AURKB-mediated effects on chromatin regulate binding versus release of XIST RNA to the inactive chromosome

Lisa L. Hall, Meg Byron, Gayle Pageau, and Jeanne B. Lawrence

Department of Cell Biology, University of Massachusetts Medical School, Worcester, MA 01655

How XIST RNA strictly localizes across the inactive X chromosome is unknown; however, prophase release of human XIST RNA provides a clue. Tests of inhibitors that mimic mitotic chromatin modifications implicated an indirect role of PP1 (protein phosphatase 1), potentially via its interphase repression of Aurora B kinase (AURKB), which phosphorylates H3 and chromosomal proteins at prophase. RNA interference to AURKB causes mitotic retention of XIST RNA, unlike other mitotic or broad kinase inhibitors. Thus, AURKB plays an unexpected role in regulating RNA binding to heterochromatin, independent of mechanics of mitosis. H3 phosphorylation (H3ph) was shown to precede XIST RNA release, whereas results exclude H1ph involvement. Of numerous Xi chromatin (chromosomal protein) hallmarks, ubiquitination closely follows XIST RNA retention or release. Surprisingly, H3S10ph staining (but not H3S28ph) is excluded from Xi and is potentially linked to ubiquitination. Results suggest a model of multiple distinct anchor points for XIST RNA. This study advances understanding of RNA chromosome binding and the roles of AURKB and demonstrates a novel approach to manipulate and study XIST RNA.

Introduction

In mammalian female cells, the faithful localization in cis of XIST RNA across one X chromosome is the initiating step in its subsequent silencing. Thus, the mechanism by which this unusual chromosomal RNA localizes to, spreads across, and stably binds its chromosome of origin is essential to its function. 15 yr after the discovery of XIST RNA (Brown et al., 1992), almost nothing is known about what regulates XIST RNA binding to the chromosome. Despite long-standing attempts by many laboratories to isolate or identify proteins in a specific XIST RNA "complex" using standard biochemical approaches (Brown and Baldry, 1996; for review see Brockdorff, 2002), there still has been very little success, possibly because of the tight association of the RNA with nuclear structure. XIST RNA is so tightly bound at interphase that it remains localized under a variety of fixation and extraction conditions, including extensive nuclear matrix extraction procedures (Clemson et al., 1996). The latter observation suggests that the RNA is unlikely bound by hybridization to DNA, but even this is not known for certain. A study using mouse XIST RNA transgene constructs reported that multiple parts of the XIST RNA promote its localization (Wutz et al., 2002), which suggests that each large (14 kb) XIST RNA transcript may bind more than one site on the chromosome/chromatin. In contrast to the faithful chromosomal localization at interphase, this tight binding is lost at mitosis, during which the RNA can be seen visibly released from the inactive chromosome and appears by RNA FISH as bright punctate dots distributed throughout the cytoplasm (Clemson et al., 1996; this study). Identification of defined conditions that release XIST RNA from the chromosome at interphase or maintain binding during mitosis would be significant for breaking the impasse in understanding the underlying mechanism of XIST RNA localization and chromosome binding.

Correspondence to Lisa L. Hall: Lisa.Hall@umassmed.edu; or Jeanne B. Lawrence: Jeanne.Lawrence@umassmed.edu

Abbreviations used in this paper: AURKB, Aurora B kinase; CANTH, cantharidin; CPC, chromosomal passenger complex; HESP, Hesperadin; IF, immunofluorescence; INCENP, inner centromere protein; NOC, nocodazole; OKA, okadaic acid; SB, sodium butyrate; STSP, staurosporine; TAUT, tautomycin; TSA, trichostatin-A.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 186 No. 4 491–507
www.jcb.org/cgi/doi/10.1083/jcb.200811143

One could imagine that XIST RNA binding might be compromised during mitosis as the result of steric constraints imposed as DNA condenses, or, if XIST RNA is bound through chromosomal proteins, biochemical changes to chromatin may directly alter binding affinity. Unlike the more permanent chromatin modifications on the Xi that follow the initial spread of XIST RNA, mitotic modifications, particularly phosphorylation, are transient (Barber et al., 2004; for review see Nowak and Corces, 2004), as is the change in XIST RNA binding. Thus, we reasoned that a new approach based on investigating what controls these in situ changes in XIST RNA chromosomal association may yield inroads into what has been an intractable problem. In this study, we began by testing several manipulations that impact chromatin protein modifications, with emphasis on phosphorylation, to determine whether such perturbations alone could release XIST RNA in the absence of mitosis and chromosome condensation. We identify specific manipulations that indeed released XIST RNA at interphase and, more importantly, reciprocal manipulations that can cause XIST RNA retention on human metaphase chromosomes. These findings unexpectedly link Aurora B kinase (AURKB) activity, which has known effects on chromatin phosphorylation, as key to the regulation of this noncoding RNA's interaction with heterochromatin.

Results

Inhibitors of PP1 (protein phosphatase 1) release XIST RNA from the inactive chromosome at interphase

We began by investigating whether the mitotic conditions that release XIST RNA from its normally restricted localization (Fig. 1, A–C) could be mimicked during interphase by increasing chromatin phosphorylation. Okadaic acid (OKA), cantharidin (CANTH), and tautomycin (TAUT) are potent, specific, and cell-permeating inhibitors of Ser/Thr phosphatases whose cellular effects and specificities have been widely investigated (Knapp et al., 1998; Dawson and Holmes, 1999; Honkanen and Golden, 2002). We began by testing inhibitor concentrations previously reported to be specific for phosphatase inhibition in intact cells but not high enough to produce premature chromosome condensation (Ajiro et al., 1996; Favre et al., 1997; Zhang et al., 2005). Using our standard fixation protocols (see Materials and methods), disassociation of XIST RNA from the parental chromosome was seen in interphase nuclei within 4–6 h of introducing 2–9 μM TAUT (Fig. 1). In both Tig-1 diploid fibroblasts and HT1080 G3 cells (XIST transgene cell line; see Materials and methods), XIST RNA released from the inactive chromosome and dispersed throughout the nucleoplasm as bright punctuate dots (Fig. 1, D and E), which is similar to what is seen at mitosis (Fig. 1 C). TAUT has been shown to be specific for PP1 inhibition at 10 μM in vivo, and in this study, we find that the lowest concentration (2 μM) that still affects XIST RNA binding (∼30% of the population) is at the lower end of this reported range (Fig. 1 G). Additionally, the nuclear morphology in these cells still appears fairly normal, with no condensation of chromosomes evident (Fig. 1, D and E).

However, OKA and CANTH inhibit PP2A at low concentrations in vivo (∼100 nM OKA and 1 μM CANTH; Favre et al., 1997; Zhang et al., 2005) but also inhibit PP1 at 10–100-fold higher concentrations (Bialojan and Takai, 1988; Knapp et al., 1998). In this study, we find that concentration ranges specific for PP2A (<5 μM CANTH and <170 nM OKA) do not result in substantially mislocalized XIST RNA (Fig. 1 G and Fig. S1 B) and that only concentrations much higher than those reported to be specific for PP2A inhibition (≥300 nM OKA and ≥10 μM CANTH) produce a significant effect on XIST RNA binding. However, these high levels of CANTH and OKA also caused premature chromosome condensation (see Fig. 5 D) and showed evidence of toxicity (cell loss, debris, and severely altered nuclear morphology; Fig. 1 F), which was not seen in samples using lower and more specific inhibition of PP1 by TAUT. This suggests that the more specific effect on XIST RNA localization seen by TAUT was likely caused by the specific inhibition of PP1, whereas the effect on XIST RNA binding seen at very high, nonspecific concentrations of CANTH and OKA may be caused by the inclusion of PP1 inhibition along with PP2A.

Because many phosphatases and kinases are intimately linked in a biochemical network, we tested whether disruption of XIST RNA binding was affected by a more general disruption of overall cellular phosphorylation or by lethal levels of chromatin modifying drugs. Using the broad range kinase inhibitor staurosporine (STSP), compaction of the inactive chromosome appeared to be affected but not XIST RNA binding, even at very high concentrations (Fig. S1). Similarly, deacetylase inhibitors sodium butyrate (SB) and trichostatin-A (TSA), which dramatically increase nucleoplasmic acetylation, also had no effect on XIST RNA binding in both cell types at a broad range of concentrations, including toxic levels (0.5–20 μM), and for 6 or 18 h (Fig. S1, D and E). Thus, broad concentration ranges of several inhibitors (Fig. S1 F), including toxic levels, had no apparent impact on XIST RNA binding in the cell lines tested, and only the PP1 inhibitor TAUT induced significant release of XIST RNA at low concentrations in interphase (Fig. 1 G).

Inhibitors of PP1 may indirectly affect XIST RNA binding via activation of AURKB, which impacts chromatin phosphorylation

We next considered what proteins or processes might be affected by PP1 activity, with emphasis on chromatin changes that also occur at mitosis. However, because phosphorylation is known to impact mRNA splicing factors (Misteli and Spector, 1999; Huang and Steitz, 2005; Clemson et al., 2006), we first considered whether a block in splicing might play a role in XIST RNA mislocalization. We previously demonstrated that simultaneous two-color hybridization with intron and cDNA probes allowed discrimination of spliced versus unspliced nuclear RNA accumulations (Xing et al., 1995; Clemson et al., 1996; Johnson et al., 2000). Using this approach, we found that TAUT-treated cells show efficient splicing, as judged by the splicing of introns 1 and 2, because treated and control cells show the same small focus of intron signal within the larger cloud of mature XIST RNA (Fig. 2, A and B).

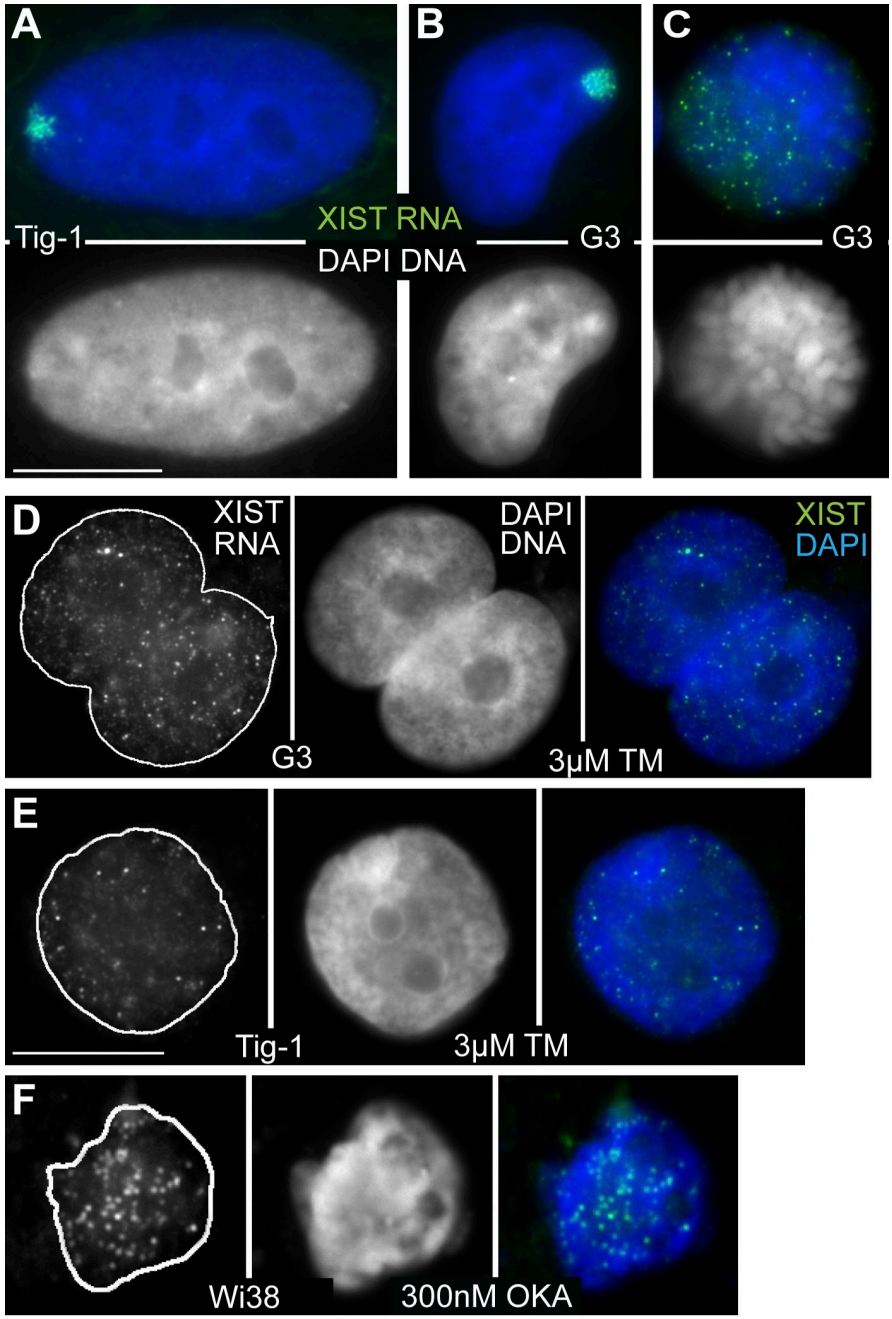

Figure 1. **PP1 inhibition causes mislocalization of XIST RNA.** (A and B) Normal female fibroblasts (Tig-1; A) and XIST transgenic HT1080 cells (G3; B) show tightly localized XIST RNA. (C) XIST RNA binding is lost at mitosis (G3). (D and E) TAUT (TM) treatment of G3 (D) or Tig-1 (E) cells causes release of XIST RNA in interphase. (F) High levels of OKA (or CANTH) can cause XIST RNA drift in interphase cells. (D–F) Using image morphometrics (MetaMorph) the edges of the nucleus (from the blue channel) are imposed over the green channel to identify the limits of the nucleus for better viewing of the drifting XIST RNA signal. Note that the XIST RNA signals remain within the nucleus defined by the white lines. (G) Only TAUT treatment effectively results in delocalization of the XIST RNA from its chromosome of origin at low (specific) concentrations. Bars, 10 μm.

Furthermore, introns were not detected in the drifting XIST RNA signal in treated cells (Fig. 2 B). Although we did not directly test all potential permutations of splicing defects, this indicates that the mislocalization of XIST RNA was not caused by a general block of splicing factor activity.

The ability to release XIST RNA at interphase using inhibitors of protein phosphorylation supports the idea that the RNA's localization depends on interaction with chromosomal proteins, which undergo modification at mitosis. During interphase, PP1 is known to directly repress the activity of the kinase responsible for histone H3 phosphorylation (H3ph) at the beginning of mitosis, AURKB (Ajiro et al., 1996; Murnion et al., 2001; Edmondson et al., 2002; Sugiyama et al., 2002). Thus, we used an antibody raised against phosphorylated H3S10, and found that in TAUT-treated interphase cells, it was sharply increased relative to CANTH-treated and untreated controls. In untreated or CANTH-treated cells, H3ph is only abundant in mitotic cells (Fig. 2, C and D; Hendzel et al., 1997), whereas many interphase cells (47%) showed high H3ph in 2 μM TAUT–treated samples (Fig. 2 E). This correlated well with the frequency of cells showing mislocalized XIST RNA in parallel samples (Fig. 1 G), suggesting that AURKB kinase may be activated in cells in which XIST RNA binding was affected. We tested this directly by doing simultaneous staining of H3S10ph and XIST RNA in single cells. Even in samples in which the less specific 1 μM OKA was used to inhibit PP1, the presence of H3S10ph in interphase cells correlated to mislocalization of XIST RNA (Fig. 2, K and L), suggesting that the presence of drifting XIST RNA was linked to the activation of AURKB.

At the same time, we found that H1ph, which is also normally elevated at mitosis (Fig. 2, F–H), was not increased in TAUT-treated H3ph-positive interphase cells (Fig. 2, I and J). This indicates that TAUT was not affecting histone phosphorylation more broadly because H1ph is controlled by another kinase at mitosis (CDC2/cyclin B). This also demonstrates that the high levels of H1ph seen at mitosis are not necessary for XIST RNA release.

These findings suggest that once PP1 is inhibited during interphase by TAUT, the known repression on AURKB is released (similar to that seen upon normal entry into mitosis), and H3ph is induced prematurely. Thus, inappropriate activation of AURKB may play a role in the destabilization of XIST RNA binding to chromatin in TAUT-treated interphase cells and during normal mitosis as well. To determine whether the effects of PP1 are via its effects on AURKB, we next examined inhibition of AURKB.

Inhibitors of AURKB block release of XIST RNA on metaphase chromosomes and counter interphase release induced by phosphatase inhibitor

If the interphase release of XIST RNA after PP1 inhibition is indirectly caused by the derepression of AURKB, the inverse should be true: the inhibition of AURKB at mitosis should maintain interphase-like binding conditions and retain XIST RNA on human metaphase chromosomes. Hesperadin (HESP) and ZM447439 (ZM) are small molecules identified as inhibitors of AURKB kinase for chemotherapy and inhibit AURKB in vivo at nanomolar concentrations (Hauf et al., 2003; Yang et al., 2005; Agnese et al., 2007; Lipp et al., 2007). Using concentrations of HESP and ZM shown to inhibit AURKB in intact cells, we find that XIST RNA was strikingly retained across the human metaphase chromosome, in a manner not seen in untreated control cells, or control cells treated with the broad kinase inhibitor STSP. In two different human cell types (Tig-1 and G3), the vast majority of cells in metaphase control preps retained essentially no XIST RNA bound to the chromosome (Fig. 3, A and B). This was true even for mitotic cells that were only fixed in paraformaldehyde and cytospun with no additional extraction step (see Materials and methods). In contrast, under identical conditions, many HESP- and ZM-treated cells had chromosomes that were completely or almost completely painted by XIST RNA well into metaphase, as was apparent on the metaphase rosette (Fig. 3 C). The retention of XIST RNA was apparent at metaphase, which is well past the point in early prophase when the binding of the RNA is normally compromised. No metaphase rosettes in control cells showed this extensive XIST RNA chromosome painting, although a few early metaphase cells in the untreated G3 line retained residual amounts, possibly because of the highly robust XIST RNA signal in this line. Because AURKB inhibition prevents normal anaphase figures and chromosome segregation (such that cells release from mitosis as polyploid), only metaphase cells were scored in both control and treated samples. Results were consistent between both cell lines and over three different experiments and were apparent even on cells extracted with detergents and cytospun onto slides, further indicating that the RNA remained tightly bound in treated cells, which is similar to what is seen at interphase.

Importantly, this mitotic retention of XIST RNA by AURKB inhibitors was not caused by general effects of mitotic perturbation because we tested whether the same effect would be seen with nocodazole (NOC), which arrests cells in prometaphase. Thus, even though NOC perturbs mitosis even earlier than AURKB inhibition, it had no effect on the normal release of XIST RNA at prophase; results were indistinguishable from controls in which only very rare cells (1 in 50) showed substantial XIST RNA still on the metaphase chromosome. XIST RNA retention by AURKB inhibitors was also not caused by inhibition of kinases in general, as STSP, which is known to inhibit numerous kinases in vivo (except Aurora kinases), did not cause mitotic retention of the RNA. AURKB not only orchestrates mitotic chromosome movements, it also induces major changes to chromatin across the chromosome arms (see Discussion). In HESP- and ZM-treated cells, H3ph (Fig. S4, C and D) was absent from mitotic chromosomes, further indicating that AURKB's normal prophase role of chromatin phosphorylation across the chromosome arms was inhibited. Like XIST RNA, HP1 was also retained under these conditions compared with nontreated mitotics (Fig. S4, A and B), confirming that HP1 release is linked to H3S10ph and AURKB activity (Hauf et al., 2003; Fischle et al., 2005; Hirota et al., 2005; Yang et al., 2005; Terada, 2006).

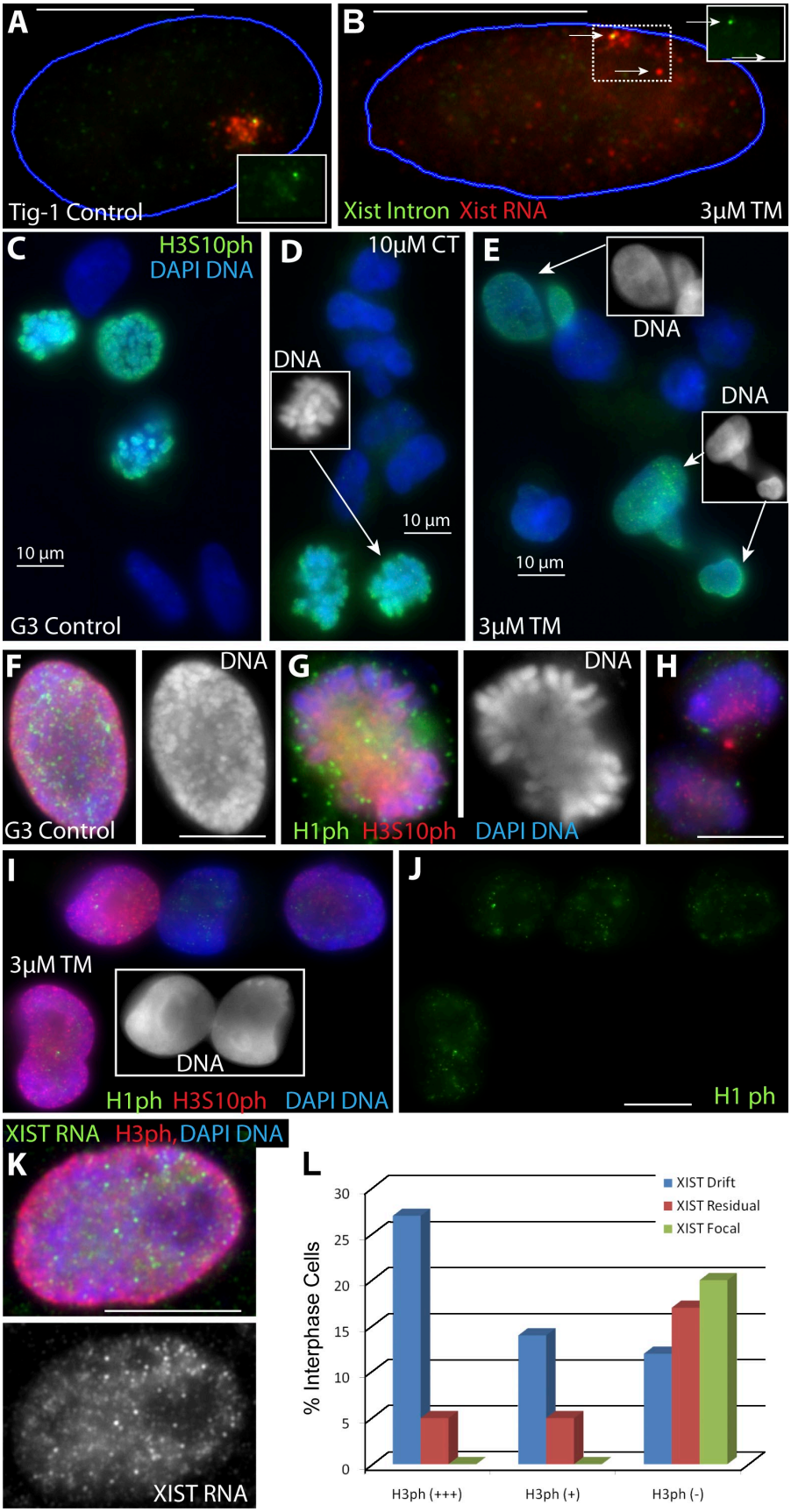

Figure 2. **Phosphatase inhibition affects H3ph not H1ph or XIST RNA splicing.** (A and B) Untreated and TAUT (TM)-treated Tig-1 cells demonstrate splicing of two XIST RNA introns within the mature XIST RNA accumulation. The green intron signal is separated as an inset. Arrows in the main image identify two regions of XIST RNA signal (red); the larger top one remains localized to the Xi, and the small bottom one is drifting. The arrows in the inset identify only the top XIST signal still contains intron 1 (green). The bottom arrows point to an example of spliced XIST RNA (lacking green intron), which is mislocalized in the nucleoplasm. Using image morphometrics (MetaMorph) the edges of the nucleus (from the blue channel) are imposed over the green channel to identify the limits of the nucleus for better viewing of the drifting XIST RNA signal. Note that the XIST RNA signals remain within the nucleus defined by the blue lines. (C and D) In untreated and CANTH (CT)-treated Tig-1 cells, only mitotic cells stained positive for H3ph, as shown by the black and white inset of DAPI DNA stain. (E) H3ph is commonly seen in interphase cells upon TAUT treatment, as shown by the black and white insets of DAPI DNA stain. (F–H) Control cells (and CANTH-treated cells) show both H1ph and H3ph in prophase (F) and metaphase (G), but H1ph is lost before H3S10ph, as in anaphase/telophase (H). (I and J) In TAUT-treated cells, H1ph does not increase (J) in cells that show marked increase in H3S10ph (I). DAPI DNA (inset) confirms interphase cells. (K) Increased H3ph in interphase cells is correlated with drifting XIST RNA in 1 μM OKA–treated G3 cells. (L) In 100 OKA-treated cells, XIST RNA tightly localized to one large focal accumulation (XIST focal) only in cells with low H3ph (−). XIST RNA was partially mislocalized (XIST residual) in cells with high H3ph (+) and completely mislocalized (XIST drift) in cells with the highest H3ph (+++). Bars, 10 μm.

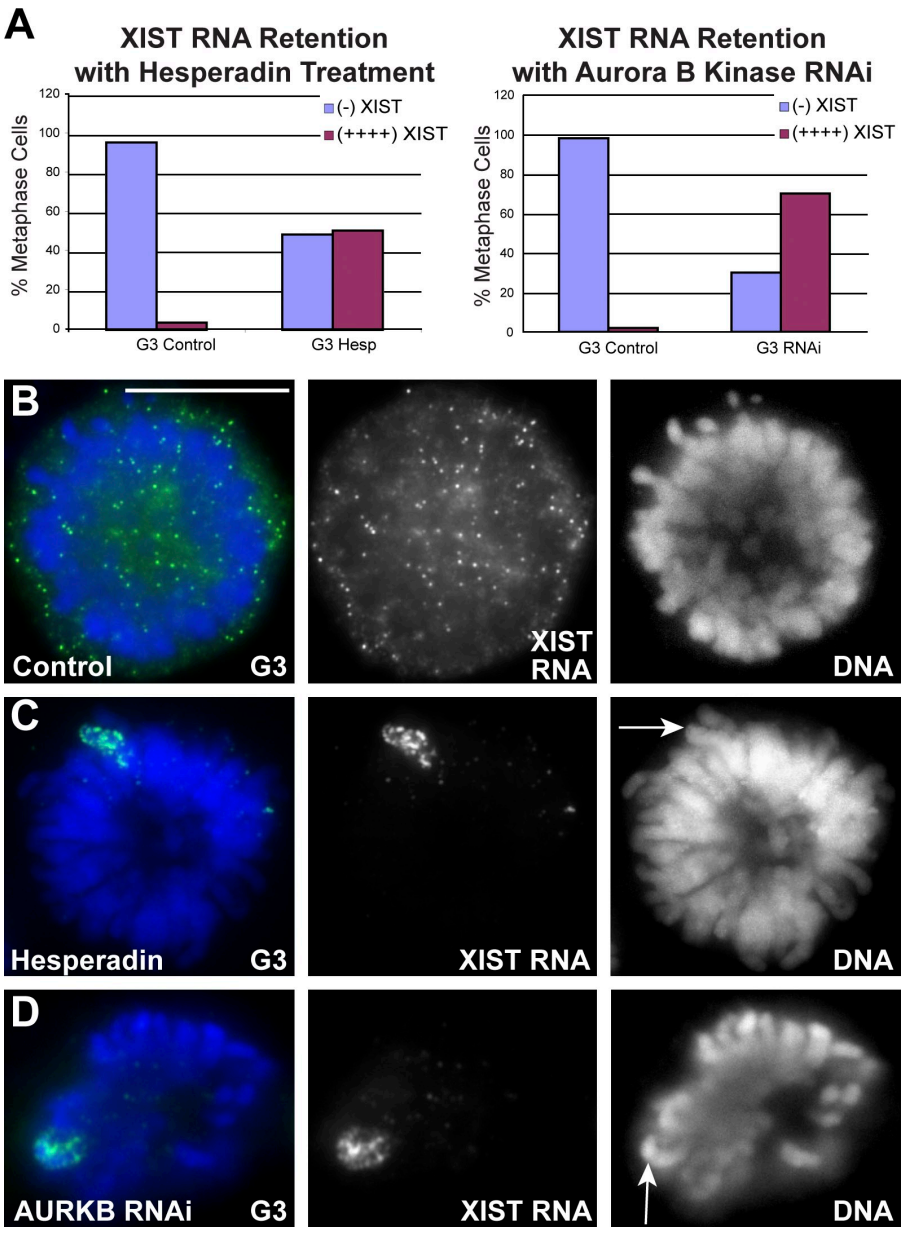

Figure 3. **XIST RNA is retained on human metaphase chromosomes when AURKB is inhibited.** (A) HESP- or AURKB RNAi–treated G3 cells show robust XIST RNA retention (++++) on metaphase cells. (B) Control G3 metaphase cells show drifting XIST RNA. (C) HESP induces marked retention of XIST RNA on the inactive metaphase chromosome (arrow). D) AURKB RNAi resulted in similar retention of XIST RNA on the metaphase inactive chromosome (arrow). Bar, 10 µm.

All of these results are consistent with the involvement of AURKB in XIST RNA binding and suggest that the release of XIST RNA at interphase by phosphatase inhibitors may occur by blocking the PP1 repression of AURKB. To test this, we attempted to rescue the interphase release of XIST RNA by simultaneously inhibiting PP1 and AURKB. Despite the potential for more nonspecific or toxic effects by dual pharmacological inhibitors, we were surprised to see that treatment with the AURKB inhibitor during interphase (for 2 h before PP1 inhibition) can suppress the release of XIST RNA, even using the more nonspecific inhibitor OKA (Fig. 4). This further supports that (a) the XIST RNA interphase release is a relatively specific effect of particular phosphatase inhibitors, (b) the interphase release likely involves PP1 inhibition and the consequent activation of AURKB, and (c) increasing the potential toxic or nonspecific effects by using two drugs simultaneously does not further degrade XIST RNA binding but still allows inhibition of AURKB to largely rescue it.

Specific inhibition of AURKB by RNAi shows that it is required for the normal prophase release of human XIST RNA

Although the aforementioned results implicate the involvement of AURKB kinase, pharmacological inhibitors are not entirely specific; for example, in some circumstances, HESP and ZM can also impact other Aurora kinases (Agnese et al., 2007). Therefore, it was important to perform RNAi specifically targeted against AURKB, which we did in both Tig-1 and G3 cells using an AURKB SMARTpool siRNA. By optimizing transfection protocols, we achieved ~50–70% transfection efficiencies in Tig-1 and G3 cells. Use of a robust immunofluorescence (IF) assay together with FISH provided the advantage that the impact of RNAi on AURKB could be assessed in individual cells simultaneously with XIST RNA. As documented in Fig. 5, RNAi eliminated any detectable AURKB in over half of metaphase cells by 24 h, with another

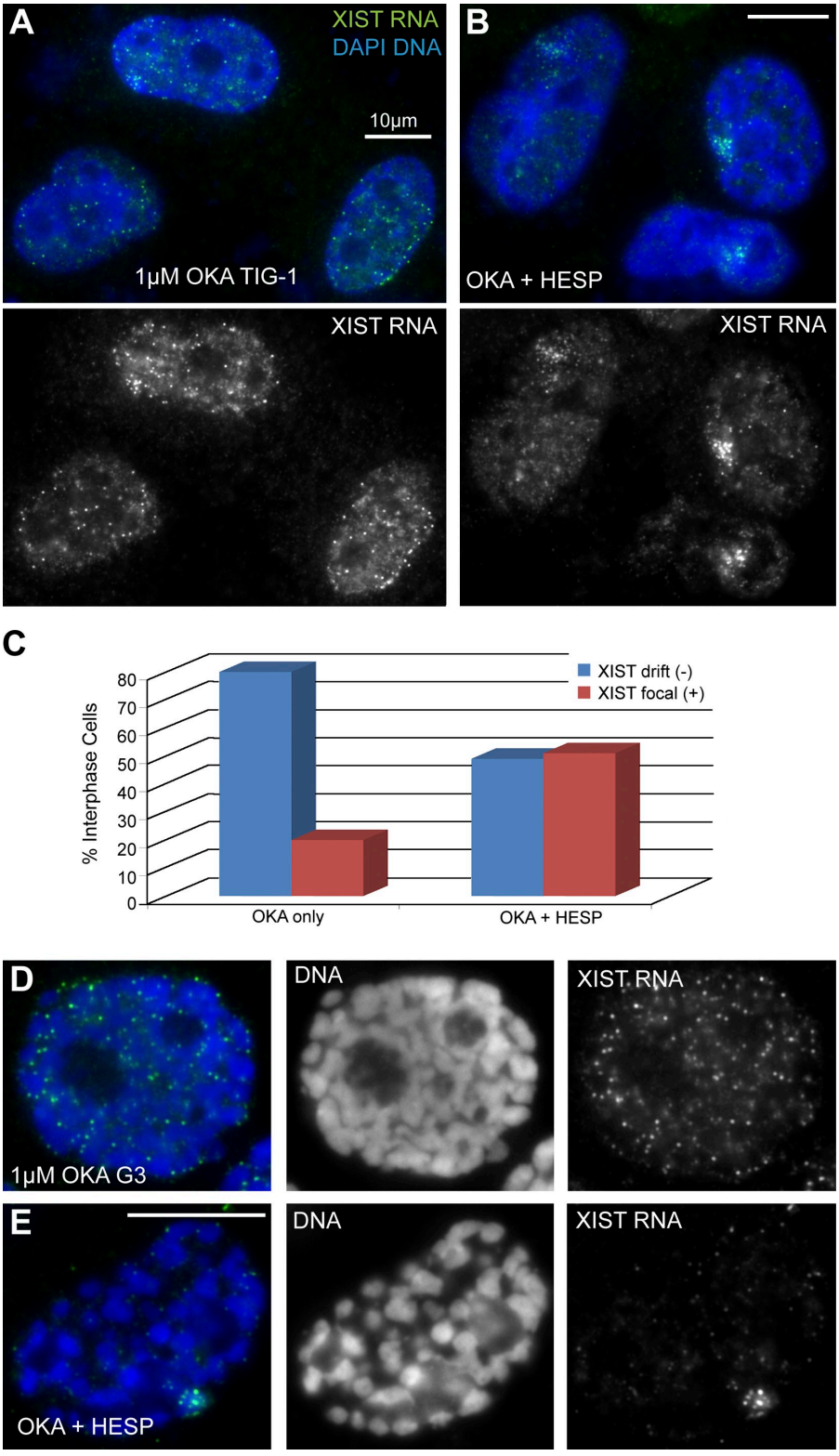

Figure 4. **Inhibition of AURKB activity impedes XIST RNA release during PP1 inhibition.** (A–C) OKA inhibition of PP1 in G3 cells causes XIST RNA release in interphase cells (A and C), whereas the double inhibition of PP1 (with OKA) and AURKB (with HESP) allowed the retention of XIST RNA in many cells (B and C). The green (XIST RNA) channel is separated below each image. (D and E) 1 μM OKA causes premature chromosome condensation in many cells (see DNA channel), but the retention of XIST RNA in OKA + HESP–treated cells occurs despite the premature chromosome condensation. Bars, 10 μm.

fraction showing significantly reduced staining. All metaphase cells in control slides exhibit bright AURKB staining (Fig. 5, A and C). Importantly, the AURKB RNAi–treated cell population showed significant retention of XIST RNA on metaphase chromosomes relative to control cells (untreated and nonspecific RNAi controls; Fig. 3, A and D). This was evident by the amount and frequency of retention and was reproducible in over six RNAi experiments (using two different cell lines). In addition to many metaphase chromosomes retaining XIST RNA, a few AURKB-inhibited cells were noted to have progressed into anaphase, with XIST RNA still associated, as illustrated in Fig. S5. These rare cells had substantial but incomplete

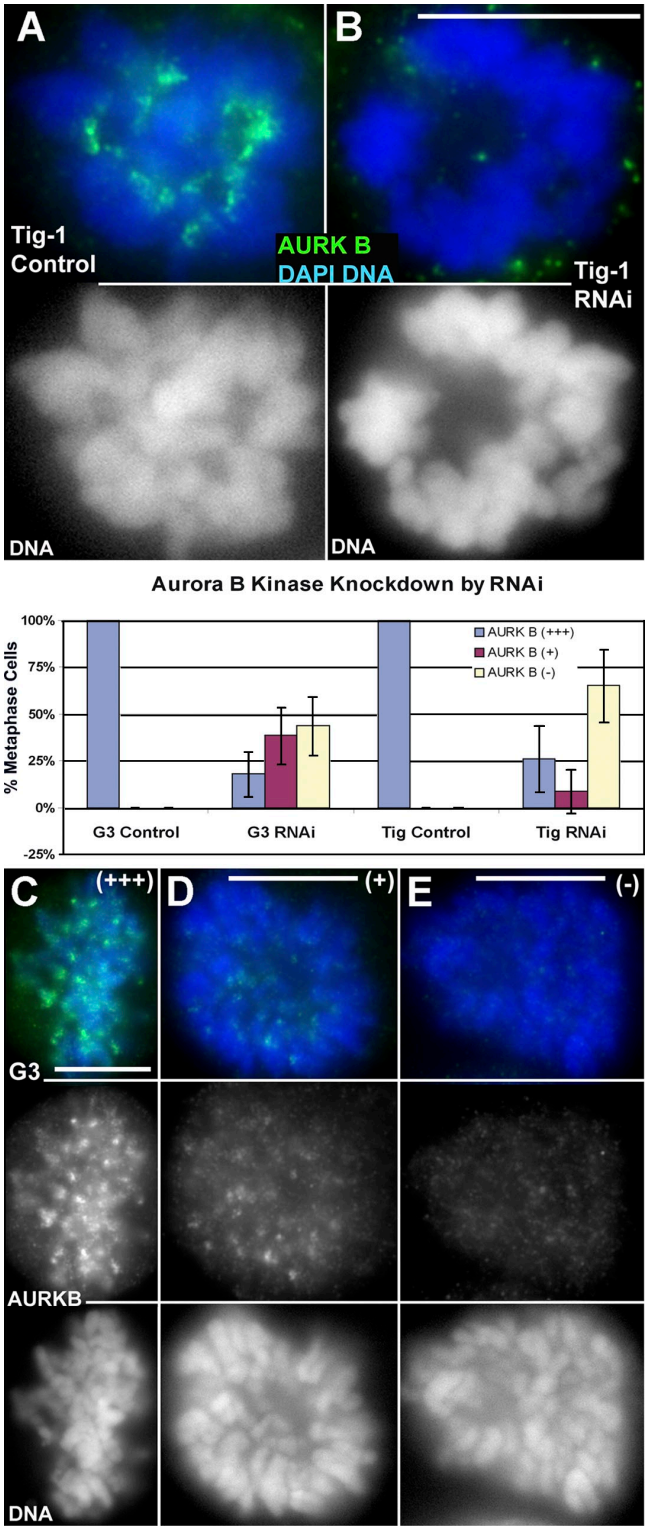

Figure 5. **RNAi effectively eliminated AURKB in two different cells lines.** (A) AURKB is abundant and localized to centromeres at metaphase in normal female fibroblasts (Tig-1). (B) AURKB is eliminated after RNAi treatment. DAPI DNA channels are separated below. (graph) AURKB was essentially eliminated (−) in most metaphase Tig-1 or G3 cells, whereas some retained a low level (+) or were unaffected (+++). Error bars indicate the SD between the two experiments performed. (C–E) As scored in the graph, examples of cells showing unaffected (+++; green), reduced low level (+), or eliminated (−) AURKB. Exposures were equal for all three images. Bars, 10 µm.

reduction of AURKB, which was sufficient to promote XIST RNA retention but not to fully block anaphase progression. This likely relates to the dynamics and relative amounts of AURKB required for different functions, as considered in the Discussion, and provides further evidence that AURKB effects on XIST RNA binding can be separated from the mechanics of mitosis.

These findings clearly establish that AURKB does specifically play a key role in the regulation of XIST RNA binding to the inactive chromosome, providing the first knowledge of the specific players controlling this interaction. Results indicate that AURKB activity is required for the normal release of human XIST RNA, which occurs in mitotic prophase, and further suggest that this role of AURKB is independent of its role in orchestrating chromosome segregation at anaphase (see Discussion).

H3ph is not sufficient for XIST RNA release

These findings now make it possible for the first time to manipulate XIST RNA chromosomal binding as a new approach to investigate chromatin-associated factors, either upstream or downstream of the change in XIST RNA's binding. Because essentially nothing is known about this and there are many factors potentially involved, any information that narrows the possibilities is valuable.

Mitotic histone phosphorylations on H1 and H3 are of particular interest because they both increase dramatically across chromosome arms at early prophase, during the same ∼5–10 min window when XIST RNA binding is altered. (Because mitosis takes <1 h, this window of prophase corresponds to just a few minutes.) However, the aforementioned results show that H1ph is not required for XIST RNA release because TAUT did not induce H1ph at interphase when XIST RNA released (Fig. 2, I and J), and H1 is not phosphorylated by AURKB. In contrast, H3ph may be required because H3ph (on both Ser10 and Ser28) is phosphorylated by AURKB (as we confirmed by inhibition with HESP; Fig. S4 D), and we consistently saw XIST RNA released under conditions that also increased H3ph.

The first indication that H3ph does not itself lead to loss of proper XIST RNA binding comes from comparing the detailed timing of each of these in human versus mouse mitotic cells. Previous studies have shown that mouse cells retain the interphase-like binding affinity of XIST RNA longer into mitosis (Fig. S2), with some cells still showing RNA associated with the chromosome into anaphase (Clemson et al., 1996; Lee and Jaenisch, 1997; Duthie et al., 1999; Smith et al., 2004), although we find that it is ultimately released before G1. This release of XIST RNA occurs significantly after the increased histone phosphorylation seen along the chromosome arms at prophase (Figs. S2 and S3), suggesting that high levels of H3ph may not be sufficient to affect XIST RNA binding. However, the clearest evidence that H3ph is not sufficient came from analysis of H3ph after RNAi to AURKB. Unlike HESP treatment, which blocked H3ph and caused XIST RNA retention at metaphase (Fig. S4, C and D), RNAi to AURKB also caused XIST RNA retention at metaphase, but, unexpectedly, the

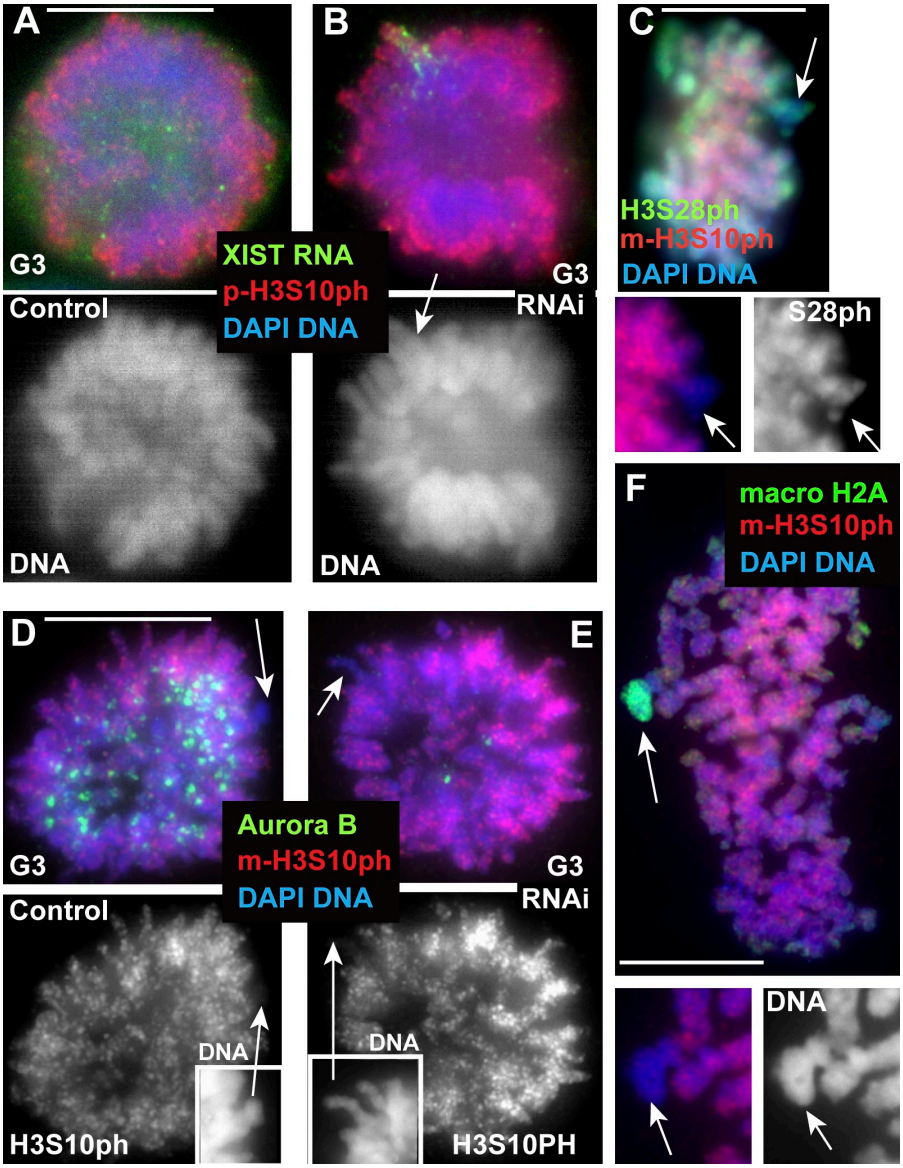

Figure 6. **H3S10ph and H3S28ph are distinct on the Xi, suggesting that H3S10 may not be involved in XIST RNA retention after AURKB inhibition.** (A and B) A polyclonal antibody raised against H3S10ph lights up control and AURKB RNAi–treated cells, including the inactive chromosome (arrow) covered by XIST RNA. (C) The inactive chromosome (arrows) labels with an antibody specific to H3S28ph but not with a monoclonal antibody specific to H3S10ph. (D and E) H3S10ph (monoclonal) is seen in both control and AURKB inhibited cells, except for a single chromosome (arrows). Small insets show DAPI DNA. (F) MacroH2A staining confirms that it is the inactive chromosome (arrows) that lacks monoclonal H3S10ph. Bars, 10 μm.

chromosomes continued to stain brightly for H3ph, which is similar to controls (Fig. 6, A and B).

The presence of H3ph after AURKB RNAi (in mitotic cells confirmed by IF to lack AURKB; Fig. 6, D and E) was surprising in light of several studies (primarily in HeLa cells), which reported that AURKB was required for mitotic H3ph (of either Ser10 or Ser28; Fischle et al., 2005; Hirota et al., 2005; Terada, 2006). We tested RNAi to AURKB in HeLa cells and confirmed that it does indeed block H3ph (Fig. S4, G and H), and HP1 remains bound (Fig. S4, E and F), as previously reported for HeLa (Dormann et al., 2006). Thus, we demonstrate a reproducible difference between cell types. It's possible that some functions of AURKB can be rescued in the primary fibroblast line (and in G3 cells) but not in HeLa cells. For example, Aurora C kinase can rescue the mitotic H3ph function in some cell types (Sasai et al., 2004) and would also be inhibited by HESP (Agnese et al., 2007) but not RNAi to AURKB. Because HeLa cells have lost the Xi, we did not examine XIST RNA in these cells; nevertheless, the results in normal fibroblasts indicate that increased H3ph can still occur under conditions that retain mitotic XIST RNA; thus, H3ph is not sufficient to release XIST RNA.

Given the aforementioned findings, we examined whether there may be unanticipated differences between the phosphorylation of H3S10 versus H3S28 because both could potentially be detected by the polyclonal antibody used (raised against H3S10ph) and both increase in concert at the onset of mitosis (via AURKB; Goto et al., 1999). To examine H3S10 and H3S28 specifically, we obtained monoclonal antibodies that detect H3S10ph and H3S28ph specifically. Surprisingly, although phosphorylation of both H3S10 and H328 increases at mitosis on all other human chromosomes, H3S10ph was not detected with the monoclonal antibody on the inactive X chromosome (Fig. 6, C–F), which was positively identified by the presence of macroH2A (Fig. 6 F). However, the inactive chromosome does still label with an antibody raised against H3S28ph (Fig. 6 C; unpublished data). Thus, if H3S10ph is not present on the mitotic Xi, it can play no role in the compromised XIST RNA binding seen at prophase, although we cannot rule out the

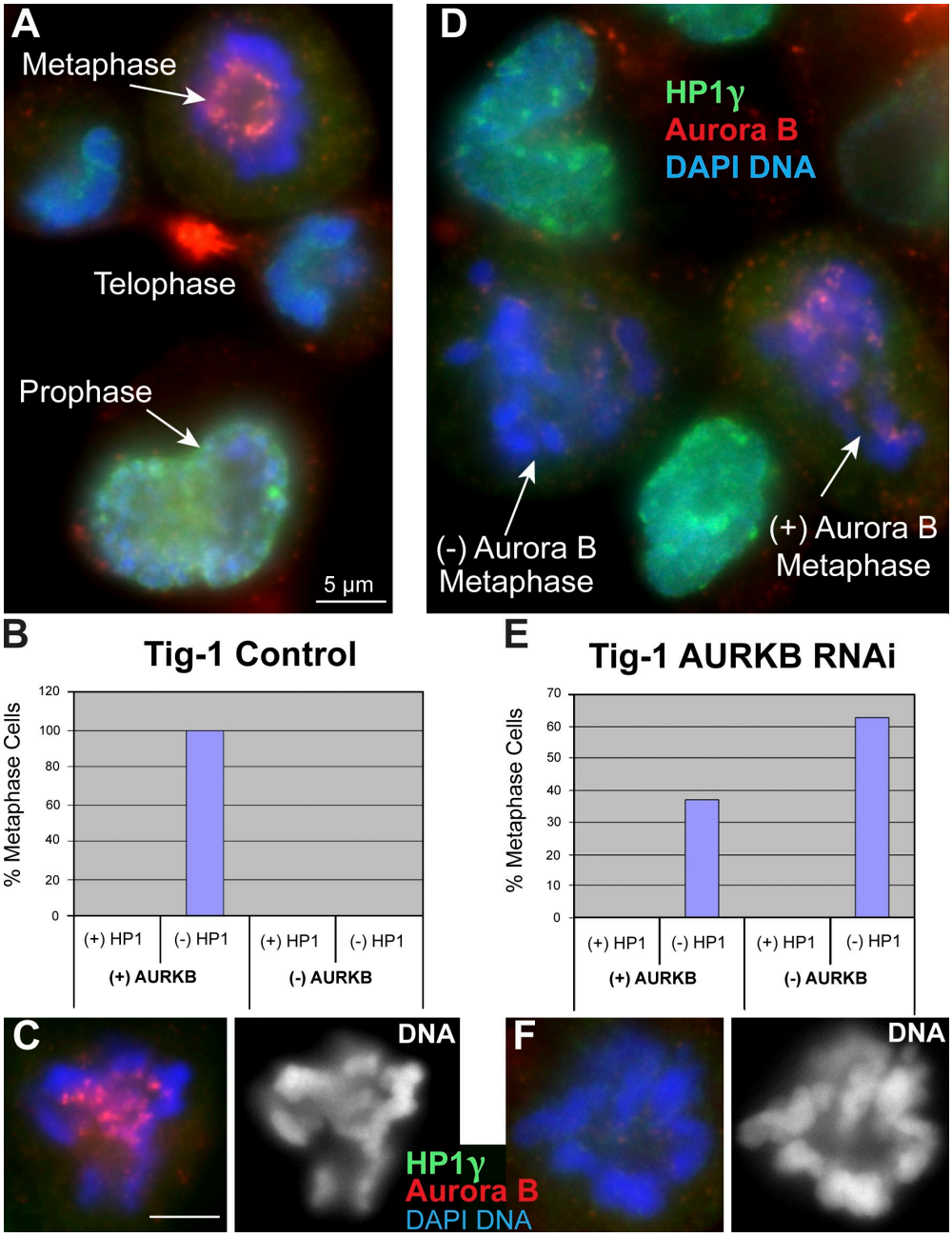

Figure 7. **HP1-γ is still released from mitotic chromosomes after Aurora B inhibition, suggesting that the retention of HP1-γ is not required to induce retention of XIST RNA at metaphase.** (A–C) Control Tig-1 cells. Metaphase cells (A, top cell) show abundant Aurora B (red) signal and absent HP1-γ (green). HP1-γ in prophase cell appears slightly yellow because AURKB (red) is also localized to the chromosomes at this time. (D–F) AURKB RNAi–treated Tig-1. Although RNAi eliminated Aurora B in some metaphase cells (−) and not others (+), both cell populations lack HP1-γ staining at mitosis (green). DAPI DNA channels are separated in C and F to confirm metaphase cells. Bars, 5 µm.

possibility that the monoclonal epitope is somehow masked. Regardless, because H3ph increases in mitotic cells treated for RNAi to AURKB, when XIST RNA is retained (Fig. 6 B), H3ph alone cannot be responsible for the normal release of XIST RNA. However, because we find that H3ph occurs before XIST RNA release, it remains possible, for example, that H3S28ph is one of multiple required events. Because XIST transcripts likely have multiple binding sites (Wutz et al., 2002) and appear to bridge chromatin with nonhistone components of nuclear substructure (see Introduction; Clemson et al., 1996), no single modification to chromatin may control XIST RNA binding (see Fig. 10).

Of the many chromatin hallmarks on Xi, only ubiquitin shows a relationship to the presence of XIST RNA along the chromosome

Given that multiple chromatin factors may be involved in XIST RNA localization, we further extended this strategy to begin examining other Xi chromatin hallmarks to determine

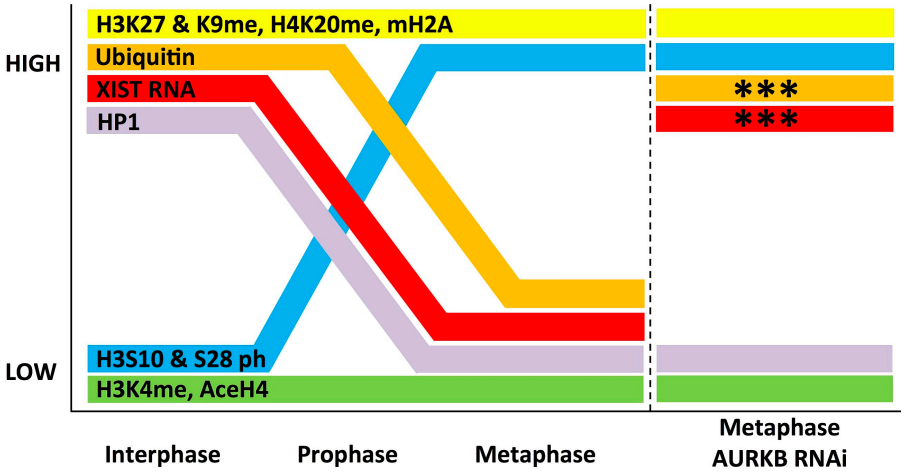

Figure 8. **Strategy to narrow the candidate factors involved in XIST RNA binding.** The ability to manipulate XIST RNA behavior in vivo provides a strategy to determine which chromosome/chromatin-associated proteins most closely mirror the binding pattern of XIST RNA. Of the 10 chromosome-associated proteins examined in this study, only 4 show the requisite fluctuations in mitosis when XIST RNA is released (H3S10, H3S28ph, HP1, and ubiquitin). Of these four, only ubiquitin was differentially affected in AURKB RNAi–treated cells coincident with XIST RNA (asterisks indicate completely concordant patterns). mH2A, macroH2A.

which are clearly separable from compromised XIST RNA binding and identify any that may be closely linked to or impacted by XIST RNA retention. These need not be direct targets of AURKB but could be impacted indirectly (e.g., HP1 chromatin binding is released at mitosis as a consequence of AURKB-induced H3ph; Fischle et al., 2005; Hirota et al., 2005). Although loss of XIST RNA in somatic cells does not result in the rapid loss of heterochromatin marks (Csankovszki et al., 2001), some chromatin properties may show a more immediate relationship to alterations in XIST RNA binding. If so, one would expect to see a change in status during mitosis, when XIST RNA binding is compromised, and a reversal of this by RNAi to AURKB.

As shown (see Fig. 8), we find that most of the Xi hallmarks remain unchanged on the Xi throughout the cell cycle and show no change in mitosis, which is consistent with prior literature (Chadwick and Willard, 2002; Plath et al., 2003; Smith et al., 2004; Karachentsev et al., 2005; Xiao et al., 2007). Therefore, compared with the behavior of XIST RNA at mitosis, it can be surmised that histone modifications such as H3K27me, H3K9me, H4ac, H3K4me, and H4K20me are not closely linked to changes in XIST RNA binding affinity in somatic cells. HP1 is a heterochromatin-associated protein that is released from all chromosomes at mitosis as a direct consequence of AURKB-mediated H3ph (Fischle et al., 2005; Hirota et al., 2005). HP1 has an RNA- and DNA-binding domain, and evidence suggests that its chromatin binding may be dependent on an unknown RNA (Maison et al., 2002; Muchardt et al., 2002). Therefore, it was of particular interest to examine HP1 in cells inhibited for AURKB. Inhibition with HESP resulted in loss of mitotic H3ph and the abnormal interphase-like retention of both XIST RNA and HP1 (Fig. S4, A and B). However, in fibroblasts and G3 cells treated with AURKB RNAi, histone H3 was still phosphorylated (using the polyclonal antibody; Fig. 6 B), which caused HP1 to be released (Fig. 7), but XIST RNA was still retained on the chromosome. Therefore, results are consistent with the published literature showing a link between H3ph and HP1 (Fischle et al., 2005; Hirota et al., 2005; Terada, 2006) but also indicate that loss of the HP1-γ isoform is not sufficient to cause XIST RNA release.

Although 9 of the 10 heterochromatin modifications illustrated in Fig. 8 were separable from the XIST RNA–binding pattern, this approach identified ubiquitination of Xi as one interesting exception. In human (Smith et al., 2004) and mouse (de Napoles et al., 2004; Fang et al., 2004), the inactive X is markedly ubiquitinated on histone H2A throughout interphase, and it is transiently lost at some point during mitosis (Smith et al., 2004). To determine whether there is a temporal separation or order to the loss of XIST RNA and ubiquitin in somatic cells, we examined the two simultaneously on individual mitotic chromosomes. Using an antibody against protein-bound ubiquitin (FK2), XIST RNA and ubiquitin are visible together along the inactive chromosome in prophase (Fig. 9 A), but as cells enter metaphase, XIST RNA releases, and only ubiquitin remains in a subset (~38%) of mitotic cells, indicating that it remains for a short time (Fig. 9, B and D). Although ubiquitinated chromosomes lacking XIST RNA were found, the converse was not seen. Ubiquitin is gone by late metaphase, when the only protein-bound ubiquitin usually visible is in the cytoplasmic proteosomes seen before the metaphase/anaphase transition (Fig. 9 C; Marston and Amon, 2004). This suggests that loss of ubiquitination on the Xi closely follows XIST RNA release.

If loss of ubiquitination in mitosis is dependent on XIST RNA removal, it should be retained together with XIST RNA into late metaphase when AURKB is inhibited. In AURKB RNAi–treated cells, both XIST RNA and ubiquitin are seen together well into late metaphase, when the proteosomes are visible at the metaphase/anaphase transition (Fig. 9, E–H). In some rare cases (<1% of cells), XIST RNA and ubiquitin can even be seen together into anaphase (Fig. S5 C). These cells appear to have only reduced AURKB sufficiently to retain XIST RNA but not to prevent anaphase (Fig. S5, A and B). Thus, conditions that induce retention of XIST RNA in AURKB-depleted cells simultaneously preserve ubiquitin on the inactive mitotic chromosome. Although there are many more chromosome-associated proteins that remain to be examined by this new approach, these results illustrate that this strategy can be used to narrow the candidate factors involved and indicates that chromatin ubiquitination is the modification studied thus far that is most closely linked to a robust presence of XIST RNA in mitotic cells (Fig. 8).

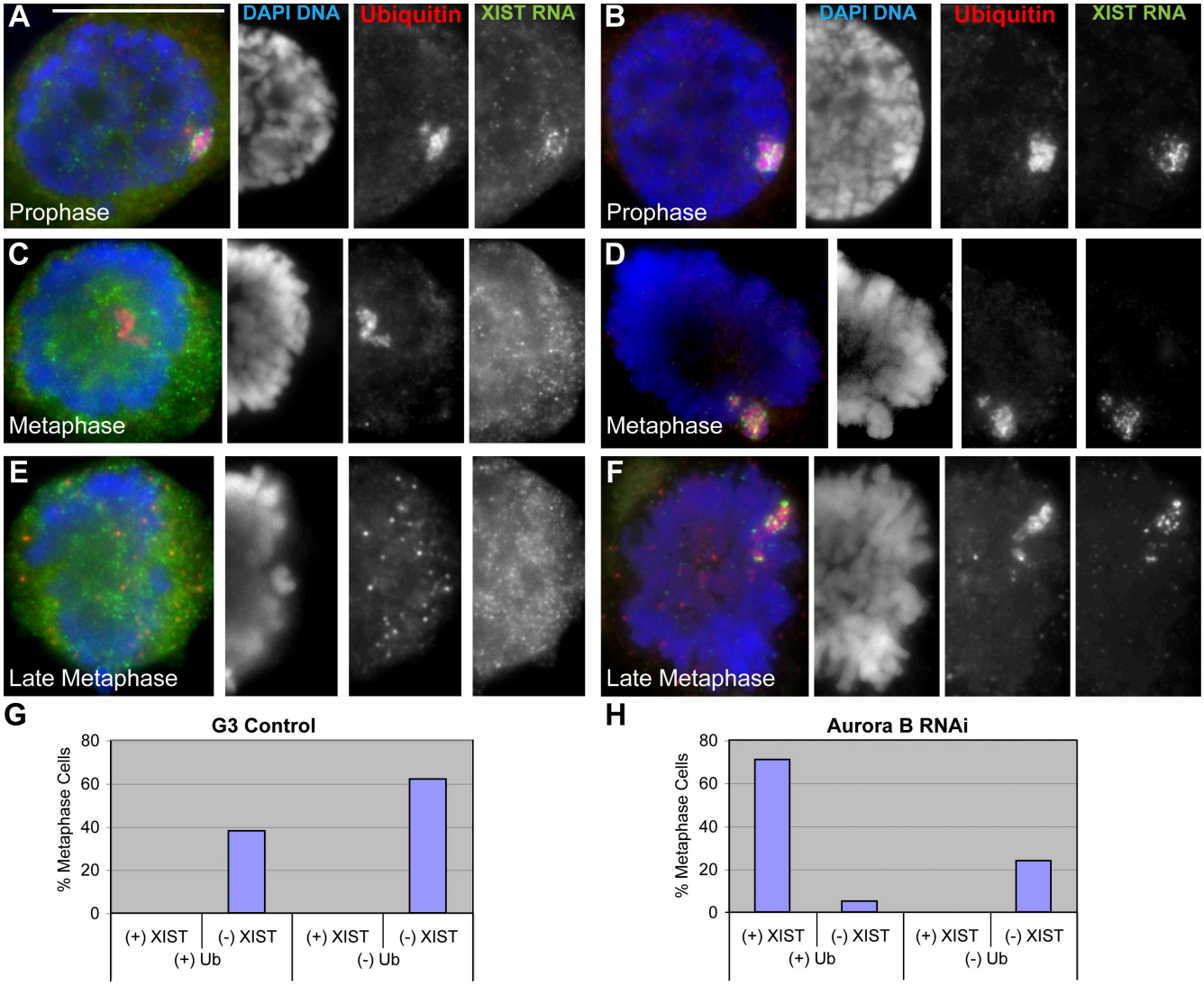

Figure 9. **After AURKB RNAi, increased retention of XIST RNA on metaphase chromosomes is paralleled by increased retention of ubiquitin.** (A, C, and E) In G3 control cells, ubiquitin and XIST RNA are shown in prophase (A), early metaphase (C), and late metaphase (E). Normally, XIST RNA detaches early in prophase, and the ubiquitin mark (on histone H2A; Smith et al., 2004) is lost shortly thereafter. (G) The graph shows quantification in control cells. (B, D, F, and H) In AURKB RNAi–treated cells, both XIST RNA and ubiquitin stay on the chromosome throughout prophase (B) and metaphase (D) and into late metaphase (F), as quantified in the graph (H). Both XIST RNA and ubiquitin (Ub) are found together in ~70% of metaphase cells after AURKB inhibition. Bar, 10 µm.

Discussion

Despite over a decade and a half of research, extremely little is known as to how XIST RNA faithfully binds and localizes across its chromosome of origin. This study provides some inroads into the mystery of how a large noncoding RNA interacts with chromatin. First, it was a significant step to define conditions by which XIST RNA's interphase binding or mitotic release can be manipulated. This approach to manipulate XIST RNA in situ may provide a means to break the impasse in understanding how a large chromosomal RNA actually interacts with complex chromosomal and nuclear structure. Second, findings in this study further support and extend earlier evidence (Clemson et al., 1996) that XIST RNA's relationship to the chromosome does not strictly depend on hybridization to chromosomal DNA but is regulated by interaction with a protein present across the chromosome arms. Third, results unexpectedly identify AURKB as a key player, independent of its role in the mechanics of mitosis, and expand our knowledge of the biological roles of AURKB, an extremely important, heavily studied enzyme which is commonly overexpressed in cancer. Although AURKB was known to phosphorylate mitotic chromatin, it was not known to have any role in maintaining noncoding RNA on heterochromatin. Fourth, our findings regarding H3S10ph, H3S28ph, HP1, ubiquitination, and several other chromatin modifications substantially advance and narrow the potential candidates for chromatin/nuclear factors that are closely linked to XIST RNA binding and identify one that appears to be closely impacted by the presence of XIST RNA.

Although AURKB's most-studied role is at the mitotic centromere and spindle, our findings point to a new role involved in RNA interaction with heterochromatin, implicating

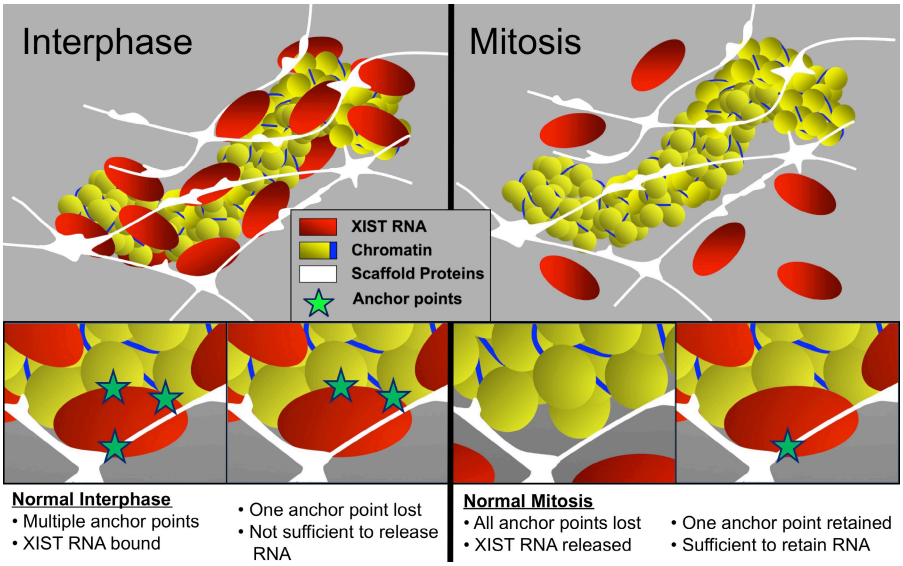

Figure 10. **Model for multiple XIST RNA anchor points impacting its localization to chromatin.** (Interphase) In normal interphase cells, XIST RNA is tightly bound to the chromosome because of protein interactions at multiple anchor points. The loss of a single anchor point may not be sufficient to release XIST RNA from the chromosome. (Mitosis) The normal loss of all anchor points at prophase in human cells releases XIST RNA from the chromosome. Inducing the retention of a single anchor point is sufficient to significantly retain XIST RNA on the chromosome. This study shows that at least one of the anchor points involved in human XIST RNA binding is regulated by AURKB.

AURKB's impact on chromosomal proteins. AURKB's multiple functions during mitosis are intimately linked to its changing cellular localization; thus, it is significant that AURKB is only present on the chromosome arms in mitosis during the same brief prophase window when XIST RNA binding changes (Crosio et al., 2002). AURKB is a key component of the chromosomal passenger complex (CPC), a four-subunit complex which promotes chromosome alignment, cytokinesis, and multiple mitotic functions (for review see Vader et al., 2006). This four-subunit complex is distinct from the two-subunit CPC complex (inner centromere protein [INCENP] and AURKB) that transiently localizes across the chromosome arms during prophase (when XIST RNA is released), where it is known to phosphorylate histone H3. By metaphase, the CPC localizes to centromeres, then microtubules, and finally the midbody. Its intracellular localization is correlated with its particular functions at each mitotic phase.

Several observations indicate that chromosome condensation or other mechanical features of mitosis are not necessary to regulate XIST RNA binding. We show that XIST RNA can be released by specific manipulation at interphase and can still be retained on fully condensed metaphase chromosomes under certain conditions and further demonstrate that mitotic arrest alone, with other agents (NOC), had no effect on XIST RNA release (unlike AURKB inhibition). Because numerous XIST transcripts bind all along the chromosome, a change in this binding necessarily involves changes that occur throughout the length of the chromosome, which is consistent with AURKB's early mitotic localization to the chromosome arms and its known effects on chromatin phosphorylation. In a small subset of cells in which AURKB had been reduced (but not eliminated) by RNAi, AURKB was sufficiently depleted across the chromosomes to cause retention of XIST RNA but was not low enough to fully prevent anaphase progression. Therefore, it is likely that more AURKB is required to coat the chromosome arms and modify proteins that impact XIST RNA than the smaller amount required to localize to just the centromeres and centriole for chromosome segregation. All of these observations support the idea that AURKB's effect on XIST RNA binding can be separated from its role in the mechanics of mitosis. Instead, AURKB impacts, either directly or indirectly, the numerous XIST RNA anchor points along the chromosome arms. Although we cannot rule out that this could occur via AURKB regulation of some other chromatin-modifying enzyme, the fact that AURKB localizes to chromosome arms when XIST RNA detaches and that AURKB is known to modify several chromatin proteins make it most likely that AURKB's effects on XIST RNA binding are related to and possibly downstream of its know modifications to chromatin.

Activation of AURKB is very complex; not only is it repressed by PP1 at interphase, but activation also requires INCENP for full activity (Ruchaud et al., 2007; for review see Andrews et al., 2003), and both AURKB and INCENP can be sequestered in the cytoplasm during parts of the cell cycle (Rodriguez et al., 2006). This may in part explain why inhibitors of PP1 did not consistently result in mislocalized XIST RNA in all cells, whereas inhibition of AURKB more reliably caused robust retention of XIST RNA at metaphase. However, this may also relate to the likely presence of multiple interphase anchor points, as further discussed in the next paragraph.

An important concept for understanding the effects of various perturbations is presented in the model in Fig. 10. This model proposes that XIST RNA has multiple anchor points that bind it to chromatin. This is likely, given the large size (14 kb) of the transcript (Brown et al., 1992), evidence that multiple parts of the RNA contribute to its chromosomal localization (Wutz et al., 2002), and further evidence that XIST RNA bridges chromatin and nonchromatin components of interphase nuclear structure (Clemson et al., 1996). This redundancy of binding sites may assure the RNA's very tightly restricted localization in cis and lack of promiscuous localization and silencing of neighboring chromatin in trans. Importantly, the perturbation of any one protein-binding site would then be unlikely to release XIST RNA at interphase, although maintenance of a single binding site could still be sufficient to retain XIST RNA at metaphase, as illustrated in Fig. 10. This in turn fits with our observations in

this study, in which it appeared more difficult to induce XIST RNA release in interphase than it was to cause atypical XIST RNA retention on human metaphase chromosomes.

Given that traditional biochemical approaches have not identified XIST RNA–associated proteins nor illuminated how transcripts bind the chromosome, this new approach to manipulate XIST RNA binding in situ can provide inroads into the complexities of the RNA's interaction with the chromosome. Although the problem is far from solved and will not likely prove tractable to a single interaction, our findings allow us to conceptualize and narrow the other potential players considerably. Candidates for substrates involved in regulating human XIST RNA binding would need to be present across the chromosome arms and change with XIST RNA both in normal prophase and with the manipulations to release or retain XIST RNA, as defined in this study (Fig. 8). Specific inhibition of AURKB by RNAi results in the preservation of at least one anchor point for XIST RNA at mitosis (Fig. 10), which normally would be directly or indirectly abrogated by AURKB. Although H3ph was initially the prime candidate, our surprising finding that the inactive chromosome appears to lack mitotic H3S10ph suggests that it may not be involved, although it will be important to evaluate this interesting distinction between X and autosome mitotic modifications more extensively (unpublished data). It is clear that increased H3S28ph alone is also not sufficient to release XIST RNA at mitosis, although it remains possible that H3S28ph is one of multiple binding sites and may still be required (Fig. 10). Additionally, the fact that H3S28 can be highly phosphorylated along the chromosome arms in mitosis without releasing XIST RNA rules out that increased negative charge across the chromosome induces mitotic release. Another strong candidate was HP1-γ, which is indirectly regulated by AURKB via H3ph to release from heterochromatin at mitosis, has an RNA-binding site (Maison et al., 2002; Muchardt et al., 2002), and is enriched on Xi in the cell types we study (Wreggett et al., 1994; Chadwick and Willard, 2003). However, our data show that XIST RNA can still remain bound when HP1-γ is released at mitosis, although this does not rule out that other HP1 subtypes (α and β) more closely mirror XIST RNA. Because XIST RNA may bridge chromatin with other nuclear structural elements (Fig. 10), nonhistone chromatin–associated factors (affected by AURKB) may also be involved. For example, the presence of two SMC (structural maintenance of chromosome) proteins, cohesin and condensin, are altered because of phosphorylation by AURKB early in mitosis when XIST RNA is released (Hauf et al., 2005; Lipp et al., 2007).

Although the inactive chromosome has numerous chromatin hallmarks that help maintain its inactive state, most can be excluded as candidates for any immediate link to XIST RNA binding, primarily because they lack the requisite fluctuation during mitotic prophase (Fig. 8). The inactive X stains brightly for ubiquitin and was shown to be markedly enriched for ubiquitination of histone H2A specifically (de Napoles et al., 2004; Fang et al., 2004). Unlike other Xi hallmarks, the ubiquitination mark was shown to be lost at some point during mitosis (Smith et al., 2004). Further analysis in this study shows that ubiquitination is lost shortly after release of XIST RNA from the mitotic chromosome in somatic cells, suggesting that ubiquitination does not directly affect XIST RNA binding but may be affected by it. Additionally, upon depletion of AURKB and retention of XIST RNA, ubiquitin retention increases concomitantly, suggesting that normal loss of ubiquitin during mitosis depends on prior removal of XIST RNA. This is distinct from the reported requirement for Xist RNA to recruit the PRC1 complex responsible for the initial ubiquitination of Xi that occurs when chromosome silencing is first enacted during differentiation of mouse embryonic stem cells (Schoeftner et al., 2006). Interestingly, a recent study suggests that H2A ubiquitination impairs the ability of AURKB to phosphorylate H3S10 (Joo et al., 2007). Thus, our results indicating that the Xi may lack H3S10ph could be related to this effect, and by the time XIST RNA and ubiquitin are both released during mitosis, AURKB is no longer available on the chromosome arms to phosphorylate H3S10 on the Xi.

This study has developed the first system to systematically manipulate XIST RNA's interaction with its chromosome and identified one major regulatory player (AURKB); however, this experimental approach could be expanded or refined in the future, as we anticipate other means will be found that release or retain XIST RNA. Although the use of pharmacological inhibitors has the obvious downside that drugs are not entirely specific (and may have different effects in different cell types), this may actually enhance the ability to release XIST RNA at interphase if multiple anchor points are indeed involved. For example, TAUT or OKA may impact more than one anchor point, only one of which is the result of PP1 inhibition. Although not explored in depth, recent preliminary attempts to use RNAi to PP1 did not release XIST RNA, in contrast to the RNAi to Aurora B which consistently caused RNA retention at mitosis. However, the fact that only certain drugs released XIST RNA indicates that the result is not just a nonspecific effect. That one of the XIST RNA anchor points is abrogated by AURKB activation in interphase by these drugs was further supported by the fact that HESP could largely rescue the mislocalization phenotype despite the fact that simultaneous treatment with two such drugs increases the likelihood of nonspecific effects and toxicity.

A final important implication of these findings is that interaction of this noncoding RNA with heterochromatin is influenced by factors (such as AURKB) that can broadly affect chromatin, and thus, any change or perturbation that impacts the epigenetic and chromatin state of the cell could potentially impact XIST RNA. This should be considered when interpreting any literature involving XIST RNA localization and could also relate to the finding that XIST RNA is sometimes mislocalized in cancer cells (Pageau et al., 2007b), and loss of the Xi is common in many tumors (Richardson et al., 2006; for review see Pageau et al., 2007a). Thus, rather than a specific effect of a single factor such as BRCA1, mislocalization of XIST RNA may occur through broad effects on chromatin, which may be impacted by other factors in addition to AURKB. Our results also raise the possibility that AURKB may impact the relationship of other regulatory noncoding RNAs with chromatin (for review see Bernstein and Allis,

2005). The role of epigenetics in cancer development has been increasingly recognized (Feinberg and Tycko, 2004; Esteller, 2007; He et al., 2008), and these and other recent studies provide a framework for thinking about potential links between cancer and broad heterochromatic instability (for review see Pageau et al., 2007a). Because AURKB has been implicated in several cancers in which overexpression can cause cell transformation (Giet and Prigent, 1999) and is currently a target for chemotherapeutics (Girdler et al., 2006), it is important to recognize the potential impact on chromosomal RNAs and heterochromatin.

Materials and methods

Cell manipulation and inhibition

Three different cell types were used to assess XIST RNA binding. WI-38 and Tig-1 are both normal female fibroblasts, and the G3 cells are a subclone of the F2-6 male HT1080 cell line containing an ectopic XIST RNA transgene that consistently silences and forms a well-defined Barr body (Hall et al., 2002). In G3 cells, the XIST RNA localization phenotype is very consistent throughout the population, and the abundant RNA is tightly localized to the chromosome 4 territory (Hall et al., 2002). This cell line provides a more robust and tighter localized XIST RNA signal than any normal female cell line we have studied. The Tig-1 and G3 cell lines were maintained in minimum essential media (Invitrogen) and 10% non–heat-inactivated fetal calf serum with supplemental antibiotics. WI-38 human female fibroblast cells were grown in Basal Media Eagle (Invitrogen) and 10% non–heat-inactivated fetal calf serum with supplemental antibiotics. For M-phase analysis of inhibitors, mitotic cells were dislodged from asynchronously growing cultures and cytospun onto coverslips before fixation in 4% paraformaldehyde.

Drug treatments

Inhibitors used were HESP (obtained from Boehringer Ingelheim through B.D. Murphy, Université de Montréal, Montréal, Québec, Canada), OKA (Invitrogen and Sigma-Aldrich), STSP (Sigma-Aldrich), SB (Sigma-Aldrich), TSA (Sigma-Aldrich), CANTH (EMD), NOC (Tocris Bioscience), ZM (Tocris Bioscience), and TAUT (EMD). Drugs were dissolved in DMSO for stock solutions (10 mM HESP, 0.5 mM OKA, 100 µg/ml STSP, 0.22 g/ml SB, 1 mg/ml TSA, 10 µM CANTH, 100 µM TAUT, and 2 mg/ml NOC) and used fresh or before 3 mo. Drugs were added directly to media over cells growing as a monolayer on coverslips typically for 4–6 h (and occasionally overnight) before being assayed. Working concentration ranges are as follows: 100 nM HESP, 0.5–1,000 nM OKA, 3–30 µM CANTH, 0.5–9 µM TAUT, 39 mM SB, 0.03–66 µM TSA, 0.2–10 µM STSP, 330 nM NOC, and 0.05–1 µM ZM. The highest ranges of the inhibitors, OKA, STSP, TSA, CANTH, and TAUT, were usually lethal to the cell, as assessed by nuclear morphology, loss of cells from the slide, and high levels of cellular debris in culture. When used at high concentrations, all cells, including any floating, were cytospun onto coverslips for in situ assessment. The broad range kinase inhibitor STSP was used as a negative control for HESP and ZM. STSP did not cause XIST RNA to be retained on mitotic chromosomes. Metaphase arrest by NOC was also use as a negative control for HESP and ZM and did not cause XIST RNA retention.

RNAi

RNAi for AURKB was performed using the AIM1/AURKB SMARTpool from Thermo Fisher Scientific. DharmaFECT siRNA transfection reagent (Thermo Fisher Scientific) was used for primary Tig-1 cells, and Lipofectamine RNAiMAX (Invitrogen) was used on G3 cells according to the manufacturers' protocols. Negative controls for XIST RNA retention with AURKB RNAi were untreated cells as well as a 27-mer T-3′ nonspecific control siRNA (sense, 5′-AACAAGGUUCUUAGUUAGACGUGACUG-3′; control antisense, 5′-GUCACGUCUAACUAAGAACCUUGTT-3′; Integrated DNA Technologies), which did not exhibit XIST RNA painting in metaphase.

Fixation

Our standard protocols for cell fixation have been described previously in detail (Johnson et al., 1991; Tam et al., 2002). In brief, for most experiments, cells were grown on glass coverslips and extracted in cytoskeletal buffer, 5% Triton X-100, and vanadyl ribonucleoside complex for 1–3 min. Cells were fixed in 4% paraformaldehyde for 10 min and then stored in 1× PBS or 70% EtOH. Loss of human XIST RNA at prophase was initially shown using four different fixation conditions (Clemson et al., 1996) and was further confirmed in this study by also testing three additional fixation procedures: (1) fixation before extraction in Triton X-100 for 3 min, (2) using proteinase K permeabilization instead of Triton X-100 (fix first, then rinse in 70% EtOH followed by PBS, treat with 25 µg/ml proteinase K for 5 min, and then fix again), and (3) cytospinning with no permeabilization step before fixation.

FISH

Hybridization to RNA, DNA, and simultaneous DNA/RNA detection was performed as previously described (Johnson et al., 1991; Tam et al., 2002) or according to manufacturer's directions (MP Biomedicals). DNA probes were nick translated with either biotin-11-dUTP or digoxigenin-16-dUTP (Roche). RNA-specific hybridization was performed under nondenaturing conditions under which the DNA was not accessible. In brief, RNA was hybridized overnight at 37°C in 2× SSC, 1 U/µl RNasin, and 50% formamide with 2.5 µg/ml of DNA probe. Cells were washed with 50% formamide/2× SSC at 37°C for 20 min, with 2× SSC at 37°C for 20 min, with 1× SSC at RT for 20 min, and with 4× SSC at RT for 5 min. Detection was performed using antidigoxigenin bound to 200 µg/ml rhodamine or 2.5 mg/ml fluorescein-conjugated avidin in 1% BSA/4× SSC for 1 h at 37°C. Postdetection washes were performed with 4× SSC, 4× SSC with 0.1% Triton X-100, and 4× SSC for 10 min each at RT in the dark. For simultaneous RNA/DNA hybridizations, RNA hybridization was performed first (as above), and then the cells were fixed in 4% paraformaldehyde for 10 min, DNA was denaturated, and DNA hybridization was performed. DNA was hybridized after denaturation. In brief, the cells were denatured in 70% formamide and 2× SSC at 80°C for 2 min before EtOH dehydration and air drying. Hybridization and detection were performed as described above. Probes used for FISH were a 10-kb human XIST RNA gene construct (XIST RNA plasmid G1A), a probe against the human XIST RNA intron 1 and 2 (gift from H. Willard [Duke University Medical Center, Durham, NC] and C. Brown [University of British Columbia, Vancouver, British Columbia, Canada]), human Cot-1 DNA (Invitrogen), and chromosome 4 paint (MP Biomedicals).

IF

IF and simultaneous protein/RNA detection were performed as previously described (Johnson et al., 1991; Tam et al., 2002). Most antibodies were used before RNA hybridization. In brief, slides were incubated in the appropriate dilution of primary antibody in 1% BSA, 1× PBS, and 1 U/µl RNasin for 1 h at 37°C (RNasin is added to protect RNA from possible RNase contamination of the primary antibody). Slides were washed, and immunodetection was performed using 1:500 dilution of appropriately conjugated (FITC or rhodamine) secondary (anti–goat, –mouse, or –rabbit) antibody in 1× PBS with 1% BSA. The antibody signal is fixed in 4% paraformaldehyde for 10 min before hybridization (performed as detailed in the previous section), and all slides were counterstained with DAPI. Vectashield (Vector Laboratories) was used as mounting media for all fluorescence imaging.

Antibodies used were anti–HP1-γ (Millipore), anti-AURKB (Bethyl Laboratories, Inc.), anti–phosphorylated histone H1 (clone 12D11; Millipore), polyclonal anti-H3S10ph (Millipore), monoclonal anti-H3S10ph (Millipore), anti-H3S28ph (Abcam), anti–acetylated H4 (Millipore), anti-macroH2A (Millipore), and anti–protein-bound ubiquitin (FK2; Thermo Fisher Scientific). Previous studies used anti–ubiquitinated H2A (Millipore) in conjunction with FK2 to delineate the Xi (Fang et al., 2004; Smith et al., 2004); however, this antibody is no longer available, and other commercial ubiquitinated H2A antibodies were not validated for IF and did not work when tested.

Microscopy and image analysis

Digital imaging analysis was performed using a microscope (Axiovert 200 or Axiophot; Carl Zeiss, Inc.) equipped with a 100× NA 1.4 Plan-Apochromat objective and multi-bandpass dichroic and emission filter sets (model 83000; Chroma Technology Corp.) set up in a wheel to prevent optical shift. Images were captured with the AxioVision software (Carl Zeiss, Inc.) and a camera (Orca-ER; Hamamatsu Photonics) or a cooled charge-coupled device camera (200 series; Photometrics). Where rhodamine was used for detection in red, a narrow bandpass fluorescein filter was inserted to correct for any bleed through of rhodamine fluorescence into the fluorescein channel. Most experiments were performed a minimum of three times, and typically 50–100 cells were scored in each experiment,

with a representative experiment shown. Key results were confirmed by at least two independent investigators. All findings were easily visible by eye through the microscope, and images were minimally enhanced for brightness and contrast in Photoshop (Adobe) for publication. Digital morphometrics was used to measure chromosome territories, where the signal intensity is digitally thresholded, and the computer defines the borders (MetaMorph program; MDS Analytical Technologies) and then measures the area within.

Online supplemental material

Fig. S1 summarizes the effects of other inhibitors of chromatin modifiers (acetylation and phosphorylation) on XIST RNA localization. Fig. S2 illustrates the link between H3S10ph and Xist RNA release in mouse mitotic cells. Fig. S3 compares the timing of H3S10ph in both mouse and human mitotic cells. Fig. S4 demonstrates that AURKB RNAi in HeLa cells does mimic what is observed in other cell lines treated with HESP. Fig. S5 shows that in rare anaphase cells partially inhibited for AURKB, XIST RNA and ubiquitin can be seen to mark the Xi. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200811143/DC1.

This research was supported by National Institutes of Health grant GM53234.

Submitted: 25 November 2008
Accepted: 29 July 2009

References

Agnese, V., V. Bazan, F.P. Fiorentino, D. Fanale, G. Badalamenti, G. Colucci, V. Adamo, D. Santini, and A. Russo. 2007. The role of Aurora-A inhibitors in cancer therapy. *Ann. Oncol.* 18:vi47–vi52.

Ajiro, K., K. Yoda, K. Utsumi, and Y. Nishikawa. 1996. Alteration of cell cycle-dependent histone phosphorylations by okadaic acid. Induction of mitosis-specific H3 phosphorylation and chromatin condensation in mammalian interphase cells. *J. Biol. Chem.* 271:13197–13201.

Andrews, P.D., E. Knatko, W.J. Moore, and J.R. Swedlow. 2003. Mitotic mechanics: the auroras come into view. *Curr. Opin. Cell Biol.* 15:672–683.

Barber, C.M., F.B. Turner, Y. Wang, K. Hagstrom, S.D. Taverna, S. Mollah, B. Ueberheide, B.J. Meyer, D.F. Hunt, P. Cheung, and C.D. Allis. 2004. The enhancement of histone H4 and H2A serine 1 phosphorylation during mitosis and S-phase is evolutionarily conserved. *Chromosoma.* 112:360–371.

Bernstein, E., and C.D. Allis. 2005. RNA meets chromatin. *Genes Dev.* 19:1635–1655.

Bialojan, C., and A. Takai. 1988. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. *Biochem. J.* 256:283–290.

Brockdorff, N. 2002. X-chromosome inactivation: closing in on proteins that bind Xist RNA. *Trends Genet.* 18:352–358.

Brown, C.J., and S.E. Baldry. 1996. Evidence that heteronuclear proteins interact with XIST RNA in vitro. *Somat. Cell Mol. Genet.* 22:403–417.

Brown, C.J., B.D. Hendrich, J.L. Rupert, R.G. Lafrenière, Y. Xing, J.B. Lawrence, and H.F. Willard. 1992. The human XIST gene: analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. *Cell.* 71:527–542.

Chadwick, B.P., and H.F. Willard. 2002. Cell cycle-dependent localization of macroH2A in chromatin of the inactive X chromosome. *J. Cell Biol.* 157:1113–1123.

Chadwick, B.P., and H.F. Willard. 2003. Chromatin of the Barr body: histone and non-histone proteins associated with or excluded from the inactive X chromosome. *Hum. Mol. Genet.* 12:2167–2178.

Clemson, C.M., J.A. McNeil, H.F. Willard, and J.B. Lawrence. 1996. XIST RNA paints the inactive X chromosome at interphase: evidence for a novel RNA involved in nuclear/chromosome structure. *J. Cell Biol.* 132:259–275.

Clemson, C.M., L.L. Hall, M. Byron, J. McNeil, and J.B. Lawrence. 2006. The X chromosome is organized into a gene-rich outer rim and an internal core containing silenced nongenic sequences. *Proc. Natl. Acad. Sci. USA.* 103:7688–7693.

Crosio, C., G.M. Fimia, R. Loury, M. Kimura, Y. Okano, H. Zhou, S. Sen, C.D. Allis, and P. Sassone-Corsi. 2002. Mitotic phosphorylation of histone H3: spatio-temporal regulation by mammalian Aurora kinases. *Mol. Cell. Biol.* 22:874–885.

Csankovszki, G., A. Nagy, and R. Jaenisch. 2001. Synergism of Xist RNA, DNA methylation, and histone hypoacetylation in maintaining X chromosome inactivation. *J. Cell Biol.* 153:773–784.

Dawson, J.F., and C.F. Holmes. 1999. Molecular mechanisms underlying inhibition of protein phosphatases by marine toxins. *Front. Biosci.* 4:D646–D658.

de Napoles, M., J.E. Mermoud, R. Wakao, Y.A. Tang, M. Endoh, R. Appanah, T.B. Nesterova, J. Silva, A.P. Otte, M. Vidal, et al. 2004. Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. *Dev. Cell.* 7:663–676.

Dormann, H.L., B.S. Tseng, C.D. Allis, H. Funabiki, and W. Fischle. 2006. Dynamic regulation of effector protein binding to histone modifications: the biology of HP1 switching. *Cell Cycle.* 5:2842–2851.

Duthie, S.M., T.B. Nesterova, E.J. Formstone, A.M. Keohane, B.M. Turner, S.M. Zakian, and N. Brockdorff. 1999. Xist RNA exhibits a banded localization on the inactive X chromosome and is excluded from autosomal material in cis. *Hum. Mol. Genet.* 8:195–204.

Edmondson, D.G., J.K. Davie, J. Zhou, B. Mirnikjoo, K. Tatchell, and S.Y. Dent. 2002. Site-specific loss of acetylation upon phosphorylation of histone H3. *J. Biol. Chem.* 277:29496–29502.

Esteller, M. 2007. Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat. Rev. Genet.* 8:286–298.

Fang, J., T. Chen, B. Chadwick, E. Li, and Y. Zhang. 2004. Ring1b-mediated H2A ubiquitination associates with inactive X chromosomes and is involved in initiation of X inactivation. *J. Biol. Chem.* 279:52812–52815.

Favre, B., P. Turowski, and B.A. Hemmings. 1997. Differential inhibition and posttranslational modification of protein phosphatase 1 and 2A in MCF7 cells treated with calyculin-A, okadaic acid, and tautomycin. *J. Biol. Chem.* 272:13856–13863.

Feinberg, A.P., and B. Tycko. 2004. The history of cancer epigenetics. *Nat. Rev. Cancer.* 4:143–153.

Fischle, W., B.S. Tseng, H.L. Dormann, B.M. Ueberheide, B.A. Garcia, J. Shabanowitz, D.F. Hunt, H. Funabiki, and C.D. Allis. 2005. Regulation of HP1-chromatin binding by histone H3 methylation and phosphorylation. *Nature.* 438:1116–1122.

Giet, R., and C. Prigent. 1999. Aurora/Ipl1p-related kinases, a new oncogenic family of mitotic serine-threonine kinases. *J. Cell Sci.* 112:3591–3601.

Girdler, F., K.E. Gascoigne, P.A. Eyers, S. Hartmuth, C. Crafter, K.M. Foote, N.J. Keen, and S.S. Taylor. 2006. Validating Aurora B as an anti-cancer drug target. *J. Cell Sci.* 119:3664–3675.

Goto, H., Y. Tomono, K. Ajiro, H. Kosako, M. Fujita, M. Sakurai, K. Okawa, A. Iwamatsu, T. Okigaki, T. Takahashi, and M. Inagaki. 1999. Identification of a novel phosphorylation site on histone H3 coupled with mitotic chromosome condensation. *J. Biol. Chem.* 274:25543–25549.

Hall, L.L., M. Byron, K. Sakai, L. Carrel, H.F. Willard, and J.B. Lawrence. 2002. An ectopic human XIST gene can induce chromosome inactivation in postdifferentiation human HT-1080 cells. *Proc. Natl. Acad. Sci. USA.* 99:8677–8682.

Hauf, S., R.W. Cole, S. LaTerra, C. Zimmer, G. Schnapp, R. Walter, A. Heckel, J. van Meel, C.L. Rieder, and J.M. Peters. 2003. The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. *J. Cell Biol.* 161:281–294.

Hauf, S., E. Roitinger, B. Koch, C.M. Dittrich, K. Mechtler, and J.M. Peters. 2005. Dissociation of cohesin from chromosome arms and loss of arm cohesion during early mitosis depends on phosphorylation of SA2. *PLoS Biol.* 3:e69.

He, S., K.L. Dunn, P.S. Espino, B. Drobic, L. Li, J. Yu, J.M. Sun, H.Y. Chen, S. Pritchard, and J.R. Davie. 2008. Chromatin organization and nuclear microenvironments in cancer cells. *J. Cell. Biochem.* 104:2004–2015.

Hendzel, M.J., Y. Wei, M.A. Mancini, A. Van Hooser, T. Ranalli, B.R. Brinkley, D.P. Bazett-Jones, and C.D. Allis. 1997. Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma.* 106:348–360.

Hirota, T., J.J. Lipp, B.H. Toh, and J.M. Peters. 2005. Histone H3 serine 10 phosphorylation by Aurora B causes HP1 dissociation from heterochromatin. *Nature.* 438:1176–1180.

Honkanen, R.E., and T. Golden. 2002. Regulators of serine/threonine protein phosphatases at the dawn of a clinical era? *Curr. Med. Chem.* 9:2055–2075.

Huang, Y., and J.A. Steitz. 2005. SRprises along a messenger's journey. *Mol. Cell.* 17:613–615.

Johnson, C.V., R.H. Singer, and J.B. Lawrence. 1991. Fluorescent detection of nuclear RNA and DNA: implications for genome organization. *Methods Cell Biol.* 35:73–99.

Johnson, C.V., D. Primorac, M. McKinstry, J.A. McNeil, D. Rowe, and J.B. Lawrence. 2000. Tracking COL1A1 RNA in osteogenesis imperfecta: splice-defective transcripts initiate transport from the gene but are retained within the SC35 domain. *J. Cell Biol.* 150:417–432.

Joo, H.Y., L. Zhai, C. Yang, S. Nie, H. Erdjument-Bromage, P. Tempst, C. Chang, and H. Wang. 2007. Regulation of cell cycle progression and gene expression by H2A deubiquitination. *Nature.* 449:1068–1072.

Karachentsev, D., K. Sarma, D. Reinberg, and R. Steward. 2005. PR-Set7-dependent methylation of histone H4 Lys 20 functions in repression of gene expression and is essential for mitosis. *Genes Dev.* 19:431–435.

Knapp, J., P. Bokník, S. Huke, H. Lüss, F.U. Müller, T. Müller, P. Nacke, W. Schmitz, U. Vahlensieck, and J. Neumann. 1998. The mechanism of action of cantharidin in smooth muscle. *Br. J. Pharmacol.* 123:911–919.

Lee, J.T., and R. Jaenisch. 1997. Long-range cis effects of ectopic X-inactivation centres on a mouse autosome. *Nature.* 386:275–279.

Lipp, J.J., T. Hirota, I. Poser, and J.M. Peters. 2007. Aurora B controls the association of condensin I but not condensin II with mitotic chromosomes. *J. Cell Sci.* 120:1245–1255.

Maison, C., D. Bailly, A.H. Peters, J.P. Quivy, D. Roche, A. Taddei, M. Lachner, T. Jenuwein, and G. Almouzni. 2002. Higher-order structure in pericentric heterochromatin involves a distinct pattern of histone modification and an RNA component. *Nat. Genet.* 30:329–334.

Marston, A.L., and A. Amon. 2004. Meiosis: cell-cycle controls shuffle and deal. *Nat. Rev. Mol. Cell Biol.* 5:983–997.

Misteli, T., and D.L. Spector. 1999. RNA polymerase II targets pre-mRNA splicing factors to transcription sites in vivo. *Mol. Cell.* 3:697–705.

Muchardt, C., M. Guilleme, J.S. Seeler, D. Trouche, A. Dejean, and M. Yaniv. 2002. Coordinated methyl and RNA binding is required for heterochromatin localization of mammalian HP1alpha. *EMBO Rep.* 3:975–981.

Murnion, M.E., R.R. Adams, D.M. Callister, C.D. Allis, W.C. Earnshaw, and J.R. Swedlow. 2001. Chromatin-associated protein phosphatase 1 regulates aurora-B and histone H3 phosphorylation. *J. Biol. Chem.* 276:26656–26665.

Nowak, S.J., and V.G. Corces. 2004. Phosphorylation of histone H3: a balancing act between chromosome condensation and transcriptional activation. *Trends Genet.* 20:214–220.

Pageau, G.J., L.L. Hall, S. Ganesan, D.M. Livingston, and J.B. Lawrence. 2007a. The disappearing Barr body in breast and ovarian cancers. *Nat. Rev. Cancer.* 7:628–633.

Pageau, G.J., L.L. Hall, and J.B. Lawrence. 2007b. BRCA1 does not paint the inactive X to localize XIST RNA but may contribute to broad changes in cancer that impact XIST and Xi heterochromatin. *J. Cell. Biochem.* 100:835–850.

Plath, K., J. Fang, S.K. Mlynarczyk-Evans, R. Cao, K.A. Worringer, H. Wang, C.C. de la Cruz, A.P. Otte, B. Panning, and Y. Zhang. 2003. Role of histone H3 lysine 27 methylation in X inactivation. *Science.* 300:131–135.

Richardson, A.L., Z.C. Wang, A. De Nicolo, X. Lu, M. Brown, A. Miron, X. Liao, J.D. Iglehart, D.M. Livingston, and S. Ganesan. 2006. X chromosomal abnormalities in basal-like human breast cancer. *Cancer Cell.* 9:121–132.

Rodriguez, J.A., S.M. Lens, S.W. Span, G. Vader, R.H. Medema, F.A. Kruyt, and G. Giaccone. 2006. Subcellular localization and nucleocytoplasmic transport of the chromosomal passenger proteins before nuclear envelope breakdown. *Oncogene.* 25:4867–4879.

Ruchaud, S., M. Carmena, and W.C. Earnshaw. 2007. The chromosomal passenger complex: one for all and all for one. *Cell.* 131:230–231.

Sasai, K., H. Katayama, D.L. Stenoien, S. Fujii, R. Honda, M. Kimura, Y. Okano, M. Tatsuka, F. Suzuki, E.A. Nigg, et al. 2004. Aurora-C kinase is a novel chromosomal passenger protein that can complement Aurora-B kinase function in mitotic cells. *Cell Motil. Cytoskeleton.* 59:249–263.

Schoeftner, S., A.K. Sengupta, S. Kubicek, K. Mechtler, L. Spahn, H. Koseki, T. Jenuwein, and A. Wutz. 2006. Recruitment of PRC1 function at the initiation of X inactivation independent of PRC2 and silencing. *EMBO J.* 25:3110–3122.

Smith, K.P., M. Byron, C.M. Clemson, and J.B. Lawrence. 2004. Ubiquitinated proteins including uH2A on the human and mouse inactive X chromosome: enrichment in gene rich bands. *Chromosoma.* 113:324–335.

Sugiyama, K., K. Sugiura, T. Hara, K. Sugimoto, H. Shima, K. Honda, K. Furukawa, S. Yamashita, and T. Urano. 2002. Aurora-B associated protein phosphatases as negative regulators of kinase activation. *Oncogene.* 21:3103–3111.

Tam, R., L.S. Shopland, C.V. Johnson, J. McNeil, and J.B. Lawrence. 2002. Applications of RNA FISH for visualizing gene expression and nuclear architecture. *In* FISH: A Practical Approach. B.G. Beatty, S. Mai, and J. Squire, editors. Oxford University Press, New York. 93–118.

Terada, Y. 2006. Aurora-B/AIM-1 regulates the dynamic behavior of HP1alpha at the G2-M transition. *Mol. Biol. Cell.* 17:3232–3241.

Vader, G., R.H. Medema, and S.M. Lens. 2006. The chromosomal passenger complex: guiding Aurora-B through mitosis. *J. Cell Biol.* 173:833–837.

Wreggett, K.A., F. Hill, P.S. James, A. Hutchings, G.W. Butcher, and P.B. Singh. 1994. A mammalian homologue of *Drosophila* heterochromatin protein 1 (HP1) is a component of constitutive heterochromatin. *Cytogenet. Cell Genet.* 66:99–103.

Wutz, A., T.P. Rasmussen, and R. Jaenisch. 2002. Chromosomal silencing and localization are mediated by different domains of Xist RNA. *Nat. Genet.* 30:167–174.

Xiao, C., J.A. Sharp, M. Kawahara, A.R. Davalos, M.J. Difilippantonio, Y. Hu, W. Li, L. Cao, K. Buetow, T. Ried, et al. 2007. The XIST noncoding RNA functions independently of BRCA1 in X inactivation. *Cell.* 128:977–989.

Xing, Y., C.V. Johnson, P.T. Moen Jr., J.A. McNeil, and J.B. Lawrence. 1995. Nonrandom gene organization: structural arrangements of specific pre-mRNA transcription and splicing with SC-35 domains. *J. Cell Biol.* 131:1635–1647.

Yang, H., T. Burke, J. Dempsey, B. Diaz, E. Collins, J. Toth, R. Beckmann, and X. Ye. 2005. Mitotic requirement for aurora A kinase is bypassed in the absence of aurora B kinase. *FEBS Lett.* 579:3385–3391.

Zhang, R., M.V. Poustovoitov, X. Ye, H.A. Santos, W. Chen, S.M. Daganzo, J.P. Erzberger, I.G. Serebriiskii, A.A. Canutescu, R.L. Dunbrack, et al. 2005. Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. *Dev. Cell.* 8:19–30.