Published in final edited form as:
Mech Dev. 2014 May ; 132: 1–12. doi:10.1016/j.mod.2014.02.004.

FoxD3 regulates cranial neural crest EMT via downregulation of Tetraspanin18 independent of its functions during neural crest formation

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Abstract

The scaffolding protein tetraspanin18 (Tspan18) maintains epithelial cadherin-6B (Cad6B) to antagonize chick cranial neural crest epithelial-to-mesenchymal transition (EMT). For migration to take place, Tspan18 must be downregulated. Here, we characterize the role of the winged-helix transcription factor FoxD3 in the control of *Tspan18* expression. Although we previously found that *Tspan18* mRNA persists several hours past the stage it would normally be downregulated in FoxD3-deficient neural folds, we now show that *Tspan18* expression eventually declines. This indicates that while FoxD3 is crucial for initial downregulation of *Tspan18*, other factors subsequently impact *Tspan18* expression. Remarkably, the classical EMT transcription factor Snail2 is not one of these factors. As in other vertebrates, FoxD3 is required for chick cranial neural crest specification and migration, however, FoxD3 has surprisingly little impact on chick cranial neural crest cell survival. Strikingly, Tspan18 knockdown rescues FoxD3-dependent neural crest migration defects, although neural crest specification is still deficient. This indicates that FoxD3 promotes cranial neural crest EMT by eliciting *Tspan18* downregulation separable from its Tspan18-independent activity during neural crest specification and survival.

Keywords

neural crest; FoxD3; tetraspanin; migration; EMT

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Author Contributions

L.S.G and C.L.F conceived and designed the experiments. C.L.F. and J.P.C. performed the electroporation and in situ hybridization experiments. A.T.S. and L.A.T. performed the QPCR experiments and analyzed those data. L.S.G. and C.L.F. analyzed the remaining data, and L.S.G. and C.L.F. wrote the paper with input from L.A.T.

1. Introduction

The neural crest is a transient population of multipotent cells that arises from the dorsal neural tube of vertebrate embryos, migrates, and eventually contributes to a wide range of adult structures, including the peripheral nervous system and craniofacial skeleton (LeDouarin and Kalcheim, 1999). Neural crest development begins during gastrulation, when secreted factors induce the expression of a cohort of transcription factors, known generally as neural crest specifiers (Betancur et al., 2010), in the neural folds (Stuhlmiller and Garcia-Castro, 2012). Neural crest specifiers converge to regulate the activity of neural crest effector genes that control cell adhesion, motility, and fate (Betancur et al., 2010). By doing so, neural crest specifiers are crucial for neural crest cells to progress through epithelial-to-mesenchymal transition (EMT), migration, and differentiation into diverse adult neural crest derivatives. Unfortunately, since few targets of neural crest specifiers have been identified, we know little about the exact mechanism behind their cellular impacts.

The winged-helix transcription factor FoxD3 is a key neural crest specifier that has been implicated in multiple steps of neural crest development. FoxD3 is initially required for neural crest formation during early stages of neural crest development, including specification, multipotency, cell fate and survival (Kos et al., 2001; Sasai et al., 2001; Lister et al., 2006; Montero-Balaguer et al., 2006; Stewart et al., 2006; Thomas and Erickson, 2009; Mundell and Labosky, 2011; Hochgreb-Hagele and Bronner, 2013; Nitzan et al., 2013). Subsequently, neural crest cells fail to migrate in FoxD3 mutant zebrafish (Stewart et al., 2006). Furthermore, FoxD3 overexpression in the chick trunk neural tube alters cell-cell adhesion (Cheung et al, 2005) and increases the number of emigrating neural crest cells (Dottori et al., 2001; Kos et al., 2001). While these results suggest that FoxD3 also regulates neural crest migration, it is not possible to distinguish whether FoxD3 is required for neural crest migration independent of its functions during neural crest formation, or whether migration is blocked as a secondary consequence of these earlier roles. Moreover, FoxD3 represses neural crest and cancer cell migration in other circumstances, leaving the role of FoxD3 in migration unclear (Drerup et al., 2009; Katiyar and Aplin, 2011). Altogether, these results emphasize the need for further investigation to reconcile conflicting observations and define FoxD3 function during migration.

At the start of neural crest EMT, altered expression of Ca^{2+}-dependent cell adhesion molecules called cadherins disrupts cell-cell adhesions (Meng and Takeichi, 2009; Nieto, 2011; Oda and Takeichi, 2011). In cranial neural crest cells, *cadherin-6B* (*Cad6B*) is directly repressed by the neural crest specifier and well-established EMT transcription factor, Snail2 (Coles et al., 2007; Taneyhill et al., 2007). However, Cad6B protein levels in cranial neural crest cells are not only subject to transcriptional control. ADAM metalloproteases cleave Cad6B, leading to its loss from the neural folds (Schiffmacher et al., 2014). Moreover, the transmembrane scaffolding protein Tetraspanin18 (Tspan18) post-translationally maintains Cad6B protein levels and must be downregulated in order for cranial neural crest cells to migrate (Fairchild and Gammill, 2013). Intriguingly, premigratory cranial neural crest cells lacking FoxD3 retain *Tspan18* mRNA expression (Fairchild and Gammill, 2013). This finding, along with the observation that ectopic FoxD3 expression in chick trunk neural tube

alters cell adhesion molecule expression (Cheung et al., 2005), suggests that FoxD3 may regulate migration by modulating cadherin levels during cranial neural crest EMT through its effects on *Tspan18*.

Although our previous study showed that FoxD3 knockdown sustained *Tspan18* expression (Fairchild and Gammill, 2013), it did not address long-term outcomes. Moreover, when FoxD3 was knocked down, it was unclear whether *Tspan18* expression persisted as an indirect consequence of altered neural crest specification, or whether *Tspan18* was downstream of FoxD3 during EMT. Thus, the aim of this study was to distinguish between these two scenarios. We report that FoxD3 is required for initial downregulation of *Tspan18*, as well as for chick cranial neural crest specification and migration. Importantly, we show that Tspan18 knockdown rescues FoxD3-dependent migration but not specification defects. Altogether, these results indicate that FoxD3 promotes cranial neural crest migration at least in part through downregulation of *Tspan18* independent of its role in other aspects of neural crest development.

2. Results

2.1. *FoxD3* and *Tspan18* expression overlap in premigratory cranial neural crest cells

The general expression pattern of *FoxD3* during chick cranial neural crest development has previously been described (Kos et al., 2001; Khudyakov and Bronner-Fraser, 2009; Simoes-Costa et al., 2012); however, for FoxD3 to regulate *Tspan18*, they must be co-expressed in neural crest cells as they are undergoing EMT, specifically before 8 somites when *Tspan18* downregulation occurs (Fairchild and Gammill, 2013). To assess expression overlap, we visualized and compared *FoxD3* and *Tspan18* mRNA levels by *in situ* hybridization in whole mount and transverse sections. At 6 and 7 somites, *FoxD3* transcripts were detected exclusively in the cranial neural tube (Fig. 1A,B, arrowheads). Transverse sections confirmed that *FoxD3* was abundantly expressed in the dorsal neural tube at these stages (Fig. 1A',B'). At 8s, emigrating cranial neural crest cells expressed *FoxD3* mRNA, which persisted in the dorsal cranial neural tube (Fig. 1C,C', arrowhead), and additionally extended into the trunk (Fig. 1C, black arrow). At 9s, *FoxD3* expression was still apparent in the cranial dorsal neural tube (Fig. 1D', black arrowhead) and in the trunk (Fig. 1D, black arrow); however, its expression was reduced in actively migrating neural crest cells (Fig. 1D,D', white arrowheads). Likewise, *Tspan18* mRNA expression in the cranial dorsal neural tube was apparent at 6 and 7 somites (Fig. 1E,F, black arrowheads), entirely overlapping with the *FoxD3* expression domain (Fig. 1H,I, black arrowheads); however, *Tspan18* expression was downregulated in the dorsal neural tube and migratory neural crest cells by 8s (Fig. 1G, white arrowhead). Neural crest cells migrating away from the neural tube expressed only *FoxD3* (Fig. 1J, K, white arrowheads) and were surrounded by *Tspan18* expression in the head mesenchyme (Fig. 1F,G,I-K white arrows; (Fairchild and Gammill, 2013)). Thus, *FoxD3* is expressed at the right time and in the correct location to regulate *Tspan18* expression.

2.2. In the absence of FoxD3, *Tspan18* mRNA downregulation is delayed

We previously reported that *Tspan18* mRNA fails to downregulate when FoxD3 is knocked down (Fairchild and Gammill, 2013). To determine the persistence and dynamics of this effect, we evaluated *Tspan18* expression over time in embryos electroporated with a FITC-tagged FoxD3 translation-blocking antisense morpholino oligonucleotide (FoxD3MO; (Kos et al., 2001)). FITC-tagged standard control MO (ContMO) or FoxD3MO was electroporated unilaterally into presumptive chick neural crest cells at stage HH4+, and resulting embryos at 8–9 or 10+ somites were processed by *in situ* hybridization to visualize *Tspan18* mRNA expression in whole mount or transverse sections. As usual (Fig. 1; (Fairchild and Gammill, 2013)), *Tspan18* mRNA was absent in the dorsal neural tube of embryos with 8 or more somites that had been electroporated with ContMO (Fig. 2A,A”,B,B”; arrows). In contrast, at 8–9 somites, *Tspan18* mRNA persisted on the targeted side of the dorsal neural tube in embryos electroporated with FoxD3MO (Fig. 2C,C”,E; arrowheads). However, by 10 somites, *Tspan18* transcripts were no longer visible in the dorsal neural tube of FoxD3MO-electroporated embryos (Fig. 2D,D”). These results suggest that FoxD3 is required for prompt, initial downregulation of *Tspan18* mRNA, but it is not the only factor regulating *Tspan18* mRNA expression.

One obvious candidate to contribute to *Tspan18* downregulation is the neural crest transcriptional repressor, Snail2. Tspan18 antagonizes EMT by maintaining Cad6B protein (Fairchild and Gammill, 2013), and *Cad6B* is directly repressed by Snail2 (Taneyhill et al., 2007). Thus, we reasoned that *Tspan18* might also be regulated by Snail2. To determine if Snail2 directly regulates *Tspan18* in cranial neural crest cells, we electroporated 5 to 6 somite embryos with either ContMO or Snail2MO, excised MO-targeted neural folds after 30 min, and quantified *Cad6B* (as a positive control) and *Tspan18* mRNA levels by QPCR. As expected, *Cad6B* mRNA levels were significantly increased in Snail2MO-electroporated embryos as compared to ContMO-electroporated embryos (Fig. 2F; approximately 2.5-fold increase, $p<0.05$, as previously described; (Taneyhill et al., 2007)), suggesting that Snail2MO was effective and efficiently reduced Snail2 protein levels. In contrast, *Tspan18* mRNA levels were similar in both Snail2MO- and ContMO-electroporated embryos (Fig. 2F). Thus, Snail2 is not one of the additional transcription factors that regulate *Tspan18* expression. The identity of these additional factors awaits further analysis.

2.3. FoxD3 minimally impacts cranial neural crest cell survival

Because FoxD3 is required for neural crest cell survival in other systems (Stewart et al., 2006; Wang et al., 2011), we next determined whether FoxD3 knockdown altered cell death in chick cranial neural crest cells. To visualize dying premigratory neural crest cells, we immunostained FoxD3MO-electroporated embryos for the activated, cleaved form of the apoptotic enzyme caspase-3 (casp3) and determined the frequency of casp3-positive cells out of the total number of cells in the dorsal neural tube. While we electroporated FoxD3MO at 1.0 mM previously (Fairchild and Gammill, 2013), cells electroporated with FoxD3MO at 1.0 mM were strongly and non-specifically apoptotic (C. Fairchild, unpublished). Electroporation of 0.5 mM FoxD3MO largely prevented this response, so that very few dying cells were apparent in the neural tube ventral to the *FoxD3* expression domain (Fig. 3A’,B’; both control- and FoxD3MO-electroporated embryos exhibited casp3-

positive cells in the lumen of the neural tube (arrowheads) that were not counted during quantification). In 0.5 mM FoxD3MO-electroporated embryos, the frequency of casp3-positive cells in the dorsal neural tube was significantly higher on the targeted side compared to the untargeted side (Fig. 3B,B',C; p=0.003). However, the absolute number of dying cells was small (averaging 2.7 ContMO-electroporated cells and 2.5 FoxD3MO-electroporated cells per dorsal neural tube), with ContMO- and FoxD3MO-targeted sides exhibiting a similar, low frequency of casp3-positive cells (roughly 1–6% of cells counted; Fig. 3C, compare green bars). As a result, the fold change of the targeted compared to untargeted side of the dorsal neural tube in ContMO- and FoxD3MO-electroporated embryos was not significantly different (Fig. 3C), suggesting that reduced cell survival is a minor factor in the FoxD3 knockdown phenotype. To ensure there was no reciprocal effect on proliferation, we immunostained for phospho-histone H3 (pH3) and found that loss of FoxD3 did not alter the frequency of proliferating cells (Fig. 3D–F). In summary, while FoxD3 knockdown does slightly increase the frequency of dying cells, consistent with results in zebrafish (Stewart et al., 2006; Wang et al., 2011), it does not affect the overall fold-change in cell death. Moreover, loss of a small number of dying cells in the dorsal neural tube cannot explain the maintenance of *Tspan18* mRNA expression throughout the neural fold (Fig. 2). Thus, we conclude that FoxD3 has a nominal role in chick cranial neural crest cell survival that is independent of its role in regulating *Tspan18* mRNA expression.

2.4. FoxD3 is required for premigratory *Sox10* expression and neural crest migration

FoxD3 knockdown leads to transiently sustained *Tspan18* mRNA expression (Fig. 2). Our previous report concluded that downregulation of Tspan18 is a requirement for neural crest cells to migrate (Fairchild and Gammill, 2013). To interpret the effects of FoxD3 knockdown in the context of early neural crest development, we electroporated stage HH4+ embryos with ContMO or FoxD3MO and visualized neural crest specification and migration by *in situ* hybridization for the key neural crest transcription factor and FoxD3-responsive gene, *Sox10* (Prasad et al., 2012). *Sox10* mRNA expression levels were unaffected at 6–7 somites in ContMO electroporated embryos (Fig. 4A,A"; arrow). However, *Sox10* mRNA expression was severely reduced on the targeted side of the neural tube in embryos electroporated with FoxD3MO (Fig. 4C,C",E; arrowhead), suggesting that cranial neural crest specification is inhibited by FoxD3 knockdown in chick embryos. This is consistent with analyses of mutant mouse and zebrafish embryos, which show that FoxD3 is required for early neural crest specification and precursor maintenance (Montero-Balaguer et al., 2006; Stewart et al., 2006; Teng et al., 2008; Wang et al., 2011; Hochgreb-Hagele and Bronner, 2013). Furthermore, the FoxD3-deficient *Sox10*-positive neural crest cells that did form failed to migrate. While ContMO-targeted and untargeted *Sox10*-positive neural crest cells migrated an equivalent distance away from the neural tube at 9–10 somites (Fig. 4B,B"; arrows), FoxD3MO-electroporated neural crest cell migration distance was severely reduced relative to the untargeted side in the majority of embryos (Fig. 4D,D",F; arrowhead). These results indicate that FoxD3 is required for chick cranial neural crest formation (specification and, to a minor extent, survival) as well as subsequent migration. However, as in previous studies of FoxD3, these results do not distinguish whether FoxD3 is required for neural crest migration, or whether migration defects are an indirect consequence of impaired neural crest specification.

2.5. FoxD3 regulates cranial neural crest migration through *Tspan18*

Given that expression of Tspan18 is incompatible with migration (Fairchild and Gammill, 2013), it is possible that cranial neural crest cells fail to migrate in FoxD3 knockdown embryos (Fig. 4) because they retain *Tspan18* expression (Fig. 2; (Fairchild and Gammill, 2013)). If this were true, we reasoned that knockdown of Tspan18 should rescue the FoxD3 loss-of-function migration phenotype. To investigate this possibility, we co-electroporated embryos with FoxD3MO plus either ContMO or TS18MO, processed the resulting embryos by *in situ* hybridization for *Sox10* and scored the distance *Sox10*-positive neural crest cells had migrated away from the neural tube. Similar to embryos electroporated with FoxD3MO alone (Fig. 4D), in embryos co-electroporated with FoxD3MO + ContMO, neural crest migration distance was severely reduced on the targeted side of the neural tube in 7/9 (Fig. 5A,F) and moderately reduced in 2/9 (Fig.5D,F). Strikingly, in embryos co-electroporated with FoxD3MO + TS18MO, only 2/10 exhibited severe migration defects (Fig.5B,F), 3/10 were moderately reduced (Fig. 5C,F), and neural crest migration in 4/10 embryos was only mildly affected (Fig. 5E,F). Fluorescence imaging verified that rescued migratory neural crest cells were FITC (MO)-positive (Fig. 5B',C',E' and data not shown). Thus, knockdown of Tspan18 partially rescues the FoxD3 loss-of-function migration phenotype. Given that Tspan18 antagonizes EMT by maintaining Cad6B protein (Fairchild and Gammill, 2013), this suggests that FoxD3 promotes cranial neural crest EMT and thus migration by eliciting *Tspan18* downregulation.

While scoring the rescued embryos, we noted that, although co-electroporation of TS18MO with FoxD3MO rescued migration, the migratory population was smaller (see for example Fig. 5E", arrow). Indeed, counting *Sox10*-positive cells adjacent to the FoxD3MO + TS18MO targeted and untargeted sides of the neural tube showed that there were 2.3-fold fewer migratory neural crest cells on the targeted side (Fig. 5G). One possible explanation for this could be that Tspan18 knockdown does not rescue FoxD3-dependent specification defects. To test this hypothesis, we once again co-electroporated embryos with FoxD3MO and either ContMO or TS18MO, but instead harvested embryos at 4–7 somites and assayed *Sox10* and *Snail2* expression by in situ hybridization. Compared to the untargeted side, *Sox10* (Fig. 6A,E) and *Snail2* (Fig. 6C, F) expression were severely reduced on the FoxD3MO + ContMO targeted side of the neural tube. Importantly, *Sox10* (Fig. 6B,E) and *Snail2* (Fig. 6D,F) expression were similarly affected by co-electroporation of FoxD3MO + TS18MO. Therefore, Tspan18 knockdown does not rescue FoxD3-dependent neural crest specification. Thus, when incubated to migration stages, neural crest cells deficient for FoxD3 and Tspan18 are able to undergo EMT and leave the neural tube, but fewer are present to do so. In sum, these results reveal that FoxD3 regulates cranial neural crest migration through its effects on *Tspan18* expression. Moreover, the failure of Tspan18 knockdown to rescue FoxD3-dependent cranial neural crest gene expression indicates that FoxD3 separably regulates cranial neural crest specification and migration through Tspan18-independent and -dependent pathways (respectively).

3. Discussion

In this study, we have investigated the regulatory relationship between FoxD3 and Tspan18 and, in so doing, gained insight into the poorly understood role of FoxD3 in neural crest migration. From our FoxD3 knockdown experiments, we conclude that FoxD3 supports chick cranial neural crest specification and migration, minimally affects neural crest cell survival, and specifically report that FoxD3 is required for initial downregulation of *Tspan18* mRNA expression. In fact, FoxD3 regulates neural crest migration through its effects on *Tspan18*, as FoxD3-dependent migration defects can be rescued by simultaneous knockdown of Tspan18, although neural crest specification is still aberrant. Together these data reveal separable requirements for FoxD3 during cranial neural crest specification and migration, and show that FoxD3 promotes EMT and migration through negative regulation of *Tspan18* expression.

3.1. FoxD3 is required for initial *Tspan18* downregulation, though other factors contribute at later stages

Tspan18 expression (overexpression or persistent endogenous transcripts) is incompatible with neural crest migration (Fairchild and Gammill, 2013), and as such, *Tspan18* mRNA is absent from cranial neural crest cells later than 7 somites (Fig. 1). In embryos electroporated with FoxD3MO, *Tspan18* transcripts are still visible at 9 somites (Fig. 2C), indicating that FoxD3 is required for the initial downregulation of *Tspan18*. Although the *Tspan18* promoter contains FoxD3 consensus binding sites, we have, to date, been unable to determine whether FoxD3 directly binds and represses *Tspan18* transcription. Experiments to address this crucial question will be the subject of future investigation.

Our results highlight the complexity of *Tspan18* transcriptional regulation. Notably, even in embryos that have been electroporated with FoxD3MO, *Tspan18* mRNA is eventually downregulated (Fig. 2D). This finding suggests that FoxD3 is not the only factor that negatively regulates *Tspan18* mRNA expression. The identity of these additional factors is unclear, though Snail2 does not appear to contribute (Fig. 2F). Regulatory complexity is further indicated by prolonged co-expression of *FoxD3* and *Tspan18* in premigratory neural crest cells (Fig. 1). This delayed action of a neural crest transcription factor is not unprecedented: Snail2, which represses *Cad6B* transcription to promote EMT, is co-expressed with Cad6B throughout premigratory neural crest development and modulated through altered rates of degradation (Vernon and LaBonne, 2006; Taneyhill et al., 2007). FoxD3 activity could also undergo differential post-translational modifications, as does Sox10 (Taylor and Labonne, 2005). Alternatively, FoxD3 could require and/or affect the activity of a co-factor, for which there is also precedent. In the trunk neural crest, FoxD3 regulates the expression of the melanocyte marker *MITF*, but not through direct binding to the *MITF* promoter; instead FoxD3 binds to the transcriptional activator Pax3 and prevents Pax3 from binding to the *MITF* promoter (Thomas and Erickson, 2009). Identifying FoxD3-interactors would provide a better understanding of its regulation of *Tspan18* mRNA expression.

3.2 FoxD3 promotes *Tspan18* downregulation to drive cranial neural crest EMT independent of its role in specification

The ability of Tspan18 to rescue FoxD3-dependent migration (Fig. 5) allowed us to separate the requirement for FoxD3 during neural crest formation and migration. Consistent with previous studies in *Xenopus*, zebrafish and mouse embryos (Sasai et al., 2001; Lister et al., 2006; Montero-Balaguer et al., 2006; Stewart et al., 2006; Mundell and Labosky, 2011; Hochgreb-Hagele and Bronner, 2013), FoxD3 knockdown in chick embryos inhibited neural crest specification (Fig. 4C). While FoxD3 is also necessary for neural crest migration (Fig. 4D; (Stewart et al., 2006)), it had been unclear whether FoxD3 regulates neural crest migration directly, or affects migration as a secondary consequence of specification and survival defects. Crucially, co-electroporation of TS18MO with FoxD3MO rescues the ability of neural crest cells to migrate (Fig. 5B,C,E,F), but not the essential role of FoxD3 during neural crest specification (Fig. 6B,D-F). In other words, fewer neural crest cells form when FoxD3 is deficient (Fig. 5G), but those that arise will emigrate as long as Tspan18 is knocked down. This indicates that FoxD3 regulates neural crest EMT/migration through downregulation of *Tspan18* and provides the first evidence that FoxD3 plays independent roles in neural crest specification and migration (Fig. 7). FoxD3 knockdown only nominally affected survival in chick embryos (Fig.3A–C), and because TS18MO does not affect cell death or proliferation (Fairchild and Gammill, 2013), FoxD3/Tspan18-dependent migration defects are likely to independent of cell death as well.

As FoxD3 has been reported to both positively and negatively regulate neural crest migration, we propose that FoxD3 promotes EMT but is incompatible with motility. FoxD3 is required for neural crest migration (Fig. 4,6; (Stewart et al., 2006)). However, expanded *FoxD3* expression in zebrafish cranial neural crest cells prevents migration (Drerup et al., 2009), and FoxD3 negatively regulates Rho-GTPase-induced cell motility in human metastatic melanoma cells (Katiyar and Aplin, 2011). These contrary observations likely reflect temporal differences. FoxD3 knockdown sustains *Tspan18* expression (Fig. 2), which in turn maintains Cad6B protein levels that inhibit neural crest EMT (Coles et al., 2007; Fairchild and Gammill, 2013). Thus, at the start of neural crest migration, FoxD3 elicits *Tspan18* downregulation, which destabilizes Cad6B-dependent cell adhesions and promotes neural crest EMT (Fig. 7). Later, in actively migrating neural crest cells, FoxD3 may disrupt Rho-based motility, as has been reported for melanoma cells (Katiyar and Aplin, 2011). This could explain why *FoxD3* expression declines after neural crest cells leave the neural tube (Fig. 1; (Khudyakov and Bronner-Fraser, 2009)).

While FoxD3 regulates cranial neural crest EMT through Tspan18, it must act through a different pathway in the trunk. Although *Tspan18* is not detectable by in situ hybridization in trunk neural tube (Fairchild and Gammill, 2013), FoxD3 overexpression leads to altered expression of cell adhesion molecules (Cheung et al., 2005). Thus, FoxD3 must regulate the expression of other gene(s) that impact EMT. To understand the role of FoxD3 in neural crest migration, and specifically in EMT, it will be crucial to identify FoxD3 targets in the neural crest.

In conclusion, this report shows that FoxD3 is required for chick cranial neural crest migration because it promotes the downregulation of *Tspan18* and thus EMT. Moreover, this pathway is independent of the requirement for FoxD3 during cranial neural crest specification (Fig. 7). Overall this study provides new insight into the dual role of FoxD3 for neural crest formation and migration, and also gives us a better understanding for how neural crest transcription factors impact the cellular behaviors that occur as neural crest cells undergo EMT and migrate.

4. Experimental Procedures

4.1. Embryos

Fertile chicken embryos were incubated in a humidified incubator (G. Q. F. Manufacturing: Savannah, GA) at 37–38°C. Embryos were staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951) or by counting somite pairs.

4.2. In situ hybridization

Whole mount in situ hybridization was performed as previously described (Wilkinson, 1992). Digoxigenin-labeled RNA probes were transcribed from the following templates: *FoxD3* (Kos et al., 2001), *Tspan18* (Gammill and Bronner-Fraser, 2002), and *Sox10* (Cheng et al., 2000). For double whole mount in situ hybridization, RNA probes labeled with digoxygenin (*Tspan18*) and FITC (*FoxD3*) were mixed during hybridization. After developing the digoxygenin probe, alkaline phosphatase was inactivated by incubating 1 hour in 4% paraformaldehyde + 0.1% glutaraldehyde and 20 minutes at 63°C in maleic acid buffer + 0.1% Tween and 10mM EDTA. Orange precipitate was generated by incubating in 87.5 μg/ml iodonitrotetrazolium chloride (Sigma Aldrich; St Louis, MO) plus 87.5 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate (Roche Applied Science; Indianapolis, IN). After processing, embryos were first imaged in whole mount using a Zeiss Discovery V8 stereoscope then infiltrated with 5% and 15% sucrose, embedded in gelatin, sectioned using a Leica CM1900 cryostat at 12–18 μm and imaged again using a Zeiss AxioImager A1. Images of whole mount embryos and transverse sections were taken with an AxioCam MRc5 digital camera and Axiovision software and assembled in Photoshop (Adobe).

4.3. Morpholino design and electroporation

The following FITC-tagged, antisense morpholinos (MO) were designed and synthesized by GeneTools, LLC (Philomath, OR): FoxD3 translation blocking MO (FoxD3MO: 5'-CGCTGCCGCCGCCCGATAGAGTCAT-3'; (Kos et al., 2001)), Tspan18 translation blocking MO (TS18MO: 5'-TGCAGCTCAGACAGTCTCCCTCCAT-3'; (Fairchild and Gammill, 2013)), and standard FITC control MO (ContMO: 5'-CCTCTTACCTCAGTTACAATTTATA-3'). For early embryo electroporations, morpholinos were unilaterally electroporated into the presumptive neural crest at HH stage 4^{+}, as previously described (Gammill and Krull, 2011), at 500 μM (FoxD3 and ContMO) and 390 μM (TS18MO) with carrier DNA (0.2 μg/μL pCS2+MycTag DNA). After electroporation, embryos were re-incubated until the desired stages and fixed in 4% paraformaldehyde at room temperature for 1 hour then washed with PBS + 0.1% Tween. Targeting was verified by fluorescent microscopy for FITC before embryos were either

immediately embedded and sectioned for immunofluorescence or dehydrated into methanol and stored at −20° for in situ hybridization.

4.4. Quantitative polymerase chain reaction (QPCR)

MO-mediated Snail2 depletion followed by QPCR was carried out as described (Taneyhill et al., 2007). Briefly, 6 somite embryos were electroporated in ovo with Snail2MO or ContMO and midbrain neural folds were excised after 30 minutes. Neural folds from six electroporated embryos were pooled and total RNA was isolated using the RNAqueous Total RNA Isolation Kit (Ambion-Life Technologies; Carlsbad, CA). cDNA was synthesized using random hexamers and the Superscript II RT-PCR system (Life technologies) according to the manufacturer's instructions. QPCR was performed using the ABI 7000 in a TaqMan or SYBR Green (Life Technologies) assay as described (Taneyhill and Bronner-Fraser, 2005). Briefly, 25 μl *Tspan18* QPCR reactions included 2X SYBR Green master mix, cDNA and 75 nM of each primer (Sense: 5´-GCTTGTTGCCAGCGAAAGCTCC-3´; Antisense: 5´-TAGCAGCCCTGCCGGTTCTGA-3´). 18S QPCR reactions included 2x SYBR Green mastermix, cDNA and 150 nM each primer as previously described (Taneyhill and Bronner-Fraser, 2005). *Cad6B* QPCR reactions included TaqMan mastermix, cDNA, 150 nM of each primer, and 150 nM each Cad6B TaqMan probe, as described (Taneyhill et al., 2007). After normalization to a standard (chick 18S rRNA), fold upregulation or downregulation for each of three replicates was determined by dividing the relative expression value for the gene of interest in the presence of the Snail2MO by that obtained for the gene of interest in the presence of ContMO.

4.5. Immunofluorescence and cell death/proliferation

Immunofluorescence was performed using either anti-cleaved caspase3 (casp3, Cell Signaling; Danvers, MD: 1:200) or anti-phosho-histone H3 (pH3, Millipore; Billerica, MA: 1:250) primary antibody diluted into PBS + 0.1% Triton-X100 supplemented with 5% normal donkey serum followed by incubation with a donkey anti-rabbit secondary antibody at 1:250 (RRX conjugated; Jackson Labs; West Grove, PA). Slides were mounted in PermaFluor (Thermo Fisher Scientific; Waltham, MA) containing 1 μg/mL DAPI and imaged on a Zeiss LSM 710 laser scanning confocal microscope. Images were assembled in Photoshop (Adobe). Cell death and proliferation were quantified as follows: casp3- or pH3-positive cells and DAPI-positive cells were counted in the dorsal quarter of the neural tube on the targeted and untargeted sides in at least 5 transverse sections per embryo (n=3 embryos). Frequency was determined by dividing the number of casp3- or pH3-positive cells by the total number of DAPI-positive cells, and fold change determined by dividing by the frequency on the targeted by the untargeted side. Statistics were performed using the paired Student's *t* test in Excel (Microsoft).

4.6. Assaying neural crest migration

To consistently assay neural crest cell migration (Fig. 4) and thus clearly determine rescue (Fig. 5), the farthest distance *Sox10*-positive cells had migrated from the midline in whole mount was measured in Photoshop (Adobe) on the untargeted and targeted sides of the

neural tube. The ratio of targeted distance / untargeted distance was then used to categorize phenotypic severity. In severely affected embryos, neural crest cells on the targeted side had migrated 0–50 percent the distance of those on the untargeted side. In moderately affected embryos, neural crest cells on the targeted side had migrated 51–75 percent of the distance of those on the untargeted side. In mildly affected embryos, neural crest cells on the targeted side had migrated 76–95 percent of the distance of those on the untargeted side. Unaffected embryos displayed neural crest migration distances within 5 percent of each other on the targeted and untargeted sides of the neural tube. To quantify effects on migratory cell number, *Sox10* and DAPI images of 5 transverse sections from each of 4 embryos (20 sections total) were superimposed in Photoshop (Adobe). For each image, an outline was drawn around all *Sox10* staining outside of the neural tube, and DAPI-positive nuclei within this outline were counted. Statistics were performed using the paired Student's *t* test in Excel (Microsoft).

Acknowledgments

We thank Yi-Chuan Cheng and Carol Erickson for the kind gift of plasmids. Many thanks to the members of the Gammill Lab for their input and support. We are grateful for the resources of the University of Minnesota Supercomputing Institute. This work was supported by the National Institutes of Health, award numbers F31 NRSA GM087951 (CLF), R00 HD055034 (LAT), and K22 DE015309 (LSG), as well as a University of Minnesota Grant-in-Aid.

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Highlights

- Factors in addition to FoxD3, but not Snail2, contribute to *Tspan18* downregulation.
- Chick cranial neural crest specification and migration require FoxD3.
- FoxD3 promotes cranial neural crest migration by downregulating *Tspan18*.
- During neural crest specification, the role of FoxD3 is Tspan18-independent.
- FoxD3 separably regulates neural crest formation and migration.

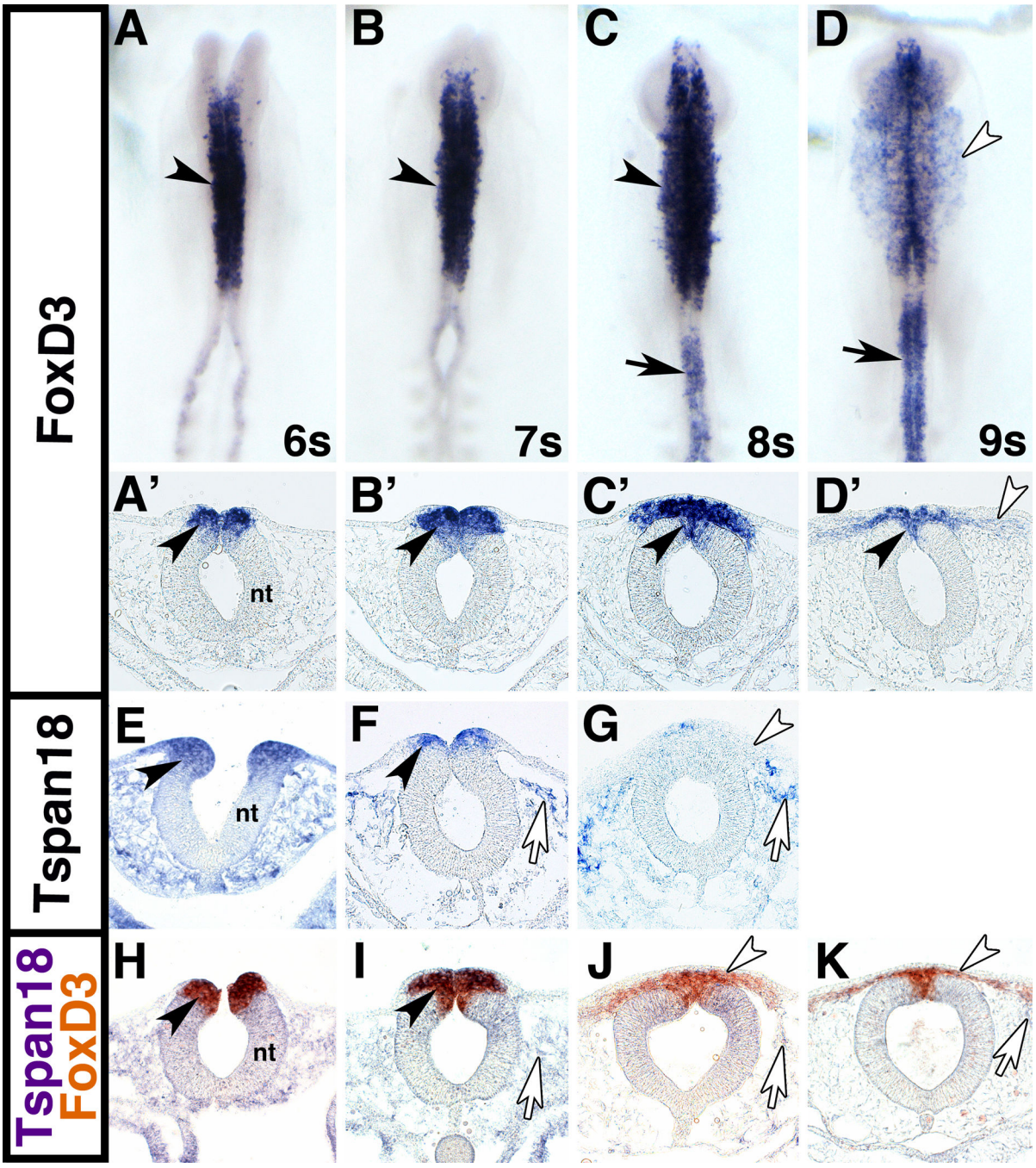

Fig. 1. *FoxD3* and *Tspan18* are co-expressed in premigratory cranial neural crest cells

Whole mount and transverse sections of chick embryos processed by *in situ* hybridization for *FoxD3* (A-D), *Tspan18* (E-G), or *FoxD3* and *Tspan18* (H-K) at 6 somites (s; A,A',E, H), 7s (B,B',F, I), 8s (C,C',G, J) and 9s (D,D', K). (A,B) *FoxD3* (purple) is expressed in premigratory cranial neural crest cells at 6 and 7s (black arrowheads). (C) At 8s, FoxD3 expression persists in early emigrating neural crest cells (black arrowheads) and extends into the trunk (black arrow). (D) At 9s, *FoxD3* expression is reduced in migrating cranial neural crest cells (white arrowhead), but retained in the dorsal neural tube (black arrowhead in D') and in the trunk (arrow). (E-G) *Tspan18* (purple) is expressed in premigratory cranial neural crest cells at 6 and 7s (black arrowheads), when its expression begins to downregulate. At 8s, *Tspan18* is absent from migratory neural crest cells (white

arrowhead). *Tspan18* is expressed in scattered cells in the head mesenchyme (white arrows). (H-K) *FoxD3* (orange) and *Tspan18* (purple) expression domains overlap in the neural folds at 6s and 7s (black arrowheads). At 8s and 9s, migratory neural crest cells express *FoxD3* but not *Tspan18* (white arrowheads); *Tspan18* expression is limited to head mesenchyme (white arrows). A-D, dorsal view; A'-D', E-K, transverse sections. nt, neural tube.

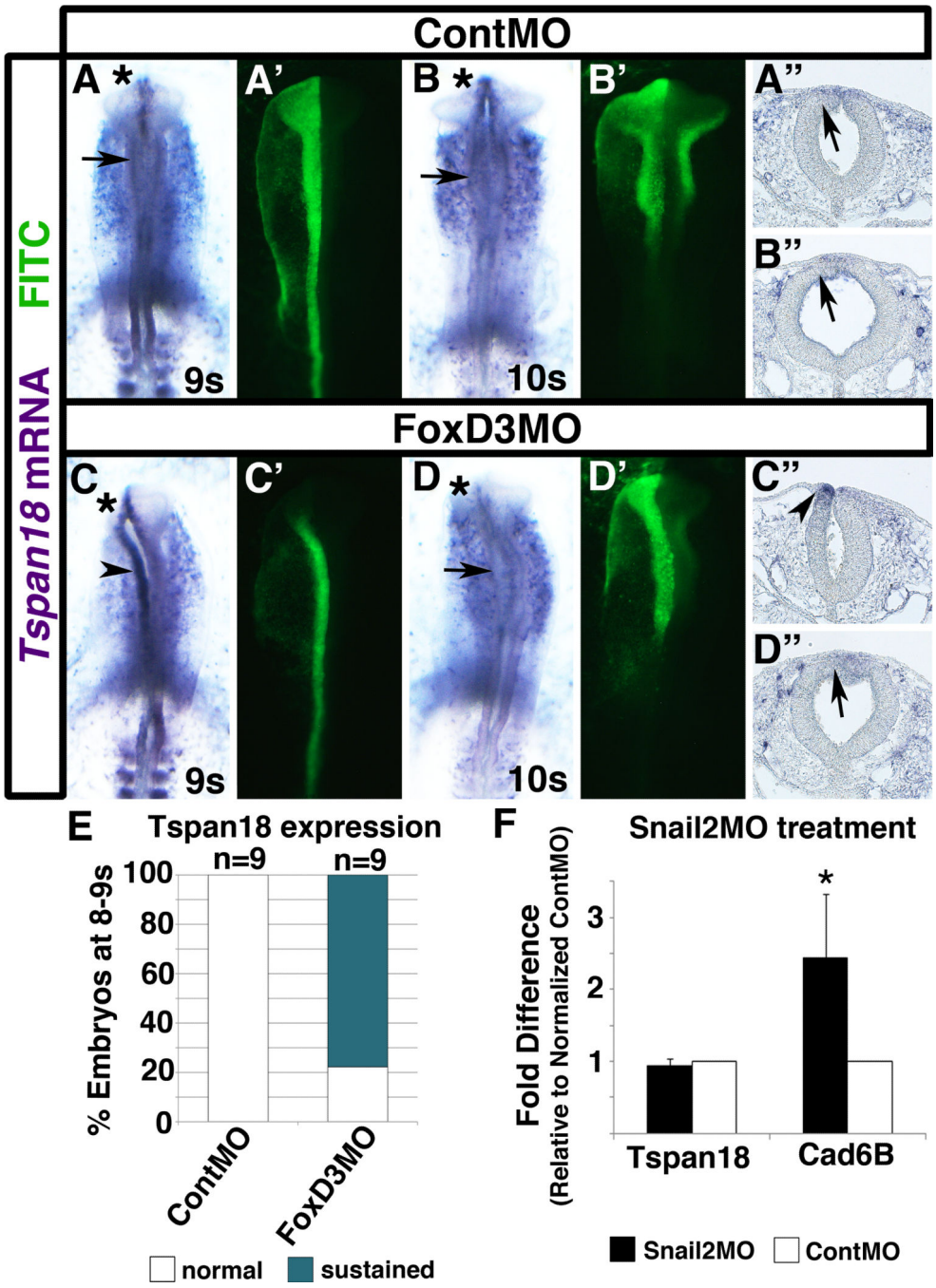

Fig. 2. *Tspan18* downregulation is initially delayed in the absence of FoxD3

Whole mount images (A-D, A'-D') and transverse sections (A"-D") of embryos unilaterally electroporated with ContMO (A,B) or FoxD3MO (C,D) at late gastrula and processed by *in situ* hybridization for *Tspan18* after harvest. At 9 somites (s; A) and 10s (B), *Tpsan18* mRNA is absent (arrows) on the targeted (asterisks; green in A',B') and untargeted sides of the dorsal neural tube in representative ContMO-electroporated embryos (A,B) and transverse sections (A",B"). At 9s, *Tspan18* expression is retained (arrowhead) on the FoxD3MO-targeted side of the neural tube (asterisk; C', green) in embryos (C) and transverse sections (C"). At 10s, *Tspan18* mRNA expression is downregulated (arrow), even on the FoxD3MO-targeted side (asterisk; F', green) of the neural tube in embryos (D) and transverse sections (D"). (E) Bar graph representing the frequency of 8–9s embryos exhibiting

sustained *Tspan18* expression. (F) Quantification of *Tspan18* mRNA levels following MO-mediated depletion of Snail2. *Cad6B* levels were analyzed as a positive control (Taneyhill et al., 2007). Results are reported as fold difference relative to that obtained with ContMO normalized to 1. Means and standard errors of fold differences were generated from 3 independent experimental cDNA replicates. The asterisk (*) located above the error bar denotes a significant difference in the fold change between Snail2MO and ContMO levels ($p < 0.05$). Black bars, Snail2 MO; white bars, ContMO.

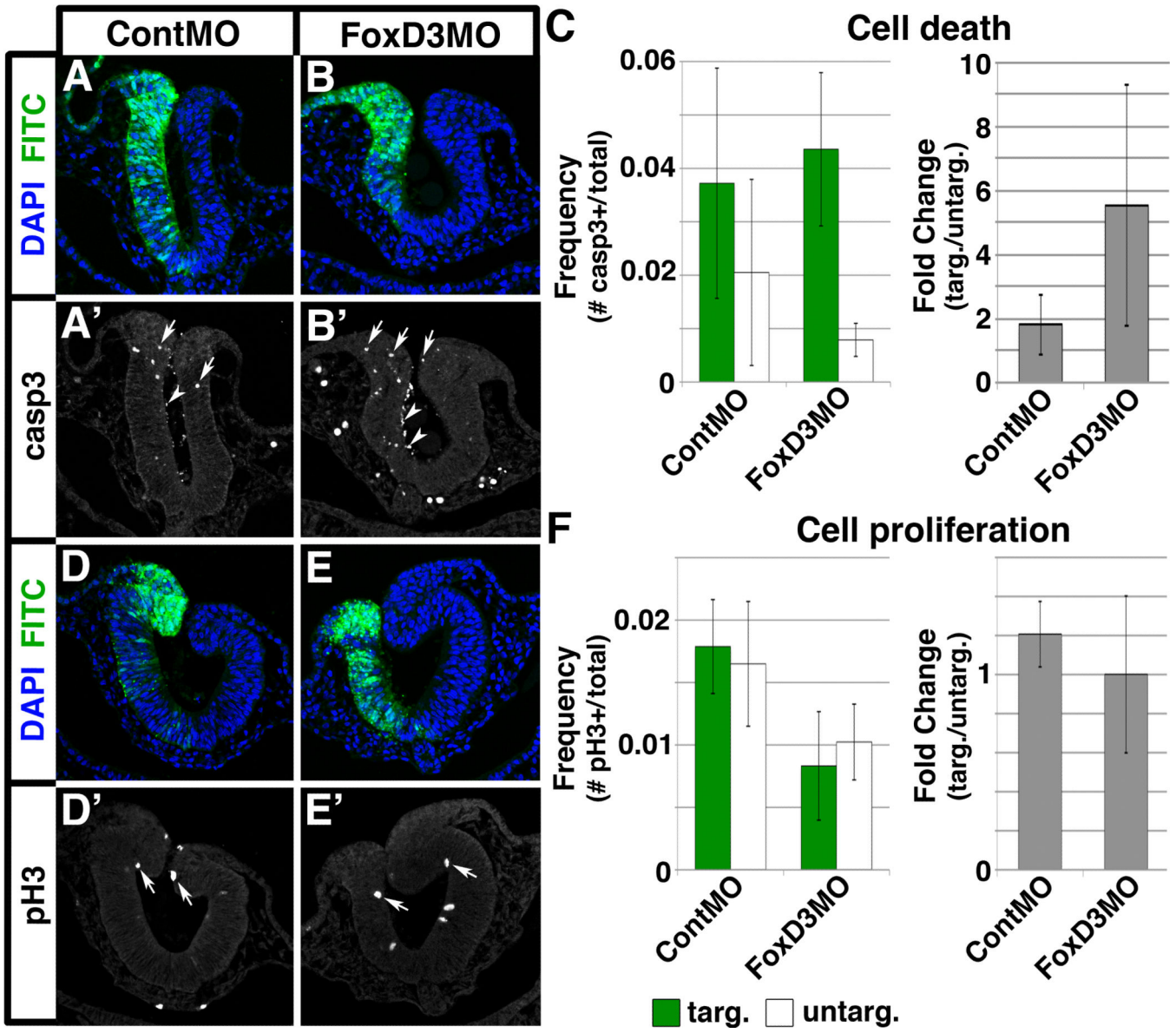

Fig. 3. FoxD3 knockdown has minimal effects on cell survival and does not alter cell proliferation

Quantification of cell death (A-C) and proliferation (D-F) in embryos unilaterally electroporated with ContMO (A,D) or FoxD3MO (B,E). (A,B) Immunostaining for cleaved caspase-3 (casp3) reveals more dying cells (arrows) on the targeted side of the dorsal neural tube (green) in FoxD3MO-electroporated embryos (B) as compared to the targeted side of control embryos (A). Casp3-positive cells were present in the lumen of the neural tube (arrowheads) under both conditions and were thus not counted during quantification. (C) Bar graphs representing the frequency and fold change of dying cells in electroporated embryos. (D,E) Immunostaining for phospho-histone H3 (pH3) reveals that proliferating cells are equally apparent (arrows) on the targeted (green) and untargeted sides of the neural tube in embryos electroporated with either ContMO (D) or FoxD3MO (E). (F) Bar graphs representing the frequency and fold change of proliferating cells in electroporated embryos.

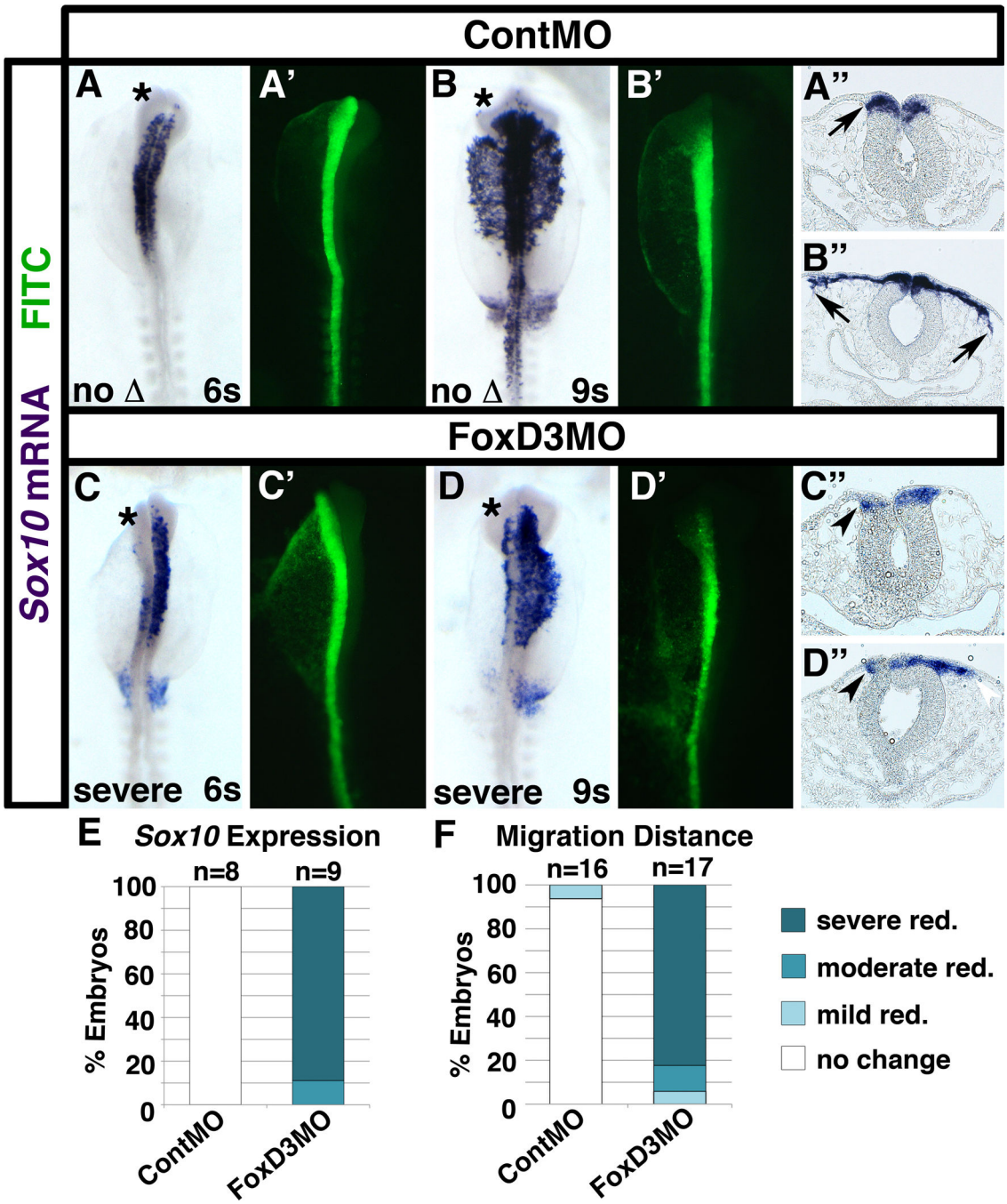

Fig. 4. Chick FoxD3 is required for premigratory *Sox10* expression and neural crest migration

Whole mount images (A-D, A'-D') and transverse sections (A"-D") of embryos unilaterally electroporated with ContMO (A,B) or FoxD3MO (C,D) at late gastrula, incubated to 6 somites (s; A,C) or 9s (B,D), and processed by *in situ* hybridization for *Sox10*. In ContMO-electroporated embryos, *Sox10* expression (A,A") and the distance that *Sox10*-positive neural crest cells migrated (B,B") was even on targeted (asterisks; A',B', green) and untargeted sides of the neural tube. In contrast, *Sox10* expression was impaired (C,C") and migration distance was reduced (D,D") on the targeted side (asterisks; C',D', green) compared to the untargeted side of the neural tube of FoxD3MO-electroporated embryos. (E,F) Bar graphs representing the frequency of electroporated embryos exhibiting reduced premigratory *Sox10* expression (E) or reduced migration distance (F).

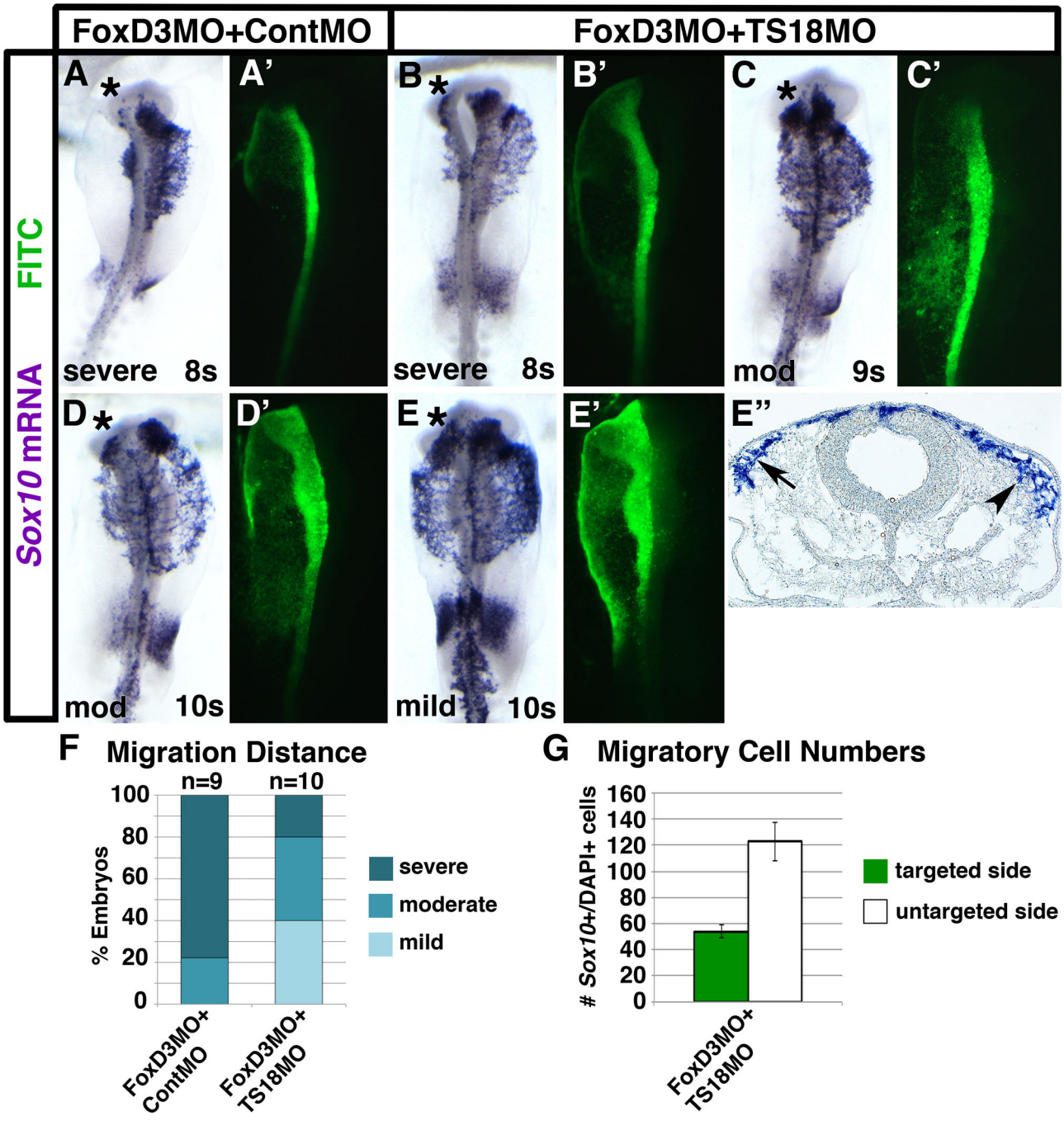

Fig. 5. Tspan18 knock down rescues FoxD3-dependent migration defects

Whole mount images and transverse sections of embryos unilaterally co-electroporated with FoxD3MO and either ContMO (A,D) or TS18MO (B,C,E) at late gastrula, incubated to 8–10 somites (s), and processed by *in situ* hybridization for *Sox10*. (A-E) Following co-electroporation with FoxD3MO and ContMO, the distance of *Sox10*-positive cells migrated on the targeted side of the neural tube (asterisks; green in A'-E') is severely inhibited (A) in nearly all embryos with only a few moderately affected (D). Following co-electroporation with FoxD3 and TS18MO, the incidence of severely affected (B) embryos is greatly reduced and most embryos are moderately (mod; C) or only mildly affected (E). (E") A transverse section of the mildly affected embryo in (E) reveals that while co-electroporation with FoxD3MO and TS18MO rescues the emigration of migratory neural crest cells,

the size of the *Sox10*-positive migratory neural crest population is reduced (arrow) on the targeted side of the embryo, as compared to the untargeted side of the embryo (arrowhead). (F) Bar graph representing the frequency of electroporated embryos that exhibit a severe, moderate, or mild reduction in migration distance. (G) Bar graph showing the reduced number of *Sox10*-positive, DAPI-positive cells adjacent to the neural tube on the targeted (green) versus untargeted (white) side of embryos electroporated with FoxD3MO and TS18MO.

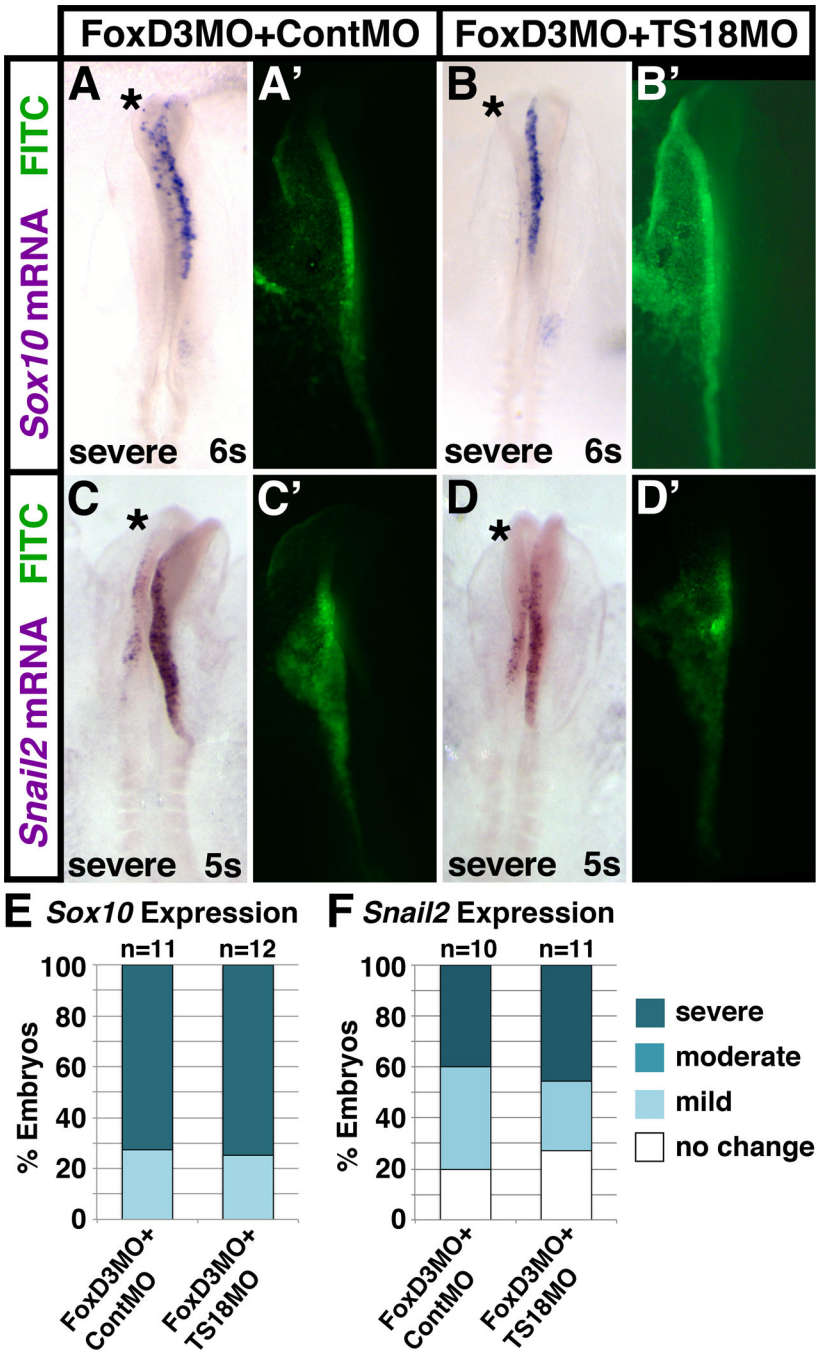

Fig. 6. FoxD3 is required for neural crest specification independent of Tspan18

Whole mount images of embryos unilaterally co-electroporated with FoxD3MO and either ContMO (A,C) or TS18MO (B,D) at late gastrula, incubated to 5–6 somites (s), and processed by *in situ* hybridization for *Sox10* (A,B) or *Snail2* (C,D). (A-D) Co-electroporation of FoxD3MO with either ContMO or TS18MO disrupts *Sox10* (A,B) and *Snail2* (C,D) expression on the targeted side of the neural tube (asterisks; green in A'-D'). (E,F) Bar graphs representing the frequency of electroporated embryos that exhibit a severe, moderate, mild, or no reduction in *Sox10* (E) or *Snail2* (F) expression, indicating that Tspan18 knockdown has no impact on the severity of FoxD3-dependent specification defects.

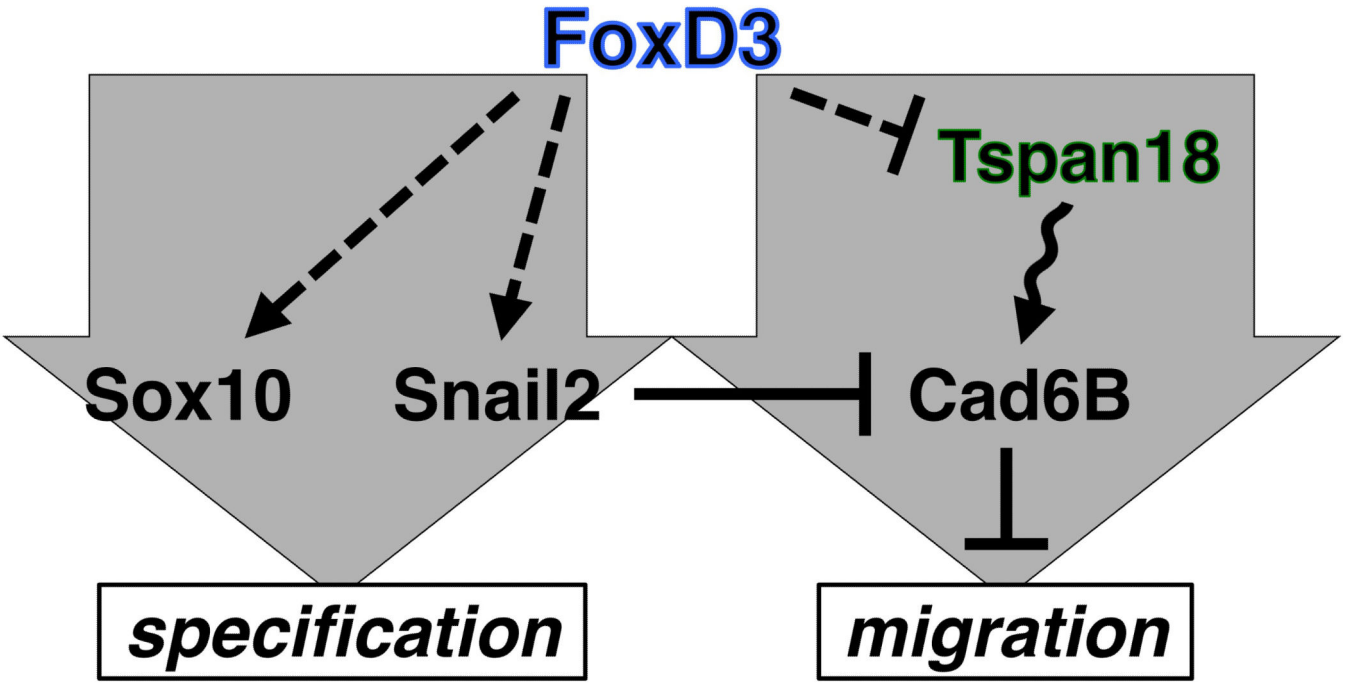

Fig. 7. FoxD3 independently regulates neural crest specification and migration

FoxD3 is required for *Sox10* and *Snail2* expression (Figs. 4, 6) in the gene regulatory network that drives neural crest specification (Betancur et al., 2010; Prasad et al., 2012). In a separate pathway (grey arrow), FoxD3 promotes neural crest migration through its effects on Tspan18 (Fig. 5), repressing *Tspan18* expression (Fig. 2) and relieving Tspan18-dependent maintenance of Cad6B (Fairchild and Gammill, 2013) that prevents EMT (Coles et al., 2007). Snail2 likewise promotes EMT by directly repressing Cad6B (Taneyhill et al., 2007) but does not affect Tspan18 expression (Fig. 2). Grey arrows, pathways; arrowhead, promotes expression; bar, represses expression; solid line, direct binding to target promoter demonstrated; dashed line, regulation not necessarily direct; wavy line, post-translational effect; box, developmental outcome.