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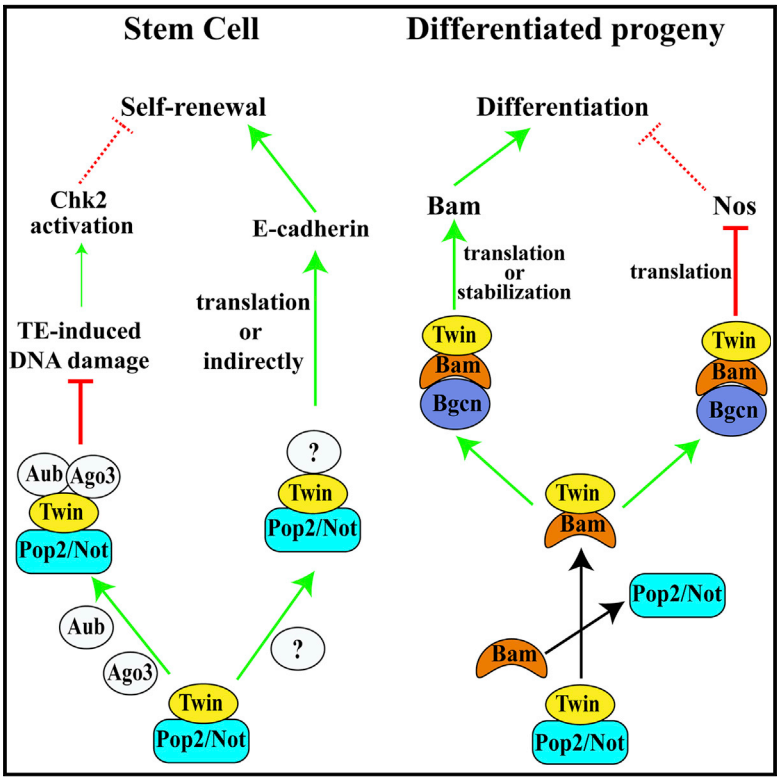
Twin Promotes the Maintenance and Differentiation of Germline Stem Cell Lineage through Modulation of Multiple Pathways

Graphical Abstract



Highlights

- Twin, a deadenylase CCR4/NOT subunit, intrinsically maintains stem cell self-renewal
- Twin sustains E-cadherin expression and prevents DNA damage in stem cells
- Twin promotes Bam-dependent stem cell progeny differentiation

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In Brief

Fu et al. find that the deadenylase complex component Twin maintains germline stem cell self-renewal in the *Drosophila* ovary by sustaining E-cadherin accumulation at the stem-cell-niche junction and preventing transposon-induced DNA damage.

Fu et al., 2015, Cell Reports *13*, 1366–1379
November 17, 2015 ©2015 The Authors
http://dx.doi.org/10.1016/j.celrep.2015.10.017

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Twin Promotes the Maintenance and Differentiation of Germline Stem Cell Lineage through Modulation of Multiple Pathways

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http://dx.doi.org/10.1016/j.celrep.2015.10.017

SUMMARY

The central question in stem cell regulation is how the balance between self-renewal and differentiation is controlled at the molecular level. This study uses germline stem cells (GSCs) in the *Drosophila* ovary to demonstrate that the *Drosophila* CCR4 homolog Twin is required intrinsically to promote both GSC self-renewal and progeny differentiation. Twin/CCR4 is one of the two catalytic subunits in the highly conserved CCR4-NOT mRNA deadenylase complex. Twin works within the CCR4-NOT complex to intrinsically maintain GSC self-renewal, at least partly by sustaining E-cadherin-mediated GSC-niche interaction and preventing transposable element-induced DNA damage. It promotes GSC progeny differentiation by forming protein complexes with differentiation factors Bam and Bgcn independently of other CCR4-NOT components. Interestingly, Bam can competitively inhibit the association of Twin with Pop2 in the CCR4-NOT complex. Therefore, this study demonstrates that Twin has important intrinsic roles in promoting GSC self-renewal and progeny differentiation by functioning in different protein complexes.

INTRODUCTION

In adult tissues, stem cell self-renewal and differentiation are controlled by concerted actions of extrinsic signals and intrinsic factors (Li and Xie, 2005; Morrison and Spradling, 2008). Some intrinsic factors are dedicated to either self-renewal or differentiation; these two classes of intrinsic factors often antagonize each other's functions to balance self-renewal and differentiation. Also, some intrinsic factors control both GSC self-renewal and differentiation, but it remains unclear how they control the two antagonizing processes at the molecular level. Germline stem cells (GSCs) in the *Drosophila* ovary offer an attractive system for studying how self-renewal and differentiation are regulated at the molecular and cellular levels (Fuller and Spradling, 2007; Xie, 2013). In this study, we have identified Twin as an intrinsic factor for promoting both GSC self-renewal and differentiation, and we have further shown that it forms distinct protein complexes in GSCs and their progeny.

Two or three GSCs in each *Drosophila* ovary niche undergo continuous self-renewing division to produce differentiating cystoblasts (CBs) (Xie and Spradling, 2001). CBs divide synchronously exactly four times with incomplete cytokinesis to form two-cell, four-cell, eight-cell, and 16-cell cysts. GSCs and CBs can be easily distinguished from cysts by their distinct fusome morphologies: spherical fusome (also known as the spectrosome) in GSCs and CBs and branched fusome in cysts (Lin et al., 1994). Although both GSCs and CBs contain a spherical spectrosome, they also can be reliably distinguished from each other by their physical locations: GSCs directly contact cap cells, whereas CBs do not (Xie and Spradling, 2000). Niche-activated BMP signaling controls GSC self-renewal by repressing the transcription of the master germ cell differentiation factor *bam* and, thus, preventing Bam-dependent differentiation pathways (Chen and McKearin, 2003; Song et al., 2004; Xie and Spradling, 1998). In addition, niche-expressing E-cadherin anchors GSCs in the niche by homophilic interactions with GSC-expressing E-cadherin (Song et al., 2002).

RNA regulators play essential roles in intrinsically controlling GSC maintenance and differentiation. GSCs are known to require the functions of the microRNA (miRNA) pathway (Jin and Xie, 2007; Park et al., 2007; Yang et al., 2007), translation regulators Pum and Nos (Forbes and Lehmann, 1998; Gilboa and Lehmann, 2004; Lin and Spradling, 1997; Wang and Lin, 2004), and translation release factor Pelota (Xi et al., 2005) for maintaining GSC self-renewal by preventing differentiation. These intrinsic self-renewing factors maintain GSCs by directly or indirectly repressing Bam-dependent and Bam-independent differentiation pathways.

Bam is the master GSC differentiation factor in the female (McKearin and Spradling, 1990; Ohlstein and McKearin, 1997). Bam directly interacts with putative RNA-binding protein Bgcn and translation initiation factor eIF4A to control CB differentiation (Li et al., 2009; Shen et al., 2009). In addition, RNA-binding

proteins Sxl and Mei-P26 control germ cell differentiation, possibly by functioning in the same protein complexes with Bam and Bgcn (Chau et al., 2009, 2012; Li et al., 2013; Neumüller et al., 2008). Sxl and Bam can repress Nos protein expression in mitotic cysts post-transcriptionally (Chau et al., 2012; Li et al., 2009), whereas Bam and Bgcn can repress E-cadherin expression via the 3′ UTR (Shen et al., 2009). RNA-binding proteins A2BP1, Aret (also known as Bruno), Brat, Pum, and Rbp9 also regulate germ cell differentiation (Harris et al., 2011; Kim-Ha et al., 1999; Tastan et al., 2010; Wang and Lin, 2007). Brat and Pum control germ cell differentiation partly by repressing the expression of BMP downstream transcription factor Mad post-transcriptionally (Harris et al., 2011), while Aret represses cytoplasmic Sxl expression (Wang and Lin, 2007). Therefore, intrinsic RNA regulators promote GSC progeny differentiation by inactivating BMP signaling and E-cadherin and enhancing the expression of other differentiation factors.

Intrinsic RNA regulators promote GSC self-renewal and differentiation via regulation of either translation or mRNA stability (Xie, 2013). The CCR4-NOT complex, which consists of NOT1–NOT3, CAF40, and CCR4 and CAF1 deadenylases, is involved in the degradation of the polyA tail, which is critical for mRNA stability and translation efficiency (Miller and Reese, 2012; Temme et al., 2010). A previous genetic study found that the *Drosophila* CCR4, which is encoded by *twin*, is required for germ cell cyst formation by regulating the polyA length of *cyclin A* (Morris et al., 2005). It has been shown recently that it also is required for GSC maintenance by interacting with Pum and Nos to repress the expression of Mei-P26 via regulation of its polyA tail (Joly et al., 2013). Similarly, Nanos proteins also can recruit the CCR4-NOT complex to control germ cell development in mice (Suzuki et al., 2010). This study shows that Twin intrinsically not only controls GSC self-renewal, at least partly by maintaining E-cadherin accumulation at the GSC-niche junction as well as preventing transposon-induced DNA damage, but also promotes GSC progeny differentiation by forming protein complexes with Bam.

RESULTS

CCR4-NOT Complex Is Required Intrinsically for GSC Maintenance

One previous study showed that *twin* mutant ovaries exhibited the defects in mitotic cyst division (Morris et al., 2005). To determine if *twin* also is required for GSC maintenance, we examined the GSC number in the *twin* heterozygous and homozygous mutant ovaries using two strong loss-of-function mutants, $twin^{ry3}$ and $twin^{ry5}$. Hts labels spectrosomes in GSCs and CBs and branched fusomes in mitotic and 16-cell cysts (Lin et al., 1994). The 3-day- and 1-week-old *twin* heterozygous control germaria maintain two or three GSCs (Figures 1A, 1B, and 1E). In contrast, 3-day- and 1-week-old $twin^{ry3}$ and $twin^{ry5}$ homozygous mutant germaria contain one GSC and 0.5 GSC, respectively (Figures 1C–1E). To further determine if the two *twin* mutants behave functionally as null mutations, we examined the GSC number in the *twin* hemizygous mutant ovaries (a *twin* mutant over the deficiency *Df(3R)Exel6198* deleting the *twin* gene region) at the ages of 3 days, 1 week, 2 weeks, and 3 weeks. In addition to Hts labeling, Vasa staining was used to label all the germ cells, including GSCs (Hay et al., 1988; Lasko and Ashburner, 1988). The *twin* hemizygous ($twin^{ry3/df}$ and $twin^{ry5/df}$) mutant germaria contain one GSC and 0.5 GSC at 3-day- and 1-week-old ages, respectively (Figures 1F and 1H). The 3-week-old germaria almost do not carry any GSCs (Figures 1G and 1H). The severity in the GSC loss phenotype in the *twin* hemizygous mutants is similar to that in the *twin* homozygous mutants, indicating that the two *twin* mutants are strong loss-of-function or null mutations (Figures 1E and 1H). These results demonstrate that Twin is required for GSC maintenance.

To determine if *twin* mutant GSCs are lost due to apoptosis, we examined the expression of cleaved Caspase 3 in *twin* heterozygous and homozygous mutant GSCs. The expression of cleaved Caspase 3 is a commonly used indicator for apoptotic cells. After examining 172 *twin* mutant GSCs, we failed to detect any cleaved Caspase 3-positive GSCs, suggesting that *twin* mutant GSCs are lost not due to apoptosis (Figure S1). Although other forms of cell death for *twin* mutant GSCs could not be completely ruled out, our results suggest that Twin maintains GSCs likely by promoting self-renewal.

To further determine if Twin is required intrinsically to maintain GSCs, we used the FLP-mediated FRT recombination and the two above-mentioned *twin* mutations to generate LacZ-negative marked *twin* mutant GSCs, and we further studied their maintenance with time in comparison with the marked control GSCs, as described previously (Xie and Spradling, 1998). As expected, most of the marked control GSCs detected 1 week after clone induction (ACI) were still maintained in the niche 3 weeks ACI, indicating that those marked control GSCs were stably maintained (Figures 1I, 1J, and 1M). In contrast, most of the marked *twin* mutant GSCs detected 1 week ACI were lost 3 weeks ACI (Figures 1K–1M). Pop2 is the second catalytic subunit in the CCR4-NOT complex. We also used the FLP-mediated FRT recombination and two P element-induced *pop2* mutations, $pop2^{DG02463}$ ($pop2^{DG}$) and $pop2^{MB11505}$ ($pop2^{MB}$), to generate the control and *pop2* mutant GSCs marked by loss of nuclear GFP expression. Consistently, the marked *pop2* mutant GSCs were lost much faster from the niche than the marked control GSCs (Figures 1N–1P). These results demonstrate that Twin and Pop2 are required intrinsically to maintain GSCs.

To examine if other CCR4-NOT components also are required intrinsically for GSC maintenance, we used *nos-gal4*-driven RNAi expression to knock down *not1* and *not3* gene expression specifically in germ cells. Not1 and Not3 are also obligate components of the deadenylase CCR4-NOT complex in *Drosophila* (Temme et al., 2010). Germline-specific *not1* and *not3* knockdown efficiently eliminated their mRNAs and led to rapid GSC loss, indicating that they also were required intrinsically for maintaining GSCs (Figures 1Q–1U; Figures S2A and S2B). Taken together, our experimental results suggest that Not1 and Not3 likely function with Twin and Pop2 in the CCR4-NOT complex to maintain GSC self-renewal.

Twin Is Required Intrinsically to Maintain E-Cadherin Accumulation at the GSC-Niche Junction, but Not BMP Signaling in GSCs

Two important pathways, BMP signaling and E-cadherin-mediated cell adhesion, are essential for GSC-niche communication

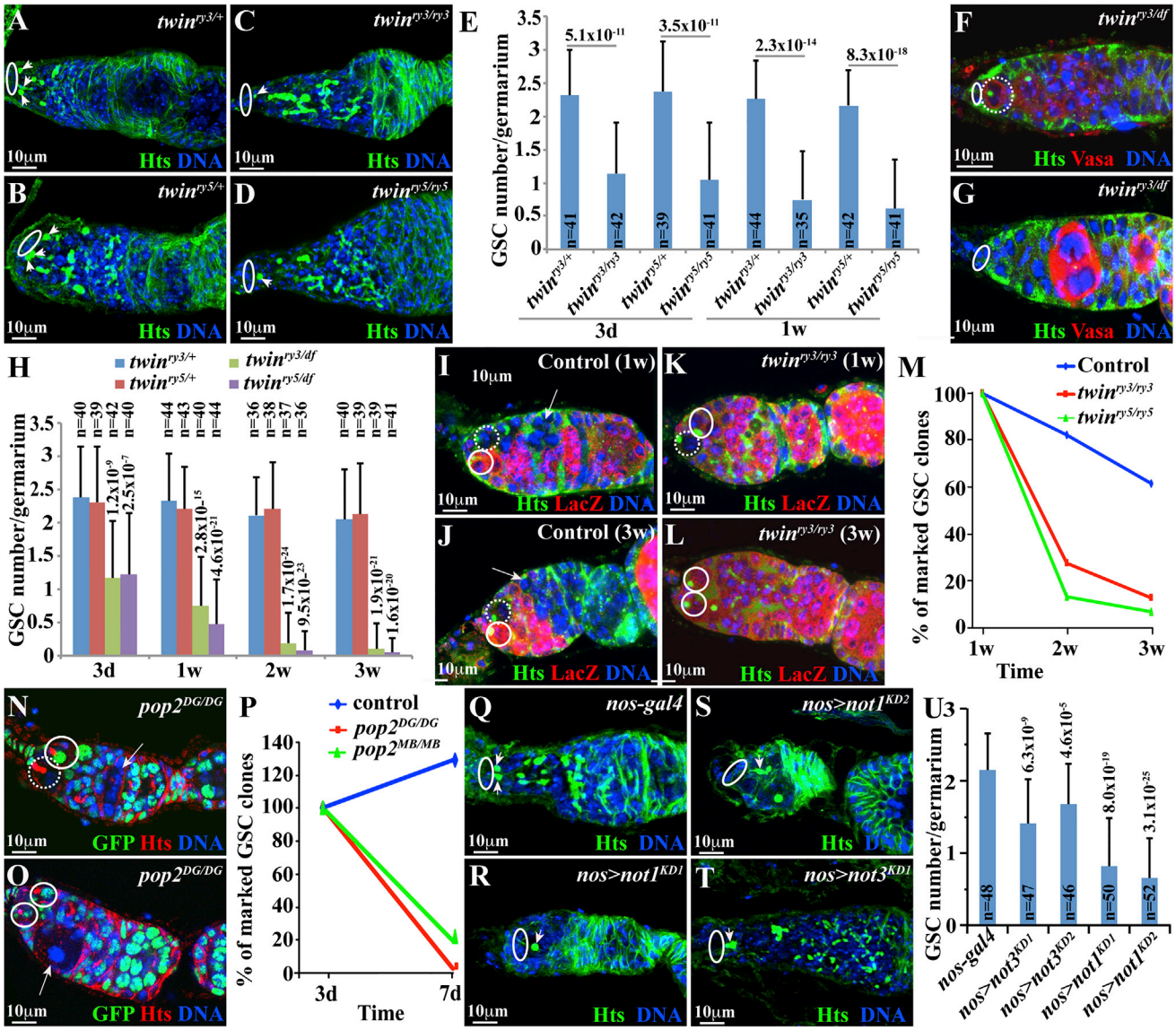

Figure 1. Twin and Other CCR4-NOT Components Are Required Intrinsically to Control GSC Maintenance

(A–E) One-week-old *twin* heterozygous control and homozygous mutant germ aria contain three GSCs and one GSC (arrowhead), respectively, located adjacent to cap cells (oval). (E) GSC quantification results are shown (n is the number of the examined germaria; all the error bars represent SDs; p values were calculated using Student's t test).

(F–H) The 3-day- (F) and 3-week- (G) old *twin* hemizygous mutant germaria contain one GSC (broken circle) and no GSCs close to cap cells (oval). (H) GSC quantification results are shown.

(I–M) LacZ-negative marked control GSC (broken circle) detected 1 week (1w) ACI (I) is still maintained 3 weeks (3w) ACI (J). In contrast, a LacZ-negative marked *twin* mutant GSC (broken circle) detected 1w ACI (K) has already been lost from the niche 3w ACI (L). (M) Quantification results are shown (the percentages of the germaria carrying a marked GSC clone at 1w ACI are normalized to 100%). LacZ-positive unmarked GSCs are highlighted by circles, whereas marked differentiated cysts are indicated by arrows.

(N–P) The GFP-negative marked *pop2* mutant GSC (broken circles) detected 1w ACI (N) is lost 3w ACI (O). Arrows indicate the differentiated GSC progeny (N and O). (P) Quantification results are shown.

(Q–U) Germline-specific *not1* (R and S) and *not3* (T) knockdown germaria retain one GSC (arrowhead) 1w after RNAi expression in contrast with a control germarium containing two GSCs (Q). (U) GSC quantification results are shown.

and GSC maintenance (Song et al., 2002, 2004; Xie and Spradling, 1998). To investigate if Twin is required intrinsically to maintain BMP signaling in GSCs, we examined phosphorylated Mad (pMad) expression in the control and *twin* mutant GSCs. pMad and *Dad-lacZ* are commonly used indicators of BMP signaling activity in GSCs, because niche-mediated BMP signaling leads to pMad production and transcriptional activation of *Dad* (Casanueva and Ferguson, 2004; Chen and McKearin, 2003; Gilboa

and Lehmann, 2004; Kai and Spradling, 2003; Song et al., 2004). Interestingly, the *twin* mutant GSCs remaining in the niche expressed comparable levels of pMad and *Dad-lacZ* to those in control GSCs (Figures 2A–2D). These results indicate that Twin is dispensable for BMP signaling activity in GSCs.

To determine if Twin is required to maintain E-cadherin accumulation at the GSC-niche junction, we used the germline-specific Gal4 Flipout system to generate GFP-positive marked *twin* knockdown GSCs, and we compared their E-cadherin accumulation at the GSC-niche junction to that in their sibling control GSCs in the same niches (Ma et al., 2014). As expected, GFP-marked control GSCs had similar levels of E-cadherin accumulation at the GSC-niche junction to those unmarked control sibling GSCs (Figure 2G). Two independent transgenic RNAi lines against different *twin* sequences (*twinRNAi-1* and *twinRNAi-2*) efficiently knocked down *twin* expression in combination with *nos-gal4* (*nos>twinKD1* and *nos>twinKD2*) in germ cells, including GSCs (Figure S2C). In contrast, GFP-marked *twin* knockdown GSCs showed significantly less E-cadherin at the GSC-niche junction than their unmarked GFP-negative control sibling GSCs (Figures 2E–2G). These results indicate that Twin is required intrinsically to maintain E-cadherin accumulation at the GSC-niche junction.

To verify if Twin controls GSC maintenance by regulating E-cadherin expression, we overexpressed E-cadherin in the *twin* knockdown GSCs. As the two *twin* RNAi lines exhibited similar knockdown efficiencies, *nos>twinKD1* and *nos>twinKD2* ovaries produced comparable GSC loss phenotypes (Figure S2C; Figures 2H and 2K). The *nos-gal4*-driven *UASp-shg* expression was used to overexpress E-cadherin in germ cells, including GSCs (*shg* encodes E-cadherin in *Drosophila*). As we reported previously (Chen et al., 2010), germ cell-specific overexpression of E-cadherin did not affect the GSC number (Figures 2I and 2K). Interestingly, germline-specific *shg* overexpression could partially, but significantly, rescue the GSC loss phenotype caused by germline-specific *twin* knockdown, suggesting that increased E-cadherin expression can partially stabilize *twin* mutant GSCs in the niche (Figures 2J and 2K). Taken together, our results indicate that Twin maintains GSC self-renewal partly via regulation of E-cadherin expression.

To further investigate how Twin regulates E-cadherin at the molecular level, we used mRNA sequencing to quantify *shg* mRNA expression levels in *twin* heterozygous and homozygous mutant ovaries. Interestingly, *shg* mRNA levels remained unchanged in the *twin* homozygous mutant ovaries in comparison with the *twin* heterozygous control ovaries (Figure 2L). Because Twin is involved in the regulation of polyA tail length, we also used the PCR-based assay to determine the polyA tail length in the *twin* heterozygous and homozygous mutant ovaries. The polyA tails of *shg* mRNAs remained similar in both *twin* heterozygous and homozygous ovaries (Figure 2M). These results suggest that Twin regulates E-cadherin expression not via regulation of polyA tails and mRNA stability.

Twin Controls GSC Self-Renewal Partly by Preventing DNA Damage-Induced Checkpoint Activation

Twin recently has been identified to be required for PIWI-interacting RNA (piRNA)-mediated transposable element (TE) repression (Czech et al., 2013; Handler et al., 2013). Elevated TE activities lead to an accumulation of double-stranded breaks, which can be recognized by phosphorylated H2AvD (γ-H2AvD) (Jang et al., 2003). In the control germaria, only meiotic germ cells were γ-H2AvD positive, but GSCs were negative (Figures 3A and 3A'). In contrast, the *twin* homozygous germaria contained γ-H2AvD-positive GSCs in addition to meiotic germ cells, indicating that Twin is required in GSCs to prevent DNA damage (Figures 3B–3C'). It is worth noting that the extent of DNA damage in *twin* mutant GSCs is not as severe as in the mutant 16-cell cysts. These results indicate that Twin is required in GSCs to prevent DNA damage accumulation.

DNA damage leads to checkpoint activation in stem cells, slowing down the cell cycle progression for DNA damage repair (Sperka et al., 2012). CHK2, which is encoded by *lok*, is known to be required for the DNA damage-invoked checkpoint activation in germ cells (Chen et al., 2007; Klattenhoff et al., 2007). We investigated if *twin* knockdown-induced GSC loss is caused by DNA damage-induced checkpoint activation. Germline-specific *lok* knockdown did not result in discernible GSC phenotypes, behaving like the control (Figures 3D and 3F). Interestingly, germline-specific *lok* knockdown partially and significantly rescued the GSC loss phenotype caused by *twin* knockdown, indicating that Twin maintains GSCs partly by preventing DNA damage-induced checkpoint activation (Figures 3E and 3F). Then we determined if both CHK2 activation and E-cadherin downregulation contribute to the *twin* mutant GSC loss phenotype. Interestingly, E-cadherin overexpression and CHK2 knockdown together could much better rescue the *twin* mutant GSC loss phenotype than either E-cadherin overexpression or CHK2 knockdown alone (Figures 3G and 2K). These results suggest that Twin maintains GSCs by promoting E-cadherin expression and preventing DNA damage.

To investigate if TE transcripts are increased in *twin* mutant ovaries, we used qRT-PCR to quantify the mRNA levels of germline-specific transposons *TART* and *Het-A*. Interestingly, both *TART* and *Het-A* transcripts increased, but the latter transcripts were upregulated more dramatically than the former ones in the *twin* homozygous mutant ovaries (Figure 3H). Interestingly, the expression levels of germline-specific piRNAs *HetA*, *AT-chX-1*, and *roo* still remained similar in both wild-type control and *twin* mutant ovaries, indicating that Twin is dispensable for general germline piRNA production (Figure 3I and 3I'). Piwi family members Ago3, Aub, and Piwi are known to be important for piRNA biogenesis and piRNA-mediated transposon repression (Aravin and Hannon, 2008; Juliano et al., 2011; Siomi et al., 2010). Our co-immunoprecipitation (coIP) results showed that Twin is capable of forming protein complexes with Aub and Ago, but not Piwi, in S2 cells (Figures 3J–3L). Taken together, our results suggest that Twin represses TEs in germ cells downstream of piRNA biogenesis, possibly by forming protein complexes with Aub and Ago3.

Twin Works with Bam to Regulate Germ Cell Differentiation and Repress Nanos Expression

It was reported previously that Twin is required to maintain Bam expression (Morris et al., 2005). Indeed, we have confirmed that Bam-positive cyst number and Bam protein levels decreased in the *twin* mutant ovaries in comparison with the control ovaries

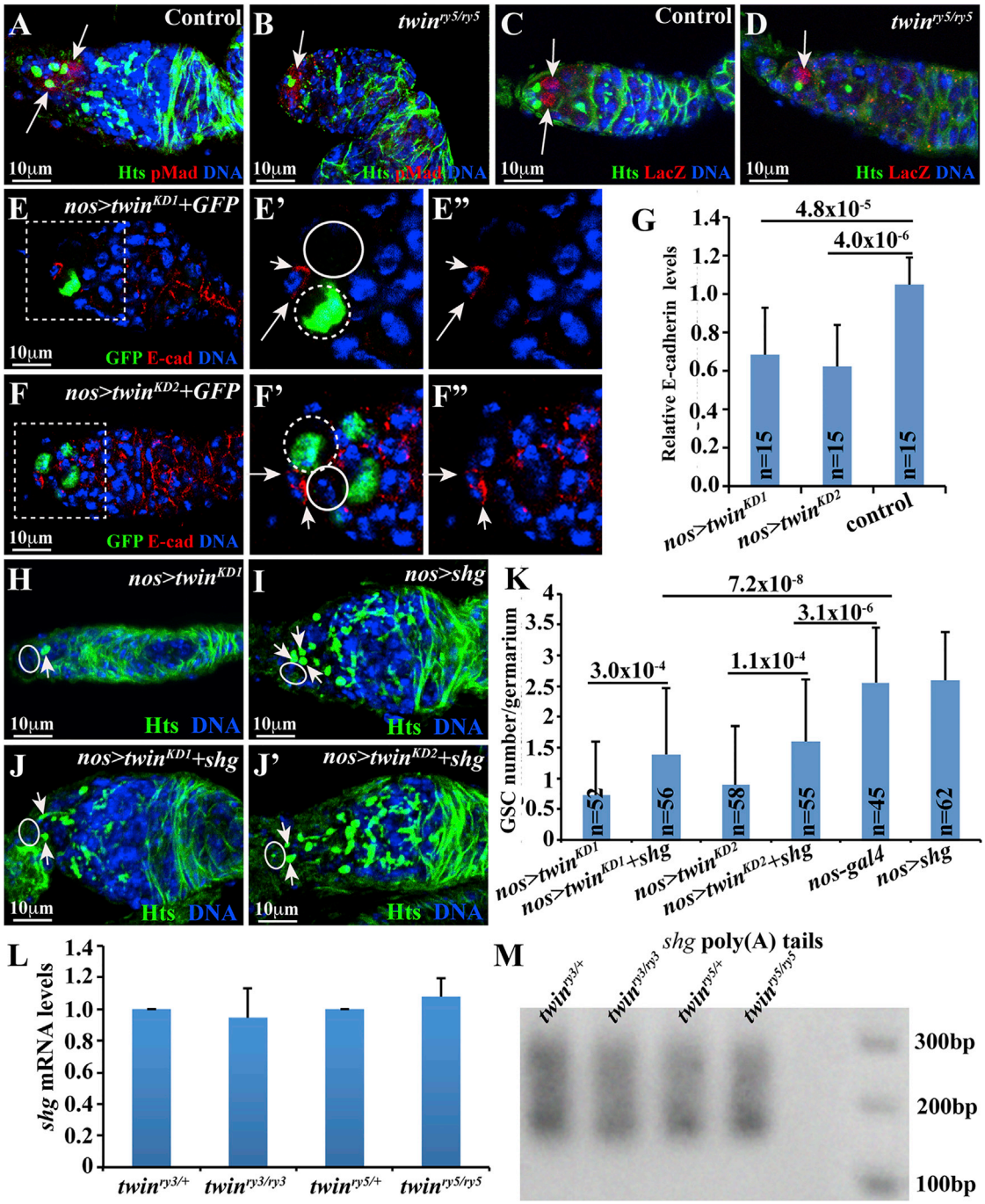

Figure 2. Twin Maintains GSCs Partly by Sustaining E-Cadherin

(A–D) The remaining GSC (arrows) in the *twin* mutant germaria (B and D) still expresses pMad (A and B) and *Dad-lacZ* (C and D) at levels similar to those of GSCs (arrows) in the heterozygous control germaria (A and C).

(E–G) GFP-positive *twin* knockdown GSCs (broken circles in E' and F') express less E-cadherin at the GSC-niche junction (arrows and arrowheads in E'' and F'') than their sibling control GSCs (GFP-negative; solid circles in E' and F'). (G) Quantification results are shown.

(H–K) Germline-specific *twin* knockdown germaria contain one GSC, whereas germline-specific E-cadherin expression can partially rescue the GSC loss phenotype caused by *twin* knockdown. Spectrosomes in GSCs and cap cells are indicated by arrowheads and ovals in (H–J'), respectively. (K) Quantification results are shown.

(L) RNA sequencing (RNA-seq) results show that *shg* mRNA levels in the heterozygous and homozygous *twin* mutant ovaries remain similar.

(M) The *shg* polyA tail length determined by PCR-based assays remains similar in the heterozygous and homozygous *twin* mutant ovaries.

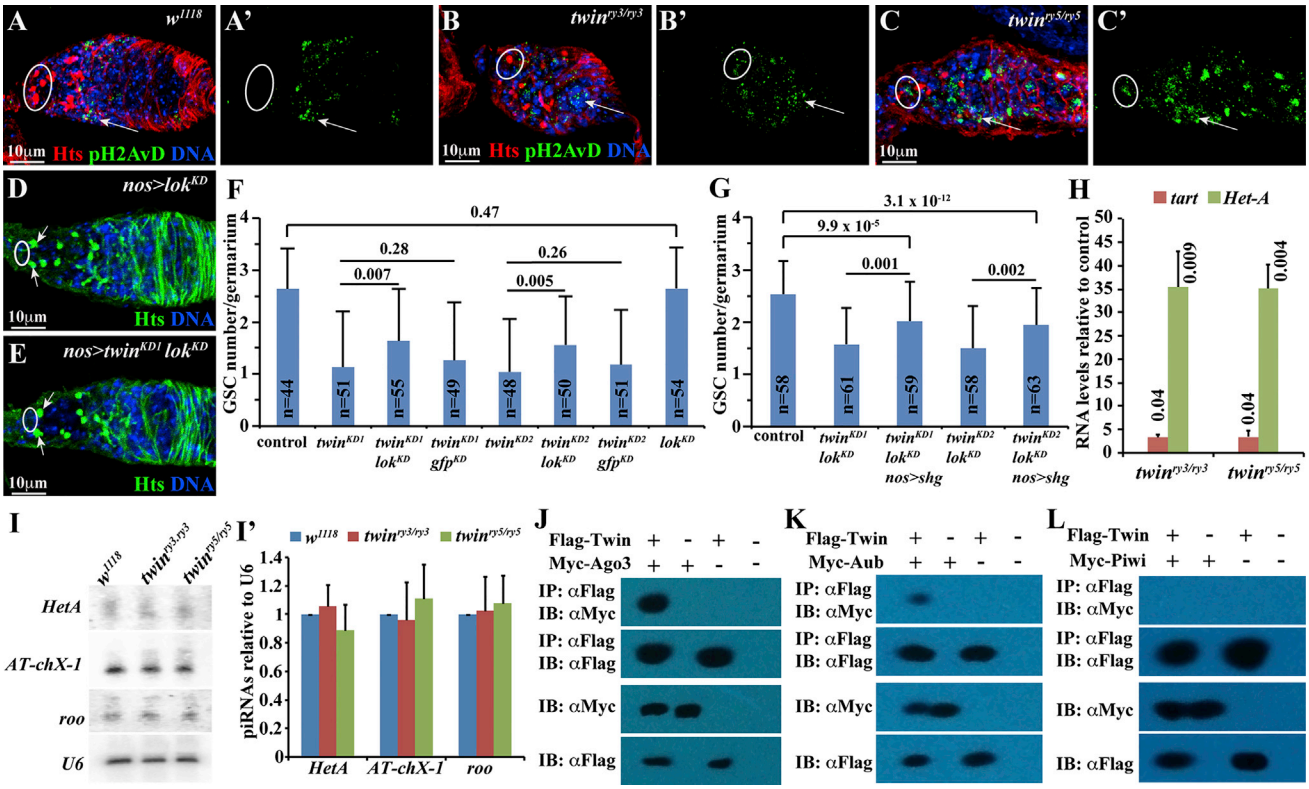

Figure 3. Twin Is Required Intrinsically to Prevent Transposon-Induced DNA Damage

(A, A') In the control germarium, DNA damage recognized by γ-H2AvD can be detected easily in meiotic germ cells (arrow), but not in GSCs (oval).

(B–C') In *twin*^{ry3} (B and B') and *twin*^{ry5} (C and C') germaria, the remaining GSCs (oval) are positive for γ-H2AvD in addition to meiotic germ cells (arrow).

(D and E) The *lok* knockdown (D) and *lok twin* double-knockdown (E) germaria still contain two GSCs (arrows).

(F) Quantification results show that *lok* knockdown can partially and significantly rescue the GSC loss phenotype caused by *twin* knockdown (*twinKD gfpKD* acts as the control for *twinKD lokKD*).

(G) Quantification results show that *lok* knockdown and *shg* overexpression work additively to rescue the GSC loss phenotype caused by *twin* knockdown.

(H) The qRT-PCR results show that *twin*^{ry3} and *twin*^{ry5} mutant ovaries express significantly more *tart* and *Het-A* transcripts than wild-type.

(I, I') Northern blot results show that *HetA*, *AT-chX-1*, and *roo* piRNA expression levels remain unchanged in *twin* mutant ovaries compared with wild-type ovaries (*U6* is an internal loading control. (I') Quantification results are shown.

(J–L) CoIP experiments show that Flag-Twin can pull down Myc-Ago3 (J) and Myc-Aub (K), but not Myc-Piwi (L), in S2 cells. IP, immunoprecipitation; IB, immunoblot; + and –, the presence and absence of a given tagged protein, respectively; αFlag, anti-Flag antibody.

(Figure S3). We used two independent genetic experiments to investigate if Twin works with Bam to regulate germ cell differentiation. First, we examined if a *twin* mutation enhances the germ cell differentiation defects of the *bam*$^{\Delta 86}$ heterozygote. As we reported previously (Shen et al., 2009), inactivating one copy of Bam by the *bam*$^{\Delta 86}$ heterozygous mutation caused a slight increase in CB number, exhibiting minor germ cell differentiation defects (Figures 4A and 4D). Interestingly, the homozygous *twin*^{ry5} ovaries also heterozygous for *bam*$^{\Delta 86}$ contained significantly more CBs than the *twin*^{ry5} homozygous and the *bam*$^{\Delta 86}$ heterozygous ovaries (Figures 4A–4D). However, the homozygous *twin*^{ry5} ovaries that also were heterozygous for *bam*$^{\Delta 86}$ still contained one GSC on the average, which was comparable to that in the *twin*^{ry5} homozygous ovaries, ruling out the possibility that excess CBs are caused by increased GSCs (Figure S4A). Second, we examined if germline-specific *twin* knockdown also could enhance the germ cell differentiation defects of the *bam* heterozygous ovaries. Consistently, germline-specific *twin* knockdown made *bam*$^{\Delta 86}$ heterozygous ovaries carry significantly more CBs than the *bam*$^{\Delta 86}$ heterozygous ovaries (Figures 4E and 4H), whereas germline-specific *twin* knockdown led to a similar GSC loss in the *bam*$^{\Delta 86}$ heterozygous ovaries and control ovaries (Figure S4B). Taken together, these results demonstrate that Twin works with Bam to control germ cell differentiation.

To determine if other components in the CCR4-NOT complex also work with Bam to control germ cell differentiation, we examined if germline-specific knockdown of *pop2*, *not1*, and *not3* also could enhance the germ cell differentiation defects of the *bam* heterozygous ovaries. As expected, germline-specific knockdown of *pop2* significantly decreased *pop2* mRNAs and also GSC number, indicating that *pop2* knockdown worked well (Figure 4F; Figures S2D and S4B). In contrast with *twin* knockdown, *pop2*, *not1*, or *not3* knockdown failed to enhance the germ cell differentiation defect of the *bam*$^{\Delta 86}$ heterozygote (Figures 4F–4H). These results suggest that Pop2, Not1, and Not3 might not work with Bam to regulate germ cell differentiation.

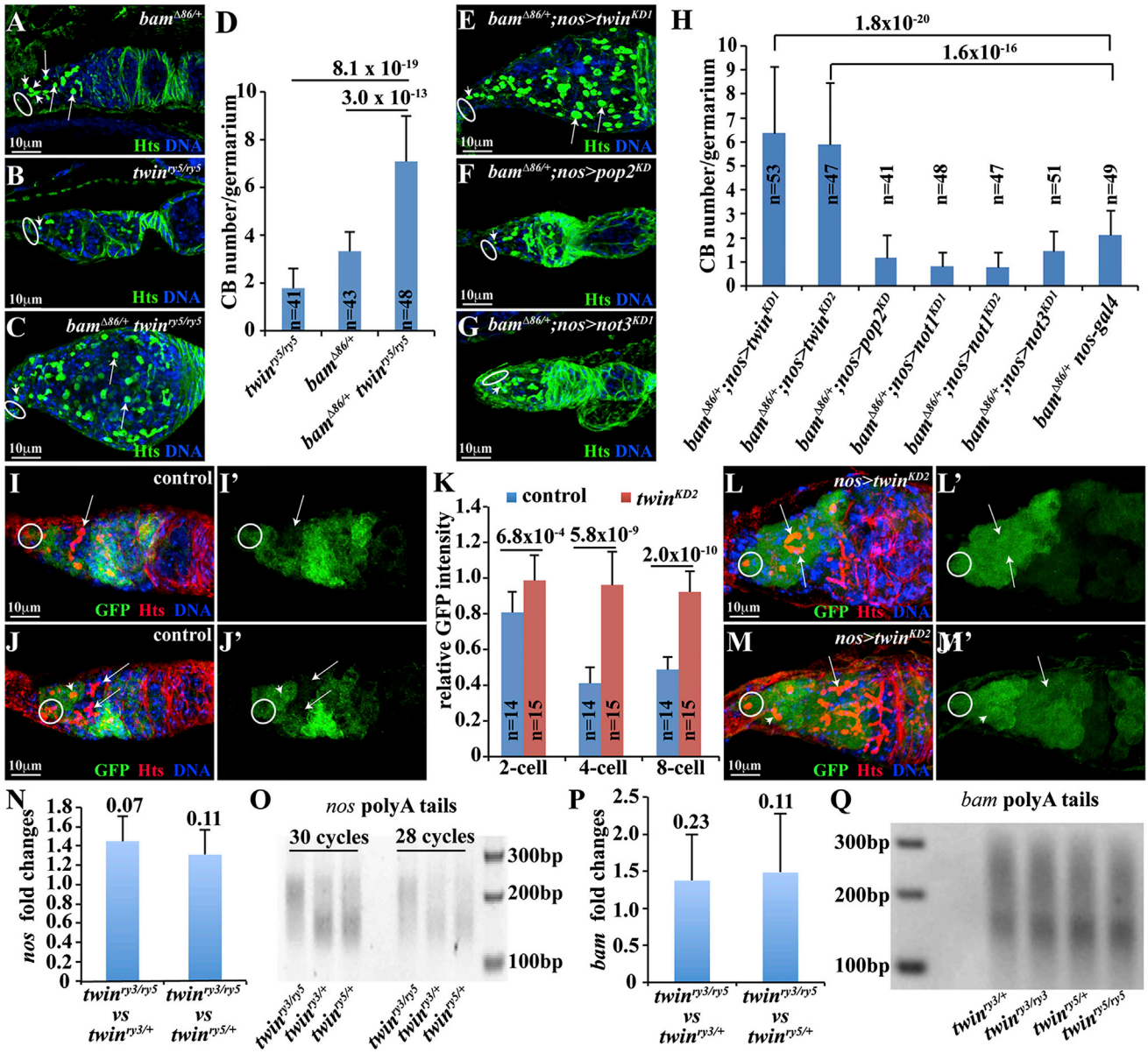

Figure 4. Twin Promotes Bam-Dependent Germ Cell Differentiation

(A–D) The *bam*$^{\Delta 86}$ heterozygous mutation significantly enhances germ cell differentiation defects of the *twin* mutant germaria (*bam*$^{\Delta 86/+}$: three GSCs and three CBs; *twin*^{ry5ry5}: one GSC; and *bam*$^{\Delta 86/+}$ *twin*$^{ry5/ry5}$: one GSC and many extra CBs). A control germarium normally contains one CB. Cap cells, GSCs, and CBs are indicated by ovals, arrowheads, and arrows, respectively. (D) Quantification results are shown.

(E–H) The *twin* knockdown *bam*$^{\Delta 86/+}$germarium (E), but not *pop2* (F) or *not3* (G) knockdown *bam*$^{\Delta 86/+}$ germarium, contains excess CBs (arrows) in addition to one GSC (arrowhead). (H) Quantitative results are shown.

(I–M') The *nos-GFP* is significantly lower in control four-cell (arrow, I, I') and eight-cell cysts (arrows, J, J') than in other germ cells, but is significantly upregulated in *twin* knockdown four-cell (arrows, L, L') and eight-cell (arrow, M, M') cysts. (K) Quantification results are shown. Circles and arrowheads indicate GSCs and two-cell cysts, respectively.

(N) RNA-seq results show that *nos* mRNA levels are not significantly changed in *twin* mutant ovaries compared with the heterozygous control ovaries.

(O) The *shg* polyA tails are longer in the *twin* homozygous mutant ovaries than in the heterozygous ovaries.

(P) RNA-seq results show that *bam* mRNA levels remain similar in *twin* homozygous and heterozygous ovaries.

(Q) The *bam* polyA tails remain similar in *twin* homozygous and heterozygous ovaries.

One of the Bam functions in mitotic germ cells is to repress Nanos (Nos) expression via its 3′ UTR (Chau et al., 2012; Li et al., 2009). The transgene *nosP-GFP-nos3′UTR* was used to monitor the post-transcriptional regulation of *nos* expression in germ cells. In the wild-type background, the reporter showed comparable GFP expression in GSCs and CBs, but its

expression gradually was downregulated in two-cell, four-cell, and eight-cell mitotic cysts (Figures 4I–4K). Consistent with the reduced Bam function in the *twin* mutant mitotic cysts, the downregulation of *nos-GFP* expression in four-cell and eight-cell cysts was completely abolished (Figure 4K). Consequently, *nos-GFP* expression was significantly higher in *twin* knockdown four-cell and eight-cell cysts than in their control counterparts (Figures 4K–4M'). These results indicate that Twin is required to repress *nos* expression in mitotic cysts via its 3′ UTR.

To determine if Twin regulates *nos* mRNA stability, we then examined *nos* mRNA expression levels and polyA tail length in *twin* mutant ovaries. Our RNA sequencing results indicated that the levels of the *nos* transcripts were not significantly changed in the *twin* homozygote in comparison with the heterozygous control (Figure 4N). The polyA tails of the *nos* transcripts in the *twin* homozygote were longer than those in the *twin* heterozygous control, indicating that Twin regulated *nos* mRNA polyadenylation but did not regulate *nos* mRNA levels (Figure 4O). Surprisingly, *bam* mRNA levels and polyA tails were not changed in the *twin* homozygote in comparison to the heterozygous control (Figures 4P and 4Q). Taken together, these results suggest that Twin represses Nos protein expression and promotes Bam protein expression in mitotic cysts, primarily at the translational or post-translational level.

Bam Is Associated with Twin in *Drosophila* Female Germ Cells

Our genetic results predicted that Bam and Twin should be expressed in mitotic cysts in the *Drosophila* ovary. Due to the lack of a suitable antibody against Twin and Pop2, we used the existing GFP-tagged BAC transgenic lines for *twin* and *pop2*, *twin-GFP* and *pop2-GFP*, to examine Twin and Pop2 expression patterns and subcellular localization, respectively (Ryder et al., 2009). In the germaria, Twin-GFP and Pop2-GFP primarily were expressed in early germ cells, including GSCs, CBs, mitotic cysts, and 16-cell cysts (Figures 5A–5D). In mitotic cysts, both Twin-GFP and Pop2-GFP were localized with Bam in the cytoplasm (Figures 5B–5B'' and 5D–5D''). These results indicate that Twin and Pop2 proteins are expressed in GSCs, CBs, mitotic cysts, and 16-cell cysts, supporting their roles in GSC maintenance and differentiation.

By pulling down protein complexes in S2 cells, we identified Twin as a Bam-associated protein. We used coIP experiments in S2 cells to confirm that the full-length Flag-tagged Twin and Myc-tagged Bam can pull down each other in S2 cells (Figures 5E and 5E'). By testing different truncations of Bam in coIP experiments with Twin, Twin was found to pull down the central (151–300 amino acid [aa]) or C-terminal (301–442 aa) region of Bam, but not the N-terminal region (1–150 aa) (Figure 5E). By testing different truncations of Twin, an N-terminal 51–100 aa deletion (d2), but not 1–50 aa and 101–150 aa regional deletions, was found to abolish the ability of Twin to pull down Bam in S2 cells, indicating that the 51–100 aa region of Twin is required to form a protein complex with Bam (Figure 5E'). The 51–100 aa region is predicted to form an α-helical structure, and three leucine residues were highly conserved (L76, L79, and L81). When the leucine residues were mutated to either alanine ($Twin^{M1}$) or glycine ($Twin^{M2}$) in the region, they no longer were associated with Bam in S2 cells, indicating that the alpha-helix is important for Twin to form a protein complex with Bam (Figure 5E''). To further confirm if Bam and Twin form a protein complex in *Drosophila* ovarian germ cells, we generated transgenic flies carrying Flag-tagged wild-type *twin*, $twin^{M1}$, and $twin^{M2}$ under the control of the germline-competent *UASp* promoters *UASp-*$twin^{WT}$, *UASp-*$twin^{M1}$, and *UASp-*$twin^{M2}$. Consistent with our coIP results in S2 cells, germ cell-expressed Flag-tagged $Twin^{WT}$, but not $Twin^{M1}$ and $Twin^{M2}$, could pull down endogenous Bam protein in ovaries (Figure 5F). Finally, we also found that Twin was associated with Bam in S2 cells and germ cells in the absence of RNAs (Figure S5). These results demonstrate that Twin forms a protein complex with Bam via its N-terminal 50–101 aa region in *Drosophila* S2 and germ cells.

Bam and Bgcn form a translation repressor complex to regulate germ cell differentiation (Li et al., 2009; Shen et al., 2009). We tested if Twin formed a ternary protein complex with Bam and Bgcn. Our coIP results showed that Flag-tagged Twin could pull down Myc-tagged Bgcn in S2 cells, but it could only do so in the presence of Bam in human 293T cells, indicating that Twin is capable of associating with the Bam/Bgcn complex via interaction with Bam (Figures 5G and 5G'). One of the potential reasons for the difference is that S2 cells expressed low levels of Bam protein. Twin also is known to interact with Pop2 via its N-terminal region (Bawankar et al., 2013). Consistent with the idea that Bam is associated with Twin, but not the CCR4-NOT complex, Bam could not pull down Pop2 in S2 cells (Figure 5H). If Bam and Pop2 are associated with Twin through the same domain, the presence of Pop2 should interfere with the interaction between Bam and Twin. Indeed, in the presence of increasing concentrations of Pop2, the ability of Twin to pull down Bam in S2 cells significantly decreased (Figure S6). To check if this is the case in vivo, we also generated the transgene carrying HA-tagged *pop2* under the control of the *UASp* promoter *UASp-HA-pop2*. As expected, germline-expressed HA-Pop2 and Flag-$Twin^{WT}$ could mutually pull down each other (Figure 5I). Indeed, germline-specific expression of HA-Pop2 also interfered with the ability of Twin to bring down endogenous Bam protein in vivo (Figures 5I and 5I'). These results suggest an interesting model that Bam inactivates the self-renewal function of the CCR4-NOT complex by taking away Twin in differentiated germ cells, thereby promoting germ cell differentiation.

The LRR Domain of Twin Is Important for Both GSC Maintenance and Germ Cell Differentiation

Consistent with the knowledge that Twin interacts with Pop2 via the LRR domain for recruitment to the CCR4-NOT complex (Bawankar et al., 2013), Flag-$Twin^{WT}$, but not Flag-$Twin^{M1}$ and Flag-$Twin^{M2}$, could pull down HA-Pop2 in germ cells (Figure 6A). To further determine if the LRR domain is required for GSC maintenance and GSC progeny differentiation, we tested if germline-expressed $Twin^{M1}$ and $Twin^{M2}$ could rescue the GSC loss phenotype of the *twin* mutant ovaries and the differentiation defect of the $twin^{ry3/ry5}$ $bam^{\Delta 86/+}$ ovaries. Germline-expressed $Twin^{WT}$, but not $Twin^{M1}$ and $Twin^{M2}$, could fully rescue the GSC loss phenotype in the *twin* mutants, indicating that the LRR domain is important for Twin to maintain GSCs (Figures 6B–6F). Then, we investigated if the LRR domain is

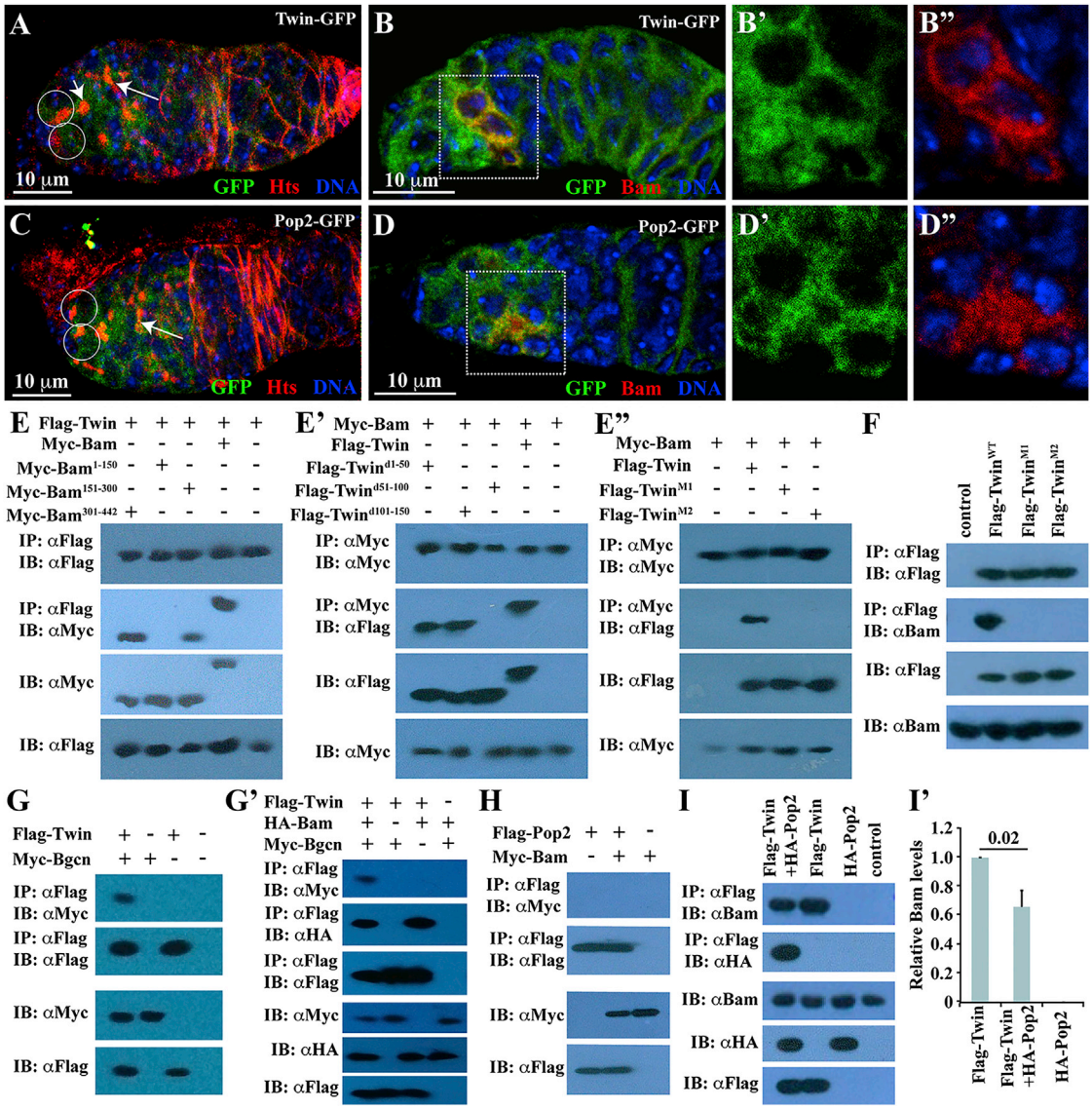

Figure 5. Bam and Twin Are Co-expressed in Mitotic Cysts to Form a Protein Complex

(A) Twin-GFP shows high expression in early germ cells including GSCs (circles), CBs (arrowhead), and cysts (arrow).

(B) Twin-GFP and Bam proteins are co-localized in the cytoplasm of mitotic cysts. (B',B'') The boxed area in (B) is highlighted at a higher magnification.

(C) Pop2-GFP shows high expression in early germ cells including GSCs (circles), CBs (arrowhead), and cysts (arrow).

(D) Pop2-GFP and Bam proteins are co-localized in the cytoplasm of mitotic cysts. (D', D'') The area in (D) is highlighted at a higher magnification.

(E–E'') CoIP results in S2 cells show that the 151–442 aa region of Bam (E) interacts with the 50–101 aa region of Twin (E'), and $Twin^{M1}$ and $Twin^{M2}$ proteins carrying the mutations in the conserved leucine residues in the 50–100 aa region lose their interaction with Bam (E''). $Bam^{1–150}$, $Bam^{151–300}$, and $Bam^{301–442}$ represent three Bam protein fragments; $Twin^{d1–50}$, $Twin^{d51–100}$, and $Twin^{d101–150}$ represent mutant Twin proteins deleting 1–50 aa, 51–100 aa, and 101–150 aa regions, respectively; the three conserved leucine residues in $Twin^{M1}$ and $Twin^{M2}$ are changed into alanine and glycine, respectively.

(F) Germ cell-expressed Flag-tagged $Twin^{WT}$, but not $Twin^{M1}$ and $Twin^{M2}$, can pull down endogenous Bam in germ cells.

(G, G') Flag-Twin can pull down Myc-Bgcn in S2 cells (G), but can bring down Myc-Bgcn in the presence of HA-Bam in human 293 cells (G').

(H) Flag-Pop2 cannot pull down Myc-Bam in S2 cells.

(I, I') The presence of germline-expressed HA-Pop2 decreases the ability of germline-expressed Flag-Twin to bring down endogenous Bam. (I':) Quantification results are shown.

required for Twin to promote germ cell differentiation. Germline-expressed $Twin^{WT}$, but not $Twin^{M1}$ and $Twin^{M2}$, could sufficiently rescue the germ cell differentiation defects of the $twin^{ry3/ry5}$ $bam^{\Delta 86/+}$ ovaries, indicating that the LRR domain also was required for Twin to promote germ cell differentiation (Figures 6G–6K). Thus, the LRR domain is required for Twin to promote GSC self-renewal and Bam-dependent germ cell differentiation.

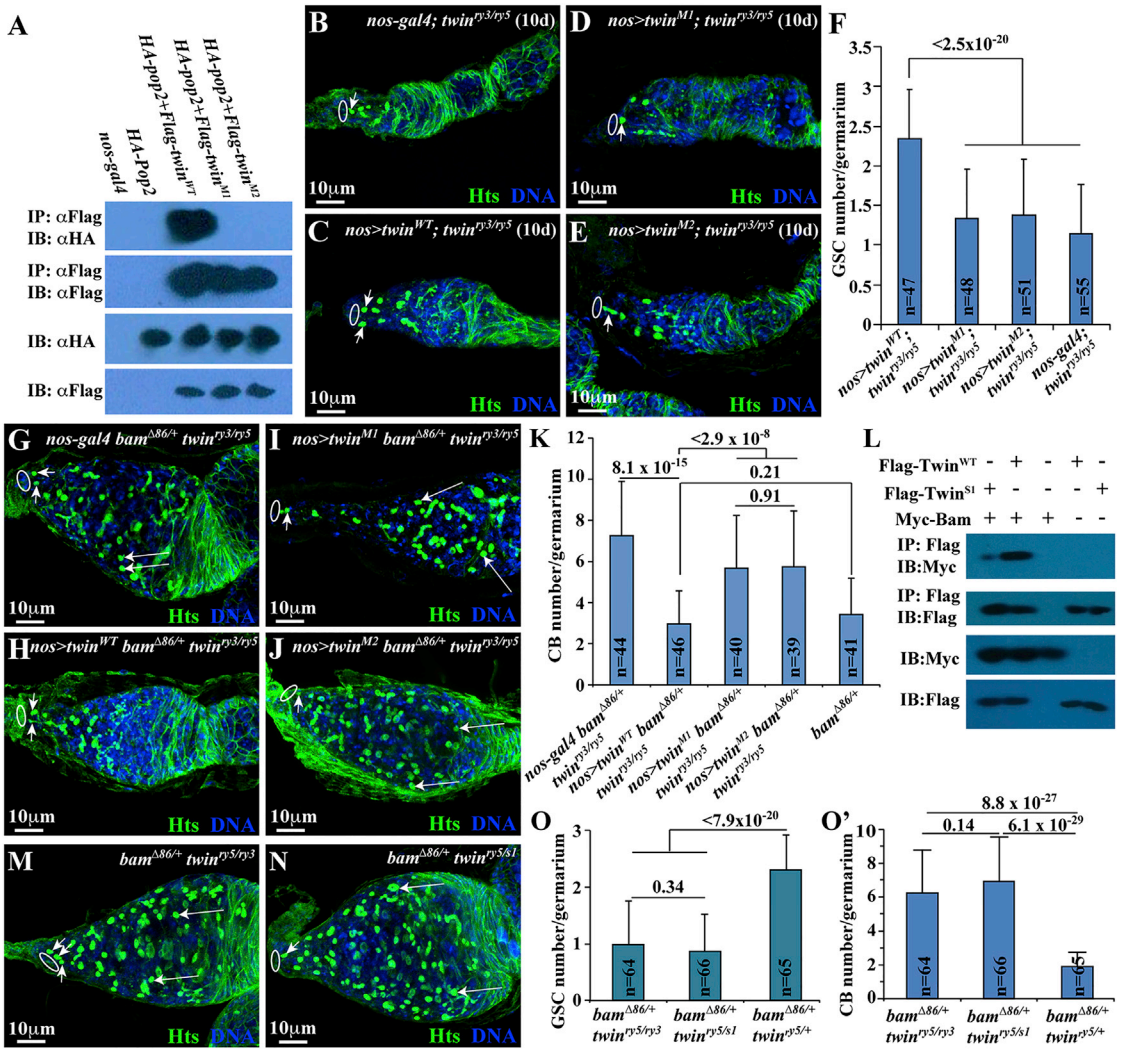

Figure 6. The LRR Domain-Mediated Protein Interaction Is Important for GSC Maintenance and Germ Cell Differentiation

In (A)–(D) and (G)–(I'), cap cells, GSCs, and CBs are indicated by ovals, arrowheads, and arrows, respectively.

(A) Germline-expressed HA-tagged Pop2 can pull down germline-expressed Flag-tagged Twin^{WT}, but not Twin^{M1} and Twin^{M2}, in the ovarian extracts (αFlag, anti-Flag antibody; and αHA, anti-HA antibody).

(B–F) A *twin* mutant germarium contains one GSC (B), while the germaria expressing *twin^{WT}* (C), *twin^{M1}* (D), and *twin^{M2}* (E) carry two GSCs, one GSC, and one GSC, respectively. (F) Quantification results are shown.

(G) A *twin* mutant germarium carrying *nos-gal4* and a *bam$^{\Delta 86}$* heterozygous mutation contains excess CBs.

(H–K) Germline-expressed *twin^{WT}* (H), but not *twin^{M1}* (I) or *twin^{M2}* (J), rescues the germ cell differentiation defect of the *twin$^{ry3/ry5}$ bam$^{\Delta 86/+}$* mutant germarium. (K) Quantification results are shown.

(L) CoIP results in S2 cells show that Flag-tagged Twin^{s1} has a lower affinity to interact with Bam than Flag-tagged Twin^{WT}.

(M and N) The *twin$^{s1/ry3}$* (N) behaves like the strong loss-of-function *twin$^{ry3/ry5}$* (M) to enhance the differentiation defect of the *bam$^{\Delta 86/+}$* mutant ovaries.

(O) GSC and CB (O') quantification results are shown.

The *twin^{s1}* carries a mutation in the LRR domain and behaves as a strong loss-of-function mutant (Morris et al., 2005). As predicted, Twin^{s1} mutant protein decreased its ability to associate with Bam based on coIP results in S2 cells (Figure 6L). Indeed, the *twin$^{s1/ry5}$* and *twin$^{ry3/ry5}$* germaria had comparable GSC numbers, but had significantly less GSCs than the *twin$^{ry5/+}$* control germaria, further confirming that the LRR domain is important for Twin to maintain GSCs (Figures 6M–6O). Similarly, *twin$^{s1/ry5}$* enhanced the differentiation defect of the *bam* heterozygotes as effectively as *twin$^{ry3/ry5}$*, indicating that the LRR domain also is required for Twin to promote germ cell differentiation (Figures 6M–6O'). These results further strengthen the idea that the LRR domain of Twin is important for GSC maintenance and germ cell differentiation.

To confirm that LRR-mediated protein association is critical for GSC maintenance and differentiation, we generated the transgene carrying the in-frame fusion of the LRR domain-coding sequence and the *gfp* gene under the control of the *UASp*

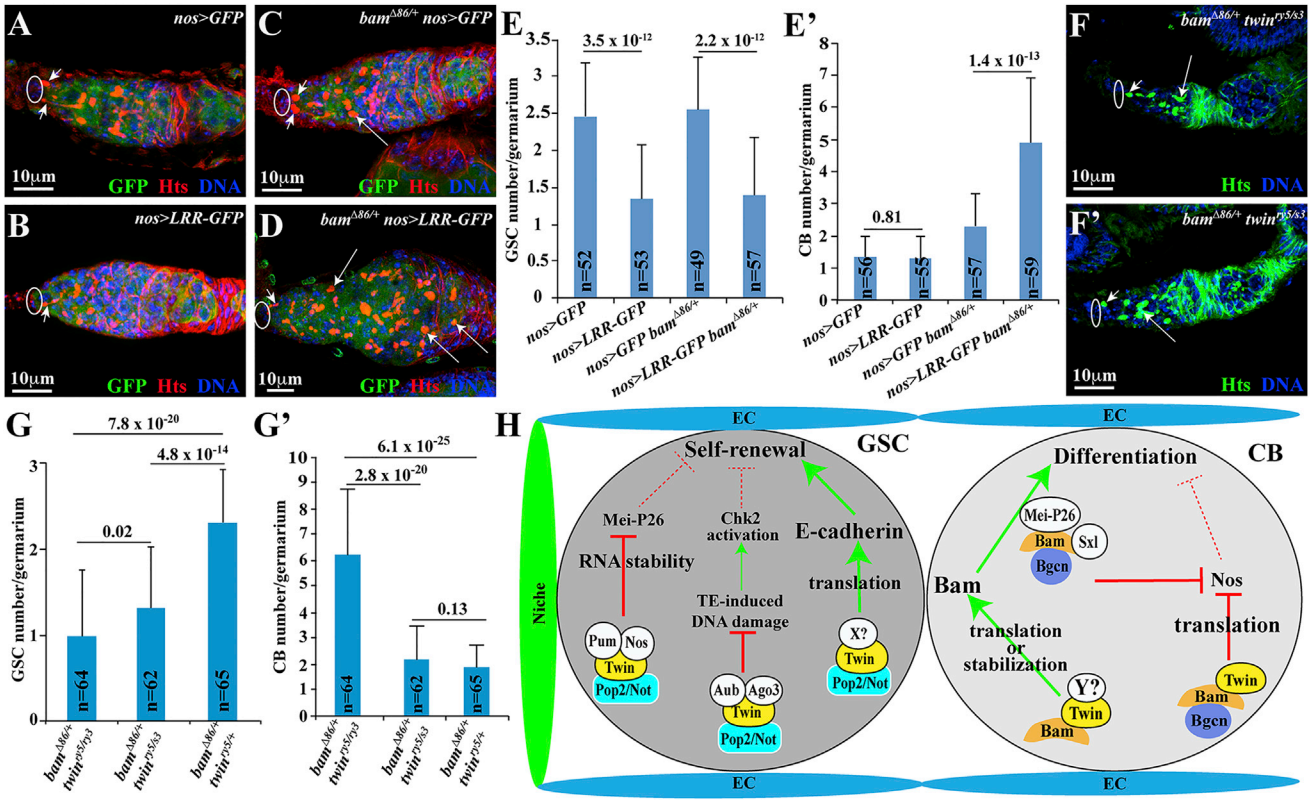

Figure 7. The LRR Domain, but Not Catalytic Domain, of Twin Is Important for Bam-Dependent Germ Cell Differentiation

(A–E') The *nos>LRR-GFP* (B) germaria carry significantly fewer GSCs (arrowheads) close to cap cells (ovals) than *nos>GFP* germaria (A), whereas *nos > LRR-GFP bam*$^{\Delta 86/+}$(D) germaria have significantly more CBs (arrows) than *nos > GFP bam*$^{\Delta 86/+}$(C) germaria. (E, E') Quantification results for GSCs and CBs are shown. (F–G') The *twin*$^{s3/ry5}$ *bam*$^{\Delta 86/+}$ germaria (F and F') contain one GSC (arrowhead) close to cap cells (oval), but do not have excess CBs. (G, G') Quantification results for GSCs and CBs are shown. Arrows in (F) and (F') indicate the branched fusomes in the cysts.

(H) A diagram shows that Twin maintains GSC self-renewal, at least by preventing DNA damage, maintaining E-cadherin expression, and repressing Mei-P26 expression, and that it also promotes germ cell differentiation, by promoting Bam expression and repressing Nos expression via protein complex formation with Bam and Bgcn.

promoter *UASp-LRR-GFP* for overexpression in germ cells in combination with *nos-gal4*. As the control, the germaria expressing GFP specifically in germ cells contained two or three GSCs (Figures 7A and 7E). In contrast, the germaria expressing LRR-GFP in germ cells contained one GSC on average (Figures 7B and 7E). Similarly, germline-expressed LRR-GFP, but not GFP, could enhance the germ cell differentiation defect of the *bam*$^{\Delta 86/+}$ females (Figures 7C, 7D, and 7E'). These results suggest that excess LRR domains could disrupt Twin's association with Bam and Pop2, thereby interfering with Twin functions in GSC maintenance and germ cell differentiation.

The Catalytic Domain of Twin Is Important for GSC Maintenance but Is Dispensable for Germ Cell Differentiation

The *twin*^{S3} mutant carries a change from an isoleucine to a serine within a conserved motif of the deadenylase catalytic domain of Twin, suggesting that this mutation could disrupt the deadenylase activity (Morris et al., 2005). The *twin*$^{ry5/s3}$ mutant germaria had slightly more GSCs than those *twin*$^{ry3/ry5}$ mutant ones, but had significantly less GSCs than the *twin*$^{ry5/+}$ control ones (Figures 7F, 7F', and 7G). In contrast with *twin*$^{ry3/ry5}$, *twin*$^{ry5/s3}$ failed to enhance the germ cell differentiation defect of the *bam*$^{\Delta 86}$ heterozygote (Figures 7F, 7F', and 7G'). Therefore, our results suggest that the catalytic domain of Twin is important for GSC maintenance, but is dispensable for germ cell differentiation.

DISCUSSION

In the *Drosophila* ovary, some important intrinsic regulators of GSC development are RNA binding proteins, including Nos, Pumilio, Bam, Bgcn, and Sxl (Xie, 2013). Nos protein interacts with the CCR4-NOT complex in the *Drosophila* embryo and in the mammalian germ cells to control mRNA stabilities (Kadyrova et al., 2007; Suzuki et al., 2010). In this report, we have shown that Twin, along with Pop2, Not1, and Not3 in the CCR4-NOT complex, intrinsically controls GSC self-renewal. In addition to repressing Mei-P26 in GSCs (Joly et al., 2013), other important functions of Twin in controlling GSC self-renewal and differentiation have been revealed by our study. First, Twin maintains E-cadherin expression at the translational or post-translational level. Second, Twin prevents transposon-induced DNA damage,

possibly by associating with Aub and Ago3. Third, both the LRR domain and the catalytic domain of Twin are required for GSC self-renewal. Fourth, Twin has an important function in promoting Bam-dependent germ cell differentiation. Twin promotes Bam protein expression and represses Nos protein expression in differentiated GSCs by forming protein complexes with Bam and Bgcn. Fifth, the LRR domain, but not the catalytic domain, of Twin is required for promoting Bam-dependent germ cell differentiation. Finally, Bam can inactivate the self-renewal function of the CRR4-NOT complex by displacing Twin from the complex via protein competition, thereby promoting germ cell differentiation. Therefore, this study has provided important insight into how Twin controls GSC self-renewal and germ cell differentiation (Figure 7H).

Twin Intrinsically Controls GSC Self-Renewal by Maintaining E-Cadherin and Genome Stability

CCR4-NOT is a highly conserved deadenylase complex regulating polyA tail length, thereby controlling mRNA translation and stability (Miller and Reese, 2012). In *Drosophila*, it contains Twin, Caf40, Pop2, Not1, Not2, Not3, Not10, and Not11 subunits, among which CCR4 and Pop2 are catalytic subunits (Bawankar et al., 2013; Temme et al., 2010). Surprisingly, Pop2, Not1, Not2, and Not3 are required for mRNA polyA tail shortening in *Drosophila* S2 cells, but Twin and Caf40 are dispensable, indicating that Pop2 is the major catalytic subunit of the complex in the cell (Temme et al., 2010). Our RNA sequencing results on control and *twin* mutant ovaries also have supported the idea that Twin is not the major catalytic subunit for mRNA degradation. Only 35 mRNAs showed more than 1.5-fold upregulation in the *twin* mutant ovaries compared with control ovaries, whereas only 10 mRNAs exhibited more than 1.5-fold downregulation, supporting the idea that Twin regulates gene expression in the ovary primarily at the translational or post-translational level (Tables S1 and S2). Although BMP signaling is critical for GSC self-renewal (Song et al., 2004; Xie and Spradling, 1998), Twin is dispensable for BMP signaling in GSCs based on normal pMad and *Dad-lacZ* expression in *twin* mutant GSCs. Therefore, these results indicate that the CCR4-NOT complex controls GSC self-renewal using BMP signaling-independent mechanisms.

A recent study revealed that Twin physically interacts with Nos and Pum to repress *mei-P26* expression in GSCs at the translational level (Joly et al., 2013). Our study found two additional important mechanisms for Twin to maintain GSC self-renewal. First, Twin maintains GSC self-renewal by sustaining E-cadherin expression at the GSC-niche junction, which is critical for GSC self-renewal (Song et al., 2002). Interestingly, E-cadherin accumulation was significantly downregulated in *twin* knockdown GSCs, and E-cadherin overexpression also could partially rescue the GSC loss phenotype caused by *twin* knockdown, indicating that Twin controls GSC self-renewal partly by maintaining E-cadherin accumulation. Based on mRNA levels and polyA tail length in *twin* mutant ovaries, we have further shown that Twin sustains E-cadherin expression in GSCs indirectly or at the translational or post-translational level. Second, Twin also controls GSC self-renewal by preventing TE-induced DNA damage. Twin recently was found to be required for piRNA-mediated TE repression (Czech et al., 2013; Handler et al., 2013). Consistently, DNA damage was dramatically elevated in *twin* mutant GSCs. Indeed, germline-specific CHK2 inactivation partially and significantly rescued the GSC loss phenotype caused by *twin* knockdown, suggesting that checkpoint activation is one of the causes for the loss of *twin* mutant GSCs. Surprisingly, Twin is dispensable for general piRNA production, and it is capable of forming protein complexes with two key piRNA pathway components Ago3 and Aub, suggesting that Twin represses TEs in germ cells downstream of piRNA production, possibly by interacting with Ago3 and Aub. Therefore, this study has revealed two important mechanisms for Twin to control GSC self-renewal.

Twin Promotes Germ Cell Differentiation by Regulating Bam Expression and Function

Recent studies have indicated that Bam works with RNA-binding partners Bgcn and Sxl to control GSC lineage differentiation (Chau et al., 2009, 2012; Li et al., 2009; Shen et al., 2009). One of the mechanisms for Bam to modulate translation is to regulate translation initiation by directly interacting with eIF4A in the translation initiation eIF4 complex (Shen et al., 2009). Consistent with the idea that Twin maintains Bam protein expression (Morris et al., 2005), this study found that germ cell-specific *twin* knockdown or *twin* mutations enhanced the germ cell differentiation defect of the *bam* heterozygous mutant. Although Bam protein levels decreased in *twin* mutant ovaries, its mRNA levels and polyA tail length remained unchanged, suggesting that Bam is regulated at the translational level and/or the post-translational level. Consistent with the idea that Bam works with Bgcn to repress Nos in mitotic cysts (Li et al., 2009), Nos expression significantly increased in *twin* mutant mitotic cysts. Interestingly, the polyA tail length of *nos* increased but its mRNAs were not significantly upregulated in *twin* mutant ovaries, suggesting that Twin regulates Nos at the translational or post-translational level. These results suggest that Twin sustains Bam protein expression in mitotic cysts, contributing to germ cell differentiation.

This study also suggests a working model that Bam can inactivate the self-renewal function of the CCR4-NOT complex by removing Pop2 via protein competition, thus promoting germ cell differentiation. First, Bam is associated with Twin in S2 cells and germ cells via the LRR domain. The mutant LRR domains cause Twin to lose its ability to interact with Bam and Pop2 in S2 cells and germ cells, whereas the Twin proteins carrying various mutated LRR domains lose, but the Twin protein carrying a mutation in the catalytic domain retains, the function in promoting Bam-dependent germ cell differentiation. In addition, overexpression of the LRR domain, which presumably disrupts the association between Bam and Twin, also interferes with Bam-dependent germ cell differentiation. These results argue that the LRR domain-mediated association between Twin and Bam is important for germ cell differentiation. Second, Bam promotes germ cell differentiation by displacing Pop2 from the CCR4-NOT complex via protein competition. Indeed, Bam and Pop2 compete for their association with Twin in an LRR domain-dependent manner in germ cells and S2 cells. Additionally, Pop2, Not1, and Not3 are required only for GSC self-renewal,

but not for Bam-independent germ cell differentiation. This is consistent with our recent finding that Bam can inactivate the self-renewal function of the COP9 complex via protein competition (Pan et al., 2014). Therefore, our findings have provided significant insight into how Twin and Bam control GSC lineage differentiation.

EXPERIMENTAL PROCEDURES

Drosophila Strains and Culture

The information of *Drosophila* stocks used in this study is available in the flybase or otherwise specified as follows: $twin^{ry3}$, $twin^{ry5}$, $twin^{s1}$, and $twin^{s3}$ (Morris et al., 2005); *Df(3R)Exel6198* (deleting the *twin* gene region; BL 7677); $bam^{\Delta 86}$ and *bam-GFP* (Chen and McKearin, 2003); *hs-FLP; nos>>stop>>gal4* (Ma et al., 2014); *FRT82B* and *UASp-shg* (Pan et al., 2007); *PBac[754.P.FSVS-0]pop2*^{CPTI002818} (*pop2-GFP*) and *PBac[681.P.FSVS-1] twin*^{CPTI002507} (*twin-GFP*) (Ryder et al., 2009); *nosP-GFP-nos 3′UTR* and the UAS-RNAi lines used in this study (*twin* [THU1091 and THU0936]; *pop2* [TH00896.N]; *not1* [THU0966 and THU3572]; *not3* [THU1254 and THU1195]; and *lok* [THU0019]). *Drosophila* strains were maintained and crossed at room temperature unless specified. To maximize the RNAi-mediated knockdown effect, newly eclosed flies were cultured at 29°C for 1 week before the analysis of ovarian phenotypes.

Immunohistochemistry

Immunohistochemistry was performed according to our previously published procedures (Song and Xie, 2002; Xie and Spradling, 1998). The following antibodies were used in this study: monoclonal rat anti-E-cadherin DCAD2 (1:3, Developmental Studies Hybridoma Bank), chicken polyclonal anti-GFP antibody (1:200, Jackson), mouse monoclonal anti-Hts antibody (1:4, Developmental Studies Hybridoma Bank), mouse monoclonal anti-Bam antibody (1:5, Developmental Studies Hybridoma Bank), rabbit polyclonal anti-pS137 H2Av antibody (1:5,000, Rockland Immunochemicals), and rabbit mAb anti-cleaved Caspase-3 (D175) (5a1E) (1:500, Cell Signaling Technology). All images were taken with a Leica TCS SP5 confocal microscope.

For experimental details, please see the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.10.017.

AUTHOR CONTRIBUTIONS

Z.F., C.G., H.W., Z.Y., C.W., L.D., and L.L. conducted experiments. H.L., N.L., J.N., and T.X. analyzed and interpreted the results. Z.F., J.N., and T.X. wrote the paper.

ACKNOWLEDGMENTS

We would like to thank Y. Yamashita, R. Lehmann, Developmental Studies Hybridoma Bank, and Bloomington *Drosophila* Stock Center for reagents, Y. Qi and X. Fang for technical advice, and T. Lu for proofreading and comments. This work was supported by the Tsinghua-Peking Joint Center for Life Sciences (Z.F.), the Ministry of Science and Technology of China (2015BAI09B03 and 2013CB35102 to J. N.) the fund from the 1000 Plan of China and the National Natural Science Foundation of China (N.L.), and the Stowers Institute for Medical Research (T.X.).

Received: April 18, 2015
Revised: August 12, 2015
Accepted: October 5, 2015
Published: November 5, 2015

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