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Mechanical synchronization of *Xenopus* tail regeneration

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Abstract

Epimorphic regeneration is a complex and synchronous process that involves three major phenotypical phases: wound healing, regeneration bud formation and a consequent regenerative outgrowth. While regeneration occurs within a multifactorial microenvironment, where biochemical and mechanical cues are in place, most studies focus in the biochemical signalling underlying the transition across these phases. Thus, despite the wide biological functions that mechanical signals, their role in regeneration remains largely unclear. Therefore, to assess the role of relevant mechanical signals in regeneration, we use the tadpole tail of *Xenopus laevis* as a regeneration model. Using a specific toxin, we demonstrate that mechanosensitive stretch-activated channels are required for proper tail regeneration. In particular, we identify that these channels are essential for the wound healing and bud formation phases. Moreover, the expression of key molecular regulators of regeneration was downregulated by the same treatment. Interestingly, this downregulated expression profile is comparable to the observed in the non-regenerative refractory period. Collectively, our data unveils the role of mechanically activated channels in regeneration and opens the discussion on the potential mechanism by which mechano-signalling may mediate transcriptional responses that favour regeneration.

Keywords: Regeneration, Mechanical cues, Stretch-activated channels, GsMTx4, *Xenopus laevis*

Resumo

A regeneração pode ser definida como a capacidade de recrescimento de um órgão ou membro após uma lesão ou amputação; este recrescimento devolve a forma, padrão e função da parte do corpo perdida. Esta área de estudo para além de poder ser investigada a diversos níveis de organização biológica varia também a nível taxonómico, suscitando ao longo de décadas o interesse de diversos investigadores. Nos vertebrados, podemos encontrar a regeneração epimórfica, um processo complexo e síncrono desencadeado por um dano de grande escala. Pode dizer-se que se desenvolve em três fases fenotípicas principais e distintas: a cicatrização do local da ferida, a formação do botão de regeneração, também designado por blastema, e o consequente crescimento regenerativo do membro perdido. As alterações morfológicas observadas no decorrer das três fases são o resultado de consecutivos rearranjos celulares tanto em posição, como em número e forma, entre outros. O comportamento das células é consequência dos sinais que esta recebe do seu microambiente, nomeadamente das interações com outras células circundantes e/ou com o ambiente onde se encontra. Ao longo de décadas, diversos estudos têm vindo a ser realizados com o objetivo de caracterizar os intervenientes moleculares nos mecanismos de comunicação e sinalização celular no desenrolar do processo regenerativo e particularmente nas transições morfologicas a ele inerentes. Destes estudos surgiram fatores como o Transforming growth factor beta (TGF-β), Sonic hedghog (Shh), Bone morphogenetic proteins (BMP), Fibroblast growth factor (FGF), entre outros, que se creem ser potenciais intervenientes no processo. Contudo, a verdade é que, a regeneração ocorre dentro de um microambiente multifatorial, isto é, sinais bioquímicos e mecânicos estão presentes, porém a investigação científica concentra-se maioritariamente no estudo da sinalização bioquímica subjacente às transições entre as fases do processo regenerativo. Apesar de amplas funções biológicas serem atribuídas aos sinais mecânicos, o seu papel na regeneração permanece ainda pouco evidente. Tendo isto em conta, o nosso estudo visa revelar um pouco do seu papel neste processo.

Um excelente modelo animal para avaliar o papel da sinalização mecânica na regeneração é a cauda do girino de Xenopus laevis. De facto, neste modelo animal a regeneração total da cauda é observável num curto período de tempo - apenas sete dias após a amputação - e implica algum nível de complexidade biológica visto que tem de ocorrer a regeneração de todos os tecidos axiais do membro - como o tecido

muscular, nervoso e da epiderme. Para além disso, o girino de Xenopus laevis é dotado de um período no seu desenvolvimento designado de refratário, durante o qual ocorre a cicatrização do local da lesão, mas não a regeneração do membro perdido.

O processo regenerativo pode ser avaliado usando dois métodos: o índice de regeneração, que avalia a morfologia da cauda regenerada; e, como medida complementar, a área da cauda pós amputação. Com o presente estudo, corroborámos que a capacidade regenerativa depende do estádio de desenvolvimento do girino. Através da comparação entre o estádio 41 - regenerativo - e o estádio 47 - não regenerativo - contatámos que efetivamente, existe uma diminuição da capacidade regenerativa no período refratário. A necessidade temporal dos sinais mecânicos foi avaliada através da incubação dos girinos, nos períodos de tempo desejados - definidos pelos eventos morfológicos da regeneração - num meio com uma toxina específica - GsMTx4 - conhecida por causar a inibição dos canais sensíveis a sinais mecânicos, ativados pela extensão elástica da membrana celular - Stretch activated channels. Conseguimos demonstrar que estes são necessários para uma regeneração eficiente e bem-sucedida da cauda. Os alvos atualmente conhecidos deste péptido são os canais: Piezo1 e Transient receptor potential channel 1 e Transient receptor potential channel 6. Neste ensaio identificamos ainda que esses canais são fundamentais nas primeiras vinte e quatro horas da regeneração, mais precisamente, as fases de cicatrização da ferida e formação do blastema, conhecidas por serem cruciais para alcançar um processo bem-sucedido em forma e função.

Um dos alvos de GsMTx4 reportado é o canal Piezo1. Para testar o seu eventual envolvimento no decorrer do processo de regeneração, diminuímos a sua expressão e avaliámos o fenótipo observado. Com a utilização de um morpholino específico para o canal em estudo verificamos que existe uma tendência em que os girinos submetidos ao tratamento apresentam um fenótipo caracterizado como "defeituoso", ou seja, é prevalente o fenótipo de ausência de regeneração ou regeneração muito defeituosa. Levando em consideração estes resultados podemos especular que o canal Piezo1 é um potencial candidato a gene regulador da capacidade regenerativa.

De modo a compreender melhor como o bloqueio por inibição dos canais mecânicos afeta o comportamento celular ao nível da expressão génica avaliámos por Polymerase chain reaction (PCR) a expressão dos principais contribuintes moleculares da regeneração. Com base em literatura da área foram selecionados marcadores para cada fase da regeneração - cicatrização do local da ferida, formação do botão de regeneração e crescimento do membro - respetivamente, TGF-β, Msh Homeobox 1 (Msx1) e Shh. Com a nossa análise da expressão temporal entre controlo e tratamento fomos capazes de corroborar a expressão destes marcadores como específicos para cada fase, e, para além disso, constatar que os seus níveis de transcrição foram reduzidos pelo tratamento com GsMTx4. Curiosamente, este perfil de expressão é comparável ao observado no período refratário, isto é, não regenerativo. Tendo em conta o conjunto dos dados adquirimos, podemos inferir que os marcadores avaliados neste estudo atuam a jusante dos canais mecânicos na sua cascata de sinalização. Assim sendo, no contexto do processo regenerativo, a mecanotransdução celular através destes canais, encontra-se a interferir em vias de sinalização bem estabelecidas e conhecidas nesta área de estudo.

É ainda importante salientar que a análise de expressão génica serviu como pré-teste para futuramente conduzir as amostras para sequenciação do ácido ribonucleico (RNA-seq). Com certeza os resultados desta análise irão abrir o leque de potenciais intervenientes alvo no processo de regeneração.

De uma maneira global, a verdade é que o impacto dos sinais mecânicos na regeneração é ainda escassamente estudado apesar de existirem evidências literárias de uma diversidade de funções biológicas desencadeadas pelos mesmos. Neste sentido, a nossa investigação tem como objetivo elucidar o seu papel e averiguar a sua necessidade ao longo do tempo. Posto isto, no presente estudo, fomos capazes, por meio da inibição dos canais previamente mencionados, de manipular como as células detetam e consequentemente respondem aos sinais mecânicos. Demonstrámos, de facto, a importância da mecanotransdução na área da regeneração e a sua necessidade ao longo do tempo. Mais

concretamente, a ativação dos canais sensíveis a estímulos mecânicos ativados pela extensão elástica da membrana celular é essencial para a fase inicial do processo de regeneração, que engloba as fases de cicatrização de ferida e a formação do blastema.
Em suma, a regeneração e o desenvolvimento embrionário, partilham as principais vias de sinalização celular e os principais mecanismos comuns, deste modo, os canais sensíveis a sinais mecânicos podem estar envolvidos em diferentes circunstâncias para além da regeneração de tal modo que as evidências demonstradas neste estudo podem ser transversais. Com a nossa investigação abrimos a discussão sobre o potencial mecanismo pelo qual a sinalização através de sinais mecânicos pode mediar as respostas transcricionais que favorecem a regeneração, e ainda despoleta a curiosidade e novas investigações na ampla área que é a biologia do desenvolvimento.

Palavras-Chave: Regeneração, Sinalização mecânica, canais mecano-sensíveis, GsMTx4, *Xenopus laevis*

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IV.List of abbreviations, acronyms and symbols

µM	Micromolar
a.u.	Arbitrary units
(RT)-PCR	(Reverse Transcriptase)- Polymerase chain reaction
BMP	Bone morphogenetic proteins
bp	Base pairs
CaCl	Calcium chloride
DNA	Deoxyribonucleic acid
ef* 1 *α	Elongation factor 1 subunit alpha
FGF	Fibroblast growth factor
Gx	GsMTx4
H_2O	Water
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hpa or dpa	Hours postamputation or days postamputation
kb	Kilobases
KCl	Potassium chloride
$MgCl_2$	Magnesium chloride
mGFP	Membrane green fluorescent protein
min	Minutes
ml	Millilitre
mM	Millimolar
mm	Millimetres
MMR	Marc's modified Ringer medium
***msx*1**	Msh Homeobox 1
NaCl	Sodium chloride
ng/µl	Nanograms per microlitre
no.	Number
nRFP	Nuclear membrane red fluorescent protein
ºC	Celsius degree
pg	Picograms
pH	Potential of Hidrogen
Piezo1-MO	Piezo1 channel morpholino
psi	Pounds per square inch
RI	Regeneration Index
RNA-seq	Ribonucleic acid sequencing
RT	Room Temperature
s	Seconds
SACs	Stretch-activated channels
st.	Stage
TGF-β	Transforming growth factor beta
TRPC	Transient receptor potential channel
vs	Versus

1. Introduction

Regeneration

Regeneration can be described as the self-renewal of the epithelial layer of the some tissues such as epidermis and in a more compelling way, the ability to regrow in form, pattern and function after a damage or amputation is inflicted to an organ or a complete body part (Bideau et al., 2021; Brockes & Kumar, 2008). Regeneration occurs across the animal kingdom at different extents, the ability to regenerate a body part is disparate through diverse phylogenetic levels (Brockes & Kumar, 2008). Years of remarkable scientific research demonstrate that embryonic development and regeneration have common processes and pathways. Furthermore, this intricate process can be seen and study at different levels of biological organization from single cell to a full body (Bely & Nyberg, 2009). Independent of the damage, regeneration implies cell behaviours like differentiation, proliferation and migration, and also large-scale form and shape alterations like wound healing, patterning and organ specification (Santos-Ruiz et al., 2002). A fundamental question in the field of regeneration is to understand why some animals are capable to regenerate and others are not, particularly mammals. To address this, developmental biologists frequently focus on understanding the role of developmental signalling pathways, like Notch, Hedgehog, Wnt, TGF-β pathways and others in later stages of the life of the organism (Poss, 2010).

Epimorphic Regeneration

Epimorphic regeneration is a type of regeneration present in some vertebrates, such as rodents and amphibians (Beck, Izpisu, & Christen, 2009; Han et al., 2008; Seifert & Muneoka, 2018; Simkin et al., 2017). This type of regeneration is triggered by a large-scale damage, like amputation, which results in migration of epithelial cells towards the amputation site to close the open wound and recruit mesenchymal cells, an important step called wound healing (Stoick-cooper et al., 2007). After this, a mass of undifferentiated cells with intrinsic morphogenetic information called blastema is formed beneath the wound epithelium (Brockes & Kumar, 2008; Iismaa et al., 2018; Seifert & Muneoka, 2018) and they proliferate, and acquire positional information contributing to the future tissues. Finally, the last phase of the process, is the outgrowth and patterning of the new body part, in which differentiation and specification of developmental pathways are deployed leading to a near-perfect recovery (**Figure 1.1**).

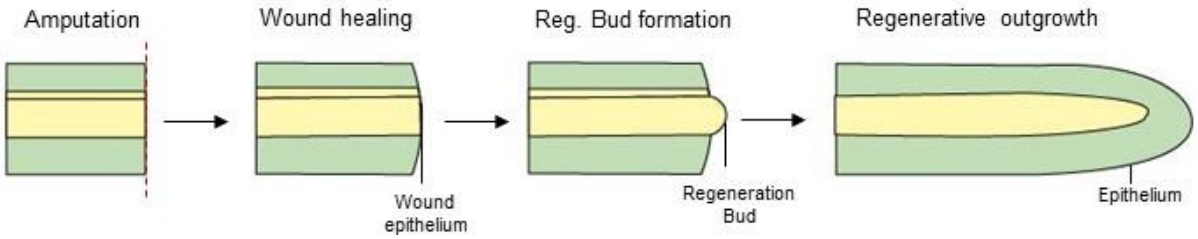

Figure 1.1 **Scheme illustrating the epimorphic regeneration phases in *Xenopus laevis* tadpole's tail regeneration after amputation.** Note the detail of wound epithelium and regeneration bud.

Transitioning from one phase to the subsequent requires large-scale rearrangements of the cells involved, not only in cell number, shape, and position across the tissue, but also in further specification in the tissue. Alteration of cellular behaviour is achieved through signalling and communication, an interaction between cell and environment, cytoskeleton dynamics and intracellular effectors (Devreotes & Horwitz, 2015; Farhadifar et al., 2007; Sun & H. Zaman, 2018). Signalling cascades and molecular components are far well-studied compared to what is currently known about the role of mechanical input impact in regenerative process (Beck et al., 2003; Sugiura et al., 2004, 2009; Taniguchi et al., 2014;

Tazaki et al., 2005; Zhang et al., 2000). Therefore, whether mechanical signalling input is required and how it affects the regeneration transitions is an emerging question in the field.

Xenopus tail regeneration

One of the simplest, easy handling and suitable organism for genetic and drug modifications in the study of epimorphic regeneration is the *Xenopus* tadpole (Levin, 2008; Phipp et al., 2020). *Xenopus laevis*, the South African clawed frog, is capable to regenerate a variety of structures, including tail, limbs and lens. Its embryonic development is well-documented, allowing the potential transference of pathways to the regenerative context (Beck et al., 2009; Slack et al., 2008; A. S. Tseng, 2017). *X. laevis*, tail can be fully regenerated after 7-12 days following its loss and the efficiency is maximum when the damage takes place between stage 40 until stage 60 (metamorphic climax). Regeneration of full tail implies axial tissues regeneration like muscle, nervous tissue (notochord and spinal cord) and epidermis. This remarkable capacity makes it a prime biomedical model of regeneration. Intriguingly, there is an exception, from stage 45 to stage 47 the tail of tadpole is able to heal but do not regenerate.

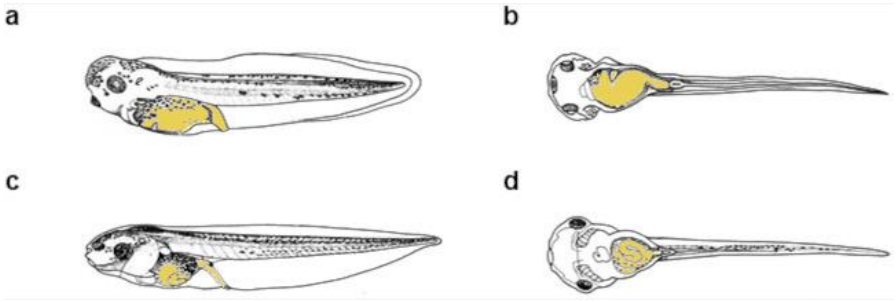

Figure 1.2 **Morphology of tadpoles at developmental stages 41 and 46**. (a-b) Developmental stage 41 lateral and ventral view, respectively. (c-d) Developmental stage 46 lateral and ventral view, respectively. Digitalized images from Nieuwkoop & Faber, 1967. Intestine highlighted in yellow as it is the morphological character to access stage of development.

Notably, this so-called refractory period offers researchers an excellent opportunity to perform loss and gain of function assays using the same animal model within the space of a week (Beck et al., 2009; Ivanova et al., 2013). The epimorphic regeneration observed in *X. laevis* tail evolves in the same three generic and distinguishable phases: wound healing, regeneration bud (equivalent to blastema) formation and the regenerative outgrow.

Molecular mechanisms in regeneration

Differential gene expression analysis studies have contributed to elucidate the molecular mechanisms in regeneration. From these arise major players in regeneration like fibroblast growth factors (FGFs), sonic hedgehog (Shh) pathway, bone morphogenetic proteins (BMPs) and also the Wnt pathway (Beck et al., 2003; Sugiura et al., 2004, 2009; Taniguchi et al., 2014; Tazaki et al., 2005; Zhang et al., 2000). In recent years, biomechanical cues and their impact on cells and their surroundings instigated the curiosity of scientists in the field of developmental biology (Heisenberg & Bellaı, 2013; Maître, 2017). Indeed, changes in gene expression profiles are well-documented when it comes to comprehending the morphological transitions between phases in regeneration. FGFs and Wnt/β-catenin signaling pathway are known for being required for numerous cell events like proliferation and further migration; tailbud patterning and overall regeneration needs some of BMPs ligands through their effector gene *msx*1; also another ligand contributing to the formation of the wound epithelium is TGF-β, required for but not sufficient to trigger regeneration (Beck, Christen, & Slack, 2003; Ho & Whitman,

2008; Lin & Slack, 2008a; Whyte, Smith, & Helms, 2012; Yokoyama et al., 2007). Taken together it's clear that regeneration field has been mostly about the elucidation of the molecular pathways.

Table 1 **Potential regeneration phase-specific markers known to regulate several processes during regeneration.**

Regeneration phase	Marker	Reference
Wound healing	*tgf-β*	Ho and Whitman (2007)
Regeneration bud formation	*msx* 1	Beck et al. (2006)
Regeneration bud formation	*wnt* 8	Sugiura et al. (2009)
Regenerative outgrow	*shh*	Sugiura et al. 2004 and Sugiura et al. 2009
Regenerative outgrow	*wnt*-5*a*	Sugiura et al. 2004
Regenerative outgrow	*bmp*2/4/7	Sugiura et al. 2009

However, to achieve a successful state of regeneration, some studies speculate that it could also be modulated by mechanical signaling (Chiou & Collins, 2018; Ferreira & Zhao, 2016; Scarpa & Mayor, 2016). Certainly, biochemical factors are far more studied than mechanical cues, although they are likely to be mutually important for understanding cellular communication and subsequently behavior in regeneration.

Mechanics in cell biology

Cells are constantly processing biochemical and mechanical inputs, both contributing directly or indirectly to the regulation of several cellular processes, such as proliferation, migration, differentiation (Cui et al., 2015; Kindberg et al., 2020; Mohammed et al., 2019). Understanding how cells sense, decode and respond or adapt accordingly to a mechanical cue– mechanotransduction – has become an area of intense focus in developmental biology. In other words, mechanotransduction is the process by which external mechanical inputs are converted to biochemical signals and consequently transmitted to the nucleus leading to an alteration in cell behaviour (Chen et al., 2017; Orr et al., 2006).

Living cells are in dynamic environments surrounded by extracellular matrix and neighbouring cells from which physical forces arise (Berrier & Yamada, 2007). One example of how physical forces impact cell behaviour is the contractile forces acting in early embryos, responsible for several cellular behaviours including, blastopore closure at the end of gastrulation (Feroze et al., 2015), neural tube closure (Zhou et al., 2009) and neural crest cell migration (Barriga et al., 2018). In fact, cells are capable to sense mechanical stimuli through membrane proteins that react to such stimuli (Goult et al., 2018; Martino et al., 2018). These membrane proteins include mechanosensitive channels, more commonly referred to as stretch activated ion channels (SACs), capable to sense several mechanical inputs, for example pressure, shear stress, stretch and rigidity of the surroundings (Goldmann, 2014; Lim et al., 2018). That said, it is clear that the extracellular matrix, the actin cytoskeleton and the interaction between them – cell matrix adhesion- and also the stretch activated channels, constitute major players in mechanotransduction (Berrier & Yamada, 2007; Goldmann, 2014; Kindberg et al., 2020). As the name implies, SACs are activated when cellular membrane undergoes stretch, the mechanical input applied to the membrane drives the channel to open, increasing the influx of ions to the intracellular environment. The SACs nomenclature englobes the channels that cause an influx of cations (Na^+, K^+, Ca^{2+} and $Mg^{2+)}$ to the cytosolic environment, this includes Piezo family (Piezo1 and 2) (Coste et al., 2010; Moroni et al., 2018) and the transient receptor potential cation channels (TRPC) family (Nilius et al., 2007; Spassova et al., 2006).

The first reported evidence of stretch activated channels was made in embryonic chick muscle cells by Guharay & Sachs in 1984. From that point on, the interest in mechanical biology has sparked

deeper interest with SACs gaining increase relevance in developmental biology. For instance the Piezo1 and 2, discovered in 2010 by Patapoutian and his team, are essential for the development of axons (He et al., 2018; Koser et al., 2016; Suslak et al., 2015) and also for the morphology of the cells in cardiovascular development in mice (Duchemin et al., 2019; Ranade et al., 2014). Regarding the mechanotransduction through TRPC family, these channels are linked to cancer progression (Y. Sun et al., 2021; Zeng et al., 2013) and also to brain development and axon growth due to the calcium needed for the synaptic development (Tai et al., 2008). In the field of regeneration not much is known about SACs impact, although *in vitro* studies show that Piezo may have a role associated with the differentiation of adult stem cells, more precisely neuronal stem cell differentiation (Pathak et al., 2014).

The previous examples capture that indeed adaptation to external stimuli can go through an alteration in gene expression (Farge, 2003; Yamada & Sixt, 2019). Activation of SACs leads to a succession of events in which recruiting cytosolic proteins triggers a cascade of events culminating with the expression of specific transcription factors and changing cellular state.

Without doubt, the role of mechanical stimuli input in cell behaviour and the unravelling of the mechanism of mechanotransduction, is a recent and consequently less explored topic. In our specific case of study that is epimorphic regeneration and its synchronous and time-defined transitions, little is known. Moreover, epimorphic regeneration is a complex process that englobes different cellular behaviours associated with the temporal transitions. Therefore, the goal of our research is to uncover whether stretch-activated channels impact the success of regeneration, specifically by changing the mechanical input to elucidate their role in the progression of this process and its major cellular events.

2. Materials and methods

Animal welfare

All animal experiments were done with the approval by the Ethics Committee and Animal welfare Body (ORBEA) of the Instituto Gulbenkian de Ciência (IGC) abiding with the Portuguese (Decreto-Lei nº113/2013) and European regulation (Directive 2010/63/EU).

Animal maintenance and embryo collection

Xenopus laevis (Daudin, 1802) embryos were obtained from *in vitro* fertilization according to standards protocols (Sive et al., 2000). In short, ovulation of mature females was induced by injection of human chorionic gonadotropin (MSD Animal Health, Chorulon) into the dorsal lymph sac. To promote spawning, female frogs were gently squeezed. The laid oocytes were further fertilized using sperm solution prepared with 1/5 of fresh testis smashed in 500 µl of Marc's modified Ringer 0.1× medium (MMR: NaCl 10 mM, $CaCl_2 \cdot 2H_2O$ 0.2 mM, KCl 0.2 mM, $MgCl_2 \cdot 6H_2O$ 0.1 mM, and HEPES 0.5 mM, adjusted to pH 7.1–7.2). Testicular tissue was previous collected from dissected mature males euthanized in tricaine (Sigma-Aldrich**)** overdose. Posteriorly embryo solution was dejellied with a cysteine solution (1g of cysteine diluted in 50 ml MMR 0.1× and 500µl of NaOH for pH≈8.0). Embryos were placed in fresh MMR 0.1× at 12 or 14ºC.

Regeneration assay

After dejelly, embryos were selected and transferred to fresh MMR 0.1× medium, and incubated between 14 and 25 ºC until the regenerative or refractory period stages (st.40/41 or st. 46/47, respectively) (Nieuwkoop & Faber, 1967). Medium was refreshed daily. At the desired stage tadpoles were anesthetized in Tricaine 1 mM (Sigma-Aldrich) diluted in MMR 0.1× for 5min (Ramlochansingh et al., 2014). Under a dissecting stereoscope (Nikon, SMZ745) half of the tail was amputated with a scalpel (blade no. 23). The anesthetic was washed with MMR 0.1× and tadpoles were kept in a 6 well

plate at a density of one tadpole per ml (5-10 tadpoles per well) at room temperature (RT) with medium refreshed daily. Tadpoles were not fed during the regeneration assay. Photomicrographs at 25× magnification were obtained using a Dino-Lite camera (Dino-lite digital microscope, AM7025X) at 5 minutes, 3, 24, 48, 72 hours and 7 days post-amputation. At the end of experiment, tadpoles were euthanized in tricaine overdose. We used two methods to assess regeneration: regeneration index and area of regenerated tail.

Regeneration Index (RI) is a morphogenetic parameter used to evaluate the efficiency of regeneration (Adams et al., 2007; Tseng et al., 2010). Tadpoles' tails were scored in four phenotypical categories: Full, regeneration occurred completely, indistinguishable from the uncut; Good, regenerated tail contains small defects (curved axis or loss of a fin); Weak, poor regeneration, major defects in regenerated tails; None, no regeneration. Then RI was computed integrating these phenotypes using the following equation:

$$Regeneration\ Index\ (RI) = F_{Full} \times 3 + F_{Good} \times 2 + F_{Weak} \times 1 + F_{None} \times 0$$

Equation 2.1 **Mathematical formula to compute Regeneration Index based on the phenotypical observations.** Frequency of the phenotype is multiplied by 3, 2, 1, or 0, for, respectively, full, good, weak and none category. The output of the equation is a value between 0 and 300 (no regeneration occurred, and all individuals regenerated perfectly, correspondingly)

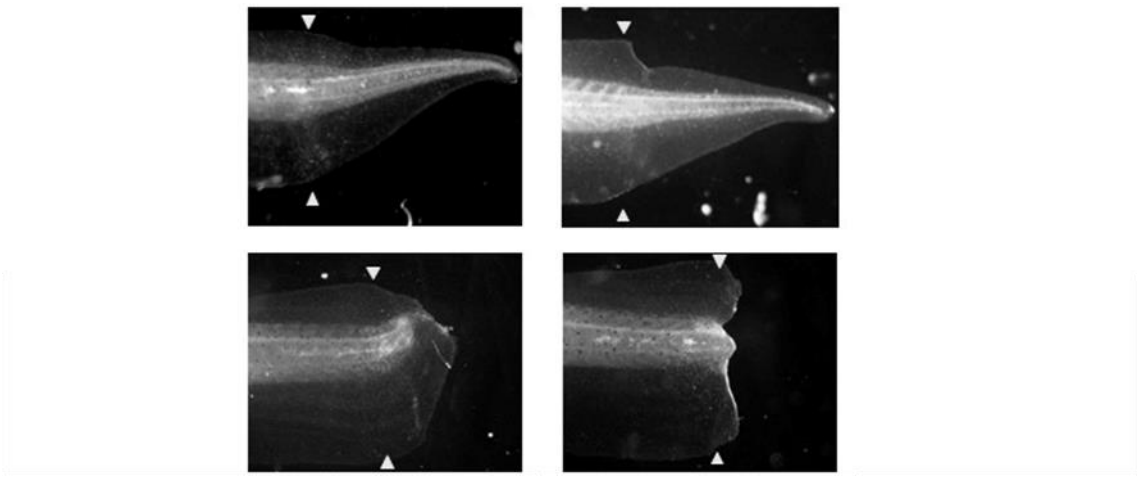

Figure 2.1 **Categorization of regeneration phenotypes used to compute the Regeneration Index**. From top left: to bottom right: Full, Good, Weak and None. White arrowheads: amputation plane.

A complementary parameter is area of regenerated tail at different time-points. Photomicrographs were loaded in Fiji (https://imagej.net/Fiji; v1.53k) and area was measured usingthe free hand tool to outline the tail from the amputation plan (Schindelin et al., 2012).

Pharmacological modulation

GsMtx4 (Smartbox Biotechnology, 08GSM001) was used to modulate mechanosensitive ion channels. Toxin was previously diluted in H_2O in small aliquots of 200 µM and stored at − 80 ºC. GsMtx4 assay solutions were prepared in MMR 0.1× for the final concentration specified. Tadpoles were incubated in this drug at the desired time windows. After exposure to the toxin tadpoles were washed 3 times, 1 minute each, in MMR 0.1× and followed until 7 days post-amputation at RT in daily refreshed MMR 0.1×. Due to biological variation each treatment was performed at least three times using embryos from different females.

Tissue harvest

Tadpoles (10 per condition) were amputated as described above and incubated in MMR 0.1× or GsMTx4 12 μM for the time required. After the exposure to the drug tails were recut 0.5 mm from the amputation plane (1 at a time). Re-cut tails were carefully pipetted into 150 μl of RNAlater (Sigma-aldrich, cat no. r0901) and saved at 4 ºC until RNA extraction.

(RT)-PCR

Total RNA was extracted and purified using a RNeasy Mini Kit (Qiagen) following the manufacturer protocol. RNA samples were further saved at -80 ºC. Complementary DNAs (cDNA) were synthetized using the SuperScript™ IV First-Strand Synthesis protocol (Invitrogen, 18090050) and oligo d(T_{20}) primer, final concentration was measured in NanoDrop™ 2000 spectrophotometer (Thermofisher scientifics). cDNA concentration loaded for PCR reaction was normalized to the same quantity in each reaction. For that, cDNA was diluted in nuclease free water to the final concentration of 440 ng/μl. Oligonucleotide primer pairs were used to amplify the cDNA. *Msx1* primer used has been previous described by Lin & Slack, 2008; the new primers designed for this work resorting to PrimerBlast were the following: *msx*1, *tgfβ* and *shh* (Ye et al., 2012). PCR target genes were amplified in a T100™ Thermal Cycler (Bio-Rad) with Q5 High-Fidelity DNA polymerase (M0493, New England Biolabs), with the following conditions: initial denaturation at 98 ºC for 30 s, succeeding 30 cycles (denaturation at 98 ºC for 10 s, annealing temperature described in Table 1 for 30s, and extension time of 25s /kb), with a final extension at 72 ºC for 2 min.

Table 2.1 **Primer pairs and respective annealing temperature (Tm) and fragment size of reverse transcription–polymerase chain reaction.**

Gene	Primer Sequence	Tm (ºC)	Size (bp)
ef1-α	FW: 5' – ACCCTCCTCTTGGTCGTTTT- 3' RV: 5' – TTTGGTTTTCGCTGCTTTCT -3'	65	121
msx1	FW: 5'- CTGGTTCCAGAACAGGAGAGCC- 3' RV: 5'- CATGCTGTATCCAAGGTGGGCTG- 3'	72	226
tgfβ	FW: 5'-GGCCGCACTGCTAAAGTAGA- 3' RV: 5'- ATCCATGTGTGGGCTGTGAA- 3'	72	251
shh	FW: 5'-ACCGGCTCATGACTCAGAGATGTA– 3' RV: 5'- TGAAGGCCTGATGGAGGACTG- 3'	72	862

After PCR reaction, it was added gel loading die (6×) (cat. B7024S, New England BioLabs) to the samples; half of the final volume was loaded in a 2% agarose gel stained with Xpert Green DNA Stain (GRiSP Research Solutions) for enhanced contrast. As a reference marker was used a 100 bp DNA ladder marker (New England Biolabs).

Quantification of PCR band intensity

A rectangle adjusted to the size of the bands was used to determine the mean grey intensity for all conditions using the ROI manager tool of FIJI (Schindelin et al., 2012). The same size rectangle was

used to quantify the background, which was then subtracted to the intensity of the bands. Values were normalized with the respective intensity of *ef*1-*α*, i.e., value of each band was obtained by dividing the mean grey intensity values of the gene by the respective values of housekeeping gene in each condition.

RNA-seq

RNA samples collected for pre-screening PCR were analyzed for RNA quality in TapeStation. Later to verifying the quality of samples , they were submitted for RNA-Seq, Smart-seq2 (Picelli et al., 2014). Both procedures were conducted by Genomic Facility of IGC (Oeiras, Portugal). At the time of thesis writing, we are awaiting the data for further bioinformatics analysis.

Piezo1 Morpholino

Embryos at 2 cell-stage were selected and placed in a Petri dish filled with a dense solution of 10 ml of methylcellulose 0.8% in MMR 0.1×. Targeted microinjections were performed using PM 1000 Cell Microinjector (MicroData Instrument, Inc) at 20 psi, injecting 750 pg of nRFP, 500 pg of mGFP per control embryo and 500 pg of mGFP and 770 pg of Piezo1 morpholino (5'-CACAGAGGACTTGCAGTTCCATCCC-3') (Koser et al., 2016) (Gene tools) per treatment embryo. In general, a control tadpole was injected with 750 pg of nRFP, 500 pg of mGFP and the treated tadpole was injected with 500 pg of mGFP and 770 pg of Piezo1 morpholino. Fluorophores were used to access the success of microinjection, and so under fluorescence microscope (Leica) embryos were selected at stage 38/39 and amputated in the following day at stage 41.

Statistical Analyses

Before any further analysis, normality assumption was tested using Kolmogorov-Smirnov, Shapiro-Wilk and/or D'Agostino tests for each condition in study. Normality assumption was validated in most of the datasets. In case of normal distribution of datasets parametric test were performed through one-way ANOVA with Sidak's multiple comparation. The equivalent non-parametric test, Kruskal-Wallis with Dunn's multiple comparation, was performed in non-verified normality grouped data of area assays. Fisher's exact test was executed to analyse regeneration index in two categorical variables for that data was assembled in two categories in which frequency of phenotypes Full and Good was added to a category *vs* the total of frequency of phenotypes of Weak and None taking together (Full + Good *vs* Weak + None). Data visualization and statistical analysis were performed using Excel, Adobe Illustrator (2021) and GraphPad Prism 6.0 software (GraphPad, San Diego, CA, USA). For clarity purposes sample size (*n*, biological replicates) as well as *p* value are indicated in figures legends. In case of RT-PCR, statistical analyses were not performed due to the number of replicates being too small to test normality.

3. Results

Regeneration of *Xenopus laevis* tail through development

The major morphologic phases of regeneration- wound healing, regeneration bud formation and the regenerative outgrow- are sequential and well-defined events. Nevertheless, each phase affects and may even triggers the next one. For example, the regeneration bud forms from signals secreted by the wound epithelium (Aztekin et al., 2019). This interdependency across phases makes it difficult to have precise temporal windows for the phases. However, the most consensual time-windows are the following: the wound healing englobes the first 6 hours post-amputation (hpa) [0–6 hpa], followed by regeneration bud formation until the 48 hpa [6-48 hpa] and from that point on until 7 days is considered regenerative outgrow [48 hpa-7 days post amputation] **(Figure 3.1c)**.

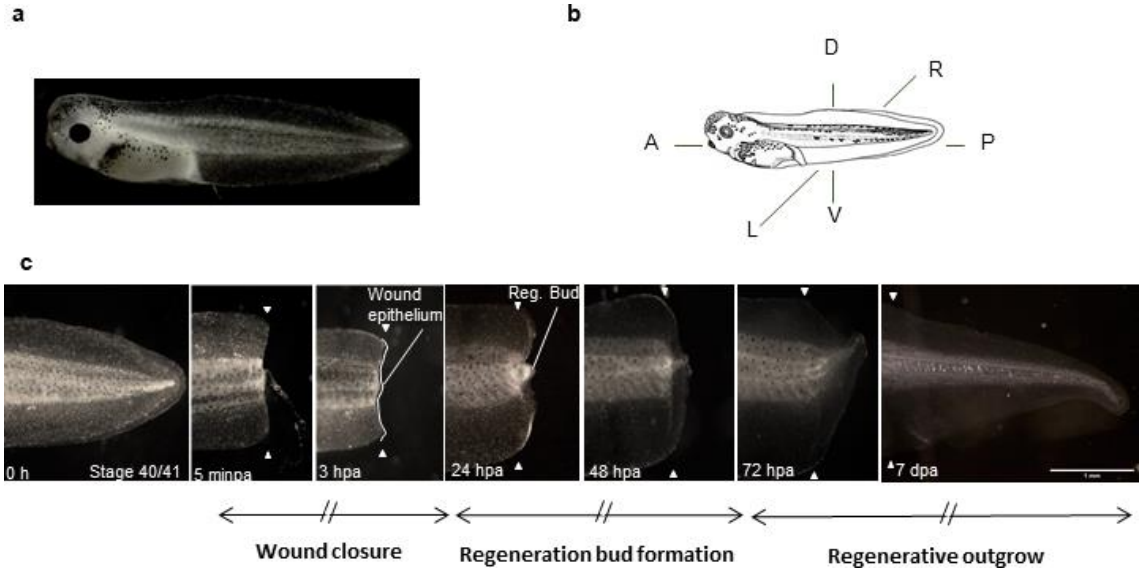

Figure 3.1 **Regeneration phases at stage 41**. (a) Xenopus laevis tadpole at stage 40/41. (b) Scheme illustrating a tadpole and indicating dorsal/ventral axis (D/V), anterior/posterior axis (A/P) and left and right axis (L/R), rebribed from Xenbase.(c) Photomicrographs of time-lapse recovery from amputation plane. White line: contour of wound epithelium. White arrowheads: amputation plane. Scale bar of 1 mm.

The African clawed frog has the capacity to regenerate the tail until its resorption at metamorphic climax. There is however and intriguing exception, the so-called refractory period. During the developmental stages 45–47 the regeneration efficiency is impaired and even totally lost (Beck et al., 2003; Ivanova et al., 2013).

Prior to any complex assay, we started by validating the regeneration assay in our hands to set up the baseline of our further experiments. We started by performing regeneration assay at two different timepoints in development of *Xenopus*, st. 40/41 and st.46/47, regenerative and non-regenerative, respectively. When compared the RI, our data shows that, indeed regeneration was significantly different. When amputated at stage 41 **(Figure 3.1a-b)**, tadpoles can easily and completely regenerate (73.56%), only presenting minor defects, the most frequent observed defect was the absence of the dorsal fin or a curved axis (12.44%). Just a minor fraction has problems in regenerating (10%). These phenotypes summed up to a RI of around 256 demonstrating an efficient regeneration when compared to stage 46 where the experimental RI was around 75, a highly impaired regenerative process **(Figure 3.2a-b)**.

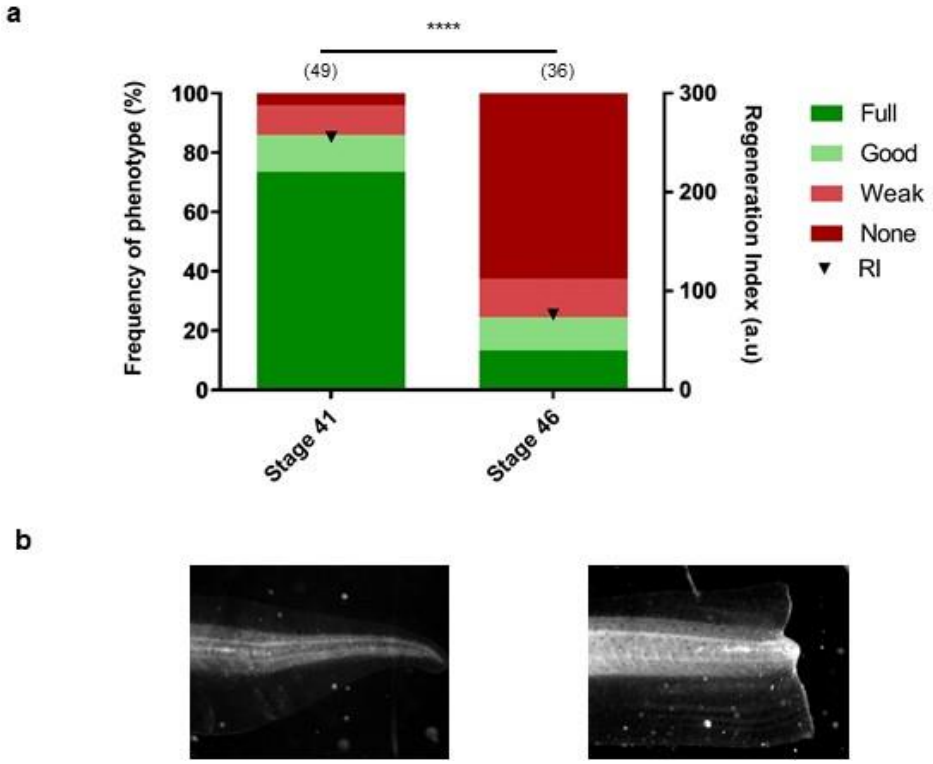

Figure 3.2 **Regeneration assay during developmental stages.** (a) Frequency of phenotypes (left) and regeneration index (right) per stage of development. Numbers between brackets indicates the n tadpoles of the experiment. ****$p < 0.0001$ (b) From left to right: Photomicrograph at 7 days post-amputation of tail amputated at stage 41 (fully regenerated) and stage 46 (non-regenerated), correspondingly.

Mechanosensitive channels are essential during tail regeneration

To understand the role of mechanical cues through time during morphologic alterations in regeneration, we tested the effect of blocking mechanosensitive stretch-activated channels. We conducted this experiment using a small peptide toxin from the spider *Grammostola spatulate*, discovered in 2000, GsMTx4 (Suchyna et al., 2000). This venom blocks cation stretch-activated channels by binding to the outer leaflet of the cell membrane. More precisely, the peptide allocates itself between the channel and the membrane, lowering the cellular membrane threshold to undergo and sense stretch, becoming more relaxed and thus precluding channel opening (Spassova et al., 2006). Importantly, GsMTx4 is a narrow-spectrum inhibitor of SACs with just three known targets, Piezo 1, transient receptor potential cation channel 1 and 6 (TRPC1 and TRPC6, respectively) (Bae, Sachs, & Philip A., 2011; Bowman et al., 2007; Gnanasambandam et al., 2017; Gottlieb & Sachs, 2012). GsMTx4 use will enlighten the role of these channels in overall regeneration phases and its time-specific transitions.

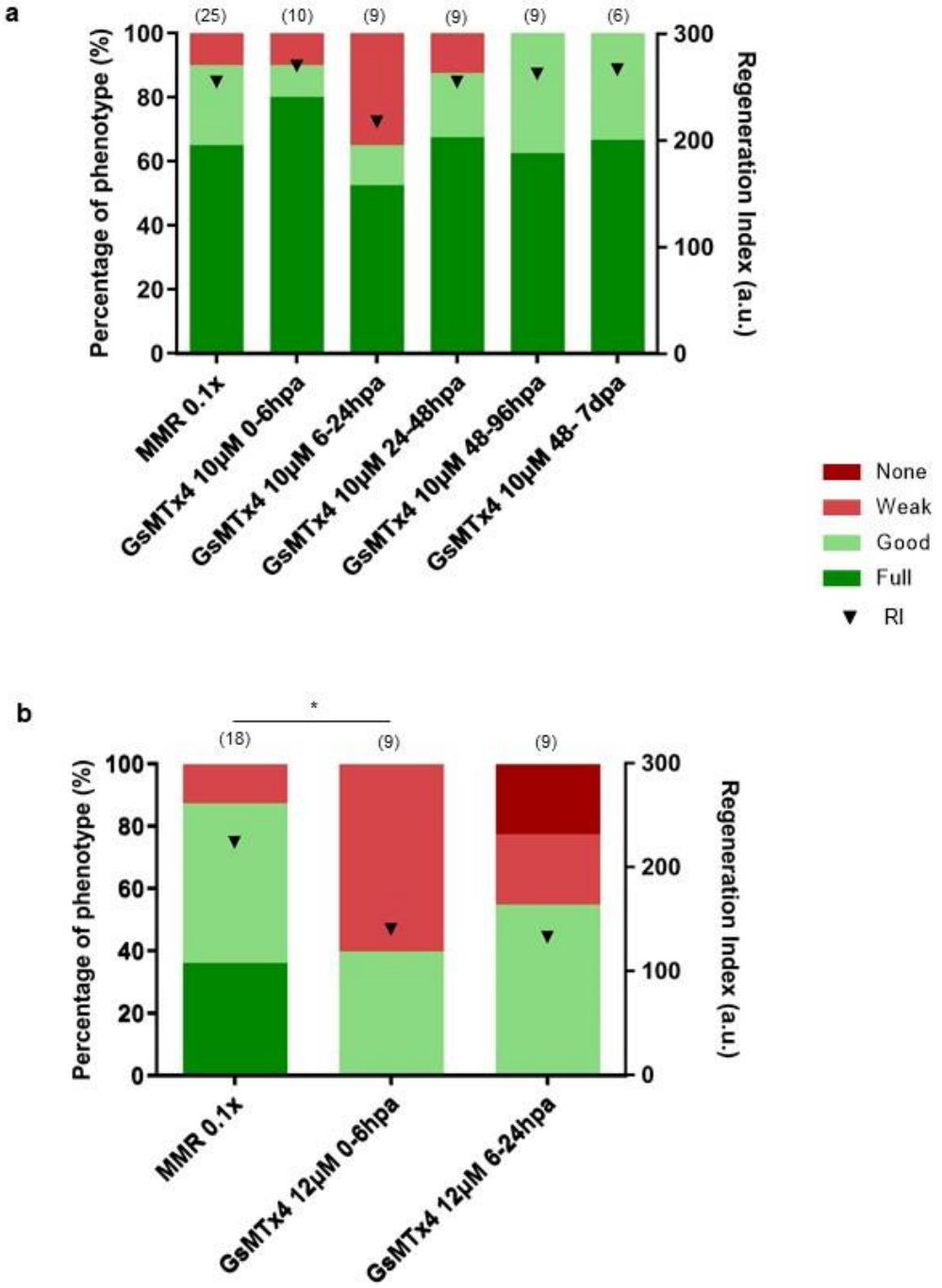

Figure 3.3 **Channel blocking effect in regeneration.** (a) Frequency of phenotypes (left) and regeneration index (right) per time-window when applying GsMtx4 10µM. Numbers between brackets indicates the n of the experiment. (b) Frequency of phenotypes (left) and regeneration index (right) per time-window when applying GsMTx4 12µM. *p-value < 0.05 MMR 0.1× medium of control embryos

Previously to the assay, we did a concentration screening for Gx (**Supplementary Figure 1**), whereby tadpoles were submitted to several concentrations of GsMTx4 in a range of 5 µM to 15 µM, to uncover not only the drug concentration with more penetrating effect and less lethality but also the time extent of exposure. Accordingly, the assay was performed one more time in which tadpoles were subjected to 10 µM during exact time-windows and, to minimize possible drug effect after exposure, they were washed 3 times and maintained in fresh medium until 7dpa. Computed at the end of the assay, regeneration index was decreased when drug treatment was applied during the period from 6 to 24 hpa,

the beginning of regeneration bud formation **(Figure 3.3a)**. This result is consequence of an increase on the number of weak, i.e deformed tails. Despite the absence of statistical significance, it is clear the decrease trend in RI, from 255 (MMR 0.1×) to 217.5 (Gx 10 μM 6-24 hpa) (p-value = 0.1023). It has been shown that affecting wound healing and bud formation robustly impairs the outcome of regeneration (Ferreira et al., 2018; Ferreira & Zhao, 2016). Thus, next we tested whether the regenerative delay was due to an insufficient inhibition of SACs. For this, we increased the dose of the toxin to 12 μM **(Figure 3.3b**). Notably, the higher concentration revealed itself more effective both during the regeneration and in the outcome of regeneration. The RI in the initial phases of regeneration, wound healing (0–6 hpa) and beginning of regeneration bud formation (6–24 hpa), decreased sharply when compared to the control. RI decreased significantly from around 224 to 140 (p = 0.0235) and also to 132 (p = 0.138), respectively. Furthermore, in both phases tadpoles were not capable to regenerate without deformities (absence of fully regenerated phenotypes).

Since regeneration index evaluates the morphological outcome, we used another measure of regeneration output, the area of regenerated tail to give us insight into the time dimension of the process. It is relevant to mention that area is considered a complementary measure as it does not provide a morphological assessment of the quality of the regenerated tail. For example, imperfect phenotypes like curved axis where tadpole's tail grows defectively will still have a normal area of regeneration when compared to control. However, it is an important tool to assess the progress of the regeneration as we can measure the area throughout regeneration, while RI only takes into account the final phenotype. In the first day, the area of regenerated tail of tadpoles exposed to GsMTx4 10 μM during the first 6 hpa and the subsequent time period of 6 hpa until 24 hpa, was statistically significantly affected by the drug treatment **(Figure 3.4a).** However, at 7 days post-amputation, after washing out the drug, tadpoles were able to recover, presenting no significant differences with the control **(Figure 3.4a and c)**. Together, our data with this dose, suggest that blocking channels significantly delays regeneration in the first two phases, while having a less efficient effect in the overall regeneration.

The temporal analysis using area of regenerated tail at 12 μM, showed, as in 10 μM treatment, a significantly decreased progress of regeneration at 24 hpa (p < 0.0001 for 0–6 hpa and 6–24 hpa) **(Figure 3.4b)**. At the end of regeneration, the effect of the toxin in the area was still very significant, confirming the RI analysis **(Figure 3.4b and c)**. Therefore, submitting the tadpoles to a higher dose of toxin translated into a more penetrant phenotype, highlighting the need to robustly inhibit mechanosensitive channels in order to obtain permanent effects on regeneration. Taken together, these results suggest that mechanotransduction conducted through SACs (Piezo1, TRPC1 and/or TRPC6) is required in wound healing and early regeneration bud formation and effectively impact the overall efficiency of regeneration.

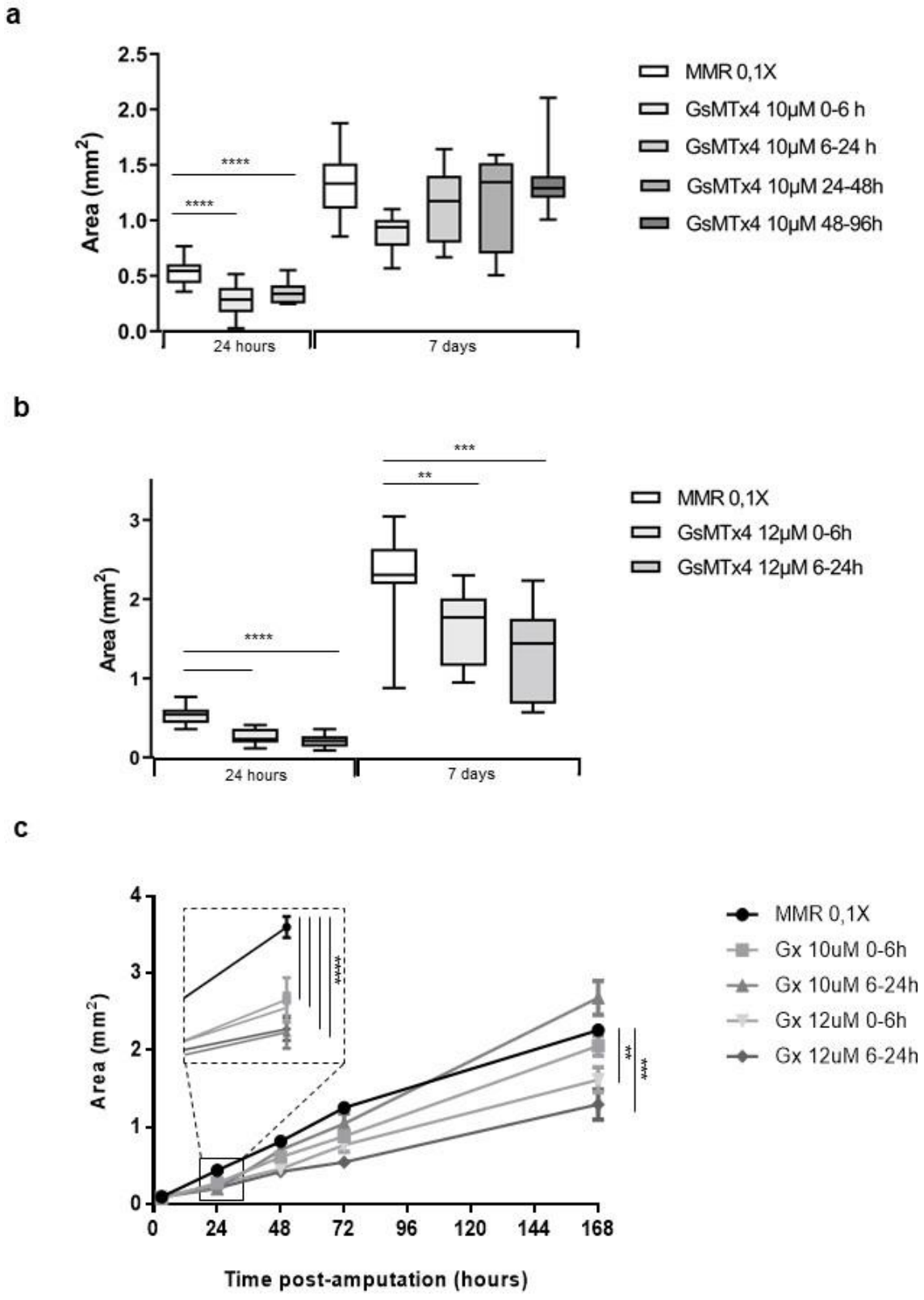

Figure 3.4 **SACs are required for regeneration by regulating the initial phases of regeneration**. (a) Area of regenerated tail measured from the amputation plane at 24 hpa, and 7 days post-amputation per time-window in GsMTx4 10 µM. (b) Area of regenerated tail measured from the amputation plan at 24 hpa, and 7days post-amputation per time-window in GsMTx4 12 µM.. (c) Time-lapse of area of regenerated tail per treatment. Dashed highlighted area of 24 hpa data. Data presented as mean with standard error ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$. MMR 0.1× medium of control embryos

A candidate SAC, Piezo1, is required for regeneration

Taking in count some unpublished data from the host lab and according to the work of Aztekin et al. on regeneration of *Xenopus* tail in which Piezo1 is downregulated at stage 46, the non-regenerative time-window of the development of the tadpole, we decided to test whether it could be the SAC candidate channel modulating regeneration. To downregulate the channel, we used a validated Piezo1 morpholino (Piezo1-MO) (Koser et al., 2016). As a preliminary experiment, we injected embryos at 2-cell stage with this morpholino and selected the ones expressing the tagged nuclei to be amputated at stage 41. After conducting the regeneration assay, we noticed a trend of strong impairment of regeneration. RI decreased from 300 to 103.8 **(Figure 3.5).** Thus, preventing the cell from sensing mechanical stretching has a similar outcome in regeneration to downregulation of Piezo1. Further, these data suggest that Piezo1 might be the SAC involved in regulating regeneration. Despite the clear trend, no statistical was conducted since the number of the tadpoles in the control group is very low and the experiment was only performed 2 times. Consequently, this experiment ought to be repeated in order to provide a more robust assessment of the channels effect on regeneration.

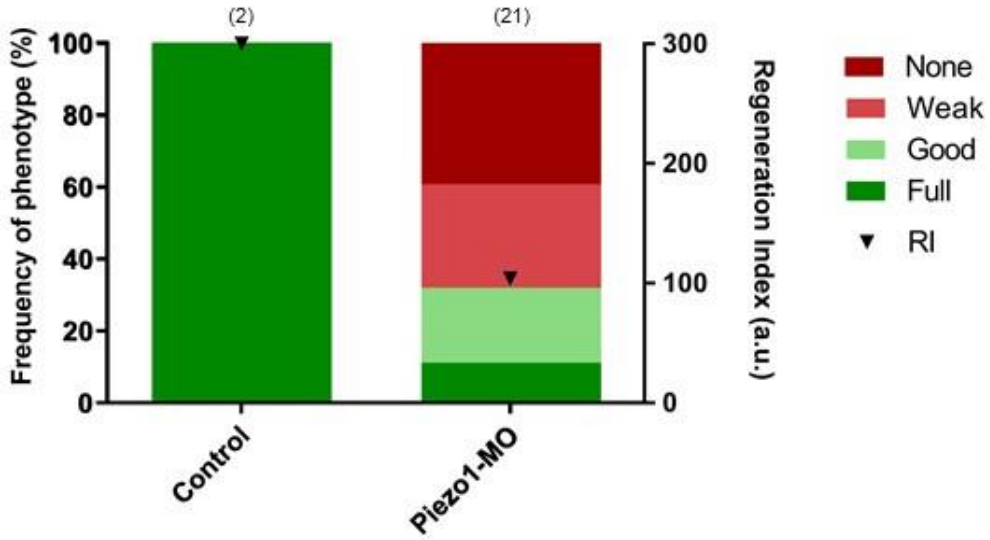

Figure 3.5 **Piezo1 candidate regulates regeneration**. Frequency of phenotypes (left) and regeneration index (right) comparing control injection (nRFP and mGFP) and Piezo1 Morpholino (770 pg and mGFP). Numbers between brackets indicates the number of tadpoles used in the experiment.

SACs regulate gene expression involved in the different phases of regeneration

According to our data, 24 hours post-amputation (hpa) is the period when SACs activation and corresponding transduction to biochemical signalling are necessary for successful regeneration. Yet, a question arises from these results: how are cells responding to these mechanosensitive channels? Tadpole's tail regeneration has some well-known signalling cascades specifically involved in wound healing and bud formation phases of regeneration. As SACs are required for these phases, we selected phase-specific markers to evaluate the downstream regulation of gene expression. From a pool of available markers **(Table 1)**, we selected *tgf-β* as wound healing and bud formation marker, and msx1 and shh as bud formation markers (Beck et al., 2003; Ho & Whitman, 2008; Lin & Slack, 2008b; Slack et al., 2004; Sugiura et al., 2004; Sugiura et al., 2009; Taniguchi et al., 2008). We collected RNA from tail regenerates and performed semi-quantitative PCR to assess fold change in gene expression. Their expression was quantified at several time points encompassing the first 24 hpa: immediately after amputation (0 hpa, baseline control), 3 hpa (wound healing), 24 hpa (bud formation). Additionally, to have a negative control of the genes *tgf-β* and *msx*1, we included the refractory period at 24 hpa. To

validate the refractory samples, we let some siblings regenerate for 7 days and computed the RI. Only the batches with low RI were processed for PCR (average RI of processed samples was ~76).

As soon as 15 minutes post-amputation the transforming growth factor, *tgf-β*, pathway is known to be activated to allow for competent regeneration. Indeed, *tgf- β* inhibition leads to alterations in size or even failure to generate regenerative structures, wound epithelium and blastema (Ho & Whitman, 2008). According to our observations, this factor undergoes a downregulation when SACs are inhibited through the action of GsMTx4 at 24 hpa. Interestingly, in the refractory period the signalling protein has its expression drastically reduced. At stage 41, when the individual is capable to regenerate, the signal of *tgf-β* is sustained indicating its requirement for a successful regenerative outcome **(Figure 3.6a)**.

The m*sx1* is a homeobox gene with functions associated to the regulation of the proliferation and growth of blastema cell. In our data, it has a consistent expression through time and development of regenerative process. Still by blocking Piezo1, TRPC1 and TRPC6 its expression is diminished at both timepoints in Gx treatment **(Figure 3.6b)** (Park et al., 2009).

The notochord synthesized ligand sonic hedgehog, *shh,* is associated with the organization and patterning of new structures (Yakushiji et al., 2009). Although the patterning of the tail is only initiated in the third phase of epimorphic regeneration (48 hpa–7 dpa), the activity of *shh* might start earlier(Taniguchi et al., 2014). Indeed, from our observations, the expression of this signalling molecule has a strong increase at 24 hpa in regenerative tails. However, the relative expression is reduced in the absence of SACs (**Figure 3.6c**).

Despite the absence of statistical significance in the results we observed a tendency in our results that lead us to further validated the three chosen phase-specific markers and, more importantly, we demonstrated that they act downstream of SACs. Therefore, the SACs-dependent mechanotransduction is reaching well-established pathways required for regeneration.

The PCRs also worked as a key validation assessment prior to RNA sequencing that we conducted (bioinformatics analysis pending) to identify further candidates and downstream signalling pathways (Bideau et al., 2021). Undeniably semi-quantitative RT-PCR is a way to verify the pattern of differential gene expression through samples. Further RNA analysis will provide us more information than the PCR and some answers to key-questions related to mechanical cues and their impact in regeneration.

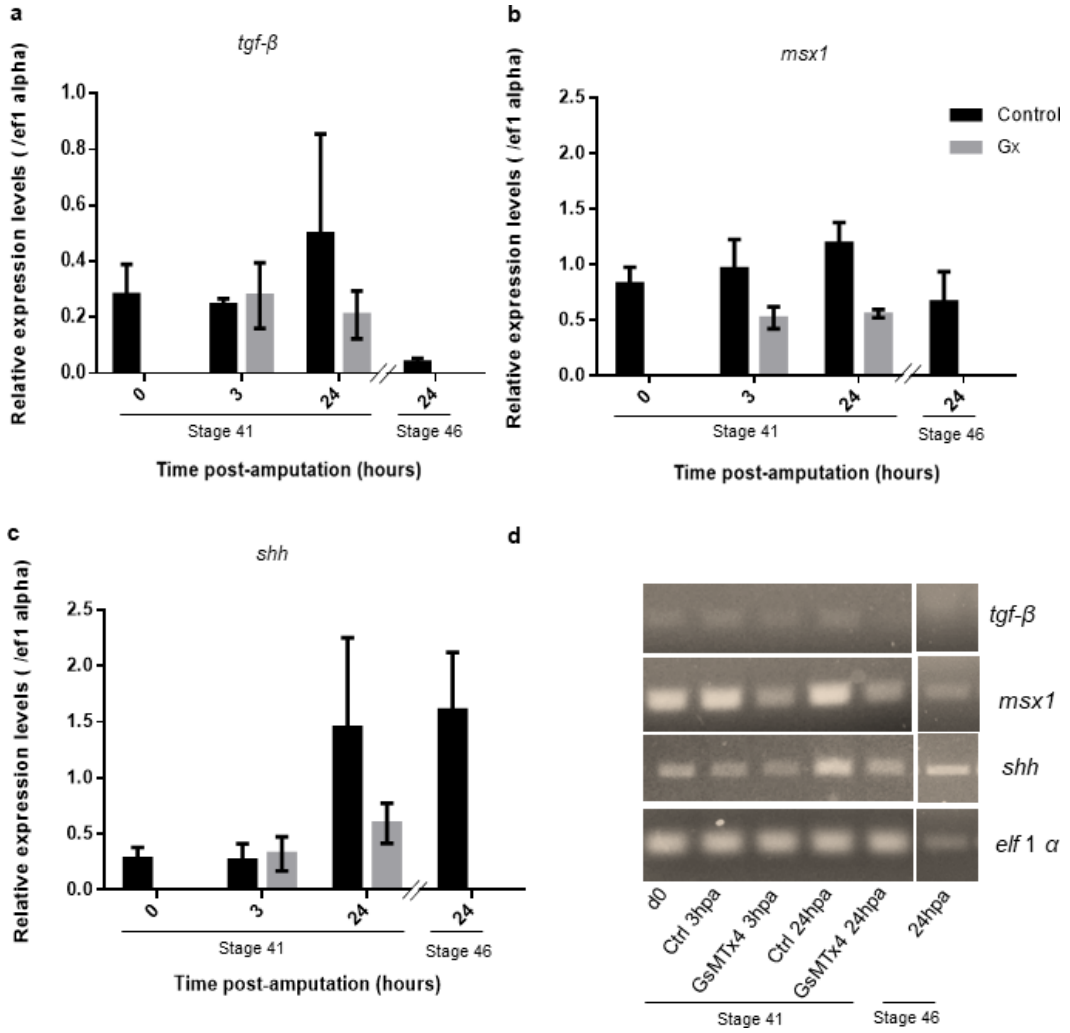

Figure 3.6 **SACs regulate the expression of important genes for regeneration**. (a) Quantitation of relative gene expression of tgf-β via reverse transcription–polymerase chain reaction (RT–PCR). (b) Quantitation of relative gene expression of msx1 (c) Quantitation of relative gene expression of shh. Data presented as mean and standard deviation. (d) Gene expression was analysed by the RT-PCR method in regenerating tails on several time points: immediately after amputation (d0), 3 hpa (wound healing), 24 hpa (bud formation) and the 24 hpa in the refractory period

4. Discussion

In the present study, we were able to manipulate how cells are sensing mechanical cues through the inhibition of SACs. Under our treatments tadpoles were not capable to regenerate properly. In recent years mechanotransduction has sparked interest in both embryonic development and regeneration fields of research (Barriga et al., 2018; Chiou & Collins, 2018; Ferreira & Zhao, 2016). Nonetheless, a gap in this field is to understand whether and which mechanical cues impact regeneration. For that reason, our work comes timely to demonstrate the transduction of mechanical cues in biochemical signaling.

Age-dependent regeneration capacity

Mostly, *Xenopus* tadpoles have a remarkably efficient and reproducible regeneration capacity throughout development. However, there is a key exception to this efficiency that only occurs in *Xenopus laevis* (Wang & Shi, 2021). In fact, the presence of this refractory period becomes an asset as a model organism to study the intricate process of regeneration. Why tadpoles at a certain age are not capable to regenerate? Our data from gene expression shows that *tgf-β* and *msx*1 have a reduced expression at 24 hpa when compared to a regenerative age. Perhaps low expression of *tgf-β* is linked to an unsuccessful activation of regeneration (Ho & Whitman, 2008). Our data also suggest that an early

activation of genes that are only required later in the successful regenerative process, like *shh* (Taniguchi et al., 2008; Yakushiji et al., 2009) .

Requirement of SACs in wound healing and early bud formation

Mechanosensitive channels are present in every cell, SACs are an integral part of this category, sensing the membrane stress and opening accordingly. These channels alternate between an open and close state depending on the extracellular environment (Sachs, 2010). Cells undergo numerous changes caused by their surroundings and SACs act as stretch sensors, allowing the influx of ions that alter cells' equilibrium and leading to further adaptation (Haswell et al., 2011; Sukharev & Sachs, 2012). Regeneration itself involves not only major phenotypical changes but also an inherent mechanical damage, making it a candidate phenomenon to address mechanotransduction. Wound healing (approximately the first 6 hours) entails epithelial cell migration from the periphery of the wound to its centre to cover the amputation site, forming the wound epithelium. Submitting tadpoles to the toxin GsMTx4, which inhibits several SACs, leads to a defective phenotype and decreased area of the regenerated tail **(Figure 3.3b; 3.4b and c)**. The first phase of regeneration is of extreme importance for the regenerative mechanism since the cells of the wound epithelium are required for regeneration to proceed by inducing the blastemal formation and maintenance (Thornton, 1957). As previously shown (Ho & Whitman, 2008), we also observed that TGF-β is expressed during early regeneration. Inhibiting SACs downregulated *tgf-β* only at 24 hpa, implying that it does not impair wound epithelium but might interfere with further sense/signalling from this structure towards the bud cells underneath. An interesting observation was the downregulation of *msx*1, the blastema marker, under the effect of GsMTx4 over time. Blastema contains the undifferentiated cells that will give rise to the lost structures (Carlson et al., 1998; Taghiyar et al., 2017). Therefore, the downregulation of this transcription repressor may be linked to the unsuccessful regeneration under the treatment (Beck et al., 2003).

Additionally, after damage the exposed structures like spinal cord and notochord are crucial elements for tail regeneration, by the secretion of several signaling molecules. The most well-known of them is Shh, which is not only important for patterning of the new member as well as for proliferation of blastema cells (Schnapp et al., 2005; Taniguchi et al., 2008). Our RT-PCR results indicates that by preventing SACs from opening results in low expression of this marker. This might be related with the low area of the tail observed at 24 hpa and following.

Together our results of channel blocking indicates that TRPC6, TRPC1 and/or Piezo1 may be responsible for some of the mechanotransduction necessary in the first steps of regeneration. These channels might be upstream of several transcription factors like Msx1, coordinating several steps in the progression of regeneration

Piezo1: potential key channel of regeneration

As a target of GsMTx4, Piezo1 become also a target of our research. Piezo1 is required in several aspects during embryonic development; yet, all the tadpoles submitted to our regeneration assay were in normal development state (He et al., 2018; Kindberg, Hu, & Bush, 2020; Koser et al., 2016; Moroni et al., 2018). Piezo1 downregulation was translated into highly defective phenotype. Indeed analysing database from previous research in *Xenopus* tail regeneration (Aztekin et al., 2019), we found that *Piezo1* is also downregulated at stage 46. Therefore, Piezo1 might be the key channel of regenerative potential and be upstream of several transcription factors related with regeneration

5. Conclusions and future research perspectives

The complex and synchronous process of epimorphic regeneration evolves in three major phenotypical phases: wound healing, regeneration bud formation and resultant regenerative outgrowth. Biochemical and mechanical cues are present in the multifactorial microenvironment where regeneration takes place, however most studies focus on the biochemical signalling characterizing the transition across these phases.

Extensive biological functions are assigned to mechanical signals and further transduction, yet their role in regeneration remains largely unclear. Our research uncovers a potential role of mechanotransduction in regeneration and its temporal requirement, in which SACs activation may be essential for the wound healing and bud formation phases. The SACs, possibly Piezo1, are pivotal for regeneration and according to our data may act upstream of core signalling pathways. Yet, his topic still is underexplored and there is much more to learn and discover. Our RNA-seq will undoubtedly elucidate us and open up a range of candidates and targets downstream the SACs in addition to those addressed in this study.

Behind the three distinguishable regeneration phases there are cellular processes like migration, proliferation and differentiation. One possibility is that by manipulating SACs, we may well be interfering with these behaviours, opening the possibilities to all the cell biology field. Furthermore, regeneration, embryonic development and regeneration share major common mechanisms, SACs may be involved in different circumstances; therefore, these findings may uncover opportunities in the broad spectrum of developmental biology.

6. References

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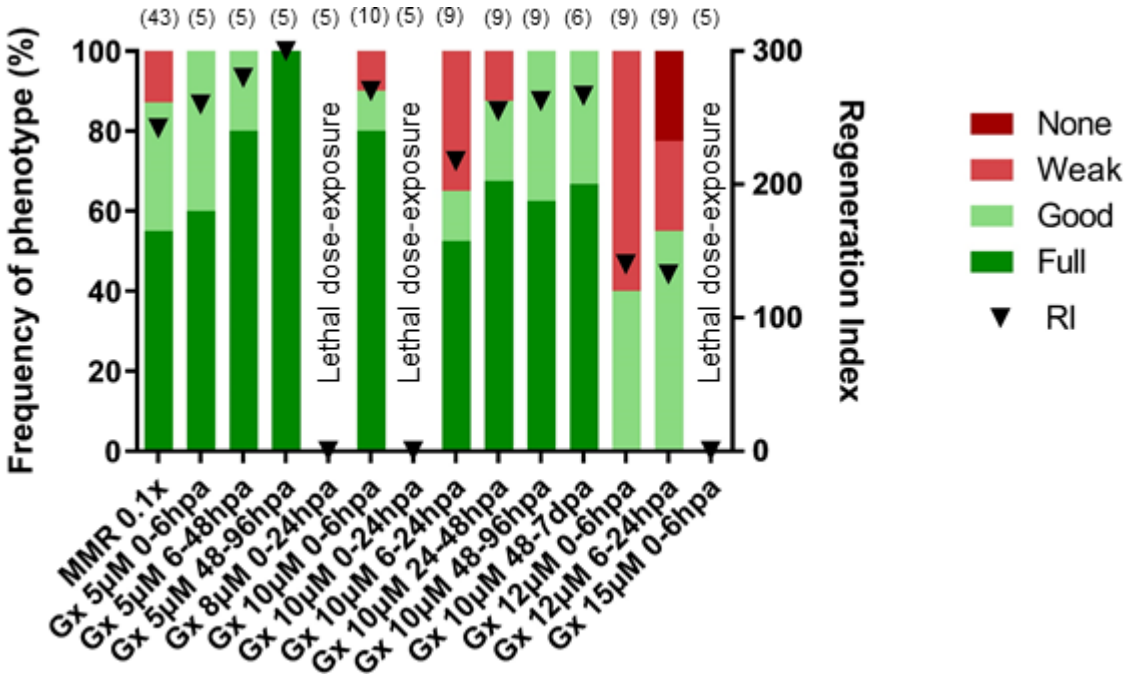
7. Attachments



Supplementary figure 1 **Dose-exposure screening to fine-tune pharmacological treatments during regeneration: GsMTx4** Drug concentration was selected based on the maximum penetrance and least mortality in the time-windows of regeneration phases. *n* biological replicates indicated in brackets.