A Mass Spectrometry Proteomics-Based Approach to Identify Target Receptors for Novel Extracellular tRNA Synthetase Fragments

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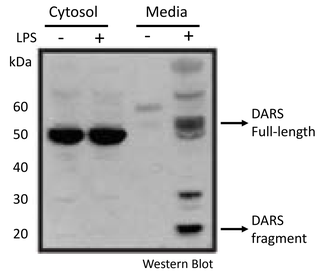
Overview

- **Purpose:** To identify target receptors and determine the biological functions of novel extracellular tRNA synthetase fragments with links to immune modulation
- **Methods:** tRNA synthetase fragments were expressed recombinantly, and their binding to various human cell lines was assessed by flow cytometry. Receptor screening was completed in three cell-lines using the ligand-receptor capture technology LRC-TriCEPS followed by mass spectrometry proteomics analysis[1,2]. Target validation was completed by siRNA knock-down and flow cytometry, and biological function was determined using a FRET-based enzyme-inhibitor assay.
- **Results:** Utilizing this workflow, we successfully identified target cell-surface receptors for tRNA synthetase fragments and have gained insight into their previously unknown biological functions. In doing so, we have also created a novel approach which can be applied more broadly to identify receptor targets of extracellular proteins in an endogenous system.

Introduction

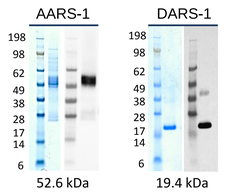
- While canonically known for their intracellular role in protein synthesis, full-length and splice or proteolytic variants of tRNA synthetases have been found to exist in the extracellular space where they may play an immunomodulatory role.
- Full-length Histidyl-tRNA synthetase (HARS) has been established as a molecule present in circulation that modulates T-cell activity, and a HARS variant has been shown to bind to Neuropilin-2 and to inhibit proinflammatory chemokines and cytokines.
- Alanyl-tRNA Synthetase (AARS) and Aspartyl-tRNA Synthetase (DARS) are also present extracellularly and have links to immune modulation; however, their receptor targets and downstream biological function remain unknown:
  - Auto-antibodies targeting AARS and other synthetases are present in rare anti-synthetase syndromes associated with inflammatory phenotypes such as myositis and interstitial lung disease[3].
  - Full-length DARS protein and a DARS fragment are secreted from THP-1 Macrophages when stimulated with LPS (shown to the right).

DARS fragment secreted upon stimulation of THP-1 macrophages with LPS:



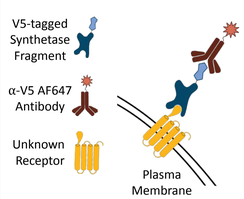
Materials and Methods

Recombinant Expression of AARS and DARS Fragments



- V5-tagged extracellular tRNA synthetase fragments AARS-1 and DARS-1 were expressed recombinantly in Expi293 or ExpiCHO cells and purified for downstream *in vitro* assays.
- Shown to the left are SDS PAGE (blue) and anti-V5 Western blots (grey) detecting purified AARS-1 and DARS-1 under non-reduced conditions.

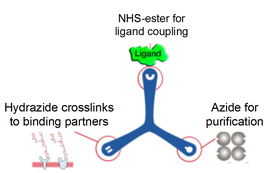
FACS Cell Binding Assay



- AARS-1 and DARS-1 binding to unknown cell surface proteins on various cell types was tested by flow cytometry.
- Cells were incubated with 300 nM protein for one hour, followed by a 30-minute incubation with a fluorescently-conjugated α-V5 detection antibody.
- Binding was quantified as median fluorescent intensity (MFI), and fold changes were calculated relative to α-V5 detection antibody alone.

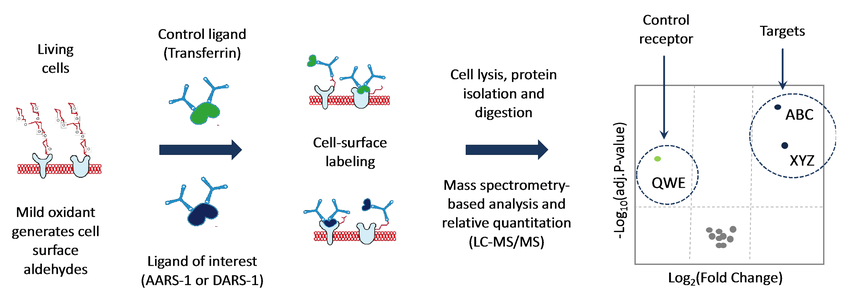
LRC-TriCEPS Mass Spectrometry Proteomics Receptor Screen

TriCEPS 3.0 Reagent



- Ligand of interest is coupled to TriCEPS reagent.
- Upon ligand binding, TriCEPS cross-links to the receptor targets via the hydrazide functional group.
- Ligand-Receptor complexes are isolated using the azide group for purification.

LRC-TriCEPS Receptor Screen



Experiments performed in triplicate;
Thresholds for significance: Log_{10} p-value > 2; Log_2 fold change > 2

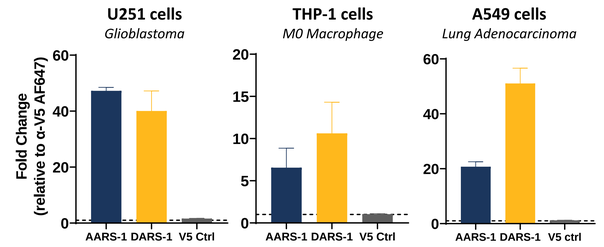
DualsystemsBiotech
LRC-TriCEPS - We identify targets on living cells

Results

AARS-1 and DARS-1 Binding to Human Cells

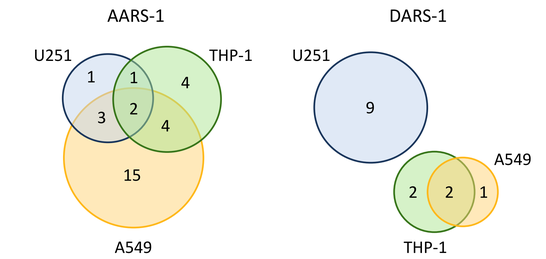
	Human cell type	Differentiation state	AARS-1	DARS-1
Innate Immune cells	Monocyte THP-1	Naïve	+	+
	Primary monocytes (classical)	Naïve	-	-
	Primary monocytes (classical)	Activated (PMA)	++	++
	Monocyte THP-1	M0 (PMA)	+	+
	Monocyte THP-1	M1 (PMA/LPS/IFNγ)	-	-
	Primary macrophages	All	-	+
	Natural Killer NK-92	Naïve	-	++
	Primary NK cells	Naïve	+	+
Adaptive Immune cells	T cell Jurkat	Naïve	+	+
	T cell Jurkat	Activated (αCD3/αCD28)	+	+
	T cell Jurkat	Activated (PMA)	-	-
	Primary CD4+ T-cells	CD4+	-	-
	Primary CD8+ T-cells	CD8+	+	+
	Primary NK-T cells	Naïve	+	+
Oncology	Glioblastoma U251	Naïve	++	++
	Glioblastoma U87	Naïve	+	+
	Lung adenocarcinoma A549	Naïve	++	++

Fold change: **≥ 20, ≥ 3, < 3**



- AARS-1 and DARS-1 binding, as well as a V5-tag control, to a panel of primary and immortalized cell-lines was assessed by flow cytometry – data overview shown in table to left.
- Three cell-lines were selected based on cell binding data for downstream receptor screening experiments – shown in graphs above (mean ± SEM; n=2-3).

Receptor Candidates Identified from 3 Receptor Screens

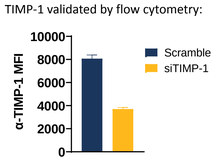


- Multiple undisclosed candidate receptors were identified for both AARS-1 and DARS-1 (as indicated by numbers above).
- Some candidates appear to be cell-type specific, while others were identified across multiple cell-types.
- The candidates identified indicate potential involvement in multiple biological pathways including TGF-β and IGF signaling, oncology and fibrosis.
- TIMP-1 protein, a regulator of extracellular matrix (ECM) degradation, was identified as a candidate for DARS-1 and selected for follow-up experiments.

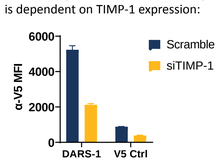
DARS-1 candidate TIMP-1: Binding validation and Functional Activity

DARS-1 binding to THP-1 M2 Macrophages is TIMP-1 dependent

siRNA knockdown of DARS-1 candidate TIMP-1 validated by flow cytometry:



DARS-1 binding to THP-1 M2 macrophages is dependent on TIMP-1 expression:

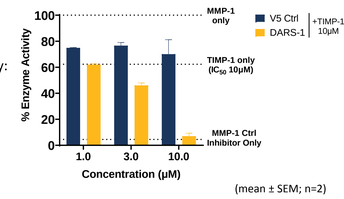


(mean ± SEM; n=2)

DARS-1 enhances TIMP-1-mediated inhibition of MMP-1

- TIMP-1 regulates ECM degradation by inhibiting matrix metalloproteinases (MMPs).
- FRET-based MMP-1 enzyme inhibitor assay: TIMP-1 inhibits MMP-1 (IC_{50} = 10.2 μM).
- DARS-1 enhances TIMP-1-mediated inhibition of MMP-1 activity in a dose-dependent manner (shown to the right).

Matrix Metalloproteinase-1 Activity

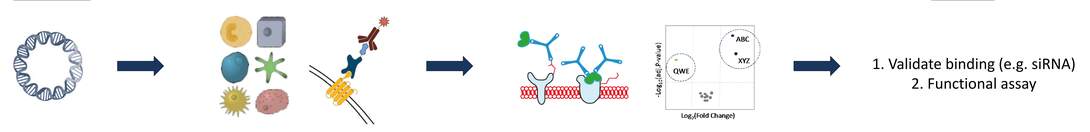


(mean ± SEM; n=2)

Conclusions and Workflow

- Extracellular AARS and DARS fragments, AARS-1 and DARS-1, were successfully expressed recombinantly, and their target receptors were identified in endogenous systems using the ligand-receptor capture technology LRC-TriCEPS and mass spectrometry proteomics.
- The receptors identified provide new insight into the biological activity of extracellular tRNA synthetases.
- DARS-1 was shown to enhance TIMP-1-mediated inhibition of MMP-1 activity, indicating it may have a regulatory role in extracellular matrix remodeling.

Workflow:



References

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3. Witt, Leah J et al. "The Diagnosis and Treatment of Antisynthetase Syndrome." Clinical Pulmonary Medicine vol. 23,5 (2016): 218-226. doi:10.1097/CPM.0000000000000171

Acknowledgements

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