Case studies from SEURAT-1
DETECTIVE

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Increasing demands on chemical risk assessment

- High failure rate of new drug candidates due to unmanageable toxicity, accounting for approximately 30% of this attrition

- The EU REACH program on industrial chemicals
  - Existing and new substances should in the future be subject to the same procedure under a single system.
  - Large amounts of additional tests required before 2018
  - 30,000 existing chemicals already placed on the market since before 1981 and sold at > 1 tonne per year

- EU-wide ban on animal use in cosmetics development.
  - European Community Cosmetics Directive (Directive 76/768/EEC) and its 7th Amendment (2003/15/EC)

- Future EU regulations on food chemicals

Safety Evaluation Ultimately Replacing Animal Testing

SEURAT-1

Towards the Replacement of in vivo Repeated Dose Systemic Toxicity Testing

BUILDING BLOCKS: construction of a solid foundation in an integrated approach of six research projects ("building blocks"), each dedicated to a specific topic:

ScreenTox	Stem cell differentiation for providing human-based organ specific target cells to assay toxicity pathways *in vitro*
HeMiBio	Development of a hepatic microfluidic bioreactor mimicking the complex structure and function of the human liver
DETECTIVE	Identification and investigation of human biomarkers in cellular models for repeated dose *in vitro* testing
COSMOS	Delivery of an integrated suite of computational tools to predict the effects of long-term exposure to chemicals in humans based on *in silico* calculations
NOTOX	Development of systems biological tools for organotypic human cell cultures suitable for long term toxicity testing and the identification and analysis of pathways of toxicological relevance
ToxBank	Data management, cell and tissue banking, selection of reference compounds and chemical repository
COACH	Cluster level coordinating and support action

SEVENTH FRAMEWORK PROGRAMME

Cosmetics Europe
the personal care association

DETECTIVE

Detection of Endpoints and Biomarkers for Repeated Dose Toxicity Using In Vitro Systems

Detection of endpoints and biomarkers for repeated dose toxicity using *in vitro* systems

- DETECTIVE will set up a screening pipeline of functional and "-omics" technologies, including high content and high throughput screening platforms, to develop and investigate human biomarkers for repeated dose toxicity in cellular *in vitro* models.
- Emphasis will be put on the systematic exploitation of functional and "-omics" readouts, including high content and high throughput screening platforms.
- While functional parameters give more insights into the effects of toxicants on specific cell functions of interest, "-omics" techniques will deliver data on the entire cellular situation at the molecular level.

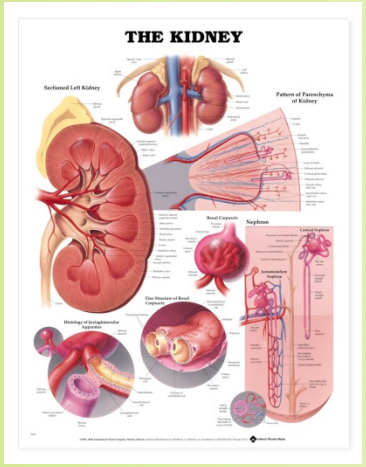
→ DETECTIVE will perform for the first time an in-depth investigation of repeated dose effects on epigenetics and microRNA (miRNA) expression thus exploring whether such analyses deepen our understanding of toxic modes of action.

SEVENTH FRAMEWORK PROGRAMME

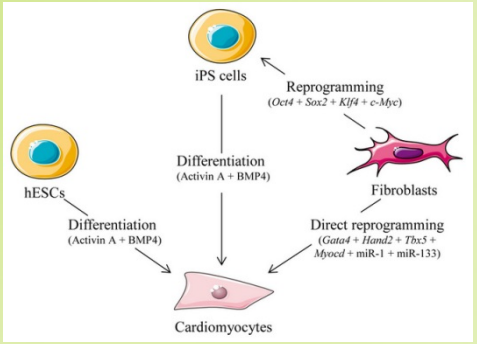
Cosmetics Europe
the personal care association

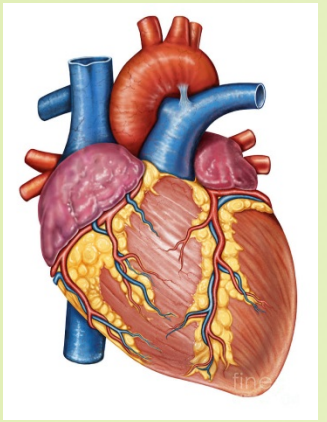
Organ models

1. Kidney model: RPTEC/TERT1 cells: human renal proximal tubule cell line

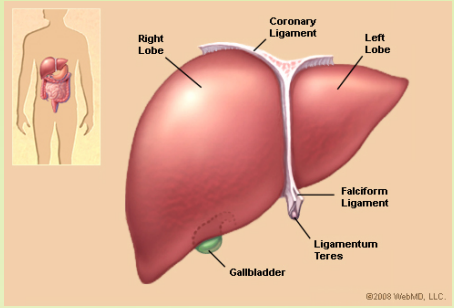


2. Heart model: human induced pluripotent stem (iPS) cardiomyocytes

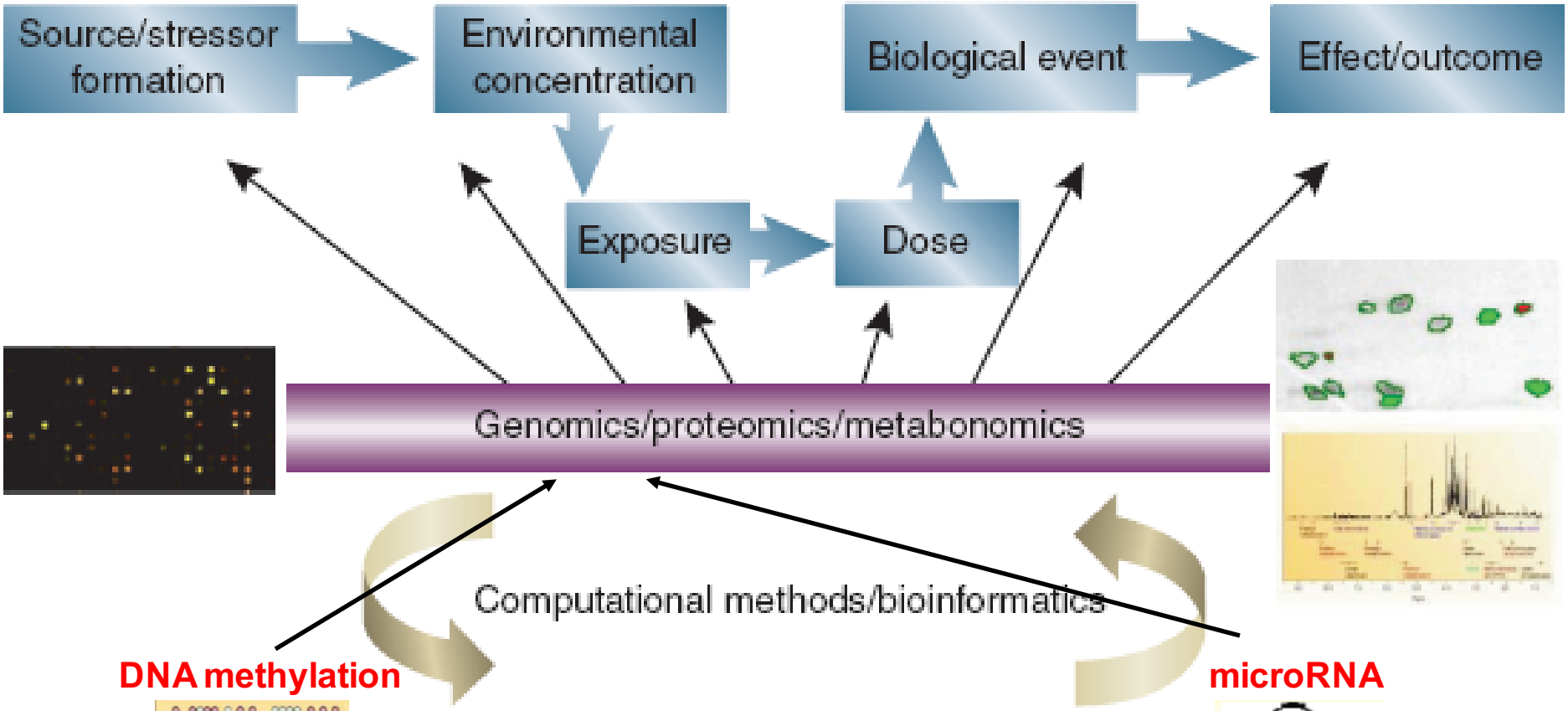




3. Liver model: primary human hepatocytes, HepaRG, HepG2



Integration of transcriptomics, proteomics, metabonomics with epigenetics and µRNA and bioinformatics in predictive toxicology



Main hypothesis:

Toxicant-induced changes in molecular networks which persist after terminating repeated dosing *in vitro*, present promising biomarkers for repeated dose toxicity in humans

Liver experiments @UM

1) "Assessment of repeated dose toxicity of valproic acid in the human liver using integrative '-omics' data analyses"

   →integrated data analyses of DNA methylation, gene expression and miRNAs in order to find novel mechanisms of VPA induced liver steatosis

2) "Assessment of repeated dose toxicity of aflatoxin B1 (AFB1) in the human liver using integrative '- omics' data analyses"

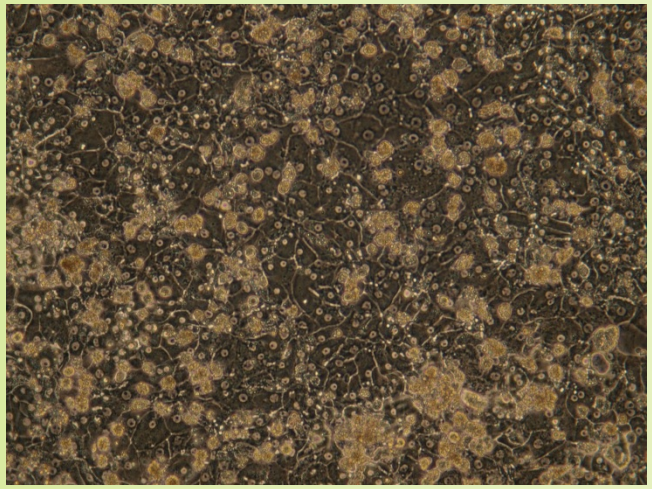
   →integrated data analyses of DNA methylation, gene expression and miRNAs in order to find novel mechanisms of AFB1 induced liver carcinogenesis

3) "Assessment of repeated dose toxicity of cyclosporin A in the human liver using integrative '-omics' data analyses"

   →integrated data analyses of DNA methylation, gene expression and miRNAs in order to find novel mechanisms of CsA induced liver cholestasis

Liver model: primary human hepatocytes:

- Commercially available
- Cryopreserved platable hepatocytes: pool of 3 different human donors
- High viability
- Cultured in two-layer collagen sandwich model
- Cells show *in vivo* like configuration
- *In vivo*-like enzyme expression levels



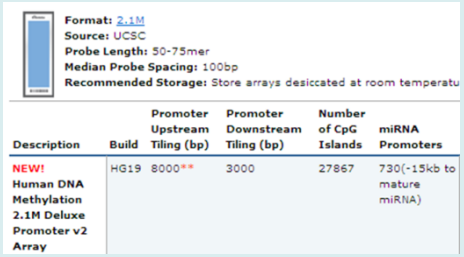
Assessment of repeated dose toxicity of valproic acid in pooled human primary hepatocytes using integrative 'omics data analyses

- VPA is known to induce liver steatosis, presumably through oxidative stress
- inhibits the enzyme histone deacetylase 1, thereby inducing histone hyperacetylation
- stimulates active demethylation in a replication independent manner by increasing accessibility of demethylase enzyme
- effects on mRNA and miRNA expression

Analyses

1. DNA methylation analyses

NimbleGen 2.1M Deluxe Promoter Array Medip-Chip



Format: 2.1M
Source: UCSC
Probe Length: 50-75mer
Median Probe Spacing: 100bp
Recommended Storage: Store arrays desiccated at room temperatu

Description	Build	Promoter Upstream Tiling (bp)	Promoter Downstream Tiling (bp)	Number of CpG Islands	miRNA Promoters
NEW! Human DNA Methylation 2.1M Deluxe Promoter v2 Array	HG19	8000**	3000	27867	730(-15kb to mature miRNA)

√

2. Transcriptomics

Affymetrix Human Genome U133 Plus 2.0 GeneChip arrays Human Genome U133 Set plus 6,500 additional genes for analysis of over 47,000 transcripts

√

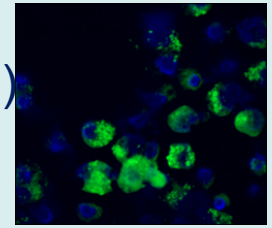
3. miRNA analyses

Agilent Human miRNA Microarray Release 19.0, 8x60K based on miRBase. 2006 human miRNAs represented.

Ongoing

4. Analysis of steatosis

collagen-sandwiched pooled PHH (3 donors) were stained with BODIPY (green) to visualize intracellular lipid droplets



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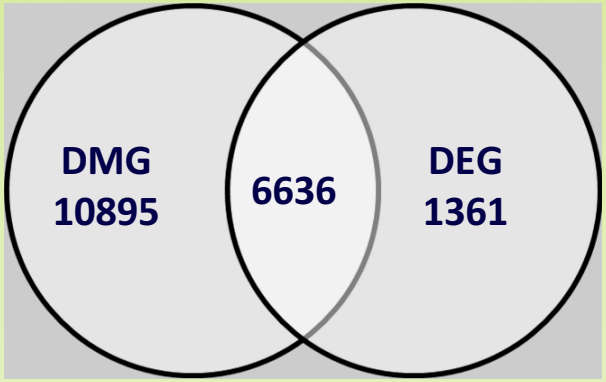
Identification of differentially methylated (DMG) and differentially expressed genes (DEG) after 5 days of VPA exposure

	DMG	DEG
Settings	Magnitude >0 or <0; p-value <0.01 FDR <0.05	P-value <0.05; FDR <0.05; FC >1.5 or <-1.5
	8226	1932
	9305	1478
Total	**17531**	**3410 (7997 no FC)**

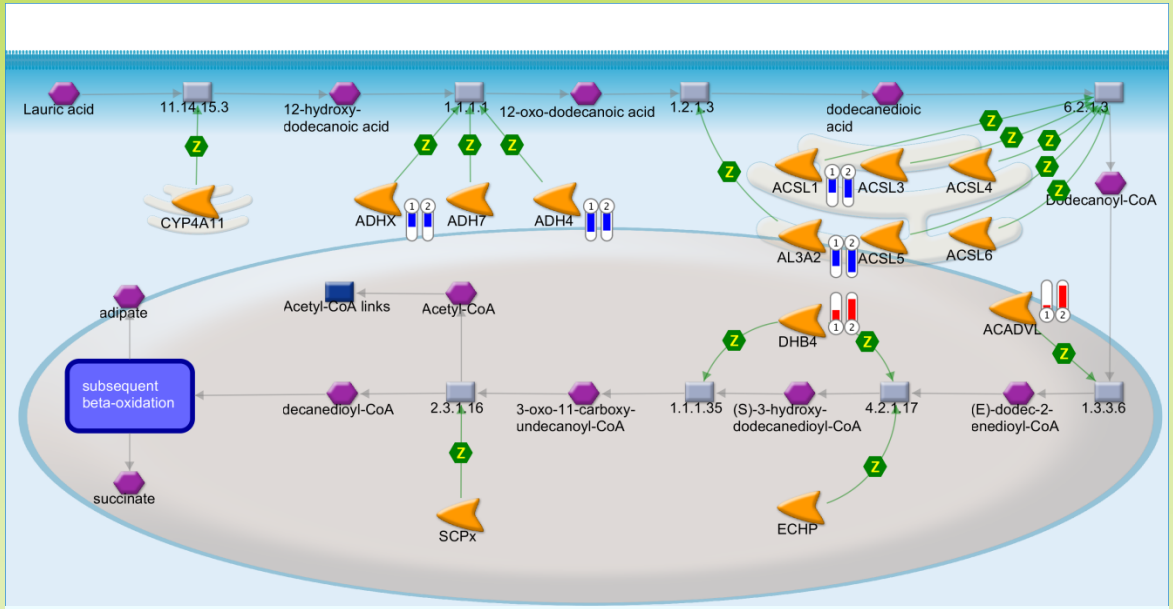


Metacore™ pathway analyses of 6636 genes: 67 significant pathways (P <0.05). Top 10:

Immune response_IL-13 signaling via PI3K-ERK
Cholesterol Biosynthesis
Development_Growth hormone signaling via PI3K/AKT and MAPK cascades
Apoptosis and survival_Endoplasmic reticulum stress response pathway
Histidine-glutamate-glutamine metabolism
Development_Angiotensin signaling via STATs
Mitochondrial ketone bodies biosynthesis and metabolism
Propionate metabolism p.2
Development_IGF-1 receptor signaling
Aminoacyl-tRNA biosynthesis in cytoplasm

Persistence of epigenetic changes: comparison of 5 days of exposure with washout after 3 days

Pathway analyses of 4082 persistently methylated genes using MetacoreTM



Bars:
1st bar : 5 days exposure: DNA methylation data
2nd bar : washout : DNA methylation data

Color:
red : hyper-methylation
blue : hypo-methylation

- Twenty pathways: P<0.05 → 2 pathways involved in lipid metabolism; but also: DNA damage, apoptosis, cytoskeleton remodeling, immune response, cell adhesion.
- Lipid metabolism: fatty acid omega oxidation

Assessment of repeated dose toxicity of AflatoxinB1 in pooled human primary hepatocytes using integrative 'omics data analyses

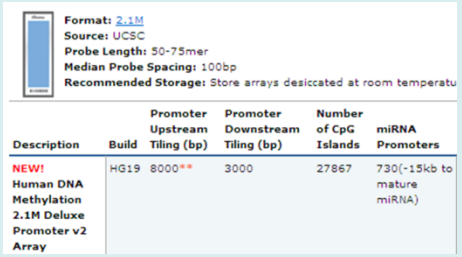
- Hepatotoxic and carcinogenic mycotoxin
  - Acute: apoptosis of liver cells and bile duct proliferation (Aflatoxicosis)
  - Chronic: hepatocellular carcinoma
- AFB1 exposure is associated with global hypo-methylation and gene specific hypermethylation

Analyses

1. **DNA methylation analyses**

   NimbleGen 2.1M Deluxe Promoter Array

   Medip-Chip



Format: 2.1M
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Description	Build	Promoter Upstream Tiling (bp)	Promoter Downstream Tiling (bp)	Number of CpG Islands	miRNA Promoters
NEW! Human DNA Methylation 2.1M Deluxe Promoter v2 Array	HG19	8000**	3000	27867	730(-15kb to mature miRNA)

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2. **Transcriptomics**

   Affymetrix Human Genome U133 Plus 2.0 GeneChip arrays

   Human Genome U133 Set plus 6,500 additional genes for analysis of over 47,000 transcripts

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3. **miRNA analyses**

   Agilent Human miRNA Microarray Release 19.0, 8x60K based on miRBase.

   2006 human miRNAs represented.

√

→ (Integrated) data analyses

Cytoscape

Bioconductor
OPEN SOURCE SOFTWARE FOR BIOINFORMATICS

√

Results: numbers of modulated genes

Number of DMGs, DEGs, and DE-miRNAs in PHH after 5 days of exposure to the high dose (1 µM) and low dose (0.3125 µM) of AFB1, and after a washout of 3 days

	High dose					Low dose					
Direction of effect*	DMG	DEG		DE-miRs		DMG		DEG		DE-miRs	
	Magnitude >0 or <0; p-value <0.01 FDR <0.05	P-value <0.05; FDR <0.05; FC >1.5 or <-1.5		P-value <0.05; FDR <0.05; FC >1.5 or <-1.5		Magnitude >0 or <0; p-value <0.01 FDR <0.05		P-value <0.05; FDR <0.05; FC >1.5 or <-1.5		P-value <0.05; FDR <0.05; FC >1.5 or <-1.5	
	5 days	5 days	washout	5 days	washout	5 days	washout	5 days	washout	5 days	washout
+	2511	1399	896	15	8	1896	4397	702	368	0	0
-	2491	1156	1069	4	8	3743	3734	788	528	0	0
Total	5002	2555	1965	19	17	5639	8131	1490	896	0	0

*direction of effect:

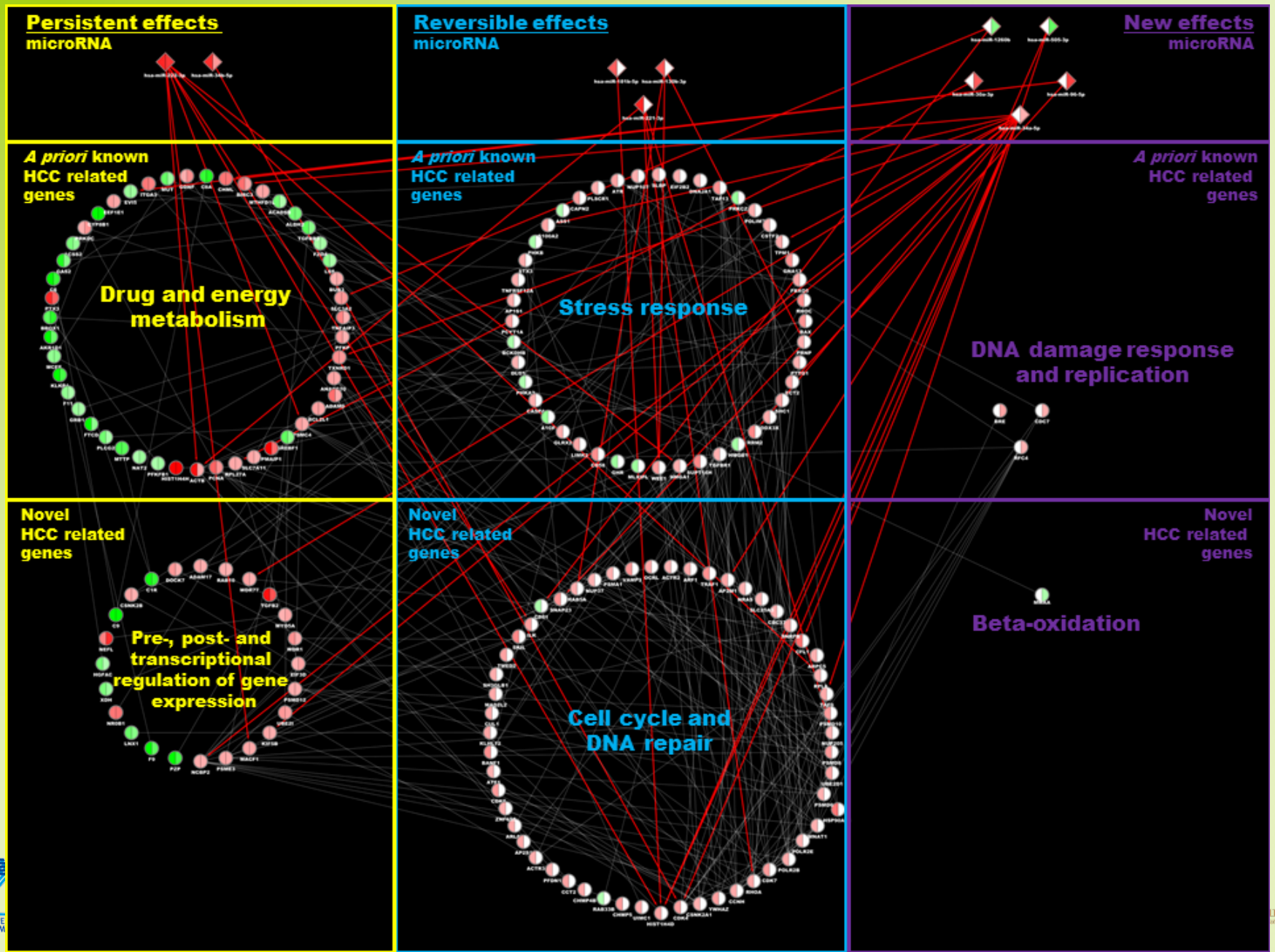
+ = DNA hypermethylation; gene expression upregulation; miRNA expression upregulation

- = DNA hypomethylation; gene expression downregulation; miRNA expression downregulation

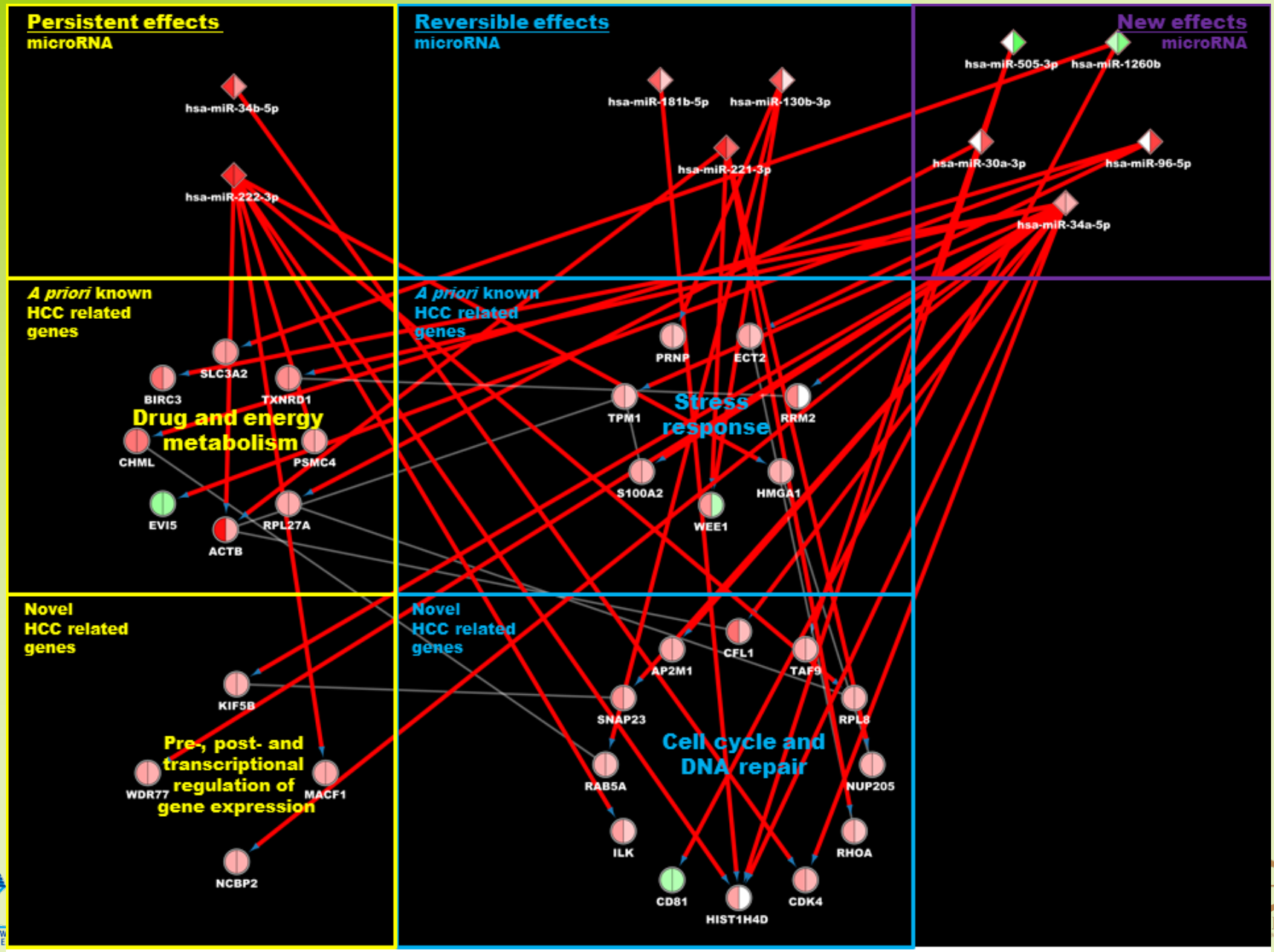
Identification of persistent, reversible and newly expressed DE-miRs

	DE-miRs persistent during washout	DE-miRs reversible during washout	DE-miRs newly emerging during washout
Total	4	15	13
Down-regulated	2	2	7
Up-regulated	2	13	6

Integrated data analysis using networks



Identification of regulatory miRNA-gene interactions



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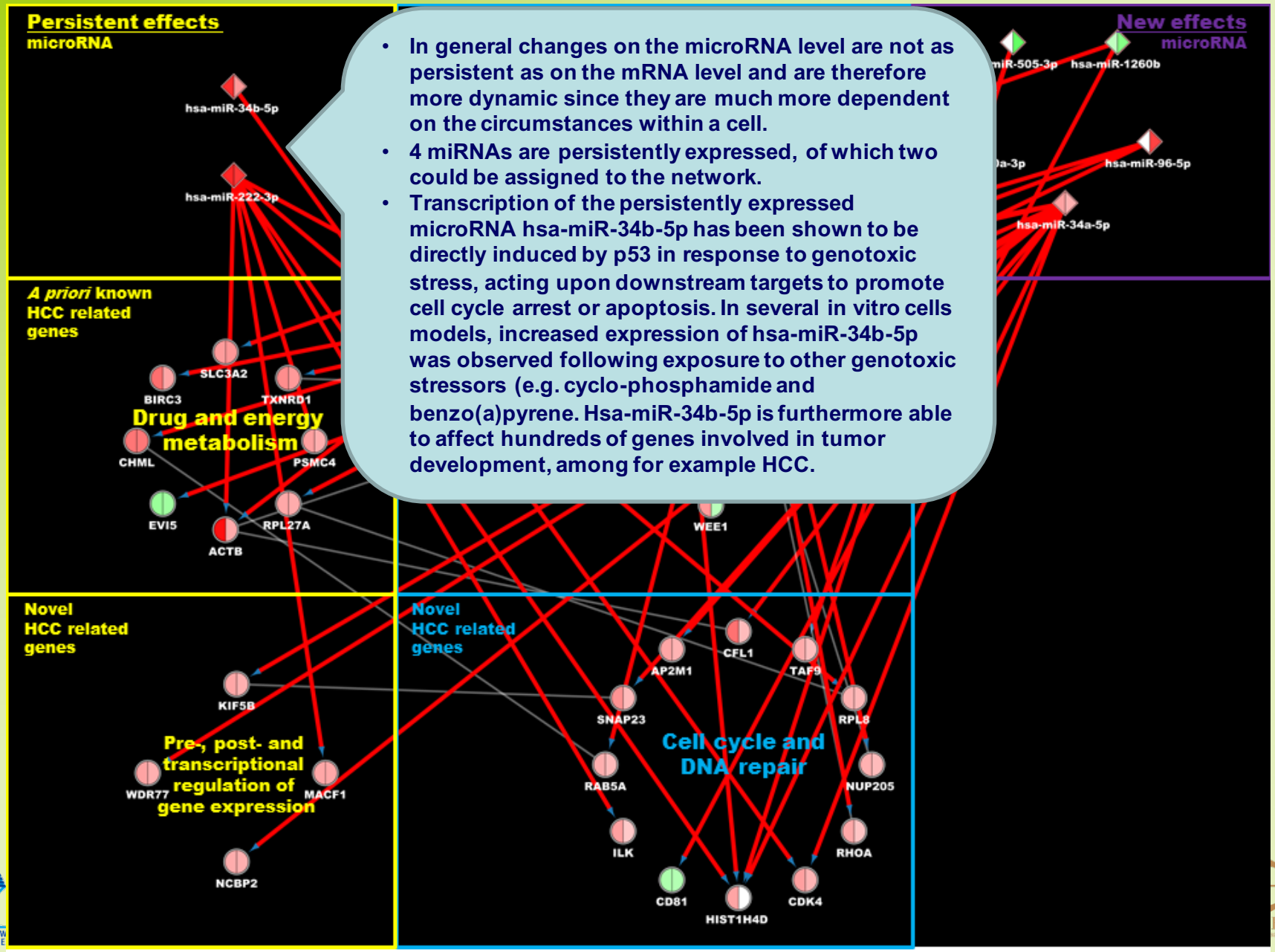
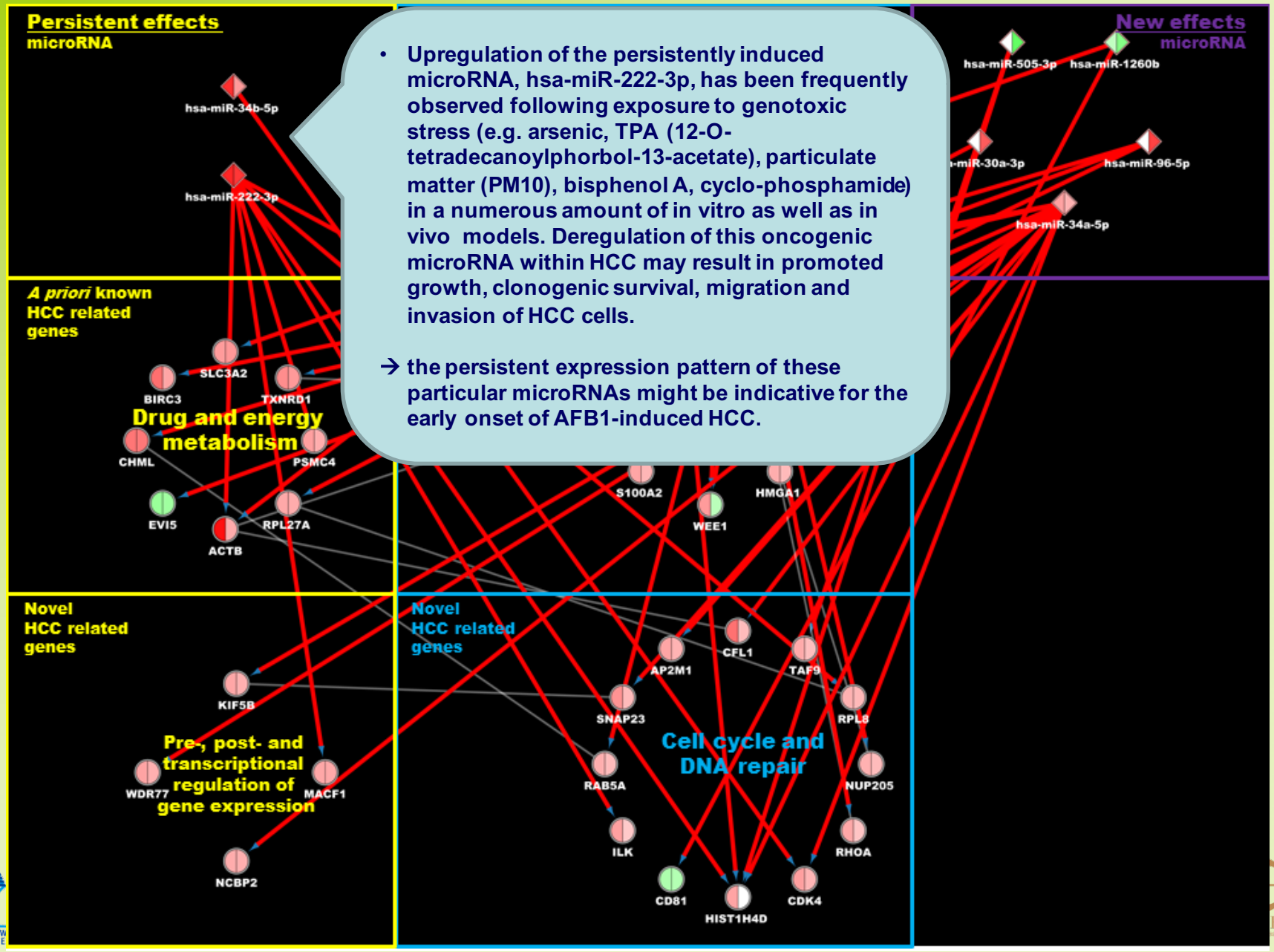


- In general changes on the microRNA level are not as persistent as on the mRNA level and are therefore more dynamic since they are much more dependent on the circumstances within a cell.
- 4 miRNAs are persistently expressed, of which two could be assigned to the network.
- Transcription of the persistently expressed microRNA hsa-miR-34b-5p has been shown to be directly induced by p53 in response to genotoxic stress, acting upon downstream targets to promote cell cycle arrest or apoptosis. In several in vitro cells models, increased expression of hsa-miR-34b-5p was observed following exposure to other genotoxic stressors (e.g. cyclo-phosphamide and benzo(a)pyrene. Hsa-miR-34b-5p is furthermore able to affect hundreds of genes involved in tumor development, among for example HCC.

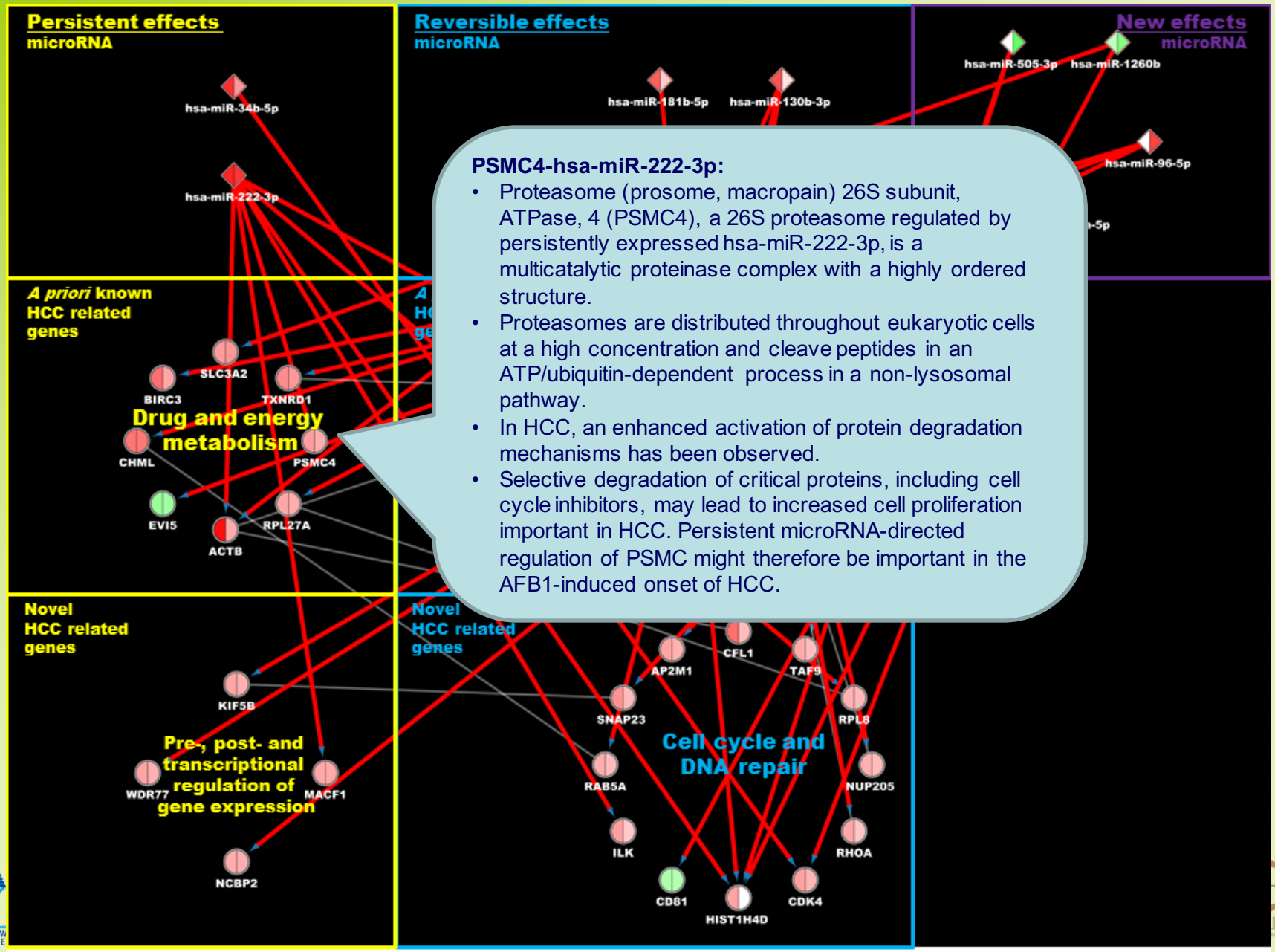
Identification of regulatory miRNA-gene interactions



- Upregulation of the persistently induced microRNA, hsa-miR-222-3p, has been frequently observed following exposure to genotoxic stress (e.g. arsenic, TPA (12-O-tetradecanoylphorbol-13-acetate), particulate matter (PM10), bisphenol A, cyclo-phosphamide) in a numerous amount of in vitro as well as in vivo models. Deregulation of this oncogenic microRNA within HCC may result in promoted growth, clonogenic survival, migration and invasion of HCC cells.

→ the persistent expression pattern of these particular microRNAs might be indicative for the early onset of AFB1-induced HCC.

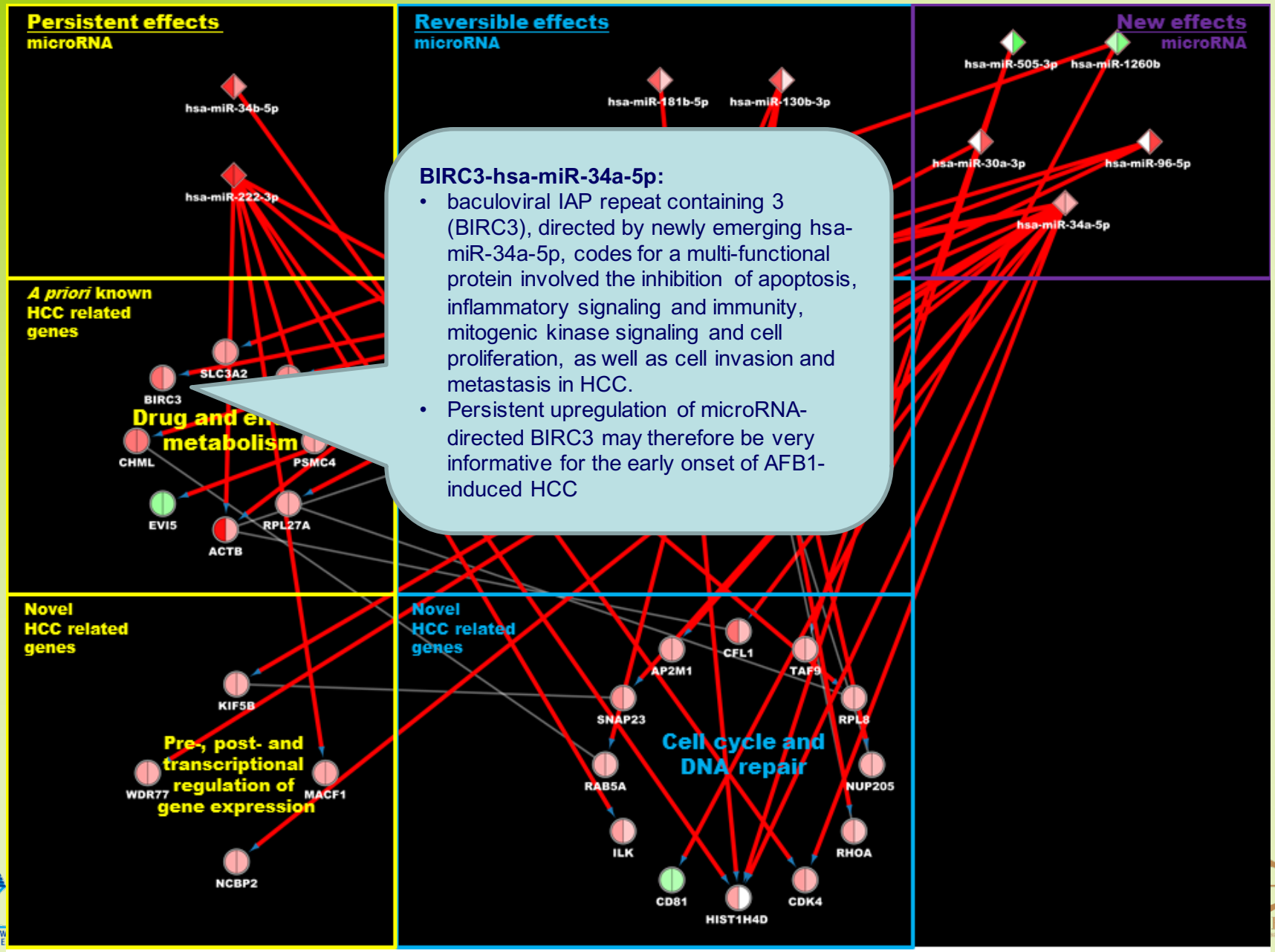
Identification of regulatory miRNA-gene interactions



PSMC4-hsa-miR-222-3p:

- Proteasome (prosome, macropain) 26S subunit, ATPase, 4 (PSMC4), a 26S proteasome regulated by persistently expressed hsa-miR-222-3p, is a multicatalytic proteinase complex with a highly ordered structure.
- Proteasomes are distributed throughout eukaryotic cells at a high concentration and cleave peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway.
- In HCC, an enhanced activation of protein degradation mechanisms has been observed.
- Selective degradation of critical proteins, including cell cycle inhibitors, may lead to increased cell proliferation important in HCC. Persistent microRNA-directed regulation of PSMC might therefore be important in the AFB1-induced onset of HCC.

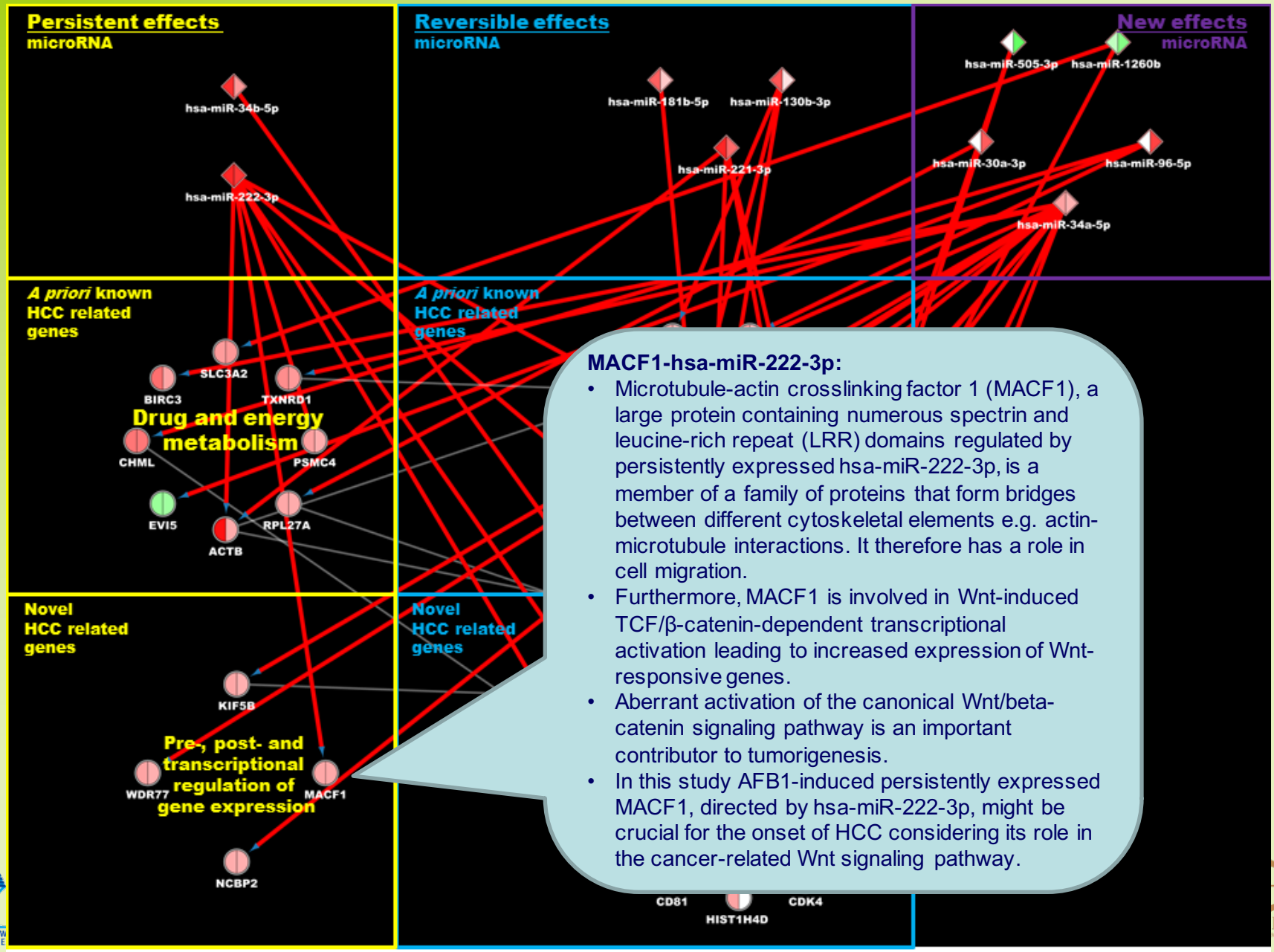
Identification of regulatory miRNA-gene interactions



BIRC3-hsa-miR-34a-5p:

- baculoviral IAP repeat containing 3 (BIRC3), directed by newly emerging hsa-miR-34a-5p, codes for a multi-functional protein involved the inhibition of apoptosis, inflammatory signaling and immunity, mitogenic kinase signaling and cell proliferation, as well as cell invasion and metastasis in HCC.
- Persistent upregulation of microRNA-directed BIRC3 may therefore be very informative for the early onset of AFB1-induced HCC

Identification of regulatory miRNA-gene interactions



MACF1-hsa-miR-222-3p:

- Microtubule-actin crosslinking factor 1 (MACF1), a large protein containing numerous spectrin and leucine-rich repeat (LRR) domains regulated by persistently expressed hsa-miR-222-3p, is a member of a family of proteins that form bridges between different cytoskeletal elements e.g. actin-microtubule interactions. It therefore has a role in cell migration.
- Furthermore, MACF1 is involved in Wnt-induced TCF/β-catenin-dependent transcriptional activation leading to increased expression of Wnt-responsive genes.
- Aberrant activation of the canonical Wnt/beta-catenin signaling pathway is an important contributor to tumorigenesis.
- In this study AFB1-induced persistently expressed MACF1, directed by hsa-miR-222-3p, might be crucial for the onset of HCC considering its role in the cancer-related Wnt signaling pathway.

A) 2 persistently hypermethylated - downregulated genes and B) 16 persistently hypomethylated – upregulated genes, following 5 days of exposure to 0.3 µM of AFB1 and 3 days of wash-out

A.

Entrez Gene ID	Gene name	FC 5D	p-val 5D	FC WO	p-val WO
10974	ADIRF	-1.7305	0.0021	-2.1523	0.0000
6768	ST14	-1.5584	0.0055	-1.5868	0.0002

B.

Entrez Gene ID	Gene name	FC 5D	p-val 5D	FC WO	p-val WO
5111	PCNA	2.1010	0.0044	1.7179	0.0000
7296	TXNRD1	2.2622	0.0031	1.6844	0.0000
8812	CCNK	1.6374	0.0021	1.5481	0.0000
81624	DIAPH3	1.6981	0.0125	1.5173	0.0002
5874	RAB27B	2.7440	0.0029	2.7740	0.0000
8343	HIST1H2BF	2.5235	0.0025	1.8298	0.0000
8351	HIST1H3D	3.5308	0.0018	2.7170	0.0000
5678	PSG9	1.9268	0.0125	1.8668	0.0030
84675	TRIM55	1.6709	0.0045	1.8220	0.0000
51232	CRIM1	1.7854	0.0066	1.7126	0.0001
169792	GLIS3	1.7870	0.0035	1.6761	0.0000
90627	STARD13	1.7390	0.0031	1.6230	0.0000
196	AHR	1.8178	0.0398	1.5874	0.0032
11167	FSTL1	1.7953	0.0019	1.5646	0.0001
3486	IGFBP3	1.7002	0.0027	1.5607	0.0000
9173	IL1RL1	2.1095	0.0064	1.5003	0.0002

- DNA damage response
- Cell growth
- Metastatic events

Conclusions

- By applying integrative cross-omics analyses to an innovative cell model in a repeated dose regime, we have unraveled molecular networks persistently affected by prototypical toxicants
  - VPA and AFB1 in the liver model
  - Doxorubincine in the heart model
  - Ochratoxin and potassium bromide in the kidney model
- Promising biomarkers for repeated dose toxicity in humans have been identified
- Follow-up is required which in particular consider
  - Larger numbers of chemicals for training and validating the predictive models
  - Physiologically relevant doses
  - Tranlsation to molecular human disease signatures

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