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Knockdown of the germ cell factor Dead end induces multiple transcriptional changes in Atlantic cod (*Gadus morhua*) hatchlings

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ABSTRACT

The RNA binding protein Dead end (DnD) is essential for maintaining viable germ cells in vertebrates and silencing of the gene has been demonstrated to cause sterility in several mammalian and fish species. Here we investigated transcriptome changes in hatched larvae of Atlantic cod induced by DnD knockdown using morpholino oligonucleotides (MO) injected in two-cell embryos. Whereas no fluorescently labeled germ cells were shown in embryos coinjected with *dnd* MO and *nanos3* 3′UTR coupled to green fluorescent protein, DnD knockdown had no visible effect on the number and location of Vasa protein positive cells in larvae. However, quantitative real-time RT-PCR (qPCR) revealed decreased *vasa, nanos3 and tudor domain containing protein 7* mRNA expression and genome-wide oligonucleotide microarray analyses indicated profound suppression of genes involved in development and regulation of the reproductive system. DnD morphants showed lowered expression of genes encoding proteins involved in lipid, retinoid, cholesterol and steroid metabolism, including those with roles in sex hormone metabolism. Biotransformation of lipophilic compounds appeared suppressed too, as evidenced by down-regulation of several key genes from the phases 1 and 2 detoxification pathways. Effects of DnD silencing were highly pleiotropic and consisted of endocrine and metabolic changes, massive induction of histones and suppression of diverse developmental processes, including erythropoiesis and formation of extracellular matrix. While transient inhibition of *dnd* mRNA translation did not block development of primordial germ cells until hatch, results suggested that ablation of DnD might have major indirect consequences, including suppression of reproductive functions.

1. Introduction

In sexually reproducing animals, male and female gametes differentiate from a small population of primordial germ cells (PGCs) set aside from all other cell lineages very early during embryonic life (Houston and King, 2000). These cells arise extragonadally and migrate toward the gonadal anlage where they remain quiescent for a prolonged period before they start to proliferate and differentiate into oogonia or spermatogonia (Balinsky, 1975; Wei and Mahowald, 1994; Braat et al., 1999; Strüssmann and Nakamura, 2002). Similar to anuran amphibians and *Drosophila*, specification of PGCs in most teleosts is directed by maternally inherited germ plasm. This

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specialized cytoplasm contains germline determinants needed for germ cell formation and survival (Extavour and Akam, 2003; Johnson et al., 2011). The essential role of germ plasm in both invertebrates and vertebrates has been demonstrated by germ plasm disruption and transplantation experiments (Illmensee and Mahowald, 1974; Lerit and Gavis, 2011). In zebrafish, physical ablation of germ plasm at the four-cell stage results in a marked reduction in the number of germ cells (Hashimoto et al., 2004). *Dead end* (*dnd*) RNA is a germ plasm component which encodes a putative RNA binding protein required for PGC migration and survival (Weidinger et al., 2003). Following germline specification, *dnd* is exclusively expressed in PGCs which is mediated through a common 3′untranslated region (UTR) dependent mechanism for the localization of maternal determinants to the germline (Zhou and King, 2004; Kloc and Etkin, 2005; Slanchev et al., 2009). Zebrafish DnD protein was shown to regulate the expression of two other germline specific genes, *nanos3* and *tudor domain containing protein 7* (*tdrd7*), presumably by protecting them from miRNA-mediated degradation of 3′UTR (D'Agostino et al., 2006; Mishima et al., 2006). In human, DnD was reported to inhibit miRNA access to *p27* and *LATS2* in germ cells by binding U-rich regions in the target 3′UTRs (Kedde et al., 2007). DnD was recently found to interact with a broad range of targets in human embryonic stem cells, including known markers of PGCs such as pluripotency factors *Oct4*, *Sox2*, *Nanog* and *LIN28* (Zhu et al., 2011).

Several knockdown studies have confirmed the importance of DnD for proper development of the gonad in teleost fish. In zebrafish, knockdown of DnD by injection of morpholino oligonucleotides (MO) during early cleavages resulted in the loss of PGC migration and their eventual death apparently without affecting somatic development (Weidinger et al., 2003). Consistently, DnD knockdown in Atlantic cod resulted in the complete loss of *nanos3* positive PGCs at late somitogenesis (Skugor et al., 2013). Wargelius and Drivenes (2012) reported a significantly reduced number of germ cells in Atlantic salmon following DnD inhibition and outlined the need for increase in MO concentration. Silencing of DnD during early development of zebrafish had a great impact on sex determination and resulted in the development of males only (Slanchev et al., 2005; Siegfried and Nusslein-Volhard, 2008). Germ cell deficient medaka also revealed the importance of germ cells for the development of ovaries (Kurokawa et al., 2007). Interestingly, loach lacking germ cells developed testicular and ovarian structures, suggesting that germ cells are not necessary for gonadal differentiation in this species (Fujimoto et al., 2010).

Cod aquaculture requires technology for reproduction control because early sexual maturation significantly increases production costs and deteriorates filet quality. *Dnd* is a potential target for manipulation of gonadal development since silencing of the gene has been successfully employed to induce sterility apparently without affecting somatic development. However, inactivation of genes with major roles in early development may cause multiple pleiotropic effects and should be examined by high-throughput analytical techniques in order to assess the direct and remote consequences of gene knockdown. We used a recently developed genome-wide oligonucleotide microarray (Krasnov et al., 2013) for gene expression profiling in newly hatched Atlantic cod larvae after DnD MO knockdown. To our knowledge, this is the first study that examined the effects of experimental silencing of a germline specific gene on the whole-genome transcriptome.

2. Materials and methods

2.1. Rearing and sample collection

Atlantic cod (*Gadus morhua*) eggs were obtained from the National Cod Breeding Centre (Kraknes, Tromsø, Norway). Eggs were hand stripped, fertilized in vitro and microinjected with MO solution at the 2-cell stage. After injection the embryos were transferred to seawater rearing tanks at an average temperature of 4.5 °C. Hatched larvae were collected and submerged in RNAlater (Ambion, Austin, Texas, USA) and used for RNA isolation for gene expression analyses.

2.2. Embryo microinjections of dnd MO

The Atlantic cod genome harbors only one *dnd* gene (ENSGMOG00000016112) and no paralogs. MOs were specifically designed to target the 5′UTR of the *dnd* transcript with *dnd* MO1 (5′-TGCAGCCGAGCAGGGCTTACCATCT-3′) and *dnd* MO2 (5′-TGCGGGAGCGAAAGAAACAAATCCA-3′) (Genetools, Philomath, OR). Two different MO concentrations (0.3 and 0.6 mM) were tested and based on the embryo survival, the lower concentration was chosen. MO1 and MO2 in 0.05% phenol red solution were microinjected in the 2-cell stage cod eggs just beneath the blastomeres to ensure the MO uptake. To evaluate the MO-mediated DnD knockdown, green fluorescent protein (GFP) fused to the 3′UTR of cod *nanos3* was coinjected as previously described (Skugor et al., 2013).

2.3. Whole mount in situ hybridization (WISH) and immunohistochemistry

Presumptive PGCs were also detected by expression of the cod *vasa* mRNA and protein, identified by WISH (Thisse and Thisse, 2008) and immunohistochemistry (Beesley, 2000), respectively. The transcript was analyzed at several developmental stages using a riboprobe synthesized from a 958 nt cod *vasa* PCR product (forward primer: GAGAGGGAAGGTGGGTCTGA-3′; reverse primer: TGGTCCGTGAAGTGACGAGT) as template (Roche Diagnostics, Mannheim, Germany). Pre-hybridization and hybridization was performed at 65 °C and 50 °C, respectively. Prior to mounting, the embryos were cleared in 100% glycerol and then viewed by Zeiss Axio Observer Z1 equipped with an AxioCam MRc5 camera and AxioVision software (Carl Zeiss Microimaging GmbH).

Whole mount immunohistochemistry was carried out on rehydrated and permeabilized embryos at segmentation and newly hatched larvae (10 min 1% Triton X-100, Sigma), followed by 3 h blocking in 5% dry milk dissolved in 1× Phosphate Buffered Saline Tween 20 (PBST; 1× PBS, 0.1%