Dual-specificity phosphatase 5 regulates nuclear ERK activity and suppresses skin cancer by inhibiting mutant Harvey-Ras ($HRas^{Q61L}$)-driven SerpinB2 expression

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Edited* by Melanie H. Cobb, University of Texas Southwestern Medical Center, Dallas, TX, and approved November 17, 2014 (received for review October 21, 2014)

Ectopic expression of dual-specificity phosphatase 5 (DUSP5), an inducible mitogen-activated protein (MAP) kinase phosphatase, specifically inactivates and anchors extracellular signal-regulated kinase (ERK)1/2 in the nucleus. However, the role of endogenous DUSP5 in regulating the outcome of Ras/ERK kinase signaling under normal and pathological conditions is unknown. Here we report that mice lacking DUSP5 show a greatly increased sensitivity to mutant Harvey-Ras ($HRas^{Q61L}$)-driven papilloma formation in the 7,12-Dimethylbenz[a]anthracene/12-O-tetradecanoylphorbol-13-acetate (DMBA/TPA) model of skin carcinogenesis. Furthermore, mouse embryo fibroblasts (MEFs) from $DUSP5^{-/-}$ mice show increased levels of nuclear phospho-ERK immediately after TPA stimulation and fail to accumulate total ERK in the nucleus compared with $DUSP5^{+/+}$ cells. Surprisingly, a microarray analysis reveals that only a small number of Ras/ERK-dependent TPA-responsive transcripts are up-regulated on deletion of DUSP5 in MEFs and mouse skin. The most up-regulated gene on DUSP5 loss encodes SerpinB2, an inhibitor of extracellular urokinase plasminogen activator and deletion of DUSP5 acts synergistically with mutant $HRas^{Q61L}$ and TPA to activate ERK-dependent SerpinB2 expression at the transcriptional level. SerpinB2 has previously been implicated as a mediator of DMBA/TPA-induced skin carcinogenesis. By analyzing $DUSP5^{-/-}$, $SerpinB2^{-/-}$ double knockout mice, we demonstrate that deletion of SerpinB2 abrogates the increased sensitivity to papilloma formation seen on DUSP5 deletion. We conclude that DUSP5 performs a key nonredundant role in regulating nuclear ERK activation, localization, and gene expression. Furthermore, our results suggest an in vivo role for DUSP5 as a tumor suppressor by modulating the oncogenic potential of activated Ras in the epidermis.

DUSP5 | MAP kinase | protein phosphatase | SerpinB2 | skin cancer

Dual-specificity phosphatase 5 (DUSP5) is one of four mammalian inducible, nuclear mitogen-activated protein kinase (MAPK) phosphatases (MKPs) (1). However, DUSP5 is unique within this group in targeting only the classical extracellular signal-regulated kinases 1 and 2 (referred to hereafter as ERK) (2). This, coupled with the finding that ERK activation is required for inducible DUSP5 expression, indicates that it acts as a negative feedback regulator of nuclear Ras/ERK signaling (3). DUSP5 overexpression also leads to nuclear accumulation of endogenous ERK (2), suggesting that DUSP5 may also act as a nuclear anchor, thus regulating both the spatial organization and activity of the pathway (4).

Ras/ERK signaling is frequently deregulated in human cancers due to activating mutations in pathway components such as growth factor receptors, Ras GTPases, and the MAPK kinase kinase, BRaf (5). *BRaf* is mutated in 40–60% of malignant melanomas as well as in thyroid, colorectal, and lung tumors, underscoring the importance of this pathway and making it a focus of anticancer drug development (6). Whereas mechanisms of Ras/MAPK pathway activation in cancer are understood, little is known about how negative feedback controls influence tumorigenesis (7). Studies suggest that MKP-mediated negative feedback is a major determinant of pathway activity in Ras/ERK-driven cancers (8). Increased MKP levels are detected in a variety of cancer cells with activated KRas or BRaf and are presumed to suppress ERK activity (9–12). Ectopic expression of DUSP5 in lung and colon cancer cells lowers ERK activity and suppresses growth (13). Furthermore, in stomach cancer, low DUSP5 expression correlates with poor prognosis and its reexpression reduces both cell growth and colony-forming ability in vitro (14). Taken together, these observations suggest that DUSP5 may act as a tumor suppressor. However, ERK signaling can also exert tumor suppressor activity by promoting cellular senescence (15). Thus, in certain contexts, MKPs may promote cancer development.

Studies of MKPs in cancer rely on either correlations between expression levels and biological/clinical endpoints or on MKP overexpression (16). Thus, there is a need for genetic experiments to define the role(s) of these proteins in tumorigenesis. To address this need, we generated $DUSP5^{-/-}$ mice and examined their susceptibility to HRas-driven 7,12-Dimethylbenz[a]anthracene/12-O-tetradecanoylphorbol-13-acetate (DMBA/TPA)-induced skin cancer. We report that $DUSP5^{-/-}$ mice are sensitized to skin

Significance

Ras/extracellular signal-regulated kinase (ERK) signaling is implicated in human cancer development and progression. ERK activation also results in the expression of MAP kinase phosphatases (MKPs) that inactivate ERK. However, it is currently unclear how MKPs regulate the oncogenic potential of the Ras/ERK pathway. Using knockout mice, we identify the MKP encoded by dual-specificity phosphatase 5 (DUSP5) as both a key regulator of nuclear ERK activity and a tumor suppressor in the DMBA/TPA model of Harvey Ras (HRas)-induced skin carcinogenesis. DUSP5 loss results in increased HRas/ERK-inducible SerpinB2 expression, which causes increased skin cancer sensitivity. Our results establish a key role for DUSP5 in the regulation of oncogenic ERK signaling and suggest that this enzyme may play a wider role in tumors containing activated Ras.

Author contributions: L.K.R., C.J.C., and S.M.K. designed research; L.K.R., A.M.K., L.D., G.S., S.v.S., J.D., C.J.B., E.C., and C.J.C. performed research; L.K.R., A.M.K., P.E., C.J.C., and S.M.K. analyzed data; and L.K.R., C.J.C., and S.M.K. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

Freely available online through the PNAS open access option.

Data deposition: Microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE62433).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1420159112/-/DCSupplemental.

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papilloma induction, displaying a doubling of tumor multiplicity. Furthermore, TPA-treated *DUSP5*$^{-/-}$ fibroblasts display a significant elevation in nuclear phospho-ERK levels compared with wild-type (WT) cells. We further identify SerpinB2, a protein previously linked with susceptibility to DMBA/TPA-induced skin cancer, as the most up-regulated gene on DUSP5 loss, and we demonstrate that combining deletion of DUSP5 and SerpinB2 completely abrogates the increased sensitivity to carcinogenesis seen on DUSP5 loss alone. Our results demonstrate that DUSP5 plays a key role in the spatiotemporal regulation of Ras/ERK signaling in mammalian cells and that DUSP5 acts as a tumor suppressor in Ras-induced skin cancer by restraining the oncogenic potential of nuclear ERK signaling and gene expression.

Results

***DUSP5*$^{-/-}$ Mice Are Sensitive to DMBA/TPA-Induced Skin Carcinogenesis.** The murine DUSP5 gene was targeted in embryonic stem (ES) cells. Chimeric mice were obtained and crossed with Rosa26-Cre mice to yield the null allele (Fig. S1*A*). Crossing *DUSP5*$^{+/-}$ mice produced litters in which *DUSP5*$^{+/+}$, *DUSP5*$^{+/-}$, and *DUSP5*$^{-/-}$ knockout (KO) animals were born at Mendelian frequency and levels of DUSP5 mRNA and protein were consistent with genotype (Fig. S1 *B* and *C*). KO mice appeared normal and are fertile, demonstrating that *DUSP5* is not an essential gene.

To study Ras-induced tumors, we used two-stage chemical skin carcinogenesis (17). Cohorts of *DUSP5*$^{+/+}$ (WT), *DUSP5*$^{+/-}$ heterozygous (HET), and *DUSP5*$^{-/-}$ (KO) animals (n = 19 for each genotype) were treated with DMBA (50 μg) followed by twice weekly promotion with TPA (12.5 μg) for 25 wk. All three groups developed skin papillomas by 5–6 wk after TPA treatment, but HET and KO mice reached 100% tumor incidence slightly earlier than WT animals (Fig. 1*A*). KO mice developed more tumors than WT animals (Fig. 1*B*) and after 25 wk, KO mice had an average of 20 papillomas per animal compared with only 10 for WT mice. *DUSP5*$^{+/-}$ mice had an intermediate number, developing on average 15 tumors per animal. Controls treated with either DMBA or TPA alone developed no tumors after 25 wk, irrespective of genotype (n = 3 for each). To assess malig-nant conversion, cohorts were aged to 48 wk. However, conversion rates are very low in C57BL/6 mice (17) and no malignant lesions developed in WT, HET, or KO animals.

Epidermal architecture and expression of differentiation markers were normal in *DUSP5*$^{-/-}$ mice, indicating that increased tumor sensitivity is not due to defective skin structure (Fig. S2*A*). Furthermore, TPA-induced skin hyperplasia, proliferation, and phospho-ERK (p-ERK) staining were unaffected by DUSP5 loss (Fig. S2*B*). Histopathologically, tumors were defined as squamous cell papillomas and morphology and tumor size were similar in all three genotypes (Fig. 1 *C* and *D*). DMBA/TPA-induced cancer is driven by a signature mutation (CAA to CTA) at codon 61 in the HRas gene (HRas^{Q61L}) (18). DNA sequencing confirmed the presence of HRas^{Q61L} mutations in ∼90% of tumors, irrespective of genotype (Fig. 1*E*), indicating that DMBA-induced mutagenesis during tumor initiation is unaffected.

Finally, comparing HRas^{Q61L}- and HRas^{G12V}-induced transformation in WT and KO immortalized mouse embryo fibroblasts (MEFs), revealed no difference in the numbers of transformed foci produced (Fig. 1*F*). Overall, we conclude that DUSP5 acts as a potent tumor suppressor in this model of skin cancer with loss of one or both copies of *DUSP5* resulting in a significant increase in tumor multiplicity.

DUSP5 Loss Deregulates Nuclear ERK Signaling. Given that DUSP5 specifically targets ERK (2), we analyzed p-ERK levels in TPA-treated MEFs from WT, HET, and KO mice by immunoblotting. No significant changes in the levels or duration of ERK phosphorylation were seen in KO cells. As expected, DUSP5 loss did not affect phosphorylation of the p38 and JNK MAPKs. The

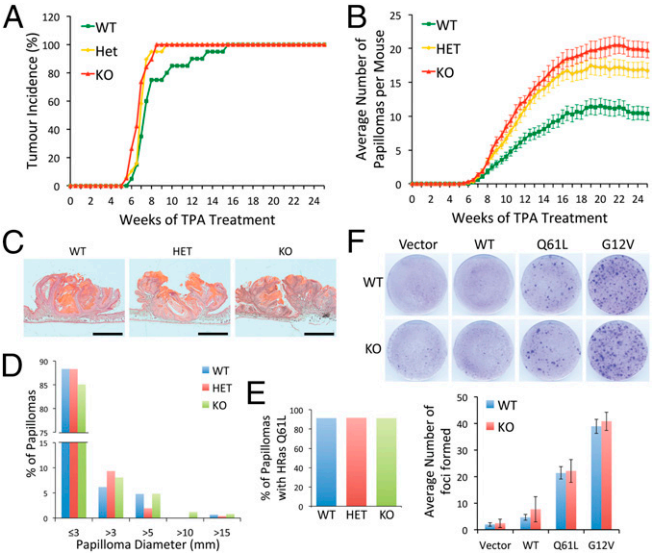

Fig. 1. *DUSP5*$^{-/-}$ mice are sensitive to DMBA/TPA-induced skin carcinogenesis. (*A*) Tumor incidence and (*B*) average number of tumors per mouse in wild-type (WT), *DUSP5*$^{+/-}$ (HET), and *DUSP5*$^{-/-}$ (KO) animals (n = 19 for each cohort) exposed to DMBA followed by TPA treatment for 25 wk. DUSP5 loss decreased latency (P values for WT/HET = 0.030460; WT/KO = 0.002957 by repeated measures multivariate ANOVA with Tukey's post hoc analysis) and increased tumor multiplicity (P values for WT/HET = 0.000761; WT/KO = 0.000002 by repeated measures multivariate ANOVA with Tukey's post hoc analysis). (*C*) H&E stained sections of representative skin papillomas from mice of each genotype at 25 wk. (Scale bar, 1 mm.) (*D*) Size distribution of tumors from mice of each genotype after 25 wk. (*E*) Percentage of tumors from mice of each genotype with HRas^{Q61L} mutations. (*F*) Numbers of transformed foci produced in immortalized wild-type (WT) and *DUSP5*$^{-/-}$ (KO) MEFs following expression of wild-type HRas (WT), HRas^{Q61L} (Q61L), or HRas^{G12V} (G12V) (n = 3, mean values ± SEM are shown). Representative images are shown (*Upper*).

levels of other MKPs (DUSP1, DUSP4, DUSP6, and DUSP9) were also unchanged, indicating that DUSP5 loss does not cause compensatory up-regulation of other MKPs known to inactivate ERK. Finally, we examined cell proliferation in primary MEFs of each genotype and found no significant differences in growth rate, even at limiting serum concentrations (Fig. S3 *A*–*C*).

As the nucleus represents only a fraction of total cell volume, changes in activity or distribution resulting from DUSP5 loss might be difficult to detect in whole cell lysates. We therefore performed fractionation of lysates from TPA-treated WT and KO MEFs. These experiments reveal a significant increase in the levels of nuclear, but not cytoplasmic, p-ERK in *DUSP5*$^{-/-}$ cells compared with WT (Fig. 2*A*). We also used confocal microscopy and high-content imaging of cells to visualize and quantitate the spatiotemporal regulation of ERK signaling in TPA-treated MEFs. These experiments reveal that DUSP5 loss has two effects. First, TPA stimulation results in higher nuclear p-ERK levels in KO MEFs compared with wild-type cells. Second, KO MEFs retain less total ERK in the nucleus (Fig. 2 *B* and *C* and Fig. S4*C*). In contrast, cytoplasmic ERK is unaffected.

To demonstrate that these effects are a direct result of DUSP5 loss, we used early growth response 1 (Egr1)-driven expression of Myc-tagged DUSP5, or a kinase interaction motif (KIM) mutant of DUSP5 to drive ERK-dependent inducible expression of both proteins in KO MEFs at endogenous levels (Fig. S4 *A* and *B*). The DUSP5 KIM mutant is catalytically active, but unable to bind to and inactivate ERK (2). Importantly, DUSP5, but not DUSP5 KIM, completely reverses the phenotypes observed in *DUSP5*$^{-/-}$ MEFs, with both levels of p-ERK and total ERK in the nucleus restored to those seen in WT cells (Fig. 2 *B* and *C* and Fig. S4*C*). We conclude that DUSP5 performs a nonredundant function in

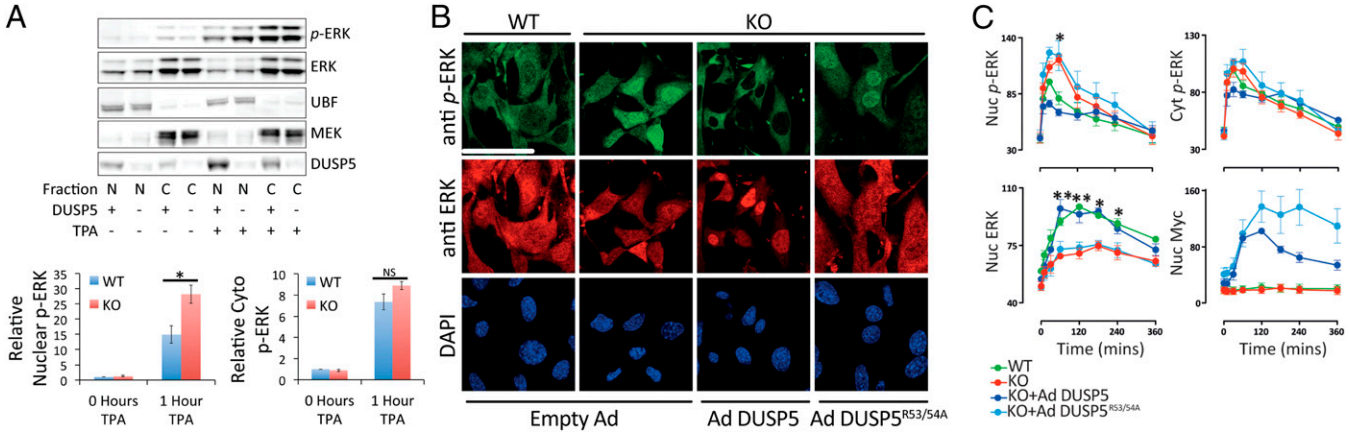

Fig. 2. DUSP5 loss increases nuclear phospho-ERK (p-ERK) after TPA stimulation. (*A*) WT and *DUSP5*$^{-/-}$ MEFs at 0 and 1 h after TPA treatment were lysed and fractionated before analysis by immunoblotting using the antibodies indicated. Upstream binding factor (UBF) and MEK were used to verify separation of nuclear (N) and cytoplasmic (C) proteins, respectively. p-ERK levels were quantified using a Li-Cor Odyssey infrared scanner. Mean values ± SEM are shown, $n = 5$. NS, not significant; $^{*}P < 0.05$ using Student *t* test (*Lower*) and a representative Western blot is shown (*Upper*). (*B*) Representative confocal images of KO MEFs are shown following infection with empty adenovirus (empty Ad), Ad Egr1 promoter-driven DUSP5-Myc (Ad DUSP5), or Ad Egr1 promoter-driven KIM mutant of DUSP5-Myc (Ad DUSP5$^{R53/54A}$) and stimulation with TPA for 1 h. (Scale bar, 60 μm.) (*C*) MEFs were infected with virus before TPA stimulation as indicated and stained for either p-ERK, total ERK, and DAPI or p-ERK, Myc, and DAPI before image analysis using high content fluorescence microscopy. Graphs represent population average values for nuclear (Nuc) and cytoplasmic (Cyt) p-ERK intensity (*Top*), nuclear (Nuc) total ERK intensity (*Bottom Left*), and Nuc Myc intensity (*Bottom Right*) derived from four separate experiments, each of which contained two to four replicate wells per condition (5,000–10,000 individual cells per condition). Mean values ± SEM are shown, $n = 4$. $^{*}P < 0.05$, $^{**}P < 0.01$ using two-way repeated measures ANOVA and Bonferroni post hoc test comparing WT and KO cells.

regulating both the activation and anchoring of nuclear ERK and that the loss of this function may contribute to increased carcinogenesis in *DUSP5*$^{-/-}$ mice.

DUSP5 Loss Affects TPA-Inducible Gene Expression. To determine the effects of DUSP5 loss on gene expression, we performed a microarray experiment comparing TPA-treated primary WT and KO MEFs derived from male littermate embryos at 0, 1 and 3 h. Data analysis revealed that only 20 genes showed altered expression when comparing KO and WT cells (Fig. 3*A*). Of these, 18 were up-regulated and 2 were down-regulated. Three members of the Egr transcription factor family were among the 18 up-regulated genes. However, the mRNA that increased the most in *DUSP5*$^{-/-}$ cells encoded SerpinB2 (also known as plasminogen activator inhibitor 2, PAI-2).

The increased expression of SerpinB2 mRNA and protein was confirmed by RT-qPCR analysis and Western blotting in an independent pair of TPA-treated WT and *DUSP5*$^{-/-}$ MEFs. This was ERK dependent, as mRNA levels were reduced to those seen in WT cells by either a MEK inhibitor (PD184352) or reexpression of DUSP5, but not DUSP5 KIM. (Fig. 3 *B* and *C*). Importantly, an increase in SerpinB2 mRNA was also seen in TPA-treated DUSP5$^{-/-}$ skin compared with WT tissue (Fig. 3*D*). RT-qPCR analysis confirmed the up-regulation of Egr1, Egr3, Egr4, DUSP2, sulfiredoxin 1 (Srxn1), and alcohol dehydrogenase 1A3 (Aldh1A3) in DUSP5$^{-/-}$ cells (Fig. S5*A*). Although DUSP2 expression increases, mRNA levels in MEFs are very low and we cannot detect DUSP2 protein in TPA-treated WT or *DUSP5*$^{-/-}$ MEFs.

We next examined the link between Egr transcription factors and expression of SerpinB2 and DUSP5. siRNA knockdown of Egr1, but not Egr3 or Egr4, reduced TPA-inducible SerpinB2 mRNA levels in both WT and *DUSP5*$^{-/-}$ MEFs, whereas ectopic expression of human Egr1 led to an increase in mRNA and protein (Fig. 3*E* and Fig. S5*B*). Similar results were obtained using WT and *Egr1*$^{-/-}$ MEFs (Fig. S5*C*), indicating that Egr1 may mediate SerpinB2 expression. We used SerpinB2 promoter–reporter constructs (19) to analyze TPA-induced transcription in WT and KO MEFs, finding that reporter activity was greatly increased in KO MEFs compared with WT cells (Fig. S5*D*). TPA-inducible reporter activity was also reduced following Egr1 knockdown (Fig. 3*F*), despite the absence of Egr1 DNA-binding sites in the SerpinB2 promoter. Reporter constructs lacking specific transcription factor binding sites revealed that two AP1 sites and a cAMP responsive element (CRE) mediate this activity, indicating that Egr1 acts indirectly on SerpinB2 expression (Fig. 3*F*). In the case of DUSP5, either siRNA knockdown or Egr1 deletion reduced basal levels of DUSP5 mRNA and protein, whereas ectopic expression of Egr1 increased SerpinB2 expression (Fig. 3*E* and Fig. S5*E*). Thus, DUSP5 may act to reduce ERK-dependent Egr1 expression as part of a negative feedback loop to regulate its own expression.

HRas^{Q61L} Synergizes with DUSP5 Loss to Increase Levels of Nuclear p-ERK and SerpinB2 Expression. As HRas^{Q61L} drives DMBA/TPA-inducible tumorigenesis, we determined the effect of HRas^{Q61L} on both p-ERK distribution and SerpinB2 expression in WT and KO MEFs. HRas^{Q61L} expression increased both the magnitude and duration of the increase in nuclear p-ERK levels seen in TPA-treated *DUSP5*$^{-/-}$ MEFs. Again, expression of DUSP5, but not the DUSP5 KIM mutant, rescues this phenotype by reducing p-ERK to levels seen in WT cells (Fig. 4 *A* and *B* and Fig. S6*A*).

These effects are mirrored in the expression of SerpinB2. Whereas either DUSP5 loss or expression of HRas^{Q61L} alone causes a significant increase in SerpinB2 mRNA and protein in response to TPA, combining DUSP5 deletion and HRas^{Q61L} expression results in a synergistic increase in SerpinB2 levels (Fig. 4 *C* and *D*). This is due to increased ERK-dependent transcription, as SerpinB2 promoter–reporter assays reveal a synergistic increase in MEK inhibitor-sensitive luciferase activity when HRas^{Q61L} is expressed in TPA-treated KO MEFs (Fig. 4*E*). This is again dependent on AP1 binding sites within the SerpinB2 promoter (Fig. S6*B*). Overall, these results suggest that the negative feedback control exerted by DUSP5 on ERK activity and *SerpinB2* expression is more potent when mutant HRas is present.

The Increased Tumor Susceptibility of DUSP5$^{-/-}$ Mice Is Mediated by SerpinB2 Expression. SerpinB2 transgenic mice are sensitized to DMBA/TPA-induced carcinogenesis (20), whereas SerpinB2 KO

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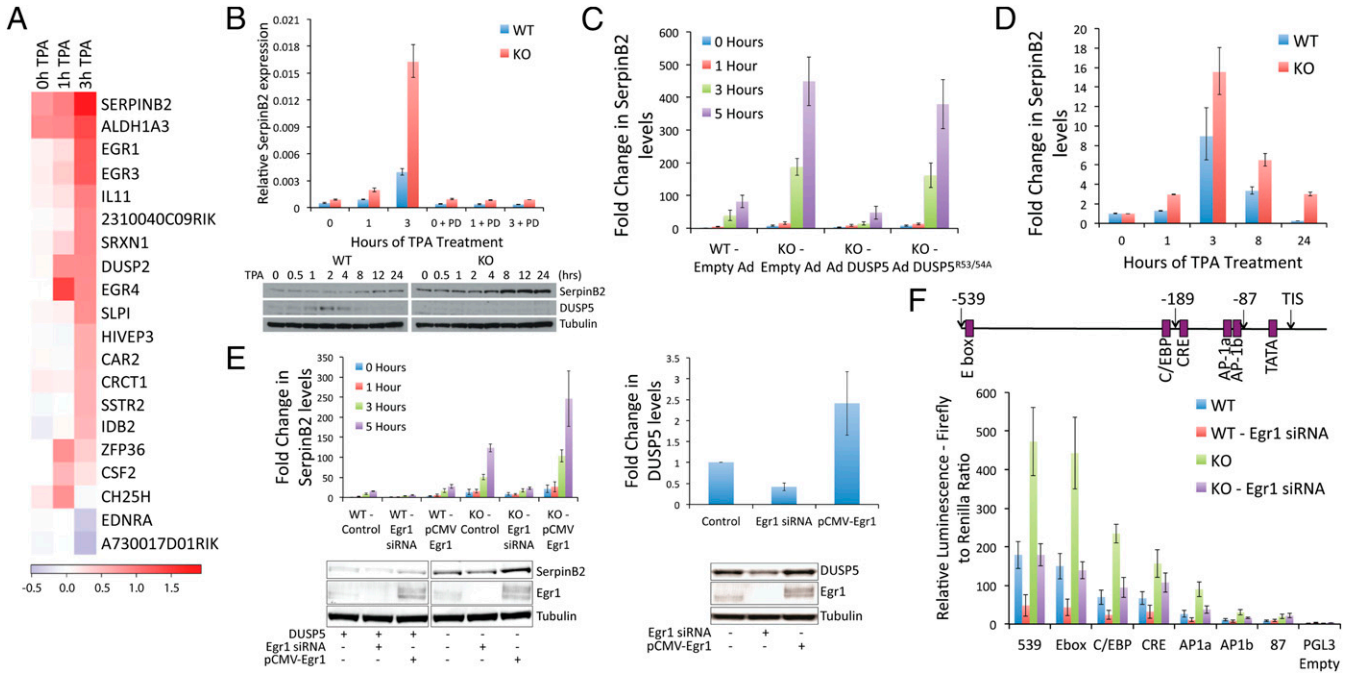

Fig. 3. DUSP5 loss increases TPA-inducible gene expression. (*A*) Heatmap showing gene expression changes at 1 and 3 h in TPA-treated wild-type (WT) and *DUSP5*$^{-/-}$ (KO) MEFs. Values are log2 ratios of KO/WT. (*B*) RT-qPCR and Western blot analysis showing SerpinB2 expression levels in TPA-treated WT and KO MEFs in the absence or presence of the MEK inhibitor PD184352 ($n = 3$). (*C*) RT-qPCR assay showing fold change in SerpinB2 mRNA levels in TPA-treated WT and KO MEFs. Cells were infected with empty adenovirus (empty Ad), Egr1 promoter-driven Ad DUSP5-Myc (Ad DUSP5), or Ad KIM mutant of DUSP5-Myc (Ad DUSP5$^{R53/54A}$) as indicated ($n = 4$). (*D*) RT-qPCR assay showing the fold change in SerpinB2 mRNA levels in TPA-treated skin from either WT or KO mice ($n = 2$). A representative result is shown as mean values ± SD of assays performed in triplicate. (*E*) RT-qPCR assay showing the fold change in SerpinB2 mRNA levels in TPA-treated WT and KO MEFs. Cells were transfected with siRNA targeting murine Egr1 (Egr1 siRNA) or an expression vector encoding human Egr1 (pCMV-Egr1) as indicated ($n = 3$). A Western blot showing the effects of either siRNA knockdown or expression of human Egr1 on SerpinB2 protein levels 24 h after TPA treatment in WT or KO MEFs is also shown (*Left*). RT-qPCR and Western blot analysis assay showing the effects of siRNA knockdown or expression of human Egr1 on basal DUSP5 expression levels ($n = 3$, *Right*). (*F*) Relative activity in WT and KO MEFs of SerpinB2 promoter–reporter constructs containing wild-type or mutant transcription factor binding sites as indicated in the absence or presence of siRNA targeting murine Egr1, following 8 h of TPA treatment ($n = 4$). Unless stated otherwise, all assay results are shown as mean values ± SEM.

mice are reported to be more resistant (21). *SerpinB2*$^{-/-}$ mice (22) were crossed with *DUSP5*$^{-/-}$ mice to generate four cohorts: WT ($n = 19$), DUSP5 KO ($n = 19$), SerpinB2 KO (SerpinB2 KO, $n = 18$), and SerpinB2/DUSP5 double KO mice (DKOSB2/5, $n = 19$). Epidermal morphology is normal in *SerpinB2*$^{-/-}$ mice (21) and skin architecture was also normal in SerpinB2/DUSP5 double KO mice. Mice were treated with DMBA/TPA as before and monitored over 25 wk (Fig. 5 *A* and *B*). In contrast to a previous report (21), we find that the incidence and multiplicity of tumor formation in *SerpinB2*$^{-/-}$ mice was indistinguishable from WT

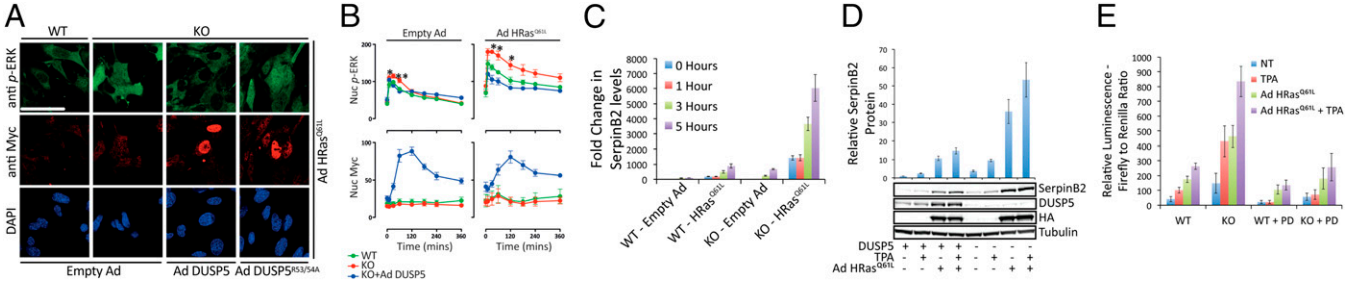

Fig. 4. Mutant HRas^{Q61L} and DUSP5 loss act synergistically to increase levels of nuclear phospho-ERK and expression of SerpinB2. (*A*) MEFs from wild-type (WT) or *DUSP5*$^{-/-}$ (KO) mice were infected with Ad HA-tagged HRas^{Q61L} (Ad HRas^{Q61L}), empty adenovirus (empty Ad), Ad Egr1 promoter-driven DUSP5-Myc (Ad DUSP5), or KIM mutant of DUSP5-Myc (Ad DUSP5$^{R53/54A}$) as indicated and stimulated with TPA for 1 h. Cells were fixed and stained for p-ERK, Myc, and DAPI. Representative confocal images are shown. (Scale bar, 60 μm.) (*B*) MEFs were infected with virus before stimulation with TPA as indicated and stained for p-ERK, Myc, and DAPI before analysis using high content fluorescence microscopy. Graphs represent population average values for nuclear (Nuc) p-ERK, and Myc intensity from four to eight experiments (5,000–20,000 individual cells per condition). Mean values ± SEM are shown, $n = 4$–8. $^{*}P < 0.05$, $^{**}P < 0.01$ using two-way repeated measures ANOVA and Bonferroni post hoc test comparing WT and KO cells. (*C*) RT-qPCR assay showing the fold change in SerpinB2 mRNA levels in TPA-treated WT and KO MEFs at the indicated time. Cells were either infected with empty Ad or Ad HRas^{Q61L} as indicated ($n = 3$). (*D*) Relative SerpinB2 protein expression in 0 or 24 h TPA-stimulated WT and KO MEFs in either the absence or presence of Ad HRas^{Q61L} as indicated was quantified using a Li-Cor Odyssey infrared scanner ($n = 3$). A representative Western blot is also shown (*Lower*). (*E*) Relative activity of a SerpinB2 promoter reporter, in WT and KO MEFs after 0 or 8 h TPA stimulation in the absence or presence of Ad HRas^{Q61L} and the specific MEK inhibitor PD184352 as indicated ($n = 4$). All assay results are shown as mean values ± SEM.

(10 and 8 tumors per animal, respectively). However, whereas *DUSP5*$^{-/-}$ mice again showed an approximate doubling in tumor multiplicity (15 papillomas per mouse) compared with WT (8 papillomas per mouse) the concomitant deletion of SerpinB2 completely abrogated this increased sensitivity, with double knockout (DKOSB2/5) mice developing an average of 10 papillomas per mouse. As before, all lesions were squamous cell papillomas and tumor morphology and size was similar in all genotypes (Fig. 5*C*). Despite previous reports that SerpinB2 is not expressed in WT skin papillomas (20, 21), RT-qPCR analysis (n = 12 for each genotype) reveals that SerpinB2 mRNA is expressed and is present at significantly higher levels in KO versus WT tumors (Fig. 5*D*). Immunohistochemical (IHC) staining also confirms that SerpinB2 protein is present in the convoluted and hyperplastic basal layer in papillomas of both genotypes, with higher levels found in tumors compared with matched normal skin (Fig. 5 *E* and *F*). We conclude that the increased sensitivity of *DUSP5*$^{-/-}$ mice to DMBA/TPA-induced carcinogenesis is mediated by a failure to regulate Ras/ERK-dependent expression of SerpinB2.

Discussion

We demonstrate that the DUSP5 nuclear ERK phosphatase is a potent tumor suppressor in the DMBA/TPA model of HRas-induced skin cancer. Specifically, we uncover a key function of DUSP5 in suppressing nuclear ERK activity and SerpinB2 expression and show that this function is responsible for tumor suppression.

We focused on SerpinB2 as a mediator of increased tumor incidence in *DUSP5*$^{-/-}$ mice upon finding that ERK-dependent TPA-inducible SerpinB2 expression is increased in *DUSP5*$^{-/-}$ MEFs. Furthermore, combining expression of mutant HRas^{Q61L} and DUSP5 loss results in a further increase in SerpinB2 expression, indicating that DUSP5 assumes greater importance in the presence of oncogenic HRas^{Q61L}. These data entirely support the proposal that negative feedback regulators of ERK such as DUSP5 form a key component of the increased transcriptional output of the Ras/ERK pathway in response to activated oncogenes and play a vital role in reducing the potency of these mutations (8). While our data demonstrate a key role for DUSP5 as a tumor suppressor in incipient HRas-driven cancers, this does not preclude a protumorigenic role for DUSP5, or other MKPs, in cases where high ERK activation can engage antiproliferative mechanisms, as seen in certain Ras mutant and BRaf^{V600E}-driven tumors (23, 24).

Egr1 is also up-regulated on DUSP5 deletion: both SerpinB2 and DUSP5 expression are decreased by Egr1 knockdown/deletion and increased by Egr1 overexpression, suggesting that this transcription factor plays a role in the expression of SerpinB2 and DUSP5 itself. This is in agreement with work demonstrating that SerpinB2 lies downstream of Egr1 in a signaling pathway driving mammary cell migration (25). Although Egr1 DNA-binding sites are absent from our SerpinB2 promoter–reporter constructs, knockdown of Egr1 inhibits AP1-dependent SerpinB2 transcriptional activity. This suggests an indirect effect of Egr1 on SerpinB2, possibly via the regulation of AP1 itself (26). *DUSP5* was recently identified as an Egr1 target gene in TPA-treated leukemia (THP-1) cells (27), which is compatible with our proposal that by regulating ERK-dependent Egr1 levels, DUSP5 acts as part of a negative feedback loop to regulate its own expression.

SerpinB2 has previously been implicated in DMBA/TPA-induced skin cancer. Transgenic mice expressing SerpinB2 are sensitized to DMBA/TPA-induced tumors (20), whereas mice lacking SerpinB2 were reported to be resistant (21). Importantly, the former study demonstrates that the effects of SerpinB2 are cell autonomous, as bovine keratin 5 promoter-driven expression was restricted to basal keratinocytes. Our experiments using DUSP5/SerpinB2 double KO mice unequivocally identify the up-regulation of SerpinB2 as causing the increased papilloma incidence in *DUSP5*$^{-/-}$ mice. Furthermore, we show that SerpinB2 is expressed in skin papillomas from WT and KO mice and that papillomas from *DUSP5*$^{-/-}$ mice express higher levels of mRNA. It is unclear why, unlike Tonnetti et al. (21), we failed to see resistance to DMBA/TPA on SerpinB2 deletion alone, but genetic background is unlikely to be a factor as both studies used inbred C57BL/6 mice.

As DMBA/TPA-induced carcinogenesis is an inflammatory process (28) and MKP deletion can lead to immune phenotypes (29) we examined immune system development in *DUSP5*$^{-/-}$ mice. However, we find no changes in T- or B-cell differentiation, nor is cytokine or chemokine synthesis in LPS-treated macrophages or skin affected by DUSP5 loss (Fig. S7*A*). Although gene knockouts that affect DMBA/TPA-induced inflammation often result in altered skin hyperplasia and immune cell infiltration (28), we saw no significant differences in the infiltration of immune effector cells in TPA-treated skin sections and papillomas from WT and *DUSP5*$^{-/-}$ mice (Fig. S7 *B* and *C*). This, coupled with our finding that TPA-induced skin hyperplasia is normal in *DUSP5*$^{-/-}$ mice (Fig. S2*B*), indicates that the increased tumorigenesis seen in *DUSP5*$^{-/-}$ mice is unlikely to be due to an altered immune response.

The mechanism(s) by which SerpinB2 exerts biological effects remains unclear. In addition to its role as a secreted regulator of the urokinase-type plasminogen activator (uPA),

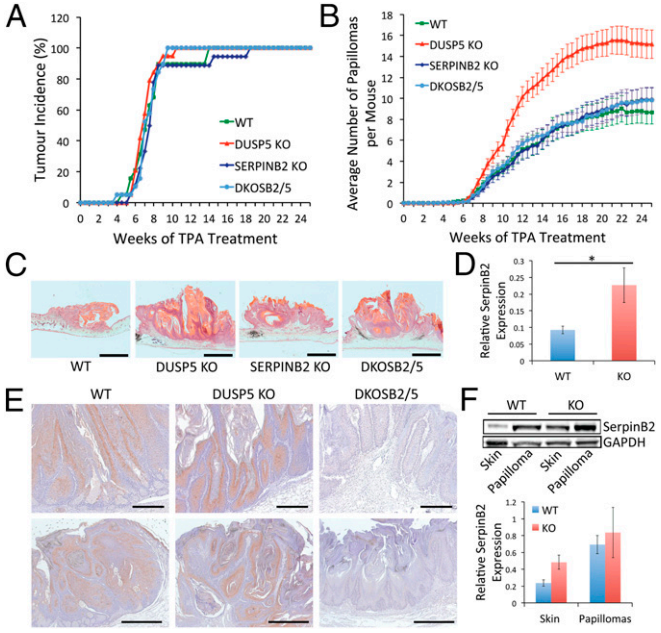

Fig. 5. Deletion of SerpinB2 abrogates the sensitivity of DUSP5$^{-/-}$ mice to DMBA/TPA-induced carcinogenesis. (*A*) Tumor incidence and (*B*) tumor multiplicity in WT (n = 19), DUSP5$^{-/-}$(n = 19), SerpinB2$^{-/-}$ (n = 18), and DUSP5$^{-/-}$/SerpinB2$^{-/-}$ (DKOSB2/5, n = 19) mice exposed to DMBA followed by twice weekly treatment with TPA for 25 wk. DUSP5 loss significantly increased tumor multiplicity compared with WT mice (P value for WT/KO = 0.001 by repeated measures multivariate ANOVA with Tukey's post hoc analysis), whereas SerpinB2$^{-/-}$ or DKOSB2/5 mice were indistinguishable from wild-type animals (P values for WT/SerpinB2$^{-/-}$ = 0.499 and WT/DKOSB2/5 = 0.851 by repeated measures multivariate ANOVA with Tukey's post hoc analysis). (*C*) H&E stained sections of representative skin papillomas excised from mice of each genotype after 25 wk of TPA treatment. (Scale bar, 1 mm.) (*D*) RT-qPCR assay showing SerpinB2 mRNA levels in WT and DUSP5$^{-/-}$ papillomas. Mean values ± SEM are shown, n = 12. *P < 0.05 using the Mann–Whitney test. (*E*) SerpinB2 protein staining in WT, DUSP5$^{-/-}$, and DKOSB2/5 papillomas. (Scale bar, 200 μm, *Upper*, and 500 μm, *Lower*.) (*F*) SerpinB2 protein expression in WT and DUSP5$^{-/-}$ mouse skin and papillomas was quantified using a Li-Cor Odyssey infrared scanner (n = 4, mean values ± SEM are shown). A representative Western blot is also shown (*Upper*).

intracellular SerpinB2 functions have been suggested. With respect to DMBA/TPA-induced tumors, inhibition of apoptosis has been proposed to promote cancer (20, 21). However, others have failed to detect any influence of SerpinB2 levels on cell growth, cell cycle progression, or apoptosis (30–32). We have not detected changes in stress-induced apoptosis in cells either lacking or overexpressing SerpinB2, or any evidence of attenuated apoptosis in *DUSP5*$^{-/-}$ skin or papillomas. SerpinB2 has also been reported to promote autophagy and is associated with senescence (33, 34). In human cancer, reports show both favorable (breast and pancreas) and poor (colorectal and ovarian) prognoses associated with high SerpinB2 levels (35). A recent study identified the extracellular protease function of SerpinB2 as a means by which lung and breast tumors evade plasmin-mediated defenses against brain metastasis (36). Thus, SerpinB2 effects during tumor initiation and development may be complex.

In conclusion, we show that DUSP5 is a nonredundant regulator of both nuclear ERK activation and localization and that the loss of this function and the subsequent failure to downregulate the ERK-dependent expression of SerpinB2 sensitizes mice to the development of HRas-induced skin cancers. Our demonstration of a tumor suppressor function for a member of the MKP family suggests that these proteins may play key roles in restraining tumor development in a range of cancers, which are driven by Ras/ERK signaling. This and the wider involvement of SerpinB2 dysregulation after DUSP5 loss in other tumor models will be the subject of future study.

Materials and Methods

Skin Carcinogenesis. Chemical carcinogenesis was performed as described (17). The dorsal skin of 7.5-wk-old mice was shaved and, at 8 wk, animals were DMBA treated (50 μg in 100 μL acetone). One week later, twice weekly treatment with TPA (12.5 μg in 100 μL acetone) was initiated. Promotion was for 25 wk to assess papilloma formation or 48 wk to assess malignant conversion. The onset, as well as the number and size of tumors, was recorded weekly. Mice were maintained under standard conditions, with free access to food and water. All work was carried out in accordance with the Animal (Scientific Procedures) Act (1986) after ethical review by the University of Dundee Welfare and Ethical Committee.

High Content Imaging and Analysis. MEFs were imaged using an IN Cell Analyzer 2000 (GE Healthcare) microscope and 10× objective lens. Cells were stained using mouse anti–p-ERK1/2 monoclonal antibody (clone MAPK-YT, 1:200; Sigma) alongside either rabbit anti-ERK1/2 monoclonal (clone 137F5, 1:200; Cell Signaling Technology), rabbit anti-Myc tag (clone 71D10, 1:200; Cell Signaling Technology), or rabbit anti-HA tag (clone C29F4, 1:200; Cell Signaling Technology). Analysis was performed using IN Cell Developer software and a custom algorithm using DAPI and p-ERK1/2 images to define nuclear and cytoplasmic regions, respectively, in each experiment. Defined regions were used as a mask for detection of changes in ERK1/2 or Myc staining.

Details of all other methods, including generation of knockout mice, microscopy, microarray analysis, tissue sectioning/histology and plasmid/adenoviral vector construction are described in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Prof. Martin Eilers (Theodor Boveri Institute) for pSUPER-retro-sh-ARF, Prof. Dan Mercola (University of California at Irvine) and Dr. Veronique Baron (Vaccine Research Institute of San Diego) for Egr1$^{-/-}$ MEFs, Prof. Toni Antalis (University of Maryland) for reporter constructs, Dr. Beverley Davidson (University of Iowa) for pacAd5, Prof. David Ginsburg (University of Michigan) for SerpinB2 antiserum, Peter Murphy (Wellcome Trust Clinical Research Facility) for Illumina array analysis, and Andrew Campbell (Beatson Institute) for immune cell staining. S.M.K. is supported by a Cancer Research UK Programme Grant (CR-UK Stress-Response Laboratory C8227/A12053). C.J.C. is supported by a Royal Society Research Grant (2010/R2). A.M.K. is supported by a Dundee Cancer Centre Studentship.

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