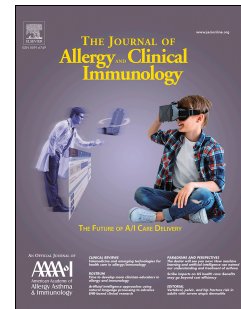


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MUTATION IN *KARS*: A NOVEL MECHANISM FOR SEVERE ANAPHYLAXIS

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ABSTRACT

Background: Anaphylaxis is a severe allergic reaction that can be lethal if not treated adequately. The underlying molecular mechanisms responsible for the severity are mostly unknown. **Objective:** This study is based on a clinical case of a patient with extremely severe anaphylaxis to paper wasp venom. This patient has a mutation in the *KARS* gene, which encodes Lysyl-tRNA synthetase (LysRS), a moonlight protein with a canonical function in protein synthesis and a non-canonical function in Ag dependent-FcεRI activation in mast cells. In this study, we characterized the mutation at the molecular level.

Methods: Analysis of the *KARS* mutation was carried out using biochemical and functional approaches, cell transfection, western blot, confocal microscopy, cell degranulation, PGD₂ secretion and proteases gene transcription. Structural analysis using Molecular Dynamics Simulations and Well-tempered Metadynamics were also performed.

Results: The mutation found, P542R (proline was replaced by arginine at aminoacid 542) affects the location of the protein as we show in biochemical and structural analyses. The mutation resembles active LysRs and causes a constitutive activation of the Microphthalmia transcription factor (MITF) which is involved in critical mast cell functions such as synthesis of mediators and granule biogenesis. Moreover, the structural analysis provides insights on how LysRS works in mast cell activation.

Conclusion: A link between the aberrant LysRS P542R function and mast cell exacerbated activation with increase of pro inflammatory mediator release after Ag-IgE dependent response could be established.

Clinical Implication: Our study highlights a signalling pathway: IgE-LysRS-Microphthalmia transcription factor (MITF) that can set the degree of severity of anaphylaxis.

Capsule summary: Mutation in *KARS* leads to an increase in MITF activity and mast cell release of proinflammatory mediators. Dysregulation of the *KARS*-MITF pathway can be an underlying mechanism resulting in anaphylaxis.

Key words: anaphylaxis, lysyl-tRNA synthetase (LysRS), *KARS*, Microphthalmia transcription factor, mast cells.

Abbreviations: LysRS: Lysyl-tRNA synthetase, MITF: Microphthalmia transcription factor, PGD₂: Prostaglandin D₂.

INTRODUCTION

Mast cells are the effector cells in anaphylaxis, a severe allergic reaction that is rapid in onset and includes signs and symptoms that involve the skin, gastrointestinal track, respiratory system and cardiovascular system ¹. The most severe form of anaphylaxis is anaphylactic shock, which is characterized by hypotension and can cause death. Anaphylaxis can be caused by several circumstances: allergies to foods, insect venoms, drugs... In the last decades the incidence of anaphylaxis has risen at an alarming rate and continues to rise ². Therefore, understanding the molecular basis of anaphylactic shock is crucial for developing new biomarkers and effective prevention and treatment.

In this study, in a patient suffering from extremely severe anaphylaxis due to paper wasp venom allergy, manifested as immediate cardiovascular collapse (refractory hypotension and loss of consciousness), we detected a mutation in one of the two copies of the *KARS* gene that caused an amino acid change in position 542 of the cytosolic form of Lysyl-tRNA synthetase, from a Proline to an Arginine.

Lysyl-tRNA synthetase (LysRS) belongs to the Aminoacyl-tRNA synthetases (ARSs) group. ARSs play key roles in charging specific tRNAs with cognate amino acids and establish the genetic code rules enabling protein translational fidelity and cellular integrity. Particularly, LysRS is the enzyme that charges Lysine to its specific tRNAs. It has two main differentiated domains, an aminoacylation domain found in the C-terminus and a tRNA anticodon-binding domain in the N-terminus. It is encoded by the *KARS* gene located on chromosome 16. LysRS has a cytosolic isoform of 597 amino acids and a longer mitochondrial isoform of 625 amino acids generated by an unusual alternative mRNA splicing ³. The cytosolic LysRS forms dimers that are bound to a p38 molecule of the Multi-tRNA Synthetase Complex (MSC). Interestingly, human cytosolic LysRS has been recognized to participate in an alternative (moonlighting) function beyond translation, connecting it to immune responses. LysRS is phosphorylated at Serine 207 through the mitogen-activated protein

kinase (MAPK) pathway in stimulated mast cells ⁴. Small-angle X-ray scattering (SAXS) data suggested that this induces a conformational change of LysRS and causes the translocation of the enzyme to the nucleus, abandoning its role in translation. Once in the nucleus, LysRS becomes the major producer of diadenosine tetraphosphate (Ap4A) during mast cell activation ⁵. As a consequence, the Ap4A produced releases the Microphthalmia Transcription Factor (MITF) from its repressor Hint-1 ⁶. Therefore, LysRS controls MITF transcriptional activity ⁴. MITF is a basic-helix-loop-helix-leucine zipper (bHLH-Zip) DNA binding protein ⁷. The primary cell types affected in MITF-deficient mice are mast cells, osteoclasts, and melanocytes ⁸.

Regarding mast cells, MITF regulates the expression of many genes critical for mast cell activation including proteases, cytokine receptors, and cell adhesion molecules ^{9,10,11,12}. MITF is also responsible for the biosynthesis of mediators such as Prostaglandin D2 (PGD₂), a mast cell specific eicosanoid, derived from arachidonic acid ¹³. PGD₂ has bronchoconstricting and vasoactive properties ¹⁴. Recently, it has been reported that MITF controls IgE/mast cell-mediated anaphylaxis by regulating histamine production by regulating histidine decarboxylase (HdC) ¹⁵.

In this study, we analysed, for the first time, the structure and dynamics of LysRS and its translation to a transcription functional mechanism at a molecular level and in addition the structure and function of a mutation in one of the two copies of the *KARS* gene that caused an amino acid change in position 542 of the cytosolic form of LysRS, from a Proline to an Arginine. Our data shows that this mutation resembles active LysRs and causes a constitutive activation of MITF which results in an increase of degranulation and proinflammatory mediator release after IgE-dependent activation.

Our study explains for the first time the switching mechanism from translation to transcription of LysRS at a molecular level, and why specific mutations in the *KARS* gene deregulate its functional mechanism. Our data indicates that dysregulation of the *KARS*-MITF pathway can be an underlying mechanism resulting in anaphylaxis.

METHODS

Patient characteristics and allergological work up

The index patient was a 29 year-old male from a rural area north of Barcelona (Spain) referred to the Hospital Clinic, Barcelona, Spain for recurrent anaphylactic shocks after paper wasp stings. He worked as a farmer and had a history of several paper wasp, yellow jacket and honeybee stings with good tolerance, until 2016 when he presented the first anaphylactic episode. Reactions were characterized by their severity (refractory hypotension and loss of consciousness), rapid onset (less than 3 minutes after a single sting) and such fast progression that the patient was unable to use his epinephrine auto injector.

Allergological work-up (see supplementary information) confirmed wasp allergy (*Polistes* spp). During skin prick tests, after performing intradermal injection with *Polistes* spp venom, the patient suffered an anaphylaxis that required repeated doses of epinephrine. This episode was milder than the original reactions with no hypotension or cardiovascular symptoms, but with palm and sole itching, dyspnoea, and impending doom. Baseline serum tryptase (ImmunoCAP (ThermoFisher, Uppsala, Sweden)) was within the normal range and did not increase during anaphylaxis. However, serum PGD2 (ELISA PGD2-MOX (Cayman Chemicals, Michigan, USA)) values increased 2.7-fold during anaphylaxis (Suppl. Table 1).

Despite having a normal baseline serum tryptase (3.62 ng/mL), REMA score¹⁶ was 4, meaning a high probability of clonal mast cell disease. Thus, a bone marrow study was performed, including immunophenotypical analysis for detection of somatic activating exon 17 KIT mutations (D816V). Genomic DNA was extracted from peripheral blood, bone marrow and purified cell populations using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). KIT mutational status was carried out by the allele-specific oligonucleotide real-time quantitative PCR method that allows quantification of as little as 0.003% KIT

D816V mutation-positive cells¹⁷. KIT D816V mutation was not identified and no morphological abnormalities or CD25/CD2 aberrant expression in mast cells were found.

Because of the loss of a daughter at a few months of age, the patient and his partner received genetic counselling. Both parents were found to have missense mutations in the KARS gene in heterozygosity that caused an amino acid substitution in the LysRS protein. The KARS mutation was identified by performing exome sequencing –next generation sequencing (MacroGen). Moreover, the exome analysis further confirm no mutations in KIT.

Molecular Dynamics Simulations

See Supplemental information

Well-Tempered Metadynamics

See Supplemental information

Plasmid construction

Human LysRS (*KARS* WT) was used for the production the LysRS mutant by site-directed mutagenesis in which proline was replaced by arginine at position 542 (*LysRS* P542R). Human LysRS WT and P542R variant were sub-cloned into the KpnI and NotI sites of the pcDNA 3.1+C-eGFP vector and the fidelity of all constructs was verified by direct sequencing (GenScript Biotech, Netherlands).

Mutant RBL-2H3 cell transfectant generation

RBL-2H3 was used as a mast cell model. Cells were cultured as described elsewhere¹⁸. RBL-2H3 cells were transfected with *KARS* WT or *P542R* or *G189D* constructs with Lipofectamine (Invitrogen) following manufacturer's instruction. Neomycin antibiotic G418 (Gibco) and sorting with FACS ARIA was performed to select transfectants. Cells were finally checked with FACS CALIBUR for GFP and FcεRI expression.

Flow cytometry

A total amount of 10^4 cells/sample were acquired using a FACSCalibur flow cytometer (Beckton-Dickinson) and the data were collected with CellQuest Alias and analysed with FlowJo V10 software. The antibodies used were: mouse primary antibody anti-rat FcεRI mAb (BD Pharmingen, San Diego, CA) and secondary antibody anti-mouse Alexa 546 (BD Biosciences).

Confocal microscopy staining and analysis

Cell transfectants were initially seeded at 10^5 cells/well on cover slips. The following day, cells were fixed with PFA 4% for 15 minutes at 4°C and then nuclei were stained with Hoechst for 5 minutes. Cells were washed and cover slips were placed on microscope slides using Prolong Gold antifade reagent. Other assays included Lysotracker Red DND-99 (Molecular Probes/L7528) for 2 hours at 37°C, prior to fixation. After, confocal microscopy images were taken with a Leica SP5 confocal microscope. Images were then analysed using the Fiji/Image J software.

SDS-PAGE acrylamide gel electrophoresis and Western Blot

Samples were obtained as cellular fractions from $5 \cdot 10^6$ cells, using a centrifuge-based fractionation method¹⁹. The samples were quantified for protein using a Bradford technique and were then loaded accordingly into NuPAGE™ 4-12% Bis-Tris Precast Protein Gels (Thermo Fisher Scientific). Western blot procedure was performed as described elsewhere¹⁸. Mouse anti-KARS (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) or Rabbit anti-Laminin B1 (Abcam, Cambridge, UK) or Mouse anti-tubulin (Sigma-Aldrich, St. Louis, MO) or pKIT (Tyr 719) (Cell Signalling Technology) antibodies were used for the immunoblot. Secondary antibodies: Goat anti-Rabbit HRP (Sigma) or Polyclonal Rabbit anti-mouse HRP (DAKO, Carpinteria, CA) were used.

β-hexosaminidase determination and Prostaglandin D₂ (PGD₂) ELISA.

The degranulation assay was performed as described previously¹⁸. Briefly, cells were seeded at $2.5 \cdot 10^4$ cells/well in 96 well plates and were sensitized with anti-DNP IgE overnight with indicated concentrations. Next day, after washing, cells were incubated with the stimuli DNP-HSA, Ionomycin and PMA or plain

medium (NS, not stimulated). Incubation was carried out at 37°C for 1 hour. β -hexosaminidase activity was measured in supernatants and the cell lysates as described¹⁸. The activity of the β -hexosaminidase enzyme was expressed as the percentage of the release: β -hexosaminidase release = [cell degranulation / (cell degranulation + total lysate)] x100.

PGD₂ release was determined from the supernatants of cells ($2.5 \cdot 10^4$ cells/well) activated as described above for 4 h using a specific competitive EIA for PGD₂ (Cayman Chemical) according to manufacturer's instructions.

Luciferase assay

Firefly luciferase under the control of TRPM1 promoter and control vector PGL3-Luciferase were a gift from David Fisher (Harvard Medical School)²⁰. 3XNFAT-luciferase was reported previously²¹. $6 \cdot 10^4$ cells were transfected with the Firefly and the Renilla reporters at a ratio of 7:1 respectively. Transfections were performed using the AMAXA program T20.

Luciferase activity was detected using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) following manufacturer's instructions. Firefly Luciferase data were normalised according to Renilla luciferase data.

Gene expression analysis in cell transfectants

See Supplemental information and Supplementary Table II.

Statistical analysis

All results are expressed as mean $\pm$ standard error of the mean (SEM). After determination of normal distribution of the samples and variance analysis, the unpaired student's t-test was used to determine significant differences (p value) between two experimental groups and two-way ANOVA with Bonferroni multiple comparison test was used to determine significant differences (p value) between several experimental groups. "*"P<0.05, "**" P<0.01, "***"P<0.001, "****"P<0.0001.

RESULTS

LysRS functional mechanisms. P542R mutant characterization.

As mentioned, a mutation in Proline 542 in LysRS was found in a patient with anaphylaxis to hymenoptera. Our first analysis through sequence alignment showed that Proline 542 is a well-conserved amino acid (Fig. 1A), which is placed at the interface between the two protein domains (Fig.1B), facing Ser207, which when phosphorylated triggers the opening of LysRS and, in turn, the release of LysRS from the MSC²². Mutations in this region, such as G540Y lead to the opening of LysRS²², suggesting that mutation of P542 to a quite different residue, such as arginine, may profoundly affect the binding of LysRS to the MSC and the entire functionality of the protein. To check this, we performed Molecular Dynamics (MD) simulations and Well-Tempered Metadynamics simulations of different LysRS systems: LysRS wild type system (LysRS-WT), LysRS where Ser207 was phosphorylated (LysRS-S207(P)) and LysRS carrying the P542R mutation (LysRS-P542R) (see Methods and Supplementary information).

Free energy profiles along the opening path (Figure 2A) obtained for the LysRS-WT and LysRS-S207(P) systems show a single minimum for LysRS-WT in the closed state, while opening is spontaneous for the LysRS-S207(P) system, a result that was reproduced by unbiased MD simulation (see Suppl. Information, Suppl. Fig. 1A and Suppl. Video 1), which confirmed the spontaneous opening induced by phosphorylation. This result agrees with qualitative estimates from small-angle X-ray scattering (SAXS) obtained by collecting spectra of the wild type and LysRS-S207D variant²². Our simulations demonstrate (see Fig. 2B)

that the major conformational changes occur at the N-terminal tRNA anticodon-binding domain which opens and moves away from the C-terminal aminoacylation domain, leading to a major global conformational rearrangement.

Mutation P542R is quite disruptive, as it introduces a positively charged residue at a region of the interdomain interface which is mainly non-polar (see Suppl. Fig 2A). In the closed state, arginine 542 interacts with both E150 and D313 (see Fig. 2C, closed state inset and Suppl. Fig. 2B). Interestingly, both residues are placed in the binding region with the MSC-p38 protein (see Suppl. Fig. 2C), and sequestering them should impact binding between LysRS and the MSC. On the other hand, in the opened state, the N-terminal tRNA anticodon-binding domain has moved far from the interdomain interface and residue R542 is found mostly exposed to the solvent and only interacts with residue D313 (see Fig. 2C, open state inset). Metadynamics simulations show that while only the “closed” state is populated for the unphosphorylated wild type protein, both open and closed states are populated (almost identically) for the LysRS-P542R system (see Fig. 2C, and Suppl. Video 2). Thus, the P542R mutation firstly alters the MSC-p38 binding region both in the closed and opened states by trapping one or two residues involved in the interaction and recognition of the MSC binding loop. Furthermore, the P542R mutation deregulates functional motions of LysRS by promoting the system to explore an open state that could unbind from the MSC triggering translocation to the nucleus. A behaviour that is only triggered in the LysRS-WT system by the phosphorylation of S207.

Transfectant generation and LysRS cellular location.

Next, we pursued the analysis of the functional consequences of this mutant in a mast cellular model. RBL-2H3 cell transfectants expressing LysRs WT-GFP or LysRS P542R-GFP were obtained after two consecutive sortings and cells were tested for FcεRI and GFP expression of the respective transfectants (Figure 3A). We checked endogenous expression of LysRs versus transfected Lysyl tRNA-synthetase-GFP (Figure 3B).

Next, we determined the cellular localization of the Lysyl tRNA-synthetase-GFP recombinant proteins, RBL-2H3 cells expressing the recombinant LysRs WT

gene or LysRS P542R by confocal microscopy. LysRs WT was expressed only in the cytoplasm as expected. As known, lysyl-tRNA synthetase is attached to the MSC in quiescent conditions. Interestingly, LysRS P542R (Figure 3C), was found mainly in the nucleus. We confirmed results by performing cell fractioning and western blot analysis. The whole cellular fraction indicates slightly lower levels of LysRS P542R compared with LysRS WT (Figure 3D). Nevertheless, in the cytoplasmic fraction *P542R* levels were severely diminished and most of the LysRS P542R was found in the nucleus (Figure 3D).

LysRs mutant P542R enhances degranulation and PGD₂ synthesis.

Having established the protein localisation, we proceeded to check whether IgE-dependent mast cell activation was altered. Cell transfectants were sensitized with the indicated concentrations of anti-DNP IgE overnight. The following day cells were stimulated for 1 hour with 50ng/ml of the antigen DNP-HSA or Ionomycin plus PMA (1 μ M+50ng/ml respectively). Our data shows that degranulation is enhanced in P542R compared with WT. Both transfectants have the same ability to degranulate after a general stimulus such as Ionomycin and PMA (Figure 4 A). Interestingly, when we use lower doses of ionomycin we observe an increase of degranulation in P542R cells, suggesting that this mutation may affect IgE independent signals (Figure 4B). Afterwards, IgE-dependent eicosanoid production was also assessed. (Figure 4 C). Prostaglandin D₂, a hallmark of *de novo* mediators' synthesis in mast cell granules, was analysed by ELISA. Our data shows that PGD₂ was significantly enhanced in P542R. In both cases the basal levels (non-stimulated conditions) of β -hexosaminidase and PGD₂ release were similar in WT and P542R indicating that proinflammatory mediators are released upon IgE crosslinking. This data agrees with the increase in PGD₂ found in the patient carrying the mutation (Supp. Information, Suppl. Table I).

Granule analysis in cell transfectants

To assess whether there was any difference in the size and amount of the granules in both transfectants, two different assays were performed (Figure 5). In both experiments, cells were stained with LysoTracker, a red-fluorescent dye for acidic organelles. First, the cells were dyed and submitted to flow cytometry

analysis (Figure 5A). Unexpectedly, the mutant cell transfectants did not show an increase in staining. To further corroborate, cells were prepared for analysis with confocal microscopy. This time cells were stained with lysotracker (granules) and Hoechst (nucleus), as depicted (Figure 5B). In order to assure an impartial analysis, we developed a macro for the Image J software. This less cumbersome approach enabled the quantification of individual cells, up to a total of 120 for each cell transfectant. Only cells within the microscope field of view were counted, hence the cells at the edge of each image were not included. The analysis was limited to the cytoplasm by subtracting the nucleus and delimiting the outer membrane of each cell unit. To evaluate any existent difference in the content of the granules, a ratio of the mean intensity of lysotracker versus the area stained with this dye was performed (Figure 5C). Additionally, a ratio of the area stained with lysotracker versus the area of the cytoplasm was carried out to examine possible variances in the number of granules (Figure 5D). This set of analyses both presented no differences between the two cell transfectants, confirming the previous result obtained by flow cytometry (Figure 5A).

MITF activity analysis

Next, we assessed whether LysRS P542R constitutive nuclear translocation was concomitant with an increase of MITF activity. For that purpose, we used a reporter gene methodology, Melastatin 1 (TRPM1) promoter-controlled firefly luciferase. MITF is a major transcriptional regulator of TRPM1²³. Thus, Firefly luciferase expression correlates with MITF activity²⁴. This reporter gene was transfected to the cells together with a Renilla luciferase reporter gene, and results were normalised to the empty vector-transfected conditions. Results show that P542R transfectants present a significant increase in MITF activity compared with WT cells in unstimulated conditions (Figure 6A) and after IgE stimulation (Figure 6B). It should be noted that overexpression of LysRS WT significantly increases TRPM1 transcription compared with control vector (PGL3-Luciferase). In parallel we transfected cells with 3xNFAT-luciferase and measured basal 3XNFAT activation in WT and P542R transfectants. As shown in Figure 6C, no activation was observed in P542R in unstimulated conditions. As expected, after PMA + Ionomycin stimulation 3xNFAT-luciferase was

induced indicating that the reporter gene was working properly and it was susceptible to activation after the right stimulus (Figure 6D). The data indicates that MITF is active in P542R unstimulated cells while other transcription factors such as NFAT are not.

MITF dependent gene expression

Next, we analysed some described MITF dependent genes involved in mast cell biology, such as mast cell protease 2, 4 and 9, chymase 1, among others (see Supplementary Table II). The levels of some of them increased significantly in P542R transfectants compared with WT. Interestingly, Chymase 1 was found particularly augmented in contrast to other proteases (Table I).

DISCUSSION

Anaphylaxis is a critical illness in emergency medicine which is described as a severe allergic reaction with an immediate onset, led by mast cells and basophils²⁵. The mediators released by mast cells and basophils upon contact of FcεRI-bound IgE to a specific allergen characterizes the pathology of this disease, which can lead to death if not controlled. Unlike other allergic diseases, patients with anaphylaxis barely have prophylactic treatments. Frequently, the only option for these patients is to avoid the stimuli that trigger these reactions. However, it becomes a huge challenge when the trigger is a ubiquitous and / or hidden allergen (foods such as milk, egg or wheat, among others) or when the trigger avoidance escapes the patient's control (hidden allergens, hymenoptera, or presence of cofactors, for example). In addition, with the exception of tryptase and the D816V mutation of the KIT receptor (CD117), we barely have reliable biomarkers that allow us to unequivocally identify those patients who have a higher risk of reaction, or serious reaction²⁶. Therefore, there is a clear interest in the field to better understand the molecular mechanism underlying severe allergic reactions. This study is based on a particular clinical case of an individual with extremely severe anaphylactic reactions in response to *Hymenoptera* stings, reacting even at concentrations used for routine allergological work-up (skin tests) and pre-treated with omalizumab (anti-IgE) in order to tolerate wasp venom immunotherapy. In this study, we found that

dysregulation of the FcεRI-LysRS-MITF pathway due to mutations in the *KARS* gene may explain a hyperreactive phenotype that can lead to anaphylaxis.

LysRS (encoded by the *KARS* gene) as a moonlight protein has a dual and crucial role in protein translation or gene transcription in mast cells⁶. Some mutations of *KARS* have been reported and the associated clinical phenotype of them is heterogeneous ranging from early onset encephalopathy to isolated peripheral neuropathy, nonsyndromic hearing impairment or progressive leukoencephalopathy^{27,28,29}. Some other mutations have been associated with suspected mitochondrial disorder³⁰, or with combined respiratory chain complex deficiencies (I and IV)³¹.

Nevertheless, no naturally occurring mutations have been identified nor have they been connected to anaphylactic reactions. Here, we attempted to link the non-canonical role of LysRS involving the activation of transcription factor MITF with the release of key mediators in anaphylaxis.

Our findings indicate that the P542R amino acid substitution in LysRS promotes the translocation of mutant LysRS to the nucleus in the absence of stimuli. This could be explained by the substitution of a Proline, an amino acid that introduces rigidity to the polypeptide backbone, for a positively-charged amino acid with a considerable side chain, like Arginine. Our findings also suggest that mutations placed in the region of the interdomain interface of LysRS are candidates to deregulate its functionalities. The constitutive nuclear location of LysRS P542R results in a higher release of both preformed and “*de novo*” mediators in mast cells. Interestingly, the LysRS P542R cell transfectant showed that MITF activity was enhanced, as well as specific MITF-dependent gene transcription, in the absence of stimulation. One of the most intriguing aspects of human anaphylaxis is that some patients with severe symptoms do not show elevated tryptase. This raises the possibility of the involvement of other, unidentified pathways and opens up the possibility of finding unidentified biomarkers. Remarkably, chymase (Cma) expression was significantly increased in P542R cells while tryptase (Tph1) was not. This result agrees with the clinical data of this patient where there was no increase of tryptase (hallmark of anaphylaxis) in the acute phase in this clinical case (Suppl. Information, patient characterization). Chymase is a serine protease basically

found in the secretory granules in mast cells. The evidence of chymase as a potential biomarker for anaphylaxis comes from the significant results found in two different studies of autopsy cases with anaphylaxis^{32,33}. One consideration is that chymase stability in serum appears to be higher than tryptase. Chymase is detectable 4h hours after the anaphylactic reaction while tryptase levels return to base line by this time point³⁴. Consequently, it would be interesting to extend the analysis of chymase in those cases of anaphylaxis where tryptase is negative.

This study is based on a particular clinical case of an individual with anaphylactic reactions in response to *Hymenoptera* stings due to such severe allergic reactions, even at concentrations used for routine allergological work-up (skin tests). Indeed, this patient requires omalizumab (anti-IgE) treatment in order to tolerate wasp venom immunotherapy administration. Because of the loss of a daughter at a few months of age, the patient and his partner received genetic counselling. Both parents were found to have missense mutations in the *KARS* gene that caused an amino acid substitution in the LysRS protein. The allergic patient has mutation P542R while the partner, with no allergic background, has mutation G189D. In the supplemental data, we compared both mutations at the structural level and also at the functional level. Our metadynamics simulations showed that the G189D mutation does not promote the opening mechanism as is the case of the P542R mutation. A single free energy minimum is found in the closed state of the LysRS-G189D system as in the LysRS-WT system (see Suppl. Fig. 3). Moreover, G189D is found far from both the interdomain interface and the S207 residue (see Suppl. Fig.3C bottom structure), and it is not found to interact with residues in charge of binding with MSC-p38, as opposed to the P542R mutation. Therefore, G189D would neither perturb the possibility of S207 being phosphorylated nor the binding of LysRS to MSC-p38.

Interestingly, the G189D LysRS protein is not observed intrinsically in the nucleus (Supplementary figure 4A) as we observed for P542R and consequently does not increase MITF activity using the TRPM1-luciferase gene reporter assay (Supplementary figure 4C). Accordingly, we found that a MITF-dependent event such as PGD₂ generation (Supplementary figure 5A) or MITF-

dependent gene expression were similar in LysRS G189D and LysRSWT (Supplementary figure 5B) indicating that this mutation does not enhance the non-canonical function in mast cells. To conciliate our results with the clinical data, where inheriting both mutations produced a deleterious phenotype, we demonstrated that mutation P542R translocates the protein to the nucleus, increasing MITF activity, thereby committing this protein to its non-canonical function. The increase of MITF activity allows crucial genes for anaphylaxis such as Hdc to be enhanced¹⁵. Upon IgE activation the proinflammatory mediator release will be more pronounced resulting in a more harmful inflammatory phenotype. It is of note that spontaneous degranulation is not enhanced in P542R transfectants indicating that the presence of IgE plus allergen would be needed in order to trigger the anaphylaxis. The preventive treatment of the patient with Omalizumab reduces the IgE-bound to mast cells³⁵ and the eventual anaphylactic shock in the presence of the allergen. Regarding mutation G189D, this substitution, based on our results, does not lead to a more susceptible anaphylactic profile upon allergen encounter. In fact, this individual has not reported any allergic episode so far. We believe that this mutation impairs the LysRS canonical function in protein synthesis. As depicted in Suppl. Fig. 3C, the G189D mutation is placed on the surface of the tRNA anticodon binding domain. Thus, this mutation might distort the mode of tRNA binding and alter the LysRS translational mechanism.

Altogether, this study shows how a mutation in LysRS triggers its translocation to the nucleus independently of stimuli and that this translocation enhances MITF activity and proinflammatory mediator release upon IgE crosslinking. Besides the MAPK-LysRS-Ap4A-HINT1 signalling pathway, multiple pathways have been reported to regulate MITF activity and/or stability in mast cells, including the c-KIT and PI3K pathways^{36,37,38}. The analysis of the MITF signalling cascade in the context of IgE and beyond IgE could provide further insight into the molecular pathways involved in anaphylaxis to *Hymenoptera* and it may be extended to food or idiopathic anaphylaxis.

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AUTHOR CONTRIBUTIONS

P.R., Y.G., and J.A. performed the experiments, E.A-E., A.N-F., M.G. provided technical support, R.M-C, M.P, C. d-C provided clinical data. P.R, J.A, M.O., R.M-C and M.M. conceived the experiments and wrote the manuscript. M.M. secured funding. All authors reviewed the manuscript.

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FIGURE LEGENDS

Figure 1. Proline 542 is a conserved residue in LysRS. Sequence alignment of LysRS domains Mega X: Molecular Evolutionary Genetics Analysis X software. Using the (A) Serine207, Glycine540 and Proline542 are highlighted as the key residues. Source: Modified from ²². (B) Structural prediction of the proximity between Proline542 and the key residues of LysRS.

Figure 2. LysRS WT and P542R functional motions at an atomic level. (A) Free energy profiles obtained for the opening process of the LysRS-WT and LysRS-S207(P) proteins. (B) Phosphorylation of S207 (shown in van der Waals representation) provokes the spontaneous opening of LysRS (shown as ice-blue and cyan ribbons) during MD simulation. (C) FES associated with the opening/closing motion of the LysRS-P542R mutated protein, isoenergetic lines are shown in kcal·mol⁻¹. The two free energy minimum structures which correspond to the opened and closed state are depicted as ribbons (in cyan and

ice-blue for each of the monomers). Insets for both states show the interaction between R542 and residues involved in MSC-p38 binding.

Figure 3. LysRS WT and P542R show distinct patterns of expression. (A) Flow cytometry histograms for GFP and FcεRI expression in WT and *P542R* RBL-2H3 transfectants. Parental RBL-2H3 cells are used as control. (B) pKIT and LysRS expression levels. (C) Confocal microscopy of WT and *P542R* cells. GFP shows the distinct pattern of LysRS expression in WT and *P542R* transfectants. Nuclei are stained with Hoechst (blue). (D) Western Blot of the whole cell lysate, cytoplasmic and nuclear fractions of WT and *P542R* cells. LysRS was detected by blot. Laminin B1 and α-tubulin are markers for nuclei and cytoplasm respectively.

Figure 4. LysRS P542R mutation enhances β-hexosaminidase and PGD₂ release upon IgE activation. (A) β-hexosaminidase assay in Non-stimulated conditions (NS), IgE+DNP-HSA or Ionomycin-PMA (I+P) (1μM+50ng/ml) stimulation in WT and *P542R* RBL-2H3 cells. (B) Ionomycin titration (from 0.01μM to 1μM) was performed in WT and *P542R* cells plus 50ng/ml of PMA (C) Prostaglandin D2 ELISA for *P542R* and WT cell transfectants with non-stimulated conditions (NS) , IgE-DNP-HSA stimulus. Data was performed in triplicate and is the mean of three independent experiments. Black and grey columns correspond to WT and *P542R* cells respectively.

Figure 5. Granule staining with LysoTracker does not show differences in LysRS WT and P542R transfectants. Mast cell granule analysis was performed with LysoTracker Red dye. (A) Flow cytometry histogram for WT and *P542R* stained cells. (B) Confocal analysis of WT and *P542R* cells including Hoechst staining for the nucleus. (C & D) ImageJ software analysis of the WT and *P542R* cells, showing intensity (C) and total amount of granules (D). Data is representative of three independent experiments.

Figure 6. LysRS P542R increases MITF activity in the absence of stimulation.

(A) LysRS WT and *P542R* were transfected with TRPM1-luciferase reporter gene or PGL3-Luciferase plus TK renilla. Next day cells were lysed and luciferase activity was measured. (B) Cells were stimulated with IgE+ DNP-HSA

(0.25 $\mu\text{g/ml}$ + 50 ng/ml). (C) The same procedure was carried out with 3XNFAT instead of TRPM1. (D) WT cells were activated with Ionomycin+PMA (1 μM +50ng/ml respectively) as a positive control of 3XNFAT. Measurements were performed in triplicate and the data is representative of three independent experiments.

TABLE I

Gene	P542R vs WT
Cdk2	-1.11
Cma	5.31 ***
Gzmb	-2.72
Kit	1.59 **
Mcpt2	1.46
Mcpt4	1.69
Mcpt9	2.0 *
Serpine 1	-1.80
Tph1	-1.49
Hpgd	-1.07
Tpsb2	1.52
Sort1	-1.47
Lyst	1.11
Cdk5r1	1.25
Hdc	1.77*

Table I. MITF-dependent gene array. Gene expression fold changes between WT and P542R are shown. Statistical significance of upregulated gene expression in P542R vs WT is shown with asterisks. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 1

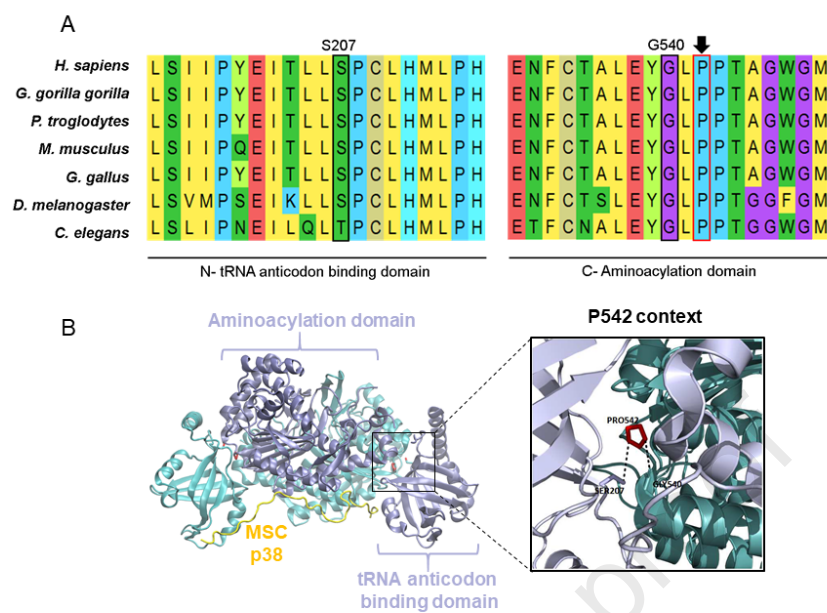


Figure 2

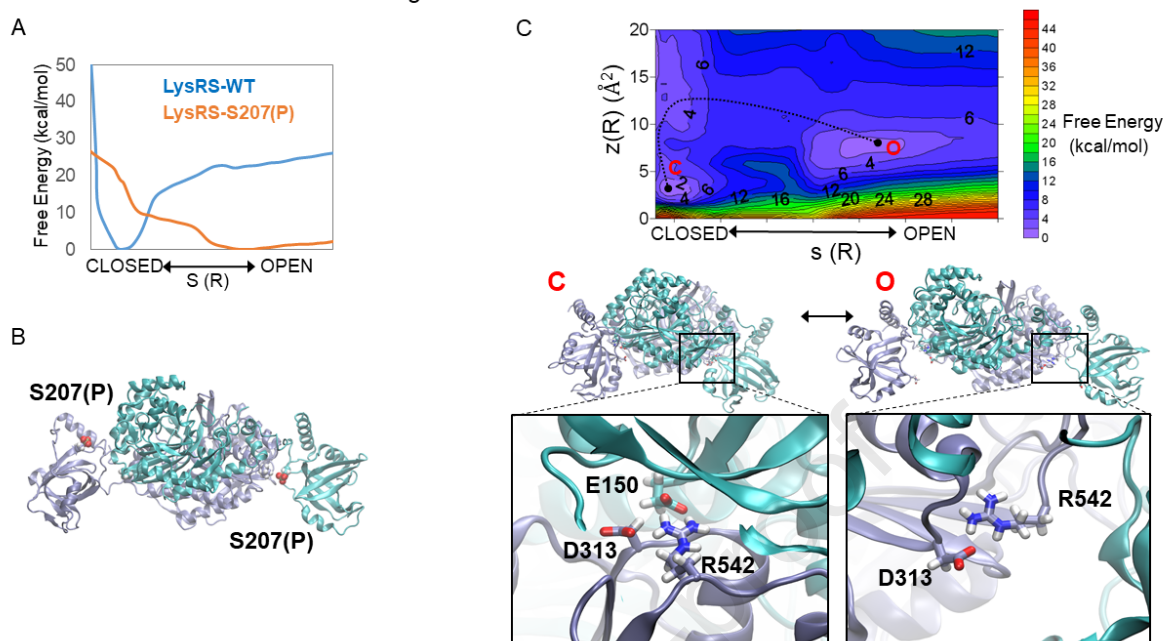


Figure 3

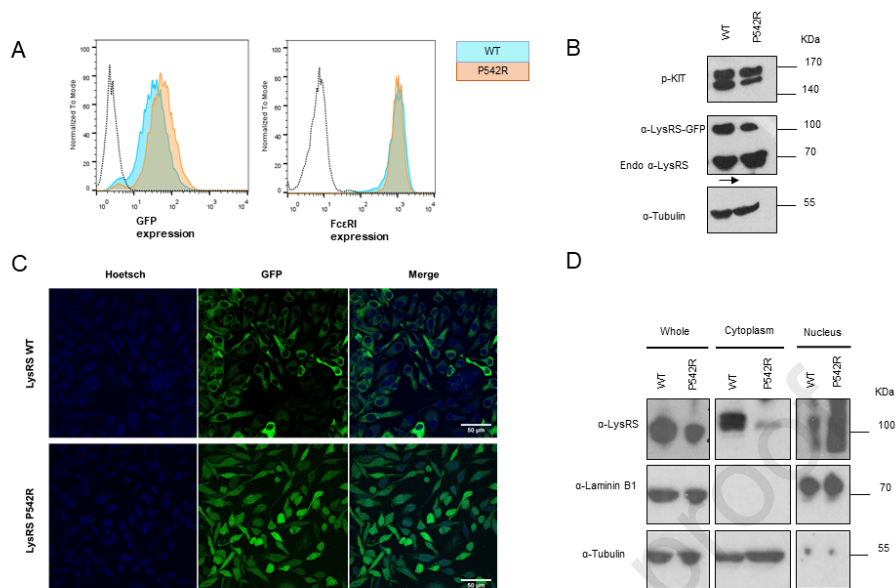


Figure 4

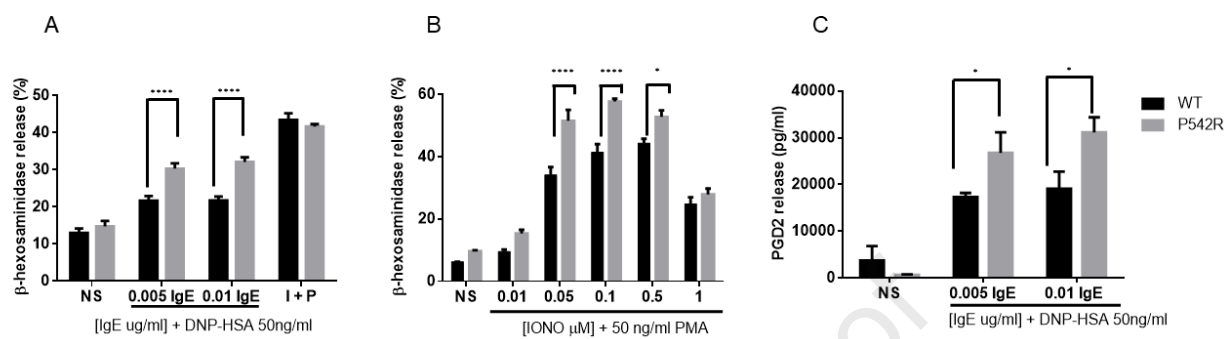


Figure 5

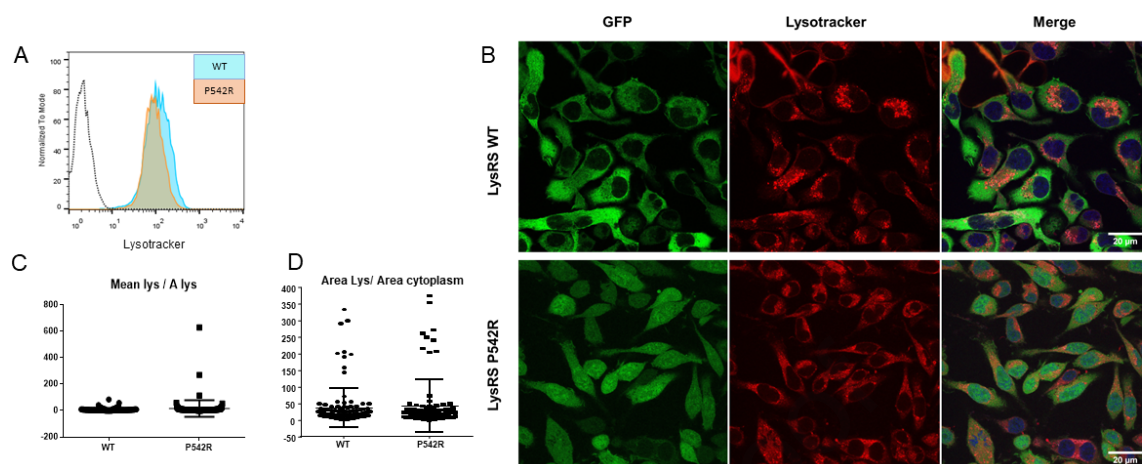
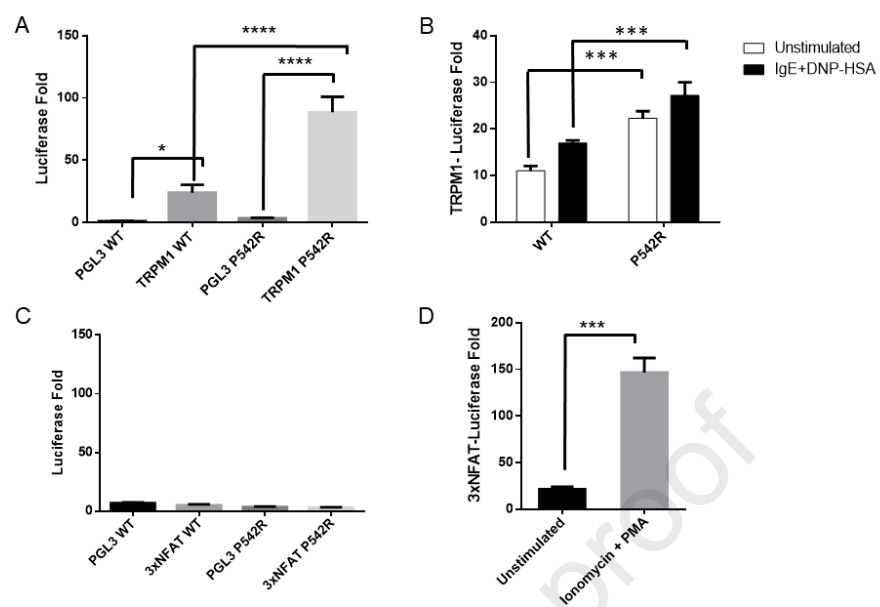
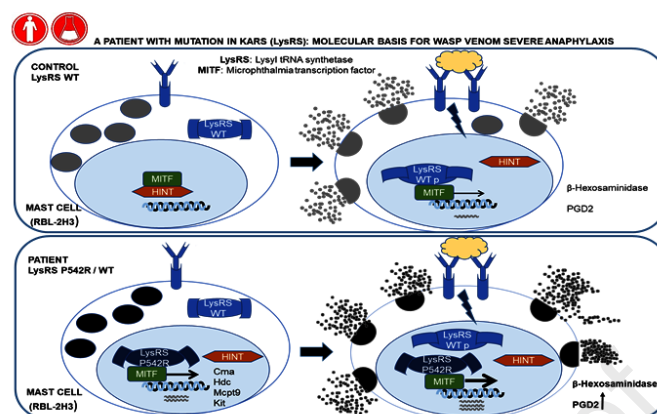


Figure 6





SUPPLEMENTARY INFORMATION

Allergological study

Skin test (prick and intradermal tests) with yellow jacket, paper wasp (*Vespula spp*, and *Polistes spp*, ALK-Abelló, Madrid, Spain) and honeybee venom, as the most prevalent hymenoptera insects in our area, were performed following the standard procedure ¹. Intradermal tests were performed at 0.001, 0.01 and 0.1 µg/ml and, were positive only at 0.1 µg/mL for both *Vespula spp* and *Polistes spp*. Specific IgE (ImmunoCAP (ThermoFisher, Uppsala, Sweden)) were positive for *Polistes spp* (2.33 kU/L) and *Vespula spp* (0.89 kU/L), and negative for bee venom (<0.1 kU/L). Basophil activation test (BAT; Flow2CAST®; Bühlmann Laboratories AG®, Schönenbuch, Switzerland), measured as expression of CD63, was positive only for *Polistes spp* at 0.25 µg/mL, and negative for *Vespula spp* at all tested concentrations (1, 0.5, 0.25, 0.1 µg/mL). Considering the severity of the reaction, venom immunotherapy (VIT) for *Polistes spp* was prescribed based on the results of the allergological study and the higher prevalence of *Polistes spp* in our area. With the first dose of VIT (0.01 µg), the patient presented again an anaphylaxis, similar to that observed with the skin test, without cardiovascular involvement, and required 0.5 mg of epinephrine. For this reason, we decided to use omalizumab 300 mg as a pre-treatment. Thus, the patient received 2 doses of omalizumab without VIT, and thereafter a monthly dose of omalizumab was administered. Using that strategy, we were able to reach 100 µg of venom using a conventional VIT schedule, with no relevant symptoms. Basophil activation test with *Polistes spp* venom was negative after 12 months of VIT and omalizumab.

Gene expression analysis in LysRS WT/ P542R and G189D transfectants,

Samples were performed in triplicate. RNA extraction was performed by following the Trizol® Reagent protocol (Thermo Fisher Scientific). Reverse transcription was performed using StaRT Reverse Transcription Kit (AnyGenes). Before qPCR array analysis, a quality control for all cDNA was performed by qPCR on a Light Cycler 480 instrument (Roche Diagnostic) with 2X Perfect Master Mix SYBR Green (PMS) (AnyGenes) and Actb primer set (Actin beta, House-Keeping Gene, known to be constitutively expressed in all tissues). Briefly, each qPCR reaction contains 10 µl of PMS, 1µl of Actb Validated qPCR Primer Sets (Reverse + Forward, 10 µM each: AnyGenes), 1µl of diluted cDNA and 8 µl nuclease-free water, for a final volume of 20 µl. All qPCR reactions were performed on the Light Cycler 480 instrument (Roche Diagnostic) with 2x Perfect Master Mix SYBR Green (PMS) (AnyGenes). All the Validated qPCR primer sets used are described in Supplementary Table II. All cDNA samples were analysed in qPCR triplicates for each studied or House-Keeping gene. The mRNA expression for each gene was normalized using the mean of Cp values for the 8 housekeeping genes, and defined by $2^{-(dCp)} \times 10000$ values. Fold changes, p value via Student Test and Standard Deviation has been calculated, to generate tables and graphs. The entire analysis was performed by the AnyGenes SignArrays Service.

Molecular Dynamics Simulations (MD)

Methods

We performed long Classical MD simulations for the LysRS-WT system, the LysRS-WT system where Ser207 was phosphorylated (LysRS-S207(P)), the LysRS-P542R mutant and the LysRS-G189D mutant. The starting conformations of the LysRS systems were modelled from the crystal structure of LysRS with PDB ID 6ILD ² which is a homodimeric structure. In order to insert the ligands in the active site of our systems we aligned the 6ILD crystal structure with the PDB ID 1E22 ³ crystal structure. 1E22 is crystallized as a monomer with a lysine residue, three Mg^{2+} ions and a cofactor analogue of ATP. The alignment showed a RMSD of less than 0.5 Å between the structures. The LysRS subfamily of enzymes are known to belong to the class II of Aminoacyl-RSs which work as homodimers and, as a first step of their canonical function, catalyse the formation of an aminoacyl adenylate in the presence of 3 Mg^{2+} cations ⁴. Thus, we inserted the missing ligands (a modelled ATP molecule and 3 Mg^{2+} ions) inside the two active sites of our homodimer. Finally, we reconstructed the missing amino acids in the 6ILD chains making use of SWISS PROT utility ⁵. The initial structures were subjected to several cycles of minimization and equilibration as explained below.

The AMBER 18 program ⁶ was used to carry out the MD simulations using the AMBER ff14SB force field ⁷ for the protein. Force field parameters of ATP were developed by Carlson et al. ⁸, Mg^{2+} parameters by Allner et al., ⁹ and parameters of phosphorylated serine by Stitch et al. ¹⁰. The protonation state of LysRS residues was assigned making use of propKa ¹¹ at pH 7. Then the system was solvated using the LEAP module of AMBER into a truncated octahedron box of TIP3P ¹² water molecules. A buffer of water molecules extending for 12 Å in every direction around the system was applied and the system was neutralized with potassium ions by adding an excess of 150 mM of KCl. Long-range electrostatic interactions were computed by using the Particle Mesh Ewald method ¹³ using standard defaults and a cut off in the real-space of 10 Å. A cut off of 10 Å was used to compute the non-bonded interactions. The SHAKE algorithm ¹⁴ was applied to restrain bond lengths involving hydrogen

atoms, and the time step of the simulation was 2 fs. The systems were energy minimized, thermalized and pre-equilibrated for 50 ns in the canonical and isobaric-isothermal ensembles before the production run. Production MD simulations were carried out in the isothermal-isobaric ensemble with a pressure of 1 atm and a temperature of 300 K making use of the Berendsen thermostat and barostat. Unrestrained MD simulations were performed for 1 μ s.

Results

Initially, we performed molecular dynamics simulations (MDS) of 1 μ s for the wild-type system LysRS-WT, the wild-type system with the S207 phosphorylated LysRS-S207(P), the protein containing the P542R mutation (LysRS-P542R) and the system containing the G189D mutation (LysRS-G189D) (see Methods sections for details). Our unbiased MD simulation for LysRS-S207(P) was able to capture the opening motion of LysRS leading to an open state in agreement with the published SAX data ¹⁵. The opening of the protein occurs in a relatively short simulation time (see Root-Mean-Square Deviation -RMSD- in Suppl. Fig. 1B) suggesting that the introduction of a negative charge in a mainly apolar interdomain interface should provoke steric clashes and the opening of the protein dimer in a short time scale. As expected, the LysRS-WT system did not open during the simulation run and remained in its closed conformation (see Suppl. Fig. 1A). In addition, we did not observe much change in the overall structure of the LysRS-P542R system (see Suppl. Fig. 1C, RMSD) which remained closed. The mutated residue P542R is found interacting with residues E150 and D313 (see Suppl. Fig. 2B) along part of the MD simulation as in the closed state free energy minimum found in the Metadynamic simulation (see Fig. 2C in the manuscript). Finally, the G189D mutated LysRS system remained in the closed state throughout the simulation. As can be seen in Suppl. Fig. 1D, where D189 is depicted as van der Waals spheres, this mutation is placed far from the interdomain interface and is exposed to the solvent and the overall structure did not suffer any conformational changes.

Well-Tempered Metadynamics (WT-MD)

Methods

The Amber18 programme patched with PLUMED 2.6¹⁶ was used to carry out metadynamics simulations with path collective variables¹⁷. This enhanced sampling methodology allows us to explore processes, such as the opening of a protein that may occur at higher time-scales not accessible by state-of-the-art unbiased MD simulations. We selected as our Collective Variable (CV), or reaction coordinate in which a bias potential is added during the simulation, the progress along the path $s(R)$ and the distance from the path $z(R)$. The path employed was the opening path obtained from the unbiased MD simulation of the LysRS-S207 (P) system where we captured the opening motion spontaneously. Thus, this path was defined on 90 equally spaced structures which were aligned on its C α atoms extracted from the MD trajectory. Finally, the free energy surface of the process was computed by using the added bias along the CV. A cumulative time of 1 μ s of Well-Tempered Metadynamics simulation for each of the LysRS systems were carried out. A wall potential with force constant of 300 kcal·mol⁻¹·Å⁻⁴ was employed to restraint $z(R)$ to a maximum value of 20 Å², thus allowing the system to explore regions different from the reference path while preserving an efficient sampling and computational cost. The addition frequency of the Gaussians was set to 1 ps. For both s and z the height and the width of the Gaussians were 0.2 kcal/mol and 0.1 respectively.

Results

To further analyse the opening motion and its thermodynamics, we performed Well-Tempered Metadynamics simulations on the aforementioned systems. This enhanced sampling methodology (see Methods) allows us to explore processes, such as the opening of a protein that may occur at higher time-scales not accessible by state-of-the-art unbiased MD simulations. In Fig. 2 and Supplementary Fig. 3B-3C, free energy surfaces monitoring the opening of the

different LysRS systems are shown. As can be observed, one single minimum is found for LysRS-WT in the closed state (see Fig. 2 and Suppl. Fig. 3A). As already seen for the LysRS-S207 (P) phosphorylated system in our MD simulation, the opening occurs spontaneously from the closed to the open state, and one free energy minimum is found in the opened state (see Fig. 2 and Suppl. Fig. 3B). We found two accessible states for the LysRS-P542R system separated by a free energy barrier of 6.3 kcal/mol and thermodynamically equally stable (see Fig. 2C). Thus, mutation P542R critically impacts the dynamical behaviour of the LysRS by promoting the system to explore an open state that could unbind from the MSC and might provoke translocation to the nucleus.

Finally, the G189D mutation does not alter the functional behaviour of the LysRS protein. As can be observed in the calculated FES, one free energy minimum is found in the closed state as for LysRS-WT (see Suppl. Fig. 3C). The G189D mutation is found far from the interdomain interface which is not affected by the mutation. If we observe the closed state in both our MD simulations (see Suppl. Fig. 1D) and the one obtained by MD simulations, we can see that D189 is found exposed to the solvent and therefore not altering the LysRS structure. Moreover, the G189D mutation is also far from the MSC-p38 binding region and no interactions were found between D189 and any of the residues that interact with the p38 protein during our simulations. These results are in agreement with our experiments which show that mutation G189D is not observed intrinsically in the nucleus and this mutation does not enhance the non-canonical function in mast cells.

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