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E4-013P
Proteomic analysis of H-RAS-mediated oncogenic transformation in a genetically defined human ovarian cancer model

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Ras is a small GTP binding protein mutated in approximately 30% human cancer. Despite its important role in the initiation and progression of human cancer, the underlying mechamism of RAS-induced human epithelial transformation remains elusive. In this study, we probe the cellular and molecular mechanisms of RAS-mediated transformation, by profiling two human ovarian epithelial cell lines, one immortalized with SV40 T/t antigens and the human catalytic subunit of telomerase (T29) while the other transformed with an additional oncogenic rasV12 allele (T29H). Thirty-two proteins associated with RAS-mediated transfromation have been identified using peptide mass fingerprinting. These protein targets are involved in several cellular pathways, including metabolism, redox balance, calcium signaling, apoptosis, and cellular methylation. One such target, the 40 kD procaspase-4 is significantly up-regulated at the protein level in RAS-transformed T29H cells, related driectly to signaling through MEK, but not PI3 kinase. Cellular caspase-4 activity is, however, suppressed in the T29H cells, suggesting that the maturation process of caspase-4 is abrogated in RAS-transformed T29H cells. Consistent with this notion, transformed T29H cells were less susceptible to the toxic effects of anti-Fas antibody than were immortalized, non-transformed T29 cells, associated with less activation of caspase-4. Our study demonstrates that functional proteomic analysis of a genetically defined cancer model provides a powerful approach toward systematically identifying cellular targets associated with oncogenic transformation.

E4-014P
Dissociation between hyperosmotic Na-K-2Cl cotransporter activation and myosin light chain phosphorylation

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Myosin light chain kinase (MLCK)-dependent increase in MLC phosphorylation has been proposed to be a key mediator of the hyperosmotic activation of the Na+-K+-2Cl- cotransporter (NKCC). To address this hypothesis we used pharmacological and genetic means to manipulate MLCK, MLC phosphorylation or myosin ATPase activity, and followed the impact of these alterations on the hypertonic stimulation of NKCC in LLC-PK1 kidney tubular cells. We found a large dissociation between the osmotically induced MLC phosphorylation and NKCC activation. The inhibition of Rho or Rho kinase strongly suppressed osmotic MLC phosphorylation, without affecting NKCC activation. The MLCK inhibitor ML-7 reduced NKCC activity independent of its effect on MLC phosphorylation. Prevention of the osmotically induced increase in MLC phosphorylation by viral induction of cells with a non-phosphorylatable, dominant negative MLC mutant did not affect the hypertonic activation of NKCC. Finally, a constitutively active MLC-mutant, which mimics the diphosphorylated form, neither stimulated the isotonic, nor potentiated the hypertonic NKCC activity. However, the inhibition of myosin ATPase by Blebbistatin significantly reduced the osmotic stimulation of NKCC, without suppressing its basal activity. These results indicate that (i) The Rho-ROK pathway is the major mediator of the osmotic MLC phosphorylation. (ii) MLC phosphorylation is neither a sufficient nor a necessary signal to stimulate NKCC in tubular cells. (iii) However, basal myosin activity plays a permissive role in the optimal osmotic responsiveness of NKCC.

E4-015P
Homodimerization of the deleted in liver cancer 2 (DLC2) is not mediated by the SAM domain

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Deleted in liver cancer 2 (DLC2) is a novel tumor suppressor gene which is found to be frequently deleted in hepatocellular carcinoma. Although DLC2 is consisted of a RhoGAP homology domain, a START domain and a sterile-α-motif (SAM) domain, the role of DLC2 in cellular function remains elusive. To gain insight on the function of DLC2, we expressed and purified a recombinant ^{13}C and ^{15}N doubled DLC2 SAM domain. Circular dichromism analysis showed that DLC2 SAM contains high helices. We further determined the structure of the DLC2 SAM domain by 2D and 3D NMR experiments together with simulated annealing calculations. Our data revealed that the structure of DLC2 SAM is slightly different from other known SAM domains and adapts a monomeric structure with four alpha helices in solution. Consistent with the structural data, cross-linking experiment also suggested that DLC SAM exists in predominantly monomeric form *in vitro*. On the other hand, co-immunoprecipitation experiments suggested that the DLC2 protein is homodimerized. Analysis of the deletion mutants revealed that the homodimerization is not mediated by the SAM domain, but through a distinct region on the DLC2 protein.

E4-016P
Hansenula polymorpha Vps1p is required for fission of uninduced constitutive peroxisomes but not of substrate-induced organelles

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Peroxisomes are single membrane bound organelles that are ubiquitously present in eukaryote cells. Characteristically, peroxisomes are inducible in nature and, upon induction, proliferate by growth and fission of existing organelles. Recent studies in bakers yeast indicated a role for Vps1p in peroxisome fission/inheritance. *VPS1* is a non-essential gene in *Saccharomyces cerevisiae* and member of the dynamin family of GTPases whose members are known to function in various membrane fission events. This study aimed at understanding the role of Vps1p in peroxisome biogenesis in the methylotrophic yeast *H. polymorpha*. We show that *H. polymorpha* Vps1p has a dual location in the cytosol and at spots at peroxisomes. Upon induction of peroxisomes by methanol, cells of the *VPS1* deletion strain (*vps1*) contained reduced numbers of peroxisomes of enhanced size, relative to

WT controls. Live cell imaging revealed that these cells were not affected in organelle partitioning over newly developing buds. However, in uninduced cells, grown on glucose, peroxisome fission and partitioning was affected similar as observed in bakers yeast. These data therefore led to conclude that Vps1p is only involved in fission of uninduced, consitutively present organelles but not of induced ones. These data are consistent with the view that uninduced peroxisomes in glucose-grown cells differ in biochemical properties with induced ones. Moreover, we observed that methanol-induced peroxisomes were strongly unstable in *vps1* cells, leading to a continuous organelle turnover via macropexophagy.; This degradation event was abolished in a double *vps1*/*atg1* mutant that was impaired in macropexophagy.

E4-017P
Simvastatin affects oligodendroglial process formation

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Statins are therapeutically used for reducing an elevated cholesterol level. Recently, several studies have reported about immunomodulatory effects of statins. Based on these findings, statins were subsequently discussed as therapeutics for multiple sclerosis (MS) [1]. Statins could, however, interfere with oligodendroglial process formation and myelin production, another challenge of MS treatment. To address this question, we exposed cultured pig oligodendrocytes (OL) to Simvastatin (Sst), which is used in ongoing MS studies. Initial experiments were performed with Sst, kindly provided by MSD (Rahway, NJ, USA). OL cultures exposed to 10 μM Sst showed diminished cholesterol content of 20–30%, when a low cholesterol FCS was used (FCS-Gold; PAA, Cölbe). The cholesterol content of FCS-Gold was 3 mg/100 ml in contrast to 30–120 mg/100 ml of normal FCS, which did not reveal an Sst effect on cellular cholesterol production. For further investigations, Sst obtained from Calbiochem (Darmstadt) was used. An obvious morphological effect was that OL process formation was retarded (+/−NGF); already formed processes were retracted. Addition of mevalonate but not PEG-cholesterol reversed this effect indicating that intermediates such as farnesylpyrophosphate (FPP) and geranylpyrophosphate (GPP) were no longer provided due to an inhibition of HMG-CoA-reductase via Sst. Indeed, MAPK activity of OL exposed to Sst was reduced compared to controls as evidenced by an in-gel-kinase assay. However, the major effect on OL process formation seemed to be mediated via GGPP: Sst exposed OL cultures returned to normal when GGPP was added, while FPP alone seemed to be insufficient for compensating the Sst effect. Preliminary results indicate that RhoG seems to be involved [1]. Wekerle H. Tackling multiple sclerosis. *Nature* 2002; **420**: 39–40.

E4-018P
PKC-dependent downregulation of NF1 in neuronal cells via non-proteasomal pathways

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Neurofibromin, the protein product of the NF1 gene, has been characterized as a negative regulator of Ras signaling via its Ras-GTPase-activating properties. It is, however, not known what regulates neurofibromin upon stimulation of Ras signaling cascades, and consequently its function as a Ras-GAP. Previous studies have indicated that phosphorylation of neurofibromin by PKC or PKA may impact on its ability to regulate Ras. Thus, we used several cell lines (SH-SY5Y, IMR32, COS-7 and HeLa) and studied the role of direct PKC activation with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) on neurofibromin regulation. We found that TPA caused a rapid (as early as 5 min) decrease of neurofibromin, as assessed by western blot analysis with C-terminus antibodies and in comparison with levels of actin, p120GAP and p-MARCKS. This downregulation was maintained at similar levels up to 24 h after treatment. In cells where $Ca2^{+}$-dependent isoforms were depleted with 24 h TPA prior to re-stimulation with TPA for 5 min, no additional neurofibromin decrease was observed. Moreover, TPA effects were reversed by the $Ca2^{+}$-dependent isoform-inhibitors Go6976 and Ro-31-8220. Taken together these studies indicated that PKC-induced downregulation of neurofibromin was likely due to degradation and not inhibition of its synthesis. We subsequently studied whether proteasome activity is involved. Lactacystin or ALLN, under conditions where both inhibit proteasome-mediated proteolysis of proteins, did not prevent TPA-induced neurofibromin decrease. On the contrary, lactacystin consistently facilitated the long-term effect of TPA probably due to partial protection of PKC alpha by proteasomal degradation. These data indicate a role of PKC in non-proteasomal neurofibromin regulation.

E4-019P
Role of the prenyl group of Rac in its interaction with p50RhoGAP

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In the present study, interaction of p50RhoGAP with Rac was investigated in a yeast two-hybrid system, by radioactive GAP assay and in an enzymatic reaction dependent on RacGTP. The yeast two-hybrid system revealed an interaction between the C-terminal GAP domain and the N-terminal part of p50RhoGAP. The first 48 amino acids play a special role both in the stabilization of the intramolecular interaction and in recognition of the prenyl tail of small GTPases. Hydrolysis of radiolabelled GTP by bacterially expressed (non-prenylated) Rac was only poorly accelerated by full-length p50RhoGAP. Removal of the N-terminal 48 amino acids increased the activity of p50RhoGAP significantly. The fragments 49–439, 121–439 and 169–439 exhibited identical GAP activity, thus amino acids 49–168 seem not to influence the interaction of Rac with p50RhoGAP. In contrast, removal of the next 20 amino acids (169–198) resulted in a further increase in the GAP activity. When prenylated Rac expressed in Sf9 cells was used, the full-length protein and all the different constructs had identical effect. The effect of p50RhoGAP was also tested in a biological system: superoxide production by the phagocyte NADPH oxidase was measured in a fully reconstituted system that consisted of purified cytochrome b558, recombinant p47phox, p67phox and bacterially expressed Rac preloaded with GTP. The effect of the various fragments in the reconstituted oxidase system fully suported data obtained in the radioactive GAP assay. We thus propose that two regions (aa 1–49 and 169–198) of the p50RhoGAP molecule form intramolecular interactions, which limit the access of non-prenylated Rac to the GAP domain. As no limitation was observed when prenylated Rac was applied, we suggest that the prenyl moiety interacts with these regions of p50RhoGAP and thereby releases the intramolecular restraint.