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Report

Bucky Ball Organizes Germ Plasm Assembly in Zebrafish

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Summary

In many animals, gamete formation during embryogenesis is specified by maternal cytoplasmic determinants termed germ plasm [1, 2]. During oogenesis, germ plasm forms a distinct cellular structure such as pole plasm in *Drosophila* or the Balbiani body, an aggregate of organelles also found in mammals [3–10]. However, in vertebrates, the key regulators of germ plasm assembly are largely unknown. Here, we show that, at the beginning of zebrafish oogenesis, the germ plasm defect in *bucky ball* (*buc*) mutants precedes the loss of polarity, indicating that Buc primarily controls Balbiani body formation. Moreover, we molecularly identify the *buc* gene, which is exclusively expressed in the ovary with a novel, dynamic mRNA localization pattern first detectable within the Balbiani body. We find that a Buc-GFP fusion localizes to the Balbiani body during oogenesis and with the germ plasm during early embryogenesis, consistent with a role in germ plasm formation. Interestingly, overexpression of *buc* seems to generate ectopic germ cells in the zebrafish embryo. Because we discovered *buc* homologs in many vertebrate genomes, including mammals, these results identify *buc* as the first gene necessary and sufficient for germ plasm organization in vertebrates.

Results and Discussion

buc Is Required for Germ Plasm Assembly in Early Zebrafish Oocytes

The *bucky ball* (*buc*) mutant was previously identified by its maternal effect phenotype, whereby embryos and oocytes from homozygous *buc* mothers show a defect in germ plasm localization and polarity [10, 11]. To determine the hierarchical order of the germ plasm and polarity defect, we analyzed the localization of mRNAs with high-resolution fluorescent in situ hybridization at the very beginning of oogenesis. *cyclinB* mRNA [12] was not localized during later oogenesis in buc^{p106re} mutant oocytes (Figures S1A and S1B available online) but showed polarized localization in early stage I mutant oocytes (Figures 1A and 1D). Contrary to *cycB* mRNA, the germ plasm markers *dazl* [13], *vasa* [14, 15], and *nanos* mRNA [16, 17], as well as the vital dye $DiOC_6$ [10, 18] labeling mitochondria and ER, failed to localize to the Balbiani body in *buc* mutants (Figures 1B, 1E, S1C–S1F, and S1I–S1K). We observed similar levels of *dazl* and *vasa* mRNA in real-time PCR experiments in mutant and wild-type ovaries (Figure S1L), demonstrating that these germ plasm mRNAs are present, but not localized in mutants. Moreover, *foxH1* and *vg1RBP* were asymmetrically localized at the animal pole in buc^{p106re} mutants instead of the Balbiani body as in wild-type oocytes (Figures 1C, 1F, S1G, and S1H), similar to their localization in wild-type during later oogenesis (Figure S2) [19, 20]. Taken together, our results show that the *buc* mutation disrupts maintenance of oocyte polarity and that Buc controls germ plasm assembly in the Balbiani body.

Buc Encodes a Novel Vertebrate-Specific Protein

To molecularly identify *buc*, the first gene required for germ plasm localization in vertebrate oocytes, we embarked upon a positional cloning strategy. The *buc* gene is located on zebrafish chromosome 2 on Scaffold215 and encodes an open reading frame of 1917 bp (GeneID: 560382) (Figures S3A and S3B). Sequence comparison of the corresponding cDNAs between mutant and wild-type females identified a nonsense mutation in exon 4 of the $buc^{p43bmtb}$ allele and exon 6 of the buc^{p106re} allele. These mutations are predicted to delete 277 ($buc^{p43bmtb}$) and 37 (buc^{p106re}) amino acids at the C terminus of the Buc protein (Figures S3C and S3D). These results identify *buc* as a novel gene in zebrafish that is required to organize germ plasm during oogenesis.

To find conserved protein domains in Buc, we looked for homologous sequences in other organisms. Based on the conserved synteny and BLAST searches, we found related genes in all vertebrate classes, such as fish, amphibian, bird, and mammalian genomes (Figure 1G and Table S1). Whereas functional studies of other *buc* homologs have not been performed before this work was carried out, the amphibian homolog was previously described as *Xvelo1* in a screen for vegetally localized mRNAs in *Xenopus* oocytes [21]. Alignment of 15 related Buc proteins revealed a conserved 100 amino acid N terminus, which we termed BUVE motif (Buc-Velo) (Figure 1H). The BUVE motif contains a highly conserved 15 amino acid peptide (Figure 1I). However, this conserved motif has no homology to known protein domains, which would provide insight into the biochemical function of the predicted Buc protein.

Buc mRNA is Specifically Expressed in the Ovary and Localizes to the Balbiani Body

To determine whether the isolated candidate gene is expressed at the appropriate time to direct germ plasm assembly, we performed real-time PCR with RNA isolated from various stages of oogenesis and embryogenesis. In stage I oocytes, *buc* mRNA is detected when the germ plasm defect of the *buc* mutant becomes visible and persists at constant levels during oogenesis (Figure 2A). *Buc* mRNA is detected in embryos after fertilization and then is not maintained after the midblastula transition (4 hpf = hours post fertilization) (Figure 2B), when most maternal mRNAs are degraded

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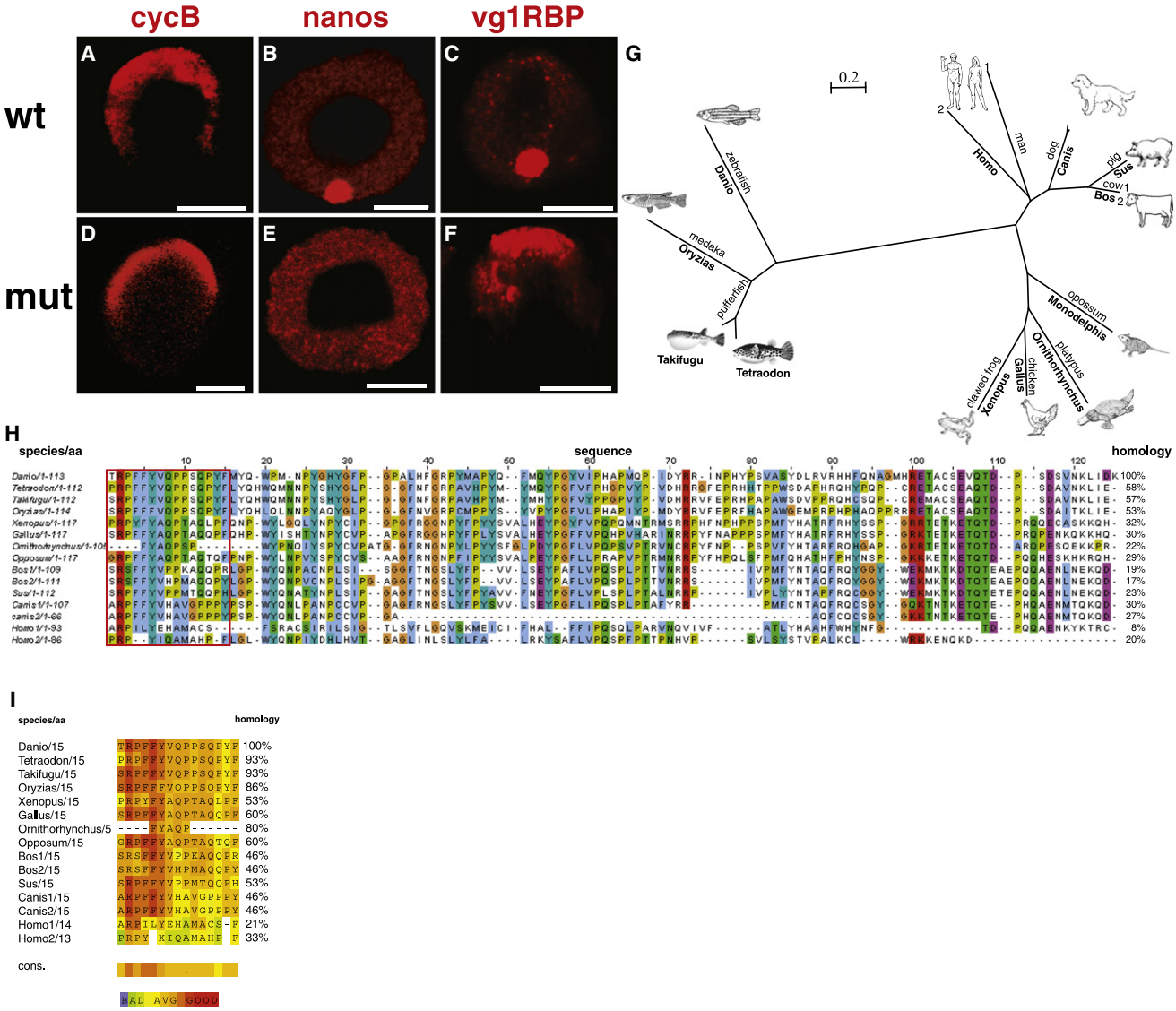

Figure 1. Germ Plasm Formation in Early *Bucky Ball* Oocytes and Evolutionary Conservation of the Buc Protein

(A–F) Fluorescent whole-mount in situ hybridization of early Ib oocytes showing mRNAs (red) of *cyclinB* (A and D), *nanos* (B and E), and *vg1RBP* (C and F) in wild-type (A–C) and *buc* ^{p106re} mutant oocytes (D–F). Note that the localization of *nanos* and *vg1RBP* mRNAs in the Balbiani body is lost in mutants (E and F), but the *cyclinB* and *vg1RBP* mRNAs still show polarized localization (D and F). Animal pole to the top. Scale bar represents 25 μm. All mutant images are of the *buc*^{p106re} allele.

(G) Unrooted star diagram displaying the phylogenetic conservation of Bucky ball proteins among vertebrates. The scale indicates the number of substitutions per amino acid residue.

(H) Alignment of Buc N terminus in 12 vertebrate species (zebrafish amino acids 23–136). The sequence homology was calculated with Kalign [45], which revealed a conserved peptide (zebrafish amino acids 23–38, red box).

(I) Alignment of conserved peptide (red box in H) with T-Coffee [46]. The color code below the alignment illustrates the level of conservation (cons.) from BAD (blue) to GOOD (red). Note that, with evolutionary distance, the sequence similarity decreases.

Gene-ID from NCBI or Ensembl-genome databases: Zebrafish, *Danio rerio*: NCBI-locus 560382; Medaka, *Oryzias latipes*: UTOLAPRE05100115054; Spotted pufferfish, *Tetraodon nigroviridis*: GSTENT00016539001; Tiger pufferfish, *Takifugu rubripes*: SINFRUT00000183089; Clawed frog, *Xenopus tropicalis*: ENSXETT00000045658; Chicken, *Gallus gallus*: ENSGALT00000019911; Platypus, *Ornithorhynchus anatinus*: GENSCAN00000155561; Opossum, *Monodelphis domestica*: GENSCAN00000062731; Cow, *Bos taurus*: GENSCAN00000042786, GENSCAN00000042790; Pig, *Sus scrofa*: Ssc.46160, Ssc.28451; Dog, *Canis familiaris*: GENSCAN00000091809, GENSCAN00000058820; Man, *Homo sapiens*: EU128483, EU128484

during the maternal-to-zygotic transition [22]. Zygotic expression of *buc* mRNA was not detected until 42 dpf (days post fertilization), when sexual differentiation in zebrafish is complete [23]. Therefore, *buc* mRNA expression coincides with the onset of gametogenesis in zebrafish and is downregulated at the end of the maternally controlled period of embryogenesis.

At 42 dpf, we observed that only 50% of the animals initiate *buc* mRNA expression, although they were the same size (20 mm, data not shown). To address whether *buc* mRNA is expressed differentially in males versus females, we analyzed the female-specific expression of the long splice form of *vasa* mRNA (*vasa-l*) [24]. We found that *vasa-l* mRNA was coexpressed with *buc*, indicating that *buc* mRNA is exclusively

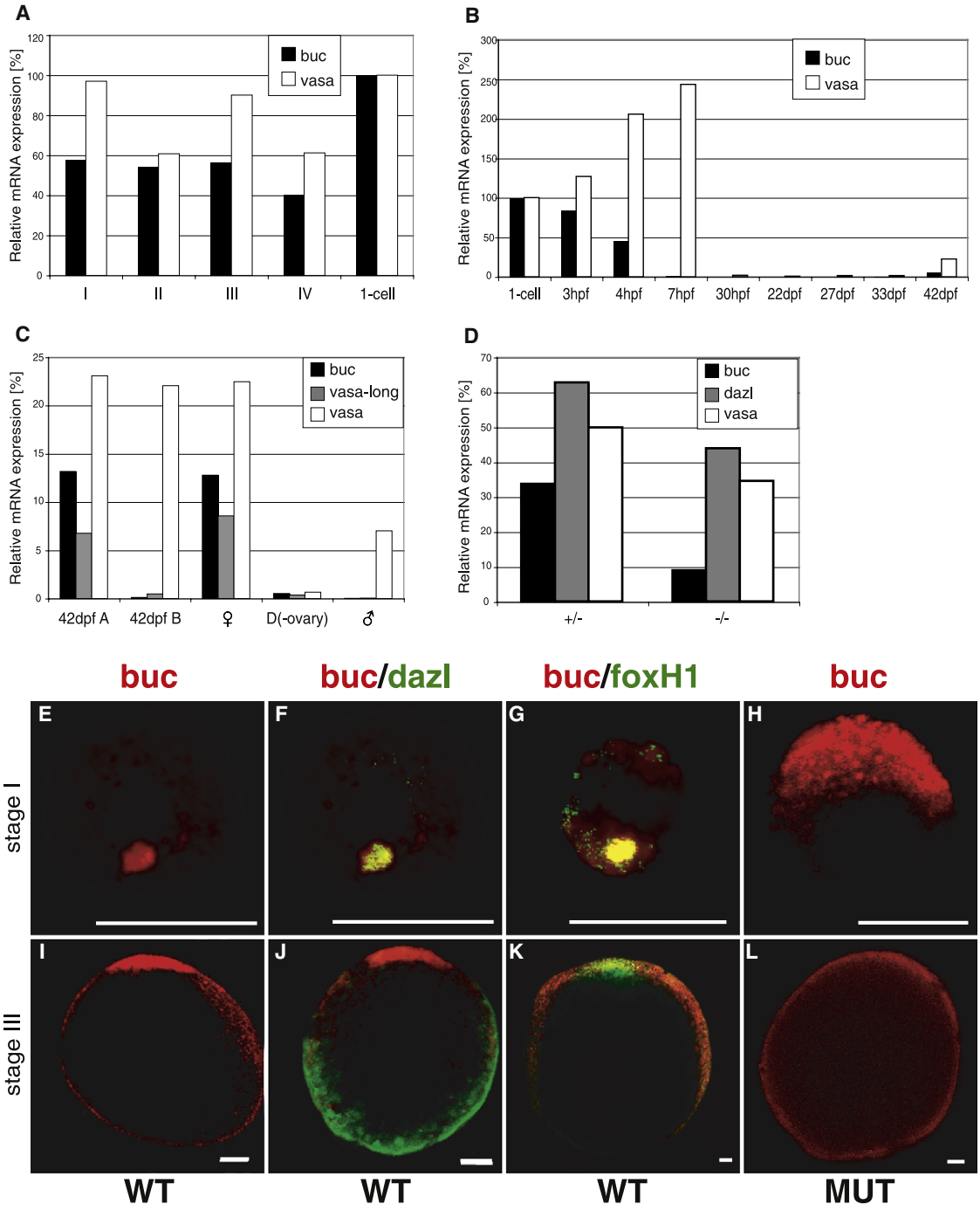

Figure 2. Expression Analysis of *buc* mRNA

(A–D) Real-time PCR analysis of *buc* and *vasa* mRNA relative to expression in the one-cell embryo during oogenesis (A), embryogenesis (B), and in sexually mature adults (C). (D) Real-time PCR comparing the levels of *buc*, *dazl*, and *vasa* mRNA in wild-type (+/−) and buc^{p106re} mutant ovaries (−/−).

(E–L) Fluorescent whole-mount in situ hybridization of *buc* (red in all pictures) during stage I (E–H) and stage III (I–L) of oogenesis. Animal to the top in all pictures. (F and J) Double in situ hybridizations showing colocalization of *buc* and the vegetal *dazl* mRNA (green) at stage I (F), but not at stage III (J). (G and K) Double in situ hybridizations showing colocalization of *buc* and *foxH1* mRNA (green) at stage I (G) and at stage III (K). (H and L) Animal localization of *buc* mRNA at stage I (H), but not after 3-fold longer exposure at stage III in buc^{p106re} oocytes (L). Scale bars represent 25 μm (E–H) and 50 μm (I–L).

expressed in females (Figure 2C, 42 dpf A), whereas prospective males show no signal (Figure 2C, 42 dpf B). Therefore, we analyzed mRNA expression in adults, when the sex of the fish can be readily distinguished. *Buc* mRNA was only expressed in females, but not in males (Figure 2C). In addition, *buc* mRNA was not observed in females after ovary removal, confirming its ovary-specific expression (Figure 2C). The lack of *buc* transcripts in males is consistent with the lack of an obvious phenotype in homozygous mutant *buc* males, which are viable and fertile (data not shown). Together, these results indicate that *buc* mRNA is an ovary-specific transcript.

By using whole-mount in situ hybridization, we studied *buc* mRNA localization during oogenesis. In stage I oocytes, *buc* mRNA is localized to the germ plasm, where it colocalizes with *dazl* and *foxH1* mRNA (Figures 2E–2G and S4A–S4C). In zebrafish, *buc* mRNA no longer colocalizes vegetally with *dazl* mRNA in stage III oocytes, whereas, in *Xenopus*, *velo1* remains vegetal [21] (Figures 2I, 2J, S4A, and S4B). Instead, it is restricted to the animal pole with *foxH1* mRNA (Figures 2I, 2K, S4A, and S4C). Thus, *buc* mRNA shows a novel and dynamic localization pattern during zebrafish oogenesis, initiating in the Balbiani body, moving vegetally, and eventually localizing to the animal pole in late-stage oocytes. The transient localization of *buc* mRNA with the germ plasm corroborates its essential function in the transient Balbiani body.

The mutant buc^{p106re} allele generates a premature STOP codon in exon 6. To investigate whether the mutant mRNA is a target of nonsense-mediated decay (NMD) [25], we examined mutant *buc* mRNA expression in *buc* mutants. In early buc^{p106re} oocytes, *buc* mRNA is still present, but not localized to the Balbiani body, and colocalizes with *cycB* and *foxH1* mRNA to the animal pole (Figure 2H; data not shown). These data indicate that the mutant *buc* mRNA is not a target of NMD during early oogenesis. Moreover, these results imply that Buc is required for its own mRNA localization in the Balbiani body.

In late-stage buc^{p106re} mutant oocytes, *buc* mRNA was almost undetectable, even after 3-fold longer exposure compared to stage I (Figure 2L). To support the whole-mount in situ hybridization results, we analyzed buc^{p106re} mutant mRNA expression in real-time PCR experiments. The level of buc^{p106re} mRNA was decreased in mutant ovaries, whereas *dazl* and *vasa* mRNAs were not affected (Figure 2D). These results show that the mutant *buc* mRNA is not stable in late-stage oocytes and is presumably degraded by NMD if it is not correctly localized. In summary, *buc* mRNA is localized during early zebrafish oogenesis to the Balbiani body, consistent with its required function in germ plasm assembly.

Buc mRNA Translation Is Required for Germ Plasm Formation

Our expression analysis detected *buc* mRNA specifically in oocytes, but not in the surrounding follicle or thecal cells, and these somatic cells were also confirmed to be *buc* negative on sections (Figures 2E and S4 and data not shown), though we detected these cells by DAPI staining after in situ hybridization (data not shown). To address in which cells of the ovary Buc activity is required, we developed a method to microinject mRNA into stage I zebrafish oocytes, cultured the oocytes for 2 days, and examined germ plasm formation by *dazl* mRNA in situ hybridization. Wild-type oocytes showed *dazl* mRNA localization after injection of wild-type and mutant buc^{p106re} mRNA, confirming that the injection and culture conditions do not disturb *dazl* mRNA localization (Figures 3A, 3C, and 3E). In contrast, mutant oocytes rarely displayed *dazl* mRNA localization after injection of mutant buc^{p106re} mRNA (Figures 3D and 3E). However, wild-type *buc* mRNA injection rescued *dazl* mRNA localization in buc^{p106re} mutant oocytes (Figures 3B and 3E). Consistent with the absence of *buc* mRNA in somatic cells, these rescue experiments suggest that Buc is sufficient in the female germline and probably acts in a cell autonomous manner to localize germ plasm in the oocyte.

The human *BUC* sequences do not predict an open reading frame, suggesting that Buc might act as a noncoding RNA. Although injection of the buc^{p106re} mRNA did not rescue the oocyte phenotype (Figure 3D), we wanted to exclude the possibility that *buc* RNA mediates germ plasm localization. When we injected *buc* mRNA that was prehybridized with a translation-blocking morpholino into mutant oocytes, rescue of *dazl* mRNA localization in *buc* oocytes was abolished (Figures 3I and 3J), similarly to injections with mutant buc^{p106re} mRNA (Figures 3F and 3H). Conversely, coinjecting a mismatch control morpholino did not inhibit rescue by *buc* mRNA (Figures 3G and 3J). Together, these data show that *buc* mRNA translation is required in zebrafish for germ plasm assembly.

Our injection results suggest that the Buc protein is localized to the Balbiani body. Because a Buc antibody was not available to detect endogenous Buc protein, we injected mRNA encoding a Buc-GFP fusion into early oocytes. We observed fluorescence in the Balbiani body of living oocytes (Figures 3K–3M), which progressively localized to more vegetal positions during oogenesis like other germ plasm components [8]. In summary, Buc protein, but not its mRNA, organizes germ plasm components in the Balbiani body (Figure 3N).

Buc Localizes to the Germ Plasm, and Its Overexpression Induces Ectopic Germ Cell Formation in Zebrafish Embryos

During zebrafish embryogenesis, germ plasm localizes to the first two cleavage furrows, which is essential for the specification of four clusters of primordial germ cells (Figure 4K) [26, 27]. Because the Buc-GFP fusion localized to germ plasm in oocytes, we injected *buc-GFP* mRNA into one-cell embryos to examine its localization during embryogenesis. As soon as the fluorescence became visible (~1.5 hr at RT), we observed aggregation of Buc-GFP at four cleavage furrows of the eight-cell embryo, demonstrating that Buc protein localizes to the germ plasm during early embryogenesis (Figures 4A and S6A–S6J). Because the *Xenopus velo* depends on its 3′UTR for localization in the oocyte [21], like the majority of localized mRNAs [28, 29], we repeated the injection experiment in the zebrafish embryo with a 3′UTR deletion of Buc-GFP. Buc-GFP was still localized to the four cleavage furrows (Figure 4B), suggesting that the 3′UTR does not contain essential germ plasm localization elements. To exclude localization elements in other parts of the *buc* mRNA, we performed whole-mount in situ hybridizations on embryos. *buc* mRNA is ubiquitously present in the blastomeres of four-cell and high-stage embryos (Figures 4C and 4D), whereas a sense mRNA control produced no signal (data not shown), suggesting that Buc localization depends on a protein motif. In summary, the Buc-GFP fusion protein localizes to the germ plasm as early as the eight-cell stage, representing the earliest in vivo marker for germ plasm in zebrafish.

Classical experiments in amphibians show that the transplantation of germ plasm from the oocyte into the embryo induces the formation of additional germ cells [30, 31]. Because Buc-GFP localizes to the germ plasm in the oocyte and the germ cells in the embryo and the overexpression of Buc-GFP generated additional spots during early embryogenesis (Figure S6), we investigated whether Buc represents a candidate for the germ cell-inducing activity. To label germ cells in living embryos, we injected *GFP-nanos* 3′UTR mRNA into one-cell embryos as a reporter [16] either alone or in combination with *buc* mRNA. After coinjection of *buc*, we frequently observed germ cells at the 13 somite stage outside of the gonad-anlagen (Figure 4F) compared to controls

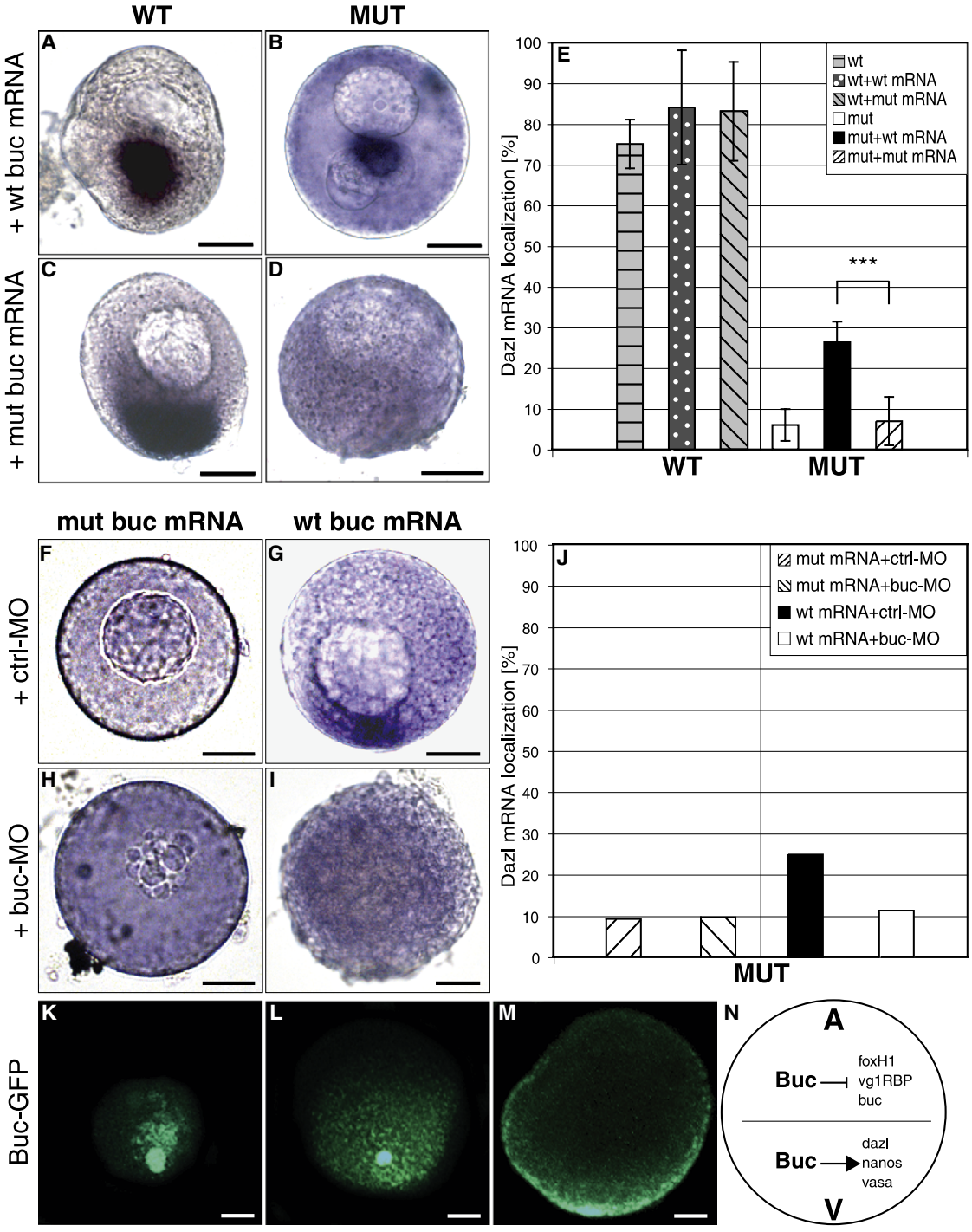

Figure 3. *buc* mRNA Translation Is Required to Assemble Germ Plasm, and Buc-GFP Localizes to the Balbiani Body

(A–E) Buc is required in the oocyte for germ plasm localization. Wild-type (A and C) and buc^{p106re} mutant oocytes (B and D) after microinjection of wild-type (A and B) or mutant buc^{p106re} mRNA (C and D) into stage I zebrafish oocytes. (E) Quantification of *dazl* mRNA localization from three independent experiments in WT oocytes, uninjected (75% ± 6.0%, n = 63), injected with WT *buc* (84% ± 14%, n = 67), or mutant buc^{p106re} mRNA (83% ± 12%, n = 47) compared to buc^{p106re} mutant oocytes, uninjected (6% ± 3.9%, n = 122), injected with WT *buc* (26% ± 5.1%, n = 103), or mutant buc^{p106re} mRNA (7% ± 6.4%, n = 26). Note the rescue of *dazl* mRNA localization in mutant oocytes after injection of wild-type *buc* mRNA (B and E). Error bars represent SD. ***p = 0.0008.

(F–I) Buc needs to be translated to assemble germ plasm. buc^{p106re} mutant stage I oocytes injected with mutant buc^{p106re} (F and H) or WT *buc* mRNA (G and I) with control (+ctrl-MO) (F and G) or translation inhibition morpholino (+buc-MO) (H, I), which inhibited translation of Buc-GFP fusion mRNA and, hence, GFP-fluorescence efficiently in embryos (Figure S5).

(A–D and F–I) Whole-mount in situ hybridization of *dazl* mRNA (blue). Scale bars: 25 μm.

(J) Quantification of *dazl* mRNA localization in mutant oocytes injected with mutant buc^{p106re} mRNA plus ctrl-MO (9%, n = 11) or plus buc-MO (10%, n = 21) and WT mRNA plus ctrl-MO (25%, n = 20) or plus buc-MO (11%, n = 27). Note that the rescue of *dazl* mRNA localization is blocked by a morpholino-inhibiting *buc* mRNA translation (I and J).

(Figure 4E). To study whether the ectopic cells originate from a defect in cell motility [32], we examined germ cell formation before the onset of their migration with *vasa* mRNA in situ hybridization at oblong stage. In contrast to control-injected embryos (Figure 4G), *buc* mRNA-injected embryos showed an increase of about 50% in *vasa*-positive cells (Figures 4H, S7A, and S7B and Tables S2 and S3). Interestingly, we observed no change of germ plasm mRNA levels at oblong stage by real-time PCR (Figure 4I), whereas we detected a moderate increase of around 50% of *vasa* and *dazl* mRNAs at the 18 somite stage (Figure 4J and Table S3), paralleling the 44% increase of additional germ cells at the 13 somite stage (Table S3). These results indicate that Buc might induce some additional germ cells after overexpression. However, the expression analysis does not support a mechanism whereby Buc forms germ cells by de novo induction of germ plasm components.

Alternatively, Buc overexpression might change the distribution of germ plasm and induce additional germ cells either by dispersing the existing four germ plasm aggregates or by recruiting germ plasm components ubiquitously present in the early embryo to novel aggregates [33, 34]. To distinguish between these two possibilities, we exploited the 16-cell embryo, a stage when germ plasm, essential for germ cell formation, is localized in four aggregates at the cleavage furrows between marginal blastomeres (Figure 4K) [26], and examined the injected embryos between the 13 and 18 somite stages (Figure 4L). In control experiments wherein we injected embryos with *GFP-nanos* 3′UTR mRNA into either the middle, marginal blastomere (Figure 4K, blue arrowhead) or the corner blastomere (Figure 4K, green arrowhead), we observed that the middle blastomere more frequently contributes to primordial germ cells (82% ± 10%; n = 92) (Table S2 and data not shown) than does the corner blastomere (26% ± 8%; n = 99) (Figure 4M), where the majority of GFP-positive embryos had one fluorescently labeled cell outside of the prospective gonad. However, coinjection of *buc* mRNA into the corner blastomere increased the frequency of embryos with GFP-positive cells (81.9% ± 14.1%; n = 118; ***$p = 3.1 \times 10^{-5}$) (Figures 4N and S7C and Table S2), similarly to injections into the middle blastomere. Furthermore, quantification of the total number of *vasa* mRNA-positive cells at the 18 somite stage after corner blastomere injection showed an increase from 17 ± 5.9 (n = 17) in controls to 26 ± 5.5 (n = 29) in *buc*-injected embryos (***$p = 9.0 \times 10^{-6}$) (Table S3). In addition, double-staining with *vasa* mRNA and a GFP antibody confirmed that the additional germ cells were coinjected with *buc* mRNA (Figures 4O and 4P). Together, our results suggest a mechanism whereby Buc overexpression probably aggregates extant germ plasm and, in this manner, can induce some additional germ cells.

This study identifies the molecular nature of the *bucky ball* gene, a vertebrate-specific maternal determinant. A key role of Buc is the organization of germ plasm during oogenesis and early embryogenesis based on its protein localization as well as its loss- and gain-of-function phenotype. However, Buc is also involved in maintaining oocyte polarity, but at present, it is unclear whether the mutant defect is a direct consequence of Buc disruption or a secondary defect of the failure to assemble the Balbiani body. Consistent with a direct role of Buc in oocyte polarity, we found its mRNA localized to the animal pole, where the loss of Buc leads to the differentiation of multiple micropyles, an animal pole structure [10]. Furthermore, Buc might have additional roles that have not been discovered yet.

In our current model of germ plasm formation, Buc localizes to the Balbiani body, where it acts molecularly upstream to (1) recruit germ plasm components, including *nanos*, *vasa*, and *dazl* mRNA and (2) inhibit the premature localization of *foxH1*, *vg1RBP*, and its own mRNA to the animal pole (Figure 4N). Moreover, after fertilization, Buc localizes to the cleavage furrows of the first two cell divisions, where it seems to be sufficient for germ plasm assembly. Interestingly, whereas Buc protein colocalizes with the germ plasm during oogenesis and embryogenesis, the RNA is only localized to the Balbiani body during oogenesis but is ubiquitous in the embryo. Furthermore, in *buc* mutants, the *buc* RNA was not localized in the Balbiani body during early oogenesis and then was degraded during late oogenesis. Because the Balbiani body is implicated in RNA storage and degradation [35], these data suggest that *buc* RNA is under tight posttranscriptional control during oogenesis.

The Buc amino acid sequence shows no functional protein domains, which would explain its biochemical function. In addition, the protein appears to evolve very fast, which has been frequently observed in sex-specific genes involved in reproduction [36, 37]. However, most of these proteins are involved in gamete recognition, which is considered to play a role in speciation. In contrast to these proteins, Buc is an intracellular protein probably not involved in gamete recognition, and, in fact, mutant oocytes are fertilized [10]. As a consequence, Buc homologs might exist outside of vertebrates, but their sequence might be unrecognizable to sequence alignment algorithms. In addition, in BLAST searches with the strongly conserved N terminus of the protein, we have not identified additional Buc homologs outside of the vertebrate subphylum (data not shown).

During oogenesis, the *buc* mRNA changes its localization from the vegetal Balbiani body to the animal pole between stages I and II. In addition to *buc*, the *foxH1*, *vg1RBP*, and other mRNAs (A.S. and R.D., unpublished observation) also show this novel localization pattern, which has not been described previously [12]. A study of the dynamics of *dazl* mRNA localization shows a continuous localization at the vegetal pole of the oocyte [8] that is similar to the germ plasm components in *Xenopus* [38, 39]. However, a careful analysis found recently that the germ plasm RNAs *nanos*, *vasa*, and *dead end* are already localized to the animal pole right after fertilization, whereas *dazl* RNA relocates from the vegetal to the animal pole, which results in distinct subdomains of these RNA groups at the two cleavage furrows [40]. Although the differential localization of these RNAs suggests different domains within the germ plasm of zebrafish, the function of this germ plasm subdivision is presently unknown.

It is striking that Buc overexpression appears to induce a few additional germ cells upon overexpression in the embryo. Other germ plasm components have been overexpressed in embryos, e.g., *vasa*, but did not induce the

(K–M) Buc localizes to the Balbiani body. Living WT oocytes injected with mRNA encoding a Buc-GFP fusion (green) at mid (K) and late-stage Ib (L), as well as stage II (M) of oogenesis. Scale bars represent 25 μm.

(N) Model of the role of Buc in early-stage I oocytes. Buc inhibits the premature localization of *foxH1*, *vg1RBP*, and *buc* mRNA at the animal pole (A) and concomitantly recruits the germ plasm RNAs *dazl*, *nanos*, and *vasa* in the Balbiani body at the vegetal pole (V).

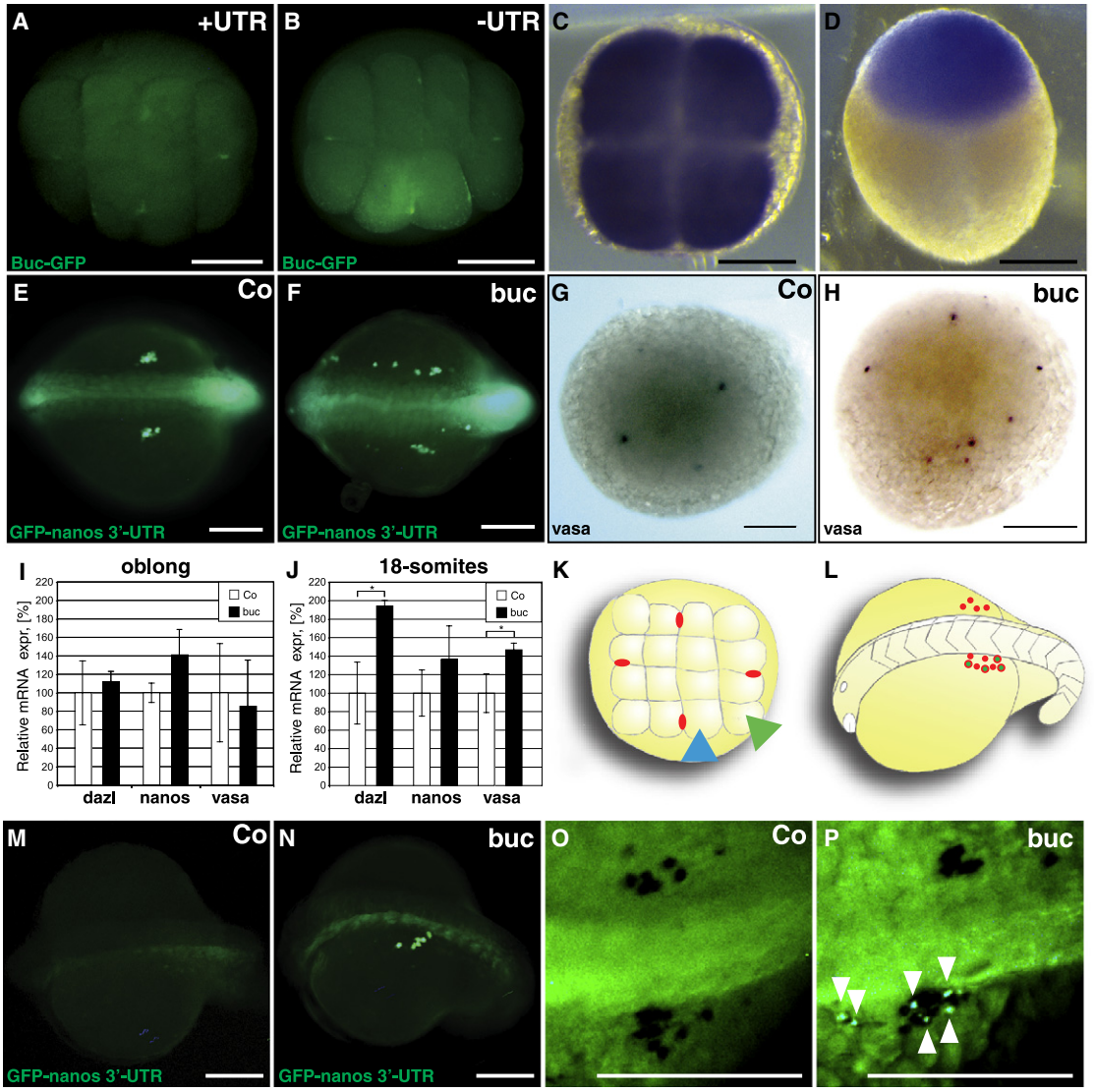

Figure 4. Buc Colocalizes with Germ Plasm and Induces the Formation of Germ Cells during Embryogenesis

(A and B) Buc-GFP localizes to the germ plasm in eight-cell embryos. One-cell embryos were injected with 200 pg mRNA-encoding Buc-GFP (green) with (A) or without 3′UTR (B). Note the localization of GFP at four cleavage furrows in living eight-cell embryos. Animal view. Scale bars represent 200 μm.

(C and D) *buc* mRNA is throughout the blastodisc during embryogenesis. Whole-mount in situ hybridization showing *buc* mRNA in the blastodisc (blue) without specific localization to the germ plasm at the cleavage furrow at four-cell (C) and blastula stages (D) (high stage). Animal view (C), lateral view, animal to the top (D). Scale bars represent 200 μm.

(E–J) Overexpression of Buc in one-cell embryos induces germ cell formation. Dorsal view of living 13 somite stage embryos (15.5 hpf), anterior to the left (E and F). Germ cells are labeled green fluorescent after coinjection with 100 pg *GFP-nanos-3′UTR* mRNA. In control embryos, germ cells accumulate in the bilateral gonad anlagen (E), whereas in *buc*-injected embryos, extragonadal germ cells are visible (F). Animal view of oblong stage embryos (3.5 hpf) after whole-mount in situ hybridization with *vasa* mRNA (blue) injected with 300 pg GFP (G) or *buc* mRNA at the one-cell stage (H). Note additional *vasa*-positive cells in *buc*-injected embryos (85.0% ± 11.5%; n = 343) compared to control injections (5.7% ± 8.7%; n = 280; ***$p = 3.4 \times 10^{-12}$). Scale bars represent 200 μm (E–H). (I and J) Real-time PCR analysis of the germ plasm RNAs *nanos*, *vasa*, and *dazl* after control and *buc* mRNA injection analyzed at oblong stage (3.5 hpf; [I]) and 18 somites stage (18 hpf; [J]). Error bars represent SD (*dazl* *p = 0.028; *vasa* *p = 0.041; Table S3).

(K and L) Experimental scheme for germ cell formation assay. (K) The 16-cell embryos (animal view) were either injected into one corner blastomere (green arrowhead) or into a middle blastomere (blue arrowhead; positive control) containing essential germ plasm (red ovals). (L) Embryos were examined between the 13 and 18 somite stage for the formation of additional germ cells (green dots) in addition to the endogenous germ cells (red dots; not visible in the experiment). Oblique dorsal view, anterior to the left.

(M and N) Live 15 somite stage embryos, similar view as in panel (L), after injection of 100 pg *GFP-nanos 3′-UTR* mRNA into a corner blastomere (M) or after coinjection of 170 pg *buc* mRNA into the corner blastomere (N) with 12 ± 5.4 (n = 21) fluorescent cells per embryo. Scale bars represent 200 μm.

(O and P) Buc-induced germ cells express *vasa* mRNA. Injection of *GFP-nanos 3′-UTR* into a corner blastomere of a 16-cell embryo does not label germ cells after *vasa* mRNA in situ hybridization at the 18 somite stage (black; [O]), whereas coinjection of *buc* mRNA generates additional *vasa*-positive cells also expressing GFP (green, white arrowheads; [P]). Dorsal view, anterior to the left. Scale bars represent 200 μm.

formation of extra germ cells [41]. In *Xenopus*, the injection of *Xpat* leads to the formation of ectopic germinal granules in the oocyte, and it would be interesting to study whether *Xpat* is also sufficient to form ectopic germ cells in the embryo. The additional germ cells show the following features of functional germ cells. They express *vasa* mRNA and the germ cell

reporter GFP-nos-3′UTR, and they migrate to the gonad anlagen, so Buc seems to be sufficient to change the fate of some cells into germ cells. However, to confirm that these cells are fully functional germ cells, it would be necessary to demonstrate their differentiation into gametes.

Several scenarios can explain the mechanism of germ cell formation by Buc. On the one hand, de novo formation of germ plasm by Buc seems unlikely because the expression of germ plasm components is not induced in the early embryo, and the one-cell injections did not transform the whole embryo into germ cells but, rather, induced a few additional *vasa* mRNA-positive cells at distinct locations. On the other hand, the localization of Buc to the germ plasm of the embryo and the unchanged position of the germ plasm in the oocyte after *buc* mRNA injection most likely excludes a mechanism whereby Buc splits germ plasm aggregates, which, after dispersal, form additional germ cells. Our data are most consistent with a mechanism whereby Buc would recruit germ plasm components already present in the early embryo, and this aggregation probably prevents their degradation during later embryogenesis.

Support for this hypothesis comes from the continuous observation of embryos that overexpress Buc-GFP and show strikingly similar spots to the *vasa* mRNA expression after *buc* mRNA injection (Figures 4H and S6). Furthermore, consistent with the recruitment model, early cleavage stage embryos seem to contain surplus germ plasm, given that the majority of germ plasm RNAs show abundant cytoplasmic signal in addition to the cleavage furrow localization [15, 16, 26, 33, 40, 42, 43]. This mechanism appears to be conserved during evolution because, in *C. elegans*, the germ cell determinant PIE-1 protein is also degraded if it is not localized to germ cells [44]. Understanding the precise mechanisms by which Buc regulates germ plasm assembly, as well as determining the functions of the mammalian *buc* genes in the Balbiani body, could provide key insights into the formation and evolution of this essential cytoplasmic structure.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at http://www.current-biology.com/supplemental/S0960-9822(09)00610-1.

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