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A *trans*-Regulatory Code for the Forebrain Expression of *Six3.2* in the Medaka Fish*[S]

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Background: The transcription factor Six3 is key element of forebrain specification, but its upstream regulators are unknown.
Results: A systematic search in medaka fish identifies and functionally characterizes novel *Six3* regulators.
Conclusion: The spatio-temporal regulation of *Six3* depends on a few *trans*-acting factors.
Significance: This study provides new information on how forebrain neuronal diversity is originated.

A well integrated and hierarchically organized gene regulatory network is responsible for the progressive specification of the forebrain. The transcription factor Six3 is one of the central components of this network. As such, Six3 regulates several components of the network, but its upstream regulators are still poorly characterized. Here we have systematically identified such regulators, taking advantage of the detailed functional characterization of the regulatory region of the medaka fish *Six3.2* ortholog and of a time/cost-effective *trans*-regulatory screening, which complemented and overcame the limitations of *in silico* prediction approaches. The candidates resulting from this search were validated with dose-response luciferase assays and expression pattern criteria. Reconfirmed candidates with a matching expression pattern were also tested with chromatin immunoprecipitation and functional studies. Our results confirm the previously proposed direct regulation of Pax6 and further demonstrate that Msx2 and Pbx1 are *bona fide* direct regulators of early *Six3.2* distribution in distinct domains of the medaka fish forebrain. They also point to other transcription factors, including Tcf3, as additional regulators of different spatial-temporal domains of *Six3.2* expression. The activity of these regulators is discussed in the context of the gene regulatory network proposed for the specification of the forebrain.

The progressive specification of the forebrain (or of any other developing structure) depends on the synchronized activity of intrinsic genetic programs and extrinsic signals, which form well integrated and hierarchically organized gene regulatory networks (GRNs).[3] Perturbation of the GRN status causes abnormal development of the involved structure, which is also considered a mechanism that enables the acquisition of morphological innovations during evolution (1, 2). Components of a GRN with a high hierarchical and central position are known as hubs. In general, hubs are transcription factors (TFs), which regulate the many other components of the network and which are, in turn, regulated by a relatively low number of genes (1). *Six3*, a homeobox-containing TF belonging to the *Six/sine oculis* family, is one of the hubs of the GRN responsible for forebrain specification (2).

In vertebrates, *Six3* is expressed from the neurula stage in the anterior-most neural plate and its derivatives (the telencephalon, hypothalamus, diencephalon, and retina) as well as in the lens and olfactory placodes (3–6). Consistent with its hub position, *Six3* overexpression induces the formation of ectopic retinal-like structures in the forebrain (7, 8), whereas inactivation of its function impairs forebrain development (9–12); alters the expression of key morphogenetic proteins, such as Wnt1, Wnt8, BMP4, Shh, and Nodal (9, 13–17); and disrupts the balance between cell proliferation and differentiation (18, 19). Progressive knockdown of the medaka fish orthologs, *Six3.1* and *Six3.2*, has also demonstrated a graded requirement of the two genes for proximal-distal and anterior-posterior specification of the forebrain (20, 21), in the latter case through the direct regulation of the TFs *Foxg1*, *Rx3*, and *Nkx2.1* (21, 22).

In contrast to the relatively well characterized downstream targets of *Six3* activity and despite the detailed characterization

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[3] The abbreviations used are: GRN, gene regulatory network; TF, transcription factor; TFBS, TF binding site; TRS, *trans*-regulation screening; Luc, luciferase; ChIP, chromatin immunoprecipitation; BS, binding site; EGFP, enhanced green fluorescent protein; CRE, Conserved Regulatory Element; DN, dominant negative; BMP, bone morphogenetic protein; CDS, coding sequence.

of the *cis*-regulatory code that controls the spatial-temporal expression of the medaka *Six3.2* and zebrafish *Six3a* paralogs (23–25), only a few of the TFs that govern *Six3* expression have been so far identified. Six3 appears to regulate its own transcription with a context-dependent positive or negative feedback loop (25, 26). There is also evidence that *Six3* expression is restrained anteriorly by BMP-mediated Lmo4 activity (26) and posteriorly by Wnt signaling (13). In both cases, it is still unclear whether the mechanism is direct.

Taking advantage of the identified *cis*-regulatory code and of available TF binding site (TFBS) prediction tools, we have recently demonstrated that the neural inducing factor Sox2 directly controls the expression of the medaka *Six3*.2 (21). However, *in silico* prediction of putative candidates has limitations because it depends on available TFBSs and does not account for transcriptional modifications imposed by the interaction with a given cofactor. Thus, to extend our search and generate a more comprehensive scenario of the relevant *Six3* regulators, we have reinforced our *in silico* prediction approach with a *trans*-regulation screening (TRS). This screening is a systematic survey of potential regulators, which has already been successfully used to identify *trans*-acting factors of the medaka *Ath5* (27) and of the zebrafish *eng2a* genes (28). The latter study used a preselected version of the library enriched in characterized developmental regulators, thus improving the time/cost efficiency of the screening. By combining this mid-scale TRS with our *in silico* prediction-based approach over a well characterized *cis*-regulatory code (23), we have identified a number of potential upstream regulators of the medaka *Six3.2* gene, further improving the time/cost efficacy of the approach over previous studies (21, 27, 28). These regulators were further validated with different combinations of expression pattern analysis, dose-response luciferase (Luc) assays, chromatin immunoprecipitation (ChIP), and functional studies. Our results indicate that Msx2, Pbx1, and Pax6 are *bona fide* direct regulators of *Six3.2* expression at early stages of medaka fish forebrain development and point to Tcf3, Etv4/5, Nkx2.2, Prdm1/Blimp1, and Vsx1 as additional TFs responsible for *Six3.2* expression at different developmental stages.

Experimental Procedures

Medaka Stocks—Wild type (WT) medaka fish (*Oryzias latipes*) of the *Cab* strain and the transgenic line *Six3.2cI*::*EGFP*, expressing the EGFP reporter under the control of the full-length *Six3.2* regulatory region (23), were maintained in an in-house facility (28 °C on a 14/10-h light/dark cycle). Embryos were staged as described previously (29).

In Silico Prediction of TFBS—Sequences of the medaka, zebrafish (*Danio rerio*), fugu (*Takifugu rubripes*), stickleback (*Gasterosteus aculeatus*), and tetraodon (*Tetraodon nigroviridis*) genomes were obtained from the USCS genome browser. Multiple alignments of the 4-kb regulatory region of the medaka *Six3.2* gene (23) were performed using the Mulan and mVista tools (Fig. 1*A*). Evolutionarily conserved TFBSs were identified combining rVista (multi-TF), Jaspar, and Matinspector. In some cases, we found BSs with partially overlapping sequences. When overlap was more than 80% and the BS belonged to closely related TFs, we considered the presence of only one BS. Candidates were further screened according to gene expression data available in the literature and in the following databases: GeneCards, OMIM, Emage, and zFIN. Candidate TFs were considered "related" if their expression was overlapping with or complementary to that of *Six3.2*. The distribution of these regulators was plotted along the *Six3.2* regulatory region using GraphPad software (Fig. 1*B* and supplemental material), and these candidates were considered for subsequent experimental validation. Thus, relevant members of each one of the identified TF families were assayed in Luc assays (Table 1).

Plasmid Construction—For the TRS, the 4-kb regulatory region of the medaka *Six3.2* gene, including the minimal promoter region and the 5′-UTR, was cloned into the pGL3b vector (Promega), carrying the firefly Luc gene to generate the *pSix3.2*-full reporter vector. Reporter constructs carrying different combinations of the *Six3.2 cis*-regulatory modules upstream of the thymidine kinase minimal promoter in the pGL3b-TK vector (Fig. 2*A*) were used to validate *in silico* predicted candidates (21) (Table 1). Information about the plasmids coding for the *in silico*-predicted candidate TFs used in the Luc assays is available upon request. The Tcf3-HA-VP16 and Tcf3-HA-En were kindly provided by Dr. E. Marti. Modified versions of the pCS2 plasmid, carrying either the HA-Eng or the HA-VP16 proteins, were generated to obtain constitutive repressive or activating forms of the TFs and then used for mRNA transcription and injection experiments. To this end, the HA-VP16 or the HA-Eng cassettes were amplified with PCR-specific primers and cloned into the pCS2+ vector using specific restriction enzymes. The CDS of the different TFs was subsequently amplified with specific primers and subcloned either in the pCS2-HA-En (Pbx1, Pax6) or pCS2-HA-VP16 (Msx2). Deletions of the *Pax* BS present in the D elements were produced by PCR amplification using the Phusion DNA polymerase (New England Biolabs) and PCR-specific primers. The obtained PCR product was subsequently purified, treated with the polynucleotide kinase, self-ligated using the T4 DNA ligase, and transformed in DH5a-competent cells.

TRS—The TRS was performed as previously described (27). Briefly, we individually tested 1064 clones (supplemental material) belonging to a Unigene library enriched in genes considered as "developmental regulators." BHK21 cells seeded in 96-well plates were co-transfected with 2 μl (10–300 ng) of each cDNA, 40 ng of the pSix3.2-full reporter vector, and 5 ng of pRL-CMV using the Fugene 6 transfection reagents (Roche Applied Science) according to the manufacturer's protocol. Cells were cultured and transfected with DMEM supplemented with 10% FCS. The quality threshold was set above 1,000 relative units for *Renilla* luminescence and above 10 times the background signal (10 raw units) for firefly luminescence. Clones that activate the reporters with lower values were discarded. Raw luminescence readings were stored in a FileMaker database. Experiments were performed in triplicate, and the median values were normalized and analyzed using MS Excel. To account for plate-to-plate variations, each firefly/*Renilla* ratio was normalized against the average of all ratios in the plate as described (27). Nevertheless, there was no significant difference in the average values of this ratio

among plates (supplemental material), supporting the reproducibility and robustness of this approach. Selected candidate clones were further tested in a dose-response Luc assay performed in triplicate by co-transfecting 20, 40, 80, or 160 ng of pCMV-Sport6.1::cDNA with the reporter and constructs. The total amount of transfected DNA was kept constant by adding the necessary amount of pCS2+ vector. Dose-response experiments were performed with both CMV- and SV40-driven *Renilla* vectors to discard clones with a possible off-target effect on the *Renilla* promoter and to avoid the influence of possible difference in the expression efficiency of the different plasmids. Each clone and dose was assayed in triplicate, and the firefly/*Renilla* ratio was statistically compared using an appropriate *t* test. Clones were considered positives when at least one of the tested doses induced a response at least 75% higher (activation) or 50% lower (repression) than that observed with the dose of 20 ng (supplemental material). Clones were also considered positive if the firefly/*Renilla* ratio at 20 ng was significantly different from the average value of the other clones at the same dose, independently of their dose response.

Cell Transfection and Luc Assay—Luc assays were performed using the undifferentiated and pluripotent P19 teratocarcinoma line cultured in minimum Eagle's medium α with Glutamax (Gibco). Cells were transfected with 310 ng of DNA/well composed of the reporter plasmid (*pSix3.2-cI-cVI*, 50 ng), a CMV-driven *Renilla* Luc control vector (pRL-CMV; 10 ng), increasing amounts (25–250 ng) of the effector plasmid, and, when needed, variable amounts of the empty vector to kept the total amount of the transfected DNA constant. Transfections were performed with Fugene HD, following the manufacturer's instructions. To minimize possible bias imposed by differences in the expression level of the various plasmids, we normalized the reporter luciferase activity to a control *Renilla* reporter and then to the basal activity of the reporter construct when co-transfected with GFP. After normalization, the raw values of basal activity of the cI-cVI constructs were statistically indistinguishable and quite reproducible among experiments (data not shown). Cells were maintained in the same medium containing 5% FCS for an additional 48 h, when the Luc activity was measured using the Dual Luc reporter assay (Promega) following the manufacturer's instructions. Experiments were performed in triplicate and replicated at least three times. Data are presented as -fold induction of the Luc activity observed in the control vector normalized to 1.

Chromatin Immunoprecipitation Assays—ChIPs were performed as described (21), using a Myc- or HA-tagged version of the selected candidate genes. Briefly, P19 cells were transfected with reporter/effector plasmids (3:1 ratio, 10 μg total). ChIP was performed using the anti-Myc 9E10 (Sigma) or the anti-HA 3B9 (Active Motif) monoclonal antibody. The relative enrichment of each one of the *Six3.2* regulatory elements was determined by quantitative PCR (Roche Applied Science) using specific primers (information available upon request). Primers designed in the Luc coding sequence and in the genomic locus of the 18S RNA were used as negative controls. -Fold enrichment was expressed as the ratio of Myc to IgG signal and calculated with the expression, $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct = Ct_{\text{c-Myc}} - Ct_{\text{IgG}}$.

mRNA Overexpression Studies—The mRNA used in overexpression experiments were synthesized with mMESSAGE mMACHINE kits (Ambion) and co-injected with the RFP reporter (10 ng/μl) into one-cell stage *Six3.2-cI::EGFP* transgenic embryos at concentrations ranging from 30 to 100 ng/μl for each one of the tested mRNA. Within this range, we selected the best working concentration as that which gave the most evident phenotype without affecting embryo survival. Changes in *Six3.2* expression were determined by *in situ* hybridization (see below) and EGFP reporter expression using a Leica fluorescent stereomicroscope. We injected and analyzed a minimum of 30 to a maximum of 250 embryos for each one of the tested mRNAs.

Whole-mount in Situ Hybridization—Antisense riboprobes for the identified candidates were synthesized from the pCMV-Sport6 clone containing the full-length mRNAs according to standard protocols. Probes for the medaka *Tcf3*, *Etv4*, *Etv5*, and *Msx2a* genes were amplified by PCR using specific primers (information available upon request) and cloned into the pSCA-Strataclone vector (Stratagene). The *Six3.2* probe used in this study is as described (23). *In situ* hybridizations were performed as described (23).

Results

Identification of Potential trans-Regulators of Six3.2 Expression—The regulatory region sufficient to recapitulate the entire spatio-temporal expression of the medaka *Six3.2* gene is composed of 10 conserved regulatory elements (CREs) distributed in a 4-kb region located upstream of the coding region (Fig. 1, *A* and *B*) (23). Two of these CREs, known as D and IL elements, act like enhancers during early and late forebrain development, respectively (23). The A element instead silences *Six3.2* expression in the hindbrain and neural tube, whereas the E, F, G, and H elements modulate D or IL function (23). *In silico* analysis with different prediction tools (see "Experimental Procedures") identified several TFBSs randomly distributed along the *Six3.2* regulatory region (Fig. 1*B*), but the addition of evolutionary conservation as a criterion reduced their number and restricted their localization to the A, C, D, E, G, and IL CRE (Fig. 1, *A* and *B*). The identified TFBSs belonged to families of known forebrain regulators, including, among others, *Pax*, *Sox*, *Gli*, *Tcf/Lef*, and *Six* (Table 1 and supplemental material).

Because *in silico* prediction is limited by the existence of defined consensus BSs, we undertook a TRS with the aim of identifying additional *trans*-acting factors for the *Six3.2* locus. To this end, we took advantage of a preselected medaka Unigene expression library, composed of 1064 full-length cDNAs mostly encoding *bona fide* "developmental regulators." These cDNAs were individually screened for their ability to modulate the expression of a Luc reporter upon co-expression in BHK21 cells. As demonstrated previously (27), with this approach, only a small number of tested cDNAs are likely to have an effect on the regulatory region of choice, and the variation of the normalized luminescence ratios around the average is expected to be random for most of the clones, thus fitting a Gaussian curve (Fig. 1*C* and supplemental material). By contrast, values outside the normal range are likely to be specific, reflecting a direct or indirect regulatory relationship of the candidate gene with the

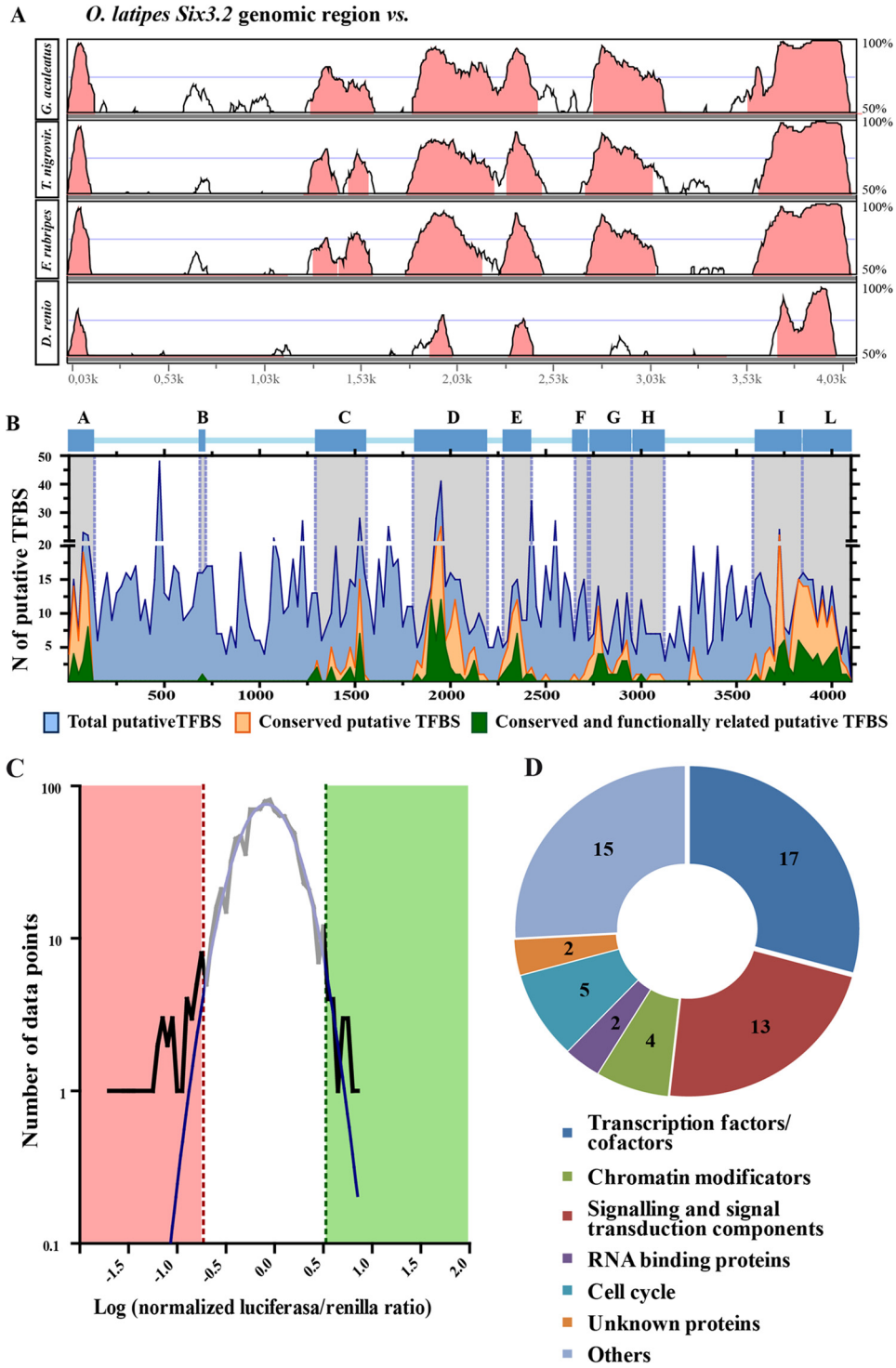

FIGURE 1. **Identification of putative *trans*-regulators of the medaka fish *Six3.2* regulatory region.** *A*, Vista multiple pairwise alignment of the 4-kb medaka (*O. latipes*) *Six3.2* regulatory region *versus* its orthologs in stickleback (*G. aculeatus*), tetraodon (*T. nigroviridis*), fugu (*F. rubripes*), and zebrafish (*D. renio*). *Pink peaks* indicate regions with at least 75% conservation over a 100-bp window. *B*, plot of the frequency and distribution of the total number of TFBSs (*blue*) found along the entire 4-kb regulatory region of *Six3.2*, which is schematically depicted at the *top* with a *light blue line*. The A-L CREs are represented by *blue boxes*. The distribution of TFBSs that are evolutionarily conserved without or with an additional related expression pattern is indicated in *orange* and *green*, respectively. Note how these TFBSs are clustered in the CREs. *C*, frequency distribution of normalized Luc values induced by the putative *trans*-regulators identified in the TRS. The observed distribution fitted a Gaussian curve, represented with a *blue line*. The *green shaded area* indicates the clones with a deviation from the mean >2.8732, thus considered as activators. The *red shaded area* instead represents putative repressors (clones with a normalized log ratio <0.2859). *D*, representation of the gene ontology classification of the identified targets based on bibliographic searches and information available in the GeneCards database.

tested genomic region. Using this criterion, we considered that clones with a normalized ratio lower than 0.2859 or higher than 2.8732 could be considered putative repressors and activators, respectively, of the *Six3.2* regulatory region (Fig. 1*C* and supplemental material). Fifty-eight clones, with an apparent repressor (40) or activator (18) effect and representing 5.4% of

TABLE 1
***In silico* predicted candidates further tested in Luciferase assays**

Tested TF	TF family	Predicted TFBS location	Supporting evidences	Tested Six3.2 constructs	Effect
Gata1 *Gata2* *Gata3*	GATA	A, C, D, E, G, I, L	Gata TFs are implicated in non-neural ectoderm specification as BMP signalling downstream targets.	cI, cII	− − −
Foxg1	Fox	A, C, D, E, G, I, L	Foxg1 is a key factor in telencephalic specification.	cII	−
Neurod	bHLH	B, D, E, I, L	NeuroD TFs are required for amacrine cell specification.	cII, cV	−
*Lef1ca** *Tcf3ca**	Tcf	C,H,L	Tcf3 is required for forebrain specification.	cI, cII, cIII, cIV, cV	▲ ▲
Sox2	Sox	A, C, D, E, G, I, L	Sox2 is a direct regulator of Six3.2 expression.	cII	▲
Otx2 *Vsx2*	Prd-like	I	Otx2 is involved in early neural plate specification and specifies retinal pigmented epithelium *versus* neural retinal fate. Vsx TFs are required for retinal bipolar cell specification.	cI, cII, cV	− −
Pax2 *Pax6*	Paired	C, D, E, G, I, L	Six3 and Pax6 have been shown to positively regulate each other expression.	cII, cIII cI, cII, cIII, cV	− ▲
Six3 *Six3.1* *Six3.2* *Six6*	Six	A, L	Six3 has been shown to positively and negatively regulate its own transcription.	cI, cII, cV cI, cII, cV cI, cII, cV cI, cII, cV	− − − −
Meis1b *Pbx1*	TALE	A, I, L	Six3 and Irx3 repress each other expression. Pbx proteins participate in eye development and Pbx1 is involved in telencephalic specification.	cI, cII, CV	− ▲
Irx3		-		cI	−
Msx2	Antp	A, D,I,L	*Msx* genes are involved in the establishment of the neural non-neural ectoderm border as downstream effectors of BMP signalling.	cI, cII, cIII	▼
Gli1 *Gli2*	Gli/Zic	A, I	The Gli TFs are transcriptional effectors of Shh, which specifies ventral forebrain domain.	cI, cII, cV	− −
Smad1 Smad3 Smad5 Smad8 Smad4	Smad	G, I	Smad proteins are transcriptional effectors of BMP and TGF signalling pathways. BMP signalling limits *Six3* expression and promotes non-neural *vs* neural plate specification. TGF3β is involved in eye development.	cI, cII	− − − − −
Pou2	POU	A,D,E,G,I,L	Oct/ Pou TFs are determinants of stem cell maintenance and proliferation and cooperate with Sox2 in the regulation of different target genes.	cII	−

the screened clones, fulfilled this criterion. The majority of the positive clones were TFs, signaling molecules, or signaling transduction components (Fig. 1*D*). With the exception of two clones, for which no information was available, the remaining clones were classified as chromatin modifiers, cell cycle regulators, or RNA-binding proteins (Fig. 1*D*). Notably, at least one putative conserved BS within the *Six3.2* regulatory region could be identified for each one of the regulatory TFs selected in our screening for which a consensus BS had been described (supplemental material).

Dose-response Luc Assays Confirmed Several TRS Candidates as Six3.2 Regulators—Potential regulators of *Six3.2* expression should activate/repress its regulatory region in a dose-dependent fashion. To select the candidates with this behavior among the 58 identified genes, we performed Luc assays co-transfecting BHK21 cells with increasing doses of the selected cDNAs, the *pSix3.2-full* plasmid, and either the CMV- or SV40-driven *Renilla* plasmids (Table 2; see supplemental material for a full version of the data). This assay had two additional purposes: to discard clones with an off-target effect over the CMV:*Renilla* plasmid and to confirm the repressive/activating nature of the candidates. The TFs Pbx1, Nkx2.2, Etv4, Prdm1, and Hoxb1b; the signaling protein Wnt9b; Cap1, a component of the cyclic AMP pathway; the laminin receptor integrin-β4 subunit (Itgβ4); the histone deacetylase 1 (Hdac1); the Nedd4-binding partner-1 (N4bp1); and one of the unknown candidates acti-

TABLE 2

Summary of the TRS-selected candidates validated by dose-response Luciferase assays

For each of the factors, the data were normalized to the lowest dose employed in the study. Values above or below the reference value are color-coded: in graded greens for activation and in graded reds for inhibition. cDNAs were cotransfected with a CMV or a SV40 driven *Renilla* vector. The candidates were considered positive when they significantly activated or repressed the reported activity compared to their reference values at least at one of the doses in both assays. Their effects are represented with green or red arrow respectively. Gray arrows represent candidates that did not display significant changes in luciferase activity at the doses tested in this study but whose reference values were significantly higher (putative activator) or lower (putative repressor) than the average of the remaining clones.

	Renilla CMV				Renilla SV40				Graphical summary		Regulatory effect
	Dose 20ng average values	Normalized fold change (log2 scale)			Dose 20ng average values	Normalized fold change (log2 scale)					
		dose 40ng	dose 80ng	dose 160ng		dose 40ng	dose 80ng0	dose 160ng	Renilla CMV	Renilla SV40	
Rac1	0,026	0,650	0,394	0,512	0,035	-0,146	-0,609	-0,935			↓
Bcl2l10	0,019	0,250	-0,388	-0,401	0,035	-0,041	-0,046	0,077			↓
Pbx1	0,034	0,666	0,639	3,144	0,046	-0,067	0,267	3,172			↑
Vsx1	0,082	0,014	0,436	0,321	0,077	-0,094	0,728	1,303			↑
Nkx2.2	0,032	0,586	1,233	1,424	0,035	-0,214	1,384	0,750			↑
Etv4	0,059	0,056	0,531	1,314	0,067	-0,171	0,479	1,710			↑
RhoAa	0,027	0,469	0,519	1,072	0,036	-0,526	-0,745	-0,736			↓
Quaking	0,023	0,288	0,305	0,224	0,036	-0,297	-0,198	-0,664			↓
HoxB1b	0,111	-0,107	0,375	0,749	0,066	-0,012	0,341	0,705			↑
Cap1	0,094	0,468	0,428	0,661	0,062	-0,110	0,003	0,389			↑
Mink1	0,080	-0,197	-0,428	-0,509	0,051	0,035	-0,397	-0,662			↓
Homolog to Znf467	0,081	-0,508	-0,279	-0,356	0,058	-0,045	-0,418	-0,351			↓
Wnt9b	0,059	0,279	0,835	1,065	0,074	0,115	0,585	1,056			↑
Prdm1	0,069	-0,102	0,639	0,880	0,056	0,113	0,478	0,475			↑
Itgb4	0,061	-0,011	0,681	0,806	0,085	0,557	0,988	1,390			↑
Rab11b	0,055	-0,675	-0,355	-0,363	0,036	0,446	-0,101	-0,603			↓
Clns1a	0,092	-0,192	0,450	0,501	0,044	0,170	0,268	0,207			→
Unknown	0,088	0,363	0,379	0,620	0,052	0,337	0,251	0,596			↑
Hspa12a	0,063	0,213	0,838	0,943	0,050	0,060	0,297	1,094			↑
N4bp1	0,150	0,111	0,091	-0,078	0,082	0,087	0,071	0,404			↑
Hdac1	0,058	0,645	0,970	1,603	0,046	-0,069	0,182	0,532			↑

vated the *Six3.2* promoter in both assays (Table 2). Although the TF Vsx1 and the cell cycle regulator Cins1a (Cdk2) did not elicit a significant dose response, they both activated the *Six3.2* promoter above the average basal activity of the remaining clones even when used at a low dose (Table 2 and supplemental material). The Fzd7 receptor and the medaka homolog of the *Znf467* gene instead significantly reduced *pSix3.2-full* reporter activity in a dose-dependent fashion, whereas low doses of the GTPases Rac and RhoA, the apoptosis-related *Bcl2l10* gene, and the RNA-binding protein Quaking repressed the firefly/*Renilla* ratios below the average of the remaining clones (Table 2 and supplemental material).

To extend this analysis to the *in silico* predicted regulators, we transfected P19 cells with plasmids encoding the candidates and the reporter constructs harboring different combinations of the *Six3.2* CREs (Figs. 2 (*A–E*) and 3 and Table 1). These constructs were used to identify the relevant bound regions because putative BSs for the selected TFs were often found in more than one CRE (Table 1 and Fig. 2*F*). As a positive control for the assay, we co-transfected an expression plasmid for *Sox2* (Fig. 3*A*) (data not shown), previously described as activating the *Six3.2* locus (21).

Pax6, Pbx1, and a constitutively active form of Tcf3 (Tcf3ca), generated by fusion of the VP16 activator domain (30), activated the Luc reporter of the constructs harboring distinct CRE combinations (Fig. 2, *A–D*). Pax6, but not Pax2 (Figs. 2*B* and 3*C*), which recognizes a similar BS (31), induced up to a 6-fold activation of the reporter constructs carrying the D module (cII and cIII; Figs. 2*B* and 3 (*C* and *D*)), in line with the presence of conserved *Pax* BS in the D box (Table 1 and Fig. 2*F*), but, as reported for Sox2 (21), it failed to activate the cI construct (Fig. 2*B*), containing the A repressor module. Notably, deletion of the two Pax6 BSs at the 5′-end of the D element strongly decreased reporter activation, whereas elimination of the additional Pax6 BSs had only a very modest additive effect (Fig. 3*D*), suggesting that this 5′ portion of the D CRE mediates most of the Pax6 activity.

As observed for Pax6, Tcf3ca and the related Lef1ca activated in a dose-dependent manner all reporter constructs containing the H and L CREs (Figs. 2*C* and 3*E*), in which conserved BSs were found (Fig. 2*F*), suggesting that different members of the Tcf/Lef family may participate in the regulation of *Six3.2* expression.

The strong effect of Pbx1 in the *trans*-regulatory screening and the presence of evolutionarily conserved *Pbx* BS in the A and L modules (Fig. 2*F* and Table 1) made Pbx1 one of the strongest candidates for a *Six3.2 trans*-acting factor. Pbx TFs form complexes with members of the Meis family (32–36). We thus tested the effect of Pbx1 on different *Six3.2* constructs transfected in P19 cells and asked if its activity was enhanced in the presence of Meis1b. Compared with the strong activation observed in the TRS, Pbx1 was less effective in activating reporter expression in P19 cells, although activation was significant (cI, cII, and cV; Fig. 2*D*). Meis1b, which alone had little

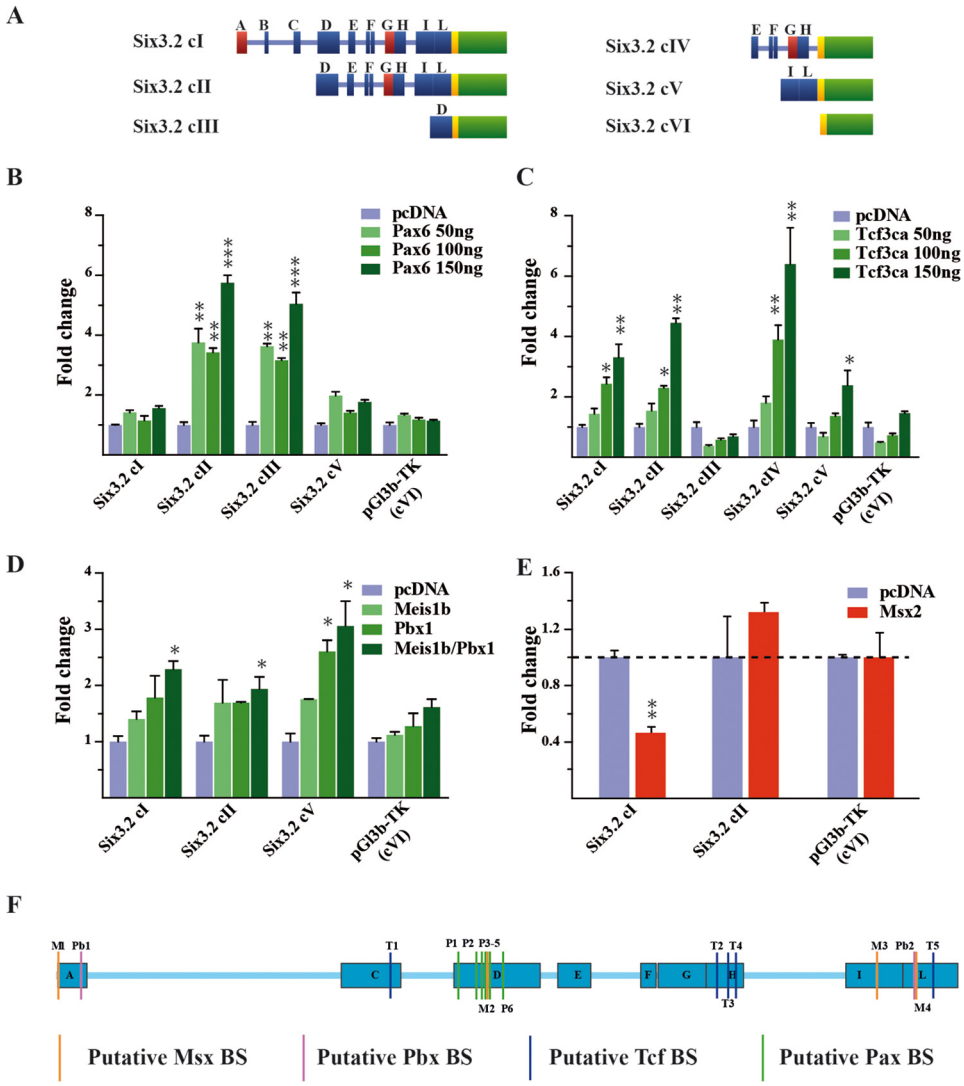

FIGURE 2. **Dose-response Luc assays validate a number of putative *trans*-regulators of the *Six3.2* regulatory region.** *A*, schematic representation of the constructs (*cI–cVI*) used in this study containing different combinations of the *Six3.2* CREs. *Boxes* represent silencers (*red*), enhancers or silencer blockers (*blue*), basal thymidine kinase promoter (*yellow*), and luciferase (*green*). *B–E*, graphs of Luc assays performed with different constructs and expression plasmids at different doses, as indicated in the *panels*. For each construct, data were normalized to control (pcDNA-transfected) values. *Bars*, mean values ± S.E. (*error bars*) normalized to control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$. *F*, schematic representation of the evolutionarily conserved *Pax*, *Tcf*, *Pbx*, and *Msx* BSs present in the 4 kb of the *Six3.2* regulatory region identified using the Jaspar, Matinspector, and/or rVista tools.

effect, enhanced Pbx1-induced reporter activation only modestly (Fig. 2*D*), raising the possibility that additional cofactors might be required for the stabilization of the Meis-Pbx1 complex, as already shown in other contexts (37, 38, 39). Alternatively, other TALE family members may be more suitable cofactors in this context. In a similar way, we observed mild or no cooperation between Sox2 and Pax6 or Otx2 on *Six3.2* regulation (Fig. 3, *F* and *G*), in contrast to reports for other target genes (40, 41). Furthermore, we could not find a previously proposed Six3 autoregulation (25, 42), neither with *Six3.2* nor with its *Six3.1* paralog or their mammalian orthologue (Fig. 3, *A* and *B*), suggesting that in medaka fish, other cofactors might be required or that the *Six3* BS might be located outside of the 4-kb region that we have characterized. Similar reasons or an indirect regulation might explain why Irx3 did not repress the *Six3.2*-cI reporter activity (Fig. 3*B*), despite the described cross-repression between Six3 and Irx3 (43). In support of an indirect regulation, Irx1, which seems to act redundantly with Irx3 (44), was identified in the TRS but failed to regulate the *Six3.2*-full construct in the dose-response Luc assays (supplemental material). Instead, Msx2 significantly reduced reporter expression but only when the A CRE was present (Fig. 2*E*), in good agreement with the observation that the A element silences *Six3.2* expression in the hindbrain and neural tube (23).

In Vivo Expression of Selected Candidates—To further narrow down the number of the predicted candidates, we searched different expression databases and followed only those genes with expression overlapping with or complementary to that of *Six3.2*. The expression pattern of these remaining genes, classified as "functionally related conserved TFs" (Fig. 1*B*), was further compared with that of *Six3.2*, using *in situ* hybridization at early (Fig. 4) and late (Fig. 5) developmental stages. Notably, *Tcf3*, a Wnt signaling effector, *Etv5a*, and to a lower extent *Etv4*, two Fgf signaling effectors, were distributed as *Six3.2* in the prosencephalon with an anterior^{high} to posterior^{low} gradient (Figs. 4 (*A–D*) and 5 (*A–D*)). *Pax6* expression overlapped with

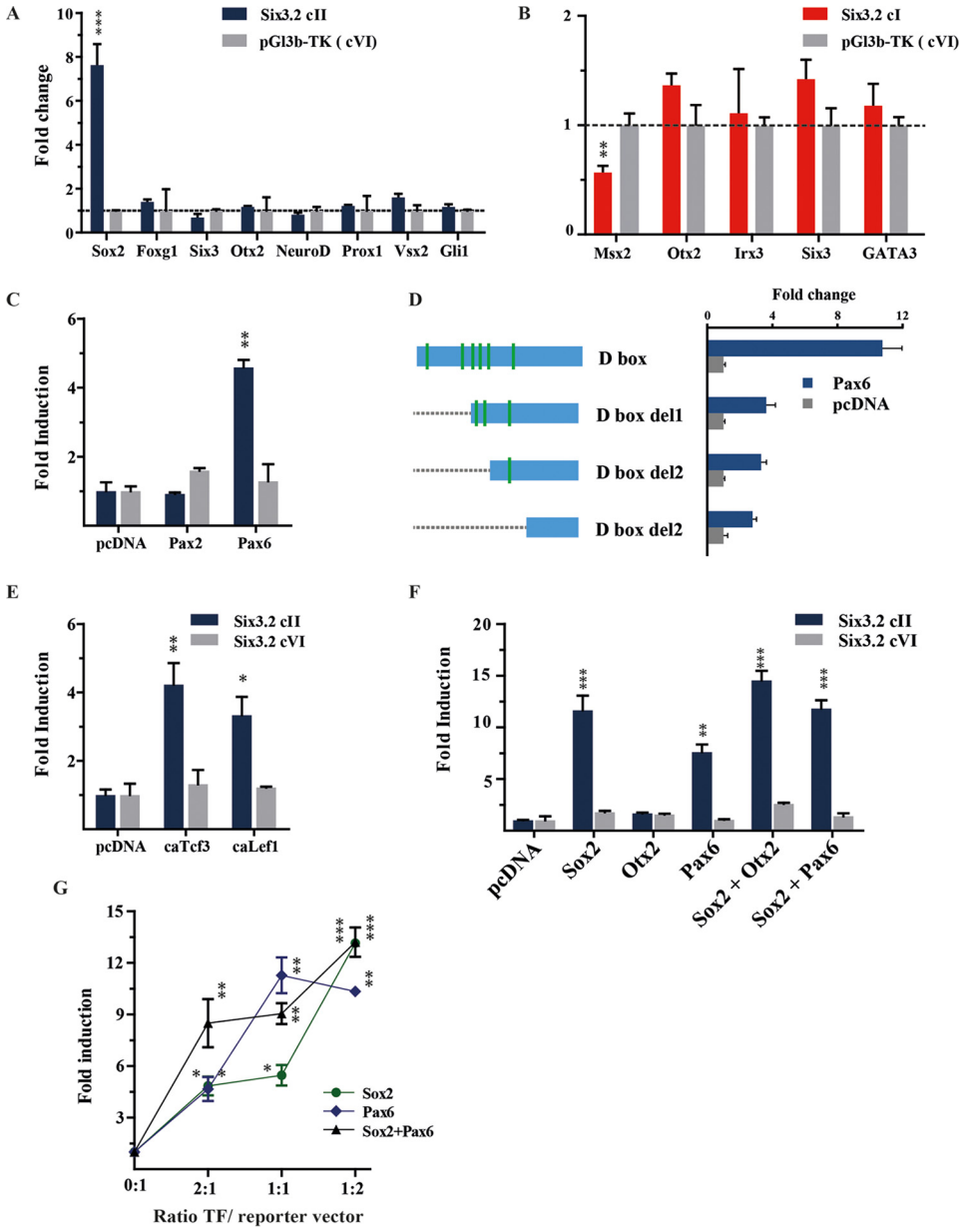

FIGURE 3. **Luc assays of the *in silico* predicted candidate.** *A–F*, graphs of Luc assays performed with the different constructs and expression plasmids at different concentrations, as indicated in the *panels*. For each construct, data are normalized to control (pcDNA-transfected) values. *Bars*, mean values ± S.E. (*error bars*) normalized to control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$.

that of *Six3.2* in the retina, diencephalon, and developing lens but not in the telencephalon, as reported previously (21, 45), whereas *Nkx2.2* coincided with *Six3.2* in the hypothalamus (Fig. 4, *E* and *F*). By contrast, the transcripts of the TFs *Msx2*, *Tgf3b*, and *Pbx1* localized to the caudal neural tube or caudal optic vesicles, thus with a pattern complementary to that of *Six3.2* at early stages (Fig. 4, *A* and *G–I*). Later, *Six3.2* expression still overlapped with that of *Pax6* and *Pbx1* in the diencephalon, lens, and amacrine and ganglion cells of the retina and with that of *Nkx2.2* in the hypothalamus and in the retinal ganglion cell layer (Fig. 5, *E–G*). The TFs *Prdm1/Blimp1* and *Vsx1* are expressed in retinal progenitors and then in photoreceptors and bipolar cells, respectively (Fig. 5, *J* and *K*), thus being likely candidates for *Six3.2 trans*-regulation during retinal neurogenesis.

Tcf3, Msx2, Pax6, and Pbx1 Are Bona Fide trans-Regulators of Six3.2 Expression—Taken together, these data indicate that the majority of the genes validated as *Six3.2 trans*-factors in Luc assays and/or with an evolutionarily conserved BS in at least one of the identified CREs also had a distribution either overlapping or complementary to that of *Six3.2*. To further define their role as *bona fide trans*-regulators, we focused on four of them: *Tcf3*, *Msx2*, *Pax6*, and *Pbx1*.

First, we further validated their binding to the *Six3.2* regulatory regions using ChIP assays in P19 cells co-transfected with the *Six3.2-cI* construct and a plasmid encoding a 3× Myc-tagged version of *Pax6* and *Pbx1* or a 3× HA-tagged version of *Tcf3ca* and *Msx2*. Precipitation with anti-Myc antibodies revealed a specific enrichment of Pax6 on the D regulatory element (Fig. 6*A*) compared with other *Six3.2* CREs or a control

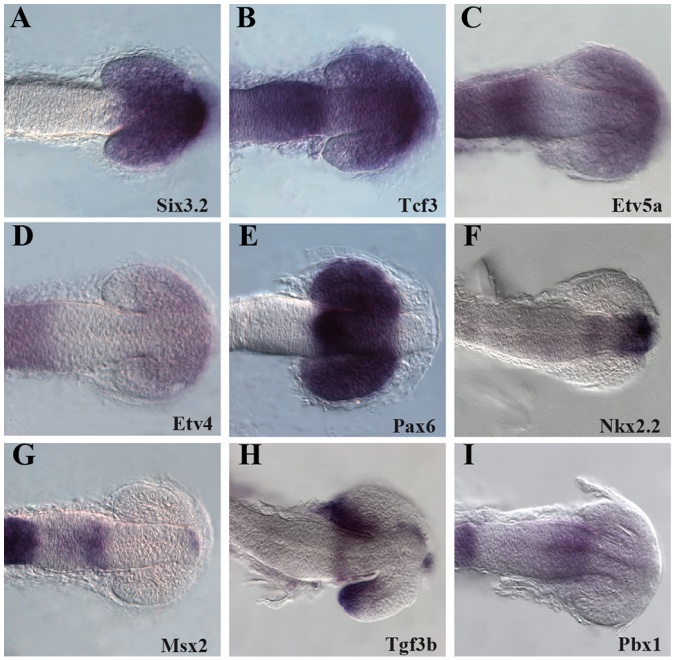

FIGURE 4. **Expression pattern of putative *Six3.2 trans*-regulators at early stages of forebrain development.** Dorsal views of medaka fish embryos at optic vesicle stage (stage 19–20) hybridized *in toto* with probes specific for *Six3.2* (*A*), *Tcf3* (*B*), *Etv5a* (*C*), *Etv4* (*D*), *Pax6* (*E*), *Nkx2.2* (*F*), *Msx2* (*G*), *Tgf3b* (*H*), and *Pbx1* (*I*). Note that *Tcf3*, *Etv5a*, *Etv4*, *Pax6*, and *Nkx2.2* present a distribution partially or completely overlapping with that of *Six3.2*, whereas the expression pattern of *Msx2*, *Tgf3b*, and *Pbx1* is complementary.

region, thus identifying the relevant BSs for the already reported Pax6-mediated regulation of *Six3* expression (20, 46, 47). We also found Pbx1 specifically enriched on the *Six3.2* regulatory region but in this case on the A and IL enhancers (Fig. 6*B*), as predicted by the distribution of its BSs (Fig. 2*F*). The binding of Pbx1 to these CREs supports its potential duality as an early repressor of *Six3.2* expression mediated by the A box and as a late activator by binding to the IL module. This is well in line with the initial complementary distribution of *Pbx1* and *Six3.2* mRNA at early stages of development (Fig. 3*I*) and with their subsequent overlap in the retina and forebrain (Fig. 5, *G* and *G*′). Precipitation with anti-HA antibodies confirmed the binding of Msx2 to the *Six3.2* A CRE, as expected by the presence of *in silico* predicted *Msx* BS within this element. In contrast, we failed to immunoprecipitate any of the *Six3.2* regulatory elements using an HA-tagged version of Tcf3, Tcf3VP16, or Lef, transfected either alone or in combination with a constitutively active version of β-catenin (data not shown). Although different conserved clusters of Tcf/Lef BS were predicted within the H box, none of them presented the reported high affinity Tcf binding sequence, in which the G of the core T(C/A)AAG motif appears to be required for strong binding (48–50). This suggests that Tcf3 might regulate the *Six3.2* promoter either indirectly or through low affinity binding to the H CRE, making it difficult to confirm this interaction by ChIP.

We next asked whether overexpression or interference with the endogenous expression of these four genes indeed impaired *Six3.2* expression, thereby interfering with forebrain development, as reported (21). In agreement with the activation seen in Luc assays, *Pax6* mRNA overexpression (100 ng/μl) increased the levels of *Six3.2* mRNA and of the *Six3.2cI*::*EGFP* reporter in a large proportion of the injected embryos (85%; $n = 250$; Fig. 7*A*). When fused to heterologous TFs, the engrailed (*Eng*) repression domain confers strong transcriptional repression, generating dominant negative (DN) forms of transcriptional activators (51, 52). We thus fused this *Eng* domain to the N terminus of the *Pax6* CDS to generate its DN version. As expected, overexpression of *Pax6-Eng* (100 ng/μl) reduced the expression of *Six3.2* and that of the *Six3.2cI*::*EGFP* reporter in a large proportion of embryos, as compared with control *HA-Eng* (100 ng/μl)-injected embryos (77%; $n = 200$; Fig. 7*A*), which was identical to *Six3.2* expression observed in WT embryos (Fig. 4*A*). The decrease in *Six3.2* expression was particularly evident in the telencephalon and optic vesicles, the latter being also reduced in size.

In contrast to what was observed with *Pax6* mRNA, *Pbx1* overexpression (50 ng/μl) resulted in down-regulation of *Six3.2* in the optic vesicle, thereby reducing their size, whereas *Six3.2* telencephalic expression appeared expanded, a phenotype that was observed in 67% of the injected embryos ($n = 50$; Fig. 7*B*). Notably, the *Pbx1-Eng* version (50 ng/μl) did not induce an opposite phenotype but rather showed a somewhat similar phenotype in a comparable proportion of the injected embryos (72%; $n = 60$; Fig. 7*B*). According to ChIP-quantitative PCR, Pbx1 was enriched on the A CRE (Fig. 6*B*), possibly explaining the reduction of *Six3.2* expression in the optic vesicles. In contrast, the increased but spatially different transcriptional output of the *Six3.2* gene/reporter after *Pbx1* and *Pbx1-Eng* overexpression can be better explained by an indirect effect, such as *Pbx1*-mediated repression of other negative regulators.

Data from different species have demonstrated that Tcf3 antagonizes canonical Wnt signaling activity, thereby enabling forebrain specification in a pathway related to that of *Six3* (11, 13, 53–55). Indeed, the forebrain phenotypes caused by *Tcf3* or *Six3* disruption are similar (13, 54, 55), and *Six3* rescues prosencephalic development in *Tcf3*$^{-/-}$/*headless* zebrafish mutants (11, 13). According to these observations, overexpression of *Tcf3* mRNA (30 ng/μl) in *Six3.2cI*::*EGFP* transgenic medaka embryos (23) enlarged the forebrain domain and induced a 3-fold increase associated with a posterior expansion of both EGFP reporter expression and the endogenous *Six3.2* mRNA distribution in the large majority of the embryos, when compared with RFP-injected controls (95%, $n = 30$; Fig. 7*C*), which, in turn, displayed a pattern identical to that previously reported for WT embryos at similar stages (23). Consistent with the idea that Tcf3 is an activator of *Six3.2*, its DN form (30 ng/μl) reduced the extension of both reporter and endogenous *Six3.2* expression (85%, $n = 40$; Fig. 7*C*), suggesting that at least part of *Tcf3* function in the medaka forebrain is mediated by the direct activation of *Six3.2*. However, in agreement with previous studies (54, 55), we also observed an overall increase of the forebrain size in the *Tcf3-Eng*-injected embryos compared with controls (Fig. 7*C*), probably due to Wnt pathway inhibition with a mechanism independent of *Six3.2*.

Overexpression of *Msx2* mRNA (100 ng/μl) caused a reduction in *Six3.2* and *Six3.2-cI*::*EGFP* expression in a large proportion of the injected embryos, compared with control injected embryos (74%, $n = 30$; Fig. 7*D*) that presented a pattern identi-

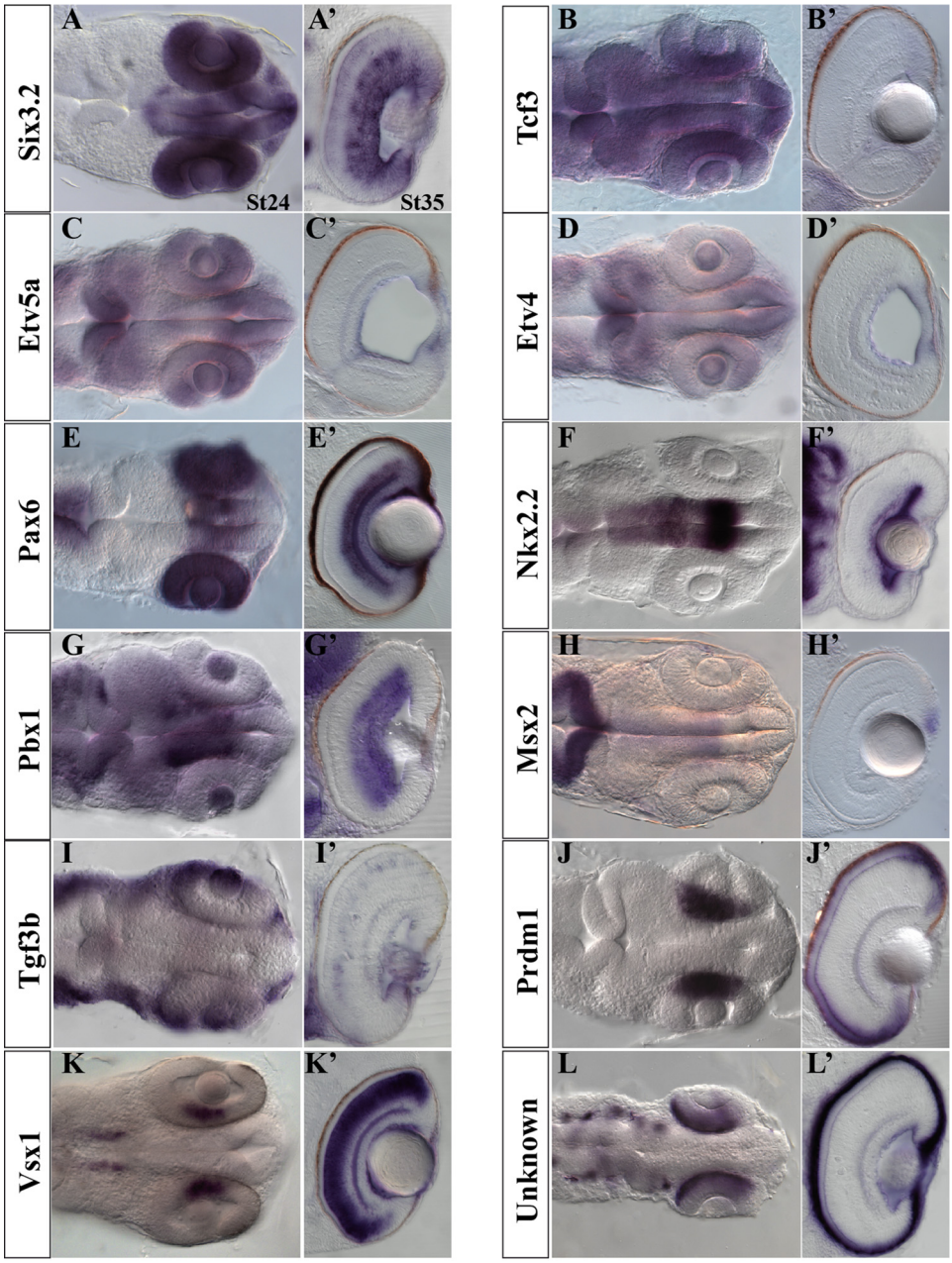

FIGURE 5. **Expression pattern of putative *Six3.2 trans*-regulators at later stages of forebrain development.** Shown are dorsal views of medaka fish embryos at the optic cup stage (stage 23–24) and frontal sections of stage 35 embryos hybridized *in toto* with probes specific for *Six3.2* (*A* and *A'*), *Tcf3* (*B* and *B'*), *Etv5a* (*C* and *C'*), *Etv4* (*D* and *D'*), *Pax6* (*E* and *E'*), *Nkx2.2* (*F* and *F'*), *Pbx1* (*G* and *G'*), *Msx2* (*H* and *H'*), *Tgf3b* (*I* and *I'*), *Prdm1* (*J* and *J'*), *Vsx1* (*K* and *K'*), and an unknown CDS (*L* and *L'*). Note that at this stage *Tcf3*, *Etv5a*, *Etv4*, *Pax6*, *Nkx2.2*, and *Pbx1* present a distribution partially or completely overlapping with that of *Six3.2*, whereas the patterns of *Msx2*, *Tgf3b*, *Prdm1*, and *Vsx1* and that of the unknown CDS are instead complementary.

cal to those of WT embryos at the same stage (21). This finding is in consonance with the repressive effect observed in Luc assays and with the binding of Msx2 to the *Six3.2* A CRE, characterized as a repressor element. *Msx* genes are downstream effectors of BMP signaling, which has been implicated in the repression of anterior neuroectodermal genes (26, 56). In agreement with this idea, overexpression of a chimeric active version of *Msx2*, generated by fusing the VP16 activation domain (57) (*Msx2-VP16*, 100 ng/μl), resulted in an increased expression of *Six3.2* mRNA and of the reporter in the *Six3.2cI*::*EGFP* line, albeit only in a fraction of the injected embryos (24%; $n = 30$; Fig. 7*D*). These findings provide a mechanism by which Msx TFs can limit the size of the neural plate by acting as redundant downstream effectors of BMP signaling during the establishment of the neural/non-neural ectodermal border (58, 59).

Altogether, the results of our study provide converging evidence indicating that Pax6 and Tcf3 are direct activators of *Six3.2* expression, whereas Msx2 limits it at both the neural/non neural border and the posterior neural tube.

Discussion

A hierarchical GRN organization progressively specifies the different structures of the prosencephalon. Central to GRN function are "hub" genes, which critically control the expres-

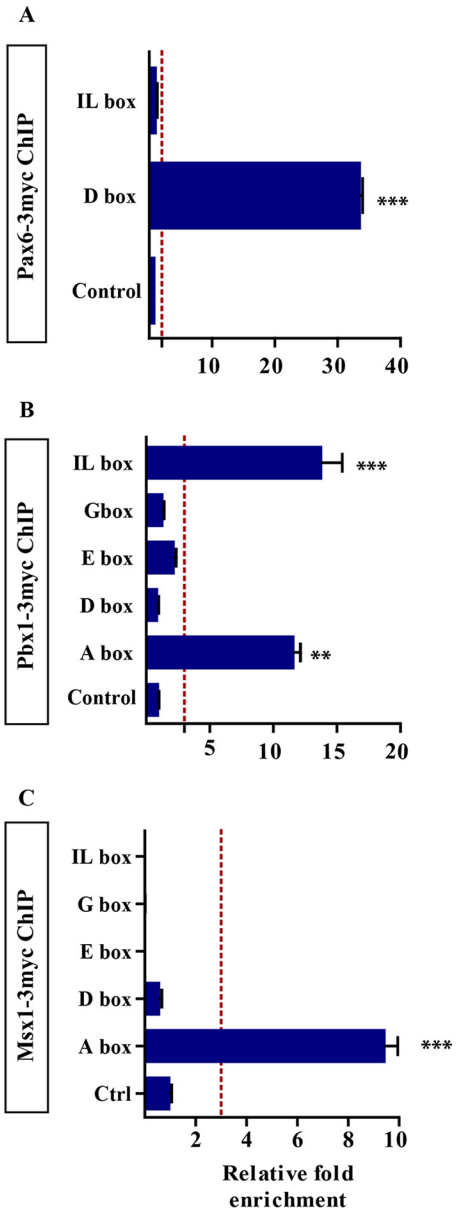

FIGURE 6. **Pax6, Pbx1, and Msx2 directly bind to different CREs of the *Six3.2* regulatory region.** ChIP of the predicted Pax6 (*A*), Pbx1 (*B*), and Msx2 (*C*) target regions was performed with anti-Myc (*A* and *B*) or anti-HA (*C*) antibodies in P19 cells. Histograms show the mean value of a representative experiment performed in triplicate. -Fold enrichments for each tested region were normalized to control IgG and to the CDS control regions. Values >3 (*red dotted line*) were considered positive. **, $p < 0.01$; ***, $p < 0.0001$. *Error bars*. S.E.

sion of many target genes but, in contrast, are regulated by few *trans*-acting factors (1). Intensive research on forebrain patterning led to the outline of a partial GRN operating from neural induction to forebrain patterning (2). This fragmented network contains most of the central actors, but many of the regulatory relationships are still ill defined, hampering our understanding of the molecular mechanisms behind forebrain specification. A case in point is the *Six3* gene. Functional studies had proven that *Six3* is, from the very beginning, essential for forebrain patterning (4, 13, 15, 18, 20, 21, 42, 60), bestowing on *Six3* a hub position within the forebrain GRN (2). Despite this central position, only a few of its direct downstream targets have been identified, and the list of its direct regulators was even shorter. Our study starts filling the latter gap and identifies Msx2 and Pbx1 as *bona fide* regulators of early *Six3.2* expression in distinct domains of the medaka forebrain (Fig. 8). It also confirms the previously proposed direct regulation exerted by Pax6 on *Six3* and points to *Etv5*, *Nkx2.2*, *Prdm1/Blimp1*, *Vsx1*, and especially *Tcf3* as additional regulators of different spatial-temporal domains of *Six3.2* expression.

This information is the result of a previously used TRS (27, 28) that overcame the limitations of and complemented the *in silico* prediction approaches based on the identification of TFBS. As described (27, 28), we took advantage of a preselected subset of the library enriched in developmentally expressed genes, which improved the effort/cost as well as the efficiency of the original screening (27). In fact, there was a 5-fold increase (5.4% *versus* 1.1%) in the proportion of positive *versus* screened clones when we compared our data with those obtained in the *Ath5* TRS (27). As in the latter study, an important proportion of the candidates activating the *Six3.2* regulatory region (23) were classified as TFs, chromatin modifiers, or components of signaling pathways, and almost half of them showed a dose-response behavior in Luc assays. In our study, the identification of *trans*-regulatory factors was further facilitated by the detailed functional characterization in enhancers, silencers, or silencer modulators of the CREs present in the *Six3.2* locus (23). The knowledge that the A element acts as a silencer seems to account, at least in part, for the differences observed, for example, when Pax6 and Tcf3 (this study) or Sox2 (21) were tested on constructs containing different CREs; the presence of the A element counteracted the otherwise activator function of the *Six3.2* regulatory region. Therefore, our approach based on a dual screening and the analysis of transcriptional regulators over different combinations of well characterized CREs seems to provide an efficient method to identify the *trans*-regulatory factors of a given gene.

In support of the efficiency of this approach, the majority of the candidates selected after this combined analysis showed expression patterns either overlapping with or complementary to that of *Six3.2*, which we interpreted as consistent with an activator or repressor role, respectively (Fig. 8, *A* and *B*). For Tcf3, Pax6, Pbx1, and Msx2, these predictions were validated by gain and loss of function assays, which, in the case of Pax6, are also supported by earlier studies (20, 46, 47, 61). The direct binding of these TFs to the predicted CREs was further validated with ChIP experiments. Notwithstanding, a few candidates obtained with the TRS and confirmed as putative activators in dose-response Luc assays, as Hoxb1 and Prdm1, displayed an expression pattern not overlapping with that of Six3.2, hinting instead at a repressor function. This discrepancy may be explained by a differential expression of specific cofactors between the Six3-positive cells of the forebrain and the cell line used in the TRS.

Regulators of Early Six3.2 Expression—In a previous study, we have shown that differential binding of Sox2 to the *Six3.2* regulatory region activates its graded expression along the anterior forebrain (21). There is also evidence that the BMP effector Lmo4 restrains *Six3* expression at the neural/non-neural border (26) and that Msx2, Prox1, Pax6, Six3 itself,

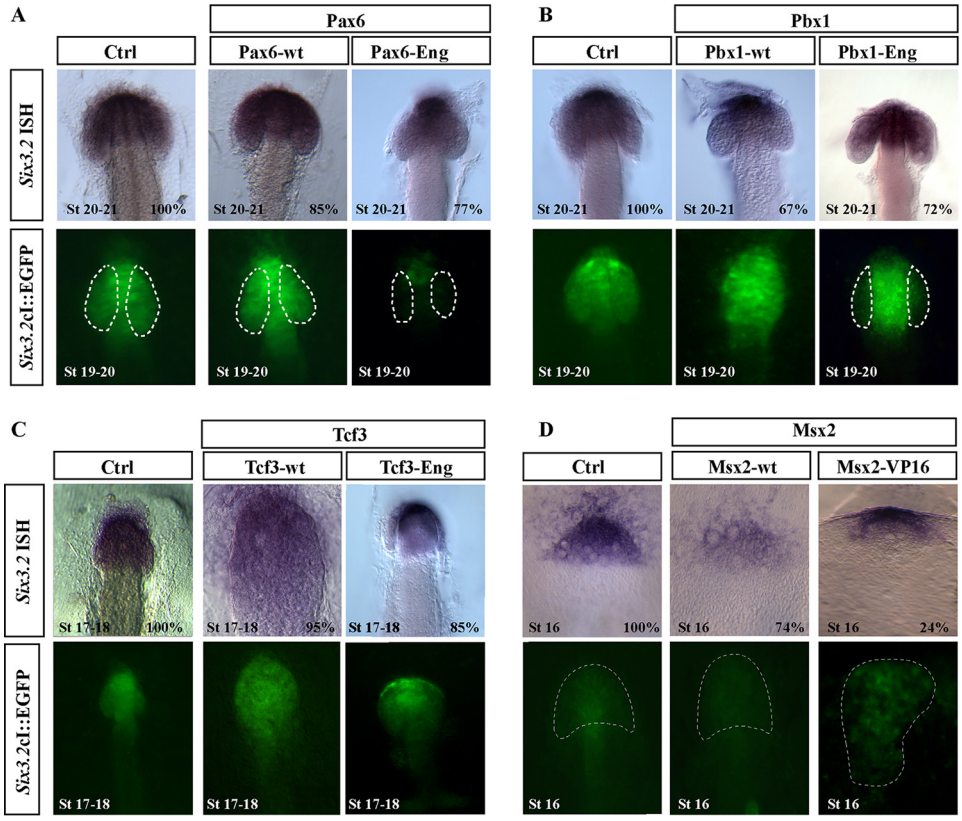

FIGURE 7. **Tcf3, Pax6, Pbx1, and Msx2 control *Six3.2* forebrain expression *in vivo*.** *A–D*, dorsal views of living *Six3.2*::*EGFP* transgenic medaka fish embryos or embryos fixed and hybridized *in toto* with a probe against *Six3.2* at the stages indicated in the *panels*. Embryos were injected with control mRNA (*RFP*, *HA-Eng*, *VP16*) *Tcf3*, *Pbx1*, *Pax6*, and *Msx2* mRNAs or with the mRNA of their respective DN forms obtained by fusing the *Eng* or *VP16* domains, as indicated in the *panels*. Note that *Pax6*, *Pbx1*, and *Tcf3* variably expand reporter expression and the distribution of *Six3.2*, enlarging the anterior neural plate (*A–C*), whereas the DN forms of *Pax6* and *Tcf3* have an opposite effect (*A* and *C*). *Pbx1-Eng* injection has instead a differential effect in the optic vesicle and telencephalon. Note that *Msx2* mRNA reduces *Six3.2* expression, whereas *Msx2-VP16* restores *Six3.2* expression (*D*). Note that *Pbx1-En* expands *Six3.2* telencephalic expression. The percentage of embryos showing the illustrated phenotype after each one of the mRNA injections is indicated at the *bottom right corner* of the respective *in situ* hybridization images.

Tcf3, and the Wnt pathway contribute to define *Six3* activity (53, 55, 62–65). Our study goes beyond these observations and shows that Tcf3, Etv4, Pax6, Pbx1, and Msx2 are most likely direct regulators of early *Six3.2* expression, thus sharpening the GRN proposed for anterior forebrain patterning (Fig. 8, *A* and *C*).

Tcf3 inactivation disrupts prosencephalic development, in part because Tcf3 probably antagonizes the posteriorizing activity of Wnt signaling (53). In the zebrafish *headless*/*Tcf3*$^{-/-}$ mutant, this phenotype is rescued by *Six3* mRNA injection (13). Complementing this notion, we show that overexpression of the *Tcf3ca* form up-regulates endogenous *Six3.2* expression, leading to an enlarged anterior neural plate, whereas a Tcf-DN has the opposite effect. Luc assays indicated that this effect could be direct and mediated by the H CRE, in which conserved Tcf BSs were identified. However, we could not confirm with ChIP assays this direct binding, probably because the clusters of BSs present in the H element do not correspond to high affinity sites (48–50), probably generating unstable interactions. Similarly, our TRS identified the Fgf signaling components Fgf3, Etv5, and the Fgf signaling effector Etv4, as upstream regulators of *Six3.2*, suggesting that the well known Fgf-mediated telencephalic patterning (66–68) may involve *Six3* activation. Notably, the anterior forebrain determinant Foxg1 appears to act downstream of Fgf signaling (68), whereas high *Six3.2* expression levels specify the telencephalic field, in part through direct *Foxg1* activation (21). Therefore, telencephalic development may require the Fgf/Six3.2/Foxg1 hierarchical organization (Fig. 8, *A* and *C*).

Reciprocal cross-regulation between Pax6 and Six3 is important for early eye specification (7, 20, 47, 69, 70). Our study shows that Pax6 directly binds the D element of the *Six3.2* regulatory region, which accounts for its early forebrain expression. Notably, although Pax6 and Sox2 have been reported to synergize in the activation of other regulatory regions (*i.e.* during lens development (40, 71)) they showed additive effects on the transactivation of the *Six3.2* D CRE only at low doses. Considering that in medaka fish, *Sox2* is predominantly expressed in the telencephalon and *Pax6* in the eye, this effect on the D element probably reflects a spatially subdivided regulation of *Six3.2* expression levels, which are critical to specify the relative size of these two domains (21). A similar mechanism could activate *Six3.2* expression also in the lens ectoderm, in which a differential inter-regulation between Sox2 and Pax6 has been proposed (72). A regulatory network among Sox2, Six3.2, Nkx2.2, and Nkx2.1 may instead lead to hypothalamic patterning, as supported by Nkx2.2 transactivation of the *Six3.2* regulatory region and the effects of impairing *Sox2* and *Six3.2* expression on the *Nkx2.1* distribution in this region (21, 22).

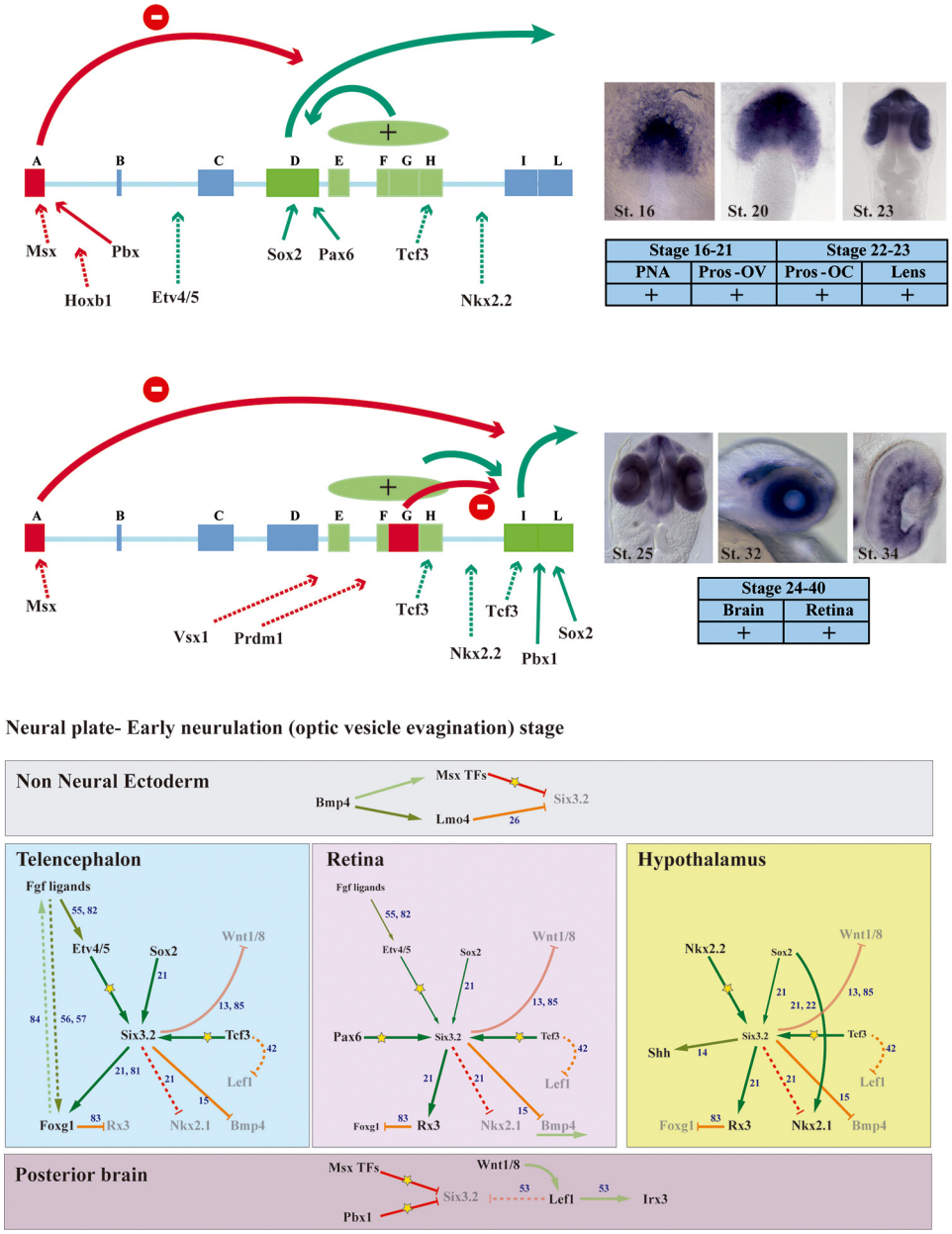

FIGURE 8. **Schematic representation of the transcriptional network in which Six3.2 operates during forebrain specification.** *A* and *B*, schematic representation of CREs controlling *Six3.2* expression at early (*A*) or late (*B*) developmental stages as illustrated in the dorsal and lateral view of the images on the *right*. *Boxes* represent enhancers (*green*) and silencers and silencer blockers (*red*). *Continuous thick arrowed lines* represent positive (*green*) and negative (*red*) interactions among CREs. The positive (*green*) or negative (*red*) activity of *Six3.2 trans*-regulators is represented with *thin continuous arrowed lines*. Indirect or not fully characterized regulatory relationships are represented with *discontinuous lines*. *C*, schematic representation of the GRN controlling early forebrain development. *Green arrows* represent activation, and *red lines* indicate repression. A *continuous line* represents known direct regulations, whereas indirect or unproven direct relationships are indicated with *dashed lines*. The new regulatory inputs identified in this work are marked with a *yellow asterisk*. *Dark green lines* represent direct activation described in medaka, *light green arrows* represent evidence obtained in zebrafish, and the *palest green arrows* represent activating relationships proven in other vertebrate species. Similarly, *dark red*, *orange*, and *pale red* arrows represent inhibitory relationships proven in medaka, zebrafish, or other vertebrate species, respectively. The references reporting these regulatory relationships are indicated with *blue numbers* according to the References.

As mentioned above, BMP signaling limits the expression of *Six3* at the neural/non-neural border of gastrulating vertebrate embryos (26). Accordingly, its downstream effectors *Msx1* and *Msx2* are expressed in the non-neural ectoderm of different species, including the zebrafish (58, 59). Our molecular and functional studies support that the *Msx2a* gene directly represses *Six3.2* expression in gastrulating embryos. Whether *Msx2a* or the related *Msx2b* is actually responsible for this function *in vivo* is unclear, because the *Msx* family is particularly diversified in medaka (six members *versus* the two reported in mammals and birds); thus, other family members may be the relevant regulators, probably acting redundantly, as reported for the regulation of other target genes. Nevertheless, *Msx2a*, localized to the mesencephalon, the hindbrain, and the posterior optic vesicles, is possibly repressing *Six3.2* activity in these domains. The direct binding of Pbx1, which, like Msx2, is distributed in the hindbrain and the caudal neural tube, on the *Six3.2cI* A CRE suggests that this TF also represses *Six3.2* in the caudal neural tube. This

idea is supported by the caudal extension of reporter expression observed in *Six3.2cII*::*EGFP* transgenic embryos, in which the A element is missing (23). However, the complex interactions of Pbx TF with other TALE family members and homeobox TFs (36, 73–75) hampers the identification of the precise transcriptional complex acting in *Six3.2* regulation.

Regulators of Late Six3.2 Expression—The role of *Six3* in forebrain differentiation is still poorly addressed, and most studies have focused on its role during retinal neurogenesis, highlighting its requirement for progenitor proliferation and amacrine and ganglion cell specification in cooperation with Pax6, NeuroD, Math3, Tcf3, and Pbx1 (28, 51, 57, 76–81), among others. The high transactivation activity, the overlapping expression domains, the presence of conserved putative BSs, and the direct binding of Pbx1 to the IL CRE, responsible for late *Six3.2* expression, support the possibility that during neurogenesis, Pbx1 directly promotes *Six3.2* retinal expression (Fig. 6*B*). This function is probably aided by Tcf3, which also transactivates the IL region. It is likely that the *Pbx1/Tcf3/Six3.2* network controls retinal progenitor proliferation because each one of these genes has been separately shown to influence the rate of retinal cell division (80). In this view, Pbx proteins would have an effect on retinal *Six3.2* regulation opposite to that we propose at early stages. However, these two roles are not incompatible, given the aforementioned complexity of TALE protein interactions. It is less clear whether Vsx1 and Prdm1 are functionally related to *Six3.2* activity in the retina, despite their significant transactivation of the *Six3.2cI* construct. The two genes are involved in bipolar *versus* photoreceptor cell specification (82, 83), and their expression is complementary to that of *Six3.2* during neurogenesis. It is thus possible that their identification in the TRS might reflect the presence of sequence similarities among the regulatory elements of *Six3.2* and those of the *Six3.1* paralog and the *Six6* ortholog, which are instead expressed in the inner nuclear layer.[4] In support of this possibility, *Six6* has been implicated in the development of photoreceptor precursors (84).

In conclusion, our study describes a time/cost-effective approach to identify *trans*-acting factors that can be applied to the study of many gene regulatory regions. More relevant, our study shows that Msx2, Pbx1, and Pax6 and probably Tcf3, Etv5, Nkx2.2, Prdm1/Blimp1, and Vsx1 are important regulators of *Six3.2* expression during anterior forebrain development. Mutation of each one of the identified BSs would be a necessary step to further evaluate the relative contribution of each single BS to the spatio-temporal regulated expression of the *Six3.2* gene. Nevertheless, altogether, our data add new elements to the complex GRNs in charge of early forebrain specification, providing new knowledge on how forebrain neuronal diversity is originated.

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A *trans*-Regulatory Code for the Forebrain Expression of *Six3.2* in the Medaka Fish

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