

# Interaction of Mesalazine (5-ASA) with Translational Initiation Factors eIF4 Partially Explains 5-ASA Anti-Inflammatory and Anti-Neoplastic Activities

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**Abstract:** 5-aminosalicylic acid (5-ASA or mesalazine) is widely used for treatment of inflammatory bowel disease and considered to be cancer preventive. Still, the molecular mechanisms explaining its properties remain largely unknown, partially due to the lack of instrumentarium needed to identify its array of molecular targets. Modern OMICs-based technologies utilized in this study may serve as a powerful and unbiased tool to search for such targets. Here we demonstrate that 5-ASA alters  $\beta$ -catenin immunocomplex formation by changing complex binding of seven proteins including translation initiation factors eIF4b. OMICs-based cross-testing by reverse in-gel chemogenomics (utilizing 5-ASA's fluorescent properties), in-silico docking and surface plasmon resonance experiments identified binding of 5-ASA to eIF4e's cap-binding pocket, a key regulatory site for protein synthesis. In-vitro translation experiments with rabbit reticulocytes confirmed a dose-dependent inhibition of protein syntheses by 5-ASA. By using two unbiased and independent OMICs-based experimental approaches two members of the cellular translation machinery, eIF4b and IF4e, were identified as targets of 5-ASA. Inhibition of protein syntheses is a previously unrecognized property of 5-ASA that may add to its anti-inflammatory and anti-neoplastic activities.

**Keywords:** 5-ASA, chemogenomics, 2D-DIGE, IBD, mesalazine, OMICs.

## INTRODUCTION

Inflammatory bowel disease (IBD) is a relapsing and remitting condition characterized by chronic inflammation at various sites in the GI tract, which results in diarrhea and abdominal pain. The current standard of care for mildly to moderately active ulcerative colitis (UC) involves the use of drugs that contain 5-ASA [1-3]. Moreover, epidemiological and *in vitro* studies suggest that 5-ASA protects from the development of CRC by lowering the incidence of colitis-associated cancer [4, 5]. CRC is the second leading cause of cancer death in North America and Europe with a natural history of 10-15 years from a precursor lesion to malignant transformation [5, 6]. 5-ASA also reduces oxidative stress, inhibits cell proliferation and cyclooxygenase-2 (COX-2)/prostaglandin E2 synthesis, decreases transcriptional activity of NF- $\kappa$ B and interferes with Wnt pathway [7, 8]. Although clinical evidences for the effects of 5-ASA in patients with IBD suggest specific anti-inflammatory or oxygen scavenging properties, this drug seems to possess multiple modes of action which, despite numerous experimental studies, remain unknown. Therefore, finding novel 5-ASA targets

or altered pathways represents a challenging task both in the field of IBD and cancer prevention.

Although the molecular basis of 5-ASA action and metabolism is critical both for understanding pharmaceutical and side effects, the direct methods to study targets for such a small molecule are still hardly achievable. This depends on several important factors: (i) inability to label 5-ASA by specific markers, either fluorescent or radioactive, without changing the pharmacophore properties of a drug [9]; (ii) difficulties in selecting major 5-ASA molecular targets from the minor ones, and (iii) difficulties of tagging 5-ASA for binding studies. However, recent progress in OMICs approaches, especially 2D-DIGE and chemical genomics, makes it possible not only to analyze changes of the proteome in a single gel [10, 11] but also identify potential binding partners [12]. In DIGE-based proteomics, the stimulated and control samples are labeled with different fluorophores and run in the same gel, thereby minimizing technical variations and allowing to perform quantitative profiles of differentially expressed proteins [11]. In turn, chemical genomics (chemogenomics) involves the combination of a compound's effect on biological targets together with modern genomics approaches [12] and is aimed either at the isolation of small molecules that exhibit specific activity against certain phenotypes (forward chemogenomics) or going from drug with known functions towards identification of the cellular targets for that drug (reverse chemogenomics) [13]. Yet the amount of data, reliability, cost effectiveness and processing time are

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obstructive to a broad implementation of OMICs in this field. Therefore novel approaches for studying molecular drug targets and effects are welcome.

Here, we developed two novel OMICS-based integrative approaches for a better understanding of the molecular basis of 5-ASA drug effects. This enabled us to detect new molecular targets of 5-ASA, in particular, initiation translation factors eIF4e,b which we further proved to be important for 5-ASA-mediated inhibition of protein synthesis.

## MATERIALS AND METHODS

### Cell Lines and Reagents

Human colorectal carcinoma cell lines HT29 and SW480 were grown in IMDM (Gibco/Invitrogen, Lofer, Austria) containing 10% fetal bovine serum (FBS; Biochrom, Berlin, Germany). For the cell treatment with 5-ASA (Sigma) the chemical was dissolved in culture medium at 20 mM final and pH adjusted to 7.2 with NaOH. The 5-ASA analogues 4-ASA, 3-ASA (Sigma) and inactive metabolite N-acetyl-5-ASA (Dr. Falk Pharma, Freiburg, Germany) were dissolved in PBS prior to use.

### Immunoprecipitation and Western Blotting

Western blotting and immunoprecipitation experiments were carried out as described [14]. Briefly, 1 µg of appropriate antibodies or unrelated (non-immune control) antibody was bound to protein A-agarose beads (Invitrogen) in the presence of ethidium bromide (20 µg/ml) and incubated with corresponding cell lysates overnight at 4°C. After extensive washing with PBS buffer, beads were eluted by boiling in SDS gel sample buffer and proteins were separated by SDS-PAGE, immunoblotted onto a nylon membrane and probed with specific primary antibodies. Protein bands were visualized with anti-rabbit or anti-mouse IgG coupled to horseradish peroxidase using the ECL kit (Amersham, Arlington Heights, IL, USA). Primary antibodies used were as follows: monoclonal antibody anti-beta catenin (Becton Dickinson), pan-actin (Santa Cruz Biotech.), anti-PPAR-γ antibody (AbCam, Cambrifge, UK).

### IP-Coupled 2D-DIGE Approach and Image Acquisition

Total cell extracts (24h and 0h 5-ASA-treated) or control (antibody alone) were subjected to immunoprecipitation. Following four washing steps with PBS, immunoprecipitates were boiled in acidic buffer containing pH 2.7 HEPES and 1.5% SDS. After spinning with 5000 x g, supernatants were precipitated with cold TCA (10% final), washed with pre-chilled 75% acetone in water. The pellet was resolubilized in 1% SDS, pH 7.2 buffer and labeled with fluorescent dyes according to the protocol from the supplier (FlashPro kit, Raytest, Germany). Samples from 0h and 24h 5-ASA treatment were labeled with blue and red dye, respectively. The antibody control samples were labeled with green. Mixture of all three samples (approximately 2.5 µg each) was either processed through a regular 1D SDS PAGE or dissolved into rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 70 mM DTT, and 0.5% ampholytes) and subjected to 13 cm pH 4-7 IPG DryStrips (GE Healthcare) for 14 h at room temperature. Isoelectric focusing was carried out on an IPGphor device (GE Healthcare) until 15 kV-h was reached. Prior to SDS-PAGE the IPG strips were equilibrated for 20 min in

equilibration solution (50 mM Tris-HCl, 6 M urea, 30% glycerol, 2% SDS, pH 8.8) containing 10 mg/ml DTT followed by equilibration for 15 min in equilibration solution containing 25 mg/ml iodoacetamide (Sigma). Thereafter SDS-PAGE was performed on homogeneous gels (12% acrylamide) using a SE-600 equipment (GE Healthcare). Electrophoresis conditions were: 50 V for 45 min., 120 V for 1.5 h, and finally 200 V for 3 h. Then gels were scanned using a Typhoon TRIO scanner (GE Healthcare) with a resolution of 100 µm and the following filter setting: 505 nm excitation and 530 nm emission for the blue dye, 555 nm and 580 nm for the green dye and 647 nm and 671 nm for the red dye. Sensitivity (photomultiplier voltage) was adjusted in a prescan with 1000 µm resolution so that no channel had saturated spots. After scanning, the gels were silver-stained according to Shevchenko *et al.* [15] and stored in 1% acetic acid at 4 °C until protein identification. In parallel, gel image warping and detection of spots were performed using the Delta-2D software (Decodoc, Germany) (pH range 4-7). Each gel image was warped to a selected reference gel. Spot detection was performed on an artificial summative gel. Spots with a molecular weight above 250 kDa or below 10 kDa and spots with a pI above 6.9 or below 4.1 were excluded from further analysis. Finally all the remaining spots were manually reviewed to ensure no false spot detection. In case of multiple spots from the stretches representing modified proteins only one spot was taken for MS analyses. The fluorescent signal of a spot was background-corrected and normalized by the Delta-2D according to the manufacturer's manual.

### Protein Identification

Protein spots were cut out of the gel by a pipette with blunt tip, the gel-pieces were de-stained with 15 mM K<sub>3</sub>Fe(CN)<sub>6</sub>/50 mM Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> and intensively washed with 50% methanol/10% acetic acid. The pH was adjusted with 50 mM NH<sub>4</sub>HCO<sub>3</sub>, proteins were reduced with 10 mM DTT/50 mM NH<sub>4</sub>HCO<sub>3</sub> for 30 minutes at 56°C and alkylated with 50 mM iodoacetamide/50 mM NH<sub>4</sub>HCO<sub>3</sub> 20 minutes in the dark. Afterwards the gel-pieces were treated with ACN and dried in a vacuum centrifuge. Between each step, the tubes were shaken for 5-10 minutes (Eppendorf Thermomixer comfort). Dry gel-spots were treated with 0.1 mg/ml trypsin (Trypsin sequencing grade, Roche Diagnostics)/50 mM NH<sub>4</sub>HCO<sub>3</sub> for 20 minutes on ice, afterwards covered with 50 mM NH<sub>4</sub>HCO<sub>3</sub> and subsequently incubated overnight at 37°C. The digested peptides were eluted by adding 50 mM NH<sub>4</sub>HCO<sub>3</sub>, the supernatant was transferred into silicon-coated tubes, and this procedure was repeated two times with 5% formic acid/50% ACN. Between each elution step the gel-spots were ultrasonicated for 10 minutes. Finally the peptide solution was concentrated in a vacuum centrifuge to an appropriate volume. For the identification of 2D spots, peptides were loaded on a Zorbax 300SB-C8 (5 µm, 0.3 mm, 5 mm) column and separated by nanoflow LC (1100 Series LC system, Agilent) with a Zorbax 300SB-C18 (5 µm, 75 mm, 150 mm) column at a flow rate of 250 nl/min using a gradient from 0.2% formic acid and 3% ACN to 0.2% formic acid and 45% ACN over 12 minutes. In case of shotgun analysis, peptides were also separated by nano-flow 1100 Series LC system (Agilent) using the HPLC-Chip technology (Agilent) equipped with a 40 nl Zorbax 300SB-C18

trapping column and a 75  $\mu\text{m}$  x 150 mm Zorbax 300SB-C18 separation column at a flow rate of 400 nl/min, using a gradient from 0.2% formic acid and 3% ACN to 0.2% formic acid and 50% ACN over 80 minutes. Peptide identification was accomplished by MS/MS fragmentation analysis with an iontrap mass spectrometer (XCT-Ultra, Agilent) equipped with an orthogonal nanospray ion source. The MS/MS data, including peak list-generation and search engine, were interpreted by the Spectrum Mill MS Proteomics Workbench software (Version A.03.03, Agilent) allowing for two missed cleavages and searched against the SwissProt database for human proteins (Version 14.3 containing 20328 entries) allowing for precursor mass deviation of 1.5 Da, a product mass tolerance of 0.7 Da and a minimum matched peak intensity (%SPI) of 70%. Due to previous chemical modification, carbamidomethylation of cysteine was set as fixed modification. No other modifications were considered here.

### PPAR- $\gamma$ Overexpression and 5-ASA Binding

Plasmid DNA encoding for full length PPAR- $\gamma$  under control of the SV40 promoter was transformed into E.coli to allow overexpression of the product. Thereafter SDS-PAGE was performed on 4-12% acrylamide gel. The gel was rinsed with PBS and incubated with 50  $\mu\text{M}$  5-ASA solution for 15 min at RT. After three consecutive washing steps in PBS (1, 3 and 3 min) the gel was UV-scanned using ProXPRESS system (PerkinElmer). After that, the gel was thoroughly rinsed with PBS and processed for Western blotting. PPAR- $\gamma$  signals were determined by Western blotting.

### In-Gel Chemogenomics Approach

2D-PAGE was performed as described above. The gel was rinsed with PBS and incubated with 50  $\mu\text{M}$  5-ASA solution for 15 min at RT. After three consecutive washing steps in PBS (1, 3 and 3 min) the gel was UV-scanned using ProXPRESS system (PerkinElmer). After that, the gel was silver-stained and stored in 1% acetic acid at 4  $^{\circ}\text{C}$  until protein identification. The spots of interest were excised manually, trypsin digested, desalted and analyzed on a MALDI-TOF/reflectron TOF instrument as above (provided by Cleveland Clinics, OH). MASCOT program was used to search the Swiss-Prot protein sequence database.

### Surface Plasmon Resonance (SPR)

The SPR assay was done as described earlier [16]. Shortly, full-length recombinant protein eIF-4e (AAH12611,1-218 a.a.) with GST tag (Novus) was immobilized by anti-GST antibody on a surface of a dock sensor chip CM-5 and the 5-ASA solution (10  $\mu\text{M}$ ) was manually injected over a single flow cell until a resonance unit value of 500 was obtained. Heat inactivated EIF4E-GST protein was immobilized on a control flow cell and used to compare specificity of 5-ASA binding and bulk changes in the refractive index.

### In vitro Translation Assay

In vitro translation assay was performed using the Retic Lysate IVT<sup>TM</sup> Kit (Ambion) according to standard protocols [17-19]. Briefly, the reaction was initiated by the addition of [<sup>35</sup>S]-methionine to the reaction mix containing freshly thawed reticulocyte lysate, incubated at 30  $^{\circ}\text{C}$  and terminated by spotting 6  $\mu\text{l}$  aliquots at 0,5,15,30,60 and 120 min

on to trichloroacetic acid (TCA)- soaked Whatman 3 filter discs. The filters were immediately dried, washed in 10% TCA at room temperature for 10 min, transferred to boiling 10% TCA for 10 min, and then to 70C 10% TCA with hydrogen peroxide (3% final v/v) for 5 min, followed by 3 consecutive washes in the ice-cold 10% TCA for 5 min, then at 1:1 absolute ethanol/diethyl ether for 5 min and finally in diethyl ether for 5 min. The discs were air-dried for 10 min, placed in vials containing scintillation fluid and the radioactivity counted in a Hewlett Packard Liquid Scintillation counter.

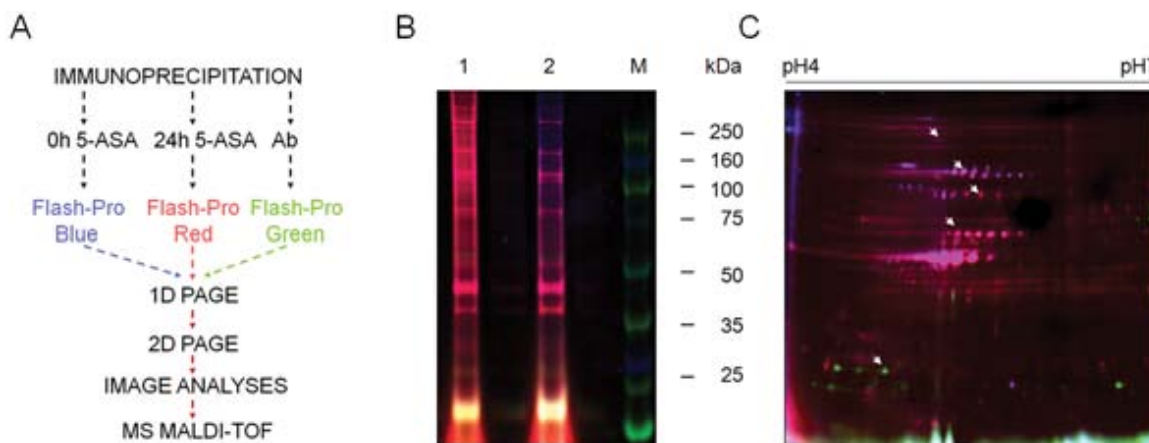
### Docking Simulation

Docking of 5-ASA to target proteins was performed using AutoDock4.0 (<http://autodock.scripps.edu>) and the AutoDockTools package. We performed 2000 runs of Lamarckian Genetics Algorithm with 0.375  $\text{\AA}$  grid spacing. Grid sizes were sufficient for blind docking. All docking parameters were set to their default values. We utilized the eIF-4e structure from crystal structure of eIF-4e complex with 7-methyl-GpppA (PDB ID 1IPB) [20] as a receptor for blind docking with AutoDock. The structure of 5-ASA with the best docking score is provided.

## RESULTS

### Differential Expression Analysis of 5-ASA Target Proteins from Wnt Pathway

Similarly to many other OMICs techniques, the conventional 2D-DIGE approach suffers from inability to limit the number of proteins that are differentially expressed under certain stimuli. In order to overcome this obstacle towards potential search of 5-ASA targets we included an immunoprecipitation (IP) step with the antibody from the 5-ASA known target protein before processing through the 2D-DIGE (Fig. 1A). Since 5-ASA was known to interfere with Wnt pathway we immunoprecipitated HT29 cell extracts with the antibody against  $\beta$ -catenin that represents a key player within Wnt signaling. Immunopellets from 24h 5-ASA-treated and non-treated HT29 cell extracts or antibody controls were carefully washed, normalized, labeled with saturation fluorochromes of different emission lengths and subjected to 1D SDS PAGE. Changes of protein patterns were obtained by laser-scanning the gel with three different wavelengths (Fig. 1B). Importantly, bands from antibodies did not mask the overall signals from IP-ed proteins, thus enabling subtraction of false-negative signals (Fig. 1B in green color). We then subjected the labeled IP pellets to the 2D gel analyses followed by the same scanning procedure (Fig. 1C). In order to narrow down the spots of interest for further protein identification, the scanned gels underwent quantitative analyses using Delta-2D software and the signals from antibodies, the highest and the lowest pH areas were consecutively excluded (supplementary Fig. 1S). The remaining spots were selected as spots of interest and annotated on the master gel. Spots representing differentially regulated proteins below or above certain threshold were identified (supplementary Fig. 1S-IV). Whenever possible, multiple spots (stretches) of the same molecular weights representing post-translationally modified proteins were narrowed down to one spot (depicted by arrows on Fig. 1C). Spots were excised from the gel, trypsin-digested and ana-



**Fig. (1). Identification of 5-ASA target proteins using proteomics approach.** (A) Experimental scheme to identify 5-ASA targets. (B) Extracts from HT29 cells treated (24h) or untreated (0h) with 5-ASA have been immunoprecipitated with  $\beta$ -catenin antibodies. Corresponding immunopellets were carefully washed, normalized and labeled with saturation fluorochromes of different emission lengths (Flash-blue for 0h, Flash-red for 24h and Flash-green for antibody along). The equal amounts of labeled probes have been mixed together and subjected to 1D SDS PAGE followed by laser scanning with three wave lengths. (C) Same as above replicas have been processed for 2D-DIGE and the laser-scanned images representing up- (blue), down- (red) or control (green, antibody signals) regulated proteins were merged to find out differentially expressed patterns. Some spots of interest (depicted by arrows) were identified by MS MALDI-TOF technique.

lyzed by the MALDI-TOF/TOF MS analysis. Seven proteins including insulin receptor, melanoma-associated antigen C1 and the eukaryotic translation initiation factor 4b (eIF4b) were identified by this approach, all of which are potential targets of 5-ASA biological activity (supplementary Table 1). Overall, this approach visualizes the dynamic changes of protein complex composition upon exposure to 5-ASA and demonstrates that 5-ASA alters the assembly of the  $\beta$ -catenin complex by reducing complex binding with eIF4b and other proteins.

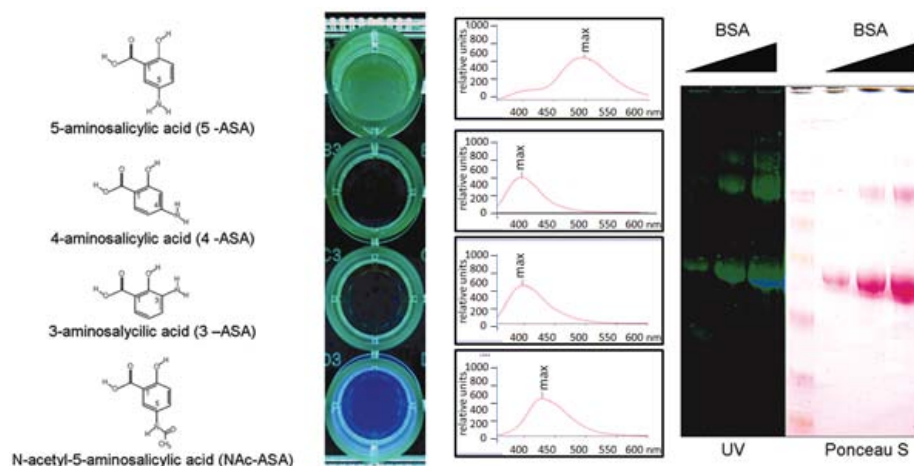
#### Reverse In-Gel Chemogenomics for 5-ASA Targets

Reverse chemogenomics [13], including, siRNA-small molecule screening [21], reverse two-hybrid system [22] or screening direct-acting compounds modifying DNA [23] represents some recent advancements of modern chemogenomics and allows identifying the cellular drug targets. Unlike high throughput screening of chemical libraries that contain thousands of small molecules against a certain target, reverse screening of chemogenomics explores biological pathways to identify phenotypic change by small molecules. As part of the reverse screening approach here we designed a novel and simple method to narrow down potential 5-ASA targets. We made use of the fact that UV-evoked emission spectrum of 5-ASA differs both from its analogues 4-ASA or 3-ASA and from its inactive metabolite NAc-ASA (Fig. 2A-C). We then asked whether any potential 5-ASA binding protein can be visualized by incubating an SDS PAGE with resolved protein of interest in 5-ASA solution followed by exposure to UV light. For that part we utilized BSA protein whose interaction with 5-ASA has been recently described [24]. When subjected to SDS PAGE, signals from BSA interacting with 5-ASA can be visualized by incubating the gel in 5-ASA solution, several washings in PBS, and exposure to UV light from the laboratory trans-illuminator (Fig. 2D, left panel). 5-ASA-BSA signals detected by UV were confirmed by staining the gel in Ponceau S (Fig. 2D, right panel). We also recognized enhanced fluorescence emission when both

5-ASA and BSA were mixed in the solution (data not shown). This may intensify signals from 5-ASA-interacting partners.

We further proved this technique by testing another protein, PPAR- $\gamma$ , that not only docks with 5-ASA but also interferes with its function as part of the Wnt pathway [25]. Following similar approach we were able to visualize signals from PPAR- $\gamma$  overexpressed in E.coli cells (supplementary Fig. 2S). This finding suggests that 5-ASA may selectively bind certain target proteins and such binding can be detected within the signals of other proteins. After multiple washing steps, the fluorescent band (green spectrum) was revealed upon exposure of the gel to UV light (supplementary Fig. 2SA). Western blot of the same gel probed with specific anti-PPAR- $\gamma$  antibody revealed signals with the size identical to fluorescent bands (supplementary Fig. 2SB), which was in line with the binding of 5-ASA to PPAR- $\gamma$ .

In attempt to cross-test the results of the IP-coupled 2D-DIGE and identify novel (non- $\beta$ -catenin-related) 5-ASA binding partners within the whole protein extract we performed similar experiments by incubating a 2D gel with resolved SW480 colorectal cancer cell lysate in 5-ASA solution. Since the amount of proteins and therefore overall concentration of small molecules that might be retained on potential targets is limited, we attempted to optimize the drug-protein signals by scanning the above gel with UV scanner (ProXPRESS system, PerkinElmer). Followed by 5-ASA incubation, rinsing and scanning procedures (Fig. 2E upper panel) the gel underwent more stringent washing steps and processed for silver staining (Fig. 2E lower panel). We also incubated the same gel in NAc-ASA solution however scanning produced weaker signals (data not shown). Spots were processed for MS analyses using MALDI-TOF system and six proteins were identified (supplementary Table 2), one of which (depicted with red arrow on Fig. 2E) turned out to be translation factor eIF-4e. The finding of these two factors, both of which play a role in initiation of translation suggests that 5-ASA may affect protein syntheses.



**Fig. (2).** In-gel visualizing of 5-ASA interaction with BSA and Identifying 5-ASA targets in 2D-gel. (A) 5-ASA, 4-ASA, 3-ASA and NAcetyl-ASA show differential fluorescence in solution upon exposure to UV light (B) which corresponds to the difference in emission spectra (C). In-gel visualization (D) of 5-ASA-BSA signals upon exposure to UV light (left) or after Ponceau S staining (right). (E) Protein cell extract from SW480 colorectal cancer cells has been resolved in 2D gel and 5-ASA solution (20  $\mu$ M) was added to the gel following 3 washing steps as in previous experiments and subjected to UV scan by ProXPRESS system (upper panel) After extensive washing in PBS, 2D gel has been silver-stained and some spots representing signals from 5-ASA target proteins have been processed for MALDI-TOF analyses (supplementary Table 2). eIF-4e identified protein is depicted by red arrow (lower panel).

### 5-ASA Interferes with eIF-4e and Inhibits Protein Syntheses

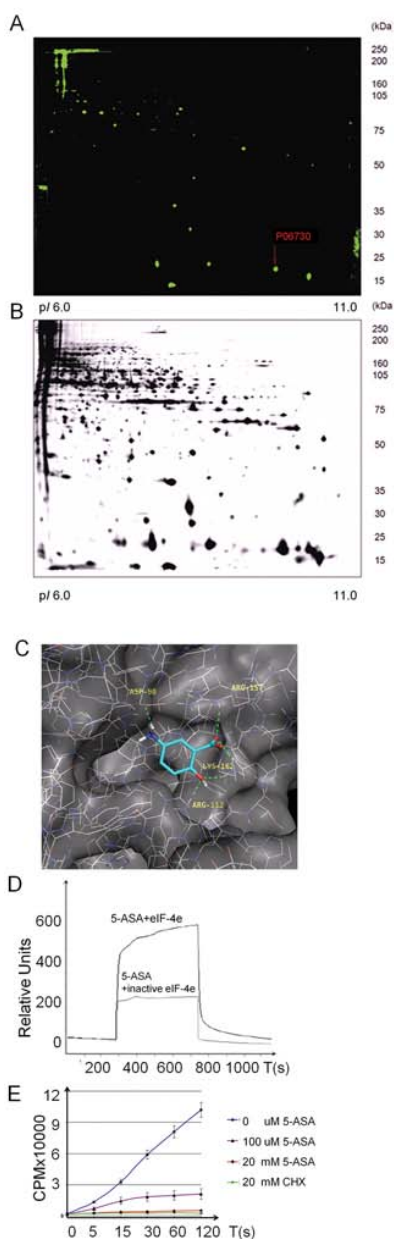
Docking simulation of the ligand (5-ASA) and the protein (eIF-4e) with AutoDock 4.0 software confirmed the above results (Fig. 3A). The most stable docking model demonstrates binding of 5-ASA in the region of eIF-4e's cap-binding pocket [20]. Residues Asp90, Arg157 and Lys162 seem to form hydrogen bonds with 5-ASA and residues Arg112 and Arg157 belong to the cap-binding pocket. Other identified targets did not have fully resolved X-ray structures and were not further investigated. In order to validate the actual binding of eIF-4e and 5-ASA we performed surface plasmon resonance (SPR) experiments using Biacore<sup>®</sup> 2000 system [16]. 5-ASA overlaid above chip-immobilized eIF-4e protein showed 2.5 times stronger binding *vs.* heat-inactivated eIF-4e protein (Fig. 3B). To test whether 5-ASA may affect the translation apparatus we decided to perform an *in vitro* translation assay using the rabbit reticulocyte system. We obtained significant inhibition of translation after 5 min of 5-ASA treatment at the concentration as little as 1 mM (Fig. 3C). These experiments provide evidence that 5-ASA interferes with de-novo protein syntheses.

### DISCUSSION

Colon carcinogenesis involves multiple mutations or epigenetic modifications followed by changes in gene expression [26]. Tumor cells further acquire an invasive and metastatic phenotype that is the main cause of death for cancer patients. Such changes need several years that open a window of opportunity to prevent the transition from normal to benign or malignant cells. Cancer prevention, in particular chemoprevention, aims for reducing cancer development by administering certain compounds that interfere with this process [27]. Because in IBD CRC development occurs faster and more frequently, colitis-associated carcinogenesis represents an important pathway to study chemopreventive chemicals, especially 5-ASA [3, 28].

To unravel alterations concerned with 5-ASA treatment, proteomic approaches can be combined to identify qualitative and quantitative changes in gene and protein composition. In line with this note, the OMICS-based techniques used in the current study may successfully fulfill the above goal. In this work we provide a proof of principle for two newly developed approaches that allow identification of potential 5-ASA binding targets on protein level. IP-coupled 2D-DIGE technique was utilized to narrow down proteins within the selected immunocomplexes of a known 5-ASA pathway (Wnt/ $\beta$ -catenin). Importantly, the eukaryotic translation initiation factor 4b which was downregulated within  $\beta$ -catenin immunopellets by 5-ASA provided the first evidence of 5-ASA involvement in protein biosyntheses. The second approach of reverse chemogenomics identified several candidates for 5-ASA targets (supplementary Table 2). Importantly, both approaches along with various validation assays revealed initiation translation factor 4 to be a target of 5-ASA, which allowed us to uncover a previously unknown activity of 5-ASA, i.e. inhibition of translation.

The cap-dependent initiation step of protein synthesis is a key regulatory element in the flow of genetic information from the genome to the proteome [29]. Increased expression of the mRNA cap-binding protein eIF4E is an early event in tumorigenesis [30]. It was shown to be upregulated in some patients with AML [31] or mantle lymphomas. Moreover, eIF4e phosphorylated form is present in many other cancers [32]. Since eIF4e activity is a key element for the production of many pro-oncogenic proteins, recent efforts were focused on targeting this protein for anti-proliferative approaches [33, 34]. However, the exact molecular function of eIF4 remains unclear. It is known that, initiator tRNA (Met-tRNA<sub>i</sub>) is delivered to the 40S ribosomal subunit to form a 43S pre-initiation complex in eukaryotes, where eIF4e is the rate-limiting component of the translation apparatus. Based on our docking model and translation assay it seems possible that 5-ASA interferes with the assembling of 48S initiating



**Fig. (3). 5-ASA binds to eIF4e and inhibits protein syntheses.** (A) One of the identified proteins, eIF-4e, was subjected to docking simulation using AutoDock 4.0 computer software. (B) SPR sensorgram of 5-ASA binding to eIF-4e protein or heat inactivated eIF-4e obtained using Biacore® 2000. (C) Translation assay in rabbit reticulocyte lysates incubated with 5-ASA (0, 1 mM and 20 mM) or cycloheximide (CHX, 10  $\mu$ M) was determined by *in vitro* incorporation of [<sup>35</sup>S]-methionine in TCA-precipitated proteins.

complexes and disrupts proper positioning of mRNA on the subunit. Indeed, binding of the 43S complex to the mRNA near the 50-7-methylguanosine (m7G) cap is promoted by the cap-binding protein eIF4e (along with eIF4G and the poly(A)-binding protein) to form the 48S complex [35]. This may partially explain 5-ASA-dependent translation inhibition at the stage of initiation. The functional role of eIF4b specifically within the  $\beta$ -catenin complex is less understood. Because of its binding to mRNA this factor may also be targeted by 5-ASA [36].

The cap-binding pocket of eIF-4e may represent a previously unknown molecular target of 5-ASA not only in eukaryotes, but also in prokaryotic systems. This may explain 5-ASA recently described antimicrobial properties [37]. Further data also suggest that 5-ASA inhibit bacterial growth *in vitro* (Gloria Luciani, personal communication). In light with a recent notion that an abnormal gut microbiota may provoke chronic inflammatory disorders it seems plausible to propose another line of defense that 5-ASA may play against IBD [38].

Both of our OMICs techniques have various practical implications. The IP-coupled 2D-DIGE approach can be applied not only to find potential targets of 5-ASA which are responsible for cancer suppression but to unravel altered pathways of any drugs. In turn, in-gel chemogenomics approach can be further improved onto different levels. First, depending on the scanning platform, one can similarly investigate other drugs which emission falls onto other range of spectrum, including infrared. Another improvement can be achieved by in gel incubation of a compound with the proteins, resolved on native conditions. Overall, this method may serve as a prescreening assay to limit the number of potential proteins during drug-target search. This would allow studying potential drugs adverse-effects ahead of animal or clinical studies drugs. Nevertheless, using this combinatorial OMICs approach we identified the translation initiation machinery as a novel target of 5-ASA in colon cells that is of great importance in understanding the mechanism of action of this commonly used drug.

## ACKNOWLEDGEMENT

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## ABBREVIATION

|     |   |                            |
|-----|---|----------------------------|
| IBD | = | Inflammatory bowel disease |
| GI  | = | Gastro intestinal          |
| CRC | = | Colorectal cancer          |

|   |   |  |
|---|---|--|
| 5-, 4-, 3- or N-acetyl-ASA - 5- (4-, 3- or N-acetyl-) | = | Aminosalicylic acid  |
| 2D-DIGE   | = | 2D-differential in gel electrophoreses                     |
| ACCN  | = | Acetonitrile   |
| MS  | = | Mass spectrometry  |
| PPAR- $\gamma$  | = | Peroxisome proliferator-activated receptor gamma           |
| MALDI-TOF   | = | Matrix-assisted laser desorption/ionization time of flight |
| SPR   | = | Surface Plasmon resonance                                  |

## SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

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