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The Hippo pathway target, YAP, promotes metastasis through its TEAD-interaction domain

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The transcriptional coactivator Yes-associated protein (YAP) is a major regulator of organ size and proliferation in vertebrates. As such, YAP can act as an oncogene in several tissue types if its activity is increased aberrantly. Although no activating mutations in the *yap1* gene have been identified in human cancer, *yap1* is located on the 11q22 amplicon, which is amplified in several human tumors. In addition, mutations or epigenetic silencing of members of the Hippo pathway, which represses YAP function, have been identified in human cancers. Here we demonstrate that, in addition to increasing tumor growth, increased YAP activity is potently prometastatic in breast cancer and melanoma cells. Using a Luminex-based approach to multiplex in vivo assays, we determined that the domain of YAP that interacts with the TEAD/TEF family of transcription factors but not the WW domains or PDZ-binding motif, is essential for YAP-mediated tumor growth and metastasis. We further demonstrate that, through its TEAD-interaction domain, YAP enhances multiple processes known to be important for tumor progression and metastasis, including cellular proliferation, transformation, migration, and invasion. Finally, we found that the metastatic potential of breast cancer and melanoma cells is strongly correlated with increased TEAD transcriptional activity. Together, our results suggest that increased YAP/TEAD activity plays a causal role in cancer progression and metastasis.

Cancer is the second leading cause of death worldwide, and more than 90% of all cancer-associated deaths are caused by metastasis. Therefore, understanding the cellular mechanisms that regulate metastasis is vital to the development of effective cancer therapies. To form metastatic tumors, cancerous cells must detach from the primary tumor, invade through the surrounding tissue, enter and survive in circulation, seed a target organ, exit circulation, and survive and proliferate in a foreign microenvironment. Transcription factors and the pathways that regulate them are well suited to influence this metastatic cascade because they can regulate the expression of multiple target genes, which in turn could regulate several of these prometastatic processes. Of particular interest are pathways that regulate transcription in response to extracellular cues. One such pathway is the Hippo pathway, which alters gene expression in response to changes in cell shape, adhesion, and density (1–5).

The Hippo pathway and its effector, the transcriptional coactivator Yes-associated protein (YAP), have emerged as major regulators of organ size and proliferation (reviewed in refs. 6–8). The core Hippo pathway was described initially in *Drosophila* and is largely conserved in vertebrates and mammals (6, 8). Although the membrane-proximal components of the mammalian Hippo pathway have not been elucidated fully, it is clear that the pathway is regulated by cell density (1, 2) as well as by changes in cell shape and in the actin cytoskeleton (3–5). In mammals, the Hippo pathway consists of a core kinase cascade in which Mst1 or Mst2 forms a complex with the adaptor protein WW45 and phosphorylates the kinases LATS1 and LATS2 as well as the adaptor protein MOB. A LATS/MOB complex then phosphorylates and represses the transcriptional coactivators YAP and TAZ (6–8). Phosphorylation by LATS kinases promotes cytoplasmic sequestration of YAP and TAZ in a manner that involves 14-3-3 proteins (1, 9) and α-catenin (10, 11). LATS-mediated phosphorylation of YAP also can promote YAP ubiquitination and subsequent proteasomal degradation (12). Several additional proteins are involved in Hippo pathway-dependent and -independent regulation of YAP and TAZ, including the FERM domain proteins Merlin/NF2 and FRMD6, the junctional proteins ZO-2 and AJUB, the polarity complex proteins Crumbs, Angiomotin, Scribble, and KIBRA, and the protein phosphatases PP2A and ASPP1 (6–8). Thus, YAP protein levels and activity are regulated tightly at multiple levels.

The importance of YAP and deregulation of the Hippo pathway during cancer development and progression is now clear. YAP is a driving oncogene on amplicon 11q22, which is amplified in several human cancers (13–15), and YAP expression and nuclear localization strongly correlate with poor patient outcome and the progression of several tumor types (15–22). Experimentally, YAP expression transforms cells (9, 23, 24), promotes an epithelial-to-mesenchymal transition (EMT), and enhances in vitro invasion (9, 14, 24). Overexpression of YAP in cancer cell lines also can promote tumor growth (13, 25–27), demonstrating that YAP acts as an oncogene in several cell types. In transgenic mice, forced tissue-specific expression of either wild-type YAP or a mutant form of YAP that is insensitive to Hippo-mediated cytoplasmic sequestration results in tissue overgrowth and tumor formation (10, 28–30). TAZ, a related protein that also is repressed by the Hippo pathway, recently was shown to confer cancer stem cell-like traits in breast cancer cells (31), suggesting that defects in Hippo signaling also play important roles in cancer. Indeed, mutations and/or epigenetic silencing of several Hippo-pathway proteins, including NF2, LATS1/2, MST1/2, WW45, MOB, and KIBRA, have been found in human cancers (6, 8, 32). Furthermore, studies in mice have demonstrated that loss of MST1/2, NF2, WW45, α-catenin, or LATS can lead to a dramatic increase in organ size and tumor formation, effects that are largely dependent on YAP (10, 11, 33–37).

Despite the studies described above, a role for YAP or deregulation of the Hippo pathway in promoting metastasis has not been explored. Furthermore, in breast cancer, it remains controversial whether YAP acts as a tumor suppressor or oncogene. Finally, the mechanisms through which YAP promotes its established protumorigenic effects are still not understood fully. We investigated the impact of aberrant YAP activity on breast

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cancer and melanoma cells and found that an activated form of YAP can potently promote tumor growth and drive metastasis. Using a Luminex-based approach to multiplex in vivo tumor growth and metastasis assays, we show that the transcriptional enhancer activator DNA-binding (TEAD)-interaction domain of YAP is essential for YAP-mediated tumor growth and metastasis. These results establish a clear role for YAP in mammary carcinoma and melanoma progression and metastasis.

Results

YAP with the S127A Mutation Enhances Mammary Carcinoma and Melanoma Growth and Promotes Metastasis. Given the evidence linking aberrant YAP activity and defects in Hippo signaling to cancer development, we sought to test the consequences of unregulated YAP activity on breast cancer progression. Serine 127 is critical for LATS-mediated cytoplasmic sequestration of YAP by the Hippo pathway (1, 9, 28), and several groups have reported increased YAP activity after mutating serine 127 to alanine (1, 10, 24, 30). Therefore, to generate an activated form of YAP, we introduced the S127A mutation into YAP (YAP^{S127A}). Expression of YAP^{S127A} dramatically enhanced the in vivo growth potential of several mammary carcinoma cell lines (Fig. 1*A*) and a human melanoma cell line (A375) (Fig. 1*B*) following orthotopic transplantation into mice. More importantly, YAP^{S127A} expression increased the number and size of metastases that formed following tail-vein injection of 67NR or 4T1 mammary carcinoma cell lines (Fig. 1 *C* and *D*) and rendered A375 cells highly metastatic (Fig. 1*E*). Strikingly, YAP^{S127A} expression also rendered a nontransformed mammary epithelial cell line (NMuMG) highly metastatic (Fig. 1*G*). However, in contrast to the mammary carcinoma and melanoma cells, YAP^{S127A} expression in NMuMG cells did not dramatically influence tumor formation (Fig. S1*B*) or in vivo growth (Fig. 1*F*), despite enhancing growth in 3D Matrigel and soft agar (Fig. S1*A*). This latter result can be explained by the fact that, although growth in soft agar often is used as a readout for in vivo tumorigenicity, altering the ability to grow in soft agar does not always result in altered growth in vivo. Thus, YAP^{S127A} expression rendered NMuMG cells metastatic without dramatically influencing in vivo growth. Taken together, these results show that unregulated YAP activity can dramatically enhance the metastatic potential of both mammary carcinoma and melanoma cells and can do so regardless of whether or not it also promotes tumor growth.

To test whether the YAP^{S127A}-dependent effects on metastasis are cell autonomous, mCherry-expressing control cells were mixed with ZSgreen-expressing YAP^{S127A} cells, and tail-vein metastasis assays were performed. For NMuMG cells the percentage of YAP^{S127A}-expressing cells in the lungs was roughly 36-fold greater

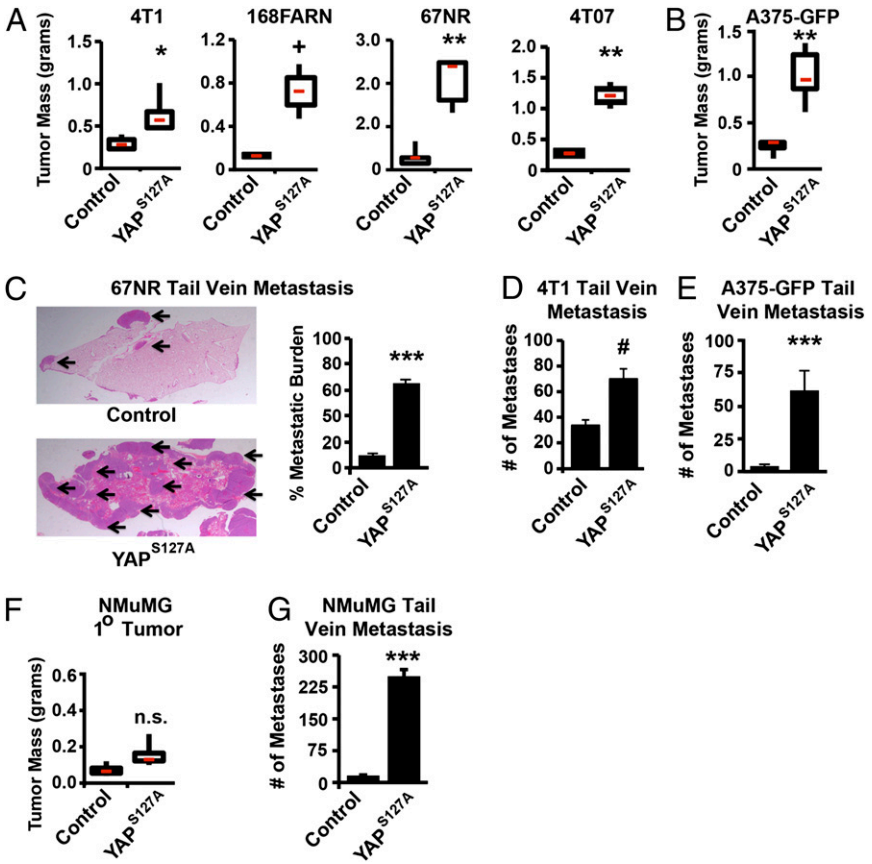

Fig. 1. YAP^{S127A} enhances tumor growth and promotes metastasis. (*A* and *B*) The indicated cells (5×10^5) stably expressing control vector or YAP^{S127A} were transplanted into the mammary fat pads (*A*) or delivered s.c. (*B*) into mice. After 3–5 wk, tumor masses were compared using box and whisker plots. $n = 8$ mice. (*C–E*) 67NR cells (2×10^5), 4T1 cells (5×10^4), or A375-GFP cells (1×10^6) expressing either control vector or YAP^{S127A} were assayed by tail-vein metastasis assays. (*C*) (*Left*) Representative H&E-stained sections at 19 d postinjection; arrows indicate metastases. (*Right*) Graph shows percentage of total lung area that was metastatic burden (± SEM) for all mice. $n = 3$ mice. (*D* and *E*) The numbers of metastases were counted in H&E-stained sections (*D*) or in whole lungs (*E*) at 7 d (*D*) or 34 d (*E*) postinjection. $n = 3$–5 mice in *D* and 5 mice in *E*. (*F* and *G*) NMuMG cells expressing control vector or YAP^{S127A} (5×10^5 for each condition) were assayed by either orthotopic mammary transplant or tail-vein metastasis assays. (*F*) Tumor masses were compared using box and whisker plots. $n = 4$ mice. (*G*) The numbers of metastases were counted in H&E-stained sections at 10 d postinjection. $n = 5$ mice. n.s., not significant ($P = 0.15$); $^{+}P = 0.06$; $^{\#}P = 0.09$; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$, two-tailed paired *t* test.

than that of control cells, and the percentage of 67NR cells in the lungs was roughly 14-fold greater than that of control cells (Fig. 2). This effect was not caused by differences in survival or detection of mCherry- versus ZSgreen-expressing cells, because ZSgreen-expressing control cells were not enriched (Fig. 2). These results suggest that YAP promotes metastasis, at least in part, in a cell-autonomous manner.

Multiplex Approach for Studying Tumor Growth and Metastasis in Vivo. The members of the transcriptional enhancer factor/TEA domain (TEF/TEAD) family of transcription factors are major mediators of YAP transcriptional activity (6, 7). Initial insight into the importance of this family of transcription factors came from work in *Drosophila* showing that regulation of proliferation by the YAP homolog, Yorkie, required interaction with the TEF/TEAD protein, Scalloped (38). Subsequent studies confirmed the importance of the four mammalian TEF/TEAD family members (39, 40), which bind to the N-terminal region of YAP in a manner that is highly dependent on serine 94 (40). YAP also interacts with numerous other transcription factors through its WW domains (reviewed in ref. 41). In addition, the WW domains, SH3-binding motif, and PDZ-binding motif (PDZbm) of YAP also can mediate interaction with several additional proteins (41). To gain insight into the mechanism whereby YAP promotes tumor growth and metastasis, we sought to determine which domains of YAP are critical for YAP-mediated effects in vivo. For this purpose, we introduced several mutations into our YAP^{S127A} construct (YAP^{S127A}-WT) (Fig. 3*A*). The S94A mutation in the TEAD-interaction domain was shown previously to disrupt the interaction of YAP with the TEADs (40). A mutation

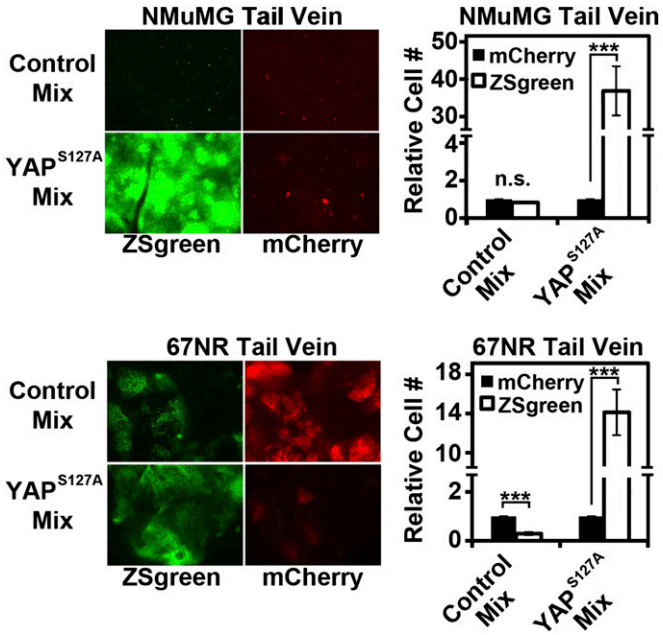

Fig. 2. YAP^{S127A} promotes metastasis through a cell-autonomous mechanism. The indicated cell lines were stably transduced with vectors expressing mCherry, ZSgreen, or YAP^{S127A}-2A-ZSgreen. Two separate cell mixtures were generated by mixing equal numbers of mCherry cells with either ZSgreen cells (Control Mix) or with YAP^{S127A}-2A-ZSgreen cells (YAP^{S127A} Mix). Then 5×10^5 cells of the indicated mixtures were assayed by tail-vein metastasis assay. (*Left*) Fluorescent images of whole lungs after 14 d. (*Right*) Lung lobes were digested to single-cell suspensions, and the relative numbers of ZSgreen- and mCherry-positive cells were counted using flow cytometry. Graphs show fold increase in ZSgreen-positive cells compared with mCherry-positive cells for each mix (± SEM). *n* = at least 6 mice for each condition; n.s., not significant; ***$P \leq 0.001$; two-tailed paired *t* test.

of a critical tryptophan residue known to block interaction with proteins that bind the WW domains (42) was introduced into the WW1, WW2, or both (1+2) WW domains of YAP^{S127A}. The Double (Dbl) mutant combined the S94A mutation and the WW domain mutation, and the PDZ mutant has a C-terminal truncation of five amino acids removing the PDZbm, which previously was shown to regulate YAP localization and function (43, 44).

Testing the effects of each of these mutant forms of YAP on tumor growth and metastasis in vivo would require a prohibitively large number of mice. Therefore we developed an approach to multiplex our in vivo tumor growth and metastasis assays (Fig. 4*A*). In this approach, cells stably expressing uniquely DNA-barcoded versions of either control vector or each mutant form of YAP^{S127A} were mixed in equal numbers, and the cell mixture was used in Luminex-based tumor growth and metastasis assays (*Methods* and Fig. 4*A*). Cells expressing YAP^{S127A} constructs that enhance tumor growth and metastasis would be enriched, relative to control cells, in tumor and lung samples, whereas cells expressing constructs that are unable to enhance tumor growth and metastasis would not be enriched. To confirm that this Luminex-based approach can detect enrichment or depletion of cell populations in vivo, we performed proof-of-principle experiments. We found that cells expressing shRNAs targeting replication protein A, subunit 3 (RPA3), a protein essential for cell division, were depleted significantly in both primary tumors and metastasis-bearing lungs of the mice compared with cells expressing firefly luciferase shRNAs (Fig. 4 *B* and *C*). This result confirms that this approach can be used to multiplex tumor growth and metastasis assays.

YAP-Mediated Tumor Growth and Metastasis Are Dependent on the TEAD-Interaction Domain of YAP. To test the requirement of key domains of YAP in tumor growth and metastasis, cells stably expressing barcoded versions of each YAP^{S127A} construct were mixed in equal numbers and used in Luminex-based tail-vein metastasis assays, tumor growth assays, and spontaneous metastasis assays. Primary tumors and metastatic lungs from mice injected with mixed populations of 67NR cells contained significantly more cells expressing YAP^{S127A}-WT than control cell populations (Fig. 3 *C–E*). Cells expressing the WW domain mutants of YAP^{S127A} or the mutant lacking the PDZbm also were significantly enriched compared with control cells, suggesting that the WW domains and PDZbm are not essential for YAP-mediated tumor growth and metastasis. However, cells expressing the YAP^{S127A}-S94A and YAP^{S127A}-Dbl mutants, neither of which can interact with the TEAD family of transcription factors, were not enriched relative to control cells, suggesting that these mutants are unable to promote tumor growth and metastasis efficiently (Fig. 3 *C–E*). To confirm these Luminex results, we generated 67NR cell lines expressing either the WT or S94A forms of YAP^{S127A} and performed individual mammary transplant and tail-vein metastasis assays. Consistent with the Luminex results, mice injected with 67NR cells expressing YAP^{S127A}-WT had significantly reduced survival, significantly larger tumors, and significantly more metastases than mice injected with control 67NR cells (Fig. S2), and this enhanced tumor growth and metastasis was lost completely in the cells expressing the S94A mutant (Fig. S2). When we reduced the number of 67NR cells transplanted into the mammary fat pad, YAP^{S127A}-WT tumors still were significantly larger than control and S94A tumors (Fig. S2 *D–F*). In addition, we now observed a significant reduction in the numbers of control and S94A tumors that developed, whereas the majority of the mice injected with YAP^{S127A}-WT cells developed tumors (Fig. S2*D*). These results suggest that, when expressed in 67NR cells, YAP^{S127A} enhances tumor formation as well as tumor growth. YAP^{S127A} also enhanced tumor growth in a manner that was

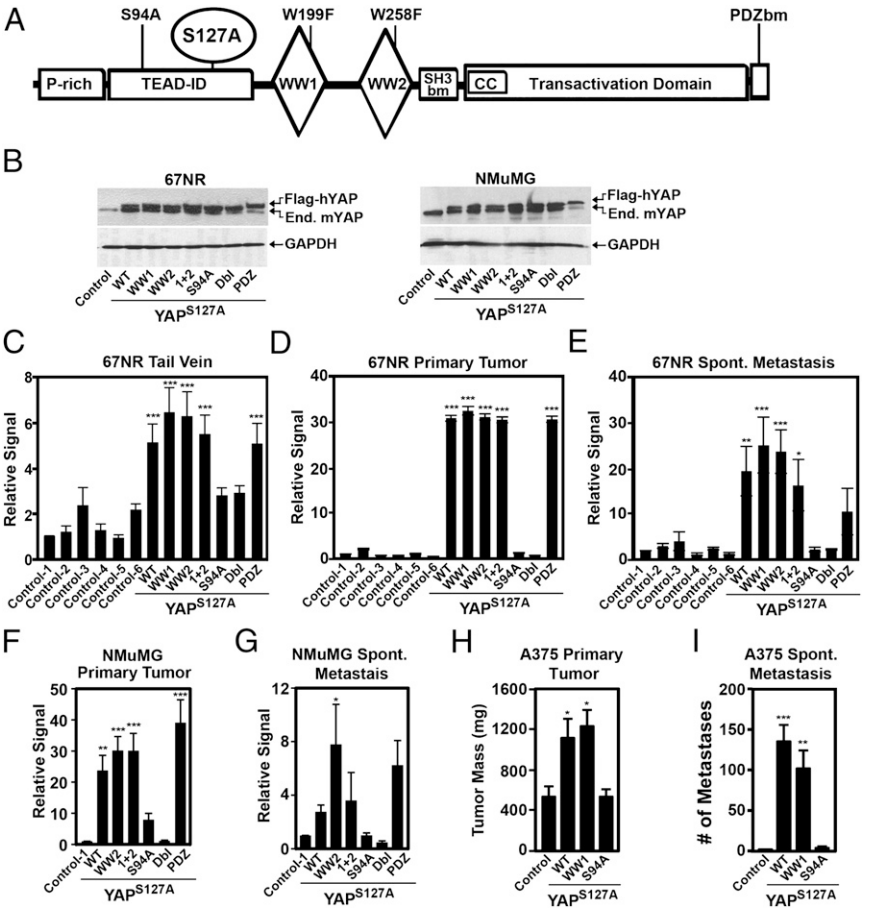

Fig. 3. YAP^{S127A}-mediated tumor growth and metastasis are dependent on the TEAD-interaction domain of YAP. (*A*) Schematic of human YAP protein showing the proline-rich N terminus (P-rich), the TEAD-interaction domain (TEAD-ID), WW domains (WW1 and WW2), the SH3-binding motif (SH3bm), the coiled-coil domain (CC), the transactivation domain, and the PDZ-binding motif (PDZbm). Several point mutations were introduced into a YAP^{S127A} construct with an N-terminal Flag-tag. (*B*) Immunoblots of lysates from 67NR and NMuMG cells stably expressing barcoded versions of either control vector or the indicated mutant forms of YAP^{S127A}. (*C*–*G*) 67NR or NMuMG cells from *B* were mixed in equal numbers and then either 5×10^5 cells (*C*, *F*, and *G*), or 2.5×10^5 cells (*D*) were used in Luminex-based tail-vein metastasis (*C*) or orthotopic mammary transplant and spontaneous metastasis assays (*D*–*G*). After 8 (*C*), 28 (*D* and *E*), or 86 (*F* and *G*) d, lungs and primary tumors were isolated, and the relative numbers of cells expressing each vector were quantified using Luminex. Graphs show mean signal (± SEM) for each cell line relative to the signal for cells expressing control vector 1. Results similar to those in *B*–*F* were obtained in a second independent experiment. (*H* and *I*) A375 cells (5×10^5) expressing the indicated constructs were injected s.c. into mice, and tumor masses and the numbers of lung metastases were compared. Graphs show mean (± SEM) of 14 mice (*C*); 10 mice (*D* and *E*); 7 mice (*F*); 5 of the mice from *F* that developed metastases (*G*); or 7 mice (*H* and *I*); $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; one-way ANOVA with Dunnett's post test.

dependent upon the TEAD-interaction domain in other mouse mammary carcinoma cell lines (Fig. S3 *A* and *B*).

We also performed Luminex-based tumor growth and spontaneous metastasis experiments with NMuMG cells. As observed with 67NR cells, primary tumors and lungs isolated from mice that received orthotopic transplantation of NMuMG cell mixtures also contained significantly more cells expressingYAP^{S127A}-WT than control cells, and mutation of the WW domains or truncation of the PDZbm did not affect significantly the ability of YAP^{S127A} to promote tumor growth or metastasis (Fig. 3 *F* and *G*). However, both the S94A and the Dbl mutants had significantly reduced ability to promote tumor growth and metastasis from the primary site (Fig. 3 *F* and *G*). Our finding that, when injected as a mixture, NMuMG cells expressing YAP^{S127A} were significantly enriched in primary tumors compared with control cells was surprising, given our finding that, when injected individually, NMuMG cells expressing YAP^{S127A} did not show enhanced tumor growth (Fig. 1*F*). This result may indicate that YAP promotes cell competition in NMuMG cells, as previously observed in cells expressing Yorkie, the *Drosophila* homolog of YAP (*Discussion*) (45, 46).

Similar results were obtained using human melanoma cells. Mice injected s.c. with YAP^{S127A}-expressing A375 cells developed significantly larger tumors and had numerous spontaneous lung metastases, whereas mice injected with control cells had smaller tumors and did not develop metastases (Fig. 3 *H* and *I*). Once again, mutation of the TEAD-interaction domain, but not the WW domain, significantly impaired YAP^{S127A}-mediated tumor growth and metastasis (Fig. 3 *H* and *I*). These results were confirmed using Luminex-based tumor growth and spontaneous metastasis assays with A375 cells (Fig. S3 *C* and *D*). Together, these findings demonstrate that YAP-mediated mammary carcinoma and melanoma growth and metastasis require a functional TEAD-interaction domain.

YAP Promotes Transformation, Proliferation, Migration, and Invasion Through Its TEAD-Interaction Domain. Previous studies have demonstrated roles for YAP in promoting proliferation and transformation of mammary epithelial cells (14, 40, 42, 47). Consistent with these reports, we found that YAP^{S127A} expression transforms NMuMG cells in a manner that is dependent on the TEAD-interaction domain but not on the WW domains or PDZbm

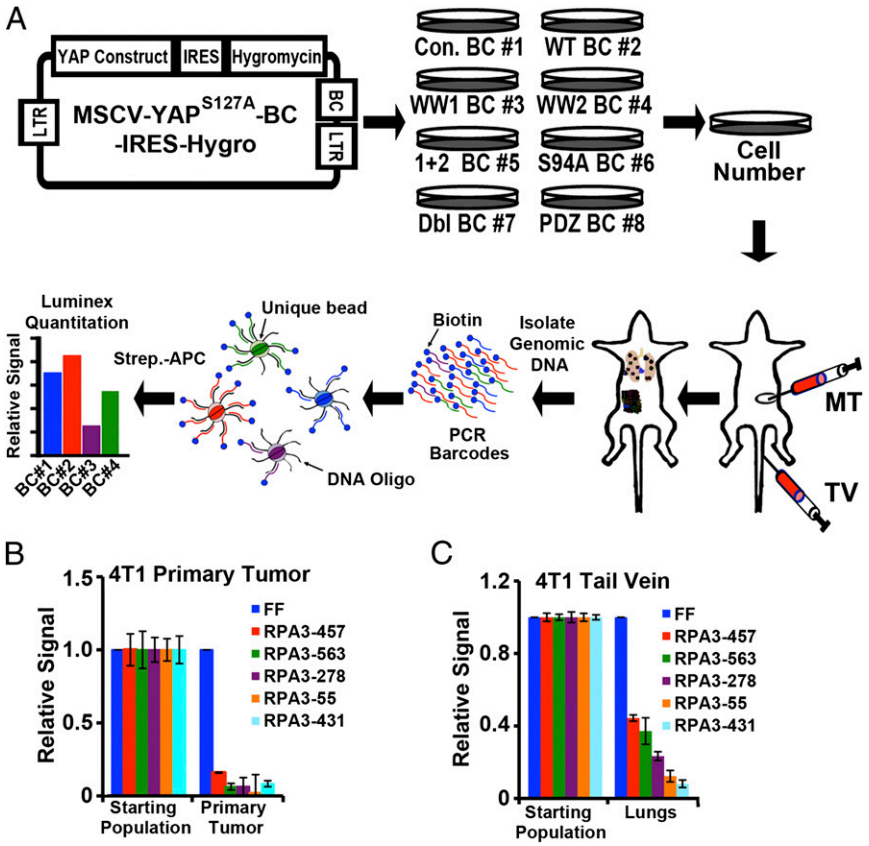

Fig. 4. A Luminex-based approach for multiplexing in vivo tumor growth and metastasis assays. (*A*) Schematic of our Luminex-based approach for multiplexing in vivo tumor growth and metastasis assays (*Methods*). Individual cell lines stably expressing barcoded (BC) versions of each YAP^{S127A} mutant were mixed in equal numbers and either injected into the tail veins (TV) or transplanted orthotopically into the mammary fat pads of mice (MT). Genomic DNA was isolated from the resulting tumors and metastasis-containing lungs, and the relative amount of each barcode was quantified using streptavidin-conjugated APC (Strep.-APC) and the Luminex FlexMap 3D system. (*B*) 4T1 mammary carcinoma cells were stably transduced with uniquely barcoded retroviral vectors expressing miR30-based shRNAs targeting either RPA3 or control firefly luciferase (FF) and were mixed in equal numbers. Then 2×10^5 cells were used in Luminex-based tail-vein metastasis assays and orthotopic mammary transplant assays. The starting population also was collected at the time of injection as a reference. After 14 d (*B*) and 28 d (*C*) lungs and primary tumors were isolated, and the relative numbers of cells expressing each vector were quantified using Luminex. Graphs show mean signal (± SEM) for each RPA3-shRNA–expressing cell line in the lungs or primary tumor (normalized to the signal for firefly luciferase) relative to the signal in the starting population. $n = 6$ mice in *B* and 4 mice in *C*.

(Fig. 5*A*). YAP^{S127A} expression also increased the in vitro proliferation of both 67NR and NMuMG cells as measured by growth in 3D Matrigel, as well as Luminex-based and flow cytometry-based proliferation assays (Fig. 5 *B* and *C* and Fig. S4 *B–E*). These YAP^{S127A}-mediated effects were severely impaired in the constructs with the mutated TEAD-interaction domain (YAP^{S127A}-S94A and YAP^{S127A}-Dbl) but not in constructs with mutated WW domains or with the PDZbm truncation (Fig. 5 *A–C* and Fig. S4 *B–E*).

Although the ability of YAP to transform cells and promote proliferation could explain the differences in tumorigenicity and may contribute to increased metastatic burden, we wondered whether YAP also promotes metastasis through additional mechanisms. Numerous studies have established links between metastasis and both increased invasiveness and the induction of an EMT. Although YAP^{S127A} induced transcriptional changes characteristic of an EMT in NMuMG cells, changes in the expression of EMT-associated proteins were not as significant (Fig. S4*A*), thus suggesting that YAP^{S127A} is not sufficient for a full EMT in these cells when assayed under these conditions. However, YAP^{S127A} expression did potently enhance invasion of 67NR, NMuMG, and A375 cells (Fig. 5 *D–G*). Again, these effects were severely impaired by the S94A mutation but not by mutation of the WW domains. Consistent with a role for YAP in invasion, 72% (8 of 11) of YAP^{S127A}-expressing NMuMG tumors had invaded from the mammary fat pad into either the overlying skin or underlying muscle of the peritoneal cavity, whereas none of the nine control tumors that formed had done so.

The finding that YAP enhances metastasis in tail-vein metastasis assays suggests that YAP also impacts processes that occur after cells enter circulation. To explore this possibility further, we tested whether the increased metastatic burden resulting from expression of active YAP was caused by increased proliferation/survival of cells at the metastatic site or by increased seeding of circulating tumor cells in the lung. Control NMuMG cells labeled with Cell Tracker red were mixed in equal numbers with YAP^{S127A}-expressing cells labeled with Cell Tracker green and were injected into the lateral tail veins of mice. We found that the numbers of control and YAP^{S127A}-expressing cells in the lungs were roughly equivalent 12 h after injection (Fig. S4*E*), suggesting that expression of YAP^{S127A} did not enhance initial tumor cell seeding. The average number of dye-containing cells per section was significantly lower at 24 h (44.83 ± 5.82) than at 12 h (73.83 ± 5.64), indicating that disseminated cells were being cleared from the lungs. We observed a 50% increase in the relative number of YAP^{S127A}-expressing cells in the lungs at 24 h; however, a similar increase was observed when the cell

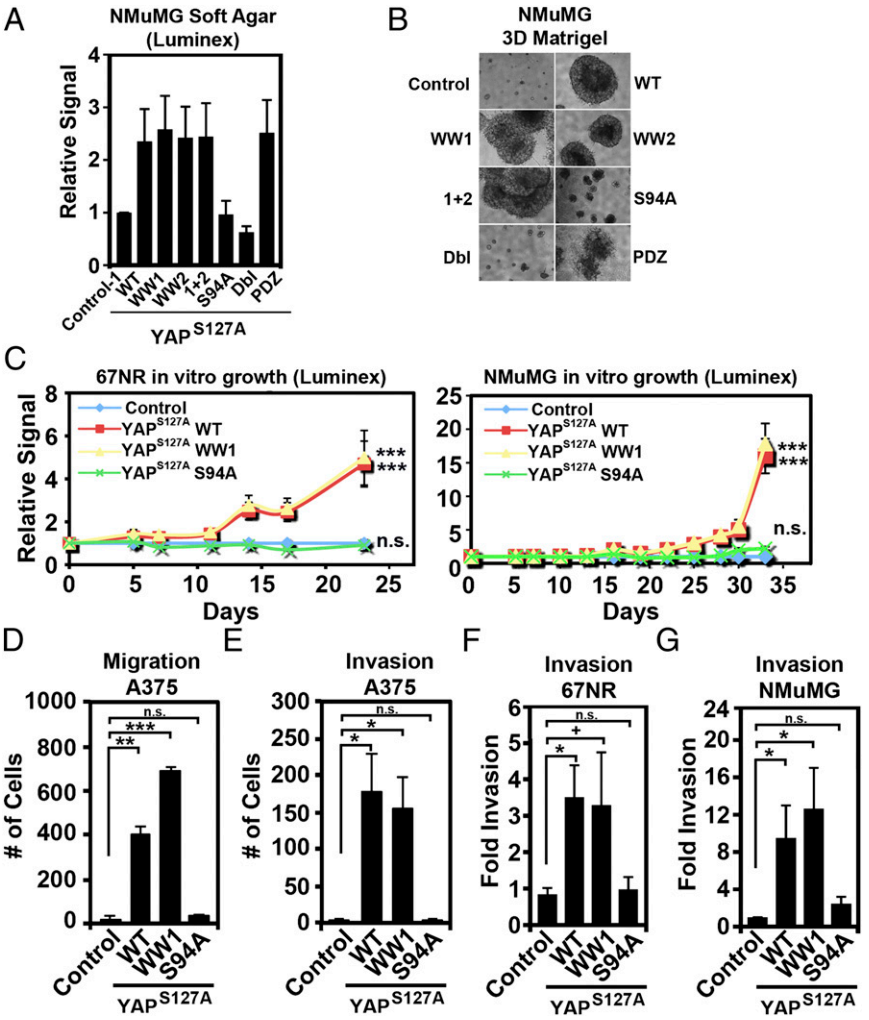

Fig. 5. YAP^{S127A}-mediated proliferation, migration, and invasion are dependent on the TEAD-interaction domain of YAP. (*A* and *B*) NMuMG cells stably expressing the indicated constructs were mixed together in equal numbers and used in Luminex-based soft agar assays (*A*) or were assayed for their ability to grow in 3D Matrigel (*B*). (*C*) 67NR or NMuMG cells expressing barcoded versions of the indicated constructs were assayed for proliferation in vitro using Luminex-based assays. Graphs show mean relative signal for each cell population relative to the control vector-expressing population (± SEM). $n = 6$ wells per cell line. (*D*–*G*) A375 (*D* and *E*), 67NR (*F*), or NMuMG (*G*) cells expressing the indicated constructs were assayed by transwell migration or invasion assays; graphs show either the numbers of cells on the bottom of the filter or mean fold invasion (relative to control cells) (± SEM). $n = 3$ triplicate wells in *D* and *E*; $n = 3$ independent experiments done in duplicate in *F*; and $n = 3$ independent experiments done in triplicate in *G*. n.s., not significant; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; repeated measures ANOVA with Dunnett's post test (*C*) or paired *t* test (*D*–*G*).

mixture was cultured in vitro over the same 24-h period (Fig. S4*E*), suggesting that YAP^{S127A}-expressing cells have increased proliferative potential in vitro and in vivo. Similar results were obtained with 67NR cells by mixing mCherry-positive control cells with ZSgreen-positive cells expressing YAP^{S127A} (Fig. S4*D*). Together, these results suggest that increased YAP activity enhances the ability of circulating tumor cells to colonize the lung without dramatically impacting initial seeding.

Metastatic Potential Is Strongly Correlated with YAP/TEAD Activity. Several studies have found that increased YAP activity can promote TEAD-dependent transcription (39, 40, 42, 48, 49) in a manner that is dependent upon the TEAD-interaction domain of YAP (39, 40, 42, 48). Therefore, we next tested whether increased metastatic ability is correlated with increased TEAD transcriptional activity. In both 67NR and NMuMG cells, expression of YAP^{S127A} significantly increased the activity of a TEAD-dependent luciferase reporter construct (Fig. 6*A*). As expected, mutation of the TEAD-interaction domain severely impaired the YAP-dependent induction of the TEAD reporter, but mutation of the WW domains or truncation of the PDZbm did not (Fig. 6*A*). Next we tested whether TEAD transcriptional activity was correlated with metastatic potential in mouse and human breast cancer cell lines. Metastatic human mammary carcinoma cells (MDA-MB-231, MDA-MB-468, BT-20, and BT-54.9) displayed dramatically higher TEAD transcriptional activity than nonmetastatic mammary carcinoma (T47D, SUM159, and MCF7) and mammary epithelial cells (MCF10A) (Fig. 6*B*). Highly metastatic mouse mammary carcinoma cell lines (4T1 and 66cl4) also displayed significantly higher TEAD transcriptional activity than the weakly metastatic 67NR cells (Fig. S5*A*). However, TEAD reporter activity also was increased in the weakly metastatic 168FARN and 4T07 cells compared with 67NR cells (Fig. S5*A*), indicating that high TEAD transcriptional activity alone is not always sufficient for metastasis. Nevertheless, these results demonstrate that highly metastatic human and mouse breast cancer cells have high TEAD transcriptional activity and suggest a strong correlation between TEAD-mediated transcription and metastasis.

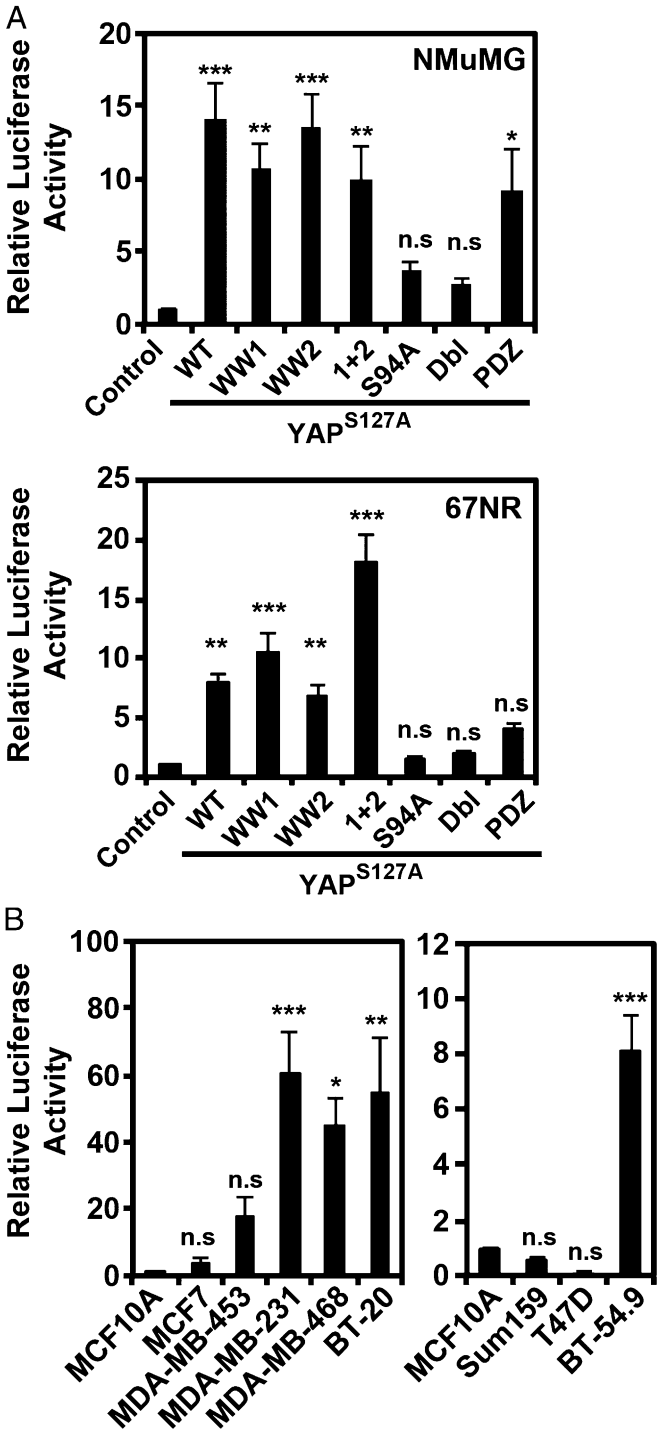

Fig. 6. TEAD transcriptional activity is strongly correlated with metastatic potential. (*A*) 67NR or NMuMG cells expressing the indicated constructs were assayed for TEAD transcriptional activity using a TEAD-dependent promoter-driven firefly luciferase reporter construct. Graphs show normalized luciferase activity (relative to control cells, ± SEM); $n = 3$ independent experiments done in duplicate. (*B*) The indicated cell lines were assayed for TEAD transcriptional activity as above. The graphs show normalized luciferase activity (relative to MCF10A cells) for each cell line (± SEM). n = at least 6 separate transfections assayed in duplicate; $^*P \le 0.05$; $^{**}P \le 0.01$; $^{***}P \le 0.001$; n.s., not significant; one-way ANOVA with Dunnett's post test.

To explore the relationship between YAP/TEAD activity and metastasis further, we tested whether the prometastatic effects of YAP could be enhanced by increasing YAP/TEAD activity further. As mentioned above, the Hippo pathway represses YAP activity both by promoting its cytoplasmic sequestration through phosphorylation of serine 127 (1, 9) and by promoting its proteasomal degradation through phosphorylation of serine 381 (12). Therefore, we compared the activity of wild-type YAP with that of YAP mutants that were insensitive to Hippo pathway-mediated sequestration (YAP^{S127A}), Hippo pathway-mediated degradation (YAP^{S381A}), or both ($YAP^{S127A,S381A}$). As expected, YAP^{S127A} and YAP^{S381A} both showed higher TEAD transcriptional activity than wild-type YAP (Fig. 7*A* and Fig. S5 *B* and *C*). Accordingly, in Luminex-based spontaneous metastasis assays, tumor cells expressing these mutants were significantly enriched in the primary tumors and metastatic lungs compared with cells expressing the control vector or wild-type YAP (Fig. 7*B* and Fig. S5 *D* and *E*). These results suggest that the Hippo pathway inhibits metastasis both by promoting YAP degradation and by YAP cytoplasmic sequestration. Consistently, cells expressing the $YAP^{S127A,S381A}$ double mutant generally had the highest TEAD reporter activity and were the most significantly enriched cells in the primary tumors and metastatic lungs of mice (Fig. 7*B* and Fig. S5 *D* and *E*). We did observe some differences in the relative activities of each YAP mutant among cell lines, suggesting that the potency of endogenous Hippo signaling in these cell lines is variable. Together, these results suggest a strong correlation between YAP/TEAD activity and metastatic potential in breast cancer and melanoma cells.

Discussion

YAP Promotes Metastasis by Influencing Events at both Primary and Metastatic Sites. Although YAP is a known oncogene in several cell types (10, 13, 25, 26, 28, 29), a role for YAP in promoting metastasis has not been described to our knowledge. Here we demonstrate that increased YAP activity drives mammary carcinoma and melanoma metastasis and potently enhances tumor growth. Mutant forms of YAP have not been described in humans, but the *yap1* locus is amplified in several human cancers (13–15), and mutations or silencing of Hippo pathway members,

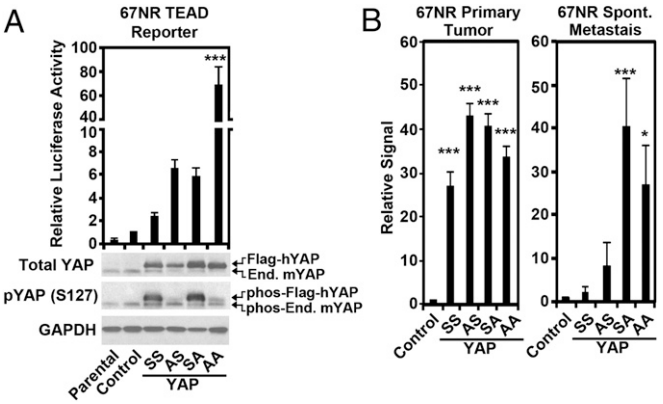

Fig. 7. Loss of Hippo pathway-mediated repression of YAP leads to increased TEAD transcriptional activity and enhanced metastasis. (*A*) 67NR cells were stably transduced with barcoded control vector or vectors expressing wild-type YAP (YAP-SS), YAP^{S127A} (YAP-AS), YAP^{S381A} (YAP-SA), or $YAP^{S127A,S381A}$ (YAP-AA) and were assayed by Western blot for the indicated proteins or for TEAD transcriptional activity. Graphs show normalized luciferase activity (relative to control cells; ± SEM); $n = 6$ independent experiments done in duplicate. (*B*) Cells from *A* were mixed in equal numbers, and then 5×10^5 cells were used in Luminex-based orthotopic mammary transplant and spontaneous metastasis assays. After 29 d, lungs and primary tumors were isolated, and the relative numbers of cells expressing each vector were quantified using Luminex. Graphs show mean signal (± SEM) for each cell line relative to the signal for cells expressing control vector; $n = 10$ mice. $^*P \le 0.05$; $^{***}P \le 0.001$; one-way ANOVA with Dunnett's post test.

which would result in increased YAP activity, have also been identified (6, 8, 32). In addition, both increased YAP protein staining and reduced LATS mRNA levels are correlated with metastasis in human patients (3, 22). Combined with these studies, our results suggest that YAP plays a functional role in the progression and spread of human cancer and that the Hippo pathway restricts tumor progression and metastasis by promoting both the cytoplasmic sequestration and the proteasomal degradation of YAP. Interestingly, YAP also has been implicated in the cellular response to DNA damage (26, 50–52) and in promoting resistance to chemotherapeutics (17, 53). Thus, disrupting YAP function in tumor cells has the potential to decrease primary and metastatic tumor burden, block further metastatic spread, and make disseminated tumor cells more susceptible to chemotherapeutics.

Although the enhanced in vitro and in vivo growth observed in cells expressing active YAP likely contributes to the increased metastatic burden, our results show that YAP also can promote metastasis through additional mechanisms. Indeed, YAP^{S127A} rendered nonmetastatic NMuMG cells highly metastatic without enhancing primary tumor formation or growth (Figs. 1 and 3), suggesting that YAP's effects on metastasis are not solely the result of its ability to enhance growth in vivo. Active YAP also enhanced invasion (Fig. 5), a process known to play an important role in promoting metastasis from the primary site. Consistently, YAP^{S127A} was able to render otherwise nonmetastatic 67NR mammary carcinoma and A375 melanoma cells metastatic from the primary site metastatic (Fig. 3 and Fig. S3). YAP also enhanced the ability of multiple cell lines to colonize the lungs in tail-vein metastasis assays (Figs. 1 and 2), which bypass the need for cells to leave the primary tumor and enter circulation, indicating that YAP also influences processes that occur at the metastatic site. Because expression of active YAP did not enhance initial seeding of tumor cells in the lungs (Fig. S4 *D* and *E*), the increased metastatic burden that we observed (Figs. 1–3 and Fig. S2) is likely the result of enhanced extravasation, survival, or proliferation of the disseminated tumor cells. Thus, increased YAP activity promotes metastasis by enhancing prometastatic processes that occur both in the primary tumor and at the metastatic site.

YAP-Mediated Tumor Growth in the Breast Is Context-Dependent, but Metastasis Is Not. To date, the role of YAP in breast cancer has been controversial. There is clear evidence that YAP can promote the proliferation and tumorigenicity of breast cancer cells (14, 25, 40, 47). In contrast, YAP expression is reduced in some human breast cancer samples (54, 55), and YAP can promote apoptosis in other cell types (43, 50, 52, 56–58). These findings suggest that YAP's ability to enhance tumor growth may be context-dependent. In support of this notion, we found that active YAP enhanced tumor growth when expressed in mammary carcinoma or melanoma cells but not when expressed in a nontransformed mammary epithelial cell line (Fig. 1 and Fig. S1). As a transcriptional coactivator, YAP can regulate multiple target genes, and it is possible that some of these genes enhance tumor growth and metastasis, whereas others promote apoptosis. Thus, the protumorigenic effects of YAP may depend upon the ability of a cell to resist the proapoptotic effects of YAP. Indeed, in addition to YAP, the 11q22 amplicon contains a known inhibitor of apoptosis, cIAP1, which collaborates with YAP to promote tumorigenesis (13). A further complication is that YAP potentially could promote cell competition in mammals, as Yorkie does in *Drosophila* (45, 46). Consistent with this idea, we found that NMuMG cells expressing active YAP were able to significantly outcompete control cells when transplanted as a mixture (Fig. 3) but did not show enhanced tumor growth when injected alone (Fig. 1). Elucidation of the exact context in which YAP promotes tumor growth instead of apoptosis is of great interest but will require extensive experimentation that is beyond the scope of this paper. Importantly, our studies show that increased YAP activity enhances the metastatic potential of cells, whether or not it also is able to enhance their in vivo growth.

YAP Promotes Tumor Growth and Metastasis Through Its TEAD-Interaction Domain. Although the TEAD-interaction domain of YAP is known to be important for several YAP-mediated processes (10, 11, 40, 59), the WW domains and the PDZbm also are important for certain YAP functions (24, 42, 43, 51). To test the importance of these domains, we developed a Luminex-based approach for multiplexing in vivo assays. This approach allowed detection of transgene or shRNA-mediated enrichment or depletion of individual cell populations, relative to control populations, in the primary tumors and metastatic tumor-containing lungs of mice (Figs. 3 and 4). Luminex-based assays also can be used to multiplex in vitro assays for transformation and cell proliferation (Fig. 5 and Fig. S4). The cost of in vivo experimentation and the lack of a reliable in vitro surrogate for the metastatic phenotype often make it difficult to explore the mechanism of prometastatic proteins. Our Luminex-based approach overcomes these limitations by providing a means to test the metastatic potential of multiple cell populations simultaneously using both in vitro and in vivo assays.

Using Luminex-based assays and traditional assays, we found that the TEAD-interaction domain of YAP is critical for the protumorigenic and prometastatic effects of YAP. Indeed, mutation of the TEAD-interaction domain severely impaired YAP^{S127A}-mediated tumor growth, metastasis, invasion, and transformation, whereas mutation of the WW domains or removal of the PDZbm did not (Figs. 3 and 5 and Figs. S2–S4). Several groups have used mutant forms of YAP containing the S94A mutation or the corresponding murine S79A mutation to demonstrate the importance of the TEAD family of transcription factors in YAP-mediated processes (10, 11, 40, 59). Our results using this mutant suggest that YAP promotes tumor progression and metastasis by regulating the expression of TEAD-target genes and that targets downstream of proteins that bind to the WW domains or PDZbm are not essential. In support of this conclusion, we found that human and mouse cancer cells with high metastatic potential have dramatically increased TEAD transcriptional activity (Fig. 6) and that disrupting the ability of the Hippo pathway to repress YAP/TEAD transcriptional activity enhances metastasis (Fig. 7 and Fig. S5). Because several processes regulated by YAP can contribute to tumor progression, it seems likely that multiple downstream TEAD-target genes are involved. However, at this time we cannot rule out the possibility that a single target gene mediates all observed YAP-mediated effects. Given the importance of the TEADs in the oncogenic functions of YAP, the YAP/TEAD complex may represent a promising target for anticancer therapies. Indeed, a recent study demonstrated that verteporfin, a small molecule that inhibits TEAD–YAP interaction, suppressed the oncogenic effects of YAP (59).

In summary, we found that aberrant YAP activity can promote mammary carcinoma and melanoma metastasis in a manner that is highly dependent on the TEAD-interaction domain. YAP exerts its prometastatic effects at both the primary site and the metastatic site and can enhance several distinct processes known to contribute to metastasis. Together, these results suggest that increased YAP activity can contribute functionally to metastatic disease, making YAP and its regulators potential therapeutic targets.

Methods

Cell Lines and Mice. The 4T1, 168FARN, 4T07, and 67NR murine mammary carcinoma cell lines (a kind gift from Fred Miller, Wayne State University, Detroit, MI) were described previously (60) and were cultured in DMEM supplemented with 5% (vol/vol) newborn calf serum, 5% (vol/vol) calf

serum, 2 mM L-glutamine, and nonessential amino acids. The human mammary carcinoma cell lines MDA-MB-231, MDA-MB-453, MDA-MB-468, BT-20, MCF7, T47D, and BT-54.9 (ATCC) and the A375-GFP human melanoma cells (61) were cultured in DMEM supplemented with 10% (vol/vol) FBS and 2 mM L-glutamine. The murine mammary epithelial cell line NMuMG (ATCC) was cultured in DMEM supplemented with 10% (vol/vol) FBS, 2 mM L-glutamine, and 10 μg/mL insulin. The human mammary epithelial cell line MCF10A (ATCC) was cultured in DMEM/F12 supplemented with 5% (vol/vol) horse serum, 20 ng/mL epidermal growth factor, 0.5 mg/mL hydrocortisone, 1 mg/mL cholera toxin, 10 μg/mL insulin, and 2 mM L-glutamine. The human mammary carcinoma cell line SUM159 (Asterand) was cultured in Ham's/F12 medium supplemented with 5% (vol/vol) FBS, 1 μg/mL hydrocortisone, 5 μg/mL insulin, and 2 mM L-glutamine.

For in vivo experiments, 6- to 10-wk-old female mice (Taconic) of the following strains were used: syngeneic Balb/C mice for the murine mammary carcinoma cells (4T1, 168FARN, 4T07, and 67NR); NCR-Nude mice for the murine mammary epithelial cells (NMuMG); and NOD-SCID mice for the human melanoma cells (A375-GFP). All animal experiments and husbandry were approved by the MIT Department of Comparative Medicine.

Vectors and Retroviral Transduction. The human YAP^{S127A} construct was cloned from pCDNA-YAP^{S127A} (a kind gift from Kun-Liang Guan, University of California at San Diego, La Jolla, CA) (1) into the indicated versions of the murine stem cell virus (MSCV) retroviral vector (Table S1). Mutant forms of YAP^{S127A} were generated via PCR-mediated site-directed mutagenesis of the pCDNA-YAP^{S127A} vector, sequenced, and then cloned into barcoded versions of the MSCV-internal ribosome entry site Hygromycin (MSCV-IRES-Hygro) retroviral vector. miR30-based shRNAs targeting RPA3, which were described previously (62), were synthesized (Integrated DNA Technologies) and then cloned into barcoded versions of the MSCV-ZSG-2A-Puro-miR30 vector, which is a derivative of the MSCV-Puro-2A-GFP-miR30 vector (63). All other vectors were generated from the base MSCV-Puro-2A-GFP-miR30 vector (63) using standard molecular biology techniques. The TEAD-dependent promoter-driven luciferase reporter construct, pGL3-5xMCAT(SV)-49 (a kind gift from Iain Farrance, Virginia Technological Institute and State University, Blacksburg, VA), was described previously (64). Packaging of retrovirus and transduction of cells was done as described previously (63).

Invasion and Migration Assays. To assay invasive potential, 4×10^4 67NR cells or 5×10^4 NMuMG cells were seeded into growth factor-reduced Matrigel invasion chambers (8-μm pore) (BD). After 24 h cells were stained with DAPI, and cells on the bottom of the filter were quantified by counting four 10× fields per filter. Assays for migration and invasion of A375 cells have been described previously (61).

Western Blots and Quantitative PCR. For immunoblotting, cells were lysed in Cell Lysis Buffer (Cell Signaling Technology), and 15–30 μg of protein was subjected to SDS/PAGE using 4–12% (wt/vol) gradient gels (Invitrogen), transferred to nitrocellulose membranes, and assayed by immunoblotting. Primary antibodies were used at the following concentrations: mouse anti–E-cadherin (BD), 1:5,000; mouse anti–N-cadherin (BD), 1:5,000; rabbit anti-fibronectin, 1:2,000; rabbit anti-vitronectin (Abgent) 1:1,000; rabbit anti-snail (Cell Signaling Technology), 1:1,000; rabbit anti-YAP1 (Cell Signaling Technology), 1:1,000; mouse anti-GAPDH (Millipore), 1:5,000. HRP-conjugated secondary antibodies were used at the following concentrations: goat anti-rabbit IgG (Jackson Laboratory), 1:5,000; goat anti-mouse IgG (Jackson Laboratory), 1:5,000. For quantitative PCR (qPCR), cells were lysed in TRIzol Reagent (Invitrogen Corporation), and RNA was isolated according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed to produce the cDNA template using the First-Strand cDNA Synthesis Kit (Promega). qPCR reactions were carried out on 2 μL of cDNA, with 12.5 μL IQ SYBR Green Supermix (Bio-Rad) and 0.4 μmol of each primer (Table S2). Analysis was performed using the MyiQ real-time PCR detection system (Bio-Rad) according to the manufacturer's instructions. PCR conditions were 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s.

Dual Luciferase Reporter Assay. For TEAD transcriptional activity assays, the indicated cells were plated on 12-well dishes in duplicate and cotransfected with 400 ng of a 20:1 mixture of pGL3-5xMCAT(SV)-49 (64) and PRL-TK (Promega) using Lipofectamine Plus (Invitrogen). After 48 h, luciferase activity was assayed using the dual luciferase reporter assay system (Promega) and the Tecan Infinite 200Pro plate reader. For each duplicate well the signal of firefly luciferase (pGL3-5xMCAT(SV)-49) was normalized to the signal for Renilla luciferase (PRL-TK).

In Vivo Tumor Growth and Metastasis Assays. For tail-vein metastasis assays the indicated numbers of cells were injected into the lateral tail veins of mice in 100 μL of HBSS. After the indicated number of days the lungs were inflated with and fixed in 3.7% (wt/vol) formaldehyde for 24 h, followed by 24 h in 75% (vol/vol) ethanol. For metastasis quantification, GFP- or ZSgreen-positive tumors were counted in whole lungs or the numbers of metastases were counted in paraffin-embedded, H&E-stained sections. Tumor growth and spontaneous metastasis were assayed by transplanting mammary carcinoma cells orthotopically into the mammary fat pad or A375 cells s.c. For orthotopic mammary transplant assays, 6- to 10-wk-old female mice were anesthetized by i.p. injection of 125–250 mg/kg body weight of Avertin (reconstituted in PBS), followed by i.p. injection of 100 μL of 12 μg/mL buprenorphine for analgesia. A small incision was made on the right flank, and the indicated numbers of tumor cells in 25 μL of HBSS were injected into the right number 4 fat pad using a 25-μL Hamilton syringe. Mice received three additional i.p. injections of 100 μL of 12 μg/mL buprenorphine at 12-h intervals following the surgery. After the indicated number of days tumor masses were measured, and lungs were fixed and assayed for metastasis as above. In some experiments tumor volumes were estimated using the formula (width × length^2)/2. For s.c. injections the indicated numbers of cells were injected into the right flank in 200 μL of HBSS, and after the indicated number of days tumor masses were measured, and lungs were fixed and assayed for metastasis as above.

Flow Cytometry-Based Assays. Control or YAP^{S127A}-expressing cells that also expressed either mCherry or ZSgreen were mixed in equal numbers as indicated in the figure legends and then were injected into the lateral tail veins of mice or were plated and cultured in vitro. For some experiments control or YAP^{S127A}-expressing cells were stained with Cell Tracker Green CMFDA (5.6 μg/mL in DMEM) or Cell Tracker Red CMPTX (5.6 μg/mL in DMEM) (Invitrogen) for 30 min, allowed to recover for 1 h, mixed in equal numbers, and then injected into the lateral tail veins of mice or plated and cultured in vitro. After the indicated number of days, cultured cells and lungs were isolated, and single-cell suspensions were generated. For this purpose, cells were collected by trypsinization, and lungs were isolated, minced, digested in 1.5 mg/mL type I collagenase for 3 h at 37 °C, pelleted, and resuspended in 0.625% trypsin-Versene for 5 min. All cell suspensions were pelleted and resuspended in PBS with 5% (vol/vol) BSA, and stained with propidium iodide. The percentages of red and green cells were quantified using the LSR II flow cytometer (BD). In all flow cytometry-based experiments, a starting population was collected at the time the cell populations were mixed, and all subsequent cell suspensions were normalized to this starting population. Raw flow data were analyzed using FlowJo version 8.6.

Luminex-Based Assays. Mutant forms of human YAP^{S127A} were cloned into uniquely barcoded versions of the MSCV-IRES-Hygromycin vector and used to transduce stably cells that subsequently were mixed in equal numbers and used in the Luminex-based assays. For tumor growth and metastasis, cell mixtures were transplanted orthotopically or injected into the lateral tail veins of mice, and lungs and primary tumors were isolated for analysis after the indicated number of days. For in vitro proliferation assays, the cell mixture was cultured for the indicated number of days, during which samples were collected every 3–5 d for analysis. For soft agar assays, the cell mixture was grown in soft agar for 2 wk, and then colonies were pelleted out of the agar for analysis. For all Luminex experiments, a starting population was collected at the time the cell populations were mixed and was used for normalization of subsequent samples. For analysis, genomic DNA was isolated from tumors, lungs, and cell pellets, and barcodes were PCR-amplified using biotinylated primers that bind to common regions in the vector flanking the DNA barcode and then quantified by the Genetic Analysis Platform at the Broad Institute (Cambridge, MA) using Luminex technology. Briefly, PCR product was hybridized to uniquely dyed Xmap beads (Luminex Corporation) that were precoupled to oligonucleotide sequences complementary to the 21-nt barcodes contained in our vectors. Barcodes were then quantified by incubating the beads with streptavidin-conjugated allophycocyanin (APC) and measuring the APC signal on each bead using the Luminex FlexMap 3D system (Luminex Corporation). The relative amounts of each unique barcode in a sample then were calculated from the raw APC signals. Addition details for Luminex-based assays are provided in *SI Methods*.

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