Thank you for the careful read!

1. **Additional species in GB1-d (Fig. 2c):**

> Could you expand the discussion regarding the presence of additional species observed in Fig. 2c? For instance, why are these species not present in GB1-1 and GB1-2? You write, "Therefore, the low-intensity peaks in the 19F-NMR spectrum of GB1-d most likely originate from the species with one valine and three diFVal residues." Does this imply that the absence of one diFVal residue causes a significant change in the chemical shifts of the other 19F-labeled valines? Additionally, could you specify which residue is missing, since the small number of low-intensity peaks suggests that not all possible combinations are present in solution?

Response: We observed similar low-intensity ^{19}F-NMR signals in our previous work with 5,5'-difluoroleucine (diFLeu; Tan et al., 2025). These signals became the dominant signals in a sample prepared with a mixture of diFLeu and Leu to yield protein containing single diFLeu residues. The resonance assignments of this sample (referred to as GB1-dd) showed that the dilution of the diFLeu residues by Leu most strongly affected the ^{19}F chemical shifts of diFLeu7 (by about 3 ppm). Leu7 is located between Leu5 and Leu12. The ^{19}F chemical shifts of diFLeu in positions 5 and 12 changed by less than 1 ppm. Furthermore, GB1 made with diFLeu gave no hint of chemical exchange between main and weak peaks.

Based on this we believe that the ^{19}F chemical shifts of diFVal are similarly sensitive to the presence or absence of fluorine atoms at other valine sites. Val39 and Val54 are next to each other, so the main peaks of these two residues should be accompanied by four weak signals of equal intensity at chemical shifts quite different from the protein containing 100% diFVal. For example, the lonely weak signal at -231 ppm almost certainly stems from diFVal in position 54 with valine in position 39 (Fig. 5c). Counting all the weak signals and peak shoulders in the 1D ^{19}F-NMR spectrum, we can account for a total of eight weak signals, which suggests that the identity of the nearest diFVal-versus-Val neighbour can matter also over greater distances. Unfortunately, as we don't have any diFVal left, we cannot assign all the weak peaks.

The abundance of protein containing two diFVal and two Val residues is insufficient for detection by NMR (Fig. S4a).

Weak signals are less prominent in GB1-1 and GB1-2, because the singly fluorinated valine analogues are less easily outcompeted for incorporation into the

protein by traces of canonical valine (FVal2 performed better than FVal1, see Fig. 2 and the mass spectra in Maleckis et al., 2022).

2. **Differences in 19F chemical shifts between variants:**

   Could you briefly discuss potential reasons why the 19F chemical shifts differ between GB1-1 and GB1-d, and between GB1-2 and GB1-d? This may become clearer later in the manuscript when you discuss how rotamers are affected by fluorine substitution, but at this earlier stage the distinction is not immediately obvious to the reader.

Response: We propose adding (in Section 2.2 after the discussion of the γ-gauche effect) the general sentence "As the ^{19}F chemical shifts depend on the rotamers populated by the CH_2F groups as well as their chemical environment, the ^{19}F chemical shifts observed in GB1-d do not simply recapitulate those of GB1-1 and GB1-2, necessitating an independent resonance assignment strategy." Revisiting the chemical shift description in lines 188–190, we noted that we need to include residue 21 as an example of swapped relative shifts in GB1-d.

3. **Formatting issue (page 17, line 314):**

   There appears to be a paragraph break and an unfinished sentence at this location. Please check and correct this formatting issue.

Response: The missing part of the sentence is "To explore the full range of the γ-effect, DFT calculations were performed". We apologize for the oversight.

4. **Fig. 12 and DFT calculations:**

   If I understand correctly, the DFT calculations do not indicate a strictly linear dependence of the FC coupling on the chemical shift, but rather a more complex relationship. Could you comment on this in the text, clarifying that the linear fit is used primarily to illustrate the presence of a correlation?

   In addition, could you discuss the relevance of the DFT calculations performed on (2R)-1-fluoro-2-methylpropane(3-13C) to the conformations of valine residues in GB1? A brief justification of this model system would be helpful.

Response: The sole purpose of the straight line is to guide the eye. We'll make this clearer in the legend of Fig. 12. In addition, we'll add a sentence to stress that the pseudolinearity of the correlation strengthens when all molecular degrees of freedom are relaxed in the DFT calculations (Fig. S8).

We used the model compound for DFT calculations to avoid obscuring the results by any site-specific effects in a protein context. The DFT calculations performed for 16 FVal1 residues in the protein PpiB suggested that the ^{13}C γ-effect is not overwhelmed by the protein context (see the first paragraph of Section 2.9).

5. **Fig. 13:**

It might be useful to include the structures of the valine residues identified in GB1-d alongside those from GB1-1 and GB1-2, to facilitate direct comparison.

Response: Without measurements of the $^3J_{FC}$ couplings and ^{13}C γ-effect, the data of GB1-d do not distinguish between g^S and g^L rotamers. A unique rotamer could be assigned only for the $C^{\gamma 2}H_2$ group of residue 54 based on a large $^3J_{HF}$ coupling.

In GB1-d, the ^{13}C γ-effect seems to be overwhelmed by other effects arising from the switch of a CH_3 to a CH_2F group: subtracting the ^{13}C chemical shifts of the valine methyl groups in the wild-type protein from those of the corresponding CH_2F groups in GB1-d yields $\Delta\delta(^{13}C)$ values that vary much more (> 4 ppm) than the $\Delta\delta(^{13}C)$ values reported in Table 1 for the GB1-1 and GB1-2 samples. If we ignore this and forcefully interpret the $\Delta\delta(^{13}C)$ values in GB1-d as solely reflecting the ^{13}C γ-effect, the preferred rotamers obtained do not correlate at all with those detected in GB1-1 and GB1-2.

6. **Clarification of 3JFC coupling statement:**

The sentence "Assigning preferential rotamers in GB1-d is more difficult, as the diFVal residues contain no CH3 group, which makes 3JFC coupling measurements difficult" is not fully clear. Could you elaborate on how the absence of a CH3 group specifically complicates the 3JFC coupling measurements?

Response: The transverse ^{13}C and ^1H relaxation is significantly faster for CH_2 than CH_3 groups. In addition, fewer protons contribute to the ^1H-NMR signals.

7. **Potential applications to protein–ligand interactions:**

> It would be valuable to expand the discussion on how these fluorinated labels might be applied to studying protein–ligand interactions. Would such interactions be detectable as perturbations in the 19F chemical shifts? Do you expect these effects to be site-specific and sensitive to local changes, or rather global, given the apparent sensitivity of the 19F shifts to the overall protein structure?

Response: Given the sensitivity of the ^{19}F chemical shifts to the presence of any canonical valine residues in the protein, we expect that chemical shift perturbations would not be limited to local interactions. In the case of the protein PpiB produced with Fleu or diFLeu, we demonstrated that ligand binding generates detectable changes in ^{19}F chemical shifts, especially for residues near the ligand binding site and competitive in size with chemical shift changes observed for fluorotryptophan (Tan et al., 2024). If valine sidechains are less flexible than leucine sidechains, however, this may render them less responsive to ligand binding. We just don't know and, in the absence of any experimental data on the effects of ligand binding on the chemical shifts of FVal or diFVal residues, prefer not to speculate.

8. **Estimating energy differences between rotamers:**

> Based on your experimental data and the DFT calculations of 3JFC couplings, would it be possible to estimate the energy differences between the various rotamers?

Response: We'll revisit the calculations of 1-fluoro-2-methylpropane to determine energy differences.