DEAD-box RNA helicase Belle/DDX3 and the RNA interference pathway promote mitotic chromosome segregation

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During mitosis, faithful inheritance of genetic material is achieved by chromosome segregation, as mediated by the condensin I and II complexes. Failed chromosome segregation can result in neoplasm formation, infertility, and birth defects. Recently, the germ-line–specific DEAD-box RNA helicase Vasa was demonstrated to promote mitotic chromosome segregation in *Drosophila* by facilitating robust chromosomal localization of Barren (Barr), a condensin I component. This mitotic function of Vasa is mediated by Aubergine and Spindle-E, which are two germ-line components of the Piwi-interacting RNA pathway. Faithful segregation of chromosomes should be executed both in germ-line and somatic cells. However, whether a similar mechanism also functions in promoting chromosome segregation in somatic cells has not been elucidated. Here, we present evidence that *belle* (*vasa* paralog) and the RNA interference pathway regulate chromosome segregation in *Drosophila* somatic cells. During mitosis, *belle* promotes robust Barr chromosomal localization and chromosome segregation. Belle's localization to condensing chromosomes depends on *dicer-2* and *argonaute2*. Coimmunoprecipitation experiments indicated that Belle interacts with Barr and Argonaute2 and is enriched at endogenous siRNA (endo-siRNA)-generating loci. Our results suggest that Belle functions in promoting chromosome segregation in *Drosophila* somatic cells via the endo-siRNA pathway. *DDX3* (human homolog of *belle*) and *DICER* function in promoting chromosome segregation and hCAP-H (human homolog of Barr) localization in HeLa cells, indicating a conserved function for those proteins in human cells. Our results suggest that the RNA helicase Belle/DDX3 and the RNA interference pathway perform a common role in regulating chromosome segregation in *Drosophila* and human somatic cells.

During mitosis, faithful inheritance of genetic material is achieved by chromosome segregation, which is mediated by the condensin I and II complexes (1, 2). Failed chromosome segregation can result in neoplasm formation, infertility, and birth defects (2). A number of proteins, such as the protein phosphatase 2A (PP2A) and the retinoblastoma family of protein 1 (RBF1), have been demonstrated to regulate condensin II localization (3, 4). Although the condensin I components exhibit dynamic localization during mitosis in *Drosophila* and HeLa cells (5, 6), the mechanism by which their localization is regulated during mitosis has not been fully elucidated.

In *Drosophila*, the Piwi-interacting RNA (piRNA) and endogenous short interfering RNA (endo-siRNA) pathways function to silence deleterious transposable elements in germ-line and somatic cells, respectively (7). Although they play similar roles in regulating transposons in various cell types, the pathways' courses of biogenesis require distinct sets of components. The piRNAs are generated in a *dicer*-independent manner and require the PIWI class of argonaute proteins, including Piwi, Aubergine (Aub), and Argonaute3 (Ago3) (8–10). Furthermore, other piRNA pathway genes, such as *vasa*, *spindle-E* (*spn-E*), *armitage*, *krimper*, *maelstrom*, *squash*, *zucchini*, *cutoff*, *rhino*, *tejas,* and *hsp90*, are required to silence transposons in the *Drosophila* gonads (11–22). Conversely, endo-siRNA biogenesis requires *dicer-2* (*dcr-2*), *argonaute2* (*ago2*), and *loquacious* (23–27). Despite the differences in their biogenesis pathways, a subset of piRNAs and endo-siRNAs are derived from the transposon-rich pericentromeric heterochromatin regions, which function as piRNA- and endo-siRNA-generating loci (9, 24, 25, 27).

Recently, Vasa, a germ-line–specific DEAD-box RNA helicase, has been demonstrated to promote mitotic chromosome segregation in the *Drosophila* germ line by facilitating robust chromosomal localization of Barren (Barr; also known as CAP-H) (28), a condensin I component. Furthermore, the mitotic function of Vasa is mediated by Aub and Spn-E, which are piRNA pathway components, suggesting that small RNA-mediated mechanisms may play active roles in organizing chromosomes during mitosis (28). A similar mitotic role for P granules (equivalent to nuage) was also observed in the *Caenorhabditis elegans* germ line in which the Argonaute CSR-1, the Dicer-related helicase DRH-3, and the Tudor-domain protein EKL-1 localize to mitotic chromosomes and promote chromosome segregation (29, 30), suggesting that the phenomenon reported in this study is conserved across species. Faithful segregation of chromosomes should be executed both in germ-line and somatic cells. Because Vasa and piRNAs are expressed predominantly in *Drosophila* germ-line and gonadal cells, respectively (28), whether analogous RNA helicase(s) and small RNA pathway components function to promote chromosome segregation in other somatic tissues remains unclear (31). In *Drosophila*, Belle, a DEAD-box RNA helicase, is the closest somatic paralog of Vasa (32, 33). Belle is expressed in both germline and somatic cells and has been indicated to regulate cell cycle progression, mRNA splicing, and RNA interference (RNAi) (33–37). Furthermore, the endo-siRNA pathway functions similarly to the piRNA pathway in somatic cells (24–27). The existence of such somatic counterparts implies a possible mechanism by which those components may promote chromosome segregation in somatic cells.

In this study, we examined whether chromosome segregation and chromosomal localization of Barr are regulated by an RNA helicase and the small RNA pathway in somatic cells. We demonstrated that Belle, the Vasa paralog, physically associates with Barr and promotes chromosome segregation in *Drosophila* somatic cells. Belle appears to function through the endo-siRNA pathway because it also interacts with Ago2 and endo-siRNA-generating loci and localizes to condensing chromosomes in a *dcr-2*- and *ago2*-dependent manner. In HeLa cells, *DDX3*, the

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human homolog of *belle,* and *DICER* are also required in promoting chromosome segregation and chromosomal localization of hCAP-H (human homolog of Barr). Thus, our results suggest that the RNA helicase Belle/DDX3 and the RNAi pathway exhibit a conserved function in regulating chromosome segregation in *Drosophila* and in human somatic cells.

Results

Belle, the *Drosophila* Vasa Paralog, Promotes Chromosome Segregation in Somatic Cells. Because Vasa is expressed specifically in germ-line cells, we investigated whether the somatic paralog of Vasa is similarly required for promoting segregation of mitotic chromosomes in somatic cells. To address this possibility, we analyzed Belle, a DEAD-box RNA helicase, which is the closest somatic paralog of Vasa (32, 33). Belle is expressed in both germline and somatic cells and has been indicated to regulate cell cycle progression, mRNA splicing, and RNAi (33–37). Consistent with the findings of previous studies, we detected high levels of Belle, but not Vasa, in *Drosophila* somatic S2 cells, although both proteins are expressed in *Drosophila* ovaries (Fig. 1*A*).

Because *vasa* mutant germ-line cells exhibit defects in Barr chromosomal localization during mitosis (28), we investigated whether *belle* is required for Barr chromosomal localization during mitosis in the wing imaginal discs of third instar larvae. Because Barr localizes to chromosomes during early prophase (6), we examined cells at metaphase to exclude the possible indirect effects caused by a delay in mitotic progression. In wild-type cells, Barr localization was concentrated in the chromosomes at metaphase during mitosis (Fig. 1*B*; 100% normal, $n = 20$). However, in *belle* mutant clones (see *Materials and Methods* for the generation and identification of the clones), the robust localization of Barr onto metaphase chromosomes was lost (Fig. 1*B*; 90% defective, $n = 20$). Conversely, the wing imaginal disk cells in *vasa* mutants did not exhibit a Barr localization defect (Fig. 1*B*; 100% normal, $n = 20$), indicating that *belle*, but not *vasa*, is required by the somatic cells of wing discs to promote Barr chromosomal localization during mitosis. In contrast to *vasa* mutants, *belle* mutant germ-line cells did not exhibit Barr localization defects and lagging chromosomes (0%, $n > 20$) (Fig. S1), indicating a specific requirement for *belle* in the somatic wing disc cells. Thus, Vasa may play a dominant role over Belle in regulating mitotic chromosomes in germ-line cells.

Because chromosomal localization of Barr is required for proper chromosome segregation during anaphase (1, 38), we further examined whether *belle* is required for proper chromosome segregation in the wing discs. In *belle* mutant clones, ≈80% of anaphase cells exhibited lagging chromosomes, which was a result not observed in wild-type and *vasa* mutant wing disc cells (Fig. 1*C*). Using S2 cells, we effectively knocked down the expression of Belle with double-stranded RNA (Fig. S2) and confirmed the requirement for *belle* in promoting the progression of chromosome condensation (as demonstrated by a delay in mitotic progression) and chromosome segregation (Fig. 1 *D* and *E*). Knockdown of Belle by double-stranded RNA resulted in a delay of mitosis at prophase from ≈10% in control cells to ≈70% in *belle* RNAi cells, which was concomitant with a reduction in the percentage of prometaphase cells from ≈50% to ≈20% in control and RNAi-treated cells, respectively (Fig. 1D). Knockdown of Belle also caused an approximately fourfold increase in the percentage of lagging chromosomes during anaphase (from ≈10% in control cells to ≈40% in *belle* RNAi-treated cells) (Fig. 1*E*). This phenotype was weaker than that exhibited by the *belle* mutant wing discs, which could be attributed to the incomplete knockdown of Belle in S2 cells (Fig. S2). Taken together, our results suggest that Belle functions in promoting chromosome segregation in somatic cells, at least in part, by facilitating robust chromosomal localization of Barr.

In germ-line cells, Vasa interacts with Barr and CAP-D2, which are condensin I components, suggesting a direct regulation of condensin I by Vasa (28). Therefore, to determine whether Belle also interacts with condensin I components in S2 cells, we performed reciprocal coimmunoprecipitation experiments. Using S2 cell lysates, endogenous Belle was coimmunoprecipitated with Barr, but not with the IgG controls (Fig. 1*F*). Furthermore, Belle and Barr also coimmunoprecipitated with CAP-D2, the other condensin I component, but not with CAP-D3, a component of the condensin II complex, indicating a specific interaction between Belle and the condensin I components Barr

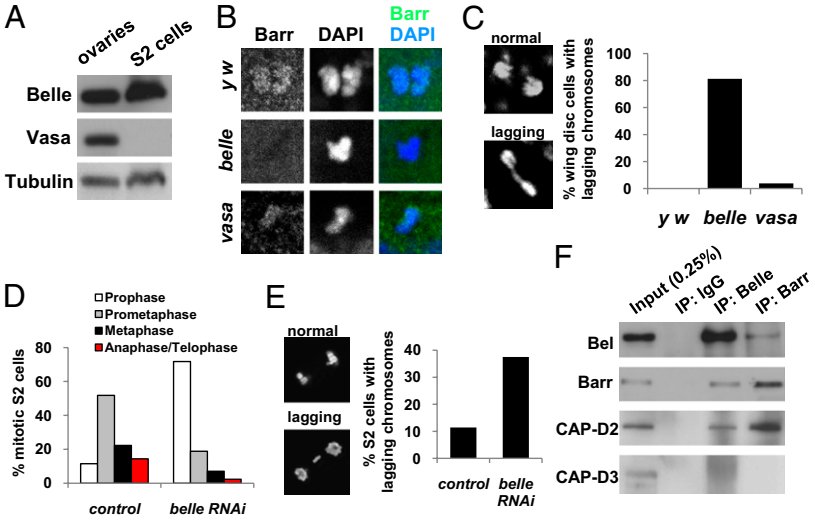

Fig. 1. Belle, the *Drosophila* Vasa paralog, promotes chromosome segregation in somatic cells. (*A*) Western blot analysis indicating the relative expression levels of Belle and Vasa in ovaries and S2 cells. Tubulin was used as a loading control. (*B*) Third instar larval metaphase wing disc cells of the indicated genotypes were stained for Barr (green) and DAPI (blue). (*C*) The percentages of wing disc cells at anaphase exhibiting lagging chromosomes. $n > 15$ for each genotype. (*D*) Percentages of S2 cells at each stage of mitosis. $n > 50$ for each experiment. (*E*) Percentages of S2 cells at anaphase exhibiting lagging chromosomes. $n > 20$ for each experiment. (*F*) Western blots depicting the coimmunoprecipitation of endogenous Belle and Barr using S2 cell lysates. Belle and Barr also coimmunoprecipitated with CAP-D2, but not with CAP-D3, confirming the specificity of the assay.

and CAP-D2 (Fig. 1*F*). Thus, similarly to Vasa, Belle may regulate condensin I loading onto chromosomes by a direct but transient interaction during mitosis.

Belle Interacts with Argonaute2 and Endogenous siRNA-Generating Loci. Next, we examined the mechanism by which Belle functions to promote chromosome segregation. We reported that, in germline cells, Vasa interacts with the piRNA pathway components Aub and Spn-E and localizes to piRNA-generating loci (28). We investigated whether Belle also interacts with any small RNA pathway components and localizes to small RNA-generating loci in somatic cells. Belle was observed to be involved in the RNAi pathway in the *Drosophila* eye and to interact with both Ago2 and siRNAs in S2 cells (34, 37). Endo-siRNAs, similar to piRNAs in the germ line, function to silence transposons in somatic cells in *Drosophila* and are derived from pericentromeric heterochromatin regions and from regions producing converging transcripts (7).

We detected the expression of the endo-siRNA pathway components Ago2 and Dcr-2 and of the piRNA pathway component Spn-E, but not Aub and Piwi, in S2 cells (Fig. 2*A*). By performing coimmunoprecipitation experiments, we determined that Belle and Barr interact with Ago2, but not with Ago1, a microRNA pathway component, or with Spn-E, a piRNA pathway component (Fig. 2*B*), which suggests that Belle may function via the endo-siRNA pathway. We, therefore, investigated whether Belle associates with endo-siRNA–generating loci in S2 cells. By performing chromatin immunoprecipitation, we found that Belle was enriched by approximately twofold at endo-siRNA-generating loci, but not at piRNA-generating and control loci devoid of any known genes (Fig. 2*C*). By immunostaining S2 cells with an anti-Belle antibody, we observed small foci near the condensing chromosomes in addition to cytoplasmic localization during prophase (Fig. 3*A*, arrowheads). The number of these foci was significantly reduced in *belle* knockdown cells (≈14 visible foci in control cells compared with ≈1–2 foci in *belle* knockdown cells) (Fig. 3 *A* and *B*), confirming specific antibody labeling. We observed similar chromosomal foci of FLAG-tagged Belle in transfected S2 cells during prophase (Fig. S3), verifying that Belle localizes to mitotic chromosomes during prophase. Taken together, the associations of Belle with the endo-siRNA pathway component Ago2 and endo-siRNA–generating loci suggest that Belle may function by localizing to condensing chromosomes through the endo-siRNA pathway to promote chromosome segregation in somatic cells.

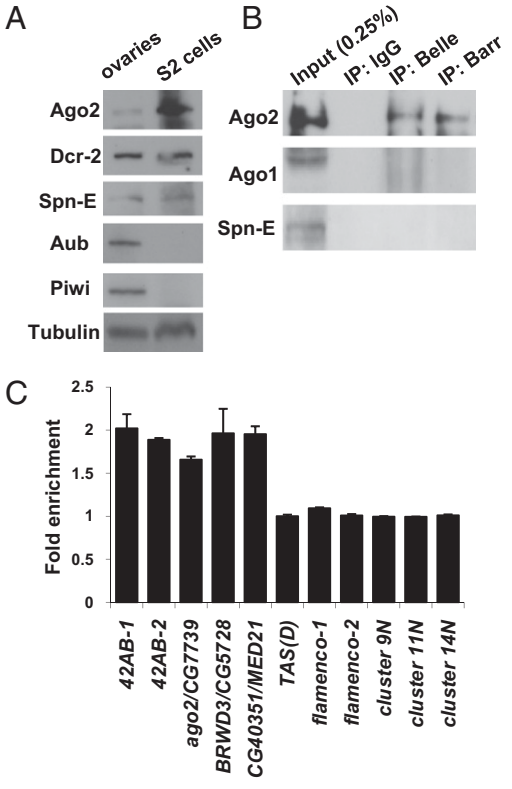

Fig. 2. Belle interacts with Argonaute 2 and endogenous siRNA-generating loci. (*A*) Western blots indicating the relative expression levels of the endo-siRNA pathway components (Ago2 and Dcr-2) and the piRNA pathway components (Spn-E, Aub, and Piwi) in ovaries and S2 cells. Tubulin was used as a loading control. (*B*) Western blots depicting the coimmunoprecipitation of endogenous Ago2 with endogenous Belle and Barr using S2 cell lysates. Belle and Barr did not coimmunoprecipitate with Ago1 and Spn-E, confirming the specificity of the assay. (*C*) A representative ChIP assay in S2 cells indicating the enrichment of endo-siRNA–generating loci in Belle immunoprecipitates normalized against bead controls. $n = 3$.

***dicer-2* and *argonaute2* Are Required for Chromosomal Localization of Belle and Proper Chromosome Segregation.** To further test our hypothesis, we investigated whether the endo-siRNA pathway components *dcr-2* and *ago2* are required for the chromosomal localization of Belle observed during mitosis in S2 cells. In cells treated with siRNA against *dcr-2* and *ago2*, the number of mitotic foci containing Belle was greatly diminished (≈14 foci in control compared with ≈4–5 in *dcr-2* or *ago2* knockdown cells), but not in the case of *spn-E* knockdown cells (≈15 foci) (Fig. 3 *A* and *B*). Moreover, knockdown of *dcr-2* and *ago2* did not lead to a reduction in the expression of Belle (Fig. S2). This finding supports our hypothesis that the endo-siRNA pathway specifically mediates the mitotic chromosomal localization of Belle.

Next, we examined whether the endo-siRNA pathway components are required for the proper chromosomal localization of Barr in mitotic wing disc cells. Indeed, in *dcr-2* and *ago2* mutants, metaphase chromosomal localization of Barr was less robust compared with that observed in wild-type cells (Fig. 3*C*; 70–75% defective, $n = 20$, compared with Fig. 1*B*). On the contrary, chromosomal localization of Barr was unaffected in the wing disc cells of mutants of the piRNA pathway components *spn-E*, *piwi* and *aub* (Fig. 3*C*; ≈94% normal, $n = 18$–21). Moreover, lagging chromosomes were frequently observed in >60% of *dcr-2* and *ago2* mutant wing disc cells at anaphase, but not in *spn-E*, *piwi,* and *aub* mutants (Fig. 3*D*). Our data are consistent with previous observations that *ago2* mutant embryos exhibit mitotic defects and reduced viability, suggesting a role for *ago2* during mitosis (39). Similarly, a delay in the progression of chromosome condensation (as indicated by a delay in mitotic progression at prophase) and an increase in chromosome segregation defects were also observed in cells treated with siRNA targeting *dcr-2* and *ago2* (from ≈10% in control cells to ≈30–40% in *dcr-2*- and *ago2*-knockdown cells) (Fig. 3 *E* and *F*). However, this result was not observed in *spn-E*–knockdown cells (≈10%) (Fig. 3 *E* and *F*), minimizing the possibility of off-target effects induced by RNAi. Taken together, our results indicate that, in somatic cells, the endo-siRNA pathway components *dcr*-2 and *ago2* are required to promote chromosome segregation, in part, by promoting robust chromosomal localization of Belle and Barr during mitosis.

Human DDX3 Promotes hCAP-H Chromosomal Localization and Chromosome Segregation. The common requirement for the Vasa/Belle class of RNA helicases in regulating chromosome segregation in both *Drosophila* germ-line and somatic cells prompted us to examine whether vertebrate somatic cells exhibit a similar requirement. Using HeLa cells, we studied the possible role in chromosome segregation played by DDX3, the human homolog of Belle that has been implicated to function as a tumor suppressor and to participate in viral RNA-sensing pathways (40).

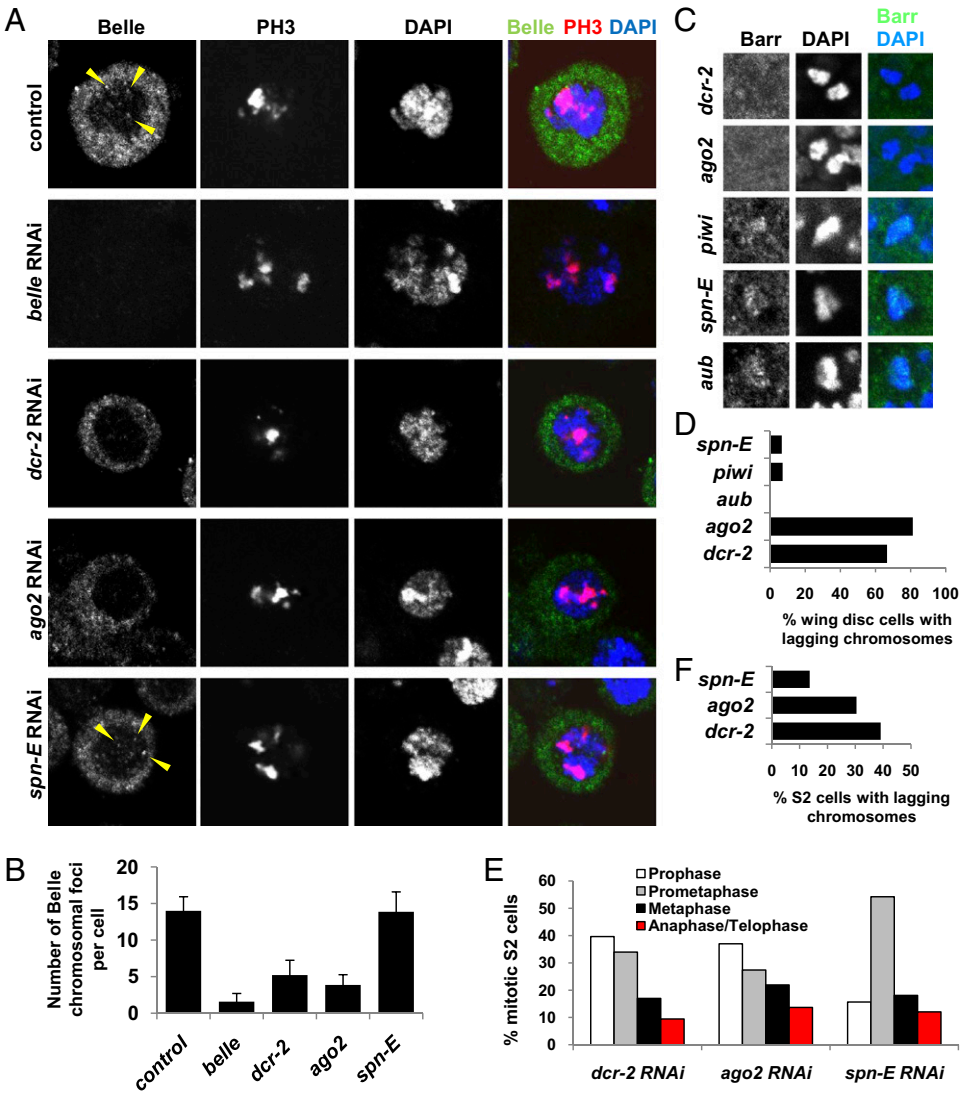

Fig. 3. *dicer-2* and *argonaute 2* are required for the chromosomal localization of Belle and proper chromosome segregation. (*A*) The localizations of Belle (green and arrowheads) during prophase in an S2 cell after the indicated siRNA knockdown treatments. Mitotic chromosomes (as determined by phosphorylated histone H3) are stained in red. (*B*) The quantification of the number of Belle chromosomal foci per cell. $n = 20$ cells. Error bars represent SDs. (*C*) Third instar larval metaphase wing disc cells of the indicated genotypes were stained for Barr (green) and DAPI (blue). (*D*) The percentages of wing disc cells at anaphase exhibiting lagging chromosomes. $n > 15$ for each genotype. (*E*) The percentages of S2 cells at each stage of mitosis. $n > 50$ for each experiment. (*F*) The percentages of S2 cells at anaphase exhibiting lagging chromosomes. $n > 20$ for each RNAi experiment.

Previous studies have demonstrated that human condensin I is required for proper chromosome segregation in HeLa cells (41, 42). In keeping with these results, during anaphase, we observed an increased incidence (>40%) of lagging chromosomes in hCAP-H knockdown cells compared with mock-treated controls (<5%) (Fig. 4 *A* and *B*), confirming a role for *hCAP-H* in chromosome segregation. Next, we investigated whether *DDX3* is required to promote the localization and chromosome segregation of hCAP-H. When the expression of DDX3 was knocked down (Fig. S4), the robust localization of hCAP-H to mitotic chromosomes was lost, and hCAP-H became dispersed compared with the localization of hCAP-H in mock-treated controls, whereas the expression of hCAP-H was unaffected (Fig. 4A and Fig. S4). Consistent with this phenotype of defective hCAP-H chromosomal localization, lagging chromosomes were frequently observed in >40% of DDX3-knockdown cells (Fig. 4 *A* and *B*). These defects were consistent with those observed after hCAP-H knockdown (Fig. 4*B*), suggesting that DDX3 functions to promote chromosomal localization of hCAP-H and to mediate chromosome segregation in HeLa cells.

In keeping with the results obtained regarding Belle in *Drosophila* somatic cells, we observed that DDX3 associates with hCAP-H through reciprocal coimmunoprecipitation experiments using HeLa cell lysates (Fig. 4*C*), further supporting a direct role for DDX3 in loading hCAP-H onto condensing chromosomes. Thus, our data suggest that the function of the DEAD-box helicases Belle and DDX3 in promoting chromosomal loading of the condensin I component Barr/hCAP-H appears to be conserved between *Drosophila* and humans.

***DICER* Promotes Mitotic Localization of DDX3 and hCAP-H.** Next, we examined the localization of DDX3 during mitosis in HeLa cells. During interphase, DDX3 localized to cytoplasmic foci, as reported (Fig. 4*D*) (43). During prophase/prometaphase, DDX3 localized in close proximity to the condensing chromosomes (Fig. 4*D*). We investigated whether this mitotic localization of

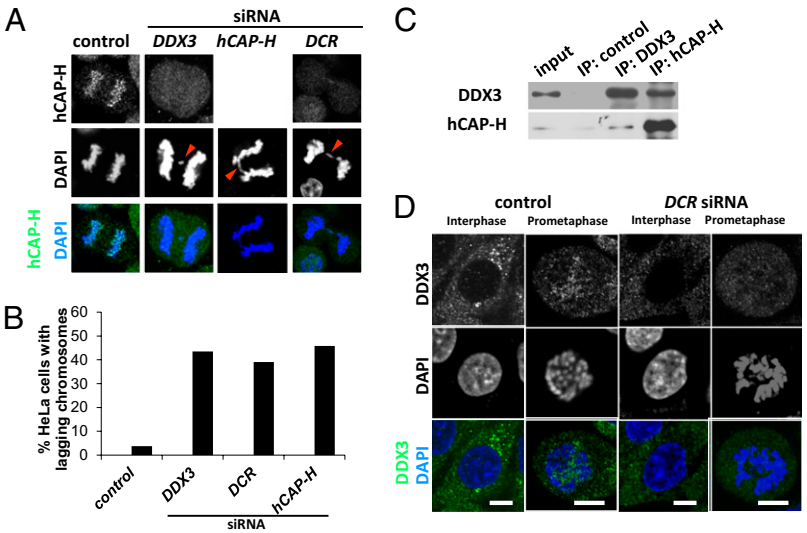

Fig. 4. *DDX3* and *DICER* are required for the robust chromosomal localization of hCAP-H and proper chromosome segregation in HeLa cells. (*A*) The localization of hCAP-H (green) in control, DDX3-, hCAP-H-, and DCR-depleted cells indicating chromosome segregation defects during anaphase (arrowheads). (*B*) The percentages of anaphase cells exhibiting segregation defects. $n > 20$ for each experiment. (*C*) Western blots depicting the coimmunoprecipitation of DDX3 and hCAP-H using HeLa cell lysates. (*D*) The localization of DDX3 (green) in control and DCR-depleted HeLa cells. (Scale bars: 10 μm.)

DDX3 depends on the RNAi pathway component DICER (DCR). When DCR was knocked down, the cytoplasmic foci associated with DDX3 during interphase became less prominent, and DDX3 did not localize near the chromosomes during prophase/prometaphase (Fig. 4*D*), although the expression level of DDX3 was unaffected (Fig. S4). This result suggested that the interphase and mitotic localizations of DDX3 were perturbed in the absence of functional DCR. Furthermore, in DCR-knockdown cells, the localization of hCAP-H to mitotic chromosomes was less robust in cells harboring lagging chromosomes (Fig. 4 *A* and *B*), although the expression level of hCAP-H was unchanged (Fig. S4). Taken together, our data suggest that DCR is required, at least in part, to promote the mitotic localization of DDX3 and hCAP-H in the regulation of chromosome segregation. Nevertheless, we cannot exclude the possibility that DCR may also simultaneously regulate other factors that contribute to chromosome stability, as reported (44).

Discussion

Although Vasa and Belle have been implicated in the piRNA and endo-siRNA pathways, respectively (7, 12, 34), we do not know whether DDX3 is also involved in the RNAi pathway. The fact that DDX3 is involved in viral RNA sensing offers the possibility that DDX3 may be a component of the RNAi pathway in humans (40). Furthermore, the DCR-dependent localization of DDX3, both during interphase and prophase (Fig. 4*D*), suggests that DDX3 may function downstream of DCR. Further investigation into the nature of the genomic loci and RNAi pathway components that associate with DDX3 and the nature of the noncoding RNAs involved in this process will provide greater insight into its molecular mechanism in human cells.

This study, in combination with our previous work (28), has indicated that the robust chromosomal localization of Barr/hCAP-H is regulated by the Vasa/Belle/DDX3 class of DEAD-box RNA helicases in both germ-line and somatic *Drosophila* cells and human somatic cells (as summarized in Fig. 5*A*). This finding suggests the possibility of a common pathway that regulates chromosome segregation by the Vasa/Belle/DDX3 class of RNA helicases. Although chromosome segregation appears to be regulated by RNAi machinery, the necessary small RNA pathway components vary notably between the germ-line and somatic cells (Fig. 5*A*). The piRNA pathway components are required in the germ-line cells, whereas the endo-siRNA pathway components function as their somatic counterparts. This finding suggests that various cell types can use the existing small RNAs and RNAi factors to achieve a common goal of robust Barr/hCAP-H localization (Fig. 5*B*). Our study also provides a framework for future studies investigating the molecular mechanism of the cooperation between the Vasa/Belle/DDX3 RNA helicases and the RNAi factors to ensure proper chromosome segregation.

Materials and Methods

Fly Strains. Fly strains and alleles used were *y w* (wild-type control unless otherwise stated), *vasa*^{PH165} (gift from P. Lasko, McGill University, Montreal, Quebec, Canada), *Df(2L)BCS299* (Bloomington Stock Center), *aub*^{N11}, *aub*^{HN2} (gifts from T. Schupbach, Princeton University, Princeton, NJ), *spn-E*^{616}, *spn-*

A

	Drosophila		Human
	germline cells	somatic cells	somatic cells
RNA helicase	Vasa	Belle	DDX3
RNAi machinery	*aub*, *spn-E*	*dcr-2*, *ago2*	*DCR*

B

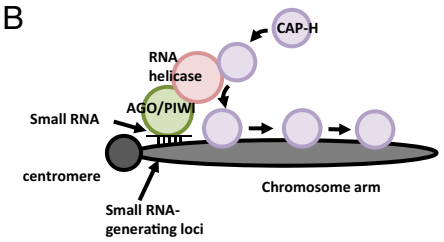

Fig. 5. Summary of results and proposed hypothetical model. (*A*) Summary of results describing the various requirements for RNA helicases and RNA interference factors in *Drosophila* germ-line and somatic cells and in human somatic cells. (*B*) Proposed hypothetical model of how RNA helicase may promote chromosomal recruitment of the condensin I component CAP-H via the small RNA pathway. AGO/PIWI, Argonaute or PIWI family proteins.

E^{hls} (gifts from D. St. Johnston, University of Cambridge and the Wellcome Trust/Cancer Research UK Gurdon Institute, Cambridge, United Kingdom), $piwi^{2}$ (gift from A. Spradling, Carnegie Institution, Baltimore, MD), $belle^{6}$ *FRT82B* (gift from N. Perrimon, Harvard Medical School, Boston, MA), $ago2^{414}$ (gift from M. C. Siomi, Keio University School of Medicine, Tokyo, Japan), $dcr\text{-}2^{L811fsX}$ (gift from R. Carthew, Northwestern University, Evanston, IL). Further information regarding symbols and genes can be obtained in flybase (http://flybase.org/).

Immunostaining. Immunostaining for wing imaginal discs was performed as described (15, 28).

Western Blotting. Western blot analysis was performed as described (15, 28).

Coimmunoprecipitation. Co-IP was performed, as described (28). Co-IP was performed with an IP buffer (50 mM Tris at pH 8.0, 100 mM NaCl, NaF, and 0.05% CHAPS) supplemented with a protease inhibitor mixture (Roche).

Detailed methods are provided in Figs. S5–S7, Table S1, and *SI Materials and Methods*.

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