

A promiscuous liaison between IL-15 receptor and Axl receptor tyrosine kinase in cell death control

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Discrimination between cytokine receptor and receptor tyrosine kinase (RTK) signaling pathways is a central paradigm in signal transduction research. Here, we report a ‘promiscuous liaison’ between both receptors that enables interleukin (IL)-15 to transactivate the signaling pathway of a tyrosine kinase. IL-15 protects murine L929 fibroblasts from tumor necrosis factor α (TNF α)-induced cell death, but fails to rescue them upon targeted depletion of the RTK, Axl; however, Axl-overexpressing fibroblasts are TNF α -resistant. IL-15R α and Axl colocalize on the cell membrane and co-immunoprecipitate even in the absence of IL-15, whereby the extracellular part of Axl proved to be essential for Axl/IL-15R α interaction. Most strikingly, IL-15 treatment mimics stimulation by the Axl ligand, Gas6, resulting in a rapid tyrosine phosphorylation of both Axl and IL-15R α , and activation of the phosphatidylinositol 3-kinase/Akt pathway. This is also seen in mouse embryonic fibroblasts from wild-type but not Axl $-/-$ or IL-15R α $-/-$ mice. Thus, IL-15-induced protection from TNF α -mediated cell death involves a hitherto unknown IL-15 receptor complex, consisting of IL-15R α and Axl RTK, and requires their reciprocal activation initiated by ligand-induced IL-15R α .

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Introduction

The cytokine interleukin (IL)-15 is a potent apoptosis inhibitor (Bulfone-Paus *et al.*, 1997b, 1999) with many immunomodulatory activities that overlap with those of IL-2 (Bulfone-Paus *et al.*, 1997a; Waldmann and Tagaya, 1999). IL-15 binds to a heterotrimeric receptor complex that shares the IL-2 receptor β (IL-2R β) and IL-2 receptor γ (IL-2R γ/γ_c)

chains, but has a unique IL-15R α chain that binds IL-15 with high affinity even in the absence of the other two receptor subunits (Giri *et al.*, 1995; Fehninger and Caligiuri, 2001).

Receptor tyrosine kinases (RTKs) are cell-surface receptors that transduce specific signals from the extracellular environment, possess an intrinsic tyrosine kinase activity, and control growth, differentiation, and survival of cells (Bennasroune *et al.*, 2004). The mammalian Axl RTK family includes Axl, Tyro3, and Mer (Graham *et al.*, 1994; Mark *et al.*, 1994), and is widely expressed (Lai *et al.*, 1994; Lu *et al.*, 1999; Healy *et al.*, 2001; Lu and Lemke, 2001). Axl, Tyro3, and Mer display an extracellular region (two immunoglobulin-related domains linked to two fibronectin type III repeats) and an intracellular region that contains an intrinsic RTK domain. The only known bona fide ligand for the Axl RTK family members is Gas6, the product of *growth-arrest-specific* gene 6 (Nagata *et al.*, 1996), a soluble member of the vitamin K-dependent protein family (Manfioletti *et al.*, 1993).

Gas6/Axl signaling modulates cell growth and inhibits apoptosis (Bellosta *et al.*, 1997; Melaragno *et al.*, 2004). Axl promotes survival of pulmonary endothelial cells (Healy *et al.*, 2001) and neuronal cells (Allen *et al.*, 1999), and protects murine fibroblasts and human endothelial cells from apoptosis induced by TNF α or other stimuli (Bellosta *et al.*, 1997; Goruppi *et al.*, 1997). Axl is overexpressed in a variety of tumor cells (Chen *et al.*, 1999; Goruppi *et al.*, 2001), and overexpression of Axl can transform fibroblasts even in the absence of ligand (O’Brian *et al.*, 1991; Burchert *et al.*, 1998). Among signaling molecules activated by Axl in various cell types are phosphatidylinositol 3-kinase (PI3K), Akt, Src, extracellular signal-regulated kinase (ERK), and nuclear factor kappaB (NF- κ B) (Goruppi *et al.*, 1997; Lee *et al.*, 2002). For example, in NIH 3T3 mouse fibroblasts, antiapoptotic Gas6/Axl signaling induces NF- κ B activity and increases protein level of Bcl-x_L (Demarchi *et al.*, 2001).

IL-15 protects mouse L929 fibroblasts from TNF α -induced apoptosis through recruitment of TRAF2 to the IL-15R α chain and NF- κ B activation (Bulfone-Paus *et al.*, 1999). In human Raji cells, IL-15 stimulation reduces ceramide-induced apoptosis, accompanied by the recruitment of Syk, a non-receptor protein-tyrosine kinase, to the IL-15R α chain, which phosphorylates a single tyrosine in the cytoplasmic tail of IL-15R α (Bulanova *et al.*, 2001). L929 cells lack Syk, yet IL-15 induces IL-15R α phosphorylation in these cells.

Therefore, the goal of this study was to investigate whether the IL-15-mediated antiapoptotic signaling in these model cells involves the activity of other, yet unknown kinase(s). Here, we unravel an unexpected alliance between IL-15R α and Axl RTK, which is indispensable for the IL-15-mediated survival of L929 cells upon TNF α stimulation. Our results point to a so far unprecedented cooperation in signal transduction between a cytokine receptor and an RTK, and demonstrate how direct interactions between heterologous receptor systems can regulate qualitatively distinct cellular responses to death-inducing and death-opposing stimuli.

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Results

Axl RTK is essential for the IL-15-mediated protection of murine fibroblasts from TNF α cytotoxicity

IL-15 protects mouse L929 fibroblasts from TNF α -mediated cell death via recruitment of TRAF2 to its high-affinity receptor chain, followed by NF- κ B activation (Bulfone-Paus *et al*, 1999). To further dissect the antiapoptotic effects of IL-15, we have established an IL-15-dependent L929 subline that is resistant to TNF α -induced cell death, referred to hereafter as L929R (see Materials and methods). The L929R cells are indistinguishable from the parental cell line, both morphologically and with respect to the expression levels of TNF α receptors, IL-15R α and IL-2R γ (Supplementary Figure 1) and the absence of IL-2R β (data not shown). However, cDNA microarray revealed a significant upregulation of an RTK, Axl, in L929R cells compared to parental L929 cells (data not shown). High expression of Axl was confirmed by RT-PCR, Western blotting (WB), and flow cytometry analysis (FACS) (Supplementary Figure 1A and B).

Given the high expression of Axl in L929R cells, we tested how IL-15 treatment affects Axl expression in L929 cells. In fact, IL-15 stimulation for 24 h induced a significant upregulation of Axl RTK mRNA and protein to a level comparable to that of L929R cells (Supplementary Figure 2A and B). In

L929R cells, IL-15 failed to further elevate Axl expression. Gas6 did not modulate the expression of its own receptor (neither at the transcriptional nor at the protein level) within 24 h (Supplementary Figure 2A and B), 48, or 72 h (data not shown). Instead, Gas6 significantly upregulated IL-15R α expression (Supplementary Figure 2A). As previously reported (Kumaki *et al*, 1996), IL-15 downregulated the expression of its own high-affinity α chain (Supplementary Figure 2A).

The fact that Gas6/Axl signaling protects murine fibroblasts from apoptosis induced by serum withdrawal or treatment with TNF α (Bellosta *et al*, 1997) raised the possibility that the observed overexpression of Axl in L929R cells was the sole cause for the acquired TNF α resistance. In this case, targeted deletion of Axl was expected to restore TNF α sensitivity in L929R cells. Thus, L929R cells, as well as the parental cells, were transfected with small interfering RNA oligonucleotides (Axl siRNA) to disrupt Axl transcripts and were analyzed by RT-PCR and WB for the knockdown of Axl 48 h post-transfection.

Transfection with Axl siRNA resulted in a significant decrease of Axl at the mRNA (Figure 1A) and protein (Figure 1B) levels in both cell lines. As expected, the targeted depletion of Axl indeed restored TNF α sensitivity in L929R cells, resulting in a dramatic increase in the number of apoptotic cells in the presence of TNF α , as assessed by

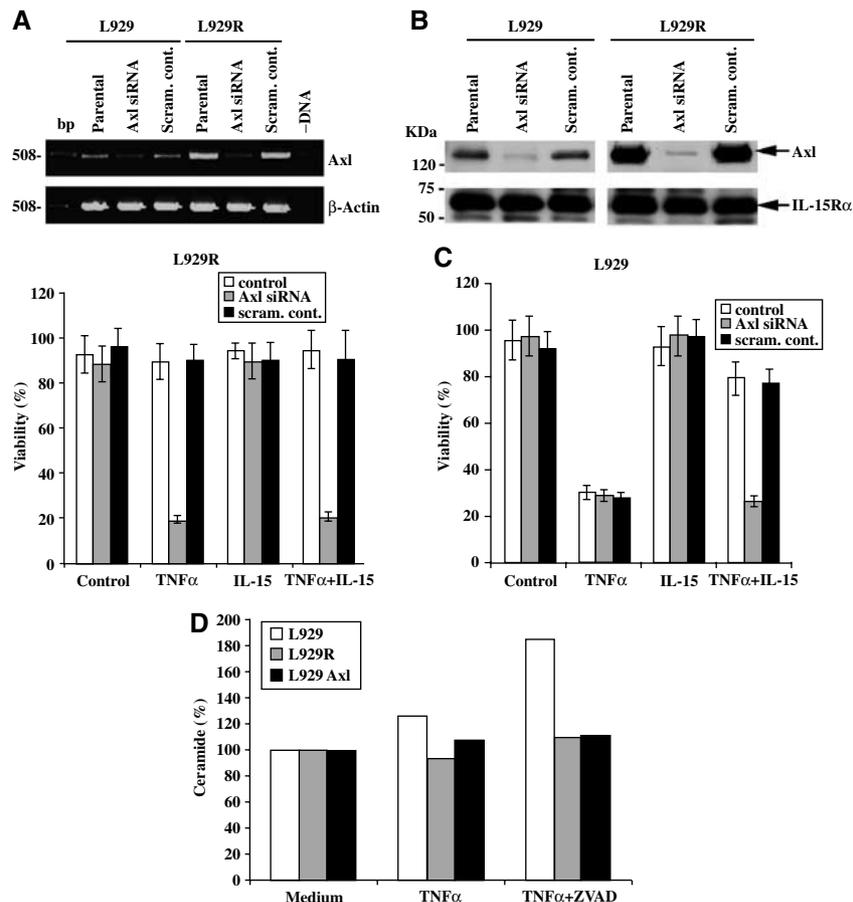


Figure 1 Axl is required for resistance to TNF α cytotoxicity. L929 and L929R cells were transfected with Axl siRNA. (A) Axl mRNA expression was analyzed by RT-PCR (upper panel). β -Actin is used as an internal control (lower panel). (B) WB analysis of Axl protein expression (upper panel). IL-15R α expression served as a control (lower panel). (C) Cells were treated for 18 h with TNF α (5 ng/ml), IL-15 (100 ng/ml), or both, and cell viability was assessed by PI and annexin V staining and FACS analysis. (D) L929, L929R, and Axl-overexpressing L929 cells were treated or not with TNF α (100 ng/ml) alone or together with 20 mM zVAD-fmk and ceramide generation was determined. All ceramide determinations were performed in duplicate.

propidium iodide (PI) and annexin V staining and FACS analysis. About 70–80% of Axl-targeted L929R cells died, whereas cells transfected with scrambled control siRNA oligonucleotides remained viable (Figure 1C). Moreover, IL-15 failed to prevent the TNF α -induced apoptosis in parental L929 cells deficient in Axl expression, but effectively rescued non-transfected fibroblasts (Figure 1C). The protective properties of Axl were corroborated by the fact that L929 cells stably overexpressing a vector coding for Axl were resistant to TNF α (Supplementary Figure 2C). Transfection of L929 cells with siRNA oligonucleotides targeting expression of IL-2R γ had no influence on the IL-15-mediated protection of these cells from TNF α -induced apoptosis (data not shown). This indicates that IL-2R γ is dispensable for the antiapoptotic function of IL-15 in L929 cells.

Since TNF α induces rapid generation of ceramide in L929 cells, which induces apoptosis in these fibroblasts (Strelow *et al*, 2000), we asked whether high Axl expression interferes with ceramide generation. Parental, TNF α -resistant, and Axl-overexpressing L929 cells were treated with TNF α for 4 h or were left untreated. In parallel, cells were cultured in the presence of TNF α in combination with the broad-spectrum caspase inhibitor zVAD-fmk, because such treatment induced a more pronounced increase in intracellular ceramide than TNF α alone (Thon *et al*, 2005).

TNF α induced a rapid accumulation of ceramide in L929 cells, whereas ceramide generation was clearly impaired in L929R fibroblasts (Figure 1D). Importantly, transfection with Axl construct also reduced ceramide generation. TNF α combined with zVAD-fmk induced a dramatic increase in the intracellular ceramide level, yet only in TNF α -sensitive fibroblasts (Figure 1D). Therefore, Axl RTK is required for the survival of mouse fibroblasts in the presence of TNF α , and Axl affects the TNF α -induced generation of intracellular ceramide. Since IL-15 treatment no longer protected Axl-deficient L929 cells from TNF α (Figure 1C), Axl must be an essential player in the IL-15-mediated protection of these fibroblasts from TNF α -induced cell death.

Axl RTK and IL-15R α associate and colocalize with each other

To study whether and how Axl RTK participates in IL-15 signaling, immunoprecipitation (IP) experiments were performed. Parental L929 cells were stimulated with IL-15 or left untreated, and IL-15R α or Axl was precipitated from the cell lysates using specific antibodies (Abs). Surprisingly, anti-Axl Abs co-immunoprecipitated IL-15R α , and *vice versa*, in L929 fibroblasts even in the absence of IL-15 stimulation (Figure 2A). This unexpected physical association between IL-15R α and Axl was confirmed by a two-site ELISA, where

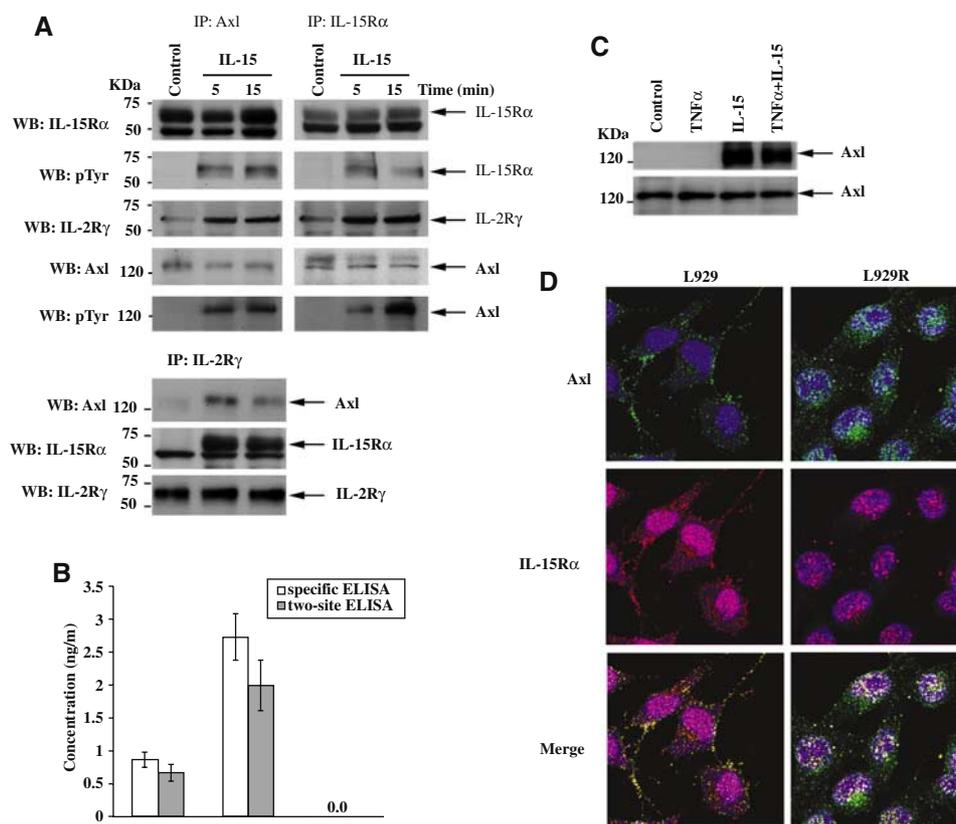


Figure 2 IL-15 induces phosphorylation of IL-15R α and Axl, which constitutively associate. (A) L929 cells were stimulated with IL-15 (100 ng/ml). Cell lysates were precipitated with specific Abs against Axl, IL-15R α , or IL-2R γ and analyzed by WB. (B) Axl/IL-15R α coassociation was detected in a two-site ELISA. Plates were coated with anti-Axl Abs. Axl (white bars) or Axl/IL-15R α complexes (gray bars) were detected in cell lysates using biotinylated anti-Axl or anti-IL-15R α Abs, respectively. COS-7 cells served as a negative control. (C) L929 cells were treated for 15 min with IL-15, TNF α , or both. Axl was precipitated from lysates and its phosphorylation was assessed with anti-pTyr Abs (upper panel). Axl detection served as a loading control (lower panel). (D) Axl and IL-15R α colocalization analysis by confocal microscopy. Cells were stained with anti-IL-15R α Abs (red), anti-Axl Abs (green), and TOTO-3 for nuclei (blue). Yellow color indicates IL-15R α and Axl colocalization (right panels).

plates were coated with monoclonal Abs directed against Axl and incubated with cell lysates to bind target protein. Next, polyclonal Abs directed against IL-15R α were added to detect Axl-associated IL-15R α (Figure 2B). This also allowed quantitative analyses, by comparing results from two-site ELISA with specific ELISA for Axl or IL-15R α (not shown). Arbitrarily defining the amount of Axl or IL-15R α detected by specific ELISA as 100%, 63.1% of Axl and 87.4% of IL-15R α molecules in L929 cells were in complex, whereas in L929R cells characterized by high Axl expression, 60.5% of Axl and 98% of IL-15R α molecules were in complex. Thus, only a fraction of Axl molecules is associated with IL-15R α in living cells, which may also depend on the cell type and may be relevant for the IL-15-mediated biological responses. To test whether the observed association occurs only in viable cells, we performed crosslinking experiments. However, no difference in the amount of IL-15R α associated with Axl was observed after crosslinking, as detected by IP followed by WB, and two-site ELISA (data not shown).

Moreover, both anti-IL-15R α and anti-Axl Abs precipitated IL-2R γ , which constitutes (together with IL-15R α) the high-affinity IL-15R complex in these L929 cells (Figure 2A). Remarkably, co-IP of IL-2R γ with either receptor was much more prominent in the presence of IL-15 (Figure 2A). To further validate these results, we tested whether anti- γ c Abs precipitate Axl or IL-15R α upon IL-15 stimulation. The γ c chain indeed associates with Axl and IL-15R α upon stimulation with IL-15, as demonstrated by the ability of anti- γ c Abs to co-precipitate these molecules (Figure 2A). Isotype-matched control Abs did not precipitate these proteins (data not shown). IL-15 treatment induced phosphorylation of both Axl and the IL-15R α chain (Figure 2A). The pattern of IL-15-induced Axl phosphorylation was rather similar in the presence or absence of TNF α (Figure 2C). The constitutive association of Axl and IL-15R α was further supported by confocal microscopy, which suggested that IL-15R α and Axl colocalize on the cell membrane in the absence of IL-15 (Figure 2D).

To analyze the physical association of IL-15R α and Axl on the cell membrane in more detail, we next tested by density gradient centrifugation and confocal microscopy whether IL-15R α and Axl associate in low-density lipid microdomains of the cell membrane (lipid rafts). These are enriched in many signaling molecules and segregate proteins to regulate signaling and membrane trafficking (Marmor and Julius, 2001). Under our experimental conditions, however, we did not detect IL-15R α or Axl in membrane microdomains by either method (data not shown).

The extracellular portion of Axl contains features that are reminiscent of cell adhesion molecules, and Axl mediates cell aggregation and adhesion by homotypic/homophilic association of its extracellular domains (Bellosta *et al*, 1995). Moreover, Axl-Fc chimeric protein, which consists of the extracellular domain of Axl (Faust *et al*, 1992) fused to the carboxy-terminal Fc region of human IgG₁, promotes the migration of L929 cells upon immobilization on plastic (Budagian *et al*, 2005). Given the ability of IL-15R α to interact with Axl, we asked whether IL-15 stimulation might provide a costimulatory signal for cell adhesion and migration through heterophilic interactions of IL-15R α with Axl. To test this hypothesis, plates coated with Axl-Fc were seeded with equal numbers of cells. After 18 h, the cells were cleared

within a defined area by scratching, photographed in phase-contrast, and allowed to migrate into the cleared area in the presence of IL-15. Unstimulated cells served as a control. However, the migratory properties of IL-15-stimulated and unstimulated cells did not differ (data not shown). These limited, initial data do not support a costimulatory role of IL-15 in the Axl-dependent mechanism(s) that mediate cell migration.

Since the other two members of the Axl RTK family, Tyro3 and Mer, share the same ligand with Axl, we also examined their potential interaction with IL-15R α . Remarkably, the expression of Tyro3 and Mer at mRNA (Supplementary Figure 3A) and protein (Supplementary Figure 3B) levels was rather low in both cell lines, compared to Axl. Also, unlike Axl, surface staining of Tyro3 and Mer was more intense in parental L929 fibroblasts than in the TNF α -resistant subline (Supplementary Figure 3C). Importantly, in line with the two-site ELISA results, no associations between IL-15R α and either of these two RTKs were observed in IP experiments (data not shown).

Thus, IL-15R α specifically interacts with Axl and not other members of this RTK family. Contrary to current views on the trimeric structure of IL-15R (Fehninger and Caligiuri, 2001), Axl RTK may serve as an integral component of the functional IL-15R complex due to a constitutive physical association of the IL-15R α chain with Axl even in the absence of ligand stimulation.

The extracellular portion of Axl is essential for its association with IL-15R α

To better understand the molecular basis for this physical interaction, we co-transfected COS-7 cells with plasmids encoding the full-length IL-15R α and one of two Axl mutant constructs, followed by IP with anti-IL-15R α Abs (Figure 3). A chimeric protein (Bek-Axl) in which the extracellular domain of Axl was substituted with that of fibroblast growth factor receptor (BEK) (Bellosta *et al*, 1995) exhibited a dramatic decrease in the ability to associate with IL-15R α in co-IP studies (Figure 3A). Conversely, Axl-DN, a dominant-negative mutant of Axl that lacks the kinase domain (Bellosta *et al*, 1995), still preserved the capacity to co-precipitate with IL-15R α (Figure 3B). Next, we performed *in vitro* co-IP of IL-15R α -Fc with different recombinant Axl proteins that contained either one of two IgG domains of Axl (Axl-Ig(1) or Axl-Ig(2), or both the domains (Axl-Ig(1+2))). However, these proteins failed to precipitate with IL-15R α -Fc (Supplementary Figure 4). These data suggest that the Axl and IL-15R α association requires the intact extracellular domain of Axl RTK.

Next, we determined whether the IL-15-induced phosphorylation of IL-15R α is mediated by Axl. COS-7 cells were co-transfected with IL-15R α and Axl constructs, followed by cell stimulation with IL-15 and assessment of IL-15R α phosphorylation. IL-15R α phosphorylation was seen only in cells expressing both constructs, whereas transfection of IL-15R α with an empty vector or Axl-DN had no effect (Figure 3C). This strongly suggests that the IL-15-induced phosphorylation of IL-15R α is mediated by Axl RTK.

IL-15 transactivates the Axl signaling pathway

Axl stimulation by Gas6 suppresses apoptosis in NIH 3T3 fibroblasts by upregulating Bcl-x_L through activation of the

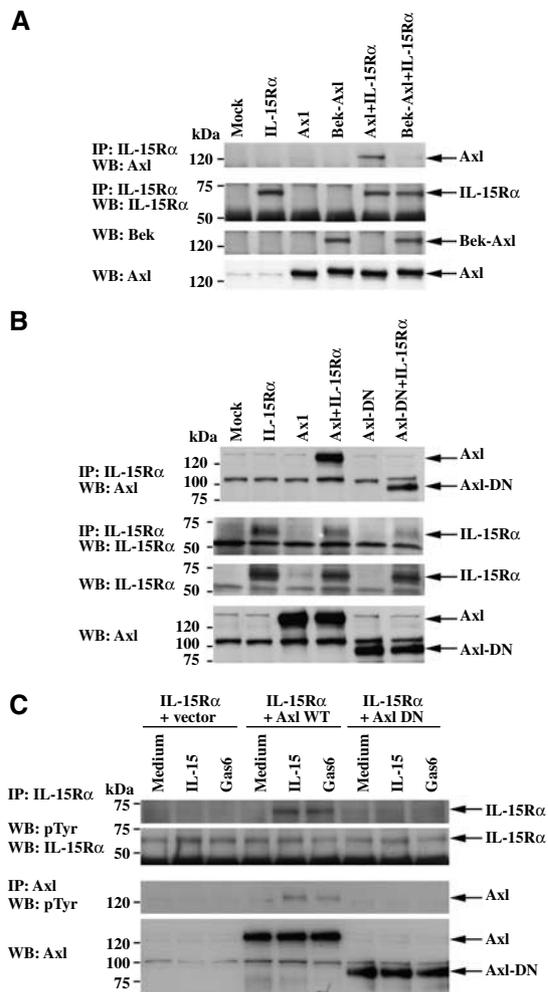


Figure 3 Mapping analysis of IL-15R α /Axl interaction. COS-7 cells were transfected with IL-15R α , Axl, Axl-Bek, and Axl-DN expression constructs in different combinations. IL-15R α (A) or Axl (B) was precipitated from the cell lysates using specific Abs and analyzed by WB using anti-IL-15R α or Axl Abs (upper two panels). Transfection efficiency was proved by protein expression analysis in lysates (lower two panels). (C) Cells were stimulated with IL-15 or Gas6 for 15 min. IL-15R α or Axl was precipitated from the cell lysates and its phosphorylation was analyzed by WB. IL-15R α or Axl was detected as a control for transfection and loading.

PI3K/Akt pathway and NF- κ B (Goruppi *et al*, 1997; Demarchi *et al*, 2001). Because IL-15 induced Axl phosphorylation, we compared the effects of IL-15 and Gas6 on L929 cells. Intriguingly, both IL-15 and Gas6 induced similar patterns of Axl phosphorylation within the first minutes of action (Figure 4A). IL-15 stimulation essentially mirrored Gas6-mediated signaling events, also resulting in the phosphorylation of PI3K and Akt. A modest phosphorylation of ERK1/2 was detected (Figure 4A), whereas the phosphorylation state of JNK and p38 kinase was unaffected (data not shown).

On analyzing Bcl-x_L and Bcl-2 protein expression in L929 cells treated with IL-15 or Gas6 for 24 h, we found that IL-15 and Gas6 upregulated these proteins in L929 cells (Figure 4B). L929R fibroblasts exhibited high basal expression of these two proteins, which could not be upregulated further by IL-15 (data not shown). To explore whether the similarity in downstream signaling events in response to Gas6 or IL-15 stimulation reflected transactivation of the

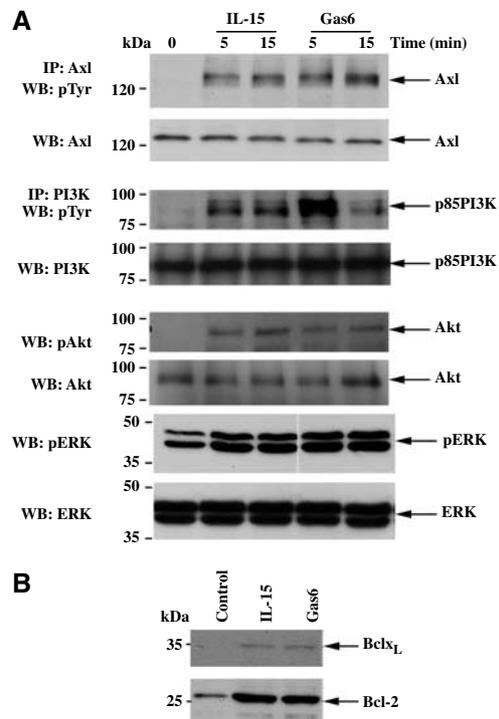


Figure 4 IL-15 mimics Gas6-mediated signaling. (A) L929 cells were activated with IL-15 or Gas6. Axl (first panel) and PI3K (third panel) were precipitated from the cell lysates and analyzed by WB using anti-pTyr Abs. Phosphorylation of Akt (fifth panel) and ERK (seventh panel) was analyzed using phospho-specific Abs. For loading control, Axl, PI3K, Akt, or ERK was detected after stripping (even panels). (B) Bcl-x_L and Bcl-2 were detected in cells stimulated for 24 h.

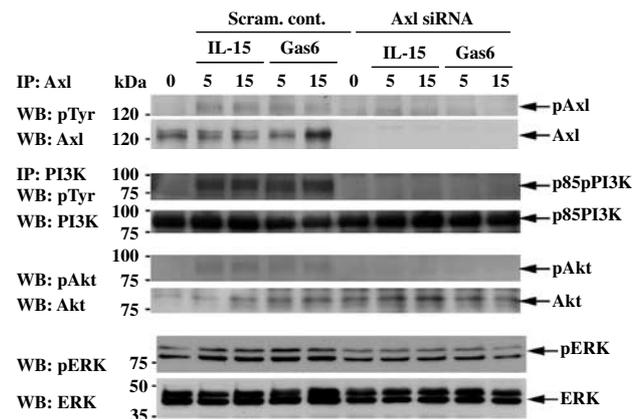


Figure 5 Axl-mediated signaling is impaired in L929 cells upon Axl depletion. L929 cells were transfected with Axl siRNA. After 48 h, cells were harvested, serum-starved for 4 h, and activated with IL-15 or Gas6. Axl (first panel) and PI3K (third panel) were precipitated from the cell lysates and analyzed by WB using anti-pTyr Abs. Phosphorylation of Akt (fifth panel) and ERK (seventh panel) was analyzed by phospho-specific Abs. For loading control, Axl, PI3K, Akt, or ERK was detected on the same blots after stripping (even panels).

Axl signaling cascade by IL-15, we repeated our experiments in Axl-depleted cells. Transfection with Axl siRNA revealed that IL-15 failed to activate PI3K, Akt, or ERK1/2 in Axl-deficient cells (Figure 5). This suggests that IL-15 transactivates the Axl signaling pathway in L929 fibroblasts.

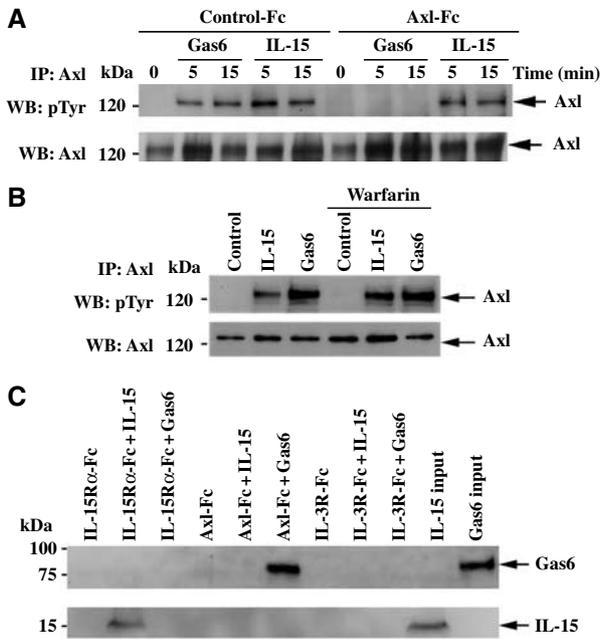


Figure 6 Gas6 does not affect IL-15-mediated Axl transactivation. (A) Cells were incubated for 10 min with 100 ng/ml Axl-Fc or control IL-3R-Fc proteins and then IL-15 or Gas6 (100 ng/ml) was added for another 5 or 15 min. Axl phosphorylation was detected by WB. (B) L929 cells were cultured in the presence of warfarin (100 μg/ml) for 24 h and stimulated with IL-15 or Gas6. Axl phosphorylation was detected by WB. (C) Recombinant Gas6 or IL-15 (10 nM) was incubated with 10 nM of Axl-Fc, IL-15Rα-Fc, or control IL-3R-Fc proteins. Complexes were captured on protein A-agarose and analyzed by WB using anti-Gas6 (upper panel) or anti-IL-15 (lower panel) Abs.

Gas6 is not involved in the IL-15-mediated Axl transactivation

Next, we assessed the role of Gas6 in the IL-15-mediated transactivation of Axl under conditions that prevented endogenous Gas6 activity, using a chimeric Axl-Fc protein that captures Gas6 and inhibits its receptor binding (Costa *et al*, 1996). However, IL-15 still induced Axl phosphorylation in the presence of Axl-Fc (20–100 nM), whereas this chimeric protein abrogated Gas6-specific signaling in both L929 and L929R cells (Figure 6A). Importantly, IL-15 also stimulated the phosphorylation of Axl in L929 cells treated with warfarin (Figure 6B). Warfarin selectively inhibits the post-translational γ -carboxylation of Gas6, which is essential for its receptor binding and growth stimulation (Yanagita *et al*, 1999). These findings essentially rule out that endogenous Gas6 participates in the IL-15-mediated transactivation of Axl. To exclude a direct binding of IL-15 to Axl, we performed *in vitro* binding assays as described (Nagata *et al*, 1996), using purified recombinant proteins. However, no interactions other than the expected binding of IL-15 and Gas6 to their respective receptors were detected (Figure 6C).

IL-15 cross-modulates the Axl signaling pathway in mouse embryonic fibroblasts

To check whether the ability of IL-15 to stimulate the IL-15Rα-mediated activation of Axl was restricted to L929 fibrosarcoma cells, mouse embryonic fibroblasts (MEFs) obtained from wild-type (WT), IL-15Rα-deficient (IL-15Rα^{-/-}) (Lodolce

et al, 1998), or Axl-deficient mice (Axl^{-/-}) were also examined (Bellosta *et al*, 1997). MEFs from WT and Axl^{-/-} mice almost equally expressed IL-15Rα, while MEFs from WT and IL-15Rα^{-/-} animals had equal expression of Axl by WB (Figure 7A) and FACS analysis (Figure 7B). All three types of MEFs had comparable levels of IL-2Rγ expression (Figure 7A). To test whether IL-15 signaling in these cells depends on the expression of Axl, WT, Axl^{-/-}, or IL-15Rα^{-/-} MEFs were stimulated with IL-15 or Gas6. Subsequently, Axl was precipitated from the cell lysates using anti-Axl Abs, and protein precipitates were analyzed by WB.

Indeed, both IL-15 and Gas6 stimulated the phosphorylation of Axl in MEFs from WT mice, and the patterns of phosphorylation of downstream signaling molecules closely resembled those observed in L929 cells, demonstrating the phosphorylation of PI3K/Akt and ERK and upregulation of Bcl-2 (and, to a lesser extent, of Bcl-x_l) also in primary fibroblasts (Figure 7C and D). Anti-Axl Abs also co-precipitated the IL-15Rα chain in both non-stimulated and stimulated WT MEFs. This indicates that IL-15Rα and Axl are constitutively associated not only in immortalized but also in primary mouse fibroblasts (Figure 7C). IL-15, and to a lesser extent Gas6, induced IL-15Rα phosphorylation within the first 15 min in WT MEFs, whereas both failed to induce IL-15Rα phosphorylation in Axl^{-/-} animals (Figure 7C, middle part). This supports the concept that IL-15Rα is directly phosphorylated by activated Axl RTK.

As expected, the IL-15-mediated activation of Axl signaling cascade was absent in MEFs of IL-15Rα^{-/-} mice, while Gas6-induced signaling remained intact (Figure 7C, right part). This confirms that activation of the PI3K/Akt pathway in WT MEFs results from the IL-15Rα-mediated transactivation of Axl-specific downstream signaling. The fact that IL-15 failed to mediate Axl phosphorylation in the absence of IL-15Rα argues for the role of the ligated high-affinity cytokine receptor chain as the initiator of Axl transactivation, and further excludes a direct interaction of IL-15 with Axl.

Next, the binding affinity of IL-15R complex in the presence or absence of Axl expression was compared by ¹²⁵I-IL-15-labeled ligand binding assays and Scatchard plot analysis. We utilized immortalized lung fibroblasts obtained from Axl^{-/-}, IL-15Rα^{-/-}, and IL-2Rγ^{-/-} (RAG^{-/-}) mice. These experiments demonstrated that neither Axl nor IL-2Rγ expression affected binding affinities of IL-15Rα toward IL-15, which remained constant in all cell types tested ($K_d \approx 10^{11}$ pM), except cells from IL-15Rα^{-/-} animals, which served here as a control (Supplementary Figure 5).

Since Gas6 supports survival of fibroblasts upon serum deprivation (Bellosta *et al*, 1997), we next examined whether IL-15 and Gas6 rescue MEFs from cell death induced by serum starvation. Gas6 and IL-15 were almost equipotent in their ability to support survival of MEFs from WT mice, but not Axl^{-/-} mice, whereas Gas6 (but not IL-15) supported survival of IL-15Rα^{-/-} MEFs (Figure 7E). Again, this argues for the use of a common signaling pathway by IL-15 and Gas6. In summary, these data suggest that IL-15 stimulation triggers transactivation of Axl, which in turn results in IL-15Rα phosphorylation, presumably by the activated tyrosine kinase domain of Axl. Although Gas6 can signal also in the absence of IL-15Rα, proper signal transduction through Axl is indispensable for IL-15-induced signaling in mouse primary fibroblasts.

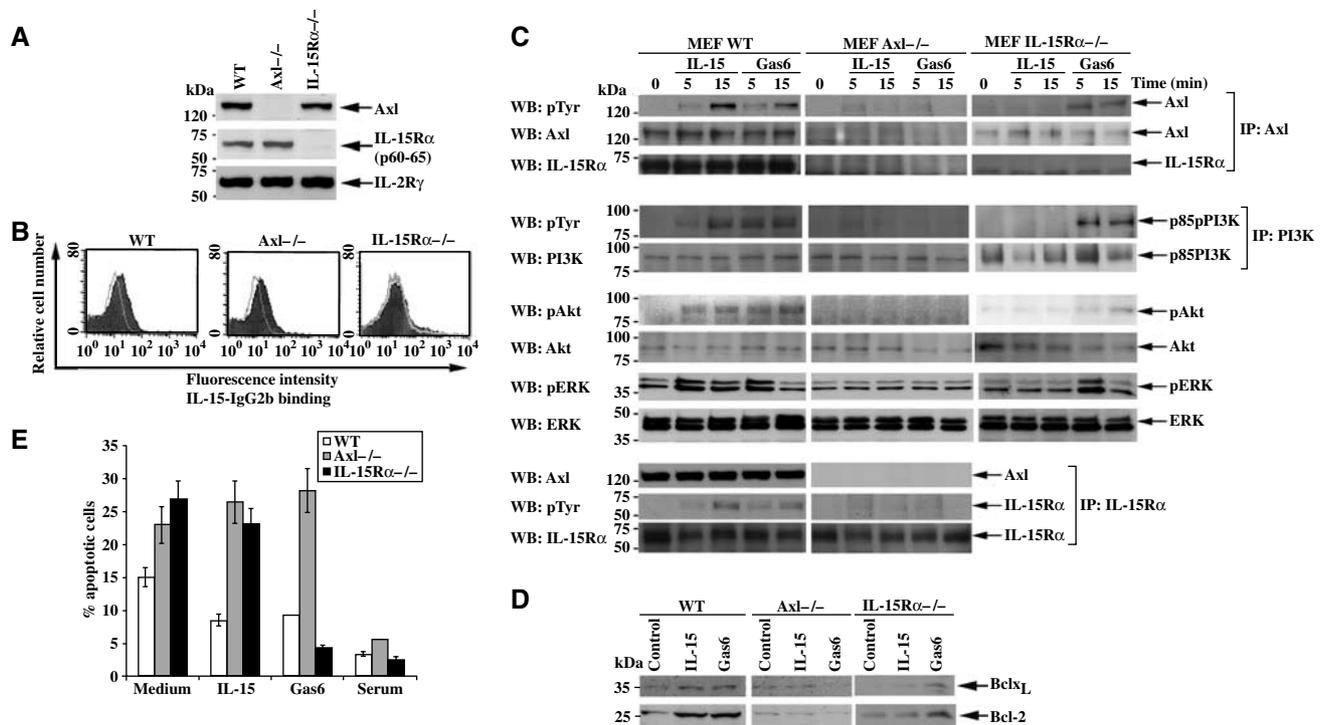


Figure 7 IL-15 and Gas6 signal in WT but not in Axl^{-/-}, whereas Gas6 but not IL-15 induces signaling in IL-15Rα^{-/-} MEFs. (A) WB analysis of Axl, IL-15Rα, and IL-2Rγ protein expression in WT, Axl^{-/-}, and IL-15Rα^{-/-} MEFs. (B) IL-15Rα expression was analyzed by FACS on cells labeled with IL-15Rα-IgG_{2b}. (C) MEFs were stimulated with IL-15 or Gas6. Axl was precipitated from the lysates and phosphorylation was analyzed using anti-pTyr Abs (first panel). Same blots were analyzed for the association of Axl with IL-15Rα (third panel). Equal loading was confirmed by re-probing of blots with anti-Axl Abs (second panel). PI3K (fourth and fifth panels) and IL-15Rα (last two panels) were precipitated and their phosphorylation was detected using anti-pTyr Abs. Phosphorylation of Akt and ERK was detected using phospho-specific Abs. Blots were re-probed with PI3K, IL-15Rα, Akt, or ERK Abs for loading control. (D) Bcl-x_L and Bcl-2 were detected in MEFs stimulated with IL-15 or Gas6 for 24 h. (E) Influence of IL-15 or Gas6 on serum deprivation-induced apoptosis in WT, Axl^{-/-}, and IL-15Rα^{-/-} MEFs. Apoptosis was induced by 48 h incubation in serum-free medium. IL-15 or Gas6 was added at a concentration of 100 ng/ml. Cells cultured in serum-free or serum-supplemented media were used as controls. Percentage of apoptotic cells was determined by PI and annexin V staining and FACS analysis. Data represent the mean ± s.d. from duplicate samples.

IL-15Rα and Axl constitutively associate in murine dendritic cells

Next, we explored whether cross-communication between the IL-15Rα and Axl signaling pathways can also be demonstrated in additional primary cells. To this end, we tested whether IL-15 associates with Axl in murine dendritic cells (DCs). DCs express Axl at the mRNA and protein level, as well as IL-15Rα and IL-2Rγ of the IL-15R complex (data not shown). These cells also release a proteolytically processed, soluble form of Axl (Budagian *et al*, 2005). Here, we show that murine DCs also display constitutive association of IL-15Rα and Axl, as detected by co-IP (Figure 8A) and two-site ELISA (Figure 8B and C). No association between Axl and IL-2Rγ was seen, even upon IL-15 stimulation (Figure 8A). However, neither IL-15 nor Gas6 was able to induce Axl, PI3K, and Akt phosphorylation in murine DCs (data not shown). This indicates that the activation of Axl signaling pathway is cell-type-dependent.

Finally, we asked whether other cytokine receptors, which share the γc chain, may also associate with Axl upon stimulation with the corresponding ligand. DCs were stimulated with IL-2, IL-4, IL-7, IL-9, or IL-21 (DCs express high-affinity receptor subunits of these cytokines; data not shown). However, none of these cytokines induced Axl phosphorylation or the association of Axl with the high-affinity subunits of the relevant cognate receptors (Figure 8A and data not

shown) or with the γc chain (Figure 8A). No phosphorylation of PI3K, Akt, and ERK1/2 was seen in response to these cytokines (data not shown). Therefore, among these cytokine receptors, only the high-affinity α chain of IL-15R associated with Axl RTK in murine DCs.

Discussion

Our study suggests a new model for how cross-communication between an RTK and a cytokine receptor can promote cell survival, and identifies Axl as a member of the RTK family that can integrate signaling from heterologous receptor systems (Figure 9). Specifically, we provide the first evidence that the Axl signaling pathway is essential for an efficient IL-15-mediated protection of mouse fibroblasts from TNFα-induced cell death. IL-15 literally transduces the Axl signaling cascade by binding to IL-15Rα. Thus, the Axl signaling pathway is activated directly by Gas6 and indirectly by IL-15. Nevertheless, IL-15 induces rapid Axl phosphorylation even when Gas6-mediated Axl activation is inhibited. Therefore, Axl transactivation is likely mediated through mechanism(s) that do not involve the interaction of Axl with endogenous Gas6.

It is increasingly appreciated that the interaction of one ligand with its high-affinity cognate receptor can activate more than one signal transduction pathway, both on the

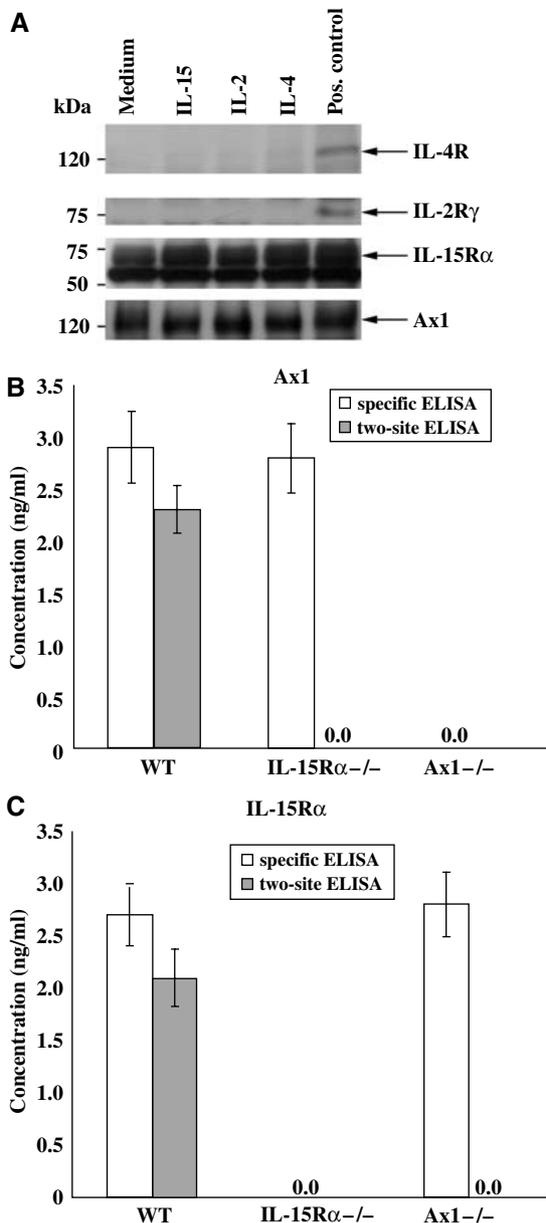


Figure 8 Axl and IL-15R α coassociate in mouse DCs. (A) DCs were stimulated with cytokines for 15 min. Axl was precipitated from the cell lysates and precipitates were analyzed by WB using anti-IL-4R α (upper panel), anti-IL-2R γ (second panel), and anti-IL-15R α (third panel) Abs. IP using specific Abs served as a positive control. For loading control, blots were developed with anti-Axl Abs (lower panel). (B) Plates were coated with anti-Axl Abs, and biotinylated anti-Axl (specific) or anti-IL-15R α (two-site) Abs were used as detection Abs. (C) Plates were coated with anti-IL-15R α Abs, and biotinylated anti-IL-15R α (specific) or anti-Axl (two-site) Abs served as detection Abs.

intracellular and the cell membrane level (Mera *et al*, 1999; Hirota *et al*, 2001).

Here, we reveal an unprecedented cooperation in signal transduction of a cytokine receptor and a transmembrane tyrosine kinase, adding yet another 'odd couple' to the ever-growing list of heterologous receptor interactions, as exemplified by the transactivation of EGF family receptors (=inter-receptor signal transmission between G-protein-coupled receptors and RTKs) (Gschwind *et al*, 2001). In addition, G-protein-coupled receptors engage in cross-talk

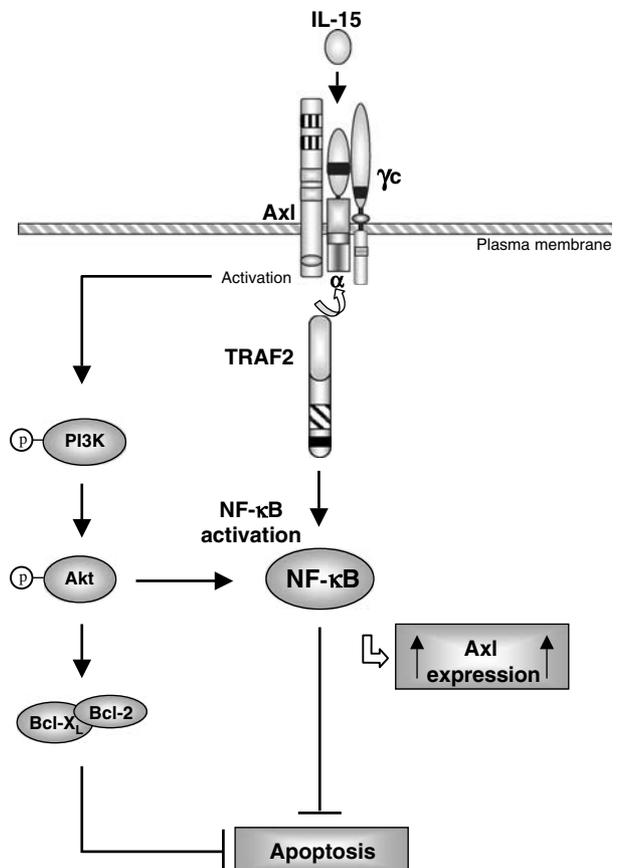


Figure 9 Hypothetical model of cross-talk between IL-15 and Axl signaling pathways suggested by the current study.

with growth hormone, prolactin, integrins, and PDGF (Prenzel *et al*, 2000), while the growth factor receptor ErbB2 forms a complex with the gp130 subunit of the IL-6R complex, and is critical for IL-6-mediated MAPK activation (Qui *et al*, 1998).

That Axl can modulate the functions of other membrane receptors is further supported by a recent report that cross-talk between Axl and vascular endothelial growth factor receptor 2 regulates activation of an angiogenic program in endothelial cells (Gallicchio *et al*, 2005). Thus, like members of the EGFR family, Axl may serve as a key signaling element for the integration of multiple, changing environmental influences. 'Promiscuous receptor liaisons' of the kind revealed in the current study appear to be only the tip of an 'iceberg' of cross-signaling complexity, whose full functional significance under physiological and pathological conditions we can only but guess.

Although this remains to be validated in other cells besides murine DCs, our data suggest that the IL-15R α /Axl interaction is specific to IL-15 and that IL-2 does not activate the Axl pathway. The specificity of the IL-15R α /Axl interaction identified here is underscored by the finding that neither IL-2 nor other cytokines, which utilize the γ c chain for signaling, induce the association of Axl with their high-affinity α receptors upon ligand stimulation. We also show that the observed cytokine receptor and RTK interaction is specific for Axl. In DCs, however, contrary to fibroblasts, IL-15 stimulation did not recruit IL-2R γ to the IL-15R α /Axl complex. This suggests cell type-specific differences in individual cross-

signaling events. Since, in DCs, considerable amounts of soluble Axl are constitutively generated from the membrane-bound form through ADAM10-mediated cleavage (Budagian *et al*, 2005), this may account for the lack of response of DCs to both IL-15 and Gas6. Therefore, activation of Axl-specific signaling and the ability of IL-15 to transactivate the Axl signaling cascade likely vary between different cell types.

We also show that an RTK can constitutively associate with the high-affinity subunit of a cytokine receptor even in the absence of ligand stimulation. The 'promiscuous liaison' between these two distinct receptor systems operates already at the cell membrane level, and Axl RTK seems to serve as an integral component of a functional IL-15 receptor complex. This may be particularly important for IL-15 effects on cells that do not express IL-2R β (such as fibroblasts) and/or γ chains, and the ability of IL-15 to transactivate Axl may primarily refer to non-immune cells.

Although our pilot data do not support that the Axl and IL-15R α interaction occurs in lipid rafts, this possibility remains to be excluded definitively. Interestingly, IL-15R α , IL-2R α , IL-2R β , and IL-2R γ subunits can form supramolecular receptor clusters in lipid rafts, which may be critical in the cross-regulation of IL-15- versus IL-2-mediated responses (Vamosi *et al*, 2004). Perhaps, the Axl/IL-15R α interaction segregates the IL-15R α subunit from membrane microdomains, thereby secluding it from IL-2R α and limiting its availability for other microdomain-associated molecules.

Axl and IL-15R α associate and colocalize on the cell surface, while no physical interaction of IL-15 and Axl is observed. Considering that IL-15R α can partially oligomerize upon ligand binding (Vamosi *et al*, 2004), it is tempting to speculate that IL-15 treatment physically affects the distribution of Axl on the cell membrane, leading to homophilic interactions between the extracellular domains of several RTK molecules. Our mapping analysis, which shows that the extracellular domain of Axl is required for the described IL-15R α /Axl interaction, further supports this notion. Activated Axl then phosphorylates IL-15R α and activates the PI3K/Akt signaling pathway. Since Syk kinase, which phosphorylates IL-15R α (Bulanova *et al*, 2001), is absent in L929 cells (E Bulanova, unpublished observation), it can essentially be ruled out that Syk interacts with Axl through its SH2 domain and mediates IL-15R α /Axl interaction in these cells. However, a transient participation of other molecule(s) in this process, especially *in vivo* and in other cell types than the ones studied here, cannot yet be excluded.

The L929 cell line has been commonly used to study TNF α cytotoxicity, since it is one of the few systems in which TNF α exerts its cytotoxic effects in the absence of inhibitors of *de novo* protein synthesis (Strelow *et al*, 2000). Given the exquisite sensitivity of L929 cells to TNF α -induced cell death, the newly acquired, IL-15-mediated resistance of L929 cells to TNF α underscores the capacity of tumor cells to activate protective mechanisms in response to negative selective pressure, which may result in the development of a more malignant phenotype *in vivo*. The observed high expression of Axl in L929 cells is in line with the ability of Axl to transform fibroblasts even in the absence of ligand upon engineered overexpression (O'Brian *et al*, 1991).

It is still unclear how TNF α kills L929 cells: apoptosis, necrosis, both (Humphreys and Wilson, 1999; Los *et al*,

2002), and/or 'atypical' apoptosis with membrane blebbing and unusual degradation of poly(ADP-ribose) polymerase in the absence of caspase activity (Strelow *et al*, 2000). Therefore, the analysis of caspase activity does not aid in the understanding of the exact cell death mode in this system. Our data support a key role for ceramide as the mediator of caspase-independent apoptosis in L929 cells (Strelow *et al*, 2000). Since Bcl-2 blocks the effects of ceramide on mitochondria (Lin *et al*, 2005), the Axl-mediated overexpression of Bcl-2 and Bcl-x_L identified here may counteract the proapoptotic effects of ceramide. It is still an open question whether Axl prevents generation of ceramide through inhibiting its production and/or accelerating its degradation.

Our data suggest that a two-pronged protective mechanism underlies the antiapoptotic properties of IL-15 on L929 fibroblasts: (a) the previously reported, immediate recruitment of TRAF2 to IL-15R α , with subsequent NF- κ B activation, by IL-15 (Bulfone-Paus *et al*, 1999); and (b) IL-15-mediated transactivation and upregulation of Axl, with subsequent activation of PI3K/Akt and upregulation of Bcl-2 and Bcl-x_L (Figure 9). While these mechanisms likely cooperate to suppress apoptosis in L929 fibroblasts, only some of them may be operative in other cell types. However, the novel concept that IL-15 and Gas6 utilize a common signaling pathway and that Axl RTK represents yet another convergence point for signal integration and diversification between heterologous signaling systems (such as RTKs and cytokine receptors) (Figure 9) also applies at least to some primary cell types.

Materials and methods

Cytokines, Abs, fusion proteins, and plasmids

Recombinant human IL-15 and TNF α were purchased from TEBU; Gas6 and biotinylated Abs and Axl-Fc, IL-15R α -Fc, and IL-3R-Fc proteins were from R&D Systems. Abs against IL-15R α , Axl, Bek, IL-2R γ , ERK, pERK, PI3K, Bcl-2, Bcl-x_L, Akt, and phospho-Akt were purchased from Santa Cruz Biotechnology. Mouse anti-phosphotyrosine (anti-pTyr) and anti-IL-4R α Abs were from BD PharMingen. IL-15-IgG_{2b} fusion protein was produced as described previously (Bulfone-Paus *et al*, 1997b). Cloning of mouse Axl, Bek-Axl, Axl-DN, and IL-15R α cDNA was described elsewhere (Bellosta *et al*, 1995; Bulfone-Paus *et al*, 1999).

Cell lines, culture, and transfection

The parental L929 cell line was purchased from ECACC. The L929R cell line, a TNF α -resistant derivative of L929 cells, was established by using IL-15 and TNF α in combination. In brief, L929 cells were cultured over a 1-month period with 5 ng/ml TNF α and gradually decreasing IL-15 concentrations (20–0 ng/ml). After that time, cells started to proliferate again despite the continued presence of TNF α in the medium. Parental and derivative cells exhibited similar rates of proliferation and same morphological appearance, as assessed by [³H]thymidine incorporation and light microscopy (data not shown).

Bone marrow-derived DCs were generated as described (Brandt *et al*, 2003). L929, L929R, DCs, and COS-7 cells were maintained in RPMI-1640. MEFs from WT, Axl^{-/-}, and IL-15R α ^{-/-} mice were generated as described (Bellosta *et al*, 1997) and maintained in DMEM. Culture medium was supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. COS-7 cells were transiently transfected using LipofectAMINE 2000 (Gibco-Invitrogen), harvested, and analyzed 48 h post-transfection.

siRNA transfection

siRNAs were purchased from Eurogentec. Double-stranded siRNA for Axl or scrambled control was obtained by annealing the

respective two RNA oligonucleotides: Axl 5'-UAUCACAGGUGCC-AGAGGA-dTdT-3' and 3'-dTdT-AUAGUGUCCACGGUCUCCU-5'; scrambled control 5'-CUGCGAACUGUAUAGUC-dTdT-3' and 3'-dTdT-GACUAUA-UACAGUUCGAG-5'. Cells were transfected with jetSI-FluoF siRNA transfection reagent (Eurogentec), using 20 nM of double-stranded siRNA. At 48 h post-transfection, cells were analyzed for Axl mRNA/protein depletion by RT-PCR/WB. Transfection efficiency was about 80–90%, as detected by fluorescent microscopy.

Two-site ELISA

A 96-well plate (Greiner) was coated with 1 µg/ml of anti-Axl or anti-IL-15R α Abs. Wells were blocked with 2% BSA in PBS. Cells were lysed with 1% NP-40 buffer. Lysates (50 µl/well) were added to the plate and incubated overnight at 4°C. Serial dilutions of murine recombinant Axl or IL-15R α were used for standardization. Bound Axl/IL-15R α complexes were detected using biotinylated IL-15R α Abs (in plates coated with anti-Axl) or using biotinylated Axl Abs (in plates coated with IL-15R α), followed by streptavidin-peroxidase incubation. Chromogenic substrate (R&D Systems) was used for visualization. Optical density was determined at 450 nm (ELISA reader, Dynatech). The specificity of two-site ELISA was demonstrated by the absence of detectable Axl using anti-IL-15R α Abs, and *vice versa*.

RT-PCR

RNA was extracted using TRIZOL reagent (Invitrogen). cDNA was synthesized from 5 µg of total RNA using random oligonucleotides and the SuperScriptII™ kit (Invitrogen). cDNA was amplified by standard PCR procedure as described (Bulfone-Paus *et al*, 1999). To evaluate mRNA expression semiquantitatively, aliquots of PCR products from 25, 30, and 35 cycles were run simultaneously. β -Actin was used for normalization. A mock PCR (without cDNA) was included to exclude contamination in all experiments. Sequences of the primers are shown in Supplementary Table 1.

IP and WB

Cell pellets were lysed for 15 min on ice in 1% NP-40 protein extraction buffer (20 mM Tris-HCl buffer, pH 8.0, 15 mM NaCl, 2 mM EDTA, 10 mM sodium fluoride, 1 µg/ml pepstatin A, 1 µg/ml leupeptin, 10 mM PMSF, and 100 µM sodium vanadate (all from Sigma-Aldrich)). Proteins were precipitated in 0.5% NP-40 buffer using 2 µg of Abs and captured on protein A/G-agarose. Proteins were analyzed by 10% SDS-PAGE as described (Bulanova *et al*, 2001).

References

Allen MP, Zeng C, Schneider K, Xiong X, Meintzer MK, Bellosta P, Basilico C, Varnum B, Heidenreich KA, Wiermann ME (1999) Growth arrest-specific gene 6 (Gas6)/adhesion related kinase (Ark) signaling promotes gonadotropin-releasing hormone neuronal survival via extracellular signal-regulated kinase (ERK) and Akt. *Mol Endocrinol* **13**: 191–201

Bellosta P, Costa M, Lin DA, Basilico C (1995) The receptor tyrosine kinase ARK mediates cell aggregation by homophilic binding. *Mol Cell Biol* **15**: 614–625

Bellosta P, Zhang Q, Goff SP, Basilico C (1997) Signaling through the Ark tyrosine kinase receptor protects from apoptosis in the absence of growth stimulation. *Oncogene* **15**: 2387–2397

Bennasroune A, Gardin A, Aunis D, Cremel G, Hubert P (2004) Tyrosine kinase receptors as attractive targets of cancer therapy. *Crit Rev Oncol Hematol* **50**: 23–38

Brandt K, Bulfone-Paus S, Foster DC, Rückert R (2003) Interleukin-21 inhibits dendritic cell activation and maturation. *Blood* **102**: 4090–4098

Budagian V, Bulanova E, Orinska Z, Duitman E, Brandt K, Ludwig A, Hartmann D, Lemke G, Saftig P, Bulfone-Paus S (2005) Soluble Axl is generated by ADAM10-dependent cleavage and associates with Gas6 in mouse serum. *Mol Cell Biol* **25**: 9324–9339

Bulanova E, Budagian V, Pohl T, Krause H, Dürkop H, Paus R, Bulfone-Paus S (2001) The IL-15R α chain signals through association with Syk in human B cells. *J Immunol* **167**: 6292–6302

Bulfone-Paus S, Bulanova E, Pohl T, Budagian V, Dürkop H, Rückert R, Kunzendorf U, Paus R, Krause H (1999) Death

Ceramide quantitation

Cells were left untreated or treated with 100 ng/ml TNF α alone or in combination with 20 µM of benzyloxycarbonyl-Val-Ala-Asp(Ome)-fluoromethylketone (zVAD-fmk) for 4 h. Ceramide was quantified by the charring method following high-performance thin-layer chromatography as described (Jensen *et al*, 1999). Thin-layer chromatography plates were scanned and analyzed using the software package PCBAS (Raytest).

FACS analysis

For analysis of surface receptors expression, L929 and L929R cells were stained with monoclonal Abs or incubated with IL-15-IgG_{2b} as described (Bulfone-Paus *et al*, 1999). Negative controls consisted of isotype-matched Abs (BD Pharmingen). The percentage of apoptotic cells was evaluated by the ApoTarget Annexin-V-FITC Apoptosis kit (BioSource) and FACS analysis was performed by FACScalibur (Becton Dickinson) using CELLQuest software.

Confocal microscopy

Cells were fixed with 2% paraformaldehyde, permeabilized by 0.25% Triton X-100 and stained with 1:100 dilutions of Abs. Alexa Fluor-488 anti-goat IgG (H + L) and Alexa Fluor-546 anti-rabbit IgG (H + L) (Molecular Probes) at a dilution of 1:100 were used as secondary Abs. Nuclei were stained using TOTO-3 dye (Molecular Probes). The specimens were mounted in DABKO (1,4-diazabicyclo [2,2,2]octane) anti-fading solution and analyzed by scanning confocal microscopy (Leica TCS SP).

Data analysis

All experiments were performed in at least three independent assays, which yielded highly comparable results. Data are summarized as mean \pm s.d. Statistical analysis of the results was performed by Student's *t*-test for unpaired samples. A *P*-value of <0.05 was considered as statistically significant.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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deflected: IL-15 inhibits TNF α -mediated apoptosis in fibroblasts by TRAF2 recruitment to the IL-15R α chain. *FASEB J* **13**: 1575–1585

Bulfone-Paus S, Dürkop H, Paus R, Krause H, Pohl T, Onu A (1997a) Differential regulation of human T lymphoblast functions by IL-2 and IL-15. *Cytokine* **9**: 507–513

Bulfone-Paus S, Ungureanu D, Pohl T, Lindner G, Paus R, Rückert R, Krause H, Kunzendorf U (1997b) Interleukin-15 protects from lethal apoptosis *in vivo*. Interleukin-15 protects from lethal apoptosis *in vivo*. *Nat Med* **3**: 1124–1128

Burchert A, Attar EC, McCloskey P, Fridell YW, Liu ET (1998) Determinants for transformation induced by the Axl receptor tyrosine kinase. *Oncogene* **16**: 3177–3187

Chen WS, Kung HJ, Yang WK, Lin WC (1999) Comparative tyrosine-kinase profiles in colorectal cancers: enhanced *arg* expression in carcinoma as compared with adenoma and normal mucosa. *Int J Cancer* **83**: 579–584

Costa M, Bellosta P, Basilico C (1996) Cleavage and release of a soluble form of the receptor tyrosine kinase ARK *in vitro* and *in vivo*. *J Cell Physiol* **168**: 737–744

Demarchi F, Verardo R, Varnum B, Brancolini C, Schneider C (2001) Gas6 anti-apoptotic signaling requires NF- κ B activation. *J Biol Chem* **278**: 31738–31744

Faust M, Ebensperger C, Schulz AS, Schleithoff L, Hameister H, Bartram CR, Janssen JW (1992) The murine ufo receptor: molecular cloning, chromosomal localization and *in situ* expression analysis. *Oncogene* **7**: 1287–1293

- Fehninger TA, Caligiuri MA (2001) Interleukin 15: biology and relevance to human disease. *Blood* **97**: 14–32
- Gallicchio M, Mitola S, Valdembrì D, Fantozzi R, Varnum B, Avanzi GC, Bussolino F (2005) Inhibition of vascular endothelial growth factor 2-mediated endothelial cell activation by Axl tyrosine kinase receptor. *Blood* **105**: 1970–1976
- Giri JG, Kumaki S, Ahdieh M, Friend DJ, Loomis A, Shanebeck K, DuBose R, Cosman D, Park LS, Anderson DM (1995) Identification and cloning of a novel IL-15 binding protein that is structurally related to the α chain of the IL-2 receptor. *EMBO J* **14**: 3654–3663
- Coruppi S, Chiaruttini C, Ruaro ME, Varnum B, Schneider C (2001) Gas6 induces growth, β -catenin stabilization, and T-cell factor transcriptional activation in contact-inhibited C57 mammary cells. *Mol Cell Biol* **21**: 902–915
- Coruppi S, Ruaro E, Varnum B, Schneider C (1997) Requirement of phosphatidylinositol 3-kinase-dependent pathway and Src for Gas6-Axl mitogenic and survival activities in NIH 3T3 fibroblasts. *Mol Cell Biol* **17**: 4442–4453
- Graham DK, Dawson TL, Mullaney DL, Snodgrass HR, Earp HS (1994) Cloning and mRNA expression analysis of a novel human protooncogene, *c-mer*. *Cell Growth Differ* **5**: 647–657
- Gschwind A, Zwick E, Prenzel N, Leserer M, Ullrich A (2001) Cell communication network: epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission. *Oncogene* **20**: 1594–1600
- Healy AM, Schwartz JJ, Zhu X, Herrich BE, Varnum B, Farber HW (2001) Gas 6 promotes Axl-mediated survival in pulmonary endothelial cells. *Am J Physiol Lung Cell Mol Physiol* **280**: L1273–L1281
- Hirota K, Murata M, Itoh T, Yodoi J, Fukuda K (2001) Redox-sensitive transactivation of epidermal growth factor receptor by tumor necrosis factor confers NF- κ B activation. *J Biol Chem* **276**: 25953–25958
- Humphreys DT, Wilson MR (1999) Modes of L929 cell death induced by TNF- α and other cytotoxic agents. *Cytokine* **11**: 773–782
- Jensen JM, Schütze S, Forl M, Krönke M, Proksch E (1999) Roles for tumor necrosis factor receptor p55 and sphingomyelinase in repairing the cutaneous permeability barrier. *J Clin Invest* **104**: 1761–1770
- Kumaki S, Armitage R, Ahdieh M, Park L, Cosman D (1996) Interleukin-15 up-regulates interleukin-2 receptor α chain but down-regulates its own high-affinity binding sites on human T and B cells. *Eur J Immunol* **26**: 1235–1239
- Lai C, Gore M, Lemke G (1994) Structure, expression, and activity of Tyro 3, a neural adhesion-related receptor tyrosine kinase. *Oncogene* **9**: 2567–2578
- Lee W-P, Wen Y, Varnum B, Hung M-C (2002) Akt is required for Axl-Gas6 signaling to protect cells from E1A-mediated apoptosis. *Oncogene* **21**: 329–336
- Lin CF, Chen CL, Chang WT, Jan MS, Hsu LJ, Wu RH, Fang YT, Tang MJ, Chang WC, Lin YS (2005) Bcl-2 rescues ceramide- and etoposide-induced mitochondrial apoptosis through blockage of caspase-2 activation. *J Biol Chem* **280**: 23758–23765
- Lodolce JP, Boone DL, Chai S, Swain RE, Dassopoulos T, Trettin S, Ma A (1998) IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity* **9**: 669–676
- Los M, Mozoluk M, Ferrari D, Stepczynska A, Stroh C, Renz A, Herceg Z, Wang Z-Q, Schulze-Osthoff K (2002) Activation and caspase-mediated inhibition of PARP: a molecular switch between fibroblast necrosis and apoptosis in death receptor signaling. *Mol Cell Biol* **13**: 978–988
- Lu Q, Gore M, Zhang Q, Camenisch T, Boast S, Casagrande F, Lai C, Skinner MK, Klein R, Matsushima GK, Earp HS, Goff SP, Lemke G (1999) Tyro-3 family receptors are essential regulators of mammalian spermatogenesis. *Nature* **398**: 723–728
- Lu Q, Lemke G (2001) Homeostatic regulation of the immune system by receptor tyrosine kinases of the Tyro 3 family. *Science* **293**: 306–311
- Manfioletti G, Brancolini C, Avanzi G, Schneider C (1993) The protein encoded by a growth arrest-specific gene (*gas6*) is a new member of the vitamin K-dependent proteins related to protein S, a negative coregulator in the blood coagulation cascade. *Mol Cell Biol* **3**: 4976–4985
- Mark MR, Scadden DT, Wang Z, Gu Q, Goddard A, Godowski PJ (1994) Rse, a novel receptor-type tyrosine kinase with homology to Axl/Ufo, is expressed at high levels in the brain. *J Biol Chem* **269**: 10720–10728
- Marmor MD, Julius M (2001) Role of lipid rafts in regulating interleukin-2 receptor signaling. *Blood* **98**: 1489–1497
- Melaragno MG, Cavet ME, Yan C, Tai LK, Jin ZG, Haendeler J, Berk BC (2004) Gas6 inhibits apoptosis in vascular smooth muscle: role of Axl kinase and Akt. *J Mol Cell Cardiol* **37**: 881–887
- Mera A, Suga M, Ando M, Suda T, Yamaguchi N (1999) Induction of cell shape changes through activation of the IL-3 common β chain by the RON receptor tyrosine kinase. *J Biol Chem* **274**: 15766–15774
- Nagata K, Ohashi K, Nakano T, Arita H, Zong C, Hanafusa H, Mizuno K (1996) Identification of the product of *growth arrest-specific gene 6* as a common ligand for Axl, Sky, and Mer receptor tyrosine kinases. *J Biol Chem* **271**: 30022–30027
- O'Brien JP, Frye RA, Cogswell PC, Neubauer A, Kitch B, Prokop C, Espinosa R, Le Beau MM, Earp HS, Liu ET (1991) *Axl*, a transforming gene isolated from primary human myeloid leukemia cells, encodes a novel receptor tyrosine kinase. *Mol Cell Biol* **11**: 5016–5031
- Prenzel N, Zwick E, Leserer M, Ullrich A (2000) Tyrosine kinase signaling in breast cancer. Epidermal growth factor receptor: convergence point for signal integration and diversification. *Breast Cancer Res* **2**: 184–190
- Qui Y, Ravi L, Kung H-J (1998) Requirement of ErbB2 for signaling by interleukin-6 in prostate carcinoma cells. *Nature* **393**: 83–85
- Strelow A, Bernardo K, Adam-Klages S, Linke T, Sandhoff K, Kronke M, Adam D (2000) Overexpression of acid ceramidase protects from tumor necrosis factor-induced cell death. *J Exp Med* **192**: 601–611
- Thon L, Möhlig H, Mathieu S, Lange A, Bulanova E, Winoto-Morbach S, Schütze S, Bulfone-Paus S, Adam D (2005) Ceramide mediates caspase-independent programmed cell death. *FASEB J*, in press
- Vamosi G, Bodnar A, Vereb G, Jenei A, Goldman CK, Langowski J, Toth K, Matyus L, Szollosi J, Waldmann TA, Damjanovich S (2004) IL-2 and IL-15 receptor α -subunits are coexpressed in a supramolecular receptor cluster in lipid rafts of T cells. *Proc Natl Acad Sci USA* **101**: 11082–11087
- Waldmann TA, Tagaya Y (1999) The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. *Annu Rev Immunol* **17**: 19–49
- Yanagita M, Ishii K, Ozaki H, Arai H, Nakano T, Ohashi K, Mizuno K, Kita T, Doi T (1999) Mechanism of inhibitory effect of warfarin on mesangial cell proliferation. *J Am Soc Nephrol* **10**: 2503–2509