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Pax3 synergizes with Gli2 and Zic1 in transactivating the *Myf5* epaxial somite enhancer

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Abstract

Both Glis, the downstream effectors of hedgehog signaling, and Zic transcription factors are required for *Myf5* expression in the epaxial somite. Here we demonstrate a novel synergistic interaction between members of both families and Pax3, a paired-domain transcription factor that is essential for both myogenesis and neural crest development. We show that Pax3 synergizes with both Gli2 and Zic1 in transactivating the *Myf5* epaxial somite (ES) enhancer in concert with the *Myf5* promoter. This synergy is dependent on conserved functional domains of the proteins, as well as on a novel homeodomain motif in the *Myf5* promoter and the essential Gli motif in the ES enhancer. Importantly, overexpression of Zic1 and Pax3 in the 10T1/2 mesodermal cell model results in enrichment of these factors at the endogenous *Myf5* locus and induction of *Myf5* expression. In our previous work, we showed that by enhancing nuclear translocation of Gli factors, Zics provide spatiotemporal patterning for Gli family members in the epaxial induction of *Myf5* expression. Our current study indicates a complementary mechanism in which association with DNA-bound Pax3 strengthens the ability of both Zic1 and Gli2 to transactivate *Myf5* in the epaxial somite.

Keywords

Myf5; myogenesis; Pax; Zic; Gli; skeletal muscle

Introduction

Development of skeletal muscle is controlled by the myogenic regulatory factors (MRFs): Myf5, MyoD, Myogenin, and MRF4. These transcription factors are essential for the determination and differentiation of skeletal muscle during embryogenesis (18, 45), and display the unique ability to convert non-muscle cell types to skeletal muscle (58). *Myf5* is the first MRF to be expressed, at E8 in the mouse (42). Transcripts are first detectable in the dermomyotome of the earliest somites, then in the newly formed myotome, followed by expression in the ventral dermomyotome and branchial arches (42). Following migration of

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muscle precursor cells from the somites to the limb buds, *Myf5* is also transiently activated in the developing limbs (42, 54). In the adult, *Myf5* expression is downregulated and only maintained in muscle satellite cells and spindles (61). Targeted disruption of *Myf5*, *MyoD*, and *MRF4* in the mouse results in a complete absence of myoblasts, underscoring the importance of these factors for myogenic commitment (9, 29, 47).

Myf5 and *Mrf4* are linked, and the shared locus is subject to complex transcriptional regulation (18). Manipulation of the locus in transgenic reporter mice has uncovered a number of discrete enhancers that direct expression of each gene in specific progenitor cell populations in the embryo (12). One of the best characterized enhancers in the locus is the *Myf5* epaxial somite (ES) enhancer. This region lies ~6.6 kb upstream of the *Myf5* transcription start site and controls the expression of *Myf5* in the epaxial muscle progenitors of the dorsal somite (53, 55). Interestingly, while the *Myf5* ES enhancer activates its own promoter, it cannot engage productively with the closer *Mrf4* promoter or with several cryptic promoters in the locus (12, 56), suggesting that expression of *Myf5* in the epaxial somite requires specific interactions with its homologous promoter.

Several signaling pathways and their downstream effectors have been implicated in activity of the *Myf5* ES enhancer. Correct expression of *Myf5* in the epaxial somite requires Sonic hedgehog (Shh) signaling via a conserved Gli motif, Wnt signaling through multiple TCF/LEF motifs, and Dmrt2 motifs in the ES enhancer (5, 21, 51, 56). Much less is known regarding control of the *Myf5* promoter, but FoxD3 binding to a conserved motif in the *Myf5* promoter of zebrafish is required for maintenance of *Myf5* expression in the somites (32).

Glis and Zics are closely related zinc-finger transcription factors, shown to have antagonistic effects in neural patterning (10), and cooperative effects in skeletal patterning and myogenesis (2, 43). Mutations in both families result in a range of developmental abnormalities (25, 28), and members of both families are important for *Myf5* expression in the epaxial somite (6, 35, 43). Expression of *Pax3*, a paired-domain transcription factor that is essential for both myogenesis and neural crest development, also overlaps with that of *Myf5* in myogenic progenitors in the dermomyotome and limb buds. Pax3 has been shown to activate several *Myf5* enhancers, both directly (3, 14) and indirectly (51), and mutations in Pax3 lead to Waardenburg syndrome types I and III, diseases characterized by defects in muscle and neural crest derivatives (24).

Given the overlapping roles of Gli, Zic, and Pax transcription factors in somite myogenesis, we asked whether these factors are capable of synergizing in activating the *Myf5* ES enhancer and homologous promoter. Here we demonstrate novel synergistic interactions between Gli2 and Pax3, and Zic1 and Pax3. This synergy is dependent on conserved functional domains of the proteins, as well as on a novel homeodomain motif in the *Myf5* promoter and the essential Gli motif in the ES enhancer. Importantly, overexpression of Zic1 and Pax3 in the 10T1/2 mesodermal cell model results in the enrichment of these factors at the endogenous *Myf5* locus and induction of *Myf5* expression. Unlike Gli2 and Pax3, Zic1 is expressed exclusively in epaxial muscle progenitors within the dermomyotome. In our previous work, we showed that Zics provide spatiotemporal patterning for Gli family members in the induction of *Myf5* expression (43). Here we show that in addition to enhancing the nuclear translocation of Gli factors (30, 43), Zic1 also associates with Pax3 on the *Myf5* promoter to drive *Myf5* expression. Likewise, the ability of Gli2 to transactivate *Myf5* is strengthened by a synergistic association with Pax3. Collectively, our data indicate novel interactions that link several well-established myogenic pathways.

Materials and Methods

Plasmids and antibodies

Mammalian expression plasmids Gli1 and Gli2 in pcDNA3.1-His, and Gli2 lacking the C-terminal domain (Gli2[ΔC2] and Gli2[ΔC4]) have been described previously (50). Zic1 and Zic2 in pCS2FLAG have also been described (43). Pax3-HA in pcDNA3 and Pax7-FLAG in pBRIT were purchased from Addgene (#27319 and #17521, respectively). Zic1 lacking the ZOC domain (Zic1[ΔZOC]) or zinc fingers (Zic1[ΔZF]), and Pax3 lacking the homeodomain (Pax3[ΔHD]) or transactivation domain (Pax3[ΔTD]) were made using standard site-directed mutagenesis on the plasmids described above. The *Myf5* ES enhancer, EpExt in (5), and *Myf5* promoter (56) were amplified from mouse genomic DNA and cloned into the firefly luciferase reporter vector pGL3-Basic (Promega) to generate E-P-luc. Mutations in the ES enhancer Gli motif (Gli-mt) (21) and *Myf5* promoter homeodomain motif (HD-mt) were made using standard site-directed mutagenesis of E-P-luc. $(Gli)_8$-TK-luc, containing eight wild-type Gli binding sites from the *Myf5* ES enhancer, has been described (21). Antibodies used in this study were: anti-HA monoclonal antibody (sc-7392, Santa Cruz Biotechnology, Inc.), anti-FLAG M2 monoclonal antibody (#F3165, SIGMA), anti-Gli2 polyclonal antibody (ab7195, abcam), anti-V5 monoclonal antibody (#R960-25, Invitrogen), and normal mouse IgG (sc-2025, Santa Cruz Biotechnology, Inc.).

Cell culture

NIH 3T3 mouse embryonic fibroblasts and C3H/10T1/2 mouse embryonic mesenchymal stem cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc.) + 10% fetal bovine serum (FBS) (Denville) and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). 10T1/2 cells were also supplemented with 10 mM HEPES.

Transient transfections and reporter assays

Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol, and harvested at 48 hours post6-transfection. For co-immunoprecipitation assays, gel-shift assays, RT-PCR, and ChIP assays, cells were transfected with expression constructs in 10 cm plates. For reporter assays, cells were reverse transfected with expression and reporter constructs in 96-well plates according to the manufacturer's protocol. To aid cell lysis, plates were frozen at −70° for 1 h, followed by 10 min at 25°, 15 min at 37°, and 10 min at 25°. Firefly and renilla luciferase activities were measured using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's protocol.

Co-immunoprecipitation and Western analysis

10T1/2 cells transfected with Pax3-HA and FLAG-Zic1 expression plasmids were harvested 48 h after transfection in PBS + protease inhibitors (#P8340, SIGMA). Cells were pelleted, washed with PBS + protease inhibitors, then lysed in lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 1 mM Na orthovanadate, 1 mM DTT, and protease inhibitors) for 30 min on ice. Lysates were mixed with equal parts adjustment buffer (50 mM Tris, 150 mM NaCl), then cell debris was pelleted and lysates transfered to new tubes. Lysates were incubated with or without antibodies for 2 h at 4°C with rotation, then incubated with Protein A Sepharose beads (#17-5280-01, Amersham) for 2 h at 4°C with rotation. Immunoprecipitates were washed 3X with wash buffer (1:1 lysis buffer + adjustment buffer), then resuspended in 2X SDS loading dye and boiled for 5 min before storing at −20°C. Proteins were separated by SDS-polyacrylamide gel electrophoresis, transfered to PVDF membranes, blocked 1 hr at RT with 5% milk diluted in PBS, and incubated at 4°C overnight with anti-HA antibody diluted 1/100 in 5% milk-PBS. Membranes were washed in PBS and incubated

with a horseradish peroxidase-conjugated anti-mouse antibody (GE Healthcare). SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used for detection.

Preparation of nuclear extracts

Crude nuclear extracts from 10T1/2 cells overexpressing Pax3-HA were prepared as previously described (15). Total protein in the extracts was quantitated by the Bradford method (8).

Gel mobility shift assays

Gel-shift assays were carried out as previously described (22) using nuclear extracts prepared as above. Incubations with antibodies or unlabeled oligonucleotide competitors were carried out at room temperature for 20 min prior to the addition of probe. Forward sequences of oligonucleotides used as probe/competitors are: consensus Pax3 motif from the −58/−56 kb distal *Myf5* enhancer: 5'-GCATGACTAATTGCATGGTAACTGGAGAAA-3' (11); wt Pax3 HD motif from the *Myf5* promoter: 5'-CTGGGCGTTATTAGCATATCCCACC-3'; mt Pax3 HD motif from the *Myf5* promoter: 5'- CTGGGCGTTATGAGGATCTACCACC-3'. Mutated bases are underlined.

RT-PCR

RNA was extracted using the Qiagen RNeasy kit, according to the manufacturer's instructions. RNA was DNase-treated and reverse-transcribed as previously described (22). PCR was performed using 20–50 ng cDNA and Pfu DNA polymerase with the following cycling conditions: 95°C for 5 min, followed by 38 cycles of 95°C for 1 min, 51°C for 1 min, 72°C for 45 sec, and a final extension at 72°C for 10 min. Primer sequences for amplifying *Myf5* and *GAPDH* are as described (43).

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed with 10T1/2 cells overexpressing FLAG-Zic1 and Pax3-HA using the Fast ChIP method (40) with some modifications. Cells were fixed in 1% formaldehyde in DMEM for 10 min and dounced 10X prior to sonication. Cells were sonicated for 8 rounds of 15-sec pulses at 90% power output on a Branson Sonifier 450 (VWR Scientific) to shear the DNA to a ladder of ~200–800 bp, and efficiency of shearing was verified by agarose gel electrophoresis. Chromatin was immunoprecipitated using 2 μg of specific antibodies or normal rabbit IgG. Quantitative PCR was performed using forward and reverse primers (300 nM) and the QuantitTect SYBR Green PCR Kit (Qiagen). Reaction conditions were 40 cycles of: 94°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec. Sequences of primers are as follows: *Myf5* promoter: F: 5'-GTCAAAGGGACCAGTAAAC-3'; R: 5'-GGGGCTCTTTATATATTCCTG-3'; ES enhancer: F: 5'-CAAAGCCCCAGAGAGAGCCGGA-3'; R: 5'-CCTGGCGTGCTTTGCTCTGC-3'. PCR products were analyzed on a 1.5% agarose gel to verify correct size of product and specificity of primer annealing.

Results and Discussion

Pax3 synergizes with Gli2 and Zic1 in transactivating the *Myf5* ES enhancer and promoter

It has been demonstrated that promoter context is critical in determining the behavior of enhancers, and the *Myf5* ES enhancer is no exception (21, 56). To rule out potentially spurious results following the use of a non-homologous promoter, we cloned the mouse ES enhancer and *Myf5* promoter (56) upstream of the firefly luciferase reporter for use in these studies. To test whether Gli and Zic transcription factors cooperate with members of the Pax family in activating the *Myf5* ES enhancer, we co-transfected expression constructs for these

factors with the reporter construct into 3T3 cells, and assayed luciferase activity (Fig 1). 3T3 fibroblasts represent a convenient system in which to test these potential interactions, since these cells express low levels of Gli2 and no Pax3 or Zic factors. While overexpression of Zic1 or Pax3 alone increased activity of the reporter construct, the combination of factors displayed a modest, but statistically significant synergy (Fig. 1A). Likewise, Gli2 and Pax3 synergized in activating the ES enhancer-promoter (Fig. 1B). Interestingly, other family members (Gli1, Zic2, and Pax7) showed no synergistic effects (Fig. 1C & D), suggesting that factor-specific contacts are required for recruitment/stabilization of coactivators.

To determine whether Gli2 and Zic1 can physically associate with Pax3 in the absence of DNA, we made cytoplasmic extracts from 10T1/2 cells transfected with FLAG-Zic1 and Pax3-HA. 10T1/2 cells were used because they produced high levels of the overexpressed proteins for co-immunoprecipitaton assays. Co-immunoprecipitations were performed using antibodies to FLAG or endogenous Gli2, and immunoprecipitated proteins were probed with HA antibodies. Pax3-HA, which runs at ~60 kD, was not detected in mock-transfected lysates, only in transfected cells (Fig. 1E). Pax3 was precipitated with antibodies to FLAG or Gli2; by contrast, only a very faint band was detected using antibodies to an unrelated V5 epitope (Fig. 1E). These results indicate a specific physical interaction between Zic1-Pax3 and Gli2-Pax3.

A novel homeodomain motif in the *Myf5* promoter is required for Pax3 synergy with Gli2 and Zic1

The Pax family is structurally defined by the presence of a DNA-binding motif called a paired domain (PD). A subset of family members, including Pax3, contain an additional DNA8-binding domain known as a paired-type homeodomain (HD) (33, 41). The PD and HD are functionally interdependent and capable of modifying Pax binding to DNA (13). Although the *Myf5* ES enhancer and promoter do not contain a consensus PD motif, multi-species sequence alignments revealed a highly conserved single HD motif (TAAT) at −79 relative to the transcription start site in mouse. To determine whether Pax3 can bind this sequence, we performed gel-shift assays using nuclear extracts from 10T1/2 cells transfected with Pax3-HA. Pax3-HA bound to the labeled probe containing a consensus Pax3 motif from the −58/−56 kb distal *Myf5* enhancer (11), and this complex was supershifted with antibodies to HA (Fig. 2A, lanes 1–2). An excess of cold competitor oligonucleotides containing the consensus Pax3 motif competed away this complex (Fig. 2A, lane 3). Importantly, the wild-type, but not the mutant *Myf5* promoter HD motif also competed for Pax3 binding (Fig. 2A, lanes 3–5), although not as well as the consensus Pax3 sequence, which contains both a paired motif and a HD motif. This indicates that Pax3 can recognize the HD motif from the *Myf5* promoter, although binding is not as strong in the absence of a paired motif.

To determine whether the HD motif is required for Pax3 synergy with Zic1 and Gli2, we performed cotransfection experiments with the *Myf5* reporter construct containing a mutated HD motif, as described above. Surprisingly, Zic1 appears to be a stronger activator of the ES enhancer-promoter construct when the HD motif is mutated, and Pax3 is still able to activate this construct, indicating an indirect effect of Pax3 on the ES enhancer or *Myf5* promoter (Fig. 2B). Importantly, despite the higher individual activity of these factors, Zic1-Pax3 synergy is completely abrogated in the absence of a functional HD motif (Fig. 2B). Likewise, Gli2-Pax3 synergy is lost on the HD-mutated construct (Fig. 2B). These results indicate that the novel HD motif in the *Myf5* promoter is required for Pax3 to synergize with both Zic1 and Gli2, in spite of the fact that these factors can interact in the absence of DNA. It is possible that association between Zic1/Gli2 and Pax3 helps to stabilize Pax3 binding to the HD motif in the absence of a paired motif in the *Myf5* promoter.

To confirm that Pax3 does not cooperate with Gli2 in the absence of a Pax binding site, we tested Pax3-Gli2 interactions on a reporter construct containing 8 Gli binding sites upstream of the *Thymidine kinase* (TK) promoter. As expected, Gli2 strongly activates this construct, whereas Pax3 does not (Fig. 2C). The combination of Gli2 and Pax3 is less potent than Gli2 alone, demonstrating that Pax3 does not behave as a cofactor for Gli2 (Fig. 2C).

The essential Gli motif in the *Myf5* ES enhancer is required for Pax3-Gli2 synergy

The conserved variant Gli motif in the ES enhancer is required for maintenance of *Myf5* expression in the epaxial somite via Shh signaling (21, 56), and Glis have been demonstrated to bind this essential site (21). To verify that this sequence is required for Gli2-Pax3 synergy, we performed cotransfection experiments with a *Myf5* reporter construct containing a mutation in the ES enhancer Gli motif. As expected, synergy between Gli2 and Pax3 is abolished in the absence of a functional Gli binding site (Fig. 2D), indicating that Gli2 binding to the ES enhancer is required for synergy with Pax3.

Both Zic and Gli family members bind to DNA via five C2H2-type zinc fingers, and Zics have been shown to recognize Gli binding sites, albeit with much lower affinity than Gli factors (37). To test whether Zic1 synergizes with Pax3 via binding to the Gli motif in the ES enhancer, we tested Zic1-Pax3 interactions on the Gli-mt reporter construct. Zic1 and Pax3 are still capable of synergizing in the absence of a functional Gli binding site, indicating that Zic1 does not require the Gli motif in the ES enhancer to synergize with Pax3 (Fig. 2D). Furthermore, Zic1-Pax3 synergy does not take place on a construct driven by multiple Gli motifs (Fig. 2C), providing further evidence that Zic1 does not synergize with Pax3 via binding to Gli motifs.

In addition to recognizing Gli motifs, Zic family members have been shown to bind a wide range of GC-rich sequences in their target genes (17, 37, 48, 60). Two conserved candidate sequences in the *Myf5* promoter were able to compete for Zic1 binding in gel-shift assays; however, mutation of either sequence had no effect on Zic1-Pax3 synergy (data not shown). This suggests that Zic1 synergizes with Pax3 by binding to functionally redundant motifs in the *Myf5* promoter; however, we cannot rule out that Zic1 acts as a transcriptional cofactor for Pax3. This is particularly difficult to test in light of the fact that Zic1 activates a wide variety of promoters through binding to degenerate GC-rich motifs (36, 37).

Conserved regions of Pax3, Gli2, and Zic1 are required for synergy

To determine regions of the proteins required for synergy, we tested various truncated forms of the three factors (Fig. 3A) in co-transfection experiments with the *Myf5* ES enhancer-promoter reporter. Interestingly, Zic1 lacking the Zic-Opa conserved motif (Zic1ΔZOC) was a more potent transactivator than full-length Zic1, suggesting that the ZOC motif may serve a repressive function (Fig. 3B). However, despite its higher activity, Zic1ΔZOC was unable to synergize with Pax3, indicating that in addition to repressing Zic1 activity, the ZOC motif is also required for cooperative interactions with other factors (Fig. 3B). This is consistent with previous studies indicating that this protein domain behaves as a context-dependent activator or repressor of transcription (38). Zic factors associate with DNA via their zinc fingers; as expected, when the zinc fingers of Zic1 were removed (Zic1ΔZF), the protein had little effect on reporter activity and was incapable of synergizing with Pax3 (Fig. 3B). Truncation of the Pax3 HD also resulted in a loss of synergy with Zic1, further confirming that DNA-binding of Pax3 is required for synergy (Fig. 3B). Likewise, when the transactivation domain (TD) of Pax3 was deleted, the remaining protein was incapable of synergizing with Zic1, suggesting that the ability of Pax3 to recruit coactivators is critical for synergy (Fig. 3B).

Interestingly, truncation of the C-terminal TD of Gli2 (ΔC2 and ΔC4) also abrogated synergy with Pax3, but deletion of the Pax3 TD had no effect on synergy with Gli2 (Fig. 3C), suggesting that in the context of Gli2-Pax3 interactions, it is the Gli2 TD that is competent to recruit transcriptional coactivators. This is consistent with studies indicating that Gli2 acts primarily as a transcriptional activator (16, 34) and is the major transducer of Shh signaling in the mouse (35, 44). As with Pax3-Zic1, Pax3-Gli2 synergy is dependent on the conserved HD of Pax3 (Fig. 3C).

Pax3 and Zic1 are enriched at the ES enhancer during induction of *Myf5* transcription

Zic1 has been shown to initiate expression of *Myf5* in 10T1/2 cells, which can be induced to form skeletal muscle in response to myogenic cues (43). To confirm that Zic1 and Pax3 occupy the endogenous *Myf5* promoter during activation of *Myf5* expression, we performed chromatin immunoprecipitation (ChIP) assays on chromatin from 10T1/2 cells overexpressing FLAG-Zic1 and Pax3-HA. *Myf5* induction in the transfected cells was confirmed by RT-PCR (Fig. 4A). Immunoprecipitation with either FLAG- or HA-specific antibodies yielded ~2-fold enrichment of the *Myf5* promoter over that obtained with non-immune IgG (Fig. 4B), demonstrating that these factors occupy the endogenous *Myf5* promoter during induction of *Myf5* expression. Interestingly, we also observed enrichment of FLAG-Zic1 and Pax3-HA at the ES enhancer, likely due to enhancer-promoter looping interactions (Fig. 4B) (49).

Cooperation among several distinct pathways promotes myogenesis in the epaxial somite

Taken together, our data suggest an intriguing new model for cooperative interactions between Pax3 and Zic1, in which Pax3 binds its recognition motif in the *Myf5* promoter via the HD and Zic1 binds GC-rich sequences via its zinc finger domain (Fig. 5A). Contacts between Pax3 and the ZOC motif of Zic1 (which is normally repressive in the absence of Pax3) prevent Zic1 from recruiting transcriptional co-repressors, while the transactivation domain (TD) of Pax3 serves to recruit transcriptional co-activators for gene expression (Fig. 5A).

Our work places Zic and Pax genes in the broader context of several well-established signaling pathways that regulate myogenesis (Fig. 5B). As shown in our earlier work, by enhancing nuclear translocation of Gli factors, Zics provide spatial patterning for the Gli family, which is expressed throughout the somite, to activate *Myf5* expression in epaxial muscle progenitors (43). In contrast to this, our current study indicates a different mechanism of cooperativity between Zic1 and Pax3, through the establishment of interactions that require DNA-binding and likely help to recruit or stabilize coactivator proteins. Interestingly, the combination of Pax3/7 and Zic genes is sufficient to induce neural crest formation in Xenopus (52), and in the mouse, Pax3 and Zic1 are both expressed in the dorsal neural tube (20, 43). This indicates that in addition to driving commitment of cells to other lineages, the presence of these factors is not sufficient to induce *Myf5* expression in non-muscle tissues.

Pax3 serves to regulate *Myf5* in the epaxial somite at multiple levels – as we have shown, through direct binding of a homeodomain motif within the promoter, as well as indirectly, via upregulation of FoxD3 and Dmrt2 (32, 51) (Fig. 5B). Although the BMP antagonist Noggin is required for Zic2 expression in the epaxial somite (43), the positive signals mediating expression of Zics in this compartment are still unknown, although Wnts secreted from the dorsal neural tube and surface ectoderm are likely candidates. Signaling by both canonical and non-canonical Wnts plays an important role in myogenesis (57), and direct binding by LEF1/β-catenin is required for full activity of the ES enhancer via synergy with Gli (5) (Fig. 5B).

It will be interesting to determine if the novel synergistic interactions described here extend to *Myf5* activation/maintenance in other muscle lineages. During embryogenesis, Pax3 is expressed in the dorsal neural tube and PSM, followed by expression throughout the somites which is subsequently restricted to the dermomyotome (19, 20). Following this, Pax3 expression is decreased in the epaxial somite and maintained in hypaxial precursors (4, 19, 59). At E12.5, Pax3 continues to be expressed in MyoD-positive regions in the trunk and the limbs before expression is lost at later stages (23). Pax3 directly regulates *Myf5* expression in the hypaxial somite and some hindlimb muscle precursors via binding to a −57.5 kb upstream enhancer (3). Recently, Pax3 was also shown to be a direct regulator of the −111 kb enhancer, which regulates expression of *Myf5* in the ventral somite and a subset of limb muscle precursors (14). Since Shh was recently shown to be required for *Myf5* expression in limb muscle progenitor cells (1, 26), it will be important to determine whether Gli2 and Pax3 synergize in driving *Myf5* expression in this muscle lineage. Although Shh is not required for *Myf5* activation in hypaxial progenitors (7, 31), Gli2 expression overlaps that of *Myf5* in this domain (35). This raises the possibility that Gli2 (activated independent of Shh signaling; possibly via FGF and PKCδ/MEK1 (27, 46)) and Pax3 synergize in activating hypaxial *Myf5* expression. Likewise, while the strong epaxial expression of Zics closely mimics that of Pax3 at E9.5 (43), Zic2/3 are also expressed in the limb buds and in the developing limbs at later stages (39). Thus, it will also be important to determine whether Zics synergize with Pax3 in activating the hypaxial *Myf5* enhancers. Understanding the spatiotemporal dynamics of these interactions and the mechanisms by which these factors cooperate to drive myogenesis remains a significant challenge for future studies.

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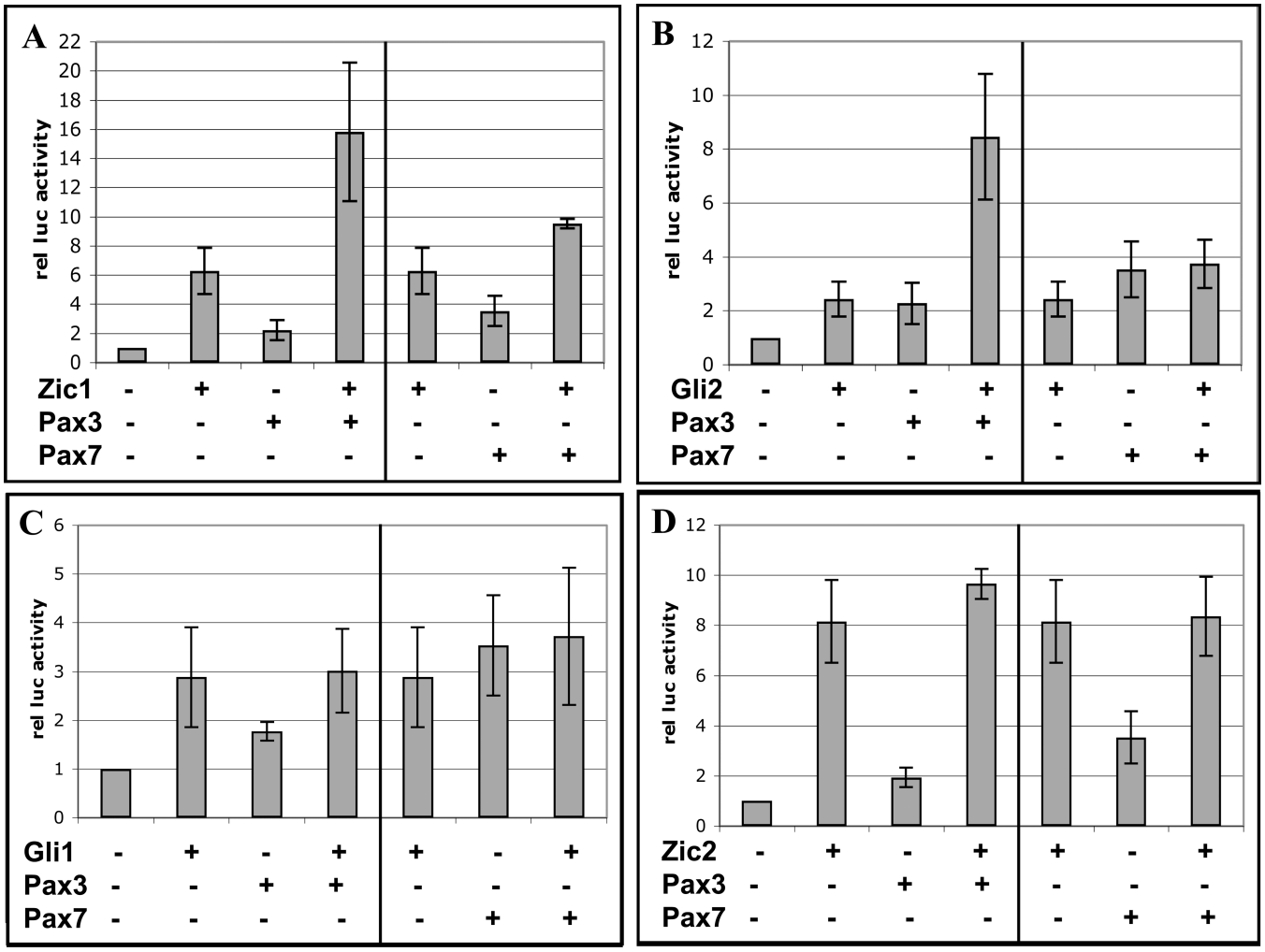
Highlights

- Pax3 synergizes with Gli2 and Zic1 in transactivating *Myf5*.
- Pax3 synergy with Gli2/Zic1 requires a novel *Myf5* promoter homeodomain motif.
- Pax3 synergy with Gli2 requires the essential Gli motif in the *Myf5* ES enhancer.
- Zic1 and Pax3 are enriched at the ES enhancer during *Myf5* induction.



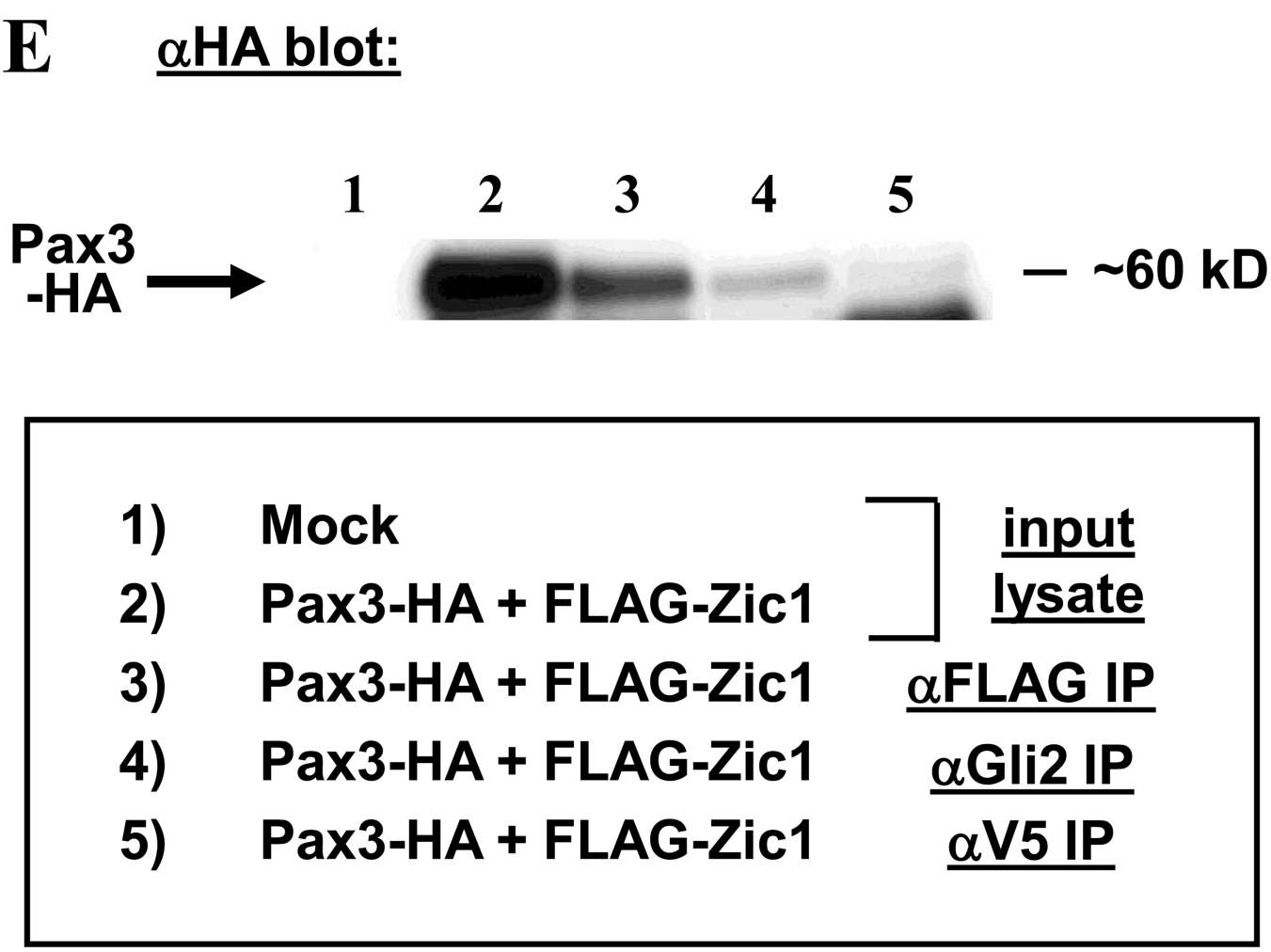
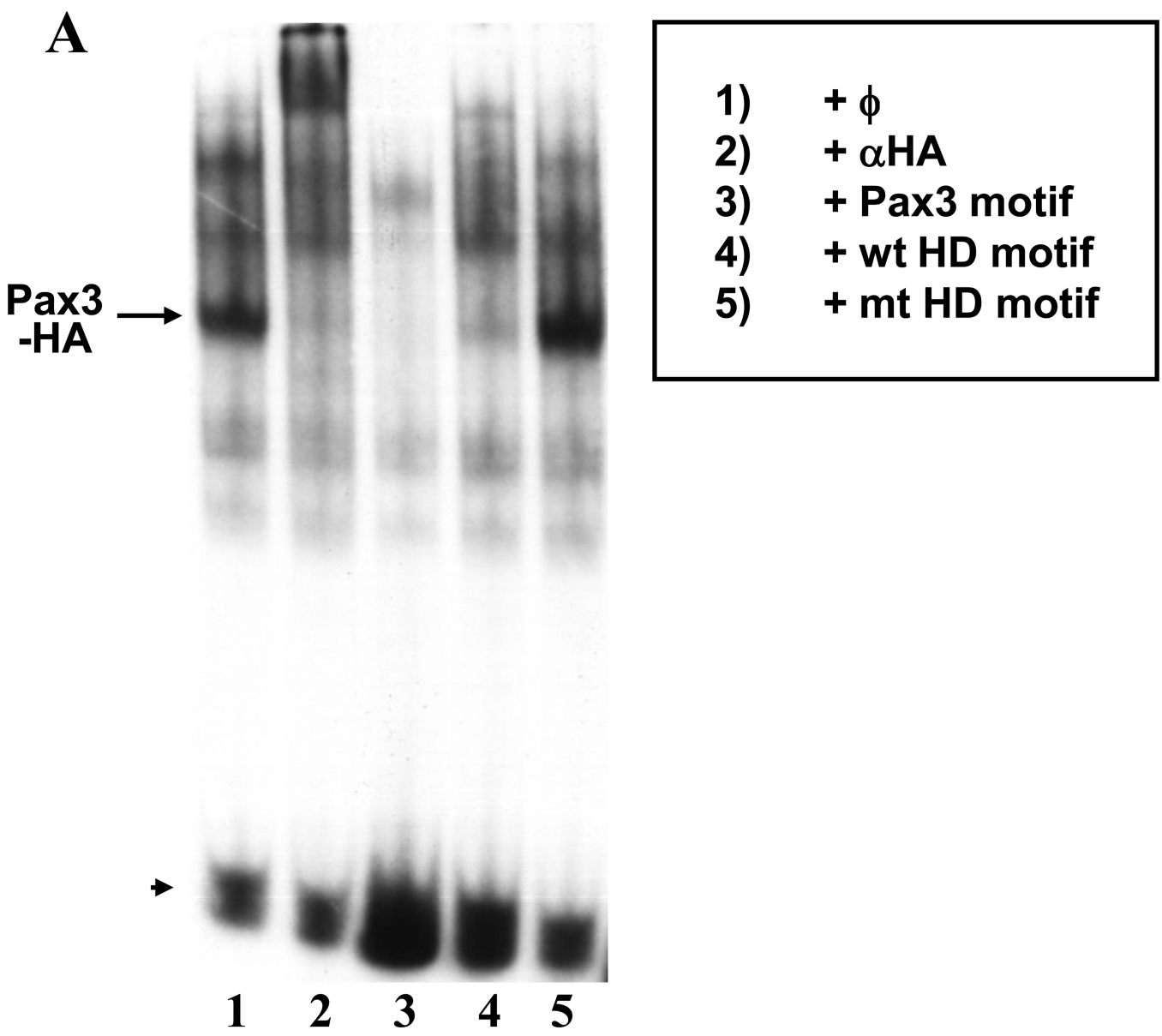
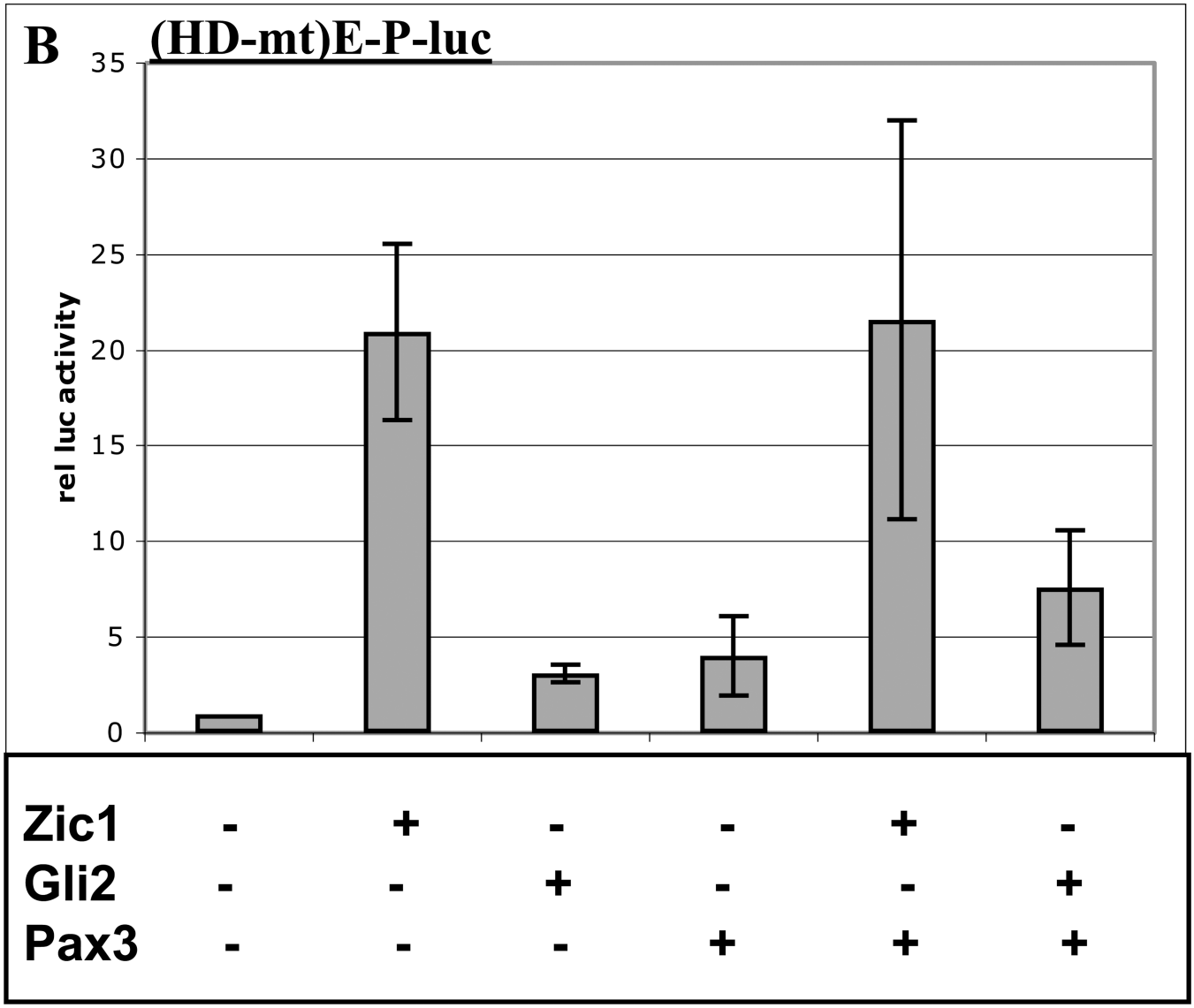
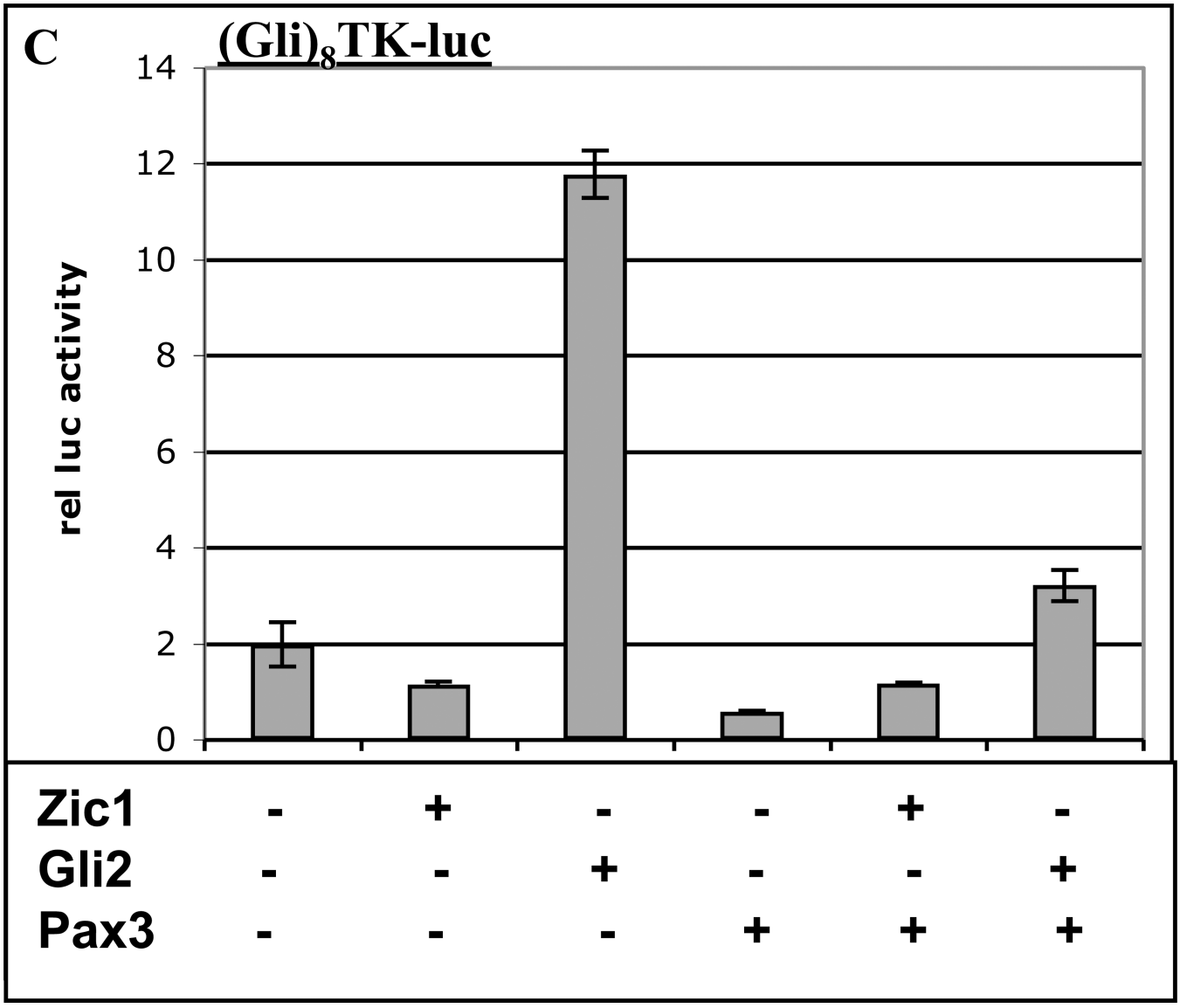


Fig. 1. Pax3 synergizes with Gli2 and Zic1 in transactivating the *Myf5* ES enhancer and promoter

A–D. A luciferase reporter plasmid containing the *Myf5* ES enhancer and minimal promoter was transiently transfected into 3T3 cells with or without expression plasmids for Zic1, Zic2, Gli1, Gli2, Pax3, or Pax7. Cells were harvested 48 h post-transfection and assayed for luciferase activity. Data are plotted as the mean value and standard deviation of relative luciferase activity, with activity of the reporter construct alone set at 1. Pax3 synergizes with Zic1 (A, asterisk = $p < 0.01$) and Gli2 (B, asterisk = $p < 0.05$) in transactivating the reporter (one-tailed, one-step t-test comparing activity of factors in combination to the sum of individual factors), whereas other combinations of factors show no synergistic effects (C-D). **E.** 10T1/2 cells were transfected with Pax3-HA and FLAG-Zic1 expression plasmids, and cell extracts were immunoprecipitated with antibodies to FLAG, Gli2, or V5 (as a negative control). Immunoprecipitates were subjected to Western analysis using HA antibodies.







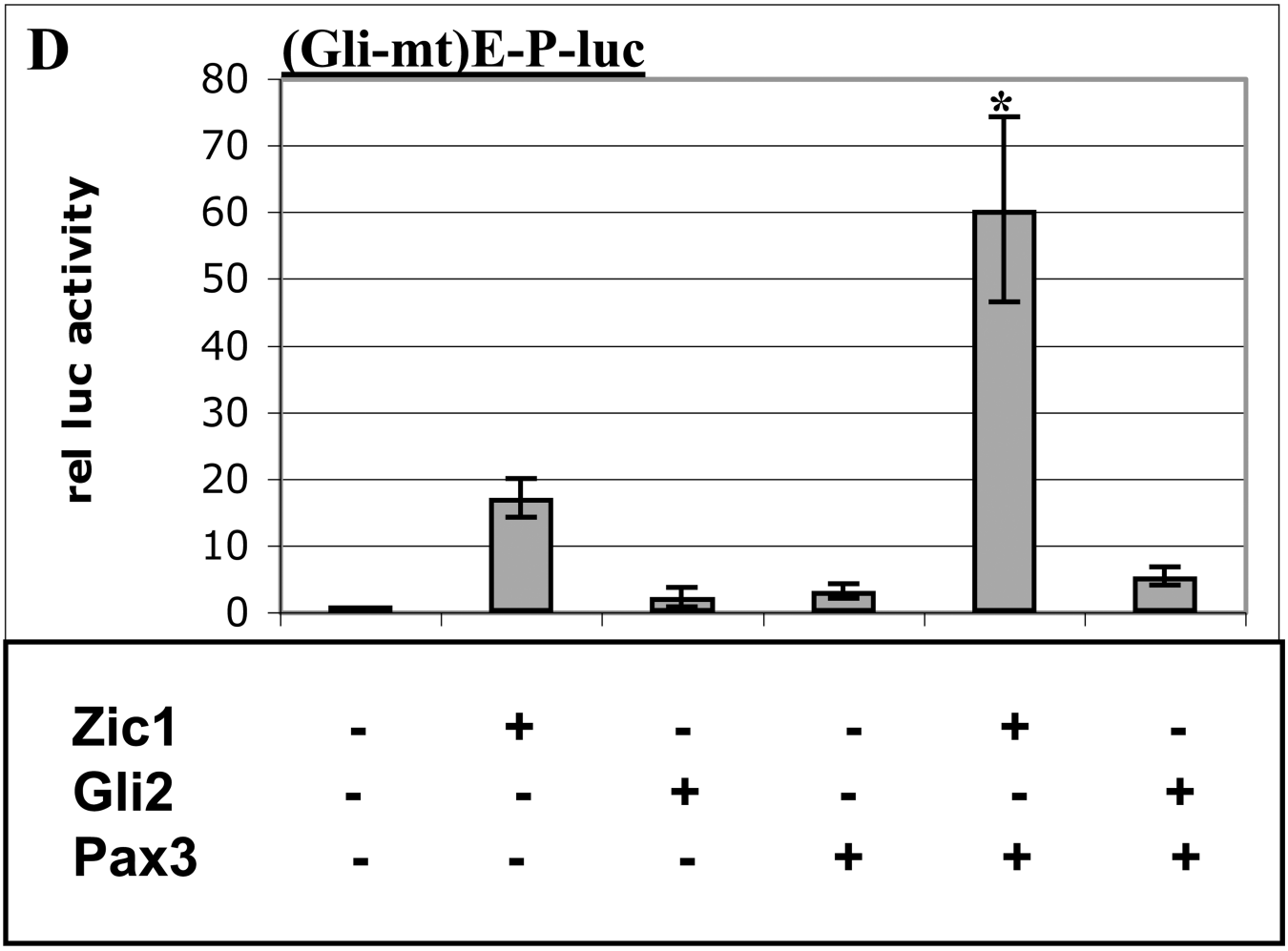
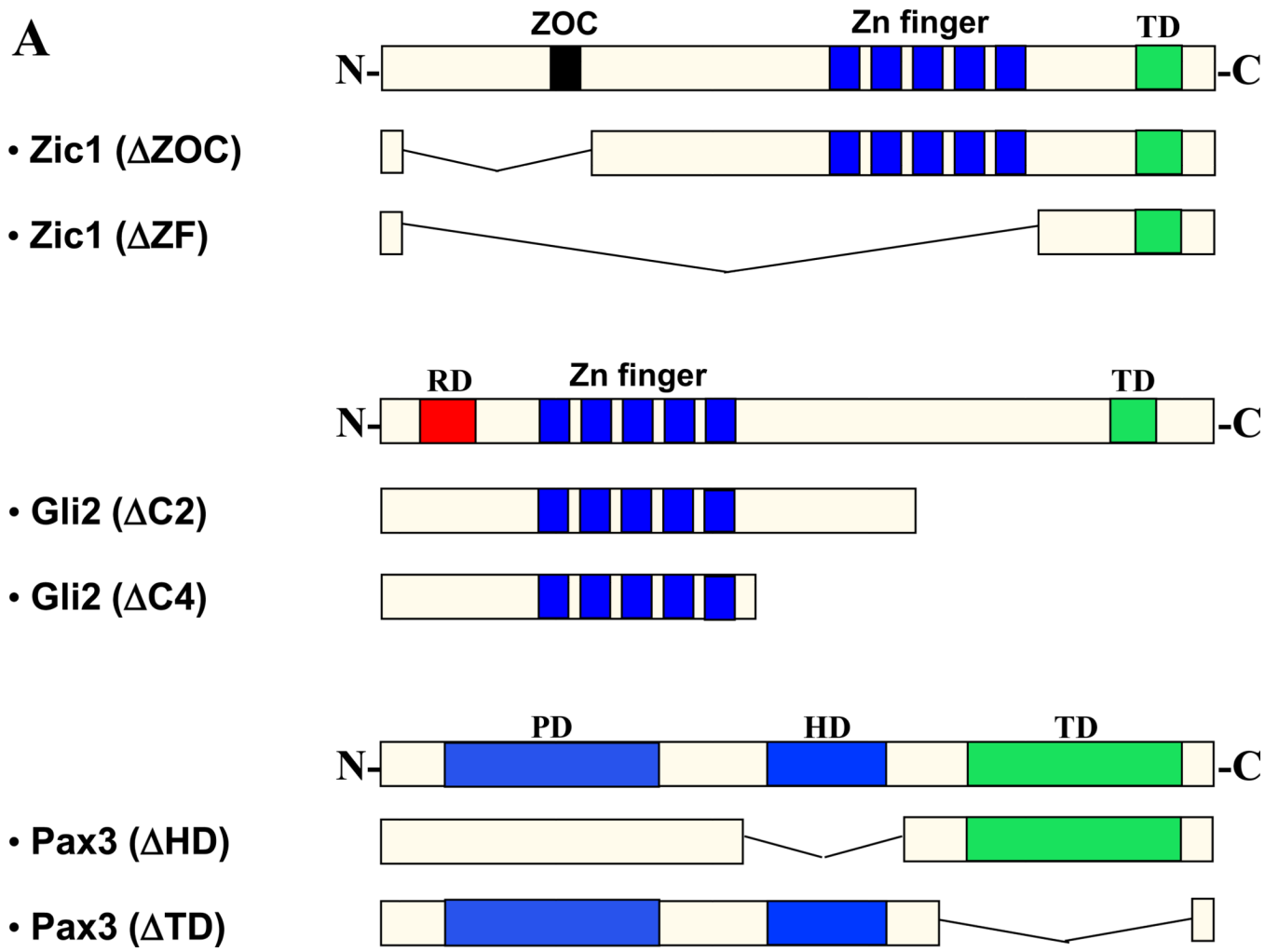
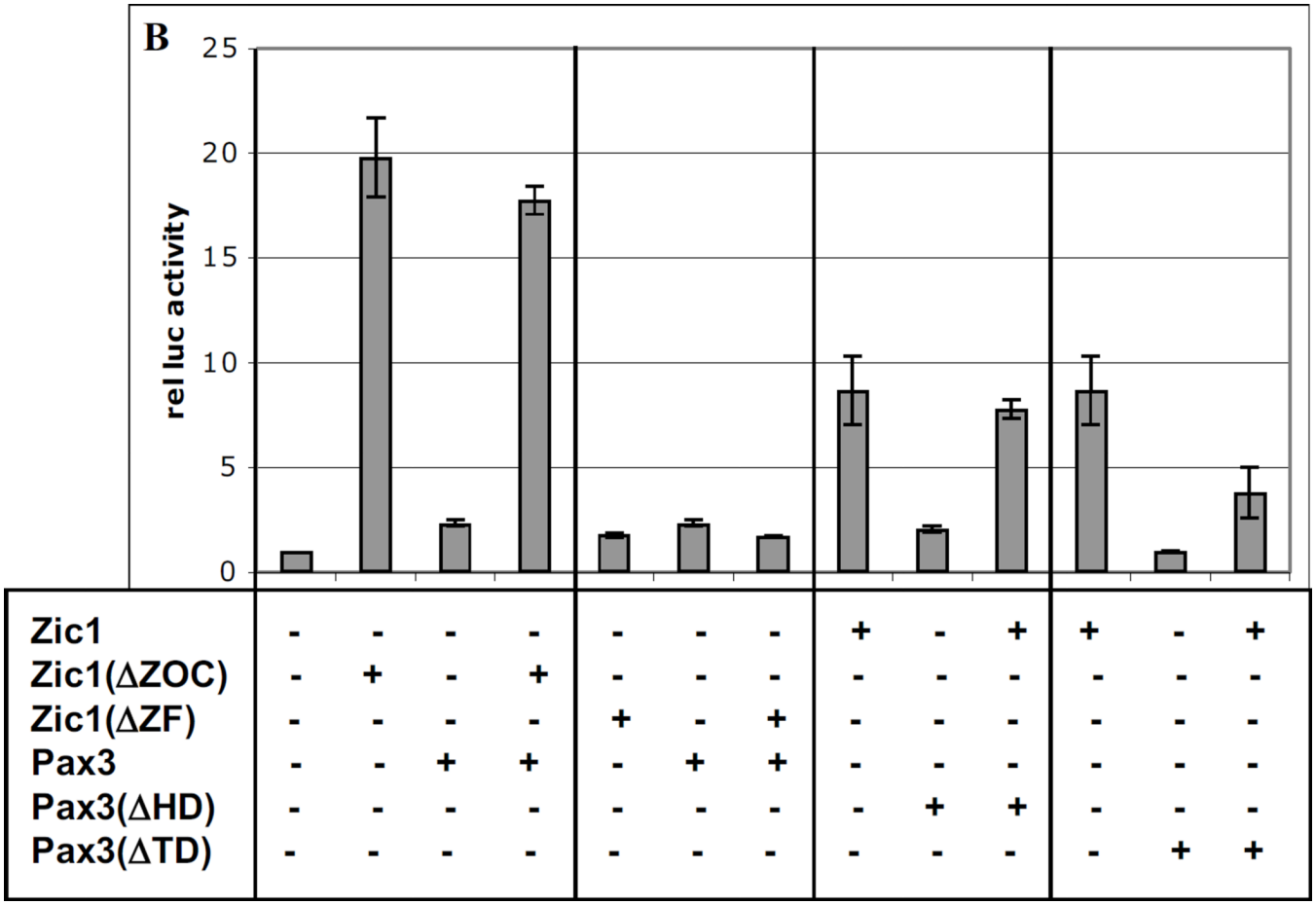


Fig. 2. Sequences in the *Myf5* ES enhancer and promoter are required for Pax3 synergy with Gli2 and Zic1

A. Labeled probe containing a consensus Pax3 binding site from the −58/−56 kb distal *Myf5* enhancer (11) was mixed with nuclear extracts from 10T1/2 cells overexpressing Pax3-HA, and analyzed via gel-shift assays. Antibodies (αHA, lane 2) or competitor oligos (Pax3 consensus motif, lane 3; wt HD motif in *Myf5* promoter, lane 4, and mt HD motif in *Myf5* promoter, lane 5) are indicated. The complex containing Pax3-HA bound to the probe (supershifted with αHA in lane 2) is labeled. Arrowhead indicates free probe. **B.** A luciferase reporter plasmid containing the *Myf5* ES enhancer and promoter with a mutation in the promoter HD motif (HD-mt) was transiently transfected into 3T3 cells with or without expression plasmids for Zic1, Gli2, or Pax3. **C.** A luciferase reporter plasmid containing 8 consensus Gli motifs upstream of the thymidine kinase (TK) promoter was transiently transfected into 3T3 cells with or without expression plasmids for Zic1, Gli2, or Pax3. **D.** A luciferase reporter plasmid containing the *Myf5* ES enhancer and promoter with a mutation in the ES enhancer Gli motif (Gli-mt) was transiently transfected into 3T3 cells with or without expression plasmids for Zic1, Gli2, or Pax3. For B–D, cells were harvested and assayed, and data was analyzed as in Fig. 1. Asterisk in D indicates synergy between Zic1 and Pax3 ($p < 0.05$, one-tailed, one-step t-test comparing activity of factors in combination to the sum of individual factors).





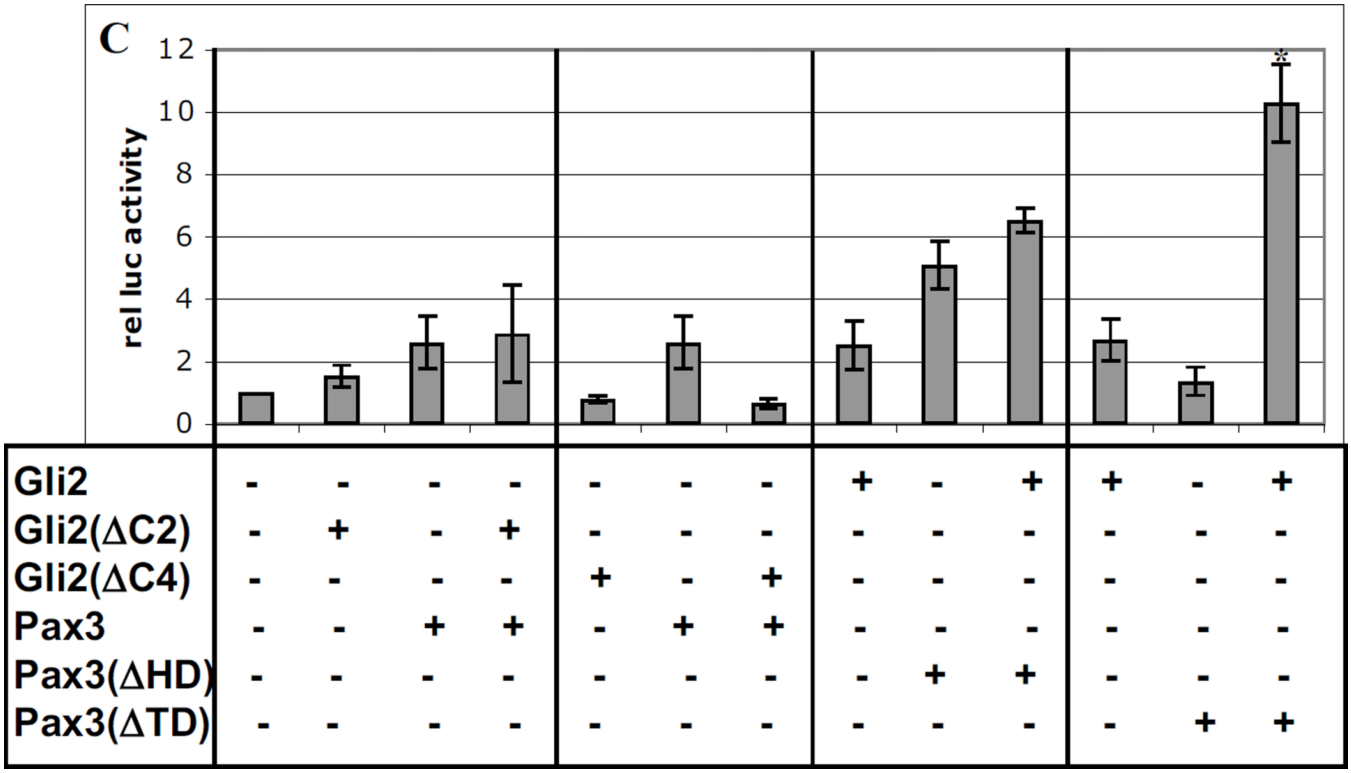


Fig. 3. Conserved regions of Pax3, Gli2, and Zic1 are required for synergy

A. Diagram of truncations in expression constructs encoding Zic1, Gli2, and Pax3. ZOC = ZOC motif; ZF = zinc finger domain; HD = homeodomain; TD = transactivation domain; C2 & C4 = C terminal domains. **B–C.** Truncated constructs in A were tested for synergy in transactivating the *Myf5* ES enhancer and promoter as in Fig 1–2. Asterisk in C indicates synergy (p = 0.01) between Gli2 and Pax3(ΔTD) (one-tailed, one-step t-test comparing activity of factors in combination to the sum of individual factors).

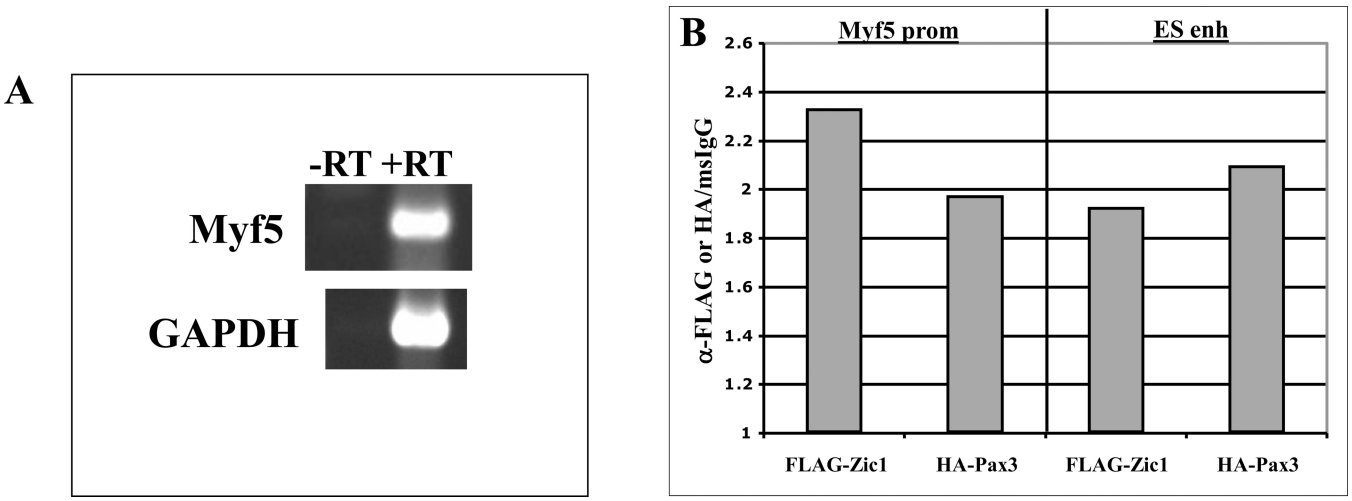


Fig. 4. Pax3 and Zic1 are enriched at the ES enhancer during activation of *Myf5* transcription

10T1/2 cells were transiently transfected with FLAG-Zic1 and Pax3-HA expression plasmids. RNA was isolated for RT-PCR or cells were fixed for chromatin immunoprecipitation (ChIP) analysis. **A.** RT-PCR was performed using primers specific for *Myf5* and *GAPDH*. **B.** ChIP assays were performed using antibodies specific for FLAG or HA or normal mouse IgG. Immunoprecipitated chromatin was analyzed by qPCR using primers specific for the *Myf5* promoter or ES enhancer. Data are represented as fold enrichment of the *Myf5* promoter or ES enhancer by αFLAG or αHA relative to normal mouse IgG.

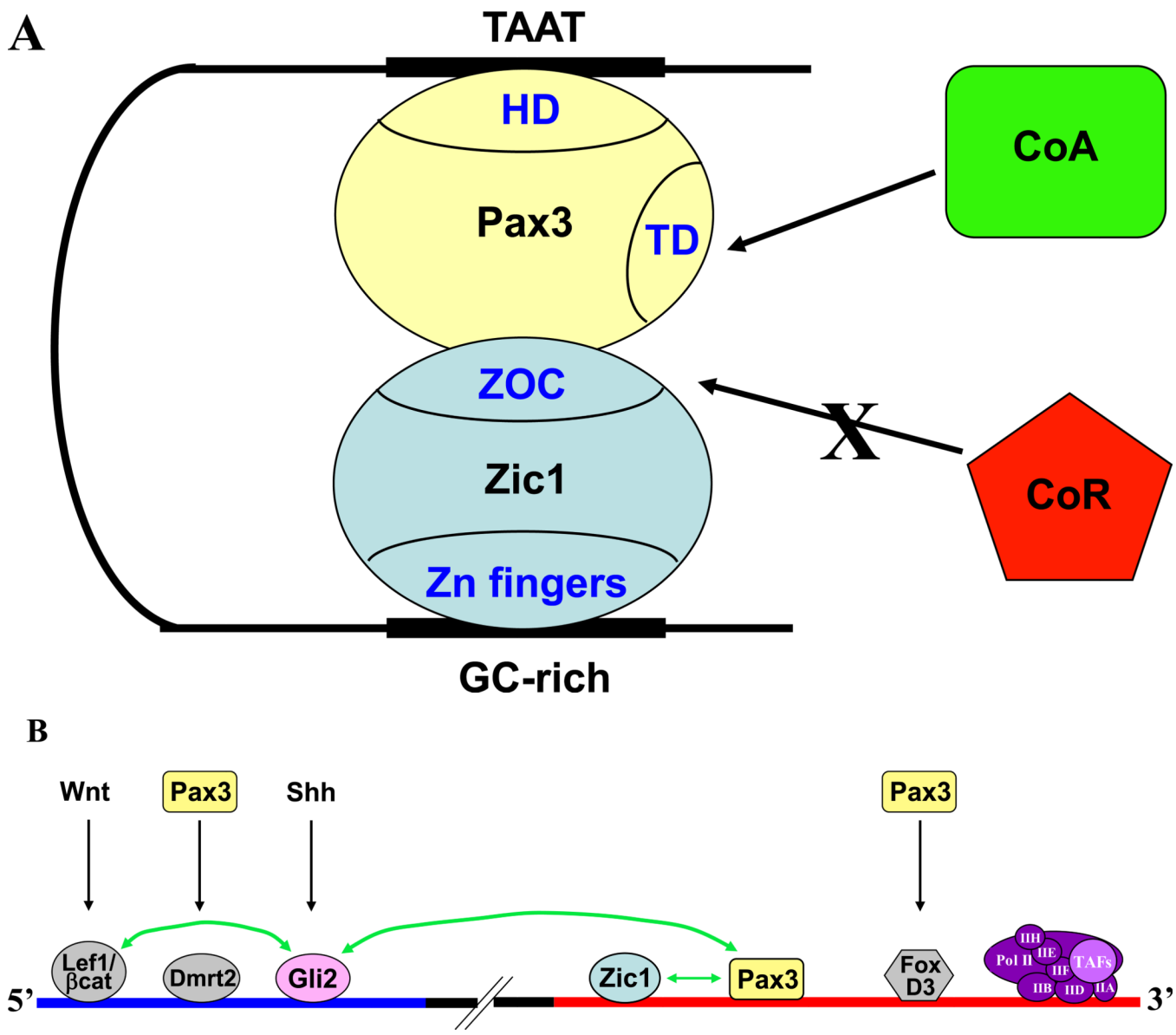


Fig. 5. Cooperation among several distinct pathways promotes myogenesis in the epaxial somite

A. Model of Pax3-Zic1 synergy. Pax3 binds the homeodomain motif in the *Myf5* promoter (TAAT) via its homeodomain (HD), and Zic1 binds GC-rich sequences via its zinc finger domain. Interaction between Pax3 and the ZOC domain of Zic1 prevents the latter from recruiting transcriptional co-repressors (CoR). The transactivation domain (TD) of Pax3 recruits transcriptional co-activators (CoA). **B.** Transcription factors and upstream signaling pathways regulating *Myf5* expression in the epaxial somite. The blue bar indicates the *Myf5* ES enhancer, and the red bar indicates the *Myf5* promoter. Green arrows indicate synergistic interactions between transcription factors. Shh signaling is required for Gli activation of the ES enhancer via a single essential motif (21, 56). Wnt and Shh signaling culminate in synergy between LEF1/β-catenin bound to three motifs (shown as a single motif for simplicity) in the ES enhancer and Gli bound downstream (5). Pax3 activates *Myf5* in the epaxial somite indirectly, by driving expression of the Dmrt2 transcription factor, which activates the ES enhancer (51), and in zebrafish, by driving expression of FoxD3, which activates the *Myf5* promoter (32). In the current work, Pax3 also activates epaxial *Myf5*

expression directly, by binding a homeodomain motif in the *Myf5* promoter and synergizing with both Gli2, bound to the ES enhancer, and Zic1, which recognizes GC-rich motifs in the promoter. Refer to the text for more details.