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Gli2 is required for the induction and migration of *Xenopus laevis* neural crest

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Running Title:

Gli2 in *Xenopus* neural crest development

Abstract

The neural crest (NC) is a multipotent migratory embryonic population that is formed during late gastrulation and gives rise to a wide array of derivatives, including cells from the peripheral nervous system (PNS), the craniofacial bones and cartilages, peripheral glial cells, and melanocyte cells, among others. In this work we analyzed the role of the Hedgehog signaling pathway effector *gli2* in *Xenopus* NC. We provide evidence that the *gli2* gene is expressed in the prospective, premigratory and migratory NC. The use of a specific morpholino against *gli2* and the pharmacological specific inhibitor GANT61 in different experimental approaches allowed us to determine that *gli2* is required for the induction and specification of NC cells as a transcriptional activator. Moreover, *gli2* also acts by reducing apoptosis in the NC without affecting its cell proliferation status. We also demonstrated that *gli2* is required cell-autonomously for NC migration, and for the formation of NC derivatives such as the craniofacial cartilages, melanocytes and the cranial ganglia. Altogether, our results showed that *gli2* is a key transcriptional activator to accomplish the proper specification and development of *Xenopus* NC cells.

Key words

Neural crest; Hh; cell signaling; cell specification, cell migration; GANT61

List of Symbols and Abbreviations used in this paper:

AP, alkaline phosphatase; NC, neural crest; NCC, neural crest cells; NCP, neurocristopathies; Hh, Hedgehog; Ihh, Indian hedgehog; Shh, Sonic hedgehog; ISH, *in situ* hybridization; IHC, immunohistochemistry; NP, neural plate, NPB, neural plate border; ng/E, nanograms per embryo.

1. INTRODUCTION

The neural crest (NC) is a multipotent embryonic cell population with stem-cell-like properties that arises at the border between the neural plate (NP) and the prospective epidermis, and migrate along the entire embryo. This cell population, which separates the craniates/vertebrates from other metazoans, has played a key role in the evolutionary development of the jaw and other cranial structures (Saint-Jeannet, 2006). Derivatives from the NC include neurons and glia of the sensory, autonomic and enteric nervous system, medullary secretory cells, bone marrow cells from the femur and other long bones, smooth muscle cells, melanocytes, bone and cartilage cells, among other cell types (Couly et al., 1998; Keyte et al., 2014; Vega-Lopez et al., 2017). Increasing research in recent years has focused on NC development based on the fact that the abnormal formation of this cell population can give rise to a diverse class of pathologies known as neurocristopathies (NCP), which affect a large proportion of newborns (Vega-Lopez et al., 2018; Watt and Trainor, 2014). Therefore, studying the mechanisms underlying NC formation is a necessary step for the isolation of multipotent NC stem cells that could act as potential sources for the treatment of several of these NCP and many NC-related cancers (Liu and Cheung, 2016).

Interactions between distinct signaling pathways such as BMP, FGF, Notch and Wnt play a key role in the development of the NC. These signals, along with other molecules, participate in establishing a gene regulatory network that is crucial to determine NC identity (Martik and Bronner, 2017; Sauka-Spengler and Bronner-Fraser, 2008). Hedgehog (Hh) ligands include Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh) (Wu et al., 2017). We have previously found in *Xenopus* embryos that the Indian Hedgehog (Ihh) signaling pathway is active in the NC tissue, and that it is required for the development of this cell population (Aguero et al., 2012). We also revealed the regulatory relationships between this pathway and the *eg5* gene in the NC (Fernandez et al., 2014). The Hh signaling pathway plays important roles in development, tissue homeostasis and tumorigenesis. Signaling is initiated when the ligand binds to the receptor Patched1 (Ptch1). When unbound, Ptch1 inhibits the activity of a second receptor called Smoothened (Smo), which is responsible for the activation of the pathway effectors, the Gli transcription factors (Gli1, Gli2 and Gli3). These transcription factors are post-translationally regulated and act in a context-dependent combinatorial fashion (Chen and Jiang, 2013; Ruiz i Altaba et al., 2007). When the Hh signal is absent, Gli2 and Gli3 are proteolytically processed into repressor forms (GliRep), whereas when the pathway is active, they act as full length transcriptional activators (GliAct) (Hui and Angers, 2011).

Gli2, the main activator of the Hh signaling pathway, stimulates the transcription of several target genes (Kim et al., 2009). In *Xenopus* embryogenesis, Gli proteins play a crucial role during neurogenesis, inducing different neuronal types through a cooperative and combinatorial action (Nguyen et al., 2005). Gli2 has been shown to participate in several developmental processes, acting as a transcriptional activator. These processes include palate development (Mo et al., 1997), lung and pituitary gland formation (Ding et al., 1998; Wang et al., 2010) and the generation of the central nervous system (Chen et al., 2018; Lepanto et al., 2016; Matise et al., 1998). However, a repressor function of Gli2 has also proved to be important for the development of the zebrafish nervous system (Karlstrom et al., 2003). Therefore, since Gli2 regulates many cellular processes, it has been associated with disease when its regulation goes awry (reviewed in (McCleary-Wheeler, 2014)). The growing number of therapeutic applications based on the function of Gli2 has generated a dramatic increase in the number of studies that analyze the function of this transcription factor. Nevertheless, despite great advances in the understanding of the mechanisms underlying the function of Gli2 during central nervous system development, little is known about the role of this transcription factor in the formation of the NC.

In this work we address the participation of Gli2 in the formation of *Xenopus laevis* NC. We made a detailed analysis of the spatio-temporal expression pattern of this gene, thus complementing previous reports. Furthermore, using gain and loss-of function studies and RT-qPCR analysis, we show that *gli2* is essential for the induction, specification and maintenance of the specification and migration of the NC. Mechanistically, our results also provide evidence that Gli2 is acting as a transcriptional activator in NC development. We also suggest that Gli2 might be required for the formation of NC derivatives such as craniofacial cartilages, melanocytes, Rohon Beard (RB) neurons and cranial ganglia primordia. By taking advantage of the Gli2-specific inhibitor GANT61, we corroborated the results obtained with the standard morpholino-based knock down approach. To summarize, by different approaches we have addressed the participation of this transcription factor in the formation of the NC.

2. RESULTS

2.1. Spatio-temporal expression patterns of *gli2* and Ptc1

Using simple and double *in situ* hybridizations (ISH), we performed a comprehensive analysis of the spatio-temporal expression pattern of the *gli2* gene during the initial development of *Xenopus laevis*. During late gastrulation (stages 12 and 13), the expression of *gli2* was observed in the dorsal region of the embryos at the anterior NP border (NPB) extending through the NP posteriorly with no expression at the dorsal midline. This gene was also expressed in the lateral posterior NPB (Figures 1A-B). At mid-neurula stages (st. 15-16), the expression increased in the NPB (Figures 1C-D, yellow arrowheads), which corresponds to the prospective NC. During the course of neurulation we detected *gli2* transcripts in the anterior NP (black arrow). As development proceeded, at stages 16-17 (Figure 1E, asterisk) *gli2* expression was also observed in the mesoderm. When NC cells (NCC) started to migrate from the dorsal region of the embryo (stage 18), the expression of *gli2* was located in the NC migratory region without apparent expression in the midline (Figures 1F-G). Additionally, at tailbud stages (Figures 1H-I), *gli2* transcripts were detected in the head region of the embryo. Stage 24-embryos showed expression in the prospective branchial arches, in particular in the mandibular and hyoid NC streams, and also in the optic vesicle. Afterwards, at stage 32, the expression became more prominent in the boundaries between the branchial arches. At this point, *gli2* expression in the optic vesicle became restricted to its borders. Additionally, *gli2* was detected in the otic vesicle and in the segmentative presomitic mesoderm.

To further delineate the expression of *gli2* in the NC, we performed double ISH with NC and NP markers. At stage 15, a double ISH of *gli2* with the NC marker *foxd3* showed a clear overlapping expression of both genes in the inner region of the NC (Figure 1J). Transversal sections of these embryos revealed that *gli2* expression occurred mainly in the internal ectodermal layer corresponding to the NP and prospective NC (Figure 1J'). Then we analyzed the NP marker *sox2* and found an overlap in the expression of both genes in the lateral region of the NP as summarized in Figure 1K-K'. Additionally, *gli2* expression (white arrowhead) expanded more anteriorly than that of *sox2* (Figure 1K'', anterior view, green arrowhead). These initial results suggest that *gli2* is mainly expressed in the NC territory during late gastrulation and throughout neurulation.

It is well known that *ptch1* serves as a readout of the Hh signaling pathway as its expression is increased in response to the activation of the Hh pathway (Goodrich et al., 1996). Therefore, analyzing the expression pattern of the Ptch1 protein could provide useful

information about the embryonic locations where the Hh signaling is active. To that end, we analyzed the expression of the Ptch1 receptor by performing a wholemount immunohistochemistry (IHC) assay. We found that Ptch1 is expressed in the ectoderm at the dorsal side of the embryo (Figure 1L) at the gastrulation stage 11. By the end of gastrulation, at stages 12.5-13, Ptch1 is expressed in the anterior NPB (Figure 1M, yellow arrowheads) and is faintly expressed in the lateral NPB. At neurulation stages the expression of Ptch1 increases in the elevating neural folds, as depicted in Figure 1N-O. Figure 1O' is a magnification of Ptch1 expression in the uprising neural folds that clearly shows Ptch1 expression in the NC cell outlines. This expression profile agrees with its role as a transmembrane receptor. To summarize, the scheme in Figure 1P shows the different domains of expression of *gli2* (blue) and Ptch1 (green) along with the neural crest (*foxd3*, light yellow) and neural (*sox2*, pink) markers.

Taken together, these results provide compelling evidence that *gli2* is expressed in the prospective, premigratory and migratory NC, thus supporting a potential role in NC formation. In addition, the analysis of Ptch1 expression shows strongly suggesting that the Hh signaling pathway is active in this cell population.

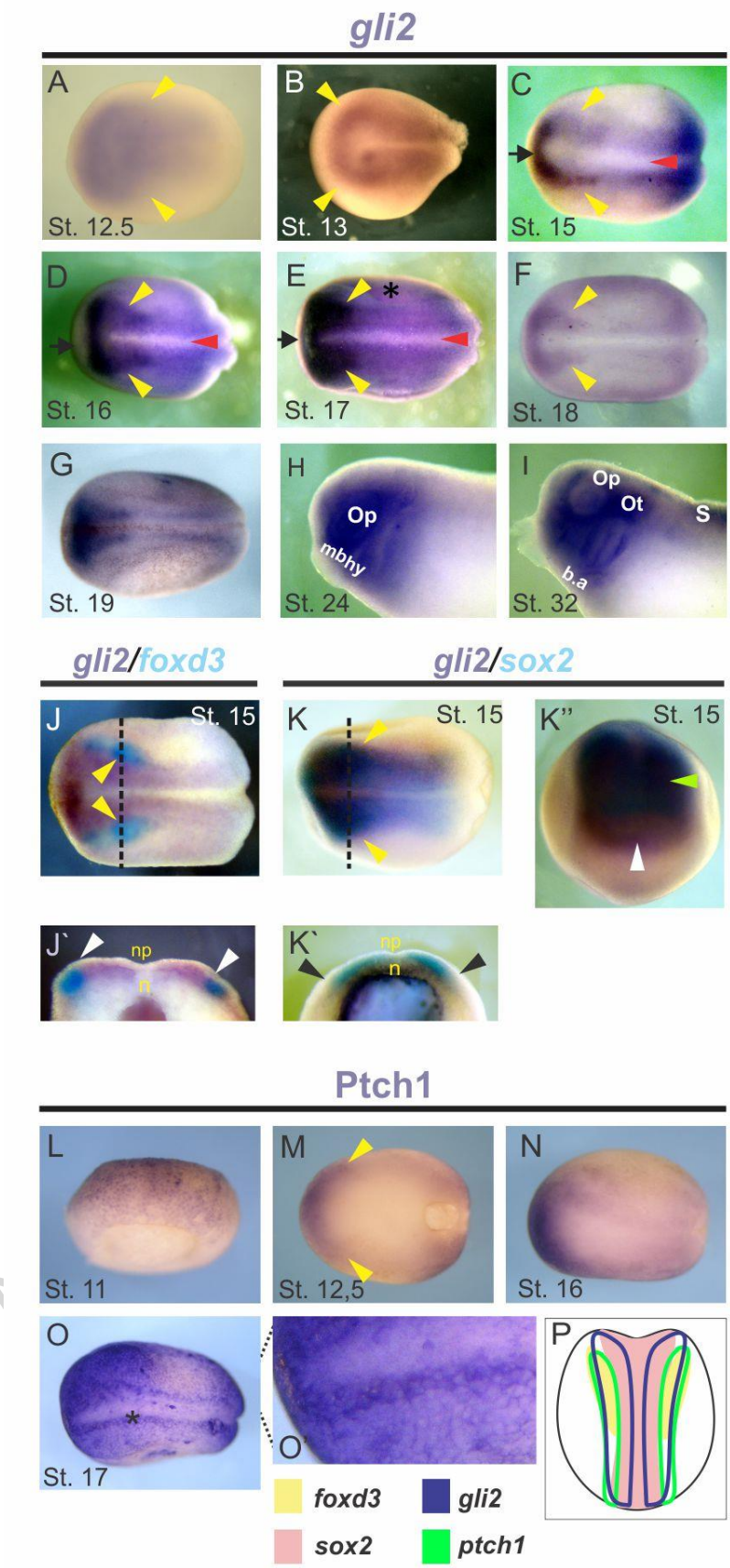

Figure 1. *gli2* and Ptc1 expression patterns in *Xenopus* embryos. **(A-G, J-K, M-O)** Dorsal views of *Xenopus laevis* embryos, anterior side on the left. **(L)** Dorsal view, anterior side at the top. **(J', K')**: Transversal sections, dorsal side at the top. **(H-I)** Lateral views, anterior side on the left. **(K'')** Anterior view. **(A-B)** *gli2* is

expressed in the dorsolateral region of the embryo at stages 12.5 and 13 (yellow arrowheads). **(C-D)** *gli2* transcripts are detected in the NPB at early neurula stage (yellow arrowheads) and in the anterior NP (black arrow). No expression can be observed in the dorsal midline (red arrowhead) **(E-G)**. From stage 17 to 19, *gli2* becomes more prominent in the lateral and anterior NPB. **(H-I)** At tailbud stages, *gli2* is expressed in the mandibular (mb) and hyoid (hy) NC streams, in the branchial arches (b.a.), in the otic (Ot) and optic (Op) vescicle and in the presomitic mesoderm (S). **(J-K)** Double ISH showing *gli2* expression with two gene markers. **(J-J')** *gli2* is expressed in an overlapping territory with the NC marker *foxd3*. **(K-K')** The domain of *gli2* expression extends laterally from the NP, as evidenced by the double ISH with *sox2* in whole embryos. **(K'')** Anterior view showing that the expression of *gli2* extends mores anteriorly than that of *sox2*. **(L)** At the late gastrula stage, Ptch1 is expressed in the dorsal side of the blastopore. **(M-O)** At neurula stages, Ptch1 can be detected in the NPB and in the neural folds. **(O')** Expression of Ptch1 in the cell membranes of ectodermal cells. **(P)** Diagram representing the expression domains of the Ptch1 protein (green) and the genes *gli2* (blue)*, foxd3* (light yellow) and *sox2* (pink).

2.2 *gli2* is required for the early induction of the NC

The process of NC induction is complex and involves several steps of signal inputs, protein-protein interactions, and the combinatorial activity of different transcription factors. More specifically, during the early steps of NC formation, several molecules including signaling peptides and transcription factors act to establish the NPB, which thereof acquires a different molecular identity from the surrounding neural and non-neural ectoderm. Together, these factors are called NPB specifiers. These specifiers include the transcription factors *pax3, snail1* and *msx1* (Mayanil, 2013).

Initially, in order to analyze the requirement of *gli2* during the early steps of NC induction, we used the directed microinjection of a morpholino oligonucleotide against the translation initiation site of the *gli2* mRNA, called *gli2MO*. As a first step we decided to evaluate the efficiency of *gli2MO* against *gli2 in vivo* and *in vitro*. A plasmid encoding a chimeric protein consisting of the first 92 aminoacid residues of Gli2 fused to a GFP protein (*gli2GFP*) was constructed. Subsequently we injected different amounts of *gli2MO* along with the *mRNA* of the *gli2GFP,* and the expression of GFP by fluorescence microscopy and Western blotting was evaluated. The injection of a control morpholino *(CoMO)* along with *gli2GFP mRNA* did not affect GFP expression in the embryo (Figure 2A, 100% of embryos, n=25). As shown in Figures 2B-C the injection of 2.5 ng/E and 5 ng/E of *gli2MO* caused a reduction in GFP expression in a dose dependent manner (100%, n=25, and 95%, n=25, respectively). At a *gli2MO* concentration of 10 and 27 ng/E the fluorescence was completely abolished (Figures 2D-E, 100%, n=25). To further analyze the effects of the morpholino on *gli2GFP* expression, we tested the highest concentration of *gli2MO b*y western blot analysis of whole

embryos protein extract and found that the band intensity was noticeably reduced by the effect of the morpholino (Figure 2F). Thus, these two approaches allowed us to confirm the efficiency of *gli2MO* against *gli2* expression.

We subsequently carried out loss-of-functions studies to analyze the requirement of Gli2 in the induction of the NC. *gli2MO* (27 ng/E) was microinjected into one dorsal blastomere of 8-cell stage embryos together with the lineage tracer fluorescein dextran amine (FDA) to identify the injected side (Figure 2G). The analysis of the expression of the NPB markers at stages 13-14 showed that the injection of *gli2MO* produced a marked reduction in the expression of *pax3* (Figure 2H, 71%, n=43)*, msx1* (Figure 2I, 61%, n=61) and *snail1* (Figure 2J, 70%, n=41). This result suggests that *gli2* might be required for the early events of NC formation. We then evaluated the participation of *gli2* in NC induction by the directed microinjection of *gli2* mRNA (2.6 ng/E) in one dorsal blastomere of 8-cell stage embryos. We observed a strong increase in the expression of *pax3* (59%, n=46)*, msx1* (66%, n=38) *snail1* (72%, n=39) as shown in Figures 2K-M. The quantification of the different phenotypes is plotted in Figures 2N-O. These results reinforce the assumption that *gli2* would participate in the early events of NC formation.

NC explant culture provides a way to specifically analyze the effect of certain chemicals exclusively on these cells, thus providing a detailed spatial and temporal control of the experiment. We have previously shown that the inhibition of Ihh signaling by the effect of Cyclopamine produces a decrease in the expression of the NC gene marker *foxd3* (Aguero et al., 2012). Based on these results, we decided to evaluate the function of GANT61, a specific Gli2 inhibitor, in the induction of the NC by analyzing its effects on the expression of the *foxd3* marker. GANT61 prevents Gli2 from binding to the DNA, thus precluding its action as a transcription factor (Agyeman et al., 2014; Lauth et al., 2007). Taking into account that the Gli1 protein is not directly modulated by Hh signaling and that it is not expressed in the NC (Nguyen et al., 2005; Suzuki et al., 2010), we can assume that the effects of GANT61 on NC are only due to the inhibition of Gli2. Consequently, we performed NC explants containing the underlying mesoderm (NC+M) at stage 12.5 and incubated them with 50 µM GANT61 (or only the vehicle as control) until stage 16, when the explants were fixed and the expression of *foxd3* was tested (Figure 2P). The inhibition of Gli2 by GANT61 produced a marked decrease in the expression of *foxd3* (Figure 2Q, 80% of explants n=35) as compared with DMSO incubation, in which no reduction in *foxd3* was detected (Figure 2R, 94% of NC+M explants with normal expression, n=16). To summarize, this combination of

approaches to block Gli2 expression and function allowed us to suggest that this transcription factor is important in the early events that regulate NC formation.

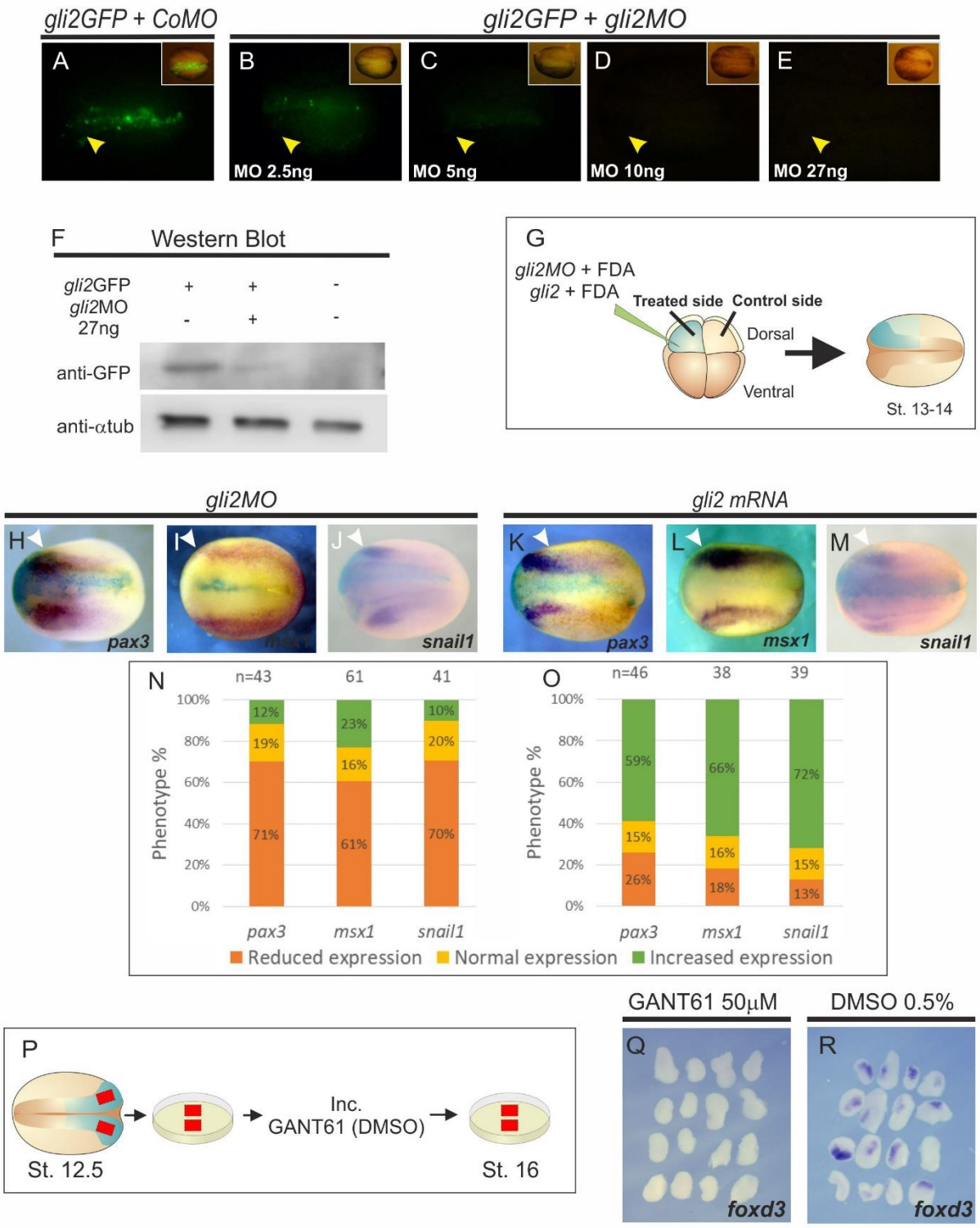

Figure 2. *gli2* participates in the early induction of the NC. (A-E, H-M) Dorsal views of *Xenopus laevis* embryos at mid-neurula stage; anterior side is on the left. Arrowheads indicate the injected side, shown in turquoise and by GFP fluorescence in A-E. The expression of gene markers is shown in purple. **(A-F)** Analysis

of *gli2MO* efficiency *in vivo* and *in vitro*. **(A-E)** *in vivo* assay. Merged fluorescence and clear field images of each embryo are shown as insets. **(A)** Normal GFP fluorescence was detected in the embryo coinjected with *gli2GFP* mRNA (2.6 ng/E) and *CoMO* (27 ng/E). **(B-E)** Embryos injected with *gli2GFP* and increasing concentrations of *gli2MO* (2.5 to 27 ng/E) evidenced a reduction in GFP fluorescence intensity. **(F)** *in vitro* analysis by Western blot shows a marked reduction in the expression of *gli2GFP*. α-tubulin expression was used as loading control. **(G)** Schematic representation of the gain- and loss-of-function experiments. **(H-J)** Analysis of *gli2* requirement on NC induction. *gli2MO*-injected embryos show a reduced expression of the NC induction markers *pax3*, *msx1* and *snail1*. **(K-M)** *gli2* mRNA-injected embryos show an increase in the expression of the NPB markers *pax3, msx1* and *snail1.* **(N-O)** Quantification of phenotypes shown in H-J and K-M, respectively. **(P-R)** The specific inhibition of Gli2 in NC explants incubated from stage 12.5 to 16 with GANT61 diminishes the expression of the NC marker *foxd3*.

2.3. *gli2* is required for the maintenance of NC specification

After NC induction occurs, these cells are specified and acquire a distinct molecular signature that separates them from other ectodermal cells. In order to assess the requirement for *gli2* in NC specification we analyzed the expression of the NC specifier genes *foxd3, snail2* and *sox9* after experimentally affecting *gli2* function (Figure 3A). *gli2MO* injection produced a reduction in the expression of the NC markers *foxd3* (64%, n=66), *snail2* (92%, n=25) and *sox9* (46%, n=13) on the injected side, as indicated in Figures 3B-D. To assess whether the effects produced by *gli2* gain- and loss-of-function on NCC affected the other ectodermal cell populations, we tested the expression of the NP marker *sox2* and the prospective epidermis marker *xk81a* (Figures 3E-F). The results showed that *gli2* knock-down produced an increase in the expression of *sox2* (45%, n=69) as well as in the epidermal marker *xk81a* (74%, n=15). To assess this phenotype in the same embryo we performed a double ISH of the *sox2* and *xk81a* markers after *gli2MO* injection. We observed a decrease in the NC territory at the expense of an increase in the expression of the markers *sox2* and *xk81a* (Figure 3G, 75%, n=16). The quantification of the phenotypes is plotted in Figure 3H. These findings may imply that changes in NC territory are not a product of an increase in cell proliferation but could be due to changes in the ectodermal cell fate instead.

Subsequently, we evaluated the effect of *gli2* mRNA overexpression on the same gene markers. Figures 3I-N show that *gli2* gain of function produced an increase in the expression of the NC specification markers *foxd3* (56%, n=81), *snail2* (58%, n=26) and *sox9* (53%, n=19). As might be expected, we found that the overexpression of *gli2* produced a concomitant decrease in the expression of *sox2* (Figure 3L; 53%, n=49) and *xk81a* (Figure 3M; 56%, n=27). These results were also found when their expressions were analyzed in

the same embryo by double ISH (Fig. 3N; 60%, n=20). Figure 3O shows the quantification of the above phenotypes. In order to target the overexpression of Gli2 directly to NCC in a temporally and spatially controlled manner we used the *α3000gli2* construct. This plasmid uses the *snail2* promoter to specifically direct the expression of downstream constructs to the NC (Vallin et al., 2001). The specific overexpression of *gli2* (Figures 3P-Q) caused a clear increase in the expression of the NC markers *foxd3* (54%, n=13) and *snail2* (50%, n=14). To determine whether the effects seen on the NC are a result of the inhibition of the Gli2 function by *gli2MO*, we prepared a rescue construct, named *CRgli2,* which encodes the full-length Gli2 protein containing 6 mismatches in the nucleotide sequence recognized by the morpholino. The co-injection of *CRgli2* mRNA along with *gli2MO* efficiently restored the disrupted expression of the markers *foxd3* (60% of embryos with normal expression, n=48) and *snail2* (53%, n=17) in the NC (Figures 3R-S). Moreover, to specifically rescue *gli2* function in the NC territory, we coinjected *gli2MO* along with the *α3000CRgli2* plasmid, which carries the mutations in the MO binding site and is additionally transcribed under the *snail2*-promoter action only during NC specification. This experiment yielded a rescue in the expression of the *snail2* marker (Figures 3T and 3V, 82%, n=22). Furthermore, as a control of morpholino toxicity, we injected *CoMO* and found that the expression of *foxd3* was not affected (Figure 3U and 3V, 88%, n=17). Altogether, these results imply that Gli2 participates in the specification of the NC.

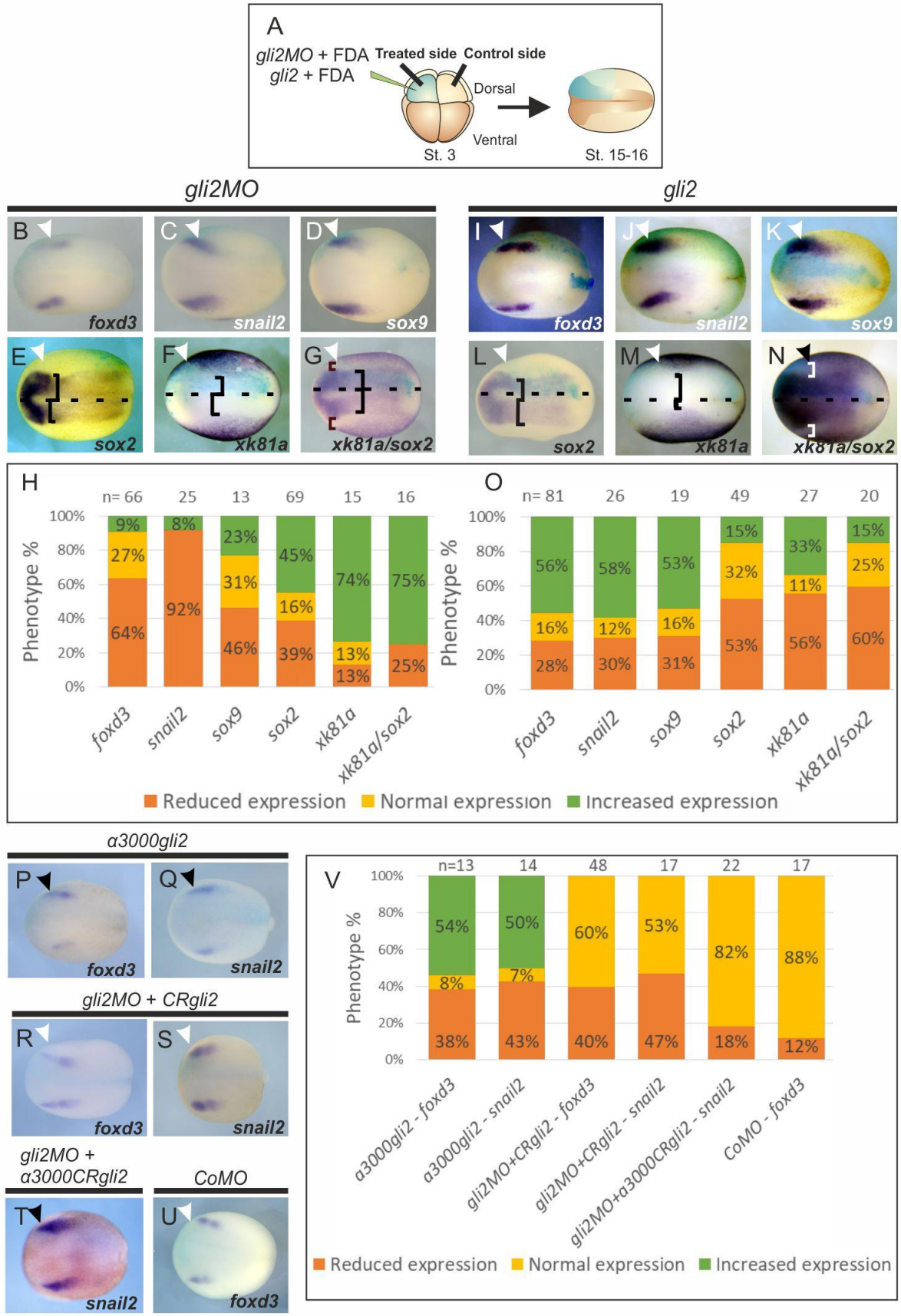

Figure 3. *gli2* requirement in NC specification. **(B-G, I-N, P-U)** Dorsal views of *Xenopus laevis* embryos at mid-neurula stage; anterior side is on the left. Arrowheads indicate the injected side (turquoise). The

expression of the genes is shown in purple. **(A)** Graphic representation of the experiments. **(B-D)** *gli2*MO-injected embryos show reduced expression of the NC markers *foxd3* (B), *snail2* (C) and *sox9* (D). **(E-G)** Increase in the expression of the NP marker *sox2* and the epidermal marker *xk81a* after *gli2MO* injection. Figure G shows a double ISH of *sox2* and *xk81a,* with brackets indicating the width of the NC domain. **(I-N)** The opposite effect is seen when *gli2* is overexpressed in the NC territory. **(H, O)** Quantification of *gli2MO* and of *gli2* mRNA effects on the expression of NC markers, respectively. **(P-Q)** Overexpression of *gli2* with the construct *α3000gli2* produced an increase in the expression of *foxd3* and *snail2.* **(R-S)** Control of morpholino specificity. Coinjection of gli2MO morpholino along with a plasmid encoding a mutated *gli2* at the morpholino binding site (*CRgli2*) rescued the expression of the NC markers *foxd3* and *snail2.* **(T)** The injection of the *α3000CRgli2* construct also rescued *foxd3* expression. **(U)** The microinjection of *CoMO* did not alter *foxd3* expression. **(V)** Quantification of the experiments shown in Figures 3P-U.

We then tested the pharmacological inhibition of Gli2 function with GANT61 during NC specification using three different approaches. The first one consisted of the injection of GANT61 at a concentration of 3 µM in one dorsal blastomere of 8-cell stage embryos (Stage 3). This low concentration was chosen to avoid possible toxic effects in the injected blastomere. Afterwards embryos were fixed at stage 16 and the expression of the NC markers *foxd3* and *snail2* was analyzed (Figure 4A). The loss of function of *gli2* produced a marked reduction in the expression of the NC marker *foxd3* (Figures 4B and 4F, 64% n=11) and *snail2* (Figures 4C and 4F, 65%, n=20) on the injected side. In agreement with the results obtained with *gli2MO*, the injection of GANT61 produced an increase in *sox2* (Figures 4D and 4F, 47%, n=19) and *xk81* expression (Figures 4E and 4F, 50%, n=8). As a control we injected the diluent of GANT61 (DMSO 0,03%) and analyzed the expression of the above mentioned markers (Figures 4G-K). We found that almost all embryos showed no change in gene expression between the control and the injected side (*foxd3,* 95%, n=20; *snail2*, 95%, n=22; *sox2*, 91%, n=23; *xk81a,* 95%, n=21)

The second approach consisted in implanting GANT61-soaked beads into the NPB of stage 12.5 embryos, which were then fixed at stage 16 for the analysis of *foxd3* expression (Figure 4L). The pharmacological inhibition of Gli2 produced a marked decrease in the expression of *foxd3* on the injected side (Figures 4M and 4O, 57%, n=37) in contrast to control embryos implanted with DMSO-soaked beads, in which the majority of the embryos presented no change in expression (Figure 4N and 4O, 73% n=34). Finally, in the third approach we incubated NC explants with the underlying mesoderm in GANT61 from stage 14 to 18, during which the specification of the NC takes place. The results showed a marked decrease in *foxd3* expression when NC explants were treated with the inhibitor (Figure 4Q, 92%, n=36) while lack of effect was observed in DMSO-incubated control explants (Figure

4R, 94% normal expression, n=36). These results showed that Gli2 is required for the maintenance of the specification of the NC during midneurula stages.

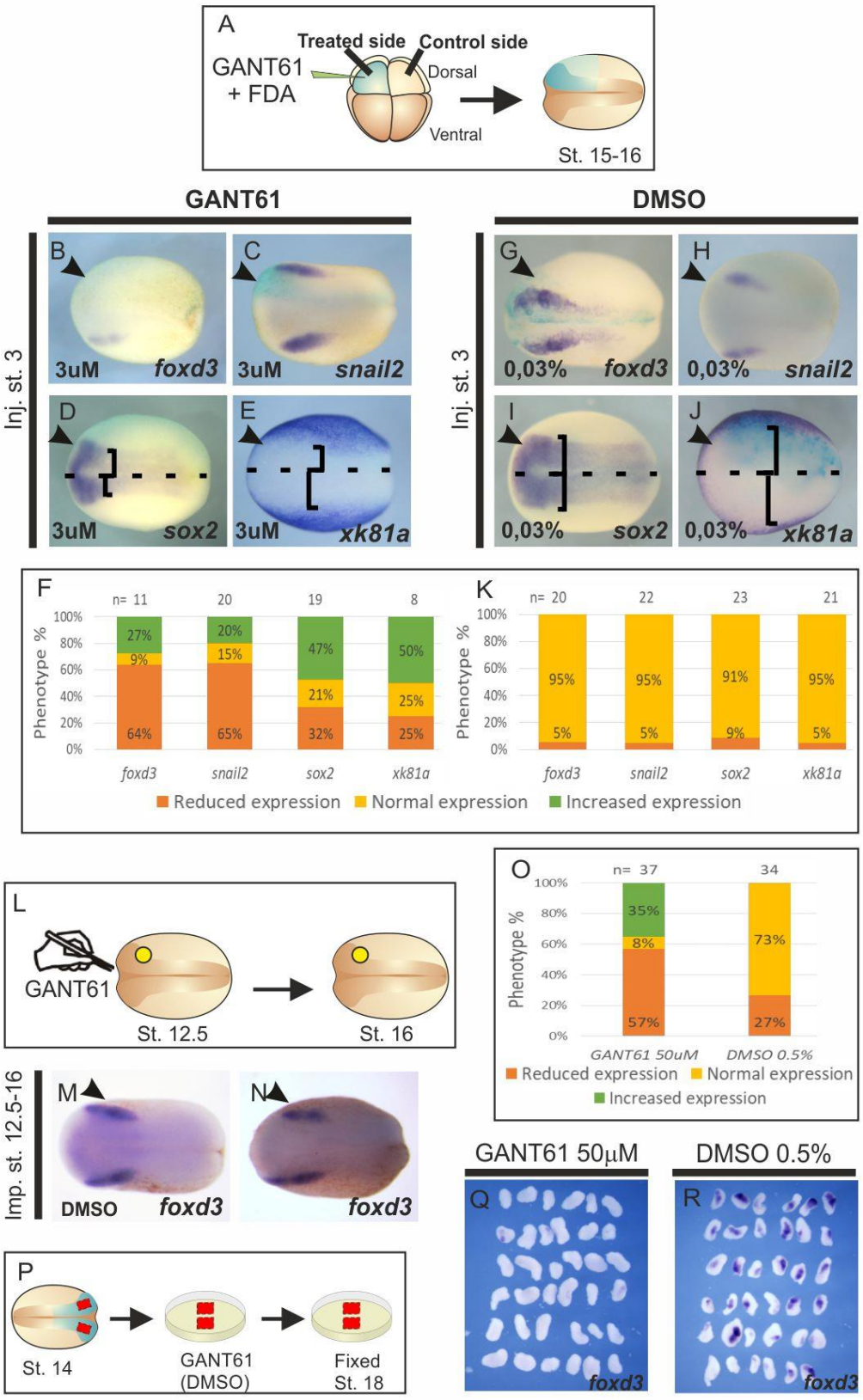

Fig 4. *gli2* requirement for the maintenance of NC specification. **(B-E, G-J, M-N)** Dorsal views of *Xenopus laevis* embryos at midneurula stage; anterior side is on the left. Arrowheads indicate the injected or implanted side. **(A-E)** GANT61 injection at stage 3 produces a decrease in the expression of *foxd3* and *snail2,* with a concomitant increase in the expression of *sox2* and *xk81a*. **(F)** Quantification of phenotypes in the injected embryos of figures B-E. **(G-J)** Injection of the GANT61 diluent (DMSO 0,03%) produced no changes in the expression of the gene markers analyzed. **(K)** Quantification of phenotypes. **(L-O)** Implanted GANT61-soaked beads from stage 12.5 to 16 produced a reduction in *foxd3* expression, as opposed to the implantation of DMSO-soaked beads. **(P-R)** NC explants with mesoderm incubated in GANT61 from stage 14-18 lose *foxd3* expression.

2.4. The transcriptional activator form of *gli2* acts on NC specification

Our next step in the elucidation of the mechanism of action of Gli2 was to determine which functional form is responsible for eliciting the effects observed in NC induction and specification. It is known that in most cases Gli2 acts as a transcriptional activator (McCleary-Wheeler, 2014), but for some developmental contexts it has been shown that Gli2 might act as a transcriptional repressor (Bowers et al., 2012). With these facts in mind we prepared DNA constructs encoding functional forms of Gli2 lacking the N-terminal repressor domain (*gli2act*) and the C-terminal activator domain (*gli2rep*) in order to discern which functional variant is operating in the NC. These functional constructs of Gli2 have already been tested in other studies and have been found to activate, for the case of *gli2act,* and to repress (*gli2rep*) the expression of Hh target genes (Pan et al., 2006; Santos and Reiter, 2014; Sasaki et al., 1999). The summarized experimental procedure is indicated in Figure 5A. The directed microinjection of the activator form of *gli2 (gli2act)* produced an increase in the expression of the NC markers *foxd3* (Figure 5B, 56%, n=32) and *snail2* (Figure 5C, 72%, n=28). Moreover, *gli2act* was also able to rescue the phenotype of the *gli2MO*-injected embryo (*foxd3,* Figure 5D, 45%, n=51; *snail2,* Figure 5E, 67%, n=15). Additionally, the NC-specific overexpression of the *α3000gli2act* construct rescued the morphant phenotype*,* producing an increased expression of *foxd3* (Figure 5F, 75%, n=16) and *snail2* (Figure 5G, 60%, n=15). Secondly, we tested the action of the repressor form of Gli2 (*gli2rep*). We found that *gli2rep* produced a consistent reduction in the expression of the markers *foxd3* (Figure 5H, 78%, n=70) and *snail2* (Figure 5I, 59%, n=54), indicating an inhibition of the specification of the NC. Therefore, as expected, the coinjection of *gli2rep* along with *gli2MO* produced no rescue in *foxd3* (Figure 5J, 62% of embryos showing reduced foxd3 expression, n=16) and *snail2* expression (Figure 5K, 53% reduced *snail2* expression, n=30). The quantification of these phenotypes is indicated in Figure 5L. These

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results strongly suggest that the transcriptional activator function of Gli2 is essential for the specification of the NC.

In the canonical signaling, when the Hh pathway is inactive, Gli2 processing takes place as a result of multi-site phosphorylation at six conserved serine residues that occurs due to the sequential action of the kinases PKA, CK1 and GSK3β. This phosphorylated version of Gli2 is ultimately processed by the proteasome and a large proportion of the protein pool is degraded, with the remaining part of the Gli2 protein acting as a transcriptional repressor (Pan and Wang, 2007). Conversely, when the Hh signal is present, the phosphorylation by PKA is prevented and therefore Gli2 is converted into a transcriptional activator (Niewiadomski et al., 2014; Riobo et al., 2006; Tuson et al., 2011). Accordingly, we created a mutant version of Gli2 in which the six PKA-target serine residues were changed into non-phosphorylatable alanine residues, thus acting as a constitutive transcriptional activator when expressed in *Xenopus* embryos. This protein, unable to be phosphorylated and degraded, was called *gli2PSM* (gli2-**P**KA **S**ite **M**utation). The overexpression of this construct produced a marked increase in the expression of the markers *foxd3* (Figure 5M and 5Q, 51% n=55) and *snail2* (Figure 5N and 5Q, 60%, n=15), whereas it reduced the expression of *sox2* (Figure 5O and 5Q, 59%, n=17) and *xk81a* (Figure 5P and 5Q, 61%, n=18). In addition, we overexpressed this construct in a NC-specific fashion through the injection of the plasmid *α3000CRgli2PSM* and found that it increased the expression of *foxd3* (Figure 5R, 73%, n=22). Moreover, this construct was able to rescue *foxd3* and *snail2* expression after *gli2MO* injection (Figures 5S-T, *foxd3* 81%, n=16; *snail2* 64%, n=14). The quantification of these experiments is shown in Figures 5Q and 5U.

In summary, the results provide compelling evidence for a role of *gli2* in the maintenance of the specification of the NC. In addition we also found that the transcriptional activator form could be the active variant participating in NC specification.

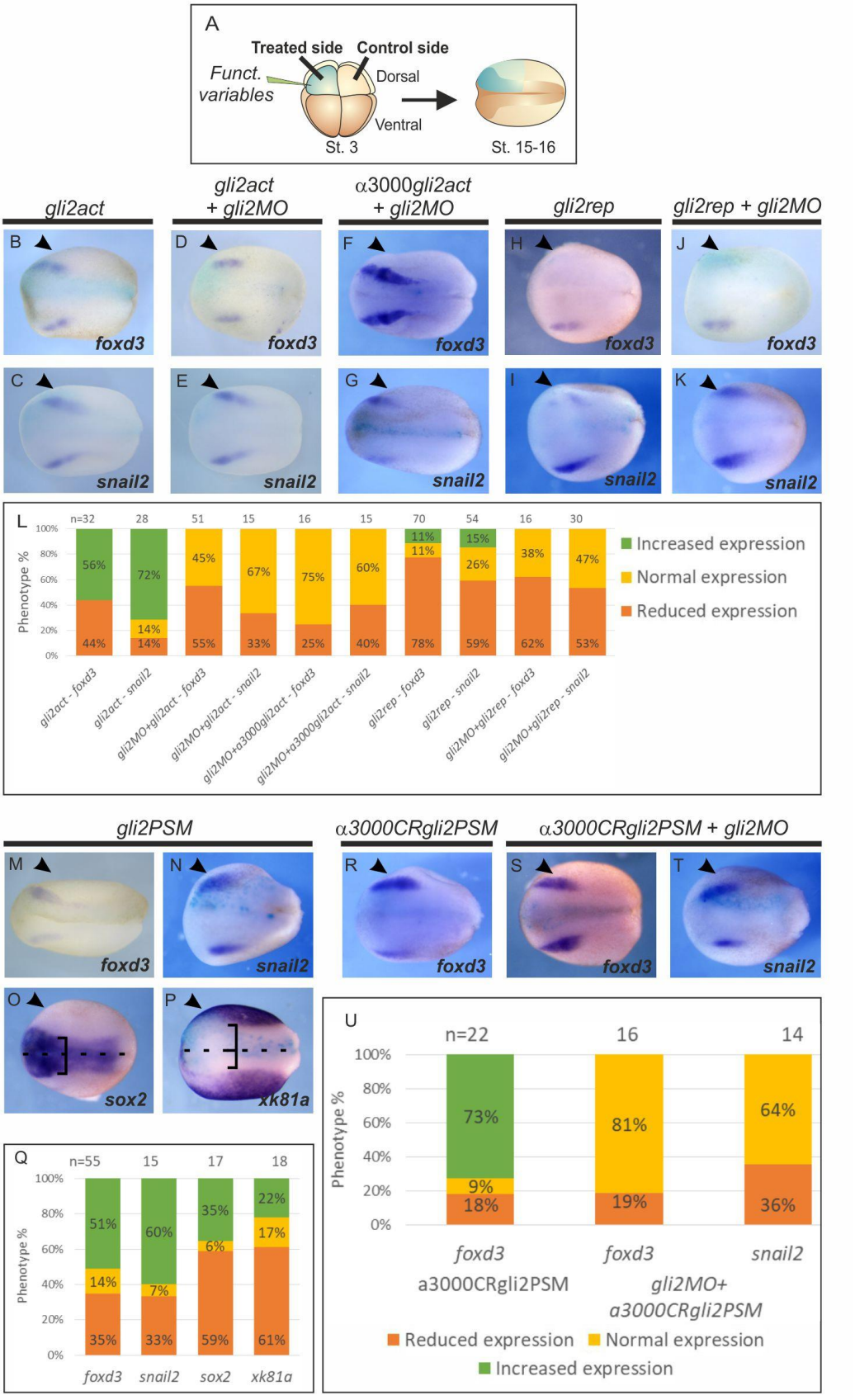

Figure 5. The activator form of *gli2* is required for NC specification. **(A)** Schematic procedure of the experiments. **(B-K, M-P, R-T)** Dorsal views of *Xenopus laevis* embryos at mid-neurula stage, anterior side to the left. Arrowheads indicate the injected side, shown in turquoise. **(B-C)** *gli2act* increases the expression of

foxd3 and *snail2.* **(D-G)** *gli2act* and *α3000gli2act* are able to rescue the expression of *foxd3* and *snail2* in the morphant embryo. **(H-K)** *gli2rep* represses the expression of *foxd3* and *snail2* and therefore is not able to rescue these expressions in the morphant embryo. **(L)** Quantification of effects shown in B-K. **(M-P)** The phosphorylation mutant *gli2PSM* increases the expression of NC markers and represses the activity of *sox2* and *xk81a*. **(R-T)** The *α3000gli2PSM* construct also increases the expression of *foxd3* and is able to rescue the effects of *gli2MO.* **(Q, U)** Quantification of the experiments shown in M-P and R-T, respectively.

2.5. *gli2*-mediated transcriptional activation modulates the expression of NC and NPB specifiers

To further explore Gli2 role on NC development we studied the specific mechanism through which Gli2 exerts its function during NC specification. We analyzed the transcriptional activity of Gli2 using a Gli-dependent genetic reporter construct known as the *8xGBS-Luciferase* plasmid (*8xGBS-LucII*; GBS, specific Gli Binding Site) (Sasaki et al., 1997). Embryos were injected in both dorsal blastomeres at stage 3 with the reporter plasmid (*8xGBS-LucII*) along with an internal control plasmid (*pGL4.74-TK-hRluc*, Promega, USA). These luciferase reporter constructs were coinjected with different *in vitro*-transcribed mRNAs and MOs in order to assess the effect of Gli2 on the transcriptional response of the reporter construct. For maximal tissue specificity and spatio-temporal control we dissected out NC explants for the luciferase assay. At stage 15-16 NC explants were dissected out and the firefly luciferase activity was assayed and normalized to the Renilla luciferase activity using a commercial kit (Dual-Luciferase Reporter Assay System, Promega, USA). The scheme of this experimental design is shown in Figure 6A. The injection of either *gli2* or *ihh* mRNA produced a significant increase in Luciferase expression (Figure 6B). By using a mutated version of the 8xGBS domain (*8xGBSmut-LucII* plasmid) along with the coinjection of *gli2* mRNA (2.6 ng/E), we found that the increased Luciferase activity produced by Gli2 overexpression was abolished. This result indicates that the increase observed in Luciferase activity was only due to a stimulation of the 8xGBS promoter caused by Gli2. On the other hand, when we knocked down *gli2* and *ihh* function with their respective MOs, a marked reduction in Gli-mediated Luciferase activity was observed. These results allow us to suggest that Gli2 acts as a transcriptional activator in the development of *Xenopus* NC.

In order to determine whether *gli2* was acting downstream of *ihh* in the gene regulatory network that participates in NC specification, we injected *gli2* mRNA and *ihhMO* and observed that Gli2 was able to rescue the Luciferase expression back to the control levels. Additionally, we coinjected *ihh* mRNA and *gli2MO* and found that the *ihh*-increased

Luciferase activity was markedly reduced. However, a small activation of the luciferase reporter still persisted, suggesting that other players could also act on the reporter construct in response to *ihh* stimulation. We speculated that Gli3 in particular could play this role in the activation of the *8xGBS* promoter. We also decided to analyze the effect that the constitutive transcriptional activator form of Gli2, *gli2PSM*, elicits during NC specification. We observed that Luciferase activity increased up to eight times compared to *Renilla* luciferase activity. This indicates that the effects of *gli2PSM* injection on the expression of the NC markers shown in the previous section were produced by the transcriptional activator function of the protein encoded by this mRNA. Finally, we observed that Gli2 inhibition by GANT61, produced by the incubation of NC explants from stage 13 to 16 with this inhibitor, caused a small, nonsignificant reduction in Luciferase activity with respect to the control embryo. Altogether, our results strongly point to a mechanism in which Gli2 stimulates the transcription of target genes during the development of *Xenopus* NC.

The expression of many genes must be tightly regulated for NC to develop properly. As noted previously, these genes are included in groups called NPB specifiers, NC specifiers and effector genes (Sauka-Spengler and Bronner-Fraser, 2008). To further investigate whether *gli2* is involved in regulating the expression of such genes we performed quantitative RT-qPCR analysis of NC explants that were injected with either *gli2MO* or *gli2* mRNA. The specific genes analyzed were the NPB specifiers *pax3, hairy2b* and *snail1*, the NC specifiers *snail2* and *twist,* and also the cell adhesion genes *e-cad* and *n-cad*. In this qPCR analysis *gapdh* was used as loading control. Embryos were injected in both dorsal blastomeres at stage 3, and at midneurula stages (st. 14-16) 200 NC explants were dissected out and the total RNA was extracted and analyzed by RT-qPCR. (Figure 6C). This gene expression analysis performed on the NC tissue showed the effect of *gli2* specifically on NC development (Figure 6D).

Regarding the expression of the NPB markers, we found that in the case of *pax3, hairy2b, and snail2* the morpholino knock-down of *gli2* produced a significant reduction in their expression, whereas the gain of function by overexpression of this gene increased the expression of these markers, with *snail1* showing the higher increase in expression of the NPB genes analyzed. This analysis also showed that *gli2* is required for their expression. The expression of the NC specifiers *snail2* and *twist*, also showed a functional interaction with *gli2* similar to the one found for the NPB genes, and *twist1* was the most sensible gene to *gli2* gain of function, showing a 14-fold increase in its expression, compared to the uninjected wildtype NC. Finally, in the case of the cell adhesion genes, we found that *e-cad*

and *n-cad* were downregulated when the *gli2* function was knocked down. Only *n-cad* increased its expression after *gli2* injection, suggesting a positive regulation of *gli2* of its expression.

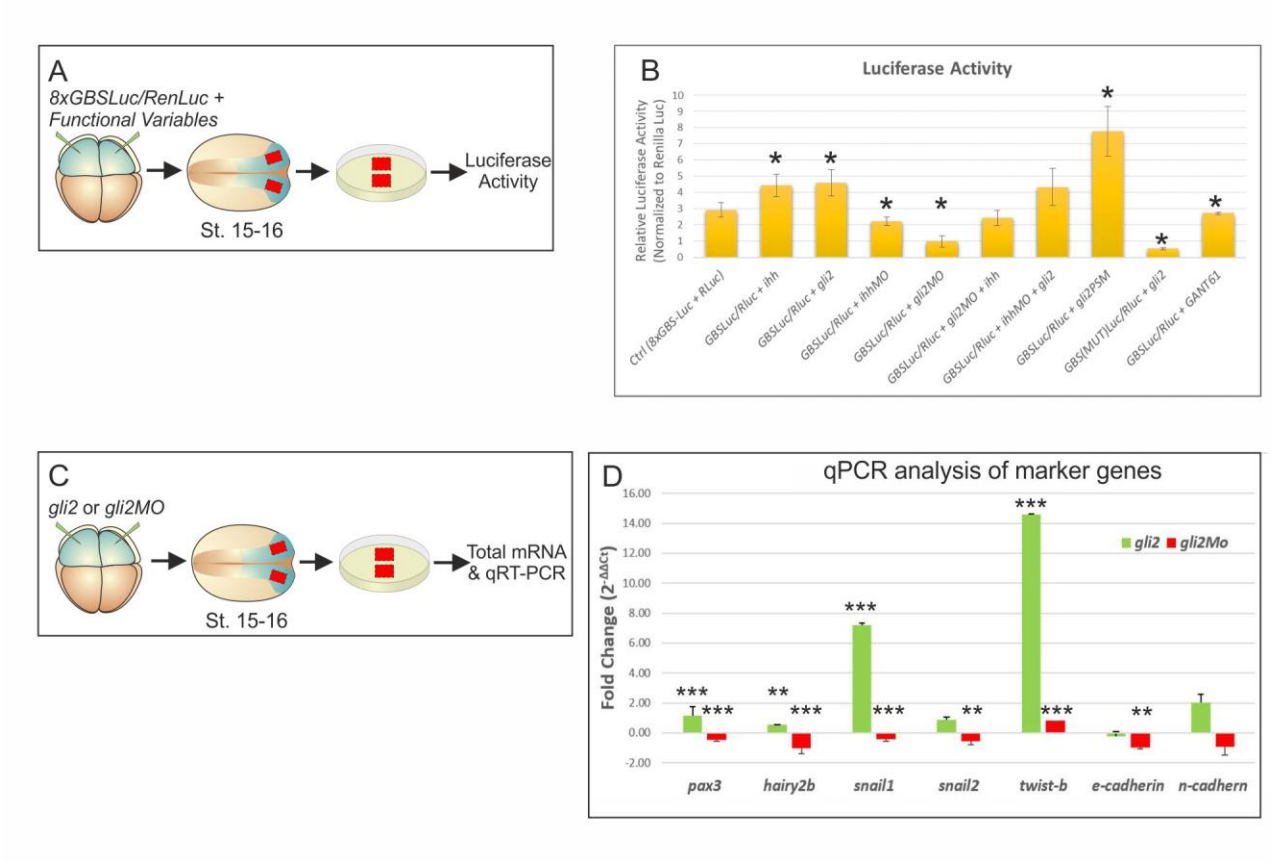

Figure. 6. *gli2* acts through a transcriptional activation mechanism and affects the expression of several NC markers. **(A-B)** Gli-Luciferase reporter assay. **(A)** Schematic representation of the Luciferase assay experiment. **(B)** Transcriptional activity of Gli2 in NC explants. Intensities are shown as relative units of Luciferase normalized to the internal control Renilla Luciferase. An increase in Gli2 and Ihh function increases the expression of the Gli-dependent reporter construct. **(C-D)** RT-qPCR analysis of Gli2 function on the expression of NPB and NC markers. Each color coded bar represents the relative expression level of the markers for each condition (gain and loss-of-function of *gli2*). All the expression levels denote Ct values that were normalized first to the expression level of *gadph,* and then they were normalized to the expression of each gene in the uninjected wildtype condition, and are presented as fold change. **(C)** Schematic representation of the grafting experiment. At stage 15-16 total RNA was isolated and then RT-qPCR was performed. **(D)** Expression levels of the markers *pax3, hairy2b, snail1, snail2*, *twist-b, e-cad* and *n-cad*. Data are presented as mean ± s.d. of triplicates of two independent qPCR experiments. Reference: **, *P*<0.05; ***, *P*<0.001 (*P*-values correspond to Student's t-Test).

2.6. *gli2* is involved in the control of apoptosis of NCC but not in their proliferation.

The above results suggest that *gli2* might act on NC development by changing cell fate. However, we considered the possibility that this protein could participate in the apoptotic

process or in the cell proliferation of NC during the specification period. In order to determine whether *gli2* participates in cell proliferation we performed an IHC assay in which we analyzed changes in cell proliferation when we knocked down or overexpressed the *gli2* gene. The detection of proliferating cells was made by IHC against phospho-histone H3, which is present in cells undergoing mitosis. Our results showed that there were no significant changes in the number of proliferating cells between the injected and the control sides of embryos (n=8) for *gli2MO* or *gli2* mRNA-injected embryos (Figures 7A-C). These results show that *gli2* is not involved in the proliferation of the NCC during their induction and specification.

We also tested whether the effects of *gli2* function on NC were due to changes in the apoptotic process. We injected either *gli2* mRNA or *gli2*MO in the dorsal blastomeres of 8-cell stage embryos, grafted NC explants at mid neurula stage (n=20) and measured the caspase 3/7 activity with a homogeneous assay (Figure 7D, see Materials and Methods). The overexpression of *gli2* produced a 2.5-fold reduction in caspase activity. This suggests that *gli2* specifically plays a role in the apoptotic state of the NC. On the other hand, the injection of *gli2MO* had no effect on the apoptosis level of NCC. To further validate the specificity of *gli2MO* we coinjected it with *CRgli2* and found that the apoptotic level of the NC was also reduced, similarly to *gli2* overexpression. We also tested the ability of the *gli2* activator construct (*gli2act*) to control apoptotic levels, and found out that it reduced caspase activity levels even when we coinjected it with *gli2MO* (Figure 7E). These results suggest that the mechanism of action of Gli2 in NC specification might not be due to the regulation of the proliferative state of NCC, but that it may prevent cells from undergoing apoptosis, thereby maintaining their survival during mid-neurula stages.

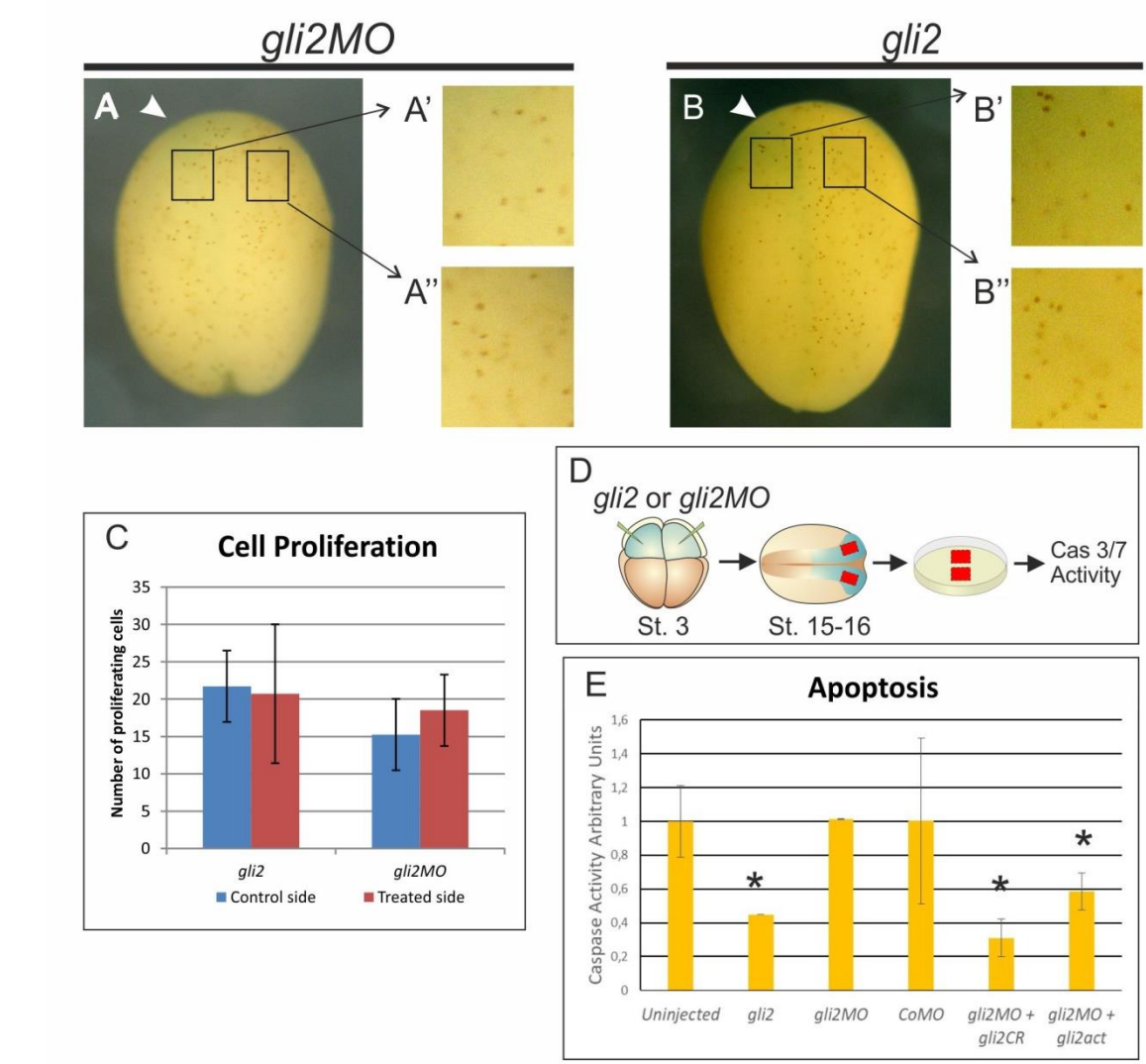

Figure 7. *gli2* is involved in the apoptosis of NCC but not in their proliferation. **(A-B)** Dorsal views of *Xenopus laevis* embryos, anterior side to the top. White arrowheads indicate the injected side. Gain- and loss-of-function of *gli2* did not alter the number of proliferating cells between the injected and the control side. **(A', A'', B', B'')** Insets showing the quantified area of each embryo. **(C)** Quantification of proliferating cells in the embryos treated with *gli2* mRNA or *gli2MO.* **(D)** Schematic diagram showing the approach used to evaluate the apoptosis status of NCC. **(E)** Graph showing that the overexpression of *gli2* prevents NCC from undergoing normal apoptosis. The inhibition of *gli2* expression did not produce any significant changes in the apoptosis level of NCC. The activator form of Gli2 also decreased apoptosis in NC explants. Values are mean ± s.d. from two independent experiments. P≤ 0.05 (Student's t-test).

2.7. *gli2* is required for the proper migration of NC in a cell-autonomous process

Migration of the NC is an essential process that allows these cells to colonize several regions of the embryo. Once the NC is already specified, effector proteins induce epithelial-to-mesenchymal transition (EMT), delamination, and finally migration of the NCC. We employed different strategies to determine the participation of *gli2* in *Xenopus* NC migration,

using *gli2MO* and the spatio-temporal control of GANT61 inhibitory action through soaked-beads grafting. The first approach consisted in the targeted injection of *gli2MO* along with different *gli2* constructs in a dorsal blastomere of stage 3 embryos, and the assessment of NC migration at stages 20-22 (Figure 8A). The loss of function of *gli2* caused a reduction in the expression of *foxd3* between the control side and the injected side (Figure 8B, 63%, n=16). This result was confirmed by the analysis of *sox10* (Figure 8C, 70% n=33). We then determined the rescue of *gli2* function by coinjecting *CRgli2* and *gli2act* along with *gli2MO.* The results show that these mRNAs were able to restore the front of migration of NC as evidenced by *foxd3* expression (Figures 8D, *gli2CR,* 69%, n=26; Figure 8E, *gli2act,* 50%, n=14).

Subsequently, we knocked down *gli2* function through the injection of the pharmacological inhibitor GANT61 at a concentration of 3 μM. We found that GANT61 produced a marked reduction in the front of migration of the NC as evidenced by the diminished expression of *foxd3* (Figure 8G, 56% n=16). We next evaluated the function of Gli2 in NC migration in a more spatio-temporal specific manner. We implanted GANT61-soaked beads into the NC of embryos at two time frames (Figure 8I and 8L). The first time frame comprised the stages of specification maintenance and onset of migration (stages 14-22). Here we observed a marked reduction in the front of migration in the treated side, as shown by the expression of *sox10* (Figure 8K, 56% n=43). Treatment with DMSO did not produce any significant changes (Figure 8J, 93%, n=28). This indicates that if *gli2* is not properly functioning at the NC maintenance of specification period, NC migration is affected. The second experiment analyzed *gli2* knockdown from stages 17 to 22. In this case GANT61 inhibition was performed at the beginning of NC cell migration and the results showed an impaired cell migration in the implanted side of the embryos (Figure 8N, 62%, n=45). As expected, DMSO treatment produced no changes in the front of migration (Figure 8M, 89%, n=37). Altogether, being that in this particular assay Gli2 was interfered during the onset of migration, we can conclude that that *gli2* is involved in the process of NC migration.

The process of NC migration is complex, with different signals acting coordinately to trigger the EMT in NCC along with a change in the cadherin molecular signature. We previously found that the Ihh signaling pathway is essential for NC migration (Aguero et al., 2012). Therefore, we decided to further explore the requirement of *gli2* in this process. To that end, we knocked-down *gli2* function by the microinjection of *gli2MO*, grafted NC explants at stages 17-18 into WT siblings and then analyzed NC migration under fluorescence microscopy following the FDA lineage tracer (Figure 8P). Initially, we grafted

uninjected NC control explants into wild type embryos. This produced a normal NC migration pattern in the host embryo as evidenced by the analysis of FDA fluorescence (Figure 8Q, 67%, n=18). When NC explants from embryos injected with *gli2MO* were grafted into wild type embryos, migration was severely affected (Figure 8R, 64%, n=11). Conversely, when uninjected NC explants were grafted into *gli2MO*-injected host embryos, migration was normal, undistinguishable from the untreated NC explants grafted into WT embryos (Figure 8S, 60%, n=10). These results show that *gli2* acted in a cell autonomous manner in NCC migration, as this process only depends on proper *gli2* function in the NC and not on external signals that could also depend on the function of *gli2.*

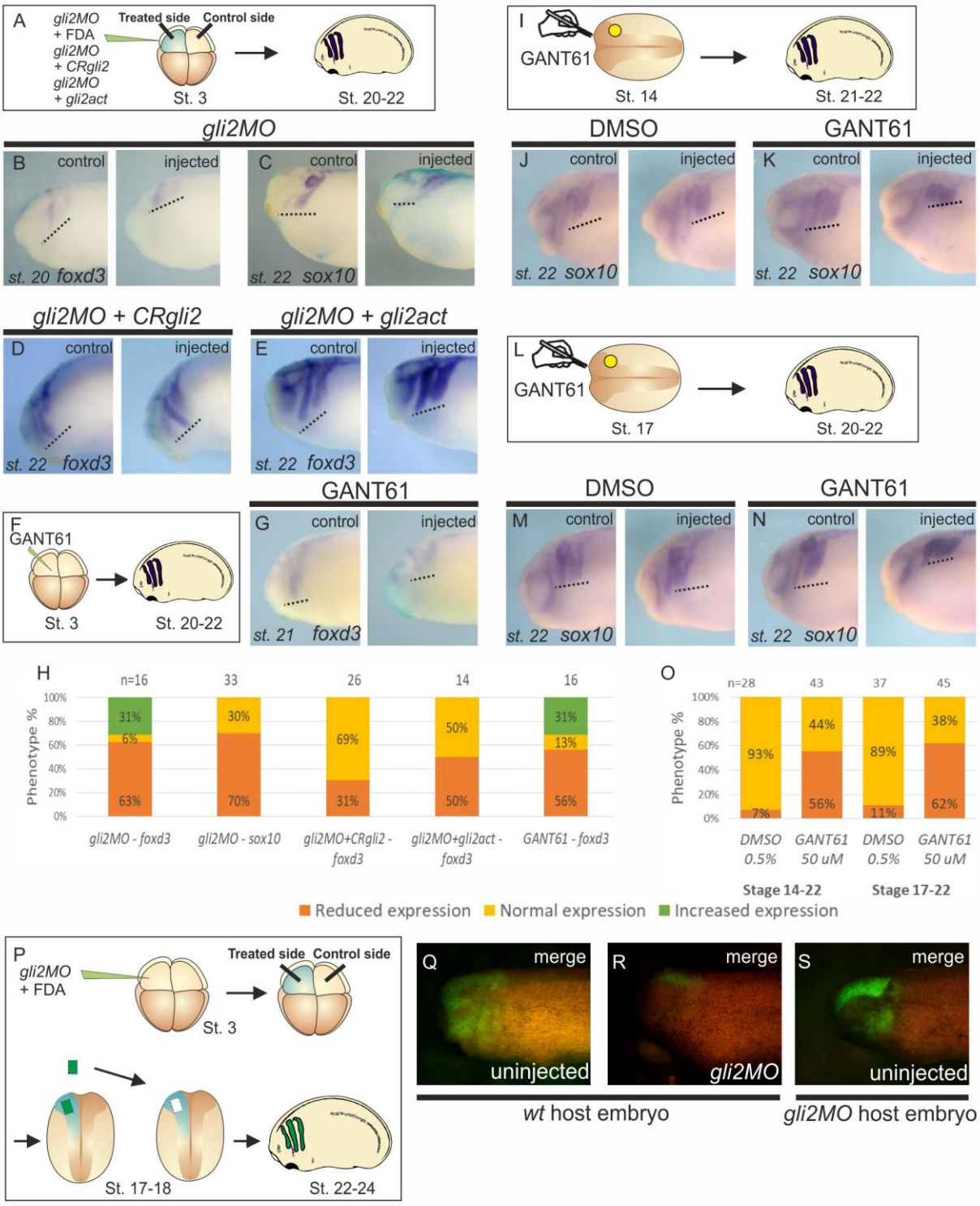

Figure 8. *gli2* acts in a cell-autonomous manner in NC migration. **(B-E, G, J-K, M-N, Q-S)** Lateral views of embryos with the anterior side on the left showing the expression of the NC migration markers *sox10* and *foxd3*. Figures Q-S show merged images of fluorescence and bright field captures. **(A, F, I, L, P)** Schematic representation of the different assays. **(B)** Stage 20 embryos showing a reduction in the migration front in the injected side evidenced by the expression of *foxd3*. **(C)** In stage 22 embryos the difference in expression between the control and the injected side is notably higher, as evidenced by the marker *sox10.* **(D-E)** Coinjection of either *CRgli2* or *gli2act* with *gli2MO* rescued NC migration as shown by *foxd3* expression. **(F-**

G) Injection of GANT61 produced a loss of *gli2* function and a noticeable decrease in NC cell migration, as shown by the expression of *foxd3.* **(H)** Quantification of phenotypes shown in B-G. **(I-N)** Implantation of GANT61-soaked beads into NC at two different time frames (stages 14-22 and 17-22) produced a reduction in the front of migration of the NC as shown by *sox10* expression. **(O)** Quantification of experiments shown in Figures I-N. **(P-S)** *gli2* acts cell-autonomously in NC migration. Grafting of uninjected NC explant into an uninjected wild type embryo produced no alteration in the front of migration of the NC. The injection of *gli2MO* in the transplanted explant altered the normal pattern of NC migration. Grafting of a wild type NC explant into a *gli2MO* injected host produced no changes in normal NC migration.

2.8. *gli2* is required for the development of NC derivatives

In order to further explore the role of *gli2* on the development of the NC, we investigated the effect of *gli2* knockout on NC derivatives. Embryos were injected with *gli2MO,* GANT61 or with a combination of rescuing constructs at stage 3 and then, at stage 45, the analysis of cartilage morphology was performed by Alcian Blue staining (Figures 9A-B). The injection of GANT61 produced a slight reduction in the size of the cartilages (52%, n=27, Figure 9C). Interestingly, *gli2*MO-injected embryos showed a severe reduction in the size of the craniofacial cartilages in the injected side (Figure 8D, 71%, n=21). The gross morphology of craniofacial cartilages revealed that NC-derived cartilages (Meckel's, ceratohyal, and mostly the ceratobranchial cartilages) were severely reduced. The coinjection of *CRgli2* was able to rescue the morphant phenotype, thereby proving the specificity of the *gli2* function on NC derivatives formation (Figure 8E, 67%, n=21). As a control we injected *CoMO* and found no effect on craniofacial cartilage size or morphology (Figure 9F, 100% n=30), indicating that the observed effect was only due to *gli2* loss of function. The quantification of the embryos is shown in Figure 9G.

With the purpose of analyzing the role of *gli2* in the development of craniofacial cartilages in a more temporally and spatially controlled manner, we grafted two GANT61-soaked beads into the NC region of embryos either at stage 12.5 or 17 and analyzed their cartilage morphology at stage 45 (Figures 9H-M). In both treatments, the structure and size of the craniofacial cartilages were severely disrupted in the grafted side (Figure 9J and 9N, st.12.5-45 with GANT61, 59%, n=29; Figure 9M and 9N, stage 17-45 with GANT61, 63%, n=24), in contrast to the embryos implanted with DMSO-soaked beads (Figure 9I and 9N, st.12.5-45, 89% normal, n=28; Figure 9L and 9N, st.17-45, 93% normal, n=28). However, taking into account the fact that we affected Gli2 function during the beginning of NC migration, we cannot decisively conclude that the impairment of derivative formation was

due to a deficit in NC migration or in the differentiation process. Therefore, future experiments are needed to address this issue.

NCC are also responsible for the formation of different kinds of pigmented cells in the vertebrate body (Lapedriza et al., 2014). To further investigate the effects of *gli2* in the formation of NC derivatives we analyzed the role of *gli2* in melanocyte development. We found a remarkable decrease in melanocyte number at stage 36 in the injected side, suggesting a strong participation of *gli2* in the development of this cell type (Figure 9O-Q, 68%, n=22). We also decided to evaluate the effect of *gli2MO* in the formation of Rohon-Beard (RB) sensory neurons and glia precursors, which in *Xenopus laevis* are marked by the expression of the *tbx2* gene (Takabatake et al., 2000b). The results indicate that *gli2* loss of function produced a reduction in these cell populations (Figure 9R-T', 69%, n=13). *tbx2* expression also allowed us to conclude that, in the cranial region of the embryo, *gli2MO* produced a severe reduction in the trigeminal, the profoundal and the lens placodes. This suggests that *gli2* might participate in cranial ganglia primordia formation. Taken together, these results suggest that *gli2* is involved in the development of NC derivatives, namely craniofacial cartilages, melanocytes, RB neurons and cranial ganglia precursors.

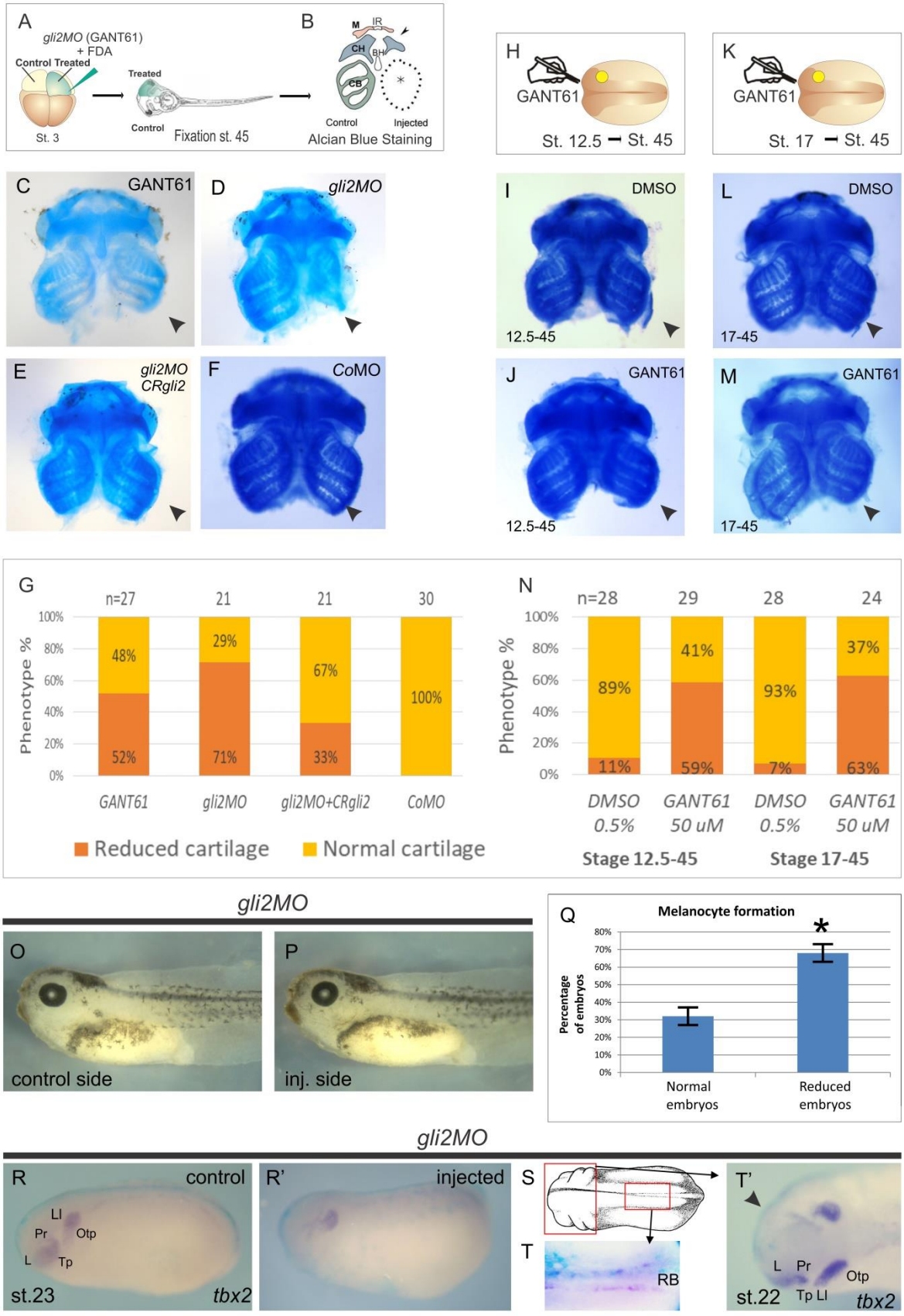

Figure 9. *gli2* is required for the proper formation of NC derivatives. **(A, H, K)** Schematic representation of the experiments performed. **(B)** Diagram indicating the different components of the

craniofacial cartilages. **(C-F, I-M)** Craniofacial cartilages preparation of stage-45 tadpoles. Alcian blue staining, ventral views of embryos, anterior side is at the top. Injected side is indicated by arrowhead. **(C)** GANT61 injection at stage 3 produced a reduction in craniofacial cartilages, with a more prominent phenotype in the ceratobranchial cartilage. **(D)** The *gli2*MO-injected side of embryos (arrowhead) presents a marked reduction in Meckel's, ceratohyal and ceratobranchial cartilages. **(E)** Rescue of cartilage morphology after coinjection of *CRgli2* along with *gli2MO.* **(F)** *CoMO*-injected embryos showed no effect on the treated side. **(G)** Quantification of phenotypes shown in C-F. **(H-N)** Grafting of GANT61-soaked beads at two different time frames (st. 12.5 to 45 and 17 to 45) produced a consistent reduction in craniofacial cartilages. **(O-Q)** Lateral view of a *gli2*MO injected embryo. On the injected side there is a reduction in the number of melanocytes in the zone of the future gut. Quantification is shown in Figure 9Q. **(R-T)** *tbx2* expression shows a reduction in RB neurons and cranial ganglia primordia on the *gli2MO* injected side. Pr, profoundal placode; L, lens placode; Ll, lateral line placode; Tp, trigeminal placode; RB, Rohon-Beard sensory neurons.

2.9. Hierarchical Relationships of *gli2* in early NC development

To assess the function of Gli2 in the gene regulatory network that is essential for the formation of the NC, we analyzed its hierarchical relationships with other components of the Hh signaling pathway as well as with other genes involved in NC development. We have previously shown that the Ihh ligand is essential for the development of the NC during early *Xenopus* development (Aguero et al., 2012). Consequently, we set out to analyze the particular hierarchical relationship between *gli2* and the *ihh* gene. Stage 3 embryos were injected with *ihh* or coinjected with *gli2MO* and *ihh* mRNA, and the expression of *foxd3* was analyzed. *ihh* overexpression alone produced an increase in the expression of *foxd3* (Figure 10B, 72%, n=50), whereas the knockdown of its function decreased the expression of *foxd3* (Figure 10C, 73%, n=51) (Aguero et al., 2012).The combination of *ihh* and *gli2MO* blocked the effect of the *ihh* overexpression and produced a strong reduction in NC population (Figure 10D, 75%, n=20), whereas the opposite combination, i.e, *ihhMO* and *gli2* mRNA, rescued the phenotype produced by the lack of *ihh* (Figure 10E, 75%, n=28). This result suggests that *gli2* acts downstream of *ihh* in the signaling cascade in *Xenopus laevis* early NC development.

Additionally, we evaluated the epistatic relationship between *gli2* and *gli3.* The overexpression of *gli3* mRNA caused an increase in NC territory (Figure 10F, 81%, n=70). Moreover, knocking down *gli3* function diminished *foxd3* expression (Figure 10G, 75%, n=60). When we coinjected *gli2* mRNA along with *gli3MO*, we observed a remarkable rescue in the expression of the NC marker *foxd3* (Figure 10H, 69%, n=16). On the other hand, when we coinjected *gli2MO* along with *gli3* mRNA, we also observed a rescue of gli2 function manifested in the rescue of the expression of the NC marker *foxd3* (Figure 10I,

55%, n=40). This might be indicative of a possible redundancy in the function of both genes during the gene regulatory network that forms the NC.

We also studied the epistatic relationship between *gli2* and the *ednra* gene. Ednra signaling is required for early NC development, as the modulation of its functions affects *foxd3* expression in the NC (Figure 10J-K, (Bonano et al., 2008)). In particular, overexpression of *ednra* causes an increase in *foxd3* expression (Figure 10J, 65%, n=40), while its knockdown reduces the expression of this NC marker (Figure 10K, 73%, n=70). In the epistatic analysis, when *ednra* mRNA was coinjected with *gli2MO,* no rescue of *foxd3* expression was observed (Figure 10L, 63% n=41). Conversely, *gli2* overexpression was able to rescue the reduced *foxd3* expression in embryos treated with *ednraMO* (Figure 10M, 69% n=59). This combinatorial analysis allowed us to suggest that *gli2* acts downstream of the *ednra* gene during NC induction and specification.

Finally we sought to establish the relationship between *gli2* and the kinesin Eg5, which has been previously shown to participate in the processes that regulate the development of the NC (Figures 10N-O, (Fernandez et al., 2014). We observed that the overexpression of *eg5* mRNA produced a marked increase in the expression of *foxd3* (Figure 10N, 77%, n=60). On the other hand, when we injected *eg5MO, foxd3* expression was reduced ion the injected side (Figure 10O, 85%, n=40). We then proceeded to the epistatic assays. The coinjection of *eg5* mRNA could not rescue *foxd3* expression produced after *gli2* knockdown (Figure 10P and 10R, 66% of reduced expression, n=29). Conversely, *gli2* mRNA coinjected along with *eg5MO* produced a striking rescue in the expression of *foxd3* (Figure 10Q and 10R, 70% n=10). Therefore, *gli2* might act downstream of *eg5* during NC specification.

In summary, in this section we showed that *gli2* is downstream of several genes that are important during NC development, indicating that these genes may possibly interact in order to establish the molecular framework for the development of the NC. The proposed model for the epistatic interactions of *gli2* and these genes is shown in Figure 10S.

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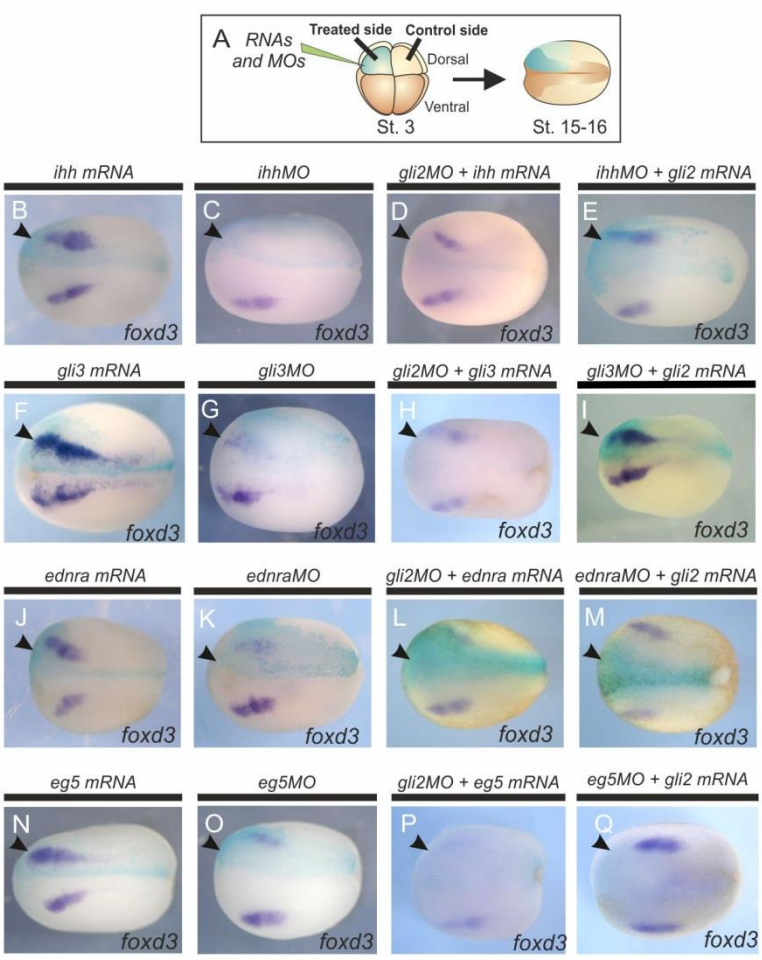

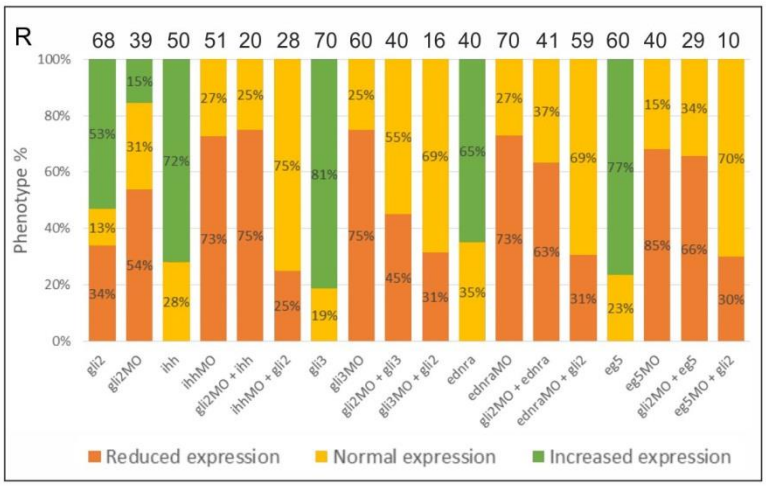

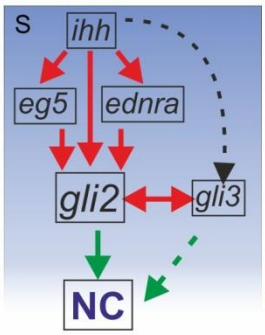

Figure 10. *gli2* epistatic relationships with *ihh, gli3, ednra* and *eg5*. **(A)** Schematic representation of the experiments performed. **(B-Q)** Dorsal views of *Xenopus laevis* embryos at mid-neurula stage; anterior side is on the left. Arrowheads indicate the injected side, shown in turquoise. The images show combinatorial experiments performed to evaluate the epistatic relationship between *gli2* and different genes involved in the gene regulatory network that induces the NC. They also show the effect of individual genes on the NC. **(B-E)** *gli2* is downstream of *ihh* during NC induction. **(F-I)** *gli2* and *gli3* rescue each other's deficiency during NC induction. **(J-Q)** Both genes *ednra* and *eg5* act upstream of the *gli2* transcription factor during NC induction and specification. **(R)** Quantification of the phenotypes shown in B-Q. **(S)** Hierarchy diagram plotting the epistatic relationships between the genes analyzed.

3. DISCUSSION

NC formation involves a complex network of molecular actors, i.e., receptor/ligand-signaling systems that translate environmental cues to specific molecules into the cell to control cellular behavior. Gli2 is a key transcription factor during vertebrate development and is also indispensable for the maintenance of homeostasis in the adult organism. Additionally, a misregulation of its function has been shown to cause a wide array of different congenital diseases and many types of cancers (McCleary-Wheeler, 2014). Therefore, it is crucial to understand the regulation of Gli2 and its function in different developmental processes in order to unveil its mechanisms of action and for the development of novel therapeutical molecules and treatments. In this study, we investigated *gli2* requirement in NC development through different molecular, pharmacological and embryological approaches. We found that Gli2 is a key regulator of early embryogenesis required for NC induction, specification, migration and differentiation acting as a transcriptional activator.

The onset of NC specification occurs during late gastrulation (Stuhlmiller and García-Castro, 2012). We found that *gli2* was expressed in the NPB and NC from late gastrulation onwards, undoubtedly indicating a role in the early events that define the NC population and the later processes required for its migration. Thus, this study provides an extensive analysis of the *gli2* expression pattern that complements previous analyses (Aguero et al., 2012; Michiue et al., 2017; Perron et al., 2003; Rankin et al., 2016). Moreover, we independently analyzed Ptch1 expression by wholemount IHC. The product of *ptch1* gene, a well-known target of Hh signaling, was found in the NC territories during neurulation in expression domains fully compatible with a role in the early NC developmental processes. These results are in agreement with previous findings that partially showed *ptch1* expression (Rorick et al., 2007; Takabatake et al., 2000a), and with its use as a readout of Hh activity in the neural fold (Aguero et al., 2012). Recently, a comprehensive transcriptome analysis of ectodermal dissected regions from *Xenopus* embryos was made available through a searchable tool called EctoMap (Plouhinec et al., 2017). The *gli2* and Ptch1 expression patterns described here were compared with this database and we found that the *gli2* L-homeologous has a higher relative expression and is the one that most closely resembles the expression pattern found in this study using ISH in the NC at neurula stages. Moreover, *ptch1* expression in EctoMap recapitulates the expression found in this work. Taken together, these results strongly support a role for *gli2* in the NC.

On the other hand, our expression pattern found *gli2* transcripts in the anterior NP throughout neurulation, which coincides with the role of *gli2* as an essential factor in the development of the telencephalon, and as a causative factor for holoprosencephaly when it is mutated (Roessler et al., 2003; Takanaga et al., 2009). During vertebrate development, NCC are essential for the formation of ocular tissues such as the corneal endothelium, the corneal stroma and the sclera (Evans and Gage, 2005; Williams and Bohnsack, 2015). The expression of *gli2* in the optic territory during tailbud stages concurs with the role of NC in ocular development, and also agrees with the role that Hh plays during the development of *Xenopus* retinal pigment epithelium (Perron et al., 2003). Finally, *gli2* was also detected in the developing somites, which is consistent with the fact that *gli2* is a direct target of the myogenic regulatory factor *myoD* in *Xenopus* development (McQueen and Pownall, 2017).

NC induction is a complex multi-step process involving many diffusible molecules, signaling pathways and transcription factors that interact to shape the specification and identity of NPB cells in order for them to become NC-fate restricted cells. Here, we provide evidence that *gli2* is required for the expression of a subset of these genes in the early specification steps. This fact puts *gli2* in a pivotal position during the initial steps that give ectodermal cells the NPB identity. We also corroborated the timing of *gli2* requirement in NC induction through two approaches using the specific inhibitor GANT61. The NPB genes particularly affected by *gli2* gain-and loss-of-function include *pax3, msx1, snail1, zic3* and *zic5* (Aybar et al., 2003; Groves and LaBonne, 2014; Monsoro-Burq et al., 2005; Nakata et al., 2000; Nakata et al., 1997; Tribulo et al., 2003). Moreover, by a RT-qPCR assay of NC explants, we found that *gli2* is required for the expression of NPB specifiers genes *pax3, hairy2b* and *snail1* (Aybar et al., 2003; Vega-Lopez et al., 2015). These findings support a role for gli2 in the early steps of NC development.

Once the NPB territory is competent to become NC, additional signals are integrated to maintain NC specification. These factors are called NC specifiers and are crucial in establishing NC identity (Duband et al., 2015; Pegoraro and Monsoro-Burq, 2013). Moreover, they are important for the EMT transition and the subsequent migration of NCC. We scrutinized the role of *gli2* in this process through microinjection and NC-explant assays and found that it modulates the expression of key NC specifiers. We found by qPCR analysis that *gli2* regulated the expression of the NC specifiers *snail2* and *twist1*, confirming the role of *gli2* in the specification process of the NC. It could be argued that *gli2* might indirectly control the expression of these genes by initially controlling the expression of the NPB specifiers, and then these genes would take over the activation of the NC specifiers.

However, considering that NC induction does not fully confer a certain fate to NPB cells but enables them to respond to other signaling cues, acting as progenitor cells (Roellig et al., 2017), it is possible that *gli2* input is indeed necessary for both processes in a direct way. However, it remains to be determined whether *gli2* acts in this fashion by directly binding to promoters of some NC specifiers to control the specification of the NC.

NC transcription factors operate by activating or repressing the expression of their target genes. Gli2 could act as a transcriptional activator or repressor depending on the particular status of the Hh signaling (Ruiz i Altaba et al., 2007). We designed and constructed different functional variants to assess which form of gli2 participates in NC development. We found that the activator form of Gli2 is responsible for the induction and specification of the NC, as evidenced by the transcriptional responses of *foxd3* and *snail2* marker genes. Additionally, we found that the repressor form of *gli2* reduced the expression of such markers. Moreover, the *gli2PSM* construct, which lacks the phosphorylation sites for PKA and behaves only as a transcriptional activator, also increased the NC markers expression during initial specification. Additionally, by performing a Gli-dependent luciferase activity assay on NC explants, which uses 8 tandem Gli-binding sites driving the luciferase construct, we confirmed that Gli2 acts as a transcriptional activator during NC development. Different combinatorial experiments on NCC showed that *gli2* responds to *ihh* for the proper activation of its target genes. Interestingly, the injection of the *gli2PSM* construct produced a dramatic increase in the expression of the reporter construct, thereby confirming the activator role of Gli2 during NC morphogenesis. Altogether, our results point to the fact that this transcription factor acts as an activator to specify the NC. These results extend the current insights of Gli2 functions in embryogenesis, specifically during NC development.

Gli2 is expressed in the NP and, as shown here, also in the NPB and the neural fold, and is essential for the D-V patterning of the NP through the control of several specific genes (Pal and Mukhopadhyay, 2015). The fact that the same molecule can control the expression of different genes in contiguous territories suggests that this transcription factor responds differently in each cell population. With respect to this question, one possibility could be argued in favor of the Hh ligand that stimulates the pathway. Based on our previous expression pattern and the functional analysis the Gli2 protein, we postulate that this protein receives the signal from the Ihh ligand in the NC, and not from Shh secreted from the notochord and the floor plate (Aguero et al., 2012). Secondly, the differential activity of Gli2 between the NC and the NP could be explained by proposing that NC-specific expression of regulatory proteins and transcription factors present in the NC tissue creates a differential

context for the action of Gli2. During NC development, one possible candidate for the differential action of Gli2 could be Pax3, one of the NC earlier specifiers (Monsoro-Burq et al., 2005), which has been shown to specifically interact with the Hh effector to promote the activation of gene enhancers in the epaxial somite, where both are coexpressed (Himeda et al., 2013). Additionally, by qPCR we have corroborated the effect of *gli2* on *pax3* expression. Moreover, other NC-expressed genes have Gli binding sites in their regulatory sequences. This is the case with *snail1* and *twist1*, which are essential for NC specification and are upregulated by Hh during cancer initiation (Kong et al., 2015). These genes were also tested by qPCR and found to be regulated by *gli2.* Therefore, our results establish a novel model for further investigations to elucidate the mechanism of action of the Ihh/Gli signaling during NC development. Additionally, we can conclude that the importance of Gli2 during development is thus not restricted only to the patterning of the neural tube, but extends to the transcriptional activation of NC inductors and specifiers during the morphogenesis of this cell population.

The NC maintains a coordinated balance between proliferation and apoptosis for the proper organization of the derived tissues and for its migration (Le Douarin et al., 2004). Previous investigations in mice have shown that Shh and Ihh increase the proliferation of NC during the differentiation of its derivatives (Everson et al., 2017; Yang et al., 2016). Gli2 in particular has been involved in the enhancement of proliferation during lung development by increasing the expression of Cyclin-D (Rutter et al., 2010). In this work we assessed whether this behavior of *gli2* applies to the development of *Xenopus* NC and found that, in agreement with previous investigations on the role of *ihh*, *gli2* did not alter the proliferative status of the NCC (Aguero et al., 2012; Tribulo et al., 2003). These findings suggest that the changes observed in the ectodermal marker expression could be due to cell fate decisions operating during *gli2* gain- and loss-of-function.

On the other hand, we found a marked reduction in the apoptotic state of NCC when *gli2* or its activator form was overexpressed. These results correlate with the proven role of the Hh signaling pathway in the survival of the NCC in chick and mouse models (Delloye-Bourgeois et al., 2014; Smoak et al., 2005). Moreover, in carcinoma cells, Gli2 elicits this function through the transcriptional upregulation of the pro-survival gene *cflip* (Kump et al., 2008). Gli2 has also been shown to promote cell survival and to interact directly with *pax3* as mentioned before (Himeda et al., 2013; Wang et al., 2011). Curiously, *pax3* is one of the transcription factors operating in NC development, suggesting that the interaction between both genes could be crucial for the survival of NCC.

NC migration occurs shortly after the cells have performed the EMT and become delaminated from the dorsal side of the embryo (Kerosuo and Bronner-Fraser, 2012; Kuriyama and Mayor, 2008). Cadherins in particular are important during the cell-cell interactions that trigger the migratory behavior of NCC (Taneyhill and Schiffmacher, 2017). One of the changes that are needed for NCC to become migratory involves the dissolution of tight cadherin-mediated adherens junctions. Our quantitative qPCR data showed that *gli2* overexpression actively increases the expression of *e-cad* and *n-cad,* which are known to be upregulated during the initial phase of NC migration (Cousin, 2017; Huang et al., 2016). This regulation highlights the role of *gli2* as a regulator of NCC delamination and migration. Moreover, we have shown that *gli2* is required for NC migration in a cell autonomous manner. This data is in agreement with the cell autonomous role that *gli2* plays in the proliferation and contact inhibition of cancer cells (Alexaki et al., 2010; Regl et al., 2004).

In most animal species, cranial NCC form the majority of the craniofacial skeleton (Schilling and Le Pabic, 2014)**.** Defects in the craniofacial apparatus underlie some of the most common human birth defects such as cleft palate, in which Hh signaling plays a central role (Xavier et al., 2016). Moreover, Gli2 has been associated with the formation of mouse temporomandibular joint articular disk (Purcell et al., 2009). Therefore it is of crucial importance to understand the factors that promote the skeletogenic differentiation of cranial NCC in order to elucidate the mechanisms that drive human birth defects. In this work we strongly related *gli2* function with the development of the craniofacial cartilages, which agrees with its role in proliferation and differentiation of chondrocytes (Joeng and Long, 2009; Thompson et al., 2017). The abnormal development of the craniofacial cartilages produced by interfering with Gli2 function may reflect a deregulation of the processes that control NC formation and the subsequent NCC migration. A probable mechanism of this process could be the direct action of Gli2 upon *sox9* to promote or regulate the chondrocytic lineage, as it is known that both proteins interact in cancer cells (Syu et al., 2016).

Melanocytes, which are derived from the NC, are formed at all axial levels and are responsible for the pigmentation of the skin and hair follicles (Mort et al., 2015). Our results indicate that *gli2* is involved in the formation of this cell lineage. Considering how *gli2* affected *sox10* expression in our explant assay, we can argue that the effect observed on the number of melanocytes could be due to a downregulation of *sox10,* which is a known promoter of the melanocytic lineage (Aoki et al., 2003; Honoré et al., 2003).

NCC give rise to the cranial ganglia precursors, to all Schwann cells and to the sympathetic neurons of the peripheral nervous system (Newbern, 2015; Sakaue and

Sieber-Blum, 2015). We found that *gli2* was required for the formation of the Rohon-Beard sensory neurons and cranial ganglia precursors (in lens, the profoundal and the trigeminal placodes), and for *tbx2* expression during the early tailbud stage. *tbx2* gene is expressed and required for the formation of the cranial ganglia precursors of the PNS glial cells (Takabatake et al., 2000b) and was found to be regulated by a Gli-dependent Hh signal transduction pathway during *Xenopus* eye development (Takabatake et al., 2002). Another possible mechanism that could explain the reduction in Rohon-Beard neurons could be the direct modulation of the expression of *ascl1* (Voronova et al., 2011), which participates in the establishment of the enteric nervous system circuitry, by itself or by repressing the function of *sox10* (Kim et al., 2003; Memic et al., 2016). It is also possible that Gli2 is directly affecting *sox10* expression, as this protein has also been shown to be essential for the development of the Rohon-Beard neurons (Olesnicky et al., 2010). However, since when analyzing the formation of NC derivatives we knocked down *gli2* during the migration of the NC, we cannot definitively rule out a role of an altered NC migration in the formation of the NC derivatives

Our results, integrated with previous studies, support a model in which *gli2* acts downstream of the *ihh* signal secreted from the NC and from the underlying mesoderm to modulate the expression of different NC essential factors. This assumption was confirmed by the luciferase assay, in which we showed that *gli2* responds to *ihh* to activate the transcription of the reporter. Additionally, in our epistatic analyses we found that *gli2* and *gli3* might act in an equivalent hierarchical position during the induction of the NC. On the other hand, our results have shown that *gli2* acts downstream of the receptor *ednra* during NC specification, and is probably responsible for the action of the Edn1/Ednra signaling in promoting the cell survival of NCC during their specification (Bonano et al., 2008). Alternatively, a relationship between both signaling pathways could operate in the NC. This positive regulation between Hh and Endothelin-2 signaling pathways was established during the onset of basal cell carcinoma (Tanese et al., 2010). Additionally, our last epistatic analysis showed that *eg5* acts upstream of *gli2.* Since Eg5 is considered an essential kinesin during microtubule organization and stability (Chen et al., 2017) with a participation in NC development different from its classical function in cell division (Fernandez et al., 2014), it is conceivable that Eg5 could act in the process required for Gli2 translocation to the primary cilium of *Xenopus* NCC. Moreover, this kinesin is highly expressed in glioblastoma cells as it is the case of Gli2 (Taglieri et al., 2017). However, future studies will be needed to specifically pinpoint the interactions between Gli2 and the proteins Eg5 and

Ednra during NC development. Our data hence support a model in which Gli2 acts downstream of the Ihh signaling pathway and also responds to the activities of the Ednra signaling and the kinesin Eg5 during the initial steps required for NC formation.

As stated above, an improper specification, migration or differentiation of the NC can result in a group of diseases called neurocristopathies (NCP), which affect an appreciable percentage of newborns (Vega-Lopez et al., 2018). It has been shown that ciliopathies, which arise due to defects in the assembly or function of the primary cilia, are linked to NCP due to similar craniofacial defects occurring in some specific ciliopathies and NCP (Chang et al., 2015; Vega-Lopez et al., 2018). However, the genetics and cellular pathogenesis linking NCP and ciliopathies are still incompletely understood. Therefore, it is essential to develop suitable experimental model systems to gather more insights into the molecular basis underlying both diseases. Since *gli2* was found to be expressed in the NC and since this transcription factor acts through primary cilia to activate target genes (Eggenschwiler and Anderson, 2007), we could speculate that this study will improve our understanding of the close relationship between ciliopathies and NCP. Moreover, this approach represents an excellent model to investigate the pathogenesis of NCP during development. Additionally, to the best of our knowledge, this work represents the first embryological usage of GANT61 for inhibiting in vivo the transcriptional activity of Gli2 and thus exploring its role during *Xenopus* development. Therefore, the use of this inhibitor could prove to be a useful tool for future studies. Altogether, our findings shed new light on the signal-mediated control of NC development by Ihh/gli signaling, and establish Gli2 as an essential factor in the development of the NC. Further investigations on *gli2* function and its close relationships with other signaling pathways during *Xenopus* NC development will improve our understanding of the regulatory mechanisms driving the formation of the NC.

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4. MATERIAL AND METHODS

4.1. Embryonic Manipulation, RNA Microinjection and Lineage Tracing

Embryos were obtained by standard procedures (Aybar et al., 2003) and staged according to the Nieuwkoop and Faber series (Nieuwkoop, 1967). Tissue microdissections, mRNA *in vitro* synthesis, microinjection and lineage tracing were performed as previously described (Aguero et al., 2012; Bonano et al., 2008; Fernandez et al., 2014; Vega-Lopez et al., 2015). *In vitro* transcribed mRNA was synthesized from *pCS2+6MT_gli2* (a gift from Muriel Perron, also known as *XGli4* (Genbank U42462, (Marine et al., 1997)) by standard procedures. Loss- and gain-of-function experiments through microinjection were conducted as follows: one dorsal blastomere of an 8-cell stage embryo (stage 3) was injected with mRNAs or antisense morpholino oligonucleotides (*MO*) at different concentrations (expressed as nanograms per embryo: ng/E) along with a lineage tracer, with the uninjected side serving as an internal control. At the desired stage the embryos were fixed with MEMFA and processed by ISH, IHC or another protocol specified in the text.

4.2. GANT61 treatment, morpholino antisense oligonucleotides and DNA constructs

In order to specifically inhibit the function of the Gli2 transcription factor in *Xenopus laevis* NC explants or whole embryos, the inhibitor GANT61 (Medchemexpress LLC, Princeton, NJ, USA) was used to microinject *Xenopus laevis* embryos and also to incubate NC explants. For incubation experiments, the NC explants with underlying mesoderm were dissected as previously described (Aguero et al., 2012). In order to spatially control Gli2 function, heparin-sepharose beads (Affi Blue Beads, Bio-Rad, USA) were soaked into 50 µM of GANT61 and grafted into embryos as previously described (Fernandez et al., 2014). GANT61-soaked beads were implanted in the right neural fold region. DMSO-soaked beads were grafted as a control in sibling embryos at the same position. In order to inhibit the translation of the *gli2* mRNA, a morpholino antisense oligonucleotide (*gli2MO*) was designed and synthesized including the initiation start site (from +1 to +24 bp) with the sequence shown in Supplementary Table 1 (Gene Tools, LLC). Doses of 5–27 ng/E were microinjected with a lineage tracer into one blastomere in 8- or 16-cell stage embryos. A control antisense morpholino oligonucleotide (*CoMO*) composed of a random sequence was injected as a control. For the knock-down of *gli3, ihh, eg5* and *ednra* mRNAs, specific morpholino oligonucleotides were used (See Table 1). The *gli2* ORF from *pCS2+6MT_gli2*

was used as a template for DNA constructs and *in vitro* mRNA synthesis. In order to test the efficacy of *gli2MO in vivo*, a *pCS2+gli2*GFP fusion was generated by high-fidelity PCR using *pCS2+MT_gli2* as the template with the primers shown in Table 2. A fragment containing the N-region of Gli2 encoding 277 amino acid residues of Gli2 was cloned into the *pTOPO-TA* vector (giving rise to *pTOPO-gli2*), amplified and then subcloned directionally into *BamHI* and *NcoI* sites in *pCS2+eGFP* vector to produce the *gli2GFP* construct. *In vitro* transcribed *mRNA* (2.6 ng/E) of this construct was coinjected with different amounts of *gli2MO* and the GFP fluorescence was observed under a Leica fluorescence stereomicroscope. For the rescue of morpholino knock-down experiments, a DNA construct was prepared by changing specifically the codon sequence targeted by the *gli2MO* antisense oligonucleotide. Special caution was taken to mutate the DNA sequence without changing the wild type *gli2* amino acid sequence. This construct, made by high fidelity PCR and named *pCS2+MT-CRgli2* (CR, Codon Replacement), introduced 6 silent mutations in the nucleotide sequence recognized by the morpholino.

In order to test the different functional variables of Gli2, we generated an activator and a repressor Gli2 construct, *gli2act* (*pCS2+MT-Gli2act*) and *gli2rep* (*pCS2+MT-Gli2rep*), respectively. The activator construct lacks the N-terminal repressor region of Gli2 and the repressor plasmid lacks the C-terminal activator domain of this protein. These constructs were generated with the primers indicated in Table 1, using *pCS2+MT_gli2* as the template.

The construct *gli2PSM* (pCS2+MT-gli2PSM-6xHIS) was generated by site directed mutagenesis and encoded a myc tag in the N-terminal region and a 6xHIS tag in the C-terminal region. Additionally, it has the six PKA-phosphorylatables serine residues mutated into non-phosphorylatable alanines. In order to specifically direct the overexpression of *gli2* functional variables to the NCC, the *α3000 snail2* promoter (Vallin et al., 2001) was fused upstream to the full length *gli2* sequence. The *α3000gli2* (*Alpha-3000-gli2*) DNA construct was prepared from *BamHI/NotI* digested pTOPO-Gli2 and ligated into *SB*XlpSIα3000-GFP* plasmid. The *α3000gli2* plasmid was purified and 200–400 pg/embryo were microinjected into one blastomere of 8- to 16-cell embryos. Fluorescence was observed since stage 13. Similarly, the constructs *α3000-CRgli2* (*Alpha-3000-gli2*), *α3000-gli2act* (*Alpha-3000-gli2act*) and *α3000-CRgli2PSM* (*Alpha-3000-CRgli2PSM*) were prepared using the primers described in Supplementary Table 2. All constructs prepared in this work were sequenced on both strands by automated DNA sequencing and are shown in Supplementary Figure 1. Their NCBI accession numbers are indicated in Table 5.

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4.3. *in situ* hybridization, immunohistochemistry and caspase activity measurement

The ISH protocol was performed as previously described (Aybar et al., 2003). Phenotypes obtained by ISH were double-blind counted by two lab members. The NC gene expression areas of treated and injected sides of at least 5 embryos for each treatment or condition were measured and quantified using the ImageJ software (NIH, USA) in 3-5 photographs per embryo. The expression of a gene marker was considered increased or decreased when compared to the control side showed a difference of at least 25% in the average values of gene expression between control and treated sides.

Antisense probes containing Digoxigenin-11-UTP or Fluorescein-12-UTP were prepared from the clones indicated in Supplementary Table 3. Apoptosis in NC was detected by Caspase 3/7 activity using the Apo-ONE homogeneous assay (Promega, Madison, WI) according to the manufacturer's instructions as described previously. In detail, NC explants were dissected from midneurula stage embryos. The explants were then resupended in 50 µl of NAM 3/8 and 50 µl of the Apo-One Caspase 3/7 reagent. After mixing by pipetting to homogenize the tissue, the samples were incubated 40 min at room temperature and the fluorescence was measured as arbitrary fluorescence units in a Perkin-Elmer LS55 fluorescence spectrometer (excitation wavelength 499 nm, emission wavelength 521 nm). The Caspase 3/7 activity was expressed as the ratio to the uninjected embryo (Caspase Arbitrary Unit = 1). (Fernandez et al., 2014). IHC for Ptch1 and phosphohistone was performed as described previously (Vega-Lopez et al., 2015). The anti-Ptch1 antibody used was obtained from the Developmental Studies Hybridoma Bank (DSHB, USA, clone Apa1) while the rabbit Polyclonal anti-phosphohistone-3 antibody was obtained from Upstate Biotechnology, Lake Placid, NY). Student's t-test was used to analyze the differences between each of the groups for Caspase 3/7 activity with respect to the corresponding control group. ANOVA analysis (InfoStat 2011, (Di Rienzo et al., 2011) was used to determine statistical differences ($P \leq 0.05$) between Caspase 3/7 activity and the control group. Tukey's test was also used ($\alpha \leq 0.05$). All experiments were repeated twice.

4.4. Cartilage staining, RNA isolation from embryos and RT-qPCR analysis, Western blot and luciferase assay.

Cartilage staining was performed as previously described (Vega-Lopez et al., 2015). For the RT-qPCR study of *gli2* function, total RNA was isolated from NC explants using the Trizol LS Reagent (Invitrogen) after microsurgery. After DNaseI treatment, reverse

transcription was performed starting from 2 µg of total RNA using the M-MLV Reverse Transcriptase (Promega) and Oligo (dT) primers. Real-time PCR was done on 1 µl of dilutional series of cDNA starting from a dilution of factor 5 using Mezcla Real (Biodynamics). PCR amplification was monitored with a CFX96 Real-Time System (Bio-Rad). After one incubation step at 95°C for 7 min, the thermal cycling protocol was as follows: 95°C for 10 seconds, 60°C for 15 seconds and 72°C for 15 seconds during 40 cycles. The primers designed for this study are shown in Supplementary Table 4. Target genes were analyzed using standard curves to determine relative levels of gene expression, and individual cDNA samples were normalized according to the levels of *gapdh.* For each primer set, the efficiency of the PCR reaction was measured in duplicate on serial dilutions (factor 5) of the cDNA sample. Real-time PCR efficiencies (E) for each reaction were calculated from the slope of the standard curve using the BioRad CFX manager V3 software, with 100% efficiency as an indicator of a robust assay. Statistics were generated using Excel and *p* values determined using paired, 2-tailed Student's t-Test. Error bars represent average ± s.d. All results reported here are averages of two independent reverse transcription reactions, which have been each tested with two qPCR reactions. For quantification, data were analyzed by the 2-DDCT method (Livak and Schmittgen, 2001), by normalizing to the housekeeping gene *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) and to the uninjected NC. Finally, qPCR results were represented showing each gene expression level in treated explants normalized to the level of expression of wildtype uninjected NC explants.

For the control of the efficiency of the *gli2MO* Western blots were performed as described (Bonano et al., 2008; Fernandez et al., 2014). Protein extracts were obtained from 10 *gli2MO*- and *pCS2+gli2GFP*-injected embryos at stage 15 for each experimental group and processed for Western blot assay using anti-GFP, 1:1000 (Invitrogen) and anti-alpha-tubulin 1:1500 (DSHB, 12G10 clone). The alpha-tubulin antibody was used as a loading control. After electrophoresis and electrotransference, nitrocellulose membranes were incubated with primary antibodies overnight at 4ºC followed by 2 h incubation with the secondary antibody. The secondary antibodies were peroxidase-conjugated (cat. # AM-P1, Endocrinology Lab, Universidad Nacional del Litoral, Argentina) and alkaline phosphatase (AP)-conjugated (cat. # A0162, Sigma Aldrich) 1:750. The substrates for colorimetric detection were BCIP (cat #94550364, Roche, USA) and NBT (cat. # 94550571, Roche, USA) as AP substrates. For chemiluminescent detection the reagent Biolumina was used

(Kalium Tech, Buenos Aires, Agentina). Labeling detection and documentation was performed using a C-Digit scanner (LI-COR Biosciences).

For the luciferase assay, stage 3 embryos were injected with the reporter plasmid *8xGBS-LucII-pCS2+* (a kind gift from Stephan Teglund) (300pg/E) and the control reporter plasmid *pGL4.74-TK-hRluc* (200pg/E). Along with the reporter constructs, mRNAs and MOs were also injected to analyze the transcriptional mechanism involving Gli2. At stages 15-16, 60 NC explants were extracted and homogenized with Passive Lysis Buffer (Promega). Then luciferase activity was measured by a commercial kit (Dual-Luciferase Reporter Assay System, Promega, WI, USA) using the Synergy HTX microplate reader (Biotek, Winooski, VT, USA). Results are representative of two independent experiments.

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Human and Animal Rights

Animal experimentation: All animal care and experimentation were conducted in accordance with institutional guidelines. The study protocol was approved by the Institutional Animal Care Committee (CICUAL, Protocol #01/17) of the Universidad Nacional de Tucumán (Tucumán, Argentina).

Declaration of Interest

Declaration of interests: none

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Highlights

- A complete spatio-temporal expression pattern of *gli2* in *Xenopus laevis* was investigated. *gli2* is expressed in the premigratory and migratory neural crest.
- Gli2 acts as a transcriptional activator in the neural crest and modulates the expression of neural plate border and neural crest genes.
- Gli2 does not affect cell proliferation but enhances survival of neural crest cells.
- Neural crest migration requires *gli2* in a cell autonomous manner.
- *gli2* participates in the formation of craniofacial cartilages, Rohon-Beard neurons, cranial ganglia primordia and melanocytes.