Microarray Evaluation for Gene Regulation Analysis

Course Tutorial and Step by Step Example

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Contents

This tutorial was compiled by Dr. Martin Seifert.

Martin Seifert has a Ph.D in Biology. After spending some time at Brunel University (London) he accepted the position of head of the microarray facility at the Department of Cell Biology of the Technical University of Munich. In spring 2004 he joined Genomatix adding his expertise in DNA-microarray analysis to Genomatix training team.

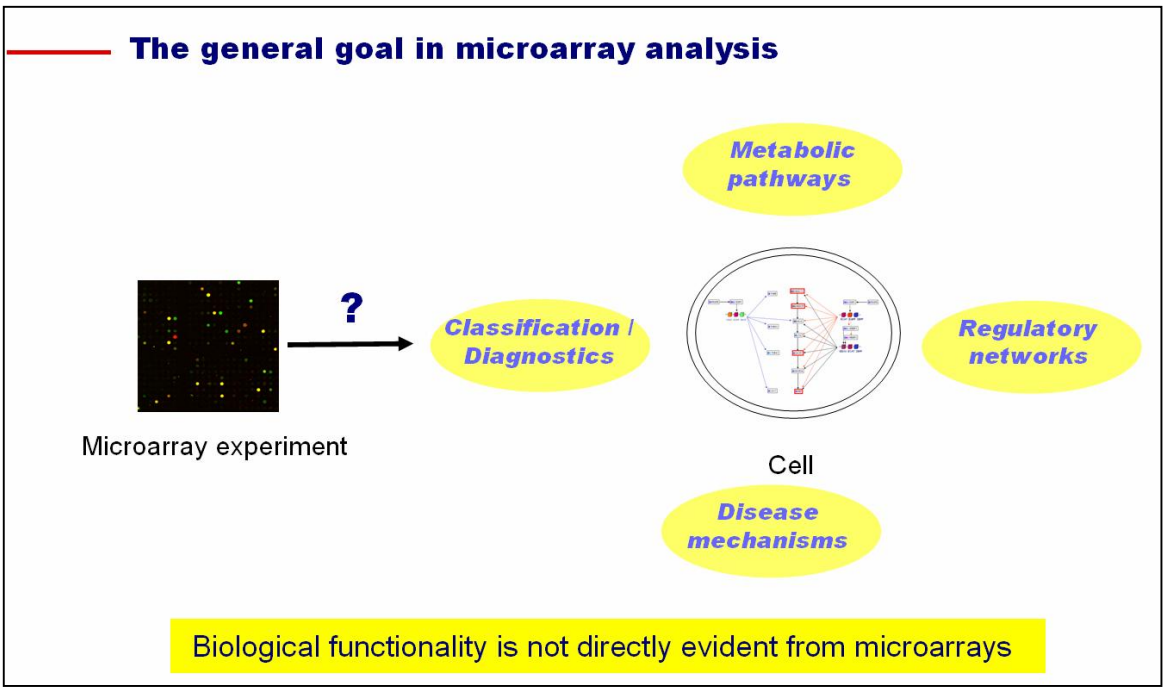
1. Introduction

Microarray mining is a challenging task because of the intrinsic superposition of several biological processes mirrored in the data. However, general aims of microarray analysis always comprise classification and diagnostics of samples, gaining insight into metabolic pathways and regulatory networks and finally learning more about disease mechanisms (Figure 1).

In this tutorial Genomatix presents a strategy for microarray mining based on the combination of statistical significance analysis of gene expression, literature-mining, and promoter analysis. The strategy is illustrated by a stepwise analysis of publicly available microarray data (a timeline experiment of PDGF-stimulated fibroblasts). The applied strategy reveals results beyond the detection of the major metabolic pathway known to be directly linked to the PDGF response: It is possible to identify cross-talking regulatory networks underlying the metabolic pathway without using a priori knowledge about the experiment. These regulatory networks involve the EGR1 factor, which is a known target of PDGF stimulation.

To reproduce the analysis all necessary data can be downloaded at:

http://www.genomatix.de/download/tutorial/



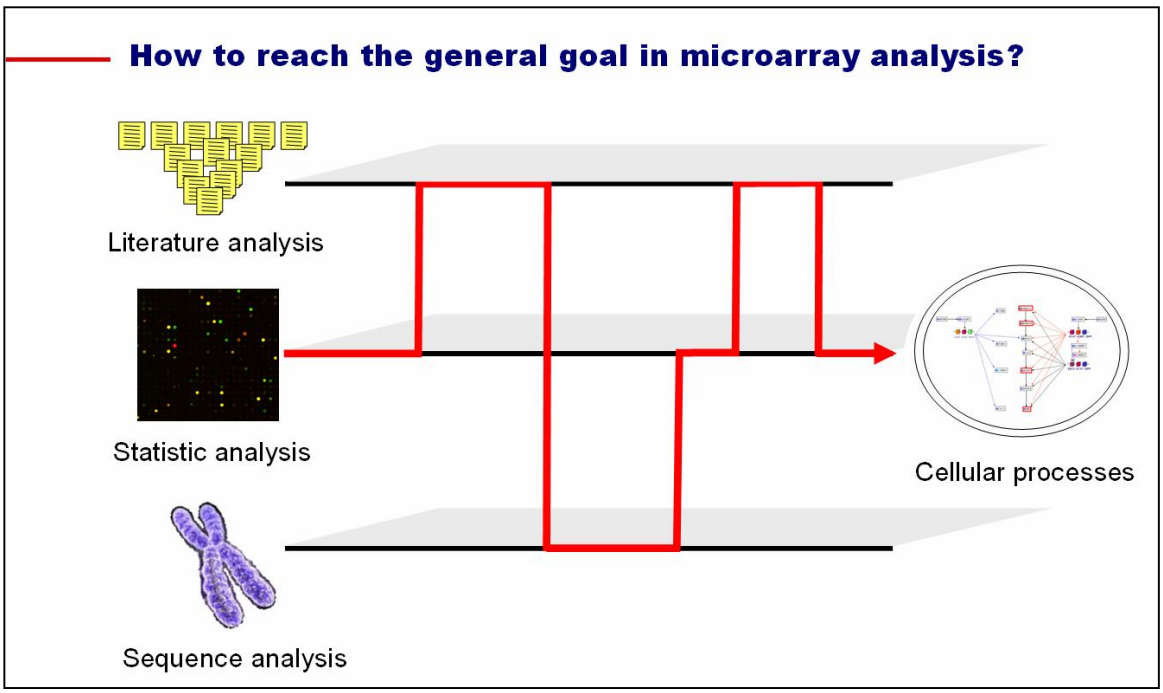
2. Theoretical Background

Microarray results reflect a multitude of simultaneous cellular processes although only subsets of expression changes are directly caused by the experimental conditions. Therefore, a major task for an in depth analysis is to identify genes whose expression changes due to the experimental setup and distinguish them from effects of biological diversity or general stress response of the cell.

In this tutorial we present a combinatorial strategy, which includes the analysis of gene promoters, for a biological evaluation of relationships between significantly regulated genes. The procedure is based on a combination of statistical-, literature- and promoter analysis and aims at establishing gene regulatory networks on molecular level.

No single method could solve the task for the following reasons:
Statistical analysis reveals mRNA with significantly changed expression levels but fails to assign these changes to biological events. Projecting microarray data onto pathway information from literature allows to associate genes with biological processes, but is restricted to current knowledge and fails to select those genes that are directly pertinent for the experimental conditions. Promoter analysis is capable of revealing targets of transcriptional coregulation but cannot discern molecular mechanisms of regulation directly from the initial microarray data.

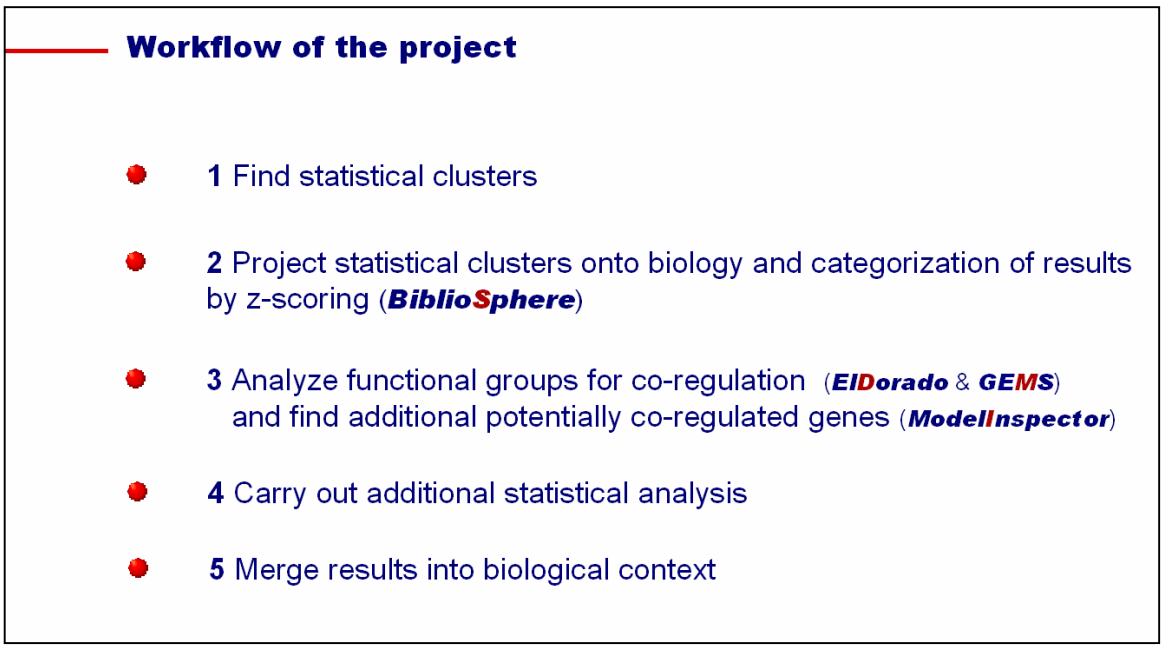
We use the principle of biological consistency and comprehensiveness to complete the picture by combination of these methods, which also allows for the integration of genes missed by individual methods.



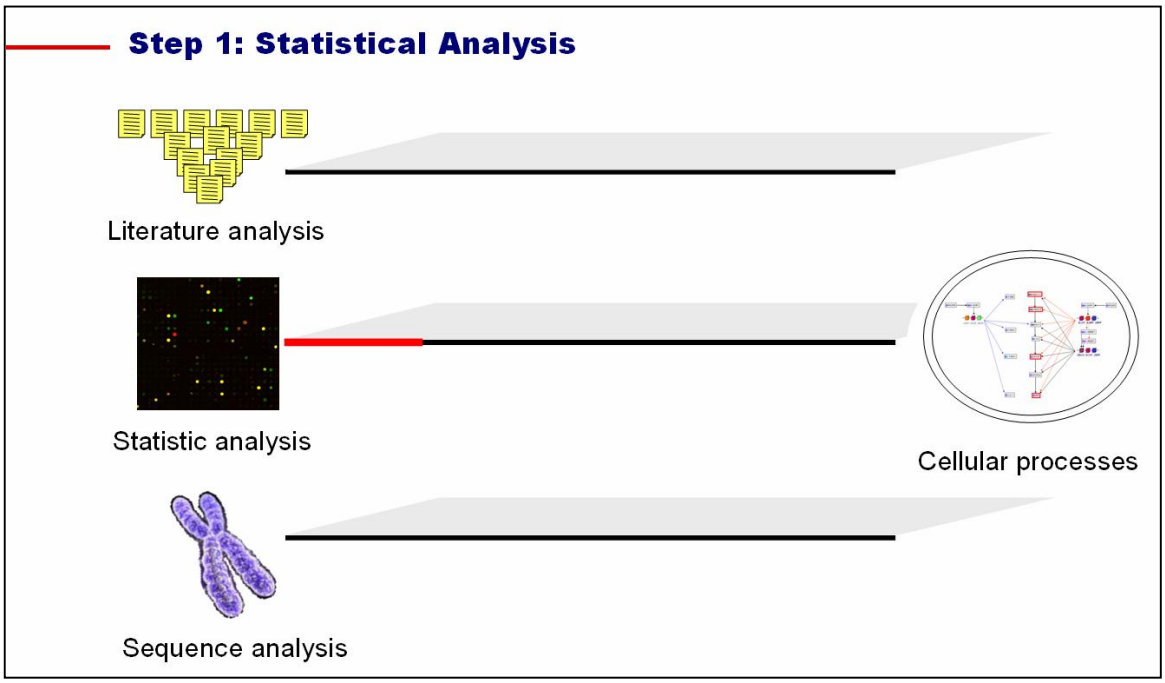
3. Practical Example: Evaluation of the Role of PDGF in Fibroblasts

To illustrate the strategy the tutorial shows the analysis of data from a study of platelet-derived-growth-factor (PDGF) stimulation of human fibroblast cells, carried out in a time series study using cDNA microarrays [1]. The data set can be retrieved from the Gene Expression Omnibus (GEO) database (dataset GSE1484). http://www.ncbi.nlm.nih.gov/geo/

The analysis follows the steps below:

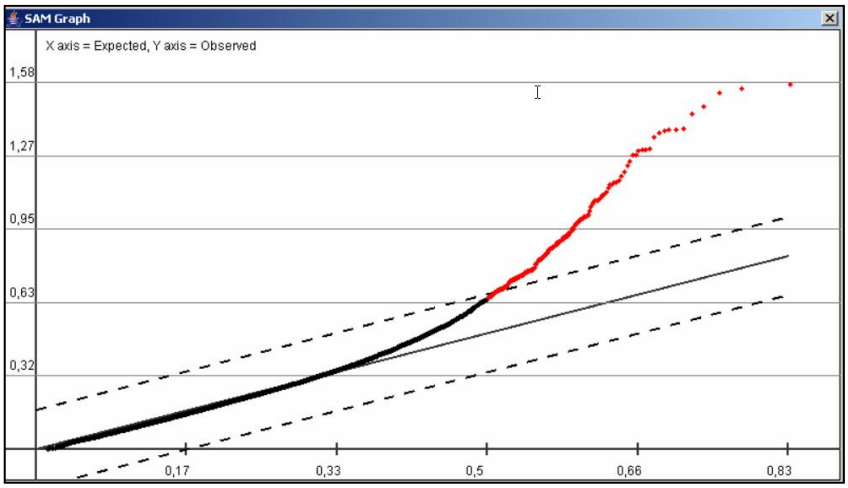


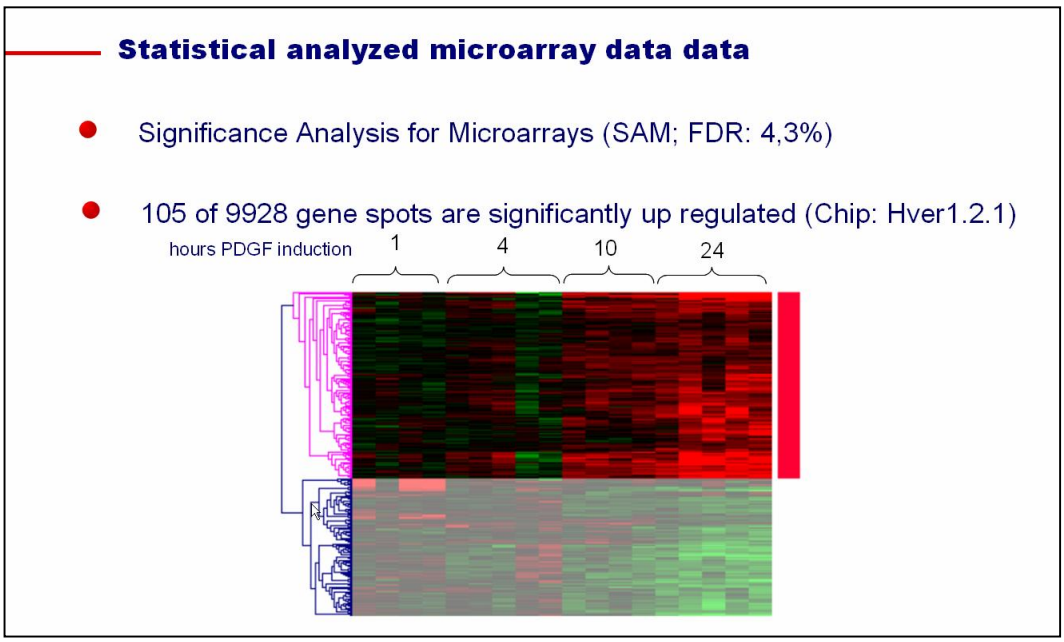
3.1. Step 1: Statistical Analysis



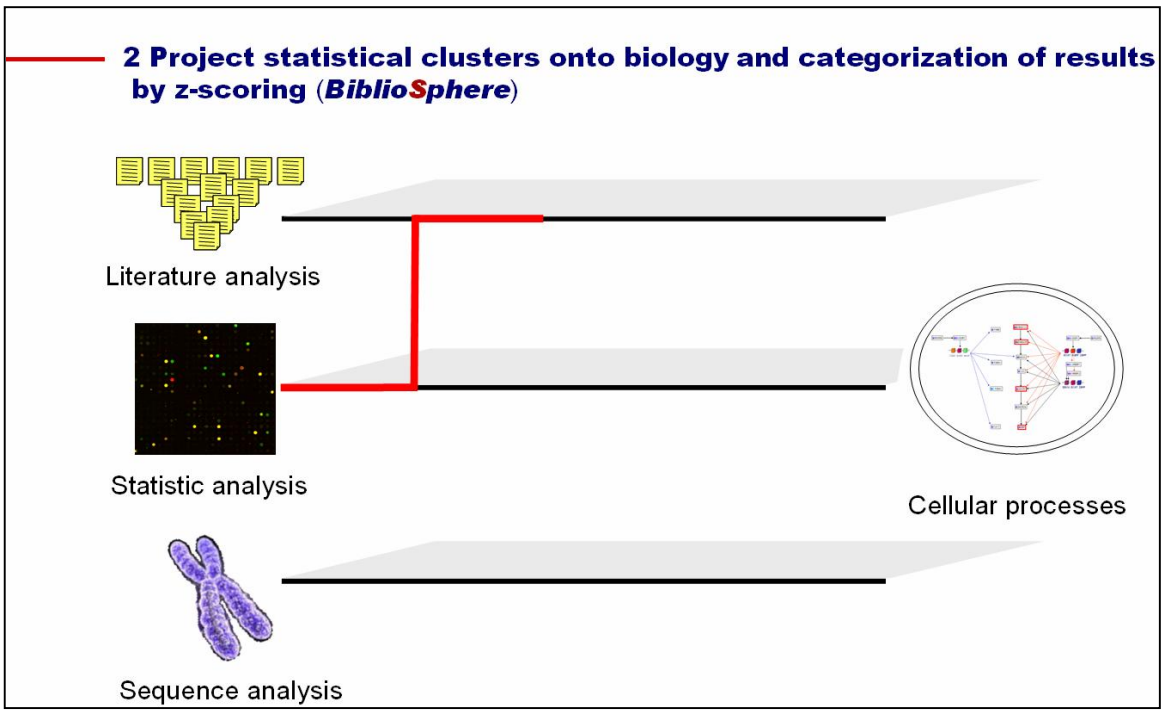
Microarray data were statistically analyzed using the "Multi Experiment Viewer - MeV included in the TM4 software package from TIGR (www.tigr.org): The Data was first normalized using total intensity normalization. Subsequently the SAM-algorithm [2] with a false discovery rate (FDR) below 5% was applied.

The resulting list contained 105 significant up regulated genes. This list of genes can be downloaded at http://www.genomatix.de/download/tutorial/PDGF_tutorial.xls for subsequent analysis to follow the strategy.





3.2. Step 2: Literature Analysis



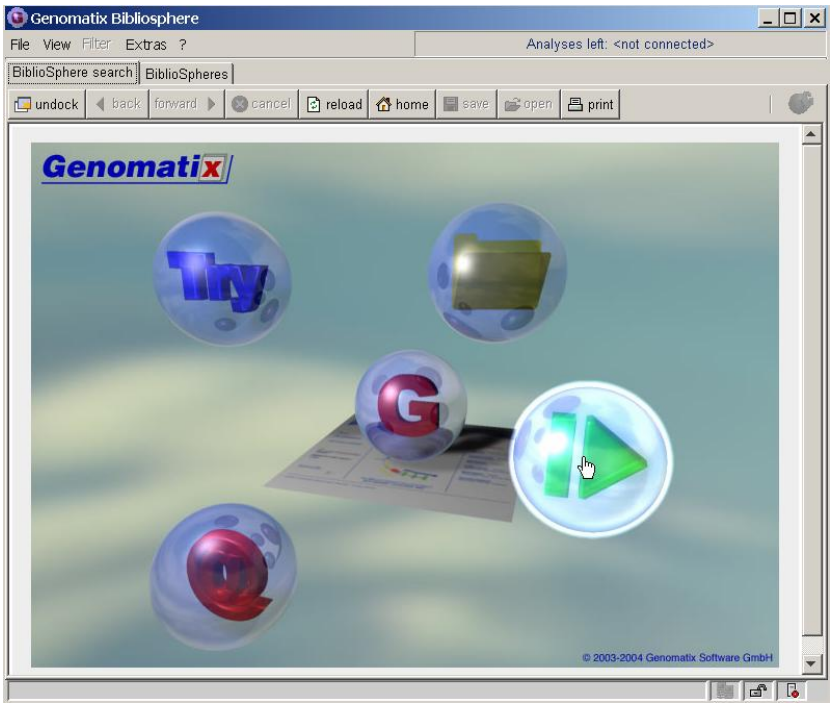
The 105 genes from step 1 are now used for a subsequent analysis using BiblioSphere.

BiblioSphere is a data-mining solution to extract and analyze gene relationships from literature databases and genome-wide promoter analysis. BiblioSphere contains literature data mining strategies using more than 350.000 quality checked gene names, synonyms and Genomatix proprietary semantic relation concepts. Based on PubMed, BiblioSphere currently searches over 12 million abstracts. The BiblioSphere program can be downloaded at:

http://www.genomatix.de/products/BiblioSphere/bibliosphere6.html

The aim of the analysis is to find whether there are functional sub-clusters within the 105 genes. And if there are transcription factors which are related with the genes.

BiblioSphere offers different entry possibilities: There is a single gene query to find the literature environment for single genes, a gene group query allows to find co-citations within a gene group plus the literature environment of the input genes. For this tutorial the third option “large cluster query” is applied. In the case of the tutorial we will first only have a look at the input genes and perform a functional filtering.



Step 2a: Functional Subcluster Analysis

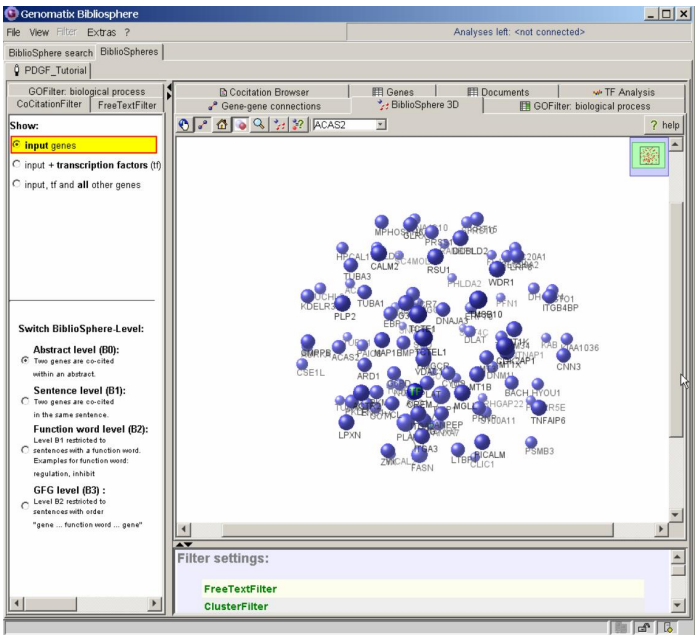
The list of 105 genes is submitted to BiblioSphere™ using the large cluster query option and the Gene Ontology filter “biological process” is applied to gain subgroups with the same GO annotation to find functionally correlated groups. This makes sense as e.g. most pathways belong to particular biological processes. Each resulting subgroup is scored by a z-score: The z-score for an item indicates how far and in what direction that item deviates from its distribution's mean. Z-scores are sometimes called "standard scores".

Box 1
To follow the strategy please take the 105 significantly up regulated genes and put them into the large cluster query input screen of BiblioSphere.

Use human as species.

After the query you will receive a 3D BiblioSphere view.

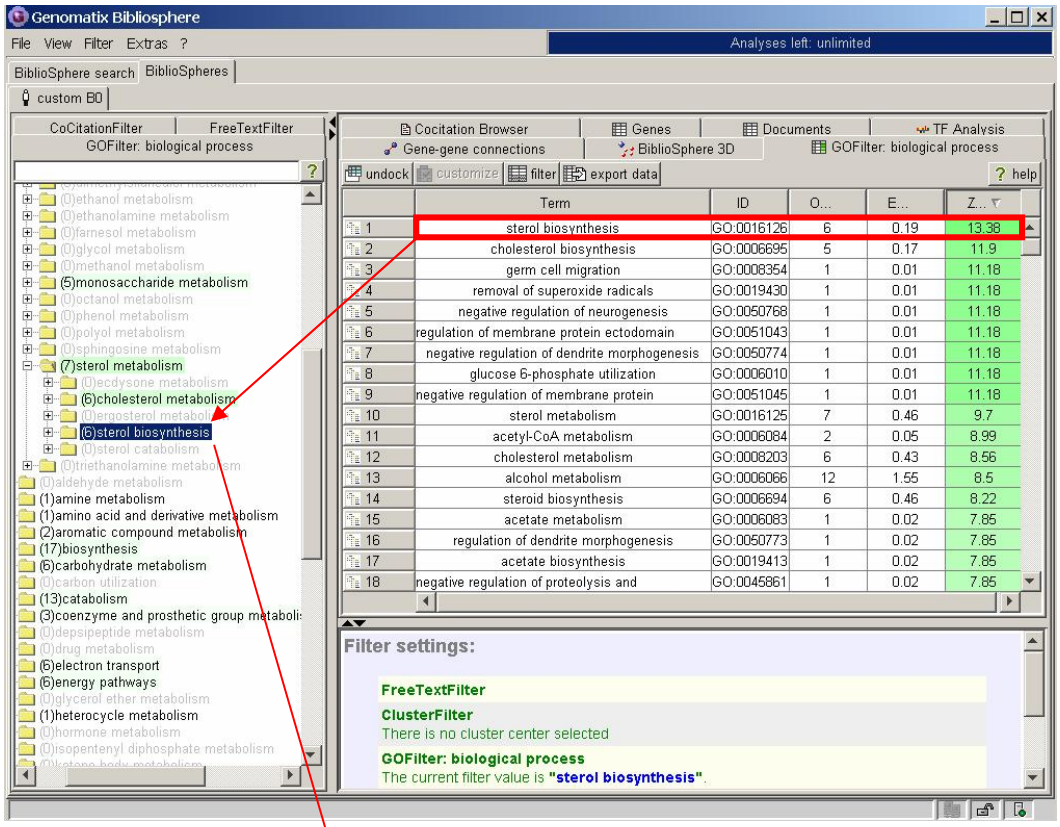
Please go to the “CoCitation Filter” in the left window and click “Show input genes”



Box2
Apply the biological process
filter from the filter pull down menu

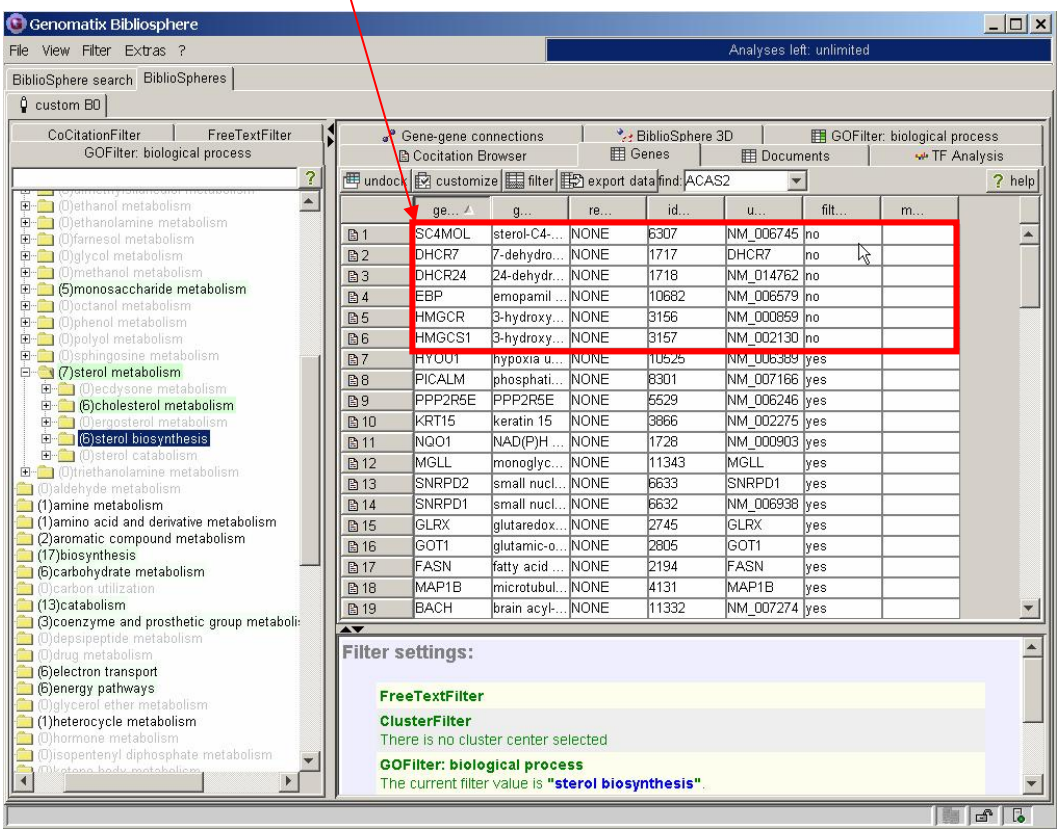
Go to the GOFilter: biological process table and sort for the highest z-score´by clicking on the column header

Click on the category with the highest z-score: sterol biosynthesis



Box 3
Go to the “Genes” table. All genes not filtered out are flagged “no” all genes filtered out are flagged “yes”.

Copy the genes flagged “no”



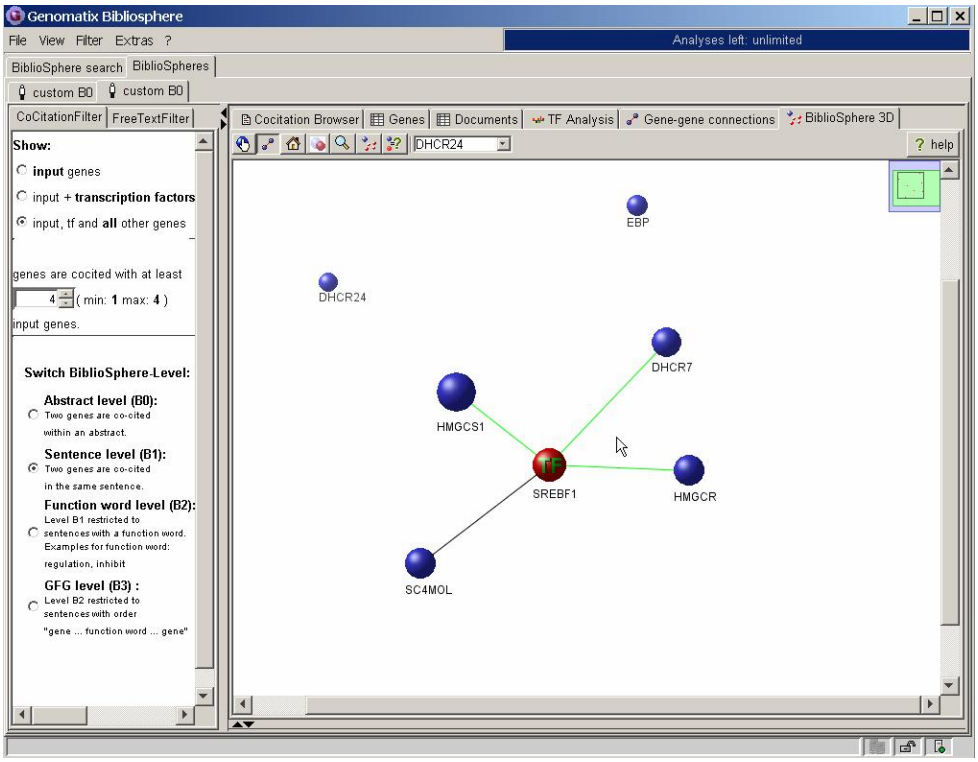
Result Using BiblioSphere genes can be categorized in different biological ontologies provided by GO or MeSH. Subgroups of genes derived from experimental results or by biological criteria are differentially associated with these categories. The degree of association is expressed by the z-score.
The top-scoring group in our experiment has a z-score of 13 and contains 6 genes, scored in the category "sterol biosynthesis". Thus, a purely data-driven approach correctly identifies a subgroup of genes known to be involved in PDGF response [1].

Step 2b Transcription Factor Analysis

Box 4
Paste the six genes from the category "sterol biosynthesis" into a new "gene group query" of BiblioSphere.

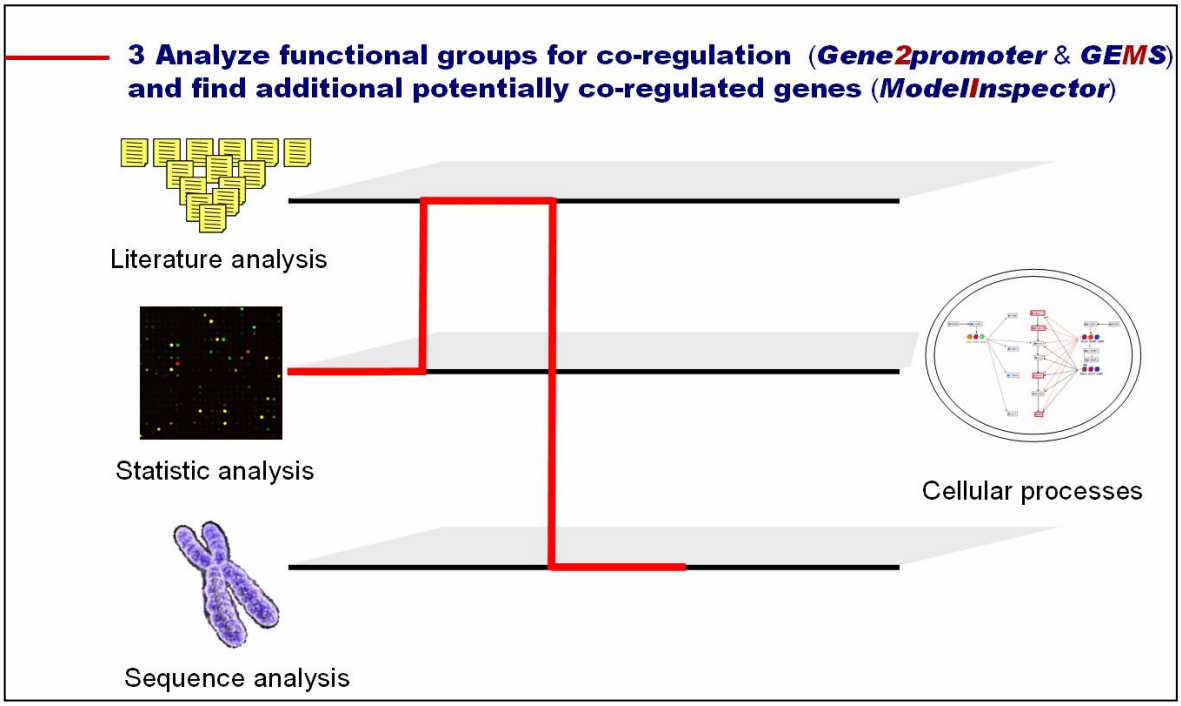
Have a look at "input genes and all other genes"

To make the search more stringent switch the BiblioSphere-CoCitation Level to "Sentence" and look for genes which are at least cocited with four of the six input genes.



The six genes from the "sterol biosynthesis" group (SC4MOL, HMGCS1, DHCR7, HMGCR, EBP, and DHCR24) are re-analyzed as a gene group and BiblioSphere is used for the identification of transcription factors which are cocited with as many of the genes of the "sterol biosynthesis" group as possible. This results in the identification of a single factor, SREBF1 (sterol regulatory element binding transcription factor 1), which is cocited with 4 input genes (SC4MOL, DHCR7, HMGCS1, HMGCR). Potential binding sites for SREBF1 (a member of the E-box family of transcription factors) can be verified on sequence level for three of the cocited genes (HMGCS1, HMGCR, and DHCR7; indicated by the green connecting lines). SREBF1 induction by PDGF had been already confirmed experimentally by Demoulin et al[1], supporting our independent finding from data analysis. In addition SREBF1 is known to be involved in the sterol-induction of HMGCS1 in hamster [3].

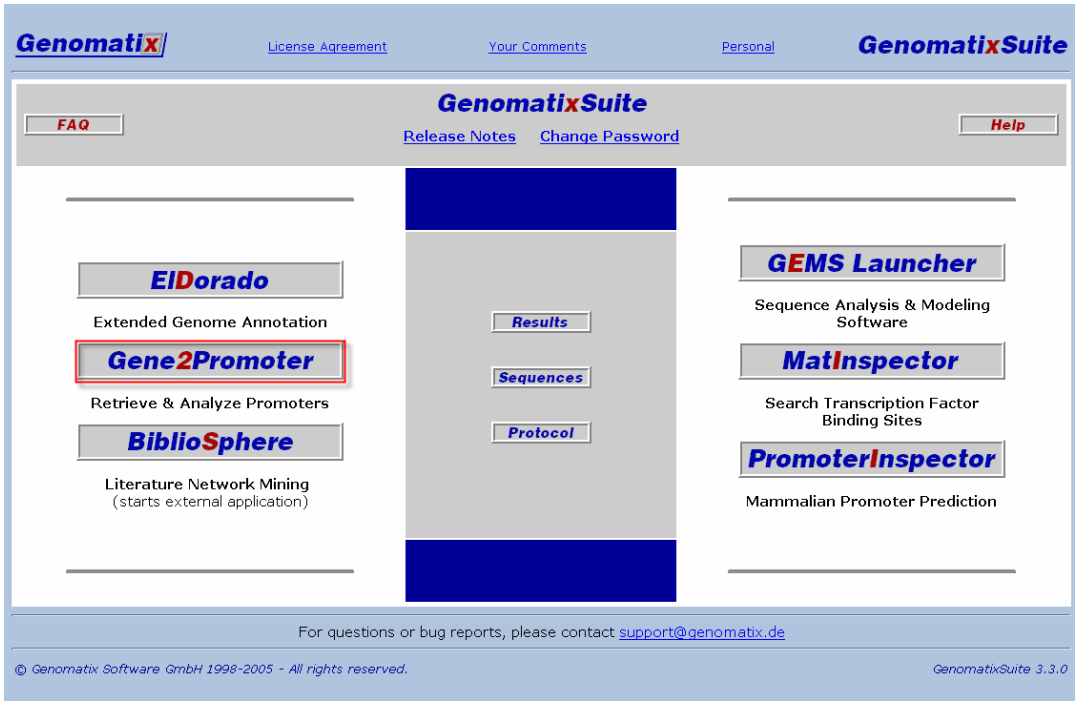
3.3 Step 3: Promoter Analysis

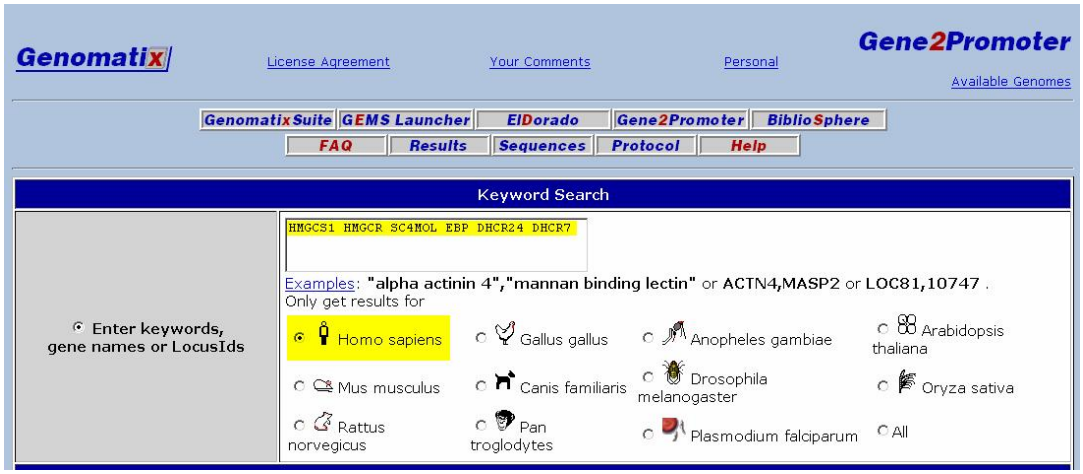


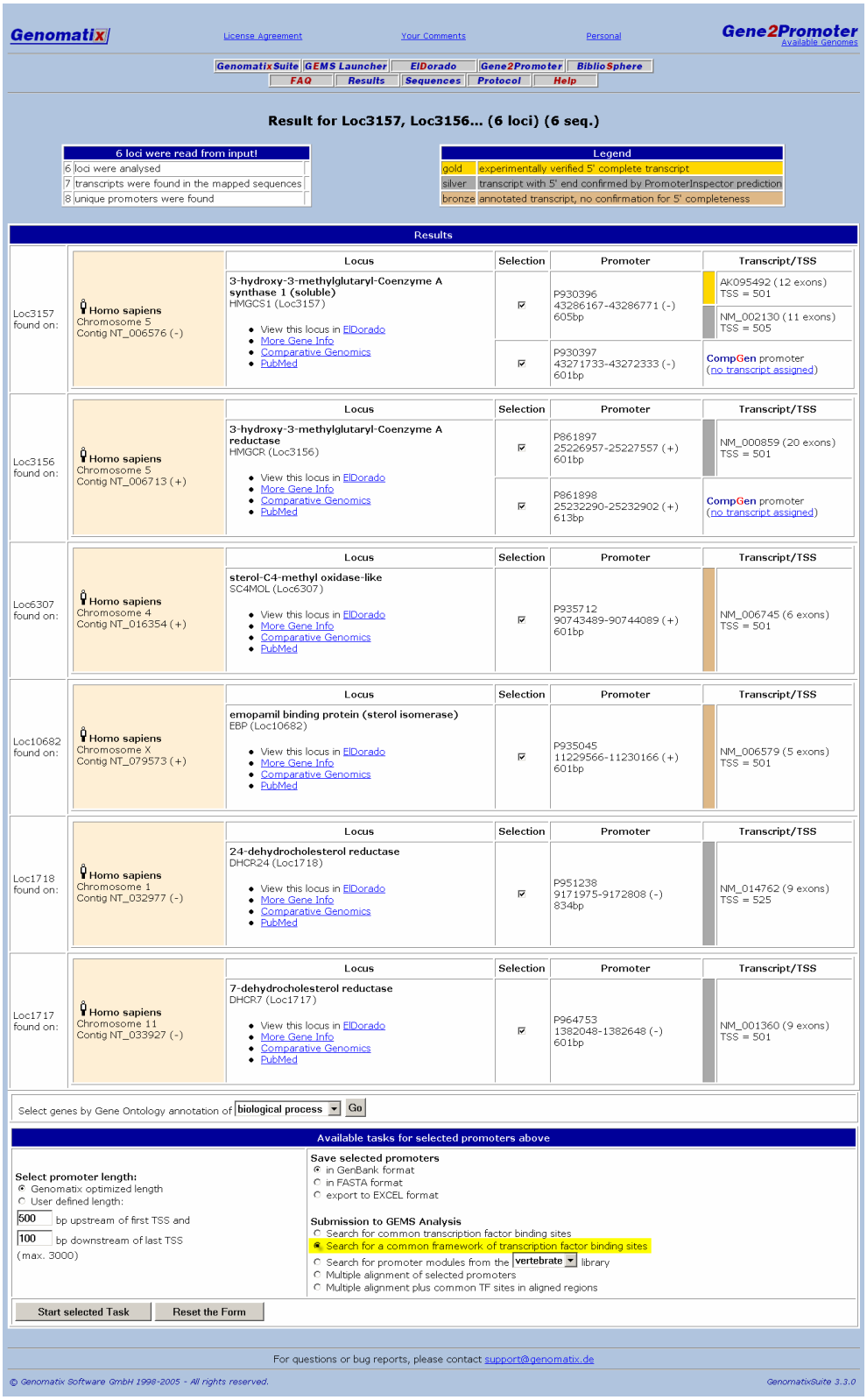
Coregulation of mammalian genes usually depends on sets of transcription factors rather than individual factors alone. Regulatory sequence elements are often organized into defined frameworks of two or more transcriptions factor binding sites and clusters of such motifs. The aim of the sequence analysis is to find such transcription factor motifs in regulated genes.

Therefore, the corresponding human promoter sequences for all six genes from step 2 are analyzed with the ElDorado™/Gene2Promoter system. ElDorado is a genome annotation database which includes promoter sequences at highest quality levels. ElDorado is based on a condensation of publicly available data plus Genomatix proprietary annotation, including promoters, transcription factor binding sites, promoter modules, scaffold/matrix attachment regions (S/MARs), and single nucleotides polymorphisms (SNPs) as well as comparative genomics. Gene2Promoter is a multiple identifier interface to query ElDorado.

Box 5
To follow the strategy, paste the six genes from the category “sterol biosynthesis” into the Gene2Promoter interface at the GenomatixSuite main page (http://www.genomatix.de/cgi-bin/eldorado/main.pl). You can either enter gene symbols or Locus IDs. This will allow you to retrieve and select the promoters for subsequent promoter analysis.

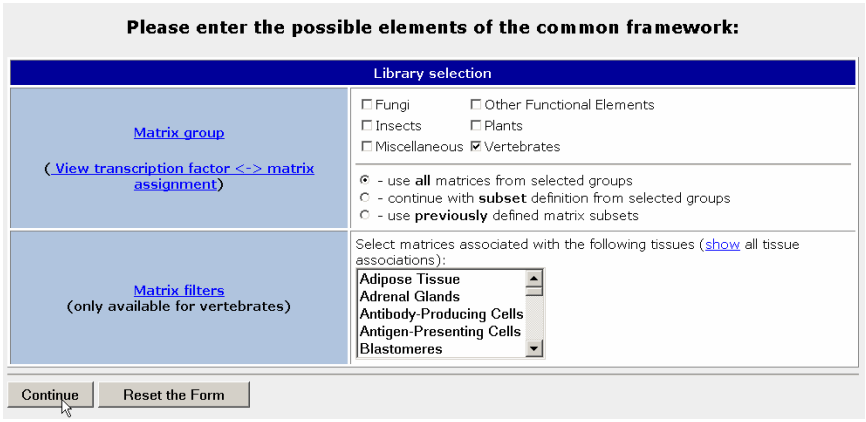




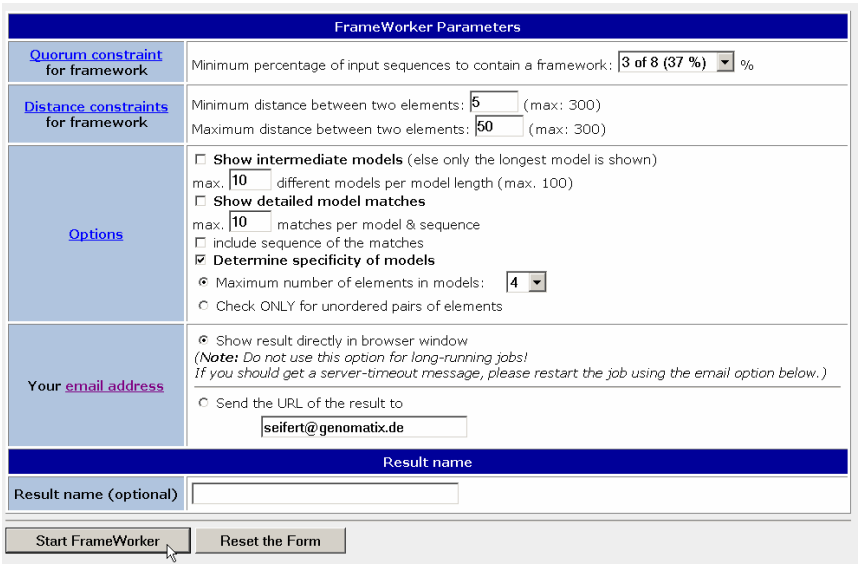


Box 6
Leave all promoters in the analysis as “Selected” and proceed with “Search for a common framework of transcription factor binding sites”. This will open the FrameWorker program of GEMS-Launcher.

Box 7
Use the “vertebrates” library and“ all matrices”.



Box 8
The following screen gives you several options for FrameWorker parameters. Change only the “quorum constraints” to 3 of 8 (37%) and check the checkbox “Determine specificity of models”. This is the most stringent setting giving a framework of at least three elements and will bring the most specific model to the top of the following results. Leave the other parameters at their default setting (you can of course play with the settings and see what happens)



Box 9
Scroll past the list of common elements to find the top-scoring framework. It consists of three elements: EBOX-ECAT-ZBPF. Please name the model and save it by clicking the “Save selected models” button at the bottom of the page.

Result Background and Explanation

For the analysis the program FrameWorker of the GEMS-Launcher package is used. GEMS Launcher is a software package for DNA analysis. GEMS includes software for transcription factor analysis, discovery of complex regulatory patterns and alignments.

FrameWorker is a software tool allowing the extraction of common motifs (frameworks) of transcription factor binding sites from a set of DNA sequences (in this case promoter sequences). The resulting top-scoring framework consists of three transcription factors: EBOX-ECAT-ZBPF.

The factor SREBF1, a member of the EBOX family was already identified from the BiblioSphere analysis. The framework was identified in the promoter of three of the six genes (HMGCS1, EBP, and DHCR7). Interestingly, while this yields further support for the binding sites already found by cocitations of SREBF1 with HMGCS1 and DHCR7, there had been no evidence for inclusion of EBP from the literature analysis.

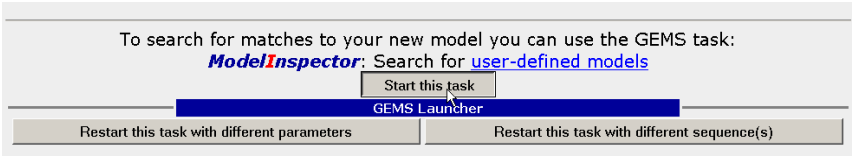
Promoter Database Scan

Subsequent to the definition of a framework, it is possible to scan DNA sequences for matches of such defined transcription factor motifs [4]. One comprehensive approach is to scan databases containing promoter sequences for matches. By this, it is possible to identify potential target genes of defined transcription factor motifs.

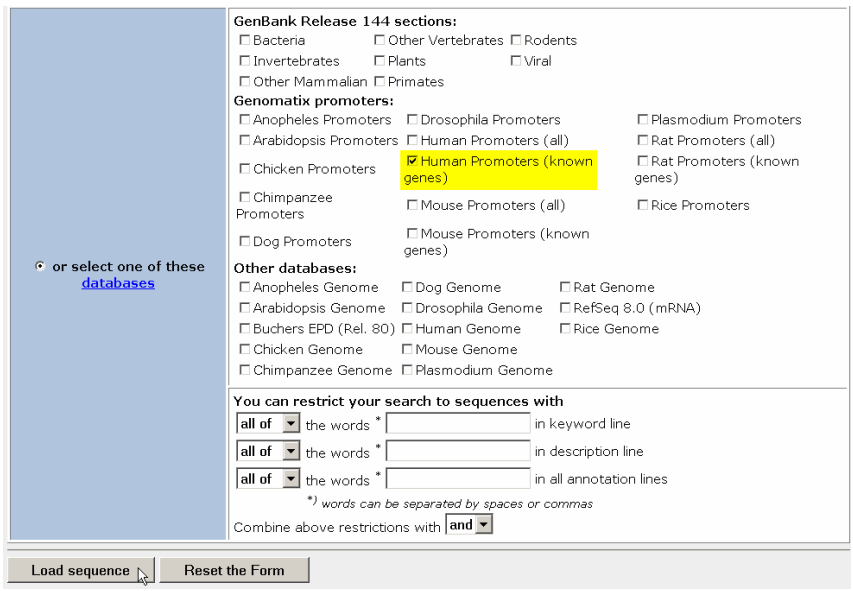
In order to find additional genes belonging to the emerging regulatory network governed by the identified framework, we can now scan all human promoters (Genomatix Promoter Database GPD, 50,109 promoters) with the task ModelInspector from GEMS Launcher.

ModelInspector uses a library of predefined models or models defined with FastM or FrameWorker to scan DNA sequences for matches to these models. A model consists of various individual elements (like transcription factor binding sites, repeats, hairpins), their strand orientation, their sequential order, and their distance ranges.

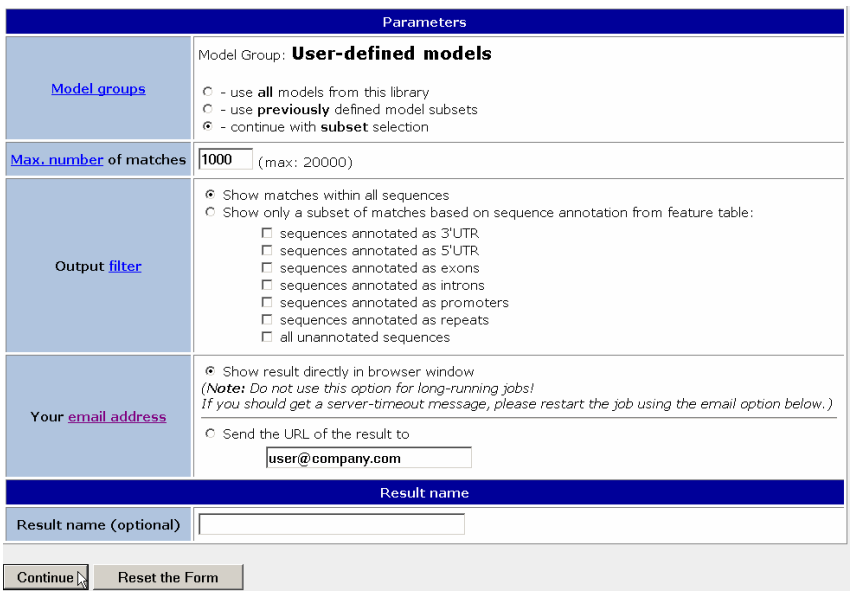
Box 10
To follow the strategy, from the last step, saving the model the program will take you directly to the ModelInspector task. By clicking the “Start this task button” the program to search the promoter databases will be launched.



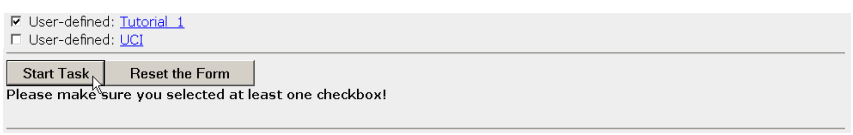
Box 11
In the database selection window please check “Human Promoters (known genes)” and proceed with “Load sequences”.



Box 12
In the following parameters screen leave the parameters default. Only change to “show results directly in browser window”



Box 13
Select the saved model on the next screen and “Start Task”



Box 14
The ModelInspector search returns with a Match List giving detailed information about the position of matches in promoters from the searched promoter database (in this case all known human promoters). For the searched model 10 matches are retrieved.

Match List:

Sequence	Model Name	Position	Strand	Model Score	Select Match
P501173 (1 - 601) [DNA] sym=STX6|loc=Loc10228|taxid=9606|spec=Homo sapiens| ctg=NT_004487|str=(-)|start=31400819|end=31401419|len=601| comm=syntaxin 6;(NM_005819/501/silver)	Tutorial_1	326 - 410	(+)	96.9 %	☐
P583520 (1 - 605) [DNA] sym=HMGCS1|loc=Loc3157|taxid=9606|spec=Homo sapiens| ctg=NT_006576|str=(-)|start=43286167|end=43286771|len=605| comm=3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble); (AK095492_1/501/gold;NM_002130/505/silver)	Tutorial_1	297 - 379	(+)	95.2 %	☐
P529035 (1 - 601) [DNA] sym=IDH2|loc=Loc3418|taxid=9606|spec=Homo sapiens| ctg=NT_010274|str=(-)|start=5611135|end=5611735|len=601| comm=isocitrate dehydrogenase 2 (NADP+), mitochondrial; (NM_002168/501/bronze)	Tutorial_1	487 - 408	(-)	90.2 %	☐
P534499 (1 - 601) [DNA] sym=NEDD4L|loc=Loc23327|taxid=9606|spec=Homo sapiens| ctg=NT_025028|str=(+)|start=3502144|end=3502744|len=601| comm=neural precursor cell expressed, developmentally down-regulated 4-like;(NM_015277/501/silver)	Tutorial_1	46 - 114	(+)	94.8 %	☐
P615672 (1 - 601) [DNA] sym=DHCR7|loc=Loc1717|taxid=9606|spec=Homo sapiens| ctg=NT_033927|str=(-)|start=1382048|end=1382648|len=601| comm=7-dehydrocholesterol reductase;(NM_001360/501/silver)	Tutorial_1	368 - 454	(+)	94.8 %	☐
P310311 (1 - 630) [DNA] sym=BOP1|loc=Loc23246|taxid=9606|spec=Homo sapiens| ctg=NT_037704|str=(-)|start=82403|end=83032|len=630| comm=block of proliferation 1;(AK024840_1/530/gold;NM_015201/501/silver)	Tutorial_1	518 - 442	(-)	94.8 %	☐
P583533 (1 - 676) [DNA] sym=HSF1|loc=Loc3297|taxid=9606|spec=Homo sapiens| ctg=NT_037704|str=(+)|start=82130|end=82805|len=676| comm=heat shock transcription factor 1;(NM_005526/563/silver)	Tutorial_1	386 - 462	(+)	94.8 %	☐
P588169 (1 - 601) [DNA] sym=EBP|loc=Loc10682|taxid=9606|spec=Homo sapiens| ctg=NT_079573|str=(+)|start=11229566|end=11230166|len=601| comm=emopamil binding protein (sterol isomerase); (NM_006579/501/bronze)	Tutorial_1	326 - 391	(+)	93.4 %	☐
P501174 (1 - 650) [DNA] sym=STX6|loc=Loc10228|taxid=9606|spec=Homo sapiens| ctg=NT_004487|str=(-)|start=31400834|end=31401483|len=650| comm=syntaxin 6;(CompGen promoter, no transcript assigned)	Tutorial_1	390 - 474	(+)	96.9 %	☐
P613030 (1 - 601) [DNA] sym=LMNA|loc=Loc4000|taxid=9606|spec=Homo sapiens| ctg=NT_004487|str=(+)|start=6596006|end=6596606|len=601| comm=lamin A/C;(CompGen promoter, no transcript assigned)	Tutorial_1	97 - 21	(-)	91.3 %	☐

A total of 10 matches was found in 10 sequences.

Sequences searched: 36776 (23930905 bp).

Evaluation of results

Model: Tutorial_1

Number of input genes: 9
Number of genes annotated in GO: 9
Number of significant GO groups found: 9

GO group	z-score	# genes (observed)	# genes (expected)	list of genes	Locus IDs
steroid metabolism	9.28	3	0.10	HMGCS1, DHCR7, EBP	3157, 1717, 10682
lipid biosynthesis	8.24	3	0.12	HMGCS1, DHCR7, EBP	3157, 1717, 10682
sterol metabolism	13.55	3	0.05	HMGCS1, DHCR7, EBP	3157, 1717, 10682
sterol biosynthesis	21.22	3	0.02	HMGCS1, DHCR7, EBP	3157, 1717, 10682
cholesterol metabolism	14.05	3	0.04	HMGCS1, DHCR7, EBP	3157, 1717, 10682
lipid metabolism	4.45	3	0.37	HMGCS1, DHCR7, EBP	3157, 1717, 10682
alcohol metabolism	7.15	3	0.16	HMGCS1, DHCR7, EBP	3157, 1717, 10682
steroid biosynthesis	13.55	3	0.05	HMGCS1, DHCR7, EBP	3157, 1717, 10682
cholesterol biosynthesis	22.71	3	0.02	HMGCS1, DHCR7, EBP	3157, 1717, 10682

Box 15
At the bottom of the results page an evaluation of results is carried out for the matched genes by z-scoring. I.e. a z-score is calculated by grouping the matched genes into GeneOntology groups applying the “biological process filter”. Only highly significant groups are displayed (z-score > 4.0)

Extract Sequences

Which matches?	○ selected matches in above list ◉ all matches
Extent of sequences	○ complete sequence ◉ match positions ± 50 bp
Output file	suite_Seifert.ext

Extract sequence of matches

Extract LocusIDs for BiblioSphere | Export matches to EXCEL format

Further Evaluation of Matches

Search PubMed for ("gene name") AND ("promoter" OR "transcription factor")
(where "gene name" is automatically extracted from the description lines)
Note: This works only for annotated genomic DNA sequences from eukaryotes!

Extract gene names

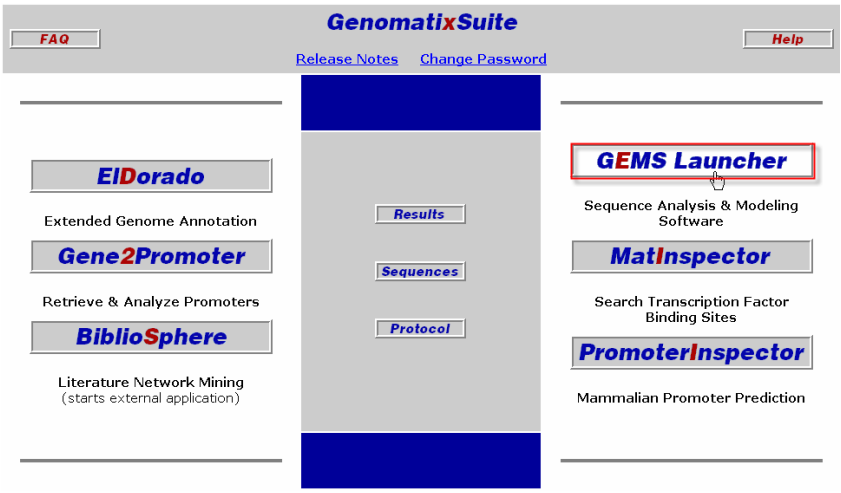
For questions or bug reports, please contact support@genomatix.de

 GEMS Launcher 4.0.2

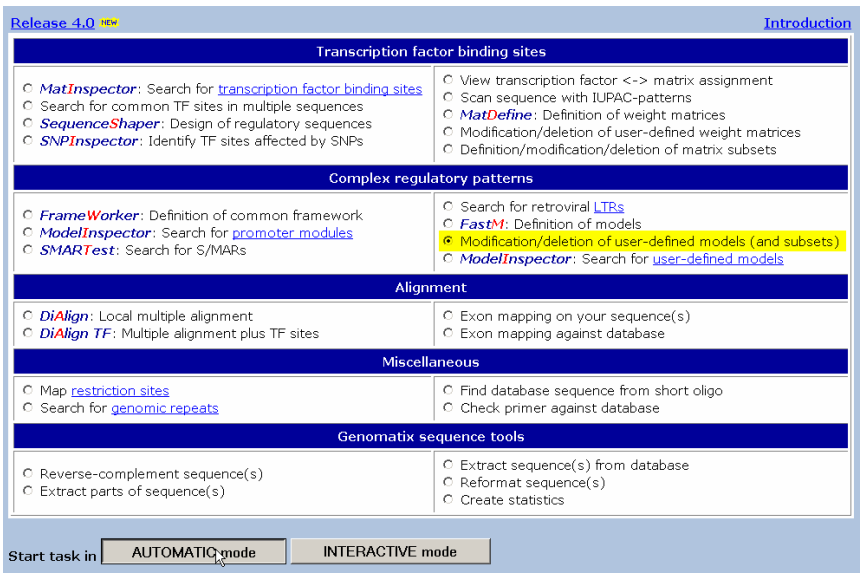
Result Evaluation 1

The matchlist contains 10 matches for the framework (this is rather specific). Regarding the original functional focus, only the genes which were used in FrameWorker for the construction of the framework were retrieved. Since the distance variability between the single transcription factors influences the selectivity, the framework can be altered to be less selective by increasing the distance variability. This is carried out with the Gems-Launcher task "FastM "Modification/deletion of user-defined models (and subsets)"

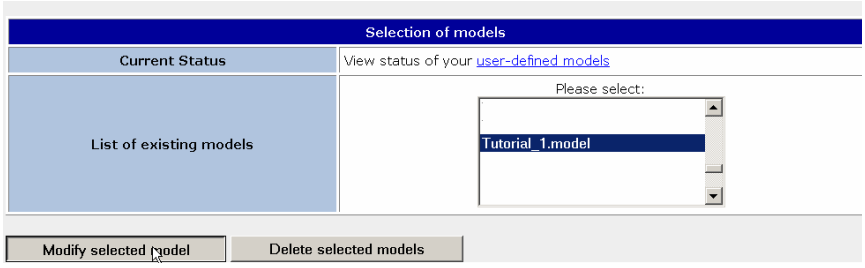
Box 16
To follow the strategy, start GEMS Launcher from the GenomatixSuite main page



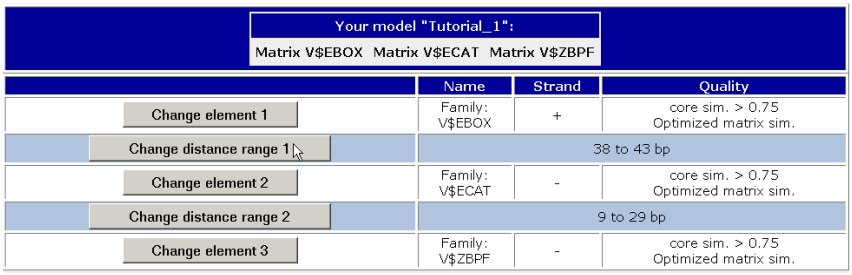
Box 17
Check "Modification/deletion of user-defined models (and subsets)" and start the program in "AUTOMATIC mode"



Box 18
Select the model you want to modify from the "List of existing models" and click "Modify selected model"

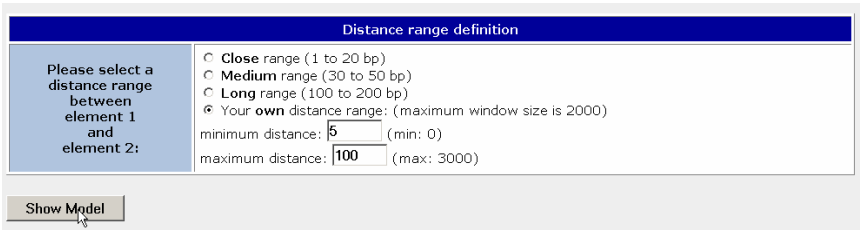


Box 19
Click on “Change distance range 1”.

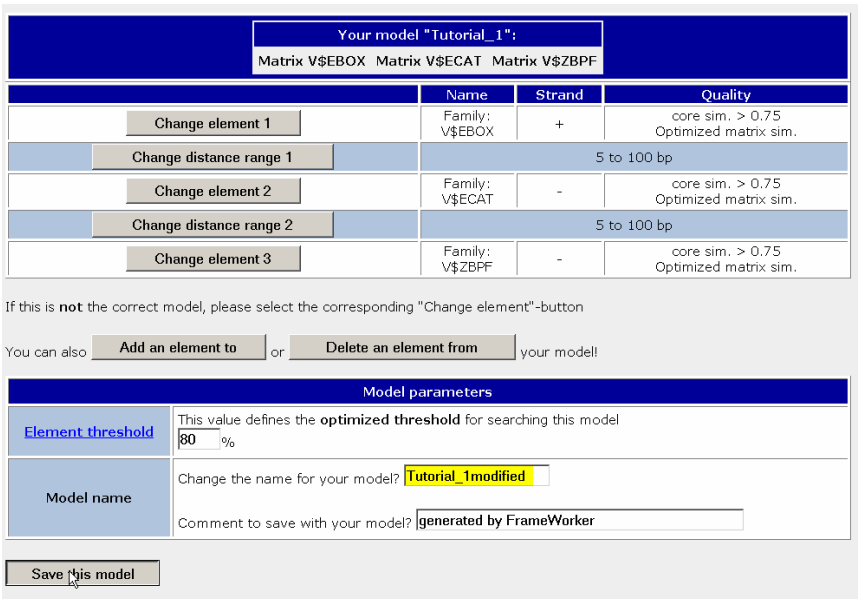


Box 20
Change distance range to “minimum distance” 5 and “maximum distance” 100. Finish with “Show model”

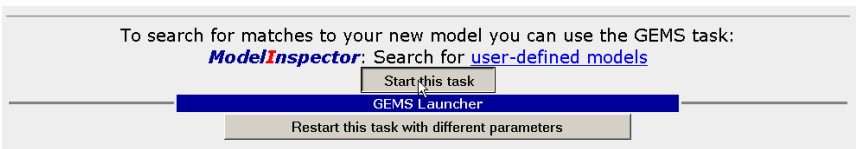
“Change distance range 2” in the same way (also use 5-100)



Box 21
“Change the name of your model” and save



Box 22
Perform the ModelInspector search with the modified model as described on page 13. (Be careful to select the modified model from the list)



Box 23
ModelInspector results.
(truncated list)

Output overview of ModelInspector matches (389 matches)

go to:[Output overview] [Detailed output] [Statistics]

ModelInspector professional Release 5.1.1 October 2004

Solution parameters:

Sequence file:	Human Promoters (known genes)
Models:	User-defined/Tutorial_1_mod.model
Model score threshold:	80.0 % (abs. 2.40)
Output sorted by:	match positions on the sequences
Maximum number of matches:	1000

Match List:

Sequence	Model Name	Position	Strand	Model Score	Select Match
P537935 (1 - 601) [DNA] sym=ARHGEF16|loc=Loc27237|taxid=9606|spec=Homo sapiens| ctg=NT_004321|str=(+) |start=686397|end=686997|len=601| comm=Rho guanine exchange factor (GEF) 16;(NM_014448/501/bronze)	Tutorial_1_mod	527 - 403	(-)	87.6 %	☐
P589365 (1 - 1073) [DNA] sym=TDRKH|loc=Loc11022|taxid=9606|spec=Homo sapiens| ctg=NT_004487|str=(-) |start=2253218|end=2254290|len=1073| comm=tudor and KH domain containing; (AK056402_1/972/gold;NM_006862/938/bronze)	Tutorial_1_mod	820 - 936	(+)	94.6 %	☐

Box 24
The Evaluation of results reveals several sterol metabolism associated categories. These categories include additional genes: LSS, MVK, SC5DL and SREBF2

GO group	z-score	# genes (observed)	# genes (expected)	list of genes	Locus IDs
tRNA aminoacylation for protein translation	4.68	4	0.55	DARS, RARSL, C20orf27, FARS1	1615, 57038, 54976, 10667
proline metabolism	5.86	2	0.11	PYCR2, PYCR1	29920, 5831
tricarboxylic acid cycle	4.43	3	0.36	FH, MDH2, IDH2	2271, 4191, 3418
tRNA aminoacylation	4.68	4	0.55	DARS, RARSL, C20orf27, FARS1	1615, 57038, 54976, 10667
cholesterol biosynthesis	5.37	4	0.45	HMGCS1, MVK, DHCR7, EBP	3157, 4598, 1717, 10682
porphyrin biosynthesis	4.43	3	0.36	ALAD, NFE2L1, FECH	210, 4779, 2235
amino acid activation	4.68	4	0.55	DARS, RARSL, C20orf27, FARS1	1615, 57038, 54976, 10667
sterol biosynthesis	6.35	5	0.51	HMGCS1, MVK, SC5DL, DHCR7, EBP	3157, 4598, 6309, 1717, 10682
response to metal ion	4.84	2	0.15	NEDD4L, MTF1	23327, 4520
response to inorganic substance	4.48	2	0.17	NEDD4L, MTF1	23327, 4520
heme biosynthesis	5.49	3	0.26	ALAD, NFE2L1, FECH	210, 4779, 2235
nucleobase, nucleoside, nucleotide and nucleic acid metabolism	4.52	73	45.86	MYOG, POU3F1, DARS, GBX2, SOX11, PTMA, ORC3L, RARSL, CREBL1, CPSF4, FLJ23311, ARNTL, ASCL2, FLJ21415, FANCA, NEUROD2, PPP1R9B, NFE2L1, ATP5G1, HOXB13, JJAZ1, TGIF, SMAD2, RUVBL2, NPAS1, ERF, ZNF540, ZSCAN1, ZNF543, FLJ14981, MBD3, POLRMT, FLJ14009, C20orf27, SUHW1, SREBF2, ECGF1, SCML1, ARX, H2AFZ, EXOSC9, HNRPDL, TRIM33, DNMT3A, ELL2, DPYSL2, GDA, POLE, HMGB1, CBX8, HEY2, ADCY4, HIF1A, YY1, ATXN3, CHX10, E2F1, HOXC13, ZNF295, JUN, POLD4, FARS1, BARHL1, BOP1, ZNF34, HSF1, COPEB, ELK1, MTF1, POLD2, LDHD, SP2, AMPD2	4656, 5453, 1615, 2637, 6664, 5757, 23595, 57038, 1388, 10898, 79733, 406, 430, 79794, 2175, 4761, 84687, 4779, 516, 10481, 23512, 7050, 4087, 10856, 4861, 2077, 163255, 284312, 125919, 84954, 53615, 5442, 79816, 54976, 129025, 6721, 1890, 6322, 170302, 3015, 5393, 9987, 51592, 1788, 22936, 1808, 9615, 5426, 3146, 57332, 23493, 196883, 3091, 7528, 4287, 338917, 1869, 3229, 49854, 3725, 57804, 10667, 56751, 23246, 80778, 3297, 1316, 2002, 4520, 5425, 197257, 6668, 271
growth hormone secretion	9.58	2	0.04	GAL, LTBP4	51083, 8425
tRNA modification	4.25	4	0.64	DARS, RARSL, C20orf27, FARS1	1615, 57038, 54976, 10667
heme metabolism	4.79	3	0.32	ALAD, NFE2L1, FECH	210, 4779, 2235
sterol metabolism	4.34	6	1.24	HMGCS1, MVK, SREBF2, SC5DL, DHCR7, EBP	3157, 4598, 6721, 6309, 1717, 10682
protein complex assembly, multichaperone pathway	5.29	2	0.13	RUVBL2, SURF1	10856, 6834
peptide hormone secretion	5.86	2	0.11	GAL, LTBP4	51083, 8425
response to unfolded protein	4.47	5	0.87	CREBL1, DNAJA1, HERPUD1, TRA1, HSF1	1388, 3301, 9709, 7184, 3297
proline biosynthesis	7.74	2	0.06	PYCR2, PYCR1	29920, 5831
glutamine biosynthesis	6.63	2	0.09	PYCR2, PYCR1	29920, 5831
steroid biosynthesis	4.34	6	1.24	HMGCS1, MVK, LSS, SC5DL, DHCR7, EBP	3157, 4598, 4047, 6309, 1717, 10682

Result Evaluation 2

Within the total list of 389 hits in 375 promoters, four categories related to "steroid metabolism" were overrepresented (z-scores ranging from 4.43 - 6.35). In addition to the three genes used to define the framework, LSS, MVK, SC5DL and SREBF2 were identified. LSS is the lanosterol synthetase, which belongs to the same metabolic pathway (synthesis of cholesterol) as the three initial framework genes. LSS is present and up-regulated on the microarray. However, the gene failed to pass the statistical test with SAM and was selected only on molecular evidence. For SREBF2 and MVK the situation is the same as for LSS: they are on the chip and up-regulated but not statistically significant. SREBF2 is the "sterol regulatory element binding transcription factor 2". Interestingly SREBF2 is an EBOX factor. MVK (mevalonate kinase) is an enzyme also belonging to the cholesterol synthesis chain. SC5DL is the sterol-C5-desaturase which also belongs to sterol metabolism (ergosterol). SC5DL is not on the chip therefore no information about expression change can be retrieved.

In total we have now identified seven genes belonging to the sterol metabolism to be co-regulated by the EBOX-ECAT-ZBPF framework!

Reiteration of the Framework Analysis

To see whether matches for additional frameworks can be retrieved, the complete group of 10 genes (6 from the literature analysis, four additional from the database search, (categories related to "steroid metabolism") are subjected to an additional FrameWorker analysis. The ten genes are:

HMGCS1, MVK, SC5DL, DHCR7, EBP, SREBF2, LSS, HMGCR, SC4MOL, DHCR24

Box 24

To follow the strategy enter the 10 genes identified by BiblioSphere and sequence analysis into Gene2promoter as described in Box 5 and Box 6. Proceed as described in Box 7 and Box 8.
Set the "Quorum constrains" to at least 8/19. Set the distance constraints to 5-100 bp.
Start the analysis.

Box 25

Frameworker will display this error message. This is due to an overlap in promoter sequences in the sequence pairs. Please go back to the Gene2promoter results window (hit your browser's "Back" button three times) and uncheck one sequence each for the overlapping promoters, e.g. P920801 and P936407. Then restart the framework analysis. For "Quorum constraints" now use 8/17, for "Distance constraints" 5-100.

```
Sorry, but the GEMS Launcher task
" FrameWorker: Definition of common framework"
returned with the following error:

>
> Sorry, but the sequence pair(s)
>
> P920801 & P920799
> P936407 & P936405
>
> are identical in at least 50 percent of the shorter sequence.
> Please remove one sequence of each pair from your input and start FrameWorker again!
>
```

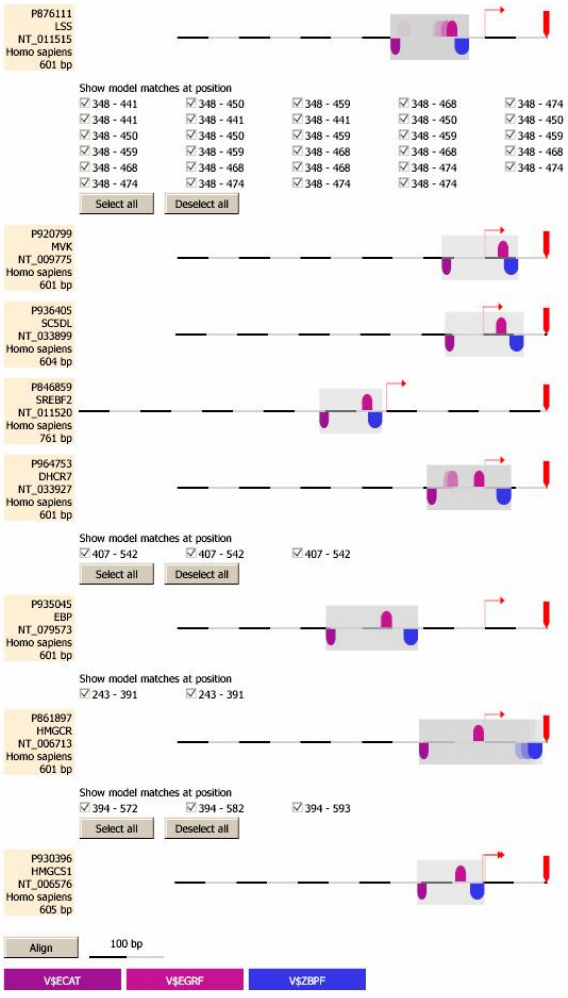
Box 26
You will receive a FrameWorker result as displayed. Please save the model and proceed with a Modelinspector search as described in Box 10-13.

Model "model_3el_1":

Save this model as Tutorial_2

	Element	Strand	Matrix sim.	Distance to next element	Common to	FW-Scores
1	V$ECAT	-	Optimized (min. 0.95)	10 - 92 bp	8 sequences (47 %) 41 matches, 8 non-overlapping	0.20 / 1.00 2.40701e-13
2	V$EGRF	+	Optimized (min. 0.77)	6 - 98 bp		
3	V$ZBPF	-	Optimized (min. 0.74)	---		

Graphical output:



Sequences searched: 17 (10630 bp).

Save selected models

Box 27
Modelinspektor Results.
total number of matches: 961

Output overview of ModelInspector matches (961 matches)

go to:[Output overview] [Detailed output] [Statistics]

ModelInspector professional Release 5.1.1 October 2004

Solution parameters:

Sequence file:	Human Promoters (known genes)
Models:	User-defined/Tutorial_2.model
Model score threshold:	80.0 % (abs. 2.40)
Output sorted by:	match positions on the sequences
Maximum number of matches:	1000

Match List:

Sequence	Model Name	Position	Strand	Model Score	Select Match
P537842 (1 - 637) [DNA] sym=HSPC150|loc=Loc29089|taxid=9606|spec=Homo sapiens| ctg=NT_004487|str=(-)|start=52719887|end=52720523|len=637| comm=HSPC150 protein similar to ubiquitin-conjugating enzyme; (AK000504_1/536/gold;NM_014176/501/bronze)	Tutorial_2	335 - 485	(+)	89.8 %	☐
P622046 (1 - 894) [DNA] sym=C1orf22|loc=Loc80267|taxid=9606|spec=Homo sapiens| ctg=NT_004487|str=(-)|start=35132543|end=35133436|len=894| comm=chromosome 1 open reading frame 22; (AK023095_1/501/gold;NM_025191/794/bronze)	Tutorial_2	407 - 511	(+)	92.0 %	☐

Box 28
The Evaluation of results reveals significant sterol metabolism associated categories (truncated list).

Please export the whole match list with the “export to excel function” at the bottom of the page

Evaluation of results

Model: Tutorial_2

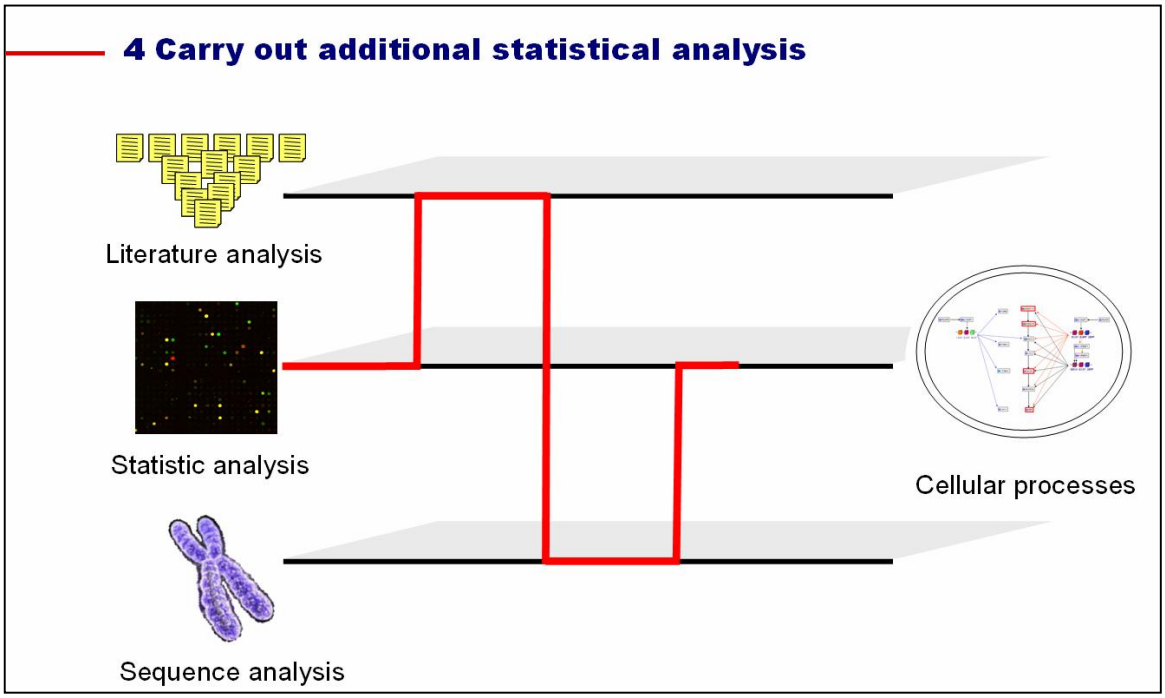
Number of input genes: 828
Number of genes annotated in GO: 529
Number of significant GO groups found: 26

GO group	z-score	# genes (observed)	# genes (expected)	list of genes	Locus IDs
steroid metabolism	4.36	16	5.81	HMGCS1, HMGCR, HSD17B8, OPRS1, MVK, STARD5, SREBF1, LSS, SREBF2, SC4MOL, CYP46A1, DHCR24, SC5DL, DHCR7, EBP, FDPS	3157, 3156, 7923, 10280, 4598, 80765, 6720, 4047, 6721, 6307, 10858, 1718, 6309, 1717, 10682, 2224
sterol metabolism	6.25	13	2.81	HMGCS1, HMGCR, OPRS1, MVK, SREBF1, SREBF2, SC4MOL, CYP46A1, DHCR24, SC5DL, DHCR7, EBP, FDPS	3157, 3156, 10280, 4598, 6720, 6721, 6307, 10858, 1718, 6309, 1717, 10682, 2224

Result Evaluation 3

The second framework analysis results in a single framework consisting of three TFBSs (ECAT, EGRF, ZBPF), matching 8 of 10 genes (HMGCS1, DHCR7, HMGCR, EBP, LSS; MVK, SC5DL, SREBF2). The framework notably does no longer contain the SREBF1 binding site (EBOX). Database analysis (ModelInspector) with this framework yields a total of 961 matches in 828 genes and again the “sterol metabolism” associated categories were overrepresented (among others), this time 16 genes could be significantly related to sterol/steroid metabolism. The genes are falling into the categories associated to “sterol metabolism” are: **CYP46A1**, DHCR24, DHCR7, EBP, **FDPS**, **HMGCR**, HMGCS1, **HSD17B8**, LSS, MVK, **OPRS1**, SC4MOL, SC5DL, **SREBF1**, SREBF2 and **STARD5** (the 7 new identified genes are marked bold). SREBF1 was already identified as a likely regulator of the first framework and now as a target of the new framework!

3.4. Step 4: Additional Statistical Analysis

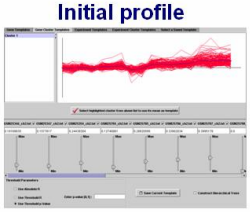


We already learned that some genes (MVK and LSS) which were found by the analysis strategy were not in the original cluster of 105 significantly up regulated genes. However after re-evaluation a sub-statistical up-regulation could be confirmed. Using the framework and its model matches as a complementary line of evidence, it is possible to revisit the statistical analysis with relaxed stringency. To relax the statistics PTM - template matching was used [5].

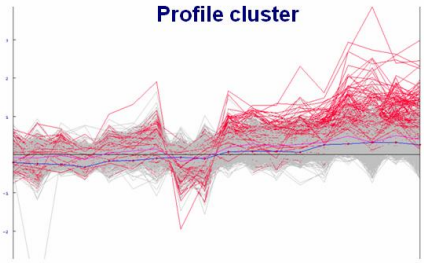
Clustering by profile of the initially selected 105 genes

- Expression cluster is extended by Pavlidid Template Matching (PTM)
- Cluster of 105 significant regulated genes is taken as template
- The threshold p-value is 0.1

Initial profile



Profile cluster



- Cluster is extended to 798 genes (including all 105 initial genes)

The underlying profile was derived from the 105 significantly regulated genes from step 1. 798 genes were retrieved with a threshold p-value of 0.1. This list of 798 genes can be downloaded at:

http://www.genomatix.de/download/tutorial/PDGF_tutorial_2.xls

52 of these genes also were in the match list of the ECAT, EGRF, ZBPF framework (this can e.g. be confirmed by the "Query" function in MS Excel. Alternatively, download the match list at

http://www.genomatix.de/download/tutorial/ PDGF_tutorial_3.xls

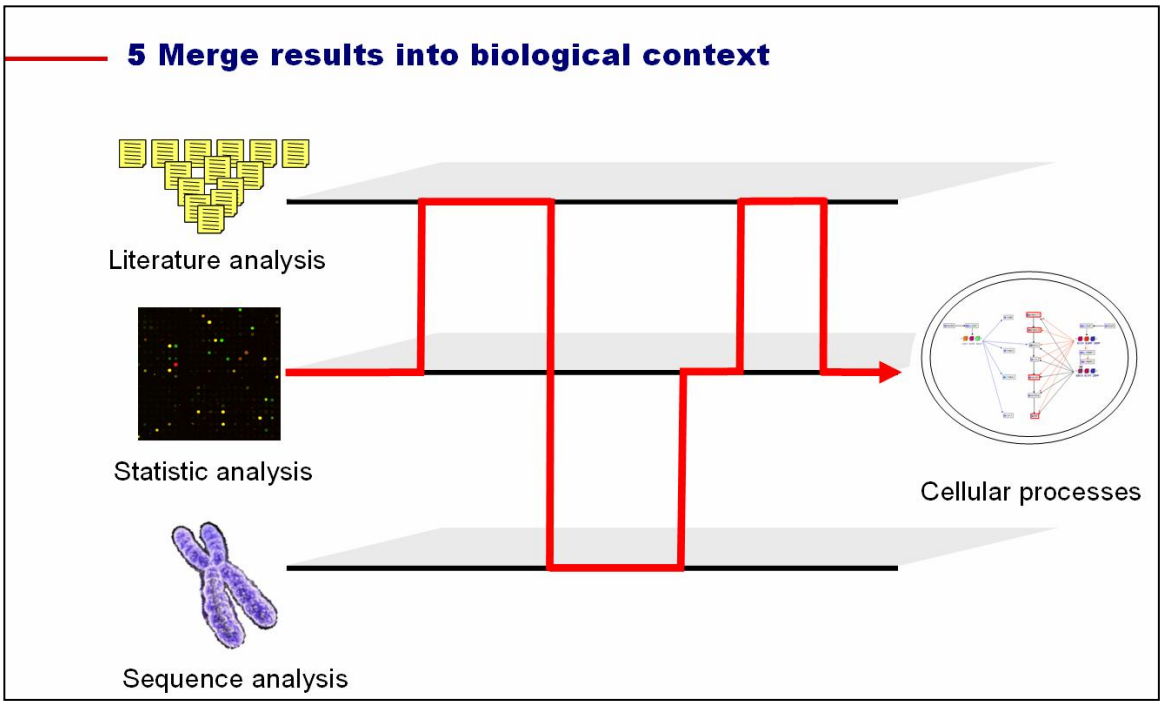
These 52 genes can now be submitted to BiblioSphere (cf. Box 1) and filtered with the GO-Filter "Biological Process". Eight of the 52 genes belong to the GO-category "steroid metabolism".

- 52 genes share a common framework and are co-expressed
- 8 genes belong to the GO-category "steroid biosynthesis":
  DHCR24, DHCR7, EBP, HMGCR, HMGCS1, LSS, MVK, SC4MOL

Eight genes are associated with steroid metabolism are supported by three lines of evidence:

1. Common up-regulation
2. Common framework
3. Common functional class (GO-annotation)

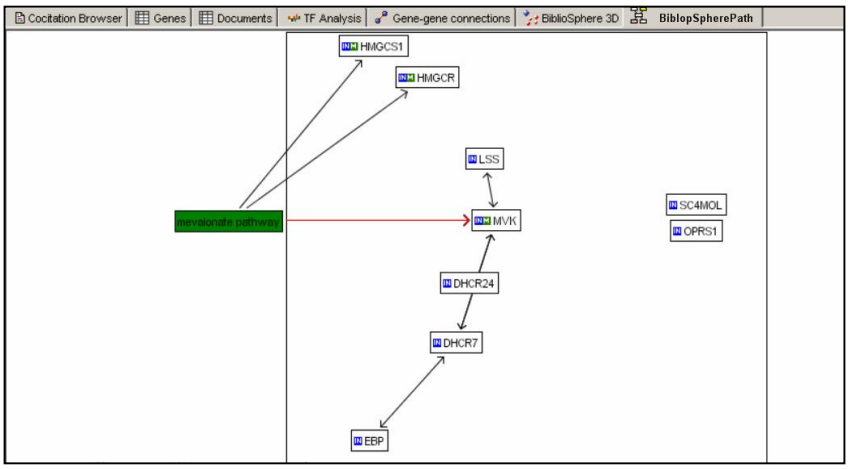
3.5 Step 5: Merging of Results Into Biological Context



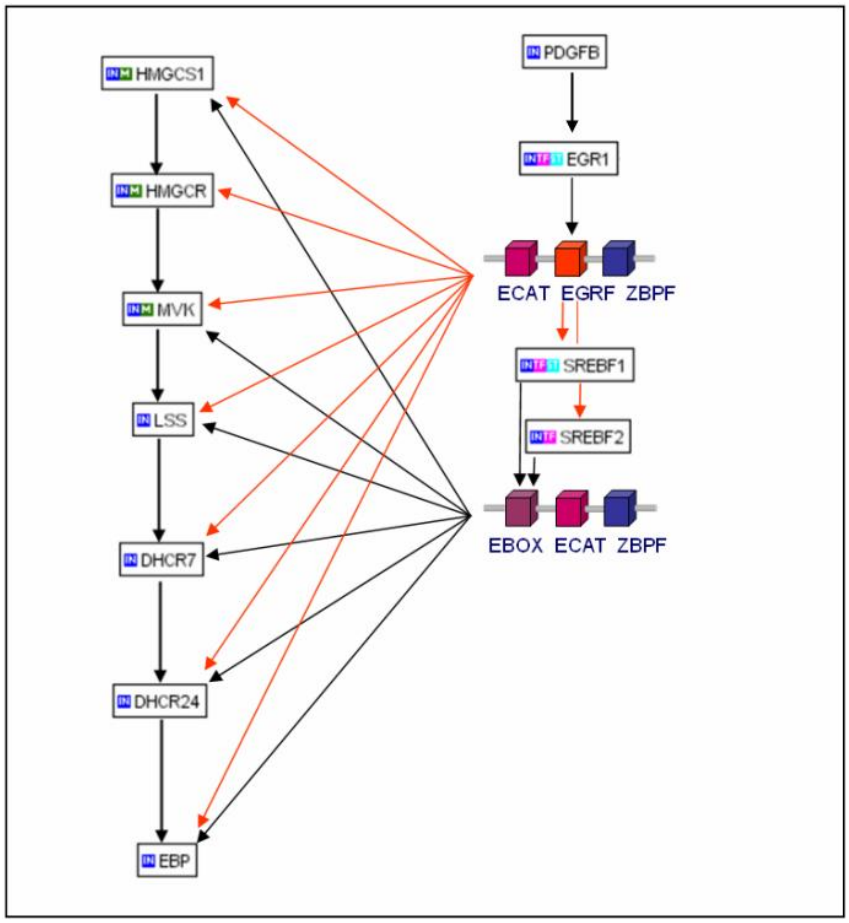
Pathway Mining Using BiblioSphere Pathway Edition

The 54 genes supported by three lines of evidence were used as input for the BiblioSphere GO analysis. 9 of the 54 genes belong to the category “steroid biosynthesis” with a z-score of 17.51. The biological connection between the six top-scoring genes was analyzed with BiblioSphere Pathway Edition which will be released in March 2005. This reveals that seven genes are part of the mevalonate pathway which is closely linked to the sterol metabolism. SC4MOL and OPRS1 are not directly linked, they belong to the ergosterol synthesis.

Box 29
You can already use BiblioSphere for the GO-categorization. Please enter the list of 54 genes into BiblioSphere and proceed as described in Box 1-3
The BiblioSphere Pathway Edition will be released in March 2005.



Box 30
Merging results into biological context. Displayed are the metabolic steps of the sterol biosynthesis and the referring signalling pathway. The results of the sequence analysis are integrated in this view.



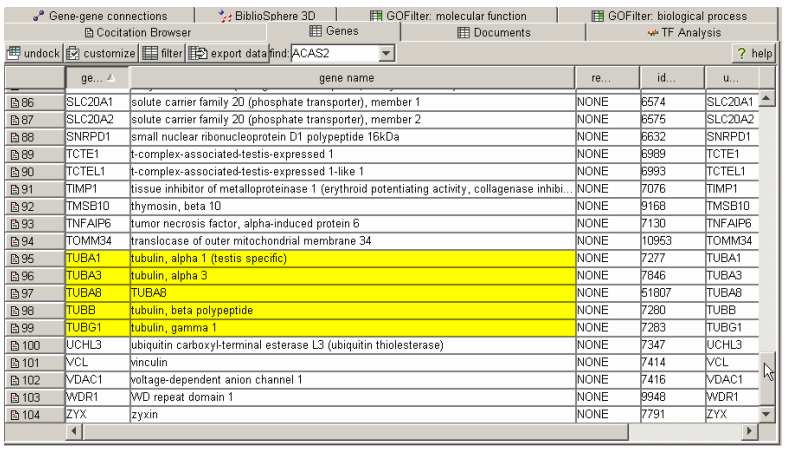
The biological connection between the six top-scoring genes was analyzed with the BiblioSphere pathway mining tool. This reveals that seven genes are part of the mevalonate pathway which is closely linked to the sterol metabolism. SC4MOL and OPRS1 are not directly linked, they belong to the ergosterol synthesis.

Additional Analysis Cycle: Tubulin Genes

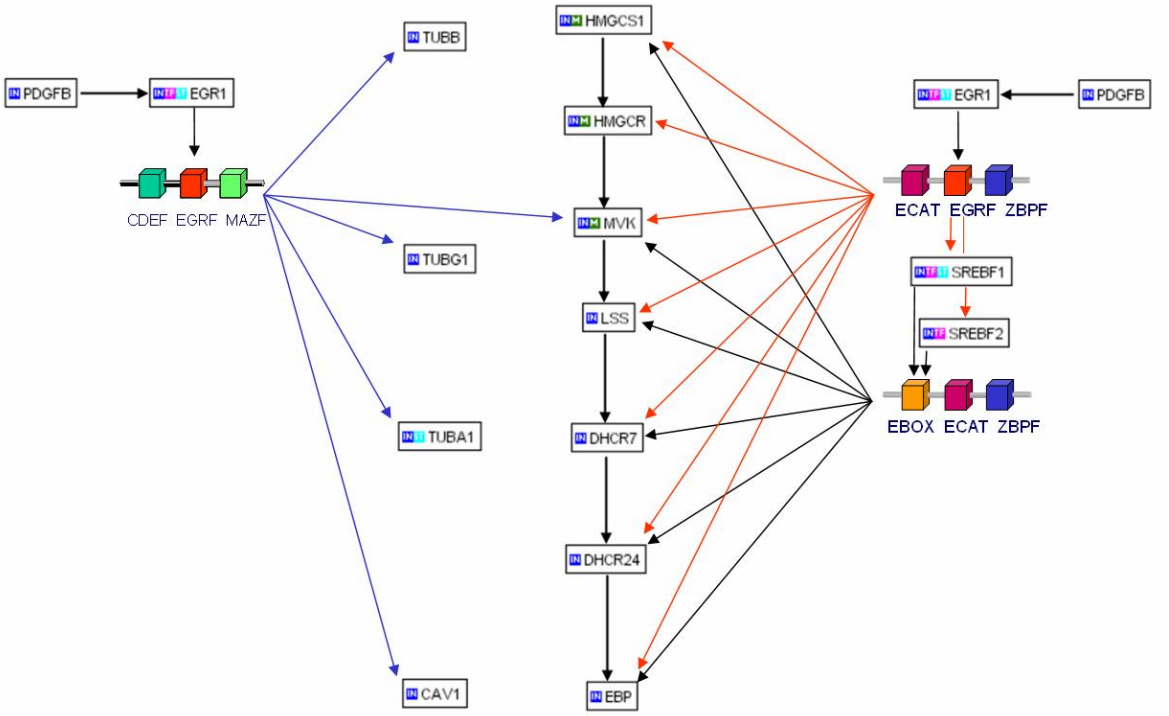
From the literature analysis it is evident, that several more functional groups can be derived from the experimental data. Among others, there is a group of tubulin genes up-regulated under the experimental conditions. Please try the presented strategy and see what you can find out.

The genes for further analysis are: TUBA1, TUBA3, TUBAB, TUBB, TUBG1

Box 31
To further follow the analysis go back to the to the BiblioSphere of the 105 original up-regulated genes. Go to the genes table, sort genes alphabetically by clicking on the genes column and select the 5 tubulin genes.
Carry out the subsequent analysis according to the steps from Box 5-15



	ge...	gene name	re...	id...	u...
86	SLC20A1	solute carrier family 20 (phosphate transporter), member 1	NONE	6574	SLC20A1
87	SLC20A2	solute carrier family 20 (phosphate transporter), member 2	NONE	6575	SLC20A2
88	SNRPD1	small nuclear ribonucleoprotein D1 polypeptide 16kDa	NONE	6632	SNRPD1
89	TCTE1	t-complex-associated-testis-expressed 1	NONE	6989	TCTE1
90	TCTEL1	t-complex-associated-testis-expressed 1-like 1	NONE	6993	TCTEL1
91	TIMP1	tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibi...	NONE	7076	TIMP1
92	TMSB10	thymosin, beta 10	NONE	9168	TMSB10
93	TNFAIP6	tumor necrosis factor, alpha-induced protein 6	NONE	7130	TNFAIP6
94	TOMM34	translocase of outer mitochondrial membrane 34	NONE	10953	TOMM34
95	TUBA1	tubulin, alpha 1 (testis specific)	NONE	7277	TUBA1
96	TUBA3	tubulin, alpha 3	NONE	7846	TUBA3
97	TUBA8	TUBA8	NONE	51807	TUBA8
98	TUBB	tubulin, beta polypeptide	NONE	7280	TUBB
99	TUBG1	tubulin, gamma 1	NONE	7283	TUBG1
100	UCHL3	ubiquitin carboxyl-terminal esterase L3 (ubiquitin thiolesterase)	NONE	7347	UCHL3
101	VCL	vinculin	NONE	7414	VCL
102	VDAC1	voltage-dependent anion channel 1	NONE	7416	VDAC1
103	WDR1	WD repeat domain 1	NONE	9948	WDR1
104	ZYX	zyxin	NONE	7791	ZYX



In the final picture, regulatory links between different functional gene groups can be discovered. Here, cell structure proteins TUBB, TUBA1, TUBG1, and CAV1 (which is involved in tubule formation) can be linked to genes of the sterol metabolism via a framework (CDEF EGRF MAZF) derived from the tubulin genes: There is a match in the promoter of MVK. Notably this framework also contains EGRF. Egr-1 is a transcription factor fitting to EGRF known to be a target of PDGF treatment in several cells [6, 7].

Summary

The iterative combination of complementary lines of evidence, independent of prior knowledge, enables us to gain new insights about the underlying regulatory networks and cascades of PDGF signaling. Regulatory links between different functional groups, cell structure proteins (tubulins) and genes of the sterol metabolism are discovered.

It becomes clear that only relatively small sub-groups from all co-expressed genes can be related to co-regulation (typically less than 10). This is not a contradiction, since co-expression may be based on a variety of co-regulatory mechanisms and reflects the natural flexibility and complexity of living organisms.

Literature

1 Demoulin, J.B. et al. (2004) Platelet-derived growth factor stimulates membrane lipid synthesis through activation of phosphatidylinositol 3-kinase and sterol regulatory element-binding proteins J Biol Chem 279, 35392-402

2 Tusher, V.G., Tibshirani, R. and Chu, G. (2001) Significance analysis of microarrays applied to the ionizing radiation response Proc Natl Acad Sci U S A 98, 5116-21

3 Dooley, K.A., Millinder, S. and Osborne, T.F. (1998) Sterol regulation of 3-hydroxy-3-methylglutaryl-coenzyme A synthase gene through a direct interaction between sterol regulatory element binding protein and the trimeric CCAAT-binding factor/nuclear factor Y J Biol Chem 273, 1349-56

4 Frech, K., Danescu-Mayer, J. and Werner, T. (1997) A novel method to develop highly specific models for regulatory units detects a new LTR in GenBank which contains a functional promoter J Mol Biol 270, 674-87

5 Pavlidis, P. and Noble, W.S. (2001) Analysis of strain and regional variation in gene expression in mouse brain Genome Biol 2, RESEARCH0042

6 Kaufmann, K. and Thiel, G. (2001) Epidermal growth factor and platelet-derived growth factor induce expression of Egr-1, a zinc finger transcription factor, in human malignant glioma cells J Neurol Sci 189, 83-91

7 Hjoberg, J. et al. (2004) Induction of early growth-response factor 1 by platelet-derived growth factor in human airway smooth muscle Am J Physiol Lung Cell Mol Physiol 286, L817-25

Further reading and other tutorials: http://www.genomatix.de/science/index.html