

Title

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Authors: Pieter J. Peeters, Hinrich W. Göhlmann, Ilse Van den Wyngaert, Sigrid M.

Swagemakers, Luc Bijmens, Stefan U. Kass and Thomas Steckler.

Primary laboratory of origin: Johnson & Johnson Pharmaceutical Research and Development,
a division of Janssen Pharmaceutica N.V., Turnhoutseweg 30, 2340 Beerse, Belgium

Running title

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b) Corresponding author: Pieter Peeters, Johnson & Johnson Pharmaceutical Research and Development, a division of Janssen Pharmaceutica N.V., Turnhoutseweg 30, 2340 Beerse, Belgium. Tel. +32(0)14606648, Fax. +32(0)14605737, e-mail ppeeter3@prdbe.jnj.com

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Abstract

Corticotropin-releasing factor (CRF) plays a central role in regulation of the hypothalamic-pituitary-adrenal axis, mediating endocrine and behavioral responses to various stressors. Two high affinity receptors for CRF have been described. Although many of the intracellular signaling pathways activated by CRF have been extensively studied, our knowledge of transcriptional responses downstream of the CRF receptor 1 (CRFR1) is still limited. To elucidate gene networks regulated by CRF and CRFR1, we applied microarray technology to explore transcriptional response to CRF stimulation. Therefore mouse pituitary derived AtT-20 cells were continuously exposed to CRF either in the presence or absence of a specific CRFR1 antagonist R121919. Transcriptional responses to different treatments were studied in a time course ranging from 0.5 to 24 hours. Microarray data were analyzed using classical microarray data analysis tools such as correspondence factor analysis, cluster analysis and fold change filtering. Furthermore spectral map analysis was applied, a recently introduced unsupervised multivariate analysis method. A broad and transient transcriptional response to CRF was identified that could be blocked by the antagonist. This way several known CRF-induced target genes and novel CRF responsive genes were identified. These include transcription factors such as cAMP responsive element modulator (*CREM*, 7X increased), secreted peptides such as cholecystokinin (1.5X) and proteins involved in modulating intracellular signaling, for example regulator of G-protein signaling 2 (*Rgs2*, 11X). Upregulation of many of these genes can be explained as negative feedback, attenuating CRF activated pathways. Additionally, spectral map analysis proved to be a promising new tool for microarray data analysis.

Introduction

Corticotrophin releasing factor (CRF), a 41-amino acid polypeptide plays a central role in the regulation of the hypothalamic-pituitary-adrenal axis, mediating neuroendocrine, autonomic and behavioural responses to various stressors. Hypothalamic neurons from the parvocellular part of the paraventricular nucleus release CRF into the hypophyseal portal system in response to stress, stimulating the secretion and biosynthesis of adrenocorticotropin (ACTH) from pituitary corticotrophs, subsequently leading to increased adrenal glucocorticoid release (Steckler, 2001). Two high affinity receptors for CRF have been described, CRFR1 and CRFR2, both of which exist in several splice variant forms (Dautzenberg *et al.*, 2001). CRFR1 expression is abundant in the brain and pituitary and is limited in the periphery (Steckler and Holsboer, 1999). In contrast to CRFR1, the expression pattern of CRFR2 is discrete in the brain and more widespread in the periphery. The intracellular signaling pathways activated by CRF in the anterior pituitary have been extensively studied (Bilezikjian and Vale, 1983; Aguilera *et al.*, 1988; Rossant *et al.*, 1999). Activation of the receptor by CRF results in G_s-mediated stimulation of adenylyl cyclase leading to increased levels of intracellular cAMP (Stalla *et al.*, 1988). These high levels of cAMP lead to the activation of the cAMP dependent protein kinase A (PKA) (Litvin *et al.*, 1984). In addition to augmenting production of cAMP, it has been shown that CRF triggers a persistent membrane depolarization via PKA-dependent closure of K⁺ channels (Lee and Tse, 1997). This membrane depolarization in turn activates Ca²⁺ entry through L-type and P-type voltage-gated Ca²⁺ channels and causes a continuous cytosolic Ca²⁺ concentration increase. As a result of this, exocytosis via secretion vesicles located near the plasma membrane is stimulated (Tse and Lee, 2000).

Increased cytosolic levels of cAMP and Ca²⁺ lead to the activation of several other downstream effectors of CRF receptors. In addition to the above mentioned PKA, additional kinases such as Ca²⁺/calmodulin-dependent kinase II (CAMKII) and p42/p44 mitogen activated kinases are activated upon stimulation with CRF. As a result Ca²⁺/cAMP response

element-binding protein (CREB) transcription factor is phosphorylated, in turn regulating the transcription of genes containing the Ca²⁺/cAMP response element (CRE) (Rossant *et al.*, 1999). Examples of such genes include *c-fos* (Boutillier *et al.*, 1991), macrophage migration-inhibitory factor gene *Mif* (Waeber *et al.*, 1998), orphan nuclear receptors *Nur77* and *Nurr1* (Murphy and Conneely, 1997). For the latter it has been recently shown that CRF signalling leads to the induction of mRNA through PKA and CAMKII dependent mechanisms whereas *Nurr77* transactivation through phosphorylation is dependent on MAPK regulation (Kovalovsky *et al.*, 2002).

In addition to intracellular protein kinases, Ca²⁺ and cAMP, CRH receptors modulate a plethora of other important signaling molecules such as nitric oxide synthase (NOS), guanylyl cyclase, Fas and Fas ligand, procaspase-3 pathways and suppress activities of glycogen synthase kinase 3 and nuclear factor-kappaB (Dermitzaki *et al.*, 2002; Cantarella *et al.*, 2001; Kostic *et al.*, 2001; Radulovic *et al.*, 2003). It is not always clear whether these effects are mediated via the CRHR1 or CRHR2 subtype. In case of nitric oxide synthase it seems that the divergent effects of CRH on different cell lines can be explained by different expression of either subtype of its receptor (Cantarella *et al.*, 2001). Along the same line, accumulating evidence suggests tissue-specific coupling to G proteins and activation of different signaling cascades. In some tissues CRFR1 may couple to Gq and activate the phospholipase C-protein kinase C cascade (Grammatopoulos *et al.*, 2000).

AtT-20 cells have been studied intensively as a cellular model of corticotrophs. As such, many of the important players of the signaling cascade downstream of CRFR1 have been identified and studied in this cell line. Although several aspects of CRFR1 signaling have been extensively studied, at a transcriptional level our insight is limited. The aim of this study was to explore the transcriptional response to continuous CRF exposure in the mouse pituitary corticotroph-derived adenoma cell line AtT-20 at the genome level using oligonucleotide microarrays and quantitative PCR. We report on novel CRF-responsive genes that are transiently induced after CRF exposure, an induction that can be suppressed by a CRFR1 specific antagonist R121919. Many of the induced transcripts encode proteins that would

exert a negative feedback on the CRFR1 signaling possibly contributing to the transient nature of the induction. The use of spectral map analysis of microarray data revealed the presence of both time and CRF related effects in transcriptional responses to prolonged CRF exposure. Our results demonstrate the power of this projection method in microarray data analysis.

Material and Methods

Cell culture and sample preparation. AtT-20 cells were purchased from ATCC and were maintained at 37°C in 5% CO₂ in humidified air in Dulbecco's modified Eagle's medium (Invitrogen, Groningen, The Netherlands) containing 10 % fetal bovine serum, 5% horse serum and 4.5 g/L D-glucose. For experiments cells were seeded in 25 cm² flasks. The medium was replaced 48 h later and cells were treated with either 0.1% DMSO, 1μM ovine CRF (Sigma, St. Louis, MO) in DMSO, 1μM R121919 (3-[6-(dimethylamino)-4-methylpyrid-3-yl]-2,5-dimethyl-N,N-dipropyl-pyrazolo[2,3-a]pyrimidin-7-amine, Neurocrine Biosciences, San Diego, CA) in DMSO or 1μM CRF +1μM R121919 in DMSO for 0, 0.5, 1, 2, 4, 8 and 24h in fresh medium. The dose of 1μM CRF was chosen because it was shown to induce maximal response in an transactivation assay in AtT20 cells transfected with a luciferase reporter construct (data no shown). The incubation was stopped by aspirating the incubation medium and adding 3 ml Trizol (Invitrogen) for lysis of the cells. Total RNA was extracted using Trizol according to the instructions of the manufacturer. 100μg total RNA was further purified using Qiagen Rneasy kit (Westburg, Leusden, The Netherlands) with DNaseI treatment on column.

Microarray hybridisation. cRNA was prepared as follows. Reverse transcription was performed on 10μg of total RNA for 1h at 42°C using a T7-oligo(dT)₂₄-primer and SuperscriptII RT (Invitrogen). Second strand cDNA synthesis was done for 2 h at 16°C using *Escherichia coli* DNA Polymerase I, DNA ligase and RNaseH (Invitrogen). After phenol-chloroform extraction using phase-lock gel (Eppendorf, Hamburg, Germany) in vitro transcription was performed for 6 h at 37°C using the Bioarray high-yield RNA transcript labelling kit with Biotin labelled ribonucleotides (Enzo Diagnostics, Farmingdale, NY). cRNA samples were purified on Qiagen Rneasy columns followed by fragmentation for 35 min at 95°C. cRNA yields were between 50 and 100μg. Samples were processed on GeneChips (Affymetrix, Santa Clara, CA). In order to check the quality of each sample, 5μg of labelled cRNA was run on Test2-arrays. Actual experiments were performed on Murine

Genome U74Av2a arrays, containing probe sets interrogating approximately 12,000 full-length mouse genes and EST clusters from the UniGene database (Build 74). Hybridisation was performed using 15µg cRNA for 16 h at 45°C under continuous rotation. Arrays were stained in Affymetrix Fluidics stations using Streptavidin/Phycoerythrin (SAPE) followed by staining with anti-streptavidin antibody and a second SAPE staining. Subsequently arrays were scanned with a Agilent Laserscanner (Affymetrix) and data were analysed with the Microarray Suite Software 5.0 (Affymetrix). No scaling or normalization was performed at this stage. Quality of the experiment was assessed based on the percentages present calls across all samples which was on average $47.06 \pm 2.45\%$. The cytoplasmic β -actin and GAPDH 5'/3' ratios were 1.10 ± 0.08 and 0.93 ± 0.05 respectively.

Data analysis and selection of genes.

Normalization. Genes which were called absent in all samples according to Affymetrix' MAS 5.0 software (p-value of >0.06) were removed from further analysis. Raw intensities from each chip were \log_2 transformed and all data from the sample of one time point were quantile normalized using the method described by Amaratunga and Cabrera (Amaratunga and Cabrera, 2001). Following the group-wise quantile normalization, a second quantile normalization was carried out across the data of all samples. Basically this alignment sets the average intensity of one array to the average measured across all arrays, compensating for array to array variations in hybridization, washing and staining, ultimately allowing a reasonable comparison between arrays.

Correspondence factor analysis and spectral map analysis. Correspondence factor analysis and spectral map analysis are special cases of multivariate projection methods that help to reduce the complexity (dimensions) of highly dimensional data (n genes versus p samples). In essence these so called unsupervised methods allow the reduction of the complexity of large microarray datasets and provide means to visually inspect and thereby identify clusters of genes and/or subjects in the data. Typically, projected data are displayed in a biplot combining genes and samples in the same plot. Multivariate projection methods are based on

a derived space with k orthogonal axes. These k axes are linear combinations of the p original measurements (intensities) on the n genes. The axes are constructed in such a way that the first axis lies in the direction (in the multivariate data space) with the largest variability and the last axis in the direction with the smallest variability. In conventional principal component analysis the first axis (principle component) that maximizes the variability is often related to the size of the intensities. Clusters that can be identified based on the first axis will simply differ in absolute size of the intensities. Correspondence factor analysis (CFA) was originally developed for contingency tables and in a sense decomposes the chi-square statistic. Therefore, distances between objects in CFA have a chi-square distribution. In spectral map analysis (SPM) of log-transformed data, the distances are proportional to ratios of genes or samples. In microarray data, we are mainly interested in contrasts and not in simple high or low intensities. For that reason both CFA and SPM have the appropriate properties (double closure for CFA and double centering for SPM) that remove the size component from the data. CFA and SPM will look for contrasts in intensities between genes without the nuisance effect of the absolute values of the intensities. Microarray data tend to be more reliable with increasing intensity. Therefore, re-introduction of the size component via variable weighting proportional to the mean intensities of genes and samples is required. A biplot (Chapman *et al.*, 2002) created by the first two axis displays the maximal separation of both the genes and the samples. Coinciding clusters of samples and genes on the biplot indicate the genes (signatures) that are responsible for the separation of the samples. Genes that are located in the general direction of a sample on the biplot should be looked at as potential signatures for the separation of that sample versus the others. Since, in micro-array data there is a large difference in row- and column-dimension, an asymmetric factor scaling is recommended in constructing the biplot. This operation pulls the genes away from the center of the biplot, while leaving the samples at their original places (Wouters *et al.*, 2003). Both methods were carried out in R (version 1.6.1) using the functions described by Wouters *et al.* (<http://users.pandora.be/luc.wouters/spm.htm>). R is an open source implementation of the statistical programming language S (<http://www.r-project.org>).

k-means clustering, hierarchical clustering and fold change. Additional analysis based on k-means clustering, hierarchical clustering and fold change criteria was carried out using the OmniViz program (OmniViz, Maynard, MA). Gene expression fold differences for CRF, R121919 and CRF+R121919 treatments were calculated at each time point. For those calculations signals at corresponding time points in DMSO treated samples were used as controls to calculate ratios. K-means clustering (By magnitude and shape; 79 clusters) was done on all \log_2 transformed ratios. Using the numeric query tool genes were selected which were two fold up or down-regulated in any of the experiments. 975 genes out of 6393 showed a two fold change in expression level in at least one of the experiments. 846 genes were selected which were differentially expressed and present in at least one of the experiments. For those 846 genes a subset view was created and hierarchical clustering (By magnitude and shape; 29 clusters) was done on \log_2 transformed ratios. Using the OmniViz Treescape view (see figure 3), 88 genes which showed a difference in expression after treatment with CRF compared to treatment with the antagonist, were selected (see supplementary table 1).

Quantitative RT-PCR. Microarray data were confirmed using real time PCR analysis. First strand cDNA synthesis was performed on 0.5 μ g total RNA using random hexamer primers and SuperscriptII RT (Invitrogen). Quantitative PCR was performed on a ABIPrism 7700 cyclor (Applied Biosystems, Foster City, CA) using a Taqman PCR kit (Applied Biosystems). Serial dilutions of cDNA were used to generate standard curves of threshold cycles versus the logarithms of concentration for β -actin and the genes of interest (see table 1 for sequences of primers (Eurogentec, Seraing, Belgium)). Other quantitative assays were obtained from Applied Biosystems (Nurr1, Mm00443056_m1; Nur77, Mm00439358_m1; Per1, Mm00501813_m1; Atf3, Mm00476032_m1; Fyn, Mm00433373_m1; Snk, Mm00446917_m1; Pim3, Mm00446876_m1; Ptpn, Mm00436138_m1; Dusp1, Mm00457274_g1). A linear regression line calculated from the standard curves allowed the determination of transcript levels in RNA samples from the different time points. RT-PCR for CRFR1 and CRFR2 was performed using Amplitaq (Applied Biosystems) on a 9600 cyclor (Applied Biosystems) with 35 cycles and annealing temperature of 55°C.

Results

***c-fos* expression after CRF exposure.** Transcriptional response to continuous CRF exposure was studied in the CRFR1-expressing murine AtT-20 pituitary corticotroph-derived adenoma cell line in a time course ranging from 0.5 to 24 hours. Whereas CRFR1 was readily detectable by RT-PCR, CRFR2 expression could not be discerned in AtT-20 cells (figure 1). In order to identify CRFR1 specific responses, cells were exposed to CRF either in the presence or absence of the specific CRFR1 antagonist R121919 (Heinrichs *et al.*, 2002). To this end cells were incubated in fresh culture medium containing either a maximal stimulatory concentration of 1 μ M CRF (Xiong *et al.*, 1995), 1 μ M CRF in the presence of 1 μ M of R121919 and 1 μ M R121919 alone. Maximal stimulatory concentrations were used in order to enhance differences between CRF and antagonist treated samples. Transcriptional responses were followed over time, starting from 30 minutes until 24h after administration. In order to assess treatment efficacy, *c-fos* mRNA levels were determined by quantitative PCR on RNA from the different treatments and time points before array experiments were carried out. In agreement with previous reports, exposure to CRF elicited a transient surge in *c-fos* transcription, with levels already going down after 30 minutes to 1h (see figure 2) (Boutillier *et al.*, 1991). This response was almost completely suppressed in the presence of R121919. Interestingly, 0.1% DMSO induced *c-fos* expression, however levels were between 5 to 10 times lower compared to CRF induced expression.

Graphical exploration of array data using projection methods. All time points were analyzed on oligonucleotide microarrays interrogating expression levels of approximately 12000 mouse genes and ESTs. Overall analysis of the expression profiles at the different time points and different treatments was performed using multivariate projection methods such as spectral map analysis (SPM) and correspondence factor analysis (CFA). In the SPM biplot showing the first two principal components, samples seemed to be clustered more according to time point (see figure 3A). This is exemplified by the genes that cluster with the samples. The gene with identifier 95632_f_at (mevalonate kinase) clustered together with samples

isolated at 4 and 8 hours. This implies that the gene is responsible for the separation of the 4 and 8 hours samples from the other time points. Indeed high levels of expression of the mevalonate kinase gene (95632_f_at) are observed at 4 and 8 hours after the beginning of the exposure, irrespective of treatment (see left inset figure 3A). At the opposite site of the biplot the RIKEN cDNA 4930429H24 gene with identifier 96494_at, is located together with samples exposed for 0.5, 1 and 24 hours indicating that this gene is high expressed at these time points (see right inset figure 3A). Clearly the first two principal components of SPM indicate progressing time to account for most of the observed changes in gene expression. When applying CFA, a similar clustering by time point was observed when plotting the first two principal components. However, in contrast to the SPM biplot, CRF treated samples seemed to be separated from the other treatments at the corresponding time point. This is mainly the case for the early time points (1h until 4h). More striking is the SPM biplot of the first versus third principal component, where all CRF treated samples are separated from other samples (Figure 3B). This points towards an overall difference in expression for CRF treated samples compared to the other treatments. After 8 hours of exposure this overall difference is diminishing. Of interest is the observation that R121919 treatment suppresses CRF effects as indicated by the clustering of CRF+R121919 samples with either untreated samples or compound only treated samples. The clear separation of the time and CRF exposure effect was not observed in the CFA, even when looking at all principal components, demonstrating a clear difference between the algorithms. One important feature of both correspondence analysis and spectral map analysis is that they allow the identification of genes that contribute to differences in samples. As indicated in the CFA biplot some genes are clustered together with the CRF treated sample. As shown in the inset in figure 3C the transcription factor JunB (102362_i_at) is readily upregulated at 0.5 h exposure to CRF with a maximum at 1h. As a consequence this gene clusters together with the CRF 1h treated sample. Similarly cAMP responsive element modulator CREM-ICER (100533_s_at) and cholecystokinin (96055_at) are clustered with the CRF treated samples at respectively 2 and 4 hours of exposure. In this

way SPM, and to a lesser extent CFA, allowed the identification of genes specifically induced by CRF (see table 2).

Cluster and fold change difference analysis. Other methods to identify genes regulated by CRF include k-means clustering and fold change difference analysis. Because of the obvious influence of time, expression measurements were analyzed relative to those observed in the corresponding time point in DMSO treated control samples. Regulated genes were defined as those showing a greater than 2-fold change in transcript levels at any one time point. Using the OmniViz Treescap view, 88 genes that showed a difference in expression after treatment with CRF compared to treatment with the antagonist, were selected (see material and methods). 17 out of the 88 genes were “early responders”, showing at least a 2-fold change already after 30 minutes treatment with CRF. 48 genes were “intermediate responders”, responding between 1 to 2 hours after start of the treatment and 23 genes were “late responders”, showing a response after 2 hours or more (figure 4). These responses were all suppressed by the CRFR1 antagonist R121919 (figure 4). Among the early responders were known players in the pathways downstream of the CRFR1 such as the transcription factors *Nurr1*, *Nurr77*, *Jun-B*, validating the assay. Of interest is the observation that 50 of the 88 genes that were identified using this arbitrary fold change criterion were also identified in the unsupervised spectral map and correspondence factor analysis. A direct comparison of the genes identified by the different methods is shown in table 2.

Genes induced by CRF exposure. Remarkable novel players identified using methods outlined above include transcription factors (e.g. hairy/enhancer-of-split related 1 (*Hey1*), nuclear factor regulated by interleukin 3 (*Nfil3*), cAMP responsive element modulator (*Crem*) and prostate specific ets transcription factor (*Pse*)), receptor and channel regulators (e.g. Ras-related GTP-binding protein (*Gem*) and receptor (calcitonin) activity modifying protein 3 (*Ramp3*)), secreted peptides (e.g. calcitonin, cholecystokinin) and proteins involved in intracellular signaling (e.g. regulator of G-protein signaling 2 (*Rgs2*), cAMP specific phosphodiesterase 4B (*Pde4b*) and the regulatory subunit phosphatidylinositol 3-kinase, p85). Other regulated genes comprise Period homolog *Per1*, fibroblast growth factor receptor 2

(*Fgfr2*), serum/glucocorticoid regulated kinase and serum-inducible kinase (figure 5). Intriguingly, all responders identified according to above mentioned criteria were up regulated after exposure to CRF. This induction was transient and nearly all of the induced genes returned to baseline after 4 to 8 hr. Many of the induced transcripts encode proteins that would exert a negative feedback on the CRFR1 signaling (e.g. *Pde4*, *Rgs2*, *CREM*, etc...), possibly contributing to the transient nature of the induction. In addition to this negative feedback, other mechanism such as desensitization of CRFR1 through phosphorylation and internalization contribute to the transient nature of transcriptional induction (Hauger *et al.*, 2000; Dautzenberg *et al.*, 2002). In this respect it is of interest to note that challenge with CRF quickly downregulates CRFR1 mRNA in rat pituitary cells (Pozzoli *et al.*, 1996). Although array experiments did not reveal obvious alterations in CRFR1 mRNA, quantitative PCR showed an increase in CRFR1 mRNA in AtT-20 cells exposed to 1 μ M CRF after 1 (3.4 fold) and 2 (2.2 fold) hours of exposure (figure 6). Of interest is the observation that even in the presence of R121919 an upregulation of CRFR1 mRNA is observed although less pronounced and with some delay to the response in CRF treated samples.

Quantitative PCR. Confirmation of microarray data was carried out using quantitative PCR on the same samples used for hybridization experiments and furthermore on an independent experiment. Levels of regulation and the time course identified by microarray experiments were in agreement with those observed by quantitative PCR. For those genes that have been tested, levels of induction compared to the untreated sample are indicated in figure 5. A comparison between the time course identified by microarray and quantitative PCR is shown in figure 7, demonstrating the regulation of *Rgs2*.

Discussion

To identify transcriptional pathways downstream of the CRFR1 we applied oligonucleotide microarray analysis on AtT-20 exposed to CRF and the CRFR1 specific antagonist R121919. A transient induction of negative regulators of CRFR1 signaling was observed. Several previously unknown CRF inducible genes were identified such as *Rgs2*, *Pde4b* and *Crem*. The use of microarray data to explore the mechanism of action of a compound requires efficient analysis tools that allow rapid identification of specific signatures in gene expression across different samples. Projection methods allow this type of graphical exploration by reducing complex multidimensional data to a two-dimensional subspace, in which samples and genes are plotted together such as principal component analysis (PCA) and correspondence factor analysis (CFA) (Alter *et al.*, 2000; Hastie *et al.*, 2000; Holter *et al.*, 2000; Fellenberg *et al.*, 2001). Recently spectral map analysis (SPM) was introduced as an alternative unsupervised multivariate projection method. Weighted SPM outperforms PCA, and is at least as powerful as CFA, in finding clusters in the samples, as well as identifying genes related to these clusters (Wouters *et al.*, 2003). When applying SPM and CFA to our dataset we identified exposure time to treatment as important discriminator between the different samples. Synchronization of cell cycle in these cultures induced by addition of new medium and serum could possibly account for this phenomenon although accumulation of metabolites and progressing cell culture conditions are additional contributing factors. Besides time, SPM identified an effect of CRF treatment in the early time points. These effects of CRF are in agreement with activation of several second messenger such as cAMP and Ca^{2+} upon stimulation with CRF. Moreover all of these effects were blocked by the CRFR1 specific antagonist R121919. Some of the transcriptional responses can be explained by the phosphorylation of CREB and the subsequent transcription of genes downstream of cAMP responsive elements. These elements have been found for example in the promoters of *Per1*, *Nurr1*, *Crem-icer*, *c-fos*. Furthermore the kinetic profile of the induction of these genes corresponds with the observed maximal transcription rate by CREB after 0.5 hr of initial

cAMP formation. The induction of *Crem-icer* constitutes a negative feedback mechanism in attenuating transcriptional response to cAMP (Lamas *et al.*, 1997). Of interest is the reported induction of *Crem-icer* in response to acute stress in the intermediate lobe of the pituitary gland. Mice deficient for *Crem-icer* show a chronic increase of beta-endorphin levels suggesting that *Crem-icer* induction may be involved in the modulation of gene expression in response to stress (Mazzucchelli and Sassone-Corsi, 1999). Our results suggest that *Crem-icer* is directly involved in the modulation of CRF signaling and as a result ablation of *Crem-icer* could lead to an altered response to stress signals.

Another novel putative negative feedback regulator of CRF signaling is Rgs2. We identified two single CRE motifs in the promoter of the human *RGS2* gene, providing a possible explanation for the early response behavior of this gene upon stimulation with CRF. In support of our findings is a recent report showing that both phosphoinositide signaling and cAMP induce a rapid and transient increase in *Rgs2* mRNA in human astrocytoma and neuroblastoma cells (Zmijewski *et al.*, 2001). The Rgs2 protein is a selective inhibitor of G_q function (Heximer *et al.*, 1997). It has been shown that Rgs2 reduces odorant-elicited cAMP production, not by acting on G_α but by directly inhibiting the activity of adenylyl cyclase type III (Sinnarajah *et al.*, 2001). Although *Rgs2* was originally identified as an immediate early response gene in activated T lymphocytes, studies in *Rgs2* deficient mice indicate that it also plays a role in the modulation of stress related behavior as these mice show increased anxiety and aggression (Oliveira-Dos-Santos *et al.*, 2000).

The induction of the cAMP specific phosphodiesterase 4B gene (*Pde4b*), encoding an enzyme responsible for hydrolysis of cAMP (particularly in nerve and immune cells) can also be categorized as negative feedback, directly attenuating the cAMP signal.

Another important second messenger generated upon stimulation with CRF is Ca²⁺. It has been shown that CRF triggers a steady-state depolarization stimulated extracellular Ca²⁺ entry via voltage-gated Ca²⁺ channels and raises intracellular Ca²⁺ concentration through release from inositol 1,4,5-triphosphate (InsP3) sensitive Ca²⁺ pools (Tse and Lee, 2000). This leads

to the secretion of ACTH. Wortmannin has been shown to inhibit both calcium-independent and -stimulated secretion from permeabilized AtT-20 cells, indicating a role for phosphatidylinositol-3 kinase (PI-3K) in determining the size of the readily releasable pool of ACTH (Wilson and Guild, 2001). The p85 regulatory alpha subunit of PI-3K is upregulated in CRF treated AtT-20 cells. This upregulation of the regulatory unit might constitute an attenuation of PI-3K activity. In addition to its role as second messenger, intracellular Ca^{2+} has also been shown to play a critical role in regulating gene expression. Of interest is the regulation of NFIL3/E4BP4 by calcineurin/NFAT and CaM kinase signaling (Nishimura and Tanaka, 2001), accounting for an increase in NFIL3 mRNA levels upon CRF treatment. In B lymphocytes expression of NFIL3 is induced by interleukin 3 through both the Raf-mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways. In this cell type NFIL3 inhibits apoptosis in synergy with Bcl-xL dependent pathways (Kuribara *et al.*, 1999). Our data suggest a role for NFIL3 in prevention of apoptosis in AtT-20 cells, further supporting a cytoprotective effect of CRF by suppression of pro-apoptotic pathways (Radulovic *et al.*, 2003).

CRF is the most efficacious ACTH secretagogue. Several other prepropeptides mRNAs were found to be upregulated after CRF administration such as cholecystokinin (CCK) and calcitonin (CT). Of interest in this respect is the upregulation of RAMP3. RAMPs control the transport and glycosylation of the calcitonin receptor-like receptor (CRLR). In the case of RAMP3, it has been shown that together with CRLR it generates an adrenomedullin (ADM) receptor (McLatchie *et al.*, 1998). Upregulation of this gene might play a role in regulating the responsiveness of AtT-20 cells to ADM after CRF exposure. In addition regulation of RAMP3 could influence responses to other extracellular stimuli such as CRF, as it is known that RAMPs might regulate other G-coupled receptors for example vasoactive intestinal polypeptide receptor and parathyroid receptor (Christopoulos *et al.*, 2003).

Although CCK is secreted by AtT-20 cells, induction of its expression by CRF has not been previously reported (Beinfeld, 1992). Interaction between CCK and CRF has however been intensively studied and demonstrated in panic attacks, depression, anxiety and gastric

emptying (Coskun *et al.*, 1997;Geraciotti, Jr. *et al.*, 1999;Kellner *et al.*, 2000). Most of these experiments point towards a role for CRF in mediating the central effects of CCK. Our data indicate that CRF in addition might function as a CCK secretagogue.

In agreement with previous reports we could find a upregulation of CRFR1 mRNA after prolonged CRFR1 exposure (Iredale *et al.*, 1996). It is tempting to speculate that this upregulation compensates for the loss of receptor at the surface after internalization and restores responsiveness of the cells. An interesting observation is the similar upregulation with a shift in time in the cells treated with CRF in the presence of the antagonist. This suggests that also these receptor complexes are internalized.

In conclusion, we have unraveled part of the corticotropin-releasing hormone receptor-1 activated gene network and have identified several novel targets of this signaling cascade. In addition we have demonstrated the power of spectral map analysis for microarray data exploration.

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Footnotes

b) Reprint requests: Pieter Peeters, Johnson & Johnson Pharmaceutical Research and Development, a division of Janssen Pharmaceutica N.V., Turnhoutseweg 30, B-2430 Beerse, Belgium

Legends for Figures

Figure 1

Title: expression CRFR1 and CRFR2 in AtT-20 cells.

Legend: RT-PCR reaction were performed on two isolates of AtT-20 cells, mouse heart and mouse brain RNA with primers specific for mouse CRFR1 and CRFR2. Both receptors are expressed in brain whereas only CRFR2 is expressed in heart. As a control for RNA quality primers for β -actin were used.

Figure 2

Title: normalized *c-fos* expression after CRF treatment.

Legend: *c-fos* mRNA levels as assessed by quantitative RT-PCR normalized against β -actin mRNA levels in AtT-20 cells continuously exposed to DMSO, 1 μ M CRF, 1 μ M CRF+R121919 or 1 μ M R121919 in a time course ranging from 30 minutes to 24h.

Figure 3

Title: Spectral Map and Correspondence Analysis of Microarray data.

Legend: A – First two principal components (PC) of the weighted Spectral map analysis (SPM) applied on normalized microarray data for all time points and treatments. On the spectral map squares depict different samples whereas circles depict genes (size of circle correspond to intensity). Distances between squares are a measure for similarity between samples. A positive association of a gene with a given sample (i.e. an upregulation of that gene in that particular sample) results in the positioning of the gene and sample on a common line through the centroid (depicted by a cross). Genes contributing significantly (measured by their distance from the centroid) to difference between samples are annotated with their Affymetrix identifier (www.affymetrix.com/analysis/netaffx) (see table 2 for annotation). First two PCs identified time as the major discriminator between the samples. Inserted heat

maps (insets) show representative genes (indicated in bold in biplot) that are maximally induced either after 4 and 8 h or at 0.5, 1 and 24h, corresponding to their position on the biplot. B – biplot of first and third PCs. Third PC identifies the specific CRF effect on AtT-20 cells over time. C – Summary views on the first three dimensions of spectral map, showing how this technique identified time and CRF effects in the microarray dataset. D - Correspondence factor analysis (CFA) applied on the same data as in A. The interpretation of the CFA output is similar to that of SPM. In addition to time, CFA identified the effect of CRF exposure as most prominent in the early time points (1-4h). Inserted heat maps show representative genes that are maximally induced either after 1, 2 or 4 h of exposure to CRF, corresponding to their position on the map.

Figure 4

Title: Heat map of selected genes affected by CRF exposure.

Legend: A heat map depicting 88 genes that are changed upon CRF treatment as identified by a combination of cluster analysis and fold change criteria (for list of 88 genes see supplementary table 1). Values were calculated by dividing the intensity of each sample by the intensity of the DMSO sample at the corresponding time point. These calculated ratio are converted into a colour ramp on a \log_2 scale. In this way the different timing of induction of expression becomes apparent. Genes showing a 2-fold change after 30 minutes of treatment with CRF were called “early responders”, “intermediate responders” show a change after 1 to 2 hours of treatment and “late responders” show a response after 2 hours or more.

Figure 5

Title: Overview of regulated pathways.

Legend: Overview of a selection of genes induced by CRF grouped by pathway or function. Values were calculated by dividing the intensity of each sample by the intensity of the DMSO sample at the corresponding time point. These calculated ratio are converted into a color ramp on a \log_2 scale and depicted in a heat map. Next to the heat maps the maximal fold change

over all timepoints as detected by quantitative PCR is shown for each gene tested. These PCR data were obtained on a repeated experiment. For an overview of all quantitative PCR data see supplementary table 2. At the right Affymetrix identifiers for each gene are depicted. (ND, not done)

Figure 6

Title: Induction of CRFR1 mRNA by CRF in AtT-20 cells.

Legend: Quantitative PCR results with CRFR1 specific primers. Induction is calculated in comparison to levels observed in AtT-20 cells before any treatment.

Figure 7

Title: Induction of *Rgs2* mRNA by CRF in AtT-20 cells.

Legend: Induction is calculated in comparison to levels observed in AtT-20 cells before any treatment. On top, array data obtained for *Rgs2* are shown. Below, levels of *Rgs2* mRNA as measured by quantitative RT-PCR on the same samples as used for array experiments and as measured in an independent experiment.

Tables

Table 1

Sequence of oligonucleotides used for quantitative PCR

β -actin forward	5'-CATCTTGGCCTCACTGTCCAC-3'
β -actin probe	5'-TGCTTGCTGATCCACATCTGCTGGA-3'
β -actin reverse	5'-GGGCCGGACTCATCGTACT-3'
c-fos forward	5'-GGGAGGACCTTACCTGTTCGT-3'
c-fos probe	5'-CACCAGGCTGTGGGCTCAAGG-3'
c-fos reverse	5'-CCAGATGTGGATGCTTGCAA-3'
Crf2 forward	5'-GGGAGAACAGAAGCGCCTG-3'
Crf2 probe	5'-AGAAGGGTGAGGATCCCCCAAATCAGAGT-3'
Crf2 reverse	5'-CCCTTGTTTCAATCACTCCCA-3'
Crf1 forward	5'-TTTCTGAACAGTGAGGTCCGC-3'
Crf1 probe	5'-CCGGAAGAGGTGGCGGCGA-3'
Crf1 reverse	5'-GGGCTCTGATGGAGTGCTTG-3'
Rgs2 forward	5'-TTGGAAGACCCGTTTGAGCTA-3'
Rgs2 probe	5'-TCTTGCAGAATTCCTCTGCTCCTGGG-3'
Rgs2 reverse	5'-TTTCTTGCCAGTTTTGGGCT-3'
Fgfr2 forward	5'-AGACTTCCATGGGAATGATAGCA-3'
Fgfr2 probe	5'-CCTCTCGTCCGGCAGCTGGC-3'
Fgfr2 reverse	5'-AATGTGTAAGCCGGGCAGAA-3'
Mig6 forward	5'-AATCCTTTGTCCAATACTGTACACACA-3'
Mig6 probe	5'-GAAAAATGCACTGATCTCCGCA-3'
Mig6 reverse	5'-GTATGAACTAAATGAAGGTTAAAACATGCT-3'
Pi3k forward	5'-CCATGGTGCTTGTTAACGCTTT-3'
Pi3k probe	5'-CCCAACTTGTAAGCTGGTAAAGCTTCA-3'
Pi3k reverse	5'-CCTGTCTACCTTCTGGTCTCCAA-3'

Crem forward	5'-CTTGCTGATCGTCTGGAGAGTTT-3'
Crem probe	5'-TGCTGATGACCCTCCATTGTGA-3'
Crem reverse	5'-TTAACATTCCTGAGGTTGCAAGAA-3'
Pde4b forward	5'-GCCGTGTGTATGGCTGCAT-3'
Pde4b probe	5'-CAGCCCCCAGGCCACTGTGG-3'
Pde4b reverse	5'-AGGAGGGATAACAGGTGCTGTGT-3'
CCK forward	5'-CCTGGACCCCAGCCATAGA-3'
CCK probe	5'-AGCCCATGTAGTCCCGGTCACTTA-3'
CCK reverse	5'-TGCGCCGGCCAAAA-3'
CT forward	5'-GCTTGGACAGCCCCAGATC-3'
CT probe	5'-GGTACTCAGATTCCCACACCGCTT-3'
CT reverse	5'-TGTGTGTACGTGCCCAGCAT-3'
Nfil3 forward	5'-GCGAGTTTGAAGGCATGCA-3'
Nfil3 probe	5'-CTCTCTTCACCCGCCGATGCGAT-3'
Nfil3 reverse	5'-CCATGTTTCTCCAGGTCAAAATG-3'
Ramp3 forward	5'-TGGCAGACTCGGCTTCTGT-3'
Ramp3 probe	5'-TTTGCTTTGGCCACACCCTACCTGG-3'
Ramp3 reverse	5'-CTGGTCGGGAGGACTTTGG-3'
Sgk forward	5'-TGGACCAATGCCCCAGTT-3'
Sgk probe	5'-TCAGTCAAAGCCGTTGGTGTTCATTG-3'
Sgk reverse	5'-GCCCGTTTTATAGGTGACATTTTAA-3'

Table 2

Genes identified on SPM and CFA biplots

SPM PC1 vs PC2

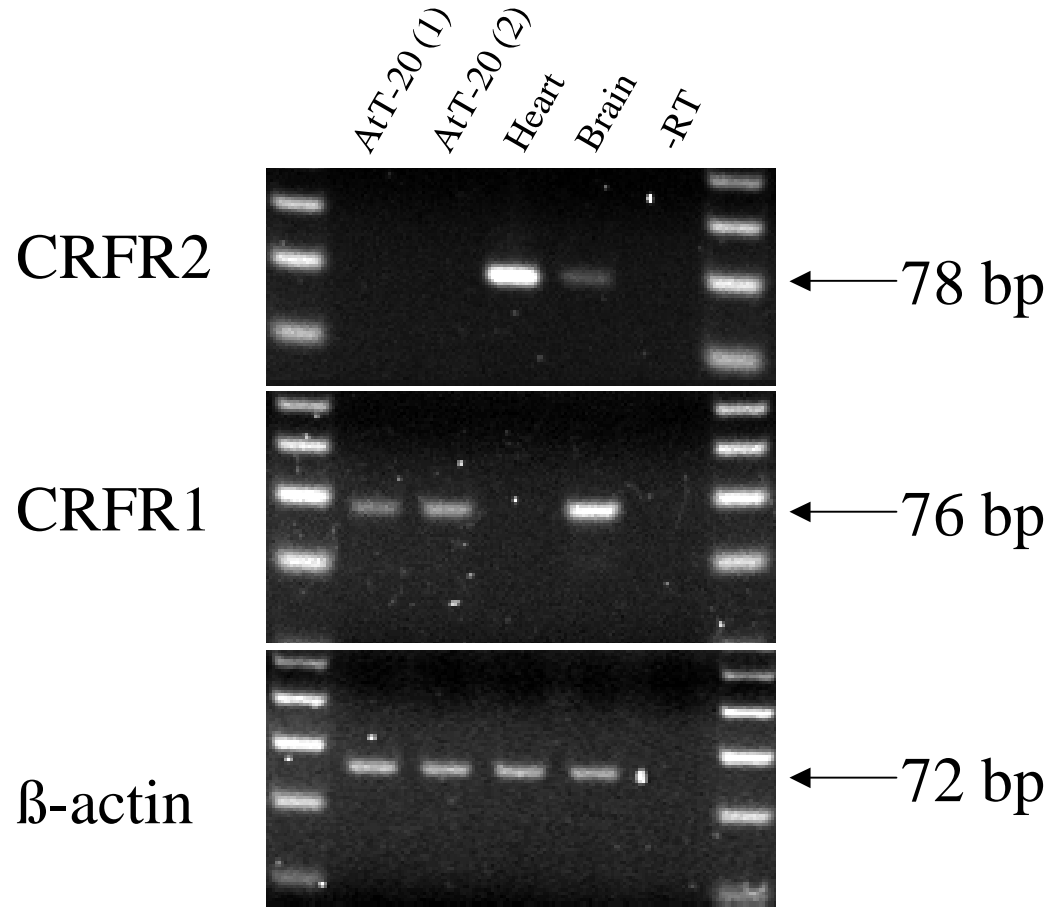
Probe Set ID	Gene Symbol	Gene name
100050_at	Irb1	inhibitor of DNA binding 1
102362_i_at	Junb	Jun-B oncogene
102397_at	Cbfa2t3h	core-binding factor, runt domain, alpha subunit 2; translocated to, 3 homolog
102410_at	Hs3st1	heparan sulfate (glucosamine) 3-O-sulfotransferase 1
103748_at	4933407C03Rik	RIKEN cDNA 4933407C03 gene
104588_at	1810073K19Rik	RIKEN cDNA 1810073K19 gene
94428_at	Ilvb1	ilvB (bacterial acetolactate synthase)-like
95632_f_at	Mvk	mevalonate kinase
96494_at	4930429H24Rik	RIKEN cDNA 4930429H24 gene
96803_at	Gbe1	glucan (1,4-alpha-), branching enzyme 1
98478_at	Ccng2	cyclin G2
98855_r_at	---	weak similarity to melanoma-associated chondroitin sulfate proteoglycan 4
99077_at	Thra	thyroid hormone receptor alpha

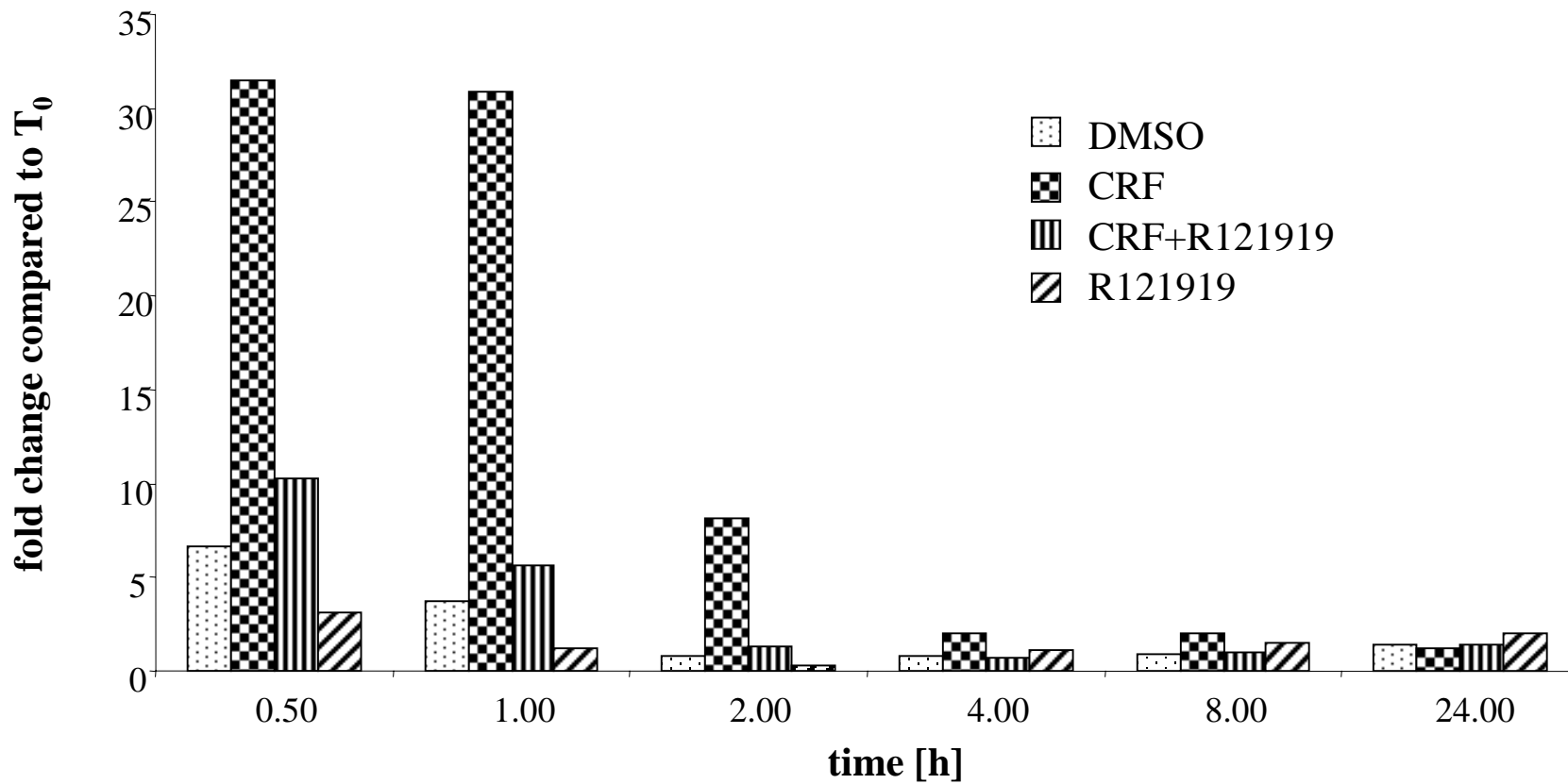
SPM PC1 vs PC3

Probe Set ID	Gene Symbol	Gene name
100533_s_at	Crem	cAMP responsive element modulator
102362_i_at	Junb	Jun-B oncogene
102410_at	Hs3st1	heparan sulfate (glucosamine) 3-O-sulfotransferase 1
102907_at	C820004H04Rik	RIKEN cDNA C820004H04 gene
104588_at	1810073K19Rik	RIKEN cDNA 1810073K19 gene
104712_at	Myc	myelocytomatosis oncogene
92205_at	---	moderate similarity to insulin receptor substrate 2
94428_at	Ilvb1	ilvB (bacterial acetolactate synthase)-like
95632_f_at	Mvk	mevalonate kinase
96055_at	Cck	cholecystokinin
96326_at	Tat	tyrosine aminotransferase
96494_at	4930429H24Rik	RIKEN cDNA 4930429H24 gene
97844_at	Rgs2	regulator of G-protein signaling 2

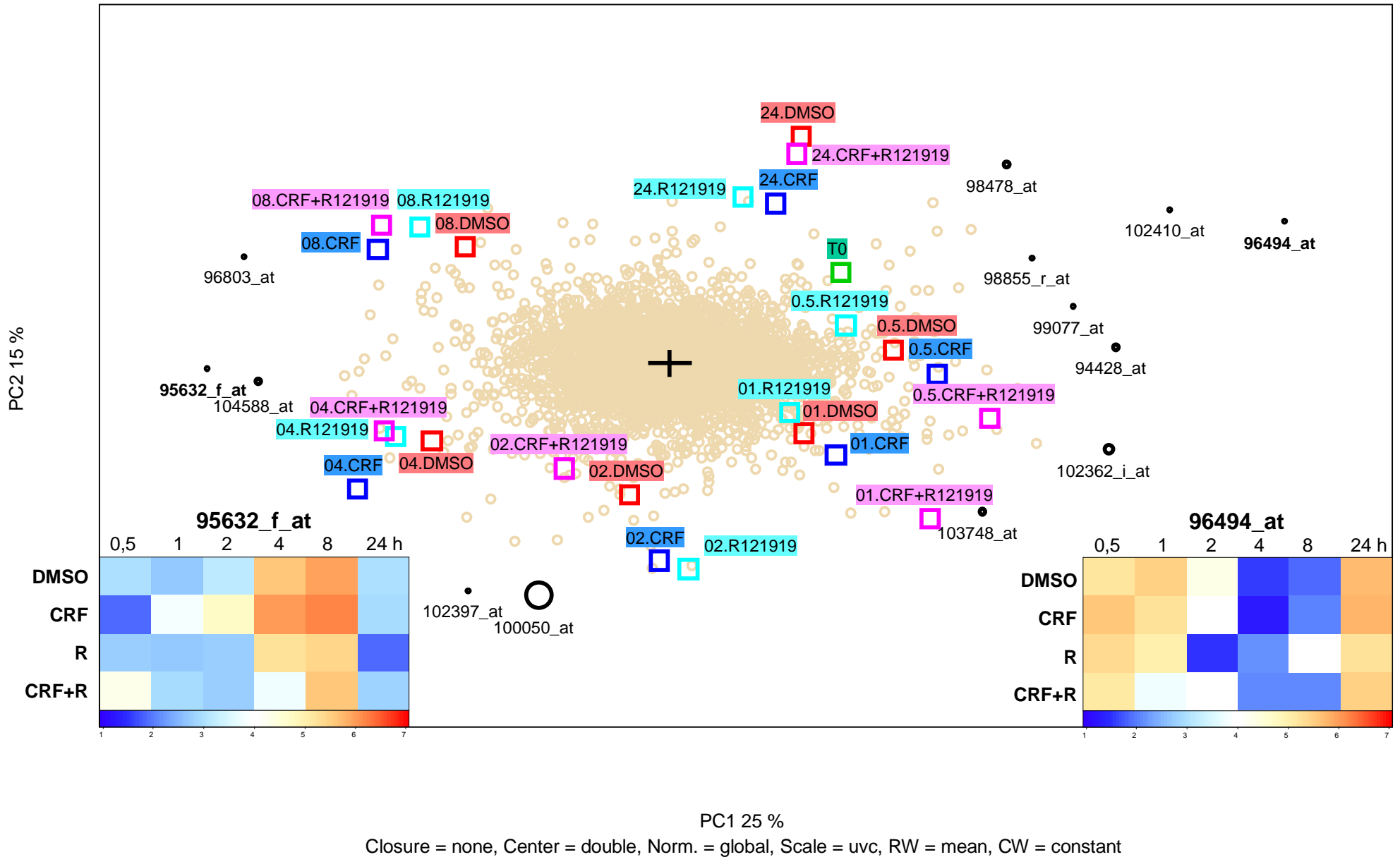
CFA

Probe Set ID	Gene Symbol	Gene name
100533_s_at	Crem	cAMP responsive element modulator
102362_i_at	Junb	Jun-B oncogene
102363_r_at	Junb	Jun-B oncogene
102371_at	Nr4a1	nuclear receptor subfamily 4, group A, member 1
104712_at	Myc	myelocytomatosis oncogene
92248_at	Nr4a2	nuclear receptor subfamily 4, group A, member 2
93090_at	Fgfr2	fibroblast growth factor receptor 2
93091_s_at	Fgfr2	fibroblast growth factor receptor 2
93974_at	1300002F13Rik	RIKEN cDNA 1300002F13 gene
96055_at	Cck	cholecystokinin
96326_at	Tat	tyrosine aminotransferase
97844_at	Rgs2	regulator of G-protein signaling 2
99329_at	Abcc1	ATP-binding cassette, sub-family C



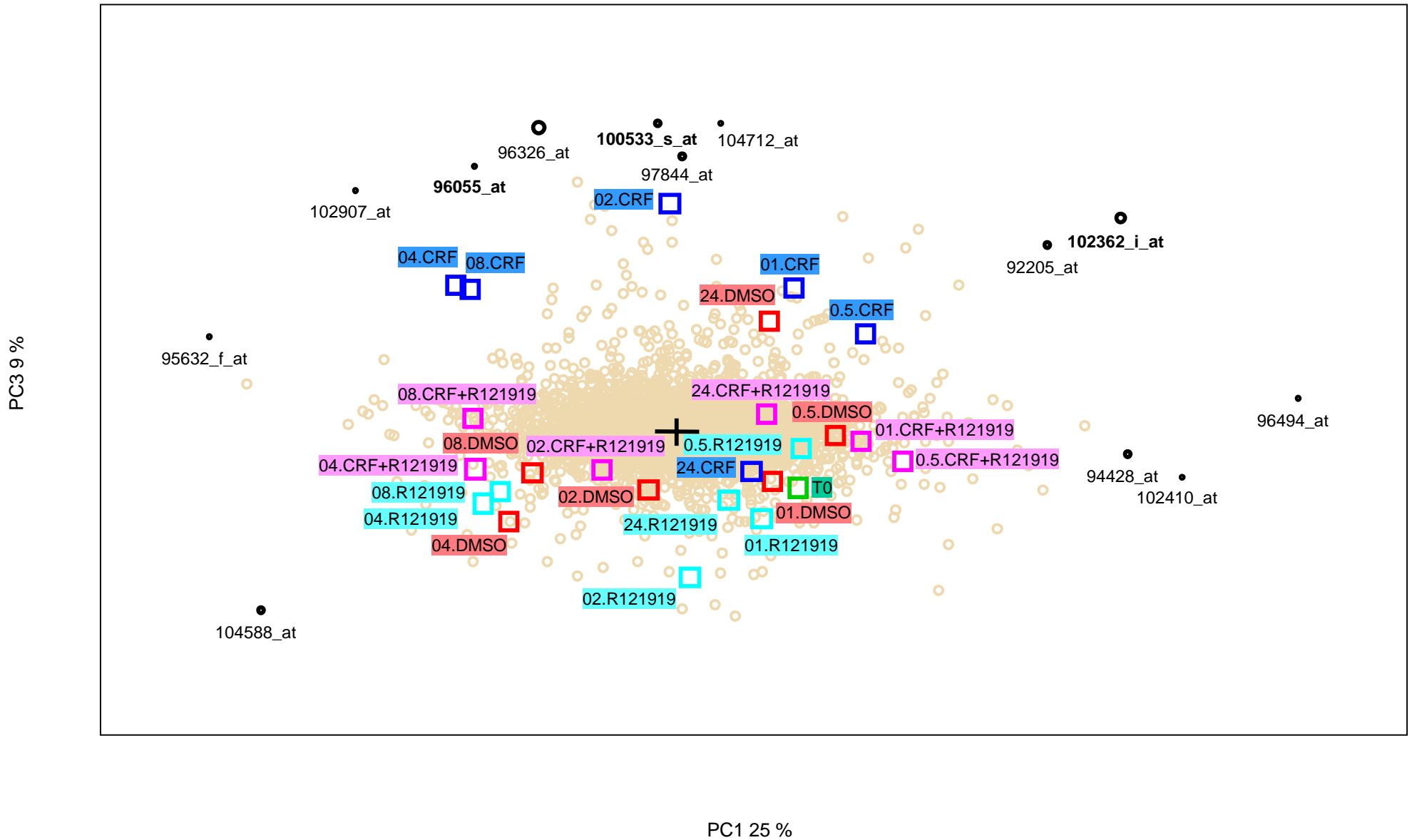


SPM - PC1 vs. PC2

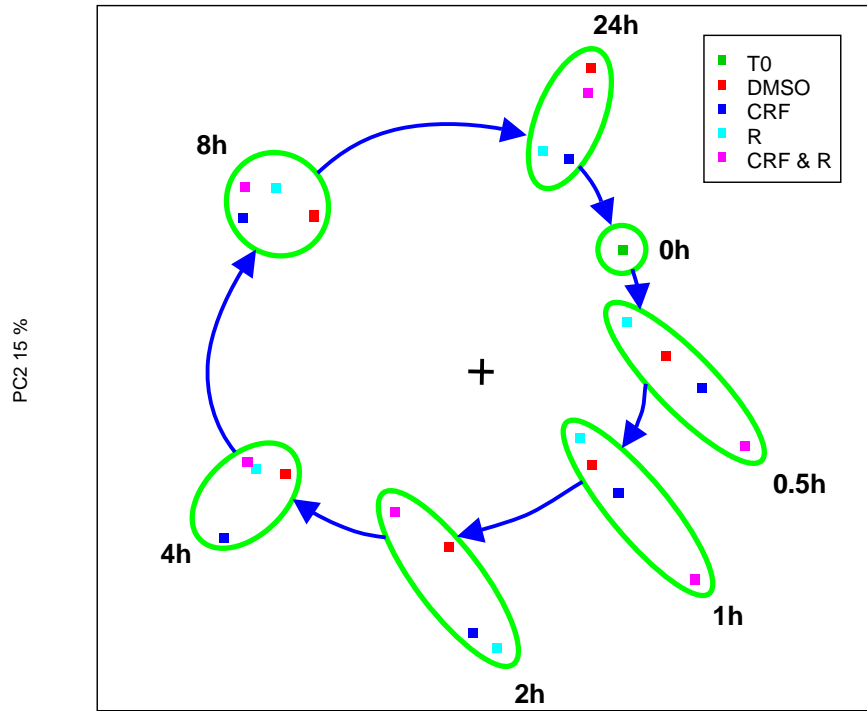


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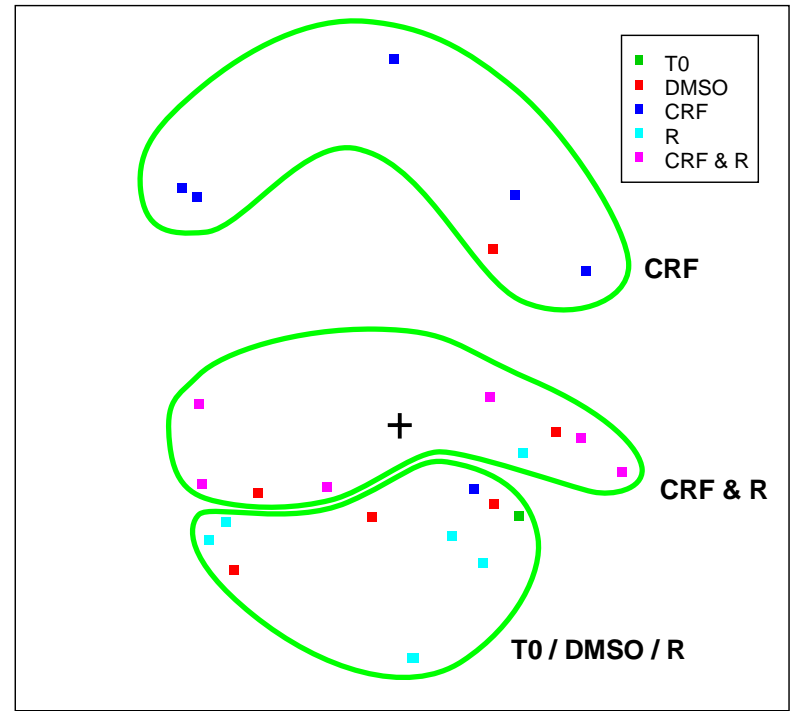
SPM - PC1 vs. PC3



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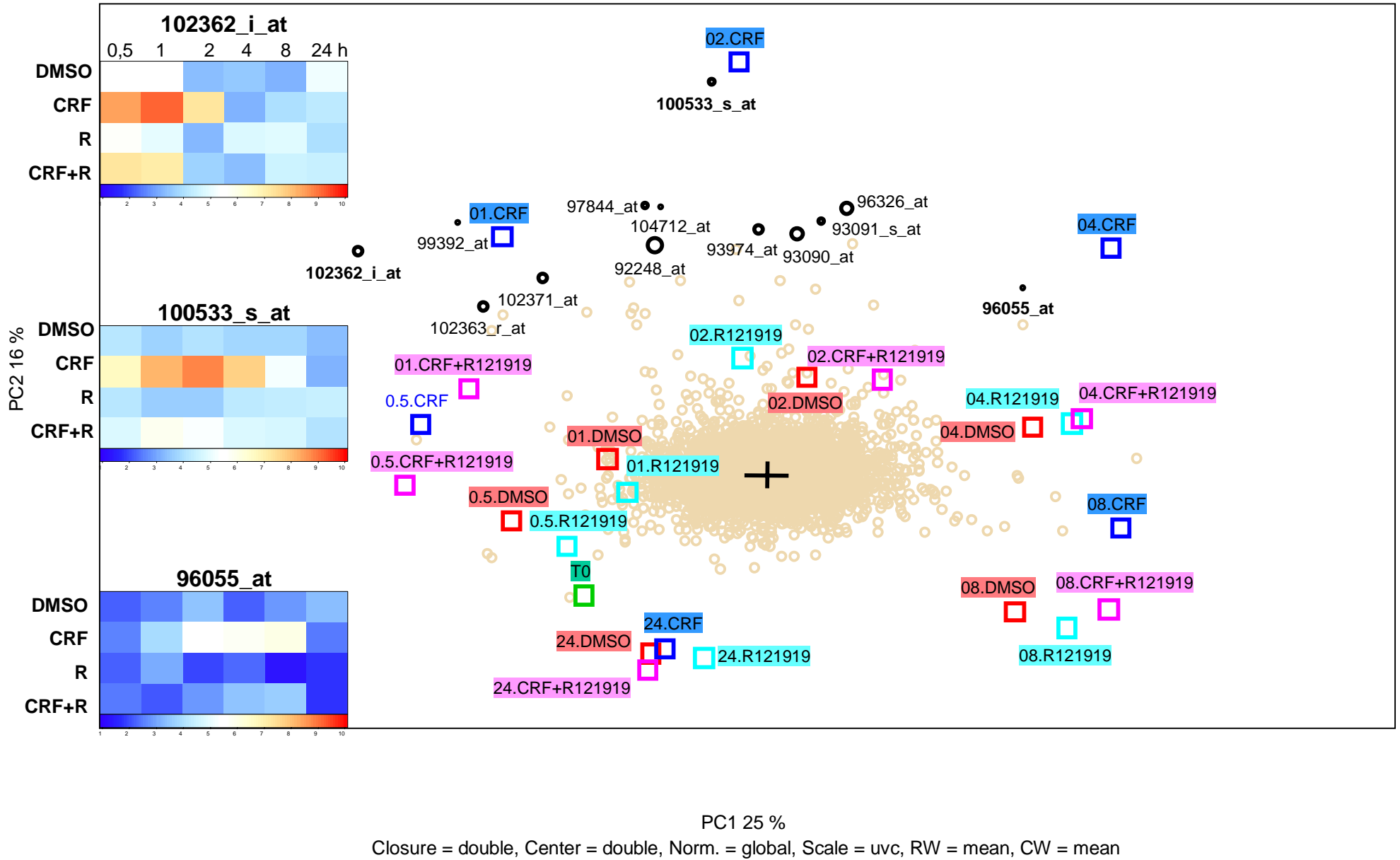


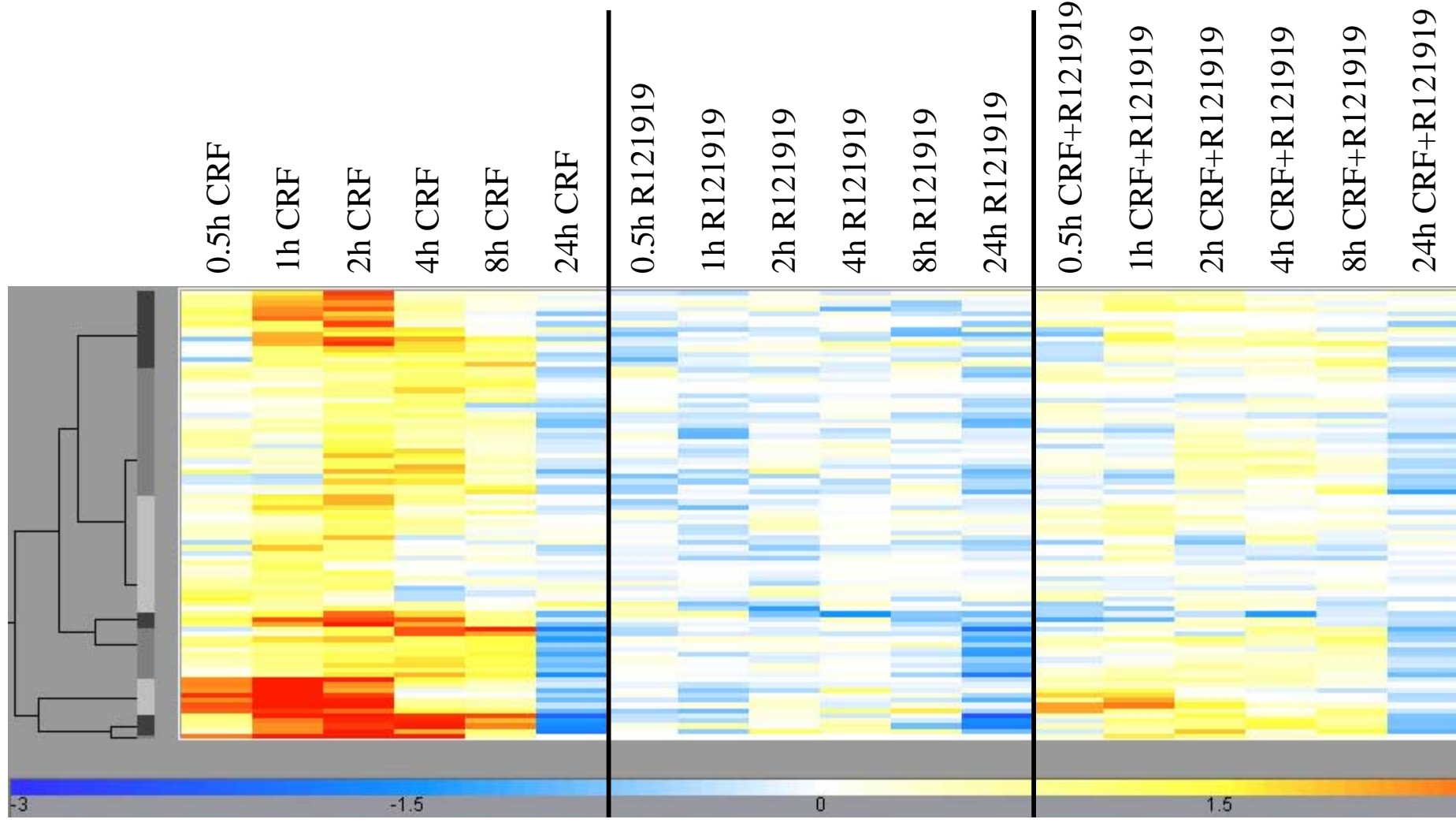
PC1 25 %
 Closure = none, Center = double, Norm. = global, Scale = uvc, RW = mean, CW = mean



PC1 25 %
 Closure = none, Center = double, Norm. = global, Scale = uvc, RW = mean, CW = mean

CFA





Transcription factors

prostate specific ets transcription factor

nuclear receptor subfamily 4, group A, member 1

nuclear receptor subfamily 4, group A, member 2

Jun-B oncogene

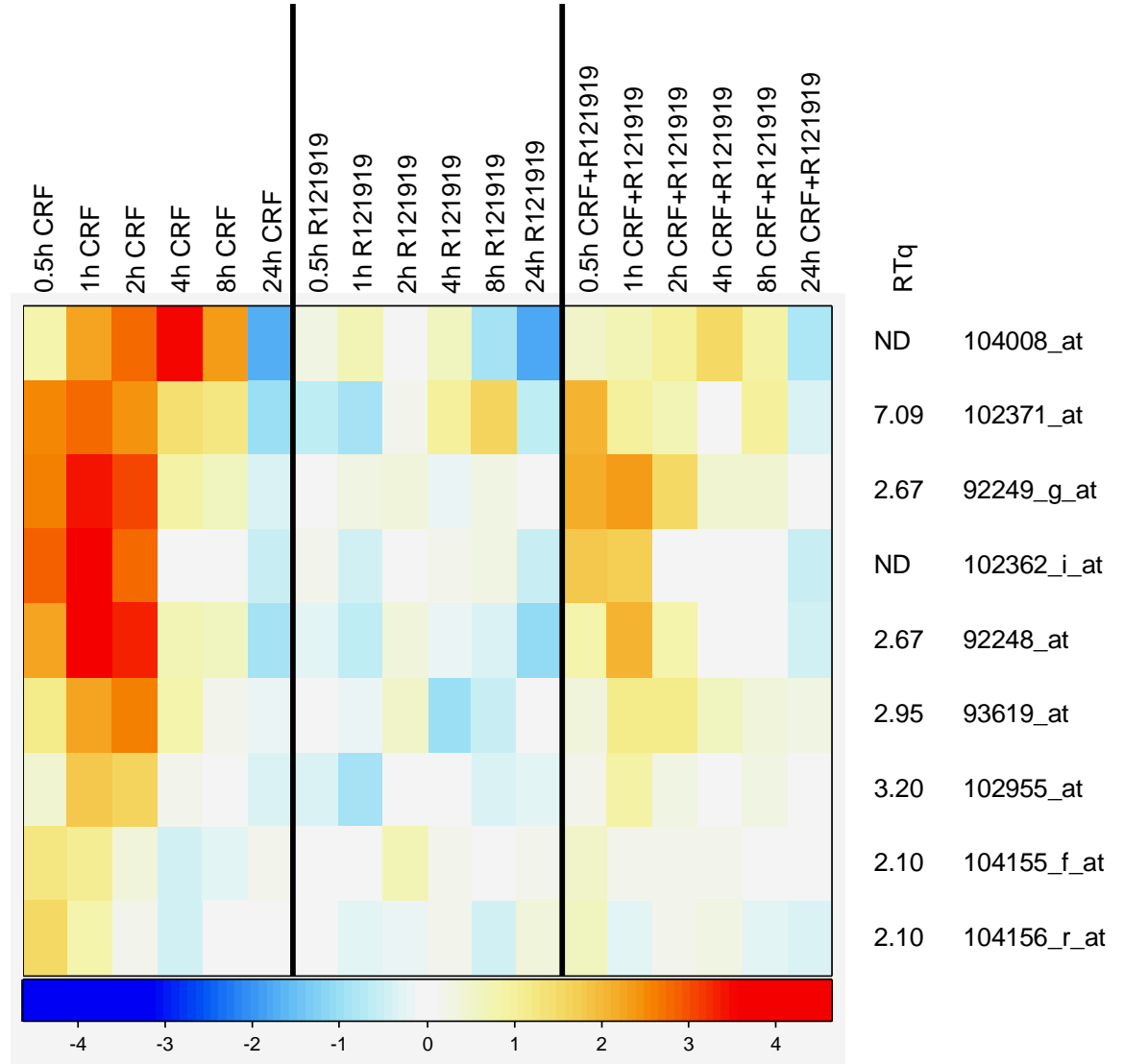
nuclear receptor subfamily 4, group A, member 2

period homolog

nuclear factor, interleukin 3 regulated

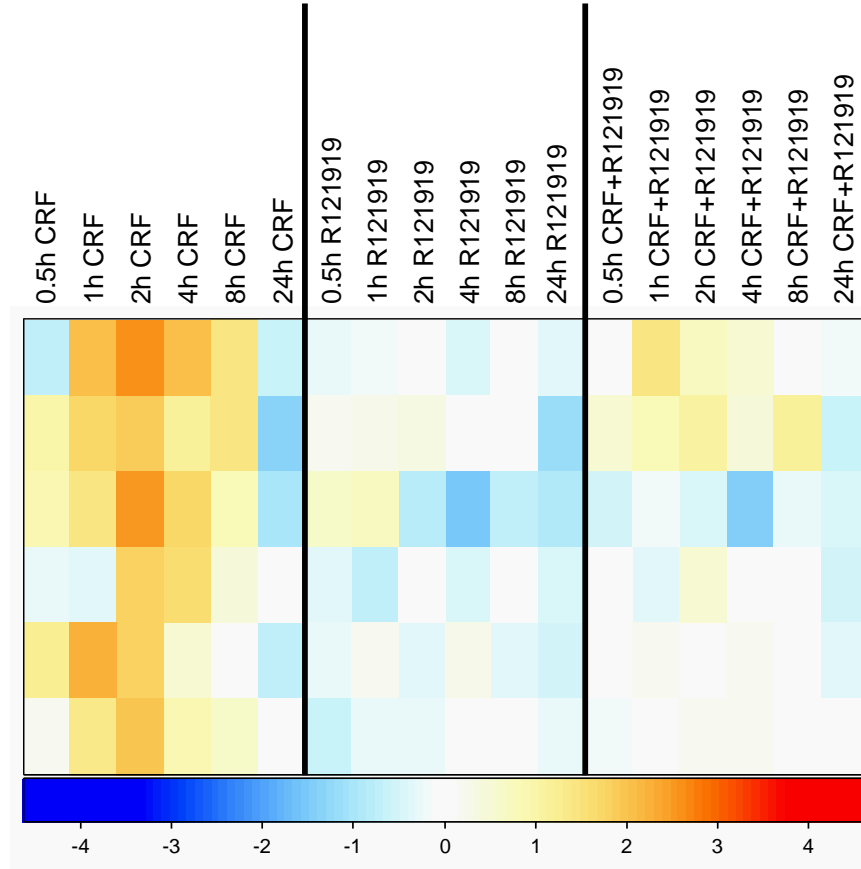
activating transcription factor 3

activating transcription factor 3



Kinases

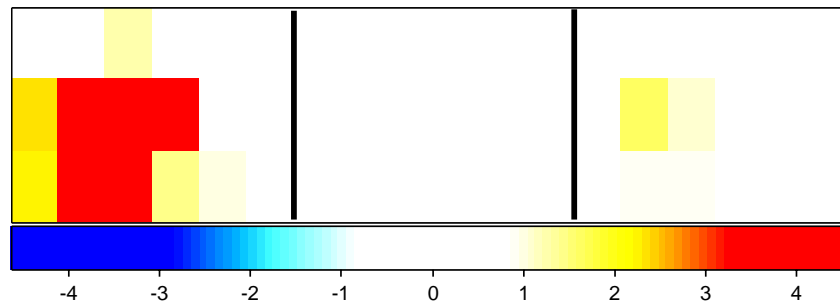
fibroblast growth factor receptor 2, Fgfr2
 serum/glucocorticoid regulated kinase, Sgk
 fibroblast growth factor receptor 2
 Fyn proto-oncogene, Fyn
 serine threonine kinase, Pim3
 serum inducible kinase, Snk



RTq
 9.71 93090_at
 6.18 97890_at
 9.71 93091_s_at
 1.84 100133_at
 10.23 96841_at
 3.25 92310_at

cAMP signaling

phosphodiesterase 4B, cAMP specific
 cAMP responsive element modulator
 regulator of G-protein signaling 2, Rgs2

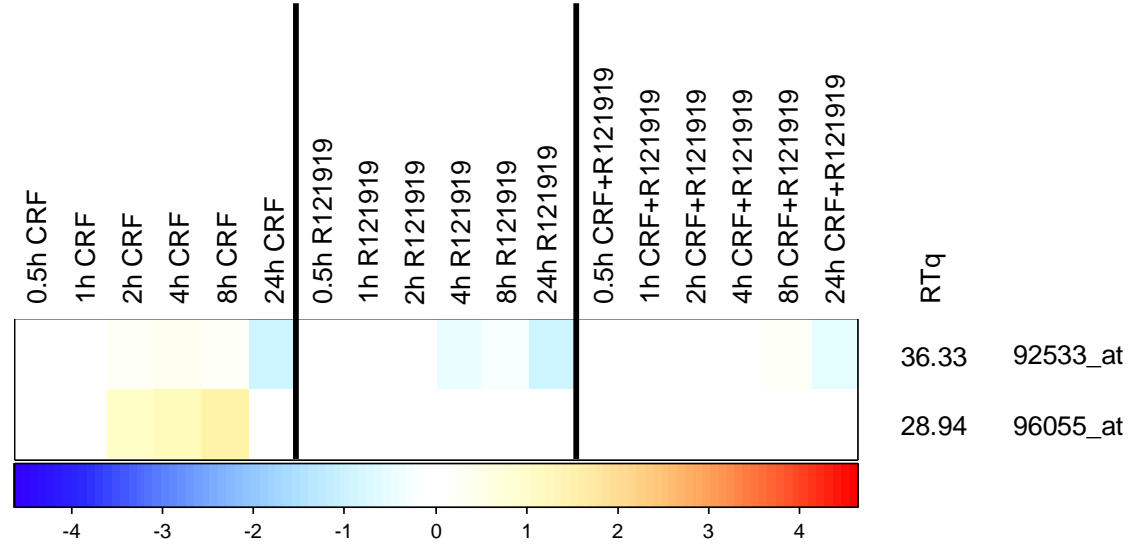


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Secreted peptides

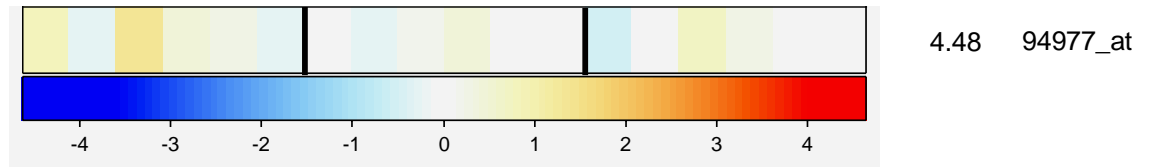
Calcitonin, Ct

Cholecystokinin, Cck



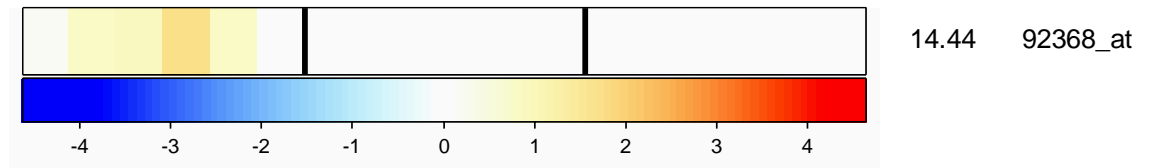
Inositol signaling

phosphatidylinositol 3-kinase, regulatory subunit



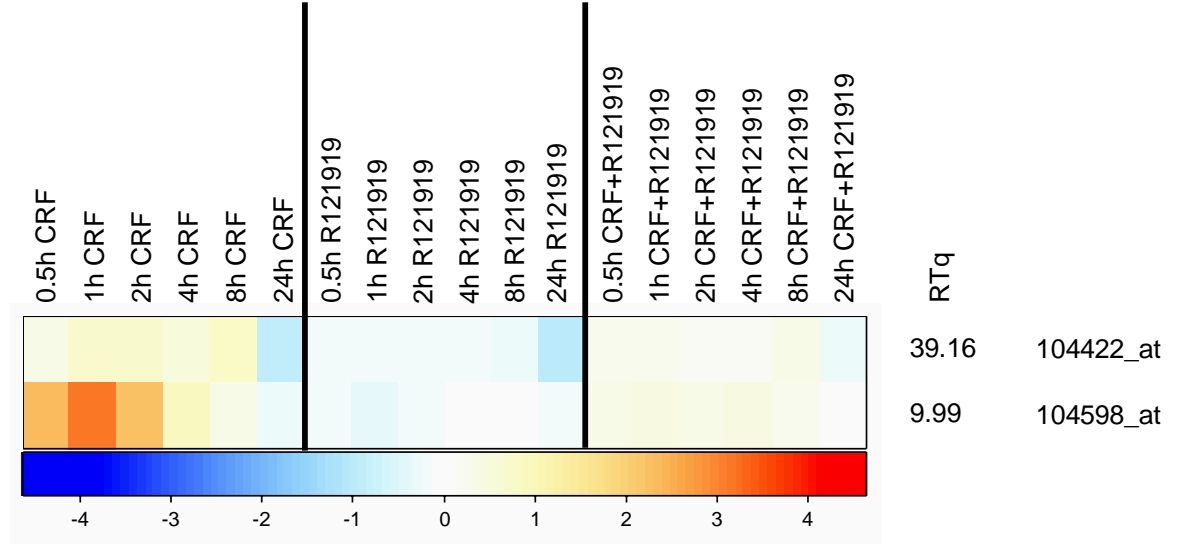
Receptor and channel regulators

receptor (calcitonin) activity modifying protein 3



Phosphatases

protein tyrosine phosphatase, receptor-type, N
 dual-specificity phosphatase 1, Dusp1



Unknowns

MGC:37790 IMAGE:5097591
 RIKEN cDNA 1300002F13 gene (MIG-6)
 tumor necrosis factor, alpha-induced protein 3

