ORIGINAL ARTICLE

YES1 Drives Lung Cancer Growth and Progression and Predicts Sensitivity to Dasatinib

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Abstract

Rationale: The characterization of new genetic alterations is essential to assign effective personalized therapies in non–small cell lung cancer (NSCLC). Furthermore, finding stratification biomarkers is essential for successful personalized therapies. Molecular alterations of *YES1*, a member of the SRC (proto-oncogene tyrosine-protein kinase Src) family kinases (SFKs), can be found in a significant subset of patients with lung cancer.

Objectives: To evaluate *YES1* (v-YES-1 Yamaguchi sarcoma viral oncogene homolog 1) genetic alteration as a therapeutic target and predictive biomarker of response to dasatinib in NSCLC.

Methods: Functional significance was evaluated by *in vivo* models of NSCLC and metastasis and patient-derived xenografts. The efficacy of pharmacological and genetic (CRISPR [clustered regularly interspaced short palindromic repeats]/Cas9 [CRISPR-associated protein 9]) YES1 abrogation was also evaluated. *In vitro* functional assays for signaling, survival, and invasion were also performed. The association between *YES1* alterations and prognosis was evaluated in clinical samples.

Measurements and Main Results: We demonstrated that YES1 is essential for NSCLC carcinogenesis. Furthermore, YES1 overexpression induced metastatic spread in preclinical *in vivo* models. YES1 genetic depletion by CRISPR/Cas9 technology significantly reduced tumor growth and metastasis. YES1 effects were mainly driven by mTOR (mammalian target of rapamycin) signaling. Interestingly, cell lines and patient-derived xenograft models with *YES1* gene amplifications presented a high sensitivity to dasatinib, an SFK inhibitor, pointing out YES1 status as a stratification biomarker for dasatinib response. Moreover, high YES1 protein expression was an independent predictor for poor prognosis in patients with lung cancer.

Conclusions: YES1 is a promising therapeutic target in lung cancer. Our results provide support for the clinical evaluation of dasatinib treatment in a selected subset of patients using YES1 status as predictive biomarker for therapy.

Keywords: dasatinib; YES1; Src family kinases; predictive biomarker; lung cancer

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At a Glance Commentary

Scientific Knowledge on the Subject: Identifying a subset of patients with specific druggable mutations is critical to developing personalized treatments that will benefit these specific patients over receiving current state-of-the-art nontargeted therapies. In this context, we identify YES1 (v-YES-1 Yamaguchi sarcoma viral oncogene homolog 1) genomic alteration as a potential stratification biomarker that predicts benefit from SFK (SRC [proto-oncogene tyrosine-protein kinase Src] family kinases) inhibitors.

What This Study Adds to the Field: In this article, we address both of these challenges. First, we demonstrate that YES1 genetic alterations constitute a potential therapeutic target in both adenocarcinoma and squamous cell carcinoma subtypes. Second, we have shown that YES1 status (amplification and overexpression) is a predictive marker of response to the SFK inhibitor dasatinib in non–small cell lung cancer cells and patient-derived xenografts. This study suggests that YES1 inhibition may represent a novel therapeutic target to control lung cancer dissemination in a selected subset of patients with genetic alterations of this gene.

The treatment landscape for patients with non–small cell lung cancer (NSCLC) with advanced-stage disease has deeply changed over the last years. Next-generation sequencing techniques have allowed the discovery of driver mutations that can be targeted with specific drugs (1). The identification of gain-of-function mutations in *EGFR* (epidermal growth factor receptor) set the basis for the use of molecular-targeted therapies with EGFR tyrosine kinase inhibitors (2). The discovery of *ALK* (anaplastic lymphoma kinase) rearrangements accelerated approval of specific inhibitors that led to dramatic improvements in the outcome of selected subgroups of patients (1). However, although lung cancer is the paradigm of genomics-driven oncology, there is still a high proportion of patients with NSCLC (around 50%) who cannot benefit from targeted therapies (3). Therefore, the identification of new genetic alterations may expand the population who could benefit from these therapeutic strategies.

Previously, we showed the potential clinical relevance of gene amplification of *YES1* (v-YES-1 Yamaguchi sarcoma viral oncogene homolog 1) as part of a prognostic signature for patients with stage I or II lung adenocarcinoma (ADC) on the basis of copy number (CN) alterations and clinical profiles (4). YES1 is a nonreceptor cytoplasmic tyrosine kinase that belongs to the SRC family kinases (SFKs). This family consists of nine proteins sharing similar structural architecture (5). SRC has been the most studied member of the family (6). Although several SFK inhibitors have been investigated in a variety of tumors, dasatinib is the only one currently approved in clinical practice, indicated for the treatment of patients with leukemia (7). However, SFK inhibitors have not yet demonstrated benefit in the treatment of solid tumors (1).

YES1 is the only member of the SFKs regulated mainly by gene amplification, and a high correlation has been described between gene CN and mRNA expression in different tumors (8). Furthermore, *YES1* gene amplification is a recurrent alteration in lung squamous cell carcinoma (SCC) (8) as well as in other solid tumors (9–11), together with elevated YES1 expression (12–16). However, its role in lung cancer has not been deeply explored yet. Interestingly, *YES1* gene amplification has been recently linked to resistance to EGFR inhibitors in NSCLC cell lines (17) and patients (18, 19).

In the present study, we report that *YES1* amplification, which correlates with YES1 overexpression, is an oncogenic driver alteration in NSCLC that induces tumor growth and metastatic spreading. Accordingly, high YES1 protein expression is an independent predictor of poor outcome in NSCLC. In addition, we show that YES1 status is a predictive marker of response to the SFK inhibitor dasatinib in NSCLC cells and patient-derived xenograft (PDX) models. Therefore, we propose that genetic alterations of *YES1* drive NSCLC progression and define a subset of patients who may potentially benefit from dasatinib treatment.

Some of the results of these studies have been previously reported in the form of abstracts (20–22).

Methods

See the online supplement for additional details.

Patient Samples

A series of 116 patients with NSCLC who underwent surgical resection at the Clínica Universidad de Navarra (CIMA-CUN) from 1999 to 2016. A validation series of 222 patients with NSCLC diagnosed from 2003 to 2008 at the University of Texas MD Anderson Cancer Center was evaluated. Clinicopathologic features of the patients are listed in Table E1 in the online supplement. The inclusion criteria were NSCLC histology, stage I to III, no neoadjuvant or adjuvant chemotherapy or radiotherapy, and absence of cancer within the 5 years previous to lung cancer surgery.

Cell Lines

YES1 status of all cell lines is listed in Table E2.

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Author Contributions: L.M.M. and J.A. conceived the experiments and supervised the work. I.G., M.J.P., C. Behrens, A.B.R., E.F., M.S.-C., and I.I.W. performed immunohistochemistry experiments and analyses. F.H.-P., R.G.-D., J.P.R., and J.M.G.-P. developed the invasion experiments and analyses. I.G., D. Ajona, C. Bértolo, C.S., A.L., K.V., H.M., I.G.-B., D. Alameda, F.L., A.C., and R.P. performed *in vitro* and/or *in vivo* experiments. I.G., I.F., C. Bértolo, C.S. and L.P.-A. developed the PDX experiments. A.L., M.C., and X.R.B. evaluated the YES1 downstream signaling analysis. All authors critically reviewed the manuscript.

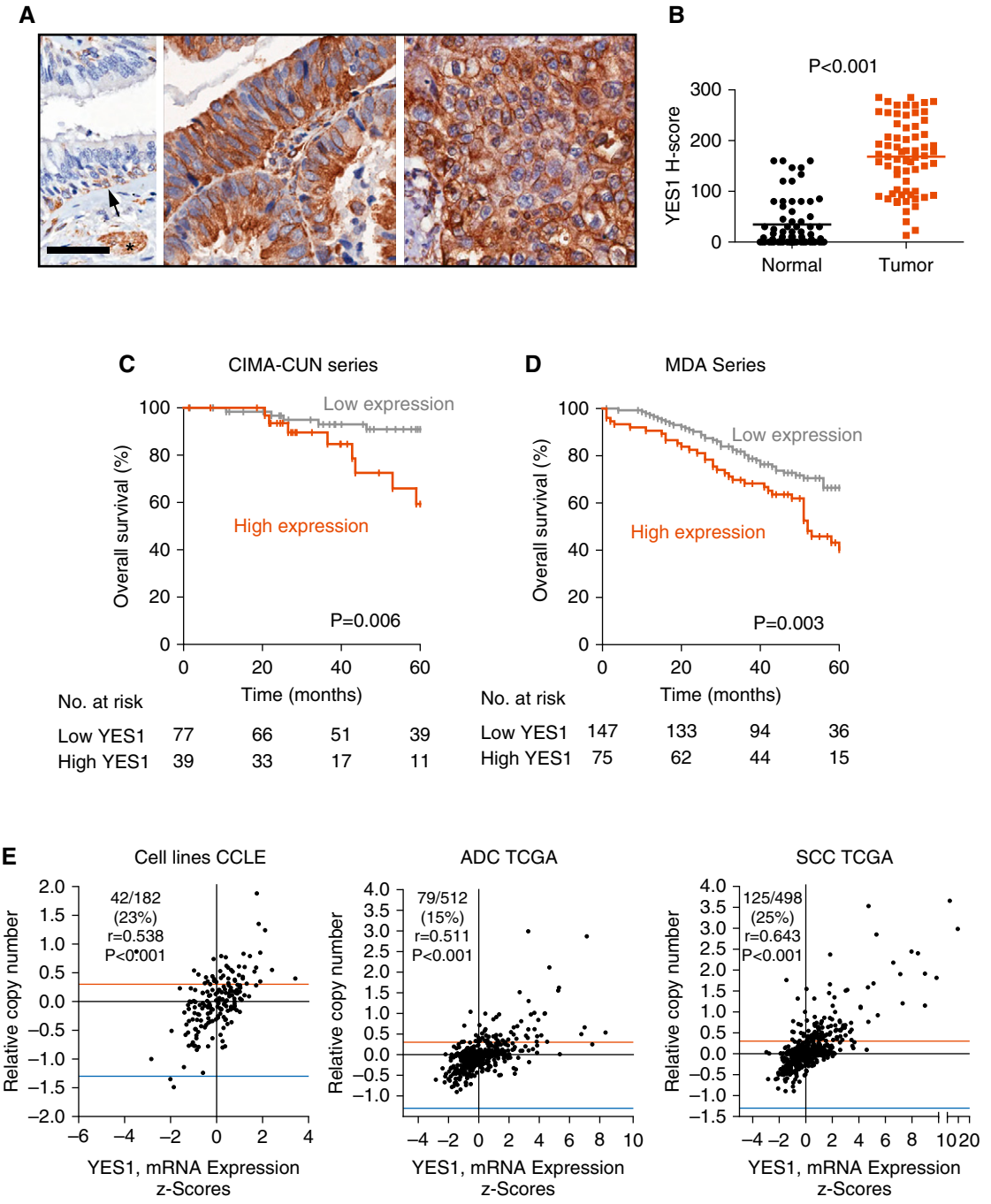

Figure 1. YES1 (v-YES-1 Yamaguchi sarcoma viral oncogene homolog 1) is associated with adverse prognosis in patients with non–small cell lung cancer (NSCLC). (*A*) Representative images of immunohistochemical analysis of YES1 immunostaining in bronchial epithelium (left panel), lung adenocarcinoma (ADC) (middle panel) and lung squamous cell carcinoma (SCC) (right panel). Basal cells (arrow) and smooth muscle cells (asterisk) showed YES1 immunoreactivity. High cytoplasmic (middle panel) and membrane (right panel) YES1 staining can be found in tumor cells. Scale bar, 50 μm. (*B*) YES1 expression was significantly higher in tumor cells than in paired nontumor tissue. (*C* and *D*) High YES1 expression is associated with adverse prognosis in NSCLC: Kaplan-Meier overall survival curves for YES1 expression and log-rank test in the Clínica Universidad de Navarra (CIMA-CUN) series (*C*) and MD Anderson Cancer Center series (*D*). (*E*) *In silico* analysis based on Cancer Cell Line Encyclopedia (CCLE) and The Cancer Genome Atlas (TCGA) data demonstrated a high correlation between gene copy number (CN) and mRNA expression of YES1 in lung cancer cell lines and both patients with ADC and patients with SCC. Red lines define the limit of CN gains ($\log_2$ CN/2 = 0.3; CN > 2.5 copies), and blue lines define the limit of homozygous deletion ($\log_2$ CN/2 = −1.3; CN < 0.8 copies). The Spearman coefficient (*r*) was calculated to evaluate correlation between CN and mRNA expression.

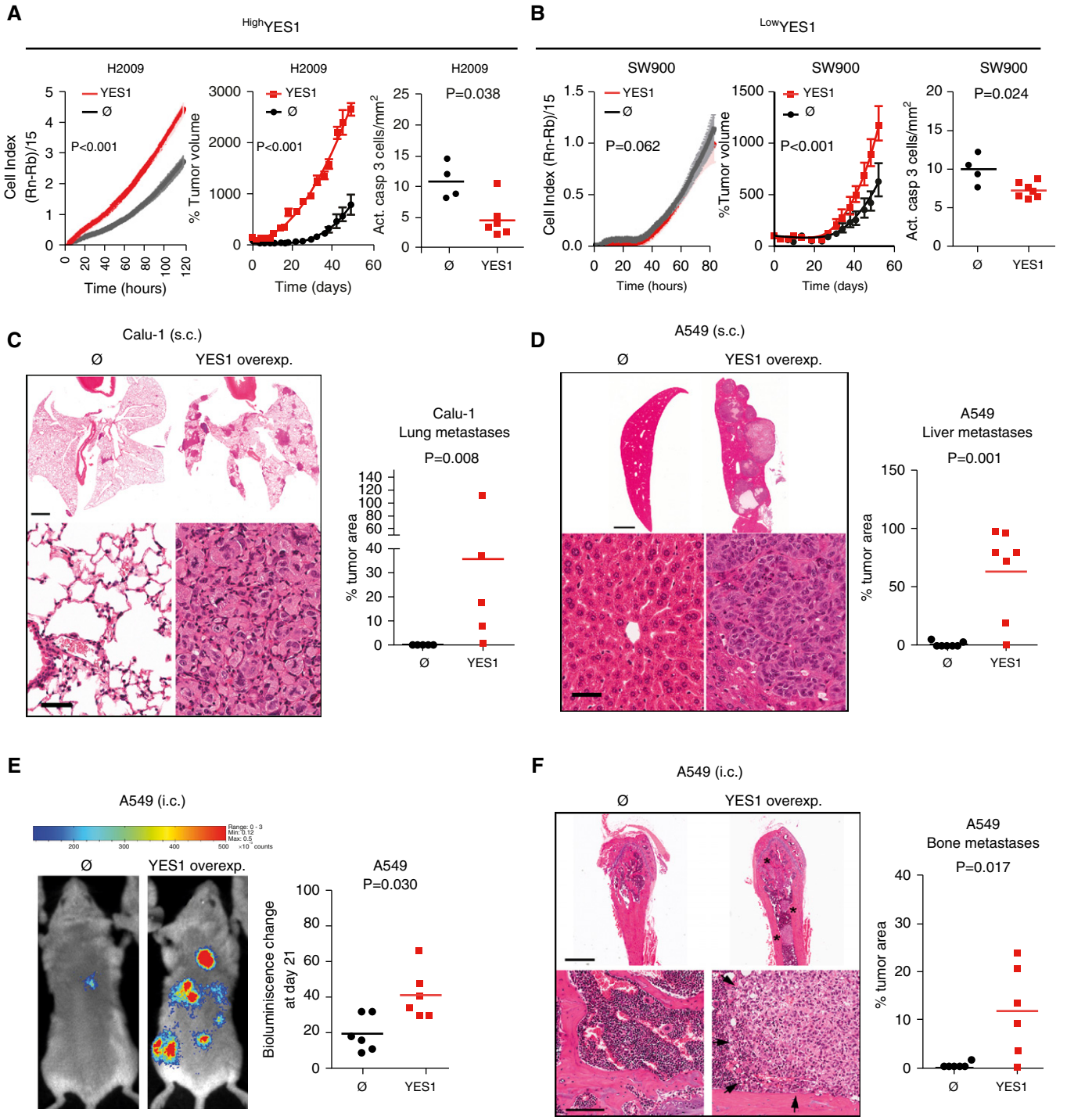

Figure 2. YES1 (v-YES-1 Yamaguchi sarcoma viral oncogene homolog 1) overexpression induces tumor growth and metastatic spreading. (*A* and *B*) Effect of constitutive YES1 overexpression in H2009 (*A*) and SW900 (*B*) cell lines. Left panels: YES1 overexpression induces *in vitro* cell proliferation only in ^{High}YES1 cells as measured by xCELLigence real-time cell analyzer system. The experiments were done in triplicate and repeated three times. Middle panels: *in vivo* tumor growth of subcutaneously injected cells overexpressing YES1 was induced in both ^{High}YES1 and ^{Low}YES1 cell lines. Data are presented as mean ± SE ($n \geq 4$ for all groups), and differences among groups were compared with the extra-sum-of-squares *F* test. Right panels: YES1 overexpression reduced the number of apoptotic cells in subcutaneous tumors as measured by the number of positive activated (cleaved) caspase 3 cells per area. Empty vector transduced cells (Ø) were used as controls. $n \geq 4$ for all groups. (*C*) Subcutaneous (s.c.) tumors of Calu-1 cells overexpressing YES1 were able to metastasize to the lungs. Left panel, top: Representative histological images of metastatic growth in the lungs (scale bar, 2 mm). Left panel, bottom: higher-magnification images (scale bar, 50 μm). Right panel: metastatic area in the lungs of mice subcutaneously injected with control or YES1-overexpressing Calu-1 cells ($n = 5$ for all groups). Tumor area was measured by using ImageJ analysis program. (*D*) YES1-overexpressing A549

Immunohistochemistry

Two independent observers evaluated the intensity (1: weak staining; 2: moderate staining; 3: strong staining) and extensiveness (the percentage of positive cells) of staining in all of the study samples. The evaluation of YES1 expression was performed using the H-score system as previously described (23). Briefly, H-score was calculated by adding the products of each intensity and the corresponding area. The range of possible scores was from 0 to 300, and expression level was categorized as low and high using the upper tertile of the H-score.

Real-Time Quantitative PCR and Western Blotting

Details are provided in the online supplement.

YES1 Overexpression

Details are provided in the online supplement.

YES1 Stable Knockdown by CRISPR/Cas9 Editing

Details are provided in the online supplement.

siRNAs

Cells were transfected with two commercial siRNAs against YES1 (Invitrogen).

MTT and Clonogenic Assays

Details are provided in the online supplement.

xCELLigence Proliferation Assay

The xCELLigence real-time cell analyzer system (Roche Applied Science) was used to analyze cell proliferation. Each condition was performed in triplicate.

Apoptosis Analyses

Apoptosis was measured by annexin V–propidium iodide double staining as previously described (24).

Three-Dimensional Spheroid Invasion Assay

The procedure was performed as previously described (25).

In Vivo Mouse Models

Cells were subcutaneously injected in $Rag2^{-/-}IL2R\gamma^{-/-}$ mice and treated daily by oral gavage with 60 mg/kg dasatinib (kindly provided by Bristol-Myers Squibb) or vehicle (80 mM citric acid, pH 2.1). PDX models were generated in Dr. Paz-Ares' laboratory and selected depending on histology and YES1 expression (Table E2). PDX tumors were treated with dasatinib or vehicle as previously described.

Analysis of Metastatic Progression

Metastatic potential of the A549-TM-YES1 (bioluminescence reporter vector) was evaluated by subcutaneous or intracardiac injection, as previously described (26).

Statistical Analysis

Correlation between *YES1* CN and mRNA expression was evaluated by the Spearman rank test. Data obtained from cleaved-caspase 3 and Ki67 immunohistochemistry, analyses of metastases, and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and clonogenic assays were analyzed by the Mann-Whitney *U* test. Tumor growth and cell proliferation data were fitted and compared using the extra-sum-of-squares *F* test (27). Kaplan-Meier survival curves and the log-rank test were used to examine differences in overall survival (OS) (defined as the date of first surgery to the last follow-up visit or to disease-related death). Follow-up period was restricted to 60 months in all cohorts. Multivariate analysis was performed using the Cox proportional hazards model. Statistical analysis was performed using Prism 5 software (GraphPad Inc.) and SPSS 22.0 (IBM).

Results

YES1 is Amplified in Lung Cancer and Its Expression Correlates with Poor Clinical Outcome in Patients with NSCLC

We first evaluated the expression of YES1 by immunohistochemistry in a cohort of 116 patients with NSCLC from CIMA-CUN. Low or negative YES1 staining was found in the cytoplasm of normal lung bronchiolar cells, with slightly higher levels in basal cells of the epithelium (Figure 1A, left panel). In tumor cells, cytoplasmic YES1 staining was found in all tumors (Figure 1A, middle and right panels), although membrane expression was also detected in some cases (Figure 1A, right panel). YES1 expression was significantly higher in tumor cells than in paired nonmalignant tissue (Figure 1B; $P < 0.001$).

To determine whether YES1 expression predicted lung cancer–related survival, univariate and multivariate analyses were performed in the CIMA-CUN series. Patients with high YES1 protein expression showed significantly shorter OS ($P = 0.006$; Figure 1C). Multivariate Cox regression analysis revealed that high YES1 protein expression was an independent predictor of shorter OS (hazard ratio, 0.198; confidence interval, 0.063–0.629; $P = 0.006$) (Table E3). We validated the association between YES1 protein expression and OS in an independent cohort of 222 patients from the MD Anderson Cancer Center (Figure 1D). A strong association between high YES1 protein expression and poor survival was found ($P = 0.003$). More importantly, the multivariate analysis validated high YES1 expression as an independent poor prognostic factor in patients with NSCLC (hazard ratio, 0.541; confidence interval, 0.337–0.869; $P = 0.011$) (Table E3).

We next evaluated the frequency of *YES1* alterations in NSCLC cell lines and patients using public data from the Cancer Cell Line Encyclopedia project (28) and The Cancer Genome Atlas study (TCGA) (29) (Figure 1E). In the Cancer Cell Line Encyclopedia dataset, 23% of cell lines showed *YES1* amplification. Moreover, in the TCGA dataset of patients with NSCLC, *YES1* gene amplification was found in 15% of ADC tumors and 25% of SCC. Interestingly, we found a significant positive correlation between *YES1* gene CN

Figure 2. (Continued). subcutaneous tumors showed a significant increase in liver metastasis. In the left panels, representative hematoxylin and eosin staining showed the presence of tumor metastases only in the livers of mice harboring YES1-overexpressing cells (scale bars: top panel, 2 mm; bottom panel, 50 μm). Right panel: metastatic burden was measured as reported in C. $n \geq 7$ for all groups. (*E*) In the intracardiac (i.c.) injection model, bioluminescence images evidenced the presence of distant metastases in A549-TM-YES1 inoculated mice. Left panel: representative bioluminescence images after i.c. inoculation of A549-TM-YES1 or control A549-TM-Ø cells. Right panel: tumor burden assessment of i.c. injected mice ($n = 6$ per group). (*F*) Histological analyses of the bones demonstrated the induction of bone metastasis in the i.c. YES1 A549 model. Left panel: representative histological images of bones from control mice (empty vector A549-TM-Ø) or A549-TM-YES1 mice. Bone metastases (asterisks and arrows) were only present in mice bearing A549 cells overexpressing YES1. Scale bars: 1 mm (top), 200 μm (bottom). Right panel: quantification of bone metastases in i.c. inoculated mice ($n = 6$ per group).

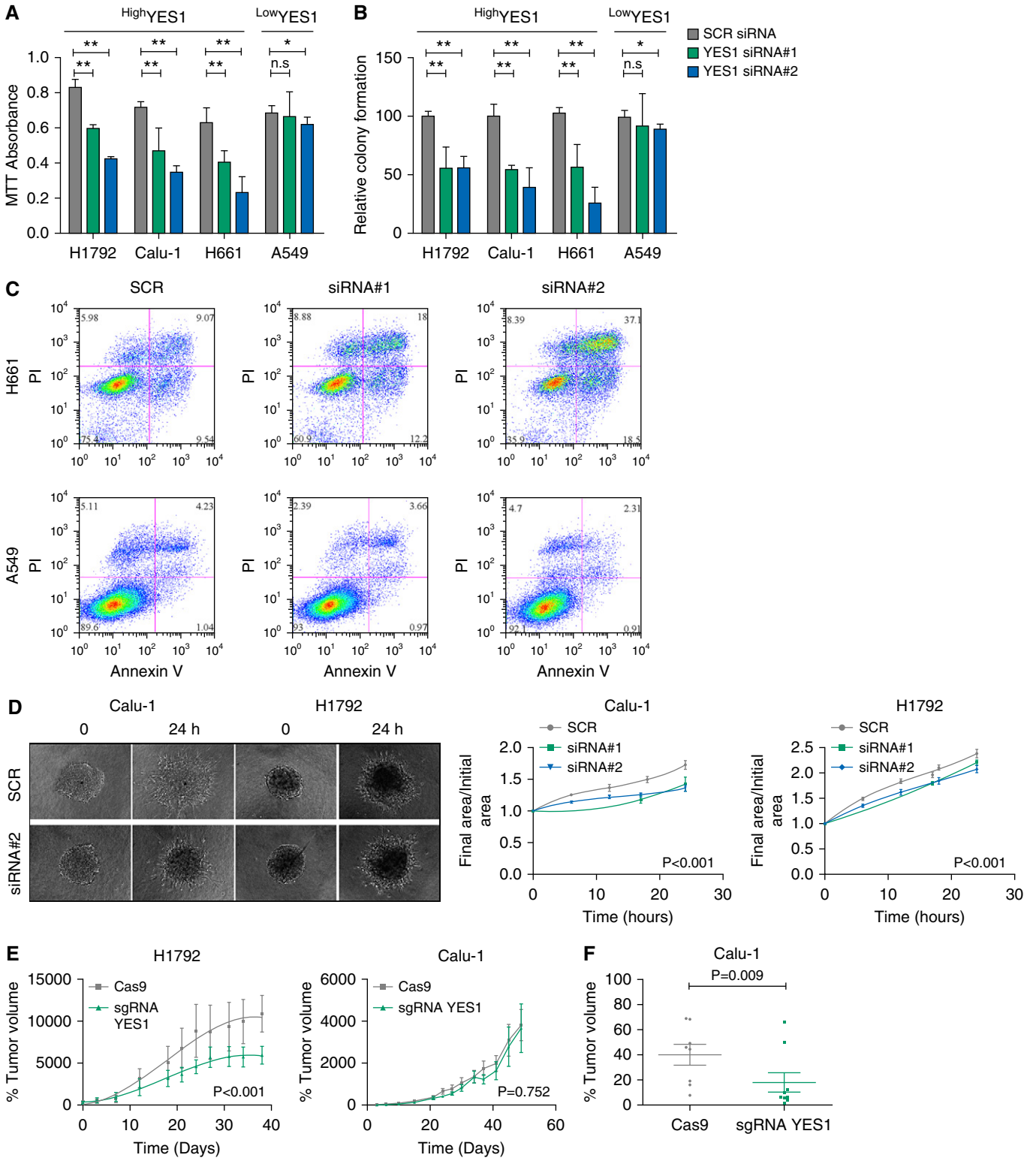

Figure 3. YES1 (v-YES-1 Yamaguchi sarcoma viral oncogene homolog 1) knockdown selectively regulates cell proliferation, apoptosis, and invasion in non–small-cell lung cancer (NSCLC) cell lines. (*A* and *B*) Knockdown of YES1 expression by two specific siRNAs selectively impairs cell proliferation (*A*) and colony formation (*B*) in ^{High}YES1 NSCLC cell lines. Data are presented as median ± interquartile range from at least three independent experiments. $*P < 0.05$ and $**P < 0.001$. (*C*) Representative images of flow cytometry analysis showing annexin V and propidium iodide (PI) staining. In the ^{High}YES1 cell line H661, siRNA treatment induced an increase in the percentage of early (annexin V^+/PI^-) and late apoptotic or necrotic cells (annexin V^+/PI^+). Conversely, no differences were found in the ^{Low}YES1 A549 cell line treated with control siRNA (SCR) or YES1-specific siRNAs (#1 and #2). (*D*) YES1

and mRNA expression in lung cancer cell lines ($r = 0.538$; $P < 0.001$) and in patients, in both ADC ($r = 0.511$; $P < 0.001$) and SCC ($r = 0.643$; $P < 0.001$). To analyze whether *YES1* amplification co-occurred with the most frequent genomic alterations found in NSCLC, an *in silico* analysis of data from cBioPortal (30) was performed. No significant co-occurrences were found between *YES1* amplification and other studied alterations in genes, including *ALK*, *ROS1* (ROS proto-oncogene 1), *BRAF* (serine/threonine-protein kinase B-raf), *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog), *FGFR1* (fibroblast growth factor receptor 1), and *TP53* (tumor protein p53), with the exception of *DDR2* (discoidin domain receptor tyrosine kinase 2) amplification, which was found in 4 out of 2,621 patients with ADC ($P = 0.002$), and *EGFR* ($P = 0.009$) and *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) ($P = 0.008$) amplifications in 8 and 26 out of 1,176 patients with SCC, respectively.

These results demonstrate the prognostic value of YES1 protein expression in patients with NSCLC and indicate that genetic alterations of *YES1* can be found in a highly significant subset of patients with NSCLC.

YES1 Overexpression Significantly Induces Tumor Growth and Metastatic Spreading in *In Vitro* and *In Vivo* NSCLC Models

To evaluate the protumorigenic role of YES1, stable YES1 overexpression was induced in lung cancer cell lines of different histological subtypes (ADC: H1792, H2009, A549, and SCC: Calu-1, SW900) (Figure E1). Constitutive YES1 overexpression significantly enhanced proliferation in cells harboring basal *YES1* amplification and high protein expression (^{High}YES1 cell lines, Table E2) (Figure 2A, left panel; Figure E2A). In marked contrast, no effect on cell proliferation was observed in cells without basal *YES1* amplification (^{Low}YES1 cell lines, Table E2) (Figure 2B, left panel; Figure E2A).

We next evaluated the effect of YES1 overexpression *in vivo*. Subcutaneous models showed larger tumors from cells constitutively overexpressing YES1 as compared with control cells in all but one cell line (Calu-1, which showed an inverse correlation), independently of the basal status of YES1 (Figures 2A and 2B, middle panels; Figure E2B). Although no differences were observed in tumor proliferation (Figure E2C), YES1-overexpressing tumors showed a significant reduction in apoptosis, measured by cleaved-caspase 3 staining (Figures 2A and 2B, right panels; Figure E2D).

We further evaluated the presence of distant metastasis in NSCLC subcutaneous xenografts. In YES1-overexpressing Calu-1 cells, a clear induction of metastatic growth was found in the lungs (Figure 2C; $P = 0.008$). In fact, no metastases were found in the control group. In the case of A549, both control and YES1-overexpressing cells metastasized to the lungs (Figure E3A). Notably, a significant induction of liver metastases in YES1-overexpressing cells was observed (Figure 2D; $P = 0.001$). No metastatic growth was found in the mice injected with H2009, H1792, and SW900 cell lines. We next monitored metastatic burden by bioluminescence using A549 cells overexpressing YES1 and luciferase (A549-TM-YES1). We confirmed that both control and YES1-overexpressing cells displayed lung metastases (Figure E3B). Furthermore, we verified the relevance of YES1 in metastatic spreading and found a significantly higher bioluminescence signal in the livers of A549-TM-YES1 mice than in control mice (Figure E3C; $P = 0.041$). To further investigate the prometastatic activity of YES1 in lung cancer cells, we assessed an additional model of intracardiac injection and bone metastasis. Intracardiac inoculation of A549-TM-YES1 cells induced an increase in total tumor burden (Figure 2E; $P = 0.030$). Notably, bone metastases were only present in mice inoculated with YES1-overexpressing A549 cells (Figure 2F; $P = 0.017$).

These findings show that YES1 facilitates the spreading of tumor cells and suggest that YES1 expression may have a relevant role in the regulation of the metastatic potential of NSCLC.

YES1 Inhibition Selectively Impairs Proliferation, Survival, and Invasion of NSCLC Cells

We then explored the therapeutic potential of YES1, hypothesizing that ^{High}YES1 cells would be more sensitive to the depletion of the endogenous YES1 transcript. We first validated YES1 knockdown by two specific siRNAs (siRNA#1 and #2) (Figures E4A and E4B) and verified that SRC, FYN (FYN oncogene related to SRC), and LYN (v-YES-1 yamaguchi sarcoma viral-related oncogene homolog) expression was unaffected (Figures E4B and E4C). We found that YES1 inhibition significantly reduced cell proliferation and colony formation in ^{High}YES1 lung cancer cell lines, in clear contrast to the effect observed in ^{Low}YES1 cells (Figures 3A and 3B). Accordingly, in ^{Low}YES1 A549 and SW900 cell lines, apoptosis was not affected by YES1 downregulation, in contrast with ^{High}YES1 H2009, H1792, and H661 cells (Figure 3C and Table E4). In ^{High}YES1 Calu-1 and HCC95 cell lines, although apoptosis was not induced by YES1-specific siRNA transfection, this treatment seemed to be associated with increased autophagy levels, as measured by LC3 expression (Figure E5). We then measured the effect of YES1 downregulation on cell invasion by three-dimensional spheroid invasion assays, selecting those cell lines that were able to form regular spheres. YES1 knockdown inhibited the invasive potential in Calu-1 and H1792 cell lines (^{High}YES) (Figure 3D). We next sought to evaluate whether specific YES1 knockout by CRISPR

Figure 3. (Continued). downregulation inhibited three-dimensional (3D) invasion. Representative images from the 3D invasion assays of NSCLC cell spheroids embedded into a collagen matrix treated with control (SCR) or YES1-specific siRNAs (#1 and #2). The invasive area was determined by calculating the difference between the final area and the initial area ($t = 0$) using ImageJ analysis program. All data were normalized to control-transfected cells. Data are presented as mean ± SE from at least three independent experiments, and the differences were compared with the extra-sum-of-squares *F* test. (*E*) YES1 abrogation by CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated protein 9) editing reduced *in vivo* tumor growth of subcutaneously injected ^{High}YES1 H1792 cells, whereas no effect was observed in ^{High}YES1 Calu-1 cell line. Data are presented as mean ± SE ($n \geq 5$ for all groups) and the extra-sum-of-squares *F* test was used to compare tumor growth differences among groups. (*F*) Metastatic area in the lungs of mice subcutaneously injected with Calu-1 cells ($n \geq 8$ for all groups). Tumor area was measured by using ImageJ analysis program. Metastatic growth in the lungs of Calu-1–injected mice was significantly inhibited by YES1 depletion as compared with control cells (Cas9). MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; sgRNA = single-guide RNA.

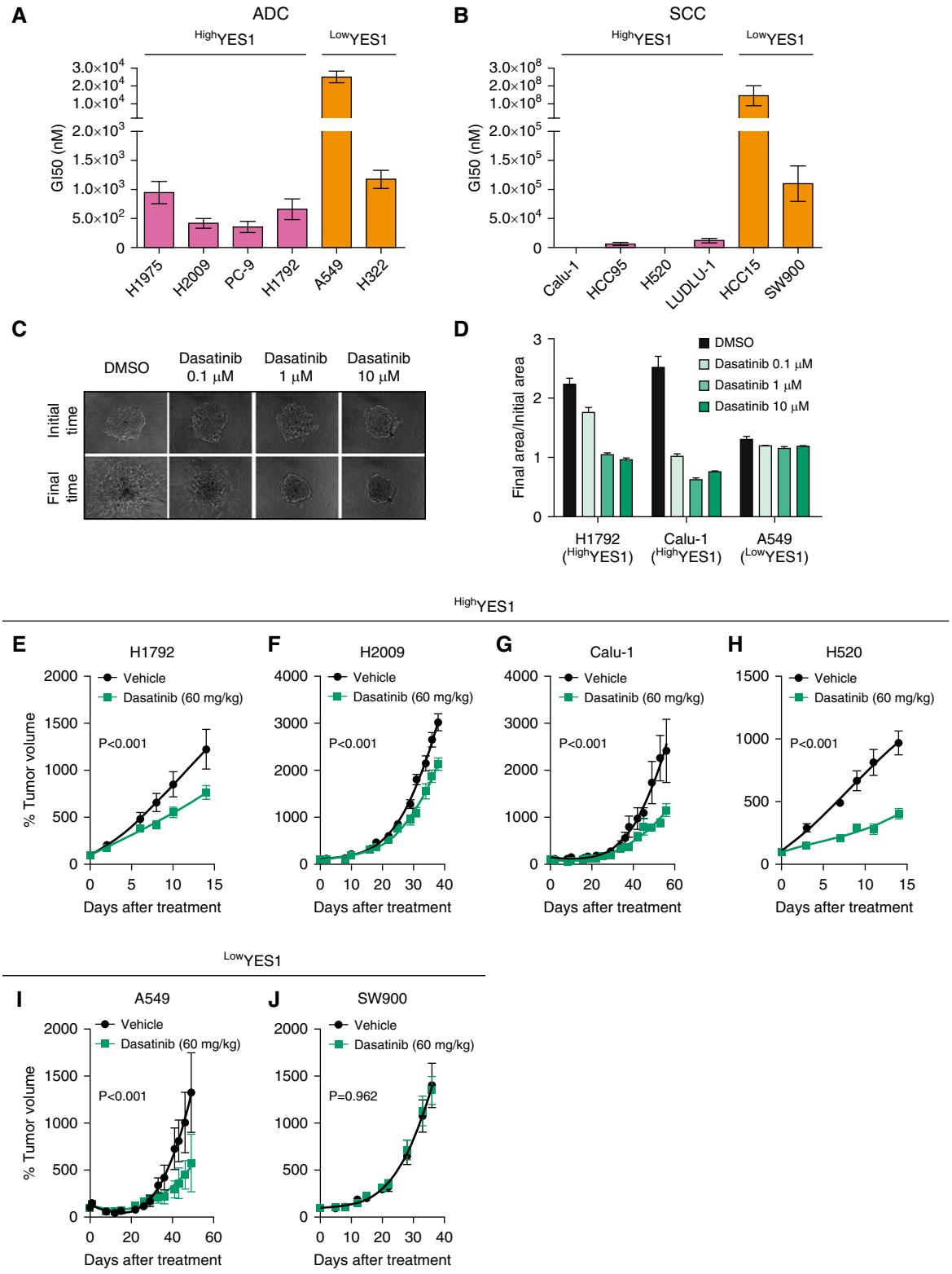

Figure 4. Dasatinib selectively and effectively blocks high YES1 (v-YES-1 Yamaguchi sarcoma viral oncogene homolog 1) cell line proliferation and invasion, as well as tumor growth in xenograft models. (*A* and *B*) Pharmacologic inhibition of YES1 by dasatinib significantly inhibited proliferation in ^{High}YES1 cell lines, whereas ^{Low}YES1 cells were more resistant to the treatment in both adenocarcinoma (ADC) (*A*) and squamous cell carcinoma (SCC) (*B*) subtypes. Cells were treated with increasing concentrations of dasatinib (10, 100, 1,000, 10,000, and 50,000 nM), and GI50 was calculated based on cell proliferation curves obtained from MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays (Figure E8). Data are presented as

(clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated protein 9) editing technology affects tumor growth *in vivo*. YES1 knockdown in ^{High}YES1 H1792 and Calu-1 cell lines was confirmed by real-time quantitative PCR and Western blotting (Figure E6). YES1-depleted H1792 cell line significantly reduced tumor growth in subcutaneously injected mice (Figure 3E). In contrast, YES1 abrogation did not affect tumor growth of the Calu-1 cell line (Figure 3E). Interestingly, the histological examination of lungs and liver of these mice demonstrated a significant reduction of metastatic growth in the lungs of Calu-1–injected mice (Figure 3F); no metastases were found in the rest of analyzed samples. All these results strongly suggest that basal YES1 levels determine the antiproliferative and anti-invasive effects of YES1 downregulation.

To characterize the molecular mechanisms underlying the tumor-driving capabilities of YES1, we investigated the downstream signaling events triggered by YES1 (Figures E7A and E7B). We focused on the mTOR (mammalian target of rapamycin) pathway, as YES1 has been reported to be involved in AKT (protein kinase B [PKB]/Akt) phosphorylation (31). Although p4EBP1 expression was not affected by YES1 expression, AKT phosphorylation (serine 473) and phosphorylation of the mTOR downstream effector S6K were downregulated in siRNA-treated cells (Figure E7A). Consequently, YES1 overexpression led to an increase in phospho-S6K (Figure E7B). Together, these data suggest that YES1 sustains mTOR pathway activity. The RAS/MAPK (Ras- or Mitogen-activated protein kinase) pathway was not altered by YES1 expression, because no ERK phosphorylation alterations were found.

Pharmacological Inhibition of YES1 Impairs *In Vitro* and *In Vivo* Lung Cancer Growth

To evaluate the feasibility of YES1 as a therapeutic target, we tested the effect of the SFK inhibitor dasatinib, a currently approved multitarget tyrosine kinase inhibitor that inhibits YES1 at nanomolar concentrations (32).

We first assessed the *in vitro* effect of dasatinib on cell proliferation in high and low YES1 cell lines (Figures 4A and 4B). Interestingly, in both ADC and SCC cell lines, dasatinib treatment inhibited proliferation in ^{High}YES1 cell lines, whereas ^{Low}YES1 cells were more resistant to dasatinib treatment (Figures 4A and 4B; Figure E8). We also investigated the effect of YES1 pharmacological inhibition on *in vitro* cell invasion. Consistently, we found that dasatinib significantly blocked three-dimensional migration in ^{High}YES1 cells (Calu-1 and H1792) in a dose-dependent manner (Figures 4C and 4D), whereas invasive capabilities of ^{Low}YES1 A549 cell line were not affected by dasatinib treatment (Figure 4D). These results suggest that YES1 status predicts the efficacy of dasatinib *in vitro*.

We next evaluated the pharmacological effectiveness of dasatinib in mice bearing subcutaneous tumors of a panel of ^{High}YES1 and ^{Low}YES1 human lung cancer cells. In ^{High}YES1 tumors, a significant tumor volume decrease was observed in dasatinib-treated mice when compared with vehicle-treated control mice (Figures 4E–4H). However, in ^{Low}YES1 cell lines, differences in tumor growth were found only in one out of the two tested cell lines (Figures 4I–4J).

To assess whether *YES1* amplification may be a predictor of response to SFK inhibitors, and to gain further insights into the potential therapeutic application of YES1 inhibition in patients carrying ^{High}YES1 tumors, we used NSCLC PDX to explore the efficacy of dasatinib. Congruently, dasatinib significantly impaired tumor growth in ^{High}YES1 ADC or SCC PDX (Figures 5A–5C). Moreover, two out of three ^{Low}YES1 PDX did not respond to dasatinib treatment (Figures 5D–5F), both in ADC and SCC models. In summary, these data suggest that YES1 status may be used as a stratification biomarker to select patients with NSCLC who may respond and benefit from SFK inhibitors.

Discussion

Although lung cancer is the archetype of genomics-driven oncology, a high proportion of patients cannot benefit from targeted therapies yet. This is especially relevant in patients with SCC who currently lack targeted molecular therapies. Our study provides evidence for the protumorigenic role of YES1 in NSCLC and reports the preclinical rationale for targeting this kinase, using its expression as an independent predictive marker of response to treatment. *YES1* amplification has been recently reported as a new mechanism of resistance to EGFR inhibitors (17–19), suggesting that YES1-mediated signaling may exert an important role in lung cancer pathogenesis. However, the specific role of YES1 in lung cancer progression has not been explored yet. In this report, we show that *YES1* alterations drive lung cancer progression and that high YES1 protein expression is associated with shorter overall survival in patients with NSCLC. We have also demonstrated that specific genetic and pharmacological YES1 inhibition reduces cell proliferation and invasion *in vitro* and tumor growth *in vivo* in ^{High}YES1 tumors.

A high proportion of patients with NSCLC harbor *YES1* DNA CN alterations. In the TCGA series (29), 15% of patients with ADC and 25% of patients with SCC show gains in the *YES1* gene and a high mRNA expression. Interestingly, in a recent comprehensive analysis of NSCLC exome sequences and CN profiles, *YES1* amplification was described as a recurrent alteration of lung SCC (8). In a previous study, we described a prognostic signature for lung ADC composed of seven-gene genomic alterations (4). Among the seven genes, *YES1* and *TYMS* (thymidylate synthetase) presented the highest prognostic power. In the present study, we demonstrate that YES1 protein expression is an independent prognostic biomarker in lung cancer.

We have also demonstrated the driving role of YES1 in NSCLC carcinogenesis. Stable YES1 overexpression significantly induces cell proliferation *in vivo* and

Figure 4. (Continued). mean ± SE from at least three independent experiments. (*C* and *D*) Dasatinib treatment blocked three-dimensional invasion of ^{High}YES1 Calu-1 (*C* and *D*) and H1792 (*D*) cells in a dose-dependent manner. Invasion capability was not affected in ^{Low}YES1 A549 cell line by dasatinib (*D*). (*E–J*) Effect of dasatinib treatment in ^{High}YES1 (*E–H*) or ^{Low}YES cell lines (*I* and *J*) subcutaneously injected mice. Dasatinib significantly inhibited tumor growth in ^{High}YES1 tumors, whereas no differences were found in the SW900 ^{Low}YES1 tumors. Data are presented as mean ± SE ($n \geq 6$ for all groups). The extra-sum-of-squares *F* test was used to compare tumor growth differences among groups.

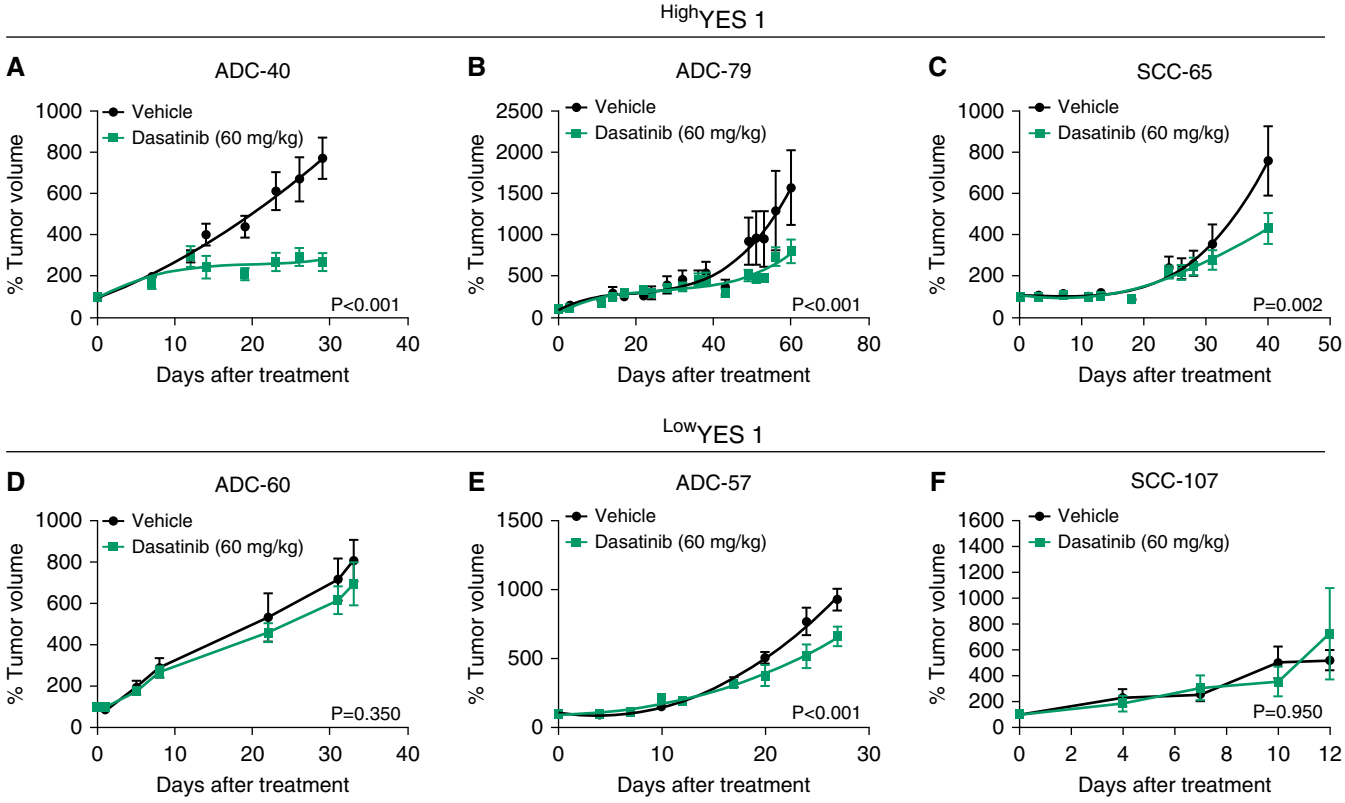

Figure 5. Dasatinib inhibits tumor growth in patient-derived xenograft (PDX) models depending on YES1 (v-YES-1 Yamaguchi sarcoma viral oncogene homolog 1) status. PDX models were developed and treated with dasatinib. Dasatinib treatment in ^{High}YES1 adenocarcinoma (ADC)-40, ADC-79, and squamous cell carcinoma (SCC)-65 PDX significantly inhibited tumor growth (*A–C*). In contrast, in ^{Low}YES1 ADC-60 and SCC-107 PDX models, dasatinib did not affect tumor growth (*D–F*) but reduced tumor growth in ^{Low}YES1 ADC-57 PDX. Data are presented as mean ± SE ($n \geq 6$ for all groups). Tumor growth differences among groups were compared with the extra-sum-of-squares *F* test.

in vitro, supporting the tumorigenic effect of this kinase. The increase on both proliferation and tumor growth is more prominent in NSCLC cell lines with basal high *YES1* CN and protein expression. In fact, in the ^{Low}YES1 A549 cell line, exogenous YES1 induction led to an increase in tumor growth in mouse models but not *in vitro*, suggesting that YES1 tumor expression may also regulate the microenvironment. In this regard, it has been reported in pancreatic tumors that the hyperactivation of the YES1 target FAK (focal adhesion kinase) induces a tumor-protective fibrotic and immunosuppressive microenvironment by inducing stromal expansion and tumor infiltration of immunosuppressive cells (33). It could be argued that, in ^{Low}YES1 cell lines, the higher influence of YES1 induction *in vivo* may be due to its effect on the microenvironment more than on tumor cells *per se*. In addition, using two lung cancer mouse models, we have shown that YES1 promotes metastatic spread. Subcutaneous tumors of NSCLC cells overexpressing YES1 developed metastasis to the lungs and liver. Moreover, intracardiac injection of cells harboring *YES1* CN alterations led to increased tumor burden and induced metastases to the lung, liver, and bones. Conversely, YES1 abrogation by CRISPR/Cas9 gene editing was associated with reduced tumor burden and lower metastasis rate. In other tumor types, such as melanoma (34), colon carcinoma (35), and prostate cancer (36), YES1 and other SFK members have also been correlated with metastasis. Furthermore, we have shown that YES1 knockdown selectively impairs cell growth and invasion and induces apoptosis in lung cancer cell lines harboring CN gains and high YES1 expression. In contrast, none of these effects were observed in cell lines with low expression and normal CN of this kinase, suggesting a genomic alteration–dependent effect.

Regarding molecular signaling, YES1 kinase has been previously reported to be implicated in FAK (36), YAP1 (37), and mTOR (31) pathways among others. In this work we found that the PI3K/AKT/mTOR pathway is an important mediator of YES1 signaling in NSCLC, as YES1 promotes S6K and AKT phosphorylation.

Given that our results determined that YES1 is a therapeutic target in high YES1-expressing tumors, we evaluated the activity of the SFK inhibitor dasatinib in NSCLC models according to their YES1 status. Dasatinib is a multitarget tyrosine kinase inhibitor that inhibits SFKs at nanomolar concentrations, and it is also active against BCR-ABL (breakpoint cluster region–Abelson murine leukemia viral oncogene homolog 1), c-KIT (stem cell growth factor receptor Kit) and PDGFRβ (platelet-derived growth factor receptor, beta polypeptide) (32). In lung cancer, unselected dasatinib trials were developed based on preclinical data on SRC inhibition (38). In this trial of 34 patients with advanced NSCLC, a striking response to first-line dasatinib therapy was found in

one patient and long-lasting disease stabilization in four others, suggesting a potential subpopulation of patients sensitive to dasatinib. Unfortunately, no predictive markers were found, and a great effort has been made to identify biomarkers of response to dasatinib. Mutations in the *DDR2* kinase were proposed to predict dasatinib treatment in patients with SCC (39), but clinical trials failed to demonstrate this issue (40–42).

Our hypothesis is that dasatinib treatment may have therapeutic activity in those tumors harboring *YES1* CN gains and high expression, functioning as a predictive biomarker for this therapy. This hypothesis is also supported by computational studies (43) suggesting that CN gains of a selected list of genes (which includes *YES1*) may serve as predictive markers for dasatinib treatment. However, the specific contribution of *YES1* gene CN has so far never been investigated. In the present work, we report that the NSCLC (both ADC and SCC subtypes) sensitivity to dasatinib was closely associated with YES1 status. Indeed, cell proliferation and invasion were impaired by dasatinib in a dose-dependent manner mostly in ^{High}YES1 cells. Consistently, dasatinib also demonstrated to be an effective treatment for ^{High}YES1 cells in our *in vivo* NSCLC models, being effective only in one out of two ^{Low}YES1 cells, A549, probably because of its high SRC expression. Expanding on these findings, we evaluated the efficacy of dasatinib in NSCLC PDX, confirming that this inhibitor led to robust tumor shrinkage in ^{High}YES1 tumors. Therefore, our results suggest that *YES1* amplification may be considered a potentially useful predictive biomarker for dasatinib response. Basal high YES1 status is associated with dasatinib efficacy, although this association could not be excluded in low YES1 tumors, probably because of the expression of other SRC kinases in these tumors. We are currently exploring the potential interaction between YES1 overexpression and the immune landscape of the tumors as well as potential epistatic influences between *YES1* amplification and other gene alterations. Importantly, *YES1* amplification has been recently proposed as a new mechanism of resistance to EGFR inhibitors (17–19). Specifically, Fan and colleagues have demonstrated by transposon-based mutagenesis that acquired *YES1* amplification is a mechanism of acquired resistance to EGFR TKIs (tyrosine-kinase inhibitors) afatinib, erlotinib, and osimertinib in lung ADC (19). Accordingly, patients with *YES1* amplification may be selected for dasatinib treatment. The evaluation of YES1 status as a predictive marker of response to dasatinib in patients with NSCLC could also contribute to better selecting and stratifying patients. Ultimately, the refinement in the patient selection criteria may allow for clinical trials in which more adjusted working dosages of the SKF inhibitors would be tested to maximize treatment efficacy while minimizing toxicity. Furthermore, the development of specific YES1 inhibitors, rather than general SFK pan-inhibitors, may also increase the therapeutic efficacy in patients carrying ^{High}YES1 tumors. Finally, in clinical trials exploring the combination of dasatinib and immune checkpoint inhibitors such as the FRACTION (Fast Real-Time Assessment of Combination Therapies in Immuno-Oncology)-lung study, YES1 expression may be used to assess efficacy of the combined treatment.

In summary, the results presented herein not only support the prognostic significance of YES1 but also, more importantly, demonstrate that YES1 is a driver of tumor growth and progression in a high percentage of patients with NSCLC. Thus, YES1 targeting emerges as a potential therapeutic strategy in patients with NSCLC harboring *YES1* genetic alterations. Indeed, we have presented a comprehensive preclinical evaluation of the SFK inhibitor dasatinib, demonstrating its efficacy in ^{High}YES1 tumors both *in vitro* and *in vivo*. Altogether, our studies provide support for the clinical evaluation of YES1 as a stratification biomarker to define a subset of patients who may potentially benefit from current or novel SFK inhibitors. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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