# PI3Kγ Adapter Subunits in Mast Cells

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# 1 Summary

The incidence of allergy and related diseases such as asthma increased rapidly over the last century, especially in western societies. The allergic symptoms impair the quality of life of patients and can even be life threatening. The symptoms vary in strength and location, leading to sneezing, allergic sinusitis, itching, broncho-constriction, diarrhoea or anaphylaxis. Allergy is an acquired hyperreactivity of the immune system towards non-harmful substances, which leads to the activation of mast cells as a central component in the disease development and progression. Thus a detailed understanding of how mast cells are activated could be beneficial for the development of novel anti-allergic medication. For proper mast cell differentiation, proliferation, tissue distribution and their activation the lipid kinase family of Phosphoinositide 3-kinase (PI3K) has been proven fundamental, which are thus putative pharmacologic targets.

Class I PI3K activation by cell surface receptors facilitates the production of the lipid second messenger phosphatidylinositol(3,4,5)-trisphosphate (PtdIns(3,4,5)$P_3$) which is central for the integration of effector proteins, equipped with PtdIns(3,4,5)$P_3$ binding PH domains. In turn activation of serine/threonine kinases, tyrosine kinases, GEFs and GAPs of small GTPases modulate mast cell survival, proliferation, migration and degranulation.

Subclass IA PI3K serves receptor tyrosine kinases (RTK) and non-receptor tyrosine kinase (NRTK) as well as immunoglobulin receptors by direct or indirect association, through their p85 family of regulatory subunits. In contrast the only class IB member, PI3K$\gamma$, is primarily activated downstream of G-protein coupled receptors (GPCR). Thereby PI3K$\gamma$ interaction with released G$\beta\gamma$ subunits from heterotrimeric G proteins is required to transmit signals from the extracellular milieu.

PI3K$\gamma$ is a heterodimeric complex, consisting of the catalytic subunit (p110$\gamma$) and a adapter subunit, either p101 or the recently identified p84 (or p87$^{PIKAP}$). In line with PI3K$\gamma$'s predominant expression in immune cells, genetic ablation of the catalytic subunit p110$\gamma$ causes defects in inflammatory and allergic responses, such as leukocyte chemotaxis, mast cell migration, and the synergistic amplification of IgE and antigen mediated degranulation.

While the requirement of class IB PI3K activity in mast cell function was proven in murine disease models, there is still a lack of knowledge on the physiologic function of its adapter subunits, dubbed p101 and p84. Based on in vitro experiments both adapters support p110$\gamma$ activity by sensitizing p110$\gamma$ for the G$\beta\gamma$-mediated activation downstream of GPCRs.

Expression analysis performed here determined mast cells most appropriate to study the physiologic requirement of p84. Mast cells have high abundance of p84 and p110$\gamma$ protein, while p101 protein was undetectable. In particular the observed destabilization of p84 protein in p110$\gamma$ null mast cells was beneficial to conduct elegant complementation experiments. To this end, p84 complexed with the catalytic subunit p110$\gamma$ (p84:p110$\gamma$), was essential for all PI3K$\gamma$ dependent cell responses, such as adenosine driven PtdIns(3,4,5)$P_3$ production, phosphorylation of PKB/Akt, cell migration and the adenosine enforced degranulation. Of note, increased abundance of p110$\gamma$ was ineffective in compensating lack of p84.

Moreover, the replacement of p84 by p101 in complex with p110γ (p101:p110γ) unraveled a non-redundant function for the two adapter subunits, as p101:p110γ failed to support degranulation, while cell migration and phosphorylation of PKB/Akt were intact.

A possible explanation was provided by adapter dependent spatiotemporal differences of PtdIns(3,4,5)$P_3$ production. Both PI3Kγ complexes produced PtdIns(3,4,5)$P_3$ at the plasma membrane, which was however rapidly endocytosed via microtubule dependent process, when derived from p101:p110γ signaling. Especially during co-stimulation with adenosine and IgE and antigen, p84:p110γ derived PtdIns(3,4,5)$P_3$ significantly prolonged localization to the plasma membrane was observed as compared to PtdIns(3,4,5)$P_3$ of p101:p110γ origin. Moreover the two PI3Kγ complexes have differential sensitivity to cholesterol depleting agents. Altogether this implies adapter dependent production of distinct pools of PtdIns(3,4,5)$P_3$ at the plasma membrane, eliciting specific cell responses.

Thus it is exclusively the p84:p110γ complex, that amplifies mast degranulation and allergic responses. This adds an additional pathway for specific treatment of allergic diseases, e.g. by particular disruption of this complex.

# 2 Introduction

The incidence of allergic diseases increased dramatically over the last decades, now reaching one third of the german population, thus recognized as the widespread disease. Allergic symptoms have a very broad range, which is in particular dependent of the site of the allergic reaction. Thus allergic skin reactions induce itching redness, urticaria and edema formation, while inhaled allergens leads to sneezing, rhinitis, the swelling of the respiratory tract mucosa, resulting in bronchoconstriction and asthmatic symptoms. Moreover diarrhoae or life threatening systemic anaphylaxis can occur.

The initial trigger for the development of allergy is an imbalanced immune response to non-harmful substances, which under healthy condition would induce immune tolerance, but which are misleadingly recognized dangerous by the immune system of pre-disposed individuals.

Although the exact mechanisms of allergy development are unclear, it is certainly connected with a skewed ratio of type1 (Th1) and type2 (Th2) T helper cells. In developing allergy, dendritic cells present antigen to naïve $CD4^+$ T cells under the influence of interleukins IL-4, IL-5, IL-9, IL-10 and IL-13, which promote differentiation of naïve T cells toward Th2 cells. The consequently enhanced Th2 response increases the release of IL-4, -5, -9, -10 and -13, and thus favours the isotype switch in B cells, to produce immunoglobulin E (IgE), proliferate and differentiate into antibody secreting plasma cells. In parallel differentiation of naïve $CD4^+$ T cells to Th1 cells is decreased. This further contributes to the detrimental process, as Th1 derived expression of IL-12 and interferon $\gamma$ (INF$\gamma$) is reduced. Under normal conditions these cytokines inhibit Th2 differentiation (Romagnani, 2000).

The secreted IgE is captured by the high affinity receptors for IgE, Fc$\varepsilon$RI, especially on mast cells, but also basophils and eosinophils, sensitizing these for the antigen. A subsequent second exposure of IgE decorated cells to polyvalent antigens, cross-links IgE-Fc$\varepsilon$RI complexes leading to mast cell activation and consequently to allergic symptoms (Wills-Karp, 1999; Wedemeyer et al., 2000).

## 2.1 Treatment of allergic disease

Currently treatment of allergy is for the most part restricted to symptomatic medication, thereby antagonizing the function of released mast cell mediators. In contrast the treatments recognized etiologic by the World Health Organization (WHO) is only allergen prevention and allergen desensitizing treatment (personal communication with Dr. med. K.-H. Bohnacker, allergologist and pulmonary physician).

### 2.1.1 Symptomatic treatment

Symptomatic treatment of allergies includes the application of second-generation histamine receptor 1 ($H_1$) antagonists, such as Loratadin or Cetirizin. These prevent binding of histamine to its cognate receptors. Thus they decrease vascular permeability and itching and function antispasmodic. In contrast to first generation H1 antagonist adverse effects, such as sedation are reduced, nevertheless they can still cause cardiac arrhythmia and cross-react with a variety of other medications.

Singulair (Montelukast) and Accolate (Zafirlukast) are a second class of receptor antagonists, implicated in allergy treatment, targeting leukotriene receptors, e.g. $CysLT_1$ receptor on smooth muscle and endothelial cells or B-$LT_1$ receptors on granulocytes. Thereby patients benefit from reduced smooth muscle contraction, vascular permeability in the respiratory tract and from their anti-inflammatory capacity reducing the chemotactic response of granulocytes, expressing the B-$LT_1$ receptors.

“Mast cell stabilizers”, derivates of sodium-cromoglycate, such as Tilade (Nedocromil), are commonly used relieving patients from allergic symptoms. In contrast to the above, cromoglycate prevents inflammatory mediator release from mast cells, by inhibiting the essential $Ca^{2+}$ mobilization required for mast cell exocytosis. Many other symptomatic medications are known, e.g. corticosteroids or antagonists for cholinergic receptors (Merk and Ott, 2008).

Reducing the allergic symptoms is certainly convenient for the patients, while the medications above share also unfavourable traits. First, adverse effects, e.g. sleepiness, vomiting, headache and mucosal irritation, to name a few, hamper the acceptance of the treatment by the patients. Second the short-term effectiveness requires continuous dosage throughout the time of allergen exposure and raises health care cost tremendously. In addition cromoglycate derivates and corticosteroids are not implicated in acute reactions and thus require the constant intake in advance.

### 2.1.2 Etiologic treatment of allergies:

An etiologic treatment of allergy is the prevention of allergen exposure, which can lead to extended asymptomatic periods. Dependent on the allergens nature and distribution prevented allergen exposure can however be impossible and it is obviously necessary to know the specific allergen. The second etiologic treatment is the specific immunotherapy against a known allergen, dubbed hyposensitization. Thereby a solution of the isolated antigen is repeatedly injected subcutaneously or orally (Deutscher Allergie- und Asthmabund e.V.; http://www.daab.de/hypo.php). The positive long-term effects, e.g. symptom remission and protection from disease progress to more severe pathologies, are the consequence of desensitization of mast cells towards the allergen, a reduced IgE production by B-cells, restoration of the Th1/Th2 ratio and the activation of regulatory T-cells (James and Durham, 2008). Disadvantageous is that hyposensitization does not result in immediate disease improvement and requires symptomatic treatment initially, as well as long-term medical attendance, for up to three years, raising the costs for the health care system.

Recently a humanized recombinant monoclonal anti-IgE antibody was launched on the market (Xolair [Omalizumab]). Administration of this $IgG_{1k}$ prevents the association of free IgE to the high affinity receptor for IgE, FcεRI, on mast cell, eosinophils and basophils, as it recognizes and thus shields the epitope for FcεRI association. Consequently the reduced number of IgE-bound FcεRI on mast cells reduces exocytosis, when exposed to allergen. Moreover the observed down-modulation of FcεRI on immune cells is beneficial (Lin et al., 2004; Winchester et al., 2006). Xolair medication is however mostly implicated in individuals with increased serum IgE levels, which not all allergic individuals share. Moreover Xolair administration requires, as hyposensitzation, long-term medical attendance and its considerably high costs, as compared to the other treatments, prevent its comprehensive application (personal communication with Dr. med. K.-H. Bohnacker, allergologist and pulmonary physician).
The positive effects of IgE and FcεRI in the expulsion of parasites (Marshall, 2004) can also be contraindicative for an anti-IgE treatment.

Thus there is still a need to investigate for alternative allergy treatments, which requires a detailed understanding of mast cell function. To unravel molecular aspects of mast cell activation and of mast cell migration to IgE occupied tissues could be fundamental for new therapeutic approaches.

## 2.2 Mast cells

Mast cells are tissue resident immune cells, especially located in the perivascular space in tissues close to the host-environment interface, e.g. the connective or mucosal tissues of the skin, the gut, or in the respiratory and gastrointestinal tract. They are characterized by dense cytoplasmic granules, which contain neutral proteases, histamine, heparin, carboxy-peptidases and preformed inflammatory mediators, to be released upon activation by exogenous stimuli as an immediate response, followed by *de novo* synthesis and release of inflammatory mediators, chemokines and cytokines (Tab.1).
The vast array of surface receptor types, including Toll-like receptors (TLR-1,-2,-3,-4,-6 and -9), complement receptors (CR2, CR4, C3aR and C5aR) and immunoglobulin receptors (FcεRI, FcγRI, FcγRIII) allow mast cells to modulate innate and acquired immunity as well as pathologic conditions as allergy, asthma or autoimmune diseases, either with initiator or effector function (Marshall, 2004).

**Table 1: The repertoire of mast cell mediators and their functions**

| **Mediators** | **Function** |
|---|---|
| **Granule associated** | |
| Histamine (Serotonin in rodents) | Modulate vascular permeability; induces adhesion molecule expression on endothelial cells; increase migration of DCs (Jawdat et al., 2004) and polarizes immune reponse to Th2 responses via DCs (Mazzoni et al., 2001) |
| Heparin, chondroitin sulphate | Improved cytokine and chemokine function |
| Tryptase, Chymase, Carboxypeptidase, mast cell proteases (MCPs) | Effector cell recruitment, tissue remodeling, cleavage of exogenous and endogenous toxins (Schneider et al., 2007) |
| Tumor necrosis factor (TNF$\alpha$)**, Vascular endothelial growth factor (VEGF) | Effector cell recruitment, angiogenesis, adhesion molecule expression (ICAM-1, VCAM-1) on endothelial cells |
| **Lipid mediators** | |
| Leukotriene $C_4$, $B_4$, Prostaglandin $D_2$, $E_2$ | Recruit effector cells (e.g. $LTC_4$: eosinophils; $LTB_4$: neutrophils, subset of $CD4^+$ T cells), regulate immune responses, smooth muscle contraction |
| Platelet-activating factor (PAF) | Effector cell activation |
| **Cytokines** | |
| TNF$\alpha$, IL-1$\alpha$, IL-1$\beta$, IL-6, IL-18, INF$\alpha$,$\beta$, GM-CSF; LIF | Inflammatory response |
| IL-3, IL-4, IL-5, IL-9, IL-13, IL-15, IL-16 | Th2 type cytokines. In allergy with a detrimental feedback to increase the Th2 response. Eosinophil recruitment |
| IL-12, INF$\gamma$ | Th1 type cytokine |
| IL-10, TGF-$\beta$, VEGF | Regulate inflammation, angiogenesis, but also tissue remodeling (TGF-$\beta$; fibrosis) |
| **Chemokines** | |
| CCL2, 3, 4, 5, 11 and 20 | Effector cell recruitment (including DCs), regulated immune response |
| CXCL 1, 2, 8, 9, 10, 11 | Recruitment of effector cells, modulation immune response |
| **Superoxide radicals, nitric oxide, antimicrobial peptides** | Antibacterial capacity |

Mediators released from mast cells, adapted from (Marshall, 2004), additional functions included here are given with references. DCs: dentritic cells; ICAM: intercellular adhesion molecule; VCAM: vascular cell adhesion molecule; IL: interleukin; INF: interferon; GM-CSF: granulocyte/macrophage colony stimulating factor; CCL: ligand of the chemokine of the CC family, CXCL: ligand of the chemokine of the CXC family; TGF: transforming growth factor, ** TNF$\alpha$ is partially stored in granules, but also *de novo* synthesized after mast cell activation

## 2.2.1 Mast cell development and homeostasis

Mast cells originate from c-KIT$^{hi}$ FcεRI$^{-}$ Sca I$^{+}$, Thy1.1$^{+}$ (human CD34$^{+}$) bone marrow progenitor cells of the haematopoietic lineage (Rottem et al., 1994; Kirshenbaum et al., 1999; Kirshenbaum et al., 1991). The differentiation and maturation to FcεRI$^{+}$ mast cells and their survival are largely dependent on stem cell factor (SCF), which activates the proto-oncogenic receptor tyrosine kinase (RTK) c-KIT, and can be modulated by the presence of T cell derived cytokines, such as IL-3, -4, -9 and -10. Preceding is the weakly characterized, but eventually SCF dependent, distribution of progenitors through the circulation, their adhesion (Lorentz et al., 2002), migration (Nilsson et al., 1994) and extravasation mainly to mucosal or connective tissues (Boyce, 2003).
Mast cells are very heterogenous population of cells with different histamine contents and type of secreted protease. Dependent on tissue localization mast cells show different surface receptors expression and different responsiveness to T cell derived cytokines (Marshall, 2004).

## 2.2.2 Mast cells in immune responses

Mast cells were mainly connected with their harmful effects in allergic disease. The discovery of i) mice carrying cKIT mutations or carrying modulations in positive regulating elements of the c-Kit locus (W/W$^{v}$ or W$^{sh}$/W$^{sh}$ mice; (Kitamura et al., 1978; Lyon and Glenister, 1982)), with almost complete lack of mast cells, ii) the successful *in vitro* cultivation of isolated, bone marrow- or embryonic stem cell- derived mast cells, and iii) their adoptive transfer to mast cell deficient mice were fundamental for the assignment of physiologic responses to mast cells, and the cellular components required (Wedemeyer et al., 2000; Nakano et al., 1985; Tsai et al., 2000). This elegant *in vivo* reconstitution approach broadened knowledge on the physiologic requirement of mast cells and improved their reputation not only being detrimental, as in allergy, but also beneficial.

***Innate immunity***
In innate immunity the mast cell's repertoire of surface receptors, such as TLR, complement receptors and immunglobulin-receptors, and their location determines mast cells as very effective sentinels for the detection of pathogens, like bacteria, viruses, parasites, helminthes, or their respective components and products. Subsequently they promote the clearance of the parasites or their toxic compounds (Marshall, 2004; Marshall and Jawdat, 2004; Wedemeyer et al., 2000; Dawicki and Marshall, 2007).
TLR signaling, except for TLR2, does not induce degranulation but TNFα and IL-6 expression and release, together with a variable pattern of cyto- and chemokines to especially attract neutrophils. Signaling of LPS via TLR4 and peptidoglycan via TLR2 are confirmed *in vivo*, while other TLRs were only investigated *in* vitro (Supajatura et al., 2002; Marshall, 2004). Indirect pathogen recognition is based on complement receptors or immunoglobulin receptors, which either recognize specific antigens, or super-antigens, e.g. gp120 from

HIV, the *S.aureus* protein A, or the Fv protein after hepatitis B viral infection. Interestingly dengue virus infected mast cells change pattern of inflammatory mediators, as compared to mast cells exposed to bacterial components, resulting in T- and natural killer (NK) cell recruitment instead of neutrophils. A fundamental life-saving role was recently ascribed to granule stored mast cell proteases, which degrade and thereby detoxify snake and bee venoms, as well as high concentrations of Endothelin-1, during bacterial peritonitis and sepsis (Maurer et al., 2004; Metz et al., 2006; Schneider et al., 2007).

***Acquired immune responses***

Of course, effector functions of mast cells in allergy and asthma are very well documented and known since long. To date their initiating function in acquired immunity is gaining more and more acceptance. Thereby the release of IL-4, -6 and -13 activates differentiation and migration of B- cells, with which they can also communicate by expression of CD40 ligand. Their effect on T cells is also mediated by released factors, such as leukotrienes, prostaglandins, histamine, TNF$\alpha$ and various chemokines and cytokine to modulate differentiation into T cell subsets, induce migration proliferation and activation. Moreover mast cells were also reported to have antigen presenting capacity by expression of MHC molecules and co-stimulatory molecules. Both cells types can also be indirectly affected by mast cell signaling, in that this also modulates dentritic cells (DC) migration maturation and differentiation, especially of peripheral DC (Sayed and Brown, 2007; Galli et al., 2005).

***Autoimmunity***

A down side of mast cells is the contribution to the initiator and effector phase of autoimmune diseases. Thus mast cells have detrimental effects in multiple sclerosis (MS) or its animal model experimental allergic encephalomyelitis (EAE) (Secor et al., 2000; Brown et al., 2002), by a myriad of possible pathways driving development and progression of the disease, see (Sayed and Brown, 2007). Murine models for rheumatoid arthritis also links to activated mast cells (Lee et al., 2002; Nigrovic et al., 2007), as well as atherosclerosis (Sun et al., 2007), chronic urticaria, Bullous pemphingoid and many more (Rottem and Mekori, 2005).

***Allergy***

The development of allergic diseases was outlined above. Hereby the enhanced secretion of IgE to specific antigens integrates mast cells in disease development and progression. Mast cell capture the IgE by Fc$\varepsilon$RI receptors expressed on the cell surface and are thus sensitized for the antigen. A subsequent second exposure of IgE decorated mast cells to polyvalent antigens, cross-links IgE-Fc$\varepsilon$RI complexes, inducing intracellular signal transduction cascade leading to immediate mast cell degranulation and the delayed production and release of de novo synthesized inflammatory mediators (Fig.1). Thus mast cell induce the immediate type 1 hypersensitivity reaction, in which released histamine causes itching of the skin by binding to histamine receptors on nerve cells, bronchospams by inducing smooth muscle contraction and modulates endothelium permeability and adhesion molecule expression on the latter. As consequence the blood pressure can drop dramatically and effector cells are recruited through the combined action with the released TNF$\alpha$. The release of proteases can further drive immune cell migration to the site of the allergic response, but also results in mucus production and tissue remodeling. The subsequent late phase response

in allergy can also be linked to mast cells, in that the de novo synthesized compounds further increase vascular permeability and smooth muscle contraction. They also recruit and activate an increasing amount of neutrophils, eosinophils and T-cells. Consequently inflammation is prolonged and leading to increased tissue damage and remodeling, which can promote disease progression, e.g. from rhinitis to asthma. The increased production of Th2 polarizing cytokines further enhances IgE production, which exacerbates the allergic response in a subsequent allergen exposure (James and Durham, 2008; Wills-Karp, 1999; Wedemeyer et al., 2000).

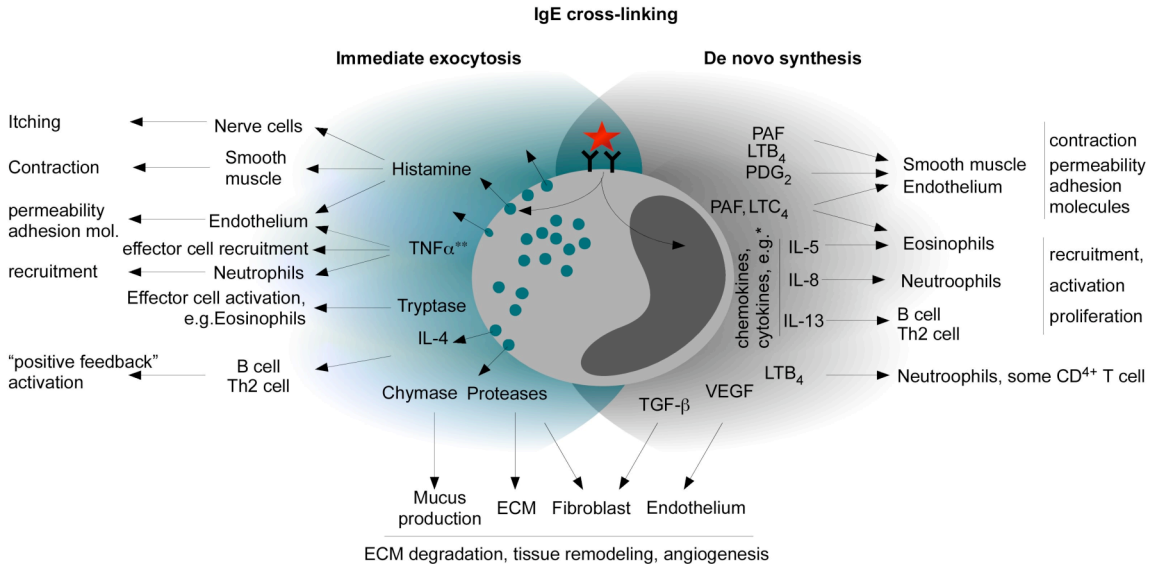


**Fig. 1: Inflammatory mediators of mast cells in allergy and asthma:** Activated mast cells, e.g. by FcεRI cross-linking, release preformed granule stored mediators (left, bluish cloud,) as an immediate response. *De novo* synthesized mediators of the late response are depicted on the right (grayish cloud, only a selection is displayed) Both responses affect multiple cell types or tissue components. Thereby mast cells drive typical allergic and asthmatic symptoms, such as smooth muscle contraction, increased endothelial permeability, recruitment of lymphocytes to inflamed tissues and mucus production, tissue remodeling and fibrosis. ECM: extracellular matrix; * Different chemo- and cytokines recruit different effector cells; ** Place restrictions do not allow a complete list of effector functions of TNFα. For abbreviations see Tab. 1

## 2.3 The Phosphatidylinositol 3-kinase (PI3K) family

In multi-cellular organisms the extra-cellular milieu is mainly composed of neighboring cells, which need to communicate to each other in order to guarantee a well-balanced homeostasis in healthy individuals. This is facilitated by direct cell-to-cell contacts using adhesion molecules or indirectly by the release of signaling molecules such as hormones, growth factors, cytokines, chemokines or neurotransmitters. Receptors recognizing such molecules link to an intracellular, tightly regulated signaling network, which transduces, amplifies and integrates the plurality of inputs to orchestrate a cellular response according to the environmental requirements. An imbalanced regulation of extracellular or of its intracellular components is however connected to the development and progress of disease. A well-known protein family of intracellular signal transducers within these signaling networks is the Phosphatidylinositol 3-kinase (PI3K) family. Alterations of PI3K signaling were consequently linked to cancer development, progression and metastasis, and immunological disorders, such as autoimmune diseases, chronic inflammation and allergy.

PI3Ks are a protein family of lipid kinases, which catalyze the phosphorylation of the D3 position in the D-myo-inositol ring of phosphoinositides (PtdIns) and can thus produce key lipid second messenger molecules such as PtdIns(3)P, PtdIns(3,4)$P_2$ and PtdIns(3,4,5)$P_3$ (Fig.2). The PI3-Kinase activity was initially connected with oncogenic products (Macara et al., 1984), but then shown to be fundamental in non-transformed cells, as only activated but not resting neutrophils produced PtdIns(3,4,5)$P_3$ (Traynor-Kaplan et al., 1989). Subsequently PI3K and its lipid products have gained much attention in several vital cell responses, such as cell survival, cell migration, chemotaxis, glucose metabolism, vesicular trafficking. As consequence deregulated PI3K activity supports disease development and progression, such as oncogenic transformation, chronic inflammation, allergy or cardiovascular diseases (Wymann and Marone, 2005).
According to their *in vitro* substrate specificity, their structural and functional homologies the PI3K family is subdivided into three classes (class I, II and III) (Wymann and Pirola, 1998; Vanhaesebroeck et al., 2001) (Fig. 3).

### 2.3.1 Class I PI3Ks

Class I PI3Ks are the best characterized members of the PI3K family and are most importantly the only ones that phosphorylate phosphatidylinositol (4,5)-bisphosphate (PI(4,5)$P_2$) yielding in the second messenger phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)$P_3$), although they also metabolize PI and PI(4)*P in vitro*. PtdIns(3,4,5)$P_3$ production requires extracellular stimuli and is restricted to the plasma membrane.

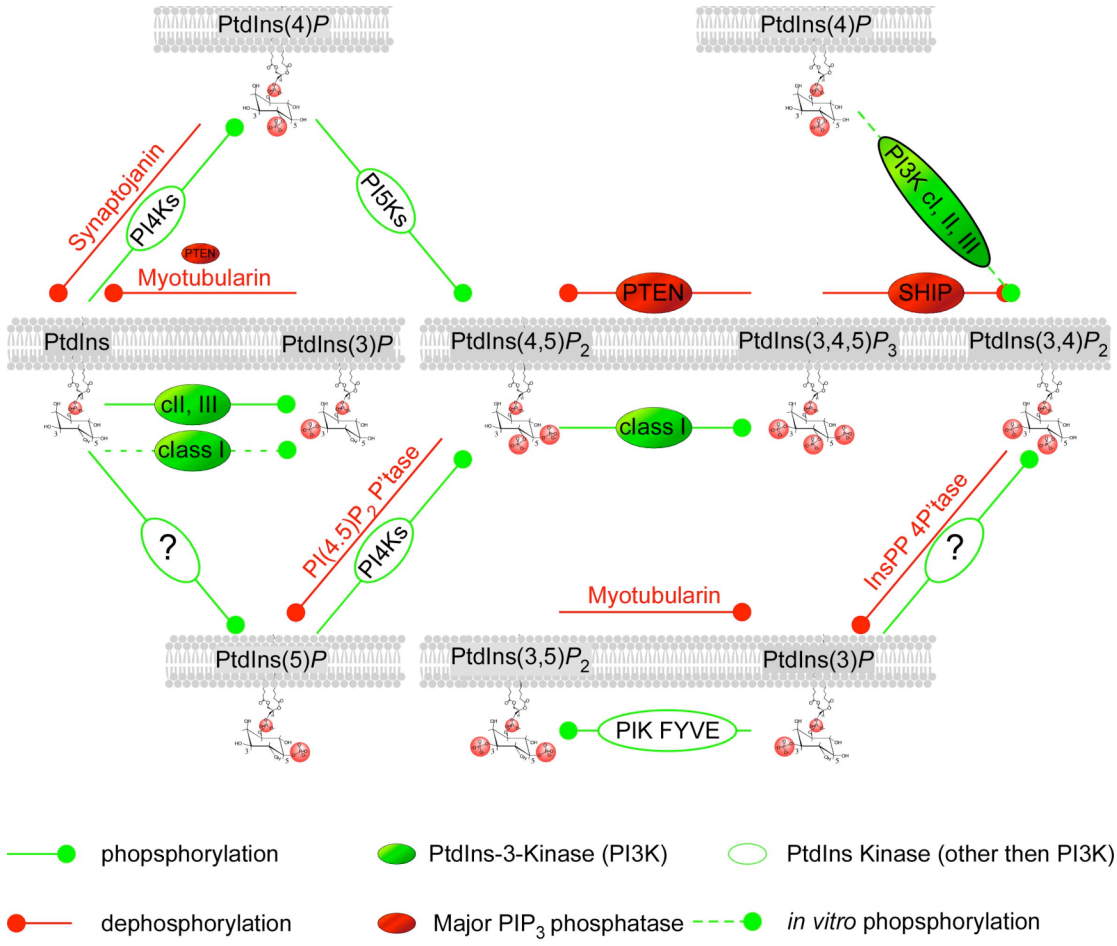


**Fig.2 Phosphoinositide metabolism (simplified):** Phosphoinositides (PI), components of the inner leaflet of lipid bilayers, are phosphorylated by lipid-kinases (green), or dephosphorylated by lipid-phosphatases (red) at the 3-, 4- or 5-OH position of the D-*myo*-inositol ring, regulating the recruitment of phosphoinositide interacting proteins. PI3Ks are labeled with filled circles, as well as the major phosphatases, which degrade the class I PI3K product (PtdIns(3,4,5)$P_3$; PTEN and SHIP) downstream of cell surface receptors. Known components are indicated with names, others indicate the catalytic activity only (PI4K, PI5K). InsPP 4P'tase: Inositol-Polyphosphate 4-phosphatse; P'tase: Phosphatase; class I and cI: Class I PI3K; cII: class II PI3K; cIII: class III PI3K). **Note:** PI's are also converted by other enzymatic activities, e.g. hydrolysis by phospholipases to generate the second messenger diacylglycerol (DAG) and soluble inositol-poly-phosphates ($IP_x$), not depicted here.

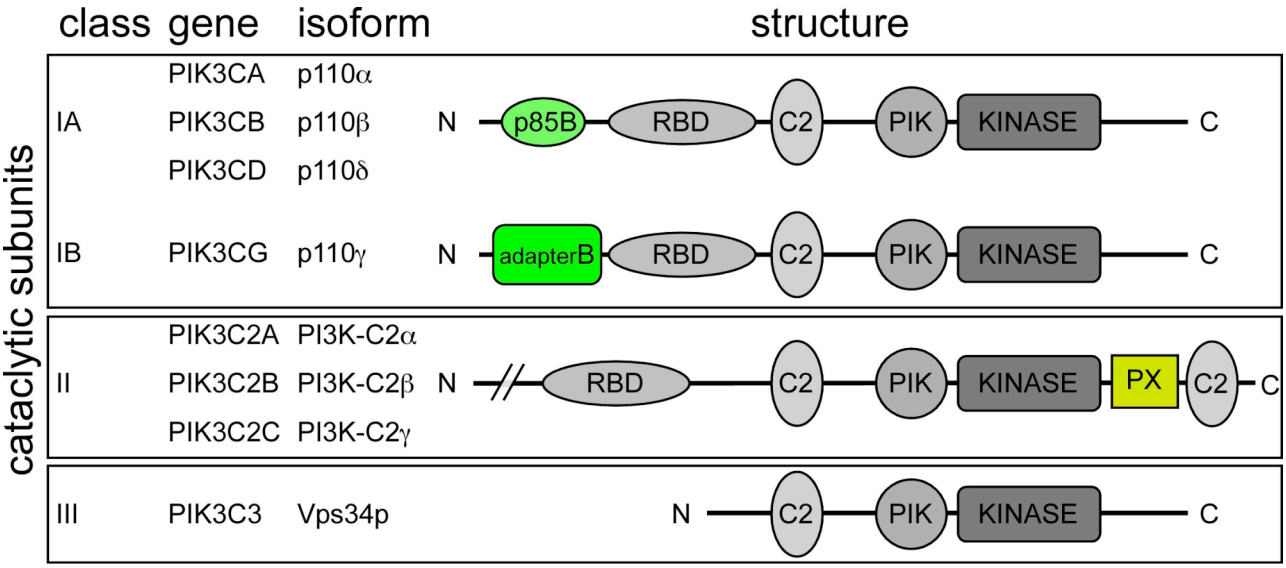


**Fig. 3: Nomenclature and linear domain organization of PI3K catalytic subunits:** The PI3K family consists of eight catalytic subunits (all encoded by different genes), which are divided into three classes (cI, cII and cIII; according to their homology), of which the first is sub-devided into class IA and IB. They share centrally located homologous domains, e.g. the C2 domain, the PIK (also known as accessory or helical) domain, as well as a c-terminal kinase domain, but differ in N- and C-terminal extensions. Class I and II have an additional Ras binding domain (RBD), and class II is c-terminally extended with a Phox homology domain (PX) and a second C2 domain. The N-terminal region of class I PI3K is central for the differential activation, as it defines the adapter subunits to associate with: class IA binds p85 family members while class IB associates with either p101 or p84 (p85B and adapterB).

### 2.3.1.1 Class IA PI3Ks

***Structure:***

Class IA PI3Ks are heterodimeric complexes consisting of a catalytic subunit of about 110 kDa molecular mass, dubbed p110α, β or δ (Fig. 3), which bind to a regulatory subunit of one of the five p85 family members (Fig. 4) (Wymann and Pirola, 1998; Vanhaesebroeck et al., 2001; Klippel et al., 1993). Class IA catalytic subunits contain all four PI3K homology regions, namely a Ras-binding-, a C2-, the PI3K accessory- (PIK) and a C-terminal kinase domain, but are unique for the N-terminal p85-binding domain.

The members of the regulatory p85 family contain two Src homology 2 (SH2) domains N- and C- terminal (nSH2 or cSH2, respectively) of the interSH2 domain (iSH2), which is crucial for binding the catalytic subunit p110. In addition proline rich region were mapped for all members and p85α and β have a N-terminal src homology 3 (SH3) domain, a second proline rich region and a breakpoint cluster region homology domain (BH domain), which allows association with Rho GTPases (Wymann and Pirola, 1998).

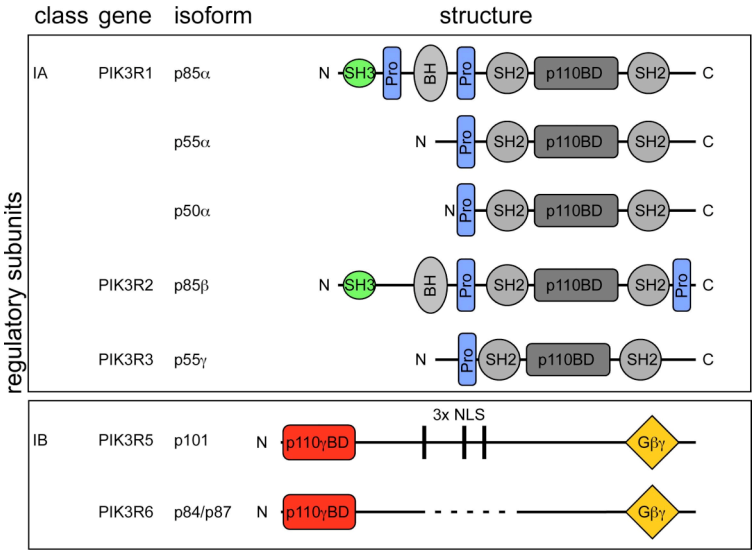


**Fig. 4: Structural organization of class I regulatory subunits:** The five known class IA regulatory subunits are encoded in 3 genes, as PIK3R1 gene yields 3 different gene products. Their common core module for receptor association are the two SH2 domains interlinked through the coiled-coiled p110-binding domain (p110BD), and 1-2 proline-rich repeat (Pro). Isoforms of 85 kDa (p85α,β) contain additionally a n-terminal SH3 domain and a BH domain (Breakpoint cluster [BCR] homology domain), which allows association with Rho GTPase family members. The class IB sub-units p101 and p84 (dubbed after their calculated molecular weight), are encoded by two genes, located in close proximity. Domains indicated for p110γ binding (p110γBD) and association to Gβγ subunits are only an approximation derived from deletion mutants, as their primary amino acid sequences lack similarity to any known proteins domains. NLS: nuclear localization sequences.

### *Class IA PI3K activation*

Class IA PI3K are activated downstream growth factor receptors, cytokine receptors or antigen receptors, which are categorized either as receptor- or non-receptor tyrosine kinases (RTK, NRTK respectively). Membrane recruitment is mainly driven through the constitutively bound p85 family regulatory subunits due to their protein-protein interaction domains, especially their SH2 domains.

Monomeric RTKs are dimerized through ligand binding. Due to their intrinsic kinase activity, tyrosine residues in their cytoplasmic tails or in associated adaptor molecules, such as the insulin receptor substrate-1 (IRS-1, (Backer et al., 1992)) are auto- or transphosphorylated. In particular the phosphorylated YxxM (pYxxM) motif within the receptors or adaptors has been identified as recognition site for the SH2 domains of p85 (Fantl et al., 1992). Besides direct association by pYxxM recognition class IA PI3K recruitment is facilitated by additional SH2-domain-containing adapter molecules, which associate with phosphorylated tyrosines (pY) different from YxxM motifves (e.g. Shc or Grb2), which is especially important for RTKs not harbouring a YxxM motif (Schlessinger, 2000;Wymann et al., 2003)(Fig. 5).

Here association of the SH2 domains of p85 with pYxxM motifs has a dual function. First class IA PI3K is recruited to the plasma membrane, providing lipid substrate availability, second the interaction relieves partial inhibition of the p110 activity (Yu et al., 1998), through a conformational change in the linker region of the nSH2 and the iSH2 domain (Shekar et al., 2005).

Receptors, lacking intrinsic kinase activity, are associated with tyrosine kinases, which tyrosyl-phosphorylate the receptor, non-covalently associated adaptor proteins or co-receptors. So are receptors that employ the beta-common chain or gp130 for signal transduction associated to Janus kinases (JAK; Fig. 5), while antigen receptors, like the B cell receptor (BCR), the T cell receptor (TCR), or the FcεRI in mast cells, depend on src kinase activity (Wymann et al., 2003).

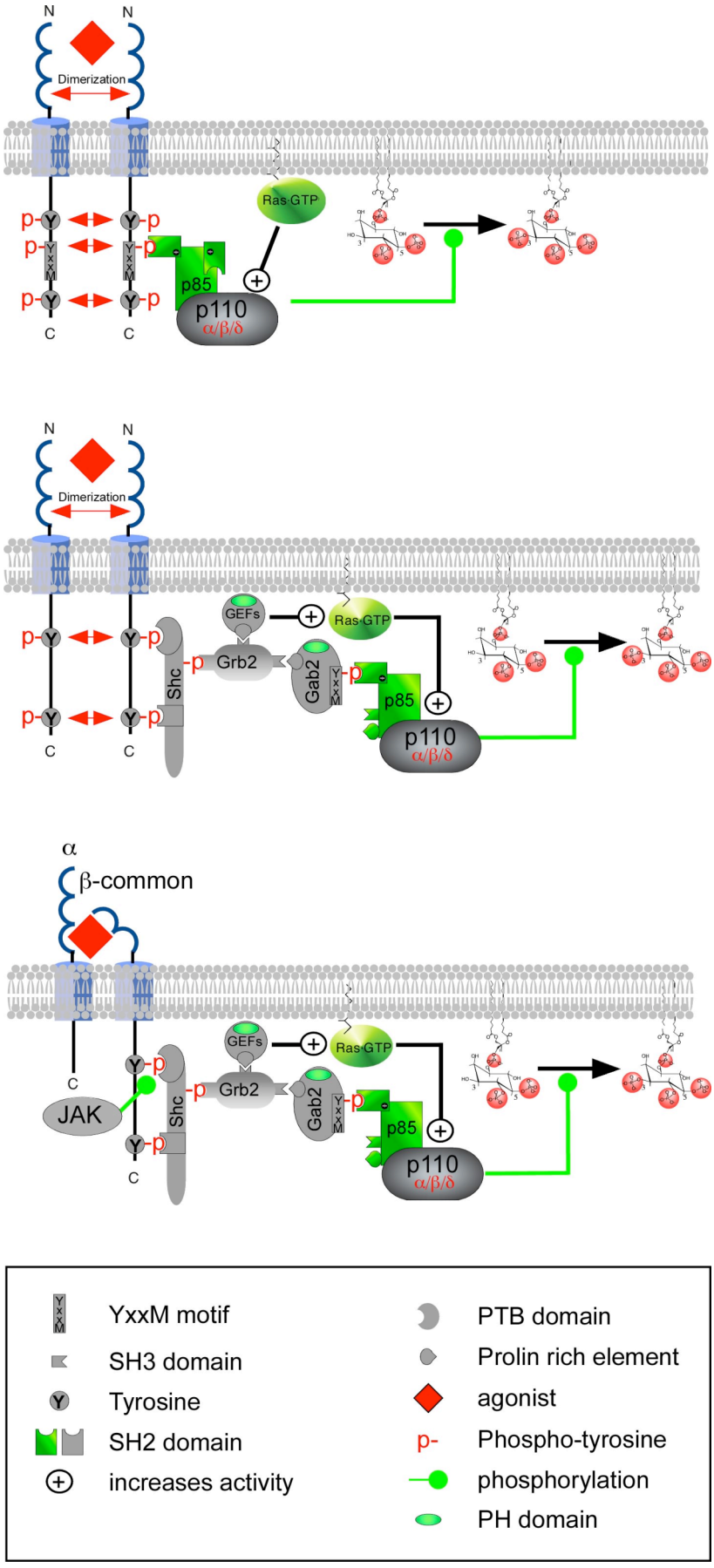


**Fig. 5:Activation of class IA PI3K:** Agonist binding to recpetor tyrosine kinases (RTKs) dimerizes receptors, which leads to tyrosine autophos-phorylation (pY) in their cytoplasmic tails.

***Top:*** pY within YxxM motifs directly recruit p85/p110 complexes. via p85's SH2 domain thus activating catalytic p110α, β or δ , which then phosp-horylate PtdIns(4,5)$P_2$. Association of GTP loaded Ras enhances activity. **Examples**: cKIT, EGF-R, PDGF-R, CSF1-R, VEGF-R.

***Middle:*** Both, RTKs with or without YxxM motif (not shown) recruit adapter molecules (e.g. Shc, Grb2 and Gab1,2 ; as shown here, or Cbl) to pY other than pYxxM. In consequence YxxM on Gab or Cbl is phosphorylated and class IA PI3K recruited. The adapters also provide binding site for various other molecules e.g. GEFs, which increases RasGTP thus further recruiting and activating PI3K. **Examples:** NGFR, CSF1R, HGFR, PDGFR.

***Bottom:*** Non-receptor tyrosine kinases require receptor associated tyrosine kinases such as JAK or src, to phosphorylate the receptors cytoplasmic tails to transduce signaling as described in the middle section.

**Examples:** Receptors acquiring the β-common chain or gp130 (IL-3, -5R, GMCSF-R or IL-6R respectively).

**Not depicted**: RTKs and NRTK signal by phosphorylating YxxM on insulin receptor substrates (IRS) for recruitment of class IA PI3K. **Examples**: IR, IGFR, IL4R, INFα,βR

Antigen receptor signaling is exemplified for the FceRI in Fig. 8

The commonalities of BCR, TCR and FcεRI (discussed in more detail for FcεRI in chapter 2.6.6 and Fig. 8) are receptor aggregation and a suggested re-localization to lipid rafts (Pierce, 2002; Xavier et al., 1998; Holowka and Baird, 2001; Field et al., 1995;Fattakhova et al., 2006) thus inducing close proximity to src kinases (Lck, Fyn for T cells; Lyn in B cells; Lyn, and Fyn in mast cells). Subsequently the phosphorylation of tyrosines within the cytoplasmic receptor tails increases the recruitment and the activation of more src kinases and of other SH2 domain containing kinases, such as Syk in B- and mast cells or Zap70 in T cells (Beitz et al., 1999; Benhamou et al., 1993; (Iwashima et al., 1994). In consequence the phosphorylation cascade is expanded to co-receptors, like CD19 in B cells or CD28 in T cells, to transmembrane adaptor proteins, e.g. LAT in mast cells and T cells or NTAL in mast cells and B cells, which are core to large signalosome complexes of cytosolic adapters and enzymatic proteins, providing multiple sites for interaction with class IA PI3K (Okkenhaug et al., 2007). A more direct class IA PI3K translocation occurs via pYxxM motifs in T-cell interacting molecule (TRIM), the B-cell adaptor protein (BCAP) (Okada et al., 2000) or Gab2 in mast cells, which phosphorylation via Fyn is required for PI3K activity (Parravicini et al., 2002). Moreover phosphorylated Syk or ZAP70 were reported to directly recruit class I PI3K (Yu et al., 2006; Moon et al., 2005). The clustered protein-protein interaction domains of p85 family members provide a plethora of alternative or cooperative modes of class IA PI3K membrane translocation and activation as many of the docking/adaptor proteins of RTKs or NRTKs contain the complementary motifs required for binding.

***Ras in class IA PI3K activation***

The catalytic subunits p110α and p110δ (the latter with weak affinity and restricted to few Ras-proteins) contribute to PI3K membrane localization by interacting with GTP-loaded Ras (Rodriguez-Viciana et al., 1994; Rodriguez-Viciana et al., 1996; Rodriguez-Viciana et al., 1997; Rodriguez-Viciana et al., 2004). Still, the $p85_{SH2}$ - pYxxM interaction precedes Ras-induced PI3K activation, as unbound cSH2 and parts of iSH2 of p85 sterically hinder the Ras-association to p110 (Jimenez et al., 2002).

Nevertheless the importance of Ras interaction with class IA PI3Ks was underlined recently in *Drosophila melanogaster,* carrying a Ras binding deficient transgene of Dp110, the sole orthologue of class IA PI3Ks in flies, which resulted in decreased insulin signaling, size and egg numbers (Orme et al., 2006).

Earlier studies however suggested, that only mutated constitutively active Ras was able to augment Dp110 activity (Prober and Edgar, 2002). Analysis of transgenic mice with a Ras-binding deficient p110α were largely protected from Ras-oncogene mediated tumorigenesis, further supporting connection of, at least constitutive active Ras with some class IA PI3Ks (Gupta et al., 2007).

### 2.3.1.2 Class IB PI3K

Class IB PI3K is restricted to vertebrates and consists of the catalytic subunit p110γ (Stoyanov et al., 1995) and two regulatory subunits, p101 (Stephens et al., 1997), and the recently identified p84 (Suire et al.,

2005). In contrast to class IA catalytic subunits, p110γ cannot associate with p85 family members and is consequently not activated by RTKs but by GPCRs (Stoyanov et al., 1995).

### 2.3.1.2.1 Class IB structure

***The catalytic subunit p110γ***

The p110γ subunit was prototypical for understanding the organization of PI3K catalytic subunits, the mode of PI3K inhibitor action, and its association with Ras, as it was the first resolved in its crystal structure (Walker et al., 1999), including small molecule inhibitors (Walker et al., 2000), or co-crystalization with H-Ras (Pacold et al., 2000). The C-terminal catalytic domain, the C2 domain and the Ras-binding domain surround the central helical PIK domain. Surface exposure of the catalytic and the C2 domain allow the interaction with phospholipids either required as substrate or for protein translocation. The catalytic domain shows similarity to protein serine/threonine kinases, in accordance with the dual specificity kinase character of p110γ (Bondeva et al., 1998), composed of a N- and C-terminal lobe, that form the catalytic cleft. Co-crystals with wortmannin, Ly294002 and protein kinase inhibitors provided insight into their mechanistic action, binding into the ATP binding pocket, thus providing invaluable information for the successful design of PI3K inhibitors (Walker et al., 2000). The mode of Ras mediated PI3K activation, which is essential for PI3Kα (Gupta et al., 2007) and PI3Kγ (Suire et al., 2006) function, was also addressed by co-crystallizing p110γ and Ras (Pacold et al., 2000). Binding of GTP-loaded Ras induces a conformational change in the catalytic domain of p110γ, thus most likely relieving it from an allosteric inhibition.

The most profound aberration of p110γ compared to class IA p110 is its N-terminal sequence, which excludes the binding of p85 family members, but instead associates the adapters p101 (Stephens et al., 1997) or p84 (Suire et al., 2005). Krugmann et al. localized the interaction of p101 with p110γ to the N-terminus of p110γ (Krugmann et al., 1999), while Maier et al. reported the N-terminus dispensable for association with p101 (Maier et al., 1999). To date this discrepancy was not re-evaluated. Interaction of p110γ with Gβγ subunits of heterotrimeric G-proteins was mapped N- and C-terminal (Leopoldt et al., 1998), which could set membrane association into operation.

***Structure of the regulatory subunits***

The sound knowledge on the molecular structure and physiologic functions of p110γ contrasts the little information on its poorly explored adapter subunits p101 (Stephens et al., 1997), and p84 (Suire et al., 2005). Structure-wise both adapters do neither share homologies with other proteins, when aligned to the known proteome, nor can protein domains be detected by domain knowledge databases. The adapters themselves however have similarity especially in N- and C-terminal stretches of about 50%, separated by a rather non-homologous intermediate stretch, with three potential nuclear localization signals in p101 (Voigt et al., 2005) but not in p84. Thus over-expressed p101, not associated to p110γ, resides nuclear, while its interaction with p110γ renders it cytosolic (Brock et al., 2003).

Interestingly, the p110γ binding region in p101 (Krugmann et al., 1999), (Voigt et al., 2005) covers the N-terminal region homologous to p84 and the Gβγ association localizes to the C-terminal homology region. Both, the overlap of functional regions in p101 with regions of high similarity to p84, and the fact that p101 and p84, associate via their N-terminal regions with the N-terminus of p110γ (Brock et al., 2003), (Voigt et al., 2005), (Voigt et al., 2006) indicate equivalent adapter association to overlapping regions of p110γ- and indeed their binding is mutually exclusive (Voigt et al., 2006).

### 2.3.1.2.2 *Class IB activation*

#### *G-protein coupled receptors (GPCR)*

GPCR are seven transmembrane helix receptors, which associate with GDP loaded heterotrimeric G-proteins in an inactive state. The signal transduction downstream is channeled and diversified by the assembly of associated trimeric G-proteins, of which up to 20 Gα-, 5 Gβ- and 12 Gγ- subunits have been described. Extracellular ligand binding, including light photons, ions, neurotransmitters, chemokines, nucleotides and adenosine, results in a conformational change of the GPCR, thereby decreasing GDP-affinity of the Gα subunit and favouring GTP-bound state of the associated heterotrimeric G-protein. The spatial reorganization of GTP-bound Gα-subunit dissociates Gβγ subunits from the complex, and the Gα-subunit as well as the Gβγ-complex signal to downstream effectors (Pierce et al., 2002). Four distinct Gα-subunit families have been classified, due to their downstream signaling: Simplified, $G\alpha_i$-subunits couple to the inhibition of adenylyl cyclase, thereby reducing cAMP signal transduction, while $G\alpha_s$ proteins stimulate adenylyl cyclase. $G\alpha_q$ subunits activate the phospholipase Cβ (PLCβ) branch, signaling through Inositiol-(1,4,5) trisphosphate ($IP_3$), Diacylglycerol (DAG) and consequently $Ca^{2+}$, and $G\alpha_{12}$ stimulate Rho-GEF signaling (Pierce et al., 2002). Gβγ complexes also gained attention to actively transduce signals from GPCR (Clapham and Neer, 1993). The multiple combinations of Gβ and Gγ subunits diversify signaling to preferential downstream targets. *In vitro,* the activity of the p101:p110γ complex is modulated with different efficacy by different Gβγ combinations, e.g. $G\beta_5\gamma_2$ fails to activate the complex, is however effective in activating PLCβ, while $G\beta_1\gamma_2$ efficiently activates PI3Kγ (Maier et al., 2000; Kerchner et al., 2004).
Still all Gβγ subunits share essential structural traits: a common β-propeller motive consisting of WD40 repeats required for effector binding on the Gβ-subunit, and as for the Gα-subunits, a C-terminal prenylation of the Gγ-subunits (Clapham and Neer, 1997). Thus interaction of p110γ with Gβγ complexes targets the kinase to plasma membrane, where it has access to its substrate PtdIns(4,5)$P_2$.

#### *The function of p101 and p84 in p110γ activation*

Complexes of p110γ in tight association with its adapter p101 were first reported in lysates from pig neutrophils (Stephens et al., 1997). *In vitro* p101 or p84 association increases the affinity of monomeric p110γ to recombinant Gβγ, resulting in enhanced PI3Kγ activity (Stephens et al., 1997; Suire et al., 2005). In

contrast, Stoyanov et *al*. and Leopoldt et al. reported, that already the sole presence of Gβγ readily enhances the activity of monomeric p110γ in *in vitro* kinase assays (Stoyanov et al., 1995; Leopoldt et al., 1998). The contradiction is most likely due to the substrates used in the studies (PtdIns(4,5)$P_2$ in Stephens et al. and PtdIns in the latter ones), thus p101 might define substrate specificity of p110γ (Maier et al., 1999). In non-haematopoietic cells over-expression studies on the localization of p110γ with or without p101 suggested, that p101 is mainly required for p110γ translocation to the membrane, due to its higher affinity for co-expressed Gβγ, rather then being activating *per se*, as constitutively membrane targeted p110γ (p110γ-CAAX) compensated the lack of p101 (Brock et al., 2003). The interaction of p84 and its requirement in Gβγ or GPCR mediated p110γ activation was addressed similarly with identical outcome (Voigt et al., 2006).
The necessity of the p101 adapter subunit of p110γ was however challenged in the haematopoietic cell line U937, in which retinoic acid increased p110γ expression, while p101 levels were unaffected, thus arguing, that p110γ could function without p101, dependent on the cellular context (Baier et al., 1999). Moreover cytosol depleted neutrophils were fMLP sensitive by the addition of p110γ only (Kular et al., 1997).
The physiologic requirement of p101 at least in some PI3Kγ mediated cell responses was reported recently, as p101$^{-/-}$ neutrophils were defective in migration towards fMLP or C5a (Suire et al., 2006) (Fig.6). The oxidative burst induced by the same stimuli was intact, although PI3Kγ dependent (Hirsch et al., 2000). From the data presented by Suire et al. (2006) it is however not possible to ascribe the migration of neutrophils specifically to the p101:p110γ complex. Certainly low agonist concentrations resulted in severely reduced migration capacities, but high concentrations reestablished the migratory capacity to 50% of the wild type controls. Thus it could be either monomeric p110γ or the minor portion of p84:p110γ complexes that support migration at high agonist concentration. Consequently it needs to be validated whether the overall PI3Kγ activity rather than the p101 adapter specific function is the basis of the reported effects. A physiologic role p84 is currently not known and it will be highly exciting to know, if both PI3Kγ adapter proteins are mutually replaceable in physiologic contexts. The little comparative information of both adapter:p110γ complexes is thus far based on *in vitro* data, which only included the $G\beta_1\gamma_2$ but no other combinations of Gβγ subunits (Suire et al., 2005; Voigt et al., 2006), or the over-expressed fMLPreceptor system (Voigt et al., 2006). In addition only PtdIns(3,4,5)$P_3$ production was monitored, while physiologic responses were excluded from the studies. Thus it is impossible to conclude, whether either of the adapter:p110γ complexes has preferential receptor systems, Gβγ complexes or can even promote distinct physiologic responses.

***Ras dependent p110γ activation***

Ras association to p110γ was resolved in crystal structure, in which constitutively GTP loaded H-RasG12V induced an activating conformational change of p110γ, which was confirmed in cellular over-expression systems. Activating effects of Ras were inhibited by multiple amino acids exchanges in the Ras binding domain of p110γ (T232D, K251A, K254S, K255A and K256A; dubbed DASAA mutant) (Pacold et al., 2000). The physiologic relevance was confirmed in neutrophils derived from transgenic mice (p110γ-DASAA mice), in which migration and the oxidative burst was impaired after fMLP or C5a challenge (Suire et al., 2006).

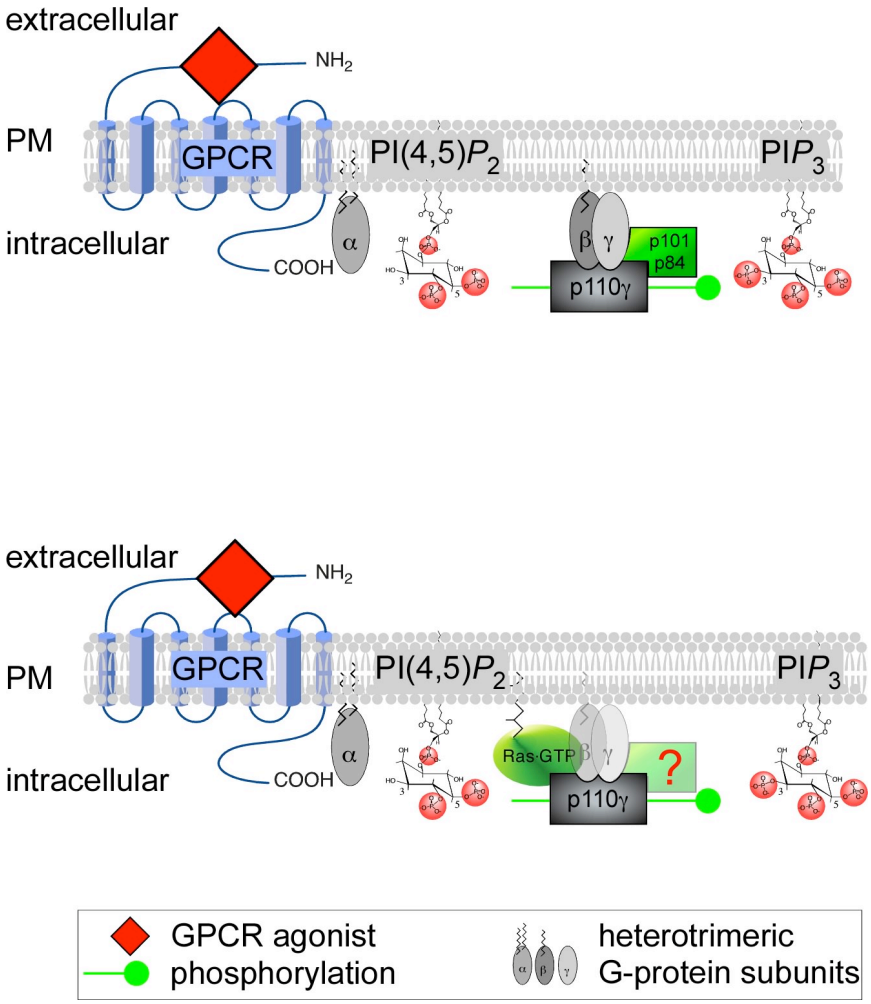


**Fig.6: Class IB PI3K activation:** PI3Kγ is activated downstream of agonist bound G-protein coupled receptors (GPCR). Released βγ subunits of trimeric G-proteins support p110γ membrane targeting, to access its substrate PI(4,5)$P_2$. ***Top:*** the adapter subunits of p110γ were reported to increase the affinity to βγ subunits *in vitro* and within cells to mainly support membrane translocation of p110γ instead of being primarily activating. The physiologic requirement is however only proven for p101, but not for p84. *Bottom:* the identical receptor system, as used to prove p101's requirement, revealed an essential role of Ras mediated p110γ activation. The requirement of the PI3Kγ adapter subunits and/or βγ subunits was not addressed in the context of Ras mediated PI3Kγ activation (thus shaded here).

## 2.4 Downstream Effectors of Class I PI3K Signaling

Class I PI3K activity results in increased PI(3,4,5)$P_3$ levels, which provide docking sites for the pleckstrin homology (PH) domain containing effector molecules. Consequently the effector molecules are recruited to PI(3,4,5)$P_3$ rich membrane areas. Thereby a spatial proximity is provided by the concomitant recruitment of proteins, with enzymatic activity, and their substrate, thus facilitating signal transduction. In addition binding of the PH domain to PtdIns(3,4,5)$P_3$ can, through conformational changes, enhance the enzyme activity, substrate affinity or accessibility to the substrate.

The *PH domain* is one of several phosphoinositide binding protein domains. Beside FYVE, PX, ENTH [Epsin N-terminal Homology] and PROPPINS [β-Propellers that bind phosphoinositdes] domains, which bind phosphoinositides of non-class I PI3K activity *in vivo*, and are thus not discussed here (Lemmon, 2007).

PH domains were first described by Haslam et *al.* (Haslam et al., 1993) and Mayer et *al.* (Mayer et al., 1993) being homologous to sequences to N- and C-terminal regions of the pleckstrin protein. However it is rather the overall structure than the primary amino acid sequence, which makes them homologous. They all form a β- sandwich in the core and a C-terminal α–helix (Lemmon and Ferguson, 2000). Although PH domains were reported to bind all phosphoinositides with variant and weak affinity, strong association requires phosphorylation of at least at two adjacent phosphate groups, like PtdIns(3,4,5)$P_3$, PtdIns(4,5)$P_2$, PtdIns(3,4)$P_2$.

The observation that the PH domain containing serine/threonine kinase PKB/Akt recognizes the PI3K lipid product finally established PtdIns(3,4,5)$P_3$ as an important second messenger molecule (Franke et al., 1995). Since then various PI3K effectors were identified, which can be roughly grouped according to their relation to protein families, such as serine/threonine- or tyrosine-kinases, regulators of small GTPases (GEFs and GAPs) or adapter proteins, providing platforms to transduce, sustain or amplify signals (Fig. 7).

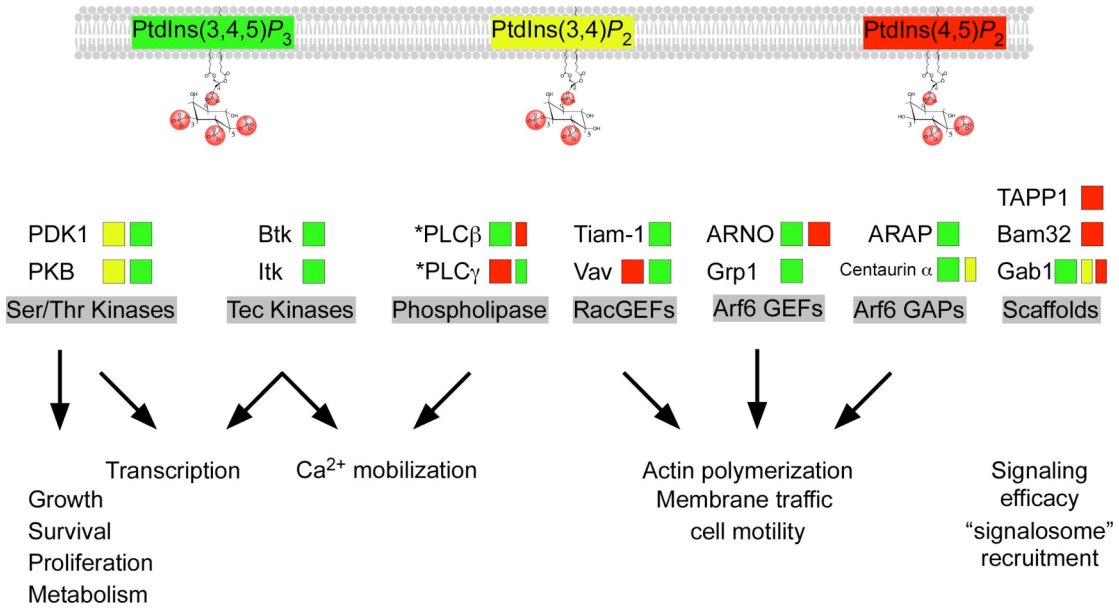


**Fig. 7: Protein families downstream of class I PI3K activity:** Major protein families, containing a PH domain, are given in grey boxes, with typical members listed above. Below major physiologic functions of each familiy are indicated. PH domains preferentially bind phosphoinositides with 2-3 adjacent phosphate groups. Still PH domains differ in the affinity (size of coloured boxes) and specificity (colour code).

***Serine/Threonine Kinases:***

PtdIns(3,4,5)$P_3$ dependent effects on cell survival, cell growth, proliferation, metabolism, translation and transcription are mainly channeled through the PI3K downstream effectors which have serine/threonine kinases. Most importantly the phosphoinositide dependent kinase 1 (PDK1), as master kinase in the PI3K-PDK1-PKB axis, and its substrates such as PKB/Akt transduce signaling to the above cell responses. The

PtdIns(3,4,5)$P_3$ binding dependent spatial proximity of PDK1 and its substrates, thereby facilitates phosphorylation of the activation- or T-loop, in case of PKB at position Thr308 (Alessi et al., 1997).
Conserved for all members of the AGC family of protein kinases (cAMP-dependent kinases, PKAs; cGMP-dependent kinases, PKGs; PKCs or serum- and glucocorticoid induced kinase SGK) full activity of PKB requires a second PDK2 dependent phosphorylation in the regulatory hydrophobic motif, for PKB residue Ser473. PDK2 activities are attributed to mTORC2 (summarized in (Polak and Hall, 2006) and DNA-PK (Feng et al., 2004). Its vast array of protein substrates, allows activated PKB/Akt to modulate their activity, with tremendous effects on the cellular state (Fig. 7).

***The Tec family of tyrosine kinases:***

Members of the Tec (tyrosine kinase expressed in hepatocellular carcinoma) family of protein tyrosine kinases (Tec, Btk, Itk, Bmx, Txk in mammals) are another prominent example, known to be recruited by PtdIns(3,4,5)$P_3$. They are most abundant in, but not restricted to immune cells. Beside the kinase and the PH domain, except for Txk, which has a palmitoylated Cys-rich repeat instead, they additionally harbour SH3 and SH2 domains, and the family-specific TH domain, which consists of the Btk homology domain (BH) and proline rich regions. This gives rise to a variety of possible protein and lipid interactions, which synergistically activate Tec kinases and orchestrate activation of multiple effectors. Simplified it is the interplay of i) PtdIns(3,4,5)$P_3$ binding, for translocation of Tec-kinases to the plasma membrane and positively modulating their activity (for Btk see Saito et al., 2001), ii) the resulting close proximity to membrane bound protein tyrosine kinases (PTK) of the src family, which in turn phosphorylate Tec kinases in their activation loop, and iii) the subsequent auto-phosphorylation (Park et al., 1996, Wilcox and Berg, 2003) (Fig.8), which are required for full kinase activity. Tec kinases then phosphorylate and activate their substrates, amongst them PLC$\gamma$, hydrolyzing PtdIns(4,5)$P_2$ to $IP_3$ and DAG, leading to $Ca^{2+}$ mobilization, and activation of PKC. Aside $Ca^{2+}$ mobilization, the Tec kinases, as part of a big “signalosome” complex, modulate/activate small GTPases, MAPK signaling, leading to actin-reorganization, gene-expression, cell growth or proliferation (reviewed in Smith et al., 2001 and Takesono et al., 2002).

***Small G-protein signaling:***

Small G-proteins or GTPases, especially of the Rho family, e.g. Rho, Rac, Cdc42, are crucial enzymes for cytoskeletal remodeling, during cell migration, chemotaxis, processes essential in development, immunity but also metastasis (Sasaki and Firtel, 2006). Alike class IB PI3K were implicated in cell migration (Hirsch et al., 2000; Sasaki et al., 2000; Li et al., 2000), indicating a connection of PI3K and Rho GTPases.
GTPases cycle between an inactive GDP- and an active GTP-loaded state. The exchange of GDP to GTP is supported by guanine nucleotide exchange factors (GEFs), while GTP hydrolysis is catalyzed by an intrinsic GTPase activity, which is additionally supported by GTPase activating proteins (GAPs, Schmidt and Hall, 2002).
Rho family GTPases are by far not only PI3K-dependent, but all GEFs contain a PH domain i) for membrane recruitment and ii) for those, binding to PtdIns(3,4,5)$P_3$, to increase activity. For Sos1 and Vav1 association with PtdIns(3,4,5)$P_3$ relieves the GEF from an auto-inhibitory state, as PtdIns(3,4,5)$P_3$ binding prevents an auto-inhibitory binding of the PH domain to the conserved Dbl homology domain (DH). The autoinhibition

prevents phosphorylation by tyrosine kinases (Han et al., 1998) and consequently also the association of the GEF with Rac and the exchange of the guanine-nucleotide in Rac (Das et al., 2000).

***Adapter proteins:***

Adapter or scaffolding proteins lack enzymatic activity, but possess besides their PH-domain additional protein-protein interaction motifs, for association with membrane-localized proteins or for recruitment of proteins to assemble large protein platforms, facilitating signal amplification. Although signal initiation can be independent of its PH domain, its association to PtdIns(3,4,5)$P_3$ is required for sustained signaling.

For example the Gab (Grb2-associated binder)/Dos family of adapter proteins, (consisting of Gab1-3 in mammals, Dos in flies or Soc-1 in worms), which additionally contain tyrosine or serine/threonine phosphorylation motifs and proline rich stretches, are almost exclusively membrane recruited through association of its SH3 domain with Grb2, which itself interacts with pY motifs on activated receptors or their adapters (e.g. Shc; Gu and Neel, 2003; Lock et al., 2000; Ravichandran, 2001). Gab2, being phosphorylated in its YxxM motif (e.g. by Fyn) targets PI3K activity to the plasma membrane. In consequence the production of PtdIns(3,4,5)$P_3$ provides additional binding site to retain the Grb2-Gab1 complex membrane associated and sustains signaling. This applies to EGFR signalling (Rodrigues et al., 2000), to BCR signal transduction, which is completely compromised with PH-domain deletion mutants of Gab1, and to multiple vital receptor systems, reflected by the embryonic lethality of Gab1 gene-targeted mice (Itoh et al., 2000). Gab2 deficient mice are viable but are compromised FcεRI and IL-3R signaling in mast cells (Gu et al., 2001)

An essential role of PI3K induced membrane translocation also has been reported for the B-cell specific Bam32 (B-lymphocyte adapter molecule of 32 kDa) and its closest homologues TAPP1 and 2 (Tandem PH domain-containing protein 1 or 2) (Marshall et al., 2002; Marshall et al., 2000), which PH domains preferentially bind to PtdIns(3,4)$P_2$ rather then PtdIns(3,4,5)$P_3$ (Ferguson et al., 2000; Thomas et al., 2001) and translocation thus most likely requires PI3K activity followed by activity of the phosphatidylinositol 5-phosphatase SHIP.

## 2.5 Determination of class I PI3K signaling by Phosphatidyl-inositol phosphatases:

Class I PI3K signaling, downstream of many receptor systems modulate cell growth, differentiation, proliferation, the migratory capacity. On a systemic level, these cellular features are essential for proper tissue homeostasis, development and immune responses. Deregulated PI3K signaling is thus linked to autoimmune disease, chronic inflammation, allergy, as well as cancer development, progression and metastasis (Wymann and Marone, 2005). A tight regulation of PI3K action is therefore required, realized through lipid phosphatases, which degrade PtdIns(3,4,5)$P_3$. Best characterized are PTEN (phosphatase and

tensin homologue deleted on chromosome 10), which dephosphorylates the 3' position (Myers et al., 1998), (Stambolic et al., 1998) and SHIP (SH2-containing inositol 5-phosphatase) family members.

***Phosphatase and tensin homologue deleted on chromosome 10***

PTEN was identified and dubbed due to its location on chromosome 10 q23 as a tumor suppressor gene, which mutations and loss of heterozygocity are strongly abundant in sporadic tumors, like glioblastoma, prostate and breast cancer (Li et al., 1997; Steck et al., 1997), but also present in inherited proliferative diseases, e. g. the cowden syndrome. Genetic targeting results in embryonic lethality for homozygous mice (Di Cristofano et al., 1998; Suzuki et al., 1998), while heterozygous mice or mice with T-cell specific loss of PTEN have increased susceptibility for lymphomas and autoimmune disorders, such as glomerulonephritis (Di Cristofano et al., 1999 Suzuki et al., 2001). Elevated PtdIns(3,4,5)$P_3$ due to loss of PTEN, with all its consequences, is conserved throughout species including *D. melanogaster* (Stocker et al., 2002) and *C.elegans* (Rouault et al., 1999). Accessibility of PtdIns(3,4,5)$P_3$ to PTEN is mediated by its C2 domain (Lee et al., 1999) or by interaction with membrane associated proteins via its PDZ binding domain, e.g. MAGI (Wu et al., 2000)

## *SH2-containing inositol 5-phosphatases*

The SHIP1 phosphatase was simultaneously identified by three independent research groups. Today two mammalian isoforms, SHIP1 and SHIP2, are known, with various splice variants or truncated forms of SHIP1 (Rohrschneider et al., 2000). Interestingly non-mammalian orthologues of SHIP proteins are missing. SHIP1 expression is restricted to the haematopoietic system and spermatogenic cells, while SHIP2 is broadly expressed (Bruyns et al., 1999; Muraille et al., 1999). In contrast to PTEN, SHIP metabolizes PtdIns(3,4,5)$P_3$ to PtdIns(3,4)$P_2$ (Damen et al., 1996; Lioubin et al., 1996; Pesesse et al., 1997), which allows to modulate PI3K mediated signal transduction, e.g. by recruiting TAPP1 and Bam32. In accordance with SHIP1's haematopoietic expression, SHIP1$^{-/-}$ mice suffer from myeloid infiltration, hyperplasia of granulocytes or macrophages (Helgason et al., 1998). On a molecular basis SHIP's N-terminal SH2 domain preferentially binds ITIM (immunreceptor tyrosine-based inhibition motif) based pY on inhibitory receptors like in B cells the FcγRIIB or the FcγRIIB and gp49B1 in mast cells (Ono et al., 1996), (Kuroiwa et al., 1998). Association with ITAM (immunreceptor tyrosine-based activation motif) based pY of the FcεRI (Osborne et al., 1996) or the monocytic FcγRIIa (Maresco et al., 1999) was reported, too. Multiple alternative protein interactions, either mediated by the SH2 domain, e.g. with class I PI3K (Gupta et al., 1999), the C-terminal NPXY motifs, if tyrosyl-phosphorylated interacting with Shc (Lamkin et al., 1997)), or the proline rich motifs (binding to Grb2; Damen et al., 1996), position SHIP1 proximal to activating receptors, to counteract their activity.

Association of SHIP with inhibitory (co-) receptors that co-ligate with BCR or FcεRI after antigen association is a critical process for negative signaling in B cells and mast cells. Thus SHIP1$^{-/-}$ B cells and mast cells have increased Btk membrane association and $Ca^{2+}$ fluxes, which in mast cells enhances degranulation (Huber et al., 1998) and cytokine production (Kalesnikoff et al., 2002). Thus SHIP1 is a "gatekeeper", which regulates the threshold level of PtdIns(3,4,5)$P_3$ to avoid constitutive degranulation already after sensitization

with IgE or weak affinity antigen (Huber et al., 2002).In addition SHIP1 controls proliferation and anti-apoptotic signaling downstream of the IL-3 or the GM-CSF receptor (Liu et al., 1999)

## 2.6 Class I PI3K signaling in immune cells

### 2.6.1 Isoform expression

The two catalytic subunits p110α and β are ubiquitously expressed and homozygous ablation results in early embryonic lethality (p110α E9-E10; p110β E3-7) (Bi et al., 1999; Bi et al., 2002). Viable mice can however be generated, in case of a combined heterozygous genotype, thus 50% of protein and kinase activity is sufficient for viability. Severe impacts on development thus far impaired clear studies of immunologic phenotypes of both isoforms. PI3Kδ and PI3Kγ are mainly expressed in leukocytes, and gene targeted mice are viable, fertile, with immunological defects only if acutely stressed (Hirsch et al., 2000; Sasaki et al., 2000; Li et al., 2000; Okkenhaug et al., 2002; Clayton et al., 2002; Jou et al., 2002).

### 2.6.2 Genetic Targeting of Phosphoinositide 3-Kinases

To define the physiologic functions of class IA PI3K either the regulatory or the catalytic subunits were eliminated or inactivated by gene targeting. Elimination of the regulatory subunits by disruption of i) the PIK3r1 gene, thus deleting p85α, p55α, p50α or ii) the first exon thus expressing p55α, p50α protein but not p85α, or iii) the PI3Kr2 gene (p85β) (Fruman et al., 1999; Suzuki et al., 1999; Ueki et al., 2002) were approached to inactivate all class IA catalytic subunits and to define isoform-specific functions of the regulatory subunits.

Attempts for isoform specific targeting of class IA catalytic subunits yielded viable mice only for p110δ (PI3K$\delta^{-/-}$), but not for p110 α and β (Bi et al., 1999; Bi et al., 2002; Clayton et al., 2002; Jou et al., 2002). Transgenic mice with kinase inactivated PI3Kβ (PI3K$\beta^{KR/KR}$; Ciraolo et al., 2008) and PI3Kδ (PI3K$\delta^{KD/KD}$; Okkenhaug et al., 2002) are viable, however only the latter were investigated for immunologic phenotypes, yet. Except for p85β null and PI3K$\delta^{KD/KD}$ mice, cell-type specific modulations of non-targeted class IA PI3K protein levels hamper data interpretation. Compensatory effects of non-targeted subunits can cover loss of protein effects of the targeted one, while down-modulated expression of the remaining subunits can cause effects, which are ascribed to the targeted subunit but are primarily independent of the latter one.

Class IB PI3K activity was genetically engineered by either disrupting the PIK3CG gene, p110$\gamma^{-/-}$ (Hirsch et al., 2000; Sasaki et al., 2000; Li et al., 2000) or by insertion of a catalytic inactive variant, p110$\gamma^{KR/KR}$ (with replacement of the essential Lys(K) 833 by Arg(R)). Both share comparable immunologic defects, while cardiac protection after aortic constriction was lost only in p110$\gamma^{-/-}$ mice (Crackower et al., 2002; Patrucco et al., 2004), which suggests a kinase independent function of PI3Kγ as a scaffold protein. As for class IA targeting, down-modulated adapter protein levels, e.g. p101 and p84, were reported in p110$\gamma^{-/-}$ neutrophils

(Suire et al., 2005). As p110γ does neither compete with class IA PI3Ks for activated receptors, which was proposed for class IA (Okkenhaug and Vanhaesebroeck, 2003), nor any of the adapters associate with class IA catalytic subunits, one can assume that despite modulated adapter protein levels neither receptor- nor adapter-availability to other PI3Ks is affected. More recently elimination of the p101 adapter subunit was reported (Suire et al., 2006), while the PIK3r6 gene was not targeted yet.

### 2.6.3 Pharmacological Targeting

The discovery of first generation PI3K inhibitors (Arcaro and Wymann, 1993; Vlahos et al., 1994) and especially the understanding of their molecular action (Wymann et al., 1996; Walker et al., 2000) allowed the development of many 2$^{nd}$ generation compounds, with decreased toxicity, increased stability and specificity (Marone et al., 2008). These pharmacological tools in combination with gene-targeted mice emphasized PI3Kγ and PI3Kδ as excellent targets in human immune disease related animal models (Tab.2). That all inhibitors are based on their competition with ATP for binding to the catalytic core, however risks that other lipid- or protein kinases are inhibited, due to structural homologies in their catalytic domains. Thus alternative therapeutic approaches, e.g. by allosteric inhibition or disruption of required interactions of PI3Kγ with other proteins, could minimize adverse effects. Therefore a detailed knowledge of how e.g. PI3Kγ is activated is required

**Table 2: Reported roles of PI3K isoforms in murine disease model**

| | | PI3K isoforms | | | | |
|---|---|---|---|---|---|---|
| **Disease** | **Murine Model** | **α** | **β** | **γ** | **δ** | **Effects of PI3K inhibition (GT= gene targeting; PH= pharmacological treatment)** |
| **Inflammation** | | | | | | |
| Pancreatitis | •acute cerulein induced pancreatitis<br>•CDE diet | | | | | PI3Kγ deficient mice are more resistant to cerulein induced acute pancreatitis, with reduced acinar cell injury/necrosis, diminished neutrophil infiltration and increased acinar cell apoptosis. The non-invasive CDE (choline-deficient, ethionine supplemented) diet model confirmed positive effects of PI3Kγ ablation. Thus PI3Kγ deficient mice showed reduced mortality after CDE diet (GT; (Lupia et al., 2004) |
| Peritonitis | •septic (*E.coli* injection) and aseptic (Thiol-glycollate) peritonitis | | | | | PI3Kγ deficient mice challenged with peritoneal injection of either thiolglycollate or bacteria (*E.coli* or *S. aureus*) showed strongly reduced leukocyte infiltration and bacteria clearance (GT; (Hirsch et al., 2000)). Selective loss of gastrointestinal mast cells in "PI3K deficient" mice (by deletion of p85α) show reduced bacteria clearance in septic peritonitis. This was however not attributed to a specific catalytic subunit (p110α, β or δ; GT; (Fukao et al., 2002b)) |
| **Airway Inflammation, Allergy, Asthma, Anaphylaxis** | | | | | | |
| Allergy, Anaphylaxis | • passive cutaneous or systemic anaphylaxis (PCA or PSA) | | | | | PI3Kγ null mice are protected from IgE/antigen driven PSA, due to interruption of a paracrine/ autocrine adenosine feedback loop. In accordance adenosine induced vascular permeability was decreased (GT; (Laffargue et al., 2002)). PI3Kδ targeted mice are protected from PCA, due to a blocked FcεR I signaling in mast cells (GT, PH; (Ali et al., 2004)). However "class IA deficient" mice (p85α null mice) were not resistant to PSA and showed anaphylactic shock induced death as wild type mice (GT; (Fukao et al., 2002b)) |
| Asthma, Airway inflammation | •intra-nasal KC, MIP-2<br>• Ovalbumin induced allergic asthma<br>• Metacholine induced hyperresponsivness | | | | | Neutrophil numbers in BAL fluids post intra-nasal chemokine administration are strongly reduced in PI3Kγ deficient mice (GT; (Thomas et al., 2005)). In LPS induced airway inflammation PI3Kγ and PI3Kδ are required for neutrophil extravasation, however PI3Kγ appears to be dominant in neutrophils, while PI3Kδ could have functions in the endothelium (GT, (Puri et al., 2004)). Pharmacological inhibition of PI3Kδ reduces leukocyte infiltration (mainly eosinophils) into ovalbumin challenged lungs, mucus production and levels of inflammatory cytokine as well as expression of cell adhesion molecules (PH; (Lee et al., 2006b), (Lee et al., 2006a)). Genetic targeting of PI3Kδ suppresses type 2 cytokine response and is required for Th2 mediated airway inflammation (GT; (Nashed et al., 2007)) |

| Disease | Murine Model | α | β | γ | δ | Effects of PI3K inhibition (GT= gene targeting; PH= pharmacological treatment) |
|---|---|---|---|---|---|---|
| **Autoimmune disease** | | | | | | |
| Rheumatic arthritis (RA) | • Collagen induced arthritis (CIA)<br>• passive CIIα 1A model (T-, B-cell independent) | | | | | Both models result in improved histological and clinical scores for RA in PI3Kγ deficient mice or mice treated with PI3Kγ specific inhibitor AS-605240. Mild disease progression is characterized by reduced paw swelling, impaired neutrophil infiltration and cartilage erosion (GT, PH; (Camps et al., 2005)). Pharmacological inhibition of PI3Kγ improved the disease state even after the onset of RA, indicating therapeutic relevance. |
| Systemic lupus erythematosous, Glomerolu-nephritis | • MPR-*lpr* mice<br>• Class1A PI3K mediated SLE ($p65^{PI3K}$) | | | | | MPR mice carrying homozygous lymphoproliferation (*lpr*) mutation develop spontaneous lupus like disease. Abrogation of PI3Kγ activity results in reduced $CD4^+$ T-cells reduced autoantibody production and thus ameliorates renal failure and improves clinical scores of glomerulonephritis (GT, PH; (Barber et al., 2005)). PI3Kγ inactivation protects from SLE like disease induced by constitutive activation of PI3K class IA, by the truncated activating $p65^{PI3K}$ (GT, (Barber et al., 2006)). |
| Crohn's disease | •Inflammatory bowels disease | | | | | Genetic targeting of PI3Kδ results in the development of mild inflammatory bowels disease in the rectum and cecum, characterized by mucosal hyperplasia, crypt abscesses and leukocyte infiltration (GT; (Okkenhaug et al., 2002)) |
| Sjörgen's syndrome | • r1ΔT/r2n mice (Oak, 2006) | | | | | T-cell specific targeted PIK3R1 gene products in p85β null mice develop immune cell infiltration (mainly $CD4^+$) into lacrimal glands and their destruction. Mice show increased autoantibody levels, altered $T_{helper}$-cell differentiation and cytokine production (GT; (Oak et al., 2006)) |
| **Others** | | | | | | |
| Atherosclerosis | • Apolipoprotein E $(ApoE)^{-/-}$ mice<br>• LDL-receptor $^{-/-}$ mice | | | | | $ApoE^{-/-}$ mice develop spontaneous atherosclerotic lesions, $LDL\text{-}receptor^{-/-}$ mice after fat rich, cholesterol-depleted diet. PI3Kγ inactivation resulted in decreased macrophage activation after pro-atherosclerotic or acute inflammation stimuli (oxidized LDL, C-peptide, CRP)(GT; (Chang et al., 2007)). PI3Kγ null mice and PI3Kγ-inhibitor treated wild type mice had reduced atherosclerotic lesions, macrophage and T-cell ($CD4^+$) invasion and increased stabilization of formed plaques. (GT, PH; (Fougerat et al., 2008)) |
| Thrombosis | • ADP induced thrombosis | | | | | PI3Kγ null mice are protected from ADP induced thrombosis, as ADP induced platelet aggregation is impaired (GT; (Hirsch et al., 2001)) |

No Shading: No reported effect on disease

Green: Isoform inhibition has a positive effect on disease

Orange: Class IA inhibition has a positive effect on disease, however catalytic isoform has not been further defined

Red: Negative effect or cause of disease after isoform inhibition.

## 2.6.4 *In vivo* verified functions of PI3K isoforms in immune cells:

***T-cells***

Class IA PI3K signaling in T-cells was studied with all viable gene-targeted mice described above. Neither p85α nor p110δ null T-cells showed any defects, whereas p85, 55, and 50α null mice displayed an altered Th1:Th2 balance *in vivo*, however mediated by T cell extrinsic, dentritic cell mediated effects (Fukao et al., 2002a). Loss of p85β protein in T cells, rather surprising, increased proliferation upon anti-CD3 and IL2 treatment and reduced cell death *in vivo* and *in vitro* (Deane et al., 2004). Interestingly PI3K$\delta^{KD/KD}$ mice, in contrast to PI3K$\delta^{-/-}$ mice, were impaired in proliferation upon TCR cross-linking with anti-CD3 antibodies, so were $Ca^{2+}$ fluxes and phosphorylation of PKB impaired *in vitro*. Co-stimulation with IL-2 or CD28, however, eliminated proliferative defects (Okkenhaug et al., 2002). Inactive PI3Kδ impaired also proper differentiation into effector T cells, memory T cells, and Th1 and Th2 helper cells while T cell populations were normal in the periphery (Okkenhaug et al., 2006). Elimination of all class IA regulatory subunits in T cells and thymocytes could however only partially confirm the PI3K$\delta^{KD/KD}$ phenotype, as their normal development and differentiation challenges the requirement of class IA PI3K in T cell development (Deane et al., 2007).

PI3Kγ deletion perturbs T cell development, reducing double positive cells and altering the $CD4^+$: $CD8^+$ single positive ratio. Peripheral T cell proliferation and cytokine production in response to TCR/CD28 co-stimulation (Sasaki et al., 2000; Alcazar et al., 2007; Rodriguez-Borlado et al., 2003) is negatively affected, too. Defects in chemokine induced T cell migration are present, but not as prominent as in neutrophils or macrophages defective for PI3Kγ signaling (Reif et al., 2004).

Similar and mild defects in T cell development were observed by inactivation of either PI3Kδ or PI3Kγ. Combined inactivation of both isoforms, however, led to complete block of thymocyte development. Thus signaling through one isoforms could compensate for loss of the other, or partial PI3K activity could be sufficient for T cell development (Webb et al., 2005).

***B cells***

B cells a highly dependent on class IA PI3K downstream the B cell receptor (BCR). Deletion of PIK3r1 gene products (p85α, p55α, p50α), or manipulated p110δ impairs BCR signaling, while defects in p85β or PI3Kγ deleted B cells were absent (Suzuki et al., 1999; Okkenhaug et al., 2002; Jou et al., 2002). Mice with disrupted or defective PI3Kδ or p85α have profoundly reduced total number of mature B cells, due to blocked transition from pro-B cell to the pre B cell stage during development. Proliferation upon anti-IgM treatment was completely compromised, while anti-CD40 or LPS treatment resulted in partial proliferation defects. So was the T cell-dependent and -independent antibody production defective in PI3Kδ or PIK3r1 targeted B cells.

***Neutrophils and macrophages***

Neutrophils and macrophages are required for immediate clearance of bacterial pathogens and cause acute and chronic inflammatory processes. Therefore they are dependent on their ability to migrate towards sites of inflammation along shallow chemotractant gradients, including blood vessel extravasation, degranulation, phagocytosis, superoxide production and chemokine release. Neutrophilic migration and superoxide production post chemokine stimulation are strongly impaired by elimination of PI3Kγ activity by either gene targeting (Hirsch et al., 2000) or isoform specific inhibition of PI3Kγ *in vitro* and in murine disease models such as chemokine induced neutrophilia in airway inflammation (Thomas et al., 2005) or septic peritonitis (Hirsch et al., 2000; Camps et al., 2005).

Although none of the above-mentioned neutrophil-intrinsic functions require PI3Kδ, *in vivo* its cooperative activity with PI3Kγ was required for $LTB_4$-induced extravasation of neutrophils (Puri et al., 2004). Since then, approaches using combinations of isoform specific inhibition and gene targeting proposed a biphasic process for chemokine driven neutrophil extravasation and ROS production in TNFα primed human neutrophils: in an early phase PI3Kγ is indispensable, which is prerequisite for the second phase, in which PI3Kδ is involved (Liu et al., 2007; Condliffe et al., 2005).

## 2.6.5 Class I PI3K in mast cells:

### 2.6.5.1 PI3K signaling in mast cell development

Class IA PI3Ks are commonly critical signal transducers in growth factor receptor signaling (Fig. 3). In mast cells c-KIT is substantial for survival, proliferation and differentiation. Inhibitory experiments with wortmannin supported the involvement of PI3K in SCF mediated survival, proliferation, adhesion of BMMC (Serve et al., 1995). Both, mice with kinase inactive PI3Kδ and mice with deleted p85α, confirmed the vital function of class IA PI3K and in particular of PI3Kδ downstream c-KIT *in vivo*. Thus reduced mast cell numbers and a complete lack of mast cells in the peritoneum and the gastrointestinal tract were reported for either of the gene-targeted mice (Ali et al., 2004; Fukao et al., 2002b). In line doubling times of cultured $PI3K\delta^{KD/KD}$ BMMC are prolonged (Ali et al., 2004).

### 2.6.5.2 PI3K signaling in mast cell migration

Mast cell precursors have to evade the circulation in order to become tissue resident. As outlined before the sequential steps in this process were suggested to be SCF/c-Kit mediated (Lorentz et al., 2002; Nilsson et al., 1994; Boyce, 2003). In line with the role of class IA PI3K downstream c-Kit, mice with inactivated p110δ or deleted p85α show tissue specific reductions in mast cell numbers. Further BMMC of either of the mice were defective to migrate in response to SCF stimulation. Thus the tissue specific loss of mast cells is most likely a convoluted result of decreased survival and impaired migration towards SCF gradients for initial

mast cell distribution to tissues, provided that SCF gradients between the circulation and tissues exist (Ali et al., 2004; Fukao et al., 2002b). During the development of allergic disease IgE is secreted from B cells in inflamed tissues. Thus IgE gradients can be expected (Cameron et al., 1998; Smurthwaite et al., 2001). Under allergic conditions the chemotactic capacity of IgE can thus locally enhance mast cell numbers. Interestingly migration towards IgE is PI3Kγ dependent *in vitro* (Kitaura et al., 2005) and *in vivo*, while PI3Kδ was dispensable (Collmann et al., in preparation). Moreover IgE-mediated BMMC migration is amplified in a PI3Kγ dependent autocrine and paracrine feedback signaling of GPCR agonists, which are gradually distributed from the inflamed tissue (Kitaura et al., 2005).

### 2.6.6 PI3K signaling in mast cell degranulation

***Mast cell activation via the FcεRI***

The FcεRI is a multidomain receptor, with four membrane spanning subunits: the α-subunit for extracellular ligand binding, a tetra-spanning β-subunit and the dimeric, di-sulfide linked γ-subunit for signal transduction and amplification, due to the ITAM motifs present in cytoplasmic tails the β- and γ-subunits. FcεRI mediated signal transduction requires sensitization by bound IgE, which in a monomeric form positively modulates receptor expression (Hsu and MacGlashan, 1996; Yamaguchi et al., 1997) and cell survival (Asai et al., 2001; Kalesnikoff et al., 2001) but also lymphokine synthesis and release (Gonzalez-Espinosa et al., 2003).
When IgE-decorated FcεRI is however cross-linked by polyvalent antigens (Ag-IgE-FcεRI; Kinet, 1999) signal transduction is transiently enhanced, leading to an immediate release of secretory granules, followed by a late phase response, through the release of *de novo* synthesized inflammatory mediators, such as prostaglandins ($PGE_2$, $PGD_2$), leukotrienes ($LTB_4$, $LTC_4$), cytokines and chemokines (Tab.1). Both phases thereby contribute to the typical, location dependent, allergic indications.
Mechanistically, Ag-IgE-FcεRI complexes re-localize to lipid raft compartments (Holowka and Baird, 2001), which was suggested to induce close proximity to the Lyn tyrosine kinase of the src family. Lyn phosphorylates the β and γ chain located ITAM motifs, further recruiting and activating Lyn itself as well as the PTK Syk. Fyn is a second src family kinase to be activated by cross-linked Ag-IgE-FcεRI complexes, but independent of Lyn activity (Parravicini et al., 2002).
Lyn and Fyn subsequently induce an inconceivable signaling network through a tyrosine phosphorylation cascade and the assembly of signalosome complexes, consisting of trans-membrane, membrane-associated and cytosolic adapters, as well as signal transduction enzymes (Kalesnikoff and Galli, 2008). In the end various physiologic responses are induced, amongst others, $Ca^{2+}$ mobilization which is required for degranulation (Blank and Rivera, 2004). Simplified, the Lyn-Syk axis hereby acquires LAT and NTAL as signaling platform, which is phosphorylated at multiple tyrosine residues to attract cytosolic adapters, such as SLP76, Grb2 or Gads, the Rac-GEF Vav and Rac itself. PLCγ is additionally recruited and the whole signalosome orchestrates $Ca^{2+}$ mobilization, due to positive modulation of PLCγ activity.

Fyn is also implicated in the $Ca^{2+}$ response, however through affecting a different set of molecular targets as Lyn. The association of Fyn to the β-chain ITAMs of the FcεRI is required initially (Parravicini et al., 2002). Being activated, Fyn phosphorylates the "signaling platform" Gab2 to recruit class IA PI3K and SHP-2.

There is a lot of evidence for the requirement of PI3K activity downstream FcεRI aggregation, as both the Lyn-Syk-LAT and Fyn-Gab2-PI3K pathways include multiple proteins that associate with PI3K and can thus integrate class IA PI3K activity. The gatekeeper role of SHIP in mast cell degranulation (Huber et al., 1998) further supports PI3K activity downstream of FcεRI aggregation.

Mast cells from Gab2 deficient mice are blocked in the transmission of signals from FcεRI to PI3K, and have thus tremendous reduction of PI3K activity, reduction in $Ca^{2+}$ fluxes and show diminished degranulation, which translates into protection from anaphylaxis in Gab2 deficient mice (Gu et al., 2001). The reduced PI3K activity and thus the inactive Btk might be causal for diminished $Ca^{2+}$ fluxes. Although the regulatory subunit p85 associates with Gab2 in BMMC lysates these data do not define the class IA PI3K isoform, which is associated. Comparable defects were observed in PI3K$\delta^{KD/KD}$ mice and BMMC (Ali et al., 2004) with complete inhibition of signaling to PKB/Akt, a 30-50% inhibition of granule and cytokine release in BMMC and partial protection from passive cutaneous anaphylaxis (PCA) in mice, which challenged the previously reported role of PI3Kγ in systemic anaphylaxis (Laffargue et al., 2002). This indicates that Gab2 could be associated with PI3Kδ activity. Similar to PI3K$\delta^{KD/KD}$ mice, Gab2 deficient mice have a tissue specific loss of mast cells, and BMMC of both genotypes are defective in SCF-mediated signaling (Nishida et al., 2002; Ali et al., 2004).

Confusing herein is however that mice and mast cells lacking all class IA PI3K activity, through deletion of p85α or p85, p55 and p50α, were fully functional in IgE/Ag induced degranulation and passive systemic anaphylaxis was as severe as in wild type controls (Fukao et al., 2002b ;Lu-Kuo et al., 2000).

PI3Kγ deficient mice are resistant to passive systemic anaphylaxis (PSA) after IgE/antigen challenge, highlighting this isoform *in vivo*. Reduced IgE/Ag dependent granule exocytosis in BMMC supplemented to observed protection *in vivo* (Laffargue et al., 2002). Mechanistically PI3Kγ is however not to directly engaged downstream of FcεRI. In accordance with the model of PI3Kγ activation here PI3Kγ was involved in a GPCR dependent autocrine/paracrine positive feedback loop, which is essential for full-scale mast cell activation (Laffargue et al., 2002). Thereby increased PtdIns(3,4,5)$P_3$ levels overshoot a threshold, for which SHIP activity can not compensate anymore (Fig. 9). The feedback was shown to involve adenosine, as adenosine deaminase treatment blocked signal amplification. Similar effects were observed with pertussis toxin treatment, thus signaling through the $G\alpha_i$ coupled $A_3$ adenosine receptor. Interestingly increased adenosine levels were reported in brocho-alveolar lavage fluids from asthmatic patients, and those showed increased sensitivity to inhaled adenosine compared to healthy controls (Fozard, 2003). Extrapolation of murine models to human disease like asthma or allergy could thus be possible. The contradictions on which PI3K is involved in mast cell activation led to a side-by-side analysis of PI3Kδ and PI3Kγ inhibition (pharmacologically and genetically; Ali et al., 2008). First this study could not confirm the complete block of IgE/Ag signaling in PI3Kδ inhibited BMMC, as it was reported before. In contrast PI3Kγ inactivation impaired IgE/Ag mediated signaling to phosphorylation of PKB/Akt to a great extent.

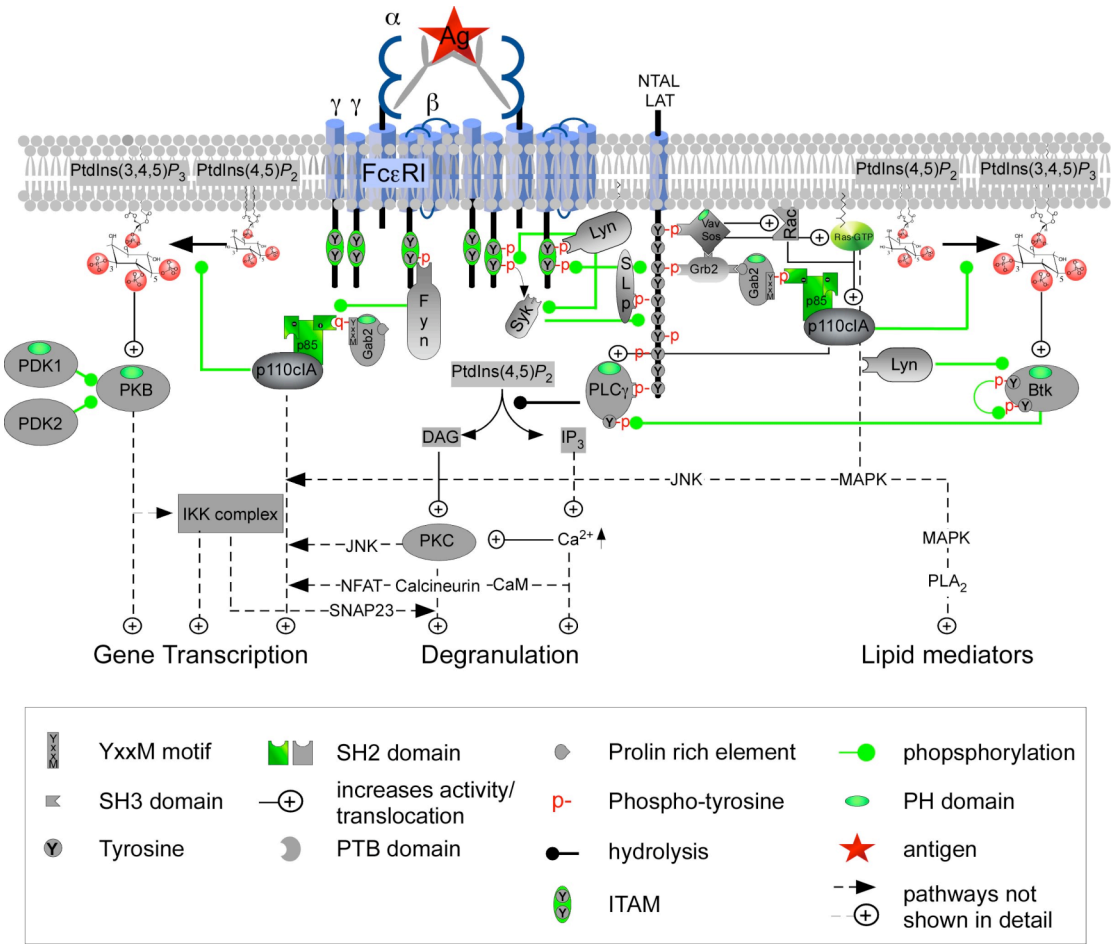


**Fig. 8: Simplified network of activating signaling events downstream of FcεRI cross-linking by IgE/antigen complexes:** Aggregated FcεRI requires Lyn or Fyn kinases for initial phosphorylation of its ITAM motifs, which are then bound by more Lyn or Fyn molecules as well as by the tyrosine kinase Syk. The associated and in turn activated kinases phosphorylate adapter proteins, like Gab2, LAT and NTAL, for further recruitment of pY binding adapter proteins or enzymes. Class IA PI3Ks (p110clA complexed with p85) are associated to the signaling complexes, mainly via p85's SH2 domains binding to the pYxxM motifs on Gab2, but also by additional or co-operative protein interactions, e.g. with GTP loaded Ras or direct association with Lyn (not shown). PI3Ks can here directly activate phospholipase Cγ (PLCγ) or by PtdIns(4,5)$P_2$ phosphorylation, which increases Btk membrane translocation and its activation through phosphorylation by Lyn and auto-phosphorylation. Btk then activates PLCγ, which hydrolyses PI(4,5)$P_2$ to Diacylglycerol (DAG) and Inositol(1,4,5)$P_3$ ($IP_3$). DAG directly activates protein kinase C (PKC) in synergy with $Ca^{2+}$, mobilized from the ER (via $IP_3$ regulated $Ca^{2+}$ channels) and the coupling to extracellular $Ca^{2+}$ currents via stromal interaction molecule1 (Stim1) activity, to increase $Ca^{2+}$ levels (not shown) and to drive degranulation. PtdIns(3,4,5)$P_3$ also recruits PDK1 and PKB to the membrane thus activating PKB, which indirectly induces gene transcription and degranulation (via IKK [IκB kinase] complex phosphorylation and subsequent SNAP23 [synaptosomal associated protein, 23 kDA] phosphorylation). Some indirect interconnections are indicated by dashed lines, others are not shown to increase clarity, e.g. the positive effect of PI3K activity on GEFs, as Vav, or Sos, and consequently Ras and Rac GTPases. SLP: SH2 domain containing leukocyte protein of 76kDa; Grb2: Growth factor receptor bound protein 2; JNK: Janus kinase; CaM: Calmodulin; NFAT: Nuclear factor of activated T-cells; MAPK: Mitogen activated protein kinase pathway; $PLA_2$: Phospolipase A2, References see main text)

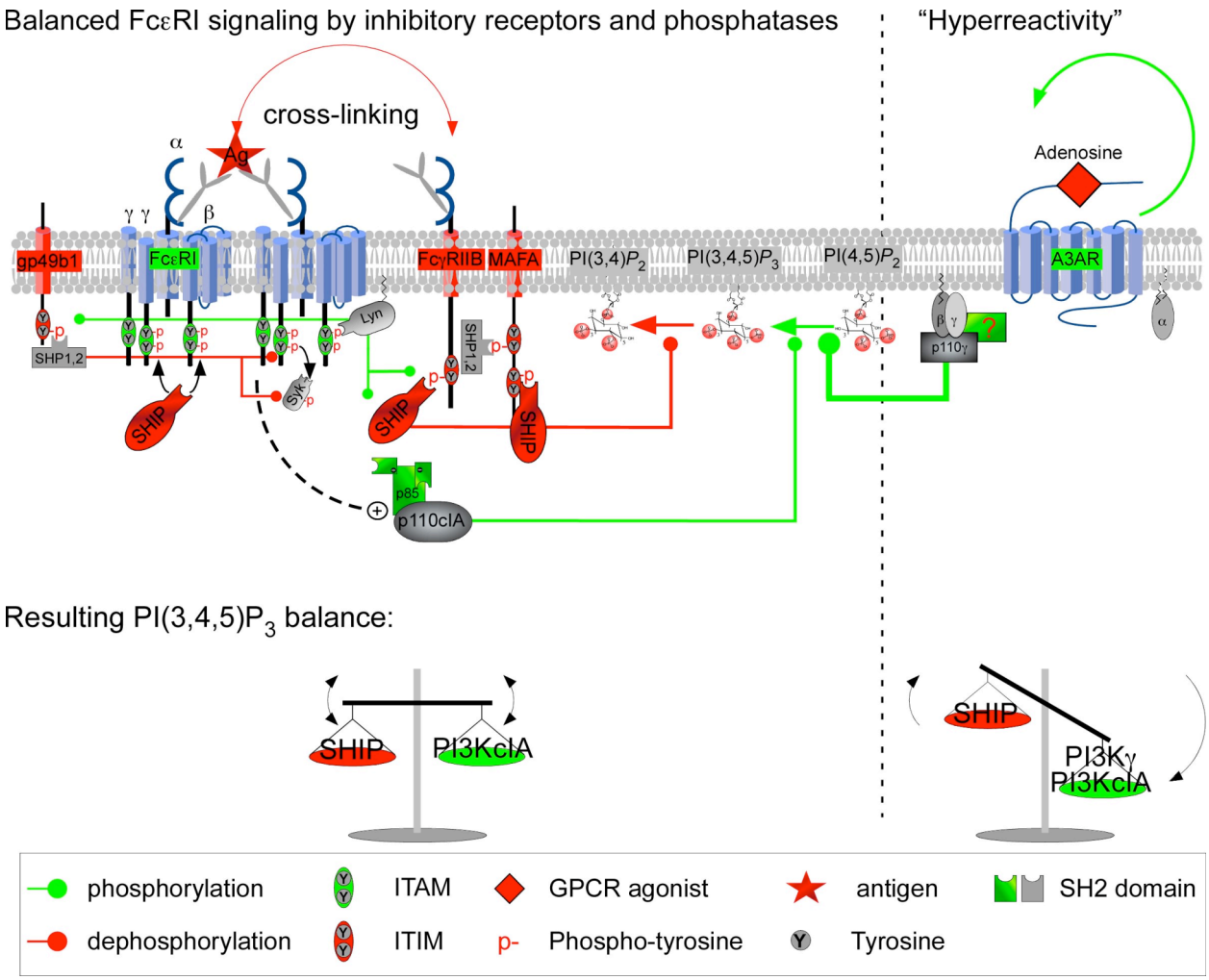


**Fig. 9: Inhibitory events in FcεRI signaling and the PI3Kγ dependent bypass driving hyperreagibility:**
***Left:*** Antigen cross-linking leads to the activating events shown in Fig. 8 (indicated with the dashed line here for class IA PI3K). It however also integrates (red arrow) inhibitory immune receptors (in red; FcγRIIB), containing ITIMs, into the signaling complex, and other inhibitory receptors (gp41b, MAFA [mast cell function associated antigen]) are close to the FcεRI as well, which drives phosphorylation of their ITIMs by Lyn. Phosphorylated ITIMs recruit protein tyrosine phosphatases (SH2 containing tyrosine phosphatase 1 and 2 [SHP1, 2], which de-phosphorylate Syk and ITAMs (thus inactivating the PTK cascade), and/or the SH2 domain containing inositol-5-phosphatase 1 and 2 (SHIP), which convert PtdIns(3,4,5)$P_3$ to PtdIns(3,4)$P_2$, leading to the redistribution of essential signaling components (e.g. Btk) to the cytosol. Thus signaling is finely balanced. **Note:** there are alternative routes for down-modulation of FcεRI signaling such as endocytosis and degradation of FcεRI and Syk by interaction with Cbl and subsequent ubiquitination (not shown). ***Right:*** PI3Kγ mediated autocrine/paracrine feedback loop (green arrow) provides an additional PtdIns(3,4,5)$P_3$ source, which overshoots SHIP's capacity to regulate PI3K activity, leading to imbalanced signaling, hyperreactivity of mast cells and maximal degranulation. Here, especially adenosine, with increased levels in inflamed and hypoxic tissue is very effective in driving this signal amplification. Notably the requirement of PI3Kγ adapters is undefined herein.

The inhibition of either PI3Kδ or PI3Kγ yielded in equal reductions of granule exocytosis from BMMC. From this it is difficult to construe how then inhibition of PI3Kδ, but not of PI3Kγ, protected mice from PCA, as reported here.

Accepting this observation, an explanation could be provided by indirect effects on mast cells in their natural context, rather than by a direct effect in IgE/antigen mediated mast cell activation. Notably both isoform equally contributed to IgE/antigen dependent mast cell degranulation *in vitro*. Thus impaired c-Kit and IL-3 signaling could reduce the concentration of tissue resident mast cells, which was reported for non-IgE treated mice (Ali et al., 2004), but which was thus far not assessed after local tissue sensitization with IgE. Moreover PI3Kδ inhibited mast cells are certainly impaired the signal transduction of co-stimulatory effectors, such as SCF (Hundley et al., 2004), and apart from this PI3Kδ inhibition deprives signaling from cytokine and growth factor sources, changing the overall state of the cells.

Recent work in our laboratory (Collmann *et al.*, in preparation) reevaluated the functional requirement of PI3Kδ, compared to PI3Kγ, in FcεRI mediated mast cell activation: in short our results showed that the role of PI3Kδ diminishes to its vital function in mast cell differentiation, while signaling from aggregated FcεRI to activation of PKB/Akt and to granule exocytosis were unaffected in PI3K$\delta^{KD/KD}$ BMMC or PI3Kδ inhibitor treated wild type mast cells. Moreover, genetic and pharmaceutical inhibition of PI3Kδ was neither effective to protect mice from PCA, nor to prevent mast cell precursor recruitment to sites of IgE injection or to sites of TNFα simulated inflammation *in vivo*. In contrast all the physiological processes, and their simulative *in vitro* experiments, were impaired by genetic of pharmaceutical PI3Kγ targeting.

# 3 Aims

As outlined above PI3Kγ is an excellent target for treatment of allergic disease. First inhibition of PI3Kγ protected mice from mast cell degranulation after allergic challenge and thus reduced allergic symptoms, even when the PI3Kγ inhibitor was acutely administered. Second PI3Kγ inhibition prior sensitizing tissue with IgE prevented mast cell recruitment to the site of IgE injection (Collmann *et al.*, in preparation). Thus prolonged administration of PI3Kγ inhibitors could be beneficial in reducing disease progression, due to reduced mast cell numbers in tissues leading to decreased release of inflammatory mediators. Similarly the physiologic importance of PI3Kγ signaling was also very well documented in other haematopoietic cells, e.g. neutrophils, macrophages (Hirsch et al., 2000).
In recent years many promising compounds for PI3Kγ inhibition were tested in human disease related animal models (Tab. 2). All inhibitors act here by competing with ATP for binding to the catalytic core. Consequently there is still a risk to inhibit other lipid- or protein kinases, due to structural homologies in their catalytic domains.
Thus alternative therapeutic approaches, e.g. by allosteric inhibition or disruption of required interactions of PI3Kγ with other proteins, could minimize adverse effects. Therefore a detailed knowledge of how PI3Kγ is activated is required. But even the physiologic requirement of the only known PI3Kγ adapter subunit p101 was disputed until Suire et al. (Suire et al., 2006) could show that p101 was required for PI3Kγ mediated neutrophil migration. An adapter independent PI3Kγ-mediated migration could however not be excluded. Our work and work of others identified a second adapter subunit of PI3Kγ, dubbed p84 or $p87^{PIKAP}$ (Suire et al., 2005; Voigt et al., 2006), which further complicated interpretation of p101's function in neutrophils, as cross-compensatory effects of both adapters could not be excluded.

1st we thus aimed to define a clean system with physiologic relevance in which to analyze the role of p84- if PI3Kγ adapters are required at all. Expression analysis presented mast cells as most appropriate to study the function of p84.

2nd by developing a mast cell complementation system we aimed to show and could show that PI3Kγ activity completely depends on the presence of p84 in mast cells.

3rd we aimed to analyze if the two adapters have non-overlapping functions, although *in vitro* data did not suggest so.
Distinct functions of either of the two adapter:p110γ complexes would add additional complexity to PI3Kγ signaling, but would certainly be highly interesting for therapeutic aspects. Indeed we were able to define a non-redundant function of either of the adapter:p110γ complexes.

4th we approached to explain the distinct signal capacities of both of the adapter:p110γ complexes.

# 4 Results

## PI3K$\gamma$ adapter subunits define coupling to degranulation and cell motility by distinct PtdIns(3,4,5)$P_3$ in mast cells

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CELL BIOLOGY

# PI3Kγ Adaptor Subunits Define Coupling to Degranulation and Cell Motility by Distinct PtdIns(3,4,5)$P_3$ Pools in Mast Cells

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**Phosphoinositide 3-kinase γ (PI3Kγ) plays a major role in chronic inflammation and allergy. It is a heterodimer of a catalytic p110γ subunit and an adaptor protein, either p101 or the p101 homolog p84 (p87$^{PIKAP}$). It is unclear whether both PI3Kγ complexes specifically modulate responses such as chemotaxis and degranulation. In mast cells, the p84:p110γ complex synergizes with immunoglobulin E (IgE)– and antigen-clustered FcεRI receptor signaling and is required to achieve maximal degranulation. During this process, PI3Kγ is activated by ligands of heterotrimeric guanine nucleotide–binding protein (G protein)–coupled receptors (GPCRs), in particular adenosine receptors, through autocrine and paracrine pathways. Here, we show that p110γ needs p84 to relay signals from GPCRs to formation of phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)$P_3$], phosphorylation of Akt, migration of cells, and synergistic adenosine-enforced degranulation. Furthermore, the absence of adaptor subunits could not be compensated for by increased p110γ abundance. Differentiated, p110γ null cells also lost adaptor proteins. Complementation of p110γ null mast cells with p101 and p110γ restored the activation of Akt and cell migration, but failed to support degranulation. Lack of degranulation was attributed to a change in the spatiotemporal localization of PI3Kγ-derived PtdIns(3,4,5)$P_3$; although both p84:p110γ and p101:p110γ complexes initially deposited PtdIns(3,4,5)$P_3$ at the plasma membrane, p101:p110γ–derived PtdIns(3,4,5)$P_3$ was rapidly endocytosed to motile, microtubule-associated vesicles. In addition, p84:p110γ, but not p101:p110γ signaling was sensitive to disruption of lipid rafts. Our results demonstrate a nonredundant function for the p101 and p84 PI3Kγ adaptor proteins and show that distinct pools of PtdIns(3,4,5)$P_3$ at the plasma membrane can elicit specific cell responses.**

## INTRODUCTION

Phosphoinositide 3-kinases (PI3Ks) are implicated in cancer (*1*–*4*) and inflammation (*5*, *6*), and their net activity can modulate the transition from normal cell functions to the progress of disease. Regulatory subunits translocate the 110-kD catalytic subunits—consisting of class IA PI3K members p110α, β, and δ and class IB p110γ—to their substrate phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)$P_2$] at the plasma membrane. For class IA PI3Ks, it is well documented that both Src homology 2 (SH2) domains of p85-like regulatory subunits (encoded by *PIK3R1*, *PIK3R2*, and *PIK3R3*) direct the PI3K complexes to phosphorylated Tyr-X-X-Met motifs on receptor protein tyrosine kinases or their substrates (*5*, *7*) and that interactions between p85 and p110 regulate enzyme activity (*8*, *9*). In spite of this knowledge, the physiological consequences of genetic ablation of regulatory p85 subunits have often been surprising. Whereas targeting of p85α (by deletion of exon 1 of *PIK3R1*) attenuated the function of PI3K downstream of the B cell receptor as expected (*10*–*12*), loss of p85α or p85β increased insulin signaling (*13*–*15*). Due to the multilevel complexity of the PI3K network, however, a full description of the physiological roles of the regulatory subunits of PI3Ks is far from being complete.

The sole member of the class IB family, p110γ (encoded by *PIK3CG*) operates downstream of heterotrimeric guanine nucleotide–binding protein (G protein)–coupled receptors (GPCRs) (*5*, *16*) and is activated by βγ subunits of G proteins (Gβγ) in vitro (*17*, *18*). The catalytic subunit p110γ forms heterodimers with either of the adaptor subunits p101 (encoded by *PIK3R5*) (*18*) and a p101 homolog, p84 (also called p87$^{PIKAP}$, encoded by *PIK3R6*) (*19*, *20*). N- and C-terminal sites of p110γ bind to Gβγ subunits directly (*21*), but p101 and p84 are required for efficient Gβγ-stimulated phosphorylation of PtdIns(4,5)$P_2$ in vitro (*19*, *22*, *23*). The physiological importance of p101 was demonstrated convincingly in p101 null mice, in which GPCR ligands failed to trigger neutrophil chemotactic responses, whereas the induction of neutrophil NADPH oxidase was not affected (*24*), even though both responses are dependent on PI3Kγ (*16*). The p101 and p84 adaptor subunits perform equally well in vitro (*19*), but a physiological role for p84 is yet to be defined. It is still unclear whether p84 and p101 have overlapping or nonredundant functions in vivo.

We have therefore addressed the requirement and actions of PI3Kγ adaptor proteins in the physiologic context of mast cell activation, which provides a superb model system in which to study the function of PI3Kγ. In mouse mast cells, PI3Kγ signals downstream of A3 adenosine receptors (A3ARs) and drives cell migration (*25*), as well as synergistically enhancing IgE- and antigen-mediated degranulation in vitro and IgE- and antigen-induced passive systemic anaphylaxis in vivo (*26*). In this context, adenosine signaling through GPCRs is well known for activating mast cells in atopic, but not normal, patients (*27*, *28*).

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The clustering of FcεRI receptors through antigen-IgE complexes triggers a protein tyrosine kinase cascade that leads to the phosphorylation of immunoreceptor tyrosine–based activation motifs (ITAMs) on FcεRI receptor chains, multiple tyrosines on linker of activated T cells (LAT), non–T cell activation linker [(NTAL), also known as linker for activation of B cells (LAB), also known as LAT2] (*29*), and Tyr-X-X-Met motifs on growth factor receptor–bound protein 2 (Grb2)–associated binding protein 2 (Gab2) (*30*). Class IA PI3Ks, and in particular p110δ (*31*), thus produce a first wave of phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)$P_3$] as a consequence of the clustering of FcεRI receptors. This signal is, however, quenched by the action of the SH2 domain–containing inositol 5′-phosphatase (SHIP), which degrades PtdIns(3,4,5)$P_3$ to PtdIns(3,4)$P_2$ (*32*). In this context, GPCR-triggered activity of PI3Kγ delivers a superimposed pulse of PtdIns(3,4,5)$P_3$, which is integrated with the FcεRI protein tyrosine cascade and produces a full-scale activation and degranulation response. Its exclusive dependence on PI3Kγ makes this an attractive model in which to unravel the functions of the PI3Kγ adaptor proteins.

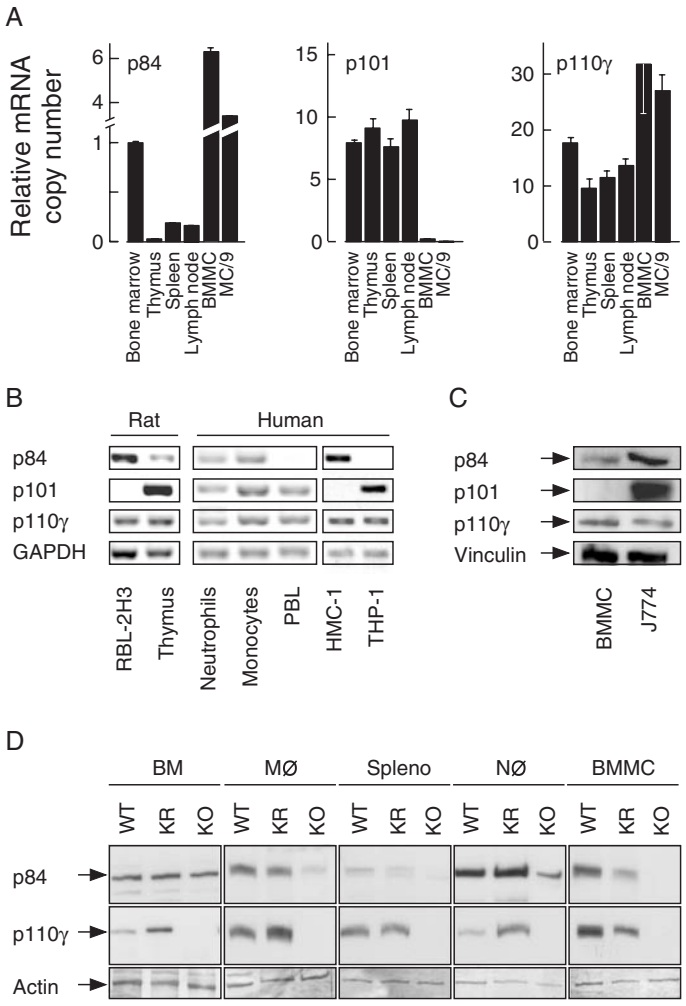


Fig. 1. Tissue distribution of p110γ and its adaptor subunits. (**A**) Relative copy numbers of mRNAs for p110γ, p84, and p101 were assessed by qPCR in the indicated murine tissues (for additional tissues, see fig. S1), as well as in BMMCs and a mouse mast cell line (MC/9). Data shown represent mean ± SEM of more than three experiments. All values are normalized to the copy number of p84 mRNA in BM. (**B**) p84, p101, p110γ, and GAPDH (internal standard) mRNAs were amplified from the RBL-2H3 and HMC-1 mast cell lines and the indicated rat and human cells and tissues by RT-PCR, separated by agarose gel electrophoresis, and visualized with ethidium bromide. (**C**) p101 protein is undetectable in murine BMMCs. Total lysates of murine BMMCs and J774 cells were subjected to SDS-PAGE and the indicated proteins were detected by Western blotting. (**D**) p84 protein is stabilized in the presence of p110γ. Bone marrow (BM), macrophages (MØ), splenocytes (Spleno), neutrophils (NØ), and mast cells (BMMC) from WT mice (WT), mice deficient in p110γ (KO), or mice with a catalytically inactivated p110γ (Lys$^{833}$→Arg mutation; KR) were probed for the presence of p110γ and p84 proteins by Western blotting. Blots in (B) to (D) are representative of more than three experiments each.

Here, we show that differentiated hematopoietic cells that lack the p110γ catalytic subunit also lose their PI3Kγ adaptor proteins, which was exploited to produce mast cells devoid of both p110γ and p84. Complementation assays showed that p110γ could not operate without its adaptor proteins. Moreover, p101 and p84 had nonredundant functions in a physiological context and produced distinct pools of PtdIns(3,4,5)$P_3$ with different fates, sensitivities to depletion of cholesterol, and capacities to promote release of mast cell granules. PI3Kγ adaptor subunits thus controlled spatiotemporal signaling of p110γ catalytic subunits to yield diverse physiologic outputs. Because PI3Kγ is an important component in chronic inflammation and allergy, a full understanding of its mechanism of activation might open previously unknown avenues of context- and tissue-specific targeting of PI3Kγ. Spatial organization might also apply to mammalian p85 class IA PI3K adaptor proteins, which have physiologically poorly explored interaction domains, such as the SH3 and breakpoint cluster region homology (BH) domains (*7*).

## RESULTS

### Mast cells: a model in which to study the functions of PI3Kγ adaptor proteins

To evaluate and compare the tissue distribution of p84:p110γ and p101:p110γ complexes, we measured the abundance of messenger RNAs (mRNAs) for mouse p110γ, p84, and p101 by quantitative polymerase chain reaction (qPCR) assays. mRNAs for p110γ, p84, and p101 were detected at high abundance in murine hematopoetic tissues and cells (Fig. 1A and fig. S1), corresponding to the reported role of PI3Kγ in the recruitment of leukocytes (*16*) and full-scale activation of mast cells (*26*). In all tissues investigated, p110γ mRNA correlated with the presence of that of at least one of the adaptor subunits, supporting the importance of the regulators to the function of PI3Kγ. The mRNA for p101 was prominently expressed in bone marrow (BM), thymus, spleen, and lymph nodes but was at low abundance in BM-derived mast cells (BMMCs) (see Fig. 1C for protein abundance) and mast cell lines of mouse (MC/9), rat (RBL-2H3), and human (HMC-1) origin. In contrast, the abundance of p84 mRNA was high in mast cells (Fig. 1, A and B). Similarly, we examined the abundance of PI3Kγ subunit proteins in hematopoietic cells derived from wild-type (WT) mice, p110γ null mice (KO) (*16*, *33*), or genetically targeted mice with catalytically inactive p110γ (KR, which contains a Lys$^{833}$→Arg mutation) (*34*). In BM samples, the abundance of p84 protein was not affected by manipulations of the p110γ locus (Fig. 1D). However, the abundance of p84 was lower in differentiated cells such as mast cells, macrophages, splenocytes, and neutrophils from KO mice than from WT mice, whereas the abundance of p84 was normal in cells from KR mice (Fig. 1D and fig. S2). This implies that the stability of p84 was dependent on the presence of p110γ but not on its kinase activity. That other, yet unexplored mechanisms control the abundance of

PI3Kγ adaptor proteins is illustrated by the stability of p84 in BM samples from KO mice and the lower abundance of p84 mRNA in KO BMMCs compared to that in WT BMMCs (fig. S2C).

Because BMMCs from KO mice lack both adaptor and catalytic subunits, we used them for complementation assays and functional studies. We first explored the physiological output of the p84:p110γ complex and then tested whether exogenous p101 could substitute for p84. Both adaptor proteins interact with p110γ and Gβγ subunits in vitro, although p101 sensitizes PI3Kγ for activation by Gβγ subunits at even lower concentrations of Gβγ subunits (*19*).

Combinations of WT or catalytically inactive (KR) p110γ proteins with either green fluorescent protein (GFP)–tagged p84 (GFP-p84) or p101 (GFP-p101) were introduced into KO BMMCs by nucleofection. Complementation of WT p110γ with GFP-p84 or GFP-p101 restored adenosine receptor signaling, as detected by the presence of phosphorylated Akt, but the isolated introduction of p110γ did not (Fig. 2A). Under these conditions, introduction of p110γ with or without GFP-p84 or GFP-p101 did not increase the abundance of endogenous p84 (fig. S3). As for WT BMMCs (*26*), adenosine-mediated phosphorylation of Akt in KO BMMCs reconstituted with either p110-adaptor complex was prevented by the PI3K inhibitor wortmannin (Fig. 2B) and by pretreatment with pertussis toxin (PTX) (fig. S4). The A3AR-specific agonist IB-MECA and agonists targeting all adenosine receptors (adenosine and NECA) stimulated phosphorylation of Akt to a similar extent independently of the PI3Kγ adaptor protein used, indicating that PI3Kγ adaptors did not alter adenosine receptor signaling (fig. S4).

A role for PI3Kγ in GPCR-induced chemotaxis has been established in various cell types (*16*, *24*, *35*, *36*). Mast cells migrate toward adenosine through activation of A3AR in a PI3Kγ-dependent, PTX-sensitive manner (*25*). When reconstituted with p110γ only, BMMCs derived from KO mice were unable to migrate toward adenosine, whereas reconstitution with WT p110γ and either hemagglutinin (HA)-tagged p84 (HA-p84) or p101 (HA-p101) resulted in the cells having an increased migratory capacity (Fig. 2C). Neither the presence of the individual adaptor proteins nor reconstitution with either adaptor protein and catalytically inactive p110γ (KR) restored

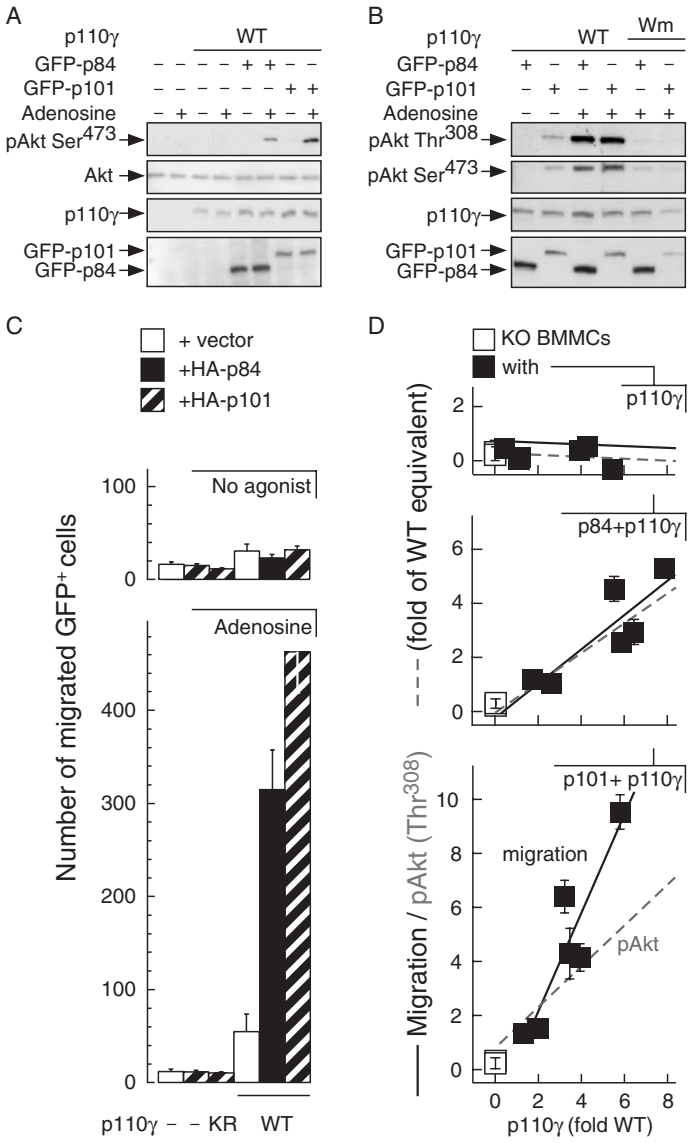


Fig. 2. Adenosine-induced phosphorylation of Akt and cell migration is restored in KO BMMCs by reconstitution with p110γ and either p84 or p101. KO BMMCs were transfected with plasmids encoding functional p110γ (WT) and either GFP-tagged p84 (GFP-p84) or p101 (GFP-p101). One day later, cells were starved in IL-3–free medium containing 2% FCS for 3 hours and stimulated with 2 μM adenosine for 3 min. (**A**) Phosphorylation of Akt at $Ser^{473}$ was determined by analysis of Western blots of cell lysates with an anti–Akt-p$Ser^{473}$. The abundance of p110γ was assessed with an anti-p110γ, whereas GFP-p84 and GFP-p101 were detected and their relative abundance was compared with an anti-GFP. One representative experiment of three is shown. (**B**) Stimulations performed as in (A) were tested for their sensitivity to 30 min of preincubation with 500 nM wortmannin (Wm). Phosphorylation of $Thr^{308}$ of Akt was assessed in parallel with the appropriate antibody. (**C**) KO BMMCs were reconstituted with WT or KR p110γ and either HA-p84 or HA-p101 as indicated, as well as with GFP to select for transfected cells. Migration of GFP-positive BMMCs was assayed in Transwell chambers for 6 hours in the absence (top) or presence of 1 μM adenosine (bottom) in the lower well. Subsequently, GFP-positive cells were quantified. Data shown are the mean number ± SEM of migrated GFP-positive cells from $n = 3$ to 9 experiments. (**D**) Comparison of activation of Akt and migration of reconstituted KO BMMCs. KO BMMCs were transfected with plasmids encoding GFP, p110γ, and either HA-p84 or HA-p101 (as indicated, with variable DNA concentrations), whereas WT BMMCs were transfected with a plasmid encoding GFP as a control. Experiments to measure adenosine-stimulated Akt activity and cell migration were performed as described for (A) and (C), respectively. In parallel, the abundance of p110γ protein was determined by Western blotting. Cell responses (phosphorylation of $Thr^{308}$ of Akt and cell migration) of complemented KO BMMCs are expressed as the fold difference to those of WT BMMCS and were correlated with the fold difference in the abundance of p110γ protein compared to that of WT BMMCs. Data shown are from $n = 3$ experiments, with two to three different DNA inputs in each experiment. Migration events were corrected by subtracting the number of migrating unstimulated cells to determine the number of cells that migrated in response to adenosine.

mast cell migration, showing that a functional p110γ:adaptor complex is required to sustain motility (Fig. 2C). This is underlined by the extent of adenosine-dependent phosphorylation of $Thr^{308}$ of Akt and the efficiency of cell migration both being proportional to the amount of PI3Kγ complex present, whereas even when the abundance of p110γ was greater than six-fold higher than that of the endogenous protein in WT cells, the lack of adaptor proteins could not be compensated for (Fig. 2D).

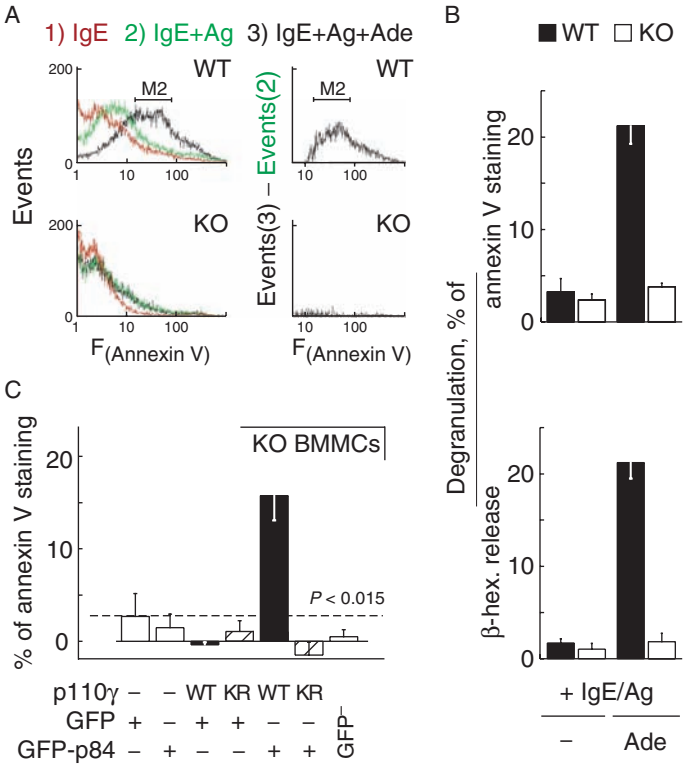


Fig. 3. Mast cell hyperreactivity requires p84. (**A** and **B**) Mast cell degranulation was assessed by annexin V staining and flow cytometry. WT and KO BMMCs were exposed overnight to anti-DNP IgE (100 ng/ml). (**A**) Left: Cells were stained with annexin V after loading with IgE (red), additional stimulation with DNP-HSA (1 ng/ml; Ag) for 20 min (green), or with DNP-HSA (1 ng/ml) and 2 μM adenosine for 20 min (black). Right: Adenosine-dependent activation is represented by the difference in histograms from [IgE+Ag+Ade] and [IgE+Ag] stimulations, given as Events(3) – Events(2). (**B**) Top: Quantification of the experiments shown in (A). Degranulation was calculated from events within region M2 after stimulation with IgE+Ag or IgE+Ag+Ade, as indicated. Data shown are the mean ± SEM of $n = 4$ experiments with two populations of BMMCs and are expressed as the percentage of annexin V–positive cells in the M2 gate (Degranulation, % of annexin V staining). Bottom: For comparison, β-hexosaminidase (β-hex.) release assays were performed under identical conditions. Data shown are the mean ± SEM of $n = 3$ experiments. (**C**) Restoration of adenosine-enforced degranulation in transfected KO BMMCs. KO BMMCs were transfected with expression plasmids for GFP or GFP-p84 in combination with p110γ (WT or KR). The adenosine-dependent component of degranulation in the reconstituted BMMCs is shown (ΔDegranulation; see Materials and Methods). Here, % of annexin V staining represents the difference [Events(3) – Events(2)] as explained for (A). Data shown are the mean ± SEM for $n > 3$ experiments. The dotted line sets $P < 0.015$ for comparison with p110γ:GFP-p84. The bar labeled with $GFP^-$ denotes the degranulation of GFP-negative, electroporated, but nontransfected KO BMMCs, which served as an internal control. The setting of regions ($GFP^+$, M2, etc.) is explained in detail in fig. S5.

## Nonredundant functions of PI3Kγ adaptor proteins

To investigate adaptor-dependent PI3Kγ signaling in the degranulation of mast cells, we adapted a flow cytometric assay first described by Demo *et al.* (*37*), which is based on the degranulation-induced, extracellular exposure of phosphatidylserine on the cell surface and the subsequent staining of cells with annexin V. Many groups (*37*–*40*) have established a direct correlation between the extent of release of β-hexosaminidase and the extent of annexin V binding in various contexts. With WT and KO BMMCs, we confirmed that the assay sensitively detected IgE- and antigen-mediated degranulation and we distinguished this from a full-scale response that involved costimulation of cells with adenosine (Fig. 3, A and B). In agreement with previous β-hexosaminidase assays, the stimulation of WT BMMCs with IgE and a low concentration of antigen resulted in little degranulation, which was even less so for KO BMMCs. As expected, adenosine-triggered hyperactivation of mast cells was completely abrogated in the absence of PI3Kγ (Fig. 3B). With the annexin V staining assay, we could separately analyze transfected and nontransfected cells through the detection of GFP or GFP fusion proteins by flow cytometry. GFP-negative cells served as an internal standard. When KO BMMCs were transfected with plasmids that encoded GFP-p84 and p110γ, responsiveness to adenosine was restored to that of WT BMMCs, and only the GFP–p84:p110γ complex could enhance degranulation after costimulation with adenosine. As was observed for phosphorylation of Akt and cell migration, the individual subunits GFP-p84 and p110γ, or the catalytically inactive p110γ-adaptor complex, failed to reconstitute adenosine-dependent degranulation of mast cells (Fig. 3C). These results underline that p110γ was incapable of signaling or contributing to degranulation in the absence of an adaptor subunit.

As shown above, p84 was indispensable for adenosine receptor–mediated PI3Kγ signaling that led to enhancement of IgE- and antigen-mediated degranulation (Fig. 3C). KO BMMCs reconstituted with GFP-p101 and WT p110γ, however, did not produce an adenosine-dependent increase in degranulation above that observed in response to IgE and antigen, whereas cells reconstituted with GFP-p84 and p110γ exhibited maximal degranulation (Fig. 4A). The abundance of p110γ, p84, and p101 proteins were also quantified. When the abundance of p110γ was normalized to that of the p84:p110γ complex, it was found that reconstitution of cells with p110γ alone resulted in a fivefold lower abundance of p110γ protein than that in cells reconstituted with both p110γ and p84. The p101 protein stabilized p110γ somewhat better than p84 did (Fig. 4B). There was no significant difference in the abundance of GFP-p84 and GFP-p101 (Fig. 4D), and both GFP-tagged adaptor proteins were localized to the cytosol (Fig. 4E). Because p101 translocates to the nucleus in the absence of p110γ (*41*), we assumed that cytosolic GFP-p101 was tightly bound to p110γ and was thus in a functional state. This is also in agreement with mediation of adenosine-induced activation of Akt and migration by the p101:p110γ complex. Similar results were obtained in experiments with cells reconstituted with GFP-fused p110γ and HA-tagged adaptor subunits (Fig. 4A). Consistent with the previous experiments, KO BMMCs reconstituted with HA-p84:GFP-p110γ complexes exhibited adenosine-dependent degranulation, whereas those reconstituted with HA-p101:GFP-p110γ complexes did not. In the latter experiments, fusion of GFP to the N terminus of p110γ stabilized the kinase when expressed alone, so that its abundance approached that observed in the presence of its adaptor proteins (Fig. 4C). Despite this increased abundance, GFP-p110γ alone was incapable of rescuing adenosine signaling in KO BMMCs.

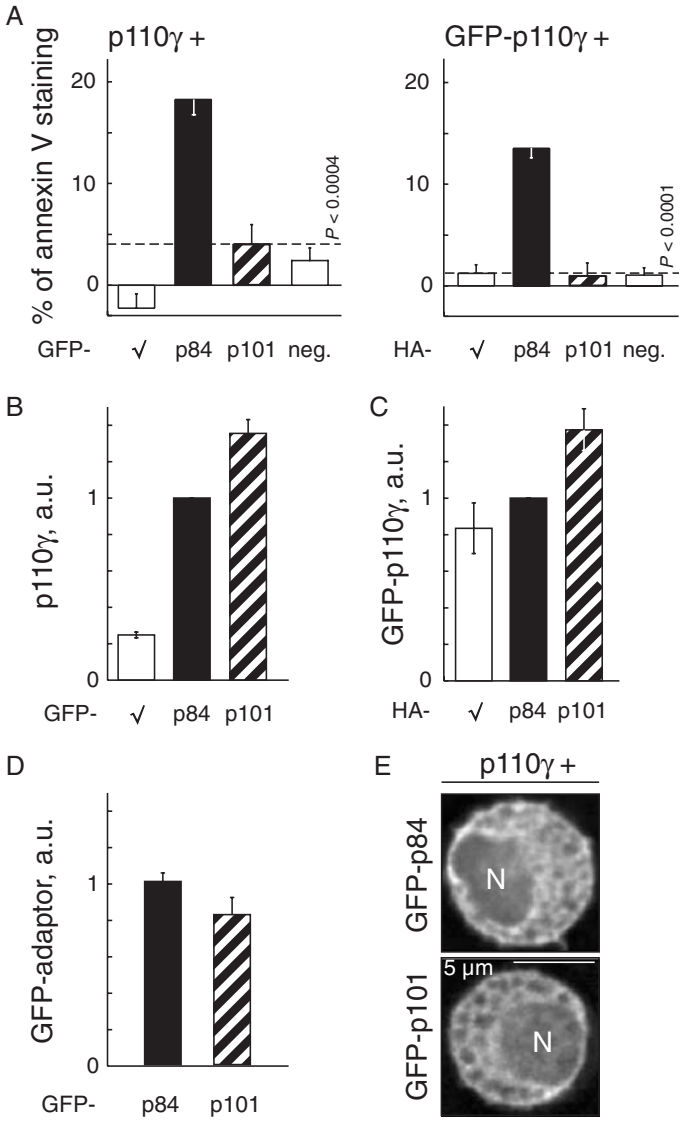


Fig. 4. The p101 adaptor protein does not compensate for p84 in degranulation responses. (**A**) Left: KO BMMCs were transfected with plasmids encoding p110γ in combination with plasmids encoding GFP (√), GFP-p84, or GFP-p101. Degranulation was measured after stimulation of cells with IgE and antigen with or without adenosine as in Fig. 3C. Data shown are the mean ± SEM for $n > 5$ experiments with $P < 0.0004$ for p84 compared to p101. "Neg." denotes the degranulation of GFP-negative, electroporated, but nontransfected KO BMMCs. Right: Experiments were performed as in the left panel but with GFP fused to p110γ instead of the adaptor proteins. (**B** to **D**) Quantification of restored p110γ and adaptor subunits on an Odyssey infrared-detection system. The abundance of p110γ is shown normalized to that of p110γ when coexpressed with p84. Data shown are the mean ± SEM of $n = 3$ experiments. (**B**) Quantification of data from the left panel of (A). (**C**) Quantification of data from the right panel of (A). (**D**) Quantification of the abundance of GFP-p84 and GFP-p101 proteins normalized to that of GFP-p84. Data shown are the mean ± SEM from $n = 2$ experiments. (**E**) Cellular localization of GFP-p84 and GFP-p101 in KO BMMCs reconstituted with either fusion protein and p110γ. Images are deconvoluted. N, nucleus.

## Differences in p84- and p101-mediated localization of PtdIns(3,4,5)$P_3$

PI3Kγ-derived PtdIns(3,4,5)$P_3$ was visualized with the PtdIns(3,4,5)$P_3$-specific pleckstrin homology (PH) domain of Bruton's protein tyrosine kinase (Btk) (*42*) fused to GFP ($PH_{Btk}$-GFP). Although adenosine-triggered phosphorylation of Akt occurred with both types of PI3Kγ complex, the localization of $PH_{Btk}$-GFP was distinguishable depending on the adaptor protein used. Whereas adenosine caused the transient translocation of $PH_{Btk}$-GFP from the cytosol to the plasma membrane in cells containing HA–p84:p110γ complexes, in adenosine-treated BMMCs that contained HA–p101:p110γ complexes, $PH_{Btk}$-GFP displayed a vesicular distribution below the plasma membrane at later time points (Fig. 5A and movies m1 and m2). These endocytosed vesicles were separated from the plasma membrane and did not contain PtdIns(4,5)$P_2$, as visualized with a red fluorescent probe made from the PH domain of phospholipase C δ ($PH_{PLCδ}$-RFP) (Fig. 5B). Whereas the $PH_{PLCδ}$-RFP probe was constitutively localized at the plasma membrane, the membrane docking of $PH_{Btk}$-GFP required stimulation of the cells with adenosine, an active PI3Kγ complex, and an intact $PH_{Btk}$ domain, because the $Arg^{28}$→Cys (R28C) mutation of $PH_{Btk}$ blocked its translocation (Fig. 5, A and B). Therefore, PtdIns(3,4,5)$P_3$ was indeed present in the p101-dependent, internalized vesicles, whereas PtdIns(4,5)$P_2$ was excluded.

To test a correlation between the occurrence of intracellular PtdIns(3,4,5)$P_3$ and the abundance of p101, we performed an analysis of a population of cells to assess the extent of plasma membrane (rim) or internal, vesicular staining for PtdIns(3,4,5)$P_3$ (Fig. 6A). Within 20 s of stimulation with adenosine, cells that contained HA–p84:p110γ complexes displayed translocated $PH_{Btk}$-GFP at the plasma membrane and attenuated rim staining could be detected until 2 min later. Cells that contained HA–p101:p110γ complexes showed rim staining at early time points (<15 s), but intracellular speckles of PtdIns(3,4,5)$P_3$ also became visible. By 120 s after the addition of adenosine, most p101-containing cells displayed speckles, whereas p84-containing cells did not.

Live-cell microscopy was applied as an alternative method to monitor the relocalization of PtdIns(3,4,5)$P_3$. In single cells that contained HA–p84:p110γ complexes, translocation of $PH_{Btk}$-GFP to the plasma membrane was maximal at 40 to 50 s after the addition of adenosine and was maintained for less than 2 min after stimulation before moving from the rim region to the cytosol. In BMMCs that contained HA–p101:p110γ complexes, the intensity of fluorescence at the rim showed similar dynamics, but diminished because of endocytosis of PtdIns(3,4,5)$P_3$ (Fig. 6, B and C, fig. S6, and movies m3 and m4). Quantification of this process illustrated that PtdIns(3,4,5)$P_3$ was produced at the plasma membrane by both PI3Kγ complexes at the same rate, and that the pace of degradation of PtdIns(3,4,5)$P_3$ within the plasma membrane was comparable to the removal of PtdIns(3,4,5)$P_3$ by endocytosis (Fig. 6C). Costimulation of mast cells with IgE-antigen and adenosine dramatically prolonged and accentuated the localization of $PH_{Btk}$-GFP to the plasma membrane in cells containing p84:p110γ and delayed the onset of translocation and p101:p110γ-mediated endocytosis of $PH_{Btk}$-GFP to >2 min after stimulation with adenosine (Fig. 6, D and E, and movies m5 and m6). The prolonged retention times of $PH_{Btk}$-GFP at the plasma membrane might reflect a spatial synergy between p84- and class IA–derived PI3K activities.

Cytosolic, PtdIns(3,4,5)$P_3$-containing vesicles were mobile, and endocytosis of PtdIns(3,4,5)$P_3$ was followed by fast transcellular movements in a fraction of the speckles (fig. S6; see alternative analysis exemplified with movie m4). The curvature of the transport trajectories and the often perinuclear destination of the speckles suggested that a subset of the speckles was associated with microtubules. Indeed, pretreatment of BMMCs that contained HA–p101:p110γ with the microtubule disruptor nocodazole

attenuated the mobility of PtdIns(3,4,5)$P_3$-containing speckles, but not the initial endocytosis to a submembrane localization (fig. S6B and movies m8 and m9).

### Disruption of cholesterol-rich domains affects the signaling of p84:p110γ, but not of p101-containing PI3Kγ

PI3Kγ complexes consisting of p101 were capable of driving a subset of cellular responses even though PtdIns(3,4,5)$P_3$ was endocytosed. The only response not promoted by p101 was degranulation, which requires the integration of signals derived from costimulation with IgE and antigen. Because cross-linked FcεRI receptors accumulate and signal in cholesterol-rich, plasma membrane microdomains called lipid rafts (*43–45*), we set out to test the sensitivities of both types of PI3Kγ complex to methyl-β-cyclodextrin (MβCD), a compound that depletes cholesterol from membranes. To monitor the process, we stained cells with fluorescently labeled cholera toxin subunit β (CTβ), a marker of lipid rafts. In untreated cells, CTβ showed a punctuate pattern, which was disrupted on exposure to MβCD (Fig. 7A). Of note, pretreatment with MβCD selectively attenuated adenosine signaling mediated by p84:p110γ in WT BMMCs (Fig. 7A) or by p84:p110γ in reconstituted KO BMMCs (Fig. 7, B and C). In contrast, p101:p110γ–mediated or interleukin-3 (IL-3)–stimulated phosphorylation of Akt was unaffected by disruption of lipid rafts (Fig. 7, B and C).

The above data add to the evidence that suggests that the PI3Kγ adaptors p84 and p101 serve a nonredundant function and can specifically diverge PI3Kγ signals downstream of GPCRs. Altogether, our results are compatible with a model in which both p110γ complexes integrate upstream signals from GPCRs and PTX-sensitive heterotrimeric G proteins, but differ in downstream processing toward a localized signal output.

### Cells with endogenous p101 internalize PtdIns(3,4,5)$P_3$

Having shown the nonredundant role of PI3Kγ adaptor subunits in mast cells in which p84 is the predominant adaptor protein, we investigated translocation of $PH_{Btk}$-GFP in the J774 macrophage cell line, in which p101 is the predominant PI3Kγ adaptor protein (Fig. 1C and fig. S8C). It can therefore

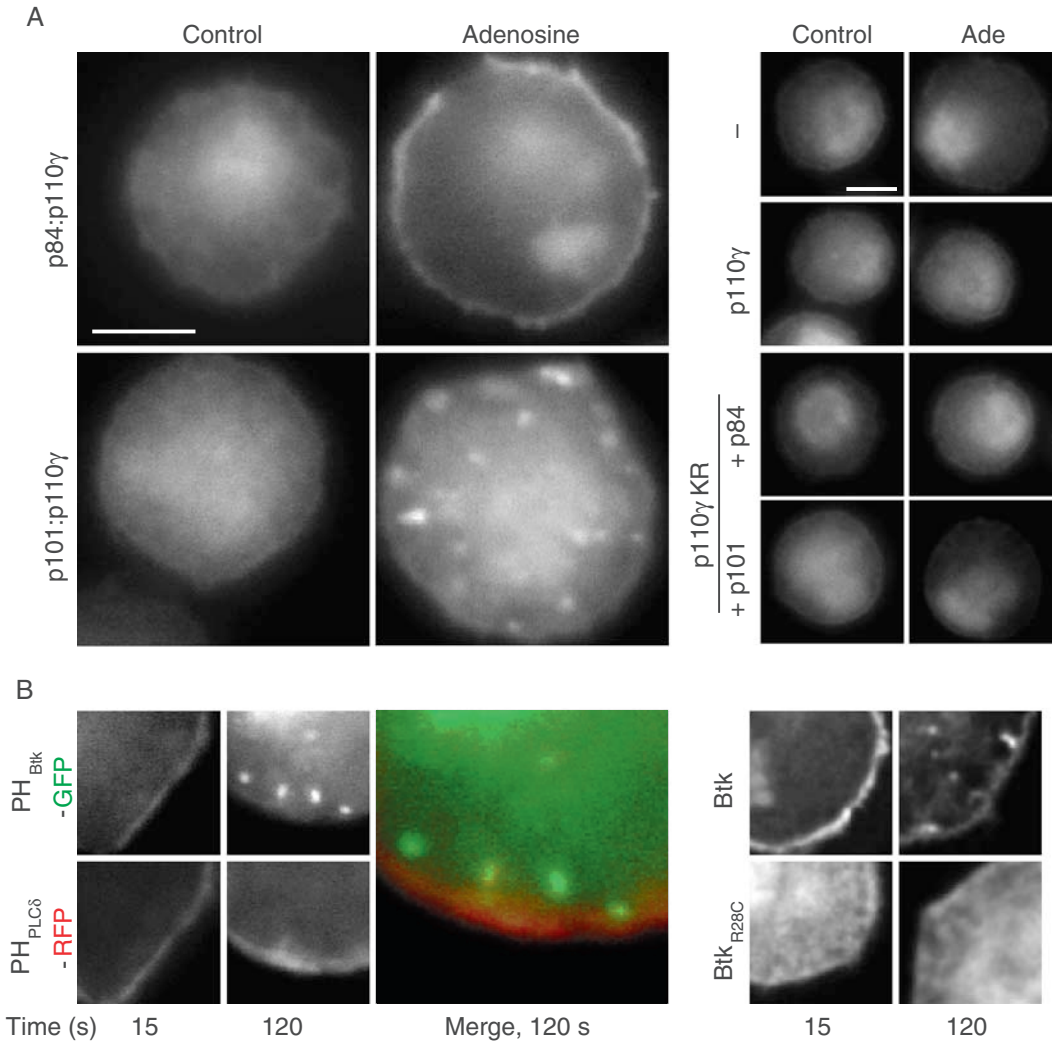


Fig. 5. Adaptor-specific, spatiotemporal differences in the localization of PtdIns(3,4,5)$P_3$. KO BMMCs were transfected with plasmids encoding p110γ (WT or KR) with or without plasmids encoding HA-p87 or HA-p101, as well as $PH_{Btk}$-GFP to monitor PtdIns(3,4,5)$P_3$ production. Twenty-four hours after transfection, cells were depleted of IL-3 and serum-starved for 3 hours before stimulation with 2 μM adenosine. (**A**) At 0 s (control) and 120 s (adenosine, Ade) after stimulation with adenosine, cells were fixed in 4% *p*-formaldehyde and prepared for microscopy. The localization of PtdIns(3,4,5)$P_3$ was visualized with $PH_{Btk}$-GFP. For adenosine-stimulated cells containing p84:p110γ or p101:p110γ complexes, deconvoluted, high-resolution z stacks can be found in movies m1 and m2, respectively. (**B**) p101:p110γ–driven internalization is specific to PtdIns(3,4,5)$P_3$-binding PH domains. KO BMMCs were reconstituted with p101:p110γ and the indicated fluorescently labeled lipid-binding probes. Stimulation with adenosine (2 μM) was stopped after 15 and 120 s and the localizations of the lipid probes were visualized. Only portions of cells are shown. Left: $PH_{Btk}$-GFP (Btk) and $PH_{PLCδ}$-RFP (PLCδ) were co-expressed. Middle: Merged picture of PtdIns(3,4,5)$P_3$-bound (green) and PtdIns(4,5)$P_2$-bound (red) PH domains after 120 s of stimulation with adenosine. Right: Cells were also transfected with either $PH_{Btk}$-GFP (Btk) or a lipid-binding defective mutant $PH_{Btk(R28C)}$-GFP ($Btk_{R28C}$). z stacks were acquired, and images were subsequently deconvoluted.

be assumed that the major output of PI3Kγ in these cells is derived from p101:p110γ complexes. J774 cells could be stimulated with either C5a or C3a to phosphorylate Akt (fig. S8A). C5a and C3a signaling to Akt was PI3Kγ dependent, because the PI3Kγ-specific inhibitor AS252424 efficiently blocked phosphorylation of Akt, whereas TGX-221, which targets PI3Kβ, was ineffective at blocking phosphorylation of Akt (fig. S8A). In J774 cells transfected with a plasmid encoding $PH_{Btk}$-GFP, stimulation with C5a and C3a led to the association of the PtdIns(3,4,5)$P_3$ probe with the plasma membrane at early time points (<15 s), but at >45 s, $PH_{Btk}$-GFP was internalized, as had been observed in adenosine-stimulated KO BMMCs reconstituted with p101:p110γ complexes. Internalization of PtdIns(3,4,5)$P_3$ thus occurs in cells with endogenous p101:p110γ complexes and might be a pathway alternative to that of the lipid phosphatases to remove PtdIns(3,4,5)$P_3$ from the plasma membrane (fig. S8B).

## DISCUSSION

PI3Kγ is the major PI3K isoform that operates downstream of GPCRs, such as receptors for chemokines, *N*-formyl-Met-Leu-Phe, complement fragments (*16*, *33*, *46*), and murine A3AR (*26*), in hematopoietic cells. GPCRs mediate the activation of PI3Kγ through the release of Gβγ subunits from heterotrimeric G proteins (*18*, *21*, *47*). Biochemical and over-

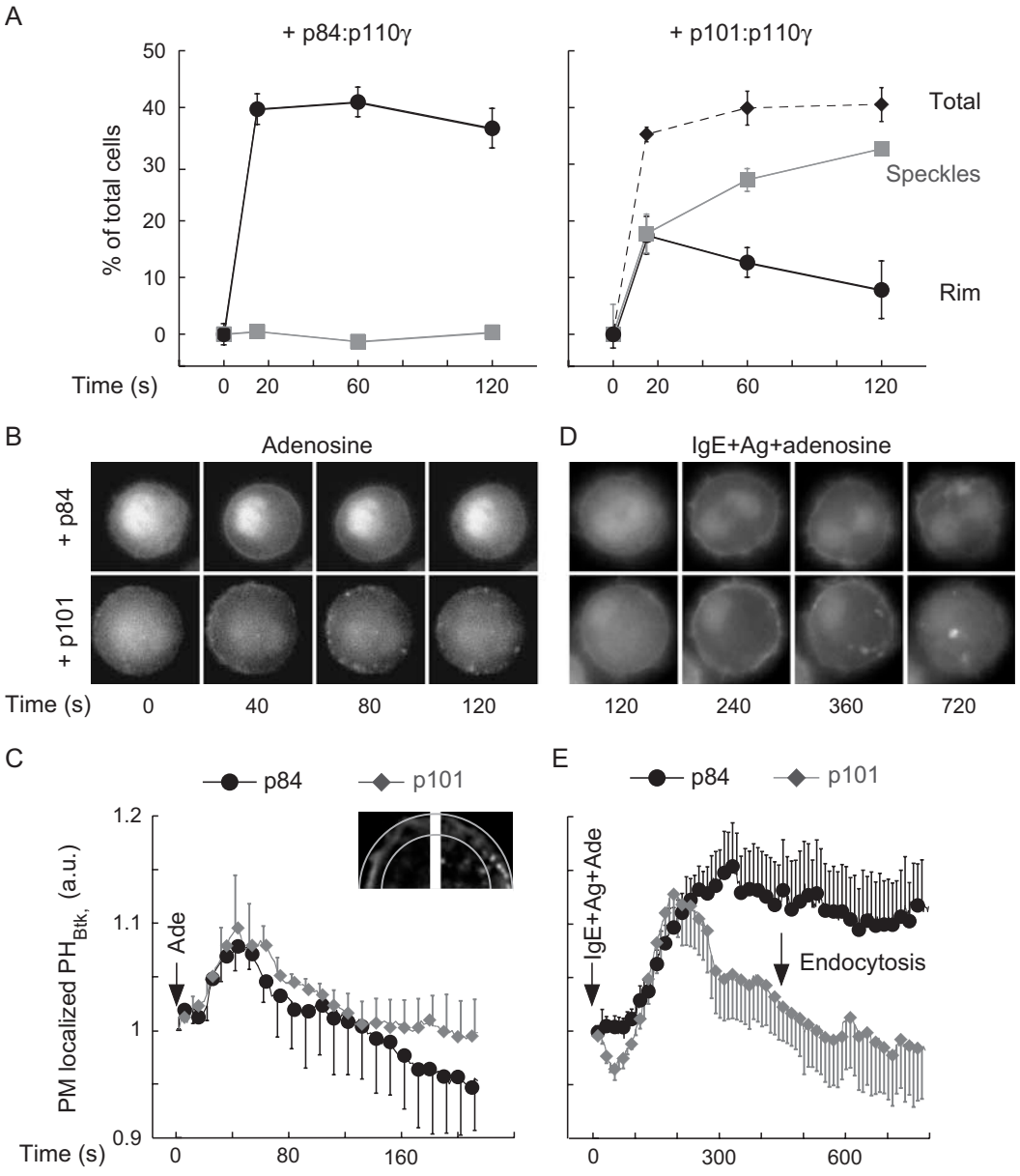


Fig. 6. Time course of the localization of PtdIns(3,4,5)$P_3$ visualized with $PH_{Btk}$-GFP after stimulation with adenosine. (**A**) Performed as a population analysis: The localization of the probe was differentiated between membrane staining (rim) and speckles. Quantification of $n = 3$ experiments was performed blindly and the results are expressed as a percentage of the total number of transfected cells. (**B**) Selected time points of live-cell imaging experiments in adenosine-stimulated KO BMMCs reconstituted with p110γ and either p84 or p101 (see also movies m3 and m4). (**C**) Quantification of plasma membrane–localized $PH_{Btk}$-GFP calculated from live-cell imaging experiments. a.u., arbitrary units, every fifth frame is marked with a symbol. Cells were stimulated with 5 μM adenosine. Data shown are the mean ± SEM for $n = 6$ experiments. Inset is an image that illustrates the region defined as plasma membrane–localized $PH_{Btk}$-GFP located between the outer circle and the inner circle, for a sample of transfected cells containing p84:p110γ (left) or p101:p110γ (right) complexes. (**D**) Selected time points of live-cell imaging experiments in KO BMMCs reconstituted with p110γ and either p84 or p101. Cells were loaded overnight with anti-DNP IgE (100 ng/ml), washed, and stimulated with DNP-HSA (5 ng/ml; Ag) and 5 μM adenosine for the indicated times. Data shown represent excerpts from movies m5 and m6. (**E**) Quantification of membrane-bound PtdIns(3,4,5)$P_3$ in IgE+Ag+adenosine–stimulated cells. Data shown are the mean ± SEM of nine experiments for cells containing p84:p110γ complexes, and of seven experiments for cells containing p101:p110γ complexes, and were analyzed as in (C). The low concentration of antigen used here did not trigger translocation of $PH_{Btk}$-GFP in the absence of adenosine (see fig. S7).

expression studies in nonhematopoietic cells have shown that p101 augments the translocation of PI3Kγ (*41*) and the Gβγ-dependent turnover of PtdIns(4,5)$P_2$ (*18*, *22*), but the necessity for a PI3Kγ adaptor protein in physiological processes has remained disputed until recently (*24*).

Studies of the expression profiles of PI3Kγ across multiple tissues show that mRNAs encoding p110γ, p84, and p101 are highly expressed in hematopoietic tissues. Whereas the abundance of p101 mRNA was high in tissues and cells that contain lymphocytes [thymus, spleen, and peripheral blood lymphocytes (PBLs)], we could not detect p84 mRNA in PBLs, and the abundance of p84 protein was low in splenocytes. Both adaptor proteins were found in neutrophils and monocytes or macrophages, whereas only p84 was found in mast cells. Overall, p101:p110γ complexes dominate the lymphoid lineage, whereas p84 is found in myeloid cells with varying penetrance. When p110γ was eliminated by gene targeting, p84 protein was destabilized in differentiated cells. Similar reductions were observed for p101 in neutrophils isolated from BM of p110γ null mice (*19*). Because catalytically inactive p110γ (KR) was sufficient to maintain p84 protein in BMMCs, the lipid kinase activity of p110γ is apparently not required for the stabilization of adaptor proteins.

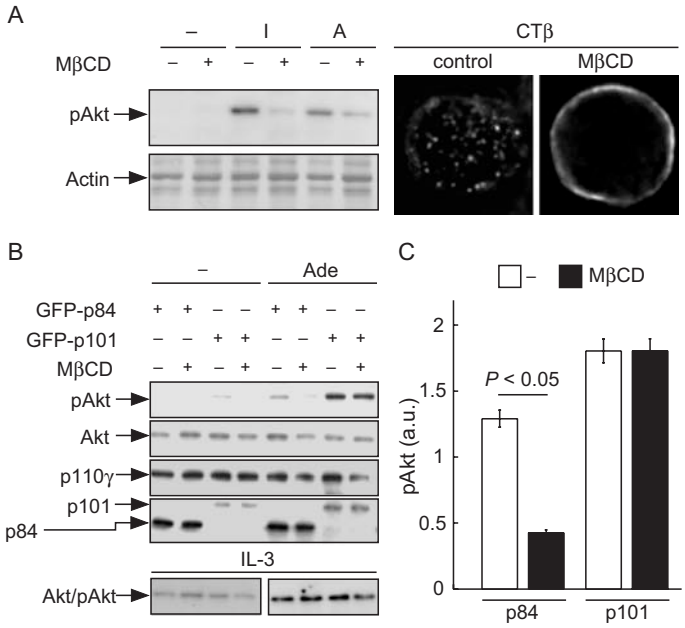


Fig. 7. The function of p84:p110γ, but not p101:p110γ, is sensitive to disruption of lipid rafts. (**A**) Left: WT BMMCs were starved in IL-3–free medium containing 2% FCS for 3 hours with or without 30 min of pretreatment with 5 mM MβCD at 37°C, and then stimulated with 2 μM adenosine or 10 nM IB-MECA for 2 min. Phosphorylation of Akt was determined by Western blotting analysis with an anti-pSer473. Right: Precooled WT BMMCs were incubated at 4°C for 30 min with $Alexa_{555}$-conjugated choleratoxin β-subunit (CTβ) with or without MβCD. Deconvoluted images were acquired after fixation and sedimentation of cells. (**B**) p110γ and GFP fusions of p84 or p101 were expressed in KO BMMCs as indicated (indicators refer to Ade and IL-3 stimulations). Cells were starved as in (A) before phosphorylation of Akt was detected after stimulation with adenosine (Ade) or IL-3 (20 ng/ml for 2 min). One representative experiment of four is shown. (**C**) Quantification of adenosine-triggered phosphorylation of Akt from experiments as shown in (B). Data shown are the mean ± SEM of $n = 4$ experiments.

In mast cells lacking functional p110γ, adenosine was incapable of activating Akt or of triggering the translocation of a probe of PtdIns(3,4,5)$P_3$ ($PH_{Btk}$-GFP) to the plasma membrane. In this setting, the combination of adaptor protein with active p110γ was required to restore the responsiveness of KO BMMCs to adenosine. These results show conclusively that p110γ must interact with adaptor protein to generate PtdIns(3,4,5)$P_3$ downstream of GPCRs.

To date, functional comparisons of p101 and p84 in cells that normally express p110γ have not been performed; in vitro studies have, however, convincingly shown that p101 and p84 activate p110γ by the same basic mechanism (*19*, *22*, *23*). In mast cells, PI3Kγ controls physiological responses in vitro and in vivo (*26*), and both the loss of adaptor protein in p110γ null cells and the inability of monomeric p110γ to rescue GPCR signaling exclude the interference of cross-compensatory effects of the PI3Kγ adaptors in this model.

With the above biochemical studies in mind, it was a surprise that the coexpression of p101 with p110γ did not reconstitute adenosine-dependent degranulation in IgE- and antigen-primed KO BMMCs. The analysis of the abundance of p110γ, p84, and p101 proteins, as well as of their cellular localization, suggested that both types of PI3Kγ complexes were in a position to signal, but that only p84:p110γ did so successfully. In contrast to degranulation, both p84 and p101 could be combined with p110γ to reconstitute adenosine-induced cell motility of KO BMMCs, whereas p110γ alone was insufficient. It is thus evident that GPCRs can link to the chemotactic machinery through both adaptor isoforms. Important roles for p110γ (*16*, *48*) and p101 (*24*) in the chemotaxis of neutrophils have been described previously. That migration of mast cells was proportional to the amount of PI3Kγ complex present strongly suggests that the loss of migratory capacity in p101 null neutrophils (*24*) is due to a reduction in the extent of PI3Kγ signaling rather than to an exclusive link between p101 and cell motility.

The selective promotion of degranulation by p84 suggests that the two adaptor proteins selectively redirect PI3Kγ downstream signaling into separate response patterns. Such selectivity is unlikely achieved through the deployment or interference of different adenosine receptor types, because an A3AR-specific ligand (IB-MECA) and ligands that stimulate all four adenosine receptors (adenosine, NECA) caused phosphorylation of Akt to an equivalent extent in conjunction with both types of PI3Kγ complexes. The observation that PtdIns(3,4,5)$P_3$ remained at the plasma membrane when it originated from the activity of p84:p110γ, but was endocytosed when its source was the p101:p110γ complex, strongly indicates that the two PI3K complexes produce two distinct PtdIns(3,4,5)$P_3$ pools at the plasma membrane. That p84:p110γ could signal in lipid microdomains was also supported by its sensitivity to disruption of cholesterol-rich domains by MβCD, whereas adenosine-mediated signaling through p101:p110γ to activation of Akt resisted cholesterol depletion. Cross-linking of FcεRI concentrates the activated receptor in lipid rafts (*43*, *44*), and one could thus speculate that p84:p110γ locally enhances the abundance of PtdIns(3,4,5)$P_3$ in the vicinity of clustered FcεRI receptors and the associated protein tyrosine kinase cascade. This would imply that degranulation is under the control of a system that integrates the status of the protein tyrosine kinase cascade output with local concentrations of PtdIns(3,4,5)$P_3$ and relays downstream signals only when the two inputs occur coincidently. Moreover, for efficient degranulation to occur, high threshold concentrations of PtdIns(3,4,5)$P_3$ have to be reached (*26*), which could be achieved through the selective targeting of p84:p110γ complexes. Such a localized but high-amplitude signal emerging from PI3Kγ could thus overrun the gatekeeper function of the 5′-lipid phosphatase SHIP (*32*, *49*), which continuously attenuates the low-level signal of PtdIns(3,4,5)$P_3$ produced by p85-associated class IA PI3K under conditions of low concentrations of antigen. If PtdIns(3,4,5)$P_3$ is localized outside of these areas, as assumed here for p101:p110γ–derived PtdIns(3,4,5)$P_3$, it has a different route of degradation and can be endocytosed.

Binding of p101 to p110γ has been reported to reduce the basal catalytic activity of PI3Kγ (*50*), and the adaptor proteins can thus be considered as inhibitors and stabilizers of cytosolic PI3Kγ complexes. Here, we showed that these complexes seem to be fully activated only in the correct context and cellular localization. Due to technical limitations, it cannot be concluded presently whether the main action of the adaptors is to restrict access of PI3Kγ to certain sites and responses or to direct PI3Kγ to specific cellular docking sites. The whole process shows similarity to the activation of class IA PI3Ks, in which p85-like regulators block the activity of the catalytic p110 subunit as long as they do not interact with phosphorylated tyrosines on growth factor receptors (*9*, *51*).

The processes in mast cells presented here illustrate that the p84:p110γ and p101:p110γ complexes serve nonredundant but also overlapping functions. In allergy, only the p84:p110γ complex cooperates with IgE- and antigen-dependent signaling in mast cell activation. Our elucidation of adaptor-specific PI3Kγ signaling has a potential therapeutic value, because loss of PI3Kγ function not only attenuates chronic inflammation and allergy, but also modulates cardiovascular parameters (*34*, *52*). Although inhibition of PI3Kγ has been regarded as cardioprotective, such convolution might complicate chronic pharmaceutical targeting of PI3Kγ in inflammatory diseases. Based on our results, tissue selective targeting of PI3Kγ by modulation of the function of adaptor molecules might become possible in the future.

## MATERIALS AND METHODS

### Cloning of p84, plasmids, and antisera

Murine p84 complementary DNA (cDNA) was obtained from murine 32D cells by reverse transcription PCR (RT-PCR) (for details and primers see Supplementary Materials). Human full-length p84 cDNA was amplified from neutrophils with Pwo polymerase (Roche Diagnostics, Mannheim, Germany) (accession data for p84: *Mus musculus*, AY753194; *Homo sapiens*, AY753192; UniProt: PI3R6_Human; GenBank: *PIK3R6*). The p101 coding sequence (*18*) and p84 were transferred to pcDNA3, pcDNA-HA, and pEGFP vectors for expression. Plasmids for the expression of p110γ were previously described (*53*). The PH domain of Btk fused to GFP (pEGFP-$PH_{Btk}$, donated by T. Balla) was used as a sensor of PtdIns(3,4,5)$P_3$. The cDNA of the PH domain of PLCδ was subcloned into pTagRFP-N1. Antisera against murine p84 fragment ($Met^1$ to $Glu^{162}$) were raised in rabbits and goats and antisera against human p101 fragment ($Pro^{575}$ to $Pro^{880}$) were raised in rabbits. Protein fragments were expressed as $(His)_6$ fusion proteins from bacterial pQE vectors (Qiagen, Hilden) and purified on $Ni^{2+}$-NTA beads (Qiagen) according to the manufacturer's instructions.

### Cell culture, isolation, and differentiation

To isolate, derive, and culture BMMCs from C57BL/6J mice and mice without functional PI3Kγ [$KO_1$ (*16*), $KO_2$ (*33*), and KR (*34*)], cells from fresh BM were resuspended in complete Iscove's modified Dulbecco's medium (IMDM) with 10% heat-inactivated fetal calf serum (HIFCS), 2 mM L-glutamine (Gln), 1% penicillin-streptomycin solution (PEST), 50 μM β-mercaptoethanol (β-ME), and recombinant murine IL-3 (2 ng/ml; Peprotech, Rocky Hill, NJ) and cultured at 37°C and 5% $CO_2$ for 4 days. Subsequently, BMMCs were diluted weekly to $0.5 \times 10^6$ cells/ml with a mixture of 80% fresh, complete IMDM and 20% recycled medium, with IL-3 added every second day (*26*). Nonadherent cells were monitored for the presence of FcεRI [with a phycoerythrin (PE)-conjugated hamster antibody to mouse FcεRIα; clone MAR-1, eBioscience, San Diego, CA] and c-kit (rat $IgG_{2B}$ anti-mouse CD117/c-kit; clone 3c1, ImmunoKontact, Bioggo, Switzerland) by fluorescence-activated cell sorting (FACS) analysis. BM-derived macrophages were differentiated in bacterial dishes (Greiner bio-one, Kremsmünster, Austria) at $1 \times 10^6$ cells/ml in RPMI 1640 (Sigma) supplemented with HIFCS/Gln/PEST/β-ME and 20% L-929 cell-conditioned medium. Nonadherent cells were collected 5 days later for experiments (*54*). Neutrophils and splenocytes were isolated as described (*16*). The murine macrophage cell line J774 and the rat mast cell line RBL2H3 were cultured in complete DMEM (HIFCS, Gln, PEST), the human mast cell line HMC-1 was cultured in IMDM containing HIFCS/Gln/PEST/ β-ME, and the human monocytic cell line THP-1 was cultured in complete RPMI 1640 (HIFCS/Gln/PEST/β-ME).

### Transfections

BMMCs were transfected with the Amaxa Nucleofector according to the manufacturer's protocol (Amaxa). BMMCs ($7 \times 10^6$ to $10 \times 10^6$) were resuspended in 100 μl of nucleofection solution T (Amaxa) to which was added 10 to 15 μg of plasmid DNA (in 15 μl of 10 mM tris and 1 mM EDTA, pH 8.0). Immediately after electroporation, cells were cultured in 5 ml of complete IMDM at 37°C and 5% $CO_2$ for 5 hours. After a medium change with complete IMDM, transfected cell populations were cultured for 24 hours, after which they were sensitized with mouse anti-DNP IgE (100 ng/ml; mAB SPE-7, Sigma-Aldrich) for degranulation experiments. Stimulations occurred 24 hours after transfection.

### Stimulation of BMMCs with adenosine

Twenty-one hours after transfection, BMMCs were collected by centrifugation (160*g* for 3 min), washed, and starved in IL-3–free medium containing 2% fetal calf serum (FCS) for 3 hours ($0.5 \times 10^6$ to $1 \times 10^6$ cells/ml), and were then stimulated with 2 μM adenosine for the indicated time (at 37°C, 5% $CO_2$). For Western blotting analysis, stimulation of cells was stopped on ice, the cells were collected by centrifugation (16,000*g* for 1 min at 4°C), washed in 1× phosphate-buffered saline (PBS) and lysed at $1 \times 10^7$ cells/ml in 2× sample buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 10% β-ME, 20% glycerol, bromphenol blue]. Proteins were denatured at 95°C for 7 min, and then subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and Western blotting. For microscopy experiments, stimulation was stopped by the addition of an equal volume of ice-cold 10% *p*-formaldehyde in PBS. Wortmannin (500 nM), PTX (100 ng/ml, Sigma), MβCD (5 mM, Sigma), AS252424 (1 μM, Merck-Serono, Geneva), and TGX-221 (1 μM, Cayman Pharma, Neratovice) were added to cells 30 min before stimulation, whereas nocodazole (1 μM, Sigma) was added 3 hours before stimulation.

### Western blotting analysis

Proteins were separated by SDS-PAGE and transferred by Western blotting to Immobilon PVDF membranes (Millipore). Mouse monoclonal antibody (mAb) to p110γ (clone H1, Alexis), rabbit mAbs to pSer473-PKB/Akt and to pThr308-PKB/Akt (both from Cell Signaling Technology, Danvers, MA), mouse mAbs to GFP (Roche Diagnostics) and to HA (HA.11, Babco), and the antisera mentioned above were used to detect proteins in the PI3Kγ pathway. Mouse mAb to vinculin was produced from the hybridoma clone VII-F9 (a gift of V. Kotelianski). Secondary antibodies such as horseradish peroxidase (HRP)–conjugated rabbit antibody to mouse IgG and goat antibody to rabbit IgG (Sigma, St. Louis, MO) were visualized by enhanced chemiluminescence (Millipore).

### Quantitative Western blotting

After SDS-PAGE, proteins were transferred to Immobilon FL membranes (Millipore) and blocked with Odyssey blocking buffer (LI-COR Biosciences, NE), followed by incubation with primary antibodies in the same buffer. Matched secondary antibodies were goat anti-rabbit IgG-IR Dye 800 (Rockland, Gilbertsville, PA) and goat anti-mouse IgG-Alexa Fluor 680 (Molecular Probes, Eugene, OR). Membrane-bound fluorescence was detected on the Odyssey Infrared Imaging System (LI-COR Biosciences).

### Isolation of total RNA, RT-PCR, and qPCR

RNA was isolated from TRIzol-lysed tissues as described by Invitrogen. cDNA was generated with 2 μg of total RNA with M-MLV reverse transcriptase (RT buffer and protocol, Invitrogen) and RNAsin (Promega) and stored at –80°C until ready for use. An ABPrism 7000 (Applied Biosystems, Foster City, CA) and SYBR GREEN PCR Master Mix (Applied Biosystems) was used for qPCR analysis of p84, p110γ, and p101 mRNAs. GAPDH mRNA served as an internal standard to generate calibration curves in which the cycle numbers at the crossing point (CP) were given by

$$\mathrm{CP} = b + a\log([\mathrm{cDNA}])$$

where $b$ was 15.136 and $a$ was –3.2877. Variations in total cDNA content were compensated for with the GAPDH CP values for a given tissue:

$$\mathrm{CP}_{\text{tissue corrected}} = \mathrm{CP}_{\text{tissue}} - (\mathrm{CP}_{\text{GAPDH tissue}} - \mathrm{CP}_{\text{GAPDH external standard}})$$

The relative copy number ($R^{c\#}$) was then calculated as:

$$R^{c\#} = 10^{(4.872 - 0.30354 \times \mathrm{CP}_{\text{sample corrected}})}$$

The efficiency $E$ of PCR was 2.01 or 101%. The above calculation was cross-checked with calibration curves established with linearized plasmids for p110γ and p84. Deviations from the GAPDH-based calculations were in the range of 1.1- to 1.3-fold. cDNA of rat or human origin was amplified by conventional PCR by Taq Polymerase (New England Biolabs, Ipswich, MA) in a T3 Thermocycler (Biometra, Göttingen, Germany). PCR products were separated in 2% agarose gels and visualized with ethidium bromide in a Geneflash imaging system for gel documentation (Syngene, Cambridge, UK). Primers are listed in the Supplementary Materials.

### Fluorescence microscopy

After stimulation, cells were fixed by the addition of an equal volume of ice-cold 10% *p*-formaldehyde in PBS. For staining of F-actin, cells were permeabilized in PBS, 1% BSA, 0.1% Triton X-100 and incubated with rhodamine-phalloidin (Molecular Probes) (*55*). $\mathrm{Alexa}_{555}$-conjugated cholera toxin staining (Invitrogen) was performed according to the supplier's instructions. Subsequently, cells were spun onto microscopy slides (at 100*g*) and mounted in Mowiol (Plüss-Stauffer). Images were acquired on an Axiovert 200 M microscope (Zeiss) fitted with a Plan-Achromat 100×/1.4 oil objective and an Orca ER II camera (Hamamatsu) with OpenLab software (Improvision). High-resolution images were generated from 0.2 μM image z stacks, which were then deconvoluted with Volocity 4.0 software.

### Live-cell microscopy

Transfected cells ($0.5 \times 10^6$ to $1.0 \times 10^6$/ml, in 0.5 ml) were plated in IMDM containing 2% HIFCS, L-Gln, PEST, 50 μM β-ME on poly-Lys–coated (Sigma-Aldrich) coverslips for 3 hours in live-microscopy chambers (Life Imaging Services, Basel, Switzerland). Live imaging was performed on a Zeiss Axiovert 35 microscope (with a 100× plan-Neofluar 1.30/oil objective), equipped with Micromax heating (to 37°C) and a Princeton Instruments camera system (Trenton, NJ), or on an Axiovert 200 M microscope (Zeiss) fitted with a Plan-Achromat 63×/1.4 oil objective and an Orca ER II camera (Hamamatsu), equipped with a microscope temperature control system (Life Imaging Services). For stimulations, 0.5 ml of modified Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 20 mM Hepes, pH 7.4) containing 5 μM adenosine or 5 μM adenosine and DNP-HSA [10 ng/ml; where cells were presensitized with anti-DNP IgE (100 ng/ml) overnight] were added carefully to avoid displacement of cells. To monitor the translocation of GFP-$\mathrm{PH}_{\mathrm{Btk}}$, images were acquired every 2 s over the indicated time with either Metamorph 4.01 software (Axiovert 35 microscope) or OpenLab software (Axiovert 200 M microscope). Movies were assembled with Volocity 4.0 software. Plasma membrane–localized $\mathrm{PH}_{\mathrm{Btk}}$-GFP was determined by measuring the integrated fluorescent intensities of two circular regions of interest (ROIs) with ImageJ software. The first ROI ($\mathrm{ROI}_{\mathrm{out}}$) included the whole cell, whereas the second ROI ($\mathrm{ROI}_{\mathrm{in}}$), which had a reduced radius, was set such that plasma membrane staining was excluded. The sizes and locations of the ROIs were constant in all frames. Plasma membrane–localized $\mathrm{PH}_{\mathrm{Btk}}$-GFP was calculated as $(\mathrm{ROI}_{\mathrm{out}} - \mathrm{ROI}_{\mathrm{in}})/\mathrm{ROI}_{\mathrm{out}}$ for each acquired frame. Division by $\mathrm{ROI}_{\mathrm{out}}$ in this formula corrects for sample bleaching, because values (in arbitrary units) of all frames were normalized to the first (unstimulated) frame and plotted over time. Intensity surface plots were calculated with ImageJ software.

### Single cell–based degranulation assay

BMMCs were incubated in complete IMDM with mouse anti-DNP IgE (100 ng/ml) overnight and then stimulated with DNP-HSA (1 ng/ml; Sigma-Aldrich) with or without 2 μM adenosine for 20 min at 37°C. Cells were then washed in PBS, transferred to annexin V–binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and stained with Cy5-conjugated annexin V (BD Biosciences) for 15 min on ice. FACS analysis was performed on a FACSCalibur with CellQuest software (BD Biosciences). FACS events were gated according to cell size, GFP-positive and GFP-negative cells, and annexin V–Cy5 staining as a measure of degranulation [see Demo *et al*. (*37*) and Martin *et al*. (*56*) for assay validation]. It must be noted that the correlation between annexin V staining and degranulation required validation for each of the stimuli and conditions used (*57*). For experiments with transfected KO BMMCs, degranulation was calculated for GFP-positive and GFP-negative cells separately, as GFP-negative cells define basal degranulation of KO BMMCs. For reconstituted cells, adenosine-induced degranulation was expressed as the difference in the percentage of total cells in M2 (annexin V–positive) after stimulation with IgE and antigen either with (IgE+Ag+Ade) or without (IgE+Ag) adenosine (ΔDegranulation, % of annexin V staining). The borders of the M2 gate were set to discriminate the synergistic action of adenosine from stimulation with IgE and antigen and from apoptotic cells after nucleofection, which were also stained with propidium iodide (PI). See fig. S5 for more information.

### Release of β-hexosaminidase

The release of histamine-containing granules was quantified by the determination of β-hexosaminidase in the cell supernatants with a method slightly modified from that of Laffargue *et. al*. (*26*). BMMCs were incubated with anti-DNP IgE (100 ng/ml) overnight and resuspended in modified Tyrode's buffer at $0.5 \times 10^6$ to $1.0 \times 10^6$ cells/ml at 37°C and 5% $CO_2$. Degranulation was induced with DNP-HSA (1 ng/ml) with or without 2 μM adenosine for 20 min at 37°C and 5% $CO_2$. The reaction was stopped and β-hexosaminidase activity was measured with *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma). Results are given as the percentage of total Triton X-100–releasable β-hexosaminidase.

### Cell migration assays

Migration of transfected BMMCs was assayed in 24-well Transwell Supports (Corning) with 5.0-μm pore polycarbonate membranes for 6 hours. Before the experiment, Transwell membranes were coated with fibronectin (1 μg/ml) in PBS at 4°C overnight, blocked with 4% BSA in PBS for 1 hour at 37°C, and equilibrated in migration medium (IMDM supplemented with L-Gln, PEST, 1% BSA, 50 μM β-ME, and 20 mM Hepes, pH 7.4) for 30 min at 37°C. Transfected BMMCs were washed twice in migration medium.

Cell suspensions ($0.5 \times 10^6$ cells in 200 μl) were applied to the upper compartment of the Transwell inserts already containing migration medium supplemented with or without 1 μM adenosine in the lower well. Fluorescent cells that reached the lower well were quantified with the fluorescent microscope.

## SUPPLEMENTARY MATERIALS

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Supplementary Text
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# PI3Kγ adapter subunits define coupling to degranulation and cell motility by distinct PtdIns(3,4,5)$P_3$ in mast cells

***-Supplementary Data-***

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## Supplementary Text

### Quantification of p84 and p110γ protein in WT and KO BMMCs and bone marrow

We observed a dramatic loss in p84 protein in hematopoitetic cells derived from p110γ null (KO) mice. To determine the amount of residual p84 protein in these cells, we quantified the abundance of p84 and p110γ protein on an infrared fluorescence imaging system (Odyssey, LI-COR). Quantification revealed a residual amount of p84 in BMMCs derived from two p110γ null mouse strains generated by different gene targeting strategies [fig. S2; for $KO_1$ see (Hirsch et al., 2000), for $KO_2$ see (Sasaki et al., 2000)] that was <20% of that in WT BMMCs. The abundance of p84 protein in bone marrow isolated from these mice was identical to that of bone marrow from WT mice.

### Exogenous p110γ does not alter the abundance of endogenous p84 protein in KO BMMCs

Because p110γ stabilizes p84 in differentiated hematopoietic cells (Fig. 1D), one could speculate that endogenous p84 protein is stabilized by exogenous p110γ in KO BMMCs. Hence, we determined the abundance of endogenous p84 protein in KO BMMCs 24 hours after transfection with a plasmid encoding p110γ (time point of stimulation). Neither the expression of p110γ alone, nor of p110γ in combination with its adaptor proteins yielded significant alterations to the reduced abundance of endogenous p84 protein, as compared to mock-transfected KO BMMCs (fig. S3). Thus, possible confounding effects of increased p84 abundance on cellular functions can be excluded, which is further supported by the inability of exogenous p110γ to restore adenosine-dependent signaling in transfected KO BMMCs.

## Materials and Methods

### Culture, transfection, and stimulation of J774 cells

The murine macrophage cell line J774 (kindly provided by J. Pieters, Basel) was cultured in complete DMEM (HIFCS, Gln, PEST). For passaging, cells were washed with complete DMEM, detached with a cell lifter (Corning), and seeded on fresh cell culture dishes (at a 1:5 dilution). For nucleofection, J774 cells ($1x10^6$) were resuspended in 100 μl of nucleofection solution V (Amaxa), to which was added 1 to 2 μg of the appropriate plasmid DNA. After electroporation, cells were seeded in complete DMEM onto 12-mm coverslips, after which cell attachment medium was changed to eliminate the transfection solution. For stimulation, J774 cells were serum-starved in DMEM (Gln, PEST) for 3 hours. Wortmannin (500 nM), AS252424 (1 μM), or TGX-221 (1 μM) were added 30 minutes prior to stimulation with 10 nM C5a (R&D systems, Minneapolis) or 50 nM C3a (a kind gift of C. Dahinden, Bern). For fluorescence microscopy, stimulation was stopped by the addition of an equal volume of ice-cold 10% *p*-formaldehyde in PBS. For Western blotting analysis, cells were washed with ice-cold PBS on ice, followed by lysis in 20 mM Tris-HCl, 138 mM NaCl, 2.7 mM KCl, 5% glycerol, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% NP-40, 20 μM Leupeptin, 18 μM pepstatin, 1 mM sodium-*ortho*-vanadate, and 2 mM NaF. Equal amounts of protein (measured with BioRad Protein assay) were denatured and subjected to SDS-PAGE and Western blotting.

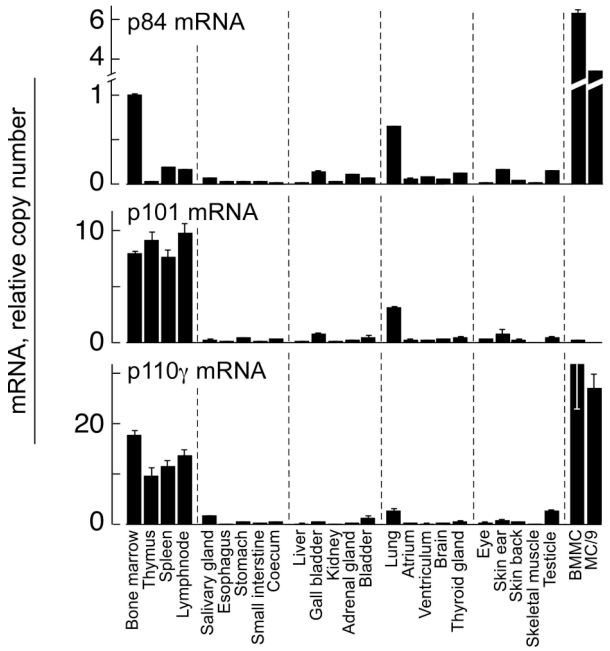


**Fig. S1.** Tissue distribution of p110γ and its adaptor subunits (additional tissues). Relative mRNA copy numbers for p110γ and adapters in the indicated mouse tissues, BMMCs, and the MC/9 mouse mast cell line were assessed by qPCR (n>3, mean ± SEM). Values were normalized to the copy numbers of p84 mRNA in bone marrow.

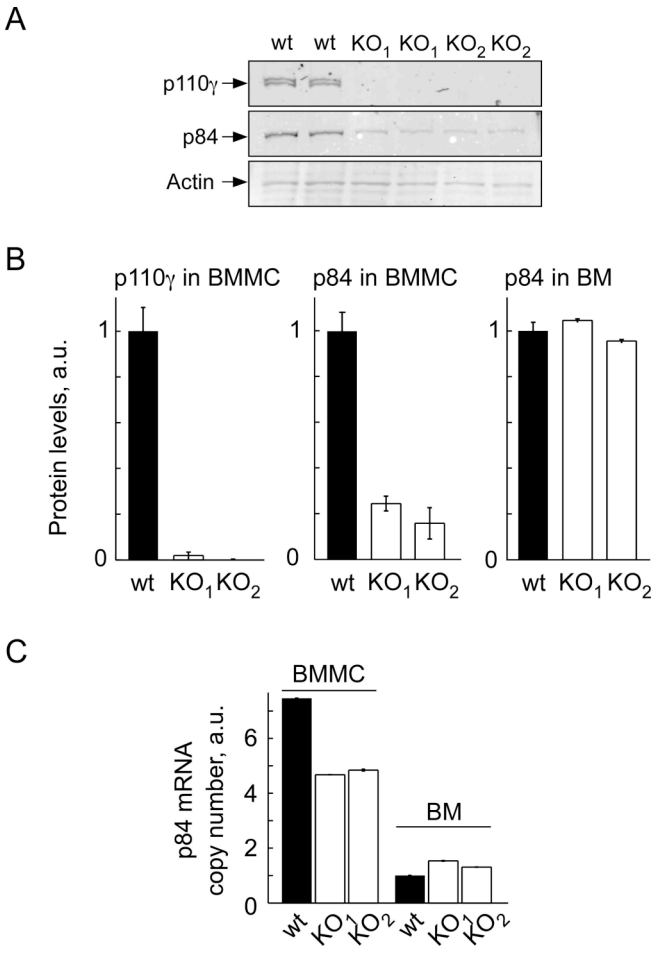


**Fig. S2.** p84 is stabilized by p110γ in mast cells. (**A**) p110γ and p84 proteins were separated on SDS-PAGE and detected with fluorescent secondary antibodies. (**B**) Quantification of signals was performed on an Odyssey Infrared Imaging device in samples from individual BMMC populations and total bone marrow (BM). Tissue was isolated and differentiated from WT mice and from two independent p110γ null mouse strains [$KO_1$ (Hirsch et al., 2000) and $KO_2$ (Sasaki et al., 2000)]. Values are normalized to those of WT samples (n = 2, mean ± SEM). (**C**) p84 mRNA was quantified (as described for fig. S1) in BMMCs and BM isolated from mice with the indicated genotypes.

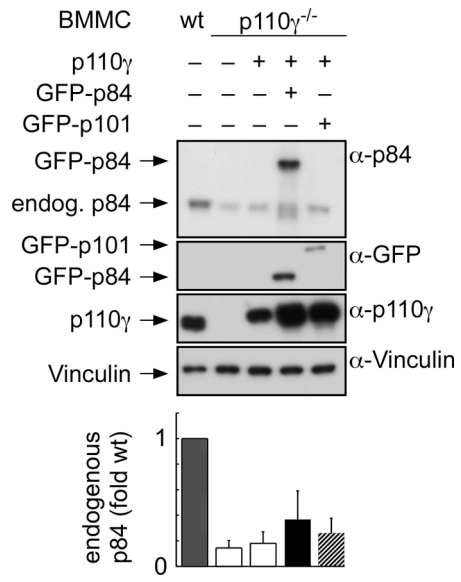


**Fig. S3**. Restoration of p110γ does not affect the abundance of endogenous p84 protein. *Top* Functional p110γ (WT) and GFP-tagged p84 or p101 were expressed in KO BMMCs (as indicated), whereas WT BMMCs were mock transfected. Twenty-four hours post-nucleofection, total lysates were subjected to SDS-PAGE and Western blotting analysis with the indicated antibodies (right labels). *Bottom* Quantification of the abundance of endogenous p84 protein arranged as in upper panel, depicted as percentage relative to the abundance of endogenous p84 in WT cells (n = 3, mean ± SEM). Statistics among KO BMMCs are not significantly different.

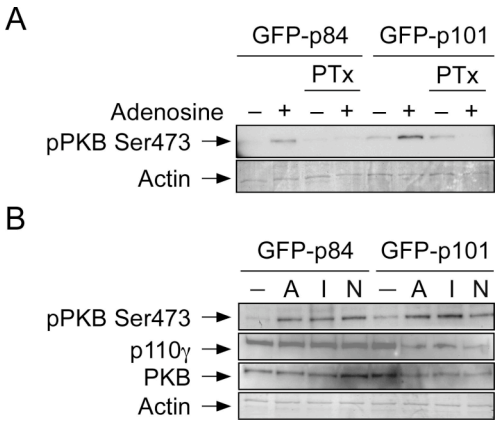


**Fig. S4.** Adenosine receptor ligands and PI3Kγ signaling lead to the phosphorylation of Akt. KO BMMCs were transfected with plasmids encoding functional p110γ (WT) and GFP-tagged p84 or p101. After starvation in IL-3-free medium containing 2% FCS for 3 hours (24 hours post-nucleofection), phosphorylation of Akt was determined with anti-pSer$^{473}$ antibodies. (**A**) Stimulation of BMMCs occurred with 2 μM adenosine for 3 minutes with or without pertussis toxin pretreatment (PTX, 100 ng/ml, for 30 min.). (**B**) BMMCs were stimulated as indicated with either adenosine (A, 2 μM), a stable, broad spectrum adenosine receptor agonist (N, NECA, 1 μM), or an A3AR-specific agonist (I, IB-MECA, 10 nM) for 3 minutes before the extent of phosphorylation of Akt was detected as described earlier.

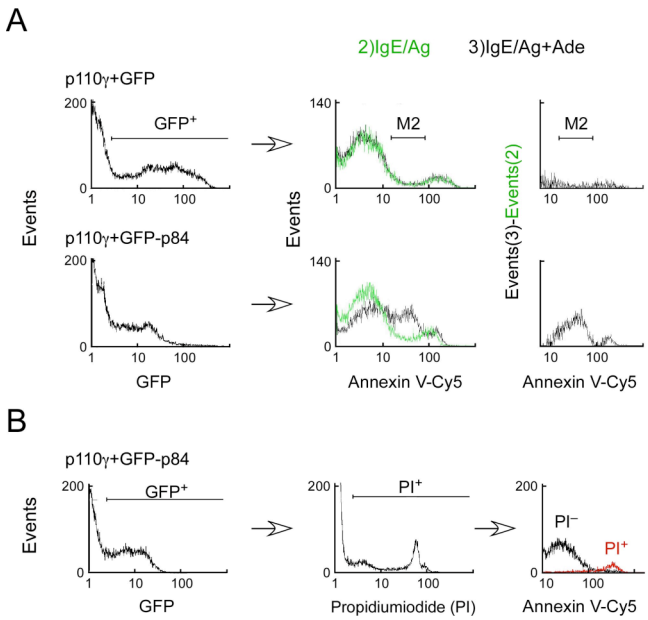


**Fig. S5.** Illustration of the gating procedures used in single-cell degranulation assays. KO BMMCs were transfected with the indicated expression plasmids for GFP, GFP-p84, and p110γ, and later stimulated with IgE+Ag or IgE+Ag+adenosine as described in the legend for Fig. 3. (**A**) GFP+ cells were selected as shown, and monitored for binding of Cy5-conjugated Annexin V. The M2 region was defined to include annexin $V^+$ cells, but to exclude dead cells (see B). Histograms obtained after stimulation of cells with IgE+Ag+adenosine or with IgE+Ag were subtracted from each other to display the adenosine-dependent part of degranulation response, which is given by [Events(3) – Events(2)]. (**B**) To avoid the false positive detection of dead cells in the annexin V-Cy5 channel, region M2 was limited to the right side so as to exclude cells staining positive for propidium iodide (PI).

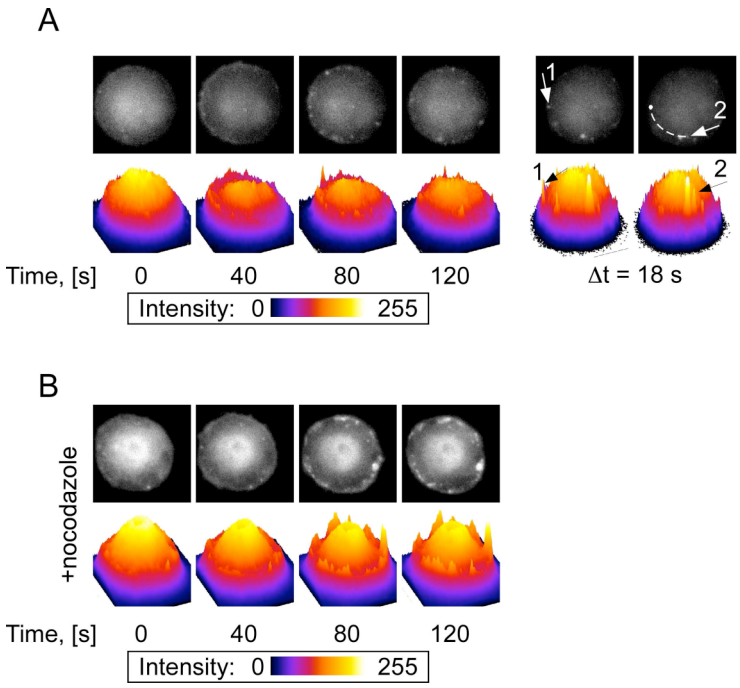


**Fig. S6.** p101:p110γ-dependent endocytosis of PtdIns(3,4,5)$P_3$ is associated with microtubules. KO BMMCs were reconstituted with p101:p110γ and $PH_{BTK}$-GFP to monitor the production of PtdIns(3,4,5)$P_3$. One day after nucleofection, cells were IL-3-starved for 3 hours prior to stimulation with 5 μM adenosine (t = 0). (**A**) *Top left* Selected time points of live-cell imaging experiments in KO BMMCs reconstituted with p101:p110γ (taken from movie m4) are aligned with plots of the surface intensity of GFP-$PH_{Btk}$. (*Bottom left*; see also movies m4 and m7). *Top right* The trajectory of a cytosolic PtdIns(3,4,5)$P_3$-GFP-$PH_{Btk}$ speckle is indicated. Arrows point to a motile speckle in a KO BMMC reconstituted with p101:p110γ. (**B**) Selected time points of live-cell imaging experiments (*top*) in a KO BMMC reconstituted with p101:p110γ after pretreatment with nocodazole (1 μM for 3 hours; see movies m8 and m9). *Bottom* Intensity surface plots as in (A).

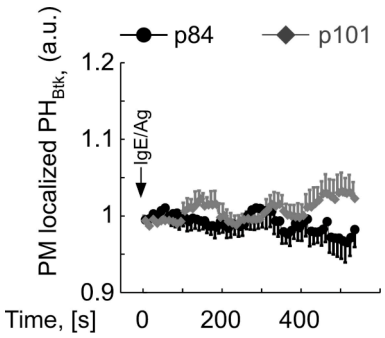


**Fig. S7.** Quantification of $PH_{Btk}$-GFP probes at the plasma membrane after low-level stimulation of BMMCs with IgE and antigen. Cells were loaded with IgE overnight and stimulated with 5 ng/ml DNP-HSA (Ag) only. Analysis and conditions as described in the legend for Fig. 6 (n=>8, mean ± SEM).

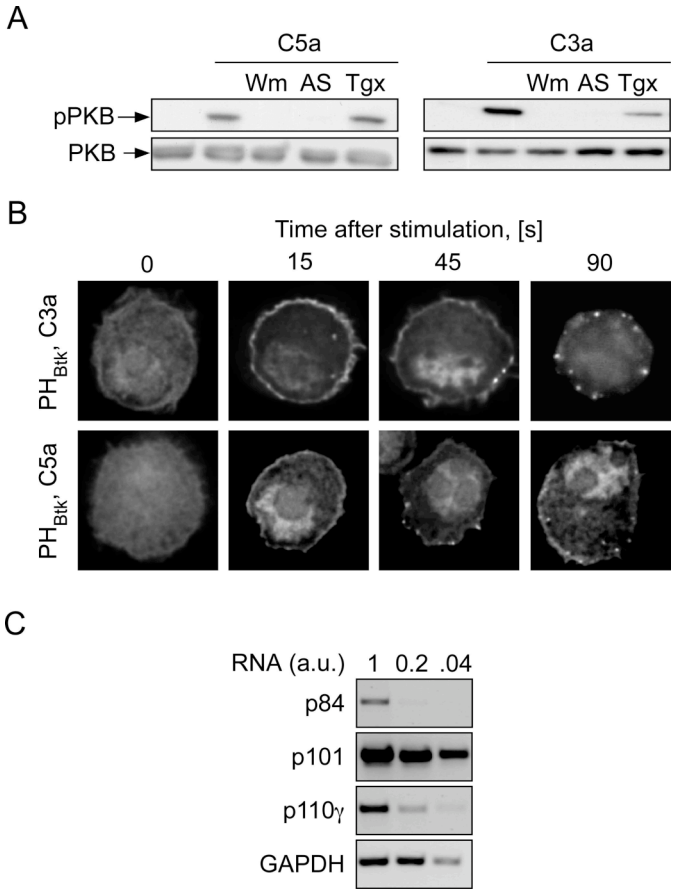


**Fig. S8.**Activation of Akt and localization of PtdIns(3,4,5)$P_3$ in J774 cells. (**A**) J774 cells were starved in serum-free DMEM for 3 hours and stimulated with 10 nM C5a (*left*) or 50 nM C3a (*right*) for 3 minutes, after a 30-minute pretreatment with or without wortmannin (Wm, 500 nM), AS252424 (AS, 1 μM) or TGX-221 (Tgx, 1 μM). Total cell lysates were subjected to SDS-PAGE followed by Western blotting. Phosphorylation of Akt was determined with an anti-pSer$^{473}$ antibody. (**B**) J774 cells were transfected with a plasmid encoding $PH_{BTK}$-GFP and one day later were stimulated with either 50 nM C3a (*top*) or 10 nM C5a (*bottom*). Cells were fixed in 4% *p*-formaldehyde after stimulation at the indicated times and mounted for analysis by fluorescence microscopy. (**C**) Transcripts for p84, p101, p110γ and GAPDH (internal standard) were amplified in J774 cells by RT-PCR with 3 different concentrations of mRNA input (a.u., arbitrary units).

## Movie descriptions (movies can be found on the provided CD-ROM)

**Movie m1 (m1.p84_z_stack)**

A stack of deconvoluted images of a KO BMMC reconstituted with WT p110γ and HA-p84 was taken 2 minutes after stimulation with 2 μM adenosine, and runs through adjacent focal planes to visualize the distribution of $PH_{BTK}$-GFP (green) and subcortical F-actin stained with rhodamine-phalloidin (red).

**Movie m2 (m2.p101_z_stack)**

KO BMMCs were complemented with WT p110γ and HA-p101. Subsequently experiments were performed exactly as for movie m1.

**Movie m3 (m3.p84_ade.mov)**

Time-lapse video microscopy was used to visualize PtdIns(3,4,5)$P_3$ in KO BMMCs reconstituted with p84 and p110γ. Images were acquired immediately after stimulation with adenosine over 360 frames (1 frame / 2 s). The movie runs at 15 frames / s.

**Movie m4 (m4.p101_ade.mov)**

Live-cell video microscopy for visualization of PtdIns(3,4,5)$P_3$ with $PH_{BTK}$-GFP in KO BMMCs reconstituted with p101 and p110γ. Images were acquired immediately after stimulation with adenosine over 360 frames (1 frame/ 2 s). The movie runs at 15 frames / s.

**Movie m5 (m5.p84_DNP_ade.mov)**

Live-cell video microscopy for visualization of PtdIns(3,4,5)$P_3$ with $PH_{BTK}$-GFP in KO BMMC reconstituted with p84 and p110γ. Cells were sensitized overnight with 100 ng/ml anti-DNP IgE. Images were acquired immediately after stimulation with DNP-HSA + adenosine over 400 frames (1 frame/ 2 s). The movie runs at 15 frames / s.

**Movie m6 (m6.p101_DNP_ade.mov)**

Live-cell video microscopy for visualization of PtdIns(3,4,5)$P_3$ with $PH_{BTK}$-GFP in KO BMMC complemented with p101 and p110γ. Cells were sensitized overnight with 100 ng/ml anti-DNP IgE. Images were acquired immediately after DNP-HSA and adenosine were added (1 frame/ 2 s; 400 frames). The movie runs at 15 frames / s.

**Movie m7 (m7.p101_ade_SIP.mov)**

Surface intensity plots (SIPs) of the cells monitored in movie m4. Intensity of GFP-$PH_{Btk}$ is shown. The intensity scale matches the one in fig. S5.

**Movie m8 (m8.p101_ade_NOC.mov)**

Live-cell video microscopy for visualization of PtdIns(3,4,5)$P_3$ with $PH_{BTK}$-GFP in KO BMMC reconstituted with p101 and p110γ. Cells were treated with the microtubule disruptor nocodazole (1 μM for 3h) prior to

stimulation with adenosine. Images were acquired immediately after adenosine stimulation over 360 frames (1 frame/ 2 s). The movie runs at 15 frames / s.

**Movie m9 (m9.p101_ade_NOC_SIP.mov)**

Surface intensity plots of the cells monitored in movie m8. Intensity of GFP-$PH_{Btk}$ is shown. Intensity scale used is the same as that in fig. S5.

**Table S1.** Sequences of primers used for cloning, antigen production, qPCR and RT-PCR.

| ***Gene**** | ***Accession-No.*** | ***Primer sequence 5' to 3'*** [$] | ***Usage*** [%] |
|---|---|---|---|
| Mm_GAPDH | NM_001001978 | F: CTGCACCACCAACTGCTTAG | qPCR, RT-PCR |
| | | R: AGATCCAC GACGGACACATT | |
| Mm_p84 | AY753194 | F: ATAGAGCAGGTGGCTAGCGA | qPCR |
| | | R: CGGAGGAAGAGTACCAGTTC | |
| Mm_p101 | NM_177320 | F: AAGCCCTGGGAAACTCCACA | qPCR |
| | | R: TCAGCGCAATGCCTGTCCAT | |
| Mm_ p110γ | NM_020272 | F: CCCTGGTGATCGAGAAATGC | qPCR |
| | | R: AGGCACAGGTCCAGAGATTC | |
| Mm_p84 | AY753194 | F: ATAGAGCAGGTGGCTAGCGA | RT-PCR |
| | | R: TGCTTTCATACCATGGGTCGACTC | |
| | | F: TCGGGATCCAAGATGGAGAGCTCAGATGTGGAGCTG | $His_6$ -antigen |
| | | R: GAGGGTACCTGCCTCAGGGTCCACGAACAGAAACAC | |
| Mm_p101 | NM_177320 | F: TACAGATTATTTACAGCAAGGGAGCC | RT-PCR |
| | | R: ACACTTCACACCTGACCACACTC | |
| Mm_ p110γ | NM_020272 | F: CCAGCTGGTACAGGCTGTGAAATT | RT-PCR |
| | | R: TGCATTTCTCGATCACCAGGGTAC | |
| Hs_GAPDH | NM_002046 | F: CTGCACCACCAACTGCTTAG | RT-PCR |
| | | R: AGGTCCACCACTGACACGTT | |
| Hs_p84 | AY753192 | F: CAGACACCAGAGACCTGCATGC | RT-PCR |
| | | R: CCTCCAAGTCAGCACTGAGGC | |
| | | F: CGACTAAGCTTCCATGGAGAGCTCAGATGTGGAGCTG | Cloning |
| | | R: CCATGCGGCCGCTCACTGGACAATACCAGAGAATGTG | |
| Hs_p101 | NM_014308 | F: CTTCATCACTGGGGAGAAGACG | RT-PCR |
| | | R: GAGACCTCACATCTGACTACACTC | |
| Hs_ p110γ | NM_002649 | F: GTGATGATCTGCGCCAAGACATG | RT-PCR |
| | | R: GGAAGTCAGGGGTTAGCACAAATGG | |
| Rn_GAPDH | Not available | F: CTGCACCACCAACTGCTTAG | RT-PCR |
| | | R: CCTGCTTCACCACCTTCTTG | |
| Rn_p84 | Not available | F: CTGTCACACAGGTGCTCTGAACAG | RT-PCR |
| | | R: GTCTGGCCTAAATCCTTGTAGATCC | |
| Rn_p101 | Not available | F: CCCACTGTGACCTTCCTAAACTGC | RT-PCR |
| | | R: CCTGTCAATCTCCAAGTCCTCCTC | |

| | | | |
|---|---|---|---|
| Rn_p110γ | Not available | F: CTGATTGGCTACGACGTCACTGAC<br>R: GGGACTTCTTCTTGGCCATCTTGG | RT-PCR |

*Mm, *mus musculus*; Hs, *homo sapiens*; Rn, *rattus norvegicus*; $F, forward primer; R, reverse primer. %see also main text.

# 5 General discussion and conclusions

PI3Ks transduce extracellular signaling immediately downstream of growth factor, cytokine and chemokine receptors. Hereby they control a plethora of cellular responses, by which the homeostasis of the organism is maintained. Consequently modulated PI3K signaling was connected to various diseases such as cancer development and progression, inflammatory disease, allergy and asthma (Marone et al., 2008). As outlined above, the impressive development of PI3K specific inhibitors yielded many highly effective compounds, of which some are already in clinical trials. All compounds prevent ATP binding to the catalytic core of PI3K. Although inhibiting the kinase activity directly is currently very successful, it however risks the inhibition of structurally related kinases or similar domains. Thus alternative approaches such as allosteric inhibition or disruption of essential protein interactions with PI3Kγ should not be neglected. Therefore a detailed knowledge of how PI3Kγ is activated is required.

## 5.1 The p84 adapter increases complexity of PI3Kγ signaling

Until recently p101 was the only PI3Kγ adapter subunit (Stephens et al., 1997; Suire et al., 2005). The existence of one adapter only should have minimized complications for analyzing its physiologic role, due to the lack of cross-compensatory effects. Nevertheless controversial *in vitro* data were available on whether p101 was dispensable for PI3Kγ activity or not. The reduced chemotaxis of p101 null neutrophils towards low concentration of GPCR agonists contributed only partially to assess the physiologic role of p101, as high agonist concentrations were still able to induce half maximal migration and PtdIns(3,4,5)$P_3$ production in absence of p101 (Suire et al., 2006). Thus p110γ may partially promote migration without p101. Similarly the p110γ mediated neutrophilic oxidative burst, elicited by the same stimuli, was p101 independent (Suire et al., 2006), and p110γ might thus be able to signal independent of an adapter subunit. The discovery of p84 as an additional p110γ adapter protein provides however also the possibility that p84 compensated for the deleted p101 in migration and was required to transduce signaling to the oxidative burst, which was unfortunately not addressed. Thus a clean, but physiologic relevant system, in which cross-compensatory effects of either of the adapters can be excluded, is fundamental for defining physiologic adapter functions.

In this report we show that BMMC from p110γ null mice qualify as such a system, in that these do not express p110γ, p101 and only little p84, with completely abrogated adenosine signaling. In wild type mast cells adenosine signaling, which is PI3Kγ mediated, is essential for mast cell migration and degranulation (Kitaura et al., 2005; Laffargue et al., 2002). Thus p110γ null BMMC were exploited in complementation experiments to identify the components required for adenosine induced PI3K signaling. The complementation studies provided the first clear-cut demonstration, that p110γ, even at high abundance, is incapable to transduce GPCR stimuli to PtdIns(3,4,5)$P_3$ production, phosphorylation of PKB, mast cell migration. The abundance of either p84 or p101 together with p110γ was required for PI3Kγ signaling.

## 5.2 p84 - the sole PI3Kγ adapter in allergic responses:

Expression analysis of p110γ, p101 and p84 developed p84 as the unique PI3Kγ adapter subunit abundant in mast cells. In line with the above mentioned complementation studies we also determined p84 to be essential in adenosine enhanced mast cell degranulation. Integrating the gained knowledge, that PI3Kγ mediated mast cell migration and degranulation are strictly dependent on the abundance of p84, with the fundamental role of PI3Kγ activity in mast cell recruitment to and their activation in inflamed tissue (Collmann et *al.*, in preparation) highlights p84 as an important component in allergic responses. Thus efforts should be undertaken to understand how p84 and p110γ interact, and how to disrupt the p84:p110γ complex, which could open alternative avenues for therapeutic approaches. A specific disruption of the p84:p110γ complex could specifically inhibit mast cells, due to its the predominant expression of p84 in those, while other immune cells might be unaffected. Alternatively modulating the localization of the membrane targeted p84:p110γ complex could be an additional attempt, as our data ishow that the p84:p110γ complex produces PtdIns(3,4,5)$P_3$ in membrane micro-domains, which was essential for adenosine enhanced degranulation. Thus it is likely that p84:p110γ is in proximity to the FcεRI input, which is lipid raft dependent, and locally enhances PtdIns(3,4,5)$P_3$ In contrast p101:p110γ complexes, which were not sensitive to the lipid composition were also not able enhance degranulation. However it has to be noted, that currently too little is known on how p84 and p110γ interact and which are the driving forces. Thus many molecular details have identified before rationalizing such a strategy

## 5.3 Molecular basis of adapter specific PI3Kγ functions

In the present study we demonstrated that p110γ signaling in mast cells is strictly dependent on its adapters. Under physiologic conditions p84 is dominant here, as p101 protein was not detectable in mast cell lysates. Nevertheless replacement of p84 by p101 still led to functional PI3Kγ complexes, which were capable to produce PtdIns(3,4,5)$P_3$, promote phosphorylation of PKB and even mast cell migration. The p101:p110γ complex was however unable to restore adenosine enhanced mast cell degranulation.

It is of major interest to elucidate the molecular basis of this first non-redundant function of both PI3Kγ complexes, and maybe also of additional nor-redundant functions which are yet undefined. The little information on the structure of both adaptors cannot provide an explanation, and their *in vitro* performance was similar (Suire et al., 2005). However it must be noted, that in comparative studies of p84 and p101, the adapter dependent increased sensitivity of p110γ to Gβγ subunits included only one combination of Gβγ ($G\beta_1\gamma_2$) subunits (Suire et al., 2005; Voigt et al., 2006), while at least p101 increases p110γ activity towards other combinations of Gβγ subunits, too (Kerchner et al., 2004). Similarly, over-expression studies only applied the fMLP receptor system in non-relevant cell types to assess PtdIns(3,4,5)$P_3$ production, but without emphasis on physiologic responses (Voigt et al., 2006). Future studies should thus address, whether preferential combinations of Gβγ or GPCR exist for both of the complexes especially in cell types harbouring

both adapters. Given that Gβγ specificity could be determined, this may allow an immediate prediction, which PI3Kγ complex transduces signals from which GPCR.

Alike above, in our system both PI3Kγ complexes served the same type of adenosine receptor, and both integrated signaling from $G\alpha_i$-coupled receptors. It is thus unlikely that the cause of non-redundant functions is upstream PI3Kγ in our system. Differential PtdIns(3,4,5)$P_3$ localizations and the differential sensitivity to cholesterol depletion of either of the PI3Kγ complexes rather suggested that the adapters facilitate the differential localization of p110γ to distinct plasma membrane micro-domains. Biochemical confirmation is required, e.g. by density- gradient centrifugation techniques, which might however fail due to transient and non-convalent association of the PI3Kγ complexes to the membrane compartments. An analysis of the interactomes of both PI3Kγ complexes could considerably contribute to understand the differential PtdIns(3,4,5)$P_3$ localization and degradation pathways, we observed. Thus p84 might interact with a micro-domain localized protein, while p101 does not. Unknown adapter intrinsic domains could similarly drive the differential localization of the two complexes for PtdIns(3,4,5)$P_3$ production.

## 5.4 "Sub-Isoform" specific PI3Kγ inhibition?

The potential therapeutic value of the disruption of the p84:p110γ complex in allergy was discussed above. The non-redundant function of p101:p110γ and p84: p110γ shown here should be rapidly extended to functional studies in immune cells harbouring both complexes, with the attempt to isolate more adapter specific functions. Such a possible adapter specific response could be the neutrophilic oxidative burst (discussed above). Defining more adapter specific PI3Kγ functions in combination with the selective inhibition of either of the complexes could provide sub-isoform specific targeting. Hereby inhibition might be achieved by complex disruption but could also be mediated by inhibiting proper localization of the complexes.

## 5.5 PI3Kγ is p84:p110γ or p101:p110γ - and Class IA PI3Ks?

All class I PI3Ks are heterodimeric complexes of a catalytic and a regulatory subunit. Fifteen combinations are possible for class IA, two for class IB. Nevertheless they are often referred to as PI3Kα, β, δ or PI3Kγ, which only accounts for the catalytic subunit but not for the regulatory one. We however defined non-redundant functions of p84:p110γ or p101:p110γ and to me its seems necessary to clearly distinguish the complexes in the future.

For p110δ and class IA regulatory subunits studies in gene targeted mice unraveled some isoform specific functions in immune cells, which is indeed important knowledge to pharmacologically modulate (patho-) physiologic responses. Whether the effects are due to intrinsic functions of the specific subunit or whether it is "only" the total loss of class IA PI3K activity was however often unclear. Complementation experiments as performed here could establish isoform specific functions, namely if an "equivalent" subunit cannot rescue the defects of the targeted one.

Moreover the molar ratios of the 5 regulatory subunits and 3 catalytic subunits of class IA have to be determined for each cell type in the future, in order to define real isoform specific functions. Thus it can

currently not be excluded, that the “isoform specific” defects are only based on the predominant expression of the targeted subunits, which results in a total block of class IA PI3K signaling. Thus it is highly interesting, that male PI3K$\delta^{KD/KD}$ mice are fertile (Ali et al., 2004), although SCF/c-Kit signaling is essential for spermatogenesis, while SCF/c-Kit signaling is blocked in mast cells. Male PI3K$\beta^{KR/KR}$ mice show however defects in spermatogenesis (Elisa Ciraolo, in preparation), while extrapolated from Ali et al. 2004, p110$\beta$ is negligible in SCF/c-Kit signaling in mast cells.

Adapter specific signaling of the p85 family members were also addressed in knock-out approaches, but not combined with functional restoration. However already the fact that spliced the forms of p85$\alpha$ (p55 and p50$\alpha$ ) and p55$\gamma$ do neither contain the SH3 domains nor the BH domain present in p85 $\alpha$ and p85$\beta$, suggest that there might differential modes of activation or different localizations, as we have observed it for the class IB complexes, e.g. by specific protein-protein interactions.

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# 7 Appendix

## 7.1 Protocols:

### 7.1.1 Mast Cell Protocols

Sterile conditions are obligatory, with exception of bone isolation and post stimulation of BMMC (which do not re-enter the incubator). Common tissue culture equipment is not separately listed, e.g. lamina flow, incubators, centrifuges, tissue culture flasks in materials.

**Isolation of BMMC from the bone marrow**

**Material:**

- Various knock-out and wild-type mice,
- Sterile scissors and tweezers,
- Sterile 50 ml Falcon tubes,
- Sterile 1.5 ml Eppendorf tubes,
- Sterile, **punctured** 0.5 ml Eppendorf tubes:
  Prepare 0.5 ml Eppendorf tubes by puncturing a hole at the bottom with flame heated sterile tweezers or a G21 syringe needle. Cut off the caps. Put these small tubes into **intact** 1.5 ml sterile Eppendorf tubes.
- Sterile tissue culture flask,
- Complete IMDM = IMDM$^c$ (add to 500 ml Iscove's Modified Dulbecco's Medium, + 50 ml 10% heat-inactivated FCS [Amimed], 5 ml 200mM L-Gln [Sigma, G7513],
  + 5 ml Penicillin-Streptomycin 10k U-μg/ml [Sigma, N°109],+ 0.5 ml 50mM β-mercaptoethanol. Store at 4°C.
- **Murine** Interleukin-3 (**m**-IL3; from Peprotech, CatNo. 213-13). The Stock solution is 10 μg/ml m-IL3 in PBS/BSA 0.1%. Store sterile at -20°C.
- Optional: stem cell factor (SCF; from Peprotech, CatNo. 250-03, 5 ng/ml final).
- *Note:* human IL-3 and SCF do not promote murine mast cell growth.

**Procedure:**

- To disinfect, spray $CO_2$-euthanized mice externally with 70% ethanol
- Excise the femurs and clean them with a scalpel. Remove all soft tissue.
- Immerse the thigh bones in 10 ml sterile IMDM$^c$ in a 50 ml Falcon tube, keep on ice.
  → **Transfer to sterile bench from here!**
- Pour the femurs and IMDM$^c$ into a Petri dish. Cut the heads of the femurs and place into the punctured 0.5 ml Eppendorf tubes.

- Clean the centrifuge with 70% EtOH. Centrifuge for eppendorf tubes 30 seconds at 5000 rpm and then 2 min at 2000 rpm.
- Resuspend the pellet in the 1.5 ml Eppendorf tube with 1 ml IMDM$^c$. Transfer the cells to 20 ml IMDM$^c$ (heated to 37°C)
- Supplemented cell suspension with 2ng/ml m-IL3 and (optional: 5 ng/ml m-SCF) and cultivate at 37°C, 5% $CO_2$.

## Bone Marrow-derived Mast Cell Culture

**Material:**

- see Isolation of BMMC from bone

**Procedure:**

- Isolate bone marrow as described above.
- Incubate 3-5 days at 37°C, 5% $CO_2$ to allow macrophages and fibroblasts to adhere.
  *Note:* watch density, if too dense change flask after 2-3 days.
- Collect non-adherent cells in a 50 ml Falcon tube and centrifuge, 3 min at 900 rpm (approx. 160 g).
- Remove and keep the supernatant.
- Resuspend cells in fresh IMDM$^c$ and count them.
- Resuspend the cells at 0.5 x $10^6$ cells/ml in a mixture of 20 % old and 80% fresh medium supplemented with 2 ng/ml m-IL3, and cultivate at 37°C, 5% $CO_2$.
- Supply fresh m-IL3 every 2-3 days; passage cells weekly when they reach about 1-2x $10^6$ cells/ml and dilute to 0.5x$10^6$ cells/ml (keeping 20% of old medium).
  Lazy way: grow cells to 2.0 x $10^6$ cells/ml (8 ml), add 32 ml fresh medium supplemented with 2 ng/ml m-IL3. Readd IL-3 every 2-3 days. This gives a passaging cycle of about 9 days.

  The cells show a mast cell-like phenotype after 4-8 weeks. Test for FceRI and c-kit expression.

## FACS Analysis of FceRI and c-kit Expression

**Additional Material, Antibodies:**

- Hamster anti-mouse FceRI : R-PE-labeled, 0.2 mg/ml (=stock; from eBioscience, CatNr. 12-5898-83, lot #E011195; directed against FceRIa chain, clone MAR-1).
- Rat anti-mouse CD117/c-kit: R-PE-labelled, 0.1 mg/ml (=stock), ImmunoKontact, lot #212-AB-407V, rat $IgG_{2b}$.

R-Phycoerythrin (R-PE): excitation 488 nm, emission 575 nm

- 2.4G2 Fc$\gamma$RII exo, Rat IgG1, Karl Matter, Geneva.
- FACS tubes
- PBS supplemented with 3% FCS

**Procedure:**

- Resuspend the bone marrow cells of 2 femurs in 20 ml of IMDM$^c$; during mast cell differentiation repeat experiment after 2 and 4 weeks of cell cultivation. Take 3x1 ml of each cell population and transfer to FACS tubes.
- Centrifuge (400g, 3 min.).
- Aspirate medium and resuspend in 100 µl PBS supplemented with 3% FCS and 1:100 diluted 2.4G2 FcγRII exo and incubate for 15 min.
- Add to tube 1-3 of each population

  1) Nothing (unstained control)

  2) 0.5 µl R-PE-labeled anti-mouse FcεRI (from 0.2 mg/ml stock),

  3) 0.5 µl R-PE-labeled anti-mouse CD117/c-kit (from 0.1mg/ml stock,

  → incubate for 25 minutes on ice in the dark.
- Analyze immediately by FACS. Acquisition was performed with FACS Calibur or Cantor II (BD).

## BMMC transfection

**Materials:**

- BMMC, especially of p110γ null genotype
- 10-15 µg plasimd DNA (total), isolated with Genelute high performance endotoxin-free Maxi-Prep KIT (Sigma, #NA 0410), DNA concentration adjusted to 0.5 or 1.0 mg/ml in 1xTE buffer
- Nucleofector device II (Amaxa)
- Cell Line Nucleofector Kit T (Amaxa, # VCA-1002)
- Complete IMDM (Sigma, I3390; HIFCS, Gln, PEST, 50 µM β-mercaptoethanol), supplemented with 2ng/ml recombinant murine IL3 (Peprotech, #213-13)
- 25 cm$^2$ cell culture flasks (BD$^{TM}$ Falcon)

**Important !**

- Prepare everything before cells are re-suspended in solution T. Solution T is somewhat toxic to the cells.
- Everything is performed sterile under the laminar flow, except the nucleofection. During nucleofection, cuvettes are covered with the caps provided in the kit.

**Procedure:**

- Pipette 10-15µg plasmid DNA into 1.5 ml Eppendorf tubes, adjust the total DNA content to equal amounts were necessary (using base vector without expression insert).
- Add 7 ml prewarmed complete IMDM supplemented with 2 ng/ml IL-3, to 25cm$^2$ cell culture flasks and label appropriately.
- Keep 10 ml complete IMDM, containing IL-3, in a 15 or 50 ml Falcon tube to flush the cuvettes and collect the cell suspension post nucleofection
- Unwrap nucleofection-cuvettes and plastic pasteur pipettes provided in the cell line nucleofector kit (1 cuvette and 1 pipette for each nucleofection reaction)
- Turn on the nucleofector device and choose program X_001
- Count BMMC (CASY-Cell Counter, Analyser System, Model TT; Schärfe System, program 01).

- Collect the required amount (8-12x$10^6$/ nucleofection) of BMMC by centrifugation (900 rpm, 3-5 min.)
- Aspirate supernatant of the cells, and add 100µl solution T per nucleofection reaction to the cells, mix by pipetting.

**From here work as quick as possible**

- Distribute 100µl of cell suspension to each of the DNA-containing Eppendorf tubes
- Mix DNA and cell suspension and transfer to the nucleofection cuvette. Cap the cuvette.
- Place cuvette into the nucleofection device, press ok- nucleofection starts!
- Post nucleofection flush the cuvette with complete IMDM using the provided plastic pasteur pipette. Aspirate the cell suspension with the same pipette and transfer to the prepared tissue culture flasks, containing the growth medium.
- Repeat for all the other nucleofection reactions
- Incubate the flasks at 37°C, 5% $CO_2$
- 5 hours post nucleofection, warm complete IMDM, and transfer the samples to 15 ml Falocn tubes
- Centrifuge cells, 900 rpm, 3-5 min
- In the meanwhile, label new tissue culture flasks
- Post centrifugation aspirate the supernatant, add 7ml complete IMDM to each falcon tube and transfer cell suspension to the tissue culture flasks. Add IL-3 (2ng/ml final) and incubate overnight (37°C, 5% $CO_2$)For degranulation experiments add additionally 100 ng/ml IgE anit DNP (SPE-7 clone, Sigma, #D8406)

## Annexin V binding as measure for MC degranulation

**Material:**

- Non transfected, or transfected BMMC, include GFP or GFP-fusion proteins, for gating of transfected cells
- Phosphate buffered saline (PBS)
- Annexin binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4)
- Annexin-Cy5 (BD Pharmingen, Cat. No. 559934)
- Optional: propidiume iodide (PI), Sigma P4170, our stock is 2.5 mg/ml
- FACS tubes
- IgE anti DNP, stock solution (SPE-7 clone, Sigma, #D8406)
- DNP-HSA (Sigma, #A-6661) in PBS (stock solution 1 mg/ml)
- Adenosine (Sigma, # 01890) in $H_2O$ (stock solution 10 mM)
- Complete IMDM (Sigma, I3390; HIFCS, Gln, PEST, 50 µM β-mercaptoethanol), supplemented with 2ng/ml recombinant murine IL3 (Peprotech, #213-13)
- 24-well tissue culture plates (BD Falcon™), do not use our 12-well plates!

**Procedure:**

- The day before the degranulation assay, and the day of transfection for- complementation assays respectively, add 100 ng/ml (final) IgE anti DNP, to sensitize BMMC (in complete IMDM with IL-3, cell concentration 0.5-1.0x $10^6$/ml)

- At the day of the degranulation experiment prepare dilutions of 1 μM DNP-HSA with or without the addition of 2 mM adenosine → final concentrations will be 1 ng/ml DNP-HSA and 2 μM adenosine in PBS.
- Count cells and if required adjust concentration to 0.5- 1.0 x $10^6$/ml with $IMDM^C$
- Label 24-well plate and pipette 1 μl PBS or stimuli to the wells.
  **Work quick to avoid evaporation of the PBS or stimuli**
- Mix cell suspensions by pipetting up and down and distribute 1 ml (for each sample) of the cell suspension to the 24 well plate.
- Incubate for 20 min. at 37°C, 5% $CO_2$, in the meanwhile label FACS tubes
- Post incubation mix each sample (pipetting up and down) and transfer each sample to one of the FACS tubes
- Sediment cells by centrifugation (400g, 5 min.)
- Aspirate supernatant and wash with 1xPBS
- Sediment cells once more, during sedimentation prepare 1x AnnexinV binding buffer with 1:100 dilution of AnnexinV-Cy5 (each samples requires 100 μl), optional: add 1:100 Propidium Iodide solution (PI)
- Aspirate PBS and resuspend cells in 100μl AnnexinV binding buffer, containing AnnexinV-Cy5
- Incubate for 15 min. on ice in the dark
- Perform FACS analysis using FacsCalibur (Becton Dickinson) and Cell Quest pro software. Settings were as followed **(only an approximation for new experiments, please include controls of single stained samples to adjust settings if you do a new set of experiments)**:

| | | |
|---|---|---|
| FSC: E00 | 1.0 | |
| SSC: 399 | 1.0 lin | |
| FL-1: 420 | log | (GFP detection) |
| FL-4: 522 | log | (Cy5 detection) |
| **If co stained with PI:** | | |
| FL-3: 396 | log | (PI detection) |
| Compensation required: | | |
| FL-4% -FL3%: 22% | | (Cy5 and PI signal separation) |
| FL-3%-FL4%: 6% | | |

## 7.1.2 Microscopy:

### Preparation of mounting solution:

**Material:**

- 50 mM Tris-HCl, pH7.5-8.0
- Mowiol (Plüss-Staufer, Oftringen, Hoechst 4-88/cmB339161)
- Glycerol (Fluka, 49770)
- n-Propylgallat (Sigma, P3130)

**Procedure:**

- Stir 5 g Mowiol in 20 ml Tris-HCl buffer (0.05M, pH7.5-8.0) on a magnet stirrer (30-40°C, 16h).
- Add 10 ml 100% Glycerol and stir 16 h.
- Spin 10 min. at 1500 rpm at room temperature.
- Add 10 mg/ ml n-propylgallat as anti-fading agent.
- Aliquot the Mowiol solution
- Spin aliquots to remove air bubbles, store at -20°C.
- Thaw the aliquot at least 30 min. before usage at 37°C

### Preparation of 10% *para*-formaldehyde in PBS (10% PFA/PBS):

**Material:**

- Weigh 100 g PFA to 400 ml water.
- Boil in a waterbath under the extractor hood
- Shake from time to time.
- Add cautiously drops of 5N NaOH to completely dissolve the PFA (PFA is not soluble at that pH without NaOH).
- After complete solution of the PFA add 100 ml 10x PBS.
- Fill up to 1 liter with water.
- Solution can be further diluted to 4% PFA/PBS. Store at 4°C

### Preparation of adherent cells:

**Material**

- PBS
- 4% *para*-formaldehyde in PBS pH = 8.0
- Mounting solution, Mowiol, see above
- Hoechst 33342 (Molecular Probes, H-1399), cell permeable
- 12- well, or 24- well plate
- 18 mm or 12 mm diameter coverslips
- Microscopy slides
- dd$H_2O$

**Procedure:**

- Grow (transfected) cells on sterile coverslips in a 12 well or 24-well plate.
- Perform your experiment of interest.
- a) if the experiment is time-dependent, add an equal volume of 10% PFA/PBS to the sample directly to stop the reaction
  b) otherwise wash cells twice with PBS, aspirate PBS, add ice cold 4% PFA in PBS.
- Incubate on ice for 30 min.
- Wash with PBS.
- Optional nuclear staining: add PBS with 1:1000 Hoechst 33342 (stock solution 1 mM in $ddH_2O$), incubate (15 min., 4°C, in the dark).

Preparation of the microscopy slides:

- Put one drop of mounting solution onto a microscopy slide
- Take the coverslip with fine forceps and wash with $H_2O$, quickly.
- Soak residual liquid from the edge of the coverslip with a fine paper towel.
- Place coverslip upside down onto the drop of mounting solution on the microscopy slide.
- Dry for min. 30 min. at room temperature in the dark.
- Then store at 4°C in the dark.

## Preparation of suspension cells

**Material:**

- (Transfected) suspension cells (e.g. BMMC)
- 10% PFA/PBS
- Mounting solution
- Fine forceps
- Coverslips
- Microscopy slides
- Cytospin device
- Cytospin filter cards for cyto-centrifugation (Thermo, 3424)
- Cytospin microtube 0.75 ml, with 0.5 mm bottom hole (Thermo, 1153)
- PBS containing 1-2% BSA (BSA reduces shear forces and protects from cell rupture)

**Procedure:**

- Perform your experiment of interest
- Fix cells by addition of 1:2.5 volume of 10% PFA/PBS
- Incubate 30 min.
- Transfer samples to eppendorf tubes
- Sediment cells (400 g, 3 min.)
- **Permeabilize the cells for additional intracellular stainings! Procedures see below**
- Wash cells twice with 1 ml PBS/1-2% BSA
- Collect cells (400 g, 3 min.)

- Aspirate supernatant

Cytospin:

- Resuspend cells in 150 µl PBS/1-2% BSA for cytospin
- Assemble cytospin device
- Transfer cell suspension to the cytospin microtube
- Spin cytospin devices in a centrifuge equipped with a swing out rotor, with a buck for multi-well plates (100g, 1min.)
- Disassemble cytospin device carefully.
- Overlay cells with a drop of mounting solution
- Place coverslip on top of the mounting solution
- Dry for several hours at room temperature in the dark.

Staining of intracellular components:

- Post fixation, permeabilize the cells with PBS with 0.1% saponine, 1-2% BSA (or 5% goat serum for antibody staining) (15 min., room temperature)
- Pellet cells (400 g, 3 min.)
- Resuspend cells in a small volume (100-200 µl) of PBS/1-2% BSA (or 5% goat serum)
- For F-actin staining add rhodamine labeled phalloidin 1:1000 (Molecular Probes, R415), for staining of microtubuli add 1:5 anti tubulin antibody (see antibody table below), slightly shaking on a rotary wheel or a thermoblock.
- For F-actin staining proceed with washing steps in PBS/1-2% BSA, followed by cytospin.
- For microtubuli staining proceed with washing in PBS/5% goat serum, shake slightly for 5 min.
- Pellet cells (400g, 3 min.), aspirate supernatant, repeat washing step
- Aspirate supernatant.
- Resuspend cell in 100-200 µl PBS/5% goat serum
- Incubate (1h, dark, shaking) with 2$^{nd}$ antibody (1:100 goat anti rat, Alexa$_{546}$)
- Wash 3x with PBS/1-2%BSA, with 5 min. washing intervals
- Prepare for cytospin afterwards.

## Staining with Alexa$_{555}$- labeled Choleratoxin

**Materials:**

- BMMC
- Vybrant Lipid Raft Labeling Kits (Alexa Fluor 555) (Invitrogen, V-34404)
- 10% PFA/PBS
- Cytospin device
- Mounting solution
- Coverslips
- Microscopy slides

**Procedure:**

- Collect BMMC by sedimentation (900g, 3 min.). Aspirate supernatant.
- Resuspend cells in complete IMDM (0.5-1.0x $10^6$ cells/ ml). Place on ice.
- BMMC were labeled according to the manufacturer's instructions, see http://probes.invitrogen.com/media/pis/mp34403.pdf.
- Afterwards prepare cells for cytospin (see above).
- **Note: incubation at 4°C "freezes" the original membrane structure, incubation at 37°C increases endocytosis. The additional incubation with the provided antibodies could induce artificial aggregation of the choleratoxin subunits.**

## Live microscopy

**Material:**

- 5N NaOH
- 5N HCl
- Poly-L-Lysine coated coverslips (12 mm diameter)
- transfected BMMC
- IMDM (PEST, Gln 2% FCS, without IL-3)
- 6-slot holder for Ludin chambers (Life Imaging Services [LIS], 10622
- Ludin chamber type III (LIS, 10920)
- Reconfiguration tool for Ludin chambers (LIS, 10910)
- 1 ml syringe, with 21Gx2" needle
- Modified Tyrode's Buffer (MT buffer; 137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 20 mM HEPES, pH 7.4)
- Stimulant (e.g. adenosine, DNP-HSA)
- Temperature conditioned microscope equipped with "The Cube+ Box temperature control system", LIS)
- Silicon tube for perfusion (Fine Bore Polyethylene Tubing 0.76mm ID, Portex, 800/100/240)
- Silicon grease

**Procedure:**

- **The day before the experiment:**
- BMMC transfection (see above)
- Place coverslips into 24-well plate
- Add 1 ml 5N NaOH to the coverslips, leave for 2 hours.
- Aspirate 5N NaOH and wash once with $ddH_2O$, aspirate
- Add 1 ml 5N HCl for 2 hours.
- Aspirate 5N HCl and wash with $ddH_2O$, aspirate
- Add 1 ml $ddH_2O$ with 1:10 dilution of poly-L-Lysine solution, 0.1% (Sigma, 8920), leave overnight
- **At the day of the experiment:**
- Grease the O-ring seals of the Ludin chambers
- Assemble the Ludin chambers including the poly-L-Lysine coated coverslips using the configuration tool. Assure yourself that the assembly is leak-proof (by filling with water for a while).

- Heat IMDM (PEST, Gln 2% FCS, without IL-3) and MT-buffer
- While preparing the samples UV-irradiate the Ludin chambers, then wash once with IMDM (PEST, Gln 2% FCS, without IL-3)
- Collect cells by centrifugation (900 rpm., 3 min.), aspirate supernatant
- Wash once in IMDM (PEST, Gln 2% FCS, without IL-3) and collect cells.
- Aspirate the supernatant and resuspend in IMDM (PEST, Gln 2% FCS, without IL-3) at 0.5-1.0x $10^6$ cells/ml, leave for 3h at 37°C, 5% $CO_2$.
- 1 hour before the experiment warm polystyrene box (for transport) and the syringe with the attached needle and tubing to 37°C, thaw stimuli. Switch on temperature control of the microscope. Start computer, OpenLab Software and set up time-lapse microscopy automation.
- Prepare stimuli 2x concentrated in 1 ml MT buffer (no IMDM/ 2%FCS due to possible instability of adenosine) and aspirate to the syringe.
- Place Ludin chamber into polystyrene box and connect the syringe-tubing system to the perfusion line.
- Close polystyrene box
- Carefully (avoid floating of the cells!) transfer samples to the microscope and fit Ludin chamber into the 6-slot holder for Ludin chambers.
- Once the microscope is set up and the acquisition automation is running, **very carefully** inject 500 µl stimuli/MT solution (final concentration is then 1x).

### 7.1.3 Molecular Biology

#### RNA isolation

**Materials:**

- Various tissues and cells
- Trizol (Invitrogen, 15596-026) or Tri-Reagent (Sigma, T9424)
- Chloroform (Fluka, 25690)
- Isopropanol
- 75% (v/v) Ethanol
- RNAse free water
- Filter tips

**Procedure:**

- Clean bench and pipettes carefully, wear gloves and lab coat to avoid contamination with RNAse.
- RNA isolation was performed according to the manufacturer's instruction, details see: http://tools.invitrogen.com/content/sfs/manuals/15596018%20pps%20Trizol%20Reagent%20061207.pdf
- Measure RNA concentration in a spectrophotometer.
- Use 2 µg total RNA for cDNA synthesis

## cDNA synthesis:

**Materials:**

- RNA
- Oligo-$(dT)_{15}$ primer (0.5 μg/μl; Microsynth)
- DTT (supplied with M-MLV RTase)
- 5x RT buffer (supplied with M-MLV RTase)
- 10 mM dNTPs (Invitrogen, 10297-018)
- RNAsin (Promega, N2111)
- M-MLV RTase (Invitrogen, 28025-013)

**Procedure (one reaction):**

- For primer annealing mix 2 μg RNA and 8 μl oligo$(dT)_{15}$
- Fill up to 23μl final volume
- Incubate for 10 min. at 70°C
- In the meanwhile mix 4 μl 0.1M DTT, 8 μl 5xRT buffer, 2 μl 10 mM dNTPs, 1 μl RNAsin and 2 μl M-MLV RTase.
- Place annealing reaction on ice (2 min.)
- Add 4 μl 0.1M DTT, 8 μl 5xRT buffer, 2 μl 10 mM dNTPs, 1 μl RNAsin and 2 μl M- MLV RTase to the annealing reaction.
- Incubate at 37°C, 90 min.
- Heat to 95°C for 10 min.
- store at -20°C until used for PCR.

## Plasmid DNA amplification and isolation:

**Materials:**

- LB-media (5 g NaCl (LB Miller = 10 g NaCl), 5 g yeast extract; 10 g bacto-tryptone, 5 ml of NaOH 1M, add 1 liter with $H_2O$ (autoclave)
- LB-agar (LB media + 12.5 g Agar [DIFCO, 281230]), autoclave.
- For antibiotic selection add 100 μg/ml Ampicilline or 25μg/ml Kanamycin to the LB media or LB-agar
- Chemically competent bacteria (usually *E.coli* XL-1 Blue strain)
- Template plasmid DNA or ligation reaction
- TE- buffer (10 mM Tris-HCl, pH 7,6; 1mM EDTA, pH 8.0)

**1. Preparation of chemically competent cells ($CaCl_2$ method)**

**Procedure:**

- Take bacteria from -80°C stock by scratching the frozen tube content with a sterile tip and plate on LB agar solid media overnight at 37°C.
- Pick single colony and inoculate a 100 ml LB-Miller, grow overnight (shaking in 37°C, 300 rpm)
- Inoculate 1 liter LB-Miller with the overnight culture, inoculation 1:100.

- Grow in shaker (37°C, ca. 300 rpm) until $OD_{600}$= 0.6. Then put the flask on ice. Wait 20 min until culture is really cold. Pre-cool $CaCl_2$ solutions on ice!
- **From now keep at 4°C (coldroom)**
- Transfer bacteria to the centrifuge bottles and spin in the pre-cooled centrifuge about 20 min. 3000 rpm at 4°C.
- Discard the supernatant, resuspend the pellet in 500 ml ice-cold $CaCl_2$ ($^1/_2$ of initial volume). Mix strongly initially, then gently.
- Leave the bacteria for few hours at 4°C, mix gently occasionally. Cool down solution of $CaCl_2$/10-15% glycerol on ice.
- Spin 15 min at 4°C, 3000 rpm, discard the supernatant and resuspend very gently in 100 ml of pre-cooled $CaCl_2$/10-15% glycerol.
- Immediately aliquot the bacteria in 500 μl portions to Eppendorf tubes and freeze in liquid $N_2$ immediately.
- Store tubes at -80°C.

**2. Transformation of competent bacteria**

**Procedure**

- Thaw tube with bacterial suspension on ice.
- Aliquot bacterial suspension to 100 µl portions in pre-cooled tubes. Add DNA (plasmid [100 ng] or ligation [up to15 µl]).
- Mix and incubate for 30 min. on ice
- Heat shock for 45- 90s at 42°C. Place tube on ice.
- Add 900 μl of SOC media and shake for (1 hour, 37°C)
- Collect cells by centrifugation (1 min. 3000g)
- Remove 800 μl supernatant, resuspend cells in the remaining 200 μl.
- Plate suspension on LB agar plate with the respective antibiotic (for plasmid selection). When the fluid is absorbed, incubate plate up side down overnight at 37°C.

**3. Plasmid amplification and purification:**

**Material:**

- Transformed *E. coli*
- LB media, LB agar, supplemented with antibiotics
- Endofree plasmid maxi kit (Qiagen, 12362) or Genelute high performance endotoxin-free (Sigma, NA 0410) or Genelute Plasmid Miniprep kit (Sigma, PLN-350)
- TE-buffer
- Isopropanol
- 70% Ethanol

**Procedure:**

- Pick colonies form the transformed *E.coli* plates, streak back up on LB agar and inoculate LB media, both supplemented with the selective antibiotic.
- Growth conditions and plasmid isolation were performed according to the manufacturer's instructions.
- Plasmid DNA was eluted with 1xTE buffer

- The concentration and purity of the plasmid was measured in a spectrophotometer
- (Optional: ethanol precipitation, according to the manufacturer's instructions, to increase plasmid concentration and purity)
- Adjust plasmid DNA to 0.5 or 1.0 μg/μl in 1x TE

## Agarose gel-electrophoresis

**Material:**

- Agarose, standard (Eurobio, 018054)
- 1x TAE buffer (40 mM Tris-acetate pH 8.0, 1mM EDTA)
- ethidium bromide
- Loading buffer (0.4% bromphenol, 0.4% xylene cyanol FF, 50% glycerol)
- Lambda marker, l HindIII/EcoRI (Labforce, 1695/1),
- pBR322 DNA/AluI Marker (Fermentas, SM0121)
- GenElute Gel Extraction Kit (Sigma, NA1111)

**Procedure:**

- Prepare 1-2% agarose gels.
- Melt agarose in TAE (40 mM Tris-acetate pH 8.0, 1mM EDTA)
- Add 0.3 µg/ml ethidium bromide.
- Pour liquid agarose on glass plates
- Place the combs and wait until agarose has solidified.
- Mix DNA samples (PCR reactions, restriction digests) with 0.1 volume loading buffer and load on the gel together with a DNA marker
- Separate DNA by electrophoresis (constant current 70 V)
- Visualize DNA under UV-light.
- For preparative electrophoresis excise DNA fragments of interest and purify with GenElute Gel Extraction Kit according to the instructions
- Run another gel for purified fragments to approximate the DNA amount, if used for ligation

## Restriction endonuclease digests

**Materials:**

- PCR product or plasmid DNA 1-5μl
- Reaction buffers (2μl, NEB, type depends on restriction enzymes)
- NEB restriction enzymes (0.5μl)
- (Optional add 1:100 BSA, supplied with NEB enzymes)
- dd$H_2O$, up to 20μl final volume.

**Procedure:**

- Mix all required components (see material)
- Incubate 1h at 37°C, if required incubate additionally at 65°C afterwards
- Analyse and isolate digest products by agarose gel electrophoresis.

## Ligation:

**Material:**

- Purified restriction endonuclease digest products (approx. equimolar concentrations, with compatible ends, 0.5- 2μl)
- 10x T4 Ligase buffer (1.5μl, NEB)
- T4 DNA Ligase (0.5μl, NEB, 102378)
- dd$H_2O$, fill up to 15μl final volume

**Procedure:**

- Thaw T4 Ligase buffer on ice.
- Mix all required components (see material)
- Incubate for 5 min. on ice, then for 16 h at 16°C or for 1h at room temperature
- Transform competent *E. coli* with ligation products.

## Polymerase Chain Reaction (PCR)

**Material:**

- Template DNA (plasmid DNA [10-100 ng/ml, 1 μl], cDNA [1μl])
- 10x PCR reaction buffer (5 μl)
- 50 mM $MgCl_2$ stock solution (1.5 μl)
- Taq-DNA Polymerase (NEB, 102373) or Pwo-DNA Polymerase (Roche, 1164495500; 0.5 μl)
- 10 mM dNTPs (form dNTP set PCR grade, Invitrogen, 10297-018 [2.5 mM of dATP, dCTP, dTTP, dGTP; 1μl])
- Forward primer (10μM, 1μl)
- Reverse primer (10μM, 1μl)
- dd$H_2O$ (fill up to final volume of 50 μl)
- 0.5 ml PCR tubes (Molecular BioProducts, 3430)
- T3 Thermocycler (Biometra, Göppingen)

**Procedure:**

- Thaw all frozen components on ice, except for DNA-Polymerase
- Combine all components on ice, except for the DNA Polymerase in 0.5 ml PCR tubes, volumes are given in material for one PCR reaction)
- Example of a PCR protocol:
  - Heated lid (105°C)
  - 95°C for 5 min, add DNA-Polymerase after 4 min (hot start)
  a) Denaturing: 95°C, 45 s
  b) Annealing: 62-65°C, 40 s
  c) Elongation: 72°C, time dependent on size of the PCR product
  d) Repeat step a)-c) 30 times
  e) 72°C, 10 min.
  f) 4°C
- Prepare PCR samples for agarose gel electrophoresis.

## 7.1.4 Protein methods:

### SDS-PAGE Electrophoresis

**Solutions**

- Ultra pure Accu Gel 29:1 (40%), National Diagnostic, EC852
- Tris-HCl, pH8.8 (1.875 M)
- Tris-HCl, pH 6.8 (1.25M)
- 10% SDS (Sodium Dodecyl Sulfate solution):
- 10% Ammonium Persulfate solution
- 10x Electrode Buffer (Tris-Glycine):
  Glycine (144.2 g), Tris (30.3 g), SDS (10 g). Dissolve in 800 ml $H_2O$.
  1x: Dilute 100 ml of 10xElectrode Buffer with 900 ml distilled water.
- Cell lysis buffer (20 mM Tris/HCl, pH 8.0, 138 mM NaCl, 2.7 mM KCl, 5% glycerol), store at -20°C,
  **prior usage finish lysis buffer by addition of (final conc.):**
- 1% NP 40 or 0.1% Triton-X 100; Protease and phosphatase inhibitors (20 μM Leupetin,18 μM Pepstatin,1 μg/ml Aprotinin, 1 mM PMSF, 20 mM Sodiumfluoride, 1 mM Sodium-ortho-vanadate,1 mM DTT)
- 5x Loading Buffer: 1.25 M Tris-HCl pH 6.8 (2.5 ml), SDS (1g), 2-Mercaptoethanol (2.5 ml) Glycerol 87% (5.8 ml), Bromophenol blue (5 mg), $H_2O$ distilled (35 ml)
- Staining solution: Coomassie Brillant Blue G250 (0,1%), 50%Methanol, 7%Acetic acid, 43% dd$H_2O$ -
- Destaining solution: Methanol (20%), Acetic acid (5%), Distilled water (75%)
- PBS
- Cell lifter (Corning, F21222F)

**Sample preparation:**

**For adherent cells:** place cell culture dishes on ice

- Aspirate medium, wash 2x with cold PBS
- Add 100-200 μl cell lysis buffer and incubate on ice
- Scrape cells from the dishes and transfer cell lysates to eppendorf tubes
- Incubate 15 min. on ice
- Spin (16000g, 10 min., 4°C)
- Transfer supernatant to new eppendorf tubes, measure protein concentration with BioRad Protein Assay (BioRad, 500-0006), store at -20°C or -80°C, or proceed by addition 1/5$^{th}$ of 5x loading buffer. Heat to 95°C for 7 min.
- Store at -20°C until usage for SDS-PAGE

**For BMMC:**

- Collect cells by centrifugation (16000g, 1 min., 4°C).
- Aspirate supernatant, wash with PBS.
- Pellet cells by centrifugation (16000g, 1 min., 4°C).
- Aspirate PBS
- Dissolve mast cells in 2x Loading Buffer at a concentration of 1.0x $10^7$ cells/ml and vortex.
- Heat to 95°C for 7 min., Store at -20°C until usage for SDS-PAGE

**Gel preparation:**

- Clean alumina and glass plates (Hoefer, SE202N-10 and SE202P-10), the spacers and the combs
- Assemble 10 gel units in the multicasting cassette with a plastic plate in between the single units (do not place the combs at this time)
- Prepare the resolving gel mixture (see table next page) and fill the multicasting cassette
- Overlay each gel unit with 1ml isopropanol. Polymerization takes about 30 min.
- Discard the isopropanol, wash gel surface with water and remove residual water with whatman paper
- Prepare the stacking gel solution and overlay the resolving gel with the stacking gel solution. Insert combs.
- After polymerization disassemble the cassette and store each gel in a plastic bag with 1 ml 1xTris, pH 8.8 at 4°C until usage.

**For ten 0.75 mm gels in multicasting cassette**

| | Resolving gel (75 ml) | | | | | Stacking (30 ml) |
|---|---|---|---|---|---|---|
| | 7.5 % | 10 % | 12% | 15 % | 20 % | 5 % |
| Acrylamide Stock - ml | 14.1 | 18.8 | 22.5 | 28.1 | 37.5 | 4.8 |
| Distilled water - ml | 44.9 | 40.2 | 36.5 | 30.9 | 21.5 | 21.6 |
| TRIS-HCl pH 8.8 - ml | 15 | 15 | 15 | 15 | 15 | --- |
| TRIS-HCl pH 6.8 - ml | --- | --- | --- | --- | --- | 3 |
| Degas solution, then add | | | | | | |
| 10%-SDS -µl | 750 | 750 | 750 | 750 | 750 | 300 |
| TEMED -µl | 37.5 | 37.5 | 37.5 | 37.5 | 37.5 | 30 |
| 10% Amm.Persulfate - µl | 250 | 250 | 250 | 250 | 250 | 102 |
| Mix carefully and cast gel | | | | | | |

Parameter settings for one 0.75 mm gel

- Constant current: 20 mA
- Set voltage: 250 V
- Time: approx. 60 min.

## Electrophoretic Transfer / Semi-dry blotting

**Material and solution:**

- Transfer buffer (25 mM Tris, 192 mM Glycine, 20% methanol, pH 8.3)
- Immobilon FL (Millipore, IPFL00010), Immobilon P PVDF (Millipore, IPVH00010) cutted to gel size (9x6.5 cm)
- For one gel 6x whatman paper (9x6.5 cm)
- Semidry blotting device
- Methanol

**Procedure:**

- **NOTE: For infrared imaging with Odyssey use Immobilon FL and always use fresh methanol. Label membrane with a pencil, not with a pen**
- Label and activate membrane in methanol for 1 min.
- Equilibrate in Transfer buffer.
- Mount SDS-PAGE/membrane sandwich.
- Blot proteins to the membrane with constant current of approx. 10 mA per $cm^2$ for 75 min (0.75mm gel) or 120 min. (1.5 mm gel).
- Disassemble sandwich, incubate in blocking buffer for 30 min.
- Add $1^{st}$ antidbody (see antibody table below for their dilution, blocking buffer, time).
- Wash 3x 5 min. with TBS, 0.1%Tween
- Add $2^{nd}$ antibody (HRP or IR-Dye conjugated, see antibody table for details) for 1 hour
- Wash 3x 5 min. with TBS, 0.1%Tween
- Detection: either enhanced chemoluminiscence (ECL) or by infrared detection (Odyssey)

## Immunoprecipitation:

- Described for anti PI3K$\gamma$ 641 here. Might also be used for other antibodies, but eventually modifications are necessary.

**Materials:**

- GammaBind Plus Sepharose (Amersham; 17-0886-02)
- Ascites fluid Mm-$\alpha$-p110$\gamma$ 641 (ASC Eurogentech)
  Corresponds to Alexis JBS-ABD-027 (clone 641, Jena Bioscience)
  www.axxora.com
- lysis buffer, recipe see SDS-PAGE electrophoresis protocol
- Wash buffer 1: lysis buffer (20 mM Tris/HCl, pH 8.0, 138 mM NaCl, 2.7 mM KCl, 5% glycerol) **+ 1% NP40, no protease inhibitors**
- Wash buffer 2: 50mM Tris/Hcl, pH 8.0, 150 mM NaCl, 1% NP40
- 1x PBS
- Optional 0.5 M LiCl solution
- 10- 20 x $10^6$ bone marrow derived mast cells

**Procedure**

**Important: all steps are on ice, in cooled centrifuges or in the cold room**

- **Cell lysis**
  - Collect cells by centrifugation (400g, 5 min), aspirate medium, wash with 1x PBS
  - Collect cells (400g, 5 min) and aspirate PBS,
  - Lyse cells in 2 ml lysis buffer (15 min, on ice, pipet up and down or vortex once in a while).
- **GammaBind Sepahrose preparation**
  - Transfer 50 µl 50% slurry into 3x 2ml Eppendorf tubes
  - Wash with lysis buffer (1ml), pellet beads (1000g, 1 min.)
  - Aspirate lysis buffer.
  - Repeat washing step.
- **Preclearing of the lysate:**
  - Centrifuge cell lysate (4°C, 15 min., max. speed).
  - Take 100-200 µl for usage as total lysates. Add to the 100-200µl 20-40 µl 5x concentrated SDS-sample buffer, heat at 95°C for 7 min., then place on ice (Calculate cell concentration for this; around $1x10^4$/ µl is fine).
  - The remaining cell lysate (1.8-1.9 ml) is transferred to one of the 3 2ml eppendorf tubes, containing the prewashed GammaBind beads.
  - Rotate the lysate with the beads for 30-45 min. in the cold room to pre-absorb unspecific binding proteins.
- **Immunoprecipitation**
  - Spin precleared sample (1000 g, 1 min.). Transfer ½ of the supernatant into each of the 2 other Eppendorf tubes containing pre-washed GammaBind beads.
  - Add to one tube 2µl of ascites fluid Mm-α-p110γ 641
  - Add isogenic control antibody to the second tube (for 6.4.1 it is IgG2a).
  - Rotate samples for 1h in the cold room (4°C).
  - Wash 2x with 1 ml wash buffer 1 and sediment beads (1000 g, 1 min.), aspirate supernatants
  - Wash twice with wash buffer 2 (1000 g, 1 min.) aspirate supernatants
  - (optional wash once with 0.5 M LiCl solution)
  - Wash once with 1x PBS.
  - Add 2x concentrated SDS-sample buffer (adjust volume that you have a final concentration of 0.5x10- $1x10^5$/ µl; include beads volume (50µl) in your calculations). Heat at 95°C for 7 min.

    Samples can then be loaded for SDS-PAGE.

## Purification of bacterial recombinant protein with $(His)_6$ tag

**Materials**

- Handbook "The QIAexpressionist", can be found online: http://www1.qiagen.com/literature/handbooks/PDF/Protein/Expression/QXP_QIAexpressionist/1024473_QXPHB_0603.pdf
- pQE vector expression system
- *E. coli* host strain M15
- $Ni^{2+}$-NTA beads (Qiagen, 30410)
- Solutions see handbook

**Procedure, details see "The QIAexpressionist" and especially the protocols given below:**

- Expression of $(His)_6$-tagged recombinant proteins (derived from plasmids #915 and #1105) were tested for solubility, using protocol 6 (Handbook "The QIAexpressionist")
- Insolubility of the $(His)_6$-tagged recombinant proteins required protocol 10 and 17 (Handbook "The QIAexpressionist") for purification.

## Protein precipitation

**Material**

- Recombinant protein
- Trichloroacetic acid (TCA) 20% in $H_2O$
- Acetone, -20°C

**Procedure:**

- Add an equal volume of 20% TCA to the protein solution
- Keep on ice, 30 min.
- Sediment protein precipitate by centrifugation (5 min, 16000 g, 4°C)
- Wash protein pellet 3x with Acetone
- Dry pellet and resuspend in denaturation buffer

## 7.2 Consumables

Restriction enzymes were purchased from New England Biolabs (not separately listed). Chemicals from Sigma, if not stated different.

| **Product** | **Supplier** | **Cat. No.** |
|---|---|---|
| 2- Mercaptoethanol | Sigma | M 7522 |
| AccuGel 40% | National Diagnostics | EC-852 |
| Adenosine | Sigma | 01890 |
| Agar Technical | DIFCO/BD | 281230 |
| Agarose, standard | Eurobio | 018054 |
| Albumin bovine, serum fraction V | Sigma | A7906 |
| Albumin human Dinitrophenyl (DNP-HSA) | Sigma | A-6661 |
| Ammonium persulfate (APS) | Bio-RAD | 161-0700 |
| Ampicillin trihydrate | Sigma | 10045 |
| Annexin V Cy5 | BD bioscience | 559934 |
| Aprotinin | Fluka | 10820 |
| AS-605240 | Serono | non commercial |
| C3a | Clemens Dahinden | non commercial |
| C5a human recombinant carrier free | R&D systems | 2037-c5/CF |
| Calciumchlorid dihydrate | Merck | 1.02382.0500 |
| Cell Line Nucleofector Kit T | Amaxa | VCA-1002 |
| Cell Line Nucleofector Kit V | Amaxa | VCA-1003 |
| Chloroform | Fluka | 25690 |
| Cytospin filter cards for cytocentrifugation | Thermo | 3424 |
| Cytospin Mikrogefässe 0.75 ml mit 0.5 mm Bodenöffnung | Thermo | 1153 |
| Diisopropyl fluorophosphate | Sigma | D12600-4 |
| Dimethyl sulfoxide | Sigma | 154938 |
| Dithiothreitol | Eurobio | GAUDTT00-71 |
| dNTP set PCR grade | Invitrogen | 10297-018 |
| ECL Western Blotting Detection | Amersham | RPN2106 |
| Endofree plasmid maxi kit | Qiagen | 12362 |
| FITC:rat anti-mouse IgE (R35-72) | Pharmingen | 553415 |
| GammaBind Plus Sepharose | Amersham | 17-0886-01 |
| GenElute Gel Extraction Kit | Sigma | NA1111 |
| Genelute high performance endotoxin-free | Sigma | NA 0410 |
| GenElute Plasmid Miniprep | Sigma | PLN-350 |
| Glucose D(+) Monohydrate | Sigma | 49159 |
| Glutathione Sepharose 4B | Amersham | 17-0756-01 |
| Guanidine Hydrochloride | Fluka | 50590 |
| HEPES | Sigma | H3375 |

| | | |
|---|---|---|
| HOECHST 33342 | Juro supply | H-1399 |
| Hyperfilm ECL | Amersham | RPN3103 |
| IB-MECA | Sigma | I-146 |
| Immobilon Western substrate reagents | Millipore | WBKLS0500 |
| Immobilon-FL | Millipore | IPFL00010 |
| Immobilon-P PVDF membrane | Millipore | IPVH00010 |
| IPTG | Axon Lab | 102124 |
| Iscove's Modified Dulbecco's Medium | Sigma | I3390 |
| Jet PEI transfection reagent | Polyplus Transfection | 101-40 |
| Kanamycin-sulfat | Fluka | 60615 |
| Lambda marker, l HindIII/EcoRI | Labforce | 1695/1 |
| Leupeptin | Alexis | 260-009-M025 |
| L-Glutamine (200mM) | Sigma | G7513 |
| Lithium chloride anhydrous | Fluka | 62476 |
| Luria Broth Base | life technologies | 127-95-027 |
| LY294002 | Calbiochem | 440202 |
| LysoTracker® Red DND-99 | Invitrogen | L7528 |
| Magnesium chloride | Fluka | 63068 |
| Magnesium sulfate | Fluka | 63140 |
| Methyl-beta-cyclodextrin | Sigma | C4555 |
| M-MLV RTase | Invitrogen | 28025-013 |
| Mouse monoclonal anti-DNP IgE | Sigma | D8406 |
| Murine recombinant SCF | Peprotech | 250-03 |
| N,N,N,N'-Tetramethyl-ethylenediamine, TEMED | Bio-Rad | 161-0800 |
| NECA, 5'-n-ethylcarboxamidoadenosine | Sigma | E 2387 |
| N-formyl-Met-Leu-Phe | Sigma | F3506 |
| Ni-NTA Superflow | Qaigen | 30410 |
| Nocodazole | Sigma | M1404 |
| Oligo$(dT)_{15}$ primer | Microsynth | non commercial |
| Optiprep | Axon Lab | 1114542 |
| p85a/p110a complex | Upstate | 14-602 |
| Penicillin-Streptomycin 100x | Sigma | P0781 |
| Pepstatin A | Fluka | 77170 |
| Percoll | Amersham | 17-0891-01 |
| Pertussis Toxin | Sigma | P7208 |
| Phycoerythrin (PE) anti-mouse FcεRIα | eBioscience | 12-5898-83 |
| p-Nitrophenyl N-Acetyl-β-D-Glucosaminide | Sigma | N-9376-1G |
| poly-L-Lysine solution, 0.1% | Sigma | P 8920 |
| Potassium chloride | Fluka | 60128 |
| Propidium Iodide | Sigma | P4170 |

| | | |
|---|---|---|
| Pwo DNA Polymerase | Roche | 11644955001 |
| Rantes CCL5 carrier free, human recombinant | R&D systems | 278-RN/CF |
| rat anti-mouse CD117 (c-kit):PE conjugated | ImmunoKontakt | 212-AB-407V |
| recombinant murine Interleukin-3 | PeproTech | 213-13 |
| recombinant murine Stem Cell Factor | PeproTech | 250-03 |
| Rhodamine phalloidin | Molecular Probes | R415 |
| RNasin | Promega | N2111 |
| SERVA Blue R | Serva | 35051 |
| siStrike U6Hairpin Cloning system (human)-hMGFP | Promega | C3550 |
| Sodium chloride | Fluka | 71376 |
| Sodium dihydrogen phosphate | Fluka | 71500 |
| Sodium dodecyl sulfate | Fluka | 71729 |
| Sodium orthovanadate | Sigma | S6508 |
| Super RX film | Fuji | F57164052 |
| T4 DNA ligase | NEB | 102378 |
| Taq DNA Polymerase | NEB | 102373 |
| Taq DNA Polymerase recombinant | Invitrogen | 10342-020 |
| TGX-221 | Cayman | 10007349 |
| TOPO TA Cloning kit | Invitrogen | K4575-01 |
| Trichloroacetic acid | Fluka | 91230 |
| Tri-Reagent | Sigma | T9424 |
| Triton X-100 | Fluka | 93420 |
| Trizma base | Fluka | 93304 |
| TRIZOL Reagent | Invitrogen | 15596-026 |
| Urea purum p.a., crystallized, ≥99.0% (N) | Fluka | 51461 |
| Vybrant Lipid Raft Labeling Kits (Alexa Fluor 555) | Invitrogen | V-34404 |

## 7.3 Antibodies

| # | Detected Protein | Supplier/ Cat.-No. | Species | Usage, additional information |
|---|---|---|---|---|
| - | Vinculin | Gift of Victor Kotelianski | Rouse, mAb, clone VII-F9 | 1:5000 in 5% skim milk TBST, 1-3 hours RT, overnight 4°C, **WB** |
| ** 99 | mouse IgG | Sigma/ A4416 | Goat | 1:5000 in 5% skim milk TBST, HRPO-conjugated. **WB** |
| - | Tubulin | Gift from Romina Marone | Rat | 1:5, 2nd antibody goat anti rat-$Alexa_{546}$ (obtained from Christofori lab) **IF** |
| 1 | GST | Babco/MMS-112R | Mouse, mAB | **IP, WB** |
| 7 | HA-tag | Lucernachem/ MMs-101R | Mouse, mAb clone HA.11 | 1:2000 in 5% skim milk TBST, for complemented mast cells(MC) (4°C overnight or 1-3 hours, RT); more diluted in other experiments, **WB** |
| 45 | ACTIVE® MAPK | Promega/ V8031 | Rabbit , polyclonal | 1:5000 in 5% BSA/TBST, **WB** |
| 75 | Phospho-Akt (Thr308) (244F9) | Cell signaling technology / #4056 | Rabbit, mAB | 1:500 in 5% BSA TBST for complemented MC, overnight, 4°C, ideally increase MC number to $3x10^5$ per slot. For non-transfected cells 2-3 hours incubation, RT, 1:1000 works. **WB** |
| 78 | Phospho-Akt (Ser473) (193H12) | Cell signaling technology / #4058 | Rabbit, mAB | 1:2000 in 5% BSA TBST for complemented MC, overnight, 4°C, or for 2-3 hours, RT. **WB** |
| 90 | PI3Kγ (Russian) | Eurogentech, gift from R.Wetker | Mouse, mAB ascites | 1:5000 in 5% BSA TBST for complementation experiments, 1 hour RT. In other experiments 1:10000 is possible, too. Now commercially available from Alexis 804-230-L001, clone H1. **WB** |
| 91 | PI3Kγ (641) | now Alexis JBS-ABD-027 (clone 641, JenaBioscience) | Mouse, mAB | 1:500 for **IP** only, no western blotting |
| 115 | (PE) anti-mouse FcεRIα | eBioscience/ 12-5898-83 | Hamster, mAB, MAR-1 | **1: 150 in FACS,** PE-conjugated |
| 119 | p84, p87 | This work/ flask labeled Rabbit #1 Antiserum/Max, p87 N-term, T. Bohnacker | Rabbit total serum | 1:2000 in 5% skim milk TBST (4°C overnight or 1-3 hours, RT). Antisera were raised by Zymed in 2 rabbits (#1, #2). Both detect p84. Used here: flask labeled “Rabbit #1 Antiserum/Max, p87 N-term, T. Bohnacker Lot |

| | | | | |
|---|---|---|---|---|
| | | Lot 50802 | | 50802. Part of the serum was absorbed against human serum. Recombinant protein originate from **plasmid #915** (Met1 to Glu162 of murine p84) |
| 121 | goat IgG | Jackson ImmunoResearch #705-035-147 | Donkey, polyclonal | 1:5000 in 5% skim milk TBST, HRPO-conjugated. **WB** |
| 122 | rabbit IgG | Sigma-Aldrich/A 6154 | Goat, IgG | 1:5000 in 5% skim milk TBST, HRPO-conjugated. **WB** |
| 132 | GFP (Green Fluorescent Protein) | Roche/ #11814460001 | Mouse, mAB | 1:1000 in 5% skim milk TBST for detection of GFP fusion proteins in complemented overnight. **WB** |
| 155 | rabbit IgG | Rockland(Bioconcept)/ 611-132-122 | Goat | 1:5000 in 5% skim milk TBST, IRDye800- conjugated for **quantitative WB** with Odyssey, usage in the dark, 2-3 hours, RT. |
| 212 | mouse IgG | LICOR Biosciences/926-32220 | Goat | 1:5000 in 5% skim milk TBST, IRDye 680- conjugated for **quantitative WB** with Odyssey, usage in the dark, 2-3 hours, RT. |
| 216 | p101 | This work, **tube label** p1105-p101, RB11537, date 070704 | Rabbit, total serum | 1:1000 in 5% skim milk TBST, 1 hour RT, **wash extensively**, **WB**, recombinant protein was expressed from plasmid #1105, thus the antiserum is against Pro575 to Pro880 of human p101 |

# : Inventory ID (Antibody database); ** ID Consumables; WB Western Blot; IF: Immunofluorescence; IP Immunoprecipitation; RT room temperature; TBST 0.1% Tween/TBS

## 7.4 Plasmids

| # | Name | Protein | Origin | Notes |
|---|---|---|---|---|
| 9 | pcDNA3 | - | Invitrogen | www.invitrogen.com, |
| 64 | pSTC-tkGST<pi3kg BamHI/PmeI> | GST-p110γ | Previous work | |
| 147 | pCDM8-β1 | β1 G-protein subunit | Garritsen and Simonds, JBC 1994, 269:24418- | |
| 148 | pCDM8-γ2 | γ2 G-protein subunit | | |
| 378 | pDNA3<p110g wt> | Hsp110γ | Previous work | |
| 530 | eGFP_BTKPH wt | GFP_$PH_{Btk}$ | Obtained from T. Balla | PtdIns(3,4,5)$P_3$ sensor |
| 531 | eGFP_BTKPH R28C | GFP_$PH_{Btk}$ | | PtdIns(3,4,5)$P_3$ binding deficient mutant |
| 537 | pcDNA3 HA<p101> | HA-Ssp101 | Previous work, | |
| 592 | pcDNA3<p110g KR KI> | Hsp110γ(kinase inactive) | Previous work | |
| 594 | pcDNA3<p110g wt-CAAX KI> | Hsp110γ with c-terminal CAAX box | Previous work | Constitutively membrane targeted p110γ, due to c-terminal CAAX box |
| 601 | pEGFP-C1 | GFP | Clontech | www.clontech.com |
| 868 | pcDNA3<p101-YFP> | Scp101-YFP | Obtained from P.Voigt | |
| 884 | pEYFP<Mm_p101β> | YFP-Mmp84 | Previous work, Ronan Calvez | Introduce Mm_p101β coding sequence (witout polyA signal) in 2 pieces:<br>- XhoI/BstEII PCR fragment from TOPO #881 (sequence 100% correct)<br>- BstEII/HindIII fragment from #875 |
| 893 | pcDNA3-HA<Mm_p87> | HA-Mmp84 | Previous work, Ronan Calvez | Originally dubbed p101β or p87; excised from #885 (which is original p101β cDNA deleted of 3'-UTR and poly-A) in 2 fragments BamHI/BstEII (818bp) and BstEII/NotI (1461bp). Inserted into #515 digested BamHI/NotI; contains N-terminal HA-tag. |

| | | | | |
|---|---|---|---|---|
| 912 | pEGFP<Mm_p87> | GFP-Mmp84 | This work | Cloned from pEGFP (#601) and #884 pEYFP<Mm_p87>. Digested XhoI/ Hind III. |
| 915 | pQE<p87Frag2-1> | N-terminus Mmp84 (Met1 to Glu162) | This work | For recombinant protein expression and antisera production, $(His)_6$ tag for purification |
| 999 | pEGFP<Sc_p101> | GFP-Scp101 | This work | Derived from #602, #866. Digestion NdeI/ EcoRI digested. Strategy: replacement of YFP (in #866) by a GFP (isolated from #602) |
| 1000 | peGFP<p110g> | GFP-Hsp110g | This work | Originates from # 862 and # 912. Digestion with BamHI, XhoI. Strategy: Replacement of p87/84 insert by p110$\gamma$ ORF |
| 1105 | pQE30<Hs_p101CTl> | C-terminus Hsp101 (Pro575 to Pro880) | This work | For recombinant protein expression and antiserum production, $(His)_6$ tag for purification |
| 1139 | pTagRFP-N1 | RFP | Evrogen | http://www.evrogen.com/products/vectors/pTagRFP-N/pTagRFP-N.shtml |
| 1189 | pTagRFP<PH-PLCd> | RFP-$PH_{PLCd}$ | This work | Cloned by excision of the PH-BTK domain from plasmid 1140 (EcoRI/ BamHI). Ligation of this backbone with the PH-PLCd ORF obtained from plasmid 528 (EcoRI/ BamHI). |

## *Abbreviations used in introduction and discussion:*

| | |
|---|---|
| Ag | Antigen |
| Arg | Arginine |
| ATP | Adenosine Trisphosphate |
| Bam32 | B cell adaptor molecule of 32 kD |
| BCAP | B cell activating protein |
| BCR | B cell receptor |
| BH | Breackpoint homology |
| bLT1 | Leukotriene B receptor |
| BMMC | Bone marrow derived mast cell |
| Bmx | Tec familiy tyrosine kinase |
| Btk | Bruton's tyrosine kinase |
| C5a | Complement factor 5a |
| $Ca^{2+}$ | Calcium ions |
| cAMP | cyclic Adenosine monophosphate |
| Cbl | E3 ubiquitin ligase |
| c-Kit | CD117 cytokine receptor |
| CR | Complement Receptor |
| CSF-1R | Colony stimulatingfactor-1 receptor |
| Cys | Cystein |
| CysLT | Cysteinyl leukotrienes (receptor) |
| DAG | Diacylglycerol |
| DC | Dentritic cell |
| DH | Dbl homology |
| DNA-PK | DNA-activated protein kinase |
| EAE | Experimental autoimmune encephalomyelitis |
| ECM | Extracellular matrix |
| EGFR | Epidermal growth factor receptor |
| FceRI | High affinity receptor for IgE |
| fMLP | Formyl-Methionyl-Leucyl-Phenylalanine |
| Fyn, Lyn, Lck | Src familiy kinases |
| FYVE | Zinc finger domain as present in Fab1, YOTB/ZK632.12, Vac1, and EEA1 |
| g | Gram |
| Gab | Grb2 associted binding protein |
| Gads | Grb2 related adapter protein |
| GAP | GTPase activating protein |
| Gbg | $\beta\gamma$ subunits of heterotrimeric G-proteins |
| GDP | Guanosine bisphosphate |
| GEF | Guanine- nucleotide exchage factor |
| GMCSF, GM-CSF | Granulocyte- macrophage colony stimulating factor |
| GPCR | G-protein coupled receptor |
| Grb2 | Growth factor receptor-bound protein 2 |
| GTP | Guanosine Trisphosphate |
| HGFR | Hepatocyte growth factor receptor |
| HIV | Human immunodeficiency virus |
| ICAM | Intercellular adhesion molecule |
| IgE | Immunglobulin E |
| IGFR | Insulin like growth factor receptor |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| IL | Interleukin |
| Inf | Interferon |
| $IP_{3,}$ | Soluble inositol (1,4,5)$P_3$ |
| IR | Insulin receptor |

| | |
|---|---|
| IRS | Insulin receptor substrate |
| ITAM | Immunreceptor based activation motif |
| ITIM | Immunreceptor based inhibition motif |
| Itk | IL2-inducible T-cell kinase |
| JAK | Janus kinase |
| kDa | Kilodalton |
| kg | Kilogram |
| l | Liter |
| LAT | Linker of activated T cells |
| LPA | Lyophosphatidic acid |
| LPS | Lipopolysaccharide |
| LTB, LTC | Leukotriene B, C |
| Lys | Lysine |
| MAPK | Mitogen activated protein kinase |
| MCP1 | Monocyte chemotactic protein-1 |
| mg | Milligram |
| MHC | Major histocompatibility complex |
| ml | Milliliter |
| MS | Multiplesclerosis |
| mTOR | Mammalian Target of Rapamycin (TOR) |
| ng | Nanogram |
| NGFR | Nerve growth factor receptor |
| NK | Natural killer |
| NRTK | Non receptor tyrosine kinase |
| NTAL | Non-T-cell activation linker |
| PCA | Passive cutaneous anaphylaxis |
| PDGF-R | Plateled derived growth factor receptor |
| PDK1 | 3-Phosphoinositide-dependent kinase 1 |
| PDZ | Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1) |
| PGD, PGE | Prostaglandin D, E |
| PH | Pleckstrin homology |
| PI | Phosphoinositide |
| PI3K | Phosphatidylinositol 3-Kinase |
| PKA | Protein kinase A (cAMP dependent) |
| PKB/Akt | Protein kinase B, V-akt murine thymoma viral oncogene homolog 1 |
| PKC | Protein kinase C |
| PKG | Protein kinase G (cGMP dependent) |
| PLC | Phospholipase |
| PSA | Passive systemic anaphylaxis |
| PtdIns | Phosphoinositide |
| PTEN | Phosphatase and tensin homolog on chromosome 10 |
| PTK | Proteintyrosine kinase |
| PX | Phox homology |
| pY | Phosphorylated tyroisne |
| Rab | GTPase |
| Ras | GTPase |
| RBD | Ras binding domain |
| Rho, Rac, Cdc42 | Small GTP of the Rho- family |
| ROS | Reactive oxygen species |
| RTK | Receptor tyrosin kinase |
| SCF | Stem cell factor |
| SGK | Serum and glucocorticoid-inducible kinase |
| SH2 | Src homology 2 |
| SH3 | Src homology 3 |
| Shc | Src homology 2 domain-containing adaptor protein |
| SHIP | SH2 Domain-containing Inositol 5-Phosphatase |
| Shp | SH2 domain containing proteinphosphatase |

| | |
|---|---|
| SLP76 | SH2 domain containing leukocyte protein (76kDa) |
| Sos | Son of sevenless |
| Src | v-src homologous protein tyrosine kinase |
| Syk | Spleen tyrosine kinase |
| Tapp | Tandem-pleckstrin-homology-domain-containing protein |
| TCR | T cell recepotr |
| Tec | Tyrosine kinase expressed in hepatocarcinoma |
| TGFβ | Transforming growth factor |
| Th | T helper |
| TLR | Toll-like receptor |
| TRIM | TCR-interacting molecule |
| Vav | GEF of the Rho family members |
| VCAM | Vascular cell adhesion molecule |
| VEGF-R | Vascular endothelial growth factor receptor |
| Vps | Vacuolar protein sorting |
| YxxM | Tyrosine-Xxx-Xxx-Met motif |
| ZAP70 | Zeta-chain-associated protein kinase of 70 kDa |
| μg | microgram |
| μl | Microliter |

# Curriculum Vitae

**Personalia**

| | |
|---|---|
| **Name:** | Thomas Bohnacker |
| **Date of Birth:** | 08.11.1978 |
| **Place of Birth:** | Heilbronn |
| **Nationality:** | german |

**Current position:**

| | |
|---|---|
| 26.06.09 | Doctoral examination, Faculty of Science, University of Basel.<br>Examinators: Prof. Dr. M.P. Wymann (Uni Basel), Prof. Dr. M. Huber (RWTH Aachen), Chair: Prof. Dr. C. Arrieumerlou |
| Since 09.2004 | PhD student position at the Institute of Biochemistry and Genetics, Department Biomedicine, University of Basel, Switzerland.<br>Supervisor Prof. Dr. M.P. Wymann<br>Project: " PI3K$\gamma$ adapter subunits in mast cells" |

**Education:**

| | |
|---|---|
| University:<br>10.1998-07.2004 | Studies "Technische Biologie", University of Stuttgart<br>Graduation: Dipl. Biologe (t.o.), Mit Auszeichnung bestanden |
| | Diploma Thesis, Institute of Biochemistry, with Prof. Dr. D.H. Wolf:<br>"Viral Gly-Ala repeats, as tools for ER- associated degradation research". |
| | Student research project at the Australian National University, Canberra, with Dr. R.T. Baker:<br>"The Deubiquitinating Enzyme USP2- Cellular Localization an Protein Interactions" |
| Civillian service<br>08.1997- 09.1998 | Klinik Löwenstein, Intensive Care |
| Grammar school<br>08.1988- 07.1997 | Justinus Kerner Gymnasium, Weinsberg, Germany<br>Graduation: General qualification for university entrance (Abitur) |

**Courses:**

27.02.06- 03.03.06 Animal Experimentation Course, Module 1, Institut für Labortierkunde (Uni Zürich)
19.02.07- 21.02.07 FACS application training, BD Biosciences, Heidelberg

**Scientific Communications:**

1. Thomas Bohnacker. Romina Marone, Ronan Calvez, Philipp Voigt, Michael Schäfer, Matthias P Wymann (2005), "p87$^{PIKAP}$, a novel Phosphoinositide 3-Kinase γ (PI3Kγ) adaptor protein" Main student retreat, Gwatt, April 30- May, **oral and poster presentation**
2. Thomas Bohnacker (2005), "A novel regulatory subunit of Phosphoinositide 3-Kinase γ in allergy, DKBW monthly group meeting, October 4, **oral presentation**
3. Thomas Bohnacker (2006), "p84/p87$^{PIKAP}$, a PI3Kγ regulatory subunit in mast cell hyperreactivity". 40th Annual Scientific Meeting of the European Society for Clinical Investigation, Prague, March 15-18, **oral presentation**.
4. Thomas Bohnacker (2006), "Distinct role of p84/p87$^{PIKAP}$, a Phosphoinositide 3-Kinase gamma regulatory subunit, modulating allergy". Biochemical Society Annual Symposium - The Cell Biology of Inositol Lipids and Phosphates, Birmingham, March 29-31, **poster presentation**
5. Thomas Bohnacker, "Distinct role of p84/p87$^{PIKAP}$, a Phosphoinositide 3- Kinase gamma regulatory subunit, modulating allergy" Main student retreat, Gwatt, May 13-16, **poster presentation**
6. Thomas Bohnacker (2006), "PI$P_3$ not equal PI$P_3$ – adapter-specific signaling of PI3Kγ", Targeting the kinome, Basel, 4-6 December 2006, **poster presentation**
7. Thomas Bohnacker (2007), "PI$P_3$ not equal PI$P_3$ – adapter-specific signaling of PI3Kγ", USGEB, Basel, 13-14 March, **poster presentation**
8. Thomas Bohnacker (2007), "PI$P_3$ not equal PI$P_3$ – adapter-specific signaling of PI3Kγ", DKBW monthly group meeting, May 15, **oral presentation**
9. Thomas Bohnacker (2007), "Adaptor-specific signalling of PI3Kγ - to novel strategies targeting allergy?" BioValley, Basel, 23.10.2007, **poster presentation**
10. Thomas Bohnacker (2008), "Adaptor-specific signalling of PI3Kγ - to novel strategies targeting allergy?" 42nd Annual Scientific Meeting of the European Society for Clinical Investigation, Geneva, March 26-29, **oral presentation.**
11. Thomas Bohnacker (2008), "PI3K signaling in allergy", Monthly CBM research group presentations, Basel, 3$^{rd}$ June, **oral presentation**

**Peer-reviewed publications:**

1. **Targeting Melanoma with Dual Phosphoinositide 3-Kinase/Mammalian Target of Rapamycin Inhibitor (Mol Cancer Res April 2009 7:601-613)**
   Romina Marone, Dominik Erhart, Ann C. Mertz, Thomas Bohnacker, Christian Schnell, Vladimir Cmiljanovic, Frédéric Stauffer, Carlos Garcia-Echeverria, Bernd Giese, Sauveur-Michel Maira, and Matthias P. Wymann

2. **PI3K$\gamma$ Adaptor Subunits Define Coupling to Degranulation and Cell Motility by Distinct PtdIns(3,4,5)P3 Pools in Mast Cells (Science Signaling, in press)**
   Thomas Bohnacker, Romina Marone, Emilie Collmann, Ronan Calvez, Emilio Hirsch, Matthias P. Wymann

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