Functions of block of proliferation 1 during anterior development in *Xenopus laevis*

**Corinna Gärtner**, **Annika Meßmer**, **Petra Dietmann**, **Michael Kühl**, **Susanne J. Kühl**

1 Institute of Biochemistry and Molecular Biology, Ulm University, Ulm, Germany, 2 International Graduate School in Molecular Medicine Ulm, Ulm, Germany

☯ These authors contributed equally to this work.

* susanne.kuehl@uni-ulm.de

**Abstract**

Block of proliferation 1 (Bop1) is a nucleolar protein known to be necessary for the assembly of the 60S subunit of ribosomes. Here, we show a specific *bop1* expression in the developing anterior tissue of the South African clawed frog *Xenopus laevis*. Morpholino oligonucleotide-mediated knockdown approaches demonstrated that Bop1 is required for proper development of the cranial cartilage, brain, and the eyes. Furthermore, we show that *bop1* knockdown leads to impaired retinal lamination with disorganized cell layers. Expression of neural crest-, brain-, and eye-specific marker genes was disturbed. Apoptotic and proliferative processes, which are known to be affected during ribosomal biogenesis defects, are not hindered upon *bop1* knockdown. Because early *Xenopus* embryos contain a large store of maternal ribosomes, we considered if Bop1 might have a role independent of *de novo* ribosomal biogenesis. At early embryonic stages, *pax6* expression was strongly reduced in *bop1* morphants and synergy experiments indicate a common signaling pathway of the two molecules, Bop1 and Pax6. Our studies imply a novel function of Bop1 independent of ribosomal biogenesis.

**Introduction**

Before cell division, cells need to grow so that daughter cells are similar in size to their parental cells. Cell growth occurs during the G1 phase of the cell cycle and requires a sufficient number of ribosomes. Ribosomes are involved in the cellular protein synthesis machinery that translates the genetic information from intermediate mRNA into a protein and are therefore crucial for cellular function [1]. Ribosomes are generated mainly in the nucleoli, and this process requires the synthesis of ribosomal proteins for the small and large ribosomal subunits and the transcription and processing of the 47S rRNA precursor (45S in *Xenopus*). The processing of the precursor is a complex step and requires numerous proteins to be organized into different pre-ribosomal multiprotein complexes. Defects in ribosome assembly or function can lead to a group of diseases called ribosomopathies. The best-known ribosomopathies are Diamond Blackfan anemia, Shwachman Diamond syndrome, and Treacher Collins syndrome [2,3].
Block of Proliferation 1 (Bop1) is one of the 200 factors known to be necessary for ribosomal synthesis [4–6]. Bop1 forms a protein complex with WD repeat domain 12 (WDR12) and Pescadillo homologue 1 (Pes1). This complex is called the PeBoW (Pes1-Bop1-WDR12) complex, and its integrity is required for rRNA processing and thus ribosomal biogenesis, as well as for cell proliferation [7,8]. Bound into the PeBoW complex, Bop1 is involved in the maturation of 28S and 5.8S rRNA and is therefore required for the assembly of the 60S subunit of the eukaryotic 80S ribosome [6]. Consistently, in mouse-derived cell lines dominant-negative mutants of Bop1 lead to cell cycle arrest in the G1 phase, resulting in impaired proliferation [9,10]. bop1 knockout also leads to drastically decreased protein synthesis in human cell lines [11].

*Xenopus laevis* oocytes can rely on a large store of $10^{12}$ maternal ribosomes, proteins, and mRNAs [12,13]. Therefore, their early development does not require the zygotic synthesis of new ribosomes, as shown in the anucleolate mutant. In homozygous anucleolate mutants, no rRNA precursors can be synthesized because of the lack of the rRNA gene cluster. Nevertheless, these embryos survive until the swimming tadpole stage [14]. During *Xenopus* embryogenesis, the nucleoli are reformed in the gastrula stage, which is accompanied by rRNA precursor synthesis [15] and ribosomal protein mRNA synthesis, both at low levels [16]. Significant ribosomal protein synthesis subsequently starts at stage 26 [16].

We previously analyzed the function of Pes1, one protein of the PeBoW complex, during early *Xenopus laevis* development, in particular during neural [17] and pronephric [18] development. We also analyzed the function of Peter Pan (Ppan), a Pes1-interacting protein, during *Xenopus* development [18,19]. We found a tissue-specific expression of these two genes, in e.g., the anterior neural plate, developing neural crest cells (NCCs), the eye and the pronephros. Loss-of-function studies indicated a crucial role of these proteins during early neural and pronephros development [17–19]. In neither neural nor pronephric tissues, the early phenotypes could be mimicked by interfering with 45S rRNA precursor processing, indicating a function of these proteins beyond their role in rRNA processing. In further studies, we were able to show that Ppan defines a tumor protein p53 (Tp53) independent of the nucleolar stress response pathway [20].

The above findings raised the interesting question of whether interfering with Bop1 function would also affect early *Xenopus* development. First evidence of a tissue-specific expression of bop1 during early development in *Xenopus laevis* was provided by Neilson and colleagues [21]. To obtain further evidence, we therefore investigated the role of Bop1 during early *Xenopus* development. A detailed expression analysis of bop1 revealed a specific expression during early anterior development, and loss-of-function studies revealed a function of Bop1 during neural development, in particular during eye and cranial cartilage development. This role of Bop1 involves the transcription factor Pax6 (Paired box 6), at least in part.

**Materials and methods**

*Xenopus laevis*

All procedures were approved by the German state administration Baden-Württemberg (Regierungspräsidium Tübingen) and performed according to the German animal use and care law. Embryos of *Xenopus laevis* were generated and cultured as described in previous protocols [22]. Embryos were cultivated in 0.1 x Modified Barth’s saline with HEPES buffer (MBSH) at 12.5 to 20°C, and staging was done according to Nieuwkoop and Faber [23]. Fixation was performed with MEMFA(T) (0.1 M MOPS (pH 7.4), 2mM EGTA, 1 mM MgSO$_4$, 4% formaldehyde, (0.1% Tween20)).
Synteny analysis and protein alignment

Synteny analysis and protein alignment of Bop1 were performed with the NCBI Gene Bank for *Homo sapiens* (NP_056016; Gene ID: 23246), *Danio rerio* (NP_001071203; Gene ID: 777627), and *Mus musculus* (AAB19223; Gene ID: 12181) and the Xenbase platform (xenbase.org) for *Xenopus laevis* (NP_001080358; Gene ID: XB-GENE-6254226 (bop1. L); NP_001079852; Gene ID: XB-GENE-6255479 (bop1. S)). For protein alignments, the software CLC Main Workbench 8 (Qiagen bioinformatics, 2017) was used.

Whole mount *in situ* hybridization

Whole mount in situ hybridization (WMISH) was performed with a digoxygenin-labeled probe generated via *in vitro* transcription with T7, SP6, or T3 RNA polymerase (Roche) against the mRNA of different antisense probes in accordance with previous protocols [24,25]. We cloned the open reading frame of *Xenopus laevis* bop1 into the pSC-B vector (Stratagene) with the cloning primers bop1_l 5’ GACAGGGAAAAACGTGTTTCT 3’ and bop1_r 5’ TTAATGTGAA TATCCGATTGTTGG 3’. *In vitro* transcription with T3 RNA polymerases (Roche) resulted in digoxygenin-labelled antisense RNA probes. We used the following RNA anti-sense probes: celf1 (CUGBP Elav-like family member 1) [26], cryba1 (crystallin beta A1) [26], emx1 (empty spiracles homeobox 1) [27], en2 (engrailed homeobox 2) [28], egr2 (early growth response 2) [27], foxd3 (forkhead box D3) [29], otx2 (orthodenticle homeobox 2) [30], pax6 [31,32], pou4f1 (POU class 4 homeobox 1) [33], prox1 (prospero homeobox 1) [34], rax (retina and anterior neural fold homeobox) [35], sox2 (sex determining region Y-box 2) (sequence ID: LC164013.1), sox3 (sex determining region Y-box 3) [36], vsx1 (visual system homeobox 1) [37], and rho (rhodopsin) [38]. For analyzing the expression of genes upon knockdown of bop1 via WMISH, only the described tissues were evaluated.

Morpholino oligonucleotide specificity and effectiveness test

Morpholino oligonucleotides were designed to the sequence of the bop1 S homeologue. To show the specificity of the morpholino oligonucleotide (MO) binding, we cloned the MO binding sites and the binding sites as they appear on the bop1 L homeologue in front of and in frame with the green fluorescent protein (GFP) gene by following an established protocol [17]. The cloning primers were as follows:

- bop1_MO1_bs_GFP_l, 5’ GATCCACCACGAGGCTGAGTGAAGATGAAG 3’;
- bop1_MO1_bs_GFP_r, 5’ AATCTTCATGGTTCACCTAGGGGTCCTG 3’;
- bop1_MO2_bs_GFP_l, 5’ GATCCAGAGACGGAAAAACGTGTTTGTGCG 3’;
- bop1_MO2_bs_GFP_r, 5’ AATTCGTAGAAACACGTTTTTCCCTGTCTCG 3’;
- bop1_MO1_bs_ChrL_GFP_l, 5’ GATCCCCCGAGTCCTGAGTGAAACATGAAG 3’;
- bop1_MO1_bs_ChrL_GFP_r, 5’ AATCTTCATGGTTCACCTAGGGGTCCTG 3’;
- bop1_MO2_bs_ChrL_GFP_l, 5’ GATCCGAGAGACGGAAAAACGTGTTTGTGCG 3’;
- bop1_MO2_bs_ChrL_GFP_r, 5’ AATTCGTAGAAACACGTTTTTCCCTGTCTCG 3’;
- Δ5’UTRBop1_MO bs_GFP_l, 5’ GATCCAGAGACGGAAAAACGTGTTTGTGCG 3’;
- Δ5’UTRBop1_MO bs_GFP_r, 5’ AATTCGTAGAAACACGTTTTTCCCTGTCTCG 3’.

To test the MO binding specificity, we injected 1 ng of the respective GFP construct bilaterally together with 10 ng of bop1 MO, bop1 MO2, or Control MO into embryos at the two-cell stage and checked for GFP expression with an Olympus MVX10 fluorescence microscope.

Morpholino oligonucleotides, cloning, injection mRNAs, and microinjections

We designed two bop1 MOs that bound to the following sequences: bop1 MO, 5’-CCCAGGCC-CUGAGUGAGACUGA-3’; and bop1 MO2, 5’-GAGACAGGGAAAAACGTGTTTGTGCG-3’.
pax6 MO and tp53 MO were used as previously described [39,40]. All gene-specific and a standard Control MO were obtained from Gene Tools (Philomath, OR, USA). MOs were diluted in water treated with diethyl pyrocarbonate (DEPC) and injected into one of the two animal-dorsal blastomeres of eight-cell embryos to specifically target the anterior tissue. As an injection control, 0.5 ng of GFP RNA was co-injected, and the fluorescence was assessed with a fluorescence microscope (Olympus MVX10, U-RFL-T, Japan). The uninjected side served as an internal control and Control MO injections, as an injection control. If not indicated differently, the following amounts of MO were injected: 15 ng bop1 MO, 15 ng bop1 MO2, 15 ng Control MO, 15 or 30 ng pax6 MO, and 2.5 or 5 ng tp53 MO. For rescue experiments Xenopus Δ5’UTR-bop1 RNA was cloned into the pCS2+ vector (Rupp and Weintraub) using XhoI and XbaI for restriction and the following primers: Δ5’UTRbop1_l: 5’ ATGAAGAGAGGGAGCCAGG 3’ and Δ5’UTRbop1_r: 5’ TTATGTGAAATAATCGGATTGTGG 3’. Generation of injection mRNAs was accomplished by in vitro transcription using T3, T7, or SP6 polymerase. Rescue experiments of Bop1 were performed by injecting bop1 MO along with 0.5 ng of Xenopus Δ5’UTR-bop1 RNA, which is not targeted by the bop1 MO and bop1 MO2, into one animal-dorsal blastomere (Δ5’UTR-bop1 RNA is not targeted by the bop1 MO and bop1 MO2 because of the altered sequence as shown in S2B Fig). bop1 RNA for gain of Bop1 function experiments was cloned into the pCS2+ vector using XhoI and XbaI for restriction and the following primers: bop1_l: 5’ GACAGGGAAAAACGTGTTTCT 3’ and bop1_r: 5’ TTATGTGAAATAATCGGATTGTGG 3’. Gain-of-function experiments were implemented with injections of bop1 RNA ranging from 0.5 to 1 ng. For synergy experiments, 5 ng pax6 MO and 5 ng of bop1 MO were injected unilaterally alone or in combination. Co-injections of bop1 MO and foxd3 RNA [29] were carried out with 50, 250, or 750 pg foxd3 RNA. Co-injection of bop1 MO and c-myc (MYC proto-oncogene) RNA [19] were carried out with 0.5 ng c-myc RNA. Co-injections of bop1 MO together with pax6 RNA were performed with 0.2 ng, 0.5 ng, or 1 ng pax6 RNA. GFP RNA was used to adjust RNA levels for all experiments in which RNA was injected and Control MO was used to adjust MO levels for all experiments in which MOs were injected.

Quantitative real-time PCR

Embryos were bilaterally injected with 20 ng Bop1 MO or 20 ng Control MO at two-cell stage. At stage 13, total RNA was isolated from whole embryos using peqGOLD RNAPure Kit (PEQLAB) according to the manufacturers’ protocols. cDNA synthesis was performed using SuperScript II reverse transcriptase and random primers (Invitrogen). Primers for qPCR were designed using the online tool NCBI primer blast. The following primers were used:

- slc35b1_qPCR_l: 5’ CGCATTTCCAAACAGGCTCC 3’ [41]
- slc35b1_qPCR_r: 5’ CAAGAAGTCCCAAGGCTGC 3’ [41]
- odc_qPCR_l: 5’ TGCAATGCTCAAGGCACTTC 3’ [42]
- odc_qPCR_r: 5’ GCCCATCACAACGTGGTC 3’ [42]
- glyceradehyde-3-phosphate dehydrogenase (gapdh)_qPCR_l: 5’ GCCGTGTATGTGGTGGAATCT 3’ [19]
- sox2_qPCR_r: 5’ AACTCTGCTTGGATGACCTTTGC 3’ [19]
- sox2_qPCR_r: 5’ TGTCATCTTTGGGTCTCCTCC 3’
- sox3_qPCR_l: 5’ GGTCAAGATCGGTTGGAAGC 3’
- sox3_qPCR_r: 5’ GGTGACATCCAGGATGGCA 3’

For quantitative real-time PCR, the QuantiTect SYBR Green PCR Kit (Qiagen) and a BioRad CFX Connect Real Time System was used. gapdh, slc35b1, and odc served as housekeeping genes. Each experiment was measured in triplicates and qPCR products were verified.
via gel electrophoresis. Relative gene expression was calculated using the ΔΔCT method and the three housekeeping genes as previously described [43].

**Knockout using the CRISPR/Cas9 system**

For gene editing experiments using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system, *bop1* gRNA #1 was designed using CRISPRscan (crisprscan.org) [44] targeting exon 8 of the *bop1* S and *bop1* L homeologue with the following CRISPR site: 5’ TGTACCTGTGCCCGCCAG 3’. The gRNA template was synthesized using the oligo extension reaction [45]. Transcription of gRNA was performed using the MEGAscript T7 Transcription kit (Invitrogen) and the miRNAeasy Micro kit (Qiagen) for purification. Cas9 protein (PNA Bio) and gRNA were assembled to the ribonucleoprotein complex by 5 min incubation at 37˚C. 1 ng Cas9 protein was injected together with 300 pg gRNA. For phenotype analysis, embryos were injected at two-cell stage. Cas9 protein alone and injection of 0.5 ng GFP RNA into the second blastomere served as injection control. For sequencing experiments, embryos were injected at one-cell stage and lysed (50 mM Tris [pH 8.8], 1 mM EDTA, 0.5% Tween 20, 200 μg/ml proteinase K) at stage 43. DNA was extracted as previously described [45]. DNA pools of 10 control embryos or 10 *bop1* gRNA #1 injected embryos were combined. The following primer were used for confirming gene editing via direct sequencing:

- *bop1* gRNA #1 6L-l: 5’ GTATGTTCCATCTCACCCTTCTGC 3’
- *bop1* gRNA #1 6L-r: 5’ GTACCAGTGCAGGGAAACAAT 3’
- *bop1* gRNA #1 6S-l: 5’ TCTCTTCCCCTGTTGGCTCCT 3’
- *bop1* gRNA #1 6S-r: 5’ AAGACATGTAGCGGCAGTAA 3’.

**Alcian blue staining**

Cranial cartilage staining was performed according to Gessert et al. [17]. *bop1* MO-injected embryos were fixed at stage 45 in 1x MEMFA for 1 hour and stained overnight at room temperature in a staining solution containing 1% Alcian blue and 0.5% acetic acid diluted in water. To wash the embryos, we used 80% EtOH / 20% acetic acid, changing the solution several times. Bleaching was performed with 30% H2O2. Manual isolation of cartilage was reached with fine tweezers.

**Imaging**

*Xenopus* embryos were imaged with an Olympus MVX10 (fluorescence) or Olympus SZX12 microscope and an Olympus UC50 camera. Sections were imaged with an Olympus BX60 microscope and an Olympus DP70 camera. Images were processed with ImageJ64, Affinity Designer 1.10.4, Adobe Photoshop CS6, and Adobe Illustrator CS5.

**Quantitative tissue measurements**

The head width (distance between midline and the lateral edge of the embryo), the area of the eye, and the coloboma (apex angle in degree) were measured with the software ImageJ64 (Wayne Rasband, NIH). For the analysis of brain size, embryos were fixed at stage 42, the brain was isolated, and pictures were taken. ImageJ was used to measure the brain area. For the analysis of the width of *sox3* expression, embryos were fixed at stage 13 and stained with a *sox3* antisense probe. After imaging, the width of *sox3* expression was measured with ImageJ. To analyze the area of *sox3* and *pax6* expression, *bop1* MO and Control MO-injected embryos were photographed after WMISH. By using ImageJ, the area of expression was selected and
measured. The intensity of GFP expression was analyzed using ImageJ. Here, the mean of the grey values was analyzed at indicated areas and normalized to the control group.

**Histology sections**

A vibrating microtome (Vibratome 1500 Classic, The Vibratome Company) was used to slice 25 μm-thick sections of the *Xenopus* embryos. Before sectioning, the embryos were embedded in blocks containing gelatin and glutaraldehyde.

**Phospho histone 3 staining**

For analysis of cell proliferation, phospho histone 3 (pH3) staining was performed in unilaterally injected embryos at stage 23 that were fixed with MEMFAT for 2 hours at room temperature. First, embryos were washed in 1x PBS for 5 x 10 minutes and then blocked for 1 hour in 1x PBS / 10% Horse Serum before being treated with a rabbit-anti-pH3 antibody (1:100; Millipore, Temecula, CA, USA), diluted in 1x PBS / 10% Horse Serum and incubated overnight at 4°C. The next day, embryos were washed in a solution of 1x PBS / 0.1% Tween-20 for 2 hours, whereby the medium was changed 4 times. For blocking, embryos were incubated in 1x PBST / 20% Horse Serum for 1 hour. Incubation with the secondary antibody was performed for 5 to 6 hours with 1x PBST / 20% Horse Serum / anti-rabbit-IgG-AP (1:1000; Sigma-Aldrich, Munich, Germany). Overnight, embryos were kept in 1x PBS / 0.1% Tween at 4°C. On the last day, embryos were transferred into a 24-well plate after several washing steps with 1x PBS / 0.1% Tween and then incubated with AP buffer for 10 minutes. Staining was performed with 500 μl of BM purple, and fixation, with MEMFAT. 30% H₂O₂ was used to bleach the. Then, pH3-positive cells were counted on both sides of the eye area.

**Terminal deoxynucleotidyl transferase dUTP nick end labeling assay**

Cell apoptosis was detected via terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. TUNEL staining was performed at stage 23 according to established protocols [17,27,46]. After fixation, embryos were bleached in 30% H₂O₂. TUNEL-positive cells were counted on both sides of the embryo.

**Statistics**

Data were analyzed with the software GraphPad Prism 9 (San Diego, CA, USA). P values were calculated with a one-tailed, non-parametric Mann-Whitney rank sum test. For real-time qPCR analysis a paired t-test was performed upon checking for normal distribution using a Shapiro-Wilk test. Error bars represent standard error of the means (SEM). Statistical significance was indicated as follows: *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; and ****, p ≤ 0.0001.

**Results**

**Genomic analysis of bop1**

To investigate the genomic region of *bop1*, we performed an *in silico* synteny analysis in which we compared the genomes of *Homo sapiens*, *Mus musculus*, *Xenopus laevis* (both homoeologues), and *Danio rerio* (S1A Fig). This approach showed that the neighboring genes of *bop1* are highly conserved across species, although some deletions and inversions were observed upstream and downstream of *bop1*. A protein alignment showed that the proteins are similar between the various species, especially at the BOP1 N-terminal (NT) domain and the WD40 repeat (WD 40) domain (S1B–S1D Fig). Taken together, these data suggest a strong conservation of Bop1 across species.
Spatiotemporal expression of bop1 during early Xenopus embryogenesis

To investigate the expression pattern of bop1 in Xenopus laevis embryos, we used WMISH approaches with a bop1-specific antisense RNA probe (Fig 1). bop1 is expressed at the animal pole in very early stages of development (Fig 1A). During gastrulation, bop1 expression was detected in the ectoderm and the invaginating mesoderm surrounding the blastoporus (Fig 1A). At stage 13, bop1 expression was found in the anterior neural plate and in the optic field, where rax and pax6 are expressed as well (Fig 1B and 1H). At stage 17, bop1 expression was located in the neural plate border, where NCCs are induced as shown by comparison with foxd3 expression (Fig 1I). Stage 23 embryos showed bop1 transcripts in the eye anlage and the migrating NCCs (Fig 1B). In later stages, bop1 mRNA was visualized in the developing eye, the brain, the NCCs of the mandibular, hyoid and branchial arches, the blood islands, the otic vesicle, and the tailbud (Fig 1B and 1C). Vibratome sections confirmed these findings (Fig 1D–1G). At stage 30 and 35/36, expression of bop1 was localized in the ciliary marginal zone and the lens, as confirmed by expression of rax and prox1, respectively (Fig 1F and 1J).

bop1 knockdown leads to defects during NCC development

Since bop1 was shown to be specifically expressed in the early anterior tissue (Fig 1), we analyzed the function of Bop1 in Xenopus embryos by loss-of-function experiments with a potent antisense MO-based approach. Since Session and colleagues have shown by RNA sequencing data that both homeologues (S and L) are expressed in the here addressed tissues [47], two different bop1 MOs against Xenopus bop1 mRNA were used; bop1 MO and bop1 MO2, both of which showed a high binding specificity to their binding sites on both bop1 homeologues S and L (S2A–S2D Fig).

To target anterior (neural) tissue, we injected bop1 MO unilaterally into one animal-dorsal blastomere of Xenopus embryos at eight-cell stage [48]. The uninjected side served as an internal control, and injection of a Control MO, as an injection control. To examine the phenotype upon loss of Bop1 function, embryos were analyzed at stage 42. Head width measurements revealed a significant decrease of the head width in bop1 MO-injected embryos; Control MO-injected embryos showed normally developed heads (Fig 2A and 2B). Injection of bop1 MO2 led to an identical phenotype in Xenopus embryos (S3A and S3B Fig). For rescue experiments, we generated a Xenopus bop1 RNA (Δ5'UTR-bop1 RNA) that is not targeted by bop1 MO and bop1 MO2 (S2B and S2C Fig). For the sake of simplicity, Xenopus Δ5'UTR-bop1 RNA is referred to as bop1 RNA in the figures. Co-injecting 15 ng bop1 MO or 15 ng bop1 MO2 together with 0.5 ng Xenopus Δ5'UTR-bop1 RNA led to a rescue of the NCC-derived phenotype and thus demonstrated the specificity of the bop1 MO-induced phenotype (Figs 2B and S3).

We also performed bop1 gain-of-function experiments. Overexpressing bop1 in Xenopus embryos did not result in a smaller head or any other phenotype of the anterior tissue (S4A and S4B Fig).

Since bop1 expression was found in the NCCs (Fig 1) and NCCs migrate into cranial cartilage structures as one derivative [49], we investigated a potential role of Bop1 during NCC development. Hence, we analyzed cranial cartilage development by Alcian blue staining to detect cranial cartilage structures, at stage 45. In line with the finding of smaller head widths, bop1 morphants showed a smaller cranial cartilage upon bop1 knockdown (Fig 2C). Here, especially the Meckel’s cartilage and the tectum anterius were affected.

To analyze the molecular basis of the NCC-derived cranial cartilage phenotype, we first analyzed the expression of the pan-neural marker gene sox3 at stage13. Upon bop1 knockdown, sox3 expression was significantly increased (Fig 2E). The sox3 expression domain was
found to be significantly broader upon Bop1 deficiency, as shown by analysis of the width as well as area of sox3 expression (Fig 2D and 2F–2H). An increase in relative sox3 expression was confirmed by real-time qPCR analysis (Fig 2I). Another pan-neural marker gene, sox2, was unaffected upon bop1 knockdown, which was analyzed by WMISH and real-time qPCR approaches (Fig 2J–2L). Next, we examined the expression of the NCC-specific marker gene foxd3 at stage 16, when NCCs are induced in the neural plate border. foxd3 expression was significantly downregulated upon bop1 MO injection. Control MO-injected embryos showed normally expressed foxd3 (Fig 2M and 2N).

To investigate whether the NCC phenotype solely originates from disturbed foxd3 expression, we performed rescue experiments by co-injecting bop1 MO and foxd3 RNA. The cranial cartilage phenotype was not rescued by co-injecting foxd3 RNA (S4C–S4E Fig).

In conclusion, in *Xenopus laevis* loss of Bop1 function leads to smaller heads and cranial cartilage as well as defects in NCC induction.

**bop1 knockdown affects proper brain development**

Since bop1 was also found to be specifically expressed in the developing brain, we investigated brain development of *Xenopus* upon *bop1* knockdown (Fig 1). Therefore, we dissected brains of bop1 MO- or Control MO-injected embryos at stage 42 and measured the area of the brain. Indeed, brains were significantly smaller on the bop1 MO-injected side, whereas Control MO injection had no effect on brain development (Fig 3A and 3B).

The molecular basis of the brain phenotype was analyzed by WMISH and the well-known brain-specific marker genes pax6 (forebrain and posterior neural tube), en2 (fore- and midbrain), en1 (hindbrain boundary, isthmus), and egr2 (hindbrain). At stage 13, pax6 expression was reduced in the anterior neural tube upon *bop1* knockdown (Fig 3S and 3D). A quantitative analysis confirmed the decrease of *pax6* expression (Fig 3E and 3F). At stage 23, the expression of the analyzed marker genes, *otx2*, *en2*, *egr2*, and *pax6* was reduced in *bop1* MO-injected embryos, whereas Control MO injection did not affect expression of analyzed genes (Fig 3G and 3H).
Taken together, Bop1 function was shown to be required for brain development in *Xenopus laevis* embryos.

**Bop1 is required for proper *Xenopus laevis* eye development**

As shown in Fig 1, *bop1* expression was found in the developing eye of *Xenopus laevis* embryos. Hence, we analyzed the eyes of *bop1* morphants at stage 42, and found eyes to be smaller and...
Fig 3. Bop1 is necessary for brain development in *Xenopus laevis*. A Sides of embryo brains injected with bop1 MO and Control MO and uninjected sides were compared (ventral view). B Statistical evaluation showed that bop1 knockdown led to a significantly smaller area of the brain. C *Xenopus* embryos were injected with either 20 ng bop1 MO or Control MO, and pax6 expression in the anterior neural tube was investigated at stage 13 (black arrow indicates location of reduced gene expression). pax6 expression was decreased upon bop1 knockdown. D Statistical evaluation of data given in C. E Area of pax6 expression was investigated at stage 13 embryos via ImageJ. Expression domain was photographed and injected side was compared to uninjected side. Yellow boxes indicate area in which pax6 expression (red area) was measured. F Statistical analysis confirmed reduction of pax6 expression upon bop1 knockdown. G Brain-specific marker genes were analyzed in stage 23 embryos. Embryos were injected with 20 ng bop1 MO or Control MO. Black arrows indicate location of reduced gene expression. H As shown by statistical evaluation, upon bop1 knockdown expression of otx2, en2, egr2, and pax6 was significantly decreased. Control MO-injected embryos showed normal gene expression. Abbreviations: bop1 MO, block of proliferation 1 morpholino oligonucleotide; CoMO, Control MO; inj., injected side; n, number of independent experiments; N, number of injected and analyzed embryos; n.s., non-significant; uninj., uninjected side. Error bars indicate standard error of the means; *, p ≤ 0.05; **, p ≤ 0.001; ***, p ≤ 0.0001. 

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deformed. The uninjected side and Control MO-injected embryos showed no phenotype (Fig 4A). This phenotype was induced in a MO dose-dependent manner (Fig 4B). Vibratome sections revealed an impaired retinal pigmented epithelium (RPE) upon Bop1 deficiency (Fig 4A). By co-injecting Δ5′UTR-bop1 RNA, the eye defects were rescued (Fig 4A and 4B).

As a quantitative evaluation, we measured the eye area after bop1 and Control MO injections and detected a significant decrease of the eye area in bop1 MO-injected embryos. The phenotype was rescued by co-injecting 0.5 ng Δ5′UTR-bop1 RNA (Fig 4C and 4D). In addition, we measured the angle of the eye fissure and observed a severe coloboma phenotype after bop1 MO injection but a normal phenotype after Control MO injection. Again, co-injection of Δ5′UTR-bop1 RNA resulted in a rescue of the coloboma phenotype (Fig 4E and 4F). Injection of bop1 MO2 led to identical eye phenotypes in Xenopus embryos, which were also rescued by co-injecting Δ5′UTR-bop1 RNA (S3C–S3H Fig).

Since sections of the eye showed an impaired RPE in bop1 morphants, we performed WMISH experiments with well-known retina cell type-specific marker genes [27]. In detail, we used rho for photoreceptor cells, prox1 for horizontal cells, vsx1 for bipolar cells, pax6 for amacrine and ganglion cells, and pou4f1 for ganglion cells. Vibratome sections revealed a massive disruption of the retinal layering upon bop1 knockdown but normal development in the uninjected retinas. In particular, photoreceptor cells formed rosette-like structures (Fig 4G).

bop1 is specifically expressed in the developing lens, hence we investigated lens development upon bop1 knockdown by WMISH with the specific marker genes celf1 for lens fiber cells and cryba1 for lens stem cells. Neither celf1 nor cryba1 expression was downregulated in Bop1-depleted lenses in comparison with the uninjected lenses (Fig 4H).

In addition to the knockdown using MOs, the CRISPR/Cas9 system was used to induce a knockout of bop1. Therefore, the synthesized bop1 gRNA #1 was pre-assembled with the Cas9 protein to form the ribonucleoprotein complex (Fig 5A). The complex was either injected at one-cell stage for further sequencing analysis or at two-cell stage for phenotypic evaluation (Fig 5A).

bop1 gRNA #1 targets exon 8 of the bop1 gene (Fig 5B). The wildtype sequence pool showed the CRISPR target site which is followed by the protospacer adjacent motif (PAM) sequence. The DNA sequence of bop1 CRISPants depicted a heterogeneous sequence starting 4 bases upstream of the PAM sequence indicating a successful gene editing (Fig 5C). bop1 knockout led to smaller eyes and smaller heads on the injected side (Fig 5D and 5E). Measurements of the head width and the eye area confirmed these findings (Fig 5F–5I). In contrast, Cas9 protein alone did not result in eye or head phenotypes (Fig 5D–5I).

Knockdown of bop1 alters the expression of eye marker genes

To analyze the molecular basis of the bop1 MO-induced eye phenotype, WMISH with well-established eye-specific marker genes was performed. First, we used the eye field-specific marker genes rax and pax6 in stage 13 embryos, when the eye field is induced [50,51]. rax expression was not altered upon either bop1 or Control MO injection (Fig 6A and 6B). pax6 expression was significantly decreased in the eye field (Fig 6A and 6B), which was also confirmed by expression area measurements (Fig 6C and 6D). At stage 23, rax, otx2, and pax6 were used to investigate eye cell differentiation [27]. Here, Bop1 suppression led to a decrease of all three marker genes (Fig 6E and 6F). Control MO-injected embryos showed normally expressed marker genes.

In summary, bop1 knockdown led to a severe eye phenotype including microphthalmia, coloboma, and disturbed retinal lamination as well as defects in eye-specific marker gene expression during eye field induction and eye cell differentiation.
Fig 4. *bop1* knockdown impairs proper eye development. A Sides injected with *bop1* MO and Control MO were compared with uninjected sides of embryos. Knockdown of *bop1* led to a severe eye phenotype with underdeveloped or malformed eyes (white arrows). A detailed view of the deformed eyes is depicted (black arrows). Vibratome sections showed a deformed retinal pigmented epithelium (RPE) (orange arrows). Control MO-injected embryos showed no eye phenotype. B Statistical evaluation of data given in A. Embryos with *bop1* suppression developed a significantly smaller eye than Control MO-injected embryos. The phenotype increased in a dose-dependent manner and was rescued by co-injecting 0.5 ng of Δ5'UTR-*bop1* RNA. C The area of the eye was measured (red dotted circles). Control MO- and *bop1* MO-injected sides were compared with uninjected sides. D *bop1* knockdown led to significantly smaller eyes. The size of the eye was rescued by co-injecting Δ5'UTR-*bop1* RNA. E Measurement of the angle of eye fissures (indicated by red angle). F Statistical analysis of the angle of eye fissures showed that *bop1* MO-injected embryos developed colobomas. The coloboma phenotype was rescued by co-injecting *bop1* MO with Δ5'UTR-*bop1* RNA. G Vibratome sections of *bop1* MO-injected embryos showing a severe eye phenotype. Red arrows point to disrupted cell layers of indicated cell types, and black arrows point to disrupted RPE. Specific marker genes for cell layers were used, as described in the main text. Most of the cell types were displaced and disorganized. H Lens-specific cells were unimpaired upon suppression of *Bop1*. Vibratome sections of embryos showing a severe eye phenotype. The lens-specific marker genes *celf1* and *cryba1* were used. Some lens fiber cells and stem cells were mildly displaced, but they showed no reduction in expression. Numbers below the columns indicate the number of embryos showing the depicted phenotypes per number of embryos analyzed. Abbreviations: *bop1* MO, block of proliferation 1 morpholino oligonucleotide; CoMO, Control MO; GCL, ganglion cell layer; inj., injected side; INL, inner nuclear cell layer; le, lens; n, number of independent experiments; N, number of injected and analyzed embryos; ONL, outer nuclear cell layer; RPE, retinal pigmented epithelium; uninj., uninjected side. *bop1* is the Δ5'UTR-*bop1* RNA used for rescues. Error bars indicate standard error of the means; Whiskers in B indicate minimum and maximum. *, p ≤ 0.05; **, p ≤ 0.01; †††, p ≤ 0.001; ††††, p ≤ 0.0001.

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Translation, cell proliferation and Tp53 mediated-apoptosis are not affected by *bop1* knockdown

Bop1 is a factor necessary for ribosomal biogenesis, therefore we investigated whether *bop1* knockdown possibly affects the translational machinery. Therefore, 1 ng of GFP RNA was co-injected with either *bop1* MO or Control MO and GFP intensity was analyzed at stage 15. GFP intensity did not differ between *bop1* morphants and Control MO-injected embryos (Fig 7A and 7B), indicating that—in early *Xenopus* embryos—the translational machinery is not in general affected upon *bop1* knockdown.

Previous studies showed that upon knockdown of ribosomal proteins or factors, proliferative and apoptotic pathways are impaired [17,19,52,53]. Therefore, we investigated the number of proliferative cells via pH3 staining and apoptotic cells using TUNEL staining at stage 23. Neither *bop1* MO nor Control MO injection affected proliferation (Fig 7C and 7D) or apoptosis (Fig 7E and 7F).

In a previous study investigating the ribosomal protein L5 (*rpl5*), we have shown that the expression of *c-myc*, which is an important player during ribosomal biogenesis as it enhances the performance of all three polymerases I-III, is reduced in embryos upon *rpl5* knockdown. Furthermore, the co-injection of *c-myc* RNA partially rescued the eye phenotype which occurred upon *rpl5* MO injection [53]. Additionally, Bellmeyer and colleagues showed that *c-myc* knockdown results in a deformed and smaller cranial cartilage similar to the here observed phenotype [54]. Hence, we investigated whether the co-injection of *bop1* MO together with *c-myc* RNA can rescue the *bop1* MO-induced phenotype. The phenotype was not rescued (Fig 7G and 7H).

In defective ribosomal biogenesis, the number of free ribosomal proteins increases. These free proteins bind to MDM2 (Mouse double minute 2 homolog), a crucial regulator of Tp53, thereby stabilizing Tp53 which results in an activation of apoptotic pathways [55]. Furthermore, several studies showed that craniofacial defects can be rescued upon co-knockdown of Tp53 in mice, frog, and zebrafish [52,56–58]. Therefore, we attempted to rescue the *bop1* MO-induced phenotype at stage 42 by co-injecting *tp53* MO, what failed (Fig 7I and 7J). This finding is in line with the above mentioned TUNEL experiments and indicates that the Bop1 deficiency phenotype is not a result of Tp53 pathway activation.
Conclusively, upon bop1 knockdown, pathways crucial during defective ribosomal biogenesis, e.g., proliferative and apoptotic pathways, are not impaired. This leads to the hypothesis of Bop1—additionally to its role in ribosomal biogenesis—implementing a function independent of ribosomal biogenesis during early Xenopus development.
Bop1 functions together with Pax6

We further investigated Pax 6 since 1) Pax6 is a master control gene for eye development [59] and 2) 
pax6 expression was strongly reduced upon loss of Bop1 function at the early stage 13 (Figs 3 and 6) when 
Xenopus laevis embryos are independent of de novo ribosomal biogenesis due to a maternal supply of ribosomes, proteins, and mRNAs.

Previous studies have shown that Pax6 loss of function leads to a severe eye phenotype in 
Xenopus [40] which we confirmed. 15 ng and 30 ng pax6 MO injected into one animal-dorsal 
blastomere of eight-cell stage embryos led to smaller eyes and heads in more than 50% of the 
embryos similar to the bop1 knockdown phenotype. 30 ng Control MO injection did not 
reduce eye or head size (Fig 8A and 8B).

To analyze a possible rescue mechanism, bop1 MO was injected alone and in combination 
with different amounts of pax6 RNA. A rescue via pax6 RNA co-injection was not possible

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size, and malformed and underdeveloped eyes. Our study also found changes in gene expression upon loss of Bop1 and thus provides first clues about the underlying molecular mechanisms.

In our in-depth expression analysis, we provide the first detailed expression pattern of *bop1*. We showed that *bop1* is expressed in neural tissue such as the NCCs, the brain, and the eyes. Of note, *bop1* expression overlaps with the expression of *rax* and *pax6* in the early eye anlage and with *foxd3* at the neural plate border where the NCCs are induced. The here described detailed expression pattern in *Xenopus* is in accordance with earlier findings from Neilson et al. describing *bop1* expression at stage 15 and 28 in *Xenopus tropicalis* [21]. In developing mice, *bop1* expression was also found in the central nervous system, whole brain, and liver [60]. In human fetal tissue, *BOP1* is highly expressed in the brain [61–63]. In conclusion, the conserved expression of *bop1* suggests a role of *bop1* in early neural development.

Here, we showed that knockdown of *bop1* in the anterior tissue of developing *Xenopus laevis* embryos leads to a smaller head width and cranial cartilage structures, a diminished brain size, and malformed and underdeveloped eyes. Our study also found changes in gene expression upon loss of Bop1 and thus provides first clues about the underlying molecular mechanisms. We detected *bop1* expression in *Xenopus* anterior NCCs, and *bop1* knockdown resulted in a broader expression domain for the pan-neural marker gene *sox3*, which was also confirmed using real-time qPCR. Rogers and colleagues already showed that overexpression of *sox3* leads to a severe phenotype in cranial cartilage structures and disturbed formation and migration of NCCs in *Xenopus laevis* embryos [64]. In contrast, expression of *sox2* was not
affected upon bop1 knockdown, showing a specific affect for sox3. In our experiments, bop1 knockdown also resulted in a reduced foxd3 expression during induction of the neural plate border. Foxd3 has been shown to be required for the epithelial-mesenchymal transition.
Interestingly, BOP1 has been shown to be upregulated in patients with hepatocellular carcinoma as well as gastric cancer and that BOP1 induces EMT, which leads to higher invasiveness and metastasis potential [66,67]. In summary, these data suggest that proper function of Bop1 is required for EMT in general.

The expression of the brain marker genes otx2, en2, and egr2 is reduced upon bop1 knockdown. Besides its role in eye development, Otx2 is also essential for craniofacial development, and mutations in this gene have been associated with brain defects [68,69]. En2 plays an important role in the development of the brain, especially the cerebellum [70], and was found to be associated with infantile autism [71,72]. En2 knockout mice show dysregulation in monoamine systems and defects in forebrain structures [73]. Expression of Egr2 is associated with myelination of the peripheral nervous system and the hindbrain region [74], and mutations in the human EGR2 lead to hereditary myelinopathies [75]. Taken together, the reduction found in marker gene expression in Xenopus are in line with the observed NCC and brain phenotypes upon loss of Bop1 function.

Upon bop1 knockdown, retinal lamination is disturbed and different cell layers are disorganized. In a previous study, we showed that retinal lamination defects can result from inhibited cell adhesion [76], which might also be a possible reason for this phenotype in bop1 MO-injected embryos. Furthermore, we observed a downregulation of rax and otx2 during eye cell differentiation. Earlier studies by others indicated that mutations in rax lead to the problems in eye formation in Xenopus and mice [77]. Additionally, previous studies showed that mutations in human OTX2 and RAX lead to anophthalmia, microphthalmia, and coloboma [77–81], findings that are in line with the eye phenotype upon bop1 knockdown in our study.

Upon bop1 knockdown using the CRISPR/Cas9 system, Xenopus laevis embryos showed eye phenotypes (microphthalmia) and head phenotypes. This is in accordance with our previous results using the antisense-based MO approach.

Our findings are also in line with defects observed upon changes in Bop1 interaction partners. In the developing zebrafish, mutations in Pes1, as part of the PeBoW complex, lead to a smaller brain, eye, gut, and liver and a failed expansion of the pancreas [82,83]. In Xenopus laevis, the knockdown of both pes1 and ppan (interaction partner of Pes1), also interferes with craniofacial cartilage and eye development [17,19]. The knockdown of the ribosomal biogenesis factor nucleolar protein 11 leads to a late craniofacial cartilage malformation [52]. Recently, we also have shown that loss of Rpl5 leads to craniofacial cartilage as well as eye defects [53]. In summary, suppression of these investigated ribosomal biogenesis factors (as shown in earlier studies) results in comparable phenotypes as shown for Bop1 in this study, i.e., a disrupted development of the cartilaginous head structures, the brain, and the eye. Interestingly, although the characteristics of these phenotypes differ somewhat from those found in ribosomopathies, they have a few features in common, including eye abnormalities and craniofacial dysmorphology [3,84–90].

Knockdown of bop1 affects marker gene expression in very early stages of development, i.e., even before exhaustion of the maternal ribosome pool. Xenopus embryos possess a pool of around \(10^{12}\) maternal ribosomes, as well as mRNAs and proteins, which together are sufficient for development until approximately stage 26 [16,19]. Therefore, in their first days of development Xenopus embryos are not dependent on zygotic ribosome biosynthesis. In line with this finding, we showed in previous work that interfering with the processing of the 45S rRNA precursor does not affect early development of Xenopus, in particular during neural and pronephric development [19].

At stage 15, we found GFP expression not to be affected in bop1 morphants, indicating that the translational machinery is not disturbed in general. This strengthens the hypothesis that Bop1 might have an additional, ribosomal independent function as the knockdown of this
A ribosomal factor does not affect the translational machinery and de novo ribosomal biogenesis is less important during early development due to the maternal store which is sufficient until swimming tadpole stage [12].

Analyzing proliferative and apoptotic processes via pH3 and TUNEL staining, we found that bop1 knockdown did neither affect proliferation nor apoptosis. This is not in line with the fact that ribosomes are crucial for cell proliferation. In a previous study, we were able to partially rescue an eye phenotype, which occurred upon knockdown of a ribosomal protein, with c-myc RNA [53]. Hence, we analyzed whether c-myc RNA, as transcriptional enhancer important for proliferation, can rescue the bop1 MO-induced phenotype. The phenotype was not rescued, again hinting towards a ribosomal independent function of Bop1.

In previous studies, Tp53 was found to be activated during pathological processes of ribosomal biogenesis, e.g., during Diamond-Blackfan anemia [91–93]. Wu and colleagues demonstrated that lower BOP1 levels lead to decreased ribosome biogenesis, which results in Tp53-dependent proliferative inhibition, oxidative stress, and apoptosis in a human cell line [94]. This finding is in contrast with our findings because we found that the tp53 knockdown did not rescue the bop1 knockdown-associated phenotype. The divergent results can be explained by the theory that, as explained above, Xenopus embryos rely on maternal ribosomes during early development and that zygotic ribosome biogenesis does not start at a relevant rate until the mid-20 stages at the earliest [16]. Accordingly, as mentioned in the introduction, for the ribosomal factor Ppan we were able to show a Tp53-independent nucleolar stress response pathway [20]. In conclusion, we propose that the early defects observed in Xenopus in our study can probably not be explained by defective ribosomal biogenesis. Therefore, understanding the phenotype upon bop1 knockdown in Xenopus might provide novel insights into the pathogenesis of ribosomopathies.

Our findings of altered gene expression provide an indication of the underlying mechanism. We found that the expression of pax6 was reduced upon bop1 knockdown. Previous studies demonstrated that downregulation of Pax6 leads to smaller or absent eyes in Xenopus and mice [40,95,96], which is in line with our result that pax6 knockdown in anterior tissue leads to a similar phenotype as bop1 knockdown. A rescue using pax6 RNA was not possible. This might be due to the reason that other molecules and pathways, e.g., sox3- or foxd3-related pathways are affected upon bop1 knockdown as well. Nevertheless, by co-injecting bop1 MO and pax6 MO, we showed a synergistic relationship between Bop1 and Pax6. These results strengthen the hypothesis that Bop1 and Pax6 act in the same signaling pathway. Previous studies found that in mice and Xenopus, the overexpression of pax6 leads to several eye defects, including microphthalmia, ectopic eyes, and anophthalmia [97,98]. Also, mutations in human PAX6 lead to numerous eye defects, including aniridia, corneal opacification, and cataract [99]. In conclusion, our results alongside with previous studies by others, indicate that Pax6 expression needs to be finely balanced to ensure proper eye development and that the knockdown of bop1 and the resulting changes in pax6 expression might contribute to the bop1 MO-induced phenotype.

**Conclusion and outlook**

Taken together, we showed that Bop1 is crucial for Xenopus anterior development. The results demonstrated that loss of Bop1 phenotypes closely resembles the clinical manifestations of ribosomopathies. However, the early effect on embryonic development suggests an extra-ribosomal function of Bop1 via a Pax6-mediated mechanism. Because Bop1 is a member of the PeBoW complex and the stability of each protein is interdependent, it would be of high interest to analyze the expression and behavior of the two...
complex partners Pes1 and WDR12 upon bop1 knockdown. How Bop1 regulates gene expression on a molecular level awaits further elucidation.

Supporting information

S1 Fig. Synteny analysis and comparison of block of proliferation 1 protein domains between different species. A Synteny analysis of bop1 in Homo sapiens, Mus musculus, Xenopus laevis L, Xenopus laevis S, and Danio rerio. The genomic region next to bop1 is conserved across the different species. B, C Protein domains of block of proliferation 1 (Bop1). The BOP1 N-terminal (NT) domain is depicted in orange and the WD 40 repeat (WD 40) domain, in beige. D The protein length (number of amino acids), the overall homology (%), the Bop1 NT domain homology (%) and the WD40 domain homology (%) of Homo sapiens, Mus musculus, Xenopus laevis L, Xenopus laevis S, and Danio rerio were compared. Bop1 is highly conserved across species. Abbreviations: aa, amino acid; Bop1, block of proliferation 1.

(TIFF)

S2 Fig. MO specificity and effectiveness test. A Binding sites of bop1 MO (blue) and bop1 MO2 (turquoise) are highlighted in the Xenopus bop1 gene (L and S- form). The start codon is indicated in orange. B Binding sites of bop1 MO (on both homeologues), bop1 MO2 (on both homeologues), and the Δ5'UTR-bop1 construct on Xenopus bop1. Start codon is indicated in orange. Blue letters indicate differences between binding sites of chromosome S and chromosome L. Red letters indicate differences between binding sites of bop1 MO and Δ5'UTR-bop1. C Binding specificity test of bop1 MO. Injection of 10 ng Control MO along with 1 ng bop1 MO bs-GFP or 1 ng bop1 MO bs chr. L-GFP led to GFP translation. Co-injection of 10 ng bop1 MO along with 1 ng bop1 MO bs-GFP or with 1 ng bop1 MO bs chr. L-GFP efficiently blocked GFP expression. However, co-injection of Δ5'UTR-bop1 MO bs-GFP with bop1 MO led to GFP translation. D Binding specificity test of bop1 MO2. Injection of 10 ng Control MO together with 1 ng bop1 MO2 bs-GFP or with 1 ng bop1 MO2 bs chr. L-GFP resulted in GFP translation. In contrast, injection of bop1 MO2 together with either 1 ng bop1 MO2 bs-GFP or 1 ng bop1 MO2 bs chr. L-GFP blocked GFP translation. Numbers below fluorescence photos describe number of fluorescent embryos. Abbreviations: bop1 MO, block of proliferation 1 morpholino oligonucleotide; bs, binding site; CoMO, Control morpholino oligonucleotide; GFP, green fluorescent protein; UTR, untranslated region.

(TIFF)

S3 Fig. Eye and head phenotype upon bop1 MO2 injection. A Comparison of the head width of injected (red line) to un-injected (blue line) sides after bop1 MO2, Control MO, or bop1 MO2 together with 0.5 ng of Δ5'UTR-bop1 RNA injection at stage 42. B Statistical evaluation of data in A. The head size of the bop1 MO2-injected side was significantly reduced compared to the Control MO-injected and un-injected side. Co-injection of 0.5 ng of Δ5'UTR-bop1 RNA rescued the head phenotype. C Knockdown of bop1 by bop1 MO2 led to a severe eye phenotype, with underdeveloped or malformed eyes (black arrows) in stage 42 embryos. This eye phenotype was rescued upon co-injection of 0.5 ng Δ5'UTR-bop1 RNA. D Statistical evaluation of data in C. bop1 MO2, bop1 MO2 + Δ5'UTR-bop1 RNA and Control MO-injected side was compared to un-injected side of embryos. E The area of the eye was measured (red dotted circle). F Statistical analysis showed significantly smaller eyes in embryos injected with bop1 MO2. The phenotype was rescued in embryos injected with bop1 MO2 together with 0.5 ng Δ5'UTR-bop1 RNA. G The angle of eye fissure (red angle) was measured and bop1 MO2, bop1 MO2 + Δ5'UTR-bop1 RNA and Control MO-injected embryos were compared to the un-injected side. H Embryos developed colobomas upon bop1 MO2 injection, whereas co-injection
of 0.5 ng Δ5'UTR-bop1 RNA rescued this coloboma phenotype. Abbreviations: bop1 MO2, block of proliferation 1 morpholino oligonucleotide 2; CoMO, Control MO; inj., injected side; MO, morpholino oligonucleotide; n, number of independent experiments; N, number of injected and analyzed embryos; uninj., un-injected side. bop1 is the Δ5'UTR-bop1 RNA used for rescues. Error bars indicate standard error of the means; Whiskers in D indicate minimum and maximum. **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.

(TIFF)

S4 Fig. Overexpressing bop1 does not affect anterior development in Xenopus laevis. bop1 knockdown-associated phenotype is not rescued by co-injecting foxd3 RNA. A Overexpression of bop1 did not result in a phenotype of anterior neural tissue in stage 42 embryos. bop1 RNA and GFP RNA-injected side was compared to un-injected side of embryos. B Statistical evaluation of data given in A. C Co-injection of bop1 MO and foxd3 RNA did not rescue the cranial cartilage phenotype. bop1 MO, Control MO, and bop1 MO + foxd3 RNA-injected side was compared to un-injected side of stage 45 embryos. Black arrows indicate a smaller cranial cartilage. D Statistical evaluation of data in C. E Alcian blue stained cranial cartilages of stage 45 embryos showed a reduced cartilage upon bop1 MO and bop1 MO + foxd3 RNA injection. Branchial arches (ba), Meckel’s cartilage (mc), tectum anterius (ta) were mostly affected (black arrows). Abbreviations: ba, branchial arches; bop1 MO, block of proliferation 1 morpholino oligonucleotide; CoMO, Control MO; GFP, green fluorescent protein; inj., injected side; mc, Meckel’s cartilage; MO, morpholino oligonucleotide; n, number of independent experiments; N, number of injected and analyzed embryos; n.s., non-significant; ta, tectum anterius; uninj., un-injected side. Error bars indicate standard error of the means; ****, p<0.0001.

(TIFF)

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Ethical approval

All applicable international, national and institutional guidelines for the care and use of animals were followed.

Author Contributions

Conceptualization: Corinna Gärtner, Michael Kühl, Susanne J. Kühl.
Data curation: Corinna Gärtner.
Formal analysis: Corinna Gärtner, Annika Meßmer.
Investigation: Corinna Gärtner, Annika Meßmer, Petra Dietmann.
Project administration: Susanne J. Kühl.
Supervision: Petra Dietmann, Michael Kühl, Susanne J. Kühl.
Validation: Corinna Gärtner, Michael Kühl, Susanne J. Kühl.
Visualization: Corinna Gärtner, Annika Meßmer.
Writing – original draft: Corinna Gärtner, Susanne J. Kühl.
Writing – review & editing: Corinna Gärtner, Annika Meßmer, Michael Kühl.

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