

REVIEW

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G_{12/13} signaling in asthma

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Abstract

Shortening of airway smooth muscle and bronchoconstriction are pathognomonic for asthma. Airway shortening occurs through calcium-dependent activation of myosin light chain kinase, and RhoA-dependent calcium sensitization, which inhibits myosin light chain phosphatase. The mechanism through which pro-contractile stimuli activate calcium sensitization is poorly understood. Our review of the literature suggests that pro-contractile G protein coupled receptors likely signal through G_{12/13} to activate RhoA and mediate calcium sensitization. This hypothesis is consistent with the effects of pro-contractile agonists on RhoA and Rho kinase activation, actin polymerization and myosin light chain phosphorylation. Recognizing the likely role of G_{12/13} signaling in the pathophysiology of asthma rationalizes the effects of pro-contractile stimuli on airway hyperresponsiveness, immune activation and airway remodeling, and suggests new approaches for asthma treatment.

Keywords Airway hyperresponsiveness, Airway remodeling, Anticholinergic agents, Asthma, Bronchoconstriction, Calcium sensitization, G_{12/13}, Inflammation, Muscarinic 3 acetylcholine receptor, RhoA

Background

G Protein Coupled Receptors (GPCRs) comprise the largest family of cell surface receptors in the human genome (>800 members) [1–3] and coordinate physiological responses to everything from photons to proteins. In contrast to this diversity of receptors and ligands, the heterotrimeric G proteins that mediate intracellular signaling downstream of GPCR activation seem deceptively simple: sixteen G α subunits that fall into four mechanistic subfamilies (G_s, G_{q/11}, G_{i/o} and G_{12/13}) and five G β subunits that form obligate heterodimers with one of twelve G γ subunits [4]. GPCR signaling is typically described as resulting from specific coupling between a GPCR and a particular G protein, but most GPCRs couple to multiple G proteins [5]. The structural determinants of G protein

selection by GPCRs are not well understood [6, 7]. Differential G protein recruitment (G protein bias) is often governed by ligand binding [8, 9] with signaling outcomes determined by the ensemble of G proteins that are activated. Elucidating the structural and mechanistic basis of G protein bias and using it as a tool for improving drug properties is the focus of considerable research [10–12].

Ligand binding to GPCRs promotes conformational changes that catalyze guanine nucleotide exchange within the α subunits of associated heterotrimeric G proteins [13]. GTP binding dissociates G protein heterotrimers, revealing protein interaction sites that mediate downstream signaling [14]. Although both G α and G $\beta\gamma$ complexes contribute to signaling downstream of GPCR activation, signaling pathways are typically categorized by their G α subunit. Activation of G_s stimulates adenylyl cyclase, which increases the cytosolic concentration of cyclic AMP (cAMP) thereby activating protein kinase A (PKA), while activation of G_{i/o} inhibits adenylyl cyclase [15]. Activation of G_{q/11} stimulates phospholipase C β (PLC β), which generates inositol trisphosphate (IP3) and

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diacylglycerol (DAG), thereby promoting intracellular calcium flux and activation of protein kinase C (PKC) [16].

In 1991, the $G_{12/13}$ subfamily was identified as the fourth class of $G\alpha$ subunits. This subfamily consists of G_{12} and G_{13} subunits, which share 67% amino acid sequence homology [17]. Unlike other $G\alpha$ protein subfamilies, activated G_{12} and G_{13} do not regulate enzymes that produce small molecule second messengers such as cAMP, IP₃ or Ca^{2+} . Instead, GTP-bound $G_{12/13}$ subfamily members bind to and regulate the Ras homology (Rho) family of guanine nucleotide exchange factors (RhoGEFs) [18, 19]. RhoGEF complexes activate small Rho GTPases, such as RhoA, which play critical roles in regulating cytoskeletal dynamics [20]. Rho GTPases also stimulate numerous downstream signaling pathways through activation of Rho kinase (ROCK), Lim kinase (LIMK) and c-Jun NH₂-terminal kinase (JNK; see Fig. 1) [21]. Deactivation of $G_{12/13}$ occurs through hydrolysis of GTP to GDP via their intrinsic GTPase activity, which can be accelerated by GTPase-activating proteins (GAPs) including regulators of G protein signaling (RGS) domains of RhoGEF family members [22]. $G_{12/13}$ couple

to more than 30 GPCRs, including angiotensin II receptors, cysteinyl leukotriene receptors (CysLTR), histamine (H) receptors, lysophosphatidic acid (LPA) receptors, protease-activated receptors (PAR), sphingosine-1-phosphate (S1P) receptors, and thromboxane A₂ receptors [5].

Asthma is an obstructive airway disease characterized by airway hyperresponsiveness (AHR), inflammation and airway remodeling (AR). GPCRs play critical roles in regulating bronchomotor tone, and, as such, are the targets of numerous therapeutics that are used to treat asthma and other obstructive pulmonary diseases. GPCRs coupled to G_s , such as the β_2 adrenergic receptor (β_2 AR), regulate relaxation of airway smooth muscle, while GPCRs coupled to $G_{q/11}$, such as histamine, leukotriene and muscarinic acetylcholine receptors, promote airway constriction. Notably, $G_{12/13}$ couples to many $G_{q/11}$ coupled receptors in vascular and airway smooth muscle [5], and the RhoA signaling pathway downstream of $G_{12/13}$ activation potentiates smooth muscle contraction [21]. Inflammation plays a fundamental role in the etiology of asthma, thus biologics and corticosteroids are mainstays of therapy. $G_{12/13}$ dependent signaling plays an

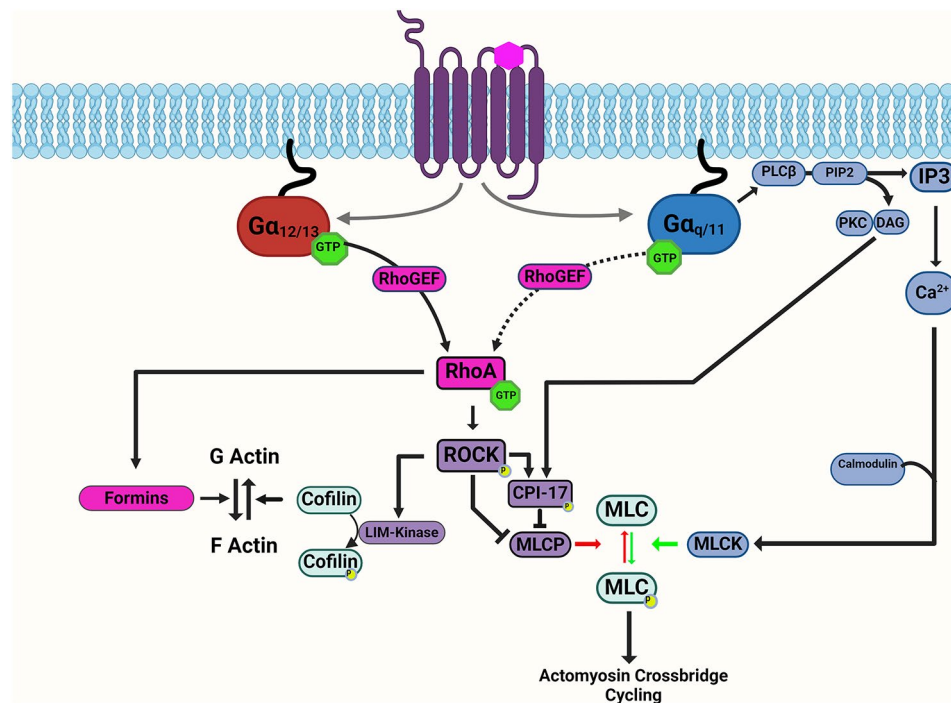


Fig. 1 G protein signaling in smooth muscle contraction. Two G protein signaling pathways contribute to airway smooth muscle contraction. Activation of $G_{q/11}$ upon receptor-dependent guanine nucleotide exchange stimulates the calcium-dependent contractile pathway, whereby GTP-bound $G_{q/11}$ allosterically activates PLC β -dependent hydrolysis of phosphoinositide bisphosphate (PIP₂) into IP₃ and DAG, which promote intracellular calcium flux, thereby activating MLCK-dependent phosphorylation of myosin light chain and actomyosin cross-bridge cycling (blue nodes on right side of figure). Calcium sensitization (fuchsia and violet nodes in the center and left of the figure) is mediated by GTP-bound RhoA, which can be generated downstream of activation of either $G_{q/11}$ or $G_{12/13}$, although $G_{12/13}$ are more potent activators [29]. PKC phosphorylation of CPI-17 promotes inhibition of myosin light chain phosphatase, which increases net MLC phosphorylation. GTP-bound RhoA stimulates actin polymerization through activation of formins (fuchsia nodes) and ROCK activity (violet nodes), which inhibit both filament severing and MLCP. ROCK also phosphorylates CPI-17, which further inhibits MLCP. (Created with BioRender.com)

important role in lymphocyte activation and migration. Finally, airway thickening and angiogenesis are stimulated in patients with asthma. Increased expression of $G_{12/13}$ has been reported in animal models of asthma [23], numerous proliferative pathways (mitogen-activated protein kinase (MAPK) [24], Hippo [25], non-receptor tyrosine kinases [26]) are downstream of $G_{12/13}$ activation, and G_{13} has been implicated in both airway smooth muscle (ASM) proliferation and angiogenesis in a variety of physiological and pathological settings, including development and cancer [27, 28].

As the most potent upstream modulator of RhoA [29], $G_{12/13}$ and its downstream signaling pathway constitutes an intriguing target with the potential to impact myriad aspects of the pathophysiology of asthma by inhibiting AHR, inflammation and AR. Since there are limited publications on $G_{12/13}$ signaling in ASM and asthma, each section of this review summarizes relevant findings from non-muscle tissues first and concludes with smooth muscle or ASM data where available. The preponderance of the evidence suggests that targeting pathways that activate the $G_{12/13}$ subfamily and its downstream signaling axis may provide a complementary therapeutic approach for managing asthma and other obstructive pulmonary diseases.

G protein stimulation of myosin light chain phosphorylation regulates smooth muscle contraction and airway hyperresponsiveness

Airway smooth muscle shortening induces narrowing of airways, evoking wheezing, difficulty breathing and chest tightness, which, when coupled with mucus buildup and airway inflammation, is characteristic of asthma [30]. Bronchoconstriction is thought to be mediated by agonist binding to $G_{q/11}$ coupled receptors. The guanine exchange activity of agonist-bound GPCRs enables GTP-bound $G_{q/11}$ to allosterically activate PLC β -dependent production of IP₃ and DAG, thereby stimulating IP₃ receptor-dependent Ca^{2+} release from the sarcoplasmic reticulum and PKC-dependent activation of Ca^{2+} channels, which cooperate to increase the concentration of calcium in the cytosol (blue nodes in Fig. 1). Cytosolic Ca^{2+} binding to calmodulin evokes allosteric activation of myosin light chain kinase (MLCK), which phosphorylates the 20 kDa light chain of myosin to promote actomyosin cross-bridge cycling and muscle contraction [31, 32]. Calcium-bound calmodulin in smooth muscle also activates calmodulin-dependent kinase II, which cooperates with PKC to relieve tonic inhibition of the interaction between myosin and actin by calponin [33]. Interestingly, however, the $G_{q/11}$ decapeptide inhibitor FR900359 (FR) failed to inhibit carbachol-dependent contraction of mouse precision-cut lung slices at 30 nM concentration [34]. A higher concentration of FR (1 μ M)

only partially inhibited carbachol-dependent contraction of human precision-cut lung slices [35] suggesting that actomyosin cross-bridge cycling still occurs even when $G_{q/11}$ -stimulated calcium flux is fully inhibited. If $G_{q/11}$ -dependent Ca^{2+} flux is insufficient for actomyosin cross-bridge cycling, what regulates the effects of acetylcholine on smooth muscle tone?

$G_{12/13}$ activates the RhoA/ROCK signaling pathway to regulate actin polymerization and inhibit MLCP

The interaction between myosin and actin in smooth muscle is regulated through phosphorylation of myosin light chain, and by maintaining a large pool of depolymerized actin [36]. Both myosin light chain phosphorylation and actin polymerization are necessary for smooth muscle contraction (Fig. 1) [37, 38].

The actin cytoskeleton is a highly dynamic structure that is regulated by polymerization and depolymerization processes interconverting globular (G) and filamentous (F) actin. This mechanism is particularly true in smooth muscle, where the ratio of F actin to G actin is three to four times less than what is observed in cardiac and skeletal muscle [36]. The Rho family of small GTPases -- Cdc42, Rac1, and RhoA -- regulate a diverse array of cytoskeletal dynamics [39, 40]. GTP-bound RhoA activates formins, which stimulate actin polymerization (fuchsia nodes in Fig. 1) [37]. Since each myosin head interacts with two actin monomers in F actin, polymerization of actin is essential for actinomyosin complex assembly and muscle contraction [41]. Microinjection of activated G_{12} or G_{13} into Swiss-3T3 cells is sufficient to induce actin polymerization in a RhoA-dependent manner [42].

GTP-bound RhoA also activates ROCK, which phosphorylates the myosin targeting subunit (MYPT1) of myosin light chain phosphatase (MLCP). MYPT1 phosphorylation inhibits MLCP binding to myosin light chain (MLC), thereby increasing net MLC phosphorylation (this is referred to as calcium sensitization – see violet nodes in Fig. 1) [43]. ROCK therefore acts synergistically with MLCK to prolong and strengthen muscle contraction [21]. Both ROCK and PKC phosphorylate and activate CPI-17, a smooth muscle specific MLCP inhibitor, which enhances the pro-contractile effect [44]. Polymerized actin is further stabilized through inhibitory phosphorylation of cofilin by LIMK [45], which is activated upon phosphorylation by ROCK. Cofilin phosphorylation impairs binding to F actin [46] thereby inhibiting filament severing and actin depolymerization [47, 48]. In tracheal smooth muscle, acetylcholine-induced stiffness is independent of MLC phosphorylation and calcium flux, but dependent on ROCK [49]. ROCK inhibition blocks contraction of airway smooth muscle both in vitro and in vivo [50–52].

Small GTPases of the Ras superfamily, such as RhoA, must be activated by guanine nucleotide exchange factors to stimulate downstream signaling. RhoGEFs can be activated by either GTP-bound $G_{q/11}$ or $G_{12/13}$, but GTP-bound $G_{12/13}$ are the more potent activators [29]. RhoA and other Ras family members are also regulated by guanine nucleotide dissociation inhibitors (GDIs). GDIs prevent small GTPases from participating in nucleotide exchange, holding them in an inactive state and in some cases blocking them from trafficking to the membrane [53, 54]. In vascular smooth muscle, constitutively activated G_{12} or G_{13} promote vasoconstriction, which can be inhibited by botulinum C3 toxin or ROCK inhibition, while dominant-negative forms of G_{12} or G_{13} inhibit vasoconstriction [55]. Similarly, conditional knockdown of $G_{12/13}$ or leukemia-associated RhoGEF (LARG) normalized age-related hypertension in mice [56], and blocked salt-induced hypertension [57], while loss of either $G_{12/13}$ or the smooth muscle specific RhoGEF, ARHGEF12, in small arteries induced loss of RhoA activation and vasodilation [58]. In a rat model, chronic administration of angiotensin II resulted in increased expression of G_{12} and elevated blood pressure, while co-administration of GNA12 antisense lowered mean blood pressure [59].

$G_{12/13}$ couples to several pro-contractile GPCRs in airway smooth muscle cells (H1, CysLTR2, PAR 1 & 2) [5], and G_{12} and G_{13} are overexpressed in AHR rats compared to healthy animals [23]. Controversy exists in the optical biosensor literature concerning whether muscarinic 3 acetylcholine receptors ($M3_R$) interact with $G_{12/13}$ [5, 8, 34], but functional assays suggest interactions between $M3_R$ and $G_{12/13}$ in both human embryonic kidney (HEK293) and human airway smooth muscle (HASM) cells. $M3_R$ -dependent activation of G_{12} in HEK293 cells stimulates phospholipase D activity [60, 61]. Knockdown of GNA13 in primary bronchial smooth muscle cells blocked methacholine-induced phosphorylation of myosin light chain [62]. In HASM cells, interaction between $M3_R$ and G_{12} was confirmed by co-immunoprecipitation, siRNA knockdown of GNA12 reduced phosphorylation of MYPT1 and MLC, expression of a G_{12} inhibitor (p115RhoGEF-RGS) suppressed carbachol-mediated HASM cell contraction, and RhoA inhibition induced dilation of human precision cut lung slices [63]. Collectively, these data suggest that $G_{12/13}$ couples to most therapeutically relevant pro-contractile GPCRs in both HEK293 and HASM cells.

Levels of TGF- β 1, a cytokine that regulates extracellular matrix formation, cell growth, inflammation, and the epithelial-mesenchymal transition (EMT) are increased in airways of patients with severe asthma [64]. Published research suggests that $G_{12/13}$ regulates TGF- β 1 expression, although the specifics of the regulatory mechanism are unclear. In HASM cells, pretreatment with TGF- β 1

increases RhoA translocation via p115RhoGEF. In $G_{12/13}$ deficient cells, TGF- β 1 expression was restored after transfection with constitutively active $G_{12/13}$ QL mutants, suggesting that $G_{12/13}$ play a role in modulating TGF- β 1 expression levels [65]. TGF- β 1 increases bronchomotor tone and enhances carbachol- and histamine-induced excitation-contraction coupling in human airway smooth muscle and precision cut lung slices, respectively. In isolated HASM cells, methacholine-induced cytoskeletal stiffness was increased after TGF- β 1 pre-treatment in a dose-dependent manner. Inhibition of ROCK attenuated TGF- β 1 induced single-cell contraction, while having no impact on intracellular calcium release [64]. These data indicate that TGF- β 1-mediated physiological effects in HASM occur in a $G_{12/13}$ -dependent manner.

In summary, smooth muscle contraction is regulated by $G_{q/11}$ dependent activation of MLCK, which is enhanced by ROCK dependent inhibition of MLCP, and by RhoA-dependent actin polymerization, which is stimulated more robustly by activated $G_{12/13}$ than by activated $G_{q/11}$. The pro-contractile and AHR-inducing effects of TGF- β 1 also appear to be regulated by $G_{12/13}$ signaling. The complex interplay between the $G_{q/11}$ and $G_{12/13}$ signaling pathways leads to impaired muscle shortening when RhoA/ROCK activation is compromised, and in elevated resting tone unless both pathways to RhoA/ROCK activation are inhibited.

$G_{12/13}$ signaling regulates immune activation and infiltration

Asthma is a chronic disease mediated by immune infiltration in which both symptoms (e.g., mucus production) and pathology (AHR, AR) are driven by inflammatory processes [66]. Asthma patients display heterogeneous clinical phenotypes, in part due to the spectrum of immune mechanisms that are dysregulated in patients with asthma. In approximately half of all patients, asthma is characterized by type 2 immune response and sensitization to allergens [67]. Immune responses in these patients result from production of IgE and Th2-associated cytokines (IL4, IL5, IL13), which stimulate dendritic and mast cell activation, eosinophil invasion and mucus production [68]. As such, these patients are often responsive to inhaled corticosteroids and biologics that antagonize Th2-associated cytokine signaling. In contrast, patients with minimal production of Th2-associated cytokines are often poorly responsive to inhaled corticosteroids. Inflammation in these patients is driven by macrophages, neutrophils and cytokines like TGF- β , IL-1 β and IL-6 [69].

G_{12} and G_{13} have been implicated in various aspects of immune cell activation and trafficking (Fig. 2). S1P is released by mast cells in response to IgE stimulation. S1P receptors on mature dendritic cells coordinate expression

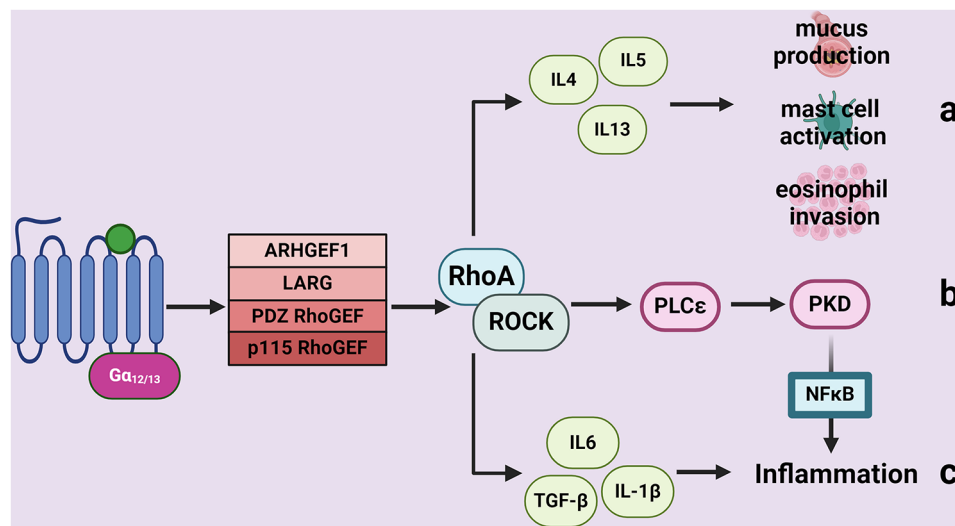


Fig. 2 $G_{12/13}$ signaling in inflammation. $G_{12/13}$ -dependent activation of RhoA and ROCK promotes inflammation in both Th2-high (a) and Th2-low asthma (c). $G_{12/13}$ can also stimulate NF κ B-dependent production of inflammatory cytokines (b). (Created with BioRender.com)

of Th2 cytokines in a $G_{12/13}$ -dependent manner (Fig. 2a) [70, 71], and both S1P concentration and GNA12 expression predict asthma control [72]. In the central nervous system, S1P activates receptors on astrocytes that signal via $G_{12/13}$, RhoA, phospholipase C-epsilon and protein kinase D to stimulate NF κ B dependent transcription of inflammatory genes (Fig. 2b) [73]. Biased inhibition of $G_{12/13}$ signaling downstream of PAR2 activation blocks inflammation in vivo [12]. Diminished follicular helper T (Tfh) response was observed in a T-cell specific $G_{\alpha_{13}}$ deficient mouse model, and G_{13} knock-out Tfh cells showed an inability to transduce signal through RhoA mediated ROCK activation, highlighting the regulatory role of the G_{13} -Rho-Rock signaling axis in Tfh cell differentiation [74].

The expression level of activating protein 1 (AP-1), a transcription factor that is necessary for the proliferation and differentiation of Th2 cells, was reduced in $G_{12/13}$ knockdown cells [62]. Myeloid-specific knockdown of $G_{12/13}$ results in increased expression of anti-inflammatory genes in macrophages, which protect against atherosclerosis in wild-type mice [75]. GNA12 regulates C5a-mediated migration in macrophages, indicating an anti-inflammatory role for GNA12 in inflammatory bowel disease [76, 77]. Loss of RGS-containing RhoGEFs such as ARHGEF1, LARG, PRG or p115 that activate RhoA and ROCK downstream of $G_{12/13}$ compromise immune function (Fig. 2c) [78, 79]. G_{13} knockdown in microglial cells inhibits LPS-induced activation and Rac-dependent migration [80]. In platelets and leukocytes, outside-in signaling between integrins and G_{13} regulates Rho-dependent, pro-inflammatory secretion and thrombosis [81–84]. $G_{12/13}$ are also implicated in cytokine

signaling and inflammation in prostate cancer [85] and mediate renal ischemia-reperfusion injury [86–88].

Published evidence suggests that $G_{12/13}$ and downstream RhoA/ROCK signaling mediate inflammation in diverse physiological and pathological settings. Inhibiting this pathway may cooperate, or even synergize, with biologics and inhaled corticosteroids in Th2 high asthma, and provide a novel approach to suppress inflammation in patients who are refractory to corticosteroid therapy.

$G_{12/13}$ regulation of airway remodeling and hyperplasia

AR is a hallmark of the pathophysiology of asthma [89]. In airways, hyperproliferation evokes thickening of both smooth muscle and the extracellular matrix, which narrows bronchi and predicts poor patient outcomes [90]. HASM cells from patients with asthma proliferate faster than ASM cells from control patients [91, 92]. Activation of $G_{q/11}$ -coupled receptors stimulates MAPK, which promotes proliferation of ASM and contributes to hyperplasia, angiogenesis and tissue remodeling in a wide variety of contexts [93]. Proliferation downstream of the $G_{12/13}$ -RhoA signaling axis is regulated by kinases, proliferative transcription factors, and alterations in gene expression (Fig. 3).

G_{12} was the first $G\alpha$ subunit that was shown to be capable of oncogenic transformation upon overexpression [94, 95]. Introduction of mutations that inhibit the intrinsic GTPase activity of G_{12} (Q229L) resulted in focus forming activity in NIH-3T3 cells that was similar to the most potent viral oncogenes [95, 96]. Similarly, overexpression of G_{13} is sufficient to transform NIH-3T3 fibroblasts and to promote xenograft growth in nude mice, and the GTPase deficient mutant (Q226L) is a potent

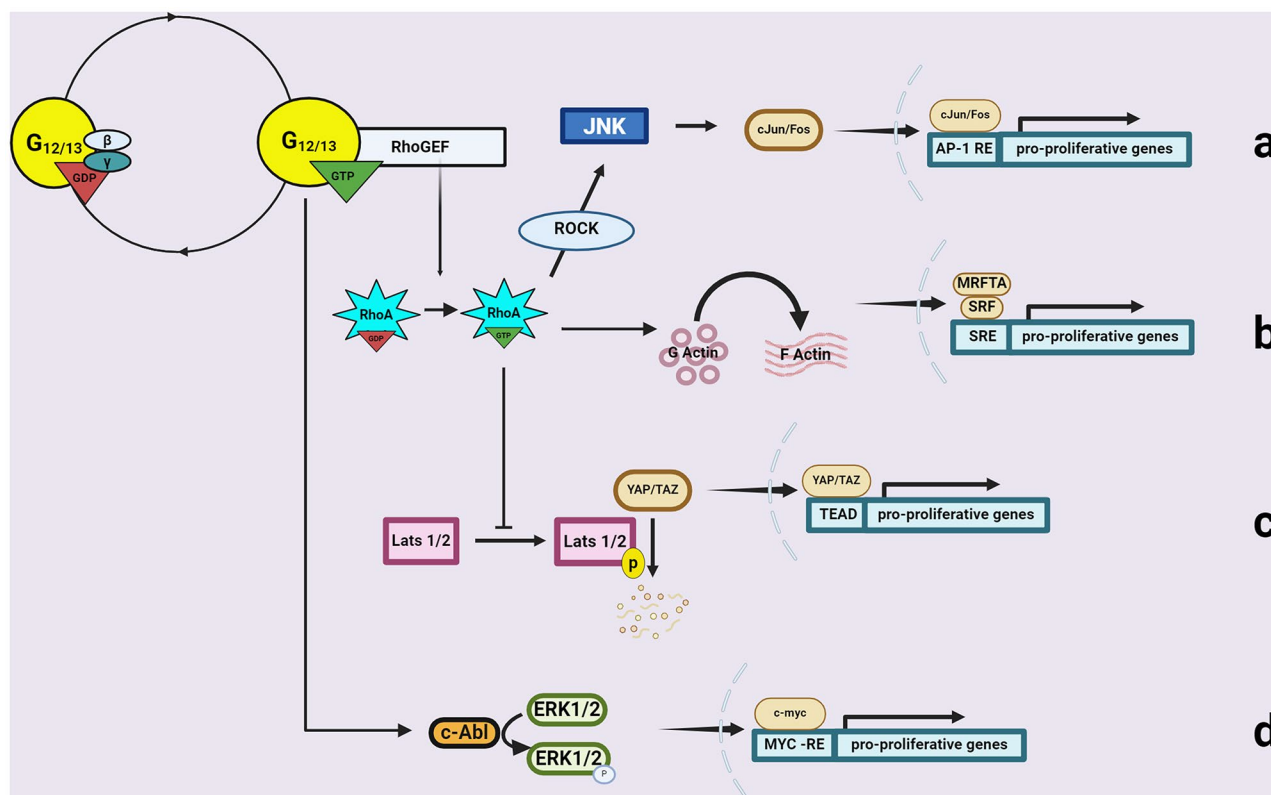


Fig. 3 $G_{12/13}$ signaling in proliferation. $G_{12/13}$ activate RhoGEFs that generate GTP-bound RhoA, activating ROCK and stimulating proliferative gene transcription via AP-1 (a) and SRE (b) promoters. GTP-bound RhoA inhibits phosphorylation of Lats 1/2, thereby blocking negative regulation of the Hippo pathway (c). $G_{12/13}$ bind to c-Abl, affecting ERK-dependent proliferation (d). (Created with BioRender.com)

oncogene [96, 97]. These genes, which were cloned from a Ewing sarcoma plasmid library (gop oncogenes), are dysregulated in numerous human cancers (reviewed in [98]). Dysregulation of $G_{12/13}$ signaling has been implicated in cardiac remodeling, angiogenesis and hypertrophy [99–102], in growth factor-stimulated cell migration [103], and in hepatic, cardiac and pulmonary fibrosis [104–106].

The proliferative effects of dysregulated $G_{12/13}$ signaling were originally attributed to activation of the JNK pathway [24]. Expression of constitutively active G_{12} leads to increased JNK activity and c-Jun phosphorylation (Fig. 3a) [107]. $G_{12/13}$ -dependent c-fos activation via the serum-response element (SRE) has also been implicated in gep oncogenesis [108]. Serum response factor (SRF) is a transcriptional activator that binds SRE in the promoter region of genes involved in pro-proliferative signaling pathways. Actin polymerization stimulated by RhoGEF/RhoA activation induces translocation of myocardin-related transcription factor A (MRTFA) to the nucleus, where it functions as a co-activator with SRF (Fig. 3b) [109–111]. AP-1 dependent transcription serves as an integrated read-out of the actin polymerization (c-fos/SRE) and ROCK stimulation (JNK/c-Jun) functions of activated RhoA [112]. AP-1 activation stimulates

expression of MDM2, which promotes degradation of p53 and FOXO1, thus driving malignancy and EMT [113, 114]. $G_{12/13}$ -dependent activation of the JNK pathway also promotes cell migration [78] and invasion [115]. In smooth muscle, S1P dependent activation of $G_{12/13}$ stimulates growth and proliferation in a RhoA and SRE-dependent manner [116].

The Hippo signaling pathway can modulate cell proliferation downstream of $G_{12/13}$ induced RhoA signaling. LPA, S1P, and PAR signaling through $G_{12/13}$ and RhoA inhibits phosphorylation of Lats 1/2, which blocks negative regulation of the oncogenic transcription factors YAP and TAZ (Fig. 3c) [117, 118]. In glioblastoma, S1P/ $G_{12/13}$ /RhoA-mediated YAP activation promotes cellular invasion and metastasis [110], while LPA activation of $G_{12/13}$ and RhoA is implicated in YAP-mediated hepatocellular carcinoma [119]. In addition to affecting Lats 1/2 phosphorylation, $G_{12/13}$ and RhoA-dependent activation of YAP and TAZ is also significantly regulated by actin polymerization [120]. In vascular smooth muscle cells, knockdown of $G_{12/13}$, RhoA inhibition, and/or disruption of actin cytoskeleton formation all prevented YAP/TAZ activation, thereby inhibiting tissue remodeling [25]. The response of smooth muscle to mechanical stretch is mediated by the interaction between G_{13} and integrins,

which inhibits $G\alpha_{13}$ -dependent RhoA activation and YAP/TAZ transcription, thereby preventing proliferation, inflammation, and angiogenesis [84]. G_{13} binding to integrins stimulates the non-receptor tyrosine kinase c-Src, which promotes cell attachment and spreading [121]. In contrast, focal adhesion kinase (FAK) is activated in a G_{13} -dependent fashion downstream of PAR [29], LPA [122], GRP4 [123] and CCK2 [124], causing sustained RhoA activation that promotes cell migration in ovarian, skin and colon cancer.

Abelson tyrosine kinase (c-Abl) is a primary regulator of actin dynamics and contraction in airway smooth muscle [125]. c-Abl inhibition attenuates actin polymerization, but fails to impact pro-contractile myosin phosphorylation [126]. In platelets, proline-rich tyrosine 2 (Pyk2) activation downstream of $G_{12/13}$ regulates shape change. Co-immunoprecipitation studies have shown that G_{13} interacts directly with c-Abl tyrosine kinase in endothelial cells, regulating actin cytoskeletal dynamics and inducing cell remodeling and cell migration [26]. In smooth muscle cells, knockdown of c-Abl inhibited ERK_{1/2} phosphorylation, a key mediator of smooth muscle proliferation (Fig. 3d) [127].

$G_{12/13}$ -mediated signaling through JNK, YAP/TAZ and non-receptor tyrosine kinases is implicated in proliferation, angiogenesis and tissue remodeling in diverse disease pathologies, including vascular and airway smooth

muscle hypertrophy. Antagonizing pathways that activate $G_{12/13}$ and its downstream signaling axis may suppress several hallmarks of AR, including increased smooth muscle mass.

$G_{12/13}$ pathway pharmacology

Therapeutic suppression of $G_{12/13}$ signaling can be achieved by balanced or biased antagonism of upstream receptors, direct G protein inactivation, blocking of activation-dependent protein-protein interactions or through inhibition of downstream signaling pathways (Fig. 4).

Humankind has been treating symptoms of asthma with anticholinergic agents for millennia. The Ebers Papyrus (1550 BC) recommends burning of henbane, which produces anticholinergic tropane alkaloids, to “remove phlegm, alleviate coughs and ease breathing” [128]. Extracts from a variety of other nightshade family plants figure prominently in treatment of dyspnea throughout Western medicine [129], including atropine, which was purified from deadly nightshade (*Atropa belladonna*) in 1833 [130], and was used to treat symptoms of asthma until the 1970s, when tropane derivatives with improved side effect profiles became available [131]. Despite (or, perhaps, because of) this long, empirical history of asthma treatment with anticholinergic agents, the effects of muscarinic antagonists have largely been evaluated physiologically rather than pharmacologically. This is particularly true for the effects of muscarinic antagonists on $G_{12/13}$ or RhoA signaling (highlighted in red in Fig. 4). For GPCRs like the M3R that display promiscuous G protein binding, antagonists of one G protein signaling pathway can function as agonists of another [132, 133], or can be biased to prefer one G protein signaling pathway over another [134]. For example, G_{12} biased agonists of the apelin receptor demonstrate sustained cardiac response [135]. The relative importance of $G_{q/11}$ versus $G_{12/13}$ signaling in ASM contraction, the effects of these pathways on therapeutic response to M3R agonists and antagonists, and the utility of modulating G protein signaling bias in obstructive airway diseases remains to be elucidated. Detailed study of the effects of muscarinic antagonists on $G_{q/11}$ versus $G_{12/13}$ signaling may provide insights that can improve disease management for asthma patients, or inform development of novel anticholinergics with enhanced potency, efficacy, and tolerability.

Agonists of the β_2 AR, which stimulate PKA-dependent activation of MLCP and smooth muscle relaxation, are the standard of care for asthma patients of every age and every stage of disease. Short-acting beta agonists (SABA) are used symptomatically to relieve acute asthma exacerbations, while long-acting beta agonists (LABA) are used as maintenance therapy by patients with persistent

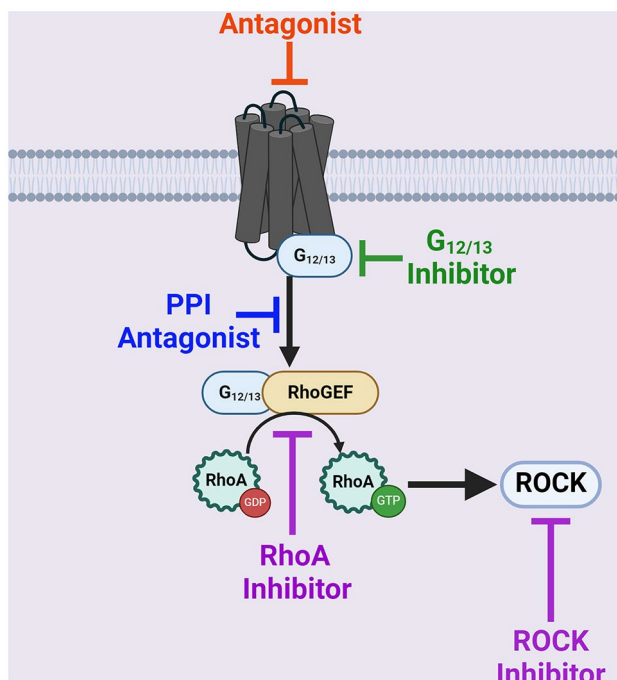


Fig. 4 Targets for regulating $G_{12/13}$ signaling. Hyperactive $G_{12/13}$ signaling in airway smooth muscle can be attenuated by antagonizing upstream receptors (red), by developing direct inhibitors of G_{12} , G_{13} (green) or complexes between $G_{12/13}$ and RhoGEFs (blue), or by inhibiting RhoA or ROCK (purple). (Created with BioRender.com)

asthma [136]. Although agonism of relaxation is a common approach to treat acute smooth muscle constriction (e.g., asthma, premature labor), other chronic diseases are often treated with antagonists (e.g., angiotensin converting enzyme inhibition or angiotensin receptor blocking in hypertension [137]; anticholinergics or alpha-blockers in detrusor hyperreflexia [138, 139]). Activated G_s is the least oncogenic $G\alpha$ subunit [97], and cAMP is generally antiproliferative [140], but chronic, unbiased beta-agonism causes iatrogenic eosinophilia, mucus production and MAPK activation [141], which may contribute to the increased risk of fatal asthmatic attacks observed in patients who are treated with LABAs [142, 143]. In principle, antagonism of hyperresponsive constrictive pathways (bronchoprotection) would circumvent the adverse therapeutic effects associated with LABA treatment [144], and anticholinergics are considered safe and effective for the treatment of chronic obstructive pulmonary disease (COPD) [145], but FDA-approved, long-acting muscarinic antagonists (LAMAs) display minimal benefit as add-on treatment for patients whose asthma is not well controlled with standard therapies [146, 147]. Notably, this conclusion is based on acute (bronchoconstriction) rather than chronic (AR) endpoints. Given the chronic activation of oncogenic $G_{q/11}$ and $G_{12/13}$ signaling that is characteristic of AHR, it would be worthwhile to assess whether anticholinergics that are able to suppress both $G_{q/11}$ and $G_{12/13}$ signaling might be effective in reducing AR when co-administered with beta agonists and inhaled corticosteroids.

Unlike bronchorelaxation, which is regulated by a single receptor (β_2 AR), multiple receptors can evoke constriction of ASM. Inhibiting the downstream G protein (highlighted in green in Fig. 4) through which multiple bronchoprovocational agents signal might provide a unified mechanism for relieving bronchoconstriction, but there are no FDA approved drugs that target G proteins. Toxins, natural products and their derivatives regulate several subfamilies of G proteins [148–152], but no natural product activators or inhibitors that are specific to $G_{12/13}$ subfamily members have been reported. As essential signaling proteins that are expressed in most tissues, G proteins are typically considered to be poor therapeutic targets, but G_{12} may be an exception. G_{12} knockout mice are viable, fertile and display minimal developmental abnormalities [153]. Nevertheless, G_{12} knock-down inhibits contraction of vascular and airway smooth muscle [59, 63] and GNA12 expression predicts asthma control [72]. Direct antagonism of G_{12} signaling may have considerable therapeutic value.

To transduce downstream signals, $G_{12/13}$ relies on protein-protein interactions (PPIs) with RhoGEFs. The avidity that dominates the free energy of PPIs is difficult to outcompete with the affinity of protein-ligand

interactions, which is why PPIs are considered challenging pharmacologic targets [154]. However, a variety of emerging approaches have succeeded in generating small molecules that can disrupt PPIs (highlighted in blue in Fig. 4) [155–157]. A recent study characterized the PPI between G_i and its GEF, Girdin, as a druggable interaction by performing high-throughput screening. The authors identified a small molecule, NF023, that disrupts GEF activity by binding to a site that overlaps with the G_i -Girdin interface but fails to disrupt formation of the inactive $G\alpha\beta\gamma$ heterotrimer [158]. Analysis of the $G_{12/13}$ interactome may reveal druggable PPI interfaces and enable discrimination of the distinct biological functions of G_{12} and G_{13} . Indeed, the G_{13} interactome has already been validated as a therapeutic target. M3mP6, a peptide derived from the G_{13} binding ExE motif of the integrin beta3 cytoplasmic domain, has antithrombotic effects without off-target adverse symptoms. In a mouse model, post-ischemic injection of M3mP6 protected the heart from myocardial ischemia-reperfusion injury [159].

Receptor-activated $G_{12/13}$ signals primarily through the RhoA/ROCK pathway. As such, inhibitors of RhoA or ROCK offer an alternative approach to interdict pro-contractile, pro-inflammatory and/or proliferative $G_{12/13}$ -mediated signaling downstream of multiple, pro-contractile GPCRs (highlighted in purple in Fig. 4). Although RhoA inhibitors (including C3 toxin from *Clostridium botulinum*) have been described [160, 161], these agents are used as research tools rather than therapeutics. In contrast, ROCK inhibitors are FDA approved for treatment of open-angle glaucoma and ocular hypertension [162] and for graft versus host disease [163], and the ROCK inhibitor fasudil is approved in Japan for treatment of cerebral vasospasm and subarachnoid hemorrhage. ROCK inhibitors have also been investigated in clinical trials as treatments for a variety of indications, including amyotrophic lateral sclerosis [164], Parkinson's disease [165], solid malignancies [166], psoriasis [167], chronic kidney disease [168] and pulmonary hypertension [169]. Clinical trials in which ROCK inhibitors are administered systemically typically report few significant adverse events [170]. Hypotension and syncope are commonly observed, but these side effects were minimized in pulmonary hypertension trials by delivering the inhibitor (fasudil) to the lung by inhalation [171]. No human clinical trials evaluating the efficacy of ROCK inhibitors for the treatment of asthma have been registered to date, but ROCK inhibitors are effective in reducing inflammation [172, 173] and airway constriction [174, 175] in cellular and animal models of asthma. Clinical trials of ROCK inhibitors as asthma therapeutics are feasible, and their potential benefits to patients are well supported by ample pre-clinical data.

Numerous small molecules can interact with the downstream $G_{12/13}$ signaling pathway to promote bronchorelaxation. PI3K inhibitors promote anti-inflammatory responses in mouse models, reducing mucus production and airway remodeling [176, 177]. Small molecule inhibitors of class I PI3Ks can act as bronchodilators in human airway smooth muscle cells, highlighting this pathway as a promising target for pharmacologic intervention [178, 179]. In mouse asthma models, the selective PI3K p110 δ inhibitor IC87114 attenuated AKT phosphorylation and mitigated airway hyperresponsiveness [180]. Inhibiting class I isoforms directly with p110 α knockdown, wortmannin, or the small molecule LY294002 resulted in diminished RhoA and MYPT1 activation, which suppressed MLC phosphorylation [181]. These inhibitors also diminished cell depolarization, a common trait of HASM contraction [182], and reduced glucocorticoid insensitivity in patients with severe asthma by restoring HDAC2 or inhibiting the activation of pro-inflammatory transcription factors [183].

Upstream receptors and downstream kinases are the most tractable targets for suppressing chronic $G_{12/13}$ signaling. However, the effects of anticholinergics on $G_{12/13}$ signaling have never been characterized, and the extent to which RhoA/ROCK or PI3K inhibition can mitigate the effects of $G_{12/13}$ pathway activation is unknown.

Conclusions

The canonical model for smooth muscle contraction posits that actomyosin cross-bridge cycling is regulated primarily by calcium-dependent activation of myosin light chain kinase downstream of M3R and $G_{q/11}$. The intuitive satisfaction of this model benefits from the convergence of two conventional wisdoms from muscle and cell physiology: that muscle contraction is calcium dependent; and that kinase activity is induced while phosphatase activity is constitutive. It's unclear whether either of these conventional wisdoms holds in airway smooth muscle. The calcium dependence of cardiac and skeletal muscle contraction is largely due to the need to relieve tonic inhibition of the interaction between myosin and actin by troponin, which isn't expressed in smooth muscle [184]. The need for troponin in cardiac and skeletal muscle and calponin in smooth muscle suggests that the basal activity of MLCK is sufficient to promote constriction in the absence of tonic inhibition. The efficacy of β -agonists in asthma pharmacotherapy, which activate MLCP, indicates that bronchomotor tone may be regulated primarily by phosphatase activity rather than kinase activity. The inability of FR to block M3R-dependent contraction of airway smooth muscle [34, 35] is consistent with this view, and indicates the existence of a signaling pathway besides $G_{q/11}$ that is able to transduce signals from M3R through RhoA to inhibit MLCP

and promote parasympathetic actomyosin cross-bridge cycling. The preponderance of the evidence strongly indicates that this critical signaling pathway is $G_{12/13}$ [61–63], although additional experiments to resolve inconsistencies between functional and biosensor assays would be worthwhile.

Pathologic constriction of airway smooth muscle is the defining symptom of asthma [185]. Smooth muscle contraction relies on actin polymerization and phosphorylation of myosin light chain, both of which are mediated by hyperresponsive acetylcholine signaling to RhoA and ROCK [37], likely through $G_{12/13}$ [29]. $G_{12/13}$ signaling also stimulates production of Th2 cytokines [70], which promote airway inflammation, and activates numerous proliferative pathways (JNK [112], Hippo [84], c-Abl [26]) that may drive AR in hyperresponsive airways. The role of $G_{12/13}$ signaling in airway constriction, inflammation and remodeling has been underappreciated despite compelling direct evidence for its significance [63]. Consequently, the effect of antagonists of constrictive and inflammatory pathways on $G_{12/13}$, RhoA and ROCK activation has never been adequately characterized. Therapeutic strategies that antagonize upstream receptors or that inhibit $G_{12/13}$ or its downstream signaling partners should benefit patients with asthma and likely synergize with current asthma treatments.

Abbreviations

GPCR	G protein-coupled receptor
cAMP	cyclic AMP
PKA	Protein Kinase A
PLC β	Phospholipase C β
IP β	Inositol Trisphosphate
DAG	Diacylglycerol
PKC	Protein Kinase C
Rho	Ras Homology
GEF	Guanine Exchange Factor
ROCK	Rho-Associated Kinase
LIMK	Lim Domain Kinase
JNK	c-Jun NH $_2$ -terminal kinase
GAP	GTPase Accelerating Protein
RGS	Regulator of G Protein Signaling
CysLTR	Cysteinyl Leukotriene Receptors
H	Histamine
LPA	Lysophosphatidic Acid
PAR	Protease-Activated Receptor
STP	Sphingosine-1-Phosphate
AHR	Airway Hyperresponsiveness
AR	Airway Remodeling
β_2 AR	Beta-2 Adrenergic Receptor
MAPK	Mitogen-Activated Protein Kinase
ASM	Airway Smooth Muscle
MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinase
FR	$G_{q/11}$ -selective depsipeptide inhibitor FR900359
G	Globular
F	Filamentous
MLCP	Myosin Light Chain Phosphatase
MYPT	Myosin Phosphatase Targeting Subunit
GDI	Guanine Nucleotide Dissociation Inhibitor
LARG	Leukemia-Associated RhoGEF
ARHGEF	Rho Guanine Exchange Factor

GNA12	Gene Encoding Guanine Nucleotide Binding Protein Subunit Alpha 12
GNA13	Gene Encoding Guanine Nucleotide Binding Protein Subunit Alpha 13
M3R	Muscarinic 3 Acetylcholine Receptor
HEK	Human Embryonic Kidney
HASM	Human Airway Smooth Muscle
siRNA	Short Interfering RNA
TGF	Tumor Growth Factor
EMT	Epithelial-Mesenchymal Transition
IgE	Immunoglobulin E
IL	Interleukin
Th2	T-helper 2
NFkB	Nuclear Factor kB
Tfh	T Follicular helper
AP-1	Activator Protein 1
PRG	PDZ Domain-Containing RhoGEF
Gep	Gene from Ewing Sarcoma Plasmid Library
SRE	Serum Response Element
SRF	Serum Response Factor
MRTFA	Myocardin-Related Transcription Factor A
MDM2	Mouse Double Minute 2
FOXO1	Forkhead Box Protein O1
Lats1/2	Large Tumor Suppressors 1 & 2
YAP	Yes-Associated Protein
TAZ	Transcriptional Activator with a PDZ Binding Motif
c-SRC	Cellular Rous Sarcoma Virus Homolog
FAK	Focal Adhesion Kinase
GRP	Gastrin-Releasing Peptide
CCK	Cholecystokinin
c-Abl	Cellular Abelson Murine Lymphosarcoma Virus Homolog Tyrosine Kinase
Pyk2	Protein Tyrosine Kinase 2
ERK	Extracellular Signal-Regulated Kinase
SABA	Short-Acting Beta Agonist
LABA	Long-Acting Beta Agonist
COPD	Chronic Obstructive Pulmonary Disease
FDA	Food and Drug Administration
LAMA	Long-Acting Muscarinic Antagonist
PPI	Protein-Protein Interaction
PI3K	Phosphoinositide-3 Kinase
AKT	Protein Kinase B/Gene that Causes Thyomas in the Akr Mouse Strain
HDAC	Histone Deacetylase
PIP2	Phosphoinositide Bisphosphate

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

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Competing interests

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