Gene network analysis leads to functional validation of pathways linked to cancer cell growth and survival.

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Supporting informations

Table S1 : Human gene sets retrieved from published experiments and used to determine gene sets detected in HepG2 cells (Full), liver (set 1) or deregulated in hepatocellular carcinoma HCC (set2).

	Number of genes	PMID	References
Full	20839	1345164	Okubo *et al* , 1992
		11466695	Tackel Hornes *et al* , 2001
		15013840	Butura *et al* , 2004
		17967932	Liguori *et al* , 2008
		20571111	Berger *et al* , 2010
		127558421	De Gottardi *et al* , 2007
Liver (set 1)	4918	1577423	Shyamsundare *et al* , 2005
		1676090	Das *et al* , 2006
		10694486	Yamashita *et al* , 2000
		11580139	Yano *et al* , 2001
HCC (set 2)	806	19171046	Okabe *et al* , 2001
		18171335	Wong *et al* , 2008
		181711335	Zender *et al* , 2010

Table S2: Drugs used in experiments

Drug	Activity	Highest Concentration tested	Reference (PMID)	Vehicle	Purchased by
2,5 DDA	Adenylate cyclase inhibitor	100 µM	21444924	Dimethylsulfoxide (DMSO)	SIGMA ALDRICH, France
A6355	P42/P44 inhibitor	90 µM		DMSO	SIGMA ALDRICH, France
Butein	Janus kinase (JAK1) inhibitor	20 µM	9571170 (HepG2)	DMSO	SIGMA ALDRICH, France
Compound C	AMPc activated kinase (AMPK) inhibitor	40 µM	22674626	DMSO	SIGMA ALDRICH, France
GW7647	Peroxisome-proliferator activated receptor alpha (PPARA) activator	10 µM	188520	DMSO	SIGMA ALDRICH, France
KT5720	Protein kinase AMPc dependant (PKA) inhibitor	30 µM		Methanol (MetOH)	SIGMA ALDRICH, France
LY294002	PI3Kinase inhibitor	250 µM	22025081 (HepG2)	DMSO	CELL SIGNALING, Ozyme, St Quentin-en-Yvelines, France
P3115	Protein kinase Calcium dependant (PKC) inhibitor	50 µM	8420972	H2O	SIGMA ALDRICH, France
Pertussis Toxin	G protein subunit Gi/G0 inhibitor	1 µg/ml		H20	SIGMA ALDRICH, France
Rapamycin	TOR/P70S6Kinase inhibitor	50 nM	15870276; 21830446	H2O	CELL SIGNALING, Ozyme, St Quentin-en-Yvelines, France
RU 486	PPARA inhibitor	5 µM		H2O	SIGMA ALDRICH, France
SD169	P38 MAP Kinase (P38MAPK) inhibitor	300 µM	16603672	DMSO	SIGMA ALDRICH, France
SP600125	Jun-NH2 kinase (JNK) inhibitor	100 µM	14766793 (HepG2)	DMSO	SIGMA ALDRICH, France
Tyrphostin 490	Janus kinase 2 (JAK2) inhibitor	50 µM	18448488	Ethanol (EtOH)	SIGMA ALDRICH, France
U0126	MEK1/2 MAPK inhibitor	100 µM	18448488	DMSO	CELL SIGNALING, Ozyme, St Quentin-en-Yvelines, France
U73122	Phospholipase C (PLC and Phospholipase A2 (PLA2) inhibitor	5 µM	18629476	EtOH	SIGMA ALDRICH, France
Wedelolactone	Nuclear factor kappa B, p65 subunit (NFKBp65) inhibitor	10 µM	17942463	DMSO	SIGMA ALDRICH, France
ZM39923	Janus kinase 3 (JAK3 inhibitor	50 µM	10741557	DMSO	SIGMA ALDRICH, France

Table S3: References of human gene sets retrieved from published experiments and used to determine pathway representativity in either human genes detected in HepG2 cells (Full), liver or deregulated in hepatocellular carcinoma (HCC). Gene number (n) in data sets represent the number of genes modulated by at least one pathway in either Full, liver or HCC sets. Gene sets modulated by external factors (Stimulus) and intracellular pathways were retrieved from experiments performed in human cells (PubMed Identifiers, PMID) and sets obtained on HepG2 cells are reported separately. Only significant Z-test scores are reported (confidence level > 90).

STIMULUS										
Pathway	Full	HepG2 & liver		HCC			References			
	n	n	Z-score versus Full	n	Z-score versus Full	Z-score versus HepG2 & Liver	Cell type	Treatment	PMID	Reference
Data set	4231	2097		401						
Adiponectin	1013	964	17.74	151	6.117	2.898	HepG2, HeLa, Panc01 cell lines	Stimulation by human recombinant adiponectin (full lengh; 2.5 μg/ml 3 hours)	20571111	Berger et al, 2010
Androgens	69	40		9			Prostate epithelial cell line (M12)	AR positive versus AR negative cells exposed in parallel to Dihydroxy testosterone (6h)	16240454	York et al, 2005
							Prostate cancer cell line (LnCaP)	R1881 (16h)	16751804	Wang et al, 2006
							Prostate cancer cell lines androgen sensitive (LNCaP) versus aggressively metastatic (AI C4)		16500022	Chen et al, 2006
Apoptosis	1206	641	1.618	145	3.311	2.312	Gene set		16959035	Huang et al, 2006
							Human leukemia HL-60	homoharringtonin 4.64 mg/ml	15000885	Jin et al, 2004
BMP	138	73		19	1.442		Prostate cancer cells (DU-145)	BMP6 (24H)	1554869	Haudenschild et al, 2004
							Prostate cancer cells (LNCAP)	Dihydroxyandrostenone (DHT) 1nM and rhBMP2 100nM	16391828	Kumagai et al, 2005
							Mammary cancer cell line (MCF7)	BMP2, 4h	18446370	Steinert et al, 2008
							Bone marrow stroma cell line	BMP2 1-21 days	15778851	Sekiya et al, 2005
							Bone marrow stroma cell line	BMP2 1-72h	11760832	Locklin et al, 2001
Estrogens	1138	483	2.543	91	1.675		Mammary cancer cell line (MCF7)	Estradiol (E2)-16h	17515612	Kininis et al, 2007
							Myeloma cell line (JJN3)	Raloxifene (2h)	16497877	Olivier et al, 2006
							Mammary cancer cell line (MCF7)	Estradiol (E2), tamoxifen, raloxifen and/or ICI 182,780 (12h)	16298037	Glidewell-Kenney et al, 2005
							Mammary cancer cell line (MCF7) with or without Akt overexpression	Estrogen (4h)	18838536	Bhat-Nakshatri et al, 2008
							Mammary cancer cell line (MCF7)	Estradiol (E2), 4h	12959972	Frasor et al, 2003
Fatty acids	541	293		80	3.855	3.012	Database	http://wwwmgs.bionet.nsc.ru/mgs/papers/ignatieva/lm-trrd		
							Breast cancer cell line (T47D)	Linoleic acid	15145518	Reyes et al, 2004
							Enterocyte cell line (Caco2/TC7)	Dietary apical lipid micelles or basal albumin-bound lipids (24h)	18755805	Beaslas et al, 2008
HepG2	214	145	3.018	39	3.844	1.812	Hepatoma cell line (HepG2)	Palmitate, oleate or linoleate (24h)	17925029	Srivastava et al, 2007
							Hepatoma cell line (HepG2)	0leate, arachidonic acid, eicosapentanoic acid (EPA) or docosahexanoic acid (EPA) (24h)	12887159	Fujiwara et al, 2003
							Hepatoma cells (HepG2)	Oleate (24h)	19083476	Vock et al, 2008
							Hepatoma cells (HepG2)	Palmitate, oleate or eicosapentanoic acid (1,5h)	17707340	Swagell et al, 2007
HGF	75	41		10			Prostate cancer cells (DU-145) transfected by mock or Dominant-negative Akt	HGF (6h)	17823924	Xu et al, 2007
							Colon cancer cell lines (DLD-1) with invalidated versus normal Ki-Ras and tumor xenograft tissues	HGF (6h)	16158056	Seiden-Long et al, 2006
							Mammary epithelial cell line (MFC-10)	HGF (2h)	17384262	Leroy et al, 2006)
Hypoxia	51	37	1.485	13	3.055	1.77	Renal carcinoma cells (786-0)	1.5 vs 21% 02, 16h	14645546	Hu et al, 2003
							HEK293	1.5 vs 21% 02, OVN	16002434	Wang et al, 2005
IGF1	676	391	2.965	81			Mammary cancer cell line (MCF7)	Constitutive IGF1 overexpression	16774935	Pacher et al, 2007
							Mammary cancer cell line (MCF7)	IGF1 (3h)	18757322	Creighton et al, 08
							Prostate epithelial cell line (M12)	AR positive versus AR negative cells exposed in parallel to IGF1	16240454	York et al, 2005
IGF2	23	15		4			Mammary cancer cell line (MCF7)	Constitutive IGF2 overexpression	16774935	Pacher et al, 2007
INFa	68	41		7			Multiple myeloma cell line(U266-1984)	INFalpha with or without Ly294002 (24h)	17141757	Hjortsberg et al, 2007
Insulin	322	200	2.537	30			Vastus lateralis muscle of healthy humans	3-h hyperinsulinemic euglycemic clamp	12621037	Rome et al, 2003
(liver)	109	78	2.575	30	5.344	3.204	Primary hepatocytes	Insulin (18h)	17628011	Kotokorpi et al, 2007
Melatonin	57	32		5			Peripheral blood mononuclear cells	melatonin 0.5 nM 24h		Ha et al, 2006
OxLDL	31	19		8	2.437	1.688	Macrophage cell line (THP1) stimulated with	Chylomicron remnants or oxidized LDLs	15156288	Batt el al, 2004
OxPARPs	54	46	2.595	16	4.021	1.948	Primary culture of aortic endotehlial cells	oxPAPC 10, 30, 50 mg/ml (4h)	16912112	Gargalovic et al, 2006
Retinoic acid	18	15	1.374	8	3.749	2.212	Primary monocytes	Beta-carotene, 9-cis retinoic acid or ATRA	15949682	Langmann et al, 2005
T3	35	26	1.396	8	2.122		HCC cell line HepG2 stably expressiong TR1alpha	T3, 3-48h	14977860	Shih et al, 2004
							Fibroblasts	T3, 24h	15507505	Moeller et al, 2005
TGFb	267	151		23			Lung epithelial (HPL1D) and adenocarcinoma (A549) cell lines	TGF beta	17425807	Ranagathan et al, 2007
TNFA	61	39		11	1.8		Neuroblastoma cell line (HeLa)	TNFalpha in presence or absence of NF- B signaling (3h;6h)	15722553	Tian et al, 2005
HepG2							Hepatoma cell line (HepG2)	TNFalpha (24h)	17925029	Srivastava et al, 2007
VIT D	778	309	3.936	68			Mammary cancer cell lines (MCF7 and MDA MB31)	Vitamin D3	12889598	Swami et al, 2003
							Mouth/base of tongue squamous tumor cell line (SCC25)	1,25-Dihydroxyvitamin D3 (12h)	16002434	Wang et al, 2005
VIT E	45	30		6			Differenciated and undifferenciated colonocyte cell lines (CRL-1807)	Vitamin E	15753143	Lunec et al, 2004

Intracellular pathways

Pathway	Full	HepG2 & liver		HCC			References			
	n	n	Z-score versus Full	n	Z-score versus Full	Z-score versus HepG2 & Liver	**Cell type**	**Treatment**	**PMID**	**Reference**
Data set	5690	2529		453						
Akt	1009	418		79			Prostate cancer cells (DU-145)	Transfection DN-Akt and stimulation by HGF (6h)	17823924	Xu et al, 2007
							Umbilical vein endotheliual cells (HUVECs)	Infected with adenoviral constitutively active Akt (24h)	15784720	Kim et al, 2005
							Mammary cancer cell line (MCF7)	Constitutive Akt overexpression	18838536	Bhat-Nakshatri et al, 2008
							Retinoic pigment epithelial cells (RPE) expressing constitutively active Akt fused to the hormone binding domain of the oestrogen receptor	4-hydroxytamoxifen (4-OHT)	16007182	Portsmann et al, 2005
Akt Translation	92	84	4.831	11			Polysom microarray analysis of protsate cancer LAPC-4 cell line stably transfected with a constitutively active AKT and treated by rapamycin		14576155	Gera et al, 2004
Gb/g	22	14		4			Knock out beta and gamma G protein subunit		15983374	Hwang et al, 2005
JNK	74	49	1.969	18	4.184	2.473	Epidermal keratinocytes	SP600125 (4h)	16648634	Gazel et al, 2006
Ki-RAS	73	44	1.665	6			4 pancreatic cancer cell lines	Infection by adenoviral Ki-Ras antisens	16446406	Spence et al, 2006
LKB1	36	15		4			Lung adenocarinoma cells (A549)	LKB1 overexpression (6h)	12649203	Jimenez et al, 2003
Mek/Erk1/2	103	50		9			Immortalized retinal pigment epithelial cells (RPE)	Transient MKK1 or ERK2 overexpression	16735500	Schweppe et al, 2006
							Lung carcinoma cells (H157)	U0126 and/or plaxitel (6h)	12941840	Taxman et al, 2003
Methylation (HepG2)	3972	1719		265	4.818	3.684	HCC cell line HepG2	5-AzaC	16649225	Arai et al, 2006
							HCC cell line HepG2	5-AzaC, TSA	16854234	Dannenberg et al, 2006
MKK5/Erk5	26	18	1.556	5	1.675		Immortalized retinal pigment epithelial cells (RPE)	Transient MKK5 or ERK5 overexpression	16735500	Schweppe et al, 2006
mTOR	540	269		50			Hepatic cell line (HepaRG)	Constitutively active mTOR overexpression	17483347	Parent et al, 2007
							Jurkat T cells	Rapamycin (3 days)	11943782	Grolleau et al, 2002
mTOR translation	280	221	6.596	31	1.664		Proteomic Profiling by 2D-electrophoresis of Rapamycin-treated Jurkat T Cells		11943782	Grolleau et al, 2002
							Polysom microarray analysis of protsate cancer LAPC-4 and glioblastoma U87MG cell line treated by rapamycin		14576155	Gera et al, 2004
N-RAS	400	281	6.028	79	7.548	3.531	Cancer cell lines (SHEP, A549, NCIH929, U87 and LaNR)	Salirasib	17409441	Blum et al, 2007
							Cord blood CD34+ cells	Constitutive N-RAS expression	17533045	Shen et al, 2007
P38MAPK	91	71	3.526	25	5.69	2.854	Follicular lymphoma cell line (OCILY-1)	SB203580	15169874	Lin et al, 2004
Pi3K	72	38		6			Gioblastoma cell line (T98G)	LY294002 (3h)	18226221	Terragni et al, 2008
							Multiple myeloma cell line(U266-1984)	Ly294002 (24h)	17141757	Hjortsberg et al, 2007
PKA	89	39		10	0.945		Prostate cancer cell line (LnCaP)	Forskolin (16h)	16751804	Wang et al, 2006
							Endometrial stromal cells	8-Br-cAMP (2h)	14532334	Tierney et al, 2003
PTEN	123	95	4.092	26	4.635	1.858	Endometrial cancer cell line (HEC-151)	Infection by adenoviral PTEN Dominant negatif	11325847	Matsushima-Nishiu et al, 2001
PTEN translation	92	69	3.235	13	1.708		Polysom microarray analysis of U87MG glioblastoma cell line transfected with wild-type PTEN and treated by rapamycin		14576155	Gera et al, 2004
Rac1	34	23	1.369	7	1.964		Colorectal adenocarcinoma cell line (SW620)	Stably transfected shRNA versus wild type Rac1	17766170	Gomez del Pulgar et al, 2007
TSC1/2	41	27		0	1.461	1.8	Neuroblastoma cell line (HeLa)	Transient TSC1/2 overexpression	12894220	Rosner et al, 2003
Wnt/bCAT	222	142	3.399	31	2.867		Neuroblastoma cell line (HeLa)	Stably integrated, inducible RNA interference (RNAi) for beta-catenin	16959035	Huang et al, 2006
							Database		http://www.stanford.edu/~rnusse/pathways/targets.html	
							Hepatoma carcinoma cell lines with constitutive versus minimal beta-catenin activity (HepG2, SNU-182, SNU-354 versus Hep3B, PLC/PRF/5, SNU-387, SNU-449, SNU-475)		17157329	Lee et al, 2007

Table S4: Nomenclature, primers and experimental conditions designed for real-time analysis of human gene transcription. Standards corresponding to fragments of human genes used as at several known concentrations were used to calibrate mRNA quantifications.

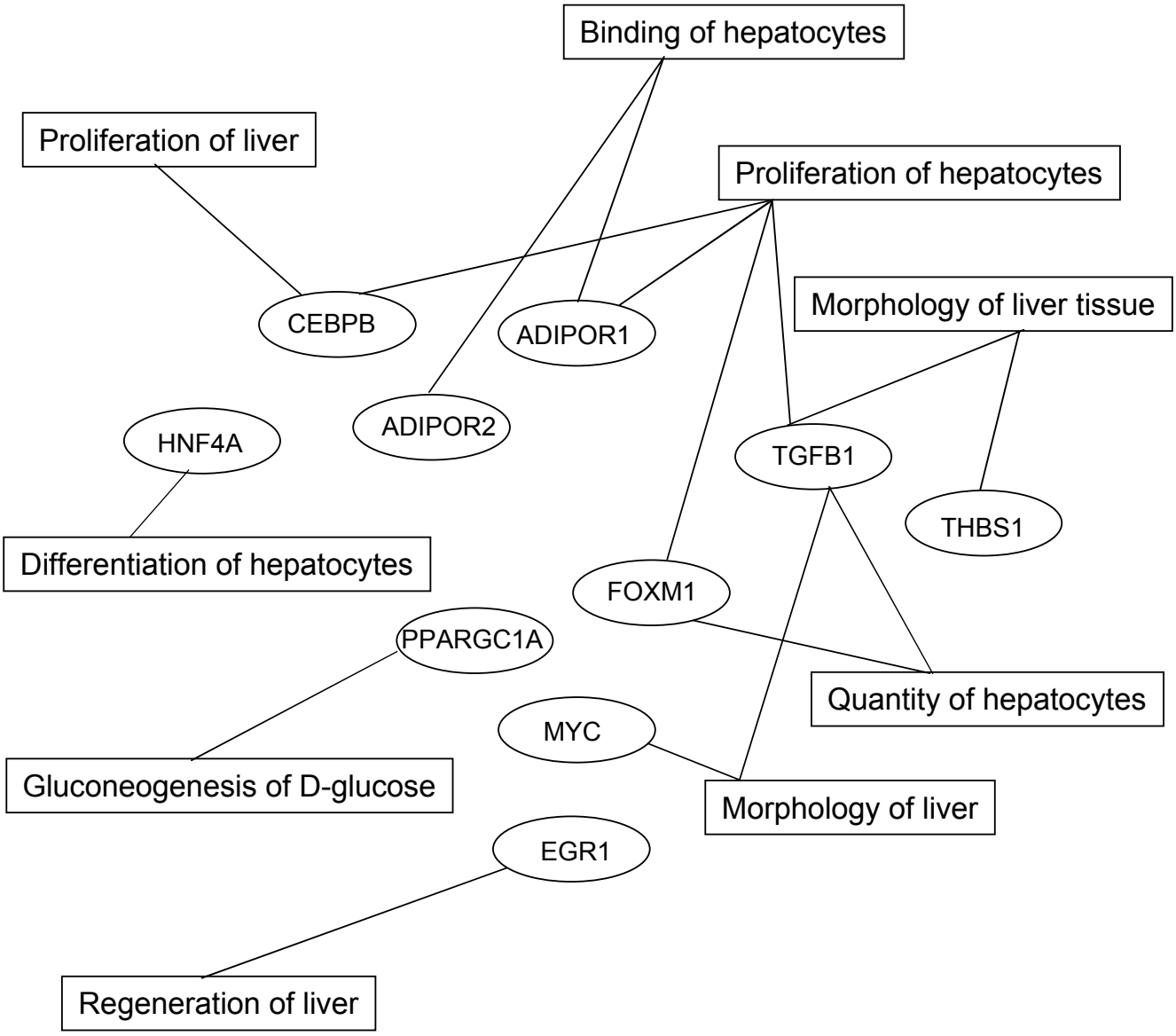
Symbol	Name	Gene Id	Primers	Hybridization (°C)	Amplicon size (bp)	Standard
ADIPOR1	Adiponectin receptor 1	51094	Sens: 5' AAGCACCGGCAGACAAGAGC Antisens: 5' AGGAAGAACCAGCCCATCTG	60	133	ADIPOR1
ADIPOR2	Adiponectin receptor 2	79602	Sens: 5' CTGTGTGCTGGGCATTGCAG Antisens: 5' AGCCTATCTGCCCTATGGTG	60	180	ADIPOR2
AFP	Alpha fetoprotein	174	Sens: 5' TCTCAGGCCTGTTGGAG Antisens: 5' CCAAAGCAGCACGAGTT	60	98	HPRT1
BHLHB2 / BHLHE40	Basic helix-loop-helix domain containing, class B, 2	8552	Sens: 5'CTGAGCAGAACATCTCTTGAC Antisens: 5'GCAGTGGTTCTTGAACTTACC	60	154	BHLHB2
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	1050	Sens: 5'TGCCCATGGCCTTGACCAAGGAG Antisens: 5'GCAAGGCCAAGAAGTCGGTGGAC	60	254	CEBPA
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	1051	Sens: 5'GCTTGAACAAGTTCCGCAGG AACATCGCCGTGCGCAAGAG	60	156	CEBPB
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	6043	Sens: 5'GCAATGCGCAGGAATAAG AGGCTTCTTGGGCGTCT	60	135	HPRT1
CREBBP	CREB binding protein (Rubinstein-Taybi syndrome)	1387	Sens: 5'CAAACGCCTTGTCCAGCATC Antisens: 5'GCCGTTTACCATGAGATCC	58		CREBBP
CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1	1588	Sens: 5'ACGTGGTCAGAGTAGAACCC Antisens:GGTGTTTGGAAAGTTCCTCC	60	179	HPRT1
EGR1	Early growth response 1	1958	Sens: 5'GTTGTTCAGAGAGATGTCAGG Antisens: 5'CAGCACCTTCAACCCTCAG	60	88	EGR1
FABP1	fatty acid binding protein 1, liver	2168	Sens: 5'GCTGGAGACAATGACAGG Antisens: 5'CATTGGTGATTATGTCGCC	62	125	HPRT1
FOXM1/HNF3a	forkhead box M1	2305	Sens: 5'TTCTTTAGTGGCGATCTGCG Antisens: 5'AAGAAAGAGGAGCTATCCCCTC	60	111	HPRT1
FOXO1A	forkhead box O1	2308	Sens: 5'AGACATCTTTGGACTGCTTC Antisens: 5'TCAAGACAACGACACATAGC	60	115	FOXO1A
HES1	hairy and enhancer of split 1, (Drosophila)	3280	Sens: 5' GAAATGACAGTGAAGCACCTC Antisens: 5' GTTCATGCACTCGCTGAAG	55	113	HPRT1
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	3091	Sens: 5'GCTTTGCAGAATGCTCAGAG Antisens: 5'TGATCGTCTGGCTGCTGT	60	98	HPRT1
HK2	Hexokinase 2	3099	Sens: 5'TGTTGCAGGATGGCTCGGAC Antisens: 5'CAAGCGTGGACTGCTCTTCC	60	131	HK2
HNF4A	hepatocyte nuclear factor 4, alpha	3172	Sens: 5'GAGATCCATGGTGTTCAAGG Antisens: 5'ATTGTCATCGATCTGCAGC	60	150	HPRT1
HPRT	hypoxanthine phosphoribosyltransferase 1	3251	Sens: 5' TTGCTGACCTGCTGGATTAC Antisens: 5' AGTTGAGAGATCATCTCCAC	60	151	HPRT1
IKBKAP	inhibitor of kappa light polypeptide gene enhancer in Bcells, kinase complexassociated protein	8518	Sens: 5' CAGCAACAGAAGACTTCGG Antisens: ATCTAGCAGGCTCAGCTTC	58	95	HPRT1
IRF1	interferon regulatory factor 1	3659	Sens: 5'GCTGGGACATCAACAAG Antisens: 5'AAAGTTGGCCTTCCACGT	58	119	HPRT1
LDLR	low density lipoprotein receptor	3949	Sens: 5'TCACTCCATCTCAAGCATCG Antisens: 5'TGGCACTGAAAATGGCTTCG	60	149	HPRT1
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	4609	Sens: 5'GTAGTTGTGCTGATGTGTGG Antisens: 5'GAGGAGGAACAAGAAGATGAG	60	171	MYC
PPARG	Peroxisome proliferator-activated receptor gamma	5468	Sens: 5'GCATTATGAGACATCCCCAC Antisens: 5'TCTCTCCGTAATGGAAGACC	65	474	PPAR
PPARGC1A	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	10891	Sens: 5'CTTGGTTGGCTTTATGAGGAGG Antisens: 5'TCCTCTGACCCCAGAGTCAC	60	143	PPARGC1A
TBP	TATA box binding protein	6908	Sens: 5' TGGTGTGCACAGGAGCCAAG Antisens: 5' TTCACATCACAGCTCCCCAC	60	139	TBP
TGFB1	Transforming growth factor, beta 1	7040	Sens: 5'GGTTATGCTGGTTGTACAGG Antisens: 5'AGGGCTACCATGCCAACTTC	55	229	TGFB
THBS1	thrombospondin 1	7057	Sens: 5'CTACAGATGGCGTCTCAGC Antisens: 5'CCCTAGTCTACCACCAGCATAG	60	126	HPRT1
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	7132	Sens: 5'CAATCTGGGGTAGGCACAAC Antisens: 5'CCTGCCAGGAGAAACAGAAC	60	129	TNFRSF1A

Table S5: Real-time quantification of gene expression in HepG2 cells incubated with specific inhibitors during 5 hours in serum free standard media. Fold changes for samples treated versus controls (i.e. vehicle) were normalized to HPRT1. Significant modulation was considered through ANOVA test, i.e. p-value < 0.01 (n>3 independant experiments). Gene names, sequence references and primers are reported in Table S4, drug informations in Table S2.

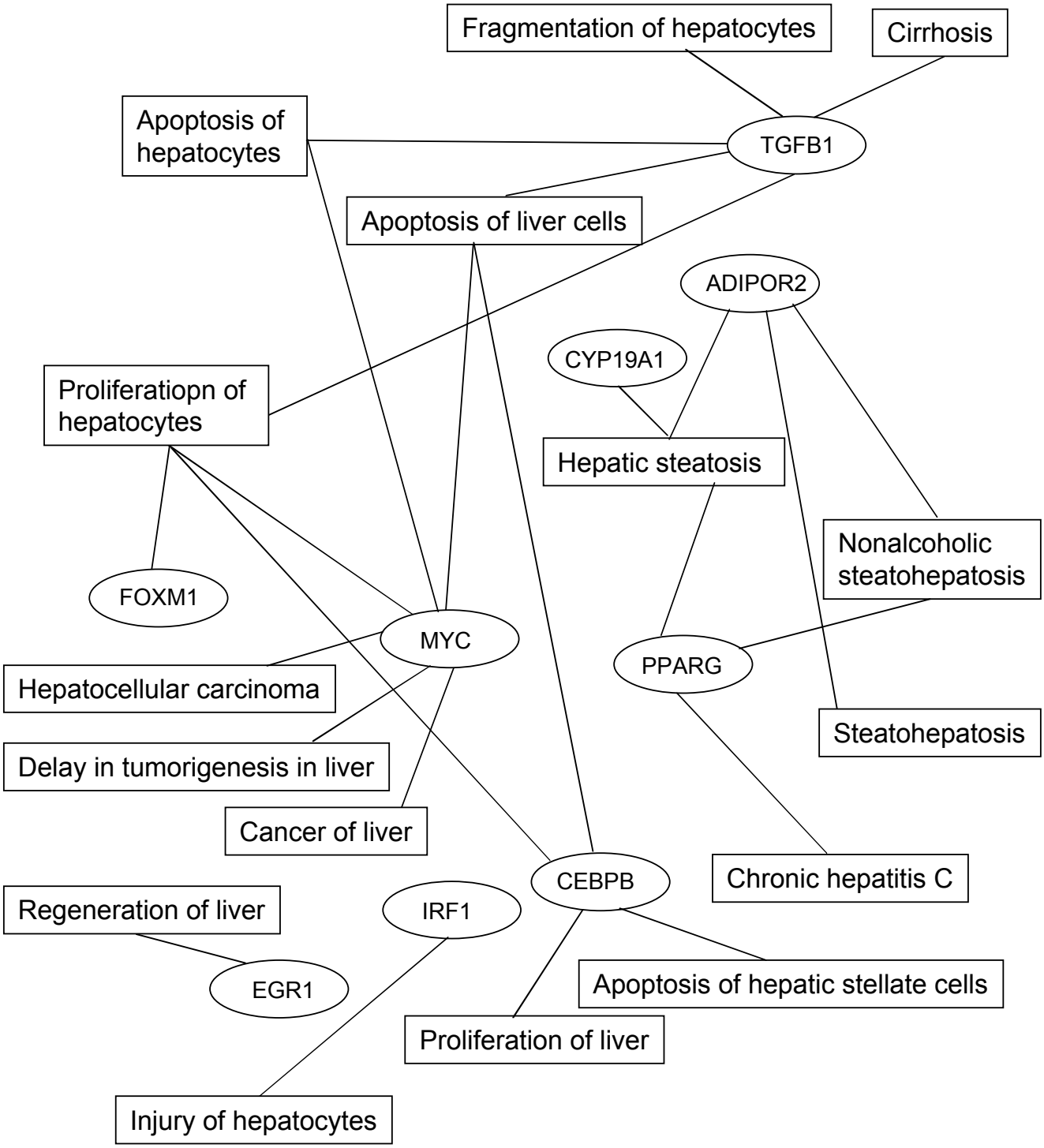
Genes up-regulated		Genes down-regulated		Genes not modulated	
Gi/G0 (Inhibition by Pertussis Toxin, 500 ng/ml)					
CEBPA	-1.96 ± 0.03	BHLHB2	2.77 ± 0.10	ADIPOR1	1.01 ± 0.05
CREBBP	-1.37 ± 0.09	HES1	2.47 ± 0.10	AFP	1.02 ± 0.06
		HNF4A	2.23 ± 0.38	CEBPB	0.93 ± 0.04
		TGFB1	1.80 ± 0.02	CYP19A1	1.03 ± 0.16
		THBS1	1.69 ± 0.20	EGR1	0.95 ± 0.01
		FOXM1/HNF3	1.54 ± 0.11	FABP1	1.31 ± 0.08
		ADIPOR2	1.49 ± 0.05	IKBKAP	0.84 ± 0/04
		IRF1	1.48 ± 0.05	PPARG	1.17 ± 0.09
		LDLR	1.44 ± 0.02	TBP	0/98 ± 0.06
		TNFRSF1A	1.44 ± 0.07		
Mek1/2 (Inhibition by U0126, 10 μM)					
CEBPA	-1.75 ± 0.02	IRF1	3.31 ± 0.67	ADIPOR1	1.21 ± 0.05
EGR1	-1.59 ± 0.07	FOXO1A	1.57 ± 0.10	ADIPOR2	-1.09 ± 0.04
HK2	-1.53 ± 0.02	CYP19A1	1.45 ± 0.06	AFP	1.18 ± 0.08
LDLR	-1.44 ± 0.02	HES1	1.42 ± 0.03	BHLHB2	-1.13 ± 0.02
MYC	-1.38 ± 0.03			CEBPB	-1.08 ± 0.13
				CREBBP	1.15 ± 0.13
				FABP1	1.21 ± 0.07
				FOXM1/HNF3	1.28 ± 0.06
				HNF4A	1.20 ± 0.07
				IKBKAP	-1.18 ± 0.07
				PPARG	1.31 ± 0.10
				PPARGC1A	1.31 ± 0.14
				TBP	1.23 ± 0.05
				TGFB1	-1.22 ± 0.12
				THBS1	-1.04 ± 0.03
				TNFRSF1A	1.07 ± 0.17
P42/44 (inhibition by A6355, 90 μM)					
CEBPA	-8.07 ± 0.00	EGR1	17.34 ± 0.39	ADIPOR1	1.23 ± 0.06
PPARGC1A	-2.31 ± 0.05	LDLR	6.63 ± 0.44	CEBPB	1.06 ± 0.13
MYC	-2.15 ± 0.05	BHLHB2	5.84 ± 0.11	FABP1	1.29 ± 0.06
CREBBP	-2.10 ± 0.04	HES1	3.98 ± 0.09	HK2	1.07 ± 0.05
IKBKAP	-2.09 ± 0.00	FOXO1A	2.93 ± 0.42	TBP	1.02 ± 0.04
HNF4A	-1.62 ± 0.04	IRF1	2.17 ± 0.15	TGFB1	1.29 ± 0.16
THBS1	-1.48 ± 0.07	CYP19A1	1.94 ± 0.17		
TNFRSF1A	-1.40 ± 0.08	ADIPOR2	1.92 ± 0.05		
AFP	-1.38 ± 0.04	FOXM1/HNF3	1.89 ± 0.11		
		PPARG	1.45 ± 0.12		
PKC (Inhibition by P3115, 50 μM)					
CEBPA	-2.17 ± 0.03	BHLHB2	3.85 ± 0.07	ADIPOR1	1.08 ± 0.03
		LDLR	3.23 ± 0.78	AFP	-1.24 ± 0.08
		HES1	2.50 ± 0.05	CEBPB	-1.22 ± 0.07
		THBS1	2.00 ± 0.32	CREBBP	-1.33 ± 0.01
		HNF4A	1.83 ± 0.08	FABP1	1.16 ± 0.06
		EGR1	1.67 ± 0.09	FOXO1A	1.23 ± 0.16
		MYC	1.61 ± 0.10	TBP	1.01 ± 0.06
		FOXM1/HNF3	1.57 ± 0.20	TGFB1	1.33 ± 0.01
		ADIPOR2	1.50 ± 0.07	TNFRSF1A	1.04 ± 0.07
		PPARG	1.44 ± 0.12		
JAK1 (Inhibition by Butein, 20 μM)					
		EGR1	2.8 ± 0.12	ADIPOR1	1.29 ± 0.00
		BHLHB2	2.67 ± 0.16	ADIPOR2	1.26 ± 0.01
		AFP	2.33 ± 0.10	CEBPA	1.18 ± 0.09
		FOXM1/HNF3	2.31 ± 0.03	PPARG	-1.02 ± 0.03
		CEBPB	1.70 ± 0.16	TBP	1.26 ± 0.04
		HES1	1.64 ± 0.02		
		LDLR	1.57 ± 0.08		

Figure S1 : Search of links between genes selected for mRNA qunatificatioon and (A) hepatic functions (B) hepatic disorders (C) canonical pathways as defined by Ingenuity Systems Library. Gene nomenclature is reported in Table S4.

A-Healty liver



B-Hepatic diseases



C-Canonical pathways

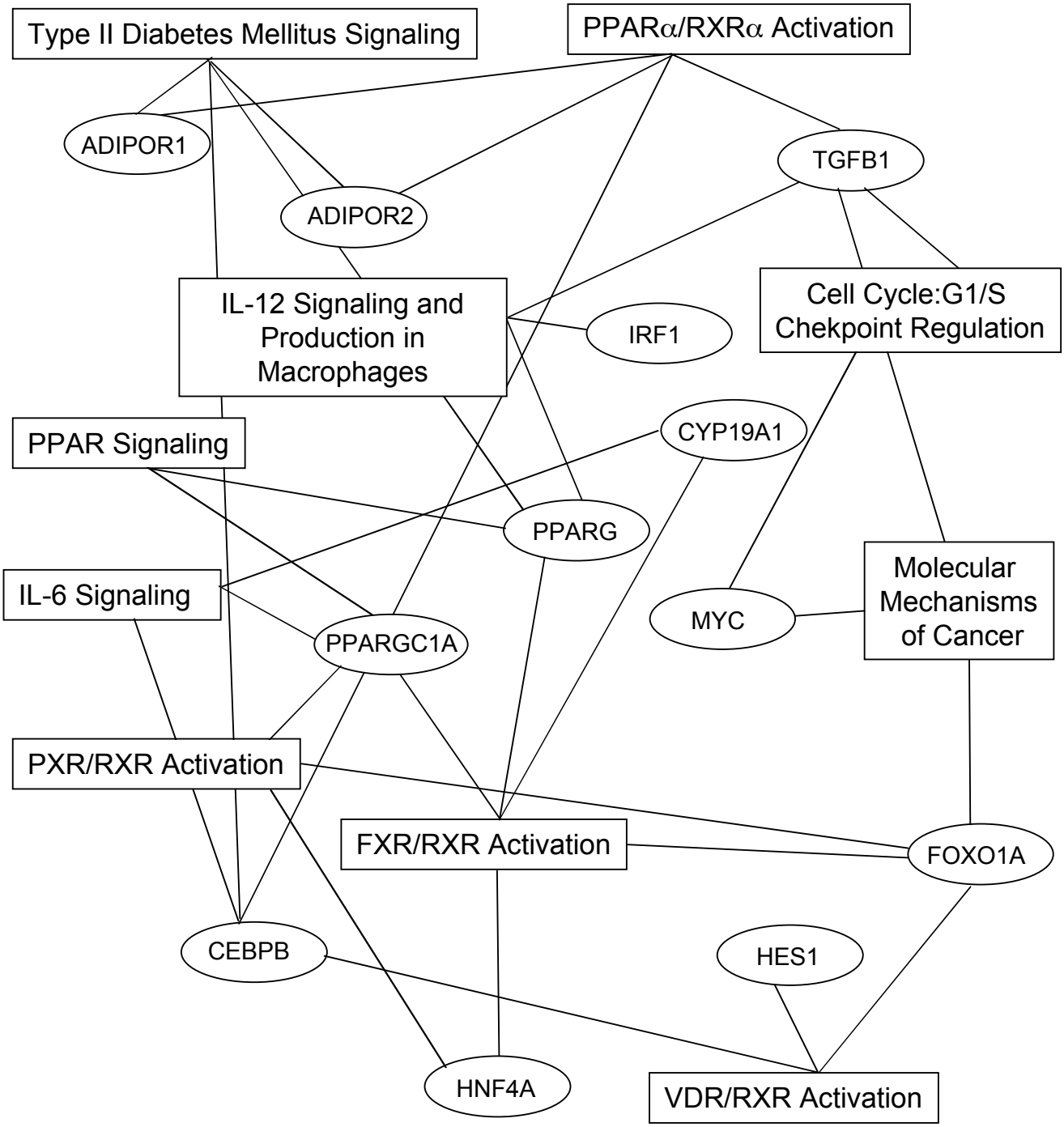
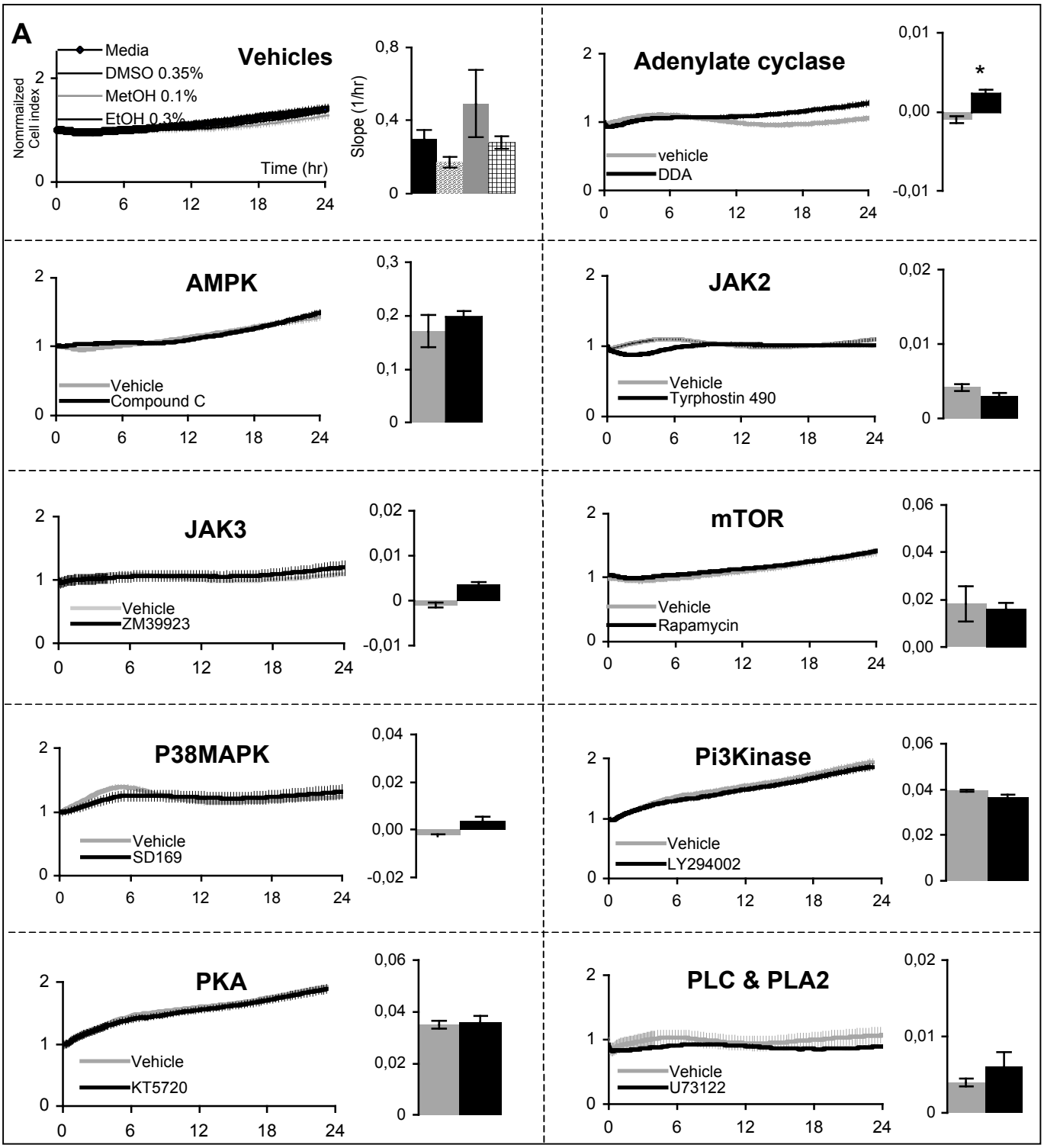
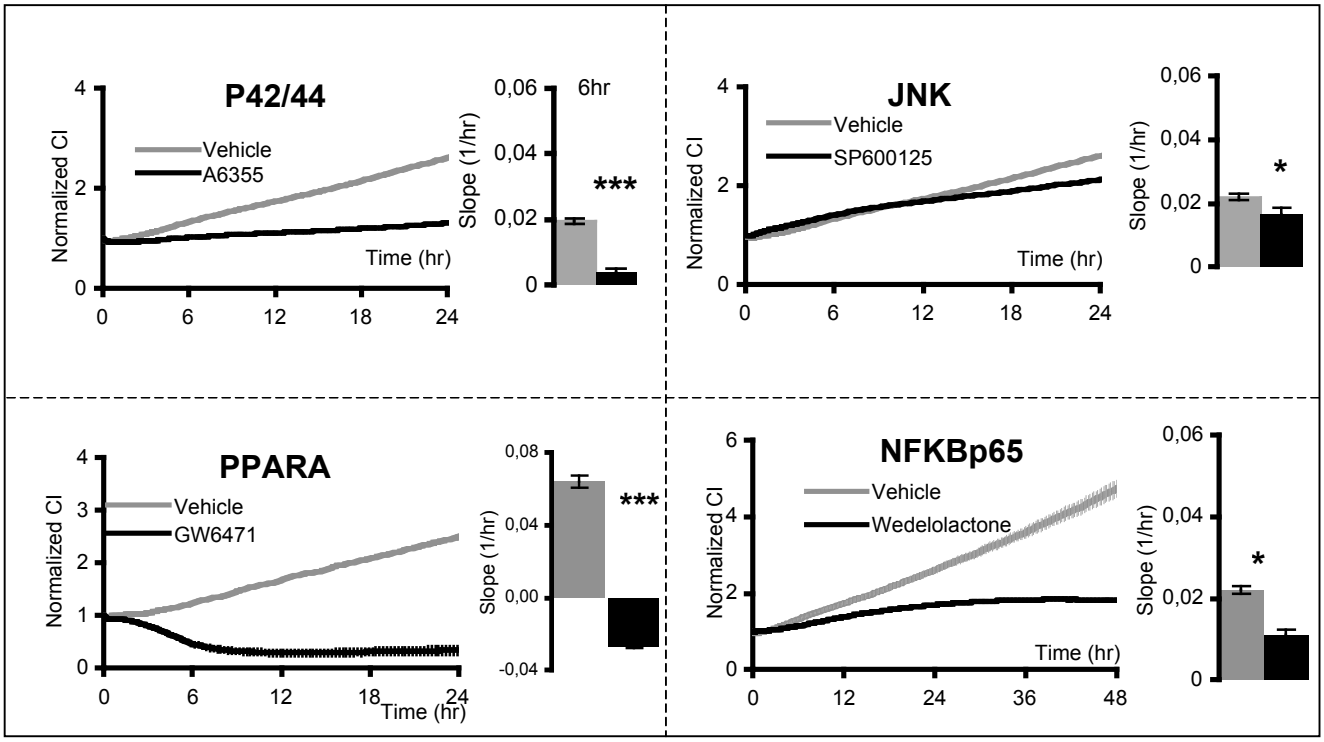


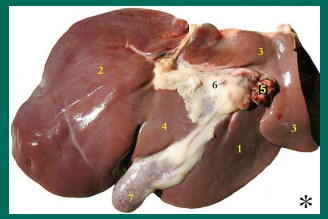
Figure S2: Real Time Cell Analysis of signaling pathways involved in cell growth and/or proliferation by using specific inhibitors on (A) HepG2 cells (B) HuH7 cells (during one day); name of pathways and concentrations are reported in Support. Information, Table S2. Left panel represents the cell index normalized at time of treatment, right panel corresponding slopes (representative experiments, mean values +/- SEM, n>6; Student test t p-value*< 0.05; **<0.001;***<0.0001).



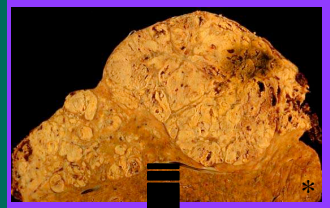
B


P42/44
Vehicle
A6355
Normalized CI
Time (hr)
Slope (1/hr)
6hr
***
JNK
SP600125
*
PPARA
GW6471
***
NFKBp65
Wedelolactone
*

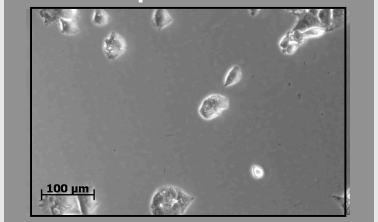
Liver



Hepatocellular carcinoma

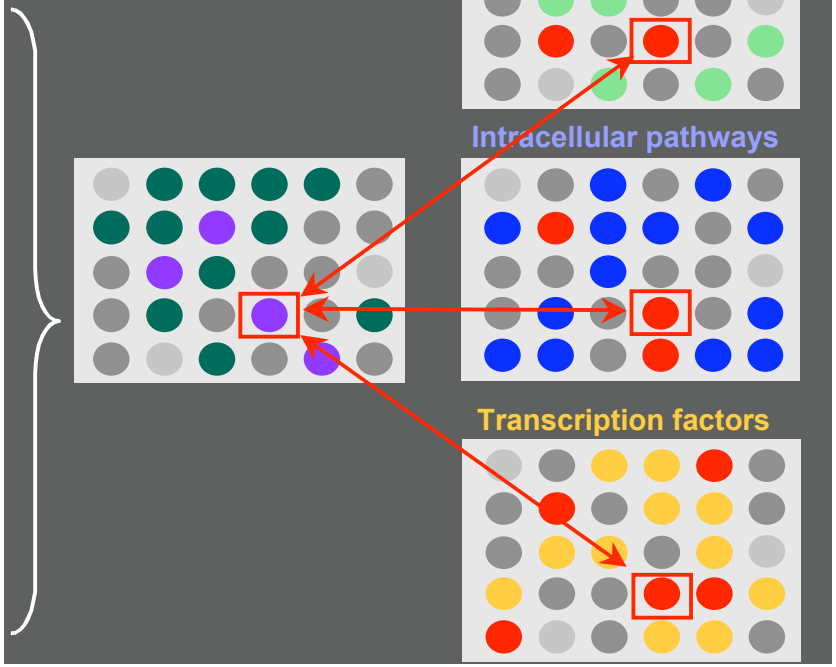


HepG2 cell line

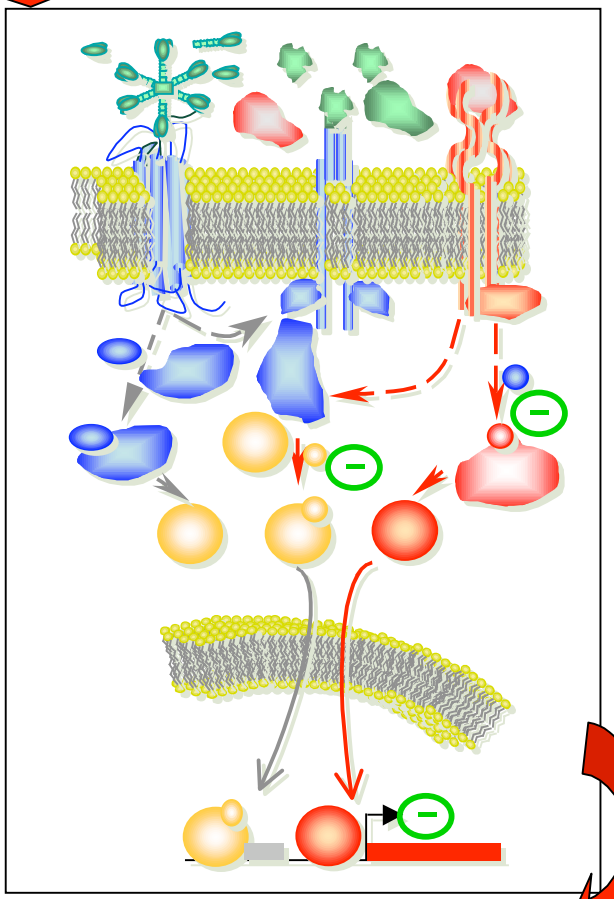


Bioinformatic analyses

Gene data sets:

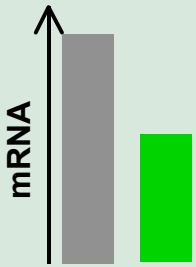


Identification

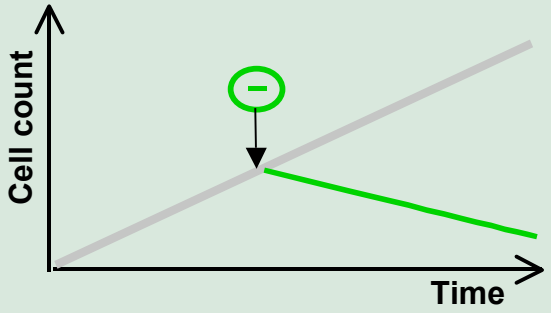


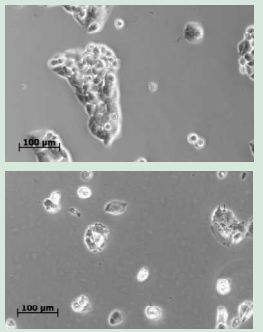
Validation

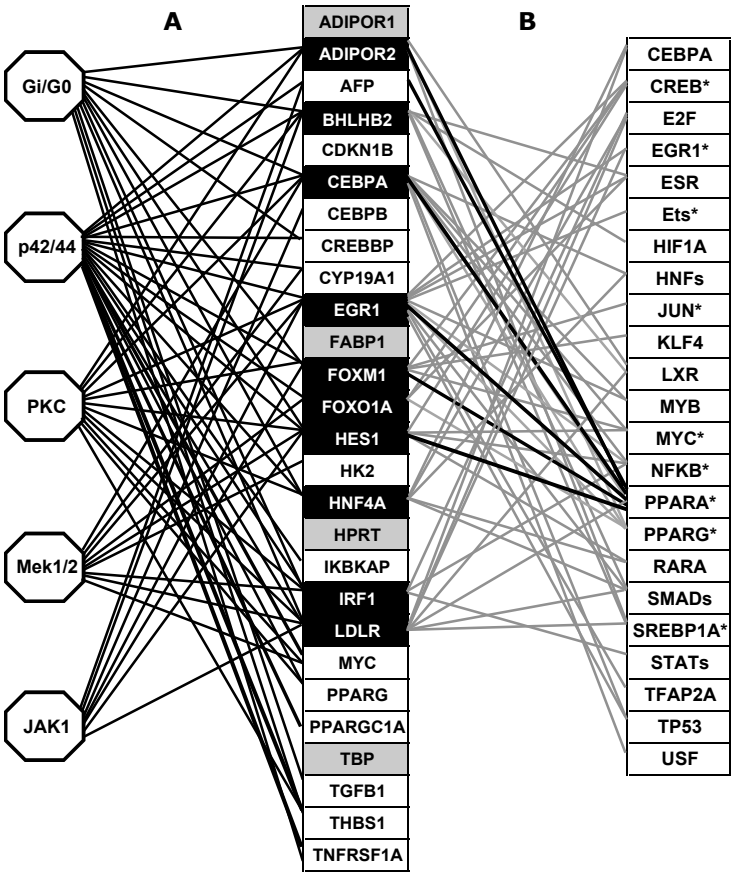
Quantitative RT-PCR



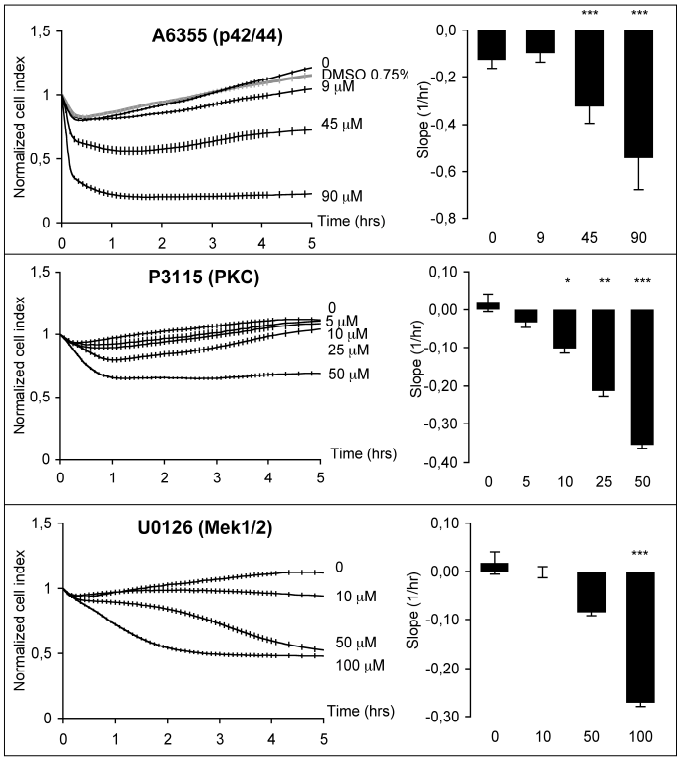
Real time proliferation assay (xCELLigence®)



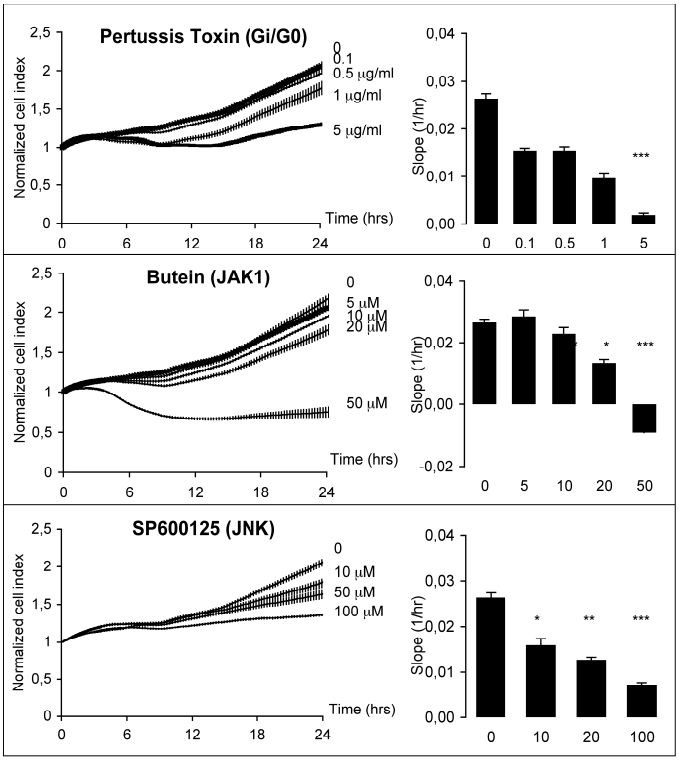



A
B
Gi/G0
p42/44
PKC
Mek1/2
JAK1
ADIPOR1
ADIPOR2
AFP
BHLHB2
CDKN1B
CEBPA
CEBPB
CREBBP
CYP19A1
EGR1
FABP1
FOXM1
FOXO1A
HES1
HK2
HNF4A
HPRT
IKBKAP
IRF1
LDLR
MYC
PPARG
PPARGC1A
TBP
TGFB1
THBS1
TNFRSF1A
CEBPA
CREB*
E2F
EGR1*
ESR
Ets*
HIF1A
HNFs
JUN*
KLF4
LXR
MYB
MYC*
NFKB*
PPARA*
PPARG*
RARA
SMADs
SREBP1A*
STATs
TFAP2A
TP53
USF

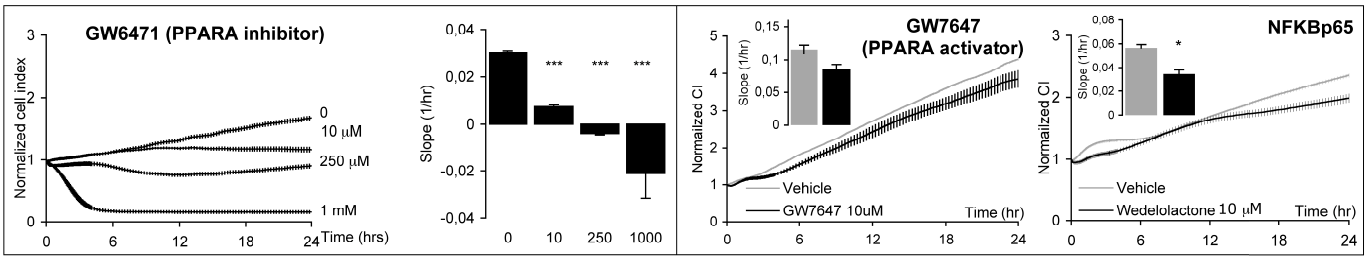
A- HIGHLY SENSITIVE PATHWAYS

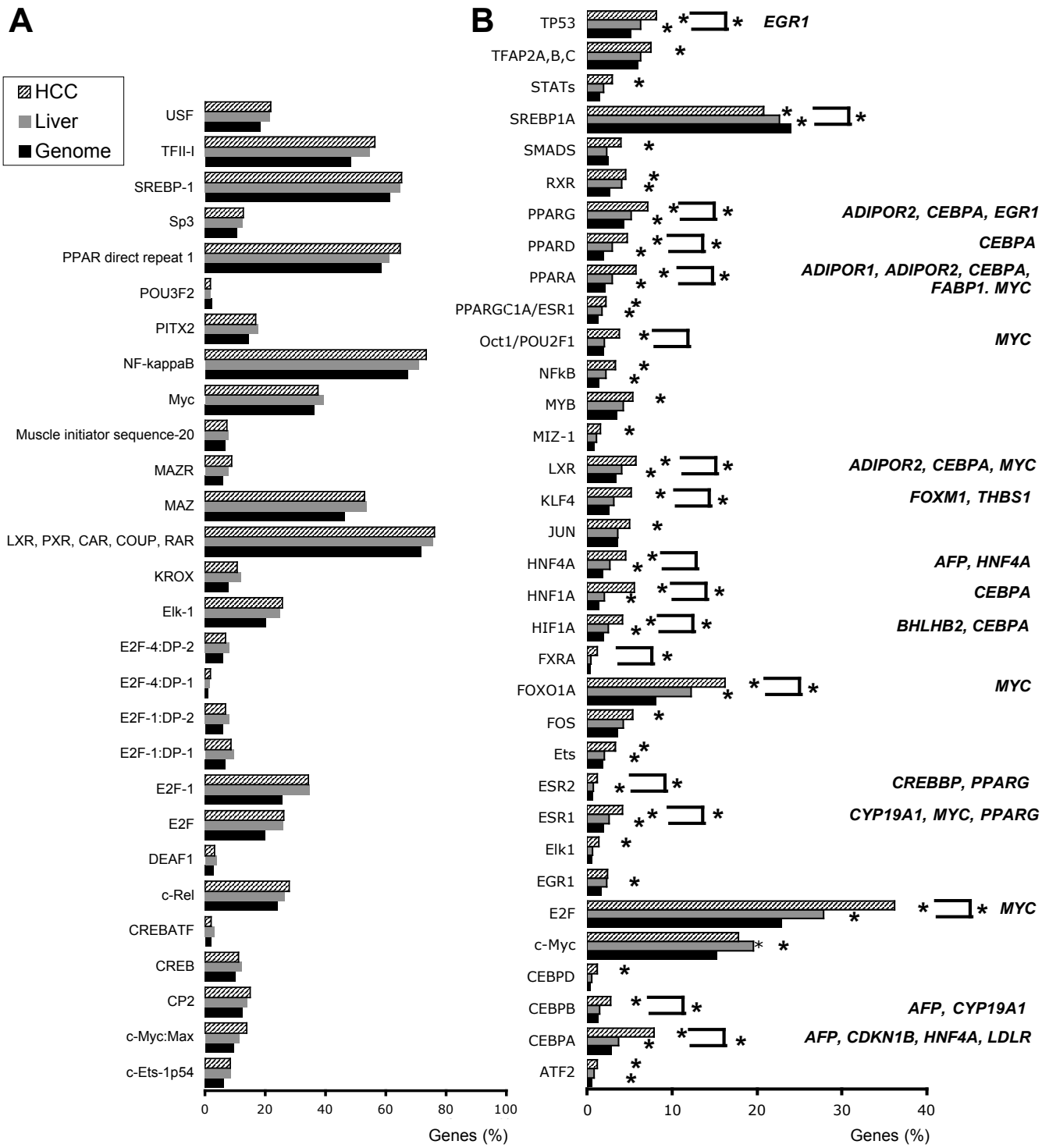


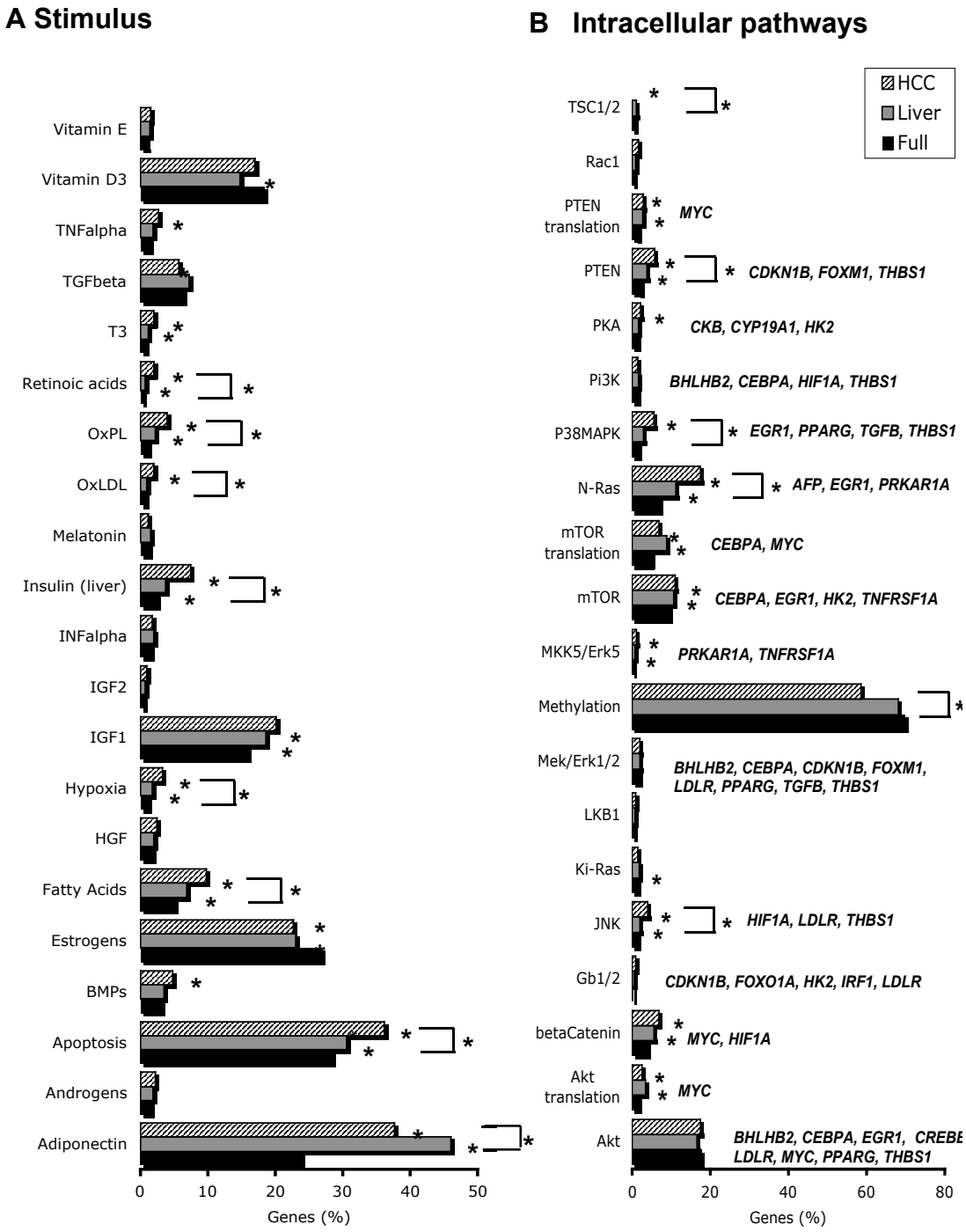
B- SLIGHT SENSITIVE PATHWAYS

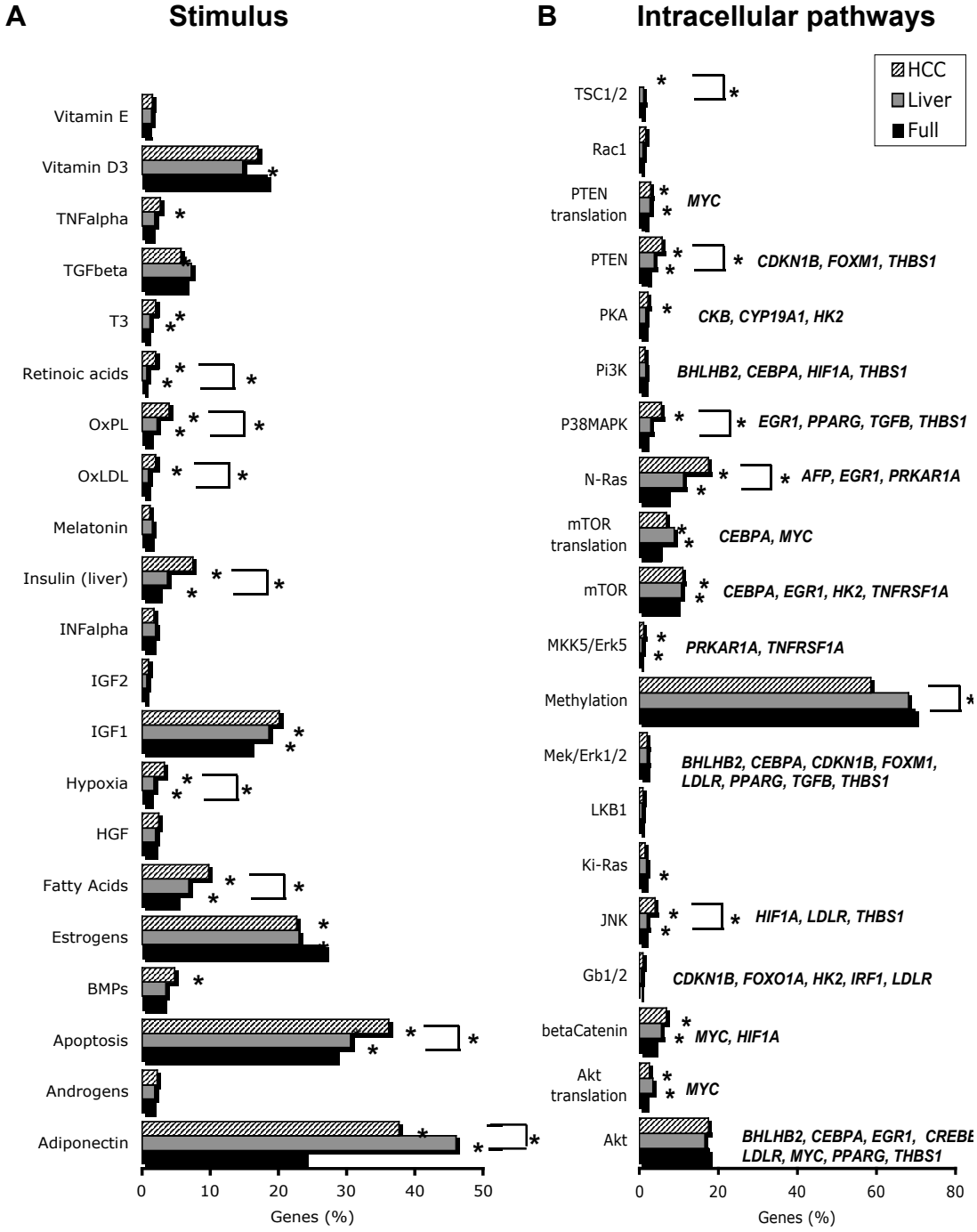


C- TRANSCRIPTION FACTORS




A
HCC
Liver
Genome
USF
TFII-I
SREBP-1
Sp3
PPAR direct repeat 1
POU3F2
PITX2
NF-kappaB
Myc
Muscle initiator sequence-20
MAZR
MAZ
LXR, PXR, CAR, COUP, RAR
KROX
Elk-1
E2F-4:DP-2
E2F-4:DP-1
E2F-1:DP-2
E2F-1:DP-1
E2F-1
E2F
DEAF1
c-Rel
CREBATF
CREB
CP2
c-Myc:Max
c-Ets-1p54
0
20
40
60
80
100
Genes (%)
B
TP53
TFAP2A,B,C
STATs
SREBP1A
SMADS
RXR
PPARG
PPARD
PPARA
PPARGC1A/ESR1
Oct1/POU2F1
NFkB
MYB
MIZ-1
LXR
KLF4
JUN
HNF4A
HNF1A
HIF1A
FXRA
FOXO1A
FOS
Ets
ESR2
ESR1
Elk1
EGR1
E2F
c-Myc
CEBPD
CEBPB
CEBPA
ATF2
EGR1
ADIPOR2, CEBPA, EGR1
CEBPA
ADIPOR1, ADIPOR2, CEBPA, FABP1. MYC
MYC
ADIPOR2, CEBPA, MYC
FOXM1, THBS1
AFP, HNF4A
CEBPA
BHLHB2, CEBPA
MYC
CREBBP, PPARG
CYP19A1, MYC, PPARG
MYC
AFP, CYP19A1
AFP, CDKN1B, HNF4A, LDLR
0
10
20
30
40
Genes (%)


A Stimulus
Vitamin E
Vitamin D3
TNFalpha
TGFbeta
T3
Retinoic acids
OxPL
OxLDL
Melatonin
Insulin (liver)
INFalpha
IGF2
IGF1
Hypoxia
HGF
Fatty Acids
Estrogens
BMPs
Apoptosis
Androgens
Adiponectin
0
10
20
30
40
50
Genes (%)
B Intracellular pathways
HCC
Liver
Full
TSC1/2
Rac1
PTEN translation
MYC
PTEN
CDKN1B, FOXM1, THBS1
PKA
CKB, CYP19A1, HK2
Pi3K
BHLHB2, CEBPA, HIF1A, THBS1
P38MAPK
EGR1, PPARG, TGFB, THBS1
N-Ras
AFP, EGR1, PRKAR1A
mTOR translation
CEBPA, MYC
mTOR
CEBPA, EGR1, HK2, TNFRSF1A
MKK5/Erk5
PRKAR1A, TNFRSF1A
Methylation
Mek/Erk1/2
BHLHB2, CEBPA, CDKN1B, FOXM1, LDLR, PPARG, TGFB, THBS1
LKB1
Ki-Ras
JNK
HIF1A, LDLR, THBS1
Gb1/2
CDKN1B, FOXO1A, HK2, IRF1, LDLR
betaCatenin
MYC, HIF1A
Akt translation
MYC
Akt
BHLHB2, CEBPA, EGR1, CREBE
LDLR, MYC, PPARG, THBS1
0
20
40
60
80
Genes (%)


A
Stimulus
Vitamin E
Vitamin D3
TNFalpha
TGFbeta
T3
Retinoic acids
OxPL
OxLDL
Melatonin
Insulin (liver)
INFalpha
IGF2
IGF1
Hypoxia
HGF
Fatty Acids
Estrogens
BMPs
Apoptosis
Androgens
Adiponectin
0
10
20
30
40
50
Genes (%)
B
Intracellular pathways
HCC
Liver
Full
TSC1/2
Rac1
PTEN translation
MYC
PTEN
CDKN1B, FOXM1, THBS1
PKA
CKB, CYP19A1, HK2
Pi3K
BHLHB2, CEBPA, HIF1A, THBS1
P38MAPK
EGR1, PPARG, TGFB, THBS1
N-Ras
AFP, EGR1, PRKAR1A
mTOR translation
CEBPA, MYC
mTOR
CEBPA, EGR1, HK2, TNFRSF1A
MKK5/Erk5
PRKAR1A, TNFRSF1A
Methylation
Mek/Erk1/2
BHLHB2, CEBPA, CDKN1B, FOXM1, LDLR, PPARG, TGFB, THBS1
LKB1
Ki-Ras
JNK
HIF1A, LDLR, THBS1
Gb1/2
CDKN1B, FOXO1A, HK2, IRF1, LDLR
betaCatenin
MYC, HIF1A
Akt translation
MYC
Akt
BHLHB2, CEBPA, EGR1, CREBE
LDLR, MYC, PPARG, THBS1
0
20
40
60
80
Genes (%)

	Genome (Fatigo +)			
		HepG2		
			Liver (set 1)	
Datasets				**HCC (set 2)**
Full	31524	20839	4918 (23.6%)	806 (16.4%)
Stimulus (24 sets)		4231 (20.3%)	2096 (42.6%)	401 (49.8%)
Intracellular pathways (21 sets)		5690 (27.3%)	2529 (51.4 %)	453 (56.2%)
Transcription factors (53 sets)		4736 (22.7%)	2473 (50.3%)	505 (62.7%)

Research Article

Gene network analysis leads to functional validation of pathways linked to cancer cell growth and survival.

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Keywords: human hepatocellular carcinoma, signal transduction, real-time proliferation assay.

Abbreviations:

ADIPOR1 and 2, adiponectin receptors 1 and 2 ; AMPK , 5'-AMP-activated protein kinase; BHLHB2, basic helix-loop-helix family, member e40 cAMP responsive element binding protein 1 ; CAR, constitutive androstane nuclear receptor ; CEBPA, CCAAT/enhancer binding protein alpha; CEBPs, CCAAT/enhancer binding proteins ; COUP, chicken ovalbumin upstream promoter-transcription factor ; DMEM : Dubbelco's minimum essential media ; FCS : fetal calf serum, FOXM1, forkhead box M1 ; FOXO1A, forkhead box O1 ; GO, gene ontology ; HCC, hepatocellular carcinoma ; HES1, hairy and enhancer of split 1 ; HIF1A, hypoxia inducible factor 1, alpha subunit ; HNF4A , hepatic nuclear factor 4-alpha; HPRT1 : hypoxanthine phosphoribosyltransferase 1 ; JAK, Janus kinase ; JNK, Jun-NH2 kinase ; KROX or EGR, early growth response factor; CREB, LXR, liver X nuclear receptor ; MAPK, mitogen activated protein kinase ; MYC, v-myc myelocytomatosis viral oncogene homolog ; NFKBp65, Nuclear factor kappa B p65 subunit; PKC, protein kinase C ; PPARA and G, peroxisome-proliferator activated receptors alpha and gamma ; PTEN,

phosphatase and tensin homolog ; PXR, pregnane X nuclear receptor ; RAR , retinoic acid receptor; ROS, reactive oxigen species ; RTCA: real-time cell analyser ; RT-qPCR, real time quantitative polymerase chain reaction ; SREBP1, sterol regulatory element binding protein 1.

ABSTRACT

Hepatocellular carcinoma (HCC) represents one of the most frequent human cancers and efforts are needed to find alternative treatments to surgical resection, like intense screening of druggable targets. In this study we performed bioinformatic analyses of previously published transcriptomic data in order to characterize liver specific networks, *i.e.* biological functions, signaling pathways and transcription factors, potentially deregulated in HCC. Then, by using real-time proliferation assay with specific inhibitors, we validated the *in silico* results. We found that G protein subunits Gi/G0, Protein kinase C, mitogen activated kinases Mek1/2, and Erk1/2 (P42/44), Janus kinase JAK1, Peroxisome-proliferator activated receptors alpha (PPARA) and Nuclear factor kappa B p65 subunit, were the major signaling pathways required for survival and proliferation in human HCC cell lines. We also found that these pathways regulate the expression of key hepatic transcription factors involved in cell differentiation, such as CCAAT/Enhancer binding protein alpha (CEBPA), Early growth response factor (EGR1), Forkhead box M1 (FOXM1) and the PPARs. Thus, our study shows that the combination of bioinformatic analyses and functional analyses provided the opportunity to find the major signaling pathways related to tumorigenicity.

1. Introduction

Hepatocellular carcinoma (HCC) represents more than 80% of primary, malignant liver tumors and many efforts are needed to find alternative treatments to surgical resection, including intensive screening of druggable targets [1]. It is well established that HCC are characterized by major alterations in gene expression, mainly related to cell growth and maintenance, cell cycle and cell proliferation as well as metabolism [2-4]. Several signaling pathways are also affected, like Wnt and MAPK pathways [5-7] with elevated expression and activation of P42/44 (Erk1/2) [8-9] and reduced P38 MAPK activity [10] in HCC. In addition a number of other kinases at the crosstalk between cell growth, stress and metabolism have also been associated to hepatocarcinogenesis, such as the energy sensor AMPK [11], Pi3kinase [12] and JAK/STATs [13]. These alterations are potentially associated with deregulation of key transcription factors, such as CEBPs and HNF4A [14-15]. However, despite this important bulk of data, it is still difficult to draw a clear picture of the molecular mechanisms deregulated in HCC. Given the availability of large amount of data in public databases, our objective in the present study was to find the major cellular pathways deregulated in human HCC. To this aim, we first analysed previously published transcriptomic data of normal liver, in HCC and in HepG2 cells, in order to define the major biological functions, signaling pathways and transcriptions factors related to human HCC. Then, by using real-time proliferation assay in the presence of specific inhibitors of these signaling pathways, related to cell proliferation and/or survival, we validated the *in silico* approach in human HCC-derived cell lines.

2. Materials and methods

2.1. Data analysis

The sets of genes expressed in human normal liver, HCC and HepG2 cells were obtained from previous published studies (**Supporting information, Table S1)**. Intracellular pathways or transcription factors modulated by various stimuli, were identified and retrieved from previously published experiments performed on different human cancer cell lines, and HepG2 cells (**Supporting information, Table S2** and [16]).

Significantly over-represented biological functions were retrieved by using Fatigo+ software from http://babelomics.bioinfo.cipf.es. The putative transcription factors associated with these biological functions were found by using Fatigo+ software. Gene networks were built using the Ingenuity Systems Pathway analysis at www.ingenuity.com. The online TRED database was used to define the potential target genes for relevant transcription factors at http://rulai.cshl.edu/cgi-bin/TED/tred.cgi?process=home.

2.2. Cell culture, treatments and analyses

The human hepatocarcinoma-derived cell lines HepG2 and HuH7 were provided from the European Collection of Cell Cultures (ECACC, Salisbury, UK). HepG2 cell line originated from human HCC is widely used as *in vitro* model to study hepatic functions as well as their deregulations in hepatic disorders. Cells were grown at 37°C in 5% CO2 in DMEM containing 10% fetal calf serum, complemented with streptomycin (100 mg/ml) and penicillin (100 units/ml). Cell proliferation and/or survival was monitored with the xCELLigence Real-time Cell Analyser (RTCA) System (Roche Diagnosis, Meylan, France), which allows label-free monitoring changes of cell number, viability, morphology and quality of cell attachment by measurement of cell-to electrode responses of cells seeded in E96-well plates manufactured with integrated microelectronic sensor arrays. Analyses were first performed to select accurate plating density (10 000 cells per well), reaching stable proliferation rate within one day (cell index 0.5 to 1). For signaling pathway analyses, specific inhibitors were applied in serum-free media one day after plating. Drug concentrations were optimized for each compound according to IC50 and dose-response analyses (**Supporting information, Table S3)**. As we found that HepG2 cells are highly sensitive to serum deprivation, we had to adapt the conditions to maintain cells in stable proliferating rate and thus the duration of the treatments was limited (not more than 24 hours) when studying the effects of drugs and inhibitors. The results are representative of at least three independent experiments and each condition was tested in at least 5 replicates.

2.3. Messenger RNA quantification by real time PCR

Total RNA purifications from HepG2 cells were performed according to standard protocol (Qiagen Quick prep mRNA, Qiagen, Courtaboeuf, France) including a DNase treatment. RNA integrity was assessed with the Agilent 2100 Bioanalyzer and RNA 6000 LabChip Kit (Agilent Technologies, Massy, France). First strand cDNAs were synthesized from 500 ng of total RNA in the presence of 100 U of Superscript (Invitrogen-Life Technologies, Eragny, France) and random hexamers and oligodT primers (Promega, Charbonnières, France). Real-time PCR was performed using ABsolute™ QPCR SYBR® Green ROX Mix (Abgene, Courtaboeuf, France) with a Rotor-GeneTM 6000 system (Corbett Life Science, Cambridgeshire, UK). Levels of target mRNAs were normalized to hypoxanthine phosphoribosyltransferase 1 (HPRT1) expression measured in all samples by RT-qPCR. All quantifications were performed at least on three independent experiments and data are presented as mean ± SEM. Gene names, references, functions, primers and respective qPCR conditions are reported in **Supporting information, Table S4**.

3. Results

3.1. Bioinformatic analysis of hepatic genes related to normal and HCC phenotypes.

We first define a list of genes commonly expressed in HepG2 cell line and in human normal liver (**Figure 1**) from published global transcriptomic data. Then, we selected a second set of genes with altered expression in human HCC (806 genes). These two lists of genes were compared by using Fatigo+ software in order to identify significantly enriched biological functions in human HCC. **Figure 2**A shows that they were linked to metabolism, cell proliferation and cell death. An analysis of biological processes over-represented in HCC *versus* hepatic HepG2 genes revealed a significant deregulation of genes involved in metabolism and coagulation (**Figure 2B**). Genes representative of these biological functions were selected for further analyses (**Supporting information, Table S4**). Using Ingenuity software, we constructed the relationships between genes involved liver functions (**Supporting information, Figure S1A**), hepatic diseases (**Supporting information, Figure S1B**) and in hepatic canonical pathways (**Supporting information, Figure S1C**). We found a number of links suggesting the implication of these genes in HCC and in liver cell proliferation and survival.

3.2. Signaling pathways over-represented in set of genes deregulated in human HCC

We next identified by computational analyses, different groups of genes regulated by external stimuli (21 different conditions) or intracellular signaling pathways (20 different conditions) expressed in normal liver (gene set 1) but deregulated in HCC (gene set 2). (**Figure 3;** references of gene sets are reported in **Supporting information, Tables S2**). We found that the list of genes deregulated in HCC is significantly enriched in genes also regulated by insulin, by fatty acids and adiponectin, by retinoic acids, by stress conditions (such as oxidized lipids and hypoxia) and during apoptosis. Furthermore, there is a specific enrichment in genes under the control of the Ras-coupled pathways Mek/Erk and P38MAPK, the JNK pathway and the PTEN/Pi3K pathway.

3.3. Transcription factors regulating hepatic and HCC gene expression

In order to determine the transcription network involved in hepatic function in HepG2 (set 1) and deregulated in HCC (set 2) , we searched for transcription factors having putative binding sites in the promoter regions of these 2 sets of genes by using Fatigo+ software. Twenty-eight significant binding sites appeared to be over-represented in the promoter region of both sets in comparison to the human genome (**Figure 4A**) and are linked to transcription factors known to be involved in the regulation of cell cycle (E2Fs, MYC), to MAPK-regulated pathways (KROX, Elk1, CREB), to metabolic pathways, such as PPARs,

SREBP1 and other nuclear receptors (LXR, PXR, CAR, COUP, RAR), and to inflammatory response (NFKB and c-Rel).

In order to determine whether the 28 transcription factors with significant over-represented binding sites in the promoter region of gene sets 1 and 2 were functional, we analysed the lists of genes regulated by 53 different transcription factors in human tissues (**Figure 4B**). This second approach thus took into account experimental data obtained mainly in cancer cell lines, including HepG2 cells, and have been previously published [16]. A set of 4736 genes (i.e. 22.7% of human genome) regulated by at least one of these transcription factors was retrieved, also regulating 50.3% of genes in set 1 and 62.7 % in set 2 (**Figure 1**). We found an over-representation of genes regulated by 34 transcription factors in either normal liver or HCC gene sets, including CEBPs, E2Fs, EGR1, FOXO1A, HNFs, MYC, NFKB and PPARs. Importantly, 16 of these transcription factors regulate expression of genes present in HCC gene set, including hepatic genes regulated by CEPBA and CEBPB, FOXO1A, HNF4A, PPARs, steroid responsive transcriptions factors (ESR1 and 2, LXR), and stress and apoptosis linked transcription factors such as TP53 or HIF1A. NFKB and E2Fs.

3.4. HepG2 cell growth, proliferation and survival depend on basal MAPK activities.

We then investigated the potential implication of the signaling pathways identified by computational analyses (**Figure 3**) on hepatic cell growth, proliferation and survival. HepG2 cells were treated with a panel of specific kinase inhibitors and corresponding vehicles as controls (**Supporting information, Table S3)** and their growth was monitored with xCELLigence System using Real-time Cell Analyser (RTCA) Instrument. Cell indexes, which represent cell surface occupancy, and thus cell number, were normalized at time of treatment (one day after plating) and slopes of resulting curves were calculated after treatment (**Figure 5 ; Supporting information, Figure S2**). Among the 15 pathways explored, only 6 inhibitors were found to significantly affect HepG2 cell proliferation. Inhibition of adenylate cycle slightly but significantly increased HepG2 cell proliferation. HepG2 cell survival was affected in less than one hour in a dose-dependent manner by specific inhibitors of P42/44, PKC and Mek1/2 respectively (**Figure 5A**). HepG2 cell proliferation was found to be reduced after one day by specific inhibitors of Gi/G0 and JAK1 (not JAK2 and JAK3) and in a lesser extend JNK (**Figure 5B**). Similar effects were observed for both P42/44 and JNK inhibitors when applied on HuH7 cells (**Supporting information, Figure S2B**).

3.5. Characterization of genes and transcription factors linked to HepG2 cell proliferation and survival

Finally, in order to experimentally validate the implication of the different signaling pathways identified, we measured the expression level of a selected number of genes expressed in

HepG2 cells treated with specific inhibitors of either Gi/G0, P42/44, Mek1/2, PKC and JAK1 pathways. The whole RT-qPCR data are reported in **Supporting information, Table S5.** An integrative view of the results obtained by both computational analyses of gene data sets and RT-qPCR is provided in **Figure 6**. We found that ADIPOR2, BHLHB2, FOXM1, HES1 and LDLR were repressed and CEBPA was induced by basal activity of either Gi/G0, P42/44 and PKC. These 6 genes were further used to identify candidate transcription factors involved in their regulation. According to transcriptional data in human cells (cf **Figure 4**), these genes might be regulated by at least 23 types of transcription factors, 9 of them being involved in P42/44 signaling pathway (i .e CREB, EGR1, Ets, JUN, MYC, NFKB, PPARA and G, and SREBP1A), 4 of them could be regulated by CREB, NFKB, PPARA and PPARG. Interstingly, genes regulated by either NFKB, PPARA and PPARG are also over-represented in both normal liver and in HCC gene sets (cf **Figure 4**). As an example, we validated that PPARA, a major nuclear factor controlling hepatic metabolism, was a transcriptional activator of EGR1, FOXM1, HES1 and ADIPOR2, and inhibited CEBPA gene transcription in HepG2 cells (**Table 1**). RTCA monitoring revealed that PPARA specific inhibitor GW6471 significantly affected HepG2 survival in a dose-dependent manner, while its specific activator GW7647 had no significant effect (**Figure 5C**). In addition, by using its specific inhibitor Wedelolactone we also found that NFKBp65 was required for HepG2 cell proliferation (**Figure 5C**). The effects of both GW6471 and Wedelactone were also observed on HuH7 cell line (**Supporting information, Figure S2B**).

4. Discussion

In this study, we report the identification of transcription networks and signaling pathways involved in normal hepatic functions being deregulated in human HCC. These results were obtained by computational analyses of published data sets. They were then validated by using real-time cell proliferation assays on HepG2 cells treated by specific inhibitors of signaling pathways and by quantification of gene expression levels. The present study provides therefore a comprehensive global picture of the complex networks including specific signaling pathways, transcription factors and gene regulations potentially contributing to the proliferation of transformed liver cells. As expected, most of the major features already described in the literature were found and thus confirmed in this study, such as hepatic specialization in regulation of metabolism and deregulation of cell growth and survival pathways in HCC.

The process of tumorigenesis involves deregulations of pathways controlling cell growth, proliferation and survival in normal conditions. These are due to abnormal responses to external stimuli and/or to defective intracellular regulations of these pathways. Using computational analyses of published data sets obtained in human cells or tissues we

identified biological pathways and gene networks over-represented in HepG2 and in HCC and we confirmed their implication in cell proliferation using Real-time cell Analyser Instrument (xCELLigence system).

Among the signaling pathways involved in HepG2 cell survival, which are either over-expressed or up-regulated in HCC, we identified PKC [17], P42/44 [10], Gi/G0 [18] as well as NFKBp65 [19] and JAK1 (but not JAK2 nor JAK3). JAK/STAT pathways are involved in the progression of cell cycle and to confer resistance to apoptosis [20, 21]. Our result can be explained by the fact that JAK1 inhibitor butein may also activate JNK and ROS production in HepG2 cells, leading to cell cycle arrest and apoptosis [22]. The lack of effect of the JAKs could be a consequence of the well described constitutive activation of STAT3 in most HCC as well as in HepG2 cells [23].

PKC is known to activate NFKB by isoform alpha and P42/44 by isoform beta in HCC [24]. In our study, NFKB was identified by both computational analyses of enriched transcription factor target genes and binding site enrichment in HepG2 and HCC. Moreover basal NFKBp65 activity appeared to be required for HepG2 cell growth. These results are in accordance with other studies showing that inhibition of NFKB as well as PKCalpha isoform in HepG2 cells can induce apoptosis [25] and that p65 subunit is involved in HepG2 cell survival [26, 27]. The second pathway regulated by PKC is linked to P42/44. High levels of basal P42/44 activated form in HCC has been shown to involve the Ras/Mek/Erk/FOXM1 pathway [28]. We found here that P42/44 inactivation led to cell death in HepG2 and to cell cycle arrest in HuH7 cells. Moreover we found a regulation of FOXM1 gene expression by PKC and by P42/44 itself. In other studies, FOXM1 has been found to exert an anti-apoptotic activity in HCC and is up-regulated in liver cancer [29, 30]. P42/44 is known to regulate the activity of the transcription factor MYC, which was also regulated at the transcriptional level in HepG2 by both PKC and P42/44 pathways in our experiments. MYC is considered as a tumor suppressor because of its pro-apoptotic activity and its down-regulation in both HCC and liver cancers [31]. EGR1 is another tumor suppressor known to be regulated by the P42/44 signaling pathway. We found that inhibition of P42/44 activity in HepG2 cells strongly induced expression of EGR1 gene. In the same way, its transcription is down-regulated in HCC and liver cancers [32], suggesting that EGR1 may play important roles in liver cancers. PKC and P42/44 also regulate the transcriptional activity of nuclear receptors PPARs [33]. Whether the PPARs are involved in hepatocellular carcinogenesis remains elusive. In several studies, activation of PPARG has been shown to induce apoptosis in HepG2 cells [34-37] and to reduce tumor growth in HCC [38, 39], and microarrays data suggest that PPARG may regulate BHLHB2, CEBPA, EGR1 and FOXO1A in HepG2 cells [40]. In the present study, we found that PPARA also regulates transcription of EGR1, FOXO1A and FOXM1. PPARA is highly expressed in liver where it is a key regulator of lipid metabolism

and its regulatory activity on fatty acid oxidation has been established in HepG2 cells [41-43]. In both HepG2 and HuH7 cell lines we did not found any effect of PPARA activation on cell growth and/or survival, however its inactivation greatly affected their survival. These results are in accordance with the hypothesis that PPARA basal activity may protect liver cancer cells from death. Recent experiments on HepG2 cells support the hypothesis that PPARG activation may be related to cell proliferation while activation of PPARA leads to apoptosis [44]. In liver PPARA appears to regulate cell cycle progression and cell survival through P38MAPK pathway [45] [45]. This nuclear receptor has also be found to stimulate hepatic carcinogenesis in mice liver [46]. Taken together, these data point out a key role of PPARA in HCC.

Interestingly, we found that transcription of CEBPA, another important hepatic transcription factor, was induced by the basal activity of PKC, Gi/G0, Mek1/2 and P42/44. In humans CEBPA is highly expressed in liver [47] where it regulates hepatic cell differentiation by strong arrest of cell proliferation [48, 49]. CEBPA gene expression is deregulated in HCC [6] and recently, epigenetic mechanisms have been proposed to explain how CEBPA could be deregulated in HepG2 cells [50, 51]. Our computational analyses also support the hypothesis of deregulation in methylation status in HCC genes (**Figure 3B**). We also found that CEBPA gene expression was induced by PPARA in HepG2 cells. Thus altered expression of CEBPA in many HCC might be the consequence of deregulations in the process of chromatine methylation, in activity or in transcriptional regulation of transcription factors, such as MYC [52] or PPARs [40]. Therefore a finely tuned regulation of CEBPA may represent a key event involved in the process of hepatic tumorigenesis.

In conclusion, combinatorial analyses of the global gene transcription networks, real time proliferation assay analysis of selected signaling pathways and gene expression analyses converged to identify key signaling pathways deregulated in human HCC. First we identified pathways related to cell survival (P42/44, PKC, Mek1/2, PPARA) and pathways involved in cell growth and proliferation (Gi/G0, JAK1, JNK and NFKB) in HCC cell lines. Second these pathways commonly control the expression of key transcription factors (i.e. they inhibit several tumor suppressors and they activate liver cell differentiation factor CEBPA). Most of the results are in agreement with published data, and this approach gives the opportunity to depict in few and rapid experiments an overall view of the molecular mechanisms involved in cell growth and proliferation.

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The authors declare that they have no conflict of interest with the present work.

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LEGENDS OF FIGURES

Table 1: mRNA levels of target genes modulated by peroxisome-proliferator activated receptors alpha activator GW7647 (1 μM) in HepG2 cells (cultured for 5 hours without serum). Fold changes (stimulated *versus* control) were normalized to HPRT1, significantly modulated genes identified by ANOVA test ($p < 0.001$). For complete gene names and sequence references, see Supporting information, Table S4.

Genes activated by GW7647:

EGR1	8.45±0.12
PPARGC1A	3.76±0.60
HES1	1.81±0.02
ADIPOR1	1.79±0.04
FOXM1/HNF3	1.75±0.04
AFP	1.73±0.41
CREBBP	1.65±0.05

Genes repressed by GW7647:

CEBPA	0.42±0.01

Genes not modulated by GW7647:

ADIPOR1, BHLHB2, CEBPB, FABP1, FOXO1A, LDLR, MYC, PPARG, TBP

Figure 1: The 31 525 of human genes contained in the Fatigo+ database were separated into subsets. Set 1 contained the hepatic genes detected in HepG2 cells; set 2 the hepatic genes deregulated in hepatocellular carcinoma and present in HepG2 cells. Then we determined, among these 2 subsets of genes, the percentage of genes affected by different external stimuli**,** or modulated by known intracellular pathways and transcription factors by using previously published data which are reported in the Supporting information, Tables S1 and S2.

Figure 2 : Functional enrichment analysis of the biological functions by using Fatigo+ software in (**A**) Hepatic genes expressed in HepG2 (gene set 1) and deregulated in hepatocellular carcinoma (HCC, gene set 2) compared to all human genes (31 524 genes). Only most representative biological functions. i.e. highest hierarchical clustering Level 6, are presented and were found to be significantly over-represented in both gene sets (Fisher's exact test, adjusted p-value <0.001). (**B**) Biological functions significantly over-represented in the list of HCC genes (gene set 2) in comparison to hepatic genes (gene set 1) (highest hierarchical clustering Levels 3-5). X-axis represents the number of genes involved in these functions in either healthy liver and in HCC. Genes selected for further analyses are indicated on the right side of the panel. For complete gene names, see Supporting information, Table S4.

Figure 3 : Identification of signaling pathways specifically over-represented in human healthy liver and in hepatocellular carcinoma (HCC) from analysis of previously published transcriptional data. Frequency of genes regulated (x-axis) by either **(A)** external signals (Stimulus, 21 data sets) and **(B)** intracellular signaling pathways (20 data sets) were calculated with sets of genes detected in HepG2 cells (Full set), specific to healthy liver and deregulated HCC**.** Significant differences (i.e. Z-test scores with confidence level > 90) indicated by asterisks represent pathways over-represented in either liver and HCC sets potentially linked to hepatic functions, significant differences in HCC *versus* liver (barrels and asterisks) identify pathways which may be affected in HCC. Target genes selected for validation by qRT PCR analysis are shown in italics. For complete gene names and references, see Supporting information, Table S4. Sets of human genes retrieved from published global gene analyses (According to the procedure previously described [16]). Complete data and references are provided in Supporting information, Table S2.

Figure 4: Identification of transcription factors potentially involved the regulation of healthy liver gene transcription and deregulated in hepatocellular carcinoma (HCC). (**A**) Bioinformatic analysis through statistical Fatigo+ screening of binding site enrichment : frequency of given binding site in either liver (hepatic genes detected in HepG2 cells, gene set 1, 4918 genes) and HCC (gene set 2, 806 genes) was compared to the complete set of genes detected in HepG2 cells (20 942 genes). Only binding sites with significant differences in representativity in either healthy liver and HCC are shown ($p<0.05$). (**B**) According to the procedure previously described [16], sets of human genes modulated by transcription factors were retrieved from published experiments (53 sets). For each transcription factor, frequency of genes (x-axis) modulated was calculated for either genome, liver and HCC gene sets. Z-test calculation was applied on adjusted gene sets (restricted to sets of genes modulated by at least one transcription factor) to compare these frequencies in either liver (4918 genes) and HCC (505 genes) *versus* rest of genome (4736 genes) (represented by an asterisk), or HCC *versus* liver profiles (barrel with asterisk). Only transcription factors sets with significant differences in representativity are shown, i.e. 34 sets (confidence level > 90). Full data sets and references are provided in [16]. Genes selected for validation by qRT PCR are indicated in italics (for gene names and references, see Supporting information, Table S4).

Figure 5: Analysis of signaling pathways involved in HepG2 cell survival and/or proliferation by real time monitoring of cell number on xCELLigence system. HepG2 cells were seeded at 5 000 cells/E-plate well and grown in FCS containing medium for 24 h. Then medium was replaced by FCS-deprived medium containing either vehicle or specific inhibitors at several concentrations. Left panels represent cell indexes calculated at time x (tx) normalized at the time of the inhibitor was applied (t0), i.e. cell index at tx divided by cell index at t0. The cell index was measured every 5 min in 50 cycles, followed by every 15 min for 48 h (x-axis). Right panels report corresponding curve slopes calculated at indicated times, depanding on inhibitor activity: (**A**) highly sensitive intracellular pathways (slopes calculated for cell indexes measured during 1 hour after treatment); (**B**) slight sensitive intracellular pathways (slopes calculated for cell indexes measured during 24 hours). (**C**) Selective activation and/or inhibition of transcription factors (slopes calculated for cell indexes measured during 24 hours).

Data are presented as mean values ± SEM (representative experiments with at least 5 replicates). Significant differences were determined through Student's test, i.e. p-values $p<0.05$ (*), $p<0.001$ (**), $p<0.0001$ (***). For inhibitor complete nomenclature see Supporting information, Table S3. Abbreviations: JNK: Jun-NH2 kinase; JAK1: janus kinase 1; NFKBp65: nuclear factor kappa B, p65 subunit; PKC : protein kinase C; PPARA: peroxisome-proliferator activated receptor alpha.

Figure 6 : Transcriptional networks regulated by signaling pathways involved in HepG2 cell growth and/or survival. The central panel represents the set of genes analyzed by qRT PCR. The grey stone genes were not modulated by any drug tested. In the left panel are reported the signaling pathways involved in cell growth and/or survival and in bold black lines their target genes (panel **A**). Genes regulated by at least 3 pathways (white fonts) were selected for further analysis. In the right side (grey lines) are linked the transcription factors regulating these genes as reported in previously published microarray data sets [16] (panel **B**). Black lines represent links validated by qRT PCR in HepG2 cells using specific activator of peroxisome-proliferator activating receptor alpha (PPARA). Transcription factors modulated by P42/44 in human cells are indicated by asterisks. Full qRT PCR data are included in Supporting information, Table S5 and Table 1.