Substrate recognition by the *Pseudomonas aeruginosa* EF-Tu–modifying methyltransferase EftM

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The opportunistic bacterial pathogen *Pseudomonas aeruginosa* is a leading cause of serious infections in individuals with cystic fibrosis, compromised immune systems, or severe burns. *P. aeruginosa* adhesion to host epithelial cells is enhanced by surface-exposed translation elongation factor EF-Tu carrying a Lys-5 trimethylation, incorporated by the methyltransferase EftM. Thus, the EF-Tu modification by EftM may represent a target to prevent *P. aeruginosa* infections in vulnerable individuals. Here, we extend our understanding of EftM activity by defining the molecular mechanism by which it recognizes EF-Tu. Acting on the observation that EftM can bind to EF-Tu lacking its N-terminal peptide (encompassing the Lys-5 target site), we generated an EftM homology model and used it in protein/protein docking studies to predict EftM/EF-Tu interactions. Using site-directed mutagenesis of residues in both proteins, coupled with binding and methyltransferase activity assays, we experimentally validated the predicted protein/protein interface. We also show that EftM cannot methylate the isolated N-terminal EF-Tu peptide and that binding-induced conformational changes in EftM are likely needed to enable placement of the first 5–6 amino acids of EF-Tu into a conserved peptide-binding channel in EftM. In this channel, a group of residues that are highly conserved in EftM proteins position the N-terminal sequence to facilitate Lys-5 modification. Our findings reveal that EftM employs molecular strategies for substrate recognition common among both class I (Rossmann fold) and class II (SET domain) methyltransferases and pave the way for studies seeking a deeper understanding of EftM’s mechanism of action on EF-Tu.

Protein post-translational modifications (PTMs) add an additional level of complexity to the proteome and are critical to the biological functions of proteins in all domains of life. In bacteria, PTMs such as methylation are used to respond to environmental cues and may be critical for adaptation to or evasion of host immune systems during infection (1–3).

The bacterial methyltransferase CheR serves as the paradigm for environmental adaptation behavior arising from protein methylation (4). In many enteric bacteria, response to chemical stimuli occurs via O-methylation of glutamic acid residues on chemotaxis receptors by CheR. Bacterial methyltransferases can also directly influence host gene expression and host/pathogen interaction. For example, *Mycobacterium tuberculosis* expresses a secreted 5-methylcytosine–specific DNA methyltransferase (Rv2996c) that directly modifies the host epigenetic machinery to alter transcription (5). Establishment of infection can also be both negatively and positively regulated by methylation of bacterial surface proteins by host- or pathogen-derived methyltransferases. The human protein SUV39H1, for example, methylates the crucial surface-exposed mycobacterial protein HupB, which reduces bacterial adhesion and survival inside the host cell (6). In *Rickettsia*, methylation of outer membrane protein B (OmpB) directly controls virulence (7): virulent species display lysine trimethylation of OmpB, which mediates host adhesion, attachment, and invasion, whereas avirulent strains possess monomethylated OmpB. Finally, our previous work has revealed a role in the establishment of *Pseudomonas aeruginosa* infection for the trimethylation of translation elongation factor thermo-unstable (EF-Tu) on its Lys-5 residue by the EF-Tu methyltransferase, EftM (8–10).

Whereas the roles for protein methylation in bacterial virulence and host/pathogen interactions are emerging, there is

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3 The abbreviations used are: PTM, post-translational modification; DSF, differential scanning fluorimetry; EftM, EF-Tu methyltransferase; EF-Tu, elongation factor thermo-unstable; IPTG, isopropyl β-D-thiogalactopyranoside; ITc, isothermal titration calorimetry; LB, lysogeny broth; NMA, normal mode analysis; PAHM4, *P. aeruginosa* strain HMA; PAO1, *P. aeruginosa* strain PAO1; SAH, S-adenosylhomocysteine; SAM, S-adenosyl-L-methionine; PDB, Protein Data Bank; ChoP, choline phosphate; SUMO, small ubiquitin-like modifier.
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generally less known about the molecular mechanisms that control substrate recognition and catalysis by the bacterial methyltransferase enzymes that incorporate these PTMs. The Gram-negative environmental bacterium *P. aeruginosa* is an opportunistic pathogen that causes serious respiratory, eye, and skin infections in vulnerable individuals, such as those with cystic fibrosis, compromised immune systems, or severe burns. *P. aeruginosa* is also intrinsically resistant to many antibiotics making infections difficult to treat once established. However, precisely how *P. aeruginosa* establishes infection in a host and adapts to its new environment is still not fully understood. A more complete understanding of this process could potentially identify novel strategies to restrict the establishment of infection.

Toward this goal, we previously described trimethylation of EF-Tu on Lys-5 as a PTM that enhances *P. aeruginosa* host-cell adherence and infection (8, 9). In *P. aeruginosa* isolates from acute infections, as well as the laboratory strain PAO1, this EF-Tu modification is present in bacteria cultured at 25 °C but absent at 37 °C (8, 9, 11). This thermoderegulation of PTM incorporation was found to occur at the level of transcription initiation as well as the direct thermolability of the modifying enzyme, EftM (8, 10). In contrast, some chronic isolates, such as PAHM4, retain their ability to methylate EF-Tu at 37 °C (11). However, whether this arises from increased thermal stability of EftM has not been tested. Together, these observations suggest that the major role of the Lys-5 PTM may be to modulate a “moonlighting” function of EF-Tu in host-cell adhesion by *P. aeruginosa*.

Here, we define the mechanism of EF-Tu substrate recognition by EftM. Evolutionary sequence analyses and computational modeling of EftM and the EftM/EF-Tu complex were used to direct mutagenesis and functional studies to identify the protein/protein binding interface, revealing that EftM requires more than just the N-terminal sequence of EF-Tu to bind and methylate its substrate. We also identify conserved residues in a substrate-binding channel that position the N-terminal sequence of EF-Tu to bind and methylate its substrate. We also identify conserved residues in a substrate-binding channel that position the N-terminal sequence of EF-Tu for trimethylation on Lys-5. Collectively, our findings lead to a new mechanistic model for EftM/EF-Tu recognition reliant on both protein/protein docking and protein conformational changes to accommodate the EF-Tu N-terminal sequence, which together direct specific substrate recognition and modification. These functional studies represent an important step toward a better understanding of the molecular recognition mechanism of EftM and bacterial lysine methyltransferases in general.

Results

EftM<sup>HMM4</sup> is functionally equivalent to EftM<sup>PAO1</sup> but with increased thermostability

We previously reported that the temperature sensitivity of EF-Tu methylation by EftM in *P. aeruginosa* strain PAO1 (EftM<sup>PAO1</sup>) is due, at least in part, to the direct unfolding of the methyltransferase upon transition from environment-like (25 °C) to host-like (37 °C) temperatures (8). This direct thermolability also likely contributed to challenges we encountered in expression, purification, and handling of EftM<sup>PAO1</sup> for biochemical and biophysical studies. Therefore, we decided to examine the folding and functional properties of the same enzyme (EftM<sup>HMM4</sup>) from the chronic infection strain of *P. aeruginosa* PAHM4 in which EF-Tu methylation was previously observed at 37 °C (9).

Using differential-scanning fluorimetry (DSF) we found that the *T*<sub>m</sub> of EftM<sup>HMM4</sup> is increased by ~5 °C, to 37 °C (Fig. 1A), compared with the 31.5 °C that we determined previously for EftM<sup>PAO1</sup> (8). Additionally, as observed for EftM<sup>PAO1</sup>, S-adenosyl-L-methionine (SAM) stabilizes EftM<sup>HMM4</sup> by ~5 °C resulting in an unfolding *T*<sub>m</sub> of 41.5 °C in the presence of the cosubstrate. Next, we asked whether this additional thermal stability of EftM<sup>HMM4</sup> directly correlates with its activity at an elevated temperature. As expected, EftM<sup>HMM4</sup> retained enzymatic activity after incubation at either 25 or 37 °C, as observed previously, but its activity was ablated after incubation at 42 °C for 5 min or more (Fig. 1B). Collectively, these data show the retained methylation of EF-Tu in PAHM4 at 37 °C is due to an increase in the thermostability of EftM methyltransferase by ~5 °C, which allows the enzyme to remain active at the elevated temperature. Critically, these findings also show that EftM<sup>PAO1</sup> and EftM<sup>HMM4</sup> are functionally equivalent enzymes and that the latter still exhibits thermoderegulation but shifted to higher temperature.

As there is no known structure of any EftM family member, we searched for structures of the closest paralogs and identified the *N,N*-dimethyltransferase DesVI from *Streptomyces venezuelae* (PDB code 3BHO), which methylates a sugar intermediate in macrolide antibiotic biosynthesis, dTDP-3-amino-3,6-dideoxy-α-D-glucopyranose (12). DesVI is 25.3% identical and 36.7% similar to EftM<sup>HMM4</sup> as calculated in EMBL EMBOSS, with both values ~1% higher compared with EftM<sup>PAO1</sup>. A combined approach of homology modeling and threading was used, followed by energy minimization, to generate structural models of EftM<sup>PAO1</sup> and EftM<sup>HMM4</sup>. This combination of methods, which is applicable to modeling in instances of relatively mod-
EftM model (the core methyltransferase fold and the auxiliary domain in the worthy that the changes involving the most physicochemically coupled with its greater ease of expression, purification, and thermostabilization of EftMHM4.

In summary, EftMHM4 exhibits conserved activity, retained thermoregulation but with increased thermostability, and is predicted to have identical structure compared with the previously characterized thermolabile methyltransferase EftMPAO1. Coupled with its greater ease of expression, purification, and handling, these factors lead us to use EftMHM4 as the optimal context to complete the detailed study described herein of EF-Tu recognition by EftM.

**EftM requires more than just the EF-Tu N-terminal peptide for binding**

To begin defining EF-Tu recognition by EftM, we first asked whether important interactions are made with the surface of the globular protein fold of EF-Tu or whether the isolated N-terminal peptide (KMAKEKFERNP11; trimethylated Lys-5 is in bold font and underlined) alone is a suitable substrate for modification. First, a variant of *P. aeruginosa* EF-Tu lacking the first 11 amino acids of the N terminus (Δ11-EF-Tu) was generated to assess contributions of the EF-Tu surface to binding by EftM. To qualitatively assess binding, we compared the ability of EftM to form a stable complex with EF-Tu or Δ11-EF-Tu using gel-filtration chromatography (Fig. 4, A and B). Complexes of both EftM/EF-Tu and EftM/Δ11-EF-Tu were observed to elute from the column before either free component due to their larger size, suggesting that EftM is able to bind the truncated EF-Tu protein.

We next quantified the binding affinity of EftM to WT *P. aeruginosa* EF-Tu and the Δ11-EF-Tu truncated construct using isothermal titration calorimetry (ITC). The binding affinity (K_D) measured for EftM and WT EF-Tu was relatively weak (∼27 μM; Table 1) as would be anticipated given the very high levels of the translation factor in bacterial cells. Remarkably, a similar affinity was also obtained with the truncated EF-Tu protein (Table 1), consistent with retained interaction even in the absence of the EF-Tu N-terminal sequence. Interestingly, however, although the weak binding precludes accurate determination of binding enthalpy (ΔH), binding of EftM to Δ11-EF-Tu appears to have a significantly lower ΔH (>10-fold lower) than for WT EF-Tu (Fig. 4C). This difference suggests binding-induced changes in the EF-Tu N-terminal peptide or EftM structure occur upon protein/protein interaction even though the EF-Tu N-terminal peptide does not appear to directly contribute to the binding affinity of the complex.
In a complementary approach, we used mass spectroscopy (MS) to discern whether peptides corresponding to the isolated N-terminal peptide sequence of *P. aeruginosa* EF-Tu could be methylated by EftM. Samples containing 10-mer peptide (1MAKEKFERNK10) alone or after incubation with EftM/SAM were subject to MALDI-TOF MS analysis. In both spectra, peaks corresponding to the unmethylated peptide (m/z 1280) were identified, but no methylated peptides were detected (predicted m/z 1294, 1308, and 1322 for mono-, di-, and trimethylated peptide, respectively), even in the presence of enzyme and SAM (Fig. 4D). We additionally tested a shorter peptide (1MAKEKMe3F6) that corresponds more precisely to the sequence we predict is accommodated within the EftM peptide-binding channel (see below). Although synthetic trimethylated peptide (1MAKEKMe3F6) could be detected, demonstrating our ability to measure Lys-5-modified peptides by MS, only unmethylated peptide was detected after treatment with EftM and SAM (Fig. S1), as for the longer sequence.

In summary, gel-filtration and ITC data show that EftM can bind EF-Tu lacking its N-terminal sequence, whereas MS analysis also reveals that EftM cannot methylate an isolated EF-Tu N-terminal peptide sequence. Together, these observations point to a critical role for an extended surface of the globular body of EF-Tu in substrate recognition by EftM.

| Table 1  | EF-Tu and SAM/SAH-binding affinities of wildtype and tryptophan variant EftM<sup>HM4</sup> |
|----------|---------------------------------------------|
| Substrate/Co-substrate | EF-Tu | Δ11-EF-Tu | SAM | SAH |
| EF-Tm | 27.9 ± 16.0 | 25.9 | 17.9 ± 7.0 | 23.4 ± 2.7 |
| EF-Tm-W170F | 19.3 ± 9.5 | 19.9 ± 6.2 | 26.0 ± 0.2 |
| EF-Tm-W196F | 24.0 ± 12.7 | | | |

Figure 3. Differences between EftM<sup>PAO1</sup> and EftM<sup>HM4</sup> are distributed throughout the protein structure. A. amino acid sequence alignment of EftM<sup>PAO1</sup> and EftM<sup>HM4</sup> with differences indicated by colored shading, and the different regions of the EftM structure (see Fig. 2) denoted by the colored bar above the sequence. The two sequences share 88.7% identity and 93.1% similarity. B. homology model of the EftM<sup>HM4</sup>/SAM complex with amino acid differences between EftM proteins from strains PAHM4 and PAO1 shown as spheres. In both panels, color coding indicates differences in amino acid physicochemical properties at each site: most distinct (red), intermediate (orange), and most similar (yellow).

Figure 4. EftM recognizes and binds to more than just the EF-Tu N terminus. A. gel-filtration chromatography of EftM/EF-Tu complexes: EftM/EF-Tu (black), EF-Tu alone (gray-shaded), and EftM/Δ11-EF-Tu (green). B. Coomassie-stained SDS-polyacrylamide gel of gel-filtration fractions confirming elution of the EftM/EF-Tu in the first peak in the chromatogram (fractio ns at 13 and 14 ml). C. Time course of binding of EftM and EF-Tu/Δ11-EF-Tu. Although weak-binding affinity precludes accurate determination of ΔH, i.e. the intercept of the fit curve on the y axis in the lower plot for each titration, note the difference in the scale of this plot for each complex, which suggests that enthalpy of binding is significantly reduced in the absence of the EF-Tu N-terminal peptide in Δ11-EF-Tu. D. MS analysis of a 10-mer peptide corresponding to the N-terminal peptide without (black) or with (orange) treatment with EftM and SAM in a methylation reaction.
EftM interacts with an extensive surface of EF-Tu

We next generated a computational model of the EftM/EF-Tu complex using the protein/protein docking software HEX 6.0 (13). To generate this model, a structure corresponding to Δ11-EF-Tu was first created by deletion of N-terminal residues in the structure of P. aeruginosa EF-Tu (PDB code 4ZV4). Potential models of Δ11-EF-Tu bound to EftMHM4 generated by HEX 6.0 were ranked according to calculated energies for shape and electrostatic complementarity. The top-scoring prediction was manually adjusted, e.g. by selection of optimal side-chain rotamers, to remove obvious clashes before energy minimization to generate the final model of the EftM/Δ11-EF-Tu complex (Fig. 5, A and B).

The final model was assessed based on amino acid evolutionary conservation within the EftM family, revealing a moderately-conserved surface of EftMHM4 that is predicted to interact with EF-Tu (Fig. 5, B and C). In contrast, the predicted solvent-exposed surface of EftM exhibited lower overall conservation, whereas the predicted openings to the EF-Tu N-terminal peptide-binding channel have much higher amino acid conservation as would be expected for this functionally critical region of the enzyme (Fig. S2). The putative EftM/Δ11-EF-Tu binding interface is stabilized by multiple electrostatic interactions made by EftMHM4 residues Asp-76, Arg-81, Asp-91, and Asp-98. To experimentally test the role of these residues in EF-Tu binding, each was altered with a charge-reversal substitution (i.e. Arg to Glu or Asp to Lys). Additionally, His-86 within the same surface was substituted with Ala. This group of residues is distributed across most of the predicted interaction interface (Fig. 5B). Variant EftMHM4 proteins were expressed and puri-
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Figure 6. N-terminal sequence of EF-Tu (MAKEKF) is stabilized by conserved residues in EftM's substrate-binding channel. A, substrate-binding channel of EftMHM4 based on solvent space calculations using the CAVER plugin of PyMOL. EftMHM4 residues Tyr-7, Asp-15, Met-74, Trp-170 and Trp-196 line the substrate tunnel. B, same view but showing SAM (yellow) and three modeled EF-Tu residues Glu-4 (modeled as Ala), Lys-5, and Phe-6 within the cavity. The approximate path of the additional N-terminal peptide backbone is marked with a dotted line. C, representative trimethyllysine immunoblots of methylation reaction with the indicated EftMHM4 variants and F6A substitution in EF-Tu; D, quantification of immunoblot data from two replicate experiments.

fied as for the WT protein, and their folding was assessed using thermal denaturation monitored by intrinsic fluorescence. Each protein had a similar unfolding profile and inflection point melting temperature (T_m; within ±5 °C) compared with WT EftMHM4 consistent with their correct folding. Additionally, because each substituted residue is located on the EftMHM4 protein surface and distant from both the SAM-binding pocket and EF-Tu N-terminal peptide-binding channel, we therefore ascribe any observed decrease in trimethylation activity to weakening of the binding interaction between EftMHM4 and its EF-Tu substrate at this predicted surface.

To measure trimethylation of EF-Tu by the variant EftMHM4 proteins, we used the established immunoassay with an anti-trimethyllysine antibody. First, to determine the optimal reaction conditions for comparison with WT EftMHM4, a time-course activity assay was performed using WT EftMHM4 (0.25 mM) in the presence of excess EF-Tu substrate (10 μM) and SAM cosubstrate (1 mM). This experiment revealed that under the conditions used, EF-Tu trimethylation neared completion at ~30 min (Fig. 5D), and this single time point was selected for use in all subsequent methylation assays. D91K, D98K, and H86A substitutions in EftMHM4 resulted in a severely reduced ability to trimethylate EF-Tu (Fig. 5E), with activity decreased to ~16, 13, and 17%, respectively, compared with WT EftMHM4 (Fig. 5E). D76K and R81E substitutions also caused a reduced ability to methylate EF-Tu, to ~40, and 15%, respectively, although double substitution of these residues in EftMHM4-D76K/R81E further lowered activity to ~7%.

We next made individual substitutions of residues Glu-69 and Arg-208 in EF-Tu, which are predicted by the model of the complex to be near EftMHM4 residues Asp-76 and Arg-81, respectively. Consistent with the effects of the corresponding residues in EftMHM4, charge-reversal substitutions E69K and R208E in EF-Tu also resulted in reduced methylation activity by WT EftM, dropping to ~55 and 20%, respectively (Fig. 5E). The double mutant, EF-Tu-E69K/R208E, again showed a more pronounced decrease in methylation activity (to ~11% of WT), mirroring the effects of the corresponding substitutions in EftM. Collectively, the activities of the EftMHM4 variants with targeted substitutions, and of WT EftM on variant EF-Tu proteins with corresponding changes, experimentally validate the predicted protein/protein interaction interface in our computational model of the EftM/Δ11/EF-Tu complex.

EF-Tu N terminus is accommodated in a conserved substrate-binding channel within EftM

Using our model of the EftM/EF-Tu complex, we next asked how the EF-Tu N-terminal peptide might be positioned in EftM's substrate-binding channel. The EftMHM4 homology model has its substrate-binding channel in a similar location to that observed in the DesVI structure but with an overall larger volume and distinct surface shape and charge distribution. The residues lining the substrate-binding channel, Tyr-7, Asp-15, Met-74, Trp-170, and Trp-196 (Fig. 6A and Fig. S2B), are highly conserved (>90%), whereas Asp-20 near the opening of the channel is also conserved (84.2%) in EftM family proteins. Additionally, although these residues are conserved in the EftM family, they are less highly conserved in DesVI and other paralogs, highlighting their potential role in interactions with the EF-Tu N-terminal peptide. Near the center of the channel, EftM also accommodates the SAM cosubstrate in an orientation consistent with other Rossmann fold methyltransferases, in which the methyl group is oriented toward the opening of the substrate channel adjacent to the modeled EF-Tu N terminus (Fig. 6B).

The cavity within EftM that forms the EF-Tu N-terminal peptide-binding channel appears large enough to accommodate the first six residues of EF-Tu (MAKEKF) with some distinctive surface pockets that can fit specific EF-Tu residues. We therefore next attempted to model this N-terminal sequence backbone path through the channel, positioning specific residues where possible (Fig. 6B). Two EF-Tu residues, Lys-5 and Phe-6, could be readily positioned with minimal clashes; in other cases, although the backbone path is clear, expansion of the channel would be necessary to accommodate the residue side chains. As discussed further below, we propose that interaction of EftM and EF-Tu may induce such widening of the peptide-binding channel.
To test the predicted roles of EftM residues within the substrate-binding channel in recognizing and stabilizing the EF-Tu N-terminal peptide, further EftM variants were generated. Tyr-7 and Met-74 are located next to the predicted path of the first three residues (\(^{1}\text{MAK}\)) of EF-Tu and may orient and stabilize the N-terminal residue. Consistent with such a role, individual alanine substitution of each residue reduced EftM activity to 6.5 and 3.5%, respectively (Fig. 6, C and D). EF-Tu Lys-5 is placed in the model near the EftM catalytic center, as required for its modification, and proximal to Asp-15. Substitution of this EftM residue with alanine (EftM-D15A) also dramatically reduced EF-Tu methylation activity to \(~5\%\) of the WT enzyme (Fig. 6, C and D). The modeling also places EftM Trp-170 adjacent to EF-Tu Phe-6. EF-Tu residue Phe-6 to precisely orient Lys-5 for modification. Trp-170 interacts with the highly-conserved EF-Tu residue Phe-6 to precisely orient Lys-5 for modification. Consistent with this idea, alteration of EF-Tu with an F6A substitution also results in a dramatic loss of methylation by EftM (Fig. 6, C and D).

**EftM Trp-170 and Trp-196 flank the EF-Tu N-terminal peptide-binding channel and are critical for EftM activity**

The highly-conserved EftM residues Trp-170 and Trp-196 are positioned at each end of the EF-Tu N-terminal peptide-binding channel suggesting they may perform “gatekeeping” functions at the channel openings. We therefore investigated their potential roles in cosubstrate binding and EF-Tu recognition and modification. EftM\(^{1\text{MAK}}\) Trp-170 and Trp-196 were individually substituted to phenylalanine or alanine, and the activity of each variant was assessed as before. First, in the immunoblot methylation assay, both Trp-170 substitutions, W170A and W170F, completely ablated EftM\(^{1\text{MAK}}\) activity, whereas the equivalent changes at Trp-196 (W196A and W196F) resulted in a very low level of trimethylation activity, \(~4\%\) and \(~10\%\), respectively, compared with WT EftM\(^{1\text{MAK}}\) (Fig. 6, C and D).

We next used ITC to establish whether substitution of either residue disrupts SAM binding. The binding affinities (\(K_D\)) of SAM and S-adenosylhomocysteine (SAH) (Table 1) for WT EftM\(^{1\text{MAK}}\) were first determined and found to be essentially identical to those previously measured for EftM\(^{\text{PAO1}}\) (8). Similarly, no major difference was observed in SAM-binding affinity to either tryptophan to phenylalanine variant (Table 1), indicating that neither Trp-170 nor Trp-196 contribute meaningfully to cosubstrate binding.

To address whether either Trp-170 or Trp-196 contributes to EF-Tu binding affinity, the interaction of the translation factor with each phenylalanine substitution variant of EftM\(^{1\text{MAK}}\) was also assessed by ITC. As discussed above, WT EftM\(^{1\text{MAK}}\) binds EF-Tu with a \(K_D\) of \(~28 \mu\text{M}~\), and the binding affinities of both W170F and W196F are essentially identical (Table 1). However, as observed for EftM\(^{1\text{MAK}}\) interaction with the \(\Delta\text{EF-Tu}~\) variant, a large decrease (>10-fold) in \(\Delta H\) was again apparent for EftM-W170F and EftM-W196F interaction with WT EF-Tu (Fig. S3). These findings are also consistent with our earlier interpretation that a large enthalpic component of binding arises from binding-induced re-organization of the EF-Tu N terminus into the substrate channel and, furthermore, that these two EftM Trp residues play an important role in this process.

Together, these data reveal that Trp-170 and Trp-196 play functionally critical roles in EftM activity, with alteration of the former residue, in particular, fully ablating EF-Tu methylation. These residues do not, however, contribute directly to the binding affinity for either substrate or cosubstrate. The proximity of Trp-196 to a conserved opening on the EftM surface (Fig. S2B) suggests this residue could be required for interactions or conformational changes necessary for exchange of SAM/SAH during catalytic turnover or in stabilizing the most N-terminal residues of EF-Tu, together with Tyr-7 and Met-74. The absolute requirement for Trp-170 in EftM activity, together with our modeling of residues in the peptide-binding channel, suggests that Trp-170 interacts with the highly-conserved EF-Tu residue Phe-6 to precisely orient Lys-5 for modification. Consistent with this idea, alteration of EF-Tu with an F6A substitution also results in a dramatic loss of methylation by EftM (Fig. 6, C and D).

**Binding of the EF-Tu N-terminal peptide for Lys-5 methylation**

We next addressed the question of how the N terminus of EF-Tu accesses the EftM substrate-binding channel. At least two distinct potential mechanisms can be envisaged. First, following EftM/EF-Tu interaction, the EF-Tu N-terminal sequence could enter via an adjacent opening on the EftM surface (Fig. S2B) and thread into the substrate channel until fully accommodated (a “threading” model). Alternatively, binding to EF-Tu could induce conformational changes in EftM that open the peptide-binding channel allowing “placement” of the \(^{1}\text{MAKEKF}\) sequence directly into its final position (Fig. S4A). Given that EftM can methylate an N-terminally His\(_6\)-tagged EF-Tu construct with similar efficiency to EF-Tu with an authentic N terminus (Fig. S5), the latter scenario appears most plausible, and we sought further evidence to more fully support this placement model.

To gain initial insight into potential points of flexibility in EftM that could be influenced by interaction with EF-Tu, we used the El Nemo server (14) to perform a normal mode analysis (NMA) on the EftM\(^{1\text{MAK}}\) homology model structure. NMA provides a coarse model of large-scale protein movements and can identify potential hinge points and the directionality of domain movements. The NMA suggests that EftM\(^{1\text{MAK}}\) can undergo a large-scale conformational change between the methyltransferase core fold and the \(\beta\)/6 linker of the auxiliary domain (Fig. 7A and Fig. S4B). This motion could potentially generate a more open conformation as well as a more closed conformation of the peptide-binding channel in EftM (Fig. 7A), compared with the starting homology model structure. We speculate that this movement toward the “open” conformation could be induced by EF-Tu binding and thus expand the substrate-binding channel to allow direct accommodation of the \(^{1}\text{MAKEKF}\) peptide sequence.
To further experimentally test the placement model, we used a construct of EF-Tu fused at its N terminus to the SUMO domain (Fig. 7B, SUMO-L0-EF-Tu). Our expectation was that if the channel is rigid and does not open upon EF-Tu binding then the relatively large, folded SUMO domain attached to the N terminus of EF-Tu would preclude entry through the opening on the EftM surface (Fig. S4C). Although methylation was reduced, EftMHM4 is still able to trimethylate Lys-5 in SUMO-L0-EF-Tu with reasonable efficiency (~30% of WT EftM activity; Fig. 7, C and D). We next increased the linker length between SUMO and EF-Tu domains, to adjust the distance between the SUMO domain and the EftM/EF-Tu complex. Two constructs with either a three-residue (SUMO-L3-EF-Tu) or a six-residue (SUMO-L6-EF-Tu) glycine/serine-rich linker both showed a further modest recovery of activity compared with SUMO-L0-EF-Tu (~50% compared with WT EF-Tu; Fig. 7, C and D). Collectively, these results support the model of placement of the EF-Tu N terminus into a widened peptide-binding channel in EftM upon its binding to EF-Tu.

**Discussion**

Some bacteria that infect the host respiratory tract present the phosphorylcholine (or, choline phosphate; ChoP) modification on their cell surface to enhance airway epithelial cell adhesion and thus promote the establishment of infection (11, 15, 16). *P. aeruginosa*, in contrast, does not present ChoP but instead uses the surface-exposed protein synthesis factor EF-Tu with trimethylated Lys-5 as a molecular mimic of this PTM that can similarly bind to the epithelial cell platelet-activating factor receptor as a key adhesin in infection (9, 11). How EF-Tu is exported is unclear as it lacks a canonical secretion signal, suggesting an alternative mechanism of selective transport to the cell surface. In this regard, EF-Tu’s association with the *P. aeruginosa* type VI secretion system (17, 18) is intriguing and warrants further investigation. Whether trimethylation of Lys-5 is required for transport to the cell surface is also not definitively known. However, EF-Tu is cell-surface–associated in numerous microbes (19), including many that lack EftM. Furthermore, studies of intact *P. aeruginosa* PAO1 have revealed that EF-Tu is cell-surface–associated in both trimethylated and unmethylated forms as it is found in this moonlighting role regardless of growth temperature and thus Lys-5 methylation status (11, 20). Collectively, these results suggest that, unlike the established role of the Lys-5 PTM in host cell adherence once on the cell surface, transport of EF-Tu to that extracellular location is likely not dependent on trimethylation.

Prior to this work, the mechanism of EF-Tu substrate recognition by EftM was unclear. More broadly, the mechanisms of action of bacterial lysine methyltransferases are currently not well-defined in sharp contrast to their eukaryotic counterparts, which play a prominent role in epigenetics via histone protein modification (21). Here, we showed that EftM from the chronic infection strain PAHM4 possesses conserved activity and retained thermostability, albeit with modestly (~5 °C) increased thermostability, compared with the previously studied EftMPAO1 enzyme, making it suitable for detailed structural and mechanistic studies of this enzyme family. Our studies revealed that the isolated EF-Tu N-terminal peptide sequence is not a suitable substrate for EftM, but instead, specific sub-
strate recognition and modification are reliant on interaction via an extended protein/protein docking interface. Overall, our results evoke a model in which protein/protein docking via an extended surface is required for interaction of enzyme and substrate, and subsequent modification of EF-Tu Lys-5 (Fig. 8). Furthermore, this interaction may induce conformational changes that open EftM peptide-binding channel to allow access to the EF-Tu N-terminal peptide sequence. Within this channel, several highly-conserved residues, including two functionally critical Trp residues, appear to act in concert to organize the 1MAKEKFG sequence and thus precisely orient the Lys-5 target residue for modification (Fig. 8).

The mechanism of EF-Tu recognition and Lys-5 methylation by EftM revealed by our work may also have broader implications for our understanding of other lysine methyltransferases belonging to both the Class I Rossmann fold- and Class II SET domain-containing methyltransferases. Specifically, our findings suggest that EftM may exploit features of molecular mechanisms common to both the Rossmann fold methyltransferases, in its requirement for an extended protein surface for substrate recognition, and the SET domain methyltransferases, with its substrate-binding channel that orients the modified residue within a flexible loop or disordered protein region (22).

Despite recent discoveries of extensive methylation in bacterial proteins, including lysine methylations, and their roles in bacterial physiology and infection (4, 7, 23, 24), there still exists a significant gap in our mechanistic understanding of bacterial lysine methyltransferases. The majority of Class I Rossmann fold protein methyltransferases recognize the globular body of their protein substrate and possess a shallow and relatively surface-exposed catalytic center. For example, the ribosomal protein L11 methyltransferase, PrmA, modifies three target sites in its substrate, including two lysine residues (Lys-3 and Lys-39), exploiting recognition of the globular L11 protein fold and accommodating its structurally distinct target sites in a shallow catalytic center (24). This enzyme–substrate recognition mechanism thus allows the necessary flexibility in target selection for both Lys-3, which resides in an unstructured N-terminal region, and Lys-39, which is located within an α-helix. The OmpB methyltransferase in Rickettsia also recognizes the globular body of its substrate to allow catalysis of multiple lysine methylations within its shallow catalytic site (25). A similar recognition mechanism is also observed in eukaryotic Class I Rossmann fold methyltransferases such as DOT1L, which methylates histone H3 Lys-79 within the nucleosome using its N-terminal domain to bind the substrate (26). The recognition mechanism and subsequent catalysis of these methyltransferases follows a “catch and catalyze” strategy, which is mostly dependent on globular body recognition, associated conformational changes, and orienting substrate in the shallow catalytic site for methylation (26). Although our studies have shown that EftM also requires an extensive protein/protein interface to bind EF-Tu, its catalytic center is located within a deep substrate-binding channel. This may reflect the fact that, unlike the bacterial lysine methyltransferases noted above, EftM is very specific with a single trimethylation target site at Lys-5. As such, in this latter aspect of substrate recognition, EftM functions in a manner more similar to the SET domain methyltransferases.

Many SET domain methyltransferases can bind and catalyze modification of short, isolated peptide sequences containing their target site residue. The histone-lysine N-methyltransferase SETD2 specifically trimethylates Lys-36 of histone H3 (H3-K36me3), and the enzyme is active on a 14-residue dimethylated (H3-K36me2) peptide as substrate (27). Upon binding to the H3 peptide, the catalytic tunnel is proposed to undergo stepwise conformational changes consistent with a placement model, accommodating and orienting the peptide (27). Similarly, EHMT1, which specifically mono- and dimethylates Lys-9 of histone H3 (H3K9me1/me2), can recognize and methylate an 11-residue peptide from its target as the substrate, again with a mechanism consistent with the placement model (28). Other SET domain methyltransferases have additional domains fused to the catalytic SET domain or act as a part of multiprotein complexes, in which both the recognition of globular domain of protein(s), induced conformational rearrangements, and placement of substrate motif into deep channels are observed (29). Our findings reveal that EftM has a deep peptide-binding channel like the SET domain methyltransferases, and the EF-Tu N-terminal sequence is accommodated directly, likely with the aid of conformational changes induced by protein/protein interaction (Fig. 8).

Our current studies have revealed important details of substrate recognition by EftM and suggest that its mechanism of substrate recognition and modification are more complex than other known bacterial lysine methyltransferases. Specifically,
EF-Tu recognition by EftM

EftM appears to more closely resemble the SET domain methyltransferases of eukaryotic origin in key aspects such as its EF-Tu N-terminal peptide-binding channel. We also note that the likely catalytic center of EftM is composed of multiple tyrosine and histidine residues, which are also found in the SET domain methyltransferases. Further investigation using high-resolution structural and careful biochemical analyses will be required to fully define the molecular basis of specific substrate recognition as well as other aspects of EftM’s mechanism of catalysis (Fig. 8).

Experimental procedures
Computational modeling of EftM structure and the EftM/EF-Tu complex

A hybrid molecular modeling strategy was used to generate homology models of the EftMPAO1 and EftMHM4 protein structures. First, homology modeling was performed using SWISS-MODEL (30) to generate complete structures for each protein. Inspection of these structures revealed likely incorrect modeling of the β5/6 linker region (e.g. multiple aromatic residues exposed to solvent), and this region was thus remodeled using protein threading with ITASSER (31). The model for each EftM protein was further examined in Coot (32) to confirm favorable side-chain conformations and minimize clashes before a round of energy minimization in UCSF Chimera (33).

A structure corresponding to Δ11-EF-Tu from *P. aeruginosa* PAO1 was generated by deletion of the residues 8–11 from PDB code 4ZV4 (note, the first seven residues are disordered and were already absent in the deposited structure). This Δ11-EF-Tu structure and the EftMHM4 homology model were used as target and ligand, respectively, in the protein/protein docking software HEX 6.0 (13) to identify an unbiased docking interface. The models were ranked based on their total energy score, which combines calculated energies derived from both from shape complementarity and electrostatics. The top-scoring model of the complex (*E* = 212.6 kcal/mol) was energy-minimized in the UCSF Chimera (33) and then examined in Coot (32) to confirm favorable side-chain conformations. This model exhibits high electrostatic and surface shape complementarity between EftMHM4 and Δ11-EF-Tu, and the close placement of the EF-Tu N-terminal sequence to EftM gives further confidence in its likely accuracy.

The CAVER 3.0.1 plugin (34) in PyMOL with shell depth and a radius of 4 Å each, clustering threshold of 3.5 Å, and the channel origin set at the SAM binding pocket, was used to visualize substrate-binding channel in EftM.

NMA, a fast and simple method to calculate vibrational modes and protein flexibility, was performed on the EftMHM4 homology model using the El Nemo server (14). NMA is an implementation of an elastic network model in which the protein residues are modeled as point masses or pseudo-atoms (Cα only) connected by springs, which represent the interatomic force fields. The springs connecting each node to all other neighboring nodes are of equal strength, and only the atom pairs within a cutoff distance (within 8 Å to each other, its interaction sphere) are considered connected. The normal modes of this “ball and spring” system are calculated using a Hessian matrix. Although this treatment is inherently simple and does not capture detailed protein dynamics, comparisons of low-frequency normal modes and the directions of large-amplitude fluctuations in molecular dynamics simulations have revealed qualitatively similar results (35). The first six NMA modes are rotational and translational, whereas modes 7–12 reflect overall long-range motions within the protein. For EftM, the resulting modes 7–12 were analyzed to identify potential regions of flexibility and possible conformational changes in the protein structure.

EftM evolutionary sequence analysis

EftM sequences were retrieved by BLAST search using EftMPAO1 as the query in UniProt. A set of 177 unique amino acid sequences was then selected by applying a 90% sequence identity cutoff in CD-HIT (36). Calculations of site-specific conservation were made using Geneious Prime (37) for all EftM sequences and used to calculate the position-specific Shannon entropy of the EftM protein family. The normalized Shannon entropy is given by Equation 1,

$$S(l) = -\sum_{i=1}^{n} p_i(l) \log_2 p_i(l) \frac{1}{\log_2 n}$$

where $p_i(l)$ represents the frequency of *i* class of residues at position *l* in the multiple sequence alignment, and *n* represents the number of amino acid groups depending the classification criteria. For our calculation, *n* = 20 was chosen (considering each amino acid unique). The higher entropy implies higher variability in the given position in multiple sequence alignment and vice versa. The calculated amino acid conservations were mapped onto the surface of the EftMHM4 homology model using the PDB B-factor column with a white to dark green gradient representing increasing evolutionary conservation.

EftM and EF-Tu cloning and site-directed mutagenesis

Plasmids encoding EftMPAO1/EftMHM4 (pColdII vector) and EF-Tu (pJP04, pDEST14-N-His6-tufB) for recombinant protein expression in *Escherichia coli* or *P. aeruginosa* were previously reported (9). Site-directed mutagenesis of EftMHM4 and EF-Tu was performed using a mega-primer whole-plasmid PCR approach (38, 39), and the variant proteins were expressed from the same parent vectors as the WT proteins. A construct for expression of tag-free EF-Tu was generated by PCR amplification and subcloning the *P. aeruginosa* PAO1 EF-Tu (tufB) coding sequence from plasmid pJP04 (9) into the pE-SUMO vector (LifeSensors). This construct produces His6*-SUMO-EF-Tu protein (“SUMO-L0-EF-Tu”) from which the authentic EF-Tu N terminus is generated by cleavage of the His6*-SUMO tag by the ubiquitin-like protease (Ulp). Equivalent constructs with linker sequences extended by three or six additional amino acids between the SUMO domain and EF-Tu (“SUMO-L3-EF-Tu” and “SUMO-L6-EF-Tu”, respectively) were generated using the mega-primer whole-plasmid PCR mutagenesis approach.
EftM and EF-Tu expression and purification

Cultures of *E. coli* BL21 (DE3) transformed with pColdII-EftM<sup>HM4</sup> or pColdII-EftM<sup>PAO1</sup> were grown at 37 °C in lysogeny broth (LB) supplemented with ampicillin (100 μg/ml) to mid-log growth (A<sub>600</sub> 0.4–0.6). After cold-shock in an ice bath for 30 min, protein expression was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG; 0.5 mM), and cultures were grown for an additional 18 h at 15 °C. Cell pellets were resuspended in 50 mM Tris, pH 7.5, lysis buffer containing 250 mM ammonium chloride, 10 mM magnesium acetate, 20 mM imidazole, 2 mM β-mercaptoethanol, and 20% glycerol. Following cell lysis by sonication, soluble proteins were applied to a HisTrap-FF column (GE Healthcare) pre-equilibrated with 50 mM Tris, pH 7.5, buffer containing 150 mM sodium chloride, 5 mM magnesium chloride, 2 mM β-mercaptoethanol, 20% glycerol, and 20 mM imidazole. Bound proteins were eluted using a linear gradient of imidazole (20–250 mM) in the same buffer solution. Fractions containing EftM<sup>HM4</sup> or EftM<sup>PAO1</sup> were pooled, and the protein was further purified by gel-filtration chromatography on a Superdex200 10/300 column (GE Healthcare) equilibrated with gel-filtration buffer containing 50 mM Tris, pH 7.5, 150 mM sodium chloride, 5 mM β-mercaptoethanol, and 20% glycerol.

For expression of His<sub>6</sub>-EF-Tu, *P. aeruginosa* PAO1ΔeftM cells were freshly streaked on an LB-agar plate. A single colony was used to inoculate LB (50 ml), and the culture was incubated at 37 °C for ~8 h (to A<sub>600</sub> ~0.8). Cells were then pelleted in microcentrifuge tubes and washed three times with 300 mM sucrose (1 ml) at room temperature. Sucrose-washed cells (50 μl) were transformed with pJPO04 plasmid (1.5 μl) by electroporation and allowed to recover in SOC medium (1 ml) at 37 °C for 1 h before plating on LB-agar plates containing 300 μg/ml carbenicillin. A single colony was then grown in LB supplemented with 0.1% arabinose and 300 μg/ml carbenicillin for 18 h at 25 °C before harvesting. Expression of His<sub>6</sub>-Δ11-EF-Tu was accomplished in *E. coli* BL21-Δ1 cells transformed with pDEST14-Δ11tuBP N-His<sub>6</sub> fusion in ZYM-5052 medium supplemented with 0.2% arabinose (w/v) and carbenicillin (100 μg/ml) and incubated with shaking at 25 °C for 14 h. The His<sub>6</sub>-EF-Tu and His<sub>6</sub>-Δ11EF-Tu proteins were extracted and purified using the same chromatographic procedures as described above for the EftM proteins.

SUMO-fusion EF-Tu proteins were expressed in *E. coli* BL21 (DE3) transformed with the p-E-SUMO-EF-Tu vector (L0, L3, or L6) and grown at 37 °C in LB supplemented with ampicillin (100 μg/ml) to mid-log phase (A<sub>600</sub> ~0.4–0.6). Protein expression was induced by addition of IPTG (1 mM) and cultures were grown for 4–5 h at 37 °C. SUMO-fusion EF-Tu proteins were purified using the same chromatographic procedures as described above for EftM. Where needed, the His<sub>6</sub>-SUMO tag was removed from partially purified His<sub>6</sub>-SUMO-L0-EF-Tu following the Ni<sup>2+</sup>-affinity chromatography step by overnight digestion with Ulp (0.1 μg of protease per 1.25 mg of fusion protein) at 37 °C. The free His<sub>6</sub>-SUMO tag was removed from EF-Tu by passing the protease cleavage reaction back over a HisTrap-FF column before further purification of EF-Tu by gel-filtration chromatography.

In vitro EF-Tu methylation assay

For EftM<sup>PAO1</sup> and EftM<sup>HM4</sup> time-course methylation assay at different temperatures (25 and 37 °C), methylation reactions contained 10 μM of both EftM and EF-Tu and 1 mM SAM in gel-filtration buffer (50 mM Tris, pH 7.5, 150 mM sodium chloride, 5 mM β-mercaptoethanol, and 20% glycerol). For assays investigating the thermolability of EftM<sup>HM4</sup> (Fig. 1B), enzyme was preincubated at 25, 37, or 42 °C for 0, 2, 5, 10, or 20 min prior to performing the methylation assay. Reactions were quenched in 2× SDS-PAGE buffer and resolved on a 12% SDS-polyacrylamide gel and immunoblotted as described below.

Methylation assays for testing the substrate recognition model were performed at 25 °C and contained EF-Tu (10 μM), EftM (0.25 μM), and 1 mM SAM in the same buffer as above. For the methylation reaction time course with WT EftM<sup>HM4</sup> (Fig. 5D), samples were removed at each time point (2, 4, 8, 15, 30, and 60 min) for analysis. All subsequent EF-Tu methylation assays with WT and variant EftM<sup>HM4</sup> and EF-Tu proteins were performed for 30 min. Reactions were quenched in 2× SDS-PAGE buffer and resolved on two 10% SDS-polyacrylamide gels, one for immunoblotting and a second for staining with Coomassie. After transfer onto a polyvinylidene difluoride membrane, blots were blocked using 5% nonfat milk in PBS with 2% Tween 20 at room temperature and then probed overnight at 4 °C with a rabbit polyclonal anti-trimethyllysine antibody (Immunchem, ICP0601) diluted 1:10,000 in the same buffer. Blots were probed for 1 h at room temperature with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Sigma; A0545) diluted 1:10,000 in the same buffer. The blots were treated with enhanced chemiluminescence reagent (Thermo Fisher Scientific), and the intensity of bands was analyzed using a Bio-Rad ChemiDoc™ Imager and quantified using ImageQuant software (GE Healthcare). Two replicates using independent preparations of protein for each methylation reaction were performed and quantified, and figures show one representative blot for each protein variant.

Protein stability measurement

DSF was used to compare the thermal stability of EftM<sup>PAO1</sup> and EftM<sup>HM4</sup>. Proteins samples (24 μM) were subject to a linear temperature gradient (0.5 °C/min from 25 to 75 °C), and fluorescence of the hydrophobic residue-binding dye SYPRO Orange (5000-fold dilution) measured on a Step One Plus Real-Time PCR instrument (Applied Biosystems). DSF measurements were performed for each protein in the absence and presence of SAM (150 μM) in three replicates, together with corresponding control experiments without protein under each condition. The first derivative of the melting curve from each replicate was calculated using GraphPad Prism software to determine the mid-point melting temperature (T<sub>m</sub>). The data for EftM<sup>PAO1</sup> were reported previously (8) and are shown here for comparison with EftM<sup>HM4</sup>.

For protein quality control and analysis of variant protein folding compared with the corresponding WT protein, intrinsic fluorescence (at 350 and 330 nm) was monitored during unfolding over a linear temperature gradient (35–95 °C) over 3 min on a Tycho NT.6 instrument (NanoTemper). The temper-
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at courage at which a transition occurs in a plot of the first derivative of the fluorescence ratio (350/330 nm), termed inflection temperature (T_i), was determined by the instrument software for each protein.

Analysis of protein binding by gel-filtration chromatography

Gel-filtration chromatography was performed to qualitatively assess the binding of EF-Tu or Δ11-EF-Tu with EftM_H9251. EftM and EF-Tu were mixed in a 500-μl reaction and resolved on a Superdex 200 column (GE Healthcare) using 50 mM Tris, pH 7.5, buffer containing 150 mM sodium chloride, 5 mM β-mercaptoethanol, and 20% glycerol. One-mL fractions were collected, and a sample of each was run on an SDS-polyacrylamide gel and stained with Coomassie to confirm coelution of EftM and EF-Tu in the first peak.

Isothermal titration calorimetry

SAM and SAH were each dissolved in gel-filtration buffer to 1 mM final concentration for EftM-cosubstrate binding experiments. EF-Tu and Δ11-EF-Tu were concentrated to ~500 μM in the same buffer for protein/protein binding experiments. These ligands (SAM, SAH, or EF-Tu proteins) were titrated into EftM (30–50 μM) in 16 × 2.4-μl injections using an Auto-iTC200 microcalorimeter (Malvern/MicroCal) at 25 °C. After accounting for the heat of dilution by subtraction of the residual heat measured at the end of the titration, the data were fit using a model for one set of sites to determine the binding affinity (K_d).

Mass spectrometry analysis of EF-Tu peptide modification

Reactions containing 10 μM EftM, 10 μM peptide, and 1 mM SAM were incubated at 25 °C for 30 min. Peptide or methyltransfer reaction was spotted onto the source target with 10 mg/ml SAM were incubated at 25 °C for 30 min. Peptide or methylation was analyzed by lysine methyltransferase. Sci. Rep. 11, 20109 –20121

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