The mechanism of signal transduction by the GPI anchored Prod 1 protein

Robert A Blassberg

Thesis submitted to UCL for the degree of

Doctor of Philosophy 2009

Department of Structural and Molecular Biology,
University College London

I, Robert A Blassberg confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis

Abstract

Salamanders such as the red spotted newt *Notophthalamus viridescens* and the axolotl *Ambystoma mexicanum* regenerate a number of anatomical structures following injury. Prod1 is believed to guide patterning processes operating during limb regeneration, however the molecular mechanism through which it operates is unclear. Being glycosylphosphatidylinositol (GPI) anchored, Prod1 does not make direct contact with the cytoplasm, raising questions as to how it functions in the transfer of information across the cell membrane.

The transmembrane epidermal growth factor receptor was shown to associate with Prod1, initiating MAPK signalling and resulting in the induction of matrix metalloprotease 9 expression (MMP9). MMP9 is known to be rapidly upregulated in the hours following amputation in the wound epithelium, a structure essential for regeneration formed by the migration of epidermal cells across the surface of the amputation plane. Patches of newt limb skin explanted into culture were used as a model for this process. A sheet of cells expressing MMP9 was seen to migrate out from skin patches, and this was shown to be sensitive to MMP inhibitors. Further to this, upregulation of MMP9 was seen to occur in the dermis of explanted skin patches, a layer of the skin known to be instructive to the patterning of the limb.

The relationship of Prod1s structure to its MMP9 inducing function was investigated through the creation of a series of point mutants, and it was shown that amino acids located on the α-helix of the protein were essential for this function. Axolotl Prod1 lacks a GPI anchor, however despite the requirement of newt Prod1 for GPI anchorage in order to induce MMP9 expression in either newt or axolotl cells, axolotl Prod1 was fully functional in cells from either species. There was some indication that amino acids on the α-helix may confer this ability to axolotl Prod1.

First and foremost I thank Jeremy Brockes for giving me the opportunity to develop my abilities as a member of his research group, for being generous with resources and allowing me to find my own way at the bench, and for all of his support and guidance. For these things I shall always be grateful.

I also thank Philip Gates for showing me the ropes, sharing his invaluable expertise in molecular biology and for his time making the constructs that made this project possible; Anoop Kumar for teaching me all that I now know about microscopy and immunohistochemistry, for his generosity with primary cultures, and for all of his software tuition; Azara Janmohamed for all the energy she put into deriving stably-expressing cell lines and Acely Garza-Garcia for the insights provided by her structural analysis. Thanks also go to all of the members of the Brockes group past and present who made the lab an enjoyable place to do science, for their stimulating discussion on topics science-related or otherwise, in particular James Godwin for his interest in my project and input to the thought that has gone with it.

This thesis is dedicated to all the friendly people who played their part in keeping me sane during its creation

Table of contents

List of Figures

List of tables

List of abbreviations

ADAM- a disintegrin and metalloprotease
AEC- apical epidermal cap
AER- apical ectodermal ridge
AMEM- amphibian minimal essential medium
APBS- amphibian phosphate buffered saline
ATP- adenosine triphosphate
cAMP- cyclic adenosine monophosphate
cDNA- complementary DNA
CREB- cAMP response element binding protein
CSKA- cytoskeletal actin
DAG- diacylglycerol
DEPC- diethylpyrocarbonate
DIG- digoxigenin
DMEM- Dulbecco's modified Eagles medium
DMSO- dimethylsulfoxide
DNA- deoxyribonucleic acid
DNAse- deoxyribonuclease
dNTP- deoxyribonucleotide triphosphate
DPBS- Dulbecco's phosphate buffered saline
ECM- extracellular matrix
EDTA- ethylenediaminetetraacetic acid
EGF- epidermal growth factor
EGFR- epidermal growth factor receptor
ER- endoplasmic reticulum
ERK1/2- extracellular signal-regulated kinases 1 and 2
ES cell- embryonic stem cell
EST- expressed sequence tag
FAK- focal adhesion kinase
FGF- fibroblast growth factor
GAPDH- glutaraldehyde phosphate dehydrogenase
GDP- guanosine diphosphate

GEF- guanine nucleotide exchange factor
GPCR- G protein-coupled receptor
GFP- green fluorescent protein
GPI- glycosylphosphatidylinositol
GTP- guanosine triphosphate
GTPase- guanosine triphosphate hydrolase
HB-EGF- heparin binding epidermal growth factor
JAK- janus kinase
JNK- c-jun N-terminal kinase
kD- kilodaltons
KGF- keratinocyte growth factor
nEGFR- newt epidermal growth factor receptor
MAC- membrane attack complex
MAPK- mitogen-activated protein kinase
MEK- mitogen-activated protein kinase kinase
MEKK- mitogen-activated protein kinase kinase kinase
MEM- minimal essential medium
MMP- matrix metalloprotease
mRNA- messenger ribonucleic acid
mTOR- mammalian target of rapamycin
nGFP- nuclear localised green fluorescent protein
NRTK- non-receptor tyrosine kinase
PBS- phosphate buffered saline
PCR- polymerase chain reaction
PD- proximodistal
PDGFR- platelet derived growth factor receptor
PDK1- phosphoinositide dependent kinase 1
PFA- paraformaldehyde
PI3K- phosphatidylinositol 3-kinase
PIPL-C- phosphatidylinositol phospholipase-C
PIP2- phosphatidylinositol 4,5-bisphosphate
PIP3- phosphatidylinositol 3,4,5-trisphosphate
PKA- protein kinase A
PKC- protein kinase C
PLA2- phospholipase A2
PLC- phospholipase C
PMF- plethodontid modulating factor
PTB- phosphotyrosine binding domain
qRT-PCR- quantitative real-time polymerase chain reaction
RA- retinoic acid
RACE- rapid amplification of cDNA ends
RNA- ribonucleic acid
Rnase- ribonuclease
RTK- receptor tyrosine kinase
SEM- standard error of the mean
SH2- Src homology domain 2
SH3- Src homology domain 3
SDS- sodium dodecyl sulphate
STAT- signal transducer and activator of transcription
TE- tris-EDTA

TFP- three-fingered protein
TGF- transforming growth factor
TIMP- tissue inhibitor of matrix metalloprotease
tRNA- transfer ribonucleic acid
TSA- tyramide signal amplification
UTR- untranslated region
uPAR- urokinase-like plasminogen activator receptor

1 Introduction

1.1 Regeneration: A process fundamental to life?

Defined by the Oxford Dictionary as regrowth, regeneration operates on all known levels studied in biology, and is arguably central to the essence of life itself. Scientific investigation in the 21st century has been facilitated by a technological explosion providing opportunities for the study of the nature of all things at levels previously unimaginable, practically or conceptually. When put in the context of our growing understanding of the truly staggering enormity of the universe, the number of possible worlds on which life could evolve according to the principles formulated by Darwin are so many that it is arguably harder to accept that its occurrence be unique to earth than that all the examples we observe have come about as a result of pure chance. Advances allowing the imaging of biological molecules, and the biochemistry that has developed alongside has pushed the scope of biology to a level of resolution whereby life of all kinds can be described at the fundamental level without reference to logic other than that formulated in physics and chemistry. Enquiry into topics as diverse as the cause of disease or the manifestation of mind can now be addressed in the context of the now seemingly unquestionable established principles describing the mechanics of the operation of the cell, the basic unit of any free-living organism. It is at this level, the level of information storage by DNA, and the transfer of this information by the cell into the diversity of proteins determining the higher order biological processes of an organism through their physical and chemical properties, that regeneration can be seen to be central to life. Even in the absence of growth and division, a cell requires that it should be able to replace proteins no longer fit for purpose, due to the damage inevitably sustained in any environment capable of supporting the chemical reactions required for life. In order to accurately replace damaged proteins, the cell must equally maintain the fidelity of its blueprint. Without such mechanisms, any form of life would not exist for long. Even with the ability to regenerate damaged proteins and DNA, a cell will eventually die. Although the specifics of the basis of cellular mortality are widely debated, limited life-span is clearly observed to be a characteristic of all known cells, and it is not hard to accept that eventually the systems repairing damage themselves become

irreparable. Life has found a strategy for overcoming the inevitable eventual failure of cellular processes in the form of reproduction. The distribution of a DNA blueprint able to support life into independent cells, and mechanisms to pass this blueprint on before repair mechanisms become overwhelmed, ensures the potential for continued existence of a life form, even if each individual will eventually cease to be. In order for a life-form to have a chance of indefinite existence it must be able to maintain the integrity of the DNA blueprint and all cellular processes associated with reproduction for long enough to reproduce the next generation, placing the ability to generate anew essential damaged cellular components, even if for only a limited period, at the heart of our current conception of what it is to be alive.

1.2 A study of regeneration across phyla

1.2.1 Regenerative capability and reproduction

This broad definition of regeneration may be better referred to as cellular-repair than regeneration, however in their essence the processes are indistinguishable. The field of regeneration research concerns itself with the mechanisms acting to replace tissues lost by an organism, however at this level it is still difficult to make clear definitions of what constitutes regeneration. In the case of some organisms capable of asexual reproduction, detachment of a section or sections of tissue from the main body results in the generation of new organisms. For example, strains of planarians, species of the phylum Platyhelminthes (flatworms), are able to detach sections of their body in order to reproduce asexually whilst others reproduce sexually (Kobayashi and Hoshi, 2002) (Fig.1.1A). Hydra (Fig.1.1 B), species of the phylum Cnidaria studied for their regenerative abilities, reproduce asexually by budding, however strains exist which can reproduce sexually (Galliot and Schmid, 2002). These organisms posses a pool of stem cells which are constantly proliferating and differentiating to replace lost cells, and when cut into two pieces (or more in the case of planarians), both planarians and hydra, be they of sexual or asexually reproducing strains, regenerate the structures missing from each piece (Galliot et al., 2006) (Reddien and Sanchez Alvarado, 2004). The precursor cells for this process are located in the body column in hydra and are composed of

interstitial cells, endodermal and ectodermal epithelial cells, each of which regenerates specific cell lineages (Galliot et al., 2006), whereas the stem cells of planaria, the evenly distributed neo-blasts, are considered to be totipotent (Reddien and Sanchez Alvarado, 2004). Asexually reproducing planarians can be induced to develop sexual characteristics and mate (Kobayashi and Hoshi, 2002), and the interstitial cells found in hydra produce gametes in the sexually reproducing strain (Galliot et al., 2006). That the asexual strains of these species have the underlying potential to reproduce sexually implies that they have undergone a mutation event whereby sexual reproduction was lost at some point in the history of their evolution. Podocoryne, another regenerating species of Cnidaria, reproduces sexually. The larvae metamorphose into a polyp, similar to hydra, however instead of budding off polyps as in the case of hydra, it instead forms buds that differentiate into the sexually reproducing medusa stage of the life cycle. This process of medusa formation requires that the polyp cells forming the bud de-differentiate, as the cell types and tissues of the medusa are distinct from those of the polyp (Galliot and Schmid, 2002).

We see then from these examples that i) asexually reproducing species such as planarians and hydra capable of budding to produce progeny are also highly regenerative ii) these regenerative capabilities are also seen in the sexually reproducing strains of these species iii) as sexual reproduction is ancestral, the ability to reproduce asexually may have evolved as a consequence of an acquisition of regenerative ability iv) the process of budding in Podocoryne involves the loss off differentiated characteristics by cells followed by re-differentiation whereas in planarians and hydra it does not.

Teleost fish such as the zebrafish Danio rerio are also studied for their regenerative abilities. Similar to the planarians and hydra, the tails of teleosts grow continually over the life of the fish, and when amputated rapidly regenerate all the missing structures (Akimenko et al., 2003). Unlike planarians and hydra however, a teleost will not regenerate from both pieces when cut in half. This example is put forward to illustrate that even in species with the underlying mechanisms required for regeneration, regenerative potential is limited by other factors. In the case of teleost fish, the animal would die from

blood-loss long before it stood a chance of initiating the substantial regenerative response that would be required to replace all of the structures lost. Even if one half of a fish containing both the brain and the heart were able to stay alive, the complexity of the anatomy of fish compared to flatworms would require the replacement of a significantly greater number of tissues, and consequently a source of a far greater diversity of cell types in order to completely regenerate. The most complex anatomical structure known to regenerate in any animal is the limb of urodele amphibians (Fig.1.1C) (Brockes, 1997). Limb regeneration requires the replacement of bone, cartilage, muscle, connective tissue, vasculature, nerves and skin. Unlike previously mentioned species however, adult urodeles are not continually growing and do not have a large pool of stem cells.

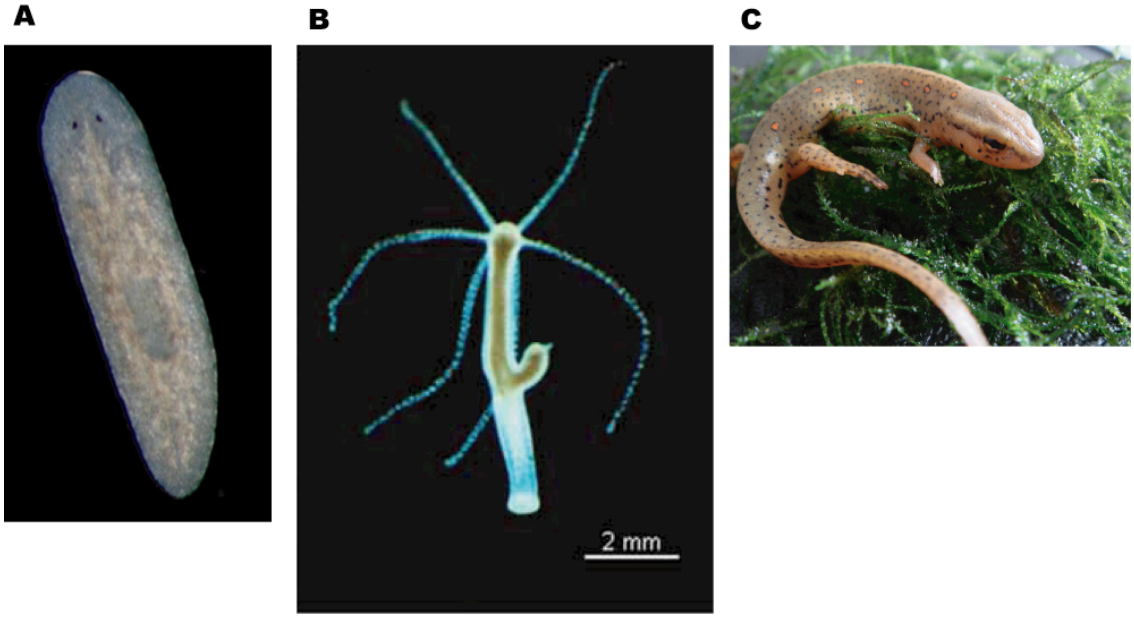


Figure 1.1 Regenerative model organisms

(A) The planarians are species of the phylum Platyhelminthes. Schmidtea mediterranea are a commonly studied species and it has been claimed that they can regenerate anew from a fragment 1/279th the size of their body. Picture courtesy of A.Aboobaker. (B) The hydra are species of the phylum Cnidaria with a defined body axis and will regenerate from both halves following bisection. Hydra reproduce by asexual budding; A bud is forming in this example. Picture adapted from (Bosch, 2009) with permission (C) The red spotted newt Notophthalmus viridescens is a urodele amphibian of the family salamandridae. N. viridescens and other salamanders are the most regenerative adult vertebrates. Picture courtesy of J.Godwin.

In mammals, regeneration also occurs, albeit to a lesser extent than in the species previously discussed. Precursor cells of limited developmental potential are found in the tissues of the body, and are able to regenerate skin (Jones et al., 1995), intestinal epithelium (Potten et al., 1997), blood cells (Broxmeyer and Williams, 1988), immune cells (McCune et al., 1988), bone (Caplan, 1991), muscle (Yablonka-Reuveni et al., 1987), liver (Michalopoulos and DeFrances, 1997) and nerve (Gage, 2000) (Stocum, 2001). These cells are able to replace cells lost due to turnover associated with the cells function, for example repairing damage to skin or gut epithelia which are exposed to environments in which some degree of damage is inevitable, or the replacement of muscle cells damaged by over exertion, however the extent of regeneration of these tissues is limited when compared to the ability of the urodele limb to regenerate. For the sake of clarity, the replacement of cell types from existing precursor cells as it occurs in mammals may be better termed tissue repair when comparing it with the process of regeneration as occurs in the urodele, however as discussed shortly, there may be no basis for drawing such a distinction. The most regenerative tissue in mammals is the liver, which is able to regenerate through a process involving both the proliferation of differentiated liver cells (compensatory hyperplasia) (Michalopoulos and DeFrances, 1997) and the mobilisation of resident precursor cells (Alison et al., 1996). Here a distinction can be drawn which underlies the difference in the regenerative capabilities of urodeles compared to mammals. Although urodeles (Morrison et al., 2006) and mammals are similar in that both have limited populations of adult precursor cells able to proliferate and produce new cells when required and are not in a continual state of growth like planarians and hydra of which a high proportion of the total cells of the body are proliferative, unlike mammals, urodeles are able to mobilise many of their existing differentiated cell types to become proliferating stem cells by a process of de-differentiation (Brockes and Kumar, 2002) (discussed in greater detail below). The closest process to de-differentiation occuring in mammals is arguably the loss and subsequent re-expression of myelin by Schwann cells as they divide during axon regeneration (Harrisingh et al., 2004).

1.2.2 Categorising mechanisms of regeneration

Regeneration of missing structures can occur by seemingly distinct mechanisms. When hydra are cut into two pieces, regeneration is preceded by a halt in the proliferation of its usually actively dividing population of cells (Holstein et al., 1991) and results from the re-organisation and differentiation of existing precursor cells to produce two regenerates half the size of the original (Galliot and Schmid, 2002), a process termed morphallaxis by T.H.Morgan (Morgan, 1901). During urodele limb regeneration, the stump remains largely unchanged and a structure termed the blastema forms (discussed in more detail below) and grows by the proliferation of undifferentiated cells, eventually producing sufficient new tissue to differentiate and replace the missing structures (Stocum, 2004), a process termed epimorphosis by T.H.Morgan (Morgan, 1901).

Comparisons between the different regenerative response can therefore be made on the basis of i) whether proliferation of cells mobilised from the remaining stump tissue regenerate the missing structures (epimorphosis) or whether the remaining stump tissue becomes respecified to regenerate the missing structures in the absence of proliferation (morphallaxis) ii) whether de-differentiation occurs to generate proliferative precursor cells, as in the formation of the urodele limb blastema (Brockes and Kumar, 2002), Schwann cell de-differentiation during axon regeneration (Harrisingh et al., 2004) or Podocoryne medusa budding (Galliot and Schmid, 2002), or whether precursor cells exist prior to the regenerative response as seen in urodeles (Morrison et al., 2006), mammals (Stocum, 2001), planarians (Reddien and Sanchez Alvarado, 2004) or Cnidaria (Galliot et al., 2006). When planaria are cut into many pieces, the process of regeneration involves the extensive remodelling of the body-plan and also the formation of a blastema. Neoblasts are observed immediately to change their differentiation characteristics without dividing, and division of cells in the blastema is also not observed (Agata et al., 2007) indicating morphallaxis as a more appropriate classification of planarian regeneration. Agata et al argue however that as the differentiated tissues of the stump remain differentiated long after the blastema has formed, that the process of regeneration is also arguably epimorphic and that these terms

should therefore be abandoned. They view the blastema instead as an organising centre rather than being the source of the regenerating tissue. This is a clear example of how analysis of biology at a molecular level, rather than at the gross level permitted at the time that many classical experiments were carried out, can change the way a problem is viewed.

1.2.3 The evolutionary relationship between regenerating species

Thus far we have looked at regeneration both in terms of its relationship to reproduction, which as argued at the beginning of this essay is of central importance when considering the concept of regeneration from an evolutionary perspective, and in terms of the origin of the tissue comprising the regenerate. Such categorisation of the regenerative attributes of species in an attempt to rationalise the evolutionary relationships of the regenerative responses does not make things much clearer however, as we see from the inability to precisely categorise the planarian regenerative response as morphallactic or epimorphic whereas for urodeles and hydra it seems clear, or that planarians and hydra group with mammals in that regeneration results from pre-existing precursor cells, and may in fact be seen as tissue repair rather than 'regeneration', whilst at the same time being so different in terms of their reproductive cycle. Similarly cnidarians, planarians and teleosts all exhibit continual growth, however teleosts do not posses the ability to reproduce asexually.

It is a long-standing question whether all metazoans shared a common ancestor with the ability to regenerate, which was subsequently lost in some species and maintained and elaborated upon in others, or whether the ability to regenerate has arisen through convergent evolution in different species. In support of the idea that at least some regenerating species share a regenerating common ancestor is the wide distribution of regeneration across phyla (Sanchez Alvarado, 2000), making it unlikely that the ability arose independently in all of these cases. An alternative view is that regeneration is instead a by-product of the processes maintaining the integrity of a given structure of an organism. The nematodes are an example of metazoans that are unable to carry out tissue repair or regeneration. Nematodes reproduce sexually and exhibit cell constancy (Hughes, 1989), that is to say, once

development has occurred, the number of somatic cells is fixed and cells do not turn over, consistent with the possibility that regeneration, rather than being ancestral to all metazoans, is instead a function which has emerged from tissue repair mechanisms, which themselves are not present in all phyla (Brockes and Kumar, 2008). Whether 'regeneration' should really be seen as distinct from tissue repair is debatable as mentioned previously.

If we accept that there is a spectrum of regenerative capabilities ranging from that seen in nematodes, to mammals, to urodeles, to hydra to planarians on the basis of how much 'stump' tissue is required to permit regeneration then we see there is a broad correlation with the degree of cell renewal (tissue repair) occurring. The underlying mechanisms facilitating tissue repair may represent the evolutionary relationship between regenerating metazoans and help to explain its widespread distribution, whilst allowing for the possibility that the specifics and degrees of the different regenerative processes evolved independently in different species. Although de-differentiation is not unique to urodeles, as exemplified by budding of Podocoryne medusa or Schwann cell proliferation, it underlies their ability to regenerate extensively. As discussed previously with the teleosts as an example, other features of an animal may antagonise its regenerative abilities. As the limb of a urodele is highly specialised in terms of its structure and contains largely differentiated cell types, it can be argued that it requires a different mechanism to regenerate missing structures compared to, for example, hydra, as it is unable to undergo morphallaxis, whilst basing the process on underlying cellular mechanisms facilitating tissue regeneration. The fact that closely related species exist with markedly different regenerative abilities indicates that once acquired, the mechanisms for regeneration may have subsequently been lost in a number of examples (Brockes and Kumar, 2008).

The widespread ability to regenerate at some level throughout metazoans opens up the possibility that it may be possible to in some way augment or manipulate existing biological systems in humans in order to bring about enhanced regeneration. The lifespan of hydra (Bosch, 2009) and planarians (Newmark and Sanchez Alvarado, 2002) have been argued to be effectively

limitless due to their ability to continually turnover the cells of their body. Theoretically, regeneration research may then, for better or for worse, produce methods to drastically increase the life-span of human beings, or at the extreme, even put an end to human mortality. It has to be questioned why the majority of animals have limited life spans however. It has been suggested that evolutionarily, limiting the potential of adult cells to divide may act as a balance against cancer (Sharpless and DePinho, 2005), which is perhaps too complex a phenomenon to be manifested or cause problems in lower metazoans such as platyhelminthes or cnidarians.

1.3 Applying principles of regeneration in wider biological contexts

Despite their ability to de-differentiate somatic cells to produce proliferative precursor cells, the incidence of cancer in urodeles is low compared to mammals (Brockes, 1998). The re-entry into the cell cycle of newt myotubes in response to serum has been shown to involve the inactivation of the tumour suppressor protein Rb, and this can be inhibited by mammalian p16 (Tanaka et al., 1997), a cyclin D CDK4 inhibitor (Serrano et al., 1993). Mouse myotubes do not re-enter the cell cycle in response to serum unless they lack Rb (Schneider et al., 1994), indicating a distinct difference between the ability of newts and mice to regulate the withdrawal of cells from the cell cycle. The study of regeneration may therefore shed light on the fundamental processes of the cell cycle and their relationship to cancer. The study of regenerative processes in model organisms may also prove complementary to the field of embryonic stem-cell (ES cell) research. As argued earlier, links between regeneration and reproduction are evident, and it is hoped that totipotent cells from the early embryo will prove a useful source of biological material for use in regenerative therapies. A number of issues overhang this field of research, both practical and ethical in nature. Due to their undifferentiated state, ES cells are capable of becoming cancerous when implanted into tissue (Evans and Kaufman, 1981) and therefore require accurate differentiation prior to implantation. Although many cell-types have been differentiated in culture as proof-of-principle, the technology is still held back by an inability to produce absolutely pure

populations (Keller, 2005). The source of ES cells for therapy poses possibly the greatest problem for both technical and practical reasons. Firstly, as ES cells are derived from an embryo, for them to be widely available for therapeutic purposes would require a large, reliable source of human embryos. Whether embryo donation on this scale is practical or ethical is debatable. As ES cells are from a donor and not the patient, they are not genetically matched and therefore subject to immune-rejection. This can in theory be overcome by replacing the nucleus of a donor embryo with one from a patient (Wilmut et al., 1997). Whether society is prepared to open the door to therapeutic reproductive cloning is another matter however.

Whether implanted stem cells will integrate properly into the tissue of a patient is likely to depend on a number of factors. Firstly, the extent of differentiation, or specific type of the implanted cells, and secondly, whether the appropriate signals exist to appropriately pattern cells in the location of implantation. It is these purely practical points which may be overcome through applying knowledge of the processes occurring during regeneration in model organisms and during normal development to the field of ES cell therapy.

Ideally, ES cells would not be used at all due to the variety of ethical issues they pose. Knowledge of the processes occurring in highly regenerative organisms may in theory lead to a means of either making use of the resident populations of stem cells present in adults, or of manipulating post-mitotic adult cells to de-differentiate and proliferate. Clearly, such interventions will still require the correct cues to produce appropriately patterned regenerated tissue, however carrying out these processes in situ may allow the native environment to be made use of and reduce the requirement for cues to be provided by external sources. The urodele limb is an ideal model within which to study both the processes of de-differentiation and patterning (Brockes and Kumar, 2005), and represents the focus of the research conducted in this thesis.

The ability to regenerate limbs varies across a spectrum. Adult urodeles are able to regenerate whole limbs (Brockes, 1997), as are metamorphosing anurans (Stocum, 2004), whilst fetuses of mammals such as humans are able

to regenerate only the tips of digits (Allan et al., 2006). In this sense, limb regeneration in urodeles is distinct from that in anurans and mammals, as in urodeles, differentiated adult cells contribute to the regeneration by de-differentiating and proliferating (Brockes, 1997), whereas in anurans (Dent, 1962) and mammals (Allan et al., 2006), it is only whilst cells still exhibit some of the plasticity associated with the embryonic state that regeneration is possible. As the nature of the plasticity of de-differentiated newt cells is unlikely to be fundamentally different to that observed of embryonic cells, and indeed it has been demonstrated earlier that de-differentiation is not unique to urodeles, it raises the possibility, at least theoretically, that adult mammalian cells might be manipulated to loose some of their differentiated characteristics and then drive regeneration under the guidance of the normal processes governing development (discussed in detail below). It must be considered however that the length of time it takes for an adult human to grow a fully sized limb is significantly longer than that taken by urodeles, making it unlikely to be realistic practically, if not in principle, for a human to regenerate an entire limb. Even if the regeneration of an entire adult human limb is unrealistic however, there is likely much to learn from this fascinating ability of the urodele which may guide future therapeutic interventions.

1.4 The process of urodele limb regeneration

The molecular processes governing urodele limb regeneration are beginning to be understood, adding much needed detail to the models formulated from the observations of classical experiments. Limb regeneration can be considered to occur in three distinct phases (Endo et al., 2004) each of which shall be described in detail:

i) Wound closure and formation of the apical epidermal cap (AEC)
ii) Formation of blastema cells
iii) Limb growth and morphogenesis

1.4.1 Wound healing

Wound healing in urodeles is distinct from the process occurring in mammals in that it is not accompanied by scarring (Roy and Levesque, 2006). In mammals, wounding is rapidly accompanied by the formation of a fibrin clot, resulting from the release of blood plasma coagulation factors from the vasculature and their contact with tissue factor expressed on the surface of stromal cells (Daubie et al., 2007). Once this clot is formed, granulation tissue fills in the wound, and epidermal epithelial cells migrate through the fibrin clot and over the surface of the wound to repair the skin (Singer and Clark, 1999). During these processes, fibroblasts are continually depositing collagen (Clark et al., 1995) into the wound site, and as healing progresses, fibres of collagen form (Welch et al., 1990), giving the repaired tissue tensile strength and contributing to the lasting scar tissue (Bailey et al., 1975). The re-establishment of new and the re-modelling of old extracellular matrix occurring during wound healing requires the activity of a variety of matrix metalloproteases (MMPs) (discussed in greater detail below), which proteolyse ECM proteins and allow the migration of invading cells into tissue (Bullard et al., 1999) (Pilcher et al., 1997). Collagen synthesised by fibroblasts and deposited into the wound can also be degraded by MMPs (Pilcher et al., 1997), providing a mechanism by which to regulate the level of collagen present in the wound as it heals.

Skin healing in urodeles on the other hand is not accompanied by the formation of a significant fibrin clot, with wounds instead being closed by the rapid migration of epidermal keratinocytes across their surface. These migrating cells do not divide and have been shown to rapidly upregulate the expression of matrix metalloprotease-9 (MMP9) (Satoh et al., 2008). Like mammalian wound healing, collagen fibres form beneath the epidermis, however remodelling of the matrix restores the original structure of the skin without leaving a scar (Endo et al., 2004). When MMPs are inhibited however, a scar-like structure can be seen to result which correlates with a complete inhibition of regeneration (Vinarsky et al., 2005). MMPs cleave fibrin, fibrinogen and other factors involved in blood clot formation as well as matrix proteins (Sternlicht and Werb, 2001), thus the regulation of MMPs in urodeles may be a factor in the differences in clot formation and subsequent scarring seen between them and mammals, and this

difference may in turn contribute to the ability of urodeles to establish an environment able to support the accurate regeneration of tissue by avoiding the formation of an inhibitory scar.

That mammals require the formation of a fibrin clot to precede wound healing whilst urodeles do not must reflect some difference in the selection they have undergone during evolution. The human clotting cascade leading to fibrin clot formation is tightly regulated and highly complex. When compared to turtles, species which diverged from mammals far back in evolutionary time, a higher degree of complexity is observed in the mammalian clotting cascade (Soslau et al., 2004), indicating that it has been highly developed by evolution and is of significant importance. One possible explanation for the difference in requirement between humans and urodeles is that urodeles are able to rapidly constrict blood vessels to prevent large-scale blood loss (Schmidt, 1968).

1.4.2 The apical epidermal cap

Amputation of the urodele limb results in the same processes occurring during wound healing, with a sheet of MMP9 expressing epithelial cells rapidly migrating across the surface of the stump (Satoh et al., 2008). The single layer of epithelium covering the wound, known as the wound epidermis, begins to thicken by proliferation and forms a structure termed the apical epidermal cap (AEC) (Fig.1.2 A) (Christensen and Tassava, 2000). It is beneath this AEC that the structure of the blastema (Fig.1.2B) will form. Figures 1.2C and 1.2D show the stages of regeneration from amputation, through blastema formation, to full digit stage regeneration.

Few molecular markers exist for the AEC, however it is observed that MMP9 expression persists for 72hrs in epidermal cells fated to form the AEC, whereas in skin wounds that will not form blastemas, MMP9 expression is downregulated within 24hrs (Satoh et al., 2008). The expression of the transcription factor Sp9, a molecule known to be expressed in the apical ectodermal ridge (AER) of the developing limb bud involved in limb outgrowth in developing chick, mouse and zebrafish embryos (Kawakami et al., 2004) is also expressed by the AEC (Satoh et al., 2008), indicating some similarity between these structures. Sp9

expression is upregulated in the keratinocytes of the AEC by exogenous FGF7/ keratinocyte growth factor (KGF) (Satoh et al., 2008). Exogenous KGF has also been shown to induce AER formation in chick limb buds (Yonei-Tamura et al., 1999), further indicating similarity between these structures. Likewise, ectopic FGF2 induces expression of the transcription factor Dlx3 in the AEC of denervated limbs (Mullen et al., 1996). Dlx genes are homologs of drosophila distal-less which are expressed in the AER and regulated by fibroblast growth factors (FGF) during limb development and known to be involved in the outgrowth of the limb (Pueyo and Couso, 2005), indicating further similarity between the role of FGFs during development and limb regeneration, and the AER and the AEC.

The early processes occurring to establish the AEC and the blastema have been investigated using the 'accessory limb model' in which a blastema can be initiated at a wound that would usually regenerate only skin by deflecting a nerve to the vicinity (Endo et al., 2004). A threshold level of innervation is required in order for a blastema to develop at an amputation plane (Singer, 1952), and as mentioned previously, it has been shown by the accessory limb model that the collagen matrix that accumulates below a skin wound does not form in the presence of a deflected nerve, the indication of this being that an early distinction between blastema formation initiated by the nerve and wound healing is the arrangement of the ECM beneath the AEC.

The AEC has been shown to be essential for the growth of the blastema, as when its formation is prevented either by grafting a piece of skin over the amputation plane or inserting a freshly amputated limb into the body cavity regeneration does not occur (Mescher, 1976) (Goss, 1956a). Removal of the AEC at different times during the regeneration process and inserting the limb into the body cavity results in truncation of the limb along the proximodistal (PD) axis (Goss, 1956b), demonstrating it plays a role in the maintenance of limb outgrowth**.** Denervation after the blastema has begun to grow leads to a halt in the proliferation of blastema cells (Maden, 1979). The cells of the blastema are able to undergo differentiation following denervation, resulting in a miniature limb being regenerated from the undersize blastema (Singer and Craven, 1948).

1.4.3 The origin of blastema cells

Up until this point the blastema structure has not been considered in greater detail than a mound of proliferating cells from which the limb regenerates, which requires signals from the nerve and the AEC in order to grow. The origin of blastema cells has previously been cited as resulting from the de-differentiation of stump cells and this shall be discussed now in greater detail. One question is whether the adult stem cells present in the limb also contribute to the blastema along with de-differentiated cells. Another important question is whether, once de-differentiated, blastema cells are restricted in their potency, or whether they can transdifferentiate into cell types other than those form which they originated.

A study tracing the fate of muscle cells by green fluorescent protein (GFP) genetic labelling indicated that like muscle, cartilage is also restricted to only produce cartilage in the regenerate and that nerve associated Schwann cells produce only Schwann cells (Kragl et al., 2009). This highlights a general principle that blastema cells are not homogenous, and are instead composed of a range of cell types, each with restricted potential. It had been demonstrated previously that blastema cells are not uniform in their characteristics through the identification of a subpopulation of blastema cells derived from Schwann cells that express the blastemal marker 22/18 (Kintner and Brockes, 1985), but what is now clearer is that different populations of cells have distinct potentials.

Interestingly, cells derived from the dermis (likely to be fibroblasts) are observed to have wider potential to contribute to different tissues (Kragl et al., 2009). Following the onset of blastema formation, cells from the dermis are observed to migrate into the blastema and become widely distributed throughout it as regeneration proceeds (Gardiner et al., 1986). These cells have been shown to regenerate cartilage, connective tissue and tendons. Cells of dermal origin are observed within muscle tissue, however they do not contribute to muscle fibres or satellite cells (Kragl et al., 2009), indicating that although they become widely dispersed throughout the growing blastema, their potential is still limited. To this point therefore, no individual totipotent blastema cell type has been identified.

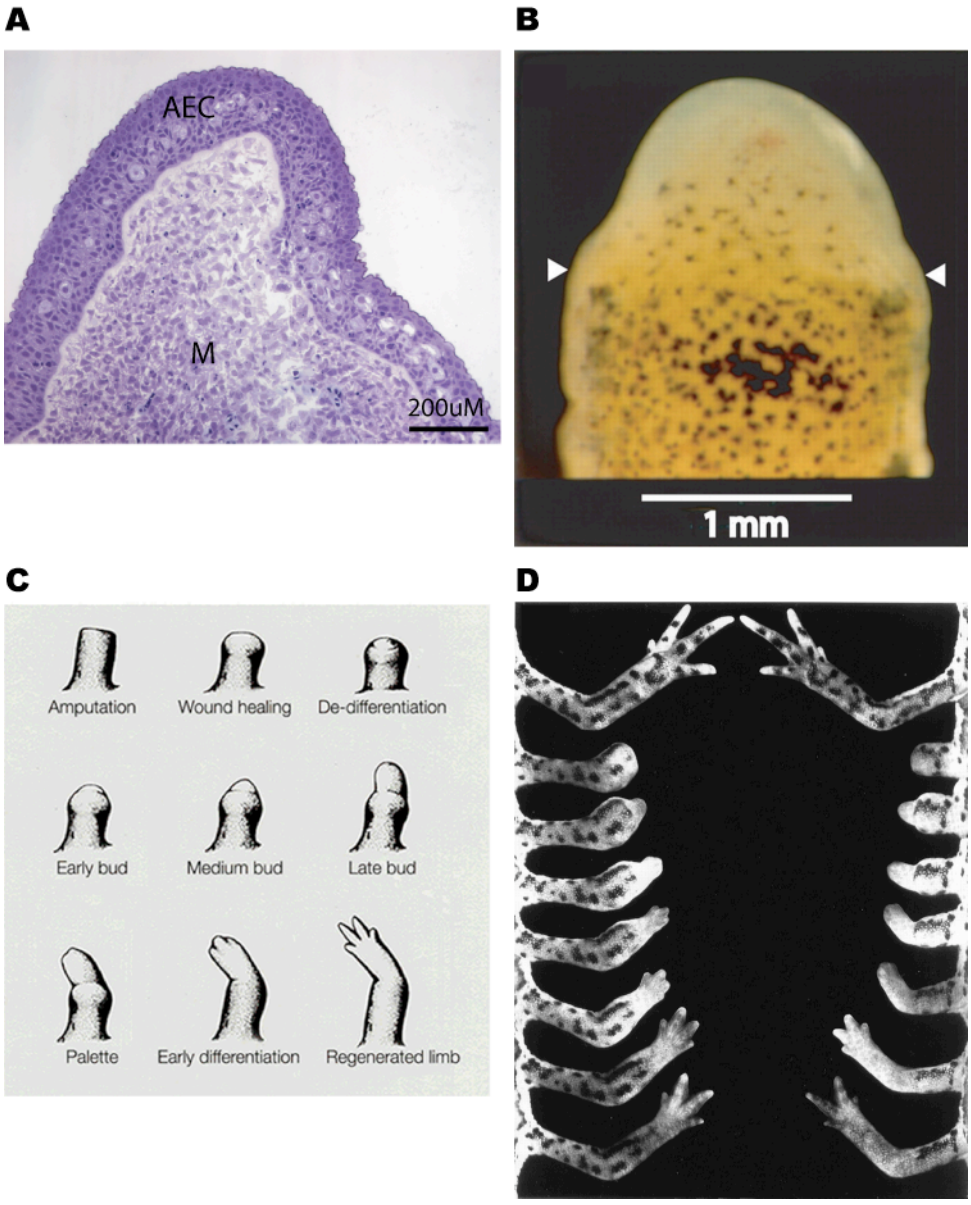


Figure 1.2 Urodele limb regeneration

(A) Morphology of the apical epithelial cap, AEC in relation to the blastema mesenchyme, M. Following migration of the single layer of cells forming the wound epithelium, the AEC forms by proliferation of epithelial cells. Image courtesy of A.Kumar. (B) Limb blastema from N.viridescens. The amputation plane is marked with arrows. Image from (Brockes and Kumar, 2005). (C) The stages of urodele limb regeneration. From (Iten and Bryant, 1973) with permission (D) Regeneration of the forelimb of N. viridescens. Distal amputation is shown on the left, proximal on the right. Original limbs are shown at the top. Photographs were taken at 7,21,25,28,32,42 and 70 days post amputation. Note that only structures distal to the amputation plane regenerate and the similar length of time required to reach digit stage following proximal and distal amputation (Goss, 1969).

1.4.3.1 Extra-cellular matrix re-modelling and matrix metalloproteases

MMPs have been mentioned as being involved in the initial wound healing event of regeneration for their role in permitting cell migration through tissue (Bullard et al., 1999) (Pilcher et al., 1997), and MMP9 expression in the cells of the wound epidermis is correlated with whether the AEC structure will form (Satoh et al., 2008). Furthermore, the expression of a number of MMPs is seen to be regulated in the mesenchymal cells of the blastema during regeneration (Vinarsky et al., 2005).

1.4.3.1.1 Regulation of MMP activity

Numerous MMPs exist in different species with a range of substrate specificities and they have been shown to be involved with a wide variety of biological processes (Page-McCaw et al., 2007) (Sternlicht and Werb, 2001). The enzymes are synthesised as pro-MMP zymogens (Harper et al., 1971). MMP8 and MMP9 secretion from neutrophils has been shown to be regulated by their release from intracellular granules (Hasty et al., 1990), whereas other MMPs are secreted in an unregulated way following translation. The secreted pro-enzyme is subsequently activated by enzymatic cleavage, either by other MMPs (Kerkela and Saarialho-Kere, 2003), or in the case of some MMPs, by other serine proteases associated with wounding such as plasmin (Carmeliet et al., 1997) and thrombin (Lafleur et al., 2001), which has itself been implicated in the process of de-differentiation preceding regeneration (Tanaka et al., 1999). The serine protease elastase is also able to activate MMP9 (Ferry et al., 1997) and is seen to be upregulated in the blastema in a similar temporal pattern to MMP9 (Vascotto et al., 2006).

MMPs can themselves be freely diffusing, membrane associated by GPI anchorage or via a C-terminal transmembrane domain (Sternlicht and Werb, 2001). Freely diffusing MMPs can also become membrane localised through the interaction with specific receptors. MMP9 can be membrane localised through interaction with the hyaluronic acid receptor CD44, facilitating the migration of tumour cells (Bourguignon et al., 1998).

Once activated by enzymatic cleavage, MMP activity is regulated by tissue inhibitors of metalloproteases (TIMPs), which interact stoichiometrically with the active site of MMPs to reversibly inhibit their catalytic activity. Different TIMPs exist, each with a different specificity for the various MMPs (Gomez et al., 1997). α2-Macroglobulin which is found in blood plasma also inhibits MMPs (Sottrup-Jensen and Birkedal-Hansen, 1989), as do some protein sub-domains with homology to TIMPs (Mott et al., 2000). α2-Macroglobulin complexes are endocytosed and degraded providing a mechanism for the irreversible removal of MMP activity from the extracellular environment (Yamashiro et al., 1989). Along with their activation, proteolytic cleavage of MMPs can also regulate their localisation (Imai et al., 1996) and sensitivity to inhibition by TIMPs (Itoh et al., 1998). It can therefore be seen that MMP catalytic activity is regulated through the induction of its expression, however once the protein is synthesised, its activity and localisation is also highly regulated and subject to modification.

1.4.3.1.2 MMPs and de-differentiation

The expression of a number of MMPs becomes widely distributed throughout the blastema and if their activity is inhibited using a pharmacological inhibitor, hypomorphic limbs are regenerated, consistent with a role for MMPs in the de-differentiation or proliferation of blastema cells. MMP inhibition reduced the length of the regenerated radius/ulna and humerus to a similar extent, but not the width of the regenerating bones, indicating that the effect was specific to the proximodistal axis of the limb (Vinarsky et al., 2005). In support of the hypomorphic phenotype resulting from defective de-differentiation is the observation that the level of de-differentiation of blastema cells is correlated with the level of MMP2 and MMP9 activity. Retinoic acid, which affects the proximodistal patterning of the limb (discussed in detail below), is seen to upregulate these MMPs (Park and Kim, 1999) and increase the degree of dedifferentiation (Ju and Kim, 1994), indicating that the initial size of the pool of dedifferentiated blastema cells may influence the patterning of the limb along the PD axis.

It has been shown in hydra that MMP activity is induced in the stump following amputation, and inhibition of MMP activity either promotes the de-differentiation or inhibits the trans-differentiation of stump cells, which blocks foot regeneration. The ECM is in a constant state of turnover in hydra and MMP and laminin expression is highest at the extremities of the animal where trans-differentiation is continually taking place during normal growth (Leontovich et al., 2000). These observations further link the interaction of cells with the ECM and the regulation of this by MMPs to the processes governing cell de-differentiation. It seems however that in hydra, MMP activity is involved in the promoting the trans-differentiation of stump cells or maintaining their differentiated state, whereas in urodele expression of MMPs is correlated with de-differentiation. MMPs have also been shown to be involved in tissue remodelling in flies, worms and sea-urchins (Page-McCaw, 2008), and during liver regeneration in rats (Kim et al., 1997).

1.4.3.1.3 Regulation of cell behaviour by MMPs

That MMPs may influence de-differentiation is not surprising. Extracellular matrix proteins contain binding motifs for integrin receptors (Mould and Humphries, 1991) (discussed in greater detail below), and the disruption of integrin ECM interactions as well as the cleavage of adherens junctions between epithelial cells can be catalysed by MMPs (Symowicz et al., 2007), and is involved in the loss of phenotypic specialisation seen in epithelial-mesenchymal transition (Orlichenko and Radisky, 2008). The cleavage of matrix proteins exposes otherwise concealed ‘cryptic’ motifs which can activate integrins and regulate cellular behaviour (Mott and Werb, 2004). The ECM also sequesters numerous growth factors which can be released upon its degradation (Mott and Werb, 2004), and a class of MMP domain containing enzymes known as adamalysins are involved in the shedding of membrane tethered growth factors such as TNFα (English et al., 2000) and various EGFR ligands (Blobel, 2005) which may influence the processes occurring in the blastema. MMPs are also able to cleave growth-factor receptors such as the FGF receptor type 1 (Levi et al., 1996), which may modulate the sensitivity of cells to FGFs. Substance P, a neurotransmitter, is also cleaved by MMPs

(Diekmann and Tschesche, 1994). It is particularly interesting to note that the activity of FGF and substance P may be regulated by MMPs as both are possible mitogens for blastema cells. Reviewed in (Stocum, 2004). Thus MMP activity is able to regulate cell signalling through a variety of mechanisms that may influence the differentiation state of stump cells.

1.4.4 Patterning the regenerating limb

Similarities between the AEC and the AER have been demonstrated in terms of some of the transcription factors expressed in response to growth factors in the two structures and their outgrowth promoting functions. Further similarity exists between the limb bud and the blastema in terms of the expression of FGF by the AEC and AER and the blastema and limb bud mesenchyme. Reviewed in (Stocum, 2004). Although not identical, the indication of some similarity between the blastema and the limb bud therefore makes it worthwhile to consider the models of limb outgrowth and patterning established through investigation in other model systems in relation to limb regeneration.

1.4.4.1 A study of the developing limb bud

The relationship between limb bud outgrowth driven by the AER and proximodistal patterning has been the subject of extensive research.

In a recent review Tabin and Wolpert have proposed a model of limb patterning based upon current molecular level information whereby the AER promotes bud growth whilst a second signalling centre in the flank is involved in the specification of proximal identities (Fig.1.3) (Tabin and Wolpert, 2007), which is described below as a framework for considering the process of proximodistal patterning of the regenerating urodele limb.

1.4.4.1.1 The role of fibroblast growth factors and retinoic acid

At the heart of the model is the idea of a proliferating undifferentiated zone of cells maintained by FGF signalling from the AEC (Globus and Vethamany-Globus, 1976) and a progressively moving ‘differentiation front’. In addition to maintaining the pool of dividing progenitors, FGF signalling from the AEC also

specifies distal fates (Fallon et al., 1994) (Niswander et al., 1993). As FGF signalling has only a limited range, as the limb bud grows, only the distal most cells remain under the influence of the AEC, with more proximal cells being free to enter differentiation pathways and assume proximal fates, consistent with the observed proximal to distal wave of differentiation that occurs during development.

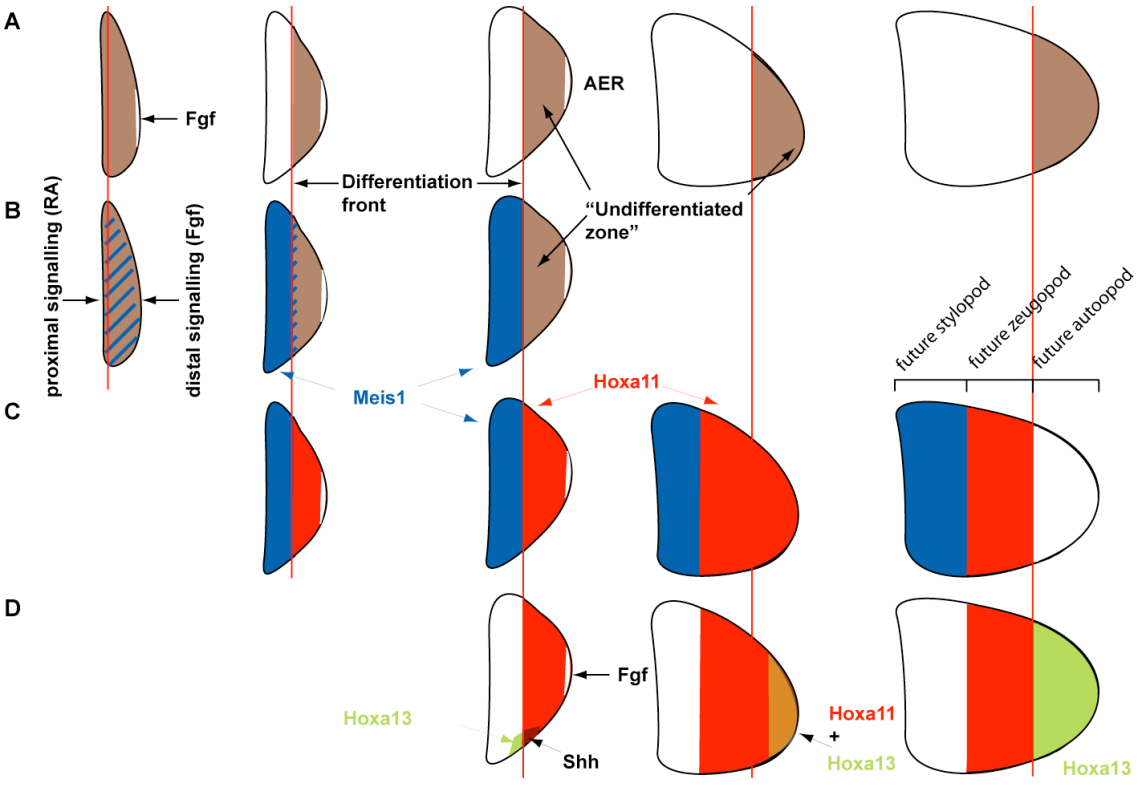


Figure 1.3 The role of retinoic acid and fibroblast growth factors in the patterning of the developing limb bud

(A) An undifferentiated zone is maintained throughout outgrowth of the limb bud through the activity of FGF from the AER (B) A retinoic acid gradient originating in the flank induces Meis expression, which initially overlaps with the undifferentiated zone maintained by FGF signals from the flank. Once out of range of distalising AEC signals, the proximal compartment of the limb differentiates under the sole influence of the proximal determinant Meis. (C) Meis and the zeugopod marker Hoxa11 expression initially overlaps, but becomes refined to mutually exclusive compartments as the limb bud develops. (D) Hoxa13 expression becomes localised to the most distal compartment. Hoxa11 and Hoxa13 expression initially overlap, but become refined to mutually exclusive compartments as the limb bud develops. Other signals such as Shh may serve to regulate the refinement of markers to mutually exclusive compartments. Adapted from (Tabin and Wolpert, 2007).

The model proposes that opposing gradients of retinoic acid (RA) and FGF signalling (Mercader et al., 2000) result in the establishment of mutually exclusive domains of expression of PD determinants timed to occur as cells cross the differentiation front, thereby establishing the segments of the limb as it develops. Initially RA signalling from the flank and FGF signalling from the AEC overlap as the limb bud is small, however as the limb bud grows two zones resolve, the undifferentiated zone under exclusive influence of AEC signals expressing distal determinants, and a zone under the exclusive influence of flank signals fated to become the proximal regions of the limb. Further growth of the limb bud pushes cells out of the range of both signalling centres, which will assume distal fates as they are neither under the proximalising influence of the flank nor maintained as undifferentiated by signals from the AEC. FGF signalling from the AEC therefore plays a dual role in that in concert with RA it is involved in the establishment of clear boundaries of expression of PD determinants spatially in the limb bud whilst also maintaining a region of the limb bud in an undifferentiated state. This mechanism therefore integrates the specification of progenitor cell identity with the process of differentiation via FGF signalling.

1.4.4.1.2 Markers expressed during limb patterning: The Hox genes

A number of gene products are seen to be confined to specific limb compartments, providing markers that can be followed during limb development. Meis1 is expressed in the proximal regions of the limb (the humerus or stylopod), upregulated by RA and downregulated by FGF (Mercader et al., 2000). Hoxa13 is expressed in distal regions of the limb (the hand or autopod) (Yokouchi et al., 1991), upregulated by FGF (Vargesson et al., 2001) and downregulated by RA (Mercader et al., 2000). Meis1 and Hoxa13 also act to repress each other's expression (Capdevila et al., 1999). Hoxa11 is expressed in the radius/ulna or zeugopod (Yokouchi et al., 1991). RA downregulation of Hoxa13 in the autopod extends Hoxa11 expression distally (Mercader et al., 2000), however Hoxa11 and Hoxa13 do not appear to regulate each other's expression (Yokouchi et al., 1995). Tabin and Wolpert suggest that

Hoxa11 and Hoxa13 may respond reciprocally to a common signal such as Shh, which may establish the sharp boundary between their locations of expression. Although good markers for the limb compartments, out of the genes mentioned only Meis has been shown to reprogram proximodistal identity when overexpressed (Capdevila et al., 1999), although it is not essential for normal limb patterning (Azcoitia et al., 2005), indicating some degree of redundancy. In the absence of Hox11 gene expression, the zeugopod compartment is formed but does not grow correctly (Boulet and Capecchi, 2004), demonstrating that although Hox11 genes are involved in the growth of the zeugopod, they are not required to establish its initial formation in the limb bud.

1.4.4.2 Hox gene expression in the regenerating urodele limb

Studies in axolotls have shown that Hoxa13 expression becomes restricted to distal regions of the blastema during regeneration and is downregulated by RA (Gardiner et al., 1995) as described previously in the developing limb bud of other model organisms. Meis genes are also upregulated in blastemas and developing limbs exposed to RA (Mercader et al., 2005), indicating essentially the same underlying mechanisms specifying PD identity may be in place in the blastema as in the developing limb bud. RA treatment of blastemas progressively respecifies them to more proximal identities over a range of concentrations, such that at high concentrations a wrist level blastema regenerates an entire new limb including the shoulder girdle (Fig.1.4A) (Maden, 1982). Overexpression of Meis2 is able to re-locate distal blastema cells to more proximal locations and knockdown of Meis is able to block the proximalising effects of RA (Mercader et al., 2005), indicating that the proximalising effect of RA on blastemas is indeed mediated through the upregulation of the proximal determinant Meis.

1.4.4.3 The autonomy of the blastema

Developing limb buds can be transplanted to a host limb at the mid-stage and develop normally, indicating that any requirement for RA in specifying the progenitor pools of limb segments must occur early (Tabin and Wolpert, 2007). Consistent with this, RA is only able to proximalise the blastema during the de-

differentiation phase (Thoms and Stocum, 1984). Likewise, a 10 day blastema grafted to a remote location will regenerate structures according to its origin along the limb axis (Stocum, 1968) demonstrating that after this stage it is autonomous and requires no instructive cues from the stump or nerve in order to pattern its tissues or grow. Whether RA plays any role in regulating the PD identity of regenerating blastemas is unclear. In the developing limb bud, all cells are initially in the range of signalling from the flank however in the case of a distal blastema it is significantly remote from the flank to not realistically be under significant influence of such a morphogen gradient. In the regenerating limb however, the stump tissues are already specified to proximal identities, thus it is conceivable that only the distal AEC signalling centre is necessary to direct the process. It has been suggested that regeneration is distinct from development in that a distal boundary is established by the AEC and that missing structures are 'filled in' or intercalated between the proximal and distal boundaries (Stocum, 1984). If cells are maintained in an undifferentiated pool beneath the AEC and progressively differentiate into ever more distal structures as they cross the 'differentiation front' as in the model of limb development proposed by Tabin and Wolpert, then the specification of the first blastema cells to differentiate to appropriate PD values could be achieved if the progenitor cells had some form of 'memory' of their origin along the PD axis. This has been proposed to be mediated by a mechanism involving a graded expression of a cell-surface determinant of PD identity expressed by progenitor cells (Stocum, 1984). Arguments against a mode of fate specification whereby structures differentiate in a proximal to distal wave exist however due to the observation that the most distal cells of the blastema are fated to form the most distal structures of the limb very early after the blastema is established (Echeverri and Tanaka, 2005) (discussed in greater detail below).

1.4.4.4 A cell-surface determinant of proximodistal identity?

1.4.4.4.1 Classical experiments: Engulfment, affinophoresis and intercalation

Arguments for a surface determinant of PD identity come from a number of classical experiments. When a proximal and a distal blastema are cultured in contact with one another, the proximal blastema will tend to engulf the distal blastema (Fig.1.4Ba) (Nardi and Stocum, 1983) and this has been interpreted as being due to a difference in adhesiveness of the surface of proximal compared to distal blastema cells. If a distal blastema is grafted onto the dorsal surface of a proximal blastema it will remain undifferentiated and translocate distally as the regenerating limb grows, until it reaches the level of its origin along the PD axis, at which point it will differentiate hand structures (Fig.1.4Bb) (Crawford and Stocum, 1988a), a phenomenon termed affinophoresis. Similar to this, if a distal blastema is labelled and grafted onto a proximal stump, the stump tissue will intercalate the missing tissue between itself and the blastema (Fig.1.4C). If a proximal blastema is grafted onto a proximal stump however, regeneration occurs by growth of the blastema without mobilising stump tissue (Pescitelli and Stocum, 1980). If distal blastemas are treated with RA prior to grafting onto a proximal stump, they behave as if they were proximal blastemas, with no intercalation from the stump occurring and regeneration being driven purely from cells of the blastema. Likewise, if an RA treated distal blastema is grafted onto the dorsal surface of a regenerating limb, it does not translocate distally and differentiates at the proximal position at which it was grafted (Crawford and Stocum, 1988b). These observations argue that the surface determinant of PD identity is regulated by RA. The authors of these studies have suggested that distal blastema cells may be more adhesive than proximal cells, providing a means by which cells can sort themselves according to PD identity.

It is observed that a distal blastema takes the same length of time to reach the digit stage as a proximal blastema (Iten and Bryant, 1973). Although the reason for this is unclear, this observation taken together with the autonomous nature of blastemas help to explain why a grafted blastema completes regeneration

co-ordinately with the blastema of the amputated limb onto which it was grafted. As the blastema is autonomous at this point, the interaction with the host limb relates only to its translocation along the axis until it reaches its level of origin, suggested to be mediated by the affinity of cells for others of like PD identity.

1.4.4.4.2 Sensing disparity between cells of different proximodistal origin

Some mechanism of sensing local disparity in PD values between neighbouring cells is proposed to drive intercalation to restore the missing positional values (Stocum, 1996). The positional value of cells of the stump could be sensed in this way, ensuring that only the missing distal structures are intercalated between it and the AEC. This observation that a stump will produce only missing distal structures has been termed the rule of distal transformation. It has been shown that labelled distal blastema cells implanted into a proximal region of an upper arm blastema translocate distally (Echeverri and Tanaka, 2005), in similarity to distal blastemas grafted onto the dorsal surface of a regenerating limb. Experiments with dissociated chick wing bud cells have indicated that distal cells express higher N-cadherin than proximal cells and that this expression correlates with a tendency to associate with other distal cells (Yajima et al., 2002). The authors suggest that this may play a role in patterning along the PD axis of the limb.

1.4.4.5 The role of Prod1 in proximodistal patterning

1.4.4.5.1 The identification of Prod1

RA has been used to screen for potential surface determinants of PD identity regulated by it with a pattern of expression consistent with its proximalising effect. The expression of genes encoding cell-surface expressed molecules was compared between RA treated and normal blastemas, and selected on the basis of them either being upregulated by RA and expressed at a higher level in proximal limbs and blastemas compared to distal or downregulated by RA and expressed at a lower level in proximal limbs and blastemas compared to distal. A single molecule was identified on this basis and named Prod1. Prod1 is expressed to a higher level in proximal blastemas and sections of the limb and

is upregulated by RA, making it a possible candidate for marking the relative extent of proximal identity of stump or blastema cells (da Silva et al., 2002).

1.4.4.5.2 What does Prod1 do?

Prod1 antibodies block the engulfment of distal blastemas by proximal blastemas (da Silva et al., 2002) indicating it may mediate the differential adhesiveness proposed by Nardi and Stocum. When Prod1 is focally electroporated into the most distal cells of a distal blastema, which would usually differentiate to form the hand structures, cells are found at more proximal locations in the regenerating limb (Fig1.4C) (Echeverri and Tanaka, 2005), similar to results seen with Meis2 electroporation (Mercader et al., 2005) and RA receptor activation in individual blastema cells (Pecorino et al., 1996).

1.4.4.5.3 A link between Prod1, retinoic acid and Meis?

As noted previously, Meis is a determinant of proximal identity positively regulated by RA. Prod1 is also positively regulated by RA, and there is some indication that the Prod1 promoter may have Meis response elements within it (N. Shaikh, P.Gates unpublished), raising the possibility that Meis may regulate its expression. Unlike Prod1, Meis expression is not graded along the PD axis of the limb, however it is expressed more highly in proximal blastemas (Mercader et al., 2005). That Meis expression is not higher proximally in the intact limb is not inconsistent with it regulating PD identity via Prod1 expression during regeneration, as it is higher in proximal blastemas, and this is when its PD specifying activity is required. It will be interesting to understand further the relationship between the proximal determinant of development Meis, and Prod1, a potential determinant of PD identity of the regenerating blastema.

1.4.4.5.4 Prod1 as the cell-surface determinant of proximodistal identity

A model to explain the replacement of missing distal structures based upon the level of cell surface Prod1 expression as the PD determinant has been proposed such that when stump cells first de-differentiate, Prod1 expression is

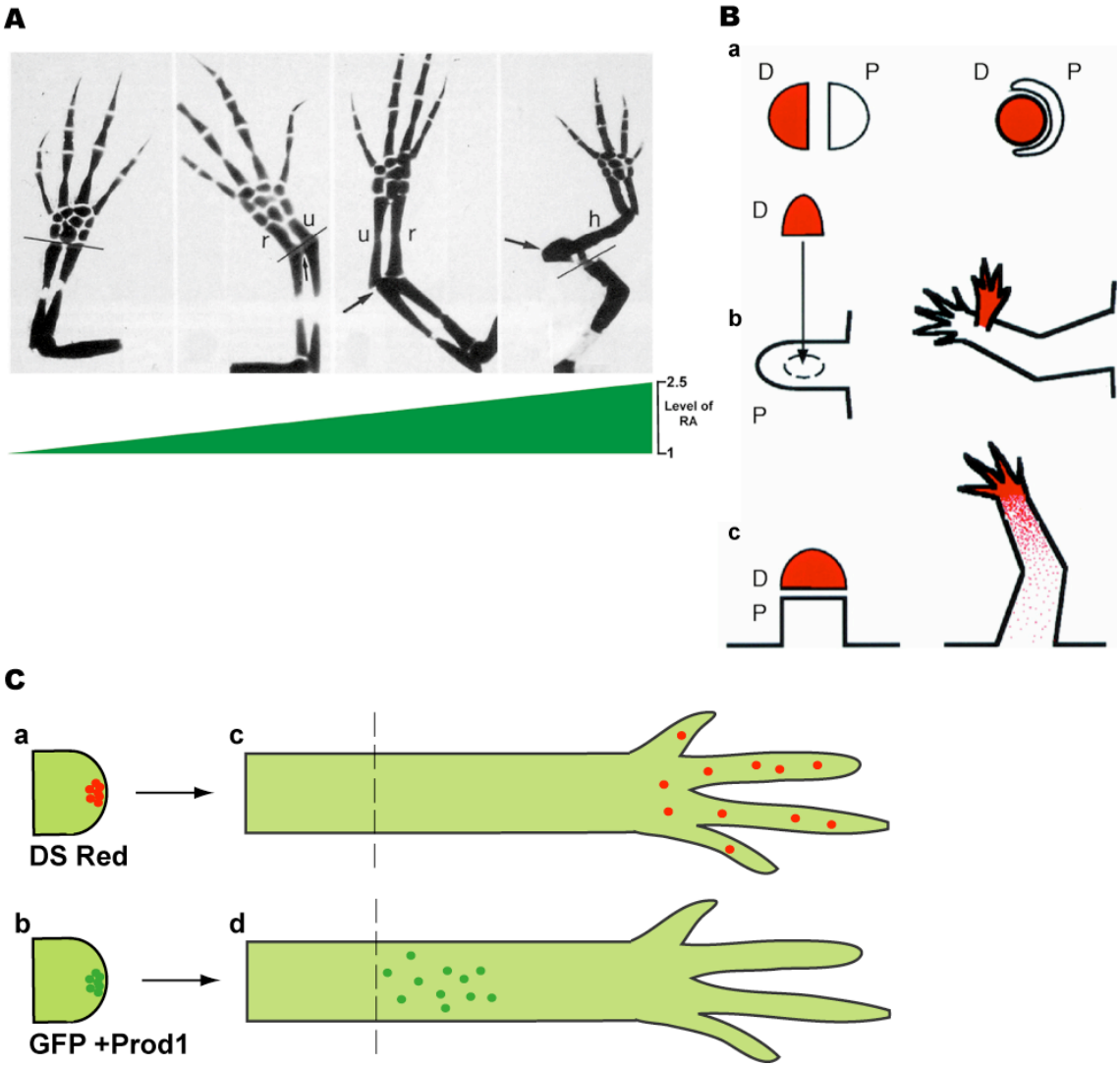


Figure 1.4 Proximodistal patterning of the regenerating urodele limb

(A) Distal blastemas treated with increasing concentrations of RA show duplications of increasingly proximal limb structures as they regenerate. Adapted from (Thoms and Stocum, 1984). (B) Classical experiments with blastemas. a) When juxtaposed in culture, proximal blastemas engulf distal blastemas. b) When a distal blastema is grafted onto the dorsal surface of a proximally amputated limb, the blastema translocates to its level of origin before differentiating, a process termed affinophoresis. c) When a distal blastema is grafted onto the amputation surface of a proximally amputated limb, the stump regenerates the majority of the limb by intercalation, with the grafted distal blastema contributing predominantly to distal structures. (Brockes, 1997) (C) Prod1 expression proximalises distal blastema cells. a) Distal blastema cells electroporated with DS Red are localised distally in the regenerated limb, b). c) Distal blastema cells electroporated with GFP and Prod1 become more proximally located than those in b) in the regenerated limb d). Adapted from (Echeverri and Tanaka, 2005).

lost, producing a disparity between the initial blastema cells to de-differentiate and the stump cells, which will have a Prod1 encoded PD value corresponding to their position along the limb axis. Intercalation then fills in the missing values between these two boundaries by progressive respecification of the level of Prod1 expression of blastema cells as they divide, which then determines the fate of cells once they differentiate (Echeverri and Tanaka, 2005).

1.4.4.6 Specification of blastema cell-fate

Labelling experiments with early blastemas indicate that PD identity of blastema cells is specified very early, as distal cells are not respecified by moving them to more proximal positions in the blastema, and labelling of the most distal cells of an early blastema indicates that these cells always contribute to the hand (Echeverri and Tanaka, 2005). The authors suggest that this rules out the possibility of models whereby the blastema grows at the distal tip with proximal structures are specified first as they exit the undifferentiated zone as in the model of limb development proposed by Tabin and Wolpert. They suggest that either populations of cells corresponding to the three limb compartments are established very early on, with each expanding and eventually differentiating, or that the early blastema is divided into proximal and distal regions, with the stump continually providing cells to the blastema during the de-differentiation phase of regeneration with progressively more proximal identities, producing progenitors with the appropriate identities to intercalate the structures missing between the distally specified region of the blastema and the stump. Any contribution by the stump must none the less be complete by the time at which a grafted blastema will develop autonomously. Even if specification of positional identity is not coupled to outgrowth, there must be some mechanism by which the blastema regulates the differentiation of the specified progenitors. It is possible that once pools of progenitors are established they regulate their development through entry into cell autonomous differentiation programs, interactions between neighbouring cells, the establishment of local signalling centres or a combination the three.

Consistent with a difference in the specification of distal cells between development and regeneration is that the distal marker HoxA13 is not observed in the initial stages of limb bud development in the axolotl, however is seen to be expressed after only 24hrs in the blastema (Gardiner et al., 1995). Nuclear localised Meis2 protein was seen to be expressed throughout the early limb bud and subsequently refined to a clear proximal domain as the bud developed (Mercader et al., 2005) consistent with observations in other models of limb development (Tabin and Wolpert, 2007). As mentioned previously, Meis expression is not observed to be graded along the PD axis of the limb, however it is expressed more highly in proximal compared to distal and in RA treated blastemas. These observations are consistent with the proposal that distal fates are established early in all blastemas, as shown by the Hoxa13 marker, and that it is the degree of proximal identities that are regulated by some means, as shown by differential Meis expression between proximal and distal blastemas. Although growth of the blastema is unlikely to occur by proliferation of an unspecified distal zone of cells that subsequently differentiate in a proximal to distal wave, the data is not inconsistent with the involvement of the AEC in setting up the pools of progenitors that will form the discrete limb compartments early on.

Some caution must be exercised in interpreting data from axolotl regeneration due to their paedomorphic character, as the recapitulation during regeneration of gene expression seen during development may be misleading when interpreted with data from newts, which are true adults. It is possible that axolotl limb regeneration is comparable to limb regeneration by metamorphosing anurans (Dent, 1962) in which tissues have not fully differentiated. Classical experiments with grafted blastemas show the same results with newts and axolotls, however it has not been ruled out that different patterning mechanisms may exist between the different species. The similarity observed between the AER and limb bud in models of limb development and the AEC and blastema suggests that any difference between axolotls and newts is likely to be subtle though.

1.4.4.6.1 Retinoic acid in the regenerating blastema

Proximal blastemas are observed to have higher RA activity than distal blastemas (Scadding and Maden, 1994), consistent with a role for it in conferring proximal identity to blastemas. The basis for this is unknown but must reflect some underlying difference encoded by the stump cells contributing to either the AEC or the blastemal mesenchyme. An alternative model to one based on the stable expression of a graded cell-surface determinant of PD identity by limb cells based on this difference in RA between proximal and distal blastemas and the observation that RA treated blastemas show a greater degree of de-differentiation (Ju and Kim, 1994) would be that the size of the pool of de-differentiated cells in some way determines their response to signals from the AEC. A small pool may produce only the distal most structures whereas increasingly larger pools of progenitors may adopt increasingly proximal fates upon specification to distinct limb compartments due to increased distance from distalising AEC signals, which subsequently expand and differentiate according to their PD identity. In proximal blastemas where there is greater de-differentiation, the stump would produce progenitors of increasingly proximal values as they come in 'at the back' of the blastema out of range of the AEC. Consistent with this is the observation that RA only acts during de-differentiation (Thoms and Stocum, 1984). If RA is involved in the extent of de-differentiation of the stump and this is a primary determinant of the fates that populations of blastema cells will adopt, it seems logical that RA be unable to respecify the blastema after de-differentiation has occurred. Also, RA does not have the same proximalising effects during development, during which patterning occurs by processes distinct from those tied to the process of de-differentiation occurring during regeneration (Scadding and Maden, 1986).

1.4.4.6.1.1 Retinoic acid and matrix metalloprotease expression

The increased de-differentiation in RA treated blastemas is also correlated with increased MMP production (Park and Kim, 1999). Inhibition of MMPs produces hypomorphic limbs rather than proximal truncations however (Vinarsky et al., 2005), which is hard to reconcile with a model in which the degree of de-differentiation determines the PD compartmentalisation of the early blastema, if

it is indeed MMP activity regulating this de-differentiation. The indication from this data is that MMPs determine the size of the compartments, possibly through the regulation of precursor pool size, rather than the number of compartments. Due to the exponential nature of cell division, even small differences in size of the early pools of de-differentiated cells fated to different compartments could have a large effect on the eventual size of those limb segments once proliferation is halted and differentiation is initiated. If MMP activity is behind the increased de-differentiation seen in RA treated blastemas, the fact that it does not affect patterning per se suggests that RA may coordinate both the production of extra de-differentiated cells in order to provide a sufficiently large pool of precursor cells to differentiate into the extra structures whilst independently regulating the number of limb compartments formed**.** It would be interesting to address this question either by overexpression of MMPs in the blastema, to see if either extra limb segments are produced or larger than ordinary limb segments are produced. The combination of MMP inhibition and RA administration may also provide some insight into the problem.

Late denervation of the blastema results in a miniature limb (Singer and Craven, 1948) similar to the effect of MMP inhibition, indicating that the nerve regulates growth rather than patterning. On the other hand, late removal of the AEC results in distal truncation of the regenerate (Goss, 1956b). This could be interpreted as indicating that the AEC is required for maintaining distal fates as the blastema grows, however evidence from AER removal from limb buds indicates that this may be due to the death of underlying mesenchyme (Rowe et al., 1982). As distal cells are specified early and retain their distal character when grafted proximally (Echeverri and Tanaka, 2005) it seems unlikely that AEC signals are required to maintain distal cell fate and therefore likely represents the death of the most distal precursors (Rowe et al., 1982), which cannot be reestablished from the more proximally determined cells.

1.4.4.6.1.2 Retinoic acid and Meis expression

Meis itself has been shown to be regulated by RA directly (Mercader et al., 2005), however its regulation in the blastema is likely to be different from in the limb bud due to the presumed lack of an RA gradient from the flank. It is possible that Meis expression is induced by RA in the blastema and that opposing signals from the AEC define the distal boundary of its expression, providing a means of establishing early proximal and distal zones in the blastema. It is conceivable that only in blastemas with a sufficiently large pool of de-differentiated cells is Meis stably induced by RA. This model puts RA in a dual role, in both inducing Meis expression and determining the size of the pool of progenitors which will in turn determine whether any cells are sufficiently distant from the AEC to stably express Meis and form the proximal most compartment of the limb**.** There is some indication that Meis activity in axolotls is regulated through its nuclear localisation (Mercader et al., 2005), raising the possibility that this may also play a role in the mechanism specifying PD identity of blastema cells.

1.4.4.6.2 Prod1, Meis, and cell sorting in the blastema

Proximal respecification by Meis is limited at later stages of the blastema and the authors suggest that this may be because cells are 'trapped' in their compartment at this point (Mercader et al., 2005). Perhaps by this point cell associations have been developed that do not permit movement to more proximal regions of the blastema. It is also conceivable that by later stages of blastema development, cells become refractory to Meis upregulation. In support of this, RA is unable to proximalise blastemas after de-differentiation has occurred (Thoms and Stocum, 1984). That distally localised cells do become more proximally localised by early Meis (Mercader et al., 2005) or Prod1 expression and relocate distally when grafted more proximally (Echeverri and Tanaka, 2005) does suggest that some degree of cell migration or sorting can occur within the early blastema. Unless de-differentiating cells are specified to random proximal and distal identities and subsequently sort into compartments it is unclear whether cell sorting would be necessary during the normal situation in the blastema. Perhaps the affinity of cells for others with the same PD identity

represents a mechanism to ensure robust compartment formation early in regeneration in order to prevent specified cells from becoming mislocated.

One interesting point is that evidence from affinophoresis and cell grafting suggests that it is distal cells which tend to desire to make contact with other distal cells (Echeverri and Tanaka, 2005), whereas proximal blastemas have higher Prod1 (da Silva et al., 2002) and Prod1 causes cells to relocate proximally (Echeverri and Tanaka, 2005), indicating that high Prod1 reduces a cell's affinity for other distal cells. The interpretation of engulfment of distal blastemas by proximal blastemas has also been interpreted as being due to the higher adhesiveness of distal blastema cells (Nardi and Stocum, 1983), and if this is taken as being adequate explanation then the block of this process with Prod1 antibodies (da Silva et al., 2002) is again consistent with high Prod1 expression reducing the adhesiveness of proximal blastemas. Nardi and Stocum suggests that the process is analogous to placing a drop of liquid on another with which it is immiscible, however in this process gravity act on the drop being placed to 'pull' it over the surface of the other (Nardi and Stocum, 1983). With blastema confrontation, although distal cells may have a higher tendency for self-association and therefore maintain the integrity of the distal blastema to a greater extent than the cells of the proximal blastema, none the less it is unclear what causes the proximal blastema to spread over it unless active cell migration is occurring. It is conceivable that proximal cells are more migratory due to looser association with either each other or blastema ECM proteins.

1.5 Signal transduction

In this section, a number of well-characterised signal transduction pathways will be examined as a prelude to a final investigation of how Prod1 may function at the molecular level to control cell behaviour.

1.5.1 Transmembrane receptors

1.5.1.1 Heterotrimeric G-protein coupled receptors

Heterotrimeric G-protein coupled receptors (GPCRs) are a large family of transmembrane receptors with a diverse array of ligands (Hauser et al., 2006). The structure of GPCRs are composed of seven transmembrane spanning domains linked by cytoplasmic and extracellular loops, with the extracellular regions being responsible for ligand binding and the intracellular domain mediating the interaction with, and activation of the GTPase activity of their associated heterotrimeric G-protein (Rosenbaum et al., 2009). Ligand binding to the extracellular region of GPCRs leads to a conformational change in the cytoplasmic loops, stimulating the exchange of GDP for GTP by the α subunit of the associated G-protein, leading to its dissociation for the $\beta\gamma$ subunit (Morris and Malbon, 1999).

G-proteins come in a number of flavours. All are composed of α, β and γ subunits, however the activity of the dissociated α and $\beta\gamma$ subunits depend upon their type. Two classes of α subunits, α_s and $\alpha_{i,}$ stimulate or inhibit the activity of the enzyme adenylate cyclase respectively, which produces the second messenger cyclic adenosine monophosphate (cAMP) (Malbon, 2005). Many possible β and γ subunits exist, and depending upon the subunit composition of the dimer they are also able to activate adenylate cyclase along with a large array of other downstream signalling pathways components including subtypes of phospholipase C (PLC), phosphatidylinositol 3-kinase (PI3K) and Src kinases (Schwindinger and Robishaw, 2001). Activated heterotrimeric G-proteins are also able to activate other small G-proteins of the Ras family through either direct activation of their regulatory guanine nucleotide exchange factors (GEF) as in the case of Rho GTPases (Whitehead et al., 2001), or, most likely,

through the activation of non-receptor tyrosine kinases (NRTK) such as Src in the case of Ras (Kranenburg and Moolenaar, 2001). Src activation by G-proteins is also responsible for their ability to activate signal transducer and activator of transcription 3 (STAT3) (Ram and Iyengar, 2001), a regulator of gene expression also activated by the epidermal growth factor receptor (EGFR).

1.5.1.2 Integrins

Integrins are heterodimeric transmembrane receptors composed of various combinations of α and β subunits, each with different preferences for binding ECM proteins, providing both a means of anchoring a cell and also sensing the ECM environment (Hynes, 2002). Although the cytoplasmic domains of integrins do not posses intrinsic kinase activity, ECM protein binding to the extracellular regions of the receptor leads to activation of kinases associated with the cytoplasmic tails of the α and β subunits (Giancotti and Ruoslahti, 1999). Focal adhesion kinase (FAK) is commonly activated by integrin signalling (Miyamoto et al., 1995) (Schaller et al., 1995) leading to Src activation (Schaller et al., 1994) and the activation of PI3K (King et al., 1997) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) mitogen activated protein kinase (MAPK) signalling by recruitment of the Grb2-SOS complex (Schlaepfer et al., 1994) (discussed in greater detail below). Some integrins are also able to interact directly with Src kinases, either via their α or β subunits. $\alpha_6\beta_4$ integrin is phosphorylated on the β_4 subunit by Src kinases, leading to the recruitment of the adaptor protein Shc (Mainiero et al., 1997). Downstream pathways and adaptors are discussed in greater detail below.

ECM protein activation of downstream signalling from integrins promotes cell survival via the PI3K pathway (Khwaja et al., 1997), and is linked, via Src and SHC as mentioned, to receptor tyrosine kinase (RTK) signalling (Roovers et al., 1999). The integration of integrin signalling with RTK signalling provides a means by which the ECM encountered by a cell can influence the response of a cell to other growth factors, determining whether a given growth factor will stimulate cell proliferation (Roovers et al., 1999) (Oktay et al., 1999) or

migration via the remodelling of the cytoskeleton (Cary et al., 1998) (Sieg et al., 2000).

1.5.1.3 Epidermal growth factor receptor signalling

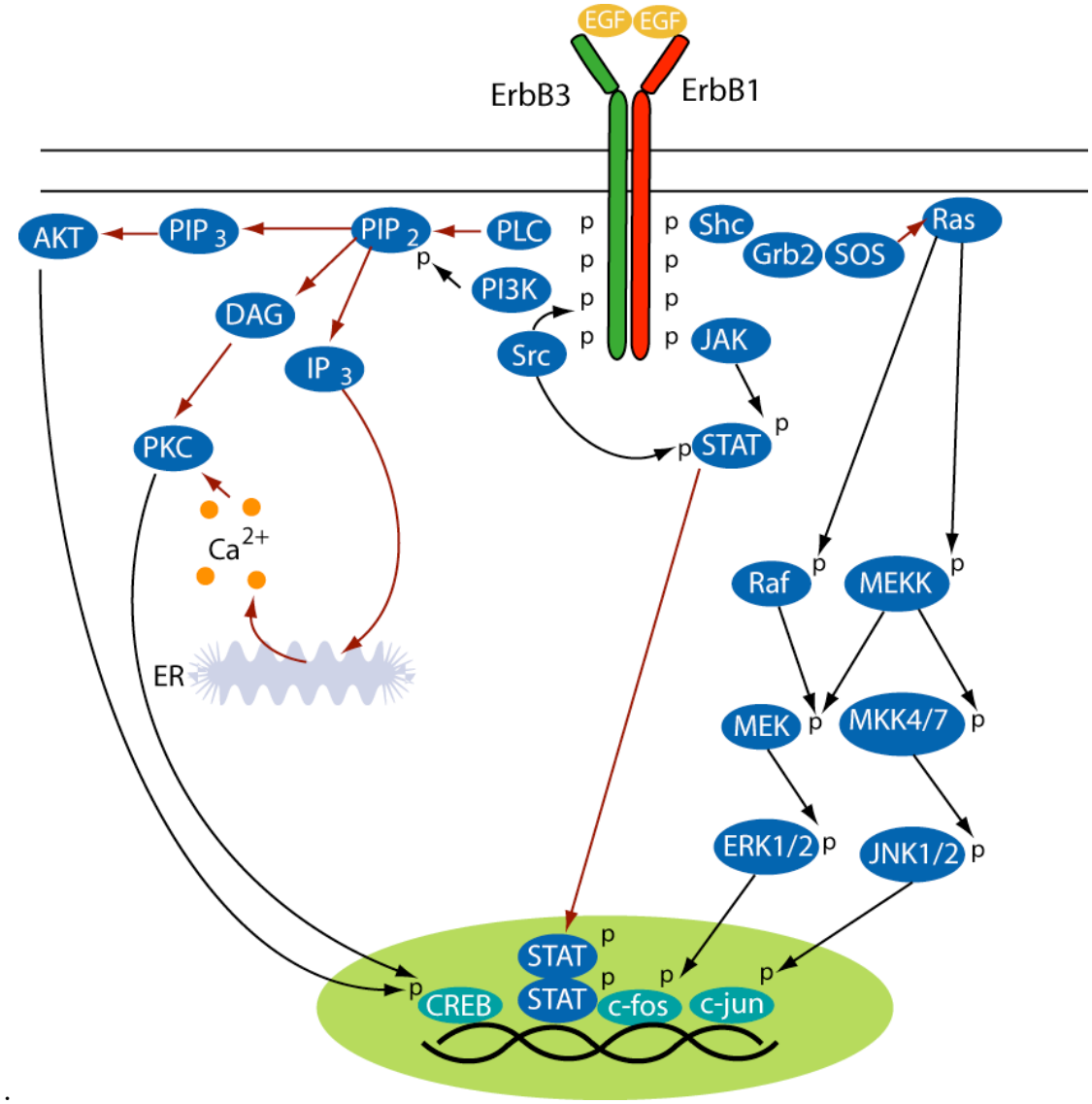
The EGFR has great potential for mediating and integrating signalling events. It provides sites for the activation of both kinases and adaptor proteins through which it is able to regulate the activation of a number of signalling pathways. The EGFR is activated by ligands such as epidermal growth factor (EGF) and transforming growth factor (TGF), which are initially synthesised as membrane anchored precursors (Schneider and Wolf, 2009). Four EGFR family members exist denoted ErbB1-4 with different downstream targets and ligand binding potential (Yarden and Sliwkowski, 2001). Of these only ErbB1, commonly referred to as EGFR, binds EGF, whereas only ErbB4 binds neuregulin 4 (NRG4) (Jones et al., 1999). Ligand binding to the extra cellular domains of ErbB monomers exposes dimerisation interfaces, leading to the formation of homo- or hetero-dimers (Schlessinger, 2002). The ErbB2 extracellular domain adopts a conformation primed for dimerisation without the ability to bind ligand (Klapper et al., 1999) and is the preferred partner of the other ErbB family members (Tzahar et al., 1996) and binds a greater number of downstream signalling components than the other monomers (Jones et al., 2006), whereas ErbB3 does not have a functional kinase domain (Guy et al., 1994). When in a monomeric state the kinase activity of EGFR, ErbB 2 and ErbB4 is auto-inhibited by self-association of regions of the cytoplasmic domains, with activation resulting from a phosphorylation independent, asymmetric interaction between the two members of a dimer (Zhang et al., 2006). Once activated, the kinase domain can phosphorylate residues on the C-terminal domain of the receptor, producing docking sites for components of downstream signalling pathways (Yarden and Schlessinger, 1987). See Figure 1.5 for a cartoon depicting major components of the EGFR pathway.

Along with regulation of kinase activation, EGFR activity is controlled at the level of receptor internalisation and degradation, a process which occurs through regulated endocytosis following activation, with different heterodimers

having different propensities for endocytosis due to their different abilities to interact with regulatory proteins (Baulida et al., 1996). Endocytosis of the activated EGFR is thought to occur largely through the clathrin-dynamin mediated pathway as inhibition of its phosphorylation prevents its accumulation in clathrin coated pits (Sorkina et al., 2002) and clathrin or dynamin knock-down has been shown to inhibit its internalisation (Huang et al., 2004). Following receptor activation and phosphorylation of the C-terminal domain, the ubiquitin ligase Cbl marks the receptor for internalisation and subsequent degradation (Levkowitz et al., 1998) (Dikic, 2003) (Marmor and Yarden, 2004), although it is of note that the phosphorylated C-terminal of the activated EGFR is exposed to the cytoplasm whilst in the endocytic pathway and is likely to be able to continue signalling whilst in endosomes (Wiley, 2003). Inactive EGFRs are also slowly internalised at a rate comparable to bulk membrane and recycled back to the cell membrane (Wiley et al., 1991); some examples also exist of pinocytic endocytosis of the activated EGFR by A431 carcinoma cells (Haigler et al., 1979), though the physiological relevance of these results is debated due to the extremely high level of EGFR expressed by this cell line (Sorkin and Goh, 2008).

1.5.1.4 Attenuation of receptor activation

The inactivation of receptor signalling has not been examined in detail here. Activated G-proteins hydrolyse GTP (Kleuss et al., 1994), leading to their re-association with $\beta\gamma$ subunits and inactivation (Meij, 1996), whereas receptor tyrosine kinases such as the EGFR can be inactivated by dephosphorylation catalysed by the regulated activity of phosphatases (Tiganis, 2002). Prolonged activation of GPCRs can also lead to their desensitization via their phosphorylation by activated downstream kinases such as protein kinase A (PKA), leading to their endocytosis (Ferguson, 2001).



.

Figure 1.5 The epidermal growth factor receptor: A paradigm for transmembrane receptor signalling

Ligand binding to the EGFR leads to the activation of a number of downstream signalling pathways. Black arrows denote phosphorylation (p). Tan arrows represent all other interactions. Shc and Grb2/SOS link EGFR activation to the MAPK pathways via Ras. Activated ERK1/2 and JNK1/2 translocate to the nucleus and phosphorylate a number of transcription factors, including c-fos and c-jun. STATs can be phosphorylated by EGFR activated JAKs, Src or by the EGFR itself (not shown), leading to their dimerisation and translocation to the nucleus where they regulate gene expression. PLCγ can be activated directly by ErbB3, or via Ras activation (not shown), leading to cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 releases Ca^{2+} from the endoplasmic reticulum (ER), which acts as a co-factor with DAG in the activation of protein kinase C (PKC). PIP_2 phosphorylation by PI3K produces PIP_3, which activates AKT. Activated AKT and PKC regulate gene expression by phosphorylating transcription factors such as the cyclic-AMP response element binding protein (CREB).

1.5.1.5 General principles of receptor signalling

The examples presented here of three distinct types of transmembrane receptors illustrate the diverse mechanisms through which extracellular signals can be transmitted across the cell membrane. Although the mechanisms of those described are of course not representative of all known receptors, and of those mentioned, description of all their known mechanistic details is incomplete, they do serve to illustrate some fundamental aspects of receptor signalling. The EGFR is either a hetero- or homo-dimer with intrinsic kinase activity, integrins are hetero dimers and require associated cytoplasmic kinases for signalling activity, whereas signals from GPCRs are transduced by the exchange of guanine nucleotides by their associated heterotrimeric G-proteins.

1.5.2 Adaptor proteins, non-receptor tyrosine kinases and small G-proteins

In many cases adaptor or scaffolding proteins form a link between receptors and downstream signalling pathways by providing a means by which to bring proteins together into functional signalling complexes. Individual NRTKs and small G-proteins are activated following the activation of a number of transmembrane receptors. As these classes of proteins can both be activated by various inputs and lead to the activation of a variety of downstream pathways, they serve to integrate the effects of receptor activation. Figure 1.6 shows the involvement of some of the proteins described below in relation to EGFR signalling.

1.5.2.1 SH2, PTB and SH3 domains

The adaptor proteins Shc (Pelicci et al., 1992) and Grb2 (Lowenstein et al., 1992) contain the Src homology 2 (SH2) domain, which mediates their interaction with phosphorylated tyrosine residues (Mayer and Baltimore, 1993). Shc can also contains a second phosphotyrosine interacting domain, the phosphotyrosine binding (PTB) domain (Blaikie et al., 1994) whereas Grb2 contains two Src homology 3 (SH3) domains (Lowenstein et al., 1992), which facilitate its interaction with both proline-rich motifs on other proteins (Sparks et al., 1996) and other sequence motifs (Lewitzky et al., 2001). Shc binds to

phosphorylated tyrosines on many proteins including the EGFR (Pelicci et al., 1992), the T-cell receptor (Ravichandran et al., 1993), the platelet derived growth factor receptor (PDGFR) (Yokote et al., 1994) via its SH2 domain, and the neurotrophin receptor TrkA (Zhou et al., 1995) via its PTB domain, leading to its phosphorylation. As mentioned previously, Shc also interacts with Src phosphorylated integrins (Mainiero et al., 1997). Phosphorylated Shc provides a docking site for Grb2 (Sasaoka et al., 1994), though it has been shown that Grb2 can also become localised to the EGFR directly via its SH2 domain (Batzer et al., 1994). These proteins containing multiple different interaction domains therefore facilitate the regulation of protein complex formation in response to receptor stimulation, linking the activated receptor to downstream components in the signalling pathway.

1.5.2.2 Ras, Rho and Src

Grb2 is constitutively associated with the Sos GEF in the cytoplasm. Upon recruitment to a transmembrane receptor, Sos initiates the exchange of bound GDP for GTP by the small G-protein Ras (Olivier et al., 1993), which is anchored to the inner leaflet of the cell membrane by covalently attached prenyl and palmitoyl groups (Pechlivanis and Kuhlmann, 2006). Targets of Ras include upstream components of the MAPK pathways Raf (Hallberg et al., 1994; Hamilton and Wolfman, 1998) and MEKK-1 (Lange-Carter and Johnson, 1994), PI3K (Rodriguez-Viciana et al., 1994) and protein kinase C ζ (PKCζ) (Diaz-Meco et al., 1994), all of which are described below in greater detail.

The Rho GTPases, of which Rac, Cdc42 and the Rho sub-categories are family members, are a sub-family of the Ras super-family of molecules responsive to growth factor receptor (Schiller, 2006) and integrin stimulated signals (Keely et al., 1997) (Ren et al., 1999) (Shaw et al., 1997). Like Ras, their activation requires the catalysed exchange of bound GDP for GTP by GEFs (Rossman et al., 2005). This sub-family has been shown to regulate cell shape and motility through their ability to interact with cytoskeletal associated proteins, modulating the dynamics of the turnover of the cytoskeleton (Heasman and Ridley, 2008) ,

as well as functioning in the activation of other signalling pathways such as MAPK (Joneson et al., 1996).

The NRTK Src contains an SH2 and an SH3 domain. In its inactive state it is auto-inhibited through the binding of its SH2 domain to an inhibitory phosphorylated tyrosine (Liu et al., 1993), leading to the association of the SH3 domain with a stretch of proline residues (Xu et al., 1999), disrupting its kinase activity (Young et al., 2001).

Thus Src can be activated by de-phosphorylation of the inhibitory tyrosine bound by the SH2 domain, association with phosphorylated tyrosines residues on proteins such as the EGFR (Stover et al., 1995) and integrin associated focal adhesion kinase (FAK) (Schaller et al., 1994), which compete for binding of its SH2 domain, or similarly, association with proline residues on other proteins which compete for binding with its SH3 domain (Gonfloni et al., 2000). When associated with the EGFR, activated Src phosphorylates tyrosine residues on the receptor, providing new binding sites for proteins (Biscardi et al., 1999) whilst also phosphorylating other EGFR associated proteins such as STATs (Quesnelle et al., 2007).

1.5.3 Feedback and crosstalk: An added level of complexity to receptor signalling

The activation of a cell-surface receptor can transactivate other pathways through either direct or indirect production of other ligands. For example, GPCR activation can lead to elevated MMP activity, which in turn leads to an increase in available EGFR ligand and EGFR activity through cleavage and release of cell-membrane anchored heparin-binding EGF (HB-EGF) precursor (Prenzel et al., 1999). A similar mechanism operates following the activation of the uPAR receptor by its ligand uPA (Guerrero et al., 2004).The MMP involved may be of the ADAM (a disintegrin and metalloprotease) family, known to regulate EGFR activity in a number of contexts through the release of membrane tethered HB-EGF (Blobel, 2005). EGFR activation can lead to the production of its own ligands (Schulze et al., 2001), providing a positive feedback loop through autocrine signalling. In addition, NRTKs such as the Src family can be activated

by integrins leading to phosphorylation of tyrosine residues on the EGFR (Moro et al., 2002), providing an example of how activation of one receptor can modulate the activity of another.

Src family NRTKs are activated by a number of receptors, such as the EGFR (Stover et al., 1995) and integrins (Schaller et al., 1994), and have been shown to participate in down-stream signalling from both of them (Leu and Maa, 2003) (Playford and Schaller, 2004). Such crosstalk between pathways originally thought of as independent is emerging as the rule rather than the exception. In light of the fact that compared to the possible cellular outcomes of signalling the number of receptors involved is small, that they are able to interact and modulate each other explains the observed complexity they generate. Figure 1.6 illustrates some of the crosstalk between EGFR and integrin signalling as an example.

1.5.4 Signal transduction pathways

1.5.4.1 Mitogen activated protein kinase pathways

The MAPK pathways provide a clear illustration of the principles of cell signalling via kinase cascades. Three or more tiers of kinases are activated by phosphorylation, with each kinase phosphorylating the next leading to the eventual activation of one or more MAPKs (Pearson et al., 2001). Figure 1.7 illustrates some of the points described below in the text.

1.5.4.1.1 MAPK kinase kinases

At the top of the cascade are MAPK kinase kinases (MAPKKK), which can be activated by signals originating from G-protein coupled receptors (Kranenburg and Moolenaar, 2001), RTKs (English et al., 1999) and integrins (Giancotti and Ruoslahti, 1999). Of these, Raf is specific for the ERK1/2 MAPK pathway (Kyriakis et al., 1992), whilst MEKKs 1-4 are able to activate multiple MAPK pathways (Hagemann and Blank, 2001). MEKK1 can be activated by Ras (Lange-Carter and Johnson, 1994), which is a target of EGFR activation

(Lowenstein et al., 1992), providing a link between the EGFR and multiple MAPK pathways.

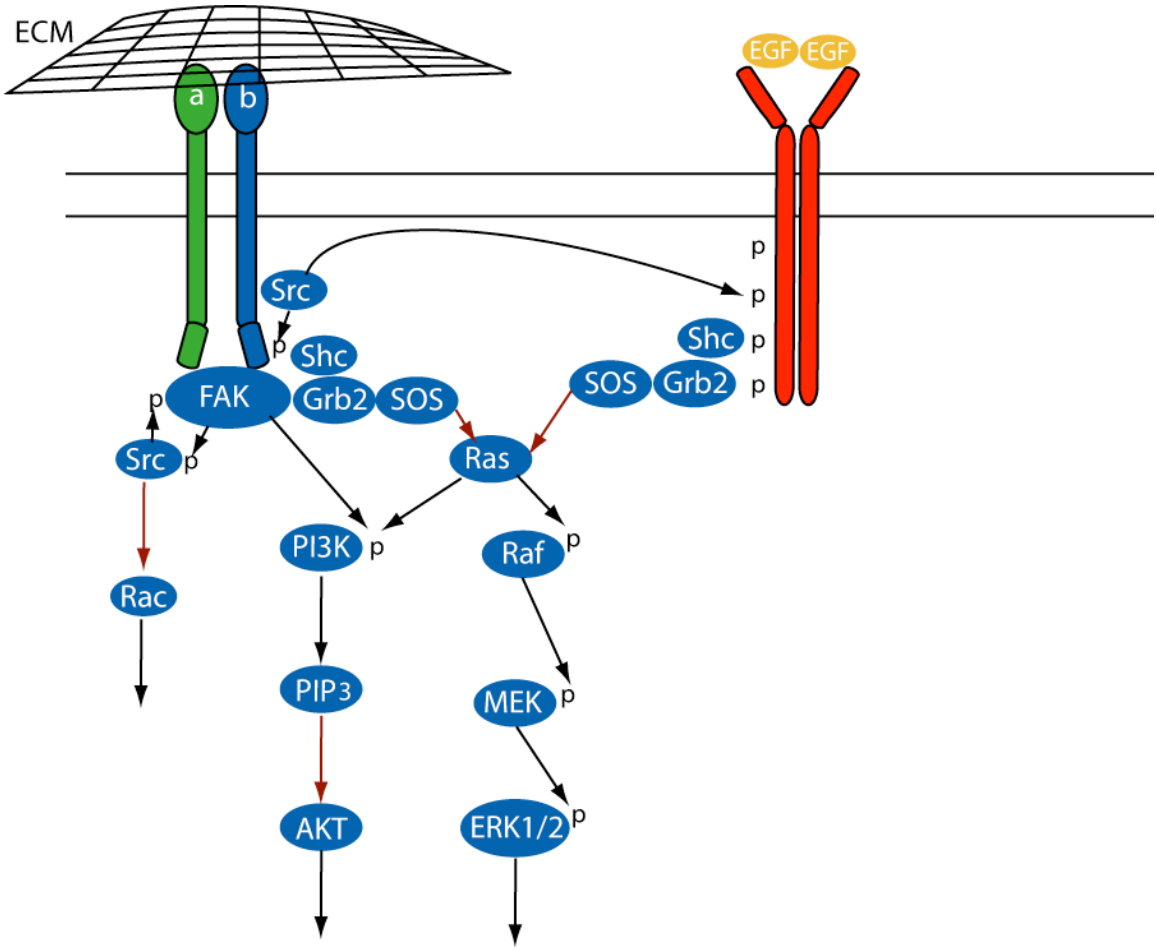


Figure 1.6 Crosstalk between epidermal growth factor receptor and integrin signalling

Ligand activated EGFR recruits the Grb2/SOS complex directly or via Shc. Integrin contact with the ECM activates FAK, leading to the recruitment of the Grb2/SOS complex. FAK also activates Src, which can phosphorylate FAK, providing a docking site for components leading to Rac activation, some integrin β subunits, providing a docking site for Shc, or tyrosine residues on the C-terminal domain of the EGFR, modulating its activity. FAK activation also activates PI3K, either directly or via Ras. Black arrows denote phosphorylation (p). Tan arrows represent all other interactions

1.5.4.1.2 MAPK kinases

MAPKKKs activate MAPK kinases (MAPKK) (Hagemann and Blank, 2001). The specificity of Raf for ERK1/2 lies in its own specificity for the ERK1/2 MAPKKs MEK1 and MEK2 (Kyriakis et al., 1992), whereas MEKKs 1-4 are able to phosphorylate and activate MEK4/7, the MAPKK of c-Jun N-terminal kinase (JNK) MAPK, MEK3/6, the MAPKK of p38 MAPK as well as MEK1 and MEK2 (Hagemann and Blank, 2001). In addition to their activation by Ras, MEKKs1 and 4 can be activated by Rho family kinases (Fanger et al., 1997) (Gerwins et al., 1997), providing a means of integrating these signals into the ERK1/2, JNK and p38 pathways. As well as performing an integrating function, the tiered kinase cascade provides a means of signal amplification as in many cases the number of target kinases at one level in the pathway greatly exceeds the number of effector kinases (Pearson et al., 2001).

1.5.4.1.3 Extracellular signal regulated kinase: An example of the MAPKs

MEKs are activated by serine/threonine phosphorylations (Zheng and Guan, 1994). ERK1/2 activation by MEK1 and MEK2 involves an initial tyrosine phosphorylation followed by a threonine phosphorylation (Ferrell and Bhatt, 1997) (Pearson et al., 2001). Tyrosine phosphorylation does not itself activate ERK1/2 and a threshold level of tyrosine phosphorylated molecules must be reached before phosphorylation of the activating threonine can occur (Ferrell, 1997). Activated ERK1/2 can be inactivated by tyrosine, serine/threonine, or dual specificity phosphatases which can themselves be activated by MAPK phosphorylation, providing a negative feedback loop regulating the duration of MAPK activation (Keyse, 2000). The expression of other phosphatases can be induced by extracellular stimuli, (Guan et al., 1992) providing further means of regulating MAPK pathway activity.

1.5.4.1.4 Achieving specificity in the MAPK pathways

The promiscuity of MEKKs 1-4 illustrates a puzzle central to the study of cell signalling, as although in principle their activation by different inputs would be expected to lead to the same downstream effects this is of course not the case.

Specificity of downstream effects in response to different stimuli is proposed to be achieved through scaffolding proteins that regulate the ability of MEKs to engage with their MAPKs or through proteins which inhibit kinases in specific MAPK pathways (Hagemann and Blank, 2001). For example, the activity of MEKs can also be regulated by Rho family G-proteins, which may affect their ability to participate in protein complexes (Frost et al., 1997). The ability of MAPKs to engage their targets or to themselves become activated is also regulated through their subcellular localisation (Pouyssegur and Lenormand, 2003).

1.5.4.1.5 Targets of the MAPKs

Targets of MAPKs include other cytoplasmic effector kinases, cytoskeletal components, phospholipase A_2 (PLA2), and nuclear transcription factors (Pearson et al., 2001). Mnk1 and Mnk2 are kinases that are phosphorylated by ERK1/2 and p38, but not JNK, and function to stimulate translation via activation of eukaryotic elongation factor eIF-4E (Waskiewicz et al., 1997). MAPKAP kinase 2 is also a target of both p38 and ERK1/2 (Stokoe et al., 1992) which phosphorylates the F-actin binding proteins hsp27 (Larsen et al., 1997) and LSP1 (Huang et al., 1997) and the transcription factors CREB (Xing et al., 1998) and ATF-1 (Tan et al., 1996), as are Msk1 and Msk2 (Deak et al., 1998) which can phosphorylate histone H3 (Thomson et al., 1999) and cyclic AMP response-element binding protein (CREB).

Translocation of MAPKs to the nucleus allows them to directly phosphorylate transcription factors. The AP-1 transcription factors c-fos (Chen et al., 1993) and c-jun (Hibi et al., 1993) are phosphorylated directly by ERK1/2. ERK1/2 phosphorylation of c-jun inhibits its activity whilst phosphorylation by JNK stimulates it (Hibi et al., 1993). The transcription factor Elk-1 is phosphorylated by ERK, JNK and possibly p38 (Janknecht et al., 1993), whilst SAP-1 and SAP-2 are phosphorylated only by ERK1/2 and p38 (Strahl et al., 1996). Thus it can be seen at both the level of MAPK activation and the downstream targets of the MAPKs that some components of the pathways are specific to one MAPK, whereas others may be integrated into two or more.

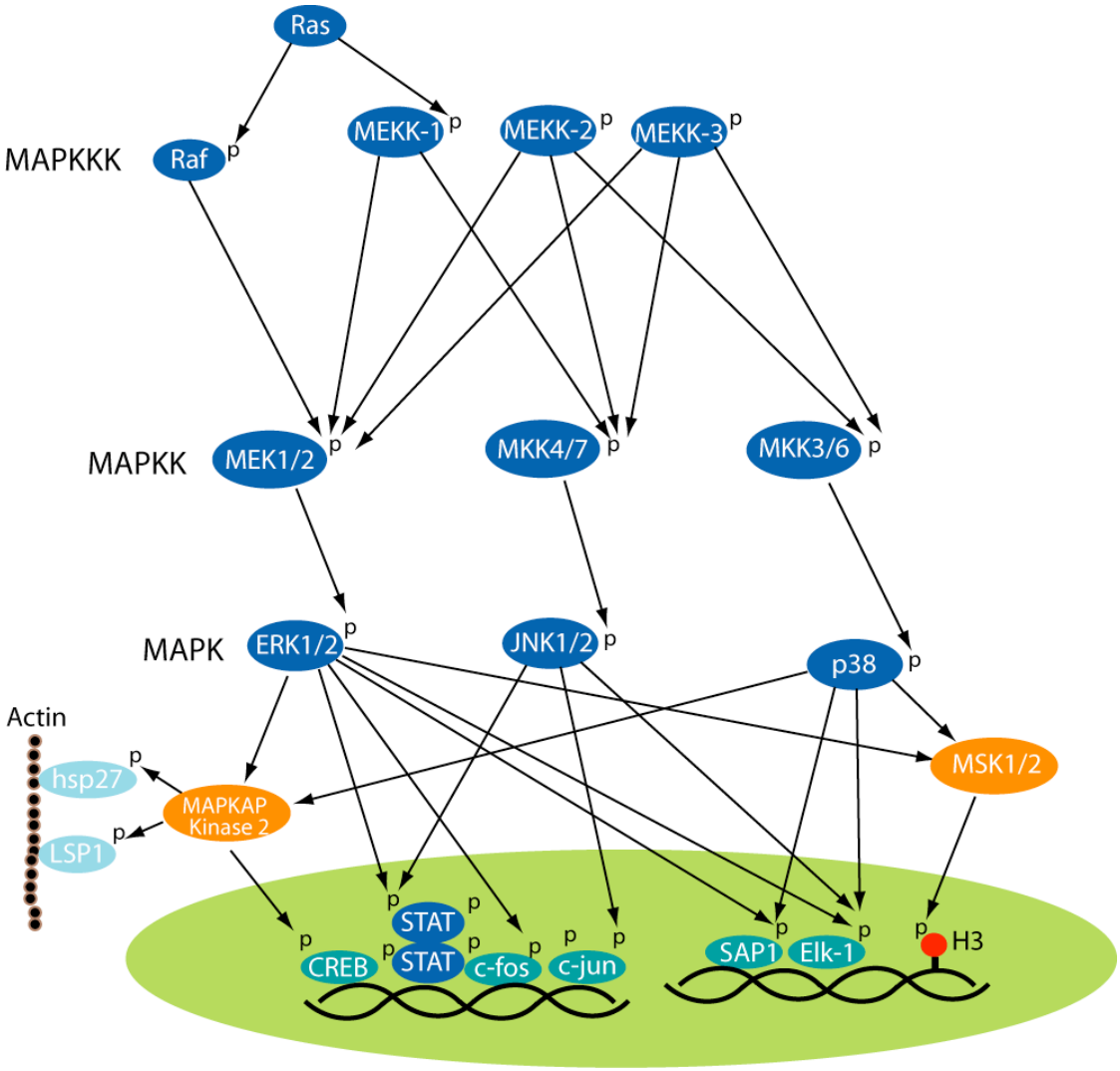


Figure 1.7 The MAPK pathways

A number of MAPKKKs exist with overlapping substrate specificity, each of which has different specificity for MAPKKs. The MAPKKs on the other hand are more specific for their respective kinases; Regulation of MAPKK activation via their interaction with scaffolding proteins (not shown) facilitates the activation of specific MAPKs in response to stimuli such as receptor activation. The MAPKs themselves also have overlapping substrate specificity and regulate transcription via the direct phosphorylation of transcription factors or via other kinases such as MSK1/2 and MAPKAP kinase 2. MSK1/2 also regulates gene expression by remodelling chromatin structure via the phosphorylation of histone H3. MAPKAP kinase 2 regulates actin structure via the phosphorylation of cytoskeleton associated proteins hsp27 and LSP1. Black arrows denote phosphorylation (p). See (Hagemann and Blank, 2001) and (Pearson et al., 2001) for detailed reviews of the MAPK signalling cascades.

1.5.4.2 Janus kinase/ signal transducer and activator of transcription pathway

A number of STATs are activated by phosphorylation through the binding of their SH2 domains to phosphorylated tyrosines on receptor tyrosine kinases such as EGFR (Silvennoinen et al., 1993), or through activation of cytokine receptors and GPCRs via NRTKs such as Src (Ram and Iyengar, 2001) (Silva, 2004), leading to their dimerisation and translocation to the nucleus where they regulate gene expression. EGFR signalling can regulate STAT mediated gene expression through the direct phosphorylation of STATs, via Src or via the activation of janus kinases (JAKs), which phosphorylate and activate a number of STAT proteins (Quesnelle et al., 2007). In addition to the activation of the STAT pathway through different routes originating from the EGFR, JAKs and the ERK1/2 MAPK pathway converge in the regulation of STAT transcriptional activity (Chung et al., 1997).

1.5.4.3 Phosphatidylinositol 3-kinase pathway

PI3K can be activated by receptor tyrosine kinases such as the EGFR. Unlike ErbB3 (Ram and Ethier, 1996), EGFR does not directly activate PI3K, however it is able to do so via Ras activation (Marte et al., 1997). This ability of ErbB3 to activate PI3K directly and via Ras highlights the non-linearity of cellular signal-transduction pathways. The PI3K pathway can also be activated via G-protein coupled receptors (Murga et al., 1998) and integrins (Clark et al., 1998).

The target of PI3K is the lipid phosphatidylinositol (PI) in the inner leaflet of the cell membrane. Multiple phosphorylations of PI lead to the production of phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP2 serves as a docking site at the membrane for the kinase Akt, localising it appropriately for its activation by phosphoinositide dependent kinase 1 (PDK1) (Chan et al., 1999). Akt is involved in the regulation of a wide diversity of cellular processes including proliferation, survival, inhibition of apoptosis through a variety of effector molecules (Chan et al., 1999), and the stimulation of translation through the mammalian target of rapamycin (mTor) pathway (Mamane et al., 2006). Akt also phosphorylates

transcription factors including CREB (Du and Montminy, 1998). The PI3K pathway illustrates how signalling pathways can involve the phosphorylation of lipids as an intermediate step between the activation of two kinases. Akt can also phosphorylate and inactivate Raf in the ERK1/2 MAPK pathway (Lee et al., 2008).

1.5.4.4 Phospholipases, protein kinase A and protein kinase C

The activation of phospholipases is another mechanism through which the membrane lipid phosphatidylinositol can act as a signal-transducing molecule. PLCγ can be activated by the EGFR (Margolis et al., 1989) and cleaves PIP2 to yield IP3 and diacylglycerol (DAG) (Kamat and Carpenter, 1997). DAG functions as a co-factor for the activation of PKC, whereas IP3 releases Ca^{2+} from the endoplasmic reticulum, another co-factor in the activation of PKC, from intra-cellular stores (Pettitt et al., 1997) (Weiss et al., 1991). A subclass of PKCs named the atypical PKCs also exist which do not require Ca^{2+} for their activation (Spitaler and Cantrell, 2004). As mentioned, PI3K catalyses the phosphorylation of PI to PIP2 and PIP3, providing a link between PI3K and IP3 signalling (Chan et al., 1999). As with many of the pathways discussed, activated PKC leads to phosphorylation of transcription factors such as CREB (Yamamoto et al., 1988), thereby regulating gene expression. PLCγ is also involved in the re-organisation of the cytoskeleton in response to EGFR activation, and has been shown to do so via interaction with the Rho GTPases (Li et al., 2009).

PLA2 is Ca^{2+} dependent (Burke and Dennis, 2009) and becomes activated following phosphorylation by MAPKs and PKC (Nemenoff et al., 1993). Activated PLA2 translocates to the cytoplasmic surface of the cell membrane where it catalyses the cleavage of phospholipids to produce arachidonic acid (Burke and Dennis, 2009), which subsequently becomes modified into active compounds such as prostaglandins and leukotrienes which regulate cell behaviour via the activation of GPCRs, and may also regulate gene transcription via nuclear hormone receptors (Funk, 2001).

PKA is activated by intracellular cAMP produced in response to activated G_S-protein coupled receptor mediated stimulation of adenylate cyclase (Meinkoth et al., 1993). PKA has a number of targets, amongst which are transcription factors such as CREB (Thomson et al., 2008).

1.5.4.5 General principles of signal transduction pathways

The signal transduction pathways described, although by no means complete in terms of those that exist in the eukaryotic cell, illustrate many of the general principles of signal transduction. Phosphorylation of tyrosine, serine and threonine occurs widely throughout the various pathways, leading to either activation or inhibition of a given molecule's activity. Components of different pathways can interact, providing the cross-talk necessary to establish specific outcomes from pathways with many potential outputs. Signalling can be initiated via phosphorylation events originating from receptor or non-receptor tyrosine kinases or through mechanisms that do not require kinase activity such as in the case of heterotrimeric G-protein coupled receptors. Following receptor activation, signal transduction can also be mediated either via further kinase activity, the exchange of phosphorylated guanine nucleotides by small G-proteins, or through distinct mechanisms involving second messengers such as DAG, Ca^{2+} or cAMP, which in many cases results in signal amplification at some stage of the pathway. The end points of many pathways are the activation by phosphorylation of transcription factors controlling gene expression, many of which are the targets of multiple pathways. As well as regulating transcription, receptor mediated signalling is also able to control translation and cell growth, the balance of cell survival and apoptosis and cell motility via the modification of cytoskeletal components.

1.6 Glycosylphosphatidylinositol anchored proteins

Prod1 is a glycosylphosphatidylinositol (GPI) anchored cell-membrane localised protein (da Silva et al., 2002). GPI anchored proteins are synthesised with an N-terminal signal peptide shared by all secreted proteins which is cleaved co-translationally, targeting the molecule into the endoplasmic reticulum (ER) (Rapoport, 1991), and a C-terminal peptide which is a recognition sequence for the GPI anchor attachment machinery (Eisenhaber et al., 1998).

1.6.1 GPI anchor attachment

The consensus C-terminal peptide has been defined in terms of the biochemical properties of the amino acids rather than specific residues and can be described as a region of 4 small amino acids followed by a moderately polar spacer region of 7 amino acids and a mostly hydrophobic tail region (Eisenhaber et al., 1998). The GPI anchor is covalently attached to the second small amino acid in the recognition sequence in a concerted reaction occurring on the inner surface of the ER membrane, during which the C-terminal peptide is cleaved (Eisenhaber et al., 2003). The C-terminal peptide of Prod1 conforms moderately to the consensus and it has been demonstrated that Prod1 mediated effects on blastema engulfment can be blocked by phosphatidylinositol-phospholipase C (PIPL-C) (da Silva et al., 2002), demonstrating its attachment to the cell membrane via a GPI anchor is required for its biological activity.

1.6.2 Trafficking of GPI anchored proteins

After GPI anchor attachment, proteins are linked to the inner leaflet of the ER membrane (Eisenhaber et al., 2003). The GPI anchor itself is composed of a highly conserved glycan core modified with a number of side-chains and attached to a phospholipid anchor (Fig.1.8) (Paulick and Bertozzi, 2008). The primary lipid anchor is modified subsequent to GPI anchor attachment yielding an anchor of variable lipid composition. In yeast, lipid anchor modification occurs in the ER (Fujita and Jigami, 2008), and it has been proposed that GPI anchored proteins become concentrated at specific regions of the ER

membrane through association with these lipid anchor remodelling enzymes. These regions then bud to produce vesicles that are trafficked to the Golgi. Transmembrane proteins are concentrated at regions of the ER membrane distinct from where GPI anchored proteins tend to accumulate, and tend to be trafficked to the Golgi in separate vesicles from those carrying GPI anchored proteins, though sorting is not absolutely efficient as a population of vesicles are observed containing both GPI anchored and transmembrane proteins (Castillon et al., 2009). In mammals it is not until proteins reach the Golgi that the lipid anchor is modified (Kinoshita et al., 2008) and thus it is likely that this will be the location of GPI anchored protein sorting if it occurs .

GPI anchored proteins are internalised by a pathway independent of clathrin or caveolin mediated endocytosis. Internalisation of GPI anchored proteins is instead mediated by pinocytosis, delivering them to recycling endosomes rather than the Golgi (Sabharanjak et al., 2002).

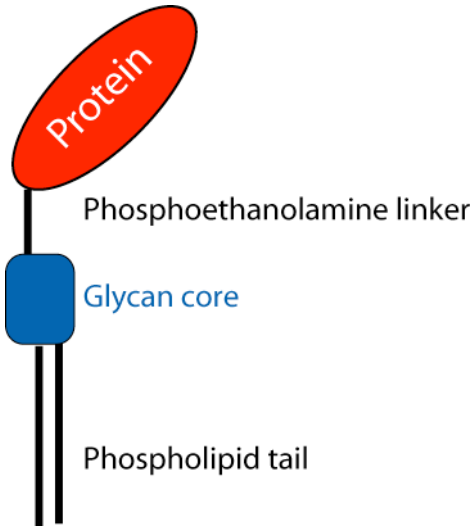


Figure 1.8 The glycosylphosphatidylinositol anchor

The GPI anchor is composed of a glycan core, which can be decorated with a variety of side groups and a phospholipid tail of variable composition. Proteins are linked to the GPI anchor via a phosphoethanolamine linker.

1.7 'Lipid rafts', or detergent insoluble microdomains

1.7.1 Cell membrane localisation of GPI anchored proteins

Once trafficked to the cell membrane by exocytosis, the GPI anchor becomes inserted into the outer leaflet of the lipid bilayer (Eisenhaber et al., 2003). Thus GPI anchored proteins do not make direct contact with the proteins in the cytoplasm, posing questions as to how they function in a signal transducing capacity. It has been proposed that the sequence of the C-terminal peptide can determine the specific composition of the GPI anchor and thereby regulate the localisation of a molecule on the cell membrane (Nicholson and Stanners, 2006). It has also been indicated that the activity of molecules may rely on their GPI anchorage, as when they are instead anchored proteinaceously, their activity is altered (Robinson et al., 1989). These results tend to be explained in the context of detergent insoluble membrane microdomains or 'lipid rafts' (Helms and Zurzolo, 2004).

Cholesterol alters the biophysical properties of lipid bilayers by causing the acyl chains of lipids to become more closely packed and ordered. This ordered state of the membrane is further enhanced by sphingolipids (Brown and London, 2000) and it has been proposed that stable local regions, or 'rafts', rich in cholesterol and sphingolipids exist within the membrane, which as a whole is less ordered (Pike, 2004). These rafts are proposed to concentrate GPI anchored proteins in the outer leaflet of the membrane (Brown and Rose, 1992) and lipid anchored NRTKs and G-proteins in the inner leaflet (Resh, 1999). Transmembrane proteins such as receptors are proposed to be included or excluded from rafts depending on their identity (Simons and Toomre, 2000). Although GPI anchor modification has been proposed to be a mechanism for sorting of proteins into detergent resistant microdomains (Brown and Rose, 1992), it has been shown with the GPI anchored prion protein PrP that deletion of the C-terminal GPI anchor attachment sequence does not affect its localisation (Walmsley et al., 2003).

1.7.2 Lipid rafts and signal transduction

The localisation of GPI anchored proteins to microdomains in the outer leaflet of the cell membrane and NRTKs and G-proteins to the inner leaflet has been proposed as a means of facilitating signal transduction (Simons and Toomre, 2000). It is observed that antibodies against GPI anchored proteins can activate downstream signalling, and this is interpreted as being the result of GPI protein clustering (Murray and Robbins, 1998). This clustering is then proposed to result in the transduction of a signal across the cell membrane to NRTKs and G-proteins localised to the inner membrane surface (Simons and Toomre, 2000). This can be explained with reference to the participation of a transmembrane adaptor protein in the process, however it has been suggested that in the case of some molecules, rather than the protein itself, it is instead the anchor that mediates biological effects (Nicholson and Stanners, 2006). Such a mode of signal transduction suggests that the protein domain acts only to facilitate clustering of anchors, leading to the activation of molecules localised to the inner surface of the membrane by the interaction of lipids between the two leaflets. Thus a 'soft' interpretation of lipid rafts would be that the localisation of proteins to the inner leaflet of the cell membrane positions them appropriately to maximise their potential to be activated by events involving GPI anchored proteins occurring in an adjacent region of the outer membrane, transduced across it by transmembrane proteins. A 'hard' interpretation of the function of lipid rafts would be that without the co-ordination of the composition of adjacent regions of the inner and outer leaflets of the membrane, signal transduction across the cell membrane initiated by events involving GPI anchored proteins could not occur due to the requirement for the interaction between the lipid anchor of the GPI anchored protein and some the signalling proteins involved.

1.7.3 A continuous model of the cell membrane

In contrast to the microdomain organisation of the cell membrane, a continuous model proposes that cholesterol and sphingolipids are evenly distributed throughout the membrane. See Figure 1.9 for a comparison between lipid raft containing cell membranes and continuous membranes. The observation that an insoluble fraction enriched in GPI anchored proteins, NRTKs, G-proteins,

cholesterol and sphingolipids is seen after centrifugation of cell membranes lysed in Triton X-100 at 4°C in a density gradient (Yu et al., 1973) has been taken as evidence for lipid rafts. As evidence for the existence of rafts, this observation is not sufficient however as it is open to alternative interpretations. Detergent solubilisation of the membrane occurs as a result of the insertion of individual detergent molecules into the membrane until holes form, causing its fragmentation (le Maire et al., 2000), during which membrane components may become re-arranged in various ways (Munro, 2003) giving a false impression of its natural composition. Likewise, the effects of depleting cholesterol from membranes on cell signalling taken as supporting the lipid raft model of signalling (Tansey et al., 2000) can be interpreted as being the result of interfering with the permeability and physical state of the membrane without definitively implicating the existence of lipid rafts (Munro, 2003). The dynamics of lateral diffusion in the cell-membrane of the GPI anchored protein CD59 has also been seen to be very similar to a non-raft phospholipid (Subczynski and Kusumi, 2003), in contrast to expectations were it raft localised and adding further cause for scepticism of the model.

Even if lipid rafts do not truly exist, the concentration of GPI proteins into regions of either the ER or Golgi membrane during their synthesis, and their sorting into specific vesicles prior to trafficking to the cell membrane (Castillon et al., 2009) may play a role in determining potential molecular interactions. Membrane proteins linked to the cytoskeleton may concentrate other proteins in membrane microdomains by limiting lipid lateral diffusion (Fujiwara et al., 2002), thus if multi-protein complexes are formed at specific locations on membranes within the cell, trafficked in specific vesicles and subsequently become situated in regions of the cell membrane with limited diffusion, proteins may still become localised to microdomains.

In light of current evidence, it seems unwise to place rigid constraints based on the GPI anchored nature of Prod1 on the potential molecular interactions it may participate in. As no molecular level information exists on Prod1 other than its structure, this investigation of its signal transducing activity has remained open-minded to the possibility that it may or may not be localised to specific domains

of the cell membrane, that its GPI anchor may or may not play a functional role in the mechanism of its action and that it may or may not interact with other transmembrane proteins.

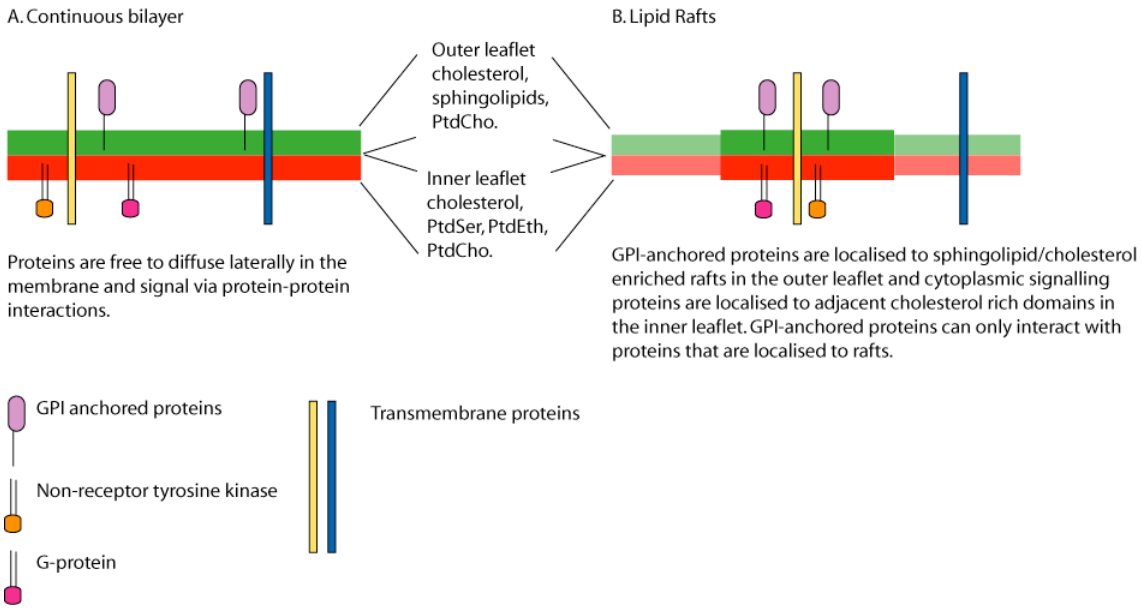


Figure 1.9 Lipid rafts or a continuous membrane?

It has been proposed that cholesterol and sphingolipids form micro-domains in the cell membrane termed 'lipid rafts' enriched in GPI anchored proteins in the outer leaflet, lipid anchored signalling molecules in the inner leaflet and specific transmembrane receptors. Alternatively, cholesterol and sphingolipids may be uniformly distributed throughout the membrane. In this case, if GPI anchored proteins are localised to micro-domains it is due to factors other than the membrane composition. Phosphatidylcholine (PtdCho), phosphatidylserine (PtdSer), phosphatidylethanolamine (PtdEth). Image adapted from (Munro, 2003).

1.8 How does Prod1 function at the molecular level? Insights from Prod1 homologs

Prod1 is a member of the large three-fingered family of proteins (TFPs) and is structurally most similar in structure to CD59 and domain 3 of the urokinase plasminogen activator receptor (uPAR), both of which are also GPI anchored proteins (Garza-Garcia et al., 2009). Structural similarity between TFPs is shown in Figure 1.10.

1.8.1 CD59

CD59 is expressed on the surface of a wide variety of cells and functions to protect cells from the innate immune system by disrupting the formation of the membrane attack complex (MAC) and thereby inhibiting cell lysis (Rollins and Sims, 1990). In addition to physically disrupting the formation of the MAC, CD59 has been shown to initiate downstream signalling in response to complement factor binding and antibody cross-linking (Murray and Robbins, 1998).

CD59 has also been shown to immunoprecipitate with the α subunit of heterotrimeric G-proteins, which are known to be associated with detergent resistant microdomains (Resh, 1999), though the signalling events resulting from this are uncharacterised (Solomon et al., 1996).

In haematopoietic cells, cross-linking of CD59 with antibodies or binding of complement factor 8 leads to phosphorylation of Shc (Murray and Robbins, 1998). When activated by phosphorylation, Shc dimerises with the Grb2-SOS complex leading to the activation of Ras and the ERK1/2 MAPK pathway as described previously. CD59 cross linking also led to Src phosphorylation and Src kinase activity dependent phosphorylation of other cytoplasmic kinases (Murray and Robbins, 1998). As described previously, Src family kinases are associated with phosphorylated tyrosine residues on other proteins such as the EGFR (Stover et al., 1995) and are able to phosphorylate an number of downstream targets including Shc (van der Geer et al., 1996). An unbiased screen for potential EGFR interacting proteins indicated a physical interaction between CD59 and the EGFR (Blagoev et al., 2003). Shc and Src are known to

be involved in EGFR signalling as described previously, thus the EGFR may represent the link between phosphorylation of Shc, Src, and CD59 dimerisation.

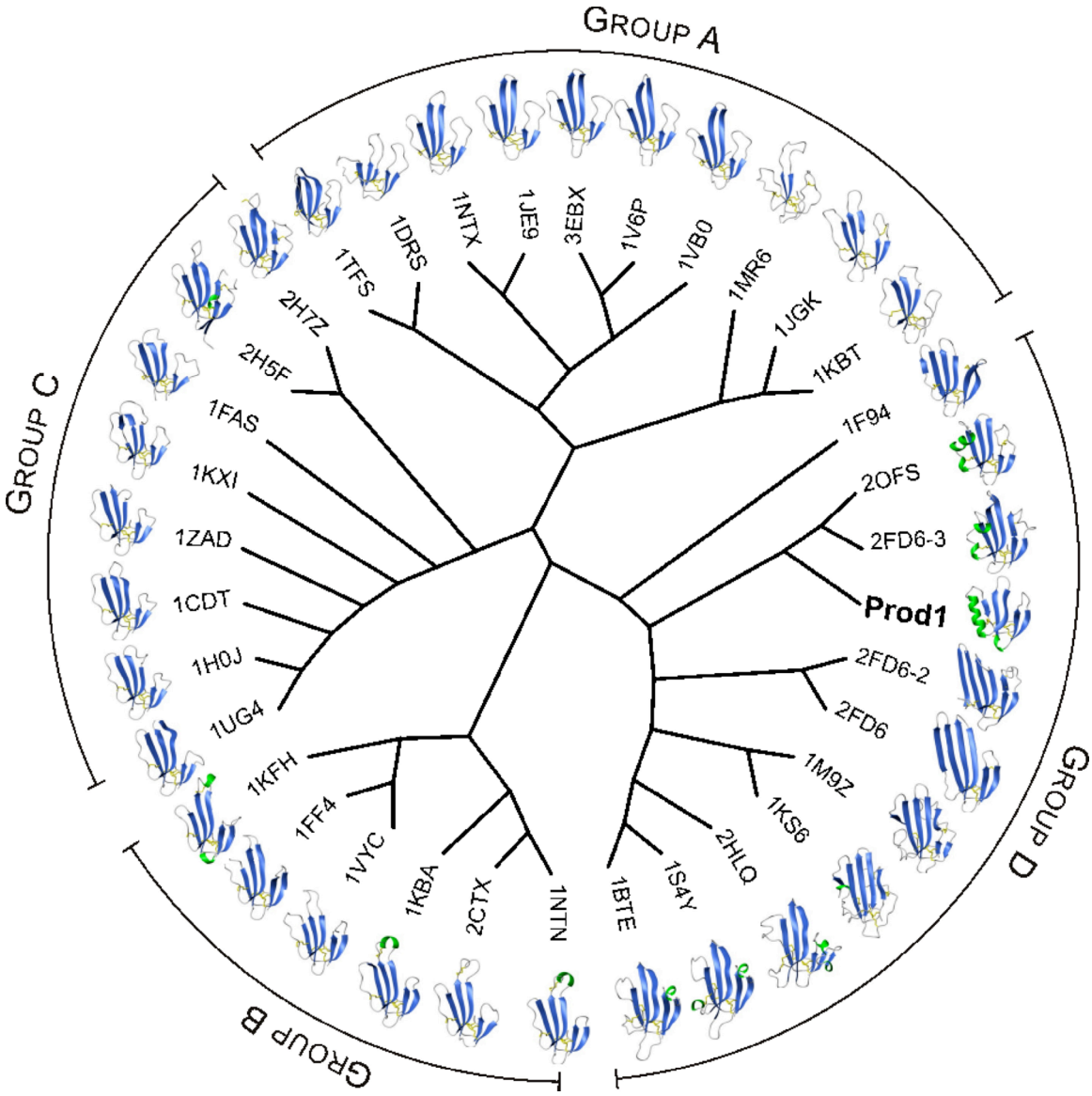


Figure 1.10 The three fingered family of proteins

Three-fingered proteins can be categorised into groups A-D based upon their protein structure. Structural comparison groups Prod1 most closely with 2OFS (CD59) and 2FD6-3 (domain 3 of the urokinase-like plasminogen activator receptor) (Garza-Garcia et al., 2009). Image courtesy of A. Garza-Garcia.

1.8.2 The urokinase-type plasminogen activator receptor

uPAR is another member of the three-fingered family of proteins structurally similar to CD59 and Prod1 (Garza-Garcia et al., 2009) with a significantly more detailed body of literature surrounding its participation in cell-signalling. High expression of uPAR leads to ligand-independent phosphorylation of the EGFR in response to fibronectin binding $\alpha 5\beta 1$ integrin, via the activity of cytoplasmic FAK, leading to cell proliferation (Liu et al., 2002). Likewise, activation of uPAR by its ligand uPA has been shown to result in the phosphorylation of EGFR by Src, leading to increased fibronectin secretion by fibroblasts (Monaghan-Benson and McKeown-Longo, 2006). The phosphorylation of sites on EGFR by uPAR dependent Src activity has also been shown to modulate the response of cells to EGF. In this example EGF activates ERK1/2, but does not stimulate cell proliferation in the absence of uPAR. Interactions between uPAR and integrins mediate Src dependent phosphorylation of sites on the EGFR in response to EGF that result in STAT5b activation in addition to ERK activation, leading to a stimulation of proliferation (Jo et al., 2007). As well as activating downstream signalling pathways via integrins and the EGFR, uPAR has also been shown to initiate signalling via interaction with a GPCR, leading to the activation of tyrosine kinases and cell migration (Resnati et al., 2002).

1.8.2.1 A link between Prod1 and MMP9?

uPAR integrin interactions leading to Src dependent activation of ERK1/2 have also been shown to regulate the activity of MMP9 (Wei et al., 2007), which as discussed previously, is highly regulated during blastema formation (Vinarsky et al., 2005) and may contribute to cell de-differentiation (Park and Kim, 1999) and the establishment of the wound epidermis (Satoh et al., 2008). The similarities between the structures of Prod1 and uPAR (Garza-Garcia et al., 2009) and the demonstrated involvement of MMPs with the processes of regeneration (Vinarsky et al., 2005), taken together with the observations that RA increases the level of de-differentiation of a blastema (Ju and Kim, 1994) and upregulates both Prod1 (da Silva et al., 2002) and MMP9 (Park and Kim, 1999) discussed previously make the investigation of a role for Prod1 in the regulation of MMPs

a seemingly worthy point at which to begin the investigation of the molecular mechanisms through which this little studied protein regulates cell behaviour.

1.8.2.2 A role for the EGFR in Prod1 signal transduction?

The observed interaction of CD59 (Blagoev et al., 2003) and uPAR (Mazzieri et al., 2006) with the EGFR warrants investigation of the possibility that it may serve as a transmembrane signal transducing protein able to relay a signal from Prod1 across the cell membrane. In cancer biology, upregulation of uPAR and MMPs is prognostic of a metastatic phenotype (Inuzuka et al., 2000). As discussed above, previous studies of Prod1 may indicate it as having a role in cell migration, a process with clear analogy to metastasis. Due to its similarities with uPAR, in addition to the relevance of this investigation to understanding the processes regulating regeneration, study of the potential regulation of MMP9 by Prod1 may yield insights with wider implications in the field of cancer biology.

2 Materials and methods

2.1 Constructs

2.1.1 EGFR and β1 integrin

Notophthalmus viridescens Prod1 was cloned previously (da Silva et al., 2002). Notophthalmus viridescens EGFR and β1 integrin were cloned by rapid amplification of cDNA ends (RACE) from blastemal cDNA, as were Ambystoma mexicanum and Ambystoma maculatum Prod1 (P. Gates).

2.1.2 Epitope tagging

Epitope tags were added by polymerase chain reaction (PCR) with primers containing a tag sequence and restriction sites to produce fragments that were subsequently cloned back into the PCI-Neo vector.

2.1.3 Prod1 Flag deletion constructs

Prod1 Flag deletion constructs were cloned by PCR of the original Prod1 Flag construct with restriction site containing primers to produce truncated fragments that were subsequently cloned back into the PCI-Neo vector.

2.1.4 Prod1 Flag site-directed mutagenesis

Computer algorithms were used to select residues for mutation following inspection of the three-dimensional structure of Prod1 and the alignment of its amino acid sequence with other TFPs. Care was taken to ensure that mutation sites were distributed across the whole protein surface to sample all possible interaction surfaces. A residue was selected as a candidate for mutation if:

a) more than 30% of the side chain is solvent accessible
b) by inspection of the structure it does not appear to fulfil a role in the attainment of the Prod1 three-dimensional fold
c) it is not conserved across the TFP superfamily.

Point mutations were generated using the Stratagene Quick Change site-directed mutagenesis kit (Stratagene 200519) according to the manufacturer's

instructions. Prod1-Flag, Prod1-Flag LFA and Axolotl Prod1 cloned into the N2 vector were PCR amplified with Pfu turbo enzyme using mis-matched primers at the site of mutation and subsequently degraded using Dpn1 prior to cloning the mutated product.

2.2 Cloning

2.2.1 Fragment purification

PCR fragments were electrophoresed on an agarose gel, bands of appropriate size were cut from the gel and DNA was purified using a Quiagen gel extraction kit. After restriction digestion of insert/ vector, DNA was purified by organic phase separation. Equal volumes of phenol chloroform isoamyl alcohol (25:24:1) were mixed with DNA in TE buffer (see below) and spun for 5 minutes. The aqueous phase was then collected and mixed with an equal volume of chloroform isoamyl alcohol (24:1) and spun for 10 minutes to pellet the DNA. Pellets were washed in 70% ethanol and re-suspended in TE.

2.2.2 Ligation/transformation

Ligations were performed for 1hr at 11°C with T4 DNA ligase in 10ul reactions using a 20:1 ratio of fragment to vector. XL2 Blue E.coli (Stratagene 200150) were transformed according to manufacturer's instructions and plated under antibiotic selection. Although not confirmed extensively, there was some indication that constructs cloned with RecA- XL2 Blue E.coli gave better expression in B1H1 cells than when cloned in RecA+ SURE E.coli (Stratagene). Cultures were grown from single transformed colonies and DNA was prepared by Quiagen Maxi-prep according to the manufacturer's instructions.

See Appendix for construct sequences, primers used for site-directed mutagenesis and a more detailed discussion of the different constructs.

2.3 Cell culture

2.3.1 Conditions

B1H1 cells were grown in tissue culture plastic flasks coated with gelatin.
B1H1 cells were cultured in 70% minimal essential medium (MEM) + Earles (21090 GIBCO), 30% H_2O (hereafter referred to as AMEM), supplemented with 10% FBS, 100U-100U/ml penicillin-streptomycin (15140 GIBCO), 2mM glutamine (25030 GIBCO) and 10ug/ml insulin (Sigma I-5500).

HEK293T cells were grown in uncoated tissue culture plastic flasks.
HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) + 4.5g/L glucose, + 580mg/L L-glutamine, -pyruvate (41965 GIBCO) supplemented with 10% foetal calf serum and 100U-100U/ml penicillin-streptomycin (15140 GIBCO)

2.3.2 Passage

B1H1 cells (Ferretti and Brockes, 1988) were rinsed twice using 70% Dulbecco's phosphate buffered saline (DPBS) 30% H_2O (hereafter referred to as APBS) and incubated at room temperature in trypsin (15090 GIBCO) diluted to 1x concentration (0.25%) in APBS until cells detached. Trypsin was then neutralised using growth media and cells were spun down in conical-bottom tubes at 1000rpm for 3 minutes. Media were aspirated, pellets were re-suspended and cells were either plated onto tissue culture dishes or into tissue culture flasks pre-coated with gelatin. B1H1 cells were split at no greater than 1:3 and were passaged after 7-14 days.

HEK 293T cells were rinsed twice using PBS and incubated at 37°C in trypsin (15090 GIBCO) diluted to 1x concentration in PBS until cells detached. Trypsin was then neutralised using growth media and cells were spun down in conical-bottom tubes at 1000rpm for 3 minutes. Media were aspirated, pellets were resuspended and cells were either plated onto 'Primera' tissue culture dishes (Falcon) or into tissue culture flasks. HEK 293T cells were passaged routinely every 3-4 days.

2.3.3 Incubation

HEK 293T cells were incubated at 37°C in 7% CO_2.

B1H1 cells and explanted skin patches were incubated at 25°C in 2% CO_2.

2.4 Transfection

2.4.1 B1H1 cells

Cells were plated on gelatin 7 days prior to transfection. Confluent dishes of cells were transfected for 18hr using a 1:2 ratio of DNA to Lipofectamine 2000 (11668-019 Invitrogen) according to manufacturer's instructions in AMEM without penicillin/streptomycin. 35mm dishes were transfected in 2ml medium plus 4ug DNA. 60mm dishes were transfected in 5ml medium with 10ug DNA.

2.4.2 HEK 293T cells

Cells were plated 24hr prior to transfection on 'Primera' dishes (Falcon). Cells were transfected at a density of 30% for 7hr using a 1:2 ratio of DNA to Lipofectamine 2000 (11668-019 Invitrogen) according to manufacturers instructions in DMEM without penicillin/streptomycin. 60mm dishes were transfected in 5ml medium with 6ug DNA.

2.4.3 Transgenic HEK 293T cells

HEK 293T cells were infected with a lentiviral vector containing the coding region for Prod1-Flag or Prod1-Flag Q59A and a puromycin resistance gene. Cells were grown at clonal density in the presence of puromycin and transgenic strains were derived from individual colonies (A.Janmohamed)

2.5 RNA preparation

2.5.1 Tissue culture cells

Cells were collected in conical-bottom tubes as described under 'cell passage', resuspended in cold APBS and then pelleted in 1.5ml tubes at 4°C. Cells were lysed on ice in appropriate volumes of cold Ambion Lysis Buffer (Ambion Cells-to-cDNA II Kit AM1723), samples were heated to 75°C for 10 minutes, cooled on ice and then treated with deoxyribonuclease (DNAse) (Ambion) at 37°C for 15 minutes. DNAse was inactivated at 75°C for 5 minutes prior to cDNA synthesis.

2.5.2 Tissue samples

Skin and other tissue samples were mechanically dissociated in Tri reagent (Sigma T9424) and RNA was extracted by phase separation according to manufacturers instructions. RNA samples were treated with DNAse in Ambion Lysis Buffer (Ambion Cells-to-cDNA II Kit AM1723) prior to cDNA synthesis.

2.6 cDNA synthesis

Complementary DNA (cDNA) was synthesised in 20ul reactions including 1ul Superscript II Reverse Transcriptase (Invitrogen 18064-014), 1ul RNAse out RNAse inhibitor (Invitrogen 10777-019), 5uM random hexamers (Invitrogen N8080127), 1uM dNTP's (Invitrogen18427-013) and <500ng RNA in Ambion Lysis Buffer (Ambion Cells-to-cDNA II Kit AM1723).

2.7 Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was used to assay gene expression in cultured cells and explanted skin-patches. mRNA was reverse transcribed using random primers to produce cDNA representative of the transcriptome under investigation. Genes of interest were amplified from cDNA using gene specific primers and the accumulation of product measured over the course of the reaction. The intercalation of SYBR Green into double-stranded DNA produces

fluorescence, which was used to quantify the accumulation of PCR product for all target genes other than Prod1. As Prod1 is expressed at low levels in most tissues, the more sensitive TaqMan PCR approach was employed, during which a fluorescent dye attached to a gene specific primer becomes un-quenched as the primer becomes incorporated into product.

The PCR cycle at which product begins to accumulate exponentially is denoted the cT value. As at this point in the PCR product is doubling each cycle, cT values can be compared to calculate the relative number of target sequences between samples based upon the premise that a difference in cT value of 1 represents a two-fold difference in the number of target sequences, which holds true under optimal PCR conditions with efficient primers. cT values were used to calculate the relative difference in expression of a gene between two samples using the equation:

Relative difference = $2^{\Delta cT}$

In order to take into account unequal amounts of total cDNA in samples either due to their being synthesised from unequal amounts of RNA or slight differences in efficiency of cDNA synthesis, the level of a housekeeping gene was determined and taken to bear the same relationship to the total cDNA in each sample to serve as a normalisation factor. The normalised relative difference of expression of gene X between sample 1 and sample 2 is:

$$2^{(cT\ Sample\ 1 - cT\ Sample\ 2)}{}_{Gene\ X} / \ 2^{(cT\ Sample\ 1 - cT\ Sample\ 2)}{}_{Gene\ Normaliser}$$

cDNA was amplified using a two-step protocol. A melting temperature of 95 °C was used in all reactions. Primer-pairs were characterised by determining the temperature that gave the lowest cT value whilst also producing a clear, sharp peak on the melt-curve. This temperature was used for annealing/ extension.

All quantitative real-time PCR was performed using the Biorad Chromo 4 detector and analysed using Biorad Opticon Monitor software. iQ Supermix with

SYBR green (Biorad 170-8882) was used for all PCR reactions with conventional primers. iQ Supermix (Biorad 170-8862) was used for TaqMan PCR reactions.

In initial experiments results were verified using two independent normalising genes, GAPDH plus either L27, Actin or EF1a. Subsequent repeats were carried out using GAPDH only. In cultured cells, two normalising genes were used to ensure that drug treatments truly affected MMP9 expression and not the expression of the normalising housekeeping gene. When comparing between different limb tissues, the use of multiple normalising genes was of particular importance, as the level of expression of housekeeping genes compared to the total messenger RNA (mRNA) in a cell cannot be assumed to be the same between different cell types (Vandesompele et al., 2002).

RNA used for cDNA synthesis was included as a control in all experiments to ensure that signal from cDNA samples did not result from contaminating genomic DNA that had evaded DNAse degradation prior to cDNA synthesis. If RNA controls generated traces emerging at cT values within 4 cycles of cDNA, therefore representing 1/16 (2^4=16) of total signal, RNA was further treated with DNA and cDNA was re-synthesised for a repeat PCR reaction, or samples were discarded.

2.8 Western-blotting

Western-blotting was used for detecting overexpressed proteins and protein phosphorylation in cultured cells. Cultured cells were washed with PBS and lysed by scraping in ice-cold cell lysis buffer (see below). 35mm dishes were lysed in 100ul and 60mm dishes in 200ul. Lysates were incubated on ice for 30 minutes prior to clearing debris by spinning at 5,000g for 10 minutes at 4°C. For analysis of EGFR phosphorylation, cells were lysed in ice-cold RIPA buffer (see below) with protease inhibitors (Roche 04906837001).

Protein concentration was determined by bicinchoninic-acid assay using a Dynex Technologies MRX spectrophotometer to read absorbance. Samples of equal total protein were prepared by heating lysates with SDS-buffer (see below) and reducing agent (Invitrogen NP0009) at 90°C for 10 minutes.

Protein samples were run on appropriate NuPage Bis-Tris pre-cast SDS gels according to manufacturer's instructions in MES buffer (Invitrogen NP0002) with antioxidant (Invitrogen NP0005). 12% Gels (NP0341BOX) were used for analysing Prod1 Flag expression and ERK1/2 phosphorylation. 4-12% gels (NP0321BOX) were used for analysing EGFR-Myc and β1-Integrin-Myc pull-down and 3-8% gels (EA03755BOX) were used for analysing EGFR phosphorylation.

Protein was blotted from gels onto nitrocellulose membrane (1041191-Whatman) for 3hr at 150mA constant current immersed in transfer buffer (see below). Membranes were then rinsed in TBS (see below), blocked in Odyssey Block (Li-Cor 927-40000) for 10 minutes and incubated with primary antibody at 4°C overnight in Odyssey Block or TBST (see below). Unbound primary antibody was removed by washing membranes three times for 10 minutes in TBST. Membranes were then incubated for 1hr at room temperature with infra-red labelled secondary antibodies diluted 1:10,000 in TBST 0.005% SDS, and unbound secondary was removed by washing three times for ten minutes in TBST followed by two washes for two minutes in TBS.

2.9 Zymography

Zymography was used to quantify the level of matrix metalloprotease enzyme activity secreted by cells into culture medium. Conditioned serum-free medium was collected from cells, debris was pelleted at 5,000rpm for 10min at 4°C and SDS sample buffer was added (see above). Enzyme-containing samples were electrophoresed on pre-cast NuPage polyacrylamide zymography gels impregnated with either 10% gelatin (Invitrogen-EC6175BOX) or 12% caesin (Invitrogen EC6405BOX) using NuPage Tris-Glyceine buffer (Invitrogen-

LC2675) for 2hr at 125V. After electrophoresis, enzymes in the gels were renatured (Invitrogen-LC2670) and developed overnight at room temperature in the presence of metal ion co-factors (Invitrogen-LC2671), allowing enzymatic degradation of the substrate at positions on the gel determined by the electrophoretic mobility of enzymes present in the sample. Gels were then stained for 3hr using Coomassie Blue Safe Stain (Invitrogen-LC6065) then washed overnight in H_20, staining the substrate protein impregnated in the gel uniformly and producing reverse stained bands at positions on the gel to which an MMP had migrated, proportional in intensity to its level of enzymatic activity. Gels were scanned at 700nm on a Li-Cor Odyssey scanner.

2.10 Band quantitation

Western-blot membranes and zymogram gels were imaged using a Li-Cor Odyssey scanner and software. Scanning intensity was set at a level to produce a visible band compared to background fluorescence whilst avoiding over-exposing pixels of images.

Band intensities were quantified using Fuji ImageGauge software. Pixel values were summed along a line perpendicular to the width of the band and background pixel intensities above and below the band were subtracted. To be able to compare between experiments, arbitrary relative values for each band were normalised against a chosen band in each experiment, the value of which was set at unity.

2.11 Co-immunoprecipitation

Protein-G Dynabeads (Invitrogen-10003D) were loaded with mouse anti-Flag antibody as instructed by manufacturer at a ratio of 5ug antibody per 75ul beads. 60mm dishes of HEK 293T cells were transfected for 7hr and split 1:2 the following day. 48hr post transfection two 60mm dishes of HEK 293T cells were lysed in 200ul of ice-cold IP lysis buffer (see below) and incubated on ice for 30 minutes. Lysates were spun at 5,000g for 10 minutes at 4°C to pellet debris. 200ul volumes of cleared lysates were incubated with rotation with 75ul

of antibody-loaded beads for 45 minutes at 4°C after which beads were washed 3 times with 200ul of ice-cold PBS. Captured protein was eluted from beads by heating 75ul beads in 15ul H_2O, 15ul SDS-Sample buffer (see below) and 3ul Reducing Agent (Invitrogen NP0009) to 90°C for ten minutes after which samples were analysed by Western-blotting.

2.12 Skin patch preparation

Before preparation of skin patches for culture, animals were anaesthetised in 0.1% tricane (Sigma A5040), bathed in 0.01% Virkon for 30 seconds, then washed three times in sterile water. Limb skin was dissected from amputated limbs under a Leica MZ8 stereo microscope by removing the hand, making an incision along the length of the limb then peeling the skin away from the muscle. Patches were then plated onto type1 collagen coated plastic dishes with 200ul Skin culture medium (70% MEM + Earles (21090 GIBCO), 30% H_20, 10% serum, 2mM glutamine (25030 GIBCO),10ug/ml insulin (Sigma I-5500), antibiotic-antimycotic solution (Sigma A5955), 50ug/ml gentamycin. A further 200ul was added the following day after attachment to the substrate.

Upper epidermal layers were removed by incubating patches in 0.025% trypsin in 70% L15 medium (Gibco 21083), 30% H_20, 2mM glutamine (25030 GIBCO) 10ug/ml insulin (Sigma I-5500), antibiotic-antimycotic solution (Sigma A5955), 50ug/ml gentamycin for 1hr, followed by mechanical separation of the two layers under a Leica MZ8 stereo microscope.

2.13 In-situ hybridisation

In situ hybridisation was used to analyse the expression pattern of MMP9 in cells migrating from skin patches. All reagents were RNAse free and solutions prepared with diethylpyrocarbonate (DEPC) treated water.

2.13.1 Cloning region of gene for use as probe

Probes were prepared by first cloning a region of the gene of interest from newt cDNA by performing PCR with the gene-specific primers ACTACGATACAGACAGGAAG and CGTCACAGCTGGGTATAGCA. A second PCR was then performed on the initial PCR product using primers TGAATTCGGGCACGCCATTGGCCT and AGAGGAGCGTTGATTGTCCCA. pBluescript SK vector (Stratagene) was cut with Sma1, the fragment was blunt ligated into it and transformed into E.coli as described previously. Insert containing colonies were identified by blue-white selection on X-gal coated agar plates and PCR was performed to identify inserts of correct size. Colonies were then sequenced and an appropriate clone identified.

2.13.2 Probe synthesis

The vector containing the probe fragment was cut either side of the insert in separate reactions to create a template for anti-sense and sense strand synthesis, and DNA was purified by organic phase separation as described previously. Precipitated DNA pellet was resuspended in DEPC water for use as a template for probe synthesis. Dioxigenin (DIG) labelled RNA probe was synthesised for 3hr at 37°C using T3 (Stratagene 600111-51) or T7 polymerase (Stratagene 600123-51) and DIG labelled deoxynucleotide triphosphates (dNTP's) (Roche 1277073), and then DNA was degraded using RNAse free DNAse (Stratagene 600031-51). DIG labelled RNA probe was then precipitated in cold ethanol, 50mM EDTA, 80uM LiCl, and 0.1mg/ml yeast transfer RNA (tRNA). The pellet was then washed in 75% ethanol, resuspended in TE and precipitated again in 80uM LiCl and ethanol at -30°C. The pellet was once again washed in ethanol, dried and resuspended in TE with RNAse Inhibitor (Invitrogen 10777-019). The RNA concentration was determined and an appropriate volume was added to hybridisation solution (see below) to give a probe concentration of 1ug/ml.

2.13.3 Sample preparation

Skin patches prepared as described previously were cultured for a defined period, rinsed with PBS and then fixed for 15 minutes with fresh 4% paraformaldehyde (PFA). Samples were then rinsed twice with PBT, digested for 10 minutes at 37°C with 10ug/ml proteinase K (Roche 03115887001) and fixed again with 4% PFA for 5 minutes. A PBS rinse was followed by two 10 minutes washes with 2mg/ml glycine solution, a further PBT rinse and a PBS rinse.

2.13.4 Hybridisation

Samples were then rinsed with hybridisation solution pre-heated to 45°C, and after a further 1hr incubation in hybridisation solution (see below) at 45°C incubated overnight at 45°C with DIG labelled probe denatured at 80°C for 10 minutes then cooled to 45°C.

2.13.5 Washing

Unbound probe was washed off at 45°C for 20 minutes with wash buffer 1 (see below), 20 minutes with wash buffer 2 (see below) and a further 10 minutes with wash buffers 1 and 2 mixed in equal quantities. All buffers were pre-heated to 45°C.

2.13.6 Antibody

Samples were blocked for 40 minutes in blocking solution (Roche 11096176001) then incubated at 4°C overnight with 1:500 sheep anti-DIG antibody (Roche 11093274910) in blocking solution.

2.13.7 Colour Development

The sample was washed 5 times for 10 minutes in 100mM Tris 150mM NaCl pH 7.5 to remove unbound antibody, rinsed in alkaline phosphatase (AP) substrate buffer (see below) and incubated with AP developing reagents BCIP and NBT (Promega S771) in AP buffer with 10% polyvinyl alcohol (Mw 31,000-

50,000) until colour was visible. The reaction was stopped by washing in 1% Triton PBS for 1hr, rinsing with PBS three times and then post-fixing with 4% PFA. Samples were then mounted in VectaMount AQ (Vector laboratories H-5501).

2.14 Immunofluorescence

Tyramide signal amplification (TSA) used to visualise Prod1 expression on cell membranes was performed using the TSA-Plus Cyanine 3 System Kit (Perkin Elmer NEL744) according to manufacturers instructions by J. Brockes. Mouse α-Flag (Sigma F 3165) was used for the first layer antibody and biotin-conjugated rabbit α-mouse (Zymed 61-0140) for the second layer.

Migrating cells were analysed for markers of epidermal and dermal character by A. Kumar. In all cases, 10% goat serum APBS was used to block samples prior to and when reacting with primary antibody.

For keratin identification, explants were fixed with ice cold methanol/acetic acid (95:5) for 5 minutes, washed with PBT and reacted overnight at 4°C with 1:100 LP34 mouse α-pan-keratin IgG (Abcam ab17153). The next day samples were washed with PBS and reacted for 1hr with 1:500 Cy3-conjugated goat α-mouse (Jackson Laboratories 115-163-03).

For vimentin identification, explants were fixed with PFA, reacted overnight at 4°C with 1:100 13.2 mouse IgM α-vimentin (Sigma V5255), and reacted for 1hr with 1:500 texas red-conjugated class specific goat α-mouse (Southern Biotechnology) the following day.

B1H1 cells were reacted in parallel as a negative control, and for both antibodies, no staining was observed (not shown).

Longitudinal limb sections were analysed for Prod1 expression by A.Kumar.

Whole limbs were fixed with 0.5% PFA in APBS containing 0.1% glutaraldehyde overnight at 4 °C. Limbs were washed in APBS, embedded in TissueTEK OCT compound (Sakura FineTEK 4583) and 12uM longitudinal sections were cut in a Leica CM1850 cryostat. Air dried sections were rehydrated in PBS for 3 minutes then washed for 6 minutes in freshly prepared PBS 0.1% $NaBH_4$ (Sigma 21346-2) and then rinsed in PBS. Sections were reacted overnight with

1:200 683 affinity purified rabbit α-Prod1 antibody (custom peptide antibody produced by Eurogentec) (da Silva et al., 2002) then reacted with 1:1000 goat α-rabbit Alexa 488 (Invitrogen) for 1hr the following day.

2.15 Microscopy

2.15.1 Fluorescence microscopy

Live cell imaging was carried out using a Carl Zeiss Axiovert 200M microscope and Cascade 2 camera (Photometrics). Stage movement, objective selection, filter selection and shutters were controlled automatically via Axiovision software, which was also used for image manipulation and analysis.
Fixed samples were imaged using a Carl Zeiss Axioskop 2 microscope and a Hamamatsu C4742-95 camera**.** Image manipulation and analysis was carried out using Improvision Openlab software.

2.15.2 Bright field microscopy

Fixed samples were imaged using a Carl Zeiss Axiophot 2 microscope and an Axiocam HRc camera**.** Image manipulation and analysis was carried out using Axiovision software.

2.16 Reagents

2.16.1 Buffers

TE

10mM Tris, 1mM EDTA pH 8.0

Cell Lysis Buffer (Western-blot)

1% NP40,150mM NaCl, 50mM Tris HCl pH 7.5, 1mM sodium orthovanadate, 2mM AEBSF, 1.6uM aprotinin, 40uM leupeptin, 80uM bestatin , 30uM pepstatin A, 28uM E-64

RIPA buffer (Western-Blot)

100mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP40, 1% NaDoc, 0.1% SDS

Sample Buffer (Western blot/ Zymography)

100mM Tris pH 8.0, 4% SDS, 0.02% Bromophenol Blue, 20% Glycerol

Western Transfer Buffer

40mM glycine, 50mM Tris base**,** 0.07% SDS, 20% methanol

TBS

25mM Tris pH 8.0, 0.1M NaCl

TBST

2mM Tris HCl pH 8.0, 0.1M NaCl, 0.1% Tween 20

IP Lysis Buffer

1% Triton X-100, 150mM NaCl, 50mM Tris, 2mM MgCl2, 2mM EGTA, 1mM NaVO4, 10% glycerol, 2mM AEBSF, 1.6uM aprotinin, 40uM leupeptin, 80uM bestatin, 30uM pepstatin A, 28uM E-64

PBS

137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.4mM KH_2PO_4 pH 7.4

PBT

137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.4mM KH_2PO_4 pH 7.4

0.3% Triton X-100

Wash Buffer 1

50% deionised formamide, 5X SSC, 1% SDS

Wash Buffer 2

50% deionised formamide, 2X SSC, 1% SDS

Hybridisation Buffer

50% deionised formamide, 5X SSC, 1mg/ml tRNA, 0.1mg/ml heparin, 1x Denhardts solution, 0.2% Tween 20, 5mM EDTA

Alkaline Phsophatase (AP) Buffer

100mM Tris pH 9.5, 100mM NaCl, 50mM $MgCl_2$, 0.1% Tween 20

Post Hybridisation Wash (Tris-NaCl) Buffer

100mM Tris pH 7.5, 150mM NaCl

2.16.2 Western-blotting antibodies

- Mouse a-Flag, M2 monoclonal (Sigma F 3165)
- Rabbit a-Flag, affinity purified (Sigma F7425)
- Rabbit a-Myc, affinity purified (Cell-Signalling 2272)
- Mouse a-Myc, 9E10 monoclonal (Sigma M4493)
- Mouse 4G10 a-pY (gift from Ivan Gout)
- Rabbit a-pERK, affinity purified IgG (Upstate 07-467)
- Mouse a-pERK (Sigma M 9692)
- Rabbit a-ERK (Gift from J. Ladbury)
- Rabbit a-pJNK, affinity purified (Cell-Signalling 9251)
- Rabbit a-Y845, affinity purified (Cell-Signalling 2231)

2.16.3 Inhibitors

- AG1478 (Invitrogen PHZ1034)
- AG879 (Calbiochem 658460)
- PD153035 (Calbiochem 234490)
- U0126 (Calbiochem 662005)
- JNK Inhibitor II (Calbiochem 420119)
- P38 Inhibitor (Calbiochem 506126)
- PP2 (Calbiochem 529573)
- AG490 (Calbiochem 658401)
- GM6001 (Biomol El-300)
- MMP9 Inhibitor I (Calbiochem 444278)
- Recombinant mouse DKK-1 (R&D Systems 1765-DK)

2.16.4 Primers

2.16.4.1 RT PCR primers

EGFR
GATTCAGGATCAAGAATCCCA
TCCTTAATGGCTACAGGGAT

Prod1
TTCCCTAGAATTTGGGAACG
GGCAGCTAGTTCACGTGTTG
TGACTGGTGTCCTCACACAACCACC Fam labelled probe

GAPDH
TGTGGCGTGACGGCAGAGGTG
TCCAAGCGGCAGGTCAGGTCAAC

Actin
CCACTGCTGCTTCTTCATCCTCTC
GGGCACCTGAACCGCTCATTG

L27
TACAACCACTTGATGCCA
CAGTCTTGTATCGTTCCTCA

EF1α
TAGAGTGCAGGTGACGATCC
AGTCACCAAGTCTGCCATCA

MMP9 newt
CATCGTAGGATTCACCATCG
ACCACGACCGACTATGACAA

MMP9 axolotl
GCATCGTAGGATTCTCCATCA
ACCAGTGAAGGCCGTTCCGAT

2.16.4.2 Site-directed mutagenesis primers

(mismatches are marked in bold)

D28A
CACCAGAAACGGAG**C**CGACAGGACTGTG

CACAGTCCTGTCG**G**CTCCGTTTCTGGTG

T33A
GGAGACGACAGGACTGTG**G**CCACCTGCGCCG

CGGCGCAGGTGG**C**CACAGTCCTGTCGTCTCC

E37A
GTGACCACCTGCGCCGC**G**GAACAGACTCG

CGAGTCTGTTC**C**GCGGCGCAGGTGGTCAC

Y49A
GCCTCTTCGTACAACTGCCA**GC**TTCTGAGATACAAGAATG

CATTCTTGTATCTCAGAA**GC**TGGCAGTTGTACGAAGAGGC

E54A
CTGAGATACAAG**C**ATGCAAGACGGTGCAACAGTG

CACTGTTGCACCGTCTTGCAT**G**CTTGTATCTCAG

Q59A
GAATGCAAGACGGTG**GC**ACAGTGTGCTGAGGTGT

ACACCTCAGCACACTGT**GC**CACCGTCTTGCATTC

E66A
GTGTGCTGAGGTGTTAG**C**GGAAGTCACTGCC

GGCAGTGACTTCC**G**CTAACACCTCAGCACA

Y73A
GTCACTGCCATTGGA**GC**TCCAGCAAAGTGCTGCTGCG

CGCAGCAGCACTTTGCTGGA**GC**TCCAATGGCAGTGAC

K76A
GGATATCCAGCA**GC**GTGCTGCTGCGAGGAT

GATCCTCGCAGCAGCAC**GC**TGCTGGATATCC

C79S
CCAGCAAAGTGCTGC**A**GCGAGGATCTCTGCAAC

GTTGCAGAGATCCTCGC**T**GCAGCACTTTGCTGG

R85A
GCTGCGAGGATCTCTGCAAC**GC**GAGTGAGCAAG
CTTGCTCACTC**GC**GTTGCAGAGATCCTCGCAGC

2.16.5 Expression constructs

2.16.5.1 A discussion of vector choice for amphibian cell expression constructs

All Prod1 constructs are derived from the original Prod1 construct 70/71, which was cloned from cDNA (da Silva et al., 2002) complete with 5' and 3' untranslated region (UTR) into PCI-Neo. The entire 70/71 insert was subcloned into a vector driven by the cytoskeletal actin (CSKA) promoter. All other Prod1 constructs lack the 5' and 3' UTR sequences of 70/71 and express only vector UTR.

Both the 70/71 and the CSKA constructs showed only very weak MMP9 induction, possibly due to the newt 5' UTR, whereas the N2 and PCI-Neo constructs contained only vector UTR. It is unclear why this was the case but a number of possible explanations exist. The N2 5' UTR sequence may be more favourable than that in PCI-Neo or from the Prod1 gene for promoting message stability or protein translation in B1H1's. The 5' UTR in the Prod1 gene may suppress its expression either constitutively or in a regulated fashion. Also, PCI-Neo contains intronic sequence in the 5' UTR designed to enhance the correct processing of mRNA's in mammalian cells and this may perturb expression in newt cells, as both N2 and CSKA vectors, which do not contain an intron, were seen to drive expression of other genes to a significantly higher degree, as seen by Western-blotting (J.Godwin, not shown).

2.16.5.2 A discussion of the origin of constructs used

Expression cassettes are shown below with 5' and 3' restriction sites used for cloning. Prod1-Flag, Prod1-Flag ΔC-terminal anchor, Prod1-Flag ΔN-terminal signal and Axolotl CD59-Flag were originally cloned into the PCI-Neo vector (Promega E1841, GenBank Acc # U47120) with N-terminal Flag tags; however poor expression of PCI-Neo constructs was seen in B1H1 cells and expression

cassettes were subsequently subcloned into the peGFP-N2 vector (Clontech 6081-1, GenBank Acc #U57608) replacing the GFP sequence. As a non-specific GPI anchored protein negative control, GPI anchored GFP was employed (GPI GFP). The construct contains an enhanced green fluorescent protein (eGFP) with the C-terminal GPI anchor attachment sequence from lymphocyte function associated antigen 3 (LFA3) and the N-terminal signal peptide of lactase phlorizin hydrolase (Keller et al., 2001). Prod1-Flag LFA anchor was cloned by replacing the GFP sequence of GPI GFP with Prod1-Flag, producing a Prod1-Flag construct with both N- and C-terminal peptides from original the GPI GFP construct. Point mutations at Q59 and E66 were generated in Prod1-Flag LFA in order to express detectable levels of the proteins on the cell membrane.

Fragments of newt EGFR and newt β1 integrin were both cloned from newt genomic DNA by P.Gates and assembled in pTL-1 vector with a C-terminal Myc tag. pTL-1 is a pSG5 plasmid (Stratagene 216201) with an extended polylinker. For technical reasons, it was not possible to subclone these constructs into the N2 vector.

2.16.5.3 Constructs used

- Newt Prod1-Flag (WT)
- Newt Prod1-Flag ΔC-terminal anchor sequence (NA)
- Newt Prod1-Flag ΔN-terminal secretory sequence (NS)
- Newt Prod1-Flag LFA anchor (LFA)
- Axolotl Prod1-Flag (Axo)
- Axolotl CD59-Flag (CD59)
- Newt EGFR-Myc (EGFR-Myc)
- Newt β1 integrin-Myc
- GFP anchored with LFA3 GPI anchor (GPI GFP) (Keller et al., 2001)

Sequences are found in the Appendix at the end of the thesis

3 Prod1 regulates MMP9 expression and ERK1/2 activation

3.1 Introduction

Since Prod1 is GPI-anchored (da Silva et al., 2002), and thus unable to make direct contact with the cytoplasm of the cell in which it is expressed, it is hypothesised that a transmembrane protein binding partner may facilitate signal transduction. Prod1 exhibits structural similarities with uPAR and CD59 (da Silva et al., 2002) (Garza-Garcia et al., 2009) both of which have been shown to interact with the EGFR (Blagoev et al., 2003) (Mazzieri et al., 2006), prompting an investigation to determine whether Prod1 also interacts functionally with the EGFR. uPAR activates the ERK1/2 pathway via its ligand-independent activation of the EGFR (Liu et al., 2002), however little is known of the downstream effects of CD59 EGFR interaction. uPAR is also known to regulate the expression of MMP9 via the ERK1/2 MAPK pathway activation (Wei et al., 2007).

MMP9 is highly up-regulated in the wound epidermis in a temporally defined manner during early blastema formation (Satoh et al., 2007) and its expression is seen to be spread throughout the blastema as it grows, with MMP activity being essential for normal regeneration to take place (Vinarsky et al., 2005). The involvement of Prod1 in this process could provide a basis for the mechanisms contributing to patterning of the limb during regeneration. It was hypothesised therefore that Prod1 might regulate the expression of MMP9 through a mechanism involving the EGFR.

In order to establish a suitable experimental system within which to study the mechanism of action of Prod1, a number of alternatives were considered. The types of experiments under consideration required a simple system that lent itself to genetic manipulation and allowed appropriate controls to be built in. Due to the lack of genetic tools and the complexity of working with animals, experiments with salamanders were ruled out at an early stage. During the time of this thesis a protocol was developed by A. Kumar and J. Godwin in which primary blastemal cells could be derived and maintained temporarily in culture (Kumar et al., 2007b). The availability of these cells was limited due to the time

required to derive them from limb blastemas and the heavy requirements they placed on the animal stocks, as they were not expandable in culture. Two cell lines, B1H1 and A1, derived from newt limb and capable of extensive passage and expansion were available (Ferretti and Brockes, 1988). B1H1 cells were favoured, as a protocol for effective transfection had been developed previously by J. Godwin, providing a genetically manipulatable, and fast growing model system. B1H1 cells had been shown by P. Gates not to express Prod1 as assayed by RNAse protection. This indicated their potential as a suitable model system within which to investigate the hypothesised functional interaction between Prod1 and the EGFR, providing the opportunity to compare the characteristics of cells in the presence and absence of Prod1 expression.

3.2 Results

3.2.1 Characterising Prod1 and EGFR expression in B1H1 cells

In B1H1 cells, no Prod1 signal could be detected after 40 PCR cycles. This represents a level of expression at least $4.5*10^5$ fold lower than that observed in the skin (Table 3.1) and indicates that Prod1 is not expressed in B1H1's, thus confirming previous observations.

In view of the potential interest in the newt EGFR for the study of the mechanism of action of Prod1, this cDNA was cloned by P. Gates. Sequence alignment showed it to be the newt ortholog of ErbB1, the stereotypical EGFR (Fig. 3.1). Alignment with mouse and human ErbB1 is shown in Supplementary Figure 1. Expression of EGFR mRNA in B1H1 cells was confirmed by qRT-PCR (Fig. 3.2) and their ability to respond to EGF was demonstrated in order to confirm the expression of the EGFR protein on the cell surface. A well-established down-stream target of EGFR signalling is the ERK1/2 MAPK (Jorissen et al., 2003). ERK1/2 phosphorylation was analysed by Western-blotting the proteins extracted from B1H1 cells incubated in the presence and absence of recombinant human EGF with phospho-ERK1/2 specific antibodies. Serum-starvation conditions were optimised and it was found that a 24hr

	B1H1 cT	Skin cT	Relative difference Expression Skin vs. B1H1
Prod1	>40	24.2	>5.7*10^4
GAPDH	15.76	18.73	0.13
Normalised relative difference Prod1 expression Skin vs. B1H1			>4.5*10^5

Table 3.1 Prod1 expression in B1H1 Cells

cT values of a representative qRT-PCR analysis of cDNA prepared from B1H1 cells and newt limb skin. Comparison of cT values allows the determination of the relative difference between the number of target sequences in two samples, and can be normalised to the level of expression of a housekeeping gene. The relative difference in expression of Prod1 between skin and B1H1 cells is normalised to GAPDH in this example. For details of cDNA synthesis, qRT-PCR and quantitation of relative differences in gene expression from cT values, see Material and Methods.

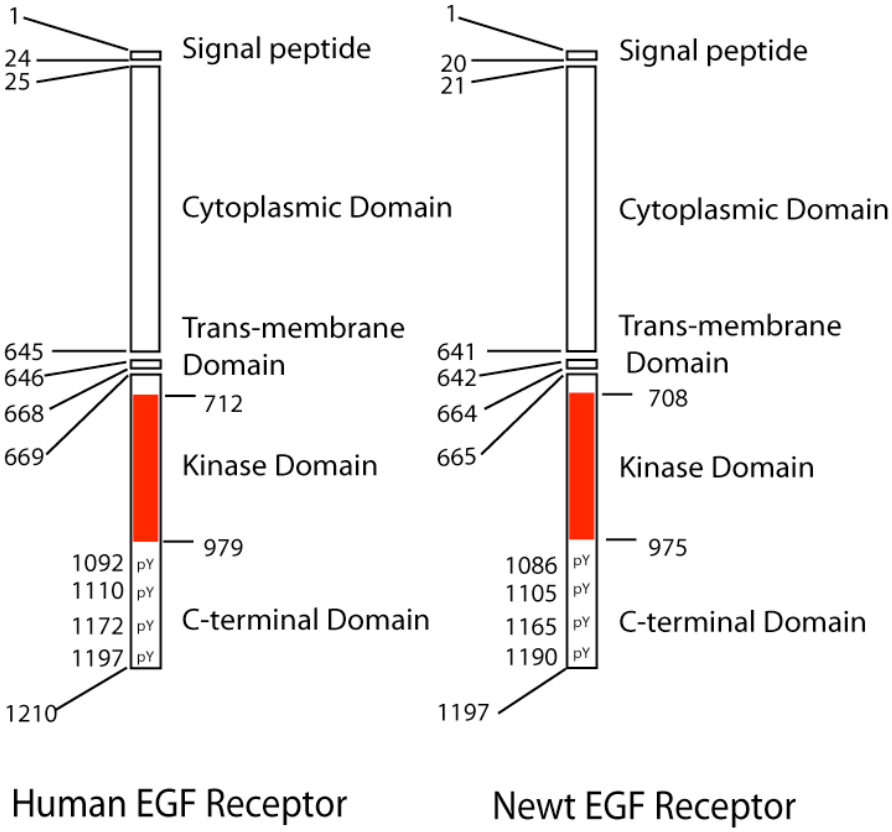


Figure 3.1 Comparison of human and newt epidermal growth factor receptors

Schematic representations of the human and newt EGFRs based upon alignment of amino-acid sequences. Selected phosphorylation sites and boundaries of domains are shown for comparison, indicating a high degree of similarity between the two proteins, with no major insertions or deletions. For detailed comparison of sequences see Supplementary Figure 1.

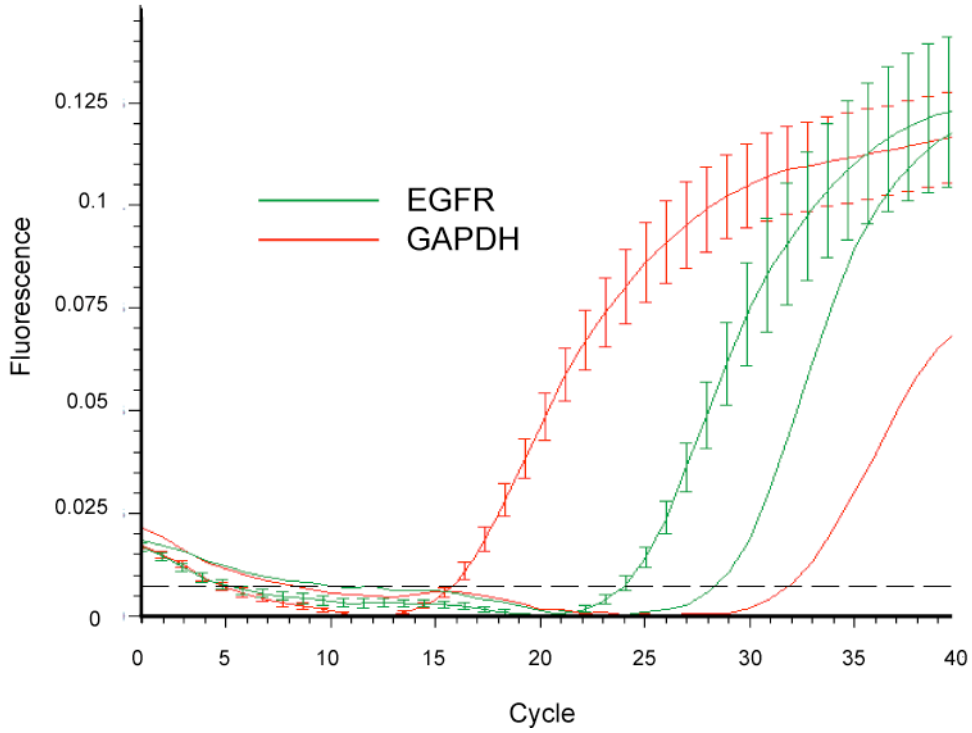


Figure 3.2 EGFR expression in B1H1 cells

qRT PCR traces of EGFR and GAPDH signal in cDNA and RNA prepared from B1H1 cells. Traces for cDNA are the average of three samples with bars showing the standard deviation. Traces for RNA are single samples, shown without bars. The fluorescence signal for cDNA traces can be seen above the background signal of the RNA control, demonstrating the expression of EGFR and GAPDH mRNA in B1H1 cells. Note that the GAPDH fluorescence signal increases exponentially significantly earlier than the EGFR signal, suggesting that GAPDH expression is higher in B1H1 cells, however direct comparisons between signals from different primer pairs cannot be made. For an explanation of qRT-PCR data output, see Materials and Methods.

starvation period gave the largest and most reproducible EGF response in EGF treated cells compared to untreated controls (Fig. 3.3a.). The ERK1/2 response to EGF was seen to be dose-dependent and the optimal concentration of EGF was determined (Fig. 3.3b). The ability to respond to EGF confirmed not only the presence of the EGFR on the surface of B1H1 cells but also demonstrated that the EGFR/ ERK1/2 pathway in newt cells is similar to the well-characterised mammalian pathway.

Commercial antibodies generally give little indication of whether they will be cross-reactive with salamander proteins, and as little genomic information is available for N.viridescens in order to make comparisons with the antigen to which antibodies were raised, antibodies require validation experimentally. The ability to detect ERK1/2 phosphorylation in response to EGF validated the phospho-ERK1/2 antibodies used. An attempt to detect the phosphorylation of the EGFR in response to EGF using an anti-phosphotyrosine was unsuccessful in B1H1 cells, (Fig. 3.3c.) compared to in high EGFR expressing A431 human tumour cells, where a good response was seen.

3.2.2 Investigating the effect of Prod1 expression on MMP9 secretion

Having established a suitable system in which the EGFR is expressed and Prod1 is not, investigation of the effects of Prod1 expression and its relationship to the EGFR could begin. As the Prod1 antibody used in the laboratory was in limited supply, and it was anticipated that large amounts of antibody would be used over the course of the project, all experiments were carried out using Flag-tagged Prod1 constructs in order to allow their immuno-detection using commercially available anti-Flag antibodies. GPI anchored GFP (GPI GFP) was used as a general non-specific GPI anchored protein control throughout the experiments in order to control both for effects of transfection and overexpression of a GPI anchored molecule.

The ability of Prod1 to regulate the expression of MMP9 was investigated by analysing culture medium from Prod1 and GPI GFP expressing B1H1 cells by gelatin zymography, a standard method of analysing MMP enzyme activity (see Materials and Methods for a detailed description of the technique). The linearity and range of detection of gelatin zymograms was first established by running a dilution series of conditioned medium collected from B1H1 cells (Fig. 3.4) demonstrating their linearity and ability to detect small differences in enzyme activity.

The requirement for serum in culture medium was investigated, as experiments would ideally be conducted under serum-free conditions in order to simplify interpretation of results. Analysis of conditioned medium by gelatin zymography

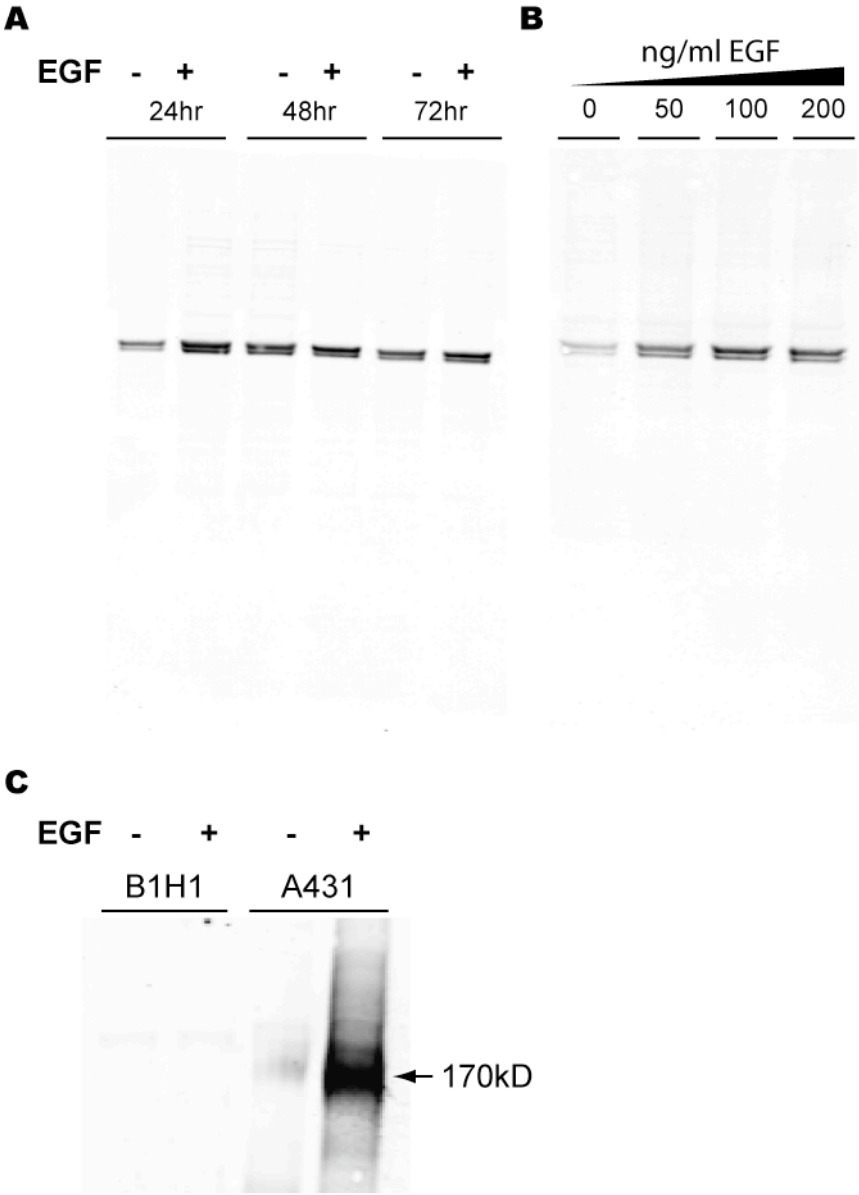


Figure 3.3 EGF response of B1H1 cells

(A) B1H1 cells were serum starved for the indicated duration and stimulated with 50ng/ml EGF or (B) serum starved for 24hr and stimulated with the indicated concentration of EGF for 20min. Cell lysates were Western-blotted using anti phospho-ERK1/2 antibody. (C) B1H1 cells were serum starved for 24hr and stimulated with 200ng/ml EGF for 5min. A431 cells were serum starved for 8hr and stimulated with 100ng/ml EGF for 5min. Cell lysates were Western-blotted with an anti-phosphotyrosine antibody. Note increased phosphorylation of the 170kD EGFR in EGF stimulated A431 cells. Note that despite activating ERK1/2 phosphorylation, no EGFR phosphorylation was detectable in response to EGF in B1H1 cells. ERK1/2 response to EGF was observed to diminish after extended starvation periods.

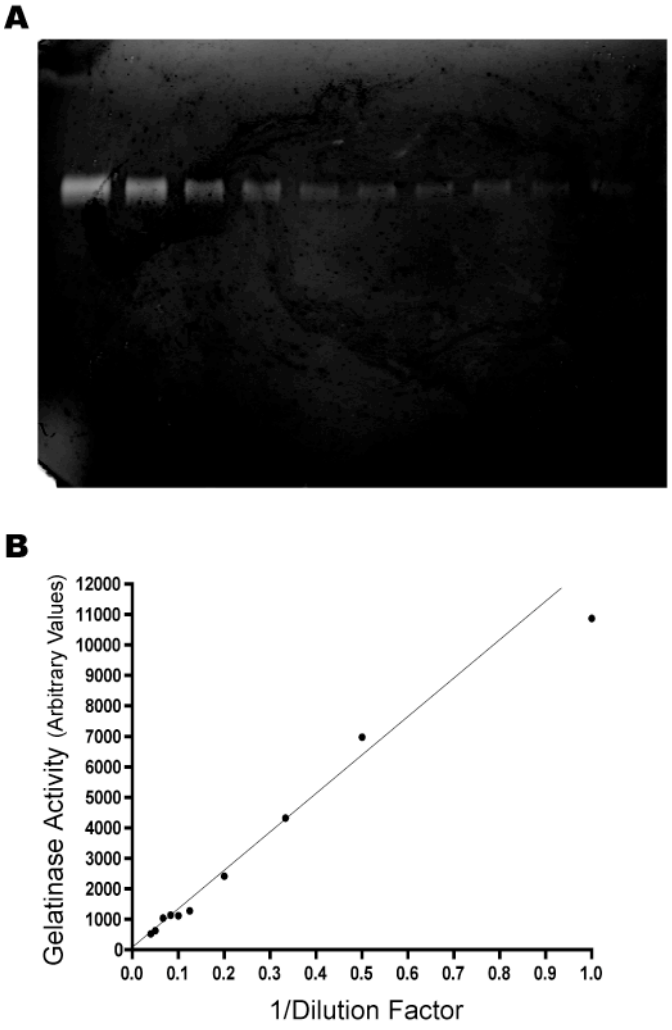


Figure 3.4 Establishing the linearity of gelatin zymography

(A) Cell-culture medium was collected from B1H1 cells and a range of dilutions was run on a gelatin zymogram. (B) Band intensity was quantitated as detailed in Materials and Methods, and plotted as a function of the dilution factor, revealing a good linear relationship between the actual level of gelatinase activity in a sample and the level of gelatinase activity as determined by zymography.

revealed that expression of Prod1 led to an increased gelatinase activity in cell culture medium (Fig. 3.5) both in presence and absence of serum. Inclusion of serum appeared to raise both the level of MMP9 secretion in GPI GFP and Prod1 expressing cells.

A number of considerations were taken into account in light of the known functions of MMP9 and the uPAR paradigm. uPAR regulates the expression of MMP9 in response to RGD motifs through interplay with the fibronectin receptor $\alpha5\beta1$ integrin (Wei et al., 2007), and as MMP9 is involved in re-modelling the ECM, preliminary investigations were mindful of the possibility that any mechanism involving MMP9 regulation by Prod1 may also involve extracellular matrix (ECM) proteins. Standard culture conditions for B1H1 cells involve growth on gelatin also raising the question of whether the experimental conditions should include such substrates. It was observed that Prod1 expressing cells cultured in the absence of substrate exhibited the same pattern of enhanced gelatinase secretion compared to the GPI GFP control as when cultured on gelatin (Fig. 3.5a) or fibronectin (Fig. 3.5b), though a mild enhancement in MMP9 secretion in both Prod1 and GPI GFP expressing cells was observed when cells were cultured on these ECM substrates. In light of the lack of requirement for either serum or ECM substrate, neither was included in future experiments in favour of simplifying experimental conditions and aiding ease of interpretation.

Culture medium was analysed on gelatin and casein gels so as to identify the gelatinase activity increasing in response to Prod1 expression. The approximately 80Kd molecular weight in combination with an inability to degrade casein indicated it to be newt MMP9 based upon previous characterisation (Vinarsky et al., 2005) (Fig. 3.6a). Gelatinase activity was also inhibited by the broad-spectrum MMP inhibitor GM6001 and an MMP9 specific inhibitor (Fig.3.6b), indicating it genuinely to be MMP9.

MMP9 was also seen to be secreted by cultured blastema cells along with a second unidentified MMP of roughly 55Kd able to degrade both gelatin and casein (Fig. 3.6c). This 55Kd enzyme was also observed in B1H1 cell conditioned medium (Fig. 3.5a, marked *), however its activity was not seen to increase in response to Prod1 expression, indicating the effect of Prod1 expression is acting through a pathway specific to MMP9 and not a result of a non-specific up-regulation of MMPs or secreted proteins in general.

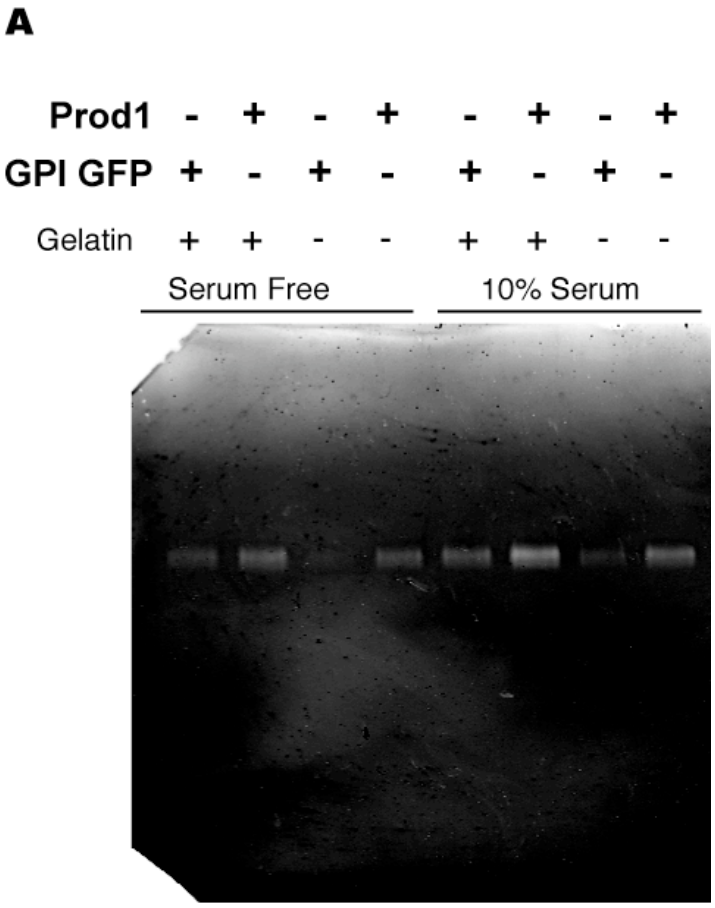


Figure 3.5 Gelatinase regulation by Prod1

The effect of culturing B1H1 cells expressing Prod1 or GPI GFP on gelatin (A) and fibronectin (B) compared to culture on plastic was assessed in the presence and absence of serum. Cells were serum starved for 24hr then culture medium was replaced for either serum free or serum-containing medium. Culture medium was collected after 48hr of conditioning and analysed by gelatin zymography as described in Materials and Methods. Note that gelatinase activity was upregulated by Prod1 expression in the absence of serum, gelatin or fibronectin, and that fibronectin and gelatin increase the level of gelatinase expression in control GPI GFP expressing cells and Prod1 expressing cells in both the presence and absence of serum.

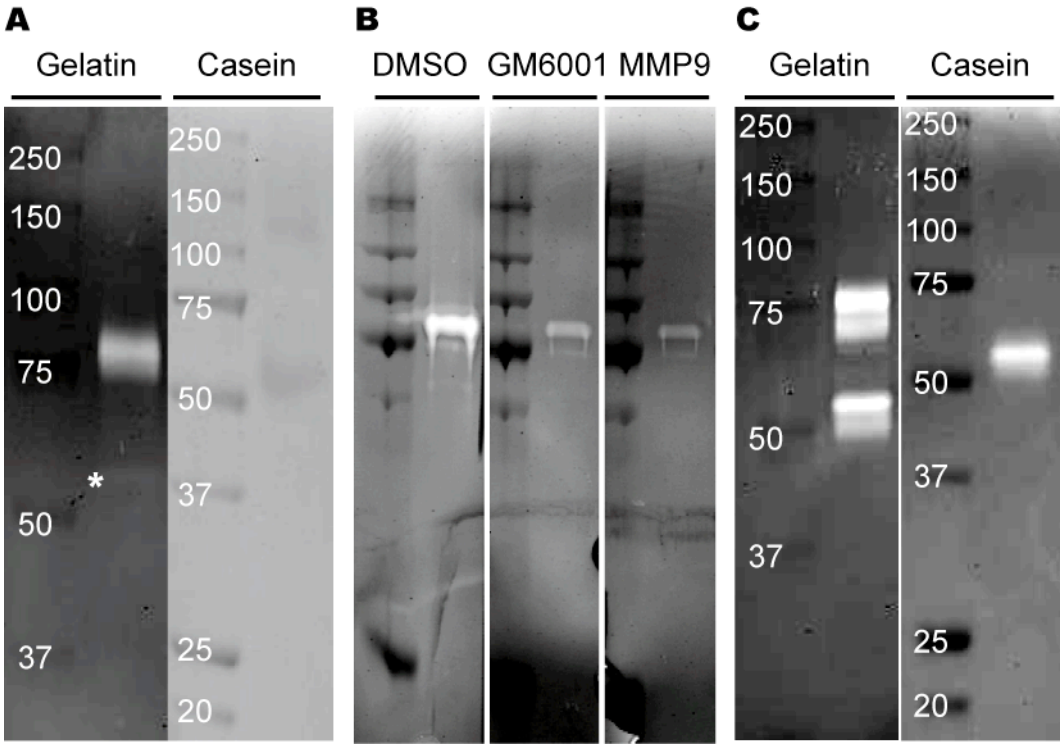


Figure 3.6 Characterisation of the gelatinase regulated by Prod1

(A) Culture medium from B1H1 cells expressing Prod1 was analysed by gelatin and casein zymography. A faint band is marked *, however the secretion of this enzyme is not regulated by Prod1 expression. (B) Equal volumes of culture medium from B1H1 cells expressing Prod1 were run on a gelatin zymogram and developed in the presence of either DMSO vehicle, MMP inhibitor GM6001 or an MMP9 specific inhibitor. The gelatinase activity is seen to be sensitive to both inhibitors. (C) Culture medium from cultured blastema cells was analysed by gelatin and casein zymography, revealing the expression of two distinct enzymes, one of which degrades both substrates.

3.2.3 The effect of Prod1 expression on MMP9 transcription

In order to determine if the increase in extracellular MMP9 activity brought about by Prod1 expression was a consequence of increased MMP9 gene expression, cDNA was prepared from cultured cells and analysed by qRT-PCR as detailed in Materials and Methods. Prod1 constructs cloned into a variety of vectors were tested in order to compare MMP9 gene induction relative to the GPI GFP control (Fig. 3.7a). Construct N2 gave a level of MMP9 gene induction at least 6 times greater than any of the other constructs, and was adopted for use in all further experiments. For further discussion of constructs see Materials and Methods. The effect of the Flag-tag expressed at the N-terminal of Prod1

constructs on MMP9 gene induction was investigated by comparison of Flag-tagged N2 Prod1 to a construct derived from it lacking the tag, showing it to have no detectable effect on the process and validating its use in future experiments (Fig. 3.7b).

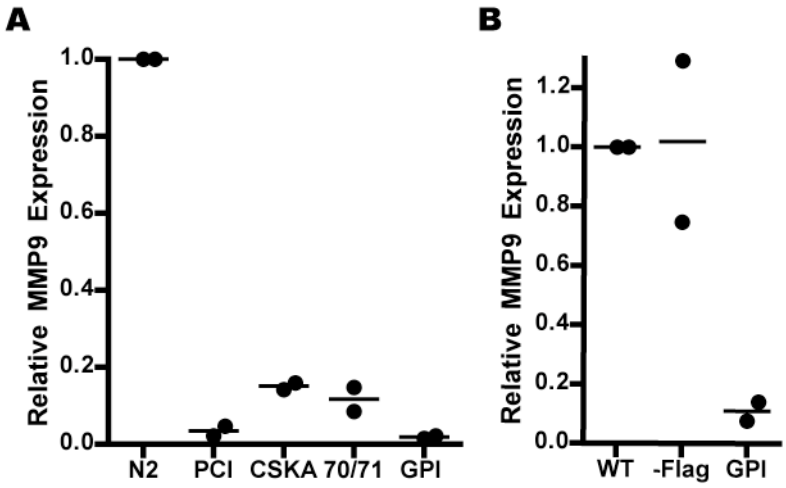


Figure 3.7 MMP9 gene regulation by Prod1

B1H1 cells were transfected with (A) Prod1 constructs N2, PCI, CSKA and 70/71, and GPI GFP, and (B) Prod1+Flag tag (WT), Prod1 -Flag tag (-Flag) and GPI GFP. cDNA was prepared from transfected cells after 72hrs serum starvation and expression of MMP9 was analysed by qRT-PCR. MMP9 expression in individual experiments is relative to N2 and WT in (A) and (B) respectively. Values for relative MMP9 expression are normalised to GAPDH in all experiments. Note that Prod1 was most able to induce MMP9 expression when itself expressed from the N2 vector, and that the Flag tag did not affect this activity.

Having determined that Prod1 expression in B1H1 cells results in an increase in both MMP9 secretion and transcription when compared to a non-specific GPI anchored protein, more rigorous controls were introduced. The hypothesis was that Prod1 would be bringing about this effect through its expression on the cell membrane, and to test this Prod1 constructs unable to localise to the cell surface membrane were employed. Prod1 is synthesised as a polypeptide with both N- and C- terminal peptides that are cleaved post-translationally (da Silva et al., 2002), the N-terminal peptide being a hydrophobic sequence which targets proteins into the secretory pathway (Rapoport, 1992) and the C-terminal

peptide being a recognition sequence for GPI-anchor attachment (Eisenhaber et al., 1998). The lack of a C-terminal sequence would be expected to produce a protein that entered the secretory pathway and was secreted because of the absence of a GPI anchor, whilst the lack of an N-terminal peptide would prevent a protein from entering the secretory pathway resulting in its accumulation within cells. Expression of these two constructs resulted in enzyme secretion (Fig. 3.8a,b) and gene expression (Fig. 3.8c) equal to that seen for the non-specific GPI anchored protein control, consistent with a requirement that Prod1 must indeed be expressed at the cell membrane in order to regulate MMP9 expression. Expression of Prod1-Flag and Prod1-Flag lacking the C-terminal anchor attachment sequence was shown by Western-blotting samples of cell lysate and conditioned medium (Fig. 3.8d), and cell-surface expression of Prod1-Flag was confirmed by immunofluorescence (Fig. 3.8e). A significant proportion of expressed Prod1-Flag was observed in conditioned medium, with a higher molecular weight than that seen in cell lysates. This may be an unanchored species retaining the C-terminal anchor attachment sequence. The higher apparent molecular weight of Prod1-Flag in cell lysates compared to the construct lacking a C-terminal anchor sequence is taken to be due to a retardation of mobility resulting from the attachment of a GPI anchor.

3.2.4 The effect of Prod1 expression on ERK1/2 activation

As demonstrated, ERK1/2 MAPK is a downstream effector of newt EGFR (nEGFR), and due to the lack of availability of an antibody to directly detect activated nEGFR, the effect of Prod1 expression on ERK1/2 activation was investigated. uPAR is known to regulate MMP9 expression via ERK1/2 (Wei et al., 2007) and it was shown that expression of Prod1 on the cell surface of B1H1 cells raised the level of ERK1/2 phosphorylation when compared to control constructs in serum starved cells. The largest and most reproducible ERK1/2 response to Prod1 expression compared to controls was seen after starvation for 24hrs (Fig. 3.9).

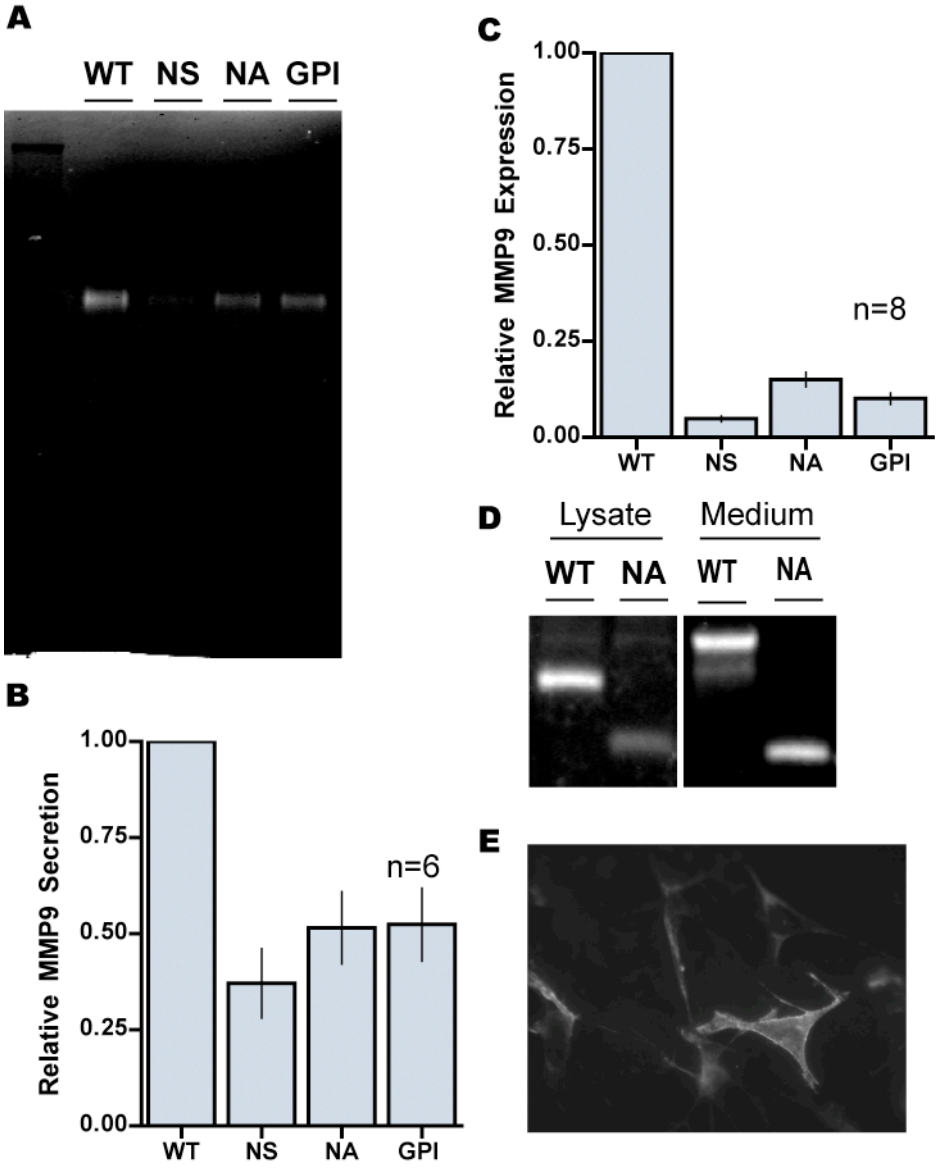


Figure 3.8 Requirement of membrane localisation of Prod1 for MMP9 induction

B1H1 cells were transfected with Prod1 (WT), Prod1 ΔN-terminal signal peptide (NS), Prod1 ΔC-terminal anchor attachment peptide (NA) and GPI anchored GFP (GPI). Cells were serum starved for 72hr and MMP9 activity in conditioned medium was assayed by zymography (A). Secreted MMP9 enzyme activity was quantitated relative to Prod1 in 6 independent experiments. Bars represent SEM (B). Cells were serum starved for 72hr and MMP9 gene expression was quantitated relative to Prod1 by qRT-PCR. MMP9 expression is normalised to GAPDH and bars represent SEM (C). Expression of Prod1 and Prod1 ΔC-terminal anchor attachment peptide in cell lysate relative to cell culture medium was analysed by Western-blotting with anti-Flag antibodies (D). Expression of Prod1-Flag on the membrane of B1H1 cells was confirmed by immunofluorescence (E).

Note that MMP9 secretion and gene induction occurred only when Prod1 was localised to the cell membrane by a GPI anchor.

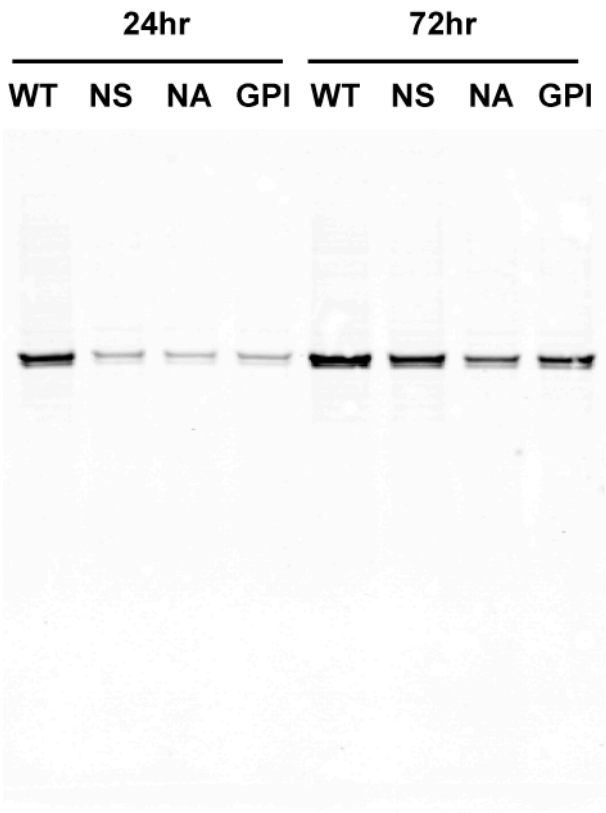


Figure 3.9 Prod1 activation of ERK1/2

B1H1 cells were transfected with Prod1 (WT) Prod1 ΔN-terminal signal peptide (NS), Prod1 ΔC-terminal anchor attachment peptide (NA) and GPI anchored GFP (GPI). Cells were serum starved for the indicated duration and cell lysates were western blotted with anti phospho-ERK1/2 antibodies. Note that ERK1/2 activation occurred only when Prod1 was localised to the cell membrane by a GPI anchor and that ERK1/2 background was seen to rise in controls after extended serum starvation.

3.3 Discussion

3.3.1 Implications of ERK1/2 activation

The observation that Prod1 expression on the cell membrane both induces MMP9 gene expression and activates ERK1/2 MAPK invites further investigation of whether a link exists between the two in light of the known requirement for ERK1/2 signalling in the regulation of MMP9 by uPAR (Wei et al., 2007). Activation of the ERK1/2 pathway is a pre-requisite of a number of cellular effects originating from uPAR signalling including cell migration and proliferation (Pulukuri et al., 2005) (Jo et al., 2003) (Aguirre-Ghiso et al., 2001)

(Jo et al., 2005) (Liu et al., 2002) (Tarui et al., 2003) (Wei et al., 2007) and given that ERK1/2 regulates the activity of a variety of transcription factors (Pearson et al., 2001) it is highly probable that Prod1 regulates a variety of other target genes and processes.

3.3.2 MMP9 induction in the absence of extracellular matrix proteins

The lack of a requirement for serum raises the possibility that the mechanism by which Prod1 regulates the expression of MMP9 may be EGFR ligand independent, and this is in agreement with the known mechanism of uPAR activation of EGFR signalling (Liu et al., 2002). It was observed when optimising conditions for detecting differences in ERK1/2 phosphorylation in response to both Prod1 and EGF however that shorter incubation periods in serum-free medium generally saw lower background ERK1/2 phosphorylation. This indicated that after an initial decrease, phosphorylation levels rose again, pointing to either a cellular response to prolonged serum starvation or the accumulation of growth factors in culture medium over time.

Fibronectin and gelatin are not required for Prod1 to induce MMP9 gene expression, and although this does not rule out the involvement of integrins in the mechanism, it is in contrast to some previous observations with uPAR (Wei et al., 2007) (Liu et al., 2002) (Madsen and Sidenius, 2008), which co-operates in processes requiring the binding of ECM proteins to integrins in order to regulate downstream signalling. uPAR, integrins and the EGFR have been shown to form a dynamic complex (Mazzieri et al., 2006), and the EGFR has been shown to transduce uPAR dependent signals originating from $\alpha 5\beta 1$ integrin binding to fibronectin (Liu et al., 2002). It cannot be ruled out that integrins may still be being activated by binding to motifs on the coating of culture dishes or ECM substrate laid down by the cells themselves, however. A mild enhancement in MMP9 secretion was observed both in control and Prod1 transfected cells plated on either gelatin or fibronectin. Cells plated on these substrates have a more ‘spread’ morphology compared to those plated on plastic (not shown), and as B1H1 cells do express MMP9 even in control transfected cells, this enhancement in MMP9 secretion may simply reflect a

more general effect of cells being healthier, or exhibiting a general increase in gene expression or protein secretion. As this effect was observed not to be specific to Prod1 expressing cells however, it was not investigated further. Similarly, the general increase in MMP9 secretion seen in the presence of serum may be due to a general increase in protein synthesis or secretion. This was investigated further in Chapter 4 when addressing the role of the EGFR and its ligand dependence in the process of MMP9 gene regulation.

3.3.3 MMP9 in the blastema

Why would Prod1 be involved in regulating MMP9 expression and how does this relate to proximodistal patterning in the regenerating limb? These questions are not easy to address other than at a superficial level. MMPs are known to be involved in tissue re-modelling (Page-McCaw, 2008) and cell-migration during metastasis (Liotta et al., 1980) and wound healing (Pilcher et al., 1997), both specific processes inevitably contributing to the broader process of 'patterning'. Whether MMP9 is more highly expressed in proximal blastemas than distal blastemas may shed some light on this question. Perhaps higher MMP9 expression in proximal blastemas produces a more plastic environment to facilitate the regeneration of a greater amount of tissue. Over-expression of Prod1 in cells of distal blastemas re-specifies them to more proximal identities in the regenerate (Echeverri and Tanaka, 2005) raising the possibility that cell migration within the early blastema may be playing a role in the mechanism by which Prod1 directs patterning during limb regeneration.

It was observed that cultured blastema cells express two MMP's, MMP9 and another unidentified enzyme. B1H1 cells also express a low level of this enzyme, however Prod1 expression and the other experimental manipulations employed such as serum addition and culture on fibronectin and gelatin showed no effect on its expression. Although a number of MMP's are expressed in the blastema (Vinarsky et al., 2005), the specific regulation of MMP9 by Prod1 may point to it having a role in regeneration associated with the mechanism through which Prod1 directs patterning rather than a more gross matrix degrading function.

The fact that MMP9 is first induced in the migrating cells of the forming wound-epidermis and that expression persists past the time-point observed in cells contributing to wound healing (Satoh et al., 2008) points to a role for it in the establishment of the different properties of wound epidermis compared to skin. Also, the dermis is known to posses properties instructive to limb-patterning during regeneration (Namenwirth, 1974).These cells migrate into the blastema as it progresses through the regenerative process (Gardiner et al., 1986), and MMP9 may play a role in the regulation of this.

3.3.4 Quantitative real time PCR for the analysis of the MMP9 regulatory pathway

Compared to qRT-PCR, zymography is a less quantitative and less robust assay. MMP9 upregulation in response to Prod1 expression was an order of magnitude higher when assayed at the level of gene expression compared to secreted enzyme activity. This may accurately reflect the situation, or result from the experimental protocol employed to assay gelatinase activity. However, zymograms were not overloaded with gelatinase when assaying activity in culture medium and showed good linearity, indicating that accurate detection and quantitation were not a limitation. It seems unlikely that the small difference in secreted enzyme activity between Prod1 and control expressing cells when compared to gene expression is due to MMP9 degradation or clearing from the medium as this would have to occur preferentially in Prod1 expressing cells, otherwise such an effect would affect absolute levels rather than the relative difference in levels. It was observed that Prod1 expression inhibited the secretion of alkaline phosphatase (not shown) raising the possibility that although Prod1 expression induces MMP9 gene expression, it somehow also inhibits its secretion by some general effect under the experimental conditions employed. Whatever the reason for the discrepancy between gene expression and enzyme secretion, assaying MMP9 by qRT-PCR was shown to be significantly more sensitive to the effect of Prod1 expression, making it more suitable for further analysis of the specific mechanism through which Prod1 acts. Once initial experiments with zymography had defined the presence of serum or ECM substrate as unnecessary in order to observe Prod1 stimulated

MMP9 secretion, as assaying in their absence was desirable in order to ease interpretation, these conditions were adopted for further, more accurate measurement with qRT-PCR, which confirmed the lack of a requirement for either.

It has not been ruled out at this point that Prod1 regulates the processes of MMP9 gene expression and secretion through independent mechanisms. The possibility exists that despite the upregulation of MMP9 mRNA expression, no increase in MMP9 secretion directly results from this, instead being contingent on a second mechanism controlling its release. Evidence presented in Chapter 4 makes this unlikely however.

3.3.5 Issues of detection

Overexpression of Prod1 in B1H1 cells by transfection results in a level of expression in cell lysates at the limit of detection by the Western-blotting protocol used. Care was taken to guard against proteolysis in cell lysates, however this has not been definitively ruled-out as the cause of the low signal. The transfection efficiency consistently averaged ~40% as indicated by nuclear GFP (nGFP) co-transfection across experiments (not shown), a level not expected to give such low levels of protein expression in cell lysates. It was also observed that other salamander proteins, for example a transglutaminase-like protein and tissue factor, were more readily detectable by Western-blotting when expressed in B1H1 cells by lipofection (not shown). In order to demonstrate by Western-blotting the expression of the Prod1 constructs used throughout the experiments described, electroporation with Anaxa nucleofector was used to overexpress transgenes, as the technique achieves a higher transfection efficiency and more reproducible detection, however this protocol was only established after functional data had been collected. Nucleofection delivers vector DNA straight to the nucleus unlike Lipofection, which simply allows it to cross the plasma membrane. The enhanced reproducibility seen with nucleofection may therefore reflect a difference in the rate or level of delivery of vector DNA to the nucleus.

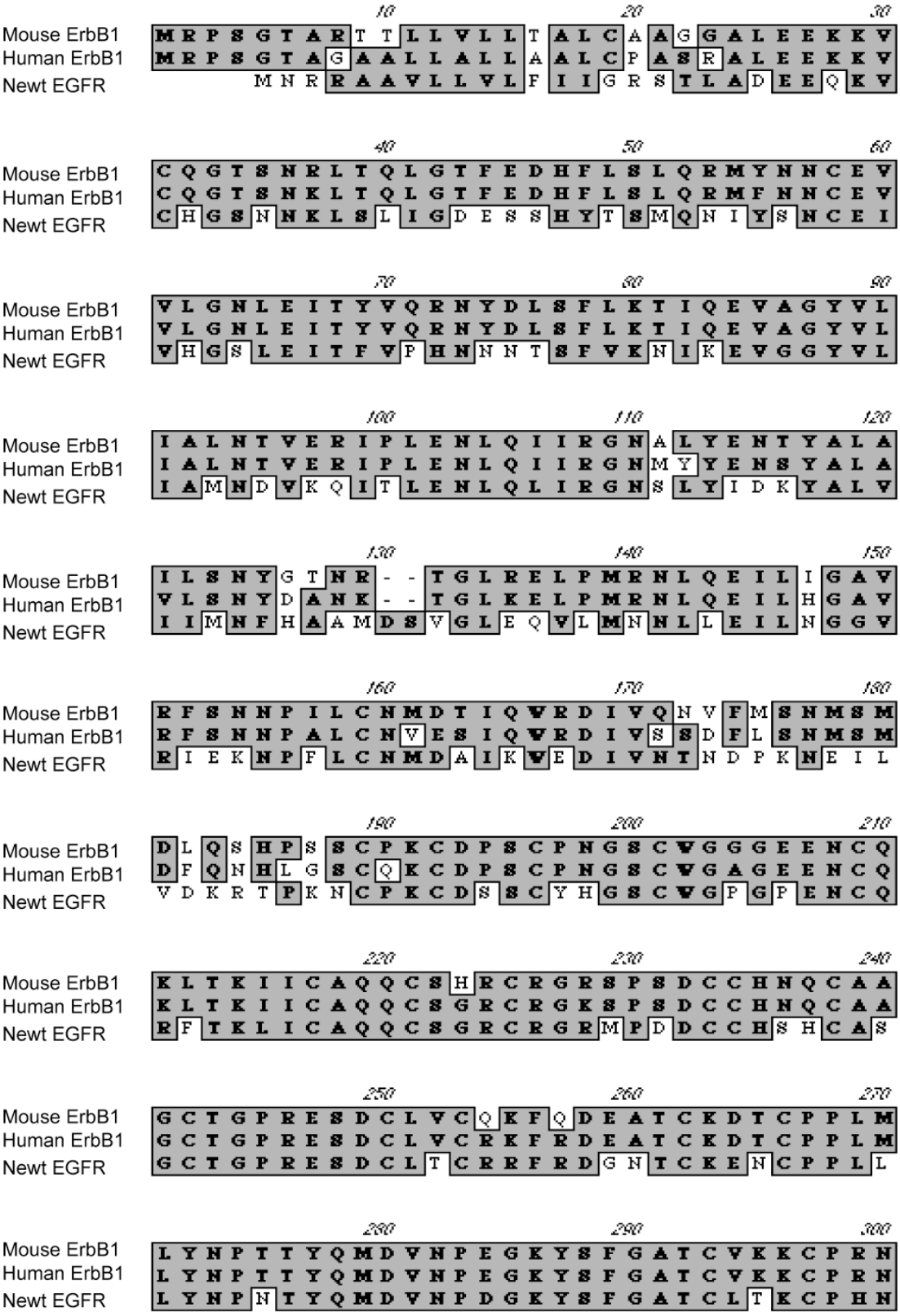
Prod1 was detectable at the cell surface by immunofluorescence in a far higher proportion of transfected cells when engineered to contain the more active C-terminal GPI anchor sequence from LFA-3 (not shown). The presumption is that the native Prod1 anchor sequence is only inefficiently recognised by the GPI anchor attachment machinery hence the GPI anchored protein is expressed at a relatively low level on the cell surface. It was observed that a significant proportion of Prod1 with the native anchor attachment sequence was found in cell culture with a higher molecular weight than that found in cell lysates, possibly representing unanchored protein with the C-terminal peptide un-cleaved. Conversely, Prod1 with the LFA3 anchor attachment sequence was not seen in culture medium (not shown). Perhaps the inefficient GPI anchor attachment sequence represents a form of regulation involved in ensuring appropriate levels of Prod1 are expressed at the cell membrane.

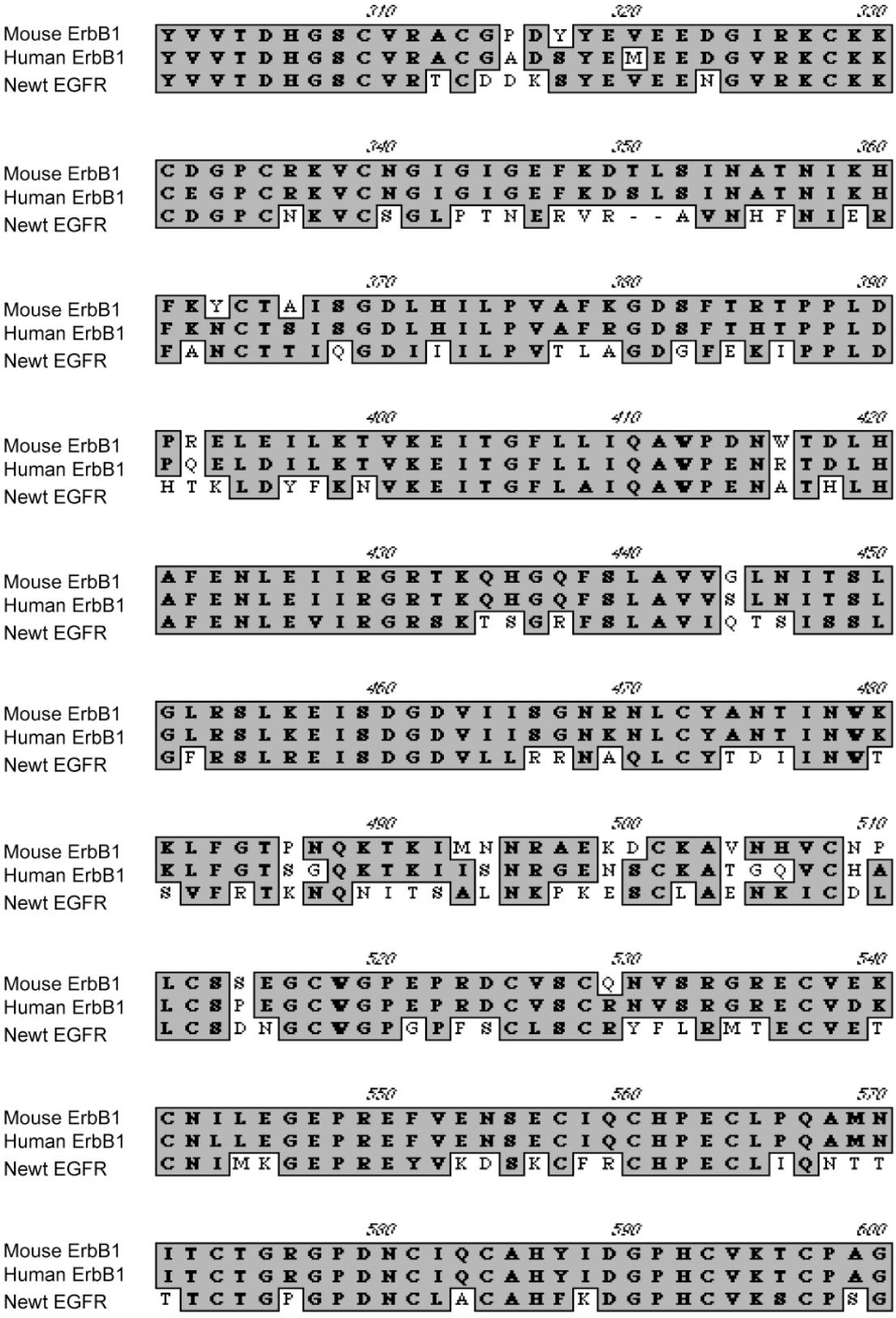
Despite the inability to readily detect the wild-type Prod1 protein by Western-blotting or immunofluorescence under the experimental conditions used in the assays described, expression of wild-type Prod1 in B1H1 cells consistently led to ERK1/2 pathway activation and MMP9 gene induction when compared to constructs of Prod1 rendered unable to be expressed on the cell membrane, inviting speculation that the molecule is highly biologically active and requires relatively low levels of cell-surface expression to bring about ERK1/2 pathway activation and MMP9 gene induction.

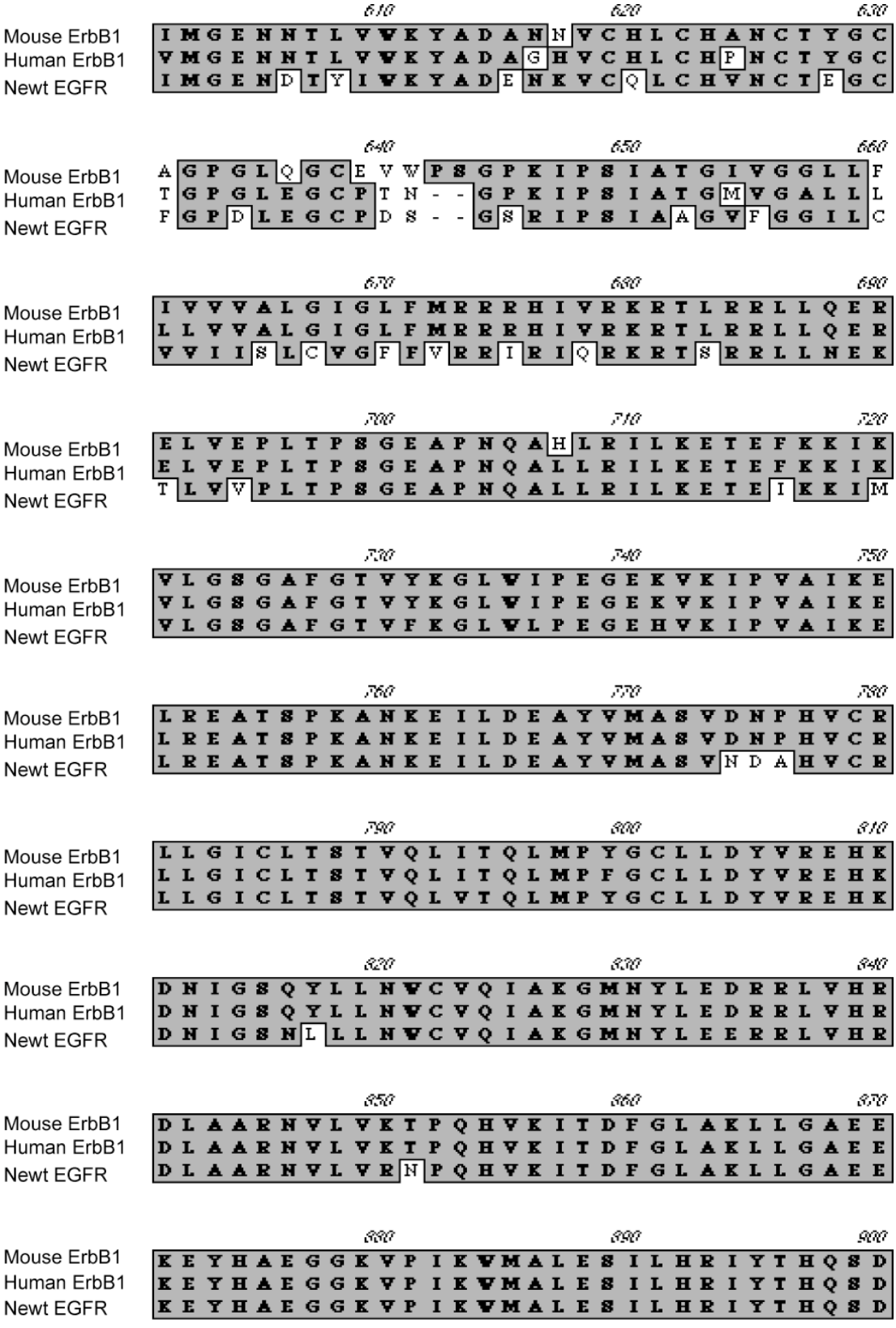
As shown by their ERK1/2 response to EGF, B1H1 cells do express the EGFR. The ERK1/2 response is not particularly great, however B1H1 cells do exhibit a level of ERK1/2 background under the starvation conditions used which may make it difficult to detect an EGF response over background. This will be discussed further in Chapter 4. The possibility exists that nEGFR has a low affinity to human recombinant EGF (hrEGF), however even at high concentrations of hrEGF the ERK1/2 response is still not large. The fact that a phosphotyrosine antibody could not detect phosphorylation of the EGFR in response to EGF is perhaps the most telling point, as the EGFR was clearly being activated, revealed by ERK1/2 phosphorylation in response to EGF. The phosphotyrosine epitope recognised by the antibody cannot exhibit species

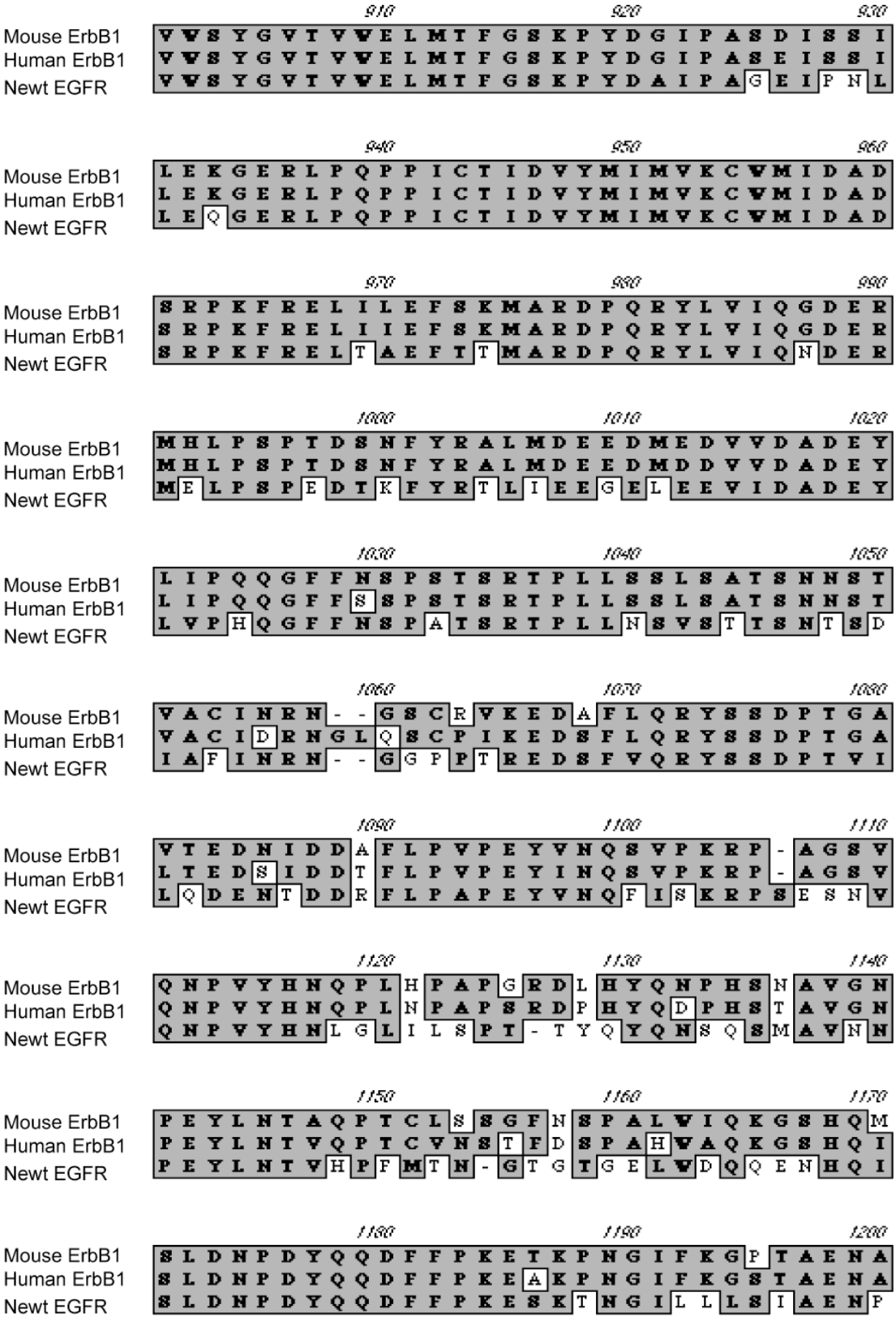
specificity as phosphorylated tyrosine residues are identical in all proteins and therefore the inability to detect it is likely to reflect a particularly low level of EGFR compared to other species. qRT-PCR of B1H1 cells also indicates a low level of EGFR transcript due to signal emerging at a relatively high cycle number compared to other genes, however as no absolute quantitation of transcript number was carried out it cannot be ruled out that this is due to a function of the annealing properties of the primers.

3.3.5.1 Supplementary Figure 1: Alignment of newt EGFR with human and mouse ErbB1




310 320 330
Mouse ErbB1 Y V V T D H G S C V R A C G P D Y Y E V E E D G I R K C K K
Human ErbB1 Y V V T D H G S C V R A C G A D S Y E M E E D G V R K C K K
Newt EGFR Y V V T D H G S C V R T C D D K S Y E V E E N G V R K C K K
340 350 360
Mouse ErbB1 C D G P C R K V C N G I G I G E F K D T L S I N A T N I K H
Human ErbB1 C E G P C R K V C N G I G I G E F K D S L S I N A T N I K H
Newt EGFR C D G P C N K V C S G L P T N E R V R - - A V N H F N I E R
370 380 390
Mouse ErbB1 F K Y C T A I S G D L H I L P V A F K G D S F T R T P P L D
Human ErbB1 F K N C T S I S G D L H I L P V A F R G D S F T H T P P L D
Newt EGFR F A N C T T I Q G D I I I L P V T L A G D G F E K I P P L D
400 410 420
Mouse ErbB1 P R E L E I L K T V K E I T G F L L I Q A W P D N W T D L H
Human ErbB1 P Q E L D I L K T V K E I T G F L L I Q A W P E N R T D L H
Newt EGFR H T K L D Y F K N V K E I T G F L A I Q A W P E N A T H L H
430 440 450
Mouse ErbB1 A F E N L E I I R G R T K Q H G Q F S L A V V G L N I T S L
Human ErbB1 A F E N L E I I R G R T K Q H G Q F S L A V V S L N I T S L
Newt EGFR A F E N L E V I R G R S K T S G R F S L A V I Q T S I S S L
460 470 480
Mouse ErbB1 G L R S L K E I S D G D V I I S G N R N L C Y A N T I N W K
Human ErbB1 G L R S L K E I S D G D V I I S G N K N L C Y A N T I N W K
Newt EGFR G F R S L R E I S D G D V L L R R N A Q L C Y T D I I N W T
490 500 510
Mouse ErbB1 K L F G T P N Q K T K I M N N R A E K D C K A V N H V C N P
Human ErbB1 K L F G T S G Q K T K I I S N R G E N S C K A T G Q V C H A
Newt EGFR S V F R T K N Q N I T S A L N K P K E S C L A E N K I C D L
520 530 540
Mouse ErbB1 L C S S E G C W G P E P R D C V S C Q N V S R G R E C V D K
Human ErbB1 L C S P E G C W G P E P R D C V S C R N V S R G R E C V D K
Newt EGFR L C S D N G C W G P G P F S C L S C R Y F L R M T E C V E T
550 560 570
Mouse ErbB1 C N I L E G E P R E F V E N S E C I Q C H P E C L P Q A M N
Human ErbB1 C N L L E G E P R E F V E N S E C I Q C H P E C L P Q A M N
Newt EGFR C N I M K G E P R E Y V K D S K C F R C H P E C L I Q N T T
580 590 600
Mouse ErbB1 I T C T G R G P D N C I Q C A H Y I D G P H C V K T C P A G
Human ErbB1 I T C T G R G P D N C I Q C A H Y I D G P H C V K T C P A G
Newt EGFR T T C T G P G P D N C L A C A H F K D G P H C V K S C P S G
Mouse ErbB1
Human ErbB1
Newt EGFR


610 620 630
Mouse ErbB1 IMGENNTLVVKYADANNVCHLCHANCTYGC
Human ErbB1 VMGENNTLVVKYADAGHVCHLCHPNCTYGC
Newt EGFR IMGENDTYIVKYADENKVCQLCHVNCTEGC
640 650 660
Mouse ErbB1 AGPGLQGCEVWPSGPKIPSIATGIVGGLLF
Human ErbB1 TGPGLEGCPTN--GPKIPSIATGMVGALLL
Newt EGFR FGPDLEGCPDS--GSRIPSIAAGVFGGILC
670 680 690
Mouse ErbB1 IVVVALGIGLFMRRRHIVRKRTLRRLLQER
Human ErbB1 LVVALGIGLFMRRRHIVRKRTLRRLLQER
Newt EGFR VVIISLCVGFFVRRIRIQRKRTSRRLLNEK
700 710 720
Mouse ErbB1 ELVEPLTPSGEAPNQAHLRILKETEFKKIK
Human ErbB1 ELVEPLTPSGEAPNQALLRILKETEFKKIK
Newt EGFR TLVVPLTPSGEAPNQALLRILKETEIKKIM
730 740 750
Mouse ErbB1 VLGSGAFGTVYKGLWIPEGEKVKIPVAIKE
Human ErbB1 VLGSGAFGTVYKGLWIPEGEKVKIPVAIKE
Newt EGFR VLGSGAFGTVFKGLWLPEGEHVKIPVAIKE
760 770 780
Mouse ErbB1 LREATSPKANKEILDEAYVMASVDNPHVCR
Human ErbB1 LREATSPKANKEILDEAYVMASVDNPHVCR
Newt EGFR LREATSPKANKEILDEAYVMASVNDAHVCR
790 800 810
Mouse ErbB1 LLGICLTSTVQLITQLMPYGCLLDYVREHK
Human ErbB1 LLGICLTSTVQLITQLMPFGCLLDYVREHK
Newt EGFR LLGICLTSTVQLVTQLMPYGCLLDYVREHK
820 830 840
Mouse ErbB1 DNIGSQYLLNWCVQIAKGMNYLEDRRLVHR
Human ErbB1 DNIGSQYLLNWCVQIAKGMNYLEDRRLVHR
Newt EGFR DNIGSNLLLNWCVQIAKGMNYLEERRLVHR
850 860 870
Mouse ErbB1 DLAARNVLVKTPQHVKITDFGLAKLLGAEE
Human ErbB1 DLAARNVLVKTPQHVKITDFGLAKLLGAEE
Newt EGFR DLAARNVLVRNPQHVKITDFGLAKLLGAEE
880 890 900
Mouse ErbB1 KEYHAEGGKVPIKWMALESILHRIYTHQSD
Human ErbB1 KEYHAEGGKVPIKWMALESILHRIYTHQSD
Newt EGFR KEYHAEGGKVPIKWMALESILHRIYTHQSD


910
920
930
Mouse ErbB1
Human ErbB1
Newt EGFR
940
950
960
Mouse ErbB1
Human ErbB1
Newt EGFR
970
980
990
Mouse ErbB1
Human ErbB1
Newt EGFR
1000
1010
1020
Mouse ErbB1
Human ErbB1
Newt EGFR
1030
1040
1050
Mouse ErbB1
Human ErbB1
Newt EGFR
1060
1070
1080
Mouse ErbB1
Human ErbB1
Newt EGFR
1090
1100
1110
Mouse ErbB1
Human ErbB1
Newt EGFR
1120
1130
1140
Mouse ErbB1
Human ErbB1
Newt EGFR
1150
1160
1170
Mouse ErbB1
Human ErbB1
Newt EGFR
1180
1190
1200
Mouse ErbB1
Human ErbB1
Newt EGFR

The newt EGFR protein sequence was first aligned against all non-redundant protein sequences at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The top hit was ErbB1. Newt EGFR protein sequence was then aligned against human and mouse ErbB1 protein sequences using MacVector software.

4 Investigating the mechanism of Prod1 signalling

4.1 Introduction

In similarity to uPAR, Prod1 was shown in Chapter 3 to both activate signalling leading to ERK1/2 activation and induce the expression of MMP9. The structurally similar proteins CD59 and uPAR (Garza-Garcia et al., 2009) have been shown to interact physically with the EGFR (Blagoev et al., 2003) (Mazzieri et al., 2006). uPAR has also been shown to activate ERK1/2 signalling through it's interaction with the EGFR in a ligand-independent manner (Liu et al., 2002). These similarities prompted investigation of whether Prod1 also signals via the EGFR and ERK1/2 in order to induce MMP9 expression, and whether it does so by physical interaction with the EGFR leading to its ligand-independent activation. In addition to its interaction with the EGFR, activation of downstream signalling by uPAR has been shown to be mediated by its interaction with β1 containing integrins (Ghosh et al., 2006) (Wei et al., 2001) (Wei et al., 2007). β1 integrin was identified in a yeast-two-hybrid screen as interacting physically with Prod1 (P. Gates, unpublished), prompting investigation of its possible physical interaction with Prod1 and participation in the mechanism leading to MMP9 induction.

qRT-PCR analysis of MMP9 induction in response to Prod1 expression provides a highly quantitative assay suitable for further characterisation of the specifics of the molecular mechanism through which it acts. Considerable attention has been paid to the development of inhibitors for specific components of cellular signalling pathways, in particular those known to be involved in the oncogenic processes, which serve as targets for chemotherapy. These small molecules are in general analogues of ATP engineered to show a high degree of specificity for the ATP binding site of their target, thereby acting as competitive inhibitors at the active site of the kinase. Small molecule kinase inhibitors were employed to further investigate the mechanism through which Prod1 signals to induce MMP9 expression. Whether a link exists between the observed ERK1/2 activation and MMP9 induction was investigated through use of an ERK1/2 inhibitor. The roles of a number of other down-stream effectors of EGFR signalling were also investigated using specific kinase inhibitors to

further characterise the intracellular signalling pathways through which Prod1 acts. As inappropriate EGFR activity is known to be associated with a variety of cancers, a number of inhibitors against its activity have been developed. In particular, inhibitors specific to ErbB1 or ErbB2 exist (Levitzki and Gazit, 1995), allowing investigation of the involvement of both isoforms in the mechanism of Prod1 signalling.

The most notable similarity between the structure of Prod1 and the two most structurally similar members of the three-fingered family of proteins, uPAR and CD59, is that all three have an α-helical third finger. The role of this and other regions of the structure of Prod1 in the activation of ERK1/2, induction of MMP9 and interaction with the EGFR were investigated in order to better understand the functional relationship between these structures, as well as to gain further insight into the molecular mechanism through which Prod1 regulates MMP9 expression.

4.2 Results

4.2.1 Prod1 signalling pathway analysis

In order to investigate the role of the EGFR in Prod1 activation of ERK1/2 signalling, the EGFR inhibitor AG1478 was employed. The minimum concentration of AG1478 required to completely inhibit EGF stimulated ERK1/2 activation was first established. Cells were treated with increasing concentrations of AG1478 and it was found that 25uM was sufficient to completely inhibit ERK1/2 phosphorylation in response to EGF (Fig. 4.1). Cells treated with AG1478 showed inhibited ERK1/2 phosphorylation compared to untreated starved cells indicating a background level of EGFR signalling occurs in B1H1 cells in the absence of serum.

Prod1 and GPI GFP expressing cells were treated with AG1478 and the ERK1/2 inhibitor U0126. U0126 completely inhibited ERK1/2 phosphorylation in Prod1 and GPI GFP expressing cell as expected, and inhibition of EGFR also inhibited ERK1/2 phosphorylation in both Prod1 and GPI GFP expressing cells (Fig. 4.2a). The subtraction of the pERK1/2 background levels seen in control

GPI GFP transfected cells from those in Prod1 expressing cells revealed the decreased ERK1/2 phosphorylation resulting from inhibition of EGFR was of greater magnitude in Prod1 expressing cells (Fig 4.2.b). This indicated that there was a component of ERK1/2 activated by EGFR signalling above the level of background in these cells, which could only be attributed to the expression of Prod1.

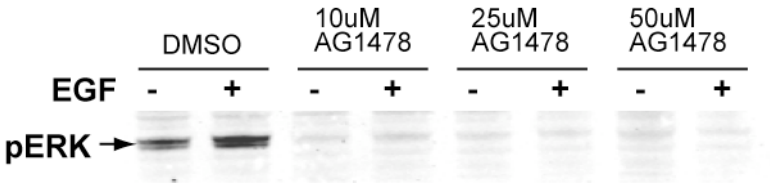


Figure 4.1 Optimising AG1478 concentration

B1H1 cells were serum starved for 24hr, then treated for 30min with indicated concentrations of AG1478 or dimethylsulfoxide (DMSO) vehicle control. Cells were stimulated for 20min with 100ng/ml EGF and cell lysates were subsequently Western-blotted with anti-phospho ERK1/2 antibody

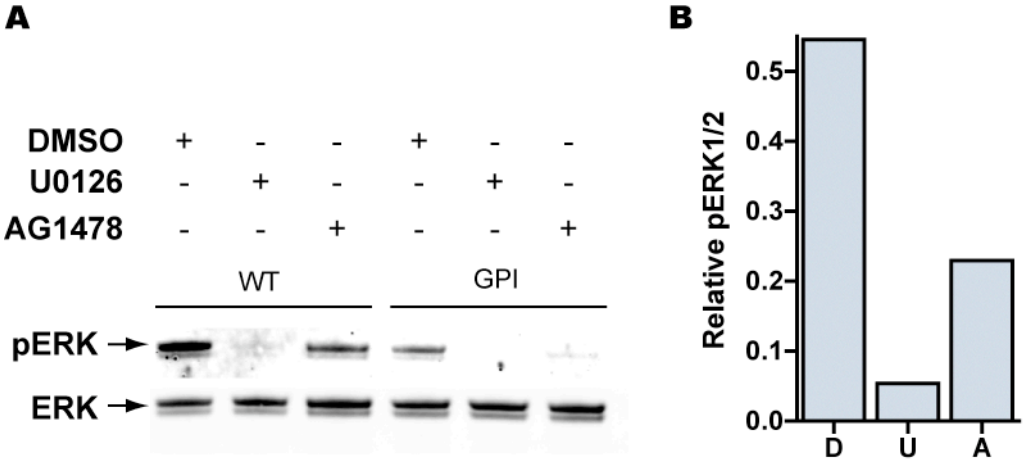


Figure 4.2 Effect of EGFR inhibition on Prod1 induced ERK1/2 activation

B1H1 cells transfected with Prod1 (WT) or GPI GFP (GPI) were serum-starved for 24hrs, then treated with 5uM U0126 [U], 25uM AG1478 [A] or DMSO vehicle [D]. (A) Cell lysates were Western-blotted with anti-phospho ERK1/2 and total ERK1/2 antibodies. (B) ERK1/2 phosphorylation was quantitated and normalised to the level seen in DMSO treated Prod1 expressing cells. GPI GFP ERK1/2 background was subtracted from Prod1 ERK1/2 levels for each treatment, and adjusted ERK1/2 phosphorylation was plotted.

Whether inhibition of ERK1/2 or EGFR activity affected induction of MMP9 expression by Prod1 was investigated by zymography and qRT-PCR. B1H1 cells expressing Prod1 and GPI GFP were treated with the ERK1/2 inhibitor U0126 or the EGFR inhibitor AG1478. Protein secretion was analysed by zymography (Fig. 4.3 A) and quantitated (Fig. 4.3 B), and gene expression was assayed by qRT-PCR (Fig. 4.3 C). Assaying by qRT-PCR indicated an approximately 30% inhibition of Prod1 induced MMP9 expression with the EGFR inhibitor AG1478, and ~50% with the ERK1/2 inhibitor U0126. The EGFR inhibitor PD153035 showed a similar degree of inhibition as AG1478 (data not shown).

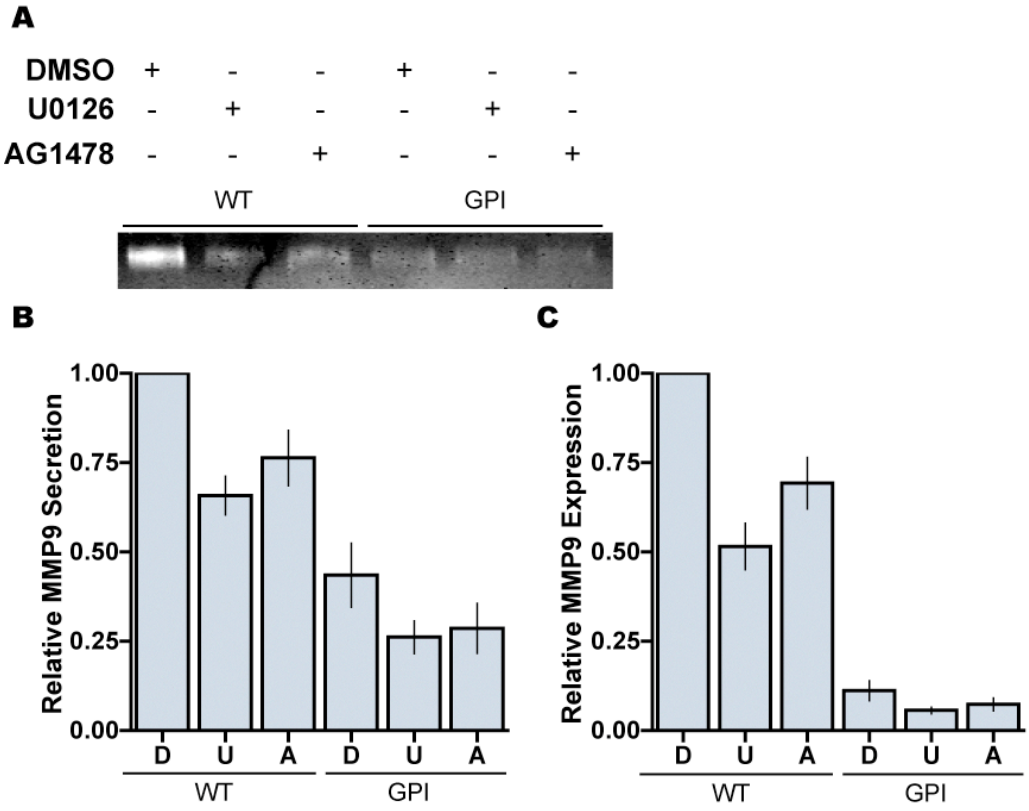


Figure 4.3 Effect of ERK1/2 and EGFR inhibition on Prod1 induced MMP9 expression.

Prod1 (WT) and GPI GFP (GPI) expressing B1H1 cells were serum starved for 24hr then treated with U0126 [U], AG1478 [A] or DMSO [D] for 48hr. (A) MMP9 activity in culture medium conditioned for 48hr in the presence of inhibitors was analysed by gelatin zymography, and (B) results of seven independent experiments were plotted on a histogram. (C) cDNA was prepared from cells and MMP9 expression was analysed by qRT-PCR. Results of nine independent experiments were plotted on a histogram. Values in each experiment are normalised to that seen in DMSO treated Prod1 expressing cells. Bars represent SEM.

The duration of EGFR and ERK1/2 inhibition was extended (Fig. 4.4 A) and ERK1/2 inhibitor concentration was raised (Fig. 4.4 B), showing no greater effect on MMP9 expression. The observation that ~30% inhibition of MMP9 induction was the maximum possible with the EGFR inhibitor AG1478 suggested that other transmembrane signal-transducers were involved in Prod1 regulation of MMP9 expression. The role of ErbB2, another member of the EGFR family was investigated by treating Prod1 expressing cells with the ErbB2 inhibitor AG879, resulting in ~40% inhibition of MMP9 induction (Fig. 4.4 C). No greater inhibition was observed at higher concentrations (data not shown). Higher concentrations of AG879 were seen to be toxic to the cells, as seen by light microscopy (not shown). A combination of AG879 and AG1478 was unable to completely inhibit MMP9 induction (Fig. 4.4 D), despite exceptionally strong inhibition being observed with individual inhibitors in these experiments. Combined inhibition resulted in cytotoxicity however.

As inhibition of ERK1/2 gave only 50% inhibition of MMP9 expression, the effect of inhibiting the other MAPKs, p38 and JNK, both of which are linked by MEKK (Lange-Carter et al., 1993) to EGFR signalling (Lange et al., 1998) (Minden et al., 1994) and have been shown to be involved in MMP9 regulation (Wang et al., 2009), was analysed. It was seen that inhibition of p38 showed no MMP9 inhibition, even at high concentrations (Fig 4.5 A). Prod1 expression was seen to induce phosphorylation of JNK (Fig. 4.5 B) and inhibition of JNK activity with the drug SP600125 inhibited Prod1 MMP9 induction by ~50%, similar to ERK1/2. Inhibition of both simultaneously did not have an additive effect on Prod1 induced MMP9 transcription however, with no greater inhibition of MMP9 transcription being seen than when they were inhibited singly (Fig. 4.5 C). It was observed that ERK1/2 phosphorylation was inhibited by SP600125 and likewise, that JNK phosphorylation was inhibited by U0126 (Fig. 4.5 D). SP600125 inhibited ERK1/2 phosphorylation by ~75% compared to ~95% seen with U0126 (Fig 4.5 E). Simultaneous inhibition of Prod1 expressing cells with SP600125 and AG1478 showed no greater inhibition of MMP9 induction than when JNK was inhibited singly (Fig. 4.5 F), indicating that JNK is downstream of EGFR signalling and not some other, as of yet unidentified, Prod1 interacting receptor.

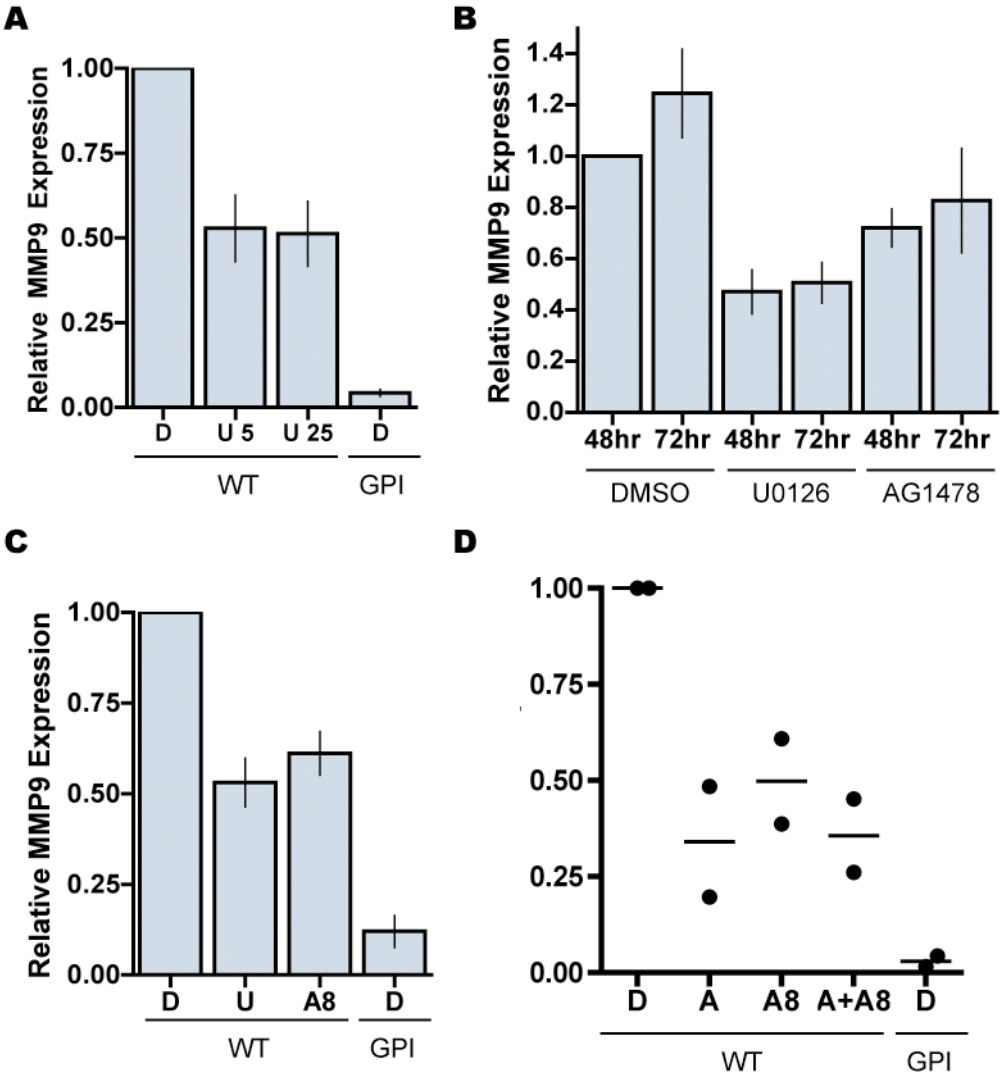


Figure 4.4 Contribution of ERK1/2 and EGFR to Prod1 induced MMP9 expression

(A) B1H1 cells expressing Prod1 (WT) or GPI GFP (GPI) were serum starved for 24hr then treated with DMSO vehicle [D], 5uM U0126 [U5] or 25uM U0126 [U25] for 48hr. cDNA was prepared from cells, and MMP9 expression was analysed by qRT-PCR. Histogram represents results of four independent experiments. (B) B1H1 cells expressing Prod1 were serum starved for 24hr then treated with 5uM U0126 or 25uM AG1478 for either 48 hr, or 48hr + 24hr with fresh inhibitor = 72hr. cDNA was prepared from cells and MMP9 expression was analysed by qRT-PCR. Histogram represents results of three independent experiments. (C) B1H1 cells expressing Prod1 (WT) or GPI GFP (GPI) were serum starved for 24hr then treated with DMSO vehicle [D], 5uM U0126 [U] or 5uM AG879 [A8] for 48hr. cDNA was prepared from cells and MMP9 expression was analysed by qRT-PCR. Histogram represents results of six independent experiments. (D) B1H1 cells expressing Prod1 (WT) or GPI GFP (GPI) were serum starved for 24hr then treated with DMSO vehicle [D], 25uM AG1478 [A],

5uM AG879 [A8] or 25uM AG1478 + 5uM AG879 [A+A8] for 48hr. cDNA was prepared from cells and MMP9 expression was analysed by qRT-PCR.
Values in each experiment are normalised to that seen in DMSO treated Prod1 expressing cells. Bars represent SEM.

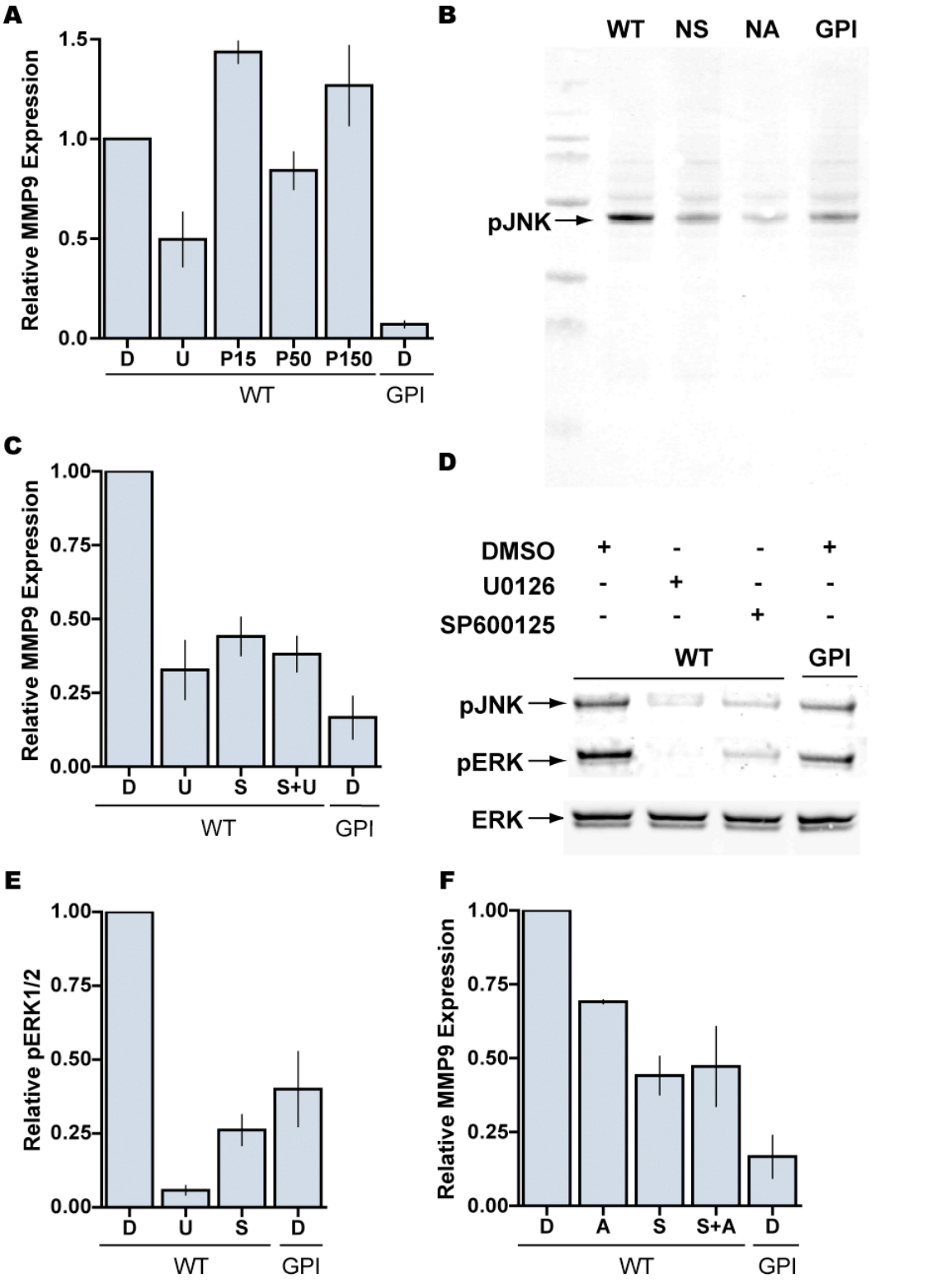


Figure 4.5 Role of MAP kinases in Prod1 regulation of MMP9 expression

(A) B1H1 cells transfected with Prod1 (WT) or GPI GFP (GPI) serum starved for 24hr then treated with 5uM U0126 [U], 15uM, 50uM or 150um P38 inhibitor [P15], [P50], [P150] or DMSO vehicle [D] for 48hr. cDNA was prepared from cells, and MMP9 expression was analysed by qRT-PCR. Histogram represents results of two independent experiments for 15uM and 50uM P38 inhibitor, and 5 independent experiments for 150uM P38 inhibitor. (B) B1H1 cells were transfected with Prod1 (WT) Prod1 ΔN-terminal signal peptide (NS), Prod1 ΔC-terminal anchor attachment peptide (NA) and GPI anchored GFP (GPI). Cells were serum starved for 24hr and cell lysates were Western-blotted with anti phospho-JNK antibodies. (C) B1H1 cells transfected with Prod1 (WT) or GPI GFP (GPI) serum starved for 24hr then treated with 5uM U0126 [U], 10uM SP600125 [S], 5uM U0126 +10uM SP600125 [S+U] or DMSO vehicle control for 48hr [D]. cDNA was prepared from cells, and MMP9 expression was analysed by qRT-PCR. Histogram represents results of five independent experiments for SP600125 and four independent experiments for U0126+SP600125. (D) B1H1 cells transfected with Prod1 (WT) or GPI GFP (GPI) were serum-starved for 24hr, then treated with 5uM U0126, 10uM SP600125, or DMSO vehicle for 30 minutes. Cell lysates were Western-blotted with anti phospho-ERK1/2, anti phospho-JNK and total ERK1/2 antibodies, and results of three independent experiments were represented in a histogram (E). B1H1 cells transfected with Prod1 (WT) or GPI GFP (GPI) serum starved for 24hr then treated with 25uM AG1478 [A], 10uM SP600125 [S], 25uM AG1478 + 10uM SP600125 [S+A] or DMSO vehicle [D] for 48hr. cDNA was prepared from cells, and MMP9 expression was analysed by qRT-PCR. Histogram represents results of four independent experiments for AG1478 + SP600125. Experimental values for SP600125 are as in (D). Values in each experiment are normalised to that seen in DMSO treated Prod1 expressing cells. Bars represent SEM.

The inability to completely inhibit Prod1 induction of MMP9 with MAPK inhibitors indicated another distinct pathway may be involved. In addition to the MAPKs, the JAK/STAT pathway regulates transcription downstream of EGFR signalling. STATs are transcription factors which become phosphorylated either by JAKs activated by the EGFR (Andl et al., 2004) or by the EGFR itself (Silvennoinen et al., 1993), leading to their nuclear localisation. The MMP9 promoter has been shown to contain a STAT3 response element (Kim et al., 2008), therefore Prod1 expressing cells were treated with the JAK2/STAT3 inhibitor AG490, resulting in ~60% inhibition of MMP9 induction (Fig. 4.6). No greater inhibition was seen at higher inhibitor concentration (not shown). Whether JAK/STAT signalling was

regulating MMP9 expression independently of the ERK1/2 pathway was addressed by treating Prod1 expressing cells with both U0126 and AG490. The simultaneous inhibition of both pathways showed no greater effect than inhibition of them singly (Fig. 4.6) indicating their interaction at some level in the regulation of MMP9 expression.

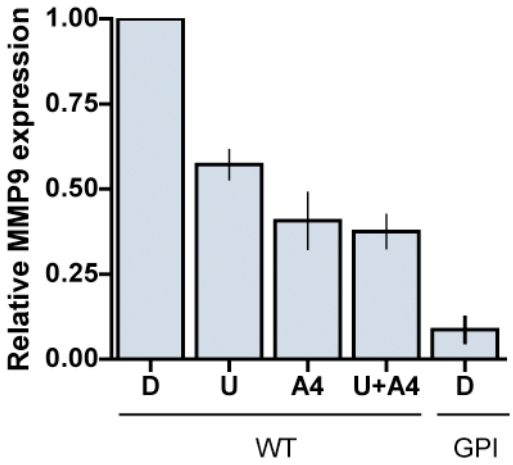


Figure 4.6 Role of JAK/STAT pathway in Prod1 regulation of MMP9 expression

B1H1 cells transfected with Prod1 (WT) or GPI GFP (GPI) serum starved for 24hr then treated with 5uM U0126 [U], 20uM AG490 [A4], 5uM U0126 + 20uM AG490 [U+ A4] or DMSO vehicle control [D] for 48hr. cDNA was prepared from cells, and MMP9 expression was analysed by qRT-PCR. Histogram represents results of five independent experiments. Values in each experiment are normalised to that seen in DMSO treated Prod1 expressing cells. Bars represent SEM.

The inability to completely block MMP9 induction with combined inhibition of either ERK1/2 and JNK or ERK and JAK/STAT indicates that another pathway downstream of Prod1 is involved with the regulation of MMP9 expression. PI3K is a known transducer of both integrin (Hruska et al., 1995) and EGFR signalling, in particular ErbB2 ErbB3 heterodimers (Ram and Ethier, 1996). Reports of the involvement of PI3K co-operation with ERK1/2 in MMP9 regulation (Guo et al., 2007), and in MMP9 regulation by EGFR (Wang et al., 2006) prompted investigation of its possible role in MMP9 regulation by Prod1. mTor has also been shown to interact with ERK1/2 signalling, negatively regulating MMP9 expression in response to lipopolysaccharide in a manner

independent of PI3K (Mendes Sdos et al., 2009). Rather than inhibiting MMP9 induction in Prod1 expressing cells, treatment with the PI3K inhibitor LY294002 (Fig.3.7 A) lead to increased MMP9 expression in a dose-dependent manner. The mTor inhibitor rapamycin increased the induction of MMP9 seen to result from Prod1 expression (Fig. 4.7 B). LY294002 treatment also raised MMP9 expression in GPI GFP control expressing cells (Fig. 4.7 C), as did rapamycin to a lesser extent (Fig. 4.7 D).

4.2.2 Relating the structure of Prod1 to its function

In order to identify structural elements of Prod1 relevant to its function, a series of point-mutants were generated by site-directed mutagenesis. Amino-acids were selected for mutation based on computational prediction, which was carried out by A. Garza-Garcia. See Materials and Methods for details of the mutation strategy.

B1H1 cells were transfected with the series of point mutants, MMP9 expression was assayed by qRT-PCR and compared to the level seen for the wild-type Prod1 protein (Fig. 4.8 A). The point mutations either showed no effect, a mild effect or a complete inhibition of MMP9 induction. These three categories of mutation were represented on the Prod1 tertiary structure (Fig. 4.8 B), indicating amino-acids glutamine 59 (Q59) and glutamate 66 (E66) on the α-helix of finger three to be essential for MMP9 induction. Substitution of Q59 or E66 to alanine was also observed to abolish the ability of Prod1 to activate ERK1/2 signalling (Fig. 4.8 C). Expression of Q59A and E66A on the cell membrane was confirmed by immunofluorescence (Fig. 4.8 D).

4.2.3 Prod1 interacts physically with the EGFR

As EGFR inhibition is observed to inhibit both ERK1/2 activation and MMP9 induction, the possibility of a physical interaction between it and the EGFR was investigated. In order to further characterise the specific molecular interactions participating in the mechanism through which Prod1 activates ERK1/2 signalling and induces MMP9 expression, the effect of the Q59A mutation, which completely abolishes these activities, was investigated in the context of the physical interaction between Prod1 and the EGFR. HEK 293T cells stably

expressing Prod1-Flag or Prod1-Flag Q59A were transfected with Myc-tagged newt EGFR and proteins were immunoprecipitated from cell lysates with anti-Flag antibody. Immunoprecipitated protein was Western-blotted with anti-Myc antibody to detect Myc-tagged EGFR and it was observed that EGFR specifically co-immunoprecipitated with both wild-type Prod1-Flag and Prod1-Flag Q59A (Fig. 4.9 A).

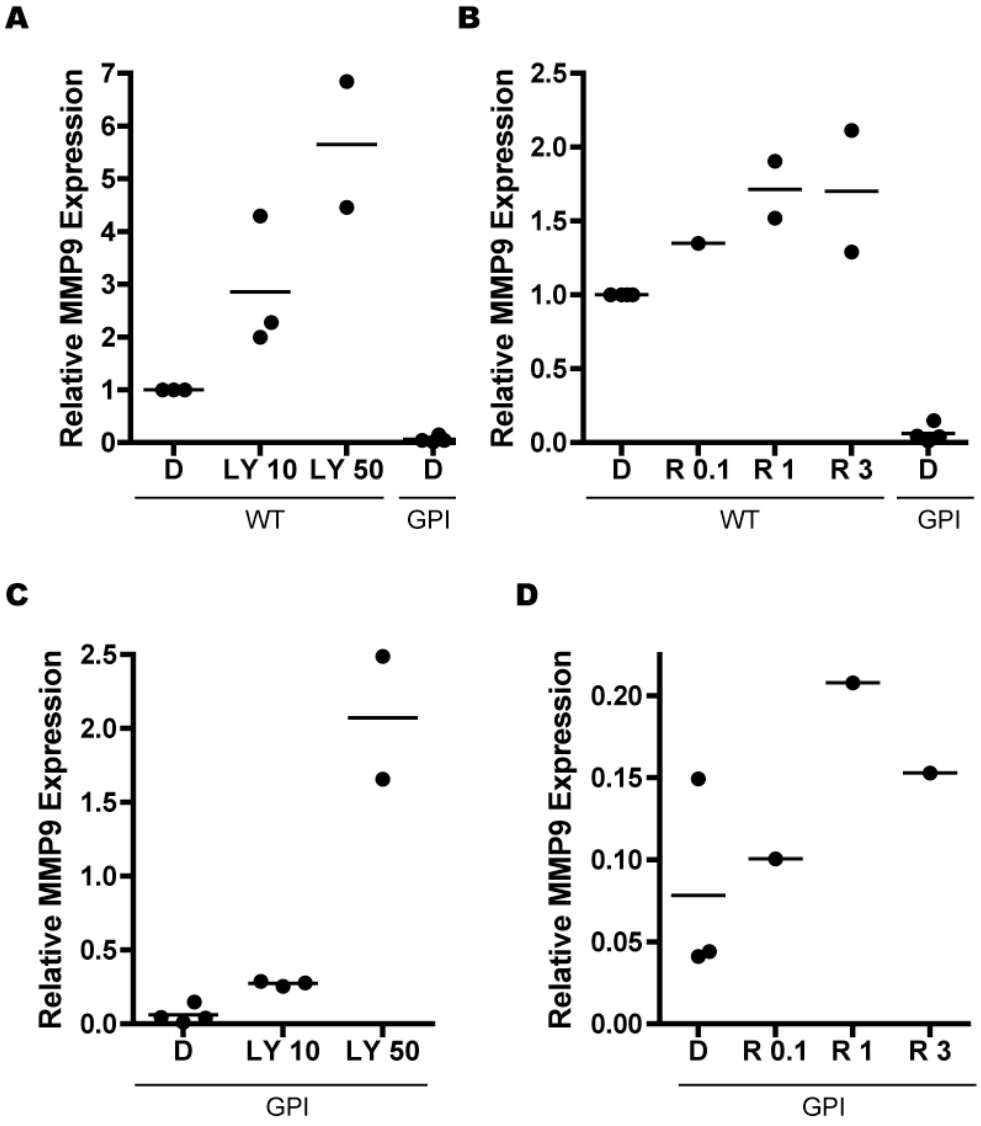


Figure 4.7 Role of PI3K and mTOR in Prod1 regulation of MMP9 expression

B1H1 cells transfected with Prod1 (WT) or GPI GFP (GPI) were serum starved for 24hr then treated with LY294002 at a concentration of 10uM [LY 10] and 50uM [LY 50], rapamycin [R] at a concentration of 0.1uM [R 0.1], 1uM [R 1] or 3uM [R 3], or DMSO vehicle control [D] for 48hr. cDNA was prepared from cells, and MMP9 expression was analysed by qRT-PCR. Values in each experiment are normalised to that seen in DMSO treated Prod1 expressing cells.

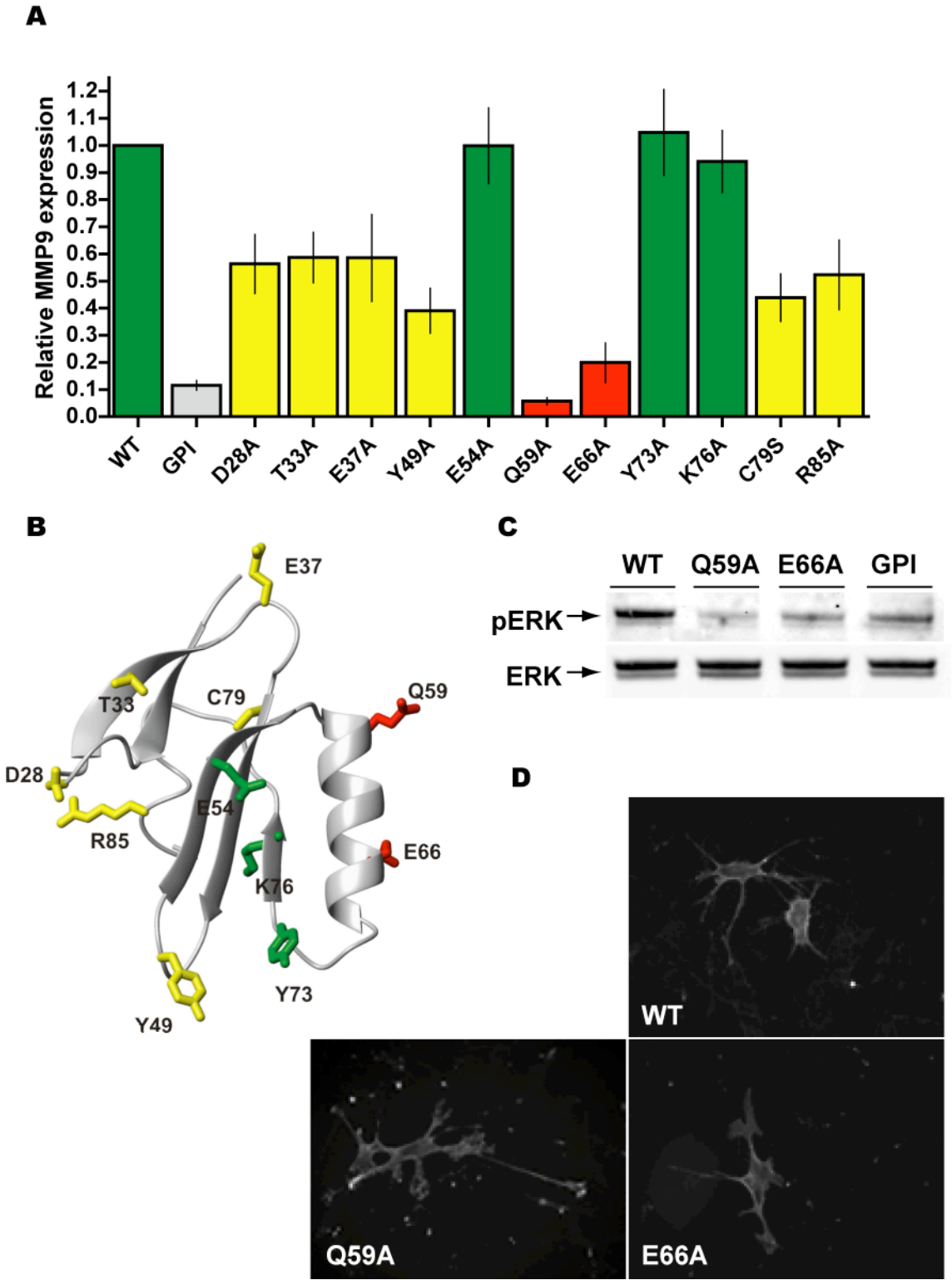


Figure 4.8 Identification of amino-acids key to Prod1 activity

B1H1 cells transfected with Prod1 constructs with single point mutations to alanine/serine at the indicated amino-acid residues were serum-starved for 72hr. (A) cDNA was prepared from cells, and MMP9 expression was analysed by qRT-PCR. Amino-acids were categorised according to the severity of the effect of mutation, red being most severe, yellow being mild and green showing the same activity as wild-type

Prod1 (WT). GPI GFP (GPI) was used as a negative control. Values in each experiment are normalised to that seen in wild-type Prod1 expressing cells. Bars represent SEM. (B) Amino-acids were colour coded on the structure of Prod1 according to the convention described. Figure courtesy of A. Garza-Garcia (C) Protein from cell lysates collected for cDNA preparation from the point-mutants with the most severe effect on MMP9 induction was Western-blotted with anti-ERK1/2 and anti-phospho ERK1/2 antibodies. Results shown are representative of three independent experiments. (D) Membrane expression of the point-mutants with the most severe effect on MMP9 induction was confirmed by immunofluorescence.

In the light of the glutamine 59 mutation showing no disruption of the EGFR Prod1 physical association, the possibility that Prod1 interacts with the EGFR at another interface other than the α-helix was raised. Point mutations at a second surface remote from the α-helix showed an intermediate effect on Prod1 MMP9 inducing activity, and as inhibition of EGFR activity also showed only an intermediate effect on MMP9 induction, the role of this interface in the activation of EGFR signalling was investigated. If disruption of this interface perturbs the physical interaction between Prod1 and EGFR, inhibition of EGFR signalling should show no effect on the MMP9 inducing activity of these point-mutants, however cells transfected with Prod1 mutated at threonine 33 showed a degree of sensitivity to EGFR inhibition comparable to wild-type Prod1 (Fig. 4.9 B).

4.2.4 Prod1 activation of the EGFR

The EGFR is co-immunoprecipitated with Prod1 and inhibition of EGFR activity perturbs the ability of Prod1 to modulate downstream effects, though whether the EGFR is directly associated with Prod1 or is indirectly associated with a multi-protein complex including Prod1 is unclear. Despite experiments being carried out under serum-free conditions, the possibility that the mechanism of Prod1 signal transduction through the EGFR may involve EGFR ligand was assessed. Evidence from use of the EGFR inhibitor AG1478 that serum-starved B1H1 cells have a constitutive level of EGFR signalling (Fig.4.1) raised the possibility that the cells may produce their own EGFR ligand.

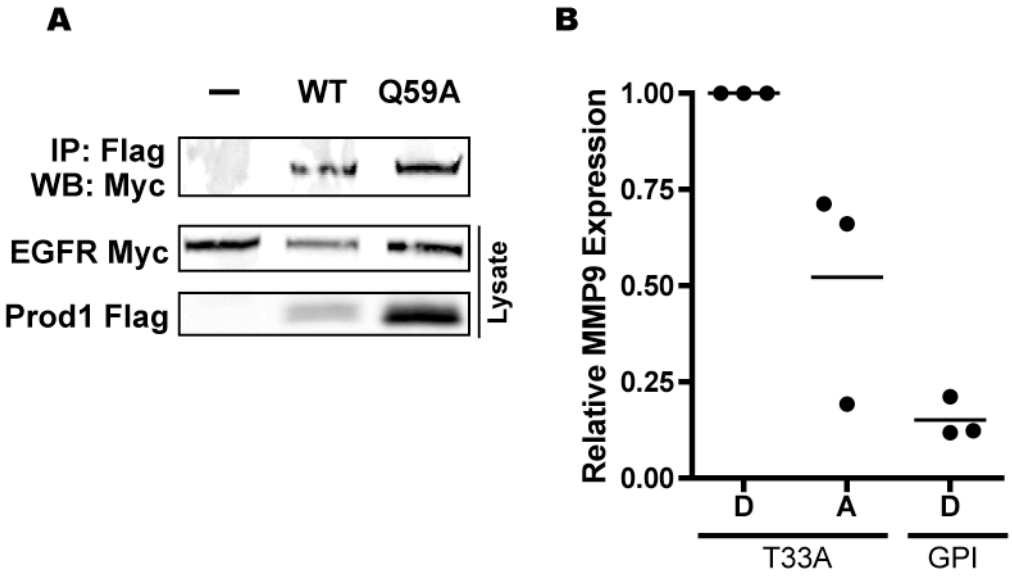


Figure 4.9 Physical interaction between Prod1 and EGFR

(A) HEK-293T cells negative for Prod1 (-), expressing Prod1-Flag (WT) or Prod1-Flag Q59A (Q59A) were transfected with EGFR-Myc. Cells lysates were immunoprecipitated with anti-Flag antibody and Western-blotted with anti-Myc antibody. Expression of EGFR- Myc and Prod1-Flag in cell lysates is shown in the bottom two panels. (B) B1H1 cells transfected with Prod1-Flag T33A (T33A) or GPI GFP (GPI) were serum starved for 24hr then treated with DMSO [D] or AG1479 [A] for 48hr. cDNA was prepared from cells, and MMP9 expression was analysed by qRT-PCR. Values in each experiment are normalised to that seen in DMSO treated Prod1 expressing cells. Bars represent SEM.

It was observed that serum-free conditioned medium collected from B1H1 cells contained an ERK1/2 stimulating activity that was inhibited completely by AG1478 (Fig. 4.10 A). Despite its physical interaction with the EGFR it had not been ruled-out that Prod1 may up-regulate the expression of EGF and thereby bring about EGFR dependent downstream effects. Conditioned medium from Prod1 expressing cells was observed to contain no more EGFR stimulating activity than that produced by GPI GFP control expressing cells however (Fig. 4.10 A) indicating that MMP9 induction by Prod1 activation of the EGFR is not a result of increased EGFR ligand production. The physical interaction between Prod1 and the EGFR may be modulating the response of the EGFR to EGF rather than directly activating it, however despite containing an EGFR ligand with ERK1/2 stimulating activity, conditioned medium did not induce further MMP9 expression in either Prod1 or GPI GFP control expressing cells, nor did ERK1/2 activation by serum stimulation (Fig. 4.10 B). Similarly, uPAR has been

shown to transactivate the EGFR in a ligand-independent manner (Monaghan-Benson and McKeown-Longo, 2006)

It has been suggested that uPAR participates in an interaction with integrins on neighbouring cells (Tarui et al., 2001) as well as on the same cell (Tarui et al., 2003). It was therefore investigated whether the mechanism of MMP9 induction by Prod1 involves the participation of molecules on adjacent cells, or whether all necessary components are present on one cell membrane, by comparing the degree of Prod1 induced MMP9 expression seen in cells close to confluence to cells plated at a density permitting minimal contact between membranes of surrounding cells. It was observed by zymography (Fig. 4.10 C) that the magnitude of MMP9 induction by Prod1 was not influenced by the degree of contact between membranes of adjacent cells. Similar results were observed with qRT PCR (not shown).

The ability of Prod1 to activate mammalian EGFR signalling was investigated by transfecting HEK 293T cells with Prod1. It was observed however that in contrast to EGF stimulation, Prod1 expression did not lead to increased EGFR or ERK1/2 phosphorylation (Fig 4.11).

4.2.5 The role of other Prod1 interacting molecules in the regulation of MMP9

Taken together with the apparent maximum 35% decrease in MMP9 induction seen with EGFR inhibition, the observation that mutation of Prod1 residue glutamine 59 does not affect its interaction with EGFR despite abolishing its MMP9 inducing activity, raised the possibility of the involvement of another signal transducing protein that interacts with Prod1 at the α-helix. A previous yeast two-hybrid screen aimed at identifying Prod1 interacting proteins indicated a potential physical interaction between Prod1 and β1 integrin (P.Gates, unpublished). uPAR regulation of MMP9 involves fibronectin binding

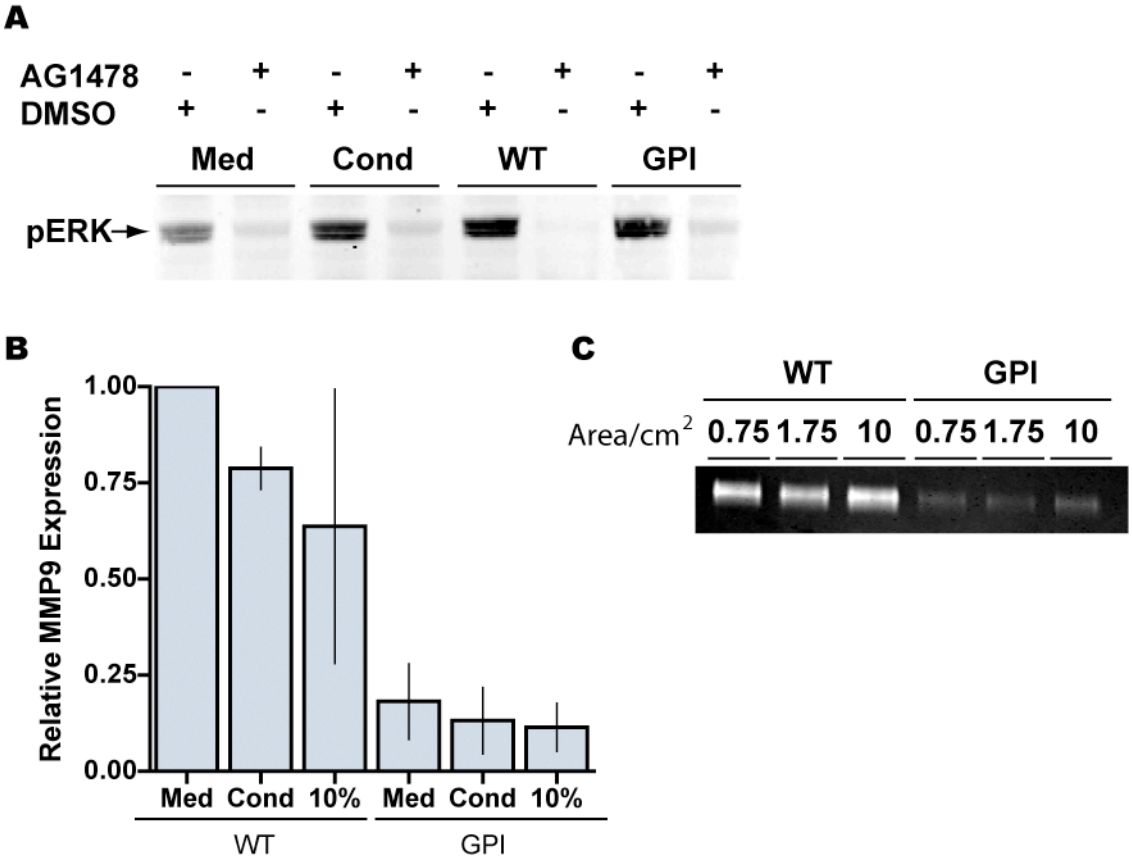


Figure 4.10 Mode of EGFR activation by Prod1

(A) B1H1 cells were serum starved for 8hr and stimulated with concentrated serum free culture medium (Med), concentrated serum free culture medium conditioned by B1H1 (Cond), GPI GFP expressing (GPI) or Prod1 expressing B1H1 cells (WT), in the presence of AG1478 or DMSO vehicle. Cell lysates were Western-blotted with anti-phospho ERK1/2 antibodies. Results shown are representative of four independent experiments. (B) B1H1 cells transfected with Prod1 (WT) or GPI GFP (GPI) were incubated for 72hr in the presence of concentrated cell culture medium (Med), concentrated conditioned cell culture medium from B1H1 cells (Cond) or 10% serum (10%). cDNA was prepared from cells, and MMP9 expression was analysed by qRT-PCR. Results shown are the average of three independent experiments. Values in each experiment are normalised to that seen in DMSO treated Prod1 expressing cells. Bars represent SEM. (C) Equal numbers of Prod1 (WT) of GPI GFP (GPI) expressing cells were plated on surfaces with a range of areas in equal volumes of medium. MMP9 activity in serum free culture medium conditioned for 48hr in was analysed by gelatin zymography. Results shown are representative of three independent experiments.

induced α5β1 integrin signalling, leading to ERK1/2 activation (Wei et al., 2007), and in light of this the role of integrins in Prod1 regulation of MMP9 was investigated, despite the apparent lack of requirement for matrix-protein binding. Prod1-Flag and Prod1-Flag Q59A expressing HEK 293T cells were transfected with Myc-tagged β1 integrin and cell lysates were immunoprecipitated with anti-Flag antibodies. β1 integrin was observed to co-immunoprecipitate with both Prod1-Flag and Prod1-Flag Q59A however (Fig. 4.12 A), indicating that the abolition of Prod1 MMP9 inducing activity resulting from mutation of glutamine 59 is also not attributable to a disruption of its physical association with β1 integrin, as with the EGFR.

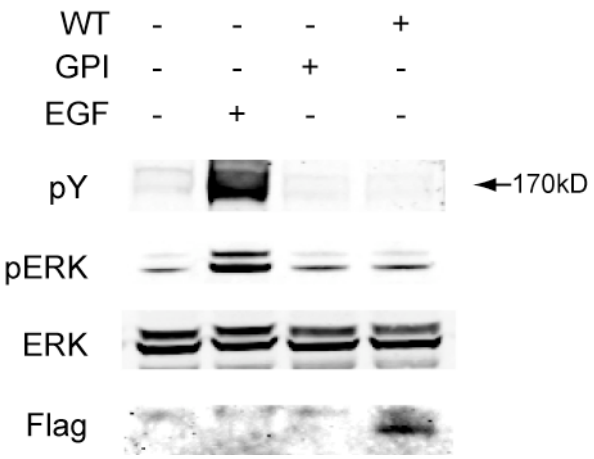


Figure 4.11 Lack of activation of mammalian EGFR by Prod1

HEK-239T cells were either stimulated with EGF, transfected with Prod1 (WT) or GPI GFP (GPI). Cell lysates were Western-blotted with anti-phosphotyrosine (pY), anti-phospho ERK1/2, total ERK1/2 or anti-Flag antibodies. Note that EGF treatment lead to phosphorylation of a 170kD band corresponding to the EGFR and ERK1/2 activation, whereas Prod1 expression did not.

Src is involved in the activation of a number of the major integrin signal transducers, reviewed in (Playford and Schaller, 2004), and is specifically involved in regulating integrin mediated ERK1/2 activation (Zhao et al., 1998). uPAR integrin activation of ERK1/2 and regulation of MMP9 requires Src

activity (Wei et al., 2007), and integrin transactivation of EGFR has also been shown to be Src dependent (Moro et al., 2002). In order to investigate whether integrin activity plays a role in MMP9 regulation by Prod1, the Src inhibitor PP2 was employed. Treatment of Prod1 expressing cells with the Src inhibitor PP2 did not inhibit MMP9 induction however (Fig. 4.12 B), though an inhibition of ERK1/2 phosphorylation was observed (Fig. 4.12 C).

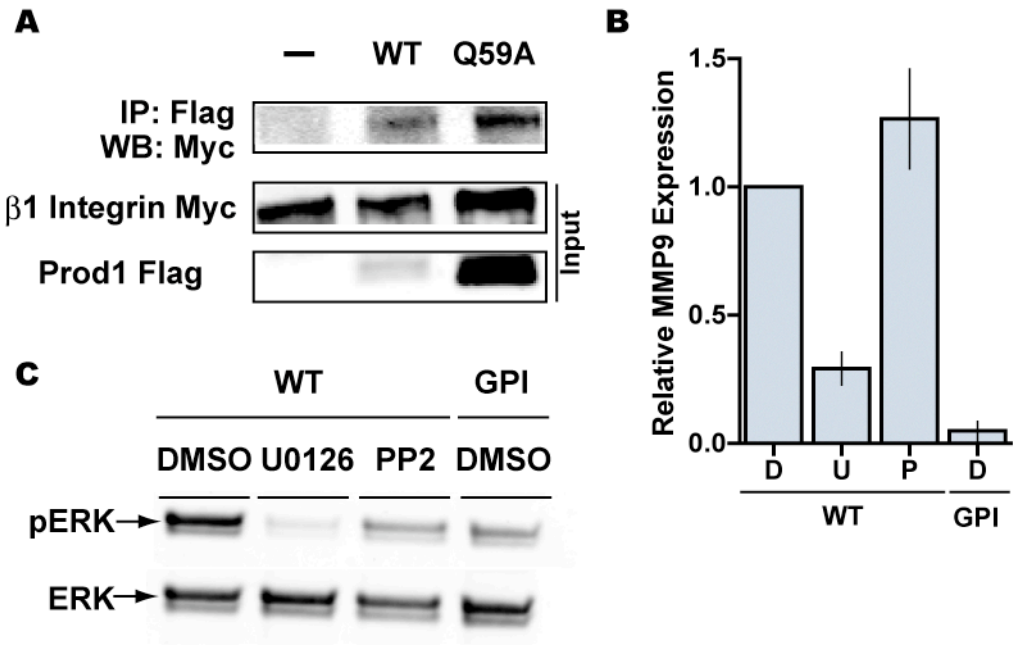


Figure 4.12 Role of integrins in MMP9 induction

(A) HEK-293T cells negative for Prod1 (-), expressing Prod1-Flag (WT) or Prod1-Flag Q59A (Q59A) were transfected with β1 integrin-Myc. Cell lysates were immunoprecipitated with anti-Flag antibody and Western-blotted with anti-Myc antibody. Expression of β1 integrin-Myc and Prod1-Flag in cell lysates is shown in the bottom two panels. (B) B1H1 cells transfected with Prod1 (WT) or GPI GFP (GPI) were serum starved for 24hr then treated with DMSO [D] or 50uM PP2 [P] for 48hr. cDNA was prepared from cells, and MMP9 expression was analysed by qRT-PCR. Results shown are the average of 4 independent experiments. Values in each experiment are normalised to that seen in DMSO treated Prod1 expressing cells. Bars represent SEM. (C) B1H1 cells transfected with Prod1 (WT) or GPI GFP (GPI) were serum starved for 24hr then treated with DMSO [D], 5uM U0126 [U] or 15uM PP2 [P] for 30min. Cell lysates were Western-blotted. Results representative of two independent experiments.

4.3 Discussion

4.3.1 Transmembrane-receptor mediated regulation of MMP9 expression by Prod1

Inhibition of EGFR activity leads to both diminished ERK1/2 activation and MMP9 gene induction in response to Prod1 expression. Similarly, inhibition of the activity of the EGFR family member ErbB2 diminishes Prod1 induced MMP9 expression. Prod1's ability to induce MMP9 is also impaired by ERK1/2 inhibition, and similarly, mutation of amino acids on the α-helix of Prod1 inhibit ERK1/2 activation and abolish MMP9 induction**,** indicating that the decrease in ERK1/2 activation by Prod1 seen when EGFR signalling is inhibited is mechanistically linked to the resulting decrease in MMP9 induction. Results of a single experiment (data not shown) in which ERK1/2 and either EGFR or ErbB2 were inhibited simultaneously showed no greater effect than when ERK1/2 alone was inhibited, consistent with the proposal that ERK1/2 is downstream of EGFR. Were the EGFR to be signalling primarily through another pathway independent of ERK1/2, simultaneous inhibition should show an effect greater in magnitude than that seen with ERK1/2 inhibition alone.

Prod1 was seen to interact physically with the EGFR, and the production of EGFR ligand was not stimulated by Prod1 expression, indicating that EGFR activation is not a result of increased autocrine signalling. Further to this, EGFR ligand showed no stimulation of MMP9 induction in either Prod1 or control expressing cells. In light of these points, it is proposed that Prod1 interacts physically with the EGFR, and in doing so, rather than modulating the effects of EGFR ligand, directly activates EGFR signalling, leading to activation of ERK1/2 and subsequently resulting in induction of MMP9 gene expression. Whether Prod1 makes direct contact with the EGFR or interacts with it via other components of a multi-protein complex has not been established however. As cell density, and therefore the degree of contact between membranes of adjacent cells, was not observed to influence MMP9 induction in response to Prod1 expression, it is proposed in addition that the interaction between Prod1

and EGFR family member occurs on the membrane of individual cells rather than between neighbouring cells.

Mutation of residues on Prod1 revealed two classes of mutants. Those on the α-helix that showed a strong inhibition of MMP9 induction when mutated, and those localised to a second surface that showed a mild inhibition. Cells expressing the second class of mutant Prod1, with an intermediate effect on MMP9 expression, were equally sensitive to EGFR inhibition as were wild-type Prod1 expressing cells, indicating that mutation at this interface does not disrupt EGFR signalling. The EGFR is also observed to associate with Prod Q59A, which is unable either to activate ERK1/2 signalling or induce MMP9 expression, raising the possibilities that the EGFR either associates with Prod Q59A without becoming activated, or alternatively, that another protein essential for any ERK1/2 activation or MMP9 induction requires this interface to associate with Prod1. In light of the inability to completely inhibit Prod1 induction of MMP9 with EGFR inhibitor or with combined EGFR ErbB2 inhibition, it seems highly likely that another protein able to induce MMP9 expression in the absence of EGFR ErbB2 signalling is also associated with Prod1, and it may be the interaction with this that is perturbed by mutation of Q59. β1 integrin was thought to be a good candidate for this in light of experiments with uPAR demonstrating the involvement of β1 integrins in its regulation of MMP9 (Mazzieri, D'Alessio 2006), and transactivation of the EGFR (Liu et al., 2002). However, β1 integrin was also observed to associate with both wild-type Prod1 and Prod1 Q59A. Attempts were made to inhibit integrin activity using the snake venom disintegrin echistatin, however results were too variable to make a reliable conclusion of whether it was able to inhibit MMP9 induction (not shown). Also, inhibition of Src kinase, a known mediator of both MMP9 induction (Wei et al., 2007), ERK1/2 activated cell migration (Nguyen et al., 2000) and EGFR transactivation by uPAR via integrins (Guerrero et al., 2004), had no effect on MMP9 induction. Src independent activation of FAK, a transducer of integrin signalling, and ERK1/2, have also been reported however (Tang et al., 1998).

Mutation of Q59 and EGFR inhibition both decrease ERK1/2 phosphorylation to the level seen in GPI GFP expressing control cells, however mutation of Q59 has a much more drastic effect on MMP9 induction than EGFR inhibition. It seems unlikely therefore that it is only ERK1/2 signalling which is perturbed by Q59A mutation, indicating the role of another signalling pathway originating from an as yet unidentified Prod1 interacting protein. Also, treatment of Prod1 expressing cells with U0126 reduced ERK1/2 phosphorylation to a level greatly below that seen in GPI GFP control expressing cells, whilst the level of MMP9 expression remained considerably higher than that seen in GPI GFP controls. In light of the inability to completely inhibit Prod1 induced ERK1/2 activation with concentrations of AG1478 shown to be sufficient to inhibit completely EGF induced ERK1/2 activation, it seems likely that this other protein, in addition to activating the hypothesised ERK1/2 independent pathway, is also able to transduce a signal to ERK1/2 independently of the EGFR. Further support for this comes from the observation that mutation of residues on the α-helix also inhibits ERK1/2 phosphorylation. It is proposed then that the ERK1/2 pathway contributes ~50% to Prod1 induced MMP9 expression, with components of this coming from EGFR signalling and the hypothesised second transmembrane signal transducer, and that this hypothesised transmembrane signal transducer also activates an ERK1/2 independent pathway contributing to 50% of Prod1 induced MMP9 expression.

Stimulation of the ERK1/2 pathway with either serum or EGFR ligand containing medium was not sufficient to induce MMP9 expression, suggesting that another pathway downstream of ERK1/2 must be activated simultaneously in order for ERK1/2 to induce any MMP9 expression, consistent with the proposal that mutation of Q59, which has a considerably greater effect than that of ERK1/2 inhibition, disrupts the activation of an as yet unidentified pathway. The inability of EGFR ligand containing conditioned medium to stimulate MMP9 expression raises the possibility that Prod1 activates the EGFR in a manner distinct from that brought about by ligand. uPAR transactivation of EGFR in cooperation with integrins is shown to result in phosphorylation of EGFR residues distinct from those resulting from ligand binding (Monaghan-Benson and McKeown-Longo, 2006), however this was also shown to be Src dependent. It is acknowledged

that serum or conditioned medium may contain factors inhibitory to MMP9 expression, though this is none the less consistent with the involvement of a level of regulation able to supersede ERK1/2 activation.

4.3.2 The role of PI3K, JNK and JAK STAT pathways in Prod1 regulation of MMP9 expression

PI3K was a candidate for the second pathway (Guo et al., 2007) contributing to the induction of MMP9 expression by Prod1, however the PI3K inhibitor LY294002 showed no inhibitory effects on Prod1 induction of MMP9 expression. Instead, inhibition of PI3K showed a stimulation of MMP9 expression both in Prod1 and GPI GFP expressing cells, indicating that it constitutively represses expression of MMP9 in B1H1 cells. To a lesser extent, the same was observed with mTor, a downstream component of PI3K signalling which has been reported previously to repress MMP9 expression (Mendes Sdos et al., 2009) through its repression of ERK1/2 activity**.** This previous report showed no effect of PI3K inhibition however.

Prod1 expression was also observed to activate JNK, and similarly to ERK1/2 inhibition with U0126, its inhibition with SP600125 diminished induction of MMP9 by Prod1 by ~50%. Simultaneous inhibition of ERK1/2 and JNK did not have additive effects on Prod1 induction of MMP9 however, indicating that they do not function in independent pathways, and instead converge on some common point. One possibility is that each kinase has a specific recognition site on a downstream target, with phosphorylation at both sites simultaneously being essential for its activity. It was observed however that inhibitors of ERK1/2 and JNK decrease the phosphorylation of each others targets to a level lower than that seen in control transfected cells. Were JNK or ERK1/2 required for the activation of each other specifically in response to Prod1 signalling, inhibition of either should merely prevent the activation of the other. However, as both ERK1/2 and JNK phosphorylation is lower than the background level seen in control transfected cells under the presence of either inhibitor, this does not appear to be the case. The inhibitors appear to have a general effect on the activation of their non-target kinase rather than being specific to the mediation

of Prod1 signalling, raising the possibility that JNK and ERK1/2 require each others activity for even the basal level of activation seen in control cells. The inhibitor concentrations used were within the range of conventional usage, however to the knowledge of the author the specificity of each for their respective kinases in newt cells has not been confirmed, raising the possibility that they are directly acting on their non-target kinases. In giving consideration to the possible non-specificity of either inhibitor, in light of their similarity in molecular weight it must also be acknowledged that the antibodies used may not specifically detect the appropriate newt kinase. However, the observation that subtle differences in the level of phosphorylation are seen with the two antibodies after treatment with either inhibitor leads to the tentative suggestion that the antibodies and inhibitors are indeed specific**.** The possibility that ERK1/2 and JNK converge at some point in the pathway by phosphorylation of unique, equally essential targets is not ruled-out by the finding that both ERK1/2 and JNK inhibitors act on their non-target kinases, however it does prevent definitive interpretation of results.

The JAK/STAT pathway inhibitor AG490 also inhibited MMP9 induction in response to Prod1 expression, however simultaneous treatment with U0126 and AG490 was also not additive**,** again implying some degree of convergence of the targets of these compounds in the pathway and further indicating that another, unidentified pathway also contributes to Prod1 induced MMP9 expression. The JAK/STAT inhibitor used, AG490, inhibits JAK2 and STAT3. It has been shown that ERK1/2 (Chung et al., 1997) (Haq et al., 2002) and JNK (Turkson et al., 1999) phosphorylate serine residues on STAT3, which has been shown to enhance its activity (Wen et al., 1995), raising the possibility that the reduced MMP9 induction seen when ERK1/2 or JNK is inhibited may be due to a lack of STAT3 activation, providing a possible explanation for the observed convergence of the two pathways. Reports of an inhibitory effect of STAT3 phosphorylation by ERK1/2 (Chung et al., 1997) and JNK (Lim and Cao, 1999) also exist however. It is also known that JAK2 is able to phosphorylate ERK1/2 (Winston and Hunter, 1995), raising the possibility that the effect of AG490 may be due to reduced ERK1/2 activation resulting from JAK2 inhibition, again providing a possible explanation for the observed convergence.

Stimulation of ERK1/2 phosphorylation in response to AG490 is also reported however (Kwak et al., 2008). Conflicting reports also exist as to whether AG490 is able to inhibit EGFR autophosphorylation (Andl et al., 2004) (Osherov et al., 1993). In some experiments, AG490 was observed to mildly inhibit ERK1/2 phosphorylation in GPI GFP transfected cells (not shown) raising the possibility that rather than affecting STAT3 activity, AG490 was actually acting by reducing ERK1/2 phosphorylation either by JAK2 or EGFR kinase inhibition. The Src inhibitor PP2 also showed an inhibition of ERK1/2 phosphorylation without inhibiting MMP9 expression in Prod1 expressing cells however. In order to explain this lack of inhibition, a difference in the perdurance of ERK1/2 inhibition is posited compared to that resulting from U0126 treatment.

A model is proposed in which ERK1/2 JNK pathway activation by EGFR signalling is placed in an ancillary role in the regulation of MMP9 expression by Prod1, requiring the cooperation of a second as yet unidentified transmembrane receptor (Fig.4.13), however serves to explain how Prod1 activation of EGFR is able to bring about effects not seen with ligand activation, adding complexity to the repertoire of the processes in which it participates. The model places Prod1 in the role of an adaptor protein able to guide simultaneous activation of multiple signalling pathways through it's participation in such a multi-receptor complex, facilitating a greater diversity of outcomes than would be possible with the activation of individual pathways.

4.3.3 Future work

In order to fully understand the mechanism by which Prod1 induces MMP9 expression, the identity, or indeed existence of the hypothesised second signal transducer should be determined. A strategy based upon co-immunoprecipitation of the Prod1 signalling complex, and subsequent mass-spectrometry to identify interacting proteins would seem the obvious approach, however in light of the lack of an annotated, or even complete genome sequence this is not a trivial exercise. uPAR is known also to interact with the GPCR FRPL-1 (Resnati et al., 2002), and this, or GPCRs in general, may serve as a potential target for future investigation.

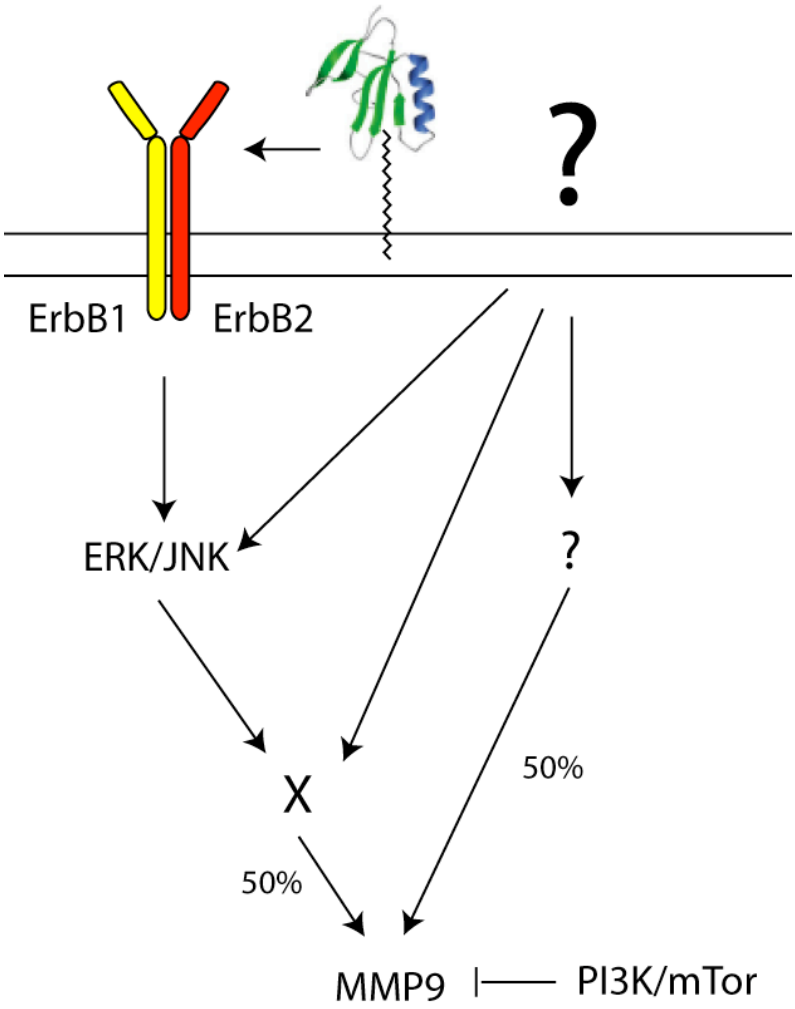


Figure 4.13 A model for MMP9 induction by Prod1

Prod1 activates ErbB1 or ErbB2 containing EGFRs by a ligand independent mechanism through direct or indirect physical association, leading to ERK/JNK MAPK pathway activation. In order for ERK1/2/JNK activation to induce MMP9 expression, 'X' must be co-activated by a signal originating from another Prod1 interacting transmembrane receptor, possibly a 7TM GPCR. The unidentified transmembrane receptor also activates ERK/JNK signalling, which in total contributes to 50% of MMP9 induction, with another independent unidentified pathway contributing the other 50%. PI3 Kinase and mTor negatively regulate MMP9 expression via a Prod1 independent mechanism.

A variety of reports, reviewed in (Carpenter, 1999) demonstrate that GPCR activation can lead to EGFR phosphorylation. This may represent an explanation as to how EGFR activation by Prod1 leads to effects distinct from those seen with EGFR ligand stimulation, through phosphorylation of the EGFR at residues not promoted by ligand binding, however the requirement for Src activity is a general theme in this mode of EGFR activation (Andreev et al.,

2001). Reports of the requirement for the cooperation of EGFR and G-proteins in promoting cell migration (Schafer et al., 2004) are interesting to consider in light of this potentially being a process regulated by Prod1 (da Silva et al., 2002) (Echeverri and Tanaka, 2005). A common approach for investigating GPCR involvement in a process is through use of pertussis toxin, which inhibits the activation of some classes of G-proteins by GPCRs. GPCRs are able to activate ERK1/2 and JNK signalling via activation of the adaptor protein Grb2 (Luttrell et al., 1997) as proposed for the hypothesised second Prod1 interacting signal transducer, as well as a number of other signalling pathways including PLC and PKC (Selvatici et al., 2006), which may represent the proposed second, ERK1/2 independent, MMP9 regulating signal transducer. PLA2 has been shown to be involved in MMP9 regulation by uPAR (Menshikov et al., 2006), and this molecule is regulated by simultaneous ERK1/2 and PKC phosphorylation (Nemenoff et al., 1993).

Q59A was seen to inhibit ERK1/2 phosphorylation to the level seen in GPI GFP expressing cells, however under the experimental conditions used, serum was present. Whether Q59A prevents Prod1 EGFR activation of ERK1/2 must be addressed using EGFR inhibitors under serum free conditions in order to determine whether the hypothesised second signal transducer also acts up-stream of ERK1/2 to inhibit ERK1/2 activation by Prod1 stimulated EGFR signalling when it is itself not activated.

Whether AG490 results in inhibition of MMP9 induction through JAK2/STAT3 signalling, or whether the effects are due to EGFR inhibition would ideally be confirmed with use of anti-phospho EGFR and anti-phospho STAT3 antibodies. Attempts were made with anti-phospho human EGFR antibodies unsuccessfully, either due to the low level of EGFR thought to be present in B1H1 cells, or due to insufficient identity of the epitope in the newt EGFR. Whether anti-STAT3 antibodies would work is unclear. Ideally, knockdown or expression of dominant negative constructs of targets identified by chemical inhibition in these studies would have followed. Each target would have to be cloned from the newt genome however, and thus far technology for gene knockdown has not been well established in the newt.

5 Axolotl Prod1

5.1 Introduction

In the previous chapters it has been shown that newt Prod1 is able to activate ERK1/2 signalling, leading to induction of MMP9 expression. These activities of Prod1 require it to be GPI anchored to the cell membrane. Axolotls are another species of salamander also studied as models of regeneration. Unlike newts, axolotls never become true adults, and instead reach sexual maturity without metamorphosing, a state termed paedomorphosis. Due to the retention of some embryonic traits, it is conceivable that axolotls may regenerate in a manner closer to that exhibited by pre-metamorphic anurans (Dent, 1962) than other adult salamanders. It is important therefore to establish whether limb regeneration in the two species occurs through the same or different mechanisms in order to be able to confidently interpret data collected in the different models. Newts and axolotls are both able to regenerate appropriately patterned limbs following amputation, and both express Prod1, indicating the likelihood of a conserved role for Prod1 in the two species. Indeed, the most compelling functional data for the role of Prod1 in limb patterning comes from the translocation of blastema cells along the proximodistal axis of the axolotl limb observed to result from overexpression of newt Prod1 (Echeverri and Tanaka, 2005).

Axolotl Prod1 has recently been isolated by P. Gates in the laboratory. Intriguingly, in contrast to newt Prod1, axolotl Prod1 is expressed as a precursor lacking the C-terminal signal peptide required for GPI anchor attachment. Extensive screening of genomic axolotl libraries consistently identified the same Prod1 gene without the C-terminal anchor attachment peptide, indicating that this is the only Prod1 molecule expressed by axolotls, and in Fig.5.1 B it can be seen that axolotl Prod1 migrates to the same position on a poly-acrylamide gel as newt Prod1 without the C-terminal anchor peptide. These observations raise the question of whether axolotl Prod1 is capable of carrying out the same functions as newt Prod1 without the requirement for GPI anchorage.

When compared to the genomes of other species, Prod1 seems most closely related to CD59 (Garza-Garcia et al., 2009), however the ongoing sequencing of expressed sequence tags (ESTs) has shown that the axolotl genome contains a three-fingered GPI anchored molecule more similar to CD59 in other species than it is to Prod1 (http://salamander.uky.edu/ESTdb/). Prod1 therefore seems to be both unique to salamanders and distinct from CD59. Prod1 has been show to interact with the EGFR, as CD59 has been shown to do (Blagoev et al., 2003), prompting investigation as to whether the similarities between Prod1 and CD59 extend to the induction of MMP9 expression.

5.2 Results

5.2.1 Characterising the axolotl Prod1 protein

Axolotl Prod1 lacks the C-terminal peptide required for GPI anchor attachment (Fig.1A), making it more similar to the inactive, ΔC-terminal Prod1 construct than the active, wild-type protein. Prod1 also lacks a C-terminal GPI anchor attachment sequence in a third salamander species more closely related to the axolotl than the newt, Ambystoma maculatum (Fig. 5.1 A).

When B1H1 cells were nucleofected with axolotl Prod1, Δ C-terminal newt Prod1 and wild-type newt Prod1, and the relative level of Prod1 released from the cell into culture medium was compared to that retained by the cell by Western-blotting it could be seen that axolotl and ΔC-terminal newt Prod1 were indistinguishable in both their apparent molecular weights and in that a high proportion of protein was observed to be in the culture medium (Fig. 5.1B). As shown previously, wild-type newt Prod1 is also found in the culture medium as well as in cell lysate. Approximately 50% of wild-type Prod1 is GPI anchored and retained efficiently by the cell, however approximately 50% of the protein is not processed and is released into the culture medium as an unanchored form with the C-terminal peptide still attached. Due to this inefficient anchor attachment to wild-type newt Prod1, similar proportions of it are found released from the cell as in the cases of ΔC-terminal newt Prod1 and axolotl Prod1.

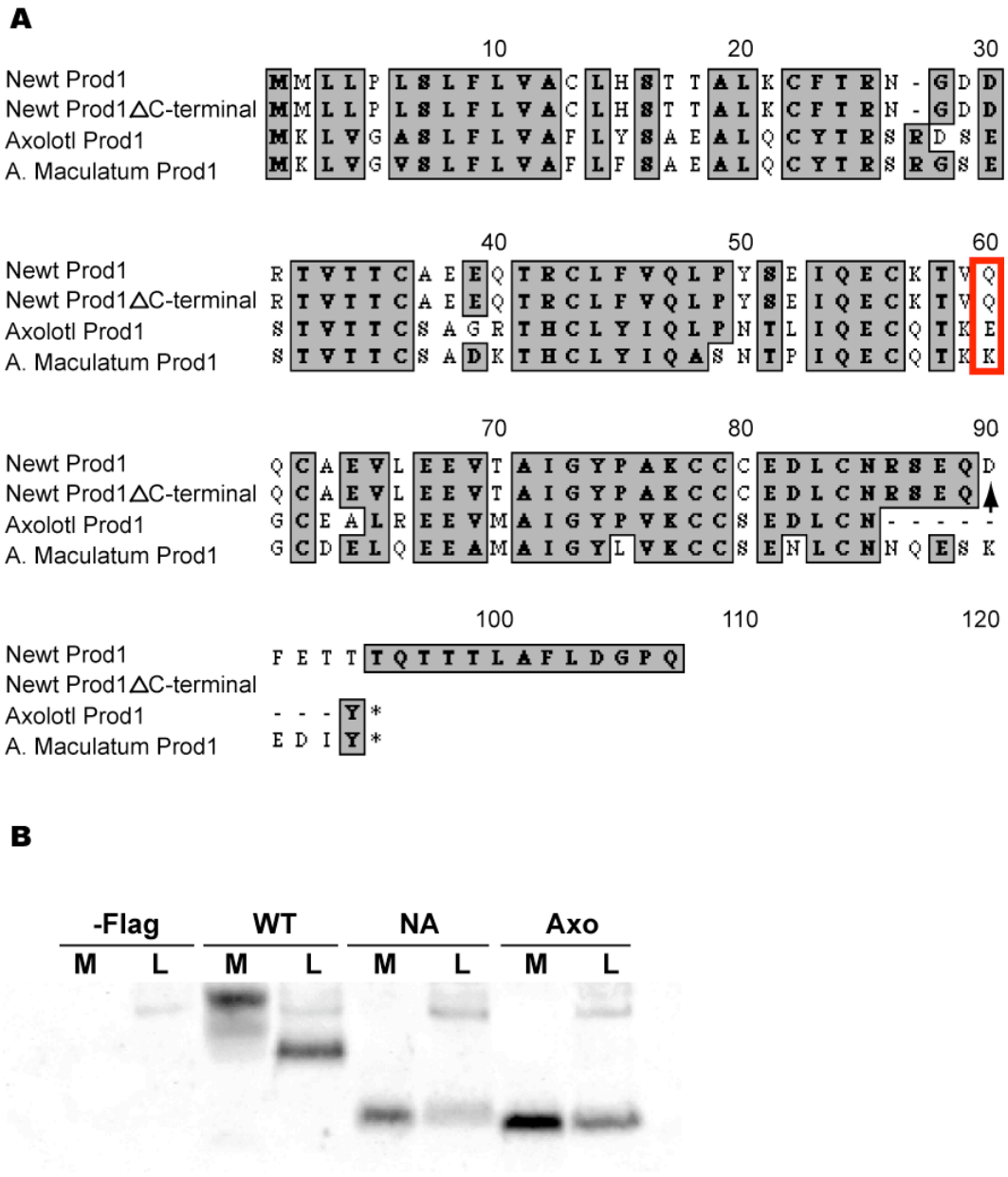


Figure 5.1 Comparison of newt and axolotl Prod1 proteins

(A) Alignment of amino-acid sequences of newt Prod1, newt Prod1 ΔC-terminal axolotl Prod1 and Ambystoma maculatum Prod1. Amino-acid 59, shown to be essential for newt Prod1 activity, is boxed. Note that this amino-acid is glutamate in the axolotl, glutamine in the newt and lysine in A. maculatum. The point of GPI anchor attachment to newt Prod1 is marked with an arrowhead. Note that in the axolotl a stop codon marked * produces a protein without a C-terminal GPI anchor attachment sequence, and that A. maculatum Prod1 has a truncated C-terminal peptide. (B) Anti-Flag Western-blot of concentrated culture medium (M) and cell lysate (L) from B1H1 cells expressing Prod1 -Flag (-Flag), newt Prod1-Flag (WT), newt Prod1 ΔC-terminal-Flag (NA) and axolotl Prod1-Flag (Axo). Equivalent proportions of total cell lysate and culture medium were loaded.

5.2.2 Characterising axolotl Prod1 activity

Whether axolotl Prod1 was more similar to the anchored or unanchored newt Prod1 was first tested by transfecting B1H1 and axolotl AL1 cells with all three Prod1 constructs and the GPI GFP control. It was observed that axolotl Prod1 induced MMP9 expression in both B1H1 (Fig. 5.2 A) and AL1 (Fig. 5.2 B) cells, and also activated ERK1/2 signalling (Fig. 5.2 C), similar to wild-type and in contrast with ΔC-terminal newt Prod1.

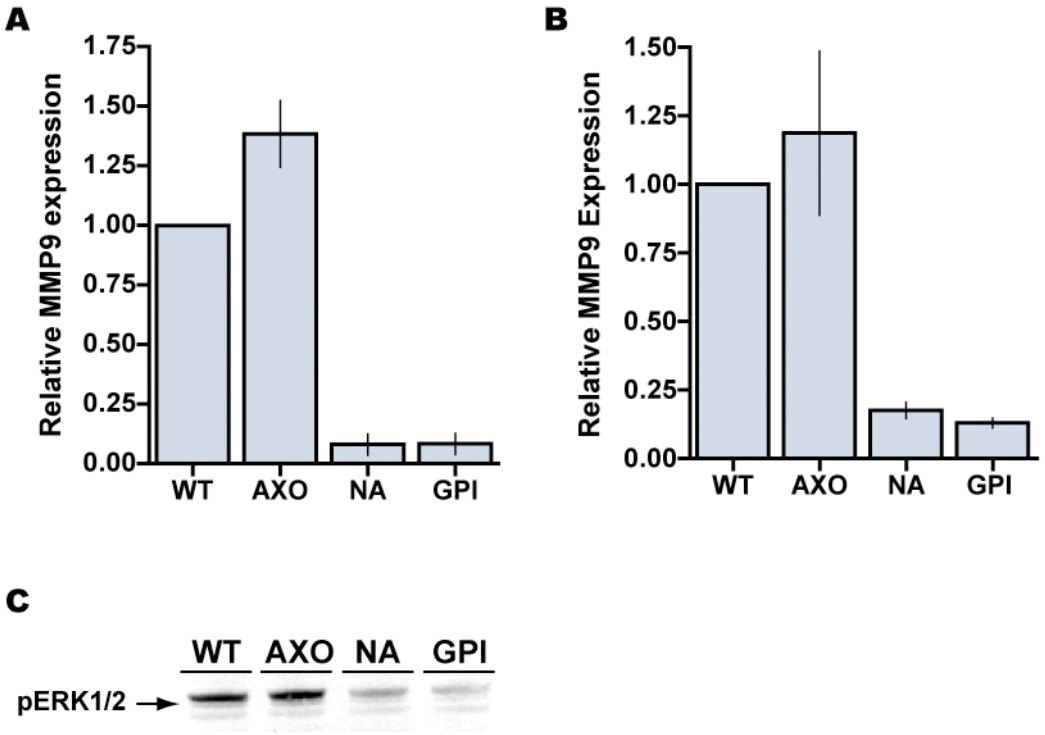


Figure 5.2 Biological activity of axolotl Prod1

(A) AL1 and (B) B1H1 cells expressing Newt Prod1 (WT), Axolotl Prod1 (Axo), newt Prod1 ΔC-terminal (NA) and GPI GFP (GPI) were serum starved for 72hr. cDNA was prepared from cells, and MMP9 expression was analysed by qRT-PCR. Results shown are the average of 4 independent experiments in (A) and 3 in (B). Values in each experiment are normalised to that seen in newt Prod1 expressing cells. Bars represent SEM. (C) Protein from axolotl cell lysate collected for cDNA preparation was Western-blotted with anti-phospho ERK1/2 antibodies. Result is from a single experiment.

In order to address the question of whether secreted axolotl Prod1 has MMP9 inducing activity when diffusing freely in the medium, cells expressing newt and axolotl Prod1 were cultured on porous membranes, sharing culture medium with untransfected cells. When compared to GPI GFP, both newt and axolotl Prod1 were seen to induce MMP9 expression in B1H1 cells as expected (Fig. 5.3 A), however those cells sharing culture medium with Prod1 expressing cells showed no significant elevation of MMP9 expression when compared to GPI GFP control expressing cells (Fig. 5.3 B). Similar results were seen in axolotl cells (not shown).

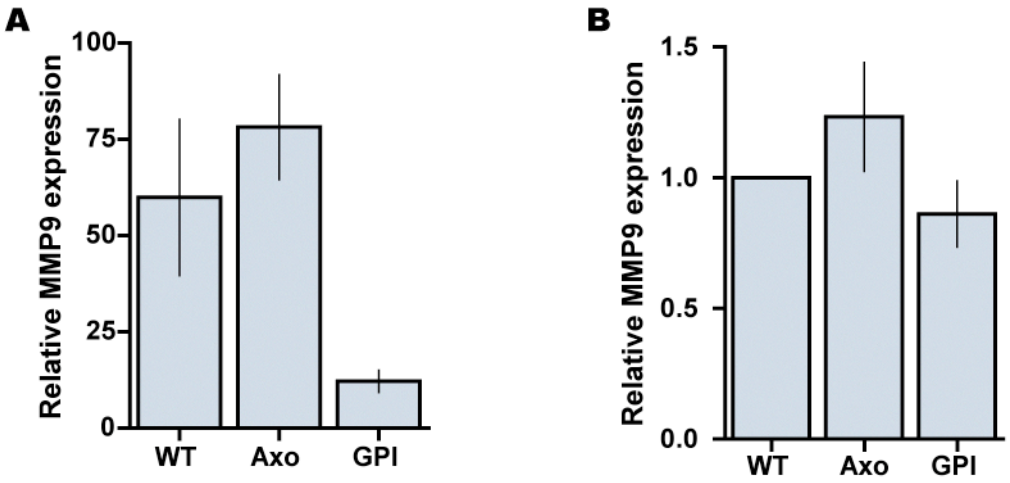


Figure 5.3 Lack of MMP9 induction by soluble Prod1

(A) B1H1 cells expressing newt Prod1 (WT), axolotl Prod1 (Axo) and GPI GFP (GPI) were plated on a porous membrane, sharing medium with corresponding untransfected B1H1 cells in (B). Cells were serum starved for 72hr, cDNA was prepared and MMP9 expression was analysed by qRT-PCR. Results shown are the average of 3 independent experiments. Values in each experiment are normalised to that of cells sharing medium with Prod1 expressing cells. Note the difference in the Y axis between A and B due to non-specific MMP9 induction resulting from the transfection method. Bars represent SEM.

5.2.3 Mutation of axolotl Prod1 activity

Axolotl Prod1 has the amino-acid glutamate at residue 59 (Fig. 5.1 A), the position on the structure of newt Prod1 shown previously to be essential for its MMP9 inducing activity. Newt Prod1 has a glutamine at this position, prompting investigation of whether this amino-acid is also essential for axolotl Prod1

activity. Glutamate 59 was substituted for either alanine or glutamine, and it was observed in newt cells transfected with these constructs that either substitution abolished both the ERK1/2 activating (Fig. 5.4 A) and MMP9 inducing (Fig. 5.4 B) activity of axolotl Prod1. These substitutions also abolished the MMP9 inducing (Fig. 5.4 C) activity of axolotl Prod1 in axolotl cells. The expression of both axolotl Prod1 mutants was confirmed to resemble that of the wild-type axolotl Prod1 by Western-blotting cell lysates and cell culture medium of B1H1 cells transfected with the various Prod1 constructs (Fig. 5.4 D).

5.2.4 Characterising axolotl CD59 activity

CD59 is another GPI anchored member of the three-fingered protein superfamily. The three-fingered fold is adopted by proteins with diverse amino-acid sequences, and indeed even orthologs may share only limited identity (Galat, 2008). When initially identified, Prod1 was classified as the newt ortholog of CD59 (da Silva et al., 2002) as this is the mammalian protein most closely related to Prod1 based upon amino-acid identity and alignment of cysteine residues responsible for maintaining the integrity of the three-fingered fold (Fig. 5.5 A). Prod1 was also predicted to share structural motifs with CD59. Subsequent identification (http://salamander.uky.edu/ESTdb/) and cloning (P. Gates, unpublished) of an axolotl protein more similar to CD59 in other genomes than it is to Prod1 cast doubt on this classification however, indicating that whereas axolotls, and possibly salamanders in general, express CD59 and Prod1, other species express only CD59. B1H1 cells were transfected with axolotl CD59 and newt Prod1, and expression of MMP9 was compared to that seen in GPI GFP expressing cells. It was observed that expression of CD59 in B1H1 cells was unable to induce MMP9 expression (Fig. 5.5 B). Protein expression of CD59 in B1H1 cells was confirmed by Western-blotting (Fig. 5.5 C).

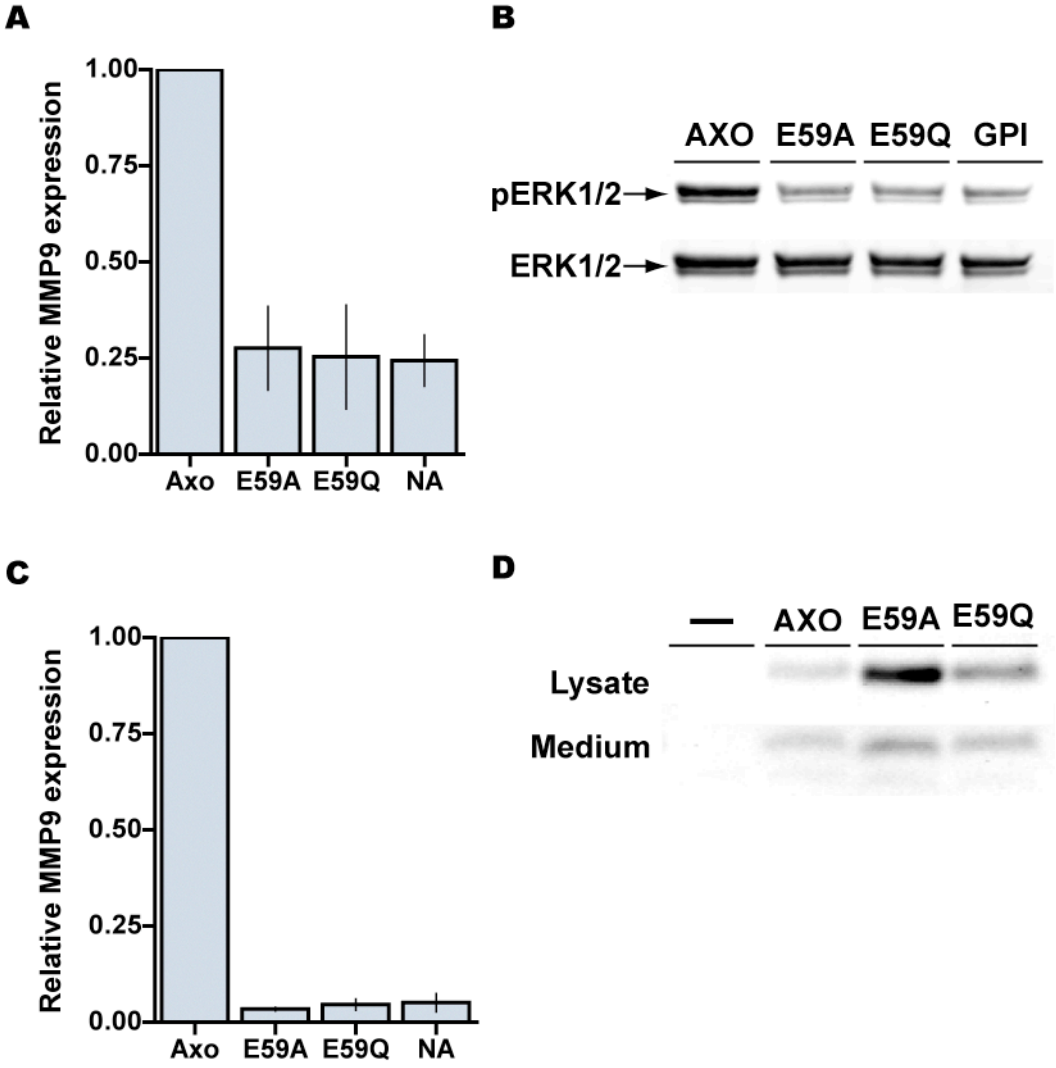


Figure 5.4 Mutation of axolotl Prod1 α-Helix

(A) B1H1 and (C) AL1 cells expressing axolotl Prod1 (Axo), axolotl Prod1 E59A (E59A), axolotl Prod1 E59Q (E59Q) and Prod1 ΔC-terminal (NA) were serum starved for 72hr. cDNA was prepared from cells, and MMP9 expression was analysed by qRT-PCR. Results shown are the average of 4 independent experiments in (A) and 3 in (C). Protein from B1H1 cell lysates collected for cDNA preparation was Western-blotted with anti-ERK1/2 antibodies and anti-phospho ERK1/2 antibodies (B). (D) Anti-Flag Western-blot of cell lysate and culture medium from untransfected (-), axolotl Prod1-Flag (Axo), axolotl Prod1-Flag E59A (E59A) and axolotl Prod1-Flag E59A (E59Q) expressing B1H1 cells.

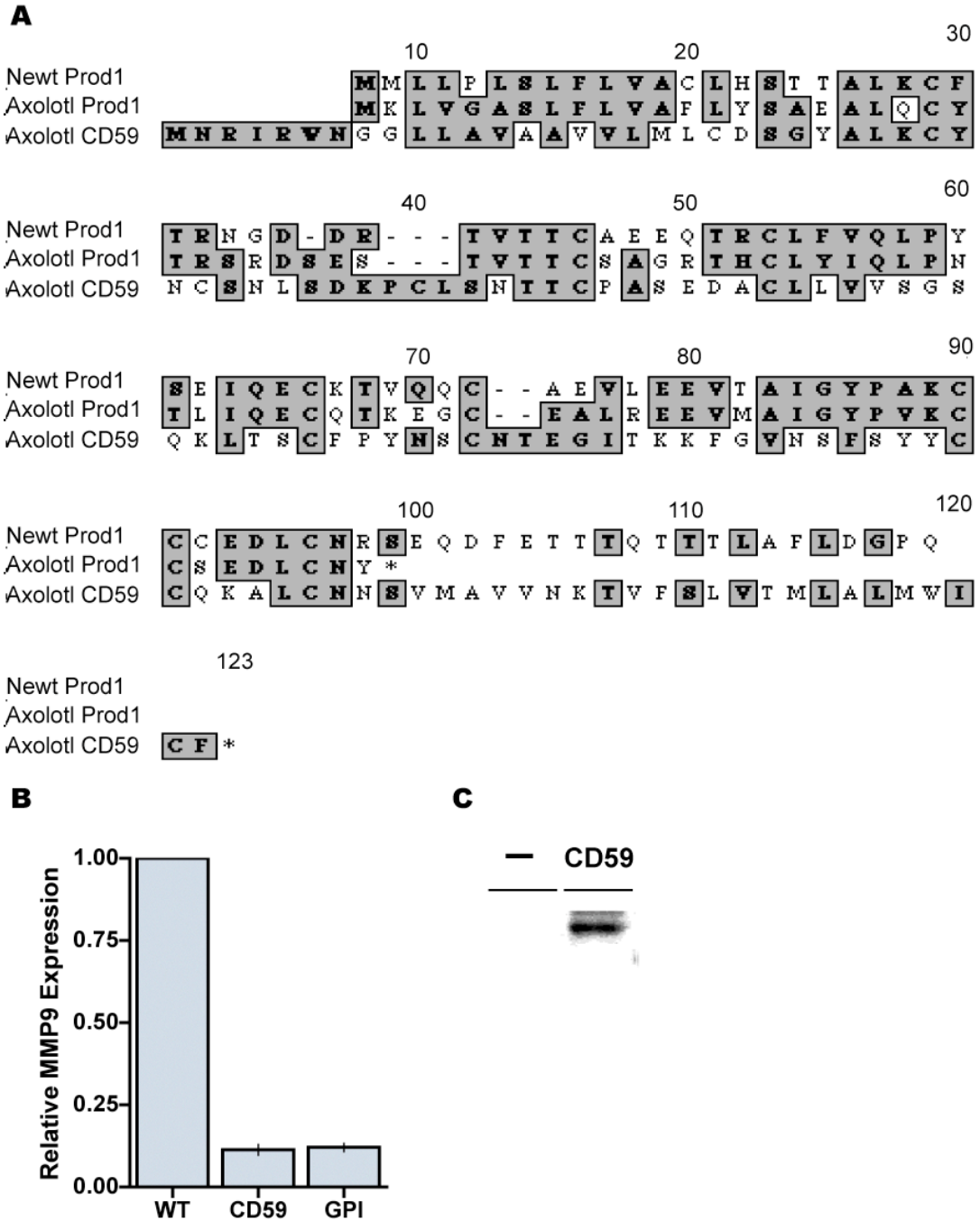


Figure 5.5 Lack of MMP9 induction by CD59

(A) Alignment of Prod1 and CD59 amino acid sequences. (B) B1H1 cells expressing newt Prod1 (WT), CD59 (CD59), and GPI GFP (GPI) were serum starved for 72hr. cDNA was prepared from cells, and MMP9 expression was analysed by qRT-PCR. Results shown are the average of 9 independent experiments. Values in each experiment are normalised to that seen in Prod1 expressing cells. Bars represent standard error of mean. (C) Anti-Flag Western-blot of cell lysate from untransfected and B1H1 cells expressing CD59-Flag.

5.3 Discussion

5.3.1 Comparing Prod1 from newt and axolotl

Axolotl Prod1 expression is seen to initiate ERK1/2 signalling and induce MMP9 expression, as is seen with newt Prod1, however, distinct from newt Prod1, it does so without the requirement for GPI anchoring to the cell membrane. A significant proportion of axolotl Prod1 is released extracellularly, however this freely diffusing form of axolotl Prod1 is unable to induce MMP9 expression. That it is inactive when freely diffusing is at first surprising, as the lack of an anchor suggests that it does not require membrane tethering to function. However, it is plausible that in order to activate ERK1/2 signalling and induce MMP9, axolotl Prod1 must become associated with another membrane-tethered protein prior to reaching the cell membrane. The presumption is that this would take place in the secretory pathway, and that unanchored newt Prod1 is unable to make this essential association, and is thus unable to become appropriately positioned at the cell membrane. In support of such a difference between unanchored newt and axolotl Prod1 is the observation that substitution of amino acid 59 on the α-helix of axolotl Prod1 from glutamate to glutamine, as seen in newt prod1, abolishes its activity, indicating that there is some significance to the difference seen between the α-helices of the two orthologs. Such a line of argument suggests that the association between newt Prod1 and the essential signal transducing protein or protein complex is significantly weaker than that of axolotl Prod1, and therefore membrane tethering is required in order to decrease its degrees of freedom and promote their encounter and association, whereas the affinity of axolotl Prod1 is sufficiently high as to occur to a significant extent without the requirement for restricted mobility.

Domains 2 and 3 of uPAR are actively shed from the cell membrane by regulated enzymatic cleavage by uPA, which activates downstream signalling via the G-protein coupled receptor FPRL, bringing about cellular effects distinct from those seen when it is tethered to the membrane (Mazzieri et al., 2006). Freely diffusing uPAR domains 2 and 3 act as a chemoattractant, promoting cell

migration through interaction with FPRL (Resnati et al., 2002), a function which Prod1 may also promote in light of evidence from blastema confrontation assays (da Silva et al., 2002) and Prod1 overexpression in blastema cells (Echeverri and Tanaka, 2005). Significant proportions of newt and axolotl Prod1 are also released from the cell, raising the question of whether these secreted forms of the protein do have some other function distinct from MMP9 regulation, or whether they merely represent a wholly inactive, inappropriately localised fraction of the total Prod1 expressed by the cell.

In contrast to B1H1 cells, axolotl cells do express Prod1, as shown by qRT PCR (not shown). It would seem to be the case therefore that in the axolotl cells used in the experiments, Prod1 signalling is not at its maximal level, and has significant potential for increasing in response to further Prod1 overexpression. Due to the method used to quantify the relative increase in MMP9 mRNA levels in response to Prod1 expression, the difference in the increase in the absolute level of transcripts between axolotl cells compared to newt cells which express no Prod1 cannot be stated.

5.3.2 The evolution of Prod1

The axolotl and the newt diverged as salamander species approximately 95 million years ago (Steinfartz et al., 2007),raising the possibility that newt and axolotl Prod1 may have independently evolved the capability to activate ERK1/2 signalling and induce MMP9 expression after the divergence of the two species. The belief that Prod1 is critical to limb regeneration, coupled with the widespread ability of salamanders to regenerate limbs, indicates it to be more likely that the properties of Prod1 seen in newt and axolotl have been maintained from those present in the common ancestral salamander however. Also, it was observed that in terms of ERK1/2 activation and MMP9 induction, both newt and axolotl Prod1 function equivalently in newt and axolotl cells, indicating a common mechanism of function for the protein. Prod1 has recently been identified in the salamander Ambystoma maculatum (P. Gates, unpublished), further indicating its presence in all regenerating salamanders

and origin in a common ancestor, however the activity of this molecule has not yet been investigated.

Although it is unsurprising that the nucleotide sequence of Prod1 should show divergence between species, that the orthologs should differ in a property as significant as membrane anchorage, whilst retaining at least some of the same functions is intriguing. The most plausible explanation for the lack of a requirement for a GPI anchor exhibited by axolotl Prod1 is that it acquired some change which released selective pressure for its direct attachment to the cell membrane in order to function, leading to acquisition of the non-sense mutation observed in the sequence 5' of the C-terminal anchor attachment peptide. That axolotl Prod1 functions equivalently in both newt and axolotl cells indicates that it is indeed a change to the protein itself rather than a difference between the proteins with which it interacts with in the two species. The difference in amino-acid 59/60 seen between the two orthologs, shown to be essential for the functioning of axolotl Prod1 without an anchor, indicates that it may be changes to the α-helical region of the protein that preceded the loss of the GPI anchor. Although the opposite is possible, that newt Prod1 acquired the property of GPI anchorage by addition of a functional anchor attachment peptide at the C-terminal, and subsequently altered in such a way as to be unable to function without it, evolutionarily this makes less sense.

Ambystoma maculatum Prod1 is more similar to Prod1 found in the axolotl Ambystoma mexicanum than to newt Prod1, which is unsurprising in light of their common genus, however there are a number of interesting differences between them. The stop codon resulting in a truncation of the C-terminal GPI anchor attachment peptide of the protein is in a different position, indicating that both Ambystoma species lost their GPI anchors at different points in evolution, subsequent to their divergence from one another. The degree of similarity between the α-helical region of the two Ambystoma Prod1 proteins, shown in the newt to be essential for MMP9 inducing activity, is greater than when either is compared individually to newt Prod1, which is again not highly surprising, however a number of amino-acids do differ, of particular note, amino-acid 59,

which in A. maculatum, rather than being a glutamine as in the newt or a glutamate as in the axolotl is instead a positively charged lysine. This is particularly interesting in light of the fact that the negatively charged glutamate has been shown to be essential for the function of unanchored axolotl Prod1, and cannot be substituted by the polar glutamine found in anchored newt Prod1. The presumption is that A. maculatum Prod1 is functional as an unanchored protein, raising the question of how such a difference in charge at such an essential amino acid is tolerated. Both Ambystoma species seem to have lost their GPI anchors subsequent to their divergence which, based upon previous reasoning, suggests that prior to anchor loss, distinct changes occurred after divergence permitting function without an anchor.

5.3.3 The three-fingered family of proteins

Being largely constrained only by the disulphide bonding between cysteine residues to form the 'palm' of the three-fingered domain, the secondary structures adopted by the fingers are flexible to evolve, facilitating the creation of diverse protein-protein interaction interfaces. Due to this, the three fingered proteins are a particularly diverse family at the sequence level (Galat, 2008) and comparison of protein structure provides a more informative means of categorisation of the members of the family. Such a structural categorisation indicates that uPAR domain 3 and CD59 are the most closely related domains found in species other than salamanders, (Garza-Garcia et al., 2009) all of which exhibit an α-helix at finger 3. The GPI anchored domain 3 of uPAR has been shown to be responsible for the interaction with $\alpha 5\beta 1$ integrin leading to ERK1/2 activation following fibronectin binding (Chaurasia et al., 2006) which has been shown to result in Src dependent MMP9 induction (Wei et al., 2007). Given the similarities in protein structure and downstream effects it is therefore surprising that Prod1 and uPAR do not seem to share integrin mediated Src activation in their mechanisms.

The three-fingered protein family has been shown to have undergone a large expansion in salamanders (pers. com. R.Voss to J.Brockes) and based upon sequence, Prod1 groups most closely with a group of pheremone proteins

named the plethodontid modulating factors (PMF) (Garza-Garcia et al., 2009), which are found only in lungless salamanders. The structures of these PMFs do not contain the α-helical region shared by Prod1, CD59 and uPAR shown to be essential for Prod1s MMP9 inducing activity however, and therefore structurally, and most likely functionally, Prod1 is more similar to CD59 and uPAR. Taken together with the absence of Prod1 from the genomes of all non-salamander species, these points indicate that Prod1 evolved after the divergence of the salamander lineage from other species, and that it, uPAR and CD59 independently evolved the structural features they share, and their ability to interact with the 'older', evolutionarily more conserved EGFR. Results presented in Chapter 4 indicate however that it is not the shared α-helix that facilitates Prod1 interaction with the EGFR, which seems a strange co-incidence in light of these features being common to CD59, uPAR and Prod1.

In this study Prod1 has been shown to regulate the expression of MMP9, as does uPAR, however CD59 was not observed to do so, despite reports that it interacts with the EGFR. It can be seen clearly from the amino-acid sequences of the two proteins that there are significant differences between Prod1 and CD59 however, and taken in light of the evidence in Chapter 4 that EGFR interaction alone is not sufficient even for Prod1 to induce MMP9, the inability of CD59 to induce MMP9 is likely to be due to its inability to interact with the full repertoire of proteins required to bring about this effect.

5.3.4 Implications for the role of GPI anchors in cell-signalling

An interesting point raised by the lack of requirement for the GPI anchor relates more generally to the broader field of GPI anchored protein biology. Reports exist that GPI anchoring plays a functional role in localising proteins to 'lipid rafts', reviewed in (Helms and Zurzolo, 2004) (Simons and Toomre, 2000) and that substitution of the lipid anchor for a proteinaceous anchor can disrupt their function (Robinson et al., 1989). It has also been suggested that NRTKs such as those of the Src family associate specifically with the inner leaflet of 'lipid rafts' (Simons and Toomre, 2000), and that they somehow transduce a signal initiated by clustering of GPI anchored proteins in the outer leaflet of the

membrane (Murray and Robbins, 1998), however as shown in Chapter 4, Src family kinases are not involved in the transduction of the Prod1 signal leading to induction of MMP9 expression. That axolotl Prod1 functions in the absence of a GPI anchor indicates that this somewhat mysterious mode of coordinating signal transduction is not in operation and that axolotl Prod1 at least is appropriately localised by a means other than through the association of lipids.

6 A primary culture model of MMP9 activity

6.1 Introduction

In the preceding chapters Prod1 expression has been shown to regulate the expression of MMP9 in cultured limb cells, but the significance of this for the processes occurring in the blastema during limb regeneration remains to be addressed. MMP9 is known to be upregulated in the skin and the wound epithelium following injury, and the persistence of MMP9 expression is one characteristic of whether wound-healing or limb regeneration results (Satoh et al., 2008). Furthermore, the processes of wound healing and the formation of the wound epithelium essential for blastema formation and subsequent regeneration both involve the migration of epithelial cells. Prod1 is expressed at a significantly higher level in the dermis (A.Kumar, unpublished) compared to other limb tissue, so with all these points in mind, a culture model to study MMP9 regulation in the skin was developed. Although a large upregulation of MMP9 was observed in this model, it appeared not to depend on the pathways delineated earlier in this thesis.

6.2 Results

6.2.1 Locating the site of Prod1 and MMP9 expression in limb tissue

It is known that RA both proximalises the regenerating limb and upregulates Prod1 in the blastema (da Silva et al., 2002). More recently, it has been shown that upregulation of Prod1 by RA is more pronounced in the dermis compared to the underlying blastemal tissue (Kumar et al., 2007a). Prod1 gene expression was also shown to be markedly higher in the dermis compared to in muscle/mesenchyme (Fig. 6.1 A) of intact limbs, although Prod1 expression in proximal dermis was not observed to be any higher than in distal dermis. Two independent normalising probes were used when comparing skin to mesenchyme (for a detailed discussion of normalisers, see Materials and Methods). Immunofluorescence labelling also showed that the level of Prod1 protein expression was markedly higher in the dermis of intact limbs compared to other tissues (Fig. 6.1 B, courtesy of A. Kumar). In order to investigate whether induction of MMP9 gene expression correlated spatially with the high

Prod1 expression seen in the dermis, a protocol was developed to remove the epidermis from skin patches (see Materials and Methods). The structure of skin prior to treatment with trypsin clearly shows the epidermis and dermis (Fig. 6.1 C.a, courtesy of A. Kumar). Subsequent to trypsin treatment, only the dermis remains (Fig. 6.1 C.b, courtesy of A. Kumar). Skin patches were explanted (see Materials and Methods), and the MMP9 expression in the dermis after 24hr of culture was compared to that seen in patches 1hr after removal from the limb (Fig. 6.1 D), and was observed to be highly upregulated following the culture period. MMP9 induction was observed in patches plated on collagen substrate, and to a lesser extent, patches cultured in suspension. MMP9 expression was also seen to be upregulated in a piece of skin with the epidermis removed prior to culture (Fig. 6.1 E), indicating that the dermis is necessary and sufficient for the response.

6.2.2 Investigating the role of MMP9 in migrating skin cells

Patches of limb skin were explanted into culture and within 12hr cells were observed to migrate out from patches, a process that proceeded for approximately 96 hr, after which migration ceased. The identity of these migrating cells was analysed with antibodies against cytokeratin, a marker of epithelial cells (Fig. 6.2 A.a, courtesy of A. Kumar) and vimentin, a marker of mesenchymal cells (Fig. 6.2 A.b, courtesy of A. Kumar). The epithelial origin of a significant proportion of cells was indicated by their cytokeratin expression however all cells also expressed vimentin. MMP9 gene expression in migrating cells was observed at various time points after plating by in-situ hybridisation, as described in Materials and Methods. A representative sample is shown (Fig. 6.2 B). In order to test whether MMPs are involved in this migration process, skin patches were plated in the presence or absence of GM6001, a broad spectrum MMP inhibitor known to inhibit limb regeneration (Vinarsky et al., 2005). Minimal cell migration was observed in the presence of the inhibitor compared to controls, and once the inhibitor was removed, migration proceeded as normal (Fig. 6.2 C).

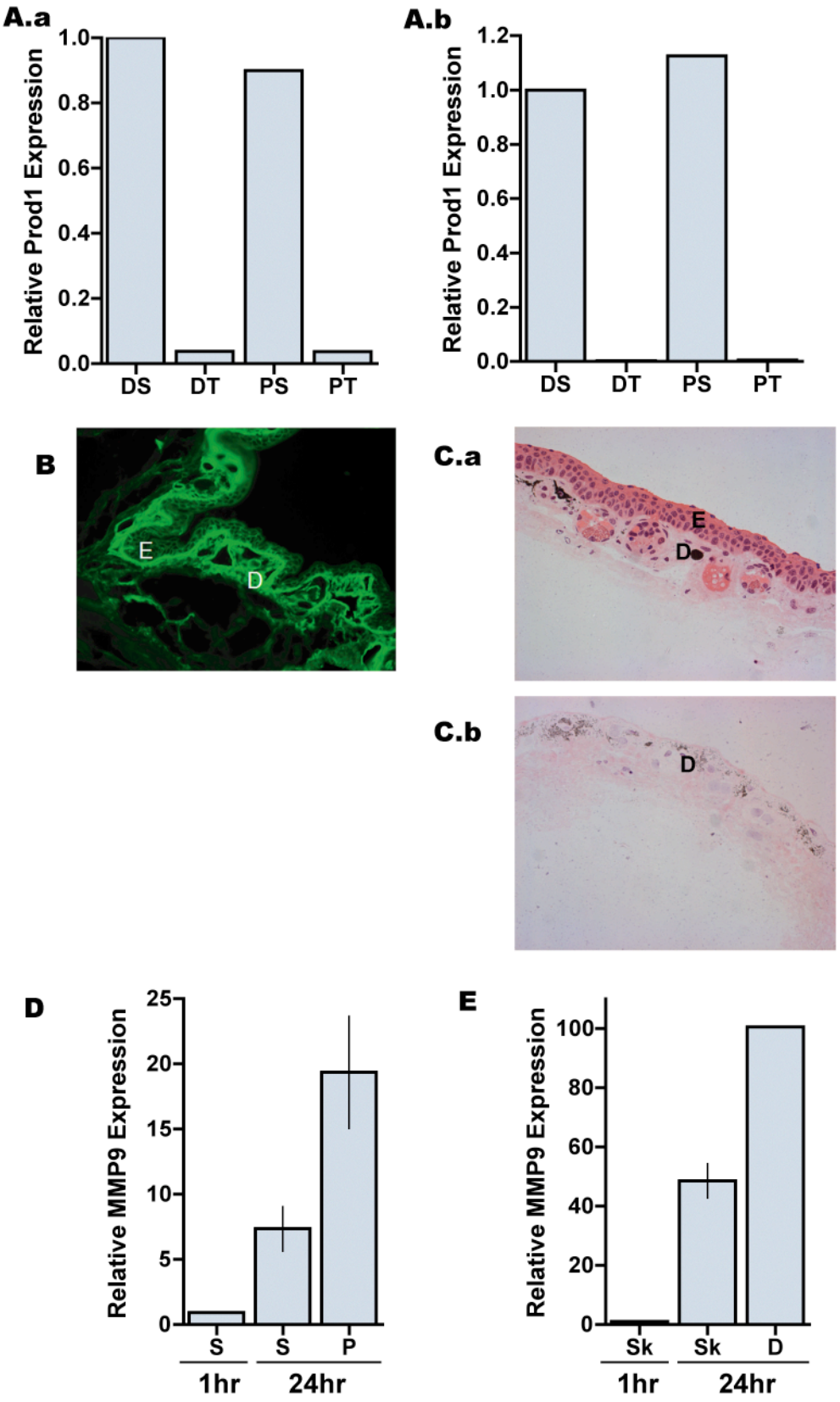

A.a
Relative Prod1 Expression
1.0
0.8
0.6
0.4
0.2
0.0
DS
DT
PS
PT
A.b
Relative Prod1 Expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
DS
DT
PS
PT
B
E
D
C.a
E
D
C.b
D
D
Relative MMP9 Expression
25
20
15
10
5
0
S
S
P
1hr
24hr
E
Relative MMP9 Expression
100
80
60
40
20
0
Sk
Sk
D
1hr
24hr

Figure 6.1 Expression of Prod1 and MMP9 in limb skin

(A) Expression of Prod1 in distal skin (DS), distal limb tissue (DT), proximal skin (PS) and proximal tissue (PT) was determined by qRT-PCR. cDNA was prepared from pooled samples of skin or tissue from 6 limbs. Values are relative to that obtained for distal skin. Prod1 expression is normalised to EF1α in (Aa) and GAPDH in (Ab). (B) Sagittal section of newt limb stained with anti-Prod1 antibody. Epidermis is marked (E), dermis (D). Note the pronounced Prod1 signal in the dermis. (C) A technique for complete removal of the epidermis from the underlying dermis was developed (See Materials and Methods). Hematoxylin eosin stain shows the presence of the epidermis (E) and dermis (D) in (C.a) prior to processing, and the presence of only the dermal layer in (C.b) after processing. (D) Skin was cultured for 1hr or 24hr in suspension culture (S), or plated on collagen (P). Epidermis was subsequently removed and expression of MMP9 in the dermis was determined by qRT-PCR. Results shown are the average of two independent samples. (E) Skin (Sk) (i.e. dermis plus epidermis) was cultured for 1hr in suspension culture or 24hr plated on collagen, or dermis alone was cultured for 24hr plated on collagen (D). Epidermis was subsequently removed and the expression of MMP9 in the dermis was determined by qRT-PCR. Results shown are the average of two independent samples for Sk and from one sample for D. In (C) and (D) values are relative to that seen in one of the patches cultured for 1hr. GAPDH was used as a normalising gene. Bars represent SEM.

6.2.3 Inhibiting Prod1 signalling in skin patches

The inhibitors shown in Chapter 4 to be effective at inhibiting Prod1 induced MMP9 expression in B1H1 cells were used to investigate the role of the Prod1/EGFR/ERK1/2 pathway in the regulation of MMP9 expression in skin patches. Cells were treated with the EGFR inhibitor PD153035 (Fig. 6.3 A), simultaneously with PD153035 and the ErbB2 inhibitor AG879, the ERK1/2 kinase inhibitor U0126, the JAK/STAT inhibitor AG490, and the Wnt pathway inhibitor Dickkopf1 (Fig. 6.3 B), and simultaneously with U0126, AG490, PD15305 and AG879 (Fig. 6.3 C). Wnt signalling was targeted as it is known to activated by skin injury and inhibition of this pathway by DKK has previously been shown to inhibit regeneration in axolotl, Xenopus and zebrafish by disrupting the formation of the apical ectodermal cap (Kawakami et al., 2006). Inhibition of EGFR signalling clearly showed no effect on MMP9 expression in the skin patches, neither did inhibition of Wnt signalling.

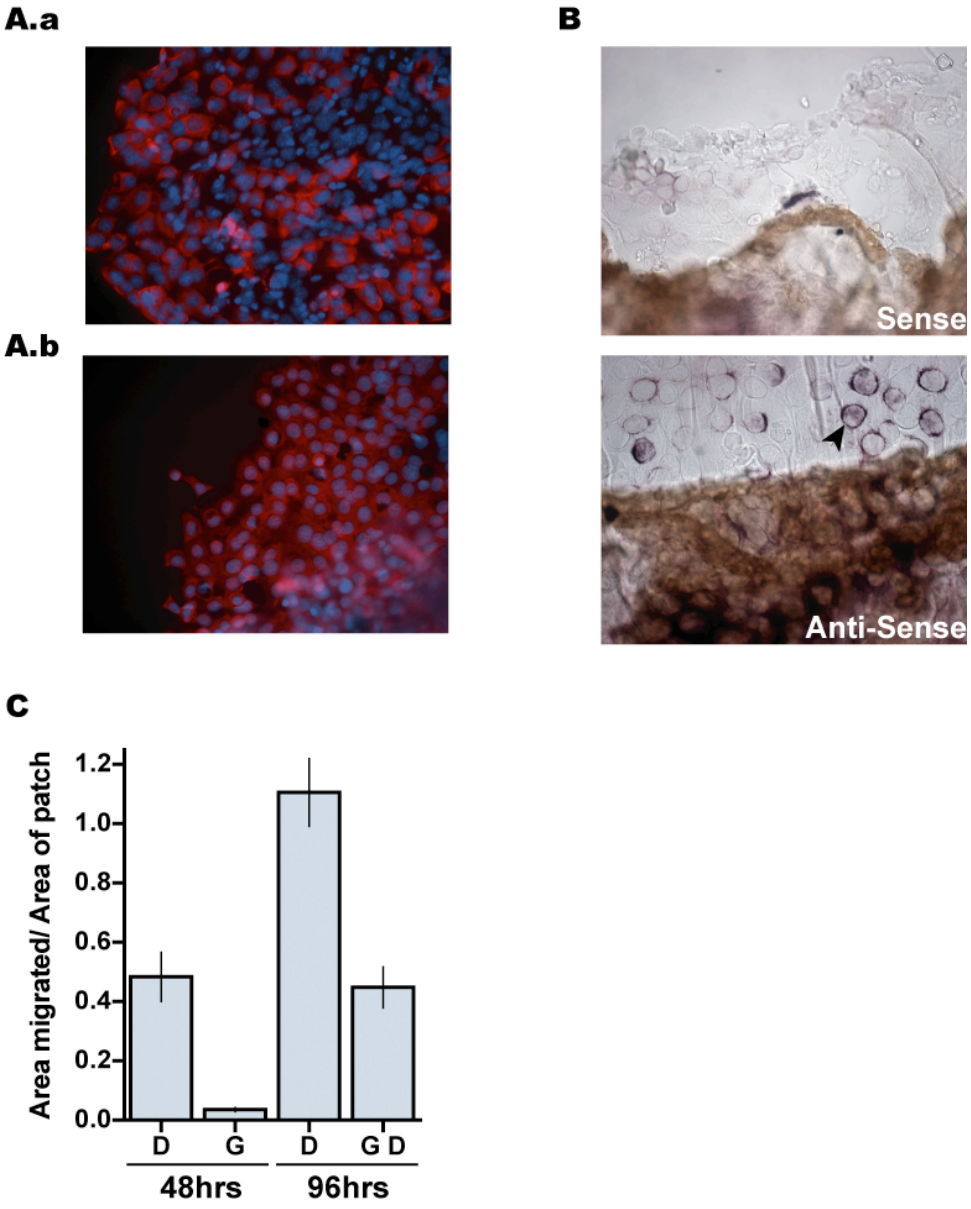


Figure 6.2 MMP9 expression in migrating cells

(A) Immunofluorescence showing cytokeratin (A.a) and vimentin (A.b) expression in cells migrating from an explanted skin patch. Nuclei are stained blue with Hoechst stain. (B) MMP9 expression in cells migrating from skin patches shown by in-situ hybridisation. The skin explant shown was fixed 48hr after culture on collagen substrate and is representative of samples fixed after 24-96hr in culture. Black arrowhead shows a migrating cell with perinuclear localisation of the anti-sense probe. (B) Skin explants were cultured for 48hr in the presence of DMSO (D) or GM6001 (G) and photographed. GM6001 was then substituted for DMSO and explants were cultured for a further 48 hr (48hr+48hr=96hr) and photographed. The area of the sheet that had migrated out from the patch at 48hr or 96hr was compared to the area of the patch itself and expressed as a ratio. Bars represent SEM. Note that little migration occurs in the presence of GM6001, however proceeds at the normal rate after its removal.

Surprisingly, there was some indication that treatment with AG490, and to a lesser extent with U0126, enhanced MMP9 induction in skin patches after 24hr, however the variability between results observed in different patches makes interpretation difficult. A similar effect was observed following treatment with a combination of inhibitors including AG490 and U0126.

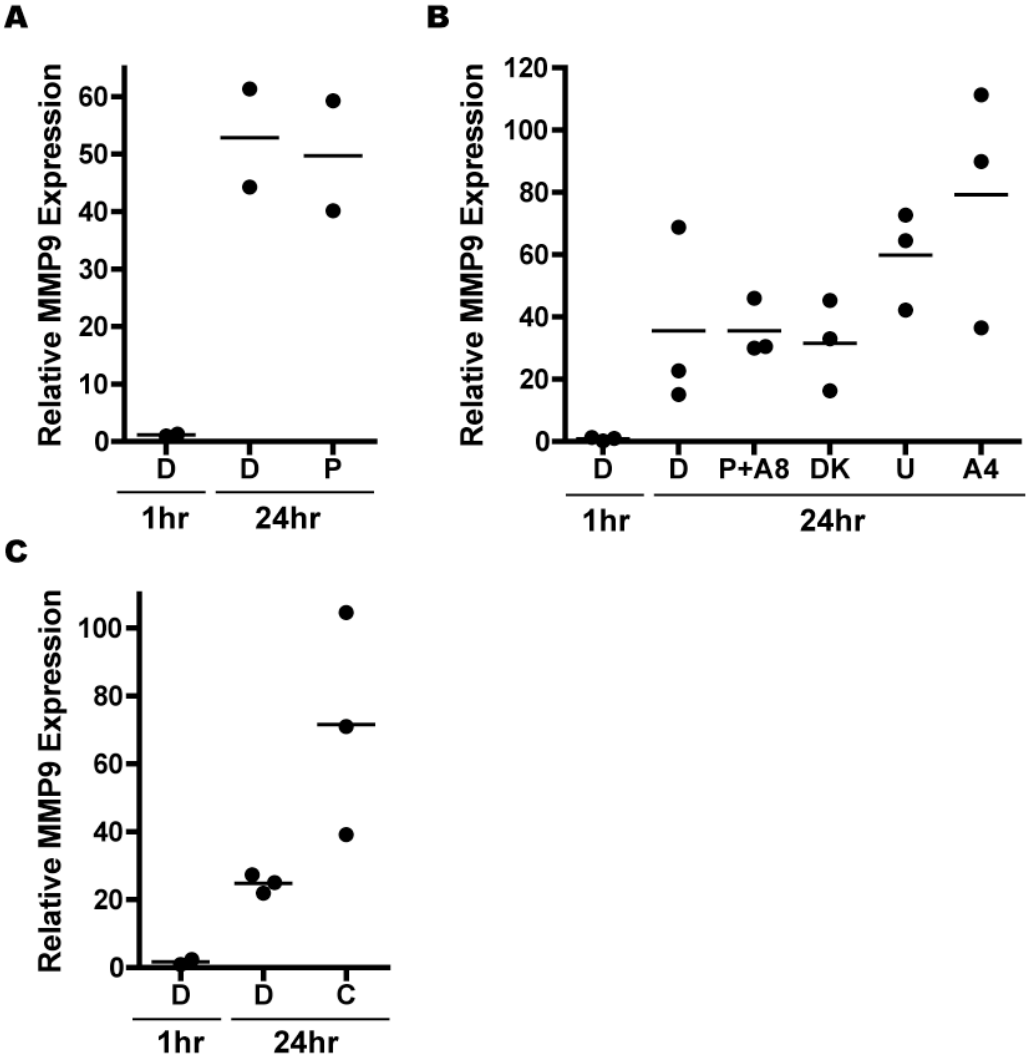


Figure 6.3 Effect of signalling pathway inhibitors on MMP9 expression in the dermis of skin patches

Skin (i.e. dermis plus epidermis) was cultured for 1hr in suspension culture or 24hr plated on collagen in the presence of (A) DMSO [D] or PD153035 50uM [P], (B) DMSO [D], PD153035 25uM + AG879 10uM [P+A8], DKK1 4ug/ml [D], U0126 30uM [U] or AG490 50uM [A4] or (C) DMSO [D] or a combination of PD153035 25uM, AG879 5uM, U0126 10uM and AG490 20uM [C]. After the culture period, epidermis was removed and the expression of MMP9 in the dermis was determined by qRT-PCR. Values are relative to that seen in one of the patches cultured for one hour in the presence of DMSO.

A peptide of sequence VQQCAEVLEEVTAI**,** representing the amino-acids constituting the α-helix of Prod1, was used in an attempt to block MMP9 induction by Prod1 in a similar approach to one employed to disrupt the interaction of uPAR and α3β1 integrins, thereby inhibiting downstream effects (Ghosh et al., 2006). It was hypothesised that as amino-acids on the α-helix of Prod1 had been shown to be essential for MMP9 induction by Prod1, such a peptide should compete with Prod1 for binding to its essential transmembrane signal transducing partner protein and prevent the formation of a functional signalling complex. The effect of the peptide on MMP9 induction resulting from Prod1 expression in B1H1 cells was investigated, but under the experimental conditions employed no effect of its inclusion in culture medium was observed (Fig. 6.4).

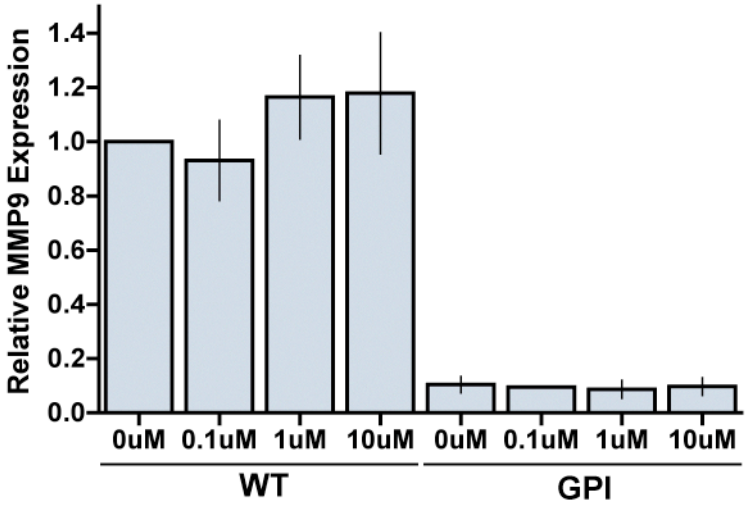


Figure 6.4 Effect of Prod1 α-helix peptide on MMP9 expression

B1H1 cells expressing either Prod1 (WT) or GPI GFP (GPI) were cultured in serum free medium containing 0.1% BSA in the presence of the indicated concentration of a polypeptide representing the amino-acid sequence of the α-helix of Prod1. After 72hr the expression of MMP9 was determined by qRT-PCR. Results shown are the average of three independent samples for 1uM and 10uM and two for 0.1uM. Bars represent SEM. Note that under the experimental conditions used, the peptide had no effect on MMP9 expression in either Prod1 or GPI GFP expressing cells.

6.2.4 Investigating the MMP9 inducing signal following injury

In the response to injury, the behaviour of cells at the wound edge can be considered distinctive in view of their boundary location. In order to gain some insight into the mechanism initiating the induction of MMP9 following explantation into culture, the role of the cells at the edge of the patch with reduced lateral cell-cell association was investigated. Patches of skin were cut into quarters, doubling the length of the exposed edge and the number of cells not fully surrounded by others in the lateral plane, and MMP9 induction after 24hr was compared to intact patches (Fig. 6.5 A). No significant difference in MMP9 induction was seen to result from this doubling of the edge length however. Whether MMP9 induction in the dermis is unique to limb skin was addressed by explanting skin from newt head, a tissue known not to support limb regeneration following amputation when grafted in place of limb skin (Tank, 1983). As with limb skin, 24hr in culture lead to a marked induction of MMP9 expression in the dermis of head skin (Fig. 6.5 B).

6.2.5 Investigating MMP9 expression in limb blastemas

In order to determine if the level of Prod1 expressed by the cells of a blastema correlates with the level of MMP9 expressed by those cells, proximal and distal blastemas were compared (Fig. 6.6 A) as well as RA to DMSO treated blastemas (Fig. 6.6 B). On average, Prod1 expression was observed to be the expected 1.7 fold higher in cells harvested from proximal blastemas, however MMP9 expression was not observed to be higher proximally. RA treatment of animals elevated Prod1 expression approximately 7.5 fold in blastema cells compared to DMSO controls, and in two out of three experiments MMP9 expression was elevated in cells harvested from the blastemas of RA treated animals.

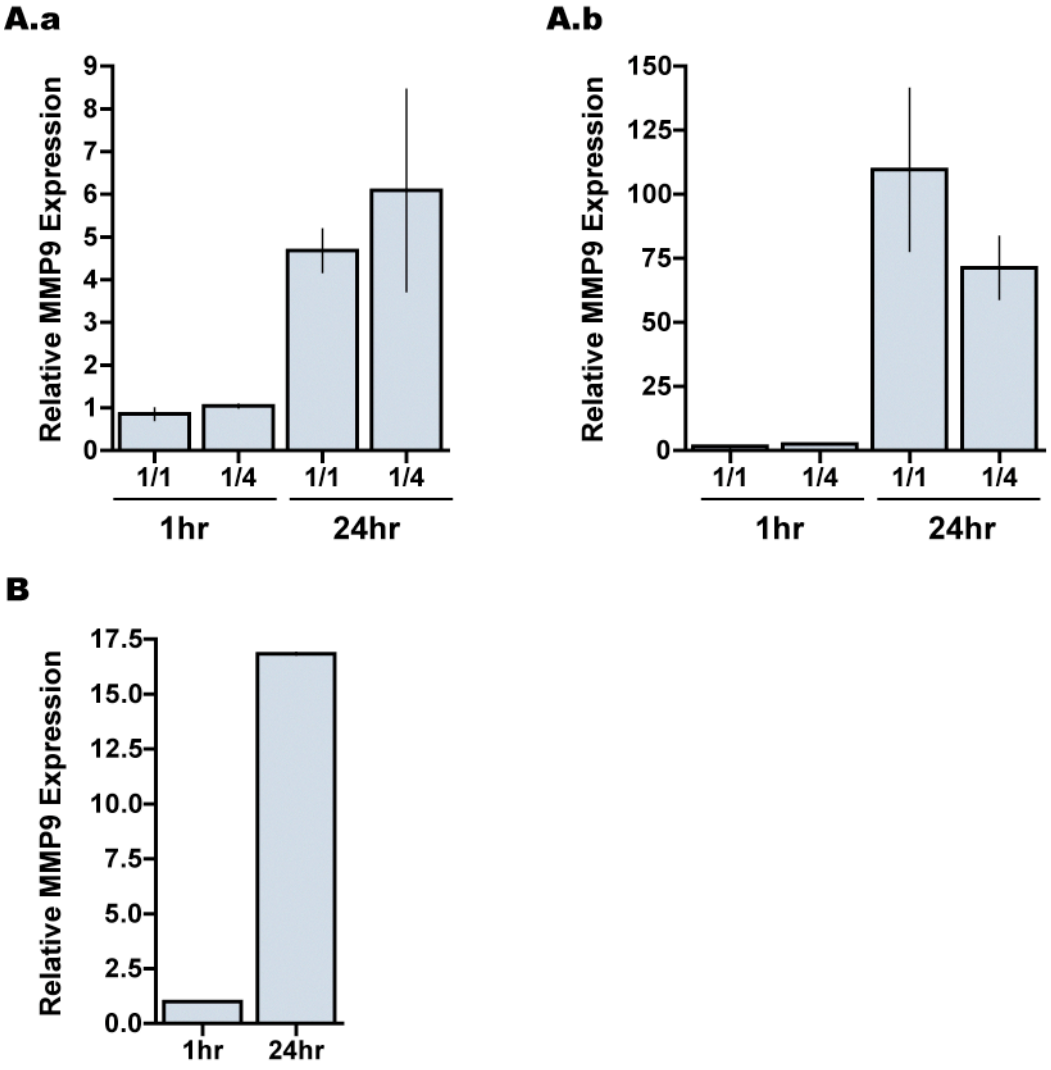


Figure 6.5 Initiation of MMP9 induction in the dermis of explanted skin patches

(A) Patches of limb skin (dermis +epidermis) were either cultured whole [1/1] or cut into quarters [1/4] for 1hr in suspension or 24hr plated on collagen. After the culture period, epidermis was removed and the expression of MMP9 in the dermis was determined by qRT-PCR. Results shown are the average of two independent samples in (Aa) and three independent samples in (Ab). (B) Patches of head skin (dermis +epidermis) were cultured for 1hr in suspension or 24hr plated on collagen. After the culture period, epidermis was removed and the expression of MMP9 in the dermis was determined by qRT-PCR. Results shown are the average of two independent samples for 24hr and one sample for 1hr. In (A) and (B), bars represent SEM.

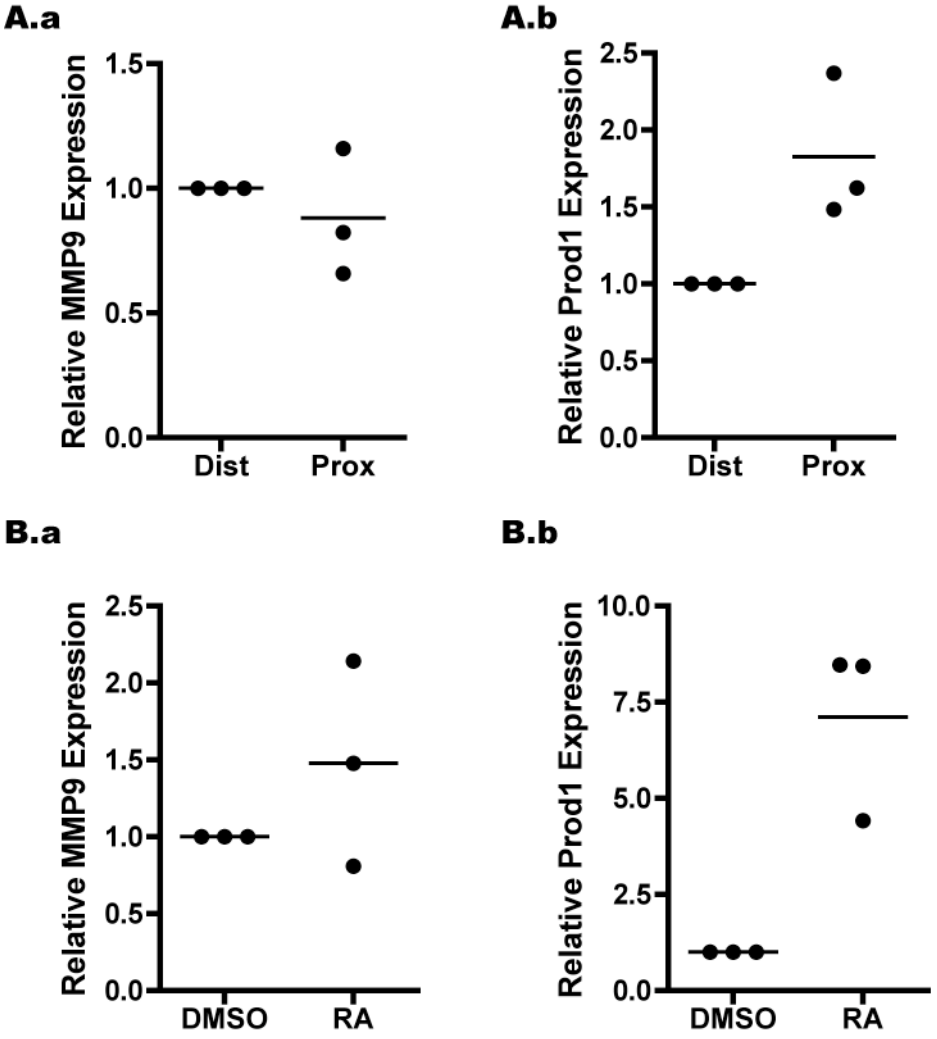


Figure 6.6 MMP9 expression in the blastema

The expression of MMP9 (A.a) and Prod1 (A.b) in distal [Dist) and proximal [Prox] blastemas was determined by qRT-PCR. Results shown are from three independent samples. MMP9 and Prod1 expression is shown relative to that seen in distal blastemas in each experiment. The expression of MMP9 (B.a) and Prod1 (B.b) in the blastemas of DMSO (DMSO) and retinoic acid [RA] injected animals was determined by qRT-PCR. Results shown are from three independent samples. MMP9 and Prod1 expression is shown relative to that seen in blastemas from DMSO injected animals in each experiment. In (A) and (B), each cDNA sample was prepared from 6 pooled blastemas.

6.3 Discussion

6.3.1 Does the Prod1 EGFR pathway regulate MMP9 expression in the dermis?

It has been shown that following surgical removal from the limb, MMP9 expression becomes upregulated in the cells of the dermis. Prod1 is observed to be expressed most highly in the dermis of intact limbs, and following RA treatment, which proximalises regenerating limbs, upregulation of Prod1 expression is most marked in the dermis (Kumar et al., 2007a). Taken in light of the observed regulation of MMP9 expression by Prod1 in cultured limb cells, it is tempting to speculate that the induction of MMP9 expression seen in the dermis following surgical removal is regulated by the Prod1 expressed there.

The α-helix of Prod1 had been shown previously to be essential for the induction of MMP9, suggesting that a peptide with the amino-acid sequence of the α-helix of Prod1 may be able to block molecular interactions between Prod1 and another protein essential for the induction of MMP9. The specific inhibition of MMP9 induction by a peptide representing the α-helix of Prod1 in skin patches would indicate a direct relationship between Prod1 expression and MMP9 induction, however the approach was unsuccessful at blocking the induction of MMP9 by Prod1 in the simpler B1H1 cell culture model and was not extended to skin patches. The possibility exists however that the peptide did not adopt the appropriate α-helical conformation in solution. Work is currently underway in the group to develop a method to knock-down Prod1 expression in the regenerating limb using morpholinos, and should this be successful it will be interesting to see whether this has an effect on the regulation of MMP9 expression in the dermis of these animals.

The inhibitors of EGFR, ERK1/2 and JAK/STAT signalling shown to inhibit MMP9 expression in cultured cells expressing Prod1 also proved unsuccessful in establishing a link between the process characterised in the cell-culture model and that occurring in the skin. The lack of efficacy of the compounds used, even when used at concentrations higher than when treating cultured

cells, or in combination to target each level of the pathway simultaneously, raises the possibility that the compounds are not gaining access to the appropriate cells in the dermis. When working with explanted skin patches in in-situ hybridisation experiments, it was not possible to gain access to the dermis layer with an oligonucleotide probe, even after enzymatic digestion of the sample, indicating the dermis to be a relatively impenetrable tissue. The signalling pathway inhibitors are significantly smaller molecules than oligonucleotides however, and there was some indication that AG490 in particular was instead able to stimulate MMP9 induction in skin patches. The number of experiments carried out were too few however, in light of the observed variability between samples, to make a firm conclusion on this. Should further experiments be conducted and confirm this result, it would be consistent with the conclusion of Chapter 4 that the EGFR ERK1/2 pathway is in fact secondary to some other as yet unidentified pathway**.** Also, as the skin has been completely removed from its normal location, dermal cells may be experiencing an overriding signal, either positive in nature or due to a release from inhibition, to induce MMP9 expression which would occur in a more regulated manner were they in their native environment. These experiments demonstrate the ability of dermal cells to rapidly upregulate MMP9 expression in response to removal from the limb, however further work will be required in order to establish unequivocally whether this process occurs in the dermis following limb amputation.

6.3.2 The origin of migrating cells

All migrating cells express the mesenchymal marker vimentin, however a sub-population of these also express cytokeratin, indicative of their epithelial origin (Fuchs and Cleveland, 1998). It is unclear therefore whether all migrating cells are undergoing epithelial-to-mesenchymal transition (Zavadil and Bottinger, 2005) and therefore essentially originate from the epidermal epithelium, or whether cells originating in the dermal mesenchyme also migrate out from explanted skin patches. A population of these cells express MMP9, but the identity of these migrating MMP9 expressing cells is not clear. Inhibition of MMP activity inhibits the migration of all cells however, indicating that its activity is required in order for any cells to exit the patch and spread across the substrate,

expressed either by themselves or by neighbouring cells. What is clear is that dermal cells that remain in the patch do markedly upregulate MMP9 expression. Cells of dermal origin are known to begin migrating into the centre of the blastema at 5 days post injury in the axolotl (Gardiner et al., 1986) and they or their progeny translocate distally as the blastema grows becoming widely distributed throughout it (Endo et al., 2004) and the subsequently regenerated structures (Kragl et al., 2009). In a 5 day blastema, MMP9 is expressed in a limited number of cells, however at 15 days its expression is seen to be widespread (Vinarsky et al., 2005), inviting speculation that the migrating dermal cells express MMP9 and thereby exit the dermis, allowing their progeny to spread through the blastema as it grows. Similarly, Prod1 expression becomes more widespread throughout the blastema as regeneration proceeds (da Silva et al., 2002). Recent experiments in which GFP labelled dermis was grafted onto a wild-type axolotl prior to amputation showed that cells of dermal origin contribute to connective tissue, tendon and cartilage (Kragl et al., 2009). In these limbs, cartilage is the precursor to bone, indicating that dermal cells contribute to all of what could be considered the structural elements of the limb. As the limb derives its complex pattern from the skeleton, it is particularly interesting to observe such a high proportion of cells of dermal origin, believed to posses innate positional identity (Tank, 1981), in these structures following regeneration.

6.3.3 MMP9 and cell adhesiveness

It has been indicated by confronting proximal and distal blastemas that distal blastema cells may be more adhesive (Nardi and Stocum, 1983), and that this property is mediated through the level of Prod1 expressed on the cell surface (da Silva et al., 2002). The regulation of MMP9 expression by Prod1 in cultured cells allows interpretation of the lower adhesiveness of high Prod1 expressing cells in terms of their potentially higher MMP9 expression and matrix degrading activity. Likewise, that distal blastemas translocate along the limb axis by affinophoresis when grafted to the dorsal surface of a proximal amputation site whereas proximal blastema do not (Crawford and Stocum, 1988a) can be interpreted as resulting from a higher affinity of distal blastemas for the ECM of the regenerating limb than proximal blastemas, which rather than being carried

along as the limb elongates instead remain at the site of grafting due to lack of adhesiveness. The proximal translocation of distal blastema cells electroporated with Prod1 (Echeverri and Tanaka, 2005) may be a result of them loosing contact with other distal cells due to their lower affinity for the ECM, and becoming re-located to more proximal regions of the limb.

Rather than regulating adhesiveness, higher Prod1 expression may promote cell migration. Proximal blastema cells may tend to migrate at a faster rate around distal blastemas due to less constraint from the ECM, or Prod1 electroporated distal blastema cells may actively migrate proximally. The affinophoresis of distal blastemas grafted proximally is less easily explained in these terms unless it is supposed that proximal blastemas remain at the site of grafting by continually migrating proximally to counteract the effect of the outgrowth of the limb displacing it distally, whereas distal blastemas lack this ability and are unable to oppose the translocating effect of limb outgrowth.

6.3.4 MMP9 regulation and proximodistal patterning

Early in limb regeneration, HoxA13, a gene known to regulate patterning in the hand, is expressed, and as limb regeneration proceeds its localisation to distal cells becomes increasingly apparent, indicating that distal cells are specified to hand fates early in the process of regeneration (Gardiner et al., 1995). When distal blastemas are grafted onto proximal stumps, it is the cells from the stump that regenerate the limb structures from shoulder to wrist, with the distal blastema only contributing to the hand (Pescitelli and Stocum, 1980). Similarly, electroporation of the most distal cells of a blastema with a GFP marker results in GFP localised only to hand structures (Echeverri and Tanaka, 2005). The cell-cycle length of cells found at more proximal locations in the blastema is significantly shorter than more distally located blastema cells (Connelly and Bookstein, 1983) (Endo et al., 2004), indicating a greater contribution of these cells to the regeneration of tissue. In light of the fact that regeneration will always produce the same hand structure, but depending upon the level of amputation, the requirement for regeneration of more proximal structures varies across a range, it makes sense that the hand would follow a defined program in all cases, and that taken with the knowledge that distal regions of the blastema are fated early to form the hand structures, that proximal regions of the

blastema would be responsive to the requirement for the amount of other limb structures to regenerate. When labelled limb dermal cells are implanted into a blastema they are found distributed along the entire proximodistal axis of the blastema. In contrast, dermal cells from head skin, which do not support limb regeneration are not (Satoh et al., 2007). The indication of this is that cells originating from the dermis of the limb actively locate distally as the blastema elongates by migration or cell division. These cells have been shown to make a large contribution to the regenerated limb (Kragl et al., 2009), indicating that they are expanding in number by proliferation. A model has been developed to induce limb regeneration from wounds that would be expected to produce only skin regeneration, and this has been shown to require the encounter of dermal cells with positional coordinates from around the circumference of the limb, which stimulate the proliferation of blastema cells and maintains the process of regeneration (Endo et al., 2004). The evident importance of dermal cells to drive the elongation of the regenerating limb raises the question of whether Prod1 regulated MMP9 induction in dermal cells somehow mediates the limb structures regenerated by regulating their exit from the dermis and migration into the blastema.

Regenerating limbs exposed to RA upregulate Prod1 expression in the dermis (Kumar et al., 2007a) and produce extra proximal limb structures (Maden, 1982). It would be interesting to establish whether this leads to an increase in MMP9 expression by dermal cells, thereby enhancing their ability to migrate into the blastema and contribute to the regenerate. There was some indication of a mild enhancement of MMP9 expression in blastema cells of animals exposed to RA, however experiments were inconclusive. There was no indication of higher MMP9 expression in proximal blastemas than in distal blastemas, however as the difference in Prod1 expression is relatively small compared to the difference seen between RA and DMSO treated blastemas, presumably any difference in MMP9 expression would be accordingly smaller and more difficult to detect.

Should the level of dermal Prod1 expression in normal intact limbs be the key regulator of the extent of regeneration of proximal structures, it would be

expected to be expressed more highly in proximal dermis than distal dermis. The limited investigation of this documented here used dermis plus epidermis samples from a number of limbs pooled together and found no difference in Prod1 expression in proximal skin compared to distal skin however. The gradient of Prod1 expression previously demonstrated in samples prepared from all limb tissue is shallow but well established (Kumar et al., 2007a), making it somewhat puzzling as to why no gradient was detected in the skin. If the skin, particularly the dermis, is a major site of Prod1 expression, a gradient might be expected to be more pronounced in skin samples as the signal would not be 'diluted' by normalisation for total mRNA taking account of all the mRNA from tissue which does not express Prod1. One possibility is that as 6 samples were pooled rather than analysed independently, a difference may have been masked by variability in the overall level of Prod1 expressed in individual limbs, for which there has been some evidence gathered by P. Gates. This seems an unlikely explanation however, as paired proximal and distal samples were all taken from the same limb, so unless the variability in Prod1 expression was specifically only in either proximal or distal skin, pooling samples should not have resulted in such sampling error. Prod1 is expressed in cells other than those found in the dermis however (da Silva et al., 2002), and if the proximodistal Prod1 gradient is truly not manifested by the cells of the dermis, the indication is that it is these other cells that express Prod1 differentially according to their position along the limb axis. One possibility is that Prod1 expressing cells associated with other tissues are distributed along the axis of the limb in such a manner that there are either more of them, or more expressing high levels of Prod1 proximally.

Prod1 expression is generally higher in the cells of proximal blastemas and its expression is upregulated by RA (da Silva et al., 2002). Dedifferentiation is also observed to be higher in RA treated blastemas (Ju and Kim, 1994), and proximal blastemas have higher RA activity (Scadding and Maden, 1994). Higher MMP9 expression has been observed in RA treated blastemas (Park and Kim, 1999) consistent with the possibility that higher expression of Prod1 induced by higher RA activity in proximal blastemas increases de-differentiation through MMP9 regulation. Discussion in the Introduction of the role of MMPs in

patterning the regenerating limb leant towards a role for them in determining the size of the regenerate rather than establishing the number of limb compartments however. Should Prod1s function during limb regeneration be to regulate the amount of stump de-differentiation according to the PD level of amputation via MMP9, the indication is that rather than being a primary determinant of PD patterning, Prod1 may instead act in a secondary capacity to ensure an appropriate quantity of precursor cells for the level of amputation.

The expression of Prod1 and of Meis homeobox protein in blastema cells respecifies them to more proximal identities (Echeverri and Tanaka, 2005) (Mercader et al., 2005). The implication of this is that cells become relocated from a region of the blastema fated to form distal structures to a region with more proximal fate. It can be imagined that the overexpression of Prod1 may upregulate MMP9 expression and allow cells to migrate through the blastema to a more proximal region, however as Prod1 is expressed in a gradient along the limb axis (Kumar et al., 2007a) but Meis is not (Mercader et al., 2005), it is seen as being upstream of Meis in specifying the proximodistal identity of blastema cells. Should this be the case, if this relocation involves MMP9 it would imply that it is regulated by Meis. Alternatively, Meis may upregulate Prod1 expression in proximal blastemas. It is conceivable that Meis may regulate both the patterning of the proximal limb compartments and the extent of de-differentiation via upregulation of MMP9 via Prod1, though it is also plausible that RA may independently regulate the level of Meis and Prod1 in the blastema as discussed in the Introduction. If either RA or Meis regulates the expression level of Prod1 in the blastema however, this indicates that Prod1 is not a primary determinant of PD identity of blastema cells and that the PD gradient of Prod1 expression in the intact limb (da Silva et al., 2002) is misleading in its suggestion that the graded expression of a cell-surface determinant underlies the patterning process during limb regeneration

6.3.5 A role for the Prod1 ligand nAG in the regulation of MMP9?

Wounding in the absence of a nerve supply results in the deposition of a collagenous matrix beginning around day 5 and becomes most evident at day 7 (Endo et al., 2004), however if a nerve is deviated to the vicinity this matrix is

not formed. Regeneration of an amputated limb requires a threshold level of innervation, below which blastemas do not develop, possibly due in part to the accumulation of collagenous matrix associated with healing which interferes with the early events in the establishment of a blastema. nAG, a protein expressed by nerve associated Schwann cells, has recently been shown to rescue limb regeneration in the absence of the nerve, and evidence exists of a physical interaction between it and Prod1 (Kumar et al., 2007b). Whether nAG is involved in promoting the migration of Prod1 positive, MMP9 secreting cells from the dermis that degrade this collagen matrix, and thereby facilitate the appropriate environment for blastema development, is an interesting point for consideration. Although in some sense it may seem counterintuitive to suppose that mesenchymal cells lay down collagen only for it to be simultaneously degraded, when taken in light of the possibility that the process of limb regeneration may have evolved subsequently to the mechanisms operating during wound healing (Brockes and Kumar, 2008), that these two opposing processes are occurring during the early stages of blastema formation becomes more plausible. Collagen degradation occurs during the process of wound healing (Bullard et al., 1999) (Pilcher et al., 1997) and the mechanisms facilitating regeneration may have adapted the role of this process. Denervated blastemas are observed to upregulate MMP9 expression to a greater extent than innervated blastemas however (Monaghan et al., 2009), making a requirement for nAG in this process unlikely. In this study Prod1 has been shown to regulate the expression of MMP9, a molecule which permits cell migration through tissue, in cultured cells, however previous work indicates that its interaction with nAG promotes cell proliferation (Kumar et al., 2007b). Prod1 may therefore play a role in the coordination of arguably the two most essential processes occurring during regeneration, one of which requires the participation of nAG and one of which does not. Denervated limbs rescued by nAG regenerate the skeleton but not the muscle (Kumar et al., 2007b) which is interesting when considered together with the fact that Prod1 expressing dermal cells contribute to the regeneration of skeletal and connective tissue but not muscle (Kragl et al., 2009).

6.3.6 Induction of dermal MMP9 expression following injury

The mechanism initiating MMP9 expression in the cells of the limb dermis is of clear interest. MMP9 was seen to be upregulated in the dermis after 24hr both in samples cultured in suspension and plated on collagen, indicating it is not the interaction with collagen initiating the signal. Patches cut into quarters, thereby doubling the number of edge cells missing lateral cell-cell contact, showed no significant increase in MMP9 induction after 24hr, indicating that the loss of these contacts are either not the source of the MMP9 initiating signal, or that the signal is propagated rapidly across all the cells of the dermis within 24hr. Perhaps it is in fact the loss of contact of dermal cells with signals from the underlying ECM that induces MMP9 expression in explanted skin. MMP9 induction was also observed in a single sample of dermis plated in the absence of overlying epidermis. Should this be repeated it would indicate that dermal cells do not require signals from the epidermis in order to induce MMP9.

MMP9 expression was induced in explanted head skin. Head skin is known to be inhibitory to limb regeneration (Tank, 1983) and dermal cells from head skin have been shown to remain at the site of implantation when grafted into a regenerating blastema rather than becoming distributed along the axis of the regenerating limb (Satoh et al., 2007). The implication of this is that head skin dermal cells differ from limb skin cells in their ability to proliferate and migrate, however their ability to initiate dermal MMP9 expression in response to surgical removal indicates that this does not underlie their inability to contribute to the regenerating limb. Instead, they may have the capacity to migrate out from the dermis due to the expression of MMP9 but then fail to proliferate as they lack the required positional co-ordinates to do so.

Induction of Wnt expression is known to result from injury to the skin (Fathke et al., 2006). The possibility that Wnt signalling may contribute to the initiation of MMP9 expression in the dermis was therefore explored using the Wnt pathway inhibitor DKK1 (Glinka et al., 1998). DKK1 showed no inhibitory effect on MMP9 expression in the dermis however. This result does not rule out Wnt signalling as an initiation signal, however the possible explanations for this are too numerous to be worthy of discussion.

7 Concluding comments

Prior to the work described in this thesis, direct molecular level details relating to the mechanism by which Prod1 regulates cellular processes had not been established. Prod1 has been shown through physical interaction with the EGFR to initiate signalling leading to activation of ERK1/2 and the induction of MMP9 expression. ERK1/2 MAPK is known to modulate the activity of a wide variety of targets including transcription factors (Pearson et al., 2001), and due to this it is highly likely that in addition to MMP9 expression, Prod1 participates in the regulation of other genes and cellular processes. The EGFR is known to feed into numerous downstream effector pathways in addition to ERK1/2 MAPK (Jorissen et al., 2003), which either act independently or via varying degrees of crosstalk, providing even greater potential for the participation of Prod1 in diverse cellular processes. The cell-culture assay of the downstream effects of Prod1 expression established during this work lends itself to the further investigation of genes regulated by Prod1. Although the lack of annotated newt genomic sequence hinders the application of micro-array technology in such an approach, modern sequencing technology may prove useful in conducting a global analysis of the regulation of gene expression by Prod1. Prod1 expression shows the same downstream effects in axolotl cells as in newt cells, and as more extensive genomic resources exist for this species, analysis of this sort may be more suited to this system.

As discussed in Chapter 4 there is some indication that the EGFR may not be the only transmembrane signal transducer through which Prod1 acts, and indeed the data can be interpreted in such a way as to suggest that regulation of MMP9 expression by EGFR signalling is dependent on some other signal originating from an independent Prod1 interacting transmembrane protein. It is therefore important to establish whether Prod1 activation of the EGFR is able to induce MMP9 expression, or even activate ERK1/2, independently of signalling from any other Prod1 interacting transmembrane protein.

The cooperation of integrins and the EGFR in mediating signalling from the Prod1 homolog uPAR has been shown to require the activity of Src kinase (Monaghan-Benson and McKeown-Longo, 2006). Src kinase activity is required for EGFR activation upon integrin clustering by uPAR as integrins themselves

do not posses intrinsic kinase activity, however Prod1 initiated EGFR signalling leading to induction of MMP9 expression has been shown in this work not to require Src activity, indicating a distinction between these two processes. Integrins can act via other kinases however (Giancotti and Ruoslahti, 1999), thus the lack of a requirement for Src in Prod1 regulation of MMP9 does not rule out the involvement of integrins. Due to its demonstrated physical association with Prod1, β1 integrin is a candidate for this second transmembrane signalling partner, however the co-expression of Prod1 with a truncated β1 integrin supposed to act in a dominant negative manner did not indicate it to play a role in the regulation of MMP9. A similar result was seen with a dominant negative EGFR construct however. As the expression of neither construct could be detected in B1H1 cells by Western-blotting, this may be due to technical problems with achieving sufficient levels of protein expression which may be overcome by more effective over-expression techniques. Similarly, experiments with integrin inhibitors were inconclusive as to their involvement due to technical problems and warrant further work (data not shown).

Uncertainties remain with respect to the involvement of some of the pathways investigated with kinase inhibitors. For example the involvement of the JAK/STAT pathway remains uncertain due to the complexity of the crosstalk between signalling pathways, and the disputed specificity of the inhibitor used. It is also not clear how the ERK1/2 and JNK MAPK pathways interact in the regulation of MMP9. More information on the phosphorylation states of these proteins and their targets should help to further establish the details of Prod1 regulation of MMP9 expression.

The EGFR can be phosphorylated on a variety of different sites depending upon the circumstances of its activation and some of these phosphorylation sites are indicative of the downstream pathways that will be activated (Jorissen et al., 2003). Detailed analysis of the sites phosphorylated on the EGFR resulting from its interaction with Prod1 should therefore prove valuable in further understanding the signalling pathways it feeds into. In this study, good

fortune permitted the use of commercially available anti-phospho ERK1/2 and anti-phospho JNK antibodies in the analysis conducted, however the anti-phospho human EGFR antibodies used did not react detectably with the newt EGFR, likely due to imperfect homology of their target epitopes. The use of commercially available antibodies is limited when analysing newt signal transduction therefore, and due to the large number of phosphorylation events that warrant investigation it is unreasonable to raise custom antibodies to all of these targets. As mass-spectrometry based proteomics becomes established in the newt, global analysis of protein phosphorylation resulting from Prod1 expression should provide further clarification of these questions and potentially identify other as yet unstudied downstream signalling components. Alternatively, due to the more extensive genomic resources, axolotl cells may be a more suitable system for such analysis. Coupling global approaches such as transcriptome sequencing and proteomics with the use of kinase inhibitors such as those used in this study should provide even greater power to identify dissect the mechanism through which signalling pathways are activated, transcription factors are phosphorylated, and genes are regulated in response to Prod1.

Site-directed mutagenesis has revealed that amino-acids on the α-helix of Prod1 are essential for its MMP9 inducing activity. Mutation of an α-helix residue with the greatest effect on the induction of MMP9 expression did not affect the physical association with either the EGFR or β1 integrin however. This raises the possibility that rather than disrupting the physical interaction between Prod1 and the EGFR or β1 integrin, mutation of α-helix residues instead affect their activation. In order to address whether the α-helix mutation does indeed affect the activation of the EGFR upon engagement with Prod1, appropriate antibodies against its many known phosphorylation sites would have to be sought, or alternatively, a mass-spectrometry based approach could be employed in order to asses the phosphorylation state of the protein. Alternatively there may be a third as yet unidentified Prod1 interacting transmembrane adaptor. As discussed in Chapter 4, it is plausible that this may

be a G-protein coupled receptor, and the involvement of such proteins warrants investigation with appropriate inhibitors.

It had been shown previously that the level of cell-surface expressed Prod1 influences the engulfment of blastemas of different PD origin (Nardi and Stocum, 1983) and that the overexpression of Prod1 in blastema cells causes them to become proximally re-located during limb regeneration (Echeverri and Tanaka, 2005), and this had been rationalised in terms of a direct function of Prod1 in regulating cell adhesivity. The demonstration that Prod1 regulates the enzyme MMP9 raises the possibility that rather than directly regulating cell adhesivity through its interaction with proteins on the surface of other cells, Prod1 may regulate the strength of association of cells within a tissue via the remodelling of the ECM. In particular, the involvement of MMPs in blastema engulfment could be addressed with relative technical simplicity through the use of MMP inhibitors such as the one used to inhibit skin cell migration in this thesis.

Experiments with explanted skin patches have shown that MMP family enzymes support cell migration as has been shown in other experimental systems. In light of this it is possible to re-interpret blastema engulfment and proximal relocation of blastema cells in terms of enhanced cell migration by cells expressing high levels of Prod1. The difference between the regulation of adhesivity and migration is subtle at best, though the term migration in some ways implies a more active process than cell adhesivity. It is unclear though, given the available experimental systems, how to distinguish between the involvement of MMPs in these two processes, if they are indeed distinct from one another.

The regulation of MMP9 by Prod1 has only been directly demonstrated in cultured cells, making it important to establish categorically whether Prod1 genuinely directly regulates the level of MMP9 in the blastema. The specific disruption of the activity of Prod1 or its expression in either explanted skin patches or in regenerating blastemas should allow this relationship to be further investigated, however reliable genetic tools to address this question are

currently unavailable. Should it become possible to knock-down gene activity it would be interesting to see whether Prod1 is required for the expression of MMP9 in skin cells migrating from explanted patches or in the cells of the blastema. Furthermore, it would be interesting to establish whether dermal cells migrating into the blastema or proximally relocating Prod1 overexpressing cells express MMP9 and whether its knockdown affects these processes. Progress is being made with transgenic technology in axolotls making this system potentially more powerful than newts when investigating questions through genetic approaches.

The initial focus of interest in Prod1 has been in relation to its potential involvement in the regulation of proximodistal patterning during limb regeneration. The controversial suggestion of a direct role of MMP9 in this process through the regulation of stump tissue de-differentiation has been discussed at length throughout this thesis. A difference between MMP9 expression in proximal and distal blastemas was undetectable, however a possible upregulation by the proximalising molecule RA was suggested. It would be interesting to establish whether RA does indeed regulate MMP9 expression, either via or independently of the upregulation of Prod1 expression, and whether manipulation of the level of expression of Prod1 and MMP9 affects de-differentiation as is seen with RA, and thereby regulates the number of limb compartments regenerated. Again, genetic tools to reliably manipulate the level of expression of these genes will be required in order to directly address these questions.

Similar to Prod1, expression of the homeobox gene Meis has been shown to proximally relocate blastema cells. Furthermore, knockdown of Meis abolishes the proximalising effect of RA, which upregulates both Prod1 and Meis expression in the blastema (Mercader et al., 2005). The relationship between Prod1 and Meis is unclear, though it has been assumed that Meis regulates the expression of Prod1. Whether proximal relocation of blastema cells by Meis involves MMP9 upregulation, as may be the case for Prod1, and if so, whether this is via Prod1 upregulation, should further clarify the role of MMP9 and Prod1 in mechanism of PD patterning during limb regeneration.

An interesting revelation has come from the comparison of Prod1 of the axolotl to that of the newt. Axolotl Prod1 is not GPI anchored, yet in all respects investigated it functions comparably to that of the newt, which is dependent on its GPI anchor in order to be functional. This finding has implications both for the evolution and mechanism of Prod1 and GPI anchored proteins in general, as it not only shows that the mechanism of Prod1 activity does not in essence require it to be GPI anchored, but also shows that orthologs can diverge in terms of this characteristic whilst retaining the same activity. A controversial area in the field of membrane biology is the existence of lipid rafts (Munro, 2003), to which it has been proposed GPI anchored proteins require localisation in order to function. The clear demonstration of a difference in GPI anchored character between two orthologs with the same function shows that the GPI anchor does not necessarily participate directly in the molecular mechanism of a protein, despite it being anchored by such means to the membrane. The elegance of this demonstration lies in the fact that in the case of previous investigations of the functional role of the GPI anchor based on experimentally engineered constructs (Walmsley et al., 2003) (Nicholson and Stanners, 2006) (Robinson et al., 1989), the results can arguably be attributed to the artificial nature of the resulting expressed proteins, whereas in this case the proteins under comparison exist naturally and have come about through evolution. As discussed in Chapter 5 the GPI anchor is likely to have been lost by Prod1 of the axolotl subsequent to the acquisition of a mutation rendering the GPI anchor superfluous, indicating that genetic drift can act to change a protein in a manner as seemingly drastic as the loss of direct membrane tethering without affecting its function. Clearly, whilst having implications for the role of GPI anchors in general, the ability of the axolotl Prod1 to function without a GPI anchor also suggests more specifically that the GPI anchor does not play the integral role in the mechanism of Prod1 suggested by the lipid raft model in localising the protein to specific microdomains of the membrane, but rather that in the case of newt Prod1, it is required in order to ensure that the protein remains associated with the cell in order to be able to form the appropriate protein-protein interactions, without itself directly facilitating these interactions.

Prod1 and uPAR share structural similarities such as an α-helix at finger three of their structure (Garza-Garcia et al., 2009), and both associate physically with and activate the EGFR in a ligand independent manner. Prod1 has evolved uniquely in salamander species (Garza-Garcia et al., 2009) indicating that it has independently evolved the ability to interact with the more ancestral EGFR. Comparison of these proteins may shed some light on the structural basis for ligand independent activation of the EGFR, which, in the case of uPAR, has implications during carcinogenesis (Inuzuka et al., 2000). Although mutation of an α-helical residue at finger three essential for MMP9 inducing activity does not appear to disrupt the interaction of Prod1 with the EGFR, the α-helix of uPAR may none the less be an interesting focus for the mechanism by which it can promote cancer via aberrant signalling. Induction of MMP9 expression by uPAR is known to promote cell migration, which can be linked to cancer metastasis, and contrary to the case of Prod1, this has been shown to require the activity of the oncogene Src (Wei et al., 2007). That Prod1 is able to induce MMP9 in the absence of Src activity may again have implications for the signalling mechanism by which uPAR promotes cancer progression.

In summary, the work presented in this thesis represents a step forward in understanding salamander limb regeneration through the first molecular level investigation of the mechanism of Prod1, providing the first insight into the signal-transduction pathways through which it operates and establishing an assay system which should prove useful for further investigations of its biological activity. Results obtained may provide new perspectives on the process of proximodistal patterning through the linking of two previously unconnected molecules studied in the field of salamander limb regeneration, Prod1 and MMP9. Furthermore, the work detailed here may prove to be of significance to fields of study outside the sphere of regeneration research, such as the cell-biology of GPI anchored proteins in general and to the study of the EGFR and uPAR in cancer.

8 Bibliography

Agata, K., Saito, Y., and Nakajima, E. (2007). Unifying principles of regeneration I: Epimorphosis versus morphallaxis. *Dev Growth Differ* **49,** 73-8.

Aguirre-Ghiso, J. A., Liu, D., Mignatti, A., Kovalski, K., and Ossowski, L. (2001). Urokinase receptor and fibronectin regulate the ERK(MAPK) to p38(MAPK) activity ratios that determine carcinoma cell proliferation or dormancy in vivo. *Mol Biol Cell* **12,** 863-79.

Akimenko, M. A., Mari-Beffa, M., Becerra, J., and Geraudie, J. (2003). Old questions, new tools, and some answers to the mystery of fin regeneration. *Dev Dyn* **226,** 190-201.

Alison, M. R., Golding, M. H., and Sarraf, C. E. (1996). Pluripotential liver stem cells: facultative stem cells located in the biliary tree. *Cell Prolif* **29,** 373-402.

Allan, C. H., Fleckman, P., Fernandes, R. J., Hager, B., James, J., Wisecarver, Z., Satterstrom, F. K., Gutierrez, A., Norman, A., Pirrone, A., Underwood, R. A., Rubin, B. P., Zhang, M., Ramay, H. R., and Clark, J. M. (2006). Tissue response and Msx1 expression after human fetal digit tip amputation in vitro. *Wound Repair Regen* **14,** 398-404.

Andl, C. D., Mizushima, T., Oyama, K., Bowser, M., Nakagawa, H., and Rustgi, A. K. (2004). EGFR-induced cell migration is mediated predominantly by the JAK-STAT pathway in primary esophageal keratinocytes. *Am J Physiol Gastrointest Liver Physiol* **287,** G1227-37.

Andreev, J., Galisteo, M. L., Kranenburg, O., Logan, S. K., Chiu, E. S., Okigaki, M., Cary, L. A., Moolenaar, W. H., and Schlessinger, J. (2001). Src and Pyk2 mediate G-protein-coupled receptor activation of epidermal growth factor receptor (EGFR) but are not required for coupling to the mitogen-activated protein (MAP) kinase signaling cascade. *J Biol Chem* **276,** 20130-5.

Azcoitia, V., Aracil, M., Martinez, A. C., and Torres, M. (2005). The homeodomain protein Meis1 is essential for definitive hematopoiesis and vascular patterning in the mouse embryo. *Dev Biol* **280,** 307-20.

Bailey, A. J., Bazin, S., Sims, T. J., Le Lous, M., Nicoletis, C., and Delaunay, A. (1975). Characterization of the collagen of human hypertrophic and normal scars. *Biochim Biophys Acta* **405,** 412-21.

Batzer, A. G., Rotin, D., Urena, J. M., Skolnik, E. Y., and Schlessinger, J. (1994). Hierarchy of binding sites for Grb2 and Shc on the epidermal growth factor receptor. *Mol Cell Biol* **14,** 5192-201.

Baulida, J., Kraus, M. H., Alimandi, M., Di Fiore, P. P., and Carpenter, G. (1996). All ErbB receptors other than the epidermal growth factor receptor are endocytosis impaired. *J Biol Chem* **271,** 5251-7.

Biscardi, J. S., Maa, M. C., Tice, D. A., Cox, M. E., Leu, T. H., and Parsons, S. J. (1999). c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr845 and Tyr1101 is associated with modulation of receptor function. *J Biol Chem* **274,** 8335-43.

Blagoev, B., Kratchmarova, I., Ong, S. E., Nielsen, M., Foster, L. J., and Mann, M. (2003). A proteomics strategy to elucidate functional protein-protein interactions applied to EGF signaling. *Nat Biotechnol* **21,** 315-8.

Blaikie, P., Immanuel, D., Wu, J., Li, N., Yajnik, V., and Margolis, B. (1994). A region in Shc distinct from the SH2 domain can bind tyrosine-phosphorylated growth factor receptors. *J Biol Chem* **269,** 32031-4.

Blobel, C. P. (2005). ADAMs: key components in EGFR signalling and development. *Nat Rev Mol Cell Biol* **6,** 32-43.

Bosch, T. C. (2009). Hydra and the evolution of stem cells. *Bioessays* **31,** 478-86.

Boulet, A. M., and Capecchi, M. R. (2004). Multiple roles of Hoxa11 and Hoxd11 in the formation of the mammalian forelimb zeugopod. *Development* **131,** 299-309.

Bourguignon, L. Y., Gunja-Smith, Z., Iida, N., Zhu, H. B., Young, L. J., Muller, W. J., and Cardiff, R. D. (1998). CD44v(3,8-10) is involved in cytoskeleton-mediated tumor cell migration and matrix metalloproteinase (MMP-9) association in metastatic breast cancer cells. *J Cell Physiol* **176,** 206-15.

Brockes, J. P. (1997). Amphibian limb regeneration: rebuilding a complex structure. *Science* **276,** 81-7.

Brockes, J. P., (1998). Regeneration and cancer. *Biochim Biophys Acta*. **1377,** M1-11.

Brockes, J. P., and Kumar, A. (2002). Plasticity and reprogramming of differentiated cells in amphibian regeneration. *Nat Rev Mol Cell Biol* **3,** 566-74.

Brockes, J. P., and Kumar, A. (2005). Appendage regeneration in adult vertebrates and implications for regenerative medicine. *Science* **310,** 1919-23.

Brockes, J. P., and Kumar, A. (2008). Comparative aspects of animal regeneration. *Annu Rev Cell Dev Biol* **24,** 525-49.

Brown, D. A., and London, E. (2000). Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J Biol Chem* **275,** 17221-4.

Brown, D. A., and Rose, J. K. (1992). Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* **68,** 533-44.

Broxmeyer, H. E., and Williams, D. E. (1988). The production of myeloid blood cells and their regulation during health and disease. *Crit Rev Oncol Hematol* **8,** 173-226.

Bullard, K. M., Lund, L., Mudgett, J. S., Mellin, T. N., Hunt, T. K., Murphy, B., Ronan, J., Werb, Z., and Banda, M. J. (1999). Impaired wound contraction in stromelysin-1-deficient mice. *Ann Surg* **230,** 260-5.

Burke, J. E., and Dennis, E. A. (2009). Phospholipase A2 structure/function, mechanism, and signaling. *J Lipid Res* **50 Suppl,** S237-42.

Capdevila, J., Tsukui, T., Rodriquez Esteban, C., Zappavigna, V., and Izpisua Belmonte, J. C. (1999). Control of vertebrate limb outgrowth by the proximal factor Meis2 and distal antagonism of BMPs by Gremlin. *Mol Cell* **4,** 839-49.

Caplan, A. I. (1991). Mesenchymal stem cells. *J Orthop Res* **9,** 641-50.

Carmeliet, P., Moons, L., Lijnen, R., Baes, M., Lemaitre, V., Tipping, P., Drew, A., Eeckhout, Y., Shapiro, S., Lupu, F., and Collen, D. (1997). Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. *Nat Genet* **17,** 439-44.

Carpenter, G. (1999). Employment of the epidermal growth factor receptor in growth factor-independent signaling pathways. *J Cell Biol* **146,** 697-702.

Cary, L. A., Han, D. C., Polte, T. R., Hanks, S. K., and Guan, J. L. (1998). Identification of p130Cas as a mediator of focal adhesion kinase-promoted cell migration. *J Cell Biol* **140,** 211-21.

Castillon, G. A., Watanabe, R., Taylor, M., Schwabe, T. M., and Riezman, H. (2009). Concentration of GPI-anchored proteins upon ER exit in yeast. *Traffic* **10,** 186-200.

Chan, T. O., Rittenhouse, S. E., and Tsichlis, P. N. (1999). AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu Rev Biochem* **68,** 965-1014.

Chaurasia, P., Aguirre-Ghiso, J. A., Liang, O. D., Gardsvoll, H., Ploug, M., and Ossowski, L. (2006). A region in urokinase plasminogen receptor domain III controlling a functional association with alpha5beta1 integrin and tumor growth. *J Biol Chem* **281,** 14852-63.

Chen, R. H., Abate, C., and Blenis, J. (1993). Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase. *Proc Natl Acad Sci U S A* **90,** 10952-6.

Christensen, R. N., and Tassava, R. A. (2000). Apical epithelial cap morphology and fibronectin gene expression in regenerating axolotl limbs. *Dev Dyn* **217,** 216-24.

Chung, J., Uchida, E., Grammer, T. C., and Blenis, J. (1997). STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. *Mol Cell Biol* **17,** 6508-16.

Clark, E. A., King, W. G., Brugge, J. S., Symons, M., and Hynes, R. O. (1998). Integrin-mediated signals regulated by members of the rho family of GTPases. *J Cell Biol* **142,** 573-86.

Clark, R. A., Nielsen, L. D., Welch, M. P., and McPherson, J. M. (1995). Collagen matrices attenuate the collagen-synthetic response of cultured fibroblasts to TGF-beta. *J Cell Sci* **108,** 1251-61.

Connelly, T. G., and Bookstein, F. L. (1983). Method for 3-dimensional analysis of patterns of thymidine labeling in regenerating and developing limbs. *Prog Clin Biol Res* **110,** 525-36.

Crawford, K., and Stocum, D. L. (1988a). Retinoic acid coordinately proximalizes regenerate pattern and blastema differential affinity in axolotl limbs. *Development* **102,** 687-98.

Crawford, K., and Stocum, D. L. (1988b). Retinoic acid proximalizes level-specific properties responsible for intercalary regeneration in axolotl limbs. *Development* **104,** 703-12.

da Silva, S. M., Gates, P. B., and Brockes, J. P. (2002). The newt ortholog of CD59 is implicated in proximodistal identity during amphibian limb regeneration. *Dev Cell* **3,** 547-55.

Daubie, V., Pochet, R., Houard, S., and Philippart, P. (2007). Tissue factor: a mini-review. *J Tissue Eng Regen Med* **1,** 161-9.

Deak, M., Clifton, A. D., Lucocq, L. M., and Alessi, D. R. (1998). Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. *Embo J* **17,** 4426-41.

Dent, J. N. (1962). Limb regeneration in larvae and metamorphosing individuals of the South African clawed toad. *J Morphol* **110,** 61-77.

Diaz-Meco, M. T., Lozano, J., Municio, M. M., Berra, E., Frutos, S., Sanz, L., and Moscat, J. (1994). Evidence for the in vitro and in vivo interaction of Ras with protein kinase C zeta. *J Biol Chem* **269,** 31706-10.

Diekmann, O., and Tschesche, H. (1994). Degradation of kinins, angiotensins and substance P by polymorphonuclear matrix metalloproteinases MMP 8 and MMP 9. *Braz J Med Biol Res* **27,** 1865-76.

Dikic, I. (2003). Mechanisms controlling EGF receptor endocytosis and degradation. *Biochem Soc Trans* **31,** 1178-81.

Du, K., and Montminy, M. (1998). CREB is a regulatory target for the protein kinase Akt/PKB. *J Biol Chem* **273,** 32377-9.

Echeverri, K., and Tanaka, E. M. (2005). Proximodistal patterning during limb regeneration. *Dev Biol* **279,** 391-401.

Eisenhaber, B., Bork, P., and Eisenhaber, F. (1998). Sequence properties of GPI-anchored proteins near the omega-site: constraints for the polypeptide binding site of the putative transamidase. *Protein Eng* **11,** 1155-61.

Eisenhaber, B., Maurer-Stroh, S., Novatchkova, M., Schneider, G., and Eisenhaber, F. (2003). Enzymes and auxiliary factors for GPI lipid anchor biosynthesis and post-translational transfer to proteins. *Bioessays* **25,** 367-85.

Endo, T., Bryant, S. V., and Gardiner, D. M. (2004). A stepwise model system for limb regeneration. *Dev Biol* **270,** 135-45.

English, J., Pearson, G., Wilsbacher, J., Swantek, J., Karandikar, M., Xu, S., and Cobb, M. H. (1999). New insights into the control of MAP kinase pathways. *Exp Cell Res* **253,** 255-70.

English, W. R., Puente, X. S., Freije, J. M., Knauper, V., Amour, A., Merryweather, A., Lopez-Otin, C., and Murphy, G. (2000). Membrane type 4 matrix metalloproteinase (MMP17) has tumor necrosis factor-alpha convertase activity but does not activate pro-MMP2. *J Biol Chem* **275,** 14046-55.

Evans, M. J., and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292,** 154-6.
Fallon, J. F., Lopez, A., Ros, M. A., Savage, M. P., Olwin, B. B., and Simandl, B. K. (1994). FGF-2: apical ectodermal ridge growth signal for chick limb development. *Science* **264,** 104-7.
Fanger, G. R., Johnson, N. L., and Johnson, G. L. (1997). MEK kinases are regulated by EGF and selectively interact with Rac/Cdc42. *Embo J* **16,** 4961-72.
Fathke, C., Wilson, L., Shah, K., Kim, B., Hocking, A., Moon, R., and Isik, F. (2006). Wnt signaling induces epithelial differentiation during cutaneous wound healing. *BMC Cell Biol* **7,** 4.
Ferguson, S. S. (2001). Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev* **53,** 1-24.
Ferrell, J. E., Jr. (1997). How responses get more switch-like as you move down a protein kinase cascade. *Trends Biochem Sci* **22,** 288-9.
Ferrell, J. E., Jr., and Bhatt, R. R. (1997). Mechanistic studies of the dual phosphorylation of mitogen-activated protein kinase. *J Biol Chem* **272,** 19008-16.
Ferretti, P., and Brockes, J. P. (1988). Culture of newt cells from different tissues and their expression of a regeneration-associated antigen. *J Exp Zool* **247,** 77-91.
Ferry, G., Lonchampt, M., Pennel, L., de Nanteuil, G., Canet, E., and Tucker, G. C. (1997). Activation of MMP-9 by neutrophil elastase in an in vivo model of acute lung injury. *FEBS Lett* **402,** 111-5.
Frost, J. A., Steen, H., Shapiro, P., Lewis, T., Ahn, N., Shaw, P. E., and Cobb, M. H. (1997). Cross-cascade activation of ERKs and ternary complex factors by Rho family proteins. *Embo J* **16,** 6426-38.
Fuchs, E., and Cleveland, D. W. (1998). A structural scaffolding of intermediate filaments in health and disease. *Science* **279,** 514-9.
Fujita, M., and Jigami, Y. (2008). Lipid remodeling of GPI-anchored proteins and its function. *Biochim Biophys Acta* **1780,** 410-20.
Fujiwara, T., Ritchie, K., Murakoshi, H., Jacobson, K., and Kusumi, A. (2002). Phospholipids undergo hop diffusion in compartmentalized cell membrane. *J Cell Biol* **157,** 1071-81.
Funk, C. D. (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* **294,** 1871-5.
Gage, F. H. (2000). Mammalian neural stem cells. *Science* **287,** 1433-8.
Galat, A. (2008). The three-fingered protein domain of the human genome. *Cell Mol Life Sci* **65,** 3481-93.
Galliot, B., Miljkovic-Licina, M., de Rosa, R., and Chera, S. (2006). Hydra, a niche for cell and developmental plasticity. *Semin Cell Dev Biol* **17,** 492-502.
Galliot, B., and Schmid, V. (2002). Cnidarians as a model system for understanding evolution and regeneration. *Int J Dev Biol* **46,** 39-48.
Gardiner, D. M., Blumberg, B., Komine, Y., and Bryant, S. V. (1995). Regulation of HoxA expression in developing and regenerating axolotl limbs. *Development* **121,** 1731-41.
Gardiner, D. M., Muneoka, K., and Bryant, S. V. (1986). The migration of dermal cells during blastema formation in axolotls. *Dev Biol* **118,** 488-93.
Garza-Garcia, A., Harris, R., Esposito, D., Gates, P. B., and Driscoll, P. (2009). Solution Structure and Phylogenetics of Prod1, a Member of the Three-Finger Protein Superfamily Implicated in Salamander Limb Regeneration. *PLos ONE* **e7123**.
Gerwins, P., Blank, J. L., and Johnson, G. L. (1997). Cloning of a novel mitogen-activated protein kinase kinase kinase, MEKK4, that selectively regulates the c-Jun amino terminal kinase pathway. *J Biol Chem* **272,** 8288-95.

Ghosh, S., Johnson, J. J., Sen, R., Mukhopadhyay, S., Liu, Y., Zhang, F., Wei, Y., Chapman, H. A., and Stack, M. S. (2006). Functional relevance of urinary-type plasminogen activator receptor-alpha3beta1 integrin association in proteinase regulatory pathways. *J Biol Chem* **281,** 13021-9.

Giancotti, F. G., and Ruoslahti, E. (1999). Integrin signaling. *Science* **285,** 1028-32.

Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C., and Niehrs, C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* **391,** 357-62.

Globus, M., and Vethamany-Globus, S. (1976). An in vitro analogue of early chick limb bud outgrowth. *Differentiation* **6,** 91-6.

Gomez, D. E., Alonso, D. F., Yoshiji, H., and Thorgeirsson, U. P. (1997). Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *Eur J Cell Biol* **74,** 111-22.

Gonfloni, S., Weijland, A., Kretzschmar, J., and Superti-Furga, G. (2000). Crosstalk between the catalytic and regulatory domains allows bidirectional regulation of Src. *Nat Struct Biol* **7,** 281-6.

Goss, R. J. (1956a). Regenerative inhibition following limb amputation and immediate insertion into the body cavity. *Anat Rec* **126,** 15-27.

Goss, R. J. (1956b). The regenerative responses of amputated limbs to delayed insertion into the body cavity. *Anat Rec* **126,** 283-97.

Goss, R. J., (1969). Principles of Regeneration. Academic Press, New York.

Guan, K., Hakes, D. J., Wang, Y., Park, H. D., Cooper, T. G., and Dixon, J. E. (1992). A yeast protein phosphatase related to the vaccinia virus VH1 phosphatase is induced by nitrogen starvation. *Proc Natl Acad Sci U S A* **89,** 12175-9.

Guerrero, J., Santibanez, J. F., Gonzalez, A., and Martinez, J. (2004). EGF receptor transactivation by urokinase receptor stimulus through a mechanism involving Src and matrix metalloproteinases. *Exp Cell Res* **292,** 201-8.

Guo, Z., Cai, S., Fang, R., Chen, H., Du, J., Tan, Y., Ma, W., Hu, H., Cai, S., and Liu, Y. (2007). The synergistic effects of CXCR4 and EGFR on promoting EGF-mediated metastasis in ovarian cancer cells. *Colloids Surf B Biointerfaces* **60,** 1-6.

Guy, P. M., Platko, J. V., Cantley, L. C., Cerione, R. A., and Carraway, K. L., 3rd. (1994). Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. *Proc Natl Acad Sci U S A* **91,** 8132-6.

Hagemann, C., and Blank, J. L. (2001). The ups and downs of MEK kinase interactions. *Cell Signal* **13,** 863-75.

Haigler, H. T., McKanna, J. A., and Cohen, S. (1979). Direct visualization of the binding and internalization of a ferritin conjugate of epidermal growth factor in human carcinoma cells A-431. *J Cell Biol* **81,** 382-95.

Hallberg, B., Rayter, S. I., and Downward, J. (1994). Interaction of Ras and Raf in intact mammalian cells upon extracellular stimulation. *J Biol Chem* **269,** 3913-6.

Hamilton, M., and Wolfman, A. (1998). Ha-ras and N-ras regulate MAPK activity by distinct mechanisms in vivo. *Oncogene* **16,** 1417-28.

Haq, R., Halupa, A., Beattie, B. K., Mason, J. M., Zanke, B. W., and Barber, D. L. (2002). Regulation of erythropoietin-induced STAT serine phosphorylation by distinct mitogen-activated protein kinases. *J Biol Chem* **277,** 17359-66.

Harper, E., Bloch, K. J., and Gross, J. (1971). The zymogen of tadpole collagenase. *Biochemistry* **10,** 3035-41.

Harrisingh, M. C., Perez-Nadales, E., Parkinson, D. B., Malcolm, D. S., Mudge, A. W., and Lloyd, A. C. (2004). The Ras/Raf/ERK signalling pathway drives Schwann cell dedifferentiation. *Embo J* **23,** 3061-71.

Hasty, K. A., Pourmotabbed, T. F., Goldberg, G. I., Thompson, J. P., Spinella, D. G., Stevens, R. M., and Mainardi, C. L. (1990). Human neutrophil collagenase. A distinct gene product with homology to other matrix metalloproteinases. *J Biol Chem* **265,** 11421-4.

Hauser, F., Cazzamali, G., Williamson, M., Blenau, W., and Grimmelikhuijzen, C. J. (2006). A review of neurohormone GPCRs present in the fruitfly Drosophila melanogaster and the honey bee Apis mellifera. *Prog Neurobiol* **80,** 1-19.

Heasman, S. J., and Ridley, A. J. (2008). Mammalian Rho GTPases: new insights into their functions from in vivo studies. *Nat Rev Mol Cell Biol* **9,** 690-701.

Helms, J. B., and Zurzolo, C. (2004). Lipids as targeting signals: lipid rafts and intracellular trafficking. *Traffic* **5,** 247-54.

Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993). Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev* **7,** 2135-48.

Holstein, T. W., Hobmayer, E., and David, C. N. (1991). Pattern of epithelial cell cycling in hydra. *Dev Biol* **148,** 602-11.

Hruska, K. A., Rolnick, F., Huskey, M., Alvarez, U., and Cheresh, D. (1995). Engagement of the osteoclast integrin alpha v beta 3 by osteopontin stimulates phosphatidylinositol 3-hydroxyl kinase activity. *Endocrinology* **136,** 2984-92.

Huang, C. K., Zhan, L., Ai, Y., and Jongstra, J. (1997). LSP1 is the major substrate for mitogen-activated protein kinase-activated protein kinase 2 in human neutrophils. *J Biol Chem* **272,** 17-9.

Huang, F., Khvorova, A., Marshall, W., and Sorkin, A. (2004). Analysis of clathrin-mediated endocytosis of epidermal growth factor receptor by RNA interference. *J Biol Chem* **279,** 16657-61.

Hughes, R. N. (1989). A Functional Biology of Clonal Animals. p331. Chapman & Hall, London

Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell* **110,** 673-87.

Imai, K., Ohuchi, E., Aoki, T., Nomura, H., Fujii, Y., Sato, H., Seiki, M., and Okada, Y. (1996). Membrane-type matrix metalloproteinase 1 is a gelatinolytic enzyme and is secreted in a complex with tissue inhibitor of metalloproteinases 2. *Cancer Res* **56,** 2707-10.

Inuzuka, K., Ogata, Y., Nagase, H., and Shirouzu, K. (2000). Significance of coexpression of urokinase-type plasminogen activator, and matrix metalloproteinase 3 (stromelysin) and 9 (gelatinase B) in colorectal carcinoma. *J Surg Res* **93,** 211-8.

Iten, L., and Bryant, S. V. (1973). Forelimb regeneration from different levels of amputation in the newt N.viridescens. Length, rate and stages. *Wilhelm Roux Arch. Dev. Biol.*, 263-282.

Itoh, Y., Ito, A., Iwata, K., Tanzawa, K., Mori, Y., and Nagase, H. (1998). Plasma membrane-bound tissue inhibitor of metalloproteinases (TIMP)-2 specifically inhibits matrix metalloproteinase 2 (gelatinase A) activated on the cell surface. *J Biol Chem* **273,** 24360-7.

Janknecht, R., Ernst, W. H., Pingoud, V., and Nordheim, A. (1993). Activation of ternary complex factor Elk-1 by MAP kinases. *Embo J* **12,** 5097-104.

Jo, M., Thomas, K. S., Marozkina, N., Amin, T. J., Silva, C. M., Parsons, S. J., and Gonias, S. L. (2005). Dynamic assembly of the urokinase-type plasminogen activator signaling receptor complex determines the mitogenic activity of urokinase-type plasminogen activator. *J Biol Chem* **280,** 17449-57.

Jo, M., Thomas, K. S., Takimoto, S., Gaultier, A., Hsieh, E. H., Lester, R. D., and Gonias, S. L. (2007). Urokinase receptor primes cells to proliferate in response to epidermal growth factor. *Oncogene* **26,** 2585-94.

Jo, M., Thomas, K. S., Wu, L., and Gonias, S. L. (2003). Soluble urokinase-type plasminogen activator receptor inhibits cancer cell growth and invasion by direct urokinase-independent effects on cell signaling. *J Biol Chem* **278,** 46692-8.

Jones, J. T., Akita, R. W., and Sliwkowski, M. X. (1999). Binding specificities and affinities of egf domains for ErbB receptors. *FEBS Lett* **447,** 227-31.

Jones, P. H., Harper, S., and Watt, F. M. (1995). Stem cell patterning and fate in human epidermis. *Cell* **80,** 83-93.
Jones, R. B., Gordus, A., Krall, J. A., and MacBeath, G. (2006). A quantitative protein interaction network for the ErbB receptors using protein microarrays. *Nature* **439,** 168-74.
Joneson, T., McDonough, M., Bar-Sagi, D., and Van Aelst, L. (1996). RAC regulation of actin polymerization and proliferation by a pathway distinct from Jun kinase. *Science* **274,** 1374-6.
Jorissen, R. N., Walker, F., Pouliot, N., Garrett, T. P., Ward, C. W., and Burgess, A. W. (2003). Epidermal growth factor receptor: mechanisms of activation and signalling. *Exp Cell Res* **284,** 31-53.
Ju, B. G., and Kim, W. S. (1994). Pattern duplication by retinoic acid treatment in the regenerating limbs of Korean salamander larvae, Hynobius leechii, correlates well with the extent of dedifferentiation. *Dev Dyn* **199,** 253-67.
Kamat, A., and Carpenter, G. (1997). Phospholipase C-gamma1: regulation of enzyme function and role in growth factor-dependent signal transduction. *Cytokine Growth Factor Rev* **8,** 109-17.
Kawakami, Y., Esteban, C. R., Matsui, T., Rodriguez-Leon, J., Kato, S., and Izpisua Belmonte, J. C. (2004). Sp8 and Sp9, two closely related buttonhead-like transcription factors, regulate Fgf8 expression and limb outgrowth in vertebrate embryos. *Development* **131,** 4763-74.
Kawakami, Y., Rodriguez Esteban, C., Raya, M., Kawakami, H., Marti, M., Dubova, I., and Izpisua Belmonte, J. C. (2006). Wnt/beta-catenin signaling regulates vertebrate limb regeneration. *Genes Dev* **20,** 3232-7.
Keely, P. J., Westwick, J. K., Whitehead, I. P., Der, C. J., and Parise, L. V. (1997). Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. *Nature* **390,** 632-6.
Keller, G. (2005). Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes Dev* **19,** 1129-55.
Keller, P., Toomre, D., Diaz, E., White, J., and Simons, K. (2001). Multicolour imaging of post-Golgi sorting and trafficking in live cells. *Nat Cell Biol* **3,** 140-9.
Kerkela, E., and Saarialho-Kere, U. (2003). Matrix metalloproteinases in tumor progression: focus on basal and squamous cell skin cancer. *Exp Dermatol* **12,** 109-25.
Keyse, S. M. (2000). Protein phosphatases and the regulation of mitogen-activated protein kinase signalling. *Curr Opin Cell Biol* **12,** 186-92.
Khwaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P. H., and Downward, J. (1997). Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *Embo J* **16,** 2783-93.
Kim, J. E., Kim, H. S., Shin, Y. J., Lee, C. S., Won, C., Lee, S. A., Lee, J. W., Kim, Y., Kang, J. S., Ye, S. K., and Chung, M. H. (2008). LYR71, a derivative of trimeric resveratrol, inhibits tumorigenesis by blocking STAT3-mediated matrix metalloproteinase 9 expression. *Exp Mol Med* **40,** 514-22.
Kim, T. H., Mars, W. M., Stolz, D. B., Petersen, B. E., and Michalopoulos, G. K. (1997). Extracellular matrix remodeling at the early stages of liver regeneration in the rat. *Hepatology* **26,** 896-904.
King, W. G., Mattaliano, M. D., Chan, T. O., Tsichlis, P. N., and Brugge, J. S. (1997). Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitogen-activated protein kinase pathway activation. *Mol Cell Biol* **17,** 4406-18.
Kinoshita, T., Fujita, M., and Maeda, Y. (2008). Biosynthesis, remodelling and functions of mammalian GPI-anchored proteins: recent progress. *J Biochem* **144,** 287-94.
Kintner, C. R., and Brockes, J. P. (1985). Monoclonal antibodies to the cells of a regenerating limb. *J Embryol Exp Morphol* **89,** 37-55.

Klapper, L. N., Glathe, S., Vaisman, N., Hynes, N. E., Andrews, G. C., Sela, M., and Yarden, Y. (1999). The ErbB-2/HER2 oncoprotein of human carcinomas may function solely as a shared coreceptor for multiple stroma-derived growth factors. *Proc Natl Acad Sci U S A* **96,** 4995-5000.

Kleuss, C., Raw, A. S., Lee, E., Sprang, S. R., and Gilman, A. G. (1994). Mechanism of GTP hydrolysis by G-protein alpha subunits. *Proc Natl Acad Sci U S A* **91,** 9828-31.

Kobayashi, K., and Hoshi, M. (2002). Switching from asexual to sexual reproduction in the planarian Dugesia ryukyuensis: change of the fissiparous capacity along with the sexualizing process. *Zoolog Sci* **19,** 661-6.

Kragl, M., Knapp, D., Nacu, E., Khattak, S., Maden, M., Epperlein, H. H., and Tanaka, E. M. (2009). Cells keep a memory of their tissue origin during axolotl limb regeneration. *Nature* **460,** 60-5.

Kranenburg, O., and Moolenaar, W. H. (2001). Ras-MAP kinase signaling by lysophosphatidic acid and other G protein-coupled receptor agonists. *Oncogene* **20,** 1540-6.

Kumar, A., Gates, P. B., and Brockes, J. P. (2007a). Positional identity of adult stem cells in salamander limb regeneration. *C R Biol* **330,** 485-90.

Kumar, A., Godwin, J. W., Gates, P. B., Garza-Garcia, A. A., and Brockes, J. P. (2007b). Molecular basis for the nerve dependence of limb regeneration in an adult vertebrate. *Science* **318,** 772-7.

Kwak, H. B., Sun, H. M., Ha, H., Lee, J. H., Kim, H. N., and Lee, Z. H. (2008). AG490, a Jak2-specific inhibitor, induces osteoclast survival by activating the Akt and ERK signaling pathways. *Mol Cells* **26,** 436-42.

Kyriakis, J. M., App, H., Zhang, X. F., Banerjee, P., Brautigan, D. L., Rapp, U. R., and Avruch, J. (1992). Raf-1 activates MAP kinase-kinase. *Nature* **358,** 417-21.

Lafleur, M. A., Hollenberg, M. D., Atkinson, S. J., Knauper, V., Murphy, G., and Edwards, D. R. (2001). Activation of pro-(matrix metalloproteinase-2) (pro-MMP-2) by thrombin is membrane-type-MMP-dependent in human umbilical vein endothelial cells and generates a distinct 63 kDa active species. *Biochem J* **357,** 107-15.

Lange, C. A., Richer, J. K., Shen, T., and Horwitz, K. B. (1998). Convergence of progesterone and epidermal growth factor signaling in breast cancer. Potentiation of mitogen-activated protein kinase pathways. *J Biol Chem* **273,** 31308-16.

Lange-Carter, C. A., and Johnson, G. L. (1994). Ras-dependent growth factor regulation of MEK kinase in PC12 cells. *Science* **265,** 1458-61.

Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J., and Johnson, G. L. (1993). A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. *Science* **260,** 315-9.

Larsen, J. K., Yamboliev, I. A., Weber, L. A., and Gerthoffer, W. T. (1997). Phosphorylation of the 27-kDa heat shock protein via p38 MAP kinase and MAPKAP kinase in smooth muscle. *Am J Physiol* **273,** L930-40.

le Maire, M., Champeil, P., and Moller, J. V. (2000). Interaction of membrane proteins and lipids with solubilizing detergents. *Biochim Biophys Acta* **1508,** 86-111.

Lee, J. T., Steelman, L. S., Chappell, W. H., and McCubrey, J. A. (2008). Akt inactivates ERK causing decreased response to chemotherapeutic drugs in advanced CaP cells. *Cell Cycle* **7,** 631-6.

Leontovich, A. A., Zhang, J., Shimokawa, K., Nagase, H., and Sarras, M. P., Jr. (2000). A novel hydra matrix metalloproteinase (HMMP) functions in extracellular matrix degradation, morphogenesis and the maintenance of differentiated cells in the foot process. *Development* **127,** 907-20.

Leu, T. H., and Maa, M. C. (2003). Functional implication of the interaction between EGF receptor and c-Src. *Front Biosci* **8,** s28-38.

Levi, E., Fridman, R., Miao, H. Q., Ma, Y. S., Yayon, A., and Vlodavsky, I. (1996). Matrix metalloproteinase 2 releases active soluble ectodomain of fibroblast growth factor receptor 1. *Proc Natl Acad Sci U S A* **93,** 7069-74.

Levitzki, A., and Gazit, A. (1995). Tyrosine kinase inhibition: an approach to drug development. *Science* **267,** 1782-8.

Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B., and Yarden, Y. (1998). c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev* **12,** 3663-74.

Lewitzky, M., Kardinal, C., Gehring, N. H., Schmidt, E. K., Konkol, B., Eulitz, M., Birchmeier, W., Schaeper, U., and Feller, S. M. (2001). The C-terminal SH3 domain of the adapter protein Grb2 binds with high affinity to sequences in Gab1 and SLP-76 which lack the SH3-typical P-x-x-P core motif. *Oncogene* **20,** 1052-62.

Li, S., Wang, Q., Wang, Y., Chen, X., and Wang, Z. (2009). PLC-gamma1 and Rac1 coregulate EGF-induced cytoskeleton remodeling and cell migration. *Mol Endocrinol* **23,** 901-13.

Lim, C. P., and Cao, X. (1999). Serine phosphorylation and negative regulation of Stat3 by JNK. *J Biol Chem* **274,** 31055-61.

Liotta, L. A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C. M., and Shafie, S. (1980). Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature* **284,** 67-8.

Liu, D., Aguirre Ghiso, J., Estrada, Y., and Ossowski, L. (2002). EGFR is a transducer of the urokinase receptor initiated signal that is required for in vivo growth of a human carcinoma. *Cancer Cell* **1,** 445-57.

Liu, X., Brodeur, S. R., Gish, G., Songyang, Z., Cantley, L. C., Laudano, A. P., and Pawson, T. (1993). Regulation of c-Src tyrosine kinase activity by the Src SH2 domain. *Oncogene* **8,** 1119-26.

Lowenstein, E. J., Daly, R. J., Batzer, A. G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E. Y., Bar-Sagi, D., and Schlessinger, J. (1992). The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. *Cell* **70,** 431-42.

Luttrell, L. M., Daaka, Y., Della Rocca, G. J., and Lefkowitz, R. J. (1997). G protein-coupled receptors mediate two functionally distinct pathways of tyrosine phosphorylation in rat 1a fibroblasts. Shc phosphorylation and receptor endocytosis correlate with activation of Erk kinases. *J Biol Chem* **272,** 31648-56.

Maden, M. (1979). Neurotrophic and x-ray blocks in the blastemal cell cycle. *J Embryol Exp Morphol* **50,** 169-73.

Maden, M. (1982). Vitamin A and pattern formation in the regenerating limb. *Nature* **295,** 672-5.

Madsen, C. D., and Sidenius, N. (2008). The interaction between urokinase receptor and vitronectin in cell adhesion and signalling. *Eur J Cell Biol* **87,** 617-29.

Mainiero, F., Murgia, C., Wary, K. K., Curatola, A. M., Pepe, A., Blumemberg, M., Westwick, J. K., Der, C. J., and Giancotti, F. G. (1997). The coupling of alpha6beta4 integrin to Ras-MAP kinase pathways mediated by Shc controls keratinocyte proliferation. *Embo J* **16,** 2365-75.

Malbon, C. C. (2005). G proteins in development. *Nat Rev Mol Cell Biol* **6,** 689-701.

Mamane, Y., Petroulakis, E., LeBacquer, O., and Sonenberg, N. (2006). mTOR, translation initiation and cancer. *Oncogene* **25,** 6416-22.

Margolis, B., Rhee, S. G., Felder, S., Mervic, M., Lyall, R., Levitzki, A., Ullrich, A., Zilberstein, A., and Schlessinger, J. (1989). EGF induces tyrosine phosphorylation of phospholipase C-II: a potential mechanism for EGF receptor signaling. *Cell* **57,** 1101-7.

Marmor, M. D., and Yarden, Y. (2004). Role of protein ubiquitylation in regulating endocytosis of receptor tyrosine kinases. *Oncogene* **23,** 2057-70.
Marte, B. M., Rodriguez-Viciana, P., Wennstrom, S., Warne, P. H., and Downward, J. (1997). R-Ras can activate the phosphoinositide 3-kinase but not the MAP kinase arm of the Ras effector pathways. *Curr Biol* **7,** 63-70.
Mayer, B. J., and Baltimore, D. (1993). Signalling through SH2 and SH3 domains. *Trends Cell Biol* **3,** 8-13.
Mazzieri, R., D'Alessio, S., Kenmoe, R. K., Ossowski, L., and Blasi, F. (2006). An uncleavable uPAR mutant allows dissection of signaling pathways in uPA-dependent cell migration. *Mol Biol Cell* **17,** 367-78.
McCune, J. M., Namikawa, R., Kaneshima, H., Shultz, L. D., Lieberman, M., and Weissman, I. L. (1988). The SCID-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. *Science* **241,** 1632-9.
Meij, J. T. (1996). Regulation of G protein function: implications for heart disease. *Mol Cell Biochem* **157,** 31-8.
Meinkoth, J. L., Alberts, A. S., Went, W., Fantozzi, D., Taylor, S. S., Hagiwara, M., Montminy, M., and Feramisco, J. R. (1993). Signal transduction through the cAMP-dependent protein kinase. *Mol Cell Biochem* **127-128,** 179-86.
Mendes Sdos, S., Candi, A., Vansteenbrugge, M., Pignon, M. R., Bult, H., Boudjeltia, K. Z., Munaut, C., and Raes, M. (2009). Microarray analyses of the effects of NF-kappaB or PI3K pathway inhibitors on the LPS-induced gene expression profile in RAW264.7 cells: synergistic effects of rapamycin on LPS-induced MMP9-overexpression. *Cell Signal* **21,** 1109-22.
Menshikov, M., Torosyan, N., Elizarova, E., Plakida, K., Vorotnikov, A., Parfyonova, Y., Stepanova, V., Bobik, A., Berk, B., and Tkachuk, V. (2006). Urokinase induces matrix metalloproteinase-9/gelatinase B expression in THP-1 monocytes via ERK1/2 and cytosolic phospholipase A2 activation and eicosanoid production. *J Vasc Res* **43,** 482-90.
Mercader, N., Leonardo, E., Piedra, M. E., Martinez, A. C., Ros, M. A., and Torres, M. (2000). Opposing RA and FGF signals control proximodistal vertebrate limb development through regulation of Meis genes. *Development* **127,** 3961-70.
Mercader, N., Tanaka, E. M., and Torres, M. (2005). Proximodistal identity during vertebrate limb regeneration is regulated by Meis homeodomain proteins. *Development* **132,** 4131-42.
Mescher, A. L. (1976). Effects on adult newt limb regeneration of partial and complete skin flaps over the amputation surface. *J Exp Zool* **195,** 117-28.
Michalopoulos, G. K., and DeFrances, M. C. (1997). Liver regeneration. *Science* **276,** 60-6.
Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Derijard, B., Davis, R. J., Johnson, G. L., and Karin, M. (1994). Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. *Science* **266,** 1719-23.
Miyamoto, S., Akiyama, S. K., and Yamada, K. M. (1995). Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science* **267,** 883-5.
Monaghan, J. R., Epp, L. G., Putta, S., Page, R. B., Walker, J. A., Beachy, C. K., Zhu, W., Pao, G. M., Verma, I. M., Hunter, T., Bryant, S. V., Gardiner, D. M., Harkins, T. T., and Voss, S. R. (2009). Microarray and cDNA sequence analysis of transcription during nerve-dependent limb regeneration. *BMC Biol* **7,** 1.
Monaghan-Benson, E., and McKeown-Longo, P. J. (2006). Urokinase-type plasminogen activator receptor regulates a novel pathway of fibronectin matrix assembly requiring Src-dependent transactivation of epidermal growth factor receptor. *J Biol Chem* **281,** 9450-9.
Morgan, T. H. (1901). Regeneration. Macmillan Co., New York.

Moro, L., Dolce, L., Cabodi, S., Bergatto, E., Boeri Erba, E., Smeriglio, M., Turco, E., Retta, S. F., Giuffrida, M. G., Venturino, M., Godovac-Zimmermann, J., Conti, A., Schaefer, E., Beguinot, L., Tacchetti, C., Gaggini, P., Silengo, L., Tarone, G., and Defilippi, P. (2002). Integrin-induced epidermal growth factor (EGF) receptor activation requires c-Src and p130Cas and leads to phosphorylation of specific EGF receptor tyrosines. *J Biol Chem* **277,** 9405-14.

Morris, A. J., and Malbon, C. C. (1999). Physiological regulation of G protein-linked signaling. *Physiol Rev* **79,** 1373-430.

Morrison, J. I., Loof, S., He, P., and Simon, A. (2006). Salamander limb regeneration involves the activation of a multipotent skeletal muscle satellite cell population. *J Cell Biol* **172,** 433-40.

Mott, J. D., Thomas, C. L., Rosenbach, M. T., Takahara, K., Greenspan, D. S., and Banda, M. J. (2000). Post-translational proteolytic processing of procollagen C-terminal proteinase enhancer releases a metalloproteinase inhibitor. *J Biol Chem* **275,** 1384-90.

Mott, J. D., and Werb, Z. (2004). Regulation of matrix biology by matrix metalloproteinases. *Curr Opin Cell Biol* **16,** 558-64.

Mould, A. P., and Humphries, M. J. (1991). Identification of a novel recognition sequence for the integrin alpha 4 beta 1 in the COOH-terminal heparin-binding domain of fibronectin. *Embo J* **10,** 4089-95.

Mullen, L. M., Bryant, S. V., Torok, M. A., Blumberg, B., and Gardiner, D. M. (1996). Nerve dependency of regeneration: the role of Distal-less and FGF signaling in amphibian limb regeneration. *Development* **122,** 3487-97.

Munro, S. (2003). Lipid rafts: elusive or illusive? *Cell* **115,** 377-88.

Murga, C., Laguinge, L., Wetzker, R., Cuadrado, A., and Gutkind, J. S. (1998). Activation of Akt/protein kinase B by G protein-coupled receptors. A role for alpha and beta gamma subunits of heterotrimeric G proteins acting through phosphatidylinositol-3-OH kinasegamma. *J Biol Chem* **273,** 19080-5.

Murray, E. W., and Robbins, S. M. (1998). Antibody cross-linking of the glycosylphosphatidylinositol-linked protein CD59 on hematopoietic cells induces signaling pathways resembling activation by complement. *J Biol Chem* **273,** 25279-84.

Namenwirth, M. (1974). The inheritance of cell differentiation during limb regeneration in the axolotl. *Dev Biol* **41,** 42-56.

Nardi, J. B., and Stocum, D. L. (1983). Surface properties of regenerating limb cells: Evidence for gradation along the proximodistal axis. *Differentiation* **25,** 27-31.

Nemenoff, R. A., Winitz, S., Qian, N. X., Van Putten, V., Johnson, G. L., and Heasley, L. E. (1993). Phosphorylation and activation of a high molecular weight form of phospholipase A2 by p42 microtubule-associated protein 2 kinase and protein kinase C. *J Biol Chem* **268,** 1960-4.

Newmark, P. A., and Sanchez Alvarado, A. (2002). Not your father's planarian: a classic model enters the era of functional genomics. *Nat Rev Genet* **3,** 210-9.

Nguyen, D. H., Webb, D. J., Catling, A. D., Song, Q., Dhakephalkar, A., Weber, M. J., Ravichandran, K. S., and Gonias, S. L. (2000). Urokinase-type plasminogen activator stimulates the Ras/Extracellular signal-regulated kinase (ERK) signaling pathway and MCF-7 cell migration by a mechanism that requires focal adhesion kinase, Src, and Shc. Rapid dissociation of GRB2/Sps-Shc complex is associated with the transient phosphorylation of ERK in urokinase-treated cells. *J Biol Chem* **275,** 19382-8.

Nicholson, T. B., and Stanners, C. P. (2006). Specific inhibition of GPI-anchored protein function by homing and self-association of specific GPI anchors. *J Cell Biol* **175,** 647-59.

Niswander, L., Tickle, C., Vogel, A., Booth, I., and Martin, G. R. (1993). FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell* **75,** 579-87.

Oktay, M., Wary, K. K., Dans, M., Birge, R. B., and Giancotti, F. G. (1999). Integrin-mediated activation of focal adhesion kinase is required for signaling to Jun NH2-terminal kinase and progression through the G1 phase of the cell cycle. *J Cell Biol* **145,** 1461-9.

Olivier, J. P., Raabe, T., Henkemeyer, M., Dickson, B., Mbamalu, G., Margolis, B., Schlessinger, J., Hafen, E., and Pawson, T. (1993). A Drosophila SH2-SH3 adaptor protein implicated in coupling the sevenless tyrosine kinase to an activator of Ras guanine nucleotide exchange, Sos. *Cell* **73,** 179-91.

Orlichenko, L. S., and Radisky, D. C. (2008). Matrix metalloproteinases stimulate epithelial-mesenchymal transition during tumor development. *Clin Exp Metastasis* **25,** 593-600.

Osherov, N., Gazit, A., Gilon, C., and Levitzki, A. (1993). Selective inhibition of the epidermal growth factor and HER2/neu receptors by tyrphostins. *J Biol Chem* **268,** 11134-42.

Page-McCaw, A. (2008). Remodeling the model organism: matrix metalloproteinase functions in invertebrates. *Semin Cell Dev Biol* **19,** 14-23.

Page-McCaw, A., Ewald, A. J., and Werb, Z. (2007). Matrix metalloproteinases and the regulation of tissue remodelling. *Nat Rev Mol Cell Biol* **8,** 221-33.

Park, I. S., and Kim, W. S. (1999). Modulation of gelatinase activity correlates with the dedifferentiation profile of regenerating salamander limbs. *Mol Cells* **9,** 119-26.

Paulick, M. G., and Bertozzi, C. R. (2008). The glycosylphosphatidylinositol anchor: a complex membrane-anchoring structure for proteins. *Biochemistry* **47,** 6991-7000.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B. E., Karandikar, M., Berman, K., and Cobb, M. H. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* **22,** 153-83.

Pechlivanis, M., and Kuhlmann, J. (2006). Hydrophobic modifications of Ras proteins by isoprenoid groups and fatty acids--More than just membrane anchoring. *Biochim Biophys Acta* **1764,** 1914-31.

Pecorino, L. T., Entwistle, A., and Brockes, J. P. (1996). Activation of a single retinoic acid receptor isoform mediates proximodistal respecification. *Curr Biol* **6,** 563-9.

Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Grignani, F., Pawson, T., and Pelicci, P. G. (1992). A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell* **70,** 93-104.

Pescitelli, M. J., Jr., and Stocum, D. L. (1980). The origin of skeletal structures during intercalary regeneration of larval Ambystoma limbs. *Dev Biol* **79,** 255-75.

Pettitt, T. R., Martin, A., Horton, T., Liossis, C., Lord, J. M., and Wakelam, M. J. (1997). Diacylglycerol and phosphatidate generated by phospholipases C and D, respectively, have distinct fatty acid compositions and functions. Phospholipase D-derived diacylglycerol does not activate protein kinase C in porcine aortic endothelial cells. *J Biol Chem* **272,** 17354-9.

Pike, L. J. (2004). Lipid rafts: heterogeneity on the high seas. *Biochem J* **378,** 281-92.

Pilcher, B. K., Dumin, J. A., Sudbeck, B. D., Krane, S. M., Welgus, H. G., and Parks, W. C. (1997). The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix. *J Cell Biol* **137,** 1445-57.

Playford, M. P., and Schaller, M. D. (2004). The interplay between Src and integrins in normal and tumor biology. *Oncogene* **23,** 7928-46.

Potten, C. S., Booth, C., and Pritchard, D. M. (1997). The intestinal epithelial stem cell: the mucosal governor. *Int J Exp Pathol* **78,** 219-43.

Pouyssegur, J., and Lenormand, P. (2003). Fidelity and spatio-temporal control in MAP kinase (ERKs) signalling. *Eur J Biochem* **270,** 3291-9.

Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., and Ullrich, A. (1999). EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* **402,** 884-8.

Pueyo, J. I., and Couso, J. P. (2005). Parallels between the proximal-distal development of vertebrate and arthropod appendages: homology without an ancestor? *Curr Opin Genet Dev* **15,** 439-46.

Pulukuri, S. M., Gondi, C. S., Lakka, S. S., Jutla, A., Estes, N., Gujrati, M., and Rao, J. S. (2005). RNA interference-directed knockdown of urokinase plasminogen activator and urokinase plasminogen activator receptor inhibits prostate cancer cell invasion, survival, and tumorigenicity in vivo. *J Biol Chem* **280,** 36529-40.

Quesnelle, K. M., Boehm, A. L., and Grandis, J. R. (2007). STAT-mediated EGFR signaling in cancer. *J Cell Biochem* **102,** 311-9.

Ram, P. T., and Iyengar, R. (2001). G protein coupled receptor signaling through the Src and Stat3 pathway: role in proliferation and transformation. *Oncogene* **20,** 1601-6.

Ram, T. G., and Ethier, S. P. (1996). Phosphatidylinositol 3-kinase recruitment by p185erbB-2 and erbB-3 is potently induced by neu differentiation factor/heregulin during mitogenesis and is constitutively elevated in growth factor-independent breast carcinoma cells with c-erbB-2 gene amplification. *Cell Growth Differ* **7,** 551-61.

Rapoport, T. A. (1991). Protein transport across the endoplasmic reticulum membrane: facts, models, mysteries. *Faseb J* **5,** 2792-8.

Rapoport, T. A. (1992). Transport of proteins across the endoplasmic reticulum membrane. *Science* **258,** 931-6.

Ravichandran, K. S., Lee, K. K., Songyang, Z., Cantley, L. C., Burn, P., and Burakoff, S. J. (1993). Interaction of Shc with the zeta chain of the T cell receptor upon T cell activation. *Science* **262,** 902-5.

Reddien, P. W., and Sanchez Alvarado, A. (2004). Fundamentals of planarian regeneration. *Annu Rev Cell Dev Biol* **20,** 725-57.

Ren, X. D., Kiosses, W. B., and Schwartz, M. A. (1999). Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *Embo J* **18,** 578-85.

Resh, M. D. (1999). Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim Biophys Acta* **1451,** 1-16.

Resnati, M., Pallavicini, I., Wang, J. M., Oppenheim, J., Serhan, C. N., Romano, M., and Blasi, F. (2002). The fibrinolytic receptor for urokinase activates the G protein-coupled chemotactic receptor FPRL1/LXA4R. *Proc Natl Acad Sci U S A* **99,** 1359-64.

Robinson, P. J., Millrain, M., Antoniou, J., Simpson, E., and Mellor, A. L. (1989). A glycophospholipid anchor is required for Qa-2-mediated T cell activation. *Nature* **342,** 85-7.

Rodriguez-Viciana, P., Warne, P. H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M. J., Waterfield, M. D., and Downward, J. (1994). Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature* **370,** 527-32.

Rollins, S. A., and Sims, P. J. (1990). The complement-inhibitory activity of CD59 resides in its capacity to block incorporation of C9 into membrane C5b-9. *J Immunol* **144,** 3478-83.

Roovers, K., Davey, G., Zhu, X., Bottazzi, M. E., and Assoian, R. K. (1999). Alpha5beta1 integrin controls cyclin D1 expression by sustaining mitogen-activated protein kinase activity in growth factor-treated cells. *Mol Biol Cell* **10,** 3197-204.

Rosenbaum, D. M., Rasmussen, S. G., and Kobilka, B. K. (2009). The structure and function of G-protein-coupled receptors. *Nature* **459,** 356-63.

Rossman, K. L., Der, C. J., and Sondek, J. (2005). GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat Rev Mol Cell Biol* **6,** 167-80.

Rowe, D. A., Cairns, J. M., and Fallon, J. F. (1982). Spatial and temporal patterns of cell death in limb bud mesoderm after apical ectodermal ridge removal. *Dev Biol* **93,** 83-91.

Roy, S., and Levesque, M. (2006). Limb regeneration in axolotl: is it superhealing? *ScientificWorldJournal* **6 Suppl 1,** 12-25.
Sabharanjak, S., Sharma, P., Parton, R. G., and Mayor, S. (2002). GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Dev Cell* **2,** 411-23.
Sanchez Alvarado, A. (2000). Regeneration in the metazoans: why does it happen? *Bioessays* **22,** 578-90.
Sasaoka, T., Langlois, W. J., Leitner, J. W., Draznin, B., and Olefsky, J. M. (1994). The signaling pathway coupling epidermal growth factor receptors to activation of p21ras. *J Biol Chem* **269,** 32621-5.
Satoh, A., Gardiner, D. M., Bryant, S. V., and Endo, T. (2007). Nerve-induced ectopic limb blastemas in the Axolotl are equivalent to amputation-induced blastemas. *Dev Biol* **312,** 231-44.
Satoh, A., Graham, G. M., Bryant, S. V., and Gardiner, D. M. (2008). Neurotrophic regulation of epidermal dedifferentiation during wound healing and limb regeneration in the axolotl (Ambystoma mexicanum). *Dev Biol* **319,** 321-35.
Scadding, S. R., and Maden, M. (1986). Comparison of the effects of vitamin A on limb development and regeneration in the axolotl, Ambystoma mexicanum. *J Embryol Exp Morphol* **91,** 19-34.
Scadding, S. R., and Maden, M. (1994). Retinoic acid gradients during limb regeneration. *Dev Biol* **162,** 608-17.
Schafer, B., Gschwind, A., and Ullrich, A. (2004). Multiple G-protein-coupled receptor signals converge on the epidermal growth factor receptor to promote migration and invasion. *Oncogene* **23,** 991-9.
Schaller, M. D., Hildebrand, J. D., Shannon, J. D., Fox, J. W., Vines, R. R., and Parsons, J. T. (1994). Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol Cell Biol* **14,** 1680-8.
Schaller, M. D., Otey, C. A., Hildebrand, J. D., and Parsons, J. T. (1995). Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains. *J Cell Biol* **130,** 1181-7.
Schiller, M. R. (2006). Coupling receptor tyrosine kinases to Rho GTPases--GEFs what's the link. *Cell Signal* **18,** 1834-43.
Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der Geer, P. (1994). Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature* **372,** 786-91.
Schlessinger, J. (2002). Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. *Cell* **110,** 669-72.
Schmidt, A. J. (1968). Cellular Biology of Vertebrate Regeneration and Repair.
Schneider, J. W., Gu, W., Zhu, L., Mahdavi, V., and Nadal-Ginard, B. (1994). Reversal of terminal differentiation mediated by p107 in Rb-/- muscle cells. *Science* **264,** 1467-71.
Schneider, M. R., and Wolf, E. (2009). The epidermal growth factor receptor ligands at a glance. *J Cell Physiol* **218,** 460-6.
Schulze, A., Lehmann, K., Jefferies, H. B., McMahon, M., and Downward, J. (2001). Analysis of the transcriptional program induced by Raf in epithelial cells. *Genes Dev* **15,** 981-94.
Schwindinger, W. F., and Robishaw, J. D. (2001). Heterotrimeric G-protein betagamma-dimers in growth and differentiation. *Oncogene* **20,** 1653-60.
Selvatici, R., Falzarano, S., Mollica, A., and Spisani, S. (2006). Signal transduction pathways triggered by selective formylpeptide analogues in human neutrophils. *Eur J Pharmacol* **534,** 1-11.
Serrano, M., Hannon, G. J., and Beach, D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* **366,** 704-7.

Sharpless, N. E., and DePinho, R. A. (2005). Cancer: crime and punishment. *Nature* **436,** 636-7.
Shaw, L. M., Rabinovitz, I., Wang, H. H., Toker, A., and Mercurio, A. M. (1997). Activation of phosphoinositide 3-OH kinase by the alpha6beta4 integrin promotes carcinoma invasion. *Cell* **91,** 949-60.
Sieg, D. J., Hauck, C. R., Ilic, D., Klingbeil, C. K., Schaefer, E., Damsky, C. H., and Schlaepfer, D. D. (2000). FAK integrates growth-factor and integrin signals to promote cell migration. *Nat Cell Biol* **2,** 249-56.
Silva, C. M. (2004). Role of STATs as downstream signal transducers in Src family kinase-mediated tumorigenesis. *Oncogene* **23,** 8017-23.
Silvennoinen, O., Schindler, C., Schlessinger, J., and Levy, D. E. (1993). Ras-independent growth factor signaling by transcription factor tyrosine phosphorylation. *Science* **261,** 1736-9.
Simons, K., and Toomre, D. (2000). Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* **1,** 31-9.
Singer, A. J., and Clark, R. A. (1999). Cutaneous wound healing. *N Engl J Med* **341,** 738-46.
Singer, M. (1952). The influence of the nerve in regeneration of the amphibian extremity. *Q Rev Biol* **27,** 169-200.
Singer, M., and Craven, L. (1948). The growth and morphogenesis of the regenerating forelimb of adult Triturus following denervation at various stages of development. *J Exp Zool* **108,** 279-308.
Solomon, K. R., Rudd, C. E., and Finberg, R. W. (1996). The association between glycosylphosphatidylinositol-anchored proteins and heterotrimeric G protein alpha subunits in lymphocytes. *Proc Natl Acad Sci U S A* **93,** 6053-8.
Sorkin, A., and Goh, L. K. (2008). Endocytosis and intracellular trafficking of ErbBs. *Exp Cell Res* **314,** 3093-106.
Sorkina, T., Huang, F., Beguinot, L., and Sorkin, A. (2002). Effect of tyrosine kinase inhibitors on clathrin-coated pit recruitment and internalization of epidermal growth factor receptor. *J Biol Chem* **277,** 27433-41.
Soslau, G., Wallace, B., Vicente, C., Goldenberg, S. J., Tupis, T., Spotila, J., George, R., Paladino, F., Whitaker, B., Violetta, G., and Piedra, R. (2004). Comparison of functional aspects of the coagulation cascade in human and sea turtle plasmas. *Comp Biochem Physiol B Biochem Mol Biol* **138,** 399-406.
Sottrup-Jensen, L., and Birkedal-Hansen, H. (1989). Human fibroblast collagenase-alpha-macroglobulin interactions. Localization of cleavage sites in the bait regions of five mammalian alpha-macroglobulins. *J Biol Chem* **264,** 393-401.
Sparks, A. B., Rider, J. E., Hoffman, N. G., Fowlkes, D. M., Quillam, L. A., and Kay, B. K. (1996). Distinct ligand preferences of Src homology 3 domains from Src, Yes, Abl, Cortactin, p53bp2, PLCgamma, Crk, and Grb2. *Proc Natl Acad Sci U S A* **93,** 1540-4.
Spitaler, M., and Cantrell, D. A. (2004). Protein kinase C and beyond. *Nat Immunol* **5,** 785-90.
Steinfartz, S., Vicario, S., Arntzen, J. W., and Caccone, A. (2007). A Bayesian approach on molecules and behavior: reconsidering phylogenetic and evolutionary patterns of the Salamandridae with emphasis on Triturus newts. *J Exp Zoolog B Mol Dev Evol* **308,** 139-62.
Sternlicht, M. D., and Werb, Z. (2001). How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* **17,** 463-516.
Stocum, D. L. (1968). The urodele limb regeneration blastema: a self-organizing system. I. Morphogenesis and differentiation of autografted whole and fractional blastemas. *Dev Biol* **18,** 457-80.
Stocum, D. L. (1984). The urodele limb regeneration blastema. Determination and organization of the morphogenetic field. *Differentiation* **27,** 13-28.

Stocum, D. L. (1996). A conceptual framework for analyzing axial patterning in regenerating urodele limbs. *Int J Dev Biol* **40,** 773-83.
Stocum, D. L. (2001). Stem cells in regenerative biology and medicine. *Wound Repair Regen* **9,** 429-42.
Stocum, D. L. (2004). Amphibian regeneration and stem cells. *Curr Top Microbiol Immunol* **280,** 1-70.
Stokoe, D., Campbell, D. G., Nakielny, S., Hidaka, H., Leevers, S. J., Marshall, C., and Cohen, P. (1992). MAPKAP kinase-2; a novel protein kinase activated by mitogen-activated protein kinase. *Embo J* **11,** 3985-94.
Stover, D. R., Becker, M., Liebetanz, J., and Lydon, N. B. (1995). Src phosphorylation of the epidermal growth factor receptor at novel sites mediates receptor interaction with Src and P85 alpha. *J Biol Chem* **270,** 15591-7.
Strahl, T., Gille, H., and Shaw, P. E. (1996). Selective response of ternary complex factor Sap1a to different mitogen-activated protein kinase subgroups. *Proc Natl Acad Sci U S A* **93,** 11563-8.
Subczynski, W. K., and Kusumi, A. (2003). Dynamics of raft molecules in the cell and artificial membranes: approaches by pulse EPR spin labeling and single molecule optical microscopy. *Biochim Biophys Acta* **1610,** 231-43.
Symowicz, J., Adley, B. P., Gleason, K. J., Johnson, J. J., Ghosh, S., Fishman, D. A., Hudson, L. G., and Stack, M. S. (2007). Engagement of collagen-binding integrins promotes matrix metalloproteinase-9-dependent E-cadherin ectodomain shedding in ovarian carcinoma cells. *Cancer Res* **67,** 2030-9.
Tabin, C., and Wolpert, L. (2007). Rethinking the proximodistal axis of the vertebrate limb in the molecular era. *Genes Dev* **21,** 1433-42.
Tan, Y., Rouse, J., Zhang, A., Cariati, S., Cohen, P., and Comb, M. J. (1996). FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2. *Embo J* **15,** 4629-42.
Tanaka, E. M., Drechsel, D. N., and Brockes, J. P. (1999). Thrombin regulates S-phase re-entry by cultured newt myotubes. *Curr Biol* **9,** 792-9.
Tanaka, E. M., Gann, A. A., Gates, P. B., and Brockes, J. P. (1997). Newt myotubes reenter the cell cycle by phosphorylation of the retinoblastoma protein. *J Cell Biol* **136,** 155-65.
Tang, H., Kerins, D. M., Hao, Q., Inagami, T., and Vaughan, D. E. (1998). The urokinase-type plasminogen activator receptor mediates tyrosine phosphorylation of focal adhesion proteins and activation of mitogen-activated protein kinase in cultured endothelial cells. *J Biol Chem* **273,** 18268-72.
Tank, P. W. (1981). The ability of localized implants of whole or minced dermis to disrupt pattern formation in the regenerating forelimb of the axolotl. *Am J Anat* **162,** 315-26.
Tank, P. W. (1983). Skin of non-limb origin blocks regeneration of the newt forelimb. *Prog Clin Biol Res* **110,** 565-75.
Tansey, M. G., Baloh, R. H., Milbrandt, J., and Johnson, E. M., Jr. (2000). GFRalpha-mediated localization of RET to lipid rafts is required for effective downstream signaling, differentiation, and neuronal survival. *Neuron* **25,** 611-23.
Tarui, T., Andronicos, N., Czekay, R. P., Mazar, A. P., Bdeir, K., Parry, G. C., Kuo, A., Loskutoff, D. J., Cines, D. B., and Takada, Y. (2003). Critical role of integrin alpha 5 beta 1 in urokinase (uPA)/urokinase receptor (uPAR, CD87) signaling. *J Biol Chem* **278,** 29863-72.
Tarui, T., Mazar, A. P., Cines, D. B., and Takada, Y. (2001). Urokinase-type plasminogen activator receptor (CD87) is a ligand for integrins and mediates cell-cell interaction. *J Biol Chem* **276,** 3983-90.
Thoms, S. D., and Stocum, D. L. (1984). Retinoic acid-induced pattern duplication in regenerating urodele limbs. *Dev Biol* **103,** 319-28.

Thomson, D. M., Herway, S. T., Fillmore, N., Kim, H., Brown, J. D., Barrow, J. R., and Winder, W. W. (2008). AMP-activated protein kinase phosphorylates transcription factors of the CREB family. *J Appl Physiol* **104,** 429-38.

Thomson, S., Clayton, A. L., Hazzalin, C. A., Rose, S., Barratt, M. J., and Mahadevan, L. C. (1999). The nucleosomal response associated with immediate-early gene induction is mediated via alternative MAP kinase cascades: MSK1 as a potential histone H3/HMG-14 kinase. *Embo J* **18,** 4779-93.

Tiganis, T. (2002). Protein tyrosine phosphatases: dephosphorylating the epidermal growth factor receptor. *IUBMB Life* **53,** 3-14.

Turkson, J., Bowman, T., Adnane, J., Zhang, Y., Djeu, J. Y., Sekharam, M., Frank, D. A., Holzman, L. B., Wu, J., Sebti, S., and Jove, R. (1999). Requirement for Ras/Rac1-mediated p38 and c-Jun N-terminal kinase signaling in Stat3 transcriptional activity induced by the Src oncoprotein. *Mol Cell Biol* **19,** 7519-28.

Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunagaran, D., Lavi, S., Ratzkin, B. J., and Yarden, Y. (1996). A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol Cell Biol* **16,** 5276-87.

van der Geer, P., Wiley, S., Gish, G. D., and Pawson, T. (1996). The Shc adaptor protein is highly phosphorylated at conserved, twin tyrosine residues (Y239/240) that mediate protein-protein interactions. *Curr Biol* **6,** 1435-44.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3,** RESEARCH0034.

Vargesson, N., Kostakopoulou, K., Drossopoulou, G., Papageorgiou, S., and Tickle, C. (2001). Characterisation of hoxa gene expression in the chick limb bud in response to FGF. *Dev Dyn* **220,** 87-90.

Vascotto, S. G., Beug, S., Liversage, R. A., and Tsilfidis, C. (2006). Expression profiles of elastase1 (NvElastaseI) and secretory leukocyte protease inhibitor (NvSLPI) during forelimb regeneration in adult Notophthalmus viridescens suggest a role in epithelial remodeling and delamination. *Dev Genes Evol* **216,** 499-509.

Vinarsky, V., Atkinson, D. L., Stevenson, T. J., Keating, M. T., and Odelberg, S. J. (2005). Normal newt limb regeneration requires matrix metalloproteinase function. *Dev Biol* **279,** 86-98.

Walmsley, A. R., Zeng, F., and Hooper, N. M. (2003). The N-terminal region of the prion protein ectodomain contains a lipid raft targeting determinant. *J Biol Chem* **278,** 37241-8.

Wang, L., Zhang, Z. G., Zhang, R. L., Gregg, S. R., Hozeska-Solgot, A., LeTourneau, Y., Wang, Y., and Chopp, M. (2006). Matrix metalloproteinase 2 (MMP2) and MMP9 secreted by erythropoietin-activated endothelial cells promote neural progenitor cell migration. *J Neurosci* **26,** 5996-6003.

Wang, Y., Shen, Y., Li, K., Zhang, P., Wang, G., Gao, L., and Bai, C. (2009). Role of matrix metalloproteinase-9 in lipopolysaccharide-induced mucin production in human airway epithelial cells. *Arch Biochem Biophys* **486,** 111-8.

Waskiewicz, A. J., Flynn, A., Proud, C. G., and Cooper, J. A. (1997). Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *Embo J* **16,** 1909-20.

Wei, Y., Eble, J. A., Wang, Z., Kreidberg, J. A., and Chapman, H. A. (2001). Urokinase receptors promote beta1 integrin function through interactions with integrin alpha3beta1. *Mol Biol Cell* **12,** 2975-86.

Wei, Y., Tang, C. H., Kim, Y., Robillard, L., Zhang, F., Kugler, M. C., and Chapman, H. A. (2007). Urokinase receptors are required for alpha 5 beta 1 integrin-mediated signaling in tumor cells. *J Biol Chem* **282,** 3929-39.

Weiss, A., Irving, B. A., Tan, L. K., and Koretzky, G. A. (1991). Signal transduction by the T cell antigen receptor. *Semin Immunol* **3,** 313-24.
Welch, M. P., Odland, G. F., and Clark, R. A. (1990). Temporal relationships of F-actin bundle formation, collagen and fibronectin matrix assembly, and fibronectin receptor expression to wound contraction. *J Cell Biol* **110,** 133-45.
Wen, Z., Zhong, Z., and Darnell, J. E., Jr. (1995). Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell* **82,** 241-50.
Whitehead, I. P., Zohn, I. E., and Der, C. J. (2001). Rho GTPase-dependent transformation by G protein-coupled receptors. *Oncogene* **20,** 1547-55.
Wiley, H. S. (2003). Trafficking of the ErbB receptors and its influence on signaling. *Exp Cell Res* **284,** 78-88.
Wiley, H. S., Herbst, J. J., Walsh, B. J., Lauffenburger, D. A., Rosenfeld, M. G., and Gill, G. N. (1991). The role of tyrosine kinase activity in endocytosis, compartmentation, and down-regulation of the epidermal growth factor receptor. *J Biol Chem* **266,** 11083-94.
Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J., and Campbell, K. H. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature* **385,** 810-3.
Winston, L. A., and Hunter, T. (1995). JAK2, Ras, and Raf are required for activation of extracellular signal-regulated kinase/mitogen-activated protein kinase by growth hormone. *J Biol Chem* **270,** 30837-40.
Xing, J., Kornhauser, J. M., Xia, Z., Thiele, E. A., and Greenberg, M. E. (1998). Nerve growth factor activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation. *Mol Cell Biol* **18,** 1946-55.
Xu, W., Doshi, A., Lei, M., Eck, M. J., and Harrison, S. C. (1999). Crystal structures of c-Src reveal features of its autoinhibitory mechanism. *Mol Cell* **3,** 629-38.
Yablonka-Reuveni, Z., Quinn, L. S., and Nameroff, M. (1987). Isolation and clonal analysis of satellite cells from chicken pectoralis muscle. *Dev Biol* **119,** 252-9.
Yajima, H., Hara, K., Ide, H., and Tamura, K. (2002). Cell adhesiveness and affinity for limb pattern formation. *Int J Dev Biol* **46,** 897-904.
Yamamoto, K. K., Gonzalez, G. A., Biggs, W. H., 3rd, and Montminy, M. R. (1988). Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. *Nature* **334,** 494-8.
Yamashiro, D. J., Borden, L. A., and Maxfield, F. R. (1989). Kinetics of alpha 2-macroglobulin endocytosis and degradation in mutant and wild-type Chinese hamster ovary cells. *J Cell Physiol* **139,** 377-82.
Yarden, Y., and Schlessinger, J. (1987). Self-phosphorylation of epidermal growth factor receptor: evidence for a model of intermolecular allosteric activation. *Biochemistry* **26,** 1434-42.
Yarden, Y., and Sliwkowski, M. X. (2001). Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* **2,** 127-37.
Yokote, K., Mori, S., Hansen, K., McGlade, J., Pawson, T., Heldin, C. H., and Claesson-Welsh, L. (1994). Direct interaction between Shc and the platelet-derived growth factor beta-receptor. *J Biol Chem* **269,** 15337-43.
Yokouchi, Y., Nakazato, S., Yamamoto, M., Goto, Y., Kameda, T., Iba, H., and Kuroiwa, A. (1995). Misexpression of Hoxa-13 induces cartilage homeotic transformation and changes cell adhesiveness in chick limb buds. *Genes Dev* **9,** 2509-22.
Yokouchi, Y., Sasaki, H., and Kuroiwa, A. (1991). Homeobox gene expression correlated with the bifurcation process of limb cartilage development. *Nature* **353,** 443-5.
Yonei-Tamura, S., Endo, T., Yajima, H., Ohuchi, H., Ide, H., and Tamura, K. (1999). FGF7 and FGF10 directly induce the apical ectodermal ridge in chick embryos. *Dev Biol* **211,** 133-43.

Young, M. A., Gonfloni, S., Superti-Furga, G., Roux, B., and Kuriyan, J. (2001). Dynamic coupling between the SH2 and SH3 domains of c-Src and Hck underlies their inactivation by C-terminal tyrosine phosphorylation. *Cell* **105,** 115-26.

Yu, J., Fischman, D. A., and Steck, T. L. (1973). Selective solubilization of proteins and phospholipids from red blood cell membranes by nonionic detergents. *J Supramol Struct* **1,** 233-48.

Zavadil, J., and Bottinger, E. P. (2005). TGF-beta and epithelial-to-mesenchymal transitions. *Oncogene* **24,** 5764-74.

Zhang, X., Gureasko, J., Shen, K., Cole, P. A., and Kuriyan, J. (2006). An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. *Cell* **125,** 1137-49.

Zhao, J. H., Reiske, H., and Guan, J. L. (1998). Regulation of the cell cycle by focal adhesion kinase. *J Cell Biol* **143,** 1997-2008.

Zheng, C. F., and Guan, K. L. (1994). Activation of MEK family kinases requires phosphorylation of two conserved Ser/Thr residues. *Embo J* **13,** 1123-31.

Zhou, M. M., Ravichandran, K. S., Olejniczak, E. F., Petros, A. M., Meadows, R. P., Sattler, M., Harlan, J. E., Wade, W. S., Burakoff, S. J., and Fesik, S. W. (1995). Structure and ligand recognition of the phosphotyrosine binding domain of Shc. *Nature* **378,** 584-92.

9 Appendix- DNA sequences of constructs used

Newt Prod1-Flag
Cloned EcoRI/Xba into PCI-Neo, then subcloned into EcoRI/NotI of peGFP-N2

GAATTCCCGCCGCCACCATGATGCTTCTACCACTCTCCTTGTTTCTGGTGGCATG
CCTGCACTCAACTACAGCGTTAGACTACAAGGACGACGATGACAAGCTTAAATGC
TTCACCAGAAACGGAGACGACAGGACTGTGACCACCTGCGCCGAGGAACAGACT
CGATGCCTCTTCGTACAACTGCCATATTCTGAGATACAAGAATGCAAGACGGTGC
AACAGTGTGCTGAGGTGTTAGAGGAAGTCACTGCCATTGGATATCCAGCAAAGTG
CTGCTGCGAGGATCTCTGCAACCGGAGTGAGCAAGATTTTGAGACCACCACCCA
GACCACAACACTAGCATTCTTGGATGGACCACAGTGATCTAGA

Newt Prod1-Flag ΔC-terminal anchor sequence
Cloned NheI/EcoRI into PCI-Neo, then subcloned into NheI/NotI of peGFP-N2

GCTAGCCCGCCGCCACCATGATGCTTCTACCACTCTCCTTGTTTCTGGTGGCATG
CCTGCACTCAACTACAGCGTTAGACTACAAGGACgACGATGACAAGCTTAAATGCT
TCACCAGAAACGGAGACGACAGGACTGTGACCACCTGCGCCGAGGAACAGACTC
GATGCCTCTTCGTACAACTGCCATATTCTGAGATACAAGAATGCAAGACGGTGCA
ACAGTGTGCTGAGGTGTTAGAGGAAGTCACTGCCATTGGATATCCAGCAAAGTGC
TGCTGCGAGGATCTCTGCAACCGGAGTGAGCAATGAGAATTC

Newt Prod1-Flag ΔN-terminal secretory sequence
Cloned NheI/EcoRI into PCI-Neo, then subcloned into NheI/NotI of peGFP-N2

GCTAGCCCGCCGCCACCATGGACTACAAGGACGACGATGACAAGCTTAAATGCTT
CACCAGAAACGGAGACGACAGGACTGTGACCACCTGCGCCGAGGAACAGACTCG
ATGCCTCTTCGTACAACTGCCATATTCTGAGATACAAGAATGCAAGACGGTGCAAC
AGTGTGCTGAGGTGTTAGAGGAAGTCACTGCCATTGGATATCCAGCAAAGTGCTG
CTGCGAGGATCTCTGCAACCGGAGTGAGCAAGATTTTGAGACCACCACCCAGAC
CACAACACTAGCATTCTTGGATGGACCACAGTGACACGAATTC

Newt Prod1-Flag LFA anchor
GFP sequence of GPI GFP replaced by Prod1 Flag (N and C terminal signal sequences of Prod removed)

ATGGAGCTCTTTTGGAGTATAGTCTTTACTGTCCTCCTGAGTTTCTCCTGCCGGGG
GTCAGACTGGGAATCTCTGCAGTCGACGGTACCGCGGGCCCGGGATCCAGACTA
CAAGGACGACGATGACAAGCTTAAATGCTTCACCAGAAACGGAGACGACAGGACT
GTGACCACCTGCGCCGAGGAACAGACTCGATGCCTCTTCGTACAACTGCCATATT
CTGAGATACAAGAATGCAAGACGGTGCAACAGTGTGCTGAGGTGTTAGAGGAAGT
CACTGCCATTGGATATCCAGCAAAGTGCTGCTGCGAGGATCTCTGCAACCGGAGT
GAGGATCCGCGCCCAAGCAGCGGTCATTCTAGACACAGATATGCACTTATACCCA
TACCATTAGCAGTAATTACAACATGTATTGTGCTGTATATGAATGTTCTTTAAT

Axolotl Prod1-Flag
Cloned HindIII/XbaI into bluescript SK, then subcloned into EcoRI/Not I of peGFP-N2

AAGCTTGATATCGAATTCCCGCCGCCACCATGAAGCTCGTCGGCGCCTCC
CTGTTTCTGGTGGCGTTCCTGTACTCAGCTGAAGCTCTGAAGCTTTACAAG
GACGACGATGACAAGGTATGCTATACCAGGAGCAGGGATAGCGAGAGCA
CTGTGACCACATGCAGTGCAGGCAGGACACACTGCCTCTACATACAGCTA
CCAAATACTCTGATACAGGAATGCCAAACCAAGGAAGGTTGTGAGGCGTT
GCGAGAAGAGGTCATGGCCATTGGCTATCCAGTAAAATGCTGTTCGGAGG
ATCTTTGCAACTACTAAGAATCGAAGGAGGATATCTACTAAAAAGGCAACA
AAATCGTTAGAAGGCAGAAAAGACTAAATCCGCAGATTCCCCAAATGGTCC
TGAGCACCCACTAGTTCTAGA

Axolotl CD59-Flag
Cloned EcoRI/Xba1 into PCI-Neo, then subcloned into NheI/NotI of pEGF- N2

GAATTCCCGCCGCCACCATGAACCGGATCAGATGGAACGGTGGTCTATTGGCCG
TGGCAGCTGTGGTGCTGATGCTTTGTCAGTCTGGATATGCACTGAAGCTTGACTA
CAAGGACGACGATGACAAGTGCTACAACTGCTCCAATCTAAGTGATAAACCATGTT
TGAGCAATACAACTTGCCCTGCCTCAGAAGATGCCTGTCTACTGGTTGTATCTGGT
TCCCAAAAACTGACAAGCTGTTTCCCATACAACTCGTGCAACACGGAAGGTATTAC

AAaGAAATTTGGTGTCAACAGCTTCAGCTACTACTGTTGCCAAAAAGCACTCTGCA
ACAACAGTGTGATGGCCGTTGTCAATAAGACCGTTTTCAGCCTAGTCACAATGTTG
GCCCTCATGTGGATATGTTTCTGAAACGTCTAGA

Newt EGFR-Myc
Cloned EcoRI/NotI into PTL-1

GAATTCGAGCTCGGTACCCCTAATACGACTCACTATAGGGCAAGCAGTGGTATCA
ACGCAGAGTACGCGGGGTGTCACGACCTGTGCCGGTAAACGTTTATATTGGATGC
TCCTGTGAAGCCCCATTACTCCTGCCCGGGCAGGACAGAAGCATGGGTTTGTACA
GCGTGAGCCCGGAGGAGAGACCGAGGAGCCGGGCTGAGGATTGAGGCTGAGTT
GTGAAGAAGCGGACAGCAGGACAGAAATCAGCTTTGGGAGATTCAAAGAGGGGA
TTTAGGACACAATACGTTCACAAAGTCTGCAATTGGGCGTCAGGAGCAGACACTG
TGGGACAGCGATAATGAACCGCCGGGCTGCGGTGCTGCTGGTGCTGTTTATCAT
CGGACGGAGCACCTTAGCCGATGAAGAGCAGAAAGTGTGCCACGGTTCAAACAA
TAAACTCAGCTTGATTGGCGATGAGAGTTCCCATTACACCAGCATGCAAAATATAT
ATAGCAACTGTGAAATCGTGCATGGGAGTTTGGAGATCACCTTTGTGCCACACAA
CAACAATACATCGTTCGTGAAGAACATAAAGGAGGTTGGAGGCTATGTCCTTATTG
CAATGAATGACGTGAAACAAATAACGCTAGAAAATCTACAGCTAATTCGAGGAAAC
TCACTTTATATTGACAAGTACGCTTTGGTAATTATAATGAATTTTCATGCAGCGATG
GATTCGGTTGGTCTGGAACAAGTATTAATGAATAATTTATTAGAAATTCTTAACGGC
GGCGTGAGAATAGAAAAGAACCCATTTCTCTGTAATATGGATGCAATCAAATGGGA
GGACATTGTGAATACAAACGATCCAAAAAATGAAATTCTAGTTGATAAAAGAACTC
CAAAGAACTGTCCAAAATGTGATTCAAGTTGCTATCACGGATCTTGCTGGGGACC
GGGTCCTGAAAATTGTCAACGTTTTACCAAGTTGATCTGTGCCCAGCAGTGTTCC
GGGCGCTGCAGAGGAAGGATGCCAGATGACTGCTGCCACAGCCACTGCGCATCT
GGCTGCACAGGACCTCGAGAAAGCGACTGCTTGACCTGTCGCCGGTTTCGTGAT
GGTAATACATGTAAGGAGAACTGTCCACCGTTGCTGTTGTATAACCCTAATACATA
TCAAATGGATGTAAACCCAGATGGAAAATATAGTTTCGGAGCCACTTGTTTAACAA
AATGTCCACACAATTATGTTGTAACGGATCATGGCTCCTGTGTTCGCACTTGTGAT
GATAAATCCTATGAAGTAGAAGAAAATGGTGTAAGAAAATGCAAAAAATGTGATGG
ACCGTGCAACAAAGTTTGCAGTGGACTACCTACTAATGAGCGTGTCCGTGCTGTA
AATCATTTCAATATTGAGAGATTTGCAAACTGCACCACGATCCAGGGAGATATTAT
AATCCTTCCTGTTACACTAGCTGGTGATGGCTTTGAAAAAATTCCGCCTTTGGACC
ACACTAAACTCGACTATTTTAAAAATGTAAAAGAAATAACAGGGTTTTTGGCAATTC
AGGCCTGGCCTGAAAATGCAACTCATCTCCATGCTTTTGAAAACCTGGAAGTTATC
CGCGGGAGATCAAAGACTTCTGGTCGTTTCTCTCTTGCTGTGATCCAAACAAGCA

TATCATCCCTTGGGTTCCGGTCCCTCCGTGAAATAAGTGATGGAGATGTGCTCCT
GAGAAGGAATGCTCAGCTCTGTTATACAGATATAATAAATTGGACAAGTGTCTTCA
GAACAAAGAATCAAAATATTACATCTGCTCTTAATAAACCTAAAGAGAGTTGTTTGG
CAGAAAACAAAATATGTGACCTCTTATGTTCTGACAATGGCTGCTGGGGCCCAGG
ACCTTTCAGCTGCCTGTCCTGCCGCTATTTCCTTCGAATGACAGAATGTGTGGAAA
CCTGCAACATTATGAAAGGGGAGCCAAGAGAGTATGTTAAAGACTCGAAGTGTTT
TCGATGCCATCCTGAATGCCTAATTCAGAACACAACTACGACTTGCACTGGACCG
GGCCCAGATAATTGTTTGGCCTGTGCCCACTTTAAGGATGGTCCTCATTGCGTAA
AATCCTGCCCAAGTGGAATAATGGGAGAGAATGACACATACATCTGGAAATACGC
AGATGAAAACAAAGTTTGCCAGCTGTGTCATGTTAATTGCACCGAAGGGTGCTTTG
GACCAGATTTGGAAGGCTGTCCGGATTCAGGATCAAGAATCCCATCCATTGCTGC
TGGAGTGTTTGGAGGCATCCTGTGTGTGGTCATTATAAGCTTATGTGTTGGATTTT
TCGTTCGCCGAATCCGTATACAGAGGAAACGCACTTCGCGGAGGTTACTCAATGA
AAAAACGTTAGTTGTGCCTTTAACTCCCAGCGGTGAAGCACCAAATCAAGCACTTC
TGAGAATCCTAAAAGAAACGGAAATAAAAAAAATCATGGTTCTTGGTTCAGGAGCT
TTTGGCACTGTATTTAAGGGATTGTGGCTTCCAGAAGGGGAACATGTAAAAATCCC
TGTAGCCATTAAGGAATTGAGAGAGGCTACATCCCCTAAAGCCAACAAGGAAATT
CTGGATGAGGCATATGTGATGGCCAGTGTGAACGATGCCCACGTCTGTCGTTTGC
TGGGTATCTGCCTTACCTCCACCGTCCAGCTGGTCACCCAACTCATGCCCTATGG
TTGTCTTCTTGACTACGTACGCGAGCACAAGGATAACATTGGCTCAAACCTTCTCC
TGAACTGGTGTGTTCAGATTGCAAAGGGAATGAATTACTTGGAAGAACGTCGACT
GGTACATCGTGACCTTGCAGCTAGAAATGTGCTTGTCCGAAATCCACAGCATGTC
AAGATCACAGATTTTGGACTTGCCAAACTGCTGGGTGCTGAGGAGAAAGAATACC
ATGCAGAGGGTGGCAAGGTGCCCATTAAATGGATGGCTTTAGAATCTATTCTTCAT
CGGATTTATACACACCAAAGTGATGTCTGGAGTTATGGTGTAACAGTTTGGGAGTT
GATGACTTTTGGCTCAAAACCTTATGATGCAATTCCTGCAGGTGAAATTCCAAATC
TTTTGGAACAAGGGGAACGCCTGCCACAGCCCCCCATCTGCACTATTGATGTTTA
TATGATCATGGTCAAATGCTGGATGATTGACGCAGACAGCCGTCCCAAATTCCGT
GAGCTGACTGCCGAATTCACCACAATGGCTCGTGATCCCCAGCGCTATTTGGTCA
TACAGAATGACGAGAGGATGGAGCTACCTAGTCCAGAAGACACCAAGTTTTATCG
CACCTTGATAGAGGAGGGTGAACTAGAGGAAGTAATTGATGCAGACGAGTATCTT
GTACCACACCAGGGCTTTTTCAACAGCCCGGCCACATCCCGCACTCCACTTCTCA
ACTCAGTGAGTACCACCAGTAATACTTCAGACATTGCCTTCATCAACAGAAACGGG
GGACCACCCACGAGAGAAGATAGCTTTGTTCAAAGGTACAGCTCGGATCCGACTG
TAATCCTGCAAGACGAAAACACCGATGATAGATTCCTGCCTGCACCAGAATACGT
GAACCAATTTATTTCCAAAAGGCCAAGTGAATCAAATGTACAGAATCCAGTCTACC
ACAATCTGGGACTTATTCTGTCTCCGACAACTTACCAGTACCAGAACTCCCAAAGC

ATGGCCGTGAACAACCCTGAGTACTTAAACACAGTTCATCCTTTCATGACGAATGG
CACTGGCACTGGTGAATTATGGGACCAGCAAGAGAACCACCAGATTAGTCTTGAC
AACCCAGACTACCAACAAGACTTCTTCCCCAAGGAATCTAAGACTAATGGCATTCT
GTTGCTTTCCATAGCAGAAAACCCTGAGTATTTGGAAGCGGGAGCACCCGAACAA
AAACTCATCTCAGAAGAGGATCTGTAGCGGC

Newt β1 integrin-Myc
Cloned EcoRI/NotI into pTL-1

GAATTCCGCTGCCTGCCAAGATGGCTTACAAGCTGGCCTTCCTGGCAGTACTCTT
TTGTTCTGTTTCGAATGGACTTGCTCAACAAGGTGGCAGTGAGTGTCTAAAAGCG
AATGCAAAGTCGTGTGGAGAATGTATACAAGCAGGGCCAAACTGTGGATGGTGCA
CAAATTTGGACTTTTTACAAGAAGGAAAACCAACTTCAGCAAGATGTGATGATATA
GCAACGTTAAAGAGTAAGGGATGTAAAGAATCAGAAATTGAAAATCCCAGAGGTA
GCCAAAGAATGCGTGAAAATAAACCATTGACAAACCGCAGCAAAGACACTGCAGA
AAAACTGAAACCAGAGGACATCACTCAGATCCGCCCTCAAAAGATGGAATTAAAA
CTTCGATCAGGAGAACCACAAACTTTTACTTTAAAATTCAAGAGAGCTGAAGACTA
CCCAATTGATCTGTATTATCTGATGGACTTGTCCTTTTCAATGAAGGATGATTTAGA
GAATGTGAAAAGTCTTGGAACTGCTCTCATGAGTGAAATGAAGAAAATTACCTCCG
ATTTTAGAATTGGTTTCGGTTCTTTTGTGGAGAAAACAGTGATGCCTTACATCAGTA
CGACCCCTGCTAAGCTCCTGAACCCTTGCACTGGTGACCAAAACTGCACTAGCCC
CTTTAGCTACAAAAATGTGCTTAATCTTACGAGCGATGGCAGTCTATTCAATGAGC
TTGTAGGAAAACAGCACATTTCTGGCAATTTGGATTCTCCTGAAGGTGGATTTGAT
GCCATAATGCAGGTTGCTGTTTGTGGTGAACAAATCGGTTGGAGAAATGTTACTC
GTTTATTGGTATTTTCCACTGATGCTGGTTTTCATTTTGCTGGCGATGGTAAACTTG
GTGGAATCGTTTTACCAAATGATGGCAAATGTCACCTGGAAGACAATATGTACACA
ATGAGCCATTATTACGATTATCCTTCCATTGCTCATCTTGTGCAGAAGCTAAGTGA
AAACAATATTCAAACCATATTTGCCATTACAGAGGAATTTCAGCCTGTGTACAAGG
AGTTGAAAAATCTTATTCCAAAATCTGCTGTGGGAACGCTTTCATCAAATTCCAGC
AATGTGATTCATCTGATTATCGACTCTTATAATTCTTTATCTTCAGAGGTCATCTTG
GAAAACAGCAAGCTTCCGGAGGGTGTTACCATAAGCTACAAGTCCTTTTGTAAAAA
TGGTGTGAATGGTACTGGAGAAAACGGAAGAAAGTGTGCCAACATATCAATTGGA
GATTCGGTGCAATTTGAAATTAGTATCACTGCTCATAAGTGCCCAAAGAAAGGACA
AGATGAAACTATTAAAATCAAGCTGCTGGGATTCACTGAGGAAGTGGAGGTTGCA
CTTCAGTTCATTTGTGAATGTGACTGCCAAAATACAGGCATTCCAAATAGTGCAGA
CTGTTTCAATGGGAATGGAACATTTGAATGTGGAGCATGCAGATGCAATGAAGGA
CGTATTGGTAGAATCTGTGAATGCAGAACTGACGAAGTGAATAGTGACAATATGG

ATGCTTTCTGTAGAAAAGACAATGCTTCAGAAATTTGCAGTAATAATGGAGAGTGT
ATTTGTGGCCAATGTGTATGCAAAGAACGGGAAAATCCAAACGAAATCTACTCTGG
AAAGTTCTGTGAATGTGACAACTTTAACTGTGATAGATCGAACGGTTTGATTTGTG
GAGGAAATGGAATTTGTGATTGTCGAGTATGTAAGTGCTTCCCTAACTTCACTCGT
AGTGCCTGTGACTGTTCACTGGACACAACTAGTTGTGTTGCAACAAATGGCCAGA
TTTGTAATGGTCGTGGTACCTGTGATTGTGGACGGTGTAAATGCACAGATCCAAAA
TTCCAAGGAGCCACCTGCGAATTGTGTCAGACGTGTCCTGGTGTATGTGCTGAAC
ACAAGGAATGTGTGCAGTGCAAAGCTTTCAACAAAGGAGAAAAGAAAGATGTTTG
TGAACAGGAGTGCAAGCATTTCAACACTACAATTGTAGAAAGTCGGGACAAGTTA
CCACAGCCAGGCCAGGCTGATGCTCTGACGGTCTGTAAAGAGAAAGATGCTGAT
GACTGTTGGTTCTACTTCACATACTCTGTAAATGGAACCAATGAAGTTGTTGTCCA
CGTGGTAGACAAACCAGAGTGTCCCAGTGGTCCTGACATAATTCCCATTGTAGCT
GGTGTAGTTGCTGGAATTGTTCTCATTGGCCTTGCATTGCTGCTGATCTGGAAGCT
ACTAATGATCATTCATGACAGAAGAGAGTTTGCTAAGTTCGAAAAGGAGAAATCGA
ATGCCAAGTGGGACACAGGTGAAAATCCAATTTATAAAAGTGCGGTGACAACAGT
GGTGAATCCTAAATACGAGGGAAAAGAACAAAAACTTATTTCTGAAGAAGATCTGT
AGGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGA

GFP anchored with LFA3 GPI anchor (GPI GFP) (Keller et al., 2001)

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGA
GCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGG
GCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGC
TGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCT
TCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCC
CGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAA
GACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGC
TGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGT
ACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCAT
CAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGC
CGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGA
CAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCG
CGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCAT
GGACGAGCTGTACAAGTATGATCCGCGCCCAAGCAGCGGTCATTCTAGACACAG
ATATGCACTTATACCCATACCATTAGCAGTAATTACAACATGTATTGTGCTGTATAT
GAATGTTCTTTAA