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Jak1-STAT3 signals are essential effectors of the USP6/TRE17 oncogene in tumorigenesis

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Abstract

Bone and soft tissue tumors are relatively poorly understood, hampering the development of effective therapies. Here we report a critical effector pathway for the ubiquitin-specific protease 6(USP6)/TRE17, which is overexpressed upon chromosome translation in various human tumors, including aneurysmal bone cyst and the related benign lesion nodular fasciitis. Ectopic expression of USP6 is known to drive formation of tumors which recapitulate key features of ABC and NF, however, the identity of USP6's relevant substrates has been obscure. Here we report that the Jak1-STAT3 signaling pathway serves as an essential effector of USP6 in BSTT formation. We found that USP6 directly de-ubiquitinated Jak1, leading to its stabilization and activation of STAT3. The tumorigenic potential of USP6 was attenuated significantly by CRISPR-mediated deletion of Jak1 or STAT3, or by administration of a Jak family inhibitor. Analysis of primary clinical samples of NF confirmed the activation of a Jak1-STAT3 gene signature in vivo. Together, our studies highlight Jak1 as the first identified substrate for USP6, and they offer a mechanistic rationale for the clinical investigation of Jak and STAT3 inhibitors as therapeutics for the treatment of bone and soft tissue tumors along with other neoplasms driven by USP6 overexpression.

Keywords

USP6; TRE17; Jak1; STAT3; ubiquitin; aneurysmal bone cyst; nodular fasciitis

INTRODUCTION

Protein ubiquitination plays a central role in diverse cellular processes, including the DNA damage response, vesicular trafficking, and transformation (1). Reversal of ubiquitination is catalyzed by de-ubiquitinating enzymes (DUBs), which comprise five sub-classes: the ubiquitin-specific protease (USPs), UCH, OTU, Josephin, and JAMM subfamilies (2, 3).

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The USP subfamily is the most populous, with over 50 members in humans, and a key goal is to elucidate their substrate specificity and biological functions.

Multiple USPs have been implicated in human tumorigenesis, but particularly notable is *USP6/TRE17* (4, 5). *USP6* is a recurrent target of chromosomal translocation in several bone and soft tissue tumors, including aneurysmal bone cyst (ABC) and nodular fasciitis (NF) (6–8). In all cases, rearrangement causes promoter swapping and high level expression of wild type *USP6*. Notably, *USP6* is absent or expressed at exceedingly low levels in most normal adult human tissues (9).

Remarkably little is known regarding ABC and NF pathogenesis. ABC is a benign but locally aggressive bone tumor that can cause bone destruction, fracture, neurological symptoms, and soft tissue damage (10–12). It was long hypothesized to originate from a localized hemodynamic disturbance, leading to formation of a dilated vascular bed, inflammatory recruitment, and neovascularization. However, identification of *USP6* translocation in ~70% of ABCs revealed that they are neoplasms. Translocated cells are disseminated throughout the lesion. While the affected lineage has not been definitely determined, candidate cells are pre-osteoblasts, fibroblasts, and mesenchymal progenitor cells (13). ABCs are typically treated by curettage, but recurrence is not uncommon. In addition, they can arise in inoperable locations, where they can cause significant morbidity. Development of targeted chemotherapeutic agents would therefore be of great value.

NF was also long believed to be a reactive process, but our identification of *USP6* translocation in ~90% of cases revealed its neoplastic origins (14, 15). The translocation drives overexpression of WT *USP6* (6). Again, the cell lineage harboring the translocation has not been conclusively identified, but fibroblasts and myofibroblast progenitors are likely candidates. NF manifests as a rapidly growing subcutaneous lesion. Because of its histology and rapid growth, it can be mistaken for a sarcoma (16), resulting sometimes in overtreatment of NF patients with radiation and chemotherapy. Since *USP6* translocation is not found in sarcomas, our findings provided a novel diagnostic marker for NF, preventing unnecessarily aggressive treatment.

While identification of *USP6* translocation in ABC and NF provided a significant advance, until recently little was known about how *USP6* overexpression contributes to tumor pathogenesis. Though initially cloned over twenty years ago (17), its molecular functions in normal physiology remain elusive, due in large part to the absence of cell lines or primary tissues expressing appreciable levels of *USP6*. However, we recently established cellular and animal models to investigate its functions in ABC and NF pathogenesis (18–20). Overexpression of USP6 in fibroblasts or pre-osteoblasts (candidate cell lineages for harboring the translocation) led to tumor formation in xenografted immunodeficient mice. Molecular, histological, and clinical features of ABC and NF were recapitulated in these systems (18–20). USP6's ability to activate NF-κB was essential for tumorigenesis (19). Furthermore, USP activity was required, since USP6 alleles lacking USP activity failed to induce tumor formation (20). However, a vital question that remained unanswered was the identity of USP6's relevant substrates. Previous studies indicate that USP6 promotes both de-ubiquitination of itself and of clathrin-independent endocytic pathway cargoes *in vivo*

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(21), (22). However, it remained unknown whether any of these proteins are direct substrates of USP6, and what their relevance is to transformation. Thus, identifying substrates that mediate USP6 tumorigenesis has remained a critical outstanding goal.

Jak family tyrosine kinases play an essential role in cytokine signaling (23–25). They function to phosphorylate STAT family transcription factors, which triggers their dimerization and nuclear translocation. The STAT3 family member is activated in numerous cancers, where it often acts in concert with NF-κB to promote tumor cell proliferation and survival (26, 27). In addition, they cooperate in establishing a pro-tumorigenic microenvironment by inducing angiogenesis and stimulating production of inflammatory cytokines, such as interleukin-6 (IL-6) (28, 29).

In the current study, we identify Jak1-STAT3 as essential mediators of USP6's pathogenic mechanism. USP6 activates this pathway by de-ubiquitinating Jak1, leading to its dramatic stabilization and STAT3 activation. Inactivation of this pathway by CRISPR-mediated gene editing or pharmacological means dramatically attenuates tumorigenesis by USP6. This work identifies the first de-ubiquitinating enzyme for a Jak family member, and defines a novel target for treating neoplasms driven by *USP6* translocation.

MATERIALS AND METHODS

Cell lines and reagents

NIH3T3 and MC3T3 cell lines expressing USP6 alleles are the major biologically relevant cells used in this study (18–20). Both parental cell lines were purchased from the ATCC in 2008 and 2007 respectively; certificates of analysis were provided. Authenticity of MC3T3 cells as osteoblasts was confirmed by validating expression of osteoblastic markers upon differentiation. Both lines were confirmed as murine by qPCR of multiple genes, including GAPDH and Jak1.

Generation of Jak1 and STAT3 deleted cell lines

CRISPR target sequences for JAK1 (GTGGTCCCTGAGCCTGGAG) and STAT3 (CAGCTGGACACACGCTACC) were designed with ZiFit Targeter software (http://zifit.partners.org/ZiFiT/; Zinc Finger Consortium) and inserted into pCR-Blunt II-TOPO. TOPO plasmids were co-transfected with a plasmid encoding CAS9 and G418 resistance (Dr. Paul Bates, University of Pennsylvania).

Reagents

Cycloheximide (C104450) and bafilomycin A (B1793) were from Sigma-Aldrich. MG132 (#474790); Jak Inhibitor I (CAS 457081-03-7; #420099), and Src inhibitor PP2 (#529573) were from EMD. Doxycyclin (#8634-1) was from Clontech. IL-6 was from R&D Systems (#206-IL-010). Lipofectamine and Oligofectamine were from Life Technologies.

Plasmids and siRNA

USP6 constructs were described previously (18, 20, 21, 30, 31). FLAG-tagged WT and kinase dead Jak1/pRK5 were provided by Dr. Warren Leonard (NHLBI). Myc-Ub was

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provided by Dr. Roger Greenberg (University of Pennsylvania). USP6 SMARTPool siGenome siRNA (M-006096-03-0050) was from Dharmacon.

Cell lysis, immunoprecipitation, immunoblotting, cell fractionation and EMSA

Cell lysis, immunoprecipitation, cell fractionation and EMSA were performed as described (18, 20, 21, 30, 31). Jak1 (#3332), Jak2 (#3230), phospho-STAT3 (#9145), STAT3 (#9139) antibodies were from Cell Signaling Technologies. Actin (sc-8432), p65 (sc-372), c-Myc(9E10) (sc-40), and HDAC2 (sc-7899) antibodies were from Santa Cruz Biotechnologies. FLAG (M2) beads (A2220) were from Sigma-Aldrich. USP6 antibody was described previously (20).

Monitoring Jak1 ubiquitination in vivo and in vitro

To monitor Jak1 ubiquitination *in vivo*, HeLa were co-transfected with FLAG-Jak1 and Myc-Ub, with indicated USP6 constructs. Cells were treated for 2.5h with MG132 (20 μM), lysed in RIPA buffer, then immunoprecipitated with FLAG beads for 4h at 4C. Samples were washed three times, fractionated by SDS-PAGE, and blotted with anti-Myc. For *in vitro* DUB assays, FLAG-Jak1 and Myc-Ub were co-transfected, then subjected to FLAG immunoprecipitation. Samples were washed in RIPA buffer, then DUB Assay Buffer (20 mM Tris pH 7.5, 100 mM NaCl, 0.05% Tween-20, 0.5 mg/ml bovine serum albumin, and 5 μM β-mercaptoethanol). The sample was divided into equal portions, one of which was immediately boiled. The remaining replicate samples were incubated with GST or GST-tagged USP6 (WT or catalytically inactive mutant; Ubiquigent) for 1.5 hr at 37C. Samples were washed in RIPA, then immunoblotted with Myc.

Tumorigenesis assays

NOD-SCID or NOG-SCID mice (4–8 weeks) were injected subcutaneously in the flank with 2.5E6 cells; tumors were harvested after 3–4 weeks, with animals maintained on water containing doxycyclin (1 mg/ml; BioWorld, 40410005-2) and 5% sucrose. Jak inhibitor CYT387 (50mg/kg; Selleckchem, S2219) or vehicle was administered twice daily by oral gavage where indicated. All animal procedures were performed under IACUC-approved protocols.

Immunohistochemistry (IHC)

IHC staining was performed at the Pathology Research Core of the Mayo Clinic on 40 primary tumor samples whose USP6 translocation status was confirmed. Formalin fixed paraffin sections were probed with phospho-STAT3 (Clone D3A7, Cell Signaling Technologies) at 1:400. The Polymer Refine Detection System (Leica, Buffalo, IL) was used, which includes hydrogen peroxidase block, post primary and polymer reagent, DAB, and Hematoxylin. Slides were counterstained with Schmidt hematoxylin.

Gene expression profiling and pathway analyses

Microarray analysis was performed on 36 tumors: 9 NF tumors with *USP6* translocation, and 27 others (alveolar rhabdomyosarcoma [n=3]; dermatofibroma or benign fibrous histiocytoma [n=3]; dermatofibrosarcoma protubera [n=3]; gastrointestinal stromal tumor

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[n=1]; malignant peripheral nerve sheath tumor [n=3]; melanoma [n=3]; neurofibroma [n=3]; embryonal rhabdomyosarcoma [n=3]; synovial sarcoma [n=3], and schwannoma [n=2]). Total RNA was extracted from formalin-fixed, paraffin-embedded material using miRNeasy FFPE kit (Qiagen). Microarray analysis was performed on 200ng RNA using Human WG-DASL Assay with Human HT12 v4.0 BeadChips (Illumina), which contain 29,377 probes. Microarray analyses were performed using Illumina GenomeStudio and Partek Genomics Suite software. Pre-processing of data at the probe level was performed within GenomeStudio software using quantile normalization with no background subtraction. 'Non-expressed' probes were defined as those with Illumina detection $p > 0.05$ in all 36 samples. 20,818 probes remained after filtering, and log2-transformed expression values were analyzed using Partek Genomic Suite software tools. Differentially expressed genes in NF compared to other tumor types were identified using ANOVA to calculate P values and fold enrichments. In order to control the False Discovery Rates (FDR) from the multiple testing, we calculated the adjusted p values using the Benjamini-Hochberg method (32). According to Source of Variance analyses, the microarray batch (array slide of 12 samples) was also included as a variable in the ANOVA. Gene Set Enrichment Analysis was performed using the Hallmark molecular signature database (MSigDB) from the Broad Institute, which contains well-defined pathways curated from other gene sets. Additional analysis was done using Canonical Pathway MSigDB, which contains pathways from well known databases such as KEGG and Biocarta typically compiled by domain experts. For both analyses, the permutation type was set to "geneset" and the metric for ranking genes was set to "Signal2Noise" (33, 34). Microarray datasets can be accessed at (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=cnejeuggnxifnmx&acc=GSE78991), and in Supplemental Table 1.

RESULTS

USP6 activates STAT3 in cellular models and *USP6*-translocated tumors

We previously reported that USP6 activates NF-κB, and that NF-κB plays an essential role in USP6-mediated transformation (19, 20). Given that NF-κB and STAT3 are often activated coordinately during tumorigenesis, we examined whether STAT3 was activated by USP6 in our established cellular models of ABC and NF. USP6 was stably expressed in a doxycycline (dox)-inducible manner in NIH3T3 fibroblasts or MC3T3 pre-osteoblasts (18–20). Upon dox treatment of USP6/NIH3T3 or USP6/MC3T3 cells, robust activation of STAT3 was observed, as measured by phosphorylation on tyrosine residue 705 (Y705) (Figure 1A).

To confirm STAT3 activation in human tumors harboring *USP6* translocation, immunohistochemistry (IHC) was performed on 24 primary NF and ABC samples, as well as 16 other mesenchymal tumors. Moderate to strong nuclear P-STAT3 staining was observed in 22/24 NF and ABC tumors (Figure 1B, Supplemental Table 2, and Supplemental Figure 1). In ABC, both neoplastic cells and other cells in the microenvironment exhibited P-STAT3 activation (Figure 1B, **left**), suggesting that USP6 might induce paracrine signaling (as was confirmed below). In NF (Figure 1B, **right**), all of

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the neoplastic cells (with their characteristic plump spindle morphology, bundled in short fascicles) (6) stained positively for P-STAT3.

Transcriptome survey reveals induction of NF-κB and STAT3 signatures in *USP6*-translocated tumors

To confirm whether STAT3 is activated by *USP6* translocation in an unbiased manner, microarray analysis was performed on NF tumors. The optimal cell type to use as a counterpart for comparison was unclear, since the cell of origin in NF has not been definitively identified. However, since the affected lineage is mesenchymally derived, transcriptome analysis was performed on 27 tumors predominantly of mesenchymal origin that lacked *USP6* translocation, and an "averaged" expression profile was generated. An NF transcriptome (derived from 9 independent *USP6*-translocated NF tumors) was then compared to this averaged data set. Using this strategy, we sought to exclude genes that are general mesenchymal markers or common indicators of the transformed state, and instead identify those that are selectively induced by USP6. Gene set enrichment analysis (GSEA) revealed that signatures reflective of IL-6/Jak/STAT3 and NFκB activation were activated in NF (Figure 1C/D and Supplemental Figure 2, and Supplemental Tables 3–4). This validates our cellular model systems, and indicates that USP6 expression in human tumors triggers STAT3 and NF-κB signaling responses.

USP6 activation of STAT3 occurs independently of NF-κB

We sought to dissect the mechanism by which USP6 activates STAT3. We speculated that STAT3 activation might arise as a secondary consequence of NF-κB activation, since NF-κB can induce expression of multiple STAT3 agonists. To test this, we examined STAT3 activation in USP6/NIH3T3 cells in which NF-κB was inhibited through expression of IκB-alpha super repressor. Activation of NF-κB by USP6 was completely suppressed in these cells (Figure 2A, left), in accordance with our previous results (19). Nevertheless, activation of STAT3, as monitored by its nuclear localization, occurred unimpeded (Figure 2A, right), indicating that its activation is not dependent on NF-κB.

Jak kinases mediate STAT3 activation by USP6 and are required for tumorigenesis

Jak and Src family kinases are capable of phosphorylating STAT3 (23, 35). To determine which family mediated activation of STAT3, pharmacological inhibitors were utilized. A pan-Jak inhibitor completely blocked USP6-induced phosphorylation of STAT3, while the pan-Src PP2 inhibitor was ineffectual (Figure 2B). To exclude the possibility that the pan-Jak inhibitor non-specifically affected Src or receptor tyrosine kinases, a range of doses was tested. As shown in Figure 2C–E, the pan-Jak inhibitor blocked USP6-induced phosphorylation of STAT3 in a dose-dependent manner, but had no effect on tyrosine phosphorylation induced by Src or the EGF receptor. Together, these results demonstrate that USP6 activates STAT3 exclusively through a Jak family kinase.

We next tested whether a Jak family inhibitor could prevent tumor formation by USP6. For these studies we used CYT387, a pan-Jak inhibitor that is efficacious *in vivo* (36, 37). USP6/NIH3T3 cells were subcutaneously injected into NOD-SCID mice. One cohort of animals was subjected twice daily to treatment with CYT387, while the other was treated with

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vehicle. As we previously reported, after 3 weeks large, highly vascularized tumors were visible in the control group (Figure 2F). In contrast, tumors harvested at the same time point from CYT387-treated animals treated were significantly reduced in size and avascular (Figure 2F). Together, these results indicate that Jak-STAT3 is activated in neoplasms harboring *USP6* translocation, and that targeting this pathway may be an effective approach for controlling growth of tumors driven by *USP6* overexpression.

Jak1 is upregulated by USP6 in a USP-dependent manner

To determine which Jak kinase is responsible for STAT3 phosphorylation, we examined expression of the various family members in our cells. Strikingly, we found that Jak1 levels were significantly increased in NIH3T3 and MC3T3 cell lines expressing USP6, in a dox-dependent manner (Figure 3A/C). This effect was highly specific, as other Jak family members (Jak2, Jak3, and Tyk2) were unaffected (Figure 3A–C).

Analysis of various USP6 alleles revealed that upregulation of Jak1 required USP activity. Jak1 levels were not elevated in NIH3T3 cells stably expressing an inactive point mutant with a critical cysteine residue mutated to serine (USP6/CS) (Figure 3D). Similar results were obtained in transiently transfected HeLa cells: neither USP6(CS) nor USP6(short), a naturally occurring C-terminally truncated isoform that lacks USP activity, were competent to upregulate Jak1 or activate STAT3 (Figure 3E), confirming that USP6's USP activity can activate Jak1-STAT3 in different cellular contexts. In contrast, a triple point mutant in the TBC domain that ablates USP6's ability to activate Arf6 (USP6/A6-) (18) promoted Jak1 upregulation comparably to WT USP6 (Figure 3E).

USP6 stabilizes Jak1 protein via its USP activity

RT-qPCR revealed that *Jak1* mRNA levels were not increased by USP6 (Supplemental Figure 3). We therefore explored whether USP6 acts through a post-translational mechanism. To monitor USP6's effects on Jak1 protein halflife, HeLa cells were co-transfected with Jak1 and various USP6 alleles, then treated for various times with the protein synthesis inhibitor, cycloheximide (CHX). In vector control samples, Jak1 was rapidly degraded, with minimal protein remaining after 2 hours (Figure 4A). Co-expression with USP6 led to dramatic stabilization of Jak1. Stabilization required USP activity, since Jak1 half-life was comparable in cells expressing USP6(short) and control vector (Figure 4A). Conversely, we found that siRNA-mediated knockdown of USP6 in HeLa cells led to significant reduction in steady state levels of Jak1 protein (Figure 4B).

Cytosolic proteins are typically degraded via the proteasome, whereas membrane proteins are typically degraded mediated via the lysosome. While Jak1 is cytosolic, it has the capacity to associate with transmembrane receptors (24), and could potentially be degraded by either route. To determine its mode of degradation, Jak1 stability was monitored in cells treated with the proteasome and lysosome inhibitors, MG132 and bafilomycin A (BafA), respectively. MG132, but not BafA, significantly prolonged the half-life of Jak1 (Figure 4C). Control blots of the EGFR, which is degraded via the lysosome, confirmed proper functioning of BafA (Figure 4D). Thus, Jak1 turnover is controlled through proteasome-mediated degradation.

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Since activated kinases sometimes trigger their own ubiquitination and degradation, we explored whether Jak1 kinase activity affected its stability. Two approaches were taken: first, cells were transfected with a kinase inactive mutant of Jak1. As shown in Figure 4E (top panel), USP6 was able to stabilize this mutant as well as it did WT Jak1. As a second approach, cells were treated with the pan-Jak inhibitor. USP6's ability to prolong the half-life of Jak1 was unaffected by the Jak inhibitor (Figure 4E, bottom panel). Together, these data indicate that Jak1 kinase activity does not modulate its halflife, either basally or in the presence of USP6.

USP6 associates with Jak1, and mediates its de-ubiquitination *in vivo* and *in vitro*

Our data are consistent with a model whereby Jak1 is normally maintained at low levels through proteasome-mediated degradation, but becomes stabilized through de-ubiquitination by USP6. To test whether USP6 is capable of directly de-ubiquitinating Jak1 *in vitro*, ubiquitinated Jak1 was immunopurified from HeLa cells co-expressing FLAG-Jak1 and Myc-Ub using anti-FLAG. Immunoprecipitates were incubated with recombinant USP6, and ubiquitin remaining on Jak1 was detected by anti-Myc immunoblotting. The isolated catalytic domain of USP6 efficiently de-ubiquitinated Jak1, while a catalytically inactive point mutant and control GST protein did not (Figure 5A). Furthermore, consistent with USP6 serving as a DUB for Jak1, USP6 co-immunoprecipitated with endogenous Jak1 *in vivo* (Figure 5B).

To test whether USP6 can induce de-ubiquitination of Jak1 *in vivo*, HeLa cells were co-transfected with FLAG-Jak1, Myc-Ub, and HA-USP6 alleles. Jak1 was immunoprecipitated using anti-FLAG, and its ubiquitination monitored by anti-Myc immunoblotting. Co-expression of USP6, but not USP6(short), completely abolished ubiquitination of Jak1 *in vivo* (Figure 5C). Although the level of Jak1 ubiquitination in cells expressing USP6(short) was clearly higher than that in cells expressing WT USP6, they were consistently reduced compared to vector control cells. The underlying reason is unclear, but may be due to interaction of USP6(short) with endogenous USP6. Regardless, together these results establish Jak1 as the first substrate identified for USP6.

Jak1 is required for STAT3 activation by USP6, and both are required for USP6-mediated cell survival and tumorigenesis

We sought to determine whether the Jak1-STAT3 signaling axis is required for USP6-mediated transformation. Toward this end, Jak1 and STAT3 were deleted from USP6/NIH3T3 by CRISPR-Cas9-mediated genome editing. Clonal cell lines were established with complete or mono-allelic deletion of Jak1 (Figure 6A), or complete deletion of STAT3 (Figure 6B). Immunoblotting confirmed that the Jak1 and STAT3 CRISPR constructs did not target other family members (Supplemental Figure 4A). USP6-induced STAT3 phosphorylation was completely abrogated in cells depleted of Jak1 (USP6/J1#17). Strikingly, STAT3 activation was also significantly reduced in cells with mono-allelic Jak1 deletion (USP6/J1#11) (Figure 6A). Jak1 in this clone was reduced to levels approximating those in control NIH3T3 cells (Supplemental Figure 4A), demonstrating that upregulation of Jak1 by USP6 is required for full activation of STAT3 by USP6.

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Next, we examined the role of Jak1 and STAT3 in signaling and survival downstream of USP6. Upon serum withdrawal NIH3T3 cells undergo growth arrest followed by apoptosis, which can be monitored by Erk inactivation and PARP cleavage, respectively (Figure 6C and D). USP6 was competent to induce Erk activation and prevent PARP cleavage upon serum starvation, and this effect was abrogated by depletion of Jak1 or STAT3 (Figure 6C and D). The protective effects of USP6 were also compromised in USP6/J1#11 cells, again supporting the notion that upregulation of Jak1 is required. We also examined the effects of Jak1/STAT3 depletion on proliferation under serum-replete conditions. Interestingly, USP6/NIH3T3 proliferated more slowly than vector control cells, and growth rates were not further reduced by Jak1/STAT3 depletion or Jak inhibitor (Supplemental Figure 4B/C).

Xenografting of the CRISPR cells into immunodeficient mice revealed a critical role for Jak1 and STAT3 in USP6-induced tumorigenesis. Strikingly, both complete and partial depletion of Jak1 significantly reduced the mass of USP6-induced tumors (Figure 6E). Deletion of STAT3 attenuated USP6's tumorigenic potential even more dramatically than Jak1 (Figure 6E). While 10/10 mice injected with USP6/NIH3T3 cells developed tumors, only 5/10 injected with USP6/S3#1 did. Furthermore, tumors that did form in STAT3-deficient cells were drastically reduced in size. Together, these data confirm that Jak1 upregulation is an essential component of USP6's transformation mechanism, and that STAT3 is a critical downstream target.

Jak1 levels dictate sensitivity to and production of STAT3 agonists

Simply elevating Jak1 levels would likely be insufficient to elicit its activation; rather, agonists would still be required. Since STAT3 was potently activated in USP6/NIH3T3 under serum-free conditions, this suggested that the cells were producing autocrine/paracrine factors. We confirmed that conditioned medium (CM) from USP6/NIH3T3 but not control cells induced STAT3 activation in naïve cells, to levels on par with IL6 (Figure 7A). Production of paracrine factors was abolished in Jak1- and STAT3-deleted cells, and significantly reduced in USP6/J1#11 heterozygously deleted cells (Figure 7A).

In addition to regulating the production of cytokines, we surmised that Jak1 levels might also dictate sensitivity to them. To test this, we examined STAT3 activation in response to exogenous Jak1 agonists. We found that not only was basal phosphorylation of STAT3 increased in USP6/NIH3T3 cells, but its activation was also enhanced in response to IL6 and IL4 (Figure 7B/C). Deletion of Jak1 reversed this enhancement in a dose-dependent manner (Figure 7B,C). Together, this group of experiments indicates that upregulation of Jak1 by USP6 is required not only for the production of autocrine/paracrine factors, but also for heightened STAT3 activation in response to them.

DISCUSSION

Our signaling studies and analysis of primary human tumors identify Jak1-STAT3 as critical effectors of USP6 in oncogenesis. We identify Jak1 as the first direct substrate of USP6, and demonstrate that it is required for STAT3 activation by USP6. We further show that this pathway is essential for USP6-mediated tumorigenesis. Our data support a model in which Jak1 levels are increased through de-ubiquitination by USP6, rendering cells hyper-sensitive

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to low levels of Jak1 agonists present in the microenvironment. This leads to activation of STAT3, which drives production of autocrine/paracrine factors by USP6-expressing cells; the elevated Jak1 levels sensitize USP6-expressing cells to the autocrine factors, thereby amplifying STAT3 activation in a positive feedback loop. In addition, the paracrine factors induce STAT3 activation in neighboring non-translocated cells in the tumor microenvironment. Whether autocrine/paracrine factor production involves pathways in addition to Jak1-STAT3 remains to be determined. This work summons the first targeted therapy (namely Jak-STAT3 inhibitors) for the treatment of neoplasms driven by *USP6* translocation/overexpression.

Our studies illustrate an atypical mode of kinase regulation. Kinases are most commonly regulated through site-specific phosphorylation. While ubiquitination and proteasomal degradation have been reported for multiple kinases, they are typically triggered by the activated kinase as a means of auto-downregulation (38). However, degradation of Jak1 is not dependent on its activation: inhibition of Jak1 kinase activity neither affects basal turnover, or stabilization by USP6. This mode of regulation is reminiscent of another kinase, NIK, a central regulator of non-canonical NF-κB. Like Jak1, NIK is maintained at low levels through constitutive ubiquitination (39). Future studies will determine if physiological agents regulate Jak1 levels through modulation of USP6.

ABC and NF can be added to the growing list of neoplasms harboring Jak1 dysregulation. Activation of Jak1 has been reported in leukemias, hepatocellular carcinoma, and gynecologic cancers (40–43). In these cases, activation arose through mutation of Jak1, usually within the kinase domain. To our knowledge, this is the first example of Jak1 being regulated at the protein level, through modulation of its turnover.

Most importantly, our work identifies potential novel therapeutic strategies for the treatment of ABC and other neoplasms driven by USP6 overexpression. Operable cases of ABC are typically treated by curettage of the affected bone. However, there is no standard of care upon recurrence (which affects up to 70% of patients), or for inoperable cases (10, 11). Treatment strategies, which involve prevention of recurrence, repair of damaged tissue, and pain management, can be associated with serious complications or morbidity. Our current and prior work suggests that inhibitors of Jak kinases, STAT3, and NF-κB would be highly effective in the management of ABC, since inactivation of all of these effectors dramatically attenuated USP6's tumorigenic potential (19). Notably, such inhibitors are being avidly explored for treatment of other cancers and inflammatory diseases (44–49), paving the way for their testing in ABC patients. We posit that regimens that simultaneously inhibit Jak-STAT3 and NF-κB might be particularly effective. Moreover, since USP activity is required for activation of Jak1-STAT3 and NF-κB, development of USP6-specific inhibitors would also be a highly desirable approach. Given USP6's highly restricted expression in normal tissues (9), such an inhibitor would likely have minimal side effects. In sum, our work identifies several new avenues for the treatment of recurrent and inoperable ABC, and other neoplasms driven by USP6 overexpression.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Varshavsky A. The ubiquitin system, an immense realm. Annu Rev Biochem. 2012; 81:167–176. [PubMed: 22663079]
2. Komander D, Clague MJ, Urbe S. Breaking the chains: structure and function of the deubiquitinases. Nat Rev Mol Cell Biol. 2009; 10:550–563. [PubMed: 19626045]
3. Reyes-Turcu FE, Ventii KH, Wilkinson KD. Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. Annu Rev Biochem. 2009; 78:363–397. [PubMed: 19489724]
4. Sacco JJ, Coulson JM, Clague MJ, Urbe S. Emerging roles of deubiquitinases in cancer-associated pathways. IUBMB Life. 2010; 62:140–157. [PubMed: 20073038]
5. Oliveira AM, Chou MM. The TRE17/USP6 oncogene: a riddle wrapped in a mystery inside an enigma. Front Biosci (Schol Ed). 2012; 4:321–334. [PubMed: 22202063]
6. Erickson-Johnson MR, Chou MM, Evers BR, Roth CW, Seys AR, Jin L, et al. Nodular fasciitis: a novel model of transient neoplasia induced by MYH9-USP6 gene fusion. Lab Invest. 2011; 91:1427–1433. [PubMed: 21826056]
7. Oliveira AM, Hsi BL, Weremowicz S, Rosenberg AE, Dal Cin P, Joseph N, et al. USP6 (Tre2) fusion oncogenes in aneurysmal bone cyst. Cancer Res. 2004; 64:1920–1923. [PubMed: 15026324]
8. Oliveira AM, Perez-Atayde AR, Dal Cin P, Gebhardt MC, Chen CJ, Neff JR, et al. Aneurysmal bone cyst variant translocations upregulate USP6 transcription by promoter swapping with the ZNF9, COL1A1, TRAP150, and OMD genes. Oncogene. 2005; 24:3419–3426. [PubMed: 15735689]
9. Paulding CA, Ruvolo M, Haber DA. The Tre2 (USP6) oncogene is a hominoid-specific gene. Proc Natl Acad Sci U S A. 2003; 100:2507–2511. [PubMed: 12604796]
10. Cottalorda J, Bourelle S. Current treatments of primary aneurysmal bone cysts. J Pediatr Orthop B. 2006; 15:155–167. [PubMed: 16601582]
11. Cottalorda J, Bourelle S. Modern concepts of primary aneurysmal bone cyst. Arch Orthop Trauma Surg. 2007; 127:105–114. [PubMed: 16937137]
12. Saccomanni B. Aneurysmal bone cyst of spine: a review of literature. Arch Orthop Trauma Surg. 2008; 128:1145–1147. [PubMed: 17922280]
13. Oliveira AM, Perez-Atayde AR, Inwards CY, Medeiros F, Derr V, Hsi BL, et al. USP6 and CDH11 oncogenes identify the neoplastic cell in primary aneurysmal bone cysts and are absent in so-called secondary aneurysmal bone cysts. Am J Pathol. 2004; 165:1773–1780. [PubMed: 15509545]
14. Dijoux P, Mesturoux L, Charissoux A, Pommepuy I, Layre B, Marcheix PS. Nodular fasciitis: case report and review of the literature. Chir Main. 2013; 32:341–344. [PubMed: 24035527]
15. Singh S, Paul S, Dhall K, Khichy S. Nodular fasciitis: a diagnostic challenge. Indian J Pathol Microbiol. 2013; 56:288–290. [PubMed: 24152513]
16. Scourtas A, Pitman MB, Chebib I. Nodular fasciitis clinically presenting as a sarcoma. Diagn Cytopathol. 2014; 43:488–489. [PubMed: 25363486]
17. Nakamura T, Hillova J, Mariage-Samson R, Onno M, Huebner K, Cannizzaro LA, et al. A novel transcriptional unit of the tre oncogene widely expressed in human cancer cells. Oncogene. 1992; 7:733–741. [PubMed: 1565468]
18. Lau AW, Pringle LM, Quick L, Riquelme DN, Ye Y, Oliveira AM, et al. TRE17/ubiquitin-specific protease 6 (USP6) oncogene translocated in aneurysmal bone cyst blocks osteoblastic maturation

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via an autocrine mechanism involving bone morphogenetic protein dysregulation. J Biol Chem. 2010; 285:37111–37120. [PubMed: 20864534]

19. Pringle LM, Young R, Quick L, Riquelme DN, Oliveira AM, May MJ, et al. Atypical mechanism of NF-kappaB activation by TRE17/ubiquitin-specific protease 6 (USP6) oncogene and its requirement in tumorigenesis. Oncogene. 2012; 31:3525–3535. [PubMed: 22081069]
20. Ye Y, Pringle LM, Lau AW, Riquelme DN, Wang H, Jiang T, et al. TRE17/USP6 oncogene translocated in aneurysmal bone cyst induces matrix metalloproteinase production via activation of NF-kappaB. Oncogene. 2010; 29:3619–3629. [PubMed: 20418905]
21. Shen C, Ye Y, Robertson SE, Lau AW, Mak DO, Chou MM. Calcium/calmodulin regulates ubiquitination of the ubiquitin-specific protease TRE17/USP6. J Biol Chem. 2005; 280:35967–35973. [PubMed: 16127172]
22. Funakoshi Y, Chou MM, Kanaho Y, Donaldson JG. TRE17/USP6 regulates ubiquitylation and trafficking of cargo proteins that enter cells by clathrin-independent endocytosis. J Cell Sci. 2014; 127:4750–4761. [PubMed: 25179595]
23. Leonard WJ. Role of Jak kinases and STATs in cytokine signal transduction. Int J Hematol. 2001; 73:271–277. [PubMed: 11345192]
24. Leonard WJ, Lin JX. Cytokine receptor signaling pathways. J Allergy Clin Immunol. 2000; 105:877–888. [PubMed: 10808165]
25. Leonard WJ, O'Shea JJ. Jaks and STATs: biological implications. Annu Rev Immunol. 1998; 16:293–322. [PubMed: 9597132]
26. Grivennikov SI, Karin M. Dangerous liaisons: STAT3 and NF-kappaB collaboration and crosstalk in cancer. Cytokine Growth Factor Rev. 2010; 21:11–19. [PubMed: 20018552]
27. He G, Karin M. NF-kappaB and STAT3 - key players in liver inflammation and cancer. Cell Res. 2011; 21:159–168. [PubMed: 21187858]
28. Grivennikov SI, Karin M. Inflammatory cytokines in cancer: tumour necrosis factor and interleukin 6 take the stage. Ann Rheum Dis. 2011; 70(Suppl 1):i104–i108. [PubMed: 21339211]
29. Taniguchi K, Karin M. IL-6 and related cytokines as the critical lynchpins between inflammation and cancer. Semin Immunol. 2014; 26:54–74. [PubMed: 24552665]
30. Martinu L, Masuda-Robens JM, Robertson SE, Santy LC, Casanova JE, Chou MM. The TBC (Tre-2/Bub2/Cdc16) domain protein TRE17 regulates plasma membrane-endosomal trafficking through activation of Arf6. Mol Cell Biol. 2004; 24:9752–9762. [PubMed: 15509780]
31. Masuda-Robens JM, Kutney SN, Qi H, Chou MM. The TRE17 oncogene encodes a component of a novel effector pathway for Rho GTPases Cdc42 and Rac1 and stimulates actin remodeling. Mol Cell Biol. 2003; 23:2151–2161. [PubMed: 12612085]
32. Benjamini YHY. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Scoiety. 1995; 57:289–300.
33. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A. 2005; 102:15545–15550. [PubMed: 16199517]
34. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, et al. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet. 2003; 34:267–273. [PubMed: 12808457]
35. Silva CM. Role of STATs as downstream signal transducers in Src family kinase-mediated tumorigenesis. Oncogene. 2004; 23:8017–8023. [PubMed: 15489919]
36. Pardanani A, Lasho T, Smith G, Burns CJ, Fantino E, Tefferi A. CYT387, a selective JAK1/JAK2 inhibitor: in vitro assessment of kinase selectivity and preclinical studies using cell lines and primary cells from polycythemia vera patients. Leukemia. 2009; 23:1441–1445. [PubMed: 19295546]
37. Tyner JW, Bumm TG, Deininger J, Wood L, Aichberger KJ, Loriaux MM, et al. CYT387, a novel JAK2 inhibitor, induces hematologic responses and normalizes inflammatory cytokines in murine myeloproliferative neoplasms. Blood. 2010; 115:5232–5240. [PubMed: 20385788]
38. Lu Z, Hunter T. Degradation of activated protein kinases by ubiquitination. Annu Rev Biochem. 2009; 78:435–475. [PubMed: 19489726]

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39. Vallabhapurapu S, Matsuzawa A, Zhang W, Tseng PH, Keats JJ, Wang H, et al. Nonredundant and complementary functions of TRAF2 and TRAF3 in a ubiquitination cascade that activates NIK-dependent alternative NF-kappaB signaling. Nat Immunol. 2008; 9:1364–1370. [PubMed: 18997792]
40. Poussin K, Pilati C, Couchy G, Calderaro J, Bioulac-Sage P, Bacq Y, et al. Biochemical and functional analyses of gp130 mutants unveil JAK1 as a novel therapeutic target in human inflammatory hepatocellular adenoma. Oncoimmunology. 2013; 2:e27090. [PubMed: 24501689]
41. Jeong EG, Kim MS, Nam HK, Min CK, Lee S, Chung YJ, et al. Somatic mutations of JAK1 and JAK3 in acute leukemias and solid cancers. Clin Cancer Res. 2008; 14:3716–3721. [PubMed: 18559588]
42. Yu H, Lee H, Herrmann A, Buettner R, Jove R. Revisiting STAT3 signalling in cancer: new and unexpected biological functions. Nat Rev Cancer. 2014; 14:736–746. [PubMed: 25342631]
43. Wen W, Liang W, Wu J, Kowolik CM, Buettner R, Scuto A, et al. Targeting JAK1/STAT3 signaling suppresses tumor progression and metastasis in a peritoneal model of human ovarian cancer. Mol Cancer Ther. 2014; 13:3037–3048. [PubMed: 25319391]
44. Wu ZH, Shi Y. When ubiquitin meets NF-kappaB: a trove for anti-cancer drug development. Curr Pharm Des. 2013; 19:3263–3275. [PubMed: 23151140]
45. Calzado MA, Bacher S, Schmitz ML. NF-kappaB inhibitors for the treatment of inflammatory diseases and cancer. Curr Med Chem. 2007; 14:367–376. [PubMed: 17305539]
46. Baud V, Karin M. Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls. Nat Rev Drug Discov. 2009; 8:33–40. [PubMed: 19116625]
47. Tefferi A. JAK inhibitors for myeloproliferative neoplasms: clarifying facts from myths. Blood. 2012; 119:2721–2730. [PubMed: 22279053]
48. Tefferi A, Pardanani A. JAK inhibitors in myeloproliferative neoplasms: rationale, current data and perspective. Blood Rev. 2011; 25:229–237. [PubMed: 21742423]
49. O'Shea JJ, Kontzias A, Yamaoka K, Tanaka Y, Laurence A. Janus kinase inhibitors in autoimmune diseases. Ann Rheum Dis. 2013; 72(Suppl 2):ii111–ii115. [PubMed: 23532440]

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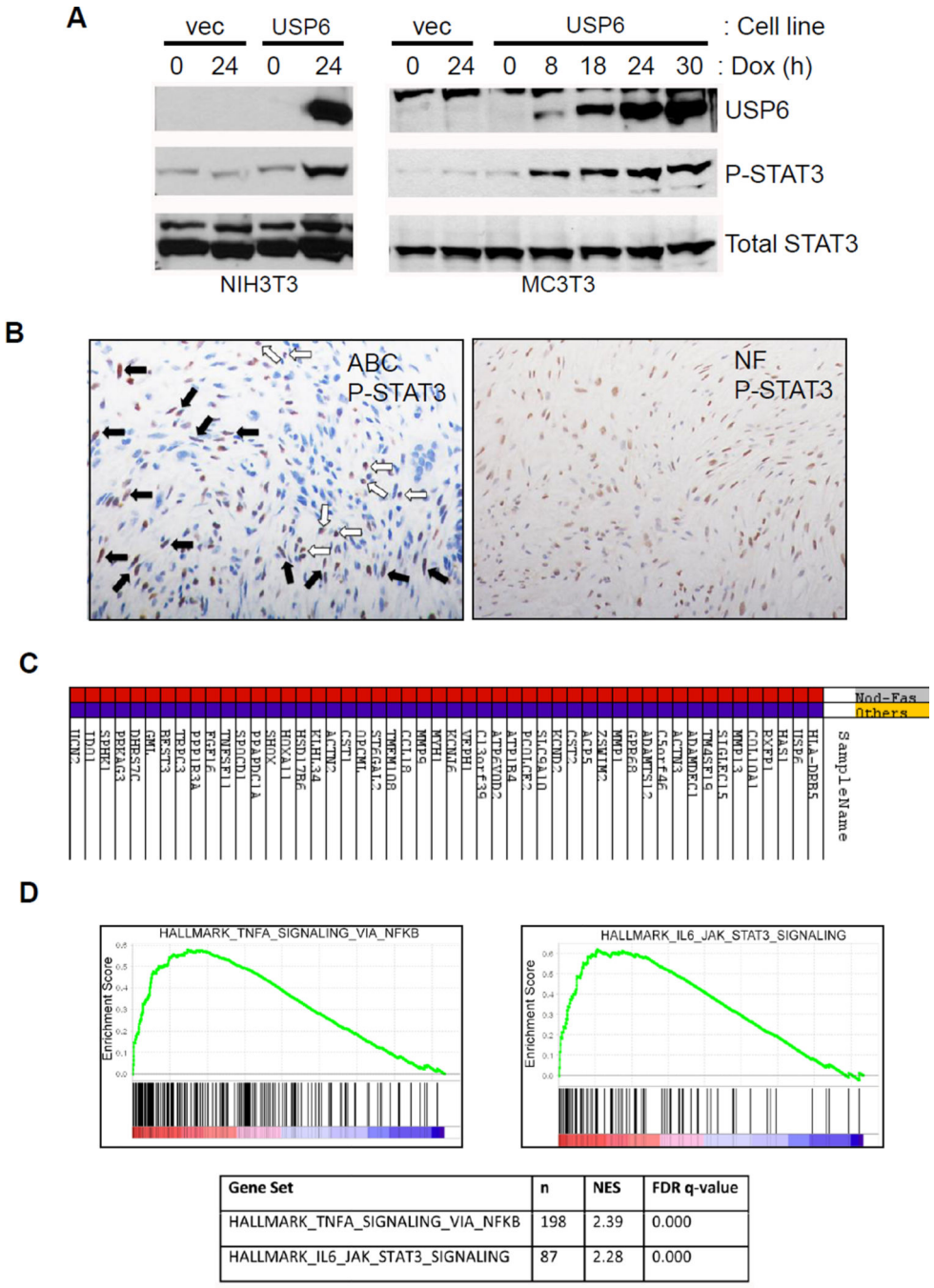

Gene Set	n	NES	FDR q-value
HALLMARK_TNFA_SIGNALING_VIA_NFKB	198	2.39	0.000
HALLMARK_IL6_JAK_STAT3_SIGNALING	87	2.28	0.000

Figure 1. Activation of STAT3 and NF-κB by USP6 in human tumors

A) Stable NIH3T3 or MC3T3 cell lines expressing USP6 or vector were starved, then treated with dox to induce USP6 expression. Lysates were immunoblotted as indicated. P-STAT3 antibody recognizes STAT3 phosphorylated on tyrosine 705 (Y705).

B) ABC (left) and NF (right) with confirmed translocation of *USP6* were subjected to immunohistochemistry using antibody for P-STAT3. In ABC (left), neoplastic cells and inflammatory cells are marked with filled and empty arrows, respectively. In NF, essentially

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all the positively-stained cells are neoplastic cells (with their characteristic plump spindle morphology, bundled in short fascicles).

C) Heatmap of top 50 upregulated genes in NF relative to other mesenchymal tumors (see text for details). Fold-induction of genes in Supplemental Table 2.

D) GSEA confirms activation of STAT3 and NF-κB in NF. The number of genes in each set (n), normalized enrichment score (NES), and statistical significance (FDR-q value) are shown.

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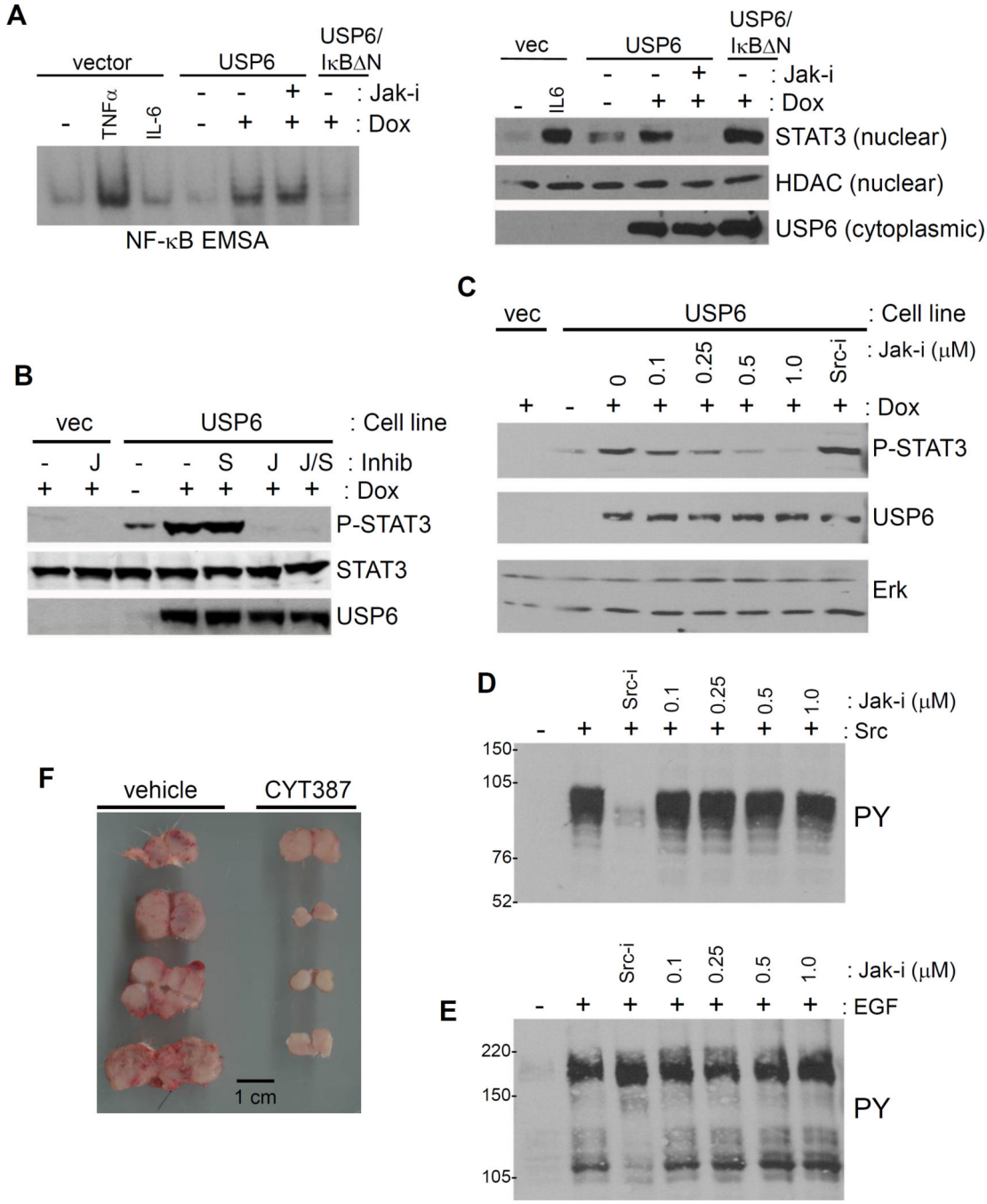

Figure 2. Jak kinases mediate STAT3 activation and tumorigenesis induced by USP6

A) Left: stable NIH3T3 cells expressing USP6 alone or with IκBα Super Repressor (IκBΔN) were subjected to cell fractionation, followed by EMSA to monitor NFκB activity. Cells were treated as indicated. Right: nuclear and cytoplasmic extracts were immunoblotted as indicated. HDAC was used as a nuclear loading control.

B) Control and USP6/NIH3T3 cells were starved in the absence or presence of dox. Inhibitors for Jak family (J; 1uM) and Src family (S; 10 uM) were added for 1 hour prior to lysis.

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C) The indicated NIH3T3 cell line was starved overnight, then treated with the indicated dose of Jak or Src inhibitor for 1h.

D) Hela cells were transfected with constitutively active Src cDNA, then treated with Src inhibitor or indicated dose of Jak inhibitor for 1h. Lysates were blotted with anti-phosphotyrosine (PY).

E) Hela cells were pre-treated with Src inhibitor or indicated dose of Jak inhibitor for 1h, then stimulated with EGF (100ng/ml) for 10min. Lysates were blotted with anti-PY.

F) Pan-Jak inhibitor attenuates USP6-mediated tumorigenesis. NOD-SCID mice were injected subcutaneously with USP6/NIH3T3 cells. Commencing the following day, animals were treated twice daily with vehicle or CYT387 (50 mg/kg). Tumors were harvested after 3 weeks.

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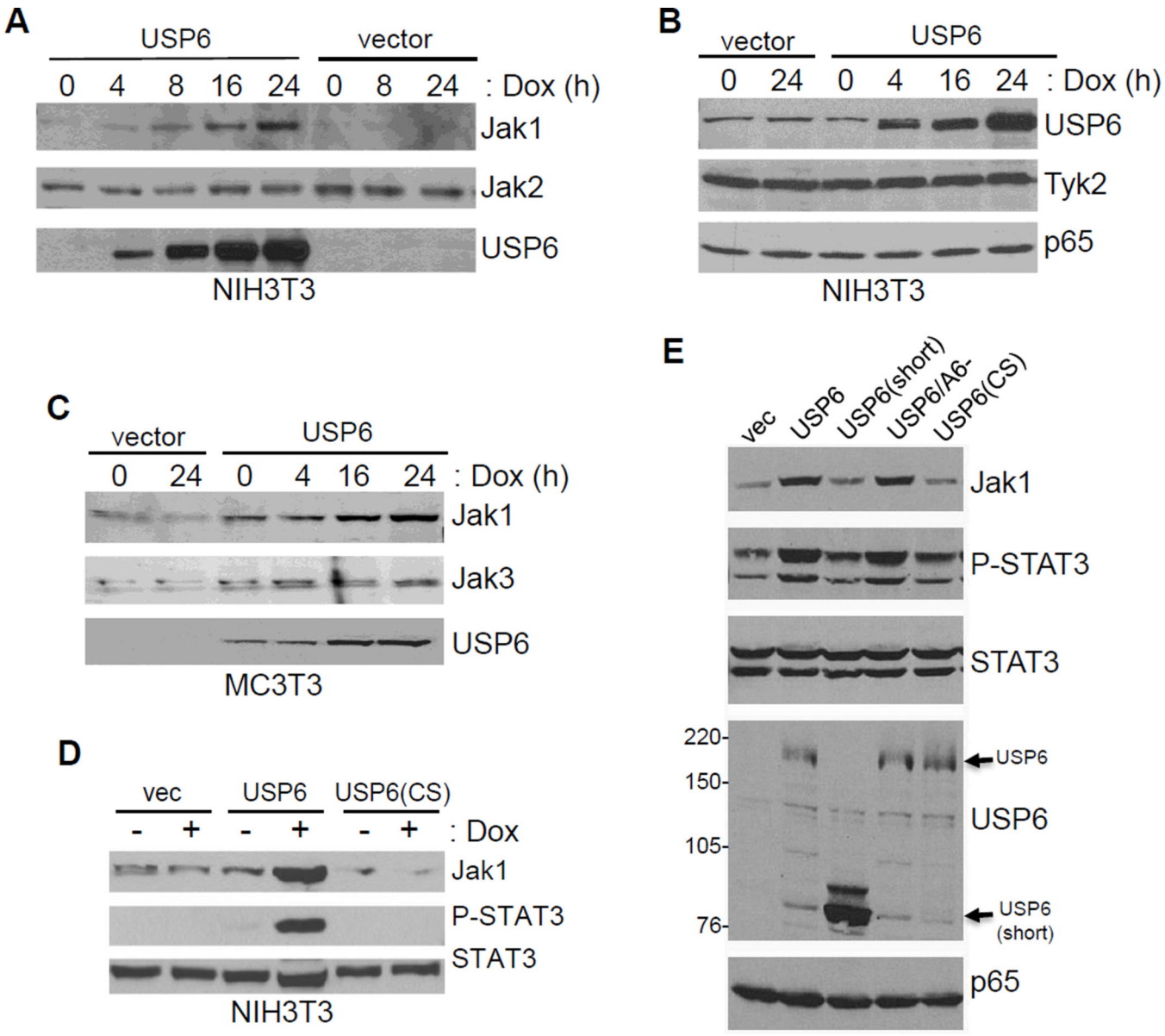

Figure 3. USP6 selectively upregulates Jak1 in a USP-dependent manner

A–C) Stable NIH3T3 or MC3T3 lines expressing USP6 or not were treated with dox and immunoblotted as indicated.

D) Stable NIH3T3 lines expressing WT USP6 or inactive point mutant USP6(CS) were immunoblotted.

E) Hela cells were transiently co-transfected with Jak1 and the indicated USP6 allele, then immunoblotted.

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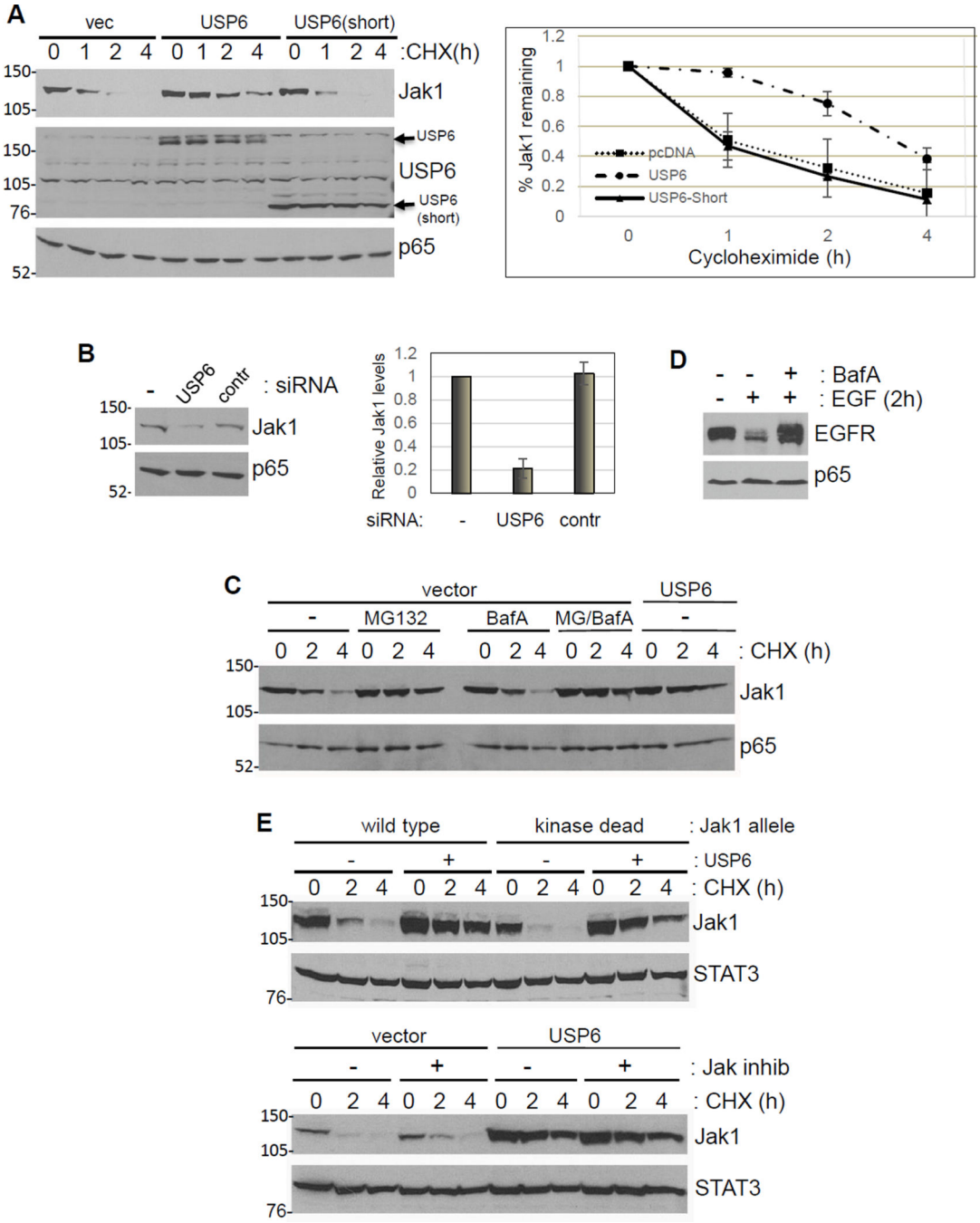

Figure 4. USP6 prolongs Jak1 protein half-life

A) Hela cells were co-transfected with Jak1 and the indicated USP6 allele. Cells were treated with cycloheximide (CHX; 25 μg/ml) for various times. LICOR-based quantification on right.

B) Endogenous Jak1 levels are reduced upon USP6 depletion. Hela cells were transfected with control or USP6 Smartpool siRNA, and Jak1 levels were quantified.

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C) Jak1 turnover is regulated by proteasome. Hela cells were co-transfected with Jak1 plus control vector or USP6. Cells were treated with CHX in the presence of MG132 or Bafilomycin A (BafA) for the indicated times.

D) To confirm functionality of BafA, its ability to block ligand-induced downregulation of EGFR was verified.

E) Stabilization of Jak1 does not require kinase activity. Hela cells were co-transfected with WT or kinase inactive Jak1, together with vector or USP6 as indicated. Cells were treated with CHX in the presence of pan-Jak inhibitor as shown.

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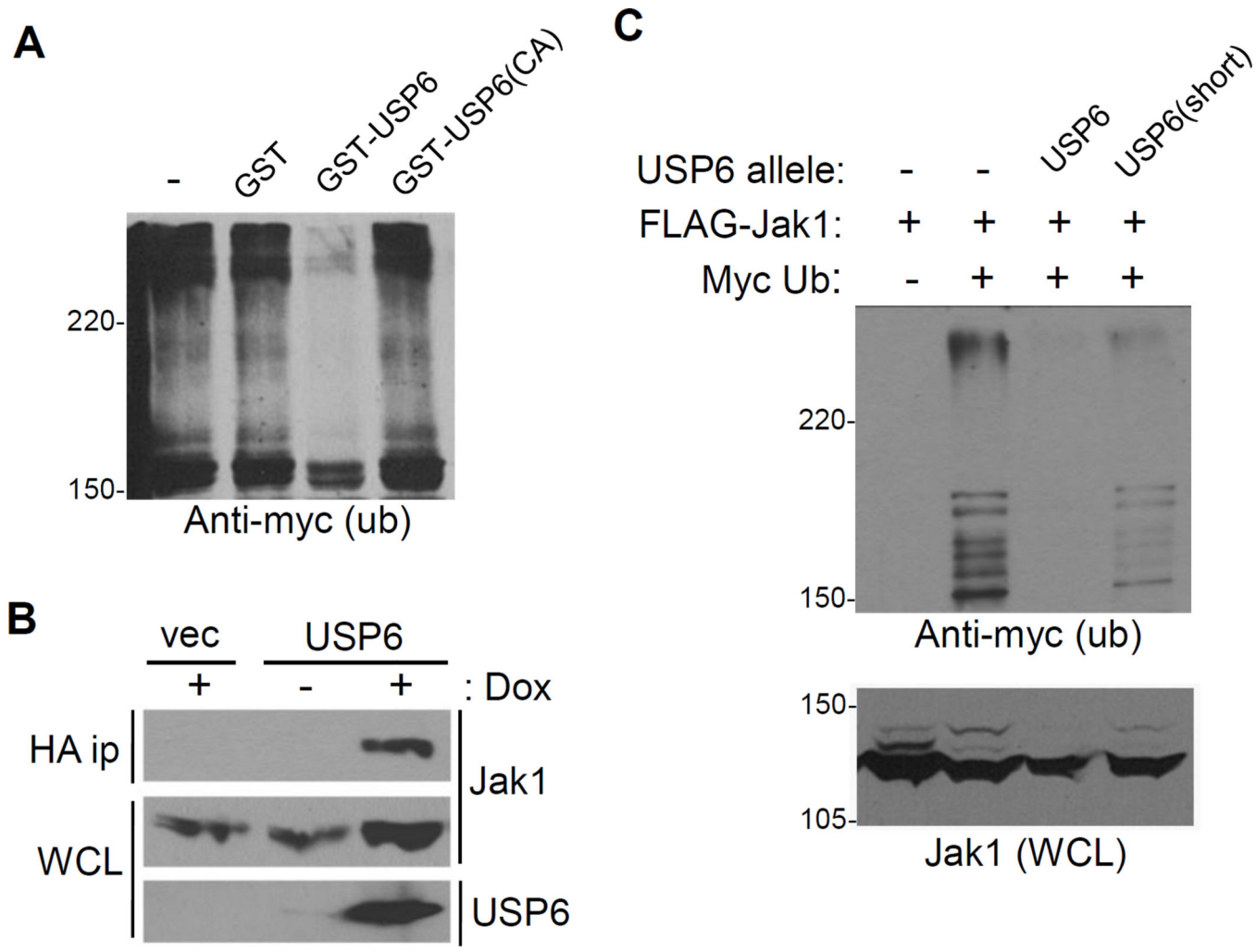

Figure 5. USP6 mediates de-ubiquitination of Jak1 *in vivo* and *in vitro*

A) USP6 directly de-ubiquitinates Jak1 *in vitro*. Hela cells were co-transfected with FLAG-Jak1 and Myc-Ub. After 2.5h MG132 treatment, Jak1 was immunoprecipitated with anti-FLAG. The sample was divided into aliquots, one of which was immediately boiled (−), while the remaining aliquots were incubated with indicated recombinant protein for 1.5h. Ubiquitination remaining on Jak1 was detected by anti-Myc immunoblotting. USP6(CA) is a catalytically inactive point mutant.

B) USP6 co-immunoprecipitates with endogenous Jak1. USP6-expressing or control NIH3T3 cells were treated with dox, subjected to anti-HA immunoprecipitation, then blotted. WCL, whole cell lysate.

C) USP6 promotes de-ubiquitination of Jak1 *in vivo*. Hela cells were co-transfected with FLAG-Jak1 and Myc-Ub, with the indicated USP6 allele. Cells were treated with MG132 (20 μM) for 2.5h, then lysed and subjected to anti-FLAG immunoprecipitation followed by anti-Myc immunoblotting.

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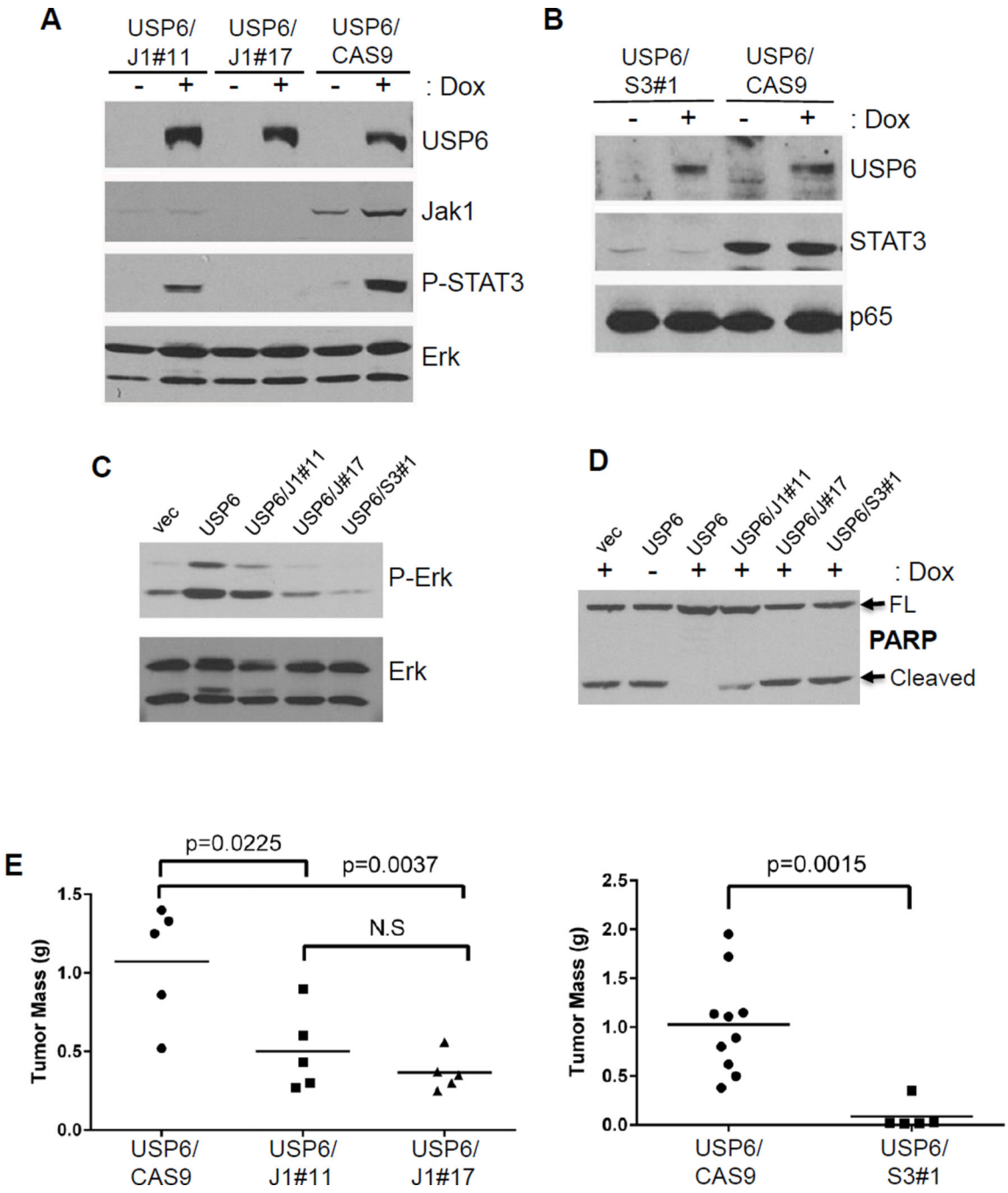

Figure 6. Jak1 and STAT3 are required for USP6-mediated cell survival and tumorigenesis

A,B) Jak1 (left) and STAT3 (right) were targeted in USP6/NIH3T3 using CRISPR; two independent clones (#11 and #17) were analyzed for Jak1. CAS9 alone was introduced as a control. The weak band recognized in STAT3 blot of the STAT3-CRISPR clone is non-specific.

C,D) The indicated cell lines were serum-starved for 1 day, then blotted as shown.

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E) The indicated cell lines were subcutaneously xenografted into NOG/SCID mice. Tumors were harvested 4 weeks later. Left, 5 mice were injected per cell ine; right, 10 mice were injected per cell line.

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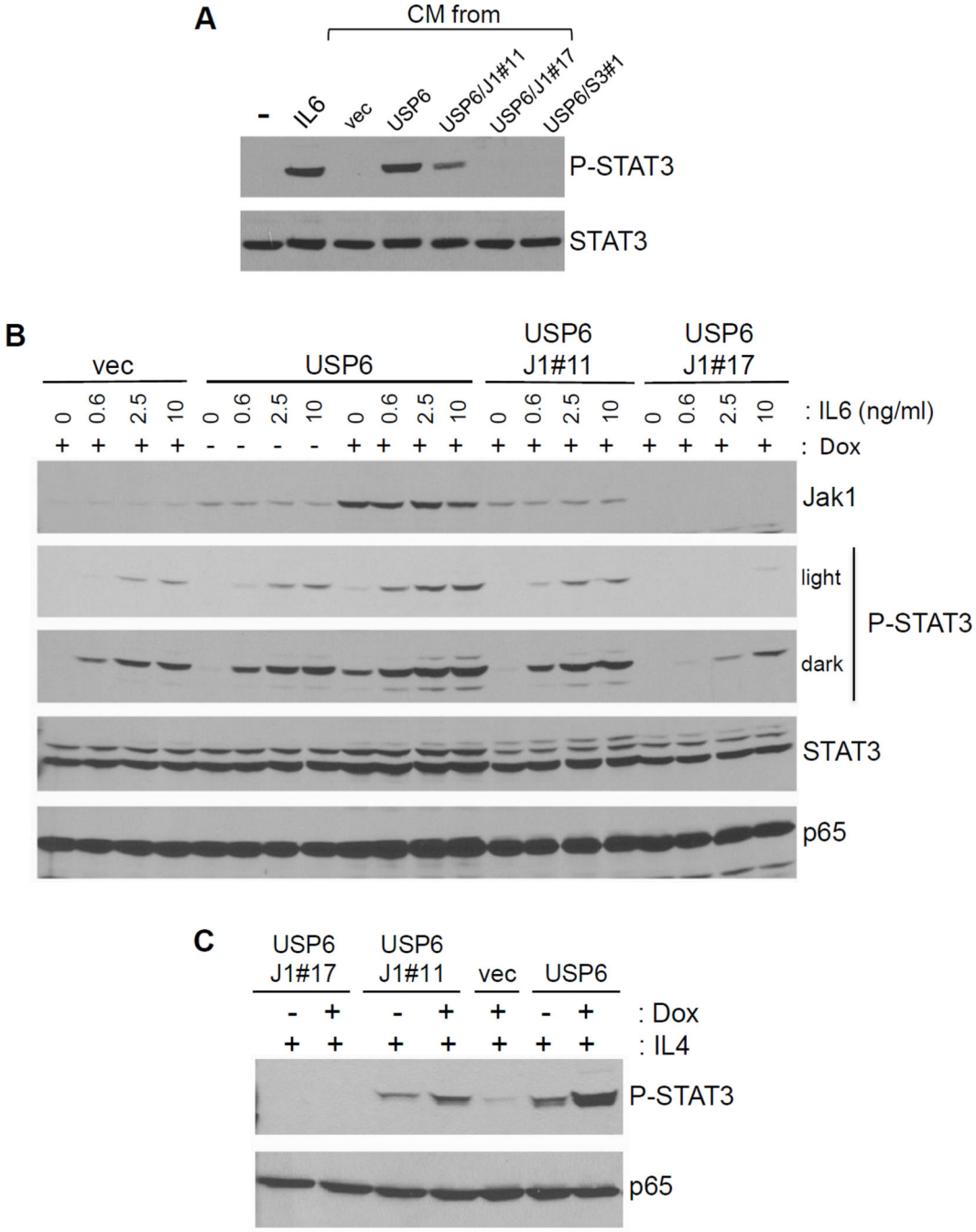

Figure 7. Jak1 levels dictate production of and responsiveness to cytokines

A) Conditioned medium (CM) was collected from the indicated cell lines treated with dox in the absence of serum. Naïve NIH3T3 cells were treated for 30min with the CM or IL6 (10 ng/ml).

B,C) The indicated cell lines were serum-starved for 24h in the absence or presence of dox, then stimulated for 30 min with the indicated cytokine.