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CRISPR-Based Knockout Screening in Primary Neurons

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By

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ABSTRACT

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Axon growth is coordinated by multiple interacting proteins that remain incompletely characterized. High content screening (HCS), in which manipulation of candidate genes is combined with rapid image analysis of phenotypic effects, has emerged as a powerful technique to identify key regulators of axon outgrowth. Here we explore the utility of a genome editing approach referred to as CRISPR (Clustered Regularly Interspersed Palindromic Repeats) for knockout screening in primary neurons. In the CRISPR approach a DNA-cleaving Cas enzyme is guided to genomic target sequences by user-created guide RNA (sgRNA), where it initiates a double-stranded break that ultimately results in frameshift mutation and loss of protein production. Using electroporation of plasmid DNA that co-expresses Cas9 enzyme and sgRNA, we first verified the ability of CRISPR targeting to achieve protein-level knockdown in cultured postnatal cortical neurons. Targeted proteins included NeuN (RbFox3) and PTEN, a well-studied regulator of axon growth. Effective knockdown lagged at least four days behind transfection, but targeted proteins were eventually undetectable by immunohistochemistry in more than 80% of transfected cells. Consistent with this, anti-PTEN sgRNA produced no changes in neurite outgrowth when assessed three days post-transfection. When week-long cultures were replated, however, PTEN knockdown consistently increased neurite lengths. These CRISPR-mediated PTEN effects were achieved using multi-well transfection and automated phenotypic analysis, indicating the suitability of PTEN as a positive control for future CRISPR-based screening efforts. Combined, these data establish an example of CRISPR-mediated protein knockdown in primary cortical neurons and its compatibility with HCS workflows.

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Ben Callif

In no particular order, I would like to thank my family, my friends, Dr. Blackmore, my initial qualifying exam committee, and my revised Master's committee for their constant support and love. In addition, I would like to thank everyone in the Neuroscience department for being such a collaborative family of scientists. Finally, I would like to thank all the Graduate School and Biological Sciences office staff who put up with my many missed deadlines.

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I. INTRODUCTION

As the name implies, Neuroscience is the study of neurons and the nervous system. However, within the field there seems to be a bias whereby researchers focus on either the brain or the body, while ignoring the interaction between these two inseparable systems. This is exemplified by the Society for Neuroscience which, per their website, is "the world's largest organization of scientists and physicians devoted to understanding the brain and nervous system" (https://www.sfn.org/About/Mission-and-Strategic-Plan). This contrast between the brain and the nervous system is likely a reflection of the differentiation between the central (CNS) and peripheral nervous systems (PNS), which are distinguished by protective barriers that isolate the cerebrospinal fluid (CSF) from the blood. Unfortunately, this puts the spinal cord in a precarious position, since it is clearly a part of the CNS, but is almost entirely ignored in Neuroscience education. For example, the blood-brain barrier (BBB), which isolates the brain from the rest of the body, is a popular topic of research and teaching curriculums, but the blood-spinal cord barrier, which isolates the spinal cord from the blood, is rarely mentioned and is intuitively thought of by researchers as being synonymous with the BBB (Bartanusz et al., 2011).

This theoretical separation between the brain and the nervous system in Neuroscience is unfortunate and unnecessary. The brain is undoubtedly a part of the nervous system – despite the isolation by the BBB, the brain and the body are intrinsically connected and neither functions without the other. The connection and interface between the brain to the body is mediated by the spinal cord: a long bundle of nerves extending from the base of the brain down to the pelvic region. The spinal cord receives information from the peripheral tissues of the body, like the skin,

muscles, and joints, and transmits this information to the brain where it is processed as sensory input and re-transmitted back to the spinal cord in the form of motor commands (Schwab, 2002). The focus on the brain as the centralized control center for the rest of the body and nervous system runs contrary to the thoughts of the great thinker, Leonardo da Vinci, who stated: "The frog instantly dies when the spinal cord is pierced; and previous to this it lived without head, without heart or any bowels or intestines or skin; and here therefore would seem lies the foundation of movement and life" (Lifshutz & Colohan, 2004). Although humans do not technically need a fully functional spinal cord to survive, the spinal cord is a necessary feature of vertebrate existence (Romer & Parsons, 1986).

The spinal cord is the connection between the brain and the body, and spinal cord injury (SCI) is a perfect metaphor for the gap between Neuroscience and bodily physiology. The interruption of information flow from the periphery to the brain, and vice versa, is debilitating for bodily function, just like the theoretical distinction between studying the 'brain' and the 'nervous system' is holding back the field. Despite the exponential advances in the complexity of Neuroscientific research and our understanding of the brain there have been few, if any, effective cures for mental health disorders (Grohol, 2009). Neuroscience, the study of the brain to improve psychological well-being, is a relatively new field, but SCI has been studied for thousands of years and has a similar story. The first historical mention of SCI was in ancient Egypt in 2500 BC, and a shortened translation of the papyrus reads: "If thou examines a man having a dislocation of the neck … thou shouldst say concerning him … an ailment not to be treated" (Lifshutz & Colohan, 2004). From this translation, we can see how it has taken over 4,000 years to advance medicine enough to even have hope for a cure to SCI. To make Neuroscience more productive it will be necessary to metaphorically unify the relationship between the CNS and the rest of the body, much like it is necessary to bridge the literal gap between the brain and the body by healing the spinal cord to cure SCI.

SCI is broadly defined as a disruption of the spinal cord which causes changes in bodily function below the injury site. This definition is quite broad because the presentation of SCI is widely varied based on the magnitude, location, and cause of the injury. There are two major categories of injury to the spinal cord: complete and incomplete. These labels refer to the severity of the injury, with a complete injury causing a total loss of function and sensation below the injury site and an incomplete injury causing only a partial loss (Ho et al., 2007). Furthermore, SCI is also designated by the location of the injury site. Lumbro-sacral injuries occur near the base of the spine and can cause changes in the function of everything from the hips down. This can cause problems with the urogenital system and lead to a toxic buildup of urine due to loss of functional bladder control (O'Sullivan et al., 2014). Thoracic injuries occur in the abdominal region and affect the function of everything from the chest down. This can be a serious issue for sympathetic homeostatic controls like body temperature and blood vessel dilation which can lead to neurogenic shock which is characterized by blood pooling in the limbs, low blood pressure, and low heart rate (Holtz & Levi, 2010). Finally, cervical spinal cord injuries occur in the region of the neck and can affect the entire body leading to tetraplegia, or loss of function in all four limbs. This can cause all the problems previously mentioned for lumbro-sacral and thoracic sites of injury in addition to an extremely serious paralysis of the muscles that control breathing (Sabharwal, 2014).

The consequences of SCI can be very severe, but luckily it has a relatively low prevalence. It is difficult to gauge the global impact of SCI, since the numbers vary drastically from country to country. For example, in Africa and many regions of Asia there is very little data to about the number of SCIs and the causes of the injuries (Witiw & Fehlings, 2015). However, in the United States approximately 12,000 people receive SCIs every year and in China there are nearly 60,000 new cases of SCI every year (Liu et al., 2015; Qiu, 2009). These may seem like low incidence rates, but in the United States there are up to 300,000 people living with a spinal

cord injury (Field-fote, 2009). This is a huge problem because with proper treatment these individuals can live a long time following their injuries and the medical costs are staggering. There are initial medical expenses that must be dealt with immediately following the injury, like surgery, rehab, and mobility equipment such as electric wheelchairs. There are also subsequent expenses for every year of life following the injury, since these patients may never entirely recover bodily function. These include follow-up surgeries and medical expenses related to complications from the injury as well as the cost of full or part-time caretakers, since SCI can cause a partial or total loss of mobility and self-reliance. For an incomplete injury, the first year can cost upwards of $300,000 and every year of life following the injury can cost up to $40,000. For a complete injury, the first year can cost anywhere from $500,000 up to $1,000,000 and each following year of life can cost anywhere from $60,000 to $180,000 (https://www.nscisc.uab.edu). The financial and physiological destruction that these injuries cause becomes even more apparent when the monetary costs are stacked on top of the debilitating nature of most SCIs and the inability of many SCI sufferers to continue working following their injury. The devastating nature of these injuries to everyday life is even more clear when patients are surveyed about how their lives could be enhanced: the top priority for quadriplegics was to simply regain arm and hand function, while paraplegics wanted to regain sexual function. All SCI patients agreed that a close second place for quality of life improvement would be simply having control over their bowels and bladders again (Anderson, 2004).

Along with its high cost, SCI has many seemingly mundane causes that could happen to anyone. Physical trauma is the most likely way to receive an injury that disrupts the spinal cord (Peitzman et al., 2013). For example, some of the most common traumas that cause SCI are motor vehicle accidents and sports (Sabharwal, 2014). Depending on the geographic region falls and violence, like gunshot wounds, are also leading causes of SCI (Holtz & Levi, 2010). Although physical trauma is the leading cause, SCI can also occur due to degenerative diseases and

infections. Interestingly, in developing countries the most common causes of SCIs are infectious diseases like HIV and tuberculosis (Sabharwal, 2014). Compared to developing countries, people in developed countries have a higher chance of SCI due to degenerative conditions like multiple sclerosis and even due to diseases like meningitis and cancerous tumors which can cause damage by putting pressure on the spinal cord (New et al., 2014).

Due to the severity of the injury, the high cost of living, and the variety of presentations of SCI there is research being done that attacks the problem from all angles. Since there are many people currently living with an SCI, much of the research is focused on how we can improve the quality of life and increase bodily function following an injury. For example, the best technology currently available for improving mobility and function following SCI essentially turns patients into cyborgs. Power wheelchairs allow for mobility without the use of the leg muscles or nursing aids and there are even wheelchairs that can be controlled with tongue motions to help individuals with complete tetraplegic injuries (Kim et al., 2013). Amazingly, some research has even started to utilize robotic exoskeletons as a method of rehabilitating and restoring function to paralyzed individuals (Aach, et al., 2014). Restoring function and rehabilitating injured individuals is a worthy goal, and while some patients spontaneously recover after an injury many patients will never fully regain function after SCI (Behrman & Harkema, 2000). Due to the role of the spinal cord as a bridge between brain function and bodily behavior one of the main goals of SCI research is to induce the regrowth of axons: the long projecting processes of neurons that encode outgoing information in the form of action potentials (Schwab, 2002; Waxman et al., 1995).

One method of achieving this goal is to use exogenously applied agents to enhance the growth of the axons that compose the spinal cord. For example, many studies have focused on disrupting molecular signaling pathways that are inhibitory for growth, like the myelin associated Nogo-A. This has been attempted through application of Nogo-A neutralizing antibodies, which have shown promise for disinhibiting axonal regrowth in several animal models of SCI (Fouad et

al., 2004; Liebscher et al., 2005). Other studies have attempted to apply growth promoting agents to the injured cord to enhance axonal growth. Direct injections of hormones, such as progesterone, have been tested for neuroprotective effects following SCI (Thomas et al., 1999). There are also pumps available to constantly supply factors directly into the CSF, but a more popular method of growth factor application is implantation of genetically engineered cells, like fibroblasts, that produce and excrete specific factors into the injured spinal cord (McTigue et al., 1998; Tuszynski et al., 2003). More recently, direct application of mitochondrial-targeted ceria nanoparticles has also emerged as a possible means of neuroprotection following SCI (Das et al., 2007).

In recent years, direct genetic modification of cortical neurons has become another method of coaxing axons to grow. The use of Adeno-associated viruses (AAVs) provides a straightforward and consistent method of expressing target genes with a relative amount of spatial specificity (Blackmore et al., 2012). Genetically modified animals have also been used to model the role that specific factors play in axonal regrowth following an injury (Sun et al., 2011). But there are some issues with this. For example, while direct application studies showed a role for factors such as Nogo-A in regeneration following SCI, there are conflicting reports about whether mutant mice altogether lacking Nogo-A mount a stronger regenerative response than naïve, wild-type animals (Simonen et al., 2003; Zheng et al., 2003). Similarly, it is now clear that targeted manipulation of gene expression in injured neurons is an effective means to improve axon growth, but improvements remain partial and no studies have yet induced spinal cord regeneration to the point of actually growing axons past the injury site (Belin et al., 2015; Blackmore et al., 2012; Sun et al., 2011; Wang et al., 2015).

These studies and others have shown that the growth of axons and dendrites depends on numerous cellular processes including cytoskeletal remodeling, membrane expansion, intracellular signaling, regulated transport, and transcription. Thus, the coordinated activity of

hundreds of proteins is needed for effective neurite extension. Identifying the full set of proteins that regulate axon growth remains a key objective in regenerative neuroscience and is critical for ongoing efforts to improve regenerative ability in neurons that have lost axons to injury or disease. Forward genetic screens performed in primary neurons have emerged as a powerful tool to discover novel regulators of neurite growth (Blackmore, 2012). These screens generally rely on two components. First, approaches such as electroporation or viral transduction in multi-well format are used to achieve high throughput genetic modification of primary neurons. Second, automated image analysis in multi-well format rapidly quantifies effects on neurite extension. This readout can be performed on the overall population of neurons (e.g. "well-based" quantification), or can assess neurite length from individual neurons (e.g. "cell-based" analysis). A variety of labs have now harnessed this genetic screening approach, leading to a rapid expansion of the set of known regulators of axon growth (Blackmore, 2012; Blackmore et al., 2010; Buchser et al., 2012; Simpson et al., 2015; Zou et al., 2015).

Many of these screens involve gene overexpression and thus address the sufficiency of ectopic gene expression to evoke phenotypic responses. For example, we in the Blackmore lab have previously performed an overexpression screen of 69 transcription factors (Simpson et al., 2015). There are an overwhelming number of candidate factors we could have picked to include in the screen, so we decided to focus on transcription factors that are related to the development of cancer. The idea behind this was that neurons are post-mitotic, and therefore, cannot divide. We hypothesized that induction of a cancerous state in these cells would cause a phenotype somewhat like cancerous growth, but through the extension of neurite processes rather than cell division. This strategy turned out to be quite effective, as 9 of the 69 factors we overexpressed significantly altered the outgrowth of cortical neurites in culture. However, overexpression studies such as this one are only useful to delineate the complexities of systemic interaction with

exogenously applied factors. In order to clarify the role of endogenous protein in neurite outgrowth, knockout approaches are necessary (e.g. (Zou et al., 2015).

Genome editing methods such as zinc finger nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and Clustered Regularly Inter-Spaced Palindromic Repeats (CRISPR) represent potentially improved tools for knockout screening. Unlike previous RNAi approaches, which act post-transcriptionally and may induce only partial protein knockdown, genome editing can modify DNA at specific loci to abolish protein production completely and permanently. CRISPR, in particular, has been widely adopted and enthusiastically embraced by the public and the media. CRISPR is an emerging technique that avoids many of the problems associated with RNAi. The tool itself is borrowed from an adaptive immune system found in approximately 40% of bacterial genomes and almost all archaeal genomes (Kunin et al., 2007). CRISPR has evolved to recognize invading viral DNA and excise it from the host's genome. It performs this complicated task through a series of nucleases known as the Cas (CRISPR-associated) proteins.

There are a variety of CRISPR/Cas systems, but they generally operate through the same principles. Briefly, the first Cas enzymes in the pathway recognize a short sequence of nucleotides in the viral DNA, known as a PAM (protospacer adjacent motif), that will never be expressed by the host. This PAM acts to ensure that the CRISPR/Cas system will not act upon any endogenous bacterial DNA sequences. The PAM, along with a consistent number of downstream nucleotides known as a spacer, is then removed from the invading DNA and inserted into a specific locus in the host's genome. The spacer is placed into the genome in such a way that it is flanked on either side by palindromic repeats. This sequence of spacers and palindromic repeats is then transcribed into a single RNA molecule, which is further processed into short, single-stranded RNAs called CRISPR RNAs (crRNAs). The crRNA, along with a partially complementary tracrRNA (trans-activating crRNA), binds to the Cas9 enzyme creating a

ribonucleoprotein effector complex. Watson-Crick base pairing will ensue if this ribonucleoprotein complex encounters dsDNA (double-stranded DNA) that is complementary to its crRNA. Once the complex is tightly bound to the dsDNA Cas9 will recognize the PAM sequence upstream of the target sequence and induce a double-stranded break (Terns & Terns, 2011).

The endogenous CRISPR system is quite complex, but a recent paper by Cong et al. (2013) showed that only a few pieces of the pathway are necessary to efficiently and accurately knock out targets at the genomic level. To simplify the way that the Cas9 enzyme handles RNA they created a crRNA/tracrRNA hybrid called sgRNA (short guide RNA). They showed that expression of the Cas9 enzyme and an sgRNA was sufficient to induce targeted double-stranded breaks with high specificity and efficiency. Without a template the double-stranded breaks are repaired by the highly error prone NHEJ (non-homologous end-joining machinery), which frequently causes indel (insertion or deletion) mutations. If the Cas9/effector complex is targeted to the ORF (open reading frame) of a gene the ensuing NHEJ repair will often cause a frameshift mutation due to indels, functionally knocking out the gene. Targeted genomic knock out is extremely useful, but it only begins to scratch the surface of CRISPRs capabilities. It is now well established that Cas proteins are functional when expressed in mammalian cells and can be directed to specific loci by supplying nothing more than an sgRNA targeting a user-specified gene. Importantly, examples now exist to demonstrate the efficacy of protein-level knockout by the CRISPR strategy in neurons (Platt et al., 2014; Straub et al., 2014; Swiech et al., 2015; Zuckermann et al., 2015).

There may be unique challenges, however, to adopting the CRISPR approach to screening in post-mitotic neurons. First, previous CRISPR-based screens in non-neuronal cells have relied on purifying and expanding affected cells with antibiotic resistance genes or fluorescent reporters to obtain knockout clones (An et al., 2014; Chen et al., 2015; Schmid-Burgk

et al., 2016; Wang et al., 2014). Without the option of proliferation-based purification, however, gene delivery methods for neurons must be rapid and efficient. Second, no studies have yet determined how quickly CRISPR causes knockdown in neurons. There must be a defined timecourse of knockdown to rationally plan experimental endpoints. Finally, the few publications that have utilized CRISPR in cultured neurons have used lentiviral-based approaches to force expression of a Cas enzyme and an sgRNA (Incontro et al., 2014; Telese et al., 2015). Large-scale screening, however, may be more feasible and economical using plasmid-based transfection (Ebert et al., 2008; Moffat et al., 2006; Root et al., 2006).

Here we propose to perform validation and optimization experiments to assess the utility of CRISPR for high content screening in neurons. Using delivery of Cas9 and sgRNA by plasmid electroporation in multi-well format, we will confirm the timecourse of protein knockdown using a previously validated targeting sequence for NeuN (Rbfox3), a ubiquitously expressed neuronal protein (Platt et al., 2014). We will next deliver sgRNA against Phosphatase and Tensin homolog (PTEN), a well-studied inhibitor of neurite outgrowth. Initial data suggests that for both targets the majority of transfected neurons will not be affected by CRISPR knockdown until after 3 days in vitro. By this point the neurons in the culture have created an extensive web of synaptic connections, which makes neurite outgrowth analysis difficult, if not impossible. Therefore, we also propose to create and optimize a replating technique, which will either mechanically or enzymatically disentangle neurons from the plate they were initially cultured in so that we can remove the cells and re-culture them on a new plate. We hope to use this replating technique in conjunction with our neurite outgrowth assay to validate a positive control for future screens.

II. THE APPLICATION OF CRISPR TECHNOLOGY TO HIGH CONTENT SCREENING IN PRIMARY NEURONS

Background

The growth of axons and dendrites depends on numerous cellular processes including cytoskeletal remodeling, membrane expansion, intracellular signaling, regulated transport, and transcription. Thus, the coordinated activity of hundreds of proteins is needed for effective neurite extension. Identifying the full set of proteins that regulate axon growth remains a key objective in regenerative neuroscience and is critical for ongoing efforts to improve regenerative ability in neurons that have lost axons to injury or disease. For instance, it is now clear that targeted manipulation of gene expression in injured neurons is an effective means to improve axon growth (Belin et al., 2015; Blackmore et al., 2012; Sun et al., 2011; Wang et al., 2015). Improvements remain partial, however, motivating the search for additional regulatory proteins that might act as therapeutic targets.

Forward genetic screens performed in primary neurons have emerged as a powerful tool to discover novel regulators of neurite growth (Blackmore, 2012). These screens generally rely on two components. First, approaches such as electroporation or viral transduction in multi-well format are used to achieve high throughput genetic modification of primary neurons. Second, automated image analysis in multi-well format rapidly quantifies effects on neurite extension. This readout can be performed on the overall population of neurons (e.g. "well-based" quantification), or can assess neurite length from individual neurons (e.g. "cell-based" analysis). A variety of labs have now harnessed this genetic screening approach, leading to a rapid expansion of the set of known regulators of axon growth (Blackmore, 2012; Blackmore et al., 2010; Buchser et al., 2012; Simpson et al., 2015; Zou et al., 2015). Many of these screens involve gene overexpression and thus address the sufficiency of ectopic gene expression to evoke

phenotypic responses. To clarify the role of endogenous protein in neurite outgrowth, however, knockout approaches are necessary (e.g. (Zou et al., 2015).

Genome editing methods such as zinc finger nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and Clustered Regularly Inter-Spaced Palindromic Repeats (CRISPR) represent potentially improved tools for knockout screening. Unlike previous RNAi approaches, which act post-transcriptionally and may induce only partial protein knockdown, genome editing can modify DNA at specific loci to abolish protein production completely and permanently. CRISPR in particular has been widely adopted. CRISPR evolved as a bacterial adaptive immune system in which Cas (CRISPR-associated) nucleases are directed to degrade foreign DNA (Kunin et al., 2007). DNA binding by Cas nucleases is limited to sequences adjacent to a specific 3-nucleotide protospacer adjacent motif (PAM) (Terns and Terns, 2011). Critically, Cas nucleases are guided to specific PAM-adjacent regions by RNAs that are complementary to the target DNA sequence. It is now well established that Cas proteins are functional when expressed in mammalian cells and can be directed to specific loci by supplying RNA sequences called single-guide (sgRNA) that contain a user-designed targeting domain. Cas nucleases initiate double-stranded breaks followed by error prone non-homologous end-joining (NHEJ), resulting in insertion or deletion mutations that disrupt the production of functional protein when directed to coding regions. Importantly, examples now exist to demonstrate the efficacy of protein-level knockout by the CRISPR strategy in neurons (Platt et al., 2014; Straub et al., 2014; Swiech et al., 2015; Zuckermann et al., 2015).

There may be unique challenges, however, to adopting the CRISPR approach to screening in post-mitotic neurons. First, previous CRISPR-based screens in non-neuronal cells have relied on purifying and expanding affected cells with antibiotic resistance genes or fluorescent reporters to obtain knockout clones (An et al., 2014; Chen et al., 2015; Schmid-Burgk et al., 2016; Wang et al., 2014). Without the option of proliferation-based purification, however,

gene delivery methods for neurons must be rapid and efficient. Second, no studies have yet determined how quickly CRISPR causes knockdown in neurons. There must be a defined timecourse of knockdown in order to rationally plan experimental endpoints. Finally, the few publications that have utilized CRISPR in cultured neurons have used lentiviral-based approaches to force expression of a Cas enzyme and an sgRNA (Incontro et al., 2014; Telese et al., 2015). Large-scale screening, however, may be more feasible and economical using plasmid-based transfection (Ebert et al., 2008; Moffat et al., 2006; Root et al., 2006).

Here we performed validation and optimization experiments to assess the utility of CRISPR for high content screening in neurons. Using delivery of Cas9 and sgRNA by plasmid electroporation in multi-well format, we first confirmed the timecourse of protein knockdown using a previously validated targeting sequence for NeuN (Rbfox3), a ubiquitously expressed neuronal protein (Platt et al., 2014). We next designed and delivered sgRNA against Phosphatase and Tensin homolog (PTEN), a well-studied inhibitor of neurite outgrowth. For both targets the majority of neurons were unaffected by CRISPR knockdown until 4 to 6 days post-transfection and during this time they extended lengthy processes that were not amenable to neurite outgrowth analysis. Therefore, we developed a replating approach to disentangle overgrown neurites. Following replating automated microscopy was able to detect a robust outgrowth phenotype from PTEN knockout, thereby establishing a positive control for CRISPR-based phenotypic screening.

Time course of CRISPR-mediated knockout of target proteins in primary cortical neurons

CRISPR has been extensively used to decrease protein expression in mitotically active cells and recently, knockdown in post-mitotic neurons has been achieved. For example, an sgRNA targeting sequence against neuron-specific NeuN was shown to reduce NeuN protein expression in adult cortical neurons in vivo upon transgenic co-expression of spCas9 (Platt et al., 2014). We therefore asked whether this same targeting sequence affected NeuN expression in primary cortical neurons that were transfected to express spCas9 and, if so, over what time course. The validated anti-NeuN targeting sequence was cloned into the Px330 plasmid, which co-expresses spCas9 and sgRNA (Cong et al., 2013). Using previously established techniques, plasmid was then delivered by electroporation to cortical neurons prepared from postnatal (P3-P5) rat pups (Blackmore et al., 2010; Buchser et al., 2006). To identify transfected cells, an EGFP reporter was co-transfected at a 1:4 ratio, a strategy shown previously to result in >90% co-expression of test plasmid and reporter (Blackmore et al., 2010). Neurons were plated at high density (100,000 cells plated per well in 24-well format) in media containing 5-Fluoro-2'-deoxyuridine (FuDR) to limit glial proliferation, and then fixed every two days between 4 and 14 days in vitro (DIV). Then, NeuN immunofluorescence was quantified in transfected neurons, which were identified by EGFP and immunohistochemistry for neuron-specific BIII-tubulin (Fig. 1A-C). After 4 days, a significant but modest decrease in average NeuN immunofluorescence was detected in anti-NeuN treated cells compared to control cells transfected with SpCas9 and non-targeted sgRNA (Fig. 1D). Importantly, only 20% of anti-NeuN treated cells showed NeuN levels that fell below a threshold set by the dimmest 5% of control cells. Two days later, however, anti-NeuN displayed a larger reduction in average NeuN immunofluorescence, and more than 50% of transfected cells were dimmer than the lowest 5% of control cells. Later time points showed a trend toward lower NeuN expression, with more than 75% of transfected cells dimmer than 95%

of control cells (Fig. 1D). These data confirm that CRISPR-mediated knockout of NeuN is

effective in cultures of primary cortical neurons, but requires approximately a week to strongly
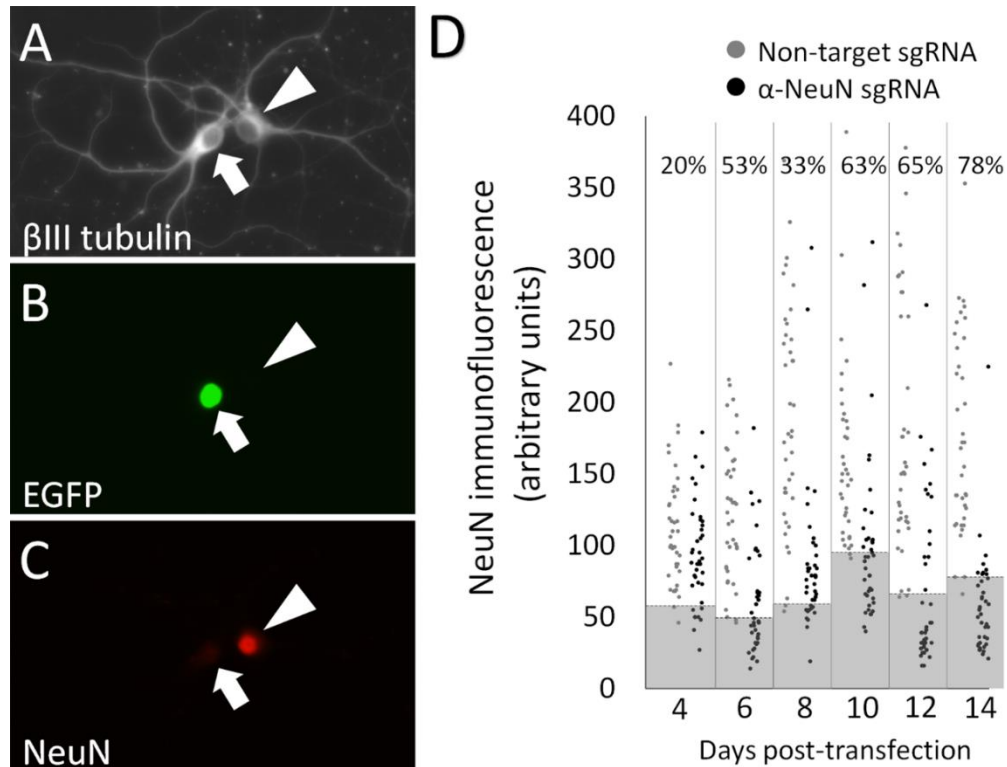
affect a majority of transfected neurons.

Figure 1. CRISPR effectively decreases NeuN protein levels in primary neurons. P3-P5 cortical neurons were transfected with plasmid DNA co-expressing spCas9 and anti-NeuN or non-targeted sgRNA. Neurons were cultured, fixed between 4 and 14 days in vitro (DIV), and immunostained for neuron-specific βIII tubulin and NeuN. (**A-C**) show anti-NeuN treated neurons 14 days after transfection. EGFP (**B**) identifies transfection (arrow). (**C**) Immunohistochemistry detects NeuN protein in a EGFP-negative neuron (arrowhead), but not in a transfected neighbor (arrow). (**D**) Each dot represents NeuN immunofluorescence intensity in a single transfected (EGFP+) neuron between 4DIV and 14DIV. Grey boxes indicate a threshold set by the dimmest 5% of control-transfected neurons at each timepoint, and the percent of anti-NeuN cells that fall below that threshold is quantified above. Significant differences existed between the population-level average intensity at all timepoints (p=0.033 4DIV, p=7.4x10^{-9} 6DIV, p=1.8x10^{-8} 8DIV, p=3.7x10^{-5} 10DIV, p=1.6x10^{-7} 12DIV, p=7.9x10^{-12} 14DIV; unpaired t-test), and the percent of anti-NeuN cells below threshold increases between 4 and 6DIV. N=40 cells per group per timepoint.

We next sought to determine the efficacy and time course of CRISPR-based knockout of a second protein, which had not previously been targeted with a CRISPR approach. We selected PTEN for knockdown based on its well-established role in neurite outgrowth in other types of neurons (reviewed in Ohtake et al., 2015). Using publicly available algorithms to maximize selectivity and efficacy (Doench et al., 2014; Hsu et al., 2013), short oligonucleotides containing anti-PTEN targeting sequences were synthesized, annealed, and ligated into the PX330 plasmid (Cong et al., 2013). As before, P3-P5 rat cortical neurons were electroporated with 4 µg of anti-PTEN or control PX330 plasmid and plated in FuDR-treated media, then fixed every 2 days starting at 4DIV and ending at 14DIV. Neurons were identified by immunohistochemistry for NeuN and transfected cells were identified by the expression of EGFP (Fig. 2A-C). PTEN expression was manually quantified by drawing a region of interest around the cell body of transfected neurons and recording average PTEN immunofluorescence, as measured by NIS-elements software. Four days after transfection, average PTEN fluorescence was significantly reduced in the anti-PTEN group, and 67% of transfected neurons fell below the dimmest 5% of control neurons. Two days later, average PTEN fluorescence was further reduced in the anti-PTEN group, and 85% of neurons fell below the 5% threshold (Fig. 2D). Thus, user-designed CRISPR targeting sequences effectively decreases protein expression of PTEN in primary cortical neuron cultures on a time scale of four to six days.

One potential strategy to accelerate protein knockdown may be to use multiple sgRNAs that target discrete coding regions of the same gene. To test this possibility, four sgRNA sequences against PTEN were designed and delivered singly or in pair-wise combinations to early postnatal cortical neurons. In order to facilitate comparison with established shRNA-based approaches, cells were transfected with anti-PTEN shRNA shown previously to be effective in CNS neurons (Yungher et al. 2015). Cells were maintained between 2 and 14 days in culture, fixed, and visualized with immunohistochemistry against PTEN and neuron-specific βIII tubulin.
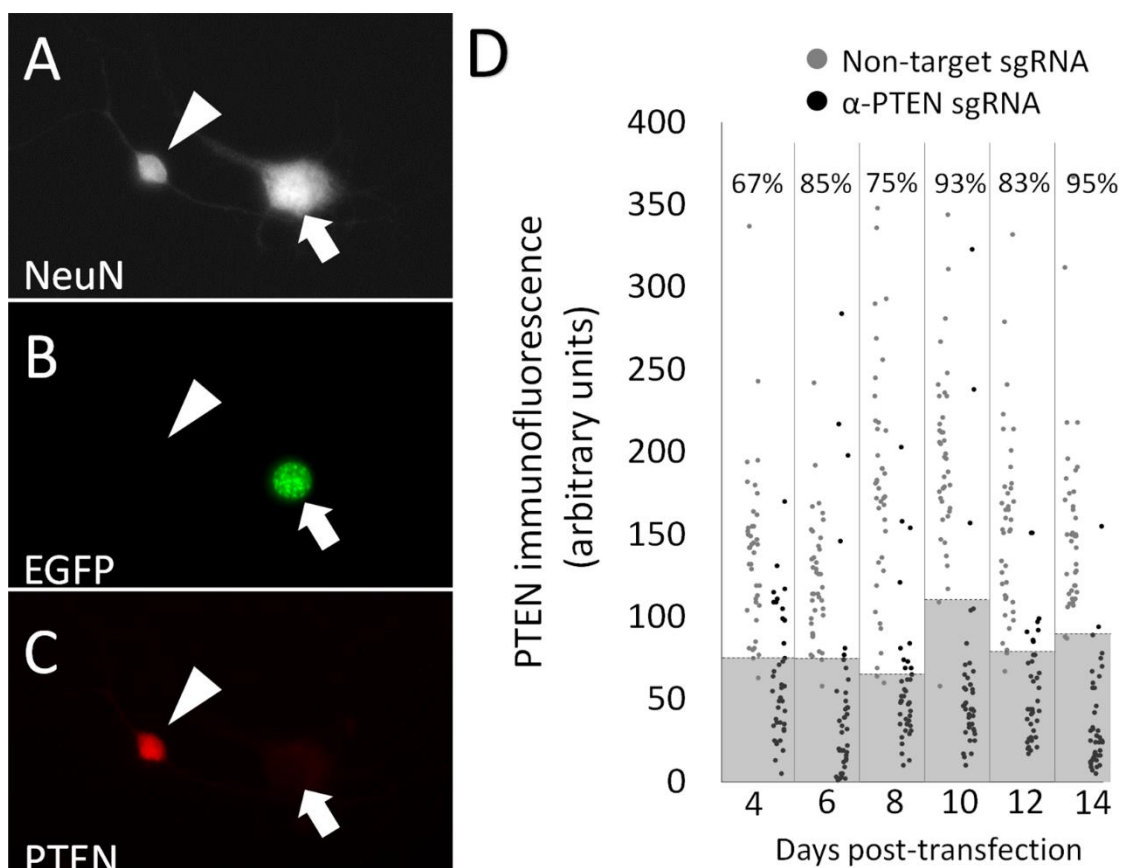
Figure 2. CRISPR effectively decreases PTEN protein levels in primary neurons. P3-P5 cortical neurons were transfected with plasmid DNA co-expressing spCas9 and anti-PTEN or non-targeted sgRNA. (**A-C**) show neurons, identified by NeuN (**A**), 14 days after transfection with anti-PTEN. (**B**) EGFP identifies transfection (arrow). (**C**) Immunohistochemistry detects PTEN protein in a EGFP-negative neuron (arrowhead), but not in a transfected neighbor. (**D**) Each dot represents PTEN immunofluorescence intensity in a single transfected (EGFP+) neuron between 4DIV and 14DIV. Grey boxes indicate a threshold set by the dimmest 5% of control-transfected neurons at each timepoint, and the percent of anti-PTEN cells that fall below that threshold is quantified above. Significant differences existed between the population-level average intensity at all timepoints ($p=6.9 \times 10^{-11}$ 4DIV, $p=1.4 \times 10^{-8}$ 6DIV, $p=5.0 \times 10^{-13}$ 8DIV, $p=3.3 \times 10^{-15}$ 10DIV, $p=4.8 \times 10^{-14}$ 12DIV, $p=3.7 \times 10^{-17}$ 14DIV; unpaired t-test), and by 6DIV 85% of anti-PTEN treated cells fell below the 5% threshold. N=40 cells per group per timepoint.

In contrast to the manual quantification performed above, in this experiment we utilized a Cellomics high content screening microscope to assess PTEN intensity, which enabled analysis of hundreds of cells per treatment at each timepoint. In this analysis, the 10% of cells with the brightest EGFP signal were quantified, in order to focus the analysis on effectively transfected

neurons. In support of this, we found that reduction in PTEN expression was greatest in the top 10% of EGFP-expressing cells (Fig. 3). As shown in Figure 4, all four anti-PTEN sgRNA sequences produced significant knockdown of PTEN in transfected neurons. Consistent with the previous manual quantification, the percent of neurons with PTEN intensity below the 5% control threshold increased from 8-10% at 2DIV to 51-66% at 14DIV. Mixtures of two sgRNAs did not accelerate the onset of knockdown or increase the percent of affected cells at later timepoints. Interestingly, knockdown of PTEN protein via shRNA was much more rapid, with 58% of cells below the 5% threshold after only 2DIV. Overall, these data confirm a lag in the onset of protein knockdown by Cas9 compared to shRNA. Moreover, combined sgRNA sequences did not shorten this lag.
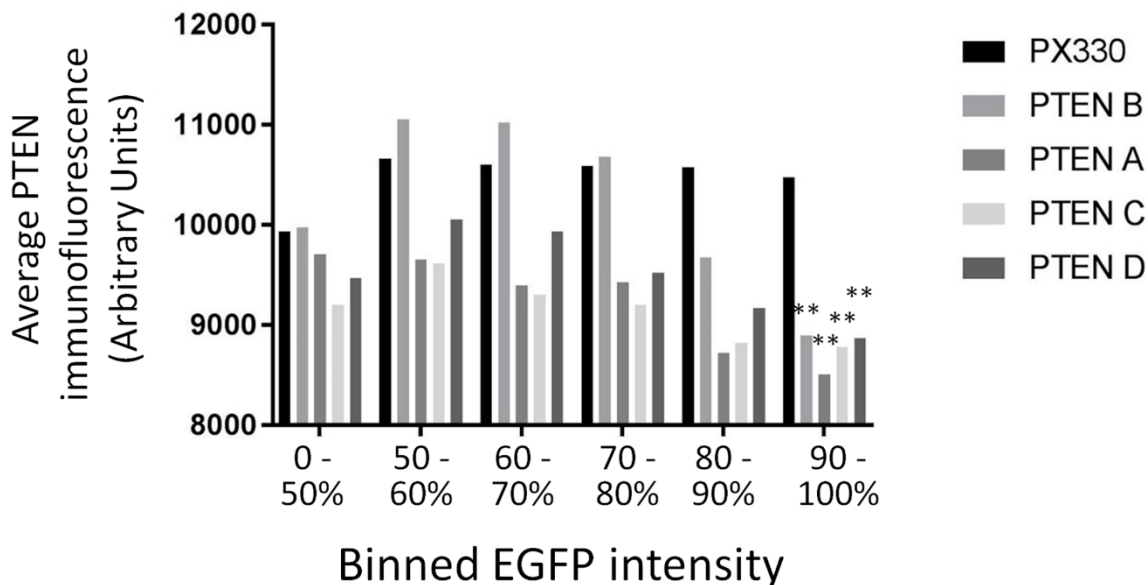
Figure 3. The degree of CRISPR-mediated knockdown of PTEN correlates with EGFP-reporter intensity. P3-P5 cortical neurons were co-transfected with EGFP reporter plasmid and plasmid co-expressing spCas9 and one of four distinct sgRNA sequences targeting PTEN labeled in the figure as PTEN A-D, or non-targeting sgRNA labeled in the figure as 'PX330' (See 'Chapter III: Methodology' for specific nucleotide sequences). At 14 days post-transfection cells were fixed and immunostained for PTEN and Cellomics automated microscopy quantified immunofluorescence and EGFP reporter intensity. Then, the PTEN fluorescence of all cells was binned by EGFP intensity to determine the relationship between the fluorescence of the transfection marker and the level of PTEN knockdown. In all anti-PTEN sgRNA treatments 10% of cells with the brightest EGFP signal show a large and consistent reduction in PTEN fluorescence compared to the non-targeting control treatment (p<.001, ANOVA with post-hoc Dunnett's).
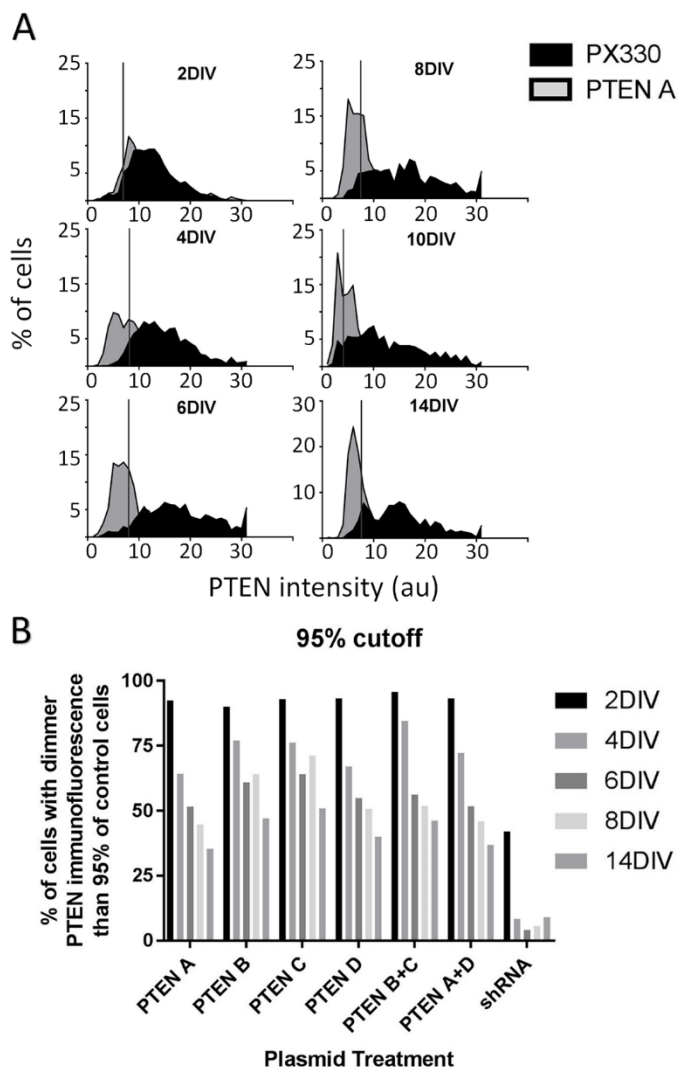
Figure 4. Four distinct anti-PTEN CRISPR constructs display similar timecourse of protein knockdown, which is delayed compared to shRNA-mediated knockdown. P3-P5 cortical neurons were co-transfected with EGFP reporter and plasmid co-expressing spCas9 and one of four distinct sgRNA sequences targeting PTEN labeled in the figure as PTEN A-D, or mixtures of two sequences B+C and A+D. In parallel, neurons were also transfected with anti-PTEN shRNA. Between 2 and 14 days later, cells were fixed and immunostained for PTEN protein, and Cellomics automated microscopy was used to quantify immunofluorescence in EGFP+ cells. (**A**) shows example histograms comparing PTEN immunofluorescence in populations of neurons transfected with anti-PTEN sgRNA and non-targeting control. At two days post-transfection the populations mostly overlap, but in subsequent days the percent of cells with low PTEN expression increases in the sgRNA treatment. Vertical lines indicate a threshold of PTEN signal set by the dimmest 5% of control cells. (**B**) quantifies the percent of EGFP+ cells with PTEN intensity below threshold at the same timepoint. At two days post-transfection, single and mixed sgRNA-treated populations display only 5% of cells below this threshold, while >50% of shRNA-treated cells fall below threshold. By six days, between 40 and 50% of sgRNA-treated cells fall below threshold. More than 500 cells were analyzed in each treatment at each timepoint.

Functional testing CRISPR-mediated phenotype in an HCS workflow

Having established effective CRISPR-mediated protein reduction in primary neurons, we next asked whether this approach could yield reproducible effects on neurite outgrowth using approaches compatible with HCS screening. Previous screens have utilized plasmid electroporation and culture in multi-well

format, followed by automated image acquisition and quantification of neurite outgrowth with Cellomics instrumentation (Blackmore et al., 2010; Simpson et al., 2015). This endpoint analysis is typically performed no more than three days in culture, after which time extensive overlap of neurites makes cell-based quantification very difficult. Although the previous timecourse data suggested it might be unlikely that phenotypic effects of CRISPR-mediated knockdown would be detectable within three days of transfection, we nevertheless tested the effects of anti-PTEN sgRNA using this lab standard protocol. As before, P4 cortical neurons were transfected with PX330-based plasmids encoding spCas9 and non-target, anti-PTEN, or anti-NeuN sgRNA as well as an EGFP reporter at a 1:4 ratio. Neurons were then cultured on PDL/laminin substrate for three days, fixed and stained with neuron-specific BIII tubulin, and neurite lengths quantified in transfected neurons. As shown in Figure 5, neurite length in cells transfected with anti-PTEN
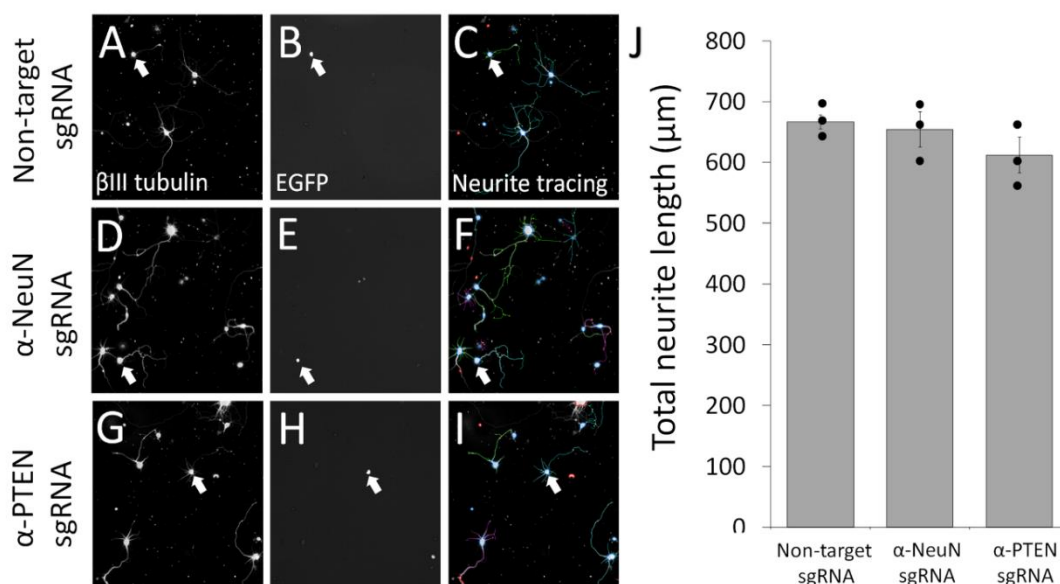

Figure 5. Short-term treatment with anti-PTEN CRISPR does not affect neurite length in primary cortical neurons. P3-P5 cortical neurons were transfected with plasmid DNA co-expressing spCas9, anti-NeuN, anti-PTEN or non-targeting sgRNA, along with plasmid encoding EGFP reporter. (**A-I**) Example images from ThermoScientific Cellomics CX5 high content screening platform. Neurons are identified by βIII-tubulin (**A,D,G**) transfection by EGFP (arrows, **B,E,H**)*,* and neurite are traced (**C,F,I**) using βIII-tubulin fluorescence (**J**) Quantification of total neurite length shows no significant differences between any groups (p>.05, matched pair t-test with Bonferroni's correction). N>100 cells per group in three replicate experiments.

sgRNA did not differ significantly from anti-NeuN or non-target controls.

One possible explanation for these negative results is that during the first three days post-transfection the degree of protein reduction was insufficient to affect neurite lengths. To test this possibility we adopted a strategy of maintaining longer term, high-density cultures, followed by replating. As before, P3-5 cortical neurons were transfected in multi-well format, but were plated and maintained at a relatively high density (100,000 cells plated per well in 24-well format) for seven days. As a positive control, additional cells were transfected with plasmid to overexpress Jun, shown previously to enhance neurite outgrowth in this cell type (Lerch et al., 2014; Simpson et al., 2015). FuDR was added to the media to block glial proliferation, resulting in more than 95% pure neuronal cultures. After seven days, neurons were dissociated with Accutase and replated at a density of 10,000 cells per well on PDL/laminin substrates. After 24 hours of neurite extension the replated neurons were fixed, stained with BIII tubulin, and analyzed with Cellomics instrumentation as above (Fig. 6A-I). Although this procedure resulted in significant cell death, several hundred transfected neurons survived and extended neurites that were readily quantified. JUN overexpression enhanced neurite outgrowth by an average of 33.5% (±12.8% SEM) across three replicate experiments, similar to the 26.2% (±6.9% SEM) seen in previous short-term screening experiments (Fig. 6J) (Simpson et al., 2015). These data confirm the sensitivity of this replating approach for detection of known growth phenotypes. In replated neurons treated with anti-PTEN sgRNA, neurite lengths were significantly elevated compared to non-targeted controls across four replicate experiments (136.8 ±8.0% SEM, p<.01, ANOVA with post-hoc Dunnett's) (Fig. 6J). Immunohistochemistry for PTEN protein in the replated neurons confirmed effective knockdown, as 75% of cells fell below the threshold set by the dimmest 5% of control neurons (Fig. 7). These data indicate that reduction of PTEN expression in early postnatal cortical neurons results in increased neurite lengths.
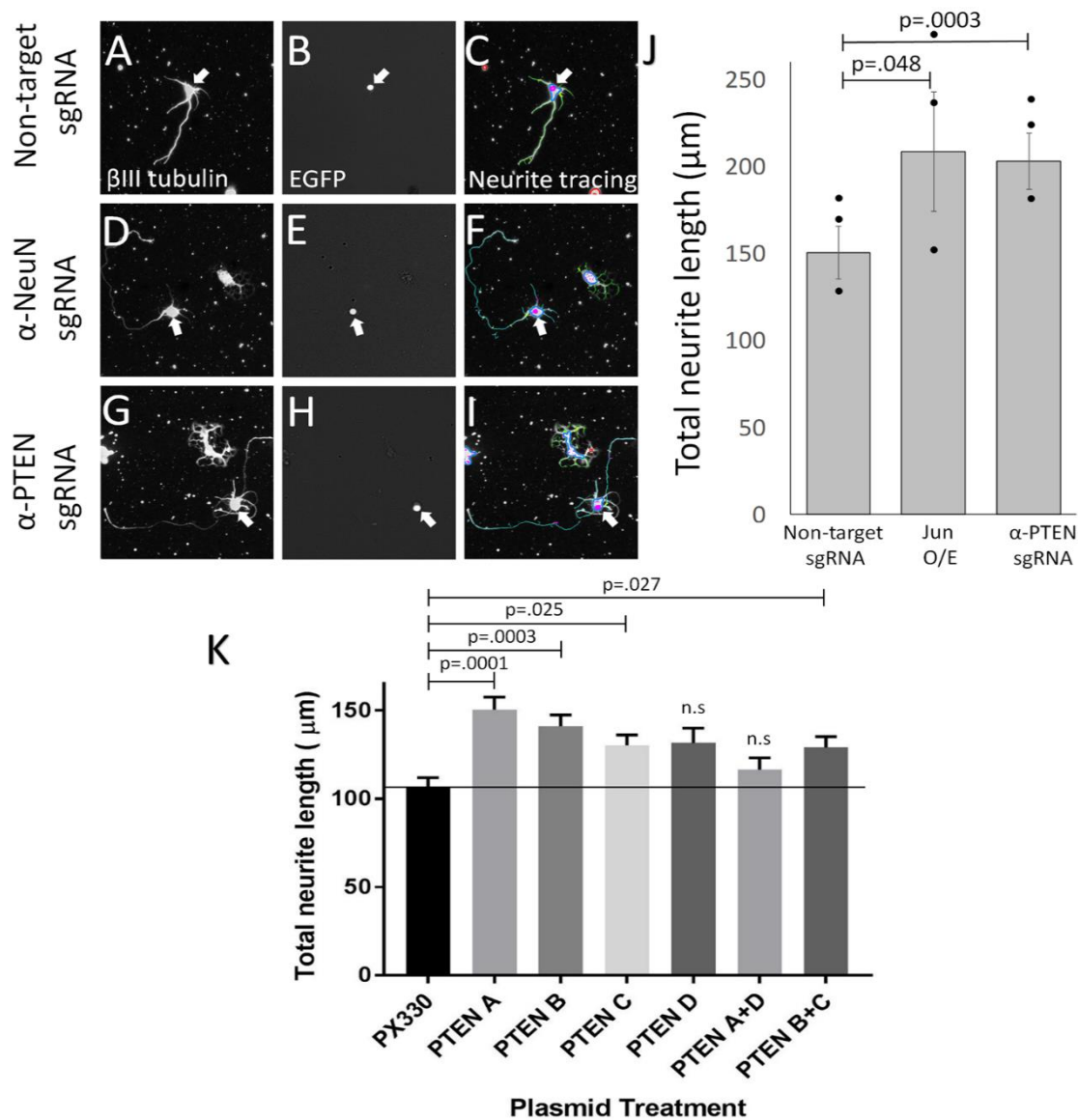
Figure 6. CRISPR-mediated knockdown of PTEN increases neurite length at a 7DIV timepoint. P3-P5 cortical neurons were transfected with plasmid DNA expressing Jun or spCas9 and sgRNA (anti-PTEN or non-targeting sgRNA), along with plasmid EGFP reporter. (**A-I**) show example images from ThermoScientific Cellomics CX5 high content screening platform. The automated microscope identifies neurons by βIII-tubulin (**A,D,G**) and transfection by EGFP (arrows) (**B,E,H**). CellInsight Cell Analysis software then automatically traces neurites (**C,F,I**) using the βIII-tubulin fluorescence. Compared to non-targeted sgRNA control (**A,B,C,**), increases in neurite length of transfected cells are apparent after Jun overexpression (**D,E,F**) or PTEN knockout (**G,H,I**). (**J**) Quantification of total neurite length shows significant increases in neurons treated with Jun overexpression and anti-PTEN sgRNA ($p<.05$, matched pair t-test with Bonferonni's correction). N≥100 cells in 3 experimental replicates. (**K**) In a separate experiment, neurons were transfected with four distinct anti-PTEN sgRNA sequences, or pairwise combinations, maintained 7DIV, and then replated. 24 hours later, Cellomics-based quantification showed that three constructs caused significant increases in total neurite length, and the fourth showed a similar trend. $P<.05$, ANOVA with post-hoc Dunnett's, N>500 cells per treatment.
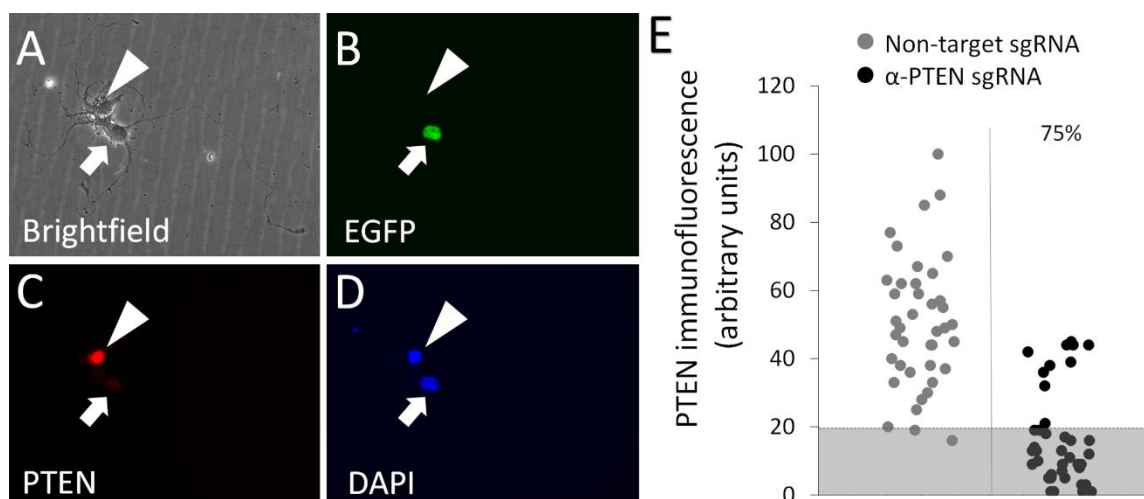
Figure 7. CRISPR-mediated decreases in PTEN expression persist following replating at 7DIV. P3-P5 cortical neurons were transfected with plasmid DNA co-expressing spCas9 and anti-PTEN or non-targeting sgRNA, along with plasmid EGFP reporter. (**A-D**) Show example image of replated cortical neurons. (**A**) Brightfield shows two neurons extending neurites 24 hours following replating. (**B**) EGFP identifies transfection (arrow). (**C**) Immunohistochemistry detects PTEN protein in an EGFP-negative neuron (arrowhead), but not in a transfected neighbor (arrow). (**D**) DAPI staining identifies both objects as nuclei containing cells (**E**) Each dot represents PTEN immunofluorescence intensity in a single transfected (EGFP+) neuron at 7DIV plus 24 hours post-replating. Grey boxes indicate a threshold set by the dimmest 5% of control-transfected neurons, and the percent of anti-PTEN cells that fall below that threshold is quantified above. Anti-PTEN sgRNA induces a significant reduction in the population average PTEN intensity (p=2.5×10^{-13}, unpaired t-test), with 75% of cells below threshold. N=40 cells per group.

It is possible that increased neurite outgrowth produced by treatment with anti-PTEN CRISPR sequences could result from off-target effects. Although off-target mutations can be detected through approaches like next-generation sequencing, a more practical solution in a screening experiment is to employ multiple targeting sequences against distinct regions of the same gene. Because different sgRNA constructs are unlikely to affect common off-target sequences, consistency of the evoked phenotype increases confidence that on-target knockdown explains the observed effects. Indeed, we found that three of the four validated anti-PTEN sgRNA sequences significantly increased neurite length compared to non-specific sgRNA

(p<0.05, ANOVA with post-hoc Dunnett's), and the fourth sequence displaying a similar trend

(Fig. 6K). Thus it is most likely that CRISPR-mediated knockdown of PTEN protein, as opposed

to off-target effects, was responsible for the increased neurite length. Importantly, these data also

provide positive control sequences for CRISPR-mediated effects on neurite outgrowth, laying

critical groundwork for the establishment of CRISPR-based screening approaches in primary

neurons.

<u>Discussion</u>

We have assessed the suitability of CRISPR-based genome editing for use in phenotypic screening in primary neurons. Using cultured cortical neurons, we have established the time course and efficacy of CRISPR knock down for two proteins, NeuN and PTEN. We also established a replating technique for postnatal cortical neurons in order to assay neurite outgrowth when an extended period in culture is required. Finally, we show that CRISPR-based knockout of PTEN, delivered by electroporation in multiwell format, causes an increase in neurite outgrowth that is readily detectable by an automated screening platform. Overall, these results demonstrate the feasibility of performing a CRISPR-based high content knockout screen.

The role of PTEN in early postnatal neurite outgrowth

PTEN, an upstream inhibitor of PI3K signaling and the mTOR pathway, was selected for this validation study because it has been shown to act in a variety of cell types as a negative regulator of axon extension. For example, transgenic knockout and shRNA-mediated knockdown of PTEN cause increased regenerative axon growth in retinal ganglion cells (Park et al., 2008; Yungher et al., 2015), corticospinal tract neurons (Liu et al., 2010; Zukor et al., 2013), rubrospinal neurons (Geoffroy et al., 2016), and dorsal root ganglion neurons (Christie et al., 2010). The timing of knockdown appears to affect the rate of axon extension triggered by PTEN ablation, with stronger regenerative responses observed when PTEN is pre-ablated early in postnatal development and injuries performed after maturation (Geoffroy et al., 2016). Indeed, despite the clear role for PTEN in limiting axon growth in adult CNS neurons, it was previously unclear whether PTEN is similarly inhibitory in the cellular context of immature neurons, which possess a higher innate growth ability and lower levels of endogenous PTEN. The current data clearly indicate that PTEN is present in cortical neurons during early postnatal periods, and that PTEN ablation increases total neurite extension.

Challenges and unanswered questions

An important question for any knockdown approach is the degree of specificity. In the case of CRISPR, knockout of off-target proteins by sgRNA could potentially produce phenotypic changes, leading to false positive screening results. Cleavage efficiency by CRISPR is strongly reduced by mismatches in the 20nt sgRNA/DNA binding site, particularly if multiple mismatches are located in PAM-proximal region (Hsu et al., 2013). Publically available algorithms now exist to scan genomes and identify sgRNA sequences with minimized potential for off-target cleavage; the sequences used here utilized the method of (Hsu et al., 2013). Nevertheless, the potential remains for off-target DNA cleavage, and a variety of techniques have been developed in non-neuronal cells to detect them (reviewed in (Wu et al., 2014). For example, potential off-target sequences in the genome can be PCR-amplified and sequenced or analyzed by mismatch-specific nucleases (e.g. Surveyor). The most comprehensive approach is to perform whole genome sequencing of cell populations clonally derived from single sgRNA-treated cells (Smith et al., 2014; Veres et al., 2014). Genome-wide sequencing is likely not feasible in individual post-mitotic neurons, and even PCR-based analysis of predicted off-target cleavage is likely impractical for most screening projects. Thus, the best safeguard against off-target, false-positive hits will likely be to test multiple sgRNA sequences against the same protein target, similar to the strategy adopted by previous RNAi screens (Nieland et al., 2014; Sharma et al., 2013; Welsbie et al., 2013). Importantly, our data indicate that in the case of PTEN knockdown, three of four sgRNA targeting sequences produced a significant increase in neurite length. Setting requirements for consistent phenotype across multiple targeting sequences greatly increases the probability that on-target effects are responsible.

Several groups have shown that RNAi approaches can cause non-specific effects through an innate immune response resulting in the expression of interferon proteins (Bridge et al., 2003; Sledz et al., 2003). Of particular relevance, delivery of lentiviral-mediated delivery of shRNA to

primary neurons has been shown to elevate expression of interferon-stimulated genes such as

Oas1, with attendant decreases in viability and neurite outgrowth (Bauer et al., 2009; Hutson et

al., 2012). Incorporation of microRNA flanking sequences and designs that prevent the formation

of perfect double stranded RNA helixes have been used to alleviate this interferon response

(Bauer et al., 2009; Cullen, 2006). It is unknown whether Cas9 and sgRNA induce a similar

interferon, although the single-stranded nature of the sgRNA may make it less likely.

Nevertheless, in future work it will be important to directly test for interferon responses and other

potential non-specific effects of CRISPR.

Another potential challenge is presented by the finding that CRISPR-mediated

knockdown can take up to six days to affect the majority of transfected cells. After plasmid

transfection, protein knockout can occur only after 1) production of Cas9 enzyme and sgRNA 2)

Cas9-mediated cleavage at both genomic copies of PTEN and 3) clearance of existing protein

pools. Which step contributes most to the six-day delay? We observe plasmid-driven expression

of reporter proteins within 16 hours of transfection, making it unlikely that delayed expression of

Cas9 protein is a major culprit. Similarly, the half-life of PTEN has been estimated at about 7.5

hours (Trotman et al., 2007), and using shRNA we observe knockdown that is strong at 2DIV and

nearly complete by 4DIV (Fig. 4), consistent with previous use of this shRNA targeting sequence

(Yungher et al., 2015). Thus, a slow rate of PTEN turnover is also unlikely to explain the delay.

Combined, these observations suggest a model in which DNA cleavage by Cas9 is a stochastic

event that affects an expanding fraction of the population over the course of several days.

Expanded experimentation is needed to confirm that a six day lag to knockdown is a general

feature of the CRISPR strategy in primary neurons. Nevertheless, it is notable that we observed

very similar timecourses for five distinct sgRNAs against two protein targets. Thus potential users

should be alert to the possibility that screens involving CRISPR manipulation of primary neurons

may require approximately a week in culture to achieve protein knockdown in a majority of

transfected cells, followed by additional time in culture for the phenotypic consequences to manifest.

One consideration is that the timing of knockout may vary between individual cells (i.e.Cas9 cleavage may have affected some cells at 2DIV, others not until 6DIV). This range of time-since-knockout could act as a source of phenotypic variability. In addition, the lag between transfection and protein knockout may present a technical hurdle for some endpoint measurements, including screens of neurite outgrowth considered here. Indeed, we empirically tested and ruled out the possibility that CRISPR-based approach might be compatible with previous HCS workflows, in which neurite outgrowth was quantified at a 3DIV timepoint (Blackmore et al., 2010). We therefore adopted a replating technique, similar to procedures that we and others have used previously to disentangle dorsal root ganglion (DRG) neurons after extended culture (Simpson et al., 2015; Zou et al., 2009). This approach is feasible but introduces an extra step into the workflow and results in significant cell death, which increases the number of cells needed for the initial transfection step. In addition, the replating procedure likely introduces an increase in variability in subsequent neurite outgrowth, potentially reducing screen sensitivity. Indeed, when compared to prior data from non-replated neurons, we find a significant increase in variance of neurite length (standard deviation approximately 25% higher, f-test p<.01). Despite these caveats, however, this approach did produce consistent enhancement of neurite outgrowth after JUN overexpression and CRISPR-mediated PTEN knockout. The establishment of a consistent positive control is a promising step toward the use of CRISPR knockout in neurite outgrowth screens.

Now that we have established positive controls it will be important to reduce variability and increase the sensitivity of future screens. One source of variability is imperfect co-transfection between separate Cas9 and EGFP plasmids, although our previous data predict a greater than 90% co-expression at the 1:4 ratios used here (Blackmore et al. 2010). The PX330

vector we tested here does not include a reporter, and the large size of Cas9 creates challenges for reporter co-expression from a single plasmid. As a potential future solution, CRISPR technology allows for targeted homology-directed repair (HDR) (Cong et al., 2013). In this way a repair template containing either a fluorescent reporter or an antibiotic selection marker could be inserted when a target gene is nicked by Cas9. In principle, this approach would specifically identify cells affected by Cas9 enzyme. Challenges to this strategy include the fact that HDR has an extremely low efficiency, and the difficulty in distinguishing homozygous from heterozygous insertion, which could leave one copy of the target gene intact (Maruyama et al., 2015). Nevertheless, as CRISPR technologies rapidly evolve, HDR-based strategies may become more practical in a HCS environment.

Future prospects for CRISPR-based screening in primary neurons

One factor that may favor the adoption of CRISPR-based screening is the simplicity of creating effective reagents. In the current study, publically available algorithms were used to design targeting sequences ranked on the basis of specificity and efficacy. Following procedures described in (Cong et al., 2013), short oligonucleotides were synthesized, annealed, and ligated into PX330 plasmid which co-expresses Cas9 and sgRNA. This process proved to be simple and highly economical; two features that make it portable to high content screens. This raises the prospect that large-scale, custom-designed CRISPR knockout libraries could be quickly and inexpensively created.

A second potential advantage is the prospect of multiplex screening, e.g. simultaneous knockdown of multiple target genes. It has been shown that in the presence of multiple sgRNAs, Cas nucleases can cleave diverse DNA sequences in the same cell (Cong et al., 2013). This strategy has been used to achieve multiplex knockdown in a variety of systems, including zebrafish, mouse embryonic stem cells, human immortalized myoblasts, HEK293T cells, and yeast (Jakociunas et al., 2015; Jao et al., 2013; Kabadi et al., 2014; Ousterout et al., 2015; Yang et

al., 2013). Notably, simultaneous knockdown of three proteins (DNMT1, DNMT3a, and DNMT3b) was recently demonstrated using a CRISPR approach in neurons (Swiech et al., 2015). Combined with the high economy of sgRNA production these studies pave the way for a combinatorial screening approach in which multiple genes are concurrently targeted for knockout.

Third, future screening projects could take advantage of the precision of CRISPR gene targeting without the ensuing DNA disruption. This is because Cas nucleases can be rendered catalytically inactive and unable to cleave DNA while still maintaining the ability to bind specific genomic loci under the control of user-designed sgRNA. One simple example is the fusion of the transcriptional activation domain VP64 to dCas9 to create a tool for targeted upregulation of specific genes (Perez-Pinera et al., 2013). The practical applications of targeted gene manipulation are nearly limitless and include the possibility of performing simultaneous, combinatorial knockout and overexpression screening.

Finally, CRISPR-mediated knockout has now been demonstrated to be highly effective in CNS neurons in vivo. Although the large size of the spCas9 enzyme can present challenges to viral delivery, strategies to circumvent this problem are being developed. For instance, the use of smaller Cas9 family members or transgenic animals that conditionally express spCas9 are proving to be effective (Cong et al., 2013; Ran et al., 2015). Viral delivery of multiple sgRNA may therefore represent a rapid and flexible alternative to current transgenic approaches to achieving simultaneous knockout of multiple genes. Moreover, transgenic animals with constitutively expressed Cas9 will likely be useful as a source of cells for in vitro screening by simplifying sgRNA delivery and perhaps accelerating the onset of knockdown by eliminating the lag between transfection and Cas9 expression. It is clear that axon regeneration is regulated by many interacting genes and combinatorial gene manipulations have proven most effective in promoting growth (Belin et al., 2015; Jin et al., 2015; Sun et al., 2011). Thus the prospect exists that CRISPR-based approaches may accelerate both in vitro discovery of gene interactions that

regulate axon growth, and the transition to combinatorial testing of lead hits in vivo. Overall it is clear that CRISPR-based approaches have enormous potential to expand understanding of interactive gene function in a variety of cell types. In this context, the current demonstration of CRISPR efficacy and compatibility with high content screening in cultured CNS neurons is a promising indicator that this potential can be harnessed for neuroscience research.

III. METHODOLOGY

Cloning and plasmid preparation

pX330-U6-Chimeric_BB-CBh-hSpCas9 was a gift from Feng Zhang (Addgene plasmid # 42230). The targeting sequence for NeuN was taken from (Platt et al., 2014). Targeting sequences for PTEN were designed using the publicly available Benchling CRISPR design tool (http://www.benchling.com). The overhang 'CACC' was added to the 5' end of the two sgRNA sequences with the highest on-target score and the overhang 'CAAA' was added to the 5' end of the reverse complements of these sequences. Anti PTEN sequences were GGTGGGTTATGGTCTTCAAA (PTEN A), TTTCCTGCAGAAAGACTTGA (PTEN B), CACCGGTTTGATAAGTTCTAGCTG (PTEN C), and CACCGTTTGTGGTCTGCCAGCTAA (PTEN D). The anti-NeuN sequence was CAGCAGCCCAAACGACTACAT, and the non-targeting control sequence was GGGTCTTCGAGAAGACCTGT. Oligonucleotides of the targeting sequences and reverse complements with overhangs were ordered from ThermoFisher and annealed. Bbs1 enzyme was used to digest the PX330 plasmid and the annealed oligonucleotides were ligated into the cut backbone using T4 ligase as described in (Cong et al., 2013). The PX330 plasmid containing sgRNA targeting sequences were then transformed into E. cloni Chemically Competent Cells (VWR 95040-456) and DNA was prepared by QIAprep Spin Miniprep Kit (Qiagen 27106). The reporter plasmid expressed EBFP-2A-H2B-EGFP from a CMV promoter, and was constructed by replacing the 2A-mCherry sequence from a reporter previously described in (Wang et al., 2015) with EGFP fused to H2B to drive nuclear localization.

Cortical cell dissociation, transfection, and culture

All animal procedures were approved by the Marquette University Institutional Animal Care and Use Committee. Cortical neurons were prepared from early postnatal (P3–P5)Sprague Dawley rat pups (Charles River) and transfected as in Blackmore et al., 2010. Frontal cortices were placed in ice-cold Hibernate E (Life Technologies A12476-01), their meninges removed, finely minced and transferred into 10 ml dissociation media for 30 min at 37 °C with constant shaking (Hibernate E containing 20 U/ml papain (Worthington 3126) and 2.5 μg/ml DNAse (Sigma D4527)). Cells were then rinsed with Hibernate E + 2% SM1, then rinsed in Hibernate E (no SM1), and incubated 30 min at 37 °C with constant shaking in 10 ml trypsin solution (Hibernate E containing 0.25% trypsin (Invitrogen/Gibco 15,090–046) and 2.5 μg/ml DNAse (Sigma D4527)). Cells were centrifuged (1 min, 10g), washed with Hibernate E+ 2% SM1, and triturated three times in 1.5 ml Hibernate E+ 2% SM1 containing 2 μl DNAse (Sigma D4527). The suspension was

allowed to settle for 2 min and the supernatant was collected; these steps were repeated until no cell pellet was visible. This process typically yielded 2 million cells/mL in 6-7 ml Hibernate E. For transfection, cortical neurons were centrifuged (4 min, 80g) and resuspended to 2×10^6 cells/ml in Internal Neuronal Buffer (INB) (KCl 135 mM, CaCl2 0.2mM, MgCl2 2mM, HEPES 10 mM, EGTA 5 mM, pH 7.3). 25 μl were placed into wells of a 96-well electroporation plate (BTX Harvard Apparatus 45–0450) and mixed with 25 μl of INB containing 1 μg EGFP plasmid and 4 μg test plasmid. A 350 V, 300 μs pulse was delivered to each well by an ECM square wave pulse generator (Harvard Apparatus ECM-830) attached to a plate handler (BTX Harvard Apparatus HT-200). After electroporation each well received 100 μl of Hibernate E. For short-term cell culture experiments, 10,000 cells/well, or for long-term cell culture experiments 100,000 cells/well, were placed in 24-well plates (Greiner Bio-One 662,160) pre-coated with 100 μg/ml poly-D-lysine hydrobromide (Sigma P7886) (N12 h, 37 °C), followed by 10 μg/ml laminin (Sigma L2020) (N2 h, 37 °C). Cells were maintained at 37 °C, 5% CO2 in Enriched Neurobasal (ENB) media, modified from (Meyer-Franke et al., 1995), consisting of Neurobasal A (Invitrogen 10,888–022), 50X NeuronCult SM1 Neuronal Supplement (Stemcell Technologies 5711), 1 mM sodium pyruvate (Invitrogen 11360-070), 2 mM GlutaMAX (Invitrogen/Gibco 35050-061), 50 U/ml Penicillin–Streptomycin (Invitrogen 15070-063), 5 μM Forskolin (Sigma F6886),

To enrich for neurons in cultures maintained for longer than 3 days, 100 nM 5-Fluoro-2'-deoxyuridine (FuDR) (Sigma-Aldrich F0503) was added to each well 1 day post-transfection. To facilitate long-term survival the media was replaced every 3 days, with fresh cortical media in the replating experiments and with FuDR-containing media in the time course experiments.

Cortical cell culture replating

Primary cortical neurons were transfected and cultured as previously described (Blackmore et al., 2010). Wells were rinsed twice with 400 μl of Hibernate E and then incubated for 10 minutes at 37 °C in 400 μl of StemPro Accutase Cell Dissociation Reagent (ThermoFisher A1110501). Accutase was then diluted with 400μl of fresh cortical media, cells were detached by pipetting three times, with half the well volume aspirated and dispensed each time. Dissociated cells collected by centrifugation (4min, 80G). Cells were resuspended in one mL, counted, and plated at 10,000/well in 24-well plates (Greiner Bio-One 662,160) pre-coated with PDL/laminin using conditioned media from the original plate. Replated cells were fixed after 24 hours in 4% PFA.

Cortical cell culture immunohistochemistry and quantification

Cultures were fixed in 4% paraformaldehyde (Electron Microscopy Sciences 15710) for 30 min at room temperature, rinsed five times, blocked and permeabilized (PBS, 20% goat serum (Invitrogen 16210- 064), 0.2% Triton X-100 (G-Biosciences 786-513) for 30 min at room temperature, and incubated in primary antibody solution (PBS, 10% goat serum, 0.2% Triton X-100, primary antibodies: rabbit anti-βIIItubulin (1:500, Sigma T2200), mouse anti-NeuN (1:500, Millipore MAB377), rabbit anti-PTEN (1:500, Cell Signaling 138G6) (overnight, 4 °C). After five washes, cultures were incubated in secondary antibody solution containing DAPI nuclear stain (PBS, 10% goat serum, 0.2% Triton X-100, 300 nM DAPI, dilactate (molecular probes D3571) secondary antibodies: Alexa Fluor 647 goat anti-rabbit IgG (1:500, Invitrogen A-21245), Alexa Fluor 546 goat anti-mouse IgG (1:500, Invitrogen A-11003)) for 2 h at room temperature. Plates were washed five times, and left in 1 ml PBS for imaging.

For the time course of PTEN knockdown experiments, a fluorescent inverted microscope was used to identify EGFP + NeuN + cells as transfected neurons. For the time course of NeuN knockdown experiments, a fluorescent inverted microscope was used to identify EGFP + BIII-tubulin + cells as transfected neurons. A circle was drawn around the cell body of each transfected cell and the fluorescence level of either NeuN or PTEN staining was recorded as quantified by NIS-Elements Basic Research software. For all treatments and time points n = 40 transfected cells. Statistical analysis was paired t-test using Graphpad Prism Software.

Neurite outgrowth analysis

For neurite outgrowth assays, Cellomics Cell Insight NXT (Thermo Scientific) acquired images of 3 channels: nuclei (DAPI), neurite staining (βIII-tubulin), and reporter (EGFP), and Cellomics Scan v6.4.0 (Thermo Scientific) traced neurites and quantified reporter intensity. Average total neurite length was quantified for the 10% of cells with greatest EGFP intensity per plate (n > 100 cells per treatment). Average neurite length was normalized to PX330 non-target control for each plate. Each treatment was tested at least three times. Statistical analysis was paired t-test using Graphpad Prism Software.

IV. CONCLUSION

The aim of this thesis was to determine the utility of CRISPR-mediated gene knockout for high content screening in primary cultures of cortical neurons. This technique has been applied in other systems to perform high content screens, but has never been tested in primary neurons. We first showed that CRISPR/Cas9 is an effective means of selectively knocking down proteins of interest in primary neurons. We then optimized the technique for use in our high content screening platform to test the function of endogenously expressed genes through targeted knockout. This included determining the time course of protein knockout, devising a strategy for replating following an extended time in culture, and performing some initial neurite outgrowth experiments to provide a positive control for future studies. In this work, I have shown that the CRISPR/Cas9 system can be effectively harnessed as a knockout tool in primary cortical neurons as well as our high content screening platform.

We have previously performed overexpression screens in primary cortical neurons with great success. In one of our previous screens we focused on transcription factors that are dysregulated in cancerous states and found significant effects on neurite outgrowth through overexpression of 9 out of 69 genes tested – a hit rate of 13 percent (Simpson et al., 2015). However, since we used overexpression methods in these previous screens it is difficult to make conclusions about the efficacy of manipulating these genes in vivo. One reason for this is that exogenous introduction of a gene could have unforeseen effects if the system does not inherently express that gene product. Knockdown screens are required to test the necessity of endogenously expressed genes and studies have previously used methods such as RNAi and TALENs to test the necessity of gene expression. Unfortunately, these tools have several limitations that prevent their wide applicability in high content screens using primary neurons (Bridge et al., 2003; Sledz et al., 2003).

This work builds upon previously published research in several ways. First, CRISPR/Cas9 is becoming a popular tool which has been tested in the neurons of living animals through the delivery of viral vectors ((Platt et al., 2014; Straub et al., 2014; Swiech et al., 2015; Zuckermann et al., 2015). To our knowledge, this study provides the first example of utilizing CRISPR/Cas9 through plasmid delivery to produce targeted, specific gene knockout in cultures of primary cortical neurons. Second, this study also provides a platform to test the necessity of genes that have been previously discovered through sufficiency screens. In addition, we hope that this newly optimized tool can discover the endogenous functions of genes that overexpression screens could not test. Finally, this work provides in vitro evidence to support the idea that the mTOR pathway, and specifically PTEN, plays an important role in the control of neurite outgrowth. Overall, this work has made several contributions to the spinal cord injury field and literature, which is evidenced by the publication that arose from this study (Callif et al., 2017).

Although this research made a clear contribution to the literature there are still some limitations of the results. One major limitation of this study is that all the experiments were performed using cortical neurons, so the results may not be generalizable to neurons harvested from other brain regions. Another problem with this work is the extended time in culture required for effective CRISPR/Cas9-mediated knockout. This timing restriction could make it difficult to utilize this knockout screening platform as a flexible model of development. To overcome some of the other limitations of this extended culture time we developed a method of replating these post-mitotic neurons. Although replating the cells works well for analyzing neurite outgrowth after a week in culture, many of the cells die during this procedure. This likely introduces some selection into the experiments, since the final assay can only provide data about the growth of cells that survived the replating. It may end up that this selection pressure more accurately models growth after an injury, but only more experiments will be able to determine that.

Despite the limitations, these findings imply that a CRISPR/Cas9-based knockout strategy for neurite outgrowth screens is possible in primary cortical neurons. In this study, we provide data showing that PTEN knockout can be used to enhance growth (Callif et al., 2017). Future studies can make use of this information to run high content screens with a verified positive control. Performing a full-scale screen on hundreds of genes will be the immediate next step, but this platform provides many other avenues of research to explore. The CRISPR/Cas9 system is rapidly becoming a flexible, modular tool that can be used for much more than simple genetic knockouts. There are studies which have used a catalytically inactive Cas9 (dead Cas9; or dCas9) to achieve a variety of other goals. For example, by fusing dCas9 to transcription factor activation or repression domains it is possible to modify gene expression without disrupting the underlying nucleotide sequence (Perez-Pinera et al., 2013). As another example of the flexibility of this technique, several groups have used dCas9 fusion proteins to manipulate the epigenetic environment of specific genes (Choudhury et al., 2016). Finally, there is research which suggests that dCas9 can even be tagged with a fluorescent molecule and exported into the cytoplasm where it can track mRNA molecules in real-time (Nelles et al., 2016). The optimization of these techniques may be far in the future, but they show that the platform we have created opens the doors to a wide range of possibilities. Furthermore, the applications of CRISPR/Cas9 extend in vivo, which provides an efficient and straightforward way to take the genes discovered through screening and apply them directly to a functional model of spinal cord injury.

This work has been an amazing experience. I feel that I have been a productive member of both the Blackmore lab and the spinal cord injury field. In the lab I performed a high content overexpression screen which resulted in a publication and the discovery of a role for HHEX in the developing brain. A hopeful avenue for future studies is to determine the specific mechanisms of action for the inhibitory effect of HHEX in growing neurons. Within the field I have developed a novel CRISPR/Cas9 knockout screening platform which is compatible with the use of primary

cortical neurons. This includes a replating technique, which has been developed for peripheral neurons like dorsal root ganglion, but has never been accomplished for central nervous system neurons. Overall, I feel as though I have made a reasonable contribution to the field and I am proud of this work and the productivity I have achieved over the last several years. I look forward to seeing the future of the spinal cord injury field and how others can build on my work.

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