MARCKS–like protein is an initiating molecule in axolotl appendage regeneration

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Identifying key molecules that launch regeneration has been a long-sought goal. Multiple regenerative animals show an initial wound-associated proliferative response that transits into sustained proliferation if a considerable portion of the body part has been removed1–3. In the axolotl, appendage amputation initiates a round of wound-associated cell cycle induction followed by continued proliferation that is dependent on nerve-derived signals4,5. A wound-associated molecule that triggers the initial proliferative response to launch regeneration has remained obscure. Here, using an expression cloning strategy followed by in vivo gain- and loss-of-function assays, we identified axolotl MARCKS-like protein (MLP) as an extracellularly released factor that induces the initial cell cycle response during axolotl appendage regeneration. The identification of a regeneration-initiating molecule opens the possibility of understanding how to elicit regeneration in other animals.

To identify a regeneration-initiating molecule in the salamander Ambystoma mexicanum (axolotl), we aimed to functionally screen6,7 axolotl complementary DNAs using an in vitro salamander myotube cell cycle re-entry assay (Notophthalmus viridescens; newt)8 with the aim of performing in vivo analysis in the axolotl that is convenient for molecular analysis9–11. To establish if axolotl blastema tissue expresses a myotube cell–cycle–entry inducing factor, we injected Xenopus oocytes with messenger RNAs from tail blastema, limb blastema or mature limb and assayed the extracellular media on myotubes (Fig. 1a). Tail or limb blastema mRNAs scored positively, comparable to serum, whereas the mature tissue mRNAs showed little inducing activity. We next screened an arrayed 6-day tail blastema cDNA eukaryotic expression vector library for the activity12. Transfection of DNA representing the entire library as a single pool into HEK293 cells (Fig. 1a, sample WL) yielded cell media that stimulated myotube cell cycle entry (Fig. 1b). This library was fractionated into 12 superpools, which yielded four positive superpools (superpool numbers 6, 9, 10 and 12; Fig. 1b and Extended Data Fig. 1a–f). Sib-selection of superpool 9 through three subfractionation steps resulted in identification of a single clone responsible for the activity (Extended Data Fig. 2a–c).

The positive clone encoded a 224-amino-acid protein containing three conserved domains (myristoylated N terminus, MARCKS homology domain and effector domain) similar to MLP (Extended Data Fig. 3a) showing 74.1%, 68.0% and 80.0% amino acid sequence identity to human MLP (also known as MARCKSL1), including the glycine G2 in the myristoylated domain and two serines in the effector domain (S94 and S105) important for plasma membrane binding (for review see ref. 13). The C-terminal region showed low (14.4%) sequence conservation. Phylogenetic analysis showed that the axolotl sequence clustered with other vertebrate MLPs (Extended Data Fig. 3b).

Previous work in other species has indicated that MLP is an intracellular substrate for protein kinase C (PKC) associated with the plasma membrane, phagocytic vesicles and actin, while phosphorylation by PKC induced dissociation to the cytoplasm (for review see ref. 14). We asked whether axolotl MLP (AxMLP) acted on myotubes as a secreted factor or whether it induced expression of a secreted factor in the HEK293 cells. To determine if AxMLP was extracellularly released, we transfected an expression plasmid encoding an AxMLP–C-terminal fusion with enhanced GFP (eGFP) or the pEGFP-N1 control construct into HEK293 cells (Extended Data Fig. 3c). Increasing levels of GFP fluorescence intensity were observed in AxMlp-eGFP-transfected but not eGFP-N1-transfected culture media (Fig. 1c). The percentage of GFP+ cells and the total cell number in AxMlp-eGFP- and eGFP-N1-transfected samples remained equivalent over time (Extended Data Fig. 3d, e). Time-lapse imaging further showed that AxMlp-eGFP-transfected cells grew similarly to the control cells (Supplementary Videos 1 and 2), indicating that extracellular AxMLP did not derive from dying cells. When comparing expression of AxMLP to zebrafish, Xenopus, mouse, newt and human MLPs in HEK293 cells, we observed that the AxMLP yielded a higher proportion of extracellular protein compared with other species (Fig. 1d). Bioassay of the axolotl versus newt MLP media induced a myotube response corresponding to the amount of protein seen by western blot (Fig. 1e).

To establish necessity, we exposed AxlMPl-containing culture media to a polyclonal antibody against AxMLP that inhibited the myotube response, indicating that extracellular AxMLP is required for the activity (Fig. 1f and Extended Data Fig. 3f). To test sufficiency, we purified AxlMPl–His, which displayed a characteristic high gel mobility (Extended Data Fig. 3g, h; for review see ref. 13). Exposure of myotubes to purified AxMLP in serum-free conditions yielded a robust myotube response with an approximate half-maximal response at 50.5 ng μl–1 (Fig. 1g and Extended Data Fig. 2d–f). We conclude that extracellular AxMLP is sufficient to induce myotube cell cycle re-entry.

To determine the in vivo function of extracellular AxMLP, we first queried whether purified AxMLP protein injected into uninjured axolotl tail (Fig. 2a and 2b) and limb (Extended Data Fig. 4a) was sufficient to induce cell cycle re-entry. We injected 270 ng of AxMLP followed by injection of 5-bromodeoxyuridine (BrdU) at 3 days post-amputation (dpa) (Fig. 2a and Extended Data Fig. 4e). AxMLP-injected tails contained significantly more BrdU-positive cells (18.9 ± 2.59%) than control tails injected with media depleted of AxMLP (flow-through, 3.20 ± 0.863%; PBS, 3.04 ± 1.00%) (Fig. 2b–d). AxMLP injection caused increased BrdU uptake in all counted cell types in limbs and tails except for myocyte enhancer factor 2C (MEF2C)+ muscle nuclei (Fig. 2b–d and Extended Data Fig. 4a–d, F–N). Interestingly, it was recently found that muscle fibres can dedifferentiate during newt limb regeneration, but not in axolotl15. The responsiveness of axolotl Pax7+ satellite cells but not MEF2C+ muscle nuclei to AxMLP corresponds with Pax7+ satellite cells being the main contributors to muscle regeneration in axolotl16.

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As we had used the newt myogenic cell line for the original screen, we asked whether AxMLP could promote in vivo cell cycle entry during muscle dedifferentiation in the newt. AxMLP protein was injected either into uninjured newt limbs or after limb amputation during the muscle dedifferentiation phase (Fig. 3). Injection of AxMLP into uninjured newt tissue was not sufficient to induce a cell cycle response (Fig. 3a). Injection during regeneration, however, resulted in an increased 5-ethynyldeoxyuridine (EdU) uptake in PAX7 + satellite cells as well as dedifferentiating myofibre-derived cells (Fig. 3b, c) 15. These data indicate that AxMLP can also promote cell cycle entry of at least two cell types in newt, including dedifferentiating muscle. The requirement for an additional injury signal to induce cell cycle entry in newt correlates with a higher propensity of axolotl stem cells to cycle in homeostasis compared with their newt counterparts 16,17.

We next asked if AxMLP is important for cell proliferation during axolotl regeneration. Microarray and quantitative reverse transcription polymerase chain reaction (qRT–PCR) detected expression in mature limb and tail tissue followed by upregulation with a peak of expression at 12 to 24 h post-amputation (hpa), returning to basal levels at 2 dpa in the tail and 4 dpa in the limb (Extended Data Fig. 5a, e). These observations are consistent with a role for AxMLP in early events of regeneration. Protein localization using immunofluorescence showed that in uninjured tissue, AxMLP was cytoplasmically localized in epidermis and in tail spinal cord cells, including radial glia and axonal tracts (Extended Data Fig. 5b, f, high-magnification images). At 1 and 6 dpa, expression was maintained in the epidermis and spinal cord (Extended Data Fig. 5c, d, g, h, high-magnification images). However, the protein in the regenerating wound epidermis at 1 dpa was plasma-membrane associated (Extended Data Fig. 5c, g, high-magnification images). Such localization changes have previously been described for MARCKS proteins and are dependent on phosphorylation state 18. In summary, AxMLP protein is
AxMlp/FT (days 1, 3) ENU (days 1–5, daily) Sections (day 5)

Figure 3 | AxMlp induces cell cycle re-entry of muscle-derived cells in the newt limb. a–c. Top, schematic representation of the experimental paradigm testing the effect of AxMlp on PAX7⁺ satellite cell proliferation in uninjured limb (a), on PAX7⁺ satellite cell activation in injured limb (b) or myofibre dedifferentiation after injury (c). a. Bottom, transverse section of uninjured limb injected with purified AxMlp shows no induction of EdU incorporation. Graph showing no difference in either purified AxMlp- or flow-through- (FT) injected uninjured sections of uninjured limb injected with purified AxMlp shows no induction of EdU incorporation. Graph showing no difference in either purified AxMlp- or flow-through- (FT) injected uninjured limbs (right; n = 4: biological replicates). b. Bottom, transverse section of the regenerating limb injected with AxMlp shows increased EdU incorporation of PAX7⁺ cells. Graph showing more EdU⁺/PAX7⁺ satellite cells in AxMlp-injected limbs (right; n = 5: biological replicates; mean ± standard error of the mean (s.e.m.)). c. Bottom, transverse section from myofibre-labelled, regenerating limb injected with AxMlp. Graph showing more EdU⁺/yellow fluorescent protein (YFP)⁺ myofibre progeny in AxMlp-injected blastemas (right; n = 5: biological replicates; mean ± s.e.m.). NS, not significant; *P < 0.05 with Student’s t-test. Scale bars in lower-magnification images, 200 μm; in higher-magnification images, 20 μm. Arrowheads indicate marker⁺/EdU⁺ cells; arrows indicate marker⁺/EdU⁻ cells. DAPI, 4′,6-diamidino-2-phenylindole.

cytoplasmically localized in mature tissue. Upon amputation, mRNA levels rise by at least eightfold. Concomitantly the wound epidermis shows juxtamembrane localization, consistent with its N-terminal myristoylation sequence and suggestive of extra-cellular release. These data suggest that both the level and intracellular localization are critical in the role of AxMlp as a non-autonomous inducer of initial cell cycles. A detailed understanding of the different cytoplasmic pools and their relationship to the extracellular form will require further study.

To test the function of endogenous AxMlp during regeneration, we implemented two different fluorescein isothiocyanate (FITC)-conjugated morpholinos directed against 5’ sequences of the AxMlp mRNA. We validated the effectiveness of the morpholinos in vitro by co-electroporation with plasmid encoding full-length AxMlp or ΔN-terminal-AxMlp. His lacking the target sequence into cultured newt cells (Extended Data Fig. 6a, k). Immunostainings and western blots showed that the morpholinos strongly reduced AxMlp expression but did not affect the expression of ΔN-AxMlp (Extended Data Fig. 6b–j, l–s). No effects were observed with two different negative control morpholinos including a five-mismatch morpholino (Extended Data Fig. 6a, k). Immunostainings and western blots showed that the morpholinos strongly reduced AxMlp expression but did not affect the expression of ΔN-AxMlp (Extended Data Fig. 6b–j, l–s). No effects were observed with two different negative control morpholinos including a five-mismatch morpholino (Extended Data Fig. 6b–j, l–s).

To knockdown AxMlp in vivo, we electroporated AxMlp or control morpholinos into the tail epidermis and spinal cord 3 days before amputation. Reduction of protein levels in electroporated cells was confirmed by immunostaining (Extended Data Fig. 7). To test whether exogenously provided AxMlp protein would rescue the knockdown phenotype, the electroporated tails were injected at 1 dpa with purified...
AxMLP or inactive flow-through. Blastema length was measured at 1, 3, 6, 10 and 14 dpa, and BrdU incorporation was assayed at 3 dpa (Fig. 4a and Extended Data Fig. 8f, i). Incorporation of BrdU in the AxMLp morpholinoo-electroporated samples was significantly reduced (Fig. 4d and Extended Data Fig. 8a-e). Correspondingly, at 6 dpa, the blastema length of the AxMlp-morpholinoo/flow-through-injected tails was 59% smaller than that of the control morpholinoo/flow-through-injected ones (specific/flow-through, 546.7 ± 81.0 μm; control/flow-through, 1,318 ± 206 μm; Fig. 4b, c). In contrast, AxMlp-morpholinoo-electroporated tails injected with purified AxMLP protein showed a 50% rescue in blastema length and 85% rescue of BrdU incorporation (Fig. 4c, d). The partial rescue in blastema length is probably due to the limited amounts of AxMLP provided by a single injection. The specificity of the phenotype was confirmed by implementing the second morpholinoo and the control five-mismatch morpholinoo (Extended Data Fig. 8g-j). These results show that knockdown of AxMLP via morpholinoo results in reduced cell proliferation that can be rescued by provision into the muscle/blastaema tissue of exogenous AxMLP protein.

To corroborate the morpholinoo experiments, we injected the anti-AxMLP blocking antibody into the tail before and during regeneration (Extended Data Fig. 9a), which strongly reduced BrdU incorporation in multiple tissues (Fig. 4e-g and Extended Data Fig. 9b). These results show that in vivo knockdown of AxMLP activity by two methods resulted in reduced cell proliferation during early regeneration. To determine if an excess of AxMLP could accelerate regeneration, we performed multiple injections of AxMLP protein before and during early phases of regeneration (Extended Data Fig. 10a). The oversupply of protein resulted in a larger blastema at 4 dpa (Extended Data Fig. 10b, c).

This work represents the first identification of a molecule, AxMLP, by functional expression cloning and in vivo testing in appendage regeneration and therefore sets an experimental paradigm for future studies. Previous work indicated that spinal cord neural stem cells accelerate their cell cycle kinetics resulting in increased mitoses between 3 and 4 dpa (ref. 19). Our work indicates that AxMLP is a protein showed a 50% rescue in blastema length and 85% rescue of BrdU incorporation (Extended Data Fig. 10b, c).

To determine if an excess of AxMLP could accelerate regeneration, we

Supplementary Information is available in the online version of the paper.

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Author Contributions R.B. performed oocyte injection assay and established expression cloning. T.S. designed and performed expression cloning, in vitro cell assays, biochemical experiments, in vivo axolotl experiments, analysed experiments and data, and wrote the manuscript. E.M.T. conceived of the project, analysed experiments and data, wrote the manuscript and secured funding. H.W. designed and performed in vivo newt experiments, analysed the data and wrote the corresponding parts of the manuscript. A.S. supervised and designed in vivo newt experiments, analysed data, edited the manuscript and secured funding.

Author Information The sequence of AxMLP has been deposited in the NCBI GenBank database under accession number KT367888. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to E.M.T. (elly.tanaka@crt-dresden.de) or T.S. (takui.sugiura@crt-dresden.de).

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**METHODS**

**Animals.** All animal experiments were performed in accordance with the European Community and local ethics committee guidelines. *Xenopus laevis* were purchased (Nasco) and maintained in our animal facility. *Ambystoma mexicanum* (axolotls) were bred and maintained in our facility, where they were kept at 18°C in Dresden tap water and fed daily with artemia or fish pellets. Five-to-six centimetre (snout to tail tip) axolotls were used for all the experiments. Animals were anesthetized for all the surgical process as previously described21. Labelling of connective tissue was achieved by transplanting lateral plate mesoderm from GFP transgenic embryos to normal host embryos as previously described20,22. Briefly, mature oocytes were defolliculated with collagenase (Sigma). Purified mRNA (5.0 ng) was injected into the selected healthy oocytes after the defolliculation. Eight injected oocytes were cultured together in one well of a 96-well plate (Nunc) for 48 h and the supernatants were harvested supernatants were concentrated approximately tenfold with a Vivaspin 10,000 MWCO (Sartorius). These concentrated supernatants were tested on A1 myoblasts for control and the cultures were harvested around OD600 nm 0.6. Superpool plasmids were purified with QIAGEN Plasmid Midi Kit (QIAGEN) according to the manufacturer’s manual. To reconstitute the whole library, 5 μg of each superpool’s plasmids were pooled in one tube before transfection. HEK293FT cells (Invitrogen) were maintained with the standard protocol from Invitrogen.

**Preparation of superpools.** To obtain superpool supernatants, 8.0 × 105 of HEK293 cells were plated on one well of a 6-well plate (Nunc) and 1 μg of each individual superpool plasmids were transfected into HEK293 cells with FuGen 6 (Roche; Extended Data Fig. 1e) according to the manufacturer’s manual. For the first 24 h, the transfected HEK293 cells were kept in the 10% fetal calf serum (FCS) medium. Then the cells were rinsed with FreeStyle 293 expression medium ( Gibco) that is a serum-free medium and cultured in the medium at 72 h after transfection. Individual harvested supernatants were concentrated approximately tenfold with a Vivasis 10,000 MWCO (Sartorius). These concentrated supernatants were tested on A1 myoblasts (Extended Data Fig. 1f). It should be noted that given the injury-specific extracellular activity of AxMLP, we infer that the Xenopus oocyte and HEK293 cell systems are likely to be in ‘wound-epithelium-like’ signalling states that permit at least some extracellular release of AxMLP and that the 6-day regenerating tail blastema cDNA had a sufficient number of AxMPL clones for detection in the expression cloning system. We only detected 1 AxMPL clone among 100,000 clones, and this may reflect the levels of mRNA present at later regeneration time points. Maintenance of A1 myoblasts, myotube differentiation from A1 myoblasts, myotube purification and subsequent myotube assays were performed essentially as previously described20,21. Briefly, concentrated supernatants were transfected into HEK293 cells with the Poly (A) Quick mRNA isolation kit (Stratagene). Xenopus oocyte preparation and microinjection were performed essentially as previously described20,21. Briefly, mature oocytes were defolliculated with collagenase (Sigma). Purified mRNA (5.0 ng) was injected into the selected healthy oocytes after the defolliculation. Eight injected oocytes were cultured together in one well of a 96-well plate (Nunc) for 48 h and the supernatants were harvested supernatants were concentrated approximately tenfold with a Vivaspin 10,000 MWCO (Sartorius). These concentrated supernatants were tested on A1 myoblasts for control and the cultures were harvested around OD600 nm 0.6. Superpool plasmids were purified with QIAGEN Plasmid Midi Kit (QIAGEN) according to the manufacturer’s manual. To reconstitute the whole library, 5 μg of each superpool’s plasmids were pooled in one tube before transfection. HEK293FT cells (Invitrogen) were maintained with the standard protocol from Invitrogen.

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**For the third screen from pool number 212, 384 single clones were arrayed into 24 wells using pin replicators (Genetix) on 96-well plates (SARSTEDT) filling 150 μL LB medium per well (Extended Data Fig. 2b; groups A–D). Individual clones on the 96-well plates were statically cultured until they were saturated and 24 clones were pooled together (Extended Data Fig. 2b; sub-pools A1–D4). Plasmids from each pool were purified with QiAprep spin miniprep kit (QIAGEN). For the fourth screen from A1, 24 clones were individually cultured in LB medium and the plasmids were purified with QiAprep spin miniprep kit (QIAGEN). To confirm plasmid copy number, the AxMLP ORF was amplified by sequencing.

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regenerating tail. These limb and tail blastemas were immunostained as previously described. Briefly, limb blastemas amputated at the wrist level were collected from the level of the shoulder, and tail blastemas amputated essentially as previously described. Purified AxMLP protein was injected into the mature (not regenerating) right lower limbs at the centre between the elbow and the wrist. The total number to the number of all the other specific cell types.

Purified AxMLP, depleted media (flow-through) (see earlier) or PBS as a negative control were showed in Supplementary Table 2 (for oligonucleotides numbers 20, AxMlp was normalized with that of β-actin measured by RT–PCR). β-actin was used as a control. Total RNA was purified with RNeasy Mini or Midi Kit (QIAGEN) according to the manufacturer's manual. cDNA was synthesized from 300 ng of total RNA using SuperScript III First-Strand Synthesis System (Invitrogen) and qRT–PCR was performed with Power SYBR Green Master Mix (Invitrogen) in total volume of 12 μl with the final primer concentration of 300 nM on the LightCycler 480 (Roche). To obtain the values of fold change for each time point, the relative concentration of the PCR products was calculated by the standard curve method. To obtain the standard curves of the limb time course or the tail time course respectively, the dilution series (1/4, 1/16, 1/64, 1/256 and 1/1,024) were made from the mixture of cDNAs that were equally collected from the cDNA samples in all the different time points. These dilution series were used as the template for the PCR and the relative concentrations were calculated by LightCycler 480 Software (Roche) based on the standard curves. The concentration of AxMlp was normalized with that of Rpl4 (large ribosomal protein 4). Primers used for PCR were showed in Supplementary Table 2 (for AxMlp, oligonucleotides numbers 20, 21; for Rpl4, oligonucleotides numbers 22, 23). The raw values of qPCR data are shown in Supplementary Table 1. Protein injection into axolotl tail and limb. The dialysed protein samples: purified AxMLP depleted media (flow-through) (see earlier) or PBS as a negative control were injected into mature (not regenerating) tails with a pressure injector, PV830 Pneumatic Picopump (World Precision Instruments). These protein samples were co-injected with tetramethylrhodamine dextran MW 70,000 (Molecular Probes, final 2.5 mg ml$^{-1}$) as a tracer. A glass capillary (Harvard Apparatus) for the injection was pulled with P-97 Micropipette Puller (Sutter Instrument) and sharpened manually (external tip diameter: 30 μm). The injection efficiency was confirmed based on the intensity of the rhodamine under the fluorescence dissecting microscope (SZX 16, OLYMPUS). No animals were excluded from the analysis. In total, 270 ng of purified AxMLP or equivalent volume of controls were injected into one side of the tail. Injected animals were kept in clean tap water for 3 days at room temperature. The animals were injected intraperitoneally with 30 μl of 2.5 mg ml$^{-1}$ BrdU (Sigma) 4 h before collecting the tails (Fig. 2a). The injected part of the tails was identified by rhodamine-positive myotomes and these tails were fixed, embedded, cryosectioned and immunostained as described earlier. The cells in spinal cord, epidermis and notochord were separately counted based on morphology. The label "Other tissues" in Fig. 2d, contained mainly mesenchymal cells and endothelial cells and was calculated by the subtraction from the total number to the number of all the other specific cell types.

Antibodies and immunohistochemistry. For the preparation of anti-full-length AxMLP polyclonal antibody, a glutathione S-transferase (GST) fusion protein with full-length amino acids of AxMLP was expressed in bacteria and purified by standard methods. The resulting protein was used without further purification. GST fusion protein as an antigen was used to immunize rabbits (Charles River). Anti-serum was affinity purified using mouse-anti-GST protein coupled with full-length AxMLP conjugated to NHS-Sepharose resin (GE Healthcare). To raise C-terminal AxMLP polyclonal antibody, keyhole limpet haemocyanin (KLH)-tagged peptides, PVPEPQVEEEAAP, was used to immunize rabbits and the affinity-purified polyclonal antibody was provided (Eurogentec). Both anti-full-length and anti-C-terminal AxMLP polyclonal antibodies were tested on the cell lysate from AxMLP-transfected HEK293 cells (Extended Data Fig. 3f).

For the in vivo antibody blocking assay, anti-full-length AxMLP polyclonal antibody (see later), anti-GFP polyclonal antibody (MPI-CBG antibody facility) or PBS as a negative control were injected into mature (not regenerating) tail as the first injection (3 h before amputation) and into the blastema as the second injection (2 h after amputation) and as the third injection (1 day post-amputation) (Extended Data Fig. 9a). These samples were co-injected with tetramethylrhodamine dextran MW 70,000 (Molecular Probes; final 2.5 mg ml$^{-1}$) as a tracer. The injection efficiency was confirmed based on the intensity of the rhodamine under the fluorescence dissecting microscope (SZX 16, OLYMPUS). No animals were excluded from the analysis. In each injection 500 ng, then, in total 1.5 μg antibody or equivalent volume of PBS were injected. Injected animals were kept in clean tap water for 3 days at room temperature. The animals were injected intraperitoneally with 30 μl of 2.5 mg ml$^{-1}$ BrdU (Sigma) 4 h before collecting the tails. The injected blastemas were fixed, embedded, cryosectioned and immunostained as described later. For the imaging, the tiled images of the entire cross-section of the tails taken on a Zeiss Observer.Z1 (Zeiss) were then stitched by Axiovision software or Zen 2 (Zeiss). For the quantification at least a total of 1,000 cells per one animal were counted from four different animals in each condition (PBS, anti-GFP antibody or anti-AxMLP injection, respectively), and the marker-positive nuclei (BrdU$^+$, PAX7$^+$, MEF2$^+$ or Hoechst$^+$) on the sections were counted by hand. The cell nuclei were stained with Hoechst 33342 (Sigma, final 0.5 μg ml$^{-1}$). Imaging for the stained sections was performed with Zeiss Observer.Z1 (Zeiss) controlled by Axiovision software or Zen 2 (Zeiss).

For the histagged AxMLP purification, AxMLP-3C-His-pCMV-SPORT6 plasmid was transfected into HEK293 cells and the supernatant was harvested at 72 hpt. Histagged protein in the supernatant was purified in native conditions on a 1 ml HisTrap HP column (GE Healthcare), Purified AxMLP was 1.31 μg ml$^{-1}$. Both concentrated eluate (purified AxMLP) and flow-through fractions were dialysed with Spectra/Por Dialysis Membrane MWCO 6-8000 (Spectrum Laboratories) in AMEM (MEM medium (Gibco) diluted 25% with distilled water) for biological assays. The fractions from the purification were tested by silver staining and western blotting (Extended Data Fig. 3g, h). The washing fraction was concentrated about tenfold to load the same volume as other fractions on 4–20% gradient SDS–PAGE gels (anamed Elektrophorese). Western blotting and silver staining were performed with a standard protocol. Briefly, the fractions were treated with 2× Sample Buffer including dithiothreitol (DTT, Sigma, final 0.2 M) and boiled at 95°C for 10 min. The proteins were blotted onto PROTRAN nitrocellulose membrane (Whatman) by TE 77 Semi-Dry Transfer Unit (Amersham). The membrane was blocked with 5% skim milk. Primary antibodies used: mouse anti-His (QIAGEN, 1/5,000), mouse anti-β tubulin (MPI-CBG, anti-β tubulin antibody facility, DM1A 1/5,000), rabbit anti-AxMLP-full length (1/2,500), rabbit anti-AxMLP-C-terminus (1/2,500). Secondary antibodies used: goat anti-mouse Alexa Fluor (AF) 647 (Molecular Probes), goat anti-mouse AF 647 (Jackson ImmunoResearch Laboratories), donkey anti-rabbit AF 647 (Molecular Probes), donkey anti-rat AF 488 (Molecular Probes). The cell nuclei were stained with Hoechst 33342 (Sigma, final 0.5 μg ml$^{-1}$). Imaging for the stained sections was performed with Zeiss Observer.Z1 (Zeiss) controlled by Axiovision software or Zen 2 (Zeiss).

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the limbs. The animals were injected intraperitoneally with 30 µl of 2.5 mg ml−1 BrdU (Sigma) 12 h before collecting the limbs (Extended Data Fig. 4e). For the quantification, at least a total of 1,000 cells per one animal were counted from four different animals in each condition (PBS, flow-through or purified AxMLP injection, respectively), and the marker-positive nuclei (BrdU+, PAX7+, MEF2+, MBP+ or Hoechst+) on the sections were counted by hand. The cells in epidermis and bone/periosteum were separately counted with their morphology. The label “Other tissues”, contained mainly mesenchymal cells and endothelial cells, and was calculated by the subtraction from the total number to the number of all the other specific cell types.

For the acceleration experiment, purified AxMLP flow-through or PBS as a negative control were injected into mature (not regenerating) tail as the first (3 days before amputation) injection and as the second (1 day before amputation) injection and injected into the blastema as the third (2 days post-amputation) injection (Extended Data Fig. 10a). These samples were co-injected with tetramethylrhodamine dextran MW 70,000 (Molecular Probes, final 2.5 mg ml−1) as a tracer. The injection efficiency was confirmed based on the intensity of the rhodamine under the fluorescence dissecting microscope (SXZ 16, OLYMPUS). No animals were excluded from the analysis. The samples were injected into both side of the tail and in each injection, 600 ng, then, in total 1.8 µg protein or equivalent amount of controls were injected. Injected animals were kept in clean tap water for 4 days at room temperature. The length of the blastema was measured from the amputation plane to the tip at the spinal cord level at 4 dpa based on the stereoscope images (SXZ 16, OLYMPUS).

**Morpholino electroporation.** In vitro assay. A1 myoblasts were transfected with original clone BL21a101, AxMLP-3C-His, ΔN-AxMLP-3C-His or empty pCMV-SPORT6-3C-His plasmids and co-transfected with AxMLP-specific morpholinos (Gene Tools; Supplementary Table 2: oligonucleotides numbers 24, 26, 27) or control morpholinos (Gene Tools; Supplementary Table 2: oligonucleotides numbers 25, 27) using Microporator (Digital Bio) according to the manufacturer’s manual with some modifications. All morpholinos were modified with FITC at the 3′ end. A1 myoblasts were re-suspended in 1 × Steinberg solution at a density of 5.0 × 10^6 cells per ml following incubation of 10 µl cell suspension with 0.5 µg of plasmid and 1 µl of the morpholino (final 100 µM in the incubation). Electroporation was performed at 1,000 V, 35 ms pulse length and 3 pulses and the electroporated cells were spread in 10% FCS AMEM media on a 24-well plate (Nunc), immediately after the electroporation. The culture medium was replaced by new media at 24 h post-electroporation and the cells were kept in culture at 72 h post-electroporation. The electroporated cells were fixed with 1.5% PFA/PBS, and the cells lysates were prepared for western blotting. Primary antibodies used for immunostaining: mouse anti-His (QiAGEN, 1:200), mouse anti-FITC (Jackson ImmunoResearch Laboratories, 1:400), rabbit anti-FITC (Invitrogen, 1:400), rabbit anti-AxMLP-full length (1/1,000). Secondary antibodies used for immunostaining (all in 1/250 dilution): goat anti-mouse Cy3 (Jackson ImmunoResearch Laboratories), goat anti-mouse Af488 (Jackson ImmunoResearch Laboratories), donkey anti-rabbit Af488 (Molecular Probes), goat anti-rabbit Cy3 (Jackson ImmunoResearch Laboratories). Images of the stained cells were taken with Zeiss Observer.Z1 (Zeiss) controlled by Axiovision software (Zeiss).

In vivo assay with rescue protein injection. Electroporation to the spinal cord was performed as previously described with some modifications. To deliver morpholino into the spinal cord and immediately electroporated (first electroporation) the tail was performed as previously described with some modifications. All morpholinos were modified with FITC at the 3′ end. For the spinal cord and one side (left) of the epidermis, the tail required electroporation twice with NEPA 21 electroporator (Nepa Gene). The first electroporation was for the spinal cord and one side (left) of the epidermis, and the second electroporation was for the other side (right) of the epidermis. 1.5 µl of morpholino (1.0 mM) was loaded onto a small piece of tissue paper on the left side of the epidermis. Approximately 3.5 µl of morpholino (1.0 mM) was injected into the spinal cord and immediately electroporated (first electroporation). Sequentially, 1.5 µl of morpholino (1.0 mM) was loaded onto a small piece of tissue paper on the right side of the epidermis and electroporated (second electroporation). The first electroporation conditions: poring pulse: 70 V, 5.0 ms pulse length and 1 pulse; transfer pulse, 55 V, 55 ms pulse length, 5 pulses and 15% decay. The second electroporation conditions: poring pulse, 70 V, 10 ms pulse length and 1 pulse; transfer pulse, 30 V, 30 ms pulse length, 7 pulses and 5% decay. FITC dextran MW 70,000 (Molecular Probes, final 5 mg ml−1) was used as a negative control, since morpholinos were labelled with FITC. The electroporation efficiency in the spinal cord and epidermis was examined based on the intensity of the FITC under the fluorescence dissecting microscope (SXZ 16, OLYMPUS). The animals with low FITC intensity were excluded from the next step of the experiments. Three days post-electroporation, the tails were amputated at the level of the maximal morpholino electroporated part. One day post-amputation, a total of 360 ng (180 ng for the spinal cord and 180 ng for blastema) of purified AxMLP or equivalent volume of control flow-through fraction was injected into the spinal cord and the blastema to rescue the morpholino effect. The length of the blastema was measured from the amputation plane to the tip at the spinal cord level on 1, 3, 6, 10 and 14 dpa based on the stereoscope images (SXZ 16, OLYMPUS). To detect BrdU incorporation, the animals were injected intraperitoneally with 30 µl of 2.5 mg ml−1 BrdU (Sigma) 4 h before collecting the tails at 3 dpa. Fixation, embedding, cryosection, staining and imaging were described earlier. For the quantification, 3 cross-sections of the blastema (200–300 µm posterior to the amputation plane) were cut from different animals in each condition (FITC, purified AxMLP, control morpholino/flow-through, control morpholino/purified AxMLP, AxMLP-specific morpholino/flow-through, AxMLP-specific morpholino/purified AxMLP, respectively) were taken, and the marker-positive nuclei (BrdU+, PAX7+, MEF2+ or Hoechst+) on the sections were counted by hand.

**Newt experiments.** Animals. Red-spotted newts, Notophthalmus viridescens, were supplied by Charles D. Sullivan Co. Animals were anaesthetized in 0.1% ethyl 3-amino benzoate methanesulfonate (Sigma) for 15 min. Forelimbs were amputated above the elbow, and the bone and soft tissue were trimmed to produce a flat amputation surface. Animals were left to recover overnight in an aqueous solution of 0.5% sultamerazine (Sigma). At specified time points, the uninjured or regenerating limbs were collected. All surgical procedures were performed according to the European Community and local ethics committee guidelines.

**Protein injection and cell cycle assays in newt limbs.** The general condition in the newt experiments: 2 µl of 5 mg ml−1 purified AxMLP protein or equivalent volume of flow-through (AxMLP depleted fraction) was injected into the newt limbs. For EdU labelling, animals were injected intraperitoneally with 50–100 µl of 1 mg ml−1 EdU. To investigate the effect of AxMLP on intact newt limbs, purified AxMLP or flow-through was injected into the uninjured limb twice at day 1 and day 3. EdU was administered daily from day 1 to day 5 (Fig. 3a, top). To investigate the effect of AxMLP on regenerating limbs, purified AxMLP or flow-through was injected into the regenerating limbs at 7 and 10 dpa (Fig. 3b, top). EdU was administered daily from 8 to 13 dpa. For labelling myofibre progeny, a H2B-YFP reporter construct was introduced into myofibres before amputation as previously described (Fig. 3c, top). Cell cycle re-entry was quantified by EdU incorporation in the YFP+ myofibre progeny at 13 dpa.

**Immunohistochemistry.** Frozen sections (5–10 µm) were thawed at room temperature and fixed in 4% formaldehyde for 5 min. Sections were blocked with 5% donkey serum and 0.1% Triton-X for 30 min at room temperature. Sections were incubated with anti-GFP (Abcam 6673), anti-PAX7 (DShB) or anti-MHC (DShB) overnight at 4°C and with secondary antibodies for 1 h at room temperature. Antibodies were diluted in blocking buffer and sections were mounted in mounting medium (DakoCytomation) containing 5 µg ml−1 DAPI (Sigma). EdU detection was performed as previously described. An LSM 700 Meta laser microscope with LSM 6.0 Image Browser software (Carl Zeiss) was used for confocal analyses. One in every eight sections was selected and labelled. For PAX7+ satellite cell counting, three sections were randomly selected and counted. For blastema YFP+ cell counting, all the sections in the region from regenerate tip to the bone were counted.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software). Student’s t-test, parametric, two-tail testing was applied to populations to determine the P values indicated in the figures. Significance was considered to have been reached at P values from < 0.05. No statistical methods were used to predetermine sample size. In vivo axolotl experiments were not randomised and no blind tests were applied.
Extended Data Figure 1 | Schematic illustration of the expression cloning approach. a, 110,592 clones from a 6-day tail blastema library were arrayed on 288 x 384-well plates. b, One 384-well plate was pooled into one conical tube and called a 'pool.' In total, 288 pools were prepared from the library. c, Twenty-four pools were combined in one conical tube and called a 'superpool' (SP) containing 9,216 clones. In total, 12 superpools were prepared. d, Bacteria of each superpool was cultured and plasmid was prepared. e, The superpool plasmids were transfected into HEK293 cells. f, Individual supernatants were tested on A1 myotubes for cell cycle re-entry activity (myotube assay) (see Fig. 1b). Positive superpools were successively subfractionated and the assay process was repeated back from the positive superpool (first screen) to come to a single clone (fourth screen) (a–c, right) (see Extended Data Fig. 2a–c).
Extended Data Figure 2 | Expression cloning of AxMLP as a myotube cell cycle inducer. a, The results of the second-round screen of superpool 9 (see Fig. 1b and Extended Data Fig. 1) and its sub-pooling diagram (right). Sub-pool D and sub-pool 2 showed higher BrdU incorporation activity than the others, identifying pool number 212 as positive (n = 12: 4 biological, 3 technical replicates each; mean ± s.d.). b, The result of the third-round screen of pool number 212 from superpool 9 and its sub-pooling diagram (right). Sub-pool A1 showed activity (n = 6: 2 biological, 3 technical replicates each; mean ± s.d.). c, Fourth-round screen of SP9 identified a single active clone (c1), AxMlp (n = 12: 4 biological, 3 technical replicates each; mean ± s.d.). The pooling diagram is shown on the right side. d, AxMLP supernatant induces an S-phase response in a dose-dependent manner in the newt myotube assay. Different amounts of AxMLP-containing supernatant (30 μl, 20 μl, 10 μl and 5.0 μl, respectively) were provided to the myotube cell culture medium. The myotube BrdU incorporation correlated with the amount of supernatant provided, whereas pCMV-SPORT6 supernatant did not provoke cell cycle entry at any dose (n = 6: 2 biological, 3 technical replicates each; mean ± s.d.). e, f, Newt myotubes treated with purified AxMLP (e) or flow-through (f) were immunostained for BrdU and MHC. More BrdU-incorporated nuclei (red) in myotubes (green) were observed in culture supplied with purified AxMLP compared with flow-through-treated cultures. Scale bar, 1 mm.
Extended Data Figure 3 | AxMLP is classified as a member of the MARCKS family and characterization of its extracellular release in HEK293 cells. a, Amino-acid sequence alignment of AxMLP with sequences from other vertebrates, human, mouse, rat, chick, newt, Xenopus and zebrafish. AxMLP contains three conserved domains: (1) myristoylated N terminus domain; (2) MARCKS homology domain; and (3) effector domain. b, A phylogenetic tree of vertebrate MARCKS family proteins. The tree was constructed by the neighbour-joining method with the ClustalW program. The percentage beside the nodes shows that a node was supported in 1,000 bootstrap pseudo replications. The scale bar indicates evolutionary distance. c, Schematic illustration of His-tagged AxMlp (left) and eGFP-fused AxMlp (right). 3C protease PreScission site was inserted between AxMlp and the tag for both constructs. d, e, AxMLP does not induce significant cell death. The percentage of GFP-expressing HEK293 cells (d) and absolute number of the cells (e) (n = 16; 4 biological, 4 technical replicates each; mean ± s.d.; centre values as median; whiskers as maximum and minimum, respectively) at the indicated time points of culture. There was no significant difference with Student’s t-test between AxMlp-transfected cells and the control in any time points. f, Characterization of anti-AxMLP antibodies by western blot. Cell lysates from HEK293 cells transfected with the indicated plasmids were tested for the full-length AxMLP polyclonal antibody (left) and C-terminal AxMLP polyclonal antibody (right). g, Silver staining of the fractions from AxMLP–His purification. Bovine serum albumin (BSA) was added to purified fraction as a carrier protein. h, AxMLP–His purification analysed by anti-His-tag western blotting. NS, not significant. © 2016 Macmillan Publishers Limited. All rights reserved
Extended Data Figure 4 | AxMLP is sufficient to induce cell cycle entry in axolotl tail and limb. a–d, Sections from AxMLP-injected tails immunostained for BrdU/PAX7 (a, b) and BrdU/MEF2C (c, d) (refers to data in Fig. 2). Scale bars, 100 μm. e, Schematic illustration of the protein injection into axolotl limb. f, Quantification of BrdU+ cells in the limbs injected with PBS, flow-through or purified AxMLP (n = 4: biological replicates; centre values as median; points represent each sample). g–n, Transverse sections from purified AxMLP-injected (g–j) or flow-through-injected limbs (k–n). Scale bars: lower-magnification images, 200 μm; higher-magnification images, 50 μm. Sections were immunostained for BrdU (g, k), BrdU/myelin basic protein (MBP) (h, l), BrdU/PAX7 (i, m) and BrdU/GFP (j, n). GFP+ cells represent connective tissues in lateral plate mesoderm (LPM)-GFP transplanted axolots. All molecular markers used except MBP had nuclear expression, and therefore allowed one-to-one colocalization of nuclear BrdU with nuclear staining of the marker. Therefore, we refer to the MBP data as ‘MBP-associated’. White boxes highlight the magnified images. Yellow circles indicate two bones in the lower limb. NS, not significant; *P < 0.05, **P < 0.005, ***P < 0.0005, ****P < 0.00005 with Student’s t-test. White arrowheads indicate marker+/BrdU+ cells.
Extended Data Figure 5 | Upregulation of AxMlp transcript during early regeneration and alteration of AxMLP protein localization in wound epidermis cells. a, e, Measurement of AxMlp expression by qPCR at the indicated time points during tail (a) and limb (e) regeneration (n = 3: biological replicates; mean ± s.d.). To obtain the values of fold-change for each time point, the relative concentrations of the PCR products were calculated by the standard curve method. The concentration of AxMlp was normalized to that of large ribosomal protein 4 (Rpl4). b–h, Immunostaining with anti-AxMLP antibody (white) on tails (b–d) and limbs (f–h) of intact (b, f: transverse sections), 1 dpa (c: sagittal; g: horizontal) and 6 dpa (d: sagittal; h: horizontal) samples. By 6 dpa, the epithelial organization and AxMLP expression appeared to be returning to a less tightly adherent, less membrane-associated appearance (d, h). Scale bars: left, 200 μm; right 50 μm. Red arrowheads indicate spinal cord; green arrowheads indicate wound epidermis; yellow arrowheads indicate normal epidermis; yellow circles indicate two bones in the lower limb.
Extended Data Figure 6 | AxMLP morpholinos specifically and efficiently reduce AxMLP translation in cultured cells. a, Schematic illustration of wild-type (WT) AxMlp (top) and N-terminal deletion AxMlp (bottom) constructs used to characterize AxMlp morpholino 1. The N-terminally deleted AxMlp lacks the morpholino-binding site. Both constructs have a His-tag on their C terminus (a). ED, effector domain; M, myristoylated N terminus domain; MH, MARCKS homology domain. b–e, Electroporated A1 myoblasts were stained with the indicated markers. b, Wild-type AxMlp plasmid was co-electroporated with the control morpholino (c) or the AxMlp-specific morpholino 1 (d), whereas wild-type AxMlp plasmid only (b) or wild-type AxMlp only without any primary antibody staining were used as negative controls (e). f–h, ΔN-AxMlp plasmid was co-electroporated with the control morpholino (g) or the AxMlp-specific morpholino 1 (h), whereas ΔN-AxMlp plasmid only (f) or pCMV-SPORT6-3C-His (empty vector) plasmid only served as negative controls (i). j, Western blotting for the cell lysates from the experiment above. AxMLP morpholino 1 specifically reduced AxMLP protein expression. k, Schematic illustration of the constructs used to characterize AxMlp morpholino 2. The original AxMlp expression clone from the cDNA library (BL212a101: top) was used as it included the 5′ untranslated region (UTR) target site for AxMlp morpholino 2. The subcloned AxMlp-His construct lacks the binding site for AxMlp morpholino 2 and was used as the control construct. l–r, Electroporated A1 myoblasts were stained with the indicated markers. l, BL212a101 plasmid was co-electroporated with the five-mismatch control morpholino (m) or the AxMLP-specific morpholino 2 (n), or BL212a101 plasmid only (l) or pCMV-SPORT6-3C-His (empty vector) plasmid only served as negative controls (o). AxMLP was detected using an anti-AxMLP antibody (red), and morpholinos were detected via FITC conjugation (green). p–r, AxMlp-His plasmid was co-electroporated with the five-mismatch control morpholino (q) or the AxMlp-specific morpholino 2 (r) or AxMlp-3C-His plasmid only (p). AxMLP was detected using an anti-His-tag antibody (red), and morpholinos were detected via FITC conjugation (green). s, Western blotting for the cell lysates from the experiment above. AxMLP morpholino 2 specifically reduced AxMLP protein expression. Scale bars, 100 μm.
Extended Data Figure 7 | AxMLP morpholinos knockdown endogenous AxMLP in vivo. a–j, The morpholinos shown in were used in Fig. 4 and Extended Data Figs 6a–j, 8a–f. The morpholinos shown were used in Extended Data Figs 6k–s, 8g–j. Transverse sections from AxMLP-specific morpholino 1 (a–e) or control morpholino (f–j) electroporated tail. b, The spinal cord (SC) boxed in a. c, The higher-magnification images of the spinal cord boxed in b. AxMLP expression was detected in morpholino-negative cells (red asterisks), whereas it was reduced in morpholino-positive cells (yellow asterisks). d, The epidermis boxed in a, e, AxMLP expression was unaffected in morpholino-negative cells (red asterisks), whereas it was reduced in morpholino-positive cells (yellow arrowheads). In the control morpholino-electroporated tail (f) there was no morpholino-specific knockdown phenotype in either spinal cord (g, h) or epidermis (i, j). The same experiments were performed with AxMLP-specific morpholino 2 (k–o) and the corresponding five-mismatch control morpholino (p–t). k–t, The data sets were the same as a–j. Scale bars, 200 μm (a, f, k, p); 50 μm (b–e, g–j, l–o, q–t).
**Extended Data Figure 8 | AxMLP is necessary for initial cell proliferation during tail regeneration.**

**a–d,** Representative transverse sections of the morpholino-electroporated/protein-injected blastemas that were used for quantification of BrdU incorporation in Fig. 4d. Rhodamine was co-injected with the protein samples.  

**e,** Quantification of BrdU+ cells in blastema sections of morpholino-electroporated/protein-injected tails at 3 dpa (n = 4: biological replicates; centre values as median; points represent each sample).  

**f,** The length of the blastema during tail regeneration. The data at 6 dpa were plotted in Fig. 4c. By 14 days the difference in total regenerate length among the samples was not statistically significant.  

**g–j,** The same experimental scheme (shown in Fig. 4a) as was used for AxMLP morpholino 1 was implemented for a second specific morpholino (AxMLP-specific morpholino 2).  

**g,** Bright-field images of the morpholino-2-electroporated/protein-injected tails at 6 dpa. Red bars indicate amputation planes. Dashed lines delineate the shape of the mesenchymal blastema.  

**h,** Blastema length at 6 dpa (n = 4: biological replicates; centre values as median; points represent each sample).  

**i,** The length of the blastema during tail regeneration. The data at 6 dpa were plotted in **h.**  

**j,** Transverse sections immunostained for BrdU from morpholino-electroporated/protein-injected tails at 3 dpa. AxMLP-specific morpholino 2 combined with flow-through (FT) injection shows reduction of BrdU incorporation, whereas AxMLP protein injection rescues the phenotype. The corresponding five-mismatch control morpholino does not affect BrdU incorporation. Yellow circles indicate spinal cord (top) and notochord/cartilage (bottom). NS, not significant; **P < 0.005,** ***P < 0.0005,** ****P < 0.00005 with Student’s t-test. Scale bars, 200μm (**a–d, j); 500μm (**g).
Extended Data Figure 9 | Anti-AxMLP antibody significantly blocks BrdU incorporation during tail regeneration. a, Schematic illustration of antibody injection into axolotl tail. b, Quantification of BrdU+ cells in blastema sections of antibody-injected tails at 3 dpa (*n* = 4: biological replicates; centre values as median; points represent each sample). NS, not significant; **P < 0.005, ***P < 0.0005, ****P < 0.00005 with Student’s t-test.
Extended Data Figure 10 | Exogenous AxMLP accelerates normal tail regeneration. a, Schematic illustration of the protein injection into axolotl tail and blastema. b, Bright-field images of the protein-injected tails at 4 dpa. c, Blastema length at 4 dpa (n = 6: PBS, FT; n = 8: AxMLP, biological replicates; centre values as median; points represent each sample). The blastema from purified AxMLP injected tails significantly increased the regenerate length. Scale bar, 500 μm. Red bars indicate amputation planes; dashed lines delineate the shape of the mesenchymal blastema. NS, not significant; ***P < 0.0005 with Student’s t-test.