Generation and validation of recombinant antibodies to study human aminoacyl-tRNA synthetases

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Aminoacyl-tRNA synthetases (aaRSs) have long been viewed as mere housekeeping proteins and have therefore often been overlooked in drug discovery. However, recent findings have revealed that many aaRSs have noncanonical functions, and several of the aaRSs have been linked to autoimmune diseases, cancer, and neurological disorders. Deciphering these roles has been challenging because of a lack of tools to enable their study. To help solve this problem, we have generated recombinant high-affinity antibodies for a collection of thirteen cytoplasmic and one mitochondrial aaRSs. Selected domains of these proteins were produced recombinantly in Escherichia coli and used as antigens in phage display selections using a synthetic human single-chain fragment variable library. All targets yielded large sets of antibody candidates that were validated through a panel of binding assays against the purified antigen. Furthermore, the top-performing binders were tested in immunoprecipitation followed by MS for their ability to capture the endogenous protein from mammalian cell lysates. For antibodies targeting individual members of the multi-tRNA synthetase complex, we were able to detect all members of the complex, co-immunoprecipitating with the target, in several cell types. The functionality of a subset of binders for each target was also confirmed using immunofluorescence. The sequences of these proteins have been deposited in publicly available databases and repositories. We anticipate that this open source resource, in the form of high-quality recombinant proteins and antibodies, will accelerate and empower future research of the role of aaRSs in health and disease.

Aminoacyl-tRNA synthetases (aaRSs) are a group of enzymes that play an important role in the protein translation machinery. However, during the last decades, these proteins have also been connected to several diseases. Their main function is to catalyze the production of aminoacyl-tRNAs by attachment of each amino acid to its cognate tRNA (1, 2). There are 17 cytoplasmic aaRSs in human cells (including the bifunctional EPRS (GluRS and ProRS)), 17 mitochondrial, and 2 that occur both in the cytoplasm as well as mitochondria (3, 4). All aaRSs consist of an aminoacylation domain (2, 3), but higher organisms have added new domains throughout evolution (2), and during the last decade, multiple new functions of these proteins have been discovered (5). Interestingly, the discoveries and research of these noncanonical functions have shown their involvement in inflammation, tumorigenesis, angiogenesis, neurological disorders, and other physiopathological processes (3, 6–9).

Although the cytoplasmic aaRSs are carrying out their aminoacylations in the cytoplasm, full-length aaRSs or truncated versions generated by proteolytic cleavage or alternative splicing have been detected in the nucleus and even outside the cell (10, 11). The secretion of aaRSs have been observed in many different cell types, and together with their observed activity in cytokine signaling pathways, an extracellular signaling role of this enzyme family has been implied (12). One example is TyrRS, which can be cleaved into two parts subsequently acting as cytokines (13). Another example is TrpRS, which has been shown to be rapidly secreted upon pathogen infection and subsequently prime innate immunity by inducing phagocytosis and chemokine production (14, 15). The mechanism for the secretion of aaRSs is not fully understood, but it has been reported that LysRS, for example, is secreted via exosomes in cancer cells (16).

The presence of aaRSs outside the cell, is further supported by the existence of autoantibodies against at least eight aaRSs. These autoantibodies have been detected in idiopathic inflammatory myopathies (IIMs, collectively called myositis), a group of rare chronic autoimmune diseases, and specifically in antisynthetase syndrome, a subgroup of IIM (17, 18). HisRS represents the major autoantigen in these diseases (19), and autoantibodies against this antigen have been reported in up to 20–30% of IIM patients (20). Recently, we also demonstrated the presence of HisRS in the circulation, and its immunomodulatory role was characterized (11).

The aaRSs have long been viewed as housekeeping proteins and disregarded within drug discovery, but the list of noncanonical functions of this protein class, as well as the connections to various disease processes, is steadily increasing, demonstrating their relevance in the clinical setting. We are convinced that many more interesting findings within this field are still to be discovered, and through the open science initiative ULTRA-DD (RRID:SCR_01899), we are committed to
providing researchers with well-validated tools to accelerate such findings. Here, we describe the establishment of a toolbox of renewable and specific high-affinity recombinant antibodies to a large panel of the cytoplasmic aaRSs. The antibodies have been validated for their ability to capture their endogenous target protein through IP-MS, and many of them have also been tested and confirmed in immunofluorescence.

Results

Antigen production

Based on clinical relevance and availability of 3D-structures, we selected 14 aaRSs and produced biotinylated recombinant proteins of these. For AlaRS, more than one version was generated to allow selection of binders to different domains, as indicated in Table 1. To achieve site-specific biotinylation, Avi-tagged target proteins were co-expressed in Escherichia coli with the bacterial protein–biotin ligase BirA (21). The target proteins were then purified from soluble cell extracts by a two-step procedure including immobilized metal affinity chromatography (IMAC) followed by size-exclusion chromatography (SEC) (Fig. 1). All selected antigens were purified to >90% purity and monodispersity and had biotinylation levels close to 100% as indicated by MS analysis.

Antibody generation and characterization

The produced proteins were used as antigens in phage display selections. Individual scFv clones from selection rounds 3 and 4 were characterized in a panel of binding assays, as depicted in Fig. 1. The number of clones that were considered positive in a certain assay and that progressed to the next validation assay can be found in Table S1. Initially the generated scFv were screened with ELISA and homogenous time resolved fluorescence (HTRF). The specificity of the binders was further assessed using a suspension bead assay (Luminex), in which all sequence-unique binders were tested against their target aaRS, as well as all the other produced aaRS antigens. Fig. 2, which includes a subset of the analyzed binders, clearly demonstrates that all of them bind their cognate aaRS and show no cross-reactivity to any of the other included antigens. For unknown reasons, this assay did not give any signals for any of the binders specific for LysRS, LeuRS, or AsnRS. Instead, these binders were tested in ELISA (Fig. S1) and HTRF against a panel of different aaRSs, showing no cross-reactivity.

Based on the specificity screen, binders were selected for affinity ranking before small-scale purification, and kinetic measurements using surface plasmon resonance (SPR). The results of the kinetic measurements for some of the validated scFv can be seen in Fig. 3. Based on the affinity results, we selected the scFv candidates most likely to pass “gold standard” binder criteria (see below for definition), to be tested in IP-MS. Specifically, binders with slow off rates were prioritized. A slow off rate is important for the binder to capture the occasional low levels of endogenous protein in the cell lysate and to retain the binding during the harsh washing steps used in the IP-MS procedure (22). Selections on all antigens generated binders with a measured binding strength in the low nanomolar range (Table 2), with favorable off rates that would usually be enough for passing IP-MS. Still, it is important to be aware that approximately half of the aaRSs combine and form quaternary structures, usually in the form of dimers. This formation takes place both in the natural environment within the cell (7) and when produced recombinantly. Binding strength is traditionally reported by the affinity constant (Kd). However, this constant is used to describe the strength of a monovalent interaction, and being aware that several of the aaRS most likely interact with the scFv surface in a bivalent fashion, thereby potentially giving rise to synergy and an apparent increase in affinity, we have here instead decided to report the apparent affinity (denoted as App Kd). The avidity contribution of an interaction will depend not only on the antigen oligomeric state but also on the scFv itself. Binders may have very different avidity effects because of differences in binding kinetics (23). Despite the avidity contribution for some of the binders, we have chosen a 1:1 binding model for calculation of App Kd for all scFv clones. Although one might argue that this procedure is not correct from a kinetic

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Table 1

List of aminocyl-tRNA synthetases with antigen details

Clinical names are used for descriptions of aarS and autoantigens in idiopathic inflammatory myopathies. Antigen aa indicates the antigen amino acid coverage. Yield indicates the yield of purified antigen in mg/liter of E. coli culture as determined from absorbance at 280 nm after IMAC and SEC purification. The Addgene ID column shows the expression vectors of each of the constructs are available through Addgene accession numbers 153046–153060.
perspective, we believe it provides an average picture of the binding interaction that allows us, in a pragmatic way, to select binders most suitable for IP-MS.

**Antibody validation**

The top-performing scFv for each antigen were subjected to IP-MS validation against endogenous proteins from HEK293 cell lysate. This method ensures that the antibody is able to bind to its native antigen in cell lysates among thousands of other proteins, DNA, RNA, and other cellular components. In addition, the IP-MS method identifies other proteins the antibody is able to immunoprecipitate allowing for the assessment of antibody specificity and selectivity. Furthermore, the method allows detection of proteins co-precipitating with the target. Thus, potential interaction partners can be identified. For all 14 targets, we found antibody fragments that passed IP-MS validation (Table 2) according to the gold standard criteria. Gold standard binders are here defined as antibodies for which the target antigen or a member of its known protein complex provides the highest normalized spectral abundance factor (NSAF), i.e. similar to the rating used previously for IP-MS validation of antibodies (24). One example of a gold standard binder is the anti-HisRS scFv J-HARS-4. For HisRS, mitochondrial and cytoplasmic versions are closely related and share high sequence similarity (76% sequence identity, BLAST entries P12081 and P49590), and as expected we detected spectral counts for both (Fig. 4), even though the spectral counts for mitochondrial HisRS are significantly lower. As mentioned above, the binders tested in IP-MS were selected based on their affinity and particularly on their off rate. However, because we do not know the binding epitope of the antibodies, it is possible that some of the antibodies that fail in this assay target an epitope of the aaRS that is blocked in the cell lysate, because of interaction with another partner, or alternatively because of potential post-translational modifications of the targeted epitope, thus resulting in none or low NSAF values despite advantageous off rates (22). Examples of such binders could be J-HARS-3 and J-HARS-6, which both have favorable off rates (0.005 s⁻¹) but did not IP any significant levels of HisRS in IP-MS (Fig. 4). Therefore, these scFv are not counted as validated antibodies according to the set criteria. The J-HARS-1 scFv also has an affinity in the low nanomolar range, but because this clone also immunoprecipitated a protein not known to be a binding partner to HisRS (Fig. 4, green), this binder needs further analysis before it can be counted as gold standard (beyond the scope of this paper) and is therefore not included in Table 2.

It has been shown that nine of the aaRSs, together with three scaffold proteins, jointly form the so-called multi-tRNA synthetase complex (MSC) (25, 26). To our knowledge, there is not much known about the expression levels of aaRSs, in complex versus in free form, for different cells or cell lines. We chose scFv binders specific for four of the MSC members (ArgRS, AspRS, LeuRS, and MetRS) and investigated whether they could immunoprecipitate the complex in different cells or cell lines. We selected HEK293, A549 (human lung cancer epithelial cell line), and peripheral blood mononuclear cells (PBMC)...
for this assay and used the same total protein amount for all samples. Indeed, binders against ArgRS and LeuRS were successful in immunoprecipitating the whole MSC, including the three core structural components AIMP1, AIMP2, and AIMP3 from several cell types (Fig. 5). The absence of cross-reactivity of the selected scFv, as shown by the results obtained in the specificity screen (Fig. 2 and Fig. S1), suggests that we have co-immunoprecipitated true interaction partners. For binders targeting ArgRS and LeuRS, we recorded the highest NSAF for the cognate antigen and, as expected, lower numbers for the other complex components. Unfortunately, because MS, as used here, is not quantitative only general assumptions can be made from the NSAF values. It is noteworthy, however, that MetRS has the lowest NSAF value of the immunoprecipitated MSC members for both ArgRS and LeuRS binders, possibly indicating that this protein is quite loosely attached to the complex. It has been suggested that MetRS could be easily released from the MSC, without interrupting the overall organization (27). In contrast, a binder targeting MetRS itself resulted in a high NSAF value, proving that the protein is present in large amounts. It has been suggested that MetRS is overexpressed in non-small cell lung cancer (28), but it is to our knowledge not known whether the overexpression leads to increased expression of only the free but not the complexed form of MetRS, something our data suggest (Fig. 5). The overall low NSAF values of the MSC members in PBMC, compared with the cell lines HEK293 and A549, could be explained by the lower proliferation rate of these cells.

**Immunofluorescence**

To broaden the usefulness of the toolbox presented in this paper and to look further into the localization of the aaRSs, we decided to set up a method for immunofluorescence (IF) using a subset of the generated gold standard validated scFv binders (Table 2). The IF was performed using U-2 OS cells (human osteosarcoma cell line), using different fixation methods (methanol and paraformaldehyde) and different scFv concentrations. The results of a representative set of binders can be found in Fig. 6. All scFv tested in IF stained as expected: the scFv binding cytoplasmic aaRSs were only visible in the cytoplasm (Fig. 6B), the scFv binding mitochondrial aaRSs showed staining only in mitochondria (Fig. 6C), and the scFv binding to dual-localized aaRSs demonstrated staining in both the mitochondria and the cytoplasm (Fig. 6D).
Discussion

Antibodies are crucial tools, used in basic research, but also in clinical assays. Unfortunately, the quality of research antibodies has been a concern and is widely discussed by the scientific community (29). During the last years, standards for proper antibody validation have been suggested (30–32) to meet the high demands for well-validated and renewable research tools (29). In the same spirit, with a focus on under-explored novel targets in autoimmune and inflammatory diseases, the IMI-project ULTRA-DD (RRID:SCR_01899) was initiated to, among other things, generate high-quality antibodies and make them available without restrictions on use for the scientific community.

The accumulation of evidence that many of the aaRSs carry out noncanonical functions beyond translation (1, 10) has spurred the interest to study this protein family further. Specifically, our motivation for including this group of enzymes on the list for antibody generation was mainly based on their reported role in inflammation and autoimmunity (11, 19, 33). There is clearly a need for well-validated antibodies to investigate these novel functions, both inside and outside of the cell.

Although there are existing antibodies targeting the aaRS family available on the market, many of these are polyclonal and not renewable. Furthermore, many of these commercial antibodies are only validated in methods where the antigen is completely or partly denatured, e.g., Western blotting or immunohistochemistry. Thus, there is a risk that these antibodies might not work in other applications where the antigen in its native conformation is targeted, such as IP-MS. Being aware of the fact that one antibody binding a particular antigen format will not fit all purposes or assays, there is of course a need for different categories of antibodies, e.g., recognizing antigens in both their native and denatured form. Accordingly, it is essential that the end user is aware of the validation context for a specific antibody to ensure successful execution of an experiment (32).

We have, as described in this paper, produced recombinant aaRS proteins and used these as antigens to generate a toolbox of validated recombinant antibodies. The diverse panel of specific and high-affinity antibodies generated here illustrates the quality of the established pipeline, including antibody library design, antigen production, and the different assays of the screening cascade (Fig. 1). We have generated the binders in...
scFv format, but one of the strengths of using a recombinant method, as compared with for example hybridoma technology, is that the sequences are easily accessed, and hence, the scFv can swiftly be converted to other preferable formats, such as IgG.

It is important to acknowledge that all validation methods have their advantages and disadvantages. IP-MS, one of the proposed validation standards (30) and here used as a final validation method, is, as described under “Results,” a very powerful technique, but it comes with limitations. Not only does it depend on the antibody targeting an available epitope in a very complex sample, it also relies on finding cells or cell lines that express the target protein at high enough concentration. Fortunately, because the aaRSs are essential proteins, the latter is not

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<td>&lt;1</td>
<td>113</td>
<td>Yes</td>
<td>MT074322</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4. IP-MS results of binders targeting HisRS. Shown are five scFv (J-HARS-1, -3, -4, -6, and -58) binding HisRS, and five unrelated scFv (x axis), measured by NSAF (normalized spectral abundance factor) (y axis). NSAF values for cytoplasmic HisRS are highlighted in red, with mitochondrial HisRS in blue and an additional protein, a potential binding partner of HisRS, in green. Gray represents background signals that are similar in all samples.

EDITORS’ PICK: Recombinant antibodies targeting aminoacyl-tRNA synthetases

Table 2

Binders validated as gold standard

aaRS indicates aaRS to which the validated scFv is binding to. The scFv column shows the names of validated scFv. $^{App}K_D$ is the apparent affinity range for the specific binder measured by SPR, and NSAF is the normalized spectral abundance factor from IP-MS using HEK293. The immunofluorescence column shows scFv tested in IF and how it performed (Yes indicates tested and with expected localization confirmed, and NA indicates not tested). The sequences of the listed scFv are available in Table S2 and in GenBank™ under accession numbers MT074296–MT074325. The 12 scFv in bold type were converted to human IgG1. As indicated, similar binding constants of these were obtained ($^{App}K_D$, IgG).
an issue here. Nevertheless, for antibody validation in general, it is important to take into consideration that the validation of an antibody is dependent not only on the method but also on sample preparation (32).

It has previously been reported that IP-MS is a good method for identifying antibodies that may also be used in IF applications (24). Indeed, all of the 21 tested scFv antibodies worked beautifully in IF and showed staining in their expected target location. Also, when converted to IgG, the same staining pattern was observed (data not shown).

Although this is encouraging, to use IF as a validation method for specificity on its own, it would be desirable to modify expression levels of the targeted protein by gene silencing and make side-by-side comparisons (30). Nevertheless, the obtained IF results combined with the generated IP-MS data suggest that this set of binders has the potential to provide a standardized toolbox to study aaRSs localization that has so far been lacking in the field (34).

Interestingly, and as already addressed under “Results”, some of the aaRSs in human cells form a large complex, the so-called MSC (25, 26). It has been suggested that the components of the MSC (Fig. 5E) can be associated and dissociated depending on the environment in the cell and that the MSC thus functions as a depot system (5, 10, 35). However, much still remains unknown about the MSC, its members, and their involvement in various biological processes (8). The antibodies generated in this study could readily be used to continue the studies of this very interesting complex. In addition, by generating binders to even more components of the MSC, using the recombinant antibody generation pipeline presented here, one could elucidate the complex composition further. Our data (Fig. 5) suggest different protein expression levels of aaRSs in different cells and cell lines. Furthermore, it indicates that the MSC also exist in different organizational assemblies in different cells. The binders we have generated in this study, in combination with a quantitative method for IP-MS, could be used to further investigate the expression levels of aaRSs and the composition and dynamics of the MSC. To date, only subcomplexes of the MSC structure are available (8, 36) and no high-resolution 3D structure of the complete complex (27). It is our hope that this toolbox will be used to address some of these fundamental biological questions.

As discussed above, it has been reported that several aaRSs are secreted, and previous studies have indicated that many of the aaRSs have a large number of splice variants (37, 38). This toolbox could potentially be used to investigate the different aaRSs and their isoforms in circulation and also to study possible extracellular binding partners, which could indirectly be addressed using IP-MS and the generated set of antibodies. Such data could be extremely useful in defining new diagnostic
Much still remains unknown of aminoacyl-tRNA synthetases and their roles in various diseases. By making this toolbox of validated and renewable antibodies accessible, we hereby reach out to the scientific community with the long-term ambition of contributing to more reproducible research results within this fascinating field.

**Experimental procedures**

**Antigen production**

Constructs for antigen production in *E. coli* were made using expression vectors pNIC-Bio3 and pNIC-CTB10H (GenBank™ accession nos. JN792439 and KX139199). pNIC-Bio3 contains an N-terminal His$_6$ tag followed by a TEV protease cleavage site and a C-terminal Avi-tag, whereas pNIC-CTB10H carries a C-terminal Avi tag and a His$_{10}$ tag, preceded by a TEV protease cleavage site. Coding DNA sequences were subcloned by ligation-independent cloning as previously described (42), using Mammalian Gene Collection cDNA clones as templates. The constructs were designed using 3D structures deposited in the Protein Data Bank to ensure native folded antigens. If structural information was available for the full-length protein this was preferentially selected, otherwise shorter variants with putative intact native conformation and oligomeric state were chosen. See Table 1 for details on amino acid coverage and vector setup.

To allow in vivo biotinylation, cloned expression plasmids were transformed into *E. coli* strain BL21(DE3) R3 pRARE2 carrying a plasmid for co-expression of BirA ligase (pCDF-BirA). Based on results obtained in small-scale screening test for soluble expression as previously described (43) (data not shown), clones were selected for scale up.

Selected clones were grown at 37 °C to an $A_{600}$ value of 1.5–2.0 in Terrific broth medium supplemented with kanamycin (50 μg/ml), chloramphenicol (35 μg/ml), spectinomycin (50 μg/ml), and biotin (100 μM). After lowering the temperature to 18 °C, protein expression was induced by addition of isopropyl-β-d-thiogalactopyranoside to a final concentration of 0.5 mM. Cultures were incubated for ~20 h and then harvested by centrifugation at 4500 × g for 15 min. Spun-down cells were resuspended in buffer containing 50 mM HEPES, pH 8.0, 500 mM NaCl, 5% (v/v) glycerol, 10 mM imidazole, 0.5 mM TCEP, and Complete EDTA-free protease inhibitor (Roche), frozen, and stored at −80°C. Frozen resuspended cells were thawed briefly in water and sonicated on ice followed by centrifugation at 44,000 × g for 50 min. The soluble fractions were decanted, filtered (0.45 μm), and subsequently loaded onto 1-ml HiTrap Chelating HP columns (GE Healthcare) loaded with Ni$^{2+}$ ions on a ÄKTA Xpress system (GE Healthcare). After washing with 20 mM HEPES, pH 7.5, 500 mM NaCl, 5% (v/v) glycerol, 10 mM imidazole, and 0.5 mM TCEP, the proteins were eluted in 20 mM HEPES, pH 7.5, 500 mM NaCl, 5% (v/v) glycerol, 250 mM imidazole, and 0.5 mM TCEP. The eluates were applied to HiLoad XK16/60 Superdex 200 columns (GE Healthcare) equilibrated with 20 mM HEPES, pH 7.5, 300 mM NaCl, and 0.5 mM TCEP. Relevant SEC fractions were pooled and analyzed by SDS-PAGE and MS. After assessment of quality and protein

**Figure 6. Immunofluorencence of four scFv.** Each panel shows a representative image of each scFv. A, controls were employed to access background and signal validity including no scFv, a scFv binding to ThrRS (L-TARS-11), or a negative control scFv (G-Strep-1, a streptavidin-binding scFv) in combination with or without secondary anti-FLAG-Cy3 antibody. B, cytoplasmic staining of SerRS using scFv L-SARS-11. C, mitochondrial staining of mitochondrial TyrRS using scFv L-YARS2-9. D, staining of LysRS, one of the dual-localized tRNA synthetases, present in both the cytoplasm and the mitochondria, using scFv L-KARS-9. proteins are used as markers for the cytoplasm and mitochondria, respectively.

and prognostic markers in both IIM and ASS, something strongly wished for by both clinicians and researchers in the field (39, 40).

It has also been reported that several aaRSs have cytokine-like activities (10). Altogether, this indicates a therapeutic potential of human aaRSs, and one could speculate in the possibilities of modulating the immune system, by interfering with some of the aaRS, activities (10). Altogether, this indicates a therapeutic potential of human aaRSs, and one could speculate in the possibilities of modulating the immune system, by interfering with some of the aaRS, with target-specific antibodies or other molecules (5, 10, 41).

To conclude, we have successfully generated recombinant antibodies to 14 aaRSs, and we are currently in the process of making antibodies against the remaining cytoplasmic ones. During the writing of this article, binders to three additional aaRS have been generated: CysRS, PheRS (β-subunit), and GlnRS. These binders have also been validated according to the pipeline presented in this article. All validated gold standard binders will be available at the ULTRA-DD website (https://ultra-dd.org/antibodies), including the last binders to complete the set. Human IgG1 versions for most of the binders are also available through the website.
identity, protein batches were flash-frozen in liquid nitrogen and stored at \(-80\, ^\circ\text{C}\) until use.

**Phage display selections**

The produced recombinant aaRSs were used as antigens in phage display selections using the human synthetic scFv library SciLifeLib, similar in design and construction to previously reported (44). The antigens were selected on in three different campaigns, indicated by the letters J (HisRS), L (GlyRS, LysRS, AsnRS, ArgRS, ThrRS, SerRS, TrpRS, TyrRS, and TyrRS mitochondrial), and O (AlaRS, AspRS, LeuRS, and MetRS). Each selection campaign included four rounds of selections on biotinylated antigens immobilized on magnetic streptavidin beads (Dynabeads M-280 streptavidin, Invitrogen). Prior to the phage-antigen incubation step in rounds 1 and 2, the phage stocks were incubated with streptavidin-coated beads without biotinylated antigens to remove nonspecific or streptavidin binders. In selection round 1, the scFv-phage particles were incubated with antigen-coated streptavidin beads in PBT + (2% (w/v) BSA, 0.05% (v/v) Tween 20 in PBS) for 3 h at room temperature and gentle rotation. In rounds 2, 3, and 4 the antigen-phage incubation time was decreased to 1.5 h. In selection rounds 1 and 2, phage library stocks were added to already antigen-coated beads, whereas phage stocks were added to antigen in solution before capture on magnetic beads in rounds 3 and 4.

The selection pressure was increased for each selection round by decreasing the amount of antigen and by increasing the intensity and the number of washes. The amount of antigen was decreased as follows; 200–260 pmol of antigen in round 1, 100–130 pmol in round 2, 50–65 pmol in round 3, and 10–32 pmol in round 4. For campaigns L and O, five washes were carried out in rounds 1 and 2, six washes in round 3, and nine washes in round 4. In selection campaign J, five washes were made in rounds 1 and 2, six washes in round 3, and seven washes in selection round 4. All washing steps were performed in 800 \(\mu\text{l}\) of PBS-T (0.05% (v/v) Tween 20 in PBS), in a 96-well plate using a KingFisher Flex robot (Thermo Fisher Scientific), except in round 1 of selection campaign J, which was performed manually. Slow washing speed was used in the first two rounds, whereas medium speed was used in the last two rounds.

Bound phages were recovered using digestion with trypsin (Gibco, Life Technologies), which cleaves at a specific site located between the phage protein III and the displayed scFv, followed by inactivation using aprotinin (AppliChem). Recovered phages were amplified overnight by infection of XL1-Blue E. coli, either on agar plates at 37\(\, ^\circ\text{C}\) (round 1) or in solution at 30\(\, ^\circ\text{C}\) (rounds 2–4). To produce an amplified phage stock, M13K07 (New England Biolabs) was used as helper phage. Precipitation of amplified phages was performed using PEG/NaCl, and the pellets were resuspended in PBT + and used in the next round of selection.

**Cloning and expression of binders**

Phagemid DNA from selection rounds 3 and 4 was purified (GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific)), and the genes encoding scFv fragments were digested, ligated into a screening vector (in house constructed), and subse-

**ELISA**

Binding of the selected scFv was initially assessed in two rounds of ELISA experiments. Each clone was tested against its target antigen and a nonrelated antigen, all in duplicate. Streptavidin diluted in PBS was coated in 384-well plates, and biotinylated antigens were added in PBT (0.5% (w/v) BSA, 0.05% (v/v) Tween 20 in PBS), followed by the addition of scFv (culture supernatant). Bound scFv were detected using horseradish peroxidase–labeled anti-FLAG M2 antibody (Sigma–Aldrich A8592). TMB-ELISA substrate solution (Thermo Fisher Scientific) was used as a chromogenic substrate, and the reaction was stopped by adding 1 \(\text{M}\) \(\text{H}_2\text{SO}_4\). The absorbance was measured at 450 nm. The plate was washed four times between each step using PBS-T (0.05% (v/v) Tween 20 in PBS). scFv with a high enough ratio between target and nontarget were cherry-picked, cultured, and re-expressed in a new 96-well plate and assessed in a secondary ELISA screen. The secondary screen had an almost identical setup as the primary, but instead of using a nonrelated biotinylated target as control, the scFv were tested against streptavidin only. Clones considered positive also after this second ELISA were sent for sequencing (GATC Biotech). Sequence-unique clones were given individual names containing the letter indicating the selection campaign and the target gene name followed by a serial number.

**HTRF**

Sequence-unique clones were tested in a HTRF energy transfer assay. Bacterial culture supernatants containing scFv diluted in assay buffer (0.1% (w/v) BSA in PBS) were mixed with biotinylated target to a final concentration of 50 \(\text{nm}\), donor anti-FLAG M2 antibody labeled with Terbium (Cisbio 61FG2TL), and acceptor streptavidin labeled with XL665 (Cisbio 610SAXL) in a total reaction volume of 20 \(\mu\text{l}\) in a 384-well plate. After incubation for 2 h at room temperature in the dark, the binding signal (665 nm) and background/noise signal (615 nm) was measured using EnVision (PerkinElmer).

**Specificity screening**

To further study the specificity of the selected scFv, we set up a bead-based multiplexed assay using the Luminex technology. 500,000 carboxylated color-coded magnetic beads (Magplex Luminex Corp.) per ID were activated by adding a solution of 5 \(\text{mg/ml}\) sulfo-\(\text{N}\)-hydroxysulfosuccinimide and 5 \(\text{mg/ml}\) 1-ethyl-3-(3-dimethylamino- propyl) carbodiimide hydrochloride in activation buffer (0.1 \(\text{m}\) \(\text{NaH}_2\text{PO}_4\), pH 6.2) to a 96-well plate and incubated for 20 min at room temperature with shaking. The beads were washed twice with MES buffer (0.05 \(\text{m}\) MES, pH 5.0) before addition of NeutrAvidin and incubation for 2 h
at room temperature with shaking. Subsequently, the beads were washed twice with PBS-T and storage buffer (blocking reagent for ELISA (Roche) with 0.1% (v/v) ProClin 300 (Sigma–Aldrich)) was added before incubation at 4°C overnight. The storage buffer was removed, and biotinylated proteins (1 μm in PBS) were added to the NeutrAvidin-coupled beads before incubation for 1 h at room temperature with shaking. The plate was washed three times with PBS-T before storage buffer was added, and the plate was incubated at 4°C overnight.

scFv in supernatant was diluted in PBS with 3% (w/v) BSA, 0.05% (v/v) Tween 20, and 10 μl/ml NeutrAvidin before incubation for 1 h at 4°C. A beadstock were prepared with the coupled beads in storage buffer to a concentration of 100 beads/μl of each ID. 5 μl from the beadstock was mixed with 45 μl of scFv in a 384-well plate and incubated for 1 h at room temperature with shaking. The plate was washed three times in PBS-T, and the samples were analyzed on FLEXMAP3D (Luminex Corp.).

Affinity screening

The selected scFv were exposed to an affinity screening by SPR using Biacore T200 (GE Healthcare) to get an estimation of the affinity. By using a capture assay and by using only one concentration of antigen, we were able to screen a high number of samples (in 96-well plates), using the same chip and immobilized surface. The affinity screen also allows us to screen for scFv with good expression levels and to eliminate low expressing clones. An anti-FLAG M2 antibody (Sigma–Aldrich F1804) was immobilized in all four channels of a series S CM5 chip (GE Healthcare) using amine coupling according to the manufacturer’s instructions. Three channels were used to capture different scFv, whereas channel 1 was used as a reference surface. The running buffer used was HBS-EP+ (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.05% (v/v) Tween 20), and all samples were diluted in this buffer before injection. Filtered supernatants (expressed in the same way as described above) containing the scFv were diluted 20 times in running buffer before capturing on the anti-FLAG M2 antibody surface. Antigen with a concentration of 50 nM was added, with a flow rate of 30 μl/min. The surface was regenerated with 10 mM glycine HCl, pH 2.5. Before each cycle with antigen, scFv was captured, and running buffer was injected instead of antigen. The binding was analyzed using Biacore T200 evaluation software 3.1.

Binder expression and purification

scFv selected based on the previously described methods were purified in small scale. E. coli containing the DNA encoding the selected scFv was inoculated in 15 ml of 2X YT supplemented with 0.2 M sucrose and 25 μg/ml kanamycin and allowed to grow until exponential phase. 0.25 mM isopropyl-β-D-thiogalactopyranoside was added, and the cultures were incubated overnight at 30°C with shaking. The purification was automated using KingFisher Flex (Thermo Fisher Scientific) and magnetic protein A beads (Pierce). Bacteria from the overnight culture were spun down, resuspended, and lysed using B-PER bacterial protein extraction reagent (Thermo Fisher Scientific) with 0.01% (v/v) Tween 20 and benzonase, and samples were spun again to remove cell debris before being transferred to a 96-well deep-well plate. The KingFisher Flex program included binding of protein A beads to scFv for 1 h with slow mixing, four washes in TBS with 0.05% (v/v) Tween 20 for 30 s with slow mixing before elution in 100 μl of 0.1 mM glycine pH 2.7 with 0.05% (v/v) Tween 20. After purification, 1 mM Tris-HCl, pH 8.8, was added to neutralize the samples. The purified samples were buffer-exchanged to PBS using a Zeba 96-well desalt spin plate (Thermo Fisher Scientific), and purity and integrity were confirmed by SDS-PAGE.

Kinetic measurements

Kinetic measurements of the purified scFv were performed using SPR, Biacore T200 (GE Healthcare), and the same capture-based setup as described for the affinity screening. Here, however, single-cycle kinetics experiments were run; meaning five concentrations of antigen were injected in the same cycle with a flow rate of 30 μl/min before regeneration with glycine HCl, pH 2.5. Five-fold serial dilutions of 0.08–50, 0.16–100, or 0.4 to 250 nm, depending on the affinity of the interaction, were used. For some of the antigens, to prevent nonspecific interactions with the chip surface, 0.1% (w/v) BSA was included in the running buffer. Before and after each cycle with antigen, scFv were captured, and running buffer was injected instead of antigen. Reaction rate kinetics constants were calculated using the Biacore T200 evaluation software 3.1 using the 1:1 Langmuir binding model. Response curve sensorgrams were obtained after removing the response from the reference channel and a reference cycle (running buffer instead of antigen). Transient spikes, for example from air bubbles, were also removed. All binders were purified as described above before kinetic measurements, except for binders against LysRS, AsnRS, SerRS, and TyrRS, which were analyzed in supernatant. Binders targeting these four aaRSs were also analyzed in a running buffer containing 0.1% (w/v) BSA.

Cell-based validation (IP-MS)

HEK293 cells were cultured as described in Ref. 45. The non-small cell lung cancer cell line A549 (ATCC) was cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 6 mM glutamine (Sigma–Aldrich) at 37°C in a humidified atmosphere containing 5% CO2. The cells were seeded in 175-cm² flasks with ventilated caps at a density of two million cells/flask. After reaching 85% cell confluence, the cells were washed once with PBS and detached using trypsin–EDTA (Sigma–Aldrich), and viable cells were counted by staining with trypan blue (Sigma–Aldrich). The cells were pelleted by centrifugation at 300 × g for 5 min and then stored at −80°C until further processing. PBMC were isolated from heparin-treated whole blood from a healthy donor by dilution of blood sample with equal volume of sterile PBS and adding blood cell density gradient media Ficoll-Paque.
MS raw files were analyzed by MaxQuant software (version 1.6.3.4) and searched against 20,523 human protein sequences in Uniprot (reviewed part: UP00005640, download date: February 21, 2019), with enzyme specificity set to trypsin, allowing two missed cleavage sites. Carbamidomethylation was set as fixed modifications, and oxidation of M and N-terminal acetylation of protein were set as variable modification. Precursor mass tolerance was set to 4.5 ppm (after recalibration, 20 ppm tolerance for first pass search) and fragment mass tolerance to 20 ppm. The false discovery rate was set to 0.01 at both PSM and protein level, and a minimum of two unique peptides were required for identification. NSAF values were calculated as described earlier (45), and the protein identification results are provided in Table S3.

**Immunofluorescence**

U-2 OS cells (ATCC), a human osteosarcoma cell line, were cultured in Dulbecco’s modified Eagle’s medium high-glucose GlutaMAX medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37 °C with 5% CO2 in a humidified incubator before seeded in black, clear-bottomed 96-well imaging microplates at ~5000 cells/well and allowed to grow overnight. The cells were washed with warm PBS, aspirated, and fixed in either: 1) fresh 4% (v/v) formalin with 0.5% (v/v) Tween 20 in PBS at room temperature for 10 min, or 2) ice-cold 100% methanol at −20 °C for 5 min. Following either fixation, the plates were washed three times for 5 min in PBS, and nonspecific binding sites were blocked in 5% (v/v) normal donkey serum, 0.5% (v/v) Tween 20, and 4% (w/v) BSA in PBS for 1 h at room temperature with shaking. For multilabeling in the cytoplasm, 10 μg/ml of each scFv was preincubated in a 96 deep-well plate with mouse anti-FLAG M2-Cy3 (Sigma–Aldrich A9594) and rabbit anti-α-tubulin antibodies (Abcam ab18251) in diluent buffer (2.5% (v/v) normal donkey serum, 0.25% (v/v) Tween 20, and 2% (w/v) BSA in PBS) for 1.5 h at room temperature with shaking. Subsequently, 100 μl was transferred to each well in duplicate on both formalin- and methanol-fixed plates and incubated overnight at 4 °C with shaking. The plates were washed twice with 0.01% (v/v) Tween 20 in PBS for 10 min and incubated with donkey anti-rabbit Alexa Fluor 488 (Invitrogen A21206) in diluent buffer for 2 h at room temperature on the shaker. For multilabeling in the mitochondria, 10 μg/ml of each scFv was preincubated in a 96-well deep-well plate with mouse anti-FLAG M2-Cy3 (Sigma–Aldrich A9594) and rabbit anti-α-tubulin antibodies (Abcam ab18251) in diluent buffer for 1.5 h at room temperature with shaking. Subsequently, 100 μl was transferred to each well in duplicate on both formalin- and methanol-fixed plates and incubated overnight at 4 °C with shaking. The plates were washed twice with 0.01% (v/v) Tween 20 in PBS for 10 min and incubated with donkey anti-rabbit Alexa Fluor 488 (Invitrogen A21206) in diluent buffer for 2 h at room temperature on the shaker.

**Sample cohort II: all scFv analyzed using HEK293—**After tryptic digestion, the samples were cleaned using Phoenix columns (Preomics) according to the manufacturer’s recommendations and dried in SpeedVac prior to LC–MS/MS analysis. Peptide identification data were acquired using an EvoSep One (Evoq) Q-Exactive Orbitrap HF (Thermo Fisher Scientific) instrument combination. The 60 samples/day method of the EvoSep One was used to elute the samples over a 150 μm × 150 mm EASY-Spray™ column (ES806, Thermo Scientific). Data acquisition was performed with a Top20 data-dependent acquisition method.
once more for 5 min in PBS. The plates were imaged on an InCell Analyzer 2200 high-throughput microscope (GE Healthcare) with a 20× objective. Image analysis and processing was performed with CellProfiler 3.1.8 (Broad Institute), Zen 2.1 SP3 (Zeiss), and Adobe Photoshop CC 2019.

**Data availability**

The MS proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE (46) partner repository with the data set identifier PXD020104. GenBank™ under accession numbers MT074296–MT074325.

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: aaRS, aminoacyl transfer RNA synthetase; HTRF, homogenous time resolved fluorescence; IF, immunofluorescence; IP-MS, immunoprecipitation followed by mass spectrometry; MSC, multi-transfer RNA synthetase complex(es); NSAF, normalized spectral abundance factor; PBMC, peripheral blood mononuclear cell(s); scFv, single chain variable fragment; SPR, surface plasmon resonance; IMAC, immobilized metal affinity chromatography; SEC, size-exclusion chromatography; IIM, idiopathic inflammatory myopathy; TCEP, tris(2-carboxyethyl)phosphine.

**References**


