "EV") %>%
    select("Eccentricity") %>%
    pull()

WT <- data %>%
  filter(Sample == "WT") %>%
  select("Eccentricity") %>%
  pull()

W284A <- data %>%
  filter(Sample == "W284A") %>%
  select("Eccentricity") %>%
  pull()

#Display p-values
```

```r
WT_ttest <- t.test(EV, WT, paired = FALSE)
print(WT_ttest)
```

```
##
##  Welch Two Sample t-test
##
## data:  EV and WT
## t = -13.448, df = 5.8203, p-value = 1.327e-05
## alternative hypothesis: true difference in means is not equal to 0
## 95 percent confidence interval:
##  -0.07711196 -0.05321969
## sample estimates:
## mean of x mean of y
## 0.7381309 0.8032967
```

```r
W284A_ttest <- t.test(EV, W284A, paired = FALSE)
print(W284A_ttest)
```

```
##
##  Welch Two Sample t-test
##
## data:  EV and W284A
## t = -9.2655, df = 4.1978, p-value = 0.0005987
## alternative hypothesis: true difference in means is not equal to 0
## 95 percent confidence interval:
##  -0.08984317 -0.04900003
## sample estimates:
## mean of x mean of y
## 0.7381309 0.8075525
```

```r
#Barplot Eccentricity Score
plot <- ggplot(data, aes(Sample, Eccentricity, fill = Sample)) +
          geom_boxplot() +
          theme_classic() +
          theme(axis.text.x = element_blank(),
                axis.ticks.x = element_blank()) +
          scale_y_continuous(limits = c(0.65, 0.85)) +
          ylab("Eccentricity Score") +
          xlab("")
```

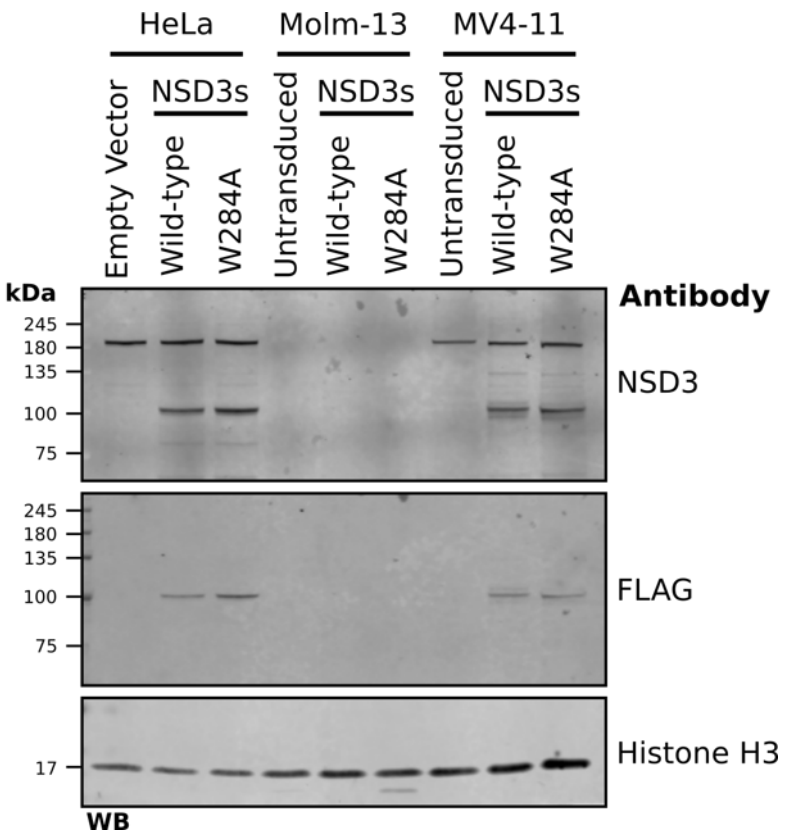

Figure 2: Western blot of NSD3, FLAG and Histone H3 in HeLa, Molm-13, and MV4;11 cells transduced and puromycin selected.

ExpID-018