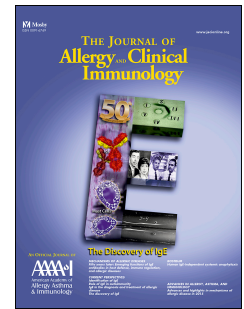


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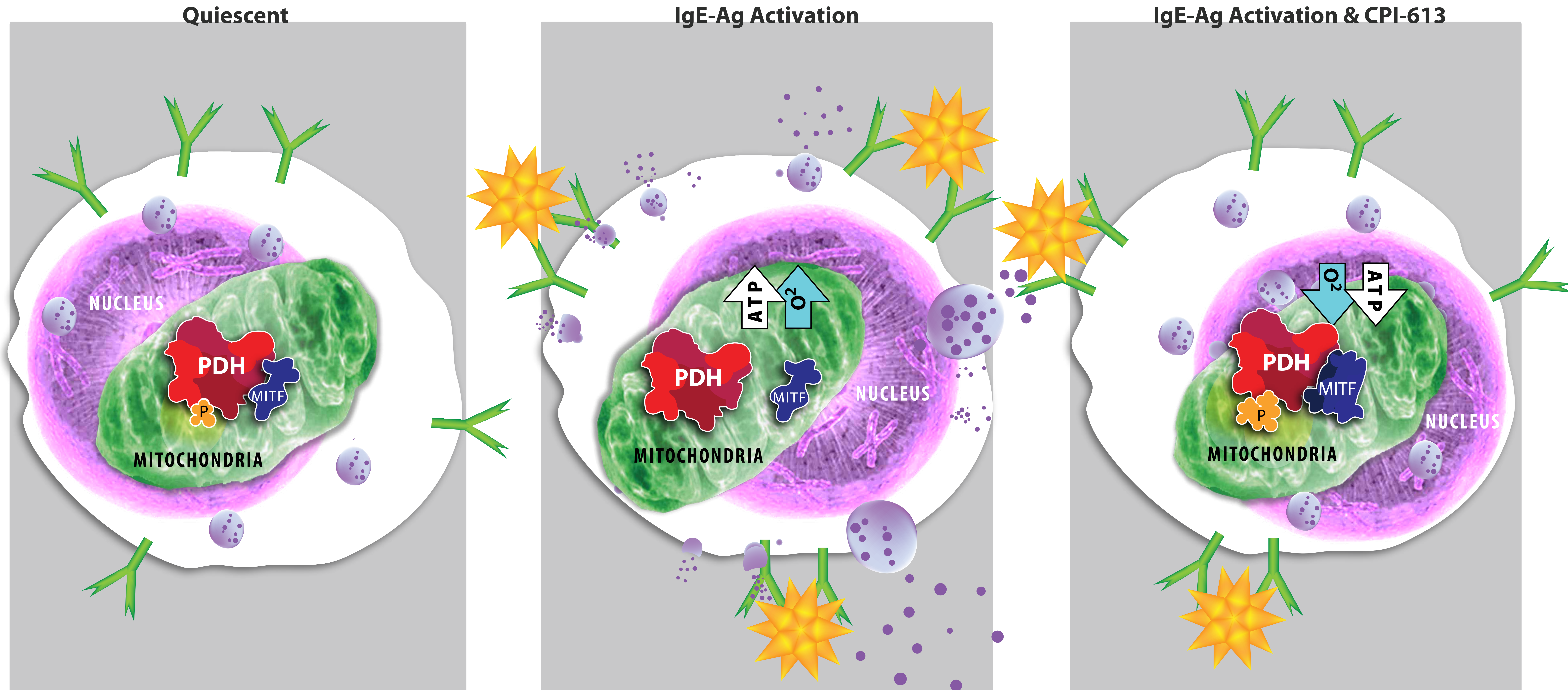
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MITF – Microphthalmia Transcription Factor • PDH – Pyruvate Dehydrogenase • Ag – Antigen • IgE – Immunoglobulin E
 P – Phosphate Group • CPI-613 – Small Molecule that Inhibits PDH.

Pyruvate dehydrogenase has a major role in mast cell function and its activity is regulated by mitochondrial MITF

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Summary

Background: We have recently observed that OXPHOS mediated ATP production is essential for mast cell function. Pyruvate dehydrogenase (PDH) is the main regulator of the Krebs cycle and is located upstream to the electron transport chain. However, the role of PDH in mast cell function has not been described. Microphthalmia transcription factor (MITF) regulates the development, number and function of mast cells. The localization of MITF to the mitochondria and its interaction with mitochondrial proteins has not been explored.

Objective: To explore the role played by PDH in mast cell exocytosis and to determine whether MITF is localized in the mitochondria and is involved in the regulation of PDH activity.

Methods: Experiments were performed *in vitro* using human and mouse mast cells as well as RBL cells, and *in vivo* in mice. The effect of PDH inhibition on mast cell function was examined. PDH interaction with MITF was measured before and after immunological activation. Furthermore, mitochondrial localization of MITF and its effect on PDH activity were determined.

Results: PDH is essential for immunologically mediated degranulation of mast cells. Following activation PDH is serine dephosphorylated. In addition, we show for the first time that MITF is partially located in the mitochondria and interacts with PDH. This interaction is dependent on the phosphorylation state of PDH. Furthermore, mitochondrial MITF regulates PDH activity.

Conclusion: The association of mitochondrial MITF with PDH emerges as an important regulator of mast cell function. Our findings indicate that PDH could arise as a new target for the manipulation of allergic diseases.

Key Messages:

- PDH is a key regulator of mast cell function and therefore could emerge as a new target for the manipulation of allergic diseases.
- MITF is localized to the mitochondria where it interacts and regulates PDH's activity.
- IgE-Ag activation of mast cells elevates the activity of PDH and causes its detachment from MITF.

Key words

Allergy, Mast cells, Degranulation, Cytokines, PDH, MITF, Asthma, Mitochondria.

Capsule summary

PDH inhibition impaired mast cell degranulation and cytokine secretion, suggesting that PDH could emerge as a new target for the manipulation of allergic diseases. MITF is located in the mitochondria and regulates PDH activity.

Abbreviations

Pyruvate dehydrogenase complex (PDC)
Pyruvate dehydrogenase (PDH)
Oxidative phosphorylation (OXPHOS)
microphthalmia associated transcription factor (MITF)
Electron transport chain (ETC)
Rat basophil leukemia (RBL)
Bone marrow derived mast cells (BMMC)
Cord blood derived mast cells (CBMC)

INTRODUCTION

We have recently shown that mitochondrial ATP production is essential for mast cell function¹. Oxidative phosphorylation (OXPHOS) activity was elevated during mast cell activation and inhibition of mitochondrial ATP production led to a significant decrease in mast cell degranulation. Since OXPHOS activity is dependent on NADH and FADH₂ produced during the Krebs cycle, in the present work we determined the role played by pyruvate dehydrogenase (PDH), the main regulator of the Krebs cycle, in mast cell activation. The PDH complex (PDC) catalyzes the oxidative decarboxylation of pyruvate with the formation of acetyl-CoA, CO₂ and NADH². This complex occupies a key position in the oxidation of glucose by linking the glycolytic pathway to the oxidative pathway of the tricarboxylic acid cycle. In mammalian cells, PDC serves as a gatekeeper in the metabolism of pyruvate in order to maintain glucose homeostasis during the fed and fasting states³. The activity of the PDC is tightly regulated in tissues under different metabolic conditions. This is accomplished by covalent modification of the rate-limiting component of the PDC involving dedicated kinases and phosphatases^{4,5}. The PDCs in prokaryotes and eukaryotes are composed of multiple copies of three catalytic enzymes: PDH (E1), dihydrolipoamide acetyltransferase (E2), and dihydrolipoamide dehydrogenase (E3)². The complex's activity is regulated by phosphorylation on three serine residues (S293, S302, S232) on the E1- α subunit, which causes reduction in the complex's activity⁶. Here we explored the role played by PDH in mast cell function by determining its phosphorylation state and activity following mast cell activation. Our yeast two hybrid assay revealed that microphthalmia transcription factor (MITF), a master regulator of mast cells, interacts with the E1 subunit of PDH. MITF is a basic helix-loop-helix leucine zipper (bHLH-Zip) DNA-binding protein⁷. Its gene resides at the *mi* locus in mice⁸ and mutations of this gene result in deafness, bone loss in dominant negative *mi/mi*

mice, small eyes, and poorly pigmented eyes and skin⁹. In humans, heterozygous mutation in this gene causes Waardenburg Syndrome type II¹⁰, resulting in hypopigmentation and deafness.

MITF is not expressed in all types of cells; however it is a key protein in the function of mast cells and melanocytes. Mast cells are widely recognized as playing an important role in allergy which is a hypersensitivity disorder of the immune system. MITF regulates the development and number of mast cells. Mice with a mutated MITF gene have fewer mast cells and these cells have a defective phenotype¹¹. Interestingly, MITF was found to promote expression of oxidative phosphorylation genes via direct regulation of PGC1 α gene expression, a mitochondrial master regulator¹². However, the expression of MITF in the mitochondria and its interaction with mitochondrial proteins has not been explored.

In the present work we show that PDH regulates mast cell function and that this regulation is controlled by its association with mitochondrial MITF.

METHODS

The methods used in this study are described in the Methods section in this article's Online Repository.

RESULTS

Inhibition of PDH decreases the degranulation and cytokine secretion levels in RBL and CBMC

Previously we have shown that OXPHOS plays a key role in mast cell degranulation¹. In addition, mitochondrial STAT3 was found to specifically regulate complex 3 of the electron transfer chain (ETC) during this physiological process¹. Since NADH and FADH₂, both products of the Krebs cycle, serve as cofactors for ETC activity, we wondered whether PDH, the main regulator of Krebs cycle, is involved in this process. In order to determine if PDH is

activated following immunological activation, rat basophil leukemia (RBL) cells and bone marrow derived mast cells (BMMC) were sensitized with IgE-anti DNP for 2 h and then triggered with DNP-BSA for 5, 15 and 30 min. Afterwards, the levels of phospho-S293 PDH were determined by Western blot analysis. As shown in Fig.1A, a significant reduction in the levels of phospho-S293 PDH was observed after 5 min activation in RBL cells. A raise in the phosphorylation levels was observed after 15 minutes activation and a reduction after 30 minutes. Indicating an increase in PDH activity after 5 and 30 min, similar results were observed in BMMC (Fig.1B). Densitometry of the results is shown in Fig.E1A.

We next investigated the effect of inhibiting the PDH complex on degranulation. RBL cells were sensitized with IgE-anti DNP for 2 h followed by 30 min incubation with DNP-BSA. One hour prior to the addition of DNP-BSA, cells were incubated with 300 μ M CPI-613, a PDH complex inhibitor¹³, and degranulation as well as TNF- α secretion were assessed by measuring β -hexosaminidase release and ELISA respectively. (Calibration curve of CPI-613 is shown in Fig.E1B) As shown in Fig.1C, PDH inhibition resulted in a significant 50% reduction in degranulation levels (n=3; p=0.007). CPI-613 also significantly inhibited the release of the cytokine TNF- α (n=5, P=0.006) as can be seen in Fig.1D. Incubation of RBL cells with 300 μ M CPI-613 for 1 h resulted in elevated phospho-S293 PDH levels as determined by Western blot analysis shown in (Fig.1E) densitometry of the results can be seen in Fig.E1C, showing that CPI-613 indeed inhibited PDH activity in these cells.

Since our hypothesis is that mitochondrial ATP is essential for mast cell degranulation, we determined if the inhibition of mast cell degranulation by CPI-613 correlates with a reduction in mitochondrial ATP. RBL cells were cultured for 24h in a glucose-free, OXPHOS dependent medium supplemented with dialyzed serum as an energy source or in complete medium used as control. Afterwards cells were incubated with 50, 200 or 300 μ M of CPI-613 for 1h and ATP levels were measured using ATPlite Luminescence Assay System. AS shown

in Fig.E1D CPI-613 reduced mitochondrial ATP levels in a dose dependent manner, which correlates to the reduction in degranulation levels. Therefore, the reduction in the degranulation levels is a result of impaired mitochondrial ATP production. CPI-613 slightly reduced the total ATP levels of RBL cells cultured in complete medium only when incubated with 300 μ M for 1h. (Fig.E1E).

Since a reduction in ATP content is also a marker for apoptosis, we determined whether the inhibitory effect of CPI-613 initiated apoptosis in these cells. As shown in Fig.E1F, no change in cleaved caspase3 levels was observed after 1h incubation with 300 μ M of CPI-613. To further examine if this reduction in mast cell degranulation is a result of specific PDH inhibition, RBL cells were transfected with siRNA to PDH. 48h later, these cells were sensitized with IgE-anti DNP for 2 h followed by 30 min incubation with DNP-BSA. As shown in Fig.1F silencing of PDH inhibited mast cell degranulation in a similar way to CPI-613. Western blot analysis of the silencing of PDH is shown in Fig.1G (n=5, P=0.01) and densitometry of the blot is shown in Fig.E1G. Thus, the reduction of mast cell degranulation is a result of specific PDH inhibition.

In order to determine if the effect of CPI-613 on mast cell degranulation is specific for IgE-Ag activation, RBL cells were activated for 30 min with high and low concentrations of the calcium ionophore A23187. One hour prior to A23187 treatment cells were incubated with 300 μ M CPI-613 and degranulation was assessed by measuring β -hexosaminidase release. As shown in Fig.E1H CPI-613 significantly inhibited mast cell degranulation in low levels of A23187 activation (n=3, p<0.005), however had no effect on high levels of A23187 activation (n=3, P=0.41). Next we determined whether PDH has a similar role in human mast cells as that described above for rodent mast cells. Human cord blood-derived mast cells (CBMC), previously sensitized with human myeloma IgE (4 days), were exposed to CPI-613 or DMSO vehicle (final concentration of DMSO in all experiments using CPI-613 or DMSO

was 0.8%) for 3 h before activation with rabbit α -human-IgE. Degranulation levels were determined after 30 min by measuring β -hexosaminidase release. As seen in Fig.1H, the degranulation seen in cells activated by anti-IgE after pre-incubation with CPI-613 was reduced by 30% (n=3; p=0.008). We also measured the levels of the inflammatory cytokine IL-6 in supernatants from CBMC that were treated either with CPI-613 or DMSO. As can be seen from Fig.1I, there was no IL-6 in the supernatants from cells that were treated with CPI-613(n=3; p=0.01). No cell toxicity was observed.

Inhibition of PDH decreases histamine secretion and lung resistance but not airway inflammation in mice.

PDH's role *in vivo* was investigated by evaluating the response to allergic stimulation in CPI-613-treated mice. The passive systemic IgE-mediated anaphylaxis model was used. Mice were injected i.v. with IgE anti-DNP and i.p. with CPI-613 (50 mg/kg) or saline, which was used as a control, twenty four hours later they were challenged with DNP and sacrificed 1.5 min later. Plasma was collected, and histamine levels were measured. As shown in Fig.2A, there was 22% less histamine in the plasma of CPI-613-treated mice than that in control mice (control n=4, CPI-613 n=5; p=0.002). Allergic asthma, the most common form of asthma, is a chronic inflammation disease of the airways which results in airway hyperresponsiveness (AHR)¹⁴. The effect of PDH inhibition on allergen-induced AHR was investigated by assessing lung resistance after methacholine challenge in ovalbumin (OVA) or saline exposed mice treated with CPI-613 or saline i.p and the results can be seen in Fig.2B-C. Exposure to OVA resulted in a significant increase in airway resistance compared to saline exposure (7.1 $\pm$ 0.5 vs 2.3 $\pm$ 0.1 respectively, p<0.001 at 64 mg/ml). Mice treated with CPI-613 still had a significant increase in airway resistance in response to OVA (5.6 $\pm$ 0.4 vs 2.3 $\pm$ 0.1, p<0.001), but this was significantly lower than the OVA only group (5.6 $\pm$ 0.4 vs 7.1 $\pm$ 0.5, p=0.005).

CPI-613 treatment had no effect on the saline exposed mice. Next we examined airway inflammation in bronchoalveolar lavage fluid (BALF) and bronchial submucosa by analyzing the total number of cells and the percentage of eosinophils in these mice (Fig.2D-E). OVA treatment increased the total cell number and percentage of eosinophils in the BALF of both control and CPI-613 treated mice (saline-saline $1.3 \pm 0.2 \times 10^4$; saline-CPI-613 $1.67 \pm 0.17 \times 10^4$; OVA-saline $18.67 \pm 1.8 \times 10^4$; OVA-CPI-613 $25.2 \pm 1.9 \times 10^4$ cells/ml BALF, $p < 0.001$). The percentage of eosinophils was slightly lower in CPI-613 treated mice, but this was not statistically significant (saline-saline 0%; saline-CPI-613 0%; OVA-saline $67.5 \pm 4\%$; OVA-CPI-613 $60.3 \pm 3.7\%$).

The role played by PDH phosphorylation on MITF-PDH interaction in IgE-Ag activation of RBL cells.

A yeast two hybrid assay performed using a mouse heart library and full-length MITF heart isoform as bait, revealed that MITF interacts with the E1 subunit of PDH (Table E.1). In order to further explore this interaction in RBL cells, MITF was immunoprecipitated using MITF specific antibody and its interaction with PDH was determined by Western blot analysis with an anti PDH-E1 α . As shown in Fig.3A, PDH-E1 α precipitated with MITF. In addition, HEK293T cells were transfected with MITF-H-flag tag plasmid, 48h later the cells were lysed and PDH-E1 β was immunoprecipitated using PDH-E1 β specific antibody. Its interaction with MITF was determined by Western blot analysis with an anti-flag tag antibody. Fig.3B shows that MITF co-immunoprecipitated with PDH-E1 β . We also performed co-immunoprecipitation in BMDC cells, whereby PDH-E1 β was immunoprecipitated with antibody specific to PDH-E1 β and its interaction with MITF determined by Western blot analysis with anti-MITF. MITF co-immunoprecipitated with PDH-E1 β antibody (Fig.E2A). Thus, MITF is associated with the E1 subunit of PDH.

Furthermore, this interaction was determined by using a proximity ligation assay named in situ PLA (materials and methods section). As shown in Fig.3C interaction (red dots shown by arrowheads) between MITF and PDH is observed outside the nucleus. Since we have previously shown that after 5 min activation, PDH-E1 α is dephosphorylated on serine 293 (Fig.1A), we determined the effect of mast cell activation on the interaction between MITF and PDH-E1. RBL cells were sensitized with IgE for 2 h followed by 5 min incubation with DNP-BSA. Unsensitized cells were used as control. Cells were then lysed and co-immunoprecipitated with anti-MITF antibody. As shown in Fig.3D, co-immunoprecipitation levels of PDH were reduced after 5 min activation, showing that activation causes PDH to detach from MITF (densitometry of these results is shown in Fig.E2B). We then determined whether PDH phosphorylation could affect the interaction between the two proteins. RBL cells were incubated with 300 μ M of CPI-613, which elevates PDH-E1 phosphorylation levels (Fig.1E), or with DMSO as control for 1h. MITF was immunoprecipitated and the levels of PDH-E1 α were determined by Western blot analysis. As shown in Fig.3E, PDH phosphorylation resulted in an increased association between MITF and PDH-E1 α (densitometry of the results is shown in (Fig.E2C)).

To determine that our MITF constructs used later in Fig.5 and Fig.6 indeed interact with PDH E1, 293T cells were transfected with PDH-E1 β and one of two MITF constructs; either MITF-H-flag, which is the full length MITF, or MITF-CE-flag, an MITF form which lacks the leucine zipper domain and cannot bind DNA. Anti-flag-tag antibody was used for the immunoprecipitation and for Western blot analysis in addition to PDH-E1 β antibody. As can be seen in Fig.E2D, only cells expressing full length MITF or MITF-CE together with PDH resulted in successful precipitation.

MITF is localized to the mitochondria of RBL cells.

Although MITF was previously shown to affect mitochondrial activity through gene regulation of PGC1- α ¹², its presence in the mitochondria and its direct interaction with mitochondrial proteins has not yet been reported in the literature. Therefore we determined whether MITF is localized in the mitochondria. RBL cells were fractionated into cytosol and mitochondria, furthermore, mitoplasts were prepared from the mitochondrial fraction by proteinase K treatment. The purity of the mitochondrial fraction was determined by Western blot analysis using antibody against GAPDH, a cytosolic marker. Mitoplast purity was determined by following the reduction of a Bcl-xl, an outer mitochondrial membrane marker. As shown in Fig.4A, MITF expression was detected in both the cytosolic and mitochondrial fractions as well as in the mitoplasts. The presence of MITF in the mitoplasts indicated that it is localized within the mitochondria and is not derived from either the contaminating cytoplasm or the mitochondrial intermembrane space.

To further evaluate and to quantify the expression of MITF in the mitochondria, imaging flow cytometry was used. The mitochondria were stained with an anti-complex1 antibody and MITF was stained with C5 specific antibody. Cell nuclei were stained with the common DNA stain, Hoechst. As the results show, two fractions of MITF were found in the cells. The main fraction was localized in the nucleus (Fig.4B) and the minor one in the mitochondria (Fig.4C). The staining method was verified using HDAC as a nuclear marker Fig.4D shows that HDAC1 is mainly localized to the nucleus and no expression was found in the mitochondria (Fig.4E). HSP60 was used as control for the mitochondrial localization. As can be seen in Fig.4G, the main fraction of this protein is in the mitochondria and a minor fraction was observed in the nucleus (Fig.4F). We further confirmed our data using HEK293T cells which do not express MITF. These cells were transfected with EV or either MITF-H-flag or MITF-CE-flag plasmids, 24 h later the cells were fractionated into

mitochondrial and cytosol fractions. MITF content was determined by Western blot analysis with an anti-flag antibody. As can be seen from Fig.E3A, MITF was detected in both the mitochondrial and the cytosolic fractions in cells transfected with MITF-H and MITF-CE, but not in cells transfected with empty vector. The purity of the mitochondrial fractions was determined by Western blot analysis with anti-caspase3 antibody as a cytosolic marker (Fig.E3B).

Overexpression of MITF-H and MITF-CE lowers PDH activity and increases pyruvate levels in RBL cells.

Since MITF affects mitochondrial function through transcriptional regulation of PGC1- α ¹², RBL cells were overexpressed with MITF-H (full length) or MITF-CE, which lacks the leucine zipper domain and therefore cannot bind DNA and EV was used as control. Forty eight hours later PDH activity was assessed by ELISA (Fig. 5A). Dichloroacetic acid (DCA), a PDH kinase inhibitor, was used as a positive control (Fig. E4A) and CPI-613 was used as a negative control (Fig. E4B).

Overexpression of MITF-H and MITF-CE resulted in a significant reduction in PDH complex's activity (MITF-H n=3, p=0.03; MITF-CE n=3, p=0.01). No effect was observed on PDH expression in MITF transfected cells (data not shown). As can be seen from Fig. 5A, the effect of MITF-H was greater than MITF-CE. To further evaluate the effect of MITF on PDH complex activity, we checked the levels of pyruvate, the substrate of PDH, which should accumulate if PDH is inhibited. RBL cells were transfected with either MITF-H or MITF-CE and total pyruvate levels were measured with a pyruvate ELISA kit. As shown in Fig. 5B, overexpression of MITF-H elevated the levels of pyruvate by nearly 40% whereas cells transfected with MITF-CE pyruvate levels were elevated by 6% (MITF-H n=3, p=0.007; MITF-CE n=3, p=0.006).

Overexpression of MITF-H and MITF-CE lowers ATP and oxygen consumption levels in RBL cells.

Since PDH activity directly affects mitochondrial function, it was determined whether changes in MITF levels affect ATP levels and oxygen consumption. RBL cells were transfected with MITF-H or MITF-CE and 48 h later ATP levels and oxygen consumption were measured. Overexpression of either one of the two constructs of MITF significantly reduced ATP levels (Fig. 6A) (MITF-H n=3, p=0.02; MITF-CE n=3, p=0.01), and oxygen consumption (Fig. 6B) (MITF-H n=3, p=0.03; MITF-CE n=3, p=0.005).

DISCUSSION:

The role played by mitochondria in both innate and adaptive immune systems is of great interest. In macrophages, mitochondria are involved in antiviral signaling via the production of reactive oxygen species (ROS)¹⁵. Aside from ROS, the TCA cycle intermediate succinate has also been implicated in LPS-induced inflammatory cytokine signaling¹⁶. In addition, mitochondrial proteins in macrophages are involved in the Toll-like receptor 4 signaling pathway, which regulates bactericidal activity¹⁷. Furthermore, mitochondrial signaling dictates macrophage polarization and function¹⁸. In the adaptive immune response, glutaminolysis in the mitochondria was found to be an important energy source for T and B cell maturation^{19, 20} and mitochondrial metabolism is required for T cell activation through the generation of mitochondrial ROS^{21, 22}. Mitochondrial localization to the immune synapse is part of T cell activation²³ and complex 3 activity was found to be essential for this activation²⁴. In B cells, both aerobic glycolysis and mitochondrial oxygen consumption are upregulated upon stimulation via either TLR4 or B cell receptors²⁵. We reported that mast

cell activation enhanced ATP levels and oxygen consumption¹ and mitochondrial translocation to exocytosis sites was also observed as part of the processes of mast cell degranulation and TNF secretion²⁶. In this paper we further explore the mechanism in which mitochondria are activated to produce more ATP during mast cell degranulation. PDH is a gatekeeper in the metabolism of pyruvate which determines whether it is glycolysed or enters the TCA cycle. Although it seems that mitochondrial function is elevated during adaptive immunity activation, the role of PDH in this process is not clear. Especially its role in mast cell function hasn't been delineated before.

In the present study we have shown the pivotal role played by PDH in immunologically activated mast cells. During mast cell degranulation PDH activity is enhanced, as evidenced by de-phosphorylation of PDH on serine 293. Inhibition of PDH by CPI-613 impaired mast cell degranulation and cytokine secretion in RBL and CBMC indicating the physiological relevance of PDH in the response to mast cell activation. It is important to emphasize that this effect is not a result of apoptosis as no caspase3 cleavage or cell toxicity were observed. Furthermore, this effect is selective for PDH inhibition as knockdown of PDH reproduced the degranulation results and there is a correlation between the reduction in mitochondrial ATP production and inhibition of degranulation. In addition, the fact that PDH depletion inhibited both degranulation and cytokine secretion clearly indicates that this is a general phenomenon of mast cell exocytosis and not specific to a certain mediator, Since CPI-613 inhibited TNF- α and IL-6 in a more extent way than degranulation, we could conclude that cytokine secretion is more sensitive to the loss of mitochondrial ATP production. Since PDH inhibition by CPI-613 reduced degranulation induced by calcium ionophore, its effect on degranulation isn't limited only to IgE-Ag activation. CPI-613 failed to reduce degranulation in high levels of calcium ionophore, probably due to the fact that calcium specifically activates PDH activity.

341 PDH activity is regulated by 4 PDH kinases²⁷ which inhibit its activity and two PDH
342 phosphatases, PDP1 and PDP2²⁸ which enhance its activity. PDP1 has a calcium dependent
343 subunit which elevates its activity²⁹. It is well known that mast cell degranulation is
344 dependent on an increase in intracellular calcium concentration, which derives from
345 intracellular stores and from calcium influx^{30, 31}. The release of calcium from the ER is
346 mediated by the PLC γ signaling pathway³². Since calcium ions increase the association of
347 the calcium dependent subunit of PDP1 with the phosphorylated E1 α subunit of PDH, the
348 activation of PDH in mast cells could be calcium mediated. Interestingly, we have observed
349 two phases of enhanced PDH activity during mast cell activation (5 and 30 min). The second
350 phase could be a result of changes in calcium influx during mast cell activation^{33, 34} or an
351 unknown mechanism. Since after 30 min, mitochondria are known to reside next to the
352 nucleus²⁶, the purpose of the second phase of PDH activation could be to supply ATP for the
353 transcription process at the end of mast cell activation.

354 Having established the importance of PDH in exocytosis of mast cells, we investigated the
355 effect of PDH inhibition *in vivo*. In the acute systemic anaphylaxis model in mice, a marked
356 decrease in histamine secretion was observed when PDH was inhibited with CPI-613. We
357 also used a murine allergic asthma model, which is a more physiological model of allergy.
358 Allergic asthma involves airway obstruction and inflammation that is partially reversible with
359 medication. In this model, asthma is triggered by inhaled allergens such as dust mite or OVA.
360 Mast cells lie adjacent to nerves, blood vessels and lymphatics, which highlights their pivotal
361 importance in regulating allergic inflammatory processes, and mediators from degranulating
362 mast cells are critical to the pathology of the asthmatic lung. To determine the effect of
363 inhibition of PDH on allergic asthma, we assessed lung resistance after methacholine
364 challenge in OVA or saline exposed mice in the presence or absence of CPI-613. CPI-613
365 treatment significantly reduced the allergic response to methacholine challenge in sensitized

366 mice. Surprisingly, CPI-613 had no effect on the inflammation state of BALf and bronchial
367 submucosal. It would seem that the inhibition of mast cell degranulation by CPI-613 was not
368 enough to reduce lung inflammation and may be mediated by an effect on other mast cell
369 related pathologies in asthma such as tissue remodeling, enhanced mucus secretion and
370 smooth muscle cell contraction. Since CPI-613 treatment in non-sensitized mice had no effect
371 on airway resistance, CPI-613 has no direct effect on lung tissue. It is important to mention
372 that no toxicity following CPI-613 administration was observed both *in vitro* and *in vivo*
373 during the time frame of our experiments. CPI-613 originally was developed as an anticancer
374 drug with selectivity for tumor cells¹³ and it was determined well tolerated in a phase 2
375 study³⁵. This could be due to the fact that although the flow of pyruvate to the mitochondria is
376 inhibited, cells could still produce energy through alternative mitochondrial pathways such as
377 amino acid catabolism and beta oxidation of fatty acids. Therefore, CPI-613 may only affect
378 cells in which the phosphorylation state of PDH is essential for their activity.

379 Unexpectedly, we have discovered that MITF is a regulator of PDH activity. PDH associated
380 with MITF in 3 different protein interaction assays: Yeast two hybrid assay, co-
381 immunoprecipitation experiments and in-situ PLA assay. Over expression of MITF reduced
382 PDH activity, indicating that MITF is a negative regulator of PDH. In addition, we have
383 clearly shown that MITF is expressed in the mitochondria, the same cell compartment in
384 which PDH is active, as shown by mitochondrial fractionation and imaging flow cytometry.

385 Most importantly, since a form of MITF that lacks transcriptional activity also reduced PDH
386 activity we can conclude that the effect of MITF on PDH function is unlikely to be through
387 MITF's canonical role in transcription and that MITF plays a role outside the nucleus.

388 Interestingly, although both MITF-H and MITF-CE bind PDH, the leucine zipper domain of
389 MITF could play a role in PDH regulation, since the effect of MITF-H was greater than
390 MITF-CE. Overexpression of MITF-CE reduced the mitochondrial functions of ATP

production and oxygen consumption, presumably by inhibiting PDH. Since MITF detaches from PDH during mast cell activation at the same time as PDH is de-phosphorylated, we hypothesize that de-phosphorylation of PDH is involved in the detachment of MITF from PDH. This hypothesis is supported by the finding that treatment with DCA, which phosphorylates PDH, increased the association between MITF and PDH. Further research is needed to determine if modifications of mitochondrial MITF are involved in the interaction with PDH. In addition it will be interesting to determine if mitochondrial MITF has additional targets in the mitochondria besides PDH.

To conclude, the association of mitochondrial MITF with PDH emerges as an important regulator of mast cell function. Inhibiting PDH either by small molecules or by MITF down regulates mitochondrial activity which is essential for mast cell function. Together these findings indicate that PDH has a critical role in mast cell function and therefore could emerge as a new target for the manipulation of allergic diseases.

References

1. Erlich TH, Yagil Z, Kay G, Peretz A, Migalovich-Sheikhet H, Tshori S, et al. Mitochondrial STAT3 plays a major role in IgE-antigen-mediated mast cell exocytosis. *J Allergy Clin Immunol* 2014; 134:460-9.
2. Patel MS, Nemeria NS, Furey W, Jordan F. The Pyruvate Dehydrogenase Complexes: Structure-based Function and Regulation. *J Biol Chem* 2014; 289:16615-23.

- 414 3. Kaplon J, Zheng L, Meissl K, Chaneton B, Selivanov VA, Mackay G, et al. A key role
415 for mitochondrial gatekeeper pyruvate dehydrogenase in oncogene-induced senescence.
416 Nature 2013; 498:109-12.
- 417 4. Korotchkina LG, Sidhu S, Patel MS. Characterization of testis-specific isoenzyme of
418 human pyruvate dehydrogenase. J Biol Chem 2006; 281:9688-96.
- 419 5. Patel MS, Roche TE. Molecular biology and biochemistry of pyruvate dehydrogenase
420 complexes. FASEB J 1990; 4:3224-33.
- 421 6. Holness MJ, Sugden MC. Regulation of pyruvate dehydrogenase complex activity by
422 reversible phosphorylation. Biochem Soc Trans 2003; 31:1143-51.
- 423 7. Hodgkinson CA, Moore KJ, Nakayama A, Steingrimsson E, Copeland NG, Jenkins
424 NA, et al. Mutations at the mouse microphthalmia locus are associated with defects in a
425 gene encoding a novel basic-helix-loop-helix-zipper protein. Cell 1993; 74:395-404.
- 426 8. Hughes MJ, Lingrel JB, Krakowsky JM, Anderson KP. A helix-loop-helix transcription
427 factor-like gene is located at the mi locus. J Biol Chem 1993; 268:20687-90.
- 428 9. Moore KJ. Insight into the microphthalmia gene. Trends Genet 1995; 11:442-8.
- 429 10. Tassabehji M, Newton VE, Read AP. Waardenburg syndrome type 2 caused by
430 mutations in the human microphthalmia (MITF) gene. Nat Genet 1994; 8:251-5.
- 431 11. Kitamura Y, Morii E, Jippo T, Ito A. Effect of MITF on mast cell differentiation. Mol
432 Immunol 2002; 38:1173-6.
- 433 12. Haq R, Shoag J, Andreu-Perez P, Yokoyama S, Edelman H, Rowe GC, et al.
434 Oncogenic BRAF regulates oxidative metabolism via PGC1alpha and MITF. Cancer
435 Cell 2013; 23:302-15.
- 436 13. Zachar Z, Marecek J, Maturo C, Gupta S, Stuart SD, Howell K, et al. Non-redox-active
437 lipoate derivatives disrupt cancer cell mitochondrial metabolism and are potent anticancer
438 agents in vivo. J Mol Med (Berl) 2011; 89:1137-48.

14. Forster O, Stoklasa E. [On the mechanism of action of histamine and serotonin antagonists in experimental paw edema]. *Arch Int Pharmacodyn Ther* 1968; 174:144-51.
15. Zhou R, Tardivel A, Thorens B, Choi I, Tschopp J. Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat Immunol* 2010; 11:136-40.
16. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, et al. Succinate is an inflammatory signal that induces IL-1 β through HIF-1 α . *Nature* 2013; 496:238-42.
17. West AP, Brodsky IE, Rahner C, Woo DK, Erdjument-Bromage H, Tempst P, et al. TLR signalling augments macrophage bactericidal activity through mitochondrial ROS. *Nature* 2011; 472:476-80.
18. Jin Z, Wei W, Yang M, Du Y, Wan Y. Mitochondrial complex I activity suppresses inflammation and enhances bone resorption by shifting macrophage-osteoclast polarization. *Cell Metab* 2014; 20:483-98.
19. Chang CH, Curtis JD, Maggi LB, Jr., Faubert B, Villarino AV, O'Sullivan D, et al. Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* 2013; 153:1239-51.
20. Doughty CA, Bleiman BF, Wagner DJ, Dufort FJ, Mataraza JM, Roberts MF, et al. Antigen receptor-mediated changes in glucose metabolism in B lymphocytes: role of phosphatidylinositol 3-kinase signaling in the glycolytic control of growth. *Blood* 2006; 107:4458-65.
21. Gill T, Levine AD. Mitochondria-derived hydrogen peroxide selectively enhances T cell receptor-initiated signal transduction. *J Biol Chem* 2013; 288:26246-55.
22. Kaminski MM, Sauer SW, Klemke CD, Suss D, Okun JG, Krammer PH, et al. Mitochondrial reactive oxygen species control T cell activation by regulating IL-2 and

- IL-4 expression: mechanism of ciprofloxacin-mediated immunosuppression. *J Immunol* 2010; 184:4827-41.
23. Quintana A, Schwindling C, Wenning AS, Becherer U, Rettig J, Schwarz EC, et al. T cell activation requires mitochondrial translocation to the immunological synapse. *Proc Natl Acad Sci U S A* 2007; 104:14418-23.
24. Sena LA, Li S, Jairaman A, Prakriya M, Ezponda T, Hildeman DA, et al. Mitochondria Are Required for Antigen-Specific T Cell Activation through Reactive Oxygen Species Signaling. *Immunity* 2013; 38:225-36.
25. Dufort FJ, Bleiman BF, Gumina MR, Blair D, Wagner DJ, Roberts MF, et al. Cutting edge: IL-4-mediated protection of primary B lymphocytes from apoptosis via Stat6-dependent regulation of glycolytic metabolism. *J Immunol* 2007; 179:4953-7.
26. Zhang B, Alysandratos KD, Angelidou A, Asadi S, Sismanopoulos N, Delivanis DA, et al. Human mast cell degranulation and preformed TNF secretion require mitochondrial translocation to exocytosis sites: relevance to atopic dermatitis. *J Allergy Clin Immunol* 2011; 127:1522-31 e8.
27. Rowles J, Scherer SW, Xi T, Majer M, Nickle DC, Rommens JM, et al. Cloning and characterization of PDK4 on 7q21.3 encoding a fourth pyruvate dehydrogenase kinase isoenzyme in human. *J Biol Chem* 1996; 271:22376-82.
28. Huang B, Gudi R, Wu P, Harris RA, Hamilton J, Popov KM. Isoenzymes of pyruvate dehydrogenase phosphatase. DNA-derived amino acid sequences, expression, and regulation. *J Biol Chem* 1998; 273:17680-8.
29. Turkan A, Gong X, Peng T, Roche TE. Structural requirements within the lipoyl domain for the Ca²⁺-dependent binding and activation of pyruvate dehydrogenase phosphatase isoform 1 or its catalytic subunit. *J Biol Chem* 2002; 277:14976-85.

- 488 30. Tshori S, Razin E. Editorial: Mast cell degranulation and calcium entry--the Fyn-
489 calcium store connection. *J Leukoc Biol* 2010; 88:837-8.
- 490 31. Sanchez-Miranda E, Ibarra-Sanchez A, Gonzalez-Espinosa C. Fyn kinase controls
491 FcepsilonRI receptor-operated calcium entry necessary for full degranulation in mast
492 cells. *Biochem Biophys Res Commun* 2010; 391:1714-20.
- 493 32. Ryu SD, Lee HS, Suk HY, Park CS, Choi OH. Cross-linking of FcepsilonRI causes
494 Ca²⁺ mobilization via a sphingosine kinase pathway in a clathrin-dependent manner.
495 *Cell Calcium* 2009; 45:99-108.
- 496 33. Matthews G, Neher E, Penner R. Second messenger-activated calcium influx in rat
497 peritoneal mast cells. *J Physiol* 1989; 418:105-30.
- 498 34. Hide M, Beaven MA. Calcium influx in a rat mast cell (RBL-2H3) line. Use of
499 multivalent metal ions to define its characteristics and role in exocytosis. *J Biol Chem*
500 1991; 266:15221-9.
- 501 35. King C Lee CP, Inc. An Open Label, Phase 2 Trial to Evaluate the Safety, Tolerability
502 and Efficacy of CPI-613 in Cancer Patients. *ClinicalTrials.gov* [Internet]. National
503 Library of Medicine (US), 2015
- 504

Figure Legends

Fig.1 Inhibition of PDH decreases the degranulation and cytokine secretion levels in RBL cells and CBMC

(A-B) RBL cells **(A)** and BMMC **(B)** were sensitized with IgE and triggered with DNP-BSA for 5, 15 or 30 minutes. Afterwards the levels of the total and phospho-S293 PDH were determined by Western blot analysis. One representative out of 3 independent experiments is shown.

(C-D) RBL cells were sensitized with IgE, followed by 30 min DNP-BSA challenge. One hour before the addition of DNP-BSA, 300 μ M of CPI-613 was added to the medium. β -hexosaminidase **(C)** or TNF- α **(D)** release were then measured. Results represent mean $\pm$ SEM, n=3, *p<0.05.

(E) RBL cells were treated as in C and the levels of phospho-S293 PDH were determined by Western blot analysis. One representative out of 3 independent experiments is shown.

(F-G) RBL cells were transfected with siRNA targeted to PDHE1- α , 48 h later β -hexosaminidase release was measured (F). WB showing the levels of PDHE1- α in cells transfected with siPDHE1- α compared to cells transfected with non-targeted siRNA.

(H-I) IgE- sensitized CBMC were incubated with 300 μ M of CPI-613 for 3h and then activated with 5 μ g/ml rabbit α -human-IgE 30min (β -hexosaminidase) or overnight (IL-6 release). % β -hexosaminidase release **(H)** and IL-6 (pg/ml) release **(I)** were assayed. Results represent mean $\pm$ SEM, n=3, *p<0.05.

Fig.2 Inhibition of PDH decreases histamine secretion and lung resistance but not inflammation in mice.

(A) Mice were sensitized with IgE anti-DNP through injection to the tail vein, and injected i.p with 50 mg/kg of CPI-163, saline was used for control. Twenty four hours later, mice were injected with DNP-BSA for 1.5 min. Plasma was collected, and histamine levels were

measured by using competitive ELISA. Results represent mean $\pm$ SEM, n (Control)=4; n (CPI-613)=5, *p<0.05.

(B-C) Increase in lung resistance (R) in response to methacholine measured in mice sensitized and challenged with OVA with and without CPI-613 during the challenge periods compared to that in non-sensitized, non-challenged mice (saline control). **(B)** Response to various doses of MCH, **(C)** Response to maximal dose of MCH. Values represent means $\pm$ SEM, 10 mice per group; p<0.05 compared to OVA group or saline.

(D-E) Inflammatory cell counts in OVA-induced allergic asthmatic mice administered CPI-613 (75 mg/kg) or saline i.p. during the challenge period compared to those in non-sensitized mice (saline control). **(D)** Represents the total cell number/ml in the BAL fluids and **(E)** represents the differential count of the BALf cells. Values represent means $\pm$ SEM, 10 mice per group; *p<0.05 compared to OVA or saline group.

Fig.3 The role played by PDH phosphorylation on MITF-PDH interaction in IgE-Ag activation of RBL cells.

(A) Immunoprecipitation of MITF in RBL lysates using anti-MITF antibody for immunoprecipitation (IP) and anti PDH-E1 α antibody for Western blot analysis. One representative out of 3 independent experiments is shown.

(B) Immunoprecipitation of PDH-E1 β in 293T cells that were transfected with MITF-H-flag tag lysates using anti PDH-E1 β antibody for IP and anti-Flag tag antibody for Western blot analysis. One representative out of 3 independent experiments is shown.

(C) Proximity ligation assay (PLA). Fixed and permeabilized RBL cells were incubated with anti-MITF and anti-PDHE1- α antibodies and detected by DuoLink probe (Sigma-Aldrich, St Louis, Mo). Scale bar=1 μ m. The blue dye (DAPI) represents the nucleus and the red dots indicate the positive PLA signal. The red arrows indicate the

representative positive PLA signals. One representative out of 3 independent experiments is shown.

(D) Immunoprecipitation in quiescent or 5 min IgE-DNP activated RBL cell lysates using anti-MITF antibody for immunoprecipitation (IP) and PDH-E1 α antibody for Western blot analysis. One representative out of 3 independent experiments is shown.

(E) Immunoprecipitation in control or CPI-613 treated RBL cell lysates using anti-MITF antibody for IP and anti-PDH antibody for Western blot analysis. One representative out of 3 independent experiments is shown.

Fig.4 MITF is localized to the mitochondria of RBL cells

(A) RBL cells were fractionated into cytosol and mitochondria. Mitoplasts were prepared by proteinase K treatment. The levels of MITF, GAPDH, and Bcl-XL were determined by Western blot analysis. One representative out of 3 independent experiments is shown.

(B-G) Quantification of mitochondrial MITF localization - To visualize MITF, RBL cells were stained with mouse anti MITF (C5) antibody followed by CY2-conjugated anti-mouse secondary antibody. Hoechst staining was used to visualize nuclei. To visualize mitochondria rabbit anti complex1 antibody followed by rhodamine conjugated anti rabbit secondary antibody were used. To visualize HDAQ1, a nuclear marker, mouse anti HDAQ1 and CY2-conjugated anti-mouse secondary antibody were used. To visualize, Hsp60, a mitochondrial marker, mouse HSP60 antibody and anti-mouse CY2-conjugated secondary antibody were used. Afterwards, mitochondrial localization of MITF was evaluated by ImageStream flow cytometry. The localization of HDAQ1 and HSP60 were used as a validity of the staining method. For each treatment, 30,000 cells were collected. The cells that were included in the quantification were not in the mitotic state. Positive single cells were gated, and mitochondrial expression of MITF was plotted. In order to reduce unspecific plotting the nuclei staining area was omitted. **(B)** Quantification of the similarity between the CY2

stained MITF and Hoechst stained nuclei, which represents the localization of MITF to the nuclei. **(C)** Quantification of the similarity between the CY2 stained MITF and Rhodamine stained mitochondria which represents the localization of MITF in the mitochondria. **(D)** Quantification of the similarity between the CY2 stained HDAQ1 (nuclear marker) and Hoechst stained nuclei, which represents the validity of the nuclear staining **(E)** Quantification of the similarity between the CY2 stained HDAQ1 and rhodamine mitochondria, which represents the validity of the mitochondrial staining **(F)** Quantification of the similarity between the CY2 stained HSP60 and Hoechst stained nuclei which represents the validity of the mitochondrial staining **(G)** Quantification of the similarity between the CY2 stained HSP60 (mitochondrial marker) and the rhodamine stained mitochondria.

Fig.5 Overexpression of MITF-H and MITF-CE lowered PDH's activity and increases pyruvate levels in RBL cells.

(A) RBL cells were transfected with EV, MITF-H or MITF-CE. 48 h later cells were lysed and PDH activity was determined by PDH activity ELISA kit-was used to determine PDH activity in the different lysates. Results represent means $\pm$ SEMs (n = 3); *p < 0.05.

(B) RBL cells were transfected with EV, MITF-H or MITF-CE, 48 h later the cells were lysed and the levels of pyruvate were determined using an ELISA kit. Results represent means $\pm$ SEMs (n = 3), *p < 0.05.

Fig.6 Overexpression of MITF-H and MITF-CE lowered the ATP and oxygen consumption levels in RBL cells.

(A) RBL cells were transfected with EV, MITF-H or MITF-CE, 48 h later the cells were lysed, and then total cell ATP levels were measured. Results represent means $\pm$ SEMs (n = 3), *p < 0.05.

604 **(B)** Oxygen consumption analysis. Cells were transfected as for A and then intracellular
605 oxygen consumption was determined with the Seahorse Extracellular Flux Analyzer.
606 Results represent means $\pm$ SEMs (n = 3) *p < 0.05.

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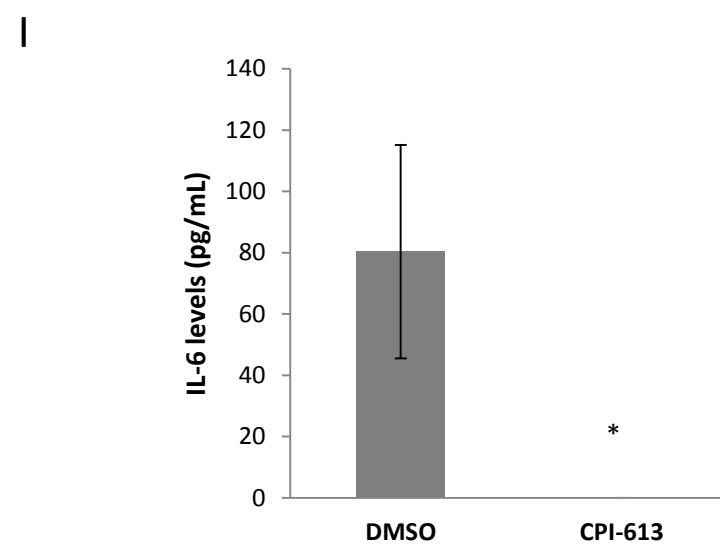
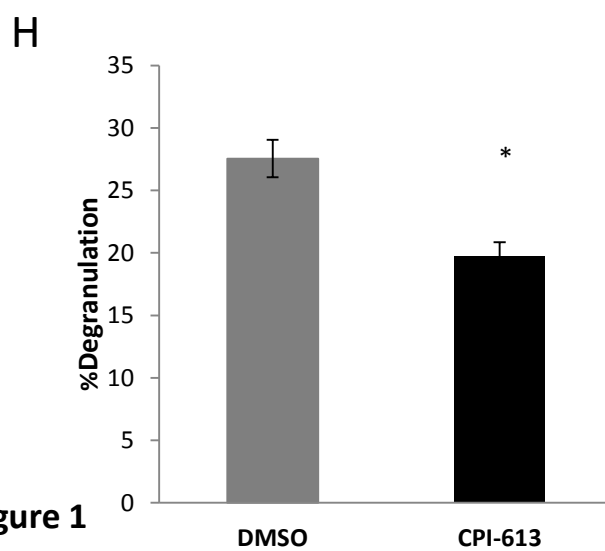
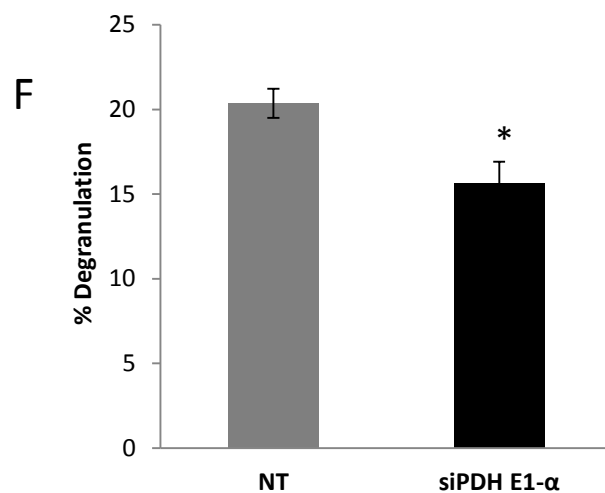
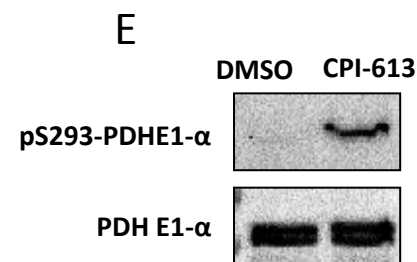
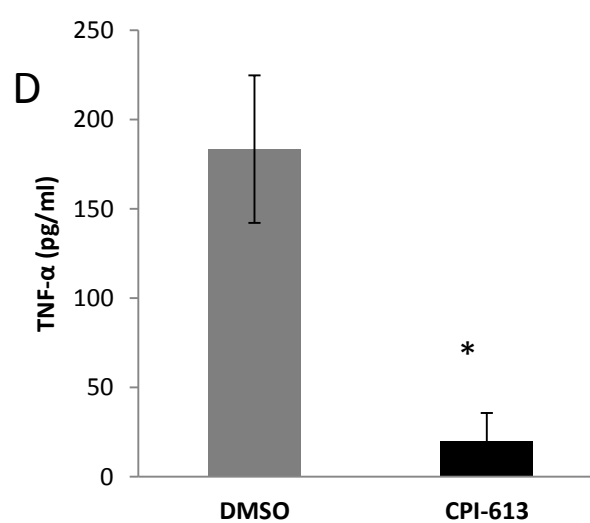
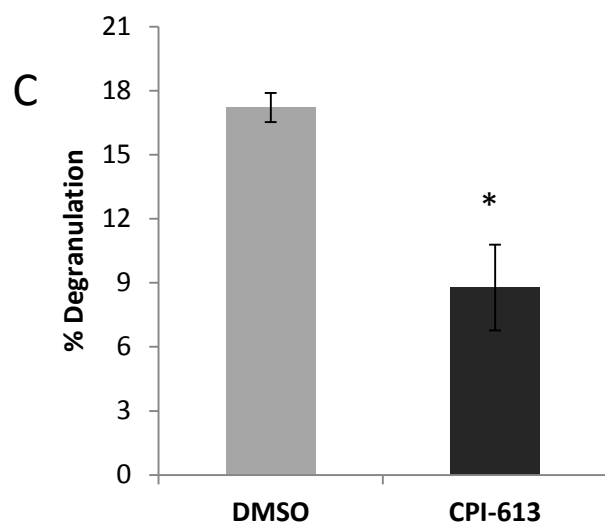
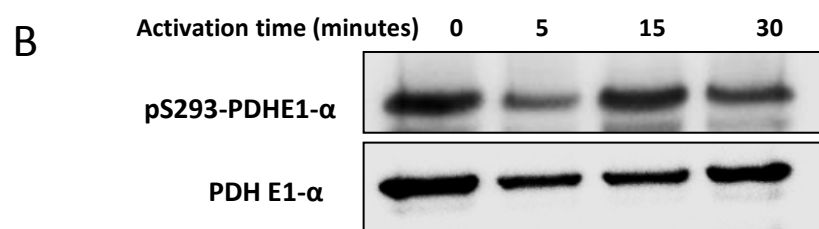
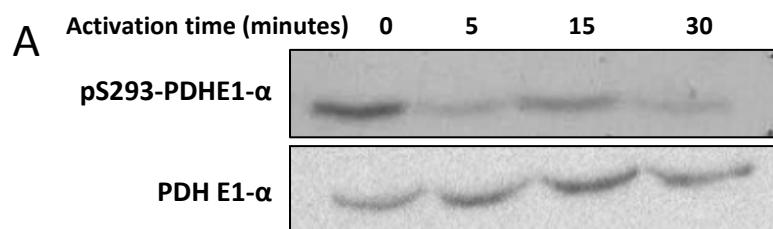


Figure 1

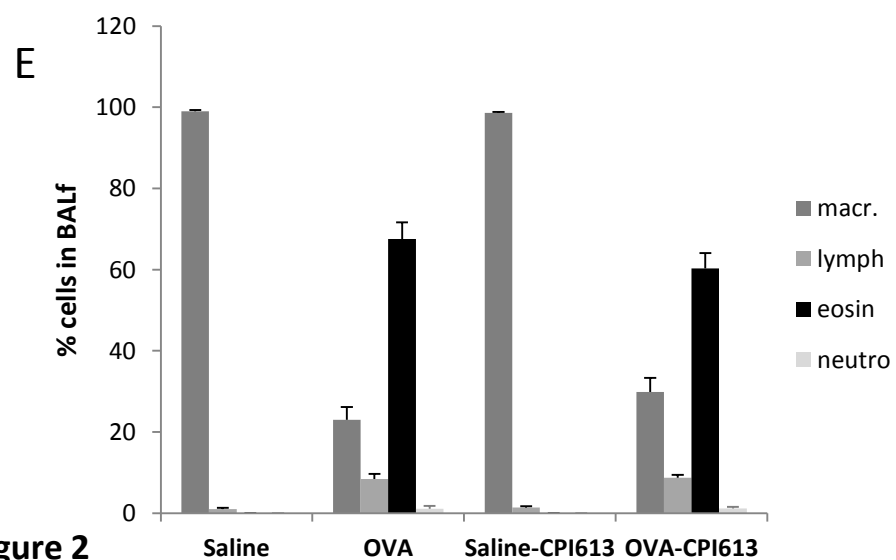
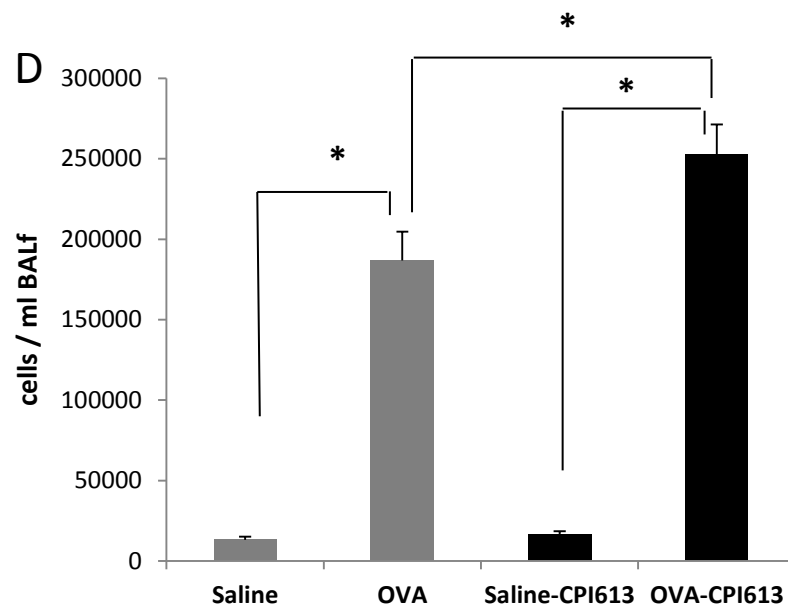
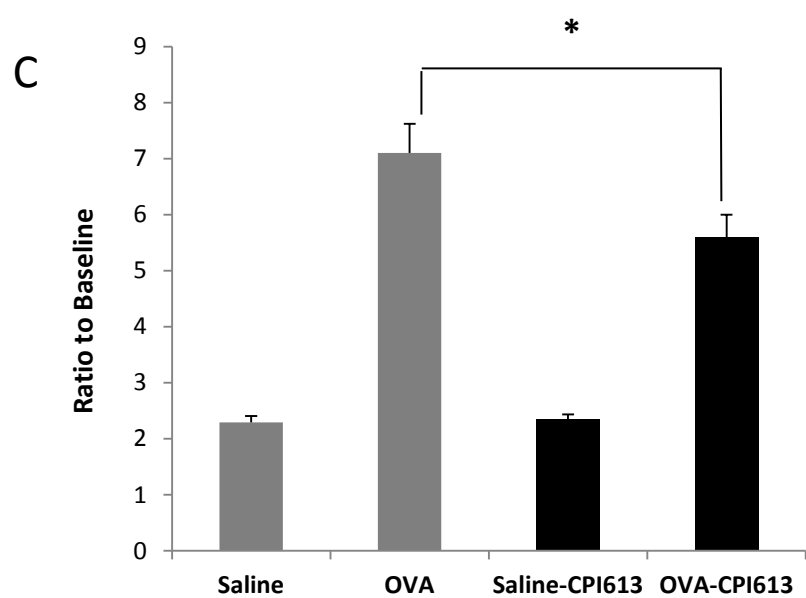
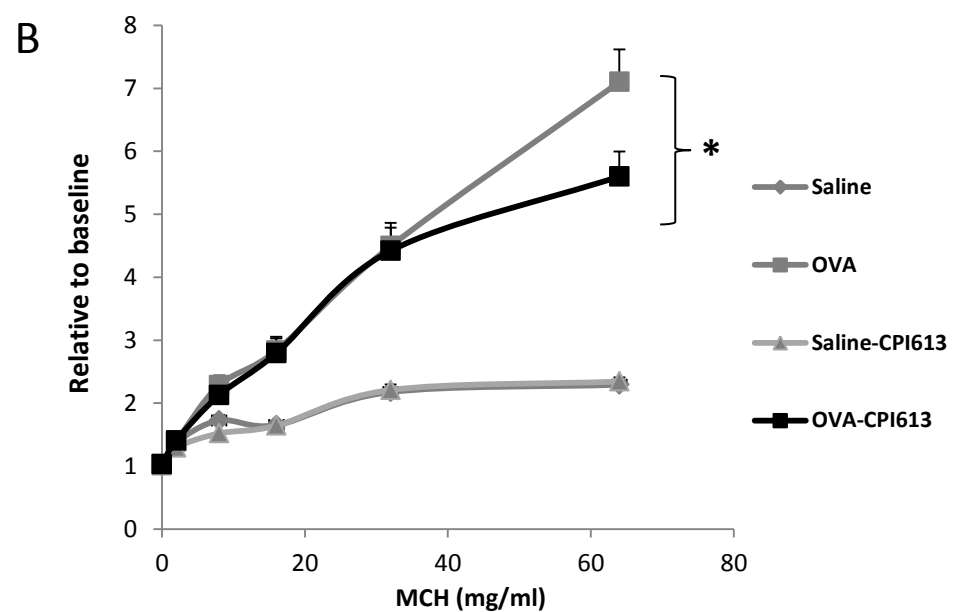
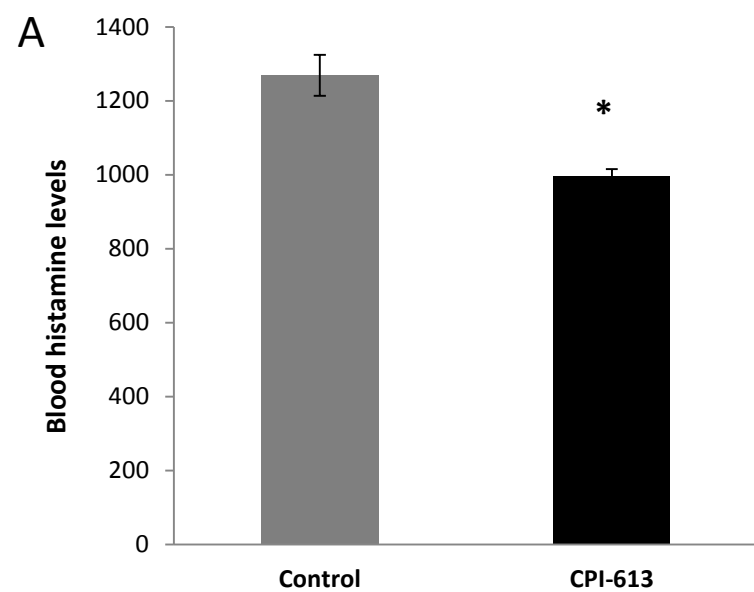


Figure 2

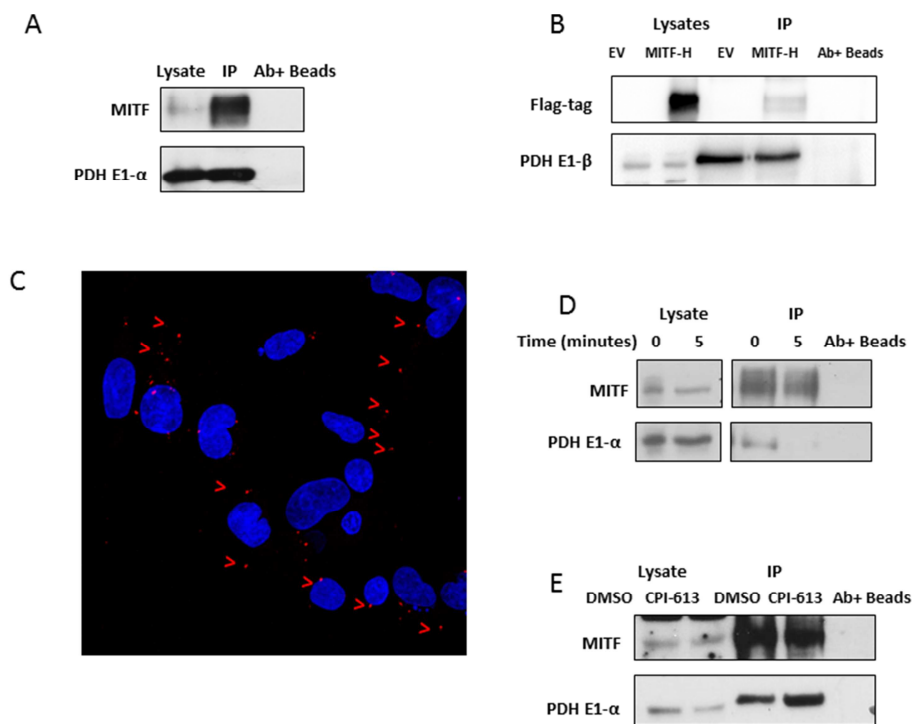


Figure 3

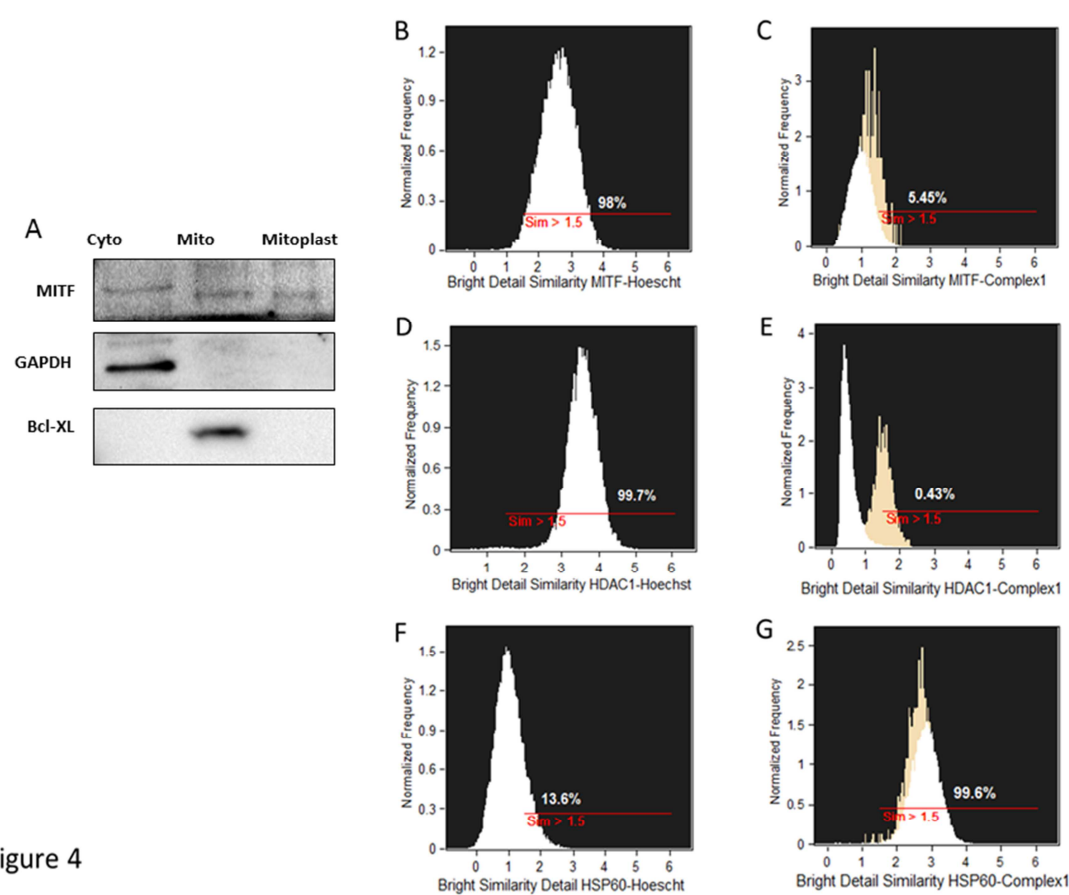


Figure 4

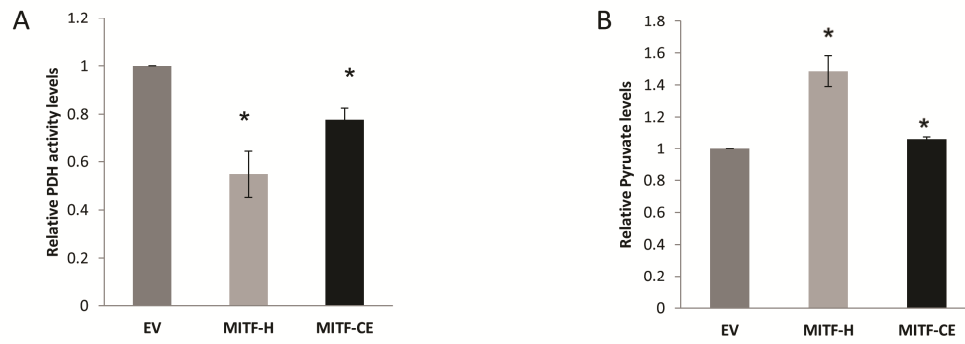


Figure 5

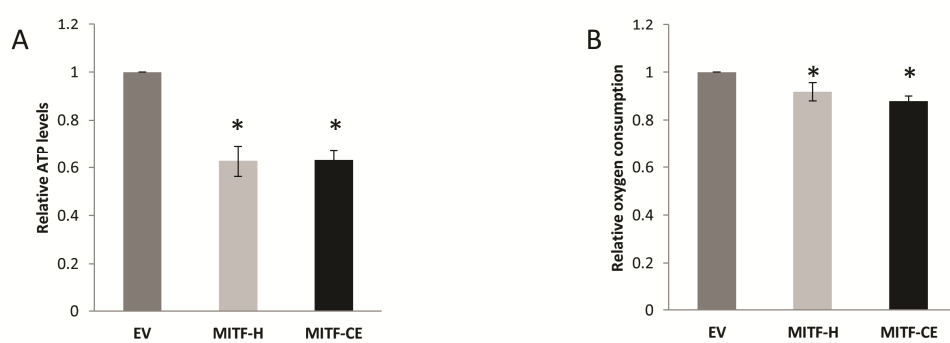


Figure 6



Materials and Methods:

Mice

Male and female C3H wild-type mice aged 6 to 7 weeks and Balb/c male mice aged 8 to 10 weeks were purchased from Jackson Laboratories (Cambridge, Mass). Mice were fed a regular diet and given drinking water *ad libitum*. Experiments were approved by the Hebrew University Ethical Committee for Animal Experimentation.

Antibodies

Anti-MITF C5 was provided by David Fisher (Harvard Medical School). Rabbit anti-MITF was purchased from Sigma-Aldrich (St Louis, Mo). Rabbit anti PDH-E1 α (used for WB), rabbit anti-Bcl-XL, rabbit anti-flag tag and rabbit anti-Capase-3 antibodies were purchased from Cell Signaling Technologies (Beverly, Mass). Mouse anti- β -actin antibody, Rabbit anti-phospho-S293 (used for WB in BMMC) and mouse anti-HSP60 was purchased from Abcam (Cambridge, Mass). Goat anti GAPDH antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, Calif). Rabbit anti-NUSD1 (complex1), Rabbit anti PDH-E1 α (used for PLA), and PDH-E1 β antibodies were purchased from Proteintech (Rosemont, IL.). Rabbit anti-phospho-S293 antibody was purchased from Merck-Millipore (Billerica, Mass).

Chemical inhibitor treatment

CPI-613 was purchased from Sigma-Aldrich. The inhibitor was dissolved in 100% DMSO (Sigma-Aldrich), according to the manufacturer's instructions, for *in vitro* experiments and in saline for *in vivo* experiments.

Cell culture

RBL-2H3 cells were maintained in RPMI 1640 medium without sodium pyruvate or L-glutamine, supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL of penicillin, and 100 μ g/mL of streptomycin (Biological Industries), as previously described.¹ Unless stated

otherwise complete medium has been used for the experiments. For mitochondrial ATP determination, a galactose (5 mmol/L), containing, glucose free RPMI 1640 medium without sodium pyruvate or L-glutamine, supplemented with 10% Dialyzed FBS, 2 mM L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin (Biological Industries) was used. BMBCs were cultured in RPMI conditional medium, as previously described.^{2, 3} Cells were generally grown for a minimum of 4 weeks and used when greater than 90% of the population was positive for toluidine blue staining. BMBCs and RBL cells were sensitized first with anti-DNP IgE mAb (SPE-7, Sigma-Aldrich), washed in PBS and then challenged with DNP (Sigma-Aldrich).

CBMC culture and activation

CBMCs were prepared from umbilical cord blood of healthy donors according to a well-described method.⁴ Briefly, fresh cord blood diluted in Hank's solution was loaded on Ficoll-Hypaque (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and centrifuged (350 *g* for 25 min). Mononuclear cells were washed twice and resuspended in MEM- α medium supplemented with 10% heat-inactivated FBS, 100 U/mL of penicillin, 100 µg/mL streptomycin, and 10 µg/mL ribonucleosides. All media and supplements were purchased from Biological Industries (Beit Haemek, Israel). CBMC growth media also contained recombinant human stem cell factor (100 ng/mL; PeproTech, Rocky Hill, NJ), human IL-6 (10 ng/mL, PeproTech), and prostaglandin E₂ (0.3 µmol/L, Sigma-Aldrich). Cells were maintained in a humidified incubator (37°C at 5% CO₂), with media replaced on a weekly basis. After a 6- to 8-week culture period, the CBMCs were examined for maturity by using acidic toluidine blue staining (positive >90%) and for viability by using Trypan blue exclusion (negative >95%) and used thereafter.

CBMCs were sensitized by the addition of human myeloma IgE (0.3 µg/mL; Calbiochem-Merck, Schwalbach, Germany) and human IL-4 (0.10 ng/mL, PeproTech) to cell cultures for

4 days in a humidified incubator (37°C at 5% CO₂). CBMC (1 × 10⁵/100 µL MEM-α medium with supplements and recombinant human stem cell factor) were washed in PBS and pre-incubated with 300 µmol/L CPI-613 in DMSO or DMSO vehicle for 3 h at 37°C. Then cells were centrifuged, washed in PBS and activated by addition of rabbit α-human-IgE (5 µg/mL; Dako, Glostrup, Denmark) for 30 min (beta hexosaminidase) or overnight (IL-6 release) in T++ buffer (137 mmol/L NaCl, 12 mmol/L NaHCO₃, 5.5 mmol/L L-glucose, 2 mmol/L KCl, 0.3 mmol/L Na₂HPO₄, 1.8 mmol/L CaCl₂, and 0.9 mmol/L MgCl₂). Overnight activation was performed in CBMC medium. Supernatants and cell pellets were collected for assessment of β-hexosaminidase release and supernatants for IL-6 release.

Proximity ligation assay

Intracellular protein–protein interactions were examined using the Duolink PLA kit (Olink Biosciences, Sigma-Aldrich). In brief, cells were cultured on coverslip slides, fixed with 4% paraformaldehyde for 20min at room temperature, and permeabilized with 0.5% (vol/vol) Triton X-100 in phosphate-buffered saline. Primary antibodies were incubated overnight at 4°C and samples processed according to the instructions provided. The cells were visualized using a Zeiss LSM 710 Axio Observer.Z1 laser scanning confocal microscope.

Mitochondrial isolation

RBL cells were washed once in cold PBS before homogenization. Cells (20 × 10⁶) were homogenized in buffer A (320 mmol/L sucrose, 5 mmol/L Tris, and 2 mmol/L ethyleneglycol-bis-[β-aminoethylether]-N,N,N',N'-tetraacetic acid, pH 7.4) with 10 strokes of a Teflon Dounce homogenizer. The procedure was performed at 4°C. The homogenate was centrifuged for 3 min at 2000 *g*, and the supernatant was kept. This was repeated twice, and the supernatants were combined and then centrifuged for 3 min at 2000 *g* to remove the

nuclei and cell debris. The supernatant was transferred to a clean tube and centrifuged for 10 min at 12,000 *g* to pellet mitochondria, washed twice, resuspended in a small volume of buffer A, and stored at -70°C. The mitochondrial pellet was resuspended in buffer A with the addition of 0.015 mg/ml proteinase K to prepare mitoplasts. The pellet was resuspended and washed twice in large volumes of digitonin-free buffer A.

Measurement of pyruvate dehydrogenase complex activity

Pyruvate dehydrogenase complex was measured at room temperature by using an ELISA kit from Mitoscience (Cambridge, Mass).

Expression constructs

MITF-H was amplified by PCR and then excised and ligated into pCMV-Tag 4A vector (Agilent Technologies) using XbaI and HindIII (New England Biolabs, Inc.).

MITF deletion construct was created by using Q5 site-directed mutagenesis kit (New England Biolabs, Inc.) on the original pCMV-MITF-H vector. The following primers were used to generate the mutated MITF-CE from the full length MITF-H: ceDel F GTCGACCTCGAGGATTAC and ceDel R TTGCTGTTCCCGTTGCAA.

The PDH-E1 β subunit plasmid was purchased from the Harvard plasmid bank (clone ID:MmCD00315349).

Transfection

RBL cells: Amaxa Nucleofector technology (Amaza, Cologne, Germany) was used to transfect cells. For plasmid transfection a total of 2×10^6 RBLs 2 μ g of each plasmid was used. Briefly, the cells were resuspended in 100 μ L of Ingenio solution (Mirus, Madison, Wisconsin), DNA (plasmid) was added, and the mixture was transferred to an electroporation cuvette. Electroporation was performed with the T-20 program for RBLs and T-17 for BMBCs.^{3, 5}

HEK293T cells: The cells were co-transfected using Jetprime reagent (Invitrogen) with 5 µg pCMV-EV as control or pCMV MITF-H or pCMV MITF-CE mutant. All cells were transfected with additional 5 µg pCMV-SPORT PDH-E1β. Twenty four hours after the transfection the cells were lysed and immunoprecipitation was conducted.

Coimmunoprecipitation (co-IP)

BMMC, RBL cells and HEK293T cells were used in various co-immunoprecipitation experiments. Cells ($5-10 \times 10^6$) were lysed by the addition of 400 µl cold RIPA lysis buffer (0.01 M Tris-HCl [pH 7.4], 1% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 0.15 M NaCl, 0.25 µM phenylmethylsulfonyl fluoride) and 25 µl of protease inhibitor (Sigma-Aldrich). Cells were then homogenized, and their supernatants were collected after 15 min centrifugation in a microcentrifuge at 14000 *g* at 4°C. Recovered lysates were incubated overnight at 4°C with anti-rabbit flag tag, anti-mouse MITF C5 or with anti-rabbit PDH-E1β. The next day, 20 µl protein A/G agarose beads (Pierce) were added to the lysate prebound to the antibody and incubated with agitation 2 h at 4°C. The recovered immune complexes were washed three times with cold PBS, 40 µl of sample buffer (5 ml Glycerol, 1 g SDS, 2.56 ml β-mercaptoethanol, 2.13 ml 0.5 M Tris HCL pH 6.8, bromophenol blue for color) was added to the prewashed beads and then the beads with the sample buffer were incubated at 100°C for 10 min.

β-hexosaminidase release assay (degranulation assay)

RBL cells (1×10^4) were immunologically activated, as described above. After IgE incubation, the cells were washed 3 times with 5 mL degranulation buffer (130 mmol/L NaCl, 5 mmol/L KCl, 5.6 mmol/L glucose, 1 mmol/L MgCl₂, 1.2 mmol/L CaCl₂, 10 mmol/L HEPES [pH 7.4], and 0.1% BSA) and resuspended in 200 µL of the same buffer with DNP (5 ng/ml) for 30 min. β-hexosaminidase release was determined in triplicates in a 96-well plate. Aliquots (20 µL) of supernatants and cell lysates were incubated for 30 min with 100 µL of

substrate solution (1.3 mg/mL *p*-nitrophenyl- β -D-2-acetamido-2-deoxyglucopyranoside in 0.1 mol/L citrate, pH 4.5). The reaction was stopped by the addition of 200 μ L of 0.2 mol/L glycine (pH 10.7). The plate OD was read in an ELISA reader at a wavelength of 405 nm. Percentage release values for each experimental condition were calculated.

ATP determination

RBL cells were transfected with either empty vector (EV), MITF-H or MITF-CE, and cultured for 48 h. Twenty four hours prior to the ATP assay, the growth medium was changed to a glucose-free medium (RPMI 1640 without D-glucose, sodium pyruvate, or L-glutamine; Biological Industries), supplemented as above.

Mitochondrial ATP levels were measured using the ATPLite luminescence-based assay according to the manufacturer's instructions (Perkin Elmer, Waltham, MA, USA) and are expressed as levels relative to control cells that were transfected with p-CMV empty vector.

Oxygen consumption

RBL cells were transfected with either empty vector (EV), MITF-H or MITF-CE, and cultured for 48 h. Twenty four hours prior to the oxygen measurement, the growth medium was changed to a glucose-free medium (as above). The oxygen consumption rate was measured with an XF24 extracellular flux analyzer (Seahorse Biosciences, North Billerica, Mass.)

Gel electrophoresis and Western blots

RBL and HEK293T cells and mitochondrial pellets were lysed by the addition of lysis buffer (50 mmol/L Tris-HCl [pH 7.4], 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, and 1 mmol/L phenylmethylsulfonyl fluoride) supplemented with 15 μ L protease inhibitor (Sigma-Aldrich). Cells and mitochondria were vortexed and incubated on ice for 15 min. The lysates were then centrifuged at 14,000 *g* for 15 min, and pellets were discarded. The protein concentration of each sample was determined by using Bradford

reagent (Sigma-Aldrich). Proteins were resolved by using 10% to 15% SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, Mass). The membranes were incubated in 5% fat-free milk and TBST (10 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, and 0.1% Tween 20) for 1 h and then in 5% BSA and TBST containing various dilutions of primary antibodies for 18 h at 4°C. The membranes were washed 3 times with TBST for 5 min before and after each incubation with secondary antibody. The proteins were detected with the appropriate secondary antibody (one hour at room temperature) coupled to horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody and visualized by means of chemiluminescence, according to the manufacturer's instructions (Pierce, Cheshire, United Kingdom).

ImageStream flow cytometry

For ImageStream analysis (Amnis, part of EMD Millipore, Seattle, Wash), RBL cells were washed in PBS, fixed in 4% paraformaldehyde, permeabilized with PBS with 90% cold methanol, and blocked in PBS containing 0.5% BSA. Anti-complex1 antibody and donkey anti-rabbit IgG(H+L) were used to stain the mitochondria. Anti-MITF C5 antibody and donkey anti-mouse IgG(H+L) were used to stain the MITF. Hoechst 33258 (Sigma-Aldrich) was used to stain nuclei. Fluorescence imaging was carried out with the ImageStream flow cytometer (Amnis). Data analysis was performed with IDEAS software, version 6.10, with compensation according to the software standards. Cells were gated for single cells, using the area and aspect ratio features, and for focused cells, using the Gradient RMS feature, as previously described.⁶ The colocalization of MITF1 and the mitochondria (labeled by complex1) was quantified using the bright detail similarity feature (the log transformed Pearson's correlation coefficient of the localized bright spots in the two input images).⁶ Images of cells with similarity values above 1.5 were considered to display a localized MITF1 in the mitochondria. To identify mitotic cells, The G2/M population was further gated

using 2 parameters: the bright detail intensity of Hoechst staining (intensity of localized bright areas, subtracted for the local background), and the area of the 50% highest intensity pixels of the nuclear staining; Mitotic cells show higher intensity and lower area.

***In vivo* histamine release assay**

Wild-type C3H mice were sensitized with 3 µg murine IgE anti-DNP mAb SPE-7 by means of intravenous injection in the tail vein on the same day mice were injected i.p. with 50 mg/kg CPI-613 dissolved in saline or saline alone for 24 h, and then mice were challenged with i.v. injection of DNP (250 µg/mouse). 100 µL blood was collected immediately and added to an equal volume of pre-cooled Alsever solution. Blood was centrifuged at 2000 *g* for 20 min, and the plasma supernatant was collected. Plasma histamine levels were determined using a competitive ELISA (IBL International GmbH, Hamburg, Germany).

Allergen challenge protocol

C3H male mice aged 8 to 10 weeks were sensitized and challenged with OVA to induce allergic inflammation. Mice received i.p. injections of 10 µg OVA and 1 mg Al(OH)₃ in 0.5 mL saline at days 1 and 10. One week after the last sensitization (on days 14-18), the mice were challenged with OVA aerosols (2% in saline) for 20 min each day for 3 days while animals of the control group were challenged with saline.

Mice were fed *ad libitum* with standard mouse chow. The PDH inhibitor CPI-613 was administered as a single i.p. injection (180 µL, 75 mg/kg) 24 h prior to the end of the experiment.

Assessment of airways hyper-responsiveness (AHR)

Twenty four hours after the last challenge, mice were anesthetized using ketamine/xylazine, tracheostomized and ventilated with a FlexiVent ventilator system (SCIREQ, Montreal, Quebec, Canada). After baseline determination of airway resistance, mice were challenged with 0 to 64 mg/ml methacholine (MCH) nebulized directly into the ventilatory circuit using

an AeroNebLab nebulizer (SCIREQ). The resistance parameter (R) was used to assess airway hyperresponsiveness. Results were expressed as relative increase in R over base line values.

Analysis of the bronchoalveolar lavage fluid (BALf)

BALf was centrifuged at 200 *g* for 10 min and the supernatants were stored at -20° C for further examination. Cell pellets were resuspended in 1 mL saline and aliquots of 100 µL were used for cytospin preparations using a Shandon cytospin (Thermo Fisher Scientific, Waltham, MA, USA), and stained using Diffquik stain (Dade Diagnostica, Munich, Germany). A total of 200 cells were counted and classified for each slide. Total BALf cells were counted using a Newbauer Chamber (Marien-feld, Lauda-Königshofen, Germany). Results are expressed as percent of different cell types in BALf.

Yeast two hybrid assay:

The assay was performed by Dualsystems Biotech AG. Grabenstrasse, Switzerland. The assay was conducted using MITF-H as bait.

Yeast two hybrid screen -the bait's sequence (5' → 3'):

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ATGGAGGCGCTTAGaTTTGAGATGCTCATCCCCTGCTCCTTTGAAAGCCTGTGTCTCAG
TTCTGCAGAGCATTCTGGGGCTCCAAGCCTCCGTTAAGCTCCTCCACTATGACATCAGC
CATCTTGCTACGCCAGCAACTCATGCGTGAGCAGATGCAGGAGCAGGAGCGCAGGGAGCA
GCAGCAGAAGCTGCAGGCAGCCAGTTTCATGCAACAGAGAGTGGCCGTGAGTCAGACACC
AGCCATAAACGTCAGCGTGCCCAACACCTTCCCTCTGCCACCCAGGTGCCGATGGAAGT
CCTTAAGGTGCAGACCCACCTGGAAAACCCACCAAGTACCACATACAGCAAGCTCAGAG
GCACCAGGTAAAGCAGTACCTTTCTACCACTTTAGCAAATAAACATGCCAGCCAAGTCCT
GAGCTCACCATGTCCAAACCAGCCTGGCGACCATGCCATGCCACCAGTGCCGGGGAGCAG
CGCACCCAACAGCCCTATGGCTATGCTCACTCTTAACCTCCAAGTGTGAAAAAGAGGCATT
TTATAAGTTTGAGGAGCAGAGCAGGGCAGAGAGTGAGTGCCAGGTATGAACACGCACTC
TCGAGCGTCGTGCATGCAGATGGATGATGTAATTGATGACATCATCAGCCTGGAATCAAG
TTATAATGAAGAAATTTTGGGCTTGATGGATCCGGCCTTGCAAATGGCAAATACGTTACC
CGTCTCTGGAACTTGATCGACCTNTACAGCAACCAGGGCCTGCCACCGCCAGGCCTTAC
CATCAGCAACTCCTGTCCAGCCAACCTTCCCAACATAAAAAAGGGAGCTCACAGAGTCTGA
```

228 AGCAAGAGCATTGGCTAAAGAGAGGCAGAAAAAGGACAATCACAACCTTGATTGAACGAAG
 229 AAGAAGATTTAACATAAACGACCGCATTAAAGGAGCTAGGTACTCTGATCCCCAAGTCAAA
 230 TGATCCAGACATGCGGTGGAACAAGGGAACCATCTCAAGGCCTCTGTGGACTACATCCG
 231 GAAGTTGCAACGGGAACAGCAACGAGCTAAGGACCTTGAAAACCGACAGAAGAAGCTGGA
 232 GCATGCGAACCGGCACCTGCTGCTCAGAGTACAGGAGCTGGAGATGCAGGCTAGAGCGCA
 233 TGGACTTTCCCTTATCCCATCCACCGGTCTCTGCTCGCCTGATCTGGTGAATCGGATCAT
 234 CAAGCAAGAACCAGTTCTTGAGAACTGCAGCCAGGAACTTGACAGCACCAGGCAGACCT
 235 GACATGTACGACAACCTCTGGATCTCACGGACGGTACCATCACCTTTACCAACAACCTCGG
 236 CACCATGCCGGAGAGCAGCCCGCCTACAGCATCCCCAGGAAGATGGGCTCCAACCTTGA
 237 AGACATCCTGATGGACGATGCCCTCTCACCTGTTGGAGTCACCGACCCACTGCTGTCATC
 238 AGTGTGCCAGGAGCTTCAAAAACAAGCAGCCGGAGGAGCAGTATGAGCGCAGAAGAAAC
 239 GGAGCATGCGTGT

240 Statistical analysis

241 Either the 1-sample *t* test or the 2-tailed Student *t* test was performed, when appropriate. Data
 242 are reported as means $\pm$ SEMs.

243

244 Supplementary Figure Legends:

245 Figure E.1 :

246 **(A)** Densitometry of pS293PDH/ PDH-E1 α levels in quiescent (0 min) or immune-activated
 247 for 5, 15 or 30 minutes in RBL and BMMC cells. Results represent means $\pm$ SEMs (*n* = 3),
 248 **p* < 0.05.

249 **(B)** RBL cells were incubated with DMSO or 50, 200 or 300 μ M of CPI-613 for 15, 30 or 60
 250 minutes then degranulation levels were estimated by measuring β -hexosaminidase release.

251 Results represent means $\pm$ SEMs (*n* = 3), **p* < 0.05.

252 **(C)** Densitometry of pS293PDH/ PDH-E1 α levels in RBL cells that were treated with 300
 253 μ M CPI-613 for 1 h or DMSO as control. Results represent means $\pm$ SEMs (*n* = 3), **p* < 0.05.

(D) RBL cells were cultured in OXPHOS dependent medium for 24 h and then incubated with DMSO or 50, 200 or 300 μ M of CPI-613 for 15, 30 or 60 minutes then mitochondrial ATP levels were measured by ATP-lite kit. Results represent means $\pm$ SEMs (n = 3), *p < 0.05.

(E) RBL cells were cultured in complete medium for 24 h and then incubated with DMSO or 50, 200 or 300 μ M of CPI-613 for 15, 30 or 60 minutes then ATP levels were measured by ATP-lite kit. Results represent means $\pm$ SEMs (n = 3), *p < 0.05

(F) RBL cells were incubated with 300 μ M CPI-613 for 1h or with DMSO, cells were lysed and the levels of β -actin, caspase 3 or cleaved caspase 3 by Western blot analysis. One representative experiment out of three is shown.

(G) Densitometry of PDH-E1 α / β -actin levels in RBL cells that were transfected with either siPDH E1a or non-targeted siRNA and cultured for 48h. Results represent means $\pm$ SEMs (n = 3), *p < 0.05.

(H) Degranulation levels in RBL cells that were activated with different concentrations (1.25, 2.5, 5 or 40 μ g/ml) of calcium ionophore A23187 for 30 minutes, 1h prior to the activation, the cells were incubated with 300 μ M of CPI-613. DMSO was used as control. Results represent means $\pm$ SEMs (n = 3), *p < 0.05

Figure E.2:

(A) Immunoprecipitation of PDH-E1 β in BMMC lysates using anti PDH-E1 β antibody for IP and anti-MITF antibody for Western blot analysis. Corresponding lysates in RIPA buffer were analyzed by Western blot analysis using MITF and PDH antibodies. One representative out of 3 independent experiments is shown.

(B) Densitometry of precipitated PDH-E1 α /precipitated MITF levels in quiescent (0 min) or immune-activated (5 min) RBL cells. Results represent means $\pm$ SEMs (n = 3), *p < 0.05.

(C) Densitometry of precipitated PDH-E1 α /precipitated MITF levels in RBL cells that were treated with 300 μ M of CPI-613 for 1 h or DMSO as control. Results represent means $\pm$ SEMs (n = 3), p=0.12.

(D) Immunoprecipitation of MITF in 293T lysates. Twenty four hours after transfection with EV, MITF-H or MITF-CE each were overexpressed with PDH-E1 β plasmid. For IP anti-flag tag antibody was used and for Western blot analysis anti-flag tag and anti PDH-E1 β antibody were used. Corresponding lysates in RIPA buffer were analyzed by Western blot analysis of MITF and PDH. One representative out of 3 independent experiments is shown.

Figure E.3:

(A) 293T cells were transfected with MITF-H-flag or MITF-CE-flag or empty vector as control, and 24 h later the cells were fractionated to cytosolic and mitochondrial fractions and Western blot analysis was conducted. Anti-flag tag antibody was used to show the different constructs of MITF in the cellular fractions and caspase3 **(B)** was used as cytosolic marker to show the purity of the mitochondrial fraction. One representative experiment out of three is shown.

Figure E.4:

(A) RBL cells were treated with 300 μ M CPI-613 or DMSO for 1 h, then cells were lysed and protein levels were measured by Bradford and the samples were diluted for equal protein concentration. The pyruvate dehydrogenase complex activity ELISA kit was used to determine PDH activity in the different lysates. Results represent means $\pm$ SEMs (n = 3), *p<0.05.

(B) RBL cells were treated with DCA or Nuclease free water for 30 min, then cells were lysed and protein levels were measured by Bradford and the samples were diluted for equal protein concentration. The pyruvate dehydrogenase complex activity ELISA kit was used to determine PDH activity in the different lysates. Results represent means $\pm$ SEMs (n = 3).

Table E.1:

Yeast two hybrid assay data in which MITF-H was used as a bait, revealing interaction with the pyruvate dehydrogenase complex E1- β subunit.

Prey-ID	uniprot ID	Protein name (description)	Subcellular localization	Length (aa)	Query start	Query end	Match start	Match end
MITF-H_pre_077	Q9D051	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial OS=Mus musculus GN=Pdhb PE=1 SV=1	Mitochondrion matrix	359	21	842	1	274

References

1. Razin E, Zhang ZC, Nechushtan H, Frenkel S, Lee YN, Arudchandran R, et al. Suppression of microphthalmia transcriptional activity by its association with protein kinase C-interacting protein 1 in mast cells. *J Biol Chem* 1999; 274:34272-6.
2. Razin E, Ihle JN, Seldin D, Menciahuerta JM, Katz HR, Leblanc PA, et al. Interleukin-3 - a Differentiation and Growth-Factor for the Mouse Mast-Cell That Contains Chondroitin Sulfate-E Proteoglycan. *Journal of Immunology* 1984; 132:1479-86.
3. Yagil Z, Hadad Erlich T, Ofir-Birin Y, Tshori S, Kay G, Yekhtin Z, et al. Transcription factor E3, a major regulator of mast cell-mediated allergic response. *The Journal of allergy and clinical immunology* 2012; 129:1357-66 e5.
4. Piliponsky AM, Gleich GJ, Nagler A, Bar I, Levi-Schaffer F. Non-IgE-dependent activation of human lung- and cord blood-derived mast cells is induced by eosinophil major basic protein and modulated by the membrane form of stem cell factor. *Blood* 2003; 101:1898-904.
5. Sonnenblick A, Levy C, Razin E. Immunological trigger of mast cells by monomeric IgE: effect on microphthalmia transcription factor, STAT3 network of interactions. *J Immunol* 2005; 175:1450-5.
6. George TC, Fanning SL, Fitzgerald-Bocarsly P, Medeiros RB, Highfill S, Shimizu Y, et al. Quantitative measurement of nuclear translocation events using similarity analysis of multispectral cellular images obtained in flow. *J Immunol Methods* 2006; 311:117-29.

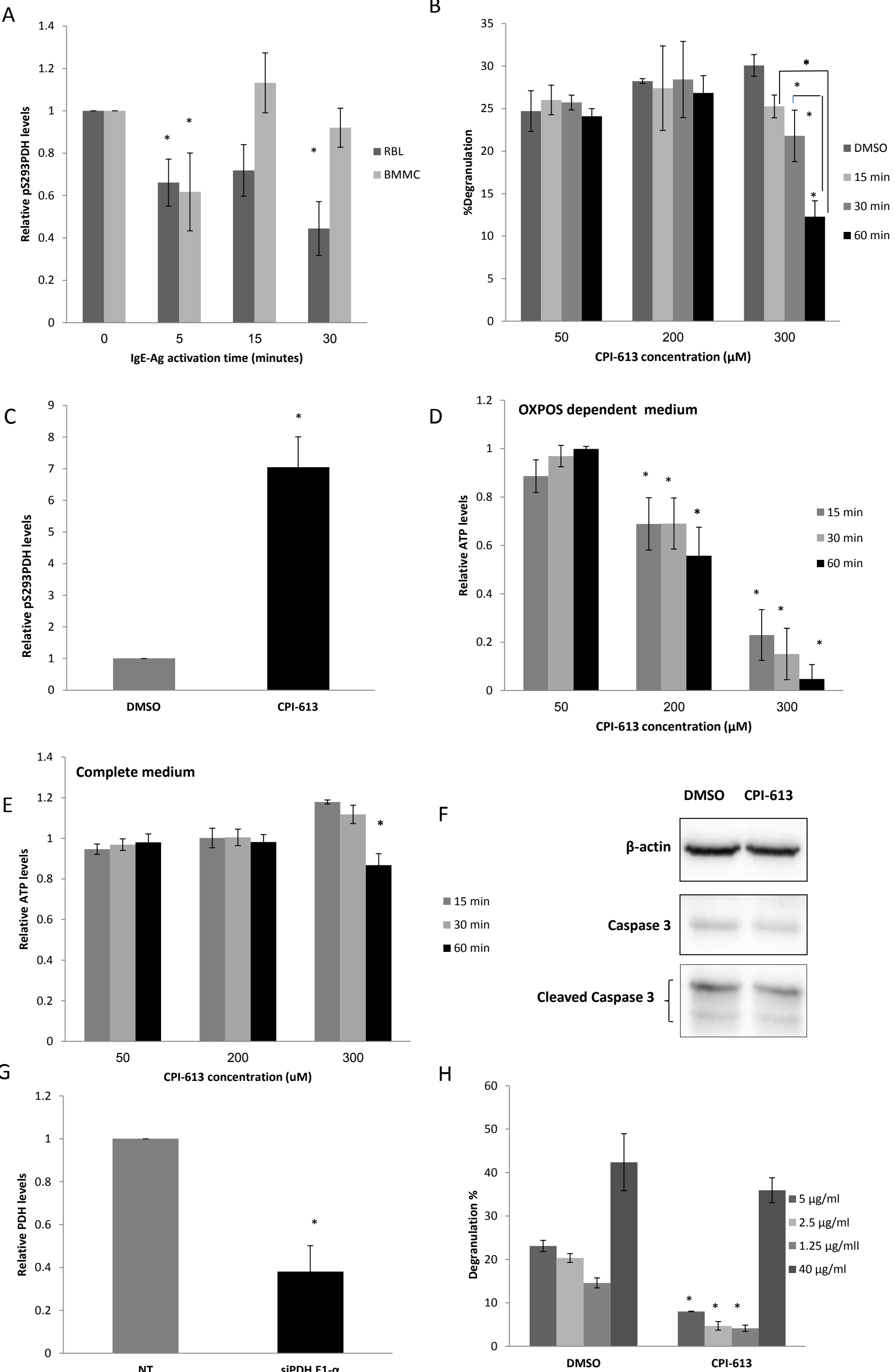


Figure E.1

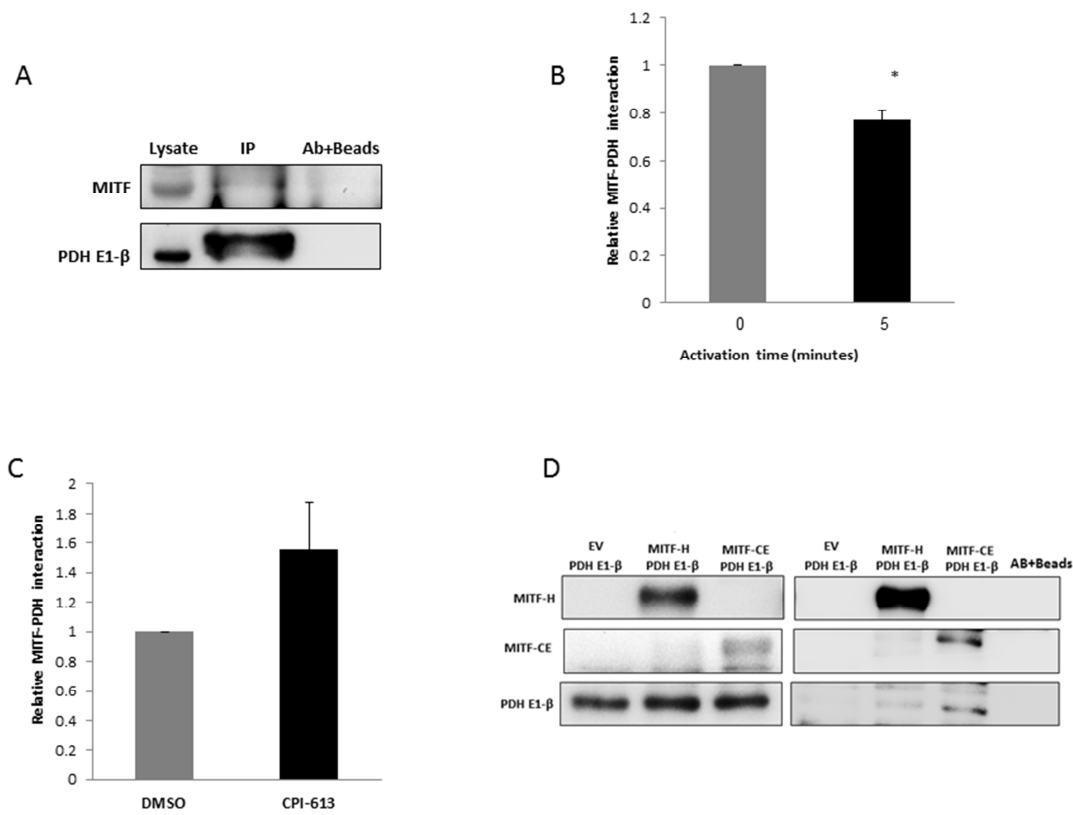


Figure E.2

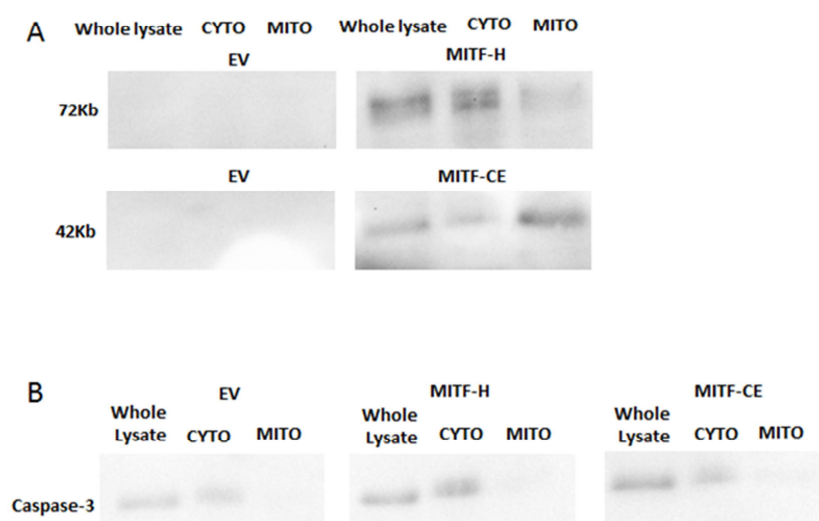


Figure E.3

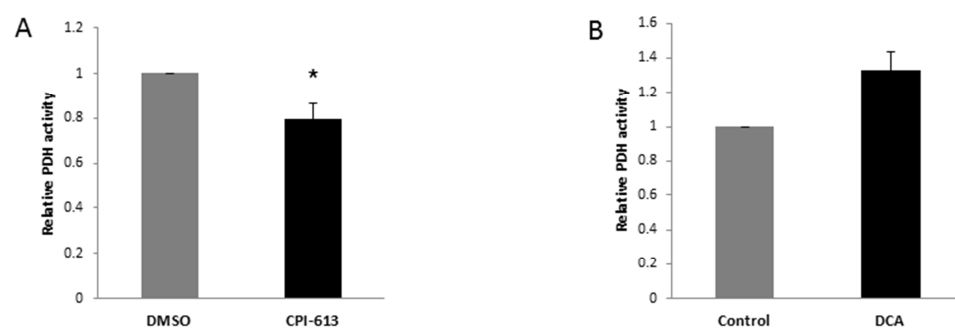


Figure E.4