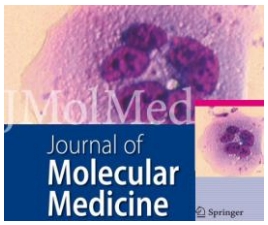
# Supplementary Material




## Comparative kinome analysis to identify putative colon tumor biomarkers

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### Supplementary Methods

*Unsupervised hierarchical clustering*

Average-linkage hierarchical clustering was done using Cluster 3.0 and the results were visualized using TreeView 1.6 software (http://rana.lbl.gov/eisen). Sample and protein kinases clusters were calculated using the Euclidean distance. Before the cluster analysis, the expression level of each gene was standardized by subtracting the mean value and dividing it by the standard deviation.

*Total protein isolation for kinases in vitro studies*

The total fraction of cellular proteins was isolated from pooled cryostat sections of collected specimens, same as for proteomic study [6]. Tissue samples were 4 times vigorously washed with cold PBS with protease and phosphatase inhibitors, to remove residual blood, and centrifuged 3 min at 15000×g. Protein pellets were dissolved in M-PER Mammalian protein extraction reagent (Pierce, Rockford, IL) containing 150 mM NaCl, inhibitors of proteases and phosphatases. Then, samples were ultrasonic homogenized using Cole-Parmer 500W (ten cycles of 3 sec. pulses with 3 sec. gaps, at Amp. 23%), centrifuged 10 min at 15000×g, and filtrated through 0.45 µM filters (Millipore). The protein concentration was measured with BCA assay (Pierce) and stored at -80$^{o}$C in 50 µl aliquots.

*Immunoprecipitation and kinase activity assay*

Protein amount was equalized for NC, AD and CRC samples with immunoprecipitation (IP) buffer (150 mM NaCl, 50 mM Tris-HCl pH=7.5, 5 mM EDTA, 0.5% NP-40, 1.0% Triton X-100) supplemented with protease and phosphatase inhibitors, and 75 µg of protein extract was used in IP reaction with 3 µg of antibody (STK24/MST3 - ab51137 – Abcam; IgG - I-1000 – Vector

Laboratories) in a volume of 150 µl. Samples were floated in ultrasonic water bath (30 min, 4$^{o}$C) to accelerate protein-antibody binding, then centrifuged for 10 min at 10000 rpm/4$^{o}$C and kinase-antibody complexes were captured on Dynabeads® Protein A (20 µl) for 60 min with a rotation in a cold room. Dynabeads were washed three times with ice cold IP buffer followed by magnetic separation.

For the kinase *in vitro* assay Dynabeads-Ab-protein complexes were resuspended in 20 µl reaction solution comprised of kinase buffer (50 mM Tris-HCl pH=7.5, 5 mM $MgCl_2$; 5 mM $MnCl_2$, 0.05 mM DTT, 0.02% Triton X-100), 5 µg PKCtide (ERMRPRKRQGSVRRRV - Millipore) and 2.5 µCi [$\gamma$-$^{32}$P] ATP followed by incubation at 30$^{o}$C for 15 min. Reaction was terminated by spotting 20 µl of the reaction mixture onto phosphocellulose P81 paper. The P81 paper was air dried, washed with 1% phosphoric acid 3 times and transferred to scintillation tube. Then it was overlaid with 3 ml scintillation solution and samples were measured in liquid scintillation spectrophotometer.

*Kinome profiling*

The kinome profiling analysis was performed by Pepscan Presto B.V. (Lelystad, Netherlands), using PepChip Kinomics peptide microarray, containing 1,024 different kinase substrates spotted in triplicate, with 48 negative and positive controls. Samples of protein concentration of at least 5 µg/µl were shipped to Pepscan Presto B.V. on dry ice.

A kinase activity was defined as the relative level of phosphorylation of peptide substrates on the array, after incubation with ~150 µg of the protein in the presence of [$\gamma$-$^{33}$P] ATP, according to Protocol Pepchip Kinase For Lysates (http://pepscan.nl/). The array scans were acquired by a scanner and quantified using standard ScanAlyze software. Background was subtracted for all individual spots and Kolmogorov-Smirnoff statistic at a cut-off $p$<0.001 was used to exclude spots with a value distribution that cannot be distinguished from the background. The average signal for each peptide was calculated based on correctly defined good spot values from all 3 replicates. Before averaging, the replicated measurements of the samples were normalized by fitting a robust locally weighted regression smoother (LOESS). The numbers are expressed as ratios of a specific sample mean over the reference. As a threshold for significance only ratios corresponding to means differing 1.96 x (standard error of mean 1 + standard error of mean 2) were considered.

## Supplementary Figures

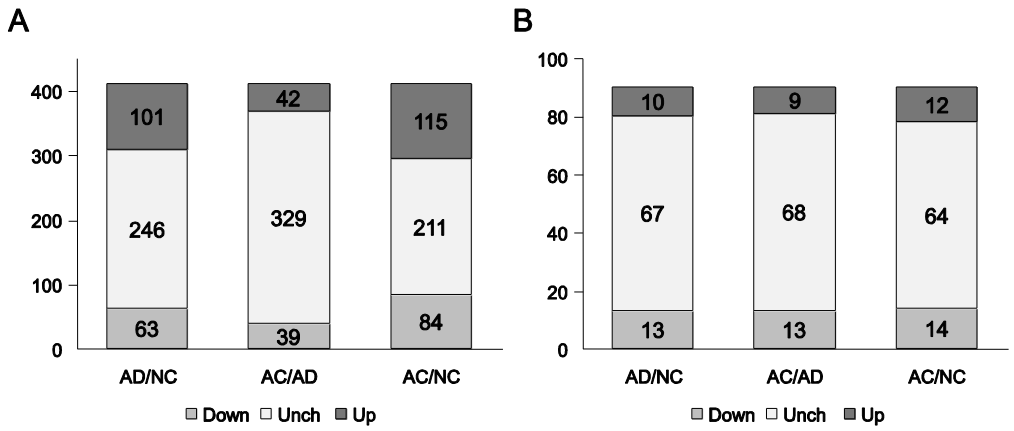


**Figure S1.** Differentially expressed protein kinases in CRC. Shown is the number of PKs that were up-regulated, down-regulated or unchanged (Unch) at the mRNA (A) and protein (B) levels. AC, adenocarcinoma; AD, adenoma; NC, normal colon.

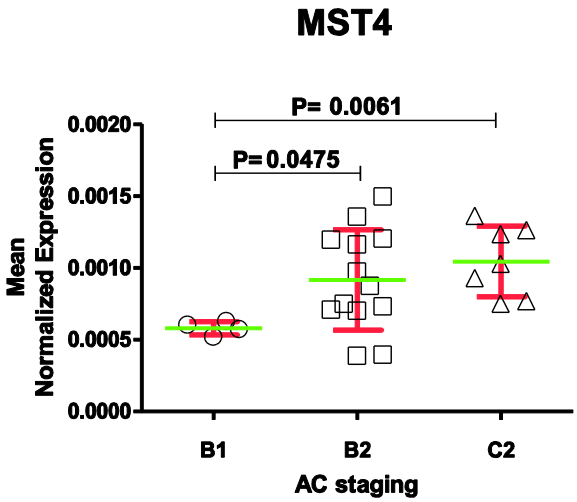


**Figure S2**. *MST4* kinase mRNA expression differentiates adenocarcinoma (AC) stages. One microgram of total RNA was reverse-transcribed to generate cDNA and then q-PCR was performed using SYBR Green I chemistry. Green horizontal bars indicate means and red whiskers indicate standard deviation. Differences were analyzed using the Mann-Whitney test. B1 (n=4), B2 (n=13), C2 (n=7). AC stages according to Astler-Coller's classification.

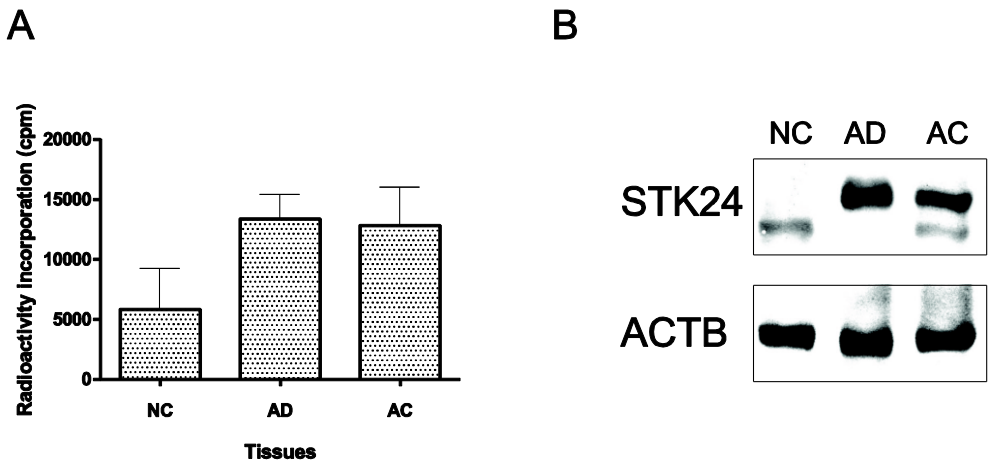


**Figure S3.** STK24 kinase *in vitro* activity and immunoblot of colon tissue extracts. (A) Equal amounts of whole tissue extracts were used to immunoprecipitate STK24 for *in vitro* kinase assays. The reaction mixtures were spotted onto P81 paper and washed three times with 1% phosphoric acid, and the bound $^{32}P$ was quantified in counts per minute (cpm) by liquid scintillation counting. Results are expressed as the difference in $^{32}P$ incorporation for an immunoprecipitation reaction with control IgG or anti-STK24, and represent the mean values ± standard deviation of two separate experiments. (B) Ten micrograms of tissue protein extract were resolved by SDS-PAGE and electrotransferred to PVDF membrane. Separated proteins were assessed by immunoblot analysis using an anti-STK24 antibody (ab51137 - Abcam) at a 1/1000 dilution and an anti-beta-actin antibody (ab6276 - Abcam) at a 1/4000 dilution. AC, adenocarcinoma; AD, adenoma; NC, normal colon.

**Supplementary Tables 1-11 (see enclosed xls file)**