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Long-acting β_2-adrenoceptor agonists enhance glucocorticoid receptor (GR)-mediated transcription by gene-specific mechanisms rather than generic effects via GR

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a) Running title (60 characters): LABA enhancement of GR-dependent transcription

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d) Abbreviations: Genes, mRNAs or proteins, unless otherwise indicated, are referred to by the official gene symbol, as supplied by The National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). Other abbreviations are: ChIP, chromatin immunoprecipitation; CRE, cAMP response element; DAPI, 4′,6-diamidino-2-phenylindole; GBS, GR binding site; GR, glucocorticoid receptor; GRE glucocorticoid response element; ICS, inhaled corticosteroid; LABA, long-acting β_2-adrenoceptor agonist; PCR, polymerase chain reaction; pHBECs, primary human bronchial epithelial cells; qPCR, quantitative real-time PCR; PKA, protein kinase A; siRNA, small interfering RNA.

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Abstract

In asthma, the clinical efficacy of inhaled corticosteroids (ICSs) is enhanced by long-acting β_2-adrenoceptor agonists (LABAs). ICSs, or more accurately, glucocorticoids, promote therapeutically-relevant changes in gene expression and, in primary human bronchial epithelial cells (pHBECs) and airway smooth muscle cells, this genomic effect can be enhanced by a LABA. Modelling this interaction in human bronchial airway epithelial BEAS-2B cells transfected with a 2×glucocorticoid response element (2×GRE)-driven luciferase reporter showed glucocorticoid-induced transcription to be enhanced 2- to 3-fold by LABA. This glucocorticoid receptor (GR; NR3C1)-dependent effect occurred rapidly, was insensitive to protein synthesis inhibition and was maximal when glucocorticoid and LABA were added concurrently. The ability of LABA to enhance GR-mediated transcription was not associated with changes in GR expression, serine (Ser^{203}, Ser^{211}, Ser^{226}) phosphorylation, ligand affinity or nuclear translocation. Chromatin immunoprecipitation demonstrated that glucocorticoid-induced recruitment of GR to the integrated 2×GRE reporter and multiple gene loci, whose mRNAs were unaffected or enhanced by LABA, was also unchanged by LABA. Transcriptomic analysis revealed glucocorticoid-induced mRNAs were variably enhanced, unaffected or repressed by LABA. Thus, events leading to GR binding at target genes is not the primary explanation for how LABAs modulate GR-mediated transcription. As many glucocorticoid-induced genes are independently induced by LABA, *gene-specific* control by GR- and LABA-activated transcription factors may explain these observations. Since LABAs promote similar effects in pHBECs, therapeutic relevance is likely. These data illustrate the need to understand gene function(s), and the mechanisms leading to gene-specific induction, if existing ICS/LABA combination therapies are to be improved.

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Introduction

Known as inhaled corticosteroids (ICSs), synthetic glucocorticoids act on the glucocorticoid receptor (GR; gene symbol NR3C1) to reduce inflammatory gene expression and are generally effective in controlling mild-to-moderate asthma (Oakley and Cidlowski, 2013; Barnes, 2011). However, in a subset of severe asthmatics, ICS provide insufficient control and add-on therapies are recommended (Newton and Giembycz, 2016; Reddel et al., 2015). Probably the most widely prescribed option are long-acting β_2-adrenoceptor agonists (LABAs), which are administered in combination with ICS, to improve lung function, reduce exacerbation frequency and improve quality of life, more than increasing the ICS dose (Newton and Giembycz, 2016). Such data suggest that ICSs and LABAs interact at a molecular level to improve therapeutic outcomes (Giembycz et al., 2008). Several mechanisms have been advanced to explain this effect, including the ability of LABAs to enhance, in a cAMP-dependent manner, glucocorticoid-driven transcription from a simple glucocorticoid response element (GRE)-dependent reporter (Kaur et al., 2008). In this simple system, LABAs alone are without effect, yet synergistically increase the maximal glucocorticoid-induced response. This mimics the clinical situation where LABAs are steroid-sparing (Newton and Giembycz, 2016).

Mechanistically, LABAs have been suggested to promote translocation of GR to the nucleus (Roth et al., 2002; Usmani et al., 2005; Mortaz et al., 2008; Haque et al., 2013), even independently of a glucocorticoid (Eickelberg et al., 1999). Alternatively, the cAMP pathway may increase GR expression, agonist affinity and/or increase GR DNA binding (Dong et al., 1989; Rangarajan et al., 1992; Korn et al., 1998). However, other studies do not corroborate these effects (Gruol and Altschmied, 1993; Moyer et al., 1993; Zhang et al., 1993; Loven et al.,

2007). Nevertheless, expression of many glucocorticoid-induced genes is markedly enhanced by a LABA in bronchial epithelial BEAS-2B cells (Kaur et al., 2008; Rider et al., 2015; Joshi et al., 2015b; BinMahfouz et al., 2015). As similar effects occur in primary human bronchial epithelial cells (pHBECs) (Kaur et al., 2008; Moodley et al., 2013; Holden et al., 2014), a key regulator of inflammatory responses (Knight and Holgate, 2003), this mechanism may be physiologically relevant. Furthermore, there is increasing evidence that gene induction by GR (i.e. transactivation) contributes to the anti-inflammatory effects of glucocorticoids (Clark and Belvisi, 2012; Newton, 2014; Oh et al., 2017) and, *in vivo*, airway epithelial cells are an indispensable site of action (Klassen et al., 2017). Indeed, analysis of inflammatory gene expression suggests that GR transactivation operates alongside transrepression to produce repression (King et al., 2012). As many genes are induced *in vivo* in the human airways following ICS inhalation (Kelly et al., 2012; Leigh et al., 2016), or ICS/LABA inhalation (Lee et al., 2016), transcriptional activation likely contributes to the clinical efficacy of these drugs.

LABAs induce expression of numerous genes (Yan et al., 2018). Some, including the bronchoprotective, regulator of G-protein signalling, RGS2 (Holden et al., 2011; Holden et al., 2014), and the anti-inflammatory genes, CD200 and CRISPLD2 (Moodley et al., 2013; Joshi et al., 2015b; BinMahfouz et al., 2015; Himes et al., 2014; Vaine and Soberman, 2014), are potentially beneficial. Others (CXCL2, IL6, IL11) may be undesirable (Ammit et al., 2002; Yan et al., 2018). Regardless, the impact of glucocorticoids on these genomic effects of LABAs are gene-dependent. For example, LABA-induced IL6 expression is repressed by glucocorticoid (Ammit et al., 2002; Holden et al., 2010), while RGS2, CRISPLD2 and CD200 mRNAs are synergistically enhanced (Holden et al., 2011; Holden et al., 2014; Joshi et al., 2015b; BinMahfouz et al., 2015). In contrast, the phosphatase, DUSP1, is induced by LABAs and

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glucocorticoids and the effects of these two drugs in BEAS-2B cells and airways smooth muscle cells are additive (Kaur et al., 2008; Manetsch et al., 2012; Manetsch et al., 2013).

Patients with asthma requiring high-dose ICS, or oral corticosteroid, may suffer from multiple side-effects including oral candidiasis, hypothalamus-pituitary-adrenal axis suppression, decreased bone density and osteoporosis. Accordingly, GR agonists showing an improved therapeutic index are urgently needed (Newton et al., 2010; Adcock et al., 2012). However, the current positioning of ICS/LABA combination therapy reveals an unmet need to elucidate the molecular interactions between these two drugs. Such analyses should promote discovery of novel GR agonists that best capture therapeutic benefits, while avoiding undesirable outcomes. Since BEAS-2B cells behave similarly to pHBECs (Kelly et al., 2012; Moodley et al., 2013; Rider et al., 2015; Holden et al., 2014), they were used to interrogate potential mechanisms by which LABAs enhance GR-dependent transcription.

Materials and Methods

Cell culture and compounds. Human bronchial epithelial (BEAS-2B) cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's/Ham's F12 medium (DMEM/F12) supplemented with 14 mM $NaHCO_3$, 2 mM L-glutamine and 10% fetal calf serum (all Invitrogen; Burlington, ON). Cells were cultured at 37°C in 5% CO_2/95% air and were incubated in serum-free medium overnight prior to experiments. Dexamethasone and RU486 (mifepristone) (both Sigma-Aldrich, Oakville, ON), budesonide and formoterol fumarate (formoterol) (gifts from AstraZeneca Sweden; Mölndal, Sweden), salmeterol xinafoate (salmeterol) and fluticasone propionate (gifts from GlaxoSmithKline, Stevenage, UK), Org

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34517 (11β-(1,3-benzodioxolo)-17β-hydroxy-17-(1-propynyl)-oestra-4,9-dien-3-one (gift from Chiesi Farmaceutici, Parma, Italy)(Bachmann et al., 2003), and dexamethasone 21-mesylate (Dex-Mes) (Steraloids; Newport, RI) were dissolved in DMSO (Sigma-Aldrich). Final DMSO concentrations on cells were ≤ 0.1%. Cycloheximide (Sigma-Aldrich) was dissolved in Hank's balanced salt solution (Invitrogen).

Luciferase reporters and assay. BEAS-2B cells stably transfected with a glucocorticoid response element (GRE) reporter plasmid, pGL3.neo.TATA.neo, which contains 2 simple GRE sites upstream of a minimal TATA box driving luciferase expression, or pADneo2-C6-BGL, which contains six tandemly repeated cAMP response element (CRE) sites upstream of a minimal β-globin promoter driving a luciferase gene were as described (Chivers et al., 2004; Meja et al., 2004). Cells were maintained in media containing 0.25 mg/ml G-418 (Promega, Madison, WI). Cells, grown to confluence in 24 well plates, were incubated overnight in serum-free, antibiotic-free, media prior to experiments. Cells were harvested in reporter lysis buffer and luciferase assays performed using a Firefly Luciferase Assay Kit (Biotium; Hayward, CA).

Western blotting and small interfering RNA (siRNA) silencing. Western blotting was performed according to standard procedures. Cells were lysed in 1× Laemmli buffer supplemented with phosphatase inhibitors (Sigma-Aldrich) and 1× complete protease inhibitor cocktail (Roche, Indianapolis, IN), size fractionated on 12% acrylamide gels and electro-transferred onto Hybond ECL membranes (GE Healthcare, Waukesha, WI). Membranes were blocked in 5% milk in tris-buffered saline containing 1% tween 20 (TBST) and probed with antibodies against CREB1 (#9197; Cell Signaling Technology, Danvers, MA), serine 133 phosphorylated CREB1 (#9191; Cell Signaling Technology), GR (sc-8992; Santa Cruz, Dallas, TX), serine 203 (S203), serine 211 (S211) or serine 226 (S226) phosphorylated GR (ab195703,

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ab55189 and ab195789, respectively, from Abcam Inc; Toronto, ON) and GAPDH (4699-9555(ST); AbD Serotec, Raleigh, NC or, in Fig. 4B and Supplemental Fig. 6, 14C10; Cell Signaling Technology). After washing, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit (111-035-003; Jackson ImmunoResearch Laboratories Inc; West Grove, PA) or anti-mouse immunoglobulin (115-035-003; Jackson ImmunoResearch Laboratories Inc). Detection was by enhanced chemiluminescence (Pierce ECL Western Blotting Substrate; ThermoFisher Scientific Inc., Rockford, IL) and visualized by autoradiography. GR siRNA 5 (target 5′- AAG TGC AAA CCT GCT GTG TTT -3′) and 6 (target 5′-AAG TGC AAA CCT GCT GTG TTT-3′) and control siRNA (LMNA_1, target 5′- AAC TGG ACT TCC AGA AGA ACA -3′) were purchased from Qiagen (Valencia, CA) and transfected at 25 nM into cells using RNAiMAX (Invitrogen).

Cytoplasmic and nuclear extracts were prepared by scraping cells and then resuspending cell pellets in 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1% NP-40, 1×protease inhibitors on ice for 15 min. After centrifugation (14,000 g, 5 min, 4˚C), the supernatant containing cytoplasm was removed and frozen at -20˚C prior to western blot analysis. The nuclear pellets were washed and resuspended in 20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA pH 8.0, 0.5 mM PMSF, 0.5 mM DTT, 1×protease inhibitors). Following repeated and vigorous agitation, nuclear debris was pelleted (14,000 g, 10 min, 4˚C) and the soluble nuclear fraction frozen at -20˚C prior to western blot analysis.

Quantitative real-time polymerase chain reaction (qPCR). Total RNA was extracted (RNeasy mini kit; Qiagen) and 0.5 µg used for cDNA synthesis (qScript kit; Quanta, Gaithersburg, MD). Following a 1:4 dilution, qPCR was performed on 2.5 µl of cDNA in 10 µl

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reactions using 7900HT or StepOnePlus instruments (Applied Biosystems; Foster City, CA) and SYBR GreenER chemistry (Invitrogen). Melt curve analysis was performed to confirm primer specificity. Serial cDNA dilution was used to assess primer efficiency and to obtain relative expression levels. In later experiments, the $\Delta\Delta C_T$ method was used with validated primers. Primers for qPCR are listed in Supplemental Table 1A.

Immunofluorescent microscopy. Cells in 8 well microscopy slides were placed on ice and fixed with 4% paraformaldehyde for 15 min, permeabilized (0.3% Triton-X/Hank's balance salt solution) for 10 min and then blocked overnight with 10% goat serum at 4˚C. Slides were incubated with primary anti-GR antibody (PA1-511A, ThermoFisher Scientific) for 1 h followed by 1 h with Alexa Fluor 488 goat anti-rabbit F(ab')2 fragment secondary antibody containing 1 μM 4′,6-diamidino-2-phenylindole (DAPI). Coverslips were mounted using ProLong Gold antifade reagent. Slides were visualised using an Olympus IX81 FV1000 laser scanning confocal microscope with 40× magnification at 405 (blue) and 488 (green) nm. Exported ".oif" files were analysed using Volocity software and Pearson product-moment correlation coefficient used to enumerate colocalization between the green (GR) and blue (DAPI) image channels.

Chromatin Immunoprecipitation (ChIP). GR chromatin immunoprecipitation followed by massively parallel sequencing (ChIP-seq) data from control or dexamethasone (100 nM) treated BEAS-2B cells have previously been published (Kadiyala et al., 2016). GR binding regions were subjected to bioinformatic analysis with TFBIND (http://tfbind.hgc.jp/) to identify putative GRE sites (Tsunoda and Takagi, 1999). For ChIP-PCR analysis, BEAS-2B cells were grown in complete medium in 100-mm cell-culture plates. Plates at 80% confluence, ~10^7 cells, were serum starved overnight prior to treatments. Protein-DNA crosslinking was performed by adding 16% methanol-free formaldehyde (ThermoFisher Scientific) directly to the culture medium to a final

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concentration of 1%, and ChIP was performed in the main manuscript using an anti-GR antibody (PA1-511A; ThermoFisher Scientific), or, in the supplemental data, as previously described (Sasse et al., 2013; Altonsy et al., 2014). Purified DNA was analyzed by qPCR performed in triplicate. Relative occupancy was calculated on a $\log_2$ scale by the $\Delta\Delta C_T$ method. The averaged C_T for each test region was normalized to the geometric mean of the C_T value obtained for three negative control regions as described (Sasse et al., 2013; Altonsy et al., 2014). Primer sequences used for ChIP qPCR are as listed Supplemental Table 1B.

Microarray analysis. BEAS-2B cells were either not treated or treated with budesonide (100 nM), formoterol (10 nM) or both combined for 1, 2, 6 and 18 h. Total RNA quality, prepared as above, was assessed on a 2100 Bioanalyzer using RNA 6000 Nano LabChips (Agilent Technologies, Santa Clara, CA). First and second strand synthesis was performed with GeneChip 3′ IVT Express kits (Affymetrix, Santa Clara, CA) and *in vitro* transcription generated biotin-labelled amplified RNA. After purification and fragmentation, hybridization to PrimeView microarrays (Affymetrix) was for 16 h prior to washing and scanning using a GeneChip Fluidics Station 450 and Scanner 3000 G7, respectively. Robust multiarray averaging, quantile normalization, and median polishing on logged probe set intensity values were performed using Affymetrix Expression Console software. Fold change of the probe set intensity values to the time-matched untreated controls were calculated and one-way analysis of variance (ANOVA) was performed using Transcriptome Analysis Console (TAC) software (Affymetrix, Santa Clara, CA). Data files are deposited with NCBI's Gene Expression Omnibus (GEO) (Accession # GSEXXXX (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSEXXXX). Where genes are represented by multiple probe sets, only those with the greatest overall change were retained for subsequent analyses.

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Statistical analyses and curve fitting. Data from *N* separate determinations are plotted as means ± SE. Statistical analyses generally assumed data to be normally distributed (Prism version 6.01; GraphPad Software, San Diego, CA). In some experiments, different batches of cells gave rise to different levels of reporter, or gene, induction. In such cases, non-parametric statistics were necessarily used. The tests used are stated in the figure legends. Four parameter curve fitting of agonist concentration-response data was performed in Prism version 6.01 to produce values for the maximum achievable response (E_{Max}) and pEC_{50}, which is defined as the negative log of the molar EC_{50}. Schild analysis to produce pA_2 values, defined as the negative log of the molar concentration of antagonist that is required to double the concentration of agonist in order to achieve the original response, was performed in Prism 6.01. Operational modelling (Leff et al., 1990) was performed in Prism 6.01 to produce pK_A, negative log of the molar affinity, values using the method of fractional irreversible receptor depletion, as previously described (Joshi et al., 2015b).

Results

Effect of glucocorticoid on phospho-CREB and CRE-dependent transcription induced by LABA. β_2-Adrenoceptor agonists enhance GR-dependent transcription via a β_2-adrenoceptor-mediated mechanism that is sensitized by PDE4 inhibition and involves the classical cAMP-protein kinase A (PKA) pathway (Kaur et al., 2008; Moodley et al., 2013; BinMahfouz et al., 2015). To assess effects of glucocorticoid on this pathway, BEAS-2B cells were treated with a maximally effective concentration (10 nM) of the LABA, formoterol, in the absence and presence of a maximally effective concentration, 1 μM, of dexamethasone (Kaur et al., 2008;

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Rider et al., 2011). Formoterol induced phosphorylation of the cAMP-activated transcription factors, CREB1 and ATF1, within 10 min (Fig. 1A). This was maintained at 30 min, before time-dependently declining. There were no effects of dexamethasone co-treatment and expression of total CREB1 was unaltered over this time-frame (Fig. 1A, Supplemental Fig. 1). BEAS-2B cells harbouring a 6×CRE luciferase reporter were stimulated with various concentrations of formoterol in the absence or presence of 1 μM dexamethasone (Fig. 1B). Formoterol maximally induced 6×CRE-dependent luciferase activity by 19.6 ± 0.6 fold with a pEC_{50} of 10.3 ± 0.1. Dexamethasone modestly reduced the E_{Max} for formoterol to 18.0 ± 0.6 fold, whereas the pEC_{50} remained unchanged at 10.3 ± 0.1 (Fig. 1B). Thus, glucocorticoid had little effect on the activation of cAMP-dependent transcription by β_2-adrenoceptor agonist.

Enhancement of glucocorticoid-induced simple GRE transcription by LABA requires GR and is time-dependent. In BEAS-2B cells harbouring a simple 2×GRE luciferase reporter, dexamethasone increased luciferase activity in a concentration-dependent manner (pEC_{50} 7.7 ± 0.2; E_{Max} 10.6 ± 1.1 fold) (Fig. 2A). Alone, formoterol (10 nM) had no effect on 2×GRE-dependent luciferase activity, but enhanced the maximal response to dexamethasone to 31.9 ± 3.6 fold without changing the potency (pEC_{50} 7.7 ± 0.2) (Fig. 2A). Reporter activity induced by dexamethasone and dexamethasone plus formoterol was competitively inhibited by the GR antagonist, Org34517 (Supplemental Fig. 2), with pA_2 values of 8.4 ± 0.1 and 8.5 ± 0.1, respectively. This is consistent with GR-mediated responses (King et al., 2012; Joshi et al., 2015b). A single concentration of Org34517 (1 μM) produced near complete inhibition of the responses to maximally effective concentrations of dexamethasone (Fig. 2B), and the ICSs, budesonide and fluticasone propionate (Supplemental Fig. 2D). The LABAs, formoterol and salmeterol, enhanced glucocorticoid-induced 2×GRE reporter activity and this was also

prevented by Org34517 (Fig. 2B, Supplemental Fig. 2D). To confirm GR-dependence, two independent siRNAs, which produced a near complete loss of GR, abolished 2×GRE reporter activation induced by budesonide and budesonide plus formoterol (Supplemental Fig. 3).

The effect of time of LABA addition on the enhancement of GRE-dependent transcription was investigated. BEAS-2B 2×GRE reporter cells were treated with a maximally effective concentration of dexamethasone (1 μM) and formoterol (10 nM) added at various times prior to, with, or after the glucocorticoid (Fig. 2C). Reporter activity induced by dexamethasone was maximally enhanced by formoterol co-treatment (i.e. addition time of 0), or when formoterol was added 15 min prior to, or post, the dexamethasone addition. Increasing the formoterol addition time either prior to, or post, dexamethasone addition reduced enhancement of the 2×GRE reporter by formoterol (Fig. 2C). Therefore, co-treatment is necessary for maximal cooperativity and is consistent with a rapidly-acting pathway to enhance simple GR-mediated GRE-dependent transcription.

LABA enhancement of GRE-dependent transcription occurs early and is insensitive to protein synthesis inhibition. Following dexamethasone treatment of BEAS-2B cells harbouring the 2×GRE reporter, luciferase activity time-dependently accumulated to a maximum at 4 – 6 h, after which luciferase activity declined modestly (Fig. 2D). Such data are replicated with other GR ligands and collectively suggest an early pulse of GRE-dependent transcription (Joshi et al., 2015a; BinMahfouz et al., 2015), which is largely abrogated by 4 – 6 h. This was confirmed in BEAS-2B cells harbouring the 2×GRE reporter where luciferase mRNA revealed a rapid glucocorticoid-induced kinetic with peak expression at 2 h, before returning to near baseline at 5 – 6 h (Supplemental Fig. 4A). Thus, peak luciferase activity accumulation, 4 – 6 h post-glucocorticoid, is primarily due to enhanced mRNA expression during the first 4 h post-

glucocorticoid administration. Formoterol enhanced this initial rate of dexamethasone-induced luciferase production, as revealed by the initial (0 – 4 h) slope of luciferase activity accumulation (Fig. 2D). Indeed, examination of the percentage enhancement produced by formoterol revealed a clear effect 1 h post-treatment (Fig. 2D, lower panel). Thus, not only did glucocorticoid-induced 2×GRE-dependent transcription occur over a relatively short window of time, but LABA enhancement was confined to the same, "early", time-frame. Indeed, at 2 h, when formoterol alone showed little effect on luciferase mRNA accumulation, dexamethasone-induced luciferase mRNA expression was enhanced 2 - 3 fold by formoterol (Fig. 2E, upper panel, left axis).

To examine the effect of protein synthesis inhibition on the enhancement of 2×GRE activity by formoterol, cycloheximide was first tested on the induction of luciferase mRNA by dexamethasone (Supplemental Fig 4B). Alone, cycloheximide had no effect on luciferase mRNA expression, but enhanced expression induced by dexamethasone at 1 and 2 h. This persisted until 6 h, despite dexamethasone-induced luciferase mRNA having returned to basal levels (Supplemental Fig. 4B). While not investigated further, these data raise the prospect that either simple GRE-dependent transcription is negatively regulated by a labile inhibitor that requires ongoing resynthesis or that dexamethasone rapidly induces the expression of a negative regulator of GRE-dependent transcription. The effect of cycloheximide on the enhancement of dexamethasone-induced transcription by formoterol was tested. At 2 h dexamethasone-induced luciferase mRNA expression was enhanced 2 – 3 fold by formoterol, which alone was without effect (Fig. 2E, upper panel, left axis). In the presence of cycloheximide, dexamethasone produced an elevated level of luciferase mRNA that was also enhanced 2 – 3 fold by formoterol (Fig. 2E, upper panel, right axis). Thus, formoterol enhanced glucocorticoid-induced 2×GRE-

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dependent transcription to similar extents, whether in the absence or presence of cycloheximide, (Fig. 2E, lower panel). These data support a directly-acting pathway that does not involve LABA-induced *de novo* protein synthesis to enhance GR-mediated transcription.

LABAs do not affect GR expression or GR phosphorylation. BEAS-2B cells were treated with dexamethasone in the absence or presence of LABAs prior to analysis of GR expression. As shown in figure 3A, treatment with glucocorticoid decreased GR protein expression. This effect was apparent at 6 h and was more pronounced by 18 h (Fig. 3A). However, maximally effective concentrations of formoterol and salmeterol, either alone or in combination with dexamethasone, had no effect on GR protein expression (Fig. 3A). These data were confirmed at 6 h using budesonide/formoterol and fluticasone propionate/salmeterol combinations (Supplemental Fig. 5A). Similarly, GR mRNA expression was significantly reduced following 6 h treatments of BEAS-2B cells with glucocorticoids and this was unaffected by formoterol co-treatment (Supplemental Fig. 5B). Alone, formoterol modestly, 1.21 ± 0.04 fold, increased GR mRNA.

Phosphorylation of GR at serines 203, 211 and 226 (Ser^{203}, Ser^{211} and Ser^{226}) have been previously characterised and, in the case of Ser^{211}, is maximal 1 to 2 h following exposure to glucocorticoid (Avenant et al., 2010). In initial analyses, 2 h of dexamethasone (1 μM) induced phosphorylation at each site, with increases at Ser^{211} being most robust (Fig. 3B). Similar data were obtained at 1 h (Supplemental Fig. 6B). Following siRNA silencing to reduce GR expression, the signal detected by each phospho-specific antibody was markedly reduced (Supplemental Fig. 6A). This confirms specificity for GR. While the dexamethasone-induced phosphorylation was not significant for Ser^{226}, combining these results (N = 4) with the data (N = 4) from Supplemental Fig. 6C, for the dexamethasone, formoterol, dexamethasone plus formoterol treatments, confirmed that glucocorticoid-enhanced phosphorylation at Ser^{226} was

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also significant (N = 8, Bonferroni's multiple comparison test $P \leq 0.05$). In each case, there was no effect of maximally effective concentrations of either formoterol or salmeterol on phosphorylation at these three sites at either 1 or 2 h (Fig. 3B, Supplemental Fig. 6B). While dexamethasone concentration-dependently increased GR phosphorylation at each site, no effects of formoterol were apparent (Supplemental Fig. 6C).

LABAs do not affect glucocorticoid affinity (K_A) for GR. The effect of formoterol (10 nM) on the affinity of budesonide for GR in 2×GRE BEAS-2B reporter cells was determined using the method of fractional, irreversible receptor depletion as previously described for GR (Joshi et al., 2015b). Agonist concentration-response curves were constructed in cells that had been treated for 30 min, or not, with the alkylating agent Dex-Mes (10 nM) (Simons, Jr. and Thompson, 1981), prior to operational model fitting ((Leff et al., 1990) (Supplemental Fig. 7). Budesonide produced a maximal response (E_{Max}), as defined by the upper asymptote of the concentration-responses curve, of 6.69 fold (Table 1). This was increased to 13.90 fold in the presence of formoterol, without material change, 8.50 and 8.40 respectively, to the pEC_{50} (Table 1). Pre-treatment with Dex-Mes depressed the E_{Max} produced by budesonide and budesonide plusformoterol by 37.5% and 41.7% respectively, without affecting potency (Table 1). Operational modelling of these response curves produced functionally derived pK_A values of 8.99 and 8.73 for budesonide in the absence and presence of formoterol, respectively (Table 1). Thus, formoterol did not alter the affinity of budesonide for GR.

LABAs do not affect glucocorticoid-induced nuclear translocation of GR. While enhanced GR nuclear translocation has been proposed to explain how LABAs enhance the therapeutic activity of ICS (Eickelberg et al., 1999; Roth et al., 2002; Usmani et al., 2005; Mortaz et al., 2008; Haque et al., 2013), other studies do not support this (Gruol and Altschmied,

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1993; Loven et al., 2007). To address this possibility, BEAS-2B cells were treated with a maximally effective concentration of dexamethasone prior to immunofluorescent imaging of GR. In untreated cells, GR was distributed diffusely throughout the cytosol, whereas following dexamethasone addition, translocation to the nucleus was evident by 15 min and was maintained for at least 6 h (Supplemental Fig. 8A). Salmeterol (100 nM) did not promote translocation of GR to the nucleus at 15 or 30 min, or 1, 2, 4 or 6 h (Supplemental Fig. 8B). Further analysis at 1 h confirmed these observations; using Pearson's correlation to assess the overlap between FITC-labelled GR and DAPI-stained nuclei, revealed no difference in the localization of GR between cells treated with dexamethasone or dexamethasone plus salmeterol (Fig. 4A).

Differential lysis was used to separate nuclear and cytoplasmic fractions of BEAS-2B cells. The effectiveness of separation was confirmed by western blot analysis of CREB1, a constitutively expressed nuclear transcription factor, and GAPDH, a cytoplasmic enzyme (Fig. 4B). Western blotting showed GR in the cytoplasmic extracts of untreated cells, but 1 h after fluticasone propionate or budesonide treatment there was a loss of cytoplasmic GR, with a corresponding increased of GR in the nuclear fractions (Fig. 4B). In contrast, salmeterol and formoterol alone were inactive and had no effect on the glucocorticoid-induced redistribution of GR (Fig. 4B). Similar data were produced at 2 h (Fig. 4B).

Mifepristone (RU486), which promotes nuclear translocation of GR (Chivers et al., 2004; Lewis-Tuffin et al., 2007), was essentially inactive (E_{Max} 1.6 ± 0.3 fold) on the 2×GRE reporter in BEAS-2B cells (Supplemental Fig. 9A). In the presence of formoterol (10 nM), RU486 concentration-dependently increased 2×GRE reporter activity (E_{Max} 3.52 ± 1.45 fold) and this effect was mimicked by salmeterol (100 nM), salbutamol (1 μM) and the adenylyl cyclase activator, forskolin (10 μM) (Supplemental Fig. 9B). While RU486 produced GR nuclear

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translocation, there was no additional effect of co-treatment with either salmeterol or formoterol, as determined by immunofluorescence microscopy or by cell fractionation combined with western blotting (Supplemental Fig. 9C & D). Thus, gross changes in GR localization cannot account for the ability of β_2-adrenoceptor agonists to convert RU486 into a weak partial agonist.

GR recruitment does not explain glucocorticoid/LABA synergy at the 2×GRE reporter. BEAS-2B 2×GRE cells were treated with a maximally effective concentration of budesonide in the absence and presence of formoterol prior to GR chromatin immunoprecipitation (ChIP). Initial analysis focused on a previously validated GR binding site located within an intronic region of FKBP5 (Fig. 5A) (Kadiyala et al., 2016), a gene that is highly glucocorticoid-induced, but not modulated by LABA (Kaur et al., 2008). ChIP qPCR primers were also designed to flank the 2 GRE sites in pGL3.2×GRE-luc.neo, the plasmid used to generate the stably transfected BEAS-2B 2×GRE cells (Fig. 5B) (Chivers et al., 2004; Kaur et al., 2008). Representative qPCR traces for FKBP5 and 2×GRE-luc are shown (Fig. 5C). There was no effect of formoterol alone on GR enrichment at these regions, but budesonide significantly enriched GR at the FKBP5 intronic region and the 2×GRE-luc construct (Fig. 5C & D). In the context of budesonide, formoterol was without further effect. While these data are consistent with FKBP5 mRNA not being affected by LABA (Kaur et al., 2008) (plus see Fig. 6), they indicate that increased GR recruitment to the simple 2×GRE in pGL3.2×GRE-luc does not account for the formoterol-induced enhancement of GRE-dependent transcription.

Effect of LABA on glucocorticoid-induced gene expression. As is described in a related manuscript (in preparation), BEAS-2B cells were treated with budesonide (100 nM) and formoterol (10 nM) alone or in combination for 1, 2, 6 and 18 h prior to expression analysis using Affymetrix PrimeView arrays. Following whole array normalization, data from 4

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independent experiments were combined and probe sets returning ≥ 2 fold or ≤ 0.5 fold change, with $P \leq 0.05$, for any treatment were used for subsequent analyses. While both formoterol and budesonide each increased and decreased gene expression, the current manuscript focuses on genes induced (≥2 fold, $P \leq 0.05$) by budesonide. These mRNAs showed a large range of inducibility by budesonide at each time, with the effects of formoterol varying from marked enhancement through to repression, with many mRNAs simply being unaffected. To illustrate these differences, heat maps were generated for budesonide-induced genes (budesonide: ≥2 fold, $P \leq 0.05$; but <2 fold formoterol) that were also induced ≥4 fold by budesonide or budesonide plus formoterol (Supplemental Fig. 10). Budesonide or budesonide plus formoterol induced peak mRNA expression in a gene- and time-dependent manner. In combination (compared to budesonide-treated as 100%), formoterol maximally enhanced the expression of budesonide-induced genes to 724, 406, 385 and 352 %, at 1, 2, 6 and 18 h respectively (Supplemental Fig. 10). The different mRNA kinetics are depicted for selected genes showing either enhancement by formoterol (1 or 2 h peak: PTGS2, TNFAIP3; PLAT; 6 h peak: ZFAND, PDK4, CDKN1C, KLF15; 18 peak: ADH1B, CNR1) no/little effect of formoterol (1 or 2 h peak KLF6, CEBPB, NFKBIA; 6 h peak: TSC22D3, FKBP5; 18 h peak: MAOA) or repression by formoterol (6 h peak: SLC16A12; 18 peak: SAA1) (Fig. 6A). In each case, qPCR (Supplemental Fig. 11A), or prior publications using glucocorticoid plus LABA combinations (Kaur et al., 2008; BinMahfouz et al., 2015; Joshi et al., 2015b; Altonsy et al., 2017), confirmed these data.

Examination of the heat maps in Supplemental Fig. 10, shows that many of the budesonide-induced mRNAs, which are enhanced by formoterol co-treatment, were also modestly induced by formoterol alone (albeit falling below the pre-determined 2 fold threshold). Similarly, the microarray data reveal many mRNAs to be induced by both budesonide and by formoterol

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(manuscript in preparation). Those mRNAs with ≥ 2 fold ($P \leq 0.05$) for each of budesonide and formoterol alone, but which also meet the additional criteria of being ≥ 4 fold for any treatment were therefore assembled into heat maps according to the time of peak expression and ranked according the effect of the budesonide plus formoterol treatment (Supplemental Fig. 12). Various interactions between the LABA and glucocorticoid are apparent. Multiple mRNAs showed predominantly budesonide-induced expression that was enhanced by formoterol (1 or 2 h peak: KLF4, SLC19A2; 6 h peak: SLC16A14), whereas others were predominantly formoterol-induced and enhanced by budesonide (2 h peak: CD200) (Fig. 6B). In addition, there were predominantly LABA-induced (2 h peak: RGS2; 6 h peak: CRISPLD2) or glucocorticoid-induced (6 peak: SLC16A14) mRNAs, where each stimulus alone produced a relatively modest response relative to the combination, but which yielded a marked synergy that occurred either early or later in the expression kinetic (Fig. 6B). However, glucocorticoid- plus separate LABA-inducibility does not necessarily produce synergistic interactions. Many mRNAs revealed simple additivity (DUSP1, CEBPD) and others (PER1, KLF9) showed glucocorticoid-inducibility that was essentially unchanged by LABA, this despite being LABA induced. Each of these expression profiles is either validated in the current study (Supplemental Fig. 11B) and/or in prior reports (Kaur et al., 2008; Holden et al., 2014; BinMahfouz et al., 2015; Joshi et al., 2015b).

Effect of LABA on GR recruitment to glucocorticoid-induced genes. To explore the effect of LABA on GR recruitment, ChIP-seq data was used to identify dexamethasone-induced GR binding sites at, or near to, multiple loci for glucocorticoid-regulated genes in BEAS-2B cells (Kadiyala et al., 2016). Many mRNAs (PTGS2, KLF4), which revealed robust glucocorticoid plus LABA synergy, had no major GR binding sites (GBSs) closely associated (≤ 100 kb) with

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their gene loci (Supplemental Fig. 13A). Other genes (*PLAT* or *RGS2*) revealed only weak GBSs and/or were located in gene-dense regions (not shown). In such instances direct regulation by GR cannot be assumed. However, multiple other glucocorticoid-induced gene loci revealed robust GBSs that were variously intronic (*CRISPLD2, FKBP5, KLF15, NFKBIA, TNFAIP3, SLC16A12*), closely associated with the 5′ region (*CDKN1C, KLF9, PDK4, SLC19A2, ZFAND5*), or both (*TSC22D3*) (Fig. 7A, Supplemental Fig. 13B & C) (Kadiyala et al., 2016). These are consistent with direct regulation by GR and GR recruitment was interrogated by ChIP-PCR.

Following budesonide treatment, GR was strongly recruited to GBSs at multiple loci for genes showing immediate/early (1 – 2 h peak) glucocorticoid-induced mRNA expression that was either markedly enhanced (TNFAIP3, SLC19A2) or unaffected by LABA (NFKBIA) (Fig. 7B, Supplemental 13B). In each case, GR binding to these sites was unaffected by formoterol alone or in combination with budesonide.

GR recruitment was also examined at 11 GBSs associated with loci for genes showing 6 h peak mRNA expression that was either strongly enhanced (CRISPLD2, PDK4, ZFAND5) or modestly enhanced/unaffected (FKBP5, KLF9, KFL15, TSC22D3) by LABA. In each case, the GBSs previously identified by ChIP-seq were confirmed as showing budesonide-induced enrichment of GR (Fig. 7B, Supplemental 13C). Furthermore, formoterol alone had no effect on GR recruitment to these sites and, with the exception of the R1 binding site of CRISPLD2, did not modify budesonide-induced recruitment (Fig. 7B, Supplemental 13C). While GR binding to the R2 region of *CRISPLD*2 was unaffected by formoterol, budesonide-induced GR binding to the R1 region was consistently enhanced by formoterol (Fig. 7B). This was borderline significant (ANOVA using Bonferroni's correction, $P \leq 0.05$) and raises the possibility that enhanced GR

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recruitment could contribute towards LABA-increased expression of glucocorticoid-induced CRISPLD2.

CDKN1C mRNA is also glucocorticoid-induced and synergistically enhanced by LABA. However, the *CDKN1C* locus is flanked by multiple genes, with the closest GR binding site lying within an intron of an adjacent gene (Supplemental Fig. 13C). Thus, a mechanistic link between GR binding and CDKN1C mRNA induction by glucocorticoid requires formal confirmation. Nevertheless, ChIP-PCR confirmed GR binding to this DNA region, and more modestly to the *CDKN1C 5′* promoter region, and in each case GR recruitment was unaffected by formoterol (Supplemental Fig. 13C).

Finally, SLC16A12 mRNA showed a delayed (6 h peak) kinetic that was repressed by formoterol. Inducible GR binding at two intronic sites was confirmed by ChIP-PCR (Fig. 7B). GR binding at the R2 region of *SLC16A12* was significantly induced by budesonide and was unaffected by formoterol. Conversely, budesonide induced GR binding to the R1 region showed a trend towards reduced recruitment in the presence of formoterol. While not significantly changed compared to budesonide-treated, GR binding to this RI region was also not significantly enhanced by budesonide plus formoterol when compared to untreated (Fig. 7B). Since budesonide-induced expression of SLC16A12 was reduced by formoterol, these data argue for modification of GR binding as a possible mechanism contributing to formoterol-induced repression.

LABA enhancement of glucocorticoid-induced gene expression can be insensitive to protein synthesis inhibition. To investigate a requirement for protein synthesis in the ability of formoterol (10 nM) to enhance glucocorticoid-induced gene expression, qPCR was performed for selected genes following 2 h treatment with glucocorticoid ± formoterol, each in the absence

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or presence of cycloheximide (Fig. 8). PTGS2 and KLF4 mRNAs both show 1 – 2 h peak expression and each revealed greater glucocorticoid-inducibility in the presence of cycloheximide (Fig. 8A, upper panels). However, the enhancement of dexamethasone-induced PTGS2 and KLF4 mRNA expression by formoterol co-treatment was also apparent in the presence of cycloheximide (Fig. 8A, upper panels). In respect of KLF4 mRNA, formoterol enhanced dexamethasone-induced expression by ~100% (i.e. ~2 fold) and this effect was unaltered by cycloheximide (Fig. 8A, lower right panel). With PTGS2 mRNA, cycloheximide resulted in a 7.0 ± 1.0 fold increase in basal expression, possibly due to the activating effect of cycloheximide on NF-κB and MAPK pathways, as previously reported (Newton et al., 1997). However, while formoterol co-treatment produced a significant ~300% (~4 fold) enhancement of dexamethasone-induced PTGS2 expression, this effect was limited to ~2 fold enhancement in the presence of cycloheximide (Fig. 8A, lower left panel). In the case of other mRNAs with rapid induction kinetics, KLF9, which showed a more modest, < 50%, enhancement of dexamethasone-induced mRNA expression by formoterol, and PLAT, which was more synergistically induced by dexamethasone plus formoterol, the enhancement produced by formoterol was essentially unaffected by cycloheximide (Supplemental Fig. 14A). However, such effects were not universal. Glucocorticoid-induced SLC19A2 mRNA was markedly enhanced by cycloheximide, but no enhancement was then observed with formoterol co-treatment (Supplemental Fig. 14A).

Analysis of PDK4 and CDKN1C mRNAs with peak expression at 6 h, both revealed inducibility by dexamethasone that was strongly enhanced, 4 or 7 fold, respectively, by formoterol (Fig. 8B). In the case of PDK4 mRNA, while cycloheximide increased the inducibility by dexamethasone, the 4 fold enhancement produced by formoterol co-

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administration was unaltered (Fig. 8B, left panels). For CDKN1C, there was little effect of cycloheximide on dexamethasone-induced mRNA expression and the enhancement of this by formoterol was also unaffected by cycloheximide (Fig. 8B, lower right panel). Likewise, TSC22D3 showed peak mRNA expression at 6 h. At 2 h, TSC22D3 mRNA was highly induced by dexamethasone and, consistent with prior data, showed a trend towards enhancement by formoterol. In the presence of cycloheximide, while glucocorticoid inducibility was markedly increased, the enhancement of this by formoterol was still apparent (Supplemental Fig. 14B). Similarly, glucocorticoid plus LABA synergy in respect of ZFAND5 persisted in the presence of cycloheximide (Supplemental Fig. 14B).

Discussion

ICS/LABA combination therapy is central to moderate-to-severe asthma management in patients where ICS monotherapy fails to achieve disease control. Accordingly, elucidating mechanisms by which ICS and LABA interact should aid efforts to rationally improve the clinical efficacy of this cost-effective therapy. While structural and infiltrating cells are potential targets of ICS/LABA therapy, the airway epithelium is an indispensable site of glucocorticoid action (Klassen et al., 2017). LABAs also act on the epithelium, and enhance glucocorticoid-induced gene expression in both bronchial epithelial BEAS-2B cells and in pHBECs (Newton and Giembycz, 2016). For this reason, BEAS-2B cells were selected to interrogate this therapeutically relevant interaction.

In BEAS-2B cells, glucocorticoids activate a luciferase reporter driven by two GRE sites and this is enhanced by β_2-adrenoceptor agonists (Kaur et al., 2008; Joshi et al., 2015b). In the

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present study, we confirm that these effects require GR and establish that the most productive interaction between LABA and glucocorticoid required concurrent addition. This is consistent with rapid-onset enhancement of glucocorticoid-induced luciferase accumulation and the transient glucocorticoid-induction of luciferase mRNA. Since increased transcription precedes luciferase mRNA accumulation, these data highlight a narrow window available for LABAs to enhance GR-driven transcription. LABAs rapidly activate a classical cAMP-PKA pathway, evidenced by the appearance of phosphorylated CREB1 within 10 mins, and PKA is necessary for enhancement of glucocorticoid-induced GRE activity (Kaur et al., 2008; Moodley et al., 2013). Thus, observed effects can occur rapidly and are mimicked by PKA over-expression (Yeagley and Quinn, 2005). The current demonstration that the enhancement of glucocorticoid-induced 2×GRE reporter activity, or the expression of multiple genes, by formoterol persisted under conditions of protein synthesis inhibition supports a direct PKA-mediated effect.

Several mechanisms could explain how LABAs enhance GR-dependent transcription (reviewed in (Newton and Giembycz, 2016)). In the current study, BEAS-2B cells are used to demonstrate that neither formoterol nor salmeterol significantly affected GR protein expression or the ability of glucocorticoids to down-regulate their cognate receptor, GR. Glucocorticoid-induced GR phosphorylation at Ser^{203}, Ser^{211} or Ser^{226}, which modify receptor function (Hapgood et al., 2016), was also unaffected by formoterol. Similarly, there was no effect of LABAs on the affinity of budesonide for GR. This confirms the results of a similar study that examined the effect of indacaterol on dexamethasone- and fluticasone furoate-induced GRE-dependent transcription (Joshi et al., 2015b). These data are also consistent with the lack of effect of LABAs on glucocorticoid sensitivity for the induction of the 2×GRE reporter (this study) or gene expression (Kaur et al., 2008; Rider et al., 2011; Joshi et al., 2015b). GR translocation

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necessarily precedes gene transcription and could represent a process that is regulated by LABAs. However, in BEAS-2B cells, salmeterol and formoterol had no effect alone or in the presence of glucocorticoid on nuclear translocation by GR. Furthermore, while budesonide recruited GR to a stably transfected 2×GRE luciferase reporter, this was unaffected by formoterol. ChIP-seq data from dexamethasone-treated BEAS-2B cells identified GBSs associated with multiple loci for glucocorticoid-induced mRNAs (Kadiyala et al., 2016). These may, or may not, show modulation by LABA. For example, ChIP-PCR confirmed budesonide-induced recruitment of GR to *FKBP5* and *NFKBIA*. This was unaffected by LABA and these mRNAs were not LABA-enhanced. ChIP-PCR also confirmed GR recruitment to 13 GBSs near to, or within, several budesonide-induced genes (*TNFAIP3, CRISPLD2, ZFAND5, SLC19A2, PDK4, CDKN1C, TSC22D3, KLF9* and *KLF15*), mRNAs for which were either highly, or more modestly, enhanced by formoterol co-treatment. At 12 of these sites, budesonide-induced GR recruitment was unaffected by formoterol. At one GBS, the R1 region of *CRISPLD2*, formoterol appeared to enhance budesonide-induced GR DNA binding. Conversely, glucocorticoid-induced SLC16A12 mRNA was repressed by formoterol. GR binding to the two main intronic sites for this gene showed robust budesonide-induced GR recruitment. However, binding to one site (R2) was unaffected by formoterol co-treatment, whereas the R1 region indicated reduced GR recruitment. Clearly, these data do not support general effects upstream of GR DNA binding to account for the LABA-enhancement of GR-driven transcription. This is important because LABAs do not invariably enhance GR-dependent transcription; indeed while many mRNAs were induced by glucocorticoid, only a fraction were enhanced by LABA co-treatment and some were even repressed. Similar gene expression patterns occur in pHBECs (manuscript in preparation). Thus, mechanistic explanations invoking generic effects on GR function cannot reconcile these

data. Modulation of GR expression, glucocorticoid binding affinity or GR nuclear translocation should affect all GR-induced genes. This was not apparent and is convincingly illustrated by reference to RU486, a glucocorticoid that usually behaves as a GR antagonist, but which shows transactivation in the presence of PKA activators (Nordeen et al., 1993). In BEAS-2B cells, RU486 induced GR translocation, but was transcriptionally silent on the 2×GRE reporter. However, LABAs showed no effect on GR translocation, but, nevertheless, converted RU486 into a weak partial agonist. Conversely, GR recruitment to the 2×GRE reporter and many glucocorticoid-induced gene loci was unaffected by LABA. Thus, the enhancement of GR-dependent transcription in BEAS-2B cells, and most likely in pHBECs, by LABAs must rely on pathways that modify transcriptional activation of specific genes rather than generic events leading to GR DNA binding.

While LABAs and glucocorticoids independently modulate gene expression, the gene-specific nature of synergy warrants comment. On the 2×GRE reporter, LABAs were inactive, yet they amplified glucocorticoid-induced transactivation without changing GR recruitment. This may be explained by the ability of the catalytic subunits of PKA to migrate to the nucleus (Meja et al., 2004), and phosphorylate substrates that promote GR-dependent transcription in a gene-dependent manner. Indeed, co-activators, basal and other components of the transcriptional machinery, histones, chromatin-modifying proteins and RNA polymerase II are all regulated phospho-proteins (Rowan et al., 2000; Vo and Goodman, 2001; Johannessen et al., 2004; Brookes and Pombo, 2009; Badeaux and Shi, 2013). Furthermore, many, including CREB1-binding protein and histones, are PKA targets (Vo and Goodman, 2001; Badeaux and Shi, 2013). Likewise, GR phosphorylation (other than at Ser^{203}, Ser^{211} or Ser^{226}) could produce gene-specific effects by modifying promoter-specific interactions.

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Direct modulation of GR-dependent transcription is unlikely as a sole mechanism for LABA-enhancement. Glucocorticoid-induced genes showing LABA-enhancement were either induced by budesonide (≥2 fold, $P \leq 0.05$), but not formoterol (<2 fold), or were induced by both drugs (each ≥2 fold, $P \leq 0.05$). For mRNAs showing independent LABA inducibility, one can presume roles for cAMP-activated transcription factors, such as CREB1 or related family members (Johannessen et al., 2004; Sands and Palmer, 2008). For example, CREB1 activates multiple genes, including *NR4A2*, which is highly up-regulated by LABA in BEAS-2B cells (Zhang et al., 2005; Yan et al., 2018). Similarly, CAAT/enhancer box proteins (CEBPs) are activated and/or induced by cAMP (Croniger et al., 1998; Ramji and Foka, 2002). These factors induce cAMP-dependent transcription and can participate in co-regulation of transcription with GR (Croniger et al., 1998; Ramji and Foka, 2002; Roos and Nord, 2012). Thus, transcriptional activation by GR and cAMP-activated factors represents a plausible explanation for glucocorticoid/LABA synergy at mRNAs, for example SLC19A2 and CRISPLD2, that are independently glucocorticoid- and LABA-inducible. Likewise, many budesonide-induced, formoterol-enhanced genes, for which formoterol inducibility was <2 fold were, nevertheless, significantly induced by formoterol alone. Thus, budesonide-induced expression of TNFAIP3 and PLAT was enhanced by formoterol co-treatment and these mRNAs were modestly up-regulated by formoterol alone. Similarly, while PDK4 and CDKN1C mRNAs were not significantly formoterol-induced in the current microarray data, LABA-induced increases were apparent by qPCR (Joshi et al., 2015b). Thus, induction by LABA is not readily separable from the enhancement of glucocorticoid-dependent effects and implies that independent activation by GR and cAMP-induced transcription factors could readily account for synergy. Independent LABA-inducibility was most apparent for those genes that were rapidly induced by glucocorticoid (1 - 2 h peak) when

compared to more slowly induced mRNAs. This is consistent with early induction kinetics for most LABA-induced genes, whereas transcription factors induced early could drive later-onset synergies. However, minimal, or even a lack of, induction by one stimulus is not incompatible with this model. Binding, or activation, by one factor may be necessary for binding, or activation, of another. For example, CEBPs promote chromatin rearrangements that can improve access and transcriptional activation by a second factor, such as GR (Grontved et al., 2013). While the current data do not support a general LABA-enhancement of GR binding to DNA, this is not excluded at specific sites. Indeed, such effects could underlie the apparent LABA-enhanced recruitment of GR to the *CRISPLD2* R1 region.

In summary, LABAs do not enhance expression of all glucocorticoid-inducible genes; their effects are gene-specific. Therefore, understanding the function of individual genes is necessary to appreciate those aspects of asthma pathogenesis that will respond most effectively to ICS/LABA therapy. Furthermore, knowledge of gene function is critical to optimize therapy. Knowing which glucocorticoid-inducible genes are beneficial, or undesirable, will aid screening strategies to identify novel GR ligands (steroidal or non-steroidal) that most effectively capture desirable synergies with a LABA. This may be especially important in severe asthma, where new GR agonists may be combined with a LABA to offer cost-effective alternatives to immune-modulatory biologicals.

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Declarations / conflict of interest

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Author contributions

Participated in research design: C.F.R., C.K.M., A.M.L., A.N.G., M.A.G., R.N.

Conducted experiments: C.F.R., M.O.A., M.M.M., S.V.S., S.S., M.L.M., D.Y., R.N.

Contributed new reagent or analytical tool: ANG

Performed data analysis: C.F.R., M.O.A., M.M.M., S.V.S., S.S., M.L.M., D.Y., A.N.G., R.N.

Wrote or contributed to the writing of the manuscript: C.F.R., M.O.A., M.M.M., S.V.S., S.S., M.L.M., D.Y., C.K.M., A.M.L., A.N.G., M.A.G., R.N.

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Footnotes

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Legends for Figures

Fig. 1. Effect of glucocorticoid on transcriptional activation by LABA. (A) BEAS-2B cells were treated with formoterol (Form; 10 nM) and/or dexamethasone (Dex; 1 μM) for the times indicated prior to harvesting for western blot analysis of serine 133 phosphorylated CREB1 (P-CREB1) and GAPDH. Phosphorylated ATF1 (P-ATF1), as indicated, was also detected. Data for total CREB appear as Supplemental Fig. 1. Blots representative of *N* independent experiments are shown. Following densitometric analysis, data for P-CREB1 were normalized to GAPDH, expressed as fold of untreated and are plotted as mean ± SE. Statistical significance comparing each formoterol treatment time to t = 0 and to the effect of Dex + Form, at each time, was tested by ANOVA with Dunn's multiple comparisons test. Compared to t = 0; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (B) BEAS-2B cells harboring a 6×CRE luciferase reporter were treated with various concentrations of formoterol in the absence or presence of dexamethasone (1 μM). Cells were harvested after 6 h for luciferase assay. Data from *N* independent experiments are expressed as fold of untreated and are plotted as mean ± SE.

Fig. 2. Formoterol rapidly enhances GR-mediated simple GRE-dependent transcription. (A) BEAS-2B cells harboring a 2×GRE luciferase reporter (BEAS-2B 2×GRE cells) were treated with formoterol (Form; 10 nM) and/or the indicated concentrations of dexamethasone (Dex). After 6 h, cells were harvested for luciferase assay. Data, from *N* independent experiments, expressed as fold of untreated, are plotted as mean ± SE. Statistical significance for the fold induction between naive and formoterol treated was evaluated by Wilcoxon matched-pairs signed rank test at each dexamethasone concentration. * $P < 0.05$, ** $P < 0.01$. (B) BEAS-2B 2×GRE cells were pre-

treated with Org34517 (Org; 1 μM), or not, prior to stimulation with the dexamethasone (1 μM) in the absence or presence of formoterol (Form; 10 nM). After 6 h, cells were harvested for luciferase assay. Data, from *N* independent experiments, expressed as fold of untreated, are plotted as mean ± SE. Statistical significance for the fold induction between the indicated treatments was tested by ANOVA with Bonferroni's correction. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (C) BEAS-2B 2×GRE cells were treated with dexamethasone (1 μM). Formoterol (10 nM) was added either with the dexamethasone (t = 0) or at various times prior (-6, -4, -2, -1, -½ or -¼ h) to or at various times after (¼, ½, 1, 2 or 4 h) the addition of dexamethasone. In each case, cells were harvested for luciferase assay 6 h after the addition of dexamethasone. Data, from *N* independent experiments, expressed as fold of untreated, are plotted as mean ± SE. Statistical significance relative to dexamethasone was examined by ANOVA with a Dunnett's post-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (D) BEAS-2B 2×GRE reporter cells were treated with dexamethasone (1 μM), formoterol (10 nM), or the combination (Dex + Form). Cells were harvested at the indicated times for luciferase assay. Data (upper panel), from *N* independent experiments, are plotted as fold of no stimulation (NS) as means ± SE. To more clearly show the enhancement by formoterol at each time (lower panel), the absolute increase (over NS) produced by dexamethasone + formoterol was expressed as a percentage of the absolute increase (over NS) produced by dexamethasone alone (100%, dotted line) and is plotted as mean ± SE. (E) BEAS-2B 2×GRE reporter cells were treated with dexamethasone (1 μM) in the absence or presence of formoterol (10 nM) and/or cycloheximide (CHX; 100 μg/ml) as indicated. Cells were harvested after 2 h for qPCR analysis of luciferase (Luc) and GAPDH mRNA. Data (upper panel) from *N* independent experiments, expressed as Luc/GAPDH, are plotted as fold of untreated as means ± SE. Statistical significance relative to untreated control, or cycloheximide alone, as appropriate, was examined by ANOVA

with a Dunn's post-test. To evaluate the effect of formoterol on the induction by dexamethasone, each in the absence or presence of cycloheximide, fold data were analyzed by Wilcoxon matched-pairs signed rank test. ** $P < 0.01$, *** $P < 0.001$. Data were also replotted (lower panel) to show the effect of formoterol on the induction of luciferase RNA by dexamethasone in the absence or presence of CHX. In each case, the data are expressed a percentage of dexamethasone or dexamethasone + cycloheximide, as appropriate. Comparing without and with cycloheximide, no significant difference in this percentage was apparent as assessed by paired t test.

Fig. 3. No effect of LABAs on the glucocorticoid-dependent loss of GR expression or on GR phosphorylation. (A) BEAS-2B cells were either not treated or treated with dexamethasone (Dex; 1 μM), formoterol (Form; 10 nM), or salmeterol (Salm; 100 nM) alone or in combination. Cells were harvested at the indicated times prior to western blot analysis for GR and GAPDH. Blots representative of *N* independent experiments are shown. Following densitometric analysis, data, normalized to GAPDH, were expressed as fold of untreated at each time and are plotted as mean ± SE. Statistical significance compared to untreated control was tested by ANOVA with a Dunnett's post-test. ** $P \leq 0.01$, *** $P \leq 0.001$. (B) BEAS-2B cells were treated with dexamethasone (1 μM) and/or the LABAs formoterol (10 nM) or salmeterol (100 nM). After 2 h, the cells were harvested for western blot analysis of S203, S211 and S226 phosphorylated GR (P-GR) and GAPDH. Densitometric data from *N* independent experiments were normalized to GAPDH, expressed as fold of untreated and are plotted as means ± SE. Statistical significance compared to untreated and between dexamethasone and dexamethasone plus LABAs was tested by ANOVA with Bonferroni's correction. Compared to untreated: * $P \leq 0.05$, ** $P \leq 0.01$.

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Fig. 4. No effect of LABAs on nuclear translocation by GR. (A) BEAS-2B cells at ~70% confluence were either not stimulated or treated for 1 h with dexamethasone (Dex; 1 μM), salmeterol (Salm; 100 nM) or both drugs in combination. Slides were fixed and probed for GR (green) and the nuclei counterstained with DAPI (blue) as described for Supplemental Fig. 8. (Right panel) Correlation between Alexa 488 (GR) staining and DAPI (nuclei) was performed using Perkin Elmer Volocity software. Analysis was performed on all cells in 5 images for each treatment. Data, expressed as the Pearson correlation, are presented as mean ± SE. (B) BEAS-2B cells were treated with fluticasone propionate (FP; 100 nM) and/or salmeterol (100 nM) or budesonide (Bud; 100 nM) and/or formoterol (Form; 10 nM) for 1, or 2 h, as indicated. Cytoplasmic and nuclear extracts were prepared and subjected to western blotting for GR, CREB1 (nuclear) and GAPDH (cytoplasmic). A total cell lysate was used as a positive control (Total). Blots representative of *N* such experiments are shown. Densitometry was performed on the cytoplasmic (GR/GAPDH) and nuclear (GR/CREB1) extracts to generate the figures shown in lower panels. Data, expressed as fold, are plotted as means ± SE. Multiple comparison testing between each treatment and untreated and between treatments with glucocorticoid and glucocorticoid plus LABA was performed by ANOVA with a Dunn's post-test. Compared to untreated: * $P \leq 0.05$, ** $P \leq 0.01$.

Fig. 5. Recruitment of GR to GRE is not affected by LABA. (A) BEAS-2B cells were treated for 1 h with dexamethasone (Dex; 0.1 μM) or vehicle control. ChIP-seq analysis for GR revealed robust GR DNA sites within intronic regions of the glucocorticoid-induced gene, FKBP5 (Kadiyala et al., 2016). Multiple putative GREs were identified by TFBIND and qPCR primers were designed to flank these sites. (B) Schematic showing the 2×GREs cloned upstream of a

TATA box and transcription start driving the luciferase gene in the pGL3.2×GRE-luc.neo plasmid used to generate the BEAS-2B 2×GRE-luc reporter cells. As shown, qPCR primers were designed to flank the 2×GRE. (C) BEAS-2B cells were either not stimulated (NS) or treated with formoterol (Form; 10 nM), budesonide (Bud; 100 nM) or both combined (B + F). ChIP was performed using an anti-GR antibody and qPCR was performed for three unrelated control regions of genomic DNA, FKBP5 and the 2×GRE of pGL3.2×GRE-luc.neo. Representative qPCR traces are shown for genomic DNA near the MYOD gene (Control region), FKBP5 and 2×GRE-luc. (D) Data from *N* experiments are shown for FKBP5 and 2×GRE-luc normalized to the control regions and are plotted as $\log_2$ fold. Statistical significance was tested between untreated and budesonide, formoterol, and budesonide + formoterol and between budesonide and budesonide + formoterol using ANOVA with Bonferroni's correction. ***, $P < 0.001$ verses untreated. There were no significant differences between budesonide and budesonide + formoterol.

Fig. 6. Effect of formoterol on budesonide-induced gene expression. BEAS-2B cells were either not stimulated (NS) or treated with budesonide (Bud; 100 nM), formoterol (Form; 10 nM) or both together (Bud + Form). At 1, 2, 6 and 18 h RNA was extracted and microarray analysis performed. (A) mRNAs that were induced ≥2 fold ($P \leq 0.05$) by budesonide, but not by formoterol alone (< 2 fold), were selected from Supplemental Fig. 10 and are presented according to the time (1, 2, 6 or 18 h) of peak mRNA expression. (B) mRNAs that were induced ≥2 fold ($P \leq 0.05$) by budesonide and by formoterol were selected from Supplemental Fig. 12 and are presented according to the time (1, 2, 6 or 18 h) of peak mRNA expression. In both A and B, combined array data ($N = 4$) are plotted as fold of NS for each time.

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Fig. 7. Effect of LABA on GR recruitment to GR binding sites in immediate/early and delayed glucocorticoid-induced genes. (A) GR ChIP-seq analysis in control and dexamethasone (Dex; 100 nM)-treated BEAS-2B cells, published by Kadiyala et al. (2016), reveals robust GR binding sites within intronic regions of TNFAIP3, CRIPSLD2 and SLC16A12 and immediately 5′ to SCL19A2 and ZFAND5 (left panels). GR peaks regions were checked for the existence of putative GREs using MatInspector (Genomatix) and TFBIND software. Primers for qPCR were designed, as shown, to validate single or multiple regions (R1, R2) showing robust ChIP-seq peaks. (B) BEAS-2B cells were either not stimulated or treated with formoterol (Form; 10 nM), budesonide (Bud; 100 nM) or both combined for 1 h. GR ChIP was performed and qPCR was performed for three unrelated control regions of genomic DNA and the indicated regions of TNFAIP3, SLC19A2, ZFAND5, CRISPLD2, and SLC16A12. Data from *N* experiments are shown normalized to the control regions and are plotted as $\log_2$ enrichment (right panels). Statistical significance was tested between untreated and budesonide, formoterol and budesonide + formoterol and between budesonide and budesonide + formoterol using ANOVA with Bonferroni's correction. ** $P < 0.01$, *** $P < 0.001$ verses untreated. There were no significant differences between budesonide and budesonide + formoterol.

Fig. 8. Effect of the protein synthesis inhibitor, cycloheximide, on the enhancement of glucocorticoid-induced transcription by LABA for mRNAs showing: (A) 1 – 2 h peaks; and, (B) 6 h peaks in expression. BEAS-2B cells were either not stimulated (NS) or treated with dexamethasone (Dex; 1 μM) each in the absence or presence of formoterol (Form; 10 nM) and/or cycloheximide (CHX; 100 μg/ml) as indicated. Cells were harvested after 2 h for qPCR analysis of the indicated genes. Data (upper panel) from *N* independent experiments, expressed as the gene

of interest/GAPDH, are plotted as fold of NS as mean ± SE. The effect of formoterol on the induction by dexamethasone, each in the absence or presence of cycloheximide, was assessed using fold data by Wilcoxon matched-pairs signed rank test. ** $P < 0.01$. Data were also replotted (lower panel) to show the effect of formoterol on the induction of luciferase RNA by dexamethasone each in the absence or presence of cycloheximide. Data are expressed as a percentage of dexamethasone or dexamethasone + cycloheximide, as appropriate and differences in this percentage comparing without and with cycloheximide were assessed by paired t test. ** $P < 0.01$.

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Tables

Table 1

Operational modelling using 2×GRE-dependent transcription to calculate budesonide affinity (K_A) in absence and presence of LABA.

	Treatments			
	Budesonide		Budesonide + Formoterol	
	Naive	+ Dex-MES	Naive	+ Dex-MES
E_{Max} (Fold)	6.69 ± 0.27	4.18 ± 0.26	13.90 ± 0.65	8.11 ± 0.37
pEC_{50}	8.50 ± 0.09	8.34 ± 0.13	8.40 ± 0.11	8.26 ± 0.11
pK_A	8.99 ± 0.82		8.73 ± 0.62	
K_A/EC_{50}	0.32	-	0.47	-

BEAS-2B 2×GRE reporter cells were either not treated or treated with dexamethasone-21-mesylate (Dex-MES;10 nM) for 30 min. The medium was then removed and the cells rinsed with serum free medium prior to treatment with various concentrations of budesonide, each in the absence or presence of formoterol (10 nM). After 6 h, the cells were harvested for luciferase assay. Data are plotted in Supplemental Fig. 7. GraphPad Prism 6.01 was used to generate parameters from concentration-response curves. Operational model fitting (Leff et al., 1990), as applied to GR (Joshi et al., 2015b), was used to provide pK_A values for budesonide in the absence and presence of formoterol. The K_A/EC_{50} ratio being less than 1 indicates that there is no receptor reserve.

Fig. 1.

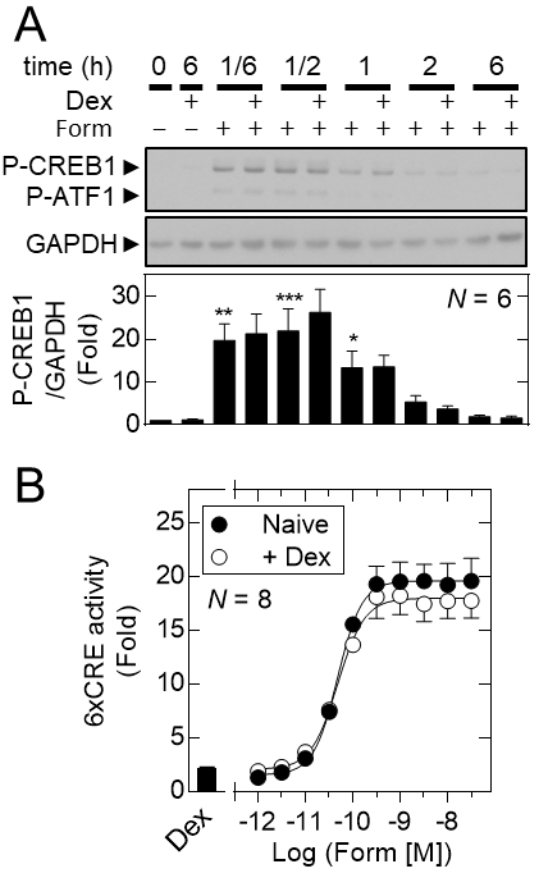

A
time (h)
0 6 1/6 1/2 1 2 6
Dex
Form
P-CREB1
P-ATF1
GAPDH
P-CREB1 /GAPDH (Fold)
N = 6
B
6xCRE activity (Fold)
Naive
+ Dex
N = 8
Dex
Log (Form [M])

Fig. 2

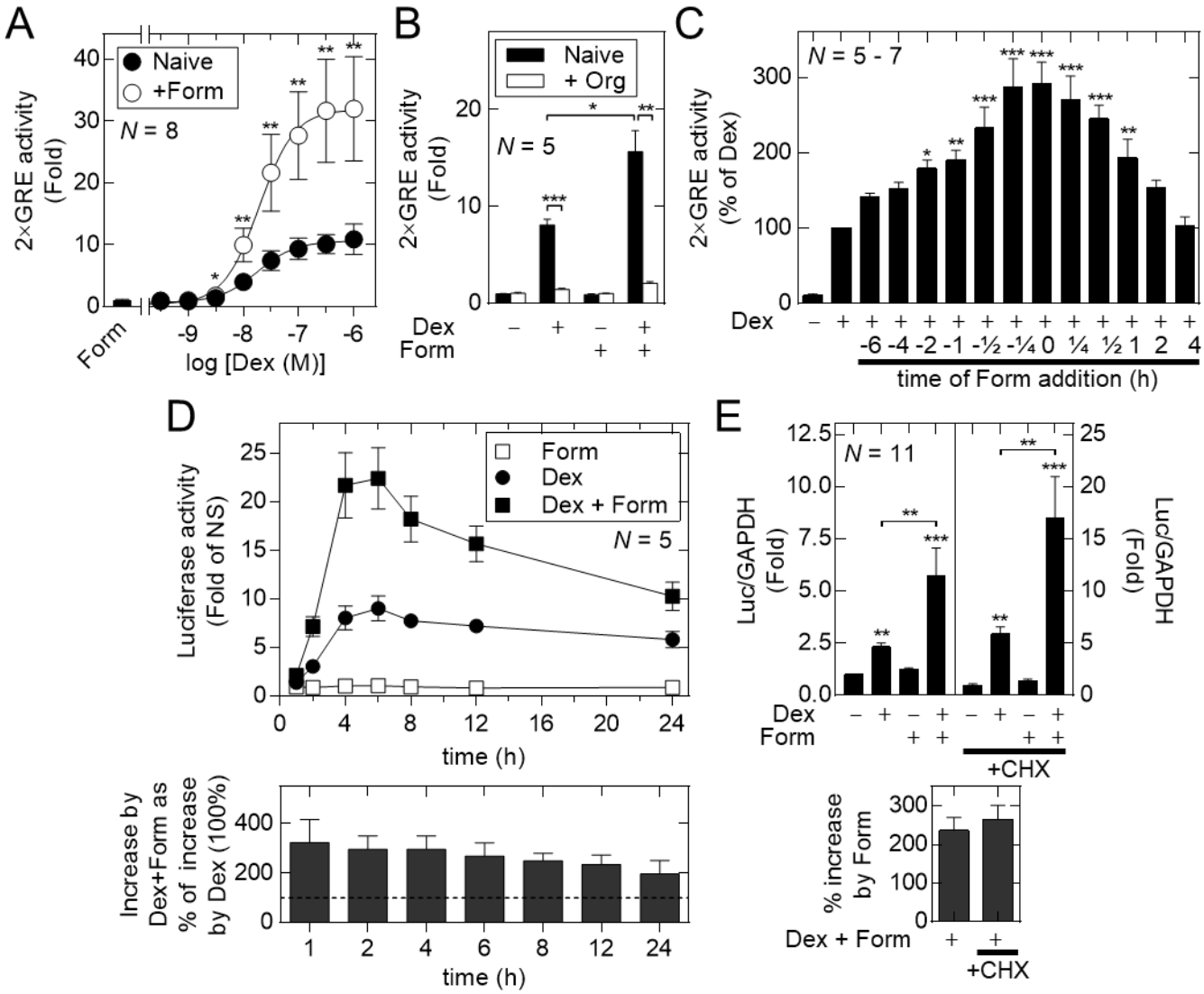

A
Naive
+Form
N = 8
2×GRE activity (Fold)
Form
log [Dex (M)]
B
Naive
+ Org
N = 5
2×GRE activity (Fold)
Dex
Form
C
N = 5 - 7
2×GRE activity (% of Dex)
Dex
-6 -4 -2 -1 -½ -¼ 0 ¼ ½ 1 2 4
time of Form addition (h)
D
Form
Dex
Dex + Form
N = 5
Lucferase activity (Fold of NS)
time (h)
Increase by Dex+Form as % of increase by Dex (100%)
time (h)
E
N = 11
Luc/GAPDH (Fold)
Dex
Form
+CHX
% increase by Form
Dex + Form
+CHX

Fig. 3.

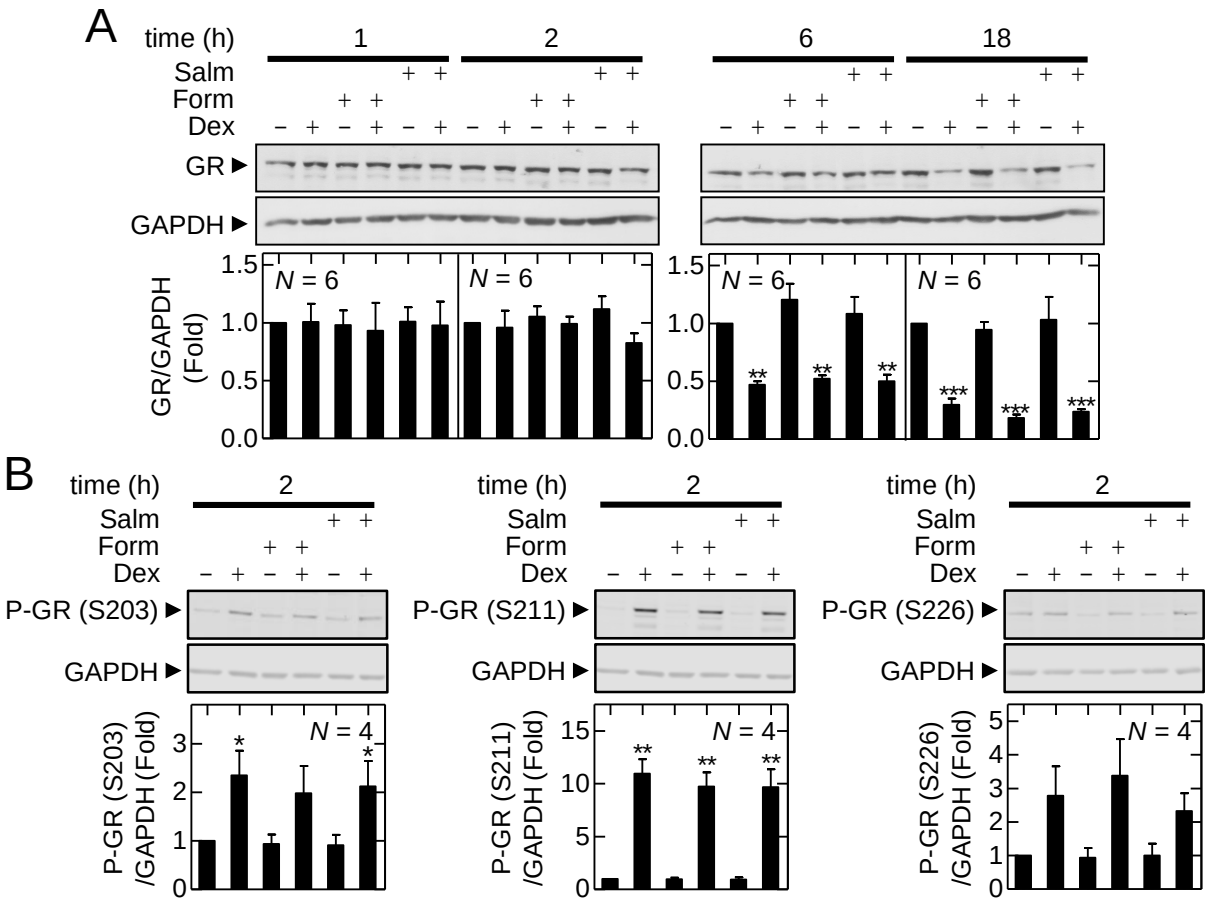

A
time (h)
1
2
6
18
Salm
Form
Dex
GR
GAPDH
GR/GAPDH (Fold)
N = 6
B
P-GR (S203)
P-GR (S211)
P-GR (S226)
P-GR (S203) /GAPDH (Fold)
P-GR (S211) /GAPDH (Fold)
P-GR (S226) /GAPDH (Fold)
N = 4

Fig. 4.

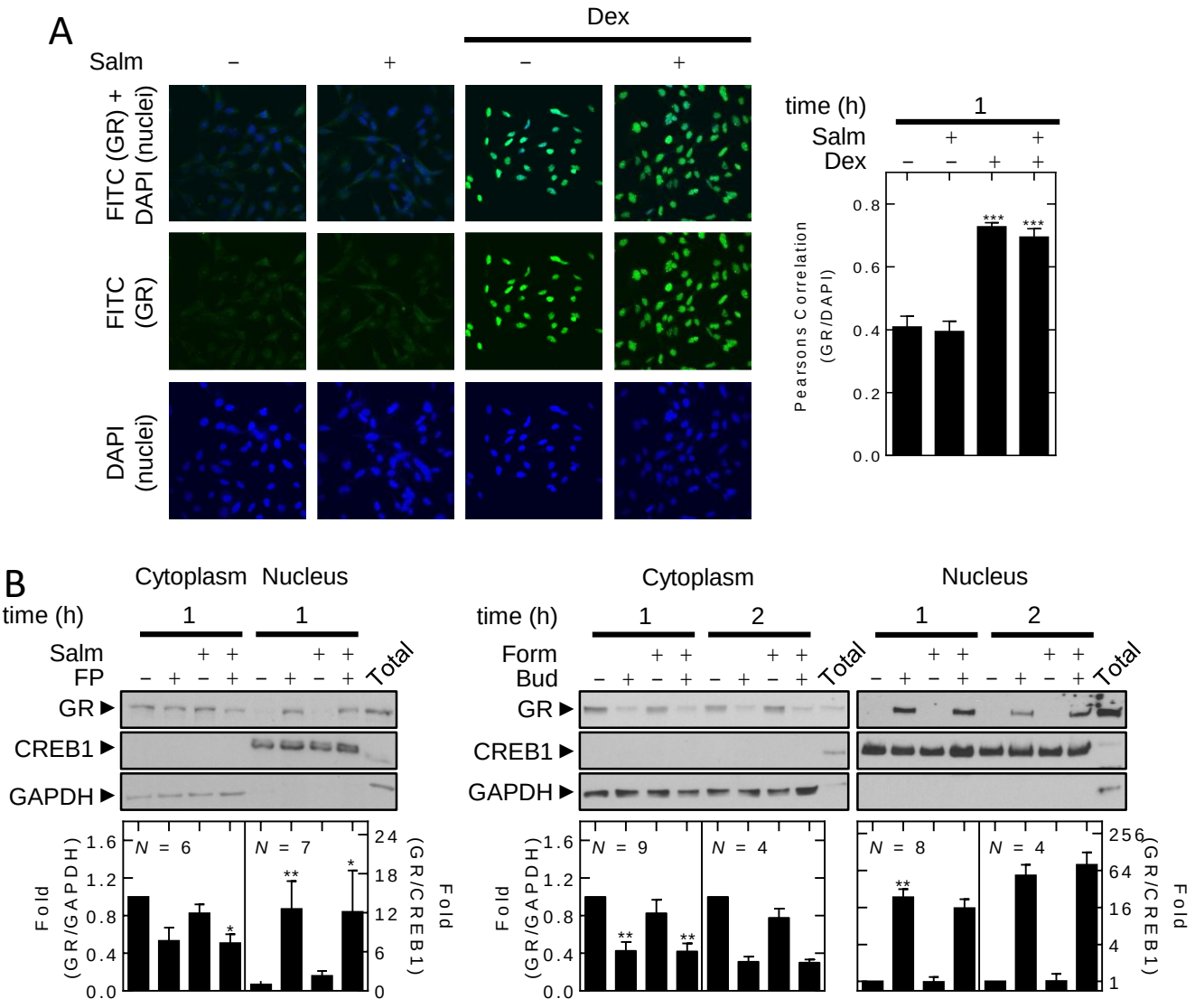
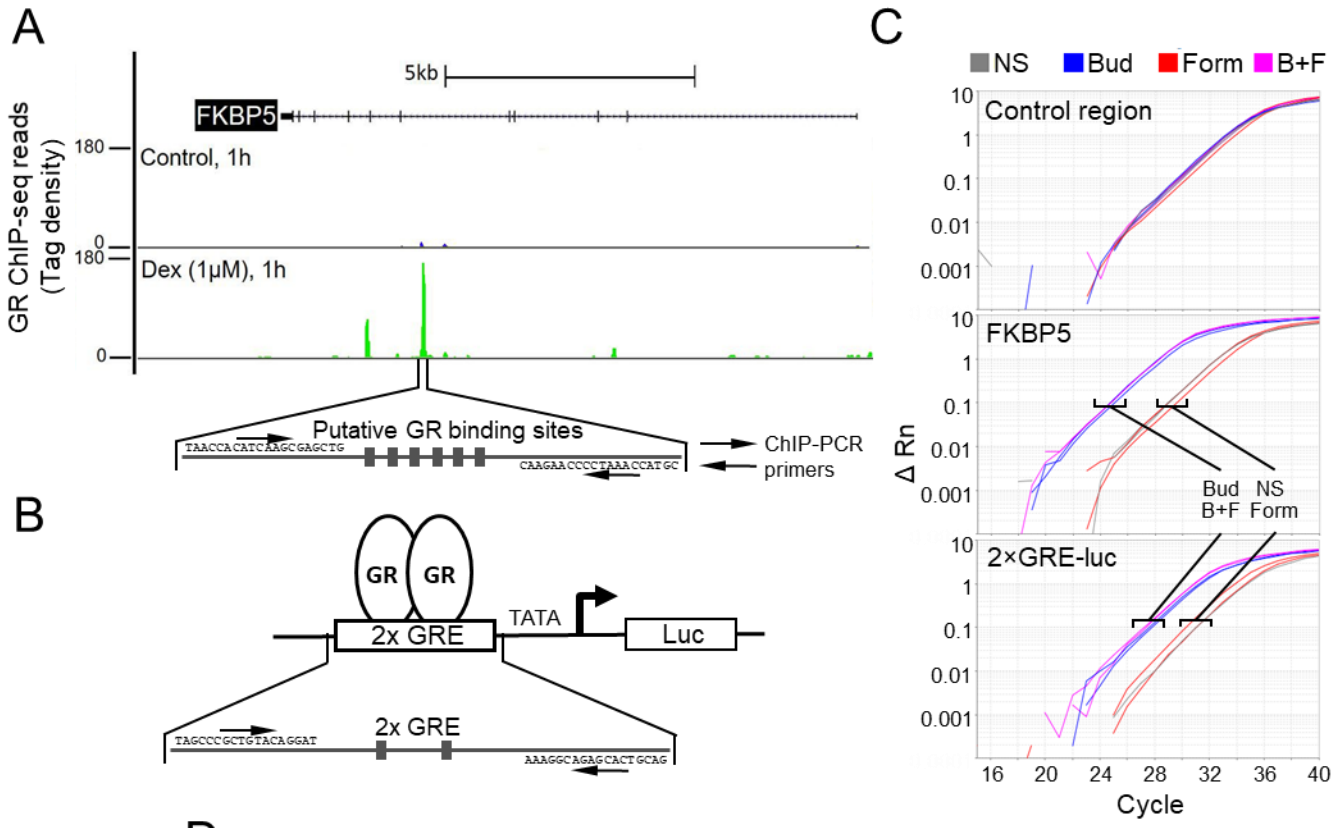

A
Dex
Salm
FITC (GR) + DAPI (nuclei)
FITC (GR)
DAPI (nuclei)
time (h)
Pearsons Correlation (GR/DAPI)
B
Cytoplasm
Nucleus
Salm
FP
Form
Bud
Total
GR
CREB1
GAPDH
Fold (GR/GAPDH)
Fold (GR/CREB1)
N = 6
N = 7
N = 9
N = 4
N = 8

Fig. 5


A
5kb
FKBP5
Control, 1h
Dex (1μM), 1h
GR ChIP-seq reads (Tag density)
Putative GR binding sites
ChIP-PCR primers
B
GR
GR
2x GRE
TATA
Luc
2x GRE
C
NS
Bud
Form
B+F
Control region
FKBP5
2×GRE-luc
Δ Rn
Bud B+F
NS Form
Cycle

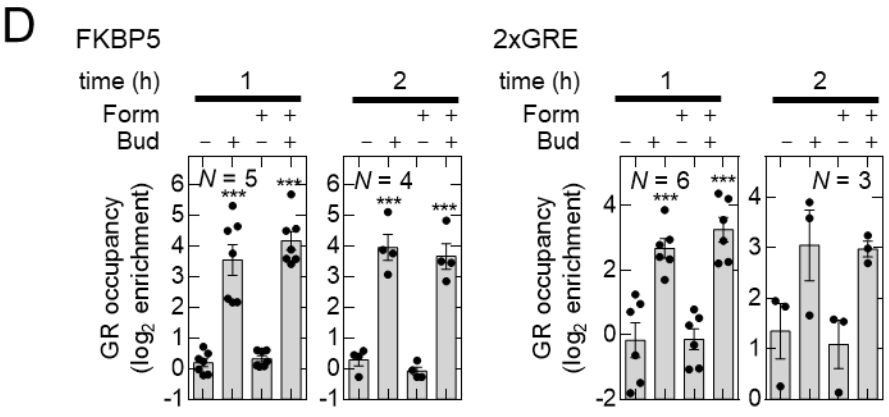
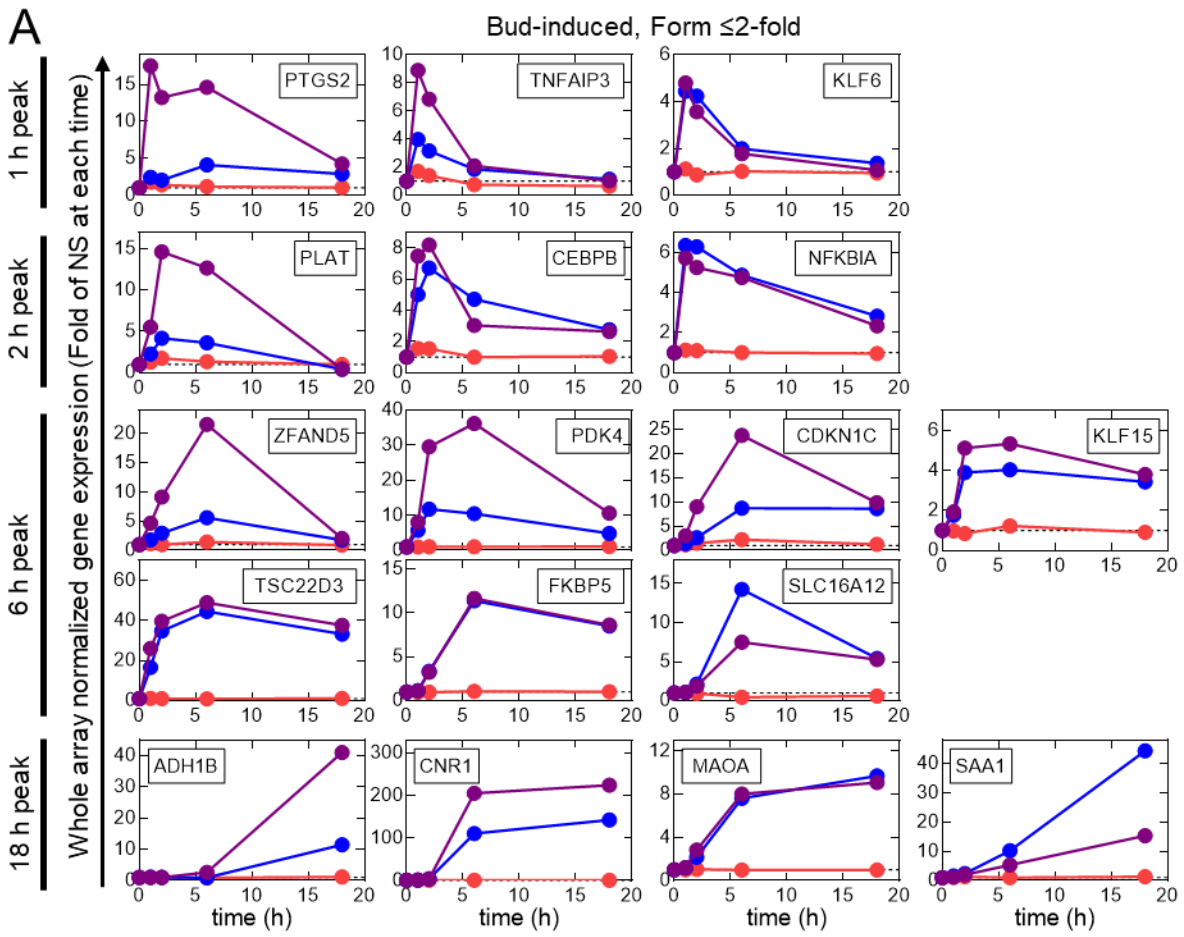

D
FKBP5
2xGRE
time (h)
Form
Bud
GR occupancy (log2 enrichment)
N = 5
N = 4
N = 6
N = 3

Fig. 6


A
Bud-induced, Form ≤2-fold
1 h peak
2 h peak
6 h peak
18 h peak
Whole array normalized gene expression (Fold of NS at each time)
PTGS2
TNFAIP3
KLF6
PLAT
CEBPB
NFKBIA
ZFAND5
PDK4
CDKN1C
KLF15
TSC22D3
FKBP5
SLC16A12
ADH1B
CNR1
MAOA
SAA1
time (h)

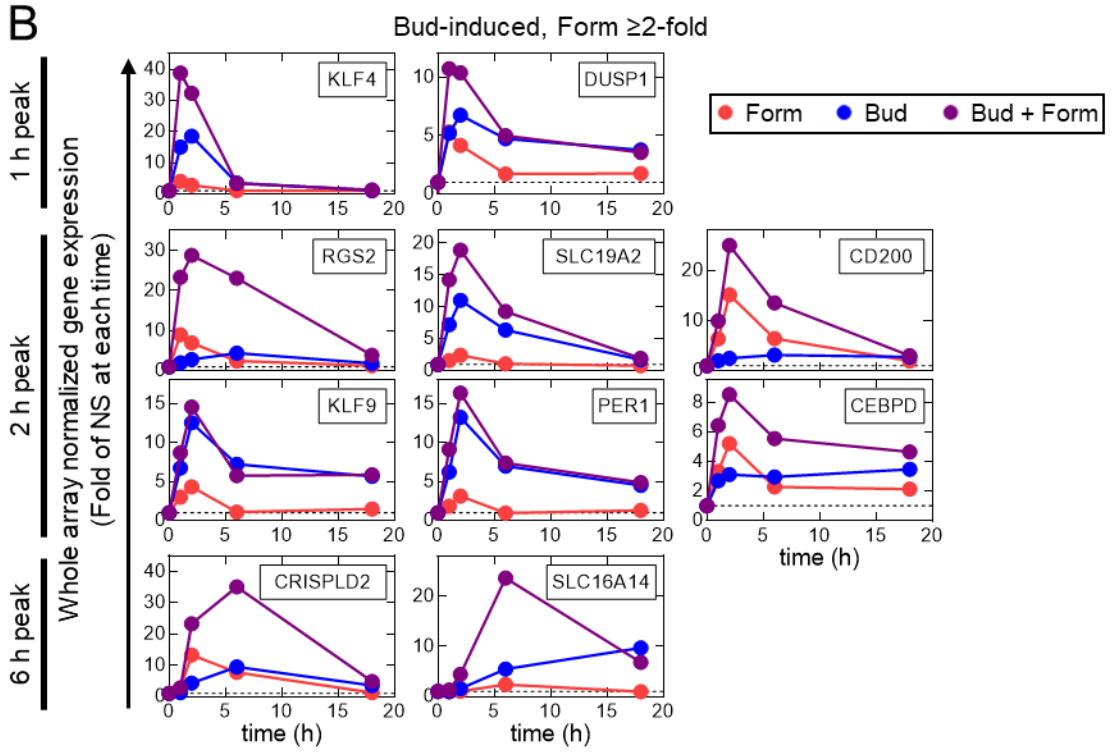

B
Bud-induced, Form ≥2-fold
1 h peak
2 h peak
6 h peak
Whole array normalized gene expression (Fold of NS at each time)
Form
Bud
Bud + Form
KLF4
DUSP1
RGS2
SLC19A2
CD200
KLF9
PER1
CEBPD
CRISPLD2
SLC16A14
time (h)

Fig. 7

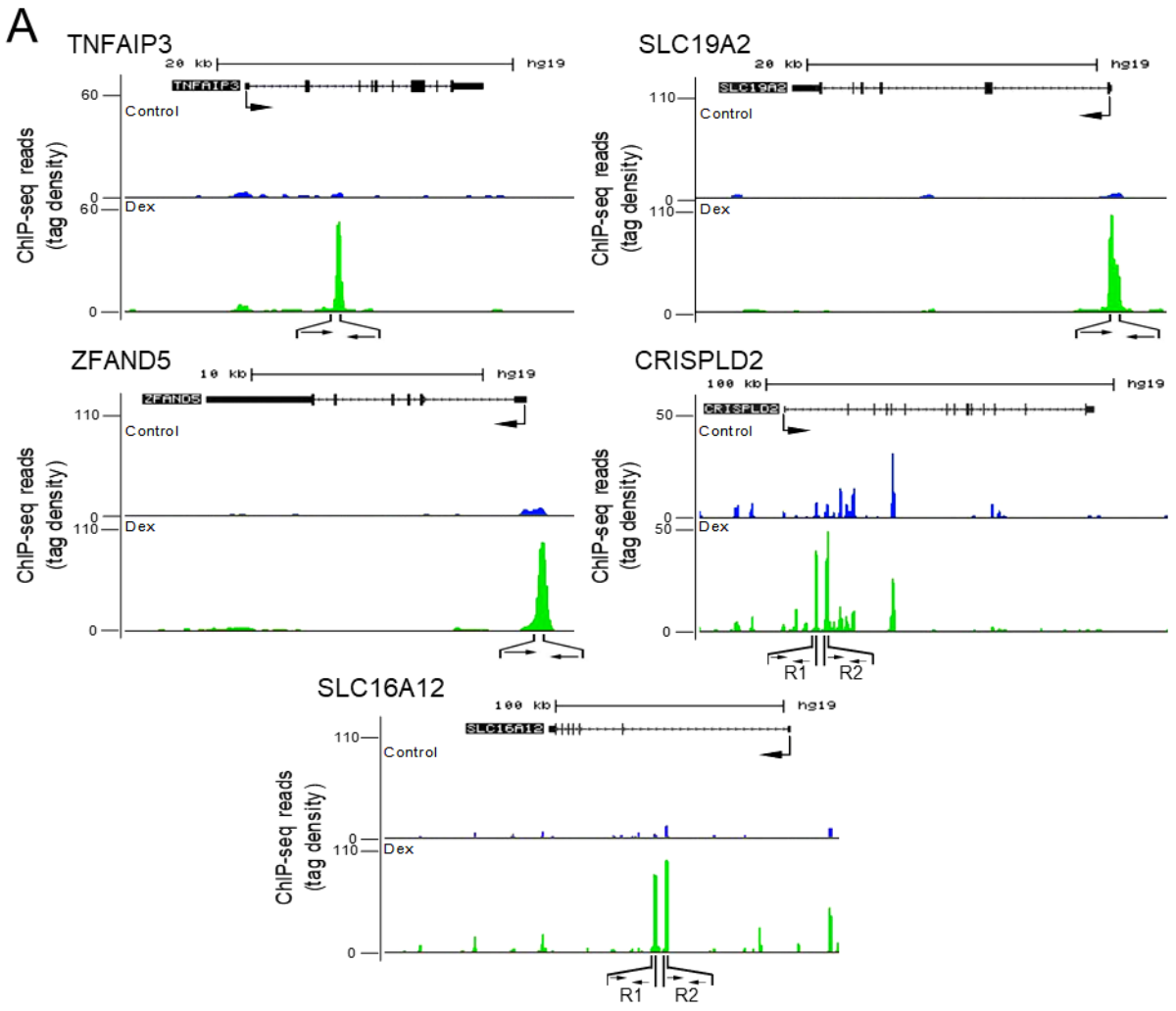
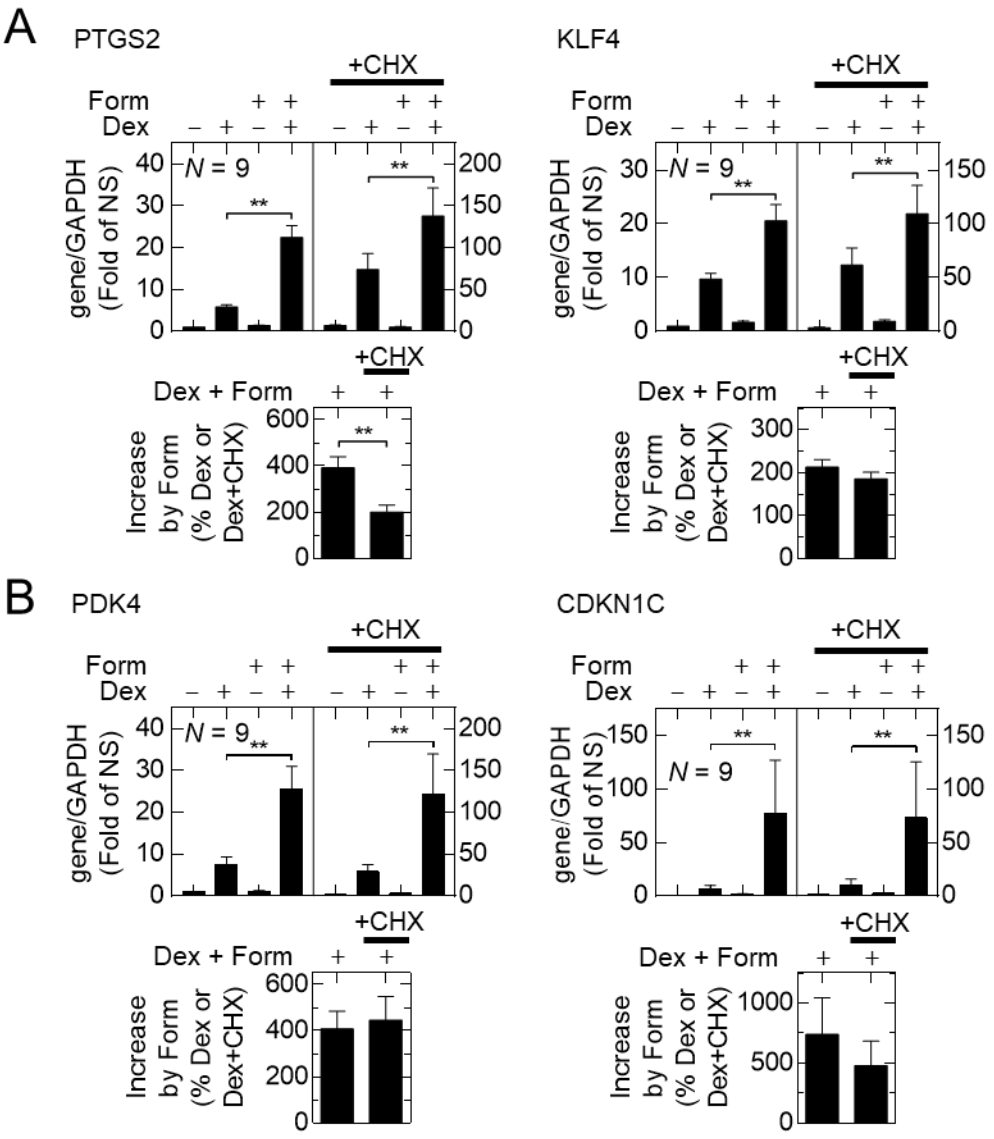

A
TNFAIP3
SLC19A2
ZFAND5
CRISPLD2
SLC16A12
Control
Dex
ChIP-seq reads (tag density)
R1
R2
B
TNFAIP3
SLC19A2
ZFAND5
CRISPLD2
SLC16A12
Form
Bud
GR occupancy (log2 enrichment)
N = 4
N = 5

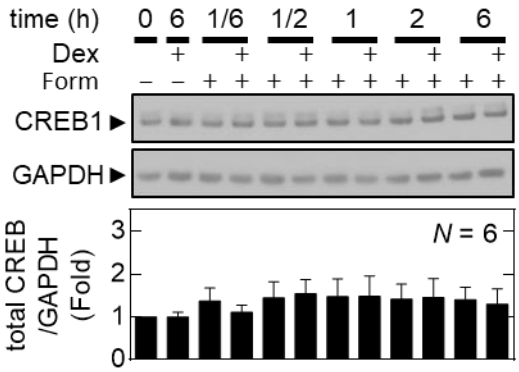
Fig. 8


A
PTGS2
KLF4
+CHX
Form
Dex
gene/GAPDH (Fold of NS)
N = 9
**
Dex + Form
Increase by Form (% Dex or Dex+CHX)
B
PDK4
CDKN1C

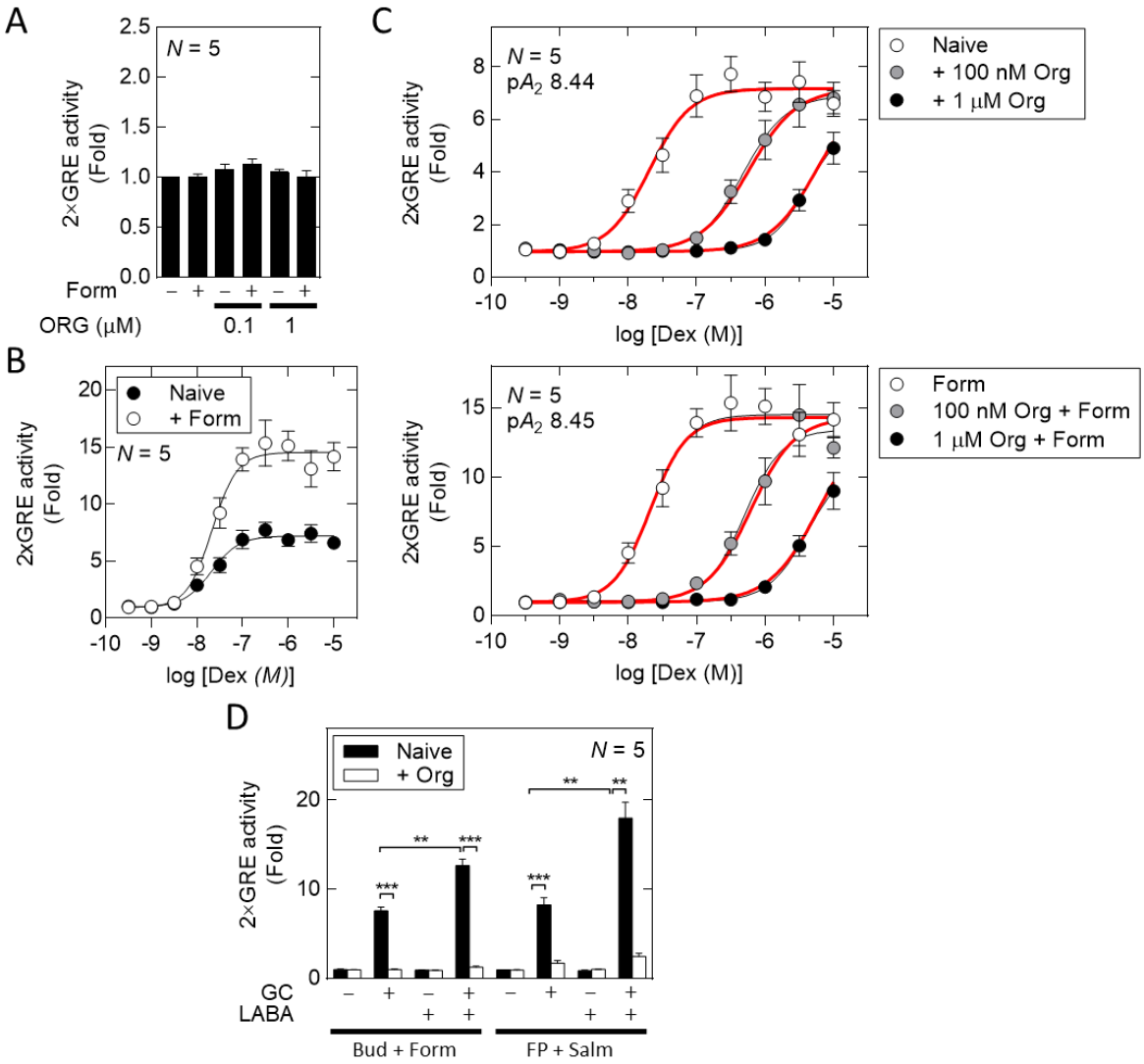
Supplementary data

Long-acting β_2-adrenoceptor agonists enhance glucocorticoid receptor (GR)-mediated transcription by gene-specific mechanisms rather than generic effects via GR

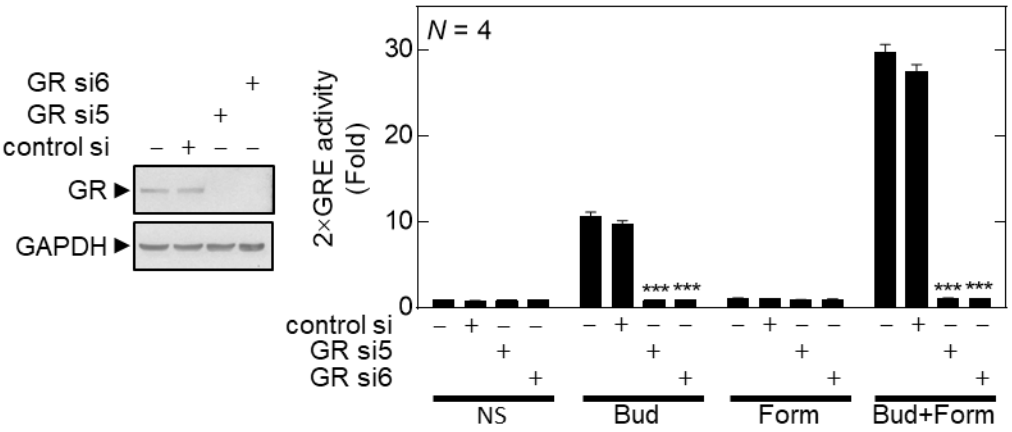
Christopher F. Rider, Mohammed O. Altonsy, Mahmoud M. Mostafa, Suharsh Shah, Sarah Sasse, Martijn L. Manson, Dong Yan, Carina Kärrman-Mårdh, Anna Miller-Larsson, Anthony N. Gerber, Mark A. Giembycz, Robert Newton



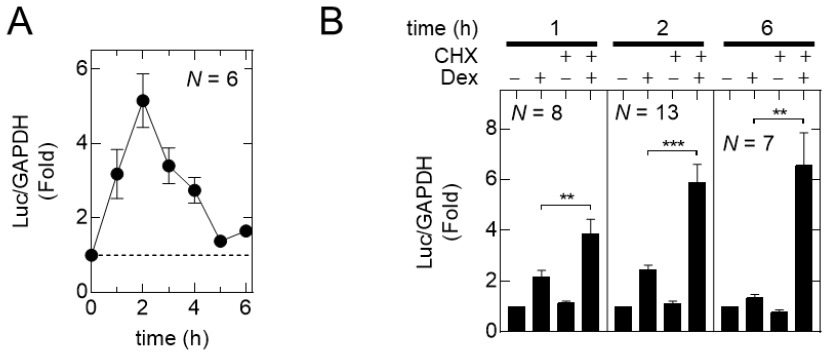
Supplemental Fig. 1. Effect of glucocorticoid and LABA on the expression of CREB1. BEAS-2B cells were treated with formoterol (Form; 10 nM) and/or dexamethasone (Dex; 1 μM) for the times indicated prior to harvesting for western blot analysis of serine 133 phosphorylated CREB1 (P-CREB1), total CREB1 (CREB1) and GAPDH. Blots representative of *N* independent experiments are shown. Data for P-CREB1 are shown in Fig 1A of the main manuscript. Following densitometric analysis, data for CREB1 were normalized to GAPDH, expressed as fold of untreated and are plotted as mean ± SE. Statistical significance comparing each formoterol treatment time to t = 0 and to the effect of Dex + Form, at each time, was tested by ANOVA with a Dunn's post-test. No significant differences were found.



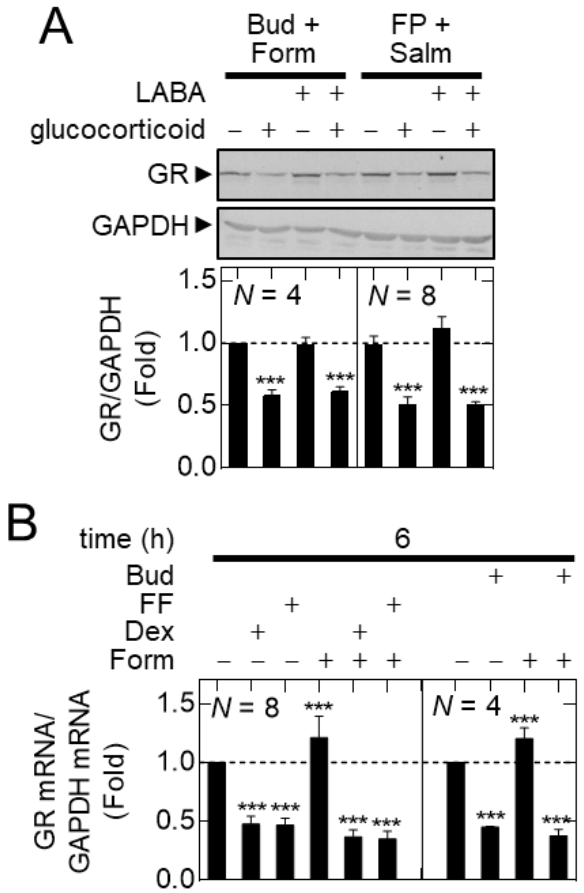
Supplemental Fig. 2. Schild analysis of simple 2×GRE-dependent transcription induced by dexamethasone and dexamethasone plus formoterol. BEAS-2B cells harboring a 2×GRE luciferase reporter (BEAS-2B 2×GRE cells) were incubated in the absence or presence of the indicated concentrations of Org34517 (Org) for 30 mins prior to treatment with the indicated concentrations of dexamethasone (Dex), each in the absence or presence of formoterol (Form; 10 nM). After 6 h, cells were harvested for luciferase assay. The effect of Org34517 and formoterol in the absence of dexamethasone (A) and the effect of dexamethasone and dexamethasone plus formoterol without Org34517 (B) are shown. The effects of 100 nM and 1 μM Org34517 are shown on the response to dexamethasone (C, upper panel) and dexamethasone plus formoterol (C, lower panel). Data, from *N* independent experiments, expressed as fold of untreated, are plotted as mean ± SE. Schild analysis was performed using GraphPad Prism 6.01 as previously described ((King et al., 2013; Joshi et al., 2015a) (Red lines). Resultant pA_2 values are stated. Four-parameter curve fitting was performed using GraphPad Prizm 6.01 (Black lines). (D) BEAS-2B 2×GRE cells were pre-treated with Org34517 (1 μM), or not, prior to stimulation with the glucocorticoids (GC), budesonide (Bud; 100 nM) or fluticasone propionate (FP; 100 nM) in the absence or presence of the LABAs, formoterol (10 nM) or salmeterol (Salm; 100 nM), as indicated. After 6 h, cells were harvested for luciferase assay. Data, from *N* independent experiments, expressed as fold of untreated, are plotted as mean ± SE. Statistical significance for the fold induction was tested by ANOVA with Bonferroni's correction. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$



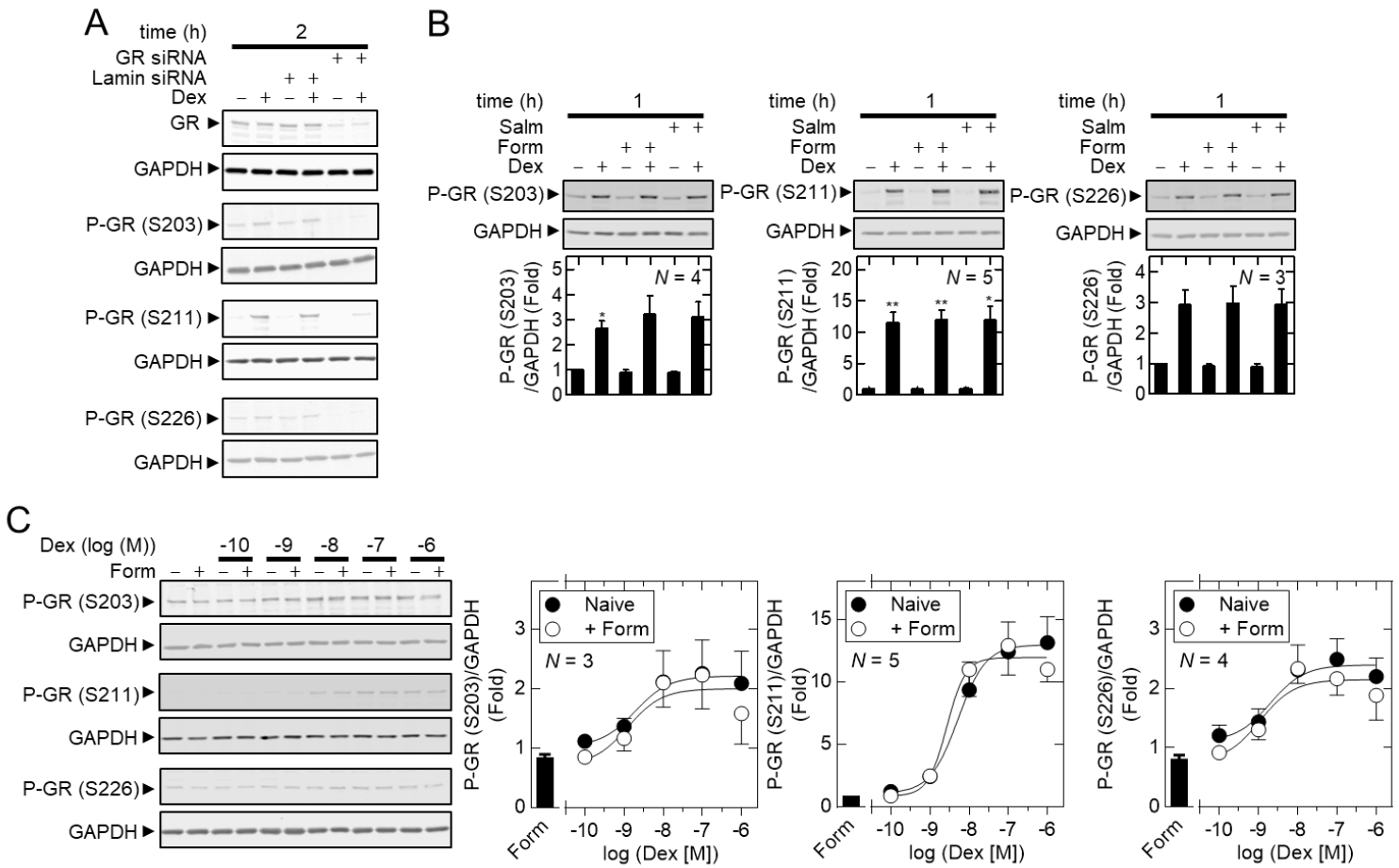
Supplemental Fig. 3. Induction of 2×GRE-dependent reporter activation by budesonide and enhancement by formoterol are prevented by GR silencing. BEAS-2B 2×GRE cells were either not treated, treated with GR targeting siRNA (GR si5, GR si6) or a control siRNA. After 24 h, cells were harvested for western blot analysis of GR and GAPDH (left panel). Representative blots are shown. Alternatively, the cells were not stimulated (NS) or treated with formoterol (Form; 10 nM) and/or budesonide (Bud; 100 nM), as indicated. After 6 h, cells were harvested for luciferase assay. Data, from *N* independent experiments, expressed as fold of untreated, are plotted as mean ± SE. Significant differences in the fold induction between control and GR targeting siRNAs for the indicated treatments was tested by ANOVA with Bonferroni's correction. *** $P < 0.001$.



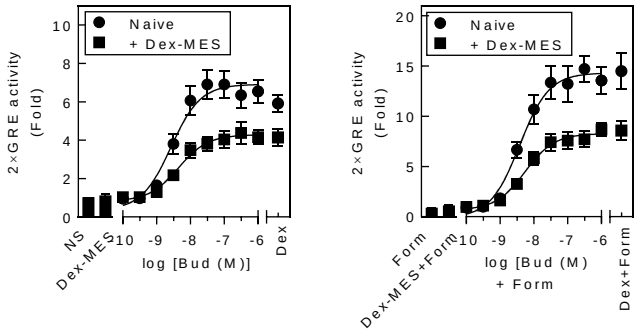
Supplemental Fig. 4. Transient accumulation of 2×GRE driven luciferase mRNA and the effect of cycloheximide. (A) BEAS-2B 2×GRE cells were treated with dexamethasone (1 μM) for the times indicated prior to harvesting for RNA extraction. Analysis of luciferase (Luc) mRNA and GAPDH was performed by qPCR. Data, from *N* independent experiments, expressed as Luc/GAPDH, were plotted as fold of untreated (t = 0) as means ± SE. (B) BEAS-2B 2×GRE cells were treated with dexamethasone (Dex; 1 μM) either in the absence or presence of a 5 – 10 min pretreatment with cycloheximide (CHX; 100 μg/ml). Cells were harvested at the indicated times for qPCR analysis of luciferase and GAPDH mRNA. Data from *N* independent experiments, expressed as Luc/GAPDH are plotted as fold of untreated, at each time, as means ± SE. Differences in fold induction between Dex and Dex + CHX treatments were assessed by paired t test. ** $P < 0.01$, *** $P < 0.001$.



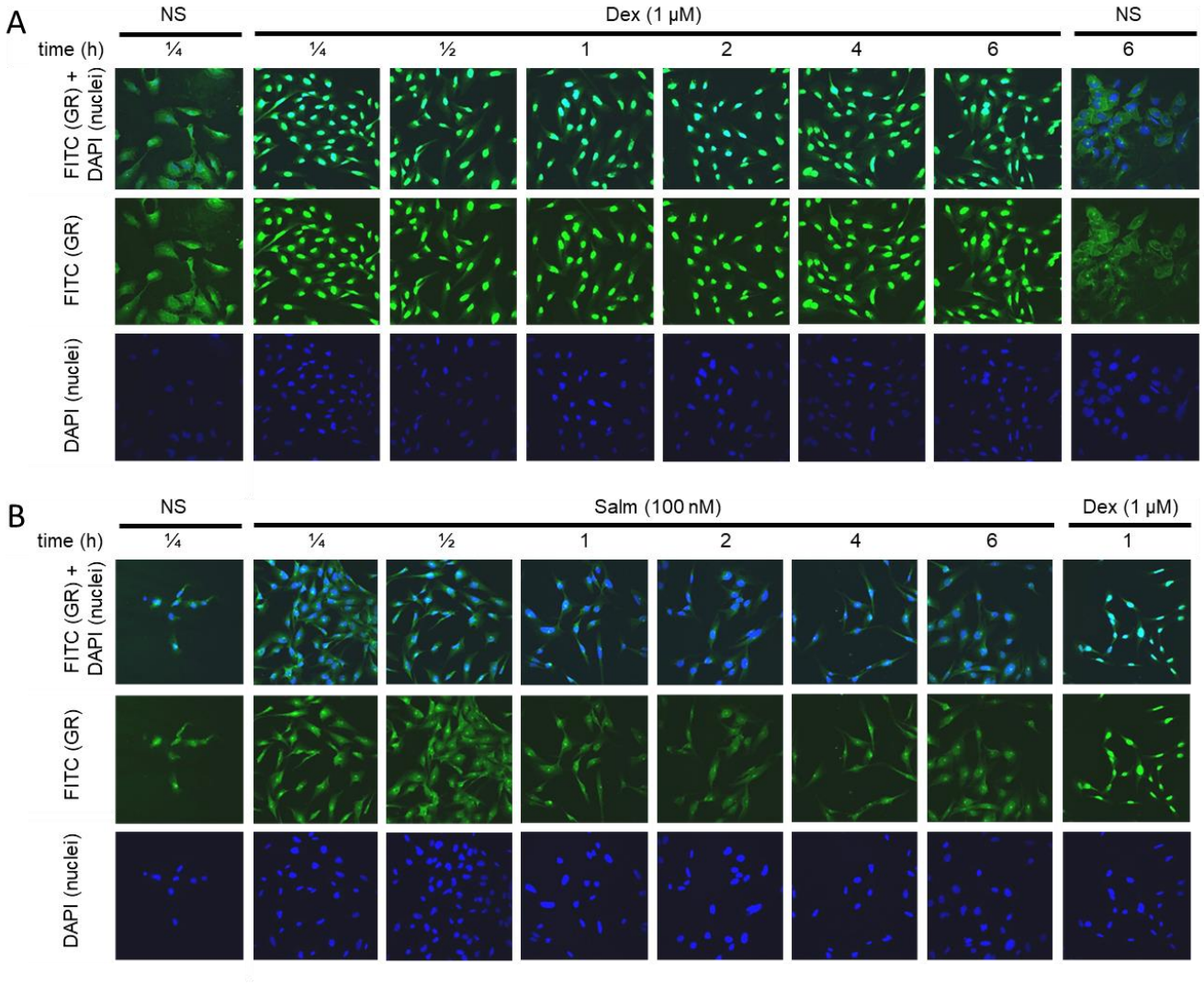
Supplemental Fig. 5. Effect of glucocorticoids and LABA on GR expression. (A) BEAS-2B cells were either not treated or treated with the LABAs, formoterol (Form; 10 nM) or salmeterol (Salm; 100 nM) and/or the glucocorticoids, budesonide (Bud; 100 nM) or fluticasone propionate (FP; 100 nM), as indicated. After 6 h, cells were harvested for western blot analysis of GR and GAPDH. Blots representative of *N* independent experiments are shown. Following densitometric analysis, data, normalized to GAPDH, were expressed as fold of untreated and are plotted as mean ± SE. Statistical significance compared to untreated control was tested by ANOVA with a Dunnett's post-test. *** $P \leq 0.001$. (B) BEAS-2B Cells were treated with dexamethasone (Dex; 1 μM), budesonide (100 nM) or fluticasone furoate (FF; 100 nM) in the absence or presence of formoterol (10 nM). After 6 h, cells were harvested for qPCR analysis of GR and GAPDH mRNA. Data, normalized to GAPDH mRNA were expressed as fold of untreated and are plotted as means ± SE. Significance compared to untreated control was tested by ANOVA with a Dunnett's post-test. *** $P \leq 0.001$.

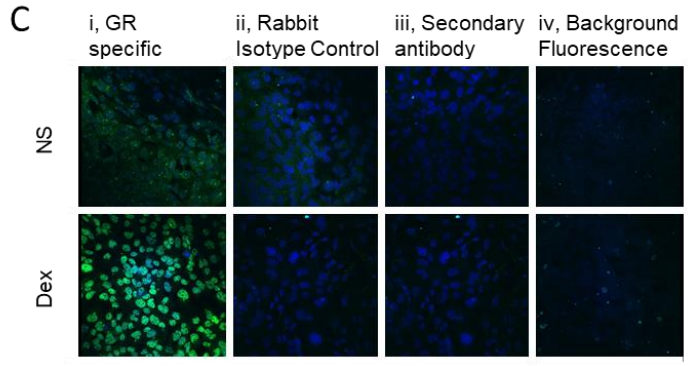


Supplemental Fig. 6. No effect of LABAs on GR phosphorylation. (A) BEAS-2B cells were treated with dexamethasone (Dex, 1 μM) following either no intervention or incubation with siRNA targeting lamin or GR, as described in Supplemental Fig. 3. Cells were harvested for western blot analysis of GR, GAPDH and serine 203 (S203), serine 211 (S211) or serine 226 (S226) phosphorylated GR (P-GR). (B) BEAS-2B cells were treated with dexamethasone (1 μM) and/or the LABAs formoterol (Form; 10 nM) or salmeterol (Salm; 100 nM). After 1 h, the cells were harvested for western blot analysis of S203, S211 and S226 phosphorylated GR and GAPDH. Densitometric data from *N* independent experiments were normalized to GAPDH, expressed as fold of untreated and are plotted as means ± SE. Significance relative to control was tested by ANOVA with a Dunnett's post-test. * $P \leq 0.05$, ** $P \leq 0.01$. (C) BEAS-2B cells were treated with the indicated concentrations of dexamethasone in the absence or presence of formoterol (10 nM). After 2 h cells were harvested for western blotting and analysis as in B. Graphs depicting data from *N* independent experiments are shown with data expressed as fold of non-stimulated and plotted as means ± SE.

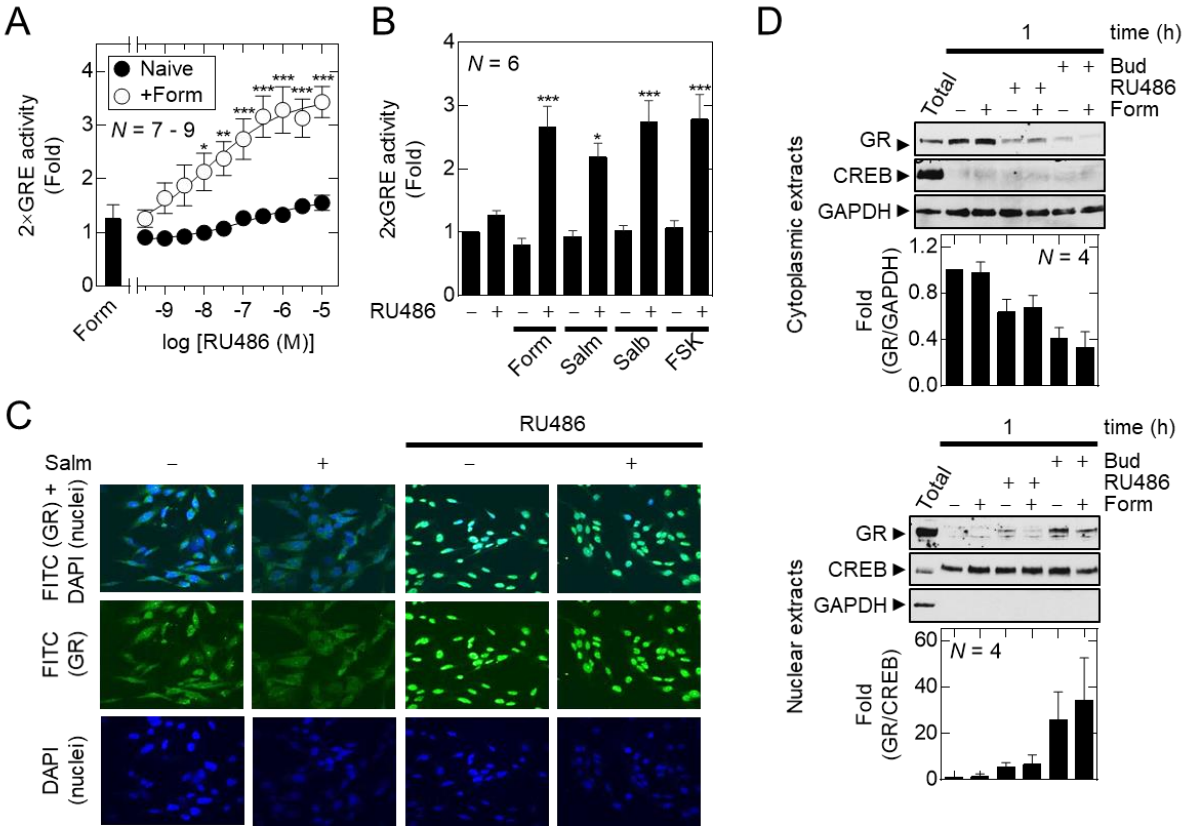


Supplemental Fig. 7. Fractional inactivation of GR following irreversible alkylation by dexamethasone-21-mesylate (Dex-MES) indicates a lack of receptor reserve and shows no effect of formoterol on functional glucocorticoid potency. BEAS-2B cells were either not treated or treated with Dex-MES (10 nM) for 30 min. The medium was then removed and the cells rinsed with fresh serum free medium prior to treatment with dexamethasone (Dex; 1 μM) or various concentrations of budesonide, each in the absence or presence of formoterol (Form; 10 nM). After a further 6 h, the cells were harvested for luciferase assay. Dex-MES, which irreversibly alkylates and inactivates GR, reduced the E_{max} for budesonide in the absence and presence of formoterol without affecting the potency. Operational model fitting (Leff et al., 1990), as applied to GR (Joshi et al., 2015b), indicated that the enhancement of budesonide-induced 2×GRE-reporter activity by formoterol was not associated with a change in the affinity of budesonide for GR, which was calculated as pK_A ~9 (Table 1).

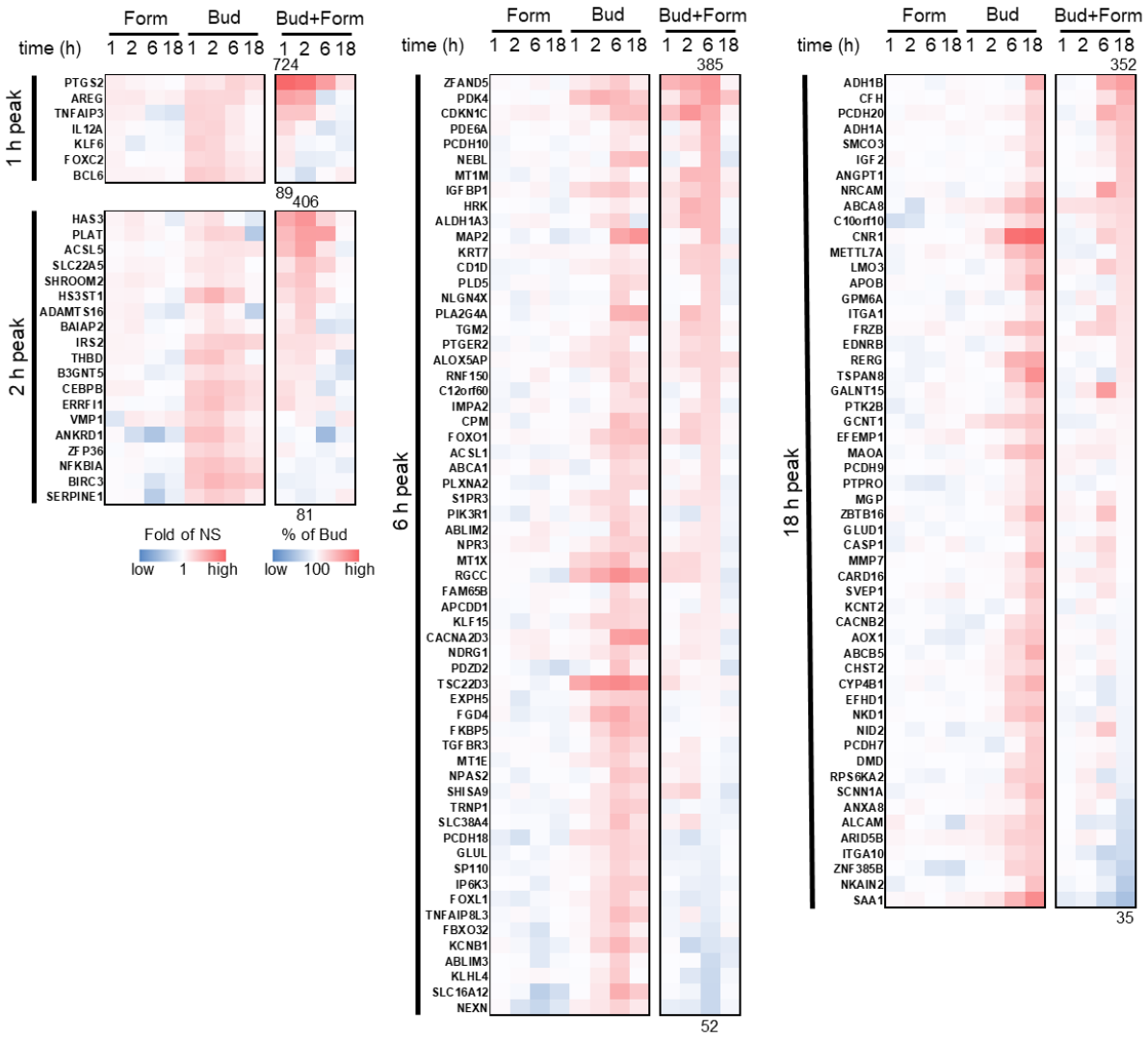




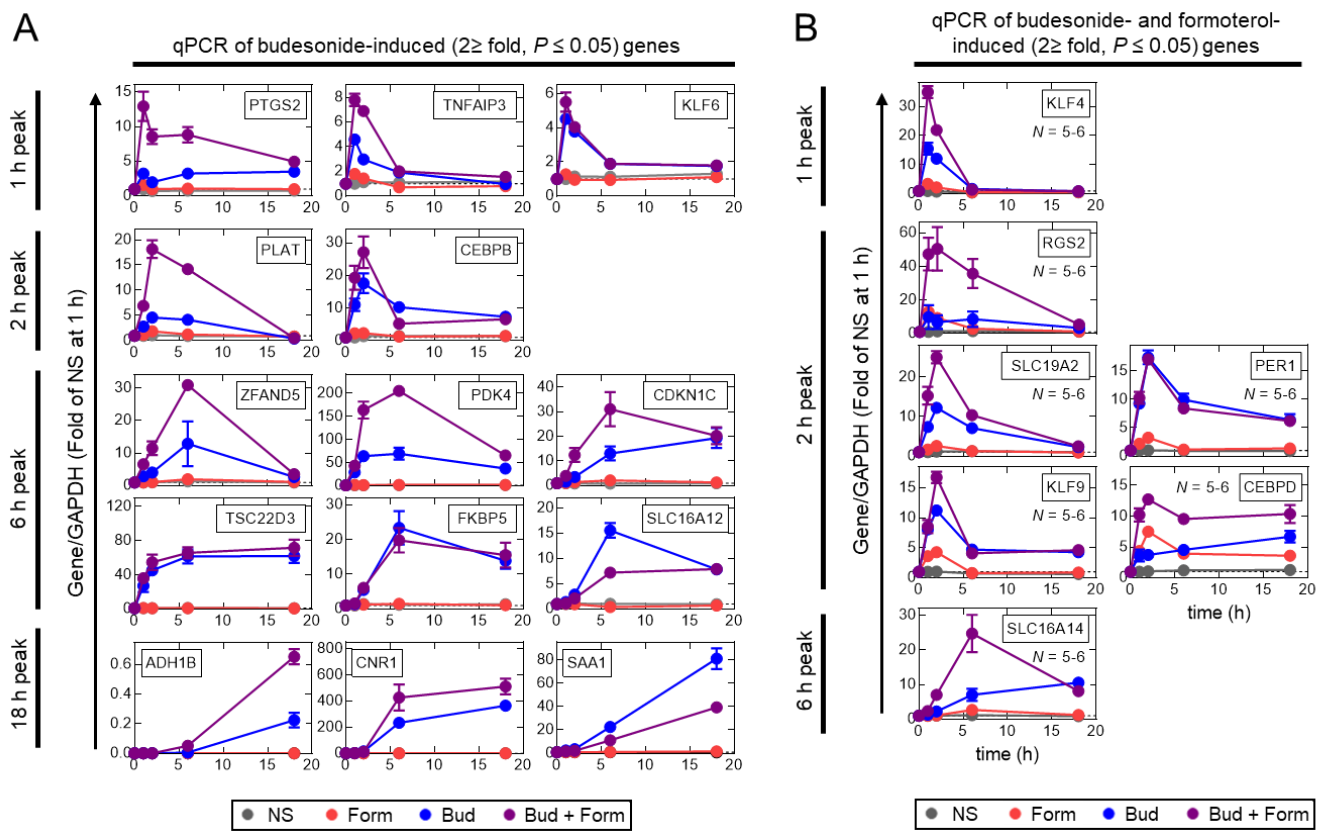
Supplemental Fig. 8. Effect of dexamethasone and salmeterol on GR localization. BEAS-2B cells grown to ~70% confluence were either not stimulated (NS) or were treated for the indicated times with (A) dexamethasone (Dex; 1 µM) or (B) salmeterol (Salm; 100 nM). Cells were fixed with 4% paraformaldehyde, blocked with 10% goat serum and incubated overnight with anti-GR antibody (1 µg/ml). Subsequently, slides were incubated for 1 h with 2 µg/ml Alexa488-conjugated F(ab′)$_2$ goat-anti rabbit secondary antibody, to visualise GR (green), and 2 µM DAPI, to stain the nuclei (blue). Slides were mounted with ProLong Gold antifade reagent and images captured on an Olympus BX81 FV1000 confocal microscope. Images are representative of 3 such experiments. All images have been treated in an identical manner. (C) BEAS-2B cells were either not stimulated (NS) or treated with dexamethasone (1 µM) for 1 h. Processing occurred as above, using i) GR-specific primary antibody, ii) isotype control as the primary antibody, iii) no primary antibody (i.e. secondary antibody alone), and iv) no primary or secondary antibody.



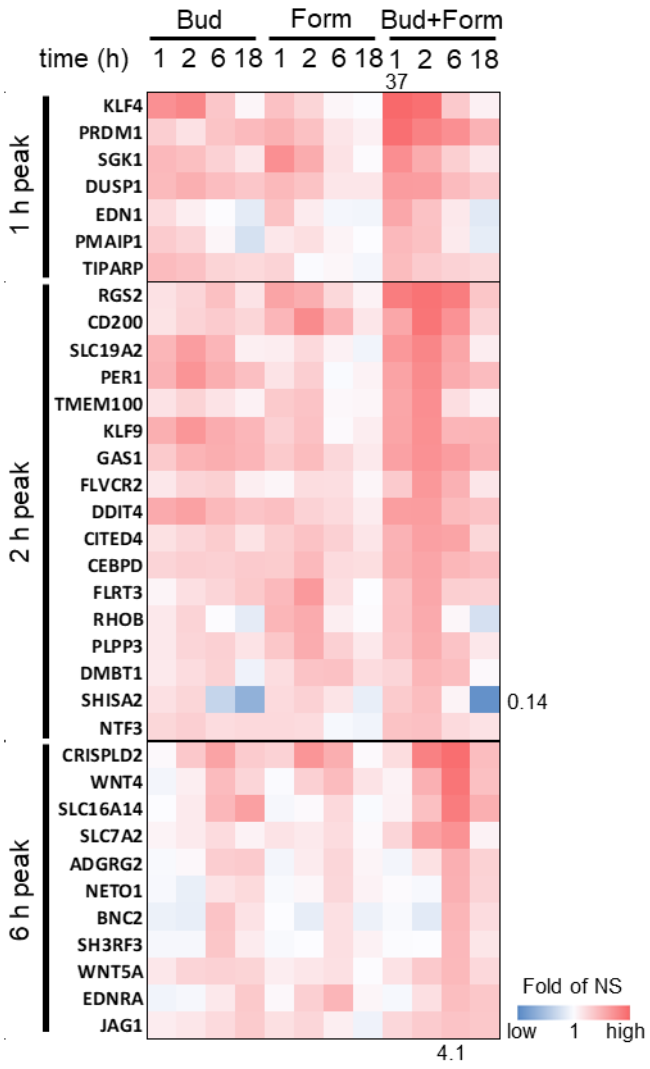
Supplemental Fig. 9. Effect of salmeterol and RU486 on GR translocation and 2xGRE-dependent transcription. (A) BEAS-2B 2×GRE reporter cells were treated with various concentrations of RU486 in the absence and presence of formoterol (Form; 10 nM). After 6 h, cells were harvested for luciferase assay. Data from *N* independent experiments are expressed as fold of untreated (naïve) and are plotted as means ± SE. (B) BEAS-2B 2×GRE reporter cells were treated with RU486 (1 µM) in the absence or presence of formoterol (10 nM), salmeterol (Salm; 100 nM), salbutamol (Salb; 1 µM) or forskolin (FSK; 10 µM). After 6 h, cells were harvested for luciferase assay. Data from *N* independent experiments are expressed as fold of untreated (naïve) and are plotted as means ± SE. (A & B) Significance was tested relative to untreated using a Dunnett's post-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C) BEAS-2B cells were grown to ~70% confluence and treated or not with salmeterol (100 nM) and/or RU486 (1 µM) for 1 hour. Cells were fixed with 4% PFA, blocked with 10% goat serum and incubated overnight with anti-GR antibody (1 µg/ml). Subsequently slides were incubated for 1 h with 2 µg/ml Alexa488-conjugated $F(ab')_2$ goat-anti-rabbit secondary antibody, to visualise GR (green), and 2 µM DAPI, to stain the nuclei (blue). Slides were mounted with ProLong Gold antifade reagent and images captured on an Olympus BX81 FV1000 confocal microscope. Images are representative of 3 such experiments. (D) BEAS-2B cells were treated with budesonide (Bud; 100 nM) or RU486 (1 µM) and/or formoterol (10 nM) for 1, or 2 h, as indicated. A total cell lysate was used as a positive control (Total). Cytoplasmic and nuclear extracts were prepared and subjected to western blotting for GR, CREB (nuclear) and GAPDH (cytoplasmic). Blots representative of *N* such experiments are shown. Densitometry was performed on the cytoplasmic (GR/GAPDH) and nuclear (GR/CREB) extracts to generate the figures shown in lower panels. Data, expressed as fold, are plotted as means ± SE.



Supplemental Fig. 10. Budesonide-induced, formoterol-enhanced mRNAs. BEAS-2B cells were either not stimulated or treated with budesonide (Bud; 100 nM), formoterol (Form; 10 nM) or both together (Bud + Form). At 1, 2, 6 and 18 h RNA was extracted and microarray analysis (N = 3) performed. Compared to time-matched controls mRNAs induced by budesonide (≥ 2 fold, $P \leq 0.05$) and not by formoterol (< 2 fold) at any time that also showed a ≥ 4 fold increase for either budesonide or budesonide + formoterol are grouped according to the time (1, 2, 6 or 18 h) of peak expression. Fold expression data for the formoterol and budesonide treatments were $\log_2$ transformed and are depicted as heat maps. Budesonide + formoterol treatments were expressed as % of budesonide-treated (100%) at each time and were then $\log_2$ transformed and expressed as heat maps. Genes within each time group are ranked (high to low) based on the budesonide + formoterol/budesonide percentage.

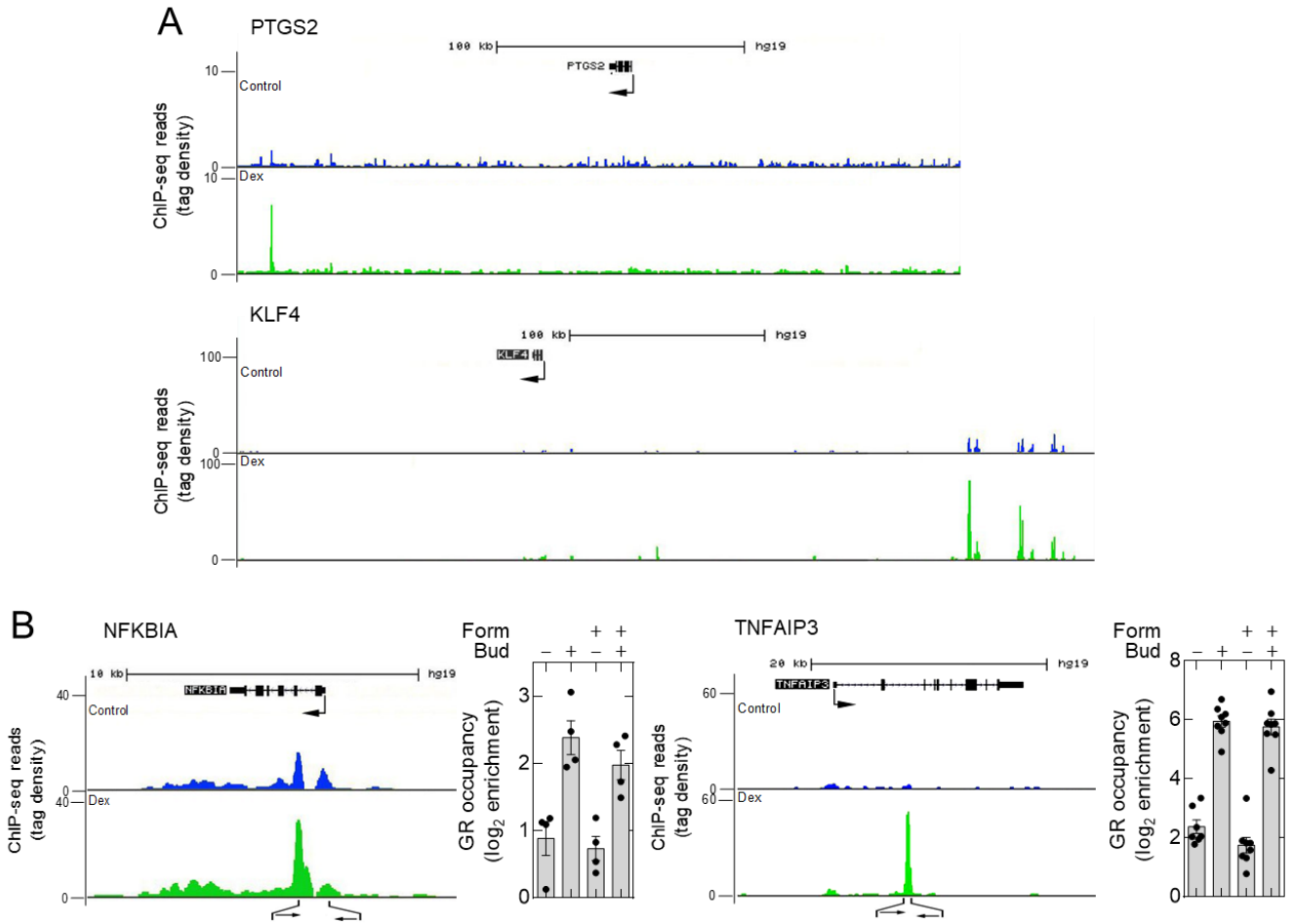


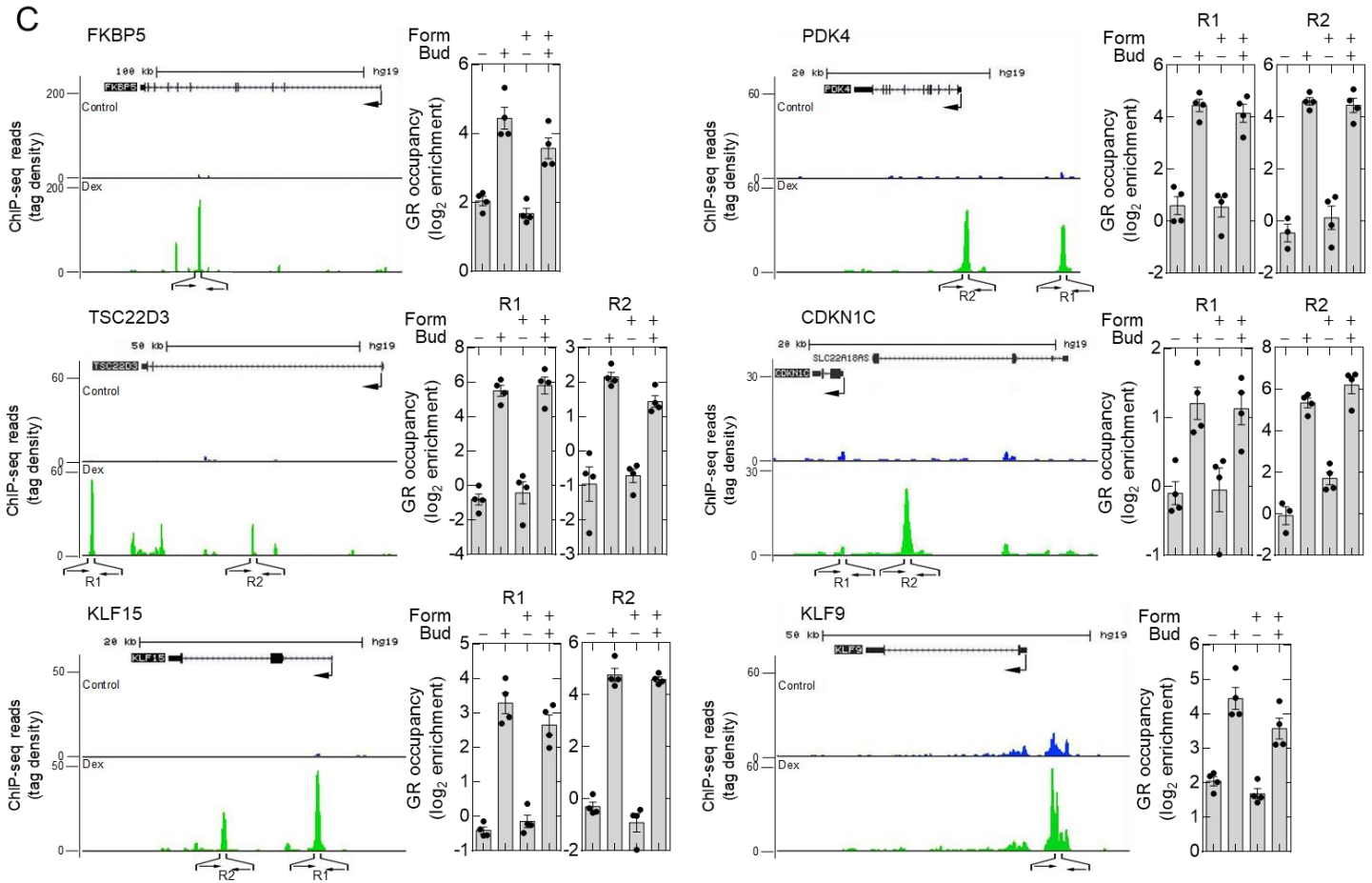
Supplemental Fig. 11. Validation of budesonide and formoterol-induced mRNAs by qPCR. BEAS-2B cells were either not stimulated or treated with budesonide (Bud; 100 nM), formoterol (Form; 10 nM) or both together (Bud + Form). At 1, 2, 6 and 18 RNA was extracted and qPCR performed for the indicated genes and GAPDH. (A) Analysis of selected mRNAs showing budesonide-induced (≥ 2 fold, $P \leq 0.05$), but not formoterol-induced (< 2 fold at any time), expression on the microarrays. (B) Analysis of selected mRNAs showing budesonide-induced (≥ 2 fold, $P \leq 0.05$) and formoterol-induced (≥ 2 fold, $P \leq 0.05$) expression on the microarrays. Data from *N* independent experiments were normalized to GAPDH, expressed as fold of NS at 1 h, and are plotted as means ± SE.



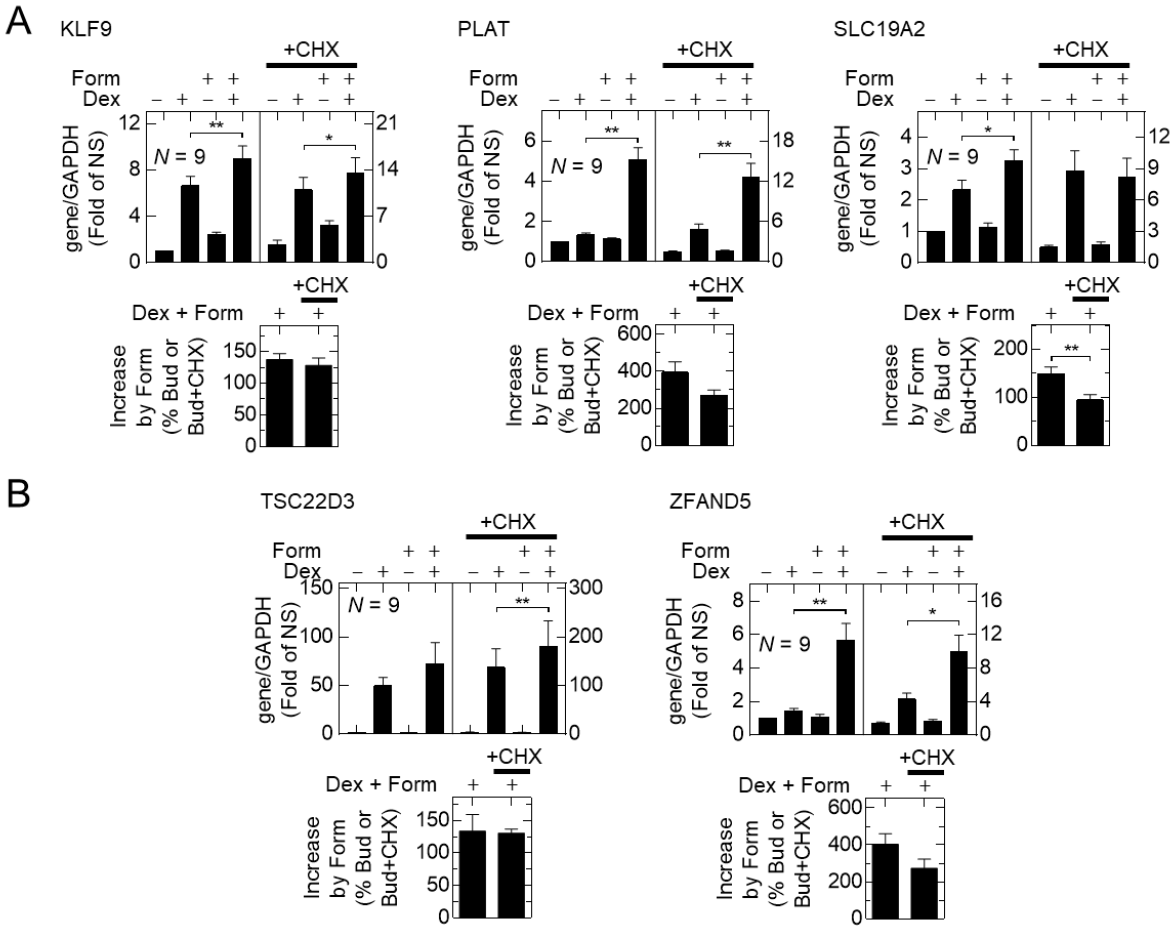
Supplemental Fig. 12. Gene expression induced by budesonide and formoterol. BEAS-2B cells were either not stimulated (NS) or treated with budesonide (Bud; 100 nM), formoterol (Form; 10 nM) or both together (Bud + Form). At 1, 2, 6 and 18 h RNA was extracted and microarray analysis ($N = 4$) performed. Genes induced by budesonide (≥2 fold, $P \leq 0.05$) and by formoterol (≥2 fold, $P \leq 0.05$) at any time that also showed a ≥4 fold increase for any treatment (Bud, Form or Bud + Form) are grouped according to the time of maximal expression by any treatment. Fold expression data were log2 transformed and depicted as heat maps. No genes met these criteria at 18 h.

Supplemental Fig. 13





Supplemental Fig. 13. GR recruitment and the effect of LABA. BEAS-2B cells were treated for 1 h with dexamethasone (Dex; 1 µM) or vehicle. ChIP-seq analysis for GR, published by Kadiyala et al., was used to identify GR binding sites at or near to gene loci of glucocorticoid-induced genes (Kadiyala et al., 2016). (Right panels – except for PTGS2 and KLF4) BEAS-2B cells were either not stimulated (NS) or treated with formoterol (Form; 10 nM), budesonide (Bud; 100 nM) or both combined (B + F). After 1 h, ChIP was performed using the GR antibody described by Kadiyala et al. 2016. Primers were designed flanking the indicated GR binding sites (regions R1 and R2 for multiple sites). qPCR was performed for each genomic region and three unrelated control regions of genomic DNA. (A) PTGS2 and KLF4 show distant GR binding sites relative to each gene locus and were not tested by ChIP-PCR. (B) Genes, NFKBIA and TNFAIP3, with peak gene expression at 1 – 2 h post budesonide or budesonide + formoterol treatment. (C) Genes; CDKN1C, FKBP5, KLF9, KLF15, PDK4 and TSC22D3, with peak mRNA expression at 6 h post budesonide or budesonide + formoterol treatment are shown. Data are shown as individual technical quadruplicates normalized to the control regions and are plotted as $\log_2$ enrichment. N = 1 experiment, or N = 2 for TNFAIP3.



Supplemental Fig. 14. Effect of the protein synthesis inhibitor, cycloheximide, on glucocorticoid-induced transcription and the effect of LABA for mRNAs showing: (A) 1 – 2 h peaks; and, (B) 6 h peaks in expression. BEAS-2B cells were treated with dexamethasone (Dex; 1 μM) in the absence or presence of formoterol (Form; 10 nM) and/or cycloheximide (CHX; 100 μg/ml) as indicated. Cells were harvested after 2 h for qPCR analysis of the indicated genes and GAPDH. Data (upper panel) from *N* independent experiments, expressed as the gene of interest/GAPDH, are plotted as fold of untreated as means ± SE. The effect of formoterol on the induction by dexamethasone, each in the absence or presence of CHX, was assessed using fold data by Wilcoxon matched-pairs signed rank test. * $P < 0.05$, ** $P < 0.01$. Data were also replotted (lower panel) to show the effect of formoterol on the induction of luciferase RNA by dexamethasone, each in the absence or presence of cycloheximide. Data are expressed as a percentage of Dex or Dex + CHX, as appropriate. Differences in this percentage comparing without and with cycloheximide were assessed by paired t test. ** $P < 0.01$.

Supplemental Table 1

(A) Primers used for PCR analysis

Forward (F) and reverse (R) primer sequences (5′ -3′) are shown in addition to the accession number for each gene. For genes with more than one splice variant, primers were designed to pick up all variants. All primers were designed using Primer Express software (Applied Biosystems) and were synthesised by the DNA synthesis lab at the University of Calgary.

Target gene	Accession Number	Primer Sequences
ADH1B	NM_000668.5	F: GCTGTGCTATGGGAGGTAAA R: TCCTACAGCCACCATCTTAATG
CEBPB	NM_005194.3	F: TCCAAACCAACCGCACAT R: AGAGGGAGAAGCAGAGAGTTTA
CEBPD	NM_005195.3	F: GGAGATGCAGCAGAAGTTGGT R: CGCGCTGGTGCAGCTT
CDKN1C	NM_000076.2	F: CGGCGATCAAGAAGCTGTC R: GGCTCTAAATTGGCTCACCG
CNR1	NM_001160259.1, NM_001160258.1, NM_001160226.1, NM_016083.4	F: ATTTCGTTCTAGCGGACAACCA R: TGACTGAGAAAGTGACCCACAG
DUSP1	NM_004417.3	F: GCTCAGCCTTCCCCTGAGTA R: GATACGCACTGCCCAGGTACA
FKBP5	NM_001145775.2, NM_001145776.1, NM_004117.3	F: CAGCTGCTCATGAACGAGTTTG R: GCTTTATTGGCCTCTTCCTTGG
GAPDH	NM_002046	F: TTCACCACCATGGAGAAGGC R: AGGAGGCATTGCTGATGATCT
GR (NR3C1)	NM_000176.2	F: TGCCAAGGATCTGGAGATGACAAC R: TGAGGAGCTGGATGGAGGAGAG
KLF4	NM_004235	F: TCGCCTTGCTGATTGTCTATT R: AATTGGCCGAGATCCTTCTTC
KLF6	NM_001160125.1, NM_001160124.1, NM_001300.5	F: ACGAGACCGGCTACTTCT R: CAGATCTTCCTGGCTGTCAAA
KLF9	NM_001206.2	F: CCTCCCATCTCAAAGCCCATT R: TCGTCTGAGCGGGAGAACTT
luciferase	M15077.1	F: CGCTGGAGAGCAACTGCATA R: CCAGGAACCAGGGCGTATCT
PDK4	NM_002612.3	F: GCGACAAGAATTGCCTGTGAG R: TCCACCAAATCCATCAGGCTC
PER1	NM_002616.2,	F: CGTCACCAGTCAGTGTAGC R: CCCACTGGACGGTAGGC
PLAT	NM_000930.4	F: TACTGTGATGTGCCCTCCTG R: GAGCCCTCCTTTGATGCGAA
PTGS2	NM_000963.2	F: GCTGGGCCATGGGGTGGACT R: CCTGCCCCACAGCAAACCGT
RGS2	NM_002923.3	F: CCTCAAAAGCAAGGAAAATATATACTGA R: AGTTGTAAAGCAGCCACTTGTAGCT
SAA1	NM_000331.5	F: CAGCGATGCCAGAGAGAATATC R: AGCAGGTCGGAAGTGATTG

SLC16A12	NM_213606.3	F: CCTGTGGTTCAGCTCCTTATT R: CTCATCAAGGCACCACATACA
SLC16A14	NM_152527.4	F: GTCATGGTGGGCAGGTATTT R: TACTTCAGCAGCACAGTCATTAG
SLC19A2	NM_001319667.1	F: CAGTGGTGCAGTCAGTGTTA R: GAAGAAGCCCTTCAGCAGTAT
TNFAIP3	NM_001270508.1, NM_001270507.1, NM_006290.3	F: AGGCGCTGTTCAGCACGCTC R: CGGGCCATGGGTGTGTCTGT
TSC22D3	NM_001015881.1	F: GGCCATAGACAACAAGATCG R: ACTTACACCGCAGAACCACCA
ZFAND5	NM_001102420.2	F: GTGGTTGAGGTGGTGACTAAA R: ACCAGCACTTGTGCATAGAA

(B) Primers used for ChIP PCR analysis

Forward (F) and reverse (R) primer sequences (5′-3′) are shown in addition to the accession number for each gene. Primer location for each gene was picked based on the presence of GR binding site as shown in the custom tracks of UCSC genome browser database. All primers were designed using primer designing software (Integrated DNA technologies) and were synthesised by the DNA synthesis lab at the University of Calgary.

Target gene	**Accession Number**	**Primer Sequences**
2xGRE	NA	F: TAGCCCGCTGTACAGGAT R: CTGCAGTGCTCTGCCTTT
CDKN1C	NG_008022.1	R1 F: GGGCTCTTTGGGCTCTAAAC R: TGCGGTGAGCCAAGTGAGTACA R2 F: GGGCCTTGGGTTAATCTGT R: CTCCACCTTCCCATCCATAAG
CRISPLD2	NC_000016.10	R1 F: TCCTGCCAAGAAGGGTTTAAG R: CTGAGCAGAACATCCCGTTAG R2 F: CCACAGAGTCATCCCGTAAAT R: TTCCTAGAAACCTCCAGTGTAATC
KLF9	NC_000009.12	F: ACCCTGTTTCATAACTCTGGCGGT R: AAGCTGATGTCATCTCGACACCCT
KLF15	NC_000003.12	R1 F: AGTGTCCTCTCTTTAATGCCGGTG R: ACAGGGTCTTAATGCTGGGCTGAA R2 F: AGAGCAGGCTTTCCCTGTTCTGAT R: ACGTTCTGCTGCAAGGAACAAAGG
FKBP5	NG_012645.2	F: TAACCACATCAAGCGAGCTG R: GCATGGTTTAGGGGTTCTTG
MYOD1(A)	NC_000011.10	F: TGCAGGAGATGAAATACTAAGCAAGTA R: AGATTGGAAACTGAGGACTTTAGTTAGAG

MYOG	NC_000001.11	F: CCAATGAGACTGAGTGGGTTTTC R: TCACCAGAGAAGACTGCTTTGC
NFKBIA	NG_007571.1	F: CTTGCAGAGGGACAGGATTAC R: GTCACGGACAGGGAACTTT
OLIG3	NC_000006.12	F: GGCAAGGACAGAGACAATCATA R: CTCTGTGTTCTCGCTTTGGA
PDK4	NG_047008.1	R1 F: CAGTGTCAGATCAGGGCTATG R: CCCAGGCATCTAGTTTCTGTT R2 F: TGGAAGAAGTGAGGAGAAGTAATC R: ACTCTGCCCTGTACGAAATG
SLC16A12	NG_021179.1	R1 F: CTTCAAGATACGGATGGTCGTT R: CAAACTAGAGGGAGGTGGAATG R2 F: AGGCTCGCATGAGGAAATC R: CTAGCTTCCGGTGAAGACAAT
SLC19A2	NG_008255.1	F: TTTAAGGGAGACCCGTCCTAA R: TGGAAGAGCAGGCAAGTATTC
TNFAIP3	NG_032761.1	F: GACCACACCCACTTGGAAA R: TTTGACTAGCAATTGAGCAACAG
TSC22D3	NC_000023.11	R1 F: CTCAGTGTTCAGCCCTTTGA R: GCATTCTCTTCTCCCAGTTGTA R2 F: GTGCCTGGAGACCAACTCAT R: ACCCTTGATGCTGAGCAAGT
ZFAND5	NC_000009.12	F: CTGGGAAAGCACCAAATGTTC R: TGAACTTCCTCTCGCAAGC

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