TEXAS WOMAN'S UNIVERSITY™

Biology Department

A Screen for modulators of N end rule pathway

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Abstract

Arginyl-tRNA-protein transferase (ATE1) is responsible for the post-translational transfer of arginine onto proteins bearing N-terminal acidic amino acids(1). The N-end rule pathway of the ubiquitin proteasome system recognizes proteins bearing N-terminal hydrophobic or basic amino acids such as arginine. These N-terminal amino acids function as degradation signals called N-degrons. Previously, we discovered that ATE1 is required for the degradation of TDP43^{247}, a specific fragment of the human TDP43 protein associated with Amyotrophic Lateral Sclerosis and other forms of dementia(4). Here, we generated a fluorescent GFP reporter bearing the N-degron of TDP43^{247} (247-DLIIKGISVHISNAEPK-263) which elicits a "digital response" with respect to degradation by the N-end rule pathway(4) Using this reporter, we are developing a highly sensitive, cell-based screen to identify chemical or genetic modifiers of the N-end rule pathway. Ultimately, this work may offer therapeutic potential in treating neurodegeneration

Introduction

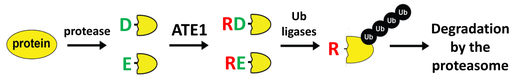

Fig 1. ATE1-dependent removal of cellular protein fragments by the N-end rule pathway. During normal as well as many disease conditions, proteins are cleaved into fragments (hatched yellow oval) by cellular proteases. Fragments bearing N-terminal acidic amino acids (Asp or Glu) are N-terminally arginylated by ATE1(1).These arginylated fragments are subsequently poly-ubiquitylated by specific Ub ligases of the N-end rule pathway. Poly-ubiquitylated proteins are ultimately degraded by the proteasome.

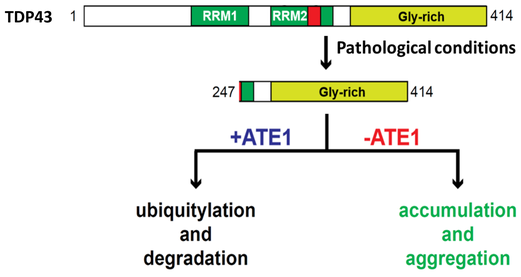

Fig 2. ATE1-dependent removal of cellular protein fragments by the N-end rule pathway. Under pathological conditions, the human TDP43 protein is cleaved into aggregation-prone C-terminal fragments. One such fragment, TDP43^{247}, begins with Asp at the 247th position. In the presence of ATE1, TDP43^{247}, is arginylated and subsequently degraded by the N-end rule pathway. In the absence of ATE1, TDP43^{247}, accumulates and form toxic aggregates(4).

Methods and Results

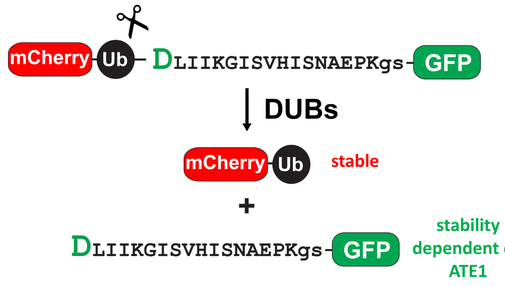

Fig 3. Generation of a dual fluorescent reporter for ATE1 activity. The Ubiquitin Fusion Technique (3) will be used to express a stable mCherry-Ub and a GFP bearing an N-terminal degradation signal (Ndeg) comprised of TDP43 residues (247-263), which is recognized by ATE1. Co-translational cleavage of mCherry-Ub-Ndeg-GFP by intracellular deubiquitylyses (DUBs) produces, at initial equimolar ratio.

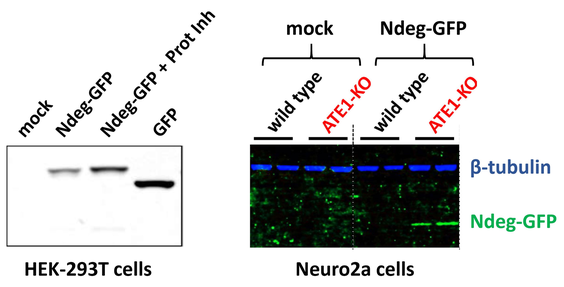

Fig 6. Validation using western blotting. A, Expression of Ndeg-GFP(3) or GFP control in HEK-293T cells. B Expression of Ndeg-GFP in wild type Neuro2a cells or Neuro2a cells that had undergone Crispr-Cas9 mediated removal of the *Ate1* gene. Expression is detected using an anti-GFP antibody.

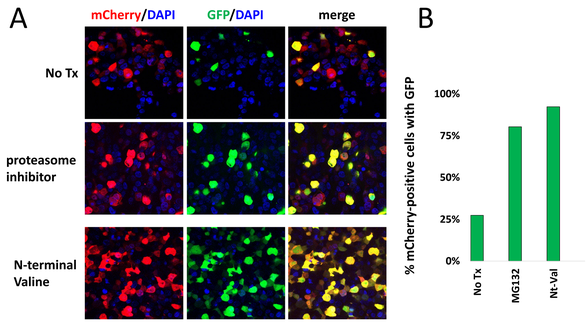

Fig 4. Validation using confocal microscopy. Representative images showing HEK293T cells expressing the dual fluorescent construct shown in Fig. 3. A, *Upper panels*, untreated cells. *Middle panels*, cells treated with the proteasome inhibitor, MG132. *Lower panels*, cells expressing reporter bearing N-terminal Val which is not recognized by ATE1. B, Percentage of mCherry positive cells also expressing GFP.

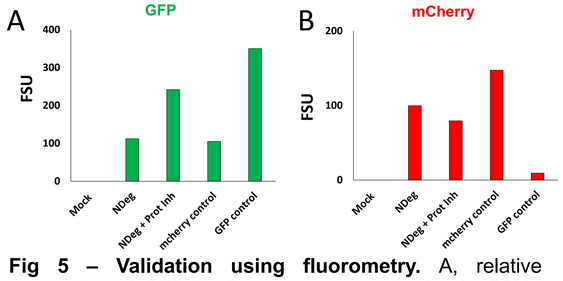

Fig 5 – Validation using fluorometry. A, relative expression of GFP (in fluorescence standard unit (FSU)) captured by blue module (Ex 460nm, Em 515–570nm). B, relative expression of mCherry (FSU) captured by green module (Ex 525nm, Em 580–640nm).

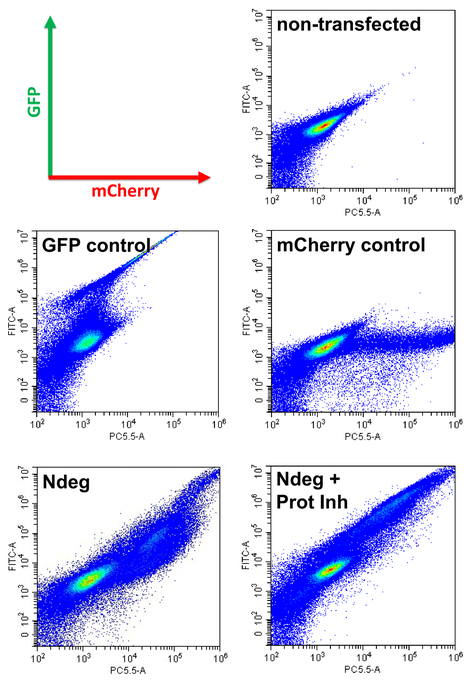

Fig 7. Validation using flow cytometry. Cells transiently transfected with either plasmids expressing GFP or mCherry alone or the dual fluorescence construct shown in Fig 3. Cells were analyzed for GFP expression (FITC-A in y-axis) and mCherry expression (PC5.5A in x-axis).

Future Direction

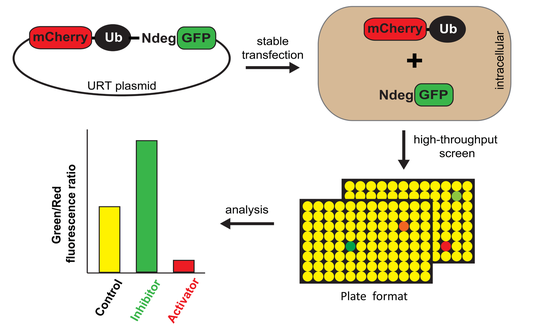

Fig 8. Design of a high- throughput screen for modulators of ATE1 activity. The Ndeg-GFP reporter can be used in screens designed to identify modulators of ATE1 activity. An Increase in GFP signal (relative to mCherry) will indicate inhibition of ATE1 activity whereas a decrease in GFP signal(relative to mCherry) will indicate activation of ATE1 activity. These can be screens of small chemical compounds to identify pharmacological modulators or in Crispr or RNAi-based screens to identify genetic modulators.

Conclusions

- Human TDP43 amino acids 247-263 can function as an N-terminal Degradation signal (NDeg) when fused to a reporter (e.g. GFP)
- Ndeg fused to GFP acts as a sensor for ATE1 activity.
- Ndeg-GFP can be incorporated into a high-throughput screen for chemical or genetic modulators of ATE1 activity.
- Such modulators may have therapeutic value in the fight against obesity(2) and/or neurodegeneration.

Acknowledgements

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References

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