Chapter 5
Conclusions & Future studies

Conclusions:

AAAS disease models: Three AAAS biallelic knockout cell lines and two homozygous point mutant cell lines were generated and validated in this study. The knockout clones were generated using a plasmid-based nuclease-assisted gene targeting strategy in human iPS cells. At a biallelic targeting efficiency of 3%, these knockout clones contained frameshift indels in one allele, introduced by NHEJ, and puro-resistant cassette in the other allele by homologous recombination with a targeting donor plasmid. The point mutant clones were generated in a protein-based nuclease-assisted gene targeting strategy by HDR using a symmetric ssODN. Two rounds of transfection were required to recover cells homozygous for the disease allele. HDR introducing the desired point mutation was more efficient when lower amount of the Cas9 protein was used. However, higher activity was seemingly demonstrated by larger amount of Cas9 probably because this larger Cas9 amount caused re-cleavage of the modified locus as well as secondary damage to the locus, resulting in more NHEJ indels. Unexpectedly, adding mutations in the PAM did not improve rates of HDR which could be explained by a poorer rate of incorporation of the ssODN when more mismatches to the target locus are present. In future experiments, this issue with the optimum amount of Cas9 to be used for cleavage can be addressed by titrating the amount of Cas9. This would potentially avoid frequent NHEJ indels as observed with higher amount of Cas9 or reduced activity if too low amount is used.

It is worth mentioning here that upon re-transfection, the damaged wild-type copy of the gene could use either the ssODN or the mutant allele as a repair template. This could be validated by carrying out further experiments using an ssODN that contains one or more silent mutations in its sequence (Skarnes & Koutsourakis, unpublished data). Presence of the silent mutation/s in the modified DNA strand would indicate that the damage was repaired using the ssODN oligo as the template, whereas their absence would indicate gene conversion using the mutant allele. Gene conversion was evidenced in this experiment by the presence of two homozygote wild-type clones which were obtained upon the second transfection of the heterozygotes. Besides, this is possible that these homozygous point mutant clones could represent the loss of one copy of *AAAS* gene. This issue can be addressed by conducting further Southern blotting experiments to confirm amplification from both the alleles and that the clones are not representing homozygosity superficially. Another approach that can be adapted to validate the presence of both the alleles is by attempting to correct the point mutation in the gene with an ssODN containing the wild-type sequence. Homozygous wild-type cells only among all the screened clones would indicate that an allele is missing in the disease model

clones. Contrarily, if heterozygotes with a mutant allele and a wild-type allele are present among the screened clones, it would indicate that both the alleles are present and that none was deleted during genome-editing.

All these AAAS disease models were validated as a complete knockout of the ALADIN protein which suggests that the disease is caused by null or complete loss-of-function mutations in the *AAAS* gene. An additional band was detected using the polyclonal antibody (Proteintech) used in this experiment which probably represents cross-reaction with an unrelated protein in the proteome. The origin of this band needs to be investigated further using monoclonal antibodies that recognize an epitope of ALADIN different to that recognized by the polyclonal antibody used in this experiment.

HTT disease model: From a screen of 192 clones, no clones containing the extended Q_{67} repeat fragment were recovered. This is most likely because the efficiency of homologous recombination was too low in unselected clones. With plasmid-based nuclease-assisted gene targeting, transient puromycin selection did not seem to sufficiently enrich for homologous recombination events. Also, in the protein-based strategy, none of the clones exhibited insertion of the extended fragment by homologous recombination. The protein delivery should have been better than the plasmid-based approach because proteins are more efficient in specific genome modification, probably due to the transient window of Cas9 activity to which each genome is exposed (Zuris et al. 2015). Cas9-RNP is expected to modify the genomic DNA almost immediately after delivery because, unlike with plasmid-based targeting, no expression of Cas9 is needed prior to cleavage. With plasmids, Cas9 expression is slow and it can take several days before any considerable level of genome modification can occur (Hu et al. 2016). Further experiments need to be conducted using cis-delivery of a drug selection cassette within the targeting plasmid containing the extended 67 Q-repeat fragment (Figure 5.1) which would potentially increase the chances of selecting the drug-resistant clones.

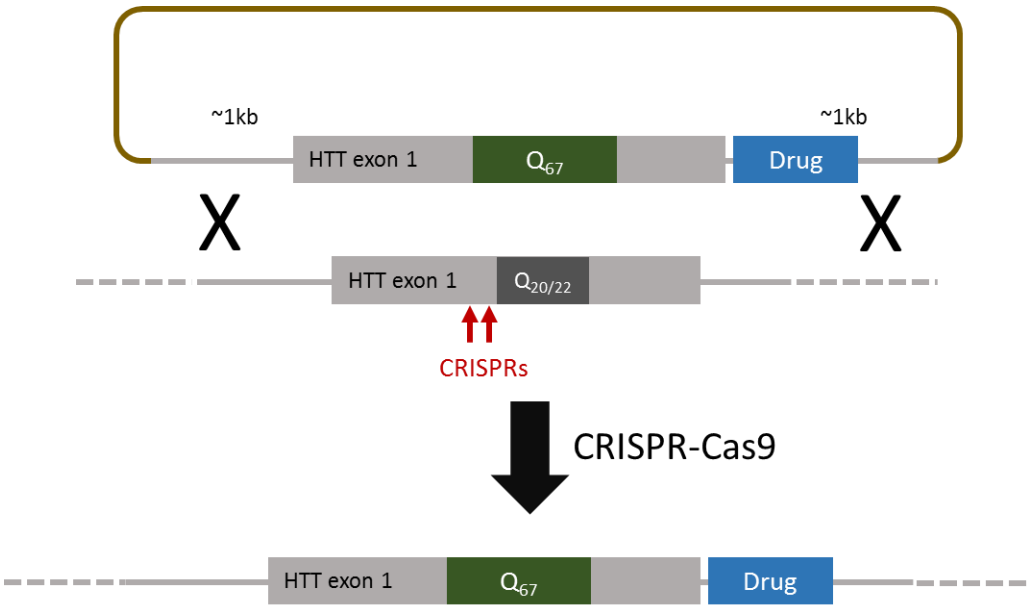

Figure 5.1: **Strategy for insertion of extended Q_{67}-repeat fragment into HTT exon 1 with cis-delivery of a drug selection cassette within the intron.** CRISPRs at which the homologous recombination occurs are indicated in red arrows. "X" indicates homologous recombination.

Also, multiple CRISPRs could be found that cleave the DNA on either side of the CAG repeat domain. Such approach would introduce multiple DSBs in the DNA and by deleting the repeat region, would increase the chances of homologous recombination between the damaged DNA and the donor plasmid.

Future studies:

The AAAS iPS cell models generated will enable us to gain a better understanding of the diverse functions of the ALADIN protein and the disease progression and pathophysiology of Triple-A syndrome. These cells can be compared to their respective isogenic wild-type control cells to identify phenotypic differences. Importantly, they can be differentiated to neurons and neural progenitor cells of the cerebral cortex, cerebellum, hippocampus, brainstem, and spinal cord, where the gene is abundantly expressed. Neural cells, affected by the disease mutations, will help us study the progressive neurological abnormalities like cognitive, motor, and sensory dysfunctions. In theory, these iPSC models can also be differentiated to adrenocortical cells, another major cell type affected in AAAS, to parasympathetic motor neurons, which mediate tear production (Dartt 2009), and to brainstem and vagal afferent neurons, which control oesophageal peristalsis (Richards & Sugarbaker 1995).

Since these *AAAS* mutants and wild-type cells are isogenic, the wild-type cells can provide us with appropriate controls to determine what other interacting genes or if any environmental factors could be confounding the phenotype of the diseased cells. Comparing the phenotypic patterns of iPSC models to patient-derived cells can also enable us gain insights into the effect of genetic background on cellular phenotypes. Moreover, these human cells complement mouse models which may not fully recapitulate the human AAAS abnormalities due to probable species-specific ALADIN functions. Further genome editing to create additional disease alleles will also help us unravel the genotype-phenotype correlation which is currently poorly understood. However, since phenotypic effects at the organism level cannot be observed with iPSC models, animal models are also needed which complement iPS cell models for studies of disease mechanisms (Merkle & Eggan 2013). An ideal animal model in many scientific studies is mouse, however, mouse models lacking the ALADIN protein have not been observed to exhibit the AAAS phenotype as humans do (Huebner et al. 2006). These mutant mice were indistinguishable from their wild-type siblings which could either be due to ALADIN functions in mice being different to that in humans or due to other external factors such as, environmental conditions or genetic modifiers. Thus, they are not suitable models to study this disease. However, other animal models genetically engineered to contain homozygous *AAAS* mutations could also be used for this disease. Such models could include primates as they are more evolutionary related to humans and have more common or shared genetic material. Thus, once the disease relevant phenotypes and their pathophysiology are identified and well-understood, these cell models can be used to perform drug screens and to develop novel therapeutics for treating this devastating disease.