Cell differentiation and cell fate during urodele tail and limb regeneration

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One of the most striking natural examples of adult tissue plasticity in vertebrates is limb and tail regeneration in urodele amphibians. In this setting, amputation triggers the destabilization of cell differentiation and the production of progenitor cells that extensively proliferate and pattern themselves to recreate a perfect replica of the missing part. A precise understanding of which cells dedifferentiate and how plastic they become has recently begun to emerge. Furthermore, information on which developmental gene programs are activated upon injury is becoming better understood. These studies indicate that, upon injury, an unusual cohort of genes are co-expressed. The future challenge will be to link the systems for studying dedifferentiation with activation of gene expression to understand on a molecular level how cells are 'pushed backward' to regenerate a complex structure such as a limb or tail.

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Abbreviation
GFAP glial acidic fibrillary protein
GFP green fluorescent protein

Introduction

The ability to regenerate an entire limb or tail, a remarkable trait of urodele amphibians, represents one of the most striking examples of cellular plasticity in biology. Although this field has had a long history of research, recent technical developments have made limb and tail regeneration an excellent system to understand how adult cells are transformed into progenitor cells that will rebuild the missing part. Cell-tracking studies indicate that mature differentiated cells can dedifferentiate and radically change their identity during the process of regeneration. In addition, molecular information on developmental genes that are re-expressed is accumulating. These results reveal unusual gene expression patterns at the start of regeneration that distinguish this process from development.

The challenge facing the field is to elucidate the molecular mechanisms that link the initial injury signal to the machinery that reverses differentiation and reactivates developmental gene expression programs. Does this involve simply turning off gene transcription associated with cell differentiation, or is there a larger scale dismantling of epigenetic, chromosomal machinery that stabilizes cell identity in adult tissue? An intriguing general issue is whether the cell reprogramming that occurs during regeneration is related to the nuclear reprogramming that occurs when a somatic cell nucleus is transplanted into an oocyte to form an entire organism.

During embryonic development, the totipotent egg divides to produce cells specified into three major germ layers. Subsequently, progenitor cells become committed to different tissue cell fates and finally differentiate into post-mitotic cells [1]. The progressive and stable commitment of progenitor cells to different tissue fates means that they acquire the ability to autonomously form their specified tissue even when placed in a new context. Such determination allows similar extracellular signaling molecules to be used in many different tissue contexts to control cell proliferation, patterning and differentiation without compromising tissue identity.

How does the process of regeneration impinge on the mechanism of commitment? Does the process of dedifferentiation create progenitor cells uncommitted to any cell lineage, or is some cell determination maintained? Cell tracking studies have started to reveal the extent of cell plasticity during regeneration, and in future, cell-transplantation studies will be required to reveal whether cells maintain any commitment to a cell lineage during regeneration. The use of rhodamine–dextran to label defined cell populations during regeneration has led to many of these new insights [2,3,4•]. More recently, the use of small, mutant axolotls that are optically transparent, combined with the development of single-cell electroporation of GFP plasmids have provided further breakthroughs for documenting the multipotency of individual cells during regeneration [5,6••].

Reprogramming adult tissue to make progenitor cells

Axolotl limb and tail regeneration proceed through the formation of a blastema — a zone of dividing progenitor cells — that will eventually differentiate into the new tissue. Cell-tracing studies of two cell types from the mature tissue, skeletal muscle and spinal cord radial glia,

have provided crucial evidence that multipotent progenitors are created from these cells during regeneration.

Muscle dedifferentiation

Early histological studies indicated that muscle fibres at the amputation plane might dedifferentiate by budding off mononucleate cells from syncitial muscle cells but needless to say, this idea was controversial [7,8]. However, this hypothesis has been confirmed recently by several cell-tracking experiments. Lo, Allen and Brockes [2] transplanted rhodamine–dextran-labeled cultured myotubes into the newt limb and found that one week later, the blastema contained mononucleated, labeled cells. A switch in cell fate was observed at 4 weeks when the regenerating tissue began to redifferentiate. Labeled cells contributed not only to muscle, but also infrequently to cartilage. These implantation results were largely confirmed by Kumar *et al.* [9], who used pseudotyped viruses to genetically label the cultured myotubes with the human alkaline phosphatase gene before implantation. The above studies left open the question of whether endogenous mature muscle fibres truly dedifferentiated during regeneration. To address this question, Echeverri, Clarke and myself labeled and followed the fate of endogenous muscle fibres in live tissue during axolotl tail regeneration [3]. Under these circumstances, the muscle cells four days post-amputation synchronously 'fragmented' into mononucleated cells that subsequently divided and populated the blastema. Because endogenous muscle fibres were tracked, we could estimate from the data that 17% of the tail blastema must derive from dedifferentiated muscle cells. The ultimate fate of the blastema cells could not be followed in the last study because of the dilution of the cell label. Therefore, it is not yet known if dedifferentiated muscle cells are capable of redifferentiating into cell types other than cartilage.

In Hay's histological observations [8], the nuclei that were apparently budding from fibres displayed dramatic changes in morphology, such as an increase in size, and an increase in nucleolar volume, indicating large-scale changes in nuclear organization. However, little else is known about the genetic events that drive these dramatic changes. Studies on mammalian muscle have demonstrated that the differentiated state is actively maintained at the transcriptional level [10]. This conclusion came from analysing heterokaryons — multinucleated cells created by inducing fusion of two different cell types. The fusion of muscle cells with various non-differentiated cells caused the non-muscle nuclei to express muscle-specific genes [11]. It is therefore plausible that the first step in dedifferentiation is simply to either turn-off or run down these transcriptional states. Heterkaryons made between urodele and mammalian muscle suggest that the dedifferentiation programme is a dominant programme that actively reverses the differentiation state [12]. Urodele myotubes that can dedifferentiate are able to re-enter S-phase in response to serum factors whereas mouse myotubes that cannot dedifferentiate do not re-enter S-phase in response to serum [13]. When urodele/mouse myotube heterokaryons were produced, the mouse nuclei in the heterokaryons were stimulated to re-enter the cell cycle, indicating that a cytoplasmic factor in the urodele myotube over-rode the mouse cell cycle block.

The heterokaryon experiments were limited in analysis to cell cycle re-entry and did not address issues of cell identity. Strikingly, Odelberg, Kollhof and Keating [14] showed that the expression of the homeodomain-containing protein Msx1 in mouse myotubes caused a small fraction to form mononucleate cells. These mononucleated cells went on to produce chondrocytes, osteoblasts, and adipocytes when given the correct inductive cues, suggesting that the expression of a single transcription factor was sufficient to drive them back to an earlier mesodermal precursor. *In vivo* confirmation of Msx1's role in dedifferentiation will provide further insight into its function.

Neural progenitors drive spinal cord regeneration and contribute to mesodermal structures

Whereas muscle dedifferentiation studies indicated that cells could revert to an earlier mesodermal progenitor, studies of neural progenitors during axolotl tail regeneration revealed that cells can even cross germ layer boundaries. During axolotl tail regeneration, the spinal cord regenerates via the proliferation of radial glial cells that line the central canal [15]. These cells migrate and then divide to form clones along the growing ependymal tube that extends out of the mature spinal cord as the regenerating tail grows [5]. These radial glial cells that act as the neural progenitors for regeneration appear to be equivalent to the neural progenitors of the embryonic and adult mammalian central nervous system [16–18]. They derive from similar anatomical cell layers, and express similar markers such as glial acidic fibrillary protein (GFAP) and vimentin [19]. Therefore, in contrast to muscle dedifferentiation that appears to be unique to urodeles, spinal cord regeneration involves the activation of a progenitor pool that is found both in urodeles and in mammals. Differentiated neurons apparently do not dedifferentiate to form progenitor cells although some differentiated neurons from the mature tissue migrate or are pushed into the regenerating tissue as differentiated cells [4•].

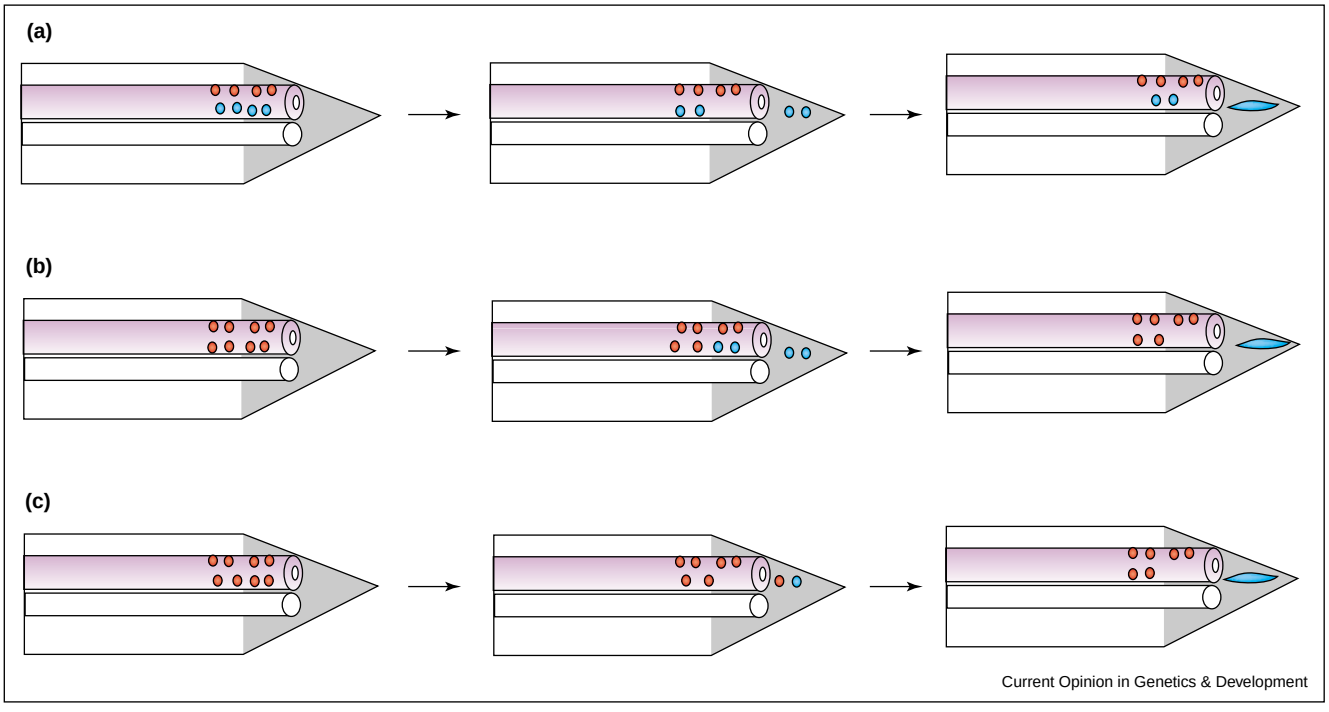
By driving green fluorescent protein (GFP) expression via a GFAP promoter, Echeverri and myself addressed whether the GFAP-positive glial cells gave rise exclusively to neural structures or if the cells also contributed to other tail tissues [6••]. Although the labeled cells produced expected cell types such as radial glia, neurons, and neural crest, surprisingly, they also frequently migrated out of the ependymal tube to form muscle and cartilage. These results indicated that during a real example of

regeneration, neural progenitor cells acquire broad multipotency and contribute to structures quite different from their embryological origin.

The distinction between ectodermal, mesodermal and endodermal lineages is made early in embryonic development, with mesoderm forming from ectoderm in response to inductive signals from the endoderm. The time in which ectoderm cells are competent to form mesoderm is restricted to a very short time window, after which they are refractory to inductive cues. In the embryo, this competence is controlled at least in part by expression of specific H1 linker histones [20]. With the finding that during regeneration, neurectoderm cells form mesoderm, the question arises, do the neurectoderm cells revert to a state where they are competent to receive mesoderm-inducing cues when they migrate into the blastema, or is mesoderm formed via a different route during regeneration?

The answer to this question is not yet known but there are several hints from embryological studies suggesting that neurectoderm may harbor a pre-potency to form mesoderm, particularly muscle. Expression of the myogenic regulatory factor Myf-5 has been described in a subset of ventro-lateral neural tube cells [21]. The action of these factors must normally be repressed, as muscle is not usually formed from the neural tube. However, if neural tube cells are placed in culture, some cells form myotubes [22]. Furthermore, ectopic expression of Pax3 in neural tube explants also leads to muscle formation [23]. These conditions appear to unleash the myogenic potential of neural tube cells. It is therefore possible that during tail regeneration, a subset of radial glia, already expressing myogenic factors, exists in the spinal cord and specifically migrates out into the blastema where their myogenic potential can be realized (Figure 1a) If this scenario is correct, it will be important to know if the 'myogenic' cells of the spinal cord are strictly restricted to form only myogenic cells during regeneration, or if this subpopulation represents a multipotent cell type capable of forming either muscle or *bona fide* neural cells. Interestingly another *in vivo* example of spinal cord cells transdifferentiating into skeletal muscle has been described. Sohal, Ali and Ali [24] infected developing chick hindbrain neural tube with B-gal virus, and later found B-gal-positive cells in head muscle. The authors in this study argue that these myogenic cells derive from the ventral

Figure 1



Models for how spinal cord progenitors switch to a muscle cell fate during regeneration. During axolotl tail regeneration, the spinal cord grows as an ependymal tube (violet) into the surrounding tail blastema (grey). Some progenitor cells from the spinal cord migrate out into the blastema to differentiate into muscle (blue spindle). A key question is: when does the conversion from a neurectodermal to mesodermal identity occur? **(a)** Mosaic model: cells within the mature spinal cord already have different, restricted potentials (blue and red). Upon tail regeneration, the muscle precursors (blue) migrate into the blastema where they can differentiate into muscle. **(b)** Progenitor cells are initially uncommitted until inductive events taking place within the ependymal tube signal some cells to assume a mesodermal potential (blue). These cells migrate out and differentiate into muscle cells in the regenerating tail. **(c)** Progenitor cells remain uncommitted while they reside in the ependymal tube. Cells that migrate out encounter mesoderm-inducing cues once they are within the blastema.

portion of the neural tube, and migrate into the surroundings via cranial nerve routes.

Another candidate subpopulation that could display such plasticity is the neural crest (see review by Le Douarin and Dupin, this issue). The ability of the neural crest to form cartilage in the head during development is well-known. Is it possible that the neural crest in the regenerating tail assumes unusual properties that allows it to form vertebrae and tail muscle, a cell fate that is normally ascribed to somite-derived cells during development? Clearly, it must be resolved whether the mesoderm-forming cells of the regenerating spinal cord derive from a specific sub-population that is already specified within the mature tissue (Figure 1a), or whether a generic neural progenitor cell is induced by regeneration-specific signals, either in the regenerating spinal cord or blastema to undergo the cell-fate switch (Figure 1b,c).

Reactivation of gene expression during regeneration

Regeneration clearly reactivates developmental gene expression programs in order for the blastema to grow and pattern itself into the proper structure. The expression of key developmental genes has been investigated during limb and tail regeneration in several studies. From these studies, it is clear that at the mid-bud blastema stage, when a sizeable population of progenitors has accumulated, the expression pattern of such genes closely resembles that in development. For example in the limb blastema, *Sonic hedgehog*, a gene that controls posterior patterning of the distal limb, is expressed in a small zone in the posterior limb blastema just as during normal development [25,26]. HoxA genes that control proximal–distal limb identity are expressed in a nested set also as in development [27].

There are, however, several observations on unusual profiles of Hox gene expression that occur early during regeneration. A key issue is whether these expression patterns either reflect or are causative of cell dedifferentiation and plasticity. *HoxA9* and *HoxA13* expression was examined during axolotl limb regeneration [27]. In contrast to the developing limb bud where these Hox genes turn on in a 3′ to 5′ sequence according to their position in the complex, during early limb regeneration these two genes are turned on coincidently in time and appear to have overlapping expression domains. These data suggests that Hox gene regulation may be quite different at the onset of regeneration when cells are being reprogrammed to enter the blastema. Gardiner *et al.* [27] argue that this expression profile reflects the patterning process of the limb blastema, where it is believed that upon limb amputation the first blastema cells formed have a finger-tip identity. Alternatively Christen *et al.* [28•] suggest that the Hox genes may themselves have a role in cellular dedifferentiation. Finally, it is also possible that reactivation of the HoxA complex from the adult state may first result in a general derepression of the complex with a subsequent re-exertion of developmental control mechanisms. In addition to the HoxA genes, the HoxC10 gene has unexpected expression profiles during regeneration [28•,29]. Whereas during development, *HoxC10* is expressed in the posterior region of the primary body axis and the hindlimb, during regeneration, *HoxC10* is also activated in the forelimb. As with the HoxA genes, it will be important to establish if this unique regulation is related to functional differences in patterning during regeneration or related to the early events of reprogramming cells.

It would be fascinating to know if the unusual Hox gene regulation is a property of the genomic organization of the axolotl Hox clusters, or is a result of unique regulatory mechanisms within regenerating cells. Would the mammalian HoxA complex display a regeneration-specific expression profile if put into axolotl cells? Studying Hox gene regulation may also provide key insights into how epigenetic states of gene regulation are dismantled during regeneration. In *Drosophila* and in mammals, the Polycomb group of gene regulators is required to propagate the repression of Hox genes during development and into adulthood [30]. The activity of Polycomb genes must presumably be deactivated upon regeneration in order to reactivate Hox gene expression. Nothing is yet known about the regulation of this gene class during regeneration.

Conclusions

The basic cellular and molecular observations that reveal plasticity have been firmly established in the urodele regeneration system. The ability to trigger reprogramming in a population of cells at the end of a limb may well make urodele limb and tail regeneration one of the most experimentally accessible systems for studying plasticity compared to mammalian stem cell engraftment into foreign tissues, or nuclear transplantation into oocytes. Furthermore, the ability to efficiently express genes in regenerating tissue via electroporation now opens the field to molecular analysis [5]. The future goals will be to use the existing knowledge and techniques to develop functional molecular assays to study reprogramming — whether this involves changes in epigenetic chromosome states or changes in transcriptional networks — and how reprogramming is triggered by injury.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Wolpert L, Beddington R, Jessell T, Lawrence P, Meyerowitz E, Smith J: *Principles of Development*. Oxford, UK: Oxford University Press; 2002.

2. Lo DC, Allen F, Brockes JP: **Reversal of muscle differentiation during urodele limb regeneration**. *Proc Natl Acad Sci USA* 1993, **90**:7230-7234.

3. Echeverri K, Clarke JD, Tanaka EM: ***In vivo* imaging indicates muscle fiber dedifferentiation is a major contributor to the regenerating tail blastema**. *Dev Biol* 2001, **236**:151-164.

4. • Zhang F, Ferretti P: **Recruitment of postmitotic neurons into the regenerating spinal cord of urodeles**. *Dev Dyn* 2003, **226**:341-348.

Spinal cord neurons in newts and axolotls were back-filled from neuromuscular junctions using rhodamine–dextran. Amputation of the spinal cord close to the labelled neurons showed that although neuronal cell bodies are 'pushed' into the regenerating spinal cord, they do not proliferate and they appear to remain differentiated. It was concluded from this study that spinal cord neurons do not dedifferentiate during regeneration.

5. Echeverri K, Tanaka EM: **Single cell electroporation as a tool to study spinal cord regeneration *in vivo***. *Dev Dyn* 2003, **226**:418-425.

6. •• Echeverri K, Tanaka EM: **Ectoderm to mesoderm lineage switching during Axolotl Tail Regeneration**. *Science* 2002, **298**:1993-1996.

In this study we traced individual GFP-expressing radial glial cells in the spinal cord and found that they migrate out into the regeneration blastema to form mesodermally derived cells: muscle and cartilage. This was the first demonstration on the single-cell level of the multipotency of regenerating cells. Furthermore, these results indicated that cells can cross from a neuroectodermal origin to a mesodermal lineage during regeneration.

7. Thornton C: **The histogenesis of muscle in the regenerating fore limb of larval *Amblystoma punctatum***. *J Morphol* 1938, **62**:17-47.

8. Hay ED: **Electron microscopic observations of muscle dedifferentiation in regenerating *Amblystoma* Limbs**. *Dev Biol* 1959, **1**:555-585.

9. Kumar A, Velloso CP, Imokawa Y, Brockes JP: **Plasticity of retrovirus-labelled myotubes in the newt limb regeneration blastema**. *Dev Biol* 2000, **218**:125-136.

10. Blau HM: **Differentiation requires continuous active control**. *Annu Rev Biochem* 1992, **61**:1213-1230.

11. Blau HM, Chiu CP, Webster C: **Cytoplasmic activation of human nuclear genes in stable heterocaryons**. *Cell* 1983, **32**:1171-1180.

12. Velloso CP, Kumar A, Tanaka EM, Brockes JP: **Generation of mononucleate cells from post-mitotic myotubes proceeds in the absence of cell cycle progression**. *Differentiation* 2000, **66**:239-246.

13. Tanaka EM, Gann AA, Gates PB, Brockes JP: **Newt myotubes reenter the cell cycle by phosphorylation of the retinoblastoma protein**. *J Cell Biol* 1997, **136**:155-165.

14. Odelberg SJ, Kollhof A, Keating MT: **Dedifferentiation of mammalian myotubes induced by msx1**. *Cell* 2000, **103**:1099-1109.

15. Egar M, Singer M: **The role of ependyma in spinal cord regeneration in the urodele, *Triturus***. *Exp Neurol* 1972, **37**:422-430.

16. Doetsch F, Caille I, Lim D, Garcia-Verdugo J, Alvarez-Buylla A: **Subventricular zone astrocytes are neural stem cells in the adult mammalian brain**. *Cell* 1999, **97**:703-716.

17. Malatesta P, Hartfuss E, Gotz M: **Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage**. *Development* 2000, **127**:5253-5263.

18. Noctor S, Flint A, Weissman T, Wong W, Clinton B, Kriegstein A: **Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia**. *J Neurosci* 2002, **22**:3161-3173.

19. O'Hara CM, Egar MW, Chernoff EA: **Reorganization of the ependyma during axolotl spinal cord regeneration: changes in intermediate filament and fibronectin expression**. *Dev Dyn* 1992, **193**:103-115.

20. Steinbach O, Wolffe A, Rupp R: **Somatic linker histones cause loss of mesodermal conpetence in *Xenopus***. *Nature* 1997, **389**:395-399.

21. Tajbakhsh S, Buckingham M: **Lineage restriction of the myogenic conversion factor myf-5 in the brain**. *Development* 1995, **121**:4077-4083.

22. Tajbakhsh S, Vivarelli E, Cusella-De Angelis G, Rocancourt D, Buckingham M, Cossu G: **A population of myogenic cells derived from the mouse neural tube**. *Neuron* 1994, **13**:813-821.

23. Maroto M, Reshef R, Munsterberg AE, Koester S, Goulding M, Lassar AB: **Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue**. *Cell* 1997, **89**:139-148.

24. Sohal G, Ali A, Ali M: **Ventral neural tube cells differentiate into craniofacial skeletal muscles**. *Biochem Biophys Res Commun* 1998, **252**:675-678.

25. Imokawa Y, Yoshizato K: **Expression of sonic hedgehog gene in regenerating newt limb blastemas recapitulates that in developing limb buds**. *Proc Natl Acad Sci USA* 1997, **94**:9159-9164.

26. Torok MA, Gardiner DM, Izpisua-Belmonte JC, Bryant SV: **Sonic hedgehog (shh) expression in developing and regenerating axolotl limbs**. *J Exp Zool* 1999, **284**:197-206.

27. Gardiner DM, Blumberg B, Komine Y, Bryant SV: **Regulation of HoxA expression in developing and regenerating axolotl limbs**. *Development* 1995, **121**:1731-1741.

28. • Christen B, Beck C, Lombardo A, Slack J: **Regeneration-specific expression pattern of three posterior Hox genes**. *Dev Dyn* 2003, **226**:349-355.

During development, *HoxC10* expression is restricted to anterior/proximal portions of the hindlimb. In contrast, during regeneration, *HoxC10* is expressed throughout the early limb blastema at the time that dedifferentiation is occurring. In the axolotl, *HoxC10* expression is also observed in the forelimb blastema even though the gene is not expressed in the forelimb during development. This result suggests a regeneration-specific regulation and/or function of *HoxC10*.

29. Carlson M, Komine Y, Bryant S, Gardiner D: **Expression of Hoxb13 and Hoxc10 in developing and regenerating Axolotl limbs and tails**. *Dev Biol* 2001, **229**:396-406.

30. Akasaka T, van Lohuizen M, van der Lugt N, Mizutani-Koseki Y, Kanno M, Taniguchi M, Vidal M, Alkema M, Berns A, Koseki H: **Mice doubly deficient for the Polycomb Group genes Mel18 and Bmi1 reveal synergy and requirement for maintenance but not initiation of Hox gene expression**. *Development* 2001, **128**:1587-1597.