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Expression and functional validation of new p38 α transcriptional targets in tumorigenesis

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Running title: p38 α targets in cell transformation

p38 α MAP kinase plays an important tumor suppressor role, which is mediated by both its negative effect on cell proliferation and its pro-apoptotic activity. Surprisingly, most tumor suppressor mechanisms coordinated by p38 α have been reported to occur at the post-translational level. This contrasts with the important role of p38 α in the regulation of transcription and the profound changes in gene expression that normally occur during tumorigenesis. We have analyzed whole genome expression profiles of Ras-transformed wild-type and p38 α -deficient cells and have identified 202 genes that are potentially regulated by p38 α in transformed cells. Expression analysis has confirmed the regulation of these genes by p38 α in tumors, and functional validation has identified several of them as likely mediators of the tumor suppressor effect of p38 α on Ras-induced transformation. Interestingly, about 10% of the genes that are negatively regulated by p38 α in transformed cells contribute to EGF receptor signalling. Our results suggest that inhibition of EGF receptor signalling by transcriptional targets of p38 α is an important function of this signalling pathway in the context of tumor suppression.

Keywords: p38 MAPK; Ras oncogene; gene expression; EGFR

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INTRODUCTION

p38 α mitogen-activated protein kinase (MAPK) is an ubiquitously expressed serine/threonine protein kinase, which is activated by many extracellular stimuli and coordinates numerous cellular processes [1, 2]. p38 α can also negatively regulate malignant cell transformation, which seems to be accounted for by its inhibitory role on cell cycle progression and its stimulatory effect on apoptosis and differentiation [3, 4].

Intriguingly, despite the recognized importance of p38 α in the regulation of gene expression [5-9], little is known about how this p38 α function impinges on tumorigenesis. In fact, most of the known tumor suppressor mechanisms coordinated by p38 α occur at the post-translational level. These include the post-translational regulation of ERK1/2 pathway activity, protein stability of cyclin D1, subcellular localization/stability of Cdc25 phosphatases, EGF receptor (EGFR) intracellular fate, and upregulation of cell cycle inhibitors such as p21^{Cip1} or p27^{Kip1} [3, 4]. There are a few examples on the regulation of gene expression by p38 α with potential relevance for malignant transformation, most notably the transcriptional control of cyclin D1 [10] or the cell cycle inhibitor p16^{INK4a} [11], and the upregulation of EGFR mRNA in lung tumors of p38 α -deficient mice [12].

We have investigated how transcriptional regulation contributes to the tumor suppressor effect of p38 α , by comparing whole-genome expression profiles of wild-type (WT) and p38 α -deficient (p38 α -/-) mouse embryo fibroblasts (MEFs) expressing oncogenic H-Ras^{G12V}. Our previous work showed that p38 α deficiency facilitates H-Ras^{G12V}-induced transformation in these cells [13-15]. Altogether, we have identified a group of genes that are regulated by p38 α in the context of malignant cell transformation, and have also functionally validated 9 of these genes in anchorage-independent cell growth assays. Interestingly, we have found that around 10% of the genes whose expression is negatively regulated by p38 α can potentially enhance EGFR signalling. These results provide further evidence for a key role of EGFR pathway down-regulation in tumor suppression by p38 α .

MATERIALS AND METHODS

Microarray procedures and data analysis

mRNA was extracted from three biological replicates of exponentially proliferating WT and p38 α -/- MEFs expressing H-Ras^{G12V} using the Qiagen RNeasy kit according to manufacturer's instructions. RNA quality was checked spectrophotometrically by its 260 to 280 nm absorbance ratio as well as by RT-PCR. RNAs were labelled with Cy5 and Cy3, pooled, hybridized and processed with the Amersham Codelink 20K mouse platform (GeneCore Laboratory, EMBL, Heidelberg).

Microarray dataset normalization was performed via cyclic loess normalization. Differentially expressed genes were obtained by applying linear models with R limma package [16] (Bioconductor project, <http://www.bioconductor.org>) and multiple hypotheses testing was accounted for by adjusting the estimated significance level (*p* value) with the Benjamini & Hochberg False Discovery Rate (FDR) correction. Those genes with FDR<0.05 and FC>1.5 (accounting to a total of 2100 genes) were selected as differentially expressed between H-Ras^{G12V}-expressing p38 α -/- and WT MEFs and subsequently discriminated with parametric ANOVA 1-way test (*p*<0.01) with the GeneSpring 6.2 software (Silicon Genetics). This latter analysis allowed to cut down the number of differentially-expressed genes to a more workable set of 202 genes, which corresponded to those genes deregulated \pm 1.5-fold between H-Ras^{G12V}-expressing p38 α -/- and WT MEFs within the 99% confidence level (*p*<0.01) and a FDR<0.05.

Gene functions were manually annotated on the basis of exhaustive searches in PubMed and Gene Ontology-based applications such as the FatiGO and Source Batch databases.

Gene set enrichment analysis (GSEA)

GSEA was performed with the EGFR-associated set of 19-genes identified in this study versus microarray data from [17] as reference. Affymetrix arrays (GEO ID: GSE9599) were normalized using Robust Multi-Array Average (RMA). Whole microarray genes were ranked based on limma moderated t statistic. After Kolmogorov-Smirnoff testing, the gene set was considered statistically significant if FDR <0.25, a widely accepted cut-off for the identification of biologically relevant gene sets [18].

mRNA isolation and RT-PCR analysis

Total RNA was extracted from MEFs or mouse tumors using the RNeasy Kit (Qiagen) according to the manufacturer's instructions. RNA concentration was determined by measuring the absorbance at 260 nm and the quality was monitored by the ratio of absorbance at 260/280 nm and by 1% agarose gel electrophoresis. cDNA was prepared using M-MLV reverse transcription (Invitrogen) and used as the template for PCR amplification. PCR primers were designed with the Vector NTI 8 software (InforMax) and used at a final concentration of 160 nM (Supplementary Table 1). RT-PCR conditions were as follows: reverse transcription at 37°C for 90 min, denaturation at 95°C for 2 min, followed by 30-35 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 45 s and elongation at 72°C for 60 s. A final step of elongation at 72°C for 10 min was performed. The products of the RT-PCR reaction were analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide and imaged in the Gel Doc 2000 system (Bio-Rad).

Quantitative Real-Time PCR (qRT-PCR) analysis was performed as previously described [15]. Primer sequences are listed in Supplementary Table 2.

Cell culture and reagents

Primary WT and p38 α -/- MEFs were immortalized using the 3T3 protocol, maintained as previously described [14] and used as the starting biological material in the experiments described. The term MEFs was used throughout the manuscript to refer to spontaneously immortalized cells or derivatives of them (i.e. H-Ras^{G12V} transformed). MEFs expressing 4-hydroxytamoxifen (OHT)-inducible H-Ras^{G12V} have been previously described [14]. HEK293 human epithelial cells were maintained in the same medium as MEFs and used for transient transfection. 293T cells were used to generate retroviruses to stably infect MEFs and were provided by M. Serrano (CNIO, Madrid).

Expression constructs and retroviral infections

pBabe-puro-H-Ras^{G12V} and pWZL-hygro-based constructs containing the *Gstm2*, *Cd9*, *Mmp3*, *Egln3*, *Pmp22*, *Aig1*, *Ctsh*, *Nck2* and *Rtn4* mouse cDNAs were used for the generation of retroviruses and subsequent MEF transduction as previously described [14]. Cloning details, cDNA origin, amplification primers and restriction sites are provided in Supplementary Table 3. Myc-tagged versions of pWZL-hygro-Gstm2 and pWZL-hygro-Cd9 were prepared by site-directed mutagenesis on the non-tagged constructs (QuickChange, Stratagene) and verified by sequencing. The retroviral construct MSCV-hygro-p38 α [19] was used to re-express p38 α in p38 α -/- MEFs for the rescue experiments.

Immunoprecipitation

Control and H-Ras^{G12V}-expressing WT MEFs were transduced with Cd9 or an empty vector and then EGFR was immunoprecipitated. Subconfluent 10-cm cell culture plates were washed twice with ice-cold PBS, scraped on ice and harvested by centrifugation at 400xg and 4°C for 10 min. The cell pellets were lysed in 100-600 μ l of modified RIPA lysis buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl₂, 1 mM EGTA, 1% TritonX-100, 1% DOC, 0.1% SDS, 20 mM NaF, 0.1 mM sodium orthovanadate, 1 mM PMSF, 2.5 mM benzamidine, 2 μ M microcystin and 10 μ g/ml leupeptin and aprotinin). Whole cell extracts

(500 µg) were immunoprecipitated with anti-EGFR antibody (Santa Cruz Biotechnology) overnight at 4°C. Immunoprecipitates were incubated with protein G-sepharose (Amersham) for 1 h at 4°C, washed 3 times, analyzed by SDS-PAGE and visualized with anti-EGFR antibody (Santa Cruz Biotechnology).

Immunoblotting, transformation and tumorigenicity assays

Immunoblot analysis was performed as previously described [14] using the following primary antibodies: c-Myc (9E10, Santa Cruz Biotechnology), p38 MAPK (C20-G, Santa Cruz Biotechnology), tubulin (clone DM1A, Sigma), Ras (clone 18, Sigma), phospho-ERK (9101, Cell Signaling Technology), phospho-AKT (9271, Cell Signaling Technology), EGFR (1005, sc-03, Santa Cruz Biotechnology), P-EGFR (Y1068, Cell Signaling), EglN3 (Novus Biologicals) and Nck2 (Upstate Biotechnology).

Cell proliferation (MTT) and anchorage-independent growth (soft agar) assays were performed as previously reported [14]. Briefly, soft agar assays were performed in duplicates with a top layer of 0.35% agar and a bottom layer of 0.5% agar. Cell colonies were stained with 0.1% crystal violet (Sigma) at the end of the experiment and counted if bigger than 0.2 mm. Colony numbers normally displayed a standard deviation of ±500 colonies. Pictures of representative fields were taken at 40x magnification with a Leica MZ6 microscope coupled to RS Photometrics camera.

Statistical analysis

Unless otherwise indicated, all results are expressed as a mean ± standard deviation (S.D.) for at least 3 independent experiments. Statistical analysis was performed using Student's *t* test with a statistically significant *p* value (*p*<0.01).

RESULTS

Gene expression profiling of H-Ras^{G12V}-transformed WT and p38α^{-/-} MEFs

In order to identify new targets of p38α in the regulation of oncogene-induced transformation, RNA was extracted from biological triplicates of exponentially proliferating H-Ras^{G12V}-transformed WT and p38α^{-/-} MEFs, labelled and analyzed with the Amersham Codelink® mouse 20K platform. Statistical filtering, retaining genes with an increased or decreased expression greater than ±1.5 fold, a false discovery rate (FDR) < 0.05 and an adjusted *p*-value < 0.01, revealed 202 genes that were significantly deregulated between WT and p38α^{-/-}MEFs (Supplementary Table 4). Among them, 139 genes (69%) were up-regulated and 63 genes (31%) were down-regulated in H-Ras^{G12V}-p38α^{-/-} MEFs (Figure 1). Genes were subsequently clustered according to their biological function using Gene Ontology (GO)-based approaches and subcategorized (Figure 1 and Supplementary Table 5). A flowchart showing all the individual steps undertaken is shown in Supplementary Figure S1.

Gene expression validation by semiquantitative RT-PCR in cultured MEFs

To validate microarray gene expression differences, 31 out of the 202 genes potentially regulated by p38α were selected for semi-quantitative RT-PCR analysis, which was performed on new mRNA preparations extracted from the two transformed MEF lines growing exponentially. Gene selection was based on the different mRNA expression levels in H-Ras^{G12V}-p38α^{-/-} versus H-Ras^{G12V}-WT MEFs, as well as on the putative involvement of each gene in Ras-induced transformation or p38α signalling. Out of the 31 genes selected, 19 were up-regulated and the other 12 were down-regulated in H-Ras^{G12V}-p38α^{-/-} cells (Supplementary Table 6).

Two independent cellular systems were used to dissect the relative contribution of p38α and H-Ras^{G12V} to the expression of these genes. The first involved established WT and p38α^{-/-} MEFs constitutively expressing H-Ras^{G12V} for at least two weeks [13, 14], which allowed

us to evaluate the steady-state gene expression levels in each cell line. The second consisted of WT and p38 α -/- MEFs expressing an OHT-inducible form of H-Ras^{G12V} (ER-HRas^{G12V}) [14]. This system allowed us to monitor gene expression in both cell lines in the absence of H-Ras^{G12V} signalling as well as early (24 h) after its activation with OHT (Figure 2A).

We found that the majority of the genes analyzed by semi-quantitative RT-PCR were deregulated between stable H-Ras^{G12V}-expressing WT and p38 α -/- MEFs (Figures 2B and 2C, two right lanes) in a way that mirrored the microarray results in more than 90% of the cases (note microarray fold change column to the right). However, the differential expression levels of *Ralgds*, *Rtn4* and *Ctsl* in H-Ras^{G12V}-WT versus H-Ras^{G12V}-p38 α -/- MEFs were less clear than expected from the microarray results. This might be related to sensitivity limitations of the semi-quantitative RT-PCR technique, at least for *Rtn4* (+2.2) and *Ctsl* (+2.4), since their moderate upregulation was detectable by qRT-PCR (see below Figures 3 and 4A). Of note, we could not detect expression of *Stac2*, *Rom1*, *Dpep1*, *Traf1* and *Lzts2*.

Concerning the remaining 26 genes, analysis of ER-HRas^{G12V}-expressing WT and p38 α -/- MEFs revealed that the expression of half of them was dependent on p38 α (Figure 2B, first and third lines marked with asterisks), whereas the other half were additionally influenced by H-Ras^{G12V} signalling (Figure 2C, second and fourth lanes marked with asterisks). Interestingly, the effect of H-Ras^{G12V} on gene expression in the latter case was already clear 24 h after the onset of H-Ras^{G12V} signalling and was usually maintained during the transformation process, in MEFs stably expressing H-Ras^{G12V}. However, the relative mRNA abundance of some genes in H-Ras^{G12V}-expressing WT versus p38 α -/- MEFs was attenuated (*Ralgds*, *Ctsl*) or enhanced (*Cd9*, *Ctsh*) with time, arguing for a kinetic component in the regulation of gene expression by p38 α and H-Ras^{G12V}. This could in principle be due either to specific changes in p38 α or H-Ras^{G12V} signalling or to non-specific alterations or selective pressure related to the transformed phenotype. For example *Cxcl1*, *Nck2* and *Tnfrsf23* showed opposite expression patterns in early transformed versus established cell lines, whereas *Rtn4* and *Snf1k* showed opposite regulation in non-transformed versus H-Ras^{G12V}-expressing cells. Interestingly, p38 α reconstitution into p38 α -/- MEFs rescued the expression levels of putative p38 α -regulated genes to about WT levels in both non-transformed (Figure 2D) and transformed cells (Supplementary Figure S2). Taken together, the results suggest that, at least the selected genes that were analysed are indeed regulated by p38 α , although some of them can be additionally modulated by the cellular context (e.g. non-transformed versus transformed cells and early versus late transformation stages).

Gene expression validation by qRT-PCR in cultured cells and mouse tumors

In order to evaluate the relevance of the observed gene expression differences between WT and p38 α -/- MEFs for *in vivo* tumorigenesis, selected genes were further analyzed by qRT-PCR in subcutaneous tumors obtained from the injection of H-Ras^{G12V}-expressing WT and p38 α -/- MEFs in nude mice [14], as well as in lung tumors induced by K-Ras^{G12V} expression in WT and p38 α -/- mice [12]. We found that all the genes analyzed were similarly deregulated in a p38 α -dependent manner in transformed MEFs, MEF-derived subcutaneous tumors and K-Ras^{G12V}-induced lung tumors, in accordance with the original results obtained from the microarray analysis (Figure 3). Although the microarray-based profiling and qRT-PCR analysis did not result in identical quantitative changes, gene expression fold changes were qualitatively conserved in the different tumor models tested (Figure 3 and Supplementary Table 7). These results link the p38 α -mediated modulation of particular mRNAs with the ability of p38 α to regulate tumorigenesis.

Functional validation of genes deregulated between H-Ras^{G12V}-transformed WT and p38 α -/- MEFs

Next, we selected 9 out of the 26 genes mentioned above, according to their level of deregulation in H-Ras^{G12V}-expressing p38 α -/- MEFs and their putative connection with the process of cell transformation, to perform functional studies (Table 1). Three genes encoded membrane (*Aig1*) or extracellular matrix (*Mmp3*, *Ctsh*) proteins and were selected due to their acute deregulation in H-Ras^{G12V}-expressing p38 α -/- versus WT MEFs. Little is known about *Aig1* (androgen-induced 1) in the context of malignant transformation. In contrast, *Mmp3* (matrix metalloproteinase 3 or stromelysin-1) and *Ctsh* (Cathepsin H) are known to be involved in tumor progression (Table 1). Four other genes encoded the tyrosine kinase signalling-modulating proteins *Cd9* and *Nck2*, the proliferation-regulating protein *Pmp22* and the pleiotropic protein *Rtn4*, and were all upregulated in the more transformed H-Ras^{G12V}-p38 α -/- MEFs, suggesting that enhanced expression of these genes might contribute to transformation. Accordingly, some of them have been described to be upregulated in tumors and potentially associated with tumor progression (Table 1). Finally, two genes that were down-regulated in H-Ras^{G12V}-p38 α -/- MEFs, namely *Gstm2* and *Egln3*, were also considered good candidates to test functionally due to their reported roles in oxidative stress and apoptosis, respectively (Table 1).

We have previously reported that the absence of p38 α enhances H-Ras^{G12V}-induced anchorage-independent growth [14]. Thus, genes found up-regulated in H-Ras^{G12V}-p38 α -/- MEFs were over-expressed in H-Ras^{G12V}-WT MEFs (Table 1) to test if they could enhance the ability of transformed cells to grow in soft agar. Proliferation rates were also measured in parallel, and over-expression was confirmed by immunoblotting or RT-PCR (Supplementary Figure S3A), depending on the availability of antibodies. Interestingly, *Mmp3*, *Cd9* and *Rtn4* enhanced the ability of H-Ras^{G12V}-WT MEFs both to proliferate and to grow anchorage-independently (Table 1 and Supplementary Figures S3B, S3C and S4). Similarly, *Nck2* stimulated the proliferation of H-Ras^{G12V}-WT MEFs to p38 α -/- levels, although it did not affect H-Ras^{G12V}-induced soft agar growth (Table 1 and Supplementary Figures S3B and S4). These results suggest that p38 α might regulate different traits of the cancer cell (i.e. proliferation and anchorage-independent growth) by specifically targeting the expression of distinct genes in each case. Overall, it appears that the down-regulation of these four genes by p38 α may contribute to its inhibitory effect on H-Ras^{G12V}-induced transformation of MEFs. In contrast, no conclusions could be drawn on *Aig1* since we did not manage to express this gene (data not shown), whereas *Pmp22* unexpectedly decreased, rather than enhanced, the transformed phenotype of H-Ras^{G12V}-WT MEFs (Supplementary Figures S3D and S4). This would agree with the pro-apoptotic and anti-proliferative functions ascribed to *Pmp22* (Table 1), but raises the question of whether the endogenous *Pmp22* is actually functional in p38 α -/- MEFs, since *Pmp22* over-expression in these cells (where it is already upregulated) attenuates their transformed phenotype as well (Supplementary Figure S3D).

In a complementary set of experiments, we over-expressed in H-Ras^{G12V}-p38 α -/- MEFs three genes found up-regulated in H-Ras^{G12V}-WT cells (Table 1) and tested if they could inhibit transformation. We found that *Egln3* attenuated the proliferation rate and the ability to grow in soft-agar of H-Ras^{G12V}-p38 α -/- MEFs to about H-Ras^{G12V}-WT levels (Supplementary Figure S5A, S5B and 5C), in agreement with its lower expression levels in p38 α -/- MEFs (Supplementary Table 6) and its reported pro-apoptotic activity (Table 1). In contrast, *Ctsh* affected neither proliferation nor soft agar colony formation in H-Ras^{G12V}-p38 α -/- MEFs (Table 1 and Supplementary Figures S5A and S5B). This is in agreement with the putative matrix remodelling function of *Ctsh*, which is likely to regulate tumor cell invasion rather than cell proliferation or anchorage-independent growth. Of note, *Gstm2* did not significantly affect H-Ras^{G12V}-p38 α -/- MEFs, but enhanced the proliferation (Supplementary Figure S5D) and soft agar growth [14] of H-Ras^{G12V}-WT MEFs. This apparent paradox could be explained if one considers that *Gstm2* over-expression interferes with the activation and subsequent pro-apoptotic function of p38 α in H-Ras^{G12V}-expressing cells [14]. Mechanistically, this involves

Gstm2 binding to ASK1, an upstream activator of p38 α , which in turn blocks the activation of the pathway by H-Ras^{G12V}-induced reactive oxygen species (ROS). Thus, cells over-expressing H-Ras^{G12V} and Gstm2 bypass the inhibitory apoptotic response mediated by p38 α , allowing the accumulation of high levels of ROS, which enhances malignant transformation. Since Gstm2 over-expression targets p38 α , it is not surprising that it has no effect on H-Ras^{G12V}-p38 α ^{-/-} MEFs.

Regulation of EGFR signalling by transcriptional targets of p38 α in malignant transformation

Experiments using mouse models of cancer suggests that EGFR transcription could be targeted by p38 α for tumorigenesis regulation *in vivo* [12], in agreement with studies using cultured cells [20-22]. Moreover, EGFR activity is enhanced in p38 α -deficient MEFs and contributes to the more transformed phenotype of H-Ras^{G12V}-p38 α ^{-/-} MEFs [15]. We found a set of 19 genes that were deregulated between H-Ras^{G12V}-expressing WT and p38 α ^{-/-} MEFs and were linked to EGFR signalling (Table 2). Most of these genes encode proteins that positively regulate EGFR signalling and were up-regulated in H-Ras^{G12V}-p38 α ^{-/-} MEFs, indicating negative regulation by p38 α . These include the plasma membrane protein Cd9 that stimulates EGFR activity by facilitating the interaction of the receptor with its ligands, the mitogenic EGFR ligand Areg previously associated with malignant cell transformation, the transcription factor Klf5 that stimulates EGFR expression, the Src-family kinase Hck that participates in EGFR recycling and signal relay as well as in cell survival, and the tyrosine kinase substrate Eps8 that amplifies EGFR signalling following mitogenic stimulation (Table 2). Validation by qRT-PCR confirmed the differential expression of these genes in H-Ras^{G12V}-p38 α ^{-/-} versus H-Ras^{G12V}-WT MEFs (Figure 4A), as predicted from the microarray results.

To strengthen the implication of the 19-gene dataset in the regulation of EGFR signalling, we performed Gene set enrichment analysis (GSEA). We found that our set of p38 α -regulated genes aligned with the EGFR-associated gene signature (GEO ID:GSE9599) of a study correlating the sensitivity of human pancreatic tumor cell lines to the EGFR inhibitor erlotinib with the expression of an EGFR-associated gene network that sustains high levels of EGFR activity [17]. Interestingly, GSEA revealed that our 19-gene cluster was significantly enriched in erlotinib-sensitive pancreatic tumors (FDR q-value = 0.165) (Supplementary Figure S6) correlating with high EGFR activity. This result supports the importance of EGFR signalling inhibition by p38 α in the regulation of cell transformation.

At the mechanistic level, we confirmed that Cd9 over-expression in H-Ras^{G12V}-WT MEFs not only enhanced their transformed phenotype to H-Ras^{G12V}-p38 α ^{-/-} levels (Supplementary Figures S3B and S3C), but also activated EGFR kinase activity, as indicated by higher levels of phospho-EGFR^{Y1068} (Figure 4B, left) and its downstream signalling pathways ERK1/2 and AKT (Figure 4B, right). This argues that p38 α negatively regulates cell transformation, at least in part, by inhibiting EGFR via down-regulation of several genes that stimulate its activity or downstream signal relay. Of note, the interplay between EGFR and p38 α in cell transformation might not be limited to a cluster of EGFR-regulating genes, but also involve direct regulation of the EGFR mRNA by p38 α . We found that the EGFR mRNA was up-regulated in the absence of p38 α in three different cellular systems: H-Ras^{G12V}-transformed MEFs growing exponentially, subcutaneous xenografts derived from H-Ras^{G12V}-expressing MEFs and K-Ras^{V12}-induced lung tumors in mice (Figure 4C).

DISCUSSION

Cancer is a genetic disease characterized by the simultaneous disruption of several cellular traits required for organism homeostasis. At the molecular level, the combination of deregulated cell proliferation and reduced apoptosis is thought to set the founding for

neoplastic progression. Accordingly, the ability of p38 α to engage cell cycle arrest and apoptosis has been shown to play an important tumor suppressor role [3, 4]. p38 α counteracts the transforming activity of oncogenic Ras in several cellular and animal models through various mechanisms, most of them occurring at the post-translational level [11, 12, 14, 23]. However, cancer progression is normally accompanied by profound alterations in gene expression, which prompted us to investigate transcriptional targets of p38 α in the regulation of tumorigenesis. We have found a number of genes not previously connected to p38 α signalling that are likely to mediate its negative effect on H-Ras^{G12V}-induced transformation. Intriguingly, our analysis has also identified various p38 α -induced genes that are related to cell migration, invasion and angiogenesis, which may belong to a family of p38 α effectors mediating some of its less characterized pro-oncogenic functions [24]. In fact, emerging evidence suggest that p38 α may have oncogenic rather than tumor suppressive functions in some advanced human tumors [4]. Further studies are needed to understand the cellular contexts in which p38 α can either suppress or promote tumor formation. We report here a set of p38 α -regulated genes that are associated with tumor suppressor functions of p38 α in different tumor models, and may be useful to identify human cancers where p38 α negatively regulates tumor formation.

New p38 α effectors in the regulation of cell survival and proliferation

Down-regulation of p38 α enhances H-Ras^{G12V}-induced transformation of MEFs, as indicated by their increased proliferation and survival, refringent morphology, loss of contact inhibition and anchorage-independent growth [14]. Not surprisingly, we have found that genes stimulating cell proliferation and survival are negatively regulated by p38 α including *Ralgds*, a Ras effector that promotes cell survival [25], and *Tnfrsf23*, a putative tumor necrosis factor receptor decoy that inhibits apoptosis [26]. Conversely, apoptosis-stimulating genes such as *Egln3* [27] are up-regulated in the less transformed H-Ras^{G12V}-WT MEFs, in line with the importance of the apoptotic activity of p38 α for tumor suppression [14]. Of note, p38 α seems to regulate *Egln3* expression at both transcriptional and post-translational levels [28]. It is possible that other genes found in our study are also post-transcriptionally regulated by p38 α , for instance through the well known p38 α -MK2-mediated stabilization of mRNAs containing AU-rich 3'UTRs [29].

Potential membrane targets of p38 α in cell transformation

Apart from survival and proliferation, p38 α can also regulate other traits of the cancer cell, such as cell morphology, contact inhibition and anchorage-independent growth, which are likely regulated by signals relid from plasma membrane proteins [14, 15]. Previous attempts to identify proteins that were differentially expressed in the membranes of H-Ras^{G12V}-expressing WT and p38 α ^{-/-} MEFs had limited success [13]. Interestingly, we now report several p38 α -regulated genes encoding integral-to-membrane or membrane-associated proteins that may be involved in such processes (Supplementary Table 5). These include *Ezr*, encoding the cytoskeleton protein Ezrin known to be important for the regulation of cell-cell contact inhibition and tumorigenesis [30], as well as various genes encoding transmembrane proteins such as tetraspanin Tspan13 or protocadherins Pcdh7 and Pcdh10. Of note, certain protocadherins (i.e. Fat1) are emerging as important regulators of contact inhibition [31], a process where p38 α plays a key role [15]. Future studies should unveil whether these proteins may link p38 α signalling with the regulation of membrane-mediated cellular processes associated to malignant transformation.

The EGFR pathway as a transcriptional target of p38 α in cell transformation

A striking finding of our studies has been the identification of 19 genes (Table 2) that impinge on EGFR signalling and whose expression seems to be co-ordinately regulated by p38 α to inhibit malignant transformation. These include proteins that enhance EGFR expression (*Klf5*), activation (*Hck*, *Cd9*, *Prkca*, *Areg*) and downstream signalling (*Nck2*, *Limk2*, *Mmp3*) or that regulate EGFR intracellular trafficking (*Ctsl*, *Lamp2*, *Arfgap3*, *Cdc42ep5*).

Interestingly, we have shown that Cd9 over-expression not only stimulates EGFR signalling in H-Ras^{G12V}-WT cells but also enhances transformation to H-Ras^{G12V}-p38 α -/- levels. The ability of p38 α to down-regulate EGFR signalling at various levels may also contribute to the inhibitory role of p38 α in Ras-induced transformation, since Ras has been shown to require EGFR signalling for full transformation [32, 33].

We have recently reported that p38 α can inhibit EGFR at the post-translational level upon cell-cell contact [15], preventing cell overgrowth at high cellular densities. The relative contribution of transcriptional versus post-translational regulation to the overall effect of p38 α on EGFR signalling is unclear and may depend on the transformed state of the cell, the cell cycle phase and the scope of the response induced by p38 α . For instance, p38 α regulates EGFR mRNA levels in proliferating, H-Ras^{G12V}-transformed cells, but no differences were found in EGFR mRNA abundance between contact-inhibited, non-transformed WT and p38 α -/- MEFs [15]. It is possible that the post-translational regulation of EGFR by p38 α in contact inhibition is aimed at eliciting a fast response, whereas the long-term inhibition of H-Ras^{G12V} transformation by p38 α requires tuning the steady-state expression levels of genes encoding proteins, which in turn impinge on EGFR signalling.

In summary, we have identified and validated in different models of tumorigenesis several p38 α targets that are functionally implicated in transformation. A significant number of the genes that are down-regulated by p38 α at the mRNA level encode positive regulators of EGFR signalling, suggesting that inhibition of this pathway probably represents an important tumor suppressor function of p38 α .

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Table 1 Genes differentially expressed between H-Ras^{G12V}-p38 α -/- and H-Ras^{G12V}-WT MEFs that were selected for functional validation

<i>Gene symbol</i> (NCBI Acc.No.)	<i>n-fold change</i> *	<i>Encoded protein</i>	<i>Functions related to cell transformation</i> ¹	<i>Proliferation</i> ²	<i>Soft agar</i> ³
<i>Aig1</i> (NM_025446)	+604.44	Androgen induced-1	Unknown	Not expressed	Not expressed
<i>Mmp3</i> (NM_010809)	+23.39	Matrix metalloproteinase 3 (MMP3) / stromolysin (Str)-1	Matrix breakdown [1]; pro-invasive, proliferative and anti-apoptotic activities [2, 3]	Enhanced in WT	Enhanced in WT
<i>Cd9</i> (NM_007657)	+5.19	CD9 antigen / motility related protein (Mrp)-1	Proliferation and tumor development through EGFR activation [4, 5]	Enhanced in WT	Enhanced in WT
<i>Pmp22</i> (NM_008885)	+4.60	Peripheral myelin protein / growth arrest specific (Gas)-3	Anti-proliferative and pro-apoptotic activity [6, 7]	Reduced in p38 α -/-	Reduced in p38 α -/-
<i>Nck2</i> (NM_010879)	+2.36	Non-catalytic region of tyrosine kinase adaptor protein 2 / Grb4 / Nck β	Proliferation and tumor progression by linking receptor tyrosine kinases to downstream signaling pathways [8-10]	Enhanced in WT	No effect
<i>Rtn4</i> (NM_024226)	+2.22	Reticulon 4	Tumor cell survival [11]	Enhanced in WT	Enhanced in WT
<i>Gstm2</i> (NM_008183)	-3.01	Glutathione S-transferase, mu 2	Oxidative stress, inhibition of apoptotic pathways [12, 13]	Enhanced in WT	Enhanced in WT
<i>Egln3</i> (NM_028133)	-6.29	EGL nine homolog 3, PHD3	Pro-apoptotic activity [14]	Reduced in p38 α -/-	Reduced in p38 α -/-
<i>Ctsh</i> (NM_007801)	-23.19	Cathepsin H	Proteolysis; tumor progression [15]	No effect	No effect

* microarray-based n-fold change ($2^{\log_2 \text{ratio}}$) in H-Ras^{G12V}-p38 α -/- versus H-Ras^{G12V}-WT MEFs.

¹ references are provided in Supplementary material

² effect of gene overexpression on H-Ras^{G12V}-induced cell proliferation (see Suppl. Fig. S3-S5).

³ effect of gene overexpression on H-Ras^{G12V}-induced soft agar growth (see Suppl. Fig. S3, S4).

Table 2 EGFR pathway-modulating genes that are regulated by p38 α

	<i>n</i> -fold change*	Gene symbol (NCBI Acc.No.)	Gene description	Effect ¹
Downregulated by p38 α	+10.41	<i>Hck</i> (NM_010407)	Hematopoietic cell kinase	EGFR activation and signal rely [1]
	+7.05	<i>Areg</i> (NM_009769)	Amphiregulin	EGFR ligand [2]
	+5.19	<i>Cd9</i> (NM_007657)	CD9 antigen	EGFR paracrine activation [3]
	+3.83	<i>Klf5</i> (NM_009769)	Kruppel-like factor 5	EGFR transcription [4]
	+2.03	<i>Eps8</i> (NM_007945)	EGFR pathway substrate 8	EGFR signaling [5]
	+1.55	<i>Prkca</i> (NM_011101)	Protein kinase C, alpha	EGFR activation and signal rely [6]
	+1.84	<i>Limk2</i> (NM_010718)	LIM motif-containing protein kinase 2	EGFR effector [7]
	+3.11	<i>Arfgap3</i> (NM_025445)	ADP-ribosylation factor GTPase activating protein 3	EGFR endocytosis [8]
	+2.36	<i>Nck2</i> (NM_010879)	Non-catalytic region of tyrosine kinase adaptor protein 2	EGFR SH2-domain adaptor protein [9]
	+2.47	<i>Ctsl</i> (NM_009984)	Cathepsin L	EGFR recycling [10]
	+2.37	<i>Cdc42ep5</i> (NM_026772)	Cdc42 effector protein (Rho GTPase binding) 5	EGFR degradation [11]
	+201.81	<i>Anxa8</i> (NM_013473)	Annexin A8	EGFR internalization [12]
	+2.04	<i>Lamp2</i> (NM_010685)	Lysosomal membrane glycoprotein 2	EGFR endocytosis [13]
	+23.40	<i>Mmp3</i> (NM_010809)	Matrix metalloproteinase 3	EGFR downstream signaling [14]
	+7.63	<i>Ralgds</i> (NM_009058)	Ral guanine nucleotide dissociation stimulator	EGFR signaling [15]
Upregulated by p38 α	-7.92	<i>Hoxa5</i> (NM_010453)	Homeo box A5	transcription of EGFR regulatory proteins [16]
	-4.63	<i>Traf1</i> (NM_009421)	TNFR-associated factor 1	EGFR expression [17]
	-3.29	<i>Aqp5</i> (NM_009701)	Aquaporin 5	EGFR signaling [18]
	-5.36	<i>Plcg2</i> (BCO23877)	Phospholipase C, gamma 2	EGFR signal rely [19]
	-25.18	<i>Cxcl1</i> (NM_021704)	Chemokine (C-X-C motif) ligand 1	EGFR signaling [20]

* microarray-based *n*-fold change ($2^{\log_2 \text{ratio}}$) in H-Ras^{G12V}-p38 α ^{-/-} versus H-Ras^{G12V}-WT MEFs.

¹ references are provided in Supplementary material

Figure Legends

Figure 1 Functional classification of genes differentially expressed between H-Ras^{G12V}-p38 α -/- and H-Ras^{G12V}-WT MEFs

Genetic deletion of p38 α significantly affected the expression of 202 genes. The number of genes within each functional category, either up-regulated (black) or down-regulated (white) in H-Ras^{G12V}-p38 α -/- MEFs, is indicated as a percentage of the total number of genes.

Figure 2 Semi-quantitative RT-PCR validation of genes differentially regulated by p38 α and/or H-Ras^{G12V}

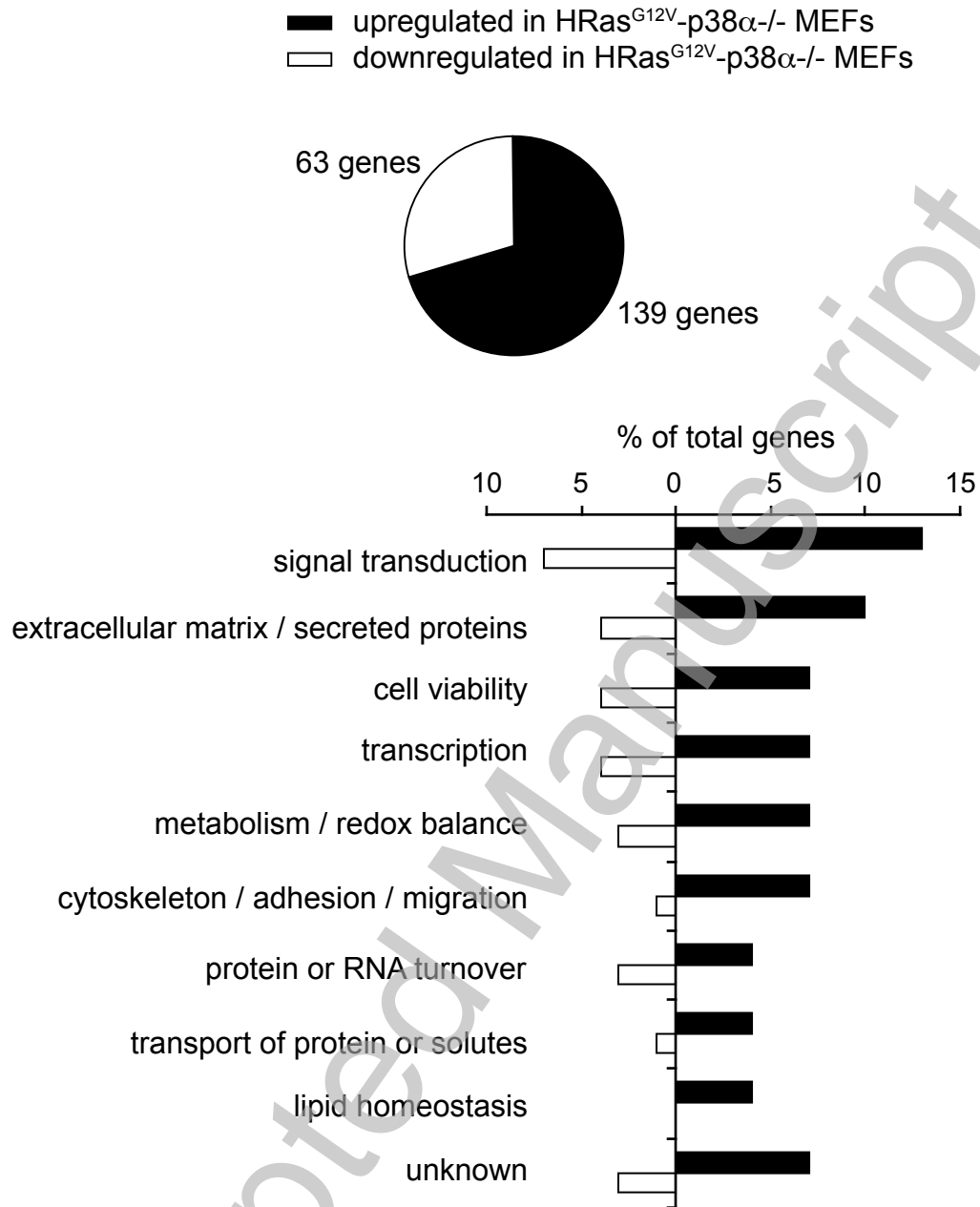
(A) Immunoblot analysis of cell extracts from ER-HRas^{G12V}-expressing WT and p38 α -/- MEFs stimulated with 1 μ M OHT for 24 h. (B) WT and p38 α -/- MEFs expressing ER-HRas^{G12V} were treated with OHT, as indicated. Established H-Ras^{G12V}-expressing p38 α -/- and WT MEFs were analysed in parallel. Asterisks indicate changes related to p38 α deficiency, but independently of H-Ras^{G12V} expression. Up and Down indicate mRNAs up-regulated or down-regulated, respectively, in established H-Ras^{G12V}-p38 α -/- MEFs according to the microarray analysis; FC, fold change in the microarray analysis. (C) Genes differentially expressed in MEFs by the combined effect of both p38 α and H-Ras^{G12V}. Samples as in (B). Asterisks indicate changes caused mainly by H-Ras^{G12V} expression. (D) Changes in gene expression were confirmed by analysing WT, p38 α -/- and p38 α add-back p38 α -/- MEFs. Immunoblot analysis is shown at the bottom. The experiments were performed at least three times with similar results.

Figure 3 Quantitative RT-PCR (qRT-PCR) analysis of gene expression in various tumor models

qRT-PCR was performed on RNA extracted from exponentially proliferating, H-Ras^{G12V}-expressing WT and p38 α -/- MEFs (light gray), subcutaneous xenografts derived from the same cells grown in nude mice for two weeks (white), or K-Ras^{G12V}-induced lung tumors in WT and p38 α -/- mice (dark grey). qRT-PCR results were compared with the respective microarray fold change value (black). Asterisks indicate fold changes greater than 28. All values together with their corresponding standard deviations are listed in Supplementary Table 7.

Figure 4 Transcriptional regulation of EGFR signalling by p38 α in oncogenesis

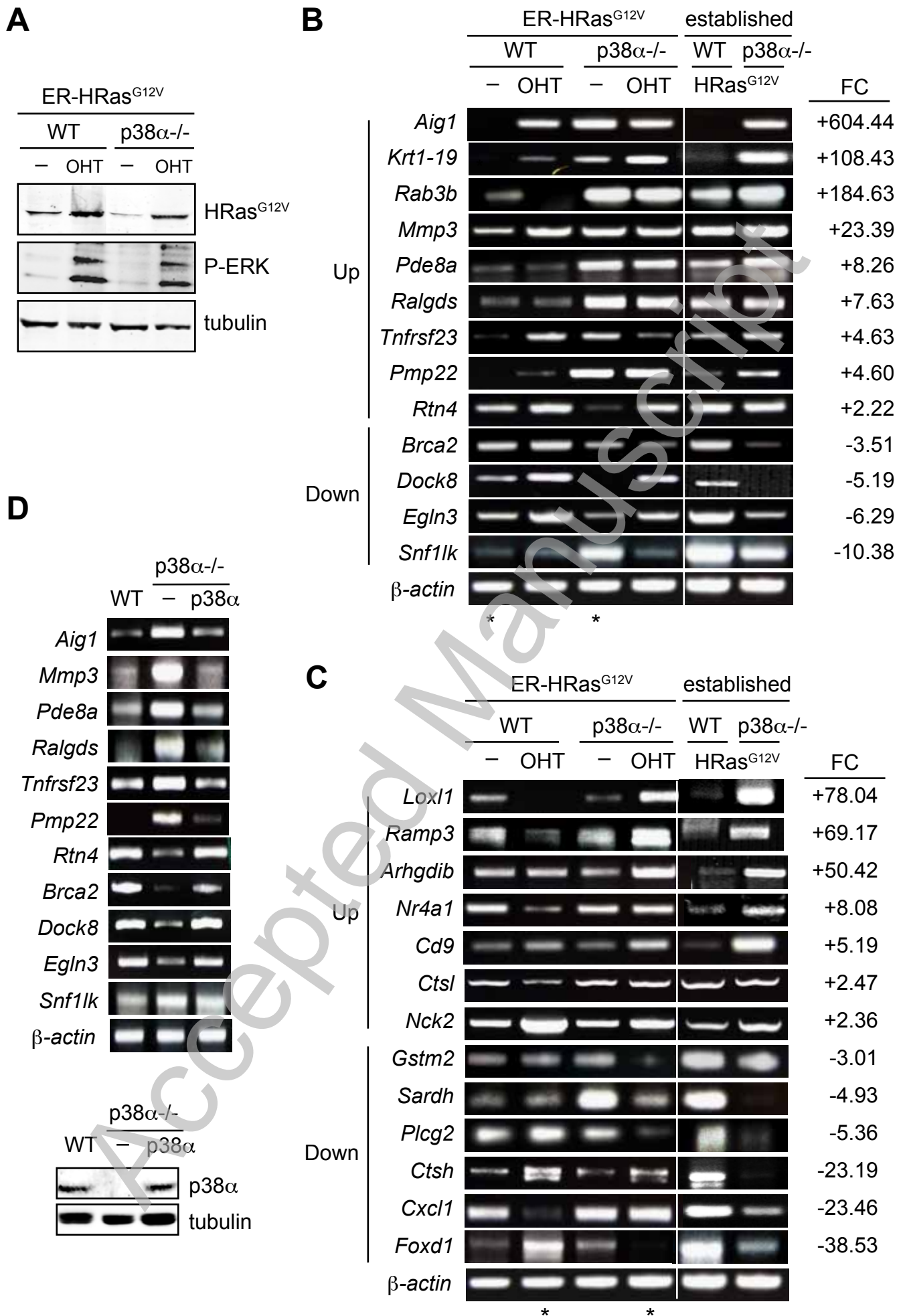
(A) EGFR signalling-related genes were validated by qRT-PCR in exponentially proliferating H-Ras^{G12V}-p38 α -/- versus H-Ras^{G12V}-WT MEFs and fold changes (white) were plotted along with the microarray fold changes for each gene (black). (B) WT MEFs were transduced with H-Ras^{G12V}, Cd9 or empty vectors (-), as indicated, and whole cell lysates were analyzed by immunoblotting with the indicated antibodies either directly (right) or after immunoprecipitation with EGFR antibodies (left). (C) qRT-PCR analysis of EGFR mRNA in exponentially proliferating H-Ras^{G12V}-expressing p38 α -/- versus WT MEFs (black), in tumor xenografts derived from the same MEFs and grown for two weeks in nude mice (grey), and in lung tumors induced by K-Ras^{G12V} expression in p38 α -/- mice versus WT mice (white).

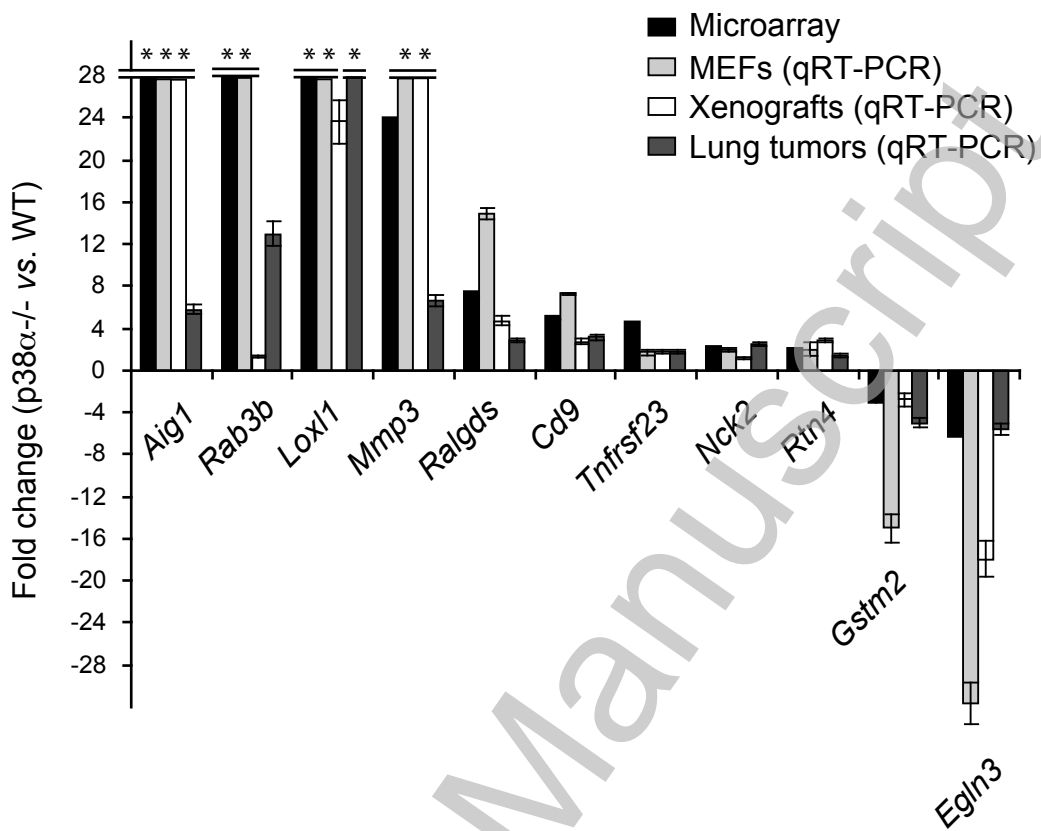


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