# Functional convergence of signalling by GPI-anchored and anchorless forms of a salamander protein implicated in limb regeneration

**Robert A. Blassberg[1], Acely Garza-Garcia[2], Azara Janmohamed[1], Phillip B. Gates[1] and Jeremy P. Brockes[1,*]**

[1]Institute of Structural and Molecular Biology, University College London, Gower Street, London WC1E 6BT, UK
[2]Division of Molecular Structure, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
*Author for correspondence (j.brockes@ucl.ac.uk)

*Accepted 6 September 2010*
*Journal of Cell Science 124, 47-56*

*doi:10.1242/jcs.076331*

## Summary

The GPI-anchor is an established determinant of molecular localisation and various functional roles have been attributed to it. The newt GPI-anchored three-finger protein (TFP) Prod1 is an important regulator of cell behaviour during limb regeneration, but it is unclear how it signals to the interior of the cell. Prod1 was expressed by transfection in cultured newt limb cells and activated transcription and expression of matrix metalloproteinase 9 (MMP9) by a pathway involving ligand-independent activation of epidermal growth factor receptor (EGFR) signalling and phosphorylation of extracellular regulated kinase 1 and 2 (ERK1/2). This was dependent on the presence of the GPI-anchor and critical residues in the α-helical region of the protein. Interestingly, Prod1 in the axolotl, a salamander species that also regenerates its limbs, was shown to activate ERK1/2 signalling and MMP9 transcription despite being anchorless, and both newt and axolotl Prod1 co-immunoprecipitated with the newt EGFR after transfection. The substitution of the axolotl helical region activated a secreted, anchorless version of the newt molecule. The activity of the newt molecule cannot therefore depend on a unique property conferred by the anchor. Prod1 is a salamander-specific TFP and its interaction with the phylogenetically conserved EGFR has implications for our view of regeneration as an evolutionary variable.

**Key words:** EGFR, GPI-anchor, MMP9, Regeneration, Salamander

## Introduction

The glycolipid glycosylphosphatidylinositol (GPI) (Paulick and Bertozzi, 2008) is covalently attached to the C-terminal amino acid of proteins that express the appropriate signal sequence (Eisenhaber et al., 1998). This process occurs at the inner leaflet of the endoplasmic reticulum (ER), leading to membrane localisation of the target protein (Eisenhaber et al., 2003) and entry into specific trafficking pathways (Castillon et al., 2009). In addition to its role as a membrane anchor, there is evidence that the GPI moiety acts as a crucial determinant of signalling activity. This might depend on the ability of the GPI-anchor to localise proteins to specific membrane microdomains through its interaction with other membrane lipids, whereas anchorless versions of the proteins would not localise in this way (Helms and Zurzolo, 2004; Pike, 2004; Lingwood and Simons, 2010). These microdomains are proposed to include lipid-anchored proteins in both leaflets of the membrane (Brown and Rose, 1992; Resh, 1999) and other specific membrane proteins, thereby determining potential signalling interactions (Simons and Toomre, 2000).

The GPI-anchored protein Prod1 is a focus of investigation into the cellular and molecular mechanisms underlying limb regeneration in salamanders. Newt Prod1 was originally identified in a differential screen for proteins regulated by retinoic acid and implicated in proximodistal (PD) identity (da Silva et al., 2002). It is expressed in a PD gradient in normal and regenerating limbs (da Silva et al., 2002; Kumar et al., 2007a), and overexpression in distal stem (blastemal) cells of the larval axolotl during regeneration converts them to more proximal cells (Echeverri and Tanaka, 2005). The division of limb blastemal cells is dependent on the presence of regenerating nerve axons (Singer, 1952; Tassava and Mescher, 1975; Satoh et al., 2009; Brockes, 1984), and Prod1 is also implicated in the mechanism of nerve dependence. A protein called newt anterior gradient (nAG), a member of the AG family (Sive et al., 1989), was identified as a binding partner of Prod1, and its overexpression is able to rescue a denervated blastema and allow it to regenerate in the absence of the nerve (Kumar et al., 2007b). The various assays for its activity suggest that Prod1 can apparently regulate cell adhesion, movement and division during regeneration (da Silva et al., 2002; Echeverri and Tanaka, 2005; Kumar et al., 2007b).

Newt Prod1 has an N-terminal signal sequence that directs the protein to the ER, and a C-terminal sequence that is cleaved upon attachment of its GPI-anchor. A fraction of the protein is released from the cell surface after digestion with phosphatidylinositol-specific phospholipase C (PIPLC) (da Silva et al., 2002). The structure of the protein has been determined by NMR and consists of a three-finger protein (TFP) domain with four of the five canonical TFP disulfide bonds (Garza-Garcia et al., 2009). The TFP fold is found in many secreted, transmembrane and GPI-anchored proteins and acts as a versatile scaffold that supports a great variety of specific protein–protein interactions (Galat, 2008; Tsetlin, 1999). One distinctive feature of the TFP domain of Prod1 is the presence of a 12-residue α-helical segment between the fourth and the fifth β-strands, forming part of the third finger. Shorter helical segments in equivalent positions are found in the mammalian GPI-anchored protein CD59, and in the C-terminal TFP domain of the urokinase plasminogen activator receptor (uPAR) (Garza-Garcia et al., 2009). CD59 was initially proposed

Journal of Cell Science

as the mammalian orthologue of Prod1 (da Silva et al., 2002), but more recent data from EST sequencing (Putta et al., 2004) and phylogenetic analyses have identified salamander CD59. At present it is considered that Prod1 is a salamander-specific protein (Garza-Garcia et al., 2009; Garza-Garcia et al., 2010).

As it is a GPI-linked protein, the question arises as to how newt Prod1 communicates with the interior of the cell to regulate cell behaviour. It is likely that it interacts with one or more transmembrane proteins that mediate signal transduction, and the identification of such components would be an important step in our understanding of Prod1. The GPI-anchored TFPs uPAR (Andreasen et al., 1997; Ossowski and Aguirre-Ghiso, 2000; Blasi and Carmeliet, 2002) and CD59 (Davies et al., 1989) share the greatest degree of structural homology with Prod1 out of all known mammalian proteins (Garza-Garcia et al., 2009). CD59 interacts with the epidermal growth factor receptor (EGFR) (Blagoev et al., 2003) and uPAR interacts with both the EGFR (Liu et al., 2002; Mazzieri et al., 2006; Jo et al., 2003; Jo et al., 2005) and integrin pathways (Wei et al., 1996; Wei et al., 2001; Aguirre-Ghiso et al., 2001; Ghosh et al., 2006) to activate ERK1/2 MAP kinase signalling and stimulate transcription of matrix metalloproteinase 9 (MMP9) (Ahmed et al., 2003; Wei et al., 2007). MMP9 is also expressed during limb regeneration in salamanders, and MMP activity has been shown to be necessary for regeneration (Yang et al., 1999; Vinarsky et al., 2005). Persistent MMP9 expression in the epidermis following wounding is also correlated with characteristic changes in the ECM associated with blastema formation (Satoh et al., 2008). We found that newt Prod1 has related activities to uPAR, and these can be mapped to residues in the α-helical segment. It is noteworthy that Prod1 in two Ambystomatid species has a closely related structure but is anchorless. Nonetheless, both also activate the MMP9 pathway, thus raising questions about the significance of the GPI-anchor for the signalling activity of these proteins. The interaction between Prod1 and the EGFR is an interesting pointer for understanding the evolution of regeneration in salamanders.

## Results

### Prod1 induces MMP9 expression in newt B1H1 cells

Newt B1H1 cells are an immortal mesenchymal line originally derived from an adult limb blastema (Ferretti and Brockes, 1988). These cells constitutively express newt EGFR (ErbB1; supplementary material Fig. S1A,B), and show activation of ERK1/2 phosphorylation after exposure to mammalian EGF (supplementary material Fig. S2), but do not express detectable

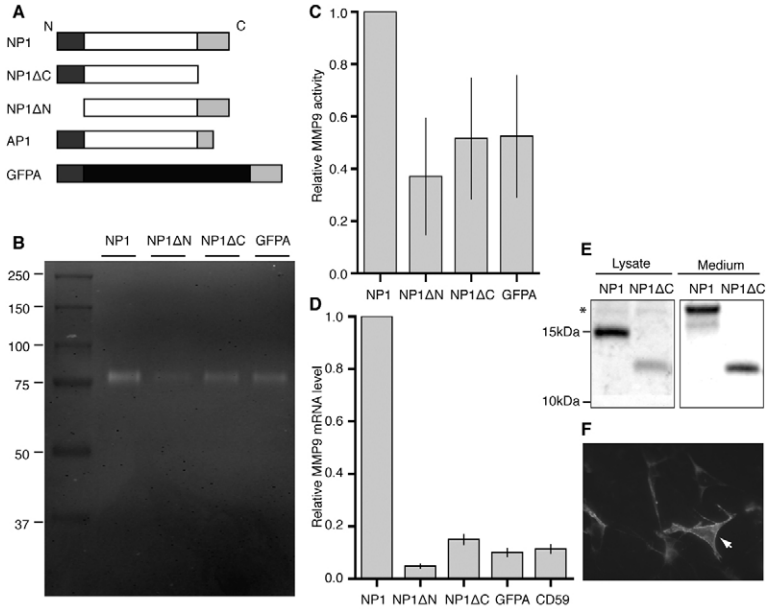


**Fig. 1. Induction of MMP9 expression in cultured cells by newt Prod1.** (**A**) Newt Prod1 (NP1) is synthesised as a precursor with N- and C-terminal sequences that are removed during post-translational processing of the protein. NP1ΔC lacks the C-terminal GPI-anchor attachment sequence, whereas NP1ΔN lacks the N-terminal signal sequence required for entry to the endoplasmic reticulum. Axolotl Prod1 (AP1) has a truncated GPI-anchor attachment sequence. GFPA is a GFP molecule with N-terminal signal and C-terminal GPI-anchor attachment sequences. B1H1 cells were transfected with Prod1 (NP1), Prod1 deleted for its N-terminal signal peptide (NP1ΔN), Prod1 deleted for its C-terminal anchor attachment peptide (NP1ΔC), CD59 (CD59) or GPI-anchored GFP (GFPA). (**B**) Cells were serum starved for 72 hours and MMP activity in the conditioned medium was assayed by zymography. (**C**) The secreted MMP9 enzyme activity normalised to NP1. The mean ± s.d is shown for six independent experiments. (Owing to variability, no single zymogram as represented by B exactly reproduces the mean quantification of MMP9 enzyme activity for all samples.) (**D**) Cells were serum starved as above and *MMP9* expression was quantified relative to NP1 by qRT-PCR, with *GAPDH* as the normalising sequence. The expression is shown relative to NP1 for eight independent experiments. (**E**) Expression of NP1 and NP1ΔC after transfection with FLAG-tagged constructs. Equivalent proportions of the total concentrated culture medium and cell lysate from each sample were analysed by immunoblotting with anti-FLAG. The NP1ΔC band in medium and lysate corresponds to the 67-residue species indicated in Fig. 5A. The NP1 band in the lysate corresponds to the GPI-anchored species (70 residues + anchor) and migrated identically to an anchored Prod1 derived from a construct with the LFA3 anchor signal sequence (Keller et al., 2001), whereas the NP1 band in the medium corresponds to the fraction of the protein (87 residues) that has retained the anchor signal and entered the secretory pathway but has not been derivatised with the anchor and hence is secreted into the medium. A non-specific band (marked *) confirms equal total protein loading of each sample. (**F**) Expression of FLAG-tagged NP1 Prod1 on the surface of B1H1 cells as determined by antibody labelling of live cells followed by immunofluorescent detection with TSA amplification (see the Materials and Methods). The arrow indicates surface labelling outlining one cell, whereas the other cells are negative for surface labelling.

levels of Prod1. After transient transfection with Prod1 expression constructs, gelatinase activity was detected in the medium by zymography and the activity could be inhibited by the MMP inhibitor GM6001. The enzyme migrated at an apparent molecular size of 89 kDa (Fig. 1A), consistent with its identification as MMP9 (Vinarsky et al., 2005). MMP9 activity after transfection with wild-type (NP1) Prod1 was higher than parallel transfections with Prod1 constructs missing either the N-terminal signal sequence (NP1ΔN), or the C-terminal anchor attachment sequence (NP1ΔN). Transfections with a GPI-anchored GFP construct (GFPA) were used as a negative control (Fig. 1A,B).

The induction of MMP9 expression was confirmed by qRT-PCR. The assay indicated an approximately tenfold difference in induction of *MMP9* mRNA between NP1 Prod1 and the other constructs. Notably, overexpression of the structurally similar salamander CD59 also failed to induce *MMP9* mRNA (Fig. 1C). The introduction of an N-terminal FLAG peptide into Prod1 had no effect on the induction of *MMP9* expression, and after transfection of newt B1H1 cells with FLAG-tagged versions of NP1 and NP1ΔC Prod1, the cell lysates and conditioned medium were analysed by immunoblotting. NP1ΔC Prod1 was predominantly secreted into the medium, whereas NP1 Prod1, because it is anchored, remained associated with cells; also, a fraction of unanchored Prod1 that had not undergone removal of the C-terminal anchor signal was detected in the medium (Fig. 1D). The expression of FLAG-tagged NP1 Prod1 on the cell surface was also detected after transfection by antibody labelling of live B1H1 cells (Fig. 1E). We conclude from this analysis that MMP9 expression was only induced above the background level seen in B1H1 cells by NP1 Prod1.

### Activation of ERK1/2 and EGFR signalling is implicated in MMP9 induction

B1H1 cells were transfected with the different Prod1 constructs, serum starved for 72 hours, and the cell lysates were analysed by immunoblotting with antibodies against phosphorylated ERK1/2 and ERK1/2. Significant activation of ERK1/2 occurred only with the NP1 Prod1 construct (Fig. 2A,B). To evaluate the significance of ERK1/2 activation and EGFR signalling for MMP9 induction, B1H1 cells were transfected with NP1 Prod1 and the GFPA control construct and exposed to an inhibitor of ERK1/2 phosphorylation (U0126), or an inhibitor of EGFR signalling (AG1478), before analysis by qRT-PCR (Fig. 3A). The results were normalised to the activation by NP1 Prod1 in the presence of DMSO (vehicle) and showed that approximately 50% of the MMP9 induction activity was dependent on ERK1/2 signalling and about 35% on EGFR signalling (Fig. 3A). In control analyses in B1H1 cells, it was found that both inhibitors could act to completely inhibit ERK1/2 activation by EGFR signalling at the concentrations used here. U0126 and AG1478 also inhibited the level of secreted MMP9 in Prod1-transfected cells, assayed by gelatin zymography (Fig. 3B). U0126 led to a statistically significant ($P$=0.0195) inhibition of the level of secreted MMP9 in control GFPA-transfected cells; however, we were unable to establish whether the background level of ERK1/2 activation observed in GFPA-transfected B1H1 cells (Fig. 2A) was linked to a low level of endogenous Prod1 expression. Consistent with this possibility is the observation that in AL1 cells, in which endogenous Prod1 is readily detectable, Prod1 transfection led to a comparable induction of *MMP9* above the background level (Fig. 6A, Fig. 8B). The magnitude of the inhibition of secreted MMP9 levels by U0126 is more than twofold greater in Prod1-transfected compared with GFPA-transfected cells, indicating that the activation of ERK1/2 by Prod1 (Fig. 2A) upregulates MMP9 secretion in Prod1-transfected cells. We conclude that ERK1/2 and EGFR activation are significant components of the Prod1 response at the level of mRNA expression and subsequent protease secretion, but can only account for part of the MMP9-inducing activity.

Signalling by a number of GPI-anchored proteins is mediated by Src kinase activation (Mouillet-Richard et al., 2000; Murray and Robbins, 1998; Wei et al., 2007). We investigated the role of Src in the Prod1 signalling cascade and found that the Src kinase inhibitor PP2 showed no inhibition of *MMP9* induction when assayed by qRT-PCR (supplementary material Fig. S3). From this we conclude that Src kinase activity is not a component of the mechanism of MMP9 induction by Prod1.

The activation of the MMP9 pathway by Prod1 on the cell surface could result from the *cis* activation of molecules like the EGFR on the same cell, or possibly by *trans* activation due to contact with an adjacent cell expressing the EGFR. When the cell density of NP1 Prod1-transfected B1H1 cells was varied from confluence (all cells in contact with at least one neighbor) to 10% confluence (most cells not in contact) this had no effect on MMP9 induction as assayed by zymography (Fig. 3C). We conclude that activation by cell contact is unlikely to be significant under our experimental conditions.

The activation of the MMP9 pathway by Prod1 could be due to the induction of secreted ligands that act on B1H1 cells in an autocrine fashion. In repeated attempts to detect such ligands by concentration of conditioned medium, or by co-culture (Fig. 6C,D;

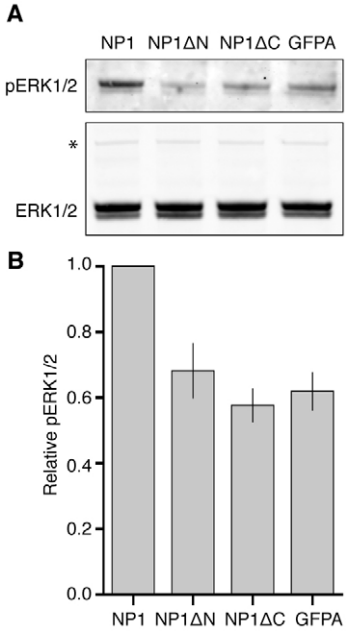


**Fig. 2. Activation of ERK1/2 signalling by Prod1.** (**A**) B1H1 cells were transfected with NP1, NP1ΔN, NP1ΔC and GFPA constructs as described, serum starved for 72 hours, and cell lysates were immunoblotted with anti-phosphorylated ERK1/2 or anti-ERK1/2 antibodies. A non-specific band (marked *) confirms equal total protein loading of each sample. Note that ERK1/2 activation over control levels occurred only with NP1 Prod1. (**B**) The results for ERK1/2 activation after starvation for 72 hours are plotted as means ± s.e.m. of seven independent experiments. Comparable results were obtained for cells subjected to 24 hour starvation.

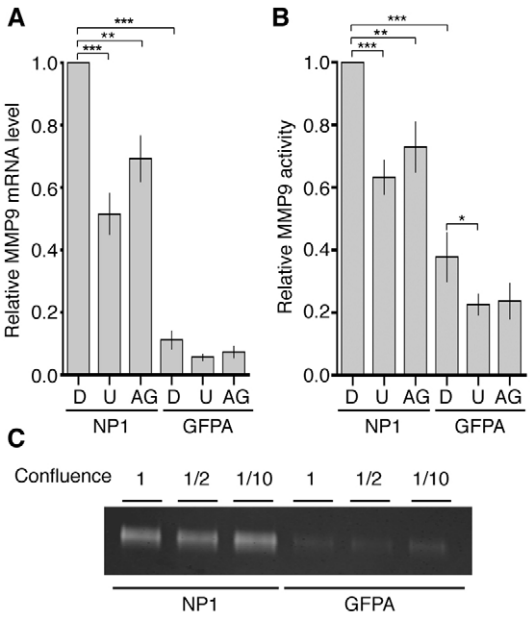


**Fig. 3. Effect of signalling inhibitors and cell density on induction of MMP9 expression by Prod1.** B1H1 cells were transfected with NP1 or GFPA, serum starved for 24 hours and treated with U0126 (U), AG1478 (AG), or DMSO vehicle (D) for 48 hours, before analysis of cDNA by qRT-PCR (**A**) or analysis of the culture medium by zymography (**B**). The value in each experiment is normalised to that for DMSO/NP1. The values for are given as means ± s.e.m. of nine independent experiments (A), or of eight independent experiments (B). **P*<0.05; ***P*<0.01; ****P*<0.001. (**C**) Equal numbers of NP1- or GFPA-transfected B1H1 cells were plated in dishes at different densities, as indicated, for 48 hours. The conditioned medium was analysed by zymography and the results are representative of three independent experiments.

supplementary material Fig. S4), no such activity was detected. We conclude that activation of transmembrane signalling by a receptor such as the EGFR is likely to proceed whereby Prod1 acts in an intermolecular complex, and direct evidence for such an association will be presented below in immunoprecipitation experiments.

### Identification of amino acid residues that are critical for Prod1 signalling activity

B1H1 cells were transfected with a series of Prod1 constructs each carrying a single amino acid substitution predicted not to disrupt the 3D structure of the TFP domain (see the Materials and Methods section). *MMP9* expression was normalised to NP1 Prod1 (Fig. 4A) and the positions of the mutations are shown in a ribbon representation of the 3D structure of Prod1 (Fig. 4B). The mutations had little or no effect on the expression of *MMP9* (shown in green in Fig. 4A,B), an intermediate effect (yellow), or reduced the expression to the level of the GFPA control (red). Strikingly, the latter two mutations are found in the α-helical segment of Prod1 (Fig. 4B) and both also reduced ERK1/2 activation to the level of the GFPA control (Fig. 4C). Several lines of evidence indicate that this is not a consequence of degradation or mislocalisation of this class of mutant. Human embryonic kidney 293T cells, which stably expressed NP1 and Q59A, showed comparable levels of Prod1 protein, whereas expression of Q59A and E66A in transiently transfected B1H1 cells was confirmed by western blotting of cell lysates (not shown). Furthermore both mutant proteins trafficked to the cell surface of B1H1 cells and were detectable by reacting live cells with anti-FLAG antibody (Fig. 4D). Finally, it is shown later that equivalent mutations at residue 60 of axolotl Prod1 rendered it unable to induce *MMP* transcription or activate ERK1/2 without diminishing the level of its expression in transfected B1H1 cells (Fig. 8A–D). These results underline the functional importance of the residues located in the α-helical segment.

### Comparison of newt and axolotl Prod1 proteins

The axolotl Prod1 sequence was identified by searching the *Ambystoma* EST database using nucleotide BLAST. Subsequent investigations have also identified the orthologue in the related species *A. maculatum*. These sequences are aligned in Fig. 5A along with newt NP1 Prod1 and newt NP1ΔC Prod1. After removal of the N-terminal signal sequence, the newt NP1 Prod1 translation product (87 residues) might have its C terminal anchor signal removed and GPI anchor added to give the mature protein (70 residues). In the axolotl and *A. maculatum*, it is striking that there are stop codons at distinct locations that yield mature proteins of 67 and 75 residues, respectively, which are predicted to be secreted. Despite extensive searches in the axolotl for an alternate anchored form, this has not been identified. Axolotl and newt Prod1 amino acid sequences are 57% identical, suggesting that they both have a very similar 3D structure that includes the long α-helix (Fig. 5B).

FLAG-tagged versions of newt NP1 Prod1, newt NP1ΔC Prod1 (69 residues) and axolotl Prod1 (AP1) were expressed in B1H1 cells and the cell lysates and conditioned medium were analysed by immunoblotting. The expression of newt NP1 and NP1ΔC constructs was as described earlier (Fig. 1D), and the axolotl AP1 Prod1, similarly to NP1ΔC, was secreted into the medium (Fig. 5C). To assay their activities, the three proteins were expressed along with the control GPI-anchored GFP in both newt B1H1 cells and axolotl AL1 cells, and the activation of the MMP9 pathway was analysed by qRT-PCR (Fig. 6A,B). The results were normalised relative to newt NP1 Prod1 and showed that newt NP1 and axolotl AP1 proteins are comparably active in both cell types (Fig. 6A,B) whereas the NP1ΔC Prod1 species had no activity relative to the GFPA control, as before (see Fig. 1C).

The activity of the axolotl Prod1 could depend on the ability of the secreted protein in the medium to bind to its co-receptors at the cell surface, or the ability of the protein to complex with its binding partners in the secretory pathway, for example in the ER or Golgi complex (Castillon et al., 2009). To investigate the potential activity of the secreted protein, B1H1 cells were transfected with newt NP1 Prod1 or axolotl AP1 Prod1, or GFPA control, and cultured on a membrane with 8 μm pores to share the medium with untransfected B1H1 cells in the same well. The Prod1-transfected cells were strongly activated for *MMP9* expression (Fig. 6C), but there was no detectable activation of the cells exposed to conditioned medium from either newt or axolotl examples (Fig. 6D). We conclude that both newt and axolotl Prod1 proteins are only able to activate the MMP9 pathway in cells that express the proteins. It is noteworthy that the secreted form of the newt protein has no activity, suggesting that there are differences in how the anchored newt and anchorless axolotl molecules react with their binding partners.

Evidence from inhibitors (Fig. 3) indicates that the MMP9-inducing activity of newt Prod1 depends in part on its interaction with the EGFR. To determine whether newt or axolotl Prod1 complexes with the EGFR, we expressed the different proteins in 293T cells, because it was not possible to obtain enough material from immunodepletion of lysates after transfection of the relatively

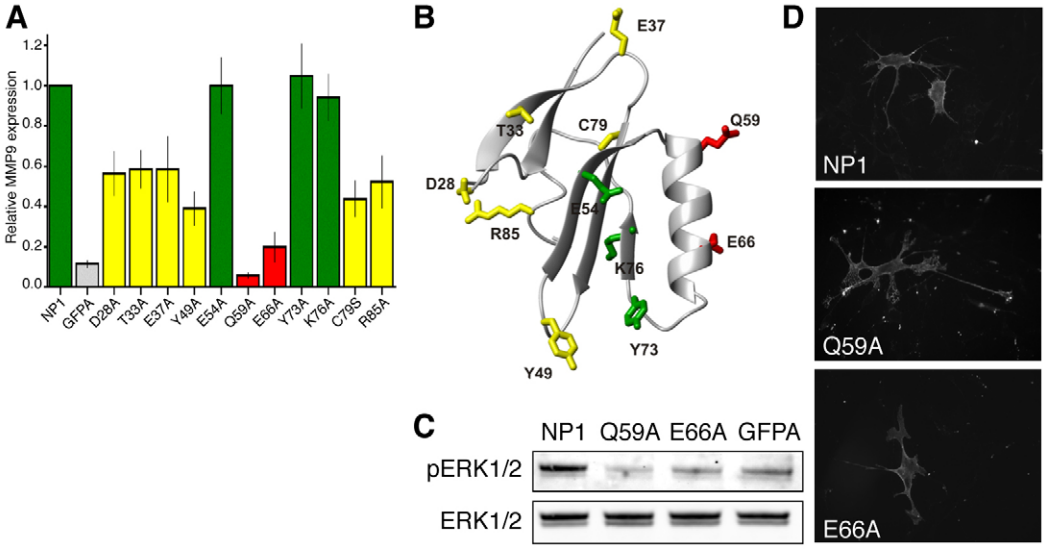


**Fig. 4. Mutational analysis of Prod1 in relation to induction of MMP9 expression or ERK1/2 activation.** (**A**) B1H1 cells were transfected with NP1, GFPA and constructs with single point mutations. After serum starvation for 72 hours, *MMP9* expression was analysed by qRT-PCR and is shown as mean ± s.e.m. of five or six determinations. The mutations had little or no effect on *MMP9* expression (green), an intermediate effect (yellow), or reduced expression to the level of the GFPA control (red). (**B**) Ribbon representation of the 3D structure of Prod1 highlighting the position of the engineered mutations; the colour of the side chains corresponds to the effect of mutations as in A. Note that Q59 and E66 are in the α-helical segment. (**C**) Selected cell lysates collected for cDNA preparation were assayed for phosphorylated ERK1/2 by immunoblotting. Note that Q59 and E66 have no significant activity over the GFPA control. (**D**) Expression of mutant proteins on the cell surface was analysed by antibody labelling of live cells followed by immunofluorescent detection with TSA amplification. The Q59 and E66 mutant proteins are expressed at the cell surface.

slow growing B1H1 and AL1 salamander cells. The 293T cells were transfected with FLAG-tagged versions of newt NP1 or axolotl AP1 Prod1 along with Myc-tagged newt EGFR. The cell lysates were immunodepleted with beads carrying antibody to the FLAG epitope, and the beads were washed and subsequently eluted with FLAG peptide. The eluates were analysed by immunoblotting with both anti-FLAG and anti-Myc antibodies. Although the lysates contained many proteins that reacted with the Myc antibody before immunodepletion (Fig. 7A, lanes 7 and 8), both newt NP1 and axolotl AP1 Prod1 specifically pulled down one band only, identified by molecular size as the newt EGFR (Fig. 7A, lanes 5 and 6). Note that this was not detectable in lysates from cells

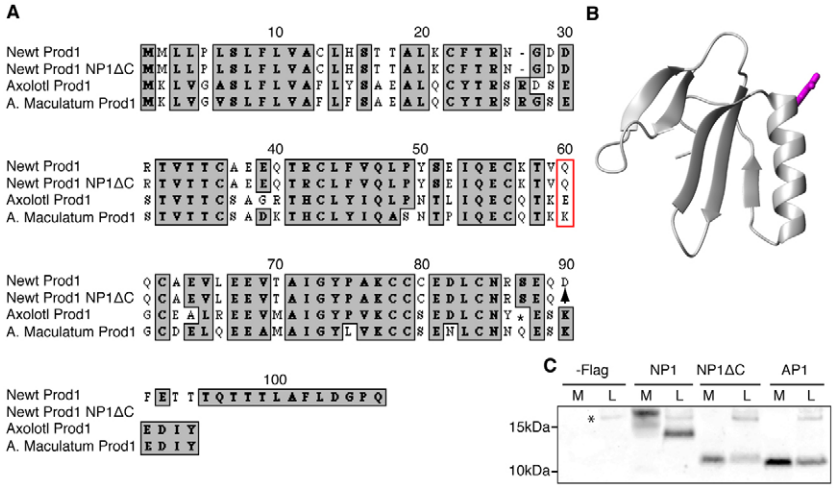


**Fig. 5. Axolotl Prod1 is a secreted protein.** (**A**) Comparison of amino acid sequences of newt Prod1, newt NP1ΔC Prod1, axolotl Prod1 and *Ambystoma maculatum* Prod1. Note residue 59 in the newt (60 in the axolotl), which is boxed in red. The predicted point of GPI-anchor attachment is indicated with an arrow for newt Prod1. Conceptual translation past the stop codon (indicated with an *) of axolotl Prod1 is shown for comparison with the C-terminal of *A. maculatum* Prod1. (**B**) Homology model of axolotl Prod1. The model was created using Modeller 9.1 and the 3D structure of newt Prod1 as template. Note the position of E60 (red) in the α-helix compared with Q59 in Fig. 4B. (**C**) Expression of FLAG-tagged Prod1 molecules. B1H1 cells were transfected with a newt Prod1 construct without the FLAG tag as a control for antibody specificity, and NP1, NP1ΔC and axolotl Prod1 (AP1), all with N-terminal FLAG tags. Equivalent proportions of the total concentrated culture medium (M) and cell lysate (L) from each sample were immunoblotted (see text for the predicted number of residues of each Prod1 construct). The NP1 and NP1ΔC results are comparable to Fig. 1D. The non-specific band marked * is seen at the same level in all lysate samples confirming equal loading of protein. Note the presence of NP1ΔC and axolotl proteins in the medium.

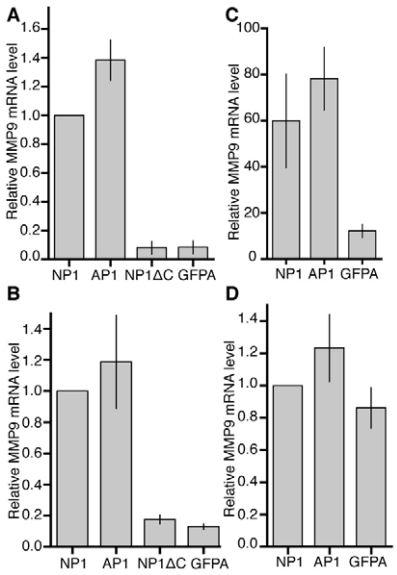


**Fig. 6. Biological activity of axolotl Prod1.** (**A**) Axolotl AL1 cells were transfected with NP1, axolotl Prod1 (AP1), NP1ΔC or GFPA, serum starved for 72 hours and analysed by qRT-PCR. The results are means ± s.e.m. of three independent experiments normalised to NP1. (**B**) Newt BIH1 cells were transfected as for A and the results are averaged over four independent experiments. (**C**) B1H1 cells transfected with NP1, AP1 and GFPA were plated on a porous membrane to share medium with untransfected B1H1 cells. The membrane and untransfected populations were harvested separately, and analysed for *MMP9* expression by qRT-PCR. The results are means ± s.e.m. of three independent experiments for transfected cells (C) and co-cultured untransfected cells (**D**). The results for C and D are normalised to the activity of NP1 in D. Therefore the activity of NP1 in C is 60-fold greater than in D. NP1 and AP1 expression induced strong expression of *MMP9* relative to the GFPA control, but there is no transfer of activity via the conditioned medium, even with AP1.

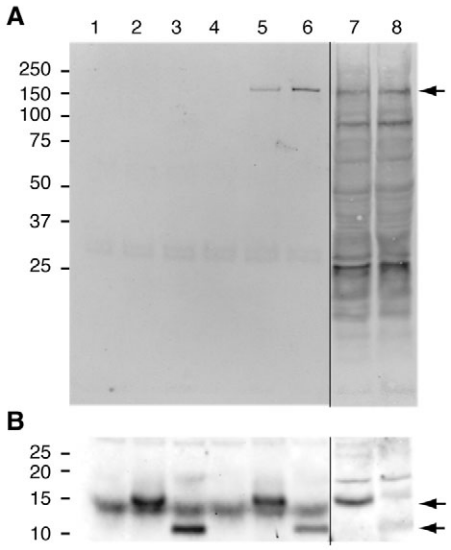


**Fig. 7. Immunodepletion of newt and axolotl Prod1 with the newt EGFR.** (**A**) 293T cells were transfected with FLAG-tagged newt or axolotl Prod1 constructs, or Myc-tagged newt EGFR, in various combinations, and lysed after 48 hours in culture. Lysates were clarified by centrifugation and reacted with anti-FLAG beads. The beads were eluted with FLAG peptide, and the eluates were analysed by immunoblotting with anti-Myc antibody. Lane 1, 293T untransfected; 2, newt Prod1; 3, axolotl Prod1; 4, newt EGFR; 5, newt Prod1 + newt EGFR; 6, axolotl Prod1 + newt EGFR; 7, input lysate for 5; 8, input lysate for 6. Note that both newt and axolotl Prod1 pull down newt EGFR (arrow). (**B**) Reaction with anti-FLAG. The lanes correspond to those in A. Note in lane 1 the FLAG reactive band present in all samples. Lanes 2 and 5 show the GPI-anchored form of newt Prod1 (arrow, see Fig. 1D), and lanes 3 and 6 show the anchorless form of axolotl Prod1 (arrow, see Fig. 5C).

transfected with either Prod1 or EGFR alone. The anti-FLAG staining shows the GPI-anchored newt NP1 Prod1 and anchorless axolotl AP1 Prod1 forms were pulled down, as expected, by the immunodepletion (Fig. 7B, lanes 2,3,5,6). The amount of EGFR pulled down was comparable for newt NP1 and axolotl AP1 Prod1. This assay suggests that both newt and axolotl molecules are able to complex with the newt EGFR after co-expression, the axolotl Prod1 doing so despite being anchorless.

It is noteworthy that residue 59 of newt Prod1 differs from the corresponding residue 60 in both the axolotl and *A. maculatum* proteins (Fig. 5A), and that this residue is located at the α-helical segment that was identified earlier as crucial for activation of the MMP9 pathway by the newt molecule (Fig. 4A,B). The axolotl E60 residue was mutated to either A or Q, the latter being the amino acid in the equivalent position in newt Prod1. The wild-type and single-mutant axolotl molecules were expressed in either newt B1H1 (Fig. 8A) or axolotl AL1 cells (Fig. 8B) along with NP1ΔC Prod1 as a control. The wild-type protein was able, as before, to activate the MMP9 pathway in both newt and axolotl cells, as well as to stimulate the phosphorylation of ERK1/2 (Fig. 8C), but neither of the residue 60 mutants had any activity relative to the NP1ΔC Prod1 negative control. All three versions of the axolotl protein were detected in both lysate and medium by immunoblotting (Fig. 8D). Equal loading of samples was confirmed with reference to a non-specific band (not shown). As the residue 60 point mutants of axolotl Prod1 did not show diminished expression, we conclude that their inability to activate ERK1/2 and induce MMP9 results from disrupting a specific activity of the α-helix. We draw the same conclusion for the equivalent residue 59 mutants of newt Prod1.

The amino acid substitutions made in the α-helix of newt and axolotl Prod1 demonstrate that this region of the protein is essential for the activity of both the anchored and anchorless proteins. Because substitution of E60 of axolotl Prod1 for glutamine as in newt Prod1 abolished the activity of the anchorless axolotl Prod1, we investigated whether the differences in the α-helices of Prod1 from the two species underlie their different requirement for a GPI-anchor. When the axolotl α-helical region was substituted for the newt α-helix in NP1ΔC Prod1, it led to a striking activation of the anchorless form, whereas axolotl Prod1 retained its activity when expressed with the C-terminal GPI-anchor attachment sequence of newt Prod1 (Fig. 8E). These results underline the differences between the newt and axolotl molecules, and demonstrate the importance of the α-helical region in conferring MMP9 induction activity to axolotl Prod1 in the absence of a GPI-anchor.

## Discussion

This work has establishes that Prod1 activates a pathway in cultured salamander cells that leads to the expression of MMP9. When the results for newt Prod1 with the qRT-PCR assay are corrected for transfection efficiency, the stimulation of this pathway in a

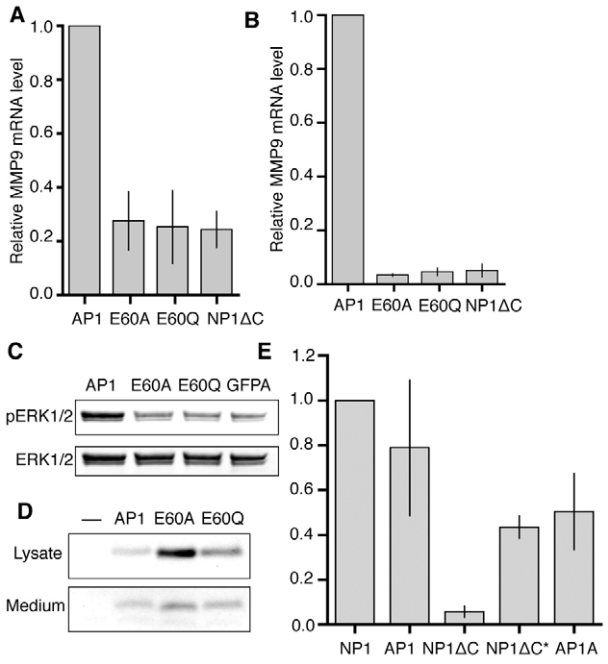


**Fig. 8. Effect of mutations in the axolotl Prod1 helical segment.** (**A**) B1H1 cells and (**B**) AL1 cells were transfected with AP1, axolotl Prod1 E60A (E60A), axolotl Prod1 E60Q (E60Q), or NP1ΔC, serum starved for 72 hours and analysed for *MMP9* expression by qRT-PCR. The results are the means ± s.e.m. of four independent experiments in A and three in B. (**C**) Lysates from B1H1 cells analysed for ERK1/2 activation by immunoblotting with anti-ERK1/2 and anti-phosphorylated ERK1/2 antibodies. The results are representative of four independent experiments. Only AP1 is able to activate above the level of the NP1ΔC control. (**D**) Expression of axolotl proteins in cell lysates and conditioned medium. Anti-FLAG immunoblots are shown for cell lysates and medium from untransfected (–), and B1H1 cells expressing AP1, E60A and E60Q. The mutant proteins are detectably expressed in both lysates and medium. (**E**) B1H1 cells were transfected with NP1, AP1, GFPA, NP1ΔC, NP1ΔC substituted with the α-helical region from axolotl Prod1 (NP1ΔC*) or AP1 with the C-terminal GPI-anchor attachment sequence of newt Prod1 (AP1A), serum starved for 72 hours and analysed for MMP9 expression by qRT-PCR. The background MMP9 expression level of negative control GFPA-transfected cells was subtracted from all values, which were then expressed relative to the level of NP1 in each experiment. The average relative level of GFPA was 0.15. Results are means ± s.e.m. of four independent experiments.

transfected cell is increased at least 30-fold. One transmembrane signalling partner implicated in this activity is the EGFR, possibly the ErbB1–ErbB2 heterodimer as suggested by sensitivity to inhibitors (see supplementary material Fig. S5). The inhibitor experiments indicate that less than half of the activity of the MMP9 pathway after transfection is attributable to signalling via the EGFR. In the case of mammalian uPAR it is clear that integrin signalling is an important additional target (Wei et al., 1996; Wei et al., 2001; Aguirre-Ghiso et al., 2001; Ghosh et al., 2006; Wei et al., 2007), a possibility that remains to be investigated for Prod1. The *Drosophila* EGFR is implicated in PD identity in limb development (Galindo et al., 2002; Campbell, 2002), whereas in the cricket, RNAi studies suggest that the EGFR influences PD identity in limb regeneration at the nymphal stage (Nakamura et al., 2008). In zebrafish fin regeneration, recent evidence from both chemical inhibition and genetic mutation has implicated ErbB2 and ErbB3 as essential for progenitor proliferation and migration (Rojas-Munoz et al., 2009). The most structurally similar proteins to Prod1 are CD59 and uPAR (Garza-Garcia et al., 2009), both of which co-immunoprecipitate with the EGFR (Blagoev et al., 2003; Mazzieri et al., 2006; D'Alessio et al., 2008). The activity of Prod1 appears to be more closely related to that of mammalian uPAR as outlined in the Introduction, because axolotl CD59 does not regulate the expression of MMP9. In some cases, uPAR seems to act in conjunction with autocrine or paracrine ligands of the EGFR (Jo et al., 2007; Guerrero et al., 2004), and this seems not to be a feature of newt Prod1. In mouse keratinocytes, for example, the responsiveness of the EGFR to ligands appears to be absolutely dependent on uPAR expression (D'Alessio et al., 2008). In view of the absence of any detectable autocrine ligand activity, we suggest that the GPI-anchored Prod1 acts as an agonist for the EGFR in respect of its activation of the MMP9 pathway. Of particular note when considering the mode of EGFR activation by Prod1 is the observation that uPAR signalling has been shown to lead to EGFR phosphorylation in a pattern that is distinct from that seen following ligand activation (Monaghan-Benson and McKeown-Longo, 2006). Similarly, Prod1 activation of EGFR signalling shows effects on MMP9 induction that are distinct from those seen following ligand stimulation (supplementary material Fig. S4). Src kinase is a crucial component of the mechanism regulating MMP9 expression in response to uPAR activation (Wei et al., 2007); however, this is not observed in the case of Prod1. Despite the similarities between Prod1 and uPAR signalling, each regulates MMP9 via a distinct mechanism. The relevance of the EGFR for other activities of Prod1, such as PD displacement (Echeverri and Tanaka, 2005) or the mitogenic response to the nAG protein (Kumar et al., 2007b), is now open to investigation.

The newt and axolotl belong to different families of the order Urodela, and current estimates suggest that newts separated from other salamanders about 100 million years ago (Steinfartz et al., 2007). Species in both families are able to regenerate their limbs and it is surprising that *Ambystoma* Prod1 is an anchorless protein. The MMP9 pathway is only activated in cells that express axolotl Prod1, and not in cells exposed to the secreted molecule in the culture medium. Furthermore, both newt and axolotl Prod1 complex with the newt EGFR upon co-expression. Since the secreted form of the newt molecule has no activity to induce MMP9, we hypothesise that the GPI-linked form complexes with the EGFR, either by association on the cell surface similarly to uPAR, or in the secretory pathway on their way to the surface, when both molecules are membrane associated (Castillon et al., 2009). The axolotl molecule must associate with the EGFR from the lumen in the secretory pathway. The ability of the axolotl α-helical region to confer activity on an anchorless version of the newt Prod1 protein strongly suggests that the sequence differences in this location allow axolotl Prod1 to engage in a functional signalling complex without requiring a GPI-anchor. The comparison of the two molecules shows that the activity of the newt molecule cannot depend on some unique property conferred by the anchor, for example, its ability to localise to lipid rafts (Simons and Toomre, 2000; Munro, 2003). Axolotl Prod1 is functional when expressed with the GPI-anchor sequence of newt Prod1, consistent with the ancestral Prod1 protein having been GPI-anchored, and the anchor being lost in the Ambystomatidae subsequent to their divergence from the Salamandridae. Conceptual translation of the sequence after the stop codon of axolotl Prod1 suggests that this codon arose after the divergence of the two *Ambystoma* species (Fig. 5A). The

changes to the *Ambystoma* proteins that underlie their anchorless activity presumably occurred before anchor loss to retain signalling activity. Anchorless variants of the GPI-anchored prion protein PrP exhibit some of the functions of the anchored protein whilst lacking others (Chesebro et al., 2005; Trifilo et al., 2008). A soluble version of uPAR with distinct activities from the GPI-anchored form is produced through alternate splicing (Pyke et al., 1993), phospholipase activity (Wilhelm et al., 1999) or proteolytic cleavage by its ligand, uPA (Mazzieri et al., 2006).

It has recently been established by detailed bioinformatic analysis of its structure and sequence that Prod1 is a salamander-specific member of the TFP superfamily (Garza-Garcia et al., 2009; Garza-Garcia et al., 2010). The family has expanded in salamanders, and new members have been recruited to functions such as pheromones (the plethodontid modulating factors) and other activities (Palmer et al., 2007). Although much research on regeneration has emphasised the role of conserved proteins and signalling pathways (Sanchez Alvarado and Tsonis, 2006; Brockes and Kumar, 2008), the example of Prod1 suggests that the salamanders have brought significant evolutionary novelty to the mechanism. This paper provides evidence that the new protein Prod1 interacts directly with an old conserved protein, the EGFR, which is found throughout the metazoa (Aroian et al., 1990; Reiter et al., 2001; Gomez et al., 2004; Alvarado et al., 2009). Previous work has shown that Prod1 also interacts with nAG, another conserved protein (Kumar et al., 2007b). The association of new and old proteins to generate evolutionary novelty has been noted previously as an important principle, for example in protein interactions in *Caenorhabditis elegans* (Li et al., 2004). The TFP family is implicated in other examples of local evolutionary change, for example, in formation of the venom apparatus in elapid snakes (Tsetlin, 1999). If regenerative ability such as limb regeneration depends on such taxon-specific activities, it seems important to take account of them in current and future attempts to increase the repertoire of regeneration processes in mammals (Brockes and Kumar, 2005; Garza-Garcia et al., 2010).

## Materials and Methods

### Cell culture

B1H1 cells were cultured as described (Ferretti and Brockes, 1988; Cash et al., 1998). AL1 cells were obtained from S. Roy (University of Montreal, Canada) and cultured as described (Roy et al., 2000; Villiard et al., 2007). 293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, penicillin 100 U/ml and streptomycin 100 µg/ml. B1H1 and AL1 cells were plated on uncoated plastic culture dishes and transfected overnight with 4 µg DNA and 8 µl Lipofectamine 2000 (Invitrogen) per 35 mm dish for all analyses of MMP9 expression, ERK1/2 activation and FLAG-tagged Prod1 expression by immunofluorescence. Lipofectamine 2000 transfection gave approximately 30% transfection efficiency of B1H1 and AL1 cells and all analysis of the salamander cells was conducted 7 days after transfection. For analysis of FLAG-tagged Prod1 expression by immunoblot, B1H1 cells were nucleofected with the Amaxa Nucleofector using program T-030 and buffer V according to the manufacturer's instructions. Nucleofection gave approximately 90% transfection efficiency of B1H1 cells and analysis was conducted 72 hours after transfection. For immunodepletion experiments, 293T cells were transfected with 3 µg FLAG-tagged Prod1, 3 µg of Myc-tagged EGFR and 12 µl Lipofectamine 2000 per 6 mm dish for 8 hours and were passaged into two 6 mm dishes on the next day. Immunodepletion experiments were conducted at 48 hours after transfection.

Signalling pathway inhibitors were all purchased from Calbiochem. The lowest concentrations giving maximal inhibition of ERK1/2 activation in response to EGF and MMP9 induction following Prod1 expression were determined as 10 µM for U0126 (MEK1/2), 25 µM for AG1478 (EGFR) and 5 µM for AG879 (ErbB2); inhibitors were used at these concentrations in all experiments described.

For co-culture experiments, B1H1 cells transfected with FLAG-tagged Prod1 constructs 7 days previously were seeded onto the membranes of cell culture inserts with 8 µm pores (BD Biosciences), untransfected B1H1 cells were plated in 24-well plates (BD Biosciences) and following a period of 12 hours to allow the cells to adhere to the growth surfaces the cell culture inserts were placed into the wells of the 24-well plates. Cells were rinsed with serum-free medium after which 100 µl of serum-free culture medium was added to cover the cells in the inserts and 250 µl was added to the cells in the well. Cells were co-cultured for 72 hours after which cDNA was prepared for qRT-PCR analysis as described below.

### DNA constructs

All newt *Prod1* constructs are derived from the original *Prod1* construct (da Silva et al., 2002). Axolotl *Prod1* was cloned from cDNA using primers GACAGA-ATTCGCGTGACTGGACGGCCACA and ATCATCTAGATAATGGGGTGC-TCAGGACCA, which are homologous to the EST sequence published at http://salamander.uky.edu/ESTdb/. A FLAG tag sequence was added by PCR immediately 5′ of the coding region of each *Prod1* construct and DNA fragments were subcloned into peGFP-n2 vector (Clontech, GenBank Acc #U57608) to replace the GFP sequence. N- and C-terminal sequences were deleted from FLAG-tagged Prod1 by PCR during subcloning to produce the truncated constructs referred to as NP1ΔN and NP1ΔC. A C-terminal Myc tag was added by PCR to the newt EGFR coding sequence and the construct was assembled in the pTL-1 vector, a pSG5 vector with extended polylinker (Stratagene). GPI-anchored GFP was obtained from M. Marsh, UCL, London, UK (Keller et al., 2001).

Residues unlikely to disrupt the three-dimensional fold of the protein were identified by manual inspection of the structure. Candidate side chains were further filtered on the basis of being greater than 30% solvent accessible. From these, residues that are unconserved across the TFP superfamily and evenly distributed over the surface of the protein were selected for site-directed mutagenesis. We used the Stratagene QuikChange site-directed mutagenesis kit according to the manufacturer's instructions to synthesise mutated product using mismatched primers and either the NP1 or AP1 FLAG-tagged Prod1 constructs as a template. For the NP1 construct most amino acids were mutated to alanine, apart form cysteine residue 79, which was mutated to serine.

### Immunodepletion

Cells were lysed in 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$ 2 mM EGTA, 1 mM $NaVO_4$, 10% glycerol and Protease Inhibitor Cocktail (Sigma). Cleared lysates were incubated for 1 hour at 4°C with Protein G Dynabeads (Invitrogen) loaded with M2 mouse anti-FLAG antibody (Sigma). Beads were washed three times with ice-cold PBS and the bound protein was eluted with excess FLAG peptide (Sigma) and analysed by immunoblotting.

### Immunoblotting

For analysis of Prod1–FLAG expression and ERK1/2 phosphorylation, cells were lysed in ice-cold 1% NP40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM $NaVO_4$ and Protease Inhibitor Cocktail (Sigma). Protein concentrations of cleared lysates were determined by bicinchoninic acid assay (Pierce), samples of equal total protein were denatured in 2% SDS, 50 mM Tris-HCl, pH 8, and reducing agent (Invitrogen), and separated on NuPAGE 12% Bis-Tris pre-cast SDS gels (Invitrogen) using MES buffer (Invitrogen) with antioxidant (Invitrogen) according to the manufacturer's instructions. Proteins were transferred to nitrocellulose membranes (Whatman) and membranes were blocked with Odyssey Block (LI-COR Biosciences) for 10 minutes before overnight incubation at 4°C with primary antibody. Prod1–FLAG was detected on blots with rabbit anti-FLAG antibodies (Sigma) at 1:1000 dilution in 25 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 0.1% Tween 20. ERK1/2 was detected with rabbit anti-ERK and phosphorylated ERK1/2 was detected with mouse anti-phospho ERK1/2 (Sigma), both of which were used at 1:1000 dilution in Odyssey Block (LI-COR Biosciences) 0.1% Tween 20. Samples from immunodepletion experiments were denatured in 2% SDS and 50 mM Tris-HCl, pH 8, and analysed using NuPAGE 4–12% Bis-Tris gels and MES buffer (Invitrogen) according to the manufacturer's instructions. Blots were probed with rabbit anti-Myc (Cell Signaling Technology) 1:1000 in Odyssey Block (LI-COR Biosciences) 0.1% Tween 20 and M2 mouse anti-FLAG (Sigma) 1:5000 in 25 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 0.1% Tween 20.

### TSA immunofluorescence of surface Prod1 expression

Live B1H1 cells, previously transfected with FLAG-tagged *Prod1* constructs, were reacted at 4°C with monoclonal anti-FLAG antibody or control monoclonal antibodies diluted in L15 medium (adjusted to urodele osmolarity) with 0.1% bovine serum albumin. The cultures were washed and reacted with biotinylated goat anti-mouse IgG under the same conditions, washed and fixed for 2 minutes at –30°C with freshly prepared 5% glacial acetic acid in absolute ethanol. Fixed cultures were washed extensively in PBS, reacted with PBS + 1% $H_2O_2$ for 20 minutes to inactivate endogenous peroxidase, washed with PBS + 1% Triton X-100, and reacted for 1 hour at room temperature with horseradish-peroxidase-labelled neutravidin (Molecular Probes) at 0.5 µg/ml in PBS-Triton. After extensive washing with PBS, cells were reacted for 2 minutes at room temperature with Cy3-tyramide diluted 600× in the supplied diluent (TSA-plus Cyanine 3 system, PerkinElmer), washed and mounted for fluorescence microscopy.

### Zymography

Culture medium was cleared by centrifugation, adjusted to 2% SDS, 50 mM Tris-HCl, pH 8.0, and samples were analysed by electrophoresis with 10%/12% (gelatin/casein) Novex zymogram gels (Invitrogen) for 90 minutes at 125 V. Gels

were developed overnight according to the manufacturer's instructions and stained using SimplyBlue Safestain (Invitrogen).

### Imaging and quantification

A Licor Odyssey imager was used for imaging all zymogram gels and immunoblots; IR-labelled secondary antibodies (Molecular Probes) were used for visualisation of bands on immunoblots. Band intensities were quantified using the profile/MW function of Fuji Image Gauge software.

### RT-PCR

cDNA was prepared from pelleted cells using the Ambion Cells-to-cDNA II kit using random primers according to the manufacturer's instructions. qRT-PCR reactions were performed with iQ Supermix with SYBR green (Bio-Rad) and gene specific primers using Bio-Rad Chromo 4 detector, and analysed with Bio-Rad Opticon Monitor software according to manufacturer's instructions. qRT-PCR reactions of RNA samples used for cDNA preparation were analysed to confirm that non-specific signal was not a significant component of the $C_T$ values obtained. *GAPDH* was used as a normalising gene for relative quantification of MMP9 expression between samples. Observed trends were confirmed with independent normalising genes (not shown). The sequences of the *MMP9* specific primers are: CATCGTAGGATTCACCATCG, ACCACGACCGACTATGACAA (Newt) and GCATCGTAGGATTCTCCATCA, ACCAGTGAAGGCCGTTCCGAT (Axolotl) and the *GAPDH* primer sequences for both species are: TGTGGCGTGACGGCAGAG-GTG, TCCAAGCGGCAGGTCAGGTCAAC. A standard curve plotting $C_T$ value against the relative number of target sequences confirmed the efficiency of all primers used.

We thank P. Driscoll, I. Gout and J. Godwin for comments, S. Roy for AL1 cells and A. Kumar for his help. Supported by a Programme Grant from the MRC and a studentship to R.A.B. from the BBSRC. MRC file reference number U117574559. Deposited in PMC for release after 6 months.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/124/1/47/DC1

## References

**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**, 863-879.

**Ahmed, N., Oliva, K., Wang, Y., Quinn, M. and Rice, G.** (2003). Proteomic profiling of proteins associated with urokinase plasminogen activator receptor in a colon cancer cell line using an antisense approach. *Proteomics* **3**, 288-298.

**Alvarado, D., Klein, D. E. and Lemmon, M. A.** (2009). ErbB2 resembles an autoinhibited invertebrate epidermal growth factor receptor. *Nature* **461**, 287-291.

**Andreasen, P. A., Kjoller, L., Christensen, L. and Duffy, M. J.** (1997). The urokinase-type plasminogen activator system in cancer metastasis: a review. *Int. J. Cancer* **72**, 1-22.

**Aroian, R. V., Koga, M., Mendel, J. E., Ohshima, Y. and Sternberg, P. W.** (1990). The let-23 gene necessary for Caenorhabditis elegans vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. *Nature* **348**, 693-699.

**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**, 315-318.

**Blasi, F. and Carmeliet, P.** (2002). uPAR: a versatile signalling orchestrator. *Nat. Rev. Mol. Cell Biol.* **3**, 932-943.

**Brockes, J. P.** (1984). Mitogenic growth factors and nerve dependence of limb regeneration. *Science* **225**, 1280-1287.

**Brockes, J. P. and Kumar, A.** (2005). Appendage regeneration in adult vertebrates and implications for regenerative medicine. *Science* **310**, 1919-1923.

**Brockes, J. P. and Kumar, A.** (2008). Comparative aspects of animal regeneration. *Annu. Rev. Cell Dev. Biol.* **24**, 525-549.

**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**, 533-544.

**Campbell, G.** (2002). Distalization of the Drosophila leg by graded EGF-receptor activity. *Nature* **418**, 781-785.

**Cash, D. E., Gates, P. B., Imokawa, Y. and Brockes, J. P.** (1998). Identification of newt connective tissue growth factor as a target of retinoid regulation in limb blastemal cells. *Gene* **222**, 119-124.

**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.

**Chesebro, B., Trifilo, M., Race, R., Meade-White, K., Teng, C., LaCasse, R., Raymond, L., Favara, C., Baron, G., Priola, S. et al.** (2005). Anchorless prion protein results in infectious amyloid disease without clinical scrapie. *Science* **308**, 1435-1439.

**D'Alessio, S., Gerasi, L. and Blasi, F.** (2008). uPAR-deficient mouse keratinocytes fail to produce EGFR-dependent laminin-5, affecting migration in vivo and in vitro. *J. Cell Sci.* **121**, 3922-3932.

**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**, 547-555.

**Davies, A., Simmons, D. L., Hale, G., Harrison, R. A., Tighe, H., Lachmann, P. J. and Waldmann, H.** (1989). CD59, an LY-6-like protein expressed in human lymphoid cells, regulates the action of the complement membrane attack complex on homologous cells. *J. Exp. Med.* **170**, 637-654.

**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**, 1155-1161.

**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**, 367-385.

**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.

**Galat, A.** (2008). The three-fingered protein domain of the human genome. *Cell. Mol. Life Sci.* **65**, 3481-3493.

**Galindo, M. I., Bishop, S. A., Greig, S. and Couso, J. P.** (2002). Leg patterning driven by proximal-distal interactions and EGFR signaling. *Science* **297**, 256-259.

**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**.

**Garza-Garcia, A. A., Driscoll, P. C. and Brockes, J. P.** (2010). Evidence for the local evolution of mechanisms underlying limb regeneration in salamanders. *Integr. Comp. Biol.* Epub ahead of print.

**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**, 13021-13029.

**Gomez, A., Volff, J. N., Hornung, U., Schartl, M. and Wellbrock, C.** (2004). Identification of a second egfr gene in Xiphophorus uncovers an expansion of the epidermal growth factor receptor family in fish. *Mol. Biol. Evol.* **21**, 266-275.

**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**, 201-208.

**Helms, J. B. and Zurzolo, C.** (2004). Lipids as targeting signals: lipid rafts and intracellular trafficking. *Traffic* **5**, 247-254.

**Jo, M., Thomas, K. S., O'Donnell, D. M. and Gonias, S. L.** (2003). Epidermal growth factor receptor-dependent and -independent cell-signaling pathways originating from the urokinase receptor. *J. Biol. Chem.* **278**, 1642-1646.

**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**, 17449-17457.

**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**, 2585-2594.

**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**, 140-149.

**Kumar, A., Gates, P. B. and Brockes, J. P.** (2007a). Positional identity of adult stem cells in salamander limb regeneration. *C R Biol.* **330**, 485-490.

**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**, 772-777.

**Li, S., Armstrong, C. M., Bertin, N., Ge, H., Milstein, S., Boxem, M., Vidalain, P. O., Han, J. D., Chesneau, A., Hao, T. et al.** (2004). A map of the interactome network of the metazoan C. elegans. *Science* **303**, 540-543.

**Lingwood, D. and Simons, K.** (2010). Lipid rafts as a membrane-organizing principle. *Science* **327**, 46-50.

**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**, 445-457.

**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**, 367-378.

**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**, 9450-9459.

**Mouillet-Richard, S., Ermonval, M., Chebassier, C., Laplanche, J. L., Lehmann, S., Launay, J. M. and Kellermann, O.** (2000). Signal transduction through prion protein. *Science* **289**, 1925-1928.

**Munro, S.** (2003). Lipid rafts: elusive or illusive? *Cell* **115**, 377-388.

**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**, 25279-25284.

**Nakamura, T., Mito, T., Miyawaki, K., Ohuchi, H. and Noji, S.** (2008). EGFR signaling is required for re-establishing the proximodistal axis during distal leg regeneration in the cricket Gryllus bimaculatus nymph. *Dev. Biol.* **319**, 46-55.

**Ossowski, L. and Aguirre-Ghiso, J. A.** (2000). Urokinase receptor and integrin partnership: coordination of signaling for cell adhesion, migration and growth. *Curr. Opin. Cell Biol.* **12**, 613-620.

**Palmer, C. A., Hollis, D. M., Watts, R. A., Houck, L. D., McCall, M. A., Gregg, R. G., Feldhoff, P. W., Feldhoff, R. C. and Arnold, S. J.** (2007). Plethodontid modulating factor, a hypervariable salamander courtship pheromone in the three-finger protein superfamily. *FEBS J.* **274**, 2300-2310.

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

**Pike, L. J.** (2004). Lipid rafts: heterogeneity on the high seas. *Biochem. J.* **378**, 281-292.

**Putta, S., Smith, J. J., Walker, J. A., Rondet, M., Weisrock, D. W., Monaghan, J., Samuels, A. K., Kump, K., King, D. C., Maness, N. J. et al.** (2004). From biomedicine to natural history research: EST resources for ambystomatid salamanders. *BMC Genomics* **5**, 54.

**Pyke, C., Eriksen, J., Solberg, H., Nielsen, B. S., Kristensen, P., Lund, L. R. and Dano, K.** (1993). An alternatively spliced variant of mRNA for the human receptor for urokinase plasminogen activator. *FEBS Lett.* **326**, 69-74.

**Reiter, J. L., Threadgill, D. W., Eley, G. D., Strunk, K. E., Danielsen, A. J., Sinclair, C. S., Pearsall, R. S., Green, P. J., Yee, D., Lampland, A. L. et al.** (2001). Comparative genomic sequence analysis and isolation of human and mouse alternative EGFR transcripts encoding truncated receptor isoforms. *Genomics* **71**, 1-20.

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

**Rojas-Munoz, A., Rajadhyksha, S., Gilmour, D., van Bebber, F., Antos, C., Rodriguez Esteban, C., Nusslein-Volhard, C. and Izpisua Belmonte, J. C.** (2009). ErbB2 and ErbB3 regulate amputation-induced proliferation and migration during vertebrate regeneration. *Dev. Biol.* **327**, 177-190.

**Roy, S., Gardiner, D. M. and Bryant, S. V.** (2000). Vaccinia as a tool for functional analysis in regenerating limbs: ectopic expression of Shh. *Dev. Biol.* **218**, 199-205.

**Sanchez Alvarado, A. and Tsonis, P. A.** (2006). Bridging the regeneration gap: genetic insights from diverse animal models. *Nat. Rev. Genet.* **7**, 873-884.

**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**, 321-335.

**Satoh, A., James, M. A. and Gardiner, D. M.** (2009). The role of nerve signaling in limb genesis and agenesis during axolotl limb regeneration. *J. Bone Joint Surg. Am.* **91**, 90-98.

**Simons, K. and Toomre, D.** (2000). Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* **1**, 31-39.

**Singer, M.** (1952). The influence of the nerve in regeneration of the amphibian extremity. *Q. Rev. Biol.* **27**, 169-200.

**Sive, H. L., Hattori, K. and Weintraub, H.** (1989). Progressive determination during formation of the anteroposterior axis in Xenopus laevis. *Cell* **58**, 171-180.

**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. Zool. B Mol. Dev. Evol.* **308**, 139-162.

**Tassava, R. A. and Mescher, A. L.** (1975). The roles of injury, nerves, and the wound epidermis during the initiation of amphibian limb regeneration. *Differentiation* **4**, 23-24.

**Trifilo, M. J., Sanchez-Alavez, M., Solforosi, L., Bernard-Trifilo, J., Kunz, S., McGavern, D. and Oldstone, M. B.** (2008). Scrapie-induced defects in learning and memory of transgenic mice expressing anchorless prion protein are associated with alterations in the gamma aminobutyric acid-ergic pathway. *J. Virol.* **82**, 9890-9899.

**Tsetlin, V.** (1999). Snake venom alpha-neurotoxins and other 'three-finger' proteins. *Eur. J. Biochem.* **264**, 281-286.

**Villiard, E., Brinkmann, H., Moiseeva, O., Mallette, F. A., Ferbeyre, G. and Roy, S.** (2007). Urodele p53 tolerates amino acid changes found in p53 variants linked to human cancer. *BMC Evol. Biol.* **7**, 180.

**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.

**Wei, Y., Lukashev, M., Simon, D. I., Bodary, S. C., Rosenberg, S., Doyle, M. V. and Chapman, H. A.** (1996). Regulation of integrin function by the urokinase receptor. *Science* **273**, 1551-1555.

**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**, 2975-2986.

**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**, 3929-3939.

**Wilhelm, O. G., Wilhelm, S., Escott, G. M., Lutz, V., Magdolen, V., Schmitt, M., Rifkin, D. B., Wilson, E. L., Graeff, H. and Brunner, G.** (1999). Cellular glycosylphosphatidylinositol-specific phospholipase D regulates urokinase receptor shedding and cell surface expression. *J. Cell. Physiol.* **180**, 225-235.

**Yang, E. V., Gardiner, D. M., Carlson, M. R., Nugas, C. A. and Bryant, S. V.** (1999). Expression of Mmp-9 and related matrix metalloproteinase genes during axolotl limb regeneration. *Dev. Dyn.* **216**, 2-9.