An ALS Therapeutic Assembly Modulator Target in Peripheral Blood Mononuclear Cells: Implications for ALS Pathophysiology, Therapeutics, and Diagnostics

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Abstract

Assembly modulators are a new class of allosteric site-targeted therapeutic small molecules, some of which are effective at restoring nuclear localization of TDP-43 in ALS cellular models, and display efficacy in a variety of ALS animal models. One of these compounds has been shown to target a small subfraction of protein disulfide isomerase, a known allosteric modulator implicated in ALS pathophysiology, within a novel, transient, and energy-dependent multi-protein complex that includes other important members of the ALS interactome, such as TDP-43, RanGTPase, and selective autophagy receptor p62/SQSTM1. Building on earlier literature suggesting PBMC dysfunction in ALS, we demonstrate here that a similar multi-protein complex drug target is present in PBMCs with signature alterations in PBMCs from ALS patients compared to PBMCs from healthy individuals. ALS-associated changes in the drug target include increased RanGTPase and MMP9, diminished p62/SQSTM1, and most distinctively, appearance of a 17kDa post-translationally modified form of RanGTPase. Many of these changes are not readily apparent from analysis of whole cell extracts, as a number of the proteins present in the target multi-protein complex, including RanGTPase, comprise a miniscule percent of their total in cell extracts. A small subset of each of these proteins appear to come together in a transient, energy-dependent fashion, to form the drug target. Furthermore, whole blood from ALS patients shows a distinctive degradation of RanGTPase not observed in blood from healthy individuals, which appears to be rescued by treatment with either of two structurally unrelated ALS-active assembly modulators. Our findings are consistent with the hypothesis that ALS is fundamentally a disorder of homeostasis that can be both detected and treated by assembly modulators.

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a devastating neurodegenerative disease that strikes approximately 6,000 American and 150,000 people worldwide annually[1,2]. Approximately 10% of ALS is familial, with a large number of specific gene mutations implicated. The vast majority of ALS cases however, are sporadic, meaning that that there is no family history of the disease and therefore is likely due to an environmental or epigenetic influence. Specific environmental factors have been implicated in causing ALS, but remain controversial and inconclusive. While the pathophysiology is not well understood, aggregates of the protein TDP-43 are almost invariably found in the brains of patients with ALS and fronto-temporal dementia (FTD)[3]. This observation and the discovery of shared genetic mutation (C9orf72) suggest that these diseases are therefore on a continuum of the same underlying disorder. Despite these commonalities, the clinical presentation as well as progression of ALS, is highly variable[4].

With regards to TDP-43 pathophysiology, controversy exists as to the triggering event. Some studies have implicated mislocalization of TDP-43 from the nucleus in healthy cells to the cytoplasm, which is observed to variable degrees in fibroblasts from many, but not all, cases of ALS[5]. An alternative view is that, in response to stress, TDP-43 forms aggregates in the cytoplasm where it is synthesized, prior to transport to the nucleus[6]. A further controversy exists as to whether the relevant TDP-43 aggregates in the cytoplasm are within, or outside of, stress granules[7]. While the TDP-43 aggregates have generally been viewed as a trigger of motor neuron death, it is possible that they are an epiphenomenon whose elimination *per se* may not necessarily be therapeutic, by analogy to the failed attempts to remedy Alzheimer's Disease by

elimination of its pathognomonic Aβ aggregates. Regardless of their natural history and role in the pathophysiology of ALS, the presence of TDP-43 aggregates are pathognomonic for the disease[8,9].

Recently we presented our studies on the role of a newly appreciated dimension of gene expression, termed protein assembly, in pathophysiology of diverse diseases, including ALS[10]. We have identified several structurally unrelated small molecule chemotypes termed assembly modulators, that are strikingly efficacious, including at both preventing neurodegeneration and correcting TDP-43 pathology, in various cellular and animal models of ALS[10]. The compounds are broadly efficacious at reversing both TDP-43 mislocalization and stress-induced TDP-43 aggregation, in both familial and sporadic ALS cellular models[10]. Efficacy was also demonstrated in various transgenic animals expressing human ALS-causing mutations including in TDP-43, FUS, c9orf72, and SOD1[10]. In view of this breadth of efficacy, we hypothesized the mechanism of action of the assembly modulator drug to target a step common to most, if not all, ALS. Analogs of some of these compounds are close to completion of a target product profile for initiation of investigational new drug (IND)-enabling studies in anticipation of human clinical trials in the near future.

The studies to be described here had several motivations. First, to explore the hypothesis that even physiological pathways thought of as well understood may have additional layers of regulation yet to be discovered, in which the small molecule assembly modulators and their novel multi-protein complex targets we have discovered, might be involved. How those

levels of control are integrated with one another and communicated from one organ system to others remains mysterious, for which the novel assembly modulator targets we have discovered are potential candidates. By corollary, what happens to other feedback controls when one level of regulation fails, remains to be determined. The argument has been made based on gene knockout experiments that back up pathway may be activated when primary regulatory controls fail[11]. Having a drug that impinges on a biochemical pathway in a novel way is a powerful means of probing biological regulation, particularly that which depends on information that does not reside simply in the sequence of the genome[12,13,14]. Thus the relevance of the compounds we have described, with their novel mechanism of action at allosteric sites[15,16], and the unconventional nature of the targeted multi-protein complex (energy-dependent for formation, transient in existence, composed of miniscule subsets of the individual component proteins in the cell)[17,18]. These tools may provide a new understanding of the molecular basis for homeostasis.

A second motivation for the present studies was to understand the meaning of the observed dysfunction of PBMCs in ALS[19,20]. These findings can be interpreted in two generally different ways. On the one hand, PBMC dysfunction might be due to ALS being a systemic disease whose most severe manifestation is motor neuron death, but which also manifests in less dramatic fashion in other organ systems, including in blood. Alternatively, dysfunction of PBMCs might be a consequence of disordered communication between organ systems, or its regulation. Thus, a disorder affecting one organ system could have a “domino effect” manifest in other organ systems. Even if ALS is initiated in motor neurons, their dysfunction may be

transmitted to other organ systems as a secondary consequence of feedback regulation intended to maintain homeostasis. In either framework, or hybrids of the two, disordered homeostasis is a fundamental feature of ALS pathophysiology. If a means of restoring homeostasis could be identified it may be effective in therapeutics of many diseases including ALS. Towards this end, we asked whether assembly modulator drugs, that are therapeutic in cell and animal models for ALS, might also detect a disordered drug target in PBMCs from ALS patients. If so, how does that target compare to the target observed in brain or patient-derived fibroblasts (PDFs) in which TDP-43 pathology is observed?

Finally, apart from intellectually interesting questions of feedback and mechanism of action, there is the practical desire to advance these compounds with remarkable therapeutic properties in cellular and animal models, to the clinic. Having a readily accessible signature in PBMCs could facilitate drug advancement in innumerable ways including for early detection of ALS (prior to severe disability), patient enrollment and tracking response to therapy. Most importantly, it could allow detection of ALS early when compounds that restore homeostasis should be most effective.

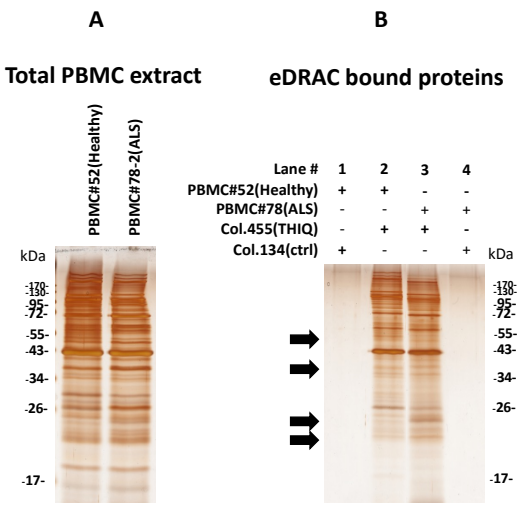
Results

Blinded blood samples were obtained from ALS patients under an IRB-approved protocol, PBMCs isolated, extracts prepared as described in methods, and analyzed for key proteins relevant to ALS. No clear and consistent different was observed in the protein pattern

between PBMCs of healthy and ALS patients by total protein silver stain (see for example, **Figure 1A**).

However when PBMC extracts were applied to the ALS-active drug resin described previously[10] versus control resin (lacking the drug ligand), washed and bound material analyzed, a distinctive subset of proteins was observed, also largely shared between healthy individuals and ALS patients, but with a few possible differences (black arrows in **Figure 1B**).

Figure 1



Legend to Figure 1. A. Silver stain of starting PBMC extract 10kxg/10min supernatant from a representative healthy individual (#52) and an ALS patient (#78-2). B. Silver stain of free drug eluate from ALS-active assembly modulator drug resin (column 455 THIQ) or control resin (column 134 lacking the drug ligand). Black arrows indicate positions at which healthy vs ALS patient samples show a suggestive difference in the free drug eluate protein pattern.

Mass spectrometry analysis of free drug eluates from PBMCs applied to the ALS-active drug resin (**Figure 2**) was carried out and compared to the pattern previous revealed from mouse

brain. A substantial proportion of tissue-specific proteins (~1/2 to 2/3 depending on the specific sample) were observed (see Supplemental Figure 1). Despite the high tissue specificity, a core set of 27 proteins were identified as shared between both PBMCs and brain, that are in the ALS interactome. Notably, this set included P4HB, a member of the PDI family, two known ALS-causing proteins (TUBA4A and VCP)[21], along with five proteins implicated in the KEGG ALS interactome[22] (ATP5A1, HSPA5, TUBA1A, TUBA4A, and VCP). Furthermore, 24 of these 33 proteins exhibited high-confidence interactions with known ALS-causing or disease-modifying proteins[21]. The 27 shared ALS interactome proteins also included multiple members of several protein families and complexes: two ATP synthase subunits (ATP5A1 and ATP6V1A), six chaperonins containing T-complex components (CCT2, CCT3, CCT4, CCT5, CCT7, and CCT8), three thioredoxin domain proteins (P4HB, PRDX1, and PRDX2), and four members of the 14-3-3 family (YWHAB, YWHAE, YWHAH, and YWHAQ) (**Figure 2A**).

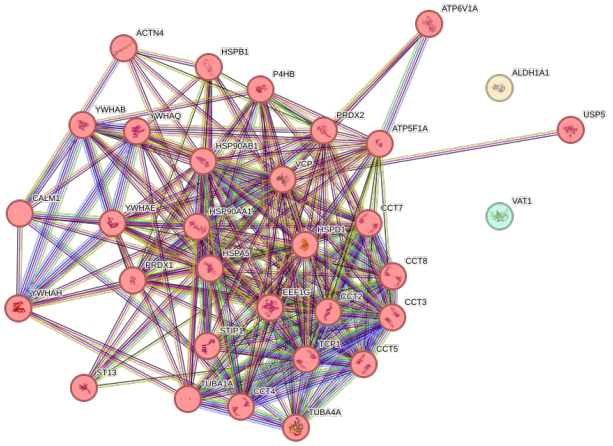
MCL clustering analysis was performed on the 33 shared proteins using the STRING database, which integrates information from various databases cataloging known and predicted protein-protein interactions. The analysis generated groups called “clusters,” characterized by a higher density of predicted associations within the clusters compared to those between clusters. Notably, 31 out of the 33 proteins formed a single, highly interconnected cluster, suggesting potential functional relationships between them. Two proteins, VAT1 and ALDH1A1, were excluded from this cluster, as there are no predicted interactions between these proteins and the other 31 proteins (**Figure 2B**).

Figure 2

A

	WT mouse THIQ eluate	WT mouse Control eluate	SOD mouse THIQ eluate	SOD mouse control eluate	Healthy PBMC 44 THIQ eluate	Healthy PBMC 52 THIQ eluate	ALS PBMC 43 THIQ eluate	ALS PBMC 53 THIQ eluate	ALS PBMC 43 Control eluate	Causitive genes	Associated and disease modifier genes	ALS protein binding partners	KEGG ALS interactome
ACTN4	7	0	4	0	64	69	78	69	0				
ALDH1A1	2	0	7	0	8	0	0	2	0				
ATP5A1	22	0	17	0	2	5	5	6	0				
ATP6V1A	13	0	7	0	3	0	5	0	0				
CALM1	11	0	10	0	13	13	11	12	0				
CCT2	8	0	13	0	7	0	3	0	0				
CCT3	6	0	2	0	8	2	6	2	0				
CCT4	3	0	6	0	8	0	3	2	0				
CCT5	9	0	8	0	9	2	5	0	0				
CCT7	4	0	6	0	8	0	4	0	0				
CCT8	8	0	7	0	11	0	7	2	0				
EEF1G	9	2	10	0	4	4	5	2	0				
HSP90AA1	37	0	44	0	20	13	15	11	0				
HSP90AB1	39	0	46	0	19	15	15	8	0				
HSPA5	11	0	15	0	0	7	0	0	0				
HSPB1	4	0	6	0	4	17	10	0	0				
HSPD1	38	2	38	0	12	21	12	9	0				
LDHB	7	0	0	0	14	0	12	0	0				
P4HB	16	0	23	0	54	48	64	43	0				
PRDX1	8	0	11	0	8	6	4	4	0				
PRDX2	7	0	10	0	18	8	9	19	0				
ST13	6	0	11	0	3	0	4	5	0				
STIP1	32	0	35	0	3	9	0	0	0				
TCP1	6	0	5	0	6	0	4	0	0				
TUBA1A	17	0	15	0	10	12	15	18	0				
TUBA4A	16	3	12	0	11	14	18	18	0				
USP5	12	0	23	0	5	0	2	0	0				
VAT1	9	0	10	0	4	0	10	3	0				
VCP	47	2	53	0	55	62	58	55	0				
YWHAB	9	0	0	0	12	6	9	0	0				
YWHAE	9	0	11	0	6	6	6	0	0				
YWHAH	6	0	7	0	6	7	8	0	0				
YWHAQ	10	0	9	0	0	5	6	7	0				

B

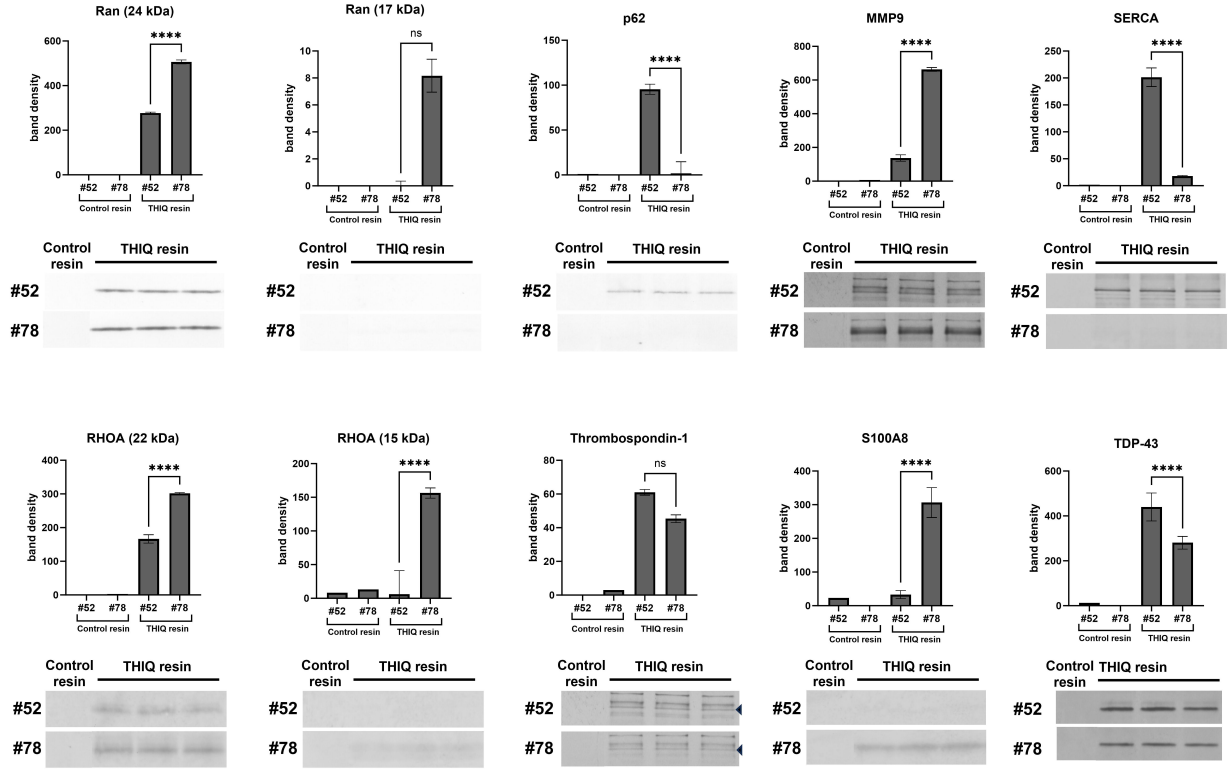


Legend to Figure 2. A**.** Spectral counts for proteins identified in THIQ drug resin eluate from mouse brain and human PBMC extracts. Colors represent the number of spectral counts for a given protein in each sample, ranging from light green (low counts) to dark green (high counts). The right panel indicates "hits" for proteins present in the ALS interactome, where yellow is a hit. B. STRING database protein-protein interaction network with MCL clustering. Each circle ("node") represents a protein, and each line ("edge") represents a predicted interaction between two nodes. Different colors represent distinct clusters identified by the MCL algorithm, with red indicating the largest cluster and yellow and green representing clusters containing only a single protein.

From the MS-MS analysis it was clear that the multi-protein complex isolated from PBMCs on the ALS-active drug resin appears related to that observed in mouse brain, based on the 27 shared proteins identified that are in the ALS protein interactome observed from both sources. However no clear differences between healthy and ALS patients were observed by MS-MS.

Recognizing that MS-MS can fail to identify specific proteins for a number of reasons, including post-translational modification and failure of peptide ionization, we analyzed the samples by western blot for particular proteins of interest including for TDP-43, RanGTPase, and P62/SQSTM1 (Figure 3). By Western blotting a number of differences in proteins bound to the assembly modulator THIQ drug resin were observed between healthy individuals and ALS patient PBMC extracts. Most notably p62/SQSTM1 was greatly diminished and a 17kDa post-translationally modified (PTM) fragment of RanGTPase detected in ALS patients compared to healthy individuals.

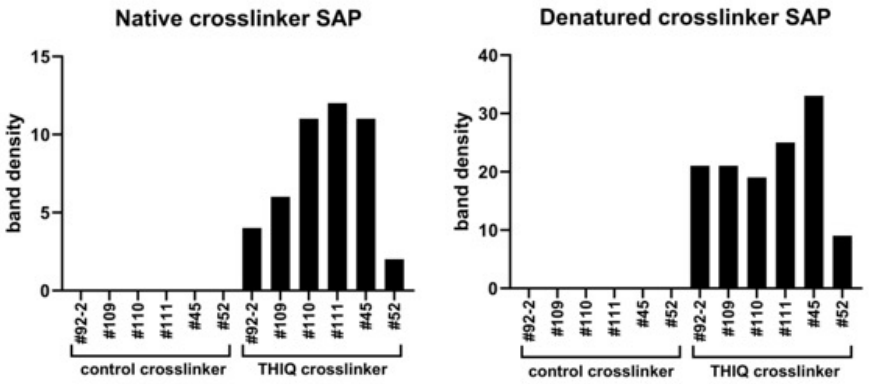
Figure 3.



Legend to Figure 3. PBMC extracts were prepared as described in methods and applied to drug resin versus control resin, washed and bound proteins analyzed by SDS-PAGE transferred to 0.2u PVDF membrane and Western blotted for the indicated proteins with quantitation of bands using Image J software.

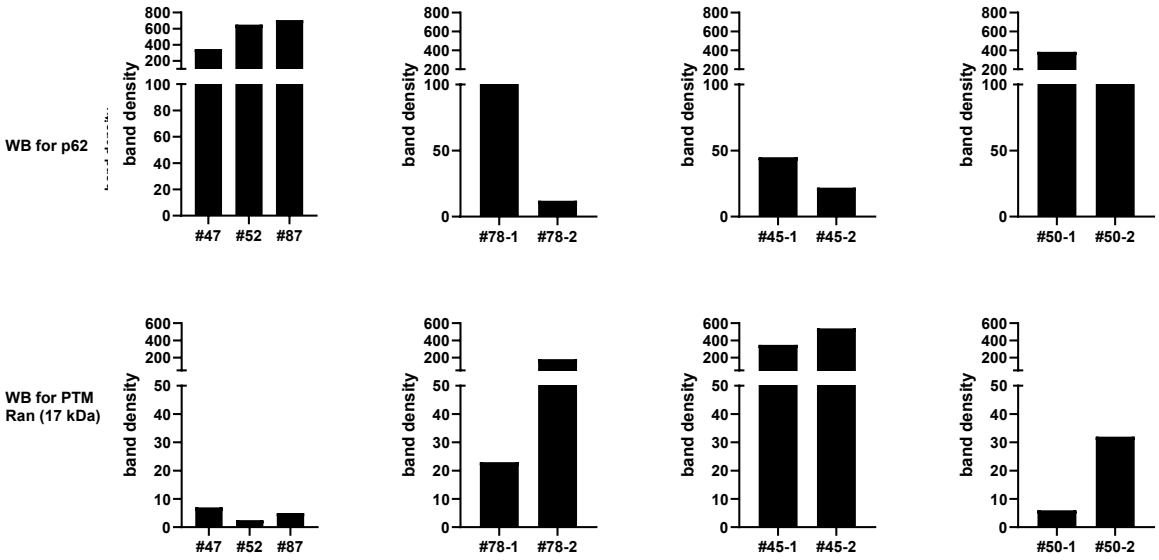
Next, photocrosslinking was carried out with PBMC extracts to which was added a drug analog in which the attachment to resin was replaced with a biotin and diazirine moiety such that exposure to UV light results in a covalent linkage to the drug binding protein(s). Under native conditions associated proteins can be precipitated with streptavidin beads. If the sample is first denatured, then only the direct drug-binding protein will be precipitated with streptavidin beads under the conditions described. Figure 4 demonstrates that PDI is the direct drug-binding protein. The PBMC extracts displayed striking similarities with the corresponding drug resin complexes from brain. In particular, the direct drug-binding protein in extracts from all three tissue sources, was PDI (Figure 4).

Figure 4.



Legend to Figure 4. Quantitation of PDI in streptavidin precipitates under native vs denatured conditions after photocrosslinking with PAV-073 analog (THIQ) or control crosslinker lacking the

211 drug ligand. Both control and THIQ crosslinkers contain biotin and diazirine as described in
212 methods and previously[10].
213
214 We then assessed whether the ALS patient vs healthy control correlations were temporal
215 in nature. As can be seen in Figure 5, with progression of disease, the amount of p62 bound to
216 the resin diminishes and intensity of the RanGTPase 17kDa fragment increases, with disease
217 progression. Notably, the diagnosis of ALS was made in pt 78 very early in her disease
218 progression. She was able to drive herself to the clinic and walk in with a cane, at the time that
219 the initial blood sample was taken.



220
221 Legend to Figure 5. PBMC extracts were prepared as previously, applied to drug resins and bound proteins analyzed
222 by Western blotting for specific proteins of interest. Bands corresponding to the correct molecular weights were
223 quantified. The presence of an ALS-specific marker at an anomalous migration (17kDa) suggested the presence of a
224 post-translational modification.
225

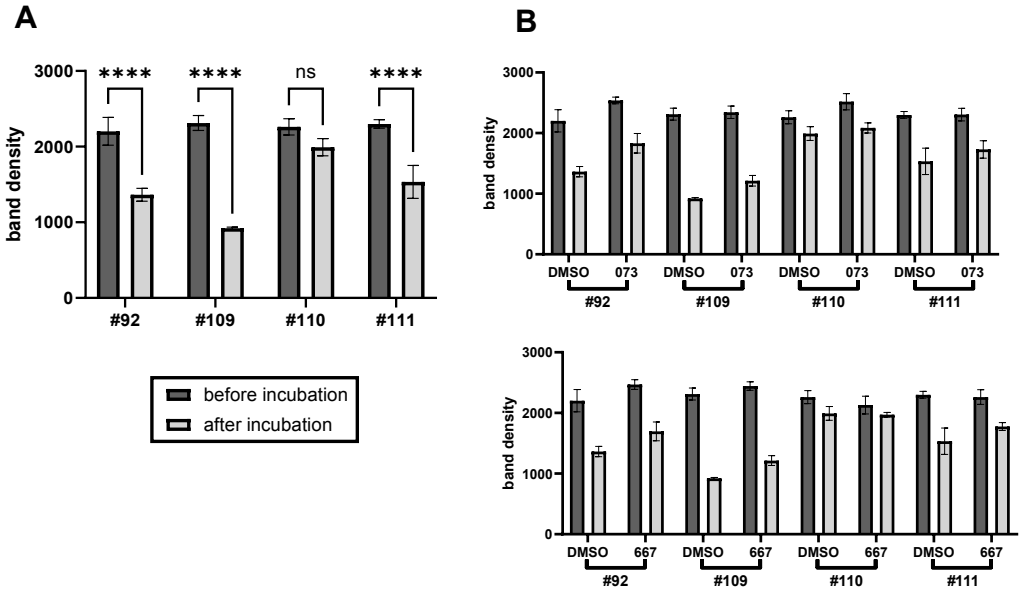
Out of 16 ALS patients and 5 healthy controls assessed in a blinded fashion, 11 ALS patients and 0 healthy controls have the ALS-associated PBMC eDRAC eluate signatures noted, namely, loss of p62/SQSTM1 and appearance of the 17kDA RanGTPase fragment. Thus, taken together, the three eDRAC signatures observed appear to have a sensitivity exceeding 90% and a specificity approaching 100%, albeit from a modest sample size.

If, as we suggest, ALS represents a breakdown in homeostasis where protective mechanisms to reboot a cellular operating system are ineffective, certain predictions follow. For one, there may be a back up mechanism activated in an attempt to correct the failure of the first line of defense, although by definition in the subset of patients with ALS, that backup has failed to correct the problem. In view of the distinctive ALS-associated appearance of a 17kDa RanGTPase fragment as noted in Figure 5 above, degradation of RanGTPase, allowing its resynthesis seemed like a plausible feedback response that could be readily assessed. A second prediction is that if, as we hypothesize, assembly modulation is a fundamental mechanism of regulation of gene expression and its activation by these compounds is therapeutic for ALS, treatment of blood from ALS patients with the drugs should result in cessation of the back up RanGTPase degradation feedback loop, upon restoration of the primary mechanism of homeostatic control.

To test these hypotheses, we took blinded blood samples from ALS patients and healthy controls and treated whole blood with two different, structurally unrelated assembly modulator compounds versus vehicle control, and assess the impact after 72hrs of incubation (Figure 6). In

248 the healthy controls RanGTPase was observed to be stable. However in blood from ALS patients,
249 there were greatly diminished levels of RanGTPase after a brief incubation, consistent with what
250 would be expected for feedback degradation of dysfunctional RanGTPase (Figure 6A).
251 Upon treatment with either of two ALS assembly modulators for 72hrs, a striking restoration of
252 RanGTPase stability was observed, with no change in the level observed in healthy subjects.

Figure 6.



253

C

Chemotype	Molecular weight (g/mol)	log P	H-bond donors	H-bond acceptors	Topological polar surface area (Å²)	Tanimoto similarity coefficient
T	443.543	4.8	1	5	49	<20%
Q	441.915	6.4	0	7	73.7	<20%

254

255 Legend to Figure 6. A. 10mls of whole blood from ALS patients or healthy controls was collected under an IRB-approved
256 protocol and shipped overnight at room temperature in EDTA-anticoagulated (purple top) tubes without any patient-specific confidential information. An aliquot of whole blood was taken, incubated in 1% SDS for one hour at 37°C, prepared for SDS-PAGE, and set aside. Another aliquot of whole blood was mixed 1:1 with glucose-supplemented MEM and incubated for 72hrs at 37oC in the presence of vehicle, at the end of which it was processed as previously described, and analyzed by SDS-
257 PAGE. (-) refers to the sample prepared for SDS-PAGE immediately, while (+) refers to the sample incubated prior to preparation for SDS-PAGE. B. Samples of whole blood incubated for 72hr with two different assembly modulator drug chemotypes (called T and Q) or vehicle (D) were incubated for 1hr at 37oC in 1% SDS and then analyzed by SDS-PAGE and WB for RanGTPase. C. The two assembly modulator chemotypes studied in B. are compared for drug-like properties and Tanimoto similarity coefficient compared to one another.

Discussion

The studies presented here demonstrate the presence, by the newly developed methods of energy-dependent ALS-active assembly modulator drug resin affinity chromatography and photocrosslinking, of a multi-protein complex in PBMCs that is related to the multi-protein complex observed by the same method in mouse brain and PDFs. As for the drug target in patient-derived fibroblasts (PDFs) where TDP-43 mislocalization and stress-induced aggregation is observed, and also for the target in mouse brain, the drug target in PBMCs has PDI as the direct drug-binding protein. The complex present in human PBMCs also shares with the target in mouse brain and PDFs the presence of RanGTPase, TDP-43, and other proteins, many of which are implicated in the ALS interactome (Figure 2). The precise role of this multi-protein complex drug target in ALS pathophysiology remains to be determined, but based on the efficacy of three structurally unrelated assembly modulators in various animal models, we hypothesize that the drug target serves a role in maintenance of homeostasis. The presence of p62 and optineurin in the complex in PBMCs from healthy individuals, and its loss in ALS patient PBMCs, exacerbated with disease progression (Figure 5), are consistent with this hypothesis.

Likewise, the appearance of a 17kDa post-translationally modified form of RanGTPase in the drug target isolated from ALS patient PBMCs (Figure 3) suggests a complex biology connected to TDP-43 mislocalization in view of the role of RanGTPase in nucleo-cytoplasmic transport. Moreover, these changes appear very early in ALS, detected in one case so analyzed, prior to onset of severe disability (Figure 4). In all three cases assessed, at a second later time point correlated to disease progression, both of these changes, namely loss of p62 and presence of

17kDa PTM RanGTPase, were exacerbated. Taken together with MMP9, which is also enriched in ALS patient PBMC eDRAC bound material, the three signatures identified had a sensitivity of 95% and a specificity of 100% for distinguishing ALS patients from healthy individuals. Of course, larger numbers of cases will need to be assessed, but this early degree of discrimination, using as an affinity ligand a drug shown therapeutically active in animal models, is promising as a novel path to an ALS biomarker highly relevant to ALS therapeutics.

This target multi-protein complex is relevant to ALS for three reasons. First, the drug that served as the ligand to bind and isolate the target is therapeutic in a wide range of ALS cellular and animal models[10]. Second, the composition of the target multi-protein complex includes a number of proteins of the ALS interactome. Third, the isolated target from ALS patient PBMCs is altered in protein composition compared to that from healthy controls. Finally, separate from the specific target in PBMCs a profound degradation of RanGTPase is observed in whole blood from ALS patients but not healthy controls and is rescued upon 72hrs of treatment with ALS-active assembly modulators.

We interpret these findings to suggest that ALS is, fundamentally, a disease of disrupted homeostasis for which assembly modulators may be therapeutic. Just as the vast majority of individuals don't have cancer for most of their lives, but a small number will develop cancer due to failure of host defenses, so also host defenses protect most of us most of the time from the consequences of various central nervous system insults. A portion of that host defense includes restoration of homeostasis following resolution of a triggering insult. Sometimes however, those

host defenses appear to be incompletely efficacious. As a result, homeostasis is not restored and more and more severe backup mechanisms are engaged in a futile attempt to restore homeostasis. By analogy, when the operating system of your computer is frozen (with appearance of the "colored wheel of doom"), simply attempting to undo the key stroke that caused it will not be effective. Instead, it is necessary to reboot the operating system. Autophagy, in part mediated by p62/SQSTM1 and OPTN, are examples of such a reboot of the operating system for cells and organisms. But what happens when the reboot fails, e.g. due to loss of p62 from the dysfunctional target? We propose the consequence, in a range specific circumstances, is ALS. The persistent deviation from homeostasis is a stress to which motor neurons appear highly sensitive, and eventually, succumb. Once motor neurons have died, the disorder can be stabilized, but no longer fully reversed by restoring homeostasis. Hence the importance of early detection to enable early treatment, which the PBMC signatures noted may provide.

The results of the whole blood experiments (Figure 6) are consistent with the hypothesis that the degradation of whole blood RanGTPase observed, is an extreme feedback response to the inability to restore the target complex back to the healthy state, where elevated RanGTPase and loss of p62 in the eDRAC eluate defined target are signatures of the dysfunctional state. Failure of more proximal feedback loops to correct that dysfunction are hypothesized to trigger a global degradation of RanGTPase in an extreme measure trying, unsuccessfully, to restore homeostasis. Treatment with either of two structurally unrelated assembly modulators results in correction of the RanGTPase degradation observed in whole blood, to a significant extent.

This suggests that a multi-step pathway with more than one potential target is involved. We hypothesize that treatment for a longer period would have completely restored RanGTPase levels to normal. Whether assembly modulator drug treatment serves to fully reboot the system, e.g. restore p62, remains to be determined and may vary on a case-by-case basis. Likewise, further studies are needed to determine whether periodic or ongoing drug treatment may be required. Regardless, drug-induced changes in target composition appear to result in restoration of homeostasis at least with respect to RanGTPase degradation. Whether this is reflective of concomitant changes in motor neurons that prevent further motor neuron loss remains to be investigated.

Our findings are significant for a number of reasons. First, these studies provide a novel window into regulatory feedback loops involved in cellular homeostasis. The precise relationship of the target multi-protein complex in healthy individuals vs ALS patients remains to be more fully understood. One hypothesis is that the target multi-protein complex in healthy individuals is the "normal" assembly machine involved in integration of diverse events of homeostasis. This includes TDP-43 localization, in which RanGTPase likely plays a critical role, perhaps along with C9orf72 and other ALS-implicated gene products. Other relevant pathways including autophagic removal of dysfunctional multi-protein complexes are naturally integrated into the physiological feedback pathways that collectively bring about homeostasis. ALS may be an example where a "stuck" pathway triggers more and more desperate feedback attempts to reboot the system without success, culminating in the mass degradation of RanGTPase observed in the whole blood experiment shown in Figure 5. In this model, the assembly modulator compounds serve

346 to reboot the cellular operating system. A future prediction to be tested, is that longer-term
347 treatment of blood results in complete normalization of RanGTPase – and restoration of
348 p62/SQSTM1 to the target complex present in PBMCs. By virtue of having therapeutic small
349 molecules in hand, the pathways discovered are likely to be productively illuminated by e.g.
350 including by fractionation and reconstitution of functional activities in extracts.
351
352 Second, these data, together with the studies shown previously, suggest that, despite the
353 heterogeneity of ALS presentation and progression, a common underlying pathway is the source
354 of the disease – and that assembly modulators are therapeutic at the underlying shared level.
355 While we hypothesize that restoration of autophagy is a crucial part of this pathway, re-
356 establishment of homeostasis likely involves far more than just restoration of autophagy. In this
357 regards the involvement of miniscule subsets of the specific gene products found together in
358 the target multi-protein complex is notable. These targets could not have been detected by
359 powerful molecular biological tools such as CRISPR or siRNA knockdown studies, because of the
360 heterogeneous roles of small subsets of many, if not most, proteins. Thus the concept of protein
361 “moonlighting” may be more pervasive than is currently generally recognized, and a level of
362 organization of gene expression accessed by protein assembly modulation may be therapeutic
363 for diverse diseases in ways not generally appreciated today.
364
365 Finally, it should be noted that many if not most patients come to medical attention long after
366 the most effective period of treatment is lost. Drugs to stop the disease process may still be
367 effective, but rely on maintenance of the underlying framework of feedback loops to restore

368 homeostasis, once disease progression is arrested. Sometimes however, those feedback loops
369 fail and homeostasis is not restored, whether or not the primary disease process has been
370 blocked. We hypothesize ALS is one such example, making it extremely difficult to treat. The
371 methods described here provide a means of detecting ALS early, prior to onset of severe
372 disability, when it is most treatable – and provide a potentially effective treatment in the form
373 of the small molecule ligands themselves. It is notable that pt 78 came to medical attention
374 early in her clinical course, prior to serious disability, and yet, the signature changes (lowered
375 p62 and elevated RanGTPase 17kDa fragment) were observed, and sadly, progressed
376 culminating in her death. To date, these findings have been observed in over a dozen ALS
377 patients and are lacking in six of six healthy controls. If extended and demonstrated to be ALS-
378 specific, they could enable rapid treatment. That PBMCs are a readily accessible tissue enables
379 early diagnosis prior to motor neuron death, essential for optimal treatment of ALS.
380
381 **Methods**
382 **Preparation of PBMC extracts**
383 Whole blood collected in purple-top EDTA tubes from healthy individuals or ALS patients was
384 shipped by overnight courier to the lab where it was diluted 1:1 with phosphate-buffered saline
385 and layered on ficoll and centrifuged for 15 minutes at 3000 rpm at room temperature. PBMCs
386 were collected at the interface, diluted with PBS 1:1 and centrifuged at 1000 rpm/15 minutes.
387 The supernatant was aspirated and the pellet dissolved in p-body buffer (PBB) consisting of
388 10mM Tris pH 7.5 ,10mM NaCl, 6mM MgAc, 1mM EDTA, and 0.35% Triton-X-100 and
389 centrifuged at 4oC 10,000xg/10min.

Drug Resin affinity chromatography

Mouse brains from wildtype or SOD1 mutant animals were homogenized in cold phosphate buffered saline (PBS) (10mM sodium phosphate, 150 mM sodium chloride pH 7.4), then spun at 1,000 rpm for 10 minutes until pelleted. The PBS was decanted and the pellet resuspended in a low salt buffer (10mM HEPES pH 7.6, 10mM NaCl, 1mM MgAc with 0.35% Tritonx100) then centrifuged at 10,000 rpm for 10 minutes at 4°C. The post-mitochondrial supernatant was removed and adjusted to a concentration of approximately 10 mg/ml and equilibrated in a physiologic column buffer (50 mM Hepes ph 7.6, 100 mM KAc, 6 mM MgAc, 1 mM EDTA, 4mM TGA). In some conditions, the extract was supplemented with an energy cocktail (to a final concentration of 1mM rATP, 1mM rGTP, 1mM rCTP, 1mM rUTP, and 5 ug/mL creatine kinase). 30 ul or 230 ul of extract was then incubated for one hour at either 4° C or 22° C degrees on 30 ul of affigel resin coupled to THIQ compound or a 4% agarose matrix (control). The input material was collected and the resin was then washed with 3 ml column buffer. The resins were eluted for 2 hours then overnight at 22°C then 4°C in 100ul column buffer containing 100uM of the cognate compound. Eluates were run on western blot or sent for mass spectrometry for analysis.

Chemical photocrosslinking

Extract from mouse brain and PDFs grown in minimum essential media were prepared as above then adjusted to a protein concentration of approximately 3 mg/ml in column buffer containing 0.01% triton. 1% DMSO or 100uM PAV-073 was added to 6ul of extract, then 3uM of PAV-073 photocrosslinker or a negative control crosslinker (comprising of the biotin and diazirine

412 moieties without compound) were added. The extract was incubated for 20 minutes then
413 exposed to UV at 365nM wavelength for 10 minutes then left on ice for one hour. After
414 crosslinking, samples were divided in two 20 ul aliquots and one set was denatured by adding
415 20 uL of column buffer 4ul of 10% SDS, 0.5 ul 1M dithiothreitol (DTT), and heating to 100^{o}C for 5
416 minutes. Both native and denatured aliquots were then diluted in 800 ul column buffer
417 containing 0.1% triton. 5 ul of magnetic streptavidin beads (Pierce) were added to all samples
418 and mixed for one hour at room temperature to capture all biotinylated proteins and co-
419 associated proteins. Samples were placed on a magnetic rack to hold the beads in placed and
420 washed three times with 800 ul of column buffer containing 0.1% triton. After washing, beads
421 were resuspended in 80 ul of gel loading buffer containing SDS and analyzed by western blot or
422 blot for affinity purified streptavidin. Samples were analyzed by western blot.
423
424 **Western blotting**
425 SDS-PAGE gels were transferred in Towbin buffer (25mM Tris, 192mM glycine, 20% w/v
426 methanol) to polyvinylidene fluoride membrane, blocked in 1% bovine serum albumin (BSA) in
427 PBS, incubated overnight at 4^{o}C in a 1:1,000 dilution of 100ug/mL affinity-purified primary IGG
428 to PDI in 1% BSA in PBS containing 0.1% Tween-20 (PBST). Membranes were then washed twice
429 in PBST and incubated for two hours at room temperature in a 1:5000 dilution of secondary
430 anti-rabbit or anti-mouse antibody coupled to alkaline phosphatase in PBST. Membranes were
431 washed two more times in PBST then incubated in a developer solution prepared from 100 uL of
432 7.5 mg/mL 5-bromo-4-chloro-3-indolyl phosphate dissolved in 60% dimethyl formamide (DMF)
433 in water and 100ul of 15 mg/ml nitro blue tetrazolium dissolved in 70% DMF in water, adjusted

to 50mL with 0.1 Tris (pH 9.5) and 0.1 mM magnesium chloride. Membranes were scanned and the integrated density of protein band was measured on ImageJ. Averages and the standard deviation between repeated experiments were calculated and plotted on Microsoft Excel.

Tandem mass spectrometry

Samples were processed by SDS PAGE using a 10% Bis-tris NuPAGE gel with the 2-(N-morpholino)ethanesulfonic acid buffer system. The mobility region was excised and washed with 25 mM ammonium bicarbonate followed by 15mM acetonitrile. Samples were reduced with 10 mM dithoithreitol and 60^{o} C followed by alkylation with 5o mM iodoacetamide at room temperature. Samples were then digested with trypsin (Promega) overnight (18 hours) at 37^{o} C then quenched with formic acid and desalted using an Empore SD plate. Half of each digested sample was analyzed by LC-MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive. Peptides were loaded on a trapping column and eluted over a 75 uM analytical column at 350 nL/min packed with Luna C18 resin (Phenomenex). The mass spectrometer was operated in a data dependent mode, with the Oribtrap operating at 60,000 FWHM and 15,000 FWHM for MS and MS/MS respectively. The fifteen most abundant ions were selected for MS/MS.

Data was searched using a local copy of Mascot (Matrix Science) with the following parameters: Enzyme: Trypsin/P; Database: SwissProt Human (conducted forward and reverse plus common contaminants); Fixed modification: Carbamidomethyl (C) Variable modifications: Oxidation (M), Acetyl (N-term), Pyro-Glu (N-term Q), Deamidation (N/Q) Mass values: Monoisotopic; Peptide

Mass Tolerance: 10 ppm; Fragment Mass Tolerance: 0.02 Da; Max Missed Cleavages: 2. The data was analyzed by spectral count methods.

Whole blood incubation and analysis. 100ul aliquots of EDTA-anticoagulated whole blood is dispensed into 24 well plates containing 100ul of room temperature 80% PBS with 10mM glucose and then treated with DMSO to 0.01% or drug in DMSO to the same dilution with final concentration of 5uM drug. After 72hrs samples are collected and aliquot 10ul into 1XLB as start material and aliquot another 10ul into 1%SDS ECT(1mM rNTPs with 0.2ug/ml CK, 50mM Hepes pH 7.6 100mK KAc, 6mM MgAc and 4mM TGA) at total volume 100ul and incubate at 37°C for 1hr, then put samples into LB. Compared by SDS-PAGE and WB for RanGTPase with whole blood aliquot taken as start material.

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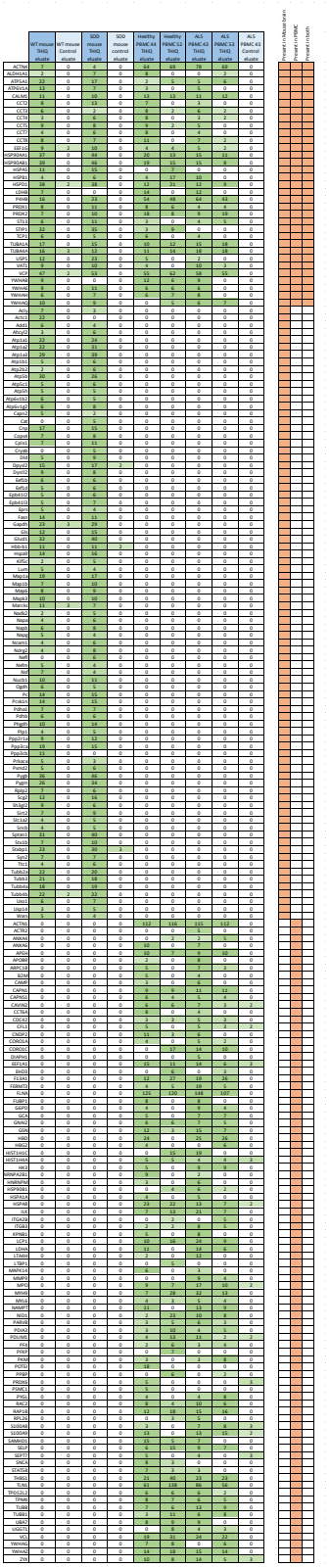
Supplemental Figure 1



Legend to Supplemental Figure 1. Spectral counts for entire dataset of mouse brain and PBMC THIQ eluates. Colors represent the number of spectral counts for a given protein in each sample, ranging from light green (low counts) to dark green (high counts). The right panel indicates whether a given protein was in one of both of the datasets, according to threshold values of >4 spectral counts in the drug eluate and <4 spectral counts in the control column eluate.

Abbreviations

ALS – Amyotrophic Lateral Sclerosis

CFPSA – Cell-free protein synthesis and assembly

CNS – Central Nervous System

DTT - dithiothreitol

DRAC – drug resin affinity chromatography

eDRAC – energy-dependent drug resin affinity chromatography

FTD – Fronto-Temporal Dementia

IND – investigational new drug

MS-MS – tandem mass spectrometry

PBMC – Peripheral blood mononuclear cell

PDF – patient-derived fibroblast

PDI – protein disulfide isomerase

PTM – post-translationally modified

SAP – streptavidin precipitation

SDS-PAGE – polyacrylamide gel electrophoresis in sodium dodecyl sulfate

WB – western blot

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546 **Competing Interests**

547 VRL is CEO of Prosetta Biosciences

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