**Drosophila Araucan and Caupolican Integrate Intrinsic and Signalling Inputs for the Acquisition by Muscle Progenitors of the Lateral Transverse Fate**

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**Abstract**

A central issue of myogenesis is the acquisition of identity by individual muscles. In *Drosophila*, at the time muscle progenitors are singled out, they already express unique combinations of muscle identity genes. This muscle code results from the integration of positional and temporal signalling inputs. Here we identify, by means of loss-of-function and ectopic expression approaches, the Iroquois Complex homeobox genes *araucan* and *caupolican* as novel muscle identity genes that confer lateral transverse muscle identity. The acquisition of this fate requires that Araucan/Caupolican repress other muscle identity genes such as *slouch* and *vestigial*. In addition, we show that Caupolican-dependent *slouch* expression depends on the activation state of the Ras/Mitogen Activated Protein Kinase cascade. This provides a comprehensive insight into the way Iroquois genes integrate muscle progenitors, signalling inputs that modulate gene expression and protein activity.

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**Introduction**

In *Drosophila* as in vertebrates the proper function of the muscular system relies on the generation of a stereotyped pattern of discrete muscles and their intimate connection with the nervous system, which together control the adequate release of contraction power to fulfill the functional requirements of the organism. The formation of a muscle pattern is therefore of great importance and consequently many efforts have been devoted to solve the central problem of the acquisition of muscle identity. The embryonic *Drosophila* muscle pattern comprises thirty elements in each abdominal hemisegment (Figure 1G). Each muscle is a syncytial fibre whose unique characteristics, i.e., position, size, attachment to tendon cells, innervation and pattern of gene expression allow its unambiguous identification [1,2]. Muscle specification is a stepwise process that ensures the local singling out of a population of myoblasts, the founder myoblasts, each of them containing the necessary information to give rise to a unique muscle. The origin of founder myoblasts can be traced to late embryonic stage 10 when groups of mesodermal cells (the promuscular clusters) start expressing the proneural gene *lethal of scute* and acquire myogenic competence [3]. Opposing activities of Notch and Receptor Tyrosine Kinase signalling pathways ensure that only one cell in the cluster will segregate as a muscle progenitor [4]. This will divide asymmetrically to generate two sibling founder myoblasts or a founder myoblast and an adult muscle precursor [3,5,6]. The unselected cells of the promuscular clusters, by activation of the Notch signalling pathway, will initiate the expression of the transcriptional regulator Myoblasts incompetent (also called Gleeceful and Lame duck) and become fusion competent myoblasts that by fusing to founders will give rise to multinucleated fibres [7–9]. Regarding muscle identity, each progenitor and founder exhibits a specific code of gene expression that confers to muscles their unique characteristics. The components of these codes are accordingly named muscle identity genes (reviewed in [2,10,11]). The identity code is transmitted to all the nuclei in the syncytium through the process of myoblast fusion [12]. According to their patterns of expression muscle identity genes can be grouped into three categories. Type I includes genes expressed by progenitors and whose expression is maintained in sibling founders and muscles. Examples are *opterous, ladybird* (lb) and *Pox meso* (*Poxm*) [13–15]. Type II identity genes are expressed in progenitors but differentially regulated in sibling founders, being lost from one of them and the corresponding muscles. Examples are *Krüppel* (*Kr*), *even-skipped* (*eve*), *collier* and *slouch* (*slou*) [3,4,16–18]. And finally type III refers to genes expressed by progenitors and founders of muscles sharing common characteristics. *vestigial* (*vg*), expressed by all internal muscles, is the only known member of this class [12,19]. Regarding the onset of their expression a few muscle identity genes, such as *Kr*, *eve* and *collier*, are already expressed in the promuscular cluster, before the segregation of muscle progenitors [4,16,18,20] whereas other genes, like *Connectin* (*Con*),
Author Summary

In Drosophila, as in vertebrates, the muscular system consists of different types of muscles that must act in coordination with the nervous system to control the adequate release of contraction power required for the proper functioning of the organism. Therefore, the acquisition of specific identities by individual muscles is a key step in the generation of the muscular system. In Drosophila, muscle progenitors (specific myoblasts that seed the formation of mature muscles) integrate positional and temporal signalling inputs, resulting in the expression of unique combinations of muscle identity genes, which confer on them specific fates. Up to now, very little was known of how this integration takes place at a molecular level and how a particular code is translated into a specific muscle fate. Here we show that the acquisition of the lateral transverse muscle fate requires the repression mediated by Araucan and Caupolican, two homeoproteins of the Iroquois Complex, of other muscle identity genes, like slouch and vestigial. The repressor or activator function of the Iroquois proteins depends on the activity of the Ras signalling pathway. Therefore, our work places Iroquois genes at a nodal point that integrates signalling inputs and regulates protein activity and cell fate determination.

In this study we identify araucan (ara) and caupolican (caup), two members of the Iroquois gene complex (Iro-C), as novel type III muscle identity genes. The Iro-C genes encode homeoproteins conserved throughout the animal kingdom. They are organized in genomic clusters of three paralogous genes, one in the case of Drosophila and usually two in most vertebrates [21]. They participate in a wide variety of developmental processes, mainly related to the specification and patterning of diverse territories of the body, including the lateral mesonotum and dorsal cephalic region of Drosophila, the neural ectoderm of Xenopus and cranial placode derivatives of zebrafish [22–30]. Here we show by means of genetic approaches that ara and caup function redundantly in the specification of the lateral transverse (LT) muscles, since in the absence of both genes LT1–4 muscles lose their LT fates and acquire those of other muscles.

At present there is compelling evidence that muscle progenitors can integrate positional and temporal signalling inputs. This promotes the expression of unique combinations of muscle identity genes, which confers on them their ultimate fate [14–18,31,32]. There has been extensive analysis on the regulation of some of these genes, such as eve and collier [4,33,34], which has allowed to propose candidate cis-regulatory modules for founder muscle specific expression [35]. However, very little is known about how progenitors integrate the activity of the transcription factors encoded by these genes, about the identity of their direct targets (save in the cases of Kr and Lb [36–38]), and of their hierarchical relationships and their putative post-transcriptional regulation. In this report we have focused on these issues in relation to the function of the ara/caup identity genes. We demonstrate that the implementation of the lateral transverse muscle fate requires the repression mediated by Ara/Caup of the muscle identity genes slou and zg, to avoid reiteration of other muscle fates regulated by these transcription factors. In addition, we identify slou as a potential direct target of Ara/ Caup. Furthermore, our tissue culture and in vivo experiments show that the repression of slou by Ara/Caup in LT precursors requires the activity of the Ras/Mitogen Activated Protein Kinase (Ras/MAPK) pathway to be kept at a very low level, since otherwise Caup is converted from a repressor to an activator of slou. This is to our knowledge the first evidence of the interplay between the Receptor Tyrosine Kinase signalling pathways and the activity of a muscle identity transcription factor. Therefore, during Drosophila embryogenesis, and for the acquisition of the lateral transverse muscle fate, the homeoproteins Ara and Caup appear to act at a nodal point in muscle progenitors, as they integrate positional and temporal signalling inputs that modulate their activity on subordinate identity genes.

Results

Expression of Iro-C genes during muscle development

The patterns of expression of ara and caup in the embryonic ectoderm have been previously reported [39,40]. In this work we focus on the embryonic ara and caup mesodermal expression. In situ hybridization showed that here both genes were similarly expressed (Figure 1 and results not shown). At early stage 11 caup (and ara) transcripts and proteins are detected in groups of cells of the presumptive visceral trunk mesoderm (Figure 1A–1A′), the available anti-Caup antibody recognises both Ara and Caup proteins. By mid stage 11 they are expressed at the same dorsoventral level in the visceral mesoderm and in the dorsolateral ectoderm (Figure 1B–1B′). Expression in the visceral mesoderm declined at late stage 11 when it became detectable in groups of cells of the somatic mesoderm (promuscular clusters [3], Figure 1C and 1C′), from where a subset of muscle progenitors (P) still expressing ara/caup, will segregate slightly later (stage 12, Figure 1D). Expression was maintained in sibling founder myoblasts (Fs in Figure 1D′) derived from ara-caup-expressing progenitors and in the muscles they give rise to (Figure 1E–1E′), namely LT1–4, dorsal transverse 1 (DT1) and segment border muscle (SBM) (Figure 1F and 1G). The expression in the somatic mesoderm of the third member of the Iroquois complex, mirr0 [41] did not overlap with that of ara-caup (not shown).

The early expression of ara/caup in all lateral muscles with vertical orientation, suggested a possible role as muscle identity genes. Therefore, we compared their expression with that of several muscle identity genes. For the LT1–4 muscles, ara/caup were co-expressed with Kr in the promuscular clusters from which progenitors LT1/LT2 and LT3/LT4 are singled out (Figure 2A). ara/caup expression was maintained at high levels in both progenitors that also express Kr (Figure 2B). Whereas Kr expression decayed in founders LT1 and LT3 before the onset of myoblast fusion and in LT2 and LT4 muscles from stage 15 onwards [18], expression of ara/caup was maintained in the four founders (Figure 2D and 2E). These also expressed Con, co-expression that was maintained in the mature LT1–4 muscles (Figure 1G). In the case of muscle DT1, the onset of ara/caup expression coincided with that of Con and slou in the progenitor of DT1 and dorsal oblique 3 (DO3) muscles (Figure 2C) and it appeared to be maintained in DT1 founder (Figure 2E) and mature muscle at low levels (Figure 1G). Finally, ara/caup co-expressed with lb in the SBM founder (Figure 2D), but were not detected in the lb-expressing progenitor and promuscular cluster. In summary, different muscle lineages expressed ara/caup at different steps of the myogenic programme (Figure 2F). In the LT1–4 case ara/caup and Kr were detected at the earliest lineage stage, that is in promuscular clusters, preceding Con expression in progenitors (not shown); in the DT1/DO3 lineage ara/caup and slou were first detected in the already singled out DT1/DO3 progenitor and in the SBM ara/caup expression was first detected in the SBM founder after lb expression.
Ara and Caup are required for specification of lateral transverse muscles

During imaginal development Ara and Caup can functionally substitute each other in all territories where their function has been investigated [22,23,28]. Thus, to analyse their role in embryonic myogenesis and evaluate the possible contribution of mirror to any phenotype we might find, we used three deficiencies: D\(\text{f}(3\text{L})\text{iroDFM3}\), which removes both ara and caup, (and probably affects mirror regulation, [23,28]), D\(\text{f}(3\text{L})\text{iroEGP6}\), which removes ara and caup without affecting mirror and its regulatory region, and D\(\text{f}(3\text{L})\text{iroEGP5}\), which only removes mirror [42]. Whereas D\(\text{f}(3\text{L})\text{iroEGP5}\) embryos did not show any detectable phenotype in the lateral region (not shown), a distortion of the lateral larval muscle pattern (visualised with antibody MAC141 to Tropomyosin) was found in both D\(\text{f}(3\text{L})\text{iroDFM3}\) and D\(\text{f}(3\text{L})\text{iroEGP6}\) embryos (Figure 3A–3C). In more than 95% of cases muscles with LT morphology were absent (Figure 3E). Instead, some fibres with abnormal orientation appeared in the lateral and ventral regions, but never inserted at the LT attachment sites (asterisks in Figure 3B and 3C). The loss of LT muscles was further verified by loss of expression of the specific LT muscle marker CG13424, recently renamed lateral muscles scarcer (\textit{lms}) [43] at stage 15 and the absence of Con expression in the lateral somatic mesoderm (Figure S1). Both DT1 and SBM fibres developed with normal morphologies (Figure 3A–3C and Figure S1). To examine the individual contribution of ara and caup to the phenotype we resorted to embryos mutant for only one of these genes (ara in ara\(^{\text{F209}}\), [28], or caup in iro\(^{\text{EGP1}}\), [42]). The larval muscle pattern was normal in both mutants (not shown). Thus, similarly to imaginal development, ara and caup appear to play redundant roles during embryonic myogenesis.

The absence of muscles with LT morphology in ara/caup mutants could be due to a failure of otherwise well specified muscles to find the right insertion to tendon cells, due to ectodermal requirement of Iro-C genes, or to a mis-specification of the muscles. Two independent results indicated that Iro-C genes are required autonomously in the mesoderm to specify the LT fate. First, the

**Figure 1. Pattern of expression of \textit{ara} and \textit{caup} during myogenesis.** Wild type embryos of the indicated developmental stages were hybridized with \textit{caup} (A, A′, B, B′, C, E, F) or \textit{ara} (F) riboprobes or sectioned after anti-Caup antibody staining (A′, A″, B″, C′, D, D′, E′). (A–A″) \textit{caup} is expressed in the visceral mesoderm at early stage 11 (arrowheads, A and A′ show the same embryo with different focus as shown in the inset). (B–B″) At mid stage 11 caup is expressed in the visceral mesoderm (arrowheads) and in the lateral ectoderm (arrows). Asterisks in A and B point to the primordium of the proventriculus. A″, B″ close-ups of the images shown in A′ and B′, respectively. (C–C′) Early stage 12/late stage 11 embryos. (C) \textit{caup} is expressed in the lateral ectoderm (arrowhead) and in groups of mesodermal cells (arrow). (C′) Cross-section showing \textit{caup} expression in ectodermal cells (Ec), visceral mesoderm (Vms) and promuscular clusters (Cl). (D, D′) At stage 12 \textit{caup} is expressed in individual muscle progenitors (P in D) and slightly later in both founders (Fs) derived from the division of progenitors (D′). (E–E′) At stage 13 \textit{Caup} is detected in a lateral stripe of ectodermal cells (arrowheads in E, E′, Ec in E′) and in muscle precursors (arrows in E, E′, M in E′). (F) Stage 15 embryo showing expression of \textit{ara} in the ectoderm and in mature muscles. (G) Stage 15 embryos double stained with anti-Caup (green) and antibodies against Con, Slou or Ladybird (red). \textit{caup} is co-expressed with \textit{Con} in LT1–4 muscles, with \textit{slou} in DT1 and with \textit{lb} in SBM. The drawing scheme summarises the wild type patterns of expression of \textit{caup} (green), \textit{slou} (red), \textit{lb} (yellow) and \textit{Con} (black contour line) in relation to the wild type complement of abdominal muscles. For muscle nomenclature see [1].

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**Figure 2.** Onset of Caup expression in muscles in relation to other muscle identity genes. All images show a detail of an embryonic wild-type abdominal hemisegment stained with antibodies against Caup (green) and different muscle identity proteins. Images show a ventral view of the embryo, with the exception of B and C that correspond to lateral views. (A–C) Stage 11 embryos. (A) caup and Kr (red) are co-expressed in a lateral transverse promuscular cluster (CLTS). (B–C) caup is co-expressed with Kr (blue) in progenitors of LT muscles (PLT1/LT2 and PLT3/LT4, B) and with slou/S59 (red) in DT1/DO3 progenitor (PDT1/DO3, C). (D) Late stage 12 embryo co-expressing caup and lb in the SBM founder (FSBM). (E) Stage 12 embryo showing co-expression of caup with slou/S59 (red) in DT1 founder (FDT1) and with Kr (blue) in LTs founders (FLT1–4). The position of LL1, LL1sib and VA1–3 founders (FLL1, FLL1sib, FVA1–3) and the ventral adult muscle precursor are also indicated. (F) Schematic representation of ara/caup expression in the LTs, DT1 and SBM lineages (SBM lineage as revised in [17]). LaPs, lateral adult muscle precursors; PC, promuscular cluster; P, muscle progenitor; Fs, founder myoblasts.

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**Figure 3.** Muscle phenotypes of Iro-C mutant embryos. (A–D) Latero-ventral region of stage 16 wild-type (A), Df(3L)iroDFM3 (B), Df(3L)iroEGP6 (C) and stage 15 Df(3L)iroDFM3 mef2-GAL4:UAS-ara (D) embryos stained with anti-Tropomyosin antibody (green). The position of ventral wild-type LT muscle tips and LT attachment sites are marked with arrowheads and brackets, respectively. Note the absence of muscles with LT morphology and insertions at LT attachment sites, and the presence of morphologically normal DT1 and SBM muscles (arrows) in the mutant backgrounds (B, C). Asterisks indicate morphological abnormal latero-ventral muscles in these embryos. This phenotype is rescued by mesodermal ara expression with the pan-mesodermal driver mef2-GAL4 (D). (E) Quantification of phenotypes produced by the loss of ara/caup in LT muscles. * Refers to changes in shape, orientation or attachment sites; n, numbers of hemisegments analysed (stages 14–16); -, not determined.

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normal development of LT muscles in Df(2L)5 embryos devoid of Iro-C gene expression at the ectoderm (Figure S2 and [44]). And second, the rescue of the muscle phenotype of Df(3L)iroDEGS mutant embryos by Ara supplied exclusively in the mesoderm (using myocyte enhancer factor 2 (mef2)–GAL4 as driver, Figure 3D).

All progenitors and founders segregate in Df(3L)iroEGR mutant embryos

We next examined whether the loss of LT muscles was due to either a failure in the segregation of muscle progenitors (absences and/or duplications) or to an early transformation of the fate of LT progenitors. To discern between these possibilities we combined the reporter line rP298, which expresses β-galactosidase in all progenitors and founders [32,43] with Df(3L)iroEGR. We focussed on the previously well-established muscle lineages labelled by Slou/S59 [3,17] and the LT1–4 lineages labelled by Kr [18]. With these markers in the lateral-ventral region of rP298 embryos we can identify the following founders (Figure 4A-4A” and insets below). In the dorsalmost lateral mesoderm we find the sibling founders DT1 and DO3 (expressing slou) and the lateral longitudinal 1 (LL1) founder and its sibling (expressing Kr). Immediately below segregate the four LT founders (expressing Kr). And more ventrally appear the sibling ventral acute 1 (VA1) and VA2 founders (which express Kr and slow) and the VA3 founder and its sibling, the ventral adult precursor (that express slow). In Df(3L)iroEGR embryos we observed the same number of identifiable founders (Figure 4B and 4B’). There were however significant differences in terms of patterns of gene expression. Namely, the presumptive LT3–4 founders now expressed slow in addition to Kr (Figure 4B and 4B’ and insets below). This code of muscle identity gene expression is similar to that of VA1 and VA2 founders (Figure 5A), suggesting an early transformation of LT3–4 to VA1–2 muscles.

Ara and Caup implement LT muscle fate by repression of muscle identity genes in progenitors

The absence of all muscles with LT morphology in ara/caup mutant embryos prompted us to examine whether, in addition to the putative transformation of LT3–4 towards VA1–2, there was a similar change of fate for LT1–2. LT progenitors express Kr, caup, Con and lb, Pll1/lll1, slou expresses Kr and zg, and PvlA1/2, slou, Con and Poum (Figure 5A and 5B). Using a combination of these markers we found in the lateral region of Df(3L)iroDEGS embryos an ectopic muscle that expressed Kr+Vg, the code of LL1 (LL1*, Figure 5C) and an ectopic muscle VA2 (VA2* in Figure 5E–5G).

This change of muscle identity could take place in founders or at the progenitor state. If this were the case, we anticipated that both genes resulting from sibling founder myoblasts should be duplicated in Df(3L)iroDEGS embryos. Indeed, using anti-Poum, which labels VA1–3 ([14] and Figure 5B), and antibodies to Kr and Slou, which are maintained only in VA2 (Figure 5B and 5D), we identified two VA2 muscles (that co-express Poum and Kr) and two Poum-expressing VA1 muscles in late stage 14 Df(3L)iroDEGS embryos (Figure 5F). The presence of the duplicated VA1 and VA2 muscles was more evident at stage 15 when Poum was only weakly expressed in VA2 muscles (Figure 5G). We concluded that Ara and Caup were required to specify LT progenitors and that implementation of this fate implies the repression of specific muscle identity genes, such as slow in Pll1/lll1 and zg in PvlA1/2. Moreover, it seemed that the only muscles affected by the lack of ara/caup were those in which these genes were already expressed in the corresponding promuscular clusters, since the fate of DT1 and SBM, visualised by the expression of slou, Con and lb, was apparently unaffected in Df(3L)iroDEGS embryos (Figure 2F, Figure 5D and 5E, and Figure S1E–S1H).

Figure 4. Changes of fate in LT founders of iro-C mutant embryos. (A–B’) Late stage 12 control (A–A’) and rP298;Df(3L)iroEGR sibling embryos (B, B’) stained with anti-βgal (red), anti-Slou/S59 (green) and anti-Kr (blue) antibodies. βgal staining is used as a marker for founders (rP298 line) and the white rectangle in A marks the individual segment shown in A’, A’. (A’, B’) Drawings indicating the relative position of the founders visualised in the corresponding (A’, B’) confocal images. The founders expressing Kr or slow/S59 are labelled by their muscle’s acronyms. Note that although founder segregation is unaffected in Df(3L)iroEGR embryos, the specification of LT founders is altered (B’, B”). Thus, two of the LT founders (LT3–4 in B’, B”), marked by expression of Kr, also express slou/S59, a property exclusive of the VA1–2 founders (see insets below for details of LT founders, the asterisks mark VT1 founder, that expresses slow/S59 but not Kr. Note that Kr is disappearing from LT1 and 3). All panels show Z projections of several consecutive confocal sections with the exception of A’ that corresponds to a combination of two Z projections, one lateral, as the one shown in B’, and other rotated ventrally to show VA4 founders. For muscle nomenclature other than ventral adult precursor (VaP) see [1]. doi:10.1371/journal.pgen.1002186.g004

Ras/MAPK cascade modulates the regulation of slou by Caup in Schneider-2 cells

Our data suggested that Ara/Caup might act as repressors of slou in the Drosophila mesoderm. Therefore we decided to investigate whether slow might be a direct target of Ara/Caup.
An "in silico" search of a previously reported slov cis-regulatory region [46] identified two putative Iro binding sites (BS) at positions +129 (BS1) and +2164 (BS2), relative to the transcription start site, which match the consensus ACAN 2–8TGT ([47] and Figure 6A). We cloned this regulatory region in a Luciferase reporter vector and measured Luciferase activity in Drosophila Schneider-2 (S2) cells transiently transfected with this construct and increasing amounts of HA-tagged Caup. Contrary to expectations, we found that addition of Caup-HA increased the basal Luciferase activity driven by the slov regulatory region in a dose dependent manner (blue bars in Figure 6B), indicating that Caup acts as a transcriptional activator of slov under these conditions. The reported regulation of the chicken Irx2 factor by MAPK (that switches it from repressor to activator) could explain this result [48]. Since Western Blot analysis of S2 cells transiently transfected with this construct and increasing amounts of HA-tagged Caup, we found that addition of Caup-HA increased the basal Luciferase activity driven by the slov regulatory region in a dose dependent manner (blue bars in Figure 6B), indicating that Caup acts as a transcriptional activator of slov under these conditions.

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depend on the binding of a MAPK-dependent phosphorylated protein, which we hypothesize might be Caup, to a so far unidentified binding site. Thus, the analysis in S2 cells confirmed the relevance of BS1, but not of BS2 on Caup-dependent regulation.

Additionally, we have analysed the evolutionary conservation of these putative Caup-BS among several Drosophila species (Figure S3). Notably, BS1 is located in a highly conserved region and its sequence is identical across the melanogaster group, whereas neither BS2 nor the adjacent sequences are conserved. These data further reinforce the relevance of BS1 for Caup-dependent regulation.

Our results are thus consistent with a direct effect of Caup on slou regulation. However, it cannot be ruled out the possibility of the existence of a transcription factor, acting downstream of ara/caup, that could repress slou through BS1 or through a still unidentified regulatory sequence of slou.

Caup integrates in vivo inputs from the Ras/MAPK cascade for its regulation of slou

To further examine in vivo the regulatory activity of Caup on slou (Figure 7B, 7C), we ectopically expressed caup or ara in VA1–3 using Con-GAL4 and checked whether they would repress slou in the VA2 muscle. This was indeed the case (Figure 7B, 7D, 7F–7F0 and not shown). Loss of slou expression caused by ectopic caup reproduced the morphological defects in VA2 previously described in slou mutants (Figure 7F–7F0 and [17]). To analyse whether the morphological effect of Caup on muscle VA2 development was only due to Caup-dependent repression of slou, we forced the expression of both genes using the Con-GAL4 driver. In this experimental condition Caup was unable to repress UAS-slou expression and the VA2 muscle and its morphology seemed unaffected (Figure 7F–7G0).

Once verified the repressor activity of Caup on slou during myogenesis, to analyse the regulatory potential of BS1 in vivo we generated transgenic flies harbouring the wild-type or the BS1 deleted version of the slou regulatory region. The wild-type regulatory region only partially reproduced the slou endogenous expression, as it drove lacZ expression in the CNS but not in the muscles (not shown and Figure S4). In contrast, the construct lacking BS1 behaved congruently with our S2 cells results, since it drove ectopic expression of lacZ in the lateral muscles (Figure S4). Curiously, up-regulation of lacZ was found in the 4 lateral muscles and not only in the ones that show slou expression in the absence of Ara/Caup (Figure 4B). Thus we interpret that this construct, while missing some of the regulatory sequences required for slou mesodermal expression, it contains those required for Caup mediated repression in the mesoderm. In addition, the absence of

Figure 6. Direct interaction of Caup with slou regulatory region and its modulation by the Ras/MAPK pathway. (A) Diagram of the 2 Kb long slou promoter region (from −1828 to +153 nt) used to drive Luciferase expression. This fragment contains two putative binding sites for Ara/Caup, BS1 and BS2. (B) Effect of increasing amounts of Caup-HA on the Luciferase activity driven by slou promoter in the absence (blue bars) and presence (red bars) of PD98059 MEK1 inhibitor. (C) Representative western blots of lysates of S2 cells expressing increasing amounts of Caup (upper panel) in the absence and presence of PD98059, showing the state of activation of the Ras/MAPK cascade (middle panel) and Tubulin expression as loading control (lower panel). (D–F) Mutagenesis analysis of slou regulatory region. (D) Binding of Caup to wild-type fragment resulted in the formation of complexes with reduced mobility (asterisk in lane 4), which was more evident in the presence of increased amounts of Caup (asterisk in lane 7). No shift was observed when fragments devoid of BS1 (Δ BS1, lanes 5, 8) or point-mutated (Mut BS1, lanes 6, 9) were used or in the absence of Caup (lanes 1–3). (E, F) Effect of Caup-HA (1 μg) on the Luciferase activity driven by wt and mutated (BS1*, BS2*) slou promoter regions in the absence (E) and presence (F) of PD98059 inhibitor. Statistical analyses for Luciferase assays were performed using the paired two-tailed Student’s t-test. The data are presented as means ± S.E.M. of 3 independent experiments. *P<0.05, **P<0.001 compared to basal (B) or wt (E, F) conditions.

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strict correlation between the phenotypes of deletion of BS1 and lack of Ara/Caup, might indicate the ability of other transcription factor(s) to regulate slou expression in LT1–2 through BS1.

To investigate whether the effect of the MAPK cascade on the transcriptional activity of Ara/Caup found in the S2 cell assay is also at work during myogenesis we examined whether there is a correlation between MAPK signalling and Caup transcriptional regulatory activity. We looked at the state of activation of this pathway in the LT promuscular cluster, where Ara/Caup repress slou, and found that it did not appreciably express dpErk (Figure 7H). Therefore, a repressor activity of Ara/Caup correlates in vivo with the absence of MAPK signalling. Next, we tested whether forced activation of the MAPK pathway using twist-GAL4; 24B-rasV12 stage 15 embryo showing ectopic expression of slou in eve-expressing muscles. doi:10.1371/journal.pgen.1002186.g007

Figure 7. Ras/MAPK modulates the transcriptional activity of Caup on slou during myogenesis. (A) Schematic drawing of muscles expressing Con in abdominal hemisegments. (B–E) Lateral views of abdominal hemisegments of stage 15–16 wild type (B), Df(3L)iro5DM3 (C), Con-GAL4;UAS-caupHA (D) and Con-GAL4;UAS-caupHA; UAS-rasV12 (E) embryos, stained with S59 antibody. Note the presence of an ectopic VA2 muscle (VA2*) in Df(3L)iro5DM3 (C), the absence of slou in VA2 when caup is ectopically expressed in this muscle (arrow, D, see also F–F”), and the failure of Caup to repress slou/S59 in VA2 muscle in the presence of the activated form of Ras, rasV12 (E). (F–G”) Lateral views of stage 15–16 Con-GAL4;UAS-caupHA; UAS-GFP (F–F”) and Con-GAL4;UAS-caupHA; UAS-slou (G–G”) embryos stained with the indicated antibodies. Note that co-expression of caup and slou in VA2 does not appreciably modify the morphology of the muscle (arrows in G–G”). As an internal control co-expression of UAS-caup and UAS-GFP still repressed endogenous slou and prevented the VA2 fate (F–F”). (H) Close-up of a lateral transverse promuscular cluster (outlined) in a stage 11 wild-type embryo showing co-expression of Caup (red) and Kr (blue) in all cells of the clusters. Note that the activation of the Ras/MAPK cascade (dpErk, green) only takes place at low levels in the segregating progenitor (yellow arrowhead) but not in the rest of the cluster. (I) Close-up of LT cluster in twist-GAL4; 24B-GAL4::UAS- rasV12 stage 11 embryo. Early activation of the Ras/MAPK pathway prevents the repression of slou by Caup in the LT cluster. (J) Close-up of the dorsal mesoderm of a mef2-GAL4;UAS-ara stage 15 embryo showing ectopic expression of slou in eve-expressing muscles.
late co-expression of rasV12 and caup (Con-Gal4 driver) blocked the repression activity of Caup on slow (Figure 7D and 7E). Finally, to test whether MAPK signalling not only prevented Caup-dependent repression of slow but also converted Caup from repressor to activator, we looked at the expression of slow after early pan-mesodermal Caup expression (me72-GAL4). As shown in Figure 7J, Ara was indeed able to ectopically activate slow in Drosophila epidermal growth factor receptor (DER)-dependent eve-expressing muscles.

Discussion

The study of myogenesis in Drosophila has increased the understanding of how the mechanisms that underlie the acquisition of specific properties by individual muscles are integrated within the myogenic terminal differentiation pathway. Thus, the current hypothesis proposes that distinct combinations of regulatory inputs lead to the activation of specific sets of muscle identity genes in progenitors that regulate the expression of a battery of downstream target genes responsible for executing the different developmental programmes [reviewed in [2,10,38]]. However, the analysis of the specific role of individual muscle identity genes and of their hierarchical relationships is far from complete since the characterisation of direct targets for these transcriptional regulators is very scarce [36,37].

Here, we report the identification of ara and caup, two members of the Iroquois complex, as novel type III muscle identity genes. We find that the homeodomain-containing Ara and Caup proteins are necessary for the specification of the LT fate. ara/caup appear to be bona fide muscle identity genes. Indeed, similarly to the identity genes Kr and slow [17,18], absence of ara/caup does not interfere with the segregation of muscle progenitors or their terminal differentiation, but modifies the specific characteristics of LT1–4 muscles, which are transformed towards VA1, VA2, LL1 and LL1 sib fates. These transformations may be due in part to the up-regulation of slow and vg in the corresponding muscles. Thus, a recent report [50] shows that forced expression of vg in LT muscles induces changes in muscle attachments similar to the ones observed in LT1 in ara/caup mutant embryos. However, it should be stressed that although in ara/caup mutants LT muscles are lost in more than 95% of cases, they are not completely transformed into perfect duplicates of the newly acquired fates. For instance, while the specific LT marker bns is lost in 91% of cases, ectopic slow expression is detected in only 75% of cases. These partial transformations might be due to differences in the signalling inputs acting in the mesodermal region from where these muscles segregate (see below). Our unpublished data also showed that forced pan-mesodermal expression of ara/caup alter the fates of many muscles both in dorsal and in ventral regions without converting them into LT muscles (i.e., they do not ectopically express bns). Similarly, Kr and slow ectopic expression is not sufficient to implement a certain muscle fate [17,18]. The failure to re-create a given muscle identity by adding just one of the relevant muscle identity proteins reveals the importance that cell context, that is, the specific combination of signalling inputs and gene regulators present in each cell, have in determining a specific muscle identity.

Our analysis of the myogenic requirement of ara/caup has revealed several features about how these genes act to implement LT fates. Thus, although they are expressed in six developing embryonic muscles, only four of them, LT1–4, are misspecified in the absence of Ara/Caup. The remaining two, DT1 and SBM, seem to develop correctly, according to morphological as well as molecular criteria. It is worth noting that the requirement for ara/caup genes in these six muscles correlates with the onset of their expression. Thus, in the affected LT1–4 muscles Ara/Caup can be first detected at the earliest step of muscle lineages, that is in the promuscular clusters. In contrast, in the unaffected muscles ara/caup start to be expressed later, in the DT1/DO3 progenitor and the SBM founder. This suggests that in muscle lineages ara/caup have to be expressed very early to repress slow and vg to implement the LT fate. Several data support this interpretation. For instance, the observation that ara/caup are co-expressed with slow in DT1, whereas they repress slow in LT3–4, may be related to the fact that slow expression precedes that of ara/caup in the DT1 lineage. Should this be so, one would expect that ectopic expression of ara using the early driver me72-GAL4 would repress slow in DT1, as it actually does (Figure S3), whereas this repression is not evident using the late driver Con-GAL4. Furthermore, the hypothesis of the relevance of the timing of muscle identity gene expression for muscle fate specification might also apply to the case of slow, where a similar correlation between the strength of the loss-of-function slow phenotypes in specific muscles and the onset of slow expression has also been found [17].

It should be stressed that the generation of the LT code depends not only on the early presence of Ara/Caup on the promuscular clusters but also on the absence (or strong reduction) of DER/Ras activity at that precise developmental stage and location (Figure 8). There is a dynamic regulation of MAPK signalling in the lateral mesoderm. Caup-expressing muscles develop from DER-independent clusters whereas the duplicated muscles observed in ara/caup mutants derive from progenitors that segregate very near the LT progenitors [3], but originate in DER-dependent promuscular clusters that are specified slightly later in development [4,51]. Furthermore we have observed both by in vivo and in cell culture that low MAPK activity is required for Caup-dependent slow repression. Therefore, we interpret the role of Ara/Caup in the implementation of LT fate as follows (Figure 8). At mid stage 11 in the myogenic mesoderm, groups of mesodermal cells acquire myogenic competence as a result of interpreting a combinatorial signalling code that reflects their position along the main body axes, as well as the state of activation of different signalling pathways [4]. Accordingly, these clusters initiate the expression of lethal of scute and a unique code of muscle identity genes, as has been shown in great detail for eve expression in the dorsal mesoderm [34,35]. In the case of the dorso-lateral mesoderm this code includes ara/caup and Kr and implements the LT fate. Since the level of activation of the Ras/MAPK cascade is low in these clusters, Ara/Caup will behave as transcriptional repressors, preventing the activation of slow or vg in LT1–2 and LT3–4 clusters, which would be otherwise activated in this location. Thus, Ara/Caup implement the LT fate by repressing the expression of the alternative fates (Kr+, slow+, Con+, Poxm+ and Kr+ and vg+ that would give rise to duplicates of PVA1/VA2 and PL1/L1sib respectively, and by allowing a different identity gene code (Kr+, Caup+, Con+, bns+) that generates the LT fate.}

Slightly later the Ras/MAPK pathway becomes active at the dorsolateral region (Figure 8). This changes the combinatorial signalling code and coincides with a change in the muscle identity genes expressed by the promuscular clusters that segregate from this position, which now accumulate Kr but not Ara/Caup. Progenitors born from them will express either slow or vg and give rise to VA1–2 and LL1/sib fates, all DER-dependent [51].

Our S2 cells experiments suggest a molecular mechanism by which the Ras/MAPK pathway modulates the transcriptional activity of Ara/Caup on slow. Thus, low MAPK activity and direct binding of Caup to BS1 site of the slow gene would favour strong repression of slow. BS1 could be embedded in a silencer regulatory element or its binding to Caup may block transcription of the downstream located luciferase gene. On the contrary, Caup-
dependent activation of slou would be dependent on MAPK signalling. We hypothesize that MAPK-dependent Caup phosphorylation could modulate its interaction with different transcriptional co-factors or/and its binding site affinity.

Furthermore, our in vivo evidence indicates a repressor function of presumably non-phosphorylated Caup on slou since forced activation of the Ras pathway allows co-expression of slou and caup. On the other hand, the ectopic expression of slou induced by caup-over-expression is suggestive of a possible activator function of phosphorylated Caup.

The role of IRO proteins in cell fate specification is conserved in both vertebrates and invertebrates (reviewed in [52]). Here we have shown that the interplay between MAPK signalling and IRO activity found in vertebrate neuroepithelium [48] is also at work in Drosophila myogenesis. We have also identified a potential direct target of Ara/Caup, slou and propose zg as a candidate gene to be regulated by Ara/Caup. In both cases the genes subordinated to ara/caup encode transcription factors that might in turn regulate the expression of other genes, genes that must be repressed in LT muscles in order to acquire the LT fate. These results, therefore, provide insights into the way Ara/Caup control lateral muscle identity and on the role of signalling pathway inputs to modulate the activity of these transcription factors, with consequences in their downstream targets. It also highlights the importance that the specific combination of muscle identity genes, their hierarchical relationships and their temporal activation have in determining the identity of a given muscle cell, very alike to what is at work during the acquisition of neural fates [53].

Materials and Methods

Drosophila stocks

The following stocks were used: Df(3L)iro^{DEL3}, ard^{F079} [28], Df(2L)J5 [54], Df(3L)iro^{DEPS}, Df(3L)iro^{EDAPI} [42], sP298 [32], mef2-GAL4 [55], Con-GALA [56], twist-GALA; 24B-GAL4 (a gift from M. Baylies), UAS-ara, UAS-caup [28], UAS-caup-HA (N. Barrios, unpublished) and UAS-mef2^{F12} [49]. Ectopic expression was generated by means of the GALA/UAS system [57].

In situ hybridisation, immunohistochemistry, and microscopy

Whole-mount in situ hybridisation with digoxigenin-labelled RNA probes and immunocytochemistry were performed as described previously [56]. Stained embryos were embedded in Araldite and sectioned (3 μm) following standard procedures. The following primary antibodies were used at the indicated dilutions: rat-anti-Caup (1:50) [23], guinea pig anti-Kr (1:500) [59], mouse anti-Lb (1:1) [15], rabbit anti-Poxm (1:10) [14], rat and rabbit S59 (that recognises Slou, 1:50) [3], rabbit anti-Allen (1:500) [60], mouse anti-Con (1:10) [61], rabbit anti-Vg (1:500) [62], rat anti-Tropomyosin [MAC141; 1:100; Babraham Tech], rabbit anti-Myosin (Myo; 1:300) [63], rat anti-HA (1:1000; Roche); rabbit anti-B-Gal (1:5000; Cappel) and mouse anti-dpErk (1:50; Sigma). Images were obtained using confocal microscopes MicroRadiance (BioRad) and LSM510META (Zeiss) and analysed using the software Zeiss LSM Image or LaserSharp and Adobe Photoshop 7.0. In most cases the figures correspond to z-projections of series of confocal sections.

Cell culture and transfections

The 5′-upstream region of slou (from −1828 to +153 nt) was amplified via PCR and cloned in pGLH43 vector, a modified version of the pGL2-basic vector (Luciferase reporter plasmid, Promega), obtained after substitution of the SV40 promoter by the Drosophila heat-shock 43 minimal promoter (a gift from A. Baonza). The putative Caup BS1 and BS2 were deleted using the “Quick Change” site-directed mutagenesis kit (Stratagene, SantaClara, CA). The sequences of the primers used to delete BS1 were 5′-GGTTCTTAAATCCACCCTGTTGTGTTGCCCTGTGGCAGTCACATAG-3′ and its reverse complement and for BS2, 5′-
CCATATACATATGTTGCATGTATGCAATGTTGAGTTGTGAGTGGG-3’ and its reverse complement. pAC5.1-Caup-HA plasmid was obtained after cloning caup ORF with an HA tag in the Drosophila expression vector pAC5.1 (Invitrogen). Drosophila S2 cells were cultured in Insect-Xpress medium (Lonza) supplemented with 7% fetal bovine serum and grown at 25°C. For Luciferase assays S2 cells were seeded at a density of 2x10^5 and co-transfected with 1 μg of the different firefly Luciferase reporter constructs DNA, 30 ng of control plasmid (expressing Renilla Lucerase driven by the promoter of Drosophila RpIII128, [64]) and either 0, 0.25, 0.5 or 1 μg of pAC5.1-Caup-HA plasmid per well using Nucleofector Technology (Lonza). Luciferase activity in the cell extracts was measured using Dual-Glo Luciferase assay system (Promega) following the manufacturer’s protocol. Briefly, 20 μl extract was added to 100 μl F-luc assay reagent, mixed gently for 5 s and placed in a luminometer. After counting F-luc activity for 10 s, 100 μl stop-and-glo reagent was added to the tube, mixed gently for 5 s and placed in the luminometer for R-luc count. The R-luc activities were used as internal control to correct for the difference in transfection efficiency of different reporter plasmids. Therefore, F-Luc/R-Luc activities were used for data analysis. To investigate whether the MEK/ERK pathway was involved in transcriptional regulation driven by the slou promoter, S2 cells were treated or not with 50 μM PD-98059 (Sigma) for 2 hrs before Luciferase activity measurement. All data reported are means from three or four independent experiments, each performed in triplicates. Primary antibodies used in immunoblots were mouse anti-dpErik (1 μg/ml; Sigma), rat anti-HA (200 ng/ml; Roche) and mouse anti-β-tubulin (1:5000; Developmental Studies Hybridoma Bank).

Generation of slou reporter transgenic lines

The 5’-upstream region of slou used in S2 cells in the Luciferase reporter assays (both the wild type sequence and that missing the putative Caup BS1) were subcloned at the EcoRI site of the C4PLZ enhancer tester plasmid that contains a weak P-element reporter plasmid [65]. These lacZ reporter plasmids were introduced into y w1118 embryos by standard P-element transformation.

Electrophoretic mobility shift assay

Caup binding activity to the slow promoter region was analyzed by EMSA. Pairs of single-stranded, Cy3c and unlabeled 40-mer oligonucleotides containing the wild-type putative Caup binding sites BS1, BS2 and their mutant or deleted versions were allowed to anneal to generate double-stranded probes. Sequences of primers are shown in Figure 6D for BS1 and in Dataset S1. Caup protein was synthesized in vitro by using the coupled transcription/translation rabbit reticulocyte lysate system (TNT Promega). The indicated amount of μl of TNT reaction mixture was incubated with 20 ng of labelled probe. Protein-DNA complexes were allowed to form at room temperature for 30 min in a total volume of 20 μl of binding buffer (50 mM HEPES, pH 7.5, 10 mM MgCl2, 10 mM KCl and 1 mM DTT). After incubation, free DNA and protein–DNA complexes were resolved by 6% non-denaturing polyacrylamide gel electrophoresis. Gel fluorescence was analyzed in a Typhoon Scanner (GE healthcare).

Supporting Information

Dataset S1 Sequences of primers used in EMSA to analyse binding of Caup to BS2. Pairs of 40-mer oligonucleotides containing the wild-type putative Caup binding sites BS2 and their mutant or deleted versions are shown.

Figure S1 Pattern of expression of muscle marker genes in Df(3L)iroDFM3 embryos. (A–D) RNA in situ hybridisation with bsu probes of stage 13 (A, B) and stage 15 (C, D) yw (A, C) and Df(3L)iroDFM3 (B, D) embryos, showing the normal early onset of bsu expression in the lateral region of abdominal segments in the mutant embryos (B, compare to A) and its absence of expression at later stages (D, compare to C). (E, F) Lateral view of stage 14 yw (E) and Df(3L)iroDFM3 (F) embryos stained with anti-Con antibodies, showing the absence of Con-expressing lateral muscles (asterisk in F) and the presence of Con-expressing DT1, VA2 and ectopic VA2 (VA2* in F) in Df(3L)iroDFM3 embryos (F, compare to E). (G, H) Lateral view of stage 15 yw (G) and Df(3L)iroDFM3 (H) embryos stained with anti-β-tub antibodies to show the presence of β-tub expressing SMB in Df(3L)iroDFM3 embryos (H, compare to G).

Figure S2 Regulation of caup expression during embryogenesis. (A, B) Lateral view of stage 15 wild-type (A) and Df(2L)5 (B) embryos stained with anti-Alien (green) and anti-Caup (red). Note that in Df(2L)5 embryos despite the absence of Caup ectodermal expression (asterisk in A), apodema specification (labelled by Alien) and Caup mesodermal expression (arrowheads) are indistinguishable from wild-type embryos.

Figure S3 Caup BS1 but not BS2 of slou cis-regulatory region is evolutionarily conserved between Drosophila species in the melanogaster group. The slou cis-regulatory region used in this study was compared between drosophilids using the VISTA Browser tool of VISTA tools for comparative genomics [http://blast.ncbi.nlm.nih.gov/ vista/index.shtml]. We found a high degree of similarity in this region between D. melanogaster and other members of the melanogaster subgroup (D. simulans, D. yakuba and D. erecta) and only partial similarity with more distant species like D. ananassae (melanogaster group) and D. pseudoobscura (obscura group). BS1 is located in a highly conserved region and its sequence is identical across the melanogaster group, whereas BS2 is located in a region of low conservation and not found in any of the related species. Significant similarities on slou coding and cis regulatory regions were only found between Drosophila melanogaster and the closer drosophilid species D. simulans, D. yakuba, D. erecta and D. ananassae. No homology was found using the BLAST tool (http://blast.ncbi.nlm.nih.gov/blast.cgi) with Anopheles gambiae, Apsi melifea, Xenopus tropicalis, Danio rerio, Mus musculus and Homo sapiens.

Figure S4 Deletion of Caup BS1 promotes lacZ expression in LT muscles driven by slou cis-regulatory region. Lateral views of stage 15 slou-lacZ (A, A’) and ABS1-slow-lacZ (B, B’) embryos stained with anti-Caup (green), anti-βgal (red) and anti-Myo (blue) antibodies. Note absence of lacZ expression in LT muscles of slou-lacZ embryos (arrows in A, A’) and co-expression of caup and lacZ in LT muscles of ABS1-slow-lacZ embryos (arrows in B, B’).

Figure S5 Repression of slou by ectopic expression of Ara. Lateral views of stage 15 wild-type (A) and mef2-GAL4:UAS-ara (B-B’) embryos stained with anti- Troponymosin (red) and anti-slow (green) antibodies. (A) Note slow expression in DT1, VA2 and VT1 muscles (arrows). (B) Early expression of ara with the panmesodermal driver mef2-GAL4 represses slou in DT1, VA2 and VT1 in many segments (arrows). A few muscles maintain slow expression (asterisks).

(DOCX)
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Author Contributions

Conceived and designed the experiments: MC-R AS MR-G. Performed the experiments: MC-R AS SP-S LP NB AL PM-RG. Analyzed the data: MC-R AS MR-G. Wrote the paper: SC MR-G.

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