Loss of Rassf1a Synergizes with Deregulated Runx2 Signaling in Tumorigenesis

Louise van der Weyden1, Angelos Papaspyropoulos3, George Poulogiannis4, Alistair G. Rust1, Mamunur Rashid1, David J. Adams1, Mark J. Arends2, and Eric O’Neill3

Abstract

The tumor suppressor gene RASSF1A is inactivated through point mutation or promoter hypermethylation in many human cancers. In this study, we conducted a Sleeping Beauty transposon-mediated insertional mutagenesis screen in Rassf1a-null mice to identify candidate genes that collaborate with loss of Rassf1a in tumorigenesis. We identified 10 genes, including the transcription factor Runx2, a transcriptional partner of Yes-associated protein (YAP1) that displays tumor suppressive activity through competing with the oncogenic TEA domain family of transcription factors (TEAD) for YAP1 association. While loss of RASSF1A promoted the formation of oncogenic YAPI-TEAD complexes, the combined loss of both RASSF1A and RUNX2 further increased YAP1-TEAD levels, showing that loss of RASSF1A, together with RUNX2, is consistent with the multistep model of tumorigenesis. Clinically, RUNX2 expression was frequently downregulated in various cancers, and reduced RUNX2 expression was associated with poor survival in patients with diffuse large B-cell or atypical Burkitt/Burkitt-like lymphomas. Interestingly, decreased expression levels of RASSF1 and RUNX2 were observed in both precursor T-cell acute lymphoblastic leukemia and colorectal cancer, further supporting the hypothesis that dual regulation of YAP1-TEAD promotes oncogenic activity. Together, our findings provide evidence that loss of RASSF1A expression switches YAPI from a tumor suppressor to an oncogene through regulating its association with transcription factors, thereby suggesting a novel mechanism for RASSF1A-mediated tumor suppression.

Cancer Res; 1–11. ©2012 AACR.

Introduction

The RASSF1A tumor suppressor exhibits epigenetic or genetic inactivation in the majority of human tumors and plays a role in a variety of key biologic processes that restrain the development of cancer, including apoptosis, cell-cycle regulation, mitosis, and microtubule dynamics (reviewed in refs. 1, 2). Although the precise mechanisms by which RASSF1A functions as a tumor suppressor are still under investigation, the most likely hypothesis is that it serves as a scaffold to modulate, localize, and perhaps integrate multiple tumor suppressor pathways. The components of these tumor suppressor pathways remain largely unknown; however, the Hippo pathway is a key downstream pathway that also restricts tumorigenesis.

The first 4 components of the Hippo pathway were discovered in genetic screens for tumor suppressor genes in Drosophila, and include the NDR family protein kinase Warts (Wts), the WW domain-containing protein Salvador (Sav), the Ste20-like protein kinase Hippo (Hpo), and the adaptor protein Mob as-tumor-suppressor (Mats; reviewed in ref. 3). Loss-of-function mutant clones for any of these 4 genes lead to a strong tissue overgrowth phenotype characterized by increased proliferation and diminished cell death. Biochemically, these 4 tumor suppressors form a kinase cascade in which the Hpo–Sav kinase complex phosphorylates and activates the Wts–Mats kinase complex (4, 5), to restrict proliferation via inactivation of the transcriptional complex formed by Yorkie (Yki) and Scalloped (Sd; refs. 6, 7).

The Hippo pathway is conserved in mammalian systems (reviewed in ref. 3) with MST and LATS kinases (orthologs of Hippo and Warts, respectively) functioning as tumor suppressors that phosphorylate the mammalian homolog of Yorkie, Yes-associated protein YAP1 (8). The regulation of the Yki-Sd (YAP1-TEAD) complex by the Hippo pathway is similarly conserved, being responsible for restricting YAP-induced overgrowth, epithelial–mesenchymal transition (EMT), and

Authors’ Affiliations: 1Experimental Cancer Genetics, The Wellcome Trust Sanger Institute, Hinxton; 2Department of Pathology, University of Cambridge, Addenbrooke’s Hospital, Cambridge; 3Department of Oncology, Gray Institute for Radiation Oncology, University of Oxford, Oxford, United Kingdom; and 4Division of Signal Transduction, Department of Systems Biology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

L. van der Weyden and A. Papaspyropoulos contributed equally to the work.

Corresponding Authors: Louise van der Weyden, Experimental Cancer Genetics, Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, CB10 1HH. Phone: 44-0-1223-834244; Fax: 44-0-1223-496802; E-mail: ldvdw@sanger.ac.uk; and Eric O’Neill, Gray Institute, Department of Oncology, ORCHB, University of Oxford, Roosevelt Drive, Oxford, OX3 7DQ. Phone: 44-0-1865-617321; E-mail: eric.onnell@oncology.ox.ac.uk

doi: 10.1158/0008-5472.CAN-11-3343

©2012 American Association for Cancer Research.
oncogenic transformation (7–9). However, in mammals YAP1 displays a more pleiotropic role serving as a coactivator of multiple transcription factors such as p73 (affecting tumor suppression; ref. 10), ErbB4 (11), and RUNX2 (inducing differentiation; ref. 12).

RASSF1A is an upstream component of the MST/LATS pathway, as it binds MST kinases and promotes active YAP1/p73 transcriptional complexes (13, 14). Therefore, Hippo pathway activation leads to opposing effects on the tumor suppressive YAP1/p73 and oncogenic YAP1/TEAD transcription factor complexes. Loss of RASSF1A in tumors leads to a failure in formation of YAP1/p73 complexes and concomitantly, Rassf1a homozygous null mice die faster than wild-type controls due to an increased incidence of tumor formation (15). Herein, we describe how deregulation of oncogenic YAP1/TEAD complexes can contribute to the tumor suppressor functions of RASSF1A. Moreover, the decrease in tumor latency following exposure to mutagens (15) suggests that additional genes collaborate with loss of Rassf1a in tumorigenesis. Thus to identify these co-operating genetic factors, we conducted a Sleeping Beauty transposon-mediated insertional mutagenesis screen in Rassf1a-null mice. This analysis allowed us to identify 10 genes potentially associated with tumor formation in the context of loss of Rassf1a. We selected the YAP1 transcriptional partner, Runx2, for follow-up analysis and show that loss of Runx2 further enhances YAP1/TEAD complex levels initiated by loss of RASSF1A. Thus, we provide evidence for RASSF1A-dependent switching of YAP1 between transcription factor complexes that regulate proliferation (TEAD), differentiation (RUNX2), and tumor suppression (p73), providing new insights into RASSF1A-mediated tumor suppression.

Materials and Methods

Mice and genotyping

Generation of the Rassf1a-null mice (Rassf1a<sup>+/−</sup>;<sup>Ref</sup> 15), mice carrying the Sleeping Beauty transposon array (T2/Onc; ref. 16), and mice carrying the Sleeping Beauty (SB) transposase (Rosa26<sup>SBI1</sup>; ref. 17) have been described previously. All mice were on a mixed 129/Sv-C57BL/6 background. Mice were housed in accordance with Home Office regulations (United Kingdom) and fed a diet of mouse pellets and water ad libitum. PCR genotyping for the Rassf1a<sup>+</sup> (T2/Onc; 16), and Rosa26<sup>SBI1</sup> (17) alleles, as well as “excision” of the transposon from the donor array (16) was conducted as described previously.

Tumor watch analysis

Heterozygous Rassf1a<sup>+/−</sup> mice were bred with mice carrying either the Sleeping Beauty transposon (T2/Onc<sup>+/−</sup>/Tg<sup>+/−</sup>) or transposase (Rosa26<sup>SBI1</sup>/SBI1). The resulting offspring (Rassf1a<sup>+/−</sup>, T2/Onc<sup>+/−</sup>/Tg<sup>+/−</sup>, Rosa26<sup>SBI1</sup>/SBI1) were intercrossed to generate offspring of each genotype (Rassf1a<sup>+++</sup>, T2/Onc<sup>+++</sup>/Tg<sup>+++</sup>, Rosa26<sup>SBI1</sup>/SBI1 and Rassf1a<sup>−/−</sup>, T2/Onc<sup>−/−</sup>/Tg<sup>−/−</sup>, Rosa26<sup>SBI1</sup>/SBI1), which were subsequently placed on tumor watch from birth. All mice on tumor watch were examined twice daily for signs of disease, at which time they were sacrificed and a full necropsy was carried out.

Histology and immunohistochemistry

Tissues were fixed in 10% neutral-buffered formalin at room temperature overnight. Samples were then transferred to 50% ethanol, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). Immunophenotyping was conducted on formalin-fixed, paraffin-embedded tissue sections that had undergone antigen retrieval (microwaving in citrate buffer pH 6 for 20 minutes) using antibodies for CD3 (clone SP7; Abcam), CD45R/B220 (clone RA3-6B2, R&D systems), and MPO (DAKO). Immunohistochemical signal was detected by secondary biotinylated goat anti-rabbit antibody (Vector Laboratories), followed by Vectorstain Elite ABC kit (Vector Laboratories) according to the manufacturer’s instructions.

Isolation and statistical analysis of transposon insertion sites

Isolation of the transposon insertion sites from tumors of both cohorts was carried out using splinkerette PCR to produce barcoded PCR products that were pooled and sequenced as described previously (18). The pooled PCRs were sequenced on the 454 GS-FLX platform (Roche) over 4 separate lanes, with one lane per restriction enzyme and a maximum of 48 tumors per lane. Processing of 454 reads, identification of insertion sites, and the Gaussian Kernel Convolution (GKC) statistical methods used to identify common insertion sites (CIS) have been described previously (18, 19). The P value for each CIS was calculated using an adjusted-by-chromosome cutoff value of P < 0.05. CIS on mouse chromosome 1 were not reported as this is the “donor chromosome” where the transposon array is located and as such there is a significantly higher than background level of transposon insertion events due to local hoping which complicates CIS analysis (16). Genotype-specific CIS analysis was conducted (i) by calling CISs on a perchromosome basis with a cutoff value of P < 0.1 and by comparing the CISs between groups to identify a discovery set of insertions and (ii) by pooling genotypes together, calling CISs on a perchromosome basis and then deconvoluting the CIS peaks using the Fishers exact test to identify genotypes enriched at each CIS peak. Only CISs that survived both calling methods were listed as Rassf1a<sup>+/−</sup>-specific CIS.

Reagents and cells

U2OS cells (ATCC HTB-96) and HCT116 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal calf serum (Gibco). Transient transfection used Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. U2OS Tet-On cells (Clontech) that inducibly express FLAG-RASSF1A upon doxycycline induction were established following puromycin selection as described in the manufacturer’s protocol (20). Authentication of the cell lines was provided with their purchase from American Type Culture Collection (ATCC) and Clontech, and the cell lines were cultured from the original stocks and maintained for no longer than 2 months.

siRNA

A total of 50 ng/ml siRNA duplexes either nontargeting or targeted against RUNX2, RASSF1A, TEAD, or p73 were
transfected with Lipofectamine 2000. To avoid unspecific effects, several different siRNAs were used for the knockdown of each protein. For detailed information, see Supplementary Table S1.

Cell assays

**Colony forming.** U2OS cells were transfected with siRNAs and 24 hours later trypsinized and replated in 6-cm dishes at a density of 350 cells per dish. Plates were stained with crystal violet (0.5% w/v crystal violet, 50% v/v methanol, and 10% v/v ethanol) 11 days later and colonies were counted.

**Viability.** U2OS cells were transfected with siRNA and 24 hours later trypsinized and replated in 6-well dishes at a density of 7.5 × 10^3/well. Cell viability was determined using the resazurin assay. The cells were incubated with media containing 10 µg/mL resazurin (Sigma) at 37°C in a humidified 5% CO2 in-air atmosphere for 2 hours. Resazurin reduction was then measured fluorometrically using a plate reader (Wallace Perkin Elmer) at excitation wavelength of 530 nm and emission wavelength of 590 nm.

**Proliferation.** Growth curves were measured by plating 5 × 10^3 cells onto specialized conductance plates and growth was determined as a steady reduction in individual well conductivity in real-time on an xCELLigence system (Roche).

Immunoprecipitation and immunoblotting

Whole-cell lysate preparation, immunoprecipitation, and Western blotting were carried out as previously described (13, 14). Nuclei were isolated as previously described (21) before incubation in lysis buffer (150 mmol/L NaCl, 20 mmol/L HEPES pH 7.5, 0.5 mmol/L EDTA, 1% NP-40). Antibodies were used for RUNX2 (M-70; Santa Cruz Biotechnology), YAP1 (H125; Santa Cruz Biotechnology), RASSF1A (3F3; Santa Cruz Biotechnology), RASSF1 (Epitomics), TEAD (TEF-1; BD Biosciences), p73 (Epitomics), FLAG (Stratagene), GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Epitomics), Hsp70 (W27; Santa Cruz Biotechnology), and Lamin B1 (Abcam).

Bioinformatic meta-analysis of RUNX2 and RASSF1 expression

Microarray expression data from 6 independent data sets were downloaded from the Oncomine repository (http://www.oncomine.org/) to examine the relative mRNA expression levels of RUNX2 between normal and cancer samples in a variety of tissue types. The distributions of log2 median-centered signal intensities were plotted using box plots and differential gene expression was computed using the Welch 2 sample t test, which is appropriate for subsets of unequal variances. Only tumor sets showing the same differential mode of expression in at least 3 independent data sets were included in this analysis. To correlate gene expression of RUNX2 with patient survival, a univariate Cox proportional hazard regression model (22) was applied to a lymphoma data set of n = 272 samples and the likelihood ratio test, Wald test, and Score (log-rank) test were all used to compute the P value (P < 5.3 × 10^-7 for all 3 tests). To visualize the result obtained from the survival analysis, the samples were ranked according to RUNX2 gene expression, and Kaplan–Meier survival curves were plotted for lymphomas with the lowest (<25th percentile) versus highest (>25th percentile) RUNX2 expression giving a P value of 1.87 × 10^-6 (log-rank test). The expression microarray data of bone marrow from precursor T-cell lymphoblastic leukemias (array ID: E-MEXP-313, n = 27) and the human colorectal data series (array ID: GSE206, n = 105) were downloaded from ArrayExpress and Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) respectively, and the data were normalized using the Robust Multi-Array method. Pearson correlation coefficient analysis was conducted to correlate RUNX2 and RASSF1 expression and the P value was computed using an asymptotic confidence interval based on the Fisher Z transform. The samples were clustered using the Euclidean distance metric and the complete linkage algorithm. Full details of the specific microarray data used are supplied in Supplementary Table S2.

Results

**Tumor watch analysis**

Mice homozygous or wild-type for the Rassf1a<Supri>Run2</Supri> allele (hereafter referred to as Rassf1a<Supri>−/−</Supri> or Rassf1a<Supri>+/+</Supri> mice, respectively) with Sleeping Beauty transposition occurring (i.e., on a T2/Onc<Supri>Tg</Supri>, Rosa26<Supri>Sbi1</Supri> background) were aged until they became moribund. Rassf1a<Supri>−/−</Supri> Sleeping Beauty mice developed tumors significantly faster than their wild-type Sleeping Beauty littermates (average life span of 35 and 44 weeks for Rassf1a<Supri>−/−</Supri> and Rassf1a<Supri>+/+</Supri> mice, respectively; Fig. 1A). As previously reported for the T20Onc transposon that carries the murine stem cell virus promoter that is preferentially active in the hematopoietic system (16), all mice in both cohorts developed leukemia/lymphoma (see Fig. 1B), although a small proportion of mice did develop an additional tumor, typically a hepatocellular carcinoma (Fig. 1B).

Immunohistochemical analysis of a selection of the leukemias/lymphomas showed that the predominant disease subtypes were poorly differentiated lymphomas, not staining positively for either T-cell (CD3) or B-cell (CD45R/B220) antigens (29/69, 42%) and CD3-positive T-cell lymphomas (27/69, 39%), with only a small amount of MPO-positive high-grade myeloid leukemias (13 of 69, 19%; Fig. 1C).

**Statistical analysis of transposon insertion sites in leukemias/lymphomas**

To identify tumor-associated genotype-enriched somatically mutated genes, that is, those genes mutated by transposon insertion found specifically in tumors on a Rassf1a<Supri>−/−</Supri> background, we identified CISs in leukemias/lymphomas from 126 wild-type Sleeping Beauty mice (25 Rassf1a<Supri>+/+</Supri> Sleeping Beauty mice and 101 wild-type mice from other Sleeping Beauty studies carried out in our facility at the same time and on the same mixed C57-129 genetic background) and 111 Rassf1a<Supri>−/−</Supri> Sleeping Beauty mice using GKC (19) statistical analysis in 2 ways. Firstly, we treated the insertions from wild-type and Rassf1a<Supri>−/−</Supri> tumors as 2 independent groups and identified 209 and 165 CISs, respectively, which we then analyzed to determine which were common to both groups and which were
found specifically on a Rassf1a−/− background. Using a chromosome-adjusted $P$ value cutoff value of $P < 0.1$ ensured that an extra degree of stringency was implemented when detecting shared CISs. This meant that the CISs found in the Rassf1a−/− group were compared with the CIS in the wild-type group that were marginally $P > 0.05$ (up to a significance of 0.1) and would otherwise have been missed. We then pooled the insertions from both groups (using a $P$ value generated by the Fisher exact test) and identified CIS found to be enriched only in the Rassf1a−/− tumors. The Rassf1a−/− CIS calls generated by both methods were compared and resulted in the identification of 10 CISs that were present in both lists (Table 1).

**Insertions in the Runx2 gene**

Given that RASSF1A is a component of the Hippo signaling pathway (13) and the transcription factor RUNX2 is activated...
Interestingly, 6 of 7 of the tumors had insertions in intron 4 of the 10 amino acid sequence (HTYLPPW) domain in YAP1 (23) and the PY motif located within a repression domain (RD), and thus be unable to carry out the functions of a full-length Runx2 protein. Importantly, although these tumors contained only heterozygous loss of Runx2 (i.e., insertions were only in one Runx2 allele), Runx2 is haploinsufficient, as human cleidocranial dysplasia (CCD) is an autosomal dominant disease that results from heterozygous inactivation of Runx2 (24) and heterozygous Runx2 mice recapitulate the CCD phenotype (25, 26).

Loss of RUNX2 in the absence of RASSF1A enhances YAP-TEAD complex formation

We first reasoned that the RASSF1A-mediated modulation of YAP1 that promotes the formation of YAP1-p73 complexes may occur at the expense of RUNX2-TEAD complexes, thus serving as a comprehensive switch away from proliferation to active tumor suppression (20). As RUNX2 is also a transcriptional partner of YAP1, the enhanced tumorigenesis indicated by the Rassf1a−/− Sleeping Beauty mice may be due to an additional layer of competition for YAP1 association. In tumors that have lost RASSF1A, YAP1 fails to associate with p73 and where this occurs in conjunction with RUNX2 loss, YAP1-TEAD complexes may be more likely and exacerbate oncogenic proliferation. We were unable to test this hypothesis in the leukemia/lymphoma samples containing transposon insertions in Runx2 due to the frequently oligoclonal or polyclonal nature of tumors induced by insertional mutagens (27). Thus, we used the Runx2 expressing human osteosarcoma cell line U2OS, that has low levels of RASSF1A and in which the association of YAP1 with TEAD was readily observed (Fig. 2A). Doxycycline-inducible expression of RASSF1A restricted the ability of YAP1 to associate with TEAD and promoted association with p73 (Fig. 2A).

To test whether RUNX2 was similarly able to compete with TEAD for YAP1 association in U2OS cells, we focused on complexes within the nuclear compartment due to alternative functions for YAP1 at cell junctions. We found that reduction of RUNX2 expression increased TEAD association with YAP1, indicating competition between transcription factors (Fig. 2B). To confirm the competition between TEAD and RUNX2, we downregulated TEAD by siRNA and observed an increase in YAP1 association with RUNX2 (Fig. 2C). Thus, low levels of either RASSF1A or RUNX2 independently elevated levels of the oncogenic YAP1-TEAD complex. We next targeted both RASSF1A together with RUNX2 and observed that knockdown of RASSF1A further enhanced YAP1-TEAD complex formation compared with loss of RUNX2 expression alone, in U2OS and the colorectal cell line HCT116 (Fig. 2D and E and Supplementary Fig. S1).

**TEAD-dependent proliferation and clonogenicity requires loss of RASSF1A-p73**

To determine whether the regulation of YAP1-TEAD observed earlier played a role in tumorigenic potential of cells, we conducted colony formation assays in U2OS cells. siRNA-mediated reduction of both RASSF1A and RUNX2 expression increased the clonogenic capacity of tumor cells to a greater extent than knockdown of either RASSF1A or RUNX2 alone.

### Table 1. GKC analysis of Rassf1a−/−-specific CIS

<table>
<thead>
<tr>
<th>CIS identification</th>
<th>Predicted affected gene</th>
<th>Other genes in CIS</th>
<th>Genomic location of CIS (chr:start-end)</th>
<th>Insertions (tumors)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIS12:100543835_75k</td>
<td>Fosn3</td>
<td>3300002A11Rik</td>
<td>12: 100477940-100609729</td>
<td>20 (16)</td>
<td>0.02848</td>
</tr>
<tr>
<td>CIS14:67677451_15k</td>
<td>Ppp2r2a</td>
<td>Bnip3I</td>
<td>14: 67657900-67692812</td>
<td>11 (11)</td>
<td>0.00026</td>
</tr>
<tr>
<td>CIS5:34769677_15k</td>
<td>Fam193a</td>
<td>Rnf4, Sh3bp2, Tnip2</td>
<td>5: 34753557-34781415</td>
<td>9 (9)</td>
<td>0.00261</td>
</tr>
<tr>
<td>CIS12:93020818_15k</td>
<td>Ston2</td>
<td>Set11</td>
<td>12: 93004760-93032497</td>
<td>9 (9)</td>
<td>0.01352</td>
</tr>
<tr>
<td>CIS3:104653212_15k</td>
<td>Capza1</td>
<td>Fam19a3, St7i, Mov10, Ppm1j, Rhoc, Wnt2b</td>
<td>3: 104639966-104660243</td>
<td>8 (8)</td>
<td>0.01081</td>
</tr>
<tr>
<td>CIS17:44818488_30k</td>
<td>Runx2</td>
<td>Supt3h</td>
<td>17: 44801068-44830102</td>
<td>8 (7)</td>
<td>0.03697</td>
</tr>
<tr>
<td>CIS4:97738853_15k</td>
<td>Nfla</td>
<td>—</td>
<td>4: 9772123-97749117</td>
<td>7 (7)</td>
<td>0.00078</td>
</tr>
<tr>
<td>CISX:39556669_15k</td>
<td>Stag2</td>
<td>Xiap</td>
<td>X: 39546415-39563993</td>
<td>7 (7)</td>
<td>0.01552</td>
</tr>
<tr>
<td>CIS16:4187397_15k</td>
<td>Crebbp</td>
<td>Adcy9, Trap1</td>
<td>16: 4175766-4197556</td>
<td>7 (7)</td>
<td>0.02029</td>
</tr>
<tr>
<td>CIS5:34056588_15k</td>
<td>Fgfr3</td>
<td>Fam53a, Letm1, Sibp, Tacc3, Tmem129</td>
<td>5: 34047785-34062457</td>
<td>7 (7)</td>
<td>0.02839</td>
</tr>
</tbody>
</table>

Published OnlineFirst June 18, 2012; DOI: 10.1158/0008-5472.CAN-11-3343
In support of a model where RASSF1A restricts proliferation by promoting YAP1-p73, targeting of either p73 or RASSF1A with RUNX2 resulted in equivalent enhanced clonogenicity and increased viability compared with controls (Fig. 3A and B). The clonogenic potential of both controls and dual RASSF1A/RUNX2 were decreased by concomitant reduction of TEAD by siRNA, confirming that the additional proliferation was due to TEAD oncogenic activity (Supplementary Fig. S2A). We reasoned that exogenous overexpression of YAP1 may resolve competition and permit all 3 complexes. However, YAP1 expression only increased clonogenicity in the absence of RASSF1A or p73 together with RUNX2 (Fig. 3A). Interestingly, while the absence of RUNX2 favored YAP1-TEAD complexes and promoted colony formation, overexpression of YAP1 suppressed the growth advantage, perhaps through simultaneous enhancement of YAP1-p73 (Fig. 3A).

To definitively address the transcription factor competition, we used combinations of siRNA that should promote YAP1-TEAD, YAP1-p73, or YAP1-RUNX2 and monitored U2OS cell growth curves in real-time. As observed earlier, reduction of RUNX2 and p73 levels (favoring YAP1-TEAD complexes) increased proliferation, whereas dual reduction of RUNX2 and TEAD (favoring YAP1-p73) suppressed cell growth (Fig. 3C). The combination of p73 and TEAD siRNA (favoring YAP1-RUNX2) surprisingly increased viability compared with TEAD loss alone, but significantly reduced cellular proliferation (Fig. 3C) and clonogenicity which is consistent with a potential...
switch to a RUNX2 differentiation program (Supplementary Fig. S2A and S2B).

Loss of RUNX2 in human tumors

Expression of RUNX2 is found in almost all types of human cancer (reviewed in ref. 2). Investigation of RUNX2 expression in microarray analysis conducted across different tumor types revealed that RUNX2 mRNA levels were significantly lower in many tumor types than their corresponding normal tissues, particularly in tumors of the brain, colon, head and neck, prostate, kidney, and leukemias (Fig. 4A), and loss of RUNX2 expression showed a strong association with poorer

Figure 3. RASSF1A and RUNX2 restrict YAP1-TEAD oncogenic behavior. A, U2OS cells were transfected with control vector or plasmid-expressing FLAG-YAP1 and indicated siRNAs before colonies were allowed to grow for 11 days, fixed and visualized with crystal violet. Whole-cell lysates were probed with indicated antibodies. Error bars indicate SEM of n = 3. Significance was determined by Student’s t test RASSF1A versus RUNX2/RASSF1A, P < 0.001; Runx2 versus RUNX2/RASSF1A, P = 0.023; p73 versus RUNX2/p73, P < 0.001. B, U2OS cells were transfected with indicated siRNAs, as shown in A, and cell viability was determined using the resazurin assay after 48 hours. Error bars indicate SEM of at least n = 4. Significance was determined by Student’s t test RASSF1A versus RUNX2/RASSF1A, P = 0.008; Runx2 versus RUNX2/RASSF1A, P = 0.0101; p73 versus RUNX2/p73, P = 0.0185. C, U2OS cells were transfected with indicated siRNAs to promote the different YAP transcriptional partner, as shown in A, and cell proliferation was monitored by xCELLigence in real-time. Error bars indicate SEM of n = 3. Arrows indicate increase or decrease in cell growth; significance determined at end point as below P = 0.05 in set of dual knockdown compared with controls.

Loss of RUNX2 and RASSF1 in human tumors

Loss of RASSF1A expression is found in almost all types of human cancer (reviewed in ref. 2). Investigation of RASSF1A expression in microarray analysis conducted across different tumor types revealed that RASSF1A mRNA levels were significantly lower in many tumor types than their corresponding normal tissues, particularly in tumors of the brain, colon, head and neck, prostate, kidney, and leukemias (Fig. 4A), and loss of RUNX2 expression showed a strong association with poorer
survival in patients with diffuse large B-cell or atypical Burkitt/Burkitt-like lymphomas (Fig. 4B). Importantly, a strong concordance in expression levels of RUNX2 and RASSF1 was observed in precursor T-cell lymphoblastic leukemias (Spearman rank correlation coefficient = 0.56, P value = 0.002; Fig. 4C). RASSF1A is frequently methylated in tumors of the gastrointestinal tract (2) and we have shown that loss of Rassf1a cooperates with loss of Apc to accelerate intestinal tumorigenesis (28). In agreement with the promotion of YAP1-TEAD in HCT116 cells (Fig. 2E), we found that loss of RUNX2

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Normal</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head and neck</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4.** Analysis of RUNX2 and RASSF1 expression across different tumor types. A, box plots indicating that RUNX2 expression is significantly lower in many tumor types compared with their corresponding normal tissues. Only the tumor types that showed significantly lower RUNX2 expression in cancer versus normal in at least 3 independent microarray data sets are included from left to right: RUNX2 expression in normal brain (white matter, n = 7) vs. brain tumor (astrocytoma, glioblastoma, oligodendroglioma, n = 35), normal colon (n = 9) vs. colorectal cancer (n = 100), normal uvula (n = 4) vs. head and neck squamous cell carcinoma (n = 34), peripheral blood mononuclear cell (n = 58) vs. B-cell acute lymphoblastic leukemia (n = 114), prostate gland (n = 21) vs. prostate carcinoma (n = 30), normal kidney (n = 10) vs. clear cell renal cell carcinoma (n = 10). B, left, ranked RUNX2 expression in a large data set of 272 lymphomas, and (right) Kaplan–Meier survival curves comparing disease-free survival between lymphomas with the lowest (<25th percentile) versus highest (>25th percentile) RUNX2 expression (log-rank test, P = 1.87 x 10^-6). C, unsupervised hierarchical clustering and heatmap of RUNX2 and RASSF1 relative transcript levels in precursor T-cell lymphoblastic leukemias (n = 27), of which 18 show concordant changes for these 2 transcripts. D, unsupervised hierarchical clustering and heatmap of RUNX2 and RASSF1 relative transcript levels indicating a strong positive correlation (predominantly concordant downregulated expression) between RASSF1 and RUNX2 expression in colorectal adenocarcinomas (n = 105; ref. 44). The color bar indicates normalized expression levels with red signifying the magnitude of upregulation and blue of downregulation. In all microarray studies, the probe(s) that detected RASSF1 were found in the part of the gene common to all RASSF1 transcripts.
expression strongly correlated with loss of RASSF1 expression in human colorectal cancers (Pearson correlation coefficient = 0.379, \( P = 9.86 \times 10^{-5} \); Fig. 4D).

Discussion

Since its discovery in 2000 (29), hypermethylation of the RASSF1A promoter, and ensuing transcriptional silencing of RASSF1A, has been frequently observed in almost all tumor types (reviewed in ref. 2). We have previously shown that Rassf1a-null mice die faster than their wild-type littermates due to increased incidence of tumorigenesis, predominantly lymphoma/leukemias (15) and that loss of Rassf1a co-operates with loss of Apc to accelerate intestinal tumorigenesis (28). In this study, we used Sleeping Beauty insertional mutagenesis to identify candidate genes that are associated with tumorigenesis in the context of loss of Rassf1a. We have shown that Rassf1a\(^{-/-}\) Sleeping Beauty mice develop tumors significantly faster than their wild-type Sleeping Beauty littermates, specifically poorly differentiated lymphomas or CD3-positive T-cell lymphomas, with a small amount of MPO-positive high-grade myeloid leukemias.

Isolating the transposon insertion sites from these Sleeping Beauty–induced tumors allowed the discovery of a set of 10 genes enriched in the Rassf1a\(^{-/-}\) Sleeping Beauty tumors (Table 1). Similar to Rassf1a, several of these genes have roles in regulating the cell cycle, mitosis, and mitotic progression, with reported loss of expression being found in tumors, including Foxn3 (30, 31), Ppp2r2a (32, 33), Stag2 (34, 35), and Runx2 (36). In addition, some of these genes are known to interact with the Ras signaling pathway, and mutations in these genes are associated with tumorigenesis, including Crebbp (37, 38) and Fgfr3 (39, 40). Given that RASSF1A is a component of the Hippo signaling pathway (13) that can activate RUNX2 (via YAP1: ref. 12), we focused our attention on the Runx2 gene.

The 3 members of the Runx family of mammalian transcription factors, Runx1-3, are related to Runt, the Drosophila pair rule gene (41) and share a highly conserved DNA-binding domain and a common DNA-binding cofactor. However, the Runx2 gene (also known as PEBP2A, AML3, CCD1, CBFA1, and OSF2) is a unique member of the family in that it produces the largest protein product which possesses 2 domains distinct from its homologues: a short stretch of glutamine-alanine (QA) repeats at the N-terminus and a C-terminal proline/serine/threonine (PST) rich tract, both regions of which are necessary for full transactivation activity (ref. 42; Fig. 1D). Members of the Runx family regulate multiple cell fate decisions and have been implicated in a wide range of cancers where there is unequivocal evidence that members of this family can act as oncogenes or as tumor suppressors according to context (43). Specifically, Runx2-deficient (Runx2\(^{-/-}\)) mouse embryonic fibroblasts (MEF) are prone to spontaneous immortalization and display an early growth advantage that is resistant to stress-induced growth arrest (44). Thus Runx2 can function as a tumor suppressor gene and loss of its expression is an important step in oncogenic transformation. On the basis of the location and orientation of these transposons in the Runx2 gene, they were predicted to result in the premature truncation of the transcript, therefore could be described as a loss-of-function allele. Interestingly, insertions in Runx2 were only significantly associated with tumourigenesis in the Rassf1a\(^{-/-}\) mice and not wild-type counterparts, indicating that loss of RUNX2 may not be sufficient for tumorigenesis.

In Hippo pathway signaling, RASSF1A ensures that YAP1 associates with the proapoptotic p73 (13), whereas loss of Hippo pathway signaling allows YAP1 to associate with TEAD, leading to oncogenic proliferation (8). We provided the first evidence that RASSF1A restricts the ability of YAP1 to associate with TEAD as part of its tumor suppressor activity (Fig. 2). YAP1 (also known as YAP65) is a key regulator of organ size and has been implicated as an oncogene due to amplification in human cancers (8). In agreement with others, we found that overexpression of YAP1 enhances oncogenic behavior. However, high YAP1 levels, in the absence of RUNX2, promoted a p73-dependent suppression of clonogenicity (Fig. 3A), therefore indicating that YAP1 amplification and RUNX2 loss are
unlikely to be sufficient to promote tumorigenesis and require deregulation of either RASSF1A or p73. Importantly, clinical correlations of outcome and YAP1 levels may benefit from further stratification of tumors displaying reduced RASSF1A or p73 (45).

Hypermethylation of the RASSF1A promoter is a frequent occurrence in a wide variety of tumors, and together with the fact that point mutations have also been found in up to 15% of primary tumors, this makes RASSF1A one of the most frequently inactivated proteins in human cancer (reviewed in ref. 2). We found that RUNX2 mRNA levels were significantly lower in many different tumor types than their corresponding normal tissues and loss of RUNX2 expression showed a strong association with poorer survival in some cancer types (Fig. 4B). Importantly, a strong concordance in expression levels of RUNX2 and RASSF1 was observed in human precursor T-cell lymphoblastic leukemias (Fig. 4C) and loss of RUNX2 expression is strongly correlated with loss of RASSF1 expression in human colorectal cancers (Fig. 4D).

In summary, we have provided evidence that loss of RASSF1A expression switches YAP1 from a tumor suppressor to an oncogene through regulating its association with the transcription factors p73 and TEAD. Furthermore, the terminal differentiation factor RUNX2 also competes with TEAD for YAP1 association, independently of RASSF1A and p73, and combined knockdown exacerbates YAP1-TEAD levels (Fig. 5). We found that the resulting enhancement in proliferative signals results in elevated tumor indices in both genetic systems and human disease. Thus loss of RASSF1A, together with RUNX2, is concomitant with the multistep model of tumorigenesis.

Disclosure of Potential Conflicts of Interest

G. Poulogiannis is a Pfizer Fellow of the Life Sciences Research Foundation. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: L. van der Weyden, A. Papaspyropoulos, D.J. Adams, E. O’Neill

Development of methodology: L. van der Weyden, A. Papaspyropoulos, E. O’Neill

Accretion of data (provided animals, acquired and managed patients, provided facilities, etc.): L. van der Weyden, A. Papaspyropoulos, E. O’Neill

Analysis and interpretation of data (e.g., statistical analysis, biosistics, computational analysis): L. van der Weyden, A. Papaspyropoulos, G. Poulogiannis, A.G. Rust, M. Rashid, D.J. Adams, M.J. Arends, E. O’Neill

Writing, review, and/or revision of the manuscript: L. van der Weyden, A. Papaspyropoulos, A.G. Rust, M.J. Arends, E. O’Neill

Acknowledgments

The authors thank Mahrokh Nohadani for conducting the tissue processing and immunohistochemistry and the staff of Team 83 at the Wellcome Trust Sanger Institute for looking after the mice.

Grant Support

The study was supported by A Kay Kendall Leukemia Fund Intermediate Fellowship KKL.309 (L. van der Weyden), a Cancer Research UK grant C20510/A6997 and Wellcome Trust grant 082356 (D.J. Adams), a Cancer Research UK grant C20510/A6997 (M.J. Arends), a Cancer Research UK grant A12932 (E. O’Neill), and a Medical Research Council UK studentship (A. Papaspyropoulos).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 10, 2011; revised May 22, 2012; accepted May 29, 2012; published OnlineFirst June 18, 2012.
Loss of Rassf1a Synergizes with Deregulated Runx2 Signaling in Tumorigenesis

Louise van der Weyden, Angelos Papaspyropoulos, George Poullogiannis, et al.

Cancer Res  Published OnlineFirst June 18, 2012.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-11-3343

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2012/06/18/0008-5472.CAN-11-3343.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.