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Requirement of a dynein light chain in transforming growth factor β signaling in zebrafish ovarian follicle cells

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Abstract

We have previously reported that the dynein light chains km23-1 and km23-2 are required for TGF β signaling in mammalian cells. Here we describe another member of the km23/DYNLRB/LC7/robl family of dynein light chains in zebrafish, termed zkm23, which is also involved in TGF β signaling. zkm23 was rapidly phosphorylated after TGF β stimulation. TGF β RII kinase activity was absolutely required for zkm23 phosphorylation, whereas a constitutively active TGF β RI did not induce phosphorylation. Further, TGF β stimulated a rapid recruitment of the zkm23 dynein light chain to the dynein intermediate chain of the dynein complex, and the TGF β RII kinase was required for this interaction. Finally, blockade of zkm23 using morpholino oligos resulted in an inhibition of TGF β -mediated transcriptional responses. Thus, our results demonstrate for the first time that the dynein light chain zkm23 is required for TGF β signaling in cultured zebrafish ovarian follicle cells.

Keywords

dynein; km23; ovary; zebrafish; TGF β

1. Introduction

Transforming growth factor β (TGF β) is the prototype for the TGF β superfamily of highly conserved growth regulatory polypeptides that are critically involved in various physiological and developmental processes, including reproduction (Knight and Glistler, 2003; Massague, 1998; Padua and Massague, 2009; Pangas and Matzuk, 2004; Roberts and Wakefield, 2003; Schmierer and Hill, 2007; Yue and Mulder, 2000b). The process of intracellular signal transduction from the membrane-bound receptors for TGF β superfamily ligands to the nucleus is conserved from worms to humans (Padua and Massague, 2009; Schmierer and Hill, 2007).

TGF β initiates its signaling by the binding of extracellular TGF β to TGF β receptor II (T β RII), followed by transphosphorylation of TGF β receptor I (T β RI). The activated receptor complex stimulates intracellular mediators of TGF β signaling, including receptor-

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activated Smads (RSmads). These activated RSmads form a complex with the common partner Smad4 and translocate to the nucleus, where they regulate transcription of a wide range of target genes (Padua and Massague, 2009; Roberts and Wakefield, 2003; Yue and Mulder, 2000b). In addition to Smad-mediated transcription signaling, the TGF- β receptors also activate non-Smad signaling pathways including the mitogen-activated protein kinases (MAPKs)-extracellular signal-regulated kinase (Erk), p38, c-Jun N-terminal kinase (JNK), resulting in activation of Erk, p38 and JNK (Kang et al., 2009; Padua and Massague, 2009; Wharton and Derynck, 2009).

We have previously identified km23-1 as a T β R-interacting protein that is also a dynein light chain (DLC) of the km23/DYNLRB/LC7/robl family (Ding and Mulder, 2004; Tang et al., 2002). It appears to play an important role in TGF β signal transduction in mammalian cells, including Smad2-dependent signaling events (Jin et al., 2007; Jin et al., 2005; Tang et al., 2002). More recently, we have reported that km23-2 has functions in TGF β signaling that are distinct from those for km23-1 in mammalian cells (Jin et al., 2009). The km23/DYNLRB/LC7/robl DLC family has also been described in lower vertebrates. For example, it has been reported that a deletion mutant found in the roadblock (robl) gene *Drosophila* (the robl² deletion mutants) shows a female sterile phenotype (Bowman et al., 1999). However, the precise role of the km23/DYNLRB/LC7/robl family of DLCs in TGF β signaling in lower vertebrates, such as zebrafish, is unclear.

We have previously noted the high degree of homology between hkm23-1, hkm23-2 and zebrafish km23 (zkm23) (Jin et al., 2005). In addition, there is evidence for the presence of the major TGF β signaling pathway molecules in the fish ovary (Calp et al., 2003; Dick et al., 2000; Hardie et al., 1998; Kohli et al., 2005; Kohli et al., 2003). Further, TGF β superfamily members (TGF β , Activin, Inhibin, GDF9, etc.) have been shown to be involved in important aspects in ovarian follicle development (Knight and Glister, 2003; Knight and Glister, 2006; Pangas and Matzuk, 2004). In the current report, we studied the effects of the zebrafish homolog of km23 on TGF β signaling in a primary cultured zebrafish ovarian follicle cells (zOFCs).

2. MATERIALS AND METHODS

2.1. Materials

The anti-Flag M2 (F3165) and mouse IgG were from Sigma. The anti-DIC monoclonal Ab was from Chemicon (Temecula, CA). The rabbit km23-1 anti-serum (hkm23-1-(27-43)-w) has been described (Jin et al., 2007; Jin et al., 2005). The mouse anti-Smad2 (610843) was from BD Biosciences Transduction laboratories (Palo Alto, CA). The rabbit IgG, mouse IgG, RII (sc-220), and protein A/G plus agarose were from Santa Cruz Biotech. 32P-orthophosphate (NEX-053) and [³H]-thymidine (NET-027X) were from Perkin Elmer (Boston, MA). TGF β 1 was purchased from R & D Systems (Minneapolis, MN). The Fugene 6 transfection reagent and anti-HA were from Roche Applied Science (Indianapolis, IN). The Lipofectamine™ 2000 transfection reagent, anti-V5 Ab, and Alexa 594 goat anti-mouse IgG were from Invitrogen (Carlsbad, CA). The Dual-Luciferase Reporter Assay System (Cat. # E1960) was purchased from Promega (Madison, MI). Morpholino oligos (MOs) (Ekker and Larson, 2001) with the following sequences (ATG MO: 5'-TAA TAG TCT CCT CCA CCT CGG CCA T-3', 5-mismatch ATG MO: 5'-TAA TAC TCT CGT CCA GCT CCG CGA T-3', 5'UTR MO: 5'-GAA GAC AAA CCG CTG TTT TCG TTG C-3', standard control ATG MO: 5'-CCT CTT ACC TCA GTT ACA ATT TAT A-3') were ordered from Gene Tools, LLC (Philomath, OR). Morpholino target sequences are marked with brackets in the mRNA sequence of zkm23:

1 atagcaacga aaacacgggt ttgtcttcaa a(atg)gccgag gtggaggaga ctatta]aaag

2.2. Cell Culture

Mv1Lu cells (CCL-64) were obtained from ATCC (Rockville, MD) and were grown in DMEM supplemented with 10% FBS. DR26 cells, a Mv1Lu derivative lacking functional T β RII (Laiho et al., 1991), were maintained as for Mv1Lu cells. 293T cells were obtained from T.-W. Wong (Bristol-Myers Squibb, Princeton, NJ) and were maintained as for Mv1Lu cells. Cultures were routinely screened for mycoplasma using Hoechst staining.

2.3. Animals

Zebrafish (*Danio rerio*) were purchased from local pet stores and maintained in our zebrafish genetics facility (Hershey, PA).

2.4. Isolation of zebrafish ovarian follicles and primary cultures of the follicle cells

Primary culture of the follicle cells were performed essentially as described previously (Wang and Ge, 2003). Briefly, the follicles from about 20 female zebrafish were carefully separated with the aid of fine forceps and blades. The follicles were measured with an ocular micrometer in a dissecting microscope and staged according to classifications by Selman et al. (Selman et al., 1993). The healthy vitellogenic follicles around 0.45 mm in diameter were selected, pooled, and washed three times with Medium 199 (Invitrogen, Cat. #11150-059) containing with 10% FBS and 100 U/ml penicillin/100 µg/ml streptomycin (Invitrogen, Cat. # 15140). The follicles were then cultured in the Medium 199 with 10% FBS at 28 °C in 5% CO₂ for 6 days to allow proliferation of the follicle cells. The proliferated follicle cells were harvested by trypsinization and plated in 24-well plates for 24 h before treatments.

2.5. Reverse transcription (RT)-PCR amplification of zkm23 from the cultured follicle cells

zOFCs were plated in 6-well plates. 24 h later, total RNA was extracted according to the manufacturer's instructions. RNA was reverse transcribed to make cDNA using Omniscript Reverse Transcriptase (QIAGEN) and Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) with random hexamer primers. PCR amplification of zkm23 was performed with the forward primer 5' GCC GAG GTG GAG GAG ACT AT 3' and the reverse primer 5' TGT GGG ATT TTG AAT GAC GA 3'. PCR conditions were as follows: 94°C 50', 60°C 60', 72°C 60' for 36 cycles.

2.6. Construction of pCMV5-zkm23-Flag plasmid

To prepare zkm23-Flag, zkm23 was PCR amplified from the above cDNA produced from the cultured follicle cells using primers containing additional suitable flanking restriction enzyme sites for BglII (5') and SalI (3'), followed by inserting into pCMV5-Flag (Sigma) after digestion with BglII and SalI restriction enzymes, respectively. DNA sequences were verified by sequencing in both directions and alignment to ZFIN (GenBank Accession number: NM_201188).

2.7

Transient transfections, immunoprecipitation (IP)/blot, Westerns, and in vivo phosphorylation assays were performed essentially as described previously (Ding et al., 2005; Ilangoan et al., 2005; Jin et al., 2007; Jin et al., 2005; Jin et al., 2009; Tang et al., 2002).

2.8. [³H] thymidine incorporation assays

[³H] thymidine incorporation assays were performed as described (Hartsough and Mulder, 1995; Jin et al., 2005).

2.9. Immunofluorescence microscopy analyses for Smad2/3

zOFCs were fixed and immunofluorescence microscopy analyses were performed as described previously (Jin et al., 2007).

2.10. Luciferase reporter assays

zOFCs were plated (1 to 3 split) in 24-well plates. 24h after plating, the cells were transfected using FuGene 6 transfection reagent with either 0.1 µg /well of 3TP-Lux (Wrana et al., 1992) or 0.1 µg /well of activin-responsive element (ARE)-Lux (Yeo et al., 1999) and 0.1 µg /well pRL-SV (control to normalize transfection efficiencies), followed by delivery of MOs by Endoport at 6 h, 12 h after transfection. The cells were then washed with serum-free (SF) medium and incubated in this SF for 30 min, prior to a 24 h incubation in the presence or absence of TGFβ1 (10ng/ml). The reporter assays were done as described previously (Jin et al., 2007; Jin et al., 2009).

2.11. Statistical Analysis

Data were analyzed and statistically significant differences determined using the Student's *t* test. The results are expressed as the mean ± SEM.

3. Results

In order to determine whether zkm23 could be detected by the rabbit km23-1 anti-serum, which has been used successfully to detect human km23-1 (Jin et al., 2007; Jin et al., 2005), we performed IP/blot analyses using an anti-Flag Ab as the IP Ab and a rabbit km23-1 anti-serum as the blotting Ab, after transfection of zkm23-Flag into 293T cells. As shown in Fig. 1A, expression of transfected zkm23-Flag was detectable using a rabbit km23-1 anti-serum (top panel, lane 2), while the empty vector (EV) control was negative (top panel, lane 1), indicating that the rabbit km23-1 anti-serum could detect exogenous zkm23. Expression of zkm23-Flag was confirmed by Western blot analyses with an anti-Flag Ab (bottom panel).

To determine whether the rabbit km23-1 anti-serum could also detect endogenous zkm23, we performed Western blot analyses on cell lysates from zOFCs. As indicated in Fig. 1B, the rabbit km23-1 anti-serum recognized a single band of 11kDa (lane 1), whereas no band was detectable when IgG control was used (lane 2). Thus, the rabbit km23-1 anti-serum could specifically detect endogenous zkm23 in zOFCs.

Since our studies relied on the responsiveness of the cells to TGFβ treatment, [³H] thymidine incorporation analyses were performed to determine whether zOFCs are responsive to the growth inhibitory effects of TGFβ. As shown in Fig. 2A, TGFβ treatment for 24 h caused a 53% inhibition of growth in zOFCs. Thus, our results demonstrate that zOFCs are responsive to TGFβ stimulation.

Smad2/3 are major mammalian TGFβ signaling components, which have also been cloned from the zebrafish ovary (Dick et al., 2000; Kohli et al., 2003). To further confirm the responsiveness of primary zOFCs to TGFβ treatment, we performed ARE-Lux luciferase reporter assays in the absence and presence of TGFβ. The ARE-lux reporter was previously shown to be activated by TGFβ or activin in a Smad2-dependent manner (Yeo et al., 1999). As shown in Fig. 2B, TGFβ induced approximately 3-fold of ARE-lux reporter activity in

zOFCs. Thus, these experiments clearly demonstrate that primary zOFCs are responsive to TGF β treatment.

TGF β receptors have serine/threonine kinase activity and can phosphorylate a number of intracellular proteins to initiate and propagate various TGF β signaling events and responses (Kang et al., 2009; Roberts and Wakefield, 2003; Yue and Mulder, 2000a). We have previously shown that km23-1 is phosphorylated on serine residues upon TGF β receptor activation, consistent with the serine/threonine kinase specificity of the TGF β receptors (Tang et al., 2002). More recently, we have shown that km23-2 is phosphorylated by TGF β (Jin et al., 2009). Thus, if zkm23 is a component of the TGF β signaling network, it is conceivable that zkm23 might be phosphorylated upon TGF β receptor activation as a mechanism for its activation.

To determine whether zkm23 is phosphorylated upon activation of TGF β receptors, we performed *in vivo* phosphorylation assays after transient transfection of zkm23-Flag, together with various TGF β receptor constructs or EV in the absence or presence of TGF β . As shown in Fig. 3A, zkm23 was not constitutively phosphorylated (lane 1, top panel), and co-expression of zkm23 with TGF β receptors in the absence of TGF β did not induce its phosphorylation (lane 2, top panel). However, TGF β □ □ □ □ □ □ □ □ for 15 min after co-expression of T β Rs with zkm23 resulted in significant zkm23 phosphorylation (lane 3, top panel). Furthermore, this phosphorylation of zkm23 was completely blocked upon co-expression of a kinase-deficient form of T β RII (KNRII) with wild-type RI (RI-V5) (lane 4, top panel). No detectable phosphorylation of zkm23 (lane 5, top panel) was observed after expression of a constitutively active T β RI (RI-T204D-HA), which has been shown to phosphorylate some TGF β signaling intermediates, such as Smad2 (Barrios-Rodiles et al., 2005). The IgG control (lane 6, top panel) indicates that the band noted is specific for zkm23. Equal expression and loading of zkm23-Flag, RII-HA, KNRII-HA, RI-T204D-HA, and RI-V5 were confirmed by Western blotting (lower panels). Thus, the results in Fig. 3A demonstrate not only that zkm23 is phosphorylated upon treatment of TGF β but also that the kinase activity of T β RII, but not that of a constitutively active form of T β RI, is required for zkm23 phosphorylation.

Our previous results have shown that km23-1 is a T β R-interacting protein; it is present in a complex with RII, even if RI is not co-expressed (Tang et al., 2002). Since km23-1 appears to directly interact with RII, this DLC may contain a direct phosphorylation site for RII. Along these lines, our results here have shown that the kinase activity of T β RII, but not that of a constitutively active form of T β RI, is required for zkm23 phosphorylation in 293T cells. Therefore, it is conceivable that overexpression of RII might have a direct effect on zkm23 phosphorylation. Accordingly, we performed *in vivo* phosphorylation assays, after transient co-expression of zkm23 with either wild-type RII (Fig. 3B, lanes 2-4 and 6) or KNRII (Fig. 3B, lane 5), or EV (Fig. 3B, lane 1) in zOFCs. As shown in Fig. 3B, ligand activation of the endogenous T β Rs resulted in a low level of zkm23 phosphorylation (lane 1). However, overexpression of wild-type RII in zOFCs resulted in a much higher level of zkm23 phosphorylation (compare lanes 1 and 4). Similarly, overexpression of KNRII increased the level of zkm23 phosphorylation in the presence of TGF β treatment for 15 min, compared to a 15-min TGF β incubation with no exogenous RII added (lane 5 versus lane 1). However, this level was lower than that observed after expression of wild-type RII with the 15-min TGF β treatment, providing further support that zkm23 is a T β RII-interacting protein, whether the complex is with wild-type RII or KNRII. The IgG control (lane 6, top panel) indicates that the band noted is specific for zkm23. Equal expression and loading of zkm23-Flag, RII-HA, and KNRII-HA were confirmed by Western blotting (lower panels). Thus, zkm23 phosphorylation is enhanced when RII levels are elevated during TGF β treatment, even if the T β RII kinase domain is not present. Similar results were previously reported for

MADR2, except that the MADR2 interaction with wild-type T β Rs was reduced relative to that of the receptor complex containing wild-type RII and KNRI, (Macias-Silva et al., 1996).

As mentioned earlier, zkm23 is a member of the km23/DYNLRB/LC7/robl family of DLCs (Jin et al., 2005). We have shown that TGF β receptor activation rapidly induces the recruitment of the km23-1 DLC to the DIC (Tang et al., 2002). Similarly, a more recent report has indicated that phosphorylation of the Tctex-1 DLC by the bone morphogenetic protein receptor type II (BMPR-II) may trigger association with the motor complex (Machado et al., 2003). Accordingly, it was of interest to determine whether TGF β could stimulate the recruitment of the zkm23 DLC to the dynein motor complex.

To determine whether TGF β could stimulate the interaction between zkm23 DLC and the DIC, we performed IP/blot analyses using anti-DIC as the IP Ab and anti-Flag as the blotting Ab after transient transfection of zkm23-Flag in Mv1Lu cells. These cells are highly responsive to TGF β , and are frequently used in studies of TGF β signaling (Calp et al., 2003; Chen et al., 2006; Jin et al., 2007; Rao and Kadesch, 2003). As shown in Fig. 4A, TGF β stimulated the recruitment of zkm23 to the DIC (lanes 3 and 4). Expression of EV only (lane 1) and the IgG control (lane 5) indicated that the interaction noted is specific for zkm23. Similar results were obtained for 293T cells, after co-expression of RI-V5, RII-HA, and zkm23-Flag in the absence and presence of TGF β (data not shown). Thus, in similarity to hkm23-1 and hkm23-2, zkm23 can also be recruited to the DIC within minutes of TGF β addition to the cultures. To further confirm the zkm23-DIC interaction and its regulation by TGF β in zOFCs, IP/blot analyses in the reverse direction were performed using the anti-DIC as the blotting Ab (Fig. 4B), which demonstrated a rapid interaction between DIC and zkm23 after TGF β stimulation with a similar pattern.

To further determine whether TGF β could regulate the recruitment of endogenous zkm23 to the DIC in zOFCs, we performed similar IP/blot analyses in the absence and presence of TGF β . As shown in Fig. 4C, there was no interaction between zkm23 DLC and the DIC in the absence of TGF β (lane 1). However, TGF β induced a rapid recruitment of zkm23 to the DIC, occurring as early as 5 min after TGF β treatment (lanes 2-6). A maximal increase in this interaction was reached after 10 min of TGF β treatment (lane 2) and appeared to remain relatively constant until at least 30 min after TGF β treatment (lane 6). The IgG control was negative (lane 7, top panel). Collectively, these data demonstrate that TGF β induced a rapid recruitment of zkm23 to the DIC in two different cell systems, and that this rapid, TGF β -mediated DIC-zkm23 interaction could occur in zebrafish cells expressing endogenous TGF β receptors.

Since we have demonstrated here that the zkm23 DLC can be phosphorylated and that the kinase activity of T β RII is required for this phosphorylation, it was of interest to determine whether the recruitment of zkm23 to the DIC required T β RII kinase activity. In order to investigate this possibility, we performed IP/blot analyses similar to those in Fig. 4A, after transient expression of zkm23 in Mv1Lu cells and DR26 cells (a Mv1Lu mutant derivative cell line lacking functional T β RII) (Laiho et al., 1991). As shown in Fig. 4D, TGF β stimulated recruitment of zkm23 to the DIC in Mv1Lu cells expressing endogenous wild type T β RII and T β RI (lanes 1-3). However, in DR26 cells, the recruitment of zkm23 to the DIC after TGF β treatment was barely detectable (lanes 4-6). No specific band was detectable in the IgG control (lane 7). Equal expression and loading of DIC was confirmed (bottom panels). Thus, the kinase activity of T β RII is required for the recruitment of zkm23 to the DIC.

The results in Figs. 3 and 4 suggest that zkm23 might function as a TGF β signaling intermediate in zOFCs. In order to more definitively establish whether zkm23 was required

for TGF β signaling events, we utilized a MO knockdown strategy to block zkm23 expression, which causes either a block to translation from the target mRNA or a blockade of target mRNA splicing (Egger and Larson, 2001; Nasevicius and Ekker, 2000). The blocking effect of zkm23-specific MOs was tested in 293T cells. As shown in Fig. 5A, the zkm23 ATG MOs specifically knocked down exogenous zkm23 expression in 293T cells (top panel, lanes 1-3), even at a concentration of 0.25 μ M. A complete knockdown effect was achieved at a concentration of 4.0 μ M zkm23 ATG MOs. However, no such effect was observed for the two control MOs (top panel, lanes 4 and 5: standard control and zkm23 ATG MO 5-mismatch control, respectively) at 1.0 μ M, or for the zkm23 5'-UTR MOs (top panel, lane 6). Because the pCMV5-zkm23-Flag plasmid did not contain the zkm23 5'-UTR MOs target sequence, the zkm23 5'-UTR MOs has no effect on the exogenous zkm23 expression in 293T cells as expected. Equal loading was confirmed by blotting with a DIC Ab (bottom panel). Since TGF β did not regulate DIC expression levels in past experiments, DIC has been used as an equal loading control in our previous publications (Jin et al., 2007; Jin et al., 2009; Pulipati et al., 2011). Thus, our results show that the translation blocking effect of the zkm23 ATG MOs was dose-dependent and specific for zkm23. The translation blocking effect of these MOs was then tested in the primary zOFCs. The results in Fig. 5B show that both zkm23 ATG MOs and zkm23 5'-UTR MOs, but not the two control MOs, blocked endogenous zkm23 expression in zOFCs in a dose-dependent manner. An optimal blocking concentration of between 0.25 μ M and 4.0 μ M was observed.

Because we have shown that zkm23 specific MOs could specifically block the expression of both transfected and endogenous zkm23 expression, we used this approach to examine the effect of zkm23 on TGF β -inducible transcriptional activity of a 3TP-Lux reporter, a TGF β -regulated reporter frequently used to evaluate TGF β signaling (Lee et al., 2004). This reporter construct contains three consecutive 12-O-tetradecanoylphorbol-13-acetate response elements and a TGF β -inducible 100-bp fragment of the plasminogen activator inhibitor-1 promoter (Wrana et al., 1992). As shown in Fig. 5C, 3TP-Lux was induced by approximately 8-fold after TGF β treatment. Both zkm23 ATG MOs and zkm23 5'-UTR MOs, at a concentration of 2.0 μ M, significantly repressed 3TP-Lux induction by TGF β , further supporting a role for zkm23 in TGF β -mediated signaling in zOFCs.

4. Discussion

In the current report, we have used a zebrafish model to gain insight into the important role of the km23/DYNLRB/LC7/robl DLC family in TGF β signaling in ovarian follicle cells. First, we have shown that endogenous zkm23 protein is expressed in zOFCs. In addition, we have demonstrated that primary cultures zOFCs are responsive to TGF β stimulation. Further, the kinase activity of T β RII is necessary for zkm23 phosphorylation and the recruitment of zkm23 to the DIC, whereas a constitutively active T β RI cannot induce zkm23 phosphorylation in zOFCs. Moreover, MOs knockdown of zkm23 decreased TGF β -mediated downstream events. Collectively, our results demonstrate for the first time that zkm23 is required for TGF β signaling in zOFCs, thereby implicating the km23/DYNLRB/LC7/robl DLC family in ovarian follicle development.

The multi-functional TGF β family signaling pathways play important roles in a wide range of processes such as adult tissue homeostasis and organogenesis of many organ systems, including the ovary (Padua and Massague, 2009; Pangas and Matzuk, 2004; Schmierer and Hill, 2007). It has been demonstrated that various TGF β superfamily members (TGF β , Activin, Inhibin, growth differentiation factor 9, etc.) are involved in important aspects in follicle development (Knight and Glister, 2006). For example, double conditional knockout of Smad2 and Smad3, which are two TGF β major signaling components, leads to severe fertility and fecundity defects in mice, supporting the involvement of an intraovarian

Smad2/3 pathway in mediating oocyte-produced signals essential for coordinating key events of the ovulatory process (Pangas and Matzuk, 2004). Similarly, recent findings suggest that TGF β may play a role in preventing premature oocyte maturation in zebrafish by down-regulating basal and human chorionic gonadotropin-induced 20 β -hydroxysteroid dehydrogenase, the luteinizing hormone receptor, and membrane progesterin receptor- β mRNA levels (Kohli et al., 2005). Our results have shown that the kinase activity of T β RII is required for zkm23 phosphorylation, and that blockade of zkm23 results in an inhibition of transcriptional activation induced by TGF β , suggesting that zkm23 is an important TGF β signaling intermediate in zOFCs. Thus, it is conceivable that zkm23 may play an important role in zebrafish ovarian follicle development via a novel zkm23-specific TGF β signaling pathway.

As mentioned above, our results have shown that blockade of zkm23 inhibited the transcriptional activity of the 3TP-lux reporter in zOFCs, suggesting that knockdown of zkm23 disrupts TGF β signaling through several transcriptional factors. The 3TP-lux reporter contains 3 of AP1-binding sites that are regulated by Ras and both JNK and ERK/MAPK pathways (Mulder, 2000; Yue and Mulder, 2001; Zhang et al., 2001). It has also been reported that TGF β can activate the Ras/ERK, as well the p38 and JNK pathways in different cell types (Yue and Mulder, 2001; Zhang, 2009). Our previous results have indicated that forced expression of hkm23-1 induced specific TGF β responses, including JNK activation, suggesting that km23-1 may be involved in a TGF β /JNK pathway. It is noteworthy that Smads can also regulate 3TP-lux transcriptional activity induced by TGF β (Zhang et al., 1996). Our results in Fig. 2 have shown that TGF β can stimulate Smad2-dependent ARE-Lux reporter activity, suggesting that TGF β can activate Smad signaling in zOFCs. In addition, our previous results have shown that km23-1 is required for Smad2-dependent TGF β signaling, whereas km23-2 is required for Smad3-dependent TGF β signaling (Jin et al., 2009). Thus, our results suggest that zkm23 might also be involved in a Smad pathway. Collectively, our results suggest that zkm23 might regulate TGF β -inducible transcriptional activity of 3TP-lux via a JNK/AP-1 and/or Smad-dependent pathway activated by TGF β .

Phosphorylation is one mechanism that regulates the functions of the km23/DYNLRB/LC7/robl DLC family. Our previous results have shown that km23-1 is serine-phosphorylated after TGF β receptor activation and binds to the DIC in response to this activation. Similarly, we show here that zkm23 phosphorylation is regulated by TGF β in both 293T cells and the primary zOFCs, requiring the kinase activity of T β RII. It has also been shown that zkm23 interacts with the DIC in zOFCs, and zkm23 phosphorylation is required for this event to occur, suggesting that non-phosphorylated zkm23 is in an inactive state until its phosphorylation and activation permits binding to the DIC and to cargoes. These data are in agreement with results for hkm23-1 and 2 (Ilango et al., 2005; Jin et al., 2009; Tang et al., 2002) and Tctex-1, a DLC interacting with BMPRII (Machado et al., 2003). However, a recent crystal structure and in vitro binding studies have shown that phosphorylation does not directly affect LC7 binding to DIC, but may modulate LC7 binding through additional proteins (Hall et al., 2010). Therefore, it will be of interest to determine in future studies how phosphorylation regulates complex formation of zkm23 with the DIC and other proteins in zOFCs in vivo.

The cytoplasmic dynein motor plays a novel developmental role in cellular determination and differentiation (McGrail and Hays, 1997). For example, a previous report has shown that the cytoplasmic dynein heavy chain is required for oocyte differentiation in *Drosophila* (McGrail and Hays, 1997). In addition, it has been shown that cytoplasm dynein-dynactin mediates poleward MEK1/2 transport; failure of poleward MEK1/2 transport causes abnormal spindles in mouse oocytes (Xiong et al., 2007). Further, it has also been reported

that the dynein motor activity is necessary to transport the centrosomal protein NuMA along microtubules to the spindle poles (Sun and Schatten, 2007) and any failure in dynein activity can result in cell and developmental abnormalities in human oocytes (Alvarez Sedo et al., 2011). Our previous results have shown that overexpression of the km23-1 DLC is aberrant in metaphase cells with altered BubR1 localization. This is associated with the formation of multipolar spindles and multinucleated cells, suggesting that hkm23-1 might be involved in the transport of cargos such as spindle checkpoint proteins (Pulipati et al., 2011). In keeping with these previous results and of our results herein, it is conceivable that the km23/DYNLRB/LC7/robl DLC family may also play an important role in oocyte maturation through mechanisms involving transport of km23 DLC-specific cargos along microtubule spindles in oocytes. Our findings indicate that this model may provide a useful tool for future studies of the role of zkm23 in oocytes. It would additionally be feasible to investigate the physiological role of zkm23 in zebrafish development by MO knockdown of zkm23 expression, followed by in vivo analysis of zebrafish development.

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Abbreviations

TGFβ transforming growth factor β

TβRII	TGFβ receptor II
TβRI	TGFβ receptor I
RSmads	receptor-activated Smads
MAPKs	mitogen-activated protein kinases
JNK	c-Jun N-terminal kinase
Erk	extracellular signal-regulated kinase
zkm23	zebrafish km23
hkm23	human km23
DLC	dynein light chain
DIC	dynein intermediate chain
zOFCs	zebrafish ovarian follicle cells
Ab	antibody
EV	empty vector
IP	immunoprecipitation
MOs	Morpholino oligos
DAPI	4,6-diamidino-2-phenylindole
KNRII	kinase-deficient form of TβRII
BMPR-II	bone morphogenetic protein receptor type II

Highlights

- Endogenous zkm23 protein is expressed in zebrafish ovarian follicle cells.
- TGF β stimulates the phosphorylation of zkm23.
- TGF β stimulates the recruitment of zkm23 to the dynein intermediate chain.
- Morpholino oligos knockdown of zkm23 decreased TGF β -mediated transcription.
- zkm23 is required for TGF β signaling in zebrafish ovarian follicle cells.

Fig. 1. Detection of both exogenous and endogenous zkm23 protein expression

A: Rabbit km23-1 anti-serum could detect exogenous zkm23. 293T cells were transiently transfected with zkm23-Flag (lane 2) or EV (lane 1). 32 h after transfection, cells were lysed, and IP/blot analyses were performed as in “Materials and Methods.” **B: Western blot analysis of endogenous zkm23 in zOFCs.** Data are representative of two independent experiments.

Fig. 2. zOFCs are responsive to TGFβ treatment

A: TGFβ inhibited [³H]-thymidine incorporation in zOFCs. 24h after plating, thymidine incorporation assays were then performed as described in the “Materials and Methods.” Data represent the mean ± SEM. of triplicate wells from three independent experiments; *n* = 3. **B: TGFβ induced Smad2-dependent ARE-lux reporter activity in zOFCs.** Luciferase reporter assays were then performed as described in the “Materials and Methods.” Transfection efficiencies were normalized by renilla luciferase. Data represent the mean ± SEM of triplicate wells from three independent experiments; *n* = 3. Numbers on top of each bar indicate the fold increase compared with control.

Fig. 3. The kinase activity of T β RII is required for TGF β induction of zkm23 phosphorylation, whereas constitutively active T β RI cannot induce zkm23 phosphorylation
A: zkm23 is phosphorylated upon activation of TGF β receptors in 293T cells and this phosphorylation is blocked by KNR11. 293T cells were transiently transfected with zkm23-Flag, together with the above constructs or EV as indicated. 28h after transfection, in vivo phosphorylation assays were performed as described previously (Tang et al., 2002). **Top panel**, in vivo phosphorylation of zkm23 was visualized by 4-12% NuPAGE and autoradiography. **Lower panels**, Controls for expression and loading of RII-HA (RII blot), KNR11-HA (RII blot), RI-V5 (v5 blot), RI-T204D-HA (HA blot), and zkm23-Flag (Flag blot). **B: zkm23 phosphorylation is enhanced when RII levels are elevated during TGF β treatment, even if the T β RII kinase domain is not present.** zOFCs were transiently transfected with the indicated plasmids. 28h after transfection, studies were performed the same as for A. Results are representative of two independent experiments.

Fig. 4. The interaction between the DIC and zkm23 is regulated by TGFβ, and a functional TGFβRII is required for this interaction

A: TGFβ stimulates the recruitment of zkm23 to the DIC in Mv1Lu cells. Mv1Lu cells were transiently transfected with zkm23-Flag or EV. 24h after transfection, Mv1Lu cells were then incubated with SF medium for 1h prior to addition of TGFβ1 for the indicated times. Cells were then lysed, and lysates were subjected to IP/blot analyses using a DIC Ab as the IP Ab, and a Flag Ab as the blotting Ab. **B: The interaction between zkm23 and the DIC is confirmed by IP/blot analyses in zOFCs.** zOFCs were transiently transfected with zkm23-Flag and were treated with TGFβ as described in A, followed by IP/blot analyses using a Flag Ab as the IP Ab and a DIC Ab as the blotting Ab. **C: The recruitment of endogenous zkm23 to the DIC in zOFCs is stimulated by TGFβ.** 24h after plating, the cells were treated with TGFβ as described in A, followed by IP/blot analyses using a DIC Ab as the IP Ab and a rabbit km23-1 anti-serum as the blotting Ab. **D: A functional TβRII is required for the TGFβ induction of the DIC-zkm23 interaction.** Mv1Lu and DR26 cells (a Mv1Lu mutant derivative cell line lacking functional TβRII) were transiently transfected with zkm23-Flag, and were treated with TGFβ as described in A. Cell lysates were subjected IP/blot analyses as indicated. Data are representative of two independent experiments.

Fig. 5. Knockdown of zkm23 in zOFCs by MOs markedly inhibited 3TP-Lux transcriptional activity induced by TGF β

A: The zkm23 MOs specifically knock down exogenous zkm23 expression in 293T cells.

293T cells were transiently transfected with 2 μ g zkm23-Flag using FuGene 6 transfection reagent. 6h after transfection, MOs were added to the each well at 0.25, 1.0 or 4.0 μ M as indicated. 40h after transfection, cells were then harvested. Cell lysates were subjected to Western blotting analyses as indicated.

B: The zkm23 MOs specifically knock down endogenous zkm23 expression in zOFCs.

24h after plating, MOs were delivered to the cells for 24 h by Endo-Porter at 0.25, 1.0 or 4.0 μ M as indicated. Cell lysates were subjected to Western blotting analyses as indicated.

C: MOs knockdown of zkm23 expression markedly inhibited 3TP-Lux activity induced by TGF β .

The luciferase reporter assays were performed as described in "Material and Methods." Transfection efficiencies were normalized by renilla luciferase. Data represent the mean $\pm$ SEM of triplicate wells from three independent experiments; $n = 3$. Asterisk (*) indicates a statistically significant difference ($p < 0.01$) compared with control.