

Transcriptome-level interactions between budesonide and formoterol provide insight into the mechanism of action of ICS/LABA combination therapy in asthma

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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: DAVID, Database for Annotation, Visualization, and Integrated Discovery; FGF, fibroblast growth factor; FOXO, forkhead box O; GPCR, G-protein coupled receptor; GR: Glucocorticoid receptor; GRE glucocorticoid response element; GO, gene ontology; HBEC, human bronchial epithelial cell; ICS, inhaled corticosteroid; JAK, Janus kinase; KEGG, Kyoto Encyclopedia of genes and genomes; LABA, long-acting β_2 -adrenoceptor agonist; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor κ B; PDGF, platelet-derived growth factor; pHBEC, primary human bronchial epithelial cell; PI3K, phosphatidylinositol 3-kinase; SABA, short-acting β_2 -adrenoceptor agonist; STAT, signal transducer of transcription; transforming growth factor β (TGF β)

Abstract

In 2019, the Global Initiative for Asthma treatment guidelines were updated to recommend that inhaled corticosteroid (ICS)/long-acting β_2 -adrenoceptor agonist (LABA) combination therapy should be a first-in-line treatment option for asthma. Although clinically superior to ICS, mechanisms underlying the efficacy of this combination therapy remain unclear. We hypothesised the existence of transcriptomic interactions, an effect that was tested in BEAS-2B and primary human bronchial epithelial cells (pHBECs) using formoterol and budesonide as representative LABA and ICS, respectively. In BEAS-2B cells, formoterol produced 267 (212 induced; 55 repressed) gene expression changes ($\geq 2/\leq 0.5$ -fold) that were dominated by rapidly (1-2h) upregulated transcripts. Conversely, budesonide induced 370 and repressed 413 mRNAs, which occurred predominantly at 6-18h and were preceded by transcripts enriched in transcriptional regulators. Significantly, genes regulated by both formoterol and budesonide were overrepresented in the genome; moreover, budesonide-plus-formoterol induced and repressed 609 and 577 mRNAs, respectively, of which $\sim 1/3$ failed the cut-off criterion for either treatment alone. While induction of many mRNAs by budesonide-plus-formoterol was *supra*-additive, the dominant (and potentially beneficial) effect of budesonide on formoterol-induced transcripts, including those encoding many pro-inflammatory proteins, was repression. Gene ontology analysis of the budesonide-modulated transcriptome returned enriched terms for transcription, apoptosis, proliferation, differentiation, development and migration. This “functional” ICS signature was augmented in presence of formoterol. Thus, LABAs modulate glucocorticoid action and comparable transcriptome-wide interactions in pHBECs imply that such effects may be extrapolated to asthmatics taking combination therapy. While repression of formoterol-induced pro-inflammatory mRNAs should be beneficial, the pathophysiological consequences of other interactions require investigation.

Significance statement

In human bronchial epithelial cells, formoterol, a long-acting β_2 -adrenoceptor agonist enhanced the expression of inflammatory genes and many of these changes were reduced by the glucocorticoid, budesonide. Conversely, the ability of formoterol to enhance both gene induction and repression by budesonide provides mechanistic insight as to how adding a LABA to an inhaled corticosteroid may improve clinical outcomes in asthma.

Introduction

As rescue medication, inhaled short-acting, β_2 -adrenoceptor agonists (SABAs) provide acute symptom relief in individuals with asthma. Conversely, inhaled corticosteroids (ICS), acting via the glucocorticoid receptor (GR, NR3C1), reduce airway inflammation and generally control mild-to-moderate disease (Barnes, 2011; Oakley and Cidlowski, 2013). However, with more severe asthma, ICS often provide inadequate control and add-on therapy, typically a long-acting β_2 -adrenoceptor agonist (LABA), is recommended (Ross et al., 2015). Despite the ability of β_2 -adrenoceptor agonists to arrest and reverse bronchoconstriction, chronic use, especially of high-efficacy ligands, increases asthma mortality (Cockcroft and Sears, 2013; Nelson et al., 2006; Reddel et al., 2019). Explanations for this include the possibility that excessive β_2 -agonist use masks underlying disease progression and severity. Alternatively, β_2 -agonists may produce detrimental effects, for example by enhancing expression of inflammatory genes. Certainly, β_2 -agonists increase the production of inflammatory mediators, particularly in the context of inflammatory stimuli (Ammit et al., 2002; Edwards et al., 2007; Holden et al., 2010; Strandberg et al., 2007). Moreover, transcriptomic analyses in human bronchial epithelial cells (HBECS) exposed to the LABAs, indacaterol and salmeterol, confirm increased expression of inflammatory genes (Yan et al., 2018).

Elevated mortality associated with chronic β_2 -agonist monotherapy is not seen in patients taking ICS/LABA combination therapy (Reddel et al., 2019). Accordingly, in 2019, the Global Initiative for Asthma (GINA) modified their treatment guidelines to recommend that ICS/LABA combination therapy, where the LABA is formoterol, should be a first-line treatment option, even for patients with mild disease (<https://ginasthma.org/reports/>). Indeed, ICS/LABA combination therapy is superior to ICS alone, irrespective of dose, at improving lung function and reducing exacerbation rates (Newton and Giembycz, 2016). Such data raise the proposition that LABAs and ICSs may interact, possibly at a molecular level, to deliver superior clinical outcomes. Certainly, LABAs can cooperatively enhance glucocorticoid-driven transcription. For example, in BEAS-2B human airway epithelial cells, LABAs

did not activate a simple glucocorticoid response element (GRE)-dependent luciferase reporter, yet enhanced, 2-3-fold, the maximum glucocorticoid-induced response (Joshi et al., 2015; Kaur et al., 2008). Similar interactions occur for multiple genes (Joshi et al., 2015; Kaur et al., 2008; Rider et al., 2018). These include the regulator of G-protein signalling, RGS2, which attenuates signal transduction from G-protein coupled receptors (GPCRs) that act via Gq (Heximer, 2004; Kimple et al., 2011). *In vivo*, including in models of lung inflammation (George et al., 2017; George et al., 2018; Jiang et al., 2015; Xie et al., 2012), RGS2 is bronchoprotective (Holden et al., 2011). Moreover, in HBECs and smooth muscle cells, LABAs synergize with glucocorticoids to enhance and prolong RGS2 expression (Holden et al., 2011; Holden et al., 2014). This suggests therapeutic relevance.

Given that GR-dependent transactivation mediates anti-inflammatory effects of glucocorticoids (Clark and Belvisi, 2012; Newton and Holden, 2007; Newton et al., 2017; Oh et al., 2017), transcriptional cooperativity with LABA could be important to realize the enhanced therapeutic efficacy of ICS/LABA combination therapy. Equally, glucocorticoid-dependent repression of LABA-induced pro-inflammatory mediators would be beneficial (Ammit et al., 2002; Holden et al., 2010). However, as not all LABA- and glucocorticoid-induced genes show co-operative effects (Rider et al., 2018), detailed transcriptomic characterization is necessary in cells relevant to asthma pathogenesis.

Airway epithelial cells promote and regulate inflammation (Knight and Holgate, 2003), are a direct target of inhaled therapy and are central to the anti-inflammatory effects of glucocorticoids in the airways (Klassen et al., 2017). Furthermore, the ICS, budesonide, induces expression of multiple genes *in vivo* in the airways of healthy and asthmatic individuals (Kelly et al., 2012; Leigh et al., 2016). Many such genes are also induced in primary HBECs (pHBECs) and the HBEC line, BEAS-2B (Mostafa et al., 2019). As pHBECs and BEAS-2B cells express functional β_2 -adrenoceptors (Kaur et al., 2008; Rider et al., 2018; Yan et al., 2018), they were selected for transcriptomic analysis using formoterol and budesonide. These represent clinically relevant LABA and ICS, which are identified by GINA as the “preferred” or reference compounds, respectively (GINA, 2020). In each case, responses to

formoterol and budesonide in epithelial cells were representative of other members of their class in modulating reporter activity and gene expression (Kaur et al., 2008; Rider et al., 2011; Rider et al., 2015; Yan et al., 2018). Following the hypothesis-free determination of differentially regulated genes by each treatment condition, gene ontology (GO) analyses were used to provide insight as to therapeutic relevance.

Materials and Methods

Cell culture, compounds and cell treatments. The human bronchial epithelial cell line, BEAS-2B (ATCC, Manassas, VA), was cultured to confluence in Dulbecco's modified Eagle's/Ham's F12 medium (DMEM/F12) supplemented with 14 mM NaHCO₃, 2 mM L-glutamine and 10% fetal calf serum (all Invitrogen; Burlington, ON). Primary human bronchial epithelial cells (pHBECs) from normal non-smokers were cultured from airways brushings obtained at bronchoscopy (Leigh et al., 2016), or were isolated from non-transplantable normal human lungs obtained through a tissue retrieval service at the International Institute for the Advancement of Medicine (Edison, NJ) (Hudy et al., 2010). In each case, protocols and consent for the bronchoscopy study were approved by the Conjoint Health Research Ethics Board at the University of Calgary and Alberta Health Services (REB IDs: 23241 and 15-0336, respectively). The pHBECs were grown as submersion culture in bronchial epithelial cell growth medium (BEGM) (Lonza, Morristown, NJ) containing SingleQuots supplements (Lonza). BEAS-2B and pHBECs were cultured at 37°C in 5% CO₂/95% air and once confluent were incubated in serum-free, or supplement-free, medium overnight prior to experiments. All cells tested negative on routine mycoplasma contamination testing. Budesonide (22RS 16 alpha, 17 alpha-butylidenedioxypregna-1,4-diene-11beta,21-diol-3,20-dione) and the active RR version of formoterol fumarate ((R', R')-(+/-)-N-(2-hydroxy-5-(1-hydroxy-2-((2-(4-methoxyphenyl)-1-methylethyl)amino)ethyl)phenyl) formamide, (E)-2-butendioate (2:), dihydrate) (gifts from AstraZeneca; Mölndal, Sweden) were dissolved in DMSO (Sigma-Aldrich) as stocks at 10 mM. Final

DMSO concentrations on cells were $\leq 0.1\%$. Based on prior data that established maximally effective concentrations (BinMahfouz et al., 2015; Holden et al., 2014; Kaur et al., 2008; Mostafa et al., 2019; Rider et al., 2011), the BEAS-2B cells were either not treated or treated with formoterol (10 nM), budesonide (100 nM), or both combined for 1, 2, 6 and 18 h. Likewise, pHBECS were treated with formoterol (10 nM), budesonide (100 nM) or both combined for 6 h. In each case, treatments were conducted in serum-free, or supplement-free medium to reduce the impact of signalling and transcriptional response due to mediators present in serum or other supplements. As is previously reported (Rider et al., 2011), this helps to ensure optimal responses to glucocorticoid and LABA.

Microarray analysis. Total RNA was extracted (RNeasy mini kit; Qiagen) and RNA quality 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 in a GeneChip Fluidics Station 450 and scanning with a Scanner 3000 G7. Robust multiarray averaging (RMA), quantile normalization, and median polishing on logged probe set intensity values were performed in Transcriptome Analysis Console (TAC) software v4.0 (Affymetrix). TAC software was then used to identify differentially expressed genes between paired samples (treated vs. untreated) using the eBayes ANOVA method, as is appropriate for microarray data (Smyth, 2004), to produce descriptive statistics for data categorization. Data files were deposited with the NCBI Gene Expression Omnibus (GEO) under accession numbers GSE115830 and GSEXXXX for BEAS-2B and pHBEC data, respectively.

Where genes were represented by multiple probe sets, only those with the greatest overall change were retained. Four independent experiments in BEAS-2Bs and pHBECS from 6 healthy individuals were analyzed for gene expression changes due to formoterol, budesonide or budesonide-plus-formoterol. In each case, data were expressed as fold of untreated for each time point and genes

showing ≥ 2 - or ≤ 0.5 -fold (each $P \leq 0.05$) relative to untreated for any treatment were considered differentially expressed. Unless otherwise stated, these criteria are henceforth used to define “induced” or “repressed” gene expression. *Supra*- and *infra*-additive or enhanced repressive effects of the formoterol-plus-budesonide combination, compared to either treatment alone, were estimated using one-way ANOVA followed by Tukey’s post-hoc test. Significant combinatorial effect is determined for genes showing adjusted P value of 0.05 or less. Positive or negative values of the difference between means defines if the significant combination effect were *Supra*- or *infra*-additive, respectively. In each case, the calculated P values for differentially regulated genes compared to untreated group or for the identification of combinatorial effects are purely descriptive. These are presented for the purpose of ranking and grouping purposes and should not be construed as testing a specific hypothesis.

Functional annotation analysis. Gene ontology (GO) analyses were performed using the functional annotation chart and functional annotation clustering tools within the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8 (Huang et al., 2009). GO categories for analysis were restricted to molecular function, biological process and Kyoto Encyclopedia of genes and genome (KEGG) pathway terms using the DAVID default cut-off, P value/EASE score (P_{EASE}) ≤ 0.1 , for enrichment. Multiple testing correction of enrichment P values, Benjamini (P_B) ≤ 0.05 , or where stated ≤ 0.01 , were used to highlight the more robustly enriched terms. For functional annotation analyses, the criterion of ≥ 3 genes per term was also required. In addition, and where specifically stated, less conservative, search criteria using the DAVID default category settings were conducted to increase GO term coverage. For functional annotation clustering, enrichment scores representing $-\log_{10}$ of the geometric mean of all enrichment P values of each term in cluster, were, as recommended (Huang et al., 2009), taken as significant where ≥ 1.3 (i.e. $-\log_{10}(0.05)$). For clarity, GO terms are referred to using quotation marks and italicized text.

Graphical presentation and χ^2 testing. Graphs were produced using GraphPad Prism version 6.01 software (GraphPad Software Inc., La Jolla, CA) or the R package “ggplot2”. The R package

“*pheatmap*” was used to produce heatmaps. χ^2 testing was performed using Prism version 6.01 and assumes 18843 unique mRNAs represented on the PrimeView arrays.

Results

Glucocorticoid- and LABA-regulated transcriptomes in BEAS-2B cells. Over an 18h timeframe in BEAS-2B cells, formoterol (10 nM), budesonide (100 nM) or budesonide-plus-formoterol modified the expression of 276, 777 and 1165 genes, respectively (Fig. 1A, Supplemental Table 1). With formoterol, this was primarily represented by transient gene induction with maximal effects occurring at 2h and relatively few mRNAs were repressed (Fig. 1A & B). By contrast, budesonide produced a modest, 1 – 2h response that was primarily due to gene induction and which progressively increased to record the highest gene count at 18h (Fig. 1A & B). Budesonide also promoted significant, but delayed, gene repression, such that at 18h the number of repressed transcripts greatly exceeded those that were induced (Fig. 1B). Similar profiles of gene expression changes were produced by budesonide-plus-formoterol except that the numbers of genes and genes with peak expression at each time were generally increased relative to either treatment alone (Fig. 1A & B). This was most apparent with the genes showing peak induction at 6 h and those showing peak repression at 6 and 18 h (Fig. 1B).

Co-regulation by glucocorticoid and LABA. With ~20,000 genes in the human genome (Clamp et al., 2007; Ezkurdia et al., 2014), and 18,843 unique genes represented on Primeview arrays, independent assortment for up- or down-regulation by formoterol and budesonide should produce only one or two mRNAs regulated by both stimuli at each time. However, “in common” up- and down-regulation by budesonide and formoterol was markedly over-represented (Supplemental Fig. 1A, Fig. 1C).

Scatterplots of the formoterol- and budesonide-regulated mRNAs also revealed over-representation of formoterol-induced genes that independently showed repression by budesonide (Supplemental Fig. 1A). Indeed, 19.3% of formoterol-induced mRNAs were independently repressed by budesonide

(Supplemental Fig. 1B), an effect that was significant and consistent with the 39.2% of formoterol-induced mRNAs that failed the criteria for induction by formoterol-plus-budesonide (Fig. 1Ci).

In addition to co-regulation, budesonide-plus-formoterol induced or repressed 196 and 198 mRNAs, respectively, that failed to meet the criteria for regulation by either treatment alone (Fig. 1C). These mRNAs represented ~1/3 of the total budesonide-plus-formoterol induced- or repressed-transcriptome and suggest extensive gene-dependent interactions between each treatment.

LABA-induced genes and the effect of glucocorticoid. While formoterol-induced genes showed variability in their mRNA kinetics (Fig. 2A), positive and negative effects of budesonide (defined as $\geq 2/\leq 0.5$ -fold further change at any time) were produced at each time of peak formoterol-induced expression (Fig. 2B). Overall, 58.5% of formoterol-induced genes were modulated by budesonide and repression (36.8%) was the dominant effect (Fig. 2C). This was also consistent with 39.2% of formoterol-induced genes not achieving inducibility by budesonide-plus-formoterol (Fig. 1Ci). Conversely, many of the 46 formoterol-induced genes that were enhanced by budesonide revealed evidence of independent inducibility by budesonide (Fig. 2B). Indeed 37 of these fell within those genes showing independent formoterol- and budesonide-inducibility (Fig. 1Ci).

Glucocorticoid-induced genes and the effect of LABA. The prevalence of delayed induction and considerable variability in kinetics was apparent among the 370 budesonide-induced genes (Fig. 2D). Further modulation ($\geq 2/\leq 0.5$ -fold further change at any time) by formoterol occurred for 20.5% of these transcripts with 3.0% and 17.5% being repressed or enhanced, respectively (Fig. 2E & F). As described above, independent induction by each stimulus was clearly apparent and 29 of the formoterol-enhanced mRNAs showed independent inducibility by formoterol (Fig. 2E & F). Furthermore, an additional 20 budesonide-induced mRNAs showed significant, but < 2 -fold, induction by formoterol. Thus, in BEAS-2B cells, 75% of the budesonide-induced mRNAs that were enhanced by formoterol were also, at least modestly, induced by formoterol alone.

Genes only up-regulated by LABA-plus-glucocorticoid. Of the 196 mRNAs uniquely induced by budesonide-plus-formoterol, 85 showed peak expression at 6 h, and overall 70.9% of these genes showed peak induction at 6 or 18 h (Fig. 2G). Furthermore, 133, or 168, of these genes were modestly (fold >1, but <2), yet significantly ($P \leq 0.05$), induced by formoterol, or budesonide alone (at any time), and 115 genes were significantly upregulated by both treatments (Supplemental Table 1). Thus, low-level inducibility may drive combinatorial effects.

LABA/glucocorticoid interactions: *Supra*- and *infra*-additive effects. To explore combinatorial interactions, the effect (i.e. fold - 1) due to each of formoterol and budesonide were summated and then compared to the effect of budesonide-plus-formoterol. Applying this to the 46 genes up-regulated in common by budesonide and formoterol revealed 20 genes where the effect of budesonide-plus-formoterol was significantly ($P \leq 0.05$) greater than the sum of the effects of budesonide and formoterol (i.e. *supra*-additivity) at one, or more, times (Fig. 3A, Supplemental Table 2). However, multiple other genes were induced in a simple additive manner and 13 of these co-regulated genes revealed significantly less than additivity (*infra*-additivity) at one or more times (Fig. 3A). For example, this was clearly apparent with SGK1 and KLF9, which were induced in common and showed 1 or 2 h peak expression, respectively. Thus, despite co-regulation, *supra*-additivity was not inevitable with co-treatment; absence of interaction and negative interactions also occurred. A similar analysis for the 196 mRNAs only induced by budesonide-plus-formoterol revealed significant *supra*-additivity between budesonide and formoterol for 107 genes (Fig. 3B; Supplemental Table 2). Examples illustrating the dramatic nature of some such effects are shown (Fig. 3C).

Extending the above analysis to all 732 genes induced by any treatment revealed 259 mRNAs showing *supra*-additivity and 196 genes with *infra*-additive effects (all ≤ 0.05) (Supplemental Table 2). Of the 212 formoterol-induced mRNAs, 45 were increased in a *supra*-additive manner, whereas 111 showed *infra*-additivity when formoterol was combined with budesonide (Supplemental Fig. 2A, Supplemental Table 2). Figure 3D reveals striking positive enhancements (upper panels), as well as

profound repression of IL6, IL11 or PDE7B (lower panels), by budesonide on formoterol-induced gene expression. For the 370 budesonide-induced mRNAs, 127 showed *supra*-additivity and 87 revealed *infra*-additivity (all $P \leq 0.05$) with formoterol co-treatment (Supplemental Fig. 2B, Supplemental Table 2). As previously reported, this illustrates the extensive enhancement of budesonide-induced genes by formoterol (Altonsy et al., 2017; Rider et al., 2018), an effect which occurred at each time of peak budesonide-induced gene expression (Fig. 3E, upper panels; Supplemental Fig. 2B). *Infra*-additivity was also apparent at early, intermediate and late times of peak budesonide-induced expression (Fig. 3E, lower panels; Supplemental Fig. 2B).

Finally, many mRNAs showed both positive and negative interactions, albeit at different times. This is illustrated by AREG (Fig. 3E), a budesonide-induced gene that was enhanced by budesonide-plus-formoterol at 1 and 2 h, but at 6 h, formoterol significantly reduced budesonide-induced expression. Similar effects were apparent for multiple other budesonide-, formoterol- and formoterol-plus-budesonide-induced genes (Supplemental Fig. 2C). While reflecting gene-specific control, this indicates the need for considerable caution when interpreting data derived from single time points.

LABA-repressed gene expression and the effect of glucocorticoid. Reduced expression of the 55 formoterol-repressed mRNA was primarily restricted to the time of peak repression and, with 60% of transcripts not showing further modulation, the overall effect of budesonide was modest (Fig. 4A-C). Thus, budesonide co-treatment further reduced expression of 15 formoterol-repressed genes and enhanced expression of 7 genes (Fig. 4C).

Glucocorticoid-repressed gene expression and the effect of LABA. Heat maps of the 413 budesonide-repressed mRNAs highlights the few genes with rapid onset kinetics compared to the more prevalent repression at 6 or 18 h (Fig. 4D). Indeed, 63.2% of these genes revealed repression that was maximal, or still increasing, at 18 h. The effects of formoterol co-treatment were restricted to few genes (Fig. 4E & F). Only 2.6% revealed further repression by formoterol, mostly due to independent repression by both treatments (Fig. 1Cii), while 8.7% showed budesonide-induced repression that was

opposed by formoterol (Fig. 4E & F). Of these latter 36 genes, 23 were formoterol-induced and, 12 were significantly induced, albeit below the 2-fold cutoff (Supplemental Table 1). These represent formoterol-induced genes whose expression was repressed by budesonide.

Genes only down-regulated by LABA-plus-glucocorticoid. The 198 mRNAs, that were only repressed by budesonide-plus-formoterol, revealed primarily late-onset effects with 49% of genes showing peak repression at 18 h (Fig. 4G). However, for each time of peak repression, effects of each treatment alone were also clearly apparent. Thus, significant formoterol-, or budesonide-, dependent repression that did not reach the ≤ 0.5 -fold cutoff occurred for 142, or 186, genes respectively and 134 of these mRNAs were significantly repressed by both treatments.

Enhanced repression by formoterol-plus-budesonide in combination. In the above sections, response additivity and either *supra*-or *infra*-additivity for formoterol and budesonide at maximally effective concentrations was used to define positive or negative cooperativity (Foucquier and Guedj, 2015). However, similar analysis is inappropriate for repressed genes where repression from fold = 1 can only approach zero. While small repressive effects due to two stimuli may combine to give greater than additive repressive effects, more highly repressed mRNAs cannot achieve this. Instead, a highest single agent analysis (Foucquier and Guedj, 2015), which simply identifies genes that display more repression by formoterol-plus-budesonide compared to either treatment alone, was performed.

Of the 641 mRNAs repressed by any treatment (Fig. 1Cii), 117 showed repression by budesonide-plus-formoterol that was significantly greater than for budesonide and formoterol alone (Supplemental Table 3). Eight mRNAs were formoterol repressed and 51 were budesonide-repressed with 7 mRNAs being repressed in common. The remaining 65 genes were all budesonide-plus-formoterol only repressed. With 0, 22, 76 and 31 genes at 1, 2, 6 and 18 h, enhanced repression was predominantly a delayed effect. Overall, 108 mRNAs were significantly repressed by formoterol alone and 114 were repressed by budesonide alone. Furthermore, while at 2 h, modest repression produced by both

treatments combined to give greater repression, at 6 and 18 h, enhanced repression was predominantly repression due to budesonide and this was enhanced by formoterol (Fig. 4H).

GO of LABA-induced gene expression and effect of glucocorticoid. As described for the LABA, indacaterol (Yan et al., 2018), functional annotation of the 212 formoterol-induced genes produced enriched GO terms ($P_B \leq 0.05$) relating to positive and negative control of transcription, cytokines, signal transduction, inflammatory signaling, Wnt signaling, differentiation and development (Table 1). Functional annotation clustering re-enforced themes for transcriptional control, cell cycle/proliferation, growth factors, development, pluripotency, epithelial-mesenchymal transition and cancer (Supplemental Fig. 3A). In addition, a strong inflammatory signal was apparent with clusters 4 and 9 (Fig. 5A, Supplemental Fig 3A).

GO analysis of the 46 formoterol-induced genes that were upregulated by budesonide showed enrichments ($P_{EASE} \leq 0.1$) for terms relating to transcription, development, and apoptosis. These genes are preferentially associated with the formoterol-induced gene clusters relating to development, growth factors, Wnt signaling and cancer (Supplemental Fig. 3A). Conversely, GO annotation of the 78 formoterol-induced genes that were repressed by budesonide showed enrichment of terms related to inflammatory signaling, including “*signal transduction*” and “*TNF signaling pathway*”, with $P_B \leq 0.05$, and “*inflammatory responses*”, “*positive regulation of GTPase activity*”, “*cytokine-cytokine receptor interaction*” and “*Jak-STAT signaling pathway*”, with $P_{EASE} \leq 0.1$. This suggests that budesonide had down-graded the inflammatory signature of formoterol. Repression of inflammatory responses was also indicated by the greater than expected fractions of budesonide-repressed genes within the formoterol-induced annotation (Table 1, Supplemental Fig. 3A). Thus, formoterol-induced inflammatory cytokines (IL6, IL11, IL20, LIF), chemokines (CCL2, CCL7, CXCL2) and receptors (ACKR3, CSF2RB), as per “*cytokine-cytokine receptor interaction*”, “*Jak-STAT signaling pathway*” and “*cytokine activity*”, were repressed by budesonide (Fig. 5A).

GO of glucocorticoid-induced gene expression and the effect of LABA. Functional annotation identified 12 ($P_B \leq 0.05$) GO terms that were enriched for the 370 budesonide-induced genes (Table 2). “*Positive regulation of apoptosis*” was most significant, while various terms related to inflammation and stress, growth factor signaling, migration, differentiation, development, insulin responses and “extracellular matrix organization” were also enriched. These themes were confirmed by functional annotation clustering, which produced clusters relating to TNF signaling/nuclear factor- κ B (NF- κ B) and apoptosis/PI3K-Akt signaling, adhesion and extracellular matrix-receptor interaction, development and morphogenesis (Supplemental Fig. 3B). Of note was that the 70 genes with 1 or 2 h peak expression revealed “*negative*” and “*positive regulation of transcription from RNA polymerase II promoter*” (14 genes each and 4.8- and 3.5-fold enrichments, respectively) as the most and third most significant terms ($P_B = 4.4 \times 10^{-3}$ & 0.019, respectively).

With only 11 budesonide-induced genes repressed by formoterol, GO was unclear. Equally, no terms with $P_B \leq 0.05$ and only 7 terms with $P_{EASE} \leq 0.1$ were identified for the 65 budesonide-induced, formoterol-enhanced genes. These included terms for apoptotic processes, development, “*angiogenesis*” and “*TNF signaling pathway*”, a number of which appear in the budesonide-induced gene GO profile (Table 2, Supplemental Fig. 3B).

Comparative GO analysis of LABA-plus-glucocorticoid-induced genes. While the 196 genes induced only by formoterol-plus-budesonide produced no GO terms meeting $P_B \leq 0.05$, terms for transcriptional control, signaling, proliferation, development, angiogenesis, growth factors, migration, and signaling pathways (PI3K-Akt, notch, wnt, hippo) all showed $P_{EASE} \leq 0.1$. To explore the overall effects of combination treatment, GO terms showing $P_B \leq 0.05$ for at least one treatment were clustered in a comparative analysis (Supplemental Fig. 4). This identified groups of terms that were: A, enriched in all treatments; B, primarily enriched with budesonide, but maintained with the combination; and, C, modestly enhanced with formoterol or budesonide, but highly enriched with the combination (Supplemental Fig. 4). Collectively, these terms related to apoptosis, transcription, development, cell

fate, angiogenesis, proliferation, migration, morphogenesis, differentiation, epithelial to mesenchymal transition and similar effects. Furthermore, significance generally increased for all these terms with combination treatment and, as illustrated by “*Pathways in cancer*” (Fig. 5Bi), this was associated with increased gene counts (Supplemental Fig. 4). A fourth group of terms (group D) were most significantly enriched by formoterol alone (Supplemental Fig. 4). Examples include: “*cytokine activity*” or “*Jak-STAT signaling pathway*”, found in formoterol-induced cluster 4, where pro-inflammatory genes induced by formoterol were repressed by budesonide (Fig. 5A). However, despite reduced enrichments with combination treatment, the gene count associated with each term was not necessarily reduced (Supplemental Fig. 4). For example, while budesonide reduced expression of many formoterol-induced genes in “*cytokine activity*” and “*Jak-STAT signaling pathway*”, (Fig. 5Bii), expression of other formoterol-induced genes was maintained. Furthermore, budesonide also induced genes that were either unaffected by formoterol (GHR, IL12A, JAK2, PIK3R1), or were further enhanced by combination treatment (AREG, BMP2, GDF6, KITLG, MYC, VEGFA, WNT5A) (Fig. 5Bii). Thus, while many inflammatory genes induced by formoterol were repressed by budesonide, budesonide separately induced signaling, cytokines and growth factor genes, such that the overall quality of the response was modified with combination treatment.

GO of LABA-repressed gene expression and effect of glucocorticoid. Functional annotation of the 55 formoterol-repressed genes produced 20 GO terms with $P_{EASE} < 0.1$, but which all failed to reach $P_B \leq 0.05$. These terms are related to the positive and negative regulation of transcription, proliferation, differentiation, and apoptosis. Functional annotation clustering produced 4 clusters containing terms for transcriptional control, cancer, proliferation/differentiation and stress responses (Supplemental Fig. 5A). However, this was insufficient for insight into the few genes showing further modulation by budesonide.

GO of glucocorticoid-repressed gene expression and effect of LABA. Inputting the 413 budesonide-repressed genes into the DAVID yielded 20 enriched GO terms ($P_B \leq 0.05$) (Table 3).

These included terms for apoptosis, transcriptional control, cell migration, chemotaxis, angiogenesis, proliferation, growth factors and related signaling pathways. These data were corroborated by functional annotation clustering, which produced 11 clusters with ≥ 1.3 enrichment scores (Supplemental Fig. 5B). Cluster 1 contained terms for transcriptional control and represented 58 genes, nearly half of which were sequence-specific transcription factors. This, along with 9 additional transcription factors in cluster 4, confirms the extensive repressive effects of budesonide on transcription. Other clusters related to growth factor signaling, proliferation, and chemotaxis.

With only 11 budesonide-repressed mRNAs that were further repressed by formoterol, GO effects were modest (Table 3, Supplemental Fig. 5B). Conversely, various budesonide-repressed terms and clusters showed higher than expected fractions of the 36 budesonide-repressed genes that were enhanced by formoterol (Table 3, Supplemental Fig. 5B). These included clusters enriched with terms for proliferation, transcription, cytokine signaling and chemotaxis. These represent functions associated with the budesonide-repressed genes that were enhanced by formoterol. This supports findings that inflammatory processes up-regulated by formoterol were reduced by budesonide.

Comparative GO analysis of genes repressed by LABA and glucocorticoid in combination. The 198 genes that were repressed only by budesonide-plus-formoterol revealed a weak GO signature, which included GO terms (all $P_{EASE} \leq 0.1$) for transcriptional control and proliferation. All GO terms with $P_B \leq 0.05$ for at least one treatment were therefore combined in a comparative analysis (Supplemental Fig. 6). This highlighted both the modest GO signature due to formoterol-repressed genes as well as the 20 terms ($P_B \leq 0.05$) enriched for the budesonide-repressed genes (Table 3, Supplemental Fig. 6). While some budesonide-enriched terms reduced in significance with combination treatment, other terms were increased. With the exception of three terms, containing formoterol-induced, but budesonide-repressed genes (e.g. IL6, IL11, LIF), the number of repressed genes associated with each term increased from budesonide treatment to the combination. Thus, despite

a modest repressed gene GO profile elicited by formoterol, effects of budesonide were largely maintained or enhanced by formoterol.

Overall GO of LABA- and glucocorticoid-modulated genes. As many GO terms were enriched in both induced and repressed gene lists, functional annotation was performed using combined lists of genes modulated (up or down) by each treatment in BEAS-2B cells to identify GO terms with $P_B \leq 0.05$ for at least one treatment (Supplemental Table. 4). These were further filtered by $P_B \leq 0.01$ to give 86 GO terms that were manually curated into 6 functional groups (Supplemental Fig. 7A). Thus, transcriptional control terms, including positive and negative regulation, were highly enriched with all treatments. This group of terms contained 77, 157 and 238 genes in respect of formoterol, budesonide and combination treatment, respectively, and represents a central feature of each response (Fig. 5C).

Numerous signaling and signal transduction terms, for example, the generic term “*signal transduction*”, were enriched with each treatment and showed the highest gene numbers with combination treatment (Supplemental Fig. 7A, Supplemental Table 4). Likewise, pathway-specific signaling terms, such as “*PI3K-Akt signaling pathway*” or “*MAPK signaling pathway*”, while containing fewer genes, revealed the same trend (Supplemental Fig. 7A, Supplemental Table 4). The greatest levels of significance were obtained for “*TNF signaling pathway*”. With 14, 20 and 27 genes regulated by formoterol, budesonide and formoterol-plus-budesonide, respectively, this term illustrates the interplay between genes induced and repressed by each treatment. Thus, formoterol induced pro-inflammatory mRNAs (e.g. CCL2, CXCL2, EDN1, FOS, IL6, IL15, LIF), many of which (e.g. CCL2, FOS, LIF, IL6) were independently repressed by budesonide and/or showed repression upon combination treatment (Supplemental Fig. 7B). However, other “pro-inflammatory”, formoterol-induce mRNAs were relatively unaffected (CXCL2, IL15) or were enhanced (EDN1) by budesonide. Budesonide also induced mRNAs for TNFAIP3 and NFKBIA, and other genes, which negatively regulate NF- κ B. In the case of NFKBIA, or I κ B α , budesonide-induced expression was unaffected by formoterol, whereas TNFAIP3, or A20, as previously reported (Altonsy et al., 2017), was induced by

formoterol alone and more highly induced by combination treatment. Thus, downregulation of cytokines and chemokines by budesonide combined with the upregulation of repressors of NF- κ B, especially in the context of LABA, may reduce pro-inflammatory effects. Furthermore, reduced expression of transcription factors (e.g. JUN, CREB3L1, CREB5) and signaling components (e.g. MAP2K3, MAP3K14, TRAF3), could also attenuate inflammatory processes. Conversely, induction of EDN1, a pro-inflammatory GPCR agonist, or MAP3K8, an upstream MAPK kinase kinase, by formoterol and budesonide, and their further enhancement by the combination implies complexity.

In terms of associated gene counts, these data show the effects of budesonide to be generally increased by combination treatment (Supplemental Fig. 7A, Fig. 5C). While signaling and transcriptional control were the second and third most highly represented groups by gene number, the proliferation, differentiation and development group of terms was represented by 354 genes with combination treatment (Fig. 5C). This represents the dominant effect, by gene and GO term number, of combination treatment. Nevertheless, cell adhesion and migration (171 genes), or inflammation and stress (142 genes) were also major components of the response to combination treatment and, in each case, showed increased gene numbers, and general significance, with combination treatment.

Formoterol- and budesonide-modulated transcriptomes in pHBECS. In pHBECS, formoterol (10 nM), budesonide (100 nM) or formoterol-plus-budesonide for 6 h induced expression of 140, 94 and 243 genes, and repressed 208, 141 and 370 genes, respectively (Fig. 6A, Supplemental Table 5). This revealed greater than predicted overlap between genes induced, or repressed, by both treatments (Fig. 6B & C). With formoterol-plus-budesonide, 50.7% of formoterol-induced mRNAs were not induced (Fig. 6Ci). Furthermore, 17.9% of the formoterol-induced mRNAs showed $\geq 50\%$ repression by budesonide, whereas 7.9% were further enhanced (≥ 2 -fold) (Fig. 6D). Thus, repression was the main effect of budesonide on formoterol-induced gene expression. Conversely, few budesonide-induced mRNAs were repressed ($\geq 50\%$) by formoterol, whereas 15.9% showed further enhancement (≥ 2 -fold). Enhancement was therefore the main effect of formoterol on budesonide-induced expression (Fig. 6D).

With combination treatment, 109 genes (44.9%) were induced, but failed the induction criteria for either treatment alone (Fig. 6Ci). Of these, 74 were significantly induced by formoterol, 86 by budesonide, with 59 mRNAs induced by both treatments, albeit below 2-fold. Thus, modest inducibility by each treatment in fact characterizes those genes only significantly (≥ 2 -fold) induced by combination treatment.

Turning to repression, 42 formoterol-repressed mRNAs were not repressed with budesonide-plus-formoterol, and eight (3.8%) of these were increased by ≥ 2 -fold (Fig. 6Cii & E). Expression of 19 (9.1%) formoterol-repressed genes was further reduced ($\geq 50\%$) by budesonide co-treatment (Fig. 6E). A more polarized effect was apparent for the budesonide-repressed genes, where only 15 failed to meet the criteria for repression with budesonide-plus-formoterol and just 1 gene (0.7%) revealed a ≥ 2 -fold increase (Fig. 6Cii & E). Conversely, 38 (27%) of the budesonide-repressed genes showed $\geq 50\%$ further repression with formoterol (Fig. 6E). Thus, while repression produced by formoterol was enhanced by budesonide, budesonide-dependent repression was more widely enhanced by formoterol. In addition, with formoterol-plus-budesonide, 162 genes reached the criteria for repression that was not reached with either treatment alone (Fig. 6Cii). Furthermore, with 125 genes significantly repressed by each treatment, and 106 being significantly repressed by both treatments, modest repression by formoterol and/or budesonide predicts combinational effects (Supplemental Table 5).

Induced genes show *supra*- and *infra*-additive effects of LABA/glucocorticoid combination in pHBECS. Of the 328 genes induced by formoterol, budesonide or by the combination, 55 showed significant *supra*-additivity, whereas 35 showed significant *infra*-additivity (Supplemental Table 5).

Of those genes showing *supra*-additivity, 5 were induced genes common to both treatments and 34 were formoterol-plus-budesonide-only induced genes. Similarly, of the 79 budesonide-induced genes that were not formoterol-induced, 11 genes showed significant *supra*-additivity, whereas only 4 revealed *infra*-additivity (Supplemental Table 5). Thus, enhancement of budesonide-induced gene expression by formoterol was more common than repression. This compared with the 125 genes that

were only induced by formoterol, where in the presence of budesonide, 6 revealed significant *supra*-additivity and 30 showed *infra*-additivity. Thus, the major effect of budesonide on formoterol-induced genes was to reduce gene expression.

Enhanced repression in pHBEs by formoterol-plus-budesonide. Assessment of combinatorial effects between budesonide and formoterol on repressed genes was restricted to identifying mRNAs that showed more repression with the combination compared to each treatment alone. Of the 429 genes repressed by any treatment, 43 were significantly more repressed by budesonide-plus-formoterol compared to either treatment (Supplemental Table 5). Forty-one of these mRNAs were significantly repressed by budesonide alone and 38 were significantly repressed by formoterol alone. Thus, repression by each treatment may combine to produce enhanced repression with combination treatment (Fig. 6F).

GO associated with budesonide- and formoterol-induced genes in pHBEs. Functional annotation of the genes induced by formoterol, budesonide or budesonide-plus-formoterol produced weak enrichments with just a single term, “*cellular response to insulin stimulus*”, in the budesonide-induced gene list, reaching $P_B \leq 0.05$. Numerous terms for each treatment met the lower, $P_{EASE} \leq 0.1$, level of significance, including terms for positive and negative transcriptional control and signaling. Formoterol-induced genes showed enrichment for development, differentiation, positive and negative regulation of proliferation, wounding responses, cancer, apoptosis and metabolism. Similarly, the budesonide-induced list revealed terms for cell cycle, differentiation and apoptosis, as well as signaling terms relating to metabolism, inflammation and proliferation. Functional annotation for the 243 budesonide-plus-formoterol-induced genes also introduced terms for development, differentiation, proliferation, apoptosis, cancer and many related pathways.

GO associated with budesonide- and formoterol-repressed genes in pHBEs. With the 208 formoterol-repressed genes, functional annotation yielded GO terms ($P_B \leq 0.05$) that related to protein phosphatase activity and “*inactivation of MAPK activity*”, plus a fifth term, “*inflammatory response*”

(Table 4). Various terms for transcription, apoptosis, development, positive and negative proliferation, migration and chemotaxis, differentiation and “*positive regulation of GTPase activity*” all met the lower $P_{EASE} \leq 0.1$ threshold.

The 141 budesonide-repressed genes produced 23 terms with enrichments of $P_B \leq 0.05$ (Table 5). Of these, “*growth factor activity*” was most significant, with other terms relating to proliferation, cell division and angiogenesis. Likewise, “*inflammatory response*” was highly enriched and this linked with “*cytokine activity*”, “*immune response*” and “*cytokine-cytokine receptor interaction*”. Together with pathway terms for “*NF-kappa B signaling pathway*” and “*TNF signaling pathway*”, these terms suggest repression of inflammatory signals. However, “*inactivation of MAPK activity*” was also highly enriched and, since the term includes multiple phosphatases, this, rather paradoxically, suggests reduced control of MAPK activity.

With the 370 budesonide-plus-formoterol-repressed genes, 8 of the 22 GO terms showing $P_B \leq 0.05$ related to transcriptional control (Table 6). Other terms, including “*growth factor activity*” correlated with cell proliferation, angiogenesis and apoptosis, while “*cytokine activity*” and “*inflammatory response*” suggest repression of inflammation. However, “*inactivation of MAPK activity*” and “*negative regulation of ERK1 and ERK2 cascade*” again indicate complexity in determining net function.

Overall GO for genes modulated by formoterol, budesonide or the combination in pHBECS.

The weak GO signatures produced by the induced gene lists, and overlap with the repressed gene GO, prompted combined functional annotation analysis of all up- and down-regulated genes. This produced 65 GO terms, with $P_B \leq 0.05$ for at least one treatment, that were manually curated into 6 functional groups (Supplemental Table 6, Supplemental Fig. 8). Positive and negative terms for transcriptional control were significantly enriched with all treatments with greatest significance and gene number with combination treatment (Supplemental Fig. 8). However, despite almost 150 genes relating to transcriptional control with budesonide-plus-formoterol, this was exceeded by proliferation,

differentiation and development, which represented 208 genes within 20 GO terms and was the largest functional group for each treatment (Supplemental Fig. 8, Fig. 7A). Again, overall significance and gene number was generally greatest with combination treatment. For example, “*growth factor activity*” contained 10, 14 and 17 significantly induced or repressed genes for formoterol, budesonide or formoterol-plus-budesonide, respectively (Supplemental Table 6, Fig. 7B). Visualization of gene expression changes revealed, not only the extent to which many growth factors were repressed by formoterol and budesonide, but also how repression can be enhanced by combination treatment (Fig. 7B). Conversely, while various factors (e.g., AREG, BDNF, EPGN) revealed formoterol-induced expression that was reduced by combination treatment, other genes (e.g., DKK1, INHBB) showed increased expression.

Related to the effects on proliferation and growth were GO terms in the adhesion and migration group (Supplemental Fig. 8). While representing fewer overall terms and genes, these showed the same trend towards greatest significance and number of genes with formoterol-plus-budesonide (Supplemental Fig. 8, Fig. 7A). This effect was less clear for the inflammation and stress group, where enrichments were consistently most significant with budesonide treatment. Furthermore, while the number of genes associated with formoterol-plus-budesonide for each term were generally higher, or at least equal, to those obtained for budesonide alone, some terms, for example “*Cytokine-cytokine receptor interaction*” and “*cytokine activity*”, showed reduced gene numbers with combination treatment (Fig. 7B). For illustration, CXCL2 and AREG were induced by formoterol and repressed by budesonide, but in combination, these effects largely cancelled out (Fig. 7B & C). Thus, multiple inflammatory genes, including cytokines (TSLP), chemokines (CXCL3, CXCL5, CXCL6), chemokine receptors (ACKR3 and CXCR4) and tyrosine kinase receptor ligands (EPHA4, EFNA1) were induced by formoterol, repressed by budesonide alone and with the combination were repressed relative to formoterol (Fig. 7B & C). Furthermore, repression of many inflammatory genes, including cytokines (IL1A, IL1B, IL11, IL24, IL36G and TSLP) and chemokines (CXCL1, CXCL2, CXCL8) was widely

observed with budesonide and in combination with formoterol repression was maintained, or enhanced (Fig. 7B). Conversely, other inflammatory products, receptors or growth factors, albeit fewer in number (see Fig. 7B), revealed budesonide- and or formoterol-induced expression that was maintained, or even increased, by combination treatment.

Finally, effects of formoterol and budesonide on GO for signaling generally conformed to the pattern of increasing significance and associated gene number with combination treatment (Supplemental Fig. 8, Fig. 7A). Furthermore, many of the signaling terms related to the other functional groupings, including cell proliferation and movement, differentiation, development, inflammation and stress. More difficult to explain are GO terms for phosphatases and inactivation of MAPK pathways that were prominent among the genes repressed by formoterol and budesonide-plus-formoterol (Tables 4 & 6). Visualization of gene expression changes associated with “*inactivation of MAPK activity*” and “*protein tyrosine phosphatase activity*” revealed 6 dual-specificity phosphatases (DUSPs), which target MAPKs (Jeffrey et al., 2007), 5 protein phosphatases and 3 protein tyrosine phosphatase (PTP) receptors (Fig. 7B). Since a majority (13/19) of these genes showed formoterol-dependent repression that, with the exception of DUSP10, was maintained, or enhanced, with budesonide-plus-formoterol, enhanced protein phosphorylation is predicted. Conversely, as DUSP1 represses MAPK activity and expression was increased by budesonide, and enhanced by formoterol, cross-regulation may be important.

Discussion

The GINA guidelines now recommend ICS/LABA combination therapy as reliever and/or maintenance in all patients with asthma (GINA, 2020). This revised approach should reduce mortality in asthmatics who take insufficient ICS, but overuse SABA for symptom relief. However, despite the clinical superiority of ICS/LABA combination therapies over ICS alone, a molecular basis remains

unclear. We hypothesised involvement of transcriptional interactions and this was tested in airway epithelial cells, a primary therapeutic target for these drugs. Widespread gene expression changes occurred in response to formoterol and budesonide with genes showing “in common” up- and down-regulation by both treatments being over-represented. Similarly, genes up-regulated by formoterol, but repressed by budesonide, were also over-represented. Such data suggest biological relevance. Indeed, the effect of formoterol-plus-budesonide was not simply a summation of the responses by each component; formoterol modified responses to budesonide, and budesonide modified responses to formoterol. Furthermore, ~1/3 of the genes modulated by formoterol-plus-budesonide only met the criteria for regulation with combination treatment. This outcome could not have been anticipated from the actions of each drug alone. Thus, effects of formoterol and budesonide alone, or in combination, are defined by gene populations, each with distinct “functional” signatures. Moreover, commonality between glucocorticoid-regulated genes, and their associated GO profiles, in BEAS-2B, pHBEs, and the human airways post-budesonide inhalation (Leigh et al., 2016; Mostafa et al., 2019), suggest that ICS/LABA interactions are pertinent to asthma therapy.

Formoterol generated a gene induction profile that reflected rapid (1-2h)-onset cAMP signaling (Mayr and Montminy, 2001; Zhang et al., 2005). GO analysis indicated transcription and signaling as major effector responses in which multiple terms specified outputs associated with inflammation. This confirms studies showing β_2 -agonists to promote inflammatory mediator expression in structural cells (Ammit et al., 2002; Edwards et al., 2007; Strandberg et al., 2007), including in HBECs (Yan et al., 2018). One example from pHBEs was TSLP, a cytokine associated with asthma pathogenesis (Mitchell and O'Byrne, 2017), which was up-regulated by formoterol but then repressed upon budesonide co-treatment. Similar effects occurred with other cytokines, chemokines, growth factors and/or their receptors. Furthermore, despite inflammatory stimuli augmenting pro-inflammatory effects of β_2 -agonists, repression by glucocorticoid persisted and suggests benefit for combination therapies in asthma (Ammit et al., 2002; Edwards et al., 2007; Holden et al., 2010).

Compared to formoterol, most budesonide-induced gene expression changes occurred after a lag of several hours. In BEAS-2B cells, expression of 70 induced genes peaked at 1-2h, whereas 300 mRNAs showed peak expression at 6-18h. Similarly, 393 of the 413 budesonide-repressed genes showed peak repression at 6-18h. Thus, the main responses to budesonide were delayed and are consistent with early GR-dependent transcription eliciting later-onset activation and repression (Chinenov et al., 2014; Newton and Holden, 2007; Reddy et al., 2009; Sasse and Gerber, 2014). Indeed, GO analysis of 1-2h budesonide-induced genes showed positive and negative regulation of transcription among the most highly enriched terms. This is consistent with transcriptional activators and repressors being glucocorticoid-inducible in multiple cell types, including epithelial cell lines (BEAS-2B, A549), pHBEs and the human airways post-budesonide inhalation (Chinenov et al., 2014; Himes et al., 2014; Kan et al., 2019; Leigh et al., 2016; Mostafa et al., 2019). Nevertheless, other control mechanisms also regulate downstream gene expression. For example, in BEAS-2B cells (this study), A549 cells and the human airways, glucocorticoids induced expression of the mRNA destabilizing protein, ZFP36 (King et al., 2009; Leigh et al., 2016; Smoak and Cidlowski, 2006). This will reduce inflammatory gene expression (Clark and Dean, 2016). However, while in BEAS-2B cells, budesonide induced negative regulators of inflammatory signaling and gene expression, including, DUSP1, NFKBIA and TNFAIP3; other glucocorticoid-induced genes, JAK2, MAP3K8, IL12A and EDN1, were pro-inflammatory. Thus, GO terms relating to inflammation may reflect mixed pro- and anti-inflammatory effects.

In BEAS-2B cells, numerous budesonide-induced genes related to growth factor responses, proliferation, differentiation, development and migration. The strength of these associations (enrichment and gene number) was increased when induced and repressed gene lists were combined suggesting that both induction and repression contribute to net biological function. When formoterol and budesonide were used in combination, these GO enrichments and associated gene numbers generally increased relative to either treatment alone. This occurred in both BEAS-2B cells and pHBEs, strengthening the idea that glucocorticoid alone, or in combination with LABA, can modulate

proliferation, differentiation, development, adhesion and migration. Indeed, growth factor expression was generally suppressed by glucocorticoids in cell lines and pHBECs (Mostafa et al., 2019), and is consistent with inhibition of proliferation (Bird et al., 2015). However, GO terms linking to both positive and negative regulation of proliferation and apoptosis were enriched with formoterol and budesonide treatments. As these were enhanced by combination treatment, overall function may be complex to unravel.

As described above, the principal effects of glucocorticoid were delayed, with the greatest numbers of budesonide-induced or -repressed genes occurring at 6-18h. At these times, formoterol-modulated gene expression changes were past their peak and, therefore, modest. However, in combination with budesonide, formoterol markedly enhanced the number of genes showing late induction or repression. Explanations for this are multiple, but as β_2 -adrenoceptor (ADRB2) mRNA expression was not markedly induced by any of the treatments in BEAS-2B or pHBECs, and glucocorticoid had no significant effect on LABA-induced signaling or CRE reporter activity (Rider et al., 2018), these effects were likely not due to enhancement of β_2 -adrenoceptor signaling by glucocorticoids. More plausible are interactions between glucocorticoid- and cAMP-activated and/or -induced transcription factors. In this respect, formoterol does not generally enhance GR binding to target genes in BEAS-2B cells (Rider et al., 2018). However, cAMP activates multiple transcription factors (Sassone-Corsi, 2012). Thus, GR and cAMP-activated factors may interact to regulate specific genes. Indeed, the over-representation of co-regulated genes supports this possibility. However, regulation by both budesonide and formoterol was not synonymous with positive cooperativity; many genes showed no more than simple additivity. For example, in BEAS-2B cells and airway smooth muscle cells, DUSP1, is independently induced by LABA and glucocorticoid and these effects summate in combination (Kaur et al., 2008; Manetsch et al., 2013; Manetsch et al., 2012). This not only illustrates how glucocorticoid-induced gene expression can promote repression of transcripts whose expression requires MAPK activity (Clark et al., 2008; Shah et al., 2014), but also shows how repression could be enhanced by

LABA. However, clinical data suggest more than simple additivity between LABAs and ICSs (Newton and Giembycz, 2016). Indeed, the current study identifies numerous genes that are regulated in a *supra*-additive manner by budesonide and formoterol. Furthermore, analysis of CRE and GRE reporters or gene expression in BEAS-2B cells shows that 10 nM formoterol and 100 nM budesonide, as used herein, are maximally effective and markedly exceed the affinities for their respective receptors (Alexander et al., 2017; Holden et al., 2014; Kaur et al., 2008; Rider et al., 2018; Rider et al., 2011; Yan et al., 2018). (Rider et al., 2018). Thus, *supra*-additive responses produced at saturating concentrations of formoterol and budesonide meet a simple definition of synergy, one that is achieved by multiple genes. These included transcription factors, solute carriers, cell cycle regulators and metabolic enzymes, for which functional roles in the context of asthma remain unclear. While such genes require investigation, one synergistically up-regulated gene that illustrates the therapeutic superiority of ICS/LABA combination therapy in asthma is, as mentioned above, the bronchoprotective gene, *RGS2*. Aside from reducing pro-inflammatory effects of LABAs, ICS acting on the airway epithelium may mimic endogenous glucocorticoids in regulating stress responses, healing and repair (Busillo and Cidlowski, 2013). Furthermore, glucocorticoid/LABA interactions may replicate physiological stress responses whereby endogenous adrenaline and cortisol interact to control the consequences of damage or insult. Focused mechanistic studies are therefore essential to tease out the functional properties of these commonly used asthma therapies, especially when used in combination. As glucocorticoids promote lung maturation, regulate proliferation and apoptosis, and may stimulate epithelial differentiation (Bird et al., 2015), such questions are important. Excessive modulation of these effects may be undesirable in a ubiquitously used therapy, whereas promotion of healing and repair could be beneficial in asthma. Since, co-operative interactions between LABAs and glucocorticoids are class specific (Newton and Giembycz, 2016), the current findings should apply to all clinically used ICS/LABA combinations. Understanding these effects may enable improvements to the effectiveness of ICS/LABA combinations indicated at each stage of the GINA guidelines. Finally,

since β_2 -agonist- and ICS-dependent gene expression changes are apparent in transcriptomic analyses of severe asthma (Djukanovic, 2019; Weathington et al., 2019), the current analysis helps distinguish therapy-dependent changes in gene expression from disease phenotype. Since the current study focused on ICS/LABAs interactions *in vitro*, further investigations are required in more physiologically and pathologically relevant systems. For instance, the influence of inflammatory stimuli, the presence of serum, and the use of primary cells from patients with mild, moderate and severe disease should be assessed. Likewise, understanding the transcriptomic signature due to ICS/LABA in patients, where pharmacokinetics, differences in severity, or endotypes of asthma may affect responsiveness, will be required for the identification of gene expression changes that associate with therapeutic benefit.

Authorship Contributions

Conducted experiments: CFR, MMM

Collection/provision of primary cells: RL

Data analysis, preparation and presentation: MMM, CFR, NDW, RN

Overall research concept and obtained funding: MAG, RL, RN

Manuscript preparation: RN, MAG, MMM; all authors contributed to reviewing and editing the manuscript.

References

- Alexander SP, Kelly E, Marrion NV, Peters JA, Faccenda E, Harding SD, Pawson AJ, Sharman JL, Southan C, Buneman OP, Cidlowski JA, Christopoulos A, Davenport AP, Fabbro D, Spedding M, Striessnig J, Davies JA and Collaborators C (2017) THE CONCISE GUIDE TO PHARMACOLOGY 2017/18: Overview. *Br J Pharmacol* **174** Suppl 1: S1-S16.
- Altonsy MO, Mostafa MM, Gerber AN and Newton R (2017) Long-acting beta2-agonists promote glucocorticoid-mediated repression of NF-kappaB by enhancing expression of the feedback regulator TNFAIP3. *Am J Physiol Lung Cell Mol Physiol* **312**(3): L358-L370.
- Ammit AJ, Lazaar AL, Irani C, O'Neill GM, Gordon ND, Amrani Y, Penn RB and Panettieri RA, Jr. (2002) Tumor necrosis factor-alpha-induced secretion of RANTES and interleukin-6 from human airway smooth muscle cells: modulation by glucocorticoids and beta-agonists. *Am J Respir Cell Mol Biol* **26**(4): 465-474.
- Barnes PJ (2011) Glucocorticosteroids: current and future directions. *Br J Pharmacol* **163**(1): 29-43.
- BinMahfouz H, Borthakur B, Yan D, George T, Giembycz MA and Newton R (2015) Superiority of combined phosphodiesterase PDE3/PDE4 inhibition over PDE4 inhibition alone on glucocorticoid- and long-acting beta2-adrenoceptor agonist-induced gene expression in human airway epithelial cells. *Mol Pharmacol* **87**(1): 64-76.
- Bird AD, McDougall AR, Seow B, Hooper SB and Cole TJ (2015) Glucocorticoid regulation of lung development: lessons learned from conditional GR knockout mice. *Mol Endocrinol* **29**(2): 158-171.
- Busillo JM and Cidlowski JA (2013) The five Rs of glucocorticoid action during inflammation: ready, reinforce, repress, resolve, and restore. *Trends Endocrinol Metab* **24**(3): 109-119.
- Chinenov Y, Coppo M, Gupte R, Sacta MA and Rogatsky I (2014) Glucocorticoid receptor coordinates transcription factor-dominated regulatory network in macrophages. *BMC Genomics* **15**: 656.
- Clamp M, Fry B, Kamal M, Xie X, Cuff J, Lin MF, Kellis M, Lindblad-Toh K and Lander ES (2007) Distinguishing protein-coding and noncoding genes in the human genome. *Proc Natl Acad Sci U S A* **104**(49): 19428-19433.
- Clark AR and Belvisi MG (2012) Maps and legends: the quest for dissociated ligands of the glucocorticoid receptor. *Pharmacol Ther* **134**(1): 54-67.
- Clark AR and Dean JL (2016) The control of inflammation via the phosphorylation and dephosphorylation of tristetraprolin: a tale of two phosphatases. *Biochem Soc Trans* **44**(5): 1321-1337.
- Clark AR, Martins JR and Tchen CR (2008) Role of dual specificity phosphatases in biological responses to glucocorticoids. *J Biol Chem* **283**(38): 25765-25769.
- Cockcroft DW and Sears MR (2013) Are inhaled longacting beta2 agonists detrimental to asthma? *Lancet Respir Med* **1**(4): 339-346.
- Djukanovic R (2019) Advancing Understanding of Mechanisms of Severe Asthma and Drug Effects Using Transcriptomics. *Am J Resp Crit Care* **200**(7): 795-796.
- Edwards MR, Haas J, Panettieri RA, Jr., Johnson M and Johnston SL (2007) Corticosteroids and beta2 agonists differentially regulate rhinovirus-induced interleukin-6 via distinct Cis-acting elements. *J Biol Chem* **282**(21): 15366-15375.
- Ezkurdia I, Juan D, Rodriguez JM, Frankish A, Diekhans M, Harrow J, Vazquez J, Valencia A and Tress ML (2014) Multiple evidence strands suggest that there may be as few as 19 000 human protein-coding genes. *Human Molecular Genetics* **23**(22): 5866-5878.
- Fouquier J and Guedj M (2015) Analysis of drug combinations: current methodological landscape. *Pharmacol Res Perspect* **3**(3): e00149.

- George T, Bell M, Chakraborty M, Siderovski DP, Giembycz MA and Newton R (2017) Protective Roles for RGS2 in a Mouse Model of House Dust Mite-Induced Airway Inflammation. *PLoS One* **12**(1): e0170269.
- George T, Chakraborty M, Giembycz MA and Newton R (2018) A bronchoprotective role for Rgs2 in a murine model of lipopolysaccharide-induced airways inflammation. *Allergy Asthma Clin Immunol* **14**: 40.
- GINA (2020) Global Strategy for Asthma Management and Prevention. <https://ginasthma.org/reports/>
- Heximer SP (2004) RGS2-mediated regulation of Gqalpha. *Methods Enzymol* **390**: 65-82.
- Himes BE, Jiang X, Wagner P, Hu R, Wang Q, Klanderman B, Whitaker RM, Duan Q, Lasky-Su J, Nikolos C, Jester W, Johnson M, Panettieri RA, Jr., Tantisira KG, Weiss ST and Lu Q (2014) RNA-Seq transcriptome profiling identifies CRISPLD2 as a glucocorticoid responsive gene that modulates cytokine function in airway smooth muscle cells. *PLoS One* **9**(6): e99625.
- Holden NS, Bell MJ, Rider CF, King EM, Gaunt DD, Leigh R, Johnson M, Siderovski DP, Heximer SP, Giembycz MA and Newton R (2011) beta2-Adrenoceptor agonist-induced RGS2 expression is a genomic mechanism of bronchoprotection that is enhanced by glucocorticoids. *Proc Natl Acad Sci U S A* **108**(49): 19713-19718.
- Holden NS, George T, Rider CF, Chandrasekhar A, Shah S, Kaur M, Johnson M, Siderovski DP, Leigh R, Giembycz MA and Newton R (2014) Induction of Regulator of G-Protein Signaling 2 Expression by Long-Acting beta2-Adrenoceptor Agonists and Glucocorticoids in Human Airway Epithelial Cells. *J Pharmacol Exp Ther* **348**(1): 12-24.
- Holden NS, Rider CF, Bell MJ, Velayudhan J, King EM, Kaur M, Salmon M, Giembycz MA and Newton R (2010) Enhancement of inflammatory mediator release by beta(2)-adrenoceptor agonists in airway epithelial cells is reversed by glucocorticoid action. *Br J Pharmacol* **160**(2): 410-420.
- Huang dW, Sherman BT and Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* **4**(1): 44-57.
- Hudy MH, Traves SL, Wiehler S and Proud D (2010) Cigarette smoke modulates rhinovirus-induced airway epithelial cell chemokine production. *Eur Respir J* **35**(6): 1256-1263.
- Jeffrey KL, Camps M, Rommel C and Mackay CR (2007) Targeting dual-specificity phosphatases: manipulating MAP kinase signalling and immune responses. *Nat Rev Drug Discov* **6**(5): 391-403.
- Jiang H, Xie Y, Abel PW, Wolff DW, Toews ML, Panettieri RA, Jr., Casale TB and Tu Y (2015) Regulator of G-protein signaling 2 repression exacerbates airway hyper-responsiveness and remodeling in asthma. *Am J Respir Cell Mol Biol* **53**(1): 42-49.
- Joshi T, Johnson M, Newton R and Giembycz MA (2015) The long-acting beta2 -adrenoceptor agonist, indacaterol, enhances glucocorticoid receptor-mediated transcription in human airway epithelial cells in a gene- and agonist-dependent manner. *Br J Pharmacol* **172**(10): 2634-2653.
- Kan M, Koziol-White C, Shumyatcher M, Johnson M, Jester W, Panettieri RA, Jr. and Himes BE (2019) Airway Smooth Muscle-Specific Transcriptomic Signatures of Glucocorticoid Exposure. *Am J Respir Cell Mol Biol* **61**(1): 110-120.
- Kaur M, Chivers JE, Giembycz MA and Newton R (2008) Long-acting beta2-adrenoceptor agonists synergistically enhance glucocorticoid-dependent transcription in human airway epithelial and smooth muscle cells. *Mol Pharmacol* **73**(1): 203-214.
- Kelly MM, King EM, Rider CF, Gwozd C, Holden NS, Eddleston J, Zuraw B, Leigh R, O'Byrne PM and Newton R (2012) Corticosteroid-induced gene expression in allergen-challenged asthmatic subjects taking inhaled budesonide. *Br J Pharmacol* **165**(6): 1737-1747.
- Kimple AJ, Bosch DE, Giguere PM and Siderovski DP (2011) Regulators of G-protein signaling and their Galpha substrates: promises and challenges in their use as drug discovery targets. *Pharmacol Rev* **63**(3): 728-749.

- King EM, Kaur M, Gong W, Rider CF, Holden NS and Newton R (2009) Regulation of tristetraprolin expression by interleukin-1 β and dexamethasone in human pulmonary epithelial cells: roles for nuclear factor- κ B and p38 mitogen-activated protein kinase. *J Pharmacol Exp Ther* **330**(2): 575-585.
- Klassen C, Karabinskaya A, Dejager L, Vettorazzi S, Van MJ, Luhder F, Meijsing SH, Tuckermann JP, Bohnenberger H, Libert C and Reichardt HM (2017) Airway Epithelial Cells Are Crucial Targets of Glucocorticoids in a Mouse Model of Allergic Asthma. *J Immunol* **199**(1): 48-61.
- Knight DA and Holgate ST (2003) The airway epithelium: structural and functional properties in health and disease. *Respirology* **8**(4): 432-446.
- Leigh R, Mostafa MM, King EM, Rider CF, Shah S, Dumonceaux C, Traves SL, McWhae A, Kolisnik T, Kooi C, Slater DM, Kelly MM, Bieda M, Miller-Larsson A and Newton R (2016) An inhaled dose of budesonide induces genes involved in transcription and signaling in the human airways: enhancement of anti- and proinflammatory effector genes. *Pharma Res Per* **4**(4): e00243.
- Manetsch M, Rahman MM, Patel BS, Ramsay EE, Rumzhum NN, Alkhouri H, Ge Q and Ammit AJ (2013) Long-acting beta2-agonists increase fluticasone propionate-induced mitogen-activated protein kinase phosphatase 1 (MKP-1) in airway smooth muscle cells. *PLoS One* **8**(3): e59635.
- Manetsch M, Ramsay EE, King EM, Seidel P, Che W, Ge Q, Hibbs DE, Newton R and Ammit AJ (2012) Corticosteroids and beta(2)-agonists upregulate mitogen-activated protein kinase phosphatase 1: in vitro mechanisms. *Br J Pharmacol* **166**(7): 2049-2059.
- Mayr B and Montminy M (2001) Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat Rev Mol Cell Biol* **2**(8): 599-609.
- Mitchell PD and O'Byrne PM (2017) Biologics and the lung: TSLP and other epithelial cell-derived cytokines in asthma. *Pharmacol Ther* **169**: 104-112.
- Mostafa MM, Rider CF, Shah S, Traves SL, Gordon PMK, Miller-Larsson A, Leigh R and Newton R (2019) Glucocorticoid-driven transcriptomes in human airway epithelial cells: commonalities, differences and functional insight from cell lines and primary cells. *BMC Med Genomics* **12**(1): 29.
- Nelson HS, Weiss ST, Bleecker ER, Yancey SW, Dorinsky PM and Group SS (2006) The Salmeterol Multicenter Asthma Research Trial: a comparison of usual pharmacotherapy for asthma or usual pharmacotherapy plus salmeterol. *Chest* **129**(1): 15-26.
- Newton R and Giembycz MA (2016) Understanding how long-acting beta2 -adrenoceptor agonists enhance the clinical efficacy of inhaled corticosteroids in asthma - an update. *Br J Pharmacol* **173**(24): 3405-3430.
- Newton R and Holden NS (2007) Separating transrepression and transactivation: a distressing divorce for the glucocorticoid receptor? *Mol Pharmacol* **72**(4): 799-809.
- Newton R, Shah S, Altonsy MO and Gerber AN (2017) Glucocorticoid and cytokine crosstalk: Feedback, feedforward, and co-regulatory interactions determine repression or resistance. *J Biol Chem* **292**(17): 7163-7172.
- Oakley RH and Cidlowski JA (2013) The biology of the glucocorticoid receptor: New signaling mechanisms in health and disease. *J Allergy Clin Immunol* **132**(5): 1033-1044.
- Oh KS, Patel H, Gottschalk RA, Lee WS, Baek S, Fraser IDC, Hager GL and Sung MH (2017) Anti-Inflammatory Chromatinscape Suggests Alternative Mechanisms of Glucocorticoid Receptor Action. *Immunity* **47**(2): 298-309.
- Reddel HK, FitzGerald JM, Bateman ED, Bacharier LB, Becker A, Brusselle G, Buhl R, Cruz AA, Fleming L, Inoue H, Ko FW, Krishnan JA, Levy ML, Lin J, Pedersen SE, Sheikh A, Yorgancioglu A and Boulet LP (2019) GINA 2019: a fundamental change in asthma management: Treatment of asthma with short-acting bronchodilators alone is no longer recommended for adults and adolescents. *Eur Respir J* **53**(6).

- Reddy TE, Pauli F, Sprouse RO, Neff NF, Newberry KM, Garabedian MJ and Myers RM (2009) Genomic determination of the glucocorticoid response reveals unexpected mechanisms of gene regulation. *Genome Res* **19**(12): 2163-2171.
- Rider CF, Altonsy MO, Mostafa MM, Shah SV, Sasse S, Manson ML, Yan D, Karrman-Mardh C, Miller-Larsson A, Gerber AN, Giembycz MA and Newton R (2018) Long-Acting beta2-Adrenoceptor Agonists Enhance Glucocorticoid Receptor (GR)-Mediated Transcription by Gene-Specific Mechanisms Rather Than Generic Effects via GR. *Mol Pharmacol* **94**(3): 1031-1046.
- Rider CF, King EM, Holden NS, Giembycz MA and Newton R (2011) Inflammatory stimuli inhibit glucocorticoid-dependent transactivation in human pulmonary epithelial cells: Rescue by long-acting beta2-adrenoceptor agonists. *J Pharmacol Exp Ther* **338**(3): 860-869.
- Rider CF, Shah S, Miller-Larsson A, Giembycz MA and Newton R (2015) Cytokine-induced loss of glucocorticoid function: effect of kinase inhibitors, long-acting beta2-adrenoceptor agonist and glucocorticoid receptor ligands. *PLoS One* **10**(1): e0116773.
- Ross EA, Smallie T, Ding Q, O'Neil JD, Cunliffe HE, Tang T, Rosner DR, Klevernic I, Morrice NA, Monaco C, Cunningham AF, Buckley CD, Saklatvala J, Dean JL and Clark AR (2015) Dominant Suppression of Inflammation via Targeted Mutation of the mRNA Destabilizing Protein Tristetraprolin. *J Immunol* **195**(1): 265-276.
- Sasse SK and Gerber AN (2014) Feed-forward transcriptional programming by nuclear receptors: Regulatory principles and therapeutic implications. *Pharmacol Ther*.
- Sassone-Corsi P (2012) The cyclic AMP pathway. *Cold Spring Harb Perspect Biol* **4**(12).
- Shah S, King EM, Chandrasekhar A and Newton R (2014) Roles for the mitogen-activated protein kinase (MAPK) phosphatase, DUSP1, in feedback control of inflammatory gene expression and repression by dexamethasone. *J Biol Chem* **289**(19): 13667-13679.
- Smoak K and Cidlowski JA (2006) Glucocorticoids regulate tristetraprolin synthesis and posttranscriptionally regulate tumor necrosis factor alpha inflammatory signaling. *Mol Cell Biol* **26**(23): 9126-9135.
- Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* **3**: Article3.
- Strandberg K, Palmberg L and Larsson K (2007) Effect of formoterol and salmeterol on IL-6 and IL-8 release in airway epithelial cells. *Respir Med* **101**(6): 1132-1139.
- Weathington N, O'Brien ME, Radder J, Whisenant TC, Bleecker ER, Busse WW, Erzurum SC, Gaston B, Hastie AT, Jarjour NN, Meyers DA, Milosevic J, Moore WC, Tedrow JR, Trudeau JB, Wong HP, Wu W, Kaminski N, Wenzel SE and Modena BD (2019) BAL Cell Gene Expression in Severe Asthma Reveals Mechanisms of Severe Disease and Influences of Medications. *Am J Resp Crit Care* **200**(7): 837-856.
- Xie Y, Jiang H, Nguyen H, Jia S, Berro A, Panettieri RA, Jr., Wolff DW, Abel PW, Casale TB and Tu Y (2012) Regulator of G protein signaling 2 is a key modulator of airway hyperresponsiveness. *J Allergy Clin Immunol* **130**(4): 968-976.
- Yan D, Hamed O, Joshi T, Mostafa MM, Jamieson KC, Joshi R, Newton R and Giembycz MA (2018) Analysis of the Indacaterol-Regulated Transcriptome in Human Airway Epithelial Cells Implicates Gene Expression Changes in the Adverse and Therapeutic Effects of beta2-Adrenoceptor Agonists. *J Pharmacol Exp Ther* **366**(1): 220-236.
- Zhang X, Odom DT, Koo SH, Conkright MD, Canettieri G, Best J, Chen H, Jenner R, Herbolsheimer E, Jacobsen E, Kadam S, Ecker JR, Emerson B, Hogenesch JB, Unterman T, Young RA and Montminy M (2005) Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. *Proc Natl Acad Sci U S A* **102**(12): 4459-4464.

Footnotes

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Figure legends

Fig. 1. Overview effects of formoterol and/or budesonide on gene expression in BEAS-2B cells. BEAS-2B cells were either not stimulated (NS) or treated with formoterol (Form; 10 nM), budesonide (Bud; 100 nM) or both together (Bud + Form) for the indicated times. Cells ($N = 4$ separate experiments) were harvested for microarray analysis. Normalized data was expressed as fold relative to time-matched NS and genes showing ≥ 2 -fold, or ≤ 0.5 fold, $P \leq 0.05$ were considered to be differentially expressed (Supplemental Table 1). (A) The number of differentially expressed genes showing up- or down-regulation at each time is shown for each treatment. Overall number of up- or down-regulated genes at any time with each treatment is shown (righthand panel). (B) For each treatment, the number of genes showing peak expression change from NS (+ or - \log_2 fold) were grouped according to the time of peak expression and the direction of change (up/down). Expression data for each group were summarized using local polynomial regression (solid line) \pm 95% confidence interval (CI; grey shading) of \log_2 fold over time (h). For groups containing 3 or less genes, the regression line of each gene (dashed line) was plotted instead of CI. (C) The number of genes meeting the expression criteria for: *i*, induction (≥ 2 -fold, $P \leq 0.05$), or *ii*, repression (≤ 0.5 -fold, $P \leq 0.05$) at any time in respect of each treatment were represented as Venn diagrams. Below each Venn, contingency tables assess the likelihood (χ^2 test) that the number of genes (induced or repressed) in common between formoterol and budesonide may have occurred by chance. O = observed, E = expected. **** $P = 0.0001$.

Fig. 2. Formoterol- and budesonide-induced gene expression and effects of combination.

(A) The 212 genes induced (≥ 2 -fold, $P \leq 0.05$) at any time by formoterol in BEAS-2B cells were grouped according to the time of peak expression (\log_2 fold) and are presented as heat maps. Red = induced, Blue = repressed, White = $\log_2(1)$, color intensity reflects increasing change in \log_2 fold. (B)

Expression of the genes showing peak formoterol-induced expression at each time were summarized using local polynomial regression (solid line) \pm 95% CI (grey shading) of \log_2 fold over time (h). For each time of peak formoterol-induced expression, the genes were stratified according to the greatest effect (+1, 0 or -1) of budesonide occurring at any time. +1 = ≥ 2 -fold, 0 = $>0.5/ < 2$ -fold and -1 = ≤ 0.5 -fold produced by budesonide addition when compared to formoterol-induced expression. Red line = formoterol, blue line = budesonide, purple line = budesonide + formoterol. (C) Graphical representation showing the gene count for formoterol-induced genes meeting the criteria (+1, 0 or -1) in (B) for the effect of budesonide (Bud) addition. (D) The 370 genes induced (≥ 2 -fold, $P \leq 0.05$) at any time by budesonide in BEAS-2B cells were grouped according to the time of peak expression (\log_2 fold) and presented as heat maps (Red = induced, Blue = repressed). (E) Expression of genes showing peak budesonide-induced expression at each time were summarized as in B. For each time of peak budesonide-induced expression group, the genes were stratified according to the greatest effect (+1, 0 or -1) of formoterol occurring at any time. +1 = ≥ 2 -fold, 0 = $>0.5/ < 2$ -fold and -1 = ≤ 0.5 -fold produced by formoterol addition when compared to budesonide-induced expression. Line colors are as in B. (F) Graphical representation showing the gene count for budesonide-induced genes meeting the criteria (+1, 0 or -1) in (E) for the effect of formoterol (Form) addition. (G) The 196 genes induced (≥ 2 -fold, $P \leq 0.05$) at any time by budesonide + formoterol, but not by either formoterol or budesonide alone in BEAS-2B cells, were grouped according to the time of peak expression and summarized as in B. Line colors are as in B.

Fig. 3. *Supra-* and *infra-*additive effects of formoterol and budesonide on gene expression. (A) The 46 genes induced (≥ 2 -fold, $P \leq 0.05$) by both formoterol and budesonide at any time in BEAS-2B cells were grouped according to the time of peak expression and expression was summarized using local polynomial regression (solid line) \pm 95% CI (grey shading) of \log_2 fold over time (h) (left panel). For each group of genes, heat maps were generated to show fold gene expression for each treatment and

time (center panel). Red = induced, Blue = repressed, White = 1, color intensity reflects fold change. B = budesonide, F = formoterol, BF = budesonide plus formoterol. For each gene (righthand main panel), the effect (fold -1) of budesonide and formoterol was summed ($B-1 + F-1 = B+F-2$) and displayed next to the effect of budesonide-plus-formoterol (BF-1) as a heat map using the same scale as the prior panel. Differences between $B+F-2$ and BF-1 were compared by one-way ANOVA followed by Tukey's post-hoc test (far right panel). *P* values presented as heat maps are shown where purple color intensity corresponds to increasing $-\log_{10}(P \text{ value})$, while values below 1.3 ($P < 0.05$) are all white. (B) The 196 genes showing induced expression by budesonide-plus-formoterol ($2\geq\text{fold}$, $P\leq 0.05$), but not by budesonide or formoterol alone were grouped, as in Fig. 2G, by time of peak expression. The effect of budesonide was summed with the effect of formoterol ($B+F-2$) and displayed as a heat map next to the effect of budesonide-plus-formoterol (BF-1) (left panel) using the same scale as in A. Similarly, significance was tested and *P* values presented as a heat map (right panel), as in A. (C) For each time of budesonide-plus-formoterol-induced peak expression, one gene showing budesonide-plus-formoterol, but not budesonide or formoterol alone induced expression from B is shown. (D) Examples of formoterol-induced genes displaying significant *supra*- (upper panels) or *infra*-(lower panels) additivity are shown for peak formoterol-induced expression at 1, 2 and 6 h. Color coding is as in C. (E) Examples of formoterol-induced genes displaying significant *supra*- (upper panels) or *infra*-(lower panels) additivity are shown for peak formoterol-induced expression at 1, 2 and 6 h. Color coding is as in C.

Fig. 4. Formoterol- and budesonide-repressed gene expression and effects of combination. (A) The 55 genes, which were repressed (≤ 0.5 -fold, $P\leq 0.05$) at any time by formoterol in BEAS-2B cells, were grouped according to the time of peak repression (\log_2 fold) and presented as heat maps (Red = induced, Blue = repressed, White = $\log_2(1)$, color intensity reflects increasing \log_2 fold change). (B) Expression of the 2, 25, 24 and 4 genes showing peak formoterol-induced repression at 1, 2, 6 or 18 h

were summarized using local polynomial regression (solid line) \pm 95% CI (grey shading) of \log_2 fold over time (h). For each time of peak formoterol-induced repression group, the genes were further stratified according to the greatest effect (+1, 0 or -1) of budesonide occurring at any time. +1 = ≥ 2 -fold, 0 = $>0.5/ < 2$ -fold and -1 = ≤ 0.5 -fold produced by budesonide addition when compared to formoterol-repressed expression. Red line = formoterol, blue line = budesonide, purple line = budesonide + formoterol. (C) Graphical representation showing the gene count for formoterol-repressed genes meeting the criteria (+1, 0 or -1) in (B) for the effect of budesonide (Bud) addition. (D) The 413 genes, which were repressed (≤ 0.5 -fold, $P \leq 0.05$) at any time by budesonide in BEAS-2B cells, were grouped according to the time of peak repression (\log_2 fold) and presented as heat maps (Red = induced, Blue = repressed). (E) Expression of the 3, 17, 132 and 261 genes showing peak budesonide-induced repression at 1, 2, 6 or 18 h were summarized as in B. For each time of peak budesonide-induced repression group, the genes were further stratified according to the greatest effect (+1, 0 or -1) of formoterol occurring at any time. +1 = ≥ 2 -fold, 0 = $>0.5/ < 2$ -fold and -1 = ≤ 0.5 -fold produced by formoterol addition when compared to budesonide-repressed expression. Red line = formoterol, blue line = budesonide, purple line = budesonide + formoterol. (F) Graphical representation showing the gene count for budesonide-repressed genes meeting the criteria (+1, 0 or -1) in (E) for the effect of formoterol (Form) addition. (G) The 198 genes, which were repressed (≤ 0.5 -fold, $P \leq 0.05$) at any time by budesonide + formoterol, but not by either formoterol or budesonide alone, in BEAS-2B cells, were grouped according to the time of peak repression. The 25, 77 and 96 genes with peak repression at 2, 6 or 18 h were summarized as in B. Red line = formoterol, blue line = budesonide, purple line = budesonide + formoterol. (H) The 117 genes that showed enhanced repression by budesonide-plus-formoterol, when compared to both budesonide and formoterol alone, were grouped according to the time of peak repression. There were 22, 76 and 31 genes showing peak repression at 2, 6 and 18 h respectively. Fold data for each group were expressed as \log_2 (fold) and are plotted as box and whiskers to show the effect of each treatment.

Fig. 5. Functional aspects of formoterol-induced gene expression with effect of budesonide and overview GO analysis of genes modulated by formoterol, budesonide and budesonide-plus-formoterol in BEAS-2B cells. (A) Functional annotation clustering of the 212 gene induced by formoterol resulted in 11 GO term clusters with enrichment scores ≥ 1.3 (Supplemental Fig. 3). *i.* GO terms making up cluster 4 were depicted along with a bar graph showing the fraction of formoterol-induced genes that were further enhanced (≥ 2 -fold) or repressed (≤ 0.5 -fold) by budesonide. Expected fractions (enhanced = 0.22, repressed = 0.37) are indicated by dotted lines. Of the 21 genes in this cluster, 3 were further enhanced (≥ 2 -fold) and 12 were repressed (≤ 0.5 -fold) by budesonide cotreatment making this cluster predominantly repressed by budesonide. *ii.* Heat map showing effects of formoterol (Form), budesonide (Bud) and Bud + Form on the 21 genes within this cluster. Red = induced, Blue = repressed, White = $\log_2(1)$, color intensity reflects increasing change in \log_2 fold. (B) Heap maps showing gene expression, as in A, in response to formoterol, budesonide and budesonide-plus-formoterol (Bud + Form) for all induced genes that were associated with the GO terms: *i)* “Pathways in cancer”, and *ii)* “cytokine activity” plus “Jak-STAT signaling pathway”, with any treatment. (C) GO terms (biological process, molecular function, KEGG) for the 267, 777 and 1165 genes modulated, either up (≥ 2 -fold, $P \leq 0.05$) or down (≤ 0.5 -fold, $P \leq 0.05$), by formoterol (Form), budesonide (Bud), or budesonide-plus-formoterol (B + F) in BEAS-2B cells, were extracted using the DAVID. The number of individual genes within each functional grouping are indicated for each treatment. prolifer, diff & dev = proliferation, differentiation & development.

Fig. 6. Effects of formoterol and budesonide alone, or in combination, on gene expression in pHBE cells. pHBE cells from 6 normal individuals were either not stimulated (NS) or treated with formoterol (Form; 10 nM) and budesonide (Bud; 100 nM) or both together (Bud + Form) for 6 h prior to RNA extraction and microarray analysis. Following RMA and normalization, data was expressed as fold

relative to NS and the genes showing ≥ 2 -fold, or ≤ 0.5 fold, $P \leq 0.05$ were considered to be differentially expressed (Supplemental Table 5). (A) The number of differentially expressed genes showing up- or down-regulation for each treatment is shown. Overall gene numbers of modulated (up- and down-regulated) genes are shown below the graph. (B) Expression of genes showing different expression by formoterol was plotted against the effect of budesonide alone and expression of the genes showing differential expression to budesonide was plotted against the effect of formoterol alone. Gene numbers in each corner quadrant is shown along with expected numbers in brackets. (C) The number of genes meeting the expression criteria for: *i*, induction (≥ 2 -fold, $P \leq 0.05$), or *ii*, repression (≤ 0.5 -fold, $P \leq 0.05$) at any time in respect of each treatment were represented as Venn diagrams. Beside each Venn diagram, contingency tables assess the likelihood (χ^2 test) that the number of genes (induced or repressed) in common between formoterol and budesonide may have occurred by chance. O = observed, E = expected. **** $P = 0.0001$. (D) The 140 formoterol-induced genes were plotted to show the number of genes that were further induced (≥ 2 -fold) or repressed (≤ 0.5 -fold) with budesonide co-treatment and the 94 budesonide-induced genes were plotted to show the number of genes that were further induced (≥ 2 -fold) or repressed (≤ 0.5 -fold) by formoterol co-treatment. (E). The 208 formoterol-repressed genes were plotted to show the number of genes that were induced (≥ 2 -fold) or further repressed (≤ 0.5 -fold) with budesonide co-treatment and the 141 budesonide-repressed genes were plotted to show the number of genes that were induced (≥ 2 -fold) or further repressed (≤ 0.5 -fold) by formoterol co-treatment.

Fig. 7. GO analysis of genes modulated by formoterol, budesonide and budesonide-plus-formoterol in pHBE cells. (A) GO terms (biological process, molecular function, KEGG) for the 348, 235 and 613 genes modulated, either up (≥ 2 -fold, $P \leq 0.05$) or down (≤ 0.5 -fold, $P \leq 0.05$), by formoterol, budesonide, or formoterol plus budesonide in pHBE cells, were extracted using the DAVID. The number of individual genes within each functional grouping are indicated for each treatment. prolifer, diff & dev =

proliferation, differentiation & development. (B) Heat map showing effects of formoterol (F), budesonide (B) and budesonide + formoterol (BF) on the expression of all those genes, within the indicated GO terms, that were significantly modulated by any treatment. Red = enhanced fractions, Blue = repressed. (C) Expression data for selected genes showing significant enhancement by formoterol. Microarray data for the indicated genes was plotted as \log_2 fold. Effects of formoterol (Form), budesonide (Bud), and budesonide + formoterol (B + F) are shown. Significance (ANOVA) is indicated where: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Tables

TABLE 1

Functional annotation of the 212 genes induced by formoterol in BEAS-2B cells

The 212 genes induced ≥ 2 -fold ($P \leq 0.05$) by formoterol in BEAS-2B cells were subjected to functional annotation analysis in the DAVID. The 24 GO terms showing $P_B \leq 0.05$ (where $1.3 = -\log_{10}(0.05)$) are listed to show fold enrichment (Fold), P_{EASE} & P_B (both as $-\log_{10}(P)$), gene count (Count) and the fraction of these genes that showed further increased (≥ 2 -fold = Fraction up) or decreased (≤ 0.5 -fold = Fraction dn) expression at any time by budesonide. Expected fractions are: Fraction up = 0.22, Fraction dn = 0.37. GO terms were manually curated into the indicated categories.

Category and Terms	Fold	$P_{EASE} (-\log_{10})$	$P_B (-\log_{10})$	Count	Fraction up	Fraction dn
<i>transcription</i>	2.92	7.35	4.12	34	0.24	0.32
positive regulation of transcription from RNA polymerase II promoter	2.92	7.35	4.12	34	0.24	0.32
sequence-specific DNA binding	3.51	5.64	3.06	21	0.14	0.33
transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	5.14	5.45	3.17	14	0.21	0.36
negative regulation of transcription from RNA polymerase II promoter	2.93	5.36	2.43	25	0.28	0.28
transcriptional repressor activity, RNA polymerase II core promoter proximal region sequence-specific binding	7.80	5.27	3.17	10	0.40	0.10
transcription factor activity, sequence-specific DNA binding	2.52	4.82	2.84	28	0.25	0.25
RNA polymerase II core promoter proximal region sequence-specific DNA binding	3.90	4.81	2.92	16	0.25	0.31
transcription from RNA polymerase II promoter	3.13	4.42	1.89	19	0.21	0.26
positive regulation of transcription, DNA-templated	2.95	3.88	1.56	18	0.17	0.33
<i>signaling</i>						
TNF signaling pathway	7.81	7.15	4.95	13	0.23	0.54
signal transduction	2.40	5.24	2.49	33	0.06	0.55
canonical Wnt signaling pathway	8.13	4.26	1.81	8	0.38	0.25
positive regulation of tyrosine phosphorylation of Stat3 protein	13.32	4.10	1.72	6	0.00	0.50
positive regulation of peptidyl-serine phosphorylation	8.44	3.76	1.49	7	0.29	0.57
negative regulation of hormone secretion	33.75	3.74	1.52	4	0.25	0.75
cytokine activity	4.92	3.69	1.89	10	0.20	0.50
signaling pathways regulating pluripotency of stem cells	4.59	3.54	1.64	10	0.30	0.10
cellular response to cAMP	9.74	3.44	1.31	6	0.00	0.33
Jak-STAT signaling pathway	4.43	3.43	1.71	10	0.00	0.60
receptor binding	3.19	3.09	1.42	13	0.15	0.46
<i>proliferation, differentiation & development</i>						
fat cell differentiation	9.25	4.62	2.00	8	0.25	0.38
palate development	7.77	3.56	1.38	7	0.57	0.14
neural crest cell development	25.96	3.37	1.30	4	0.50	0.00
<i>other</i>						
protein binding	1.23	3.29	1.56	125	0.19	0.37

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TABLE 2

Functional annotation of the 370 genes induced by budesonide in BEAS-2B cells

The 370 genes induced ≥ 2 -fold ($P \leq 0.05$) by budesonide were subjected to functional annotation analysis in the DAVID. The 12 GO terms showing $P_B \leq 0.05$ (where $1.3 = -\log_{10}(0.05)$) are listed to show fold enrichment (Fold), P_{EASE} & P_B (both as $-\log_{10}(P)$), gene count (Count) and the fraction of those genes that showed further increased (≥ 2 -fold = Fraction up) or decreased (≤ 0.5 -fold = Fraction dn) expression at any time by formoterol. Expected fractions are: Fraction up = 0.18, Fraction dn = 0.03. GO terms were manually curated into the indicated categories.

Term	Fold	$P_{EASE} (-\log_{10})$	$P_B (-\log_{10})$	Count	Fraction up	Fraction dn
<i>signaling</i>						
platelet-derived growth factor receptor signaling pathway	11.85	4.67	1.59	7	0.29	0.00
negative regulation of fibroblast growth factor receptor signaling pathway	24.55	4.50	1.60	5	0.40	0.20
positive regulation of endothelial cell migration	8.54	4.44	1.66	8	0.25	0.00
TNF signaling pathway	4.53	3.87	1.54	11	0.36	0.00
<i>proliferation, differentiation & development</i>						
positive regulation of apoptotic process	4.42	9.28	5.90	27	0.22	0.07
palate development	5.81	3.82	1.35	9	0.00	0.00
negative regulation of fat cell differentiation	8.18	3.72	1.35	5	0.00	0.00
<i>inflammation & stress</i>						
regulation of inflammatory response	7.01	4.41	1.73	9	0.33	0.00
response to lipopolysaccharide	3.89	3.87	1.34	13	0.23	0.00
response to stress	6.44	3.64	1.31	8	0.25	0.13
<i>adhesion & migration</i>						
extracellular matrix organization	3.76	4.31	1.71	15	0.13	0.00
<i>other</i>						
cellular response to insulin stimulus	5.74	3.78	1.36	6	0.00	0.00

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TABLE 3

Functional annotation of the 413 genes repressed by budesonide in BEAS-2B cells

The 413 genes repressed ≤ 0.5 -fold ($P \leq 0.05$) by budesonide in BEAS-2B cells were subjected to functional annotation analysis in the DAVID. The 20 GO terms showing $P_B \leq 0.05$ (where $1.3 = -\log_{10}(0.05)$) are listed to show fold enrichment (Fold), P_{EASE} & P_B (both as $-\log_{10}(P)$), gene count (Count) and the fraction of those genes that showed increased (≥ 2 -fold = Fraction up) or further decreased (≤ 0.5 -fold = Fraction dn) expression at any time by formoterol. Expected fractions are: Fraction up = 0.09, Fraction dn = 0.03. GO terms were manually curated into the indicated categories.

Term	Fold	$P_{EASE} (-\log_{10})$	$P_B (-\log_{10})$	Count	Fraction up	Fraction dn
<i>transcription</i>						
positive regulation of transcription from RNA polymerase II promoter	2.08	5.39	2.80	46	0.17	0.09
transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	3.31	4.22	2.08	17	0.06	0.18
<i>signaling</i>						
signal transduction	2.07	6.23	3.16	54	0.11	0.06
peptidyl-tyrosine phosphorylation	4.36	5.03	2.57	15	0.20	0.00
protein tyrosine kinase activity	4.50	4.48	2.22	13	0.15	0.00
receptor binding	2.61	3.57	1.53	20	0.20	0.00
TGF-beta signaling pathway	4.55	3.51	1.52	10	0.00	0.00
positive regulation of GTPase activity	2.20	3.71	1.43	28	0.14	0.00
PI3K-Akt signaling pathway	2.44	3.65	1.35	22	0.18	0.00
<i>proliferation, differentiation & development</i>						
wound healing	6.66	5.78	2.89	12	0.08	0.00
angiogenesis	3.78	5.52	2.85	19	0.26	0.00
negative regulation of apoptotic process	2.73	5.33	2.81	28	0.25	0.04
negative regulation of cell proliferation	2.80	4.95	2.54	25	0.08	0.08
growth factor activity	4.26	4.92	2.48	15	0.33	0.00
regulation of angiogenesis	10.03	4.25	1.89	7	0.29	0.00
positive regulation of cell proliferation	2.38	3.84	1.52	25	0.24	0.04
<i>adhesion & migration</i>						
positive regulation of cell migration	5.55	9.77	6.41	23	0.13	0.00
heparin binding	5.46	7.91	5.17	19	0.16	0.00
positive regulation of positive chemotaxis	24.23	5.64	2.88	6	0.33	0.00
collagen fibril organization	7.97	3.67	1.43	7	0.00	0.00

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TABLE 4

Functional annotation of the 208 genes repressed by formoterol in pHBECs

The 208 genes repressed ≤ 0.5 -fold ($P \leq 0.05$) by formoterol in pHBECs were subjected to functional annotation analysis in the DAVID. The 5 GO terms showing $P_B \leq 0.05$ (where $1.3 = -\log_{10}(0.05)$) are listed to show fold enrichment (Fold), P_{EASE} & P_B (both as $-\log_{10}(P)$) and gene count (Count). GO terms were manually curated into the indicated categories.

Term	Fold	$P_{EASE}(-\log_{10})$	$P_B(-\log_{10})$	Count
<i>signaling</i>				
inactivation of MAPK activity	29.36	8.02	5.04	8
MAP kinase tyrosine/serine/threonine phosphatase activity	35.10	5.04	2.49	5
protein tyrosine phosphatase activity	7.30	3.95	1.70	8
peptidyl-tyrosine dephosphorylation	7.41	4.00	1.33	8
<i>inflammation & stress</i>				
inflammatory response	3.63	4.17	1.32	15

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TABLE 5

Functional annotation of the 141 genes repressed by budesonide in pHBECs

The 141 genes repressed ≤ 0.5 -fold ($P \leq 0.05$) by budesonide in pHBECs were subjected to functional annotation analysis in the DAVID. The 23 GO terms showing $P_B \leq 0.05$ (where $1.3 = -\log_{10}(0.05)$) are listed to show fold enrichment (Fold), P_{EASE} & P_B (both as $-\log_{10}(P)$) and gene count (Count). GO terms were manually curated into the indicated categories.

Term	Fold	$P_{EASE}(-\log_{10})$	$P_B(-\log_{10})$	Count
<i>transcription</i>				
positive regulation of transcription, DNA-templated	3.65	4.23	2.21	15
positive regulation of transcription from RNA polymerase II promoter	2.43	3.14	1.34	19
<i>signaling</i>				
cellular response to mechanical stimulus	14.12	5.84	3.38	8
inactivation of MAPK activity	30.08	5.87	3.28	6
signal transduction	2.70	4.90	2.74	25
cell-cell signaling	4.93	3.72	1.74	10
NF-kappa B signaling pathway	7.38	3.49	1.71	7
TNF signaling pathway	6.00	3.01	1.41	7
<i>proliferation, differentiation & development</i>				
growth factor activity	9.92	7.49	5.06	12
negative regulation of cell proliferation	5.38	7.01	4.25	17
positive regulation of cell division	21.33	7.09	4.03	8
angiogenesis	6.74	5.79	3.43	12
positive regulation of cell proliferation	4.30	5.37	3.15	16
positive regulation of angiogenesis	7.63	3.51	1.60	7
regulation of cell proliferation	5.42	3.17	1.34	8
<i>inflammation & stress</i>				
inflammatory response	4.96	5.73	3.44	15
cytokine activity	7.61	5.19	3.06	10
immune response	4.17	4.52	2.41	14
response to molecule of bacterial origin	55.69	4.40	2.34	4
cytokine-cytokine receptor interaction	4.53	4.30	2.22	12
response to hypoxia	5.83	3.36	1.50	8
<i>adhesion & migration</i>				
induction of positive chemotaxis	33.42	3.69	1.74	4
chemotaxis	7.19	3.38	1.50	7

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TABLE 6. Functional annotation of the 370 genes repressed by formoterol-plus-budesonide in pHBEs

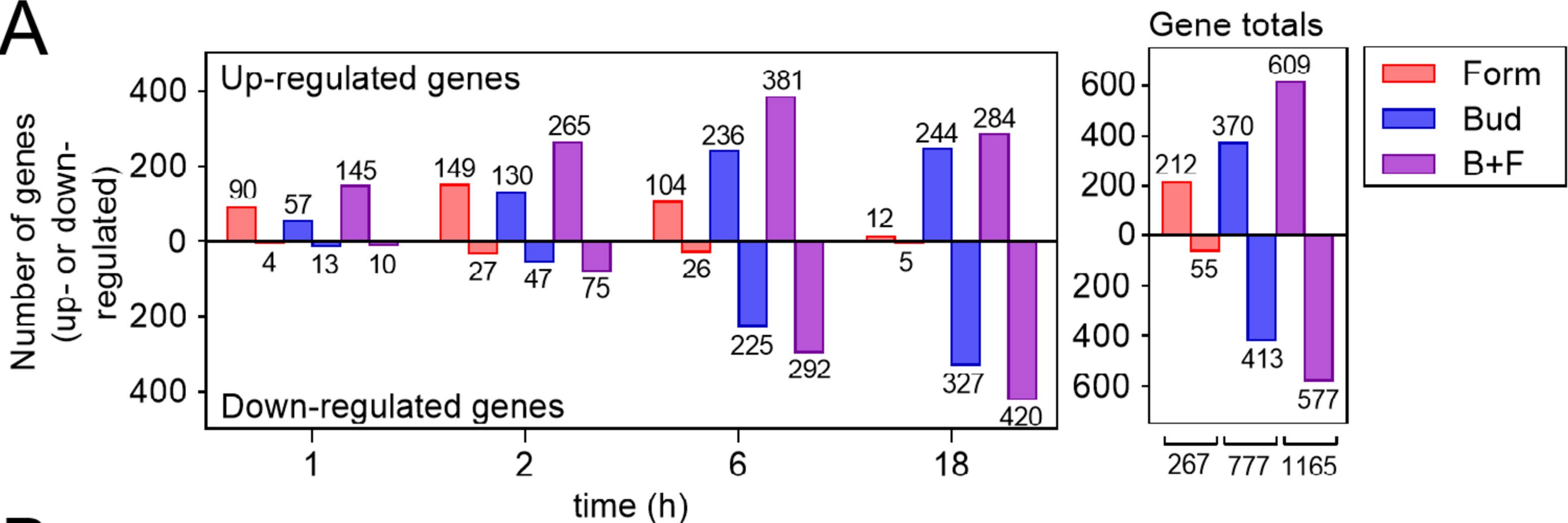
The 370 genes repressed ≤ 0.5 -fold ($P \leq 0.05$) by formoterol-plus-budesonide in pHBEs were subjected to functional annotation analysis in the DAVID. The 22 GO terms showing $P_B \leq 0.05$ (where $1.3 = -\log_{10}(0.05)$) are listed to show fold enrichment (Fold), P_{EASE} & P_B (both as $-\log_{10}(P)$) and gene count (Count). GO terms were manually curated into the indicated categories.

Term	Fold	$P_{EASE} (-\log_{10})$	$P_B (-\log_{10})$	Count
<i>transcription</i>				
positive regulation of transcription, DNA-templated	3.13	7.41	4.08	32
negative regulation of transcription, DNA-templated	2.83	5.65	2.99	28
positive regulation of transcription from RNA polymerase II promoter	2.16	5.33	2.77	42
transcription from RNA polymerase II promoter	2.65	4.93	2.44	27
transcription factor binding	3.38	4.84	2.40	19
negative regulation of transcription from RNA polymerase II promoter	2.24	4.37	2.04	32
transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	3.21	3.59	1.56	15
transcription factor activity, sequence-specific DNA binding	1.89	3.45	1.50	36
<i>Signaling</i>				
inactivation of MAPK activity	12.10	3.95	1.70	6
negative regulation of ERK1 and ERK2 cascade	6.96	3.85	1.64	8
<i>proliferation, differentiation & development</i>				
negative regulation of apoptotic process	3.21	6.94	3.91	29
positive regulation of cell proliferation	3.03	6.19	3.33	28
angiogenesis	4.07	5.65	2.92	18
negative regulation of cell proliferation	2.93	4.86	2.43	23
growth factor activity	4.37	4.70	2.44	14
positive regulation of cell division	8.58	4.45	2.08	8
positive regulation of angiogenesis	4.82	4.02	1.74	11
apoptotic process	2.31	3.78	1.60	26
positive regulation of smooth muscle cell proliferation	6.72	3.76	1.61	8
<i>inflammation & stress</i>				
cytokine activity	3.73	3.70	1.57	13
inflammatory response	2.66	3.69	1.57	20
<i>other</i>				
protein binding	1.23	5.00	2.26	213

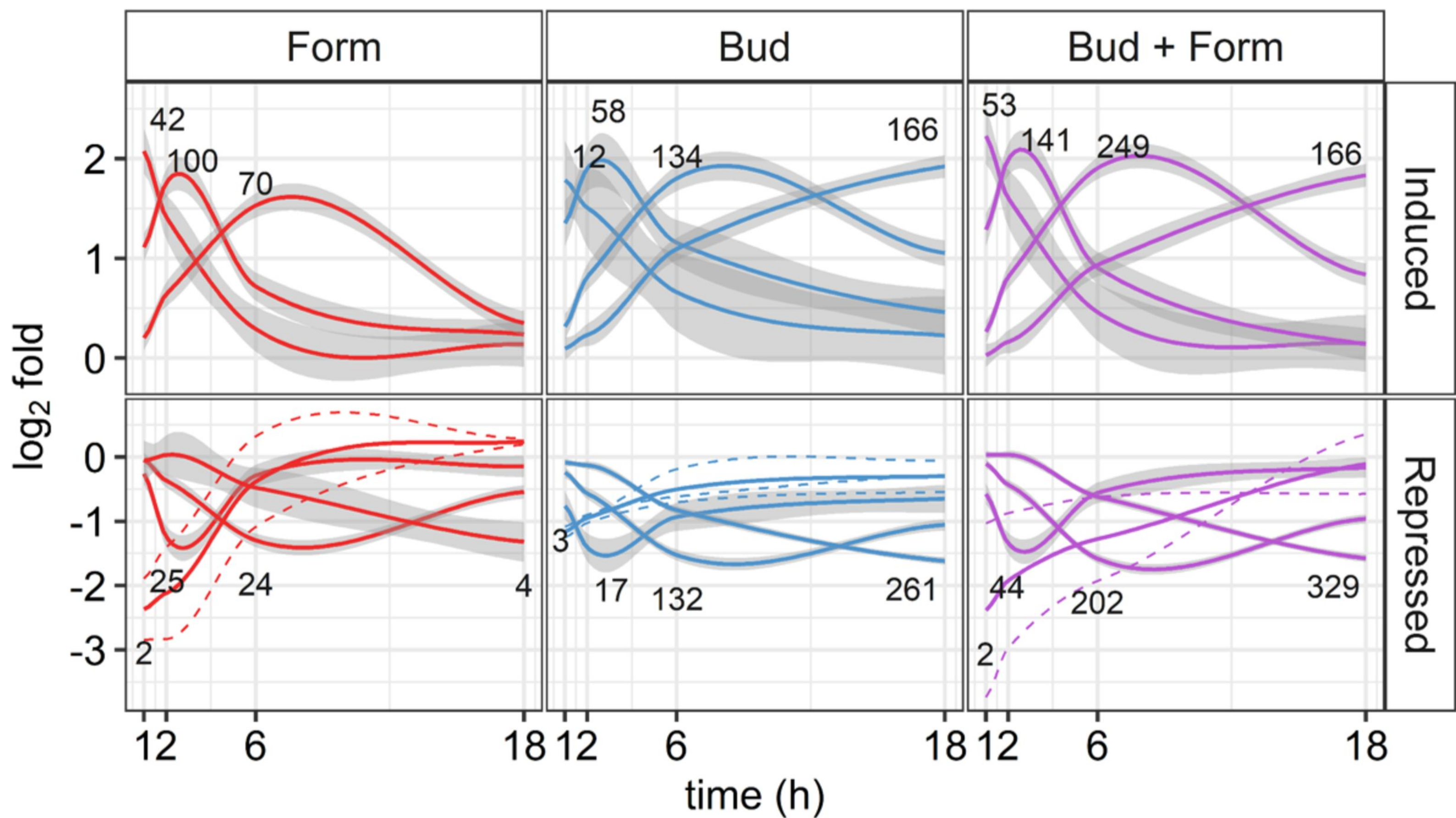
Figures

Fig. 1

A

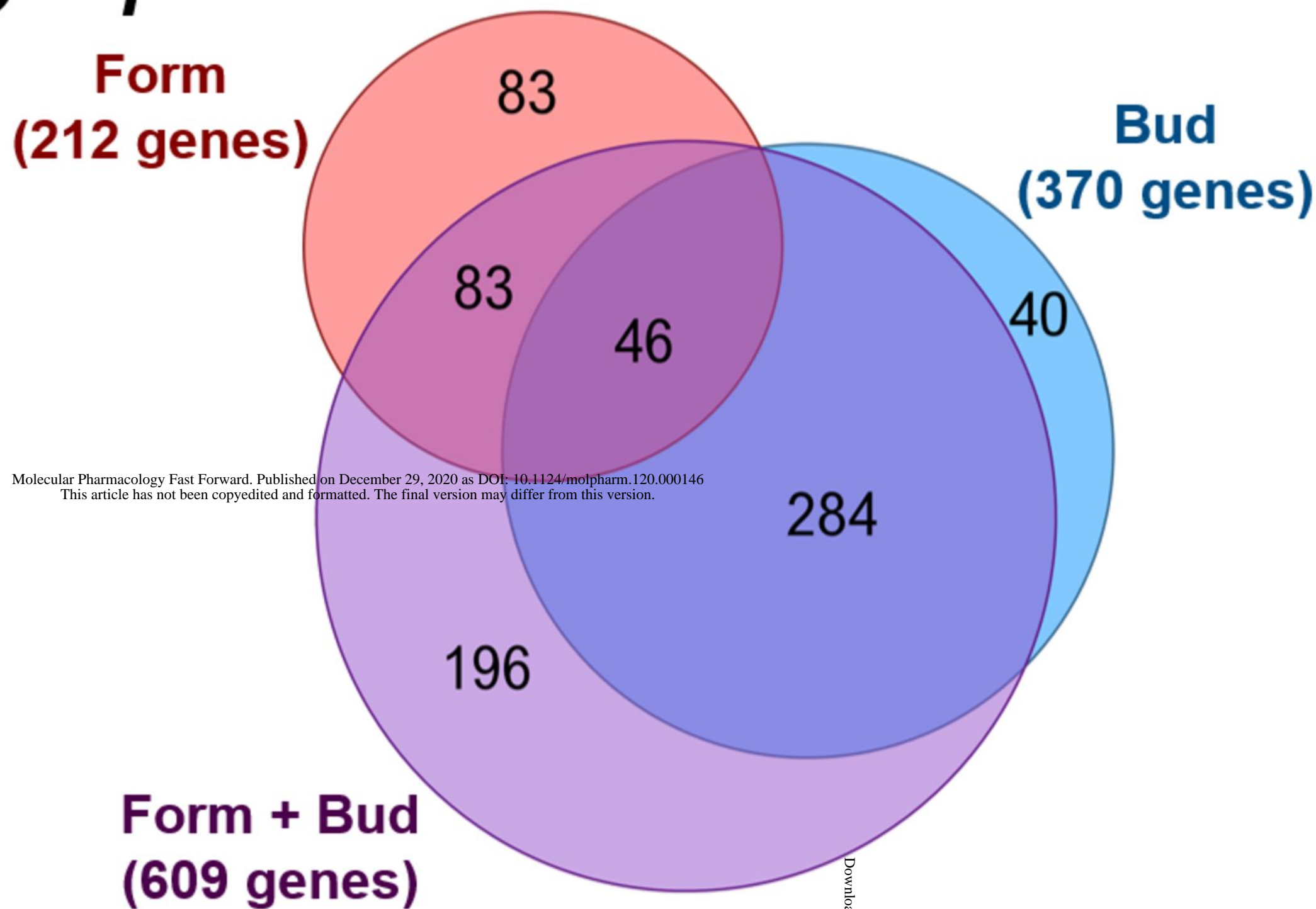


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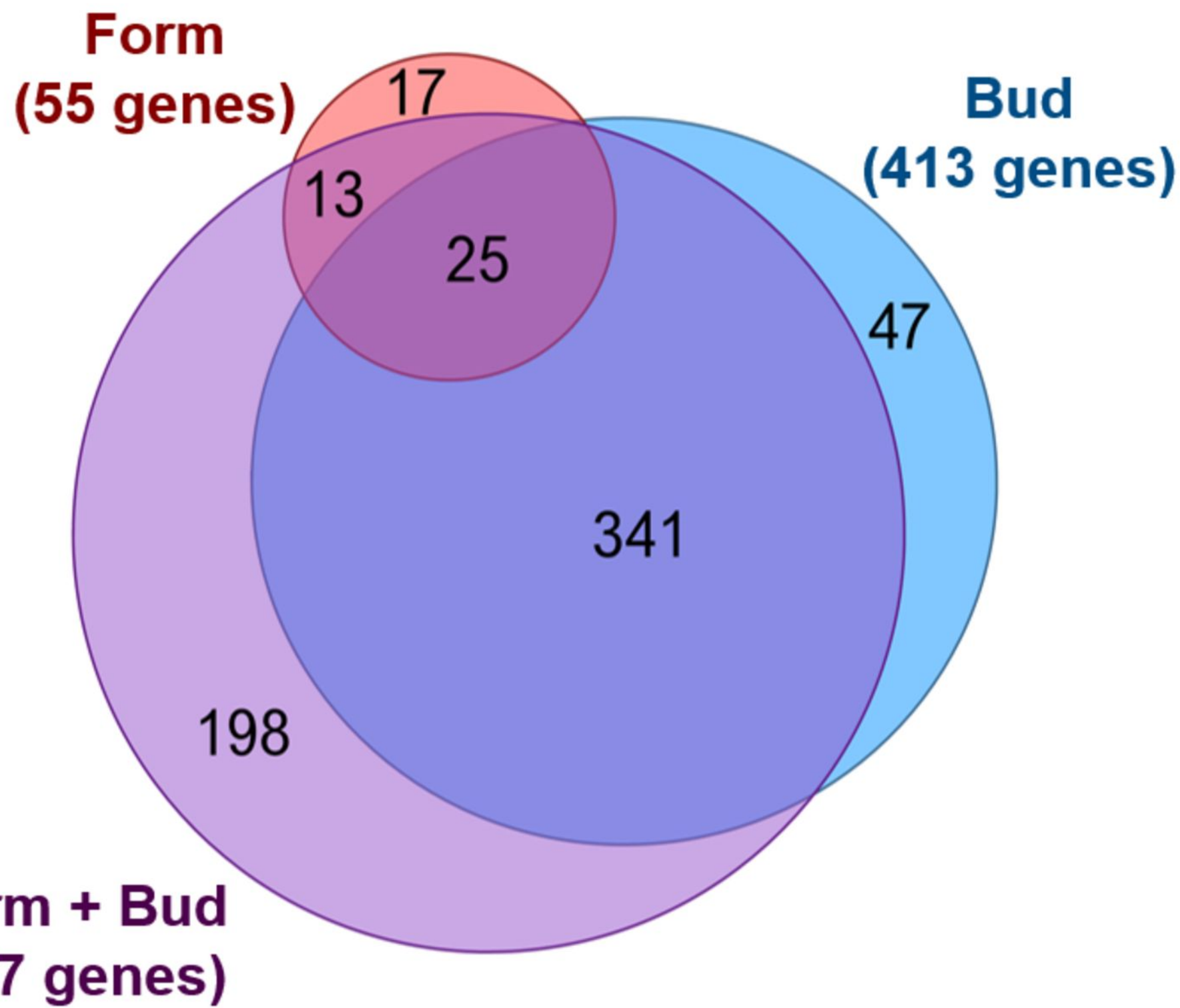


C

i



ii



χ^2 ****

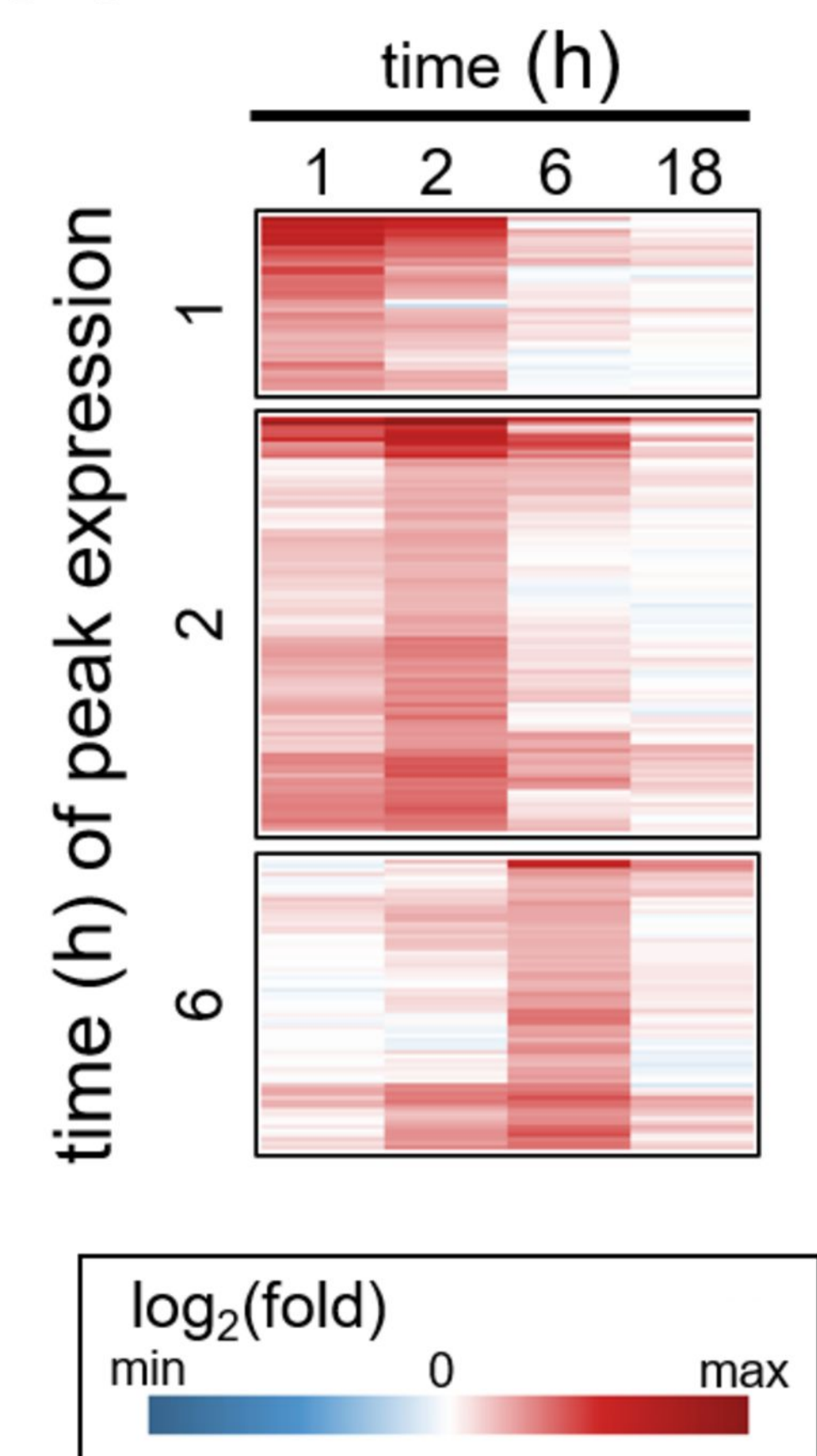
	Not induced	Induced	Totals
Not induced	18307 (O) 18265 (E)	166 (O) 208 (E)	18473
Induced	324 (O) 366 (E)	46 (O) 4 (E)	370
Totals	18631	212	18843

χ^2 ****

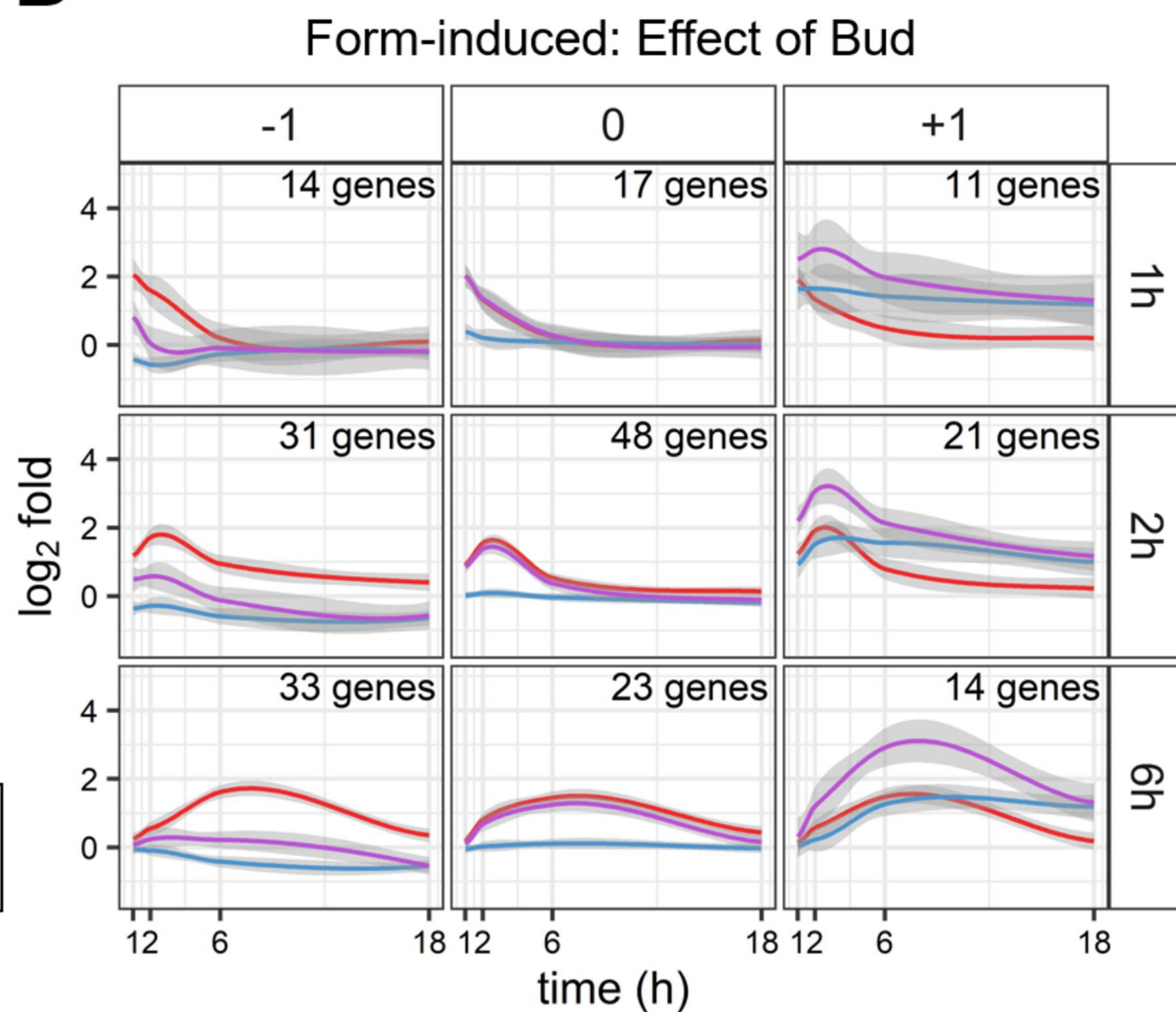
	Not repressed	Repressed	Totals
not repressed	18400 (O) 18376 (E)	30 (O) 54 (E)	18430
Repressed	388 (O) 412 (E)	25 (O) 1 (E)	413
Totals	18788	55	18843

Fig. 2

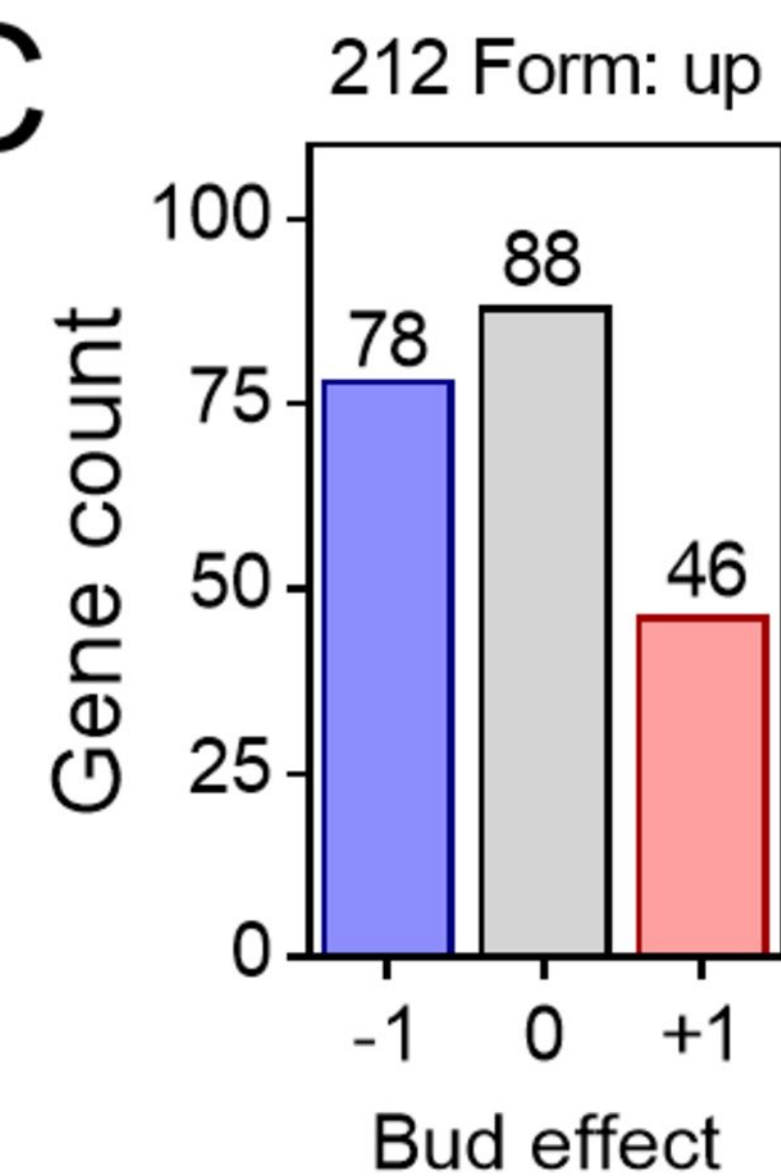
A



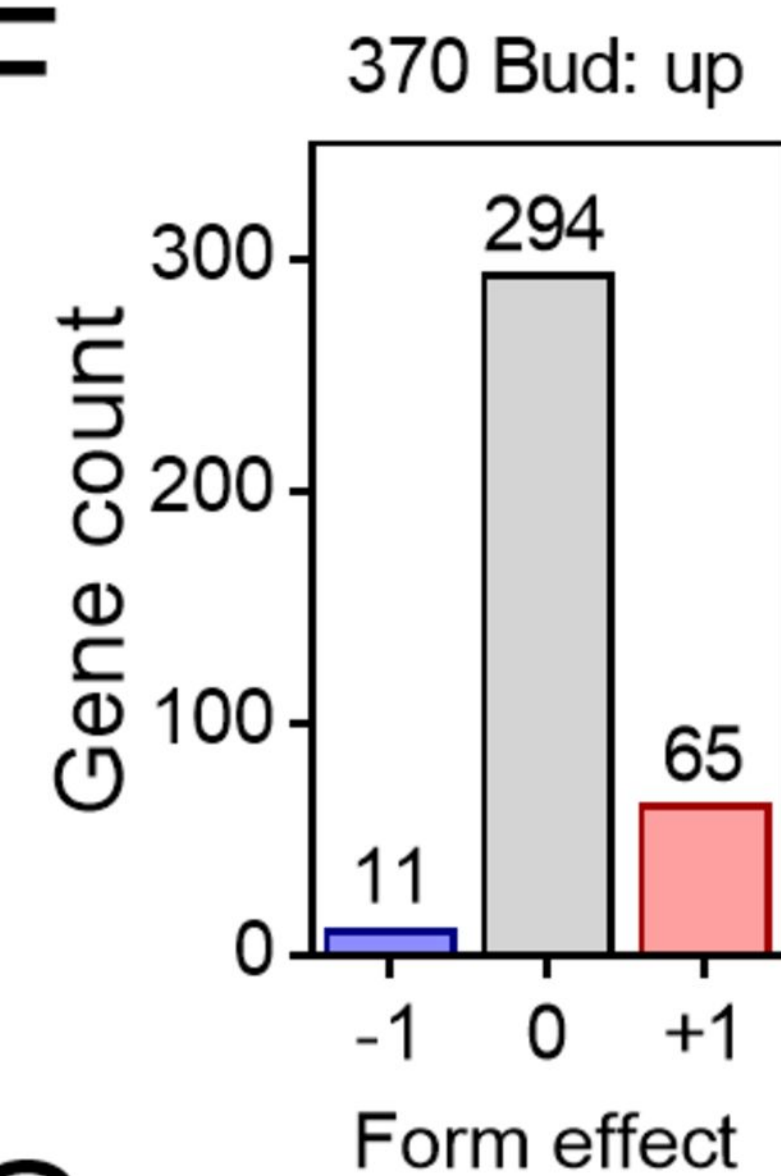
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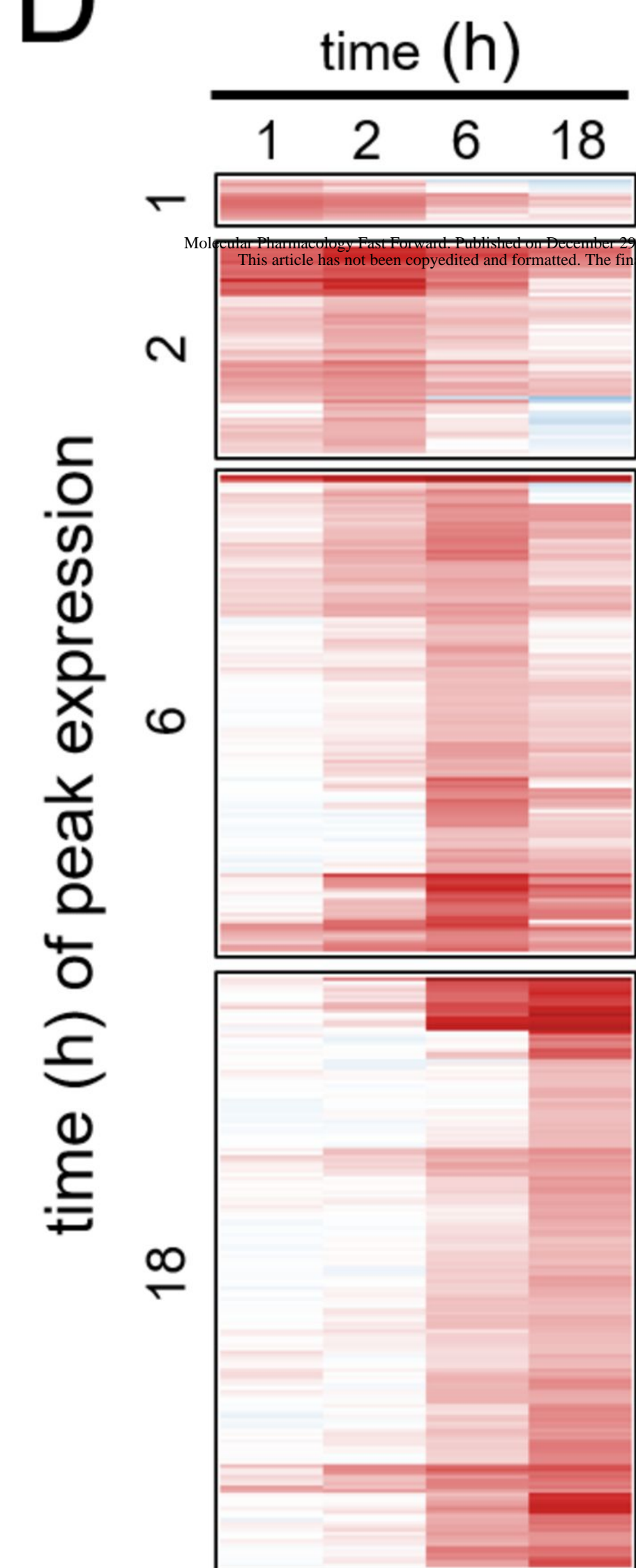
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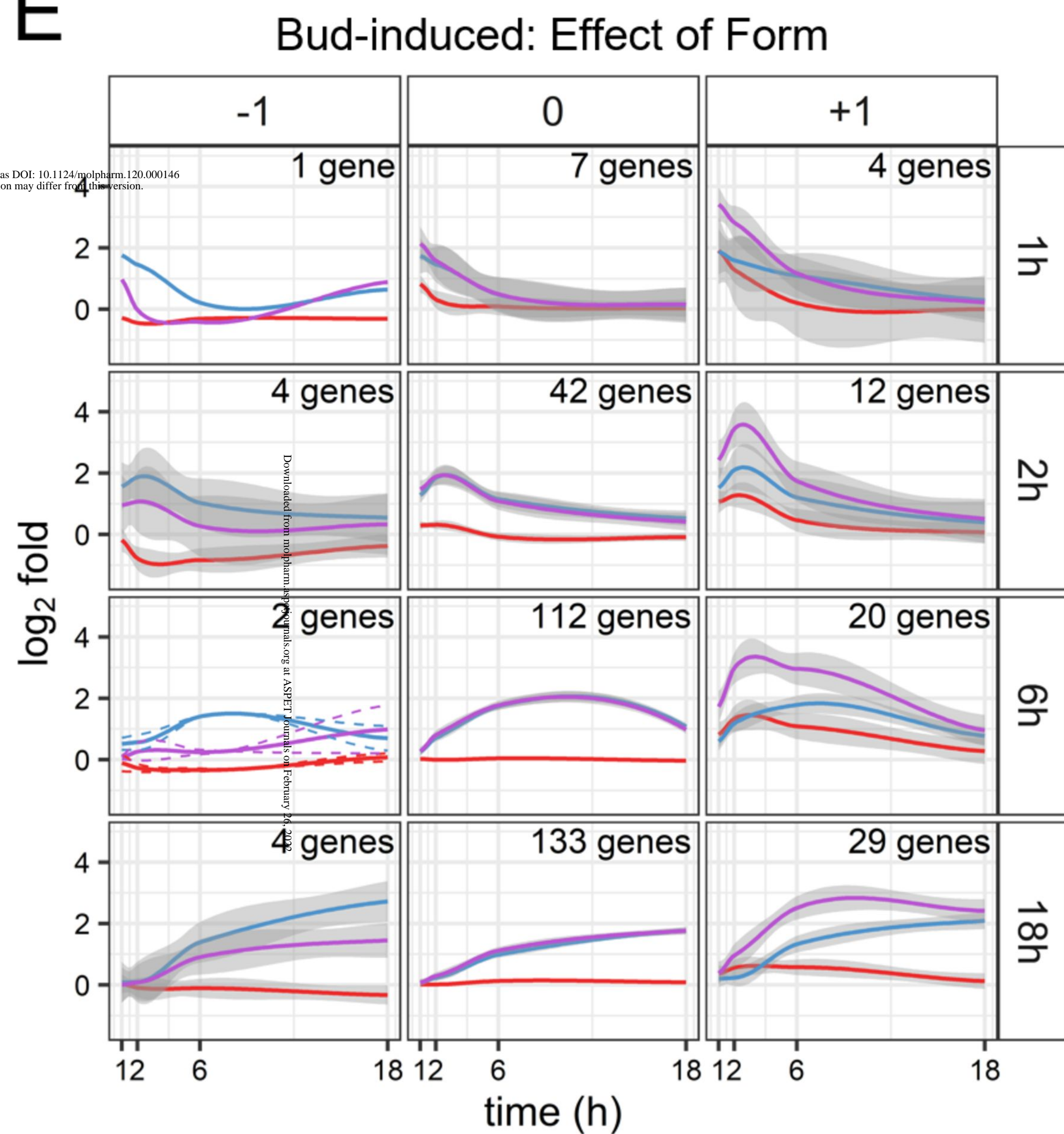
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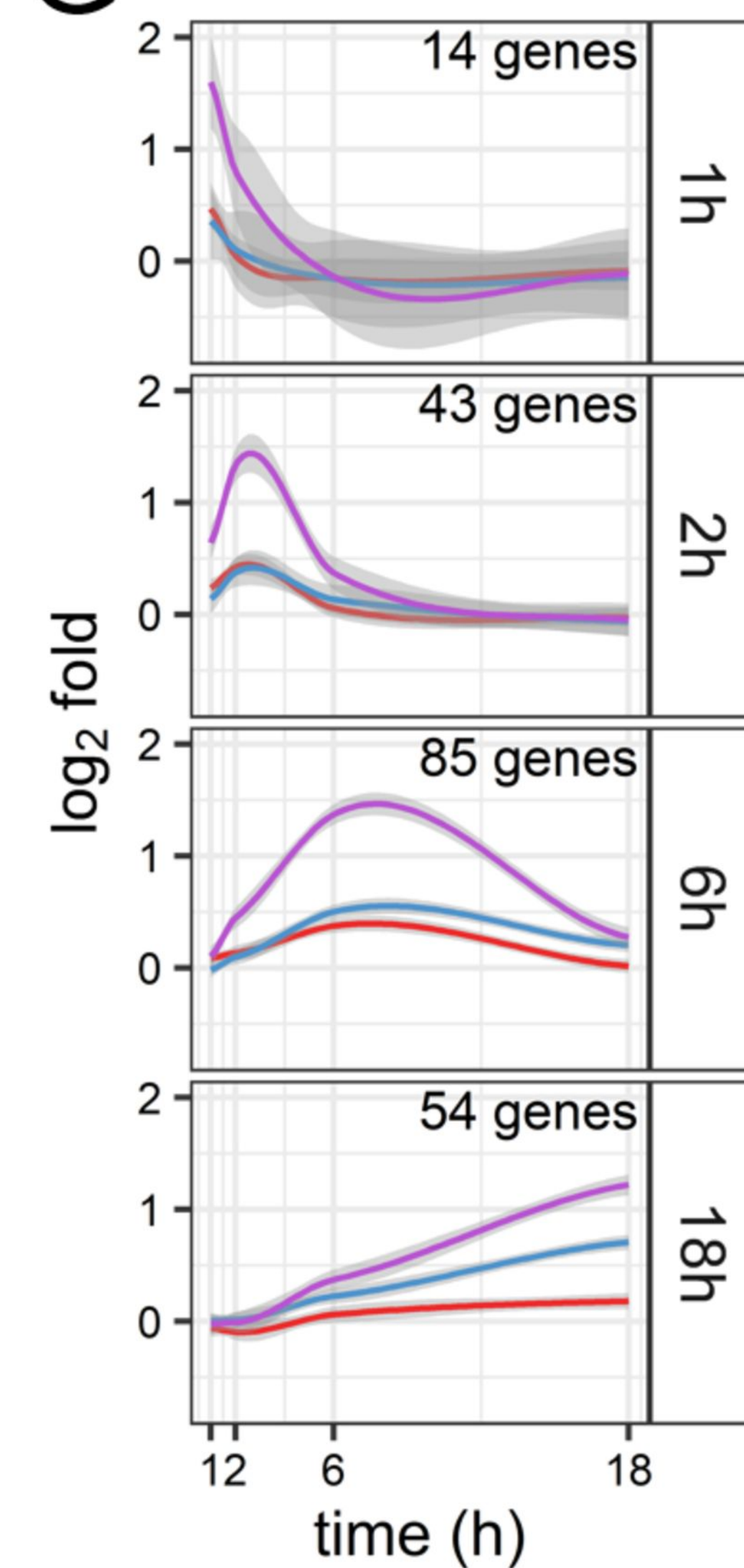
D



E



G



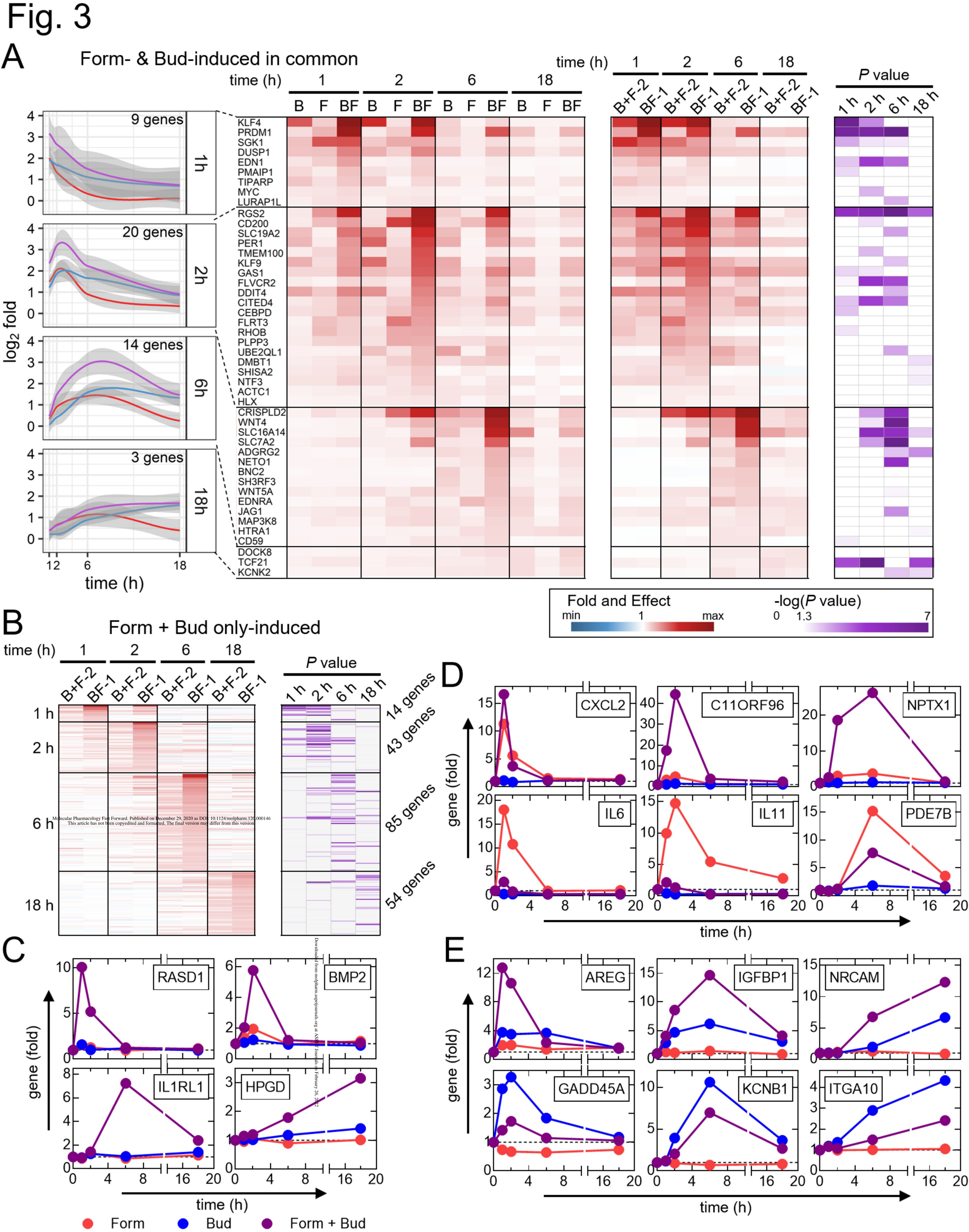


Fig. 4

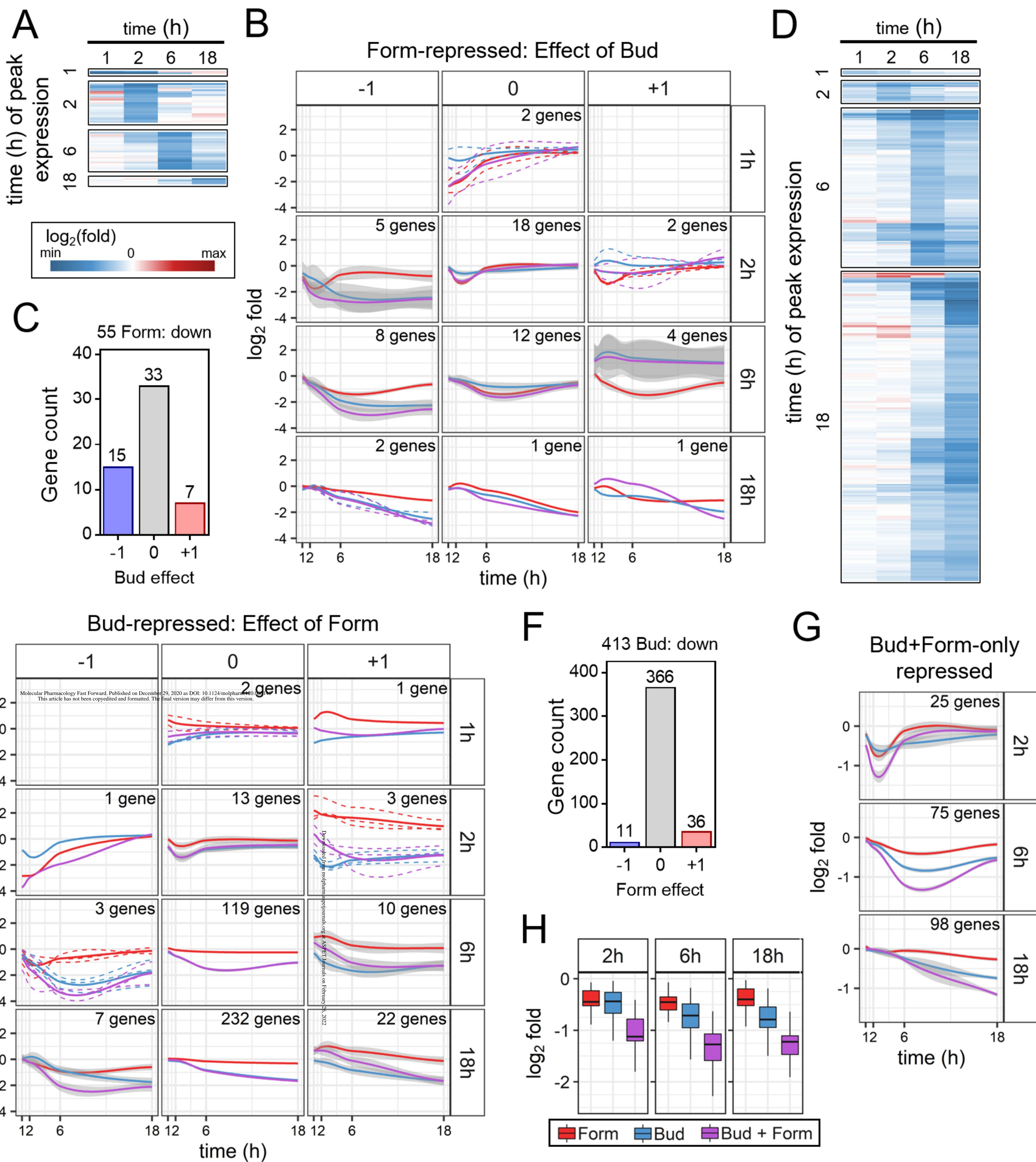
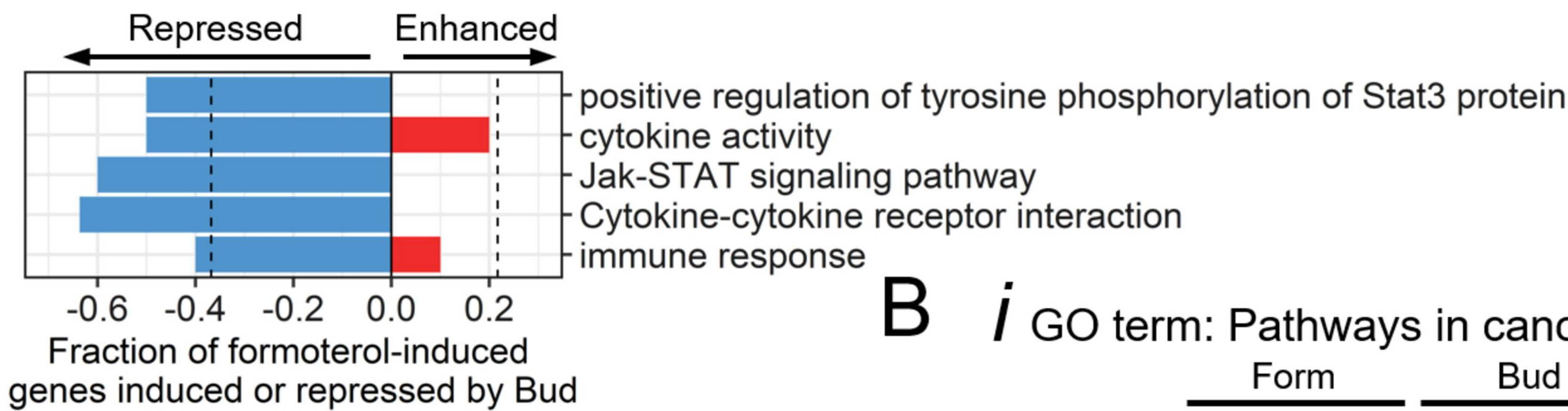


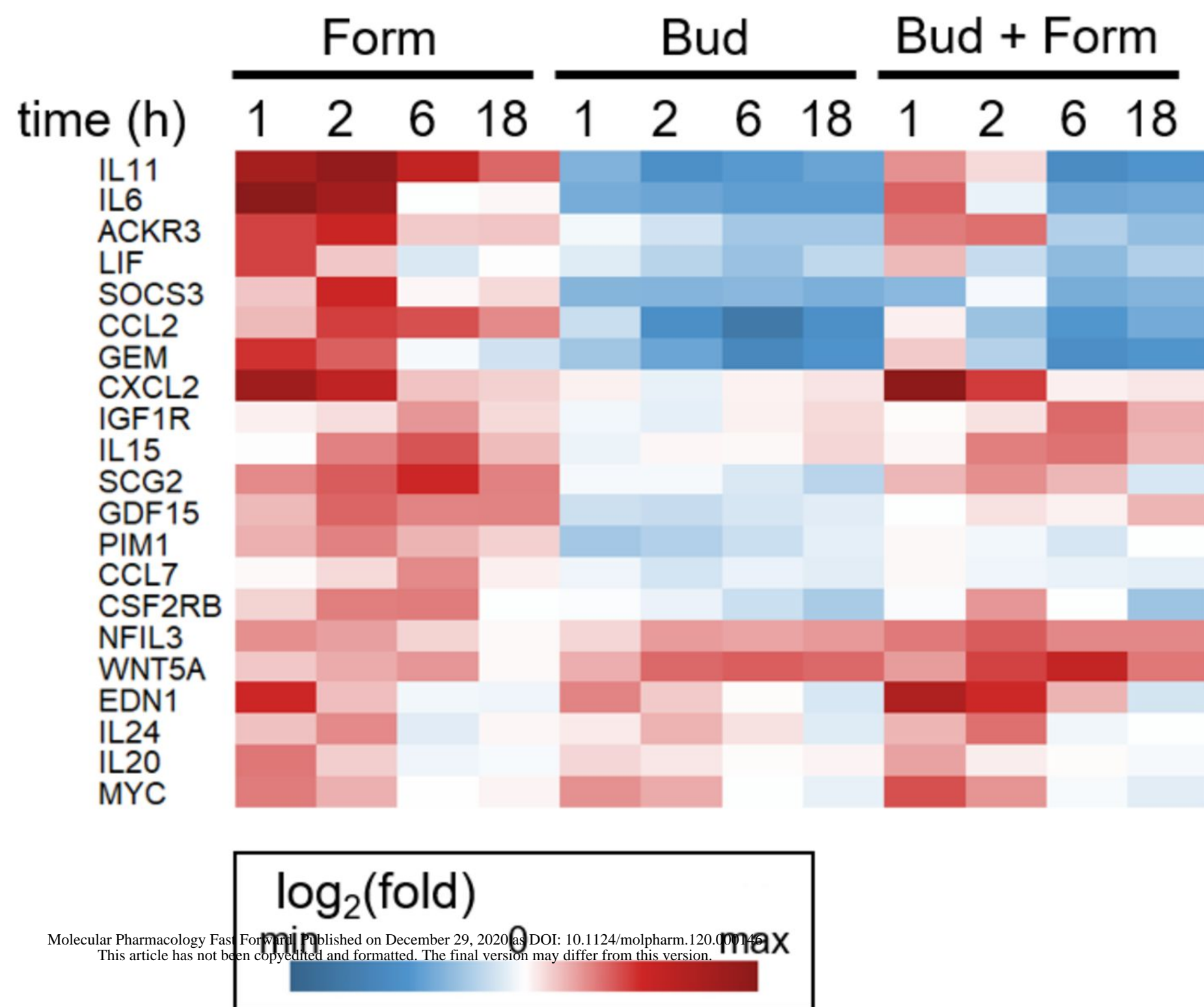
Fig. 5

A

i Form-induced annotation cluster 4

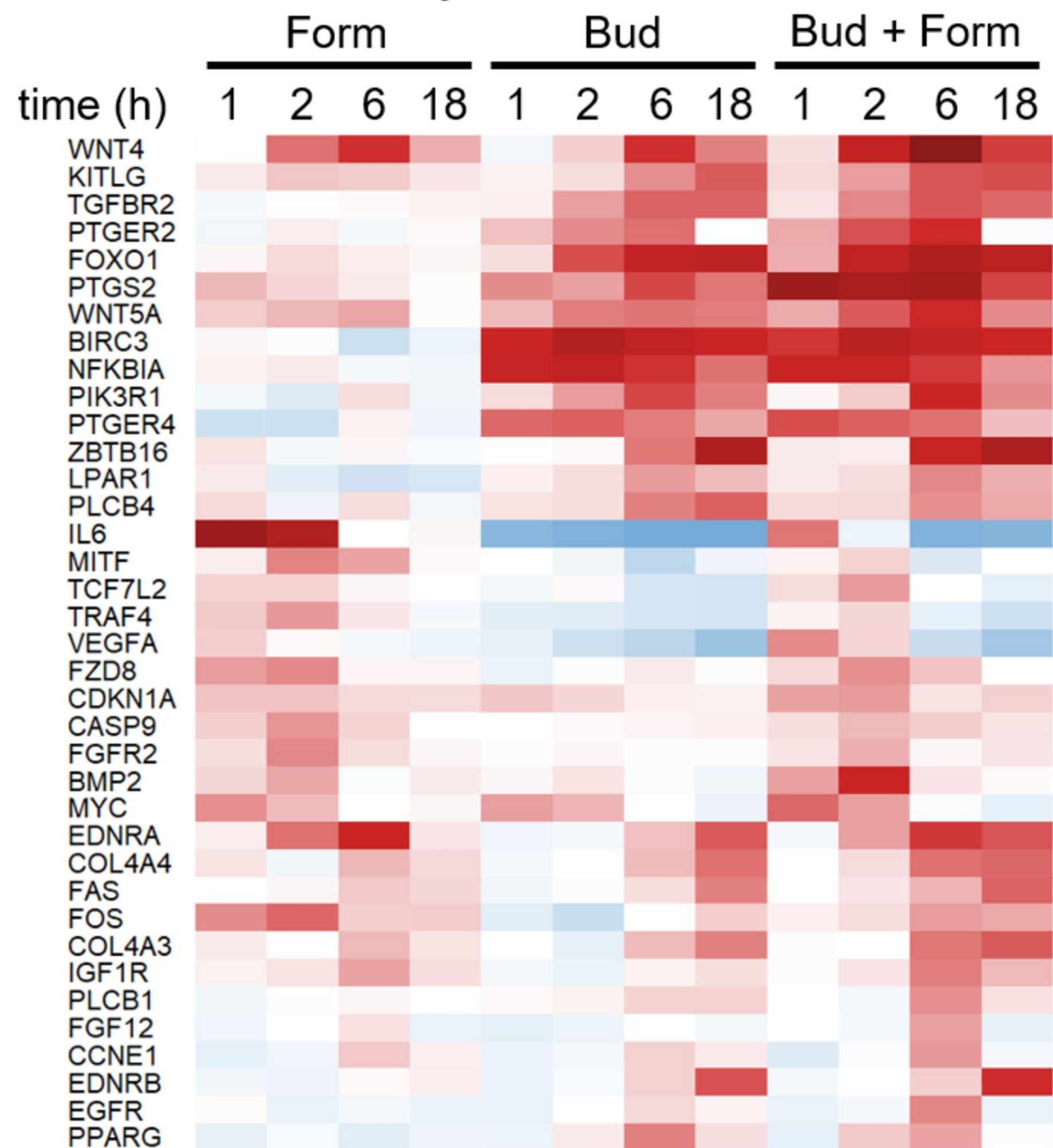


ii Form-induced annotation cluster 4

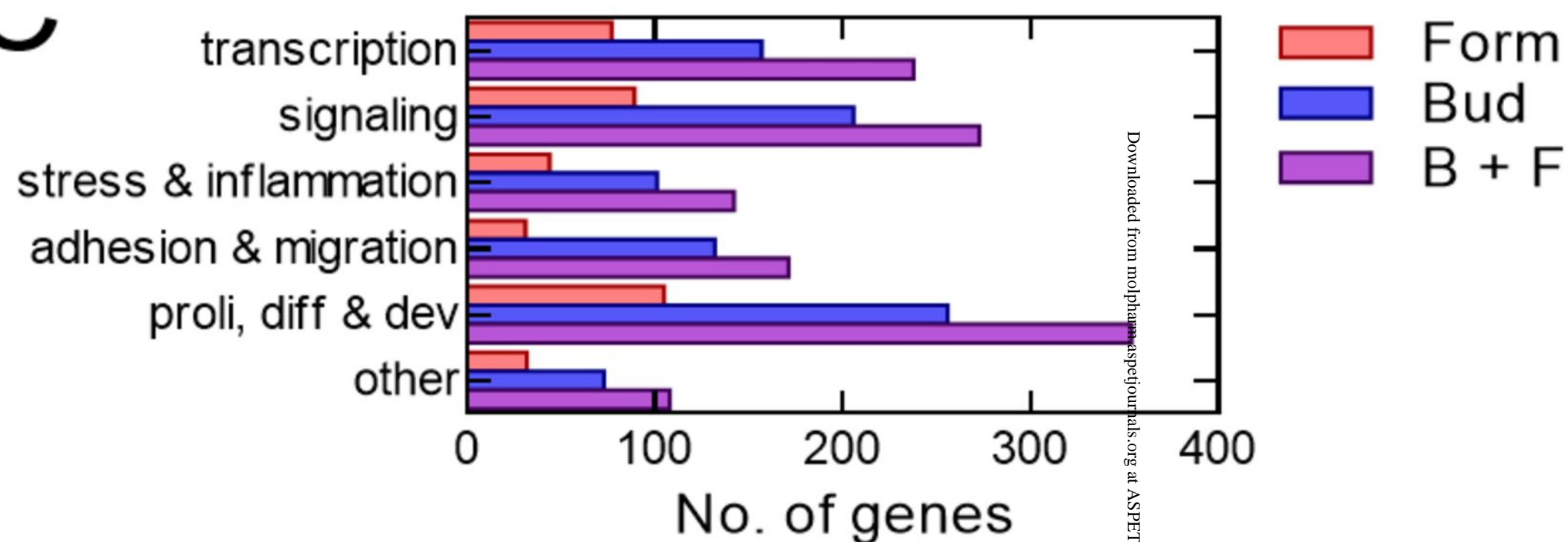


B

i GO term: Pathways in cancer



C



ii GO terms: “cytokine activity” + “Jak-STAT signaling pathway”

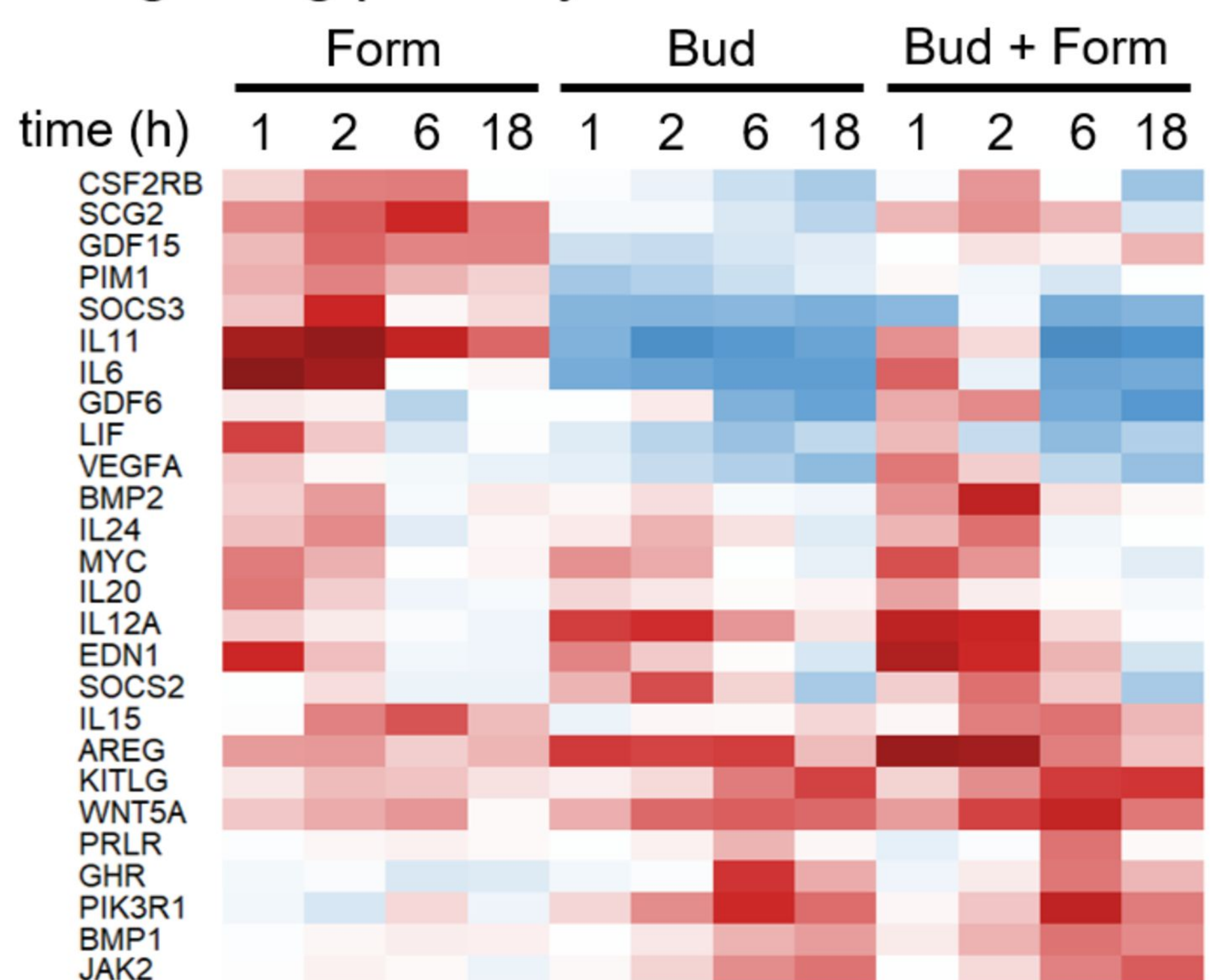


Fig. 6

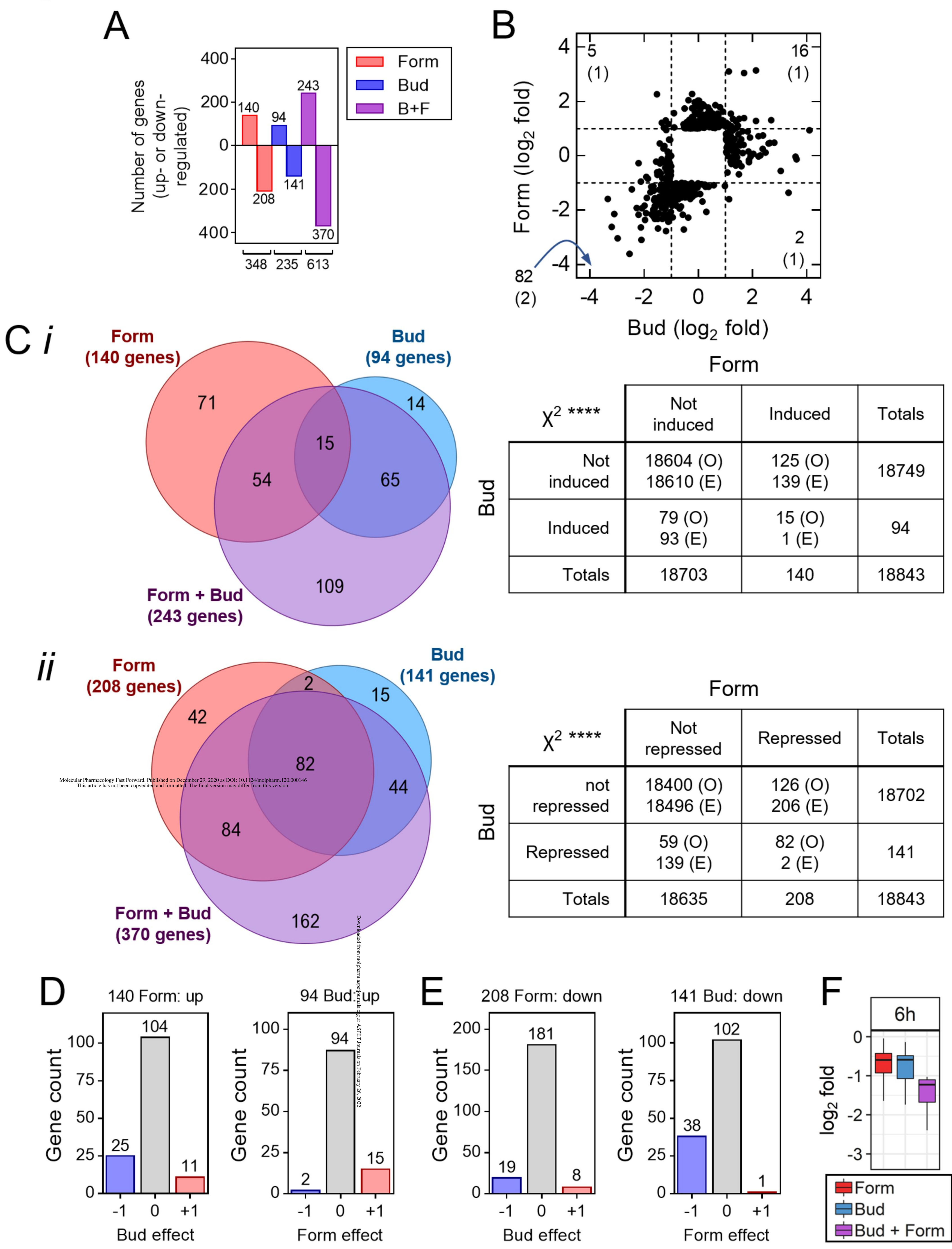
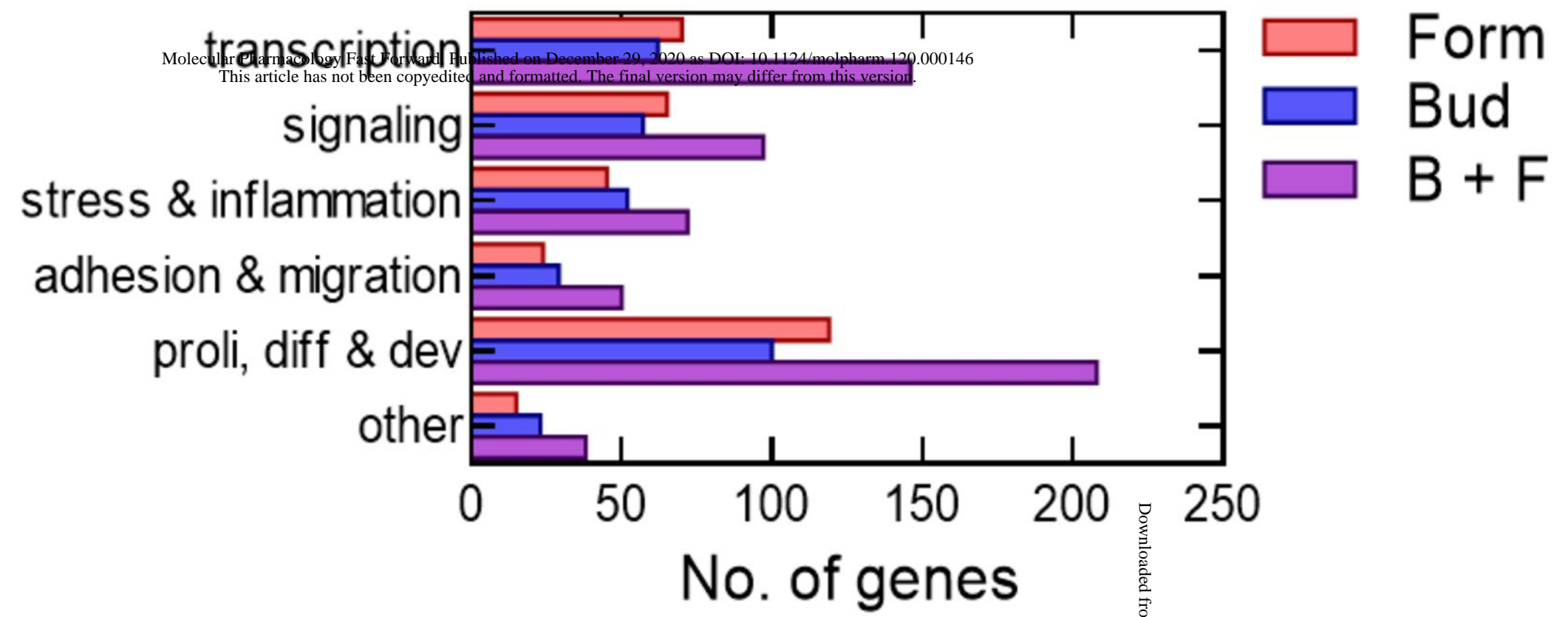


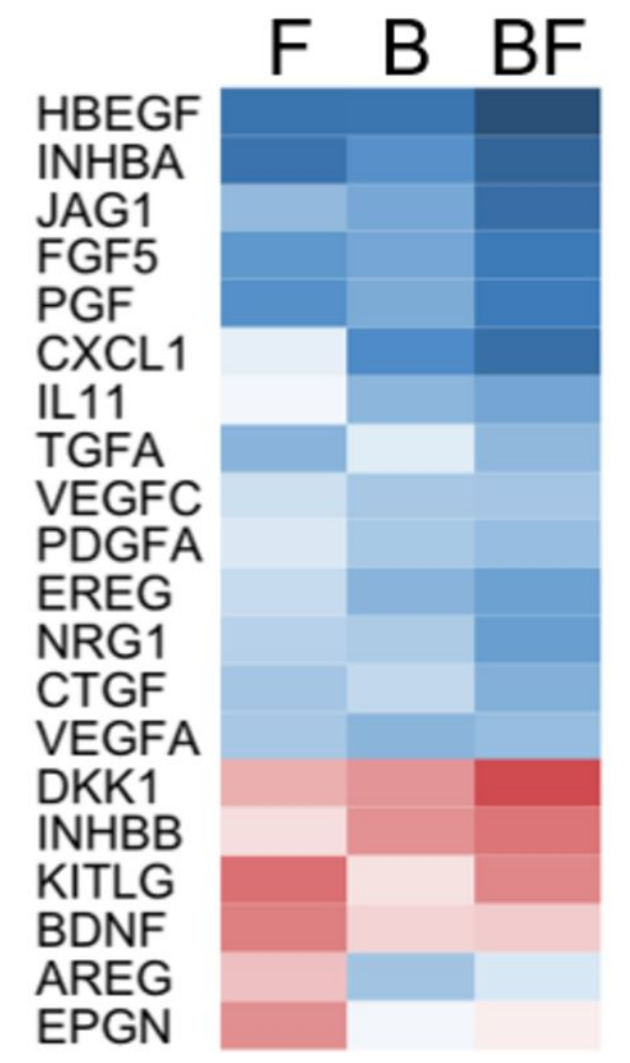
Fig. 7

A

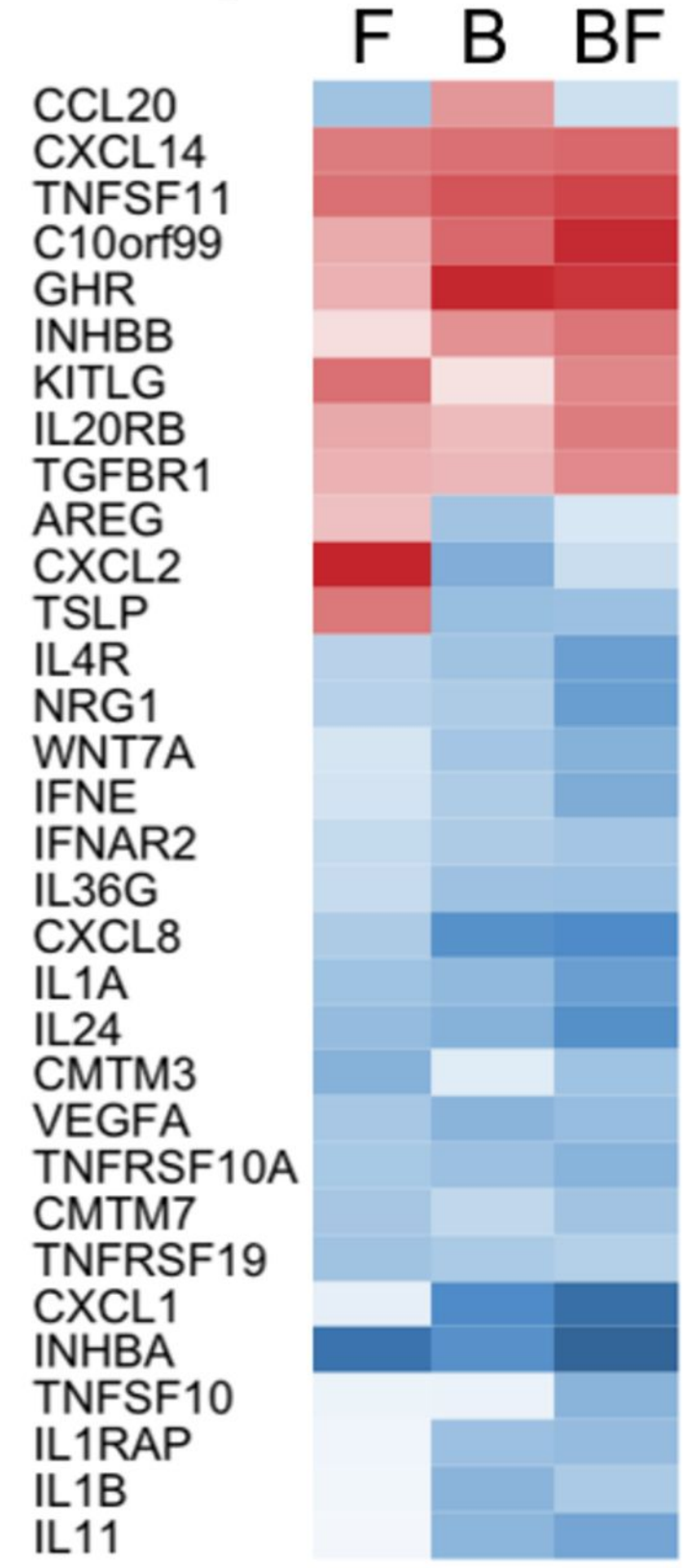


B

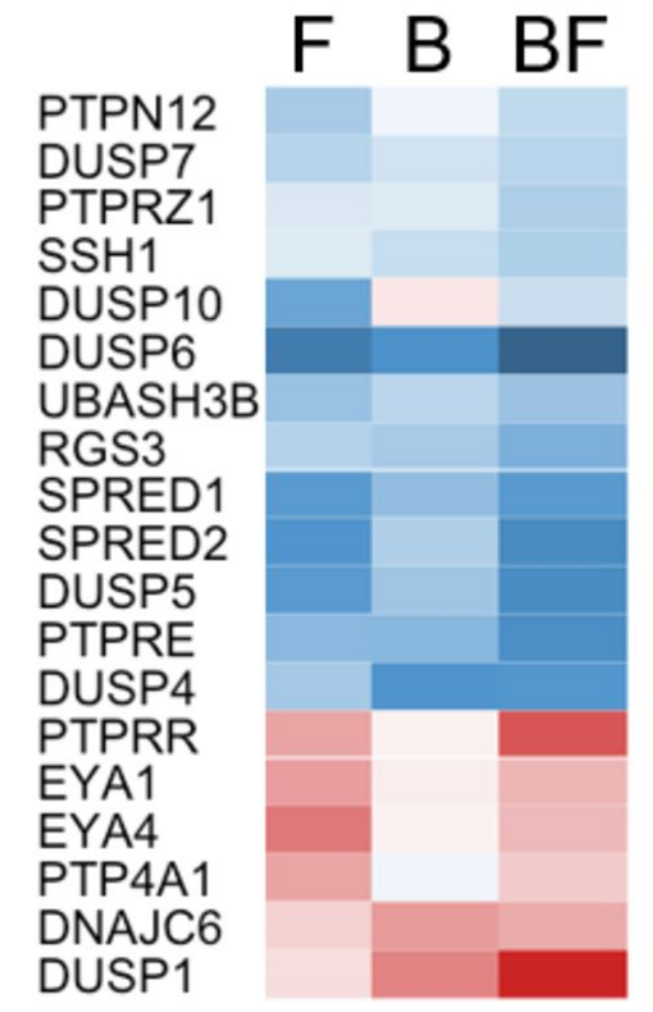
GO term: Growth factor activity



GO terms: Cytokine-cytokine receptor interaction + cytokine activity



GO terms: Inactivation of MAPK activity + tyrosine phosphatase activity



C

