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Eya1 and Eya2 proteins are required for hypaxial somitic myogenesis in the mouse embryo

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Abstract

In mammals, Pax3, Six4, Six1 and Six5 genes are co-expressed with Eya1, Eya2 and Eya4 genes during mouse somitogenesis. To unravel the functions of Eya genes during muscle development, we analyzed myogenesis in Eya2-/− and in Eya1−/− embryos. A delay in limb myogenesis was observed between E10 and E13 in Eya1−/− embryos only, that is later compensated. Compound E18 Eya1−/−Eya2−/+ fetuses present a muscle phenotype comparable with that of Six1−/− fetuses; lacking a diaphragm and with a specific absence of limb muscles, suggesting either genetic epistasis between Six and Eya genes, or biochemical interactions between Six and Eya proteins. We tested these two non-exclusive possibilities. First, we show that Six proteins recruit Eya proteins to drive transcription during embryogenesis in the dermomyotomal epaxial and hypaxial lips of the somites by binding MEF3 DNA sites. Second, we show that Pax3 expression is lost in the ventrolateral (hypaxial) dermomyotomes of the somite in both Eya1−/−Eya2−/− embryos and in Six1−/−Six4−/− embryos, precluding hypaxial lip formation. This structure, from which myogenic cells delaminate to invade the limb does not form in these double mutant embryos, leading to limb buds without myogenic progenitor cells. Eya1 and Eya2, however, are still expressed in the somites of Six1Six4 double mutant and in *splotch* embryos, and Six1 is expressed in the somites of Eya1Eya2 double mutant embryos and in *splotch* embryos. Altogether these results show that Six and Eya genes lie genetically upstream of Pax3 gene in the formation of ventrolateral dermomyotome hypaxial lips. No genetic links have been characterized between Six and Eya genes, but corresponding proteins activate key muscle determination genes (Myod, Myogenin and Mrf4). These results establish a new hierarchy of genes controlling early steps of hypaxial myogenic commitment in the mouse embryo.

Keywords: Eyes absent/Eya proteins; Six/*sine oculis* homeoproteins; Pax3; OFC syndrome; Muscle; Somite; Hypaxial

Introduction

The *eyes absent* (*eya*) gene was first characterized in *Drosophila* where it participates synergistically with *eyeless* (*Pax*), *sine oculis* (*Six*) and *dachshund* (*Dach*) in eye formation (Pignoni et al., 1997; Bonini et al., 1997). This synergy is based on a genetic feedback loop between these genes, and on biochemical interactions between *eyes absent* and *sine oculis* (Pignoni et al., 1997). *Eyes absent* is expressed not only in the eye imaginal disc, but also during other types of organogenesis. While complete absence of *eya* is lethal (Bonini et al., 1998), *eyes absent* mutants

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in *Drosophila* show multiple organ malformations including polar cell fate defects during oogenesis (Bai and Montell, 2002) and muscle defects suggesting a role for *eya* in the specification or differentiation of a subset of muscle fibers (Boyle et al., 1997). A single *eya* gene has been identified in *Drosophila*, while four Eya genes have been identified in mammals (Borsani et al., 1999; Xu et al., 1997b), Eya1 being expressed in optic placode with Pax6 and Six3. Eya genes in mammals are expressed at high levels, in particular in dorsal root ganglia and in ventrolateral extensions of the dermomyotomes of somites with Pax3 and Six1 (Heanue et al., 1999). In mammals, absence of the Eya1 gene leads to multiple organ malformations and in particular kidney, thyroid, cranial sensory ganglia and ear development is compromised (Xu et al., 1999, 2002; Zou et al., 2004). In man, Eya1 mutations are responsible for Branchio Oto Renal syndrome (Abdelhak et al., 1997) and Oto-Facio-Cervical (OFC) syndrome (Estefania et al., 2005). Eya 1 is expressed in adult skeletal muscle (Abdelhak et al., 1997; Grifone et al., 2004), and during human myogenesis (Fougerousse et al., 2002). No myopathy has yet been associated with Eya1 mutations, but cervical muscular malformations are observed in OFC (Estefania et al., 2005). Eya proteins are nuclear cofactors which possess a nuclear localization signal in *Drosophila* but lack this peptide signal in vertebrates. Eya proteins have been found in the cytoplasm and in the nucleus of cells in the embryo (Fougerousse et al., 2002), and Six proteins are among the proteins that can transport Eya to the nucleus both *ex vivo* and *in vivo* (Fan et al., 2000; Grifone et al., 2004; Ohto et al., 1999). Eya proteins are endowed with a phosphatase activity (Li et al., 2003; Rayapureddi et al., 2003), but this activity is not required for the transcriptional coactivator synergy observed in conjunction with *sine oculis* (Tootle et al., 2003). Eya proteins have also been shown to participate in a MAPK/RTK signaling pathway in *Drosophila* (Hsiao et al., 2001), but this has not been reported in vertebrates. Vertebrate orthologs of these genes are not only expressed during eye formation where Six3 acts upstream of Pax6 (Lagutin et al., 2003; Loosli et al., 1999) itself controlling the expression of Eya genes (Xu et al., 1997b), but also during development of other organs including muscle (David et al., 2001; Heanue et al., 1999; Laclef et al., 2003; Sahly et al., 1999; Spitz et al., 1998), kidney (Xu et al., 2003), cranial placodes (Schlosser and Ahrens, 2004; Zou et al., 2004) and ear (Xu et al., 1999; Zheng et al., 2003). During kidney formation, Eya genes lie upstream of Six and Pax genes, where they could cooperate with Hox genes (Wellik et al., 2002). During myogenesis, we have shown that Six1 and Six4 activate Pax3 gene in ventrolateral (hypaxial) cells of the dermomyotomes of the somites, those cells which give rise to myogenic hypaxial precursors (Grifone et al., 2005). In the posterior lip of the dermomyotome that is known to express high levels of delta (Bettenhausen et al., 1995) and to give rise to non-migrating myogenic precursors (Gros et al., 2004), Pax3 and Six1 do not control each other. These differential relationships in distinct somitic regions illustrate the heterogeneity of the genetic hierarchies involved in myogenic progenitor genesis within the somite, depending on their position and on the surrounding environment (Chen et al., 2005; Grifone et al., 2005).

Muscle regulatory factors (MRF) of the MyoD family orchestrate myogenesis in the embryo, and it has been shown that Six1 is required for MyoD and myogenin expression in the limb buds (Laclef et al., 2003). Six1 and Six4 are required for Mrf4, Myod and myogenin expression in the somite, while dispensable for the early somitic activation of Myf5 (Grifone et al., 2005). In the present study, we analyzed the involvement of *Eya1* and *Eya2* genes during myogenesis in Eya1−/−, Eya2−/− and Eya1−/−Eya2−/− gene-deleted embryos. We show that Eya1 and Eya2 genes have redundant functions during myogenesis and act genetically upstream of Pax3 in the formation of the hypaxial lip of the dermomyotome. We show that Six and Eya genes are activated independently in the ventrolateral part of somitic dermomyotomes and that induction of Pax3 in this region relies on biochemical interactions between Six and Eya proteins. Thus, synergy between Pax, Six and Eya genes in the hypaxial myogenic lineage of the mouse embryo is based on both genetic and biochemical interactions.

Results

Myogenesis is transiently delayed in Eya1−/− embryos

In order to understand the function of Eya1 and Eya2 proteins during mouse myogenesis, we analyzed the expression of several genes specifically expressed in the myogenic lineage in Eya1−/− or Eya2−/− embryos, at different development stages. It was shown previously that Eya1−/− fetuses die at birth due to the absence of rib cage closure and present several organ malformations in particular in the kidney and ear (Xu et al., 1999). Eya2−/− animals present no obvious external phenotype, and are viable and fertile. We crossed Eya1 and Eya2 heterozygous animals with either Six1−/+ animals (Laclef et al., 2003) (knock in allele with the β-galactosidase transgene at the Six1 locus) or with transgenic mice containing the Mlc3f-nlacZ-2E transgene which is specifically expressed in differentiated myogenic cells in the embryo (Kelly et al., 1995). X-gal staining of Eya2−/−Six1−/+ or Eya2−/− Mlc3f-nlacZ-2E embryos did not reveal major myogenic alterations between E10.5 and E18.5 (Fig. 1, and data not shown), and Eya2−/−Six1−/+ animals are viable and fertile. In Eya1−/− embryos on the contrary, analysis of Six1 expression or Mlc3f expression revealed transient myogenic alterations at the somitic level associated with a shorter ventrolateral extension of the dermomyotome/myotome (Fig. 1). Lack of significant Six1 downregulation in Eya1−/− embryos shows that Eya1 is not required to specifically activate Six1 at the somitic level (Figs. 1 and 3). Transient alterations of Six1 expression, detectable in the limbs until E14, cannot be attributed to a deficiency in the myogenic lineage, since Six1 and Eya1 are expressed in several cell types in the limb bud (Bonnin et al., 2005; Oliver et al., 1995; Xu et al., 1997a). β-galactosidase staining revealed that the Mlc3f-nlacZ-2E transgene is normally expressed in the forelimb bud of E11 wild type or heterozygous Eya1−/+ embryos but completely silent in the forelimb bud of E11 Eya1−/− embryos, while myogenic Pax3-positive progenitors are present (see below). Furthermore, Mlc3f-nlacZ-2E expression is undetectable in ventral muscle masses of the hindlimb until E13, which is reminiscent of