Therapeutic intervention for genetic disease by the augmented recycling of target proteins

“Prevention of protein or mRNA degradation is a potential therapeutic intervention in diseases resulting from haploinsufficiency, including lissencephaly.”

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Lissencephaly is a severe human neuronal migration defect characterized by a smooth cerebral surface, thick cortex and dilated lateral ventricles, associated with mental retardation and seizures [1–3]. *LIS1* was the first gene cloned in an organism that was found to be important for neuronal migration, as it was deleted or mutated in patients with lissencephaly in a heterozygous fashion [4]. Studies in model organisms, particularly *Aspergillus nidulans* [5,6], as well as those in the mouse [7], have uncovered an evolutionarily conserved pathway that involves LIS1 and cytoplasmic dynein. Cytoplasmic dynein is a highly complex molecular motor that transports its attached cargo towards the minus-end of microtubules. These enzymes are required for many essential motile activities within the cytoplasm [8–10]. However, until recently the underlying mechanism by which LIS1 regulated cytoplasmic dynein was unknown. We recently reported that LIS1 holds cytoplasmic dynein on microtubules in an ‘idling state’, thereby making a stable complex of microtubule–LIS1–cytoplasmic dynein [11]. This microtubule–LIS1–cytoplasmic dynein complex is thought to be transported to the plus-end of microtubules by kinesin, which is essential for the maintenance of proper cytoplasmic dynein distribution within the cell. Interestingly, a substantial fraction of LIS1 is degraded by the cysteine protease, calpain, after reaching the plus-end of microtubules. This gave us an idea: could the levels of LIS1 protein be increased by inhibiting the calpain-mediated degradation of LIS1 at the cortex of the cell? If we were able to increase the levels of LIS1 protein in this fashion, would this result

in any therapeutic benefit? It turns out, using genetic mouse models with reduced levels of LIS1, that the answer to both questions is yes.

In a recently published study, we demonstrated that inhibition of calpain restored LIS1 protein to a normal level using mouse embryonic fibroblast cells or dorsal root ganglia neurons established from *Lis1*^{+/-} mice [12]. We further demonstrated that inhibition of calpain led to the rescue of the aberrant distribution of cytoplasmic dynein and intracellular components, including mitochondria and β -coat protein-positive vesicles in *Lis1*^{+/-} mouse embryonic fibroblast cells, and improved neuronal migration of *Lis1*^{+/-} cerebellar granular neurons. Intraperitoneal injection of a calpain inhibitor to pregnant *Lis1*^{+/-} dams rescued apoptotic neuronal cell death, reduction of brain weight and neuronal migration defects in *Lis1*^{+/-} offspring. Thus, we used our knowledge of the pathogenesis and mechanism of action of LIS1 and its pathway to directly assess the effect of inhibition of calpain with quantitative phenotypic measures *in vivo* and *in vitro*.

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This approach provides proof-of-principle for a potential therapeutic intervention to a severe developmental cortical dysplasia, lissencephaly. Although extensive work has been performed in order to understand the molecular and cellular biological basis of lissencephaly due to haploinsufficiency of *LIS1*, therapeutic strategies for lissencephaly have not been addressed. This is

probably because the treatment of lissencephaly is a daunting task for several reasons. First, in lissencephaly, early brain development is affected, and so treatment would be necessary throughout development. Second, all neurons throughout the brain would have to be treated, since lissencephaly results in global defects in brain development. Third, *LIS1* mutations in humans are *de novo*, presenting a problem of detection at an early enough time point to allow therapy to commence when it can still make a difference. Despite these difficulties, there are some advantages to considering how to treat lissencephaly resulting from *LIS1* haploinsufficiency. First, LIS1 protein is present because individuals have heterozygous loss of *LIS1*; therefore, one can consider augmenting the already present but low levels of LIS1 protein in affected individuals. In addition, there are dosage-dependent effects of LIS1, and so any augmentation of LIS1 protein levels will probably have a beneficial effect. Finally, a great deal is known about the pathogenesis and mechanism of action of LIS1 and its pathway; therefore, the effects of any therapeutic modality can be assessed directly with quantitative measures *in vivo* and *in vitro*.

“...calpain inhibitors hold promise for the treatment of neuromuscular and neurodegenerative diseases in which calpains have been shown to be upregulated, for example Parkinson’s disease and Duchenne muscular dystrophy.”

Modulation of calpain activity has already been attempted therapeutically. The calpains are a class of cellular cysteine proteases that require calcium and are functionally active at neutral pH [13,14]. Calpain activation can take place in two modes: controlled activation under physiological conditions, and hyperactivation under pathological conditions that involve sustained calcium overload. The known calpain inhibitors are small molecule compounds, suggesting that it is possible to deliver calpain inhibitors to the target tissue. The major first-generation calpain inhibitors tested as anticataract agents were the epoxysuccinyl peptides, which include E64 and its analogs. These inhibitors have electrophilic centers within their structure that form an irreversible covalent bond with the thiol group of the cysteine residue in the active site of calpain, thereby blocking its proteolytic action. Structural modification of E64 produced E64d,

which showed improved membrane permeability and is readily hydrolyzed to an active form by lens enzymes *in vivo*. Moreover, a significant impetus to these approaches was provided by the recently solved structures for calpain-2 [15,16]. The detailed structural and mechanistic information gleaned from these structures opened the door to structure-based calpain inhibitor design, and, in this capacity, led to the synthesis of mercapto acrylate derivatives. One promising compound is SNJ-1945 [17–19]. SNJ-1945 is highly permeable in the CNS, which was demonstrated by its effects on retinal or neuronal protection. Moreover, SNJ-1945 showed a high selectivity for calpains, with no significant effect on the activity of many other enzymes, such as inhibition of cysteine proteases and serine proteases, which reduced the viability of previously tested calpain inhibitors. Therefore, the usage of new-generation calpain inhibitors may provide a more feasible approach for therapeutic intervention in human disease.

Besides our study using calpain inhibition to partially rescue the neuronal migration phenotype of *Lis1* mutant mice, it was recently demonstrated that postnatal conditional expression of DCX, the protein product of an X-linked gene for lissencephaly, can reduce heterotopia and restore neuronal patterning in animal models [20]. Both laminar displacement of neurons and the size of subcortical band heterotopia are reduced upon delayed expression of DCX during early postnatal periods. These encouraging findings suggest that cortical neurons maintain competence for migration after birth, and that treatment of lissencephaly after birth from perinatal stages may also provide some therapeutic benefits for the treatment of lissencephaly. In such cases, a drug that can pass the BBB will be required. Penetration of the BBB by SNJ-1945 has previously been demonstrated, making it a good choice for potential therapeutic trials in neonates [19].

The use of protease inhibitors or other agents that can increase protein levels may be generally applicable as therapeutic approaches for other genetic disorders that result from haploinsufficiency. Haploinsufficiency is defined as a dominant phenotype in diploid organisms that are heterozygous for a loss-of-function allele. The total level of a gene product (a particular protein) produced by the cell is approximately half of the normal level, which is not sufficient to permit the cell to function normally. Many of the haploinsufficient mutations in humans are observed in transcription factors [21]. For example, mutations

in the gene encoding for the CREB-binding protein (CBP) result in Rubinstein–Taybi syndrome, a rare condition that occurs in one out of 125,000 births and is characterized by mental retardation and skeletal abnormalities [22]. Loss of function of *CBP* may therefore interfere with the transcriptional activation of target genes in two ways: by preventing recruitment to the promoter of the basal transcription machinery and by blocking chromatin remodeling. Marfan syndrome and Ehlers–Danlos syndrome type IV are clinical entities characterized by vascular abnormalities that result from mutations of structural components of the extracellular matrix – another example of haploinsufficiency [23,24]. The relevance of haploinsufficiency in human disease has become increasingly apparent, and any human diseases that result from haploinsufficiency are potential targets of our strategy to boost levels of the haploinsufficient protein. Prevention of protein degradation or inhibition of mRNA degradation will be an effective method to increase levels of mutated protein in each disease. In addition to treatment of lissencephaly, calpain inhibitors hold promise for the treatment of neuromuscular and neurodegenerative diseases in which calpains have been shown to be upregulated, for example Parkinson's disease and Duchenne muscular dystrophy [25]. Application of calpain inhibitors to Duchenne muscular dystrophy might have a more direct effect via prevention of degradation of utrophin protein that has similar function with dystrophin [26]. Calpain inhibitors

might prevent the degradation of utrophin, which may then ameliorate the progression of muscular wasting. Sporadic morbidity to neurodegenerative diseases, such as Parkinson's disease or Alzheimer's disease, might be attributable to hemizygous mutations of responsible genes. Prevention of protein or mRNA degradation will also be a potential strategy for therapeutic intervention in such cases.

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