Huntington disease-like 2 (HDL2) is caused by a CTG/CAG repeat expansion on chromosome 16q24.3. Three non-mutually exclusive mechanisms have thus far been implicated in the pathogenesis of the disease: 1) loss of expression of full-length JPH3 protein [1]; 2) toxic expression of a JPH3 splice variant containing an expanded CUG repeat [2]; and 3) toxic expression, from the strand antisense to JPH3, of a cryptic transcript containing a CAG repeat and encoding polyglutamine [3].

HDL2 provides a striking opportunity to explore the pathogenesis of other neurodegenerative diseases with which it shares genetic, clinical and/or pathological features, including Huntington's disease (HD) and neuroacanthocytosis (NA) syndromes [4, 5]. For example, our studies of HDL2 and HD proteome in human brain tissue have identified a number of pathogenic pathways that may be shared between the two diseases (Ratovitski et al., submitted). In addition, bi-directional transcription in HDL2 have prompted us to examine and confirm the role of an antisense transcript, as well as toxicity of the sense transcript in HD pathogenesis [6, 7]. Based on the success of such parallel studies of HDL2 and HD, here we propose to begin exploring the hypothesis that, in a similar fashion, the loss of function of JPH3 may, at least in part, share downstream mechanistic similarities with the loss of function of vacuolar protein sorting-associated protein 13A (VPS13A) in chorea-acanthocytosis (ChAc). The experiments in this proposal are designed to 1) provide preliminary data that would allow us to develop additional projects focused on the role of JPH3 in neurons; 2) preliminarily test the hypothesis that ChAc and HDL2 may share some aspects of neuropathogenesis.

Specific Aim 1. JPH3 belongs to the four-member junctophilin family of membrane proteins. Based on what is know about the two most studied junctophilins, JPH1 and JPH2 respectively, it was proposed that JPH3 mediates cross-talk between plasma membrabe components and calcium channels in the ER/SR membranes. However, this hypothesis has not yet been experimentally confirmed. To begin to address the role of JPH3 in neurons and hence facilitate our understanding how this protein may be involved in neurodegeneration, we have first proposed to identify novel pathways that may be triggered by the loss of JPH3. To achieve this, we have used two approaches: (1) Proteomic analysis of striatal and cortical brain tissue from junctophilin 3 (JP3) knock-out mice versus controls, and (2) the identification of novel interactors of full-length human JPH3 using immunoprecipitation followed by mass spectrometry (MS).

We have used the iTRAQ approach and have identified multiple pathways, including calcium signaling, energy metabolism and protein translation, that are likely to be disrupted due to a loss of the mouse JP3 protein. Among the proteins whose levels were changed in JP3 knock-out, but not wild type, mice were TrkB and calmodulin, both of which are involved in neurodegeneration and in pathogenic processes in HD [7]. We have confirmed that levels of the two proteins are changed in JP3 knock-out, but not wild-type mice (**Fig. 1**).

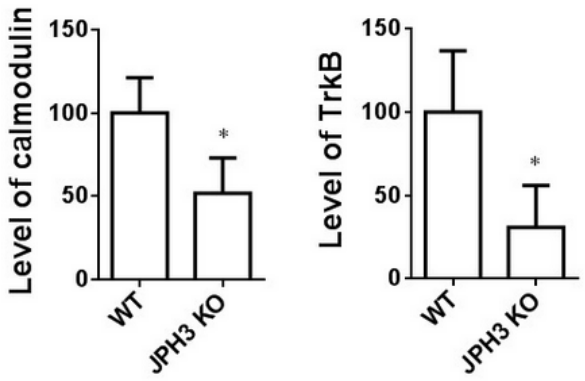


Figure 1. Immunoblotting followed by densitometric analysis confirms loss of calmodulin and TrkB expression in mice lacking JP3. N=3 JP3 vs. control mice. Experiment was repeated twice with separately prepared protein extracts.

There is accumulating evidence that shows brain-derived neurotrophic factor (BDNF) and its receptor TrkB are significantly decreased in Alzheimer's disease (AD) [8]. Moreover, calmodulin is also implicated in AD pathogenesis [9]. Hence, we speculated that the level of full-length JPH3 may be reduced in AD brains. We have performed immunoblotting analysis of protein extracts from AD and control brains and detected a significant reduction of JPH3. The reduction is unlikely to be a consequence of neuronal loss, as the levels of NeuN, a neuronal marker, are not significantly changed between AD and control brains (**Fig. 2**).

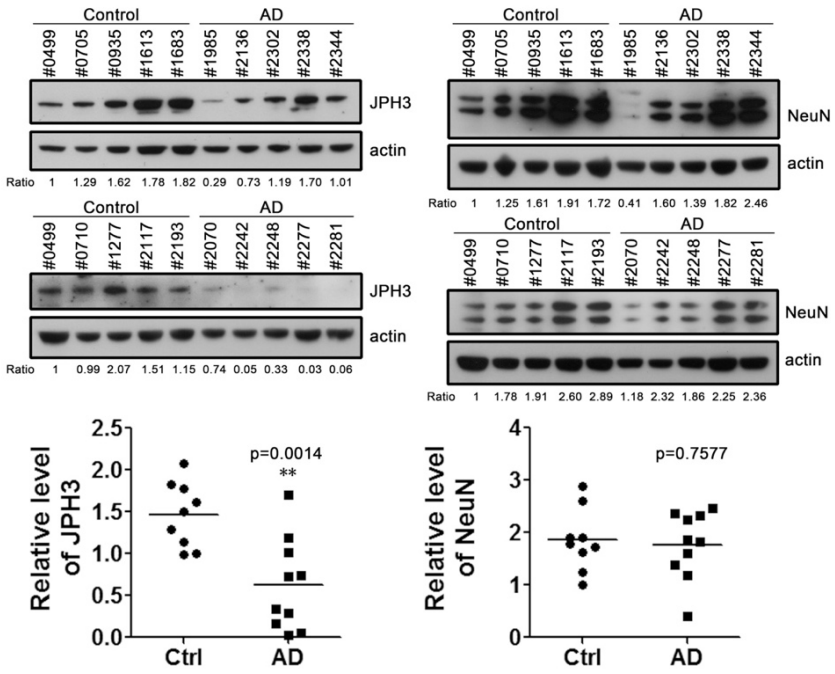


Figure 2. Full-length JPH3 is reduced in AD and not control brains. Parietal cortex was examined using immunoblotting with an anti-JPH3 antibody (developed in our laboratory [1]) followed by densitometric analysis. N=10 AD and 10 control brains. Mean ± SEM were shown; Mann Whitney test; *p < 0.05. Data were representative of three independent experiments.

Interestingly, we have recently, in collaboration with Dr. David Dyment, a geneticist at Children's Hospital of Eastern Ontario, identified a small family in which two sisters, but not their brother, have a deletion of JPH3 exon 1 and 2A that is associated with myoclonic jerks and learning disability. We have obtained lymphoblasts from unaffected mother and the three siblings and have confirmed that FL JPH3 is decreased in the affected sisters. Since previously described mouse model lacking both JPH3 and JPH4 has shown an impaired long-term potentiation and hyperactivation of Ca^{2+}/calmodulin-dependent protein kinase II, this suggest that JP3 may be involved in neural excitability fundamental to plasticity and integrated functions [10]. This is the first identification of a mutation in JPH3 that may be linked to a phenotype other than HDL2. We are collecting further genetic and phenotypic information and plan to publish a short report on the family in the near future.

In our preliminary data, we have shown that following overexpression of JPH3 in SH-SY5Y cells, very little JPH3 is localized to the membrane. To confirm this, we have performed a more detailed analysis of JPH3 expression using additional sub-cellular markers. Our data suggest that FL JPH3 localizes primarily to ribosomes, nuclear envelope and mitochondria (**Fig. 4**).

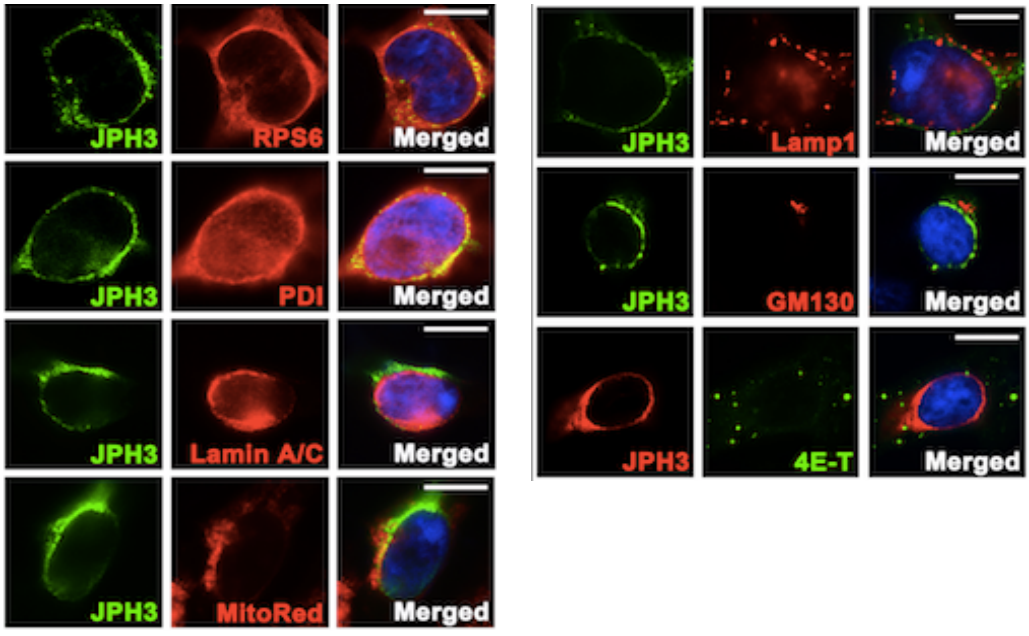


Figure 3. JPH3 co-localizes primarily with a ribosomal (RPS6), nuclear envelope (PDI) and a mitochondrial (MitoRed) marker in SH-SY5Y cells.

Future directions: We will examine the levels of calmodulin and TrkB in HDL2 versus control brains, as well as HDL2 iPSCs. We will include HD and ChAC iPSC-derived neurons (from **Aim 2**) in our analysis. We will use the siRNA approach to test if knocking down endogenous JPH3 affects the levels of calmodulin and TrkB in SK-N-MC cells. The experiment will also be performed in mouse primary striatal and cortical neurons. This will test if the loss

of FL JPH3 directly affects the levels of the proteins. We will use a lentiviral approach to overexpress FL JPH3 in mouse primary neurons from JP3 knock-out mice and examine if this rescues the levels of calmodulin and TrkB. We will examine the mechanism by which loss of JPH3 affects calmodulin and BDNF signaling. Besides FL JPH3, multiple variants of the JPH3 protein exist in human cells. Our current antibodies detect multiple variants simultaneously and are hence not suitable to examine intracellular distribution of endogenous JPH3 in human cells. We plan to develop additional anti-JPH3 antibodies that will allow us to examine the localization of endogenous JPH3 in multiple models, including HDL2 iPSCs, in more detail.

Identification of novel interacting partners of JPH3 and VPS13A. To further explore the potential role of JPH3, we overexpressed FL JPH3 in SK-N-MC cells and performed mass-spectrometry analysis to identify potential interactors of the protein. FL VPS13A construct, provided to us by Dr. Antonio Velayos-Baez, was included in the analysis. **Table 1** lists a selection of the most interesting proteins identified in at least two independent experiments.

Table 1. Potential JPH3 and VPS13A protein interactors, as identified by MS.

Cytoplasmic and nuclear lysate
unique ppetides >=2, p>95%

Unique Peptide	ID	Name	Alternative names	Localization
4	gi|209413738|ref|NP_001129243.1|	dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2 isoform 2 precursor [Homo sapiens]	Ribophorin 2	rough ER, membrane protein
4	gi|30795231|ref|NP_006308.3|	brain acid soluble protein 1 [Homo sapiens]	Nap22	membrane bound protein
4	gi|105990514|ref|NP_001448.2|	filamin-B isoform 2 [Homo sapiens]		Cytoskeleton
3	gi|9845509|ref|NP_061485.1|	ras-related C3 botulinum toxin substrate 1 isoform Rac1b [Homo sapiens]	Rac1b	
3	gi|4502205|ref|NP_001651.1|	ADP-ribosylation factor 4 [Homo sapiens]	Arf4	membrane
2	gi|11641247	Golgi-associated plant pathogenesis-related protein 1 [Homo sapiens]	GAPR-1	Golgi Body
2	gi|4507877	vinculin isoform VCL	VCL	membrane Cytoplasm
2	gi|987430343	ribosomal protein S6	RPS6	ribosome
2	gi|4506597	60S ribosomal protein L12	RPL12	ribosome
2	gi|194328685	Unconventional myosin-Ib		
2	gi|4506691	40S ribosomal protein S16 [Homo sapiens]		Cytoplasm
2	gi|13904866	60S ribosomal protein L28 isoform 2 [Homo sapiens]		Cytoplasm
3	gi|119624101	ribosomal protein S18, isoform CRA_c [Homo sapiens]		Cytoplasm
2	gi|4506707	40S ribosomal protein S25 [Homo sapiens]		Cytoplasm
2	gi|332164775	pyruvate kinase isozymes M1/M2 isoform c		
17	gi|193083120|ref|NP_001122388.1|	mitochondrial import receptor subunit TOM40 homolog [Homo sapiens]	Protein Haymaker;Translocase of outer membrane 40 kDa subunit homolog;p38.5	Mitochondrion outer membrane
6	gi|23618867|ref|NP_073591.2|	sideroflexin-1 [Homo sapiens]	Tricarboxylate carrier protein	mitochondria
5	gi|25188179|ref|NP_005653.3|	voltage-dependent anion-selective channel protein 3 isoform 1 [Homo sapiens]	Outer mitochondrial membrane protein porin 3	outer mitochondrial membrane
4	gi|21361181|ref|NP_000692.2|	sodium/potassium-transporting ATPase subunit alpha-1 isoform a [Homo sapiens]	Na/K pump	PM
3	gi|34147513|ref|NP_004628.4|	ras-related protein Rab-7a [Homo sapiens]	Rab7a	late endosome

JPH3 interactors
VPS13 interactors
Shared interactors

We confirmed that RPS6 interacts with JPH3 using an anti-JPH3 antibody (**Fig. 4**). We first selected this protein as the data was obtained prior to our optimization of the immunoprecipitation experiment with the FL VPS13A. This protein was also interesting as (1) our immunocytochemistry experiment (**Fig. 1**) indicated co-localization of overexpressed FL JPH3 and this ribosomal marker, and (2) our proteome analysis of JP3 mice supports the idea that JPH3 is a multifunctional protein that may also be involved in protein translation.

Future directions:

(1) We will confirm the interaction using an anti-RPS6 antibody. Unfortunately, we were unable to test if RPS6 also interacts with VPS13A as our experiments with Chor 1 Ab (kindly provided to us by Dr. Velayos-Baez) were inconclusive (data not included). We will next clone VPS13A into a vector with a FLAG tag and test its interaction with RPS6.
(2) We have identified Rab 7a as a possible interactor of both JPH3 and VPS13A and we will confirm the interactions.
(3) We will test if both JPH3 and VPS13A interact with sodium/potassium transporting ATPase subunit alpha 1isoform a. We have yet to identify a suitable antibody for this experiment as the two antibodies we have previously tried were not suitable for IP analysis (data not included).

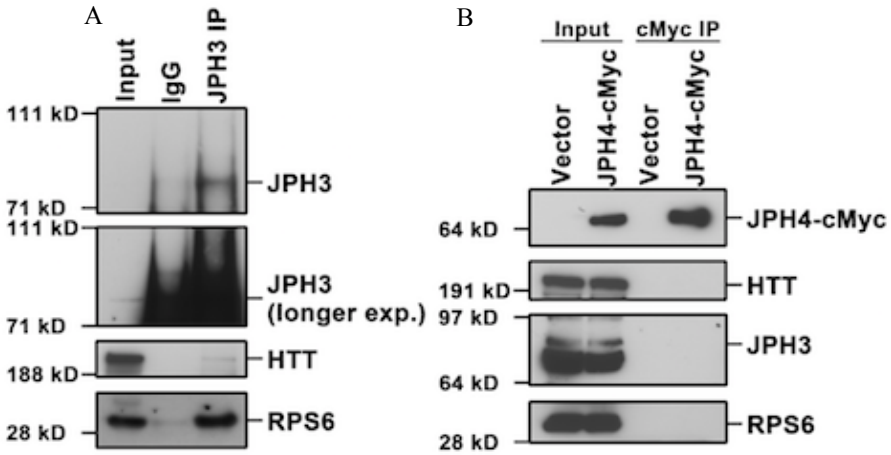


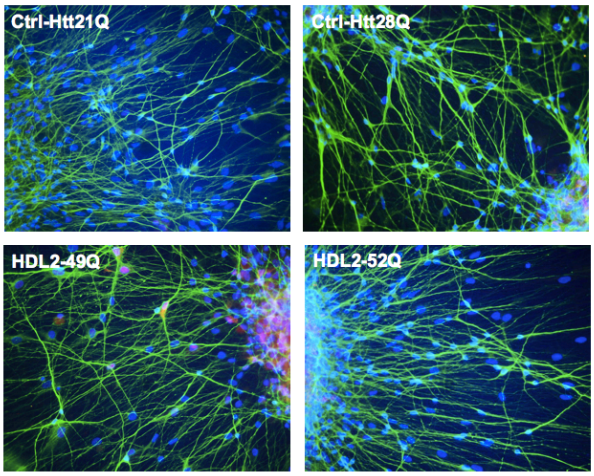
Figure 4. (A) A pull-down experiment with an anti-JPH3 antibody [1] confirms that endogenous JPH3 interacts with RPS6 in neuronal-like SH-SY5Y cells. **(B)** RPS6 does not interact with exogenously expressed junctophilin 4 (JPH4), another brain specific protein from the junctophilin family.

Specific Aim 2. Conduct quantitative proteomics assays to identify proteins whose levels change in ChAc iPSC-derived neurons compared to HDL2 iPSC- and control-derived neurons. We hypothesize that classes of proteins with altered expression in ChAc compared to control will reveal pathogenic pathways related to ChAc. We also hypothesize that at least some overlap may be observed with pathways identified as being altered in HDL2. To test this, we proposed to perform iTRAQ analysis and compare ChAc proteome to that of neurons derived from HDL2, HD and control iPSCs.

This part of the project was delayed as Drs. Hermann and Storch were unable to send existing ChAc lines and have develop new ChAc lines that we received six months after funding began. We received three ChAc (LM; lenitiviral reprogramming; D2.5, retroviral reprogramming; D1.17, retroviral reprograming) and two control lentiviral reprograming lines, WM and AK, respectively. The lines were backed up and underwent adjustment to our growth and maintenance protocols in order to allow for proper comparison with the HDL2, HD and control lines developed in our division. Unfortunately, while lines LM and D2.9 grew well in our hands, we were so far unable to propagate line D1.17. Hence, all our future experiments will be performed with lines LM and D2.9. In parallel to adapting ChAC lines to our experimental conditions, we have successfully finalized the characterization of three HDL2 lines developed in collaboration with Dr. Russell Margolis. **Figure 5.** shows differentiation of two HDL2 and two control iPSC lines into MAP2/DARP32 positive neurons, as we have proposed in our application. We have also determined that, as with HD iPSCs [11], HDL2 iPSCs show increased sensitivity to BDNF withdrawal **(Fig. 5B**). We are currently performing the differentiation experiment with ChAc lines.

Finally, we have obtained and backed up EZ spheres for each of the HDL2, HD and ChAC lines that will be subjected to the proteome analysis. We anticipate that iTRAQ analysis of the EZ spheres will be performed within the next month.

A



B

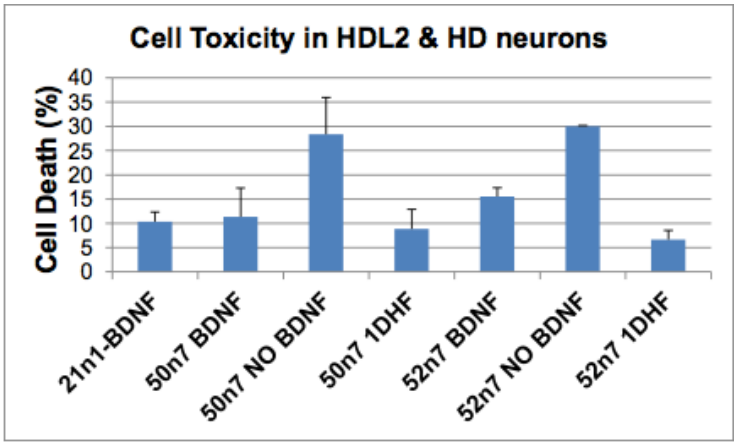


Figure 5. (A) HDL2 iPSC can be successfully differentiated into striatal neurons. (B) Both HDL2 and HD iPSC show increased sensitivity to BDNF withdrawal (No BDNF lines).

Overall summary: During the last year we have confirmed that loss of JP3 in a mouse model may disrupt calmodulin and BDNF signaling. The levels of these proteins are reduced in both HD and AD brains, indicating possible points of convergence in the pathogenesis of the diseases. In support of this, we have also observed loss of JPH3 in AD brains. Next, we will examine if reduction of calmodulin and/or TrkB is caused directly by loss of FL JPH3. To further elucidate the neuronal function of JPH3, we have performed an IP assay optimized for large proteins and have preliminarily identified novel interactors of full-length JPH3. We have also included full-length VPS13A in the analysis and have identified at least two proteins that potentially interact with JPH3 and ChAC- Rab 7a and sodium/potassium ATPase transporting subunit alpha 1 isoform a. We are currently testing antibodies that will be used to confirm the interactions using endogenously and exogenously expressed JPH3 and VPS13A. Finally, we have obtained three new ChAC iPSC lines from Dr. Hermann and were able to propagate two of the lines in our laboratory. We have completed the characterization of three HDL2 lines that we have developed in collaboration with Dr. Margolis. We are currently preparing two ChAc, two HDL2, and two HD and three control iPSC lines [11] for proteome analysis using iTRAQ.

Summary of future plans: We plan to, within the next three to four months, submit a paper describing the characterization of HDL2 iPSC lines and comparison of their phenotype and neurotoxicity to that of HD iPSC lines. Any data from iTRAQ experiments in which proteome of HDL2, HD and ChAc lines will be compared to that of controls and will be included in the paper. With regards to the protein interactor data, we plan to focus on interactors that may be involved in both HDL2 and ChAc. Our plan is to collect sufficient data that would allow us to apply for additional NIH-type funding and pursue the mechanistic comparison between the two diseases in more detail.

References:

1. Seixas, A.I., et al., *Loss of junctophilin-3 contributes to Huntington disease-like 2 pathogenesis.* Ann Neurol, 2012. **71**(2): p. 245-57.
2. Rudnicki, D.D., et al., *Huntington's disease--like 2 is associated with CUG repeat-containing RNA foci.* Ann Neurol, 2007. **61**(3): p. 272-82.
3. Wilburn, B., et al., *An antisense CAG repeat transcript at JPH3 locus mediates expanded polyglutamine protein toxicity in Huntington's disease-like 2 mice.* Neuron, 2011. **70**(3): p. 427-40.
4. Walker, R.H., et al., *Huntington's disease--like 2 can present as chorea-acanthocytosis.* Neurology, 2003. **61**(7): p. 1002-4.
5. Prohaska, R., et al., *Brain, blood, and iron: perspectives on the roles of erythrocytes and iron in neurodegeneration.* Neurobiol Dis, 2012. **46**(3): p. 607-24.

6. Chung, D.W., et al., *A natural antisense transcript at the Huntington's disease repeat locus regulates HTT expression.* Hum Mol Genet, 2011. **20**(17): p. 3467-77.
7. Sun, X., et al., *Nuclear retention of full-length HTT RNA is mediated by splicing factors MBNL1 and U2AF65.* Sci Rep, 2015. **5**: p. 12521.
8. Ginsberg, S.D., et al., *Microarray analysis of hippocampal CA1 neurons implicates early endosomal dysfunction during Alzheimer's disease progression.* Biol Psychiatry, 2010. **68**(10): p. 885-93.
9. McLachlan, D.R., et al., *Calmodulin and calbindin D28K in Alzheimer disease.* Alzheimer Dis Assoc Disord, 1987. **1**(3): p. 171-9.
10. Moriguchi, S., et al., *Functional uncoupling between Ca2+ release and afterhyperpolarization in mutant hippocampal neurons lacking junctophilins.* Proc Natl Acad Sci U S A, 2006. **103**(28): p. 10811-6.
11. Consortium, H.D.i., *Induced pluripotent stem cells from patients with Huntington's disease show CAG-repeat-expansion-associated phenotypes.* Cell Stem Cell, 2012. **11**(2): p. 264-78.