Musashi Signaling in Stem Cells and Cancer

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Abstract

How a single cell gives rise to an entire organism is one of biology's greatest mysteries. Within this process, stem cells play a key role by serving as seed cells capable of both self-renewal to sustain themselves as well as differentiation to generate the full diversity of mature cells and functional tissues. Understanding how this balance between self-renewal and differentiation is achieved is crucial to defining not only the underpinnings of normal development but also how its subversion can lead to cancer. Musashi, a family of RNA binding proteins discovered originally in *Drosophila* and named after the iconic samurai, Miyamoto Musashi, has emerged as a key signal that confers and protects the stem cell state across organisms. Here we explore the role of this signal in stem cells and how its reactivation can be a critical element in oncogenesis. Relative to long-established developmental signals such as Wnt, Hedgehog, and Notch, our understanding of Musashi remains in its infancy; yet all evidence suggests that Musashi will emerge as an equally powerful paradigm for regulating development and cancer and may be destined to have a great impact on biology and medicine.

Contents

PROLOGUE

Miyamoto Musashi was one of Japan's greatest samurais (Yoshikawa 1981). Known for his exceptional talent as a warrior, Musashi developed a unique way of fighting with two swords: a long sword, or katana, and a short sword, or wakizashi. The two-sword technique used by Musashi was termed Niten Ichi Ryu (two heavens as one) or Nito Ryu (the school of the two swords). Musashi combined his keen interest in combat with an interest in philosophy and Zen Buddhism, going on to write several books on strategy that have had an enduring appeal for both the Eastern and Western worlds.

Although iconic in Japanese history and culture, Musashi's name and unique story became part of the scientific lexicon rather serendipitously. In the early 1990s, a group of scientists led by noted neurobiologist Craig Montell discovered a new gene, which, when mutated, led to a cell fate determination defect in sensory organ precursor cells. In *Drosophila*, the sensory organ precursor cells give rise to the ectodermal system and can generate both neural and non-neural cell lineages. Normally these cells give rise to a IIa non-neuronal precursor cell, which goes on to make the socket and shaft of a bristle, and a IIb neuronal precursor cell, which goes on to make a neuron and glia. However, in this mutant, two identical non-neural precursor cells were generated, which frequently differentiated into two shaft-forming cells resulting in a double-bristle phenotype. Because the two-bristle phenotype was reminiscent of the great samurai's two swords, the gene was named *Musashi* (Nakamura et al. 1994).

MUSASHI IN STEM CELLS

Since its original discovery, it has become clear that Musashi is a critical element in controlling stem and progenitor cell function across organisms. In invertebrates, Musashi is best understood in the model organism *Drosophila*. In addition to its previously discussed role in sensory organ precursor cells, Musashi is also expressed in proliferating larval neuroblasts. Musashi overexpression triggers increased proliferation of these undifferentiated neural cells (Okano et al. 2005).

Reflecting a conserved role in stem cells of distinct tissues, Musashi loss can disrupt the normal balance between self-renewal and differentiation in *Drosophila* germline stem cells, leading to a bias toward differentiation. It was initially observed that in Musashi mutants, germline stem cells were not present adjacent to hub cells and were seemingly replaced by differentiated somatic cells. Although Musashi is normally expressed in both somatic and germ cells, its expression is intrinsically required to maintain germline stem cells. Therefore, Musashi mutant germline stem cells are unable to self-renew and differentiate prematurely (Siddall et al. 2006).

Musashi has also been implicated in stem cell function in more primitive invertebrates such as planarians, nematodes, and ascidians (Higuchi et al. 2008). Interestingly, the structure and expression pattern of Musashi is largely conserved among these highly varied species (Okano et al. 2005). These data identify Musashi as a key component in invertebrate stem cell biology and highlight its role as an ancient signal in development.

Musashi in Neural Stem Cells

In vertebrates, the Musashi gene underwent a duplication event in teleosts, giving rise to two homologs: Musashi1 (*Msi1*) and Musashi2 (*Msi2*). Both proteins play a necessary role in stem cell self-renewal and maintenance across a variety of different biological systems. Musashi's role in neural development has been primarily elucidated by Hideyuki Okano and colleagues, and appears to remain largely conserved across vertebrates and invertebrates: In zebrafish, *msi1* is strongly expressed in developing neural tissue, and its morpholino-mediated knockdown results in defects in central nervous system (CNS) formation (Shibata et al. 2012). Similarly in the mouse, *Msi1* is highly expressed in neural stem cells within the CNS in both the fetal and adult brain. The work in the nervous system revealed the specificity of Musashi expression in immature cells: Whereas *Msi1* transcripts were present in stem cells that have the potential to yield neurons and glia, its expression could not be detected in fully differentiated neuronal and glial cells (Sakakibara et al. 1996).

The lack of a strong neural stem cell phenotype in *Msi1*-deficient mice suggested that *Msi1* and *Msi2* may in part have functionally redundant roles. *Msi2* is in fact highly expressed within neural stem cells and is largely co-expressed with *Msi1*. Although *Msi1* deletion alone decreased the multipotency of neural stem cells, it did not disrupt the self-renewal ability of neural stem cells as assessed by neurosphere assays in vitro. However, the knockdown of *Msi2* in $Msi1^{-/-}$ neural stem cells significantly decreased neurosphere formation, indicating that the two proteins play compensatory roles at least to some extent (Sakakibara et al. 2002). *Msi1* and *Msi2* have independent roles in the nervous system as well. *Msi1* loss alone led to the development of hydrocephaly in a majority of *Msi1*-deficient mice (Sakakibara et al. 2002); similarly, *Msi2*, but not *Msi1*, is expressed within parvalbumin-containing GABA neurons in the neocortex and neurons in the basal ganglia (Sakakibara et al. 2001), suggestive of an *Msi2*-specific function within this lineage. Overlapping and distinct roles of *Msi1* and *Msi2* will likely be a pattern in the context of diverse tissues, and understanding the molecular and cellular basis of this specificity is essential to fully delineating how Musashi signaling influences stem cells.

Although we discuss the mechanisms of Musashi function in greater detail below, it is worth noting that Musashi's central mode of gene regulation, that of modulating translation through its RNA binding domain, was originally identified in the nervous system and has been most extensively studied in neural stem cells. This led to the identification of NUMB as a quintessential target of MSI1: MSI1 was shown to directly target the 3′ untranslated region (UTR) of *Numb* and downregulate its expression, triggering a rise in Notch signaling (Imai et al. 2001). Subsequent work indicated that MSI1 can also influence microRNA (miRNA) biogenesis during early neural

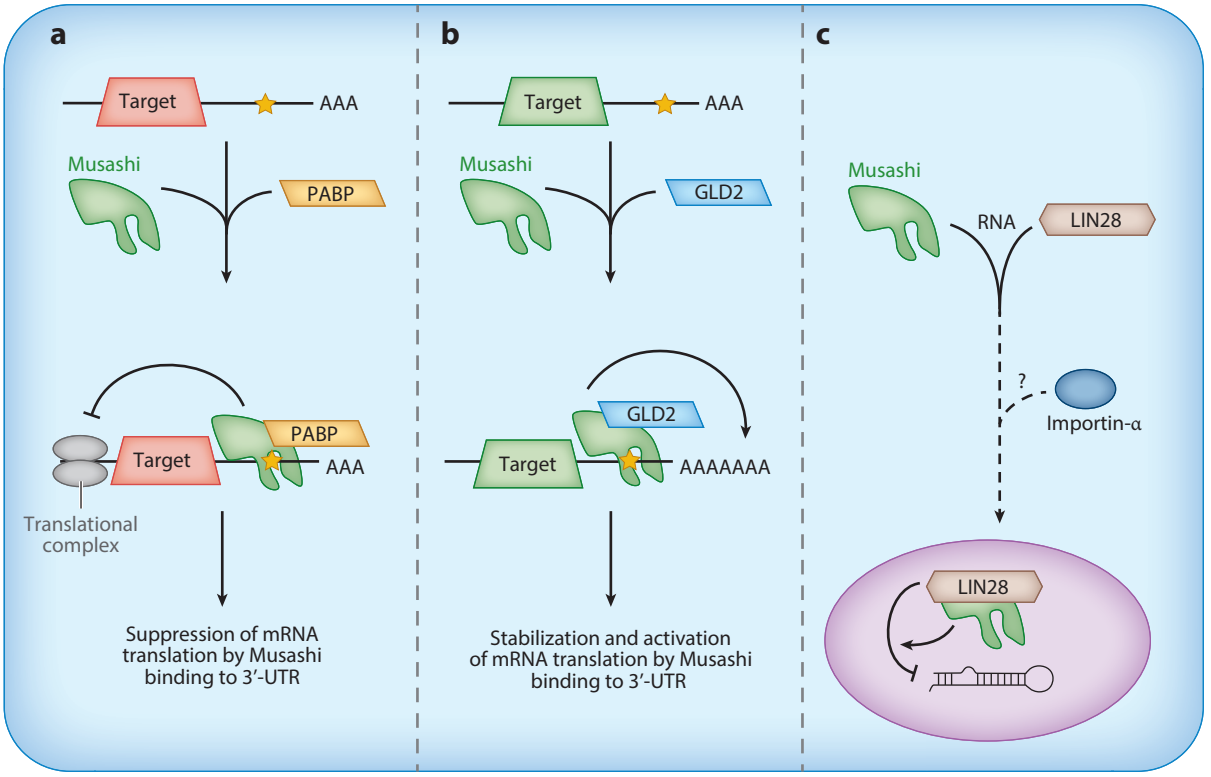

Figure 1

Potential mechanisms of action for the Musashi family of proteins. (*a*) Repression of translation. When Musashi binds to consensus sequences in the 3′ untranslated region (UTR) of a target messenger RNA (mRNA) (*star*), it can also interact with poly-A binding protein (PABP) via a separate domain in its C-terminal half. This interaction competes with eIF4G (not shown) for PABP, resulting in blockade of ribosome assembly and translation. (*b*) Activation of translation. When Musashi is bound to the poly(A) polymerase GLD2, GLD2-mediated polyadenylation stabilizes mRNA targets, resulting in increased translation. (*c*) Enhancement of LIN28 function. Musashi1 associates with LIN28 in an RNA-dependent fashion in the cytoplasm. Some evidence suggests that Musashi1 can influence nuclear import of LIN28, potentially via interaction with Importin-α. In the nucleus (*purple circle*), Musashi1 binds to LIN28 in an RNA-independent manner and may enhance the inhibition of microRNA processing.

differentiation (Kawahara et al. 2011) (**Figure 1**). Future work will undoubtedly reveal new mechanisms by which Musashi can influence cell function.

Musashi in Hematopoietic Stem Cells

The hematopoietic or blood-forming system is the organ in which the concept of stem cells was first proposed and these cells subsequently prospectively isolated; it remains in many ways a gold standard for understanding stem cell biology. Musashi signaling was linked to the maintenance of hematopoietic stem cell self-renewal independently through several related studies. In contrast to the nervous system, the hematopoietic system appears to predominantly express *Msi2* and not *Msi1*, suggesting that each family member is regulated in a context-specific manner. *Msi2* expression is highest in hematopoietic stem cells and decreases with differentiation (Hope et al. 2010, Ito et al. 2010, Kharas et al. 2010). The first genetic loss-of-function model of *Msi2* was developed to determine the role of *Msi2* in normal hematopoiesis and hematologic malignancies (Ito et al. 2010),

in part because Musashi can repress Numb, a potentially critical event in modulating asymmetric division and preserving the undifferentiated state in hematopoietic stem cells (Wu et al. 2007). This work revealed that disruption of *Msi2* expression via gene trap results in a marked decrease in the frequency of hematopoietic stem and progenitor cells (Ito et al. 2010). The observed impact of *Msi2* deletion on the stem cell pool is consistent with functional defects in the reconstitution ability of hematopoietic stem cells that occur with small hairpin RNA (shRNA)–mediated knockdown of *Msi2* (Hope et al. 2010) as well as in an independent *Msi2* gene trap mouse (de Andrés-Aguayo et al. 2011). Conversely, ectopic overexpression of *Msi2* in a transgenic model leads to an increased frequency of stem and progenitor cells (Kharas et al. 2010). *Msi2*'s influence on hematopoietic stem cells arises in part through an impact on Numb (Ito et al. 2010), implicating changes in asymmetric division (Wu et al. 2007), whereas a conditional knockout of *Msi2* in the hematopoietic system leads to an increase in commitment divisions (Park et al. 2014).

Musashi as a General Regulator of Germline, Somatic, and Embryonic Stem Cells

Although the roles of the Musashi proteins in vertebrate neural stem cells and hematopoietic stem cells have been extensively studied, the expression of these proteins in stem cells of diverse tissues suggests a much broader role. As in *Drosophila*, *Msi1* plays a critical role in development and spermatogenesis in murine germ stem cells. *Msi1* is primarily expressed within mitotic gonocytes and spermatogonia, and transgenic mice overexpressing *Msi1* display abnormal spermatogenesis. *Msi2* is also involved in spermatogenesis and expressed within the stem cell population. Whereas *Msi1* is expressed within mitotic gonocytes and spermatogonia, *Msi2* is expressed in meiotic spermatocytes and differentiating spermatids. Moreover, transgenic mice that overexpress *Msi2* are sterile (Sutherland et al. 2014). In many tissues the function of Musashi remains incompletely understood; this includes the intestine (Samuel et al. 2008, Sutherland et al. 2013, Yuqi et al. 2008), the liver (Hattori et al. 2010, Shu et al. 2002), and breast epithelial stem cells (Clarke et al. 2005).

Besides its role in germline and somatic stem cells, *Msi2* may also influence embryonic stem cells. *Msi2* mRNA and protein are expressed in mouse embryonic stem cells, and its knockdown leads to accelerated differentiation and decreased self-renewal (Wuebben et al. 2012). This could also imply a requirement during development in vivo; perhaps reflecting such a role, *Msi2* null mice are born at reduced frequencies (Ito et al. 2010). However, a clear analysis of *Msi2*'s role in development is lacking.

MUSASHI IN CANCER

Musashi in Hematologic Malignancies

During development, a finely controlled differentiation program allows stem cells to commit and diversify. However, these well-controlled events can inadvertently become part of an aggressive oncogenic process. In fact, accumulating mutations often target stem/progenitor cells and can systematically disable differentiation programs put in place during development, recreating a stem cell state. Aberrant adoption of the stem cell state can prove harmful in two ways. For many cancers, the differentiated stage represents hyperproliferative benign disease that can be controlled. However, with new genetic and epigenetic changes, tumors progressively revert to an undifferentiated stem cell state that can be extremely difficult to control; for example, astrocytoma transitions to glioblastoma multiforme, polyps to colorectal adenocarcinoma, and chronic

myelogenous leukemia (CML) to blast crisis. In addition, continued propagation of a malignant state is dependent on a subpopulation of cells that preserves self-renewal and the ability to give rise to heterogeneous, cancerous tissue. These cells, often termed cancer stem cells, can be considered the driver cells of a tumor and can also serve as the seed cells for metastasis (Reya et al. 2001).

A possible link to cancer perhaps began with observations of Musashi expression in gliomas (Kanemura et al. 2001, Toda et al. 2001), and reports of translocations involving *Msi2* and *HoxA9* in patients with CML (Barbouti et al. 2003). However, the functional significance of these observations became clear only later, as several studies contributed to the definitive demonstration that Musashi is required for cancer development. Our group used mouse models together with patient samples (Ito et al. 2010) to analyze the role of Musashi in the context of hematologic malignancies. Using mouse models of leukemia, we showed that *Msi2* was upregulated tenfold as CML progressed to a more aggressive blast crisis state. Further, using a model in which the *Msi2* gene was disrupted by gene trap, we demonstrated that genetic loss of Musashi blocked the propagation of blast crisis CML and significantly improved survival. Importantly, we found that Musashi expression is also highly upregulated during human CML progression and may serve as an early indicator of poor prognosis. This provided key genetic evidence for the requirement of *Msi2* in cancer progression and was complemented with data showing that shRNA-mediated *Msi2* inhibition blocks propagation of acute myeloid leukemia (AML) cell lines (Kharas et al. 2010). The genetic requirement for *Msi2* in primary models of AML (Kwon et al. 2015, Park et al. 2015) and in patient samples (Kwon et al. 2015) was demonstrated more recently. Loss of *Msi2* has a profound effect on leukemic stem cell propagation, leading to changes in gene expression profiles such that they more closely resemble a differentiation program than a self-renewal program. Further, increased expression of *Msi2* results in increased gene expression of *HoxA9*, *cMyc*, and *Ikzf2*; these genes were shown to be direct targets of MSI2 through binding to mRNA and regulation of translation, thus maintaining the leukemic stem cells (Park et al. 2015). An independent study linked genetic loss of *Msi2* to defects in the propagation of multiple models of primary adult and pediatric AML (Kwon et al. 2015). This study also provided a comprehensive analysis of genetic programs that are commonly regulated by *Msi2* in both CML and AML, identifying critical sets of oncogenic signals downstream of *Msi2*. These included genes such as *Flt3*, *Sox4*, *Pdgfrβ*, and *Erbb3*. Whereas some of these genes, such as *Sox4* and *Flt3*, have a known role in leukemic growth, others remain unexplored, suggesting this study can be of value in defining novel regulators of both hematologic malignancies and other cancers. As an example of this, Tetraspanin 3, a little-studied member of the Tetraspanin family of four-pass transmembrane proteins, was identified as such a target of *Msi2* in AML. *Tspan3* knockout mice revealed a genetic requirement for this pathway in de novo AML propagation, chemokine responses, and microenvironmental interaction (Kwon et al. 2015). These studies collectively show that Musashi can promote progression in hematologic malignancies and that understanding the programs it controls can open new avenues of research in the maintenance of aggressive cancers (**Figure 2**).

Musashi Signaling in Solid Cancers

Musashi's expression in multiple solid cancer lines and primary tissues (Potten et al. 2003, Shu et al. 2002) suggests that the protein may have a broader role in both solid and liquid cancers. With the knowledge that *Msi1* was overexpressed in adenomatous polyposis coli ($Apc^{min/+}$) polyps, Sureban and colleagues (2008) carried out a functional test to determine if *MSI1* is critical for tumor growth. HCT116 colon cancer cell lines were xenografted into immunodeficient recipient mice, and short interfering RNA (siRNA) against *MSI1* was delivered following the formation of measureable tumors. In these cell line–derived tumors, *MSI1* inhibition resulted in a significant

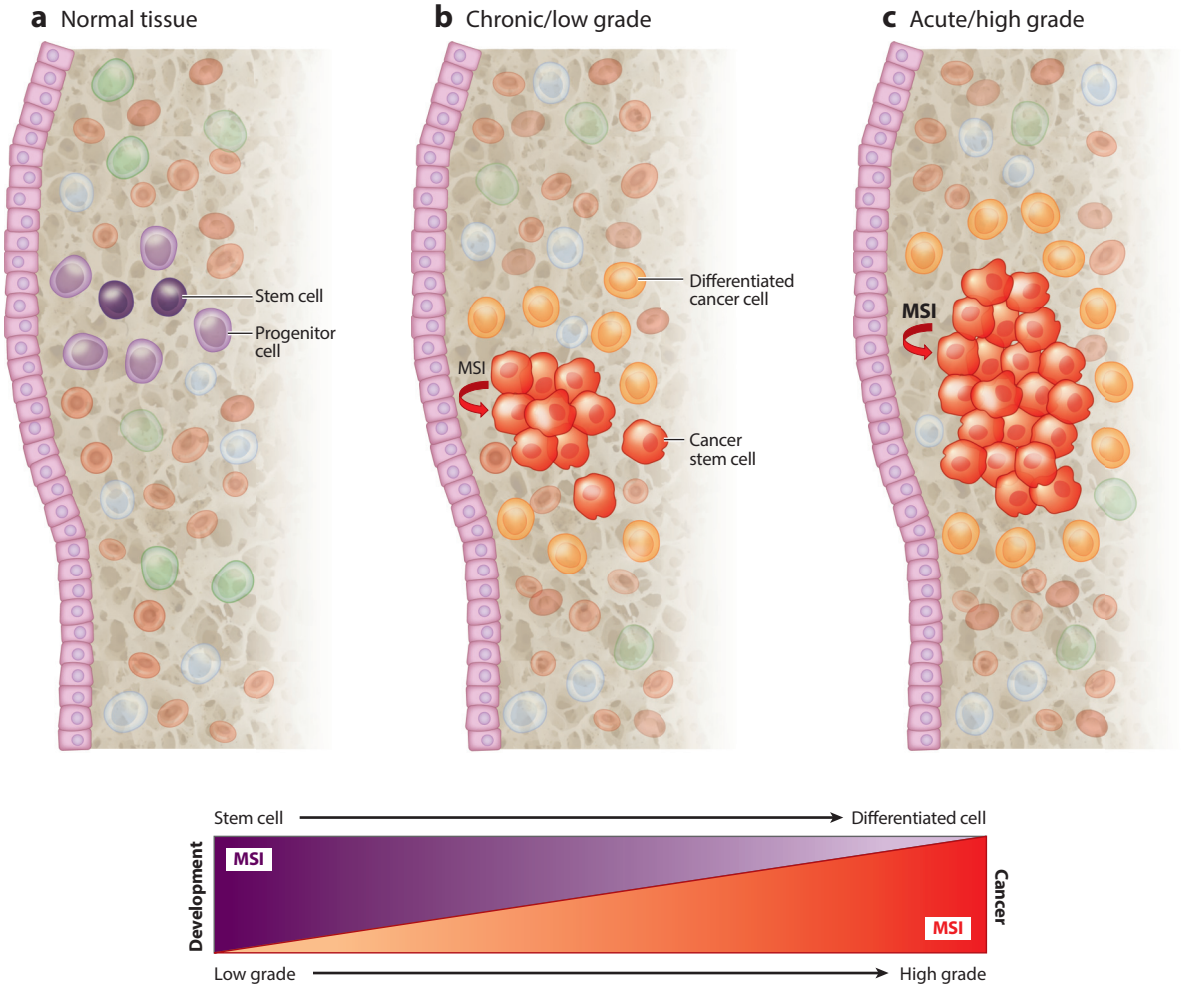

Figure 2

Musashi (MSI) mediates regulation of self-renewal in stem cells and cancer. (*a*) In normal development, Musashi is highly expressed in stem cells (*dark purple*). Musashi expression decreases in progenitor cells and differentiated cells (*lighter shades of purple*). (*b*) In the context of cancer, Musashi levels rise in response to oncogenic mutations, and Musashi expression remains elevated in cancer stem cells (*red cells*). Cancer often reflects a heterogeneous population of cells; thus, cancer stem cells can give rise to more cancer stem cells (representing the most immature fraction of the cancer), as well as more differentiated populations (*orange cells*). The balance of undifferentiated and differentiated cancer cells determines the grade and aggressiveness of disease: A shift toward differentiated cancer cells leads to less aggressive cancer. (*c*) A shift toward undifferentiated cancer stem cells (characterized by high levels of Musashi) leads to more aggressive cancer and a poorer prognosis.

slowing of tumor growth, an increase in apoptosis, and cell cycle arrest, similar to the requirement for *MSI2* in human colon cancer cell lines (Wang et al. 2015). Although the role of this signal in primary colon cancer remains to be determined, ectopic expression of *Msi2* in primary $Apc^{min/+}$ models of adenoma increased adenoma formation in vivo (Wang et al. 2015). Multiple other cancer cell lines depend on Musashi; these include the human medulloblastoma cell line Daoy (Sanchez-Diaz et al. 2008, Vo et al. 2012b) and several breast cancer (Wang et al. 2010) and lung cancer

(Rosenfeldt et al. 2013) lines. However, whether primary solid cancers depend on Musashi family genes, and the nature of this dependence, remains an important question for future investigations.

MECHANISM OF ACTION

It is clear from the discussion above that Musashi is a critically important gene, both during normal development and in oncogenesis. That it has been conserved through millions of years of evolution is a remarkable testament to its profound biological impact. How Musashi carries out its key functional role in diverse contexts has been and continues to be an active area of study.

Musashi Domain Structure and Function

The Musashi RNA-binding proteins form a subgroup within the heterogeneous nuclear ribonucleoprotein (hnRNP) A/B class of proteins (Sakakibara et al. 2001). The hnRNPs are generally composed of one or more RNA-binding modules and at least one other auxiliary domain that often mediates protein-protein interaction (Dreyfuss et al. 1993, Han et al. 2010). Like other hnRNPs of the A/B class, Musashi proteins have two RNA recognition motifs (RRMs) separated by a short linker region (Sakakibara et al. 2001). The Musashi RRMs, located in the N-terminal half of the protein, are highly conserved across diverse species. The mouse and human RRMs are essentially identical (Good et al. 1998), and those of species as divergent as *Caenorhabditis elegans* and human share 74–76% amino acid sequence identity (Yoda et al. 2000). Each Musashi RRM has the characteristic structure of RNP-type RNA-binding domains and a four-stranded, antiparallel β-sheet backed by two α-helices, with each RRM containing hallmark RNP1 octamer and RNP2 hexamer sequences (Miyanoiri et al. 2003, Nagata et al. 1999) (**Figure 3*a***).

Study of the domain structure of the mammalian Musashi proteins has shed light on the mechanisms by which these proteins regulate gene expression. Musashi RNA-binding affinity and specificity have been studied largely in relation to its interactions with its target Numb and appear to be largely determined by the more N-terminal RNA-binding domain, RRM1. Although the more C-terminal RRM2 can bind RNA weakly in isolation, its primary role is likely to enhance binding affinity in combination with RRM1: RNA binding increases ∼100-fold in the presence of RRM2 compared with RRM1 alone (Nagata et al. 1999, Zearfoss et al. 2014). In support of this idea, putative target sequences for each respective RRM are often found near one another in 3′-UTRs of known Musashi target transcripts. The weaker binding strength of RRM2 is likely a result of its less favorable surface electrostatic potential and less flexible β-sheet compared with RRM1 (Miyanoiri et al. 2003) (**Figure 3*b***).

RRM1 and RRM2 in mouse MSI1 bind the consensus sequences r(GUAG) and r(UAG), respectively (Ohyama et al. 2012). Moreover, a recent study using RNA Bind-n-Seq analysis found that human MSI1 also preferentially binds UAG-containing sequences (Katz et al. 2014). RRM1 has unique stacking interactions that confer binding specificity. These interactions involve aromatic residues (F96 and W29) that are conserved between the MSI1 and MSI2 RRM1s but are absent in other known hnRNP RRMs, including MSI RRM2 (**Figure 3*a***) (Ohyama et al. 2012). Accordingly, RRM2 does not appear to contribute to binding specificity. Although these studies have provided important insight into the binding specificity provided by the Musashi RRM domains, the short nature of these binding determinants suggests that protein-RNA affinity alone may be insufficient to determine Musashi target specificity.

Musashi proteins are usually found in the cytoplasm and are enriched in polysome fractions, consistent with their function in translational regulation (Sakakibara et al. 2001). By contrast, other hnRNPs are primarily localized to the nucleoplasm, and in certain contexts Musashi proteins have

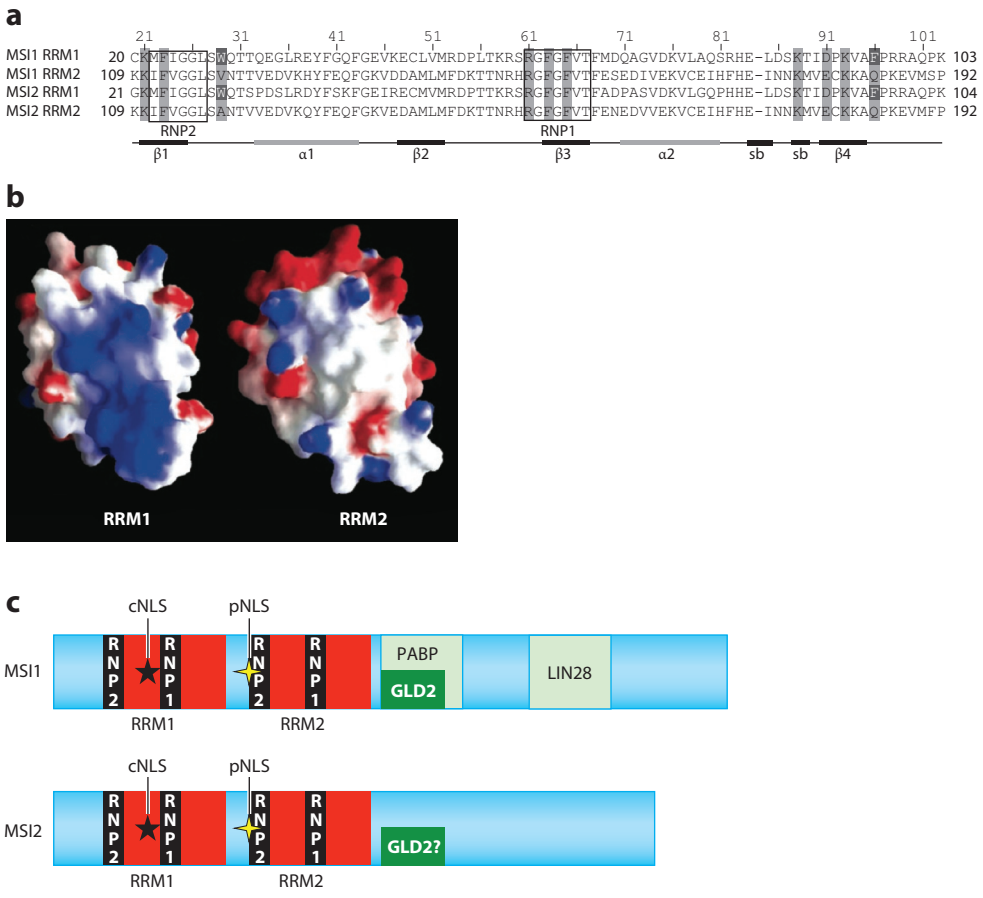

Figure 3

Schematic representation of known domains of the mammalian MSI1 and MSI2 proteins. (*a*) Amino acid sequence alignment of the RNA-binding domains, RNA recognition motifs (RRMs), of MSI1 and MSI2. Residues of MSI1 thought to interact with the r(GUAGU) target sequence are highlighted in gray. These residues are highly conserved between RRM1 and RRM2 within and between MSI1 and MSI2. In addition, residues that may confer RRM1 binding specificity are shown in white against a gray background (W29 and F96). RNP sequences are boxed, and secondary structure element positions are shown below (β, β-strand; α, α-helix; sb, short β-strands that form a β-turn). Panel *a* reproduced with permission from Ohyama et al. (2012). (*b*) Surface electrostatic potential of the MSI1 RRMs. The negative surface potential is shown in blue and the positive surface potential in red. The β-sheet surface that interacts with RNA has a negative potential in RRM1 (*left*) but a more neutral potential in RRM2 (*right*). In addition to differences in β-sheet flexibility, this may explain the large difference in RNA-binding affinity between the two RRMs. Panel *b* reproduced with permission from Miyanoiri et al. (2003). (*c*) The two highly conserved RRMs (*red*) in the N-terminal halves of the MSI proteins each contain RNP sequences (*black*). Within RRM1 is a classical nuclear localization signal (cNLS; *black star*), and at the C-terminal end of RRM2 is a peptide-like nuclear localization signal (pNLS; *yellow star*). In the C-terminal half of MSI1, a polyA-binding protein (PABP)-interaction domain is found adjacent to RRM2. More C-terminally, a LIN28-binding site is present in MSI1. Finally, a domain that binds GLD2 is present within the PABP domain of MSI1, and it may also be present in MSI2.

also been observed in the nucleus (Cuadrado et al. 2002, Kaneko et al. 2000, Nakamura et al. 1994, Nickerson et al. 2011). It is not clear how Musashi subcellular localization is determined in all contexts, but RNA-binding itself may play an important role. Putative nuclear localization signal (NLS) sequences are present in both RRMs of MSI1: a classical NLS in RRM1 and a peptide-like NLS in RRM2 (Kawahara et al. 2011) (**Figure 3*c***). These NLSs may regulate nuclear import of MSI1 via interaction with the importin-α protein. It has been postulated that when MSI1 binds mRNA in the cytoplasm, the NLSs are effectively blocked, owing to their location within the RRMs. This would explain why an RNA binding–deficient mutant form of MSI1 localizes preferentially to the nucleus (Kawahara et al. 2008).

The identification of auxiliary domains, outside of the RRMs, that mediate additional protein-protein interactions has provided important insights into the mechanisms by which Musashi regulates gene expression. The MSI1 and MSI2 RRMs show high sequence homology, with complete conservation of residues important for RNA-binding specificity. This makes it likely that the RRMs of MSI1 and MSI2 bind similar if not identical RNA target sequences (Ohyama et al. 2012, Sakakibara et al. 2001). By contrast, the C-terminal halves of the two mammalian Musashi proteins differ significantly in length and amino acid sequence, with only 56% identity. In fact, the C-terminal half of mouse MSI2 shows greater homology to the *Xenopus* Msi protein XRP1 than to mouse MSI1 (Sakakibara et al. 2001). Two key protein interaction domains in the C-terminal half of MSI1 have been identified, a polyA-binding protein (PABP) domain and a LIN28-binding domain (Kawahara et al. 2008, 2011) (**Figure 3*c***). These domains are not found in the MSI2 protein.

MSI1 binds PABP directly via a region in its C-terminal half within amino acids 190–234, and this interaction is essential for MSI1 to repress translation (Kawahara et al. 2008). MSI1 competes with eIF4G to bind PABP, blocking recruitment of the large (80S) ribosomal subunit onto mRNA and cap-dependent initiation of translation. Interestingly, in response to stress conditions, MSI1 colocalizes with PABP at stress granules, where stalled preinitiation complexes are known to accumulate. This accumulation is abolished when the RRM domains of MSI1 are mutated, providing another example of the importance of RNA interaction for appropriate Musashi intracellular localization.

MSI1 binds LIN28 in the nuclei of embryoid body cells via a 13-amino acid, C-terminal domain (Kawahara et al. 2011). Nuclear MSI1 may act sequentially and synergistically with LIN28 to block neural differentiation by inhibiting post-transcriptional biogenesis of the *Let-7* family miRNA *miR98* at the cropping step. MSI1 may also enhance LIN28 nuclear localization; knockdown of MSI1 results in a partial shift in localization from the nucleus to the cytoplasm. However, it remains unclear how nuclear import of MSI1 and LIN28 is regulated. Understanding how the domain structure, biochemistry, and signaling downstream of Musashi1 and Musashi2 are linked to their function in different contexts remains an important area for future work.

Regulation of Musashi Expression

Musashi gene expression is likely regulated at different levels and by different factors depending on the cellular and organismal context. Moreover, the expression of mammalian *Msi1* and *Msi2* may be regulated differentially to some extent. This is exemplified by the limited expression of *Msi1* in certain adult mouse tissues and developing CNS cells compared with the more widespread and persistent expression of *Msi2* in these contexts (Sakakibara et al. 1996, 2001). Interestingly, whereas *Drosophila* Musashi displays widespread expression throughout the testis, mouse MSI1 and MSI2 expression in the testis is complementary, suggesting Musashi function in spermatogenesis is divided between the two divergent mammalian paralogs (Siddall et al. 2006).

Musashi1 expression may be controlled by diverse inputs. Several signal transduction pathways may regulate Musashi1 expression. During rat brain development, the thyroid hormone T3 positively regulates *Msi1* mRNA expression (Cuadrado et al. 2002). Likewise, *Msi1* expression is induced in amphibian adult gastrointestinal precursor cells by thyroid hormone and adjacent connective tissue (Ishizuya-Oka et al. 2003). Another extracellular factor that may trigger Musashi1 signaling is TenascinC in the context of breast cancer (Oskarsson et al. 2011). At the epigenetic level, promoter methylation status may affect *Msi1* expression. Hypomethylation of regulatory CpG sites is significantly correlated with *Msi1* upregulation (Kagara et al. 2012). Finally, *Msi1* expression is likely to be regulated at a transcriptional level as well. In mouse neural cells, *Msi1* expression is controlled by the transcription factor Regulatory factor X (RFX) via a regulatory region in the sixth intron of the *Msi1* gene that is highly conserved in mammals (Kawase et al. 2011, 2014). The Wnt pathway may also regulate *Msi1* expression via a TCF/LEF-binding site upstream of the *Msi1* coding sequence (Rezza et al. 2010). In support of this hypothesis, *Msi1* mRNA is upregulated approximately fivefold in intestinal adenomas of *Apc* mutant mice. However, stimulation of the Wnt pathway alone may not be sufficient to drive *Msi1* expression, as addition of WNT3A to breast cancer oncospheres increases expression of several typical Wnt target genes but has no effect on *Msi1* levels (Oskarsson et al. 2011).

Control of *Msi1* expression at the post-transcriptional level has also been described. Neural ELAV family RNA-binding proteins stabilize *Msi1* mRNA by binding to an AU-rich element in its 3′-UTR in CNS neural stem/progenitor cells and glioblastoma cells (Ratti 2006, Vo et al. 2012a). *Msi1* mRNA stability may also be regulated by tumor suppressor miRNAs (Vo et al. 2011). Ectopic *Msi1* expression partially rescues defects in cell proliferation caused by these miRNAs. Finally, *Xenopus* Msi1 may autoregulate its translation by binding to its own 3′-UTR during oocyte maturation (Arumugam et al. 2012).

Regulation of *Msi2* expression has been studied in the hematopoietic system, where it is the predominant Musashi expressed. *Msi2* is expressed preferentially in normal HSCs and the most immature cells of blast crisis CML (Ito et al. 2010). Interestingly, NUP98-HOXA9, an oncoprotein associated with blast crisis CML, increases *Msi2* expression, and HOXA9 is found bound on the upstream promoter of the *Msi2* gene (Ito et al. 2010). Further, even in patients not carrying a *NUP98/HOXA9* mutation, *HOXA9* was routinely elevated and may be in part responsible for *Msi2* upregulation across diverse mutations (Ito et al. 2010). These results suggest that *HOX* genes can regulate Musashi2 expression in leukemia at the transcriptional level.

Considering these diverse data in varied systems, there is clearly a significant need for a more systematic and integrated analysis of how *Msi* expression is controlled. This would go a long way toward a better understanding of how *Msi* is so finely regulated in stem cells and in development and how it is aberrantly reactivated in cancer.

Downstream Targets

As one might predict from its functional domains (see above), Musashi proteins are known primarily as regulators of translation. Whether Musashi directly regulates other intranuclear processes, such as pre-mRNA splicing (Cuadrado et al. 2002, Katz et al. 2014) or *Let-7* family miRNA processing, remains to be elucidated. Genome-scale expression analyses have shown that Musashi proteins directly or indirectly control the expression of a remarkably large number of genes, indicating a powerful role in gene regulation (Kwon et al. 2015); among these, many direct targets were confirmed by RIP-PCR analysis. In addition, high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) identified multiple potential mRNA targets of MSI2 with known involvement in pathways regulating RNA translation, stem cell function, and

TGF-β signaling (Park et al. 2014). Other direct mRNA targets of MSI1 have also been identified in HEK293T and Daoy medulloblastoma cell lines by RNA-binding protein immunoprecipitation-microarray (RIP-ChIP) analysis; these are enriched for genes associated with the cell cycle, proliferation, apoptosis, DNA repair, and differentiation (de Sousa Abreu et al. 2009, Vo et al. 2012b).

The target repertoire of MSI1 and that of MSI2 are likely to overlap significantly, as would be expected from the conservation of key residues in their RRMs, and their functional redundancy has been demonstrated in the maintenance of neural stem cells (Sakakibara et al. 2002). More recently, ribosome profiling (Ribo-Seq) was used to analyze changes in mRNA expression in neural stem cells with genetic overexpression or depletion of *Msi1* and *Msi2* (Katz et al. 2014). Little change in mRNA expression was observed after 48 h of *Msi1* or *Msi2* overexpression, consistent with the primary function of these proteins being the regulation of translation rather than transcription. By contrast, changes in the translation efficiency of genes involved in pre-mRNA splicing were observed with overexpression of either *Msi1* or *Msi2*.

Although Musashi proteins appear to regulate, directly or indirectly, the expression of many genes, only a handful of these direct targets have been studied in detail. In *Drosophila*, in which the first Musashi protein was identified, Musashi controls expression of the zinc finger transcriptional repressor Tramtrack69 (*ttk69*) during asymmetric cell division of sensory organ precursors (Okabe et al. 2001). Musashi binds the 3′-UTR of *ttk69* mRNA in IIb precursor cells, inhibiting *ttk69* translation. With the downregulation of *ttk69*, a determinant of non-neuronal identity, IIb precursor cells adopt a neuronal cell fate, in contrast to their IIa precursor sister cells.

A well-validated target of Musashi is the mRNA of *CDKN1A*, which encodes the $p21^{WAF-1}$ (wild-type p53 activated fragment-1) cyclin-dependent kinase inhibitor. Musashi1 represses *CDKN1A* translation by binding to MSI1-binding consensus sequences in the *CDKN1A* mRNA 3′-UTR (Battelli et al. 2006). Ectopic expression of *MSI1* significantly reduces $p21^{WAF-1}$ levels and increases entry into S-phase and proliferation by threefold. *MSI1* regulation of *CDKN1A* may play an important role in tumor growth, as knockdown of *MSI1* in colon cancer and endometrial carcinoma cell lines results in reduced proliferation and cell cycle progression in association with higher levels of $p21^{WAF-1}$ (Götte et al. 2011, Sureban et al. 2008).

One of the best-studied Musashi targets is Numb, an inhibitor of the Notch intracellular signaling pathway. Numb was first identified as a potential target of *Msi1* because consensus sequence motifs for MSI1 binding are found in the *Numb* mRNA 3′-UTR (Imai et al. 2001). The ability of MSI1 to bind the *Numb* 3′-UTR was confirmed both in vitro and in vivo. MSI1 binding downregulates *Numb* expression at the translational but not transcriptional level in neural stem cells, and this downregulation potentiates Notch signaling activity. In leukemia, the regulation of NUMB levels by *Msi2* plays a key role in the progression of CML to blast crisis (Ito et al. 2010). In a mouse model of CML, loss of *Msi2* restores *Numb* expression and impairs the development and progression of blast crisis CML through increased differentiation. Interestingly, this corresponds to the pattern of *MSI2* and *NUMB* expression level changes observed during CML progression in leukemia patients. Microarray analysis of patient samples shows upregulation of *MSI2* and downregulation of *NUMB* in patients who have progressed to blast crisis. In this context, transcriptional levels of *NUMB* are clearly affected by *MSI2* modulation, raising the possibility that *MSI2* may influence RNA stability (Ito et al. 2010).

Although these studies provide strong evidence for the ability of Musashi proteins to repress Numb expression, repression may depend on cellular context. In one study, no change in NUMB levels was detected in the HSCs/progenitor cells of *Msi2* conditional knockout mice (Park et al. 2014). In another study, NUMB levels were higher in the cerebrum but unexpectedly lower in the stomach of *Msi1* knockout mice compared with wild-type mice (Takahashi et al. 2013). It is not clear why Numb regulation differs in these specific examples, but insights into how Musashi

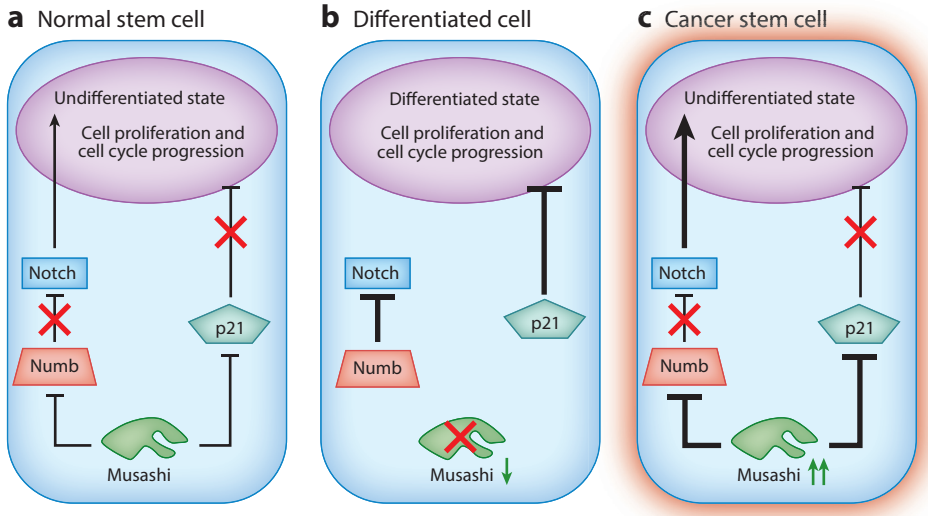

Figure 4

Model exemplifying downstream mediators of Musashi's impact in development and cancer. (*a*) In normal stem cells, Musashi expression suppresses *Numb* translation, leading to increased Notch signaling and maintenance of an undifferentiated state. Musashi can also suppress *p21* (also known as Cdnk1a), which normally blocks cell cycle progression, thus promoting cell proliferation. (*b*) As Musashi expression is extinguished, suppression of *Numb* is lost and Notch signaling is inhibited, thus triggering differentiation. In parallel, reduced Musashi expression results in increased levels of p21 and reduced cell proliferation. (*c*) In the context of cancer, increased levels of Musashi suppress *Numb* and *p21*, among other genes, leading to sustained self-renewal of the cancer stem cell pool.

proteins switch between repressing and activating translation have been gained through the study of Xenopus Musashi during oocyte maturation. *Xenopus* and mouse Musashi1 proteins are highly conserved (94% amino acid identity), and both proteins activate translation in *Xenopus* oocytes (MacNicol et al. 2011). In striking contrast, however, both *Xenopus* and mouse Musashi1 repress translation of target mRNA when expressed in mouse cells. This switch between repressor and activator of translation may be determined by differential protein-protein interactions. Musashi1 may activate translation by associating with the GLD2 poly(A) polymerase (Cragle & MacNicol 2014) (**Figure 1**). A 31-amino acid domain in the C-terminal half of both *Xenopus* and mouse Musashi1 proteins is required for GLD2 binding and subsequent mRNA polyadenylation and translational activation. Interestingly, the binding domains for GLD2 and PABP overlap, suggesting a mechanism in which Musashi1 alternatively represses translation when PABP is bound or activates translation in the presence of GLD2 (**Figure 1*a*,*b***; **Figure 3*c***). Taken together, the varied array of genes and processes regulated by Musashi proteins positions them high in the hierarchy of gene network regulatory mechanisms that control cellular identity and behavior in a variety of contexts (**Figure 4**; **Table 1**).

PERSPECTIVES

Unlike our understanding of many other key stem cell genes, such as Wnt, Notch, or Hedgehog, our understanding of Musashi remains very much in its infancy. The breadth and depth of Musashi's influence are remarkable: It is potentially a key player in building many, and perhaps

Table 1 Known Musashi targets

Target mRNA	Up-/down-regulation	Cell type	Method(s) of identification	Effect	Reference
TTK69	Down	*Drosophila* SOP precursors	Candidate gene	Asymmetric cell fate	Okabe et al. 2001
Numb	Down	NIH 3T3 cells	Msi target sequences in 3′-UTR	Notch upregulation	Imai et al. 2001
	Down	CML	Candidate gene	Blast crisis	Ito et al. 2010
CDKN1A	Down	HEK293 cells	Candidate gene	Cell cycle progression, proliferation	Battelli et al. 2006
c-mos	Up	*Xenopus* oocytes	Yeast 3-hybrid screen	Meiotic cycle progression	Charlesworth et al. 2006
Robo3/Rig1	Up	Murine precerebellar neurons	Candidate gene	Axonal crossing	Kuwako et al. 2010
arx-1, *arx-2*, *arx-3* (encode Arp2/3 subunits)	Down	*C. elegans* AVA interneurons	Candidate gene	Time-dependent memory loss	Hadziselimovic et al. 2014
doublecortin	Down	Neuro2A cells	mRNA display screen	Unknown	Horisawa et al. 2009
NF-YA	Down	SUM159PT breast cancer cell line	Msi target sequences in 3′-UTR	26S proteasome downregulation	Lagadec et al. 2014
Smad3	Down	Murine HSCs	HITS-CLIP	HSC maintenance	Park et al. 2014
Jagged1	Down	293T cells	Msi target sequences in 3′-UTR	Notch signaling upregulation	Katz et al. 2014
PTEN	Down	MSI2-overexpressing intestinal epithelium	CLIP-Seq	Crypt fission and expansion	Wang et al. 2015
Hoxa9, *Myc*, *Ikzf2*	Up	Mixed-lineage leukemia (MLL-AF9)	HITS-CLIP, RNA-Seq, RNA-IP	Maintenance of leukemia self-renewal	Park et al. 2015
Tetraspanin 3	Up	Acute myelogenous leukemia	Microarray	AML propagation, chemokine response	Kwon et al. 2015

Abbreviations: CLIP-Seq, crosslinking immunoprecipitation sequencing; CML, chronic myeloid leukemia; HITS-CLIP, high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation; HSC, hematopoietic stem cell; SOP, sensory organ progenitor; UTR, untranslated region.

most, tissues. It sustains stem cells, enables cellular diversity, and may preserve the long-term ability to replenish and regenerate exhausted or injured cells. Perhaps the greatest testament to its power in establishing and maintaining the stem cell state is reactivation of Musashi in the context of cancer. Many key questions remain unanswered and will undoubtedly be a focus of future work: How does Musashi mediate its effects in specific contexts, and does it act in distinct ways in normal stem cells and in cancer? How is its expression extinguished as differentiation occurs during development but aberrantly reactivated in cancer? Beyond its role in stem cells, is Musashi expressed and functional in progenitors and mature cells? How is it involved in regenerative processes, and do these functions explain the increased risk that injury and inflammation pose for cancer?

The role of Musashi in stem cells may have clear implications for understanding and repair of injury or degenerative states. In the nervous system, such degeneration involves the loss of function or cell death of neurons and is the underlying cause of amyotrophic lateral sclerosis (Bruijn et al. 2004), Parkinson's disease (Dauer & Przedborski 2003), Alzheimer's disease (Yankner 1996), and Huntington's disease (Cowan & Raymond 2006). As Musashi is critical for maintaining neural stem cells, it may be important to explore the impact of Musashi expression on neural stem cells and assess its potential ability to regenerate lost neural cells and restore normal function. In the short term, ectopic expression of Musashi in cultured cells could be tested for its ability to preserve the stem cell state and thus allow generation of a larger source of cells for preclinical testing. In addition to its influence on neural stem cells, Musashi clearly has an important role in stem cells of other tissues; thus, its role in regeneration and replacement of tissues such as the bone marrow and epithelium should also be investigated.

In the context of cancer, a better understanding of both the upstream regulation of Musashi activation and downstream mechanisms by which it mediates its impact could influence the development of diagnostics and therapeutics. Understanding the pathways that mark initiation of a malignancy could go a long way to improving our ability to diagnose and detect cancer at an early stage, when treatment options are greater and the disease is more responsive to therapy (Etzioni et al. 2003, Rubin et al. 2011, Thomson & Forman 2009). Musashi may serve as a particularly attractive target for diagnostic strategies because it is expressed at very low levels in most adult tissues but is highly upregulated during the initiation and progression of several cancers (Ito et al. 2010, Kharas et al. 2010, Muto et al. 2012, Nakamura et al. 1994, Okabe et al. 1997, Okano et al. 2002, Sakakibara et al. 2002). For example, our analysis of CML progression in a large cohort of patients revealed a dramatic increase in Musashi expression as patients progressed to blast crisis (Ito et al. 2010), with elevated levels of Musashi associated with poor prognosis. Although tracking Musashi expression represents a promising approach for early detection of disease, translation to clinical use awaits development of sensitive and accurate methods for monitoring Musashi levels in living cells.

As we move toward a more targeted approach to therapy and individualized medicine, the identification of new molecular targets for disease becomes increasingly relevant. Musashi is a particularly intriguing therapeutic target because it impacts a broad array of cancers, including both hematological and solid tumors. Whether drugs already in the clinic or new compounds will be effective at targeting Musashi remains to be determined. Musashi is an RNA-binding protein and hence a nontraditional target. Thus, alternative approaches to targeting it may prove to be more effective for therapy.

EPILOGUE

Toward the end of his life, Miyamoto Musashi ultimately retreated to a cave, where he wrote *The Book of Five Rings* on combat strategy and philosophy. It is ironic that although Musashi won more than 60 duels and battles, he ultimately lost his most important battle to cancer. His intense commitment to combat is marked fittingly by his words, "The only reason a warrior fights is to win," and can perhaps be an inspiration as we fight to control a powerful adversary. In many ways, Musashi is a quintessential stem cell gene, imbued with great power—enabling survival and self-renewal, and sustaining cancer growth and recurrence. The work done to date has not only brought great understanding of the ways in which Musashi triggers cancers but also begun to reveal its vulnerabilities. The next few years are bound to bring greater insight into ways to harness Musashi's great power for good in regenerative medicine and to successfully combat its role in cancer.

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