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Germline factor DDX4 functions in blood-derived cancer cell phenotypes

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Key words
DDX4/Vasa, germline factor, human cancer cells, RNA helicase, translational regulation

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The germ line and cancer cells share various cellular characteristics important for their functions. Notable shared traits include: (i) periods of hyperproliferation relative to their neighbors, such as in the gonial stem cells within the presumptive gonad, and in tumor growth; and (ii) extensive migration of the primordial germ cells from their site of formation to the gonadal ridge, and metastasis in cancer cells, which accounts for approximately 90% of human cancer deaths. It has long been proposed that similar mechanisms of regulation may be found in these otherwise disparate cell types. Several germline factors widely conserved throughout metazoans appear to be associated with tumors. For instance, Nanos (RNA-binding protein) upregulation as a consequence of Rb (Retinoblastoma) inactivation causes inhibition of apoptotic genes and promotes cell proliferation. PIWI (a small RNA regulator) has been reported to be linked to many hallmarks of cancer, including cell proliferation, anti-apoptosis, genomic instability, and metastasis. These emerging functions of germline factors led us to suggest that germline factors may contribute broadly to cancer cell regulation.

DDX4, a Drosophila Vasa ortholog, is another conserved germline factor in metazoans. It is a DEAD box RNA helicase and is implicated in translational regulation of germline-specific molecules in various organisms. In mammals, DDX4 is expressed in germ cells of both sexes and functions in germline formation and maintenance. Recent reports in several organisms, however, suggest that Vasa also functions in embryonic and adult somatic cells, in regenerative tissues, and, in some cases, in tumorigenic cells. Particularly, the finding of broad germline factor expression in a malignant brain tumor in the fly Drosophila melanogaster strongly supported the contention of not just germline factor presence, but of germline factor function in tumorigenic cells. In the Janic et al. study, Drosophila brain tumors regression when one or another of the germline factors was inactivated, indicating that these germline factors were essential for certain characteristics of tumor formation and/or maintenance, at least in this type of malignancy. Furthermore, it has been reported that DDX4 is expressed in several ovarian cancer cells and tissues, and its overexpression accelerates cell cycle progression by abrogating the G2 checkpoint. These observations, both in normal somatic cells and in cancerous cells, suggest that when DDX4/Vasa is expressed, it appears to be consistently involved in cellular proliferation, potentially through its function as a translational regulator. However, its direct contribution and detailed functional mechanisms in normal somatic cells, or in somatic cell cancers, are unclear. In this study, we report that DDX4 is consistently localized with the mitotic apparatus in various blood-derived cancer cell lines and has essential roles in cell proliferation and migration – the functionalities for which DDX4/Vasa is also involved in the germ line.

Materials and Methods

Cell lines, cell culture, and cell number counting. Multiple myeloma cell lines IM-9 (ATCC# CCL-159, Manassas, VA, USA), MM.1S (ATCC# CRL-2974, Manassas, VA, USA), KMS11 (NCiT # C3242, Baltimore, MD, USA), and OPM-2 (DSMZ # ACC 50, Leibniz Institute, Braunschweig, Germany) and acute monocytic leukemia cell line THP-1 (ATCC# TIB-
followed by heat inactivation at 96°C for 30 min. The cell pellet was subjected to genomic DNA extraction by treating with 100 μg/mL proteinase K at 50°C for 3 h, followed by heat inactivation at 96°C for 15 min. One microliter of each of these proteinase K-treated samples was used for genomic PCR to amplify a flanking region of the third exon of DDX4, where the gRNA sequences were designed, using high fidelity Platinum Taq DNA polymerase (Thermo Fisher Scientific) with primers summarized in Table S1. The resultant PCR products were either separated by agarose gel electrophoresis and visualized, or subjected to subcloning into the pGEM Easy Vector (Promega, Madison, WI, USA) for sequencing.

Reverse transcription-PCR and quantitative real-time PCR. RNA extraction was carried out using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and 1 μg each of the resultant total RNAs were subjected to RT using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) following the manufacturer’s protocol. The cDNAs (1 μL each) were then used either for conventional PCR reactions or for quantitative PCR reactions. Conventional PCR reactions were carried out with 35 cycles using high fidelity Platinum Taq DNA polymerase (Thermo Fisher Scientific) with primers summarized in Table S1. Quantitative PCR of the IM-9 cell line cDNA was undertaken using SYBR Green Master Mix (Thermo Fisher Scientific). Into each well was placed 1 μL cDNA, 12.5 μL SYBR Green Master Mix, 6 μL of 10 μM forward and reverse primer mix, and 5.5 μL water. The primers are summarized in Table S1.

Invasion assay. Invasion assays were carried out using a Corning Transwell (Corning #3422, Tewksbury, MA, USA) following the manufacturer’s protocol. Briefly, each cell line was starved in culture media lacking FBS for a day, and then each of 1 × 10⁶ cells was placed into the Transwell insert with FBS attractant at the bottom of the well. After 24 h, the number of cells that passed through the membrane were counted. For a negative control, the ORI cell line was placed into the Transwell insert without the FBS attractant.

Cell staining. 5-Ethynyl-2′-deoxyuridine (EdU) (Life Technologies, Carlsbad, CA, USA) is incorporated during DNA replication of the cell and thus works as a DNA replication marker. IM-9 cells were treated with a final 1 μg/mL EdU for 2 h to label replicative cells. L-homopropargylglycine (HPG) is a glycine derivative and is incorporated as a methionine analog during new protein synthesis and thus works as a vital method for visualizing nascent protein populations synthesized at specific times as determined by the reagent exposure. HPG (50 mM stock solution; Life Technologies) was added to cells at a 1:1000 dilution (50 μM final concentration) for 3 h in methionine-depleted DMEM culture media (#21013024; Thermo Fisher Scientific).

For both the EdU and HPG experiments, as a negative control, a set of cells with no treatment was also prepared and subjected to identical detection protocols. The cells were then fixed with 4% paraformaldehyde in PBS solution and Click-IT detection was undertaken by following the manufacturer’s protocol (Life Technologies). After detection the DNA was stained using HCS NuclearMask Blue Stain (Thermo Fisher Scientific) at 1:2000 for 30 min. Coverslips were then washed with PBS and mounted on the slide glass. Five images of each stained specimen were taken under the fluorescent microscope (Axioplan; Zeiss, Jena, Germany), and the average number of positive cells in each image was calculated by Image J (NIH, https://imagej.nih.gov/ij/). These experiments were also repeated at least three independent times.

Immunofluorescence. Cells were fixed with 4% paraformaldehyde at 4°C overnight or 30 min at room temperature, washed with PBS three times, and stained with a primary antibody against DDX4 (#13840; Abcam, Cambridge, MA, USA) diluted at 1:300 at room temperature for 3–5 h. They were washed six times with PBS and stained with a secondary antibody against rabbit IgG (1:500; 1 mg/mL; Thermo Fisher Scientific) in PBS. They were then washed six times and a Hoechst nuclear stain (10 mg/mL; Promega, Madison, WI, USA) in PBS was applied at a 1:1500 dilution. The resultant cells were visualized by wide-field fluorescence microscopy (Axioplan; Zeiss) or by confocal laser microscopy (LSM510 and LSM500; Zeiss, Jena, Germany).

Immunoblot analysis. Immunoblots were carried out by collecting approximately 10 μL packed cells from each group, and the resultant samples were prepared in 50 μL loading buffer for PAGE. Each sample (10 μL) was run on a 4–20% gradient Tris–glycine polyacrylamide gel and transferred to nitrocellulose membranes for immunoblotting with α-tubulin antibody, DDX4 antibody (#D10CS; Cell Signaling Technology, Danvers, MA, USA), MIWI antibody (#2079; Cell Signaling Technology, Dallas, TX, USA), 14-3-3τ antibody (#sc-166473; Santa Cruz Biotechnology) at 1:1000, or with peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies at 1:5000 (Life Technologies). The bound antibodies were detected by incubation in a chemiluminescence solution (1.25 mM luminol, 68 μM coumeric acid, 0.0093% hydrogen peroxide, and 0.1 M Tris pH 8.6) for 1–10 min, exposed to film, and developed. Each experiment was carried out at least three times, independently.

Results

DDX4 is expressed and localized with the spindle of human blood cancer cells. Through database searches at the Cancer Cell Line Encyclopedia(224) and Cancer Genomics Program (http://www.cancergenomicsprogram.ca/about-cgp), we identified several dozen cancer cell lines that are potentially overexpressing ddx4 transcripts. Among them, 26 of 50 cancer cell lines are...
derived from blood/lymph cells. Therefore, in this study, we tested for a potential function of this essential germ line factor on the blood cell-derived cancer cell lines IM-9 and THP-1. IM-9 (CCL-159; ATCC) is a multiple myeloma-derived B lymphocyte and THP-1 (TIB-202; ATCC) is an acute monocytic leukemia-derived monocyte. To test for D DX4 expression, we first screened for transcripts using RT-PCR for each cell line. Expression of another germ line factor PIW1-like2 (PIW1L2) that is known to function with DDX4/Vasa in the germ line(29) as well as in some cancer cells(30,31) was analyzed in parallel. We learned that both ddx4 and piwil2 mRNA were expressed in IM-9 and THP-1 cells (Fig. 1a). With the same experimental conditions, their expression levels were compared with several other myeloma-derived cell lines as well as in human embryonic stem cells. Intriguingly, human embryonic stem cells showed the most robust expression and other myeloma cells such as KMS-11 and MM1S also showed accumulation of DDX4, yet no expression was detectable in OPM-2 in this condition. As negative controls, the same amount of total RNA was used for reverse transcription without the reverse transcriptase enzyme (−RT; Fig. 1a), and the resultant “cDNA” showed no amplification of any of the target genes, including the housekeeping genes GAPDH and 36B4 used here as positive controls. These results suggest that ddx4 and piwil2 mRNAs are indeed transcribed in several blood-derived cancer cells as well as embryonic stem cells. As not all mRNAs are translated into protein to function in the cell, especially in cases of many germ line factors, we next tested by immunofluorescence whether the DDX4 protein is present in these cells. In THP-1 and IM-9 cells, DDX4 was detectable and localized throughout the cytoplasm. During M phase in these cells, DDX4 was enriched on the mitotic apparatus with a granule-like structure. In MM1S, the DDX4 signal was weak yet still enriched the most on the mitotic apparatus (Fig. 1b, arrows). Cells processed identically, but without anti-DDX4 antibody, showed no DDX4 signal anywhere in the cell, suggesting the specificity of the DDX4 signal on the mitotic apparatus. The DDX4 protein expression in DDX4−/− cells was also tested by immunoblotting (Fig. 2). From these results of protein analysis, we concluded that DDX4 associates with the spindle during M phase and might also function in cell cycle regulation in these cancer cells.

Gene targeting to reduce DDX4 protein expression. To test the possible function of DDX4 in these blood-derived cancer cells, we constructed DDX4-knockout cell lines in IM-9 cells using the CRISPR-mediated genome-editing technology. IM-9 was chosen here because it has robust DDX4 expression and proliferates every 12–24 h, allowing a better selection of knockout cell lines. Three independent gRNAs (C1, C2, and C3) were designed within the third exon of the DDX4 genomic locus, and one control gRNA with a scrambled sequence was designed (SC) in the internal cellular mechanism to repair the broken DNA double-strands caused by CRISPR/Cas9. This repair occurs in a random manner. It is thus expected to result in different patterns of deletions within the same cell population as each cell received CRISPR/Cas9. Because of this randomness within the population of cells, some of the deletions may cause a frame shift and completely inhibit protein expression, whereas others may result in truncated proteins or mutated proteins, but without a frame shift. Cells with such mutations may still express DDX4 proteins that are functional in some cases. We, therefore, were unable to acquire a complete knockout of DDX4 protein in the population immunoblot. This suggests a small population of cells may still express functional DDX4 proteins, and the DDX4 signal in C3 is likely incomplete inactivation of DDX4 protein expression in all cells of the population. A single cell culture that requires longer passaging of cells may also change cellular phenotypes, so we used a stock of cells that were stored immediately after the lentiviral infection and puromycin selection in this study. Independent knockout cell lines C1–C3, however, consistently showed decreased DDX4 expression, whereas the control cell line SC showed no reduction. These results suggest that a significant portion of DDX4 protein expression in the cell was successfully knocked out through the CRISPR genome-editing approach in these cell lines. On observation of the cells, we found that the DDX4 knockout cell lines typically formed incomplete spindles (Fig. 2e) and no longer proliferated significantly, which is also a consistent phenotype reported in germine stem cells and in some embryonic cells. In this study, however, we emphasize a population-based study.

DDX4 knockout cells have decreased cell proliferation and migration. Using the above cell lines, C1, C2, and C3, we analyzed the effects of DDX4-knockout in cellular activities. Cell counting revealed that the rate of cell proliferation in the population was decreased to 30% in C1 and C2 compared to the controls, SC and ORI (Fig. 3a). This result suggests that DDX4 is important for cell proliferation. We also tested whether DDX4 is important for DNA replication. For this experiment, each cell line was treated for 2 h with EdU (nucleotide derivative) that is incorporated into DNA during...
chromosome replication, serving as a good DNA replication marker. The proportion of the EdU-positive cells against the total number of cells was then counted under the fluorescent microscope (Fig. 3b). The C1 and C2 cells showed decreased activities in DNA replication, yet the differences compared to control cells were relatively small. This result suggests that DDX4 may be necessary for cell proliferation but less so for S-phase entry and DNA replication.

As DDX4/Vasa is also known to be involved in germ cell migration in various animals, we next tested whether DDX4 knockout affects activities of cancer cell migration by invasion assays. In C1 and C2, the number of cells migrated toward the FBS-containing well were significantly decreased, close to that of the negative control group (ORI with no FBS-containing well), whereas C3 showed relatively higher migration activities but still lower than the positive controls (Fig. 3c). These results suggest that DDX4 might be important for IM-9 cancer cell migration. Together, these results suggest that DDX4 activities function in proliferation and migration in IM-9 cancer cells.

**Fig. 1.** Germline factor DDX4 is expressed in several blood-derived cancer cells. (a) RT-PCR results of six cell lines (KMS11, THP-1, hES, OPM-2, MM1S, and IM-9) for four gene products (PIWIL2, DDX4, GAPDH, and 36B4). Left panels, negative controls (lacking RT enzyme). Right panels, positive tests. (b) (i) Immunofluorescence results showing DDX4 localization in five cell types counterstained with tubulin and DNA. As indicated by arrows, DDX4 is localized on the mitotic spindle in cancer cells expressing DDX4. Scale bar = 5 μm. (ii) Relative signal intensity of DDX4 on the spindle of each cell line. The signal level of DDX4 was calculated by Image J and normalized to that of tubulin. The average relative value of each of the five cells undergoing mitosis is presented for each cell line. The value of IM-9 is set at 1.
DDX4 knockout downregulates expression of cell cycle regulators and oncogenes. As Vasa/DDX4 is an RNA-helicase and known to function as a translational regulator, the cellular phenotypes described above may be due to changes in expression of DDX4’s downstream targets that are directly involved in cell replication and/or migration. To test whether DDX4 knockout in IM-9 cells changes the selected gene expression, we undertook quantitative RT-PCR. The potential downstream genes of interest included germline factors (ddx4 and piwil2) and oncogene/cell cycle factors (14-3-3r, cyclinB1, e2f1, and myc). Among those candidates, most transcripts were altered minimally (Fig. 4a) whereas the cyclinB1, e2f1, and chi3 l1 transcripts were found consistently downregulated in the DDX4-knockout cell lines, although still detectable.

To test whether DDX4 knockout indeed changes protein expression of the above targets, we undertook immunoblots against each molecule of interest (Fig. 4b). As a result, we found that the positive cell cycle regulators CyclinB1 and E2F1 were downregulated, whereas a negative cell cycle regulator 14-3-3r was upregulated. As DDX4 is a translational regulator, these results suggest that CyclinB and E2F1 mRNAs could be targets of DDX4 and their protein production was downregulated in the absence of DDX4. Indeed, CyclinB is essential for M-phase entry and exit and has also been reported as one of Vasa’s targets in embryonic cells.\(^{(15)}\) Its knockdown, therefore, may have blocked M-phase entry and indirectly resulted in spindle defects. E2F1 is a transcription factor and known to be highly active during the G1/S-phase transition, yet some of its target genes also function in G2/M phase.\(^{(28,29)}\)

Therefore, the reduction of CyclinB and E2F1 proteins that are critical for cell cycle progression might have caused cell proliferation defects in DDX4-knockout cell lines. It is also important to consider the phenotype observed here likely includes both direct and indirect effects of DDX4 knockout, because both DDX4 and some of its targets, including E2F1, likely regulate a wide range of translation and transcription in the cell. The further analysis of this process, including a broad screening of DDX4’s targets and cofactors by RNA immunoprecipitation sequencing and proteomics is thus necessary to parse out direct from indirect effects of DDX4 function. In contrast, 14-3-3r is a cell cycle repressor. It is unclear what increases its expression at this point in the DDX4-knockout. Intriguingly, another germline factor PIWI was
also upregulated in C1. As PIWI has been reported to target various transcripts in the germ line for destruction, the overexpression of PIWI may have resulted in degradation of mRNAs important for cellular activities, the mechanisms of which need to be tested further in the future.

Vasa has been reported to have broad targets and function as a general translational regulator in the embryo, so we tested whether DDX4 knockout inhibits general protein synthesis (Fig. 4c). Each cell line was treated in methionine-depleted media for 3 h with HPG (a tagged methionine analog) that is incorporated into newly synthesized proteins and thus serves as a good tool to measure the amount of nascent protein synthesis. We found that the level of HPG incorporation was significantly decreased in DDX4 knockout cell lines. These results suggest that DDX4 might positively function in protein synthesis as it does in embryonic or germ cells and help explain the phenotypic changes seen in the knockout cells.

Discussion

DDX4 is essential for proliferation and migration activities in somatic cancer cells. All animals tested so far appear to express Vasa/DDX4 in their germ line at one point in their life. As its knockdown often results in infertility, Vasa/DDX4 has been considered and used as a germ line marker for decades in the field. Its function in the germ line was also concluded to be “germline-specific”, essential for functions in cell proliferation and migration of germ cells and translation of the mRNAs in the germ cells. In this study, however, we reported that DDX4 is widely expressed in several blood-derived cancer cells and contributes to cell proliferation and migration. These results directly suggest that DDX4 has functionalities outside of the germ line, yet its expression may be limited to context-specific periods of cellular development, to rapidly dividing cells such as embryonic cells as reported previously, and in human cancer cells as reported in this study.

DDX4/Vasa has also been reported to be transiently expressed during regeneration, the tissues of which also undergo active cell proliferation and phenotypic changes. With the results here, we might consider that DDX4 has various downstream targets that are essential more generally for cell proliferation and migration (Fig. 5). These results are also consistent with Vasa’s activities in embryonic cells; Vasa has been reported to regulate widespread translation essential for embryonic cell division and development. Although what Vasa does on the spindle has been unclear in any cell or organism at this point, in embryonic cells, DDX4/Vasa appears to...
function as a general translational regulator rather than as a regulator of specific targets. It is thus intriguing to speculate that, in cancer cells and embryonic cells alike, DDX4/Vasa may facilitate M-phase translation (33) on the spindle to assist sufficient protein production necessary for rapid cell division. This idea needs to be experimentally tested in the future by manipulating Vasa function specifically on the spindle. Through this mechanism, DDX4 could provide a molecular environment for cancer cells to maximize proliferative activities and potentially contribute in neoplastic features of cancer cells through upregulated protein expression. The thorough screening of DDX4’s downstream targets will be essential to understand the details of DDX4 functions in cancer cell regulation.

Another important question to be addressed in the future is why DDX4/Vasa becomes essential only when it is expressed in the cells. Not all cells, neither embryonic nor cancer cells, appear to have DDX4/Vasa. Yet DDX4/Vasa expression, normally or abnormally induced, seems to change the cellular dependency for translational activity to itself. Perhaps the...
changes ensued by DDX4/Vasa expression might directly and indirectly alter the global translational landscape in the cell. We thus predict that other types of cancer cells that express the DDX4 transcript, such as germ cell cancer, breast cancer, and small-cell lung cancer (data not shown), may also depend on its activity for survival. To be noted, DDX4’s function is heavily regulated by post-transcriptional regulation. Therefore, it will be necessary to test its expression and localization at the protein level in each of these cell lines. These further exhaustive screenings as well as identification of the upstream regulator(s) of DDX4 will be essential to understand how DDX4 directly contributes to cellular activities of cancer cells.

Furthermore, as DDX4 is generally not expressed in benign adult somatic cells,(14) it could serve as a potential target to detect early steps of malignancy among heterogeneous tumors and/or for a cancer therapy in the future. Our results suggest that another germline factor, PIWI, also appears to be expressed with DDX4 in these same cancer cells, and thus may serve as important co-target for cancer therapy. The detailed functional mechanisms of DDX4 with other germline factors and its downstream targets needs to be tested further in various cancer cell types and in patient tissues.

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Disclosure Statement
The authors have no conflict of interest.

References

Supporting Information
Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Primers and guide RNA (gRNA) sequences used in this study.