BMPs Regulate msx Gene Expression in the Dorsal Neuroectoderm of Drosophila and Vertebrates by Distinct Mechanisms

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Abstract

In a broad variety of bilaterian species the trunk central nervous system (CNS) derives from three primary rows of neuroblasts. The fates of these neural progenitor cells are determined in part by three conserved transcription factors: vnd/nkx2.2, ind/gsh and msh/msx in Drosophila melanogaster/vertebrates, which are expressed in corresponding non-overlapping patterns along the dorsal-ventral axis. While this conserved suite of “neural identity” gene expression strongly suggests a common ancestral origin for the patterning systems, it is unclear whether the original regulatory mechanisms establishing these patterns have been similarly conserved during evolution. In Drosophila, genetic evidence suggests that Bone Morphogenetic Proteins (BMPs) act in a dosage-dependent fashion to repress expression of neural identity genes. BMPs also play a dose-dependent role in patterning the dorsal and lateral regions of the vertebrate CNS, however, the mechanism by which they achieve such patterning has not yet been clearly established. In this report, we examine the mechanisms by which BMPs act on cis-regulatory modules (CRMs) that control localized expression of the Drosophila msh and zebrafish (Danio rerio) msxB in the dorsal central nervous system (CNS). Our analysis suggests that BMPs act differently in these organisms to regulate similar patterns of gene expression in the neuroectoderm: repressing msh expression in Drosophila, while activating msxB expression in the zebrafish. These findings suggest that the mechanisms by which the BMP gradient patterns the dorsal neuroectoderm have reversed since the divergence of these two ancient lineages.

Introduction

In both Drosophila melanogaster and vertebrates, Bone Morphogenetic Proteins (BMPs) are expressed in the epidermal ectoderm abutting the dorsal border of the neuroectoderm [1]. The genetic network that underlies formation of a centralized nervous system consisting of segregated motor and sensory centers appears to have been conserved across bilaterians [animals with right-left symmetry] [2]. BMPs are thought to exert a common function in the early epidermal ectoderm during neural induction (i.e., suppressing expression of neural genes in epidermal regions that experience peak BMP levels). BMP signaling also acts subsequently in a dose dependent fashion to pattern dorsal versus medial regions of the neuroectoderm. For example, the trunk Central Nervous System (CNS) of both invertebrates and vertebrates consists of three primary rows of neuroblasts that are determined by the expression of three conserved transcription factors. In metazoan species spanning all three primary branches (e.g., Ecdysozoa -Drosophila, lophotrochozoa – annelids, and deuterostomes - vertebrates) “neural identity” genes [vnd/nkx2.2, ind/gsh and msh/msx] are expressed in the same relative order and orientation with respect to the dorsal-ventral axis and an epidermal BMP source. Moreover, in a broad range of organisms, BMPs and opposing antagonists have been found to play a key role in patterning the ectoderm and establishing neuronal fates. These commonalities suggest an ancestral origin for the CNS among bilateria [1–4] and raise the possibility that BMPs play a conserved role in patterning the CNS axis.
Element of SBE in the Distal BMP Regulation of msh and msh Genes

1. The previously suggested BMP-dependent pathway in vertebrates, which involves a common ancestral transcriptional regulatory mechanism for BMPs, remains to be investigated. Previous studies have suggested that a shared signaling pathway in both groups of animals is responsible for activating neural identity genes in dorsal regions and is repressed by Dpp signaling. However, the exact mechanism of this regulation has yet to be elucidated.

2. The role of BMPs in promoting epidermal over neural cell fate is consistent across species, with BMPs expressed in dorsal epidermal regions of the early embryo. This suggests a shared regulatory mechanism controlling the expression of the msh gene.

3. The BMP response in neural cells is distinct from that in epidermal cells. While BMPs act on neural cells to repress expression of neural genes, they act on epidermal cells to activate expression of epidermal genes.

4. The BMP response in neural cells is mediated by the transcriptional repressor protein and the inhibitory SMAD (CRMs), while genes that are directly activated by BMPs, such as the msh gene, remain unregulated.

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The msh CRM contains direct BMP-responsive sites involved in repression

To identify BMP responsive sites within the ME, we first scanned this element for known consensus binding sites for Mad, Med, Shn, and Brk. The two best characterized BMP responsive elements are the Silencer Element (SE), which binds a trimeric complex comprised of pMMS (GNCGNC(N)5GNCTG), and the activator element (AE), which binds pMM heteromers (GGCGCCA(N)4GNCV). Brk binding sites (T)GGCGYY overlap with a subset of AE elements [24]. Although there are no perfect consensus SE, AE, or Brk sites within the ME, we identified several candidate sites with either single base-pair mismatches to the SE or AE elements or variable spacer length (N)5–6. We defined three such candidate SE sites (SE1, SE2 and SE3) with a single nucleotide mismatch and two conserved candidate AE sites with a spacer of 6 nucleotides (conforming to the expanded consensus: GNCGNC(N)6GNCV) and tested each of these sites for direct DNA binding of pMM or pMMS complexes in vitro using Electrophoretic Mobility Shift Assays (EMSAs).

The SE1 and SE2 candidate silencer sites (Fig. 2A) both conform to the relaxed consensus of GNYGNC(N)5GNCTG (where Y can be either C or T). EMSA experiments using DNA oligonucleotide probes reveal that pMM and pMMS complexes assembled on the SE1 and SE2 sites in a BMP dependent fashion (Fig. 2B) but not on the SE3 site (Fig. S1C). As expected, mutation of the Med (SBE) motif within the SE1 (SE1\textsuperscript{SBE}) or SE2 (SE2\textsuperscript{SBE}) sites abolished binding of all BMP responsive complexes in vitro. In contrast, none of the candidate AE or Brk sites bound pMM, pMMS, or Brk complexes (Fig. S1C) (see below however, regarding effects of mutating or deleting the candidate AE sites).

In order to test the in vivo roles of the SE sites, we mutated each site (i.e., using the same SBE mutations that abolished all BMP responsive DNA binding in vitro described above) and generated a series of small deletions spanning virtually the entire ME (i.e., all but 36 bp). These mutant constructs were inserted into the same chromosomal integration site as the reference ME construct using the PhiC31 transgenesis system [28]. Deletion of the 5’ most 100 bp of ME, which contains both SE sites, led to dorsal expansion of reporter gene expression (Fig. 2C). Transgene expression, however, was also weaker within its normal neuroectodermal domain, suggesting that contributing activation sites are also present within this region. Targeted mutation of the individual SE1 and SE2 sites also led to discernable dorsal expansion of reporter gene expression, which was more pronounced for the SE2 mutant. Mutating both SE sites in combination (SE1, SE2 double mutant) resulted in more prominent dorsal expansion than observed for either mutant alone, but still less than that observed for the wild-type ME (or the endogenous msh gene) crossed into a dpp- mutant background. We conclude that SE elements mediate direct BMP-dependent repression of the ME and that additional direct or indirect BMP-dependent inputs also contribute to negatively regulating this CRM.

Figure 1. The msh CRM responds to BMPs. Lateral and dorso-lateral views (anterior to the left) of blastoderm stage Drosophila embryos showing msh, shn and ME-lacZ reporter construct expression detected by in situ hybridization, and schematic overview of the msh locus. (A) Transgenic embryos carrying the lacZ reporter gene under control of the ME were hybridized with probes against msh (green) and ME-lacZ (red). (B) The ME-lacZ construct was crossed into a dpp null background. Expression of both msh and ME-lacZ expands dorsally in dpp- homozygous mutant embryos. (C) In shn- homozygous zygotic mutant embryos, anterior msh expression expands towards the dorsal regions of the embryo compared to wild-type. A maternal contribution of shn remains intact in these embryos. (D) The msh locus depicting the location of the ME CRM. (E) Genome wide ChIP data representation depicting peaks of Mad, Med and Shn binding signal as well as DNase hypersensitivity sites reflecting regions of open chromatin in the ME CRM region.

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Differential affinities of pMMS complexes for SE sites in the ind and msh CRMs may contribute to threshold-dependent repression of these genes.

Our prior genetic studies revealed that BMP signaling is more effective in repressing expression of ind than msh [7]. One possible explanation for this differential response is that the ind CRM might contain higher affinity SE sites than those in the msh CRM. Indeed, a single perfect consensus matching SE site in the ind CRM (Fig. 2B) has been shown to be required for repression of this element dorsally [20,29]. In line with the possibility that SE sites in the ind and msh CRMs have differing affinities for binding pMMS complexes, modifying the SE2 site by one base-pair to adhere to the optimal SE consensus resulted in greater pMMS binding (Fig. 2B - SE2*), which was most evident in competition experiments (Fig. S1D). We tested whether the optimized ind-like SE2* site would result in repression of msh CRM activity in vivo. In support of this site being more effective at recruiting repressive pMMS complexes, reporter gene expression driven by the SE2* ME was greatly reduced relative to wild-type SE2 (lanes 11–13, see Fig. S1D for further verification of this effect in a competition assay). (C) Lateral and dorsal views (anterior to the left) of in situ hybridization detection of lacZ expression in ME-lacZ embryos demonstrating the in vivo effects of mutating SE1 and SE2 SBE motifs. ME: wild type embryos containing the intact ME-lacZ construct; dpp: dpp- mutant embryos show complete dorsal expansion of lacZ expression; D1: deletion of the first 100 bp of ME spanning the SE1 SE2 region leads to partial dorsal expansion accompanied by reduced levels of expression; SE1: mutating SE1 leads to modest dorsal expansion; SE2: mutating SE2 leads to significant dorsal expansion; SE1 SE2: mutating both SE1 and SE2 results in yet more pronounced dorsal expansion although not as extensive as observed for the wt-ME in a dpp- mutant background; SE2*: Converting SE2 to an optimal (ind-like) SE site results in reduced lacZ expression; SE2* dpp*: SE2* in a dpp- background shows complete dorsal expansion of lacZ.

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Figure 2. The msh CRM contains BMP Silencer Elements contributing to dorsal repression. (A) Diagram of ME showing the location of Silencer Elements SE1 and SE2 within highly conserved regions. Conservation score (Cons) is based on alignment of the ME region in 12 Drosophila species [61]. (B) Autoradiograph of electrophoretic mobility shift assay gels with radiolabeled oligonucleotide probes incubated with extracts from S2 cells over-expressing activated Tkv (to induce BMP signaling), Med and Mad in the presence or absence of over-expressed C-terminal Schnurri (Shn). EMSA probe sequences depict the location of the GC-rich (red) and SBE (blue) motifs as well as the ind SE site [20]. Mutations relative to wild type sequences are indicated by lower case. SE1 and SE2 containing probes show higher molecular weight retardation when Shn is present versus Med and Mad alone (lanes 1–3 and 6–8). When the SBE motif of either SE1 or SE2 is mutated, probe retardation is no longer observed (lanes 4/5 and 9/10). When SE2 is mutated to conform to the canonical SE consensus, SE2*, the amount of probe retained appears higher compared to wild-type SE2 (lanes 11–13, see Fig. S1D for further verification of this effect in a competition assay).
The msh CRM contains essential activation sites that resemble BMP responsive sequences

As mentioned above, in our initial search for BMP-responsive sites in the ME we identified two sites that were similar to activation elements (AE) but that did not bind pMM complexes in EMSA assays (Fig. S1C). We nonetheless tested for potential roles of these sites by deleting them or creating a point mutation in one of them (AE2). Deletions encompassing either AE1 or AE2 or the AE2 point mutation greatly reduced ME-lacZ expression (Fig. 3-B,C,E), while deletion of the 3’ most region containing a previously reported Ind site [25] resulted in ventral expansion of reporter gene expression as expected. We tested the possibility that activation of the ME via AE2 might be balanced against repression mediated by the SE1 and SE2 sites by constructing a triple mutant in which all three sites were eliminated. We reasoned that if the AE2 site, which acts as a bona fide activation site, functions in a BMP-independent manner, combining it with the double SE site mutant might result in loss of expression (the AE mutant phenotype). On the other hand, if the AE2 site were providing an important activation function in the neuroectoderm via BMP signaling, the triple mutant should at least show ectopic expression dorsally (e.g., if this was a BMP-dependent activation site, relieving repression would give rise to normalized expression since we would be removing both activating and repressing components). We found loss of expression in this triple mutant comparable to that of the AE2 single mutant (Fig. 3F), suggesting that activation via the AE2 site is BMP-independent.

Although the above analysis suggests that the AE2 site acts in a BMP-independent fashion, we further examined the possibility that BMPs might play an activating as well as repressive role in regulating msh expression. Embryos that are dorsal- (maternal); dpp- (zygotic) double mutants express msh ubiquitously [11]. To test whether there might be a threshold at which Dpp enhances rather than suppress msh expression, we attempted to augment msh expression locally by generating embryos that lack Dorsal and whose only source of Dpp is one copy of dpp driven in a narrow stripe by the eve 2 CRM (Fig. 3G,H) or by, adding progressive amounts of Dpp (by varying copy number of the dpp locus – Fig. S2A,B). In both cases, we observed only a diminution in msh expression, further arguing against any activating role for Dpp. Finally, we considered the possibility that BMPs might act indirectly to regulate msh expression via non-canonical mechanisms (e.g. via ETS or the HMG-box Ctc transcription factors) by altering EGF-R signaling. We found no evidence, however, for a role of EGF-R signaling in influencing the position of the dorsal border of msh expression (Fig. S2C-E). In aggregate, our experiments suggest that BMP-dependent regulation of the ME is mediated by SE sites and by additional inhibitory inputs, which may act either directly or indirectly.

Identification of early embryonic vertebrate msh CRMs

The above analysis of the msh CRM in Drosophila is consistent with genetic data indicating that BMPs act by dosage sensitive repression of neural identity gene expression [7]. To determine the mechanism by which BMPs regulate expression of orthologous vertebrate Msx genes we sought to identify the zebrafish (Danio rerio) mshB CRM using the powerful tol2 transgenesis system [30]. We choose to focus on regulation of the mshB gene among the zebrafish Mxs paralogs as this gene has the earliest onset and most specific pattern of expression in the dorsal neuroectoderm [31]. We identified a 2.4 Kb region of DNA immediately upstream of the zebrafish mshB coding region that drives faithful reporter gene expression in the dorsal neuroectoderm in both neural plate and early neural tube stages (i.e., 3-6 somite stage embryos) of a stable transformant line (Fig. 4A,B). This fragment has two peaks of strong sequence conservation among vertebrates, which overlap regions of predicted open chromatin [32] (Fig. 4A).

Later during neural tube stages, the early neural plate expression pattern fuses into a single dorsal zone (e.g., top panels in Fig. 4D).

We also tested a 5 Kb genomic fragment upstream of the mouse (Mus musculus) Msx1 gene, which like the zebrafish mshB CRM carries sequences lying immediately upstream of the transcriptional start site (Fig. 4A). When the mouse CRM-GFP construct was introduced into zebrafish embryos, it drove expression in a pattern (Fig. 4B) very similar to that of the fish mshB gene as well as that observed endogenously in mice. These results suggest that both the zebrafish and mouse CRMs contain sufficient information to correctly direct expression to the dorsal ectoderm despite the fact that they show only limited sequence conservation. These observations provide another clear example of the highly conserved function of vertebrate CRMs from lineages that diverged over 400 MYA in the absence of obvious sequence conservation in these non-coding regions [33,34].

The zebrafish msxB CRM contains a BMP responsive site mediating activation

We pared down the zebrafish msxB CRM in transient transformant embryos and identified a minimal 671 bp fragment containing the most conserved island that also faithfully recapitulates mshB expression in dorsal neuroectodermal/neural crest progenitor cells (Fig. 4D). Parallelizing our approach in Drosophila, we searched for BMP responsive sites within the minimal msxB CRM by first scanning bioinformatically for candidate SE or AE sites using the SMAD1/5/8 consensus GNCKNC and SMAD4 consensus GNC(T/V) with relaxed spacing constraints, and then testing by EMSA whether oligonucleotides containing these sites could indeed assemble Drosophila pMM and/or pMMS complexes in response to BMP signaling in vitro (Fig. S3). This analysis identified a single highly conserved site (zAE) to which BMP signal-dependent pMM (but no pMMS) DNA binding was observed. The zAE contains candidate SMAD1/5/8 and SMAD4 binding sites separated by an unusually long 16 bp spacer (Fig. S3A,B). These sites are also present in mouse albeit with different spacing (12 bp). Further analysis of this binding motif revealed that the SMAD1/5/8 and SMAD4 sites are each required, suggesting that the functional zAE includes both sites (Fig. S3C). Changing the sequence or length of the spacer DNA linking the two sites did not affect the ability to form pMM complexes in vitro indicating that the exceptional length of the zAE spacer is not required for SMAD complex formation in vitro. Interestingly, however, changing the linker length to 5 bp allowed the formation of trimeric pMMS complexes (Fig. S3D).

We generated a 36 base pair deletion spanning the zAE and both the SMAD1/5/8 and SMAD4 candidate binding sites – DEL mutant) in the context of the 671 bp msxB CRM and observed that GFP reporter gene expression was lost in transient transformant embryos (Fig. 4D). Similarly, mutation of two core base pairs in the GC-rich region of the zAE (GCR1 mutant), which abolished pMM binding in vitro (Fig. 4C; Fig. S3C), also reduced reporter expression in vivo in transient transformant embryos (Fig. 4D). These results indicate that a single BMP responsive site within the 671 bp zebrafish msxB CRM is required for mediating reporter gene activation by this element in vivo.
Fly and vertebrate \textit{msx} CRMs respond oppositely to BMPs.

The above dissection of BMP-responsive sequences within the \textit{Drosophila} and zebrafish \textit{msh}/\textit{msx} CRMs suggests that they are under opposing forms of BMP regulation: repression in \textit{Drosophila} versus activation in zebrafish. To test this hypothesis further, we compared the response of these CRMs to alterations in BMP signaling \textit{in vivo} (Fig. 5). In \textit{Drosophila}, we examined \textit{msh} and ME-reporter gene expression in both a \textit{dpp}-mutant background and in embryos ectopically expressing \textit{dpp} in the dorsal epidermis. As mentioned above, \textit{msh} and ME-reporter gene expression both expand dorsally in \textit{dpp}-mutant (Fig. 1B; Fig. 5A). Conversely,
ectopic dpp expressed from a Heat Shock-dpp construct (HS-dpp) resulted in loss of msh expression within its normal domain (Fig. 5C). In zebrafish, a stable transgenic line carrying the 2.4 kb msxB-CRM construct was crossed to lines carrying either a Heat Shock-chordin (HS-CHD) or a Heat Shock-BMP (HS-BMP) construct. When the BMP antagonist Chordin was induced by heat treatment (Fig. 5G), msxB-GFP reporter expression was strongly suppressed, as was endogenous msxB expression (Fig. 5D). The opposite effect was observed in HS-BMP embryos, however, where expression of endogenous (Fig. 5F) and reporter (Fig. 5I) genes was broadened compared to control embryos (Fig. 5E and 5H, respectively) that were subjected to the same conditions. Thus, consistent with the inverse effects of mutagenizing BMP-responsive sites in the Drosophila msh and zebrafish msxB CRMs, these two

Figure 4. Identification of vertebrate msx CRMs. (A) Representation of the zebrafish msxB and mouse Msx1 loci depicting the location of the CRMs and vertebrate sequence conservation (Cons) (http://genome.ucsc.edu/). For zebrafish, histone 3 lysine 4 single and triple methylation patterns indicative of open chromatin are also indicated. Block conservation tracks for select species are represented for both loci. (B) In situ expression driven by msx CRMs. Dorsal (anterior to the top) and lateral (anterior to the left) views of transgenic zebrafish embryos at the open neural plate stages (3–6 somites). Embryos were injected with either msxB-CRM or Msx1-CRM constructs driving gfp and stable transgenic lines were subsequently bred. Stable transgenic embryos were stained for msxB and gfp expression, which was detected by in situ hybridization. Both CRMs drive patterns resembling the endogenous msxB pattern. The zebrafish DNA isolated contains sufficient information to drive a pattern resembling the endogenous msxB expression pattern. The cloned mouse CRM also is capable of responding to regulatory cues in the zebrafish embryo to drive expression resembling that of the zebrafish msxB gene (as well as the endogenous Msx1 gene in mice [62]). Note that this embryo is tilted in a slightly more rostral direction than the other embryos shown from the dorsal perspective, which results in bands of anterior expression coming into view. (C) EMSA experiment in which a radiolabeled oligonucleotide probe carrying the zAE element (Msx11F in Fig. S3) was incubated with extracts from S2 cells over-expressing activated Tkv (to induce BMP signaling), Med and Mad. When the GC-rich region of the mad1 binding site is mutated (GCR1), pMM binding is abolished (the same loss of binding was also observed for the mutation in the mad2 site, GCR2 - see Fig. S3C). (D) Mutation of the pMM zAE site greatly reduces specific expression driven by the 671 bp msxB CRM. Dorsal (anterior to the top) and lateral (anterior to the left) views of injected zebrafish embryos (6–8 somites). Embryos were injected with GFP-reporter constructs under the control of the intact 671 bp msxB-CRM, a 30 bp mutant deleting the zAE (msxB-CRMDEL), or a point mutant version of the zAE that abolishes pMM binding – see panel C (msxB-CRMGCR1). Both zAE mutant constructs show greatly reduced reporter expression. Transient gfp mRNA expression was detected by in situ hybridization.

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elements respond in an opposing fashion to equivalent manipulations of BMP signaling in vivo. Our analysis strongly suggests that BMPs pattern the neuroectoderm primarily via repression in Drosophila, while in zebrafish, BMPs function, at least in part, to activate the orthologous msxB gene.

**Discussion**

BMPs play a highly conserved role in neural induction and also contribute to establishment of dorsal-ventral polarity within the CNS. In the latter case, however, it has not been established whether they act through common or distinct mechanisms to effect dose-dependent patterning of neural identity genes. Since BMPs regulate expression of highly conserved members of the ancient msh/msx family in the dorsal neuroectoderm of both Drosophila and vertebrate embryos, comparing cis-regulation of these genes by BMPs provides an excellent paradigm for addressing whether cis-regulatory processes are maintained across distant taxa. Our analysis of the Drosophila msh embryonic CRM suggests that BMPs act in part through two SE-type binding sites that mediate repression, while activation sites do not appear to mediate responses to BMP signaling. In contrast, we identified a single SMAD binding site within an embryonic zebrafish msxB CRM that is required for BMP-dependent activation. These findings suggest that BMPs act on msh/msx CRMs by opposite mechanisms in these two lineages, while nonetheless driving similar output expression patterns in the dorsal neuroectoderm.

Silencer sites mediate graded BMP responses in the Drosophila neuroectoderm

Mutational analysis of the Drosophila msh CRM in this study supports a direct role for BMP repression acting via the SE1 and SE2 sites to suppress activity of this element in the dorsal ectoderm where there are likely to be moderate levels of BMP signaling. Mutation of either of these sites results in reduced CRM activity compared to wild type embryos (B) consistent with Dpp having a repressive action on msh expression, while zebrafish HS-BMP embryos exhibit ectopic expression of both the endogenous msxB gene and the GFP reporter gene driven by the msxB-CRM when compared to wild-type.

Distinct BMP Regulation of msh and msx Genes

**Figure 5.** Manipulating BMP signaling elicits opposite responses from msh and msxB in Drosophila and zebrafish embryos. Comparison of equivalent BMP manipulations in Drosophila (Dmel) and zebrafish (Drer) embryos and their effects on msh, msxB and msxB-CRM driving GFP assayed by in situ hybridization. All embryos are oriented with dorsal at the top. Drosophila embryos are oriented with anterior to the left, while zebrafish embryos are view from a posterior perspective. Loss of BMP signaling is Drosophila was examined in dpp null mutant (dpp−) embryos (A) and ectopic BMP signaling was generated by heat induction of transgenic embryos carrying eight copies of a heat-shock dpp construct (HS-8xdpp) (C). In zebrafish, BMP signaling was reduced by heat induction of transgenic embryos carrying a Heat Shock Chordin construct (HS-CHD) (D, G), while BMP over-expression was accomplished by induction of transgenic embryos carrying a Heat Shock BMP2 construct (HS-BMP) (F, I). In dpp− embryos, msh expression expands dorsally as shown also in Fig. 1B. In contrast, in HS-CHD embryos, expression is weakened relative to the wild-type pattern for both the endogenous msxB gene (E) and msxB-CRM-gfp reporter construct (H). Drosophila HS-8xdpp embryos show reduced msh expression compared to wild type embryos (B) consistent with Dpp having a repressive action on msh expression, while zebrafish HS-BMP embryos exhibit ectopic expression of both the endogenous msxB gene and the GFP reporter gene driven by the msxB-CRM when compared to wild-type.

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the A2 site in the D3 region, Fig. 3C). If these hypothetical repressor sites act directly on the msh CRM they would presumably bind Mad, Medea and Schnurri, MAPK pathway transcriptional effectors, or possibly yet unknown BMP mediators alone or in conjunction with other transacting factors. Our detailed bioinformatic analysis and systematic experimental EMSA surveys have failed to identify any such sites, however. It is also possible that part of the BMP response of the msh CRM is mediated indirectly. For example, we have previously reported that localized overexpression of Brk can de-repress msh expression dorsally [7], yet there are no consensus Brk sites in the ME and we were unable to detect Brk protein binding to any closely related candidate Brk sites by EMSA (Fig. S1). Thus, Brk may act via regulating expression of other components required for BMP signaling such as the BMP type-1 receptor Thick veins [35]. Alternatively, activators of the ME may be under negative BMP/Brk regulation.

The SE1 and SE2 sites that play a role in repressing ME activity dorsally are imperfect matches to the consensus SE sites determined by Pyrowolakis and colleagues [36]. The ind CRM, however, which according to genetic data is more sensitive to BMP repression than msh [7], contains a perfect SE site required for repressing activity of this element dorsally [20]. When the SE2 site in the ME was mutated to similarly match the ideal SE consensus repressing activity of this element dorsally [20]. When the SE2 site in the ME was mutated to similarly match the ideal SE consensus sequence (SE2*) it repressed ME expression in its normal dorsal in vivo [7], contains a perfect SE site required for repressing activity of this element dorsally [20]. When the SE2 site in the ME was mutated to similarly match the ideal SE consensus sequence (SE2*) it repressed ME expression in its normal dorsal in vivo.

Alternatively, activators of the ME may be under negative BMP/Brk regulation.

**Evolution of conserved gene expression patterns**

In Drosophila, Evo/Devo studies of the even-skipped stripe 2 CRM have suggested that regulatory mechanisms that lead to a particular gene expression pattern are extremely flexible, i.e., the same pattern can be achieved in multiple ways [39]. Accordingly, in the current case of BMP-dependent regulation of msh/msxB expression, natural selection may have operated similarly to maintain relevant gene expression patterns that fulfill a particular function (i.e. dorsal neuroectodermal expression) while allowing the upstream mechanisms generating that pattern to change over time.

As summarized above, our analysis strongly suggests that BMPs pattern the neuroectoderm primarily via repression in Drosophila, while in zebrafish, BMPs function, at least in part, to activate the orthologous msxB gene. Genetic studies and exogenous BMP treatment in zebrafish suggest that msh gene expression may also be repressed by high levels of BMP signaling. Whether the BMP-responsive site in the 671 bp msxB CRM together with other potential BMP-responsive elements mediate such a biphasic response will be interesting to address in future experiments. In the future, it will also be important to determine whether expression of other msx paralogs in the dorsal CNS of zebrafish (e.g., msxC-F [31]) or msx genes in other vertebrates (e.g., the murine Msx1 neuroectodermal CRM identified here) are similarly regulated by BMPs. Analysis of these additional vertebrate msx CRMs should reveal whether distinct evolutionary trajectories have shaped the BMP responsiveness of these elements. Such comparative studies may also shed light on whether there is a single or multiple independent origin(s) of BMP regulation of vertebrate msx genes. Furthermore, analysis of the CRM driving BMP-dependent expression of an echinoderm Msx homolog in regions of peak BMP activity [14] will be informative since this gene is expressed in the non-neural ectoderm. In this case, one might predict finding only positively acting AE-like BMP-responsive sites.

There are two possible explanations for distinct mechanisms of BMP-regulation of msh/msxB expression in flies versus fish. One is that these genes independently evolved BMP responsiveness. Alternatively, BMP-dependent regulation may be an ancestral trait
dating back to the first bilaterians with a condensed CNS. We favor the latter alternative for the following reasons. First, the colinearity of msh-msx, ind-gsh/pax, and vnd-Nkh2.2 genes relative to the source of BMPs and the BMP responsiveness of these genes in species from all three primary branches of bilateria - flies (ecdysozoa), vertebrates (deuterostome chordates), and annelid worms [40] (lophotrochozoa) - provides a compelling argument for this arrangement reflecting the ancestral state. Second, a polarized source of BMPs was present in diploblasts (e.g., corals [41,42], jellyfish [43], and the sea anemone [44,45]) and therefore preceded evolution of bilaterian triploblasts and a condensed CNS. Thus, it is plausible that a single species evolved a condensed CNS which deployed neural identity genes along the DV axis in much the same way that Hox genes are expressed in sequential order along the AP axis. Finally, if one looks more broadly among the 30 bilaterian phyla, a striking trend is that at least some clades within most of these phyla have a condensed CNS with three primary axon bundles [46], suggestive of an ancestral tripartite subdivision of the CNS. It is true that there are also many examples of species scattered among these phyla that either secondarily lost a condensed polarized CNS or retained a prior ancestral state in which there was only a distributed nervous system. Echinoderms in which Msx genes are expressed in the non-neural ectoderm (see above) or the hemichordate Sacoglossus kowalevskii which has lost bilateral symmetry to become radially organized [47] may be examples of such derived simplifications of the nervous system. Thus, in our view, the most likely scenario is that the ancestral bilaterian CNS was a condensed nervous system partitioned into at least three DV domains and that loss of centralization has occurred numerous times in different lineages undergoing morphological simplification.

If one assumes a common ancestral origin for BMP-regulation of msx genes, one can imagine various scenarios under which BMP-mediated regulation of msh/msx genes could have switched its effect during evolution. In vertebrates, BMP targets frequently contain Drosophila SEs that activate rather than repress transcription. This might be due to Shn proteins losing their repressive activity through changes in the Shn amino acid sequence and/or the lack of components required for repression downstream of Shn. The molecular relatedness of SEs and AE sites raises the possibility that ancestral SE-mediated repressive effects on msh/Msx expression may have been relatively easy to convert into activating effects in the vertebrate lineage by the loss of the Shn repressor function. Consequently, the increased linker length of zAE could be accounted for by the lack of evolutionary pressure on the SE to meet the sequence requirements for Shn recruitment.

Since the Drosophila msh gene is weakly repressed by BMPs (e.g., relative to ind and other neural genes such as AS-C, scrt or swa [6]), while vertebrate msx genes are weakly activated by BMPs (i.e., high neuroectodermal levels of BMPs are required to activate msx genes) an intermediate CRM state may have existed in which BMPs both weakly activated msx genes expression within the neuroectoderm at moderate levels while repressing gene expression at the peak BMP levels present in the adjacent epidermis. Indeed the zebrafish msxB gene may represent such a bifunctional intermediate condition since in vivo studies indicate that high levels of BMPs can inhibit msxB expression [12]. It remains to be determined whether such proposed positive and negative inputs are mediated by a single or multiple independent CRMs [5]. Within different evolutionary lineages such biphasic responses could have then been rendered monophasic in opposing directions to account for the observed differences in the Drosophila versus vertebrate or echinoderm Msx CRMs. In vertebrates, one potential driving force for reducing the effect of BMP-mediated inhibition may have been the incorporation of BMP expression within the dorsal neural tube itself since this would be expected to generate much higher BMP levels than would result from BMPs diffusing in from the adjacent epidermal ectoderm (e.g., as is the case in Drosophila).

In future analyses it will also be important to examine BMP-mediated regulation of additional neural identity genes expressed along the dorsal-ventral axis including the Gsh = ind and Nkh2.2 = vnd genes as CRMs controlling expression of each of these genes will have undergone independent evolutionary trajectories. Since there is evidence that laterally and ventrally expressed genes in vertebrates are inhibited by BMPs [48–52], and because the more ventrally expressed ind gene in Drosophila is more sensitive to BMP-mediated repression than msh [7], one might expect to find similar, and perhaps conserved ancestral modes, of BMP-mediated repression of these genes across bilateria.

It will also be interesting to understand how flexible the ancestral metazoan state was by investigating the relationship between BMPs and msx genes in basal metazoans such as jellyfish. In these diploblastic animals, although the BMP-msx relationship has not been tested, BMP2/4 [53] and msx [54] homologues are expressed in adjacent regions during development, as is the case in the majority of triploblastic animals.

Materials and Methods

Bioinformatics

We identified candidate SE and AE sites in the msh, msxB and msx1 CRMs using binding site consensus sequences curated from the literature referenced and used Gene Palette [55]. For this analysis, we used the consensus sequence GNCNGCN(6)GNCTG to identify candidate Silencer Elements (SE) and the consensus GGGGCA[N]2GNCV for Activator elements (AE) allowing for single base-pair mismatches to these consensus sequences. We identified candidate zebrafish msxB and mouse Msx1 CRMs by using genome wide alignments for multiple vertebrate species, which indicates regions of high sequence conservation as provided by the UCSC genome browser (http://genome.ucsc.edu).

CRM-reporter construction and analysis

The 700 bp msh CRM is described in Von Ohlen et al., 2009 [25]. All primers used in this study and the corresponding constructs generated can be found in Table S1. The various Drosophila msh-CRM constructs were subcloned in pCR-TOPO vectors (Invitrogen) and subsequently cloned into the [P]acman vector [28] as NotI and KpnI restriction fragments. Site-directed mutagenesis PCR methods were adapted from [56]. The primers used to isolate the zebrafish msxB CRMs and the mouse msx1 CRM can be found in Table S1. Zebrafish constructs were cloned into pENTR-TOPO (Invitrogen), transferred to pTol2 by Gateway Recombination and injected in zebrafish embryos as previously described [30].

Genetic strains and crossing schemes

The Drosophila dppbbnull allele used in this study is Flybase stock number 2061. The 8x HS-dpp stock and its use are described in Biehs et al 1996 [6]. The schnurri mutant allele is shn. To generate the d1 dpp st2-dppembryos, females that are Ddp/dpp+/; d1+ cns’/dpp’/++ w1118 females were crossed to w/Y; dpp’/ + st2-dpp’/+,av+;CyO males. The fly strain used to inject all constructs has genotype P:Bac[yellow+] -attP-3BVK000002 and injections were outsourced to BestGeneInc (http://www.thbestgene.com/).

The zebrafish strains containing the hsp70:bmp2b [57] and hsp70:chd [38] transgenes were crossed to stable transgenic lines containing the msxB-CRM construct. Embryos at the sphere stage
(4hpf) were subjected to heat shock at 37°C for 1 hour and then returned to normal temperature of 28.5°C until they were fixed at the bud – 6 somite (10–11hpf) stage as necessary.

Electrophoretic mobility shift assays

For electrophoretic mobility shift assays (EMSA), Drosophila S2 cells were co-transfected with 50 ng Tkv:QD and 175 ng Mad- and Med-expression plasmids or with 400 ng of a Shh:CT-expression plasmid. Cells were harvested 72 hr after transfection and lysed for 10 min at 4°C in 100 μl of 100 mM Tris (pH 7.5), 1 mM DTT, 0.5% TritonX100 and 1%NP40. Radioactively labeled probes were generated by annealing and filling in partially overlapping oligonucleotides in the presence of [P]-32 ATP. Binding reactions were carried out in a total volume of 25 μl containing 12.5 μl 2x binding buffer (200 mM KCl, 40 mM HEPES (pH 7.9), 40% glycerol, 2 mM DTT, 0.6% BSA and 0.02% NP40), 10000 cpm of radioactively-labeled probe, 1 μl poly dIdC (1 mM) and 7 μl of cleared S2 cell extracts. After incubation for 30 min at 4°C, the reactions were analyzed by non-denaturing 4%polyacrylamide gel electrophoresis followed by autoradiography.

In situ hybridization

Fluorescent in situ hybridization methods were used performed according to [59] in Drosophila embryos and adapted to zebrafish embryos by increasing the hybridization temperature: 55°C in Drosophila to 65°C in zebrafish embryos. Antibodies used: dpERK (Cell Signaling Technology #3605), anti-digoxigenin (Roche), anti-biotin (Roche), Alexa fluor 488, 594, 647 (Invitrogen). We also used colorimetric staining methods performed according to O’Neill and Bier [60]. The DNA template used to generate the mRNA probe was a generous gift from the Riley lab. Histochemical stain images were acquired using a Nikon optical microscope and fluorescent stain images were collected using a LEICA SP2 confocal microscope. Images were adjusted for color, brightness and contrast using Adobe Photoshop software.

Supporting Information

Figure S1 Analysis of candidate BMP-responsive and Brk sites in the ME. (A) Diagram of the Drosophila msh CRM (ME) indicating the relative position of SEs, AEs and EMSA probes P1- P5. (B) Gel shift assay showing full length Brinker (Brk) and the Brinker DNA binding domain (Brk DNA-BD) bind to control DNA containing a Brk consensus sequence in the presence or absence of Drosophila S2 extracts containing Mad, Medea, and Schnurri (TMMS). The position of probe bound Brinker is indicated by the white arrow, the black arrow indicates the position of Brinker DNA binding domain in complex with the probe. Prior to electrophoresis, the DNA probe was incubated with lysates from Drosophila S2 cells transiently expressing Brinker (Brk). Prior to electrophoresis, the DNA probe was incubated with lysates from Drosophila S2 cells transiently expressing Brinker (Brk), constitutively active type I Dpp receptor, Mad, Medea and C-Terminal Schnurri (TMMS). The area boxed in red is a region of the same gel with less developing time. Note that the presence of the pMMS complex does not alter the position of the full-length Brk shift, while the Brk-DNA BD does (i.e., Brk-DNA BD competes with full Brk for binding to that site). (C) Gel shift assays induced by pMM and pMMS complexes on oligonucleotides containing candidate BMP-responsive sites. The ability of Brinker to bind several ME regions was also tested. The five different probes, indicated above the gel, were incubated with lysates from Drosophila S2 cells transiently expressing Brinker (Brk), constitutively active type I Dpp receptor, Mad, Medea (TMMS) and/or C-Terminal Schnurri (S). The white arrow indicates the molecular weight position of probe bound pMMS complexes while the black arrow indicates the position of probe bound pMM complexes on oligonucleotides containing the SE1 (P1) and SE2 (P3) sites. For probes P1 and P3 note that the presence or absence of Brk does not affect the retardation typical of pMMS complexes (lanes 6 and 13) as compared to controls where probes are incubated with TMM and S alone. Compare lanes 3 and 6 for P1 and lanes 15 and 18 for P3. This observation is also noted for probes incubated with extracts containing transiently expressed TMM versus probes incubated with TMM plus Brk, compare lanes 14 and 17 for example. These results suggest that despite the fact that ectopic Brk expression leads to dorsal expansion of msh expression [7], Brk does not bind the ME regions surveyed and is not directly regulating the ME via the SE1(P1) or SE2(P3) sites or the other regions surveyed. Regarding probes P4 and P5, no binding signal is detected. (D) Autoradiogram of an EMSA experiment comparing the relative affinities of SE2 and SE* for pMM and pMMS complexes. The white arrow indicates the molecular weight position of pMMS and the black arrow indicates that of pMM. The labeled oligonucleotide probe corresponding to the SE2* sequence was incubated with Drosophila S2 cell lysates transiently expressing constitutively active type 1 Dpp receptor Thick veins, Mad, Medea and C-terminal Schnurri (TMMS) in all lanes except lane 1 (labeled probe alone) and lane 2 (labeled probe with active Tkv, Mad and Med only - TM). All lanes were loaded with equimolar amounts of labeled SE2* oligonucleotide probe and cell lysate. In lanes 4 to 11, progressively greater amounts of unlabeled SE* probe were added to the mix, while in lanes 13 to 20, increasing amounts of unlabeled SE2 oligonucleotide probe was added. In these experiments unlabeled oligonucleotides act as competitors for pMMS complexes against the labeled probe. The decrease in the amount of labeled probe shifted as the competitor concentration increases is more pronounced with the unlabeled SE2* competitor than with the unlabeled SE2 competitor revealing that the wild-type SE2 probe is less effective as a competitor than the SE2* probe. Probe sequences are indicated below the gel. Bold bases indicate the GC-rich and SBE sites, lower case indicates the mutated base that differentiates SE2* from SE2.

(TIF)

Figure S2 Different Dpp doses do not elicit msh expression and changes in EGFR signaling do not affect the dorsal border of the msh expression domain. (A,B) Early stage Drosophila embryos with varying genetic dosages of dpp. In situ hybridization of embryos oriented with anterior regions to the left in both images. (A) Embryos lacking Dorsal but heterozygous for dpp+, retain slight msh expression in head regions and strong msh expression in tail regions but msh is absent from middle regions. (B) To approximate a situation where the Dpp dose is in between the wild-type and heterozygous conditions, we added the eve-stripe-2-dpp+ construct to an embryo lacking maternal Dorsal and zygotically heterozygous for dpp. In this particular genetic background, msh expression is severely reduced as well. These results reinforce the idea that Dpp does not have an activating role in msh regulation in the absence of Dorsal signaling in Drosophila melanogaster at these stages. (C) Ventral-lateral view of a wild-type embryo (this and all other embryos with anterior to the left), depicting the expression of activated ERK (detected with an anti-dpERK antibody - yellow) relative to ind mRNA (red) and msh mRNA (green). Note that dpERK staining is not detected dorsal to the ind expression domain. (D) Dorso-lateral view of an embryo, anterior to the left, ectopically expressing a secreted form of Spitz (s-Spitz) under the control of a Krüppel (Kr) driver using the GAL4/UAS system. Ectopic expression of s-Spitz leads to a localized dorsal expansion
Distinct BMP Regulation of msh and msx Genes

(white arrows of ind (red) within the Kruppel domain (detected by gal4 mRNA - yellow) while msh (green) expression is unaffected. (E) Dorso-lateral view of a cie mutant embryo. ind (red) expression expands dorsally as previously reported [63], while the msh (green) domain loses some ventral expression (presumably due to repression by Ind) but its dorsal border remains unaffected. (TIF)

Figure S3 Characterization of BMP responsive sites in the msxB-CRM. (A) Gel shift assay identifying a single site in the zebrafish 671 bp minimal msxB CRM that binds to Drosophila pMad and Medea. A diagram with the relative position of the probes within the msxB-CRM as well as a conservation map of the msxB-CRM region among selected species is shown. Labeled oligonucleotide probes corresponding to different candidate regions containing AE-related sites of the msxB-CRM were incubated with extracts from Drosophila S2 cells over-expressing activated Tkv (to induce BMP signaling), Med and Mad (TMM). The black arrow indicates the position of probe bound pMM complexes. Only probe Msx11 (lanes 1 and 2 on the gel) show a BMP-dependent shift at the pMM molecular weight position. This probe spans the most highly conserved region of the msxB-CRM in mammals and fish. (B) Map of the zAE region corresponding to the Msx11 probe within msxB-CRM. The relative position of Msx11A-F oligonucleotide probes and putative AE sites are represented. Gel shift assay demonstrating that pMM complexes can assemble within the conserved region of the msxB-CRM. Prior to electrophoresis, the DNA probes were incubated with lysates from Drosophila S2 cells transiently expressing Mad and Medea (MM), constitutively active type I Dpp receptor, Mad and Medea (TMM) or constitutively active type I Dpp receptor, Mad, Medea and C-Terminal Schnurri (TMMS). The white arrow indicates the molecular weight position of pMMs and the black arrow indicates the molecular weight position of pMM. As a positive control, the P3 probe corresponding to the SE2 region of the Drosophila msx-B-CRM (Fig. S1) was used. The Msx11 oligonucleotide is capable of assembling pMM complexes and these seem unaffected by the presence of Shn. To narrow down the binding sites of Mad and Med, sub-regions of the Msx11 probe labeled Msx11A-F were incubated with TMM lysates. Msx11F, which is contained in Msx11A, represents the minimal shifted probe, indicating that pMad/Med complexes are assembling within this sub-region. (C) Mutant versions of Msx11F probe were incubated with Drosophila S2 cell TMM lysates and gel shift assays were performed. Mutating the entire predicted 16 bp linker region (lm) did not affect the ability to assemble pMM complexes in vitro. When the GC-rich sequence is mutated at either the 5’ or 3’ end (GCR1 and GCR2, respectively) probe retardation was no longer observed. Changing only the predicted SBE site severely reduced the shifted probe signal. These results suggest complexes with the usual 2 Mad to 1 Med ratio are assembling on the Msx11F probe [9]. In addition, the linker length does not seem to be a factor in the formation of pMM complexes since changing it to 15 (l15), 12 (l12), 8 (l8), 6 (l6) or 2 (l2) base pairs did not prevent the probe from being shifted as long as the GC-rich and SBE regions remained intact. (D) Gel shift assay demonstrating that the presence of Shn does not affect the formation of pMM complexes on Msx11F in vitro. Interestingly, however, Shn can bind together with Mad/Med if the linker length is reduced to 5 base pairs (l5). (TIF)

Table S1 Primers used in this study.

| Primer Name | Description |
|-------------|-------------|
| pmx_520 | 520 bp minimal msxB CRM |
| pmx_671 | 671 bp minimal msxB CRM |
| pmx_930 | 930 bp minimal msxB CRM |

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Author Contributions

Conceived and designed the experiments: FFE AS EK GP SF EB. Performed the experiments: FFE AS EK ET. Analyzed the data: FFE AS EK ET GP SF EB. Contributed reagents/materials/analysis tools: FFE AS EK ET GP SF EB. Contributed to the writing of the manuscript: FFE AS EK ET GP SF EB.

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