UNIVERSITY OF CALIFORNIA, SAN DIEGO

Establishing Vertebrate Model Systems for the Study of Gle1 Mediated Motor Neuron Disease

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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Professor Eduardo Macagno

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The Thesis of Joseph Tsai is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

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Professor Samuel Pfaff, Chair
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Gle1 is an evolutionary conserved protein involved in both mRNA export and translation. A recent study linked mutations in Gle1 with recessive and fatal motor

neuron diseases characterized by ventral horn motor neuron degeneration before birth. This is particularly interesting in light of a growing pool of evidence indicating that a common denominator in many motor neuron disorders is defects in mRNA regulation. To investigate the role of Gle1 in motor neuron development, we used chick and mouse models to develop tools for examining Gle1‘s expression, localization, biochemical interactions, and altered activity when mutated. We have confirmed the presence of Gle1 transcripts in a variety of cell types and stages of development, including during motor neuron development, and we generated Gle1-Flag fusion constructs to study Gle1 localization and biochemistry following failure of commercial antibodies to reliably detect Gle1 through immunohistochemistry and immunoblotting. Overexpressing mutant Gle1 does not appear to noticeably affect the development of motor neurons in chick, suggesting that the mutant protein does not cause disease through a gain of function. To study the effects of Gle1 loss on development, we generated a mouse line expressing a Gene-trap allele in which Gle1 is created as a truncated protein linked to β-Geo. As animals homozygous for this allele appear nonviable, we selected a rescue strategy in which we will express Gle1 from the ROSA26 locus in a Gene-trap background, allowing us to examine the specific effects of Gle1 mutation on motor neuron survival and function.

Introduction

Neurodegenerative diseases have been heavily investigated since the dawn of medicine due to their wide range of devastating symptoms, from losses in mental capacity to uncontrollable tremors and paralysis. Despite such scrutiny from medical researchers, a lack of knowledge about the pathology of these maladies has historically allowed only the development of palliative treatments.

Advances in basic biology and medicine over the last decade have been a boon to the development of new ideas for treating such disorders. The elucidation of many molecular pathways in development and disease processes have uncovered new targets for therapies, and the proliferation of new technologies and innovations such as gene therapy have made it possible to target diseases that were previously thought intractable. Insight into diseases of the locomotor system in particular has seen enormous leaps over this time, and considerable effort has been put into understanding the mechanisms by which motor function becomes compromised.

Motor neuron development and function

Spinal motor neurons have long been the subject of intense study, in part due to the accessibility of the neuromuscular junction as a model for neuronal and synaptic function, and because of their importance in vital processes such as breathing, feeding, and locomotion. While motor neurons are often unceremoniously lumped into a single group, they exhibit remarkable diversity - fast and slow, alpha and gamma, medial and lateral, etc - which is critical to the coordinated movement of their target muscles. These

subpopulations are distinguished by various factors such as firing rate/patterns, molecular markers, and size (for review, see *Kanning et al., 2010*).

Although mammals have evolved a wide variety of locomotion patterns, many of the key steps and basic principles in spinal motor neuron development are common between species and in all spinal motor neurons. Initially, progenitor domains for motor neurons and several classes of interneurons are generated from neural precursors in response to a dorsoventral gradient of the morphogen Sonic Hedgehog (Shh), released ventrally from the notochord and floor plate. Shh activates several sets of transcription factors in the neural tube in a concentration-dependent manner, which are thought to cross-repress and sharpen the boundaries between progenitor domains. For example, motor neuron progenitors are delineated ventrally by repressive interactions between Pax6 and Nkx2.2, and dorsally by Irx3 and Olig2. Various transcriptional programs activated by these factors eventually promote the expression of neuron-specific factors, such as Ngn2, leading to a post-mitotic state. Finally, motor neurons can be identified by their expression of factors such as MNR2, HB9, and Isl1/2. For an in-depth review, see *Shirasaki & Pfaff, 2002*.

Organizationally, motor neurons are found at all levels of the ventral spinal cord, and are arranged into motor columns and pools. In mammals, they are grouped generally into the medial motor column (MMC), the hypaxial motor column (HMC), the lateral motor columns (LMC), and the preganglionic column (PGC). The MMC extends throughout the entire spinal cord and innervates axial dorsal body wall muscles, while the HMC and PGC exist generally in the thoracic region of the spinal cord and innervate abdominal/intercostal muscles and sympathetic ganglia, respectively. The LMC exist at

limb levels and are responsible for motor movements of the extremities. Once a general motor neuron identity is in place, they are further specified into pools which innervate specific targets. This columnar and pooling organization is largely determined by the expression of Hox homeodomain transcription factors, which are expressed under the influence of rostrocaudal gradients of Retinoic Acid (RA), FGF8, and Gdf11 (reviewed by *Dasen & Jessell, 2009*).

Interestingly, motor neurons are typically generated in excess during development. The neurons vie for trophic factors (such as GDNF) from their target muscles and limbs to allow for survival, and those that fail to maintain contact with a target muscle die off (reviewed by *Oppenheim, 1991 & 1996*). Additionally, each muscle fiber is initially innervated by axons from several motor neurons, and the processes are pruned until each muscle fiber only receives stimuli from one axon (reviewed by *Sanes & Lichtman, 1999*). This refinement of motor neuron number and muscle connectivity takes place largely after birth.

By studying motor neuron development *in vivo* in model systems such as chicken, frog, zebrafish, and mice, we have elucidated general principles that can now be applied to cells grown in culture. Just as cells in the inner cell mass of a blastocyst or neural tube progenitors in a developing embryo, embryonic stem (ES) cells in culture can be converted into various neuronal subpopulations by adding a cocktail of factors (such as RA and Shh) known to induce cellular differentiation at empirically determined concentrations (*Wichterle et al., 2002*). The cells can then be used for an assortment of experiments, such as collecting large amounts of neuronal material for protein activity/ binding assays, or studying intrinsic cellular and network activity of neurons in culture.

Importantly, these cultures can also be used to study diseases. For example, ES cells can be derived from mouse models of neuronal diseases and differentiated into neurons to examine possible defects. Cultured cells can also be manipulated through processes such as RNAi or homologous recombination to knock down or out genes which cannot be studied *in vivo*, as they are lethal when lost at earlier stages of development. This provides a powerful and scalable model for studying motor neuron differentiation and function, and gives us a tool to study disease states *in vitro*.

RNA processing and motor neuron diseases

While often considered as a group, the heterogeneity of motor neurons leads to very different susceptibilities between subpopulations to disease. Because the output of motor neurons are obvious and critical to life, motor neuron disorders often cause significant debilitation or death, leading to intense scrutiny and a strong demand for therapies. The two most common and well-studied motor neuron disease are spinal muscle atrophy (SMA), a childhood genetic disease, and amyotrophic lateral sclerosis (ALS, or Lou Gehrig's disease), an adult-onset neurodegenerative disorder. In both cases, motor function is progressively compromised as motor neurons degenerate. However, the patterns and areas of effect are very different.

SMA is caused by insufficient amounts of the SMN (survival of motor neuron) protein, which is involved in snRNP assembly and pre-mRNA splicing. The disease is characterized by cell death of spinal motor neurons, leading to anterior horn degeneration and skeletal muscle atrophy. The SMN protein is expressed from two loci, SMN1 and SMN2, with the SMN2 locus exhibiting a splicing defect which causes expression of only

low levels of functional protein. However, the SMN2 locus can have several copies of the defective gene. SMA patients almost invariably show deletion of the SMN1 locus, with the severity of the disease determined by the copy number of SMN2. More copies of SMN2 lead to more translated SMN protein, and the disease symptoms run a gamut from weak grip strength to paralysis and death in early childhood. For reviews, see *Monani, 2005 and Burghes & Beattie, 2009.*

In ALS, axonal degeneration and cell death of both spinal motor neurons and neurons in the motor cortex is seen, leading to progressive paralysis and eventual death almost universally (with Stephen Hawking and Jason Becker being two apparently rare exceptions). About 90% of ALS cases are sporadic, while just 10% are familial. The most studied familial form of ALS involves toxic gain-of-function mutations is superoxide dismutase 1 (SOD1), which accounts for 10-20% of familial cases (*Mitchell & Borasio, 2007*). SOD1 is ubiquitously expressed and is involved in protecting cells from toxic superoxides, converting them to harmless molecules; when mutated in ALS-model mice, they cause defects in mitochondrial oxidative phosphorylation (*Mattiazzi et al., 2002; Knott et al., 2008*). Interestingly, restricted expression of the mutation in only astrocytes causes ALS symptoms in animal models, suggesting that the mutation does not (only) cause cell-autonomous defects in motor neurons (*Di Giorgio et al., 2007; Nagai et al., 2007*).

Both SMA and SOD1-related ALS provide intriguing examples of a trend seen in neuronal diseases - ubiquitously expressed proteins causing symptoms in very specific sets of tissues (in these cases, mostly fast-fatiguable motor units; *Kanning et al., 2010*).

Other examples of a ubiquitous proteins involved in neuronal disorders are the association of A-type lamins (proteins which form the nuclear lamina) with the axonal neuropathy of Charcot-Marie-Tooth disorder type 2B1, and the implication of mutations in methyl-CpG binding protein 2 (MeCP2) in Rett syndrome (*De Sandre-Giovannoli, et al., 2002; Amir et al., 1999*).

It is unclear why motor neurons might be specifically affected by disruption of these ubiquitous proteins. An obvious possibility is that their length, size, and high metabolic requirements necessitates more stringent regulation of cellular processes than most other tissues. However, this is unlikely the whole story, as other long neurons are apparently unaffected by these diseases (e.g., sensory neurons, corticospinal tract upper motor neurons in SMA), and neurons innervating proximal muscles are often affected before distal-targeting neurons in SMA.

One hint to the general nature of many motor neuron disorders may be the recent discovery of multiple proteins involved in RNA regulation that are implicated in motor neuron disorders. Mutations and accumulations of TDP-43 and FUS/TLS, proteins implicated in mRNA transcription, splicing, transport, and translation, have been associated with ALS at extremely high rates (*Banks et al., 2008; Lagier-Tourenne & Cleveland, 2009*). Spurred by this discovery, other proteins have been linked to ALS, all with RNA-binding domains (*Elden et al., 2010*). As all the identified proteins are implicated in RNA processing pathways, along with SMN in the case of SMA, a common denominator in many motor neuron disorders may be defective mRNA regulation. This suggests the possibility that motor neuron development and function is highly dependent on proper mRNA processing. It is possible that similar cellular aberrations exist in each

of the aforementioned mutations, caused by defects in a shared pathway of mRNA regulation.

Gle1, a mRNA-regulation factor

Recently, evidence has surfaced indicating a relationship between an autosomal recessive mutation in Gle1 (for GLFG, or glycine-leucine-phenylalanine-glycine, lethal) and two Finnish-heritage motor neuron diseases characterized by prenatal ventral horn motor neuron degeneration, named lethal congenital contracture syndrome 1 (LCCS1) and lethal arthrogryposis with anterior horn cell disease (LAAHD) (*Nousiainen et al., 2008*). LCCS1 leads to death before the 32nd gestational week, while LAAHD shows a milder phenotype, with fetuses often surviving delivery but dying due to a nonfunctional diaphragm soon after. These symptoms are all associated with motor neuron death, which leads to the denervation of muscles (including the diaphragm), causing muscular spasticity and atrophy, contractures (shortening of muscles and joints), and eventual death.

The primary mutation isolated by Nousiainen et al. in Gle1 was deemed FinMajor, and is a single nucleotide substitution in intron 3 of human Gle1 (normally a 16 exon gene in humans and mice), which results in an illegitimate splice acceptor site. This causes a 9 base pair insertion in the Gle1 coding sequence, which is predicted to add three amino acids (PFQ) within a coiled coil domain responsible for protein-protein interactions. LCCS1 cases, which showed the more severe phenotypes, were almost universally homozygous for the FinMajor mutation, while LAAHD afflicted those compound heterozygous for the FinMajor mutation and another mutation of Gle1 (for

example, one affecting the domain targeting the protein to the NPC; *Nousiainen et al., 2008*). It remains unclear how the mutations affect the function of Gle1, and how this may lead to motor neuron death.

Gle1 is evolutionarily conserved and its function has been most heavily studied in yeast. It is known to be associated with the cytoplasmic fibrils of the nuclear pore complex (NPC), a 40/60 MDa complex in yeast/vertebrates, through Nup42 in yeast and hCG1 and Nup155 in humans (*Murphy & Wente, 1996; Rayala, et al, 2004; Watkins et al., 1998*). NPC's are studded throughout the nuclear envelope, which separates the cytoplasm from the nucleoplasm and its constituents (e.g., genetic material), and the NPC serves as a highly selective transporter for various proteins and ribonucleoprotein cargoes (for review, see *Wente & Rout, 2010*). Studies have shown that in yeast, Gle1 functions in tandem with inositol hexakisphosphate (IP_6) to stimulate the NPC-associated DEAD-box ATPase DBP5, which has RNA helicase activity, to control mRNA export from the nucleus (*Alcázar-Román et al., 2006; Tran et al., 2007; Weirichet al., 2006; York et al., 1999*). Specifically, Gle1-IP_6 stimulates ATP binding and ATPase activity upon binding to DBP5, acting as an ATPase activating factor, allowing mRNA binding to occur (*Noble et al., 2011*). Gle1 has also been shown to be essential to mRNA export in humans through experiments in HeLa cells, and appears to be critical to the export of all poly-adenyated RNA species in both yeast and humans (*Watkins et al., 1998*).

Apart from (but linked to) its role in mRNA export with the NPC, some Gle1 localizes to the cytoplasm and is known to participate in translation initiation and termination in yeast (*Alcazar-Roman et al., 2010; Bolger et al., 2008 ;Watkins et al., 1998*). During termination, Gle1 functions with its familiar partners, IP_6 and DBP5, with

physical interactions between Gle1 and release factors such as Sup45/35 (eRF1/3 in vertebrates) reported. However, its role in initiation appears independent of IP_6 and DBP5, with a physical interaction with initiation factors like eIF3 subunits reported. A mechanism for this function has yet to be reported, and this role has not been shown in vertebrate models to our knowledge.

Structurally, Gle1 binds the C-terminus of DBP5, while the first 29 amino acid residues of Gle1's N-terminal in humans binds to the NPC through hNup155 as shown in HeLa cells (*Dossani et al., 2009; Rayala et al., 2004*). Both these interactions are vital to Gle1's role in mRNA export at the NPC, with a lack of hNup155 resulting in a high degree of hGle1 localization in cytoplasmic foci (*Kendirgi et al., 2005*). Interestingly, two human isoforms (hGle1a and hGle1b) are generated by alternatively spliced transcripts and differ in their C-terminus (with hGle1a lacking exons 15 and 16; *Kendirgi et al., 2003*). hGle1a lacks the C-terminal sequence allowing hGle1b to attach to the cytoplasmic fibrils of the NPC through hCG1 (*Kendirgi et al., 2005*). Therefore, it is possible that the mRNA export and translation functions of Gle1 are the purview of different isoforms in humans, while in other organisms one isoform appears to perform both functions. Recently, Wente & colleagues reported that IP_6 acts as a tether between Gle1 and DBP5, strengthening their association (*Montpetit et al., 2011*). Through crystallographic studies with the yeast proteins, they discovered that the Gle1-IP6-DBP5 complex is structurally similar to the eIF4G-eIF4A complex, which is another structure involving a DEAD-box ATPase and is essential for translation initiation, providing an intriguing parallel with Gle1's function in translation. This interaction appears to be the limiting factor in DBP5-mediated mRNA export (*Hodge et al., 2011*). In yeast, DBP5

associates with the NPC through Nup159 (Nup214, or CAN in humans), and the association is known to inhibit the RNA-binding and ATPase activity of DBP5 while causing ADP release (*von Moeller et al., 2009; Noble et al., 2011*). Montpetit et al. also performed biochemical assays which suggest that Gle1 cooperates with Nup159 to stimulate the mRNA export of DBP5 through a cycling mechanism, a model further elucidated by later reports from the same group (*Hodge et al., 2011; Noble et al., 2011*).

Exploring Gle1's role in motor neurons

Despite the wealth of cellular and biochemical data available for yeast studies, it is presently unclear what specific function(s) Gle1 may have in motor neurons. Therefore, the primary goal of our work is to define how Gle1 behaves in motor neurons and how this may relate to the functions of other (motor) neuron disease related proteins (see above and discussion for examples). Importantly, while yeast Gle1 is a 538 amino acid protein, mouse, rat, chick Gle1 and human Gle1b are all within 698-726 amino acids in length. Additionally, much more homology is readily detected between the vertebrate proteins than with the yeast ortholog. This may imply that Gle1 has expanded and yet undiscovered functions in vertebrates which could account for its role in motor neuron disorders.

Because of the dearth of studies examining Gle1 in multicellular organisms, we performed experiments to characterize Gle1's expression in our model systems: chick, mice, and mES cells, and asked whether Gle1 is upregulated in motor neurons compared to other tissues. We then began to explore Gle1's role in motor neuron development/ maintenance and how mutations in the gene may contribute to deleterious phenotypes.

We hypothesized that Gle1 may interact with specific proteins or transcripts in motor neurons, and we generated Gle1-Flag fusion constructs for future use in biochemical assays to try and identify these interacting partners. Finally, experiments with a Gle1 Gene-trap animal indicate that loss of a functional copy of the gene causes death at an early embryonic stage, and we have designed a rescue strategy to model Gle1-mediated motor neuron disease in mice. We hope that studying Gle1's biology will provide unique insight into general motor neuron and cell biology, and it may eventually lead to a paradigm linking motor neuron development and function with RNA regulation.

Methods

Differentiation of mES cells to motor neurons: Protocol can be found on the Pfaff laboratory web site.

DNA purification from embryos and cultured cells: Whole embryos or tails were harvested and placed in tail lysis buffer (0.2 M NaCl, 0.1 M Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS) overnight at 55°C. 2.5x 100% ethanol was added, samples were shaken vigorously, and placed at -20°C overnight. Samples were centrifuged at maximum speed for 15 minutes at 4°C. The supernatant was discarded and the DNA pellets were allowed to dry for ~1 hour. Samples were resuspended in water or TE buffer.

Expression in chick embryos: Chicken eggs were incubated for ~62 hours at 100°C and 55% humidity and then were injected with plasmid DNA mixed with FAST Green (for visualization) into the neural tube (see Pfaff lab web site for protocol). Embryos were electroporated using five 50 ms pulses of 25V. Eggs were then incubated for an addition 72 hours, and the embryos were eviscerated for whole-mount visualization or cryosectioning.

Expression of Flagged Gle1 in mES cells: Invitrogen's Lipofectamine chemical transfection system was used to express the plasmids using 5 μg of DNA per sample.

Flagged Gle1 design: Gle1 cDNA (Clone #3990668) was ordered from NCBI's clone registry. PCR primers were designed to generate inserts for both C-terminal and N-terminal vectors. The C-terminal insert replaced the stop codon (TGA) on the 3' end of the reading frame with a BamHI site, while the N-terminal insert replaced the ATG on the 5' end of the reading frame with NotI and placed XbaI on the 3' end. The PCR products

were then TOPO-cloned using Invitrogen's TOPO-TA cloning kit. The products were then cut with the above enzymes and put into pFLAG and p3XFLAG vectors from Sigma-Aldrich. The plasmids were sequenced through Eton Biosciences to ensure that mutations would not affect the protein.

Fixing for cryosectioning: E11-12 mouse and E7 chick embryos were fixed in 4% paraformaldehyde (PFA) for 2 hours and washed them in phosphate buffered solution (PBS) overnight. Embryos were then placed in sucrose until they sank. Procedures were performed at 4°C. Sections were then cut using a Leica CM1850 Cryostat at -20°C at 20-40 μm.

Gene-trap mouse line: A Gene-trap mouse ES cell line was obtained from the International Gene trap Consortium (IGTC). The cells were sent to the Salk Institute Mouse Facility and chimaeras with ICR mice were generated. These were mated with ICR mice to spawn heterozygous mice.

Immunocytochemistry: Cells were washed for 5 minutes in PBS then fixed with 4% PFA for 10 minutes, followed by three 5 minute washes in PBS. Blocking solution as in above was applied for 30 minutes, followed by primary antibodies diluted in blocking solution overnight at 4°C. Cells were then washed in PBS 3x for 10 minutes, after which secondary antibodies diluted in blocking solution were applied for 30 minutes at room temperature. Cells were then washed in PBS 3x for 5 minutes and then mounted onto glass slides using Vectashield with DAPI.

Immunohistochemistry: Frozen slides were washed for 10 minutes in PBS then with blocking solution (0.1% Tween and 1% bovine serum albumin (BSA) in PBS) for 1 hour. Primary antibodies diluted in blocking solution were then added at 4°C overnight. Slides

were then washed with wash solution (0.1% Tween in PBS) for 10 minutes 4x, and secondary antibodies diluted in blocking solution were applied for 1 hour at room temperature. Slides were washed with blocking solution 2x, wash solution 1x, and PBS 1x 15 minutes each. Vectashield with DAPI was then added and sealed with glass coverslips.

RT-PCR: Chick embryonic motor neurons, mES cells, and differentiated motor neurons were lysed in buffer (prepared according to protocols available on the Pfaff lab web site) and RNA was again extracted using the Qiagen kit. RT-PCR was performed using Invitrogen's SuperScript III First-Strand Synthesis System.

Western blots: Invitrogen's XCell system was used with NuPAGE Bis-Tris 4-12% Gels. Protein samples were ran at 200V and transferred to Whatman Protran nitrocellulose membranes at 30V. Membranes were blocked in 5% milk for 1 hour at room temperature, primary antibodies were applied in 5% milk overnight at 4°C, GE Healthcare's ECL system was used to visualize the proteins.

X-Gal staining: Whole-mount and section X-gal staining was performed according to laboratory protocols from the Pfaff laboratory web site.

Results

Gle1 transcripts are expressed in chick and mice

While the function of Gle1 in mRNA export leads us to believe that it is expressed ubiquitously, we wished to ensure that Gle1 is expressed in motor neurons. To confirm the presence of endogenous Gle1 transcripts in our model systems, we designed primers for chick and mouse Gle1 and performed RT-PCR on transcripts collected from chick embryonic motor neurons and mES cells (both undifferentiated and those differentiated into motor neurons). The expected bands (around 240 and 210 bp, for chick and mice, respectively) were observed (*Figure 1A*).

We also obtained RNA sequencing data showing the expression of Gle1 in both mES cells and mES cells differentiated into motor neurons (*Figure 1B*). The sequencing found Gle1 at ~18 reads per kilobase of exon per million mapped reads (RPKM) in mES cells and at ~9.7 RPKM in mES cells differentiated into motor neurons. These numbers indicate that Gle1 transcripts make up a smaller percentage of the total pool of transcripts in motor neurons than ES cells; however, to our knowledge, the absolute number of transcripts in motor neurons compared to ES cells is unclear. Therefore, these numbers are inconclusive, and it is possible that there are higher levels of Gle1 transcript expression in motor neurons than ES cells. In fact, GAPDH, a transcript thought to be present at similar levels in most cell types, also has higher RPKM values in ES cells than in differentiated motor neurons (~700 compared to ~250, for a ratio of ~2.8). With the ratio of Gle1 at ~1.9, normalizing the data to GAPDH levels implies that in motor neurons, Gle1 is expressed at ~1.5 times the level of ES cells.

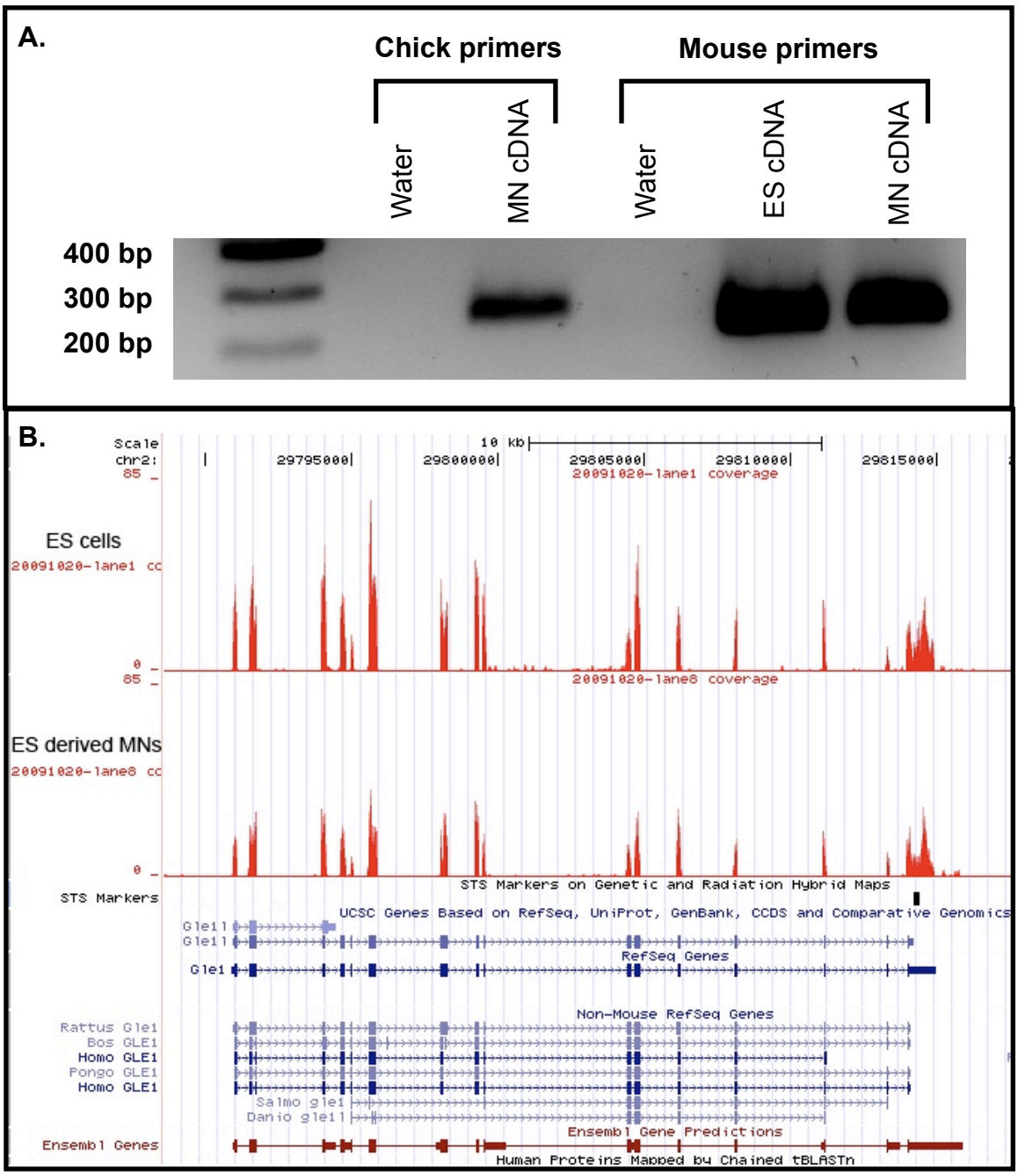

Figure 1. Gle1 transcript expression in chick and mouse. **A.** PCR products from Gle1 cDNA in chick, mES cells, and motor neurons derived from mES cells. Water indicates a no-cDNA control with the given primer set. **B.** UCSC Gene Browser plot showing RNA-sequencing results for Gle1 in mES cells and mES cell derived motor neurons. Peaks in red show exon reads mapped to each of 16 exons, which can be seen in the blue plots diagraming gene structure below. The homologous Gle1 loci of several related species are shown for comparison.

It is important to consider that transcript levels may not correspond exactly to levels of the translated protein; motor neurons may express comparable levels of Gle1 transcript, but have much higher levels of the translated protein. Future experiments will be necessary to examine this possibility (see discussion).

A screen for commercial Gle1 antibodies failed to show specificity in mice

After confirming Gle1's expression in neural tissue, we began more careful characterization of Gle1's localization both between and within cells in order to infer hints to its function. Additionally, we wished to search for novel binding partners for

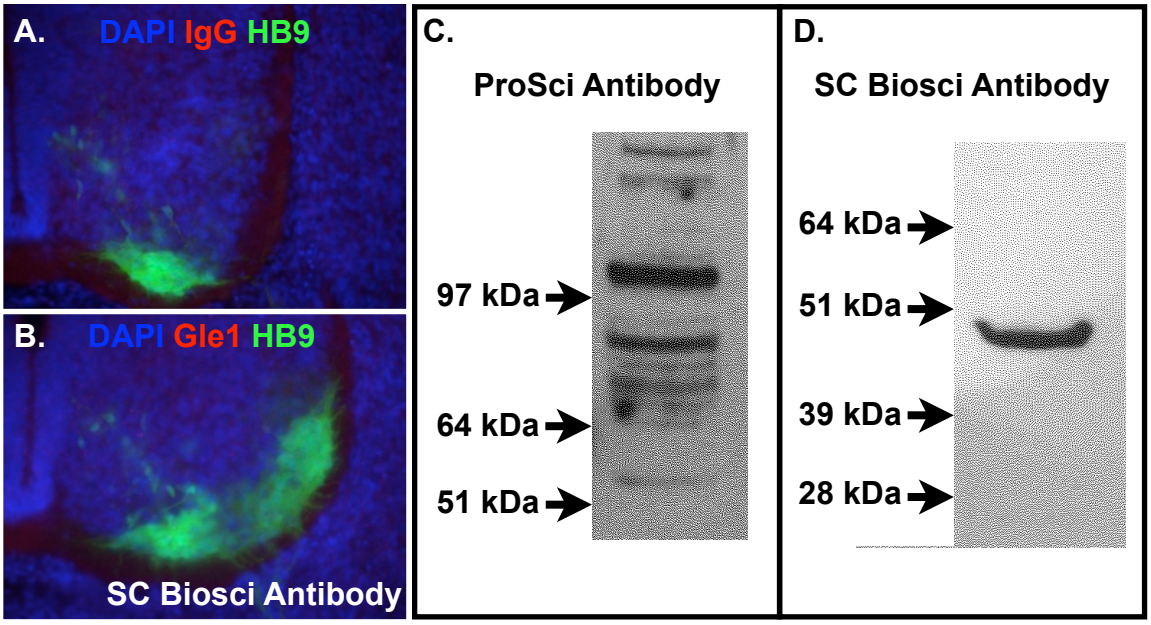

Figure 2. Non-specific staining from commercial Gle1 antibodies. **A-B.** E12.5 mouse sections stained with **A**. a rabbit polyclonal IgG antibody and **B.** a SC Biosci rabbit polyclonal Gle1 antibody. Sections were also stained with DAPI and an antibody for HB9 to visualize motor neurons. No difference between the IgG and Gle1 antibodies was seen. **C-D.** Western blots of mouse ES cell protein lysates using **C.** a Prosci rabbit polyclonal Gle1 antibody and **D.** a SC Biosci rabbit polyclonal Gle1 antibody. **C.** shows nonspecific staining with possible bands for Gle1 (expected at ~80 kDa), while **D.** shows staining for a band at ~48 kDa.

Gle1 in mouse, both globally and in targeted tissues (such as motor neurons), which may elucidate novel functions for Gle1 in vertebrates.

To these ends, we tested several commercial antibodies for application in immunohistochemistry (IHC), Western blots, and immunoprecipitation (IP) experiments (*Table 1*). In IHC, the antibodies consistently failed to show a signal above that of the secondary antibody alone (data not shown). In Western blots and IP experiments, some antibodies recognized several bands (*Figure 2C*), while the antibodies which detected only one band did not stain a band of the correct size, and did not detect the same band between antibodies (*Figure 2D*). As the antibodies were designed against human Gle1, slight differences between the mouse and human proteins could account for these inconsistent and non-specific results. Consequently, we could not use these antibodies against the mouse protein, and another strategy was required.

Table 1. Commercial antibodies for Gle1 tested in immunohistochemistry, Western blot, and immunoprecipitation.

Company	Type	Antibody
AbCam	Mouse Polyclonal	ab69968
AbCam	Rabbit Polyclonal	ab81648
ProSci	Rabbit Polyclonal	4973
SCBiosciences	Rabbit Polyclonal	sc-98363

Flagged-Gle1 fusion proteins are expressible in mES cells and chick

To compensate for a lack of specificity with commercial antibodies, we generated both N-terminal and C-terminal Flagged (Sigma) Gle1 fusion proteins, with the C-terminal fusion protein having the 3xFlag peptide (3 Flag moieties connected in series for a higher signal). We chemically transfected the vectors into mES cells and prepared them

for immunostaining with an antibody for the Flag peptide. We also transfected vectors expressing Flagged GFR-α (a GPI-anchored protein; *Jing et al., 1996*) linked with IRES-GFP to confirm transfection through GFP expression and to test the immunostaining protocol for Flag. An SMN-3XFlag fusion construct was used as a further control due to its nature as an RNA-processing protein involved in motor neuron disease. The use of a verified SMN antibody allowed us to compare the expression of the endogenous protein with the fusion protein. Finally, we used a Sox2 (a transcription factor expressed in ES cells; *Masui et al., 2007*) antibody to test for immunostaining specificity overall. The use of multiple controls allowed us to examine various subcellular localizations and to ensure that the staining protocol was effective enough to detect true signals. *Figure 3A-B* shows the nuclear localization expected for the transcription factor Sox2 (*Lee et al., 2008*). *Figure 3C-D* shows that the Flag staining for the Flagged-GFR-α appears membrane-localized, as expected. Meanwhile, *Figure 3E-H* shows the Flag staining of SMN-3xFlag overlaps well with that of endogenous SMN, which localizes cytoplasmically as shown. *Figure 3I-L* shows the expression of the Gle1-Flag fusion constructs, which is detected in the cytoplasm, consistent with its known function in translation (*Bolger et al., 2008*). The nuclear envelope can also be seen, as expected from its known interactions with the NPC (*Murphy & Wente, 1996; Rayala, et al, 2004; Watkins et al., 1998*).

To ensure that the Flagged fusion proteins can be detected in intact tissue as well as in cell culture, we also expressed the fusion proteins in chick and performed IHC. Electroporation of expression constructs into chick gives the added bonus of an internal control, as comparing the electroporated side of the spinal cord with the unaffected side allows easy visualization of any differences in signal. The Flagged-GFR-α-IRES-GFP

vector was again used as a control for electroporation efficiency as well as the Flag

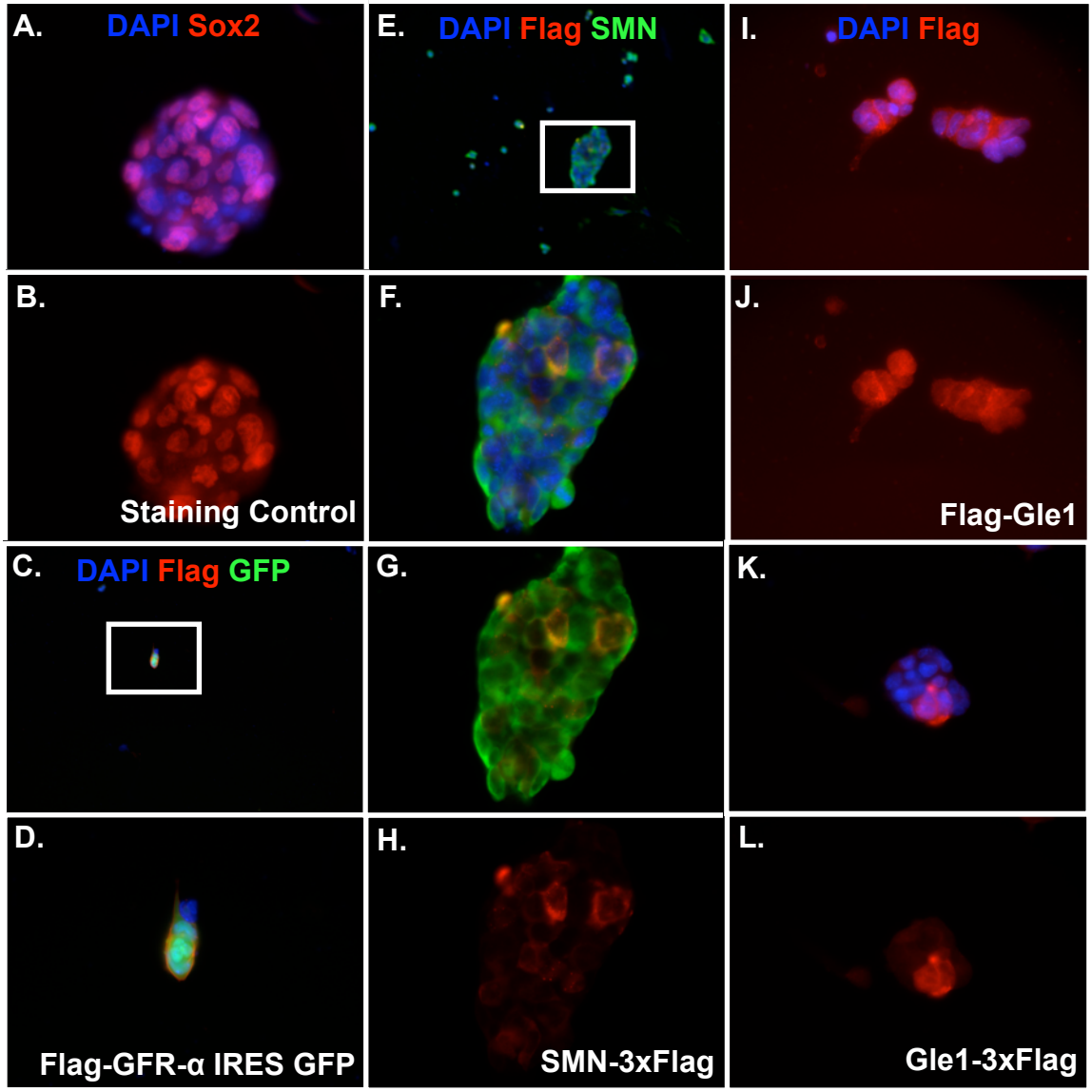

Figure 3. mES cells chemically transfected with Flag-fusion protein expression vectors and immunostained. **A-B.** mES cells stained with DAPI and anti-Sox2 (marking a nuclear ES cell transcription factor); positive control for staining protocol. **C-D.** mES cells expressing Flag-GFR-α IRES GFP stained with DAPI and anti-Flag. Positive control for transfection and anti-Flag staining. **D** shows inset area of **C. E-H.** mES expressing C-terminal 3xFlagged SMN stained with DAPI, anti-Flag, and anti-SMN. **F-H** show inset area in **E**. **I-L.** mES cells expressing N-terminal Flagged Gle1 (**I-J**) and C-terminal 3x-Flagged Gle1 (**K-L**) stained with DAPI and anti-Flag.

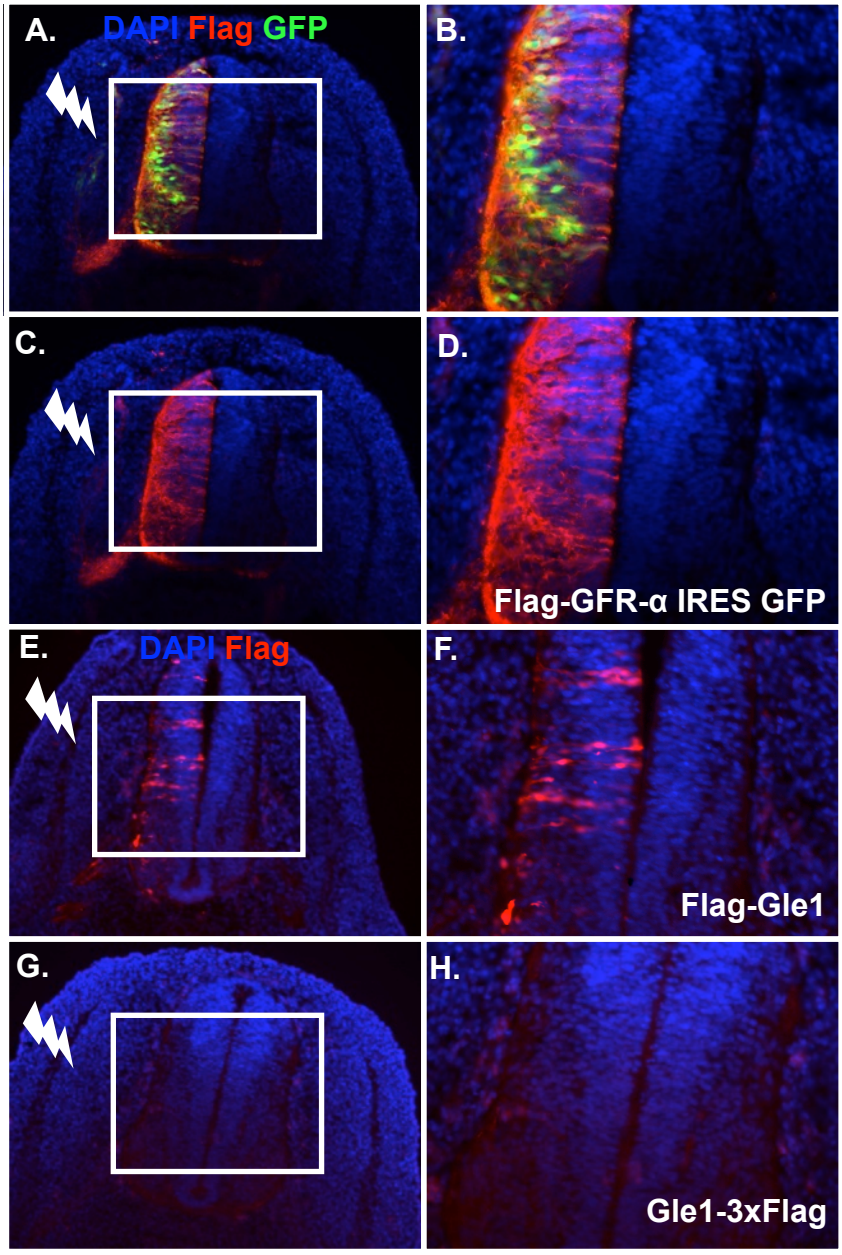

Figure 4. Electroporation of Flagged-Gle1 fusion constructs into chick. **A-D**. Electroporation of a Flag-GFR-α IRES GFP construct as a control for immunostaining efficacy and electroporation efficiency. Sections were stained with antibodies for Flag and GFP. Staining shows strong and specific signals, indicating high electroporation efficiency. **E-F.** Expression and staining of Flag-Gle1 with an anti-Flag antibody. Staining is highly specific, but the protein appears to be expressed at a lower level than that of Flag-GFR-α IRES GFP. **F** shows inset area in **E.** The lower quality of the higher magnification image is due to bleaching of the red secondary antibody. **G-H.** Expression and staining of Gle1-3xFlag with an anti-Flag antibody. No noticeable specific staining is seen. **H** shows inset area in **G.** Lightning bolts indicated electroporated side.

antibody efficacy, and was highly positive and specific in both the green channel (indicating high electroporation efficiency with the high GFP expression level, *Figure 4A-B*) and the red (indicating strong specificity and binding affinity for the Flag antibody, *Figure 4C-D*). Electroporation of the N-terminal Flagged-Gle1 vector in chicks showed specific signals after staining with an anti-Flag antibody with a red secondary (*Figure 4E-F*). However, the C-terminal Flagged-Gle1 vector showed no difference between the electroporated and unaffected sides (*Figure 4G-H*). This is in contrast to the expression seen in mES cells, where the C-terminal vector showed a brighter signal than the N-terminal vector, likely due to the 3x-Flag moiety. It is likely that this was due to technical problems in the electroporation, despite several samples examined, and more electroporations should be performed to confirm this. Alternatively, expression of the 3xFlag peptide may not be well-tolerated by the chick system as compared to the single Flag moiety. Interestingly, the Flagged-GFR-α-IRES-GFP showed much higher expression levels than the Flagged-Gle1 fusion proteins. This could be due to the different promoters (CMV promoters in the Gle1 constructs, a β-actin promoter with a CMV-IE enhancer in the GFR-α vector) in the expression vectors or differences in the way chick cells respond to the exogenous proteins. Importantly, the Flagged-GFR-α localizes very differently with the GFP expressed from the same construct, showing that the Flagged fusion proteins appear to localize similarly to the endogenous proteins. This highlights the fusion proteins as useful tools to examine the subcellular localization of Gle1 *in vivo*.

Stably expressing Flagged-Gle1 in mES cells

As mentioned above, ES cells are an exceptionally powerful tool for generating large quantities of cells and studying neuronal properties when differentiated into various subpopulations of neurons. However, normal chemical or electrical transfection of transgenes into cells encounters several problems during differentiation. First, the time frame of differentiation, as well as the multiple rounds of mitosis involved, causes the dilution and loss of the expression vectors. Additionally, any transgene that is incorporated into the cellular genome can become silenced by mechanisms such as DNA methylation or histone modification upon differentiation (for review, see *Berger, 2007; Reik, 2007*). Therefore, a strategy for stably maintaining fusion protein expression in differentiated motor neurons was necessary.

To accomplish this, we adapted a method developed by the lab of Paul Berg and took advantage of the Polyoma SV40 large T antigen, which binds to the Polyoma origin of replication (ORI) and drives DNA replication. By transfecting a plasmid expressing the large T antigen which also contains the Polyoma ORI into a cell line, the plasmid can perpetuate throughout the cell lineages in an episomal fashion (*Figure 5A*; *Gassmann et al., 1995; Camenisch et al., 1996; Aubert et al., 2002*). We obtained cells expressing this plasmid (pMGD20*neo*) from Dr. Ian Chambers and designed a vector containing the Polyoma ORI to express Flagged Gle1 fusion protein under the control of the CAG promoter, dubbed pPyCAG-FLAG-Gle1-IP (*Figure 5B*; adapted from the pPyCAG-EGFP-IP vector from Dr. Hitoshi Niwa). We then chemically transfected the large T-expressing cells with the vector to stably expressed the Flagged-Gle1 (*Figure 5C*). Thus

far, we have been able to clone and express the construct containing the N-terminally Flagged-Gle1, and we are currently at work on the C-terminus version. Western blots of

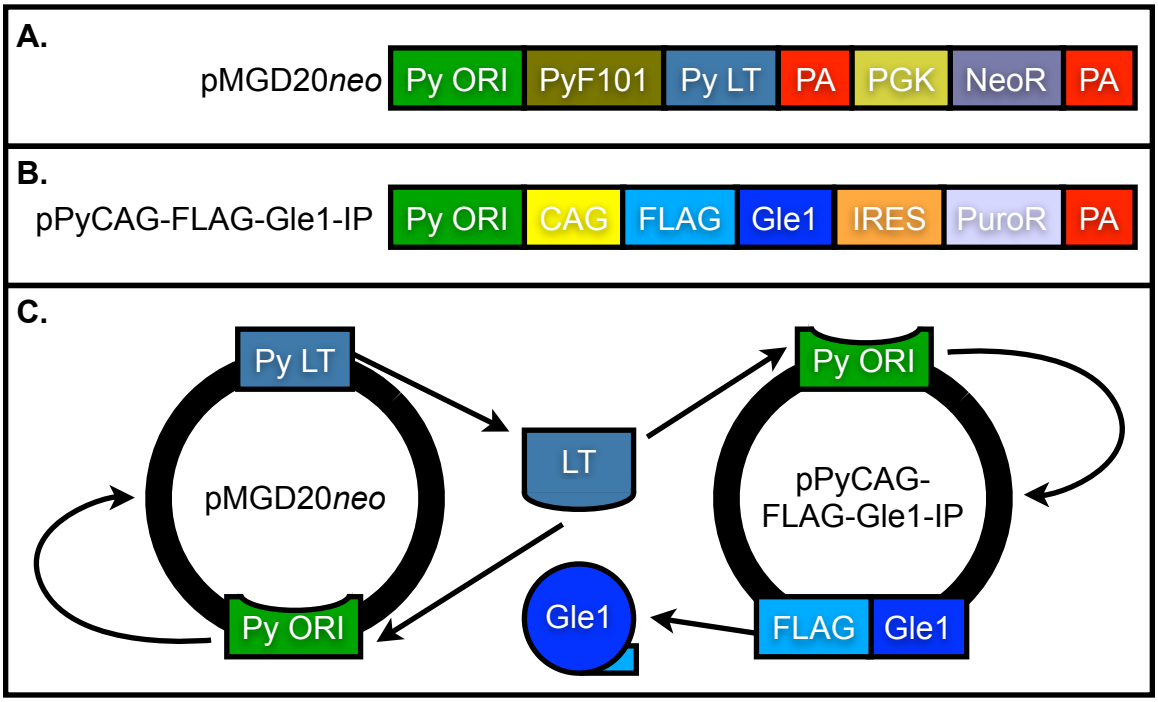

Figure 5. Stable expression of the Flagged-Gle1 fusion protein in mES cells. **A.** Key components of the pMGD20*neo* vector, and **B.** Key components of the pPyCAG-FLAG-Gle1-IP vector for stable expression of the Flag-Gle1 fusion protein. Py ORI = Polyoma Origin of Replication; PyF101 = Polyoma Enhancer; Py LT = Polyoma Large T Antigen; PA = Poly-Adenylation Site; PGK = Phosphoglycerate Kinase Promoter; NeoR = Neomycin Resistance Cassette; CAG = CAG Promoter; IRES = Internal Ribosomal Entry Site; PuroR = Puromycin Resistance Cassette. Some plasmid elements, such as bacterial selection cassettes and ORI's, are not shown. **C.** Diagram of the stable expression of Flag-Gle1 - the Py LT produced by pMGD20*neo* allows replication of its parent plasmid as well as pPyCAG-FLAG-Gle1-IP, which produces Flag-Gle1.

protein from ES cells transfected 3X-Flagged-SMN expressed from the pPyCAG construct show that the episomal vector persists through several passages as well as upon differentiation into motor neurons (data not shown). We are currently performing experiments with cells expressing pPyCAG-FLAG-Gle1-IP to confirm similar results

with Gle1, which would allow us to use these cells for various assays in the future (see discussion).

Mutant Human Gle1 elicits no noticeable phenotype when expressed in chick

While the recessive genetics of LCCS1 and LAAHD suggest that the FinMajor mutation has little or no dominant effects on Gle1 function, we wished to explore the possibility of a gain of function further. In chick, overexpressing human variants of TDP-43, which has a dominant link to ALS when mutated, causes motor neuron death, and the RNA-binding activity of TDP-43 is required for this phenotype (*Voigt et al., 2010*). Despite the difference in the genetic presentation between LCCS1 and TDP-43-mediated ALS (recessive versus dominant), TDP-43 provided a precedent for mutated RNA-processing proteins causing motor neuron phenotypes in chick. We therefore obtained plasmids containing both mutant and WT forms human Gle1 in the pEGFP-2 expression vector to perform overexpression experiments (BD Biosciences; graciously provided by Dr. Marjo Kestila). These were used in the previous study by Nousiainen and colleagues linking Gle1 with LCCS1/LAAHD, and both versions of the protein were linked to GFP at the N-terminal end for visualization upon expression.

We electroporated both constructs in chick with a CMV-Tomato electroporation control, and stained sections for GFP and MNR2 (a homeodomain transcription factor expressed in chick motor neurons; *Tanabe et al., 1998*) to visualize the motor neurons and search for abnormalities. The presence of GFP on the electroporated side along with Tomato (*Figures 6A-B,E-F*) shows that the electroporation was effective and the fusion proteins were expressed. Comparing *Figure 6A-D* with *6E-H* shows that there is no

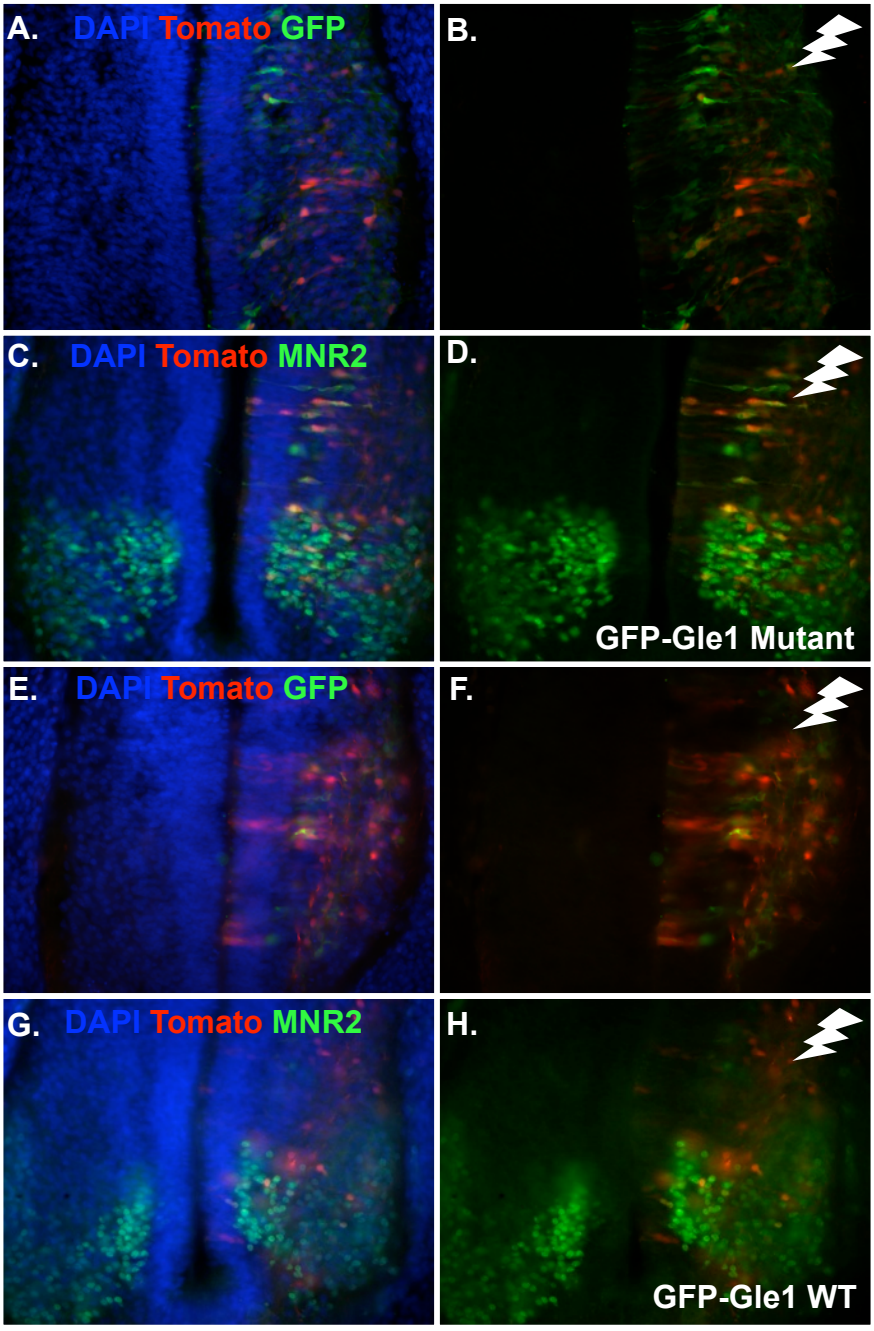

Figure 6. Electroporation of human WT and Mutant GFP-Gle1 fusion proteins into chick embryos. A CMV-Tomato vector was used as a control for electroporation efficiency. **A-D** show sections from embryos expressing the mutant vector, while **E-H** show sections from embryos expressing the WT vector. **A, B, E,** and **F** were stained with antibodies for GFP, while **C, D, G,** and **H** were stained with antibodies for MNR2 (labeling motor neurons). Lightning bolts indicated electroporated side.

significant phenotypical differences between embryos electroporated with the mutant construct versus WT. *Figures 6C-D,G-H* compare MNR2 staining and suggests in particular that motor neuron proliferation is not affected by expression of the mutant protein as the number of motor neurons appears consistent between electroporated and unelectroporated sides. While some double-labeling between tomato and MNR2 can be seen, indicating that electroporation of the constructs into motor neurons was successful, not many cells appeared to be transfected. It will be important to perform more electroporations in an attempt to attain higher electroporation efficiency, expressing the constructs in more cells, to see if a phenotype emerges with more affected motor neurons. We will also further examine motor neuron morphology through confocal microscopy to tease out subtle differences that may be caused by expression of the mutant protein, but a cursory examination using basic microscopy does not show any obvious phenotype. Although future experiments will be necessary to corroborate our findings, preliminary data does not reveal any evidence that mutant Gle1 dominantly causes any developmental defects.

The heterozygous Gle1 Gene-trap mouse expresses a β-Geo fusion protein

Mouse genetics, and in particular loss of function mutations, have long served as important systems to studying gene function. Traditionally, such mutations have been generated through homologous recombination. However, the recent generation and availability of an enormous number of Gene-trap ES cell lines have provided an alternative approach for many genes. These are generated by the transfection of a Gene-trap vector into ES cells, where the vectors integrate randomly into gene loci at introns

(*Schnütgen, et al., 2005*). A Gle1 Gene-trap mouse embryonic stem (mES) cell line was generated with β-Geo (LacZ linked with a neomycin resistance gene) cassette inserted into the intron between the third and fourth exons of the Gle1 locus (*Figure 7A-B*). This theoretically generates transcripts with a β-Geo sequence and a stop codon before the fourth exon of Gle1, truncating the translated protein and linking it with the β-Geo protein (β-Galactosidase, or β-Gal, linked to a aminoglycoside phosphotransferase conferring neomycin resistance). As function has been ascribed to the C-terminus of Gle1 in both yeast and humans, we expect the Gene-trap allele to constitute a significant loss of function mutation, if not a complete knockout (*Kendirgi et al., 2003; Kendirgi et al., 2005*). The use of this cell line allows us to model a loss of function in Gle1 in both ES cells and mice created from the line.

Using the Gene-trap mES cells, we generated chimeric mice with the Salk Transgenic Mouse Core and crossed these with WT mice to generate heterozygous Gle1 Gene-trap animals. To examine the effects of a loss of Gle1 function *in vivo*, we first ensured that the Gle1 Gene-trap mouse expressed a fusion protein with the first 145 amino acids (16 kDa) of Gle1 attached to β-Geo (1293 residues; ~146 kDa) as expected. IHC on heterozygous Gene-trap mice (E11-12) using a β-Gal antibody did not show specific staining for the reporter protein when compared to a secondary antibody-only control (data not shown). An Isl1/2 (a transcription factor labeling motor neurons and some interneurons) antibody was used as a control for our staining protocol (*Sockanathan & Jessell, 1998*). Similarly, X-Gal staining of heterozygous Gene-trap animals did not show any differences when compared to staining of WT embryos (data not shown). However, immunoblotting protein from heterozygous Gle1-Gene-trap ES

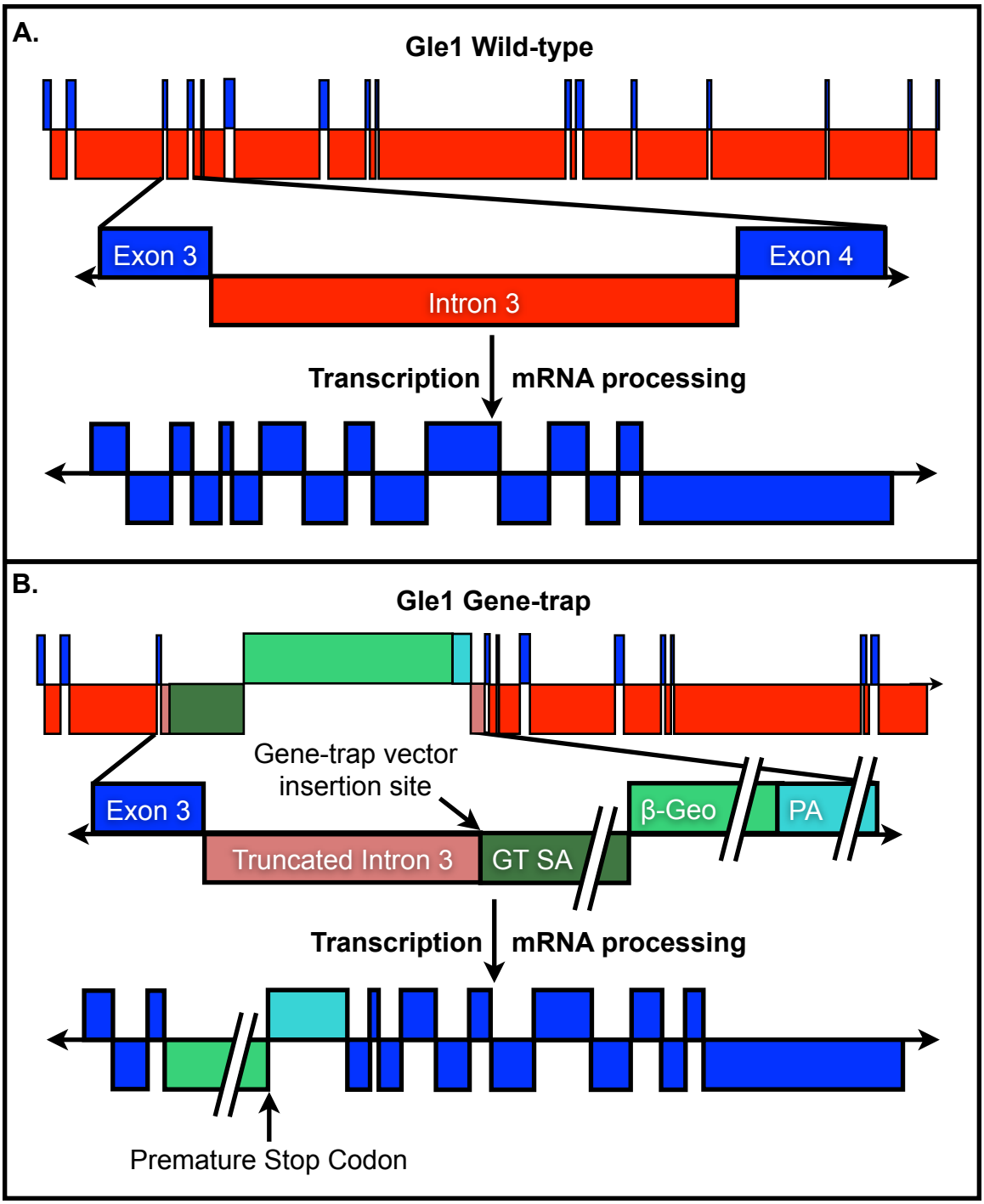

Figure 7. The Gle1 Gene-trap allele. **A.** Mouse Gle1 DNA showing the area of Gene-trap insertion, and the resulting transcript. **B.** The resulting DNA after Gene-trap vector insertion and the resulting transcript.

cell lines showed expression of the fusion protein with a band around 162 kDa as

expected, after long periods of exposure (*Figure 8*). Meanwhile, Western blots of WT samples showed no staining with the antibody, while protein from a well-characterized KDM1A Gene-trap ES cell line were positive for the KDM1A-β-Geo fusion protein (*Macfarlan et al., 2011*).

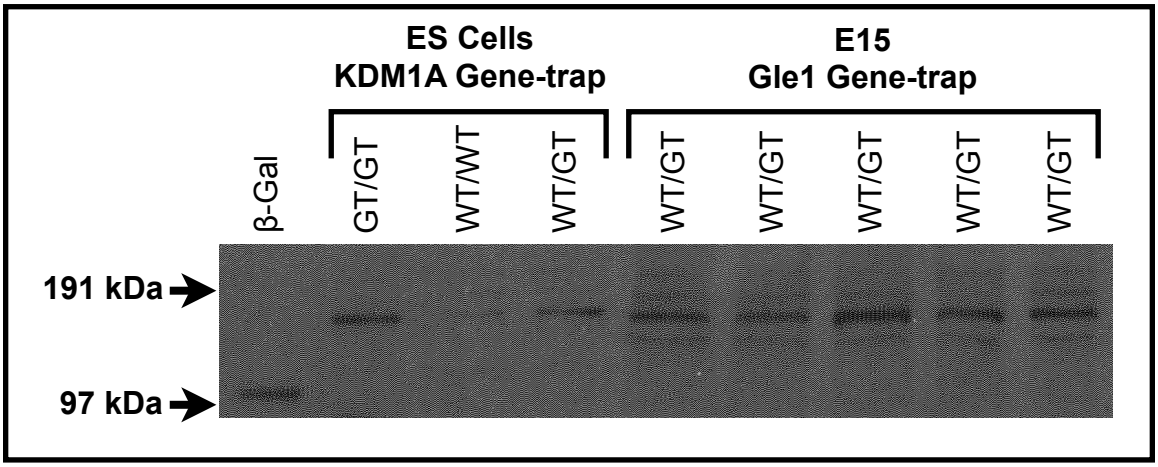

Figure 8. Expression of a Gle1-β-Geo fusion protein in heterozygous Gle1 Gene-trap animals revealed through Western blotting. Samples were immunoblotted with a Mouse Anti-Flag antibody from Sigma-Aldrich. Lane 1 shows control β-Gal protein (expected at 116 kDa), lane 3 shows protein from WT ES cells, lanes 2 & 4 show protein samples from homozygous and heterozygous KDM1A Gene-trap ES cells, respectively, and lanes 5-9 show protein samples E15 heterozygous Gene-trap mice (Gle1-β-Geo fusion protein expected at 162 kDa).

Based on the lack of X-Gal and IHC staining, along with the long exposure times required for detection in Western blots, it appears that the Gle1-β-Geo fusion protein is present at low levels in ES cells. Further experiments must be done to examine whether normal endogenous Gle1 is expressed at similar levels (see discussion).

The Gle1 Gene-trap allele is homozygous lethal

To determine how the Gene-trap mutation affects Gle1's function in mouse, we first tested whether homozygous animals were viable. As mentioned earlier, because

Gle1 is expected to serve a ubiquitous function in mRNA processing and the Gene-trap mutation eliminates numerous important protein domains, we expected homozygous animals to be inviable. We crossed the heterozygous Gle1 Gene-trap animals and

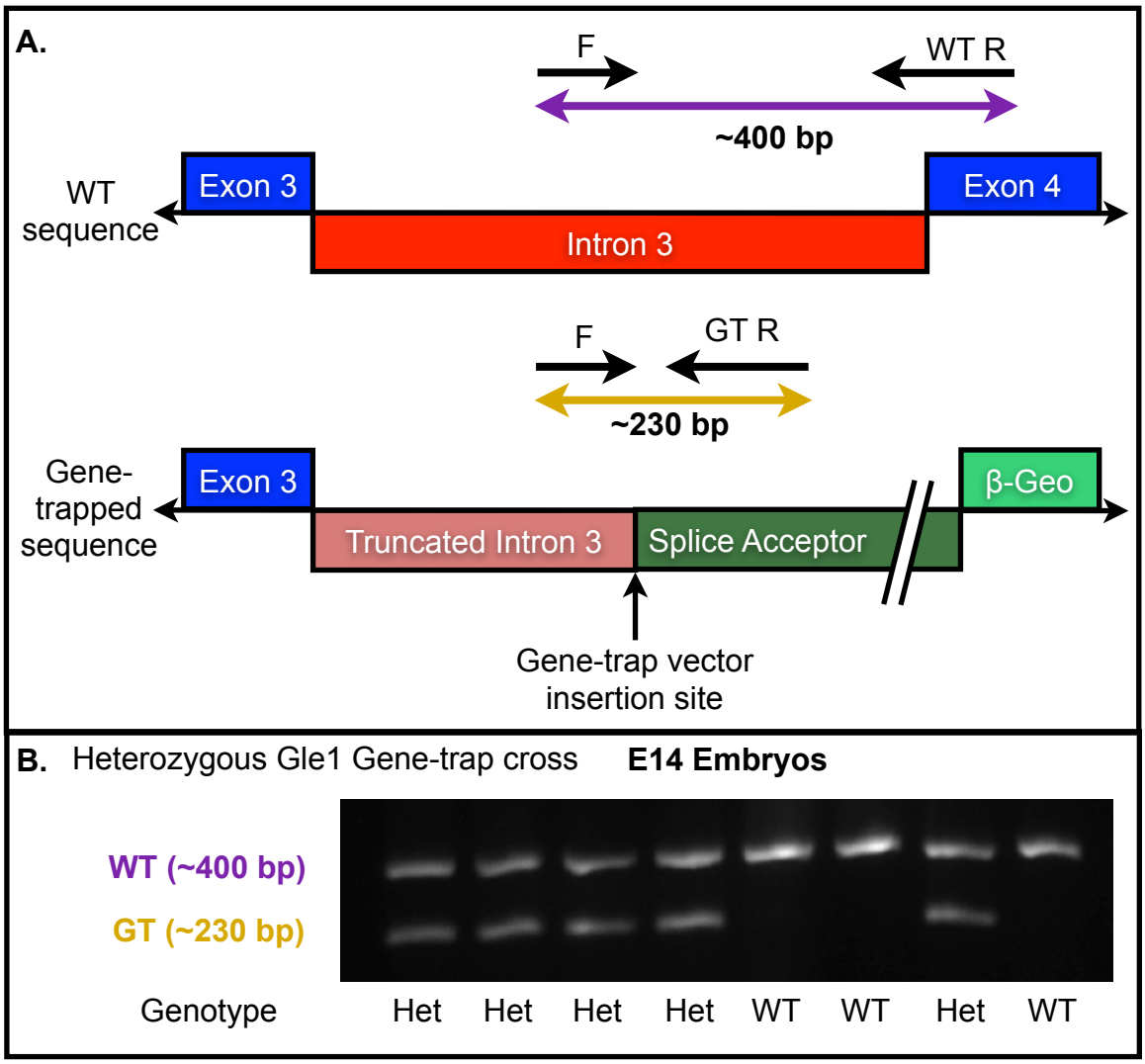

Figure 9. PCR genotyping of Gle1 Gene-trap animals. **A**. Primer placement for genotyping. WT DNA region of Gene-trap insertion is shown along with the same region after Gene-trap insertion. Single-headed arrows show primers, while double-headed arrows show PCR products for WT (purple) and Gene-trap (yellow) alleles. F = forward primer; WT R = WT reverse primer; GT R = Gene-trap reverse primer. **B**. Representative PCR results for a litter of E14 embryos from a cross of heterozygous Gle1 Gene-trap mice. 3/8 embryos shown here are WT, while 5/8 are heterozygous for the Gene-trap allele.

collected litters at various ages from E5.5 to P21 for genotyping. Additionally, ES cell lines were derived from blastocysts collected from one litter of heterozygous Gle1 Gene-trap crosses, and genomic DNA from these cultured cells was harvested for genotyping.

Genotyping primers were designed to generate PCR products around the intronic insertion site of the β-Geo transgene in the Gle1 locus, with two different possible products sharing a single (upstream) primer. The WT reverse primer is downstream of the insertion site and generates a product of ~400 base pairs in the absence of the β-Geo transgene, while the mutant reverse primer was designed against the sequence of LacZ and generates a product of ~230 base pairs in the presence of the β-Geo transgene (*Figure 9A*). *Figure 9B* shows representative genotyping results from a litter of E14 embryos. Our data (*Table 2*) suggests that the probable knockout is homozygous lethal, indicating an important role in early development and essential cellular processes.

Table 2. Genotyping results from crosses of heterozygous Gle1 Gene-trap mice. ES cell lines were derived from blastocysts at E3.5.

Age	Litters	WT/WT	WT/GT	GT/GT
ES Cell Lines	1	2	6	0
E5.5	1	1	4	0
E9-11.5	4	11	20	0
E12-15	4	6	12	0
P1	2	4	7	0
Total	12	24	49	0

We observed no homozygous Gene-trap animals, and the ratio of WT to heterozygous animals is similar to the 1:2 ratio expected for typical Mendelian inheritance (χ^2 test; $P = 0.934$). Heterozygous animals show no apparent phenotype, and the Mendelian ratio suggests that they do not die or survive preferentially compared with

WT animals. This is consistent with the recessive presentation of human LCCS1 and LAAHD (*Nousiainen et al., 2008*). We are currently performing immunohistochemistry to more closely examine possible subtle phenotypes. It will also be extremely important to determine exactly when the homozygous embryos die, as this will provide insight on the function of Gle1 (see discussion). Our current results indicate that a mouse homozygous for the Gene-trap allele is not a suitable way to model Gle1-mediated motor neuron disease, as death appears to occur long before is seen in human LCCS1 patients, preceding any neural development.

A strategy for modeling Gle1-mediated motor neuron disease via ROSA26 targeting

As homozygous Gene-trap animals appear inviable, we required another model to examine Gle1's specific effects on motor neuron survival and function. We wish to study the FinMajor mutation's effect on Gle1 function in the absence of any WT mouse Gle1, and one possibility is to use mouse genetics to reintroduce Gle1 into the Gene-trap line. We selected a strategy to rescue the animals through knocking in versions of Gle1 into the ROSA26 locus. ROSA26 has been used for numerous targeted transgenes as the locus is known to allow for moderate expression in post-mitotic differentiated cells throughout the entire animal, again allowing us to avoid the problem of potential silencing that affects traditional transgenes (*Masui et al., 2005; Zambrowicz et al., 1997*)**.** As the mouse and human proteins are 83% identical (with 90% matching similar amino acids), we decided to generate a mutant version of the mouse protein rather than use the mutated human protein as it is likely the mutation will have a similar effect on the mouse

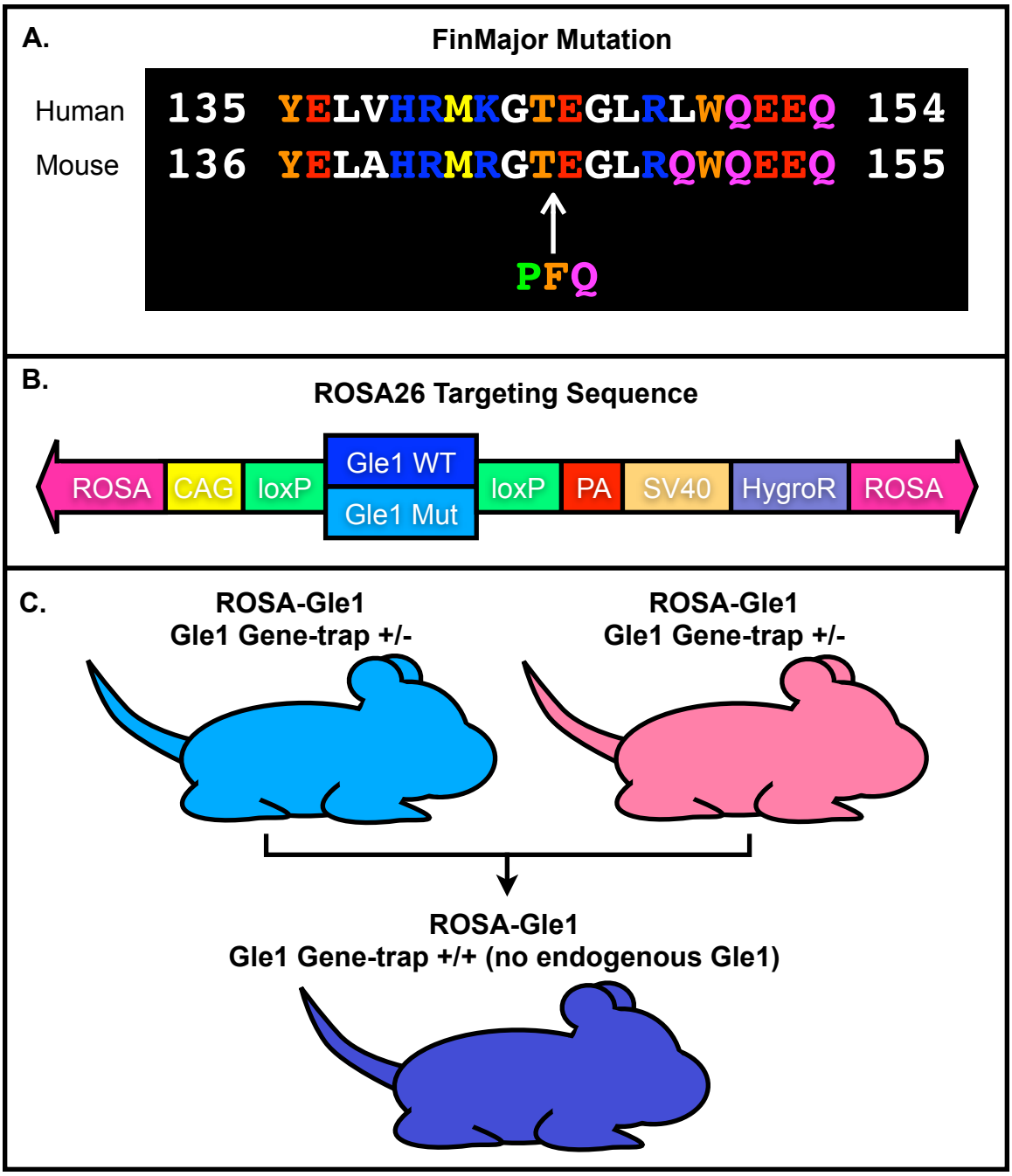

Figure 10. ROSA26-targeted rescue of the homozygous Gle1 Gene-trap animals. **A.** The FinMajor mutation (insertion of PFQ) in the context of human and mouse protein sequences. **B.** Key components of the ROSA26 targeting vector for insertion of WT and mutant Gle1. ROSA = ROSA26 homology arms; CAG = CAG promoter; loxP = loxP sites; PA = Poly-Adenylation site; SV40 = SV40 promoter; HygroR = Hygromycin Resistance Cassette. **C.** Mating strategy for generating mice expressing only Gle1 (WT or mutant) from the ROSA26 locus.

protein structure, while using the mouse ortholog will avoid problems due to possible differences in the proteins. We generated cDNA's of either WT mouse Gle1 or a mutated form which contains the 9 base pair insertion predicted by the FinMajor mutation (T145_E146insPFQ in mice, analagous to the T144_E145insPFQ mutation in humans; *Figure 10A*; *Nousiainen, et al., 2008*). We then flanked each cDNA with loxP sites, placed them under the control of a CAG promoter, and cloned them into a ROSA26 targeting construct (provided by members of the Pfaff lab) and modified it to include a hygromycin resistance cassette (*Figure 10B*). The plasmids were then linearized and electroporated into ES cells heterozygous for the Gle1 Gene-trap allele. We are currently attempting to genotype the cells using PCR and Southern blotting to confirm homologous recombination in the ROSA26 locus.

Once the recombination is confirmed, we will submit the cells to the Salk Transgenic Mouse Core to generate chimeric mice. We will then cross these mice and their offspring and attempt to generate mice that are homozygous for the Gle1 Gene-trap allele and also express Gle1 (either WT or mutant) from the ROSA26 locus, which will replace the endogenous protein (*Figure 10C*). If these animals are viable, they will provide valuable tools in the study of Gle1-mediated motor neuron disease. The mouse expressing the WT rescue will serve as a valuable control (see discussion), and we can also specifically re-knock-out Gle1 in motor neurons using a motor-neuron specific Cre driver. As we expect even targeted knockouts of the gene to cause nonviable animals (due to the likely vital function Gle1 plays), we hope that the mutant rescue recapitulates the phenotype seen in human LCCS1, providing us an animal model to explore how the FinMajor mutation causes motor neuron disease.

Discussion

With the studies by Nousiainen and colleagues, we know that recessive mutations in Gle1 can lead to motor neuron death and embryonic lethality in humans, and studies in yeast show that the protein is involved in mRNA regulation. The FinMajor mutation could disrupt one of many functions that Gle1 has, or it could make the protein less efficient at all of its functions, and it is unclear why mutations Gle1 appears to preferentially target motor neurons. It is reasonable to assume that it is ubiquitously expressed based on its role in basic cellular processes, and indeed, our data shows that the transcript is expressed in ES cells at levels comparable to those in motor neurons.

The possibility remains that there are quantitative differences in the amount of Gle1 protein in motor neurons and other cell types, which could imply that the defects in motor neurons caused by Gle1 mutations simply reflect a requirement for higher levels of Gle1 and a generalized function it serves throughout all tissues. As Gle1 is purportedly the limiting factor in DBP5-mediated mRNA export, this is possible and would indicate that motor neurons require the generation of large amounts of Gle1 protein, particularly during development (*Hodge et al., 2011*). While *in situ* hybridizations should be performed to further characterize Gle1's expression across tissues while also providing data on the subcellular localization of the transcript, our data suggests that the motor neuron phenotype seen in LCCS1 and LAAHD in humans, if the expression patterns are conserved, are not due to motor neurons requiring higher levels of a ubiquitous function, but rather the loss of a specific role Gle1 has in motor neuron development or function caused by hypomorphic mutations. This could be a role Gle1 plays in multiple cell types

that is especially vital in motor neurons, or it could be a function specific to motor neurons. However, the data may be misleading, as the consistent mRNA levels may not translate into similar protein levels across cell types; it is possible that motor neurons express translation factors which allow for specifically higher translation of Gle1 protein. Protein level assays, such as mass spectrometry experiments, are necessary to determine whether protein levels can be directly correlated with mRNA levels in this case.

In addition to more rigorous characterization of Gle1's expression pattern between tissues, it is important to consider its distribution within cells. Recently, many groups have turned their attention to the role of RNA localization and local translation in cellular functions. mRNA localization is important in various organisms for the spatial regulation of gene expression, and various examples have been found (for review, see *Martin & Ephrussi, 2009*). In neurons, such mRNA regulation is likely to be particularly important due to the compartmentalization of the cells (dendrite, axons, soma) and the huge distances neurites travel compared to the diameter of the cells. Various studies have found roles for mRNA localization and local translation in neuronal processes such as synaptic plasticity and axon guidance. For example, BDNF is locally translated in postsynaptic boutons to assist in homeostatic plasticity, and DCC (a receptor for the axon guidance molecule Netrin) helps to regulate local axonal translation (*Jakawich et al., 2010; Tcherkezian et al., 2010*).

Transcript localization is mediated by various non-coding regions of the immature or final transcript, with 3' untranslated regions (UTR's) being most heavily implicated (*Martin & Ephrussi, 2009*). The 5' UTR was also found to be important in numerous

examples as well, and a recent study showed the involvement of unspliced introns in certain transcripts as well (*Buckley et al., 2011***)**. There are precedents for mutations in proteins involved in mRNA transport causing various neural disorders. For example, the Fragile-X Mental Retardation-related Protein (FMRP), is important in the proper maintenance of mRNA localization and is also critical in neuronal function, (*Zalfa et al., 2003***)**. SMN was also recently reported to function in mRNA localization, assisting in the transport of transcripts to the axons of motor neurons (*Fallini et al., 2011*). This leads to the intriguing possibility that Gle1 may be involved in similar processes in motor neurons; for example, mutations in the gene could result in the breakdown of localized translation required in motor neuron axons, causing the breakdown seen in LCCS1 and LAAHD. This could be due to a role for Gle1 in the transport of motor neuron-specific transcripts. As Gle1 is a known regulator of mRNA in nuclear export and translation, a further function in the transcript localization would not be especially surprising and could explain the susceptibility of neurons to mutations of RNA binding proteins while apparently not affecting other tissues. An interesting possibility is that other polar cells throughout the body could be affected by these neuronal diseases, although the lack of phenotype in non-motor-neurons for the most part implies that motor neurons are very specific in their need for whatever functions are affected.

The use of the Flagged Gle1 will hopefully be informative in the future when used in biochemical assays and determining Gle1's localization within cells (and specially motor neurons with their long neurites). The fusion proteins are currently being used in experiments such as immunoprecipitation and CHIP-sequencing to elucidate the function

of Gle1 by identify possible unique DNA/mRNA/protein targets Gle1 may specifically interact with in motor neurons. An especially informative test will be to compare the targets of Gle1 between different cell types, such as generic ES cells and motor neurons. The existence of specific binding partners in motor neurons will provide much insight into specific processes Gle1 partakes in within motor neurons, while a lack of difference would imply that motor neurons stringently require a ubiquitous function of the protein. The Flagged-fusion protein may not provide a perfect answer, as the expression of the fusion protein may not match that of the endogenous protein (see below). For example, it is possible that the Flag peptide interferes with Gle1 binding to the NPC (*Rayala et al., 2004; Kendirgi et al., 2005*). We have already observed possible disruption of Gle1 function by the epitope, as electroporation of the N-terminal Flagged Gle1 vector into chick embryos shows specific signals when immunostained while preliminary tests with the C-terminal Flagged Gle1 show an absence of signal. It is possible that a critical C-terminal function is blocked by the attached Flag sequence, and this again leads to the nonfunctional protein being degraded by subcellular machinery. More testing is required to demonstrate consistency. Eventually, it will be imperative to generate an antibody to Gle1 so as to avoid the problems with the Flag tag. This will allow the characterization of the native protein expression, and comparing the two data sets will be very helpful in parsing out the functions of Gle1 that require the full genomic DNA.

To date, Gle1 has been most heavily studied in yeast, which is a eukaryotic model obviously far removed from the complexity seen in vertebrate systems. In an effort to model Gle1-mediated motor neuron disease in a higher-order organism, we

electroporated both WT and mutant human Gle1 cDNA into chicks. The GFP-Gle1 fusion proteins did not appear to interfere significantly with the function of the endogenous chick Gle1, and at a gross level, motor neuron count and morphology appeared to be normal, with no obvious defects in motor neuron development. One possibility is that the GFP linked to the protein blocked any negative activity of the mutant protein, or prevent localization to its area of effect. The expression may also not have been at a high enough dose (i.e., if the lack of a phenotype in heterozygous humans is due to a half-dose of a toxic Gle1 protein). Finally, human and chick responses to the mutant protein may be different. Regardless, this result is consistent with the disease phenotype seen in humans being caused by a recessive mutation (*Nousiainen et al., 2008*). As mentioned above, we will perform further experiments using high-resolution imaging and labeling to assess whether mutant Gle1 may have acquired different interactions and subcellular localization compared with the WT protein. For example, double labeling experiments with NPC proteins can explore any differences in Gle1's activity at the nuclear envelope.

To examine Gle1 function in a mammalian system, we utilized an available Gene-trap mouse ES cell line with an allele which appears to consitute a loss-of-function mutation in Gle1. As seen in humans and WT chicks electroporated with mutant Gle1, mice heterozygous for the Gene-trap allele lack an obvious phenotype. We have yet to observe animals homozygous for the allele, which is in line with the key functions in NPC binding reported for the C-terminus of Gle1 that has been reported in human and yeast (*Kendirgi et al., 2003; Kendirgi et al., 2005*). As we are reasonably certain that the

Gle1 Gene-trap allele is homozygous lethal, it is important to determine exactly when the animals die. It is likely that Gle1 will be essential for the maintenance of all cells, as other RNA regulation factors such as SMN, and will cause lethality at the blastocyst stage when maternal contribution of the transcript/protein ceases (*Schrank et al., 1997*). We are currently attempting to genotype blastocysts and early-stage possible homozygous Gle1 Gene-trap embryos to determine when Gle1 function becomes critical. If homozygous blastocysts are found and can form ES cell lines, various experiments could be performed using the cells. However, as mentioned above, it is likely that homozygous Gle1 Gene-trap ES cell lines cannot be cultured, as functional Gle1 is probably essential to cell survival like SMN.

To avoid the early lethality seen when Gle1 is knocked out, we designed a partial rescue via expression of mouse Gle1 with the FinMajor mutation in the ROSA26 locus. This strategy has been successful in models of SMA, where the lethality of a full SMN knockout is circumvented by a partial rescue using a mutated form of human SMN, resulting in mice that die soon after birth from motor neuron loss (*Monani et al., 2005*). It is important to note that the noncoding regions of the genomic Gle1 DNA may play important roles in transcript and protein localization, as mentioned above. The viability of the ROSA26 rescued Gene-trap mouse lines will be critical in determining the importance of these untranslated regions. As the cDNA's are being knocked in without the UTR's, a normal phenotype will indicate that the regions are unnecessary, while a specific phenotype can help tease out cell-type specific functions for the regions. For example, neuronal development may be severely hindered, which could imply that the

UTR's are critical in the nervous system (e.g., they may specifically target Gle1 to motor neuron axons, where they could serve important functions). Alternatively, the attempted rescues may fail due to the underexpression of the protein from the ROSA26 locus. This is unlikely, as ROSA26 is known to allow moderate expression in conjunction with our promoter and our X-Gal/β-Gal staining suggests that Gle1 is expressed at relatively low levels (*Masui et al., 2005*).

The design of our rescue allows us to perform targeted knockouts of Gle1 to examine its effect both spatially and temporally. Cell-type-specific Cre mouse lines can be used to abolish Gle1 in motor neurons or supporting glia, which will allow us to examine cell-autonomous effects of Gle1. Cre expression at different time points will also let us determine when Gle1 activity is critical. We can also ask whether expression of the mutant protein in the background of a WT mouse leads to any disease symptoms, which we do not expect based on what is seen in humans and our chick experiments.

Along with *in vivo* studies, we hope to be able to generate viable ES cell lines from our FinMajor mutant rescue, which we can then differentiate into motor neurons to study any defects in motor neuron development or maintenance. A possible issue in our rescue strategy is that the expression level from the ROSA26 locus may be enough to compensate for a loss of function if the phenotype is dose-dependent. In this case, the cells expressing only the mutant protein may display no noticeable phenotype, and other experiments (such as RNAi in motor neurons) will be necessary to parse out the possibilities. Alternatively, a knock-in to the endogenous Gle1 locus may be explored.

To analyze the disease phenotypes seen in LCCS1 and LAAHD, it will be vital to determine exactly how motor neurons are affected when Gle1 is mutated. During development, defects might be seen in specification, axon guidance, synaptogenesis, or other areas. Alternatively, the neurons may form normally, but Gle1 may function in maintaining their function. Interestingly, Inositol hexakisphosphate kinase-2, which is involved in IP_6 metabolism, has been found to be an effector of the Hedgehog pathway in vertebrates (*Sarmah & Wente, 2010*). As mentioned earlier, IP_6 is a key cofactor in the interaction between Gle1 & DBP5, while the Hedgehog pathway is known to be critical in neural development in the spinal cord as well as playing a role in axon guidance (for review, see *Lee & Pfaff, 2001 and Charron & Tessier-Lavigne, 2007*). Future investigation may find a role for Gle1 in a converging pathway with Hedgehog signaling, which may be a specific role it plays in motor neurons. Additionally, Tran & Wente noted in their review an interesting resemblance between Dbp5-mediated mRNA export and Ran-mediated protein export from the nucleus, in that both are NTPase-refereed processes using various cofactors for seemingly analogous steps (*Tran & Wente, 2006, Nobel et al., 2011*). This may provide yet another hint in divining the role of Gle1 in motor neurons, and analysis of similarities between the mRNA- and protein-exporting processes may provide future directions in deciphering Gle1's functions. If the ROSA-targeting rescue strategy works as we hope, we can then use the mutant mouse as a model of LCCS1 and dissect the pathology of the disease. This would then give us insight into the function of Gle1 and hopefully lead toward a broader understanding of the importance of mRNA regulation in the nervous system.

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