

1 **AMYLOIDOGENIC PROTEINS DRIVE HEPATIC PROTEOSTASIS REMODELING IN AN INDUCED**  
2 **PLURIPOTENT STEM CELL MODEL OF SYSTEMIC AMYLOID DISEASE**

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5  
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## 9 **ABSTRACT**

0 Systemic amyloidosis represents a class of disorders in which misfolded proteins are secreted by effector organs  
1 and deposited as proteotoxic aggregates at downstream target tissues. Despite being well-described clinically,  
2 the contribution of effector organs such as the liver to the pathogenesis of these diseases is poorly understood.  
3 Here, we utilize a patient-specific induced pluripotent stem cell (iPSC)-based model of hereditary transthyretin  
4 (TTR) amyloid disease (ATTR amyloidosis) in order to define the contributions of hepatic cells to the distal  
5 proteotoxicity of secreted TTR. To this end, we employ a gene correction strategy to generate isogenic, ATTR  
6 amyloidosis patient-specific iPSCs expressing either amyloidogenic or wild-type TTR. We further utilize this gene  
7 editing strategy in combination with single cell RNAseq to identify multiple hepatic proteostasis factors, including  
8 many components of adaptive unfolded protein response (UPR) signaling pathways, whose expression  
9 correlates with the production of destabilized TTR variants in iPSC-derived hepatic cells. We further demonstrate  
0 that enhancing ER proteostasis within ATTR amyloidosis iPSC-derived hepatic lineages via stress-independent  
1 activation of aforementioned adaptive UPR signaling preferentially reduces the secretion of destabilized  
2 amyloidogenic TTR. Together, these results suggest the potential of the liver to chaperone-at-a-distance and  
3 impact pathogenesis at downstream target cells in the context of systemic amyloid disease, and further highlight  
4 the promise of UPR modulating therapeutics for the treatment of TTR-mediated and other amyloid diseases.

5

6

## 7 INTRODUCTION

8 Systemic amyloid disease represents a class of devastating protein folding disorders affecting over 1 million  
9 individuals worldwide (1-5). In these diseases, proteins containing destabilizing mutations are produced and  
0 secreted from an effector organ into circulation. In the blood, these proteins undergo misfolding and subsequent  
1 aggregation into toxic oligomers and amyloid fibrils, which deposit at distal target organs resulting in cellular  
2 death and organ dysfunction. Systemic amyloid diseases can result from the pathogenic misfolding of over 15  
3 structurally distinct proteins, a majority of which are synthesized by the liver. A prominent example of this family  
4 of diseases is hereditary transthyretin amyloidosis (ATTR amyloidosis).

5 Hereditary ATTR amyloidosis is a complex autosomal dominant disorder that can result from over 100  
6 possible mutations in the transthyretin (*TTR*) gene (6-9). In normal conditions, TTR is produced chiefly by the  
7 liver, where it forms a homotetramer and is then secreted, allowing for the transport of thyroxine and retinol  
8 binding protein throughout circulation (6-9). In patients with ATTR amyloidosis, TTR mutations decrease the  
9 stability of the tetramer and result in monomerization and subsequent misfolding of TTR variants. Amyloid-prone  
0 monomers then aggregate to form proteotoxic low-molecular weight oligomers and eventually hallmark  
1 congophilic amyloid fibrils at downstream target tissues including the heart and peripheral nerves (6-9). Current  
2 standards of care for patients with the disease include small molecule kinetic stabilizers tafamidis and diflunisal,  
3 which work to stabilize the tetrameric protein and limit monomerization and downstream fibril formation (10-14).  
4 Despite some success in clinical trials for both compounds and FDA approval for the use of tafamidis in the  
5 treatment of TTR-related cardiomyopathy, not all patients respond equally and effectively to these medicines.  
6 This can be attributed to the inherited deleterious *TTR* mutation as well as the underlying genetic background of  
7 the individual (10-15). Due to their multi-tissue etiologies and age-related trajectories, systemic amyloid diseases  
8 like ATTR amyloidosis have proven difficult to study in a physiologically-relevant way. At the same time, no  
9 mouse model currently recapitulates key aspects of human TTR amyloid pathology (16-20). To better understand  
0 disease pathogenesis in the genetic context of affected patients, we leveraged our previously described, patient-  
1 specific induced pluripotent stem cell (iPSC)-based model for studying the disease. In this system, TTR amyloid  
2 disease-specific iPSCs are differentiated into effector hepatocyte-like cells (HLCs) that produce and secrete  
3 destabilized, amyloidogenic mutant TTR that can then be used to dose a myriad of iPSC-derived, disease-  
4 associated target cells (18-20).

5 Traditionally, the livers of ATTR amyloidosis patients have been thought to be unaffected throughout  
6 disease pathogenesis, as toxicity occurs at downstream target organs such as the heart and peripheral nerves  
7 (1-9). Despite this however, recent studies suggest the capacity for the liver to contribute to the deposition of  
8 amyloidogenic proteins at distal target tissues. In line with this, recipients of domino liver transplantations (DLTs),  
9 where an individual in end-stage liver failure receives a liver from an ATTR amyloidosis patient, show accelerated  
0 TTR amyloid disease pathogenesis, wherein TTR fibrils accumulate on target organs in fewer than 10 years (21-  
1 26). Furthermore, *in vivo* mouse experiments have shown that the deposition of TTR on the hearts of old mice  
2 correlates with altered expression of numerous genes in the liver associated with the regulation of hepatic  
3 proteostasis (27). These results implicate the liver in the pathogenesis of systemic amyloid diseases such as  
4 ATTR amyloidosis. *Despite these observations however, the molecular and cellular changes in the liver that*  
5 *contribute to the toxic aggregation of misfolded TTR in distal tissues remains unclear.*

6 Interestingly, significant evidence highlights an important role for endoplasmic reticulum (ER)  
7 proteostasis in regulating the secretion and subsequent aggregation of amyloidogenic proteins such as TTR (28-  
8 32). The capacity of the ER proteostasis environment to secrete destabilized, aggregation-prone TTR variants  
9 has been directly implicated in the onset and pathogenesis of ATTR amyloidosis-related disorders. Furthermore,  
0 activation of adaptive IRE1/XBP1s or ATF6 arms of the unfolded protein response (UPR) – the predominant  
1 signaling pathway responsible for regulating ER proteostasis – have been shown to reduce the secretion and  
2 subsequent aggregation of structurally diverse amyloidogenic proteins (including TTR) (29, 31). Despite this, the  
3 physiological importance of ER proteostasis and the UPR in ATTR amyloidosis remains poorly defined.

4 Here, we utilize our laboratory's previously described patient-specific iPSC-based model of ATTR  
5 amyloidosis to investigate the contribution of proteostasis and hepatic disease modifying factors to the distal  
6 toxicity observed in patients with the disease. By utilizing gene editing in combination with single cell RNA  
7 sequencing (scRNAseq), we define distinct transcriptional profiles in syngeneic corrected and uncorrected ATTR  
8 amyloidosis iPSC-derived HLCs that correlate with expression of a destabilized, amyloidogenic TTR variant.  
9 Through these efforts, we show that expression of the most proteotoxic, destabilized, disease-associated TTR  
0 mutant in HLCs increases expression of genes and pathways inversely implicated in the toxic extracellular  
1 aggregation of TTR, including transferrin and UPR target genes. To assess the consequence of functional  
2 activation of adaptive UPR signaling within hepatic cells expressing mutant TTR, we generated an ATF6-

3 inducible patient-specific iPSC line. We further utilize this tool to demonstrate that exogenous ATF6 activation  
4 preferentially reduces the secretion of mutant, amyloidogenic TTR relative to the wild-type protein.

5 Herein, we demonstrate that hepatic expression of amyloidogenic TTR results in differential expression  
6 of proteostasis factors known to protect the extracellular space from toxic protein aggregation.

## 8 RESULTS

9 *TTR is a top differentially expressed gene throughout human hepatic specification.*

0 Recent work from our group demonstrated the emergence of a stage-dependent disease signature of hepatic-  
1 specified pluripotent stem cells (PSCs) (33). In these experiments, microarray analyses were performed on cells  
2 isolated at days 0, 5, and 24 of hepatic differentiation. Post-hoc analysis of this data revealed that *TTR* was the  
3 second most differentially expressed gene comparing differentiated HLCs to PSCs (**Fig. 1A**). To confirm this,  
4 qRT-PCR was performed on RNA isolated from day 24 HLCs, demonstrating significant upregulation of *TTR* as  
5 compared to undifferentiated iPSCs (**Fig. 1B**). These data demonstrate that *TTR* is a robust marker of hepatic  
6 specification and can be used to normalize stem cell-derived hepatic differentiations.

7  
8 *Gene editing of ATTR amyloidosis iPSCs eliminates secretion of mutant TTR<sup>L55P</sup> and decreases target cell  
9 toxicity.*

0 As noted above, *TTR* is one of the most differentially expressed genes in HLC differentiations, suggesting it  
1 might serve as an excellent candidate locus to target and generate a hepatic specification reporter iPSC line. To  
2 this end, we employed TALEN-mediated gene editing to manipulate an iPSC line derived from a patient with the  
3 Leu55 →Pro (*TTR*<sup>L55P</sup>) mutation, the most proteotoxic disease-causing variant (34, 35). In order to implement a  
4 broadly applicable gene correction strategy, we targeted the ATG start site of the endogenous, mutant *TTR*  
5 allele, introducing a normal *TTR* coding sequence, followed by a 2A self-cleaving peptide and *eGFP* coding  
6 sequence (**Fig. 1C**). Inclusion of a 2A peptide allows for transcription of a single mRNA that ultimately results in  
7 two independent *TTR* and *GFP* proteins via a post-translational cleavage event. As a result of this targeting  
8 methodology, transcription and translation of mutant *TTR* is abrogated via introduction of an artificial STOP  
9 codon and polyA sequence, and replaced with a normal *TTR* coding sequence (**Fig. 1C**). Importantly, this  
0 universal gene correction strategy provides a singular technique for correcting all known *TTR* genetic lesions

1 while simultaneously obviating concerns regarding haploinsufficiency via replacement of the endogenous mutant  
2 *TTR* allele with a wild-type copy. (Additional information regarding generation and characterization of corrected  
3 iPSCs can be found in **Supplemental Fig. 1.**)

4 Using this *TTR* reporter line, we then measured the kinetics of GFP expression throughout HLC  
5 differentiation by flow cytometry. In doing so, we found that expression of GFP peaked at approximately day 16  
6 of a 24-day specification protocol (**Fig. 1D**). By day 24 of the differentiation, HLCs exhibited cobblestone-like  
7 morphology, and the majority of cells expressed GFP (**Fig. 1E**). To further validate this reporter line and ensure  
8 that GFP expression correlated with the expression of *TTR* as well as other hepatic-specification markers, day  
9 16 GFP<sup>+</sup> HLCs were sorted and assayed via qRT-PCR. GFP<sup>+</sup> cells expressed high levels of *TTR*, as well as  
0 other hepatic specification markers such as *AAT* and *ALB* (**Fig. 1F**), suggesting that our corrected reporter cell  
1 line labels maturing hepatic-lineage cells during specification.

2 We further examined the ability of this strategy to eliminate the production of destabilized, disease-  
3 causing *TTR*, as well as alleviate downstream toxicity (**outlined in Fig. 2A**). Normal, corrected, and non-  
4 targeted, heterozygous *TTR*<sup>L55P</sup> iPSCs were differentiated into HLCs. Conditioned supernatant from each line  
5 was harvested after culturing cells for 72 hours in hepatic specification media beginning on day 16 of the  
6 differentiation. We used liquid chromatography (LC)/mass spectrometry (MS) to show that *TTR* immunopurified  
7 from normal hepatic supernatants contained *TTR*<sup>WT</sup>, but not *TTR*<sup>L55P</sup>, while supernatants collected from patient  
8 iPSC-derived HLCs contained both *TTR*<sup>WT</sup> and *TTR*<sup>L55P</sup> (**Fig. 2B**). Supernatant from corrected iPSC-derived  
9 HLCs revealed complete elimination of *TTR*<sup>L55P</sup>, while levels of *TTR*<sup>WT</sup> remained unperturbed (**Fig. 2B**).  
0 Importantly, the two amino acid overhang on the N-terminal portion of *TTR*, resulting from the post-translational  
1 cleavage of the 2A peptide, was removed with the *TTR* signal peptide through normal protein processing in the  
2 ER, evident by the identical molecular weights observed for endogenous and exogenous *TTR*<sup>WT</sup>. This shows  
3 that *TTR*<sup>WT</sup> from our donor construct is indistinguishable from the endogenous protein.

4 Many studies have shown that decreasing circulating levels of destabilized *TTR* species, as in the case  
5 of liver transplantation and our novel gene editing strategy, results in decreased peripheral organ dysfunction  
6 (36-38). Therefore, we sought to determine the efficacy of our iPSC-based gene correction methodology in  
7 decreasing toxicity in a cell-based model. To accomplish this, a neuroblastoma cell line (SH-SY5Y) was dosed  
8 with conditioned supernatant generated from mutant *TTR*<sup>L55P</sup>, corrected, or normal HLCs, and surveyed for

9 toxicity. In these assays, SH-SY5Y cells dosed with mutant hepatic supernatant displayed an increase in PI<sup>+</sup>  
0 cells compared to those dosed with normal supernatant (**Fig. 2C**). Cells dosed with corrected supernatant,  
1 however, exhibited a decrease in toxicity comparable to levels observed in the normal control sample (**Fig. 2C**).  
2 These results suggest that the proposed gene correction strategy ameliorates TTR-mediated toxicity via  
3 reductions in the hepatic secretion of destabilized TTR.

4  
5 *Single cell RNA sequencing reveals a novel hepatic gene signature associated with the production of*  
6 *destabilized TTR<sup>L55P</sup>.*

7 Historically, it has been thought that the livers of patients with ATTR amyloidosis are unaffected during disease  
8 pathogenesis (1-9). Recent work however, calling into question the use of donor organs from ATTR amyloidosis  
9 patients for DLT procedures challenges this notion (21-26). Furthermore, evidence highlights a significant  
0 potential role for the liver in regulating the extracellular aggregation and distal deposition of TTR implicated in  
1 ATTR amyloidosis disease pathogenesis (27). Collectively, these results indicate that genetic or aging-related  
2 perturbations to the liver could influence the toxic extracellular aggregation and deposition of TTR aggregates  
3 on peripheral target tissues.

4 In order to define specific hepatic proteins and pathways associated with the production of destabilized,  
5 amyloidogenic TTR variants, we coupled our TTR reporter system with single cell RNA sequencing (scRNAseq)  
6 to compare mRNA expression profiles in syngeneic iPSC-derived HLCs with or without the TTR<sup>L55P</sup> mutation. In  
7 addition to our corrected TTR reporter iPSC line, we also constructed a reporter cell line where our TTR-GFP  
8 donor construct was targeted to the wild-type TTR allele in the same TTR<sup>L55P</sup> parental iPSC line. As a result, we  
9 created two syngeneic, TTR-promoter driven hepatic-specification reporter iPSC lines, where the only difference  
0 is the presence or absence of the disease causing TTR<sup>L55P</sup> mutation (henceforth referred to as uncorrected and  
1 corrected cells, respectively). To compare HLCs +/- TTR<sup>L55P</sup>, uncorrected and corrected reporter iPSCs were  
2 subjected to our hepatic specification protocol until TTR expression had plateaued (at day 16 of the  
3 differentiation) (**Fig. 1D**). To control for the inherent heterogeneity of iPSC differentiations, GFP<sup>+</sup> cells were  
4 purified by FACS to select for cells undergoing hepatic-specification (i.e. at similar stages in their developmental  
5 trajectories). Transcriptomic profiling was subsequently performed at single cell resolution via the Fluidigm C1  
6 platform (**outlined in Fig. 3A**).

7 Day 16 uncorrected and corrected HLCs formed clear and distinct groups by supervised principal  
8 component analysis (PCA), with 92 genes differentially expressed between the two groups (**Fig. 3B-3D**,  
9 **Supplemental Data File 1**) (significance determined via one-way ANOVA, FDR cutoff <0.05). These analyses  
0 identified increased expression of distinct genes and pathways previously shown to influence extracellular  
1 aggregation of destabilized TTRs in uncorrected but not corrected HLCs (*vide infra*) (28-32).

2  
3 *Transferrin expression is significantly increased in uncorrected HLCs and may represent a novel chaperone for*  
4 *destabilized TTR.*

5 The top differentially expressed gene in uncorrected HLCs is the iron transporter, transferrin (TF) (**Fig. 3C, 3D**).  
6 Although TF is a known hepatic lineage marker, no other hepatic markers (including: *TTR*, *ALB*, *AFP*, *HN4A*,  
7 *FOXA1*, *GATA4*, *SERPINA1*, *FGB*, *DUOX2*, *A2M*, *TGM2*, *HAVCR1*, *GATA6*) are differentially expressed  
8 between corrected and uncorrected HLCs, suggesting that the differential expression of *TF* is not simply due to  
9 the differentiation status of individual lines (**Fig. 3D** and **Supplemental Fig. 2**).

0 Interestingly, previous studies have demonstrated the ability of TF to act as a chaperone in the context  
1 of other amyloid disorders such as Alzheimer's Disease (AD) as well as to physically interact with TTR fibrils *in*  
2 *vivo* (39). As a result, we sought to assess the capacity for TF to act as a novel chaperone for misfolding TTR  
3 and in turn prevent TTR fibril formation. To this end, we performed an *in vitro* fibril formation assay whereby the  
4 formation of congophilic fibrils from bacterial-derived, recombinant TTR<sup>L55P</sup> was assessed with or without the  
5 addition of TF (**Fig. 4A**). In doing so, iron-free TF (apo-transferrin) at physiologically-relevant concentrations (30  
6 μM) reduced the amount of congophilic species formed by approximately 60% (**Fig. 4B**). This suggests that the  
7 increased expression of TF in HLCs containing mutant TTR<sup>L55P</sup> could represent a mechanism to suppress  
8 aggregation-associated toxicity.

9  
0 *Uncorrected HLCs show increased activation of protective UPR-associated signaling pathways.*

1 Apart from *TF*, our scRNAseq analysis also identified increased expression of multiple UPR-regulated ER  
2 proteostasis factors (e.g., *HYOU1* and *EDEM2*; **Fig. 3C, 3D**) in iPSC-derived HLCs expressing TTR<sup>L55P</sup>. These  
3 genes play important roles in regulating proteostasis within the ER. Thus, the increased expression of these ER



4 proteostasis factors suggests that the presence of the destabilized TTR<sup>L55P</sup> protein challenges the ER  
5 proteostasis environment and in turn activates the UPR.

6 Interestingly, ER stress and UPR activation can influence the toxic extracellular aggregation of  
7 amyloidogenic TTR mutants implicated in ATTR amyloidosis disease pathogenesis (28-32). Chemical toxins that  
8 induce severe, unresolvable ER stress in mammalian cells decrease the population of amyloidogenic TTR  
9 secreted as the native TTR tetramer and instead increase TTR secretion in non-native conformations that rapidly  
0 aggregate into soluble oligomers implicated in ATTR amyloidosis disease pathogenesis (28, 40). In contrast,  
1 enhancing ER proteostasis through the stress-independent activation of the adaptive UPR-associated  
2 transcription factor ATF6, selectively reduces the secretion and subsequent aggregation of destabilized,  
3 amyloidogenic TTR variants (29-32). This suggests that activation of adaptive UPR signaling pathways  
4 independent of severe ER stress is a protective mechanism to suppress the secretion and toxic aggregation of  
5 destabilized, amyloidogenic TTR mutants.

6 In order to better define the impact of TTR<sup>L55P</sup> expression on ER stress and UPR activation, we used  
7 gene set enrichment analysis (GSEA) to define the extent of UPR pathway activation in our uncorrected iPSC-  
8 derived hepatic lineages. Notably, this analysis revealed modest activation of the adaptive IRE1/XBP1s and  
9 ATF6 UPR transcriptional signaling pathways, with no significant activation of the pro-apoptotic PERK UPR  
0 pathway (**Fig. 4C**). We confirmed IRE1/XBP1s activation in two independent patient-specific iPSC-derived HLCs  
1 by monitoring IRE1-dependent *XBP1* splicing (**Fig. 4D-F**). As a positive control, cells were dosed with global  
2 UPR activator thapsigargin. These results show that expression of destabilized, amyloidogenic TTR<sup>L55P</sup> promotes  
3 adaptive remodeling of ER proteostasis through the IRE1/XBP1s and ATF6 UPR signaling pathways.

4  
5 *Hepatic activation of ATF6 signaling selectively reduces secretion of destabilized TTR<sup>L55P</sup>.*

6 We next determined the consequence of functional activation of adaptive UPR-associated signaling pathways  
7 in ATTR amyloidosis patient-specific iPSC-derived hepatic cells expressing mutant, destabilized TTR. To  
8 accomplish this, we introduced an ATF6-inducible donor construct into our previously described heterozygous  
9 TTR<sup>L55P</sup> patient-specific iPSC line. In these cells, the coding sequence for the active N-terminal bZIP transcription  
0 factor domain of ATF6 is fused to a destabilized dihydrofolate reductase (DHFR) tag as previously described  
1 (32). In the absence of chemical chaperone trimethoprim (TMP), the DHFR.ATF6 protein product is targeted for

2 degradation via the ubiquitin proteasome system (**Fig. 5A**). Upon administration of TMP, the DHFR domain is  
3 stabilized, allowing dosable, stress-independent activation of ATF6 transcriptional activity (**Fig. 5A**). ATF6-  
4 inducible iPSCs were differentiated into HLCs and subsequently dosed with TMP, beginning on day 15 of hepatic  
5 specification. Administration of TMP induced selective expression of the ATF6 target genes *HSPA5* and  
6 *HERPUD1*, but not an IRE1/XBP1s target gene (e.g., *ERDJ4*) or PERK target gene (e.g., *GADD34*) (**Fig. 5C**,  
7 **5D**), confirming selective TMP-dependent ATF6 activation in these HLCs. We then collected conditioned media  
8 for 72 hours on patient iPSC-derived HLCs dosed with or without TMP and monitored the relative populations of  
9  $TTR^{WT}$  and  $TTR^{L55P}$  by mass spectrometry. We initially showed that the relative recovery of  $TTR^{L55P}$  from IPs of  
0 media prepared on HLCs treated with TMP was reduced relative to  $TTR^{WT}$ , suggesting reduced secretion of this  
1 destabilized TTR variant induced by stress-independent ATF6 activation (**supplemental Fig. 3A, 3B**). To better  
2 quantify this reduction, we performed Tandem Mass Tag (TMT) / LC-MS/MS quantitative proteomics to directly  
3 monitor the relative amounts of peptides comprising  $TTR^{WT}$  or  $TTR^{L55P}$  in these conditioned media (**Fig. 5E**).  
4 Using this quantitative approach, we show that TMP-dependent ATF6 activation preferentially reduces levels of  
5 destabilized  $TTR^{L55P}$  25% relative to  $TTR^{WT}$  in HLC conditioned media. This demonstrates that stress-  
6 independent ATF6 activation selectively reduces secretion of destabilized, amyloidogenic  $TTR^{L55P}$  in patient  
7 iPSC-derived HLCs. We moreover found that branch-specific activation of ATF6 signaling protects iPSC-derived  
8 HLCs from morphological defects resulting from prolonged exposure to severe ER stress via addition of  
9 thapsigargin (**supplemental Fig. 4A, 4B**).

## 1 DISCUSSION

2 Herein, we describe the utilization of gene editing technology in ATTR amyloidosis patient-specific iPSCs in  
3 order to uncover a novel human hepatic gene signature resulting from the expression of destabilized, misfolding-  
4 prone TTR. Historically, the livers of patients with ATTR amyloidosis were thought to be unaffected in disease  
5 pathogenesis (1-6). This is best highlighted through the employment of DLTs, wherein the livers of patients with  
6 ATTR amyloidosis are removed and given to individuals in end-stage liver failure. Despite routine use of these  
7 transplants, recent clinical data demonstrates that non-ATTR amyloidosis DLT recipients not only go on to  
8 develop amyloidogenic TTR fibrils (and disease), but they do so at a median time of only 7.5 years post-  
9 transplant (21-26). While these clinical observations suggest a role for the liver in contributing to pathogenesis

0 of ATTR amyloidosis and potentially other systemic amyloid diseases, the cellular and molecular mechanisms  
1 for this phenomenon remain unknown.

2 Through the use of gene editing and scRNAseq, we defined distinct transcriptional profiles for hepatic  
3 cells expressing destabilized TTR<sup>L55P</sup>. We hypothesized that hepatic production of destabilized TTRs results in  
4 the upregulation of stress-responsive proteostasis factors that regulate the secretion and subsequent  
5 aggregation of destabilized TTR variants such as TTR<sup>L55P</sup>. Our scRNAseq experiment revealed that uncorrected  
6 hepatic cells exhibited differential expression of 92 genes compared to syngeneic hepatic cells where the only  
7 difference is the absence of expression of the mutant TTR. In line with our rationale, we identified many instances  
8 in which mutant hepatic cells upregulated expression of well-documented ER stress-associated proteostasis  
9 factors involved in regulating protein secretion (e.g., *HYOU1*, *EDEM2*).

0 Interestingly, *TF* was found to be the most upregulated gene in uncorrected, TTR<sup>L55P</sup>-expressing hepatic  
1 cells. Despite limited prior evidence for TF as a chaperone for misfolded TTRs, recent work implicates its  
2 chaperone capacity in other amyloid disorders such as Alzheimer's Disease (AD), noting increased protein-level  
3 expression in the prefrontal cortices of AD patients compared to elderly, non-diseased individuals (41). Moreover,  
4 recent work demonstrated the ability of TF to physically interact with and prevent the self-assembly and toxicity  
5 of amyloid  $\beta$  peptide (A $\beta$ ) oligomers, the amyloidogenic protein species in AD (42, 43). At the same time, recent  
6 *in vivo* data have demonstrated physical interactions between TF and TTR amyloid fibrils (39). Through the use  
7 of congophilic fibril formation assays, we demonstrated that iron-free TF at physiologically relevant levels,  
8 decreased *in vitro* TTR<sup>L55P</sup> fibril formation by approximately 60%, implicating TF as a novel chaperone for hepatic  
9 TTRs. These observations, together with our scRNAseq and biochemical data, suggest the possibility that TF  
0 plays a similar protective role in ATTR amyloidosis. In this model, hepatic cells producing mutant TTR may  
1 express higher levels of TF to prevent toxicity and/or fibril formation.

2 In addition to the differential expression of known and novel chaperone genes, we noted activation of the  
3 adaptive arms of the UPR (ATF6 and IRE1/XBP1s) in hepatic cells expressing mutant TTR. Together, these  
4 data indicate that the expression of TTR<sup>L55P</sup> in iPSC-derived HLCs does not induce severe ER stress, but  
5 suggests that the activation of adaptive IRE1/XBP1s and ATF6 signaling observed in these cells reflects a  
6 protective mechanism to suppress secretion and subsequent aggregation of the destabilized TTR<sup>L55P</sup> protein.  
7 Consistent with this, our IP-based LC-MS analysis of conditioned media from uncorrected HLCs showed that

8 TTR<sup>L55P</sup> levels were approximately 30% that of TTR<sup>WT</sup> (**Fig. 2B**). This result mirrors the lower levels of  
9 destabilized TTR mutants (as compared to wild-type TTR) observed in conditioned media prepared on hepatic  
0 cells expressing both variants (18-20, 28-32).

1 Our scRNAseq experiments demonstrate dynamic activation of proteostasis transcriptional networks  
2 consisting of upregulation of chaperones as well as functional activation of adaptive UPR-associated signaling  
3 pathways in mutant HLCs. Due to the gradual nature of the deposition of TTR aggregates and fibrils, with disease  
4 manifesting clinically around the 5<sup>th</sup> or 6<sup>th</sup> decade of life, ATTR amyloidosis is widely considered an aging-related  
5 disorder. It is well-understood that proteostasis factors and the ability to cope with the production of misfolded  
6 proteins decreases with age, while similarly, iron has been shown to increase in a number of organs throughout  
7 aging (41-46). Interestingly, since UPR signaling declines during normal aging (47-50), the presence of adaptive  
8 UPR signaling in the above-mentioned HLCs could reflect protective biologic pathways whose activity also  
9 decline during the aging process. Aging-dependent reductions in adaptive UPR signaling could exacerbate  
0 TTR<sup>L55P</sup>-associated ER stress and increase secretion of TTR in non-native conformations that facilitate toxic  
1 extracellular aggregation. Thus, monitoring changes in hepatic UPR activation and/or conformational stability of  
2 circulating TTR tetramers could reflect a potential biomarker to monitor progression of TTR amyloid disease  
3 pathogenesis (51) – a notoriously difficult disorder to diagnose.

4 Together, these results indicate that the expression of a destabilized, aggregation-prone protein  
5 upregulates proteostasis factors as well as functionally activates adaptive UPR-associated signaling pathways  
6 in ATTR amyloidosis patient-specific iPSC-derived hepatic cells. Moreover, we demonstrated that inducible  
7 activation of ATF6 signaling in these cells resulted in a ~25% reduction of destabilized TTR<sup>L55P</sup> while wild-type  
8 levels appeared unaffected. Despite a relatively modest decrease, this reduction could represent a shift toward  
9 stability where properly folded, mutant TTRs form stabilized heterotetramers with wild-type TTRs (52).

0 Conventional therapeutics for many systemic amyloid diseases involve decreasing circulating levels of  
1 the amyloidogenic protein. In the case of ATTR amyloidosis, for example, liver transplantation relies upon  
2 eliminating circulating levels of mutant TTR. Similarly, emerging RNAi-based therapeutics are being developed  
3 to target and eliminate wild-type and mutant TTR transcripts within liver tissue (53-56). Activating ATF6 signaling  
4 in ATTR amyloidosis hepatic cells could result in a therapeutic decrease in the secretion of misfolding-prone  
5 TTRs from the liver and thus a decrease in extracellular deposition of proteotoxic aggregates as distal target

6 tissues. Recent studies have demonstrated that stress-independent, selective activation of ATF6 signaling is  
7 achievable via addition of small molecules (29, 32, 57). At the same time, upregulation of ATF6 signaling is  
8 relatively tolerated in humans with hyperactivating mutations (38, 39, 57). Future work will aim to further the  
9 development of small molecule-based ATF6-modulating compounds for the treatment of systemic amyloid  
0 diseases such as ATTR amyloidosis.

1 Results from these experiments challenge the long-held notion that the livers of patients with ATTR  
2 amyloidosis are unaffected by the disease and do not contribute to pathogenesis. Through the use of our novel  
3 iPSC-based model, our work demonstrates that expression of amyloidogenic TTR results in transcriptional and  
4 functional changes in ATTR amyloidosis hepatic cells. Moreover, these data suggest that the liver employs  
5 protective mechanisms via adaptive UPR-associated signaling pathways in order to cope with the production of  
6 misfolding-prone TTRs. Furthermore, this work demonstrates that modulation of UPR-associated ATF6 signaling  
7 results in a selective decrease in the secretion of misfolded proteins in patient-specific iPSC-derived hepatic  
8 cells, and could potentially represent a broadly applicable therapeutic strategy for complex and diverse systemic  
9 amyloid disorders.

## 1 **MATERIALS AND METHODS**

### 2 *Patient samples.*

3 To capture the large phenotypic diversity of patients with ATTR amyloidosis, samples were procured from the  
4 Amyloidosis Center of Boston University School of Medicine. Reprogramming of material was performed on fresh  
5 samples immediately following collection or using frozen mononuclear cells that were previously collected and  
6 isolated from subjects.

### 8 *iPSC generation and maintenance.*

9 Derivation of our patient-specific iPSCs was performed as described (58-60). For all studies described here,  
0 cells were maintained either on inactivated mouse embryonic fibroblast (MEF) feeders with KSR supplemented  
1 media or under feeder-free conditions using mTeSR1 media. These studies were approved by the Institutional  
2 Review Board of Boston University.

4 *Post-hoc microarray analysis.*

5 The heatmap and differential expression analysis was performed using data from Wilson et al. 2015. Differential  
6 expression between T24 and T0 of the hepatic differentiation was performed, resulting in 12,129 differentially  
7 expressed genes (FDR<0.05). The heatmap was generated using Ward's hierarchical clustering method on the  
8 row-scaled expression values of these genes.

9  
0 *TALEN-mediated gene editing of patient-specific iPSCs.*

1 Cells were transfected via Lipofectamine with PLUS reagent (ThermoFisher, Cat. Nos. 11668019, 11514015).  
2 Briefly, iPSCs were cultured until ~60% confluent in a 6-well plate. 1.2 µg of left and right TALEN targeting  
3 vectors and 3 µg of donor vector were added to cells. 700 ng/µL puromycin selection was then performed.  
4 Puromycin cassette excision was accomplished using transient transfection of the pHAGE-EF1α-Cre-IRES-Neo  
5 plasmid followed by subsequent screening and single cell selection and expansion.

6  
7 *Directed differentiation of iPSCs to hepatocyte-like cells.*

8 iPSCs were specified to the hepatic lineage via a 2D feeder-free, chemically-defined differentiation protocol as  
9 previously described (18-20, 61, 62).

0  
1 *RNA isolation, cDNA synthesis, and qRT-PCR.*

2 RNA was extracted from cells via RNeasy Mini Kit (Qiagen, Cat. No. 74104). RNA was eluted in RNase-free  
3 H<sub>2</sub>O and further purified via treatment with DNA removal kit (ThermoFisher, Cat. No. AM1906). ~1 µg purified  
4 RNA was used to generate cDNA via High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Cat.  
5 No. 43-688-13). qRT-PCR was performed with TaqMan Universal Master Mix II, with UNG (ThermoFisher, Cat.  
6 No. 4440038). TaqMan Gene Expression Assays used include the following: β-actin (Hs99999903\_m1), AAT  
7 (Hs00165475\_m1), ALB (Hs00609411\_m1), ERDJ4 (Hs01052402\_m1), HERPUD1 (Hs01124269\_m1), HSPA5  
8 (Hs00607129\_gH), GADD34 (Hs00169585\_m1), and TTR (Hs00174914\_m1). Quantities of genes of interest  
9 were compared relative to β-actin levels. Fold-change was calculated via  $\Delta\Delta C_T$  method. Undetermined  $C_T$  values  
0 were taken to be 40. Samples were run in technical duplicate.

2 *IP-MS analysis of secreted TTR.*

3 TTR was immunoprecipitated from conditioned media prepared on iPSC-derived hepatic lineages, as previously  
4 described (20). Briefly, we incubated 5 mL of conditioned media overnight at 4°C with cyanogen bromide-  
5 activated sepharose covalently conjugated to a rabbit polyclonal anti-TTR antibody (a kind gift from Jeff Kelly,  
6 TSRI). We then washed the beads four times with 10 mM Tris pH 8.0, 140 mM NaCl, 0.05% saponin and one  
7 time with 10 mM Tris pH 8.0, 140 mM NaCl. TTR was then purified by incubating the beads with 100 µL of 100  
8 mM triethylamine pH 11.5 for 30 min at 4°C. LC/MS analysis was then performed on an Agilent single quadrupole  
9 mass spectrometer, as previously described (20).

1 *Determining downstream neuronal toxicity in response to iPSC-derived HLC supernatant.*

2 Conditioned HLC supernatant was generated by incubating hepatic differentiation media on day 16 HLCs for 72  
3 hours. Supernatant was collected and subsequently concentrated using Centrifugal Filter Units (Millipore Sigma,  
4 Cat. No. UFC901024). (After first collection, cells were refed with media for an additional 72 hours to generate a  
5 second batch of conditioned supernatant.) Supernatant was first centrifuged at 200 x g for 1 minute at room  
6 temperature to remove cell debris. Media was then collected, transferred to filter units, and spun at 2140 x g for  
7 45 minutes at room temperature. Concentrated supernatant was subsequently stored at 4°C until dosing  
8 experiment. SH-SY5Y cells were plated at  $2 \times 10^5$  cells and subsequently dosed for ~7 days with media composed  
9 of SH-SY5Y growth media and conditioned supernatant at a 1:1 ratio. Media was replaced every 48 hours until  
0 toxicity assay was performed. After dosing cells, floating and adherent SH-SY5Y cells were collected, stained  
1 with PI (BD Biosciences, Cat. No. 556463), and analyzed via flow cytometry.

2  
3 *Single-cell RNA Sequencing and Analysis.*

4 Corrected and uncorrected cells were sorted and entered into the Fluidigm C1 HT workflow, which was used to  
5 capture and lyse individual cells, reverse transcribe RNA, and prepare libraries for sequencing (See: Using the  
6 C1 High-Throughput IFC to Generate Single-Cell cDNA Libraries for mRNA Sequencing, Cat. No. 100-9886).  
7 Sequencing was performed on a Nextseq 500 using a high-output kit. This resulted in a total of 430 million paired-  
8 end, 75 bp reads. Reads were aligned to the human genome (GRCh38) and quantified using the STAR aligner  
9 (63). Outlier removal was performed (cells must have >1,500 genes detected, <3 median absolute deviations

0 away from median total reads, mitochondrial counts), in addition to removing a subpopulation determined  
1 through k-means clustering with high mitochondrial expression and low numbers of genes expressed. This  
2 resulted in 120 cells available for analysis, with a mean of 482,205 aligned reads/cell. Size factors were  
3 computed via the Scran Bioconductor package, which uses pool-based scaling factors and deconvolution,  
4 followed by log-transformation normalization using the Scater package (64). A one-way ANOVA was used to  
5 determine significantly (FDR <0.05) differentially expressed genes between corrected and uncorrected cells.  
6 Supervised PCA was performed using differentially expressed genes as factors. The heatmap was generated  
7 using Ward's hierarchical clustering method (65) on the row-scaled expression values of differentially-expressed  
8 genes determined through the aforementioned method. Raw single-cell sequencing data can be accessed from  
9 the Gene Expression Omnibus at (GSE number pending).

#### 1 *XBP1 splicing assay.*

2 RNA was isolated from HLCs and cDNA was generated via standard RT reaction (see above). XBP1 transcript  
3 was amplified via PCR reaction with forward primer 5'- A AAC AGA GTA GCA GCT CAG ACT GC-3' and reverse  
4 primer 5'- TC CTT CTG GGT AGA CCT CTG GGA G-3'. PCR program utilized included the following steps:  
5 94°C for 4 minutes, 35 cycles of 94°C (10 seconds), 63°C (30 seconds), and 72°C (30 seconds), and lastly 72°C  
6 for 10 minutes. Amplified transcripts were subsequently digested with PstI enzyme (New England BioLabs, Cat.  
7 No. R0140S) and analyzed on a 2.5% agarose gel. Relative quantities of bands were determined via ImageQuant  
8 TL software. For positive control for XBP1 activation, thapsigargin (Millipore Sigma, Cat. No. T9033) was added  
9 to undifferentiated iPSCs at a concentration of 1  $\mu$ M for 24 hours.

#### 1 *Recombinant expression and purification of TTR<sup>L55P</sup>.*

2 TTR<sup>L55P</sup> was expressed and purified as previously described (66). Briefly, TTR<sup>L55P</sup> was expressed in *E. coli* as  
3 an N-terminal histidine-tagged protein isolated from cell lysate after passage through Ni-NTA agarose (Qiagen,  
4 Cat. No. 30210). Bound TTR<sup>L55P</sup> was then eluted by competition with imidazole. Lastly, purified TTR<sup>L55P</sup> was  
5 loaded into an Econo-Pac 10DG desalting column (BIO-RAD, Cat. No. 7322010) for rapid buffer exchange to 10  
6 mM sodium phosphate buffer pH 7.8, 100 mM KCl, 1 mM EDTA. The final concentration was adjusted to 0.4  
7 mg/ml. Subsequently, purified TTR<sup>L55P</sup> was stored at 0°C and used within one week.



8

9 *In vitro fibril formation assay.*

0 TTR<sup>L55P</sup> fibril formation was triggered under mild acidic conditions. The amount of fibrils formed was measured  
1 by Congo red (CR) binding assay as reported previously (67). TTR<sup>L55P</sup> and lyophilized human plasma-derived  
2 apo-transferrin protein (Apo-TF; R&D Systems, Cat. No. 3188-AT) resuspended in 10 mM sodium phosphate  
3 buffer (pH 7.8, 100 mM KCl, 1 mM EDTA) were both filtered through 0.2 μm membranes prior to their  
4 incorporation into the reaction mixtures. Addition of 50 mM sodium acetate buffer pH 4.6, 100 mM KCl lowered  
5 the pH of the reaction to 4.9. The final concentrations of TTR<sup>L55P</sup> and Apo-TF were 0.2 mg/ml and 2500 μg/ml  
6 respectively. Fibril formation was carried out at 37°C without agitation in a mastercycler with lid temperature of  
7 80°C to avoid condensation. Reactions were halted after 24 hours with the addition of 1.5 M HEPES pH 8.0.  
8 Finally, fibril formation reaction was added to 10 μM CR (Sigma-Aldrich, Cat. No. 573-58-0) working dilution  
9 (prepared in 5 mM KH<sub>2</sub>PO<sub>4</sub> pH 7.4, 150 mM NaCl). After 15 minutes at room temperature, absorbance was  
0 taken at 477 nm and 540 nm. The amount of CR bound to amyloid fibrils was determined via molar concentration  
1 of bound CR =  $A_{540\text{nm}}/25295 - A_{477\text{nm}}/46306$ . The percentage of fibrils formed was calculated via:

2 
$$\frac{TF + TTR_{t_{24}-t_0}^{L55P}}{TTR_{t_{24}-t_0}^{L55P}} \times 100.$$

3

4 *Generation of ATF6-inducible TTR<sup>L55P</sup> iPSC line.*

5 TTR<sup>L55P</sup> iPSCs were nucleofected (Lonza) with 3 μg of previously described DHFR.ATF6 donor construct (39)  
6 using the manufacturer's protocol. 48 hours post nucleofection, cells were grown in 500 ng/μL puromycin for  
7 approximately 10 days. Successfully grown colonies were subjected to dilution cloning to ensure clonality. To  
8 assess functionality, clonal, puromycin-resistant colonies were subjected to 10 μM TMP for 48 hours. RNA was  
9 harvested from each clone and qRT-PCR was performed to assess upregulation of ATF6 target gene, *HSPA5*,  
0 in the presence of TMP.

1

2 *Tandem Mass Tag (TMT)-LC-MS/MS Analysis of TTR<sup>WT</sup> and TTR<sup>L55P</sup> in Conditioned Media Prepared on iPSC-*  
3 *derived Hepatic Lineages.*

4 Hepatic lineages were prepared from patient iPSCs expressing TTR<sup>WT</sup> and TTR<sup>L55P</sup> where TMP-regulated  
5 DHFR.ATF6 was introduced. Media was conditioned on these cells for 72 hours in the presence or absence of  
6 TMP (10  $\mu$ M). The media was collected and then subjected to chloroform/MeOH precipitation to precipitate  
7 proteins (68). Dried pellets were dissolved in 8 M urea/100 mM TEAB, pH 8.5, reduced with 5 mM tris(2-  
8 carboxyethyl) phosphine hydrochloride (TCEP), and alkylated with 50 mM chloroacetamide. Proteins were then  
9 dilute to 2 M urea/100 mM TEAB and trypsin digested overnight at 37°C. The digested peptides were labeled  
0 with TMT (ThermoFisher, Cat. No. 90309, Lot. No. UB274629). The TMT labeled samples were analyzed on a  
1 Orbitrap Fusion Tribrid mass spectrometer (ThermoFisher). Samples were injected directly onto a 30 cm, 100  
2  $\mu$ m ID column packed with BEH 1.7  $\mu$ m C18 resin (Waters). Samples were separated at a flow rate of 400 nL/min  
3 on a nLC 1200 (ThermoFisher). Buffer A and B were 0.1% formic acid in 5% acetonitrile and 80% acetonitrile,  
4 respectively. A gradient of 1–30% B over 160 min, an increase to 90% B over 60 min and held at 90% B for 20  
5 minutes was used for a 240 minute total run time. Column was re-equilibrated with 20  $\mu$ L of buffer A prior to the  
6 injection of sample. Peptides were eluted directly from the tip of the column and nanosprayed directly into the  
7 mass spectrometer by application of 2.8 kV voltage at the back of the column. The Orbitrap Fusion was operated  
8 in a data dependent mode. Full MS1 scans were collected in the Orbitrap at 120k resolution. The cycle time was  
9 set to 3 s, and within this 3 s the most abundant ions per scan were selected for CID MS/MS in the ion trap. MS3  
0 analysis with multinotch isolation (SPS3) was utilized for detection of TMT reporter ions at 30k resolution (69).  
1 Monoisotopic precursor selection was enabled and dynamic exclusion was used with exclusion duration of 10 s.

2 Protein and peptide identification were done with Integrated Proteomics Pipeline – IP2 (Integrated  
3 Proteomics Applications). Tandem mass spectra were extracted from raw files using RawConverter (70) and  
4 searched with ProLuCID (71) against Uniprot human database. The search space included all fully-tryptic and  
5 half-tryptic peptide candidates. Carbamidomethylation on cysteine and TMT labels on N terminus and lysine  
6 were considered as static modifications. Data was searched with 50 ppm precursor ion tolerance and 600 ppm  
7 fragment ion tolerance. Identified proteins were filtered to 10 ppm precursor ion tolerance using DTASelect (72)  
8 and utilizing a target-decoy database search strategy to control the false discovery rate to 1% at the protein level  
9 (73). Quantitative analysis was done with Census (74) filtering reporter ions with 20 ppm mass tolerance and 0.6  
0 isobaric purity filter.

1 Peptide quantifications corresponding to the TSESGEL/PHGLTTEEEFVEGIYKVEIDTK peptide from

2 TTR<sup>WT</sup> or TTR<sup>L55P</sup> were initially analyzed for each biological replicate. Peptides showing a m/z signal less than  
3 10,000 were excluded from the analysis. A TMP ratio for each identified peptide was quantified using the  
4 following equation: TMP ratio = peptide signal in +TMP channel replicate = n / peptide signal –TMP channel  
5 replicate = n. The TMP ratio for individual peptides was then averaged across all peptides observed for each  
6 individual biological replicate to generate the plots shown in **Supplemental Fig. 3**. The relative impact of TMP  
7 on the secretion of TTR<sup>L55P</sup> was then quantified by normalizing the average TMP ratio for the TTR<sup>L55P</sup> peptide to  
8 the TMP ratio for the TTR<sup>WT</sup> peptide to generate the plot shown in **Fig. 5D**.

#### 0 *Morphological assessment of the impact of ATF6 signaling on HLCs exposed to prolonged ER stress.*

1 To assess morphological differences under severe, prolonged stress, iPSC-derived HLCs were dosed with 50  
2 nM thapsigargin for 5 days beginning on day 15 of the differentiation. In stressed cells with ATF6 activation, TMP  
3 was simultaneously added at a concentration of 10  $\mu$ M. In instances where ATF6 signaling was inhibited in the  
4 presence of thapsigargin, 6  $\mu$ M Ceapin-A7 (CP7) (Sigma-Aldrich, Cat. No. SML2330) was added to cells. After  
5 dosing for 5 days, cell morphology was noted. To account for degradation, small molecules were supplemented  
6 to media at half-concentration every other day.

#### 8 **STATISTICAL ANALYSIS**

9 Unless otherwise stated in the text, significance was determined between experimental and control conditions  
0 via unpaired Student's t-test. All experiments were performed using technical and biological triplicates. Here, we  
1 define biological replicates as individual passages, genetically distinct iPSC lines, and/or separate  
2 differentiations. Additional statistical information can be found in individual figure legends.

#### 4 **AUTHOR CONTRIBUTIONS**

5 RMG designed research studies, conducted experiments, acquired data, analyzed data, and wrote the  
6 manuscript. DCL designed research studies, conducted experiments, acquired data, and analyzed data. TMM  
7 and NS analyzed data. CTA, JC, SG, JR, JD, and SP conducted experiments. LHC and JRY revised the  
8 manuscript. DNK, RLW, and GJM supervised and designed research studies, analyzed data, and revised the  
9 manuscript.

0

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3 P41GM103533 (JRY), R01NS092829 (RLW), F31DK121481 (RMG)), NCATS (grant 1UL1TR001430 (RMG,  
4 GJM, DNK, AAW), the Amyloidosis Foundation, and the Young Family Amyloid Research Fund.

5

## 6 **CONFLICT OF INTEREST**

7 The authors have declared that no conflict of interest exists.

8

## 9 **FIGURE LEGENDS**

### 0 **FIGURE 1. Creation of a TTR promoter-driven hepatic specification reporter iPSC line and universal gene editing strategy for hereditary ATTR amyloidosis.**

1 (A) Undifferentiated PSCs and day 24 HLCs (green and yellow columns, respectively) form distinct, independent  
2 clusters by microarray analysis. Top 10 transcripts upregulated in HLCs are labeled on the y-axis, highlighted by  
3 green box. Top 10 transcripts downregulated in HLCs are labeled on the y-axis (below). Top differentially  
4 expressed genes were determined by one-way ANOVA. (B) qRT-PCR validating microarray finding that  
5 expression of *TTR* mRNA is significantly upregulated in day 24 HLCs compared to undifferentiated iPSCs; fold-  
6 change calculated over undifferentiated iPSCs (n=3, \*p<0.05, unpaired t-test for significance, bars denote  
7 standard deviation). (C) Schematic representation of the gene targeting strategy. Black triangles flank Cre-  
8 excisable LoxP sites. (D) Flow cytometry-based time course of GFP<sup>+</sup> cells that appear throughout hepatic  
9 specification of targeted iPSCs (n=3, bars denote standard deviation). (E) Phase (left) and fluorescence (middle,  
0 right) microscopy images of day 26 reporter iPSC-derived HLCs. Images taken at 20X magnification. (F)  
1 Expression of hepatic markers in sorted day 16 GFP<sup>+</sup> HLCs; fold-change calculated over undifferentiated iPSCs  
2 (n=5, bars denote standard deviation).

4

### 5 **FIGURE 2. Gene-edited iPSC-derived HLCs no longer produce neurotoxic, destabilized TTR variants.**

6 (A) Experimental overview depicting interrogation of normal, TTR<sup>L55P</sup>, and corrected iPSC-derived HLC  
7 supernatants and determination of their requisite downstream effects on neuronal target cells. (B) LC/MS

8 analyses of supernatant from normal, TTR<sup>L55P</sup>, and corrected iPSC-derived HLCs. Red trace: TTR<sup>WT</sup>, pink trace:  
9 destabilized TTR<sup>L55P</sup> variant. Bovine TTR (green) is present in media supplements. The molecular weight of each  
0 species is denoted in daltons. (C) SH-SY5Y cells were dosed for 7 days with conditioned iPSC HLC-derived  
1 supernatant from normal, TTR<sup>L55P</sup>, or corrected conditions. Cell viability was determined via PI staining (n=3,  
2 unpaired t-test for significance comparing uncorrected and corrected conditions, bars denote standard deviation).

3  
4  
5 **FIGURE 3. Single cell RNA sequencing (scRNAseq) of corrected vs. uncorrected syngeneic iPSC-derived**  
6 **HLCs reveals a novel hepatic gene signature.**

7 (A) Experimental schematic for the transcriptomic comparison of uncorrected (TTR<sup>L55P</sup>-expressing) and  
8 corrected syngeneic iPSC-derived HLCs at day 16 of the hepatic specification protocol. (B) Uncorrected (red)  
9 and corrected (green) populations form distinct groups by supervised principal component analysis (PCA).  
0 Supervised PCA was constructed using the top 500 differentially expressed genes by FDR. (C) Heatmap  
1 depicting the 92 genes differentially expressed between uncorrected and corrected populations (one-way  
2 ANOVA, FDR cutoff<0.05). Columns represent individual cells, green bar denotes corrected cells, red bar  
3 denotes uncorrected cells. Rows represent differentially expressed genes. The top 10 genes by foldchange  
4 (uncorrected over corrected) as well as proteostasis factor *EDEM2* are highlighted on the y-axis. (D) Violin plots  
5 representing relative expression levels of *TTR*, potential mediators of TTR fibrillogenesis (*TF*), and UPR target  
6 genes (*HYOU1*, *EDEM2*). (FDR determined via one-way ANOVA, \*FDR<0.05, \*\*FDR<0.005, \*\*\*FDR<0.0005.)

7  
8 **FIGURE 4. Assessment of TF chaperone capacity and functional validation of XBP1 activation in ATTR**  
9 **amyloidosis hepatic cells.**

0 (A) Experimental outline for assessing TF's *in vitro* ability to prevent the formation of congophilic species from  
1 recombinant TTR<sup>L55P</sup>. (B) Percentage of TTR<sup>L55P</sup> fibrils formed as determined by amount of Congo red (CR)  
2 bound after 24-hour incubation of recombinant protein under fibril forming conditions (n=5, \*\*p<0.005, unpaired  
3 t-test for significance comparing apo-TF condition to TTR<sup>L55P</sup> alone). (C) GSEA depicting significant enrichment  
4 of adaptive UPR machinery (ATF6, XBP1s) but not PERK target genes in uncorrected HLCs. In these analyses,  
5 100 uncorrected and 60 corrected cells were studied. (D) Depiction of XBP1 splicing in the presence of ER stress

6 and UPR activation. (E) PstI analytical digest of amplified XBP1 transcripts from iPSCs treated with thapsigargin  
7 (Tg), wild-type iPSC-derived HLCs (WT), and ATTR HLCs differentiated from two patient-specific iPSC lines  
8 (L58H and I107M). Hybrid band represents a PstI-resistant spliced-unsliced XBP1 product generated via PCR  
9 protocol. (F) Densitometric quantitation of PstI-digested XBP1 transcripts. Ratio determined by

$$\frac{XBP1_{spliced}}{XBP1_{unsliced1} + XBP1_{unsliced2}}$$

1  
2 **FIGURE 5. Hepatic stress-independent, branch-specific activation of adaptive UPR-associated ATF6**  
3 **signaling results in the targeting and selective reduction in the secretion of destabilized TTR<sup>L55P</sup>.**

4 (A) A chemical inducible system for activating ATF6 signaling in TTR<sup>L55P</sup> iPSC-derived cell types. In the absence  
5 of chemical chaperone TMP, DHFR.ATF6 is degraded. Upon addition of TMP, DHFR.ATF6 is stabilized and  
6 targets and attenuates the secretion of misfolded TTRs. (B) ATF6-inducible iPSCs were differentiated into HLCs.  
7 TMP was added and conditioned supernatant was collected and interrogating for the presence and relative  
8 abundance of different TTR species via LC/MS-MS. (C) ATF6 target gene *HSPA5* was found to be significantly  
9 upregulated upon addition of TMP by qRT-PCR (n=4, \*p<0.05, unpaired t-test for significance comparing -TMP  
0 to +TMP conditions). (D) LC-MS/MS was used to directly detect the presence of TTR<sup>WT</sup> (upper peptide sequence,  
1 black) and TTR<sup>L55P</sup> (lower peptide sequence, orange) peptides in conditioned supernatant in the presence and  
2 absence of TMP. Abundance of TTR<sup>L55P</sup> was found to significantly decrease upon activation of ATF6 signaling  
3 by ~25% relative to TTR<sup>WT</sup>. Quantities of each peptide were normalized to TTR<sup>WT</sup> (n=3, \*\*p<0.05, unpaired t-test  
4 for significance comparing normalized quantities of TTR<sup>WT</sup> and TTR<sup>L55P</sup>). (E) Upon addition of TMP, ATF6 target  
5 gene *HERPUD1* was found to be significantly upregulated compared to DHFR.ATF6 HLCs in the absence of  
6 TMP. IRE1/XBP1s and PERK target genes *ERDJ4* and *GADD34* however, were not found to be differentially  
7 expressed in the presence of TMP. Positive control thapsigargin (Tg) was found to significantly upregulate  
8 expression of all UPR target genes tested (n=6, \*p<0.05, \*\*p<0.005, \*\*\*\*p<0.0001).

9  
0 **SUPPLEMENTAL FIGURE 1. Validation of targeted iPSC reporter line for site-specific integration of**  
1 **transgene as well as karyotypic stability.**

2 (A) Targeting construct used to perform gene correction. Red arrows denote the forward primer utilized for allele  
3 integration and puromycin cassette excision assay. Blue arrow represents the reverse primer used in the

4 targeted integration screen. Green arrow depicts the reverse primer utilized for puromycin resistance ( $\text{puro}^R$ )  
5 cassette excision. (B) PCR screen of gDNA for targeted integration of donor construct (left) and excision of  $\text{puro}^R$   
6 cassette (right). (C) Non-targeted (left) and corrected (right)  $\text{TTR}^{\text{L55P}}$  iPSCs derived from a female ATTR  
7 amyloidosis patient were karyotypically normal, post-excision of the  $\text{puro}^R$  cassette.

8  
9 **SUPPLEMENTAL FIGURE 2. Liver markers are not differentially expressed between uncorrected and**  
0 **corrected HLCs at day 16 of differentiation.**

1 Violin plots representing equivalent relative expression levels of genes known to be upregulated during hepatic  
2 specification of PSCs in uncorrected (red) and corrected (green) HLCs. None of the noted genes are differentially  
3 expressed between the two groups, suggesting competent normalization of the hepatic specification procedure.

4  
5 **SUPPLEMENTAL FIGURE 3. Activation of ATF6 signaling in ATTR amyloidosis patient-specific iPSC-**  
6 **derived HLCs results in selective decrease in the secretion of destabilized TTR.**

7 (A) Representative IP-MS data of the relative abundances of  $\text{TTR}^{\text{L55P}}$  and  $\text{TTR}^{\text{WT}}$  in non-targeted, heterozygous  
8  $\text{TTR}^{\text{L55P}}$  patient-specific HLCs. (B) Recovery of  $\text{TTR}^{\text{L55P}}$  in conditioned ATF6-inducible HLC supernatant in the  
9 presence and absence of exogenous ATF6 activation (i.e. TMP) ( $n=4$ , unpaired t-test for significance).  $\text{TTR}^{\text{L55P}}$   
0 signal denoted is normalized to  $\text{TTR}^{\text{WT}}$  in each replicate. (C) Individual experiments compiled/summarized in  
1 **Fig. 5D** of main text. Error bars represent quantification from different spectral counts (2 for  $\text{TTR}^{\text{WT}}$  and 4 for  
2  $\text{TTR}^{\text{L55P}}$ ) of the same peptide.

3  
4 **SUPPLEMENTAL FIGURE 4. Branch-specific activation of ATF6 signaling in patient-specific iPSC-**  
5 **derived hepatic cells alleviates ER stress-mediated morphological defects.**

6 (A) Inducible ATF6 iPSCs were differentiated into HLCs and exposed to global ER stress via addition of  
7 thapsigargin. In addition to the presence of thapsigargin, ATF6 signaling was either activated via addition of TMP  
8 or inhibited via addition of small molecule CP7. After 5 days of exposure to each compound, cells were observed  
9 for morphological defects. (B) In the absence of TMP, in the presence of thapsigargin, cells exhibited gross  
0 morphological defects. Upon activating ATF6 signaling (in the presence of thapsigargin), morphology appeared  
1 to be rescued. Conversely, upon inhibiting ATF6 signaling via addition of CP7 (in the presence of thapsigargin),

2 morphological defects were noted. Cells were dosed with various compounds in three independent  
3 differentiations.

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## ABBREVIATIONS

A1AT... alpha-1 antitrypsin

A $\beta$ ... amyloid  $\beta$

AD... Alzheimer's disease

AFP... alpha fetoprotein

ALB... albumin

ATTR amyloidosis... transthyretin amyloidosis

DLT... domino liver transplant

FDR... false discovery rate

GSEA... gene set enrichment analysis

HLC... hepatocyte-like cell

iPSC... induced pluripotent stem cell

LC/MS... liquid chromatography/mass spectrometry

PCA... principle component analysis

PSC... pluripotent stem cell

scRNAseq... single cell RNA sequencing

TALEN... transcription activator-like effector nuclease

TF... transferrin

TMT... tandem mass tag

TTR... transthyretin

UPR... unfolded protein response

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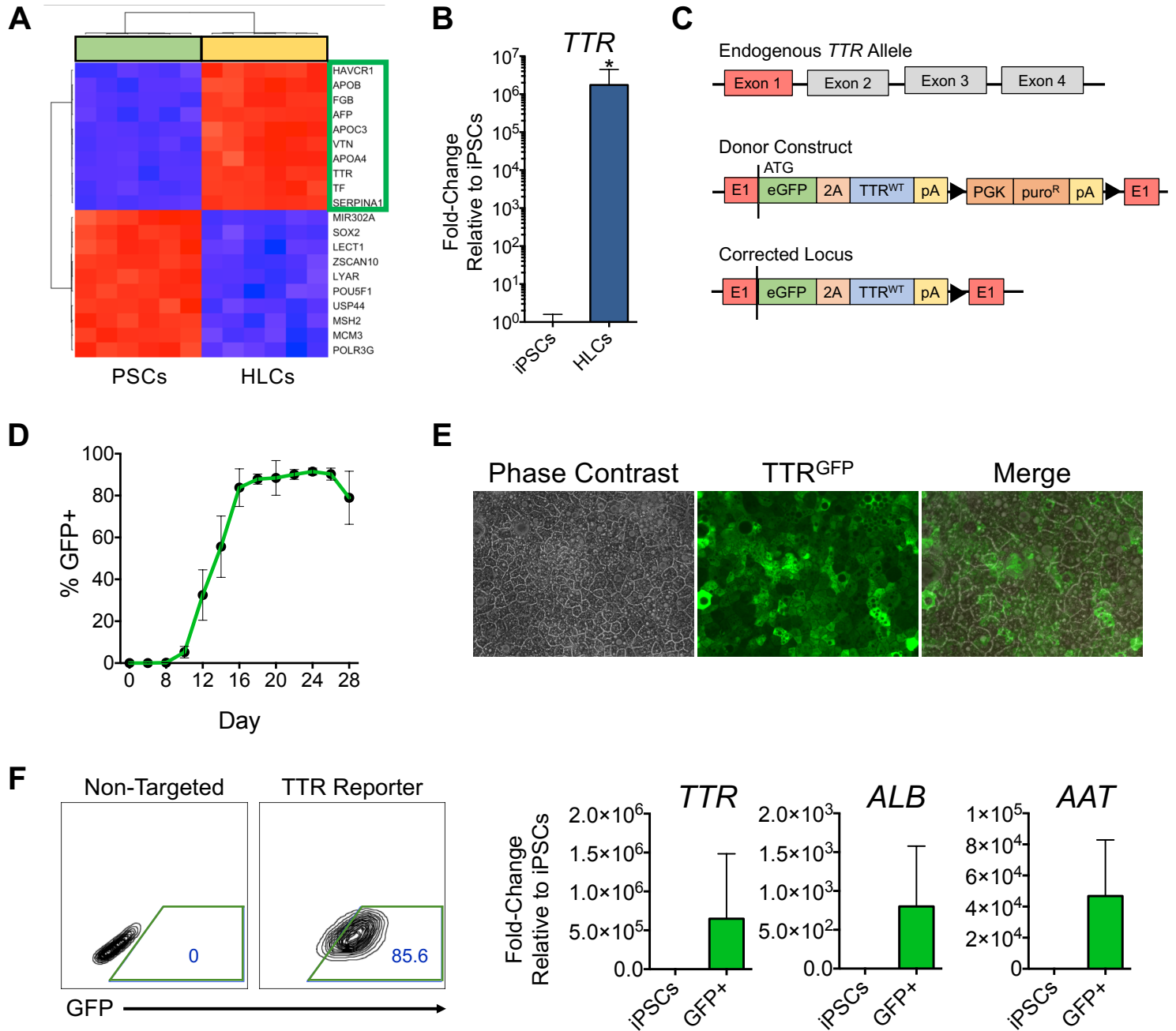
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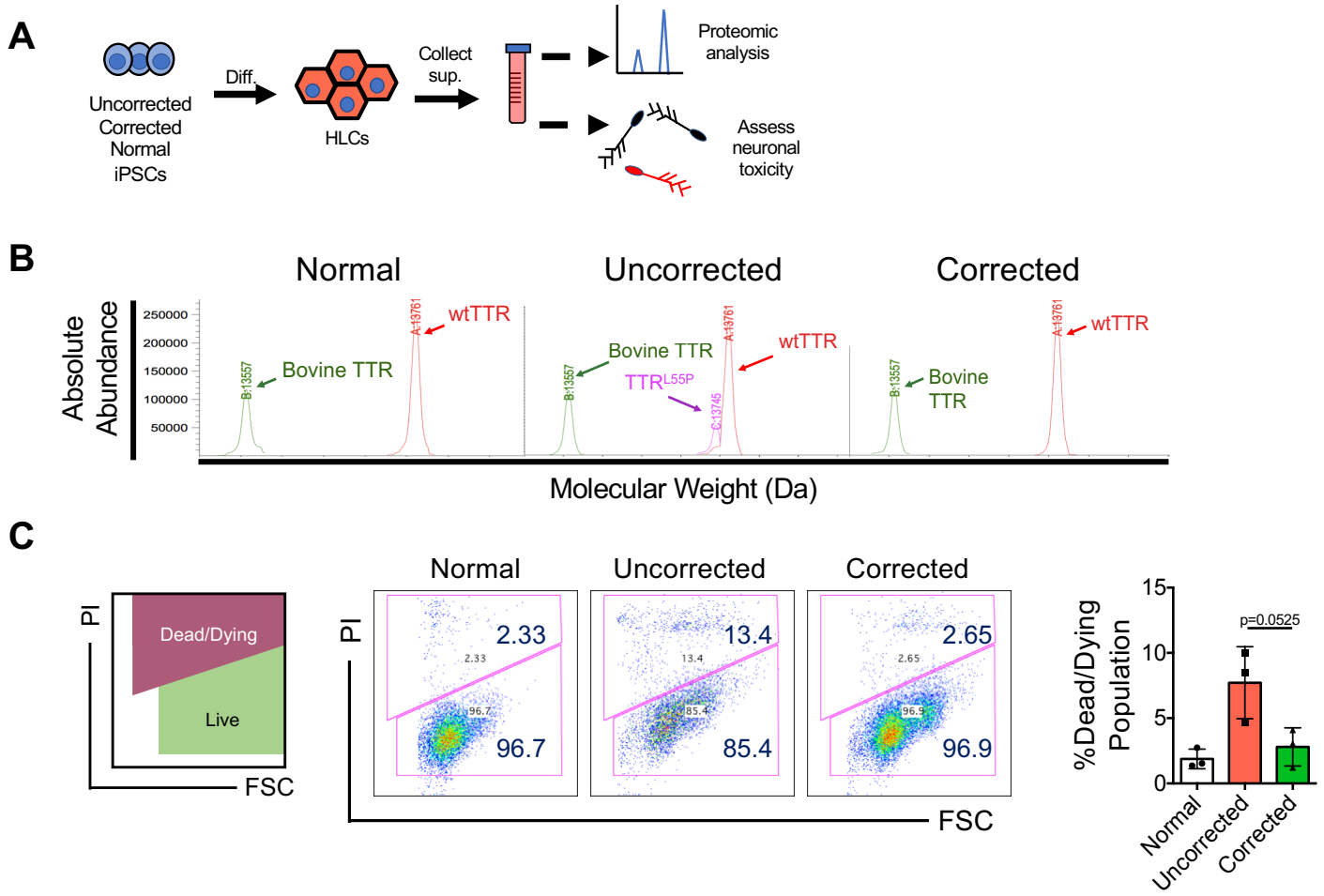
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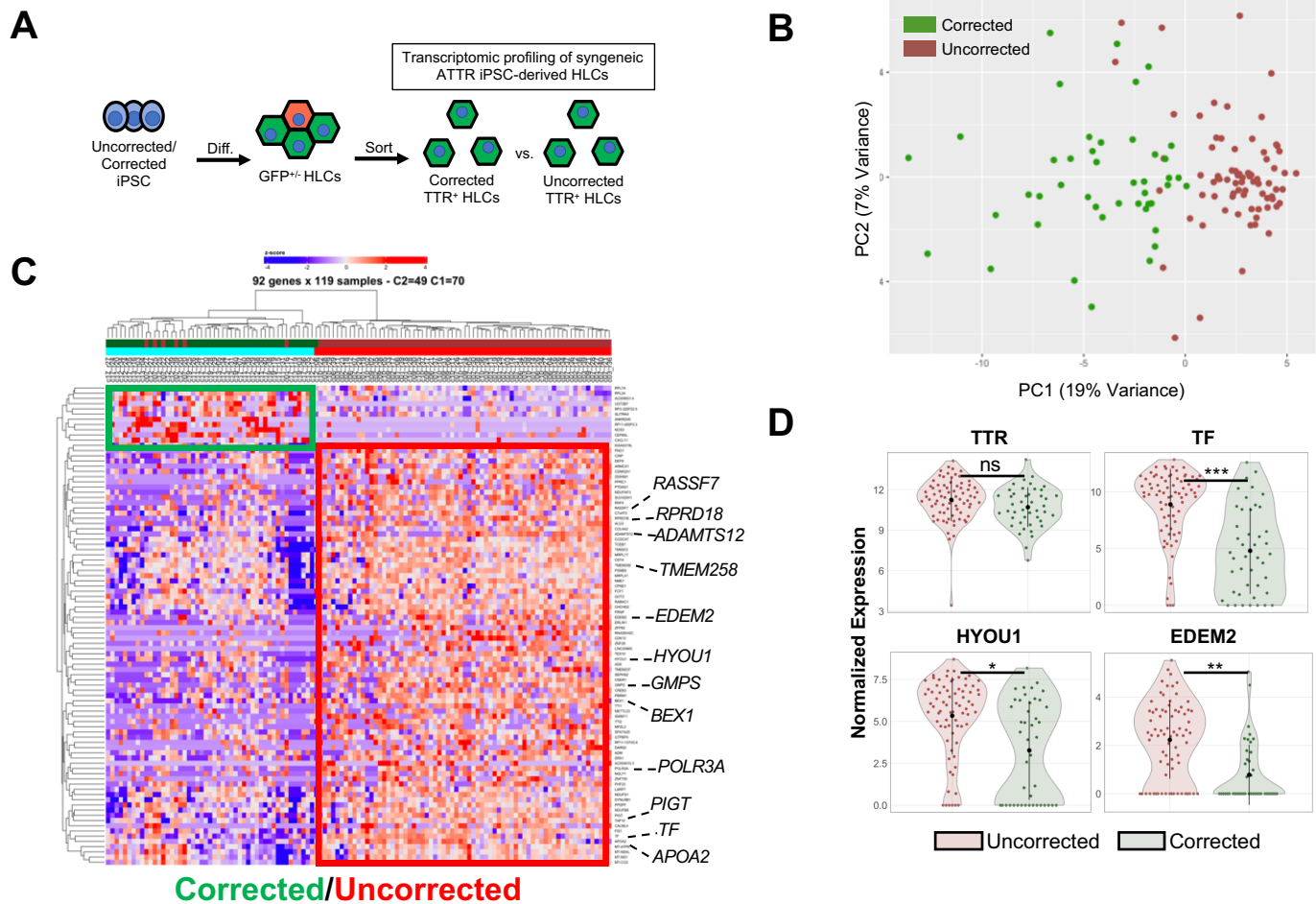
# Figure 1



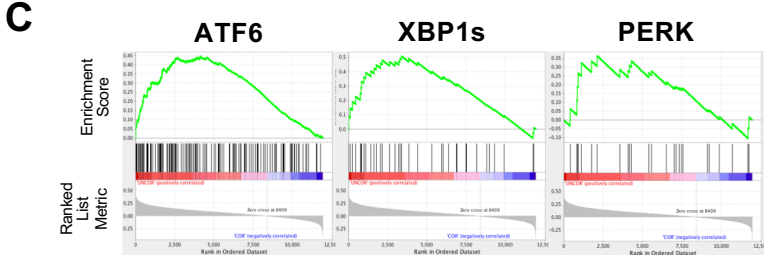
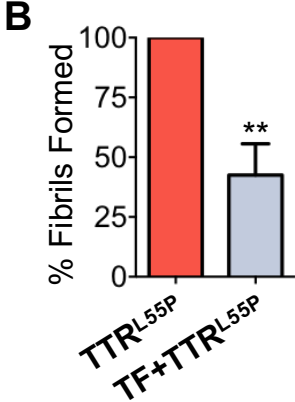
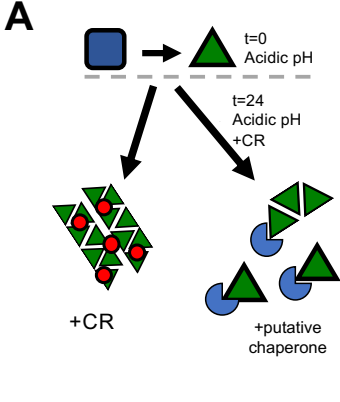
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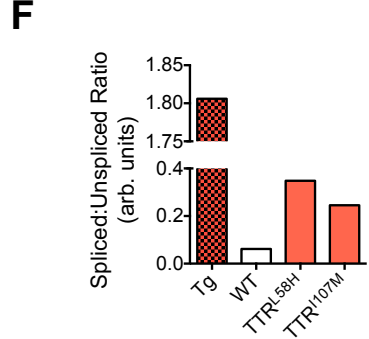
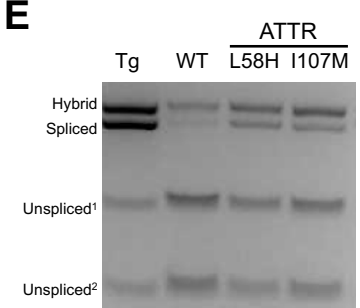
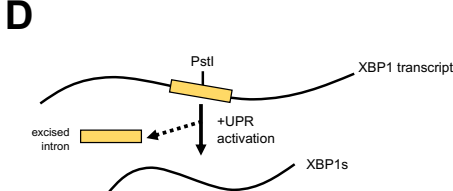
# Figure 3



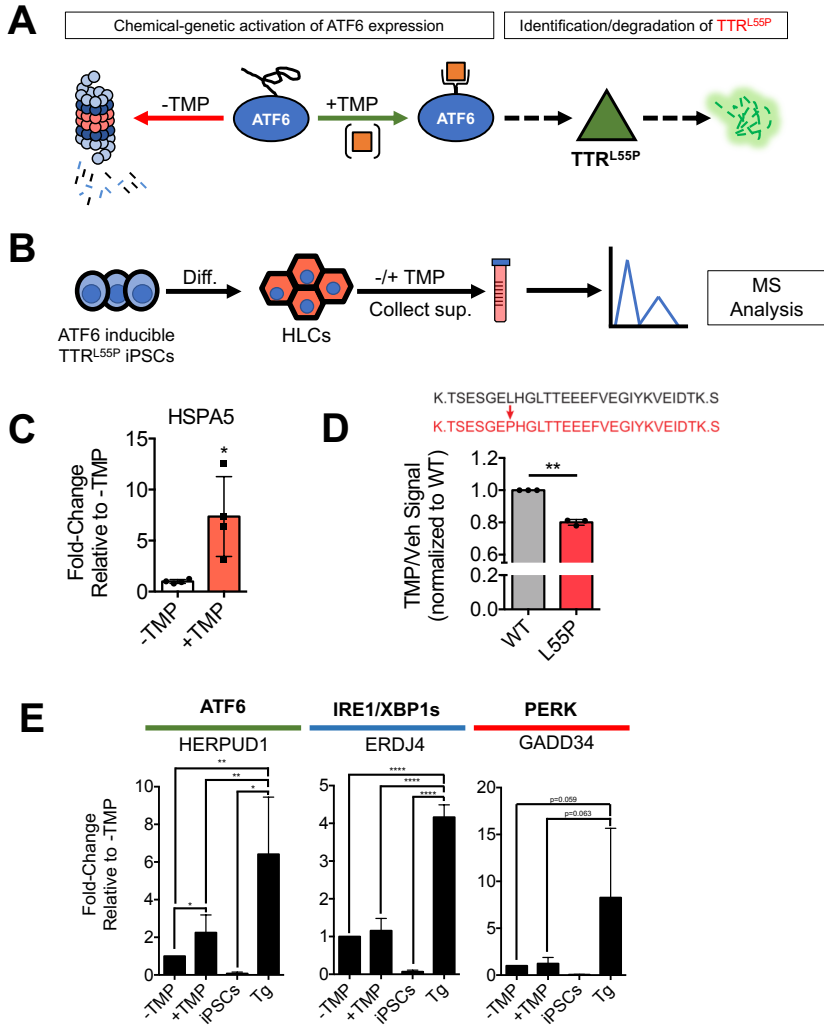
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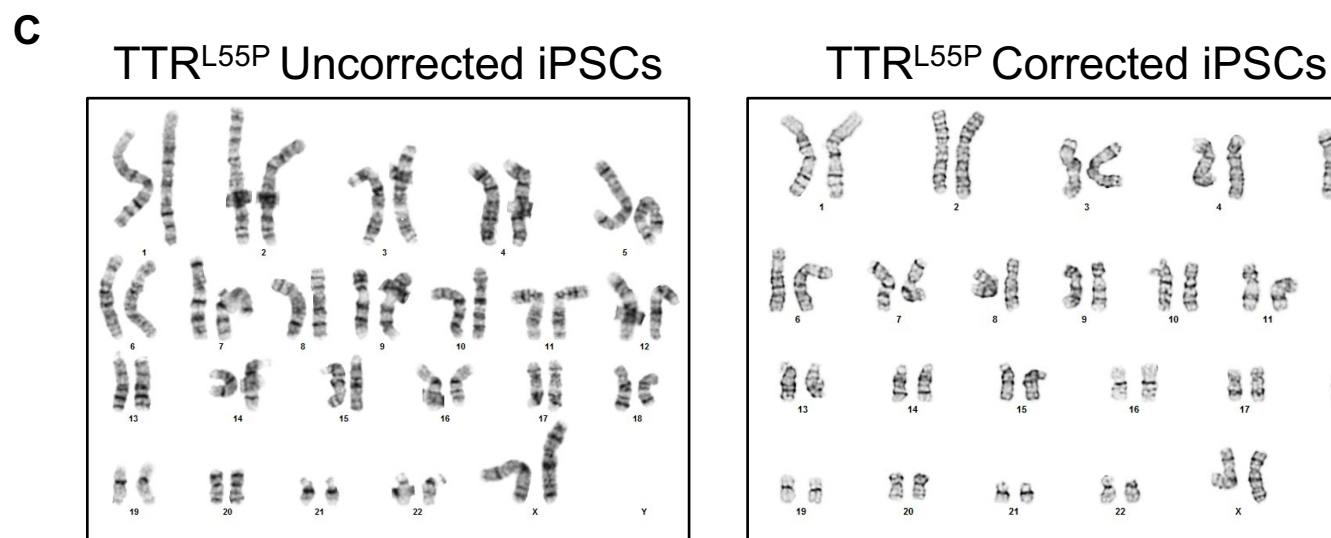
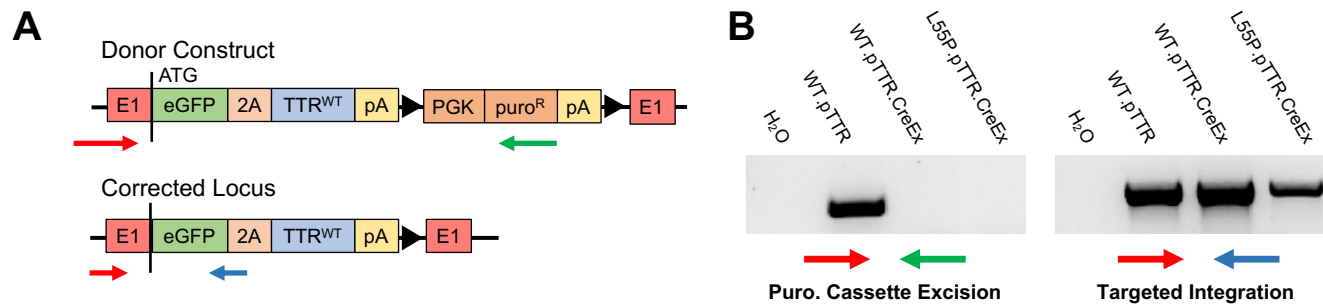
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FDR	0.035	0.0209	0.314



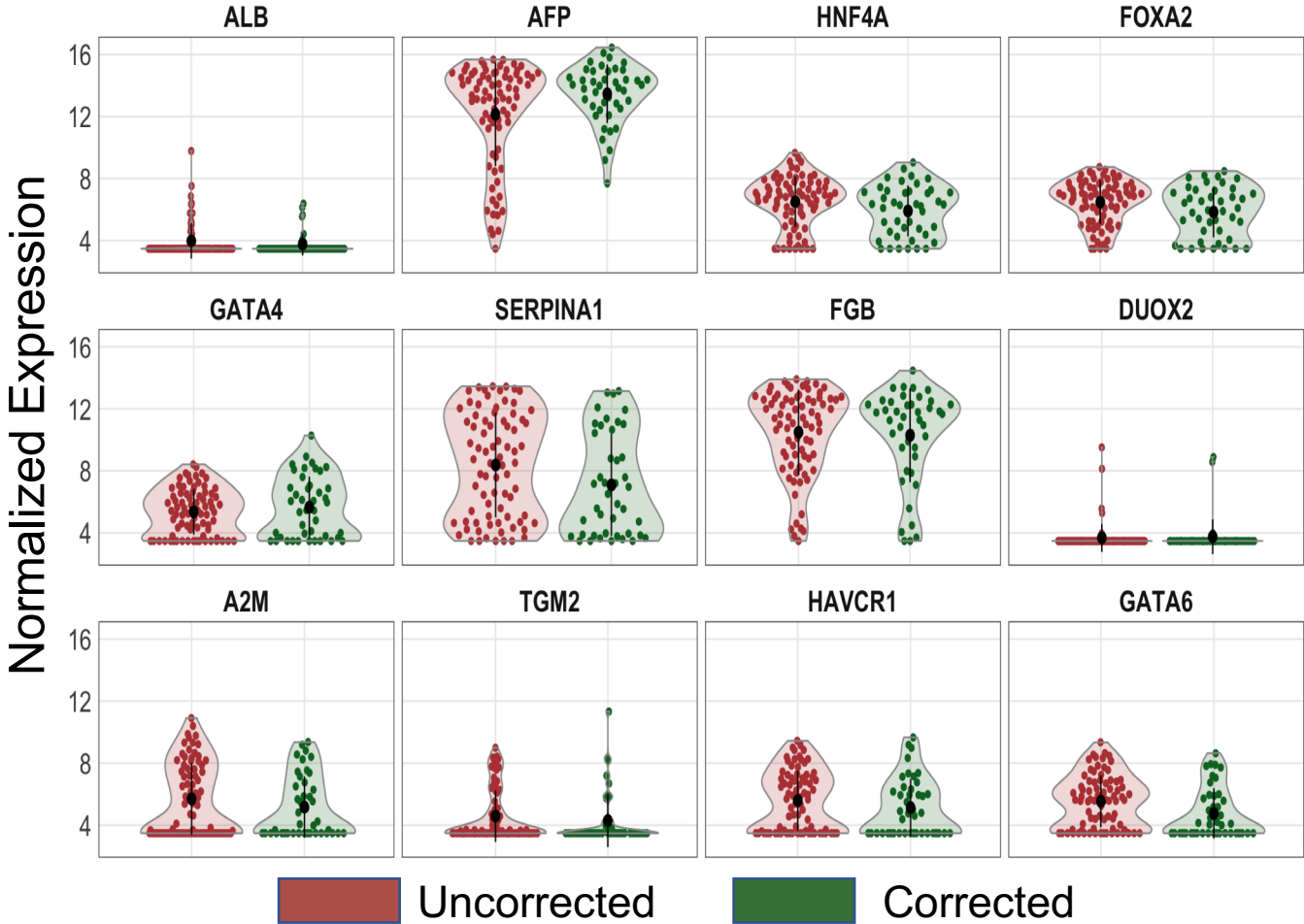
# Figure 5



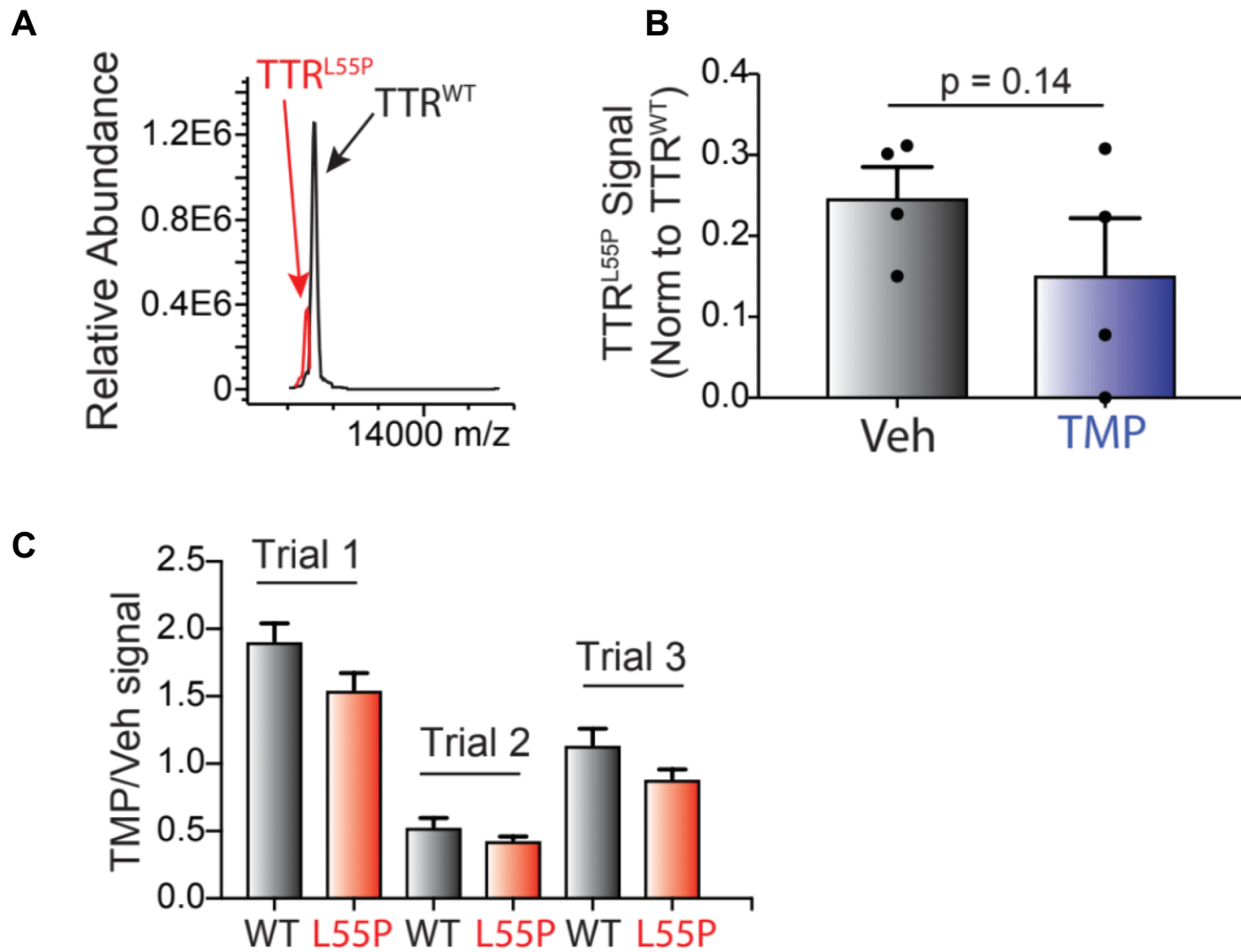
# Supplemental Figure 1



# Supplemental Figure 2



# Supplemental Figure 3





# Supplemental Figure 4

