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Zmat3 is a key splicing regulator in the p53 tumor suppression program

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Although *TP53* is the most commonly mutated gene in human cancers, the p53-dependent transcriptional programs mediating tumor suppression remain incompletely understood. Here, to uncover critical components downstream of p53 in tumor suppression, we perform unbiased RNAi and CRISPR/Cas9-based genetic screens *in vivo*. These screens converge upon the p53-inducible gene *Zmat3*, encoding an RNA-binding-protein, and we demonstrate that ZMAT3 is an important tumor suppressor downstream of p53 in mouse $Kras^{G12D}$-driven lung and liver cancers and human carcinomas. Integrative analysis of the ZMAT3 RNA-binding landscape and transcriptomic profiling reveals that ZMAT3 directly modulates exon inclusion in transcripts encoding proteins of diverse functions, including the p53 inhibitors MDM4 and MDM2, splicing regulators, and components of varied cellular processes. Interestingly, these exons are enriched in NMD signals, and, accordingly, ZMAT3 broadly affects target transcript stability. Collectively, these studies reveal ZMAT3 as a novel RNA-splicing and homeostasis regulator and key component of p53-mediated tumor suppression.

Introduction

TP53 gene is the most commonly mutated gene in human cancers, underscoring its critical role in tumor suppression (Kandoth et al., 2013). p53 is a transcriptional activator that responds to diverse stress signals by regulating gene expression programs that limit neoplastic behavior (Bieging et al., 2014; Vousden and Prives, 2009). The best characterized p53 programs are in response to acute DNA damage, when p53 transcriptionally induces the *p21* CDK inhibitor to trigger cell cycle arrest and DNA repair or pro-apoptotic BCL2 family members *Puma* and *Noxa*, to eradicate damaged cells through apoptosis. The ability of p53 to transactivate target genes is essential for tumor suppression, as *TP53* mutations in humans compromise sequence-specific DNA binding and p53 target gene induction. Moreover, knock-in mice expressing a transcriptionally-dead p53 mutant with mutations in both transactivation domains (TADs) phenocopy *p53* null mice in tumor predisposition (Brady et al., 2011; Jiang et al., 2011; Mello et al., 2017).

A variety of mutant mouse strains have been generated to illuminate the gene expression programs downstream of p53 most critical to tumor suppression. Analysis of a p53 TAD1 ($p53^{25,26}$) mutant knock-in mouse strain revealed that $p53^{25,26}$ is severely compromised in activating most p53 target genes – including many classical p53 target genes like *p21*, *Puma*, and *Noxa* – yet retains the ability to activate a small subset of primarily novel target genes (Brady et al., 2011). Interestingly, this mutant is fully competent for suppressing various cancers, including B and T-cell lymphomas, medulloblastoma, and lung adenocarcinoma (Brady et al., 2011; Jiang et al., 2011). These findings suggested that noncanonical p53 target genes may be critical for p53-mediated tumor suppression. Further support for this idea came from the analysis of mice expressing

47 $p53^{3KR}$ – an acetylation site mutant that cannot activate classical p53 target genes – and
48 *p21-/-;Puma-/-;Noxa-/-* mice, neither of which are prone to spontaneous cancers (Li et al.,
49 2012b; Valente et al., 2013). Collectively, these studies suggested that p53-mediated tumor
50 suppression does not require classical p53 target genes, including *p21, Noxa,* and *Puma*, or
51 that other genes can compensate for loss of these genes. These studies have thus prompted
52 a renewed investigation of the transcriptional programs underlying p53-mediated tumor
53 suppression (Bieging et al., 2014; Mello and Attardi, 2018).

54 Recent studies have implicated specific p53-inducible genes in p53-mediated tumor
55 suppression in different settings. In *Myc*-induced hepatocellular carcinoma, p53 induction
56 of *Abca1* and suppression of the mevalonate pathway are important for tumor suppression
57 (Moon et al., 2019b). The p53-regulated DNA repair gene *Mlh1* contributes to the
58 suppression of leukemia in an *Eμ-Myc;Puma-/-* background (Janic et al., 2018). In
59 pancreatic cancer, a p53-PTPN14-YAP axis operates to suppress the development of this
60 disease (Mello et al., 2017). Despite these recent advances, our understanding of p53
61 function in tumor suppression remains far from complete. It is unclear, for example,
62 whether different p53 pathways are involved in distinct tumor types and in response to
63 different oncogenic drivers or whether there are core p53 pathways critical for suppression
64 of tumorigenesis in multiple contexts. It would therefore be enlightening to use an unbiased
65 approach to identify p53-inducible genes most fundamental to tumor suppression.

66 Here, we investigate the p53 transcriptional programs critical for tumor
67 suppression, which will be pivotal not only for understanding the molecular underpinnings
68 of p53 function but also for ultimately gaining insights that would allow development of
69 therapies aimed at the critically important but difficult-to-target p53 pathway. We leverage

the tumor suppression-competent $p53^{25,26}$ mutant to delineate p53 tumor suppression-associated genes (TSAGs) whose expression is tightly linked to tumor suppression, and we interrogate which TSAGs display tumor suppressor activity using both unbiased RNA interference and CRISPR/Cas9 pooled screens *in vivo*. These studies, coupled with autochthonous mouse model and human cancer genome analyses, unveil the RNA binding protein ZMAT3 as a key tumor suppressor downstream of p53. Molecular analyses reveal a novel function for ZMAT3 in RNA homeostasis via modulating alternative splicing, resulting in multifaceted effects on diverse pathways. These findings establish a ZMAT3-regulated splicing program, providing critical new insight into a core mediator of p53-dependent tumor suppression.

Results

Identification of p53 TSAGs

Identifying p53 target genes critical for tumor suppression has been challenging due to the vast number of p53-regulated genes, as illustrated by the observation that >1000 genes are induced by p53 in oncogene-expressing fibroblasts (Figure 1A, S1A) (Brady et al., 2011). To identify the most relevant p53-regulated genes for tumor suppression, we sought to pinpoint p53 target genes whose expression is tightly linked to tumor suppression. We leveraged the $p53^{25,26}$ mutant, which activates only a subset of p53 target genes yet is fully competent for tumor suppression in multiple *in vivo* cancer models (Brady et al., 2011; Jiang et al., 2011). By uncovering the p53-dependent genes still induced by this mutant in neoplastic mouse embryonic fibroblasts (MEFs) expressing $Hras^{G12V}$ –one of the most commonly activated genes in human cancer– we generated a list of 87 genes,

which we term p53 tumor suppression-associated genes (TSAGs; Figure 1A, 1B). The majority of p53 TSAGs have one or more p53-bound response elements (RE) near or within their gene body, suggesting that they are direct p53 targets (Table S1, see Methods). Moreover, many p53 TSAGs are p53-inducible in different cell types, including *Eμ-Myc*-driven lymphoma and $Kras^{G12D}$-induced lung adenocarcinoma (LUAD) cells (Figure S1B, S1C). The robust expression of these genes during tumor suppression, coupled with their p53-inducibility in various contexts, supports their potential importance in the p53 tumor suppression program.

RNA Interference Screening for Functional Tumor Suppressors

To interrogate which of the 87 TSAGs are functionally important for tumor suppression, we first used an *in vivo* pooled shRNA genetic screening approach. We leveraged an *in vivo* tumor model based on subcutaneous growth of primary MEFs expressing both *E1A* and $Hras^{G12V}$ oncogenes (Jiang et al., 2011; Lowe et al., 1994). These cells provide a tractable model with an intact p19ARF-p53 tumor suppressor signaling pathway because they are derived from primary cells and cultured minimally. Importantly, p53 is critical for tumor suppression in this model (Lowe et al., 1994)(Figure S1D-S1F). We generated a lentiviral ultracomplex pooled shRNA library comprising 25 unique shRNAs targeting each TSAG, along with 1,000 negative control (NC) shRNAs (Bassik et al., 2009), a strategy that mitigates the effects of false negative and false positives to ensure sensitivity and efficacy (Figure 1C). After transduction, we subcutaneously injected *E1A;*$Hras^{G12V}$ MEFs into recipient mice and allowed 3 weeks for tumor formation. To identify genes with tumor suppressor function, we determined which shRNAs were

enriched relative to the NC shRNAs in the tumors (Figure 1D, Table S2; see Methods). The gene represented by the highest number of enriched shRNAs (10) was *Zmat3*, followed by *Ptpn14*, *Trp53inp1,* and *Dennd2c*, with at least 7 shRNAs detected per gene (Figure 1D, Table S2). We validated these hits by expressing individual enriched shRNAs in *E1A;Hras*^{G12V} MEFs, which increased anchorage-independent growth relative to control cells (Figure 1E, S1G, S1H), demonstrating that these genes suppress transformation. Interestingly, we recently independently identified *Ptpn14* as a novel p53 target gene with tumor suppressor activity in pancreatic cancer (Mello et al., 2017), and *Trp53inp1* has tumor suppressor activity in mouse models (Al Saati et al., 2013; Cano et al., 2009). Notably, the top hits encode proteins with diverse cellular functions: ZMAT3 encodes a zinc finger RNA-binding protein (RBP) (Israeli et al., 1997; Varmeh-Ziaie et al., 1997), PTPN14 is a protein tyrosine phosphatase that negatively regulates the YAP oncoprotein (Mello et al., 2017), TRP53INP1 is a negative regulator of reactive oxygen species (Cano et al., 2009), and DENND2C is a RAB-GEF (Yoshimura et al., 2010), supporting the idea that p53 modulates multiple distinct pathways to suppress cancer. Accordingly, no single gene knockdown had an effect as dramatic as *p53* knockdown (Figure 1E), consistent with the notion that activation of a network of genes by p53 is critical for tumor suppression (Andrysik et al., 2017; Bieging et al., 2014). Overall, the success of the screen is underscored both by the unbiased identification of known tumor suppressors and the discovery of potential new tumor suppressors, including *Zmat3* and *Dennd2c*.

CRISPR/Cas9 Screening for Functional Tumor Suppressors

While we identified several functional tumor suppressor genes using shRNA screening, we reasoned that a gene deletion screen using CRISPR/Cas9 technology could both reinforce our findings and unveil additional tumor suppressor genes (Morgens et al., 2016). We generated lentiviral libraries expressing 10 sgRNAs targeting each of the 87 TSAGs in two pools, with ~40-50 TSAGs and 250 NC sgRNAs per pool. Our screening approach was similar to that described above, except that we transduced *E1A;Hras*^{G12V}-expressing MEFs derived from *Cas9*-transgenic mice (Chiou et al., 2015) with the lentiviral sgRNA libraries. We injected *E1A;Hras*^{G12V}*;Cas9* MEFs subcutaneously into recipient mice and allowed 3 weeks for tumor formation (Figure 2A). For each of the 2 libraries, we quantitatively measured enrichment of individual sgRNA elements in tumors relative to input cells to identify genes with significant tumor suppressor activity in individual tumors (Morgens et al., 2016). Strikingly, in tumors derived from pool 1, sgRNAs targeting *Zmat3* dominated every tumor, bolstering our shRNA screen results (Figure 2B, S2A, S2C, Table S3). With pool 2, sgRNAs targeting *Dennd2c* were the most significantly enriched, again reinforcing the shRNA screen results (Figure 2C, S2B, S2D). Interestingly, while sgRNAs targeting other genes showed less enrichment, examination of sgRNA behavior across all tumors identified several additional putative tumor suppressors, such as *Sytl1* and *Gss,* which encode a vesicle trafficking protein (Johnson et al., 2012) and glutathione synthetase (Oppenheimer et al., 1979), respectively (Figure 2D, S2C, S2D, Table S3).

As *Zmat3* and *Dennd2c* were the most significant hits in independent screens using 2 technologies, we further probed their tumor suppressor capacity. We first performed *in vivo* competition experiments to quantitatively examine the growth advantage of *Zmat3*-

deficient cells relative to control cells using *E1A;Hras^{G12V}*;*Cas9* MEFs. Individual *Zmat3* or NC sgRNAs were expressed from vectors containing either a GFP or mCherry marker. Next, we mixed NC sgRNA and sgZmat3-expressing cells expressing different fluorophores 1:1, verified the mixed composition by flow cytometry, injected the cells subcutaneously into recipient mice, and analyzed the resulting tumors 3 weeks later by flow cytometry (Figure 2E, 2F, S2E). *Zmat3*-deficient cells consistently dominated the tumors, underscoring the *in vivo* growth advantage conferred by *Zmat3* deficiency and its tumor suppressor activity (Figure 2F, 2G). Similar experiments with *Dennd2c* sgRNAs revealed that *Dennd2c* inactivation also conferred a clear growth advantage *in vivo*, confirming its tumor suppressor capacity (Figure 2H, S2F). Thus, the combined RNAi and CRISPR/Cas9 screening approach converged on 2 potent tumor suppressors, *Zmat3* and *Dennd2c*.

Zmat3 expression is highly p53-dependent in mouse and human cells

We next sought to evaluate the role of *Zmat3* and *Dennd2c* as central components of the p53 tumor suppressor network by assessing how universally p53 regulates them. In mouse cells, both *Zmat3* and *Dennd2c* display p53-dependent expression in diverse cell types, including *E1A;Hras^{G12V}* MEFs, *Eμ-Myc* lymphoma cells, and embryonic neural crest cells, and *Zmat3* expression is p53-dependent in *Kras^{G12D}*-driven LUAD cells (Figure 3A)(Bowen et al., 2019). Strikingly, in human fibroblasts and in many human cancer types – including breast (BRCA), lung (LUAD), and liver (LIHC) cancers – *ZMAT3* expression is higher in p53-proficient samples than in p53-deficient samples, supporting the notion that *ZMAT3* is broadly a p53 target (Figure 3B, 3C). *DENND2C*, while demonstrating clear

p53-dependent expression in human fibroblasts, showed more tissue-specific p53 dependency across cancer types (Figure 3B, 3D, S3A). These findings suggest that *DENND2C* might play a more tissue-restricted role in p53-mediated tumor suppression.

The marked p53-dependent expression of *ZMAT3* in many human cancer types prompted a deeper investigation of *ZMAT3* regulation by p53. Our ChIP-seq data from both human and mouse cells indicated that the *ZMAT3* locus is directly bound by p53 (Figure 3E, 3F) (Kenzelmann Broz et al., 2013; Younger et al., 2015). Although previous sequence analysis identified several p53 response elements (RE) in the mouse *Zmat3* promoter (Wilhelm et al., 2002), our ChIP-seq analyses revealed a major p53-bound region containing a perfect p53 RE in the first intron of mouse *Zmat3* and a near-perfect p53 RE in the first intron of human *ZMAT3* (Figure 3E, 3F). To assess the importance of p53 regulation for *Zmat3* expression, we designed sgRNAs to disrupt the p53 RE at the major p53-binding peak in mouse *Zmat3* (Figure 3F). Perturbing this RE in *E1A;Hras*^{G12V};*Cas9* MEFs significantly reduced p53 binding to *Zmat3* but not to *Cdkn1a* in ChIP experiments (Figure 3G, S3B) and significantly decreased *Zmat3* mRNA and protein levels similarly to *p53* knockout (Figure 3H, 3I) suggesting that the RE is critical for p53 regulation of *Zmat3*. Together, these findings demonstrate that *ZMAT3* is broadly regulated by p53 in numerous mouse and human cell types and that robust *Zmat3* expression relies on direct induction by p53.

***Zmat3* suppresses LUAD and HCC development**

While the oncogene-expressing MEF discovery platform indicated the importance of *Zmat3* in tumor suppression in one context, we sought to interrogate whether *Zmat3*

might be more broadly relevant as a tumor suppressor using *in vivo* carcinoma models. p53 plays a key tumor suppressive role in LUAD, with nearly half of human tumors carrying *TP53* mutations (Kandoth et al., 2013). Moreover, both our cell culture data (Figure 3A) and *in vivo* data from mouse tumors (Figure 4A) (Feldser et al., 2010) indicate that *Zmat3* is robustly induced by p53 in LUAD. We thus investigated the role of ZMAT3 as a tumor suppressor using CRISPR/Cas9-mediated genome editing in an autochthonous $Kras^{G12D}$-driven mouse LUAD model (Jackson et al., 2001; Rogers et al., 2017). We induced tumors in $Kras^{LSL\text{-}G12D/+};Rosa26^{LSL\text{-}tdTomato/LSL\text{-}tdTomato};H11^{LSL\text{-}Cas9/LSL\text{-}Cas9}$ ($KT;H11^{LSL\text{-}Cas9}$) mice with lentiviruses expressing Cre recombinase and an sgRNA targeting *p53*, 1 of 3 sgRNAs targeting *Zmat3*, or either of 2 NC sgRNAs (Chiou et al., 2015) (Figure 4B). After transduction of lung epithelial cells, Cre excises the Lox-Stop-Lox cassettes, allowing $Kras^{G12D}$, *Cas9,* and *tdTomato* reporter expression. As anticipated, Cas9-mediated inactivation of p53 led to significantly larger tumors and greater total tumor burden than in NC $KT;H11^{LSL\text{-}Cas9}$ mice (Figure 4C, 4D, 4G). Interestingly, Cas9-induced *Zmat3* inactivation also drove significantly larger tumors and increased tumor burden than in control mice (Figure 4E, 4F, 4H, S4A, S4B). The increase in LUAD growth observed with *Zmat3* inactivation was less than with *p53* inactivation, supporting the idea that *Zmat3* is one critical component of the p53 tumor suppression program. Furthermore, we found that like p53, *Zmat3* impeded proliferation without inducing apoptosis, suggesting that proliferation inhibition is a mechanism for suppressing LUAD growth (Figure 4I, 4J, S4C). We did not observe a similar increase in tumor size or total tumor burden when tumors were induced in p53-deficient $KPT;H11^{LSL\text{-}Cas9}$ mice, suggesting that *Zmat3* is most relevant in the context of an intact p53 pathway (Figure S4D).

To assess the importance of *Zmat3* in suppressing tumorigenesis in an additional autochthonous carcinoma model, we used a mouse hepatocellular carcinoma (HCC) model in which p53 is tumor suppressive (Tschaharganeh et al., 2014). In this model, a recombinant transposon vector expressing $Kras^{G12D}$, along with vectors expressing *Cas9/*sgRNAs and Sleeping Beauty transposase are introduced into hepatocytes via hydrodynamic tail vein injection (HTVI). We used the HTVI model to deliver sgRNAs targeting Chromosome 8 (negative control), *p53* (positive control), or *Zmat3* (Figure 4K). Interestingly, while no tumors developed in the sgChrom8 negative control group (0/5), 5/5 mice in both sgZmat3 cohorts and the sgp53 cohort developed tumors (Figure 4L). Although the penetrance of the tumor phenotype was equivalent in the sgp53 group and both sgZmat3 groups, the tumors in the sgp53 mice were larger than in the sgZmat3 mice, again suggesting that *Zmat3* is one component downstream of p53 (Figure 4M-N). Together, these findings illuminate *Zmat3* as a p53 target gene critical for carcinoma suppression *in vivo*.

ZMAT3 is a component of the p53 pathway in human carcinomas

Given the highly conserved regulation of *ZMAT3* by p53 in human cells, and the clear tumor suppressor activity of *Zmat3* in mice, we next sought to examine the role of *ZMAT3* in the p53 tumor suppressor pathway in human cancer. We first queried the association between *ZMAT3* expression and patient prognosis in human carcinomas. We analyzed a large cohort of breast cancer patients for whom long-term follow-up data (>15 years) are available and with nearly 2000 tumors for which *TP53* status is known (Curtis et al., 2012; Pereira et al., 2016). We found that patients with tumors with relatively low

277 *ZMAT3* expression exhibited reduced disease-specific survival relative to those with higher
278 *ZMAT3* expression. Importantly, this association was only observed for patients with
279 tumors of wild-type *TP53* but not mutant *TP53* status (Figure 5A-C). As predicted from
280 our mouse studies, examination of TCGA LUAD and LIHC data revealed a similar survival
281 pattern for patients with high and low *ZMAT3* expression specifically in the context of
282 wild-type *TP53*, although the survival difference in the LUAD data did not reach statistical
283 significance (Figure 5D-F, S5A-S5C). These correlative findings further support a role for
284 *ZMAT3* particularly in cancers in which p53 is intact.

285 To further investigate the role for ZMAT3 in the human p53 tumor suppression
286 program, we leveraged TCGA data to examine patterns of *ZMAT3* mutation relative to
287 *TP53* mutations. While amplifications of chromosome 3q where *ZMAT3* resides are
288 observed in certain human cancers – potentially due to linkage to the *PIK3CA* oncogene –
289 point mutations in *ZMAT3* are also found in some cancers, such as uterine corpus
290 endometrial carcinoma (UCEC)(Cerami et al., 2012; Gao et al., 2013). Interestingly,
291 mutations and deletions in *ZMAT3* are mutually exclusive with mutations and deletions in
292 *TP53* in UCEC, supporting the notion that *ZMAT3* is a component of p53-mediated tumor
293 suppression in humans (Figure 5G).

294 To examine the functional significance of the *TP53-ZMAT3* axis in human cancer,
295 we mined data from Project Achilles, a compilation of genome-scale pooled screens from
296 485 cell lines (Meyers et al., 2017). We specifically interrogated the functional effect of
297 *ZMAT3* knockout by CRISPR/Cas9 in human carcinoma cell lines. We parsed the cell lines
298 based on *TP53* status into either wild-type or aberrant and plotted the CERES dependency
299 score (Meyers et al., 2017) for each group. Interestingly, we found a significant positive

dependency score for *ZMAT3* in cell lines with wild-type p53, suggesting faster growth of the cell lines with *ZMAT3* knockout (Figure 5H). In contrast, cell lines with aberrant *TP53* were unaffected by *ZMAT3* perturbation. Furthermore, the top *ZMAT3* co-dependencies not only include *TP53* and the p53 positive regulator *TP53BP1* (with dependency scores positively correlated with that of *ZMAT3*), but also p53 negative regulators – *MDM4* and *PPM1D* (with dependency scores negatively correlated with that of *ZMAT3*; Figure 5I). These functional studies thus further emphasize a growth-suppressive role for *ZMAT3* in human cells, particularly when p53 is intact.

ZMAT3 is sufficient to inhibit proliferation

Our findings suggesting that *ZMAT3* is particularly important for tumor suppression in the context of intact p53 may be due to a requirement for p53 for efficient *Zmat3* expression (Figure 3I), and therefore modulating *ZMAT3* in a p53-deficient context where *ZMAT3* expression is very low might have little effect. Moreover, ZMAT3 might have a more prominent role in the setting of an active p53 pathway based on its cooperation with other p53 target genes. Alternatively, ZMAT3 might act to reinforce p53 function, rendering ZMAT3 ineffective in a *p53* null context. To distinguish these possibilities, we asked whether overexpression of ZMAT3 is sufficient to inhibit proliferation in the absence of p53. We overexpressed HA-tagged ZMAT3, p53 or GFP as a negative control in $Kras^{G12C}$-expressing, *p53*-deficient H23 human LUAD cells and in *p53*-null untransformed MEFs and assessed proliferation by measuring BrdU incorporation. In both cell types, ZMAT3 overexpression inhibited proliferation, consistent with it acting downstream of p53 (Figure 5J, 5K, S5D). Importantly, one recurrent *ZMAT3* mutation found in human

cancers, R99Q, rendered ZMAT3 unable to inhibit proliferation, supporting the notion that *ZMAT3* is functionally inactivated by point mutations found in human cancers (Figure 5J, S5D). Notably, the arrest caused by ZMAT3 overexpression is not as potent as that seen with p53 overexpression, suggesting that other factors also contribute to p53-mediated cell cycle arrest. These data demonstrate not only that ZMAT3 functions downstream of p53, but also that p53 is dispensable for ZMAT3 to inhibit proliferation.

Zmat3 is an alternative splicing regulator

Given the importance of ZMAT3 as a tumor suppressor, we next explored its mechanism of action. ZMAT3 contains 3 zinc fingers, the first 2 of which mediate RNA binding (Israeli et al., 1997; Mendez-Vidal et al., 2002). To identify RNAs directly bound by ZMAT3, we performed enhanced crosslinking and immunoprecipitation (eCLIP) with ZMAT3 antibodies followed by high-throughput sequencing (Van Nostrand et al., 2016). We identified hundreds of ZMAT3-binding peaks (Figure 6A, S6A, Table S4), and analysis of bound RNAs by functional annotation revealed several enriched gene ontology (GO) terms, including RNA binding and mRNA splicing (Figure 6B). To integrate ZMAT3 binding with effects on gene expression, we performed RNA-sequencing (RNA-seq) on *E1A;Hras*^{G12V};*Cas9* MEFs expressing *Zmat3* or NC sgRNAs (Figure S6B). Comparison of the gene expression profiles of sgNC and sgZmat3-expressing cells revealed 847 significantly differentially-expressed genes (Figure 6C, Table S5). GO term annotation again uncovered various categories related to RNA biology, such as RNA binding, ncRNA processing, and mRNA splicing, suggesting a fundamental role for ZMAT3 in RNA regulation (Figure 6D). Overlapping ZMAT3-bound and regulated transcripts revealed 95

346 transcripts, again associated with RNA-related processes, including RNA binding and
347 mRNA splicing (Figure 6E). Together, these findings suggest a broad role for ZMAT3 in
348 RNA homeostasis.

349 eCLIP data can provide key mechanistic insight into RBP function by revealing
350 positional specificity of RBP binding. Analysis of ZMAT3 binding indicated a remarkable
351 stereotypical positioning, centered 95 nt upstream of 3' splice sites and 30-50 nt upstream
352 of the branch point in the majority of bound transcripts (Figure 6F, 6G, 6H). Strikingly,
353 this profile of ZMAT3 peaks is distinct from 150 RBPs profiled by the ENCODE
354 consortium (Figure 6G, S6C). For example, maximal ZMAT3 peak density was detected
355 upstream of annotated spliceosomal components, including the 3' splice site factor U2AF2,
356 which binds at the polypyrimidine tract, and branch point factors SF3B4 and RBM5
357 (Figure 6G, 6H). Moreover, alternative splicing regulatory RBPs PTBP1 and KHSRP,
358 which also bound 100 nt upstream of the 3' splice site, had far broader peak distributions
359 and significant enrichment in 5' splice site regions (Figure 6H). HOMER motif analysis
360 revealed that a subset of the ZMAT3 peaks contain a CAG adjacent to a polypyrimidine
361 tract, reminiscent of the 3' splice site consensus sequence (Figure S6D).

362 The ZMAT3 binding position, along with the enrichment of genes involved in
363 mRNA splicing in the ZMAT3-bound and regulated transcripts (Figure 6B, 6D, 6E)
364 suggested that ZMAT3 might regulate splicing. Using rMATS analysis (Park et al., 2013)
365 to compare splice variants in our RNA-seq data from ZMAT3-deficient and control MEFs,
366 we identified 719 ZMAT3-dependent alternative splicing events. The majority of the
367 alternative splicing events were skipped exon (SE) events, but we also found other types
368 of alternative splicing events (Figure 6I, Table S6). Through this analysis, we identified

alternative splicing events adjacent to ZMAT3-bound introns, including in transcripts encoding proteins involved in splicing (*Hnrnpdl*, *Dhx9*), p53 regulation (*Mdm4* and *Mdm2*), and varied additional cellular functions (*Dst*, *Sptan1*, *Bin1*) (Kemmerer et al., 2018; Kunzli et al., 2016; Lee and Pelletier, 2016; Marine et al., 2006; Sakamuro et al., 1996; Wang et al., 2018) (Figure 6J). Interestingly, splicing map analysis did not reveal a consistent pattern of ZMAT3 binding across all ZMAT3-regulated differentially spliced exons (Figure S6E), and numerous alternatively spliced RNAs did not show ZMAT3 binding (e.g. *Cask, Tia1*), suggesting that ZMAT3 not only exerts direct effects on splicing, but also indirect downstream effects.

ZMAT3 regulates splicing of transcripts involved in diverse cellular processes

Of the significant ZMAT3-bound and alternatively spliced transcripts we identified, the p53 negative regulator *Mdm4* is most prominent (Figure 6J). Interestingly, our data show that *Zmat3* knockout cells are enriched for the full-length isoform of *Mdm4* (*Mdm4-FL*), encoding the form that negatively regulates p53 by either directly blocking p53 transactivation or cooperating with the ubiquitin ligase MDM2 to promote p53 degradation (Marine et al., 2006; Toledo and Wahl, 2006) (Figure 7A-C). In contrast, the ZMAT3-expressing cells also express a short isoform of *Mdm4* (*Mdm4-S*) in which exon 6 is skipped – an event known to introduce a premature termination codon and trigger nonsense-mediated mRNA decay (NMD) (Bardot et al., 2015) (Figure 7A-C). Therefore, the *Mdm4-S* transcript does not lead to stable MDM4 protein expression, and indeed, we observe less MDM4 protein in ZMAT3-expressing cells than in ZMAT3-deficient cells (Figure 7D). Notably, we also observe a decrease in *Mdm4-S* levels in p53-deficient cells,

and in cells in which the p53 RE in *Zmat3* is disrupted, suggesting that p53 activation of *Zmat3* is required for the *Mdm4* alternative splicing event (Figure 7D). Exclusion of exon 3 of *Mdm2* - which is necessary for efficient binding to p53 – is also significantly greater in the presence of *Zmat3* (Figure 7A-B) (Giglio et al., 2010; Perry et al., 2000). Although p53 protein accumulation is not clearly decreased by *Zmat3* deficiency, expression of some p53 target genes, including *Gtse1* and *Eif4g3,* is diminished (Figure 3I, S6B, S7A). Thus, these data suggest that one component of *Zmat3* tumor suppressor function is to promote full p53 activity, but it does not fully account for *Zmat3* function, as ZMAT3 clearly can impede proliferation in the absence of p53 (Figure 5J, 5K).

Various other transcripts are bound and alternatively spliced by ZMAT3 (Figure 6J), including splicing regulators, such as *Hnrnpdl* – an hnRNP family member involved in alternative splicing (Kemmerer et al., 2018) – and *Dhx9* – an RNA helicase that interacts with other splicing factors in pre-spliceosomes (Lee and Pelletier, 2016). Both transcripts are bound by ZMAT3 upstream of a 3' splice site adjacent to alternatively spliced exons that are preferentially excluded in ZMAT3-expressing cells (Figure S7B-C, S7E, 7E). The excluded exon in *Hnrnpdl* can trigger NMD and transcript destabilization (Kemmerer et al., 2018), consistent with accumulation of this transcript in the presence of ZMAT3. *Dhx9* transcript levels are also increased in ZMAT3-expressing cells (Figure S7B, Table S5), further suggesting that differential splicing could affect transcript stability. By binding transcripts encoding splicing regulators, ZMAT3 may indirectly regulate additional splicing events. ZMAT3 binding also affects splicing of RNAs encoding proteins with roles in various cellular processes, including adhesion, cytoskeletal function and polarity (*Dst*, *Sptan, Dlg1*; Figure 7E, S7C, S7D). Collectively, our findings show that ZMAT3 drives

alternative splicing of transcripts involved in functionally distinct processes.

ZMAT3 binds Nonsense-Mediated Decay target transcripts

Alternative splicing has been reported to trigger NMD in ~30% of alternatively spliced transcripts (Lewis et al., 2003), prompting us to examine whether ZMAT3 preferentially regulates alternative splicing of known NMD targets. We compared the ZMAT3-bound transcripts with alternatively spliced transcripts from RNA-seq analysis of MEFs deficient in SMG1, a kinase that phosphorylates UPF1, a key regulator of NMD (McIlwain et al., 2010). Indeed, ZMAT3 preferentially binds introns flanking SMG1-regulated premature termination codon (PTC)-containing exons relative to non-SMG1 regulated PTC exons or random exons (Figure 7F, see methods). Thus at least a subset of ZMAT3 binding occurs on established splicing-regulated NMD targets, supporting the notion that ZMAT3 affects RNA stability through its ability to regulate alternative splicing. Interestingly, analysis of our RNA-seq data revealed significantly higher levels of ZMAT3-bound transcripts than all expressed transcripts with ZMAT3 expression, suggesting that ZMAT3 binding generally stabilizes mRNAs (Figure 7G).

Collectively, our findings support a model in which ZMAT3 binds transcripts upstream of 3' splice sites, regulates splicing to affect both isoform expression and NMD, and broadly influences gene expression programs. In addition, ZMAT3 action triggers indirect, downstream splicing events. Importantly, given the range of transcripts either differentially expressed or alternatively spliced in the presence of ZMAT3, our findings suggest that the tumor suppressive effect of ZMAT3 is not likely to be explained by a particular bound or alternatively spliced target, but rather through a more complex impact

impinging upon diverse cellular pathways (Figure 7H). These findings are in line with previous studies demonstrating that the oncogenic effects of mutations in splicing factors such as SF3B3 and SRSF2 are attributable to subtle splicing changes in a range of transcripts, rather than dramatic changes in splicing of a single transcript (Wang et al., 2016; Zhang et al., 2015).

Discussion

Here, we use a multidisciplinary approach to illuminate p53 transcriptional programs critical for p53 tumor suppressor function. By coupling unbiased ultracomplex RNA interference and CRISPR/Cas9 pooled screens *in vivo*, CRISPR/Cas9-mediated genome editing in autochthonous mouse cancer models, human cancer genome analysis, and integrative eCLIP-seq and RNA-seq analyses, we reveal an important branch of p53 tumor suppression involving an RNA splicing program. While recent studies have underscored the importance of dysregulated splicing for cancer development (Obeng et al., 2019; Zhang and Manley, 2013) and suggested a role for mutant p53 in regulating splicing in pancreas cancer (Escobar-Hoyos et al., 2020), a clear link to wild-type p53 has not been established. We show further that ZMAT3 is most active in the context of an intact p53 pathway, and that ZMAT3 controls p53 regulators, suggesting that feedback to p53 may account for some ZMAT3 tumor suppressor activity. However, ZMAT3 expression is sufficient to inhibit proliferation in p53-deficient cells, indicating that ZMAT3 can also employ p53-independent tumor suppressive mechanisms. Interestingly, a recent shRNA screen revealed that *Zmat3* knockdown in hematopoietic stem cells deficient for the p53 target genes *Puma* and *p21* promotes leukemia development (Janic et al., 2018). Together

with our data revealing a tumor suppressor role for *Zmat3* in *E1A;Hras*^{G12V} MEFs, lung adenocarcinoma, and hepatocellular carcinoma, these observations support the idea that ZMAT3 represents a core component of p53 tumor suppression across various contexts.

Although *ZMAT3* has been known as a p53 target gene for some time (Israeli et al., 1997; Varmeh-Ziaie et al., 1997), its physiological function and role in tumor suppression are not well understood. Previous studies have suggested that ZMAT3 is a double-stranded RBP that binds 3' UTRs of various mRNAs to modulate RNA levels (Bersani et al., 2016; Bersani et al., 2014; Vilborg et al., 2009). Here, we used the highly sensitive eCLIP technique (Van Nostrand et al., 2016), coupled with RNA-seq, to understand ZMAT3 function. We identified hundreds of novel ZMAT3-bound transcripts, but only ~1% of ZMAT3 binding events were at the 3' UTR. Instead, we observed dramatic enrichment of ZMAT3 binding upstream of the 3' splice site of specific introns in the majority of transcripts, strongly suggesting that ZMAT3 functions to regulate splicing. Indeed, we observed ZMAT3-dependent regulation of alternative splicing of numerous transcripts. Notably, *Zmat3* knockout affects splicing of select transcripts, suggesting that ZMAT3 is a regulator of alternative splicing rather than being a core splicing component. Intriguingly, we noted multiple instances of known 'poison exons' – regulated alternatively spliced exons containing stop codons that trigger NMD (Kurosaki et al., 2019). Depending on whether these exons are included or excluded, transcripts may be stabilized or destabilized by ZMAT3. Thus, there may be additional ZMAT3-regulated alternative splicing events that we failed to detect in our RNA-seq due to degradation of the alternative isoform.

Inclusion of cryptic exons – nonannotated exons defined by sequences similar to consensus motifs of canonical splice sites – occurs in human disease and can also trigger

NMD (Sibley et al., 2016; Ule and Blencowe, 2019). Recent studies have suggested that suppression of cryptic splice site recognition is a key function of some RBPs, such as the ZMAT3-related protein MATRIN 3, which binds and blocks the use of cryptic splice sites in antisense LINE elements (Attig et al., 2018). As with MATRIN3, ZMAT3 knockdown causes a small but significant decrease in ZMAT3-bound transcript levels. Although we do not yet know the mechanism by which ZMAT3 recognizes its specific targets, motif enrichment analysis revealed ZMAT3 binding to sequences resembling the consensus 3' splice site (CAG + polypyrimidine tract). Thus, ZMAT3 may maintain proper expression of its targets by suppressing the use of specific cryptic alternative 3' splice sites.

The direct modulation of alternative splicing by ZMAT3 promotes alterations in the expression of genes involved in p53 regulation, various cellular processes, and splicing itself. Notably, we do not observe splicing changes in the *Trp53* transcript (Figure S7F). The most dramatic effect is on the p53 inhibitor MDM4, for which different spliced isoforms have been described, including those expressing and lacking exon 6 (Bardot et al., 2015; Boutz et al., 2015). Critical evidence for the functional roles of the isoforms has come in part from mice expressing an *Mdm4* allele lacking exon 6, which display embryonic lethality provoked by diminished *Mdm4* expression and inappropriate p53 activation (Bardot et al., 2015). Moreover, inhibition of exon 6 inclusion using antisense oligonucleotides impairs melanoma growth *in vivo* (Dewaele et al., 2016). Thus, the main function of the alternative splicing event in *Mdm4* that drives exon 6 skipping is to reduce the amount of full length *Mdm4* produced and promote active p53. While specific regulators of splicing, such as SRSF proteins and PRMT5, have been genetically defined as modulators of *Mdm4* exon 6 skipping (Dewaele et al., 2016; Fong et al., 2019), we

propose that ZMAT3 binding to RNA directly dictates the occurrence of this event, as a means to maintain p53 activity.

Beyond driving this specific alternative splicing event, ZMAT3 likely impedes tumor development through combined effects on the various transcripts that it binds and directly regulates. Interestingly, ZMAT3 regulates splicing of transcripts encoding proteins involved in diverse cellular processes. For example, DST is a hemidesmosome component involved in epithelial cell-basement membrane adhesion (Kunzli et al., 2016), DLG1 is a cell polarity/signaling protein originally identified from a Drosophila larva overgrowth phenotype (Milgrom-Hoffman and Humbert, 2018), and SPTAN1 is a cytoskeletal scaffold protein (Ackermann and Brieger, 2019). Therefore, ZMAT3 regulation of such transcripts, by modulating isoform expression or levels, may influence tumorigenesis. In addition, our finding that some ZMAT3-bound and differentially spliced genes are themselves splicing regulators suggests that ZMAT3 also indirectly impinges on the alternative splicing of a broad network of transcripts. Indeed, rMATs analysis reveals hundreds of transcripts not clearly bound by ZMAT3 but nonetheless alternatively spliced in a ZMAT3-dependent fashion. Interestingly, these RNAs are involved in a wide range of cellular processes, suggesting how ZMAT3 could contribute to tumor suppression in a pleiotropic fashion.

Beyond *Zmat3*, our screening platform identified other tumor suppressor genes. *Dennd2c* is a DENN domain family member, which act as Rab-GEFs and regulate intracellular trafficking (Yoshimura et al., 2010). While not well studied, DENND2C may regulate autophagy, a process modulated by p53 and sometimes involved in tumor suppression (Jung et al., 2017; Kenzelmann Broz et al., 2013). The identification of *Ptpn14*, which we have shown suppresses both YAP and pancreatic cancer (Mello et al., 2017), as

well as *Trp53inp1*, *Ldhb*, and *Gss*, which all encode metabolic regulators, highlight additional pathways that likely contribute to p53-mediated tumor suppression. The identification of such novel functional tumor suppressor genes is critical for helping to deconvolute human cancer genome sequencing data by unveiling genes with tumor suppressor activity and is a first step toward delineating the cooperating p53 programs involved in suppressing cancer. Ultimately, deconstructing the pathways through which p53 acts may lead to new opportunities for therapeutic intervention in cancer.

Acknowledgments

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Author contributions

K.T.B-R. designed and performed experiments, interpreted data, and wrote the manuscript. A.K. and A.M.B. designed and performed experiments and analyzed data. D.W.M. and M.C.B. designed shRNA and sgRNA libraries and interpreted the screen results. J.A.S. and C.C. performed human tumor analyses. S.S.M. performed TSAG identification and ChIP-

seq analyses. J.McC. performed Western blots. M.W. generated the Zmat3 R99Q construct. C.Z, S.L.H. and S.W.L. designed, performed, and analyzed mouse HCC experiments. E.L.V., B.A.Y., and G.W.Y. performed, analyzed and interpreted Zmat3 eCLIP and splicing experiments. I.P.W. and M.M.W. assisted with mouse Lenti/Cre LUAD experiments. S.E.P. performed dependency score analysis. A.J.C. assisted with mouse lung histological analyses. L.D.A. designed experiments, interpreted data, and wrote the manuscript.

Declaration of Interests

ELVN is co-founder, member of the Board of Directors, on the SAB, equity holder, and paid consultant for Eclipse BioInnovations. GWY is co-founder, member of the Board of Directors, on the SAB, equity holder, and paid consultant for Locana and Eclipse BioInnovations. GWY is a visiting professor at the National University of Singapore. Dr. Yeo's interest(s) have been reviewed and approved by UCSD in accordance with its conflict of interest policies. The authors declare no other competing financial interests.

Figure legends

Figure 1: Identification and functional screening of p53 tumor suppression-associated genes (TSAGs). (A) Bioinformatics analyses reveal 1063 genes activated by p53 in *HRas*^{G12V} MEFs, 87 of which are also activated by the p53^{25,26} mutant (TSAGs). (B) Heat map of expression of 87 p53 TSAGs in *HRas*^{G12V} MEFs homozygous for *p53*^{wt}, *p53*^{25,26}, or *p53* null alleles. Columns represent independent MEF lines. (C) *E1A;HRas*^{G12V};*p53*$^{+/+}$ MEFs transduced with a lentiviral shRNA library were collected before transplantation

(T_0) and after 3 weeks of subcutaneous growth in *Scid* mice to assess shRNA representation. (D) Top TSAG hits (n=9 tumors). "Unique" refers to the number of unique shRNAs enriched relative to negative controls in the 9 tumors, "Total" accounts for the same shRNAs enriched in multiple tumors. p-values, hypergeometric test. (E) Soft agar assay. Mean colony number +/- s.d. of 3 independent *E1A;HRas^{G12V};p53$^{+/+}$* MEFs lines (each in triplicate) after expression of individual shRNAs. Data are relative to shLuc shRNAs. "*" indicates $p< 0.05$ and "**" indicates $p<0.01$, two-tailed paired t-test.

Figure 2: Pooled CRISPR/Cas9 screen identifies functional tumor suppressors. (A). *E1A;HRas^{G12V};p53$^{+/+}$;Cas9* MEFs transduced with lentiviral sgRNA libraries were collected before transplant at T_0 and after 3 weeks of subcutaneous growth in *Scid* mice to assess sgRNA representation. (B) and (C) Pie charts show representation of sgRNAs (grouped by gene) in each pool at T_0 and in example individual tumors. (D) Top TSAGs ranked by enrichment of sgRNAs in tumors relative to T_0 (n=6 tumors). p-values, Mann-Whitney U test. (E) *E1A;HRas^{G12V};p53$^{+/+}$;Cas9* MEFs expressing fluorescent markers and sgZmat3 or negative control sgRNAs were mixed 1:1, injected subcutaneously into *Scid* mice, and grown for 3 weeks. Dissociated tumor cells were analyzed by FACS. (F) Representative FACS plots show input and tumor populations where the negative control sgRNA cells were labeled with GFP and the sgZmat3 cells with mCherry. (G) and (H) Plots show the mean percentages +/- SEM of cells expressing the sgZmat3 fluorescent label (G) or sgDennd2c fluorescent label (H) relative to all labeled cells (either GFP or mCherry) in both input and tumor populations; n=6 tumors in (G), n=12 in (H). p-values, two-tailed paired t-test.

Figure 3: *ZMAT3* expression is highly p53-dependent in mouse and human. (A) qRT-PCR analysis of mean expression +/- s.d. of *Zmat3* and *Dennd2c* in $p53^{wt}$ and *p53* null *E1A;*$HRas^{G12V}$ MEFs, *Eμ-Myc* lymphoma cells, and $Kras^{G12D}$ LUAD cells, relative to *β-actin.* p-values, two-tailed unpaired t-test of different MEF lines (n=3), $Kras^{G12D}$ LUAD cell lines (n=3) or technical replicates (*Eμ-Myc* lymphoma cell line, n=1). (B) qRT-PCR analysis of mean expression +/- s.d. (technical replicates) of *ZMAT3* and *DENND2C* in primary human fibroblasts transfected with sip53 or a non-targeting siRNA, relative to *β-ACTIN*. (C) and (D) *ZMAT3* expression (C) or *DENND2C* expression (D) (adjusted for copy number) was mean-centered to zero to allow comparison of samples with wild-type or mutant *TP53* in TCGA datasets. The upper and lower hinges correspond to the 1st and 3rd quartiles, the upper and lower whiskers show the 1.5 interquartile ranges, and the center line is the median. p-values, two-tailed t-test. (E) and (F) p53 binding profile from human fibroblast (E) and MEF (F) ChIP-seq data shows called peaks (red triangles). The p53 RE is highlighted in yellow, with the nucleotides exactly matching the consensus motif in capital letters and the core motif in red. (G-I) *E1A;*$HRas^{G12V}$*;*$p53^{+/+}$*;Cas9* MEFs were transduced with the p53 RE, p53, Zmat3, or NC sgRNAs. Any of three sgRNAs were used to disrupt the p53 RE in the largest p53 binding peak [green lines in (F)]. (G) ChIP assays for p53 binding at the *Zmat3* or *Cdkn2a* locus. Graph shows the mean percent of input recovered +/- SEM (n = 6-12 combined technical and biological replicates). p-values, two-tailed unpaired t-test. (H) qRT-PCR analysis of mean expression +/- s.d. of *Zmat3* and *Cdkn2a* relative to *β-actin* ($n \geq 3$ per line). p-values, two-tailed unpaired t-test. (I) ZMAT3 and p53 protein levels analyzed by Western blotting with GAPDH loading control ($n \geq 3$).

Figure 4: *Zmat3* suppresses *Kras^{G12D}*-driven LUAD and HCC. (A) Published microarray analyses of grade 3 *Kras$^{LA2/+}$;Trp53$^{LSL/LSL}$;Rosa26^{CreERT2}* tumors lacking or expressing p53 (Feldser et al., 2010). (B) Lentiviral vectors expressing Cre recombinase and *p53*, *Zmat3* or NC sgRNAs were used to induce tumors in *KT;H11$^{LSL-Cas9}$* mice. Lungs were harvested after 17-20 weeks. (C) tdTomato expression in Lenti-sgNC/Cre or Lenti-sgp53/Cre mouse lungs. (D) Left, sizes of Lenti-sgp53/Cre and Lenti-sgNC/Cre tumors (n=4 mice/group; n=160 NC and 193 sgp53 tumors). Each dot represents a tumor, with area proportional to the tumor size. The bar is the mean, and p-values, two-tailed unpaired t-test. Right, representative H&E-stained lung sections. (E) tdTomato expression in Lenti-sgNC/Cre or Lenti-sgZmat3/Cre mouse lungs. (F) Left, sizes of Lenti-sgZmat3/Cre and Lenti-sgNC/Cre tumors (n=16-20 mice/group; n=546 negative control and 1059 sgZmat3 tumors). See plot description in (D). Right, representative H&E-stained lung sections. (G) and (H) Total tumor burden (% tumor area/total lung area) for Lenti-sgp53/Cre and Lenti-sgNC/Cre mice [(G) n=4 sgNC mice, 4 sgp53 mice] and Lenti-sgZmat3/Cre and Lenti-sgNC/Cre mice [(H) n=16 sgNC mice, 20 sgZmat3 mice]. Each dot represents a mouse, the bar is the mean. p-values, two-tailed unpaired t-test. (I) and (J) Left, Percentages of Ki67-positive nuclei in Lenti-sgp53/Cre and Lenti-sgNC/Cre tumors (I) and Lenti-sgZmat3/Cre and Lenti-sgNC/Cre tumors (J). Right, Representative IHC for Ki67 in tumors (n=20-45 tumors/group). Each dot represents a tumor and the bar is the mean. p-values, two-tailed unpaired t-test. (K) Hydrodynamic tail vein injection delivered Sleeping Beauty transposase, *Kras^{G12D}*, *Cas9*, and NC (Chrom8), p53, or either of 2 different Zmat3 sgRNAs to mouse livers. Livers were harvested after 7-9 weeks (sgp53) or 14 weeks

(sgChrom8 and sgZmat3). (L) and (M) Number of tumors per liver (L) and largest tumor diameter (M) in mice injected with sgChrom8, sgp53 or sgZmat3. Each dot is a mouse (n = 5 mice/group), and the bar is the mean +/- SEM. p-values, two-tailed unpaired t-test. (N) H&E of representative liver tumors or normal liver for sgChrom8.

Figure 5: *ZMAT3* has p53-dependent and -independent activities. (A) and (B) Probability of disease-specific survival (DSS) in METABRIC breast cancer patients with wild-type *TP53* (A) or mutant *TP53* (B) and high (first tertile) or low (third tertile) expression of *ZMAT3*. (C) Cox Proportional Hazard ratio plot for DSS and *ZMAT3* expression levels adjusted for age, grade, size, stage, and estrogen receptor (ER) and HER2 status in METABRIC. Cox Proportional Hazard p-value for *ZMAT3* expression: p-value: 0.0024, HR: 0.7878, CI 95% 0.67-0.91. (D) and (E) Probability of survival in TCGA LIHC patients with wild-type *TP53* (D) or mutant *TP53* (E) and high (first tertile) or low (third tertile) expression of *ZMAT3*. (F) Cox Proportional Hazard ratio plot of the association between survival and *ZMAT3* expression levels adjusted for age, stage, and gender in the TCGA LIHC dataset. Cox Proportional Hazard p-value for *ZMAT3* expression: p-value: 0.0024, HR: 0.57, CI 95% 0.39-0.82. (G) Mutations in *ZMAT3* are mutually exclusive with *TP53* mutations in UCEC. p-value, DISCOVER mutual exclusivity test. **(H)** Project Achilles cell lines were parsed on *TP53* status (wild-type or aberrant), and the CERES dependency score for *ZMAT3* plotted for each. p-value, Benjamini-Hochberg two-tailed t-test. (I) Top co-dependencies for *ZMAT3* in the CRISPR (Avana) Public 19Q4 dataset with Pearson correlations from DepMap. (J) and (K) H23 cells (J) or *p53*-null MEFs (K) expressing HA-tagged GFP, p53, wild-type ZMAT3 or ZMAT3 R99Q were analyzed for

668 BrdU incorporation by immunofluorescence. Each dot represents a replicate and the bar is
669 the mean. p-values, two-tailed t-test.
670
671 **Figure 6: ZMAT3 is uniquely positioned near the 3' splice site and drives alternative**
672 **splicing.** (A) Schematic for eCLIP experiment. (B) Enrichr analysis of top GO terms for
673 the 825 unique transcripts with eCLIP ZMAT3 binding peaks by both input and knockout
674 normalization. (C) Volcano plot of 847 differentially expressed genes identified by RNA-
675 seq in *E1A;HRas*^{G12V} MEFs expressing Zmat3 or NC sgRNAs, adjusted p-value < 0.05 (red
676 dots). (D) and (E) Enrichr analysis of top GO terms for the 847 genes identified by RNA-
677 seq (D), or the 95 genes on both the RNA-seq and eCLIP gene lists (E). (F) Percentage of
678 eCLIP peaks positioned at the indicated locations of the bound RNA in sgNC sample 2.
679 (G) Peak density heat map for ZMAT3 and other spliceosomal components and splicing
680 regulators profiled by CLIP for ENCODE across a meta-mRNA splice junction. Profiles
681 for specific RBPs are marked. Multiple rows for U2AF1 are different cell lines. Right bars
682 mark spliceosomal RBPs (blue), splicing regulatory RBPs (green) and ZMAT3 (red). (H)
683 Fraction of introns with a ZMAT3 or other RBP peak across a meta-mRNA splice junction.
684 Shaded regions indicate 5th to 95th percentile from 100 random samplings with
685 replacement. (I) rMATS analysis of RNA-seq data from *E1A;HRas*^{G12V} MEFs expressing
686 sgZmat3 or sgNC identifies 719 differential alternative splicing events. (J) ZMAT3-
687 dependent alternative splicing events in ZMAT3-bound transcripts.
688
689 **Figure 7: Multifaceted regulation of RNA splicing and stability by ZMAT3.** (A)
690 Alternatively spliced exons (yellow) from the RNA-seq data (upper tracks) and ZMAT3-

691 binding peaks (yellow) from the eCLIP data (lower tracks) in *Mdm4* and *Mdm2*. (B) Semi-
692 quantitative PCR analysis of the alternative splicing depicted in (A), with *Gapdh* control.
693 Primers were designed to flank the alternatively spliced exon. (C) qRT-PCR analysis of
694 *Mdm4* exon 6 alternative splicing (mean +/- s.d.) in *E1A;Hras^{G12V};Cas9* MEFs transduced
695 with sgRNAs targeting Zmat3, p53, the Zmat3 p53 RE, or NC sgRNAs, relative to *β-actin*.
696 p-value, 2-tailed unpaired t-test of different MEF lines (n=3-8). (D) Western blot analysis
697 of MDM4 in *E1A;Hras^{G12V};Cas9* MEFs expressing sgZmat3 or sgNC with α-TUBULIN
698 loading control. (E) Semi-quantitative PCR analysis of alternative splicing in *Dhx9*,
699 *Hnrnpdl*, and *Dlg1* with *Gapdh* control. Primers were designed to flank the alternatively
700 spliced exons. (F) Frequency of ZMAT3 binding peaks flanking SMG1-dependent exons
701 (orange triangle), non SMG1-dependent exons (blue triangle), or random exons (gray bars).
702 Significance based on comparison to random exons. (G) Cumulative fraction of changes in
703 the $\log_2$ fold change for transcripts bound by ZMAT3 near a 3' splice site (green line) or
704 all expressed genes (gray line) in the RNA-seq data. $p<0.001$, Kolmogorov-Smirnov test.
705 (H) Model for ZMAT3 action in tumor suppression. Transcripts in blue are ZMAT3-bound
706 in eCLIP data, those in black are not bound, based on statistics.
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STAR Methods

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to the Lead Contact, Laura D. Attardi (attardi@stanford.edu).

Materials Availability

Plasmids generated by this study are available upon request.

Data and Code Availability

The microarray data were published previously (Brady et al., 2011) and are available in the Gene Expression Omnibus (GSE27901). The mouse and human ChIP-seq data were also published previously (Kenzelmann Broz et al., 2013; Younger et al., 2015) and are available in the Gene Expression Omnibus (GSE46240, GSE55727). The human cancer data that support the findings of this study are available from the Genomic Data Commons, https://gdc.cancer.gov, and the European Genome-Phenome Archive, https://ega-archive.org/dacs/EGAC00001000484. The Achilles DepMap dataset (CCLE_Depmap_18q3 release) is available from the DepMap portal, https://depmap.org/portal/download/. The Zmat3 RNA-seq (GSE145430) and eCLIP data (GSE14555) have been submitted to the Gene Expression Omnibus and will be made publicly available upon publication of the manuscript. Scripts for version 1.0 of casTLE (Morgens et al., 2016) are available at https://bitbucket.org/dmorgens/castle.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Subcutaneous tumor model

All animal experiments were in accordance with the Stanford University APLAC (Administrative Panel on Laboratory Animal Care). Cells were suspended in PBS at 3 million cells per 100 µl (library experiments) or 1 million cells per 100 µl (*in vivo* competition experiments). For the library experiments, cells were mixed 1:1 in Matrigel® (Corning). 100 µl (*in vivo* competition experiments) or 200 µl (library experiments) of cell suspension were injected under the skin on the right and left flanks of 6-week-old male ICR/Scid mice (Taconic). Tumors were harvested after three weeks of growth. For library experiments, frozen tumors were ground in liquid nitrogen, followed by genomic DNA preparation (Gentra Puregene). ICR/Scid mice were group-housed (up to 5 mice per cage), and irradiated chow and water were provided ad libitum.

Mouse lung adenocarcinoma study

All animal experiments were in accordance with the Stanford University APLAC (Administrative Panel on Laboratory Animal Care). Mice were group-housed (up to 5 mice per cage), and food and water were provided ad libitum. $Kras^{LSL\text{-}G12D/+}$; $Rosa26^{LSL\text{-}tdTomato/LSL\text{-}tdTomato}$;$H11^{LSL\text{-}Cas9/LSL\text{-}Cas9}$ (*KT;H11*$^{LSL\text{-}Cas9}$) and $Kras^{LSL\text{-}G12D/+}$;$p53^{flox/flox}$;$Rosa26^{LSL\text{-}tdTomato/LSL\text{-}tdTomato}$;$H11^{LSL\text{-}Cas9/LSL\text{-}Cas9}$ (*KPT;H11*$^{LSL\text{-}Cas9}$) mice have been described (Chiou et al., 2015; Jackson et al., 2001; Madisen et al., 2010). Three unique sgRNAs targeting Zmat3 (selected from those used in the screen, sgZmat3.1: CTCCCCTGCCGTGGCAC, sgZmat3.2: CCACCACGCTGCTCACCC, sgZmat3.3:

GGCTTACACAGCTCCTCCA), one previously characterized p53 sgRNA (AGGAGCTCCTGACACTCGGA), and two previously characterized negative control sgRNAs (sgNT.1: GCGAGGTATTCGGCTCCGCG, sgNT.2: CCGCGCCGTTAGGGAACGAG) were used for these experiments (Rogers et al., 2017). Lenti-sgRNA/Cre vectors were generated using standard methods (Chiou et al., 2015). Lung tumors were induced as previously described (DuPage et al., 2009). Specifically, 6-12 week old male and female *KT;H11*$^{LSL\text{-}Cas9}$ or *KPT;H11*$^{LSL\text{-}Cas9}$ mice were randomly assigned to experimental groups, and then anesthetized by intraperitoneal injection of Avertin (2-2-2 Tribromoethanol). 90,000 particles of Lenti-U6-sgRNA/PGK-Cre virus were suspended in 50 μl sterile PBS and delivered intratracheally. Lungs were harvested 17-20 weeks after infection and fixed in formalin for 24 hours before processing and H&E staining.

Mouse HCC study

The Memorial Sloan Kettering Cancer Center (MSKCC) Animal Care and Use Committee (protocol no. 11-06-011) approved all mouse experiments. Mice were maintained under specific pathogen-free conditions, and food and water were provided ad libitum. Liver tumorigenesis was induced by delivery of plasmids via hydrodynamic tail vein injection (Moon et al., 2019a). Specifically, a mixture of sterile 0.9% NaCl solution and plasmids containing pT3-Caggs-Kras^{G12D}-IRES-GFP (5 μg), CRISPR plasmid px330 DNA (Cong et al., 2013) expressing Cas9 and a single guide RNA (sgRNA) targeting Chromosome 8 (negative control), p53 or Zmat3 (25 μg), and CMV-SB13 transposase (Huang et al., 2014) (6 μg) was prepared for each injection. sgRNA sequences used for cloning into pX330 are

as follows: Chromosome 8 oligo (GACATTTCTTTCCCCACTGG); Trp53 oligo (GACCCTGTCACCGAGACCCC); Zmat3 oligo 1 (AGAGGATTTAGCTAAGAGAG); Zmat3 oligo 2 (GCCAGGGGGCAGGGTGATCC). Female C57BL/6 mice (6-8 weeks of age) from Charles River Laboratories were randomly assigned to experimental groups to be injected with the 0.9% NaCl solution/plasmid mix into the lateral tail vein, with a total volume corresponding to 10% of body weight in 5 to 7 seconds. Upon sacrifice, numbers and diameters of macroscopic liver tumors were recorded, and liver tissues were excised and fixed in formalin for 24 hours before processing and H&E staining.

Mouse cell culture and viral infections

MEFs were derived from E13.5 embryos (Brady et al., 2011). MEFs and 293T cells were cultured in DMEM with high glucose (Gibco) supplemented with 10% FBS. $H11^{Cas9/+}$ MEFs were generated by crossing $H11^{LSL\text{-}Cas9/LSL\text{-}Cas9}$ mice to CMV-Cre mice (Schwenk et al., 1995). To generate $E1A;HRas^{G12V};p53^{+/+}$ or $E1A;HRas^{G12V};p53^{+/+};Cas9$ MEFs, wild-type or $H11^{Cas9/+}$ MEFs were transduced with *E1A* and $Hras^{GV12}$-expressing retroviruses (Brady et al., 2011). For library experiments, virus was produced in 15 cm plates, and target cells in 15 cm plates were transduced at a MOI of ~0.2, as measured by expression of the mCherry marker. Cells were selected in puromycin (2 μg/ml) for ~10 days prior to collection of time zero samples or injection into mice. Lung adenocarcinoma cell lines were generated from lung tumors dissected from 11-week-old $Kras^{LA2/+};Trp53^{LSL\text{-}wt/LSL\text{-}wt}$ mice. $Kras^{LA2/+};Trp53^{LSL\text{-}wt/LSL\text{-}wt}$ mice spontaneously recombine to express an oncogenic $Kras^{G12D}$ allele (Johnson et al., 2001). Cultures were established in N5 medium (Scheffler et al., 2005) supplemented with EGF (20 ng/ml) and FGF (20 ng/ml), then sorted by

fluorescence-activated cell sorting (FACS) (Stanford Shared FACS facility) for EpCam positivity (BioLegend), and later switched to DMEM high-glucose media supplemented with 10% FBS. For Adenovirus infections, cells were seeded at 1 x 10^5 cells/well in 6-well plates, then infected with either Ad5CMVempty (Ad-Empty, Cat# VVC-U of Iowa-272) or Ad5CMVCre (Ad-Cre, Cat# VVC-U of Iowa-5), obtained from the University of Iowa Viral Vector Core, at an MOI of 100. Cells were harvested for RNA preparation 48 hours after infection. *Eμ-Myc* lymphoma cells expressing a tamoxifen-inducible *p53*-Estrogen receptor fusion protein (Christophorou et al., 2005) ($p53^{-/ER\text{-}TAM}$) were the kind gift of Lin He. Cells were grown on a feeder layer (irradiated 3T3 cells) and treated with 1 μM 4-hydroxytamoxifen (4OHT) or Ethanol (vehicle) and harvested for RNA preparation after 6 hours. Unless otherwise indicated, all cells were maintained in DMEM high-glucose media supplemented with 10% FBS at 37ºC and 5%CO_2.

The sex of the mouse cell lines was not determined as it was not expected to impact the results. Cell line authentication was not applicable.

Human cell culture

Human fibroblasts were obtained from Coriell Cell Repositories (GM00011, GM06170) and maintained in DMEM high-glucose media supplemented with 15% FBS at 37ºC and 5%CO_2. H23 human lung adenocarcinoma cells were maintained in DMEM high-glucose media supplemented with 10% FBS at 37ºC and 5%CO_2. All human cell lines are male and were not authenticated.

METHOD DETAILS

TSAG identification

Using microarray data from mouse embryonic fibroblasts (MEFs) expressing oncogenic *Hras*^{G12V} (Brady et al., 2011), we compared the expression profiles of cells expressing p53 TAD mutants active in tumor suppression (wild-type p53, p53^{53,54}, and p53^{25,26}) with expression profiles of cells expressing p53 mutants inactive in tumor suppression (p53^{25,26,53,54} and *p53* null),and identified genes induced at least 2-fold in the active group, yielding a list of 55 activated genes. We also identified a list of 58 genes induced 2-fold or greater in *Hras*^{G12V} p53^{25,26}-expressing MEFs relative to *Hras*^{G12V} *p53* null MEFs and at least 70% as well as by wild-type p53 relative to *p53* null cells. These combined lists comprise 87 TSAGs. To determine whether the TSAGs are direct p53 targets, they were analysis for p53-bound response elements (RE) near or within their gene body using ChIP-seq datasets from mouse embryonic fibroblasts, stem cells, or splenocytes (Brady et al., 2011; Kenzelmann Broz et al., 2013; Lee et al., 2010; Li et al., 2012a; Tonelli et al., 2015) (Table S1).

Library design, amplification, and sequencing

25 shRNAs targeting each TSAG were drawn from a previously validated shRNA library (Kampmann et al., 2015), along with 1000 non-targeting shRNAs, and used as single library. 10 sgRNAs targeting each TSAG were drawn from a previously designed mouse-targeting library (Morgens et al., 2017); the guides were split into two pools along with 250 negative control safe-harbor sgRNAs each, which target predicted non-functional regions in the mouse genome to replicate the effects of DNA damage (Morgens et al., 2017). This resulted in two separate sgRNA libraries, each targeting a different set of genes

but containing the same negative controls. shRNA and sgRNA libraries were synthesized and cloned (Kampmann et al., 2015; Morgens et al., 2017). The composition of the shRNA and sgRNA libraries were monitored by amplicon sequencing on a MiSeq (for shRNA libraries) and on a NextSeq (for sgRNA libraries) (Deans et al., 2016).

Analysis of shRNA library screens

Due to the size of the shRNA library, we relied on the observation of multiple, independent shRNAs highly represented relative to negative control shRNAs in a given tumor. The 22 base pairs of each shRNA were aligned to a library index using Bowtie (Langmead et al., 2009) with zero mismatches allowed. shRNAs were then tested for enrichment over the negative controls in each individual tumor. First, shRNAs with zero or one count were removed. Then, shRNAs with a greater number of counts than at least 95% of the remaining non-targeting controls detected were considered enriched in that tumor. This analysis was repeated for each tumor, and for each gene, the number of shRNAs targeting that gene that were enriched in at least one tumor were counted. Note that shRNAs that were enriched in multiple tumors were not counted multiple times. This number of enriched shRNAs targeting each gene was then compared to the total number of shRNAs targeting that gene detected. In order to calculate significance, a similar analysis was performed with the non-targeting controls, where the top 5% of non-targeting controls from each tumor were counted (again not counting the same control shRNA multiple times) and compared to the total number of non-targeting controls detected across all tumors. The number of enriched shRNAs targeting each gene was then compared to the fraction of enriched non-targeting

controls using a hypergeometric test (i.e. sampling without replacement) to calculate a p-value.

Analysis of CRISPR/Cas9 library screens

The first 17 base pairs of the reads were aligned to the sgRNA library using Bowtie (Langmead et al., 2009) with zero mismatches allowed. Enrichment of each element was then calculated as a log base 2 ratio of the fraction of the counts in the tumor versus the time zero sample. Gene level data were then calculated with the safe-targeting guides as negative controls using version 1.0 of casTLE (Morgens et al., 2016). To identify consistent results across all 6 biological replicates for each library, the estimated casTLE effects for each gene from all 6 tumors were compared to a background of estimated casTLE effects of all genes from all 6 tumors. A Mann-Whitney U test was then used to calculate p-values.

Transformation assays

Anchorage-independent growth assays were performed by plating 3,000 cells per well in triplicate in 6-well plates. Cells were plated in phenol red-free DMEM containing 10% FBS, 50 U/ml penicillin/streptomycin and 0.3% agarose, on a layer of media containing DMEM with 10% FBS, 50 U/ml penicillin/streptomycin and 0.5% agarose, and grown for three weeks. After three weeks, cells were stained using a Giemsa solution (0.02% in PBS) and scored using ImageJ software.

***In vivo* competition assay**

E1A;Hras^{G12V};*p53*$^{+/+}$;*Cas9* MEFs were transduced with lentiviruses expressing *Zmat3*, *Dennd2c*, or negative control sgRNAs along with GFP (Hess et al., 2016) or mCherry (Han et al., 2017) fluorescent markers. Cells with sgZmat3 or sgDennd2c and GFP or mCherry were mixed 1:1 with cells expressing a negative control sgRNA and the opposite marker. This input mixture of cells was analyzed for the GFP to mCherry ratio by flow cytometery (LSR Fortessa, BD Biosciences). Cells were then injected into mice and grown as subcutaneous tumors as described above. After three weeks, tumors were harvested and incubated in RPMI media with Liberase (20 μg/ml, Roche) and DNase (400 μg/ml, Roche) enzymes, then filtered, centrifuged with 30% Percoll (Sigma), washed in RPMI and analyzed for GFP and mCherry expression by flow cytometry (LSR Fortessa, BD Biosciences).

Analysis of CRISPR indels

Genomic DNA was extracted from *E1A*;*Hras*^{G12V}*;Cas9* MEFs expressing sgRNAs using the DNeasy Blood and Tissue kit (Qiagen). The intended sgRNA target sites were PCR amplified using primers designed to flank the site. PCR products were separated on agarose gels and the amplified products extracted using a Gel Extraction Kit (Qiagen). The DNA fragments were Sanger sequenced by Quintara Biosciences (Berkeley, CA). Sequencing files were submitted to ICE v2 CRISPR analysis tool (Synthego, Menlo Park, CA).

qRT-PCR and Semi-quantitative PCR

Trizol reagent (Invitrogen) was used for RNA preparation, and reverse transcription was performed with MMLV reverse transcriptase (Invitrogen). Quantitative PCR was performed in triplicate using gene-specific primers and SYBR green (Life Technologies) in a 7900HT Fast Real-Time PCR machine (Applied Biosystems). Changes in transcript abundance were calculated using the standard curve method. Semi-quantitative PCR was performed for 27, 32, or 35 cycles (45 s 95°C; 30 s at 58°C; 40 s at 72°C) using a ~34-ng cDNA template. Products were visualized on a 2% agarose gel. Primer sequences are in Table S7.

ChIP

E1A;Hras^{G12V};Cas9 MEFs were seeded at 7.5 × 10^6 cells per 10-cm dish and ChIP was performed the following day. Briefly, cells were crosslinked at room temperature by treatment with DMEM media with 1% formaldehyde, and the reaction was quenched by addition of glycine to a final concentration of 0.125 M. After washing with cold 1X PBS, cells were harvested by scraping in lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40) and pelleted. Cell pellets were processed by passage through a 21-gauge needle 20 times. Lysates were pelleted and resuspended in RIPA buffer. Sonication was performed in a Bioruptor sonicator (Diagenode) to shear chromatin to a size range of ~200-700 bp. Anti-p53 antibody (CM5, Leica Novocastra) was coupled to ChIP-grade protein A/G magnetic beads (Thermo Scientific) overnight. After saving 10% for an input sample, samples were immunoprecipitated for one hour at room temperature and one hour at 4ºC, and washes were performed two times with low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl at pH 8.1, 150 mM NaCl), three times with high-

salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl at pH 8.1, 500 mM NaCl), and four times with LiCl wash buffer (0.25 M LiCl, 1% IGEPAL CA630, 1% deoxycholic acid sodium salt, 1 mM EDTA, 10 mM Tris at pH 8.1). Input was reverse crosslinked by treatment with ProK, RNAse A, and incubation at 65°C. All samples were purified by PCR Purification Kit (Qiagen). Chromatin-immunoprecipitated DNA was quantified by qPCR using SYBR Green (SA-Biosciences) and a 7900HT Fast Real-Time PCR machine (Applied Biosystems).

Western blots

Western blots were performed according to standard protocols. Briefly, cells were lysed in RIPA buffer, extracts were run on SDS-PAGE gels, gels were transferred to PVDF membrane (Immobilon, Millipore), and membranes were blocked with 5% milk and probed with antibodies directed against Zmat3 (1:100, Santa Cruz), p53 (CM5, 1:500 Leica Novocastra), Ptpn14 (1:100, Santa Cruz), or Gapdh (1:15,000, Fitzgerald), followed by anti-mouse or anti-rabbit HRP-conjugated secondary antibodies (Vector Laboratories). Blots were developed with ECL Prime (Amersham) and imaged using a ChemiDoc XRS+ (BioRad).

Immunohistochemistry

Hematoxylin and eosin (H&E) staining and immunohistochemistry were performed on paraffin-embedded lungs using standard protocols. Immunohistochemistry was performed using antibodies directed against Ki67 (1:100, BD Biosciences,) and Cleaved caspase 3 (1:400, Cell Signaling Technologies). Briefly, paraffin sections were re-hydrated,

unmasked in 10mM Sodium Citrate buffer with 0.05% Tween 20 in a pressure cooker for 10 minutes, the peroxidase was quenched for 15 minutes in 3% H_2O_2, sections were blocked for 30 minutes in TBS with 0.025% Triton X-100 supplemented with 10% serum and 1% BSA, and incubated overnight at 4°C with primary antibody. On the next day, the sections were incubated for 30 minutes with biotinylated antibody compatible with the primary antibody used (1:1000, Vector Laboratories) and were subsequently incubated with VECTASTAIN Elite ABC HRP Kit (Vector Laboratories), according to manufacturer's instructions. The sections were washed with TBS in between steps. Staining was performed using the DAB peroxidase kit (Vector Laboratories) and hematoxylin (H-3401, Vector Laboratories) for counter-staining. A Leica DM6000B microscope (Leica Microsystems) or NanoZoomer 2.0-RS slide scanner (Hamamatsu) was used for imaging.

Overexpression and immunofluorescence

Overexpression experiments for BrdU incorporation analysis were performed using constructs in which Zmat3 cDNA was cloned into a pCDNA vector carrying an HA-tag. The pcDNA3.1-3XHA-Zmat3 construct was generated by PCR amplification and insertion of the cDNA into AscI and PacI restriction sites in pcDNA3.1-3XHA plasmid (gift of S. Artandi). The pcDNA3.1-3XHA-p53 and pcDNA3.1-3XHA-GFP constructs have been described (Brady et al., 2011). The pcDNA3.1-3XHA-Zmat3 plasmid was used as a template for site-directed mutagenesis to generate pcDNA3.1-3XHA-Zmat3R99Q. KOD Xtreme Hot Start DNA Polymerase (71975-M, Sigma-Aldrich) was used to amplify the template with a pair of primers (forward primer 5'-catggcaagaaactacaaaattattacgcagct-3';

and reverse primer: 5'-agctgcgtaataattttgtagtttcttgccatg -3'), and the mutation was verified by Sanger sequencing. *p53* null MEFs and H23 cells were transfected with the constructs using Lipofectamine 2000 (Invitrogen), according to manufacturer instructions. Twenty-four hours later, cells were pulsed with 3 μg/ml BrdU for 4 hours, fixed in 4% paraformaldehyde for 15 minutes, washed in PBS, permeabilized in PBS + 0.25 % Triton X-100 for 15 minutes, washed in PBS, incubated with anti-HA (1:400, Cell Signaling Technologies) antibody overnight at 4°C, washed in PBS, and subsequently incubated with anti-rabbit fluorescein-labeled secondary antibody (1:200, Vector Laboratories) for 1 hour. The cells were then post fixed with 4% paraformaldehyde, washed and treated with hydrochloric acid (1.5 N) for DNA denaturation. Next, the cells were washed in PBS, incubated with anti-BrdU antibody (1:50, BD Pharmingen), washed in PBS, and incubated with anti-mouse Alexafluor 546-labeled secondary antibody (1:200, Invitrogen). Images were taken using a Leica DM6000B microscope (Leica Microsystems).

Human cancer data analysis

Expression of *ZMAT3* was evaluated in human tumors with wild-type or mutant *TP53* using TCGA data available via the Genomic Data Portal (gdc.cancer.gov) and the METABRIC breast cancer dataset (Curtis et al., 2012; Pereira et al., 2016) (www.ebi.ac.uk/ega/studies/EGAS00000000083). *ZMAT3* expression was adjusted by copy number status to control for the effect of amplifications caused by the proximity of *ZMAT3* to *PIK3CA*, a known driver of tumorigenesis, to ensure that we examined the effect of *ZMAT3* expression and not *PI3KCA* amplification. Samples were stratified based on *TP53* status. Confidence intervals were calculated at 95%. Clinical outcome analyses were

generated based on disease-specific survival for the METABRIC breast cancer dataset. A log rank test measuring the difference in survival between samples with high expression (1st tertile) and low expression (3rd tertile) of *ZMAT3* or *DENND2C* was performed. Additionally, a Cox Proportional Hazard model was built to evaluate the association between expression and outcome while adjusting for age, grade, size, lymph node number and ER and HER2 status in the breast cancer analysis and age, gender and stage in the LIHC and LUAD analysis. Mutual exclusivity between *TP53* and *ZMAT3* or *DENND2C* mutations was evaluated in uterine (UCEC) cancer using data from TCGA. The DISCOVER method, which robustly controls the false positive rate (Canisius et al., 2016), was used to test for mutual exclusivity, and the accompanying p-value is reported. For survival analysis, the packages "survival", "rms" and "survcomp" were used (Schroder et al., 2011). Oncoplots were generated with package "maftools" (Mayakonda, 2016).

ZMAT3 eCLIP and data analysis

ZMAT3 eCLIP was performed by Eclipse BioInnovations Inc (San Diego) with slight modifications to the published seCLIP protocol. Polyclonal populations of *E1A;Hras^{G12V};Cas9* MEFs expressing one of three different NC sgRNAs and one *E1A;Hras^{G12V} Zmat3*-knockout MEF sample (Janic et al., 2018) were used for the experiment. Briefly, 20 million cells were UV crosslinked (254nM, 400 mJ/cm^2) and lysed in 1 mL of 4˚C eCLIP lysis buffer (50 mM TrisHCl pH 7.4, 100 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1:200 Protease Inhibitor Cocktail III [EMD Millipore], 440 U Murine RNase Inhibitor (Vilborg et al.)). The extract was sonicated (Qsonica Q800R2, 15 cycles of 20s on/40s off), incubated with 40 U of RNase I (Ambion)

and 4 U Turbo DNase (ThermoFisher) for 5 min at 37˚C, and clarified by centrifugation (15k g, 15 min at 4˚C). Anti-ZMAT3 antibody (10504-1-AP, Proteintech) was pre-coupled to sheep anti-rabbit IgG Dynabeads (ThermoFisher) and added to clarified lysate followed by incubation overnight at 4˚C with rotation. 2% of lysate was removed as paired input, and the remainder was washed with eCLIP high- and low-salt wash buffers. Dephosphorylation (FastAP, ThermoFisher and T4 PNK, NEB) treatment and high efficiency ligation of InvRiL19 (/5Phos/rArGrArUrCrGrGrArArGrArGrCrArCrArCrGrUrC/3SpC3/) RNA adapter (T4 RNA Ligase I (NEB)) was performed as previously described. After one additional high salt buffer wash and two additional wash buffer washes, samples were denatured in 1X NuPAGE buffer with 0.1 M DTT. For chemiluminescent imaging, 10% of ZMAT3 IP and 1% of input were run on NuPAGE 4-12% Bis-Tris protein gels, transferred to PVDF membrane, probed with ZMAT3 antibody (1:1000, sc398712, Santa Cruz) and 1:8,000 EasyBlot anti Mouse IgG (HRP) (GeneTex), and imaged with C300 Imager using Azure Radiance ECL. For RNA extraction, 80% of ZMAT3 IP and 50% of input were run on NuPAGE 4-12% Bis-Tris protein gels, transferred to nitrocellulose membrane, and the region from 35 to 110 kDa (protein size to 75kDa above) was isolated from the membrane, finely fragmented, and treated with 20 µL Proteinase K (NEB) plus 130 µL PKS buffer (10 mM TrisHCl pH 7.4, 50 mM NaCl, 10 mM EDTA, 0.2% SDS). An additional 55 µL of water was added, and RNA was then purified by RNA Clean & Concentrator column cleanup (Zymo). Reverse transcription was performed with 120 U Superscript III (ThermoFisher) with InvAR17 primer (CAGACGTGTGCTCTTCCGA) at 55˚C for 20 min, followed by addition of 2.5 µL ExoSAP-IT and incubation at 37˚C for 15 min. After

addition of 1 µL 0.5M ETDA, RNA was removed by addition of 3 µL 1M NaOH and incubation at 70˚C for 10 min. 3 µL of 1M HCl was added to normalize pH, and RNA was purified with MyOne Silane beads (ThermoFisher). Ligation of InvRand3Tr3 adapter (/5Phos/NNNNNNNNNNAGATCGGAAGAGCGTCGTGT/3SpC3/) to the 5' end of cDNA was performed with T4 RNA Ligase plus the addition of 15 U 5' Deadenylase (NEB). After RNA purification with MyOne Silane beads (ThermoFisher), PCR amplification (Q5 Master Mix, NEB) was performed with standard Illumina multiplexing indexes, and samples were sequenced on the HiSeq4000 platform.

Analysis of eCLIP data was performed as previously described (Van Nostrand et al., 2016), using the UCSC GRCm38/mm10 genome build with GENCODE Release M20 (GRCh38.p6) transcript annotations. For each sample (sgNC1, sgNC2, sgNC3), ZMAT3-bound RNAs were identified by calculating enrichment of sequencing reads in the eCLIP sample relative to the reads in the input and the ZMAT3-deficient sample. Meta-exon maps were generated as previously described (Van Nostrand, 2019). Briefly, for each gene in Gencode v19, the transcript with the highest abundance in the three control *E1A;Hras*^{G12V};*Cas9* sgNC RNA-seq datasets was chosen as the representative transcript, and genes with representative transcript with TPM < 1 were discarded. Next, for all internal exons (excluding the first and last exons), the region from 500nt upstream to 500nt downstream (for introns less than 1000nt, the region was split, with half assigned to the upstream exon and half to the downstream exon) was queried for the presence of significant peaks. Finally, the number of peaks at each position was averaged over all events to obtain the final meta-exon value. To generate confidence intervals, bootstrapping was performed by randomly selecting (with replacement) the same number of transcripts and calculating

the average position-level peak coverage as above, with the 5th and 95th percentiles (out of 100 permutations) shown. Human comparison eCLIP meta-exon plots were obtained from published data (Van Nostrand, 2019).

RNA-seq

E1A;Hras^{G12V};Cas9 MEFs were transduced with lentiviruses expressing one of three unique *Zmat3* or one of three unique negative control sgRNAs, selected in puromycin for 3 days, and cultured an additional 10 days. RNA was extracted from cultured cells using RNeasy Mini kit (QIAGEN). RNA-seq libraries were prepared using the Illumina TruSeq Kit (v.2), according to the manufacturer's instructions. RNA-seq reads were aligned to the mouse genome (mm10) and analyzed using the public server Galaxy (usegalaxy.org,(Afgan et al., 2018)), which employs the STAR aligner (Dobin et al., 2013) and DESeq2 (Love et al., 2014) for differential expression analysis. Significantly differentially expressed transcripts were identified using an adjusted p-value cutoff of <0.05. Enrichr (https://maayanlab.cloud/Enrichr/) was used to identify enriched gene ontology (GO) terms (Kuleshov et al., 2016).

RNA-seq alternative splicing analysis

First, fastq files were processed using cutadapt (1.14.0) (Martin, 2011) to trim adapters and low quality sequences. Next, trimmed reads were first aligned using STAR (2.4.0i) (Dobin et al., 2013) against repeat elements (RepBase 18.05) with aligning reads removed from further analysis. Remaining non-repeat reads were then mapped against the mouse assembly (mm10) to generate corresponding bam files. Bigwigs were generated using an

in-house script (https://github.com/yeolab/makebigwigfiles) which uses bedtools (2.26.0) genomecov to generate normalized density tracks. For analysis of alternative splicing, triplicates of the *Zmat3* knockouts (*E1A;Hras^{G12V};Cas9* MEF lines transduced with lentiviruses expressing one of three unique Zmat3 sgRNAs) were compared against WT controls (*E1A;Hras^{G12V};Cas9* MEF lines transduced with lentiviruses expressing one of three unique NC sgRNAs) using rMATS (3.2.5) with Gencode (vM15) annotations. Featurecounts (1.5.3) (Liao et al., 2014)was used to count reads mapping to known transcripts using the same vM15 annotations. Differential alternative splicing events were defined by a <5% change in exon inclusion (PSI or percent-spliced-in >0.05) and a p-value <0.05.

Comparing ZMAT3 peaks & SMG1-regulated exons

To determine whether ZMAT3 binding is enriched at NMD switch exons, the frequency of ZMAT3 binding peaks was determined for a list of alternative splicing events in SMG1 wild-type and knockout MEFs that were predicted to introduce premature termination codons (PTC) (McIlwain et al., 2010) by identifying the percentage of exons that contain a ZMAT3 peak (sample WT2) anywhere in the region starting at the upstream exon and ending at the downstream exon of the queried exon. This percentage was calculated for PTC-containing exons that are ≥5% differentially spliced in the direction that would include the PTC in SMG1 wild-type versus knockout MEFs (SMG1-regulated PTC exon). As one control, the set of PTC-containing exons that are <1% differentially spliced was similarly compared against ZMAT3 peaks. As another control, sets of 232 internal exons with similar expression levels to the SMG1 regulated exons were randomly selected 10,000

times and compared against ZMAT3 peaks. Significance was calculated by identifying the number of times this random selection identified an equal or greater number of events with overlapping ZMAT3 peaks.

Analysis of DepMap samples

To evaluate genotype-specific dependencies of human cell lines, we categorized all cell lines (including breast, lung, liver, and colon cancer lines) in the Achilles DepMap dataset (CCLE_Depmap_18q3 release) into two categories: *TP53* aberrant or *TP53* wild-type. Cell lines were considered "*TP53* aberrant" if they fulfilled at least one of the following criteria: (1) mRNA expression of *TP53* was at least one standard deviation below the mean of all cell lines tested; AND/OR (2) cell lines were previously annotated as *TP53*-mutant ("is Deleterious" criteria could be either TRUE or FALSE) (n= 350 *TP53*-aberrant cell lines). Cell lines were considered "*TP53* wild-type" if they fulfilled both of these criteria: (1) mRNA expression of *TP53* was greater than one standard deviation above the mean of all cell lines tested; AND (2) cell lines were not previously annotated as having a mutation in *TP53* (n=135 *TP53* wild-type cell lines). We then calculated the mean dependency scores for each gene in the genome for all *TP53* aberrant lines and all *TP53* wild-type lines. We performed two-tailed t-tests evaluating the differences in mean dependency scores for each gene and then adjusted the P-values with the Benjamini-Hochberg procedure for multiple comparison testing (generating FDR values). Genes with FDR values < 0.1 were considered significant.

QUANTIFICATION AND STATISTCAL ANALYSIS

A hypergeometric test (i.e. sampling without replacement) was used to calculate significance in the shRNA screen. A Mann-Whitney U test was used to calculate significance in the sgRNA screen. A log-rank test and Cox Proportional Hazard model were used to calculate significance in the survival analyses. The DISCOVER method was used to test for mutual exclusivity. The Benjamini-Hochberg procedure for multiple comparison testing was used to calculate significance in the analysis of DepMap samples. The unpaired two-tailed Student's t-test was used for all the other statistical analyses. Error bars represent standard deviation or standard error of the mean (see figure legends). Significance was defined as a p value$\leq$0.05, unless otherwise stated. Details and significance values can be found in the figure legends.

Supplemental Tables

Supplemental Table 4: ZMAT3 binding peaks. (Related to Figure 6). ZMAT3 binding peaks from eCLIP data are reported for 3 samples (sgNC1, sgNC2, sgNC3) normalized to the *Zmat3* knockout sample and normalized to the input sample.

Supplemental Table 5: Differentially expressed genes in *Zmat3*-deficient versus negative control MEFs (Related to Figure 6). Gene list was generated by comparison of RNA-seq data from three *E1A;Hras^{G12V};Cas9* MEF lines transduced with lentiviruses expressing unique *Zmat3* sgRNAs and three *E1A;Hras^{G12V};Cas9* MEF lines transduced with lentiviruses expressing unique NC sgRNAs (FDR<0.05).

Supplemental Table 6: rMATs analysis of differential splicing events in *Zmat3*-deficient versus negative control MEFs (Related to Figure 6). Sample 1 is based on three *E1A;Hras^{G12V};Cas9* MEF lines transduced with lentiviruses expressing unique *Zmat3* sgRNAs and sample 2 is based on three *E1A;Hras^{G12V};Cas9* MEF lines transduced with lentiviruses expressing unique NC sgRNAs.

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