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We have recently been made aware of concerns regarding some of the data in Fig. 2A and B, and Fig. 3A. After discussion with the corresponding author, Kristi Neufeld, this matter has been referred to the authors’ institute. Journal of Cell Science is publishing this Note to make readers aware of the issue, and we will provide further information once it has been resolved.

This course of action follows the advice set out by COPE (Committee on Publication Ethics), of which Journal of Cell Science is a member.
Suppression of intestinal tumorigenesis in Apc mutant mice upon Musashi-1 deletion

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ABSTRACT
Therapeutic strategies based on a specific oncogenic target are better justified when elimination of that particular oncogene reduces tumorigenesis in a model organism. One such oncogene, Musashi-1 (Msi-1), regulates translation of target mRNAs and is implicated in promoting tumorigenesis in the colon and other tissues. Msi-1 targets include the tumor suppressor adenomatous polyposis coli (Apc), a Wnt pathway antagonist lost in ~80% of all colorectal cancers. Cell culture experiments have established that Msi-1 is a Wnt target, thus positioning Msi-1 and Apc as mutual antagonists in a mutually repressive feedback loop. Here, we report that intestines from mice lacking Msi-1 display aberrant Apc and Msi-1 mutually repressive feedback, reduced Wnt and Notch signaling, decreased proliferation, and changes in stem cell populations, features predicted to suppress tumorigenesis. Indeed, mice with germline Apc mutations (Apc^{Min}) or with the Apc_{1322T} truncation mutation have a dramatic reduction in intestinal polyp number when Msi-1 is deleted. Taken together, these results provide genetic evidence that Msi-1 contributes to intestinal tumorigenesis driven by Apc loss, and validate the pursuit of Msi-1 inhibitors as chemo-prevention agents to reduce tumor burden.

KEY WORDS: Musashi-1, Msi-1, Adenomatous polyposis coli, Apc, Intestinal tumorigenesis, Wnt, Notch

INTRODUCTION
Mutation of the tumor suppressor adenomatous polyposis coli (APC) is considered an initiating event in over 80% of all colon cancers (Vogelstein et al., 1989). APC is best characterized as a negative regulator of the canonical Wnt pathway. As such, APC acts as a scaffold for a complex that marks the transcription co-factor β-catenin for proteosomal degradation. Wnt signaling is important in the maintenance of the intestinal stem cell niche. Conditional loss of Apc in intestines of adult mice leads to an increased size of the progenitor population, and more proliferative and undifferentiated cells (Sansom et al., 2004). When Apc is conditionally deleted in intestines of adult mice, one of the most highly upregulated transcripts encodes the RNA-binding protein Musashi-1 (Msi-1) (Sansom et al., 2004). Intestinal polyps in mice with germline transcripts encodes the RNA-binding protein Musashi-1 (Msi-1) in intestines of adult mice, one of the most highly upregulated inhibitors as chemo-prevention agents to reduce tumor burden. tumorigenesis driven by Apc loss, and validate the pursuit of Msi-1

Msi-1 is an RNA-binding protein that was originally identified in Drosophila as an essential regulator of asymmetric cell division in sensory organ precursor cells (Nakamura et al., 1994). Msi-1 is also expressed in mouse neuronal stem cells and is used as a marker of stem and transit amplifying cells in the large and small intestines (Nishimura et al., 2003; Potten et al., 2003). Msi-1 binds to the 3' untranslated region (UTR) of target mRNAs and blocks translation by competing with translation initiation factor eIF4G for interaction with poly-A-binding protein (Kawahara et al., 2008). The first identified Msi-1 target mRNA was Numb, a negative regulator of Notch (Imai et al., 2001). Further research has revealed many potential Msi-1 targets. One study identified 64 Msi-1-bound mRNAs, many of which are associated with cell proliferation, differentiation, cell cycle and apoptosis (de Sousa Abreu et al., 2009). Cross-linking immunoprecipitation followed by high-throughput sequencing (CLIP-Seq) analysis of mouse intestinal epithelial cells revealed over 2200 potential Msi-1 targets (Li et al., 2015). Many human colorectal tumors display a significant increase in Msi-1 mRNA and protein compared to adjacent normal tissue (Sureban et al., 2008; Smith et al., 2015). Msi-1 overexpression can transform rat intestinal epithelial cells (Razza et al., 2010) and Msi-1 knockdown in human colon cancer cells leads to retardation of tumor growth in a xenograft model (Sureban et al., 2008). Similar opposing phenotypes for Msi-1 gain and loss of function were also seen for mammary and brain cells (Razza et al., 2010; Muto et al., 2012). Taken together, these data implicate Msi-1 in growth regulation of cell lines; however, until recently, little has been understood about the role of Msi-1 in intestinal tissue.

Our previous work revealed a mutually repressive feedback loop between Apc and Msi-1 in cultured cells (Spears and Neufeld, 2011). As a negative regulator in the Wnt signaling pathway, Apc inhibits Msi-1 transcription (Razza et al., 2010; Spears and Neufeld, 2011). Conversely, Msi-1 binds to Apc mRNA and blocks translation (Spears and Neufeld, 2011). Although in vitro evidence supports an integral role for the Apc and Msi-1 negative-feedback loop in gene regulation, the existence of this Apc and Msi-1 antagonistic relationship in intestinal tissue is unknown. Further, the consequences of disrupting this mutually repressive feedback loop in an intestinal tumor model have yet to be determined. Here, we investigate the role of Msi-1 in intestinal tumorigenesis by eliminating Msi-1 in a classic mouse model of intestinal cancer, Apc^{Min}, and also in a mouse model with an Apc mutation that closely resembles alterations in human colon cancer, Apc_{1322T} (Pollard et al., 2009). Our results provide evidence for an Apc and Msi-1 double negative feedback loop in the mouse intestine and support a key role for Msi-1 in intestinal tumorigenesis. Furthermore, our results reveal several consequences of Msi-1 loss that might contribute to intestinal polyp reduction in mice lacking Msi-1. Taken together, our findings support the pursuit of Msi-1 inhibitors as potential chemo-prevention or therapeutic agents.
RESULTS

*Msi-1*−/− mice show proliferation defects in the small intestine

To understand the role of Msi-1 in intestinal homeostasis, we investigated the intestinal phenotype in mice homozygous for an *Msi-1* deletion (Fig. S1). Because mice completely lacking Msi-1 have greatly reduced viability when in the C57BL/6 background (Sakakibara et al., 2002), experiments with the *Msi-1*−/− allele were performed in mice of the outbred stock CD-1, which show no such viability reduction. There was not a significant difference in total body, spleen and liver weight, or in colon length between wild-type mice and age-matched mice lacking Msi-1 (Fig. 1A–D). However, *Msi-1*−/− mice had small intestines that were significantly shorter than those of their wild-type counterparts (Fig. 1D). Consistent with the shorter small intestines observed in the *Msi-1*−/− mice, each of the three sections of the small intestine displayed a significant reduction in epithelial cell proliferation as assessed by determining the percentage of crypt cells that were positive for Ki-67 (Fig. 1E).

Small intestines from *Msi-1*−/− mice display reduced Wnt and Notch signaling

We focused our subsequent investigation on the small intestine, where tissue shortening and reduced proliferation in mice lacking Msi-1 was more dramatic compared to in the colon. Initially, we examined the Apc and Msi-1 mutually repressive feedback loop, previously identified and characterized in cultured cells (Spears and Neufeld, 2011) (Fig. S2). Consistent with our *in vitro* results and in support of a mutually repressive feedback loop, we observed higher levels of Apc protein in *Msi-1*−/− mice compared to wild-type mice (Fig. 2A). As would be expected to accompany higher levels of Apc, we also observed a decrease in β-catenin protein levels in *Msi-1*−/− mice (Fig. 2B). Furthermore, *Msi-1*−/− mice displayed lower levels of Wnt target genes *Myc*, *Axin2*, *Lgr5*, and cyclin D1 (*CcnD1*) mRNA than their wild-type counterparts (Fig. 2C–F). The exception to this was within the distal tissues where *Lgr5* and cyclin D1 mRNA levels were higher in *Msi-1*−/− mice than in wild-type mice.

The Notch signaling pathway is negatively regulated by another established Msi-1 target, Numb. Numb protein levels were higher in intestines from *Msi-1*−/− mice than from wild-type mice (Fig. 3A). Moreover, in intestines from *Msi-1*−/− mice, we saw a decrease in *Hes1* mRNA, a direct Notch target, and an increase in *Math1* (also known as *Atoh1*) mRNA, which is indirectly repressed by Notch signaling (Fig. 3B,C). Taken together, these results indicate that mice lacking Msi-1 show signs of reduced Wnt and Notch signaling.

*Msi-1*−/− mice show stem cell alterations

The intestine is maintained by two stem cell populations. Actively cycling crypt base columnar (CBC) stem cells are located at the crypt bottom and express *Lgr5* (Barker et al., 2007). Quiescent stem cells are located at a position ‘+4’ relative to the crypt base and express markers including Dclk-1 (Potten et al., 2003; Giannakis et al., 2006; Barker et al., 2007; Gagliardi et al., 2012). *Msi-1* is expressed within both stem cell populations and has been referenced as a stem cell marker (Kayahara et al., 2003; Potten et al., 2003; Munoz et al., 2012). We examined both active and quiescent stem cell populations for their response to loss of Msi-1.

We explored the quiescent ‘+4’ stem cell population using Dclk-1 as a marker (Fig. 4D). Only positive cells in the crypt were scored in order to distinguish quiescent stem cells from the Dclk-1-positive differentiated Tuft cells, which reside in or near the villus (May et al., 2008; Gerbe et al., 2009; Gagliardi et al., 2012). Unexpectedly, mice lacking Msi-1 showed higher numbers of Dclk-1-positive cells in all three sections of the small intestine when compared to wild-type mice (Fig. 4A).

As an initial analysis of the CBC stem cell population, we used an *ex vivo* system to culture isolated small intestinal crypts from the mouse. In this system, adapted from Sato et al. (2009), crypts are removed from surrounding mesenchymal tissue, separated from villi and grown in a three-dimensional (3D) matrix with exogenous components that enhance Wnt signaling (Ootani et al., 2009). Cultured crypts produce organoid structures, with differentiated secretory and absorptive cells present along the luminal surface. After 24 h in culture, the organoids will produce new crypt-like protrusions from stem cells (Sato et al., 2009). We used these protrusions as a marker of ‘stemness’.

To provide proof of concept that this *ex vivo* culture system could be used to assess stem cell population differences, we first compared cultured crypts isolated from an *Apc*1322T/+ mouse and a wild-type mouse. A previous study using the *Apc*1322T/+ mouse model showed that these mice have more *Lgr5*+ active stem cells than wild-type mice (Lewis et al., 2010). After culturing organoids for
5 days, those from Apc^{1322T/+} mice had significantly more protrusions than organoids from wild-type mice (see Fig. 4E, as an example, and Fig. 4B). Because Msi-1 overexpression was previously correlated with expansion of stem cell populations (Rezza et al., 2010), we expected that knockout of Msi-1 would result in organoids with fewer protrusions. Indeed, after 8 days of growth ex vivo, crypts from Msi-1-null mice had fewer protrusions per organoid than those from wild-type mice (Fig. 4C). By day nine, organoids were obscured from vision by debris in the cultures. Organoids from Msi-1-null mice that were re-plated after mechanical dispersion showed the same decrease in protrusions compared to passaged organoids from wild-type mice (data not shown).

Results gathered employing organoid culture protrusions as a marker of stemness were validated by identifying active stem cells in tissue using the marker Lgr5. Labeling Lgr5 mRNA in tissue sections allowed us to directly score the CBC stem cells (Fig. 4F). We observed that the majority of crypts in mice lacking Msi-1 had only one or two Lgr5-positive cells (Fig. 4G). In mice with wild-type Msi-1, there was a significant shift in this distribution toward crypts displaying four and even five Lgr5-positive cells. These data are consistent with the organoid culture data and indicate that mice lacking Msi-1 are compromised for Lgr5-positive active stem cells in the small intestine.

Msi-1 promotes polyps in Apc mutant mouse models
Our studies indicated that small intestines from Msi-1-null mice were defective in Wnt and Notch signaling and had decreased proliferation and altered stem cell numbers. These findings, combined with previous in vitro studies supporting a role for Msi-1...
Msi-1 in these mice led to an ~80% reduction in polyp number with an average of 2.4 polyps per mouse ($P<0.05$; Fig. 5B,C, Table 1). Moreover, over a quarter of the $Apc^{M1n}$ mice lacking Msi-1 had no polyps (Fig. 5C, incidence). Although $Apc^{M1n}$ mice are a well-utilized model of inherited intestinal tumor susceptibility, the truncated Apc protein produced in these mice is shorter than Apc truncations typically associated with human colorectal cancer (Lamlum et al., 1999). $Apc^{1322T}$ mice, on the other hand, carry a truncating $Apc$ mutation at codon 1322, within the ‘mutation cluster region’ as defined from human CRC data (Pollard et al., 2009). To further explore the requirement for Msi-1 in tumorigenesis and confirm that the tumor phenotype is not specific to the model, $Apc^{1322T}$ mice were bred with CD-1 mice for eight generations to produce mice that were predominantly CD-1 (Fig. 5D). We observed similar phenotypes in the $Apc^{1322T}$ and $Apc^{M1n}$ mice. CD-1 $Apc^{1322T}$ mice developed an average of 14 intestinal polyps by the age of 16 weeks. Msi-1-null $Apc^{1322T}$ mice displayed a significant reduction in polyp number, with an average of five polyps per mouse (Fig. 5E,F). Moreover, there was a significant decrease in polyp incidence in mice without Msi-1; nearly all $Apc^{1322T}$ mice with wild-type Msi-1 developed polyps and only 83% of $Apc^{1322T}$ mice lacking Msi-1 developed polyps (Fig. 5F). Taken together, these data demonstrate that Msi-1 loss decreases polyposis, supporting an oncogenic role for Msi-1.

Looking at polyp distribution, we found a significant decrease in polyp incidence in all three sections of small intestine of $Apc^{M1n}$ mice lacking Msi-1 (Fig. 5G). Polyp numbers within each small intestinal section were also significantly decreased in $Apc^{M1n}$ mice lacking Msi-1 (Fig. 5H). Polyps within the proximal small intestine and colon of the Msi-1-null $Apc^{M1n}$ mice were significantly smaller than in $Apc^{M1n}$ mice with wild-type Msi-1 (Fig. 5I). There were so few polyps in the medial and distal small intestines of the Msi-1-null $Apc^{M1n}$ mice that a statistical comparison of their size was not possible. Similar to the $Apc^{M1n}$ mice, $Apc^{1322T}$ mice lacking Msi-1 showed a decrease in polyp incidence in each of the intestinal sections, reaching significance in the proximal small intestine and colon (Fig. 5I). $Apc^{1322T}$ mice lacking Msi-1 also exhibited a significant decrease in polyp number in all sections of the small intestine and colon (Fig. 5K). Finally, we observed a significant reduction in polyp size in the colons of $Apc^{1322T}$ mice lacking Msi-1 (Fig. 5L). Taken together, this polyp analysis supports a role for Msi-1 in promoting $Apc$ mutation-driven tumorigenesis throughout the intestine.

### DISCUSSION

Msi-1 protein and mRNA levels were elevated in tissue from some colorectal tumors when compared to adjacent normal tissue (Sureban et al., 2008; Smith et al., 2015). Overexpression of Msi-1 in cultured normal intestinal epithelial cells resulted in tumor formation in nude mouse xenografts (Sureban et al., 2008). Conversely, knockdown of Msi-1 levels in human colon cancer cells slowed tumor growth in a similar xenograft model (Rezza et al., 2010). Taken together, these results have spurred interest in pursuing Msi-1 as a potential therapeutic target for colorectal and other cancers. To this end, we previously reported that treatment of colon cancer cells with a novel Msi-1 inhibitor slowed cell growth (Lan et al., 2015). Here, using two mouse models where intestinal tumors are initiated by different germline $Apc$ mutations, we provide proof of concept that Msi-1 loss results in a dramatic decrease in tumorigenesis (Fig. 5, Table 1). We report a significant decrease in tumor incidence, with 12–28% of the $Apc$ mutant Msi-1-null mice remaining tumor-free by the end of the 16 week study. Moreover,

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**Fig. 3.** Msi-1−/− mice display increased Notch activity in small intestinal epithelia. Proteins and RNAs were extracted from epithelial cells isolated from three regions of the small intestines of Msi-1+/+ and Msi-1−/− mice. (A) Protein samples from ten mice of each genotype were probed for the Notch inhibitor Numb (a representative blot is shown). The lower panel displays average band intensities normalized to the actin loading control and relative to Msi-1−/− mouse samples. (B,C) Total RNA was analyzed for levels of the downstream Notch target Hes1 and a transcript repressed by Math1 (C). $n$=16 per genotype. Graphs represent mean±s.e.m. *$P<0.05$ (two-tailed $t$-test).
the Msi-1-deficient mice that did display tumors displayed a ∼53–75% reduction in tumor number compared to their wild-type counterparts.

In the small intestines of both Apc models, we observed an uneven polyp distribution, with more polyps occurring in the proximal section and progressively fewer moving toward the distal portion. Regional differences were also observed in the levels of Wnt and Notch pathway component proteins and mRNA (Figs 2 and 3). These differences include higher than normal levels of cyclin D1 and Lgr5 RNA in the distal portions of Msi-1-null small intestines compared to normal, but lower levels in all other regions (Fig. 2E,F). Regional differences in gene expression patterns in the pig small intestine have been reported (Mach et al., 2014), and our data support the concept that different small intestine sections have distinct properties that might contribute to varying sensitivity to Msi-1 loss and tumorigenesis.

Our demonstration that overall Wnt and Notch signaling are diminished in mice lacking Msi-1 identifies these signaling pathways as potential contributors to enhanced tumorigenicity mediated by Msi-1. Based on results from studies performed using human colon cancer cell lines, we previously proposed a model describing Apc and Msi-1 in a mutually repressive feedback loop (Spears and Neufeld, 2011). As predicted by this model, small intestines from mice lacking Msi-1 displayed higher Apc levels, lower β-catenin levels and reduced levels of Wnt target gene transcripts compared to their wild-type littermates (Fig. 2). Small intestines from mice lacking Msi-1 also exhibited diminished cell proliferation, a reduced number of Lgr5-positive cells and fewer active stem cells when grown as organoids in 3D culture (Figs 1C and 4C,G, respectively). These changes in proliferation and stem cell number are consistent with the opposite phenotype reported for Msi-1 overexpressing mice (Cambuli et al., 2015; Li et al., 2015). Although the Msi-1 overexpressing mice did not display changes in Wnt target gene RNA levels, Apc mRNA co-purified with Msi-1 isolated from these mice (Li et al., 2015). The expected consequence of elevated Apc and reduced β-catenin levels, reduced Wnt target gene expression and proliferation, in mice lacking Msi-1 is a reduction in the opportunity for loss of heterozygosity at the Apc locus, which is typically required for tumor initiation. Moreover, the Notch signaling pathway is predicted to contribute to this phenotype by modulating proliferation and differentiation of cells near the crypt base...
The potential for Msi-1 to modulate both Notch and Wnt pathways suggests a key role for Msi-1 in crypt cell homeostasis. Continuous renewal of the epithelial cells lining the intestine depends on stem cells located near the crypt base (Stappenbeck et al., 1998). Two pools of stem cells exist: the actively cycling CBC stem cells and the quiescent ‘+4’ stem cells. The +4 stem cells are required to maintain homeostasis within the intestinal crypts (Sangiorgi and Capecchi, 2008). However, studies show that most of the characterized stem cell markers are expressed in a ‘stem zone’, and not exclusively in a single stem cell pool (Itzkovitz et al., 2012; Munoz et al., 2012). Of note, Msi-1 is reported to mark both stem cell populations in small intestine and colon (Kayahara et al., 2003; Potten et al., 2003; Munoz et al., 2012). Furthermore, Msi-1 is also expressed in a gradient within the two stem cell populations with the +4 cells having higher Msi-1 expression than the CBC cells.

(VanDussen et al., 2012). The potential for Msi-1 to modulate both Notch and Wnt pathways suggests a key role for Msi-1 in crypt cell homeostasis.

Continuous renewal of the epithelial cells lining the intestine depends on stem cells located near the crypt base (Stappenbeck et al., 1998). Two pools of stem cells exist: the actively cycling CBC stem cells and the quiescent ‘+4’ stem cells. The +4 stem cells are required to maintain homeostasis within the intestinal crypts (Sangiorgi and Capecchi, 2008). However, studies show that most of the characterized stem cell markers are expressed in a ‘stem zone’, and not exclusively in a single stem cell pool (Itzkovitz et al., 2012; Munoz et al., 2012). Of note, Msi-1 is reported to mark both stem cell populations in small intestine and colon (Kayahara et al., 2003; Potten et al., 2003; Munoz et al., 2012). Furthermore, Msi-1 is also expressed in a gradient within the two stem cell populations with the +4 cells having higher Msi-1 expression than the CBC cells.
Table 1. Polyp data from Apc mutant mice

<table>
<thead>
<tr>
<th>Polyp number</th>
<th>Penetration (%)</th>
<th>Polyp number</th>
<th>Penetration (%)</th>
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<tr>
<td></td>
<td>Apc&lt;sup&gt;1322T&lt;/sup&gt;</td>
<td></td>
<td>Apc&lt;sup&gt;Min&lt;/sup&gt;</td>
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<tr>
<td>Msi&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Msi&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Msi&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Msi&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<tr>
<td>Proximal</td>
<td>Msi&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Msi&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Msi&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.35±1.00</td>
<td>0.26±0.10</td>
<td>38.30</td>
<td>16.28</td>
</tr>
<tr>
<td>Medial</td>
<td>0.70±0.20</td>
<td>0.26±0.11</td>
<td>31.91</td>
</tr>
<tr>
<td>Distal</td>
<td>0.14±0.07</td>
<td>0.07±0.05</td>
<td>6.38</td>
</tr>
<tr>
<td>Colon</td>
<td>11.15±2.58</td>
<td>5.12±0.92</td>
<td>97.87</td>
</tr>
<tr>
<td>Total</td>
<td>14.36±3.29</td>
<td>5.70±1.04</td>
<td>97.87</td>
</tr>
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</table>

Results are shown as mean±s.e.m. for Apc<sup>1322T</sup> Msi<sup>−/−</sup> (n=25), Apc<sup>1322T</sup> Msi<sup>−/−</sup> (n=28), Apc<sup>Min</sup> Msi<sup>−/−</sup> (n=47), Apc<sup>Min</sup> Msi<sup>−/−</sup> (n=43) mice.

(Maria Cambuli et al., 2013). Here, we provide evidence that mice lacking Msi-1 display an increase in quiescent +4 cells and a decrease in active CBC cells (Fig. 4). This result is consistent with the increase in mRNA for the CBC marker Lgr5 and increased number of Lgr5-positive cells reported in mice that overexpress Msi-1 (Cambuli et al., 2015; Li et al., 2015). Taken together, these data indicate a role for Msi-1 in stem cell regulation. However, the opposing changes in the two stem cell populations point to a distinct role for Msi-1 within each population. Moreover, our finding of higher Lgr5 mRNA levels in the distal small intestine of Msi-1-knockout mice again raises the potential for varying roles of Msi-1, dependent on the intestinal region. There are reports that Msi-1 can inhibit or promote translation in a context-dependent manner (MacNicol et al., 2011; Takahashi et al., 2013). One potential mechanism for the differential role of Msi-1 in the two stem cell pools and the different intestinal regions is that features of the stem cell niche or intestinal region dictate whether Msi-1 blocks or promotes translation. In addition, the ability of Msi-1 to autoregulate potentially contributes to the disparate roles for Msi-1 in these different populations (Arumugam et al., 2012). Recently published mouse models (Maria Cambuli et al., 2013; Cambuli et al., 2015) should help clarify the role of Msi-1 in different cell populations.

Our result that Msi-1 elimination leads to a reduction in polyp number in two different Apc mutant mouse lines contrasts with those from a study published during the preparation of this manuscript that showed that elimination of Msi-1 in Apc<sup>Min</sup> mice does not result in decreased polyp formation (Li et al., 2015). Rather, a ~50% reduction in polyp number was observed only when both Msi-1 and Msi-2 were eliminated (Li et al., 2015). Several key differences in the study design and the mouse models analyzed likely underlie these contradictory results and might provide further clues regarding oncogenic Msi-1 functions. First, the present study utilized germline Msi-1-knockout mice, while the other study induced Msi-1 loss in 6-week-old mice. Intestinal tumors are thought to initiate in Apc<sup>Min</sup> mice within 2 weeks of birth (Shoemaker et al., 1995), well before the Msi-1 withdrawal at 6 weeks. Perhaps Msi-1 plays a key role in this initiation phase of tumorigenesis and at later stages, Msi-1 and Msi-2 function redundantly to support sustained tumor growth. Second, mice used for the present study lack Msi-1 in every cell, whereas in the previous study, Msi-1 was eliminated only in intestinal epithelial cells through use of a villin promoter-driven Cre recombinase. Possibly, Msi-1 tumor-promoting functions are cell not cell-autonomous. For example, Msi-1 might have a crucial tumor-promoting role in the stroma. Third, the genetic background of the mice is different, with outbred CD-1 mice used for the present study and mixed C57Bl/6:129 mice used in the other study. Mice with different genetic backgrounds are expected to contain different genetic modifiers that could alter the polyp phenotype and mask or enhance the contribution of Msi-1. Fourth, in the present study, mice were analyzed at 16 weeks of age at which time the Apc<sup>Min</sup> mice each had an average of 12 intestinal tumors (Fig. 5C). In the other study, mice were analyzed at 6 months of age at which time Apc<sup>Min</sup> mice each had, on average, 10 intestinal tumors. This difference in polyp number vs age nicely illustrates the genetic modifying effects of various mouse strains (Zeineldin and Neufeld, 2013). Finally, the age and diet of mice also differed in the two studies.

Here, we report that mice lacking Msi-1 display fewer tumors, and thus we provide evidence that Msi-1 plays an important role in intestinal tumorigenesis. This study provides proof of principle for the design and use of Msi-1 inhibitors as potential chemoprevention agents and also validates the use of Apc mutant mouse models to test Msi-1 inhibitors in tumor prevention. Finally, we provide evidence that Wnt and Notch signals are altered in Msi-1-knockout mice, suggesting that these pathways can be used as biomarkers when testing the effect of potential Msi-1 inhibitors in the small intestine.

**MATERIALS AND METHODS**

**Mouse husbandry**

Mice were maintained at the Animal Care Unit at the University of Kansas according to animal use statement number 137-02. The research complied with all relevant federal guidelines and institutional policies. Mice were maintained on a Harlan 2018 diet. The Msi-1<sup>−/−</sup> mice, ICR.129(B6)-Msi-1<sup>−/−</sup>/Apc<sup>Min</sup> (n=28), were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan (Sakakibara et al., 2002) (Fig. S1). Msi-1<sup>−/−</sup> mice were bred with CD-1 mice from Jackson Laboratories to maintain the colony. For mechanistic studies, Msi-1<sup>−/−</sup> mice were bred, and their progeny Msi-1<sup>−/−</sup>/−<sup>−</sup> and Msi-1<sup>−/−</sup>/−<sup>−</sup> littermates were killed at 12 weeks of age at which time their organs were harvested. Apc<sup>Min</sup> mice were purchased from Jackson Laboratories and bred with CD-1 mice for seven generations to produce mice that were genetically predominantly CD-1. Apc<sup>1322T</sup> mice, a generous gift from Ian Tomlinson (Wellcome Trust Centre for Human Genetics, Oxford University, UK), were bred with CD-1 mice for eight generations to produce mice that were predominantly CD-1. These Apc<sup>Min</sup> mutant mice in the CD-1 background were then bred with Msi-1<sup>−/−</sup> mice for two generations to compare Msi-1<sup>−/−</sup> and Msi-1<sup>−/+</sup> littermates, each with the mutant Apc<sup>alle</sup> allele (Fig. 5). Polyp analysis was performed on mice killed at 16 weeks of age by an individual that was blind to the genotype.

**Analysis of gross and microscopic pathology, polyp measurement**

The gross and cellular histology of intestinal tissues were examined in progeny from the bred N7 generation of mice (Apc<sup>Min</sup> Msi<sup>−/−</sup> and Apc<sup>Min</sup> Msi<sup>−/−</sup>) and in progeny from the bred N8 generation of mice (Apc<sup>1322T</sup> Msi<sup>−/−</sup> and Apc<sup>1322T</sup> Msi<sup>−/+</sup>) at 16 weeks of age. For each mouse, the gastrointestinal tract from the stomach to the anal canal was dissected, opened longitudinally and fixed in 10% buffered formalin. Using a dissecting microscope, an investigator blind to the genotype of the animal examined the intestinal luminal surface for polyps. Intestinal polyps were located and diameter was measured with the aid of a dissection microscope (MZ8; Leica, Richmond, IL) equipped with an eyepiece graticule and
Isolation of mouse intestinal epithelial cells

Intestinal epithelial cells were isolated according to a published protocol with minor modifications (Zeineldin and Neufeld, 2012). Briefly, immediately after killing the mice, their distal 3 cm of small intestine was removed, opened length-wise and rinsed with cold phosphate-buffered saline (PBS). Tissue was incubated in 0.04% sodium hypochlorite for 15 min on ice and then rinsed in cold PBS. The small intestine was then incubated on ice for 15 min in a 15 ml conical tube containing an EDTA with dithiothreitol (DTT) solution (1.5–3 mM EDTA and 0.5 mM DTT in PBS). After replacing the EDTA and DTT solution with cold PBS, tubes were shaken forcefully for 10 s to release the epithelial cells from the underlying tissue. The intestinal tissue was removed and placed in a fresh 15 ml conical tube containing the EDTA and DTT solution, and the process was repeated two additional times. The released epithelial cells were collected by centrifugation at 700 g for 5 min at room temperature. Pellets of epithelia from all three rounds of extraction were resuspended in PBS with protease inhibitors, combined and then split into two equal samples. The small intestinal epithelial cells were then pelleted and one tube was used for RNA extraction and the other for western blot analysis as described below.

Western blotting

Cells were washed twice with ice-cold PBS and then lysed in protein sample buffer (2% w/v sodium dodecyl sulfate, 10% glycerol, 50 mM Tris-HCl pH 6.8 and 0.06 mg/ml Bromophenol Blue). Proteins were separated using SDS-PAGE and 4–20% polyacrylamide gels and blotted onto nitrocellulose. Antibodies used for protein detection were against the following proteins: APC [1:3000, M2-APC (Wang et al., 2009)], Msi-1 [1:2000, 04-1041 Millipore, Boston, MA], Numb (1:1000, #2756 Cell Signal, Beverly, MA), β-catenin (1:1000, 610153 BD Biosciences, East Rutherford, NJ) and actin (1:5000, A2228 Sigma). Images acquired with Odyssey IR imager (Li-Cor, Lincoln, NE) were analyzed with Image Studio v5.0 (Li-Cor, Lincoln, NE). Results shown are representative images from 10 different mice from each genotype with all results used to calculate the average band intensity.

Real-time PCR

RNA isolated utilizing Trizol (Invitrogen) according to the manufacturer’s instructions was analyzed using quantitative real-time PCR (qRT-PCR). First-strand cDNA was prepared from 0.5 µg RNA using 200 units of M-MuLV reverse transcriptase and Random 6 primer (New England Biolabs, Ipswich, NE). Results shown are representative images from 10 different mice from each genotype with all results used to calculate the average band intensity.

Immunofluorescence staining

Immuno-fluorescence staining of formalin-fixed paraffin tissue sections was performed as described previously (Zeineldin et al., 2012). Briefly, samples were incubated with blocking buffer (5% normal goat serum, 1% bovine serum albumin, 1% cold fish skin gelatin and 0.1% Triton X-100) for 1 h at room temperature. Samples were then incubated with primary antibodies diluted in the blocking buffer at 4°C overnight, followed by three washes with PBS for 5 min each. Samples were incubated with secondary antibodies diluted in blocking buffer for 1 h followed by three washes in PBS. Coverslips were mounted on slides with Prolong Antifade with DAPI (Invitrogen). Antibodies utilized include: anti-Ki-67 (1:400, D3B5, Cell Signaling) and anti-DCLK-1 (1:100, ab31704, Abcam, Cambridge, MA) antibodies, and goat anti-rabbit-IgG conjugated to Alexa Fluor 568 (1:1000, Molecular Probes, Grand Island, NY). Negative controls included incubation with secondary antibody alone. These negative controls resulted in only minimal signal. Tissues were visualized using a PlanNeofluor 40×1.3 NA oil objective on a Carl Zeiss Axiocvert Microscope 135 (Jena, Germany).

Organoid culture

The small intestines from 5-week-old mice of each genotype were opened longitudinally, washed 3× with cold HBSS (Ca2+ and Mg2+ free) and cut into ~1 cm pieces. The pieces were placed into 30 mM EDTA in PBS and allowed to incubate on ice for 20 min. EDTA solution was removed and tissue was washed with 50 ml of cold HBSS. After the HBSS wash, 25 ml of cold HBSS was added to the tissue. Intestinal pieces were shaken for 5 min at 3 shakers/second to dissociate crypts and villi. Solutions containing crypts and villi were poured through 70 μm filters to remove villi. The solution was then centrifuged at 250 g for 5 min to pellet crypts and the supernatant was removed. Crypt pellets were resuspended in 25 ml of basal medium [advanced DMEM-F12 (Gibco cat. no. 12634-010), 200 mM L-glutamine and 10 mM Hepes pH 7.5]. Crypt solutions were centrifuged at 200 g and most of the supernatant was removed, leaving 2 ml in which the crypt pellet was resuspended. Using widebore pipettes, 40 µl of crypt solution was added to 40 µl of basal medium in 5% BSA-coated microfuge tubes (crypts diluted 1:2). 80 µl of Matrigel (BD Matrigel basement membrane matrix growth factor reduced, cat. no. 354230) was added to the crypt solution (crypts: Matrigel=1:1). 40 µl of the crypt and Matrigel solution was plated in 24-well plates and incubated for 20 min at 37°C with 5% CO2 to allow Matrigel to solidify. Once solidified, 400 µl of overlay was added to each well and incubated at 37°C with 5% CO2 for the duration of the experiment. Overlay included: 100 ng/ml Noggin (Peprotech cat. no. 250-38), 10 ng/ml EGF (Peprotech cat. no. PMG8044), 500 ng/ml R-spondin1 (Sino Biological cat. no. 50316-M08H) and penicillin-streptomycin (Gibco cat. no. 15140-148) in basal medium. Organoids and protrusions were scored beginning 1 day (24–34 h) after plating using a dissecting scope to count the number of protrusions from each organoid. For Msi-1−/− and Msi-1+/− cultures, all organoids in all wells were scored for protrusions – 10–662 organoids in Msi-1−/− cultures and 78–495 in Msi-1+/− cultures. Significance was calculated from cultures prepared from one mouse per genotype using a Student’s t-test.

In situ hybridization

Lgr5 mRNA transcripts were detected on paraffin-embedded sections from wild-type and Msi-1-knockout mice using the RNAscope 2.5 Assay according to the manufacturer’s instructions (cat. ACD-322350, Advanced Cell Diagnostics, Hayward, CA) and with probes for mouse Lgr5 (cat. no. ACD-312171), mouse PP1B (positive control, cat. no. ACD-313902) and DapB (negative control, cat. no. ACD-310043). After the Fast Red reaction, the slides were counterstained using hematoxylin and permanently mounted using Pertex mounting medium. At least 20 crypts with at least one Lgr5-positive cell were scored for each mouse. Significance was calculated using a Student’s t-test and results from at least eight mice of each genotype.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceived and designed the experiments: A.R.W., A.E., K.L.N. Performed the experiments: A.R.W., A.E., C.L., K.C., N.B., W.M. Analyzed the data: A.R.W., A.E., K.L.N. Prepared the article: A.R.W., K.L.N.

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