

Regulation of epidermal growth factor receptor signalling by inducible feedback inhibitors

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Journal of Cell Science 124, 1785–1793

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doi:10.1242/jcs.083303

Summary

Signalling by the epidermal growth factor receptor (EGFR) controls morphogenesis and/or homeostasis of several tissues from worms to mammals. The correct execution of these programmes requires the generation of EGFR signals of appropriate strength and duration. This is obtained through a complex circuitry of positive and negative feedback regulation. Feedback inhibitory mechanisms restrain EGFR activity in time and space, which is key to ensuring that receptor outputs are commensurate to the cell and tissue needs. Here, we focus on the emerging field of inducible negative feedback regulation of the EGFR in mammals. In mammalian cells, four EGFR inducible feedback inhibitors (IFIs), namely LRIG1, RALT (also known as MIG6 and ERFF11), SOCS4 and SOCS5, have been discovered recently. EGFR IFIs are expressed de novo in the context of early or delayed transcriptional responses triggered by EGFR activation. They all bind to the EGFR and suppress receptor signalling through several mechanisms, including catalytic inhibition and receptor downregulation. Here, we review the mechanistic basis of IFI signalling and rationalise the function of IFIs in light of gene-knockout studies that assign LRIG1 and RALT an essential role in restricting cell proliferation. Finally, we discuss how IFIs might participate in system control of EGFR signalling and highlight the emerging roles for IFIs in the suppression of EGFR-driven tumorigenesis.

Key words: EGFR, *ERFF11*, LRIG1, RALT (MIG6), SOCS4, SOCS5, Tumour suppression

Introduction

The epidermal growth factor receptor (EGFR) is the founding member of the ErbB receptor tyrosine kinase (RTK) family. EGFR signalling controls key cellular programmes, including survival, proliferation, differentiation and locomotion, both during development and post-natally (Sibilia et al., 2007). The execution of these diverse cellular programmes requires that receptor signals of adequate strength and duration are generated from the receptor to elicit either switch-like (e.g. cell proliferation) or graded (e.g. cell motility) responses (Citri and Yarden, 2006). EGFR signals, however, need to be carefully balanced, as excessive EGFR signalling might pose a serious oncogenic threat (Sibilia et al., 2007). As is the case for all signalling circuits (Brandman and Meyer, 2008), negative feedback regulation plays a major role in restricting the activity of the EGFR, thus ensuring the generation of stable and reproducible signal outputs (Citri and Yarden, 2006).

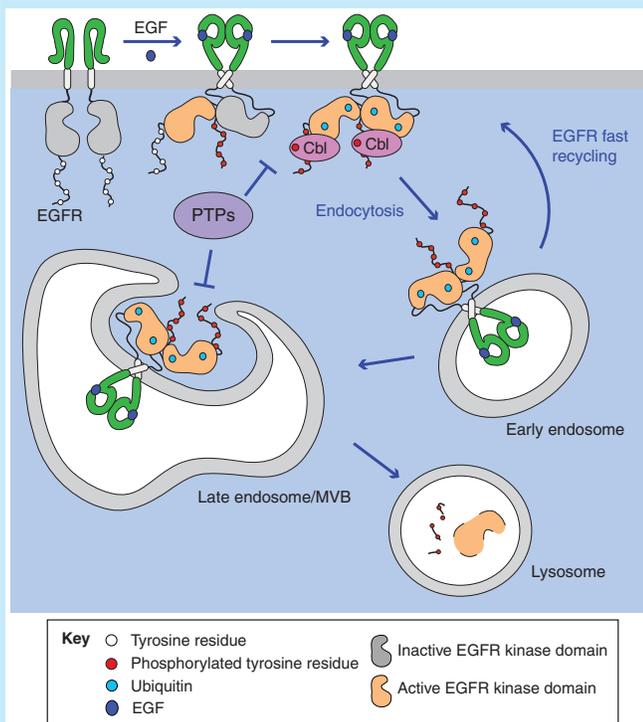
The prevailing view of negative signalling to the EGFR in mammalian organisms has been dominated for a long time by models derived from analyses of inhibitory feedback loops that act immediately and do not require de novo protein synthesis (Box 1). Pioneering studies in the fruit fly *Drosophila melanogaster*, however, have pointed to inducible feedback inhibitors (IFIs); namely inhibitors expressed de novo in the context of transcriptional programmes driven by EGFR signalling) as additional and essential regulators of developmental patterns governed by the *Drosophila* Egfr (Perrimon and McMahon, 1999; Freeman, 2000). Surprisingly, out of four IFIs acting on *Drosophila* Egfr, only one, namely Socs36e (Callus and Mathey-Prevot, 2002; Rawlings et al., 2004), seems to be conserved in mammalian organisms. Of the other three, Kekk-1 (Ghigliione et al., 1999) and Argos (Freeman et al., 1992) lack mammalian orthologues, whereas Sprouty proteins

act as enhancers, rather than suppressors, of EGFR signalling in mammals (Wong et al., 2002; Rubin et al., 2003; Haglund et al., 2005). Therefore, there was uncertainty about the existence of EGFR IFIs in mammals. A number of reports in the past few years, however, have described IFIs acting on the mammalian EGFR and demonstrated essential roles for these proteins in the control of tissue morphogenesis and homeostasis.

Here, we review molecular mechanisms underpinning the function of EGFR IFIs in mammals. The emerging picture points to IFIs as important regulators that reduce the activity and/or expression of EGFR at the cell surface. We will discuss these data in light of the genetic evidence that assigns an essential role for IFIs in restricting EGFR-driven cell proliferation. Furthermore, EGFR IFIs are candidate tumour suppressors and we will conclude the Commentary by highlighting the emerging role of IFIs as antagonists of EGFR-driven tumorigenesis.

EGFR: activation tangoes with inhibition

Signalling from the EGFR is initiated by activation of its tyrosine kinase activity. Activated EGFRs are immediately targeted by inhibitory mechanisms that do not require de novo protein synthesis. These include dephosphorylation by protein tyrosine phosphatases (PTPs) (Keilhack et al., 1998; Xu et al., 2005b; Tarcic et al., 2009) and endocytosis (Sorkin and Goh, 2009; Madhus and Stang, 2009) (Box 1). The equilibrium between ongoing EGFR kinase activity and PTP function is a key determinant of overall receptor phosphorylation and EGFR signalling potential. Endocytosis, although providing spatial resolution to forward signalling (Scita and Di Fiore, 2010), can ultimately lead to the extinction of EGFR activity through degradation of ligand–receptor complexes in the lysosome. Importantly, lack of PTP activity (Sun et al., 2011) or

Box 1. A snapshot of immediate EGFR feedback inhibition

Binding of EGF (see Figure) leads to the conversion of the EGFR kinase from an inactive (grey) into a catalytically active conformation (orange). Key to this process is the formation of asymmetric kinase dimers, in which the large C-lobe of one kinase (the 'activator'; grey) contacts the small N-lobe of the adjacent partner kinase (the 'receiver'; orange), thereby causing its activation. This process is thought to be reversible, with the receiver subsequently acting as the activator (Zhang et al., 2006). For further details on dimerisation-driven activation of EGFR and the role of the juxtamembrane domain of EGFR (not shown here for simplicity), see Jura et al. (Jura et al., 2009). In trans autophosphorylation of tyrosine residues located in the receptor C-terminal tail (red circles) generates docking sites for the phosphorylated-tyrosine-binding modules (not shown here) found on various signalling proteins, thereby igniting downstream signalling (Lemmon and Schlessinger, 2010). This is counteracted by the activity of protein tyrosine phosphatases (PTPs), which reverse EGFR tyrosine phosphorylation at the cell membrane, as well as during endocytic trafficking (Haj et al., 2002; Tarcic et al., 2009). Activated EGFRs undergo rapid endocytosis, which is orchestrated by endocytic proteins recruited directly and/or indirectly to activated receptors (Sorkin and Goh, 2009; Madhus and Stang, 2009). Internalised EGFRs reach early endosomes, from where receptors are rapidly recycled to the cell surface unless tagged robustly with ubiquitin (blue circles). Ubiquitylated EGFRs are sorted into MVBs, a step that segregates the EGFR kinase activity from the cytosol and effectively terminates signalling. The MVBs/late endosomes fuse with lysosomes, where EGF and EGFR undergo proteolysis (Sorkin and Goh, 2009; Madhus and Stang, 2009). Ubiquitylation directed by the EGFR-bound CBL E3 ligase is therefore crucial for the regulation of EGFR endocytosis and its role in signal attenuation.

the receptor being refractory to downregulation (Marmor and Yarden, 2004) might be permissive conditions for oncogenic activation of the EGFR.

An additional layer of negative feedback regulation becomes operational upon the de novo expression of EGFR inhibitors that are encoded by genes whose transcription is turned on by EGFR signalling. Four such IFIs have been identified so far in mammals, namely leucine-rich and immunoglobulin-like domains protein 1 (LRIG1) (Gur et al., 2004; Laederich et al., 2004), suppressor of cytokine signalling 4 and 5 (SOCS4 and SOCS5) (Nicholson et al., 2005; Kario et al., 2005) and receptor-associated late transducer (RALT, also known as MIG6 and ERFF1) (Fiorentino et al., 2000; Hackel et al., 2001; Xu et al., 2005a). These EGFR IFIs all bind directly to EGFR, which is key to their ability to suppress receptor signalling (Fig. 1). In addition, EGFR IFIs also bind to, and inhibit, other members of the ErbB family (Anastasi et al., 2003; Gur et al., 2004; Laederich et al., 2004; Kario et al., 2005) and therefore must be regarded as negative regulators of the entire ErbB signalling network. Because these IFIs have been studied in greatest detail in the context of EGFR signalling, in this Commentary we will focus on their role in EGFR regulation.

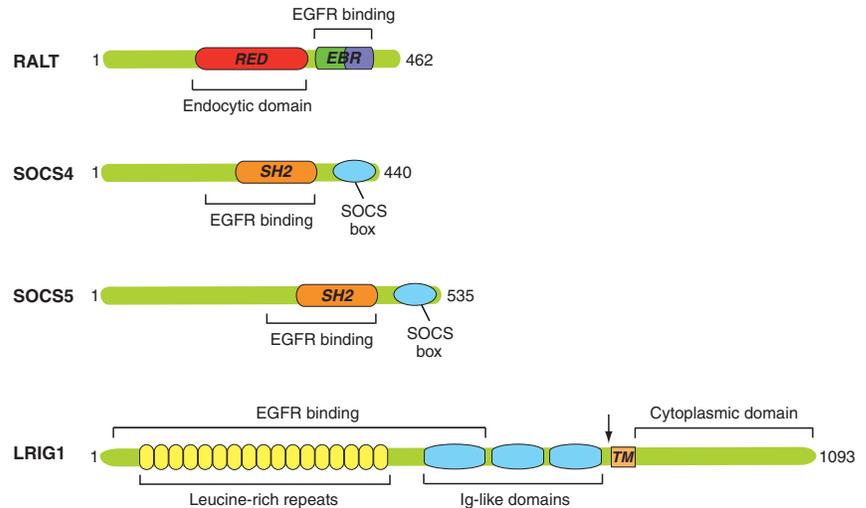
The expression of IFIs is controlled at the transcriptional and post-transcriptional level

Transcriptional activation of genes encoding the EGFR IFIs is transient. Levels of mRNAs encoding RALT (Fiorentino et al., 2000), SOCS4 and SOCS5 (Kario et al., 2005) peak within 60 minutes of the initial EGFR activation and decay thereafter. Induction of *LRIG1* mRNA is slower, reaching its maximum expression level within 3–4 hours of EGF stimulation (Gur et al., 2004). Besides these raw kinetics data, the information concerning the regulatory mechanisms involved in transcriptional activation and silencing of the genes encoding EGFR IFIs is scarce. As a word of caution, it must also be mentioned that the induction of *LRIG1*, *SOCS4* and *SOCS5* by EGF has so far only been studied in the HeLa cell line. It is interesting, though, that the *Lrig1* gene has been shown to be a transcriptional target of Myc in mouse keratinocytes (Jensen et al., 2009). This observation provides a mechanistic link between transcriptional programmes driven by growth factors and regulation of *Lrig1* expression. In the case of RALT, pharmacological and genetic analyses indicate that signalling through the RAS-ERK pathway is necessary and sufficient for EGF-dependent induction of its mRNA expression in murine fibroblasts (Fiorini et al., 2002).

In HeLa cells, SOCS5 protein expression is induced within 60 minutes of EGF stimulation and persists for at least 4 hours (Kario et al., 2005). In the same cell line, LRIG1 protein expression can be detected within 4 hours of EGF stimulation and remains high for the subsequent 6 hours (Gur et al., 2004). RALT protein expression can be induced by ErbB ligands (such as EGF, TGF α and neuregulin-1 β) in diverse cultured cell types, including fibroblasts, keratinocytes and mammary epithelial cells (Fiorini et al., 2002; Ballaro et al., 2005; Anastasi et al., 2005; Xu et al., 2005a). In these cellular models, RALT protein is expressed within 2 hours of EGFR activation and persists for a few more hours. Most importantly, subcutaneous injection of EGF leads to induction of RALT expression at the mRNA and protein levels in mouse skin, with kinetics similar to those reported in cultured cells (Ballaro et al., 2005).

The LRIG1, RALT, SOCS4 and SOCS5 proteins are labile and undergo proteasome-dependent degradation through poorly

Fig. 1. Structural features of mammalian inducible EGFR feedback inhibitors. RALT binds to the EGFR through the RALT EBR domain, which is necessary and sufficient to suppress receptor catalytic activation. The N-terminal portion of the EBR (amino acids 337–361, segment 1; depicted in darker green) binds to a surface of the EGFR kinase C-lobe that overlaps with the site through which the ‘activator’ contacts the ‘receiver’ within asymmetric kinase domain dimers (see Box 1). Residues in the C-terminal portion of the EBR (amino acids 362–412, segment 2; depicted in purple) are thought to contact the activation loop and/or the N-lobe of the (activated) receiver kinase domain. The RALT endocytic domain (RED, amino acids 143–323) couples RALT to endocytic effectors and is therefore necessary to drive EGFR downregulation. SOCS4 and SOCS5 bind to the EGFR through their respective SH2 domains, which share 87% sequence homology and a poorly defined N-terminal region. The SOCS box recruits an E3 ligase through elongin BC, and thereby leads to EGFR ubiquitylation. LRIG1 is a transmembrane protein that binds to the EGFR through its extracellular leucine-rich repeats and Ig-like domains. ADAM-mediated cleavage at, so far, unidentified sequences (the putative cleavage region is highlighted by an arrow) is proposed to release the soluble LRIG1 extracellular domain, which is capable of inhibiting EGF binding (see also Fig. 2). TM, transmembrane domain. Numbers indicate amino acid residues.



understood mechanisms. The half-life of LRIG1 has been reported to be 4 hours (Gur et al., 2004), whereas that of SOCS5 (Kario et al., 2005) and RALT (Fiorini et al., 2002) is 1 and 2 hours, respectively. EGF stimulation leads to a further reduction in LRIG1 and SOCS5 stability. By contrast, the impact of EGFR activation on the RALT half-life has not yet been determined.

In summary, these data, although still fragmentary, suggest that transcriptional and post-transcriptional control mechanisms confine the expression of IFIs to a window of a few hours, which is likely to coincide with mid-to-late G1 phase in cells undergoing EGF-driven proliferation. Details of how the individual IFIs mediate EGFR signal regulation will be discussed in the following sections.

LRIG1

LRIG1 is a cell surface transmembrane protein, whose distinctive structural feature is its extracellular domain, which contains a leucine-rich repeat (LRR) domain and three Ig-like modules (Nilsson et al., 2001) (Fig. 1). Although structurally related to the *Drosophila* Egfr inhibitor Kekk-1 (Ghiglione et al., 1999), LRIG1 is not its mammalian orthologue (Gur et al., 2004). Overexpression of LRIG1 causes inhibition of biochemical (e.g. ERK activation) and biological (e.g. cell cycle progression) responses downstream of the EGFR in a number of cultured cell lines (Gur et al., 2004; Laederich et al., 2004; Jensen and Watt, 2006).

LRIG1 interacts with the EGFR in a ligand-independent fashion. Either the LRR or the Ig-like domains of LRIG1 are sufficient to direct its binding to the extracellular region of the EGFR (Fig. 2A). In agreement with this observation, the EGFR intracellular domain is dispensable for formation of the EGFR–LRIG1 complex (Gur et al., 2004). LRIG1 promotes ligand-dependent ubiquitylation of EGFR. This is dependent on LRIG1–EGFR complex formation, binding of CBL to the intracellular domain of LRIG1 and activation of the CBL E3 ligase activity by tyrosine phosphorylation (Fig. 2A) (Gur et al., 2004). Notably, LRIG1 is capable of directing ubiquitylation and downregulation of EGFR Y1045F, a mutant receptor unable to

recruit CBL directly (Gur et al., 2004). Under physiological conditions, however, activated EGFRs probably recruit CBL proteins directly (through phosphorylated Y1045) as well as indirectly (through LRIG1), with both mechanisms acting additively to maximise EGFR ubiquitylation and its subsequent EGFR degradation (Fig. 2A). Ectopically expressed LRIG1 also affects the basal rate of EGFR degradation (Laederich et al., 2004), although it is unclear whether this requires ubiquitylation of surface-exposed EGFR molecules. In fact, expression of the dominant-negative CBL-70Z allele, which encodes a mutant form of the enzyme that is unable to direct ubiquitylation of CBL targets, did not prevent ectopic LRIG1 from reducing EGFR expression in the absence of EGF stimulation (Stutz et al., 2008). In summary, the available evidence suggests that LRIG1 overexpression accelerates basal, as well as ligand-induced, EGFR degradation. Importantly, these gain-of-function analyses are in agreement with the finding that ablation of LRIG1 expression in primary human keratinocytes is associated with increased expression of the EGFR at the cell surface (Jensen and Watt, 2006).

Proteolytic cleavage of ectopically expressed LRIG1 by members of the ADAM (a disintegrin and metalloproteinase) family releases soluble extracellular LRIG1 fragments that are capable of inhibiting EGFR signalling without inducing receptor downregulation (Fig. 1; Fig. 2A) (Yi et al., 2010). Consistent with the above study, a recombinant fragment of LRIG1 corresponding to the LRR domain was shown to attenuate EGFR signalling and EGFR-driven cell proliferation by acting as a high-affinity competitive inhibitor of EGF binding to the EGFR (Goldoni et al., 2007). It remains to be determined whether, and under which conditions, bioactive LRIG1 extracellular domain (LED) fragments are generated by processing of the endogenous LRIG1 protein.

RALT (MIG6)

RALT is a cytosolic protein (Zhang and Vande Woude, 2007), whose centrally located ErbB-binding region (EBR) (Fig. 1) allows specific binding to members of the ErbB receptor family (Anastasi et al., 2003; Zhang et al., 2007). RALT inhibits downstream EGFR

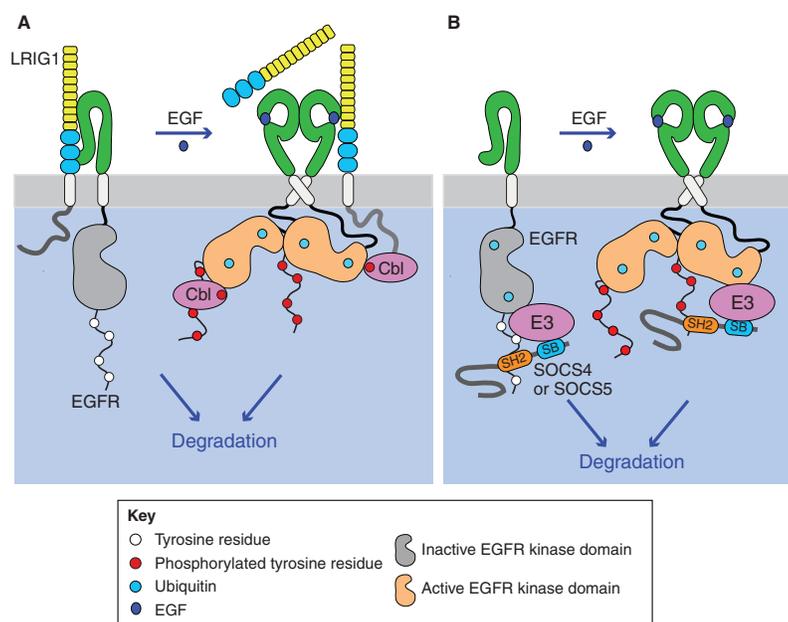


Fig. 2. Mechanisms of EGFR inhibition by LRIG1, SOCS4 and SOCS5. (A) LRIG1 binds to ligand-free EGFRs. Inactive EGFR molecules bound to LRIG1 are degraded by a poorly defined mechanism. A second EGFR inhibitory mechanism involves activated EGFR inducing tyrosine phosphorylation of LRIG1-bound CBL, which is then capable of directing EGFR ubiquitylation. Under physiological conditions, it is probable that CBL is recruited to the EGFR both directly, as well as in an LRIG1-mediated fashion, to allow for maximal ubiquitylation to take place. A third mechanism requires the LRIG1 extracellular domain to be released into the extracellular space by ADAM cleavage, where it attenuates EGFR signalling by acting as a competitive inhibitor for EGF. (B) SOCS4 and SOCS5 bind to the EGFR in a ligand-independent manner to promote EGFR degradation. This might be accomplished upon ubiquitylation of both active and inactive EGFR molecules by a, so far, unidentified E3 ligase that is recruited to the SOCS-box-bound elongin BC (not shown here). Lysine residues on the EGFR that are targeted by SOCS4- and/or SOCS5-directed ubiquitylation have not been mapped yet and are depicted arbitrarily positioned in the EGFR kinase domain by analogy to CBL-directed ubiquitylation.

signalling, including activation of ERKs and AKT, as well as biological responses regulated by the EGFR, such as cell proliferation and cell locomotion, as determined by both gain- and loss-of-function studies in a wide range of cultured cells (Hackel et al., 2001; Anastasi et al., 2003; Ballaro et al., 2005; Anastasi et al., 2005; Xu et al., 2005a; Reschke et al., 2010). RALT is recruited to the EGFR kinase domain in a ligand-dependent fashion. Overexpression of the RALT EBR is sufficient to abrogate EGFR autophosphorylation and downstream signalling (Xu et al., 2005a; Anastasi et al., 2007). The mechanism underlying RALT-mediated EGFR inhibition involves binding of the EBR to an extended surface of the EGFR catalytic domain that is involved in allosteric activation of the EGFR kinase (Zhang et al., 2007) (Fig. 1; Fig. 3). As a consequence, RALT-bound EGFR molecules remain locked in a catalytically inactive configuration, which prevents signal generation (Zhang et al., 2006; Zhang et al., 2007).

Notably, RALT-bound EGFR molecules undergo rapid downregulation and are eventually routed to the lysosome for degradation (Ying et al., 2010; Frosi et al., 2010). The latter finding is somewhat surprising, given that the EGFR directs its own endocytosis and subsequent sorting into multivesicular bodies (MVBs) through kinase-directed signalling to the endocytic machinery (Sorkin and Goh, 2009; Madhus and Stang, 2009). This conundrum was resolved by the demonstration that RALT binds to components of the endocytic machinery through the RALT endocytic domain (RED), which is positioned adjacent to the EBR (Fig. 1). The RED was shown to bind to AP-2, the major adaptor complex responsible for sorting cargo into clathrin-coated pits (Traub, 2009), and intersectins, which are SH3-domain-containing accessory proteins involved in cargo sorting and clathrin-coated pit maturation (Fig. 3) (O'Bryan, 2010; Mettlen et al., 2009). These molecular interactions allow coupling of EGFR–RALT complexes to clathrin-mediated endocytosis (Frosi et al., 2010). Interestingly, the C-terminal tail of tyrosine kinase non-receptor protein 2 (TNK2, also known as ACK-1), which is involved in clathrin-mediated endocytosis (Teo et al., 2001; Shen et al., 2011), shares a high degree of homology with the RALT EBR and the SH3-binding motifs located in the adjacent RALT RED (Fiorentino et al., 2000).

Besides promoting clathrin-mediated endocytosis of the EGFR, RALT was also shown to mediate EGFR sorting to late endosomes through its binding to syntaxin 8 (Ying et al., 2010), a component of the endosomal SNARE complexes that are involved in cargo trafficking from early to late endosomes (Jahn and Scheller, 2006; Cai et al., 2007; Prekeris et al., 1999). As RALT-driven downregulation of the EGFR takes place in the absence of robust EGFR ubiquitylation (Frosi et al., 2010), syntaxin 8 might direct ubiquitin-independent cargo sorting into late endosomes, although this has not yet been addressed experimentally. In summary, by virtue of its ability to recruit endocytic proteins, the RALT RED orchestrates EGFR trafficking from the plasma membrane to late endosomes, thus conferring full endocytic competence to RALT-bound kinase-inactive EGFR molecules.

Because EBR-mediated kinase suppression is sufficient to abrogate EGFR signalling, one might wonder why RALT also evolved the ability to downregulate EGFR. A plausible explanation is that the two mechanisms act sequentially and in concert to provide robust and long-term inhibition of the receptor. First, EGFR and RALT become concentrated on the surface of clathrin-coated pits, vesicles and endosomes. Here, binding of RALT to endocytic proteins provides EGFR–RALT complexes with multiple attachment points to membrane proteins (Schmid and McMahon, 2007; Frosi et al., 2010). This is predicted to stabilise the EGFR–RALT interaction and perpetuate EGFR catalytic repression. Second, downregulation of RALT-bound EGFR molecules switches receptor inhibition from a reversible mechanism (catalytic suppression) to an irreversible one (degradation), thus extending the duration of cellular desensitisation to EGFR ligands.

SOCS4 and SOCS5

The SOCS family comprises eight intracellular proteins [SOCS1 to SOCS7 and cytokine inducible SH2-domain protein (CIS)], which are involved in feedback inhibition of cytokine receptor signalling (Crocker et al., 2008). All SOCS proteins share a similar domain organisation, which includes an N-terminal region, an SH2 domain and a C-terminal SOCS box (Fig. 1). SOCS5 binds to the EGFR in a ligand-independent fashion. This interaction

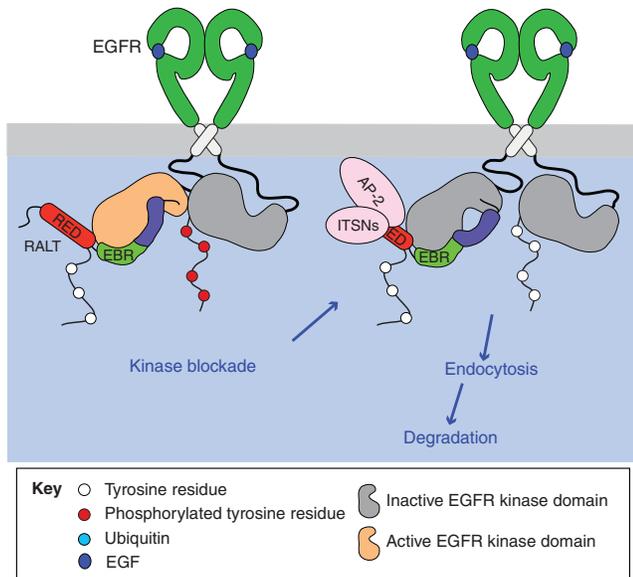


Fig. 3. Mechanisms of EGFR inhibition by RALT. RALT binds to ligand-activated EGFRs through its EBR, inhibiting allosteric kinase activation (left). As proposed by Kuriyan and colleagues (Zhang et al., 2007) the EBR docks onto the activated EGFR kinase domain (the ‘receiver’, depicted in orange) through EBR segment 1 (green), which allows for the positioning of EBR segment 2 (purple) into the kinase active site. This blocks the catalytic activity. Segment 1 subsequently impedes further propagation of EGFR activation by preventing the ‘receiver’ from acting as the ‘activator’ in the reciprocal orientation of asymmetric kinase dimers (right-hand side). Upon docking of the EBR onto the EGFR, the RED interacts with endocytic proteins [right-hand side, shown are AP-2 and intersectins (ITSNs)]; this process directs trafficking of RALT-bound EGFRs from the plasma membrane to late endosomes, thereby resulting in receptor degradation.

requires the integrity of both the N-terminal and SH2 domains of SOCS5 (Nicholson et al., 2005; Kario et al., 2005) (Fig. 2B). Structural studies have suggested that phosphorylation of tyrosine residue 1068 in EGFR is a candidate docking site for the SOCS4 and SOCS5 SH2 domains (Bullock et al., 2007). However, this has not been addressed by EGFR mutagenesis and it remains unclear how the SH2 domain of SOCS4 and SOCS5 is recruited to the EGFR in the absence of ligand-induced receptor tyrosine phosphorylation. Nonetheless, it is clear that ectopic expression of SOCS4 and SOCS5 leads to the accelerated destruction of EGFR, but not fibroblast growth factor receptor (FGFR) 1, and suppression of biological and biochemical responses downstream of EGFR in different cell types (Nicholson et al., 2005; Kario et al., 2005).

Mutagenesis studies have indicated that coupling of SOCS5 to elongin BC (the molecular complex between elongin B and elongin C) through its SOCS box is required for SOCS5-dependent EGFR downregulation. This observation supports a model in which binding of SOCS4 and/or SOCS5 to the EGFR enhances receptor ubiquitylation and, consequently its degradation, through an elongin-BC-containing E3 ubiquitin ligase complex (Kario et al., 2005) (Fig. 2B). There is disagreement as to whether EGFR molecules bound to SOCS5 do (Nicholson et al., 2005) or do not (Kario et al., 2005) require activation by EGF in order to undergo SOCS5-dependent downregulation. This discrepancy might reflect experimental differences, but it nevertheless raises the interesting

possibility that SOCS4 and/or SOCS5 might act as ‘scavengers’ that bind to ligand-free EGFRs, as has been shown for LRIG1 (Fig. 2).

It is currently unknown whether endogenous levels of SOCS4 and/or SOCS5 are sufficient to substantially downregulate EGFR and its subsequent signalling. It is noteworthy, however, that ectopically expressed SOCS2, SOCS3, SOCS6 and CIS do not affect EGFR levels (Kario et al., 2005), whereas loss of SOCS4 and SOCS5 in hepatocellular carcinomas correlates with higher EGFR expression (Calvisi et al., 2007) (see also supplementary material Table S1).

An integrated view of EGFR IFIs: facts and hypotheses

mRNA expression data available from public databases suggest that *LRIG1*, *RALT*, *SOCS4* and *SOCS5* are coexpressed in several tissues. However, a systematic immunohistochemical survey of IFI expression has not yet been undertaken. Bearing this in mind, it is nevertheless worthwhile to speculate how coexpression of different IFIs affects EGFR regulation. IFIs share the property of driving EGFR downregulation, and they seem to do so by coupling to distinct effectors. Coexpressed IFIs might therefore signal additively: each class of inhibitors might promote the downregulation of a distinct pool of EGFR molecules, the overall effect being a large reduction in the levels of EGFR. Synergy among IFIs might also be envisioned. Accordingly, LRIG1, SOCS4 and SOCS5 might reduce responsiveness to EGFR agonists by downregulating receptors not yet occupied by ligand, whereas ligand-engaged receptors would be subject to catalytic repression mediated by RALT. Thus, by limiting receptor engagement through downregulation, and ablating receptor activation through catalytic repression, IFIs working in concert would act as ‘signal erasers’ and severely limit cellular responses to EGFR agonists.

Besides acting on all ErbB RTKs, EGFR IFIs have been shown to target a limited number of non-ErbB RTKs. For example, MET signalling is inhibited by both LRIG1 (Shattuck et al., 2007) and RALT (Pante et al., 2005). LRIG1 is also capable of suppressing RET signalling (Ledda et al., 2008). These data are interesting in light of the fact that functional and/or physical interactions between MET and ErbB RTKs (Jo et al., 2000; Scheving et al., 2002; Engelman et al., 2007), as well as between RET and the EGFR (Croyle et al., 2008), have been reported. Notably, silencing of RALT by RNA interference (RNAi) has been shown not only to enhance EGFR and ERBB2 tyrosine phosphorylation, but also to promote MET activation. The latter effect was sensitive to pharmacological inhibition of EGFR (Lin et al., 2011), implying that RALT affects the lateral signalling of EGFR to non-ErbB RTKs. Thus, EGFR IFIs are able to affect, directly or indirectly, the activity of ‘foreign’ RTKs connected to the ErbB network.

RALT expression is upregulated by stimuli as diverse as growth factors, hypoxia, mechanical and osmotic stress, steroid hormones, retinoic acid (Zhang and Vande Woude, 2007) and actin dynamics (Descot et al., 2009). Although this is compatible with RALT having signalling functions independent of its role in ErbB suppression (Zhang and Vande Woude, 2007), it is notable that EGFR expression and/or signalling might be influenced by most of the above stimuli. Hence, the plethora of agents capable of inducing *RALT* expression might also reflect, albeit not exclusively, the wide range of signalling scenarios in which the EGFR has a role.

Physiological relevance of EGFR feedback inhibition

Genetic experiments in the mouse have provided compelling evidence that EGFR signalling regulates the morphogenesis and homeostasis of different tissues, including several epithelia (Sibilia et al., 2007). Thus, a relevant question is whether genetic ablation of EGFR IFIs in the mouse causes phenotypes imputable to deregulated signalling by EGFR.

Lrig1^{-/-} mice develop psoriasis-like skin lesion

The epidermis of *Lrig1*^{-/-} mice shows considerable thickening due to increased cell proliferation and reduced differentiation (Suzuki et al., 2002). These lesions are psoriasis-like and reminiscent of those observed in transgenic mice engineered to overexpress the EGFR ligands TGF α (Vassar and Fuchs, 1991) and amphiregulin (Cook et al., 1997) in the skin. In both mouse and human epidermis, LRIG1 expression is highest in keratinocytes possessing the characteristics of stem cells and is inversely correlated to the expression of EGFR (Jensen and Watt, 2006; Jensen et al., 2009). Consistently, silencing of LRIG1 increases cell surface expression of EGFR in cultured human keratinocytes (Jensen and Watt, 2006). Loss of LRIG1 in both mouse and human keratinocytes leads to increased EGFR signalling, higher expression of Myc and, consequently, expanded keratinocyte proliferation. Accordingly, LRIG1, with its capacity to reduce EGFR expression and signalling, has been proposed to maintain the quiescent state of interfollicular stem cells (Jensen and Watt, 2006; Jensen et al., 2009).

RALT restricts cell proliferation in several mouse tissues

Knockout mice lacking the gene encoding RALT (*Errfi1*) show a fully penetrant skin phenotype, characterised by epidermal hyperplasia, as a result of increased proliferation and reduced differentiation of keratinocytes (Ferby et al., 2006). In addition, *Errfi1*^{-/-} skin keratinocytes are extremely sensitive to chemical carcinogenesis, giving rise to papillomas with shorter latency and higher frequency than keratinocytes in *Errfi1*^{+/+} mice (see also supplementary material Table S1). Crucially, spontaneous and induced skin lesions in *Errfi1*^{-/-} mice regress following treatment with gefitinib, a clinically used EGFR inhibitor. In strong agreement, the skin phenotype of *Errfi1*^{-/-} mice is rescued following their cross into a hypomorphic *Egfr* background (Ferby et al., 2006). These pharmacological and genetical rescue experiments imply that the skin phenotype of *Errfi1*^{-/-} mice results from excess EGFR activity (Ferby et al., 2006). Reciprocally, targeted overexpression of RALT in mouse skin (Ballaro et al., 2005) induces a phenotype (wavy coat, curly whiskers and open eyelid at birth) similar to that of mice that either lack TGF α (Luetke et al., 1993) or are homozygous for the hypomorphic *wa2 Egfr* allele (Luetke et al., 1994).

Errfi1^{-/-} mice also show a perinatal lung phenotype associated with hyperproliferation of epithelial cells in bronchi and alveoli (Jin et al., 2009). This phenotype is probably caused by excessive EGFR signalling because the alveolar and vascular lesions of *Errfi1*^{-/-} mice, first, are reminiscent of those observed in mice with conditional perinatal overexpression of TGF α (Le Cras et al., 2004), second, develop in a timeframe characterised by a burst of expression of *Errfi1* mRNA in the lung of normal mice (Jin et al., 2009) and, third, are associated with increased EGFR signalling and overexpression of EGFR ligands in lung epithelial cells (Jin et al., 2009).

Mouse genetics of EGFR IFIs: tip of an iceberg?

Socs5^{-/-} mice do not show phenotypes imputable to altered EGFR activity (Nicholson et al., 2005) and currently there are no reports on the effects of *Socs4* ablation in the mouse. It must be noted, however, that SOCS4 and SOCS5 share extensive sequence homology (87%) in their SH2 domain and might therefore have redundant roles in EGFR regulation.

Although it is clear that RALT and LRIG1 have an essential role in restraining EGFR-driven cell proliferation in some tissues (see Box 2 for further discussion), we suspect that the information available currently might underestimate the relevance of EGFR IFIs in tissue morphogenesis and/or homeostasis. Loss of a single IFI might be tolerated in those tissues in which global IFI activity is sufficiently high. Moreover, some phenotypes could be subtle and require ad hoc experimental approaches in order to be unveiled. For instance, although liver development is normal in *Errfi1*^{-/-} mice, the initial phase of liver regeneration following partial hepatectomy is substantially faster in *Errfi1*^{-/-} mice compared with

Box 2. Inducible feedback inhibitors as gatekeepers of EGFR mitogenic signalling

Progression through G1 and entry into S phase of the cell cycle requires sustained mitogenic signals (Jones and Kazlauskas, 2001). Cell biology (Ballaro et al., 2005; Anastasi et al., 2005; Jensen and Watt, 2006) and developmental genetics (Suzuki et al., 2002; Ferby et al., 2006; Jensen et al., 2009) have placed IFIs within the regulatory circuits that determine whether EGFR signals reach the threshold necessary to commit a cell to S phase. For example, in the absence of RALT, suboptimal levels of EGF increase the fraction of cells recruited into the mitotic cell cycle (Ballaro et al., 2005; Anastasi et al., 2005). IFIs restrict EGFR mitogenic signalling through several different mechanisms (see also Figs 2 and 3). EGFR downregulation (through all IFIs) limits receptor occupancy, whereas catalytic repression (through RALT) renders ligand–receptor interactions unproductive. In addition, by routing EGFR ligands for degradation (Frosi et al., 2010), RALT also prevents renewed stimulation of surface-exposed receptors by recycled ligands.

Key to understanding the uniqueness of EGFR IFIs is the notion that they operate on the same timescale during which EGFR signalling is reinforced by the induction of autocrine ligands, such as ephregrin, TGF α and amphiregulin (Citri and Yarden, 2006). Notable features of all these ligands are their inability to drive substantial EGFR downregulation (Roepstorff et al., 2009) and their ability to elicit broad ErbB activation (Yarden and Sliwkowski, 2001). Both features, although conferring robustness to EGFR-driven mitogenic stimulation, carry the risk of causing overactivation of mitogenic pathways (Citri and Yarden, 2006). EGFR IFIs are expected to provide a key balance to these positive feedback loops, because (i) LRIG1, SOCS4 and/or SOCS5 might promote ligand-independent EGFR degradation; (ii) RALT inhibits EGFR catalytic function regardless of the activating ligand (Anastasi et al., 2007); and (iii) LRIG1, SOCS4, SOCS5 and RALT antagonise the activity of all ErbB family members, including ERBB2, which acts as a signal amplifier. Consistent with this view, levels of EGFR ligands, such as heparin-binding EGF-like growth factor (HB-EGF), which activates both EGFR and ErbB4, and amphiregulin, which stimulates EGFR without inducing receptor degradation, are increased in the lung and liver of *Errfi1*^{-/-} mice (Reschke et al., 2010; Jin et al., 2009), which might be regarded as both a consequence and cause of the unabated EGFR activity generated by unbalanced feedback control.

that in control littermates. This is correlated with increased EGFR levels and enhanced EGFR signalling in *Errfi1*^{-/-} hepatocytes (Reschke et al., 2010) and is consistent with the slower liver regeneration observed in mice carrying liver-specific *Egfr* ablation (Natarajan et al., 2007).

Inducible feedback inhibitors as tumour suppressors

EGFR and *ERBB2* are established cancer-causing genes (Futreal et al., 2004; Santarius et al., 2010), and recent work also points to *ERBB3* (Mills and Yarden, 2010) and *ERBB4* (Prickett et al., 2009) signalling as being relevant to cancer pathogenesis. This raises the question as to whether EGFR IFIs act as tumour suppressors in neoplastic disorders that rely on oncogenic ErbB signalling. This issue has been addressed by a number of studies (supplementary material Table S1). We summarise some of the most relevant findings here.

The *ERRFII* gene maps to chromosome 1p36, one of the regions most often targeted by genomic alterations in human tumours (Ragnarsson et al., 1999), including glioblastoma multiforme (GBM). *EGFR* amplification is detected in ~50% of human GBMs (Furnari et al., 2007). When analysed at the mRNA and protein level, loss of RALT is detected in ~50% of GBM samples, a fraction of which contain 1p36 deletions involving the *ERRFII* locus (Ying et al., 2010; Duncan et al., 2010). Importantly, *ERRFII* deletions are substantially more frequent in GBMs carrying *EGFR* amplification, an observation that is consistent with RALT exerting an EGFR-specific tumour suppression activity (Ying et al., 2010). In GBMs without 1p36 deletions, promoter methylation, as shown in samples of papillary thyroid carcinoma (Lin et al., 2011), might account for loss of RALT expression, although this has not been addressed experimentally. Restoration of RALT expression in glioblastoma cell lines leads to inhibition of their clonal growth. This is associated with reduced phosphorylation and accelerated degradation of the wild-type EGFR (Ying et al., 2010). Intriguingly, the half-life of EGFRvIII (a constitutively active EGFR mutant that lacks the portion of the extracellular domain encoded by exons 2–7) (Furnari et al., 2007), which is fully capable of binding RALT, is not affected by the RALT expression status in GBM cells. However, *ERRFII* deletions occur at a 2.5-fold higher frequency in GBM samples expressing the EGFRvIII mutant when compared with that in GBMs showing only *EGFR* amplification (Ying et al., 2010). It must be noted, first, that the EGFRvIII mutation occurs rarely in tumours lacking concomitant amplification of the wild-type *EGFR* allele and, second, that EGFRvIII instigates GBM cells to produce soluble factors that increase tumour aggressiveness through the cross-activation of wild-type EGFR (Inda et al., 2010). Thus, RALT might still attenuate the oncogenic potential of EGFRvIII by limiting activation and expression of concomitantly overexpressed wild-type EGFR.

Although the *LRIG1* gene maps to chromosome 3p14, a region frequently deleted in human tumours, conclusive genetic evidence implicating a role for LRIG1 in tumour suppression is not yet available. Nevertheless, a number of reports suggest that loss of LRIG1 expression does occur in human tumours and might be clinically relevant. Reduced LRIG1 expression was detected in cell lines, as well as clinical samples of head and neck squamous cell carcinomas (HNSCCs), and correlated with a higher tumour grade and cell proliferation index (Jensen et al., 2008). Similar data were reported in cutaneous squamous cell carcinomas (Tanemura et al., 2005). Although these studies did not correlate

LRIG1 expression with that of EGFR, it is intriguing that LRIG1 regulates stem cell quiescence in a prototypical squamous epithelium, such as the epidermis (Jensen and Watt, 2006; Jensen et al., 2009), and that overexpression of EGFR and its ligands might be involved in the pathogenesis of HNSCCs (Morgan and Grandis, 2009). A role for LRIG1 has been proposed in the suppression of EGFRvIII oncogenic signals in GBM cells (Stutz et al., 2008), but it is still unknown whether and how frequently LRIG1 loss occurs during GBM progression. It is interesting, however, that LRIG1, unlike RALT, is capable of targeting EGFRvIII for degradation (Stutz et al., 2008).

The studies discussed above and those cited in supplementary material Table S1 suggest that loss of EGFR IFIs is selected for in human tumours. In the majority of these studies, loss of expression of IFIs was correlated to genetic alterations of ErbB RTKs and therefore could fit in the context of ErbB-driven oncogenesis. Even in this case, however, caution needs to be exerted in drawing the conclusion that loss of IFIs is causally involved in tumour pathogenesis, unless significant correlations between loss of IFIs and relevant clinical and pathological parameters are also described. Future studies of IFI loss in human tumours should be also complemented by assessing the consequences of genetic ablation of a given IFI in mouse models of ErbB-driven carcinogenesis. Finally, from a clinical point of view, it will be crucial to understand whether the assessment of IFI expression in tumour samples could be predictive of sensitivity to ErbB-targeted therapeutics.

Conclusions and perspectives

In summary, genetic studies in mice, along with biochemical and biological experiments in cultured cells, indicate that IFIs play a crucial role in the regulation of EGFR-driven cell proliferation in a number of epithelia (see also Box 2). The role of IFIs might, however, be more pervasive than currently appreciated, potentially owing to functional redundancy among different IFIs. Much remains to be learnt about the mechanistic details of the function of IFIs, as well as the signalling pathways downstream of ErbB RTKs that regulate the expression of IFIs in tissues. It is of pressing importance that future *in vitro* studies address the structure–function relationships of individual IFIs under physiological conditions and are supported by genetic studies in mice. It will also be important to address the function of IFIs within network models that provide a more accurate representation of EGFR activity in physiological conditions. These models should take into account the compound effects that EGFR heterodimerisation with ErbB and non-ErbB RTKs has on EGFR activation, signalling and trafficking, as well as the cross-activation of EGFR by integrins, G-protein-coupled receptors and cytokine receptors (Citri and Yarden, 2006). EGFR IFIs are predicted to possess rich connectivity within these networks, given their ability to inhibit all ErbB RTKs, as well as a few non-ErbB RTKs. This implies that EGFR IFIs are likely to participate in system-level control of ErbB signalling. The daunting challenge ahead is to understand such complexity. This will require a quantitative approach to studying the expression and function of EGFR IFIs. The issue of IFI coexpression also needs to be addressed, because coexpressed IFIs might affect EGFR signalling in an additive or a synergistic fashion, thus potentially constituting an integrated platform for EGFR regulation. The generation of computational models that incorporate this information will be key to gaining a deeper understanding of the activity of IFIs in normal and pathological conditions.

We thank D. Barilà, A. Bagnato, P. G. Natali and V. Robbiati for critically reading the manuscript. O.S. is supported by AIRC and Progetto Finalizzato Oncologia and S. Alemà is supported by PRIN.

Supplementary material available online at

<http://jcs.biologists.org/cgi/content/full/124/11/1785/DC1>

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