World Journal of Stem Cells

Online Submissions: http://www.wjgnet.com/esps/
wjsc@wjgnet.com
doi:10.4252/wjsc.v5.i1.9

World J Stem Cells 2013 January 26; 5(1): 9-25
ISSN 1948-0210 (online)

ORIGINAL ARTICLE

Impact of the antiproliferative agent ciclopirox olamine treatment on stem cells proteome

Gry H Dihazi, Asima Bibi, Olaf Jahn, Jessica Nolte, Gerhard A Mueller, Wolfgang Engel, Hassan Dihazi

Gry H Dihazi, Asima Bibi, Gerhard A Mueller, Hassan Dihazi, Department of Nephrology and Rheumatology, Georg-August University Goettingen, D-37075 Goettingen, Germany
Olaf Jahn, Proteomics Group, Max-Planck-Institute of Experimental Medicine, D-37075 Goettingen, Germany
Olaf Jahn, Deutsche Forschungsgemeinschaft Research Center for Molecular Physiology of the Brain, D-37073 Goettingen, Germany
Jessica Nolte, Wolfgang Engel, Institute of Human Genetics, Georg-August University Goettingen, D-37073 Goettingen, Germany
Author contributions: Dihazi GH and Bibi A performed the majority of experiments, interpreted the data and wrote the article; Nolte J and Engel W provided the stem cell lines and were also involved in revising the manuscript; Jahn O was responsible for the acquisition of the mass spectrometry data and revised the article critically; Mueller GA helped by the study design and manuscript writing; Dihazi H designed and concepted the study and revised the article critically; Dihazi GH and Bibi A contributed equally to this work.
Correspondence to: Hassan Dihazi, PhD, Department of Nephrology and Rheumatology, Georg-August University Goettingen, Robert-Koch-Strasse 40, D-37075 Goettingen, Germany. dihazi@med.uni-goettingen.de
Telephone: +49-551-3991221 Fax: +49-551-3991039
Received: February 7, 2012 Revised: September 19, 2012
Accepted: December 20, 2012
Published online: January 26, 2013

Abstract

AIM: To investigate the proteome changes of stem cells due to ciclopirox olamine (CPX) treatment compared to control and retinoic acid treated cells.

METHODS: Stem cells (SCs) are cells, which have the ability to continuously divide and differentiate into various other kinds of cells. Murine embryonic stem cells (ESCs) and multipotent adult germline stem cells (maGSCs) were treated with CPX, which has been shown to have an antiproliferative effect on stem cells, and compared to stem cells treated with retinoic acid (RA), which is known to have a differentiating effect on stem cells. Classical proteomic techniques like 2-D gel electrophoresis and differential in-gel electrophoresis (DIGE) were used to generate 2D protein maps from stem cells treated with RA or CPX as well as from non-treated stem cells. The resulting 2D gels were scanned and the digitalized images were collated with the help of Delta 2D software. The differentially expressed proteins were analyzed by a MALDI-TOF-TOF mass spectrometer, and the identified proteins were investigated and categorized using bioinformatics.

RESULTS: Treatment of stem cells with CPX, a synthetic antifungal clinically used to treat superficial mycoses, resulted in an antiproliferative effect *in vitro*, without impairment of pluripotency. To understand the mechanisms induced by CPX treatments which results in arrest of cell cycle without any marked effect on pluripotency, a comparative proteomics study was conducted. The obtained data revealed that the CPX impact on cell proliferation was accompanied with a significant alteration in stem cell proteome. By peptide mass fingerprinting and tandem mass spectrometry combined with searches of protein sequence databases, a set of 316 proteins was identified, corresponding to a library of 125 non-redundant proteins. With proteomic analysis of ESCs and maGSCs treated with CPX and RA, we could identify more than 90 single proteins, which were differently expressed in both cell lines. We could highlight, that CPX treatment of stem cells, with subsequent proliferation inhibition, resulted in an alteration of the expression of 56 proteins compared to non-treated cells, and 54 proteins compared to RA treated cells. Bioinformatics analysis of the regulated proteins demonstrated their involvement in various biological processes. To our interest, a number of proteins have potential roles in the regulation of cell proliferation either directly or indirectly. Furthermore the classification of the altered polypeptides according to their main known/postulated functions revealed that the majority of these proteins are involved in molecular functions

like nucleotide binding and metal ion binding, and biological processes like nucleotide biosynthetic processes, gene expression, embryonic development, regulation of transcription, cell cycle processes, RNA and mRNA processing. Proteins, which are involved in nucleotide biosynthetic process and proteolysis, were downregulated in CPX treated cells compared to control, as well as in RA treated cells, which may explain the cell cycle arrest. Moreover, proteins which were involved in cell death, positive regulation of biosynthetic process, response to organic substance, glycolysis, anti-apoptosis, and phosphorylation were downregulated in RA treated cells compared to control and CPX treated cells.

CONCLUSION: The CPX treatment of SCs results in downregulation of nucleotide binding proteins and leads to cell cycle stop without impairment of pluripotency.

Key words: Stem cells; Differentiation; Hypusination; Ciclopirox olamine; Proteomics; Retinoic acid

Dihazi GH, Bibi A, Jahn O, Nolte J, Mueller GA, Engel W, Dihazi H. Impact of the antiproliferative agent ciclopirox olamine treatment on stem cells proteome. *World J Stem Cells* 2013; 5(1): 9-25 Available from: URL: http://www.wjgnet.com/1948-0210/full/v5/i1/9.htm DOI: http://dx.doi.org/10.4252/wjsc.v5.i1.9

INTRODUCTION

Stem cells (SCs) are cells, which are found in all multicellular organisms, which can continuously divide and differentiate into various specialized cell types and can also self-renew to produce more stem cells[1]. The therapeutic use of embryonic stem cells (ESCs) has been constrained by problems caused by immune rejection in the patient as well as ethical issues associated with the use of embryos[2]. Spermatogonial stem cells (SSCs) are self-renewing single cells located in the periphery of the seminiferous tubules whose continuous division maintain spermatogenesis throughout the life of a male individual[3]. SSCs were isolated from murine testis and cultured for the first time in 2006[4]. The pluripotency and plasticity of these cultured cells, named multipotent adult germline stem cells (maGSCs), were proven to be similar to ESCs. The ESC-like nature of maGSCs was confirmed on the microRNA level[5], on the transcriptome level[6] and on the proteome level[7]. In a recent study, we investigated the effects of retinoic acid (RA) treatment on the protein expression profiles of maGSCs and ESCs[8]. The study revealed the important role of Eif5a and its hypusination for stem cell differentiation and proliferation.

Eif5a is a universal translation elongation factor which is highly conserved in all cells. Eif5a has been shown to be associated with translation, viability and proliferation processes[9-12]. It is the only eukaryotic protein known to have the unusual amino acid hypusine. Hypusine is essential to the function of Eif5a and is involved in protein biosynthesis by promoting the formation of the first peptide bond and translation elongation[13]. The activation of Eif5a is controlled by a unique post-translational modification called hypusination. It occurs in two steps which are controlled by two different enzymes[14,15], which inactivation can lead to hypusination inhibition. Ciclopirox olamine (CPX), the ethanolamine salt of 6-cyclohexyl-1-hydroxy-4-methylpyridin-2(1H)-one, is a hypusination inhibitor that controls the second step of the modification, which is catalyzed by deoxyhypusine hydroxylase[14].

CPX, a synthetic antifungal agent, has been used topically to treat fungal and yeast infection of skin or mucosa for more than 20 years[16-19]. Apart from its antimycotic activity, CPX is also effective against both gram-positive and gram-negative bacteria[20]. CPX might also serve as an alternative to recombinant vascular endothelial growth factor (VEGF) treatment or to VEGF gene therapy for therapeutic angiogenesis[21]. The effect of CPX on several *Saccharomyces cerevisiae* mutants has been screened and tested, and it was suggested that CPX may exert its effect by disrupting DNA repair, DNA replication, cell division signals and a defect in mitotic spindle function. Furthermore CPX can influence the regulation of many processes, including signal transduction, transcription, cell division, and development[22]. Recent studies demonstrated CPX as a potential anti-cancer agent for the treatment of malignancies, including leukemia and myeloma[23-25]. However, the mechanism of CPX as a drug in angiogenesis and tumor treatment is poorly understood. CPX works as an inhibitor of the iron-dependent enzymes due to its role as a chelator of intracellular iron[22,23]. Other studies reported the inhibition of HIV-1 gene expression by CPX[26], the importance of Eif5a in embryogenesis and cell differentiation[27], in hepatocellular carcinoma[28] and in diabetes[29]. CPX has also been used as an inhibitor of hypusination.

In a recent study, the effect of CPX on the cellular viability and proliferation of ESCs and maGSCs was investigated. CPX treatment of the stem cells resulted in an antiproliferative effect on ESCs and maGSCs *in vitro*, but did not affect the cell pluripotency[8]. The inhibitory effect of CPX on cell differentiation was reversible and was not associated to apoptosis. The ESCs were found to be more sensitive to CPX than the maGSCs.

The aim of this study was to investigate the proteome changes of ESCs and maGSCs accompanying the treatment with CPX and subsequent inhibition of hypusination using classical proteomic techniques like 2-DE, DIGE and MS. 2D protein maps were generated from control cells and cells treated either with RA or CPX. The resulting protein maps were compared to each other and the differentially expressed proteins were investigated using bioinformatics. We could highlight that a treatment with CPX, involving proliferation inhibition, resulted in an alteration of the expression of 56 proteins compared to non-treated cells, and 54 proteins compared to RA treated cells. The majority of these proteins are involved in nucleotide binding and nucleotide biosynthetic pro-

cesses, metal binding, DNA binding, and other processes which have been linked to CPX.

MATERIALS AND METHODS

Derivation and culture of maGSC and ESC lines

The derivation and culture of maGSCs 129/Sv was described previously[4]. In brief, testes from adult mice were isolated and digested using collagenase. Single cell suspension was derived after trypsin digestion followed by the culture of the testis suspension cells on a mouse embryonic fibroblasts (MEFs) feeder layer in the presence of GDNF. After appearance of morphological ES-like cells, the colonies were picked and expanded in standard ES cell conditions. In this case, the maGSC line was generated without genetic selection, only by morphological criteria. The ESC R1 line was derived from the 129/Sv mouse[30]. To maintain maGSCs and ESCs in an undifferentiated state, the cells were cultured under standard ESC culture conditions: DMEM (PAN, Aidenbach, Germany) supplemented with 20% fetal calf serum (PAN, Aidenbach, Germany), 2 mmol/L L-glutamine (PAN, Aidenbach, Germany), 50 mmol/L β-mercaptoethanol (Gibco/Invitrogen, Eggenstein, Germany), 1 × non-essential amino acids (Gibco/Invitrogen), sodium pyruvate (Gibco/Invitrogen), and penicillin/streptomycin (PAN, Aidenbach, Germany). ESCs and maGSCs were cultured on a feeder layer of mitomycin C-inactivated MEFs in the presence of 1000 U/mL recombinant mouse leukemia inhibitory factor (LIF) (Chemicon, Temecula, United States). ESCs were isolated as described previously, and male ESC lines were identified and selected by PCR amplification of Sry gene-specific sequences[31,32]. In order to differentiate maGSCs and male ESCs, the cells were plated on gelatin-coated dishes and culture medium was supplemented with 1 μmol/L RA (Sigma-Aldrich, Steinheim, Germany) instead of LIF. Cells were cultured for 48 h before they were lysed and the proteins were extracted. For examining the effect of CPX on the proteome level, ESCs and maGSCs were treated with culture medium supplemented with 2 μmol/L CPX for 72 h.

Protein extraction

The protein extraction for 2-DE was performed as described previously[7]. Briefly, 75% confluent cultures were trypsinized and washed three times with PBS. The cells were harvested by centrifugation at 200 × *g* for 10 min, the pellet was treated with 0.3-0.5 mL lysis buffer [9.5 mol/L urea, 2% CHAPS (w/v), 2% ampholytes (w/v), 1% DTT]. Ampholytes and DTT were added shortly before use. After adding the lysis buffer, the samples were incubated for 30 min at 4 ℃. For removing the cell debris, sample centrifugation was carried out at 13 000 × *g* and 4 ℃ for 45 min. The supernatant was recentrifuged at 13 000 × *g* and 4 ℃ for an additional 45 min to get maximal purity. The resulting samples were used immediately or stored at -80 ℃ until use.

Protein precipitation

To reduce the salt contamination and to enrich the proteins, methanol-chloroform-precipitation according to Wessel *et al*[33] was performed. Briefly, 0.4 mL of methanol (100%) was added to 0.1 mL aliquots of protein samples and mixed together. 0.1 mL chloroform was added to the samples and the mixture was vortexed. Subsequently 0.3 mL water was added and the solution was vortexed and centrifuged at 13 000 × *g* for 1 min. The aqueous layer was removed, and another 0.4 mL methanol (100%) was added to the rest of the chloroform and the interphase with the precipitated proteins. The sample was mixed and centrifuged for 2 min at 13 000 × *g* and the supernatant was removed. The pellet was vacuum dried and dissolved in lysis buffer.

Total protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, United States) according to Bradford[34]. BSA (Sigma, Steinheim, Germany) was used as a standard.

2D gel electrophoresis (2-DE)

IPG strips (11 cm, pI 5-8) were passively rehydrated in 185 μL solution containing 150 μg protein in a rehydration buffer (8 mol/L urea, 1% CHAPS, 1% DTT, 0.2% ampholytes, and a trace of bromophenol blue) for 12 h. The IEF step was performed on the PROTEAN® IEF Cell (Bio-Rad, Hercules, CA, United States). Temperature-controlled at 20 ℃, the voltage was set to 500 V for 1 h, increased to 1000 V for 1 h, 2000 V for 1 h and left at 8000 V until a total of 50 000 Vhours was reached. Prior to SDS-PAGE, the IPG strips were reduced for 20 min at room temperature in SDS equilibration buffer containing 6 mol/L urea, 30% glycerol, 2% SDS 0.05 mol/L Tris-HCl, and 2% DTT on a rocking table. The strips were subsequently alkylated in the same solution with 2.5% iodoacetamide substituted for DTT, and a trace of bromophenol blue. For the SDS-PAGE, 12% BisTris Criterion precast gels (Bio-Rad, Hercules, CA, United States) were used according to manufacturer's instructions. The gels were run at 150 V for 10 min followed by 200 V until the bromophenol blue dye front had reached the bottom of the gel.

Gel staining

For image analysis, 2-DE gels were fixed in a solution containing 50% methanol and 12% acetic acid overnight and fluorescent stained with Flamingo fluorescent gel stain (Bio-Rad, Hercules, CA, United States) for minimum 5 h. After staining, gels were scanned at 50 μm resolution on a Fuji FLA-5100 scanner. The digitalized images were analyzed using Delta 2D 3.4 (Decodon, Braunschweig, Germany). For protein visualization, 2-DE gels were additionally stained with colloidal Coomassie blue, Roti-Blue (Roth, Karlsruhe, Germany) overnight.

2D-DIGE

Protein extraction and methanol-chloroform-precipitation

were performed as described above. The resulting pellet was dissolved in labeling buffer (30 mmol/L Tris-HCl pH 8.5, 9.5 mol/L urea, 2% CHAPS), centrifuged (5 min, 13 000 × *g*), and the protein concentration of the supernatant was determined as described above. The preparation of the CyDyes as well as the labeling reaction was followed according to the manufacturer's protocol (GE Healthcare).

To avoid the dye-specific protein labeling, every pair of protein samples from two independent cell extract preparations were processed in duplicate while swapping the dyes. Thereby four replicate gels were obtained, allowing to monitor regulation factors down to twofold changes[35]. An internal standard consisting of a mixture of the samples under investigation was labeled with Cy2 and included on all gels to facilitate gel matching, thereby eliminating artifacts from experimental variation. The three differentially labeled fractions were pooled. Rehydration buffer (8 mol/L urea, 1% CHAPS, 13 mmol/L DTT and 1% ampholytes 3-10) was added to make up the volume to 185 μL prior to IEF. The 2-DE was performed as described above. The CyDye-labeled gels were scanned at 50 μm resolution on a Fuji FLA5100 scanner (Fuji Photo, Kanagawa, Japan) with laser excitation light at 473 nm and long pass emission filter 510LP (Cy2), 532 nm and long pass emission filter 575LP (Cy3), and 635 nm and long pass emission filter 665LP (Cy5). Fluorescent images were acquired in 16-bit TIFF files format. Spot matching across gels and normalization based on the internal standard was performed with Delta2D software (Decodon, Greifswald, Germany). To analyze the significance of protein regulation, a Student's *t*-test was performed, and statistical significance was assumed for p values less than 0.01. For protein visualization, the 2-DE gels were post-stained with colloidal Coomassie blue (Roti-Blue) overnight. Differentially regulated proteins were excised and processed for identification by mass spectrometry.

Protein identification

Manually excised gel plugs were subjected to an automated platform for the identification of gel-separated proteins[36] as described in the framework of recent DIGE-based[37] and large-scale proteome studies[38]. An Ultraflex MALDI-TOF-TOF mass spectrometer (Bruker Daltonik) was used to acquire both PMF and fragment ion spectra, resulting in confident protein identifications based on peptide mass and sequence information. Database searches in the Swiss-Prot primary sequence database restricted to the taxonomy *mus musculus* were performed using the MASCOT Software 2.2 (Matrix Science). Carboxamidomethylation of Cys residues was specified as fixed and oxidation of Met as variable modifications. One trypsin missed cleavage was allowed. Mass tolerances were set to 100 ppm for PMF searches and to 100 ppm (precursor ions) and 0.7 Da (fragment ions) for MS/MS ion searches. The minimal requirement for accepting a protein as identified was at least one peptide sequence match above identity threshold in addition to at least 20 % sequence coverage in the PMF.

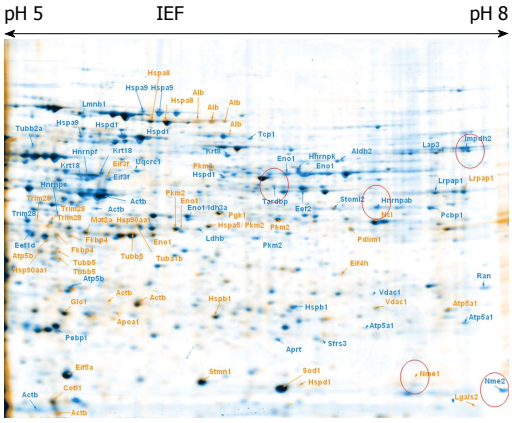

Figure 1 Embryonic stem cells control *vs* ciclopirox olamine treated cells. Overlay of 2-DE gels of samples from ciclopirox olamine treated embryonic stem cells (ESCs) (orange) compared to control ESCs (blue). The identified proteins are indicated with the gene names.

Bioinformatics

The classification of the identified proteins according to their main known/postulated functions was carried out using DAVID bioinformatics[39,40]. This classification together with the official gene symbol was used to investigate and categorize the gene ontology (GO)-annotations (biological processes and molecular functions).

RESULTS

Comparative analysis of differentially expressed proteins in RA and CPX treated SCs by 2-DE and ontogenic classification

To explore proteome changes caused by CPX treatment, we treated ESCs as well as maGSCs with CPX for 72 h. In parallel, both cell types, ESC and maGSC, were treated with RA for 48 h. 2-DE was performed from these four different samples, as well as from the corresponding non-treated cells (Figures 1-4). Proteins, which were found to be differentially expressed, were excised and subjected to in-gel-digestion and mass spectrometry analyses. A total of 316 spots were identified, which resulted in 125 non-redundant proteins (Table 1). For further interpretation of the data, only proteins, which were regulated in the same direction in ESCs and concurrently in maGSCs, were taken into consideration.

The identified proteins were classified using DAVID bioinformatics[39,40] focusing on its information considering the GO (Gene Ontology) annotations. The terms corresponding to the molecular function and biological process were regarded (Figures 5-7).

Comparison of the differently expressed proteins

Examination of all of the proteins, which expression was altered either by CPX or RA treatment, was performed regarding their involvement in biological processes. We found that seven proteins are involved in regulation of

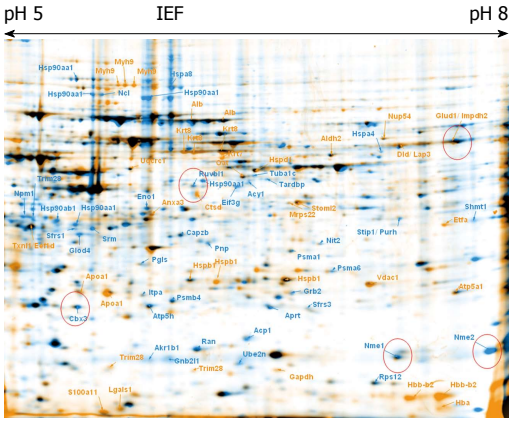

Figure 2 Multipotent adult germline stem cells control *vs* ciclopirox olamine treated cells. Overlay of 2-DE gels of samples from ciclopirox olamine treated multipotent adult germline stem cells (maGSCs) (orange) compared to control maGSCs (blue). The identified proteins are indicated with the gene names.

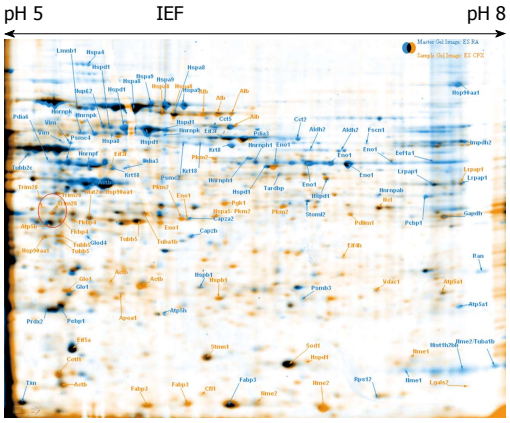

Figure 3 Embryonic stem cell control *vs* ciclopirox olamine treated cells. Overlay of 2-DE gels of samples from ciclopirox olamine treated embryonic stem cells (ESCs) (orange) compared to retinoic acid treated ESCs (blue). The identified proteins are indicated with the gene names.

transcription. Among these proteins Ube2n, Tardbp, Cbx3 and Hnrnpab were downregulated in CPX treated cells compared to control, whereas Nup62 was upregulated in CPX treated cells compared to control (Table 2). Two other proteins Trim28 and Ruvbl1 were downregulated in RA treated cells compared to control. Detailed information is given in Table 2 and the protein expression regulation folds are given in Tables 3-6.

When we looked at the molecular function of the regulated proteins, we observed that a major part of the proteins are involved in nucleotide binding (Table 7). Approximately half of these proteins were downregulated and the other half was upregulated upon CPX treatment compared to RA treated cells. About 13 proteins are involved in metal ion binding, of these five proteins (Acy1, Uqcrc1, Sfrs1, Trim28, Glo1) are involved in transition metal ion binding, like Fe^{3+}, which is known to be important in case of CPX, as CPX works as an inhibitor of the iron-dependent enzymes due to its role as a chelator of

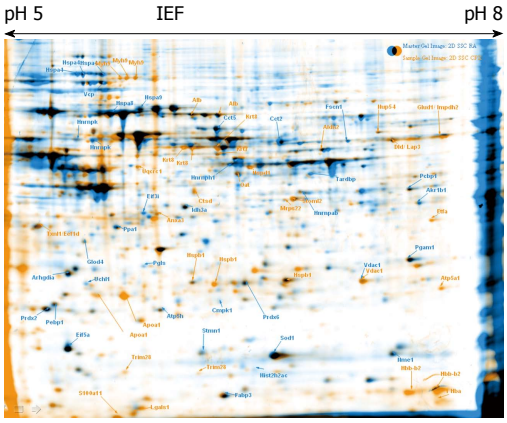

Figure 4 Multipotent adult germline stem cell control *vs* ciclopirox olamine treated cells. Overlay of 2-DE gels of samples from ciclopirox olamine treated Multipotent adult germline stem cells (maGSCs) (orange) compared to retinoic acid treated maGSCs (blue). The identified proteins are indicated with the gene names.

intracellular iron. Three of the proteins involved in transition metal ion binding (Sfrs1, Trim28 and Glo1), were up-regulated upon CPX treatment compared to RA-treated cells.

Overall, it could be observed that most of the proteins of interest were downregulated in either CPX or RA treated cells compared to control.

Treated cells compared to control

About 56 of the 125 identified proteins showed different expression as a reaction to CPX treatment compared to control. Of these, 14 proteins were upregulated as a reaction to CPX treatment (Table 3), whereas 44 proteins were downregulated (Table 4). The expression of 52 proteins was found to be altered in both cell types, ESCs and maGSCs, under RA treatment compared to control (Tables 5 and 6). Of these proteins, 11 were upregulated and 41 were downregulated as a reaction to RA treatment.

In both experiments the majority of the regulated proteins were downregulated as a reaction to one of the treatments. Although mainly different proteins were regulated, bioinformatics analysis revealed that the downregulated proteins in both experiments are primarily involved in the same molecular functions (Figure 5). The downregulated proteins upon CPX treatment are mainly involved in nucleotide binding, GTP binding, peptidase activity and metal ion binding, particularly magnesium ion binding. The proteins which were downregulated upon RA treatment are involved in transition metal ion binding instead of magnesium ion binding, and furthermore involved in enzyme binding. Proteins, which were upregulated upon CPX treatment, are mainly involved in nucleotide binding, whereas proteins which were upregulated upon RA treatment are involved in nucleotide and metal ion binding.

When we look at the involvement of the regulated proteins in biological processes, more differences were observed (Figure 6). Both treatments showed downregu-

Table 1 Non-redundant proteins

Protein name	Gene name	Swiss-prot	Nominal mass	CPI	PMF-score	PMF sequence coverage	MS/MS-score	MS/MS-sequence coverage
Low molecular weight phosphotyrosine protein phosphatase	Acp1	PPAC_MOUSE	18 636	6.4	96	65	80	24
Actin, cytoplasmic 1	Actb	ACTB_MOUSE	42 052	5.2	170	70	312	15
Aminoacylase-1	Acy1	ACY1_MOUSE	45 980	5.9	167	56	44	5
Aldose reductase	Akr1b1	ALDR_MOUSE	36 052	6.9	128	43	136	10
Aldehyde dehydrogenase, mitochondrial	Aldh2	ALDH2_MOUSE	57 015	8.6	221	54	131	7
Annexin A3	Anxa3	ANXA3_MOUSE	36 520	5.2	84	47	111	14
Adenine phosphoribosyltransferase	Aprt	APT_MOUSE	19 883	6.4	88	67	216	27
Rho GDP-dissociation inhibitor 1	Arhgdia	GDIR1_MOUSE	23 450	5	123	54	66	11
ATP synthase subunit α, mitochondrial	Atp5a1	ATPA_MOUSE	59 830	9.7	100	28	53	4
ATP synthase subunit β, mitochondrial	Atp5b	ATPB_MOUSE	56 265	5.1	90	30	167	10
ATP synthase subunit d, mitochondrial	Atp5h	ATP5H_MOUSE	18 795	5.4	122	70	169	36
F-actin-capping protein subunit α-2	Capza2	CAZA2_MOUSE	33 118	5.5	148	69	19	9
F-actin-capping protein subunit β	Capzb	CAPZB_MOUSE	31 611	5.4	117	61	129	8
Chromobox protein homolog 3	Cbx3	CBX3_MOUSE	21 013	5	38	36	67	6
T-complex protein 1 subunit β	Cct2	TCPB_MOUSE	57 783	6	248	61	75	9
T-complex protein 1 subunit epsilon	Cct5	TCPE_MOUSE	60 042	5.7	186	60	138	6
Cofilin-1	Cfl1	COF1_MOUSE	18 776	9.1	95	45	87	13
UMP-CMP kinase	Cmpk1	KCY_MOUSE	22 379	5.6	74	52	29	10
Coactosin-like protein	Cotl1	COTL1_MOUSE	16 048	5.1	86	60	116	14
Cathepsin D	Ctsd	CATD_MOUSE	45 381	6.9	160	41	95	4
Dihydrolipoyl dehydrogenase, mitochondrial	Dld	DLDH_MOUSE	54 751	9	112	48	81	2
Elongation factor 1-α1	Eef1a1	EF1A1_MOUSE	50 424	9.7	68	34	115	8
Elongation factor 1-δ	Eef1d	EF1D_MOUSE	31 388	4.8	86	54	79	9
Elongation factor 2	Eef2	EF2_MOUSE	96 222	6.4	52	26	29	1
Eukaryotic translation initiation factor 3 subunit F	Eif3f	EIF3F_MOUSE	38 090	5.2	109	45	106	14
Eukaryotic translation initiation factor 3 subunit G	Eif3g	EIF3G_MOUSE	35 901	5.6	54	35	23	7
Eukaryotic translation initiation factor 3 subunit I	Eif3i	EIF3I_MOUSE	36 837	5.3	228	78	89	16
Eukaryotic translation initiation factor 4H	Eif4h	IF4H_MOUSE	27 381	7.5	83	51	65	8
Eukaryotic translation initiation factor 5A-1	Eif5a	IF5A1_MOUSE	17 049	4.9	115	58	170	22
α-enolase	Eno1	ENOA_MOUSE	47 453	6.4	183	64	170	13
Electron transfer flavoprotein subunit α, mitochondrial	Etfa	ETFA_MOUSE	35 330	9.5	138	59	100	9
Fatty acid-binding protein, heart	Fabp3	FABPH_MOUSE	14 810	6.1	86	77	212	39
Peptidyl-prolyl cis-trans isomerase FKBP4	Fkbp4	FKBP4_MOUSE	51 939	5.4	122	38	168	9
Fascin	Fscn1	FSCN1_MOUSE	55 215	6.5	129	45	26	6
Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	G3P_MOUSE	36 072	9.2	62	38	40	8
Lactoylglutathione lyase	Glo1	LGUL_MOUSE	20 967	5.1	134	66	114	20
Glyoxalase domain-containing protein 4	Glod4	GLOD4_MOUSE	33 581	5.2	167	69	115	13
Glutamate dehydrogenase 1, mitochondrial	Glud1	DHE3_MOUSE	61 640	8.8	70	37	60	5
Guanine nucleotide-binding protein subunit β-2-like 1	Gnb2l1	GBLP_MOUSE	35 511	8.9	116	55	20	5
Growth factor receptor-bound protein 2	Grb2	GRB2_MOUSE	25 336	5.9	73	54	36	17
Histone H2B type 1-B	Hist1h2bb	H2B1B_MOUSE	13 944	10.8	52	41	93	19
Histone H2A type 2-C	Hist2h2ac	H2A2C_MOUSE	13 980	11.4	50	55	67	12
Heterogeneous nuclear ribonucleoprotein A/B	Hnrnpab	ROAA_MOUSE	30 926	8.7	83	30	107	5
Heterogeneous nuclear ribonucleoproteins C1/C2	Hnrnpc	HNRPC_MOUSE	34 421	4.8	57	32	57	6
Heterogeneous nuclear ribonucleoprotein F	Hnrnpf	HNRPF_MOUSE	46 043	5.2	163	56	207	12
Heterogeneous nuclear ribonucleoprotein H	Hnrnph1	HNRH1_MOUSE	49 454	5.9	166	61	134	15
Heterogeneous nuclear ribonucleoprotein K	Hnrnpk	HNRPK_MOUSE	51 230	5.3	144	46	251	11
Heat shock protein HSP 90-α	Hsp90aa1	HS90A_MOUSE	85 134	4.8	131	31	130	5
Heat shock protein HSP 90-β	Hsp90ab1	HS90B_MOUSE	83 615	4.8	62	25	78	6
Heat shock 70 kDa protein 4	Hspa4	HSP74_MOUSE	94 872	5	242	54	102	3
78 kDa glucose-regulated protein	Hspa5	GRP78_MOUSE	72 492	4.9	78	25	122	5
Heat shock cognate 71 kDa protein	Hspa8	HSP7C_MOUSE	71 055	5.2	234	58	154	4
Stress-70 protein, mitochondrial	Hspa9	GRP75_MOUSE	73 768	5.8	219	50	272	7
Heat shock protein β-1	Hspb1	HSPB1_MOUSE	23 057	6.1	144	55	344	24
60 kDa heat shock protein, mitochondrial	Hspd1	CH60_MOUSE	61 088	5.8	334	69	232	10
Isocitrate dehydrogenase (NAD) subunit α, mitochondrial	Idh3a	IDH3A_MOUSE	40 069	6.3	70	31	158	12
Inosine-5'-monophosphate dehydrogenase 2	Impdh2	IMDH2_MOUSE	56 179	7	173	50	107	7
Inosine triphosphate pyrophosphatase	Itpa	ITPA_MOUSE	22 225	5.5	84	72	128	15
Keratin, type Ⅰ cytoskeletal 18	Krt18	K1C18_MOUSE	47 509	5.1	199	65	58	9
Keratin, type Ⅱ cytoskeletal 7	Krt7	K2C7_MOUSE	50 678	5.6	137	52	55	4
Keratin, type Ⅱ cytoskeletal 8	Krt8	K2C8_MOUSE	54 531	5.6	245	55	237	9
Cytosol aminopeptidase	Lap3	AMPL_MOUSE	56 505	8.7	126	47	58	5
L-lactate dehydrogenase B chain	Ldhb	LDHB_MOUSE	36 834	5.6	84	46	30	6

Galectin-1	Lgals1	LEG1_MOUSE	15 198	5.2	109	70	172	25
Galectin-2	Lgals2	LEG2_MOUSE	14 984	7.9	120	88	60	14
Lamin-B1	Lmnb1	LMNB1_MOUSE	66 973	5	265	60	134	5
α-2-macroglobulin receptor-associated protein	Lrpap1	AMRP_MOUSE	42 189	7.9	139	49	177	14
S-adenosylmethionine synthase isoform type-2	Mat2a	METK2_MOUSE	44 003	6	73	41	59	3
28S ribosomal protein S22, mitochondrial	Mrps22	RT22_MOUSE	41 281	9.2	112	45	97	9
Myosin-9	Myh9	MYH9_MOUSE	227 429	5.4	73	15	103	2
Nucleolin	Ncl	NUCL_MOUSE	76 734	4.5	113	26	179	7
Omega-amidase NIT2	Nit2	NIT2_MOUSE	30 825	6.5	112	59	75	9
Nucleoside diphosphate kinase A	Nme1	NDKA_MOUSE	17 311	7.7	125	72	223	30
Nucleoside diphosphate kinase B	Nme2	NDKB_MOUSE	17 466	7.8	160	84	287	30
Nucleophosmin	Npm1	NPM_MOUSE	32 711	4.5	53	33	136	10
Nuclear pore complex protein Nup54	Nup54	NUP54_MOUSE	55 812	6.6	55	21	23	3
Nuclear pore glycoprotein p62	Nup62	NUP62_MOUSE	53 336	5.1		13	43	5
Ornithine aminotransferase, mitochondrial	Oat	OAT_MOUSE	48 723	6.2	174	64	125	9
Poly(rC)-binding protein 1	Pcbp1	PCBP1_MOUSE	37 987	6.8	175	69	115	12
Protein disulfide-isomerase A3	Pdia3	PDIA3_MOUSE	57 099	5.8	254	55	90	5
Protein disulfide-isomerase A6	Pdia6	PDIA6_MOUSE	48 469	4.9	75	40	47	2
PDZ and LIM domain protein 1	Pdlim1	PDLI1_MOUSE	36 208	6.4	200	73	56	7
Phosphatidylethanolamine-binding protein 1	Pebp1	PEBP1_MOUSE	20 988	5.1	130	79	107	11
Phosphoglycerate mutase 1	Pgam1	PGAM1_MOUSE	28 928	6.8	157	66	192	21
Phosphoglycerate kinase 1	Pgk1	PGK1_MOUSE	44 921	9	136	52	128	7
6-phosphogluconolactonase	Pgls	6PGL_MOUSE	27 465	5.5	102	49	148	17
Pyruvate kinase isozymes M1/M2	Pkm2	KPYM_MOUSE	58 378	7.9	178	49	106	9
Purine nucleoside phosphorylase	Pnp	PNPH_MOUSE	32 541	5.8	119	67	138	13
Inorganic pyrophosphatase	Ppa1	IPYR_MOUSE	33 102	5.3	126	66	26	7
Peroxiredoxin-2	Prdx2	PRDX2_MOUSE	21 936	5.1	103	62	285	22
Peroxiredoxin-6	Prdx6	PRDX6_MOUSE	24 969	5.6	156	67	101	17
Proteasome subunit α type-1	Psma1	PSA1_MOUSE	29 813	6	71	52	140	17
Proteasome subunit α type-6	Psma6	PSA6_MOUSE	27 811	6.4	72	38	108	10
Proteasome subunit β type-3	Psmb3	PSB3_MOUSE	23 235	6.2	110	51	187	30
Proteasome subunit β type-4	Psmb4	PSB4_MOUSE	29 211	5.3	60	42	109	10
26S protease regulatory subunit 7	Psmc2	PRS7_MOUSE	49 016	5.6	166	60	72	8
26S protease regulatory subunit 6B	Psmc4	PRS6B_MOUSE	47 366	5	144	55	109	9
GTP-binding nuclear protein Ran	Ran	RAN_MOUSE	24 579	7.8	124	51	139	11
40S ribosomal protein S12	Rps12	RS12_MOUSE	14 858	7.7	77	62	95	11
RuvB-like 1	Ruvbl1	RUVB1_MOUSE	50 524	6	61	35	106	10
Protein S100-A11	S100a11	S10AB_MOUSE	11 247	5.1		36	147	27
Splicing factor, arginine/serine-rich 1	Sfrs1	SFRS1_MOUSE	27 842	10.8	80	43	156	18
Splicing factor, arginine/serine-rich 3	Sfrs3	SFRS3_MOUSE	19 546	12.3			87	14
Serine hydroxymethyltransferase, cytosolic	Shmt1	GLYC_MOUSE	53 065	6.5	98	43	19	2
Superoxide dismutase [Cu-Zn]	Sod1	SODC_MOUSE	16 104	6	83	45	126	31
Spermidine synthase	Srm	SPEE_MOUSE	34 543	5.2	141	73	129	15
Stress-induced-phosphoprotein 1	Stip1	STIP1_MOUSE	63 170	6.4	184	55	89	4
Stathmin	Stmn1	STMN1_MOUSE	17 264	5.7	28	24	69	8
Stomatin-like protein 2	Stoml2	STML2_MOUSE	38 475	9.5	144	61	165	15
TAR DNA-binding protein 43	Tardbp	TADBP_MOUSE	44 918	6.3	68	30	107	7
T-complex protein 1 subunit α	Tcp1	TCPA_MOUSE	60 867	5.8	61	27	28	4
Transcription intermediary factor 1-β	Trim28	TIF1B_MOUSE	90 558	5.4		10	139	4
Tubulin α-1B chain	Tuba1b	TBA1B_MOUSE	50 804	4.8	128	39	152	9
Tubulin α-1C chain	Tuba1c	TBA1C_MOUSE	50 562	4.8	53	24	52	6
Tubulin β-2A chain	Tubb2a	TBB2A_MOUSE	50 274	4.6	126	55	111	11
Tubulin β-2C chain	Tubb2c	TBB2C_MOUSE	50 255	4.6	150	56	49	8
Tubulin β-5 chain	Tubb5	TBB5_MOUSE	50 095	4.6	169	57	237	9
Thioredoxin	Txn	THIO_MOUSE	12 010	4.6	63	67	92	22
Thioredoxin-like protein 1	Txnl1	TXNL1_MOUSE	32 616	4.7	144	78	39	2
Ubiquitin-conjugating enzyme E2 N	Ube2n	UBE2N_MOUSE	17 184	6.2	119	71	20	6
Ubiquitin carboxyl-terminal hydrolase isozyme L1	Uchl1	UCHL1_MOUSE	25 165	5	77	64	16	8
Cytochrome b-c1 complex subunit 1, mitochondrial	Uqcrc1	QCR1_MOUSE	53 420	5.7	95	40	46	6
Transitional endoplasmic reticulum ATPase	Vcp	TERA_MOUSE	89 950	5	310	61	40	5
Voltage-dependent anion-selective channel protein 1	Vdac1	VDAC1_MOUSE	32 502	9.2	159	57	80	24
Vimentin	Vim	VIME_MOUSE	53 712	4.9	218	64	47	8

CPI: Calculated isoelectric point; PMF: Peptide mass fingerprint; MS/MS: Tandem mass spectrometry.

lation of proteins involved in protein complex biogenesis, nucleotide biosynthetic process, cell death and positive regulation of biosynthetic process. Additionally, proteins involved in proteolysis and positive regulation of protein metabolic process were downregulated in SCs upon CPX treatment. Proteins which were downregulated in SCs upon RA treatment are, among others, involved in cell cycle, RNA processing, glycolysis and negative regulation

Table 2 Gene Ontology functional annotation of proteins which were regulated in this experiment according to their involvement in different biological processes

Biological process	Proteins	CPX > RA	CPX < RA	CPX > c	CPX < c	RA > c	RA < c
Monosaccharide metabolic/ catabolic processes	5	Pgls Gapdh Eno1 Pkm2	Eno1		Eno1 Pkm2		Eno1 Ldhb Pkm2
Nucleobase, nucleoside, nucleotide, and nucleic acid biosynthetic processes	7	Atp5a1	Aprt Nme2 Nme1	Atp5a1	Aprt Atp5a1 Impdh2 Nme2 Nme1 Pnp	Nme2 Nme1	Aprt Atp5a1 Atp5h Impdh2
RNA and mRNA processing	6	Sfrs1			Hnrnpk Tardbp Pcbp1		Hnrnpc Sfrs3
Regulation of transcription	7	Ruvbl1 Trim28		Nup62	Ube2n Tardbp Cbx3 Hnrnpab		Trim28 Ruvbl1
Embryonic development	5	Sfrs1 Eno1	Psmc4 Eno1	Atp5a1 Myh9	Eno1 Atp5a1	Myh9 Psmc4	Eno1
Gene expression	16	Trim28 Sfrs1 Ruvbl1	Rps12	Eif3f Eif3i	Eef1a1 Eif5a Eef1d Cbx3 Hnrnpk Hnrnpab Tardbp Pcbp1		Sfrs3 Eif3i Hnrnpc Ruvbl1 Eef1d Trim28
Cell cycle processes	6	Ruvbl1		Myh9		Krt7 Myh9	Npm1 Tubb5 Stmn1 Ruvbl1
Cell morphogenesis involved in differentiation	4	Trim28		Myh9	Uchl1 Hnrnpab	Myh9	Stmn1 Trim28
Regulation of cell proliferation	4			Nup62	Nme2 Pnp	Nme2	Npm1
Regulation of signal transduction	4			Nup62	Ube2n		Npm1 Hspa5

Ciclopirox olamine (CPX) > retinoic acid (RA): Proteins which were more than 2-fold higher expressed in CPX-treated cells compared to RA-treated cells; CPX < RA: Proteins which were more than 2-fold higher expressed in RA-treated cells compared to CPX-treated cells; CPX > c: Proteins which were more than 2-fold higher expressed in CPX-treated cells compared to control; CPX < c: Proteins which were more than 2-fold higher expressed in control compared to CPX-treated cells; RA > c: Proteins which were more than 2-fold higher expressed in RA-treated cells compared to control; RA < c: Proteins which were more than 2-fold higher expressed in control compared to RA-treated cells.

of protein metabolic process.

Proteins which were upregulated in SCs upon CPX treatment are involved in nucleotide binding, regulation of cell death and protein transport, whereas proteins which were upregulated upon RA treatment are involved in nucleotide binding, metal ion binding and proteolysis.

Proteins in CPX treated cells compared to RA treated cells

When the proteins in RA treated SCs were compared to CPX treated SCs, we observed that 54 proteins are differently regulated (Tables 8 and 9). Of these proteins, 31 were upregulated and 26 downregulated upon CPX treatment. In some cases, different forms of one protein, e.g., Actb, Eno1, and Hsp90aa1 were observed and showed different regulation.

The bioinformatics analysis of these proteins, focussing on biological processes, showed involvement of the proteins in different categories (Figure 7). Proteins which were downregulated in CPX treated cells are involved in processes like protein complex biogenesis, nucleotide biosynthetic process, proteolysis, intracellular transport and regulation of cell death. Proteins which were downregulated as a reaction to RA treatment are involved in protein complex biogenesis, cell death, positive regulation of biosynthetic process, response to organic substance, glycolysis, anti-apoptosis and phosphorylation.

To get a better focus on proteins, which may play a key role in proliferation, we also focussed on proteins, which showed contrary regulation upon CPX treatment and RA treatment compared to control. This resulted in 15 proteins, of which eight were upregulated upon CPX treatment and concurrently downregulated upon RA treatment compared to control, and seven proteins,

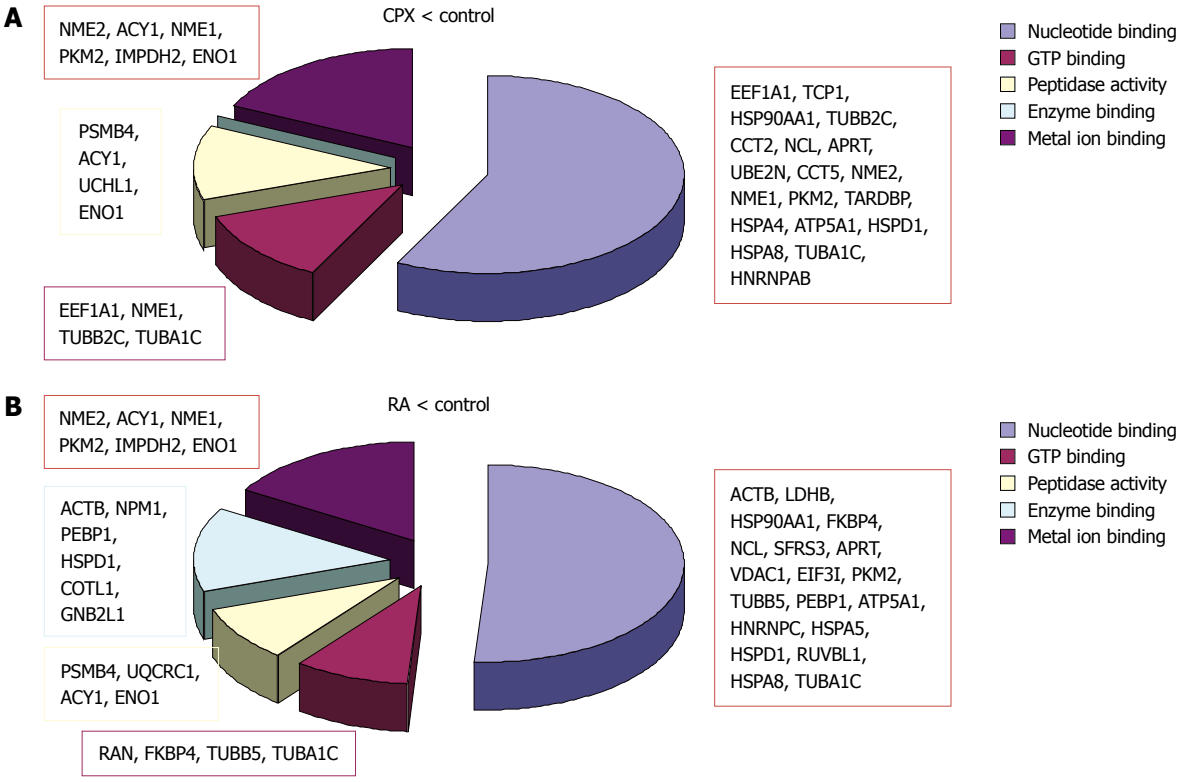

Figure 5 Molecular function. Classification of the downregulated proteins upon treatment with ciclopirox olamine (CPX) (A) or retinoic acid (RA) (B) according to their molecular functions.

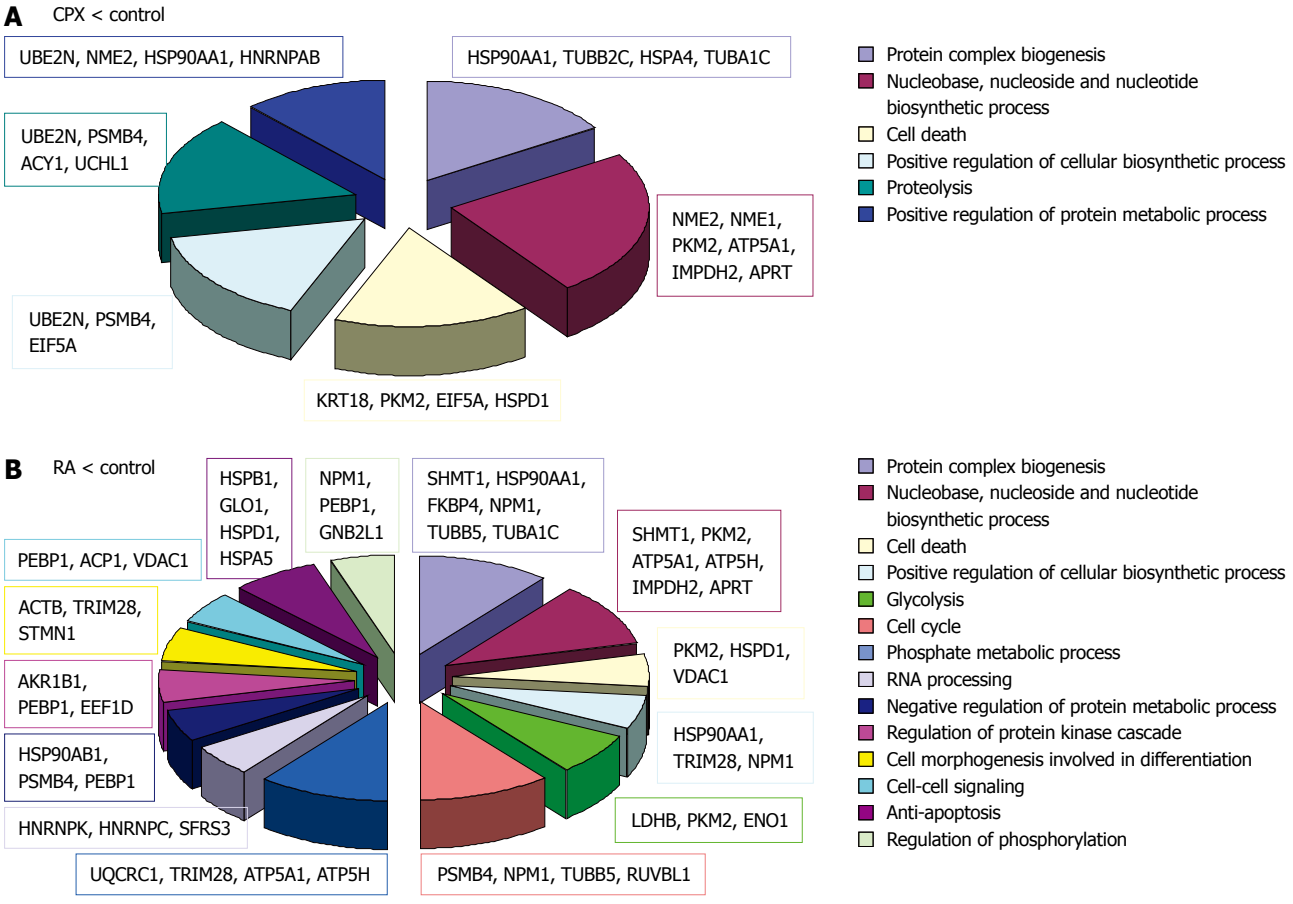

Figure 6 Biological process. Classification of the downregulated proteins upon treatment with ciclopirox olamine (CPX) (A) or retinoic acid (RA) (B) according to their biological processes.

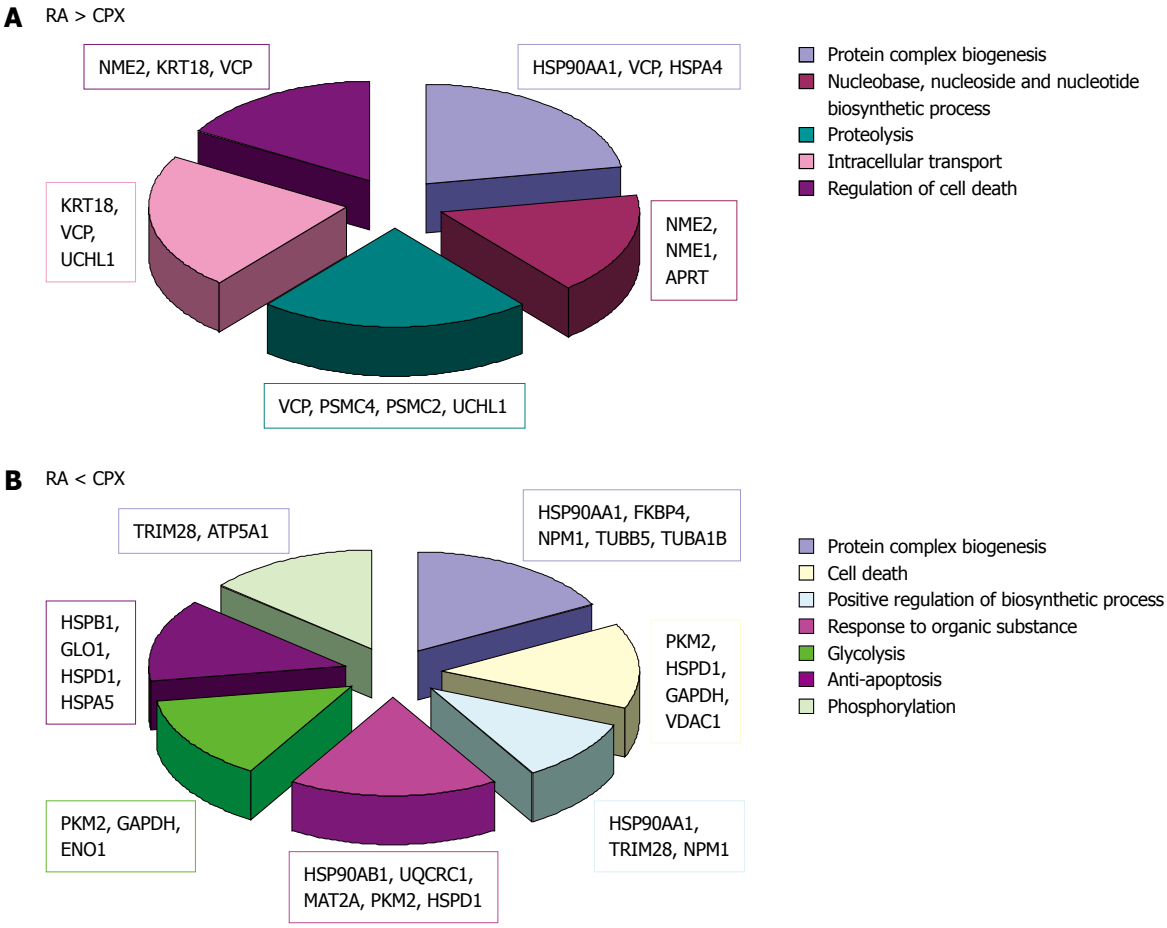

Figure 7 Biological process. Classification of the differently regulated proteins upon treatment with ciclopirox olamine (CPX) (A) or retinoic acid (RA) (B) according to their biological processes.

Table 3 Proteins which are upregulated upon ciclopirox olamine treatment compared to control

	k/CPX	
	ESC	maGSC
Actb	0.13	0.19
Atp5a1[1]	0.41	0.54
Ctsd	0.97	0.09
Eif3f[1]	0.94	0.43
Eif3i[1]	0.49	0.95
Etfa	0.63	0.39
Hspa9	0.92	0.31
Hspb1	0.63	0.05
Hspd1	0.19	0.21
Hspd1	0.36	0.69
Myh9[1]	0.63	0.09
Nup62[1]	0.30	0.60
S100a11		0.21
Tubb2a	1.00	0.21
Vdac1	0.58	0.18

[1]The proteins are referred to in the text and following tables. CPX: Ciclopirox olamine; ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell.

which were downregulated upon CPX treatment and concurrently upregulated upon RA treatment compared to control (proteins marked by asterisk in Tables 8 and 9).

Bioinformatics analysis of the proteins, which were downregulated upon CPX treatment along with upregulated upon RA treatment were primarily involved in metabolic processes (Nme2, Hsp90aa1, Psmc4, Rps12, Cct2 and Eno1) like protein folding (Hsp90aa1, Cct2), whereas proteins, which were upregulated upon CPX-treatment and concurrently downregulated upon RA-treatment were additionally involved in developmental processes (Psmc4, Eno1) and transport/localization (Vdac1, Hspa9).

Analysis of the molecular function of the differently regulated proteins upon CPX and RA treatment showed their important role in nucleotide binding (Nme2, Hsp90aa1, Psmc4, Hspa4, Cct2, Actb, Pkm2, Hspa5, Vdac1 and Hspa9) and metal ion binding (Pkm2, S100a11, Eno1).

DISCUSSION

CPX is a synthetic antifungal drug, which is currently used for the treatment of superficial mycoses[41]. Since two decades CPX has also been used as an antitumor agent[42]. It has been shown that CPX can be used to treat solid tumors due to its strong antiangiogenic activity[43,23]. CPX might inhibit the cell proliferation and work as an antitumor agent due to its iron chelating function, as iron is essential for cell proliferation and function[24]. In

Table 4 Proteins which are downregulated upon ciclopirox olamine treatment compared to control

	k/CPX	
	ESC	maGSC
Acp1	1.29	6.01
Acy1[1]	1.38	2.81
Akr1b1	2.07	13.44
Aprt[1]	4.80	3.60
Atp5a1[1]	3.50	1.21
Capzb	3.04	2.35
Cbx3[1]	1.72	2.12
Cct2[1]	12.00	1.28
Cct5[1]	1.06	2.02
Eef1a1[1]	2.47	1.74
Eef1d[1]	1.46	2.03
Eif5a[1]	1.31	2.07
Eno1[1]	3.56	1.60
Fscn1	3.31	1.49
Glod4	3.35	1.60
Gnb2l1	2.61	12.92
Hist1h2bb	2.31	2.10
Hist2h2ac	17.33	67.90
Hnrnpab[1]	2.41	3.36
Hnrnpk[1]	2.00	1.17
Hsp90aa1[1]	1.19	6.79
Hsp90aa1[1]	1.84	3.02
Hspa4[1]	3.28	1.51
Hspa4	1.13	3.14
Hspa8	1.74	7.17
Hspd1	1.67	3.07
Impdh2[1]	2.59	2.13
Impdh2[1]	> 100	27.94
Krt18[1]	2.31	1.44
Lgals2	2.82	8.45
Ncl[1]	1.33	2.61
Nit2	1.24	2.13
Nme1[1]	6.25	1.56
Nme2[1]	4.77	4.51
Pcbp1[1]	2.21	1.64
Pkm2[1]	3.75	3.27
Pnp[1]	1.20	2.62
Psmb4[1]	1.01	2.32
Ruvbl1[1]	1.02	2.14
Srm	1.64	3.63
Shmt1[1]	> 100	> 100
Tardbp[1]	1.38	3.52
Tcp1	1.47	3.76
Tuba1c[1]	1.87	3.11
Tubb2c[1]	1.38	3.41
Ube2n[1]	1.31	6.45
Uchl1[1]	2.66	1.66

[1]The proteins are referred to in the text and following tables. CPX: Ciclopirox olamine; ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell.

Table 5 Proteins which are downregulated upon retinoic acid treatment compared to control

Label	RA/k	
	ESC	maGSC
Acp1	0.61	0.50
Actb[1]	0.53	0.13
Acy1[1]	0.13	0.70
Akr1b1	0.43	0.11
Aprt[1]	0.46	0.39
Atp5a1[1]	0.76	0.38
Atp5h[1]	0.69	0.40
Cbx3	1.01	0.47
Cotl1[1]	0.50	0.44
Eef1d[1]	0.70	0.15
Eif3i[1]	0.09	0.92
Eno1[1]	0.24	0.04
Eno1[1]	0.55	0.22
Fabp3		0.45
Fkbp4[1]	0.90	0.40
Glo1[1]	0.74	0.41
Glod4	0.82	0.30
Impdh2[1]	0.76	0.35
Impdh2[1]	0.54	0.20
Gnb2l1[1]	0.66	0.15
Hnrnpc[1]	0.76	0.43
Hsp90aa1	0.75	0.08
Hsp90aa1	0.49	0.06
Hsp90aa1	0.76	0.12
Hspa5[1]	0.32	0.22
Hspa8[1]	0.69	0.50
Hspb1[1]	0.36	0.47
Hspb1[1]	0.46	0.88
Hspb1[1]	0.90	0.41
Hspd1[1]	0.16	0.67
Hspd1[1]	0.34	0.95
Itpa	0.57	0.07
Ldhb[1]	0.42	0.43
Lgals2	0.29	0.03
Ncl[1]	0.26	0.71
Npm1[1]	0.46	0.04
Pebp1[1]	0.89	0.42
Pkm2[1]	0.38	0.15
Pkm2[1]	0.32	0.65
Pkm2[1]	0.42	0.76
Pkm2[1]	0.21	0.43
Psmb4[1]	0.62	0.43
Ruvbl1[1]	0.63	0.22
Sfrs3[1]	0.41	0.46
Shmt1[1]	0.01	0.00
Srm	0.68	0.24
Trim28[1]	0.23	0.11
Trim28[1]	0.40	0.37
Tuba1c[1]	0.27	0.71
Tubb5[1]	0.70	0.25
Uqcrc1[1]	0.24	0.22
Vdac1[1]	0.30	0.52

[1]The proteins are referred to in the text and following tables. RA: Retinoic acid; ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell.

a recent study, we investigated the effect of CPX on the cellular viability and proliferation of SCs. The study demonstrated that in contrast to RA, CPX treatment resulted in a reversible antiproliferative effect[8]. The present study was conducted to understand the anti-proliferative effect of CPX on stem cells in terms of proteins and molecular processes which are involved in its mode of action.

With proteomic analysis of ESCs and maGSCs treated with CPX and RA, we could identify more than 90 single proteins which were differently expressed in both cell lines. Bioinformatics analysis of the regulated proteins demonstrated their involvement in various biological processes. To our interest, a number of proteins have potential roles in the regulation of cell proliferation either directly or indirectly.

One of the possible mechanisms of CPX action on

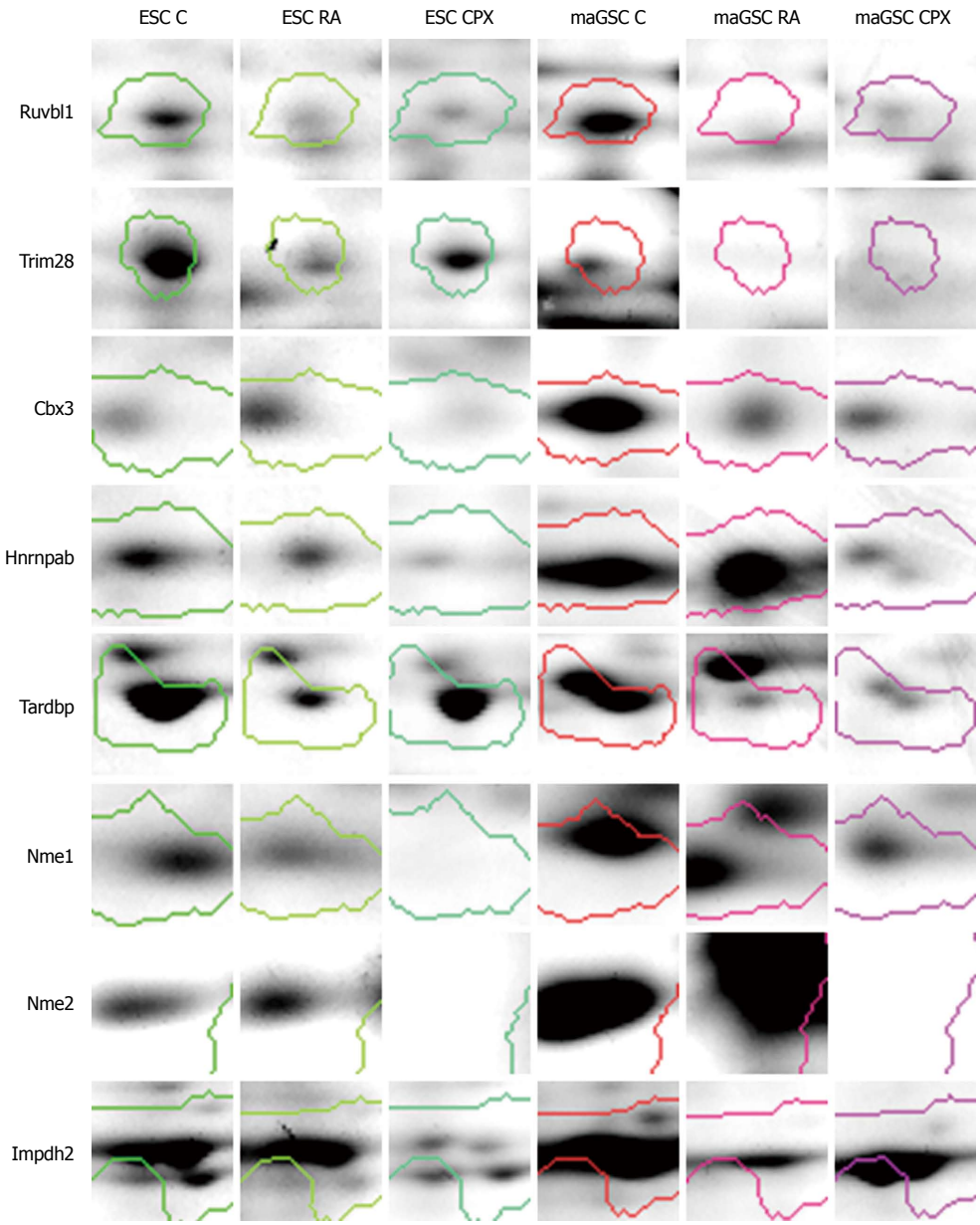

Figure 8 Enlargement of the gel spots of some proteins of interest. ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell; CPX: Ciclopirox olamine; RA: Retinoic acid.

Table 6 Proteins which are upregulated upon retinoic acid treatment compared to control

	RA/k	
	ESC	maGSC
Cct2	1.14	2.16
Hspa4	3.96	2.47
Krt7[1]	1.01	38.11
Krt8	1.97	1.78
Myh9[1]	3.06	3.24
Nme1[1]	2.52	3.81
Nme2[1]	1.20	2.48
Pdia6	1.72	20.48
Psmc4[1]	2.17	2.17
Vcp	8.30	4.13
Vim[1]	3.85	1.16

[1]The proteins are referred to in the text and following tables. RA: Retinoic acid; ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell.

cell proliferation is through controlling the progression of the cell cycle[44]. We identified a number of proteins which are involved in cell cycle processes. Ruvbl1 is one of the differentially regulated proteins which is involved in cell cycle processes, gene expression and transcription regulation. It was found to be downregulated in CPX and RA treated cells compared to control (Figure 8). Ruvbl1 is an evolutionarily highly conserved eukaryotic protein belonging to the AAA+ family of ATPase's[45]. It plays an important role in various cell cycle processes such as chromatin remodeling[46], gene activation[47], transcriptional regulation, DNA repair and transcription factor c-Myc[48]. It also controls Wnt signaling pathway through transcription-associated protein β-catenin[49,50]. Another protein, which was higher expressed in CPX treated cells compared to RA treated cells, is Trim28. Trim28 is involved in regulation of transcription and silencing gene

Table 7 Gene Ontology functional annotation of proteins which were regulated in this experiment according to their involvement in different molecular function

Molecular function	Proteins	CPX > RA	CPX < RA	CPX > c	CPX < c	RA > c	RA < c
Nucleotide binding	41	Hsp90ab1	Atp5b	Tubb2a	Cct2	Cct2	Ldhb
		Fkbp4	Cct2	Etfa	Tardbp	Hspa4	Fkbp4
		Tubb5	Tardbp	Hspa9	Hspa4	Myh9	Tubb5
		Hspa5	Hspa4	Actb	Tuba1c	Nme2	Hnrnpc
		Tubb1b	Actb	Myh9	Hspa8	Vcp	Hspa5
		Gapdh	Hsp90aa1	Vdac1	Hnrnpab	Psmc4	Tuba1c
		Etfa	Ncl	Atp5a1	Eef1a1	Nme1	Hspa8
		Hspa9	Aprt	Hspd1	Tcp1		Actb
		Actb	Nme2		Hsp90aa1		Hsp90aa1
		Hsp90aa1	Vcp		Ncl		Ncl
		Sfrs1	Psmc4		Aprt		Sfrs3
		Vdac1	Nme1		Ube2n		Aprt
		Pkm2	Psmc2		Nme2		Vdac1
		Atp5a1			Cct5		Pkm2
		Ruvbl1			Nme1		Pebp1
		Hspd1			Pkm2		Atp5a1
					Atp5a1		Ruvbl1
					Ruvbl1		Hspd1
					Hspd1		
GTP binding	8	Fkbp4	Nme1	Tubb2a	Eef1a1	Nme1	Fkbp4
		Tubb5			Nme1		Tubb5
		Tuba1b			Tuba1c		Tuba1c
ATPase activity	8	Atp5a1	Vcp	Atp5a1	Atp5a1	Vcp	Atp5a1
			Psmc4	Myh9	Hspa8	Psmc4	Atp5h
			Atp5b			Myh9	Hspa8
			Psmc2				
Enzyme binding	8	Actb	Actb	Actb	Gnb2l1	Vim	Actb
		Npm1	Gnb2l1	Hspd1			Pebp1
		Hspd1		Hspa9			Hspd1
		Cotl1					Cotl1
		Hspa9					
Cofactor binding	5	Gapdh		Etfa	Shmt1		Ldhb
		Etfa					Shmt1
Peptidase activity	6	Ctsd	Uchl1	Ctsd	Psmb4		Psmb4
		Eno1	Eno1		Acy1		Uqcrc1
					Uchl1		Acy1
					Eno1		Eno1
Metal ion binding	13	Trim28	Atp5b		Acy1	Pdia6	Acy1
		Sfrs1	Pdia6		Nme2	Nme2	Uqcrc1
		Pkm2	Nme2		Nme1	Nme1	Trim28 Pkm2
		Glo1	Nme1		Pkm2		Glo1
		Eno1	Eno1		Impdh2		Impdh2
					Eno1		Eno1

Ciclopirox olamine (CPX) > Retinoic acid (RA): Proteins which were more than 2-fold higher expressed in CPX-treated cells compared to RA-treated cells; CPX < RA: Proteins which were more than 2-fold higher expressed in RA-treated cells compared to CPX-treated cells; CPX > c: Proteins which were more than 2-fold higher expressed in CPX-treated cells compared to control; CPX < c: Proteins which were more than 2-fold higher expressed in control compared to CPX-treated cells; RA > c: Proteins which were more than 2-fold higher expressed in RA-treated cells compared to control; RA < c: Proteins which were more than 2-fold higher expressed in control compared to RA-treated cells.

expression through its ability to bind to DNA through interaction with a KRAB-ZFP protein. Other proteins, like Cbx3, Tardbp, and Hnrnpab, which are important in gene expression and regulation of transcription, were down-regulated due to treatment with CPX. Tardpb is a DNA and RNA-binding protein, which regulates transcription and splicing. It is also involved in the regulation of CFTR (Cystic fibrosis transmembrane conductance regulator), microRNA biogenesis, apoptosis and cell division. It can repress HIV-1 transcription by binding to the HIV-1 long terminal repeat. Cbx3 seems to be involved in transcriptional silencing in heterochromatin-like complexes. It recognizes and binds histone H3 tails methylated at K9, which leads to epigenetic repression. It is suggested that these proteins, which are involved in cell cycle processes, transcription regulation and gene expression, might be potential candidates for cell proliferation regulation and their repression through down-regulation might result in cell cycle stop without impact on stem cell pluripotency.

Proteins, which are involved in nucleotide biosynthetic process and proteolysis, were downregulated in CPX treated cells compared to control, as well as in RA treated cells (Figures 6A and 7A). Nucleoside diphosphatase kinases A and B (Nme1 and Nme2) are some of the

Table 8 Proteins which are upregulated in stem cells upon ciclopirox olamine treatment compared to retinoic acid treatment

	RA/CPX	
	ESC	maGSC
Actb*[1]	0.12	0.10
Actb[1]	0.14	0.15
Atp5a1[1]	0.43	0.48
Cotl1	0.19	0.67
Ctsd	0.95	0.16
Eif3i	0.04	0.87
Eno1*[1]	0.13	0.03
Eno1[1]	0.57	0.40
Etfa	0.68	0.16
Fkbp4[1]	0.46	0.43
Gapdh[1]	0.35	0.59
Glo1	0.31	0.66
Glod4	0.85	0.36
Hsp90aa1	0.28	0.38
Hsp90aa1	0.43	0.13
Hsp90ab1	0.41	0.26
Hspa5*[1]	0.17	0.13
Hspa9*[1]	0.50	0.28
Hspb1*[1]	0.29	0.04
Hspb1*[1]	0.20	0.42
Hspb1[1]	0.82	0.50
Hspd1[1]	0.49	0.74
Hspd1[1]	0.42	0.33
Itpa	0.21	0.08
Mat2a[1]	0.41	0.14
Npm1[1]	0.28	0.18
Nup62	0.57	0.26
Pgls[1]	0.40	0.68
Pkm2[1]	0.22	0.25
Pkm2[1]	0.29	0.86
Prdx6	0.46	0.94
Ruvbl1[1]	0.64	0.42
S100a11*[1]	[2]	0.17
Sfrs1[1]	0.70	0.30
Trim28[1]	0.44	0.08
Trim28[1]	0.42	0.44
Trim28[1]	0.33	0.28
Tuba1b*[1]	0.42	0.65
Tubb5[1]	0.42	0.54
Tubb5[1]	0.43	0.58
Vdac1*[1]	0.17	0.09

[1]The proteins are referred to in the text and following tables; [2]The protein was not identified in embryonic stem cells. Proteins, which are assigned an asterisk, were upregulated upon ciclopirox olamine (CPX) treatment compared to control and concurrently downregulated upon retinoic acid (RA) treatment compared to control. ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell.

Table 9 Proteins which are downregulated upon ciclopirox olamine treatment compared to retinoic acid treated stem cells

	RA/CPX	
	ESC	maGSC
Actb[1]	2.17	1.10
Aldh2	2.61	2.17
Aldh2	2.43	1.21
Aprt[1]	2.20	1.42
Atp5b	1.15	2.17
Capzb	2.79	2.07
Cct2*[1]	13.70	2.77
Eno1[1]	2.48	2.02
Eno1[1]	2.71	1.51
Fscn1	2.20	2.14
Gnb2l1	1.81	2.24
Hist1h2bb	7.53	1.62
Hist2h2ac	3.89	211.81
Hnrnpk	2.37	1.58
Hsp90aa1*[1]	2.69	6.36
Hsp90aa1	6.36	4.33
Hspa4*[1]	12.98	3.72
Hspa4	1.35	3.42
Krt7	> 100	1.14
Krt18*[1]	4.04	1.76
Ncl	1.80	3.48
Nme1[1]	2.63	1.57
Nme2*[1]	5.72	11.15
Pdia6	1.74	6.33
Psmc2[1]	2.87	1.20
Psmc4*[1]	3.06	2.26
Rps12*[1]	[2]	2.05
Tardbp	1.11	3.85
Uchl1[1]	2.02	1.10
Vcp[1]	8.94	2.57

[1]The proteins are referred to in the text and following tables; [2]The protein was not identified in embryonic stem cells. Proteins, which are assigned an asterisk, were downregulated upon ciclopirox olamine (CPX) treatment compared to control and concurrently upregulated upon retinoic acid (RA) treatment compared to control. ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell.

proteins which are involved in nucleotide biosynthetic process. These proteins are known to be involved in the synthesis of nucleoside triphosphatases[51] as well as in cell proliferation[52], differentiation[53] and development[54], signal transduction, G protein-coupled receptor endocytosis and gene expression. Nme1 was downregulated in CPX treated cells compared to control and RA treated cells (Figure 8). This may explain the slowdown of the proliferation of CPX treated SCs. Impdh2 is a rate limiting enzyme in the *de novo* synthesis of guanine nucleotides and is therefore involved in the regulation of cell growth and differentiation[55-58]. It may have a role in the development of malignancy and the growth progression of some tumors. Impdh2 was downregulated in CPX treated cells compared to control (Figure 8).

Proteins which were involved in cell death, positive regulation of biosynthetic process, response to organic substance, glycolysis, anti-apoptosis, and phosphorylation were downregulated in RA treated cells compared to control and CPX treated cells (Figures 6B and 7B).

Analysis of the molecular function of the differently expressed proteins demonstrated a potential involvement of some of these in metal ion binding, mainly iron binding. Cazzola *et al*[59] in 1990 established that iron is essential for proliferation, DNA synthesis and repair and mitochondrial electron transport. Therefore, it is assumed that CPX can stop the cell proliferation by regulating the expression of iron binding proteins.

The present study could give some insights into the mode of action of CPX in terms of expression regula-

tion of various proteins. It not only shed light on the previously discussed roles of CPX, but could also provide some further insight into their mechanism. We could identify some potential candidates which can effect the cell proliferation directly or indirectly through other cellular processes. By understanding the mode of action of CPX, this study may provide new aspect that will help in the future strategy to improve therapeutic intervention in the treatment with CPX.

ACKNOWLEDGMENTS

We thank Elke Brunst-Knoblich for technical assistance.

COMMENTS

Background

Ciclopirox olamine (CPX), a synthetic antifungal agent used in the treatment of fungal and yeast infection of skin or mucosa. Apart from its antimycotic activity, CPX is also effective against both gram-positive and gram-negative bacteria. CPX might also serve as an alternative agent for therapeutic angiogenesis. CPX was also shown to have an antiproliferative effect on stem cells without affecting their pluripotency.

Research frontiers

Although CPX is used as therapeutic for different aspect the mechanism of action is still not clear. In this study, the authors investigated the impact of CPX on stem cell proteome and identified cellular mechanisms that may explain the way of action of CPX. The authors provided evidence that CPX is involved in expression regulation of nucleotide binding proteins resulting in cell cycle arrest.

Innovations and breakthroughs

It is postulated that the CPX works as an inhibitor of the iron-dependent enzymes due to its potential role as a chelator of intracellular iron. The present study could give some insights into the mode of action of CPX in terms of expression regulation of various proteins especially nucleotide-binding proteins. It not only shed light on the previously discussed roles of CPX, but could also provide some further insight into their mechanism. We could also identify some potential candidates, which can effect the cell proliferation directly or indirectly through other cellular processes.

Applications

By understanding the mode of action of CPX, this study may provide new aspects that will be helpful in the future strategy for therapeutic intervention in the treatment with CPX.

Terminology

Multipotent adult germline stem cells (maGSCs) are spermatogonial stem cells isolated from murine testis. CPX, the ethanolamine salt of 6-cyclohexyl-1-hydroxy-4-methylpyridin-2(1H)-one, is a synthetic antifungal agent and is a hypusination inhibitor that controls the second step of the modification, which is catalyzed by deoxyhypusine hydroxylase. The hypusine is the result of a post-translational modification catalyzed by two enzymes: deoxyhypusine synthase and deoxyhypusine hydroxylase.

Peer review

This is a descriptive study in which the authors analyzed the proteome changes of embryonic stem cells and maGSCs accompanying the treatment with CPX and subsequent inhibition of hypusination using classical proteomic techniques like 2-DE, differential in-gel electrophoresis and mass spectrometry. The results are interesting and we could highlight that a treatment with CPX resulted in an alteration of the expression of 56 proteins compared to non-treated cells, and 54 proteins compared to retinoic acid treated cells. The majority of these proteins are involved in nucleotide binding and nucleotide biosynthetic processes, metal binding, DNA binding, and other processes which have been linked to CPX.

REFERENCES

1 **Weissman IL**. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *Science* 2000; **287**: 1442-1446 [PMID: 10688785 DOI: 10.1126/science.287.5457.1442]

2 **Vats A**, Bielby RC, Tolley NS, Nerem R, Polak JM. Stem cells. *Lancet* 2005; **366**: 592-602 [PMID: 16099296 DOI: 10.1016/S0140-6736(05)66879-1]

3 **Spradling A**, Drummond-Barbosa D, Kai T. Stem cells find their niche. *Nature* 2001; **414**: 98-104 [PMID: 11689954 DOI: 10.1038/35102160]

4 **Guan K**, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH, Nolte J, Wolf F, Li M, Engel W, Hasenfuss G. Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature* 2006; **440**: 1199-1203 [PMID: 16565704 DOI: 10.1038/nature04697]

5 **Zovoilis A**, Nolte J, Drusenheimer N, Zechner U, Hada H, Guan K, Hasenfuss G, Nayernia K, Engel W. Multipotent adult germline stem cells and embryonic stem cells have similar microRNA profiles. *Mol Hum Reprod* 2008; **14**: 521-529 [PMID: 18697907 DOI: 10.1093/molehr/gan044]

6 **Meyer S**, Nolte J, Opitz L, Salinas-Riester G, Engel W. Pluripotent embryonic stem cells and multipotent adult germline stem cells reveal similar transcriptomes including pluripotency-related genes. *Mol Hum Reprod* 2010; **16**: 846-855 [PMID: 20624824 DOI: 10.1093/molehr/gaq060]

7 **Dihazi H**, Dihazi GH, Nolte J, Meyer S, Jahn O, Müller GA, Engel W. Multipotent adult germline stem cells and embryonic stem cells: comparative proteomic approach. *J Proteome Res* 2009; **8**: 5497-5510 [PMID: 19810753 DOI: 10.1021/pr900565b]

8 **Dihazi H**, Dihazi GH, Jahn O, Meyer S, Nolte J, Asif AR, Mueller GA, Engel W. Multipotent adult germline stem cells and embryonic stem cells functional proteomics revealed an important role of eukaryotic initiation factor 5A (Eif5a) in stem cell differentiation. *J Proteome Res* 2011; **10**: 1962-1973 [PMID: 21344951 DOI: 10.1021/pr1012015]

9 **Schnier J**, Schwelberger HG, Smit-McBride Z, Kang HA, Hershey JW. Translation initiation factor 5A and its hypusine modification are essential for cell viability in the yeast Saccharomyces cerevisiae. *Mol Cell Biol* 1991; **11**: 3105-3114 [PMID: 1903841]

10 **Park MH**, Lee YB, Joe YA. Hypusine is essential for eukaryotic cell proliferation. *Biol Signals* 1997; **6**: 115-123 [PMID: 9285094 DOI: 10.1159/000109117]

11 **Jasiulionis MG**, Luchessi AD, Moreira AG, Souza PP, Suenaga AP, Correa M, Costa CA, Curi R, Costa-Neto CM. Inhibition of eukaryotic translation initiation factor 5A (eIF5A) hypusination impairs melanoma growth. *Cell Biochem Funct* 2007; **25**: 109-114 [PMID: 16850525 DOI: 10.1002/cbf.1351]

12 **Jakus J**, Wolff EC, Park MH, Folk JE. Features of the spermidine-binding site of deoxyhypusine synthase as derived from inhibition studies. Effective inhibition by bis- and mono-guanylated diamines and polyamines. *J Biol Chem* 1993; **268**: 13151-13159 [PMID: 8514754]

13 **Saini P**, Eyler DE, Green R, Dever TE. Hypusine-containing protein eIF5A promotes translation elongation. *Nature* 2009; **459**: 118-121 [PMID: 19424157 DOI: 10.1038/nature08034]

14 **Balabanov S**, Gontarewicz A, Ziegler P, Hartmann U, Kammer W, Copland M, Brassat U, Priemer M, Hauber I, Wilhelm T, Schwarz G, Kanz L, Bokemeyer C, Hauber J, Holyoake TL, Nordheim A, Brümmendorf TH. Hypusination of eukaryotic initiation factor 5A (eIF5A): a novel therapeutic target in BCR-ABL-positive leukemias identified by a proteomics approach. *Blood* 2007; **109**: 1701-1711 [PMID: 17008552 DOI: 10.1182/blood-2005-03-037648]

15 **Park MH**, Wolff EC, Smit-McBride Z, Hershey JW, Folk JE. Comparison of the activities of variant forms of eIF-4D. The requirement for hypusine or deoxyhypusine. *J Biol Chem* 1991; **266**: 7988-7994 [PMID: 1850732]

16 **Dittmar W**, Lohaus G. HOE 296, a new antimycotic compound with a broad antimicrobial spectrum. Laboratory re-

sults. *Arzneimittelforschung* 1973; **23**: 670-674 [PMID: 4197001]
17 **Jue SG**, Dawson GW, Brogden RN. Ciclopirox olamine 1% cream. A preliminary review of its antimicrobial activity and therapeutic use. *Drugs* 1985; **29**: 330-341 [PMID: 3158508 DOI: 10.2165/00003495-198529040-00002]
18 **Abrams BB**, Hänel H, Hoehler T. Ciclopirox olamine: a hydroxypyridone antifungal agent. *Clin Dermatol* 1991; **9**: 471-477 [PMID: 1822407 DOI: 10.1016/0738-081X(91)90075-V]
19 **Subissi A**, Monti D, Togni G, Mailland F. Ciclopirox: recent nonclinical and clinical data relevant to its use as a topical antimycotic agent. *Drugs* 2010; **70**: 2133-2152 [PMID: 20964457]
20 **Dittmar W**, Grau W, Raether W, Schrinner E, Wagner WH. [Microbiological laboratory studies with ciclopiroxolamine (author's transl)]. *Arzneimittelforschung* 1981; **31**: 1317-1322 [PMID: 7197538]
21 **Linden T**, Katschinski DM, Eckhardt K, Scheid A, Pagel H, Wenger RH. The antimycotic ciclopirox olamine induces HIF-1alpha stability, VEGF expression, and angiogenesis. *FASEB J* 2003; **17**: 761-763 [PMID: 12594177]
22 **Leem SH**, Park JE, Kim IS, Chae JY, Sugino A, Sunwoo Y. The possible mechanism of action of ciclopirox olamine in the yeast Saccharomyces cerevisiae. *Mol Cells* 2003; **15**: 55-61 [PMID: 12661761]
23 **Eberhard Y**, McDermott SP, Wang X, Gronda M, Venugopal A, Wood TE, Hurren R, Datti A, Batey RA, Wrana J, Antholine WE, Dick JE, Schimmer AD. Chelation of intracellular iron with the antifungal agent ciclopirox olamine induces cell death in leukemia and myeloma cells. *Blood* 2009; **114**: 3064-3073 [PMID: 19589922 DOI: 10.1182/blood-2009-03-209965]
24 **Zhou H**, Shen T, Luo Y, Liu L, Chen W, Xu B, Han X, Pang J, Rivera CA, Huang S. The antitumor activity of the fungicide ciclopirox. *Int J Cancer* 2010; **127**: 2467-2477 [PMID: 20225320 DOI: 10.1002/ijc.25255]
25 **Weir SJ**, Patton L, Castle K, Rajewski L, Kasper J, Schimmer AD. The repositioning of the anti-fungal agent ciclopirox olamine as a novel therapeutic agent for the treatment of haematologic malignancy. *J Clin Pharm Ther* 2011; **36**: 128-134 [PMID: 21366640 DOI: 10.1111/j.1365-2710.2010.01172.x]
26 **Hoque M**, Hanauske-Abel HM, Palumbo P, Saxena D, D'Alliessi Gandolfi D, Park MH, Pe'ery T, Mathews MB. Inhibition of HIV-1 gene expression by Ciclopirox and Deferiprone, drugs that prevent hypusination of eukaryotic initiation factor 5A. *Retrovirology* 2009; **6**: 90 [PMID: 19825182 DOI: 10.1186/1742-4690-6-90]
27 **Parreiras-e-Silva LT**, Luchessi AD, Reis RI, Oliver C, Jamur MC, Ramos RG, Oliveira EB, Curi R, Costa-Neto CM. Evidences of a role for eukaryotic translation initiation factor 5A (eIF5A) in mouse embryogenesis and cell differentiation. *J Cell Physiol* 2010; **225**: 500-505 [PMID: 20458750 DOI: 10.1002/jcp.22229]
28 **Lee NP**, Tsang FH, Shek FH, Mao M, Dai H, Zhang C, Dong S, Guan XY, Poon RT, Luk JM. Prognostic significance and therapeutic potential of eukaryotic translation initiation factor 5A (eIF5A) in hepatocellular carcinoma. *Int J Cancer* 2010; **127**: 968-976 [PMID: 19998337]
29 **Maier B**, Ogihara T, Trace AP, Tersey SA, Robbins RD, Chakrabarti SK, Nunemaker CS, Stull ND, Taylor CA, Thompson JE, Dondero RS, Lewis EC, Dinarello CA, Nadler JL, Mirmira RG. The unique hypusine modification of eIF5A promotes islet beta cell inflammation and dysfunction in mice. *J Clin Invest* 2010; **120**: 2156-2170 [PMID: 20501948 DOI: 10.1172/JCI38924]
30 **Nagy A**, Rossant J, Nagy R, Abramow-Newerly W, Roder JC. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc Natl Acad Sci USA* 1993; **90**: 8424-8428 [PMID: 8378314 DOI: 10.1073/pnas.90.18.8424]
31 **Bächner D**, Manca A, Steinbach P, Wöhrle D, Just W, Vogel W, Hameister H, Poustka A. Enhanced expression of the murine FMR1 gene during germ cell proliferation suggests a special function in both the male and the female gonad. *Hum Mol Genet* 1993; **2**: 2043-2050 [PMID: 8111372 DOI: 10.1093/hmg/2.12.2043]
32 **Cheng J**, Dutra A, Takesono A, Garrett-Beal L, Schwartzberg PL. Improved generation of C57BL/6J mouse embryonic stem cells in a defined serum-free media. *Genesis* 2004; **39**: 100-104 [PMID: 15170695 DOI: 10.1002/gene.20031]
33 **Wessel D**, Flügge UI. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal Biochem* 1984; **138**: 141-143 [PMID: 6731838 DOI: 10.1016/0003-2697(84)90782-6]
34 **Bradford MM**. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248-254 [PMID: 942051 DOI: 10.1016/0003-2697(76)90527-3]
35 **Karp NA**, Griffin JL, Lilley KS. Application of partial least squares discriminant analysis to two-dimensional difference gel studies in expression proteomics. *Proteomics* 2005; **5**: 81-90 [PMID: 15744836 DOI: 10.1002/pmic.200400881]
36 **Jahn O**, Hesse D, Reinelt M, Kratzin HD. Technical innovations for the automated identification of gel-separated proteins by MALDI-TOF mass spectrometry. *Anal Bioanal Chem* 2006; **386**: 92-103 [PMID: 16821028 DOI: 10.1007/s00216-006-0592-1]
37 **Werner HB**, Kuhlmann K, Shen S, Uecker M, Schardt A, Dimova K, Orfaniotou F, Dhaunchak A, Brinkmann BG, Möbius W, Guarente L, Casaccia-Bonnefil P, Jahn O, Nave KA. Proteolipid protein is required for transport of sirtuin 2 into CNS myelin. *J Neurosci* 2007; **27**: 7717-7730 [PMID: 17634366 DOI: 10.1523/JNEUROSCI.1254-07.2007]
38 **Reumann S**, Babujee L, Ma C, Wienkoop S, Siemsen T, Antonicelli GE, Rasche N, Lüder F, Weckwerth W, Jahn O. Proteome analysis of Arabidopsis leaf peroxisomes reveals novel targeting peptides, metabolic pathways, and defense mechanisms. *Plant Cell* 2007; **19**: 3170-3193 [PMID: 17951448 DOI: 10.1105/tpc.107.050989]
39 **Huang da W**, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2009; **4**: 44-57 [PMID: 19131956]
40 **Huang da W**, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* 2009; **37**: 1-13 [PMID: 19033363 DOI: 10.1093/nar/gkn923]
41 **Sehgal VN**. Ciclopirox: a new topical pyrodonium antimycotic agent. A double-blind study in superficial dermatomycoses. *Br J Dermatol* 1976; **95**: 83-88 [PMID: 782504 DOI: 10.1111/j.1365-2133.1976.tb15537.x]
42 **Bohn M**, Kraemer KT. Dermatopharmacology of ciclopirox nail lacquer topical solution 8% in the treatment of onychomycosis. *J Am Acad Dermatol* 2000; **43**: S57-S69 [PMID: 11051135 DOI: 10.1067/mjd.2000.109072]
43 **Clement PM**, Hanauske-Abel HM, Wolff EC, Kleinman HK, Park MH. The antifungal drug ciclopirox inhibits deoxyhypusine and proline hydroxylation, endothelial cell growth and angiogenesis in vitro. *Int J Cancer* 2002; **100**: 491-498 [PMID: 12115536 DOI: 10.1002/ijc.10515]
44 **Malumbres M**, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. *Nat Rev Cancer* 2009; **9**: 153-166 [PMID: 19238148 DOI: 10.1038/nrc2602]
45 **Neuwald C**, Heinschink A, Müller MM. Evaluation of a new screening assay, protein C pathway (PCP) test, for identification of defects in the protein C pathway. *Thromb Haemost* 1999; **81**: 992-994 [PMID: 10404784]
46 **Jónsson ZO**, Jha S, Wohlschlegel JA, Dutta A. Rvb1p/Rvb2p recruit Arp5p and assemble a functional Ino80 chromatin remodeling complex. *Mol Cell* 2004; **16**: 465-477 [PMID: 15525518 DOI: 10.1016/j.molcel.2004.09.033]
47 **Bauer A**, Chauvet S, Huber O, Usseglio F, Rothbächer U, Ar-

agnol D, Kemler R, Pradel J. Pontin52 and reptin52 function as antagonistic regulators of beta-catenin signalling activity. *EMBO J* 2000; **19**: 6121-6130 [PMID: 11080158 DOI: 10.1093/emboj/19.22.6121]

48 **Wood LJ**, Mukherjee M, Dolde CE, Xu Y, Maher JF, Bunton TE, Williams JB, Resar LM. HMG-I/Y, a new c-Myc target gene and potential oncogene. *Mol Cell Biol* 2000; **20**: 5490-5502 [PMID: 10891489 DOI: 10.1128/MCB.20.15.5490-5502.2000]

49 **Bienz M**, Clevers H. Linking colorectal cancer to Wnt signaling. *Cell* 2000; **103**: 311-320 [PMID: 11057903 DOI: 10.1016/S0092-8674(00)00122-7]

50 **Peifer M**, Polakis P. Wnt signaling in oncogenesis and embryogenesis--a look outside the nucleus. *Science* 2000; **287**: 1606-1609 [PMID: 10733430]

51 **Parks RE**, Brown PR, Cheng YC, Agarwal KC, Kong CM, Agarwal RP, Parks CC. Purine metabolism in primitive erythrocytes. *Comp Biochem Physiol B* 1973; **45**: 355-364 [PMID: 4351428 DOI: 10.1016/0305-0491(73)90070-9]

52 **Cipollini G**, Berti A, Fiore L, Rainaldi G, Basolo F, Merlo G, Bevilacqua G, Caligo MA. Down-regulation of the nm23.h1 gene inhibits cell proliferation. *Int J Cancer* 1997; **73**: 297-302 [PMID: 9335458]

53 **Rosengard AM**, Krutzsch HC, Shearn A, Biggs JR, Barker E, Margulies IM, King CR, Liotta LA, Steeg PS. Reduced Nm23/Awd protein in tumour metastasis and aberrant Drosophila development. *Nature* 1989; **342**: 177-180 [PMID: 2509941 DOI: 10.1038/342177a0]

54 **Lakso M**, Steeg PS, Westphal H. Embryonic expression of nm23 during mouse organogenesis. *Cell Growth Differ* 1992; **3**: 873-879 [PMID: 1472467]

55 **Knight RD**, Mangum J, Lucas DL, Cooney DA, Khan EC, Wright DG. Inosine monophosphate dehydrogenase and myeloid cell maturation. *Blood* 1987; **69**: 634-639 [PMID: 2879579]

56 **Itoh O**, Kuroiwa S, Atsumi S, Umezawa K, Takeuchi T, Hori M. Induction by the guanosine analogue oxanosine of reversion toward the normal phenotype of K-ras-transformed rat kidney cells. *Cancer Res* 1989; **49**: 996-1000 [PMID: 2643467]

57 **Jackson RC**, Weber G, Morris HP. IMP dehydrogenase, an enzyme linked with proliferation and malignancy. *Nature* 1975; **256**: 331-333 [PMID: 167289 DOI: 10.1038/256331a0]

58 **Zimmermann A**, Gu JJ, Spychala J, Mitchell BS. Inosine monophosphate dehydrogenase expression: transcriptional regulation of the type I and type II genes. *Adv Enzyme Regul* 1996; **36**: 75-84 [PMID: 8869741 DOI: 10.1016/0065-2571(95)00012-7]

59 **Cazzola M**, Bergamaschi G, Dezza L, Arosio P. Manipulations of cellular iron metabolism for modulating normal and malignant cell proliferation: achievements and prospects. *Blood* 1990; **75**: 1903-1919 [PMID: 2186818]

P- Reviewers Marchal JA, Tanabe S, Zaminy A
S- Editor Wen LL **L- Editor** A **E- Editor** Zheng XM