First Author: Simone Macrì, MSc

Order of Authors: Simone Macrì, MSc; Luca Simula, MSc; Ilenia Pellarin,
Msc; Silvia Pegoraro, PhD; Marco Onorati, PhD; Riccardo Sgarra, PhD;
Guidalberto Manfioletti, PhD; Robert Vignali, MSc

Abstract: HMGA proteins are small nuclear proteins that bind DNA by
conserved AT-hook motifs, modify chromatin architecture and assist in
gene expression. Two HMGAs (HMGA1 and HMGA2), encoded by distinct genes,
exist in mammals and are highly expressed during embryogenesis or
reactivated in tumour progression. We here addressed the in vivo role of
Xenopus hmga2 in the neural crest cells (NCCs). We show that hmga2 is
required for normal NCC specification and development. hmga2 knockdown
leads to severe disruption of major skeletal derivatives of anterior
NCCs. We show that, within the NCC genetic network, hmga2 acts downstream
of msx1, and is required for msx1, pax3 and snail2 activities, thus
participating at different levels of the network. Because of hmga2 early
effects in NCC specification, the subsequent epithelial-mesenchymal
transition (EMT) and migration of NCCs towards the branchial pouches are
also compromised. Strictly paralleling results on embryos, interfering
with Hmga2 in a breast cancer cell model for EMT leads to molecular
effects largely consistent with those observed on NCCs. These data
indicate that Hmga2 is recruited in key molecular events that are shared
by both NCCs and tumour cells.

Ms. No.: DBIO-15-265
Title: Hmga2 is required for neural crest cell specification in Xenopus laevis
Corresponding Author: Prof. Robert Vignali
Authors: Simone Macrì, MSc; Luca Simula, MSc; Ilenia Pellarin, Msc; Silvia Pegoraro, PhD;
Marco Onorati, PhD; Riccardo Sgarra, PhD; Guidalberto Manfioletti, PhD;

Dear Editor,

please find enclosed the revised version of our manuscript "Hmga2 is required for neural crest cell specification in Xenopus laevis" that we would like you to consider for publication in Developmental Biology.

We were happy to know that all the Reviewers find some merits in the paper.

We have performed additional experiments suggested by the reviewers and integrated and discussed new results in the present version. We think that the paper has been improved by these modifications and we hope that this new version can be accepted for publication.

Best regards,

Robert Vignali

Conflict of Interest: The authors declare that they have no conflict of interest.

Ms. No.: DBIO-15-265

Title: Hmga2 is required for neural crest cell specification in Xenopus laevis

Corresponding Author: Prof. Robert Vignali

Authors: Simone Macrì, MSc; Luca Simula, MSc; Ilenia Pellarin, Msc; Silvia Pegoraro, PhD; Marco Onorati, PhD; Riccardo Sgarra, PhD; Guidalberto Manfioletti, PhD;

Dear Editor,

please find our revision of the paper DBIO-15-265.

We were happy to know that all the Reviewers find merits in the paper.

We have performed additional experiments suggested by the reviewers and integrated and discussed new results in the present version. We think that the paper has been improved by these modifications and we hope that this new version can be accepted for publication.

We have addressed some of the issues raised by Referees as follows:

Response to Reviewer 1

1) The reviewer correctly makes the point that the "MO knock down phenotype is an early phenotype and therefore could both directly or indirectly affect many facets of

neural crest cell behavior. If neural crest induction is perturbed a lot of processes could be disrupted such as EMT and neural crest morphogenesis."

RESPONSE: We agree with the view that impairment of EMT and migration could be a consequence of earlier effects on specification. However, already in the previous version of the manuscript, we did not definitely conclude that we demonstrated that *hmga2* is directly involved in the phase of EMT and migration of NCCs, but, rather, that *hmga2* depletion impaired specification of NCCs and, as a consequence, the following EMT and migration phase. This is stated several times in the Manuscript (Abstract: "Because of *hmga2* early effects in NCC specification, the subsequent epithelial-mesenchymal transition (EMT) and migration of NCCs towards the branchial pouches are compromised"); in the Discussion we then say that "we did not uncouple the *hmga2* knockdown early effects on NCC specification from possible later effects on EMT/migration". In fact, after discussing our results in EMT cell culture models we conclude that "It is therefore feasible that HMGA2 cooperates with several gene regulators at different steps of the EMT genetic programme not only in tumourigenesis (Heldin et al., 2009) but also in NCC EMTs". So we never said we demonstrated that hmga2 is required at the EMT and migration stage, but only suggested it is feasible.

We know, however, that is an important point and the main limitation of the paper, because, as already stated in the submitted version, we did not uncouple the early effect in the specification phase from a possible later, more direct, effect on EMT and migration. There are examples where this has been done. For instance LaBonne and Bronner-Fraser, 2000 and Aybar et al., 2003, have used an inducible dominant negative approach to knockdown Snail1 and Snail2 functions and tear apart early from late requirements; these reagents have worked very nicely, presumably because the corresponding non-inducible version hade a very strong antimorphic effect. Our Hmga2EnR construct could be in principle made into a GR inducible fusion form, to be activated at later stages. However, the Hmga2EngR has a much weaker effect

compared to our MOs, and compared to the efficiency of the constructs used in those studies, both in terms of intensity of effects and in frequency of affected embryos. For this reason, we thought that results of similar experiments with an inducible, but leaky constructs, may not be sufficiently clear and interpretable and that we might need a more efficient antimorphic construct. For these reasons, given the evidence for a role of Hmga2 in orchestrating tumoral EMT (by directly acting on *snail2* and *twist* promoters among others), we thought that the breast cancer cell model would be a better system where we could tackle, in the context of EMT, the effects of *hmga2* depletion on genes of the NCC network. Consistent with the results presented here, we have added a complete new paragraph to discuss thoroughly this limitation of our study (also in connection to previous studies) (see page 22). We make clear that we cannot conclude definitely about a direct role of hmga2 at the stage when EMT starts in NCCs, though, based on our tumor cell model and on previous evidence from other papers, we still suggest this is likely. We hope this discussion may be helpful to make clear and acceptable our point of view.

2) The Referee also says that it is not clear what cell fates are lost in the neural crest region.

RESPONSE: We do not completely agree: we show that developing cranial ganglia are erased (Fig. S3H), as detected by absent *neuroD*1 expression; and that skeletal derivatives of cranial NCC are heavily affected (almost the whole pharyngeal skeleton is disrupted or missing), in agreement with downregulation of *sox9* positive scheletogenic crests (shown in Fig. 2).

3) The Referee asks for a better characterization of the neural phenotype. Considering that *hmga2* is widely expressed in the developing CNS, he/she asks for spinal cord and hindbrain markers, and for a characterization of the neural fate specificity of *hmga2* knock down phenotype.

RESPONSE: To better characterize these aspects, we have analysed hindbrain and spinal cord markers, as suggested by the Referee, as well as markers of neurogenesis. In addition to absence of effects on *otx2* and *nrp1* (already examined in the original version), we found that there is basically no alteration on the expression of positional neural markers such as *en2, krox20* and *hoxb9* within the midbrain and hindbrain region and spinal cord (interestingly, though, *krox20* positive NCCs show reduction). As for any effect on neuronal commitment, analysis of *neurog1, elr-c* and *tubb2b* (N-tubulin) shows reduction of all these markers (e.g. in primary neurons, trigeminal ganglion) a phenotype consistent with a role of *hmga2* in neural cell state. This phenotype is interesting and certainly deserves further attention. However, we feel that a full analysis of this aspect goes beyond the focus of the present paper on NCCs. Therefore we will pursue these aspects in a separate work.

All these results are displayed in two new Supplementary Figures (Fig. S4 e S5), described in Results and commented in the Discussion.

Response to Reviewer 2

A few minor points should be addressed:

1- In Figure 1, stage 13 it seems that the whole lateral neural plate border is expressing *hmga2*, thus, it is better to change the label "NCC" to "NPB" and extend the area outlined in red to the rest of the neural plate border.

RESPONSE: This has been done as suggested by the referee

2- In figure 1, it would be nice to decrease the size of the letters, so that they do not hide parts of the embryos.

RESPONSE: This has been done as suggested by the referee

3- In figure S1, the decrease in Hmga2 protein levels does not seem "strong": could

the authors perform a quantitation by densitometry to evaluate this diminished expression?

RESPONSE: a densitometry has been performed and quantification results in Hmga2 protein being abated to around half the amount (Fig. S1D).

4- In Figure 2, the two first panels could be larger, and numbers and % put in the figure legend to gain space within the figure itself.

RESPONSE: all numbers and % were removed in order to gain space for the pictures and moved into the legend of Fig. 2

5- Figure 2, Twist rescue: the embryo on the picture shows a very anterior staining for lacZ, the authors should choose another embryo, with larger injected area encompassing all the side of the head.

RESPONSE: the pictures were changed as suggested by the reviewer

6- Figure 4: Since *hmga2* expression is stronger at stage 25, could the authors add msx1 injection and MO-snail injection at stage 25, to make the phenotype more obvious.

RESPONSE: this has been done; Fig. 4 was reformatted and results of the relative experiments were included in Supplementary Tables

7- Figure 6: after *pax3* injection, *hmga2* seems reduced on the chosen picture, contrarily to what the counting on the table suggests: the authors should replace this picture.

RESPONSE: the picture was replaced with that of another embryo as suggested by the referee.

8- the fact that hmga1 is not present in frog should be mentioned in the introduction.

RESPONSE: the sentence was removed from the Discussion and mention of *hmga1*

absence in *Xenopus* genome was given in the Introduction.

Response to Reviewer 3

We are glad that the reviewer finds merits and considerable interest in our paper.

The points he/she raises are essentially the same as Reviewer 1. In order to better characterize the neural phenotype and to exclude that *hmga2* depletion in the CNS has generalized effect, but has more specific ones, we have perfomed additional experiments with other markers. We observed that patterning genes are not upset, while markers of neurogenesis were downregulated. We discuss these findings in the new version (page 20, 1st paragraph) and suggest that the results on neurogenesis markers will require further investigations in a separate piece of work.

As for the point about how direct the requirement for *hmga2* may be in subsequent behaviors of the neural crest, we agree with the referee that this may be a difficult issue to address in the *Xenopus* system due to the epistatic position of *hmga2* with respect to the NC specifier genes (see also response to Referee 1). We are aware of this limitation and we tried to make this more explicit and to discuss this more deeply than in the previous version. We have extended the Discussion on this limitation of our work (page 22). We hope we have improved the conceptual connection between part 1 on embryos and part 2 on cells, both in the Results and in the Discussion.

LIST OF MODIFICATIONS WITH RESPECT TO THE PREVIOUS MS (page number refer to the revised version)

1) ABSTRACT, page 2: "towards the branchial pouches are **also** compromised". **also** was added

2) HIGHLIGHTS, page 3: "*hmga2* knockdown early effects also impair subsequent NCC EMT and migration instead of "*hmga2* knock-down impairs NCC EMT and migration"

3) INTRODUCTION, page 6, last paragraph: the following sentence was added "In *Xenopus* only *hmga2,* but not *hmga1,* is present (Macrì et al., 2013); the frog therefore provides a unique opportunity to study *hmga2* role during embryogenesis without any possible redundancy by *hmga1*."

4) MATERIALS AND METHODS, page 8, 1st paragraph in Xenopus embryology: a simplification was made by mentioning that all the probes used in the study were previously referenced, instead of mentioning each of them one by one. Three new references were introduced, but of the previous ones were eliminated.

5) RESULTS, page 12: 1st paragraph, 2nd line : "in the area of" was replaced with "in the neural plate border region encompassing"

6) RESULTS, page 12, 1st paragraph, 3rd line from end of paragraph: "(about 50% according to densitometry)" was added.

7) RESULTS, page 13, after the 3rd paragraph: a completely new paragraph was inserted to describe the new results concerning positional neural markers and markers of neurogenesis.

8) RESULTS, page 14, 3rd paragraph: the initial sentence of the paragraph "Because HMGA2 is involved in tumour EMT and *snail2* and *twist* are key genes promoting EMT and normal cell migration in both NCCs and tumour progression (LaBonne and Bronner-Fraser, 2000; Soo et al., 2002; Thuault et al., 2006, 2008; Watanabe et al., 2009; Wu et al., 2011; Tan et al., 2012; Guo et al., 2013; Morishita et al., 2013), we asked whether Hmga2 depletion impaired EMT and migration of NCCs during development." was replaced with the following: "*snail2* and *twist* are key genes in NCC specification that also promote EMT and normal cell migration both in NCCs

and in tumour progression (LaBonne and Bronner-Fraser, 2000; Soo et al., 2002; Thuault et al., 2006, 2008; Watanabe et al., 2009; Wu et al., 2011; Tan et al., 2012; Guo et al., 2013; Morishita et al., 2013); because of the effects of Hmga2 depletion on these genes, we would expect that *hmga2* knockdown would also impair EMT and migration of NCCs during development."

9) RESULTS, page 15, 8th line from the top: the sentence "Therefore, Hmga2 plays a cell-autonomous role in NCCs that is required for their EMT and migration" was modified into "Therefore, Hmga2 plays a cell-autonomous role in NCCs that is required for their subsequent EMT and migration."

10) RESULTS, page 15, in **Hmga2 and the NCC gene network,** last three lines of the page: additions were made to introduce the results of *Msx1* injected embryos analyzed at tailbud stage.

11) RESULTS, page 16, 3rd line: additions were made, to describe the results of MO-*snail2* injection on *hmga2* expression at tailbud stage.

12) RESULTS, page 16, 3rd paragraph, 2nd line: the sentence "....but upstream of *snail2* and *twist* in the NCC genetic network. " was modified into "...but upstream of *snail2* and *twist* in the initial phase of the NCC genetic network."

13) RESULTS, page 18, in **Molecular analysis of *Hmga2* function in a mammalian EMT model of breast cancer**: The initial sentence "We then asked whether the *hmga2*-dependent molecular interactions observed in *Xenopus* NCCs could be validated in a mammalian cell model. Because HMGA2 is a crucial player in EMT and tumour progression, we chose the murine mammary epithelial NMuMG cells, a breast cancer model where EMT can be elicited by TGF-β with the determinant role of HMGA2 (Thuault et al., 2006, 2008)." was modified into "Because HMGA2 is a crucial player in EMT and tumour progression, we then asked whether the *hmga2*-dependent molecular interactions observed in *Xenopus* NCCs could be validated in a mammalian cell model for EMT. We chose the murine mammary epithelial NMuMG cells, a breast cancer model where EMT can be elicited by TGF-β with the determinant role of HMGA2 (Thuault et al., 2006, 2008)."

14) DISCUSSION, page 20, 1st paragraph: this paragraph has been rearranged as follows. After the first sentence, a sentence resuming the expression of *hmga2* in NCCs: "*Xenopus hmga2* is transcribed NCCs during their specification and in their EMT and migratory phase (Benini et al., 2006; Monzen et al., 2008; present work)." was moved here from below. The sentence concerning the absence of *hmga1* in *Xenopus*, was cut away from here and moved in the Introduction (see point 3). In addition, a few sentences were introduced to describe the effects of MOs on neural patterning genes as well as on neurogenesis markers. These sentences make up a great part of the first paragraph (up to its end) in this new version. Minor modifications were also made.

14) DISCUSSION, page 21, 2nd paragraph, 13th line from the top: the part "We also show that *hmga2* early expression at neurula stage depends on *msx1*, but not on *snail2* function. These data altogether suggest that *hmga2* acts downstream of *msx1*, but is required for the expression of *snail2* and other NCS genes." has been modified into "We also show that initial *hmga2* early expression at neurula stage depends on *msx1*, but not on *snail2* function. Our data altogether suggest that *hmga2* acts downstream of *msx1*, but is required for the initial expression of *snail2* and other NCS genes."

15) DISCUSSION, page 22, at the beginning of the page: a few sentences were added to discuss more deeply the issue of hmga2 requirement at a later stage than initial specification.

16) DISCUSSION, page 22, last paragraph, end of the page: The sentence "To get more insights about the molecular connections among *hmga2*, EMT and the NCC genes that we examined in *Xenopus* embryos," was added at the beginning of the phrase. A small modification was also made at the end of the page.

17) DISCUSSION, page 23, at the end, a new paragraph was added to summarize the conclusions, clearly stating the overall significance of the paper.

17) FIGURE LEGENDS, page 37 and following: legends to Figure 1, 2 and Figure 4 were modified according to Referees' indications and to accommodate the numbers

and % previously shown in Fig. 2 and to accommodate description of the new Results presented in the MS.

18) SUPPLEMENTARY MATERIALS AND METHODS, page 41, in Western blot analysis of proteins from *Xenopus* embryos: A part was added to describe the densitometry.

19) LEGENDS TO SUPPLEMENTARY FIGURES, page 43 and following: In legend to Fig. S1, description of the densitometry on Western Blot; then two new legends were created for two new supplementary figures (Fig. S4 and S5)

Other minor and not substantial modifications were made in the text in the various sections and are not detailed here. These concern Authors' affiliation, references in the text to Figures that have been modified according to Referees' request, modification of Tables to integrate data from the requested experiments, integration of references etc.

HIGHLIGHTS

1) *hmga2* is required for neural crest cell (NCC) specification in *Xenopus*

2) *hmga2* acts at different levels of the NCC genetic hierarchy

3) *hmga2* knockdown early effects also impair subsequent NCC EMT and migration

4) *hmga2* morphant embryos show disrupted pharyngeal skeleton

5) *hmga2* molecular interactions with NCC genes are confirmed in an EMT cancer cell model

Hmga2 is required for neural crest cell specification in *Xenopus laevis*

Authors: Simone Macrì [a,1,*], Luca Simula [a,2], Ilenia Pellarin [b,3], Silvia Pegoraro [b], Marco Onorati [a,4], Riccardo Sgarra [b], Guidalberto Manfioletti [b], Robert Vignali [a,*]

[a] Dipartimento di Biologia, Unità di Biologia Cellulare e dello Sviluppo, Università degli Studi di Pisa, SS12 Abetone e Brennero 4, 56127 Pisa, Italy

[b] Dipartimento di Scienze della Vita, Università degli Studi di Trieste, via Giorgieri 5, 34127 Trieste, Italy

[1] Present address: Institute of Biotechnology, Biocenter 3, Viikinkaari 1 (PO box 65), FI-00014 University of Helsinki, Finland

[2] Present Address: Department of Biology, University of Rome Tor Vergata, Rome, Italy; Fondazione Santa Lucia Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Rome, Italy.

[3] Present Address: Division of Experimental Oncology 2, Department of Translational Research, Centro di Riferimento Oncologico (CRO Aviano), National Cancer Institute, 33081 Aviano, Italy;

[4] Present address: Department of Neurobiology, Yale School of Medicine, New Haven, CT, USA

[*] Corresponding author: E-mail address: robert.vignali@unipi.it

ABSTRACT

HMGA proteins are small nuclear proteins that bind DNA by conserved AT-hook motifs, modify chromatin architecture and assist in gene expression. Two HMGAs (HMGA1 and HMGA2), encoded by distinct genes, exist in mammals and are highly expressed during embryogenesis or reactivated in tumour progression. We here addressed the *in vivo* role of *Xenopus hmga2* in the neural crest cells (NCCs). We show that *hmga2* is required for normal NCC specification and development. *hmga2* knockdown leads to severe disruption of major skeletal derivatives of anterior NCCs. We show that, within the NCC genetic network, *hmga2* acts downstream of *msx1,* and is required for *msx1*, *pax3* and *snail2* activities*,* thus participating at different levels of the network. Because of *hmga2* early effects in NCC specification, the subsequent epithelial-mesenchymal transition (EMT) and migration of NCCs towards the branchial pouches are also compromised. Strictly paralleling results on embryos, interfering with *Hmga2* in a breast cancer cell model for EMT leads to molecular effects largely consistent with those observed on NCCs. These data indicate that Hmga2 is recruited in key molecular events that are shared by both NCCs and tumour cells.

HIGHLIGHTS

1) *hmga2* is required for neural crest cell (NCC) specification in *Xenopus*

2) *hmga2* acts at different levels of the NCC genetic hierarchy

3) *hmga2* knockdown early effects also impair subsequent NCC EMT and migration

4) *hmga2* morphant embryos show disrupted pharyngeal skeleton

5) *hmga2* molecular interactions with NCC genes are confirmed in an EMT cancer cell model

INTRODUCTION

HMGA proteins (HMGA1 and HMGA2) are small proteins made of about 100 aminoacid residues that bind AT-rich DNA regions through conserved AT-hook motifs and modify chromatin architecture (Reeves, 2001; Harrer et al., 2004; Sgarra et al., 2010). HMGA proteins can bend the DNA double helix to assist recruitment of other factors in enhanceosomes, thus participating in the transcriptional regulation of several genes (Thanos and Maniatis, 1992; Chau et al., 2000; Monzen et al., 2008). *Hmga* genes and proteins are mainly expressed during embryogenesis in undifferentiated and/or rapidly proliferating cells from all three germ layers (Chiappetta et al., 1996; Hinring-Folz et al., 1998), but are downregulated in differentiated adult tissues. *Hmga2* inactivation in mice results in reduced body size (Zhou et al., 1995), while *Hmga2* overexpression leads to gigantism (Battista et al., 1999), suggesting its involvement in regulating proliferation. Moreover, *Hmga2* appears critical for skeletal muscle mass and fiber size (Li et al., 2012) as well as for neural and retinal stem cell state and renewal (Nishino et al., 2008; Parameswaran et al., 2014; Yu et al., 2015). On the other hand, mice homozygous or heterozygous for a null *Hmga1* allele show cardiac hypertrophy and myelo-lymphoid malignancies (Fedele et al., 2006). Finally, most of double *Hmga1/Hmga2* loss-of-function mutant mice die *in utero*; surviving pups display an extreme reduction in body size (Federico et al., 2014), suggesting involvement of both HMGAs in this aspect.

While *HMGA1* and *HMGA2* are not expressed, or expressed at low levels, in adult tissues, they are upregulated in several types of tumours (Fedele et al., 2002, 2006; Sgarra et al., 2004; Zaidi et al., 2006; Fedele and Fusco, 2010). Their association with tumourigenesis is reinforced by the observations that HMGA2 plays a key role in TGF-β induced epithelial-mesenchymal transitions (EMT), where it is activated and recruited by SMADs to turn on key genes like *Snail1* and *Twist* (Fusco and Fedele, 2007; Thuault et al., 2006, 2008; Tan et al., 2012; Morishita et al., 2013), and that also HMGA1 is involved in EMT and migration (Pegoraro et al., 2013,

2015).

EMT is a key event in carcinoma invasion and metastasis (Yang and Weinberg, 2008); however, regulated EMTs also occur during development, as in gastrulation or neural crest cell (NCC) migration. NCCs undergo EMT and successive migration to diverse regions of the embryo, giving rise to several derivatives, including skeletal and connective tissues of the head, peripheral neurons and glia, endocrine cells and melanophores (Le Douarin and Kalcheim, 1999). NCCs are induced by the combined action of BMP, FGF, NOTCH and WNT signals; these signals activate a first set of genes (the neural plate border, NPB, genes), like *Pax3/7*, *Zic*, *Msx1/2* and *Ap2,* that identify the neural crest forming region at the boundary between the epidermis and the neural plate (Nikitina et al., 2009; Milet and Monsoro-Burq, 2012; Stuhlmiller and Garcia-Castro, 2012). These initial genes in turn activate the neural crest specifier (NCS) genes that, within this boundary, specify the NCC fate and promote EMT and migration; among them, the zinc finger genes *Snail1* and *Snail2* (also named *Slug*) and the bHLH factor gene *Twist* direct the delamination and migration of NCCs (Nikitina et al., 2009; Theveneau and Mayor, 2012), driving the change from a neuroepithelial to a mesenchymal morphology and leading to a shift in cell adhesive properties (Batlle et al., 2000; Yang et al., 2004; Peinado et al., 2004; Mayor and Theveneau, 2013). During EMT, matrix metallo-proteases (MMPs) are upregulated and act on the extracellular matrix to open a route for NCCs to migrate; several MMPs of the ADAM family have been described for *Xenopus* NCCs (Alfandari et al., 2001; Harrison et al., 2004), and their expression is, again, under the control of NCS genes, like *Snail* (Kuphal et al., 2005). Significantly, these molecular switches are also employed in tumour-linked EMTs (Kuriyama and Mayor, 2008; Yang and Weinberg, 2008; Thiery et al., 2009; Nieto, 2011).

In *Xenopus* only *hmga2,* but not *hmga1,* is present (Macrì et al., 2013); the frog therefore provides a unique opportunity to study *hmga2* role during embryogenesis without any possible redundancy by *hmga1*. *Xenopus hmga2* is expressed in cells of the developing neural crest, and in other districts of the embryo, including the

developing heart and neural tube (Benini et al., 2006; Monzen et al., 2008). Because *hmga2* regulates *Snail* and *Twist* genes in tumour EMT and is expressed in NCCs, we addressed its role in NCCs and its possible relationships to NPB and NCS genes. We show that knockdown of *hmga2* function leads to downregulation of key genes, active in NCCs during their specification, EMT and migratory phases, thus impairing NCC migration and leading to severe disruption of the skeletal elements deriving from anterior NCCs. We show that *hmga2* acts downstream of *msx1* in the regulation of NCS genes; furthermore, Hmga2 may be required by Pax3 and Snail2 in the subsequent steps of the NCC genetic network. These data are extended and validated in a murine breast cancer cell model of EMT, suggesting Hmga2 as a key player both in NCCs and in tumour cells.

MATERIALS AND METHODS

Xenopus embryology

Xenopus embryos were obtained and staged as previously described (Newport and Kirschner, 1982; Nieuwkoop and Faber, 1994). *In situ* hybridizations were performed according to the protocol of Harland (1991), with modifications. All probes used in this study were referenced previously (Brivanlou and Harland, 1989; Hopwood et al., 1989; Papalopulu and Kintner, 1993; Mayor et al., 1995; Alfandari et al., 1997; Heller and Brandli, 1997; Misuzeki et al., 1998; Bang et al., 1999; Lupo et al., 2002; Spokony et al., 2002; Aoki et al., 2003; Luo et al., 2003; Monsoro-Burq et al., 2005; Benini et al., 2006; van Grunsven et al., 2006; Nichane et al., 2008).

NCC transplantations and evalution of NCC ability of migration were performed as previously described (Reisoli et al., 2010; Cousin et al., 2012). Alcian Blue staining was carried out as reported (Pasqualetti et al., 2000), with minor modifications.

Morpholinos, mRNA synthesis and embryo microinjections

Xenopus laevis has two allelic isoforms of *hmga2* (α and β), with slightly divergent 5'UTR sequences, but highly conserved coding regions (Benini et al., 2006; Hock et al., 2006). To deplete all *hmga2* variants, we used two non-overlapping morpholino oligos (MO), one targeting a conserved region in the 5'UTR (MO2: 5'-GAGGGCAAACAAGGTCATAGGGCAC-3') and the other the translation-initiation site (MO1: 5'-TTGGCGGGCTCCTTCCCTTGAGCTC-3'). 2.5-10 ng of MOs were injected into the animal region of one of the dorsal blastomeres of 4-cell stage embryos, and led to similar effects. In parallel, a standard control oligo (STD MO: 5'-CCTCTTACCTCAGTTACAATTTATA-3') and a 5 nt-mismatched MO2 (5-mis MO: 5'-GAcGcCAAAgAAGcTCATAcGGCAC-3', lower case letters indicate mismatched nucleotides) were used as controls; their injection at similar doses did not alter embryo development. MOs against *Xenopus msx1* (MO-

msx1) or *snail2* (MO-*snail2*) were described in the literature (Monsoro-Burq et al., 2005; Zhang et al., 2006). MOs were purchased from Gene Tools.

Injected mRNAs were generated from *pCS2-Xhmga2CR* (containing only the *Xenopus hmga2* coding region) or from *snail2*, *msx1* or *pax3* templates (Grammer et al., 2000; Suzuki et al., 1997; Monsoro-Burq et al., 2005) by *in vitro* transcription, using the Message mMachine kit (Ambion).

GFP or β-galactosidase mRNAs were always coinjected as lineage tracers together with MOs or mRNAs. When GFP was used, embryos were scored by fluorescence for expected distribution of injected MO or mRNA and sorted according to left/right side of injection before being processed for WISH or other analyses. β-gal staining was performed as described (Hardcastle and Papalopulu, 2000).

We tested different amounts of MO or mRNA in our microinjections to optimize their effects. For the experiments shown, unless different indications are given in the text or supplementary tables, injected amounts of MO or mRNA (either in combination or alone) were the following: MO1: 10 ng; STD: 10 ng; MO2: 2.5 ng; 5-mis: 2.5 ng; MO-*msx1*: 15 ng; MO-*snail2*: 10 ng; for mRNAs: *msx1*: 100 pg; *hmga2*: 200 pg; *pax3*: 100 pg; *snail2*: 500 pg; *GFP*: 600 pg; *β-gal*: 400 pg.

Cell cultures and reagents

Mouse mammary epithelial NMuMG cells were grown in DMEM high glucose (Euroclone), supplemented with 2 mM L-glutamine, 10% Tet free fetal bovine serum (Euroclone), penicillin (100 U/ml, Euroclone) and streptomycin (100 μg/ml, Euroclone).

NMuMG cells were stimulated with recombinant human TGF-β1 (Pepro-Tech, 2.5-5 ng/mL) for 6 and 30 hours, while corresponding control cells (CTRL) were treated with vehicle (BSA 0.1% sterile water).

The *Hmga2* siRNA (siHMGA2: 5'-GCAGTGACCAGTTATTCTT-3') and control siRNA (siCTRL: 5'ACAGTCGCGTTTGCGACTG-3') were designed with

Block It RNAI Designer tool (Invitrogen) (*Hmga2* accession number NM_010441).

Cell transfections

NMuMG were seeded at 80.000 cells per well in 24-well plates in complete medium without antibiotics (penicillin/streptomycin). Cells were transiently transfected with *Hmga2* siRNA or control siRNA using Lipofectamine® RNAiMAX reagent (Invitrogen). After two rounds of silencing every 24 hours, cells were stimulated with TGF-β, and corresponding control cells with BSA 0.1% in sterile water. After 2 hours of stimulation with TGF-β, cells were re-transfected with siRNAs for 16 hours.

Quantitative real time RT-PCR and classic RT-PCR

For quantitative real time RT-PCR (qRT-PCR), three biological triplicates for each group (siCTRL or siHMGA2 ± TGF-β) were prepared. Total RNA was extracted using TRIzol reagent (Invitrogen), subjected to DNase-I (Invitrogen) treatment and subsequently purified by phenol-chloroform extraction; mRNA was reverse-transcribed using Superscript III (Invitrogen). qRT-PCR was performed using IQ™ SYBR® Green Supermix (Bio-Rad). For relative quantification, the *Gadph* gene was used as internal standard and reference; all the primers (Table S1) were created using Primer3Plus software according to NCBI and Ensembl sequence databases. The CFX96 Real-Time PCR detection system (Bio-Rad) was used to perform quantitative PCR; all experiments were in biological triplicate and technical duplicate.

For classic RT-PCR, diluted 1st strand cDNAs were amplified using specific primers and GreenTaq mix 2X (Promega). Amplification products were analysed on 10% polyacrylamide TBE gel, and densitometric analysis was carried out using Quantity One 1-D analysis software (Bio-Rad), with endogenous *Gapdh* as internal control and normalizer.

Statistical analysis

Data were analysed by a two-tailed Student's *t*-test and results were considered significant at a *p*-value < 0.05. The results are presented as the mean ± standard deviation (SD).

For some microinjection experiments, data are reported as frequency of embryos among different phenotypic categories. In these cases, statistical analysis was performed using a chi-square test on absolute embryo number in each category; results were considered significant at a *p*-value < 0.05.

RESULTS

Xenopus hmga2 knockdown impairs NCC development and migration

During *Xenopus* development, *hmga2* localized transcripts are first detected at stage 13, in the neural plate border region encompassing the presumptive anterior NCCs and in the pre-placodal plate (Fig. 1A); successively, *hmga2* transcripts are clearly detected in the forming central nervous system (CNS), and in the developing streams of the NCCs (Fig. 1B-D; Benini et al., 2006; Monzen et al., 2008). To analyse *hmga2* potential role in NCCs, we injected MO antisense oligonucleotides (MO1 and MO2, see Materials and Methods) to block its function. To test for their effectiveness, we first injected the MOs in both blastomeres of 2-cell stage embryos (2.5-10 ng each blastomere) and used a specific antibody (Rizzi et al., 2013) to detect the amount of Hmga2 protein in stage 13 injected and control embryos by Western blot; injection of either MO1 or MO2 led to strong downregulation (about 50% according to densitometry) of the Hmga2 protein compared to control uninjected embryos or to embryos injected with a 5-mismatched MO (5-mis) (Fig. S1A-D).

In subsequent experiments, to target our MOs to the NCC region, embryos were injected unilaterally at the 4-cell stage in one of the dorso-animal blastomeres; though we mostly describe results obtained with MO2, also MO1 gave similar effects. In particular, injection of either MO resulted in a strong hypomorphism in the branchial region at tailbud stage (stage 28) (Fig. 2A), anticipating facial malformations observed at later stages on the injected side. Alcian blue cartilage staining, performed on stage 46-49 larvae, showed that Hmga2 depleted embryos failed to develop normal NCC craniofacial skeletal derivatives and otic capsules (Fig. 2B-E; Table S2). At variance, injection of the same amounts of a standard (STD) control MO or of 5-mis MO did not affect skeletal phenotype (Fig. 2F,G; Table S2).

To understand the molecular basis of these effects, we looked for possible alterations in the expression of genes involved in NCC induction, EMT and migration, as well as in early CNS and placodal development. In particular, we

analysed the effects of Hmga2 knockdown on the expression of the NCC markers *twist*, *sox9*, *dlx2* and *ap2* by whole mount *in situ* hybridization (WISH) at tailbud stage (stage 26-28). Severe downregulation or absence of expression of these genes was observed on the side injected with MO1 or MO2 (Fig. 2H; Table S3); no effect was observed upon injection of STD or 5-mis MOs (Fig. S2; Table S3).

We then monitored whether a form of *Xenopus hmga2* mRNA lacking the MO target sequence could rescue these effects. Coinjection of 5.2 ng MO2 and 6.25-12.5 pg of *hmga2* mRNA rescued normal *twist* expression in about 51-53% of injected embryos compared to 12-15% when MO2 was injected alone, while the frequency of embryos with decreased *twist* expression was reduced to 47-49% in coinjected embryos compared to 85-88% in MO2 injected embryos (Fig. 2I; Table S6A). Significantly, when grown up to tadpole stage, about 68% of similarly coinjected embryos showed a rescued normal skeletal phenotype, compared to the 35% of embryos displaying a normal skeleton when injected with MO2 alone (Fig. 2I; Table S6B).

We then checked for possible alterations in the expression of the placodal and CNS molecular markers *sox2*, *neuroD1* and *pax2*. At stage 18, *sox2* (a marker of CNS and placodes) was strongly downregulated by MO2 in its placodal expression domain, while expression in the CNS was only weakly, if at all, affected (Fig. S3A-C, Table S3). Similarly, at tailbud stage, the signal for *pax2* and *neuroD1* in placode-derived structures (otic vesicle and cranial ganglia, respectively) appeared strongly reduced, while expression in the CNS was not disturbed (Fig. S3D-H, Table S3). These same molecular markers were not affected after injection of 5-mis MO (Table S3).

Furthermore, no substantial effect was produced by *hmga2* knockdown on the expression of *nrp1* (pan-neural marker), or of patterning genes such as *otx2* (forebrain and midbrain), *en-2* (midbrain/hindbrain boundary) or *hoxb9* (spinal cord) (Fig. S3I-K; Fig. S4A,B,C,G,H; Table S4); interestingly, *krox20* (hindbrain) was unaffected in its neural tube domain in rhombomeres 3 and 5, but was reduced or contracted in its

NCC domain derived from rhombomere 5 (Fig. S4D-F; Table S4). Although we did not observe effects on these neural markers, *neurog1* (neuronal progenitors), *elr-c* and *tubb2b* (primary neurons) were severely downregulated on the injected side, either in the trunk region or in the trigeminal ganglion anlage or in both these domains, consistent with the distribution of the injected MO (Fig. S5; Table S5).

Injection of *Xhmga2EnR* mRNA, encoding for a dominant negative Hmga2 fusion to *Drosophila* engrailed repressor (Monzen et al., 2008), reproduced (though to a lesser extent) the reduction of *twist* expression observed after MO treatments, thus confirming the effects of *hmga2* knockdown on NCCs (Fig. S6A; Table S7).

On the whole these data show clear effects of *hmga2* knockdown on NCCs.

The strong reduction in the expression of NCC key genes observed in morphant embryos is not due to cell death. To prove this, we performed a time-course analysis on MO2 injected embryos: we monitored the pattern of expression of *snail2* (at earlier stages 13 and 18) and of *twist* (at tailbud stage 22 and 25), as well as the apoptotic pattern, on the injected and control sides of the embryos. Both at stage 13 and at stage 18 no apoptotic foci could be detected while, as discussed above, at these stages *snail2* expression was reduced on the injected side of embryos (Fig. S6B). At tailbud stage 22 or stage 25, *twist* expression on the injected side was strongly reduced or absent compared to control side, while cell death was comparable on both sides of MO treated embryos (Fig. S6B; Table S8).

snail2 and *twist* are key genes in NCC specification that also promote EMT and normal cell migration both in NCCs and in tumour progression (LaBonne and Bronner-Fraser, 2000; Soo et al., 2002; Thuault et al., 2006, 2008; Watanabe et al., 2009; Wu et al., 2011; Tan et al., 2012; Guo et al., 2013; Morishita et al., 2013); because of the effects of Hmga2 depletion on these genes, we would expect that *hmga2* knockdown would also impair EMT and migration of NCCs during development. To test this, we performed NCC transplantation experiments. NCCs were excised from neurula stage 13 embryos that had been injected with MO1 (or MO2 or 5-mis) along with GFP mRNA as a tracer; these NCCs were used to replace

equivalent cells in stage 13 uninjected host embryos; embryos were then grown and monitored to follow NCC migration. A clear impairment of migration was detected for MO1+GFP and MO2+GFP injected NCCs compared to control 5-mis+GFP injected NCC (Fig. 3), and was quantified as described (Cousin et al., 2012). In a reciprocal assay, RFP injected NCCs were transplanted in MO2+GFP injected hosts; in this case, transplanted NCCs normally migrated to the pharyngeal region (Fig. 3). Therefore, Hmga2 plays a cell-autonomous role in NCCs that is required for their subsequent EMT and migration. Time-lapse videorecording of transplanted embryos documents impairment of migration in MO2 compared to 5-mis MO injected NCCs (Suppl. Files 1 and 2). Consistent with this, we found that the expression of *adam13*, encoding a metalloprotease involved in NCC migrating ability (Cousin et al., 2012), and of *zeb2*, a transcription factor gene that promotes EMT (van Grunsven et al., 2007; Nieto, 2011) was downregulated by MO2 (Fig. S6C; Table S3).

Hmga2 and the NCC gene network

We next examined the position of *hmga2* in the NCC gene regulatory programme. The NPB genes *msx1* and *pax3* are among the earliest regulators of the NCC gene network, with *msx1* playing a key role upstream of *pax3* (Monsoro-Burq et al., 2005; Nikitina et al., 2009). We downregulated or overexpressed *msx1* to see how *hmga2* was affected. Injection of 15 ng of anti-*msx1* MO (MO-msx1) caused a clear downregulation of *hmga2* at both neurula (stage 13 and 16) and tailbud (stage 25) stages (Fig. 4A-D; Table S9), while resulting, as expected (Monsoro-Burq et al., 2005), in dowregulation of *pax3*, *snail2* and *twist* at neurula (Fig. 4Q-S; Table S9); no effect was produced by similar amounts of STD-MO (Fig. 4E-H; Table S9). Conversely, injection of 100 pg of *msx1* mRNA produced, as expected (Monsoro-Burq et al., 2005), an increase of *pax3*, *snail2* and *twist* expression at neurula stage (Fig. 4T,U,V; Table S9) and resulted in an enlargement of *hmga2* expression domain at neurula (Fig. 4I,J; Table S9) and in a more mature expression of *hmga2* at tailbud stage (Fig. 4K,L; Table S9). In addition, while anti-*snail2* MO injection (10 ng), as

expected, strongly downregulated *twist*, it did not substantially alter *hmga2* expression at stage 16 (Fig. 4M,N; Table S9); however, when MO-*snail2* injected embryos were analysed at tailbud stage, they showed a reduced *hmga2* expression (Fig. 4O,P; Table S9). This may not be surprising; given the crucial role of Snail2 in NCCs, it may reflect NCC disruption or fewer cells migrating after *snail2* knockdown.

We then examined the effects of Hmga2 depletion and overexpression on early key regulators of NCCs. MO2 injection did not affect expression of the NPB genes *msx1, pax3, hairy2* and *ap2*, but led to downregulation of the NCS genes *snail2* and *sox10*, as detected at stage 13 (Fig. 5A; Table S10). Consistent with this, when analysed at stage 16, MO2-injected embryos showed reduction of the expression of the NCS genes *snail2* and *twist*, while the NPB genes *msx1* and *pax3* were unaffected; no effect was produced by 5-mis MO (Fig. 5B; Table S10). On the other hand, injection of varying amounts (50 pg-2 ng) of *hmga2* mRNA did not alter the expression of *msx1, pax3, snail2* or *twist* but in a minority of embryos, and most of the embryos showed a normal expression of these genes (Fig. 5B; Table S10; and data not shown). Therefore, Hmga2 is not required for the expression of the early NPB genes *msx1*, *pax3*, *hairy2* and *ap2*, but is crucial for the expression of the NCS *snail2* and *twist*; in addition, it is not sufficient for upregulating *msx1, pax3, snail2* and *twist*.

On the whole, these data suggest that *hmga2* could be required downstream of *msx1*, but upstream of *snail2* and *twist* in the initial phase of the NCC genetic network. We therefore asked whether Hmga2 downregulation could block the effect of *msx1, pax3* or *snail2* on downstream genes.

We observed that while *msx1* mRNA injection increased *pax3, snail2* and *twist* expression, coinjection of MO2 suppressed *msx1* effect on *snail2* and *twist* (but not that on *pax3*) (Fig. 6A; Fig. S7A-C; Table S11). On the other hand, coinjection of *hmga2* mRNA did not seem to potentiate the effect of *msx1* on *snail2* or *twist* expression (Fig. 6A; Fig. S7A-C; Table S11).

When *pax3* mRNA (100 pg) was injected, both *snail2* and *twist* (but not *hmga2*) were upregulated; *pax3* effect on *snail2* and *twist* was suppressed by coinjection of MO2; instead, coinjection of *hmga2* mRNA did not enhance the effect of *pax3* on these markers (Fig. 6B; Fig. S7D,E; Table 11).

Finally, Hmga2 depletion by MO2 also suppressed the effect of *snail2* mRNA, that normally leads to the expansion of *twist* (Fig. 6C); coinjection of *hmga2* and *snail2* did not enhance significantly the increase of *twist* expression compared to *snail2* injection alone (Fig. 6C; Fig. S7F; Table 11).

These results altogether suggest that Hmga2 is required at different steps by Msx1, Pax3 and Snail2 to act on the expression of genes that come next in the NCC gene network.

Earlier results showed that some of the *msx1* effects on the NCC network are mediated by *pax3* (Monsoro-Burq et al., 2005). Our results show that the effects of *msx1* on NCS genes require Hmga2, while those on *pax3* do not; in fact, MO2 blocks *msx1*-induced expansion of *snail2* and *twist* expression, but not that of *pax3* (Fig. 6A). Because *pax3* in turn also requires Hmga2 for its effects on *snail2* and *twist*, it may be possible that Msx1 action on these two genes requires both Pax3 and Hmga2; then, MO-*msx1* effects should be rescued more efficiently if *pax3* and *hmga2* mRNAs are co-injected together. In fact, the effects of 20 ng of MO-*msx1* on *snail2* and *twist* are partially rescued by 100 pg of *pax3* mRNA (Fig. 7; Fig. S8; Table S12); interestingly, however, *hmga2* expression is not rescued by *pax3* (Fig. 7; Table S12). On the other hand, *hmga2* mRNA (50 pg) does not rescue *snail2* and *twist* expression in MO-*msx1* injected embryos (Fig. 7; Fig. S8; Table S12). However, when both *pax3* and *hmga2* mRNAs are coinjected, a more effective rescue of *snail2* and *twist* expression is obtained in *msx1* morphants (Fig. 7; Fig. S8; Table S12).

These results altogether show that *msx1* expression is independent of Hmga2; *msx1* regulates both *hmga2* and *pax3*, possibly in a reciprocally independent way, during the initial steps of NCC genetic specification (see also Monsoro-Burq et al., 2005); *pax3* and *hmga2* then cooperate downstream of *msx1* to promote expression of

the NCS genes *snail2* and *twist*. The activation of these two genes by *msx1* and *pax3* is Hmga2-dependent; in addition, the ability of *snail2* to reinforce *twist* expression also requires Hmga2 function. Therefore, *msx1, pax3* and *snail2* all require Hmga2 for full exploitation of their activities.

Molecular analysis of *Hmga2* function in a mammalian EMT model of breast cancer

Because HMGA2 is a crucial player in EMT and tumour progression, we then asked whether the *hmga2*-dependent molecular interactions observed in *Xenopus* NCCs could be validated in a mammalian cell model for EMT. We chose the murine mammary epithelial NMuMG cells, a breast cancer model where EMT can be elicited by TGF-β with the determinant role of HMGA2 (Thuault et al., 2006, 2008). As expected, TGF-β treatment significantly increased the mRNA expression levels of *Hmga2* and those of the major EMT markers, including the mesenchymal genes *Fibronectin (Fn1), Snail2* and *Zeb2*, both at 6 and 30 hours; in addition, at 30 hours, the treatment reduced the mRNA expression levels of the epithelial gene *E-cadherin* (Fig. S9A; and Thuault et al., 2006). Differently from *Hmga2*, the expression of the highly related *Hmga1* gene was not induced upon TGF-β stimulation at either mRNA or protein levels (Fig. S9A,B; Thuault et al., 2006).

After confirming *Hmga2* upregulation in this EMT cellular model, we analysed how the genes modulated by *hmga2* in *Xenopus laevis* NCCs responded to TGF-β in NMuMG cells and how they were affected upon *Hmga2* depletion.

The expression of *Sox9*, *Pax2*, *EphrinB2* and *Ap2* upon TGF-β induction was then assessed by qRT-PCR; since the expression levels of *Sox10*, *Msx1* and *Adam12* (the murine mammalian counterpart most similar to *Xenopus adam13*) were too low to be successfully analysed with qRT-PCR, these genes were studied by semi-quantitative RT-PCR. We found that *Sox9* (Fig. 8A) and *Sox10* (Fig. 8B), that are part of the NCC genetic network (Nikitina et al., 2009), were upregulated in NMuMG cells after exposure to TGF-β. Compared to untreated cells, *Sox9* mRNA levels were

increased both at 6 and especially at 30 hours after treatment (Fig. 8A); *Sox 10* mRNA was dramatically upregulated after 6 hours TGF-β treatment, whereas at 30 hours its expression was no longer detectable (Fig. 8B). *Sox2* mRNA was also analysed, but was not detectable in our conditions (not shown).

In addition to *Sox* genes, *Adam12* was upregulated after 30 hours of TGF-β treatment (Fig. 8B). The expression of *Pax2, EphrinB2, Ap2* and *Msx1* instead was not significantly influenced by TGF-β exposure (Fig. 8A,B).

Therefore, several of the different genes investigated in *Xenopus* NCCs, were consistently regulated also in the NMuMG cellular model. To understand whether the regulation of these genes was *Hmga2*-dependent, we silenced *Hmga2*. A specific siRNA (siHMGA2), designed against a sequence of the 3'UTR of the murine *Hmga2* mRNA, was transfected in NMuMG cells induced with TGF-β and the expression of the genes was analysed. As expected, *Fn1, Snail2* and *Zeb2*, that are modulated by HMGA2 following TGF-β induction (Thuault et al., 2006, Tan et al., 2012), were downregulated upon *Hmga2* silencing (Fig. 9A). *Sox9*, instead, seemed unaffected (Fig. 9A). Moreover, we found that, following *Hmga2* silencing, *Sox10* and *Adam12* mRNAs were downregulated in TGF-β stimulated cells (Fig. 9B), consistent with their downregulation in *Xenopus* NCCs by MO2.

Finally, we asked whether the genes that were not induced by TGF-β could be modulated by *Hmga2* in uninduced conditions. We found that, in absence of TGF-β, *Ap2*, *EphrinB2* and *Msx1* gene expression was not influenced by *Hmga2* silencing (Fig. S8A,B), while a significant downregulation of *Pax2* expression was detected (Fig. S8A).

DISCUSSION

This study addresses the function of the chromatin architectural factor Hmga2 in the specification of NCCs in *Xenopus laevis*. *Xenopus hmga2* is transcribed in NCCs during their specification and in their EMT and migratory phase (Benini et al., 2006; Monzen et al., 2008; present work). HMGAs have been associated with EMT, a key event in tumor progression that employs several of the molecular players acting in the genetic network of NCCs (Thuault et al., 2006, 2008; Wu et al., 2011; Tan et al., 2012; Mao et al., 2013; Guo et al., 2013; Morishita et al., 2013; Pegoraro et al., 2013). However, HMGA role in NCCs has never been investigated. For a loss-of-function approach, we injected MOs to knock down *Xenopus hmga2* function and assessed the effects of its downregulation at the early stages of NCC specification (early/mid-neurula stage), during tailbud stages when EMT and migration occurs, and finally when the NCC derivatives of the pharyngeal skeleton are formed. We also examined the effects of *hmga2* knockdown on some neural markers of early patterning and neurogenesis. While the patterning genes analysed were not affected, we found some interesting effects on markers of neurogenesis. These consisted in a clear downregulation of *neurog1*, *elr-c* and *tubb2b*, suggesting a loss of neuronal precursors and primary neurons; their expression in the forming trigeminal ganglion was very often reduced or absent, in line with the later effect on *neuroD1* expression in cranial ganglia. Although we did not pursue this interesting aspect in this paper, these results may reflect *hmga2* role in maintaining a pool of neural stem cells within the CNS (Nishino et al., 2008; Parameswaran et al., 2014). We mainly focused this paper on the role of *hmga2* in NCCs.

Knockdown of *hmga2* function in presumptive cranial NCCs leads to heavy phenotypic effects, and in particular to the disruption of NCC skeletal derivatives. Also the otic capsule and some placodal derivatives are frequently found impaired or missing, consistent with *hmga2* expression in these structures or their prospective fields. These effects are specific because: (a) MO1 and MO2, but not control MOs,

downregulate Hmga2 protein levels, as detected by an anti-Hmga2 antibody; (b) standard control or mismatched MOs do not produce similar phenotypes; (c) the MO effects are rescued by *hmga2* mRNA.

These morphological alterations are explained by the strong downregulation of key NCC regulatory genes, detected at neurula and tailbud stages. Moreover, we demonstrate that although *hmga2* is not required for the initial activation of NPB border genes, like *msx1*, *pax3* and *ap2*, it is necessary for the expression of NCS genes such as *snail2* and *sox10* at neurula stage, as well as for the expression of NCC markers like *twist*, *dlx2* and *sox9*, analysed at late neurula/tailbud stage during the EMT and migratory phase. Interestingly, initial *ap2* expression at stage 13 is unaffected by *hmga2* depletion, while its later expression and maintenance at tailbud requires *hmga2*; this may underlie the reiterative involvement of *ap2* in the NCC network (de Crozé et al., 2011). We also show that initial *hmga2* early expression at neurula stage depends on *msx1*, but not on *snail2* function. Our data altogether suggest that *hmga2* acts downstream of *msx1*, but is required for the initial expression of *snail2* and other NCS genes. In particular, we show that both *hmga2* and *pax3* are consistently modulated by *msx1*, upon its downregulation or upregulation. However, their expression may be mutually independent. In fact, on one side, *pax3* expression is not affected by *hmga2* downregulation or overexpression in wildtype embryos; in addition, *hmga2* depletion does not prevent *pax3* upregulation by *msx1* overexpression. On the other side, injection of *pax3* mRNA in wild type or Msx1-ablated embryos does not upregulate or restore *hmga2* normal expression, respectively. Coinjection experiments show that, downstream of *msx1*, *hmga2* cooperates with *pax3* for the regulation of *snail2* and *twist*: together, they can efficiently rescue the effects of MO-*msx1*. Furthermore, we show that *hmga2* function is required for the abilities of *pax3* (positive effect on *snail2* and *twist*) and of *snail2* (positive effect on *twist*). Therefore, *hmga2* plays a role in successive steps of the NCC genetic network, as a crucial node for eliciting several genetic activities that act in NCC specification and drive the programme that triggers the EMT and

migration of NCCs. Consistent with this, the compromised migration of Hmga2-depleted NCCs could result from disrupting the early specification step, though it does not exclude that *hmga2* could also play a later role in triggering the EMT/migration phase. Such a dual role, for example, has been shown for Snail1/2, that, in addition to their early role in specification, are also required from stage 16-17 for the migratory phase of NCC (LaBonne and Bronner-Fraser, 2000; Aybar et al., 2003). While we showed that initial *snail2* (and NCS gene) expression requires Hmga2, we do not know if also this later phase of *snail2* (and other NCS gene) expression requires Hmga2 at stage 16-17 and therefore is directly relevant for NCC EMT. Future work with inducible dominant negative *hmga2* constructs will be required to assess this point.

Although we did not uncouple the *hmga2* knockdown early effects on NCC specification from possible later effects on EMT/migration, the proposed sequential role of *hmga2* in *Xenopus* NCCs is consistent with observations in EMT cell culture models (our present results on NMuMG cells and following discussion). In fact, HMGA2 interacts with, and is recruited by, Smad4 for the activation of the *Snail1* promoter (Thuault et al., 2006, 2008). Similarly, HMGA2 is also involved in the activation of *Twist* by direct binding to its regulatory region, and in the epigenetic downregulation of *E-cadherin*, a target of the repressive activity of SNAIL1 and SNAIL2 factors (Thuault et al., 2006; Tan et al., 2012, 2015). It is therefore feasible that HMGA2 cooperates with several gene regulators at different steps of the EMT genetic programme not only in tumourigenesis (Heldin et al., 2009) but also in NCC EMTs; this capacity may be due to HMGA2 wide protein-protein interactive abilities (Sgarra et al., 2010), that could make it a versatile partner for different factors recruited at sequential steps during the deployment of the programme.

To get more insights about the molecular connections among *hmga2*, EMT and the NCC genes that we examined in *Xenopus* embryos, we extended our analysis to the murine breast cancer model of NMuMG cells, where EMT can be induced by TGF-β treatment with the pivotal role of HMGA2. In this EMT model we checked

whether some of the NCC network genes responded to interference of *Hmga2* function in a way consistent with our results on *Xenopus* NCCs. We found that some, though not all, of these genes (*Snail2, Twist, Zeb2, Adam12, Sox10*) were downregulated when *Hmga2* function was impaired; others, like *Sox9*, were not, in spite of their induction by TGF-β treatment. Interestingly, the relevance of *ADAM12* and *SOX10* genes in human breast cancer is emerging: the expression of *ADAM12* is associated with breast tumor-initiating phenotype and contributes to breast tumour growth (Li et al., 2012; Albrechtsen et al., 2013; Li et al., 2013) and *SOX10* is preferentially expressed in triple-negative and metaplastic breast carcinomas (Cimino-Mathews et al., 2013). Their regulation by TGF-β and by HMGA2 underlines once more the cross-talk between molecular pathways that play an important role in EMT, embryonic development and breast cancer stem cells (Takebe et al., 2011).

Among the genes examined, *Msx1* was not upregulated by TGF-β, suggesting that it does not participate in the TGF-β-induced EMT of NMuMG cells; its absence of regulation by *Hmga2,* also in basal conditions, is consistent with its higher position in the NCC network, acting at an earlier step than the actual initiation of EMT. On the other hand, though not induced by TGF-β, *Pax2* is downregulated in NMuMG by siHMGA2 in basal conditions; this suggests some modulatory ability of *Hmga2* on *Pax2*.

hmga2 cooperation in the regulation of specific genes may depend on the cellular context; indeed, in *Xenopus* embryos, we found that knockdown of *hmga2* leads to *pax2* downregulation in the otic vesicle, but not in the neural tube, where *hmga2* is also expressed. Similarly, also *sox2* is downregulated by *hmga2* ablation in its placodal domain; interestingly, evidence for direct regulation of *SOX2* by HMGA2 protein was recently shown in some cancer cell models (Chiou et al., 2013; Chien et al., 2015).

In conclusion, our results altogether show a pivotal role of *hmga2* in *Xenopus* NCC specification. Some, though not all, of the regulatory interactions of *hmga2* in

the NCC genetic network are confirmed in a murine breast cancer model of TGF-β–induced EMT (resumed in Fig. 10). These results strongly suggest, though do not definitely prove, that *hmga2* may be required for EMT also in NCCs, acting through key regulators (such as *snail2*, *twist*, *zeb2*) to which *hmga2* appears to be epistatic in a similar way to that described in tumor EMT (Thuault et al., 2006, 2006; Tan et al., 2012; Guo et al., 2013).

Besides HMGA2, several chromatin remodelers (*Xenopus* Brg1, Ajuba LIM proteins Lim1 and Wtip, Chd7, Brd7 and Lmo4; chick JMJD2A, PHD12 and LMO4) are important players in NCC development; interestingly, loss-of-function of these genes affects NCC migration and causes severe cranial skeletal malformations and/or downregulation of early NCC genes (Langer et al., 2008; Bajpai et al., 2010; Strobl-Mazzulla et al., 2010; Strobl-Mazzulla et al., 2012; Ochoa et al., 2012; Ferronha et al., 2013). Therefore, chromatin remodelers may be pivotal players in regulating NCC biology; in turn, NCCs may be a key model where to explore epigenetic aspects potentially relevant to cancer (Nieto, 2011; Hu et al., 2014).

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AUTHOR CONTRIBUTION

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FIGURE LEGENDS

Fig. 1 – *Xenopus hmga2* expression in developing NCCs as detected by WISH.
(A) *hmga2* mRNA is expressed in the prospective neural plate border (NPB, outlined in red) and in the prospective placodal plate (PPP, outlined in black) of stage 13 *Xenopus* embryo (dorsal-anterior view). (B) *hmga2* expression at stage 16 (anterior-lateral view) is visible in prospective neuroectoderm and cranial NCCs (arrowhead). (C-D) *hmga2* mRNA is detected in the anterior NCCs (arrowhead) at stage 20 (frontal view) and 28 (lateral view), respectively.

Fig. 2 – Phenotypic and molecular effects of HMGA2 depletion on *Xenopus* embryos. (A) Stage 28 morphant embryos (ventral view) show a clear reduction (red arrowhead) of the pharygeal bulges (bracket) (cg, cement gland). (B) Schematics of normal NCC migration in *Xenopus* embryos; ma, hy, br, denote mandibular, hyoid, branchial streams, respectively. (C) Schematics of skeletal derivatives of NCCs at tadpole stage; m, q, c, cb denote Meckel's, quadrate, ceratohyal, ceratobranchial cartilages, respectively. (D-G) Pharyngeal skeletal phenotypes obtained following unilateral injections of MOs, as indicated; frequencies of the displayed phenotypes (D,E, morphant phenotype; F,G, wild type aspect) were 83% (n=130) and 76% (n=122) for MO1 and MO2 injections respectively. Typical phenotypes obtained with anti-*hmga2* MOs consist in disruption or absence of branchial arch skeletal derivatives (stained in blue and indicated by arrows) and/or missing otic capsule (dashed circle) on injected side (left side in figure). (H) Effects of MO1 and MO2 on several NCC molecular markers as detected by WISH; expression was reduced by MO1 and MO2, respectively, in 75% and 76% of embryos for *twist* (n=124 and n=223); in 86% and 93% of embryos for *sox9* (n=86 and n=84); in 85% and 73% of embryos for *ap2* (n=66 and n=52); in 65% and 70% of embryos for *dlx2* (n=61 and n=47). (I) The effects of MO2 are rescued by coinjection of *hmga2* mRNA, as shown by recovery of normal *twist* expression (in 52% of embryos, n=99) and morphology

(in 68% of embryos, n=187). Injected side was determined by GFP visualization (D-G) and β-gal staining (H,I).

Fig. 3 – Hmga2 depletion impairs NCC migration in *Xenopus* embryos. Cranial NCCs from GFP or RFP labeled embryos were transplanted into host embryos (see scheme in the upper part of figure). While 5-mis MO-injected NCCs migrated normally, MO1- or MO2-injected NCCs failed to migrate properly in host embryos (left). When wild type RFP-labeled NCCs were transplanted in GFP-labeled, MO2-injected host embryos, they migrated regularly in the Hmga2-depleted environment (right). Pictures were taken at stage 18, 25, 30. Per cent inhibition of migration is indicated for MO1- or MO2-injected embryos relative to controls.

Fig. 4 - *msx1* modulates *hmga2* expression in *Xenopus* embryos. (A-P) Msx1 but not Snail2 is required for *hmga2* expression in prospective NCCs; depletion of Msx1 leads to reduction of *hmga2* expression (arrowhead) at stage 13, 16, and 25 (A-D); standard MO has no effect on *hmga2* expression (E-H); *msx1* mRNA expands *hmga2* expression, as shown at stage 13 (I, arrowhead) or at stage 16 (J, lines compare medio-lateral extension of *hmga2* expression domain between two sides of embryo), and results in stronger *hmga2* expression at stage 25 (K,L); MO against *snail2* mRNA does not affect *hmga2* (M), but downregulates *twist* (N) at stage 16, but has some effects on *hmga2* expression at stage 25 (O,P). (Q-V) Effects of *msx1* depletion or overexpression on stage 16 sibling embryos are shown on *pax3* (Q,T), *snail2* (R,U), *twist* (S,V). In all these embryos, injected side (inj) was scored by GFP fluorescence, except for (C,D,G,H,K,L,O,P) where β-gal was used as a tracer.

Fig. 5 - **Hmga2 is required for NCS, but not for NBP, gene expression**. (A) *Xenopus* embryos injected unilaterally with MO2 show downregulation of *snail2* and *sox10*, but not of *msx1, pax3, ap2* or *hairy2* at stage 13. (B) Effects of *hmga2* knockdown or overexpression in stage 16 embryos: MO2 causes downregulation of

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snail2 and *twist*, but not of *msx1* or *pax3*; a mismatched control MO (5-mis) has no effect; *hmga2* mRNA injection does not vary expression of these same genes. Injected side (inj) was scored by β-gal staining (A),or by GFP fluorescence (B).

Fig. 6 – Hmga2 is required for Msx1, Pax3 and Snail2 effects in the *Xenopus* NCC genetic network. (A-C) Embryos were injected unilaterally with *msx1, pax3*, or *snail2* mRNA (alone or in combination with MO2 or *hmga2* mRNA, as indicated) and subsequently analysed at stage 16 by WISH for expression of *hmga2, pax3, snail2* and *twist*, as indicated. GFP mRNA was coinjected to visualize injected side (inj) in all embryos.

Fig. 7 - *pax3* and *hmga2* synergize to rescue the effects of *msx1* knockdown in *Xenopus*. Embryos were injected unilaterally with the indicated combinations of MO-*msx1*, *pax3* and *hmga2* mRNAs and were subsequently analysed at stage 16 by WISH for expression of *hmga2*, *snail2* and *twist*, as indicated. Arrowheads show reduction of *hmga2* expression on the injected side. GFP mRNA was coinjected to visualize injected side (inj) in all embryos.

Fig. 8 - TGF-β stimulation regulates the expression of a novel set of genes, involved in the NCC genetic network, in a mouse breast cancer cell model. (A) Expression levels of mRNA encoding *Sox9*, *Pax2*, *EphrinB2* and *Ap2* genes were measured by real time RT-PCR at 6 and 30 hours after TGF-β treatment (5 ng/mL) in NMuMG cells, with respect to control cells (- TGF-β) set at 1 as reference. The data are represented as the mean ± SD (n=3). (B) Left panel shows representative RT-PCR amplification products of *Sox10*, *Adam12* and *Msx1* genes in NMuMG cells stimulated with TGF-β (6 and 30 hours of treatment, lanes 2 and 4 respectively) and in control cells (lanes 1 and 3) loaded on 10% TBE-PAGE. RT-PCR was carried out on the same biological triplicate samples as in A. Right panel shows densitometric analyses of RT-PCR products (n=3) of *Sox10*, *Adam12* and *Msx1* genes, comparing

cells exposed to TGF-β treatment (+TGF-β) with respect to control cells (-TGF-β) set at 1 as reference both at 6 and 30 hours. *Sox10* densitometric analysis was not reported at 30 hours (± TGF-β) because PCR products were not detectable at this time point (n.d. in the graph). In both (A) and (B) *Gapdh* is used as control and internal normalizer.

Figure 9 - Hmga2 downregulation modulates several NCC genes in the TGF-β induced EMT of mouse breast cancer cell model. (A) Relative mRNA expression levels of *Hmga2*, *Fibronectin* (*Fn1*), *Snail2*, *Zeb2* and *Sox9* genes in NMuMG cells silenced for *Hmga2* gene (si_HMGA2) with respect to cells transfected with control siRNA (si_CTRL), set at 1 as reference, in TGF-β exposed conditions (2.5 ng/mL, 16 hours after TGF-β treatment). Expression was measured by real time RT-PCR analyses and normalized against control cells (si_CTRL). The data are represented as the mean ± SD (n=3). (B) Left panel: gene expression analyses of *Sox10* and *Adam12* of NMuMG cells exposed to TGF-β and silenced (si-HMGA2) or not (si-CTRL) for the expression of *Hmga2*; a representative image of RT-PCR analyses on 10% TBE-PAGE is shown. Right panel: densitometric analyses of RT-PCR products (n=3) of *Sox10* and *Adam12* genes comparing cells silenced for Hmga2 gene (si_HMGA2) with cells transfected with control siRNA (si_CTRL), set at 1 as reference. In both (A) and (B) *Gapdh* is used as control and internal normalizer.

Figure 10 - Scheme of *hmga2* action on the NCC and EMT-promoting genes analysed in this study. In the *Xenopus* embryo, NPB genes (blue boxes), responding to earlier inducing signals, initiate the NCC genetic network; *msx1* has a key position in eliciting *pax3* and *hmga2* expression (blue arrows). *pax3* action on *snail2* and *twist* is shown by red arrows*; pax3* and *hmga2* cooperate in their modulation. *hmga2* modulates the expression of several genes of the NCC genetic network (black arrows); genes shown in green and red boxes respond to TGF-β, but only for the first group modulation by *hmga2* was confirmed in the NMuMG breast cancer cell model.
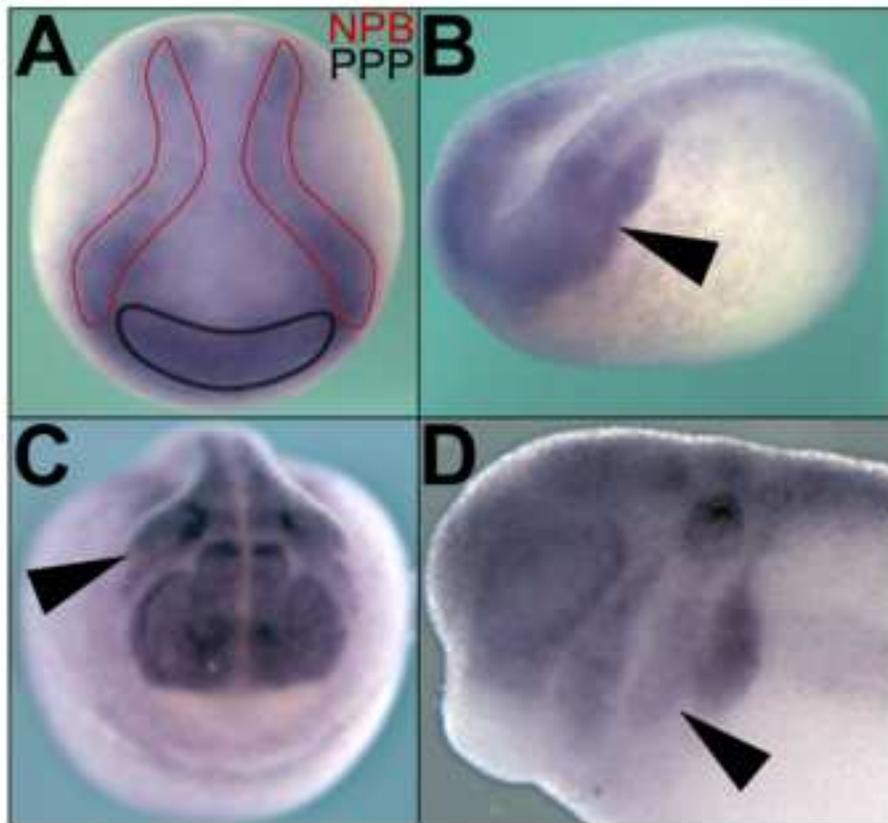
A

B

C

MO1

MO2

MO-STD

5-mis

D

E

F

G

MO1

injected side   control side

MO2

injected side   control side

twist

sox9

ap2

dlx2

H

MO2+*hmga2* mRNA

injected side   control side

twist

I

experimental design
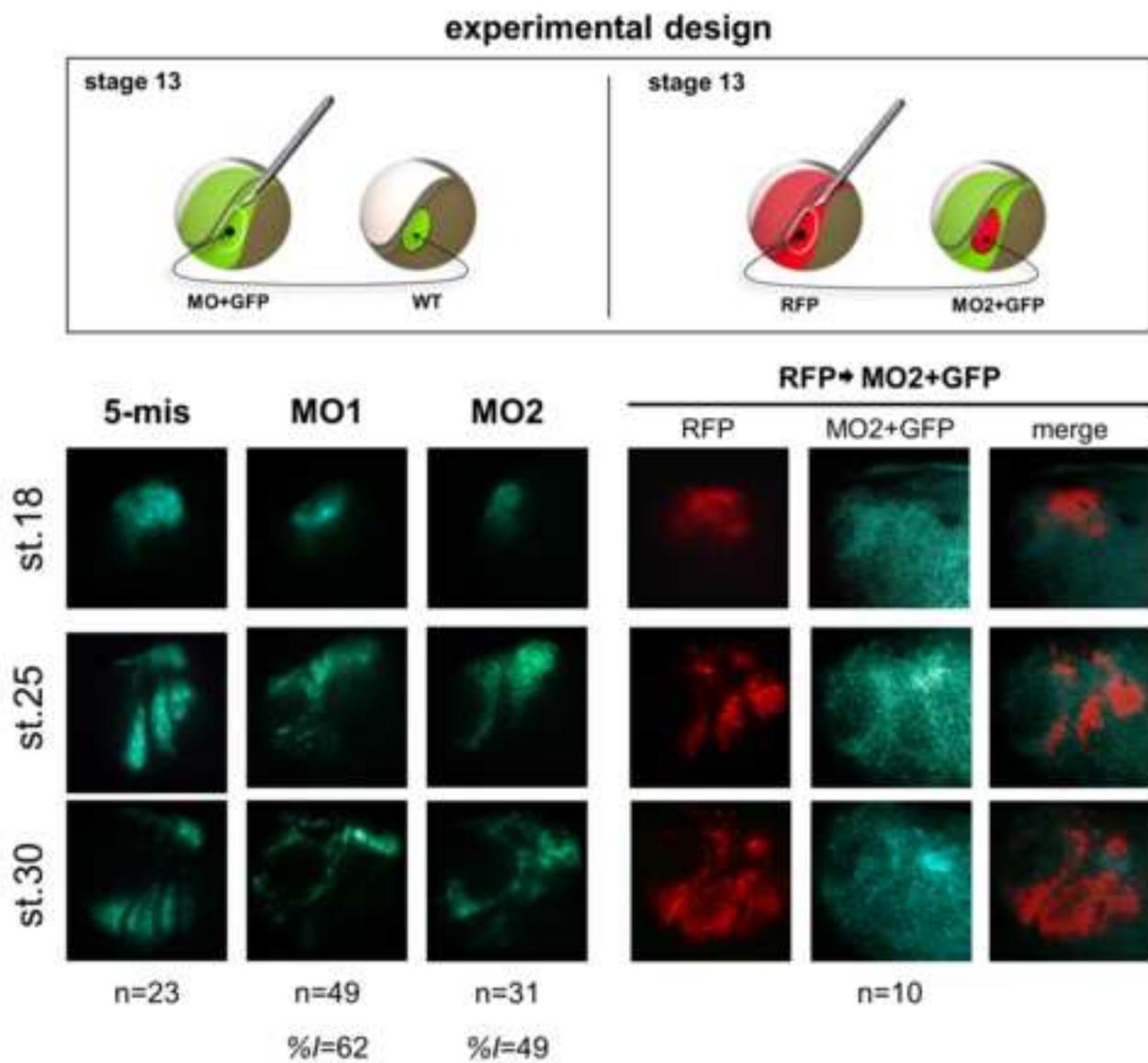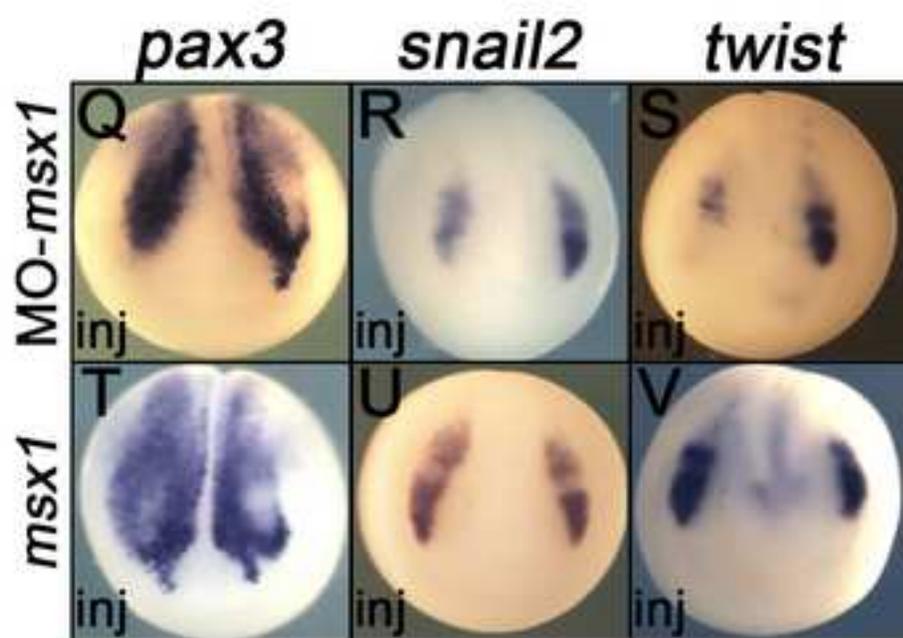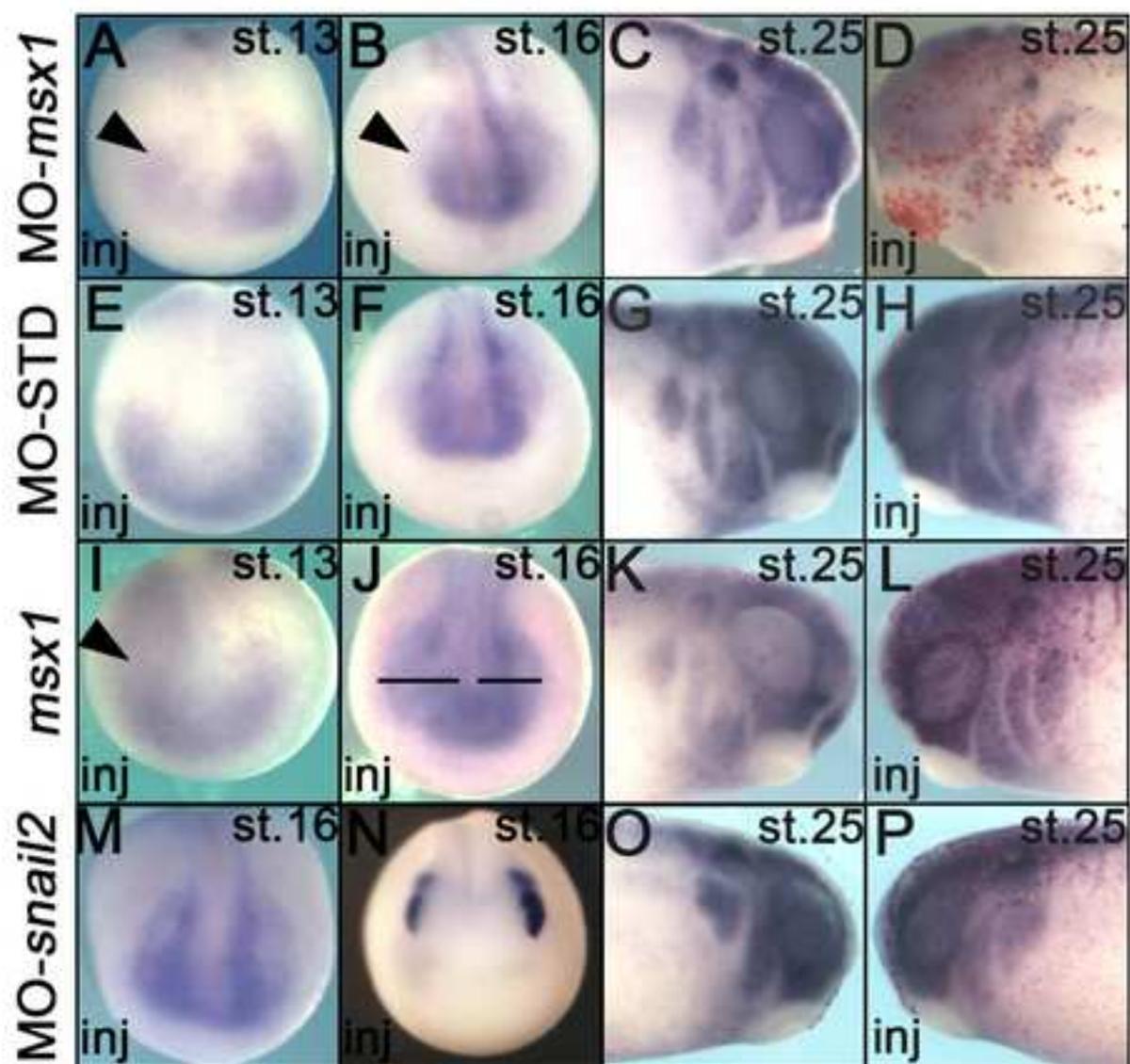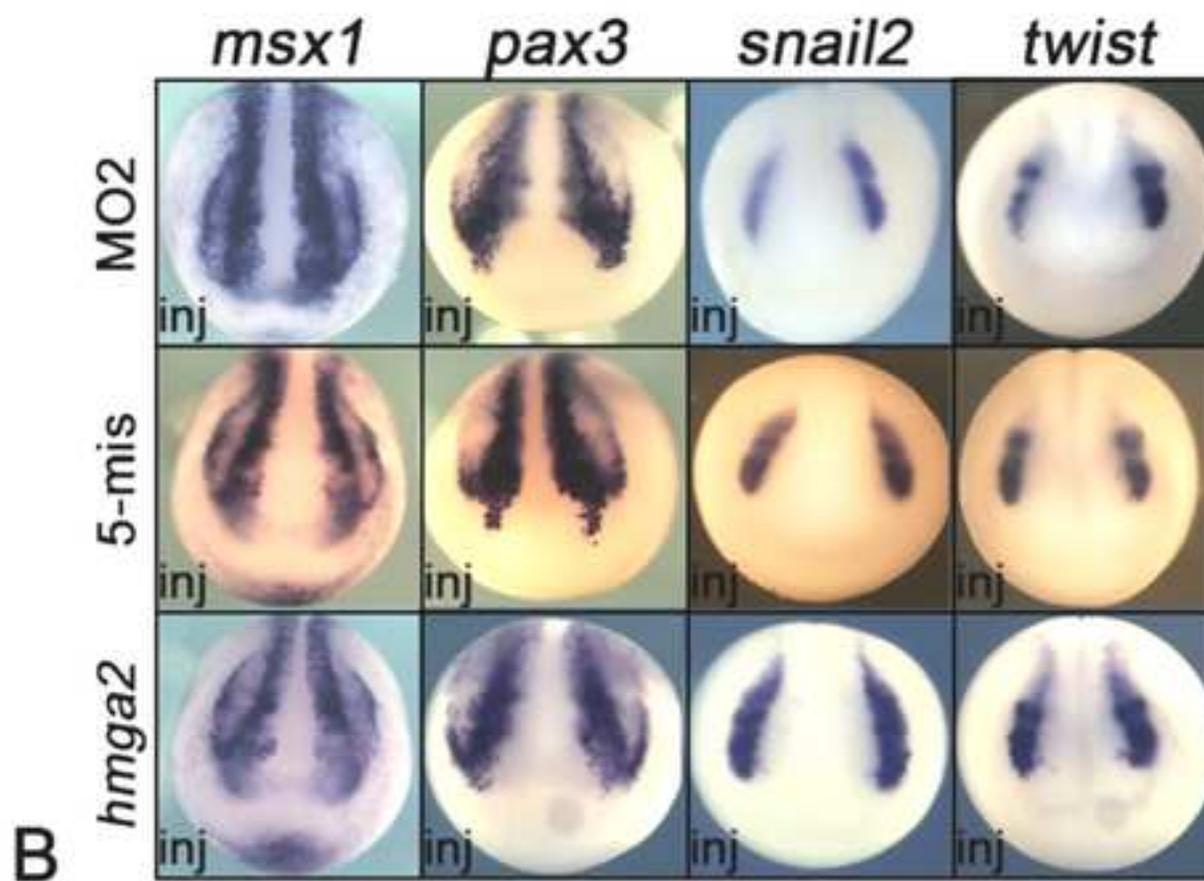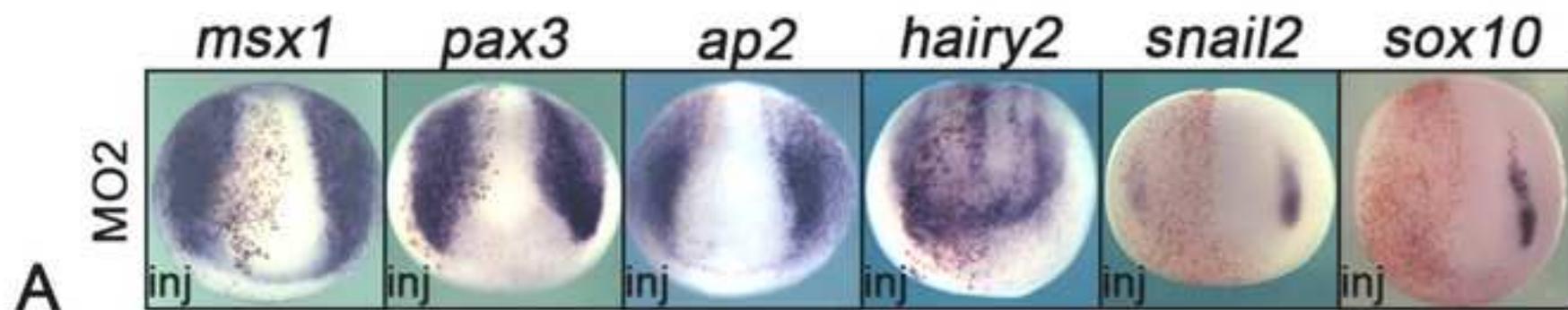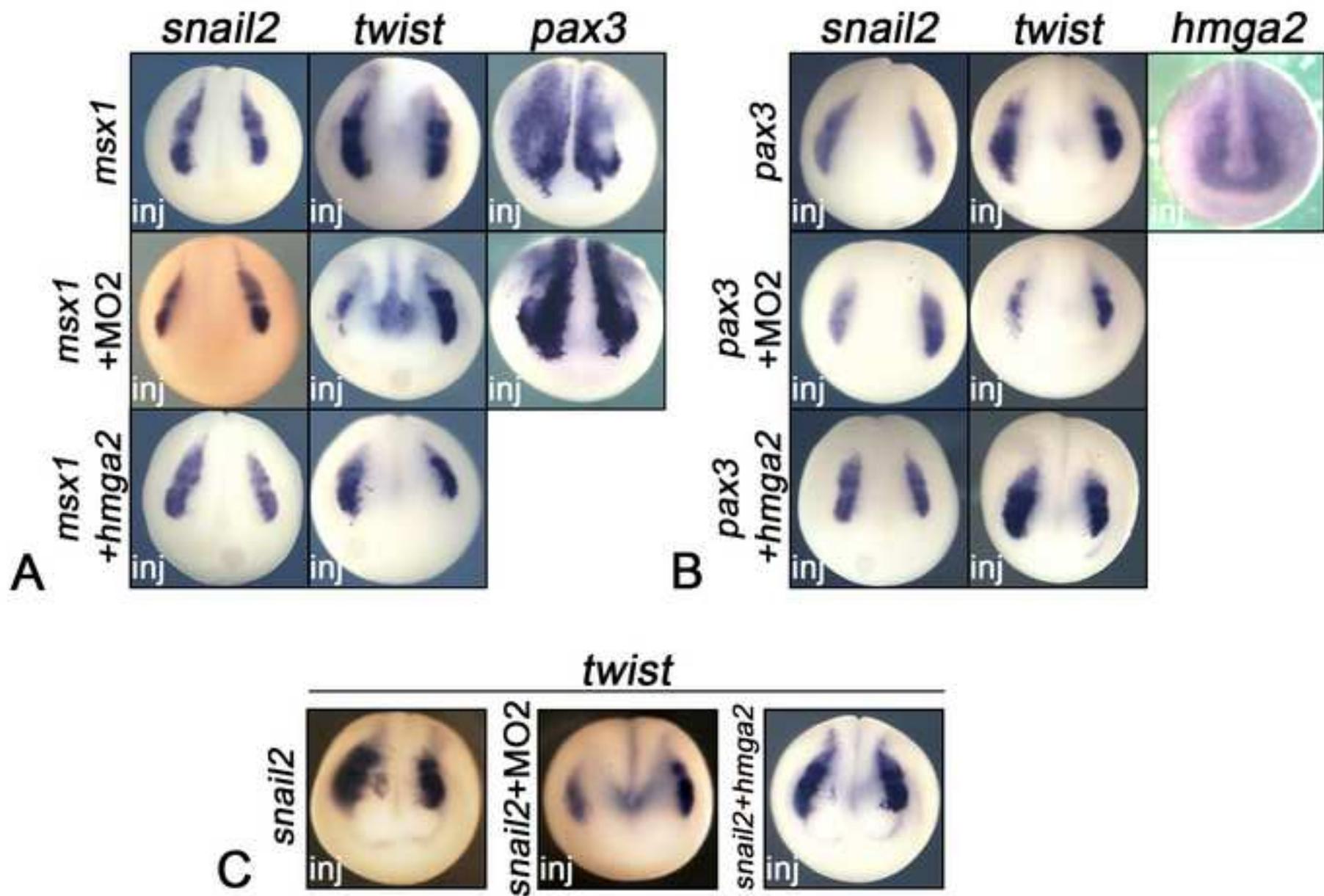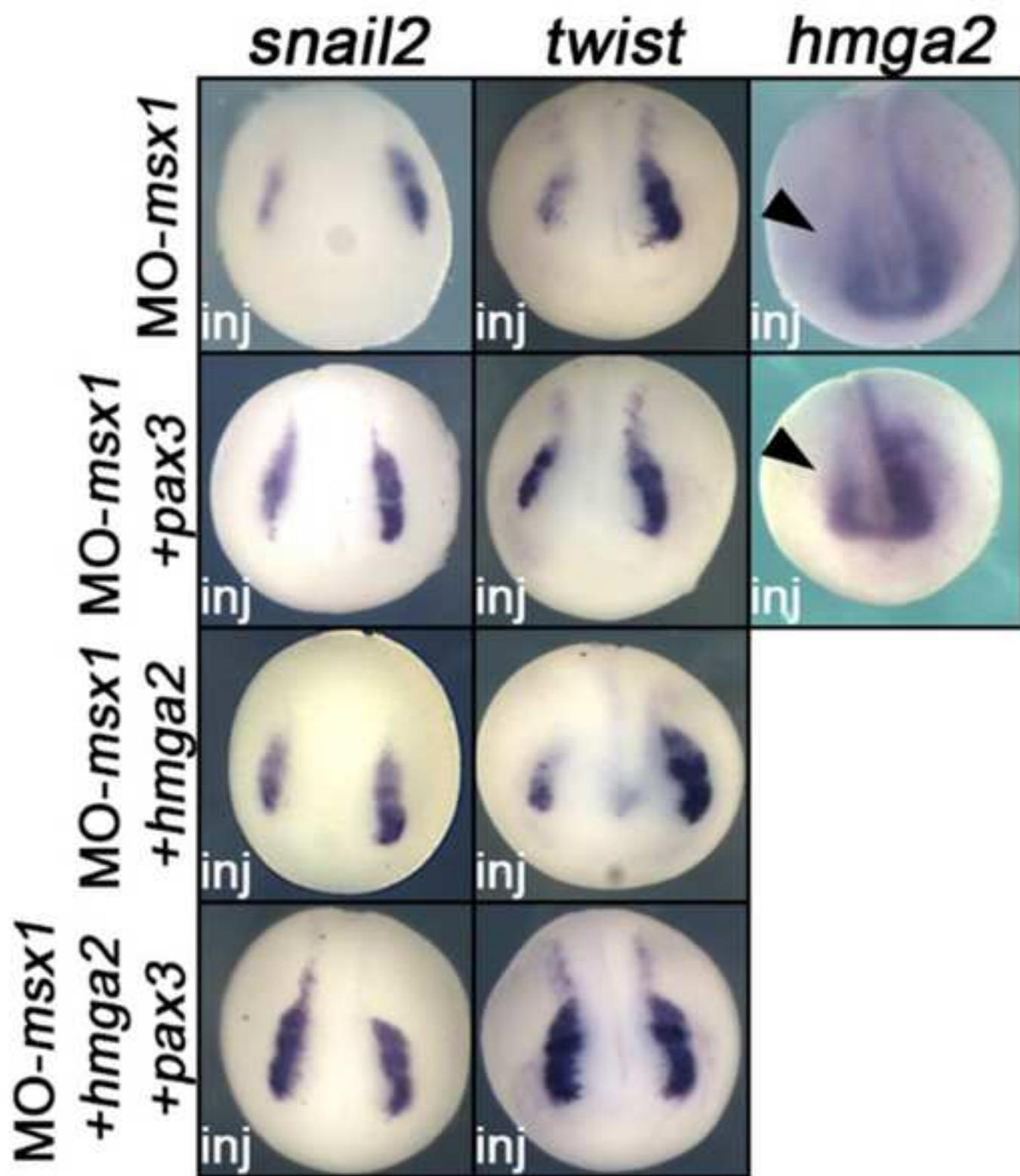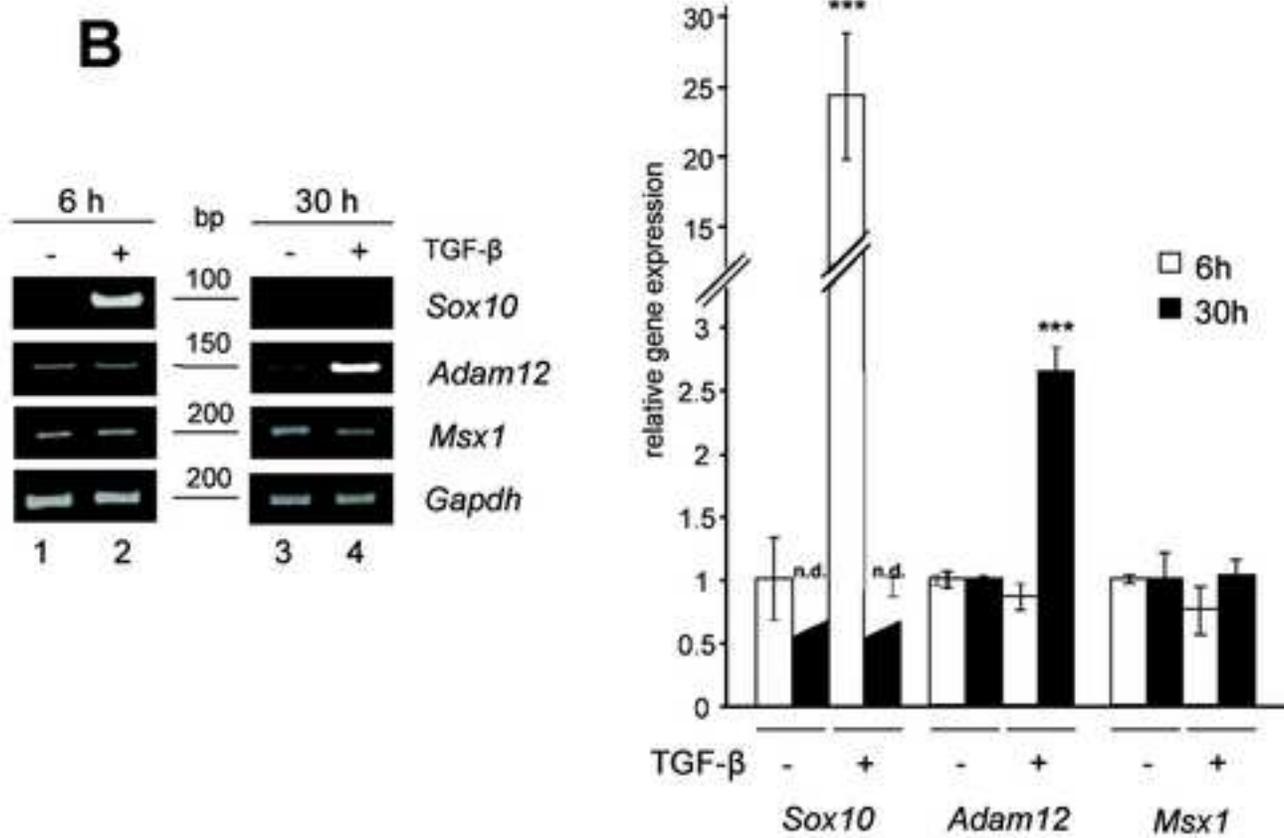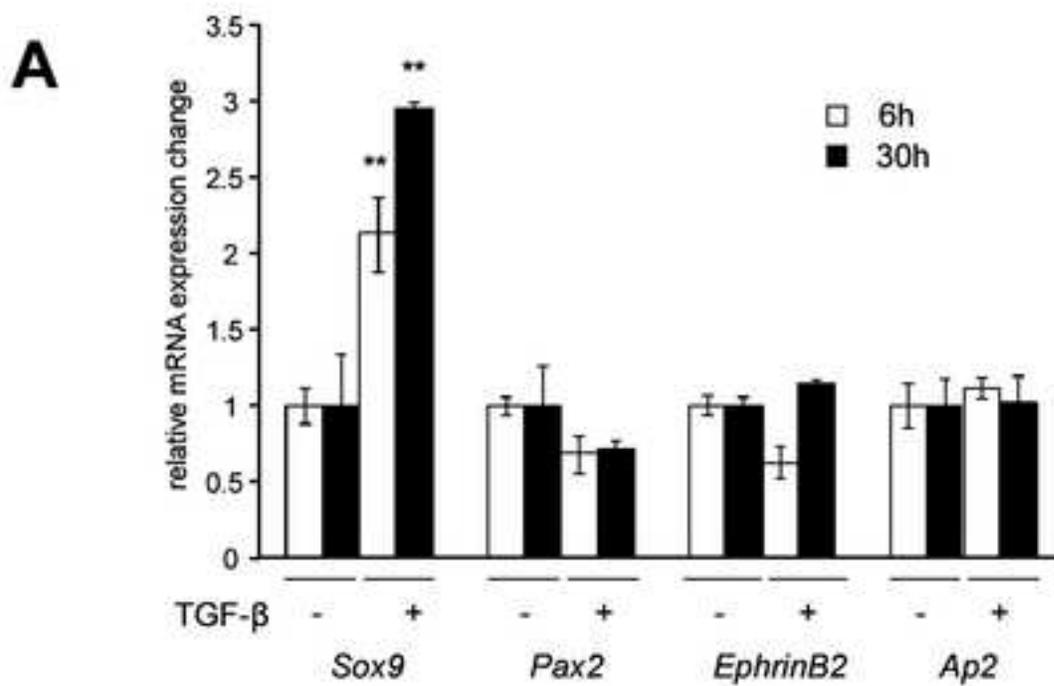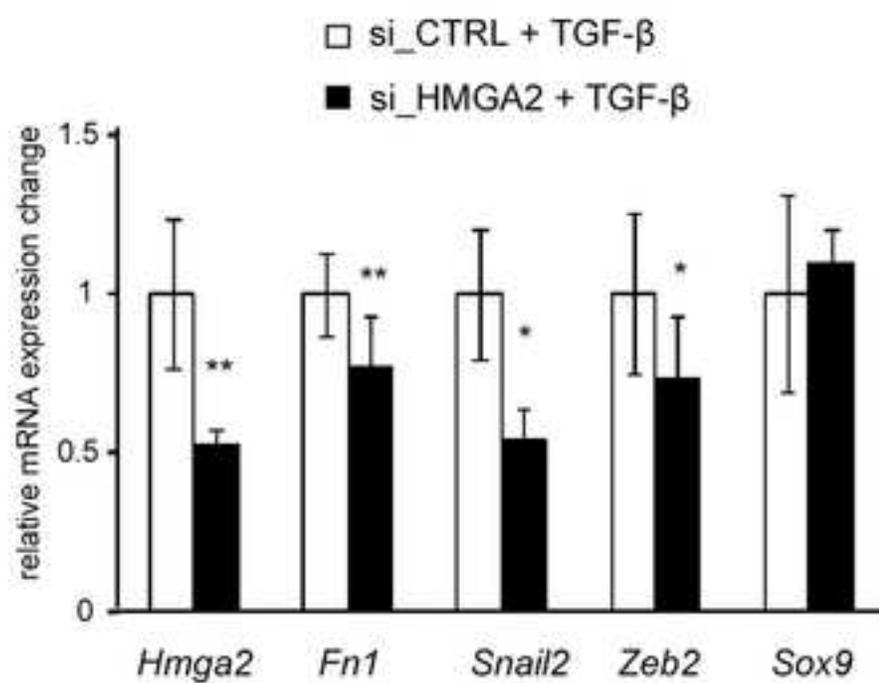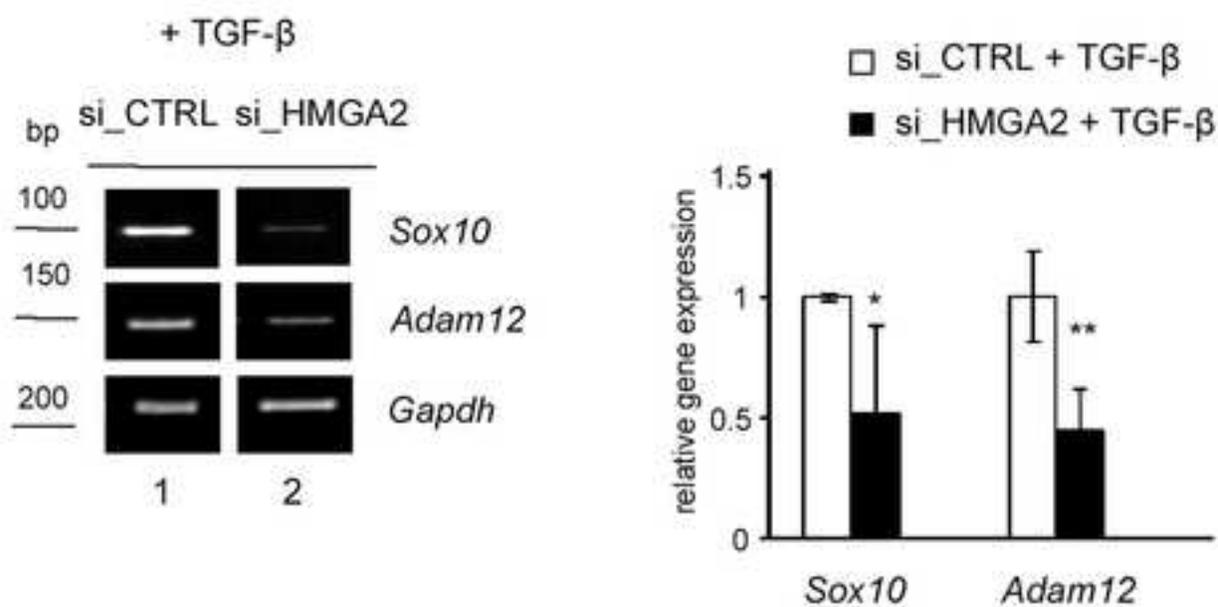
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