**Alternate RASSF1 Transcripts Control SRC Activity, E-Cadherin Contacts, and YAP-Mediated Invasion**

**Graphical Abstract**

- **Highlights**
  - Methylation of RASSF1A correlates with loss of hippo-inhibitory phospho-S127-YAP1
  - SRC, FYN, and YES are activated by RASSF1C in RASSF1A-methylated cells
  - RASSF1C promotes E-cadherin internalization and reduces cell junction integrity
  - RASSF1A loss drives RASSF1C-YAP1/β-catenin-mediated transcription and invasion

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**In Brief**

In a wide variety of sporadic malignancies, promoter methylation of the RASSF1 gene is associated with tumor invasion and metastasis. Vlahov et al. show that the clinical phenotype is driven by both RASSF1A loss and the independently transcribed RASSF1C isoform, which promotes SRC activation, pseudo-EMT, and β-catenin/YAP1-dependent invasion of tumor cells.

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Alternate RASSF1 Transcripts Control SRC Activity, E-Cadherin Contacts, and YAP-Mediated Invasion

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SUMMARY

Tumor progression to invasive carcinoma is associated with activation of SRC family kinase (SRC, YES, FYN) activity and loss of cellular cohesion. The hippo pathway-regulated cofactor YAP1 supports the tumorigenicity of RAS mutations but requires both inactivation of hippo signaling and YES-mediated phosphorylation of YAP1 for oncogenic activity. Exactly how SRC kinases are activated and hippo signaling is lost in sporadic human malignancies remains unknown. Here, we provide evidence that hippo-mediated inhibition of YAP1 is lost upon promoter methylation of the RAS effector and hippo kinase scaffold RASSF1A. We find that RASSF1A promoter methylation reduces YAP inactivation-127 kinase, which derepresses YAP1, and actively supports YAP1 activation by switching RASSF1 transcription to the independently transcribed RASSF1C isoform that promotes Tyr kinase activity. Using affinity proteomics, proximity ligation, and real-time molecular visualization, we find that RASSF1C targets SRC/YAP to epithelial cell-cell junctions and promotes tyrosine phosphorylation of E-cadherin, β-catenin, and YAP1. RASSF1A restricts SRC activity, preventing motility, invasion, and tumorigenesis in vitro and in vivo, with epigenetic inactivation correlating with increased inhibitory pY527-SRC in breast tumors. These data imply that distinct RASSF1 isoforms have opposing functions, which provide a biomarker for YAP1 activation and explain correlations of RASSF1 methylation with advanced invasive disease in humans. The ablation of epithelial integrity together with subsequent YAP1 nuclear localization allows transcriptional activation of β-catenin/TBX-YAP/TEAD target genes, including Myc, and an invasive phenotype. These findings define gene transcript switching as a tumor suppressor mechanism under epigenetic control.

INTRODUCTION

Recent advances have highlighted that YES-associated protein (YAP1) supports KRAS tumorigenicity and assists in the maintenance of transformed phenotypes [1]. YAP1 drives proliferation by acting as a cofactor for TEAD transcriptional regulators, an activity which is restricted by hippo pathway-mediated disruption of TEAD association. In model systems, genetic ablation of core hippo pathway components leads to increased tumorigenesis [1]. In human tumors, failure to activate LATS1 due to either GNAQ mutations in uveal melanoma or through inactivation of NF2/merlin in the tumor-prone neurofibromatosis syndrome prevents this inhibitory signal and makes YAP1 permissive for activation [1]. Similarly, stromal mechanics and genetic instability are reported to trigger the hippo pathway and present a tumor barrier, but as with GNAQ mutations and germline defects in NF2/merlin, these mechanisms appear to be independent of the hippo kinase/MST itself [1]. Identification of the core hippo pathway by proteomics has revealed the main direct activators of MST kinases to be SAV1 and RASSFs [2], which although infrequently mutated in cancers [3] have germline and epigenetic alterations, particularly in RASSF1A, that accelerate tumor onset and increase tumorigenicity [4, 5]. Moreover, RASSF1A activation of the hippo pathway both restricts YAP1 binding to TEAD [6] and is a direct substrate of RAS signaling in the pancreas [7], supporting the potential crosstalk in pancreatic tumor development [8, 9]. Intriguingly, methylation of the CpG island (CGI) spanning the promoter and first exon of RASSF1 has widespread prognostic value for disease-free and poor overall survival in all major sporadic cancers [10]. Thus, loss of RASSF1A expression in RASSF1-methylated tumors is likely to contribute to reduced hippo pathway-mediated repression of YAP1, due to direct activation of MST kinases and the subsequent interaction and activation of LATS1 [4, 11–14]. This RASSF1A/MST2-promoted LATS1 activity is targeted toward YAP1 [12], resulting in increased pS127-YAP [15] and decreased YAP-TEAD oncogenic behavior [6, 15].

Hahn and colleagues recently highlighted that, by using a YAP1 mutant for all LATS1 phosphorylation sites (SSA) including the inhibitory S127-YAP1 phosphorylation, while permissive, required additional phosphorylation of Y357-YAP1 by the SRC
Figure 1. Loss of RASSF1A Mediates Nuclear Localization of YAP1

(A) Correlation of RASSF1 methylation levels (meRASSF1) below 0.3, between 0.3 and 0.5, and above 0.5 with loss of inhibitory YAP1-phospho-Ser127 (pS127-YAP1) in the cancer genome atlas (TCGA) data sets (cBioportal).

(B) U2OS

(C) RASSF1 3p21.3

(D) sRASSF1_1

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family kinase (SFK) YES to activate transcription [16]. Moreover, they found that the main tumorigenic consequence of APC loss in colorectal cancer was due to a subset of β-catenin genes that were YAP1 dependent. Thus, YAP1 may serve to integrate WNT, RAS signaling in order to trigger epithelial-to-mesenchymal transition (EMT) and invasive disease [17]. Tumor progression to invasive carcinoma is associated with constitutive loss of cellular cohesion, leading to EMT, increased cellular motility, and invasion into the surrounding tissues [18]. Activation of SFKs is associated with increased tumor invasion and metastasis through initial destabilization of epithelial cell–cell junctions. Mechanistically, this occurs through direct phosphorylation of E-cadherin, p120-catenin, and β-catenin [19–21]. Phosphorylation of E-cadherin promotes internalization, whereas phosphorylation of β-catenin decreases its affinity for E-cadherin, allowing nuclear localization where it serves as a transcriptional co-activator of b-catenin [22, 23]. The identification of a YAP1–localization where it serves as a transcriptional co-activator of b-catenin promotes internalization, whereas phosphorylation of β-catenin decreases its affinity for E-cadherin, allowing nuclear localization where it serves as a transcriptional co-activator of TCF/LEF1 factors [22, 23]. The identification of a YAP1–β-catenin transcriptional program specified a novel role for β-catenin in activating transcription via T-box factors rather than TCF, particularly in cancers [16]. Importantly, YAP1 has also been shown to cooperate with β-catenin at TEAD-dependent promoters to induce cell proliferation in the heart and during tumorigenesis [24].

Here, we identify that RASSF1 CGI methylation correlates with hippo pathway inactivation and loss of pS127–YAP1 in sporadic malignancies and provide evidence for the association of this methylation with invasive behavior in cancers, such as breast, bladder, and in gliomas. CGI methylation restricts primary transcript expression but can additionally promote switching to alternative gene promoters or influence splicing, implying that epigenetic regulation may modulate the relative levels of gene isoforms rather than simply silence gene expression [25]. In tumors with RASSF1 CGI methylation, an alternative isoform, RASSF1C, is expressed from an internal promoter and has been suggested to promote motility and invasive disease [26, 27]. We find that RASSF1C actually supports tumorigenesis by promoting SRC/YES-mediated phosphorylation of E-cadherin, β-catenin, and YAP1, disrupting cell-cell contacts and initiating an EMT-like response. RASSF1A also binds to SFKs but additionally scaffolds CSK, potentially through an exon 1α-encoded C1 domain, not present in RASSF1C, which inhibits SFKs and maintains epithelial integrity. In the absence of RASSF1A, RASSF1C promotes tyrosine phosphorylation of β-catenin and YAP1, resulting in their re-localization to the nucleus and transcriptional activation of the TBX target genes, BCL2L1, BIRC5 [16], and cMyc [28]. Analyses of invasive breast tumor data sets indicate an inverse correlation of high RASSF1A methylation/low pS127–YAP1 with SRC activation and the expression of invasion-associated transcripts. To validate a role in motility and invasion, we demonstrate that RASSF1C directly promotes SFK-dependent motility, 3D invasion of mammospheres and tumor spread in vivo. These data imply that SFK activation/inactivation relies on distinct RASSF1 isoforms, presenting a mechanism for YAP1 activation in sporadic tumors and explains the clinical correlation of RASSF1 methylation with advanced invasive disease.

RESULTS

Switching of RASSF1 Isoforms Induces Nuclear Localization of YAP1

RASSF1A is a hippo pathway scaffold that switches YAP1 association from oncogenic TEAD transcriptional complexes to tumor-suppressive YAP1/p73 [6]. As RASSF1A expression is lost in multiple cancers and associates with poor outcome, we wanted to determine whether this was a route through which the hippo pathway may be inactivated in sporadic cancers. To address this, we explored YAP1 protein information in data sets of tumors where RASSF1 is known to be methylated, clinically significant [10], and for which pS127–YAP information was available. We found that methylation of RASSF1–1α (representing gene silencing) significantly correlates with low pS127–YAP1 in glioma, bladder, and breast cancer cohorts (Figure 1A), suggesting that YAP1 may be nuclear and active in RASSF1-methylated tumors. To test this correlation, we targeted RASSF1A expression in U2OS cells, unmethylated for RASSF1A, with siRNAs and observed lower pS127–YAP1 in line with the clinical data (Figures S1A and S1C). Intriguingly, in contrast to siRNA targeting exons common to all isoforms (siRASSF1), specific ablation of the RASSF1A isoform resulted in elevated nuclear localization of YAP1, indicating that reduced pS127–YAP1 appears required but insufficient for nuclear localization of YAP1 (Figure 1B). In keeping with a loss of hippo pathway activity, the loss of RASSF1 or RASSF1A led to a decrease in the phosphorylation of the core hippo kinases MST1/2 and LATS1 (Figure S1C) and was conversely increased by overexpression of RASSF1A in both U2OS and H1299 (methylated) cells (Figure S1D). RASSF1C transcripts are often present in RASSF1A-methylated tumors due to expression from a distinct promoter and are susceptible to siRASSF1, but not siRASSF1A (Figures 1C, S1B, and S1C) [27]. To determine whether the RASSF1C transcript was responsible for elevated nuclear YAP1 upon siRASSF1A, we designed derivatives of RASSF1A or RASSF1C to be resistant...
Figure 2. RASSF1C Binds to and Activates SFKs

(A) Proteomic identification of RASSF1A and RASSF1C immunoprecipitates by tandem MS/MS.

(B) HA-tag immunoprecipitation from 293T cells transfected with MYC-SRC (left), MYC-FYN (middle), or MYC-YES (right) in combination with HA-RASSF1A or HA-RASSF1C, analyzed by immunoblotting.

(C) CSK immunoprecipitation from H1299 cells transfected with FLAG-RASSF1A or FLAG-RASSF1C. The precipitates along with input fractions were analyzed by immunoblotting.

(D) RASSF1 immunoprecipitation from HeLa cells, immunoblotted for CSK, SRC, and RASSF1A.

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to the two distinct RASSF1 siRNAs, allowing a direct comparison. RASSF1C expression restored nuclear YAP1 in siRASSF1-transfected cells without affecting the pS127-YAP1 levels, whereas RASSF1A failed to do so (Figures 1D, S1E, S1G, and S1H). Thus, reduced hippo signaling and pS127-YAP levels appear effectively uncoupled from automatic nuclear localization, in keeping with the recently demonstrated requirement for tyrosine phosphorylation to mediate the transition [29]. Intriguingly, phosphotyrosine immunoprecipitates of U2OS cells demonstrate that RASSF1C promotes increased tyrosine phosphorylation of YAP1 (Figure S1F). This indicates that promoter methylation of RASSF1-1a, which inhibits RASSF1 isoform A expression, reduces hippo signaling and inhibitory pYAP-S127 but favors the nuclear localization of YAP1 via RASSF1-1a-independent transcription of RASSF1 isoform C.

RASSF1 Isoforms Interact with and Differentially Regulate SFKs

To determine the mechanism of how RASSF1C promotes tyrosine phosphorylation and nuclear localization of YAP, we performed a proteomic screen of both isoforms to identify novel protein-protein interactions (Figure 2A). We screened RASSF1 immunoprecipitates for candidates and found that both RASSF1 isoforms bind the tyrosine kinases c-SRC, FYN, and YES (Figure 2B); interestingly, however, RASSF1A had an additional unique association with the SRC inhibitory kinase, CSK (Figures 2C and S2G). The binding of endogenous RASSF1A to CSK was confirmed in HeLa cells, unmethylated for RASSF1A (Figure 2D), and could also be demonstrated to be direct using bacterially purified proteins for RASSF1A, SRC, and CSK (Figure 2E). This association was in line with a similar role for the homolog dRASSF8 in Drosophila [30]. To further confirm association, we employed proximity ligation assays and found that, whereas both isoforms bind SRC, RASSF1C associated with active pY416-SRC whereas RASSF1A bound inactive, CSK-phosphorylated, pY527-SRC (Figures 2F and S2A). We next investigated the effect of RASSF1 isoform modulation of SFK activity. Depletion of RASSF1A alone had no effect on SRC activity, whereas siRASSF1 led to reduced pY416-SRC in serum or HGF-stimulated cells (Figures S2B and S2C). Conversely, exogenous expression of RASSF1C elevated pY416-SRC in RASSF1-1a methylated colorectal, breast, and lung cancer cells but failed to do so in unmethylated cells where RASSF1A is expressed (Figures S2D and S2E) [4]. We therefore hypothesized that RASSF1C may activate SRC but is restricted by competition for association with higher-affinity RASSF1A and associated CSK. To test this, we expressed RASSF1C in H1299 cell lines in which RASSF1A expression was inducible [4] and observed increased pY416-SRC only in the absence of RASSF1A (Figure S2F). Taken together, the data indicate that RASSF1A and RASSF1C modulate SRC activity by promoting differential phosphorylation of SRC.

RASSF1C Targets SRC to the Plasma Membrane, Destabilizing Junctions

Once activated, SRC translocates to the cell membrane and phosphorylates key target proteins [31]. To determine whether the activation of SRC by RASSF1C affects the localization, we tracked GFP-SRC and pY416-SRC by fluorescence microscopy. We found that increased pY416-SRC levels in cells correlated with localization of RASSF1C, endogenous SRC, and pY416-SRC at the membrane (Figures S3A and S3B), with a significant increase at cell junctions compared to non-junctional plasma membrane (Figures 3A and 3B; red versus white arrows). Phosphotyrosine immunoprecipitates suggest that RASSF1C promotes phosphorylation of junction proteins E-cadherin and β-catenin, but not p120-catenin or FAK (focal adhesion kinase) (Figure 3C). SRC phosphorylation of E-cadherin is known to increase its internalization, subsequently destabilizing cell-cell junctions [19]. To determine whether RASSF1C-promoted phosphorylation of E-cadherin has any effect on E-cadherin junctional integrity, we first took GFP-E-cadherin-expressing cells where E-cadherin can be visualized at the cell periphery (Figure 3D). Co-expression of RASSF1C decreased intensity of GFP-E-cadherin at cell-cell junctions compared to controls but did not affect a GFP-E-cadherin derivative harboring mutations in all three SRC phosphorylation sites Y753F, Y754F, and Y755F [32] (Figure 3D). Moreover, the destabilization of eGFP-E-cadherin by RASSF1C occurs in a SRC-dependent manner (Figure S3C). It has been previously shown that loss of junctional components like α-catenin [33, 34] or E-cadherin [35] leads to YAP1 nuclear localization. Therefore, we tested whether the loss of adherens junctions in cells expressing RASSF1C similarly leads to YAP1 nuclear localization and indeed found RASSF1C promotes nuclear YAP1, but not in cells expressing E-cadherin with the SRC phosphorylation sites mutated (Figure 3E). The fidelity of E-cadherin-mediated junctions relies on continuous recycling via internalization and replacement through the late endosomal compartment [36]. Visualization of E-cadherin endosome trafficking in real-time via 4D tracking software (Imaris Bitplane; ANDOR) indicated that RASSF1C-expressing cells had reduced trafficking speed and did not register movement toward the junction, supporting the idea of increased internalization and failure to recycle (Figures 4A and 4B; Movie S1), a phenomenon which again was not observed in the case of the E-cadherin mutant (Figure S4A; Movies S2 and S3). To further determine the E-cadherin stability at cell-cell junctions, we expressed GFP-E-cadherin and monitored its dynamics by FRAP analysis, as has been shown previously [37]. We found that expression of RASSF1C increased the mobile fraction of E-cadherin and its turnover at junctions (increased half-life [1/2], but not upon inhibition of SRC with dasatinib (Figures 4C and S4B; Movies S4 and S5), suggesting that RASSF1C increased E-cadherin recycling via SRC, thus creating junctions that are less molecularly stable. To determine the physical effect on cell-cell adhesion, we employed a displace assay and observed that RASSF1C expression...
weakened cellular cohesion in a SRC-dependent manner (Figure 4D). As E-cadherin-mediated adherence relies on Ca\(^{2+}\) for stable contacts, we next wanted to test whether cell-cell disruption was indeed due to E-cadherin by employing a Ca\(^{2+}\) switch assay. We found that, after E-cadherin contacts were efficiently ablated by removal of Ca\(^{2+}\) from the media, RASSF1C-expressing cells failed to efficiently form mature contacts upon Ca\(^{2+}\) replenishment (Figures 4E, S5A, and S5B). The siRNA knock-down of both SRC and YES endowed resistance to RASSF1C expression, implicating both in RASSF1C-mediated loss of junctional strength (Figure 4F, right) and E-cadherin intensity (Figure 4F, left). Taken together, the results indicate that expression of RASSF1C destabilizes cell-cell junctions and disrupts further recycling of E-cadherin via SRC. The loss of adherens junctions can have a wide effect on the cell. Therefore, we next wanted to further investigate the effect on cells after the loss of E-cadherin.

**RASSF1C Promotes YAP and β-Catenin-Dependent Transcription**

Strong E-cadherin contacts require β-catenin, which is protected from degradation through its interaction with E-cadherin [38]. RASSF1C expression promotes the phosphorylation of β-catenin (Figure 3C). Moreover, SRC phosphorylation of β-catenin decreases its affinity for E-cadherin, leading to dissociation from the membrane [22, 23]. This allows nuclear entry and transcription of target genes. Nuclear-cytosplastic fractionation and immunofluorescence analysis confirmed that either RASSF1C expression (in RASSF1A-methylated H1299 and MCF7 cells) or specific loss of endogenous RASSF1A (U2OS and HeLa cells) increases nuclear β-catenin (Figures 5A–5C, S6A, and S6B). We also found that RASSF1C binds and activates YES1 (Figures 2B and S5D), known to promote YAP1 tyrosine phosphorylation that is required for nuclear localization and accumulation of a β-catenin-YAP complex [16, 39]. RASSF1C-induced nuclear YAP1 is also phosphorylated on Y357 (Figures 5D and S5E). Moreover, in MDA-MB-231 mesenchymal breast cancer cells that lack E-cadherin junctions, expression of RASSF1C is sufficient to drive nuclear localization of β-catenin and YAP1 (Figure S5C). β-catenin/YAP1 utilizes the T-box transcription factor TBX5 to drive a pro-tumorigenic transcriptional program including BCL2L1 and MYC (Figures 6B, 6C, and S6D). This was not a defect of attachment or spreading as increased invasion followed induction of RASSF1C expression in attached H1299 cells (Figure 6E, left) and is dependent on β-catenin, YAP, SRC, and YES (Figure 6E, right). Together, the data suggest that loss of RASSF1A promotes invasion via coordinated elevation of pY357-YAP1 and reduced pS127-YAP1, leading to transcriptional activation of specific TEAD/TBX3 genes.

**Expression of RASSF1C Promotes Invasiveness and Tumorigenesis**

To investigate the clinical relevance, we interrogated the databases described above (Figure 1A) for correlations between pS127-YAP1low and invasive signatures using total YAP1 levels and the mSigDB database YAP-TAZ signature as a control. We assessed the extent of increased gene expression from invasive, metastatic, or EMT signatures but failed to see any highly significant correlation (p < 0.0001).

We next examined a second breast data set where methylation of RASSF1A was confirmed in all cases, suggesting that RASSF1C could be expressed and that loss of pS127-YAP1 can now combine with RASSF1C-promoted pY357-YAP1 to allow YAP1/β-catenin nuclear localization. In this data set, pS127-YAP1low (independent of total-YAP1) did indeed have significantly more genes from invasive, metastatic, and EMT signatures (POLO p = 1.09e−178; BIDUS p = 4.52e−111; ANASTASIOU p = 2.62e−08; Fisher’s exact test). The fact that...
the entire data set is from invasive breast cancers negates direct mSigDB analysis in different groups; however, we could control for the increased invasive transcripts in the pS127-YAP1low group as neither the YAP-TAZ or an unrelated signature displayed variation (Figures 7A and S7A; Table S1). We interpret these data in the breast (II) cohort to imply that the pS127-YAP1low group has a YAP-TAZ signature but only an invasive signature when RASSF1A methylation is 100% penetrant (Table S2). Interestingly, this group showed significantly lower levels of the CSK substrate site pY527-SRC and elevated levels of both BIRC5 and MYC (Figure 7B). Therefore, as RASSF1C is associated with SRC activation and transcription of these genes, we decided to investigate whether RASSF1C expression could promote tumorigenesis in vitro and in vivo. MDA-MB-231 cells, stably expressing either a control plasmid or RASSF1C, readily formed mammospheres in Matrigel. However, RASSF1C-associated mammospheres were significantly larger and displayed a more-aggressive phenotype that was SRC, YES, β-catenin, and YAP dependent (Figures 7C, 7D, and S7B–S7D). In line with these observations, RASSF1A methylation has recently been associated with brain metastasis [45]. To directly address tumorigenesis and invasive spread, we adopted a brain-seeding model where spread of human cells could be readily traced. MDA-MB-231 cells stably expressing RASSF1C, injected into the left striatum of SCID mice, formed significantly larger, more-aggressive tumors than either naive or empty vector controls (Figure 7E). The results collectively indicate the role of RASSF1C in invasiveness both in vitro and in vivo. The corroboration of RASSF1C activity with loss of the RASSF1A transcript also supports increasing importance of RASSF1A promoter methylation with invasion, metastasis, and adverse outcome in multiple human tumors.

**DISCUSSION**

SRC and its associated family members play multiple roles in normal cell homeostasis controlling: cell proliferation and survival; cytoskeleton organization; cell shape; cell-cell and cell-ECM contacts; and motility. Deregulation of these activities promotes tumorigenesis, cancer cell invasion, and metastasis [46]. Evidence exists for elevation of SRC activity in tumors as a result of growth factor or cytokine signaling, but a clear somatic event is lacking [47]. In addition to mutational events and copy-number variations that drive tumors, epigenetic alterations are known to be a major contributing factor to disease progression and prognosis. We have identified that, similar to dRASSF8 binding to dCSK in Drosophila [93], RASSF1A associates with CSK and serves to keep SRC repressed. Inactivation of RASSF1A expression is the most widely observed epigenetic event across all sporadic human malignancies and has been confirmed to be a deleterious prognostic factor in meta-analyses of breast, bladder, lung, colorectal, prostate, esophageal, and ovarian cancers [10]. Therefore, the absence of CSK scaffolding to SRC, identified here, is likely to be a contributing factor in association of RASSF1A methylation with disease progression. In colorectal cancer, methylation of RASSF1A is not only associated with tumor dissemination [10], but it is found to be dramatically elevated in liver metastasis compared to primary tissue [48]. The fact that RASSF1A cooperates with APC loss to promote intestinal tumors [49] suggests that SRC activation may contribute to colorectal tumorigenesis by promoting the tyrosine phosphorylations of both β-catenin and YAP1 that are required for nuclear localization and transcriptional activation [16, 29, 39]. These data therefore provide an explanation for the prognostic value of RASSF1A methylation, while also providing a biomarker rational for treating RASSF1A-negative tumors with SRC inhibitors.

EMT is characterized by loss of cell-cell contacts through inactivation of E-cadherin and gain of mesenchymal markers. We find that RASSF1C expression in epithelial cells replicates a partial-EMT phenotype [18] where E-cadherin is expressed, together with the mesenchymal marker vimentin (data not shown), but prevented from forming stable contacts. Further, we observed that RASSF1C expression allows β-catenin and YAP1, normally sequestered at the membrane, to translocate to the nucleus. In addition to SRC-mediated destruction of cell-cell contacts in epithelial cells, we find that mesenchymal MDA-MB-231 cells also appear to require YES-mediated phosphorylation of YAP for nuclear targeting, as has been implicated previously for RUNX2 complexes [39] and in cancer-associated fibroblasts (CAFs) in response to mechanical stress [50]. Interestingly, the association of RASSF1A with filamin A and Arp3 (Figure 2A) suggests that actin dynamics and the mechanical stress response may by sensed by RASSF1A and contribute to SRC and YAP1 activation. RASSF1C also promotes nuclear accumulation of YAP1/β-catenin and upregulation of target genes that promote tumorigenesis, including BCL2L1 and

Figure 4. RASSF1C Expression Leads to Disruption of E-Cadherin Trafficking

(A and B) Representative images of the tracking of all the vesicles in control and DsRed-RASSF1C-expressing MCF7 cells, transfected with eGFP-E-cadherin (Imaris). Bar graphs (bottom right) show the analysis of the mean speed heatmap (A) or distance (B) of the vesicles in control and RASSF1C cells. For each analysis, five cells per experiment were used and an average of 700 vesicles were tracked (bars). The results are from three independent experiments including Movie S1. Inserts (top) display representative still immunofluorescence images showing the accumulation of E-cadherin and the expression of DsRed-RASSF1C. 

(C) FRAP analysis of RASSF1C-expressing MCF7 cells. (Left) Mobile fraction of the return of GFP-E-cadherin after photobleaching is shown. (Right) Half-time of the return of GFP-E-cadherin at the sites of cell-cell junctions after bleaching in MCF7 cells is shown. (Bottom) Representative still images of Movies S2 and S3 displaying junctional GFP-E-cadherin in MCF7 cells, expressing DS-Red or Ds-Red-RASSF1C, captured prebleach and following bleach. Arrows, bleached area. For each of the three independent experiments, FRAP analysis was done on ten cells.

(D) Quantification of a dispase assay in MCF7 cells expressing Ds-Red or Ds-Red-RASSF1C in the presence or absence of dasatinib treatment (50 nM; 18 hr) showing number of single cells in suspension.

(E) Quantification of total cell-cell contacts formed in calcium switch assay in MCF7 cells expressing Zs-green empty vector or Zs-green-RASSF1C. (Bottom) Representative images for ZO-1 at the sites of cell-cell junctions are shown.

(F) Quantification of a dispase assay (right) and the junctional intensity levels (left) of MCF7 cells transfected with siNT, siSRC, or siYES. (Bottom) Immunoblot indicating the level of siRNA knockdown is shown.
BIRCS. YAP1/β-catenin promote cancer cell proliferation and tumorigenesis via TBX5 [16] but also promote overgrowth of the heart through binding to TEAD [24]. Our data suggest that, in breast cancer cells, YAP1/β-catenin complex with TBX3, which phenocopies TBX5 at 8C2L2.1 and BIRCS but additionally promotes cMYC expression, potentially by combining known TEAD- and TBX3-binding elements [28, 42].

We also confirm that RASSF1A activation of the hippo pathway maintains phosphorylation of YAP1, specifically pS127-YAP1 (Figures 1B and S1A), preventing association with TEAD [6, 15]. Upon loss of the pS127, YAP1 is permissive for activation but requires additional modifications for nuclear localization and transcriptional transactivation [15, 16, 29, 39], which we find are dependent on loss of the RASSF1A transcript and expression of RASSF1C (Figure 7F). Moreover, YAP-dependent tumorigenesis, mammosphere formation, and growth in soft agar have been attributed to BIRCS expression [16, 42], which together with MYC-mediated invasion [44] is in line with the formation of larger, more-aggressive mammospheres in cells expressing RASSF1C and larger tumors in vivo. These results also explain the emerging association of both YAP1 and TBX3 with invasive cancers and motility during development [51, 52].

These phenotypes are supported by investigation of the clinical data sets that now include CGL methylation and protein phosphorylation, corroborating links of RASSF1A methylation with invasive spread and explaining prognostic association of this epigenetic event. Given the differential regulation of SFK complexes and YAP activity by RASSF1A and RASSF1C isoforms, the balance of expression of these two RASSF1 isoforms may provide an elegant mechanism for fine-tuning SFK signaling and YAP1 transcriptional activity during development and in emerging epithelial cancers. This isoform switch of RASSF1A may govern further SFK-regulatory events by the RASSF family, such as RASSF1C homolog Rap1-mediated regulation of SRC activation in innate immunity [53]. Moreover, is likely to be a common mechanism that adds complexity to the functionality of genetic information encoded by a single gene.

**EXPERIMENTAL PROCEDURES**

Proximity Ligation Assay

Proximity ligation assay was performed as described using the Duolink Starter Kit (Sigma). H1299 cells were transfected and treated as per instructions before overnight incubation with primary antibodies (SRC, SRC pY416, and SRC pY527 [Cell Signaling]; ZsGreen [Clontech]; RASSF1A [Epitomics]; RASSF1C [Abcam]; FLAG tag [Sigma]; and HA tag (Millipore)) and secondary antibodies for 1 hr. Hybridization was performed for 30 min in a humidified chamber, ligation reactions for 30 min, amplification for 100 min, and DAPI was used for cell detection. The dot-like structures were imaged using Zeiss LSM780 microscope using a 63× objective. For each cell, five z stack images were taken and analyzed with BlobFinder V3.2 (Uppsala University) [54]. The average values, SEM, and significance were calculated using Prism 6.0 (Graphpad software).

Real-Time Molecular Visualization

MCF7 cells (2 × 10^5 cells/condition) were plated on 35-mm glass bottom plates (Ibidi). Cells were grown in complete media until imaging, when media was changed to DMEM/F-12 media without phenol red and supplemented with 10% (v/v) FBS and 100 U/ml Pen/strep. Cells were imaged using Zeiss LSM780 confocal microscope using a 63× objective, for the time-lapse one image per second for a total of 200 s. Tracking of internalized E-cadherin was achieved using Imaris 7.7.1 software (Bitplane; ANDOR). Threshold was determined for each video and varied between 0.4 and 0.6 μm in size. The average distance traveled was set at a threshold of 1 μm. A Quality filter was applied and the threshold set at 15. The data were extracted using the Vantage feature and average values, SEM, and significance were calculated using Prism 6.0 (Graphpad) software. On average, five cells and 700 vesicles each were counted per experiment, and the data shown represent three independent experiments.

Fluorescence Recovery after Photobleaching

MCF7 cells (2 × 10^5 cells/condition) were plated on 35-mm glass bottom plates (Ibidi). Cells were grown in complete media until imaging, when media was changed to DMEM/F-12 media without phenol red and supplemented with 10% (v/v) FBS and 100 U/ml Pen/strep. Cells were imaged using Zeiss LSM780 confocal microscope using a 63× (NA 1.4) objective. Each bleach was done at full laser power, and two pulses were used in order to achieve 60% bleach per region of interest. Images were taken every 2 s for 5 min after the bleach. Data were analyzed using easyFRAP software [55]. The immobile and mobile fractions were calculated using double exponential formula (intensity [I] = IE - 11 × e^−(T1/IE) - I2 × e^−(T2/IE)).

Animal Experiments

Mice were anesthetized and skull burr-hole drilled. Animals were each focially injected with 5 × 10^3 MDA-MB-231 tumor cells expressing empty vector (pCDNA3) or MYC-RASSF1C in 0.5 μl PBS in the left striatum using a 75-mm-tipped glass micropipette (Clark Electromedical Instruments). At day 21, all animals were transcardially perfusion fixed under terminal anesthesia (n = 4 per group) and brains were post-fixed, cryoprotected, embedded, and frozen in isopentane at −40°C. To assess areas of tumor colonization, photomicrographs of each brain section were obtained using ScanScope CS slide scanner (Aperio) and analyzed using ImageScope (Aperio). For immunofluorescence, sections were streptavidin and biotin blocked, incubated with anti-CD34 primary antibody (Abcam; brain vessels) or anti-vimentin antibody (VectorLabs; tumor cells), washed, and incubated with a streptavidin-Cy3 fluorophore or AMCA-conjugated secondary antibody (Invitrogen; 1:100) for 30 min. Expanded animal experimental procedures are outlined in Supplemental Experimental Procedures.

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**Figure 5. RASSF1C Leads to β-Catenin Nuclear Localization and pY357**

(A) Nuclear/cytoplasmic fractionation of U2OS cells transfected with siNT or siRASSF1A (left). Representative images of U2OS cells transfected with siNT or siRASSF1A are shown (right). 

(B) Nuclear/cytoplasmic fractionation (left) and immunofluorescence detection of β-catenin (right) of HeLa cells transfected with siNT or siRASSF1A. 

(C) Nuclear/cytoplasmic fractionation of H1299 cells transiently transfected with empty vector, FLAG-RASSF1A, FLAG-RASSF1C, or both FLAG-RASSF1A and FLAG-RASSF1C to show β-catenin localization. (Right) Representative images show β-catenin localization in H1299 transfected with empty vector or FLAG-RASSF1C. 

(D) H1299 cells transfected with Zs-Green-SRC or Zs-Green-YES and either empty vector or FLAG-RASSF1C and blotted for pY146-SRC, YAP, and the SFK site, pY357-YAP1, as indicated. 

(E) Immunofluorescence detection of YAP1 and pY357-YAP1 in U2OS cells transfected with siNT, siRASSF1, or siRASSF1A (left) and immunoblot showing the nuclear/cytoplasmic distribution of YAP1 (right). All scale bars represent 20 μm. 

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Figure 6. RASSF1C Promotes YAP1-Dependent Transcription and Cell Motility

(A) Quantification of qRT-PCR for the β-catenin-YAP target genes BCL2L1, BIRC5, and cMYC as well as RASSF1A and RASSF1C expression in U2OS cells transfected with siNT, siRASSF1, or siRASSF1A.

(B) Quantification of qRT-PCR for the β-catenin-YAP target genes BCL2L1, BIRC5, and cMYC in MCF7 cells transfected with empty vector or FLAG-RASSF1C and indicated siRNAs.

(C) Scratch wound motility assay of HeLa cells transfected with non-targeting siNT, siRASSF1, and siRASSF1A (left). Wound healing motility assay of MDA-MB-231 cells transfected with empty vector, FLAG-RASSF1A, FLAG-RASSF1C, or both FLAG-RASSF1A and FLAG-RASSF1C is shown (right).

(D) Quantification of a Transwell assay with MDA-MB-231 cells expressing empty vector, FLAG-RASSF1A, FLAG-RASSF1C, or both.

(E) Migration assay in real time using ExCELLigence analyzer with H1299.ConvDox TET-ON FLAG-RASSF1C inducible cells (right) or controls H1299.ConvDox TET-ON empty-vector-inducible cells (left) in the presence or absence of 1 μg/ml doxycycline. (Right) Migration assay in real time using ExCELLigence analyzer on MCF7 cells transfected with empty vector or FLAG-RASSF1C with siNT, siYAP1, siβ-catenin, siSRC, or siYES is shown.
A

| Patients  | Low methyl RASSF1C | High methyl RASSF1C | Low p127 vs Low YAP GSEA significance [Fisher’s exact] |
|-----------|--------------------|---------------------|-----------------------------------------------------|
| Glioma    | 79%                | 21%                 | invasive breast (PODOL) metastasis (BDIXS) EMT (ANASTASSIOU) YAP TAZ |
| Bladder   | 78%                | 22%                 | invasive breast (PODOL) metastasis (BDIXS) EMT (ANASTASSIOU) YAP TAZ |
| Breast    | 84%                | 16%                 | invasive breast (PODOL) metastasis (BDIXS) EMT (ANASTASSIOU) YAP TAZ |
| Breast    | 0                  | 100%                | invasive breast (PODOL) metastasis (BDIXS) EMT (ANASTASSIOU) YAP TAZ |

B

TCGA Breast (II)

![Graph showing mRNA + Score (H3A124 and 265) vs BIRC5 and MYC](image)

C

**MDA-MB-231**

![Images showing control, RASSF1C, and MYC-RASSF1C + Dasatinib](image)

D

**Control** vs **RASSF1C**

![Bar graph showing area fold change vs control](image)

E

**Invasive Spread** (Tumour area)

![Images showing CD34 and vimentin staining](image)

F

![Diagram showing RASSF1C regulation](image)
All animal experiments were approved by the University of Oxford local animal ethical committee and were performed according to terms of a license granted by the UK Home Office, adhering to the Animals (Scientific Procedures) Act 1986.

The Cancer Genome Project Analysis

The data were downloaded from cBioPortal for Cancer Genomics [56, 57] and analyzed with SPSS 21.0 and R version 3.0.1 software. For each clinical data set, cases with missing (NA) methylation, protein, or gene expression values were excluded where appropriate. The Shapiro-Wilk test was used to assess distribution of data sets, null hypothesis of normal distribution was rejected at p < 0.05 level, and the non-parametric Spearman-Rho test was used for correlation. The non-parametric Kruskal-Wallis or Jonckheere-Terpstra test was used to compare the differences of either protein or gene expression levels between the appropriate groups analyzed. The Molecular Signatures Database (MSigDB v.4.0) [58] was used to select gene sets, and POOLA_INVASIVE_BREAST_CANCER_UP [59], BIDUS_METASTASIS_UP [60], ANASTASSIOU_CANCER_MESENCHYMAL_TRANSITION_SIGNATURE [61], CORDEMONSI_YAP_CONSERVED_SIGNATURE [62], REACTOME_YAP1_AND_WWTR1_TAZ_STIMULATED_GENE_EXPRESSION, and BLALOCK_ALZHEIMERS_DISEASE_INCIPIENT_UP [63] were selected as either test or control gene signature sets. The non-parametric Mann-Whitney test was used to compare variation in gene expression between the groups analyzed and the Fisher’s exact test used to compare differences in frequency distributions.

Statistics

For all in vitro experiments, statistical analysis was carried out using a Student’s t test. Tumor areas were compared by ANOVA followed by post hoc Newman-Keuls t tests. All data are expressed as mean ± SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, two tables, Supplemental Experimental Procedures, and five movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.09.072.

AUTHOR CONTRIBUTIONS

N.V. and S.S. helped design and performed the majority of experiments, M.S.S. and N.S. contributed to the in vivo experiments, A.M.G. performed the bioinformatics analyses with advice from F.B., L.B., A.P., and K.S.Y. contributed to nuclear localization and hippo pathway experiments, D.P. and S.S. performed the spheroid analysis. C.R.G. assisted with TBX reagents and advice, P.T. advised S.S. on imaging analysis of Src and E-cadherin. E.O. is responsible for the concept and designed and wrote the manuscript with S.S. and N.V.

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