Selective binding of MLX to triacylglycerol-rich lipid droplets: Mechanisms of cytosolic localization and proteome regulation

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Abstract:
The activation of transcription factor Max-Like Protein x (MLX) is modulated by competition between active dimerization and inactive association with cytosolic lipid droplets (LDs). This work explores how two MLX sequences inhibit activity, targeting either membranes broadly or LDs rich in triacylglycerol (TG) specifically. If applicable to other proteins in the MAX-network of transcription factors, this could offer a new direction for cancer therapeutics. The association mechanisms explain why targeting is specific, highlighting the physical properties of sterol ester-rich LDs that block association and thereby link lipid metabolic conditions to glucose metabolic regulation. Notably, MLX association is shown to be influenced by kinetics and to alter the surrounding LD monolayer. These findings demonstrate a dynamic interplay between protein association and membrane monolayer properties that enables cytosolic localization, targeting specificity, and potential kinetic and allosteric regulation of the LD proteome.

Introduction

Lipid Droplets (LDs) are cellular organelles that control lipid storage, distribution, and processing. In addition to their expected roles in lipolysis, lipogenesis, and the production of membrane building blocks, LDs have been found to play increasingly diverse roles[1–5], including in the transcriptional regulation[6] . Max like protein X (MLX) (Figure 1, SI Figure 1a) was the first transcription factor identified to associate with LDs.[6] As a member of the MYC- and MAX-centered network of transcription factors, MLX forms a dimer using a basic helix-loop-helix-leucine architecture (Figure 1a-left) that can bind onto DNA and influence expression levels. Unlike MYC and MAX, MLX has a small dimerization and cytoplasmic localization domain (DCD, Figure 1a-right) that regulates its activity. When bound to one of its dimerization partners (another MLX in a homodimer (Figure 1a) or MondoA or ChREBP as a heterodimer (Figure 1b))[7–9] MLX can translocate to the nucleus where it regulates the transcription of proteins involved in glucose metabolism.[10–15] In the presence of LDs, however, MLX can be stalled in the cytosol, where it binds LDs in monomeric form and resides for sustained periods with a slow apparent off-rate.[6] In this manner LD association alters MLX-driven gene expression, providing a link between lipid storage organelles and glucose metabolic regulation.

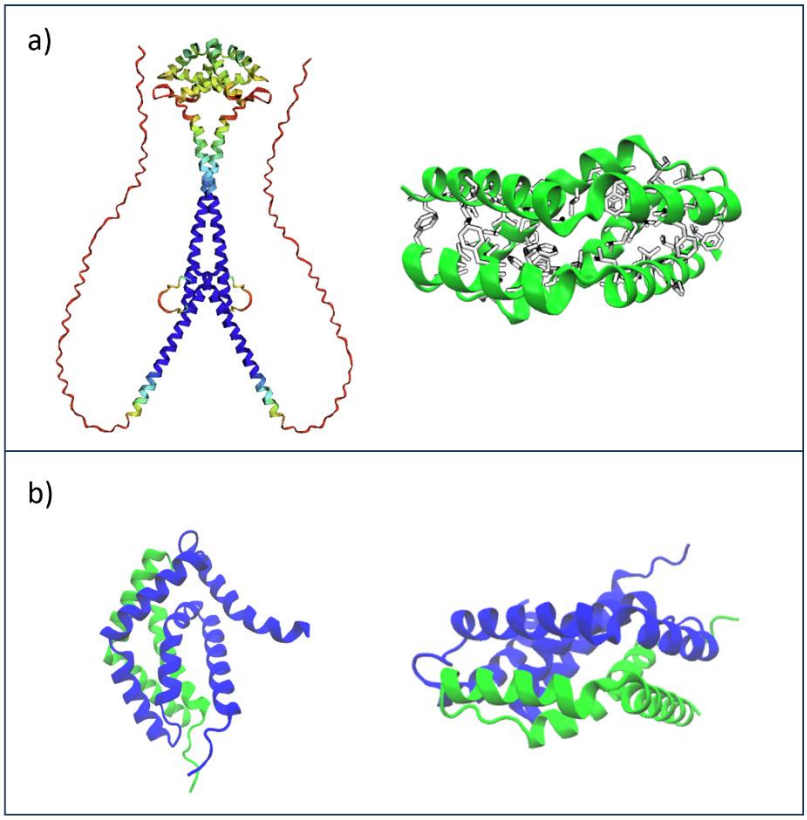

Figure 1. The predicted structures of a) the MLX homodimer (left), the homodimeric DCD (right), and b) the heterodimeric MLX-MondoA DCD from the top (left) and side (right) views.

The inactivation of MLX through LD association is interesting to consider in the context of other MAX-network transcription factors. In particular, the MYC members of this family are desirable, though challenging, cancer targets since their dysregulation is observed in ~70% of cancers and their inhibition has been linked with antiproliferation and tumor regression.[16] Additionally, high expression of MondoA, which requires MLX heterodimerization for transcriptional activity, has been correlated with both good and bad cancer prognosis.[17,18] MLX itself plays a significant role in cancer biology, particularly in osteosarcoma, where it acts as a super-enhancer-driven gene, and its high expression correlates with increased prognosis while its knockdown impairs tumor growth and metastasis.[19] Additionally, MLX is involved in the metabolic reprogramming required for MYC-driven tumorigenesis, functioning alongside MondoA to regulate critical metabolic pathways that support cancer cell survival.[20] In fact, knockdown of MondoA or MLX, blocks MYC-induced reprogramming of multiple metabolic pathways, resulting in apoptosis.[17,20] Thus, the notion of controlling the localization of MLX and MYC proteins through DCD-mediated membrane interactions, potentially with biologics containing complementary leucine zipper domains, would offer a novel approach to cancer therapeutics. This work explores the mechanisms by which MLX or MLX-like proteins can be targeted to LDs specifically or to membranes more generally.

A distinguishing feature of LDs from other organelles is the phospholipid (PL) monolayer, as opposed to bilayer, that surrounds their neutral lipid core, which is predominantly composed of triacylglycerols (TGs) and sterol-esters (SEs). Interactions with a diverse array of proteins, collectively called the LD proteome, enables various lipid-related processes.[1,2,4,5,21,22] Understanding how proteins selectively associate with LDs at different stages of their lifecycle is an emerging area of LD research, and was the primary

motivation for this research. We aim to understand how MLX specifically associates with certain kinds of LDs, and how this association may influence the association of other LD proteins.

Recent work has revealed that LDs can exist in subpopulations, distinguished by their neutral lipid composition and associated proteome.[23–28] It has been proposed that the selective protein targeting to subpopulations is at least partially driven by the unique physical features of TG- vs SE-rich LDs.[23] TG-rich LDs feature an amorphous core in which TG tails intercalate with the LD monolayer, leading to larger and more frequent membrane packing defects (regions where the PL headgroups splay apart to expose hydrophobic acyl tails to water).[25,29] Additionally, TG molecules can intercalate into the PL monolayer and adopt conformations similar to PL molecules with their glycerol group facing the cytosol and their acyl tails packing in with PL tails.[25] These surface active TGs (SURF-TGs) explain the ~15% increase in area per lipid headgroup measured experimentally[30] in LD monolayers compared to the endoplasmic reticulum (ER) bilayer, their site of origin. They additionally create chemically distinct packing defects and thus LD-specific handles for proteins to target.[2,26,31] In contrast, the core of SE-rich LDs can undergo a phase transformation[27,32] leading to significant alteration of the LD monolayer properties, including reduced size and number of packing defects.[23] MLX selectively targets TG-rich LDs and is expelled from SE-rich LDs.[6]

LD proteins are predominately grouped into two classes: ERToLD (class I) and CYToLD (class II) proteins. [2,4,29,33] ERToLD proteins transition from the ER bilayer to the LD monolayer through membrane contact sites and bridges. These proteins often have a helix-turn-helix hairpin motif that embeds in the ER bilayer. Studies to date have suggested that a thermodynamically favorable conformational change, sometimes involving sequence-specific residue positions, can drive ERToLD proteins to transition from the bilayer to the LD monolayer. [2,29,34] On the other hand, CYToLD proteins target directly from the cytosol, typically leveraging favorable interactions between amphipathic helices (AHs) and the prevalent packing defects found in LD monolayers.[2,30,33] These defects clearly play a pivotal role in protein targeting, although how they recruit a diverse array of proteins with specificity is not yet fully understood. [6,26,30,31,33,35] The importance of packing defects is further supported by changes observed in the LD proteome under stress or starvation conditions that induce the liquid-crystalline smectic phase transition in SE-rich LDs, which is characterized by concentric SE rings beneath a PL monolayer.[6,24,27,32] The SE reorganization permeates into the LD monolayer, not only decreasing packing defects by almost 50%[23], but also increasing PL packing and squeezing out SURF-TG molecules. Concomitant with these altered properties is the rearrangement of the LD proteome, kicking off certain proteins and attracting others, thereby modifying any associated cellular and metabolic pathways.[24,36] This backdrop of LD reorganization sets the stage for understanding the nuanced behavior of MLX.

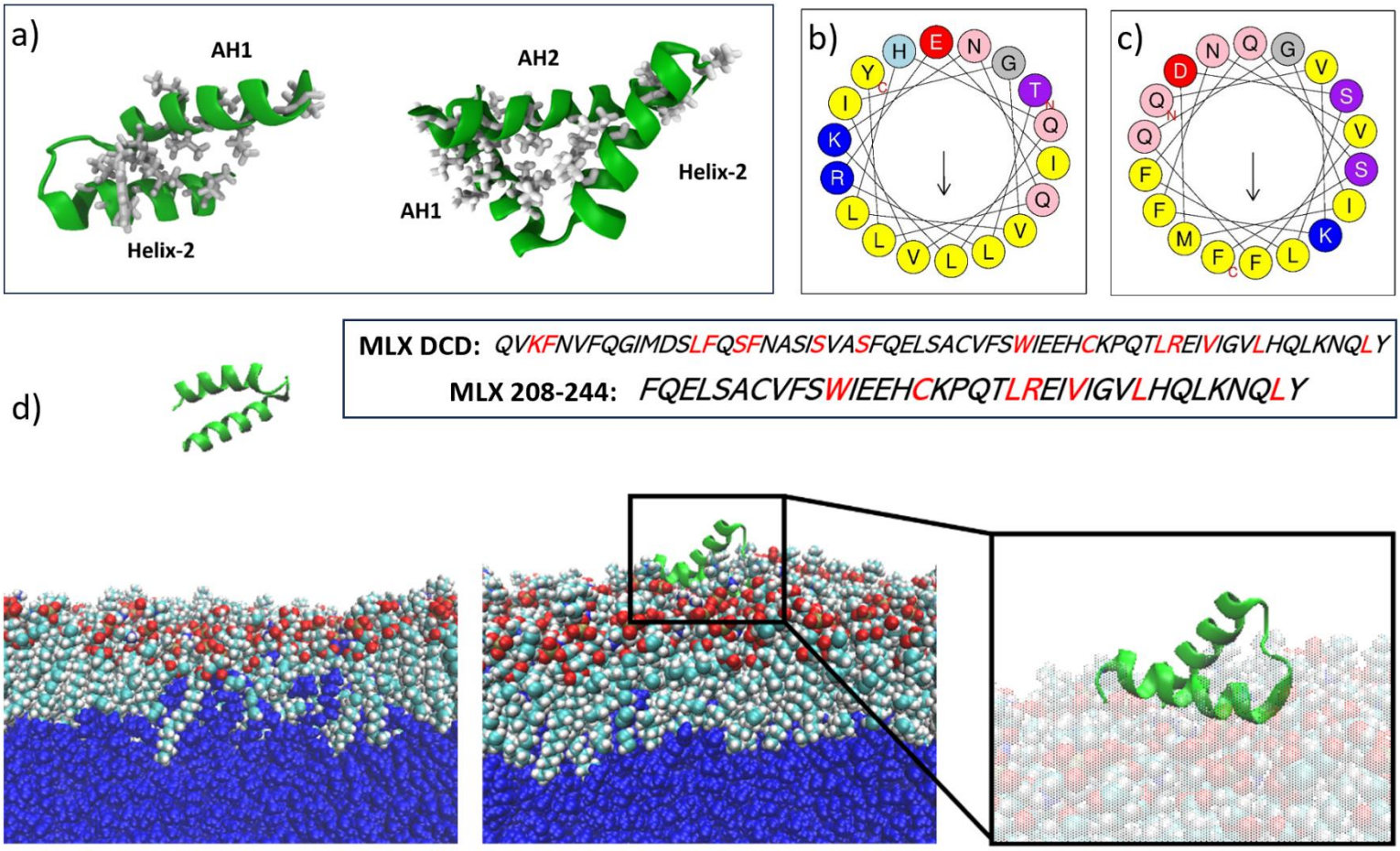

Figure 2. a) Predicted structures of MLX-hairpin (208-244) (left) and MLX-DCD (177-244) (right). b) Both sequences have a conserved AH that contains multiple Leu, Val, and Ile residues. c) MLX-DCD has a second AH with several Phe residues. d) Simulation structures of the initial cytosolic (left, water not shown for clarity) and LD-bound (right) MLX-hairpin.

Herein, we probe the mechanisms by which MLX specifically targets TG-rich LDs and how its association influences LD properties. Through long-timescale unbiased simulations and enhanced free energy sampling, we characterize the targeting mechanism of two MLX sequences. The shorter sequence (residues 208-244), herein called the MLX-hairpin (Figure 2a-left), has a highly conserved AH sequence (Figure 2b) that binds to membranes, lacking LD specificity.[37] The longer sequence (residues 177-244) spans the MLX-DCD (Figure 2a-right, Figure 1a-right); it includes a third helix (AH2, Figure 2c) and was identified as a potential minimal binding sequence that retains LD-specific targeting with an efficiency comparable to full-length MLX.[6] We find that both sequences strongly associate with TG-rich LDs through a combination hydrophobic interactions with membrane defects and polar interactions with PL headgroups, whereas the absence of defects of SE-LDs clearly inhibits association. The shorter sequence associates rapidly with a catch-anchor-embed mechanism that is amenable to smaller, less frequent packing defects, consistent with its association to membranes more broadly. In contrast, the longer sequence requires slower conformational exposure of its AHs and, subsequently, larger packing defects, consistent with specificity for LD monolayers. Interestingly, the mechanism of association is unique from the CYToLD mechanisms characterized to date,[26,38,39] partially because it involves 2-3 helices as opposed to a single AH. The AH properties and interhelical interactions that endow membrane affinity, and differences in membrane association compared to other CYToLD proteins are described. Perhaps most intriguing, we find the association of

either sequence changes the physical properties of the surrounding LD monolayer, suggesting that protein allostery through surface properties may influence LD proteome composition.

Results

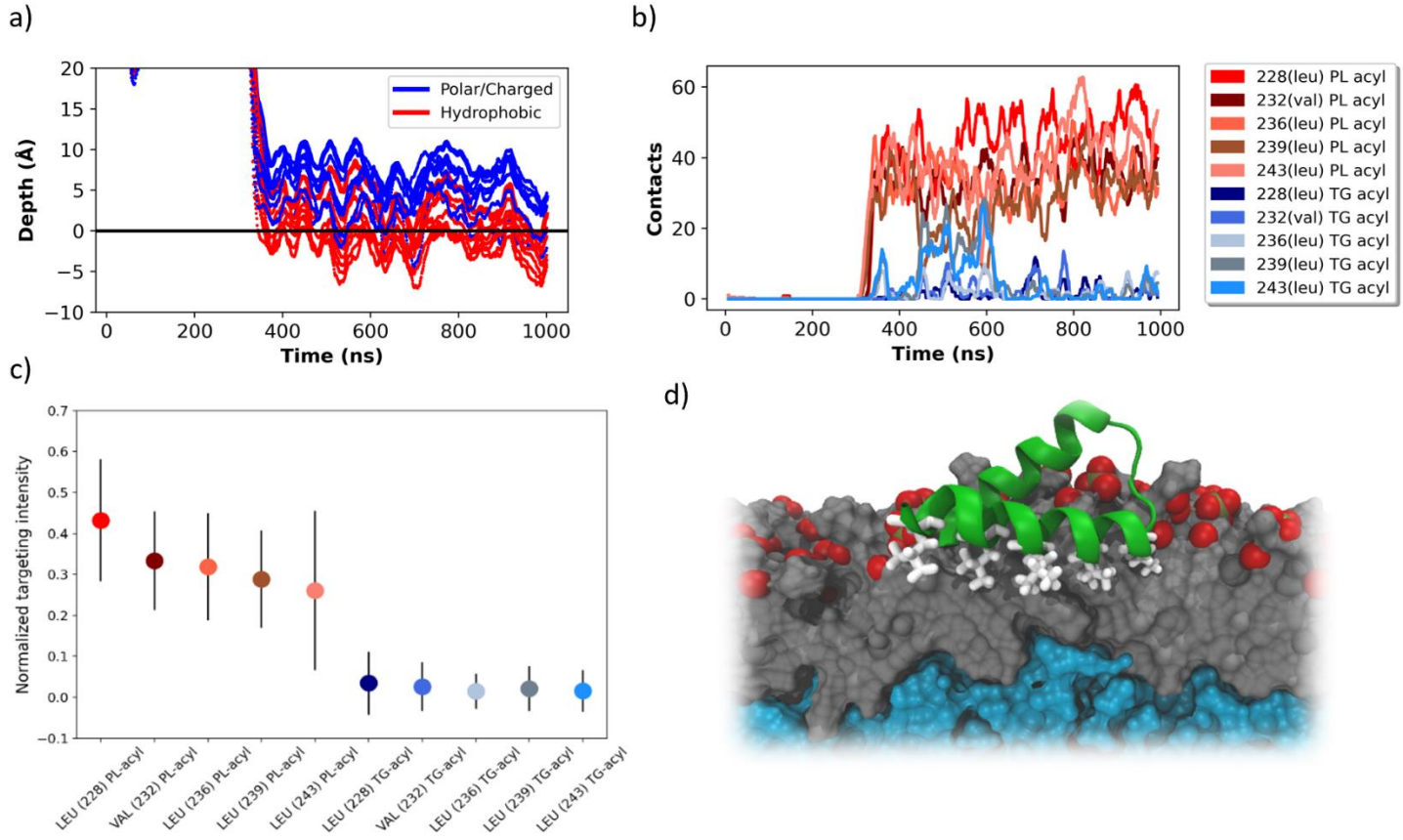

Figure 3. MLX binds to TG-rich LDs using its hydrophobic face. a) The red lines represent hydrophobic residues while the blue lines represent charged or polar residues. The black line at 0 represents the average location of the phosphorus of the PL monolayer. In all association cases, the hydrophobic residues are facing towards the membrane, meaning it is the hydrophobic face that is responsible for binding to defects. b) Contact analysis reveals which types of defects the hydrophobic face residues are interacting with. PL-acyl defects dominate. c) Normalized targeting analysis reveals contact analysis trends of all 8 bound replicas. d) The final bound conformation of MLX, with hydrophobic residues in white.

MLX targets TG-rich LDs through sequence-specific hydrophobic and charged interactions

LD association of the MLX-hairpin was first explored through parallel, long-timescale, unbiased simulations starting with the hairpin ~2 nm above the monolayer leaflet, as if in the cytosol (Figure 2d). Although observed properties are not expected to be statistically converged, unbiased simulations are essential for exploring the association mechanism with real dynamics unaltered by a Hamiltonian bias. Focusing first on AH1 (residues 228-244), the MLX-hairpin was considered fully associated once the center-of-mass (COM) of 4 or more hydrophobic residues transitioned from the cytosol to positions at or below the plane defined by PL phosphorous atoms (solid line in Figure 3a) and stabilized there. Such complete immersion occurred within 1 µs in eight out of fifteen instances in a pure TG environment, and four out of fifteen instances in the 50:50 TG:SE LD. No such association was observed in the ten 90:10 SE-rich LD simulations. Tracking the dominance of specific residue-monolayer interactions via contact analysis (Figure 3b, SI Figure 2), Leu228 had the most interactions, closely followed by Val232 and the trio of leucines (Leu236,

Leu239, and Leu243). This was confirmed by the normalized targeting intensities that determine the preference of residue-defect interaction (Figure 3c). Interestingly, these hydrophobic interactions predominantly occurred with PL acyl chains, rather than TG molecules (Figure 3b-d).

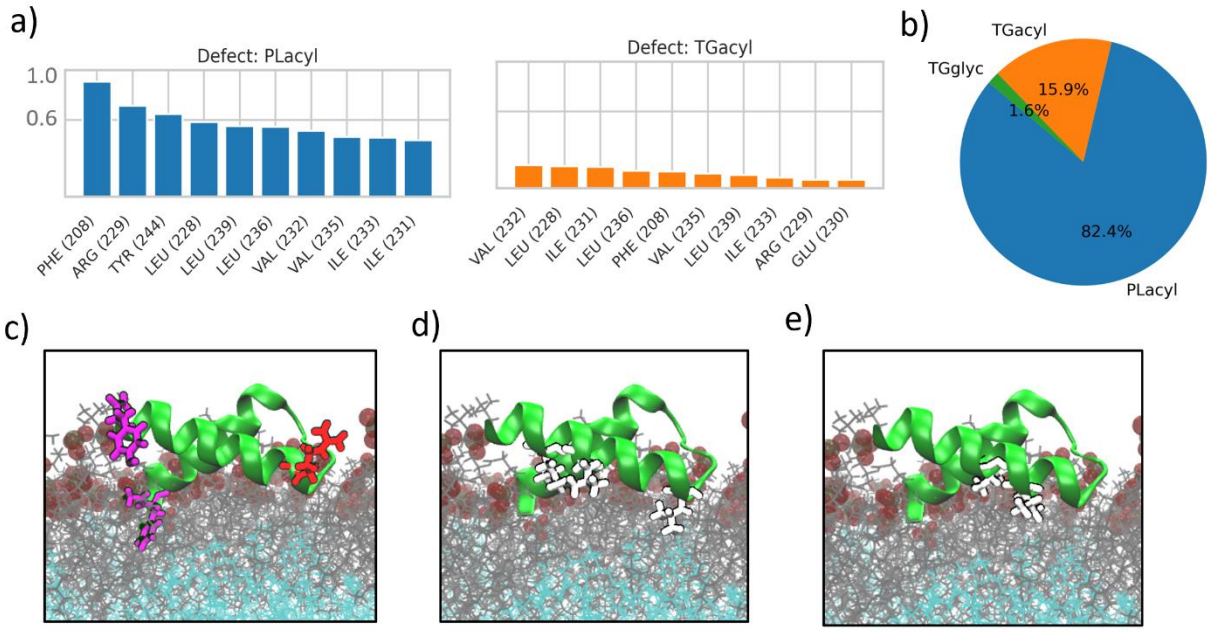

Figure 4. Defect analysis reveals the preferred interactions. a) Normalized total defect interactions of both the PL-acyl and TG-acyl induced defects. b) PL-acyl defects are the dominant defect types. c) Tyr, Phe (purple), and Arg (Red), are the dominant interacting residues. d) A series of leucines and E) valines are also critical for defect interactions.

To further refine the role of packing defects in MLX association, residue interactions within a specified cutoff of membrane defects (see Methods) (Movie 1,2) were quantified. Residues Arg229, Phe208, and Tyr244 act as anchoring residues, maintaining the most significant interactions with defects (Figure 4a,c). Following this, a series of leucines (Figure 4a-b, d), valines (Figure 4a-b, e), and isoleucines (Figure 4a-b), many of which are conserved, showed strong defect-coordination. Consistent with the contact analysis, these interactions favored PL-acyl tails, followed by TG-acyl tails, with minimal interaction with TG-glycerol headgroups (Figure 4b). Additionally, we looked at residues that might coordinate with PL-headgroups. Lys240, which is on the polar side of the AH, also acts as a stabilizing residue through electrostatic interactions with the PL headgroups (SI Figure 3a-c). Arg 229 creates similar interactions with the guanidinium group interacting with PL-headgroups, while the rest of the hydrocarbon chain is sucked into a defect shared by Leu228 (Figure 4c, SI Figure 3a-b,d).

Heterogeneous association pathways share catch-anchor-embed mechanism

Time-resolved defect analysis reveals that association occurs through multiple interaction pathways (SI Figure 4), although each converges to the same bound pose (discussed below). Several key 'catch' residues emerge as initiating and anchoring monolayer

association. Arg229, located at the hairpin kink (Figure 4c), initiates association in 5 out of 6 tracked binding events (SI Figure 4 a, b, c, e, f). This is expected given arginine's strong electrostatic attraction to PL phosphates (Figure 5a, SI Figure 3a-d), while additionally being placed next to the conserved Leu228, which buries deep into defects. In 3 out of these 5 events (SI Figure 4 a, b, f), Tyr244, which is at the other end of the AH (Figure 4c), soon thereafter forms a hydrogen bond with PL headgroups (Figure 5a) though in time it sinks below the headgroups to satisfy dispersion attraction to PL-acyl defects. Anchoring both sides of the AH with Arg229 and Tyr244 can happen concomitantly with helix 1 association (SI Figure 4a), or it can anchor the AH in the headgroup region via polar interactions until a defect sufficient for hydrophobic burial forms (SI Figure 4 b, f, 6b). In other cases, submersion happens from the kink initiated with a SURF-TG (SI Figure 4c) or via rapid hydrophobic burial (SI Figure 4e). In one case, Phe208, which is on the other side of helix 1 and often is involved in early helix-2 association, acts at the initial catch residue (SI Figure 4d, 5a). Thus, although these binding pathways vary between replicas (SI Figure 4), the strategic placement of catch residues at the ends of helices enhances monolayer association. Additionally, anchoring the protein close to the membrane provides time for the hydrophobic groups to transiently 'snorkel' above (Figure 5b) before fully embedding into defects (Figure 5c).

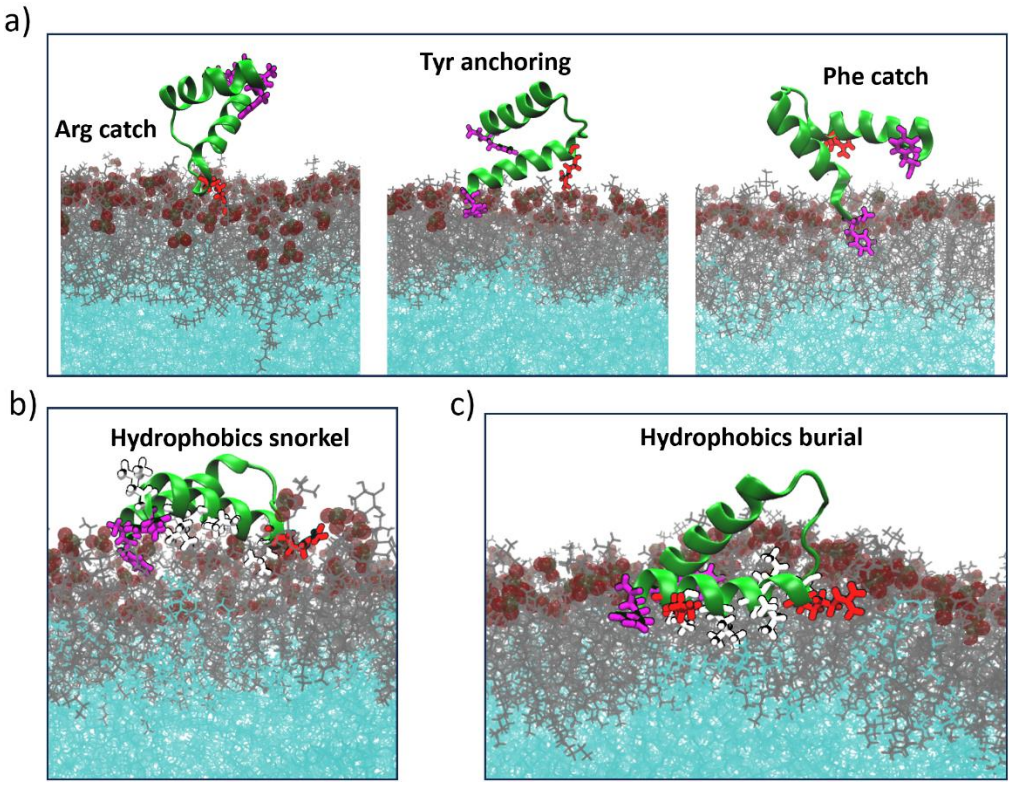

Figure 5. a) The catch residues are Arg, Tyr, and Phe on the termini and kink of the protein. b) After the initial catch, the hydrophobic residues snorkel on the surface, trying to embed into a defect. c) Finally, the hydrophobic residues find and create an adequate defect, burying the AH deep into the PL-acyl tail region (Lys240 also pictured in red on left side of AH, as it assists with coordination to PL headgroups).

MLX-DCD binding involves exchange of hydrophobic interactions

The MAX- and MYC-network of proteins share a common basic helix-loop-helix leucine zipper fold (Figure 1, SI Figure 1). Recent NMR work has shown that even the basic region, commonly thought of as disordered, retains some degree of helicity.[40] Thus, the Alphafold predicted structures of the MLX monomer (SI Figure 1) and homodimer (Figure 1) were all reported with a high degree of confidence (pLDDT scores above 90). The MLX DCD, positioned above the C-terminal domain of the leucine-zipper, is nearly identical between the dimer and monomer, forming a triangular planar structure with the AH1 and AH2 overlapping slightly to trade hydrophobic interactions (Figure 1, SI Figure 1b). Additionally, the AlphaFold predicted heterodimer with MondoA shows a similar structure, with the MLX-DCD forming a planar interface with the MondaA-DCD, maximizing the AH1 and AH2 interactions at the dimer interface (Figure 1b). In the cellular environment, the MLX distribution between monomer, dimers, and LD-bound pools likely involves a dynamic exchange of the DCD between dimer interfaces and LD monolayer. However, during simulations of the DCD monomer alone, the absence of a dimer interface or the rest of the MLX monomer results in the DCD helices folding in slightly, as captured in Figure 2a-right, to minimize exposure of the hydrophobic residues to water. This conformation is reasonably stable with minimal fluctuations. When placed above the TG-rich LD no stable binding events occurred within 1 µs in 3 replicas, which is expected given even a modest kinetic barrier for the buried hydrophobic residues to open and interact with the monolayer. To overcome this kinetic barrier, a slight bias was applied to open the DCD conformation and expose the hydrophobic AHs to the cytosol, enabling monolayer association (see Methods). Once bound, all three DCD helices form extensive interactions with the membrane (Figure 6, SI Figure 4a-c), with the two AHs burying into the PL-acyl defects. Consistent with the bound pose of the MLX-hairpin, AH1 (Figure 6a, b, SI Figure 5) makes abundant interactions with the PL-acyl defects, while AH2 has several phenylalanine's that also embed into PL-acyl defects (SI Figure 5c). The catch and anchor residues also play a similar role (Figure 5a, SI Figure 6) as they did with MLX-hairpin

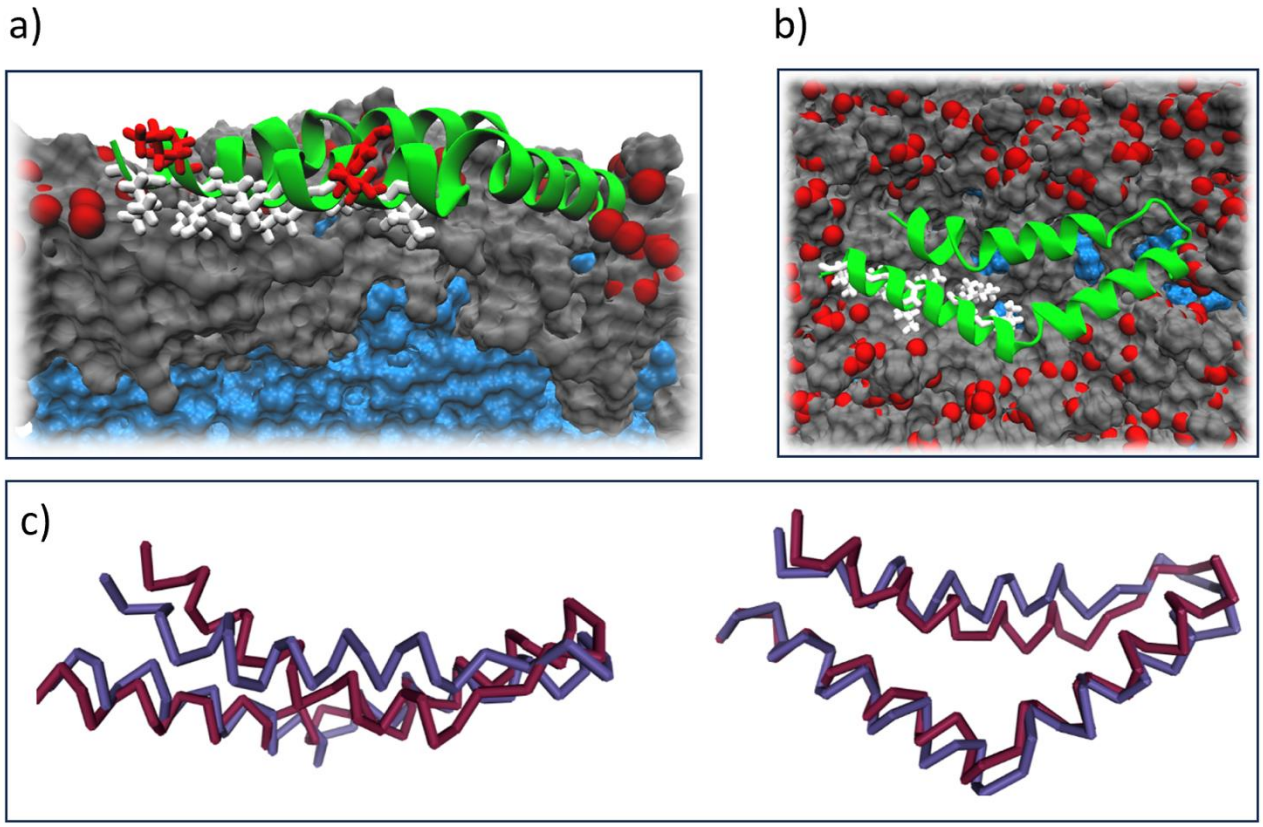

Figure 6. a) The DCD uses the same AH residues (colored in white) to bury into PL-acyl defects. The red residues are the Arg and Lys interacting with PL headgroups. b) Top-down view of the DCD embedded into the PL-acyl defects. The structure is almost identical to its dimerized form. c) Nearly perfect overlap of one of the DCD homo-dimer chains (red) to the final LD-bound structure (blue).

Interestingly, the LD-bound DCD structure is nearly identical to the DCD-dimer structure, suggesting the burial of hydrophobic groups in the dimer mimics that in the LD membrane (Figure 6b, c). The conformation is very stable once bound, as demonstrated by the backbone RMSD (SI Figure 7). Despite seemingly more interactions, the strength of association of MLX-DCD is not as strong as that of the MLX-hairpin (discussed below in Thermodynamics). This is likely due to the size of the required defect. As demonstrated previously, MLX's initial AH binding doesn't require a large defect. Catch residues allow interaction with the membrane while hydrophobic residues settle into defects. However, binding induces larger defects than those typically present on an LD over long timescales. As expected, the size of the defect tracks the size of the associating helices. Over an average of 1000 frames, the size of packing defect directly below the MLX-DCD domain is $92 \pm 17\ \text{Å}^2$, while that under the MLX-hairpin is $63 \pm 16\ \text{Å}^2$ (SI Figure 8). Thus, the larger MLX-DCD domain both requires and induces a larger defect, displacing more PL headgroups. This likely explains targeting specificity to TG-LDs since they have larger and more frequent packing defects than bilayers.[23,25]

Thermodynamics of association supports preferential targeting to TG-rich LDs

To confirm that the insights found in unbiased simulations are reflective of ensemble behavior, association free energy profiles with varying LD compositions (pure-TG, 50:50, and 90:10 CHYO:TG ratios) were computed (Figure 7a). These profiles show a clear

preference for MLX binding to the 50:50 and pure-TG LDs, with a pronounced association affinity of ~11-12 kcal/mol. Conversely, the 90:10 LD system presented an energetically unfavorable profile for monolayer interaction, favoring MLX's cytosolic location by 4 kcal/mol. These findings are congruent with the hypothesis that packing defects and monolayer density critically influence protein-LD affinity. The MLX-DCD domain also exhibits a discernible ~7 kcal/mol binding affinity for the TG-LD monolayer (SI Figure 9). This is consistent with the expected less promiscuous membrane association and the cost of inducing larger packing defects that are more easily formed in a LD monolayer than a bilayer.

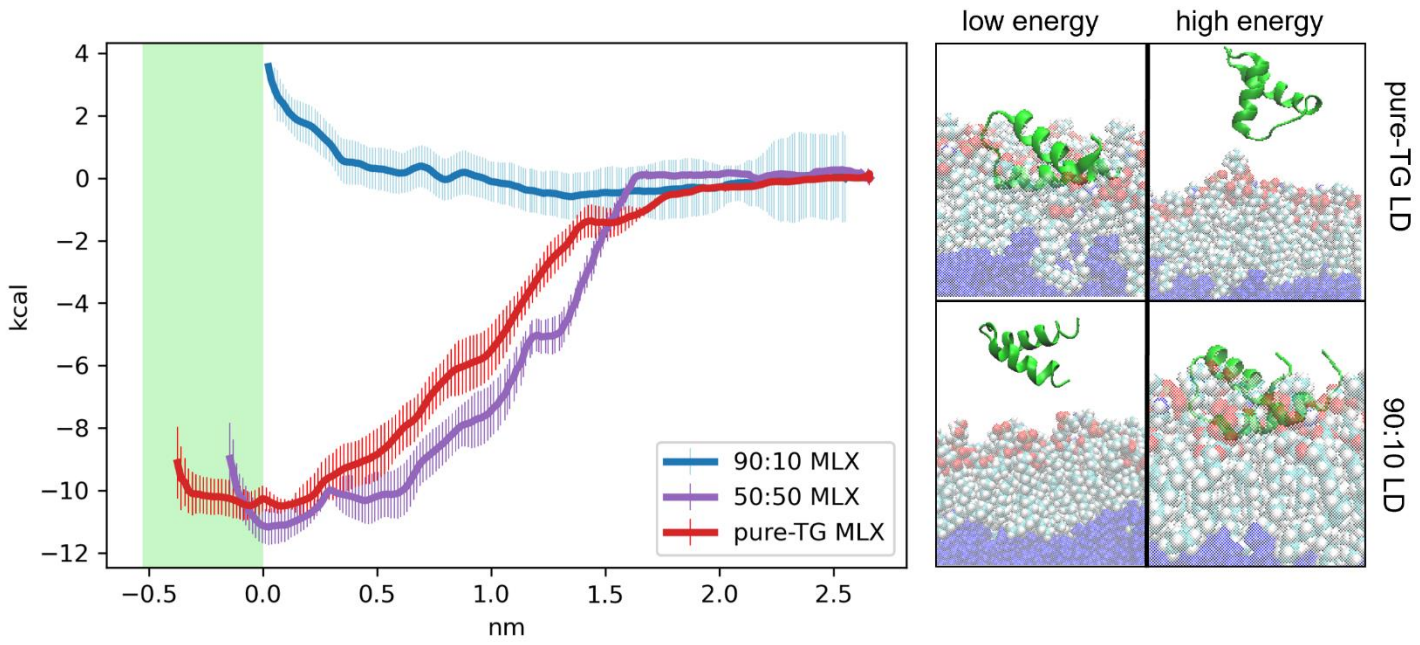

Figure 7. a) Free energy profiles for LD association and dominant stable (low energy) and unstable (high energy) conformations show that MLX has a strong affinity for the TG and 50:50 SE:TG LDs, but is repelled from the 90:10 SE:TG LD. The sampled reaction coordinate (x-axis) is the z-COM distance of MLX from the phosphate plane (x = 0) of the LD monolayers. The green shaded area from -0.5 to 0.0 represents the PL-monolayer, while x > 0.0 moves into water.

Focusing on the bound conformations from the biased simulations (z = 0 in Figure 8a), the MLX-hairpin shows consistent contacts between charged residues (Arg229, Lys240) and the PL headgroups in both the TG-rich and 90:10 CHYO:TG LD systems (SI Figure 10). In contrast, conserved hydrophobic residues engaged with the inner acyl chains (C2, C3, C4) in pure-TG LDs, but not in the 90:10 system (SI Figure 10). Thus, MLX attempts to bind to the 90:10 CHYO:TG, engaging in headgroup interactions, but fails due to the inaccessibility of the PL tail region for hydrophobic residues (Movie 3).

MLX changes conformation upon association to form stabilizing interhelical interactions.

In addition to the stabilizing interactions with the LD monolayer, both the MLX-hairpin and MLX-DCD engage in interhelical interactions once bound. This is particularly pronounced for the MLX-hairpin. Although both AH1 and helix-2 remain independently folded throughout the simulations, there is a clear inter-helical conformational change upon association with the LD monolayer. In the cytosol, the two helices are flexible, occasionally engaging in hydrophobic interactions but largely fluctuating in relative tilt angles (SI Figure 11). However, they transition to a conserved 'crossed' configuration upon membrane association

(Figure 8b-4, 8d) with the AH1 tilting upwards (~5 degrees) while helix 2 tilts downwards (-35 degrees) (Figure 8c, SI Figure 11). The final bound conformation exhibits notable stability, as confirmed by the alignment of the last bound structure frame across 8 simulation replicas (Figure 8d, 8a). This stability arises from a network of inter-helical interactions, including hydrophobic contacts and hydrogen bonds (Figure 8e, SI Figure 12a-d), enabled by complementary interactions with the LD monolayer. Notably, the number of hydrogen bonds between the helices doubles in the bound state (SI Figure 12b), with new hydrogen bonds forming between Lys and Glu residues at the sequence's kink region (Figure 8e, SI Figure 12c,d). More specifically, the Lys224 initially makes backbone hydrogen bonds with Gln226. After binding, the sidechain of Lys224 then starts to make h-bonds with the Glu230, while several other polar residues start trading h-bonding interactions as well. Collectively, this network of interhelical interactions stabilizes the bound pose. As previously demonstrated,[41] interactions between helices enhances affinity to the membrane indirectly, since the dissociation of one helix requires dislodging the other as well, making it statistically less likely. We anticipate that interhelical interactions play a central role in a strong membrane affinity, both for AH's in general and for the MLX-hairpin specifically. Thus, the binding process is dynamic with multiple specific association pathways as the MLX-hairpin finds its way to a consistent, stable bound conformation (Movie 4).

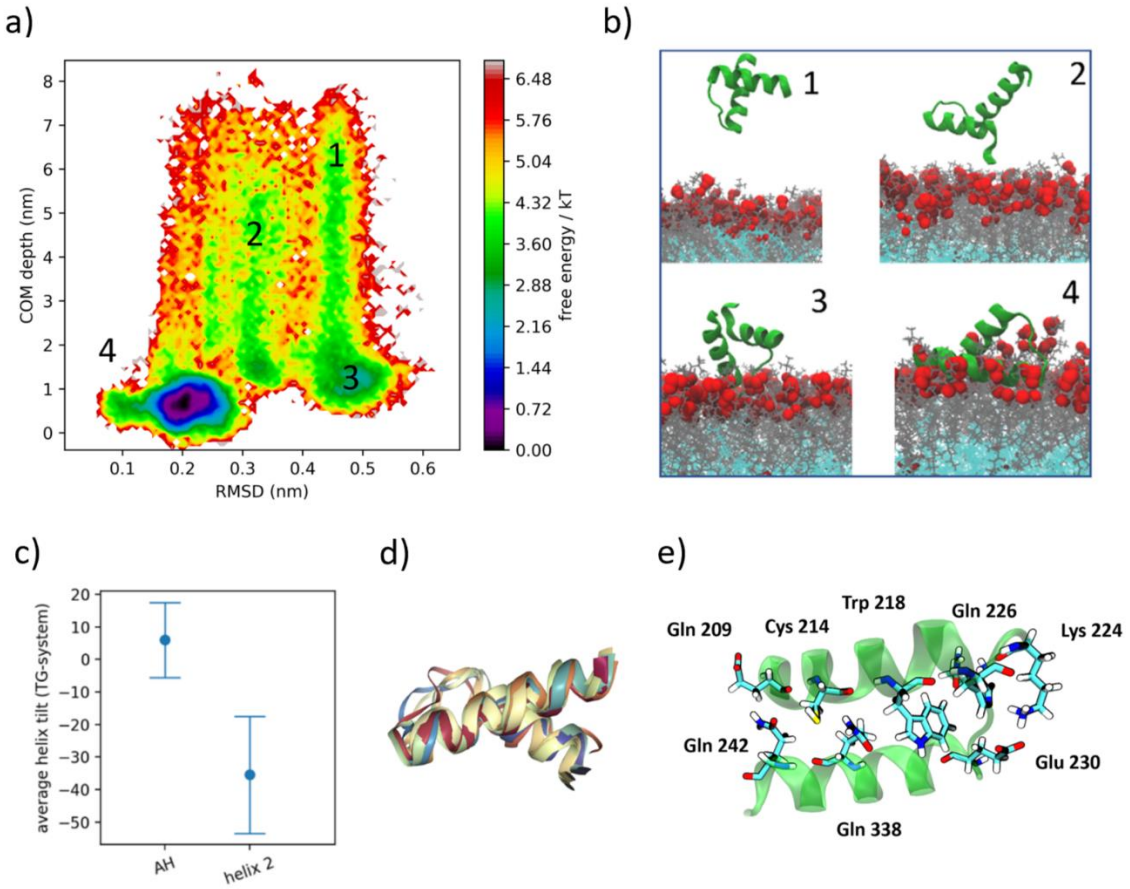

Figure 8. Stable bound conformation of the MLX-hairpin. a) RMSD vs. COM identifies three wells with the 4th being most stable, and b) corresponding structures during monolayer association. c) The final bound pose maintains a specific tilt angle between the two helices. d) Overlap of the bound conformation from the 8 trajectories that resulted in binding shows consistency in the final bound conformation, stabilized by e) multiple interhelical interactions.

Association of MLX alters LD monolayer

An unexpected finding in this work was that the association of either the MLX-hairpin or MLX-DCD, as small as they are, seemed to alter the physical properties of the LD monolayer. To quantify this impact, we focused on the monolayer composition, defect organization, PL-tail order parameters, and dynamics. To verify these properties are not artifacts due to the size of the system, we created the DCD-big system with twice as large $x - y$ dimensions, quadrupling the surface area of the LD monolayer.

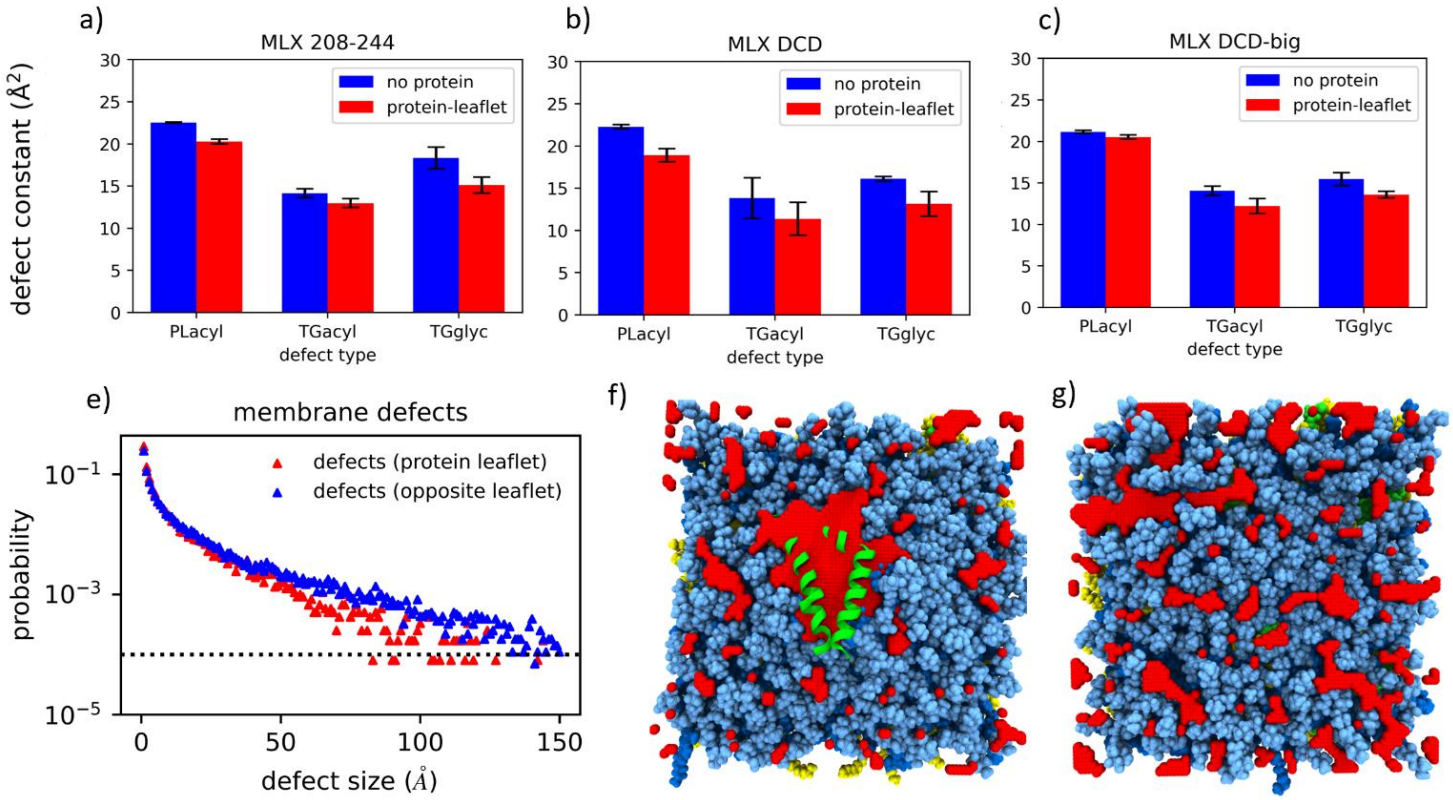

Figure 9. Packing defects of protein-bound leaflets are smaller than those of LD leaflets without association. The packing defect constants for a) the MLX-hairpin, b) MLX DCD, and c) MLX DCD-big all have smaller defects at a cut-off of more than 1 nm from the protein. e) The probability vs defect size again shows that the defects are smaller on the protein-bound leaflet for MLX DCD. The decrease in size and quantity of defects distal from the protein is apparent from the top-down view comparing the f) DCD-bound leaflet and g) free leaflet. PL-headgroups are blue and defects are red.

Defect Composition Alteration: Upon MLX association, we observed a decrease in the probability and size of defects surrounding the protein on the protein-bound leaflet in each system (MLX-hairpin, MLX-DCD, and MLX-DCD-big) (Figure 9). This was quantified by measuring probability and likelihood of defects, specifically focusing on defects more than 1 nm from the protein. Defects are smaller, on average, on the protein bound leaflet as demonstrated by the analysis of defect probability versus size (Figure 9e) and as can be seen visually in a top-down view of MLX-DCD (Figure 9f, g). We subsequently quantified the defects by calculating the defect constants. A higher packing defect constant would generally mean that there is a higher probability of finding a larger defect (see Methods for details). All defect types were smaller on the protein-bound leaflet (Figure 9a-c). As expected, the magnitude of deviations decreases from the MLX-DCD to the MLX-DCD-big system. This is consistent with the impact of the protein being both localized to the region around it and modulated by the size of free membrane. The MLX-DCD-big system is 20×20 nm. Thus, the impact of MLX-DCD must extend 15 nm to retain the same magnitude of impact as the smaller system.

SURF-TG Composition: SURF-TGs, commonly found in TG-rich LDs, decrease in number when the MLX sequences are bound, dropping to approximately 1.5% of the monolayer composition (Figure 10f). The opposite leaflet of the LD system maintained a normal SURF-TG composition (5-8%). The trend was maintained for all three systems (MLX-hairpin, MLX-DCD, and MLX-DCD-big). The number of SURF-TG molecules has been clearly correlated with the magnitude of applied surface tension.[26] Under an

applied surface tension, mimicking an expanding LD with limited PL's to grow the monolayer surface, more TG molecules are brought into the monolayer. This both modulates the magnitude of surface tension and alters the packing defects, giving LDs an expansion-specific property for proteins like CCTα to target.[26] It seems that the association of an AH-containing protein has the opposite effect, displacing PL headgroups, decreasing surrounding packing defects, and pushing SURF-TG back into the LD core. Thus, association induces a shift in composition that again could influence the association of competing proximal proteins.

Tail-Order Parameters: PL tail-order parameters are indicative of how ordered the PL tails are as a function of position, with C1 being just below the glycerol moiety and C16/C18 being the last carbon in the tail (Figure 10a-c). As anticipated, the PLs immediately below MLX-DCD were more disordered (SI Figure 13b). However, those distal from the protein by more than 2nm exhibited greater order compared to those in leaflets without protein (Figure 9a-c). This also held true for the MLX DCD-big system (SI Figure 13a). Even 1-2nm away the increase in order is apparent. From 2+ to 3+ nm the increase in order is consistent (SI Figure 13b). Thus, association of an AH containing protein displaces PL headgroups, decreasing the order in the PL tails immediately around and under it, but also increases the order in the surrounding PLs. This is not immediately obvious. It could be assumed that the increased disorder of the PL tails under the protein would spread such that all PLs within a given cutoff would be more disordered. Thus, a concern was that the observed increase in order is simply an artifact of the system size and the increased packing that protein association would cause in the membrane-bound leaflet. In a real membrane system, this would be alleviated by monolayer expansion. However, these trends were not altered when the system size was doubled (MLX-DCD-big), suggesting it is not purely an artifact of system size. However, these impacts should always be considered in the context of the system. The magnitude of increased packing order in some region surrounding the protein will be influenced by the nature of the surrounding membrane. Packed monolayer membranes with many associated proteins would likely be more impacted while empty monolayers with facile expansion would more quickly dissipate the increased proximal order.

Dynamics and Fluidity Analysis: To analyze the dynamic fluidity of the monolayer the mean square displacement (MSD) of PLs was evaluated in the DCD-big system. We found that the MSD for all PL types was larger in the leaflet without associated proteins (Figure 10d-e). This was further corroborated by the lateral diffusion coefficients (SI Table 1), indicating that protein association reduces the fluidity of membrane PLs. These findings collectively demonstrate that the association of MLX-DCD with LDs not only changes the defect composition but also influences the ordering and dynamics of the surrounding membrane.

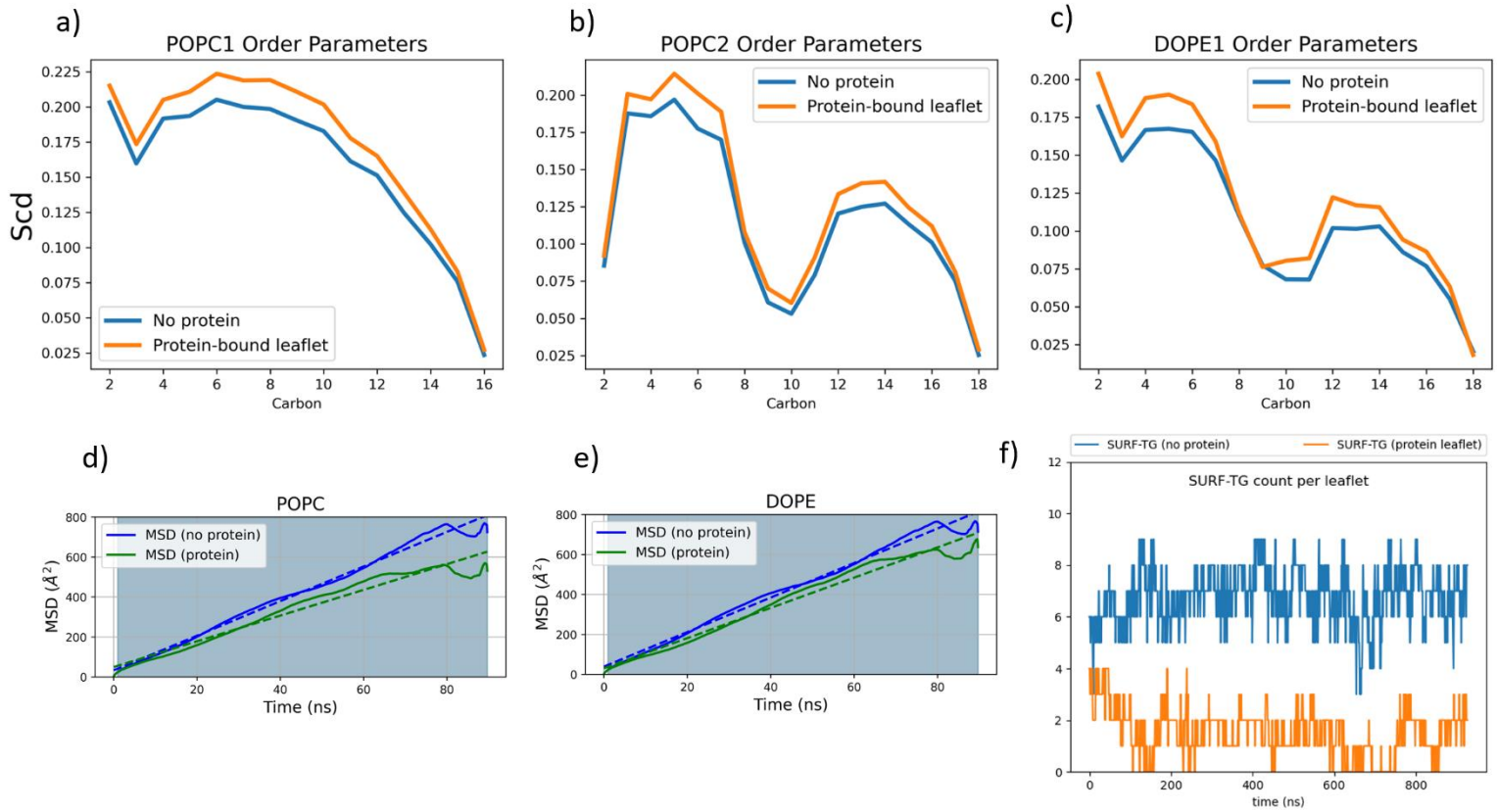

Figure 10. a-c) Tail order parameters for the protein-bound leaflets are elevated compared to those in the free leaflet. d-e) Additionally, the MSD for the protein-bound leaflets decrease in magnitude. f) Due to the changes in the LD monolayer, the number of SURF-TG decreases (data shown for MLX-DCD is representative of other systems).

Discussion:

Hundreds of proteins have been shown to dynamically associate with LDs[3,5,42–44] throughout their lifecycle, coming and going according to metabolic needs. Much like an air traffic control tower, mechanisms that determine which proteins 'land' and 'take-off' from LD 'runways' are essential to control lipid metabolism. Yet, the control handles are limited, perhaps primitive, compared to membranes with dedicated protein association machinery such as the translocon in the ER. Under the assumption that LDs have largely homogenous PL monolayers, selective association would rely upon protein properties, such as their relative binding affinities, copy number, and allostery through protein-protein interactions. Following this logic, competition for LD association has been described in terms of competitive binding affinities.[45,46] Adding to the control handle sophistication, however, is increasing recognition of LD heterogeneity. The physical properties of LD monolayers can vary with neutral lipid composition, enabling distinct LD sub-populations. LDs can also differ in curvature (size) and relative PL abundance during budding, growth, and lipolytic degrowth. Thus, selective association can also be controlled through monolayer properties. The work herein demonstrates that protein association can additionally change monolayer surface properties, suggesting allostery through protein-surface-protein interactions may also play a role in the selective association of the LD proteome.

Two central goals of this work were: 1) to explain what enables MLX to selectively target LDs based on their neutral lipid composition, and 2) to contribute to our understanding of what differentiates the numerous class II CYToLD proteins in their competition for LD association. The second goal demands unraveling CYToLD association mechanisms beyond interactions with packing defects. Thus, we present a deep dive into the interactions that govern the selective association of two peptides, one representing a model peptide with broad membrane targeting (MLX-hairpin) and the other as the minimal binding sequence for selective targeting to TG-rich LDs (MLX-DCD). We find that even the shorter MLX-hairpin is clearly excluded from the liquid-crystalline SE-rich LD surface due to the physical properties of its PL monolayer (described below). Consequently, under stress conditions such as starvation that induce SE-phase transformations in LDs, MLX will be excluded from the LD monolayer, increasing its propensity to dimerize and relocate to the nucleus. In this way, the specific targeting of MLX to TG-rich LDs acts as a switch linking extreme lipid metabolic conditions (those inducing SE-phase transformations) to a response in glucose metabolism (through transcriptional regulation).

We also find that MLX's association mechanism is distinct from other CYToLD proteins, like CCTα and CGI-58, despite all three featuring CYToLD AHs. CCTα binds to expanding LDs via Trp residues that interact with TG-glycerol defects. CGI-58, on the other hand, employs three Trp residues to penetrate deep within LD packing defects.[26,38] MLX sets itself apart by preferentially targeting superficial defects, demonstrating a versatile membrane-binding capacity especially adept at engaging shallow PL defects (Figures 3b-d, 4a-e). The selection of shallow defects can be partially explained by the sequence of AH1, which is rich in Leu, Val, and Ile, with strategically positioned Arg, Lys, Phe and Tyr residues that facilitate membrane surface interactions. Additionally, AH2, enriched with Phe residues, also supports shallow interactions with PL-acyl tail defects. The attraction to shallow defects could also be a consequence of both AH1 and AH2 forming the DCD dimer interface since the smaller hydrophobic residues (Leu, Val, Ile) with larger, aromatic Phe residues allows for a compact dimer alignment (Figure 1). Alternatively, the striking resemblance between the DCD LD-bound and dimer conformations (Figure 1, Figure 6) suggest that MLX could be evolutionarily designed to equally bind both interfaces, enabling dynamic response to the relative copy numbers of potential binding partners vs TG-rich LDs. Regardless of the evolutionary origin, the sequence-enabled engagement of shallow defects is a defining feature of MLX's specific targeting to TG-rich LDs.

Turning to the mechanism of association, although there are multiple association pathways, there is a consistent progression from an initial catch to the gradual burial of hydrophobic residues into packing defects. Both sequences display multiple catch residues on AH1, including an Arg or Lys interaction with PL phosphates (Figures 4, SI Figure 4a-c, SI Figure 3a-d), or alternatively Phe and Tyr residues engaging with PL-acyl defects or hydrogen bonding with PL oxygens (Figures 4, SI Figure 4a). The catch residues facilitate the snorkeling of conserved hydrophobic residues (Leu/Val/Leu/Leu) high in the membrane surface before embedding lower into membrane defects (Figure 5b-c). The prevalence of an initial catch phase suggests that kinetics will also influence competition since proteins with longer catch phase lifetimes will be selected over those with shorter. Similarly, monolayers with more frequent defect formation will be selected by a given protein over those with less frequent. Additionally, multiple binding pathways enabled by multiple catch residues clearly enhances MLX's affinity; this not only increases the surface states that MLX can engage with but also increases the probability that a second catch, herein called anchoring, can maintain

association, further facilitating hydrophobic burial or retaining association if the first catch interaction is lost. Collectively, MLX displays a range of association pathways, utilizing multiple catch residues strategically located at opposing ends of AH1 followed by hydrophobic burial (Movies 1, 2, 4).

In addition to protein-membrane interactions, interhelical interactions increase membrane affinity by opposing dissociation. Upon binding, the MLX-hairpin and MLX-DCD settle into stable bound structures (Figure 8a-e, SI Figure 7). The MLX-hairpin locks into a specific interhelical tilt angle (Figures 8b-d, SI Figure 11) due to protein-protein hydrogen bonds and hydrophobic contacts (Figure 8e, SI Figure 12a-d). The bound conformation inhibits detachment, echoing findings by Schaefer *et al.* in the SARS-CoV-2 Fusion Peptide, which showed similar interactions bolstering membrane affinity.[41] Such interhelical interactions essentially act like glue between two surface-associated cylinders. It is easier to pull either cylinder off in isolation because once glued, pulling one is opposed by the association of the other. Put in the context of stochastic condensed phase dynamics, the statistics of both dissociating together is far less frequent than the dissociation of either in isolation. Accordingly, MLX's interhelical interactions play a key role increasing membrane affinity and decreasing dissociation rates, consistent with MLX's slow observed off-rate.[6]

The specific targeting of MLX to TG-rich LDs is also clear from the thermodynamics of association. Consistent with the trends seen in unbiased simulations, biased simulations verify that both the MLX-hairpin and MLX-DCD have a thermodynamic preference for TG-rich LDs over the cytosol. The association free energy is approximately -12 kcal/mol for the MLX-hairpin (Figure 7) and -6 kcal/mol for MLX-DCD (SI Figure 9). The stronger association of the MLX-hairpin is consistent with targeting to membranes more broadly. The weaker association of MLX-DCD is the consequence of several factors. First, MLX-DCD has a third helix (AH2) that enables some degree of hydrophobic burial in the cytosol. Second, DCD is larger than the hairpin, requiring a defect larger than 90 $Å^2$ (SI Figure 8). This is likely the dominant factor that enables specific targeting of MLX-DCD to TG-rich LDs over other membranes since defects of this size are more common in TG-rich LDs.[23,25] In sharp contrast, even the MLX-hairpin, with its broad membrane affinity, is unable to bind with SE-rich LDs. The increased PL packing and decreased packing defects in the phase-transformed monolayer lead to a 4kcal/mol cost for the hairpin to associate (Figure 7, Movie 3). Taken together, selective association of both sequences is clearly linked to membrane properties; the selective dissociation of the hairpin and DCD from SE-rich LDs is due to a lack of even modest packing defects, while the selective association of the DCD with TG-rich LDs over other membranes is due to the need for larger packing defects.

Lastly, the binding of the MLX-hairpin and MLX-DCD are shown to alter the physical properties of the surrounding LD monolayer. Although a large packing defect is necessarily formed underneath the protein segments, there is a notable reduction in both the size and number of defects surrounding the protein (> 1-nm away) (Figure 9). This reduction coincides with fewer SURF-TGs on the protein-bound leaflet and an increase in PL tail order parameters, indicating more ordered lipid membrane beyond 1 nm from the proteins' boundaries (Figures 10a-c, SI Figure 13a-b). Additionally, there is a 34.2% reduction in lateral diffusion coefficients for POPC, showcasing diminished fluidity in the protein-bound leaflet (Figures 10d-e, SI Table 1). These findings align with broader experimental evidence that demonstrated AH binding to lipid bilayers can enhance adjacent leaflet order, potentially influencing membrane curvature and scission processes.[47,48] Our study extends such protein-induced surface effects

to LD monolayers. These altered properties could influence the co-localization of other MLX proteins, which could play an important role in blocking dimerization and dissociation. Alternatively, they could influence the proximal association of other proteins requiring either large packing defects or a direct interaction with surface-active TG.

In summary, this work reveals that MLX targets TG-rich LDs specifically by demanding large packing defects, though it does so distinctly from other CYToLD mechanisms described to date by interacting with shallow PL-acyl defects. These shallow interactions strongly mimic the competing MLX dimer interface, resulting in nearly identical bound poses, whether MLX is bound to a LD monolayer or dimer partner. The specificity for TG-rich LDs and expulsion from SE-rich LDs links lipid metabolic conditions with a transcriptionally regulated glucose metabolism response. We have also characterized the association of a shorter MLX sequence, the MLX-hairpin, that could be useful in targeting membranes more broadly. The controlled localization of MLX and MYC-like proteins through leucine zippers linked to the MLX-hairpin or MLX DCD could be an interesting strategy in cancer therapeutics. Such controlled localization could offer a therapeutic approach to metabolic disorders too, if navigated carefully. In this sense, the MLX-hairpin and MLX-DCD linked to various specific leucine zipper motifs could be tested for their ability to target membranes broadly vs TG-LDs more specifically. Of course, specific targeting should be further benchmarked and experimentally verified.

The mechanism and impacts of LD association described herein suggest that the mechanisms that regulate the LD proteome are likely more nuanced than competing binding affinities alone. The catch, anchor, burial mechanism described for the hairpin suggests that kinetic selection may play a role since the catch phase coupled to the slower formation of packing defects would endow protein selection for systems with longer, more robust catch phases. Indeed, MLX has multiple catch residues strategically placed at opposite ends of the hairpin. Additionally, the interhelical interactions enabled by membrane association disfavor dissociation, imparting increased affinity and slow off rates. Lastly, the altered surface properties proximal to the bound MLX-hairpin and MLX DCD suggest that protein-surface-protein allostery may also influence which proteins bind to LDs and the nature of their colocalization on LD surfaces.

Methods

The association mechanisms of two MLX sequences were studied: the MLX-hairpin including residues 208-244 (Figure 2a left) and the DCD including residues 177-244 (Figure 2a right) (for protein structure prediction, please refer to *Supplementary Methods*). MLX-hairpin contains two short helices, one a distinct AH (Figure 2b), with multiple conserved residues: Leu228, Arg229, Val232, Leu236, and Leu243. Several other hydrophobic residues accompany these; Ile231, Val235, and Leu239. MLX-hairpin was initially thought to be the shortest binding sequence. However, subsequent experiments revealed that it seemed to bind to a variety of membranes, such as the ER bilayer, which means it lacks the specific binding to LDs.[37] Prior work demonstrated MLX-DCD binds specifically to TG-rich LDs.[6] MLX-DCD has two AHs (Figure2b-c), separated by a middle helix (helix-2) (Figure 2a-right).

Unbiased MD to capture real dynamics of association for the MLX-hairpin

To explore the dynamics of membrane association, we first conducted long-timescale simulations of MLX-hairpin interacting with 3 LDs, each with a different neutral lipid composition: pure TG, 50:50 SE:TG, and 90:10 SE:TG. The representative LDs were previously equilibrated in long-timescale simulations from our previous work,[23,25] using the CHARMM36m forcefield.[49,50] Briefly, this involved using trilayer models, which remain essential for computational efficiency at the all-atom resolution. In these trilayers, a neutral lipid core is inserted between the two leaflets of a phospholipid bilayer. The bilayers were created with the CHARMM-GUI membrane builder[51–53] containing an ER-like composition of 88:37:10 zwitterionic phospholipids: 3-palmitoyl-2-oleoyl-D-glycero-1-phosphatidylcholine (POPC), 2,3-dioleoyl-D-glycero-1- phosphatidylethanolamine (DOPE), and phosphatidylinositol (SAPI), respectively.[54–56] Neutral lipid cores with varying ratios of SE:TG (pure-TG, 50:50 SE:TG, 90:10 SE:TG) were created and equilibrated for 500 ns at 310 K in the NPT ensemble using periodic boundary conditions to converge bulk-like properties. The bilayer was equilibrated for 10 ns before separating the leaflets to create monolayers between which the equilibrated neutral lipid box was inserted. The simulation boxes were solvated with 50 Å of a TIP3P water model[57] and 0.15 M NaCl solution on either side of the leaflets. The final systems were then simulated for 8 µs using GROMACS version 2019.4[58] with a 2 fs timestep. An energy minimization of steepest-decent over 5000 steps was used to get rid of bad contacts of TG and the monolayer leaflets. The temperatures were set to 310 K (physiological temperature) using the Nose-Hoover[59,60] thermostat and temperature coupling time constant of 1 ps. The particle mesh Ewald (PME) algorithm[61] was used to calculate the long-range electrostatic interactions with a cutoff of 1.0 nm. Lennard–Jones pair interactions were cutoff at 12 Å with a force-switching function between 8 and 12 Å, and pressure was maintained semi-isotropically using the Parrinello-Rahman barostat.[62] The pressure was set to 1.0 bar, with a compressibility of 4.5×10^{-5} bar^{-1}, and a coupling constant of 5.0 ps. The hydrogen bonds were constrained with the LINCS algorithm.[63] Trajectories were extracted every 100 ps. For a more detailed description of these simulation set-ups, please visit our previous work.[23,25] In this work, the MLX-hairpin sequence was placed at least 1 nm above the LD monolayer using MDAnalysis scripting in random placement for each replica.[64] An energy minimization of steepest-decent over 5000 steps was used to get rid of bad contacts between protein and water, followed by equilibration (noting the LD systems were already equilibrated from the previous studies). Production runs were conducted for a minimum of 1 µs. 15 replicas were run for all systems. Visualizations of the molecular systems were rendered with Visual Molecular Dynamics (VMD).[65]

<u>Association Ensemble Analyses</u>

Contact analysis

To describe the binding process, we used MDAnalysis scripting to measure the center-of-mass (COM) depth of its residue sidechains relative to the phospholipid headgroups' phosphorus atoms, providing a detailed view of the interactions between hydrophobic and polar residues and the lipid bilayer. This was complemented by counting contacts (atoms within 3 Å) between phospholipid and TG defects and residue sidechains, enriching our understanding of molecular interactions. Furthermore, we

calculated Radial Distribution Functions (RDFs) to quantitatively analyze dominant interactions within the ensembles, using the MDAnalysis Python library.[64]

Defect size below protein

To determine the size of the packing defects involved below the proteins, we calculated the convex hull area of the protein residue interactions with the defect pockets. Calculating the convex hull below the cutoff of the P atoms of the PLs allows us to determine the size, and depth of defects directly below the protein. The convex hull of a group of points S in n-dimensional space can be thought of as the smallest convex shape that encompasses all points within S. For *N* points $p_1,\ldots,p_N$, the convex hull *C* is then given the expression:

$$C \equiv \left\{ \sum_{j=1}^{N} \lambda_j p_j : \lambda_j \geq 0 \; for \; all \; j \; and \; \sum_{j=1}^{N} \lambda_j = 1 \right\}$$

Where *p* is a combination of 10 sidechains below the defect cutoff.[66] If there was an inadequate number of sidechains below the cutoff, the convex hull was not counted.[66] The size of the convex hull was averaged over the respective trajectory of 1000 frames. The MDAnalysis Python library was used for the scripting.

Packing defect analysis

We refined a Cartesian-based algorithm to quantify lipid packing defects within monolayer leaflets, building upon established methodologies.[25,33,67,68] Put briefly: For a monolayer leaflet, the lipid atoms whose positions were greater than the threshold (Z_{thr}) were projected onto a 1 Å spacing two-dimensional (x–y) grid. The threshold (Z_{thr}) was set to the average z-position of the phosphorous atoms of the leaflet minus 2 nm, which therefore encompasses most atoms of the respective leaflet. We first defined the van der Waals radii for a comprehensive set of atom types taken from the CHARMM36m[50] parameter set. This informed our overlap criteria between grid points and atoms, ensuring accurate defect localization. If a grid point overlaps with an atom (center of atom and grid point is less than atom's radius plus half of the diagonal grid, ($\sqrt{3/2}$Å), the z-position of the atom and the atom type are saved in the grid point. If the grid point overlaps with a polar PL headgroup, then it is not considered a defect. While scanning the grid using a depth-first search, if the grid point overlaps with PL-acyl groups or the LD-neutral lipids, then it is considered a defect. For each of these defect types, the neighboring elementary defects are clustered into one. If the clustered defect contains *N* elementary defects, it is considered to have a defect size of *N* Å^2.

The probability of finding a defect with size *N* was computed and fit to an exponential decay function:

$$P(N) = ce^{-N/\pi},$$

where c is the normalization constant and π is the packing defect constant. The packing defect constant represents how slowly the decay function falls off. A higher packing defect constant would generally mean that there is a higher probability of finding a larger defect. The original script for this packing defect analysis can be found at (https://github.com/ksy141/SMDAnalysis).

We additionally modified the code to create cut-offs that excluded a desired radius from the protein once the protein associated to the respective leaflet of the LD monolayer. This allowed us to identify the alteration of monolayer defects due to protein interactions. To track the protein-defect interactions over time, we included further analysis to include the protein, while isolating specific heavy-atom interactions that were below defect threshold cut-offs. These protein atoms were included in the defect analysis and depth-first grid search, but were not included as a defect type. If a heavy protein atom crossed the threshold of the defect in the x-y grid, it was counted as a defect-protein interaction. Modified packing defect script analysis scripts that account for protein cutoff distances, and identifications of top and bottom leaflets can be found at (https://github.com/jaybraunjr/PD). Error bars for packing defect constants were calculated using blocking averaging, with 10 blocks used over a 500 ns period.

Coordination analysis

The coordination of specific residues to the defects is determined by:

$$s = \sum \left[1 - \left(\frac{r_i}{r_0}\right)^6\right] / \left[1 - \left(\frac{r_i}{r_0}\right)^{12}\right],$$

where r_0=0.4 nm, and r_1 is the distance between the side chain group and defect group. Heavy atoms were used.

Tail Order Parameters

The tail order parameters are calculated with:

$$S = \langle \frac{3}{2}\, cos^2(\theta - 1/2\rangle,$$

with θ being the angle between the position vector of a carbon atom of an acyl chain bonded to a hydrogen with respect to the system normal. A larger value of S Indicates increased ordering in the membrane. The brackets represent the ensemble average.[69] The tail order parameters were calculated with MDAnalysis scripting.

Mean squared displacement

To analyze the mobility of lipids in our system, we calculated the mean-squared-displacement (MSD) of lipid molecules over time. Lipids were tracked over the course of the simulation, and their positions at each time interval were recorded. The MSD was then computed using the Einstein formula through MDAnalysis.[64,70,71]

$$MSD(\tau) = \langle \frac{1}{N} \sum_{i=1}^{N} |r_d - r_d(t_0)|^2 \rangle_{t_0},$$

where τ is the time lag, and $r(t)$ is the position of the lipid molecule at time (t). By plotting the MSD against the time τ we were able to derive a linear relationship for the diffusive regime. The lipid diffusion coefficient D was then determined from the slope of the MSD curve, using the relation: $MSD = 4D\tau$ for a two-dimensional system. This provided us with a quantitative measure of how rapidly lipids were diffusing through the monolayer, allowing for a deeper understanding of the lipid dynamics within our system.

Binding of MLX-DCD domain

After conducting three 1-µs unbiased simulations, it was found that the MLX-DCD system couldn't bind to the LD monolayer. This was due to the tendency of the three helices to fold upon each other in order to prevent the hydrophobic residues from interacting with the cytosol. Without very long-timescale simulations, it is unlikely to attain binding. A slight bias using steered MD[72] was used to open the AH1 and AH2 of the DCD to expose them to solution, and then steered MD was also used to slightly bias the COM of the protein towards the LD in the z-coordinate until initial contact with the membrane surface was observed. A bias of 1000 kJ, with a rate of 0.001 nm per ps was used. Following this initial contact, regular production MD parameters were used as previously described. The simulation was run for an additional 3 µs, and the stability was measured using root mean squared deviation (RMSD). Additionally, we created a larger MLX DCD system (DCD-big) by doubling the $x\ and\ y$ dimensions to $20\ nm\ \times\ 20\ nm$. This was done to account for any size or pressure artifacts that might appear due to the large protein with reference to the LD monolayer.[73,74]

Quantifying association affinity

Umbrella sampling (US)[75] was used to determine the free energy of association for both the MLX-hairpin and MLX-DCD segments. The potential of mean force (PMF) was calculated as a function of the z-coordinate of the COM of the respective peptide sequence with respect to the distance from the average PL phosphorous z-coordinate plane. For MLX-hairpin, 30 windows were used, and for the MLX-DCD, 34 windows were used, with 0.1 nm used as the window spacing. The windows were obtained through natural binding in the pure-TG and 50:50 SE:TG systems, and through partial association event in the 90:10 SE:TG and a DCD. Steered MD, as described in the previous section, was used to fully associate the proteins in which partial association occurred. A harmonic potential with a force constant of 1000 kJ/mol nm^{-2} was used as a distance constraint. There was no helical restraint, as the helices remained extraordinarily stable throughout the simulations. The rest of the simulation parameters were the same as described in the unbiased simulations mentioned previously. Simulations were run for a total of 100 ns with the first 70ns being used to equilibrate the systems until the fluctuations were noticeably converged. The Weighted Histogram Analysis Method (WHAM), specifically using the tool g_wham[76], was used to generate PMF, with error bars being generated from block averaging using three 10 ns chunks of the last 40ns. Simulations were run using GROMACS version 2019.4.[58]

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