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INVESTIGATING THE ROLE OF FANCD2 IN NEUROGENESIS

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BY

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ABSTRACT

Fanconi anemia (FA) is a rare human genetic disease characterized by bone marrow failure, congenital abnormalities, predisposition to cancer, accelerated aging, and premature mortality. FA is caused by mutations in any one of 23 genes. The FA proteins play a crucial role in the repair of DNA interstrand cross-links (ICLs) and the maintenance of genome stability. Recently, central nervous system (CNS) defects have become increasingly observed among FA patients. These include acute or chronic loss of neurological capabilities, accumulation of white matter lesions and large pseudo-tumor lesions with vasogenic edema, and retinal vasculopathy. These neurological manifestations have been coined Fanconi Anemia Neurological Syndrome (FANS). The molecular origins of FANS are unknown. Recent data from our lab and others shows that, under conditions of replication stress, the FANCD2 protein binds to several transcriptionally active large neural genes. Many of these genes encode for proteins that function in neuronal differentiation, migration, and cell adhesion, and many are linked to neurodevelopmental and neuropsychiatric disorders. We hypothesize that FANCD2 may play an important role in the maintenance of genome stability during neurogenesis, when neural stem and progenitor cells are undergoing rapid proliferation and expansion. To study the role of FANCD2 in neuronal differentiation, we have developed a lentiviral doxycycline-inducible shRNA system to inducibly deplete FANCD2 in the SH-SY5Y neuroblastoma line. This system will allow us to determine if loss of the

FANCD2 protein adversely impacts the ability of SH-SY5Y cells, as well as other cells, to differentiate into mature neurons.

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I would like to thank Dr. Claudia Fallini for all her help and guidance. Thank you very much for all you did and continue to do for me. I wouldn't have arrived at this point without you. I'm very indebted to you. Furthermore, I would like to extend my thanks to Michelle Gregoire for teaching me how to handle the iPSCs and many helpful discussions about differentiation experiments. Also, I'd like to thank Emily Potts and Dr. Riccardo Sirtori for their help with many experiments. Moreover, I'd like to thank Dr. Katharina Quinlan for being on my committee and guiding me and providing insightful avenues. Finally, I'd like to thank dr. Jessica Alber for agreeing to be and chairing my committee.

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ABBREVIATION

Abbreviation	Definition
FA	Fanconi Anemia
ICLs	Interstrand crosslinks
CNS	Central Nervous System
FANS	Fanconi Anemia Neurological Syndrome
iPSC	induced Pluripotent Stem Cell
BMF	Bone Marrow Failure
DDR	DNA Damage Response
HR	Homologous Repair
DSB	Double Strand Breaks
ROS	Reactive Oxygen Species
RA	Retinoic Acid
BDNF	Brain Derived Neurotrophic Factor
GFP	Green Fluorescent Protein
MAP2	Microtubule-Associated Protein 2
GAP-43	Growth Associated Protein 43
PARP	Poly (ADP-ribose) polymerase
PCNA	Proliferating Cell Nuclear Antigen
SSB	Single Strand Break
BER	Base Excision Repair
MMR	Mismatch Repair
NER	Nucleotide Excision Repair

NHEJ	Non-Homologous End Joining

INTRODUCTION

Fanconi anemia (FA) is a rare genetic disease characterized by bone marrow failure (BMF), congenital abnormalities, predisposition to cancer, and an average lifespan of approximately 29 years (Niraj et al, 2019). FA occurs in 1 out of every 100,000-160,000 people (Peake et al, 2022). FA is caused by biallelic mutations in any one of 23 genes. The FA genes encode for proteins that function in the FA pathway (Niraj et al, 2019). This pathway interacts together to repair DNA interstrand cross-links (ICLs) and to protect stalled replication forks during S-phase of the cell cycle (Nepal et al, 2017). ICLs arise from endogenous sources such as production of aldehydes as metabolic byproducts, and this could lead to genomic instability, and subsequently the depletion of hematopoietic stem cells (Rogers et al, 2020). During the DNA damage response (DDR), the FA core E3 ligase complex will monoubiquitinate FANCD2-FANCI (ID2) complex (Shigechi et al, 2012). To facilitate DNA repair, the ID2 complex will re-localize to the damaged chromatin, serving as protector of the genome (Shigechi et al, 2012). The FANCD2 protein, a master regulator of ICL repair, coordinates ICL unhooking by employing nucleases, which will lead to DNA double-strand breaks that will be repaired by homologous recombination (HR) (Ceccaldi et al, 2016). Apparently, monoubiquitinated ID2 complex permits downstream events of DNA damage. It's thought that during ICL repair, recruit HR factors, such as the tumor suppressor BRCA2 and the recom-

binase enzyme RAD51, to abrogate the DNA double-strand breaks (DSB) arising from processing DNA crosslink. Furthermore, monoubiquitinated ID2 regulates the replicative bypass of unhooked DNA crosslinks by translation DNA synthesis polymerase (Liang et al, 2019).

Recently, FA patients have shown multiple neurological malformations. Case studies have reported unique clinical manifestations in FA patients. They reported that 60% of FA patients had structural neurological anomalies (Johnson-Tesch, 2017, Aksu et al, 2020). These abnormalities range from microcephaly, small pituitary gland, cerebellar hypoplasia, to abnormalities of the corpus callosum, hydrocephalus, and malignancies (Fiesco-Roa et al, 2022). Brain imaging has shown that there are increased abnormalities of the CNS. When researchers compared brain images of FA patients, they found that 61% had at least one CNS or skull-based deformity (Johnson-Tesch, 2017, Aksu et al, 2020). Most of the frequent structural CNS anomalies localized to the midline, 46% pituitary hypoplasia, and 32% in posterior fossa consisting of cysts, vermis, and ponto-cerebellar hypoplasia (Johnson-Tesch, 2017, Aksu et al, 2020). However, CNS malformation in FA patients has been reported to be as high as 90% when brain imaging is used in patients with either neurological or endocrine manifestations, and some of these anomalies require neurosurgery (Stivaros et al, 2015). Evaluation of genotype-phenotype association in FA patients has associated those structural CNS abnormalities with the ID2 complex more specifically microcephaly (Fiesco-Roa et al, 2019).

The normal development of the brain requires faithful orchestration of massively proliferating, differentiating, and migrating neuroprogenitors (NPs) and neural stem cells before they are committed to the central nervous system (CNS). All cell types in the brain originate from those two cell types (NPs and neural stem cells), and they share a similar nuclear genome although they have epigenomic differences among cell types and developmental stages (Lui et al, 2011). During neurogenesis, for NPs to produce 80 billion neurons in proliferative regions they must undergo massive DNA replication and cell division (Dehay et al, 2007). Since NPs undergo massive proliferation, they require massive energy supply and thus generate elevated levels of reactive oxygen species (ROS) as a byproduct of respiration (Chui et al, 2020; Sai et al, 2018). Oxidative stress caused by ROS leads to DNA damage as well as the replication stress imposed by the rapid proliferation nature of NPs (Wang et al, 2020). If a mutation occurs in NPs and persists in the daughter neurons they may cause neurodegeneration in adulthood. Therefore, faithful DNA repair pathways such as homologous recombination is of utmost importance (Mitra et al, 2019).

Recent studies have found increased DNA damage in neural progenitor cells (NPs) which constitutes a threat to genome stability and the viability of NPs and in turn neuronal function. Maintaining genome integrity is critically important during active proliferation of NPs in the developing brain (Madabhushi et al, 2014; S. von Bartheld et al, 2016). The brain is a major organ targeted

by DNA damage during neurogenesis and the maintenance of adult neuronal function (Qing et al, 2022).

Patients that suffer from at deficiency exhibit human chromosome instability syndromes. Those that suffer from such syndromes, Ataxia Telangiectasia for example, exhibit various symptoms among them neurological deficits such as ataxia, microcephaly and intellectual disabilities. The overlap between the DDR and these neurological phenotypes suggests a role for the DDR in preventing neuropathology in development and adult life (Qing et al, 2022). Given the role that FANCD2 plays in the DDR (Niraj et al, 2019), and the accumulation of FANCD2 on long neural genes under conditions of replication stress (Okamoto et al., 2018; Blaize et al., 2023), we hypothesize that FANCD2 may play a key role in maintaining the stability of certain neural genes under conditions of replication stress.

NPs genome instability could cause genetic variation and diverse neurological syndromes including intellectual disability, autism symptoms, and brain malformation such as microcephaly and pontocerebellar hypoplasia (Mcconnell et al, 2017). Studies have shown that FANCD2 is involved in the maintenance of genome stability. Furthermore, FANCD2 has recently been found to accumulate at large transcribed genes under conditions of replication stress (Okamoto et al., 2018; Blaize et al., 2023). FANCD2 might play an important role in maintaining the stability of large actively transcribed neuronal genes, and in the absence of FANCD2, these genes are prone to higher levels of DNA damage

with or without replication stress (Okamoto et al., 2018). Furthermore, ChIP-seq data from our lab shows that FANCD2 binds to large actively transcribed genes under conditions of replication stress and those genes are involved in autism, neurodevelopmental or psychiatric disorders (Blaize et al., 2023). Thus, we hypothesize that FANCD2 may play a role in efficient neural differentiation under conditions of replication stress. We will test this hypothesis by investigating the differentiation of SH-SY5Y, originally derived from metastatic bone tumor biopsy, in the presence and absence of FANCD2. SH-SY5Y can be differentiated to a more mature neuron-like phenotype characterized by neuronal markers using retinoic acid (RA) as a means for differentiation. The ability to be differentiated, and the fact it is a human-derived make it suitable model to study neural differentiation. Our studies will begin to define the role of FANCD2 in the process of neuronal differentiation.

These experiments will advance our understanding of the role of FA pathway in nervous system development. It will provide insight into the origins of those emerging neurological symptoms in the FA patients and will aid in furthering our understanding of the pathophysiology of the FANS. Moreover, it will further our understanding of nervous system development and decline. This knowledge will help develop better therapeutic options for FA patients to have a better quality of life.

MATERIALS AND METHODS

Cell line maintenance and differentiation

The primary neurons derived from embryonic CNS tissue are limited in use because when they differentiate fully into mature neurons, the cells can't be cultured any longer. Thus, we use transformed neuronal-like cell lines (SH-SY5Y) in vitro to overcome this limitation. SH-SY5Y derived originally from metastatic bone tumor biopsy, SH-SY5Y (ATCC, CRL-2266) is a subline of the parental line SK-N-SH. SH-SY5Y can be differentiated to a more mature neuron-like phenotype characterized by neuronal markers using retinoic acid (RA) as a means for differentiation. The capacity for large-scale expansion of these cells prior to differentiation, with relative ease and low cost to culture compared to primary neurons offer a huge advantage.

Furthermore, SH-SY5Y cells are human-derived which means they express a number of human-specific proteins and protein isoforms (Kovalevich et al, 2013). Those cells form neurites when they differentiate to communicate with other neurons. We have confirmed that this cell line expresses FANCD2 which makes it, in addition to its ability to differentiate into neurons, a suitable biological system to test our hypothesis. We will be able to silence FANCD2 and then differentiate them in the presence of a replication stressor. This will inform us if FANCD2 is crucial for the differentiation and neurites formation in SH-SY5Y.

SH-SY5Y cells were differentiated and cultured using a previously reported protocol (Dravid et al, 2021). Undifferentiated cells were maintained in basic growth medium, comprised of Dulbecco's Modified Eagle's Medium (DMEM)

(Gibco Life Technologies, # 11965092), supplemented with fetal bovine serum (FBS) (Life Technologies, # A5256701), 1× glutamax-I (Gibco Life Technologies, # 35050061) and 1× penicillin/streptomycin (Gibco Life Technologies, # 15070063). To induce differentiation, cells were seeded onto Matrigel-coated (1:100 dilution) (Corning, #356234) dishes at the specified densities relevant to the experiment. The following day, the medium was changed to Stage I medium comprised of DMEM supplemented with FBS (2.5%), 1 × glutamax-I, 1 × penicillin/streptomycin and retinoic acid (RA) (10 μM) (Sigma, R2625, Sigma Aldrich, MO, USA). After five days, the medium was changed to Stage II medium comprised of Neurobasal media (Gibco Life Technologies, # 21103049) supplemented with brain-derived neurotrophic factor (BDNF) (50 ng/mL) (PeproTech, # 450-02), potassium chloride (KCl) (20 mM) (Sigma, #P5405), 1×B27 (Gibco Life Technologies, # 17504044), 1×glutamax-I and 1×penicillin/streptomycin for an additional five days. Cultivated cells were maintained below passage 12 at 37 °C/5% CO2 (Figure 1). HEK293T cell line (from Horizon) were grown in a DMEM (High Glucose, + Sodium Pyruvate (110 mg/L), no L-glutamine) supplemented with 10% FBS, 1x Penicillin-Streptomycin, and 1x L-Glutamine. HEK293T is used to generate the lentivirus titers.

Generating a shRNA doxycycline inducible cell line

The short hairpin RNA (shRNA) will be used to generate a doxycycline-inducible system to deplete FANCD2. shRNA inducible system reversibly turns on/off transcription in the presence of doxycycline. When doxycycline is present, the shRNA will be transcribed in the nucleus by polymerase II once the

vector has integrated into the SH-SY5Y genome. The resulting pre-shRNA is exported from the nucleus by exportin 5. This product is then processed by Dicer and loaded onto the RNA-induced silencing complex (RISC). The anti-sense strand directs RISC to mRNA that has a complementary sequence. The sense strand is degraded. This can be reversed at any time by removing doxycycline. This will allow us to study the role of FANCD2 in the SH-SY5Y differentiation in a timely manner by turning on/off FANCD2 by the addition of doxycycline. The shRNA plasmid contains a TRE-3G promoter that drives the expression of both the TurboGFP and the SMARTvector shRNA scaffold (**Figure 2**). This promoter is under the control of doxycycline which means the promoter will not be active unless doxycycline is added. When the promoter is active it will drive the expression of the TurboGFP as well as driving the expression of SMARTvector shRNA scaffold to produce the mRNA that will target the FANCD2 mRNA for degradation. The hEF1α promoter is constitutively active and drives the expression of puromycin resistance. When the SH-SY5Y integrates the cassette and puromycin is added, it will enable us to select for cells that have integrated the cassette and express the puromycin resistance. Four shRNA constructs were purchased from Horizon Discovery. Three of those are shRNA constructs that target different sequences in the FANCD2 cDNA, and the fourth is a non-silencing control shRNA (**Figure 3**). In the lab, we grew the *E. coli* that contain the plasmids in LB in the presence of 100 µg/mL carbenicillin at 37°C overnight shaking. Next day, a DNA maxiprep was performed to extract the plasmids following the manufacturer's instructions.

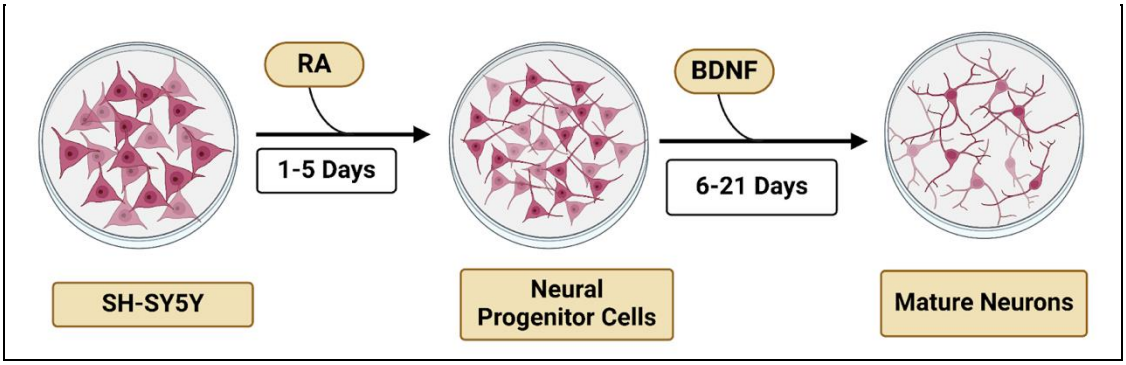


Figure 1. A schematic representation of the differentiation of SH-SY5Y. Cells are plated in a Matrigel-coated 6-, 12-, or 24-well dish vessel of desire. After 24 h incubation, the media is changed to stage I media containing retinoic acid (RA). RA promotes stem cell neural lineage specification. On day 6, the media is changed to Stage II media containing brain-derived neurotrophic factor (BDNF). BDNF is involved in neural plasticity, neurogenesis, stress resistance and survival of the cell. The cells can be cultured in Staged II media for more than 21 days.

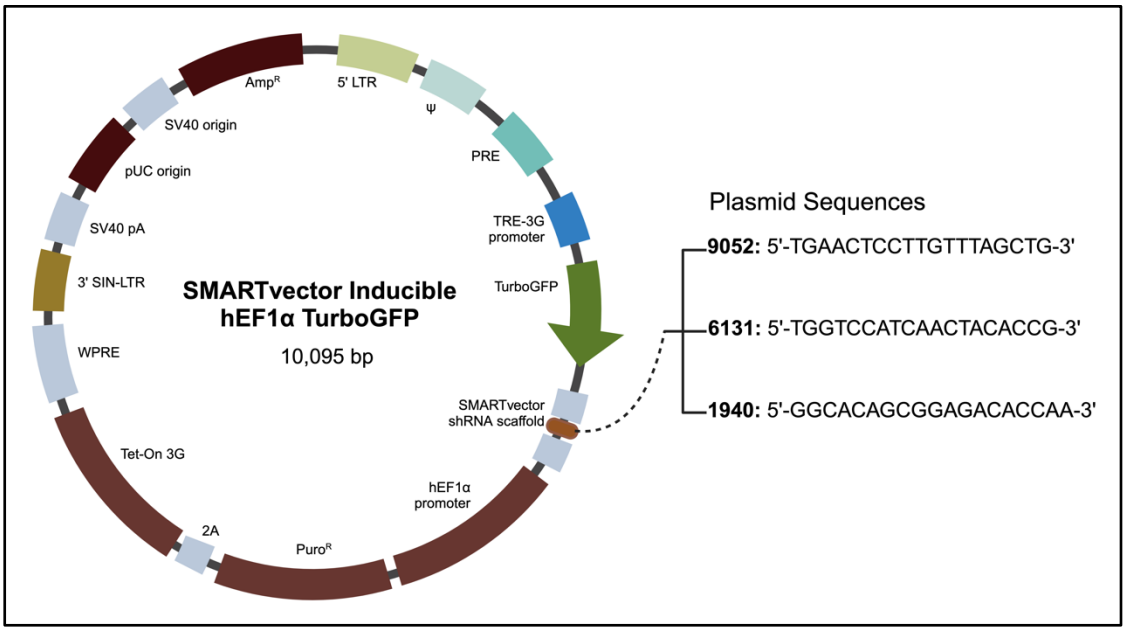


Figure 2. A map of the shRNA plasmid. This map shows the TRE-3G promoter at position 2240-2615. This promoter drives the expression of the TurboGFP (2670-3368) as well as the expression of the SMARTvector shRNA scaffold (3463-3869). This promoter is under the control of doxycycline. It's active only when doxycycline is present. The SMARTvector shRNA scaffold contains the target sequence of the plasmids. The hEF1α promoter (3890-5007) is constitutively active and drive the expression of the puromycin resistance. We have purchased three plasmids, each one expressing an shRNA that targets a different region of the FANCD2 cDNA.

After that, plasmids were confirmed by restriction enzyme digestion and by sequencing. Once confirmed, we transfected HEK293T cells with the shRNA plasmids together with the lentiviral packaging mix to generate lentivirus. Lentiviral supernatants from the transfected HEK293T cells were then used to infect SH-SY5Y cells. After infection, cells that integrated the lentivirus were selected in 2.5 μM puromycin.

Determining doxycycline dose and time required to deplete FANCD2 protein

After puromycin selection, SH-NT-1 (non-targeting control) and SH-9052-1 (FANCD2 shRNA integrated cells) were plated at 250k cell/well in 6-well plates and incubated overnight at 37°C, 5% CO2. The next day, cells were treated with 100, 500 ng/ml and 1 μg/ml doxycycline for 24 h, 48 h and 72 h. Cells were pelleted and harvested for immunoblotting confirmation of the knockdown at 24, 48 and 72 h.

Immunoblotting

To assess FANCD2 knockdown, we used immunoblotting to look at FANCD2 and other makers levels at certain point in time. For immunoblotting analysis cell pellets were washed in phosphate-buffered saline (PBS) and lysed in 2% (wt/vol) SDS, 50 mM Tris-HCl, and 10 mM EDTA followed by sonication for 10s at 10% amplitude using a Sonic Dismembrator model 500 (ThermoFisher Scientific). Proteins were resolved on NuPAGE 3-8% (wt/vol) Tris-acetate or 4-

12% (wt/vol) Bis-Tris gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes. The following antibodies were used: rabbit polyclonal antisera against FANCD2 (NB100-182; Novus Biologicals), rabbit polyclonal MAP2 (18818S; Cell Signaling), mouse monoclonal Anti-β-Tubulin III (T8660; Millipore Sigma), rabbit polyclonal GAP-43 (18818S; Cell Signaling).

Viability Assay

To determine if the doxycycline concentration is toxic to cells or not, we set to perform a viability assay. To achieve optimal knockdown, we wanted to make sure the concentration chosen will not lead to cell death. Thus, the newly generated cells were tested for toxicity at 100 ng/ml, 500 ng/ml, and 1 µg/ml doxycycline for 24, 48 and 72 h. Cells were plated at a 100k cell/well in three 12-well plates for 24, 48 and 72 hrs and grew overnight at 37°C, 5% CO2. Next day, Cells were treated with the respective doxycycline concentration in triplicate and incubated overnight at 37°C, 5% CO2. Then, used the LIVE/DEAD® Viability/Cytotoxicity Kit from Thermo Fisher at 24, 48 and 72 hrs. One the day of the assay, the cells were washed with 500 µL Dulbecco's phosphate-buffered saline (D-PBS). Then treated the positive control (dead) with 70% methanol for 30 min to kill all the cells. Then, 20 µL of supplied 2 mM ethidium homodimer-1 (EthD-1) stock solution (Component B) were added to 10 mL of sterile, tissue culture-grade D-PBS, this gives an approximately 4 µM EthD-1 solution. Then, we combined the reagents by transferring 5 µL of the supplied 4 mM calcein AM stock solution (Component A) to the 10 mL EthD-1

solution (2 µM calcein AM). The mixture was then vortexed the resulting solution to ensure thorough mixing. Then, added 150 µL of the combined LIVE/DEAD® assay reagents, so that all cells are covered with solution.

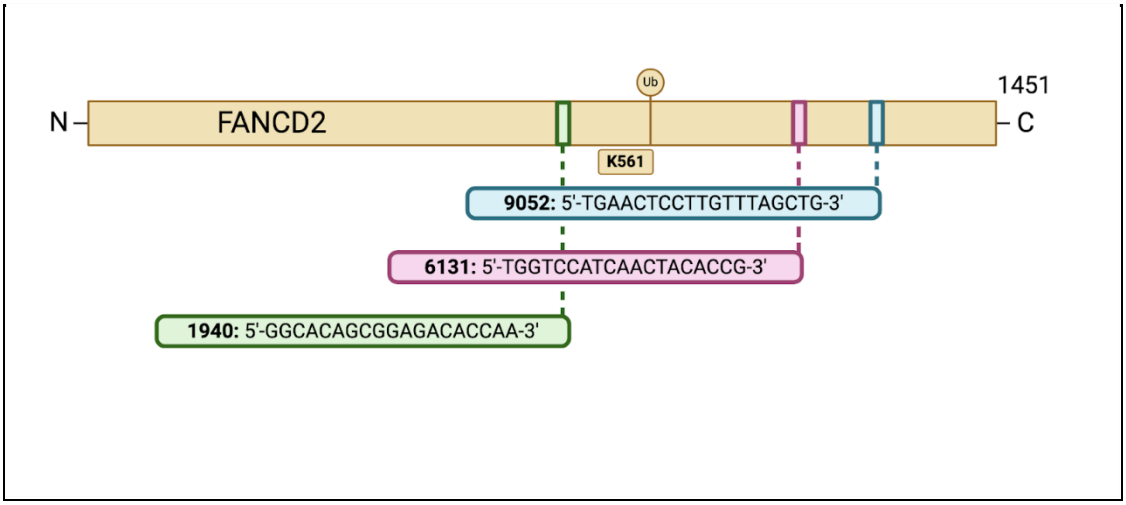


Figure 3. A schematic of the FANCD2 protein and which regions of the FANCD2 mRNA sequence the three shRNA sequences target. This is an estimation of where the three plasmids align to FANCD2 cDNA. We have chosen three different regions to determine later which location is the optimal to deplete FANCD2 using this system.

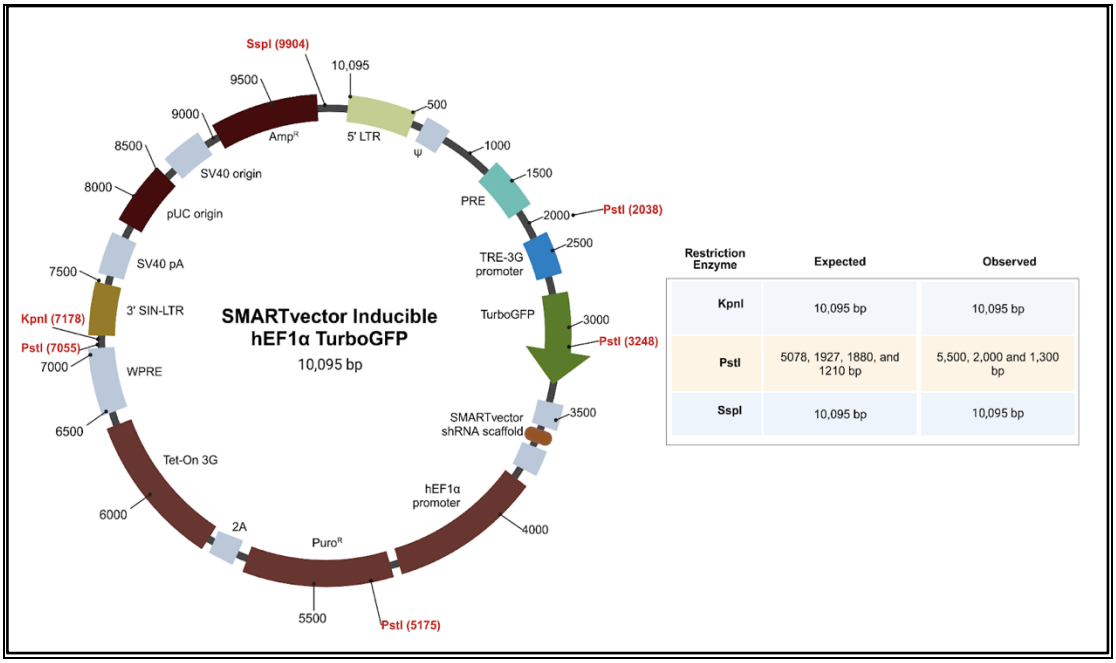
Incubated the cells for 45 min at room temperature. After 45 min, cells were imaged using EVOS FL.

RESULTS

Generation of shRNA cell lines

Plasmid extraction and confirmation

The three plasmids (plasmid# 9052, 9041, and 6131) have been extracted from the *E. coli* using Maxiprep kit and obtained a plasmid DNA concentration of (644.3, 904.4, and 66.8 ng/µL) respectively. Following that, those plasmids have been confirmed by restriction enzyme digestion. For restriction enzymes, KpnI, PstI and SspI have been used to confirm the plasmid. KpnI is a one-cutter and would produce a band at 10,095 bp and the observed band is at 10,095bp (**Figures 4 and 5**). SspI produces one band at 10,095 as well because it is a one-cutter enzyme and the observed band on the gel is 10,095 bp. A four-cutter enzyme PstI produces 4 bands at 1210, 1880, 1927, and 5078 bp and we observed bands at 5500, 2000, 1300 bp (**Figures 4 and 5**). The reason we see only 3 bands for PstI is because bands of size 1880 and 1927 are very close so they appear as one band. The other two plasmids show the same pattern because they are identical except for the shRNA scaffold that differ. This is expected for these plasmids which confirms that these are the plasmids of interest. Then, those plasmids have been confirmed via sequencing. The plasmids align perfectly to the cDNA of FANCD2 (**Figure 3**).



Restriction Enzyme	Expected	Observed
KpnI	10,095 bp	10,095 bp
PstI	5078, 1927, 1880, and 1210 bp	5,500, 2,000 and 1,300 bp
SspI	10,095 bp	10,095 bp

Figure 4. A schematic of the plasmid used. The map shows the different components of the plasmids as well as the size and location of each. The graph shows the restriction enzymes and where they cut throughout the plasmid. On the right, is a table of the restriction enzyme used with the expected and observed bands for each of them. KpnI is a one cutter that cuts at (7178 bp) and produces a band at 10,095 bp. SspI is a one cutter as well that cuts at (9904 bp) producing a band at 10,095 bp. PstI is a four-cutter enzyme that cuts at (2038, 348, 5175, 7055 bp) and produces three bands around ~ 5500, 2000 and 1300 bp. The other two plasmids show the same pattern because they are identical except for the shRNA scaffold that differ.

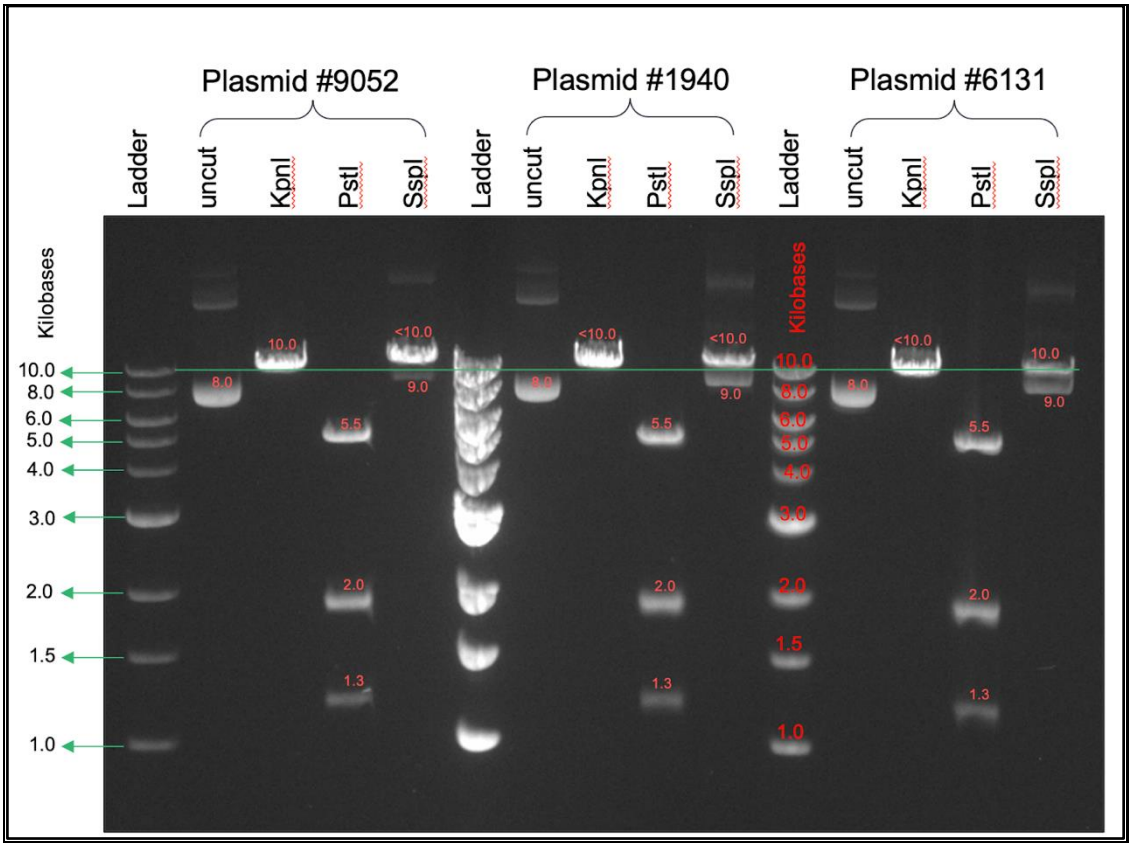


Figure 5. A confirmation gel electrophoresis image of enzyme digestion of the three plasmids with KpnI, PstI and SspI digestion enzymes.In lane 2, 3, 4 and 5 is the digestion of plasmid #9052, lane 2 is the uncut showing band at 10 kilobases, in lane 3 is the one-cutter KpnI enzyme showing a band at 10 kilobases. Lane 4 is digested with four-cutter PstI enzyme, expected bands are at 1210, 1927, 1880 and 5078 bp, observed bands at 5.5, 2.0 and 1.3 kilobases. The reason we don't see the third band is that it's very close in size to the second band. Lane 5 is digested with a one-cutter SspI that shows a band at 10,095 bp. The same thing applies for the two other plasmids.

Generating Virus in HEK293T cell

To generate a lentivirus, we used HEK293T cells. The HEK293T cells were supplemented with a mixture of the plasmid and the packaging mix. The HEK293T cell uses the five components of the virus in the packaging mix and the plasmid to generate a lentivirus that harbors the plasmid. The HEK293T cell releases the virus into their growth media (**Figure 6 and 7**). The media is collected after 48 h and used to infect the SH-SY5Y cells that have been plated the day prior. The SH-SY5Y cells are incubated with the viral media for 8 hours. Then, the cells are incubated in growth media for another 48 h. The growth media then changed to media containing 2.5 µg/mL puromycin to select for cells that have integrated the cassette.

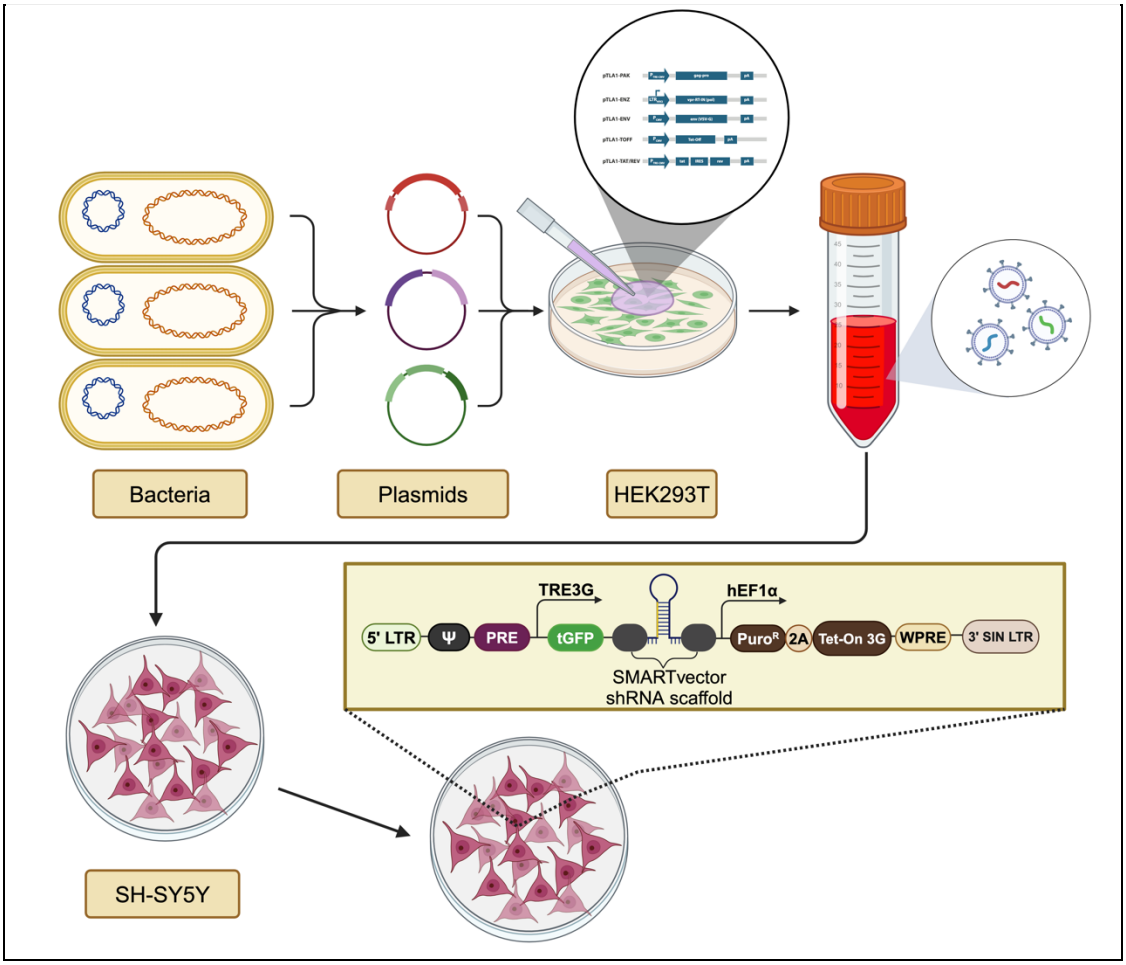


Figure 6. A schematic representation of the generation of lentivirus using HEK293T cells. Starting with *E. coli*, the plasmids are extracted from the bacteria and then confirmed via enzyme digestion and sequencing. The HEK293T cells are then used to generate the lentivirus by using the mixture of the five components of the lentivirus and the plasmid then releasing the virus into the growth media. The viral containing media is used then to infect the SH-SY5Y cell. The SH-SY5Y that have integrated the shRNA will be resistant to puromycin for selection of the shRNA integrating SH-SY5Y.

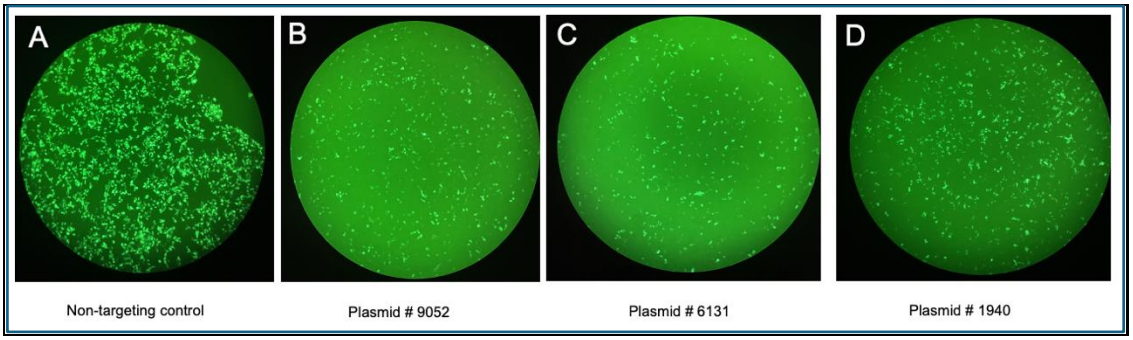


Figure 7. Microscopic images of the HEK293T cells after 48 h transfection with shRNA lentivirus. In image **A**, we see the HEK293T cells infected with the non-targeting plasmid have more cells expressing GFP. That's due to first, the non-targeting control plasmid is a different from the other three shRNA plasmids, and second, the GFP is constitutively active in the non-targeting control as opposed to the shRNA plasmid is which is under the control of the doxycycline. In image **B, C,** and **D,** we see that the HEK293T cells are expressing GFP to a lesser degree than the non-targeting.

Infecting SH-SY5Y cells with virus

SH-SY5Y were plated in a six-well plate a day prior. The collected lentivirus that was generated is then added to the SH-SY5Y cells for a duration of 8 hours. After the 8 hours, the media was changed to growth media and the cells were incubated for 48 hours. After 48 hours, the media was changed to growth media supplemented with 2.5 µg/mL puromycin and incubated for another 48 hours. Most of the cells that did not incorporate the cassette died, and the cells that integrated the shRNA cassette survived and formed colonies of cells (**Figure 8**). The cells were then cultured until they reached confluency.

Determining optimal doxycycline concentration

After the integration of the shRNA cassette, we wanted to determine what dose of doxycycline will induce FANCD2 depletion without leading to cell death in the newly generated cell lines. To determine the doxycycline concentration, we carried out an experiment whereby we exposed the newly generated cells that have integrated the shRNA cassette to (100 ng/mL, 500 ng/mL and 1 µg/mL) doxycycline and incubated the cells for 24 h, 48 h and 72 h. The cells were harvested at 24 h, 48 h and 72 h. Then lysed and ran on a western blot for FANCD2 protein (**Figure 9**).

Cell Viability

In order to determine the optimal concentration of doxycycline that is required to induce the depletion of FANCD2 and doesn't lead to cell death we have

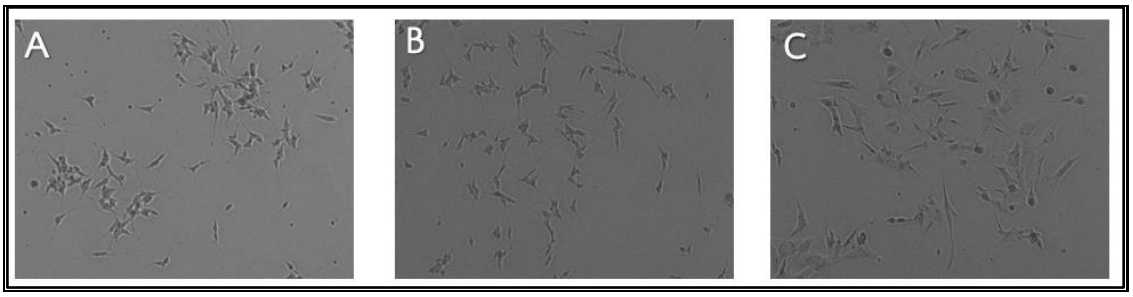


Figure 8. A 40X phase contrast microscopy image of SH-SY5Y cells after infection and puromycin selection. In image **A**, we see the SH-SY5Y cells infected with plasmid 9052, **B**, SH-SY5Y cells infected with plasmid 6131, **C**, SH-SY5Y cells infected with plasmid 1940. The survival of those cells indicate that those cells have integrated the shRNA cassette and are now resistant to puromycin by forming colonies.

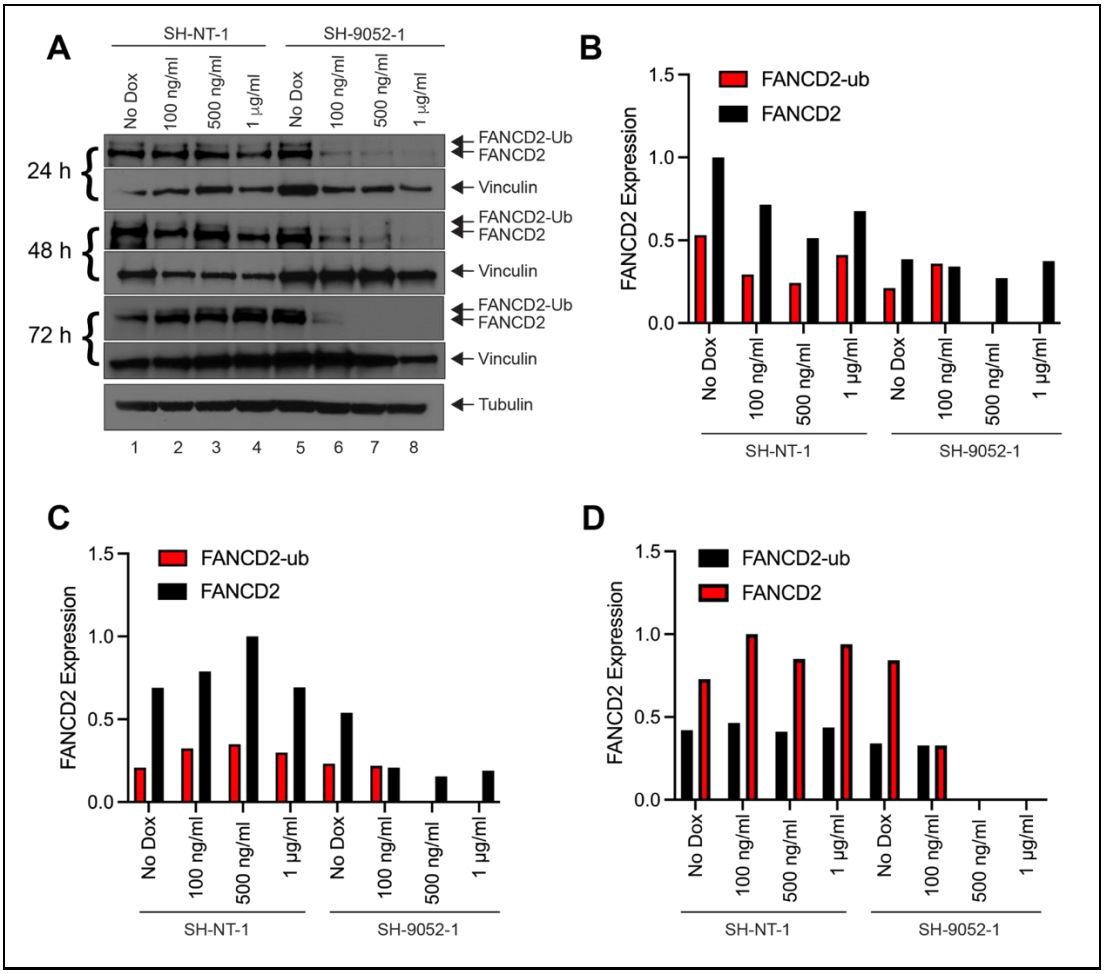


Figure 9. A western blot of the shRNA integrating SH-SY5Y after the addition of doxycycline for 24, 48 and 72 h. **A.** Top to bottom, at 24 h there is a depletion of FANCD2 after 24 h addition of doxycycline. At 100 ng/ml dose we see there is about 60% depletion of FANCD2 compared to non-targeting control (**B**). At 500 ng/ml there is about 70% FANCD2 depletion (**B**). At 1 µg/mL there is about 60% FANCD2 depletion compared to non-targeting control (**B**). At 24 h, there is about 70% FANCD2 depletion at 100 ng/ml and 1 µg/mL, and about 75% FANCD2 depletion at 500 ng/ml. At 72 h, there is about 40% depletion of FANCD2 at 100 ng/ml, and there is 100% depletion of FANCD2 at 500 ng/ml

and 1 μg/mL doxycycline. **B.** is a quantification of the 24 h western blot. **C.** is a quantification of the 48 h western blot, **D**. is a quantification of the 72 h western blot. From the quantification we see that the optimal doxycycline concentration that produced enough depletion of FANCD2 was 500 ng/mL for 48 h exposure.

have used LIVE/DEAD® Viability/Cytotoxicity assay to assess the effect of doxycycline on SH-NT-1 and SH-9052-1. To test this, we plated the cells in a 12 well plate and treated the cells with 100 ng/ml, 500 ng/ml and 1 µg/ml for 24 h, 24 h and 24 h. For the control wells, those were treated with 70% methanol. The cells were then incubated with the ethidium homodimer-1 and calcein AM. The calcein will stain the live cell green because the cell-permeant calcein AM is converted enzymatically by the intracellular esterase activity of live cells to intensely fluorescent green calcein. The polyanionic dye calcein is well retained within the live cells (ex/em ~ 495 nm/~515 nm). The ethidium homodimer-1 enters only cells with damaged cell walls and undergoes a 40-fold enhancement of fluorescence when binding to nucleic acids. The ethidium homodimer-1 will produce a bright red-light fluorescence in dead cells (ex/em ~495 nm/ ~635 nm). We saw that none of the three does lead to significant cell death. Most of the cells were stained with green color indicating that they are alive compared to the positive control (**Figure 10 and 11**).

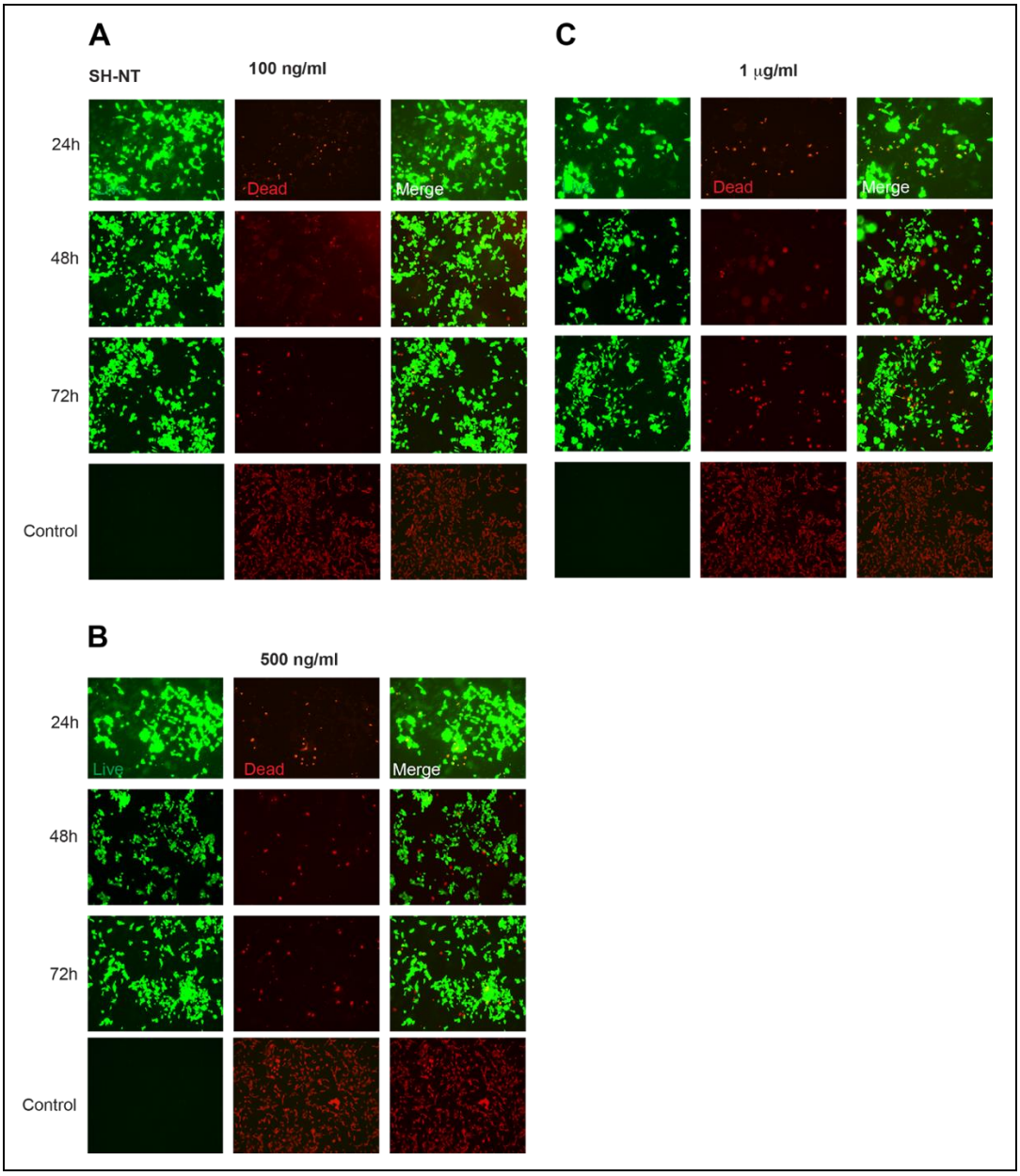


Figure 10. Viability assay of the doxycycline effect on the SH-NT-1 cells. The cells were plated and treated with (100 ng/mL, 500 ng/mL and 1 μg/mL) doxycycline and incubated for 24 h, 48 h and 72 h. On the day of the assay, the control wells were treated with 70% methanol and then the cells were treated with calcein AM and ethidium homodimer-1. In **A**, **B** and **C**, we see from the representative images that the cells were mostly green, and the red

cells were very few. Indicating that the doxycycline doses are not toxic to the cells. When comparing the control to the treated wells we see that the control had no live cells, and all the cells were stained red indicating that all the cells were dead.

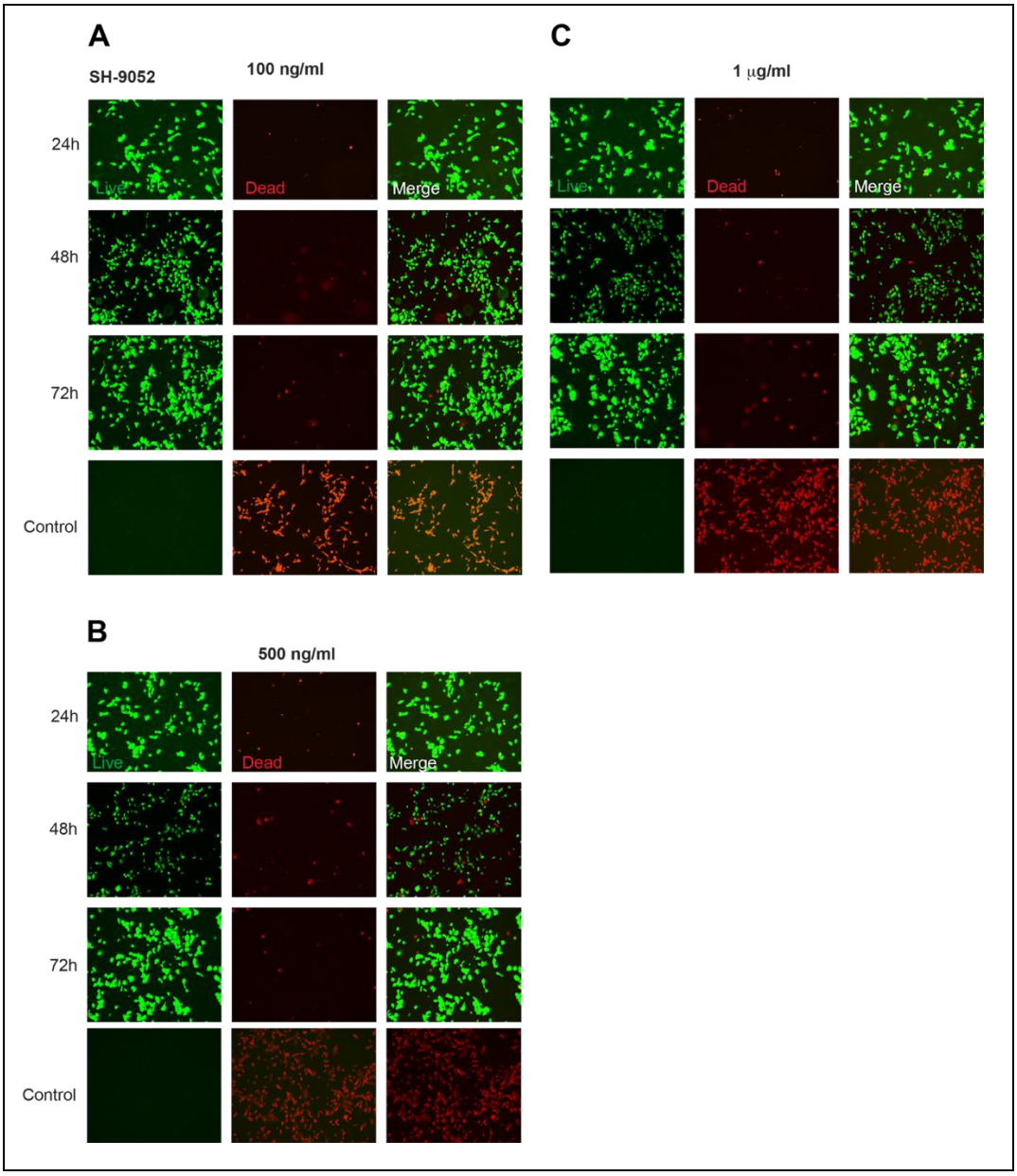


Figure 11. Viability assay of the doxycycline effect on the SH-9052-1 cells.The cells were plated and treated with (100 ng/mL, 500 ng/mL and 1 µg/mL) doxycycline and incubated for 24 h, 48 h and 72 h. On the day of the assay, the control wells were treated with 70% methanol and then the cells were treated with calcein AM and ethidium homodimer-1. In **A**, **B** and **C**, we see

from the representative images that the cells were mostly green, and the red cells were very few. Indicating that the doxycycline doses are not toxic to the cells. When comparing the control to the treated wells we see that the control had no live cells, and all the cells were stained red indicating that all the cells were dead.

How long will FANCD2 be depleted after the addition of doxycycline?

After determining the optimal doxycycline concentration that will produce the FANCD2 depletion, we wanted to know how long it would take for FANCD2 protein levels to return to normal. We carried out an experiment to test this by plating the SH-9052-1 cells in a 10 cm dish. Then, treating those cells with 500 ng/ml doxycycline for three days and then release the cells into growth media without doxycycline. The cells were harvested daily for nine days. Those lysates were then run on a gel to measure the protein levels (**Figure 12**). We notice from the results that FANCD2 levels start to drop on day three and four. From day five to day seven there is no FANCD2 expressed in those cells four days after the release of the cells. The FANCD2 levels come back to the same levels as on day three starting day eight and nine.

The role of FANCD2 in SH-SY5Y differentiation

To study the role of FANCD2 in the differentiation of SH-SY5Y. We used the two new cell lines we generated, and we differentiated them for 11 days in the presence and absence of doxycycline. The cells were exposed to 500 ng/ml doxycycline two days prior to the start of differentiation. Then the growth medium is changed to Stage I media supplemented with 500 ng/ml doxycycline until day six of differentiation. On Day six, the media was changed to Stage II media without doxycycline and the cells were cultured for up to twenty-one days. For this experiment, we have cells differentiated until day 11 (**Figure 13 and 14**). FANCD2 depletion was successful. We looked at PARP, poly-ADP

ribose polymerase, which is involved in the DNA damage response, levels as well to see if there is a difference in the DNA damage response between the two. There was no significant difference in the expression levels of PARP in SH-NT-1 cells (**Figure 13A, C**). There

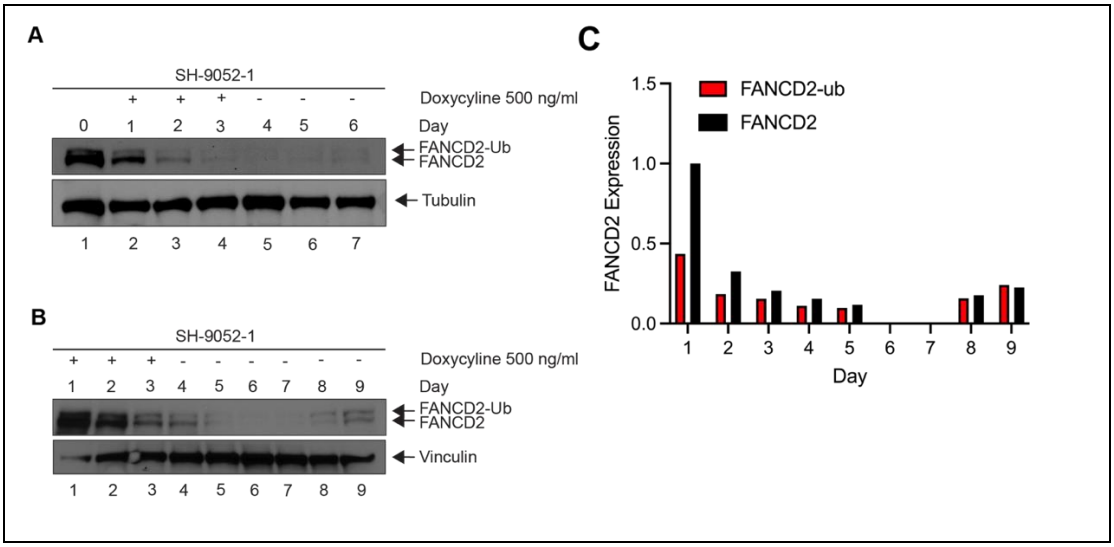


Figure 12. Western blot of SH-9052-1 after doxycycline exposure for 3 days. **A.** SH-9052 were treated with 500 ng/ml doxycycline for three days and then released into the growth medium and harvested daily up to day 6. We see from the western blot that FANCD2 depletion was successful and that it persisted up to day six. That led us to perform the same experiment with cells released into the growth medium for longer days. We performed the same experiment, and we went up to day nine (image B). **B.** We see on day three and four the FANCD2 levels get low. From day five until day seven, four days after releasing the cells into growth medium, we see that the protein is almost completely absent. On day eight and nine the protein starts to come back to cellular levels observed on day three after doxycycline addition. **C.** Is a quantification of image B. We see that FANCD2 level on day nine is close to what is one day two of the doxycycline treatment.

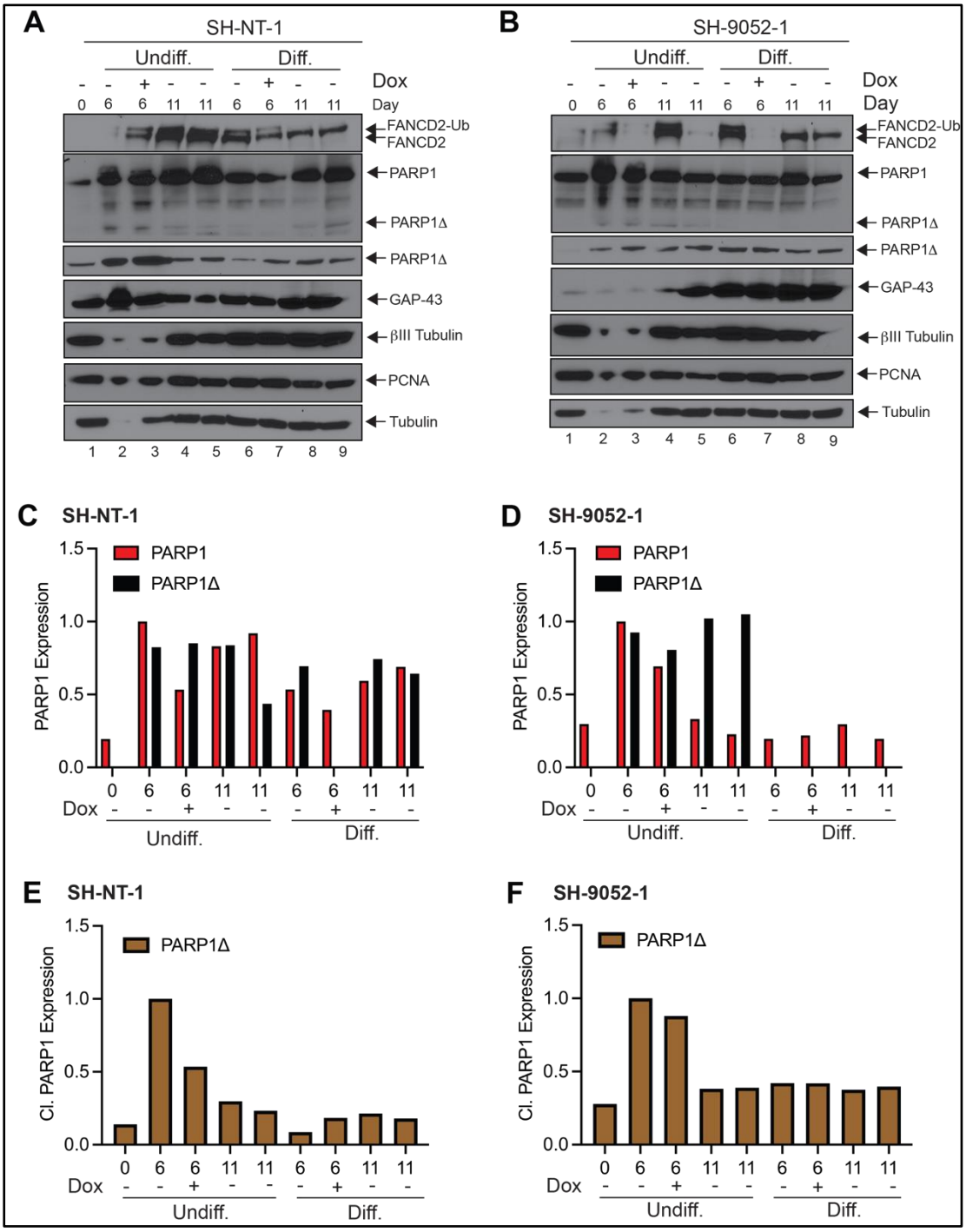


Figure 13. A western blot comparing differentiated and undifferentiated SH-NT-1 and SH-9052-1. **A.** Is a western blot of SH-NT-1. We see that FANCD2

is expressed in all the lanes. PARP1 and Cleaved PARP1 we see less of PAPR in six days differentiated plus doxycycline. The rest of the PARP levels stay similar. As for cleaved PARP1 we see less at day six differentiated and more at day six undifferentiated. Quantification shows that there is less of PARP at day 11 undifferentiated and day 6 differentiated (**C**). For GAP-43 there is more expression of it in the 6 and 11 day differentiated cells than in undifferentiated. For β-III tubulin, there is more expression of it in 6 and 11 day differentiated cells. PCNA, we see similar levels for most of the time points indicating that those cells are actively dividing. **B.** Is a western blot of SH-9052-1. FANCD2 is depleted in the time points that doxycycline was added indicating successful knockdown. The knockdown is left at day 11 because the cells were released into growth media on day 6 of differentiation. For PARP1, there is more of it on day 6 without doxycycline than on day 6 with doxycycline. We see some of Cleaved PARP1 but there is not much difference between the time points. Quantification shows low levels of it in PARP in the differentiated cells than in the undifferentiated cells (**D**). GAP-43, we see more of it expressed in the differentiated cells than in the undifferentiated. For β-III tubulin, there is more expression of it in the differentiated than in the undifferentiated. PCNA levels stay relatively the same indicating those cells are actively dividing. **C.** Is quantification of PARP1 of SH-NT-1. We see there is less of PARP at day 11 undifferentiated and day 6 differentiated. **D.** Is quantification of PARP1 of SH-9052-1. We see lower levels of it in PARP in the differentiated cells than in the undifferentiated cells. **E.** Is quantification of the cleaved

PARP1 in SH-NT-1. We see the levels of cleaved PARP less in the differentiated than in the undifferentiated. **F.** Is quantification of the cleaved PAPR1 in SH-9052-1. We see less of cleaved PARP1 in the differentiated than in the undifferentiated and the levels of clever PARP1 stay relatively the same throughout the differentiation time points.

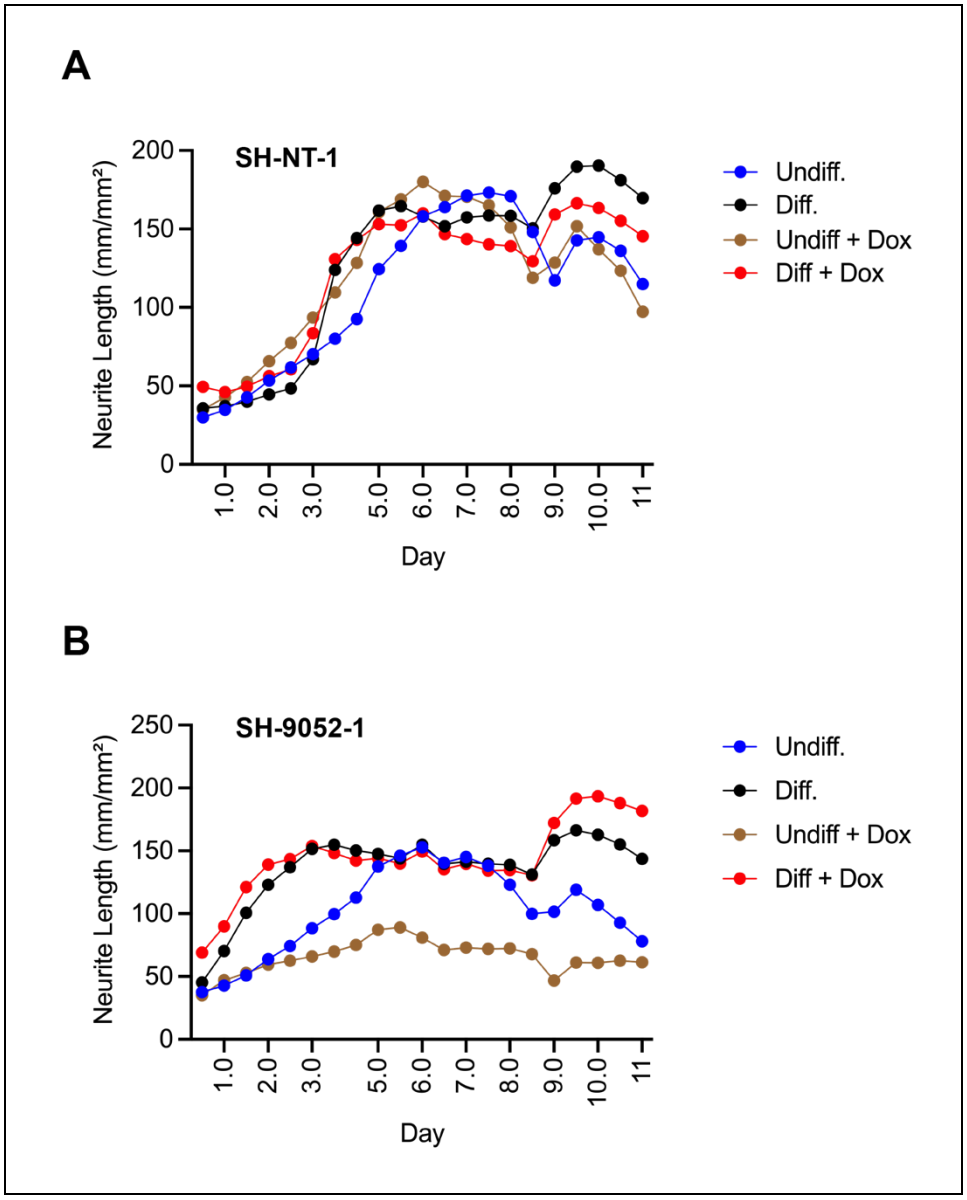


Figure 14. Result of neurite growth tracking using Incucyte Live-Cell Analysis. **A.** Is a graph of SH-NT-1 differentiated up to 11 days. As the graph shows, the neurite length increases as the cells differentiate. We see less of a difference between differentiated and undifferentiated cells. **B.** Is a graph of SH-9052-1 differentiated up to 11 days. We see more differences between differentiated

and undifferentiated cells starting at day 8 of differentiation. There was some cleaved PARP showing up on day 11 of differentiation. As for SH-9052-1, we see there are lower levels of PARP in the differentiated than in the undifferentiated (**Figure 13B, D**). When looking at the cleaved PARP alone, we see there are less expression levels in the SH-NT-1differentiated than in the undifferentiated (**Figure 13E**). The same thing is observed in SH-9052-1(**Figure 13F**). We can see a higher level of cleaved PARP in the SH-9052-1 as compared to SH-NT-1. As for the differentiation markers, GAP-43 and β-III tubulin, there is a higher level of expression of these in the differentiated cells. Indicating that those cells are differentiating and starting to express more of these markers later in the differentiation (**Figure 13A, B**). As for PCNA, we don't see a significant difference in both cell lines whether they were differentiated or not. Indicating that these cells are actively dividing. Furthermore, we don't see much of a difference between the cells that were treated with doxycycline and those that weren't. Moreover, when we tracked the neurite formation using the Incucyte Live-Cell Analysis system we saw a difference in neurite length between differentiated and undifferentiated, yet we didn't see much of a difference between the wells that were treated with doxycycline and those that weren't (**Figure 14**). These are preliminary data and further experimentation must be carried out to assess the role of FANCD2 in the differentiation of SH-SY5Y.

The expression of FANCD2 in stem cells, neural progenitor cells and mature neurons

We were interested in investigating the level of FANCD2 expression in the three stages of the cell from stem cell to a mature neuron. We wanted to determine if there is a difference in FANCD2 profile expression in those three stages that might reveal insights into the role of FANCD2 in the maintenance of genomic stability of those cells at different stages of development. We anticipated a higher level of expression of FANCD2 in the stem and NP stages of development due to the rapid proliferation of those cells and the massive expansion needed from them to produce neurons. If a mutation occurs in NPs and persists in the daughter neurons they may cause neurodegeneration in adulthood. Therefore, faithful DNA repair pathways such as homologous recombination is very critical (Mitra et al, 2019). Here is where we think that FANCD2 plays an important role in maintaining genomic stability of the stem cells and NPs. What we see from the results is that FANCD2 is expressed more at the iPSCs stage (**Figure 15, lane 5**) and less and less in the other two stages; the NPs and mature neurons (**Figure 15, lane 6 and 7**).

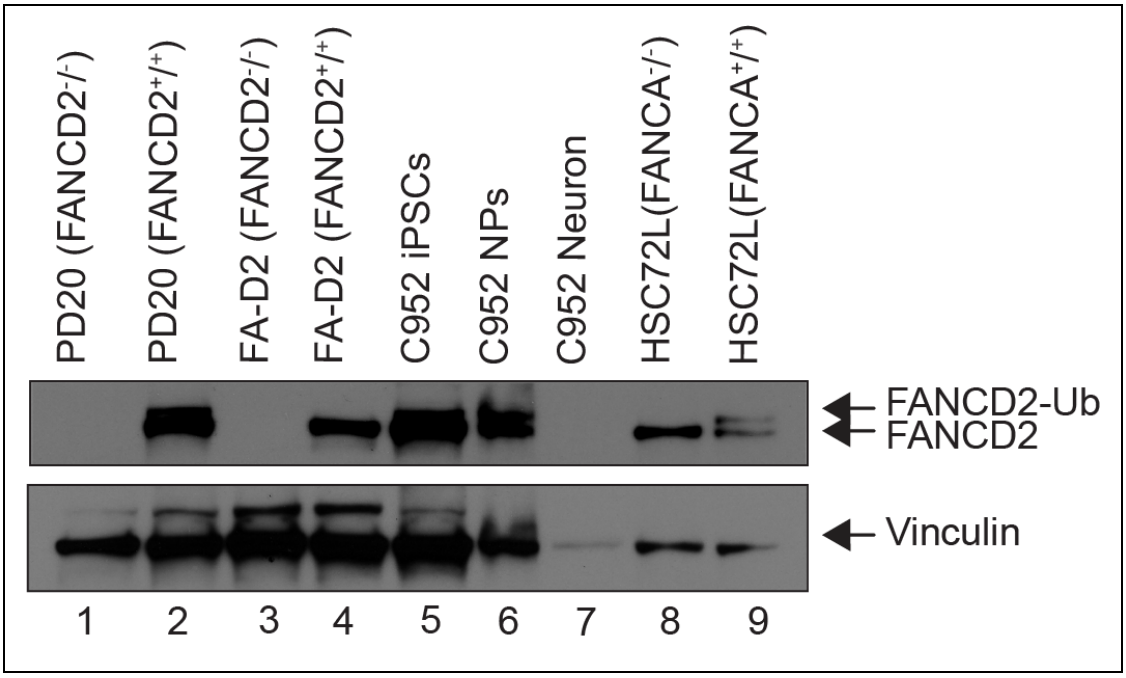


Figure 15. Western blot analysis of FANCD2 expression at different stages of stem cell development. The first two lanes are SV40-transformed FA-D2patient cells (PD20); lanes three and four are FA patient, hTRERT immortalized FA-D2 (FANCD2 $^{-/-}$) cells and FA-D2 (FANCD2 $^{+/+}$). Lanes eight and nine are from FA patient lymphoblasts. These are used as control for FANCD2 expression. C952 are iPSCs derived from an ALS patient. The C952 were differentiated and harvested at the three stages of development. FANCD2 expression in the iPSCs is more than in the NPs and neuron stages which is consistent with the role of FANCD2 in maintaining the genomic stability of those cells as they go through the development.

Is there a difference in the DNA damage markers between differentiated and undifferentiated SH-SY5Y?

After investigating the expression of FANCD2 in the three stages of development, we wanted to investigate if there is a difference in the between differentiated and undifferentiated SH-SY5Y. SH-SY5Y were differentiated for 21 days and harvested at 0, 6, 11, and 21 days. Immunoblotting analysis was carried out to assess the difference (**Figure 16**). For FANCD2, we see the expression of FANCD2 is similar at 6 and 11 days, but at day 21 we see significant reduction in the expression of FANCD2 in the differentiated and undifferentiated SH-SY5Y (**Figure 16, top panel**). As for FANCA, we see that the expression is similar throughout the days of differentiation except for day 21 in the undifferentiated SH-SY5Y the expression was significantly diminished (**Figure 16, 2nd panel**). For PARP1, we don't see a difference in the full length PARP1, but we see a difference in the cleaved PARP1. We see increased levels of cleaved PARP1 at 21 days as compared to day 6 and 11. As for the differentiation markers, GAP-43 and β-III tubulin, we don't see a difference in the undifferentiated SH-SY5Y, but we see there are higher levels of them in the 11 and 21 day differentiated SH-SY5Y. Indicating that those cells as they differentiate, they express more of GAP-43 and β-III tubulin in the differentiated as opposed to undifferentiated SH-SY5Y. For PCNA, which is a marker of active cell proliferation, we see the same levels of PCNA in the undifferentiated at 6, and 11 days, but at day 21 we see a significant reduction of it. As for the differentiated SH-SY5Y, we continue to see the same levels of PCNA at the 6 and 11 and

very little at 21 days. That indicates that the SH-SY5Y continues to proliferate even when they are forced to differentiate to mature neurons and that is seen in culturing the SH-SY5Y.

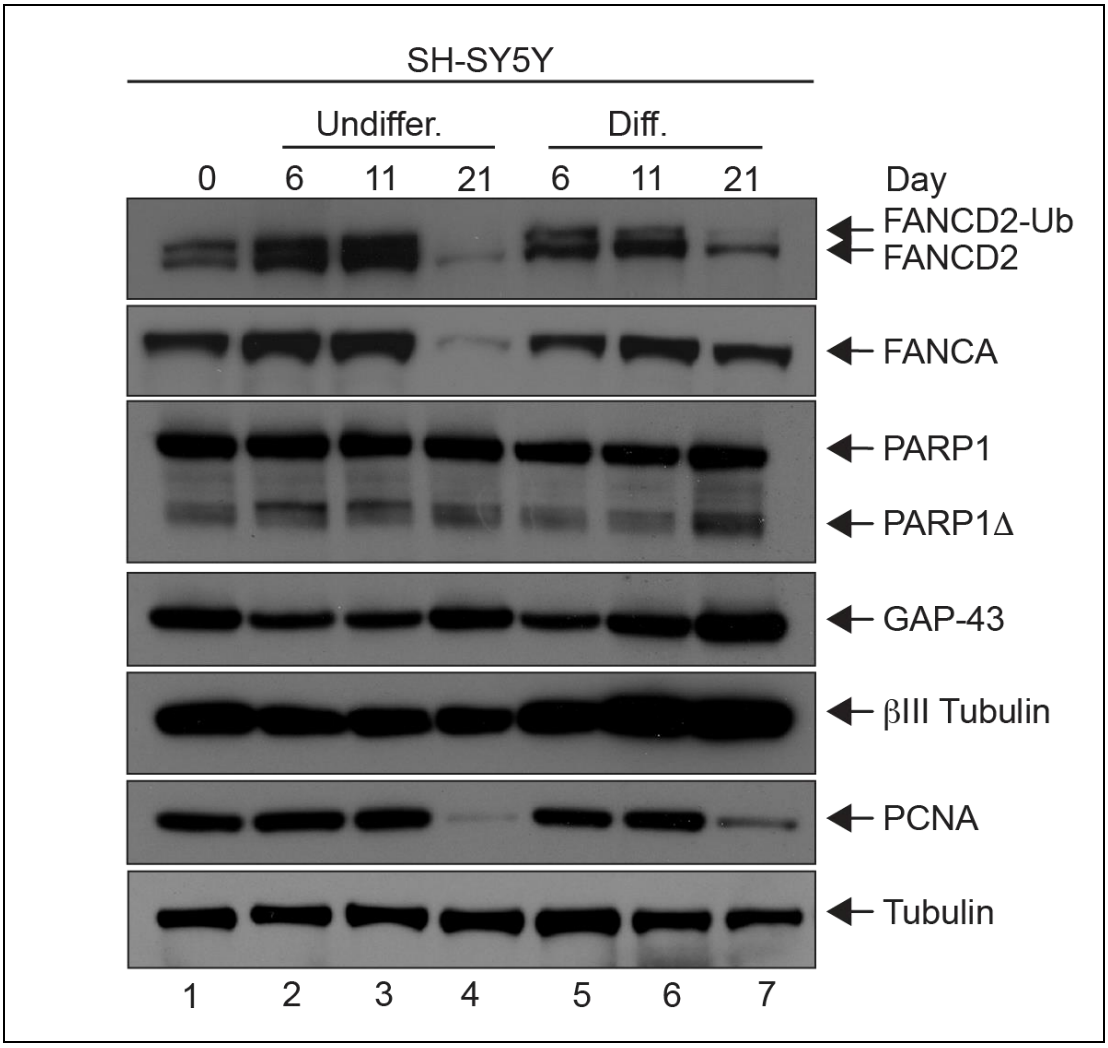


Figure 16. A western blot comparing differentiated SH-SY5Y to undifferentiated SH-SY5Y at different time points. The first figure shows FANCD2 at different time points of differentiated and undifferentiated SH-SY5Y. We see at 6 and 11 days that FANCD2 cellular levels go up compared to day 0. While at 21 days, FANCD2 is almost completely absent in the undifferentiated cells. In the differentiated cells, we see a similar pattern. For FANCA, we see the levels

of FANCA stay relatively the same except for day 21 undifferentiated cells where it is almost absent. For PARP1 and cleaved PARP, we see that PARP1 levels are the same for undifferentiated and less in the differentiated. As for cleaved PARP1 we see undifferentiated levels stay the same, as for differentiated cells we see more of cleaved PARP1 at 21 days than at 6 and 11. For GAP-43, we see higher levels of it in the differentiated cells than in the undifferentiated cells especially at day 21. For β-III tubulin, there are higher levels of it in the differentiated cells than in the undifferentiated cells, especially at day 11 and 21. As for PANCA, we see the same levels for day 0, 6 and 11 in both and day 21 we see the same for both as well.

DISCUSSION

To study the role of the FANCD2 protein in the differentiation of SH-SY5Y cells, we generated a system where we could deplete FANCD2 for extended periods of time using doxycycline. The system that will be easier and more reliable to use is the shRNA system. This system is inducible, which means we can turn on/off the expression of the shRNA that will deplete FANCD2 by simply adding doxycycline. This offers a huge advantage in controlling when to deplete FANCD2 during the twenty-one-day long differentiation of SH-SY5Y. To pick the shRNA that will produce the optimal FANCD2 depletion in the SH-SY5Y, we have chosen three different shRNA that target a different region of FANCD2 cDNA.

Development of inducible system to knock down FANCD2

shRNA is an artificial molecule with short hairpin turn used to target expression of genes of interest via RNA interference. It is a very useful and easy to manipulate system. It allows for the selective depletion of the protein and the duration of the depletion. That allows for a multitude of benefits not limited to; the time of the depletion, the duration of depletion and the extent of depletion. In addition, the shRNA system offers an advantage over small interfering RNA (siRNA). siRNA is transient, while shRNA is allowed on as long as the activating molecule is present. That allows for the depletion of FANCD2 for a 21-day long period that is required for the differentiation of SH-SY5Y. Furthermore,

CRISPR requires introducing a mutation into the gene of interest rendering the gene inactive, while the shRNA targets the mRNA of gene of interest and that allows for selective inactivation of FANCD2 expression. shRNA allows for the re-expression of FANCD2 when the activating molecule is removed. CRISPR will render FANCD2 gene inactive and thus no re-expression when needed, limiting the scope of investigating FANCD2 protein role.

One of the reasons three different shRNA have been chosen is to allow for the selective depletion of FANCD2 to gain a more comprehensive understanding of the role of FANCD2 in SH-SY5Y cells. Each shRNA will deplete FANCD2 with varying efficiency. That allows for the examining of the role of FANCD2 under varying degrees of knockdown. For example, a complete depletion of FANCD2 (i.e. 100% knockdown) might lead to dead or very sick cells given the role FANCD2 plays in the repair of DNA damage. While a moderate depletion of FANCD2 (i.e. 70 or 60% knockdown) might lead to significant effect on the cells, yet not to the point causing cell death. FA patients that are defective in FANCD2 exhibit severe phenotype and an average diagnosis age of 4.5 years as compared to 10 years median in other FA complementation groups as well as tendency for malignancies and neurological problems at an early age (Kalb, et al; 2007). That indicates that FANCD2 is very important for cells to fix DNA damage.

Our model, SH-SY5Y can be differentiated to a more mature neuron-like phenotype characterized by neuronal markers using retinoic acid (RA) as a means

for differentiation. The capacity for large-scale expansion of these cells prior to differentiation, with relative ease and low cost to culture compared to primary neurons offer a huge advantage. Furthermore, SH-SY5Y cells are human-derived which means they express a number of human-specific proteins and protein isoforms (Kovalevich et al, 2013). Those cells form neurites when they differentiate to communicate with other neurons. Using SH-SY5Y, we were able to integrate the shRNA into them generating three different cell lines with different shRNA. This robust system can be transferable to other cell lines such as stem cells. This system offers a huge advantage for studying FANCD2 in a variety of systems.

FANCD2 expression during neuronal differentiation

The expression of FANCD2 in the three stages of neural development is unknown. We aimed at determining if there is a difference in FANCD2 profile expression in the three stages that might reveal insights into the role of FANCD2. The anticipation is that there is a higher level of FANCD2 in the stem and NPs stages since those cells are highly proliferative and expansive to produce the massive network of neurons that make the human brain. That means they require a massive supply of ATP generated from cellular respiration (Qing et al, 2022). Cellular respiration leads to the formation of reactive oxygen species (ROS) as a byproduct of respiration. The ROS are genotoxic and could lead to genomic instability (Ansari et al; 2019). One of the chief characteristics of the DNA damage response processes of neural cells is to deal with different types of DNA damage, such as single strand breaks (SSBs), bulky lesions, base

mismatch and double strand breaks (DSBs) (Iyama et al; 2013). SSBs and DNA base damage can be repaired mainly by the base excision repair (BER), mismatch repair (MMR), and nucleotide excision repair (NER), DSBs are repaired by either homologous recombination (HR) or non-homologous end joining (NHEJ) mechanisms (Sáez; 2017; Jackson et al; 2009). Stem cells and NPs undergo massive DNA replication and cell division which are necessary for production of 80 billion neurons in the mature brain (Dehay et al; 2007; Chan et al; 2002). If a mutation occurs in the NPs and persists in the daughter neurons, this could lead to neurological disease and neurodegeneration in adulthood (Orii et al; 2006). Therefore, faithful DNA repair pathways such as HR are very critical (Mitra et al, 2019). Mature neurons in the adult nervous system rely mainly on NHEJ because neurons lack sister chromatid as a homologous template DNA necessary for HR (Orii et al; 2006; Mitra et al, 2019). The FA repair pathway is required for genome protection against ICLs (Niraj et al, 2019). This DNA damage is considered to be amongst the most deleterious DNA lesions, stalk replication and transcription (Kee et al; 2010). Chemotherapeutic agents (e.g. cisplatin, MMC) can induce ICLs, but there are by-product of cellular metabolism such as acetaldehyde and formaldehyde that have been identified as endogenous ICL sources that require FA pathway for repair (Langevin et al, 2011; Pontel et al, 2015). Thus, disruption in the FA pathway in stem cells and NPs will lead to DNA damage and neurodegeneration. FANCD2 fixes ICLs by creating DSBs that are then repaired via HR (Niraj et al, 2019). Since DSB is fixed via HR in only stem cells and NPs (Qing et al,

2022), and HR rely on FANCD2 to fix DSB (Niraj et al, 2019), we think that FANCD2 levels will be higher at these two stages as opposed to neurons that fix damage via NHEJ that doesn't rely on FANCD2. In figure 15, we see what we predicted. There are higher levels of FANCD2 at stem cell and NPS stages than at the neuron stage. Sequencing data from the Fallini's lab indicate that the level of FANCD2 expression in two- and three-weeks old neuron is very low.

Is FANCD2 required for efficient neuronal differentiation?

The difference between differentiated and undifferentiated SH-SY5Y in (**Figure 13A, B**) doesn't reflect what is anticipated for this assay. We see the differentiated and undifferentiated cells are very close to each other. That shouldn't be the case since the differentiated cells have neurites while undifferentiated cells do not. If the assay sensitive enough, it would show a higher degree of separation between the differentiated and undifferentiated. This assay needs to be optimized for density and sensitivity to capture the neurite formation is these different cells.

From the preliminary data collected, there is no difference between the cells that have FANCD2 protein and the cells that have the protein depleted. Looking at the differentiation markers expression (**Figure 13A, B**), there is no significant difference between differentiated and undifferentiated nor there is a difference between the cells that have functional FANCD2 and those that don't. Also, looking at the neurite formation tracking, there isn't much difference between the cells that have FANCD2 and the cells that don't (**Figure**

14A, B). From these preliminary data, we can say that absence of FANCD2 protein has no effect on the differentiation of SH-SY5Y. But the challenges that are faced with these kinds of experiments make these preliminary data in need of sufficient and careful examination to properly investigate the role of FANCD2. One of these challenges is when to start the depletion of FANCD2 during the differentiation? Is it prior to differentiation? Is it at the start of the differentiation? Or is it at some stage during the differentiation? All these questions require careful examination to assess properly the role of FANCD2 in the differentiation of SH-SY5Y. Another challenge is keeping the cells alive for longer differentiation periods. The SH-SY5Y tend to stay actively dividing, and that is evident in the expression of PCNA (**Figure 13A, B**), which means they will require splitting, differentiated SH-SY5Y are sensitive to splitting, making maintaining those cells a very dire undertaking. This is one of the reasons why there is no time point beyond 11 days differentiation. Maybe a longer differentiation is required to notice these changes? Furthermore, another challenge is what vessel will those cells be cultured. The SH-SY5Y tends to be very sensitive to 6, 12, 24 well plates. They don't grow and differentiated very well in these. So, you're limited to 96-well plates, 10 cm dishes, 25 cm2, and 75 cm2 flasks. That fact limits the scope of the experiments greatly. Furthermore, for the Incucyte Live-Cell, the cell density must be optimized. As has been shown, SH-SY5Y tends to be actively dividing when differentiating and forming clumps. Over confluent wells make the later analysis very difficult as the software is unable to differentiate between cell body and neurite. This could be an

explanation as to why we don't see much of a difference between the cells that incubated with doxycycline and the cells that weren't (**Figure 14**).

CONCLUSION

In conclusion, we were successful in generating an inducible shRNA system to deplete FANCD2 protein. This is a very easy system to manipulate as well as transferable to other models to study the role of FANCD2 in cancer. We were able to deplete FANCD2 in 48 h and it took about six days for the FANCD2 expression to come back. We have determined the concentration of doxycycline and the time points required to knockdown FANCD2. We have demonstrated that this concentration is not toxic to the cells. Our preliminary data didn't answer our question due to technical limitations that we were under. I think further research is needed to work around these limitations and investigate the role of FANCD2 protein properly.

BIBLIOGRAPHY

Aksu, T., Gümrük, F., Bayhan, T., Coşkun, Ç., Oğuz, K. K., & Unal, S. (2020, September 4). *"Central nervous system lesions in Fanconi anemia: Experience from a research center for Fanconi anemia patients".* Wiley Online Library. Retrieved March 22, 2023.

Ansari, Mohd Owais, et al. *"Evaluation of DNA Interaction, Genotoxicity and Oxidative Stress Induced by Iron Oxide Nanoparticles Both in Vitro and in Vivo: Attenuation by Thymoquinone."* Scientific Reports, U.S. National Library of Medicine, 6 May 2019.

Blaize JL, Noori BM, Hunter KP, Henrikson KA, Atoyan JA, Ardito AA, Donovan FX, Chandrasekharappa SC, Schindler D, Howlett NG. 2023. *Differential Regulation of Retinoic Acid Metabolism in Fanconi Anemia.* bioRxiv.

Chan, Wood Yee, et al. *"Proliferation and Apoptosis in the Developing Human Neocortex."* The Anatomical Record, U.S. National Library of Medicine, 2 Aug. 2002.

Chui, Angela, et al. *"Oxidative Stress Regulates Progenitor Behavior and Cortical Neurogenesis."* The Company of Biologists, Development, 11 Mar. 2020.

Ceccaldi, Raphael, et al. *"The Fanconi Anaemia Pathway: New Players and New Functions."* Nature News, 5 May 2016.

Dehay, Colette, and Henry Kennedy. *"Cell-Cycle Control and Cortical Development."* Nature News, 1 June 2007.

Dravid, Anusha, et al. *"Optimised Techniques for High-Throughput Screening of Differentiated SH-SY5Y Cells and Application for Neurite Outgrowth Assays."* Nature News, Nature Publishing Group, 14 Dec. 2021.

Fiesco-Roa , Moisés, et al. *"Genotype-Phenotype Associations in Fanconi Anemia: A Literature Review."* Blood Reviews, 16 July 2019.

Fiesco-Roa, M. Ó., García-de Teresa, B., Leal-Anaya, P., van 't Hek, R., Wegman-Ostrosky, T., Frías, S., & Rodríguez, A. (2022, August 1). *Fanconi anemia and dyskeratosis congenita/telomere biology disorders: Two inherited bone marrow failure syndromes with genomic instability. Frontiers.* Retrieved March 1, 2023.

Iyama, Teruaki, and David M Wilson. *"DNA Repair Mechanisms in Dividing and Non-Dividing Cells."* DNA Repair, U.S. National Library of Medicine, 15 May 2013.

Jackson, Stephen P., and Jiri Bartek. *"The DNA-Damage Response in Human Biology and Disease."* Nature News, Nature Publishing Group, 22 Oct. 2009.

Johnson-Tesch, Benjamin A., et al. *"Fanconi Anemia: Correlating Central Nervous System Malformations and Genetic Complementation Groups."* SpringerLink, 10 Mar. 2017.

Kalb, Reinhard, et al. *"Hypomorphic Mutations in the Gene Encoding a Key Fanconi Anemia Protein, FANCD2, Sustain a Significant Group of FA-*

D2 Patients with Severe Phenotype." Cell Press, The American Journal of Human Genetics, 1 May 2007.

Kee, Younghoon, and Alan D D'Andrea. *"Expanded Roles of the Fanconi Anemia Pathway in Preserving Genomic Stability."* Genes & Development, U.S. National Library of Medicine, 15 Aug. 2010.

Kovalevich, Jane, and Dianne Langford. *"Considerations for the Use of SH-SY5Y Neuroblastoma Cells in Neurobiology."* Methods in Molecular Biology (Clifton, N.J.), U.S. National Library of Medicine, 1 Jan. 2013.

Langevin, Frédéric, et al. *"FANCD2 Counteracts the Toxic Effects of Naturally Produced Aldehydes in Mice."* Nature, U.S. National Library of Medicine, 6 July 2011.

Liang, Fengshan, et al. *"DNA Requirement in FANCD2 Deubiquitination by USP1-UAF1-RAD51AP1 in the Fanconi Anemia DNA Damage Response."* Nature News, Nature Publishing Group, 28 June 2019.

Lui, Jan H, et al. *"Development and Evolution of the Human Neocortex."* Cell, 7 July 2011.

Madabhushi, Ram, et al. *"DNA Damage and Its Links to Neurodegeneration."* cellPress, 16 July 2014.

Mcconnell, Michael J, et al. *"Intersection of Diverse Neuronal Genomes and Neuropsychiatric Disease: The Brain Somatic Mosaicism Network."* Science, 28 Apr. 2017.

Mitra, Joy, et al. *"Motor Neuron Disease-Associated Loss of Nuclear TDP-43 Is Linked to DNA Double-Strand Break Repair Defects."* PNAS, PNAS, 5 Mar. 2019.

Niraj, J., Färkkilä, A., & D'Andrea, A. D. (2019, March 1). *The Fanconi anemia pathway in cancer - annual reviews.* Annual Reviews. Retrieved January 22, 2023.

Nepal, Manoj, et al. *"FANCD2 and DNA Damage."* International Journal of Molecular Sciences, 19 Aug. 2017.

Okamoto, Yusuke, et al. *"Replication Stress Induces Accumulation of FANCD2 at Central Region of Large Fragile Genes."* Nucleic Acids Research, U.S. National Library of Medicine, 6 Apr. 2018.

Orii, Kenji E, et al. *"Selective Utilization of Nonhomologous End-Joining and Homologous Recombination DNA Repair Pathways during Nervous System Development."* Proceedings of the National Academy of Sciences of the United States of America, U.S. National Library of Medicine, 27 June 2006.

Peake, Jasmine D, and Eishi Noguchi. *"Fanconi Anemia: Current Insights Regarding Epidemiology, Cancer, and DNA Repair - Human Genetics."* SpringerLink, Springer Berlin Heidelberg, 21 May 2022.

Pontel, Lucas B, et al. *"Endogenous Formaldehyde Is a Hematopoietic Stem Cell Genotoxin and Metabolic Carcinogen."* Molecular Cell, U.S. National Library of Medicine, 1 Oct. 2015.

Qing, Xiaobing, et al. *"DNA Damage Response in Neurodevelopment and Neuromaintenance."* Wiley Online Library, 25 May 2022.

Rogers, Cody M, et al. *"Fanconi Anemia-Independent DNA Inter-Strand Crosslink Repair in Eukaryotes."* Progress in Biophysics and Molecular Biology, Pergamon, 30 Aug. 2020.

S. von Bartheld, Christopher, et al. *"The Search for True Numbers of Neurons and Glial Cells in the Human Brain: A Review of 150 Years of Cell Counting."* Wiley Online Library, 15 Nov. 2016.

Sai Srinivas, Upadhyayula, et al. "*Ros and the DNA Damage Response in Cancer."* Redox Biology, Elsevier, 21 Dec. 2018.

Shigechi, Tomo, et al. *"ATR–Atrip Kinase Complex Triggers Activation of the Fanconi Anemia DNA Repair Pathway."* American Association for Cancer Research, 1 Mar. 2012.

Stivaros, Stavros, et al. *"Central Nervous System Abnormalities in Fanconi Anaemia: Patterns and Frequency on Magnetic Resonance Imaging."* The British Institute of Radiology, 25 Oct. 2015.

Sáez, Guillermo T. *"DNA Damage and Repair in Degenerative Diseases 2016."* International Journal of Molecular Sciences, U.S. National Library of Medicine, 16 Jan. 2017.

Wang, Meiyan, et al. *"Increased Neural Progenitor Proliferation in a hiPSC Model of Autism Induces Replication Stress-Associated Genome Instability."* Cell Press, Cell Stem Cell, 6 Feb. 2020