A genome-wide survey of RAS transformation targets

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An important aspect of multi-step tumorigenesis is the mutational activation of genes of the RAS family, particularly in sporadic cancers of the pancreas, colon, lung and myeloid system[1]. RAS genes encode small GTP-binding proteins that affect gene expression in a global way by acting as major switches in signal transduction processes, coupling extracellular signals with transcription factors[2–4]. Oncogenic forms of RAS are locked in their active state and transduce signals essential for transformation, angiogenesis, invasion and metastasis via downstream pathways involving the RAF/MEK/ERK cascade of cytoplasmic kinases, the small GTP-binding proteins RAC and RHO, phosphatidylinositol 3-kinase and others[5,6]. We have used subtractive suppression hybridization (SSH), a PCR-based cDNA subtraction technique[7], to contrast differential gene expression profiles in immortalized, non-tumorigenic rat embryo fibroblasts and in *HRAS*-transformed cells. Sequence and expression analysis of more than 1,200 subtracted cDNA fragments revealed transcriptional stimulation or repression of 104 ESTs, 45 novel sequences and 244 known genes in *HRAS*-transformed cells compared with normal cells. Furthermore, we identified common and distinct targets in cells transformed by mutant *HRAS*, *KRAS* and *NRAS*, as well as 61 putative target genes controlled by the RAF/MEK/ERK pathway in reverted cells treated with the MEK-specific inhibitor PD 98059.

The genetic complexity of cellular transformation at the level of mRNA expression was originally described more than ten years ago[8,9]. Global sequence information related to altered gene activity involved in the pathogenesis of cancer was only obtained after novel gene expression profiling methods were developed[10–12]. Several gene regulators, including ETS-domain transcription factor ELK1, serum-responsive factor (SRF), the leucine zipper protein JUN, activation transcription factor 2 (ATF2) and nuclear factor-κB (NFκB), are stimulated by signalling pathways downstream of RAS (refs 4,5), indicating that mutant *RAS* expression affects a complex set of transcriptional targets. A systematic assessment of RAS-signalling targets in transformed cells has not been performed. To provide a general catalogue of transcriptional changes related to permanent RAS signalling, we chose the preneoplastic rat 208F fibroblast line and the malignant, *HRAS*-transformed derivative FE-8 (Fig. 1*a*). These cell lines had a near-diploid karyotype without gross numerical or structural chromosomal aberrations (Fig. 1*b*). In the 208F/FE-8 cell system, chromosomal abnormalities, which frequently accumulate in cell lines established from tumours or after induction of conditional *RAS* (refs 13,14), do not mask RAS-mediated transcriptional changes. The 208F cells do not transform spontaneously[15], whereas FE-8 cells are anchorage-independent (Fig. 1*c*) and form rapidly growing tumours in athymic nude mice and newborn rats[16,17].

We recovered gene fragments representing differentially expressed mRNAs in normal 208F and transformed FE-8 cells from two cDNA libraries established after SSH (ref. 7). To isolate sequences downregulated during the transition from the normal to the transformed state, we used tester cDNA prepared from 208F fibroblasts and excess driver cDNA from malignant FE-8 cells (forward subtraction). To recover sequences upregulated upon neoplastic transformation, we used FE-8 tester and 208F driver cDNA (reverse subtraction). We determined the nucleotide sequence of 1,257 subtracted cDNA clones and identified 823 individual sequences. The list of differentially expressed genes classified with respect to selected functional properties of their products is shown (Table 1). The method used to establish expression differences between normal and transformed cells permitted isolation of genes expressed at high- and low-abundance classes. The intrinsic equalization step contained in the SSH method allowed recovery of abundantly expressed tran-

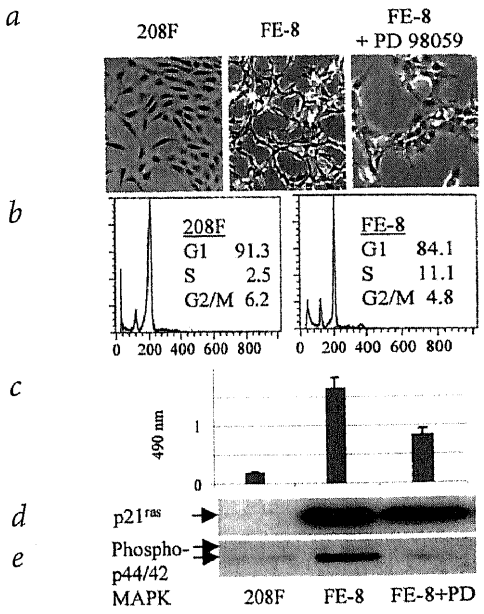

Fig. 1 Characteristics of normal 208F fibroblasts (left) and *HRAS*-transformed FE-8 cells, untreated (middle) and incubated with the MEK-inhibitor PD 98059 (right). **a**, Morphology obtained by phase contrast microscopy, magnification ×100. **b**, DNA histograms obtained by flow cytometry show the absence of gross chromosomal abnormalities in 208F and FE-8 cells. Abscissa, fluorescence intensity; ordinate, cell counts; numbers refer to the fraction of cells (%) in different phases of the cell cycle. **c**, XTT-based colorimetric proliferation assays indicate anchorage-dependent and anchorage-independent growth of cells on poly-HEME–coated surfaces. Western-blot analysis of $p21^{ras}$ (**d**) and phospho-p44/42 MAPK (**e**) is shown.

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Table 1 • Differentially expressed genes

Downregulated genes						Upregulated genes					
Sequence identity (GenBank/EMBL)	Species	Accession	Redundancy	Level of downregulation	Method to verify differential expression	Sequence identity (GenBank/EMBL)	Species	Accession	Redundancy	Level of upregulation	Method to verify differential expression
Signalling molecules											
3′,5′-cAMP phosphodiesterase (*Pde3b*)	r	Z22867	1	>100	N	A kinase anchor protein KL (*Akap2*)	m	AF033276	1	16.1	N, R
AhR repressor (*Ahrr*)	m	AB015140	1	38.0	R	B61, Eck receptor ligand (*Efna1*)	r	D38056	1	5.2	N
cAMP-dependent protein kinase type II (*Prkar2b*)	r	M12492	1	>100	R	c-Ha-ras-1 (*HRAS*)	h	V00574	1	17.0	N
colony stimulating factor 1 (*Csf1*)	r	M84361	2	5.6	N, R	c-yes oncogene (*Yes*)	m	X67677	1	12.5	N
growth arrest specific gene 6 (*Gas6*)	m	X59846	1	24.0	R	calmodulin-dependent protein kinase IId (*Camk2d*)	r	J05072	1	8.1	R
guanine nucleotide-binding protein G-s a (*Gnas*)	r	M12673	1	3.6	N	cyclooxygenase 1 (*Ptgs1*)	r	U03388	1	90.7	N, R
I-Traf (Traf-interacting protein, *Traip*)	m	MMU59864	1	38.6	N	cytocentrin, Ral-interacting protein 1 (*Rip1*)	r	U28830	1	8.3	N
inhibitor of KLP gene enhancer (*IKBKAP*)	h	AF044195	1	8.6	R	Fkbp51, T-cell-specific immunophilin (*Fkbp5*)	m	U16959	1	68.2	N
MARCKS (*Macs*)	m	M60474	2	3.3	N	Flice-like inhibitory protein (*Cash*)	m	U97076	2	>100	N
MST2 kinase (*Stk3*)	r	AJ001529	2	21.6	R	GEF-H1	h	U72206	1	32.1	N
myo-inositol monophosphatase (*Impa1*)	r	U84038	1	44.5	N	GTP-binding protein (*Rab5a*)	r	AF072935	1	>100	N
p5 protein	ha	X62678	1	3.4	R	JAK1 protein tyrosine kinase (*Jak1*)	r	AJ000556	1	55.0	N
phosducin-like protein (*Pdcl*)	r	L15354	2	>100	N, R	MAPK phosphatase cpg21 (*Dusp5*)	r	AF013144	1	27.9	N, R
phosphatidylinositol 3-kinase p110b (*PIK3CB*)	h	S67334	1	>100	N	p67 isoprenylated protein (*Gbp2*)	r	M80367	1	98.2	N
phosphatidylinositol 3-kinase p170 (*Pik3c2a*)	m	U55772	1	65.9	N, R	phosphatase 2A B56 (*P2R5A*)	h	L42373	2	50.6	N
protein tyrosine phosphatase d (*Ptprd*)	m	D13903	1	1.9	R	PkB kinase (*Pdpk1*)	r	Y15748	1	19.9	N
ROK-a (*Rock2*)	r	U38481	1	26.1	N	R-esp2, groucho-related gene 4 (*Tle4*)	r	L14463	1	>100	N
serum inducible kinase (*Snk*)	m	M96163	1	>100	N, R	Rap1B GTP binding protein (*Rap1b*)	r	U07795	1	21.0	N
SH3 binding protein 5 (*SH3BP5*)	h	AB005047	1	3.5	R	Ras-GTPase-activating protein (*G3bp2*)	m	AB001927	1	9.9	N
						RhoC (*Arhc*)	m	X80638	2	6.7	R
						SBF1 phosphatase (*SBF1*)	h	U93181	1	27.1	N, R
						Sprouty 2 (*SPRY2*)	h	AF039843	2	11.60	N, R
						T-cell death associated gene 51 (*Tdag*)	m	U44088	1	2.7	N
						tyrosine phosphatase IA-2a (*Ptprn*)	r	D38222	1	12.2	N
Nuclear proteins (transcription factors, DNA processing enzymes)											
Ahnak nucleoprotein (*AHNAK*)	h	M80902	2	>100	N	a-prothymosin (*Ptma*)	r	M60664	1	2.4	R
ATP-dependent RNA helicase	m	U46690	1	8.9	N	BRCA1-associated Ring domain protein (*Bard1*)	m	AF057157	1	3.5	N
BRG-1 (*Smarca4*)	m	S68108	1	13.1	N	Cdc-like kinase (*Clk*)	m	L29221	1	13.1	N
CCAAT/enhancer binding protein-g (*Cebpg*)	r	X64403	1	16.6	N	Flap endonuclease-1 (*Fen1*)	m	L26320	1	11.1	N
Cdc21 (*Mcmd4*)	m	D26089	1	3.9	R	Fra-1 fos-related antigen 1 (*Fosl1*)	r	M19651	3	>100	N, R
centromeric protein CENPC (*Cenpc*)	m	U03113	1	39.2	N, R	histone acetyltransferase (*GCN5L2*)	h	AF029777	1	2.7	N
chromosome-associated polypeptide C	x	AB019987	1	9.6	R	Nop56 nucleolar protein	h	Y12065	1	2.9	N
DNA polymerase-e small subunit (*POLE2*)	h	AF036899	1	5.1	R	lamina-associated polypeptide 1C	r	U19614	1	7.6	N
DNA repair protein RAD50 (*Rad50*)	m	U66887	1	3.4	N, R	Myb-binding protein p160 (*Mybbp1a*)	m	U63648	1	5.9	N
ER81 ets-related transcription factor (*Etsrp81*)	m	L10426	1	9.6	N	NF-1 transcription factor (*Nfic*)	m	U57635	1	71.8	N
ETF TEA domain transcription factor (*Tead2*)	m	D50563	1	7.4	N	p100 transcriptional coactivator	h	U83883	1	4.9	R
Gu binding, inhibitor of activated Stat1 (*DDXBP1*)	h	U78524	1	41.7	N	Pebp2b2, core binding factor (*Cbfb*)	m	D14571	2	45.4	N
HEC retinoblastoma-associated protein	h	AF017790	1	3.9	N, R	retinoblastoma protein 1 (*Rb1*)	r	D25233	1	6.5	N
helicase p68 (*DDX5*)	h	AF015812	2	>100	N, R	stromal antigen 1 (*Stag1*)	m	Z75332	1	89.1	N, R
histone H3.3B (*H3F3B*)	h	Z48950	2	5.8	R						

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Sequence identity (GenBank/EMBL)	Species	Accession	Redundancy	Level of downregulation	Method to verify differential expression	Sequence identity (GenBank/EMBL)	Species	Accession	Redundancy	Level of upregulation	Method to verify differential expression
Ki-67 antigen (*Mki67*)	m	X82786	1	>100	N, R						
lamina associated polypeptide 2 (*Tmpo*)	r	U18314	4	>100	N, R						
mouse zinc-finger protein	m	D45210	1	5.6	N						
Tfe3 X-linked transcriptional activator (*Tcfe3*)	m	S76673	1	3.6	R						
nuclear autoantigen GS2NA	h	U17989	1	31.9	R						
nucleoporin 155 (*NUP155*)	h	AJ007558	1	15.2	N						
poly(ADP-ribose) glycohydrolase (*Parg*)	m	AF079557	1	2.4	R						
Rnf4 transcription factor (*Rnf4*)	m	U95141	2	64.9	R						
single-strand DNA-binding protein (*SSBP*)	h	AF077048	1	4.9	R						
STAT5a1 transcription factor (*Stat5a*)	r	U24175	1	1.8	N						
topoisomerase I (*Top1*)	m	D10061	1	20.1	R						
topoisomerase II (*Top2*)	r	Z19552	3	2.1	R						
						Protein processing, protein transport and protein folding molecules					
26S proteasome subunit p55 (*PSMD12*)	h	AB003103	1	3.5	N	aminopeptidase P	r	AF038591	2	5.6	R
ERp99/GRP94 (*Tra1*)	m	J03297	1	2.2	R	chaperonin containing TCP-1e (*Cct5*)	m	Z31555	2	2.2	N, R
heat shock protein 105 (*Hsp105*)	m	D67016	1	15.1	N	exportin-T (*XPOT*)	h	AF039022	4	48.5	N
heat shock protein 90 (*HSPCA*)	h	X15183	1	4.8	N, R	GRP75 (*Hspa9a*)	r	S78556	2	2.1	R
MG-160 golgi sialoglycoprotein	r	U08136	1	2.3	R	Herpes ass. ubiquitin-specific protease (*USP7*)	h	Z72499	1	28.8	R
Rsec6 secretion protein	r	U32575	1	56.0	N	importin-a Q1, karyopherin (*Kpna4*)	m	AF020771	1	10.6	R
translation initiation factor 4A isoform 2 (*Eif4a2*)	m	X56953	1	3.9	R	mitochondrial processing peptidase-b (*pmpcb*)	r	L12965	1	4.3	R
translocation protein-1 (*TLOC1*)	h	D87127	1	>100	N	Ran-GTPase, toll-like receptor 4 (*Tlr4*)	m	L32751	1	19.7	N
						Sec61 (*Sec61*)	r	M96630	2	29.2	N, R
						sortilin 1 (*SORT1*)	h	X98248	1	10.5	N
						translation initiation factor 3 subunit 5 (*EIF3S5*)	h	U94855	1	5.7	N, R
						Metabolic enzymes, transporters, ion channels					
3-b-hydroxysteroid dehydrogen. isomerase (*Hsd3b*)	r	S63167	4	5.0	R	4F2hc intestinal type II glycoprotein (*Mdu1*)	r	U59324	4	2.9	N
3-hydr. 3-methylglutaryl CoA synthase (*Hmgcs1*)	r	X52625	2	12.7	R	ABC transporter Moat-B (*ABCC4*)	h	AF071202	1	10.8	N, R
aldehyde dehydrogenase (*aldh311*)	r	J03637	1	37.8	N	acyl-CoA synthetase II (*Facl3*)	r	D30666	1	4.1	R
a-mannosidase II (*Man2a1*)	m	X61172	1	6.3	R	aldehyde reductase (*Akr1a1*)	r	D10854	1	4.0	N
antioxidant enzyme AOE372	m	U96746	1	1.8	N	asparagine synthetase (*Asns*)	r	U07201	4	15.3	R
AP56, selenium binding protein 2 (*Selenbp2*)	m	S56599	1	58.7	R	ATP citrate-lyase (*Acly*)	r	J05210	2	3.1	R
Apobec-1 binding protein 1 (*HNRPAB*)	h	U76713	1	>100	N	bleomycin hydrolase (*Blmh*)	r	D87336	2	8.5	N, R
caBP1 calcium binding protein 1	r	X79328	2	4.7	N	chloride channel 6 (*CLCN6*)	h	X99473	1	19.6	R
calcium channel b-subunit-III (*Cacnb3*)	r	M88751	1	18.8	N	farnesyl diphosphate synth. (*Fdps*)	r	M34477	2	3.3	N, R
dihydropyrimidinase related protein-3 (*DPYSL3*)	h	D78014	1	2.3	R	glucose-6-phosphate dehydrogenase (*G6pdx*)	r	X07467	1	2.4	R
glutamine synthetase (*Glns*)	r	M91652	3	10.4	R	glutathione reductase (*Gr1*)	r	U73174	1	2.7	N, R
NADH dehydrogenase 5 (*mt-Nd5*)	r	X14848	1	2.5	R	Glvr-1 leukaemia virus receptor 1 (*Slc20a1*)	m	M73696	2	22.2	R
NADH dehydrogenase chain 6 (*mt-Nd6*)	r	X13220	1	5.3	R	MCT1 monocarboxylate transporter (*Slc16a1*)	r	X86216	1	7.5	R
NADP transhydrogenase (*Nnt*)	m	Z49204	1	12.3	N	mitochondrial trifunctional protein	r	D16478	1	2.4	N

Table 1 • Differentially expressed genes (continued)

Downregulated genes						Upregulated genes					
Sequence identity (GenBank/EMBL)	Species	Accession	Redundancy	Level of downregulation	Method to verify differential expression	Sequence identity (GenBank/EMBL)	Species	Accession	Redundancy	Level of upregulation	Method to verify differential expression
phosphatidate phosphohydrolase 2 (*Ppap2a*)	r	U90556	1	6.2	N	non-neuronal enolase 1 (*Eno1*)	r	X02610	5	2.5	R
selenoprotein P (*Sepp1*)	r	M63574	2	31.8	N	Niemann Pick type C1 (*Npc1*)	m	AF003348	1	3.1	R
						phosphoglycerate kinase 1 pseudogene1 (*Pgk1-ps1*)	m	M23961	1	2.9	R
						phosphoglycerate mutase 1 (*Pgam1*)	r	S63233	4	5.6	R
						stearoyl-CoA desaturase 2 (*Scd2*)	r	AF036761	1	7.5	R
						transcript ass. with monocyte differentiation	h	X85750	1	8.2	N
						transporter protein g17	h	U49082	1	4.2	R
Cytoskeletal components, molecules involved in adhesion and cell-cell interaction											
actin-binding protein-280, filamin (*FLNA*)	h	X53416	1	5.8	R	actin-related protein 3 (*ACTR3*)	h	AF006083	3	3.3	N, R
a-actin (*Actvs*)	r	X06801	5	4.2	R	calcium-binding protein pp52 (*Lsp1*)	m	M89956	2	29.7	N, R
cadherin-11 (*Cdh11*)	m	X77557	1	11.7	R	calponin 3 (*Cnn3*)	r	U06755	1	5.2	R
caldesmon (*Cald1*)	r	U18419	3	37.7	N	CD44 glycoprotein (*Cd44*)	r	M61875	1	17.0	N, R
cytohesin-2 (*Pscd2*)	r	U70728	1	>100	N	laminin receptor (*P40-8*)	m	J02870	5	4.1	R
growth arrest specific gene 1 (*Gas1*)	m	X65128	1	10.4	R	leukocyte adhesion protein p150, 95 (*ITGAX*)	h	Y00093	2	5.2	R
Hspg core fibroglycan, syndecan-2 (*Sdc2*)	r	M81687	1	61.9	N, R	melanoma antigen family B3 (*MAGEB3*)	h	U93163	2	15.3	N
EMAP microtubule-associated protein (*EMAPL*)	h	NM004434	1	26.9	N	myosin regulatory light chain	r	D14688	1	6.9	R
myosin regulatory light chain isoform C (*Mylc2c*)	r	S77900	2	2.6	N, R	TA1 oncofetal gene (*Cd98*)	r	U00995	2	1.9	N
P-cadherin (*Cdh3*)	m	X06340	1	60.1	N	thymosin-b 4 (*Ptmb4*)	r	M34043	1	2.4	N, R
podoplanin (*Gp38*)	r	U96449	1	9.4	R						
ryudocan (*Sdc4*)	r	S61868	6	27.7	N, R						
tropomyosin 4 (*Tpm4*)	r	Y00169	1	7.8	N,R						
TRPM-2, clusterin (*Clu*)	r	M64723	1	39.4	N						
vimentin (*Vim*)	r	X62952	1	1.6	R						
Extracellular proteins											
collagen a1 (*Cola1*)	r	Z78279	34	22.3	R	MMP-1, collagenase (*Mmp1*)	r	M60616	19	>100	N, R
Cyr61 immediate-early gene (*Igfbp10*)	m	M32490	4	16.0	N, R	MMP-3, stromelysin 1 (*Mmp3*)	r	X02601	7	32.3	N, R
entactin/nidogen (*Nid*)	m	X14194	14	35.8	N	MMP-10, stromelysin 2 (*Mmp10*)	m	X05083	12	33.8	R
fibrillin-1 (*Fbn1*)	m	U22493	1	3.3	R	Mob-1, interferon activated gene 10 (*Ifi10*)	r	U17035	2	2.4	N, R
fibronectin (*Fn1*)	r	X15906	25	>100	N	testin (*Tes*)	m	X78990	1	8.9	N
FISP-12 (*Fisp12*)	m	M70642	2	49.4	N						
follistatin-related protein TSC-36 (*Fstl*)	r	U06864	5	2.0	N, R						
laminin B1 (*Lamb1-1*)	m	M15525	1	5.0	R						
lysyl oxidase (*Lox*)	r	U11038	14	9.2	R						
lysyl oxidase-related protein (*LOXL2*)	h	U89942	1	59.2	N, R						
megakaryocyte potentiating factor	m	D86370	3	6.0	N						
MGF, mast cell growth factor (*Mgf*)	m	U44725	1	13.4	N						
MMP-2, gelatinase A (*Mmp2*)	r	U65656	3	50.6	N, R						
thrombospondin 1 (*Thbs1*)	m	M62470	25	42.5	R						
tissue inhibitor of metalloproteinase 2 (*Timp2*)	r	S72594	1	18.3	N, R						

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Sequence identity (GenBank/EMBL)	Species	Accession	Redundancy	Level of downregulation	Method to verify differential expression	Sequence identity (GenBank/EMBL)	Species	Accession	Redundancy	Level of upregulation	Method to verify differential expression
						Others					
activity and neurotransmitter-induced 6	r	AF030091	1	10.2	R	annexin IV (*Anxa4*)	m	U72941	1	57.8	N, R
apoptosis inhibitor 5 (*API5*)	h	U83857	2	3.1	N	annexin VII (*Anxa7*)	m	L13129	1	2.2	R
antiquitin (*ATQ1*)	h	S74728	2	7.4	N, R	B-cell receptor associated protein 37 (*Bcap37*)	m	X78683	2	42.8	N, R
ATP-dependent metalloprotease FtsH1	m	AF090430	1	21.3	R	BC-2 protein p32	h	AF042384	1	2.8	N, R
Cap-binding protein	h	X84157	2	5.0	R	BCSC-1, breast cancer suppressor candidate 1	h	AF002672	1	6.9	N
collapsin-2	c	U28240	1	>100	N	bone protein I (*Plod3*)	r	M18864	1	2.6	N
DOC-2, p96 phosphoprotein (*Dab2*)	r	U95177	1	>100	N, R	C29 keratin-I related gene (*Krt1-c29*)	m	AB013607	1	6.4	R
EI24 p53 responsive gene (*Ei24*)	m	U41751	4	5.5	N	calmodulin (*Calm*)	r	M19312	2	2.8	N
H411 precursor	ha	AF046870	1	>100	N	E1B 19k/Bcl-2-binding protein homologue (*Bnip3*)	m	AF041054	1	63.0	N
interferon induced gene	r	X61381	1	>100	N	FLS353 activated in colon tumours	h	AB024704	1	2.3	R
KIAA0045, myeloblast (*TRIP12*)	h	D28476	1	16.3	R	glycyl-tRNA synthetase (*GARS*)	h	U09510	1	12.0	R
KIAA0128, myeloblast	h	D50918	1	33.8	R	Hrihfb2216 rat fetal brain gene	r	AB015345	1	2.9	N
KIAA0235, myeloblast	h	D87078	1	4.8	R	insulinoma gene (*Rig*)	r	M19393	1	1.6	N
KIAA0259, myeloblast	h	D87448	1	3.6	R	KE04p protein	h	AF064093	1	16.0	N
KIAA0332, brain	h	AB002330	1	20.8	R	KIAA0013, myeloblast	h	D87717	1	3.2	R
L1 retroposon	r	X53581	5	20.2	R	KIAA0310, brain	h	AB002308	1	6.0	R
LxRN3, LINE 1 repetitive sequence	r	M60824	1	26.2	R	KIAA0431, brain	h	AB007891	1	10.7	R
Mama gene (*Ppicap*)	r	AF065438	1	14.5	N	KIAA0525, brain	h	AB011097	1	2.5	R
osteoglycin (*Ogn*)	m	D31951	5	2.7	R	KIAA0544, brain	h	AB011116	1	2.9	R
p53 binding protein 2 (*Mdm2*)	m	U58881	1	10.3	R	KIAA0595, brain	h	AB011167	1	9.4	R
Pebp2a1, Core binding factor a1 (*Cbfa1*)	m	D14636	1	38.4	N	KIAA0597, brain	h	AB011169	1	2.9	R
pMem2, maternal embryonic message 2 (*Mem2*)	m	X95350	1	29.4	N	LIM protein, four and a half lim domains 2 (*Fhl2*)	m	AF055889	1	4.2	R
splicing factor, arginine/serine-rich 7 (*SFRS7*)	h	L41887	2	10.4	R	LIM protein, four and a half lim domains 3 (*FHL3*)	h	U60116	1	7.3	N
Wdnm2 (*Nmor1*)	r	X17464	1	>100	N	Mam domain protein	x	XLU37376	1	>100	N, R
zinc-finger domain-containing protein	h	U90654	1	7.8	R	m-calpain large subunit (*Capn1*)	r	RNU53858	1	28.7	R
Znf216 zinc-finger protein	m	AF062071	1	6.7	R	neuritin	r	RNU88958	1	1.8	R
						oxygen-regulated 150 kD protein	r	U41853	1	9.3	N
						PHD finger protein 2 (*PHF2*)	h	NM_0053921	1	2.1	R
						spinocerebellar ataxia type 3 gene (*Mjd*)	r	Y12319	1	55.5	N
						seryl tRNA synthetase (*SARS*)	h	M88136	3	2.4	R
						transforming acidic coiled-coil protein 2 (*TACC2*)	h	AF095791	1	2.3	R
						tumour susceptibility gene 101 (*tsg101*)	m	U52945	2	2.2	N
						tyrosine phosphatase-like protein	r	U40652	11	74.9	N, R

We verified the differential expression by reverse (R) and conventional northern-blot analysis (N). Sequence annotation, species and accession number are listed according to the best match in BLAST analysis (http://www.ncbi.nlm.nih.gov/blast/blast.cgi). Official gene symbols (in parentheses) were obtained from human (http://www.gene.ucl.ac.uk/cgi-bin/nomenclature/searchgenes.pl) or mouse (http://mgd.hgmp.mrc.ac.uk) nomenclature databases. h, human; m, mouse; r, rat; c, chicken; ha, hamster; x, *Xenopus laevis*. 'Redundancy' refers to the number of individual cDNA clones matching the same gene. Densimetric analysis of mRNA levels: numbers represent the ratio of densitometric values (volume analysis) of 208F versus FE-8 mRNA (level of downregulation, left) and of FE-8 versus 208F mRNA (level of upregulation, right). A value of 30 or more indicates that the transcript was not detectable in one of the cells under comparison. Differentially expressed ESTs are not listed (library statistics, expression data and ESTs are available, see http://genetics.nature.com/supplementary_info/). The following cDNA fragments present in the libaries were not detected on reverse or conventional northern blots: 208F-specific clones, *Arhgap5, Slit2, Slugh, Sema3e, GLE1L, TID1, big1, DDX10*; FE-8-specific clones, *npr2l, Ddx5, LZTR1, Smcx, SHMT2* and *GRF2*.

Table 2 • RAS targets sensitive to MEK inhibition

Downregulated genes	Expression level			Upregulated genes	Expression level		
Sequence identity (GenBank/EMBL)	208F	FE8	FE8 +PD	Sequence identity (GenBank/EMBL)	208F	FE8	FE8 +PD
3-hydr.3-methylglutaryl coA synthase (*Hmgcs1*)	+++	+	+++	bleomycin hydrolase (*Blmh*)	+	+++	++
actin binding protein-280, filamin (*FLNA*)	+++	++	+++	BRCA1-associated Ring domain protein (*Bard1*)	0	++	+
α-actin (*Actvs*)	+++	+	+++	E1B 19k/Bcl-2-binding protein homologue (*Bnip3*)	0	+++	++
antioxidant enzyme AOE372	++	+	++	exportin-T (*XPOT*)	+	+++	++
AP56, selenium binding protein 2 (*Selenbp2*)	++	0	++	Flap endonuclease-1 (*Fen1*)	0	+++	+
Cdc21 (*Mcmd4*)	++	0	+++	Fkbp51, T-cell-specific immunophilin (*Fkbp5*)	0	+++	+
centromeric protein CENPC (*Cenpc*) (a)	+++	0	++	Flice-like inhibitory protein (*Cash*)	0	++	0
collagen α1 (*Cola1*)	+++	+	+++	GEF-H1	0	+++	+
colony stimulating factor 1 (*Csf1*)	++	0	++	lamina-associated polypeptide 1C	0	+++	0
DOC-2, p96 phosphoprotein (*Dab2*)	++	0	+++	Mam domain protein	0	+++	+
ER81 ets-related transcription factor (*Etsrp81*)	+++	+	++	MAPK phosphatase cpg21 (*Dusp5*) (c)	0	+++	+
ERp99/GRP94 (*Tra1*)	+++	+	+++	MMP-3, stromelysin 1 (*Mmp3*)	0	+++	0
ETF TEA domain transcription factor (*Tead2*)	+++	0	++	MMP-10, stromelysin 2 (*Mmp10*) (d)	0	+++	0
fibronectin (*Fn1*)	+++	+	+++	Myb-binding protein P160 (*Mybbp1a*)	+	+++	+
follistatin-related protein TSC-36 (*Fstl*)	++	+	+++	NF-1 transcription factor (*Nfic*)	0	++	0
Gu binding, inhibitor of activated Stat1 (*DDXBP1*)	+++	0	+	non-neuronal enolase 1 (*Eno1*)	+	+++	++
heat shock protein 90 (*HSPCA*)	++	0	++	oxygen-regulated 150-kD protein	+	+++	+
Hspg core fibroglycan, syndecan-2 (*Sdc2*)	+++	0	++	p67 isoprenylated protein (*Gbp2*)	0	+++	++
interferon induced gene	+++	0	++	PkB kinase (*Pdpk1*)	0	+++	+
L1 retroposon	+++	0	++	Rap1B GTP binding protein (*Rap1b*) (e)	0	+++	+
laminin B1 (*Lamb1-1*)	+++	+	++	Ras-GTPase-activating protein (*G3bp2*)	0	+++	+
lysyl oxidase (*Lox*)	+++	0	+	spinocerebellar ataxia type 3 gene (*Mjd*)	0	+++	+
lysyl oxidase-related protein (*LOXL2*)	++	0	+	stromal antigen 1 (*Stag1*)	0	++	+
Mama gene (*Ppicap*)	+++	0	+	sortilin 1 (*SORT1*)	0	+++	++
MMP-2, gelatinase A (*Mmp2*)	+	0	+++	tumour susceptibility gene 101 (*tsg101*)	++	+++	++
Tfe3 X-linked transcriptional activator (*Tcfe3*)	++	+	++				
nuclear autoantigen GS2NA	++	0	+++				
osteoglycin (*Ogn*)	++	0	++				
p5 protein	+++	+	++				
P-cadherin (*Cdh3*)	++	0	+++				
phosducin-like protein (*Pdcl*)	+++	0	+				
serum inducible kinase (*Snk*)	+++	0	++				
STAT5a1 transcription factor (*Stat5a*)	++	0	+++				
thrombospondin 1 (*Thbs1*)	+++	0	+				
tissue inhibitor of metalloproteinase 2 (*Timp2*)	+++	+	++				
TRPM-2, clusterin (*Clu*) (b)	+++	+	+++				

PD, MEK inhibitor PD 98059. Transcript levels: 0, mRNA not detectable on northern blots with total RNA; +, ++, +++, low, intermediate or high mRNA expression. We hybridized cDNA arrays comprising all differentially expressed sequences detectable by reverse northern analysis (Table 1) with ^{32}P-labelled probes prepared from RNA of untreated and inhibitor-treated FE-8 cells. In addition, we performed conventional northern analysis of 177 preferentially expressed known genes. Sequences marked a–e (in parentheses) were used as probes on northern blots shown in Fig. 2.

scripts (for example, those encoding cytoskeletal proteins) and of low-copy-number mRNAs (for example, those encoding transcription factors). The cDNA fragments obtained in this study represent a large fraction of the genes differentially expressed in the two cell lines. Others have reported a similar number of differences between normal cells and tumour cells[10,11].

The known RAS targets displaying stimulated or *de novo* expression in FE-8 cells comprise the genes encoding the metastasis-related glycoprotein Cd44, the transcription factor Fra-1, the α-chemokine Mob-1, the metallo-proteinases Mmp-1 and Mmp-3, and the myosin regulatory light chain. The known downregulated RAS pathway targets include the genes encoding α-actin, collagen α-1, entactin/nidogen, fibronectin, TGFβ-stimulated sequence TSC-36, lysyl oxidase, smooth muscle myosin light chain (Mlc)-2 and NADH dehydrogenase. We did not recover RAS targets such as those encoding the glucose transporter, Myc, Pdgf receptor and Cox-2. These divergent results may be due to the use of different cell types, growth conditions or methods of gene isolation.

FE-8 cells expressed elevated levels of genes triggering invasion and metastasis. These encode the laminin receptor, Mmp-1 (collagenase), Mmp-3 (stromelysin-1), Mmp-10 (stromelysin-2) and the Cd44 glycoprotein. There was a close link between permanent RAS signalling and gene repression. Several genes exhibiting anti-proliferative, anti-invasive or anti-angiogenic activity were simultaneously downregulated. The genes encoding syndecan-2, tissue inhibitor of metalloproteases-2 (Timp2), lysyl oxidase,

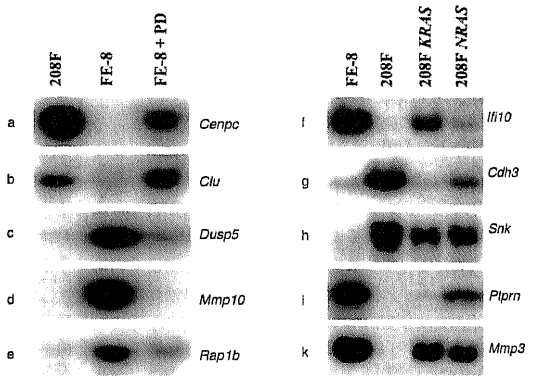

Fig. 2 Effects of the Ras/Raf/MEK signalling pathway and of different RAS isoforms on selected target gene transcription. Probes a–e, northern-blot analysis of mRNA expression in normal 208F fibroblasts, FE-8 cells and FE-8 cells treated with MEK inhibitor PD 98059. Probes f–k, northern-blot analysis of mRNA expression in normal 208F and *HRAS* (FE-8), *KRAS* and *NRAS* transformants. *Mmp3*, representative example of a gene without significant differential expression.

thrombospondin-1, protein kinase A II, the myristoylated, alanine-rich, C kinase substrate (Marcks) and the growth-arrest–specific protein Gas-1 may act as class II tumour suppressors in the RAS transformation model. Class II genes, unlike class I tumour-suppressor genes, are not targeted by tumour-initiating mutations, but rather are regulated by upstream signalling pathways and associated transcription factors[18]. Moreover, we recovered genes involved in diverse signal transduction processes regulating mitogenic activity and cell survival, cytoskeletal reorganization, stress response, oxidative phosphorylation, glycolytic energy generation, fatty acid oxidation, transport and cytotoxic drug processing (Table 1).

Besides Raf kinase, the key downstream effector of Ras, Raf-independent pathways contribute to Ras-mediated transcriptional alterations and transformation[5]. We determined to what extent the Raf signalling cascade affects target gene transcription and transformed phenotypes in FE-8 cells. On treatment with the specific Mek inhibitor PD 98059 (ref. 19), FE-8 cells displayed a more normal morphology (similar to that of 208F cells; Fig. 1*a*) and a reduced capacity of anchorage-independent proliferation (Fig. 1*c*) in the presence of unaltered levels of $p21^{ras}$ (Fig. 1*d*), but decreased levels of phospho-p44/42 Mapk (Fig. 1*e*). We found 61 RAS targets to be sensitive toward MAP-kinase inhibition by reverse and conventional northern analysis of RNA from treated FE-8 cells (Table 2 and Fig. 2*a–e*). Mapk inhibition blocked downregulation of 36 targets and upregulation of 25 targets. The inhibitor did not affect the steady-state mRNA levels of 116 genes or expressed sequences. The sensitivity of transcriptional alterations toward Map kinase inhibition defines a subset of Ras-sensitive targets regulated by substrates of Erk1/Erk2. The 116 unaffected genes are most likely controlled by Mek-independent pathways.

Although the products of *HRAS*, *KRAS* and *NRAS* are similar in structure and function, the RAS protein isoforms differ in carboxy-terminal amino acid composition, expression pattern and post-translational modification[4]. Moreover, individual isoforms are preferentially mutated in different types of cancers[1]. To determine how the two other RAS isoforms affect transcription, we compared the expression of targets in FE-8 and 208F cells expressing activated *KRAS* or *NRAS* (Fig. 3), respectively. Approximately 90% of sequences (n=237) sensitive to *HRAS* transformation showed a similar expression pattern in cells transformed by other mutated RAS isoforms (data not shown). This suggests that the oncoproteins encoded by mutated *HRAS*,

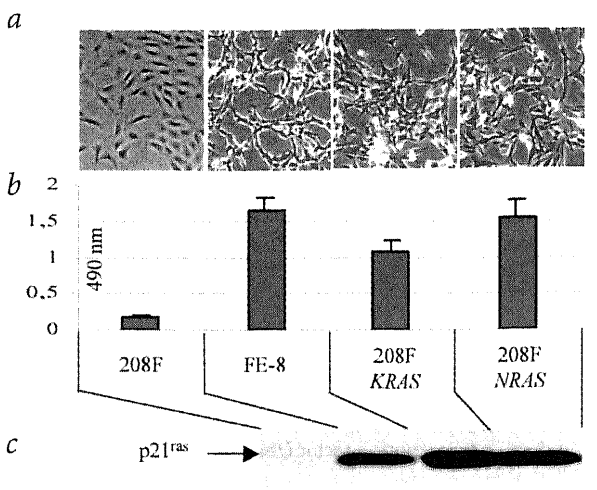

Fig. 3 The 208F cell lines transformed with oncogenic *HRAS*, *KRAS* and *NRAS* exhibit similar neoplastic characteristics. ***a***, Morphology of normal 208F fibroblasts, FE-8 cells, and *KRAS* and *NRAS* transformants, respectively. Phase contrast, magnification ×100. ***b***, Anchorage-independent proliferation of cells in cultures on poly-HEME–coated surfaces determined by XTT-based colorimetric assay. ***c***, Western-blot analysis of $p21^{ras}$.

Table 3 • RAS isoform-specific transcriptional alteration

Sequence identity (GenBank/EMBL)	Expression level 208F	FE-8 *HRAS*	208F *KRAS*	208F *NRAS*
ABC transporter Moat-B (*ABCC4*)	0	++++	0	+
BCSC-1, breast cancer suppressor candidate 1	+	++++	0	+
cyclooxygenase 1 (*Ptgs1*)	+	++++	+	+++
E1B 19k/Bcl-2-binding protein homologue (*Bnip3*)	0	++	++++	++
EST AA743557	++++	+	0	++
EST AA792426	+	++++	+	+
EST AA924000	+	++++	+	++
ETF TEA domain transcription factor (*Tead2*)	++++	0	++	++
farnesyl diphosphate synth. (*Fdps*)	+	+++	0	+
Flap endonuclease-1 (*Fen1*)	0	++++	+	0
Flice-like inhibitory protein (*Cash*)	0	+	++	++++
JAK1 protein tyrosine kinase (*Jak1*)	+	++++	+	+
MAPK phosphatase cpg21 (*Dusp5*)	0	++	+++	++++
melanoma antigen family B3 (*MAGEB3*)	0	++++	0	0
MARCKS (*Macs*)	++++	0	+	+++
MMP-10, stromelysin 2 (*Mmp10*)	0	++	++	++++
Mob-1, interferon activated gene 10 (*Ifi10*) (f)	0	++++	++	+
Tfe3 X-linked transcriptional activator (*Tcfe3*)	++++	0	+	+
Myb-binding protein P160 (*Mybbp1a*)	+	++++	++	++
novel transcript N317	++++	0	++	++++
P-cadherin (*Cdh3*) (g)	++++	0	0	++
phosphatidylinositol 3-kinase p170 (*Pik3c2a*)	+++	0	+	++
Ras-GTPase-activating protein (*G3bp2*)	0	++++	0	0
SBF1 phosphatase (*SBF1*)	0	++++	+	+
serum inducible kinase (*Snk*) (h)	++++	0	+++	+++
tyrosine phosphatase IA-2a (*Ptprn*) (i)	0	++++	0	++

Quantitation of transcript levels as in Table 2. Sequences marked f–i (in parentheses) were used as probes on northern blots shown in Fig. 2. We hybridized labelled cDNA probes derived from 208F cells transformed by mutant *KRAS* and *NRAS* to cDNA arrays comprising *HRAS*-transformation-sensitive sequences (n=233) and verified the results of reverse northern analysis by conventional northern blot. Moreover, we analysed 30 genes with low expression on northern blots. Overall, we found more targets responding to HRAS than to KRAS or NRAS signalling. The analysis may be biased, however, because we derived the expression profile primarily from *HRAS*-transformed cells.

KRAS or *NRAS* generally impinge on identical transcriptional targets. The transcript levels related to 26 cDNA fragments showed distinct differences (Table 3 and Fig. 2*f–i*). The isoform-specific downstream targets may aid in elucidating the mechanisms of how distinct phenotypic effects are generated by genetic ablation of different *RAS* genes[4]. The specificity of targets responding to the RAS isoforms may be obscured by technical differences in the selection procedures used for establishing transformed cells. Similarly, not all genes shown to be regulated by RAS signalling may turn out to be true targets. Some transcription units may be predominantly controlled by rather indirect mechanisms. Several selected targets, however, showed a very similar expression pattern in normal rat ovarian surface epithelial cells[20] (ROSE), in a *KRAS*-transformed derivative and in 208F cells carrying isopropyl-1-thio-β-D-galactoside (IPTG)-inducible *HRAS* (ref. 21, see also http://genetics.nature.com/supplementary_info/). This confirms the specificity of the gene profiles described in the 208F/FE-8 cell system.

We have provided detailed sequence information on transcriptional changes related to the activity of mutated *RAS* genes which mediate the most prevalent oncogenic signal-transduction pathways in experimental and human cancer[1,4]. RAS pathways are elevated even in tumours not carrying activating mutations[22,23]. In the absence of intrinsic mutations, the RAS signalling pathway is stimulated by inactivating mutations of the gene encoding RAS regulator NF-1 GAP in neurofibromatosis type I (ref. 24), by complex formation of upstream effector proteins with the BCR-ABL protein tyrosine kinase in chronic myelogenous leukaemia[25] and by direct association of Ras with the STP-C488 protein of the DNA tumour virus *Herpes saimiri*[26]. We verified the differential expression of 393 (47.8%) genes and gene fragments by northern-blot analysis. In addition, we recovered 236 cDNA sequences related to very low abundance transcripts, which likely exhibit differential expression at a similar proportion. Overall, of all expressed genes (estimating 5,000–15,000 different sequences), 2.6%–7.8% were transcriptionally altered in tumorigenic, *RAS*-expressing cells relative to normal cells. The gene expression profile of RAS-transformed cells provides correlative rather than causal information. The specific set of genes recovered by cDNA subtraction can be used for investigating the relationships between oncogenic signalling molecules and their targets at the level of the transcriptome. In this way, the number of targets potentially executing the repertoire of biological activities of tumorigenic cells may be narrowed to a limited set of genes which can be assayed thoroughly for causal involvement in specific aspects of tumorigenesis. The distinct effects of the Mek inhibitor on the RAS-specific gene expression profile indicate that the expression profiling approach is useful in defining novel drug targets with possible therapeutic payoff. The SSH method permitted an efficient identification of RAS target genes with known function and an unbiased isolation of novel sequences not represented among pre-selected genes available on DNA microchips[27,28]. In view of the number of putative class II tumour-suppressor genes[17,18,29] downregulated in *RAS*-expressing cells, SSH is a powerful approach for the identification of anti-cancer genes and may complement the search for deletions and mutations at the genomic level.

Methods

Cell culture and DNA transfections. We cultured cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and antibiotics. Transfections were done by calcium-phosphate precipitation[16]. The FE-8 cell line is a G418-resistant, *HRAS* (G12V)-transformed derivative of preneoplastic rat 208F cells[16]. We used FE-8 cells from stocks cryopreserved early after isolation and 208F cells maintained in culture notlonger than 30 days for RNA isolation and construction of subtracted cDNA libraries. We isolated *NRAS*-transformed 208F cells after co-transfection of the pcDneo resistance plasmid and the *NRAS* (G12D) oncogene[30] (kindly provided by J.J. McCormick) and selection in DMEM containing G418 (400 μg/ml; Gibco). We isolated the *KRAS*-transformed 208F cell line from morphologically transformed transfectants. *KRAS* (C12V) was cloned from the SW480 human colon carcinoma cell line. Transformed rat ovarian surface epithelial cells were isolated after transfection of ROSE199 cells[20] with *KRAS* (C12V). To generate cells expressing inducible *HRAS*, we co-transfected 208F cells with the plasmids pSVlacOras and pHβlacINLSneo (ref. 21). We treated cells with IPTG (20 mM; ICN) for 4 d to induce RAS expression and neoplastic transformation.

Inhibitor studies and proliferation assay. The MEK inhibitor PD98059 (Alexis; ref. 19) was dissolved in DMSO to a final concentration of 50 mM. We treated FE-8 cells for 2 d with PD98059 at a final concentration of 50 μM. Anchorage-independent proliferation was measured semi-quantitatively in cultures grown on microtitre dishes coated with poly-2-hydroxyethyl methacrylate (poly-HEME; Sigma). We added a 5 mg/ml poly-HEME stock solution (75 μl) dissolved in 95% ethanol to the wells and allowed it to dry for 72 h at 37 °C. We seeded cell suspensions (1,000 cells/well) on coated dishes and monitored growth after 5 d using a sodium 3′ [1-(phenyl-amino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)-benzene sulfonic acid hydrate (XTT)-based colorimetric assay (Roche). Formazan dye formation in metabolically active cells was measured at 490 nm.

Cloning of differentially expressed sequences by SSH. We prepared total RNA from subconfluent cultures as described[31]. Messenger RNA was isolated from 1 mg total RNA using the mRNA Separator kit (Clontech). We performed cDNA synthesis and subtraction with the PCR-select cDNA subtraction kit (Clontech) according to the manufacturer's protocol with modifications: we used a driver/tester volume ratio of 2:1 in the first hybridization. We carried out 26 cycles of primary PCR and 10 cycles of secondary PCR with the Advantage cDNA polymerase mix (Clontech). To evaluate the efficiency of cDNA subtraction, we compared the transcript levels of the housekeeping gene *Gapd* by RT–PCR in subtracted and unsubtracted cDNA populations from 208F and FE-8 RNA, respectively. Detection of *Gapd* sequences for both subtractions required 28 PCR cycles with subtracted cDNA as template, whereas only 18 cycles were sufficient to amplify *Gapd* from control cDNAs. Furthermore, transcript levels of genes known to be differentially expressed in 208F and FE-8 cells were tested by RT–PCR. As expected, *HRAS*-specific sequences were enriched in subtracted versus unsubtracted FE-8 cDNA. Lysyl oxidase levels were increased in subtracted versus unsubtracted 208F cDNA and decreased from a low level in unsubtracted FE-8 cDNA to a non-detectable level in subtracted FE-8 cDNA.

We purified subtracted cDNA sequences using the QIAquick PCR purification kit (Qiagen) and inserted 10 ng into the T/A cloning vector pCR2.1 (Invitrogen). Individual transformants carrying cDNA fragments were isolated from white colonies on X-gal/IPTG agar plates. To assess the quality of the libraries with respect to redundancy and specificity, we randomly picked 35 cDNA transformants from each library DNA and determined their sequence. We also analysed differential expression of the individual cDNA sequences on northern blots with total RNA (10 μg) from 208F and FE-8 cells.

Sequence analysis. Sequencing reactions were performed with the M13 universal primer using the BigDye sequencing kit (Perkin Elmer) according to the manufacturer's protocol. Sequences were determined on an ABI377 sequencer. We discontinued sequencing of the cDNA inserts of subtracted libraries when the number of redundant sequences exceeded that of novel clones. Clustering was performed using the gap4 software (Staden package). Sequence homology searches were done against GenBank (nr) and EST (dbEST) databases using the BLASTN program at NCBI (http://www.ncbi.nlm.nih.gov/BLAST). The last BLAST analysis was performed on 22 June 1999. Sequence management was performed with database tools developed in our laboratory.

cDNA arrays and northern-blot analysis. We transferred non-redundant plasmid DNA probes of all identified fragments to 96-well microtitre

plates. Using PCR-Select adaptor-specific primers, we carried out PCR amplification for 30 cycles (30 s 94 °C, 30 s 68 °C, 90 s 72 °C). The average size of the inserted fragments was 800 bp. The PCR amplified inserts were blotted onto duplicate 25×12 cm Nytran nylon membranes (Schleicher and Schuell). We performed reverse northern analysis as described[12], except using different hybridization conditions. For prehybridization of membranes, we used 5×Denhardt's reagent, 5×SSC, phosphate buffer (50 mM), 0.5% SDS and tRNA (100 ng/ml) at 65 °C for 3 h. Hybridization was performed in the same buffer without Denhardt's reagent and phosphate buffer (50 mM) at 65 °C for 16 h.

For conventional northern-blot analysis, total RNA (10 μg) was fractionated by electrophoresis through 1% agarose gels containing formaldehyde and blotted in 20×SSC onto Protran nitrocellulose membranes (Schleicher and Schuell). We labelled cDNA fragments with [^{32}P]dCTP using the ReadyPrime system (Amersham). Hybridization was performed in ExpressHyb hybridization buffer (Clontech) at 68 °C overnight. Membranes were washed twice in 2×SSC, 0.1% SDS at 42 °C for 20 min, twice in 0.1×SSC, 0.1% SDS at 66 °C for 30 min and exposed to X-ray film at –80 °C for 6 h–3 d.

GenBank accession numbers. *Arhgap5*, U17032; *Slit2*, AF141386; *Slugh*, U79550; *Sema3e*, AB000220; *GLE1L*, AF058922; *TID1*, AF061749; *big1*, AF023451; *DDX10*, NM_004398; *npr2l*, AF131207; *Ddx5*, X65627; *LZTR1*, D38496; *Smcx*, Z29651; *SHMT2*, L11932; *GRF2*, *E08769*.

Acknowledgements

We thank S. Petermann, J. Keil and K. Skultety for technical assistance; R. Brinckmann for sequencing; C. Schlüns for computing; F. Leenders and F. Theuring for help with phospho-image analysis; and M. Dietel for generous support. Our work was supported by Deutsche Krebshilfe (grant 10-332-Schä I to R.S.), Krebsliga des Kantons Zürich (grant to R.S.), Studienstiftung des Deutschen Volkes and Forschungsförderung der Charité (to J.Z.), and Berliner Krebsgesellschaft (grant to R.S.).

Received 30 August; accepted 13 December 1999.

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