Structural characterization of antibiotic self-immunity tRNA synthetase in plant tumour biocontrol agent

Shaileja Chopra1,*, Andrés Palencia2,*,†, Cornelia Virus1, Sarah Schulwitz1, Brenda R. Temple3, Stephen Cusack2 & John Reader1,†

Antibiotic-producing microbes evolved self-resistance mechanisms to avoid suicide. The biocontrol Agrobacterium radiobacter K84 secretes the Trojan Horse antibiotic agrocin 84 that is selectively transported into the plant pathogen A. tumefaciens and processed into the toxin TM84. We previously showed that TM84 employs a unique tRNA-dependent mechanism to inhibit leucyl-tRNA synthetase (LeuRS), while the TM84-producer prevents self-poisoning by expressing a resistant LeuRS AgnB2. We now identify a mechanism by which the antibiotic-producing microbe resists its own toxin. Using a combination of structural, biochemical and biophysical approaches, we show that AgnB2 evolved structural changes so as to resist the antibiotic by eliminating the tRNA-dependence of TM84 binding. Mutagenesis of key resistance determinants results in mutants adopting an antibiotic-sensitive phenotype. This study illuminates the evolution of resistance in self-immunity genes and provides mechanistic insights into a fascinating tRNA-dependent antibiotic with applications for the development of anti-infectives and the prevention of biocontrol emasculation.
Microbes that produce antimicrobial agents often prevent cell suicide by co-expressing self-immunity proteins that employ a variety of different resistance mechanisms. Genes encoding self-immunity proteins can also act as a source of antibiotic resistance in the environment when they are horizontally transferred to microbial pathogens. This threatens not only the successful treatment of human pathogens but also prevention of important animal and crop diseases. One mechanism of self-immunity is to encode resistant variants of the enzyme targeted by the antibiotic. A fascinating example of such an antibiotic-producing organism is Agrobacterium radiobacter strain K84, which is used as a biocontrol agent of the plant pathogen A. tumefaciens that causes plant tumours in a number of agriculturally important species. The biocontrol produces the 'Trojan Horse' antibiotic agrocin 84 (refs 7–9) that mimics a plant tumour-derived substrate to gain entry by suberfuge into the pathogen, where it is processed into a toxin termed TM84 (refs 10,12) (Figs 1 and 2a). We recently identified the molecular target of TM84 in A. tumefaciens as leucyl-tRNA synthetase (LeuRSAt) which catalyses an essential first step in protein synthesis. To prevent cell suicide, A. radiobacter K84 contains a second, non-essential LeuRS, termed AgnB2, which is resistant to TM84 (refs 12–14) (Fig. 1). The agnB2 gene is encoded on the 44 kb plasmid pAgK84, along with all the genes required to biosynthesize agrocin 84 (refs 14,15). Importantly, horizontal gene transfer of the pAgK84 plasmid from A. radiobacter K84 to the pathogen has been previously reported to occur in the field, resulting in agrocin 84-resistant strains of A. tumefaciens encoding the agnB2 gene. Thus, understanding the molecular mechanisms by which AgnB2 provides immunity to agrocin 84 is crucial for combating antibiotic resistance.

The molecular target of TM84, LeuRSAt, is an essential enzyme that specifically ligates leucine to its cognate tRNALeu isoacceptors, a critical step before protein synthesis at the ribosome. The aminoacylation of tRNALeu is catalysed in a two-step mechanism in all LeuRSs: in the tRNA-independent first step, leucine is activated by ATP to form the essential obligate intermediate leucyl-adenylate (Leu-AMP) that is stably bound to the enzyme. In the second step, a bound tRNALeu repositions its CCA 3'-end into the LeuRS active site where activated-leucine is transferred to the tRNA. The essential catalytic nature of LeuRSs, and the 19 other canonical aminoacyl-tRNA synthetases (AARSs), has led to these enzymes emerging as prominent targets for anti-pathogens ranging in the prevention of fungal diseases in humans to bacterial plant pathogens. A substantial amount of work has been performed on stable analogues of aminoacyl-adenylates that bind tightly to an AARS active site, and preventing activation of the amino acid with ATP. However, these molecules often cannot be transported efficiently into cells and can lack discrimination between microorganism and the intended host.

We recently showed that TM84 does not behave like other stable aminoacyl-adenylate analogues but, instead, employs a unique tRNA-dependent inhibition mechanism. TM84 binds only weakly to the LeuRS-active site on its own and requires the presence of tRNALeu to form a tight binding ternary inhibition complex. The X-ray crystal structure of the Escherichia coli LeuRS16•tRNALeu•TM84 ternary complex shows TM84 bound in the Leu-AMP-binding site of the catalytic domain. The tRNALeu is bound to LeuRS in an ‘aminoacylation-like’ conformation with the CCA 3'-end of the tRNALeu entering into the active site with the terminal adenosine hydrogen bonding (H-bonding) directly with TM84. The positioning of the tRNALeu acceptor stem is accompanied with a substantial movement of the catalytic K619MSK622S motif. The K619 sidechain bonds to the penultimate tRNALeu cytidine base, while K622 extends across the adenylation analogue and interacts with the phosphate of TM84 via a non-bridging oxygen. The repositioning of the Lys-Met-Ser-Lys (KMSKS) loop and tRNALeu combine to effectively bury the bound TM84, thus explaining how the presence of tRNALeu induces the tight binding of LeuRSs to TM84.

AgnB2 exhibits a high degree of resistance to TM84 (ref. 12) despite the close similarity of TM84 to Leu-AMP (Fig. 2a) and the

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**Figure 1 | Biology of agrocin 84 produced by biocontrol A. radiobacter strain K84.** Pathogenic A. tumefaciens possesses a TiC58 plasmid that upon infection of the host plant makes the plant produce a carbon and phosphate source agrocinopine. The pathogen takes up agrocinopine via an agrocinopine permease encoded on pTiC58. This transporter also recognizes the uptake moiety on agrocin 84, an antibiotic produced by the plant tumour biocontrol, A. radiobacter strain K84. Upon entry of agrocin 84 into the pathogen, it is cleaved into the toxic moiety TM84 and the transport moiety. TM84 targets the leucyl tRNA synthetase, thereby inhibiting the aminoaacylation of tRNALeu. This subsequently leads to the cessation of protein synthesis in the pathogen and leads to cell death. TM84 however has no effect on the aminoaacylation reaction of a self-immunity LeuRS called AgnB2 that is encoded by the pAgK84 plasmid in A. radiobacter K84.
need for AgnB2 to adopt a true aminoacylation complex as exhibited in a recent LeuRS●tRNA_Leu●Leu-AMS crystal structure. These observations raise the question of how does AgnB2 achieve resistance to TM84 without dramatically interfering with the catalysis of the aminoacylation reaction. This is particularly intriguing as primary sequence alignments of AgnB2 and TM84-sensitive LeuRSs show a high similarity of residues, including catalytic motifs (except a single Q instead of M at the KMSKS motif). Here, we examine the TM84 resistance mechanisms employed by AgnB2 using both thermodynamics and steady-state enzyme kinetics. We also present the X-ray crystal structure of AgnB2 in complex with tRNA_Leu and a stable leucyl-adenylate analogue and reveal tertiary interactions unique to AgnB2. We finally identify key TM84 immunity determinants in AgnB2 and the TM84-sensitive LeuRS_A and show that it is possible to partially inter-convert the TM84 sensitivity properties of these enzymes.

Results
Analysis of AgnB2 steady state enzyme kinetics. The ability of AgnB2 to discriminate between TM84 and the obligate reaction intermediate Leu-AMP prompted us to examine differences between the two molecules. As shown in Fig. 2a, TM84 contains a stable N-acyl phosphoamide bond, instead of the reactive phosphoanhydride linkage in Leu-AMP. In addition, TM84 has a deoxy-arabinose sugar group, instead of a ribose with subsequent loss of a 3'-hydroxyl (OH) and a repositioned 2'-OH above the sugar ring, and replacement of the primary amine with another OH group. For TM84-sensitive LeuRSs, these subtle differences between toxin and reaction intermediate allow TM84 to employ a tRNA-dependent inhibition mechanism where the CCA 3'-end of the tRNA_Leu binds in an ‘aminoacylation-like conformation’ into the active site of the enzyme and directly hydrogen bonds with the antibiotic. The ‘aminoacylation-like’ conformation of tRNA in the LeuRS●tRNA_Leu●TM84 ternary inhibition complex exhibits a high degree of similarity with the tRNA_Leu in the true LeuRS aminoacylation conformation. Since the tRNA_Leu bound to AgnB2 still has to reposition its CCA 3'-end into the active site during catalysis of the aminoacyl transfer reaction, we questioned whether destabilization of the TM84 ternary complex in AgnB2 might be reflected in the kinetic properties of AgnB2.

Our steady-state kinetic analysis of AgnB2 examined both the overall aminoacylation reaction and the first step, the amino acid activation reaction, using the ATP/PPi exchange reaction. We firstly determined the apparent TM84 inhibition constant (Ki) for AgnB2 in the aminoacylation reaction by altering the ATP concentrations of the substrate and pre-incubating the AgnB2●tRNA_Leu complex before initiating the reaction. The data revealed an apparent Ki of 4.72 ± 0.75 μM (Supplementary Fig. 1). We then determined the apparent TM84 Ki for AgnB2 by altering the leucine concentration and keeping the ATP fixed giving a value of 1.38 ± 0.28 μM (Supplementary Fig. 1). Both values are more than three orders of magnitude weaker than the K_0PP determined previously for the natural target LeuRS_A (ref. 23). Importantly, TM84 acted as a weak competitive inhibitor of AgnB2 with regards to both ATP and leucine (Supplementary Fig. 1), and therefore still binds to the synthetic active site and not at an alternative site. We also found that AgnB2 is resistant to TM84, but is still potently inhibited by Leu-AMS (a non-hydrolysable analogue of the Leu-AMP reaction intermediate), for both the amino acid activation (Supplementary Fig. 2) and overall aminoacylation reactions (Fig. 2b). Substantial weakening of the binding of the aminoacyl adenylate to AgnB2 did not evolve as a solution to generating resistance to TM84, as this would severely affect the catalysis of amino acid activation.

As shown in Table 1, AgnB2 and LeuRS_A share similar k_cat values for all substrates. The higher K_m for tRNA_Leu in AgnB2 may reflect an alteration in the interactions of AgnB2 with...

**Figure 2 | AgnB2 discriminates between a stable Leu-AMP analogue and TM84.** (a) Chemical structures of Leu-AMP and TM84 with differences highlighted in green. A non-hydrolysable adenylate analogue called Leu-AMS, which contains a N-sulfamoyl linkage in place of the phosphoanhydride of Leu-AMP, was used in biophysical and crystallographic experiments. (b) Effect of TM84 and Leu-AMS on the aminoacylation reaction of wt AgnB2. Aminoacylation reactions were carried out using 2 nM wt AgnB2 only (●) and wt AgnB2 in the presence of 1 μM TM84 (■) and 20 nM Leu-AMS (▲) at 28 °C, pH 7.4 and initiated using 1 mM ATP. Error bars represent s.d. (n = 3).

| Enzyme | Substrate | K_m (μM) | k_cat (s^-1) |
|--------|-----------|----------|--------------|
| **Aminoacylation** | | | |
| AgnB2 | ATP | 95.4 ± 18.3 | 0.43 ± 0.02 |
| | Leucine | 28.7 ± 7.3 | 0.35 ± 0.03 |
| | tRNA | 1.48 ± 0.25 | 0.43 ± 0.02 |
| | ATP | 396 ± 83 | 0.33 ± 0.02 |
| | Leucine | 38 ± 3.2 | 0.38 ± 0.01 |
| | tRNA | 0.94 ± 0.19 | 0.36 ± 0.02 |
| **ATP-PPi Exchange** | | | |
| AgnB2 | ATP | 1047 ± 140 | 67 ± 8.9 |
| | Leucine | 12 ± 2 | 73 ± 3.6 |
| | ATP | 357 ± 22 | 87 ± 1.5 |
| | Leucine | 9.7 ± 0.7 | 85 ± 1.6 |

All data in this table were average values obtained from three independent experiments with error ± values representing s.d. (From reference Chopra, et al.23).

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tRNA\textsubscript{Leu} has reduced effect on the affinity of TM84 to AgnB2. If resistance of AgnB2 is based on abolishing of the normal tRNA-dependent inhibition mechanism employed by TM84 (ref. 23) then one would expect to see a decrease in the affinity for TM84, tRNA\textsubscript{Leu} and/or for TM84 in the presence of tRNA or vice versa. To test these possibilities, we used isothermal calorimetry (ITC) (Supplementary Table 1) to investigate the ligand binding modes to AgnB2 (ref. 23). K\textsubscript{i} values of 1052 nM for TM84 and 310 nM for tRNA were obtained, indicating reductions in AgnB2-binding affinities for both TM84 (6.9-fold) and tRNA (3.7-fold), compared with LeuRS\textsubscript{A} (ref. 23). Further examination of TM84 binding to AgnB2 (with pre-bound tRNA) showed a K\textsubscript{i} of 332 nM, which suggests a substantial destabilization of the ternary complex (AgnB2•tRNA\textsubscript{TM84} (≈ 330-fold decrease) as compared with the LeuRS\textsubscript{A}•tRNA\textsubscript{TM84} complex (Supplementary Table 2). Interestingly, we also see no evidence of a binary isotherm for TM84 binding to AgnB2•tRNA\textsubscript{Leu} species, unlike LeuRS\textsubscript{A}. Thus, AgnB2 has a significantly weakened affinity for TM84 as compared with LeuRS\textsubscript{A}. Since TM84 and the Leu-AMP analogue bind to the same active site but potentially to different conformations, we examined if AgnB2-binding affinity for Leu-AMS had been substantially weakened. Our results show that Leu-AMS binds to AgnB2 with low nanomolar binding affinity (K\textsubscript{i} = 4.4 nM), which is slightly weaker than to LeuRS\textsubscript{A} (1.6 nM). The fact that AgnB2 binds tightly to Leu-AMS is not so surprising since, firstly, AgnB2 has highly conserved residues at the synthetic site compared with other LeuRSs and, secondly, the enzyme must bind tightly to the Leu-AMP intermediate to catalyze the aminoacylation reaction. This result was supported by our enzyme kinetic observations above which showed that Leu-AMS is a low nanomolar inhibitor of both the AgnB2-catalyzed aminoacylation and amino acid activation reactions, demonstrating that the analogue efficiently competes with ATP and leucine substrates (Supplementary Fig. 1).

Our thermodynamic results suggest that AgnB2 may achieve resistance to TM84 by not simply weakening the affinity for TM84 (and/or tRNA\textsubscript{Leu}) but most significantly by destabilizing the formation of the AgnB2•tRNA\textsubscript{Leu}•TM84 complex. Interestingly, this is accomplished with only minimal change to affinity for the adenylate, allowing the enzyme to still efficiently carry out aminoacylation.

Structure of AgnB2•tRNA\textsubscript{Leu}•Leu-AMS ternary complex. To identify structural interactions in AgnB2 that may contribute to resistance, we performed extensive crystallizations trials with AgnB2, agrobacterium tRNA\textsubscript{Leu} and multiple combinations of substrates or inhibitors. We obtained an X-ray crystal structure of AgnB2 in complex with tRNA\textsubscript{Leu} and Leu-AMS; the final model was refined to a final R-factor of 19.4% (R-free = 23.8%) and a final resolution of 2.1 Å (Table 2, Fig. 3a and Supplementary Fig. 3).

The overall AgnB2•tRNA\textsubscript{Leu}•Leu-AMS structure (Fig. 3a, and see domain architecture in Fig. 4) presents a similar conformation to a number of bacterial LeuRS structures obtained in complex with tRNA\textsubscript{Leu}, in which the CCA 3’-end is positioned into the editing active site and represents what appears to be the preferred LeuRS conformation. Another important interaction observed in the structure is the C-terminal domain interaction with the variable loop of Agrobacterium tRNA\textsubscript{Leu} (Supplementary Fig. 4a). A comparison of the aminoacylation and editing states in LeuRS\textsubscript{Ec}, shows the C-term domain coupled to the variable arm of E. coli tRNA\textsubscript{Leu} rotates significantly upwards towards the catalytic domain in the aminoacylation state. It has been hypothesized that this C-term domain movement propagates towards the main body of the tRNA\textsubscript{Leu} and towards the acceptor stem, and together with rotations of other external domains, or residues, forces the 3’-end of the tRNA into the catalytic site.

### Table 2 Data collection and refinement statistics

| Agrobacterium radiobacter strain K84 | AgnB2 LeuRS + tRNA\textsubscript{Leu} + Leu-AMS |
|-------------------------------------|---------------------------------------------|
| **Data collection**                 |                                             |
| Space group                         | P\textsubscript{2}1                         |
| Cell dimensions                     | 169.91, 50.32, 170.52                       |
| α, β, γ (°)                         | 90.00, 93.48, 90.00                        |
| Resolution (Å)                      | 50.21 (2.10-2.20)                           |
| R\textsubscript{sym}                | 7.5 (65.2)                                 |
| I/σ                                | 10.6 (2.0)                                 |
| Completeness (%)                    | 99.5 (99.8)                                |
| Redundancy                          | 3.7 (3.7)                                  |
| **Refinement**                      |                                             |
| Resolution (Å)                      | 48.6-21 (2.15-2.10)                        |
| No. of reflections work/free        | 160,330/8,487                               |
| R\textsubscript{work}/R\textsubscript{free} | 0.194 (0.309)/0.237 (0.331)               |
| No. of atoms                        |                                             |
| Protein tRNA\textsubscript{Leu}     | [6,307]\textsuperscript{A}, [6,141]\textsuperscript{B} |
| Ligand                             | [1,702]\textsuperscript{C}, [1,723]\textsuperscript{D} |
| Water/SO\textsubscript{4}\textsuperscript{2-} | 871/25 [5 × SO\textsubscript{4}\textsuperscript{2-}] |
| Mn\textsuperscript{2+}              | 2                                          |
| B-factors                          |                                             |
| Protein tRNA\textsubscript{Leu}     | [52.3]\textsuperscript{A}, [48.6]\textsuperscript{B} |
| Ligand                             | [57.7]\textsuperscript{C}, [54.9]\textsuperscript{D} |
| Water/SO\textsubscript{4}\textsuperscript{2-} | 44.4/86.2                                  |
| Mn\textsuperscript{2+}              | 87.3                                       |
| RMS deviations                     |                                             |
| Bond lengths (Å)                    | 0.016                                      |
| Bond angles (°)                     | 1.736                                      |

Values in brackets correspond to averages of the corresponding chain, indicated as superscript. Values in parentheses are for the highest-resolution shell.
Interestingly, the tRNA^{Leu} from Agrobacterium has a shorter variable stem than the E. coli tRNA, therefore questioning whether this propagation force due to coupling of tRNA with the C-term domain is also required in AgnB2 for aminoaacylation. In fact, a comparison of the C-terminal domain from AgnB2 compared with the E. coli editing structure (LeuRS\textsubscript{E.coli}•tRNA\textsuperscript{Leu})\textsuperscript{25}...
shows there is a rotation of about 18.5° and the shorter variable loop of agrobacterium only makes one contact with the phosphate of nucleotide 46f (P46f) vs the *E. coli* tRNA which makes multiple contacts with the variable domain**5** (Supplementary Fig. 4a,b). This structure may give new insights into the function of this stem-loop.

**Unique amino acid substitutions in the AgnB2 catalytic site.**

Close inspection of the Leu-AMS-binding site in the AgnB2 structure reveals that the residues at the catalytic site are very similar compared with those found in LeuRS*Ec* Fig. 3b,c. There are small differences including F39 in AgnB2, forming part of the hydrophobic pocket for the leucine, vs the corresponding L41 found in the LeuRS*Ec*. Additionally, E528 in AgnB2 exhibits a different rotamer away from ribose hydroxyl vs the corresponding E532 residue in the LeuRS*Ec* aminoacylation complex, which is situated more closely to the ribose hydroxyl. The LeuRS*Ec* E532 plays an important role in catalysis of the aminoacylation reaction, as suggested by the LeuRS*Ec*-tRNA**Leu**-Leu-AMS complex structure and confirmed by biochemical experiments**(8,9)**. The observation that TM84-sensitive LeuRS*Ec* and resistant AgnB2 showed no substantial differences between active site residues directly interacting with Leu-AMS might explain how the two enzymes can bind equally well to Leu-AMS and carry out aminoacylation with close kinetic parameters (see above). It therefore follows that no AgnB2 residues are directly in a position to interrogate the 5’ N-acyl phosphoramidate bond of TM84 when it is bound to the enzyme alone. The weaker binding of TM84 to AgnB2, with or without tRNA**Leu**, compared with LeuRS*Ec*, might be explained by residues or domains that do not directly interact with the adenylate analogue, but which could promote long range effects or conformational changes affecting the catalytic site or perturbing the interaction with tRNA**Leu**.

The KMSKS catalytic peptide motif, along with the HIGH loop, is present in all Class I aaRSs, including LeuRSs, and has been shown to play a critical role in the both the amino acid activation reaction and the aminoacyl transfer reactions**(27,28)**. Biochemical and biophysical studies on class I synthetases, including LeuRS, shows that ATP binding to the catalytic active site leads to a conformational change in the mobile KMSKS loop shifting it from an ‘open’ to a ‘closed’ conformation with the two lysine residues of the KMSKS loop interacting with the α and β phosphates of the ATP**(27-29)**. Once the aminoacyl-adenylate is formed, the KMSKS loop moves into a ‘semi-open’ conformation to allow for the entry of the CCA 3’-end of the tRNA into the active site. Finally, once the CCA 3’-end of the tRNA is in the active site, the KMSKS loop once again adopts the closed conformation**(25,27,30)**. In the case of AgnB2, the highly conserved methionine of the KMSKS motif is interestingly replaced by a glutamine residue (Q573). Our structure shows that Leu-AMS is bound in the active site with the mobile KQSKS loop present in a semi-open conformation (Supplementary Fig. 5). This loop position is in contrast to the fully closed position exhibited by the KMSKS loop in the LeuRS*Ec*-tRNA**Leu**-TM84 ternary complex where the second lysine of the KMSKS loop (K622) directly interacts with the non-bridging phosphate of the nucleotide analogue and the first lysine of the KMSKS loop (K619) interacts with cytosine 75 of the tRNA 3’-end**(12)**. In Class I aaRSs, this conserved methionine forms a number of hydrophobic packing interactions and is thought to play an important role in aminoacylation**(31-34)**. In LeuRS structures it also forms a main chain interaction with the adenine ring of the bound adenylate**(25,35)**. The equivalent position in AgnB2, Q573, forms a hydrogen bond side-chain interaction with the side chain of T581 (Fig. 5a), which is found to be a conserved hydrophobic residue in TM84 sensitive LeuRSs (I628 in LeuRS*Ec*) (Fig. 5b). It is interesting to note that the AgnB2 KQSKS loop exhibits a distinctly shifted position relative to the Leu-AMP binding pocket (Supplementary Fig. 5), perhaps due to these changes. The fact that a glutamine residue is found in this critical catalytic motif but not in any other LeuRS in nature, may indicate an important role for Q573 in TM84 resistance. It maybe that a substantial number of compensatory mutations are required to accommodate Q573 in AgnB2.

T42 and A102 are two other residues that are specific to AgnB2. T42 is positioned upstream from the catalytic H**17**G**30** motif, while the side chain of A102 is situated in close proximity to T42 on a neighbouring α-helix (Fig. 5). Importantly, the presence of A102 in AgnB2, instead of an asparagine as found in the LeuRS*Ec* structure (N104), leads to loss of an H-bond interaction between the α-helix (96–114) and a main-chain peptide bond located at T42 (P45 LeuRS*Ec*). Both these modifications could allow for subtle changes in the positioning of the neighbouring HIGH motif that binds TM84.
AgnB2 has modified tRNA elements compared with LeuRS<sub>Ec</sub>. A prominent feature of the AgnB2 structure is the lack of an idiosyncratic leucyl-specific insertion domain (LS-domain), which is present in bacterial LeuRS, except in a few cases such, as Bacillus subtilis or Mycoplasma mobile<sup>25,36</sup>, and as seen in the Helicobacter pylori LeuRS (LeuRS<sub>Hp</sub>) primary amino acid sequence in Fig. 4. The LS-domain undergoes substantial movement between the LeuRS<sub>Ec</sub> editing, and the TM84 and Leu-AMS bound ‘aminoacylation-like’ conformations, buttressing the highly mobile KMSKS loop as well as guiding the tRNA into the catalytic site, as found in the aminoacylation conformation<sup>23</sup>. The missing LS-domain in AgnB2 is replaced by a small fun domain and differs from the highly conserved residues in TM84- and also LeuRS<sub>At</sub> to test the contribution of these sites to antibiotic resistance. Because of the limited number of possible mutants and permutations that could be constructed using this approach, not all potential TM84 resistance/sensitivity determinants could be tested. We initially assayed candidate mutants using a biological assay (see Methods), followed by in vitro enzyme inhibition experiments combined with assessment of direct binding by calorimetry on promising constructs.

Three of the putative resistance determinants in AgnB2, Q573, A102 and T42 described above (Fig. 5a), reside in the catalytic domain and differ from the highly conserved residues in TM84-sensitive LeuRSs. We introduced these mutations at corresponding positions (as determined by homology modeling) in the TM84-sensitive enzyme LeuRS<sub>At</sub> (P45T, N105A and M633Q) and tested whether these mutations imparted resistance using our in vivo assay (Fig. 6a). The single M633Q mutation in the K(M/Q)SKS loop is restrictive in nature, as it was not tolerated in the LeuRS<sub>At</sub> enzyme, most likely resulting in a functionally inactive enzyme. In contrast, the P45T (Fig. 6a) or N105A (data not shown) mutations did not significantly increase the resistance to TM84. However, the double mutation P45T/N105A (Fig. 6a) did result in a 3.5-fold reduction in tRNA<sub>Leu</sub> affinity between LeuRS<sub>At</sub> and AgnB2 (Supplementary Tables 1 and 2), raising the possibility that the modification of tRNA interacting elements in AgnB2 (Supplementary Tables 1 and 2), raising the possibility that the modification of tRNA interacting elements in AgnB2 may play a role in TM84 resistance (tested below).

It has been reported that residues distant from the active site can also be crucial in forming interactions with the negatively charged backbone of the tRNA<sub>Leu</sub> CCA 3′-end and its repositioning in catalysis<sup>38</sup>. Mutation of the residue R418 to a neutral amino acid, such as glutamine, decreased the aminoacylation activity in LeuRS<sub>Ec</sub> by reduction of the residence of the 3′-end in the enzyme active site<sup>37,38</sup>. Importantly, the AgnB2 structure shows the equivalent residue to R418 in LeuRS<sub>Ec</sub> is the neutrally charged Q413 (Fig. 4). Thus, it is possible that Q413 in AgnB2 has a negative effect on binding of the tRNA-dependent inhibitor TM84.

**Identification of permissive mutations in the catalytic core.**

Having identified possible TM84 sensitivity/resistance determinants by comparison of the AgnB2 structure with other LeuRSs known to be sensitive to the toxin, we made mutants of AgnB2 and also LeuRS<sub>At</sub> to test the contribution of these sites to antibiotic resistance. Because of the limited number of possible mutants and permutations that could be constructed using this approach, not all potential TM84 resistance/sensitivity determinants could be tested. We initially assayed candidate mutants using a biological assay (see Methods), followed by in vitro enzyme inhibition experiments combined with assessment of direct binding by calorimetry on promising constructs.

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AgnB2 Triple Q413R mutant (.), AgnB2 Triple Q413R mutant (■) and AgnB2 Triple LS domain mutant (▲). Dose-response curves and the resulting IC₅₀ values were obtained at saturating leucine and (0.675 mM) ATP concentrations (see Methods). Error bars represent s.d. from three independent experiments.

![Figure 6](https://www.nature.com/naturecommunications)
shown) are permissive mutations and had a small effect on enzyme function or TM84 sensitivity. A P45T/N105A double mutant showed no increase in resistance for LeuRS\textsubscript{LP}, when the P45T was introduced with M633Q in a double mutant, the P45T acted as a permissive mutation, increasing the resistance of the mutant enzyme. Finally, when all three mutations were introduced into LeuRS\textsubscript{LP} (P45T/N105A/M633Q, termed LeuRS\textsubscript{LP} Triad) there was a significant increase in resistance to the antibiotic (Fig. 6a) compared with wild type (wt).

Kinetic inhibition experiments on LeuRS\textsubscript{LP}, Triad showed the enzyme was resistant to concentrations of TM84 up to 500 nM (Supplementary Fig. 6a). This is in contrast to wt LeuRS\textsubscript{LP}, which is sensitive to very low concentrations of TM84 (K^\text{app} = 0.3 ± 0.1 nM)\textsuperscript{23}. The introduction of the Triad mutations also negatively affected the percentage of active enzyme, presumably because these modified residues not only alter engagement of the enzyme with the antibiotic but also modify the interaction of the active site with its substrates. Subsequent thermodinamic analysis of the binding of TM84 to the LeuRS\textsubscript{LP}, Triad alone and the LeuRS\textsubscript{LP}, Triad pre-incubated with tRNALeu showed respective 10-fold and 28-fold decreases in binding affinity, as compared with LeuRS\textsubscript{LP} (Supplementary Table 2).

To see if the three reverse mutations in AgnB2 could make the enzyme more sensitive to TM84, a variant containing all three mutations (T42P/A102N/Q573M termed AgnB2 Triple) was constructed. The AgnB2 Triple showed a distinct increase in TM84 sensitivity (larger clearance zone) as detected by bioassay (Fig. 6b). An individual, Q573M mutant did not increase TM84 sensitivity (larger clearance zone) as detected by bioassay (Fig. 6b). Kinetic analysis of the AgnB2 Triple mutant showed a 1.8-fold increase in resistance using a fixed concentration of ligand (Supplementary Fig. 6b). Thermodynamic analysis of the binding of TM84 to the AgnB2 Triple alone showed no significant effect on TM84 binding, while there was a 3.5-fold increase in binding of TM84 to the AgnB2\textsubscript{LP}tRNALeu complex (Supplementary Table 1).

We also tested whether disruption of the H-bond interaction between Q573 of the KQSKS loop and T581 side chain (Fig. 5a) could affect TM84 binding by mutating the threonine residue to a valine. This single T581V mutation, as well as the double mutation (Q573M/T581V) had no detectable effect on TM84 resistance in our bioassays (Supplementary Fig. 7a). However, addition of the T581V mutation to the AgnB2 Triple construct did produce an apparent increase in antibiotic sensitivity (Supplementary Fig. 7a).

Conversion of a TM84-resistant enzyme into a sensitive form by altering key tRNA interactions. To test whether an LS domain can have a substantial effect on the TM84 sensitivity of a LeuRS, we examined recombinant LeuRS \textit{H. pylori} (LeuRS\textsubscript{HP}), which lacks an LS domain (Fig. 4). Interestingly, we found using \textit{in vitro} aminoacylation assays that the LeuRS\textsubscript{HP}, was still significantly inhibited by TM84 (IC\textsubscript{50} = 34 ± 1.14 nM; Supplementary Fig. 8) although to a lesser degree than LeuRS\textsubscript{LP} (rel. 23). Interestingly, this enzyme also contains a neutral Q413 residue in a homologous position to R418 from LeuRS\textsubscript{EC}, is thought to be involved in positioning of the tRNA CCA 3'-end in the enzyme active site\textsuperscript{25,38}. We took this one step further, by introducing all three core mutations implicated in TM84 resistance in AgnB2 into LeuRS\textsubscript{HP} (P40T, N100A and M573Q). However, our \textit{in vitro} aminoacylation analysis revealed that this enzyme was inactive under the conditions used in our study (Supplementary Fig. 8).

To pursue further a possible role of the LS domain in TM84 sensitivity, we inserted the LS domain from LeuRS\textsubscript{LP} into AgnB2 LeuRS and tested the chimeric enzyme's sensitivity to TM84 in our bioassay. In addition, we also inserted the LS domain into the background of the AgnB2 Triple variant (T42P/A102N/Q573M). Our results show that insertion of the LS domain into AgnB2 wt had no effect on antibiotic sensitivity in our bioassays but when the LS domain was inserted into the AgnB2 triple background there was a substantial increase in clearing zone size (Fig. 6b). A comparison of the TM84 inhibition of aminoacylation activity of the AgnB2 Triple mutant + LS domain construct against wt AgnB2, at a fixed ATP concentration of 0.675 mM ATP (where no substrate inhibition of either enzyme is detected) showed a 34-fold (IC\textsubscript{50} of 1.8 \mu M) decrease in the IC\textsubscript{50} of the chimeric mutant compared with wild-type (IC\textsubscript{50} of 61.3 \mu M) (Fig. 6c). ITC analysis of this construct was used to deconvolute the contribution of tRNA to the binding of TM84 to the mutant. Our results showed that TM84 binding alone (K_d = 258 nM) increased by fourfold (Fig. 7a) and TM84 binding in presence of tRNA (K_d = 19 nM) increased by 17-fold respectively, compared with wt AgnB2 (Fig. 7b).

The positively charged R418 residue in LeuRS\textsubscript{LP}, has been implicated in controlling the translocation of the negatively charged backbone of the tRNA\textsubscript{Leu} CCA 3'-end into the synthetic active site\textsuperscript{25,38}. We tested whether modification of the homologous position in AgnB2, Q413, from a neutral to positively charged arginine might modulate TM84 resistance through stabilization of the tRNA-dependent inhibition complex. Introduction of a single-point Q413R mutation into AgnB2 showed no effect on the size of clearing zones in our bioassays compared with wt (Fig. 6b). However, when we placed the Q413R mutation in the background of the AgnB2 Triple mutant (T42P/A102N/Q573M) there was a substantial increase in the clearing zone diameter suggesting an increase in TM84 sensitivity compared with the AgnB2 Triple (Fig. 6b). Analysis of the TM84 inhibition kinetics of the AgnB2 Triple + Q413R mutant showed an IC\textsubscript{50} of 2.9 \mu M, 21-fold smaller than wt-AgnB2 at a fixed ATP concentration (Fig. 6c) indicating this construct had a significant increase in TM84 sensitivity compared with wt. We then analyzed the thermodynamics of binding of TM84 to the AgnB2 Triple Q413R\textsubscript{LP}tRNA\textsubscript{Leu} complex and found it was sixfold tighter than wt AgnB2 (Fig. 7b). However, the binding of TM84 to the AgnB2 Triple Q413R enzyme alone was only 1.3-fold tighter than the wt enzyme (Fig. 7a). This further suggested the importance of tRNA interacting residues for imparting sensitivity towards TM84.

In summary, our results suggest that the absence, or modification of, idiosyncratic tRNA binding elements in AgnB2, act cumulatively with critical core mutations on the periphery of the LeuRS active site, significantly contributing to the high degree of TM84 resistance observed in AgnB2.
TM84 (agrocin 84) is a highly potent LeuRS toxin that employs a unique tRNA-dependent inhibition mechanism. During production of agrocin 84, the biocontrol A. radiobacter strain 84 expresses the self-immunity LeuRS AgnB2 to prevent cell suicide. Our data show that AgnB2 achieves resistance to TM84 by minimizing the effect tRNA^Leu has on the toxin’s affinity for the enzyme as well as weakening its affinity for tRNA^Leu and the inhibitor alone. Remarkably, this resistance phenotype is achieved without dramatically affecting the catalysis of the overall aminoacylation reaction (Table 1).

Consistent with this observation, the X-ray crystal structure of the AgnB2•tRNA^Leu•Leu-AMS ternary complex reveals that residues in the synthetic active site that directly bind Leu-AMP are not substantially different from other bacterial LeuRSs.

We identified key molecular determinants imparting TM84 resistance (or sensitivity) to LeuRSs by utilizing an approach based on the analysis of structures of the naturally resistant and sensitive enzymes AgnB2 and LeuRSc (Fig. 8). LeuRS mutants that affected TM84 sensitivity were then tested using in vitro and in vivo approaches. Our data indicate that the molecular elements in LeuRSs that contribute to TM84 sensitivity or resistance are much more complex than one would expect a priori. Surprisingly, some of these determinants were not located directly in the active site but on the periphery (with distances ~10–15 Å for most residues and 25 Å for Q413), including some residues interacting with the tRNA^Leu. The core resistance mutations in the catalytic domain of TM84 resistant AgnB2 are located in KQSKS loop (Q573), just upstream of the HIGH loop (T42) or buttressing next to T42 in the case of A102. It is possible that the nature of the amino acids at these particular sites in LeuRSs may affect the molecular dynamics of these two critical catalytic motifs and ultimately altering the LeuRSs TM84 sensitivity phenotype. The exact contribution of other determinants to TM84 sensitivity at a greater distance from the active site is more difficult to discern. For AgnB2, alterations in tRNA-binding contacts, due to lack of an LS domain, C-term domain modifications, and the effect of Q413 on CCA 3'-end localization in the active site, may all play a role in destabilizing a TM84 ternary complex leading to resistance. It maybe that evolution has traversed a narrow energetic landscape towards a resistance phenotype by exploiting small energetic differences between mutants to produce larger scale changes in only certain kinetic and/or thermodynamic parameters. Presumably, there must be other additional unconserved residues that have played subtle compensatory roles on the pathway towards evolution of TM84 resistance.

Why did nature evolve TM84 to target LeuRSc and not another AARSs from A. tumefaciens, and in particular the other Class I AARSs that have similar active site architectures such as IleRS and ValRS. TM84 does appear to be LeuRS specific, as the antibiotic does not inhibit agrobacterial IleRS12, and the single LeuRS agnb2 gene is capable of providing agrocin 84-immunity to the pathogen. In silico docking analysis of TM84, from the LeuRSc structure (PDB: 3ZGZ), into the catalytic domain of existing IleRS (PDB code: 1I7Z) and ValRS (PDB code: 1GAX) structures reveals steric clashes of the ‘leucine-like’ region of TM84 with hydrophobic side chains in the active site (Supplementary Fig. 9). This analysis may provide some understanding of the LeuRS specificity of TM84. One possible reason that agrocin 84/TM84 evolved to target a LeuRS maybe because leucine is one of the most abundant amino acids in proteins, and so translation could be particularly sensitive to interference of LeuRS activity. A more plausible reason is that agrocin 84/TM84, or its evolutionary ancestor, had other roles in Agrobacterium biology that predisposed the toxin to evolve into a LeuRS inhibitor. The recent finding that the uptake molecule of agrocin 84, when cleaved from TM84, can act as signalling molecule binding to a key agrobacterial transcriptional regulator that controls quorum-sensing signal synthesis3 provides some support for this latter view.

Horizontal gene transfer of the agnb2 gene on the pAgK84 plasmid from A. radiobacter strain K84 to the pathogen threatens emasculation of the biocontrol5,16,17. Genetic engineering of pAgK84 to prevent conjugative transfer of the plasmid to the pathogen has resulted in A. radiobacter K1026 strain39, the first bioengineered microbe approved for release into the environment40. Yet despite not containing any foreign DNA, this genetically altered strain is still only registered for use as a biocide in a limited number of countries (Gary Bullard and Allen Kerr, personal communications). In addition, as with all
antibiotic interventions, continued success against resistance can never be completely assured. Thus, understanding the resistance mechanism of agrocin 84 immunity protein AgnB2 not only provides key insights into the mechanism of a fascinating tRNA-dependent toxin but also informs research into the continued crop protection provided by this biocontrol against the spectre of antibiotic resistance.

Methods

Generation of mutants of AgnB2 and LeuRSAt. Mutations in AgnB2 and LeuRSAt were introduced using the Quikchange site-directed mutagenesis method (Stratagene). Mutagenesis was carried out in the agnB2 gene previously cloned into the pET-28a vector (Novagen) for use in recombinant protein expression, and also in the agnB2 gene subcloned into the broad host range plasmid pBRR1MCS-3 (ref. 41) for use in bioassay studies. Similarly, LeuRSAt mutants were generated in the A. tumefaciens leuS gene cloned into the pET-21b vector (for recombinant protein expression) and pBRR1MCS-3 (bioassays). Multiple rounds of mutagenesis were performed to obtain more than one mutation in an enzyme. DNA sequence analysis was used to confirm the correct construction of the entire gene sequence for all single, and multiple site mutants, used in this study.

Insertion of the LS domain of LeuRSAt into AgnB2. The LS domain of LeuRSAt (141 bp) was inserted into AgnB2 (replacing the region between amino acid no. 566 and 572 with the LS domain)41). To obtain this, the forward PCR primer was designed to contain the region of AgnB2 (~25 bp) preceding the LS domain insertion followed by the first 25 bases of the LS domain (5′–3′). Similarly, the reverse primer was designed so as to include the last 25 bp of the LS domain (3′–5′) and the gene sequence of AgnB2 after the LS domain insertion. An extension reaction was carried out to obtain mega primers, (forward and reverse) encoding the entire LS domain (of LeuRSAt) flanked by the sequence of AgnB2 on both sides using the protocol by Wang and Malcolm42. DpnI digestion was then carried out to remove the wt template. The resultant mega primer was gel purified using the Qiaex II gel extraction kit (Qiagen) to be used in the second step. The PCR amplified mega primers (generated in the first step) were then added to the extension reaction in presence of the pET-28a plasmid containing the agnB2 gene (using the Quikchange site-directed mutagenesis protocol II). Positive clones encoding AgnB2 with the entire LS domain of LeuRSAt were identified by colony PCR and finally confirmed by DNA sequencing analysis. Using a similar strategy, LS domain was added to the AgnB2 Triple mutant as well as the AgnB2 Triple Q413R mutant.

Construction of A. tumefaciens bioassay strains. A modified acc operon encoding the agrocin 84 transporter system (agrocinopine permease)43–45 was cloned into pYW15d plasmid containing a T5 promoter and an antibiotic marker (carbenicillin)46. Importantly, the accR repressor gene, responsible for the regulation of the agrocinopine permease47, had been removed from our construction in order to produce A. tumefaciens strains with maximum agrocin 84 uptake. The pYW15d-acc ΔaccR plasmid encoding the agrocin 84 transporter and the pBRR1MCS-3 plasmid containing the agnB2/leuSAt gene and a T7 promoter and a tetracycline marker were then transformed into an electrocompetent A. tumefaciens pol-Gm-FRT strain containing the DE3 lysogen encoding a T7 RNA polymerase48. Briefly, electroporation was performed at 2400 V and 400Ω and the culture was then grown in 3 ml LB broth for 2.5 h. The A. tumefaciens culture was then streaked on LB agar plates containing 100 µg/ml of carbenicillin, 15 µg/ml of gentamycin and 10 µg/ml of tetracycline. The final construct A. tumefaciens NTL4 T7pol-Gm-FRT containing both pYW15d-acc ΔaccR (encoding the agrocin 84 transporter) and a pBRR1MCS-3 plasmid (containing a gene encoding either wt or mutant AgnB2/LeuRSAt proteins was then used for bioassay analysis. Individual colonies were picked and grown in 3 ml cultures to make glycerol stocks for storage at −80 °C.

Protein expression and purification. All enzymes (mutants of AgnB2 and LeuRSAt) were overexpressed in E. coli BL21 (DE3) RIL codon plus cells (Agilent) (containing pET-28a plasmid encoding C-terminal 6X His-tagged AgnB2 gene or pET-21b plasmid encoding C-terminal 6X histidine tagged LeuRSAt gene).
LeuRS enzymes were purified in a similar manner as detailed previously for LeuRS\textsubscript{a} \cite{21,22}. In brief, His-tagged proteins were purified by affinity chromatography using Ni\textsuperscript{2+}-NTA agarose beads (Qiagen), followed by purification using a strong anion-exchange Mono-Q column (GE Healthcare) by FPLC. All proteins were confirmed to be > 95% pure using SDS–polyacrylamide gel electrophoresis analysis and the active concentration determined by active-site titration.

**Purification of TM84.** Agrocin 84 and TM84 were obtained from \textit{A. tumefaciens} NT1 (pAgK84-Tn5A1-B5) agrocin 84 secretion strain grown at 28°C in D-glucose minimal media supplemented with 50 μg ml\textsuperscript{-1} kanamycin as described previously \cite{23}. Briefly, agrocin 84 and TM84 were separated from other media components by adsorption on charcoal, and then eluted using 70–90% reagent alcohol, dried and both further purified by reverse-phase HPLC. The concentration of TM84 was determined using an extinction coefficient (\(ε\) = 260 nm) of 0.0154 M\textsuperscript{-1} cm\textsuperscript{-1}.

**Bioassays.** In vivo assays to determine the agrocin 84 sensitivity of AgnB2/LeuRS\textsubscript{a} and their mutants were performed. Stationary phase cultures of \textit{A. tumefaciens} NTL4 T7\textsubscript{pol} were grown in minimal media supplemented with 50 μg/ml of kanamycin as described previously \cite{9,23}. Briefly, agrocin 84 and TM84 were separated from other media components by adsorption on charcoal, and then eluted using 70–90% reagent alcohol, dried and both further purified by reverse-phase HPLC. The concentration of TM84 was determined using an extinction coefficient (\(ε\) = 260 nm) of 0.0154 M\textsuperscript{-1} cm\textsuperscript{-1}.

**Active-site titration assay.** Active-site titration assay was performed as described earlier \cite{23}. Briefly, the reaction buffer contained 50 mM HEPES (pH 7.4), 20 mM MgCl\textsubscript{2}, 40 mM KCl, 40 μM T7 RNA polymerase, 1 mM spermidine and 2 mM NaPPi. 1 μM tRNALeu initiator and 9 μM T7 RNA polymerase \cite{23}. After DNase I treatment for 48 h. The sensitivity of the strain towards agrocin 84 was determined by measuring the zone of clearance around the well containing agrocin 84. The control strain containing only the \textit{acs} operon, but no pBBR1MCS-3 plasmid showed a zone of clearance of around 4 cm. Therefore, any sample having a zone of clearance > 4.1 cm was considered inactive.

**tRNALeu transcription.** In this study, we used purified in vitro transcribed tRNALeu\textsuperscript{5-23} (UA) isoacceptor based on the nucleotide sequence from \textit{A. tumefaciens} NT1 (pAgK84-cis-cys) that was purified by thin-layer chromatography. The tRNALeu\textsuperscript{5-23} (UA) isoacceptor was treated with plasmids encoding the tRNALeu gene with Bst\textsubscript{NI} restriction endonuclease. The tRNA was obtained by purifying the tRNA at room temperature using 1 mM MgCl\textsubscript{2} after incubating 4 mM ATP, 1 mM tetrasodium pyrophosphate (NaPPi), 10 mM KCl, 1X TBE buffer (pH 8.3). Gel extraction of the tRNA was then performed, followed by precipitation using 0.3 M sodium acetate and absolute ethanol. The tRNA obtained was then collected over 30 min and quenched in a 10% charcoal slurry containing 0.5% HCl counting was then used to measure the amount of [32P]-PPi incorporation into tRNALeu. The data was then analyzed with Origin software (version 7) and fitted to a one-site or a two-site binding model to obtain the binding affinity (K\textsubscript{d}), stoichiometry (N) and enthalpy (AH) for each complex.

**Structure determination and refinement.** The diffraction data sets of the complex AgnB2-LeuRS-tRNALeu\textsuperscript{5-23}-Leu-AMS were obtained by mixing 1 μl of a solution containing AgnB2-LeuRS at 43 μM, tRNALeu\textsuperscript{5-23} (A. tumefaciens) at 34 μM and Leu-AMS at 2 mM, with 1 μl of reservoir solution containing 0.1 M Mes pH 6.5, 0.2 M ammonium sulfate and 27% (w/v) PEG 5000 MME. Quality and size of crystals were optimized by macroseed with protein at 2 mg ml\textsuperscript{-1}. The crystals were frozen in liquid nitrogen after transfer for a few seconds in the mother liquor plus 15% (v/v) ethylene glycol as cryoprotectant.

**Molecular modelling.** A homology model of LeuRS\textsubscript{a} was built using the Insight II Molecular Modeling System (Accelrys software.com) based on the structure of LeuRS\textsubscript{b} (PDB ID 1H3N) \cite{35}. A normalized sequence-structure compatibility score of 0.77 was calculated for the LeuRS\textsubscript{a} homology model using the Verify the three-dimensional model of Insight II \cite{99,80}.

**Data availability.** Atomic coordinates and structure factors for the AgnB2-tRNALeu-AMS ternary complex have been deposited in the RCSB Protein Data Bank with the accession code 5AH5. All other data associated with this manuscript are available from the corresponding author on reasonable request.

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Author contributions
S.Ch. and J.S.R performed project design and the data interpretation, and prepared the manuscript. S.Ch. also conducted the biochemical and biophysical experiments, in vivo mutant constructions and bioassays as well as data analyses. A.P. and S.Cu. solved the structure of the AgnB2 protein ternary complex, provided experimental suggestions and assisted in editing and figures to the final manuscript. C.V. developed the agrobacterial in vivo screen and constructed and assayed initial mutants for this project. S.S. developed AgnB2 and LeuRS\textsubscript{ag} protein expression protocols and performed initial biochemical experiments for project. B.T. assisted with structural interpretation of the data and suggestion of mutants to be tested, preparation of figures and assistance in editing manuscript.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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