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Dnd Is a Critical Specifier of Primordial Germ Cells in the Medaka Fish

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SUMMARY

Primordial germ cell (PGC) specification occurs early in development. PGC specifiers have been identified in *Drosophila*, mouse, and human but remained elusive in most animals. Here we identify the RNA-binding protein Dnd as a critical PGC specifier in the medaka fish (*Oryzias latipes*). Dnd depletion specifically abolished PGCs, and its overexpression boosted PGCs. We established a single-cell culture procedure enabling lineage tracing in vitro. We show that individual blastomeres from cleavage embryos at the 32- and 64-cell stages are capable of PGC production in culture. Importantly, Dnd overexpression increases PGCs via increasing PGC precursors. Strikingly, *dnd* RNA forms prominent particles that segregate asymmetrically. Dnd concentrates in germ plasm and stabilizes germ plasm RNA. Therefore, Dnd is a critical specifier of fish PGCs and utilizes particle partition as a previously unidentified mechanism for asymmetric segregation. These findings offer insights into PGC specification and manipulation in medaka as a lower vertebrate model.

INTRODUCTION

Primordial germ cells (PGCs) are germ stem cells capable of generating precursors of eggs and sperm and thus offer the basis for reproduction and fertility (Ko et al., 2010; Lin, 2007, 2012). PGC specification demarcates the soma-germline separation and thus sets a balance between individual life and species continuity. PGCs form early in development and migrate into the developing gonad (Wylie, 1999). In adult animals, gonadal germ cells undergo meiosis and produce eggs and sperm. PGC development has been extensively studied in several model organisms including *Drosophila*, *Caenorhabditis elegans*, and *Xenopus* (Houston and King, 2000), zebrafish (Lin et al., 1992; Raz, 2003), and mouse (Hayashi et al., 2011; Ohinata et al., 2005; Saitou et al., 2002; Tam and Zhou, 1996). In these model invertebrates and lower vertebrates, PGCs are cell autonomously preformed by maternally supplied germ plasm, a membrane-less organelle composed mainly of RNA-binding proteins and their mRNAs (Houston and King, 2000; Raz, 2003). In mouse, PGCs are epigenetically induced by cell-cell interactions in the absence of germ plasm (Tam and Zhou, 1996), where signaling molecules such as bone morphogenetic factor 4 play a critical role (Ying et al., 2001).

Dozens of genes essential for PGC development are known in the model invertebrates and lower vertebrates (Houston and King, 2000); clearly defined specifiers of the PGC fate has, however, been limited to *Drosophila*. In this organism, *oskar* acts as the PGC specifier, as it is necessary for PGC formation and more importantly, sufficient for ectopic PGC induction in a dose-dependent manner (Ephrussi and Lehmann, 1992). However, *osk* is restricted to certain insects. An evolutionarily conserved gene, *piwi*, is also necessary for PGC formation and sufficient for tripling the PGC number but incapable of ectopic PGC induction (Megosh et al., 2006). In vertebrates, *piwi* is dispensable for PGC specification but essential for subsequent PGC development, such as spermatogenesis in mouse (Deng and Lin, 2002), germ cell maintenance in zebrafish (Houwing et al., 2007), and PGC migration in medaka (Li et al., 2012). In mice, *blimp1* (encoded by *prdm1*) and *prdm14* are transcriptionally induced by BMP4 in the epiblast at E6.25, which together with *tfap2c* constitute a tripartite genetic network to induce the PGC fate in vivo (Magnusdottir et al., 2013; Ohinata et al., 2005) and in vitro from embryonic stem (ES) cells (Magnusdottir et al., 2013; Nakaki et al., 2013). In human, SOX17 has most recently been identified as a critical specifier of PGCs in ES cells (Irie et al., 2015). Accumulated data from *Drosophila*, mouse, and human suggest that PGC specifiers show remarkable diversity and do not follow the evolutionary history, although many genes involved in subsequent germ cell development are highly conserved across animal phyla.

A vertebrate-specific germ gene, *dead end* (*dnd*), was first identified in zebrafish as a germ plasm component encoding an RNA-binding protein crucial for PGC migration

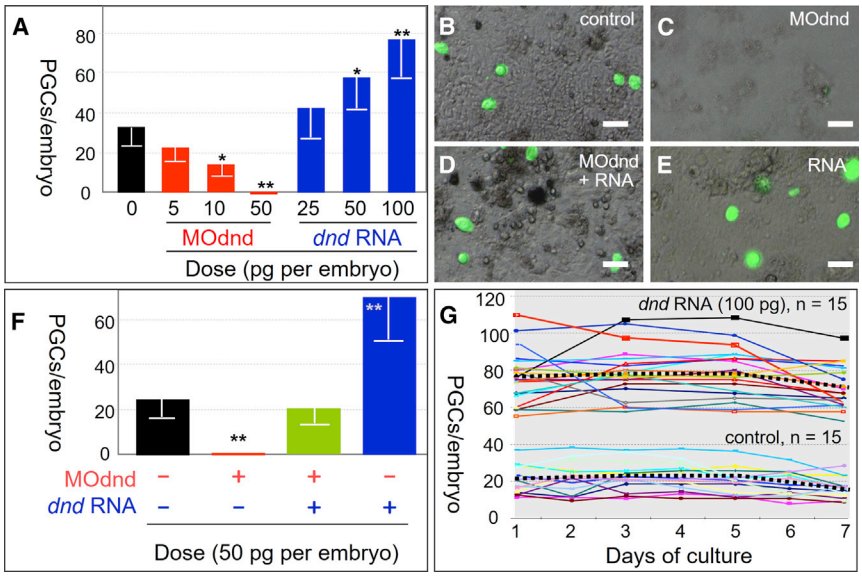

Figure 1. Dnd Dosage Determines the PGC Number

(A) Number of PGCs in embryos. Data are means ± SD (bars) of 12–21 embryos at stage 20.

(B–E) PGCs from midblastula cells at 2 days of culture. Scale bars, 20 μm.

(F) Number of PGCs at day 2 post culture from ≥12 embryos. *Significant difference ($p \leq 0.05$); **very significant difference ($p \leq 0.01$) compared with non-injected control.

(G) Time course of PGC numbers from individual blastula embryos in culture.

and survival (Tzung et al., 2015; Weidinger et al., 2003). Mouse *dnd* mutations do not prevent PGC formation (Youngren et al., 2005). The medaka fish (*Oryzias latipes*) is an excellent model for studying vertebrate development (Wittbrodt et al., 2002), stem cells (Centanin et al., 2011; Hong et al., 1996, 1998b), germ cells, and reproductive technologies. This fish has haploid ES cells capable of whole-animal production by semicloning (Yi et al., 2009), male germ stem cells capable of test-tube production (Hong et al., 2004a), and transgenic lines for PGC visualization (Li et al., 2009; Tanaka et al., 2001). Unusually, maternal germ plasm components distribute widely in medaka (Shinomiya et al., 2000; Xu et al., 2009), rather than locally as in zebrafish (Weidinger et al., 2003; Yoon et al., 1997). This suggests the presence of an unknown key factor that determines the PGC fate. Here we identify Dnd as such a critical PGC specifier in medaka. Interestingly, *dnd* RNA uses particle formation and partition as a mechanism for asymmetric segregation and cell fate decision in early developing embryos. These results provide insights into our understanding of PGC formation and manipulation in medaka as a lower vertebrate model.

RESULTS

Dnd Dosage Determines the PGC Number In Vivo

Targeted *dnd* disruption in medaka embryos led to normal survival and development to adulthood (Wang and Hong, 2014). The *dnd* mutant medaka adults are sterile and not suitable for studying its role in PGC development during embryogenesis. Conditional knockout of genes essential for early development such as PGC formation is not yet available in fish. We thus adopted direct embryo microinjection of *dnd*-targeting morpholino oligos for gene depletion and *dnd* mRNAs for gene overexpression. Two morpholino oligos were used for *dnd* depletion: MOdnd targets the medaka *dnd* mRNA and inhibits its translation, and MOddm is a mutant derivative of MOdnd by introducing four mismatches (Figure S1A). For overexpression, mRNAs *dnd:ch* and *dndΔ1:ch* were synthesized from pCSdnd:chDD and pCSdndΔ1:chDD (Figure S1B); the former encodes a cherry fluorescent protein-tagged wild-type Dnd and the latter a tagged deletion mutant Dnd. The morpholino oligos and mRNAs were microinjected alone or in combination into one-cell embryos of transgenic medaka NgVg expressing GFP specifically in PGCs (Hong et al., 2010; Li et al., 2009).

A medaka embryo at stages 18–22 has ~32 PGCs that are recognizable by GFP expression (Figure 1A). Injection of mismatch-containing MOddm had no effect on the PGC number (Figure S2A). Remarkably, *dnd* depletion by injection with 50–100 pg of MOdnd caused the complete absence of PGCs in all (n = 333) manipulated embryos (Figures 1A and S2B). When MOdnd was coinjected with *dnd:ch* RNA, the PGC number was rescued (Figure S2C), whereas *dndΔ1:ch* mRNA did not rescue (Figure S2D). Moreover, injection with 50 pg of *dnd:ch* RNA alone was sufficient to increase the PGC number (Figure S2E). Injection of *dnd:ch* RNA at 100 pg considerably boosted the PGC number, so that a significant number of PGCs were located in ectopic sites as well as numerous PGCs in the gonad (Figure S2F). Clearly, altering *dnd* expression by injection of either MOdnd or *dnd:ch* RNA altered the PGC