The role of prolines and glycine in transmembrane domain of LAT

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ABSTRACT

LAT is a critical regulator of T cell development and function. It organises signalling events at the plasma membrane. However, the mechanism, which controls LAT localisation at the plasma membrane is not fully understood. Here, we studied the impact of helix-breaking amino acids, two prolines and one glycine, in the transmembrane segment on localisation and function of LAT. Using *in silico* analysis, confocal and superresolution imaging and flow cytometry we demonstrate that central proline residue destabilises transmembrane helix by inducing a kink. The helical structure and dynamics is further regulated by glycine and another proline residue in the luminal part of LAT transmembrane domain. Replacement of these residues with aliphatic amino acids reduces LAT dependence on palmitoylation for sorting to the plasma membrane. Surface expression of these mutants is not sufficient to recover full function of non-palmitoylated LAT in stimulated T cells. These data indicate that geometry and dynamics of LAT transmembrane segment regulate its localisation and function in immune cells.

INTRODUCTION

Function of proteins (e.g., enzymes or signal transducers) is defined by their structure, but also localisation within a cell or an organism. Amino acid sequence, small molecule co-factors and interacting partners are primary determinants of the structure. However, features defining protein localisation are less well understood. The process defining protein localisation is called sorting. Short motifs within a primary sequence were found to firmly control sorting of proteins to some organelles, e.g., the ER retainment (KDEL motif), the ER exit (DxE and YxxΦ coupled motifs) or nucleus (NES/NLS motifs) [1, 2, 3]. Trafficking of proteins to the plasma membrane appears to be controlled by several unrelated determinants. Post-translational modifications, such as glycosylation and palmitoylation facilitate anterograde membrane transport of proteins to the cell surface [4, 5, 6]. On the contrary, ubiquitination regulates retrograde protein transport from the surface towards the cell interior [7]. However, the primary determinant of the plasma membrane sorting, at least for single-spanning membrane proteins, is the length and amino acid composition of the transmembrane domain (TMD) [6, 8]. A comprehensive study demonstrated that, in vertebrates, proteins with TMDs of 22 residues or longer localise to the plasma membrane [9]. Those with shorter TMDs remain in the Golgi apparatus or the ER. The voluminous amino acids (Phe, Tyr, Trp) were found to accumulate in the cytosolic part of the TMD of the plasma membrane proteins. The position of other amino acids is also non-random [9, 10]. These data indicate that physico-chemical properties of TMDs strongly influence trafficking of proteins in cells.

In vertebrates, the structure of TMD is almost exclusively formed by an α-helix. The hydrophobic core is largely composed of aliphatic (Leu, Val, Ala, Ile) and aromatic amino acids (Phe, Tyr, Trp). Weakly polar amino acids (Ser, Thr, Met, Cys) are also common components of TMDs [10]. Cysteine residues due to their propensity to form disulphide bridges in the oxidative environment, can force the protein to dimerise. Their presence is thus reduced in the exoplasmic part of the TMDs. Moreover, cysteine residues at the cytosolic end of the TMD reveal palmitoylation sites [11]. Positively charged amino acids (Lys, Arg, His) often define the cytosolic end of TMDs (*positive-inside rule*; ref. [12]). In addition, these residues can form electrostatic bridges between the TMDs of multi-spanning or multi-component membrane assemblies [13]. Interestingly, negatively charged amino acids (Asp, Glu) and their amido-derivatives (Asn, Gln) are almost absent from TMDs of vertebrate membrane proteins [10]. A weak preference for the membrane proximal part at the luminal end of the TMD was found for Asp and Glu [9, 10]. Finally, proline and glycine are the two amino acids with physico-chemical properties, which can affect the stability of TMDs.

Proline and glycine were defined as typical 'helix-breakers' in soluble globular proteins. Indeed, they are virtually absent from the helical structures of such proteins [14]. However, proline is often present in the TMDs of integral proteins, exhibiting the highest occupancy to the ends but, with a lower frequency, also to the central parts of the α-helices [10, 15, 16]. The cyclic structure of proline makes it unique among the 20 natural amino acids because its amide group lacks the proton necessary for the hydrogen bond stabilising the α-helix or the β-sheet structure [17]. Therefore, prolines in transmembrane (TM) helices are predicted to induce regions of helix distortions and/or dynamic flexibility (i.e., kinks, hinges, swivels; refs. [15, 18]). Such structural distortions may play a role in the transmission of conformational changes along the helix, establish crucial helix-helix packing interactions or geometries, but can also help the helix to adapt the optimal position [15, 19]. Proline was found in the TM helix of many membrane proteins, especially with a polytopic structure [20]. There, the presence of central proline can lead to the change in the orientation of helix [16]. Moreover, such prolines in TMDs are critical for the function of several proteins [19, 21, 22].

Glycine is also rather frequent in the TMDs of integral membrane proteins [10]. In analogy to proline, glycine helix-affecting properties depend on the local environment [23, 24]. No strong effect of a single glycine residue on basic structure of transmembrane α-helices was found experimentally [23, 24]. It functions primarily as an interface between individual helices of the polytopic membrane proteins and is involved in protein dimerisation [25, 26]. However, when positioned close to the proline, it enhances local dynamics of the helix and, thus, can affect the function of transmembrane peptides or proteins [27, 28]. The complexity of proline and glycine environment-sensitive properties suggests that it is important to characterise their role in the TMD of individual proteins separately.

LAT, linker for activation in T cells, is 36-kDa protein belonging to the family of transmembrane adaptor proteins (TRAPs). It plays an important role in membrane immuno-receptor signalling pathways of T cells, NK cells, mast cells and platelets [29, 30]. LAT is indispensable for the T-cell function and development [31]. It plays an essential role in T-cell activation by providing a platform for signal transmission initiated by the T-cell receptor (TCR) at the plasma membrane [30, 32]. T cells lacking LAT, i.e. the Jurkat mutants J.CaM2.5, fail to mobilise calcium and promote other downstream effector events upon TCR stimulation (e.g., ERK phosphorylation or IL-2 production). Human LAT is a type III transmembrane protein, i.e. it is missing a signal peptide and, thus, uses its unique TMD for its membrane insertion. Its extracellular part has only 3 amino acids, the TMD is 24 residues long and the cytoplasmic tail (235 amino acids) contains nine conserved tyrosine residues involved in T-cell signalling. The cytoplasmic tail of LAT also possesses conserved juxtamembrane cysteines required for its palmitoylation (residues 26 and 29). Palmitoylation is mainly responsible for sorting of LAT to the plasma membrane [6, 33, 34].

In this study, we have investigated the impact of highly conserved proline and glycine residues of the LAT TMD on its plasma membrane localisation and function. We demonstrate that replacement of prolines and glycine with aliphatic amino acid (alanine or leucine) partially recovers the surface localisation of LAT. However, such effect is not sufficient for LAT function. We also tested whether nanoscopic surface organisation of LAT mutants is altered and, thus, leads to their malfunction in T cells.

RESULTS

Atypical transmembrane domain of LAT with highly conserved helix-breakers at the positions 8, 12 and 17

We have previously shown that TMD controls sorting of LAT to the plasma membrane [6]. General plasma membrane sorting rules determined for other proteins are not obeyed by LAT: i) 23-residues long TMD should provide sufficient sorting signal but LAT, which lacks cysteines at the positions 26 and 29 and, therefore, cannot be palmitoylated, remains trapped in the Golgi apparatus [6, 9, 33], and ii) there are no voluminous amino acids in the exoplasmic portion of the LAT TMD (Fig. 1A). It was previously reported that prolongation of the TMD leads to the plasma membrane localisation of the proteins, which normally reside in the Golgi apparatus [8]. Nevertheless, prolongation of LAT TMD by 6 amino acids (six leucine residues or PILAML sequence) did not overcome dependence of LAT surface expression on its palmitoylation (Supplementary Fig. S1). We observed the accumulation of LAT with prolonged TMD in lysosomes and some unspecified intracellular vesicles (Supplementary Fig. S1). These data indicate that LAT TMD contains residues, which control its geometry or stability with respect to the lipid membranes of the secretory pathway.

The amino acid sequence of the LAT TMD exhibits high level of conservation with 48% identity in mammals (Fig. 1A). Among the conserved residues are two prolines, which were reported to introduce distortions into the helical structure of TMDs in several proteins [15]. One proline is positioned centrally (Pro17) and one close to the lumenal end of the LAT TMD (Pro8). Moreover, the lumenal part of LAT TMD contains glycine (Gly12) in the position i+4 with respect to Pro8 and i-5 with respect to Pro17. The motif (PxxxG) was described to control the dynamics of pore-forming peptides and proteins [27]. Importantly, helix-breaking residues were shown to exhibit cumulative effect on TM helices [24]. We, therefore, hypothesised that Pro8, Gly12 and Pro17 residues play a role in membrane stability and sorting of LAT to the plasma membrane.

All-atom MD simulations indicate kink formation in the helical structure of LAT TMD

We have first characterised the role of Pro8, Gly12 and Pro17 residues in LAT TMD structure using all-atom MD simulations. Residues 2-33, EEAILVPCVLGLLLLPILAMLMALCVHCHRLP, which form the hydrophobic core and membrane proximal parts, were used to simulate human LAT TMD in a lipid membrane composed of 128 palmitoyl-oleoyl phosphatidylcholine (POPC) molecules for 1 µs. The peptide was first enforced to adapt α-helical structure and inserted into the membrane in a transbilayer orientation. Afterwards, the system was left to adjust its optimal conformation for 500 ns. The following 500 ns of the trajectory were used for the analysis of peptide behaviour in a model membrane. The structure of LAT WT peptide exhibited high level of helicity. However, a kink could be observed in the central part of the peptide at around the position of Leu14 (Fig. 1B,C). This non-helical segment involving residues 13-15 is downstream of Gly12 (i+2 for Leu14) and upstream of Pro17 (i-3 for Leu14). Such deviation within the α-helix is in agreement with previously observed kinks induced by proline and glycine residues in the TMDs of several membrane proteins (e.g., bacteriorhodopsins, ref. [28]). The maximal impact was reported for positions i-3 or i-4 with respect to the proline residue [35]. Maximal effect of glycine was observed for residues i+1 and i+2 [24]. Another deviation from the α-helical structure, even though much smaller compared to the central non-helical segment, could be observed near Pro8 residue (Fig. 1C).

We have further used MD simulations to characterise behaviour of LAT TMD mutants, in which helix-breaking residues were replaced with alanine or leucine. Mutation of proline to alanine in the position 17 (P17A) led to complete disappearance of the kink for the full period of the simulation (Fig. 1B,C). A minor helix deviation near Pro8 was preserved in P17A mutant (Fig. 1C). On the contrary, no effect on the central kink was observed in the mutant P8A. Nevertheless, the mutant exhibited shortening of the helical TM segment (Fig. 1C) and higher dynamics of the kink, as represented by the analysis of the kink angles (Supplementary Fig. S2). The helix was 2 residues shorter compared to LAT WT peptide. No such shortening was observed for P17A mutant (Fig. 1C). MD simulations of G12L mutant of LAT TMD peptide provided similar results as for LAT WT peptide (Fig. 1C and Supplementary Fig. S2). However, the helical TM segment was 3 residues shorter than in the LAT WT peptide. The overall tilt angle of the peptides in the bilayer was similar for all variants except for the G12L mutant, probably caused by shortening of its helical fragment (Supplementary Fig. S3). The *in silico* data indicate that Pro17 is critical for the kink formation in the LAT TMD but Pro8 and Gly12 fine-tune the overall TMD structure and dynamics of the kink.

Mutation of helix-breaking amino acids in the TMD does not impact LAT sorting to the plasma membrane and its function

We have therefore investigated the impact of Pro8, Gly12 and Pro17 on LAT sorting to the plasma membrane. The coding DNA sequence was individually modified to replace Pro8 and Pro17 residues with alanine (Fig. 2A) and expressed as mutant LAT-GFP fusion proteins in LAT-deficient Jurkat T-cell

line, J.CaM2.5. Gly12 residue was mutated to leucine to prevent the disruption of leucine-rich segment of the LAT TMD (Figs. 1A and 2A). The cells were transiently transfected with the plasmid DNA and the expression was analysed 16-24h later using confocal scanning microscopy. All mutant proteins exhibited a distribution, which was comparable to the native LAT protein. The proteins prevalently localised to the plasma membrane of transfected J.CaM2.5 cells (Fig. 2B). A partial trapping of the proteins in the Golgi apparatus could be observed. This observation is in agreement with previous findings that T cells express two pools of LAT, one localized in the plasma membrane and the other in the Golgi apparatus [36]. However, no extensive accumulation of the mutant LAT proteins in intracellular membranes, e.g. the ER, was visible. These results indicate that mutating individual 'helix-breaking residues' of the LAT TMD to alanine (or leucine) does not substantially destabilise LAT protein.

We further verified whether Pro->Ala or Gly->Leu mutations influence function of LAT in T cells. It was shown previously that antigen response of T cells is LAT-dependent process [37]. High level of cytosolic calcium was rapidly mobilised in J.CaM2.5 cells transiently transfected with LAT WT stimulated with anti-TCR as measured by flow cytometry (Fig. 3A; see also **Methods**). A three-fold signal increase was observed less than a minute after stimulation, as detected by changes in fluorescence properties of the calcium-sensitive probe Fura Red (Fig. 3B). Similarly, stimulation of cells expressing LAT-P8A, LAT-G12L or LAT-P17A variants was associated with a rapid, strong, and sustained calcium response (Fig. 3B). Together, these data indicate that mutation of helix-breaking residues does not affect localisation and function of LAT in T cells.

Mutation of helix-breaking amino acids promotes sorting of non-palmitoylated LAT to the plasma membrane

As mentioned above, LAT requires palmitoylation of its membrane proximal cysteines 26 and 29 for its localization to the plasma membrane and function. We were, thus, interested whether a kink in the helix of the TMD determined using MD simulations can influence plasma membrane sorting of LAT in the absence of its palmitoylation. We prepared LAT-GFP variants (Fig. 4A), which combined alanine mutants of Pro8 and Pro17 (or leucine mutant of Gly12) with cysteines 26 and 29 replaced for serines as described before [6]. The mutant proteins were transiently expressed in J.CaM2.5 cells and analysed by confocal microscopy 16-24h post transfection. The images shown in Fig. 4B demonstrate that mutation of helix breaking amino acids in the TMD to alanine or leucine partially restores the plasma membrane localisation of non-palmitoylatable LAT in 30-50% of cells. Compared to LAT-P8A-CS, slightly stronger surface signal was observed on the plasma membrane in cells expressing LAT-G12L-CS and LAT-P17A-CS (Fig. 4B and Supplementary Figs. S5-7). Nevertheless, LAT-P8A-CS, LAT-P17A-CS and LAT-G12L-CS were found in the intracellular membranes, mainly Golgi apparatus and the ER. Such intracellular trapping is similar to LAT-CS mutant with preserved kink-forming amino acids (Fig. 4B and Supplementary Fig. S4).

When analysing response to antibody stimulation, no calcium mobilisation was observed in J.CaM2.5 cells expressing any of the non-palmitoylated LAT-CS variants (Fig. 3B). Surface expression is thus not sufficient for LAT function in antigen-induced T cell signalling. Previously, we demonstrated that addition of the CD4 extracellular domain to LAT-CS rescued plasma membrane sorting of CD4ex-LAT-CS variant ([6] and Supplementary Fig. S8). Importantly, robust calcium mobilisation is induced by antibody stimulation in J.CaM2.5 cells expressing CD4ex-LAT or its non-palmitoylatable variant - CD4ex-LAT-CS (Fig. 3B). CD4ex-LAT and CD4ex-LAT-CS contain intact LAT TMD (apart from cysteine residues 26 and 29 in the CS variant). These data indicate that replacement of helix-breaking amino acids in the TMD with helix supporting ones partially rescues LAT sorting to the plasma membrane, but such surface LAT molecules remain inaccessible for early T cell signalling molecules (e.g. Lck and ZAP-70; ref. [32]).

Nanoscopic organisation of LAT mutants on the surface of T cells

We were thus interested whether this is due to the different nanoscopic organisation of LAT-WT and the mutants, which cannot be visualized by standard confocal microscopy. We employed super-resolution (SR) microscopy called photoactivation localization microscopy (PALM) to image nanoscopic organization of the variants LAT-WT, LAT-P17A and LAT-P17A-CS at the surface of transiently transfected J.CaM2.5 cells. For this purpose, GFP tag was replaced with mEos2, which exhibits photoconvertible behaviour essential for PALM imaging [38]. Transfected cells were immobilized on glycine-coated coverslips to avoid artificial stretching of T cells caused by poly-L-lysine coated surfaces [39]. As shown in Fig. 5, LAT-WT exhibits a random distribution over the surface of unstimulated T cells. LAT-P17A variant, which generates similar calcium response in antibody-stimulated T cells, is also randomly distributed (Fig.5, upper middle panel). For J.CaM2.5 cells expressing the LAT-P17A-CS variant (Fig. 4), which do not respond to antibody stimulation, irregular distribution of molecules was observed using PALM (Fig. 5, upper right panel). This is probably caused by reduced spreading of LAT-P17A-CS-transfected cells on glycine-coated coverslips as represented by a cell footprint on the optical surface analysed using interference reflection microscopy (IRM; Fig. 5, lower right panel). The IRM images of these cells are highly non-homogenous indicating an extensive three-dimensional surface structure probably caused by the accumulation of microvilli or membrane ruffles at the contact site. Intensive spreading was observed for cells expressing LAT-WT and LAT-P17A (Fig. 5, lower panel). Similar protein distributions were detected for palmitoylated and non-palmitoylated LAT-P8A variants, respectively (Supplementary Fig. S9). These data indicate that Pro8 and Pro17 of the TMD are not directly involved in the nanoscopic organisation of LAT at the T-cell surface. However, the ability of T cells to spread on non-stimulating surface is affected by the palmitoylation state of LAT.

DISCUSSION

In this work, we demonstrate the impact of helix-breaking residues on dynamic geometry of LAT TMD and localisation of LAT in T-cell membranes. Using MD simulations, we uncovered the presence of a kink in the TMD of human LAT. The *in silico* and live cell imaging data indicate that the kink is primarily caused by the presence of proline residue in the central segment of the TMD. Replacement of this residue with alanine (P17A mutant) completely removed the kink in the LAT TMD structure. Moreover, non-palmitoylated LAT-P17A mutant localised to the plasma membrane in approximately half of the cells. No such localisation was observed for non-palmitoylated LAT with native TMD sequence, thus, indicating that Pro17 is critical for the dependence of LAT surface expression on palmitoylation. This is probably caused by a highly dynamic central region of native TMD, which is destabilised by the presence of the kink. Indeed, similar helix destabilisation was observed in TM peptides with centrally positioned proline residue [18]. In oligomeric and multispanning (polytopic) proteins, central prolines were found to facilitate tight packing of TMDs and to function as transmission elements of conformational changes required for a rapid response to external stimuli (e.g., ligands, photons or electrochemical fluctuations; refs. [19, 21, 22, 40]). Potentially, such kinked TMD can affect the stability of single-spanning protein such as LAT in membranes. We did not observe any cytosolic expression of native and mutant LAT protein, independent of its palmitoylation state. Therefore, dynamic geometry of LAT TMD must form the basis of the protein adaptability for the specific areas of cell membranes (see below).

Neighbouring amino acids were found to regulate function of prolines in TMDs [21, 22]. Indeed, our live cell imaging data indicate such role for glycine, positioned five residues upstream of the central proline, in LAT localisation in T cells. But no effect on the kink and its dynamics was observed in MD simulations. This may be caused by a subtle effect of glycine residue on TMD dynamics, which is more pronounced in the complex lipid mixture of cell membranes. Glycine was found to enhance the helix-destabilising effect of proline in channel-forming peptide alamethicin [27]. On its own, it destabilises helical structure of downstream residues, especially in the presence of additional helix-breaking residues in the TM segment [24]. The observed kink of LAT TMD spreads over 3 residues downstream of Gly12 and positions i-2 to i-4 with respect to Pro17. This is in agreement with reported destabilising effect of glycine and proline downstream and upstream, respectively, of their position in the helix [24, 35].

Interestingly, as demonstrated by MD simulations, replacement of Pro8 in the lumenal part of LAT TMD for alanine increased dynamics of the kink in the central part. Moreover, non-palmitoylated LAT-P8A mutant localised to the T-cell surface in a similar proportion of cells as observed for the LAT-P17A mutant. A minor reduction of the helicity of native LAT TMD was observed around Pro8 residue. However, similar instability was detected for all tested peptides, including P8A variant. This minor helical instability is probably not caused by Pro8. Von Heijne and Deber groups reported significant impact of prolines localised at the TMD ends on protein (peptide) function and structure [41, 42]. No effect of central proline was found using protein topology-sensitive glycosylation assay [41]. We have observed a strong effect of central proline and only a minor, if any, effect of proline present in the first loop of the helix. We speculate that local lipid environment and overall structure of the TM segment modulate the influence of TM prolines on membrane proteins.

Cellular membranes can have diverse lipid composition, which, among other things, determines their thickness [43]. Hydrophobic segment of human LAT TMD is rather long (24 residues) but the protein requires palmitoylation for its sorting to the thinker bilayer of the plasma membrane [6, 33, 34]. This contradicts previous observation that, in vertebrates, TMDs with >22 residues are sufficient for protein sorting to the plasma membrane [8, 9]. The presence of kink may thus affect the apparent 'length'

of LAT TMD. Localization of membrane proteins is also affected by the presence of specific amino acids along the oriented structure of TMDs [9, 10, 44]. For example, acidic amino acids accumulate at the luminal end of the plasma membrane TMDs [10]. Moreover, we have shown that voluminous amino acids form a rough surface of TM segments, which locally rigidifies membranes and may interfere with membrane properties [45]. In this biophysical study, a poly-leucine TM peptide and simple lipid membrane composition was used. In cell membranes, exact geometry, and dynamic structure of TMD can define protein stability and orientation in these complex structures. LAT, with its flexible TM helix, can probably adapt to membranes with diverse lipid composition [6].

Recently, Levental and colleagues reported the role of TMD asymmetry in protein sorting to the plasma membrane [44]. Using bioinformatic approach, they found that proteins at the plasma membrane have smaller surface area in the lumenal (exoplasmic) half of their TMD compared to the cytosolic half. This is in agreement with previously reported accumulation of voluminous amino acids in the cytosolic part of the TMD of the plasma membrane proteins [9, 10]. These amino acids are rather absent in the luminal part of the TMD of these proteins. Levental and colleagues argue that chemical asymmetry of the plasma membrane, which exhibit higher rigidity in its outer leaflet compared to the inner leaflet, selects proteins with asymmetric TMD in terms of surface area [44]. However, this is a static view and cellular membranes exhibit high local dynamics. LAT with evenly distributed voluminous amino acids throughout its TMD (leucine and isoleucine) does not fulfil this criterion. Phenylalanine, tryptophan, and tyrosine are absent from human LAT TMD, but could be found in other species (e.g., mouse or rat). Therefore, it must be the highly dynamic character of LAT TMD, which facilitates LAT localisation to the plasma membrane.

Additionally, LAT is one of very few integral proteins, which segregate into more ordered parts of the membrane, at least in giant plasma membrane vesicles [46]. Even though, membrane domains with similar properties have not been indisputably proven in cells yet, their existence is supported by a lot of indirect evidence [43, 47]. The ability of LAT TMD to change geometry rapidly (intrahelically) may again represent an advantage for segregation into membrane areas with more rigid organisation. Stabilisation of such structures is predicted to involve curvature [48]. The kink in LAT TMD can provide an advantage for localisation into domains with complex geometry. Indeed, our data indicate that elimination of the kink or modification of its properties in P8A, G12L and P17A mutants removes the sorting block for non-palmitoylated LAT trapped in the Golgi apparatus, but cannot recover its function. This is probably due to the sorting of native and palmitoylated LAT into membrane areas with complex chemical composition and geometry.

In summary, we demonstrate the existence of kink in LAT TMD, which controls LAT sorting to the plasma membrane. The kink is induced by centrally positioned proline but is further modulated by neighbouring glycine. Replacement of these residues with helix-stabilising amino acids partially recovers LAT sorting to the plasma membrane but not the function of this adapter. These data indicate complex control of LAT function by highly dynamic structure of its TMD.

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CONTRIBUTIONS

L.C., T.B. and M.C. conceived the study. D.G., H.M., M.C.H. and T.Ch. performed the experiments, D.G., H.M., M.C.H., T.Ch. and L.C. analyzed the data. D.G. and M.C. wrote the paper. All authors reviewed and approved the manuscript.

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Additional information
Supplementary Information accompanies this paper at …

Competing interests
The authors declare no competing financial interests.

METHODS

Cell culture and transfection

LAT-negative Jurkat T cell variant (J.CaM2.5) was cultured in RPMI-1640 media (Sigma-Aldrich), complemented with 10% fetal calf serum (FCS; Life Technologies) in a humidified incubator (Eppendorf) under controlled conditions of 37°C and 5% CO2. The cells were transiently transfected using the Neon® transfection system (Life Technologies) according to the manufacturer's instructions. One µg of vector DNA was used per shot (3 pulses of 1325 V lasting for 10 ms) per 500 000 cells, which were then incubated in 0.5 ml of media. Cells were analysed 16-20 hours post transfection.

DNA cloning

To generate LAT mutants P8A and G12L, LAT TMD coding sequence was modified using polymerase chain reaction (PCR) and the primers with the appropriate mutations (Supplementary Table S1), and cloned into pXJ41-LAT-EGFP plasmid (or its non-palmitoylatable CS mutant; ref. [6]) using EcoRI and BamHI restriction sites. The variants without the CD148 leader, c-Myc and 5′ UTR were used. LAT-P17A variants were prepared by annealing the appropriate oligonucleotides (Supplementary Table S1) and subcloning as above. LAT-P8,17A mutants were generated from LAT-P17A variant using P8A forward and reverse primers. For super-resolution microscopy, the EGFP coding sequence was changed to mEos2 using BamHI and XhoI restriction sites.

Live-cell confocal microscopy

Cells transiently transfected with GFP variants of LAT in pre-heated, colour-free RPMI-1640 medium supplemented with 10% FCS were placed on 1% gelatine-coated Ibidi µ-Slide 8-well chambers (Ibidi, Germany) and imaged using a Leica TCS SP8 laser scanning confocal microscope equipped with sensitive hybrid detectors (HyD), 488 nm (20mW) and 638 nm (30 mW) lasers and a 63×1.4 NA oil-immersion objective. Five to ten percent laser power and 10% gain were used for image acquisition. Acquired images were manually thresholded to remove signal noise detected outside of the cell using ImageJ software package [49].

Two-colour confocal microscopy of fixed cells

Cells were immobilized on poly-L-lysine-coated Ibidi µ-Slide 8 well chambers for 5 minutes, fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature and subsequently washed with PBS. Cells were permeabilized for 40min using 10x Permeabilisation Buffer (eBioscience) diluted in water, washed with PBS, and subsequently blocked with 5% BSA. Plasma membrane and Golgi apparatus of transfected cells were stained with 0.5µg/ml lectin-HPA conjugated to Alexa Fluor 647 in PBS supplemented with 1% BSA (Life Technologies) and washed twice with PBS prior imaging. Images were taken using a Leica TCS SP8 laser scanning confocal microscope equipped with a 63× 1.4 NA oil-immersion objective. Minor contrast and/or brightness level adjustments were applied, and images were processed for publishing by ImageJ software.

Single Molecule Localization Microscopy (SMLM)

Sample preparation. Round 25 mm coverslips (No 1.5H, High precision; Marienfeld) cleaned with Helmanex III solution, were coated with 500 µl of 2M glycine solution in ultrapure water for 30 minutes at room temperature. Prior adding the cells, coated coverslips were once washed with Mili Q® water (Merck Millipore). Transfected cells were harvested (3 min at 300 rcf) and transferred into PBS

prewarmed to 37°C. After 10 minutes incubation at 37°C cells were fixed for 1 hour at room temperature with 4% paraformaldehyde (Electron Microscopy Sciences) and 2% sucrose (Sigma-Aldrich) solution in PBS. Fixation was stopped with 50mM NH_4Cl in PBS followed by three rounds of washing with PBS. Coverslips were transferred into ChamLide holder (LiveCell Instruments). 200 nm gold beads (BBI) fiducial markers were loaded to the sample in 0,9% NaCl for 5 minutes. Imaging was performed in PBS.

Microscope setup and measurements. SMLM measurements were performed on a home built microscope (IX71 body; Olympus) equipped with 150mW 405nm (Cube; Coherent) and 150mW 561nm (Sapphire; Coherent) lasers, 100x 1.49 NA objective (UApoN; Olympus), EMCCD camera (iXon DU-897, Andor) and manual TIRF tilting mechanism (Thorlabs). Synchronisation of laser switching, and camera recording was performed with two acousto-optic tuneable filters (AOTF; AOTFnC-400.650-TN, AA Optoelectronics) and a home written acquisition software (LabView). To turn the fluorophores into the dark state, fast epifluorescence irradiation with 561nm laser in was applied. Data were acquired using the highly inclined and laminated optical sheet (HILO) illumination mode with 50 ms/frame collection time. The power of activation laser (405 nm) was increased gradually to collect 10 000 – 15 000 frames with a uniform emitter signal.

Data analysis. To generate super-resolution maps of LAT localisations, data were analysed using ThunderSTORM plugin of the ImageJ/Fiji software package [49].

Interference Reflection Microscopy (IRM)

IRM on living cells was recorded on modified Olympus Fluoview 1000 setup equipped with 60x 1.2 NA water immersion objective (UPlanSApo; Olympus). GFP signal was excited with 20mW 488nm laser (Sapphire; Coherent) and recorded on a single-channel PMT detector equipped with a selective dichroic mirror (DM488/543/633; Olympus). For acquisition and system control the FluoView 1000 software package (Olympus) was used. The images were processed using ImageJ/Fiji software package.

Calcium measurements by flow cytometry

Cells resuspended at 10^6 cells/ml in pre-warmed PBS containing 1 µM Fura Red AM (Invitrogen) were incubated at 37°C under 5% CO_2 in a humidified incubator for 30 minutes. Cells were then washed twice with PBS, resuspended in RPMI-1640 (Sigma-Aldrich) supplemented with 10% FCS (Life Technologies) and kept on ice. Prior measurement, cells were equilibrated to 37°C for 4 minutes in a water bath.

All experiments were performed on an LSRII flow cytometer (BD Bioscience). A steady-state (background) signal was recorded for 20 seconds. Afterwards, the cells were stimulated with an antibody: pre-warmed RPMI (Sigma-Aldrich) supplemented with 10% foetal calf serum (Life Technologies) containing anti-TCR antibody C305 (home-produced supernatant, 10 µg/ml) was added in 1:1 ratio. Changes in calcium level were recorded continuously for over 200 seconds. Transfected cells were gated for EGFP positivity. The relative calcium concentration was measured as a ratio of the Fura Red fluorescence intensity elicited by the excitation at 405 nm (emission measured at 635–720 nm) and 488 nm (emission measured at 655–695 nm). The calcium values were calculated as the increasing signal stimulated by the 405 nm laser over the decreasing signal stimulated by the 488 nm laser using the Kinetics tool in FlowJo software version 10.6.1 (Tree Star Inc., OR, USA). The fold increase in signal was calculated as a ratio between maximum peak response and a mean of the steady-state signal recorded without stimulation.

All-atom MD simulations

For atomistic MD simulations, the amino acid sequence of wild type LAT (EEAILVPCVLGLLLLPILAMLMALCVHCHRLP) was used to construct a fully helical model of the peptide employing the Protein Builder of the Molefacture plugin in the VMD software [50]. The standard GROMACS tool (pdb2gmx) was used to apply the fully atomistic AMBER99SB-ILDN force field for the peptide [51, 52]. A lipid membrane consisting of 128 POPC molecules (64 in each leaflet) was hydrated with ~5000 water molecules and pre-equilibrated. The Slipid force field was used for phospholipids and TIP3P model was employed for water [53, 54, 55]. Then, the method of Javanainen was employed to insert the LAT peptide into the membrane in a transmembrane orientation. [56]. The overall -1 charge of LAT was neutralized by inserting one Na+ cation into the water phase. The standard Dang's force field parameters were used for the sodium cation [57]. The membrane with LAT was then simulated using GROMACS software [51]. The same protocol of system preparation and simulations was used for the three LAT mutants considered in MD.

In simulations, fully periodic boundary conditions were used. The simulations were performed at the temperature of 310 K controlled by Nose-Hoover thermostat with the time constant 1.0 ps [58]. Pressure of 1 bar was controlled employing the semi-isotropic Parrinello-Rahman barostat with the time constant of 5 ps and compressibility of 4.5×10^{-5} bar^{-1} [59]. The Particle Mesh Ewald algorithm was used for accounting for long-range electrostatic interactions with the real-space cut-off set to 1.2 nm [60]. For long-range non-bonded interactions, 1.2 nm cut-off was used, switched at the distance of 1.0 nm. All covalent bonds were constrained by LINCS algorithm, and water molecules were constrained by SETTLE method [61, 62]. The equations of motion were integrated with 2 fs time step. All simulations were performed for 1000 ns. Stabilization of the root mean square displacement and peptide-lipid contacts was used as an equilibration criterion. The initial 500 ns were treated as equilibration period while the final 500 ns were used for the analysis.

The analysis of the simulated trajectories was performed using standard GROMACS tools combined with in house Python scripts. In the case of the helix tilt angle and bending, the method of Bansal et al. implemented in the HELANAL Python library was applied [63]. The numerical data were presented using Matplotlib Python library and MATLAB [64]. Molecular visualization was performed with the VMD software [50].

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FIGURE LEGENDS

Figure 1. Helix-breaking amino acids in the transmembrane domain of LAT. (A) Sequence alignment of LAT transmembrane segment and the proximal parts (hydrophobic region in bold) showing conservation of amino acids among twelve mammalian species. **(B)** Representative snapshot from MD simulations of LAT TMD peptide and its variants in POPC bilayer. Transmembrane helix is shown in magenta, phospholipid headgroups are indicated with light blue spheres. **(C)** Helicity plot showing the propensity of individual amino acids in the LAT TMD peptide and its variants to accommodate helical geometry. Standard error is indicated as shadowing of the curves.

Figure 2. Localisation of palmitoylated LAT variants in J.Cam2.5 T cell line. (A) Schematic representation of human LAT construct with attached EGFP and indicated TMD region with accompanied primary sequence. Amino acids mutated in tested LAT variants are highlighted in red. **(B)** Confocal microscopy of J.CaM2.5 T cells expressing LAT-WT-EGFP, LAT-P8A-EGFP, LAT-G12L-EGFP and LAT-P17A-EGFP stained with the plasma membrane (PM) and Golgi apparatus (GA) marker lectin-HPA AF647. Single-channel (EGFP and GA + PM marker) or two-channel overlay images (merge; EGFP – green, GA + PM – red, overlay – yellow) are shown together with brightfield image (BF). Scale bars, 10 µm. More examples in Supplementary Figs. S4-S7. Representative images of 3 measurement days are shown.

Figure 3. Calcium response of J.CaM2.5 T cells expressing LAT variants to stimulation. (A) A representative plot of calcium mobilisation (FuraRed ratio Ex405/Ex488) recorded between 0 and 200 s in transiently transfected J.CaM2.5 T cells by flow cytometry. Transfected cells were stained with Fura-Red and stimulated by the addition of c305 antibody 25 seconds after the beginning of the measurement (indicated by red arrow; y – maximum peak response, x - mean of the non-specific calcium flux without stimulation). For the analysis, transfected cells were selected using gating of GFP signal. **(B)** A bar graph showing increased intracellular calcium levels by c305 antibody in J.CaM2.5 T cells expressing indicated LAT variants (palmitoylatable LAT variants – dark grey bars; non-palmitoylatable LAT variants – light grey bars). Error bars indicate standard deviation of the mean, black puncta represent individual experiments. The data show measurements for 6 independent measurement days.

Figure 4. Localisation of non-palmitoylated LAT variants in J.CaM2.5 T cell line. (A) Schematic representation of human non-palmitoylated LAT construct with attached EGFP and indicated TMD region with accompanied primary sequence. Amino acids mutated in tested LAT-CS variants are highlighted in red. **(B)** Confocal microscopy of J.CaM2.5 T cells expressing LAT-CS-EGFP, LAT-P8A-CS-EGFP, LAT-G12L-CS-EGFP and LAT-P17A-CS-EGFP stained with the plasma membrane (PM) and Golgi apparatus (GA) marker lectin-HPA AF647. Single-channel (EGFP and GA + PM marker) or two-channel overlay images (merge; EGFP – green, GA + PM – red, overlay – yellow) are shown together with brightfield image (BF). Scale bars, 10 µm. More examples in Supplementary Figs. S4-S7. Representative images of 3 measurement days are shown.

Figure 5. Super-resolution (SR) imaging of LAT variants on the surface of T cells. (A) SR images of LAT-WT, LAT-P17A and LAT-P17A-CS in transiently transfected J.CaM2.5 T cells imaged by photoactivation localisation microscopy (PALM) of mEos2 fusion proteins. The images represent a footprint of fixed cells on glycine-coated coverslips. **(B)** IRM images of living J.CaM2.5 T cells transiently transfected with LAT-WT, LAT-P17A and LAT-P17A-CS. Dark areas represent a contact with the optical surface.

Diffraction rings (blue arrowheads) indicate complex surface morphology of an imaged cell. Scale bars, 5 µm. Representative images from 2 measurement days. At least 7 cells were analysed for each LAT variant.

Supplementary Information

For the manuscript: Glatzova et al. The role of intramembrane prolines and glycine in T cell homeostasis and function of LAT

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1. Localisation of LAT variants with prolonged TMD in J.CaM2.5 T cell line. Confocal microscopy of J.CaM2.5 T cells expressing LAT-6Leu-EGFP **(A)**, LAT-6Leu-CS-EGFP **(B)**, LAT-PILAML-EGFP **(C)** and LAT-PILAML-CS-EGFP **(D)** stained with the marker of lysosomes (Lysotracker-Red). Single-channel (EGFP and Lysosome marker) or two-channel overlay images (Merge; EGFP – green, Lysosome – red, overlay – yellow) are shown together with a brightfield image (BF). Scale bars, 10 µm.

Supplementary Figure S2. Angles formed by kinked helix of LAT peptides in POPC membrane – MD simulation data. (A) Plots representing mean angle between individual amino acids of the LAT WT peptide and indicated mutant variants. Angles were calculated by HELANAL Python library with the geometry of the α-helix characterized by computing local helix axes and local helix origins for four contiguous carbon-α atoms. Angles larger than 20˚ indicate non-helical structure [63]. **(B)** Plots representing standard deviation of mean angles between individual amino acids (as in A) of the LAT WT peptide and indicated mutant variants. Higher values of standard deviation indicate larger flexibility of the TMD peptide within kinked region.

Supplementary Figure S3. Tilt angles of LAT peptides in POPC membrane – MD simulation data. (A) A plot of tilt angles with respect to the membrane normal accommodated by the LAT WT peptide and indicated mutant variants. The data represent a distribution of tilt angles from the trajectories of the peptides in POPC membranes acquired in all-atom MD simulations. Tilt angles were calculated by HELANAL Python library [63]. The tilt angle value of zero represents a helix orientation parallel to membrane normal, and the tilt angle of 90 degree – perpendicular to the membrane normal.

Supplementary Figure S4. Localisation of LAT-WT-EGFP (A) and its non-palmitoylatable variant LAT-CS-EGFP (B). Nine examples of J.CaM2.5 cell expressing LAT variants imaged by confocal microscopy. EGFP channel is shown along the brightfield image. In total 74 cells were analysed for LAT-WT and 51 cells for LAT-WT-CS variant. Scale bars, 10 µm.

Supplementary Figure S5. Localisation of LAT-P8A-EGFP (A) and its non-palmitoylatable variant LAT-P8A-CS-EGFP (B). Nine examples of J.CaM2.5 cell expressing LAT variants imaged by confocal microscopy. EGFP channel is shown along the brightfield image. In total 80 cells were analysed for LAT-P8A and 76 cells for LAT-P8A-CS variant. Scale bars, 10 µm.

Supplementary Figure S6. Localisation of LAT-G12L-EGFP (A) and its non-palmitoyable variant LAT-G12L-CS-EGFP (B). Nine examples of J.CaM2.5 cell expressing LAT variants imaged by confocal microscopy. EGFP channel is shown along the brightfield image. In total 90 cells were analysed for LAT-G12L and 55 cells for LAT-G12L-CS variant. Scale bars, 10 µm.

Supplementary Figure S7. Localisation of LAT-P17A-EGFP (A) and its non-palmitoylatable variant LAT-P17A-CS-EGFP (B). Nine examples of J.CaM2.5 cell expressing LAT variants imaged by confocal microscopy. EGFP channel is shown along the brightfield image. In total 118 cells were analysed for LAT-P17A and 56 cells for LAT-P17A-CS variant. Scale bars, 10 µm.

Supplementary Figure S8. Localisation of LAT variants containing the extracellular domain of CD4 in J.CaM2.5 T cell line. Confocal microscopy of J.CaM2.5 T cells expressing CD4ex-LAT-EGFP **(A)** and CD4ex-LAT-CS-EGFP **(B)** stained with the plasma membrane (PM) and Golgi apparatus (GA) marker

lectin-HPA AF647. Single-channel (EGFP and GA + PM marker) or two-channel overlay images (Merge; EGFP – green, GA + PM – red, overlay – yellow) are shown together with brightfield image (BF). Scale bars, 10 µm.

Supplementary Figure S9. Super-resolution (SR) imaging of LAT variants on the surface of T cells. (A) SR images of LAT-WT, LAT-P8A and LAT-P8A-CS in transiently transfected J.CaM2.5 T cells imaged by photoactivation localisation microscopy (PALM) of mEos2 fusion proteins. The images represent a footprint of fixed cells on glycine-coated coverslips. **(B)** IRM images of living J.CaM2.5 T cells transiently transfected with LAT-WT, LAT-P8A and LAT-P8A-CS. Dark areas represent a contact with the optical surface. Diffraction rings (blue arrowheads) indicate complex surface morphology of an imaged cell. Scale bars, 5 µm. Representative images from 2 measurement days. At least 7 cells were analysed for each LAT variant.

Supplementary Table S1. List of PCR primers

Primer	Sequence	Company
P8A forward	GAATTCGCCACCATGGAGGAGGCCATCCTGGTCGCCTGC	Merck
P8A reverse	GGATCCGTTCAGCTCCTGCAG	Merck
G12L forward	TCCTGGTCCCCTGCGTGCTGCTGCTCCTG	Merck
G12L reverse	TCCTGGTCCCCTGCGTGCTGCTGCTCCTG	Merck
P17A forward	AATAATGAATTCGCCACCATGGAGGAGGCCATCCTGGTCCCCTGCGTGCTGGGGCTCCTGCTGCTGGCCATCCTGGCCATGTTGATGGCACTGTGTGTGCACTGCCACAGACTGCCAGGATCCAATAAT	Merck
P17A reverse	ATTATTGGATCCTGGCAGTCTGTGGCAGTGCACACACAGTGCCATCAACATGGCCAGGATGGCCAGCAGCAGGAGCCCCAGCACGCAGGGGACCAGGATGGCCTCCTCCATGGTGGCGAATTCATTATT	Merck
P17A-CS forward	AATAATGAATTCGCCACCATGGAGGAGGCCATCCTGGTCCCCTGCGTGCTGGGGCTCCTGCTGCTGGCCATCCTGGCCATGTTGATGGCACTGAGTGTGCACAGCCACAGACTGCCAGGATCCAATAAT	Merck
P17A-CS reverse	ATTATTGGATCCTGGCAGTCTGTGGCTGTGCACACTCAGTGCCATCAACATGGCCAGGATGGCCAGCAGCAGGAGCCCCAGCACGCAGGGGACCAGGATGGCCTCCTCCATGGTGGCGAATTCATTATT	Merck

SUPPLEMENTARY MOVIE LEGENDS

Supplementary Movie 1. The last 500 ns of the 1 µs-long MD simulation of the LAT WT peptide in POPC bilayer, related to Figure 1b. Side view (parallel to the bilayer midplane) of the simulation box is shown. The video was created based on the trajectory sampled each 500 ps. The trajectory was centered with respect to the peptide center of geometry. Phosphorous atoms of POPC are shown as cyan balls, and the peptide is depicted using NewCartoon representation (purple). The remaining atoms and molecules, including water, are not shown for clarity. The video was prepared employing MovieMaker plugin of VMD. H264-MPEG-4 encoding was used.

Supplementary Movie 2. The last 500 ns of the 1 µs-long MD simulation of the LAT P8A peptide in POPC bilayer, related to Figure 1b. Side view (parallel to the bilayer midplane) of the simulation box is shown. The video was created as described for Movie 1.

Supplementary Movie 3. The last 500 ns of the 1 µs-long MD simulation of the LAT G12L peptide in POPC bilayer, related to Figure 1b. Side view (parallel to the bilayer midplane) of the simulation box is shown. The video was created as described for Movie 1.

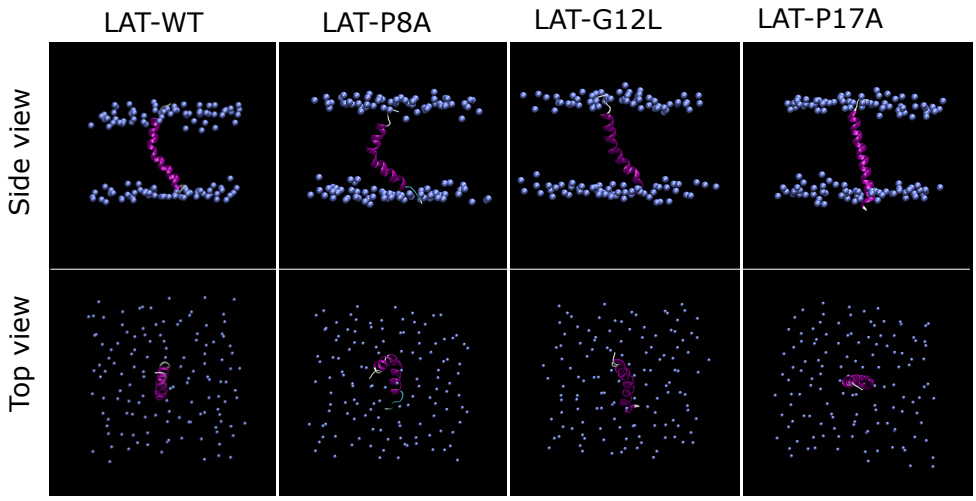
Supplementary Movie 4. The last 500 ns of the 1 µs-long MD simulation of the LAT P17A peptide in POPC bilayer, related to Figure 1b. Side view (parallel to the bilayer midplane) of the simulation box is shown. The video was created as described for Movie 1.

A

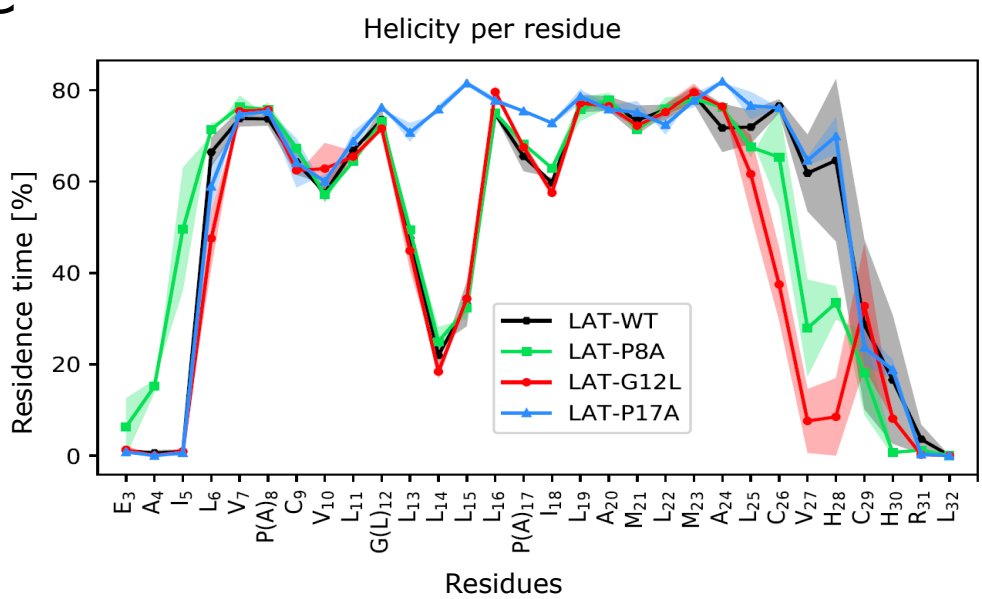
Human	M	E	E	A	I	L	V	P	C	V	L	G	L	L	L	L	P	I	L	A	M	-	L	M	A	L	C	V	H	C	H	R	L	P...
Gorilla	M	E	E	A	I	L	V	P	C	V	L	G	L	L	L	L	P	I	L	A	M	-	L	M	A	L	C	V	H	C	H	R	L	P...
Bonobo	M	E	E	A	I	L	V	P	C	M	L	G	L	L	L	L	P	I	L	A	M	-	L	M	A	L	C	V	H	C	H	R	L	P...
Bat	M	E	A	A	I	L	L	P	L	A	L	G	L	L	L	L	P	L	L	A	V	L	L	M	A	L	C	V	R	C	R	E	L	P...
Mouse	M	E	A	D	A	L	S	P	V	G	L	G	L	L	L	L	P	F	L	V	T	L	L	A	A	L	C	V	R	C	R	E	L	P...
Rat	M	E	A	D	A	L	S	P	V	E	L	G	L	L	L	L	P	F	V	V	M	L	L	A	A	L	C	V	R	C	R	E	L	P...
Dog	M	E	A	V	V	L	I	P	Y	M	L	G	L	L	L	L	P	L	L	A	V	-	-	-	V	L	C	V	R	C	R	E	L	P...
Sheep	M	E	A	V	S	L	P	V	Y	V	L	G	P	L	L	L	P	L	L	A	V	L	L	M	A	L	C	V	R	C	R	E	L	P...
Horse	M	E	A	A	I	L	I	P	S	V	L	G	P	L	L	L	P	L	L	A	V	L	L	M	A	L	C	V	R	C	R	E	L	P...
Cattle	M	E	A	V	S	P	A	V	Y	V	L	G	P	L	L	L	P	L	L	A	V	L	L	M	A	L	C	V	R	C	R	E	L	P...
Rabbit	M	E	V	N	V	L	G	H	S	A	L	G	L	L	L	G	P	L	L	A	V	L	L	M	A	L	C	V	R	C	R	R	L	P...
Whale	M	E	A	V	S	L	V	L	F	V	L	G	L	L	L	L	P	L	L	A	V	L	L	M	A	L	C	V	R	C	R	E	L	P...
	*	*									*	*		*	*		*	:	:	.				.	*	*	*	:	*	:	.	*		

*positions which have a single, fully conserved residue
: conservation between groups of strongly similar properties
. indicates conservation between groups of weakly similar properties

B

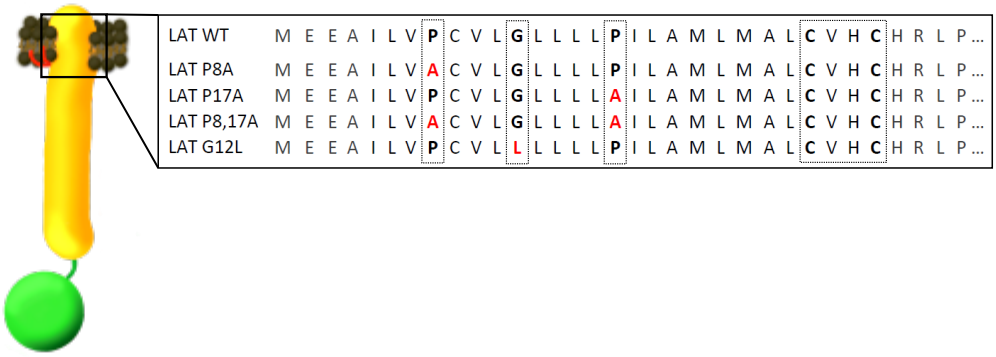


C



Glatzova 2020 Figure 1

A



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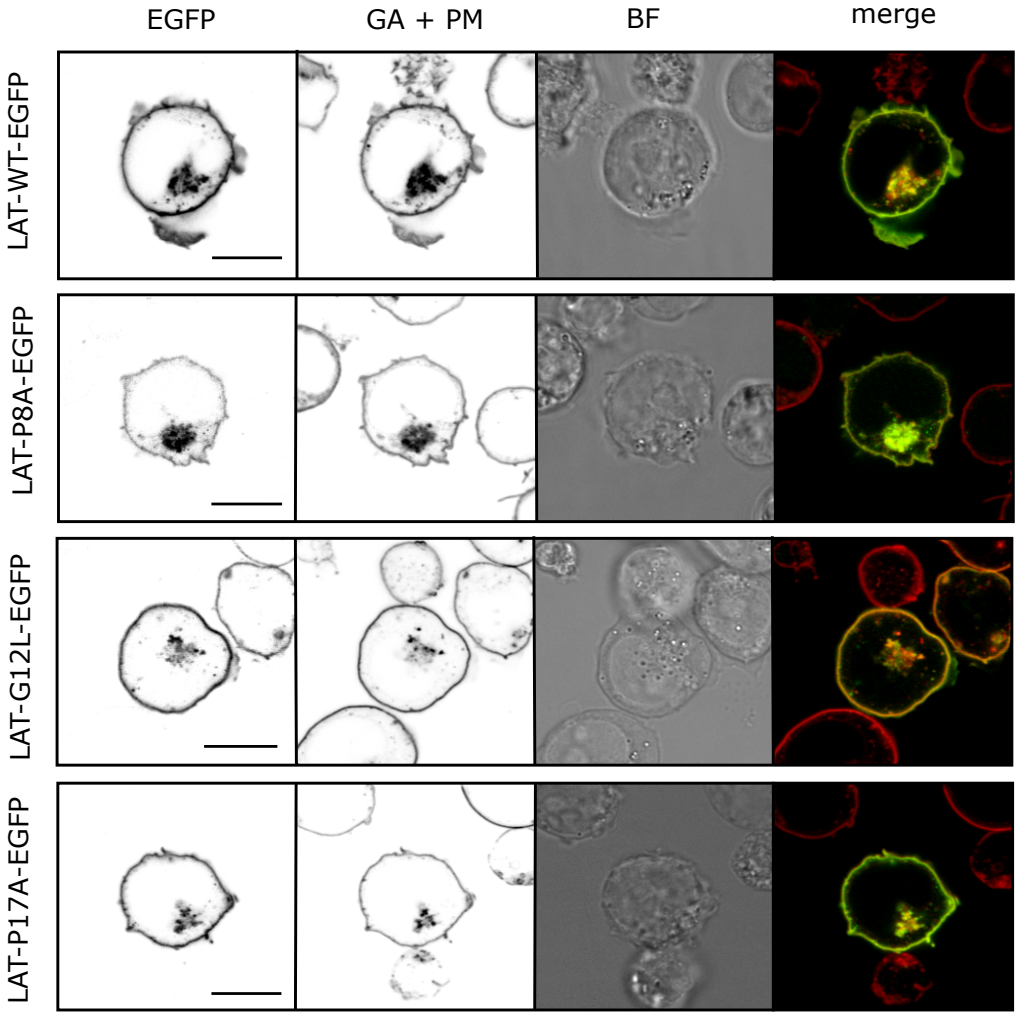
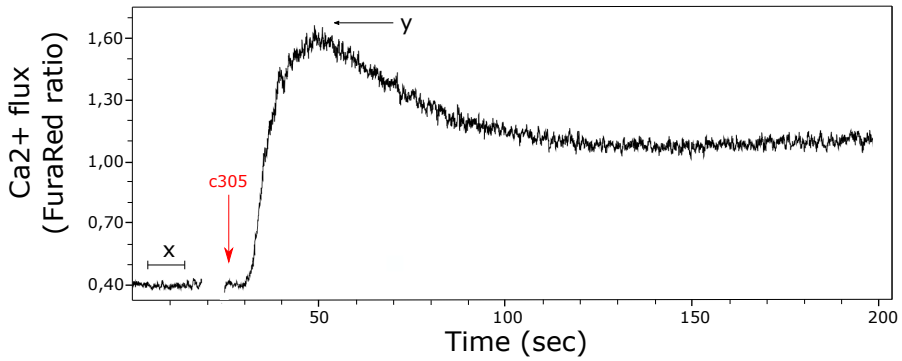


Glatzova 2020 Figure 2

A



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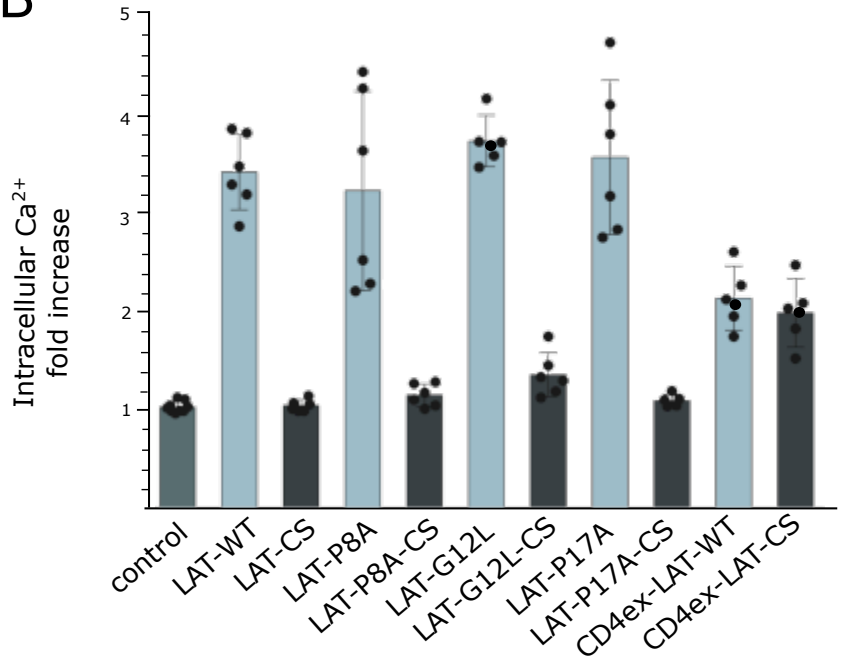
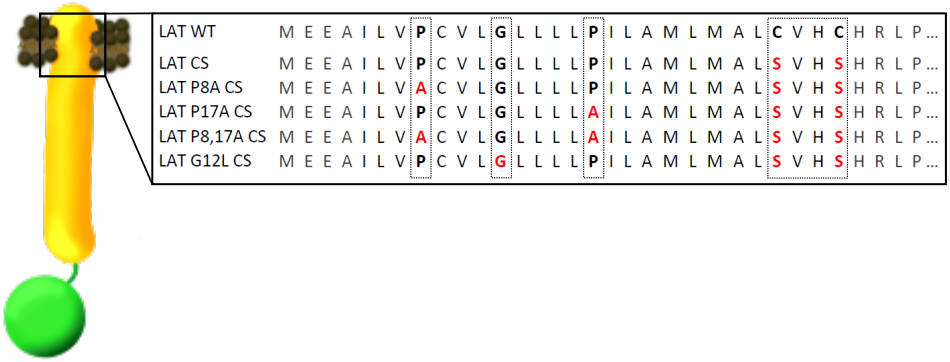


Glatzova 2020 Figure 3

A



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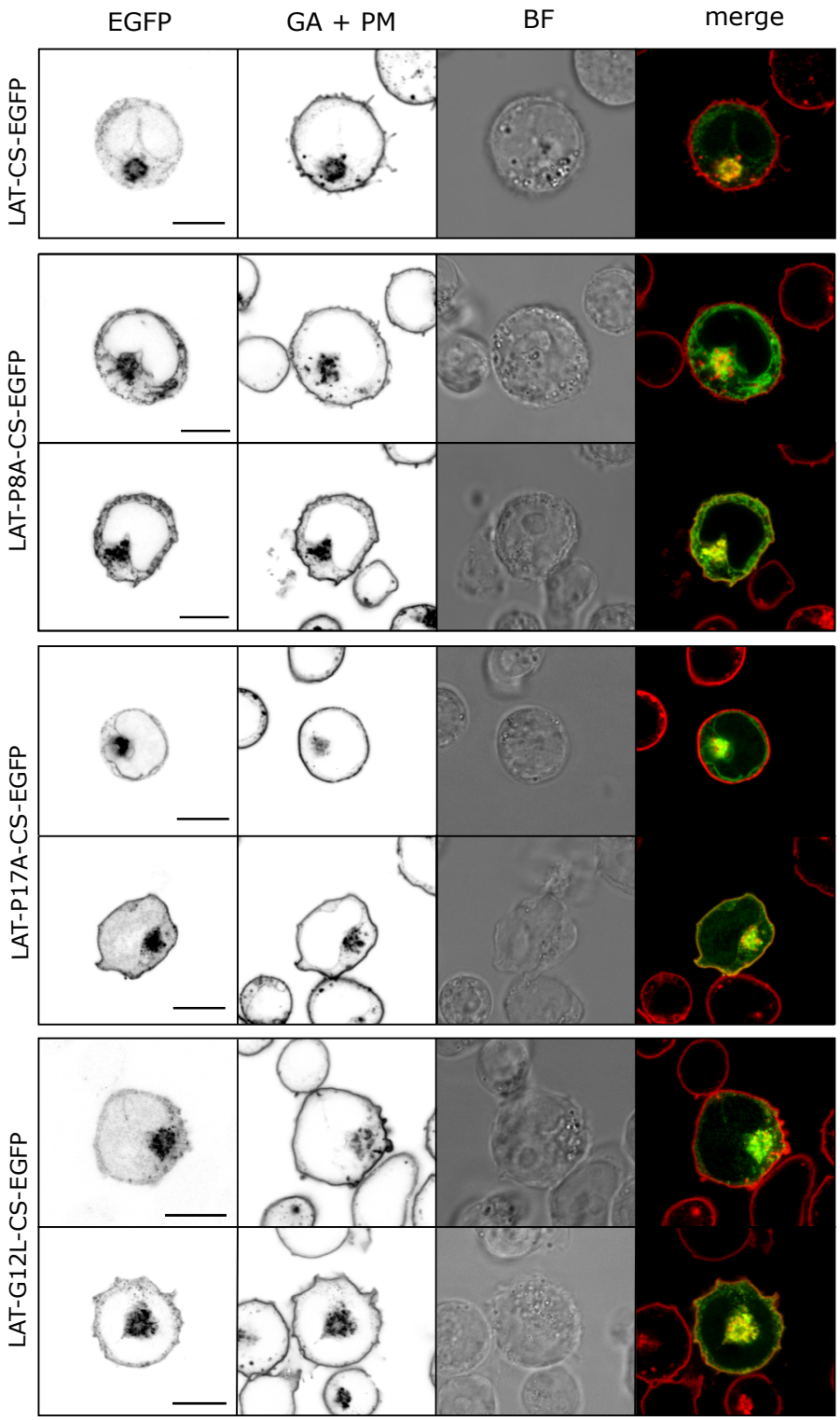
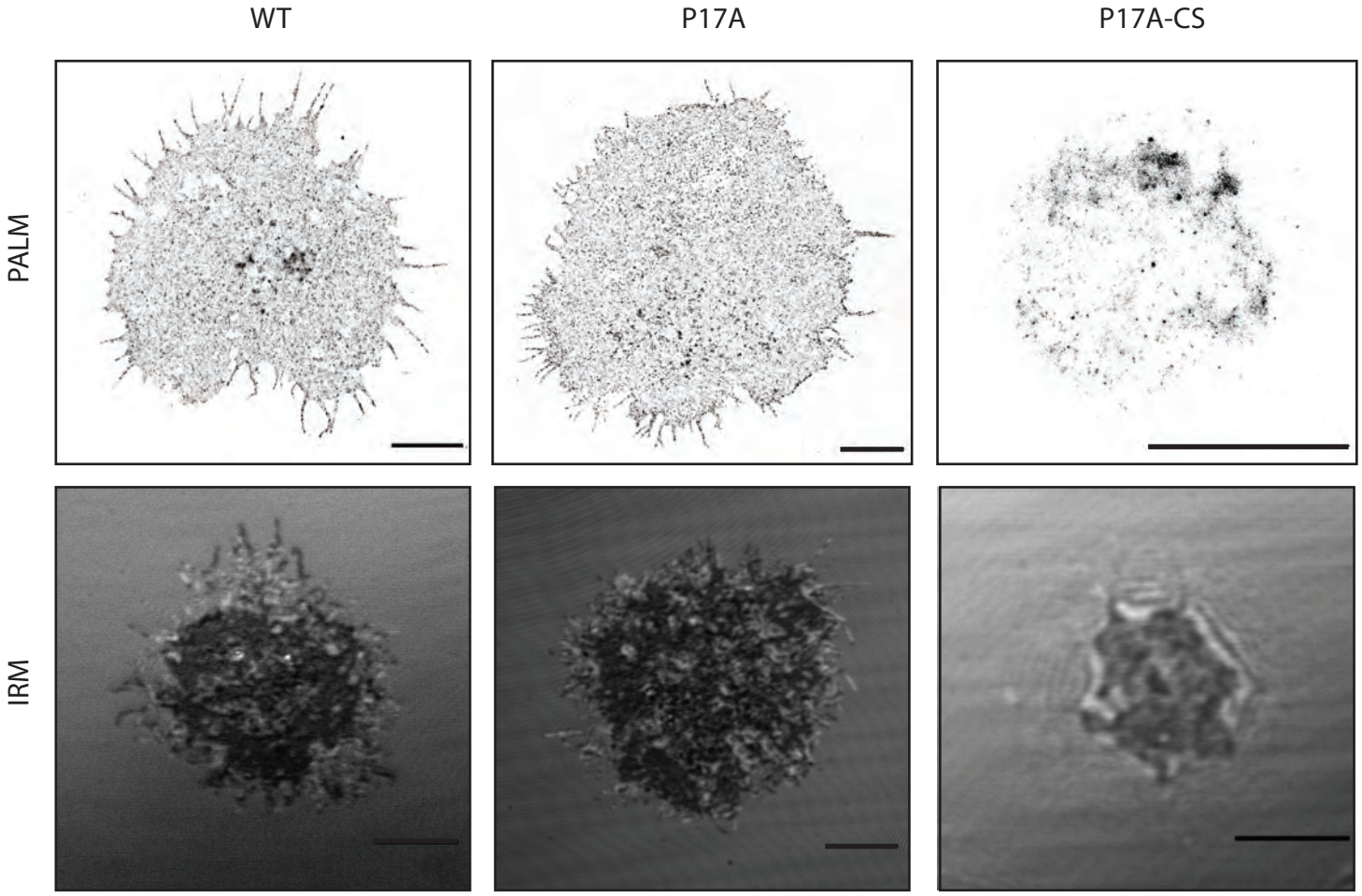


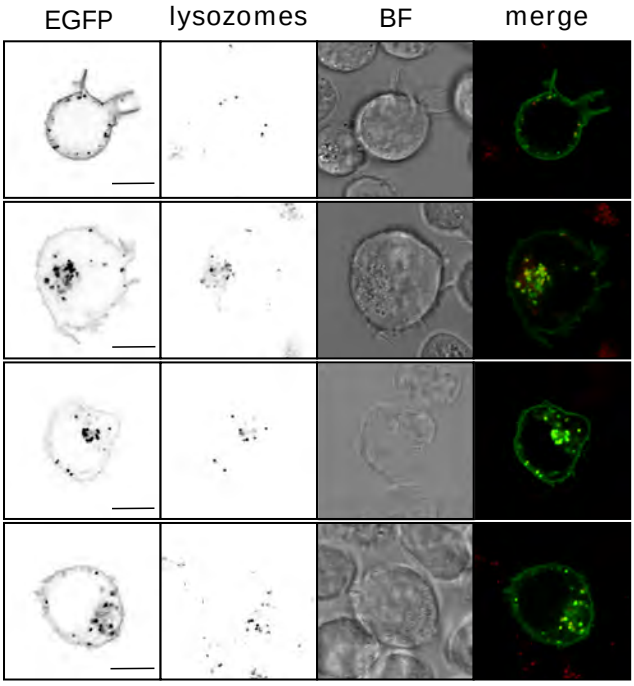
Glatzova 2020 Figure 4



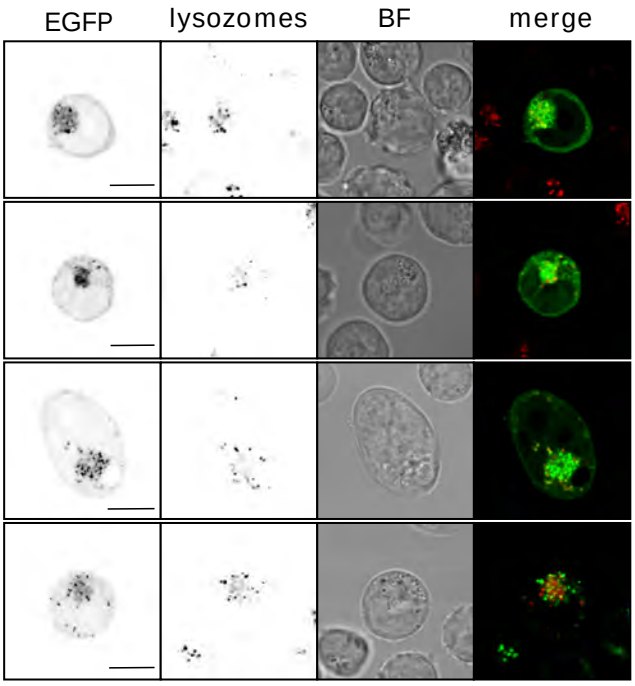
Glatzova 2020, Figure 5

A

LAT-6Leu-EGFP

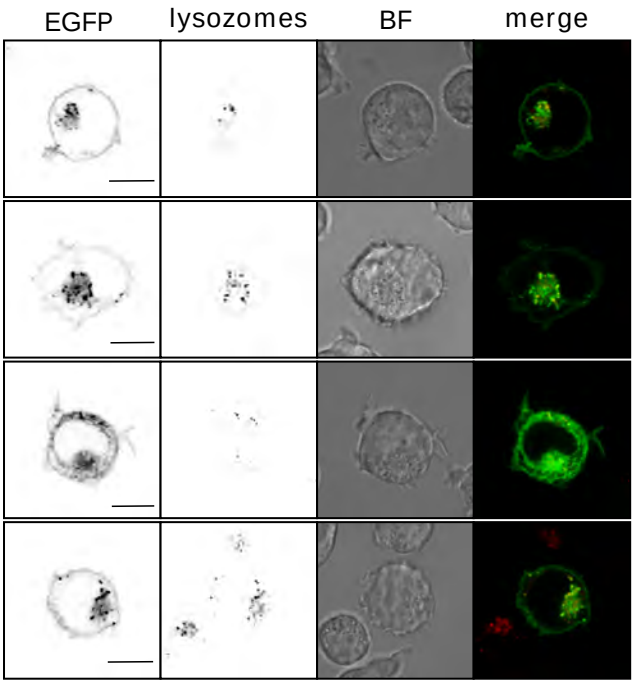


LAT-6Leu-CS-EGFP



B

LAT-PILAML-EGFP



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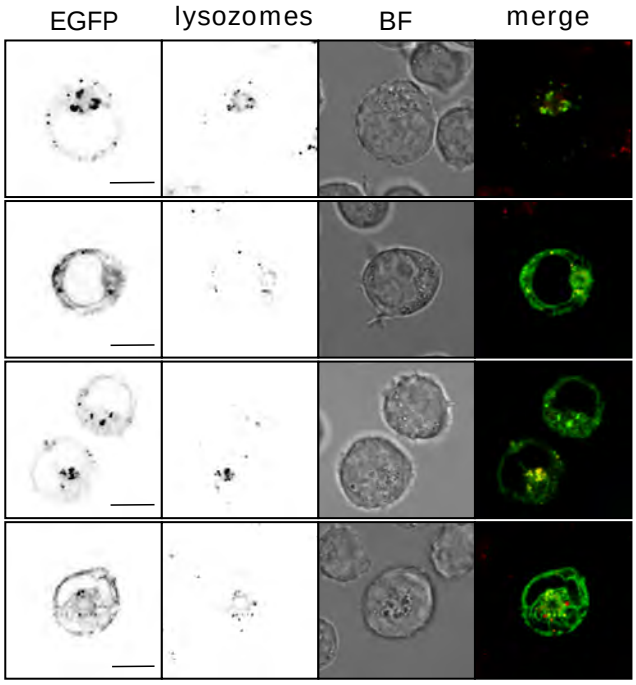
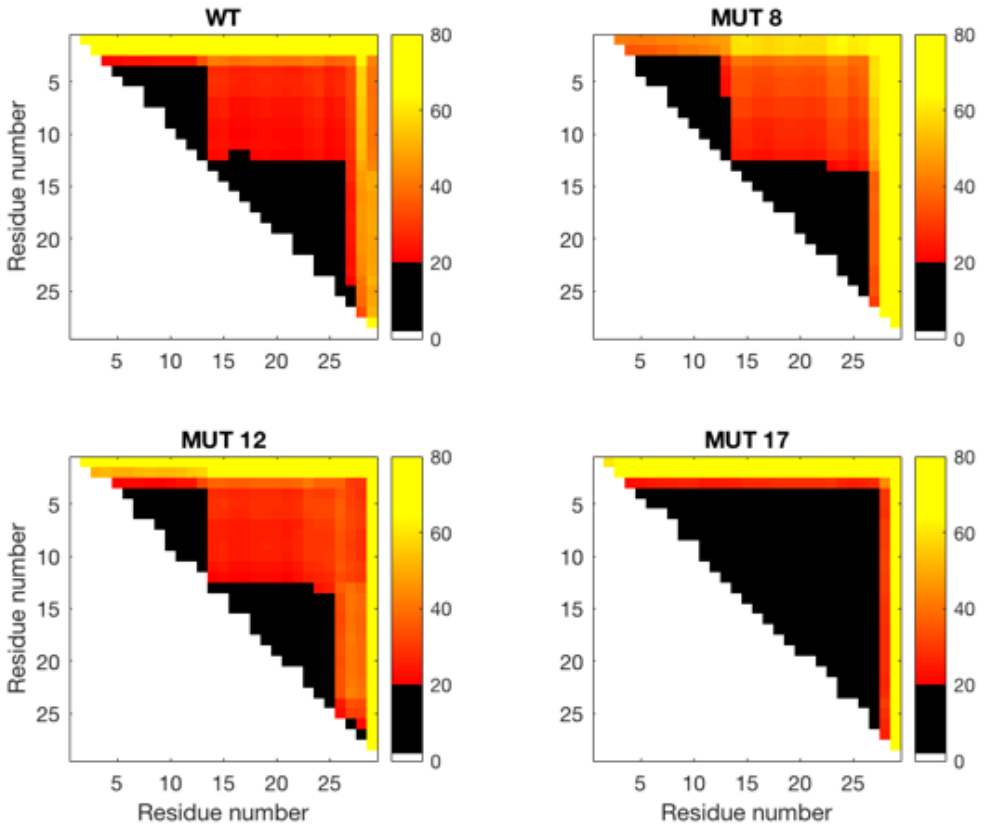


Glatzova 2020 Supplementary figure S1

A



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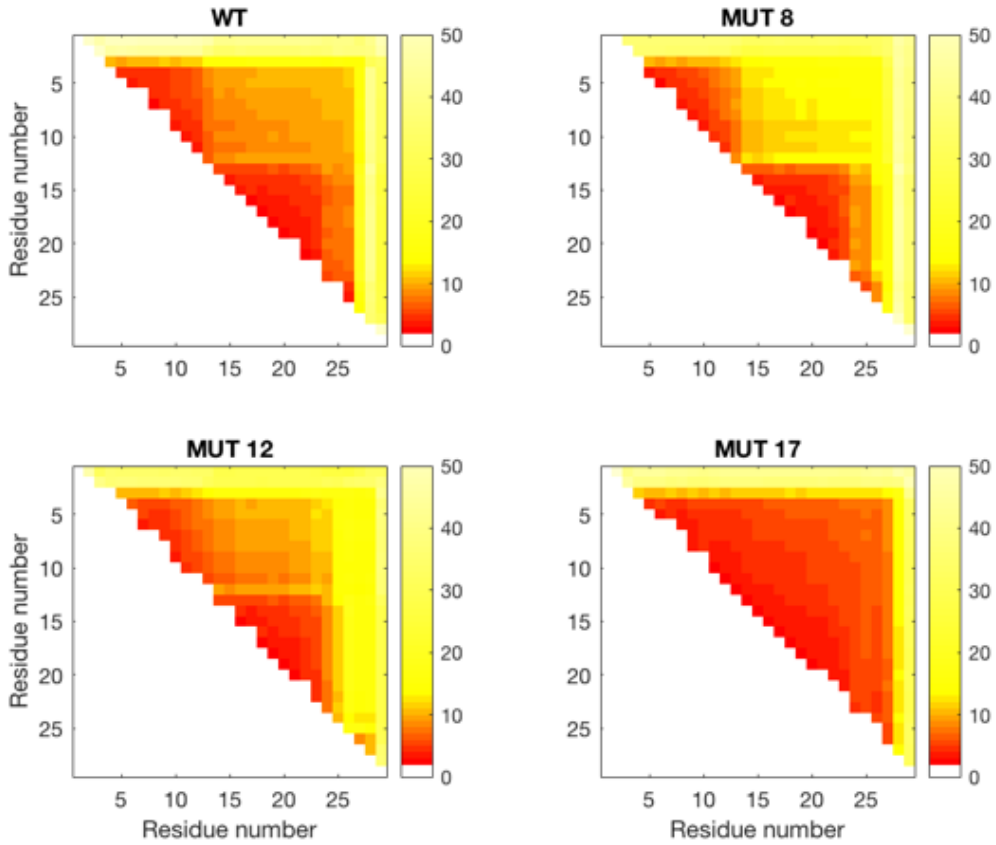
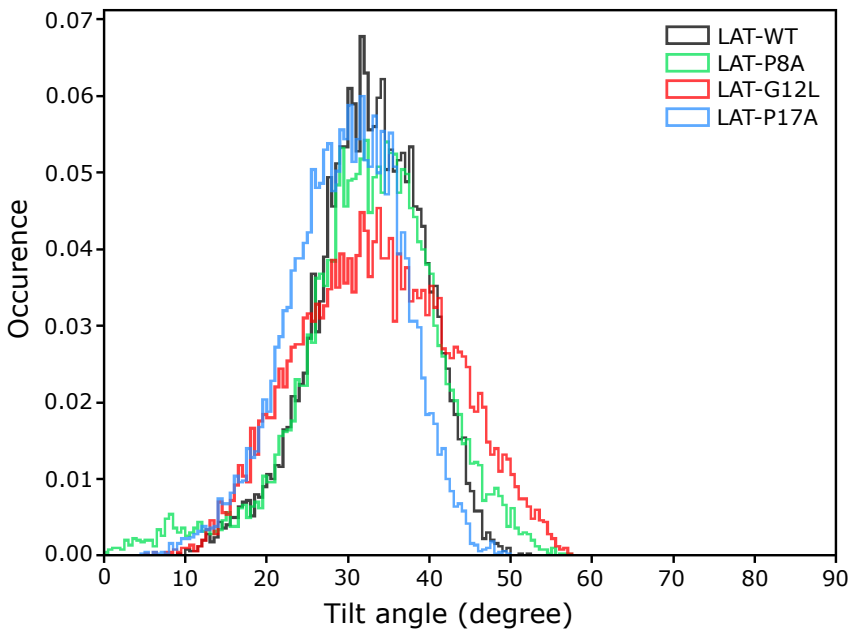


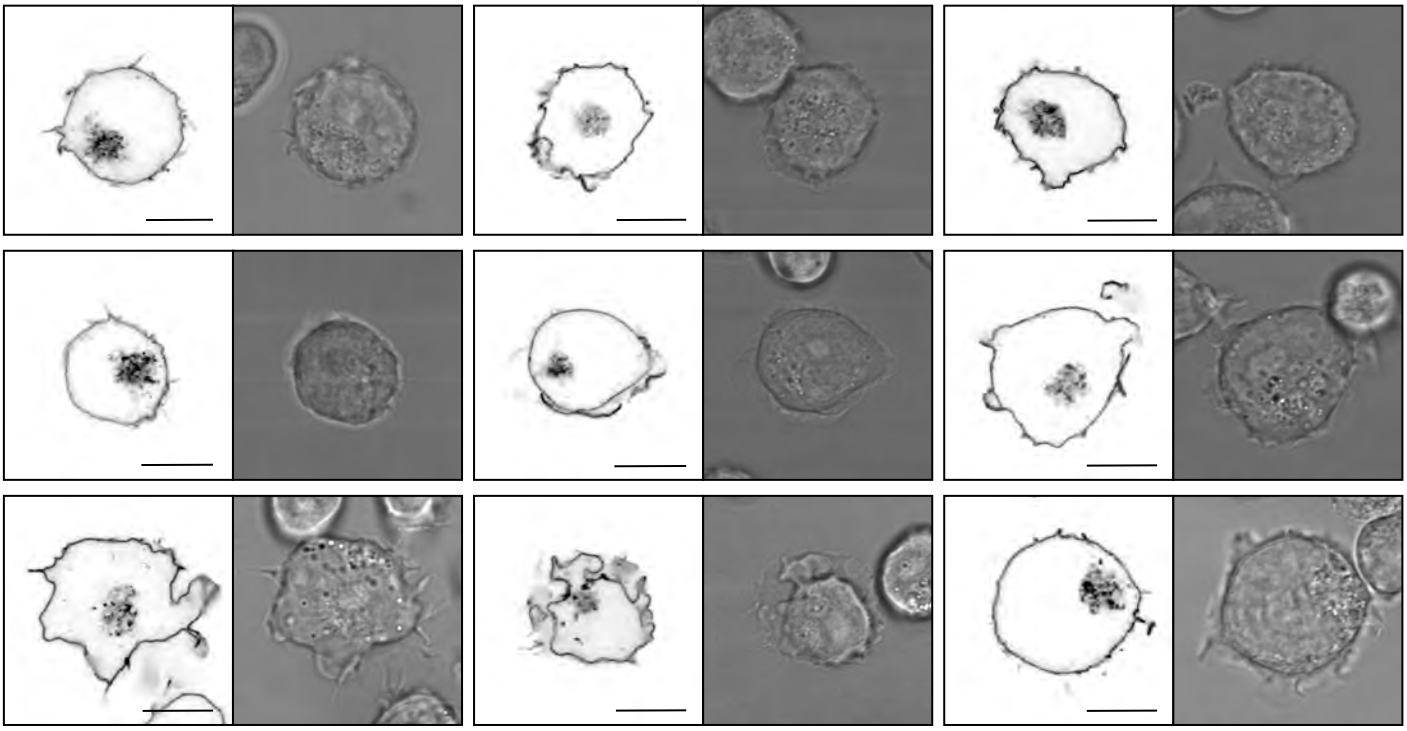
Glatzova 2020 Supplementary figure S2



Glatzova 2020 Supplementary figure S3

A

LAT-WT-EGFP



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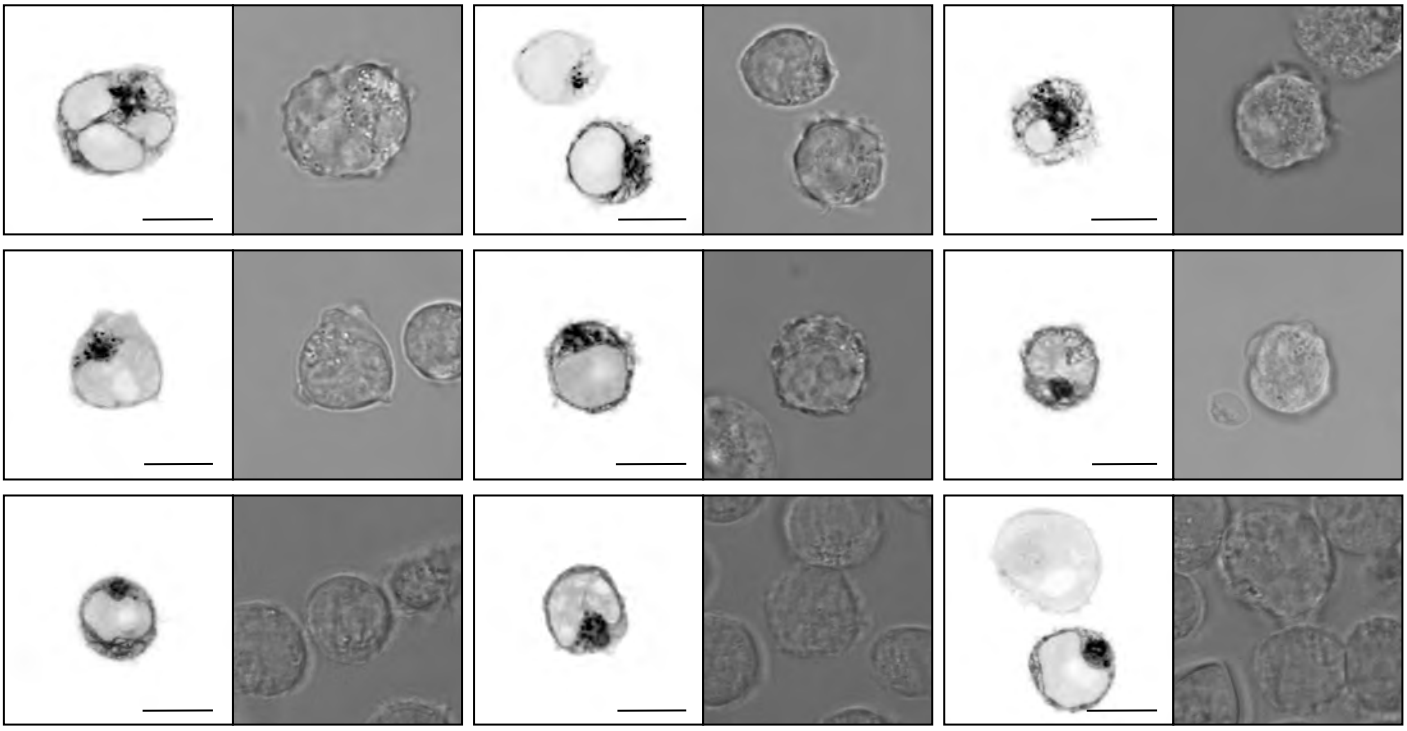
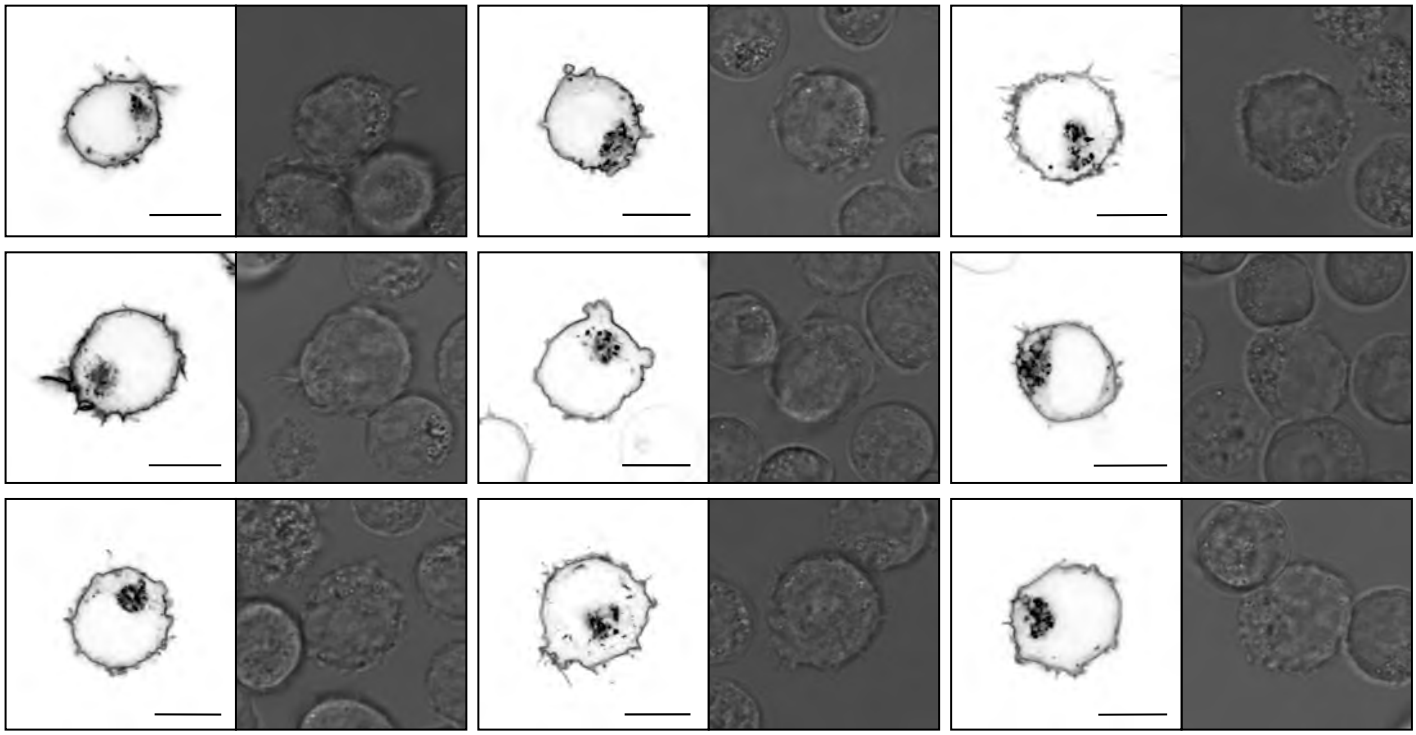
LAT-CS-EGFP



A

LAT-P8A-EGFP



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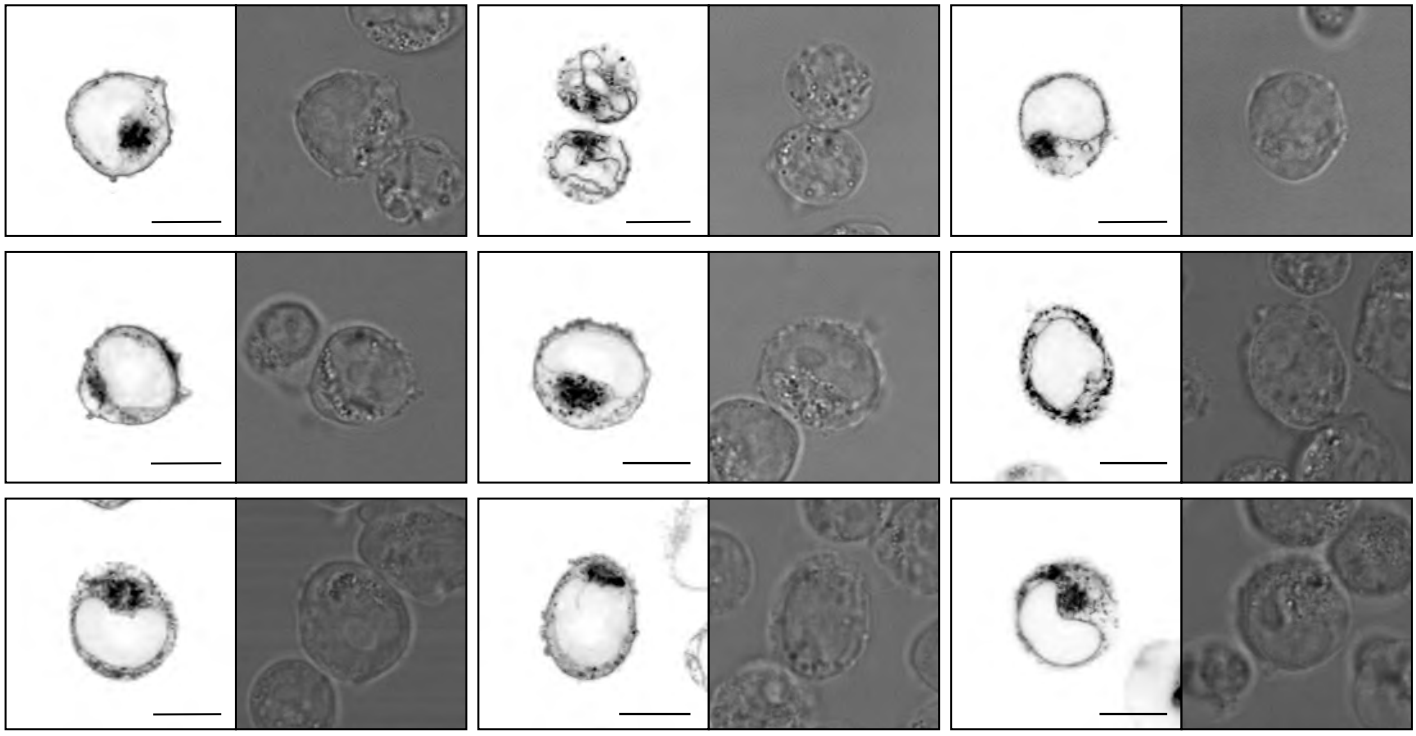
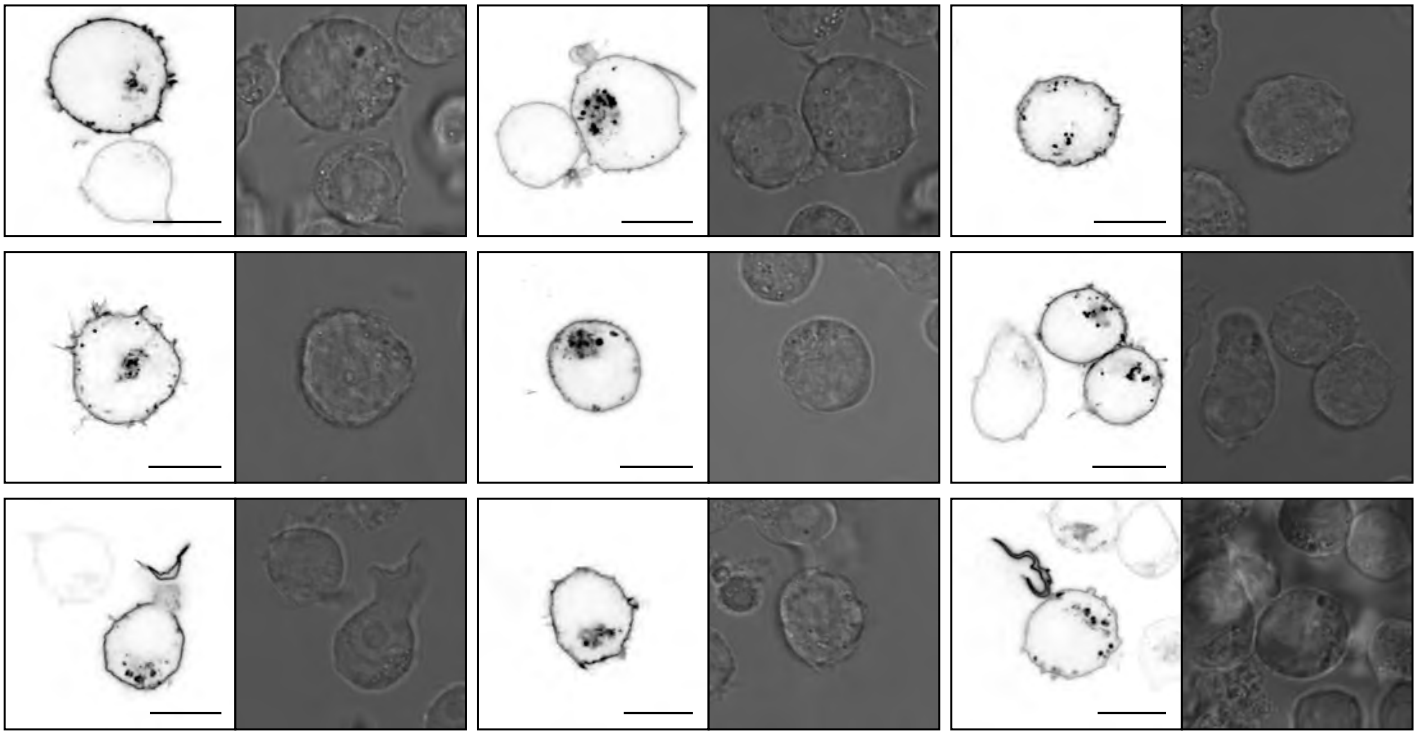


A

LAT-G12L-EGFP



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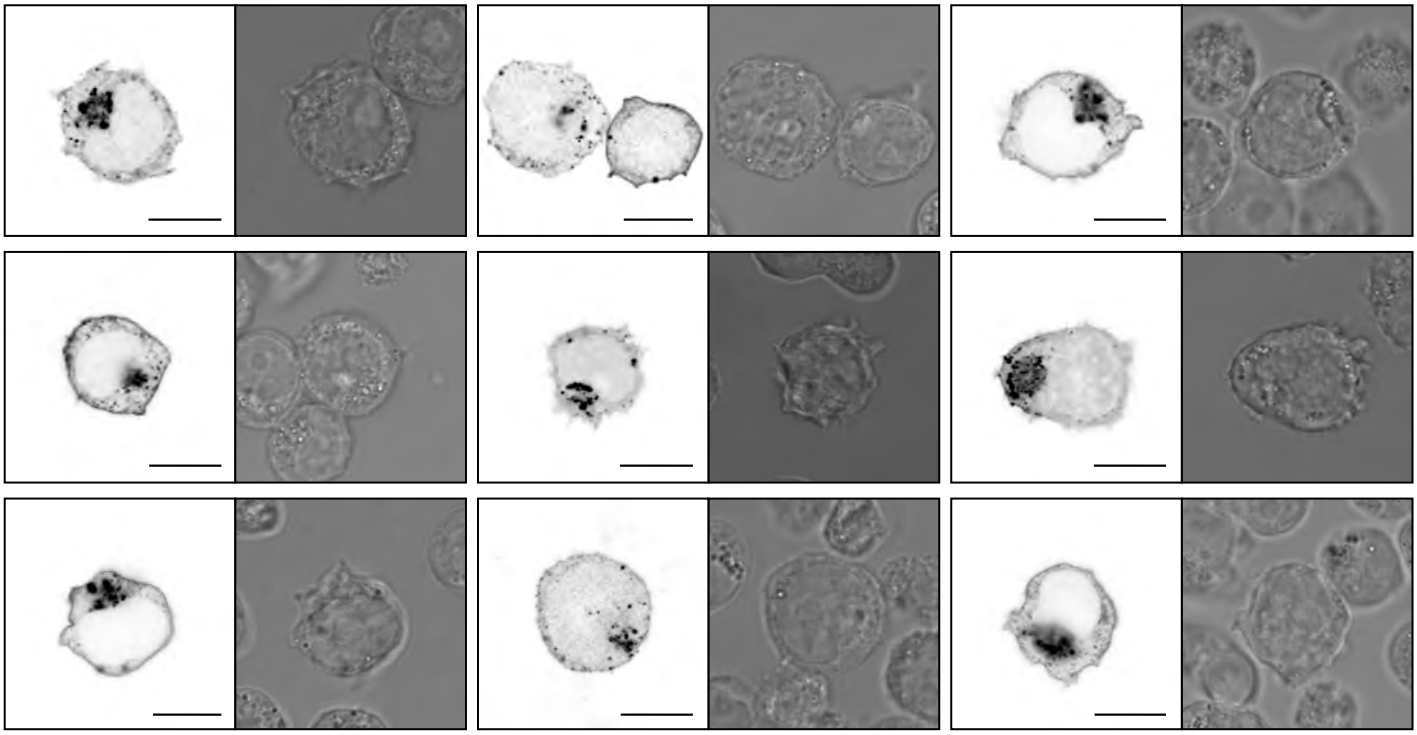
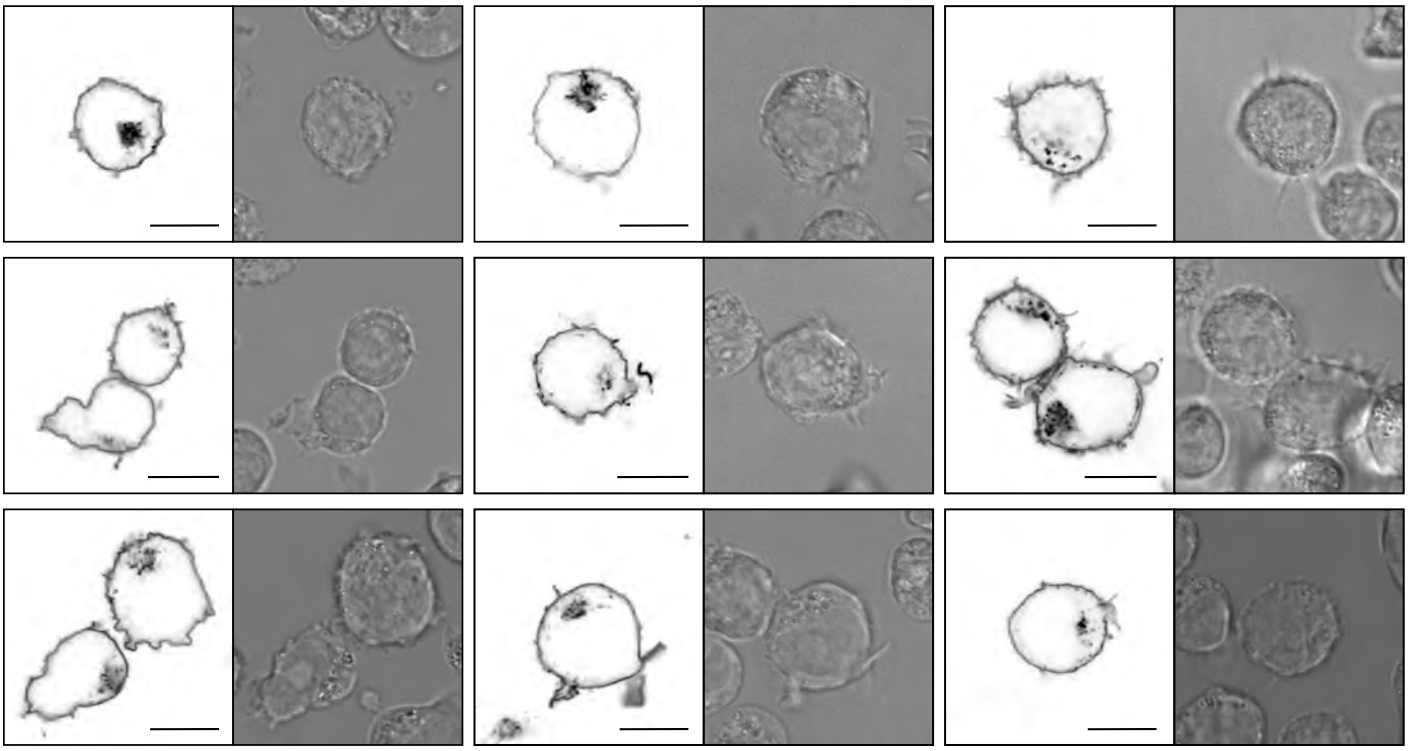


Glatzova 2020 Supplementary S6

A

LAT-P17A-EGFP



B

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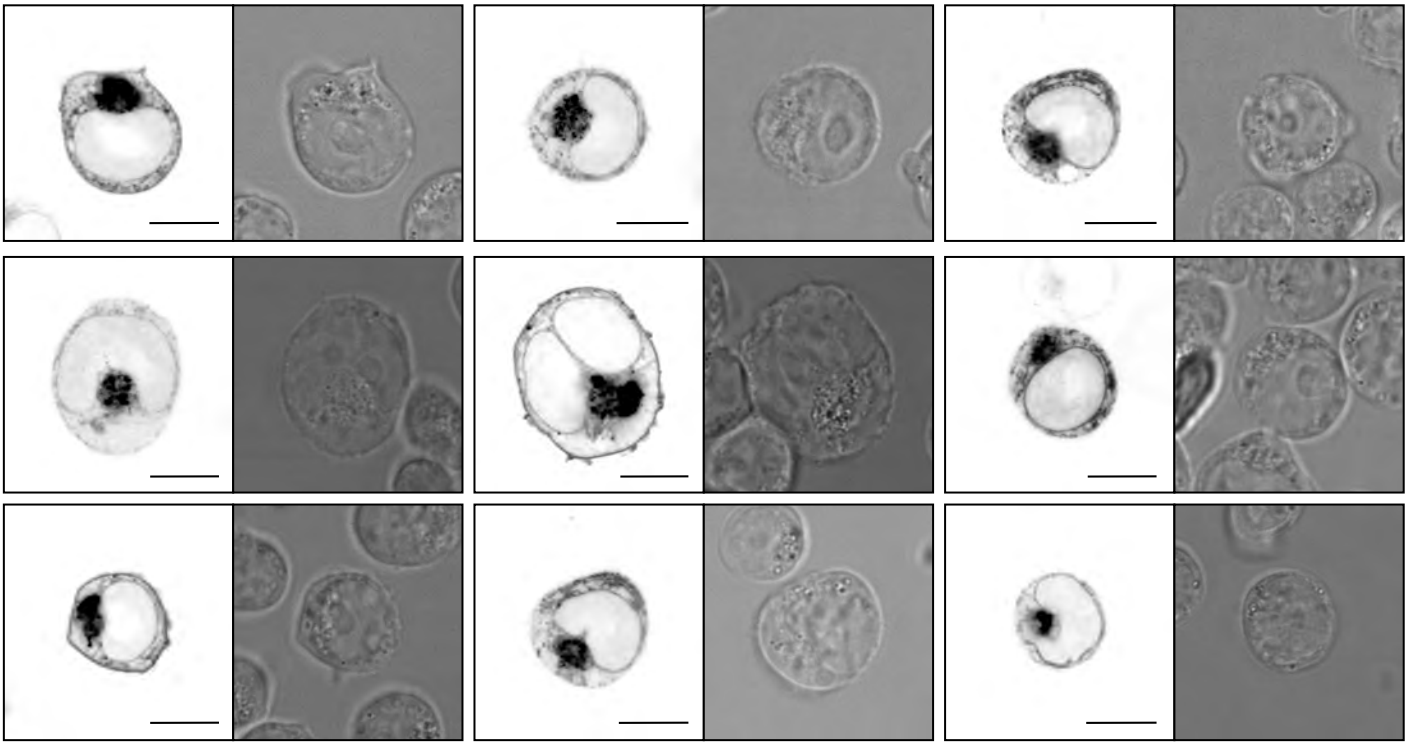
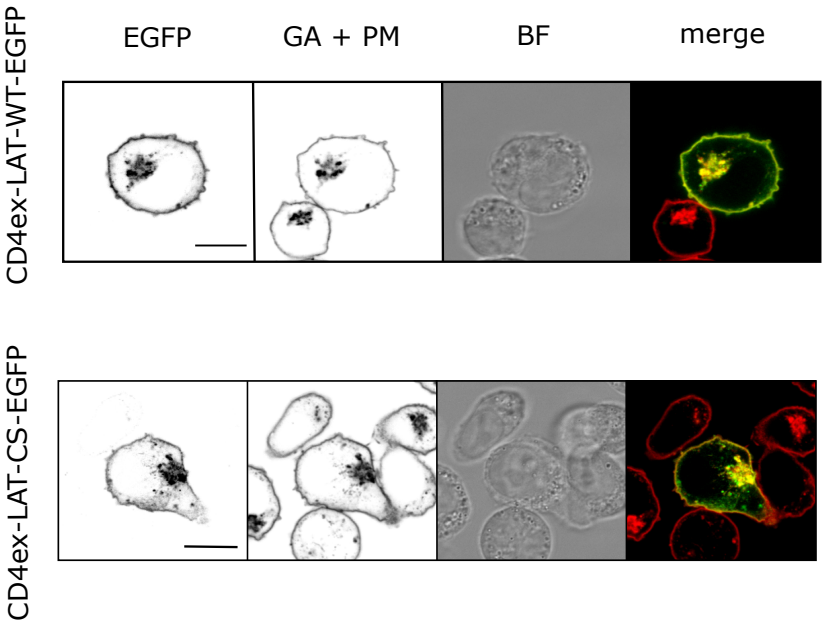
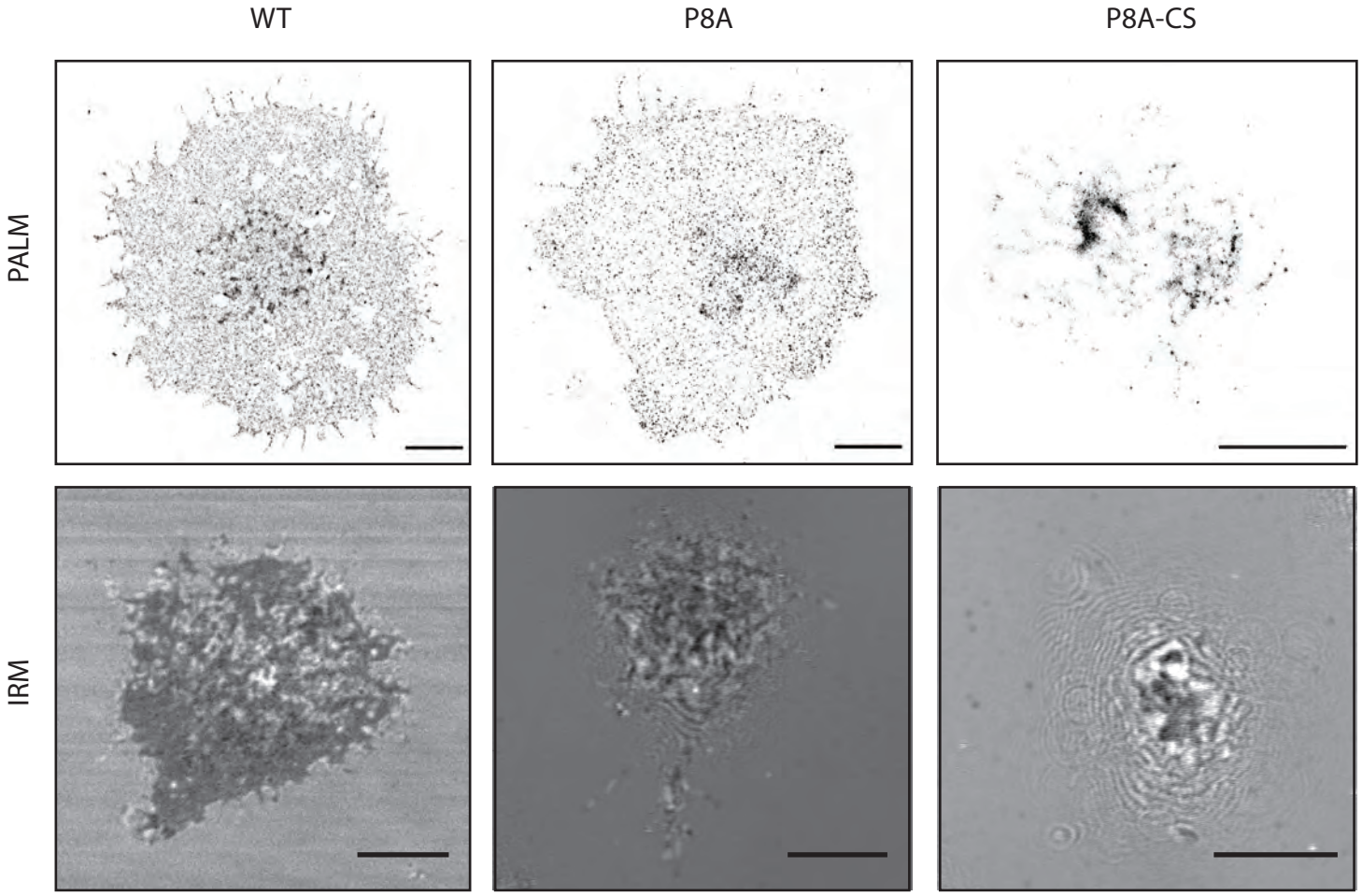


Glatzova 2020 Supplementary S7



Glatzova 2020 Supplementary figure S8



Glatzova 2020, Supplementary Figure S9